PROSTAGLANDIN RECEPTOR DISTRIBUTION AND FUNCTION IN THE RAT PERIPHERAL AND CENTRAL NERVOUS SYSTEM

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by

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Declaration

All the work in this thesis is original unless otherwise acknowledged in the text or by references. The material contained in this thesis has not been submitted for another degree at this or any other University. The thesis is based on work conducted by myself in the Department of Cell Physiology and Pharmacology at the University of Leicester, during the period between April 2002 to March 2005. In addition, I have included results from a series of cAMP experiments conducted by Raj Mistry in the Department of Cell Physiology and Pharmacology at the University of Leicester.

Abstract

Prostaglandin E_2 (PGE_2) is a chemical mediator of nociception that can evoke characteristic pain behaviour in animals after peripheral and spinal administration. The prostanoid is synthesised during tissue injury through activation of the arachidonic acid cascade, and its effects are mediated through binding to a family of specific G-protein coupled prostaglandin E (EP) receptors. The aim of the present study was to elucidate which EP receptors have an involvement in mediating the pro-nociceptive effects of PGE_2 within the rat peripheral and central nervous system.

Western blotting was used to characterise novel and commercially available EP receptor antibodies in EP receptor expressing cells to create a distribution of EP receptor subtypes present in the dorsal root ganglion (DRG) and spinal cord of the rat. A highly selective EP_4 receptor antibody was identified, which immunocytochemically localised the EP_4 receptor to small diameter ($<1000\mu m^2$) DRG neurons of a nociceptor phenotype. In contrast, EP_4 receptor expression within the spinal cord was widespread and was not restricted to any of the laminae associated with nociceptive processing.

Cultured rat DRG neurons were used to study the direct and sensitising effects of PGE_2 on sensory neurons. PGE_2 was seen to evoke reproducible increases in intracellular calcium in a small proportion of capsaicin-sensitive DRG neurons following direct application. Conversely, PGE_2 reproducibly enhanced the capsaicin-elicited calcium response in a substantial proportion of DRG neurons and sensitised *silent* neurons to become capsaicin responsive, consistent with the prostanoid having a major sensitising effect on nociceptors. With the use of novel potent and selective EP receptor compounds, the EP_3 and EP_4 receptors were identified as the key receptors responsible for mediating the sensitising effects of PGE_2 on sensory neurons.

The present study provides new evidence for PGE_2-mediated peripheral nociception being brought about via activation of EP_3 and EP_4 receptors, which identifies these two receptors as novel targets for the development of more efficacious treatment strategies for chronic pain states.

Acknowledgements

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My time in the lab will always be remembered fondly, and I will especially miss some of the *"characters"* that have come and gone over the years, especially Laura Mongan, Richard Baines, Ravinder Chandra, Bina Mistry, Steffi Mayer, Jen Eyre and Milly Thomas. Good luck to you all in the future!

To my friends from back home, Charys, Nutt, and Liz, yes it is finally finished and now I will be able to visit you all! Thanks for being so patient and understanding as to why I have been so inept at keeping in touch!

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List of Abbreviations

AA	Arachidonic acid
AHP	After hyperpolarisation
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-proprianate
ANOVA	Analysis of variance
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
°C	Degrees celsius
Ca^{2+}	Calcium
CaMKII	Ca^{2+} / calmodulin dependent kinase II
cAMP	Cyclic adenosine monophosphate
CCI	Chronic constriction injury
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene related peptide
CHO	Chinese hamster ovary cells
CNS	Central nervous system
COX	Cyclo oxygenase
CRE	cAMP response element
CREB	cAMP response element binding protein
DAG	Diacylglycerol
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DP	Prostaglandin D receptor
DRG	Dorsal root ganglion
EGR-1	Early growth response factor-1
EP	Prostaglandin E receptor
ERK	Extracellular signal-related protein kinase
EXINs	Excitatory interneurons
FCA	Freund's complete adjuvant
FITC	Fluorescein isothiocyanate
FP	Prostaglandin F receptor

GDNF	Glial cell-line derived neurotrophic factor
GSK-3	Glycogen synthase kinase-3
HBSS	Hank's balanced salt solution
HEK	Human embryonic kidney cells
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
Hsp	Heat shock protein
5-HT	5-hydroxytryptamine (serotonin)
HVA	High voltage activated
Hz	Hertz
I	Current
IB4	Isolectin B4
ININs	Inhibitory interneurons
IP	Prostaglandin I (prostacyclin) receptor
IP_3	Inositol 1, 4, 5, triphosphate
JNK	c-Jun N-terminal kinase
kDa	KiloDaltons
L	Lumbar
Li	Like-immunoreactivity
LO	Lipoxygenase
LT (A_4, B_4, C_4, D_4, E_4)	Leukotriene (A_4, B_4, C_4, D_4, E_4)
LVA	Low voltage activated
MAP	Mitogen activated protein
MKP-1	Mitogen activated protein kinase phosphatase-1
mRNA	Messenger ribonucleic acid
NADA	N-arachidonoyl-dopamine
NGF	Nerve growth factor
NK	Neurokinin
NMDA	N-methyl-D-aspartate
NON-N	Non-nociceptive neuron
NS	Nociceptor specific neuron
NSAIDs	Non-steroidal anti-inflammatory drugs
NT	Neurotrophin

PAFs	Primary afferent fibres
PBS	Phosphate buffered saline
PG (E_1, E_2, I_2, D_2, $F_{2\alpha}$)	Prostaglandin (E_1, E_2, I_2, D_2, $F_{2\alpha}$)
PIP_2	Phosphatidylinositol 4, 5-biphosphate
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PNs	Projection neurons
PNS	Peripheral nervous system
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
S.E.M.	Standard error of the mean
SP	Substance P
Th	Thoracic
TP	Thromboxane receptor
TR	Texas red
TRPV	Transient receptor potential (V for vanilloid)
TTBS	Tween-Tris buffered saline
TTX	Tetrodotoxin
TTX-R	Tetrodotoxin resistant
Tx	Thromboxane
VR1	Vanilloid receptor-1
WDR	Wide dynamic range neuron

Chapter One

Introduction

1.1 GENERAL INTRODUCTION

Pain and the perception of pain termed *nociception*, have a neuronal circuitry that is composed of both pain signalling and pain relieving pathways. These exist in a complex equilibrium, connecting the peripheral nervous system (PNS) and the central nervous system (CNS). The detection of a *noxious* (damaging) stimulus occurs primarily at the peripheral nerve terminals of specialised sensory neurons known as *nociceptors* [Millan, 1999]. These neurons have membrane receptors that act as sensitive sensors for changes in chemical or physical environment, providing nociceptors with remarkable and broad receptive properties for mechanical, thermal, and chemical signals [Pleuvry & Lauretti, 1996, Millan, 1999]. These nociceptive signals are conducted in the form of nerve impulses via the spinal cord to higher structures of the brain, where they are processed and modified to produce behavioural responses. The nociceptive input is perceived both physically and emotionally, generating an output that is primarily dependent upon the physical nature of the stimulus, but is also dependent upon the subject receiving the stimulus.

There are three distinct classes of pain, *physiological pain (acute pain)*, *inflammatory pain*, and *chronic pain*. The fundamental role of *physiological pain* is to serve as a protective system, warning of contact with potentially damaging stimuli. This leads to the motor withdrawal reaction, which is implemented to discontinue exposure to noxious stimuli and protect the affected area from further damage. This kind of pain is transient, well localised, and has a stimulus-response relationship.

Inflammation, which results from tissue injury (strains, sprains, and sunburn), produces pain as a consequence of inflammatory mediator release from damaged cells or from immune cells that infiltrate the site of inflammation. A mixture of these agents referred to as an *"inflammatory cocktail"* contributes to changes in vascular permeability, leading to redness and swelling of the affected area. In addition, the inflammatory cocktail can lead to *sensitisation* of peripheral nociceptors. This involves an alteration of ionic conductances such that the threshold for nociceptor

firing is decreased, which leads to the magnitude of the response being enhanced. This phenomenon is termed *primary hyperalgesia* and is defined as an increase in the pain evoked by a noxious stimulus. The increase in sensitivity also results in *allodynia*, which is pain evoked by a normally innocuous (non–damaging) stimulus. Therefore, it can be viewed that pain produced during the normal process of inflammation has a restorative / reparative role as it allows for normal tissue healing and repair to continue, whilst promoting hypersensitivity of the injured area, which acts to discourage further damage.

These types of adaptive pain are very different from *chronic pain*. In contrast to the protective function of physiological pain and the beneficial reparative nature of normal inflammatory pain, chronic pain sensations normally outlive their biological usefulness. This type of pain syndrome can result from neuropathies produced following trauma to the spinal cord or peripheral nerves (*neuropathic pain*), chronic inflammation (e.g. Rheumatoid arthritis), and terminal disease such as cancer. Patients suffering from chronic pain states are subject to relentless pain of a spontaneous and / or episodic nature (sometimes unrelated to extrinsic stimuli). They experience hyperalgesia and allodynia, and often enter "depressive-like" states triggered by the inescapable stress. In chronic pain states, the hyperalgesia and allodynia have both peripheral and central components, with the central components modulating the consequences of an abnormal and / or excessive primary afferent fibre input. These spinal effects are termed *central sensitisation* or *secondary hyperalgesia*, and are manifest as pain produced in response to noxious / innocuous stimuli in uninjured tissue surrounding the original area of damage (*subliminal fringe zone*). The process of central sensitisation is associated with low frequency spontaneous activity in primary afferent neurons and enlarged receptive fields. These central changes increase the responses to noxious and innocuous input [Millan, 1999].

At present the cellular mechanisms underlying chronic pain states are poorly understood. Long term, possibly irreversible changes occur at all levels of pain processing i.e. within the periphery, spinal cord, and higher brain centres. These changes involve morphological rearrangements within the nervous system, coupled with changes in cellular neurochemistry and phenotype. Unfortunately, without an in-depth knowledge of the mechanisms underlying these changes, patients suffering from conditions that are accompanied by chronic pain do not have a good prognosis

from a therapeutic perspective. This is because few of the conventional drug therapies (opioids, non-steroidal anti-inflammatory drugs (NSAIDs), local anaesthetics, anti-depressants, and anti-epileptics) are successful in alleviating the symptoms of chronic pain due to the mechanistic diversity of the syndrome. Furthermore, they are fraught with efficacy problems and numerous undesirable side effects. It is therefore imperative that new therapeutic targets are identified so that patients experiencing chronic pain states have a better outlook for the future. In this chapter, the role that inflammatory mediators, in particular prostaglandins and prostaglandin receptors, have in inflammation and chronic pain states shall be considered. Particular attention shall be given to the sensitising effects that prostaglandins have on nociceptors, a role, which underlies the exacerbation of pain states.

1.2 SUBTYPES OF PRIMARY AFFERENT FIBRE THAT DECIPHER NOXIOUS AND INNOCUOUS INFORMATION

Primary afferent nerve fibres are classified according to their axon diameter, conduction velocity, and myelination. Two classifications exist (table 1.1) and these define the groups of nociceptive and non-nociceptive fibres. In the skin, the C fibres make up the majority (70%) of all afferent fibres and are unmyelinated, of small diameter (0.4–1.2μm), and have a slow conduction velocity (0.5–2.5m.s^{-1}). The Aδ fibres are the most sparse of the various fibre types (10%), are myelinated, have a diameter of 2.5–5μm, and have an intermediate conduction velocity (12–30m.s^{-1}). Finally, the Aβ fibres constitute approximately 20% of all afferent fibres, are myelinated, have the largest diameter (>6μm), and have the fastest conduction velocity (25–75m.s^{-1}) [Belemonte and Cervero, 1996]. It is interesting to note that whilst each of the classes of primary afferent fibre can relay sensory *non–nociceptive* information, only the C and Aδ fibres transmit noxious information as a part of *normal physiological* (acute) pain processing.

Multiple subtypes of both Aδ and C-fibre receptors have been described using different experimental parameters [Julius & Basbaum, 2001]. The *first* pain that manifests itself is rapid, acute, and is produced by the faster conducting Aδ-fibres, whereas the delayed, more diffuse and dull, *second* pain is evoked by C-fibre stimulation [Julius & Basbaum, 2001]. The Aδ-fibres are predominantly high

threshold, rapidly–conducting *type I* mechanoreceptors, which are activated by high intensity mechanical stimuli in the noxious range, but are only weakly responsive to thermal stimuli [Handwerker & Kobal, 1993, Simone & Kajander, 1997]. However, if they are exposed to repetitive thermal stimulation, these receptors can become heat–responsive [Millan, 1999]. A second subset, the less prolific *type II* Aδ-fibres have also been recognised. Compared to their *type I* counterparts, these are slower conducting and have a lower threshold to noxious heat stimuli, thereby producing a more rapid response [Beydoun *et al.*, 1996]. Within the spinal cord, the majority of cutaneous Aδ fibres terminate in the superficial regions of the dorsal horn (particularly in lamina I and to a lesser extent in lamina II_o), although they are also found in deeper laminae V and VI. In these regions, the fibres synapse onto nociceptor-specific (NS) and wide dynamic range (WDR) (respond to both noxious and innocuous stimuli) neurons (sections 1.6.1 and 1.6.3) [Millan, 1999].

Class	**Function**	**Myelinated?**	**Axon Diameter (μm)**	**Conduction velocity ($m.s^{-1}$)**
Aα (or I)	Motor-somatic proprioception	Yes	12-20	70-120
Aβ (or II)	Touch Pressure	Yes	6-12	25-75
Aδ (or III)	Pain Temperature	Yes	2.5-5	12-30
C (or IV)	Pain Temperature	No	0.4-1.2	0.5-2.5

Table 1.1: Morphological and electrophysiological properties of primary afferent fibres.

The C fibres comprise a group of receptors that respond to thermal, mechanical, and chemical stimulation [Raja *et al.*, 1999]. The high threshold endings of these C fibres can transmit nociceptive information resulting from noxious thermal and / or mechanical stimuli. However, within this group of nociceptors, there are many that respond additionally to irritant chemical stimuli, such as acid or *capsaicin* (the pungent ingredient in hot chilli peppers) (section 1.4). The term *polymodal* is usually reserved for this latter type of nociceptor.

C-fibres that are sensitive to capsaicin can be further differentiated as *"peptidergic"* or *"non-peptidergic"* subpopulations [Hunt & Rossi, 1985]. The peptidergic nociceptors contain calcitonin gene-related peptide (CGRP) and

substance P (SP) and are dependent upon nerve growth factor (NGF)-mediated activation of the tyrosine kinase receptor A (TRK A) for their development [Snider, 1994, Snider & McMahon, 1998, Millan, 1999]. By contrast, the non-peptidergic nociceptors are defined by the presence of binding sites for the lectin IB4 [Silverman & Kruger, 1990], possess excitatory receptors for ATP on their peripheral terminals, and contain non-peptidergic neurotransmitters. These nociceptors are developmentally dependent on glial cell derived nerve growth factor (GDNF), which signals through a receptor complex, consisting of RET, a tyrosine kinase that is encoded by the *c-ret* proto oncogene, and the glycosyl phosphatidyl-inositol-anchored cell surface protein (GDNF family receptor (GFR) alpha) [Snider, 1994, Millan, 1999, Julius & Basbaum, 2001]. Within the spinal cord, the C-fibres project heavily to lamina II_o and less intensely to lamina I. Both of these laminae predominantly contain NS neurons. However, lamina II_o also contains interneurons (excitatory and inhibitory), which synapse onto lamina I NS neurons. The lamina I NS neurons comprise one group of projection neurons that are responsible for transmitting nociceptive information to higher structures of the brain (sections 1.6.1 and 1.6.3) [Millan, 1999].

It is important to note that one further class of C–fibre nociceptor also exists, which is commonly referred to as the *silent* nociceptor [Schaible & Grubb, 1993, Millan, 1999]. This subclass of nociceptor normally lies dormant and is unresponsive to acute noxious stimuli. However, under conditions of inflammation and tissue injury, they become mechano and / or thermosensitive, and can be sensitised by a variety of chemical mediators [Dmitrieva & McMahon, 1996, Millan, 1999].

In contrast to Aδ and C fibres, Aβ fibres are concerned with the rapid transmission of non-nociceptive impulses from peripheral proprioceptors and mechanoceptors. These large myelinated Aβ fibres synapse predominantly onto non-nociceptive (NON-N) neurons in laminae III-IV and to a lesser extent onto WDR neurons in lamina V (section 1.6.1). However, during neuropathic pain states a synaptic rewiring of Aβ, Aδ, and C fibres occurs at the level of the spinal cord [Millan, 1999]. This type of synaptic rewiring prevails due to disease / injury-induced destruction of C fibres, which results in a reduction of synaptic connections to lamina II of the spinal cord. As a result, non-nociceptive Aβ fibres begin to form collateral sprouts, which innervate the vacant areas of the superficial laminae (in

particular lamina II_o), previously occupied by C fibres [Woolf *et al.*, 1992, Millan, 1999, White & Kocsis, 2002]. These collateral sprouts undergo phenotypical changes such that they produce the same chemical messengers as nociceptive Aδ and C fibres (e.g. SP) as well as excitatory amino acids [Abbadie *et al.*, 1996, Millan, 1999]. Together, these changes result in the conversion of normal touch or movement signals to those of intense, chronic pain, creating a state of mechanical allodynia and hyperalgesia.

1.3 ROLE OF INFLAMMATORY MEDIATORS IN PERIPHERAL NOCICEPTIVE PROCESSING

Elevated levels of inflammatory mediators (prostaglandins, bradykinin, and others) have been identified in inflammatory exudates [Schaible & Grubb, 1993]. The clearest effects of these mediators are an excitation and / or sensitisation of afferent fibres, which leads to nociceptive behaviours in animals. To understand the effects of inflammatory mediators on nociceptors, it is necessary to consider the process of inflammation.

1.3.1 Inflammation

Inflammation is the body's basic response to tissue damage, injury or trauma and is characterised by redness (*rubor*), swelling (*tumor*), heat (*calor*), and pain (*dolor*). The function of the inflammatory response is to deliver effector molecules and cells to the site of injury, form a tissue barrier, and implement wound repair / healing. The inflammatory response is therefore made up of a complex series of immunological and physiological events. These consist of: an immediate vasoconstriction of blood vessels leading away from the injury site, and a vasodilation of blood vessels leading to the injury site. This gives rise to an engorged capillary network, which is responsible for the redness and increased temperature that accompanies tissue injury. There is also an increase in capillary permeability, which facilitates an influx of fluid and leucocytes from the engorged capillary network into the surrounding tissue. This fluid (plasma exudate) has a higher protein content than normal plasma fluid, which gives rise to the characteristic inflammatory swelling (oedema). The increased capillary permeability, decreased capillary blood velocity, and the increased expression of vascular endothelial adhesion molecules

facilitates further migration of leucocytes from the capillaries into the tissue. This leads to margination (cellular adherence to endothelial cells) and other essential tissue repair processes.

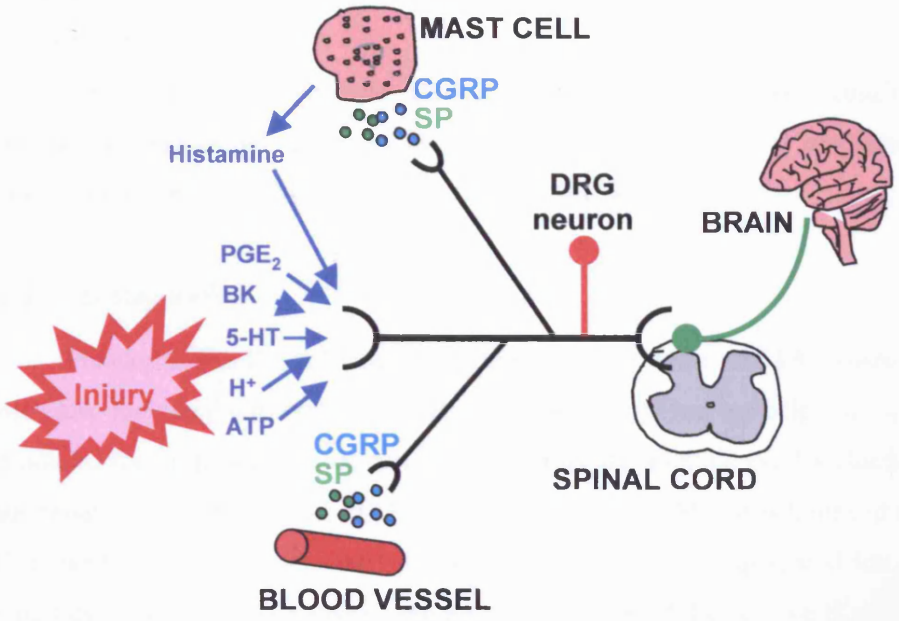


Figure 1.1: The pathogenesis of neurogenic inflammation. Following tissue injury, inflammatory mediators are released from damaged cells at the injury site including PGE_2, BK, 5-HT, H^+ (from the acidic nature of inflammation) and ATP. The action of these mediators leads to the sensitisation or direct excitation of primary afferent fibres through activation with cell-surface receptors, a process, which underlies neurogenic inflammation and causes hypersensitivity of the inflamed area. In addition, CGRP and SP are released from peripheral nerve terminals. These mediators have strong vasodilatory effects on blood vessels, which leads to plasma extravasation of proteins and fluid, a process which underlies the oedema that accompanies inflammation. CGRP and SP also act on mast cells to promote the release of histamine, which gives rise to the itch sensation. The nociceptive information is transferred from the periphery to the dorsal horn of the spinal cord in the form of action potentials. Within the spinal dorsal horn, central sensitisation can also occur, which leads to hyperalgesia and allodynia. From the spinal cord, nociceptive information is transmitted to the brain where it is ultimately processed [adapted from Julius & Basbaum, 2001].

During inflammation, chemical mediators are released from the site of injury into the periphery. These mediators are synthesised and released from both neuronal and non-neuronal cells, including circulating polymorphonuclear leucocytes and platelets, vascular endothelial cells, immune cells within the tissue (i.e. mast cells), and sensory and sympathetic nerve fibres. An "inflammatory cocktail" is produced during the release of different chemical mediators, which can have a variety of effects (figure 1.1). The chemical mediators can directly activate nociceptors to elicit depolarisation and firing of sensory neurons [Caterina & Julius 1999, McCleskey &

Gold 1999, Bhave & Gereau, 2004], they can promote the release of neuropeptides from sensory nerve terminals [Carlton *et al.*, 1996, Richardson & Vasko, 2002], and they can sensitise nociceptors to enhance noxious thermal and mechanical stimulus detection [Hu *et al.*, 2002, Bhave & Gereau, 2004]. Together, these effects not only lead to the aforementioned symptoms of inflammation, but also to hypersensitivity of the injured area.

Neurogenic inflammation accompanies inflammation and results from activation of peptidergic nociceptors and the release of chemical mediators from sensory nerve terminals [Bayliss, 1901, Holzer, 1998].

1.3.2 Prostaglandins

Prostaglandins (PGs) belong to a group of arachidonic acid (AA) metabolites, which also include the thromboxanes and leukotrienes. When specific prostaglandins are administered into the paw of conscious animals, the animals exhibit characteristic pain behaviour [Ferreira *et al.*, 1978, Taiwo & Levine, 1989]. It is believed that this PG-evoked pain behaviour occurs due to modification of voltage-gated ion channel properties, which results in lower activation thresholds of nociceptive fibres (section 1.5.1). In addition, prostaglandins can enhance other ion channel (non voltage-gated) or chemical mediator-evoked responses that underlie noxious thermal or mechanical sensation (section 1.5.2). To implement these effects they must activate second messenger system pathways via an interaction with specific prostaglandin receptors.

1.3.2.1 Arachidonic acid cascade and prostaglandin synthases

Arachidonic acid, the precursor of prostaglandins is primarily formed during the cleavage of cell membrane phospholipids by the action of phospholipase A_2 (PLA_2) or phospholipase C (PLC). These two isozymes are activated in response to a variety of non-specific stimuli, which include hormones, cytokines, and physical / chemical stimuli [Axelrod *et al.*, 1988, Rocca & Fitzgerald, 2002]. The metabolism of arachidonic acid leads to the production of prostaglandins and leukotrienes by two separate pathways.

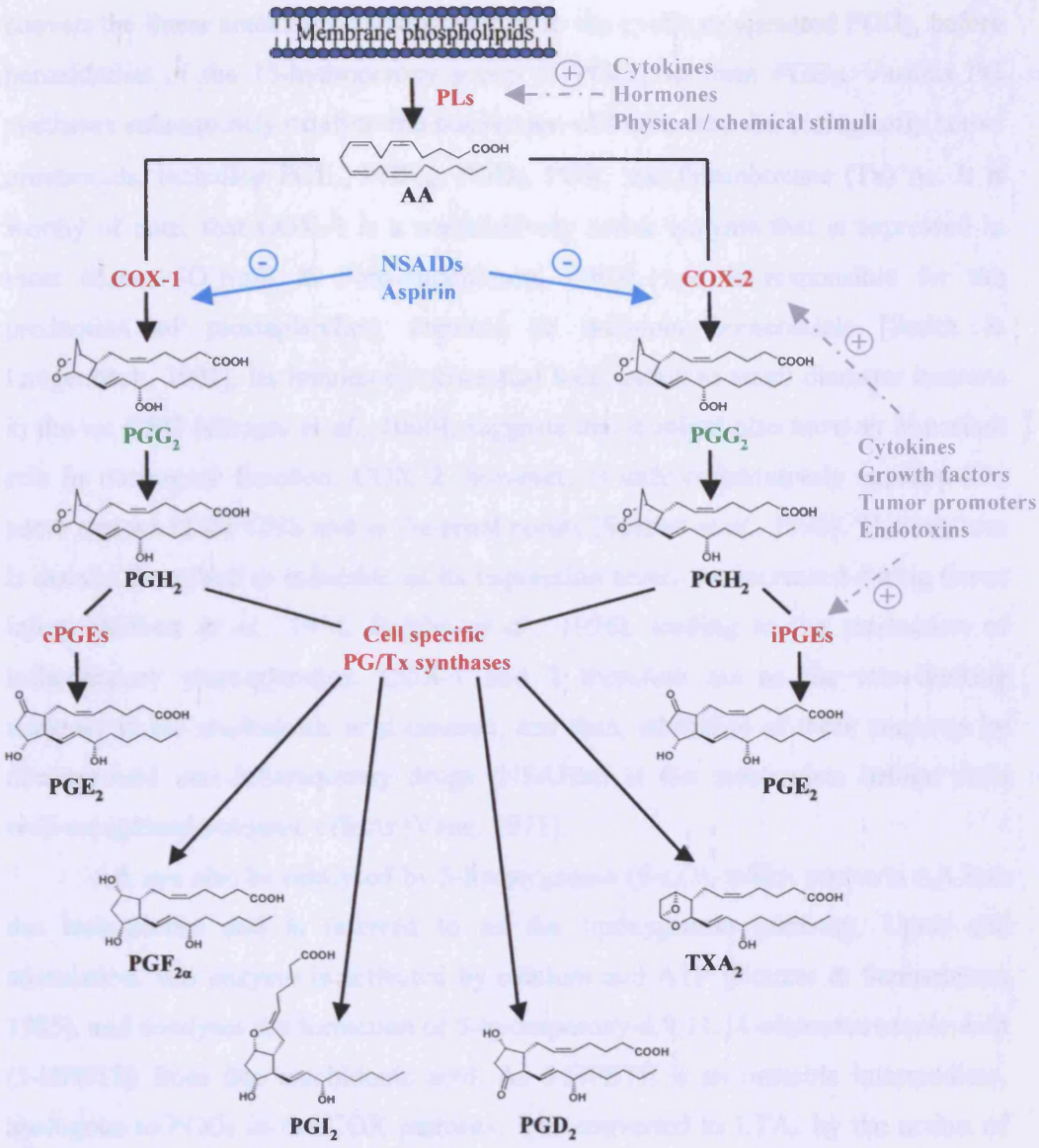


Figure 1.2: Arachidonic acid metabolism via the COX pathway. Activator (+) and inhibitor (-) agents are indicated in the scheme. The action of phospholipases (PLs) on membrane phospholipids leads to the generation of arachidonic acid (AA). AA is converted to prostaglandin (PG) G_2 by the action of cyclo-oxygenase enzymes (COXs); COX-1 is a constitutive enzyme present in most cells whereas COX-2 can be induced by a variety of stimuli. As PGG_2 is unstable it is rapidly metabolised to PGH_2. The action of constitutive PGE synthase (cPGEs) on PGH_2 is responsible for PGE_2 production required for normal physiological cellular functioning, whereas the inducible PGEs (iPGEs) is responsible for the production of PGE_2 in response to pathophysiological stimuli. $PGF_{2\alpha}$, PGI_2, PGD_2, and thromboxane (Tx) A_2 are all produced by the action of their respective synthases on PGH_2 [adapted from Rocca & FitzGerald, 2002].

In the cyclooxygenase (COX) pathway (figure 1.2), AA is metabolically converted to PGH_2 by the activity of PGH–synthase–1 or –2, more commonly referred to as cyclooxygenases (COX)–1 and –2. Cyclooxygenases first act to

convert the linear arachidonic acid precursor to the cyclic oxygenated PGG_2, before peroxidation of the 15-hydroperoxy group of PGG_2, to form PGH_2. Various PG synthases subsequently catalyse the conversion of PGH_2 into the biologically active prostanoids, including PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and thromboxane (Tx) A_2. It is worthy of note, that COX–1 is a constitutively active enzyme that is expressed in most tissues [O'Neill & Ford-Hutchinson, 1993], and is responsible for the production of prostaglandins, required to maintain homeostasis [Smith & Langenbach, 2001]. Its immunocytochemical localisation to small diameter neurons in the rat DRG [Chopra *et al.*, 2000], suggests that it might also have an important role in nociceptor function. COX–2, however, is only constitutively expressed in some regions of the CNS and in the renal cortex [Seibert *et al.*, 1994]. This isoform is usually described as inducible as its expression levels are increased during tissue injury [Seibert *et al.*, 1994, Beiche *et al.*, 1996], leading to the production of inflammatory prostaglandins. COX-1 and 2 therefore act as the rate–limiting enzymes in the arachidonic acid cascade, and thus, inhibition of these enzymes by non-steroidal anti–inflammatory drugs (NSAIDs) is the mechanism behind their well-recognised analgesic effects [Vane, 1971].

AA can also be catalysed by 5-lipoxygenase (5-LO), which converts AA into the leukotrienes and is referred to as the lipoxygenase pathway. Upon cell stimulation, this enzyme is activated by calcium and ATP [Rouzer & Samuelsson, 1985], and catalyses the formation of 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) from free arachidonic acid. As 5-HPETE is an unstable intermediate, analogous to PGG_2 in the COX pathway, it is converted to LTA_4 by the action of LTA_4 synthase. LTA_4 is then hydrolysed to LTB_4 or converted to LTC_4 by a conjugation reaction with glutathione. LTC_4 can subsequently be converted to LTD_4 and LTE_4 [Samuelsson, 1983].

1.3.2.2 The differential roles played by prostaglandins in nociception

Various arachidonic acid metabolites have been identified in either synovial effusions from patients with a variety of inflammatory joint diseases, or are produced in increased amounts by cultured synovial cells isolated from patients with rheumatoid arthritis [Dayer *et al.*, 1976, Trang *et al.*, 1977, Bombardieri *et al.*, 1981, Tokunaga *et al.*, 1981, Moilanen, 1989]. Amongst these, PGE_2 and PGI_2 are of

particular importance as they are present in inflammatory exudates at higher concentrations as compared to other prostanoids [Schaible & Grubb, 1993]. In support of their pronociceptive role, PGE_2 and PGI_2 have been shown to excite and / or sensitise nociceptive joint afferent fibres in the cat [Mense, 1981, Schaible & Schmidt, 1988, Schepelmann *et al.*, 1992] and rat [McQueen *et al.*, 1991, Grubb *et al.*, 1991, Birrell *et al.*, 1991, 1993]. Sensitisation by these prostaglandins is manifest in joint afferents as an increase in excitability evoked by mechanical stimulation [Birrell *et al.*, 1991, 1993, Schepelmann *et al.*, 1992] or as an enhancement of bradykinin-induced responses (section 1.5.2) [Mense, 1981, Schaible & Schmidt, 1988, Grubb *et al.*, 1991, Schepelmann *et al.*, 1992, Birrell *et al.*, 1993]. PGI_2 both excites and sensitises mechanonociceptors [McQueen *et al.*, 1991, Birrell *et al.*, 1991, 1993], whereas PGE_2 mainly acts as a sensitising agent [Birrell *et al.*, 1991, 1993] (section 1.5.2), suggesting that activation of the different PG receptors leads to different effects.

Behavioural experiments have also demonstrated the importance of prostaglandins in inflammation. PGE_2 and PGI_2 both cause hyperalgesia when injected into the rat paw or dog knee joint as measured by the Randall-Selitto paw withdrawal test and the degree of incapacitation, respectively [Ferreira *et al.*, 1978]. In addition, intradermal injection of PGE_2 into humans induces weal and flare responses as well as hyperalgesia [Sciberras *et al.*, 1987], and intraperitoneal injection of PGI_2 induces writhing behaviour in mice [Doherty *et al.*, 1987]. Furthermore, PGE_2 and PGI_2 (to a lesser extent) produce oedema in the rat paw and potentiate carrageenan-induced oedema [Higgs *et al.*, 1978].

1.3.2.3 Prostaglandin receptors and their distribution in the peripheral nervous system

Prostaglandin receptors belong to a family of G–protein coupled receptors with seven putative transmembrane domains [Narumiya *et al.*, 1999]. There are several subtypes of these receptors, which include the prostaglandin E (EP), prostaglandin D (DP), prostaglandin F (FP), prostaglandin I (IP), and thromboxane (TP) receptors. Upon activation by a prostaglandin or thromboxane, the receptors activate distinct second messenger pathways, which evoke their physiological effects. In the following sections, only the receptors for the E-series prostaglandins

and I-series prostaglandins, shall be considered, as at the current time, these two subsets appear to have a central role in peripheral nociceptive cellular processes.

1.3.2.3.1 EP receptors

The EP receptors constitute the largest family of prostaglandin receptors. There are four known EP receptor genes that are transcribed into EP_1, EP_2, EP_3, and EP_4 receptor subtypes (table 1.2). Of these, the EP_1 and EP_3 receptor transcripts may be further modified to give multiple receptor isoforms [Okuda-Ashitaka *et al.*, 1996, Oldfield, *et al.*, 2001]. The EP_1 receptor has two splice variants, one of which was isolated from the rat uterus and is characterised by a truncated intracellular tail [Okuda-Ashitaka *et al.*, 1996]. The EP_3 receptor gene generates multiple splice variants in human (9 variants) [Kotani *et al.*, 1997], cow (4 variants) [Namba *et al.*, 1993], mouse (3 variants) [Sugimoto *et al.*, 1993, Irie *et al.*, 1993], and rat (4 variants) [Takeuchi *et al.*, 1993, 1994, Neuschafer–Rube *et al.*, 1994, Oldfield *et al.*, 2001]. In rat, these variants are classified as $EP_{3\alpha}$ (also known as EP_{3A}), $EP_{3\beta}$, EP_{3B} (referred to as $EP_{3\gamma}$ in mouse), and EP_{3D}, and are distinguished by the length of their intracellular C-terminal tail.

Each subtype of EP receptor is coupled to a distinct second messenger system (table 1.2). The EP_1 receptor G-protein has yet to be identified but is known to mediate a PGE_2-induced elevation in intracellular calcium in EP_1 expressing CHO cells [Watabe *et al.*, 1993, Båtshake *et al.*, 1995]. Interestingly, this elevation in intracellular calcium is suppressed by co-expression of the EP_1 receptor with the variant EP_1 receptor isoform in CHO cells [Okuda-Ashitaka *et al.*, 1996]. The researchers have suggested that the variant EP_1 receptor may affect the signal coupling of prostaglandin receptors in such a way that the action of PGE_2 becomes attenuated. The EP_2 and EP_4 receptors are coupled to G_s, which activates adenylate cyclase leading to an increase in cAMP concentration [Regan *et al.*, 1994, Katsuyama *et al.*, 1995, Regan, 2003]. The $EP_{3\alpha}$ and $EP_{3\beta}$ receptor variants inhibit adenylate cyclase and cAMP generation via a pertussis toxin-sensitive G_i-coupled mechanism [Sugimoto *et al.*, 1993]. Activation of the $EP_{3B/3\gamma}$ receptor is more complicated and can result in both a decrease and an increase in cAMP concentration [Negishi *et al.*, 1996, Southall & Vasko, 2001]. Negishi and colleagues have shown that the $EP_{3\gamma}$ receptor produces full agonist-independent constitutive G_i activity but

also exhibits an agonist-dependent G_s activity. This is interesting as constitutive G_i activity could contribute to inhibiting nociceptor sensitisation during normal physiological functioning. During inflammation however, increased PGE_2 production would activate the agonist-dependent G_s activity and thereby promote nociceptor sensitisation (section 1.5). To date, there is no information in the literature, regarding the second messenger pathway that couples to the EP_{3D} receptor.

The distribution of EP receptors within the peripheral nervous system is poorly characterised, largely due to the lack of selective antibodies and cRNA probes for each of the EP receptor subtypes. A study by Donaldson and colleagues has demonstrated using reverse transcriptase polymerase chain reaction (RT-PCR) that the mRNAs for EP_1, EP_2, $EP_{3\alpha}$, $EP_{3\beta}$, EP_{3B}, and EP_4 are all present in the rat DRG [Donaldson *et al.*, 2001]. This study does not however provide any information regarding the cellular phenotype to which the receptors are localised within the DRG. To date, most studies have investigated the distribution of the EP_3 receptor (not the various splice variants) within various sensory ganglia. *In situ* hybridisation studies have revealed that EP_3 mRNA is present in approximately 50% of mouse DRG neurons [Sugimoto *et al.*, 1994, Oida *et al.*, 1995], with the majority of labelling occurring in small diameter neuronal cells (more than 70%) [Sugimoto *et al.*, 1994]. In addition, an immunocytochemical study has shown that EP_3 is expressed in the nodose, trigeminal, and dorsal root ganglia of the rat [Nakamura *et al.*, 2000]. Within these ganglia, EP_3 immunolabelling was primarily confined to small-sized neuronal cells, with a small number of medium-sized cells exhibiting a more intense labelling. With regards to the other EP receptor subtypes, expression of EP_1 and EP_4 mRNA in small-sized cells has been reported in 30% and 20% of mouse DRG neurons respectively [Oida *et al.*, 1995]. The localisation of these EP receptors to small- and medium-sized neurons suggests an involvement in PGE_2-mediated hyperalgesia.

1.3.2.3.2 IP receptors

The prostaglandin I, or *prostacyclin* receptor (IP) is primarily coupled to G_s, which leads to activation of adenylate cyclase and an increase in cAMP concentration [Namba *et al.*, 1994, Smith *et al.*, 1998, Chow *et al.*, 2003]. However, it has been reported that the IP receptor also mediates a phosphoinositol response [Namba *et al.*, 1994, Smith *et al.*, 1998, Chow *et al.*, 2003], which in IP receptor

expressing CHO cells, is not inhibited by pertussis toxin or affected by degradation of G_s by cellular incubation with cholera toxin, indicating that $G_{q/11}$ is involved [Namba *et al.*, 1994]. Whilst the cholera toxin procedure results in a loss of iloprost (non-specific IP receptor agonist)-induced cAMP formation, it does not affect the phosphoinositol response, suggesting that the IP receptor couples independently to both G_s and $G_{q/11}$.

Ligand	**Receptor Subtype**	**Terminal amino acid sequence (last 5)**	**Transduction System**	**Reference**
PGE_2	EP_1	GFSHL	Coupled to rise in $[Ca^{2+}]_i$	Watabe *et al.*, 1993
	$EP_{1\ variant}$	GNLCK	Unknown	Okuda-Ashitaka *et al.*, 1996
	EP_2	LCGQL	G_s	Katsuyama *et al.*, 1995
	$EP_{3\beta}$	SPREG	G_i	Neuschafer-Rube *et al.*, 1994
	$EP_{3\alpha}$ (EP_{3A})	DQLER	G_i	Takeuchi *et al.*, 1993
	EP_{3B} ($EP_{3\gamma}$,)	VHPGP	G_i and G_s	Takeuchi *et al.*, 1994
	EP_{3D}	LCFNR	Unknown	Oldfield *et al.*, 2001
	EP_4	SEKCI	G_s	Sando *et al.*, 1994
PGI_2	IP	ACSLC	G_s and $G_{q/11}$	Namba *et al.*, 1994

Table 1.2: Table to show the second messenger system coupling of the different EP and IP receptor subtypes. The table lists each prostanoid receptor subtype, together with any of its splice variants, and gives details of which second messenger system each receptor activates, their cloning reference, and the last 5 amino acid residues of their sequence. The latter has been included because in the case of the EP_3 receptor splice variants, the nomenclature in the literature is somewhat confusing.

IP mRNA is abundantly expressed in small (60%) and large neurons of the DRG, and co-localizes with the mRNA of preprotachykinin A, a precursor of substance P [Oida *et al.*, 1995]. This observation reinforces the involvement of the IP receptor in the mediation of pain (section 1.3.2.2). Interestingly, IP mRNA is co-expressed with the mRNA of the EP (EP_1, EP_3, and EP_4) receptor subtypes in some neurons [Oida *et al.*, 1995], which suggests that EP and IP receptors may have interrelated roles in the transmission of pain sensation.

1.3.3 Bradykinin

Bradykinin is a member of the kinin family of local hormones, which also includes kallidin, T-kinin, and their active metabolites, the des-Arg^9-kinins formed by the action of peptidases referred to as kininases [Couture *et al.*, 2001]. Following tissue damage, bradykinin is produced by activation of the kallikrein-kinin system [Ueno & Oh-Ishi, 2003]. All components of this system are present in the plasma, and are released following tissue damage. Initiation of the kinin cascade is thought to be as a result of the binding of inactive Factor XII (Hageman factor) to negative surfaces such as damaged basement membranes or to endotoxins, a process that leads to activated Factor XII (XIIa). Under normal conditions, the precursor for bradykinin, high molecular weight kininogen (HMWK), circulates bound to the inactive form of the plasma kallikrein enzyme, prekallikrein, but upon tissue damage binds to the endothelial surface. Factor XIIa then cleaves prekallikrein, to release the active enzyme, plasma kallikrein, which culminates in the liberation of bradykinin from the HMWK [Griffin & Cochrane, 1976].

Once formed, bradykinin's potent allogenic role in inflammation / nociception is mediated through activation of B_1 and B_2 receptor subtypes [Walker *et al.*, 1995, Couture *et al.*, 2001]. The B_2 receptor is constitutively expressed in many tissues and is preferentially bound by bradykinin and kallidin. In contrast, the B_1 receptor is sparsely distributed in normal tissues, but is upregulated under pathophysiological conditions such as tissue injury and upon prostanoid production, and is preferentially activated by the des-Arg^9-kinins [Couture *et al.*, 2001]. Both of the kinin receptors are coupled to $G_{q/11}$ proteins, which results in the activation of phospholipase C and the subsequent generation of the second messengers inositol-1,4,5-triphosphate (1,4,5-IP_3) and diacylglycerol (DAG) [Burgess *et al.*, 1989, Blaukat, 2003], leading to a variety of cellular effects.

Bradykinin can directly stimulate nociceptive nerve terminals and thereby produce hyperalgesia [Burgess *et al.*, 1989, Rashid *et al.*, 2004]. This occurs as a result of bradykinin receptor-induced PKC activation, which leads to an increased Na^+ conductance, and thus neuronal depolarisation [Burgess *et al.*, 1989]. In addition to directly activating sensory fibres, bradykinin sensitises them to heat and mechanical stimuli [Liang *et al.*, 2001], as well as to chemical stimuli including prostaglandins and 5-HT [Rueff & Dray, 1993, 1993b]. Bradykinin is able to mediate

a hypotensive effect on the vasculature [Lu *et al.*, 1997], and increases vascular permeability by causing contraction of vascular endothelial cells [Regoli, 1984]. Furthermore, it facilitates the release of CGRP and substance P from sensory neurons, both of which have strong vasodilatory effects on the vasculature (section 1.3.7) [MacLean *et al.*, 1990, Andreeva & Rang, 1993, Vasko *et al.*, 1994]. Bradykinin also triggers degranulation of mast cells to release histamine and other inflammatory mediators [Ishizaka *et al.*, 1985], and activates phospholipase A2, which results in an increased cellular concentration of arachidonic acid, with the subsequent production of pro-inflammatory prostaglandins (section 1.3.2.1) [Rang *et al.*, 1991].

It is now apparent that the acute effects of bradykinin are produced as a result of B_2 receptor activation, whereas the chronic effects result from stimulation of the B_1 receptor [Dray & Perkins, 1993]. This is not surprising as bradykinin has a very short half-life and is rapidly degraded to its metabolite, des-Arg^9-bradykinin [Walker *et al.*, 1995]. Therefore, during persistent inflammatory processes, des-Arg^9-bradykinin accumulates as a result of its long half-life and the rapid metabolism of bradykinin. As des-Arg^9-bradykinin preferentially binds B_1 receptors it seems that there is a trend towards B_1 receptor subtype activation during chronic pain syndromes. In line with this, intraplantar injection of bradykinin causes nociceptive behaviours, which are mediated by the B_1 receptor in nerve-injured mice and the B_2 receptor in sham-operated mice, as demonstrated using selective agonists and antagonists of the B_1 and B_2 receptors [Rashid *et al.*, 2004]. A change in receptor preference during persistent nociceptive states is further supported by the fact that B_2 receptor function is controlled by short-term mechanisms involving fast ligand dissociation, receptor desensitisation and internalisation, and during prolonged stimulation, downregulation of the receptor [Munoz & Leeb-Lundberg, 1992, Mathis *et al.*, 1996, Faussner *et al.*, 1999, Blaukat, 2003]. In contrast, the B_1 receptor elicits persistent responses that undergo limited desensitisation, and following prolonged stimulation or after nerve injury are expressed at higher levels [Mathis *et al.*, 1996, Faussner *et al.*, 1999].

1.3.4 Histamine

Histamine is released following mast cell degranulation and is responsible for triggering the well-recognised itch sensation that accompanies an inflammatory response [Shelley & Arthur, 1957]. This allergenic substance can act at H_1, H_2, H_3, and H_4 receptor subtypes, but its pro-inflammatory effects appear to be mainly mediated by H_1 receptor activation, which is positively coupled to $G_{q/11}$, and results in IP_3 / DAG formation [Hill *et al.*, 1997]. Other effects of histamine include the stimulation of inflammatory cytokines and chemokines, resulting in inflammatory cell recruitment [Albanesi *et al.*, 1998, Fumagalli *et al.*, 2004], vasodilation [Millan, 1999], and inflammatory mediator release (e.g. neuropeptides and prostaglandin E_2) [Bileviciute *et al.*, 1997, Tetlow & Woolley, 2004]. In addition, histamine can directly excite primary afferent fibres, which have been shown to be sensitive to mechanical stimulation and capsaicin, indicative of polymodal nociceptors [Handwerker *et al.*, 1991, Schmelz *et al.*, 1997]. In line with this observation, injection of histamine into the deep layers of the skin elicits pain [Rosenthal, 1949], and H_1 receptor knockout mice lack nociceptive behaviours when administered with histamine [Mobarakeh *et al.*, 2000]. Although poorly understood, one theory relating to histamine's mechanism of action is that at low concentrations, histamine-induced activity in nociceptive afferent fibres signal the itch sensation while higher levels of activity signal pain [McMahon & Kolzenburg, 1992].

1.3.5 Adenosine Triphosphate (ATP) and adenosine

Extracellular ATP directly excites nociceptive endings of sensory neurons during the tissue damage process to elicit a sensation of pain [Burnstock, 1996, Burnstock & Wood, 1996, Bland-Ward & Humphrey, 1997]. This chemical mediator produces its effects through activation of type 2 purinergic receptors. These include seven ionotropic P2X receptor subtypes ($P2X_{1\text{-}7}$) [Millan, 1999] and seven metabotropic P2Y receptor subtypes ($P2Y_{1, 2, 4, 6, 11, 12, 13}$) [Millan, 1999, Fumagalli *et al.*, 2004b].

As P2X receptors are ligand-gated cation channels that produce rapid activation of an inward current, there has been much interest in the role of these receptors in nociceptor excitation. Thus, the distribution of P2X receptors has been extensively investigated. In sensory neurons of neonatal rats, *in situ* hybridisation

studies have shown that the mRNA encoding $P2X_{1-6}$ receptors is present [Collo *et al.*, 1996]. A similar pattern of expression has been seen in adult DRG neurons, with a recent *in situ* hybridisation study demonstrating the presence of $P2X_{2-6}$ mRNAs [Kobayashi *et al.*, 2005]. These findings suggest that multiple P2X receptors are expressed in these sensory neurons. However, studies have demonstrated that only $P2X_2$ and $P2X_3$ subunits occur exclusively in small to medium-sized neurons [Vulchanova *et al.*, 1997, 1998, Ueno *et al.*, 1999, Kobayashi *et al.*, 2005,]. As these purinergic subunits are co-localised with IB4 immunoreactivity [Vulchanova *et al.*, 1997, 1998], the nociceptors expressing $P2X_2$ and $P2X_3$ subunits are believed to be a non-peptidergic phenotype.

Functional P2X receptors are comprised of at least three subunits [Nicke *et al.*, 1998, Ding & Sachs, 2000], and it is believed that two or more subunits may assemble together to constitute, not only homomeric, but also heteromeric P2X receptors [McCleskey & Gold, 1999]. Indeed, Vulchanova and colleagues have demonstrated by immunocytochemical studies in adult DRG that the expression of $P2X_2$ and $P2X_3$ subunits extensively overlaps [Vulchanova *et al.*, 1997]. In addition, it has been shown by *in situ* hybridisation that mRNA co-expression occurs between the $P2X_2$ and $P2X_3$ receptor subunits, and the $P2X_3$ and $P2X_5$ receptor subunits [Kobayashi *et al.*, 2005]. Therefore, it could be viewed that different combinations of subunits give rise to a range of P2X receptor phenotypes. In line with this, three distinct types of ATP-evoked currents have been recorded in cultured adult rat DRG neurons [Grubb and Evans, 1999]. In the majority of neurons, a fast transient inward current, with rapid inactivation kinetics was observed. This had the appropriate pharmacology and kinetics of channels assembled only from $P2X_3$ subunits ($P2X_3$ homomers). In addition, a slowly inactivating sustained current ($P2X_2$-like) and a biphasic current consisting of both fast and sustained components ($P2X_{2/3}$-like) were also witnessed. It was hypothesised that the latter current represented the existence of P2X heteromers. Indeed, evidence is rapidly accumulating to support the presence of $P2X_3$ homomers and $P2X_{2/3}$ heteromers in DRG neurons [Burgard *et al.*, 1999, Ueno *et al.*, 1999, Dunn *et al.*, 2000]. In addition, evidence suggests that $P2X_3$ and $P2X_{2/3}$ receptors mediate an acute nociceptive pathway contributing to pain in damaged tissue [North & Barnard, 1997, Tsuda *et al.*, 2000, Wu *et al.*, 2004]. It could therefore be viewed that the slow inactivation kinetics of the heteromultimeric

channel that persist during prolonged ATP exposure, represent a way in which ATP produces its pro-nociceptive effects.

Although $P2X_3$ certainly has a pro-nociceptive role, ATP-evoked nociceptive behaviour in mice is not fully abolished upon disruption of the $P2X_3$ gene [Cockayne *et al.*, 2000, Souslova *et al.*, 2000]. Therefore, the role that P2Y receptors have in nociception is now becoming a major interest. *In situ* hybridisation studies in DRG neurons have revealed that $P2Y_1$ and $P2Y_2$ mRNAs are expressed at high levels [Nakamura & Strittmatter, 1996, Molliver *et al.*, 2002, Moriyama *et al.*, 2003]. Within the DRG, co-expression occurs between $P2Y_2$ and TRPV1 (section 1.4) mRNAs, but not $P2Y_1$ and TRPV1 mRNAs [Moriyama *et al.*, 2003]. In line with this, intraplantar injection of UTP (selective agonist for $P2Y_2$ and $P2Y_4$ receptors) into the mouse hindpaw produces thermal hyperalgesia, which is blocked by suramin (blocks $P2Y_2$ not $P2Y_4$), and preserved in mice lacking the $P2Y_1$ gene [Moriyama *et al.*, 2003]. These observations therefore provide evidence for a functional interaction between the $P2Y_2$ and TRPV1 receptors in thermal nociception.

1.3.6 Protons

In inflamed tissues, pH values as low as 5.4 have been measured, and this local acidosis is believed to contribute to the generation and maintenance of pain in inflammatory disease states. In line with this, polymodal nociceptors in the rat skin exhibit stimulus-related discharges that increase with proton concentration [Steen *et al.*, 1992]. In addition, the mechanical thresholds of these nociceptors diminish when the pH drops, whereas non-nociceptive low-threshold mechanoreceptors are unaffected. Furthermore, protons are unique among allogenic substances in that they are able to drive nociceptors continuously without any apparent tachyphylaxis (section 1.4.1.1). Therefore, protons are thought to be important in maintaining pathological conditions in tissue, especially since they interact with other inflammatory mediators to enhance nociceptor firing [Steen *et al.*, 1995]. At a cellular level, protons depolarise small sized rat DRG neurons, by activating a non-selective cation current [Bevan & Yeats, 1991]. This response consists of a rapidly inactivating transient current carried mainly by the inward flow of sodium ions, followed by a sustained non-selective cation current. The ion channels that underlie this type of proton-induced response are believed to be from the acid sensing ion

channel family (ASICs), although TRPV1 (section 1.4), and certain potassium channels could also have a role [McCleskey & Gold, 1999].

1.3.7 Neuropeptides

Neuropeptides such as substance P and CGRP are released from C fibre primary afferent neurons during an inflammatory response / nociceptive process, and there is considerable evidence to suggest that they are major initiators of neurogenic inflammation [Holzer, 1988, Jang *et al.*, 2004].

Substance P together with neurokinin (NK) A, neuropeptide K, neuropeptide-γ, and neurokinin B constitute a group referred to as the *tachykinins* and act at NK_1, NK_2, NK_3, and NK_4 G-protein coupled receptors [O'Connor *et al.*, 2004]. Studies indicate that each tachykinin preferentially activates a distinct tachykinin receptor, although at high concentrations the individual tachykinins can activate all receptor subtypes. For example, most of the proinflammatory effects of substance P are mediated by NK_1 receptors, which are positively coupled to phospholipase C (PLC) [Chapman *et al.*, 1996, Traub, 1996, Wajima *et al.*, 2000]. Evidence to support the involvement of substance P in the pathophysiology of pain stems from observations, which show that nociceptive behaviours in rats can be enhanced or blocked upon intraplantar injection of NK_1 receptor agonists or antagonists respectively [Carlton *et al.*, 1996].

CGRP exists in two isoforms (α and β), both of which are known to act at, at least two different receptor subtypes classified as $CGRP_1$ and $CGRP_2$ [Quirion *et al.*, 1992]. It is believed that both of these CGRP receptor subtypes exist as a complex consisting of a seven transmembrane domain protein called a calcitonin receptor-like receptor (CRLR), a receptor activity-modifying protein (RAMP), and a receptor component protein (RCP) [McLatchie *et al.*, 1998, Evans *et al.*, 2000]. The RAMP defines the relative potency of ligands for the receptor whereas the RCP determines the G-protein to which the receptor couples. The $CGRP_1$ receptor, which binds CGRP and the CGRP antagonist, $CGRP_{8-37}$ with the highest affinity consists of CRLR, RAMP1, and RCP and couples to the production of cAMP and its downstream protein kinase A [McLatchie *et al.*, 1998]. Activation of this receptor subtype in peripheral sensory neurons has been shown to stimulate cAMP production and produce hyperalgesia in animals [Nakamura-Craig & Gill, 1991, Anderson &

Seybold, 2004]. As multiple CGRP accessory proteins exist [Li *et al.*, 2004], it is likely that some CGRP-mediated effects may be produced by other CGRP complexes, resulting in activation of signalling pathways other than cAMP production.

Substance P and CGRP are stored within a subset of small diameter sensory neuronal cell bodies [Ohtori *et al.*, 2002, von Blanchet *et al.*, 2002, Ma *et al.*, 2003] and are transported to the periphery where they are released after primary afferent fibre activation [Carlton *et al.*, 1996]. These compounds not only directly activate nociceptors but also produce a powerful dilation of microvessels and induce plasma extravasation (neurogenic inflammation). Substance P causes an indirect vasodilation through the release of nitric oxide and induces plasma extravasation by acting on endothelial cells, whilst CGRP induces a direct dilation of arterioles and facilitates plasma extravasation by increasing local blood flow. In addition, both neuropeptides activate many non-neuronal cells such as mast cells, lymphocytes, monocytes, and macrophages [Millan, 1999, O'Connor *et al.*, 2004]. Through activation of these non-neuronal cells, additional inflammatory mediators are released, which can elicit their own proinflammatory effects thereby producing a positive feedback system [Richardson & Vasko, 2002].

An additional role for peripheral neuropeptides is in neuropathic pain states. A study by Jang and colleagues has shown that intraplantar injection of CGRP or SP receptor antagonists immediately prior to spinal nerve lesion, delayed the onset of mechanical allodynia in rats [Jang *et al.*, 2004]. However, when the same injection was given after spinal nerve lesion, only the CGRP receptor antagonist reversed the mechanical allodynia. Therefore, whilst both CGRP and substance P released from the peripheral terminals of primary afferent fibres are essential in the development of neuropathic pain, it is CGRP release that is required for maintaining the pain. One explanation for this could be the emerging effect of CGRP on gene transcription. CGRP has been shown to evoke an increase in gene transcription in dorsal root ganglion neurons [Anderson & Seybold, 2004]. The authors have shown that CGRP, via activation of PKA, phosphorylates the cAMP response element binding protein (CREB), leading to an increase in cAMP response element (CRE)-dependent gene transcription. These findings are exciting as during tissue damage / nociceptive processes, CGRP released from primary afferent neurons could initiate long-term

changes in protein expression in these neurons and lead to changes in neuronal excitability or chemical transmission.

1.4 THE ROLE OF THE VANILLOID RECEPTOR IN PERIPHERAL NOCICEPTION

The vanilloid receptor has a central role in mediating thermal nociception [Caterina *et al.*, 2000, Davis *et al.*, 2000], and was originally termed vanilloid receptor 1 (VR1) when Caterina and colleagues cloned the cDNA in 1997 [Caterina *et al.*, 1997]. A homology search revealed there to be significant similarities between the VR1 receptor and members of the transient receptor potential channel (TRP) family, which were originally identified in *Drosophila* [Hardie & Minke, 1992]. This family of channels can be split into four subfamilies: TRPC (C for canonical or classical), TRPV (V for vanilloid), TRPM (M for melastatin related), and TRPP (PKD type). The TRPV family consists of six members, TRPV1-6. The VR1 receptor is now also referred to as the TRPV1 channel, and shows homology at the amino acid level with TRPV2 (46%), TRPV3 (43%) and TRPV4 (43%).

The TRP family of channels share a similar membrane receptor topology. This has been predicted from hydrophilicity analysis for TRPV1 (figure 1.3), to be that of six transmembrane domains (predicted to be mostly β-sheet) with an additional short hydrophobic stretch between transmembrane regions 5 and 6 (thought to be the pore region), and intracellular carboxy and amino termini [Caterina *et al.*, 1997]. The hydrophilic amino terminus harbours a relatively proline-rich region followed by three ankyrin repeats, which are important in linking proteins to the cytoskeleton (figure 1.3). Within the carboxy terminus, a phosphatidylinositol-4,5-biphosphate (PIP_2) binding site is contained. It is believed that PIP_2 exerts a suppressive effect on the TRPV1 channel in the resting state and thus plays a key role in TRPV1 sensitivity [Cortright & Szallasi, 2004]. As a functional channel, TRPV1 is believed to exist as homomeric or heteromeric complexes of four subunits [Kedei *et al.*, 2001]. Upon activation of the channel, a robust inward current is produced, which leads to depolarisation of the cell membrane with a reversal potential of ~0mV [Caterina *et al.*, 1997]. In transfected cells expressing the TRPV1 channel, ion substitution experiments have shown that this inward current is predominantly carried by divalent cations with a channel permeability sequence of:

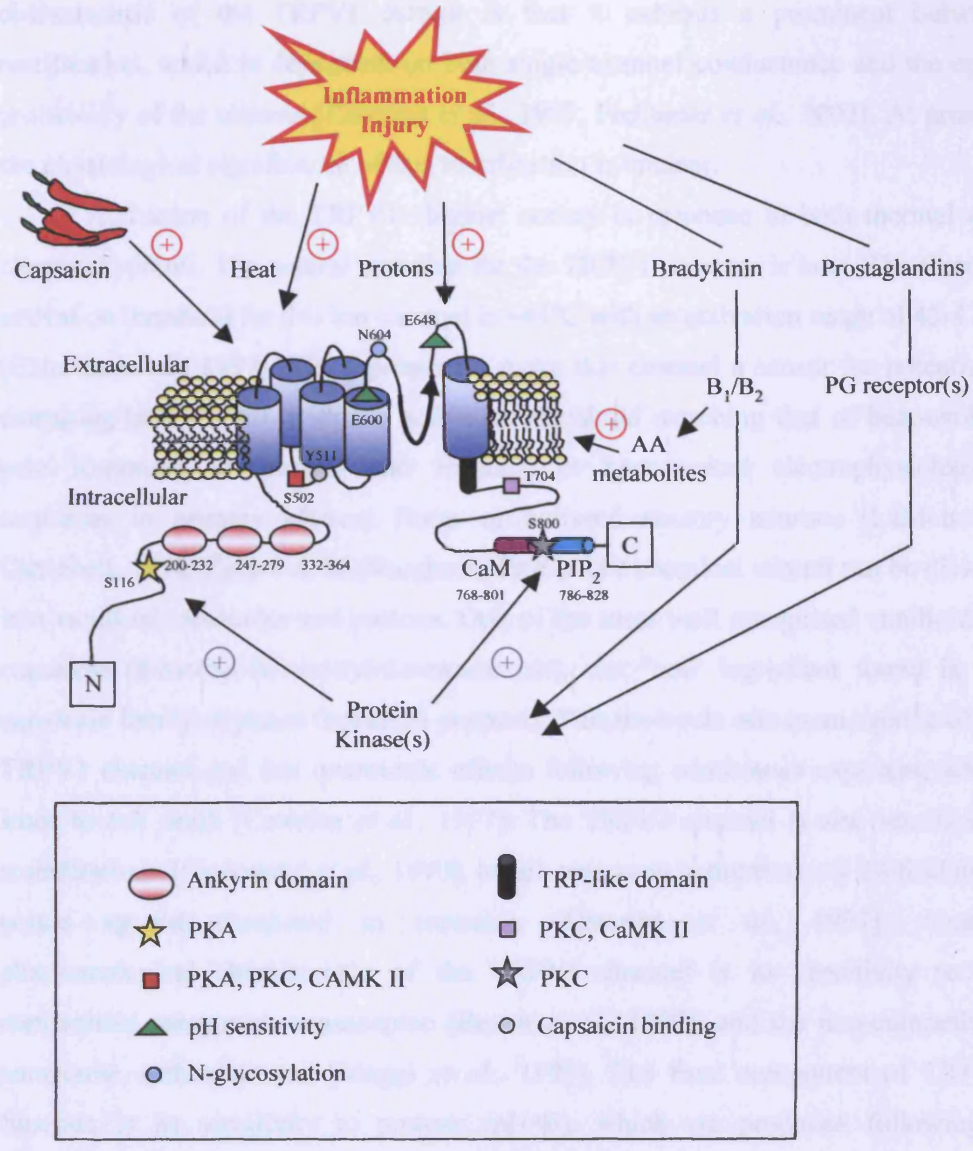


Figure 1.3: Topological organisation of the TRPV1 channel subunit and its activators. TRPV1 consists of six transmembrane spanning segments (cylinders) with a large stretch connecting the S5 and S6 segments that holds a short amphipathic fragment (curved arrow); an N-terminal domain containing three ankyrin repeats; and a C-terminus domain carrying calmodulin (CaM) and phosphatidylinositol-4,5-biphosphate (PIP_2) binding sites. TRPV1 can be directly activated by a variety of different stimuli such as capsaicin, low pH, elevated temperature, and arachidonic acid metabolites (e.g. 12-HPETE), or indirectly activated by inflammatory mediators (e.g. prostaglandins and bradykinin) via protein kinase activity. Highlighted are the various amino acid residues from the rat primary sequence that are important in TRPV1 function and activation [adapted from Ferrer-Montiel *et al.*, 2004].

$Ca^{2+} > Mg^{2+} > Na^{+} \approx K^{+} \approx Cs^{+}$ [Caterina *et al.*, 1997]. The high permeability of the channel to calcium (P_{Ca}/P_{Na}=9.60) [Caterina *et al.*, 1997] is similar to values reported for NMDA-type glutamate receptors [Mayer & Westbrook, 1987]. Another

characteristic of the TRPV1 current is that it exhibits a prominent outward rectification, which is dependent on both single channel conductance and the open probability of the channel [Caterina *et al.*, 1997, Prekumar *et al.*, 2002]. At present the physiological significance of this rectification is unclear.

Activation of the TRPV1 channel occurs in response to both thermal and chemical stimuli. The natural stimulus for the TRPV1 receptor is heat. The thermal activation threshold for this ion channel is ~43°C with an activation range of 43-47°C [Caterina *et al.*, 1997]. These properties make this channel a sensor for potentially damaging heat stimuli, with the activation threshold matching that of heat-evoked pain responses in humans and animals, or heat-evoked electrophysiological responses in primary afferent fibres or cultured sensory neurons [LaMotte & Campbell, 1978, Cesare & McNaughton, 1996]. The chemical stimuli can be divided into vanilloid molecules and protons. One of the most well recognised vanilloids is capsaicin (8-methyl-N-vanillyl-6-nonenamide), the "hot" ingredient found in the *capsicum* family of plants (hot chilli peppers). This molecule acts as an agonist of the TRPV1 channel and has neurotoxic effects following continuous exposure, which leads to cell death [Caterina *et al.*, 1997]. The TRPV1 channel is also sensitive to resinferatoxin [Szolcsanyi *et al.*, 1990], which acts as an approximately 20-fold more potent agonist compared to capsaicin [Caterina *et al.*, 1997]. Another pharmacological characteristic of the TRPV1 channel is its sensitivity to the competitive antagonist, capsazepine [Bevan *et al.*, 1992], and the non-competitive antagonist, ruthenium red [Maggi *et al.*, 1993]. The final component of TRPV1 function is its sensitivity to protons (pH<6), which are produced following a reduction in tissue pH, resulting from inflammation [Caterina *et al.*, 1997, Welch *et al.*, 2000, Wang *et al.*, 2002, Ryu *et al.*, 2003].

There is now increasing evidence to suggest an interaction between vanilloids, protons, and heat during activation of the TRPV1 channel. Several studies have shown that simultaneous applications of two or more stimuli lead to cross sensitisation of the channel [Caterina *et al.*, 1997, Tominaga *et al.*, 1998]. This is clearly demonstrated during activation of the TRPV1 channel with heat or capsaicin in the presence of protons. Whilst Caterina and colleagues have reported proton-induced inward currents in oocytes expressing the TRPV1 channel, this type of response was only witnessed in a small proportion of cells. However, upon exposure of the cells to capsaicin, a decrease in pH from 7.6 (physiological pH) to 5.5 resulted

in the capsaicin responses exhibiting a five-fold increase [Caterina *et al.,* 1997]. In addition, low pH (section 1.3.6) and vanilloids have been shown to lower the threshold for heat activation from noxious to non–noxious temperatures [Tominaga *et al.,* 1998]. A study by Ryu and colleagues has investigated using single channel kinetics in conjunction with whole-cell measurements, the interaction between capsaicin and low pH, and the possible mechanism behind the cross-sensitisation of TRPV1 channel function [Ryu *et al.,* 2003]. The authors have shown that pH has dual effects on both capsaicin binding and channel gating. A low pH enhanced the binding affinity of capsaicin, promoted long openings and short closures of the channel, and stabilised at least one of the open channel conformations. In addition, capsaicin binding and protonation of the TRPV1 channel was observed to be via an allosteric interaction, such that binding of capsaicin to the protonated channel resulted in it becoming energetically facilitated. Taken together, these findings imply that TRPV1 acts as an integrator of multiple pain producing stimuli.

In addition to TRPV1 being activated by thermal stimuli, various other members of the TRP family are also temperature sensitive. The properties of these ion channels are summarised in figure 1.4. Interestingly, a splice variant of TRPV1 named TRPV1b has recently been cloned from human and rat brain cDNA libraries by Lu and colleagues [Lu *et al.,* 2005]. The channel is activated at a temperature of 47°C but unlike TRPV1 is not activated by protons or capsaicin. A channel with similar activation properties compared to TRPV1b has been reported in trigeminal ganglion neurons [Lu *et al.,* 2005]. As well as the TRP family of ion channels, there are also acid sensing ion channels (ASICs) [McCleskey & Gold, 1999], which are ligand-gated ion channels that respond to low pH. Therefore, nociceptive sensory neurons appear to express a complement of different channels that are able to respond to thermal stimuli and acidic inflammatory conditions.

The tissue distribution of TRPV1 is fairly diverse and evidence exists for its expression in both non-neuronal and neuronal cells [Mezey *et al.,* 2000, Southall *et al.,* 2003, Birder *et al.,* 2001, Caterina & Julius, 2001]. In the DRG, TRPV1 expression has been demonstrated by northern blot analysis, *in situ* hybridisation and immunocytochemistry [Caterina *et al.,* 1997, Michael & Priestley, 1999, Guo *et al.,* 1999, Sanchez *et al.,* 2001]. TRPV1 is restricted mainly to small-sized neurons as well as to a small proportion of medium-sized neurons (section 1.2) [Michael &

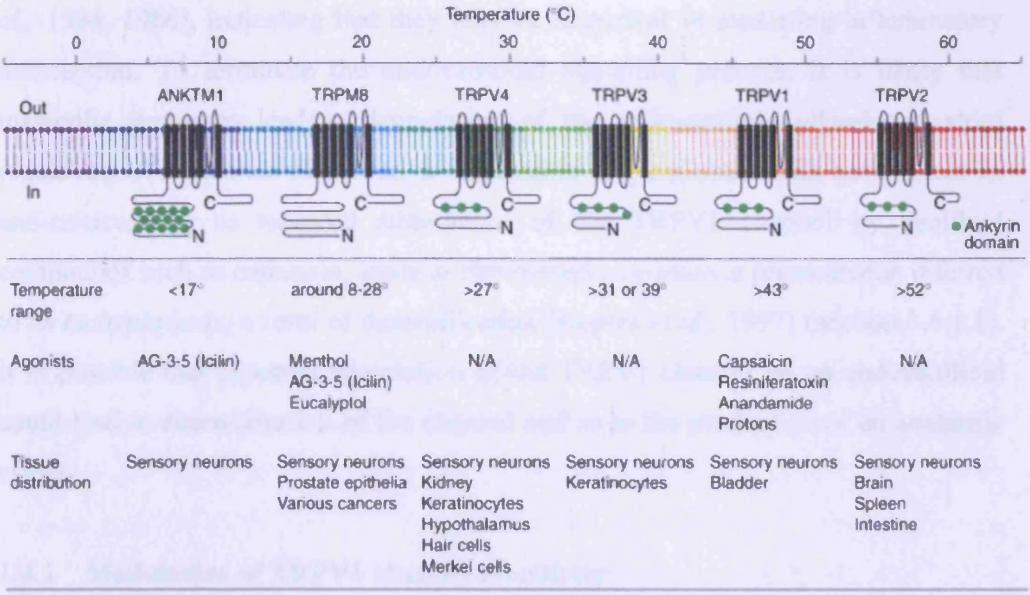


Figure 1.4: Diagram to show the various types of mammalian thermosensitive ion channels. The channels are arranged according to their thermal activation thresholds as measured in heterologous expression systems. Known agonists and modulators of each ion channel are listed as well as the various ion channel tissue distributions [Jordt *et al.*, 2003].

Priestley, 1999, Guo *et al.*, 1999], and co–localises with TRKA, TRKB, IB4, $P2X_3$, CGRP, and substance P [Guo *et al.*, 1999, Michael & Priestley, 1999].

The tissue distribution of TRPV1 outside the nervous system suggests that under normal physiological conditions, when TRPV1 is unlikely to be activated by noxious heat or low pH, there are endogenous ligands of the TRPV1 channel to carry out its as yet undefined purpose. Evidence in favour of this hypothesis is slowly emerging and potential candidates for endovanilloids include the endocannabinoid ananadamide, some lipoxygenase products of arachidonic acid (12- and 15(S)-hydroperoxyeicosatetraenoic (HPETE) acids, 5- and 15(S)-hydroxyeicosatetraenoic (HETE) acids, and leukotriene B_4 (LTB_4)), and N-arachidonoyl-dopamine (NADA) [Van der Stelt & Di Marzo, 2004]. It is envisaged that endovanilloids are synthesised within cells and are released in sufficient amounts to evoke a TRPV1-mediated response. In line with this, various lipoxygenase products and anandamide have been shown to activate the TRPV1 current in TRPV1 expressing HEK cells and cultured rat DRG neurons [Hwang *et al.*, 2000, Jerman *et al.*, 2002]. The latter observation is particularly interesting as lipoxygenase products are produced during inflammation [Samuelsson, 1983] and cause hyperalgesia when injected intradermally [Levine *et*

al., 1984, 1986], indicating that they may be important in mediating inflammatory nociception. To terminate the endovanilloid signalling process, it is likely that metabolic processes lead to degradation of the endovanilloid allowing a strict regulation of its action. However, it is possible that endovanilloids have a role in anti-nociception, as repeated stimulation of the TRPV1 channel by vanilloid compounds such as capsaicin, leads to diminished responses, a phenomenon referred to as *tachyphylaxis*, a form of desensitisation [Koplas *et al.*, 1997] (section 1.4.1.1). It is possible that repeated stimulation of the TRPV1 channel by an endovanilloid could lead to desensitisation of the channel and so to the production of an analgesic effect.

1.4.1 Modulation of TRPV1 channel sensitivity

The ability of primary afferent fibres to adapt to specific stimuli is of paramount importance in the functioning of the nervous system under both normal and pathophysiological conditions. The process of desensitisation or *tachyphylaxis* allows a neuron to diminish its overall response to signals of a chemical, physical, or electrical nature. This is of particular relevance when considering how noxious heat, protons, and capsaicin directly excite primary afferent fibres to elicit pain sensation, and how capsaicin acts as a potent neurotoxin leading to cell death during prolonged cellular exposure [Caterina *et al.*, 1997]. As TRPV1 responses are subject to desensitisation, it could be viewed that this acts as a protective mechanism to prevent cellular damage during normal physiological functioning through excessive TRPV1 stimulation. In contrast, under pathophysiological conditions, TRPV1 appears to enter a sensitised state, in which, there is an enhancement of TRPV1-mediated responses. It is this sensitisation of TRPV1 channel function that is thought to underlie thermal sensitivity in animals [Hu *et al.*, 2002], and in which several inflammatory mediators are thought to have a role.

1.4.1.1 Desensitisation of TRPV1 channel function

There are two types of TRPV1 desensitisation manifest in cells. The first, referred to as *tachyphylaxis*, results in a diminution of the maximal response amplitude during successive stimulation of the TRPV1 channel. This phenomenon is apparent in DRG neurons during sequential heat pulses of the same temperature

(noxious range) [Schwarz *et al.*, 2000, Galoyan *et al.*, 2003], and upon successive deliveries of the same concentration of capsaicin [Cholewinski *et al.*, 1993, Liu & Simon, 1996, Koplas *et al.*, 1997]. In addition, tachyphylaxis has been reported in TRPV1 expressing HeLa (human adenocarcinoma-derived cell line) [Mohapatra & Nau, 2005] and HEK [Numazaki *et al.*, 2003] cells during repeated stimulation with protons. The second type of desensitisation, referred to as *acute desensitisation*, is produced during prolonged stimulation of the TRPV1 channel, and results in an inactivation of the response despite the continued presence of the stimulus. This type of desensitisation can be observed in DRG neurons during the continuous exposure to either noxious heat [Schwarz *et al.*, 2000] or capsaicin [Liu & Simon, 1994, Koplas *et al.*, 1997, Mohapatra & Nau, 2005], and can be seen in TRPV1 expressing HeLa cells during prolonged proton exposure [Mohapatra & Nau, 2005]. The mechanisms behind such desensitisation processes have been investigated and the findings indicate that the heat-induced desensitisation may result from a different pathway to the capsaicin or proton-induced desensitisation.

Evidence suggests that capsaicin and proton-induced desensitisation (tachyphylaxis and acute desensitisation) of the TRPV1 channel is calcium-dependent, as it is abolished in the absence of extracellular calcium [Liu & Simon, 1996, Koplas *et al.*, 1997, Numazaki *et al.*, 2003], and by intracellular calcium chelators such as BAPTA or EGTA [Koplas *et al.*, 1997]. As desensitisation of the TRPV1 channel can last for up to 40 minutes [Cholewinski *et al.*, 1993], this would suggest the occurrence of a calcium-dependent modification of the channel or association with a regulatory protein that would render the channel "inactive" for prolonged periods. Indeed evidence is now coming to light to suggest that the TRPV1 channel associates with the calcium binding protein, calmodulin [Numazaki *et al.*, 2003, Rosenbaum *et al.*, 2004]. Rosenbaum and colleagues have shown using the patch clamp technique on TRPV1 expressing *Xenopus* oocytes, that a calcium / calmodulin complex contributes to the desensitisation of capsaicin-evoked currents, by reducing the open channel probability [Rosenbaum *et al.*, 2004]. The binding of calmodulin to TRPV1 occurs at, at least two distinct binding sites within the TRPV1 protein in a calcium-dependent manner. These include a region of ~30 amino acids in the amino terminus [Rosenbaum *et al.*, 2004], and a region of ~35 amino acids in the carboxy terminus [Numazaki *et al.*, 2003]. Disruption of the latter region has been shown to result in a TRPV1 deletion mutant, that when expressed in HEK cells,

exhibits currents, which are not susceptible to capsaicin and proton-induced tachyphylaxis, despite the presence of extracellular calcium [Numazaki *et al.*, 2003]. Therefore, it has been hypothesised that calmodulin acts as a calcium sensor for TRPV1 channels, and modulates channel gating in response to increases in intracellular calcium.

TRPV1 is also a target for calcineurin (protein phosphatase 2B) [Mohapatra & Nau, 2005] and protein kinases [Premkumar & Ahern, 2000, Numazaki *et al.*, 2002, Bhave *et al.*, 2003, Mandadi *et al.*, 2004, Bhave & Gereau IV, 2004]. ^{32}P incorporation experiments have shown that TRPV1 is highly phosphorylated in the resting state, and that phosphorylation is significantly reduced by a desensitising concentration of capsaicin [Bhave *et al.*, 2002]. As calcineurin is a calcium-dependent phosphatase, which acts to dephosphorylate the TRPV1 channel, it is not surprising that it has been shown to promote tachyphylaxis and acute desensitisation of capsaicin or proton activated currents in TRPV1 transfected cells [Mohapatra & Nau, 2005] and DRG cells [Docherty *et al.*, 1996]. In contrast, protein kinase A (PKA) [Bhave *et al.*, 2002, Mohapatra & Nau, 2003], protein kinase C (PKC) [Numazaki *et al.*, 2002, Bhave *et al.*, 2003, Mandadi *et al.*, 2004], and Ca^{2+}/calmodulin dependent kinase II (CaMK II) [Jung *et al.*, 2004] have all been shown to maintain TRPV1 in a sensitised state and / or rescue TRPV1 from a desensitised state by phosphorylation of the channel. Therefore, TRPV1 dephosphorylation correlates with receptor desensitisation, while TRPV1 phosphorylation reduces desensitisation.

In contrast to capsaicin- and proton-induced desensitisation of TRPV1, the desensitisation witnessed in response to heat stimulation appears to be via a calcium-independent mechanism [Vyklický *et al.*, 1999, Schwarz *et al.*, 2000]. At present, the mechanism underlying this desensitisation is unclear. However, as mentioned previously, TRPV1 can form heteromers and homomers, which could exhibit different properties. Indeed, two distinct types of TRPV1 have been described based on different structure-activity relations for ligand binding and channel opening, as well as desensitisation mechanisms: resinferatoxin-like (R-type) and capsaicin-like (C-type) [Acs *et al.*, 1996, 1997]. In addition, the vanilloid conductances in voltage clamped neurons have been shown to exhibit heterogeneity [Liu & Simon, 1996, Peterson *et al.*, 1996]. Therefore, the existence of distinct multimeric TRPV1

channels with differing properties could explain the different desensitisation mechanisms.

One potential calcium-independent mechanism of desensitisation is the induction of heat shock proteins. It has been shown that extended exposure of DRG neurons to elevated temperatures induces an enhanced synthesis of the 70kDa heat shock protein (Hsp70), which leads to a reduced sensitivity to subsequent thermal stress [Amin *et al.,* 1996]. This protein can inactivate extracellular signal-regulated protein kinases (ERK) and c-Jun N-terminal kinases (JNK) [Lee *et al.,* 2005], and activate mitogen activated protein (MAP) kinase phosphatase-1 (MKP-1) expression [Lee *et al.,* 2005]. These processes contribute to protecting cells from apoptosis during heat shock. As well as activating MKP-1, Hsp70 can increase calcineurin (phosphatase 2B) activity through a calmodulin-dependent mechanism [Someren *et al.,* 1999]. Therefore, it could be viewed that dephosphorylation of TRPV1 due to increased levels of various phosphatases, might underlie heat-induced desensitisation.

1.4.1.2 Sensitisation of TRPV1 channel function

There is now a growing body of evidence to suggest that the TRPV1 channel activity can increase or become *sensitised* during nociceptive processes by the action of inflammatory mediators. The action of prostaglandins (section 1.5.2), ATP (section 1.4.1.2.1), bradykinin (section 1.4.1.2.2), and NGF (section 1.4.1.2.3) can all lead to sensitisation of the TRPV1 channel. In addition, TRPV1 channel expression can become upregulated (section 1.4.1.2.4), a process, which contributes to nociceptor sensitisation. In the following sections, the influence of inflammatory mediators on TRPV1 channel function shall be considered together with the implications of TRPV1 upregulation.

1.4.1.2.1 ATP

It is now apparent that a functional interaction exists between ATP and TRPV1, as ATP-induced thermal hypersensitivity is abolished in TRPV1 knockout mice [Moriyama *et al.,* 2003]. In line with this, ATP has been shown to produce a PKC-dependent potentiation of the proton- or capsaicin-induced inward currents, in TRPV1 transfected HEK293 cells, and also in rat DRG neurons [Tominaga *et al.,*

2001]. In addition, ATP has been observed to lower the temperature threshold for heat activation of TRPV1, from temperatures above 42°C down to 35°C, such that normally non-painful thermal stimuli become capable of activating the TRPV1 channel [Tominaga *et al.*, 2001]. Therefore, ATP can sensitise TRPV1 currents evoked by three different stimuli: capsaicin, protons, and heat.

Whilst the work of Tominaga and colleagues points to a role for $P2Y_1$ in TRPV1 sensitisation [Tominaga *et al.*, 2001], Moriyama and co-workers have shown that there is only co-expression between $P2Y_2$ and TRPV1 mRNA, and not $P2Y_1$ and TRPV1 mRNA in rat DRG neurons [Moriyama *et al.*, 2003]. Furthermore, in $P2Y_1$-deficient mice, ATP-induced thermal hypersensitivity was preserved, and ATP-mediated potentiation of the capsaicin-evoked inward current was still present in DRG neurons from $P2Y_1$ deficient mice. A role for $P2Y_2$ has been suggested because the ATP-induced sensitisation of capsaicin-evoked currents, and the ATP-induced thermal hyperalgesia in mice could be mimicked by UTP (a semi-selective agonist for $P2Y_2$ and $P2Y_4$ receptors) and blocked by suramin (a selective $P2Y_2$ antagonist) [Moriyama *et al.*, 2003]. Taken together, these observations indicate that ATP-mediated activation of either $P2Y_1$ and / or $P2Y_2$ receptor subtypes cause sensitisation of TRPV1 channel function, and could represent the existence of a mechanism other than $P2X_3$ (section 1.3.5) activation, whereby extracellular ATP might cause pain.

The breakdown product of ATP, adenosine, has the opposing effect on TRPV1 function. Puntambekar and co-workers have shown that adenosine analogues inhibit both capsaicin-induced calcium entry and capsaicin-induced inward currents in HEK / TRPV1 and DRG cells [Puntambekar *et al.*, 2004]. These findings imply that adenosine acts as an endogenous inhibitor of TRPV1 function and provides another control, whereby the body could rein in the sensitisation process.

1.4.1.2.2 Bradykinin

The principal action of bradykinin is to lower the temperature threshold for TRPV1 channel activation. In TRPV1 and B_2 receptor expressing HEK cells and rat DRG neurons, bradykinin lowers the temperature threshold from 42°C to 24-30.6°C in a PKC dependent manner [Sugiura *et al.*, 2001]. Bradykinin also increases capsaicin-evoked ^{45}Ca uptake via activation of the B_2 receptor in rat DRG neurons,

indicating that TRPV1 channel activation is enhanced by this ligand [Tang *et al.*, 2004]. The mechanism underlying the TRPV1 sensitisation has been shown to be dependent upon the presence of cyclooxygenases, and phospholipases C and A2, which would suggest that bradykinin indirectly sensitises TRPV1 through activation of prostaglandins [Tang *et al.*, 2004]. In addition to activating PG metabolism, Shin and colleagues have shown that bradykinin excites sensory neurons by increasing TRPV1 activity via the production of 12-lipoxygenase metabolites of AA [Shin *et al.*, 2002]. These findings suggest that bradykinin can sensitise the TRPV1 channel via a number of distinct pathways.

Another feature of bradykinin-mediated sensitisation of TRPV1 is the ability of bradykinin to increase the proportion of cells that respond to capsaicin or protons [Stucky *et al.*, 1998]. This is important as any increase in the number of responsive neurons that innervate inflamed tissue could contribute to hyperalgesia via spatial summation onto spinal neurons.

1.4.1.2.3 Neurotrophins

Neurotrophins can be divided into two major families. The nerve growth factor (NGF) family, including NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and NT4/5, act through tyrosine kinase receptors (TRK) and p75 receptors to mediate their effects [Kaplan & Miller, 1997]. The second family, the glial cell-line derived neurotrophic factor (GDNF) family, includes GDNF, neurturin, artemin, and persephin [Enomoto, 2005]. These neurotrophins signal through a receptor complex, consisting of RET, a tyrosine kinase that is encoded by the *c-ret* proto-oncogene, and the glycosyl phosphatidyl-inositol-anchored cell surface protein (GDNF family receptor (GFR) alpha) [Snider, 1994, Millan, 1999, Julius & Basbaum, 2001]. Both families are critical for the development and survival of various cell types, including small diameter nociceptive neurons [Snider, 1994]. As discussed in section 1.2.1, nociceptive neurons can be divided into peptidergic and non-peptidergic phenotypes. The peptidergic neurons rely strongly on the presence of NGF for their development, whereas the non-peptidergic neurons are developmentally dependent on GDNF [Snider, 1994, Millan, 1999]. In addition, the *in vivo* and *in vitro* functional properties of these nociceptive neurons are strongly dependent on the presence of neurotrophins [Winter *et al.*, 1988, Winston *et al.*,

2001]. Therefore, if the neurotrophin levels become altered during a pathophysiological response, the function of the various ion channels / receptors that are expressed in such nociceptors could be affected.

In inflamed tissue, the levels of NGF become elevated [Weskamp & Otten, 1987, Amaya *et al.,* 2004]. As NGF produces thermal hyperalgesia in rodents [Lewin *et al.,* 1993, Shu *et al.,* 1999, Sammons *et al.,* 2000], and a hypersensitivity to noxious heat stimuli in humans [Petty *et al.,* 1994], this would suggest an interaction between NGF and TRPV1. Indeed, NGF has been shown to sensitise both the capsaicin-evoked calcium transients in mouse DRG neurons [Bonnington & McNaughton, 2003], and the capsaicin-evoked currents in rat DRG neurons [Shu & Mendell, 1999, 2001]. Bonnington and McNaughton have provided evidence to suggest that the mechanism underlying this NGF-mediated sensitisation could involve activation of phosphatidylinositol-3-kinase (PI3K), PKC, and CaMK II [Bonnington & McNaughton, 2003]. In addition to NGF, other neurotrophins could also have a role in TRPV1 sensitisation. Both BDNF and NT4/5 have been shown to produce thermal hyperalgesia in rats [Shu *et al.,* 1999], and enhance capsaicin-evoked currents in rat DRG neurons [Shu & Mendell, 1999]. Another recent observation has shown that GDNF, as well as NGF, potentiates capsaicin-, anandamide-, and arachidonyl–2-chloroethylamide-evoked CGRP release in trigeminal ganglion neurons [Price *et al.,* 2005].

1.4.1.2.4 TRPV1 channel upregulation

Evidence is now accumulating to suggest that TRPV1 is upregulated during inflammation [Ji *et al.,* 2002, Amaya *et al.,* 2003, Amaya *et al.,* 2004]. Amaya and colleagues have shown using immunocytochemistry that TRPV1 expression is restricted to small unmyelinated C-fibre neurons in normal rats, but is increased significantly following the induction of FCA-induced peripheral inflammation [Amaya *et al.,* 2003]. Furthermore, in inflammation, the authors observed co-localisation of TRPV1 with NF200 (marker of myelinated fibres) [Amaya *et al.,* 2003], indicating that TRPV1 expression patterns change during inflammation to encompass myelinated A-fibres as well as C-fibres. As mentioned in section 1.2.1, the myelinated A-fibres include type II neurons, which participate in noxious heat sensation. Therefore, an upregulation of TRPV1 in C-fibres, together with an

induction of TRPV1 expression in A-fibres, could represent an important mechanism that causes thermal hyperalgesia following inflammation.

As mentioned previously, the regulation of TRPV1 expression following inflammation has been shown to be dependent upon the presence of neurotrophins [Ji *et al.*, 2002, Amaya *et al.*, 2004]. NGF appears to be necessary for TRPV1 upregulation in medium-sized A-fibres, whereas TRPV1 upregulation in C-fibres requires the presence of GDNF [Amaya *et al.*, 2004]. Although the mechanisms that underlie neurotrophin-mediated TRPV1 upregulation remain unresolved, evidence does suggest a role for downstream signalling molecules, Ras [Xing *et al.*, 1998, Bron *et al.*, 2003] and the mitogen-activated protein kinase (MAPK), P38 [Ji *et al.*, 2002]. As NGF increases TRPV1 protein but not mRNA expression in rat DRG [Tohda *et al.*, 2001, Ji *et al.*, 2002], such downstream signalling molecules could be responsible for promoting gene transcription. Indeed, activation of P38 by NGF has been shown to phosphorylate the transcription factor CREB (cAMP response element binding protein) [Xing *et al.*, 1998]. Therefore it has been postulated that following inflammation, NGF released from damaged tissue is taken up by peripheral nerve terminals and is retrogradely transported to the cell body of the DRG. Within the DRG, NGF via the Ras/Rho cascade triggers P38 MAPK to activate transcription factors, which in turn increase TRPV1 expression. Once translocated to the peripheral nociceptor terminal, increased TRPV1 can then contribute to the maintenance of inflammatory heat hypersensitivity [Ji *et al.*, 2002].

1.5 MOLECULAR MECHANISMS OF PERIPHERAL SENSITISATION BY PROSTAGLANDINS

As a number of PGs have the ability to produce mechanical and thermal hyperalgesia (section 1.3.2.2), significant research has been focussed on deciphering the mechanism(s) by which they evoke peripheral sensitisation of nociceptor nerve terminals. The work in this thesis has been concerned with the sensitising effects of PGE_2 on nociceptors. Therefore, the following sections will discuss the way in which this prostaglandin produces peripheral sensitisation. The primary targets for PGE_2 are voltage-gated ion channels located on nociceptor terminals, and it is changes in the properties of these, which largely underlies the mode of action of this important

inflammatory mediator. Another key mechanism, by which this prostaglandin is believed to contribute to peripheral sensitisation of nociceptors, is via an enhancement of other ion channel or chemical mediator-evoked responses underlying noxious thermal and mechanical sensation.

1.5.1 Peripheral sensitisation by PGE_2-mediated modulation of voltage-gated ion channels

Since nociceptive sensory neurons function by converting noxious thermal, chemical, and mechanical stimuli into graded depolarisations, which initiate action potential firing and nerve conduction to the spinal cord, it is not surprising that inflammatory mediators such as PGE_2 are able to modulate various voltage-activated currents underlying the action potential. In the following subsections, the pathophysiological role of sodium, potassium, and calcium currents in nociception shall be considered, together with their modulation by PGE_2.

1.5.1.1 Modulation of sodium channel activity

The hyperalgesic effect produced by PGE_2 has been characterised in C-fibre nociceptors as a decrease in mechanical threshold and an increase in the number of action potentials produced in response to sustained threshold stimuli [Chen *et al.*, 1999b]. This increase in action potential firing has been attributed to the tetrodotoxin-resistant (TTX-R) Na^+ channel in the membrane of nerve endings [Gold *et al.*, 1996, Bhave & Gereau, 2004, Lai *et al.*, 2004]. To date, two TTX-R Na^+ channel, α subunits have been cloned, $Na_v1.8$ (PN3 or sensory neuron specific (SNS)) and $Na_v1.9$ (NaN or SNS2) [Akopian *et al.*, 1996, Dib-Hajj *et al.*, 1998, 1999]. Both subunits are preferentially expressed in small calibre C-fibres, and a small proportion of myelinated A-fibre DRG neurons, and are usually co-expressed with TRPV1 and each other [Akopian *et al.*, 1996, Dib-Hajj *et al.*, 1998, Fjell *et al.*, 1999, Amaya *et al.*, 2000]. As compared to other Na^+ channels, the $Na_v1.8$ current activates at relatively depolarised potentials and exhibits slow activation and deactivation kinetics, [Akopian *et al.*, 1996], whereas the $Na_v1.9$ current slowly activates close to the membrane potential and is relatively persistent [Cummins *et al.*, 1999, Priest *et al.*, 2005]. The two subunits are thought to participate in nociceptor

action potential generation, sustained repetitive firing, and resting membrane potential of the peripheral neuron [Renganathan *et al.*, 2001].

There is increasing evidence to suggest that following carrageenan or FCA-induced inflammation, the $Na_v1.8$ channel subunit is upregulated in small diameter DRG neurons projecting to the inflamed limb [Waxman *et al.*, 1999, Black *et al.*, 2004]. Following sciatic nerve chronic constriction injury, however, there is redistribution of the $Na_v1.8$ channel subunit from small diameter DRG neurons to sciatic nerve axons at the injury site [Novakovik *et al.*, 1998, Porreca *et al.*, 1999]. Such alterations in $Na_v1.8$ expression suggest a role in nociception. In line with this, intrathecal administration of specific antisense oligodeoxyonucleotides against the $Na_v1.8$ channel subunit (to knock down channel expression in SC, DRG, and peripheral nerve terminals) can reverse both neuropathic [Lai *et al.*, 2002] and inflammatory [Khasar *et al.*, 1998] pain states in rats. In addition, behavioural observations in transgenic $Na_v1.8$-null mice support a role for the channel in inflammatory pain [Akopian *et al* 1999]. This is reinforced by the observation that DRG neurons from transgenic $Na_v1.8$ (+/+) mice elicit high amplitude, all-or-nothing action potentials, compared to the smaller more graded responses recorded from $Na_v1.8$-null (-/-) mice [Renganathan *et al.*, 2001].

The inflammatory mediators serotonin, adenosine, and PGE_2 can all modulate the $Na_v1.8$ current via GPCR activation [England *et al.*, 1996, Gold *et al.*, 1996, Cardenas *et al.*, 1997]. The acute administration of PGE_2 to DRG neurons results in an increase in the TTX-R current amplitude, produces a hyperpolarizing shift of the activation curve, and causes an increase in the activation and inactivation rates of the slowly inactivating TTX-R current in these cells [England *et al.*, 1996, Gold *et al.*, 1996]. All of these effects increase the excitability of the neuron by decreasing the threshold for activation and increasing the likelihood of action potential generation, which culminates in an increased response to a fixed stimulus. This effect appears to be dependent on TTX-R Na^+ channel phosphorylation by PKA [England *et al.*, 1996, Fitzgerald *et al.*, 1999]. Indeed, PKA inhibitors injected intradermally into the rat hindpaw prevent the persistence of behavioural mechanical hyperalgesia induced by PGE_2 [Aley & Levine, 1999]. However, an alternative mechanism has also been proposed, which involves TTX-R Na^+ channel modulation by PKC [Gold *et al.*, 1998]. Upon inhibition of PKC, the PKA-induced enhancement of the TTX-R Na^+ current is blocked, indicative of PKC activity being necessary for PKA-mediated

modulation of the TTX-R Na^+ current. A similar requirement has been reported in brain type IIA channels, where it has been demonstrated that PKC-induced phosphorylation of the channel at serine 1506 is necessary to enable PKA-phosphorylation at other sites on the channel protein [Li *et al.*, 1993].

To date, there is limited evidence to suggest a role for $Na_v1.9$ in nociception, other than the knowledge that it is expressed in small diameter neurons of the DRG that bind the isolectin B4 [Dib-Hajj *et al.*, 1998, Fjell *et al.*, 1999]. However, it has recently been shown using $Na_v1.9$-null mice that the $Na_v1.9$ current contributes significantly to thermal hyperalgesia induced by peripheral inflammation [Priest *et al.*, 2005]. Furthermore, it is becoming apparent that the $Na_v1.9$ current can be modulated by PGE_2. In line with this, PGE_2-induced thermal hyperalgesia is almost absent in $Na_v1.9$-null mice [Priest *et al.*, 2005], and at the cellular level, application of PGE_2 leads to an increase in the $Na_v1.9$ current in DRG neurons from both $Na_v1.8$-null mice and wild-type mice [Rush & Waxman, 2004]. Additionally, the modulation of $Na_v1.9$ by PGE_2 has been shown to be sensitive to pertussis toxin [Rush & Waxman, 2004, Priest *et al.*, 2005], indicating that the underlying mechanism is via a G_i coupled receptor. As the $EP_{3\alpha}$ and $EP_{3\beta}$ receptor subtypes are the only receptors that fit this criterion (section 1.3.2.3.1), it seems likely that one or both of these could be involved in the modulation of the $Na_v1.9$ current. It is interesting that the modulation of the $Na_v1.8$ current should occur via a PKA-dependent mechanism, implicating a role for a G_s-coupled receptor such as $EP_{2/4}$. However, if PKC is also required, then it is more likely that a PLC coupled receptor, such as EP_1 could be involved (section 1.3.2.3.1). Currently, it appears that there could be a complex interplay of prostaglandin receptors in the modulation of $Na_v1.8$ and $Na_v1.9$ currents in neuronal excitability, an answer for which will remain elusive until selective prostaglandin receptor ligands become available.

1.5.1.2 Modulation of potassium channel activity

Another way in which PGE_2 can alter excitability of sensory neurons is by targeting potassium channels. In general, potassium currents repolarise neurons, contribute to the resting membrane potential, spike frequency adaptation, and regulate excitability. As a result, suppression of their function would be expected to enhance neuronal excitability by inhibiting hyperpolarisation of the nerve terminal

membrane, during peripheral sensitisation. There is a whole array of voltage-gated K^+ channels (K_v) or *shaker* proteins [Mathie *et al.*, 1998], and specific classes of DRG neuron express distinct combinations of K_v channels [Rasband *et al.*, 2001]. In small diameter DRG neurons, $K_v1.4$ is the sole K_v1 subunit present, implying that homomeric $K_v1.4$ channels predominate in Aδ and C fibres. The fact that $K_v1.4$ is co-expressed with TRPV1, CGRP, IB4, and $Na_v1.8$ shows that these neurons are of a nociceptor phenotype [Rasband *et al.*, 2001, Vydyanathan *et al.*, 2005]. In large myelinated DRG neurons, $K_v1.1$ and $K_v1.2$ are expressed at high levels, suggesting the formation of heteromers in these neurons. All types of K_v channels are downregulated following spinal nerve injury, an effect, which would be expected to result in neuronal hyperexcitability [Rasband *et al.*, 2001, Kim *et al.*, 2002].

The action of PGE_2 on voltage-gated K^+ currents in dorsal root ganglion neurons is still not completely understood. At least two types of voltage-dependent I_K currents have been identified in these neurons, a slowly inactivating delayed-rectifier type K^+ current (I_{DR}), and a rapidly inactivating A-type current (I_A) [Kameyama, 1983, Mayer & Sugiyama, 1988]. The former is attributed to high levels of expression of $K_v1.1$, $K_v1.2$, and $K_v\beta2.1$, whereas the latter has properties that correspond well to homotetrameric assembly of $K_v1.4$ subunits [Rasband *et al.*, 2001]. PGE_2 has been shown to inhibit an outward K^+ current in DRG neurons that is characteristic of I_{DR} [Nicol *et al.*, 1997, Evans *et al.*, 1999]. The mechanism underlying this inhibition appears to be via a cAMP-dependent pathway leading to K^+ channel phosphorylation. This is because analogues of cAMP mimic the actions of PGE_2, and PKA inhibitors abolish the suppressive effects of PGE_2 [Evans *et al.*, 1999]. This is interesting as the subunits responsible for this type of current are located in larger, non-nociceptive cells. However, it is believed that prostaglandins still might affect the rapidly inactivating component because PGE_2 can block it at voltages, which would be expected to give rise to significant amounts of rapidly inactivating I_A [Nicol *et al.*, 1997]. Furthermore, whilst mice lacking the $K_v1.1$ subunit exhibit hyperalgesia [Clark & Tempel, 1998], the PGE_2-mediated suppression of K^+ currents in $K_v1.1$ knockout sensory neurons remains intact [Jiang *et al.*, 2003]. Therefore, it has been suggested that $K_v1.1$ is not involved in peripheral sensitisation but instead plays a role in central nociceptive processes [Rasband *et al.*, 2001].

Whilst PGE_2 clearly has a well-documented suppressive effect on voltage-gated K^+ channels, it has also been reported to mediate a similar action on a type of K^+ channel that is activated by a rise in cytosolic calcium and underlies the slow afterhyperpolarisation (sAHP) current (I_{sAHP}) [Gold *et al.*, 1996b, Sah & Faber, 2002]. The sAHP current is one of three components, which contribute to an afterhyperpolarisation that follows action potentials specifically in neuronal cells. It rises to a peak over several hundred milliseconds, can last for up to 2 seconds after the action potential has ceased, and underlies the phenomenon of spike frequency adaptation. The other two components are the fast AHP (fAHP) and medium AHP (mAHP). The former is activated immediately during the action potential, lasts several tens of milliseconds, and contributes to membrane repolarisation. The latter is activated immediately following the action potential (<5ms), decays over several hundred milliseconds, and controls neuronal firing frequency. The significance of the afterhyperpolarisation is to control neuronal excitability by holding the membrane at more hyperpolarised potentials in order to prevent the generation of multiple action potentials during normal physiological functioning.

PGE_2 is known to inhibit the sAHP current (I_{SAHP}) in adult rat nodose ganglia [Fowler *et al.*, 1985] and DRG [Gold *et al.*, 1996b] neurons, thereby facilitating action potential regeneration. Gold and colleagues only observed the effect of PGE_2 on I_{SAHP} in about half of the DRG neurons sensitised by the prostanoid, and found that PGE_2 also increased the excitability of neurons not expressing the sAHP component [Gold *et al.*, 1996b]. Taken together, this would suggest that whilst modulation of the sAHP current is not critical, it does make a contribution to PGE_2-induced neuronal sensitisation. To date it has not been possible to fully elucidate, which channels underlie the I_{sAHP}. However, one possibility is the heterologous assembly of small (SK) or intermediate (IK) conductance Ca^{2+}-activated potassium channel (K_{Ca}) subunits [Sah & Faber, 2002], which have been localised immunocytochemically to non-peptidergic small diameter rat DRG neurons [Mongan *et al.*, 2005].

1.5.1.3 Modulation of calcium channel activity

Mounting evidence suggests that specific calcium channel subtypes (L, N P/Q, R, and T-type) selectively regulate cellular functions. In primary sensory

neurons, high voltage activated (HVA) N, P/Q, and R type channels modulate the release of nociceptive neurotransmitters [Tsien *et al.*, 1988], whereas low voltage activated (LVA) T-type channels generate low threshold spikes, singling them out as perfect candidates for burst firing and synaptic excitation associated with hypersensitive states [White *et al.*, 1989]. Their role in nociception is supported by the fact that both HVA (N, P/Q, R type) and LVA (T type) calcium currents, and their channel proteins predominate in small- and medium-sized DRG neurons [Scroggs & Fox, 1992, Talley *et al.*, 1999, Altier & Zamponi, 2004, Wu & Pan, 2004]. In addition, behavioural observations from knockout mice implicate N- and R-type channels in inflammatory pain states [Saegusa *et al.*, 2000, 2002]. This is supported by the occurrence of N-type channel upregulation in the DRG following carrageenan-induced peripheral inflammation [Yokoyama *et al.*, 2003]. Therefore, calcium channels represent another target for inflammatory prostaglandins in the modulation of peripheral nociceptive transmission.

Opioids produce their analgesic effects by attenuation of N, P/Q, and R-type HVA calcium currents at PAF central terminals [Bhave & Gereau, 2004, Yokoyama *et al.*, 2004]. Therefore, it could be viewed that in order for PGE_2 to produce pronociceptive effects, it would instead, have to increase the HVA I_{Ca}. Indeed, PGE_2 has been reported to increase the HVA I_{Ca} in embryonic avian sensory neurons [Nicol *et al.*, 1992]. However, there is also substantial evidence to suggest that PGE_2 actually inhibits the HVA I_{Ca} in rat sympathetic neurons [Ikeda, 1992, Ito *et al.*, 2000], bovine adrenal chromaffin cells [Currie & Fox, 2000], rat melanotrophs [Tanaka *et al.*, 1998], and mouse trigeminal ganglion neurons [Borgland *et al.*, 2002]. The individual HVA I_{Ca} components modulated by PGE_2 in the various cell types have been dissected out using various calcium channel blockers. In trigeminal sensory neurons, which are involved in the transmission of nociceptive information from the face and mouth, PGE_2 appears to target the N and P/Q-type currents [Borgland *et al.*, 2002]. The mechanism underlying this PGE_2-mediated effect is believed to be dependent upon EP_3 receptor (section 1.3.2.3.1) activation.

As the effect of PGE_2 on HVA I_{Ca} appears to be inhibitory, there are conflicting views as to whether PGE_2 produces pro- or anti-nociceptive effects through modulation of this channel. One hypothesis to support a pro-nociceptive role of PGE_2 is that reduced calcium entry via HVA Ca^{2+} channels could indirectly inhibit

the calcium-dependent afterhyperpolarisation. This would allow neurons to attain higher frequencies of action potentials and lead to an enhanced neuronal excitability.

1.5.2 PGE_2-derived peripheral sensitisation via enhancement of non-voltage activated ion channel or chemical mediator-evoked responses underlying noxious thermal and mechanical sensation

Studies have shown that prostaglandins have little excitatory action of their own on primary afferent fibres [Chahl & Iggo, 1977, Grubb *et al.*, 1991]. However, there is now abundant evidence to support the role of prostaglandins in the sensitisation of sensory neurons to chemical mediators of inflammation, such as bradykinin (section 1.3.2.2). In human subjects, injection of bradykinin into the skin has been shown to cause pain [Ferreira, 1972, Kindgen-Milles, 1995]. When preceded by an intracutaneous injection of PGE_1 [Ferreira, 1972] or PGE_2 [Kindgen-Milles, 1995] however, this bradykinin-induced pain is greatly enhanced. Similarly, an intraperitoneal injection of PGE_2 enhances the writhing response in mice, induced by a sub-nociceptive dose of bradykinin [Walter *et al.*, 1989]. Taken together, these behavioural observations indicate the occurrence of nociceptor sensitisation by the E-series prostaglandins. Supporting evidence from *in vivo* recordings of sensory nerve activity have shown that continuous infusion of PGE_1 [Chahl & Iggo, 1977] or intra-arterial injection of PGE_2 [Mense, 1981, Schaible & Schmidt, 1988, Birrell *et al.*, 1993] increases the frequency of bradykinin-elicited action potentials recorded from afferents belonging to the saphenous nerve and the medial articular nerve respectively. The significance of these observations is that an increase in afferent firing would facilitate the transmission of nociceptive information.

Sensitisation of bradykinin responses by PGE_2 has also been observed in isolated sensory neurons. In embryonic cultured rat DRG neurons, PGE_2 pre-treatment enhances three-fold the number of bradykinin-evoked action potentials, without altering the neuronal depolarisation elicited by bradykinin [Nicol & Cui, 1994]. In addition, PGE_2 can amplify the bradykinin-induced intracellular calcium responses [Smith *et al.*, 2000]. This is an interesting observation, as the calcium responses produced during repeated applications of bradykinin are known to be subject to desensitisation [Burgess *et al.*, 1989]. However, in the presence of PGE_2, this desensitisation ceases and indeed the responses are amplified compared to the

original response, indicating the occurrence of sensitisation. In relation to normal physiological functioning, the process of desensitisation represents an effective way for the prevention of excessive neuronal firing and transmission of nociceptive information. During nociceptive processing however, the increased production and interaction of inflammatory mediators, directly and indirectly activates sensory neurons to increase neuronal firing frequencies, leading to pain. An additional effect of PGE_2 is that it enhances the proportion of neurons that respond to bradykinin [Stucky *et al.*, 1996, Smith *et al.*, 2000]. Any increase in the number of nociceptors that respond to a stimulus would thereby contribute to hyperalgesia by spacial summation at the level of spinal neurons.

The mechanism underlying prostaglandin-mediated sensitisation of bradykinin-evoked responses appears to be driven by a PKA-dependent pathway as it is mimicked by cAMP analogues and blocked by adenylate cyclase or PKA inhibitors [Cui & Nicol, 1995, Smith *et al.*, 2000]. This suggests that the sensitisation is not mediated at the level of the bradykinin receptor or its ability to activate PLC. In line with this, Smith and colleagues observed that PGE_2 had no effect on bradykinin-stimulated [^{3}H] IP_3 formation [Smith *et al.*, 2000]. Therefore, it appears that PGE_2-mediated sensitisation of bradykinin responses could involve the activation of EP_2, $EP_{3B/C}$, or EP_4 receptors as they all couple through G_s. It has been hypothesised that the activation of G_s coupled receptors by PGE_2 would lead to the phosphorylation of various ion channels, thereby altering neuronal excitability (section 1.5) [Smith *et al.*, 2000].

An additional target for PGE_2-mediated sensitisation is the TRPV1 receptor. It has been shown in embryonic cultured DRG neurons that PGE_2 produces a two- to threefold enhancement of the capsaicin-evoked inward current via a PKA-dependent mechanism [Lopshire & Nicol, 1998]. This PGE_2-mediated sensitisation of TRPV1 is transient even in the continuous presence of PGE_2. A detailed examination of the response has revealed that the duration of sensitisation produced by PGE_2 is dependent upon the intracellular calcium concentration, with low intracellular calcium concentrations facilitating PGE_2-mediated sensitisation of the TRPV1 current and high concentrations limiting PGE_2-mediated sensitisation [Lopshire & Nicol, 1997]. In line with this, intracellular calcium buffering inhibits TRPV1 receptor tachyphylaxis and acute desensitisation [Koplas *et al.*, 1997] (section 1.4.1.1), and allows PGE_2 to produce maximal sensitisation of the TRPV1 current

[Lopshire & Nicol, 1997]. In addition, the cGMP pathway influences sensitisation of TRPV1 by PGE_2 as inhibition of nitric oxide synthase and PKG lengthen the duration of sensitisation of the capsaicin-evoked inward current [Lopshire & Nicol, 1997]. The significance of this is clear during an inflammatory response as activation of the cGMP pathway, and hence PKG could have an important role in suppressing sensitisation to noxious stimuli, thereby providing control over PAF sensitisation.

PGE_2-induced modulation of TRPV1 has also been studied by examining the effect of PGE_2 on $[Ca^{2+}]_i$ using Ca^{2+} sensitive dyes. Gu and colleagues have shown in vagal sensory neurons that repeated applications of capsaicin generates reproducible calcium transients, which in the presence of PGE_2 increase substantially in amplitude, indicating the occurrence of sensitisation [Gu *et al.,* 2003]. The mechanism underlying this sensitisation is again believed to be PKA-dependent. Activation of the $mGlu_5$ receptor (section 1.6.2) also produces a dramatic sensitisation of the capsaicin-evoked calcium transient in mouse cultured DRG neurons [Hu *et al.,* 2002]. The $mGlu_5$ receptor is coupled to the PLC pathway and generally to the activation of PKC. However, PKC inhibitors had no effect on $mGlu_5$-mediated sensitisation. Instead, inhibitors of PKA blocked the sensitisation, indicating the involvement of this kinase. This is somewhat confusing, as it indicates that activation of G_s is required rather than $G_{q/11}$. Since activation of $mGlu_5$ leads to the PLC-mediated metabolism of phosphatidylinositol bis phosphate (PIP_2) to the second messengers inositol triphosphate (IP_3), and diacylglycerol (DAG), it has been hypothesised that $mGlu_5$ activation could lead to the generation of arachidonic acid (AA) from DAG via the DAG-lipase pathway. Metabolism of AA by the COX pathway would subsequently lead to the generation of prostaglandins (section 1.3.2.1), which could underlie the observed TRPV1 sensitisation. Indeed, pre-treatment of the DRG cultures with indomethacin (inhibitor of COX), prevented sensitisation of the capsaicin-induced calcium responses by $mGlu_5$ activation, indicating the involvement of COX enzymes in the $mGlu_5$-mediated sensitisation process.

It is envisaged that prostaglandins produced via the DAG lipase pathway are released by the nerve terminal and activate G_s-coupled prostaglandin receptors in an autocrine fashion, leading to the generation of adenylate cyclase and the PKA-dependent phosphorylation / sensitisation of TRPV1 (figure 1.5). In support of these findings, intraplantar injection of an $mGlu_{1/5}$ receptor agonist produces thermal

hypersensitivity in mice, which is abolished in the presence of PKA inhibitors, indomethacin or aspirin [Hu *et al.*, 2002].

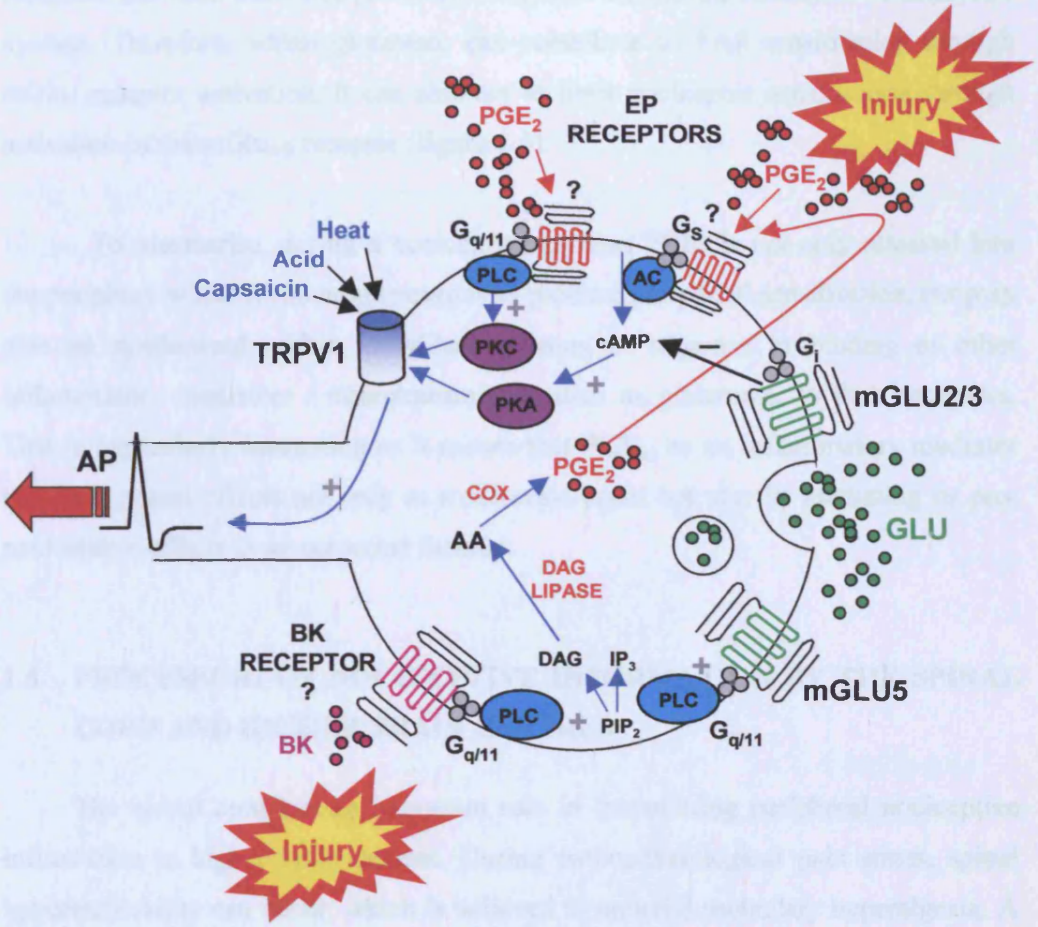


Figure 1.5: Schematic diagram showing the signalling pathways involved in the sensitisation of the TRPV1 receptor in sensory neurons. During an inflammatory insult, glutamate released from vesicles within nerve terminals or from nearby damaged cells can activate mGlu5 or mGlu2/3 receptors. Activation of mGlu5 leads to the production of IP3 and DAG. The action of DAG lipase on DAG leads to the production of AA and subsequently PGE_2 by the action of COX enzymes. PGE_2 produced from the conversion of AA or via release from damaged cells can then act at unidentified EP receptors to activate PKA or PKC, thereby phosphorylating the TRPV1 receptor, leading to sensitisation. However, glutamate can also activate the mGlu2/3 receptor, which through G_i inhibits cAMP production and so suppresses sensitisation of TRPV1. BK could have a potential role in the sensitisation of TRPV1 by activating a similar pathway to mGlu5 [adapted from Hu *et al.*, 2002].

A study by Yang and Gereau has shown that group II mGlu receptors have a role in anti-nociception [Yang & Gereau, 2002]. In mouse DRG neurons, application of an $mGlu_{2/3}$ receptor agonist blocked the PGE_2-mediated potentiation of capsaicin-induced calcium responses [Yang & Gereau, 2002]. Consistent with this, behavioural

observations in mice have shown that subcutaneous injection of an $mGlu_{2/3}$ receptor agonist inhibits PGE_2-induced thermal hyperalgesia without affecting normal thermal sensation. The inhibition of PGE_2-induced sensitisation via activation of $mGlu_{2/3}$ receptors has been attributed to the activation of G_i and the inhibition of adenylate cyclase. Therefore, whilst glutamate can contribute to PAF sensitisation through $mGlu_5$ receptor activation, it can also act to limit nociceptor sensitisation through activation of the $mGlu_{2/3}$ receptor (figure 1.5).

To summarise, during a nociceptive process PGE_2 is not only released into the periphery where it can target neurons to produce peripheral sensitisation, but may also be synthesised within individual neurons in response to binding of other inflammatory mediators / neurotransmitters such as glutamate, to their receptors. This is particularly interesting as it means that PGE_2, as an inflammatory mediator can have potent effects not only as a paracrine agent but also by mediating its pro-nociceptive effects in an autocrine fashion.

1.6 PROCESSING OF NOCICEPTIVE INFORMATION BY THE SPINAL CORD AND HIGHER BRAIN CENTRES

The spinal cord has an important role in transmitting peripheral nociceptive information to higher brain centres. During pathophysiological pain states, spinal hyperexcitability can occur, which is believed to underlie secondary hyperalgesia. A number of neurotransmitters are involved in this process including glutamate and neuropeptides. However, evidence suggests that spinal prostaglandins also play a part. The following sections shall consider the process of spinal hyperexcitability, the transmission of nociceptive information to higher brain centres, spinal cord modulation of pain, and the role of spinal prostaglandins.

1.6.1 Spinal cord structure

The spinal cord may be divided into four segments, cervical, thoracic, lumbar, and sacral regions, and consists of an outer covering of white matter and an inner structure of grey matter [Rexed, 1982]. The grey matter can be divided into 10 laminae (figure 1.6), to which small and large calibre fibres, mediating nociceptive

and non-nociceptive information respectively, project to from the periphery [Rexed, 1982, Dubner & Bennett, 1983]. Within each of these areas, there are discrete types of dorsal horn neurons, which include the *nociceptive-specific* (NS), *wide dynamic range* (WDR), and *non-nociceptive* (Non-N) type neurons [Dubner & Bennett, 1983, Cervero, 1995, Schaible & Grubb, 1993, Millan 1999]. The NS neurons are concentrated in laminae I and II_o, and are typically silent, responding exclusively to noxious stimuli mediated by C and Aδ fibres. The WDR neurons respond in a graded fashion to both noxious and non-noxious stimuli and are found predominantly in lamina V but also in laminae IV and VI. Lastly, the Non-N neurons respond to non-nociceptive information carried by Aβ fibres, and are found primarily in laminae II, III, and IV.

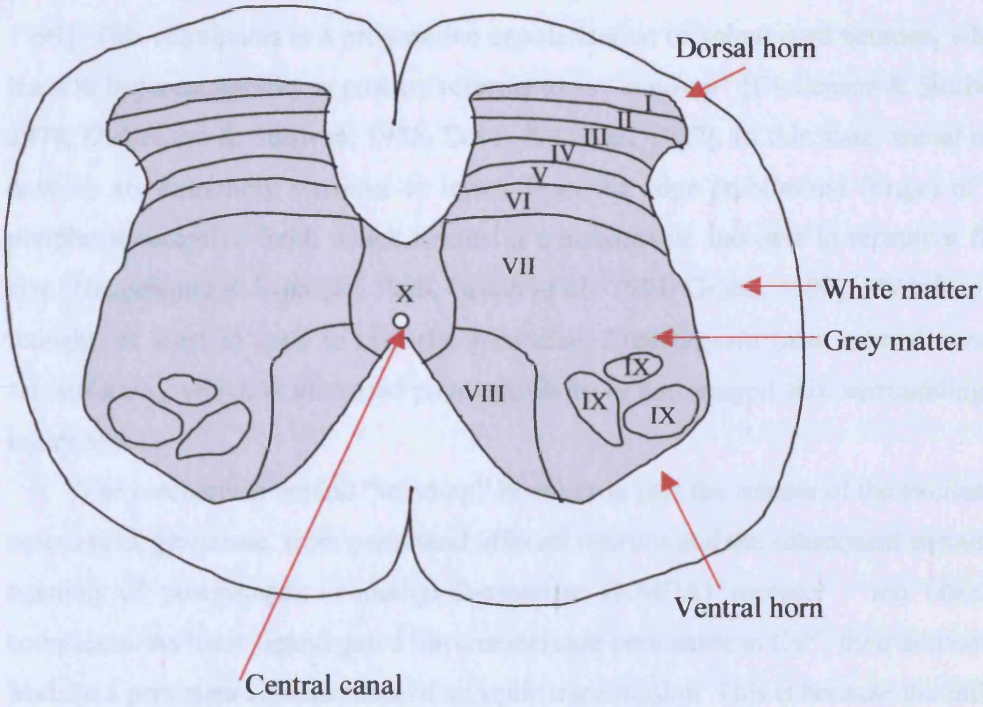


Figure 1.6: Internal organisation of the lumbar spinal cord. The spinal cord is made up of white and grey matter. The white matter contains ascending and descending nerve fibres, whereas the grey matter consists of nerve cell bodies, their dendrites and synaptic connections. The grey matter can be divided into a number of distinct laminae. Laminae I (marginal layer), II (substantia gelatinosa), III and IV (nucleus proprius), V and VI (deep layers) comprise the spinal dorsal horn, VII corresponds to the intermediate grey matter, XIII and IX make up the spinal ventral horn, and X surrounds the central canal. Nociceptive fibres predominantly innervate laminae I, II, V, VI, and X.

1.6.2 Mechanisms of central sensitisation

Dorsal horn neurons each have a receptive field, which corresponds to the region of skin from which they receive afferent input. To put this into perspective, acute noxious stimulation (physiological) of C and Aδ fibre afferents within the receptive field, leads to the generation of sub-threshold responses in dorsal horn neurons. These responses are referred to as excitatory postsynaptic potentials (EPSPs) and may summate if the stimulus is sufficiently intense or prolonged [Grubb, 1998, Millan, 1999]. Under pathophysiological conditions, sensitised PAFs result in increased afferent firing and the EPSPs summate more readily. Summation occurs because the threshold for triggering firing is reduced such that dorsal horn neurons are activated by normally sub-noxious stimulus intensities. In addition, the dorsal horn neuron response magnitudes become greater in duration and intensity [Woolf & King, 1990, Dougherty *et al.*, 1992, Baranauskas & Nistra, 1998, Millan, 1999]. This culminates in a progressive depolarisation of spinal cord neurons, which leads to hyperexcitability; a process referred to as *"wind-up"* [Dickenson & Sullivan, 1978, Dickenson & Sullivan, 1986, Davis & Lodge, 1987]. In this state, spinal cord neurons are extremely sensitive to inputs from the edge (subliminal fringe) of the peripheral receptive field, which results in a measurable increase in receptive field size [Neugebauer & Schaible, 1990, Grubb *et al.*, 1993, Grubb, 1998]. "Wind-up" is thought, at least in part, to underlie *secondary hyperalgesia* (also termed *central sensitisation*), which is increased pain sensitivity in undamaged skin surrounding an injury site.

The mechanism behind "wind-up" involves in part the release of the excitatory amino acid, glutamate, from peripheral afferent neurons and the subsequent sustained opening of postsynaptic *N*-methyl-*D*-aspartate (NMDA) receptor / ion channel complexes. As these ligand-gated ion channels are permeable to Ca^{2+}, their activation leads to a persistent enhancement of synaptic transmission. This is because the influx of Ca^{2+} acts to depolarise cellular membranes and facilitates activation of various kinases, including CaMK II and PKC, an effect, which leads to further NMDA receptor activation [Ben-Ari *et al.*, 1992, Millan, 1999]. Thus, intrathecal administration of specific NMDA receptor antagonists inhibits wind up [Davis & Lodge, 1987, Dickenson & Sullivan, 1990], reduces the size of enlarged receptive fields [Ren *et al.*, 1992], and reduces behavioural hyperalgesia [Yaksh, 1989].

The activation of NMDA receptors by glutamate requires the removal of a voltage-dependent Mg^{2+} block. This can be achieved through the glutamate-induced activation of α-amino-3-hydroxy-5-methyl-isoxazole-4-proprianate (AMPA) / kainate, receptor / ion channel complexes. These receptors are predominantly permeable to Na^{+} and their activation leads to the generation of rapid EPSPs [Yoshimura & Jessell, 1990]. Through gradual summation, these EPSPs provide the depolarising shift in voltage, necessary for relief of the NMDA receptor Mg^{2+} block, which culminates in the occurrence of slower synaptic potentials that are characteristic of NMDA receptors [Mayer *et al.*, 1984]. At present, there is controversy as to whether the AMPA / kainate receptor is able to sustain NMDA receptor activation during persistent pain states [Hunter & Singh, 1994, Hao & Xu, 1996, Chen *et al.*, 1999]. This is because AMPA receptors are subject to rapid desensitisation kinetics [Ballerini *et al.*, 1995, Fletcher & Lodge, 1996] and are downregulated under conditions of peripheral inflammation [Pellegrini-Giampietro *et al.*, 1994]. Instead, emerging evidence suggests the involvement of metabotropic glutamate receptors and neuropeptide receptors in facilitating NMDA receptor activation.

Glutamate can activate three distinct groups of metabotropic glutamate receptor. The group I metabotropic glutamate receptors (mGluRs) are comprised of $mGlu_1$ and $mGlu_5$ and are positively coupled to phospholipase C. Conversely, the group II ($mGlu_2$ and $mGlu_3$) and the group III receptors ($mGlu_4$ and $mGlu_{6-8}$) are negatively coupled to adenylate cyclase [Millan, 1999]. In the spinal cord, the group I receptors are concentrated in the superficial dorsal horn [Valerio *et al.*, 1997], which makes them obvious candidates to modulate nociceptive input from small calibre primary afferent fibres. In line with this, spinal blockade of the $mGlu_1$ receptor [Young *et al.*, 1997], or its ablation by intrathecal antisense administration [Young *et al.*, 1998] produces anti-nociceptive effects. Similarly, $mGlu_5$ selective antagonists applied spinally reduce thermal hyperalgesia [Dogrul *et al.*, 2000, de Novellis *et al.*, 2004], cold hypersensitivity [Hama, 2003], and spinal cord neuronal cell loss, which is associated with neuropathic pain states [de Novellis *et al.*, 2004]. The activation of group I mGlu receptors in the spinal cord dorsal horn increases neuronal excitability [Millan, 1999, Zhong *et al.*, 2000] and facilitates responses to NMDA and AMPA receptor activation [Bleakman *et al.*, 1992, Cerne & Randić, 1992, Zhong *et al.*, 2000].

Neuropeptides (section 1.3.7) co-localise with glutamate in small calibre nociceptive fibres that project to the dorsal horn of the spinal cord [Battaglia & Rustioni, 1988, Miller *et al.,* 1993]. During noxious thermal or mechanical stimulation, these are also released into the synapse, and make an important contribution to the induction of dorsal horn hypersensitivity [Schaible *et al.,* 1990, Hope *et al.,* 1990, Neugebauer *et al.,* 1996]. Through activation of the various neuropeptide receptors present in the spinal dorsal horn, each neuropeptide acts to induce slow membrane depolarisations in spinal neurons [Ryu *et al.,* 1988, Parker & Grillner, 1996, King *et al.,* 1997], and facilitates NMDA receptor activation. In addition, they promote a further release of glutamate and each other from primary afferent fibres [Kangrga & Randic, 1990, Schaible *et al.,* 1992, Hu *et al,* 1997], an effect, which exacerbates spinal sensitisation processes.

Whilst the mechanism underlying NMDA receptor facilitation via the activation of metabotropic glutamate receptors and neuropeptide receptors is partly explained by the production of slow membrane depolarisations in the spinal cord (eventually summating to promote NMDA receptor Mg^{2+} block removal), it is not fully understood. Evidence suggests the involvement of group I mGlu receptor and $NK_{1/2}$ (section 1.3.7) receptor-induced PLC signalling [Millan, 1999]. It is now well established that PLC leads to an IP_3-induced increase in $[Ca^{2+}]_i$ and the production of DAG, which synergistically promote activation of PKC. One of the actions of this kinase is the induction of NMDA receptor phosphorylation [Yamakura & Shimoji, 1999], a process that is closely related to pain hypersensitivity in the spinal cord [Brenner *et al.,* 2004]. Together with the IP_3-induced rise in $[Ca^{2+}]_i$, NMDA receptor phosphorylation contributes to relieving the voltage-dependent NMDA receptor Mg^{2+} block [Chen & Huang, 1992], and increases NMDA receptor / channel open probability [Xiong *et al.,* 1998]. In support of this, Guo and colleagues have shown using rat spinal cord slice preparations that group I mGluRs, IP_3, and PKC are all required for inflammation induced tyrosine phosphorylation of the NMDA receptor [Guo *et al.,* 2004].

1.6.3 Transmission of nociceptive information to higher brain centres and the influence of descending pain pathways

Noxious information is only perceived as pain following transmission to higher brain centres via dorsal horn projection neurons (PNs), which are comprised of WDR, NS, and NON-N neuronal types. PNs can be monosynaptically (directly) activated by PAFs, or polysynaptically (indirectly) activated by excitatory interneurons (EXINs) that receive input from PAFs [Price *et al.*, 1978, Dubner & Bennett, 1983, Millan, 1999]. They constitute five major ascending pathways: the *spinothalamic, spinoreticular, spinomesencephalic, cervicothalamic,* and *spinohypothalamic* tracts [Millan, 1999]. Whilst each pathway terminates in various central locations, the thalamus is still considered as the crucial relay station where nociceptive information is processed. It encodes information concerning the type, temporal pattern, and intensity of pain. Projections from the thalamus to the primary somatosensory cortex enable the body to produce a response of appropriate sensory, motor, and sympathetic output [Millan, 1999].

The presence of dorsal horn inhibitory interneurons (ININs) can limit the transmission of nociceptive information to supraspinal centres and thereby decrease pain. This is important in the transmission of normal physiological pain messages and forms the neuroanatomical pathways that explain the *gate control theory* [Melzack & Wall, 1965]. According to this view, ININs principally localised in the substantia gelatinosa (figure 1.6), act to inhibit the transmission pathway through the release of opioids, GABA, acetylcholine, and glycine, which are the major neurotransmitters involved in anti-nociception [Coggeshall & Carlton, 1997, Todd & Spike, 1993, Millan, 1986, 1999]. Activation of ININs occurs by descending inhibitory neurons or by non-nociceptive afferent input, which results in pain perception being less intense or even absent. Conversely, nociceptive C and Aδ fibre input inhibits ININs, so that persistent C-fibre activity facilitates excitation of the transmission cells by either nociceptive or non-nociceptive inputs. This auto-facilitation causes successive bursts of activity in the nociceptive afferents to become increasingly effective in activating transmission neurons, resulting in an intense pain experience.

A region of the midbrain called the *periaqueductal grey* (PAG), which receives direct input from the spinal DH and is interconnected with the

hypothalamus, thalamus, and cortex, has a pivotal role in modulating pain. It does this by activating distinct neuronal groups, which comprise various descending pathways [Millan, 2002]. Fibres from these, run down to the *rostroventromedial medulla* (RVM), which is a heterogeneous region incorporating the *nucleus raphe magnus, nucleus reticularis gigantocellularis, nucleus gigantocellularis pars alpha,* and the *nucleus reticularis paragigantocellularis lateralis* and is located in the brainstem. The RVM can respond to noxious stimuli in different ways. One subset of neurons, collectively referred to as *"on–cells"* act to increase their firing pattern such that spinal nociceptive transmission is facilitated (descending facilitation), whereas another group, the *"off–cells"* exhibit decreased activity and are believed to inhibit spinal nociceptive transmission (descending inhibition) [Millan, 2002]. The descending pathways are most notably noradrenergic and serotonergic, and project to the superficial dorsal horn and deep dorsal horn laminae of the spinal cord. Here, an action on PAFs, ININs, EXINs and PNs can modulate further nociceptive transmission such that pain perception is facilitated or inhibited.

1.6.4 Spinal involvement of prostaglandins

Prostaglandins are produced within the spinal cord during nociceptive processes. In support of this, strong electrical stimulation of the frog hindlimb has been shown to provoke the release of E-series and F-series prostaglandins into the spinal superfusion fluid [Ramwell *et al.,* 1966]. Following this early observation, a variety of other peripheral stimuli have been identified, which also evoke the release of PGs in the spinal cord. Noxious thermal stimulation (50°C) increases the levels of PGE_2, but not $PGF_{2\alpha}$ or 6-keto $PGF_{1\alpha}$ (metabolic product of PGI_2) in the rat lumbar spinal cord, whereas innocuous stimulation (35°C) has no effect [Codderre *et al.,* 1990]. In addition, the development of acute inflammation following injection of carrageenan / kaolin [Yang *et al.,* 1996, Ebersberger *et al.,* 1999], FCA [Hay *et al.,* 1997], or formalin [Malmberg & Yaksh, 1995], induces the spinal synthesis and release of PGE_2.

The increases in spinal PG levels that occur during acute nociceptive stimulation imply a role for PGs in spinal pain. In line with this, the direct application of PGE_2 [Taiwo & Levine, 1988, Uda *et al.,* 1990, Minami *et al.,* 1994, 1999, Turnbach *et al.,* 2002b], $PGF_{2\alpha}$ [Minami *et al.,* 1994b, Turnbach *et al.,* 2002b],

and PGD_2 [Uda *et al.*, 1990] to the spinal cord results in nociceptive behavioural responses in rodents, which include thermal and mechanical hyperalgesia, and touch-evoked allodynia. Furthermore, intrathecal administration of many non-selective NSAIDs (section 1.3.2.1), reduce paw flinching in rats injected with formalin into the hindpaw [Malmberg & Yaksh, 1992]. This provides indirect evidence to suggest that increased spinal PG production induced following peripheral inflammation could underlie the observed behavioural hyperalgesia. It is worthy of note, that spinal NSAIDs may only be effective in producing analgesia in models of peripheral inflammation, as they have not been shown to be effective in reducing responses to noxious thermal stimuli in the normal animal [Malmberg & Yaksh, 1992]. In support of this, an electrophysiological study in the anaesthetised rat has shown that spinal neurons receiving nociceptive input (mechanical) are typically unresponsive to spinally administered NSAIDs, unless the spinal cord has been rendered hyperexcitable by repetitive C-fibre stimulation; a situation with some similarity to afferent input in inflammation [Willingale *et al.*, 1997].

It is not fully understood how spinal PGs produce hyperalgesia and allodynia. However, intrathecal administration of NMDA, AMPA / kainate, or mGlu receptor antagonists reduce PGE_2-induced thermal and mechanical hyperalgesia in conscious rats and mice [Nishihara *et al.*, 1995, Minami *et al.*, 1997, Turnbach & Randlich, 2002]. In addition, PGE_2-induced mechanical hyperalgesia (but not thermal hyperalgesia) in the rat [Turnbach & Randlich, 2002] and PGD_2-induced thermal hyperalgesia in the mouse [Uda *et al.*, 1990] are attenuated following spinal application of NK_1 receptor antagonists. Therefore, PGs may exert their spinal effects via a presynaptic action at glutamate and / or SP receptors, leading to an augmentation of glutamate and SP release. In line with this, PGE_2 has been shown to evoke SP release from cultured avian [Nicol *et al.*, 1992] and rat [Smith *et al.*, 2000] sensory neurons, and glutamate release from rat spinal cord synaptosomes [Nishihara *et al.*, 1995b]. As substance P [Matsumura *et al.*, 1985, Takahasi *et al.*, 1987] and the excitatory amino acids NMDA, quisqualate, and kainic acid [Aanonsen & Wilcox, 1987, Raigorodsky & Urca, 1987] produce nociceptive behaviours following intrathecal application, the PG-evoked increase in these neurotransmitters could underlie the nociceptive behaviour induced by spinal PGs. An alternative mechanism is a PG-induced modification of glutamate and substance P postsynaptic receptors. Indeed, it has been shown using knockout mice that two subunits of the NMDA

receptor (ε1 and / or ε4) are important in the PGE_2 mode of action, as mice lacking these subunits exhibit no signs of PGE_2-induced hyperalgesia [Minami *et al.*, 1997].

1.6.4.1 Prostaglandin receptors in the CNS

A number of prostaglandin receptors have been detected in the CNS. In the rat spinal cord, mRNAs for EP_1, EP_2, $EP_{3\alpha}$, $EP_{3\beta}$, EP_{3B}, and EP_4 have all been identified utilising RT-PCR [Donaldson *et al.*, 2001]. In line with this, *in situ* hybridisation [Kawamura *et al.*, 1997] and immunocytochemistry studies [Beiche *et al.*, 1998, Nakamura *et al.*, 2000] performed in rats have localised the EP_2 and EP_3 receptors to laminae I-II of the spinal dorsal horn, which is the main region involved in relaying nociceptive information to higher brain centres. In the brain, various PG receptors have been localised to specific areas by *in situ* hybridisation [Båtshake *et al.*, 1995, Sugimoto *et al.*, 1994, Oida *et al.*, 1995, Zhang & Rivest, 1999, Engblom *et al.*, 2000, 2004] and immunocytochemistry [Nakamura *et al.*, 1999, 2000, 2001, Ek *et al.*, 2000] in the mouse and rat species. For the EP_1 [Båtshake *et al.*, 1995], EP_3 [Sugimoto *et al.*, 1994, Nakamura *et al.*, 1999, 2000, Ek *et al.*, 2000], and EP_4 [Zhang & Rivest, 1999] receptors, these include the preoptic area of the hypothalamus, which is associated with thermoregulatory / febrile responses and sleep induction. EP_3 [Ek *et al.*, 2000, Engblom *et al.*, 2000, 2004] and EP_4 [Engblom *et al.*, 2000, 2004] have been localised to the parabrachial nucleus, EP_2 [Zhang & Rivest, 1999] and EP_3 [Sugimoto *et al.*, 1994, Ek *et al.*, 2000, Nakamura *et al.*, 2000, 2001] to the locus coeruleus, and EP_3 to the thalamus, periaqueductal grey, and caudal raphé nuclei [Sugimoto *et al.*, 1994, Ek *et al.*, 2000, Nakamura *et al.*, 2000], all of which have a role in nociceptive processing. In addition, the IP receptor has been localised to the thymic medulla [Oida *et al.*, 1995], indicating that the IP receptor might be an important regulator of the immune system.

1.7 AIMS OF THE PROJECT

As a vast array of evidence exists that implicates a major role for prostaglandins in the progression of nociceptive states, it is imperative that the distribution and function of prostaglandin receptors within the peripheral and central nervous system is better understood. If this can be achieved, it will ultimately help

identify new therapeutic targets for the development of novel pain treatment strategies.

1.7.1 Distribution of EP and IP receptor subtypes

It is envisaged that by utilising selective antibodies raised against the EP and IP receptor subtypes, immunocytochemical techniques will enable a distribution map for each receptor subtype to be obtained in spinal cord and dorsal root ganglion sections. Once this is achieved, localisation studies will further characterise the receptor distribution, and should establish whether the prostaglandin receptors are expressed in peptidergic or non-peptidergic nociceptors.

1.7.2 Determination of prostaglandin receptor function

To determine the function of individual prostaglandin receptors within the rat nervous system, Ca^{2+} imaging shall be utilised to study 1) the PGE_2-induced calcium responses in rat DRG cells and 2) the sensitising effects of PGE_2 on capsaicin-induced calcium responses (section 1.5.2). This will represent a suitable methodology for the low-throughput screening of novel selective EP receptor agonists and antagonists developed by industrial collaborators Glaxo SmithKline (GSK). To date there has been a severe shortage of selective prostaglandin receptor compounds. Therefore, this will enable for the first time, an exploration into the different roles played by the different prostaglandin receptor subtypes in the mediation of peripheral sensitisation.

Chapter Two

Materials and Methods

2.1 REAGENTS AND SOLVENTS

Analytical or higher-grade reagents were obtained from Sigma-Aldrich Chemical Company, Fisher Scientific, or BDH Laboratory supplies. Deionised double-distilled water was obtained from a MilliQ system. The absolute ethanol and dimethyl sulphoxide (DMSO) (Sigma-Aldrich) solvents that were used in the preparation of some of the stock solutions were also of analytical or higher grade. All solutions were prepared by adding the required mass of reagent to a clean volumetric flask and making up to concentration with the required diluent.

2.2 PRIMARY ANTIBODIES

As the different prostaglandin EP receptor subtypes all show a high degree of homology at the amino acid level, there has been a lack of selective antibodies produced for each receptor subtype. An additional problem has been the question mark over the specificity of the commercially available prostaglandin receptor antibodies. Therefore, all primary antibodies were subject to specificity tests, including Western Blotting (section 2.4) and immunocytochemistry experiments (section 2.5), utilising stably transfected human prostaglandin receptor (EP_1, EP_2, EP_{3I}, and EP_4) expressing cell lines, kindly donated by Glaxo SmithKline (GSK) (Stevenage) (see section 2.3).

The antibodies that were tested came from a variety of suppliers. An array of in-house manufactured, rabbit polyclonal antibodies were a kind gift from GSK, raised against each of the prostaglandin receptor subtypes (EP_1, EP_2, EP_3, and EP_4) (table 2.1). Rabbit polyclonal antibodies raised against EP_1 (101740), EP_2 (101750), EP_3 (101760), and EP_4 (101775) receptors were sourced from Cayman Chemical Company (table 2.2). In addition, EP_2 (EP21-A) and EP_3 (EP31-A) receptor, rabbit polyclonal antibodies were sourced from Alpha Diagnostics Ltd. Finally, peptide sequences from the human EP_1 and EP_{3C} (same as rat EP_{3B}) receptors, which were

conserved in the rat and were dissimilar to other prostaglandin receptor subtypes were identified using a database search (Vector, align X, and ExPaSy) and were submitted to an antibody manufacturer, Pepceuticals for antibody generation in rabbit hosts (table 2.3).

Receptor	**Peptide Sequence**	**Rabbit numbers**
EP_1 N-terminus	SPYGPLNLSLAGEATTC	R273/292
EP_1 C-terminus	CEASSLRSSRHSGLSHF	R274/293
EP_2 N-terminus	GNASNDSQSEDC	R264/290
EP_2 C3 loop	CGPGARRRGERVSMAEETDC	R279/298
EP_2 C-terminus	CSTQSDASKQADL	R265/272
EP_3 N-terminus	KETRGYGGDAPFC	R280/299
EP_3 C3 loop	CRAKATASQSSAQWGRITTEC	R310/315
EP_3 C-terminus	CSSTLMWSDHLER	A22/A23
EP_4 C3 loop	CRMHRQFMRRTSLGTEQHHAAC	R311/319
EP_4 C-terminus	CDTTSLRTLRISETSDSSQGQC	R320/316
EP_4 C-terminus	CVTFPSETLNLSEKYI	R267/286

Table 2.1: Table showing prostaglandin receptor antibodies provided by GSK. Details of the peptide sequence against which the antibody was raised and the location of the peptide sequence within the receptor are provided. It is worthy of note that two rabbits were injected with each peptide.

Receptor	**Peptide Sequence**	**Species cross reactivity**
EP_1 (C-terminal)	GLTPSAWEASSLRSSRHSGLSHF (Residues: 380-402)	Human, baboon, murine, rat
EP_2 (C-terminal)	SLRTQDATQTSCSTQSDASKQADL (Residues: 335-358)	Human, murine, rat
EP_3 (between loop 6 and 7)	NQTSVE HCKTHT EKQKECNF (Residues: 308-327)	Human, murine, rat
EP_4 (C-terminal)	GSGRAGPAPKGSSLQVTFPSETLNLSEKCI (Residues: 459-488)	Human, murine, rat

Table 2.2: Table showing prostaglandin receptor antibodies obtained from Cayman chemical company. Details of the human peptide sequence against which the antibody was raised, the location of the peptide sequence within the receptor, and the species cross reactivity are provided. The red amino acid residues represent those that differ between the rat and human species.

Receptor	Peptide Sequence	Species cross reactivity	Rabbit number
EP_1 (between loop 3 and 4)	CTRPLLHAARVSVARAR (Residues: 139-155)	Human, rat	R13/R14
EP_{3C} (C-terminal tail)	GQKGQPISLSNE (Residues: 371-388)	Human, rat	R15/R16

Table 2.3: Table showing prostaglandin receptor antibodies obtained from Pepceuticals. Details of the human peptide sequence against which the antibody was raised, the location of the peptide sequence within the receptor, and the species cross reactivity are provided. The red amino acid residues represent those that differ between the rat and human species. It is worthy of note that the human EP_{3C} receptor is similar to the rat EP_{3B} receptor subtype.

2.2.1 Enzyme-Linked Immunosorbent Assay (ELISA)

The Pepceutical's antibodies collected from the rabbits, 3-months after the peptide injection, were tested using ELISAs. Plates (96-well, NUNC) were coated with 50µl of peptide (1µgml^{-1}) and incubated overnight at 4°C. The plates were washed with 0.05% Tween-Phosphate Buffered Saline (PBS) (PBS composition in mM: NaCl 137, KCl 2.7, KH_2PO_4 1.4, Na_2HPO_4 8.1, pH 8) three times and subsequently blocked with 0.5% Casein / 10% Calf Serum / PBS (pH 8) for 1 hour at room temperature, to ensure that non-specific binding was inhibited. The primary antibodies (10^{-1} to 10^{-7} dilutions of neat antibody / preimmune serum) were made up in PBS (pH 8) and applied (50µl per well) for 2 hours at room temperature. Addition of the peptide (used to immunise the rabbits) to the antibodies was at a concentration of 10µgml^{-1}. The plates were washed three times with 0.05% Tween-PBS prior to incubation with 50µl secondary antibody; swine anti-rabbit IgG (1:1000) (Bayer) in 0.05% Tween-PBS / 1% Calf serum / PBS (pH 8) for 1 hour at room temperature. The plates were then subjected to a further three washes with 0.05% Tween-PBS and incubated with 50µl rabbit peroxidase anti-peroxidase (1:1000) (Bayer) in 0.05% Tween-PBS / 1% Calf serum / PBS (pH 8) for 1 hour at room temperature. Following a final washing stage with 0.05% Tween-PBS (x3), positive wells were identified by the development of blue colouration following the application of 50µl of substrate (10mgml^{-1} 3,3', 5,5'-tetramethylbenzidine (Bayer) in 0.1 M sodium acetate / sodium citrate (pH 6), 0.0045% H_2O_2) for 10 minutes. The reaction was terminated with 1M hydrochloric acid and the plates were read within 30 minutes on a plate reader at a wavelength of 420nm.

2.3 MAINTENANCE OF CONTINUOUS CELL CULTURE CELLS

GSK kindly donated human prostaglandin receptor expressing cell lines. EP_1, EP_2, and EP_{3I} (similar to rat EP_{3A}) were all stably expressed in CHO (Chinese hamster ovary)-K1 cells and EP_4 was stably expressed in HEK (human embryonic kidney)-293 cells [Wilson *et al.*, 2006]. These were grown in DMEM / F12 with glutamax I (Gibco BRL) containing 10% foetal calf serum (Gibco BRL), 0.5mgml^{-1} geneticin (Calbiochem), and 1% penicillin/streptomycin (5000 units.ml^{-1}, Gibco BRL). Each cell line was grown in 175cm^2 (T175) tissue culture flasks. After 2-3 days the cells were ready to be passaged (70-80% confluent). The media was removed and the cells were incubated in 0.02% EDTA solution without calcium or magnesium (Sigma) for 5 minutes. This procedure was necessary because the proteins that anchor the cells to the flask require calcium and magnesium and in their absence, the cells do not stick to the bottom of the flask. To dislodge the cells, the flasks were given a series of sharp taps and the resulting cell suspension was triturated to avoid cell clumping. The CHO cells were passaged at a dilution of 1:20-1:40 and the HEK cells at a dilution of 1:10.

Wild type CHO K1 and HEK 293 cells were grown in MEM-α medium (Gibco BRL) containing 10% foetal calf serum (Gibco BRL), and 1% penicillin/streptomycin. These cells were also passaged at 3 days but at a 1:10 dilution for the WT CHO cells and at a 1:5 dilution for the WT HEK cells.

To ascertain whether the GSK cell lines were expressing the prostaglandin receptors of interest, the cells were subject to functionality tests. These included measurement of intracellular calcium (section 2.7) and cAMP assays (section 2.8).

2.4 WESTERN BLOTTING

2.4.1 Sample Preparation

2.4.1.1 Continuous culture cells

Prostaglandin receptor transfected cells and wild type cells were grown in T25 flasks and maintained in culture medium for at least 12 hours before an experiment. When required, cells were pelleted (200*g* for 5 minutes) and solubilized

on ice for 15-20 minutes in 500µl 2X *sample buffer* containing 0.18M Tris (base), pH 6.8, 5.7% SDS, 29% glycerol, 0.008% bromophenol blue, 50mM dithiothreitol (DTT), and 1% β-mercaptoethanol. The samples were collected in eppendorf tubes and combined with 500µl distilled water. The samples were subsequently aliquotted and stored at –20°C until usage. Immediately prior to SDS-PAGE, the samples were "cracked" at 100°C for 5 minutes and centrifuged at 13000rpm for 5 minutes.

2.4.1.2 Tissue Preparation

Adult male Wistar rats (200-350g) were killed by stunning and cervical dislocation. Tissue for SDS-PAGE was quickly excised from the animal and snap frozen on a dry ice (BOC) / hexane (Fisher) slush (-73°C) in a dewar flask for 30 minutes. The tissue was then subjected to homogenisation in order to liberate cellular proteins. Protease inhibitor cocktail (PIC) (Sigma) was added at a dilution of 1:100 to an ice-cold *homogenising buffer* (HB) (NaCl 150mM, Tris 10mM pH7.4, EDTA 1mM, EGTA 1mM, Triton X100 1%) to prevent any proteases released from the tissue during the homogenisation process from destroying the cellular proteins. As 1ml undiluted PIC inhibits the protease activity in 100ml of cell lysates, from 20g wet weight of tissue, the amount of homogenising buffer containing protease inhibitor cocktail (HB-PIC) added to the excised tissue was calculated using the following equation:

$$\frac{100}{20000} \times Xmg = Amount\ of\ HB - PIC\ to\ be\ added\ in\ ml$$

Where Xmg = weight of the excised tissue in mg

The tissue was homogenised on ice using a Dounce hand-held homogeniser. Homogenates were centrifuged for 10 minutes at 13000rpm at 4°C. The supernatant was collected and subjected to a protein assay to determine the protein content in the sample (section 2.4.1.3). Each tissue sample was subsequently diluted to a uniform concentration ($2.5 mgml^{-1}$) and stored in working aliquots at –20°C until usage. Immediately prior to SDS-PAGE, tissue sample aliquots (10µl) were prepared by dilution (1:1) with 2X *sample buffer* under reducing conditions (0.18M Tris pH 6.8,

5.7% SDS, 29% glycerol, 0.008% Bromophenol blue, 1% β-mercaptoethanol) such that 25μg protein was loaded per lane in all cases. The final samples were subsequently "cracked" at 100°C for 5 minutes and spun at 13000rpm for 5 minutes prior to use.

2.4.1.3 Bradford protein Assay

To determine the concentration of protein in each sample, a Bradford protein assay [Bradford, 1976] was used. To generate a standard curve, bovine serum albumin (BSA) protein was prepared in duplicate at concentrations of 0, 5, 10, 15, 20, and 25μgml^{-1} in 800μl of milliQ water. Tissue samples of unknown protein concentration were diluted 2μl in 800μl milliQ water (1:400), also in duplicate. Bradford reagent (Biorad) (200μl) was added to all samples and standards in 1ml plastic cuvettes and each solution was mixed thoroughly. Following a 5-minute incubation at room temperature, the protein content was read using a spectrophotometer at 595nm. A standard curve was generated using GraphPad software (GraphPad Prism version 3.02; GraphPad software Inc., San Diego, CA, U.S.A.) and was used to determine the unknown protein concentrations in each of the samples.

2.4.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using a mini protean 3 cell, gel assembly kit (Biorad). Glass plates were cleaned with ethanol to remove any debris, clamped, and assembled in the gel-casting stand. 135μl of 10% Aminopyridine (APS) and 45μl of tetramethylethylenediamine (TEMED) were added to the *resolving gel*, which was prepared from 12.5ml resolving gel buffer (1.5M Tris (base), 0.7% SDS, pH 8.8), 10ml acrylamide (30% protogel), and 6.75ml milliQ water. This mixture was then carefully poured between the plates to form a 0.75mm thick, 10% polyacrylamide gel. The resolving gel was overlaid with 0.1% SDS to avoid oxygen getting to the surface of the gel and drying it out. The resolving gel was left for 15-20 minutes to polymerise. Once the resolving gel had polymerised, any unpolymerised gel was poured off, the gel surface was washed with milliQ water several times, and any residual water blotted with filter paper. 120μl 10% APS and 36μl TEMED were then

added to the *stacking gel*, which comprised 5ml stacking gel buffer (0.5M Tris (base), 0.75% SDS, pH 6.8), 3.33ml acrylamide (30% protogel), and 11.67ml millQ water. This was poured onto the resolving gel surface and ethanol-cleaned plastic combs inserted carefully to avoid air bubbles. Once the stacking gel had polymerised, the combs were removed and the wells washed thoroughly with milliQ water. The gels were then clamped into the electrode stand and placed in the gel tank. The inner and outer reservoirs were filled with *running buffer* (24.8mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) and samples and standards loaded using gel-loading tips. The gels were run at 100V for approximately 2 hours.

2.4.3 Transfer of Proteins

Once the SDS-PAGE was complete, the stacking gel was removed from the resolving gel and discarded. The upper left corner of the resolving gel was marked so that the orientation of the gel could be deciphered. Gels were then soaked in *transfer buffer* (25mM Tris (base), pH8.3, 20% methanol) for approximately 20 minutes. In addition, 4 sheets of blotting paper and 1 piece of 0.45μm nitrocellulose (Schleicher and Schuell) were soaked in transfer buffer for each gel. Separated proteins were transferred onto the nitrocellulose membrane using a semi-dry transfer blotter (V20-SDB). Two sheets of blotting paper were positioned on the blotter with a sheet of nitrocellulose carefully overlaid. The gel was then placed onto the nitrocellulose membrane in its correct orientation with two further sheets of blotting paper laid on its surface. It is worthy of note that any air bubbles were rolled out from each layer of the gel / nitrocellulose sandwich in order to prevent interference during the transfer of the proteins. Proteins were transferred onto the nitrocellulose membrane for 3 hours at a current calculated from the total of each gel surface area: 0.9mA per cm^2 gel. Following transfer, the top sheets of blotting paper and the gel were carefully peeled back to check that the pre-stained markers had been transferred onto the nitrocellulose membrane. Cuts were made into the nitrocellulose using a scalpel blade at each of the markers so that they were easily visualised following the development process.

2.4.4 Immunodetection

Following transfer, the nitrocellulose membranes were blocked overnight at 4°C in 10% marvel in *Tween-Tris Buffered Saline (TTBS)* (50mM Tris base, 150mM NaCl, 0.1% Tween-20, pH8.0). The nitrocellulose membranes were then subject to 5x 5-minute washes in TTBS followed by incubation with the primary antibody for 2 hours at room temperature. The nitrocellulose membranes were then subject to 3x 10-minute washes in TTBS to remove any residual primary antibody. As all primary antibodies tested (section 2.2) were raised in the rabbit, this was followed by incubation in anti-rabbit peroxidase-conjugated secondary antibody (Sigma) at a dilution of 1:1333 for 1.5 hours at room temperature. Finally, the nitrocellulose membranes were subject to 5x 5-minute TTBS washes to remove any residual secondary antibody. All of the washing steps and antibody incubations were subject to gentle agitation using a rocking platform.

In order to visualise the proteins, the blots were incubated with an Enhanced Chemi-Luminescence (ECL)-plus detection (Amersham) substrate for 5 minutes. Once excess substrate had been removed from the blots, the blots were wrapped in Saran wrap and placed in an exposure cassette. The blots were then exposed to hyperfilm (Amersham) in a dark room and developed using a hyperprocessor (Amersham). The time of exposure was optimised following an initial exposure for 15 seconds.

Omission of primary antibody from the primary antibody incubation step assessed the non-specific binding of the anti-rabbit peroxidase-conjugated secondary antibody to the rat tissue and PG receptor expressing cell line proteins. No peroxidase labelling was observed following exposure of the blots to light-sensitive X-ray film for 15 minutes (a relatively long exposure time), indicating that the secondary antibody only binds to the target primary antibody.

2.5 IMMUNOCYTOCHEMISTRY

2.5.1 Subbing of slides

In order for tissue sections to remain firmly attached to the microscope slides during the numerous washing steps of the immunocytochemical process, the microscope slides were subject to a *subbing* process. The microscope slides (R A

Lamb) were racked and soaked overnight in 5% decon detergent solution and then washed in hot running water for 30 minutes. This was followed by 5 slide washing steps in milliQ water, each for 5 minutes, prior to being oven dried for 30 minutes. The subbing process commenced with the slides being submerged in a 2% solution of 3-aminopropyltriethoxysilane in acetone for 1-minute. This was followed by two 1-minute washes in acetone, and finally two 1-minute washes in milliQ water. Each of the washes was performed whilst being agitated on a rocking platform. Once oven dried, the slides were ready for usage.

2.5.2 Tissue preparation and fixation

Adult male Wistar rats (200-350g) were killed under schedule 1 by stunning and cervical dislocation under 1986 Animals Act UK. Segments of the lumbar spinal cord and whole dorsal root ganglia (L4-L6) were quickly excised and embedded in Tissue-Tek® OCT™ compound (Miles, Elkhart, CA, USA) in moulds of an appropriate size (Polysciences Europe). The tissue was rapidly frozen over dry ice (BOC) and hexane (Fisher) slush (-73°C) in a dewar flask for 30 minutes and then stored at –20°C until usage. Frozen tissue sections (10-15μm) were cut from frozen tissue blocks using a cryostat (-18°C) (Model OTF, Hacker-Bright Instruments, Fairfield, NJ, USA), mounted onto pre-subbed slides (see section 2.5.1), and air-dried. An ImmEdge™ pen (Vector Laboratories) was used to draw on the slides around the tissue sections to form a hydrophobic perimeter, which prevented run off of reagents.

Tissue sections were fixed using a freshly prepared 2% paraformaldehyde solution in *Sörensons buffer* consisting of 80mM Na_2HPO_4 and 20mM NaH_2PO_4, for 10 minutes at room temperature. The tissue sections were subsequently washed in 1X *Phosphate Buffered Saline (PBS)* (in mM: NaCl 137, KCl 2.7, KH_2PO_4 1.4, Na_2HPO_4 8.1, pH 7.4) for 15 minutes with gentle agitation (rocking platform). It is worthy of note that the PBS was filtered prior to use to remove any contaminants, which might have otherwise interfered with the quality of the immunocytochemistry.

2.5.3 Immunocytochemistry on tissue

Following the tissue fixation process, excess PBS was wiped carefully from the slides avoiding the tissue sections. The slides were then placed in a humidified

incubation box (RA Lamb) and incubated with blocking buffer [(PBS containing 10% goat serum and 0.5% triton X-100 (Sigma-Aldrich)] for 30 minutes at room temperature. The Triton X-100 enabled permeabilisation of the cell membrane so that antibodies raised against an intracellular portion of the protein of interest were able to enter the cells. Following two 15-minute washes in PBS, the sections were incubated in the humidified incubation box with primary antibody overnight at 4°C. Sections were then subjected to 6x 10-minute washes with PBS before they were incubated with host-directed fluorescein isothiocyanate (FITC) or Texas Red (TR)-conjugated secondary antibody (Jackson Immunoreseach Labs), for 2 hours at room temperature. The sections were then subjected to two final 15-minute wash steps prior to mounting using a glycerol-based aqueous antifade mountant, Citifluor (UKC Chem. Lab, Kent, UK). It is worthy of note that all the washing steps were subject to gentle agitation on a rocking platform.

The slides were subsequently viewed under a Nikon Labophot 2A epifluorescence microscope under the appropriate filter set (FITC–absorbance peak = 492nm, emission peak = 520nm; TR-absorbance = 596nm, emission peak = 620nm), and photographed. Images were collected in black and white to maximize resolution and assembled into montages for quantification using adobe photoshop 7.0 (Adobe, Mountain View, CA). In addition, high-power magnification images were taken using either the epifluorecence microscope or confocal microscope (Olympus® IX70). Black and white images taken using the epifluorecence microscope were pseudocoloured to green (FITC) and red (TR) using Adobe Photoshop. For co-localisation (sections 2.5.5 and 2.5.6), monochrome images were colour matched to reproduce the original FITC / TR fluorescence. Images were added together using the computer software, Confocal Assistant 4.02 (CAS) to determine co-localisation of the protein of interest. A yellow colour indicated the occurrence of co-localisation.

2.5.4 Immunocytochemistry on cells

For immunocytochemistry on continuous culture cells and primary culture DRG cells, cells were plated onto 26mm coverslips pre-coated with poly–d–lysine ($0.1 mg ml^{-1}$). Coverslips were removed from the culture medium, and washed briefly and gently with PBS supplemented with 100μM $CaCl_2$ and 100μM $MgCl_2$. Using forceps, the coverslips were turned on their side and blotted on a tissue to remove

excess fluid. The coverslips were placed on raised platforms in a purpose built incubation tray and fixative was pipetted onto each coverslip (enough to cover the surface), before being left for 10 minutes. Once the fixative was removed, each coverslip was subject to the same immunocytochemistry protocol used for tissue (section 2.5.3). However, it is worthy of note that the coverslips were only exposed to blocking buffer containing 0.05% triton-X for 10 minutes.

2.5.5 CGRP dual labelling

Dual labelling was achieved by incubation of the tissue sections with both the primary antibody of interest and a guinea-pig anti-CGRP antibody (1:1000) (ACRIS), overnight at 4° C. This was followed by 6 washes (10-minutes each) in PBS and a simultaneous 2-hour incubation in host-directed FITC-conjugated secondary antibody (Jackson Immunoreseach Labs) and Texas Red-conjugated anti-guinea-pig secondary antibody (Jackson Immunoreseach Labs). Finally, the sections were repeatedly washed in PBS, mounted and photographed.

2.5.6 IB4 dual labelling

Alexa Fluor 594-conjugated isolectin IB4 (Molecular Probes Inc.) was used as a known marker for non-peptidergic DRG neurons. Primary antibody and IB4 could not be applied simultaneously because the IB4 protocol produced strong auto-fluorescence in the emission wavelength of the secondary antibody used to demonstrate primary antibody staining in the dual labelling experiments [Chopra *et al.*, 2000]. In addition, the IB4 protocol required a modified PBS solution (PBS plus 100μM $MnCl_2$, 100μM $MgCl_2$, 100μM $CaCl_2$) without goat serum. To circumvent these problems, the normal immuno-labelling protocol was carried out as described in section 2.5.3, and after mounting and photography, the coverslips were carefully removed under PBS, and the sections were incubated in Texas Red-conjugated IB4 ($3\mu g ml^{-1}$) overnight. The sections were then washed 6 times in the modified PBS (10-minutes each), mounted in modified PBS and glycerol solution, and re-photographed.

2.5.7 Immunocytochemistry morphometric data analysis

For the purpose of morphometric analysis of primary antibody immunocytochemical labeling, DRG sections taken from the middle portion of L4-L6 ganglia from at least 3 different rats were used: Quantification of EP_4 immunolabelling was from 9 DRG sections. CGRP and IB4 immunolabelling was from 6 selected DRG sections. For the EP_4 co-localisation experiments with IB4 and CGRP, 6 DRG sections were used. Each section analysed within an experiment set was taken from a different ganglion.

Measurements of the cell areas and counts of labelled versus unlabeled cells were performed using a computerised image analysis system (NIH Image). Labelled cells were distinguished from unlabelled cells on their intensity of fluorescence compared to control levels, and also on the level of distinct labelling in cellular structures. To distinguish the cell size-specific distribution, DRG cells were characterised as small ($<1000\mu m^2$), intermediate ($1000\text{-}2000\mu m^2$), or large ($>2000\mu m^2$) neurons depending on their cross-sectional area.

2.6 PRIMARY CELL CULTURE

2.6.1 Preparation of coverslips

Prior to primary cell culture, 16mm coverslips (Scientific Laboratory Supplies) were pre-soaked in ethanol for at least 24 hours, flamed to sterilise, and placed into individual wells in a 12-well plate (Scientific Laboratory Supplies). The coverslips were subsequently treated with poly-d-lysine ($0.2mgml^{-1}$, Sigma-Aldrich) (enough to cover the surface of the coverslip) for 5 minutes to promote cellular adherence. The poly-d-lysine was aspirated off the coverslips and sterile water was used to wash away any residual poly-d-lysine. Following aspiration of the sterile water, the coverslips were allowed to air dry in a laminar flow hood for at least one hour. Plates were stored at 4°C for up to 2 weeks. Before usage, plates were exposed to U.V. light for 20 minutes to destroy any bacteria. Any additional opening of the plates e.g. for the seeding of cells onto coverslips or for the removal of coverslips, was restricted to the sterile conditions of the laminar flow hood.

2.6.2 Enzyme solutions and feeding media composition

Two enzymatic solutions were prepared immediately before cell culture. These were made up in sterile ice-cold Hanks balanced salt solution (HBSS) (Gibco BRL) supplemented with glucose. *Solution A* contained L-cysteine ($2mgml^{-1}$) and papain ($1mgml^{-1}$, enzyme activity=14 $units.ml^{-1}$, Sigma-Aldrich) and *solution B* contained collagenase type F ($1mgml^{-1}$, Sigma-Aldrich) and 0.8% dispase (enzyme activity=$1.17units.mg^{-1}$, Gibco).

The cellular *feeding medium* was HAM's F-12 feeding media (Sigma-Aldrich) supplemented with 10% horse serum (Gibco), 2mM glutamine (Gibco), $10ngml^{-1}$ nerve growth factor (NGF) (Sigma-Aldrich), and 1% penicillin/streptomycin (enzyme-activity=$5000units.ml^{-1}$ penicillin and $5000\mu gml^{-1}$ streptomycin, Gibco).

2.6.3 Primary Cell Culture

Adult, male Wistar rats (200–350g) were killed under schedule 1 by stunning and cervical dislocation under 1986 Animals Act UK. Whole dorsal root ganglia (DRG) were removed from all spinal levels, and were placed in a petri dish containing sterile ice-cold HBSS. DRG were chopped into small pieces using sterile scissors in order to allow a greater surface area for enzymatic digestion. The chopped DRG were collected using a fire-polished Pasteur pipette, added to pre-activated (15 minutes at 37°C) sterile-filtered ($0.2\mu m^2$) solution A, and incubated for 15 minutes at 37°C. This solution was subsequently removed and replaced with sterile filtered solution B. In this later solution, the ganglia were gently triturated with fire-polished Pasteur pipettes of decreasing size apertures to cause mechanical dissociation of the DRG neurons from the connective tissue, over a period of 30–45 minutes. To ensure that sufficient cellular dissociation had occurred, a drop of the resulting cell suspension was viewed under a light microscope. When individual DRG neurons were visible, the cell suspension was centrifuged at 200g for 5 minutes at room temperature, and the cell pellet re-suspended in feeding media then re-centrifuged to wash the cells. The final pellet was subsequently re-suspended in 1.1ml of feeding media with 90µl of the suspension being plated onto poly–d–lysine coated glass coverslips (see section 2.6.1). The plate was then transferred to a humidified incubator with a 95% O_2 / 5% CO_2 atmosphere and maintained at 37°C to allow for

cellular adherence to occur. Cells were fed 1-2 hours after plating with 2ml feeding media and then grown for 2 days.

2.7 MEASUREMENT OF INTRACELLULAR CALCIUM

2.7.1 Experimental Solutions and drugs

A normal Ringer's solution was used throughout the course of the work and was adapted to suit different experimental requirements. The normal Ringer's solution contained (in mM): NaCl 140, HEPES 10, KCl 5.4, $MgCl_2$ 0.5, $CaCl_2$ 1.8, and glucose 5, prepared in MilliQ water, titrated to pH 7.4 with NaOH. For a high KCl Ringer's solution, KCl was increased from 5.4mM to 60mM KCl, with equimolar substitution with NaCl. The solution was made up in MilliQ water, and titrated to pH7.4 with NaOH. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) (Sigma-Aldrich) and PGE_2 (Sigma-Aldrich) were prepared as 100mM and 10mM stock solutions respectively, made up in absolute ethanol. These stocks were stored at –20°C under desiccated conditions. The prostaglandin receptor compounds, kindly donated by Glaxo SmithKline (chemical names and pharmacological functions listed below, see figure 2.1 for chemical structures) were made up as 10mM stock solutions in dimethyl sulphoxide (DMSO), and were stored at –20°C. All agonist and antagonist solutions were diluted to the appropriate concentration in normal Ringer's solution (pH 7.4) prior to experimentation;

1) GW683868X (6-[({5-chloro-2-[(2-methylpropyl)oxy]phenyl}methyl) (ethyl)amino]-N-[(3,5-dimethyl-4-isoxazolyl)sulfonyl]-3-pyridazinecarboxamide) a selective EP_1 antagonist
2) GW671021X ((2E)-N-{[5-bromo-2-(methyloxy)phenyl]sulfonyl}-3-[2-(2-naphthalenylmethyl)phenyl]-2-propenamide) a selective EP_3 antagonist
3) GW627368X (2-{4-[4,9-bis(ethyloxy)-1-oxo-1,3-dihydro-2H-benzo[f]isoindol-2-yl]phenyl}-N-(phenylsulfonyl)acetamide) a selective EP_4 antagonist
4) GSK324202A ({4-[4,9-bis(ethyloxy)-1-oxo-1,3-dihydro-2H-benzo[f]isoindol-2-yl]phenyl}acetic acid) an EP_4 agonist

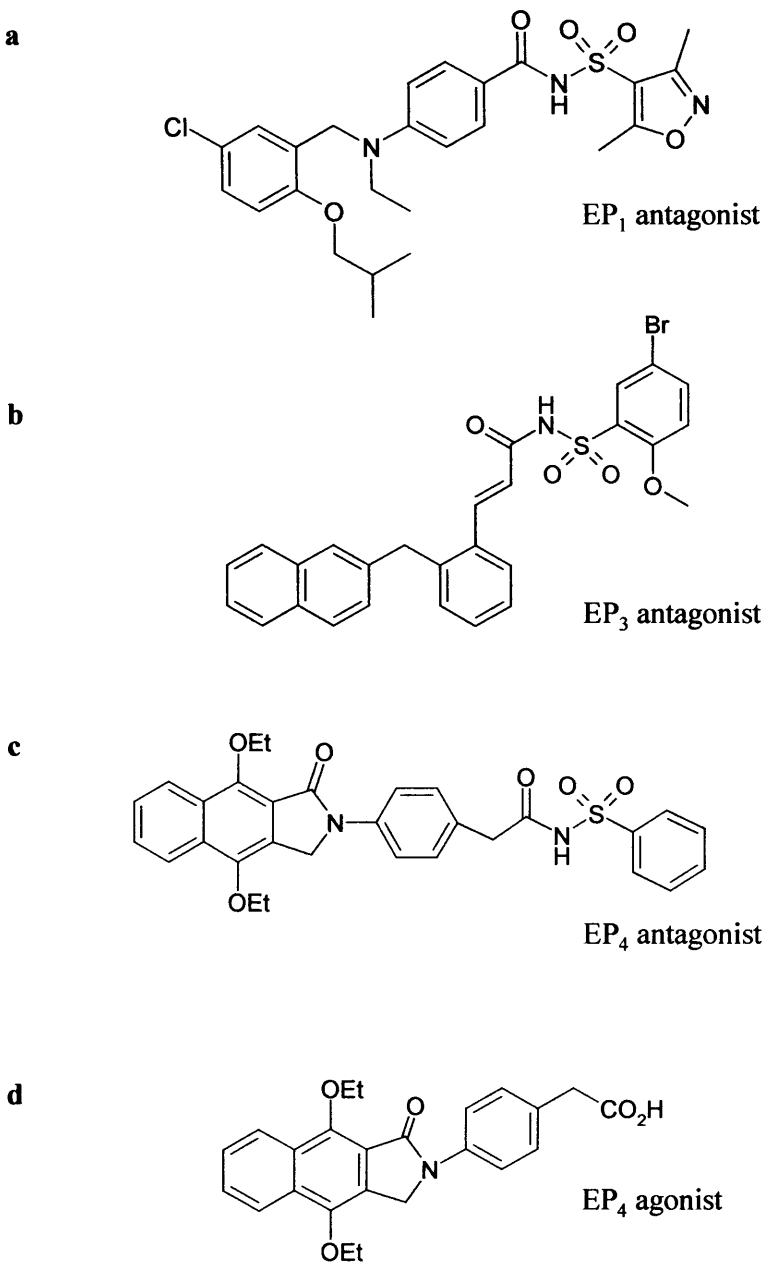


Figure 2.1: Chemical structures of novel selective prostaglandin receptor compounds.

2.7.2 Cellular perfusion and drug application

Coverslips were attached to the underneath of a purpose-built perfusion chamber using vacuum grease (Dow-Corning). The bath was screwed into a peltier device, which was mounted on the stage of a confocal microscope (Olympus® IX70). Cells were continuously perfused with normal Ringer's solution through microbore Teflon PTFE tubing ((O.D. 0.056″, I.D. 0.032″ (Cole-Parmer)) connected to Gilson

Minipuls PVC tubing ((I.D. 1.52mm (Anachem Ltd)), which was attached to a Gilson Minipuls 3 peristaltic pump (flow rate 2ml.min^{-1}). The bath efflux system consisted of a needle positioned at the edge of the bath, which sucked perfusion fluid through microbore Teflon PTFE tubing ((O.D. 0.076″, I.D. 0.052″ (Cole-Parmer)) into a large conical vacuum flask, attached to a pump (Medcalf Brothers Ltd). Drugs were applied via a fast-flow U-tube positioned directly next to the cells of interest or via the perfusion system. The U-tube was connected to a gravity-feed perfusion system using microbore Teflon PTFE tubing (O.D. 0.042″, I.D. 0.022″ (Cole-Parmer)), pre-coated with a siliconising agent (Sigmacote, Sigma-Aldrich), and via a 3-way solenoid valve (The Lee Company), which was attached to a pulse unit (High-Med) and oscilloscope (Gould) (figure 2.2). The U-tube was initially primed with normal Ringer's solution so that fluid continuously flowed through the U-tube via the solenoid to waste by gravity suction. To apply a drug, activation of the pulse unit triggered closure of the solenoid so that drug was expelled from a small hole at the U-tube apex. The pulse unit enabled fine control over the duration of drug application.

2.7.3 Fluorescent Calcium Imaging

The intracellular calcium concentration was measured using the fluorescent indicator fluo-4 (Molecular Probes, Eugene, Oregon, USA) as the cell permeant acetoxymethyl (AM) ester form. This indicator was selected as it exhibits high Ca^{2+}-dependent fluorescence enhancement, with a K_d of 345nM, making it ideally suited to measuring physiologically important cellular Ca^{2+} changes. In addition, the absorption spectrum for fluo-4 is such that the efficiency of fluo-4 fluorescence excitation is twice that of its analogue fluo-3, consequently allowing a greater emission output for confocal microscopy [Gee et al., 2000]. Fluo-4 was pre-prepared as a 1mM stock solution in DMSO and was incubated with DRG or CHO EP_1 cells in normal Ringer's solution made up to the final concentrations of 2.5μM and 5μM respectively. The loading process was carried out for 30-45 minutes at room temperature. Once inside the cell, the AM esters were cleaved by non-specific esterases to release the free impermeable acid form of the dye, which remained trapped in the cells cytoplasm [Tsien, 1981]. Fluo-4 loaded cells were washed in normal Ringer's solution for 30 minutes at room temperature to remove any fluo-4

AM that had not been taken up by the cells, and to ensure that de-esterification of the indicator had occurred.

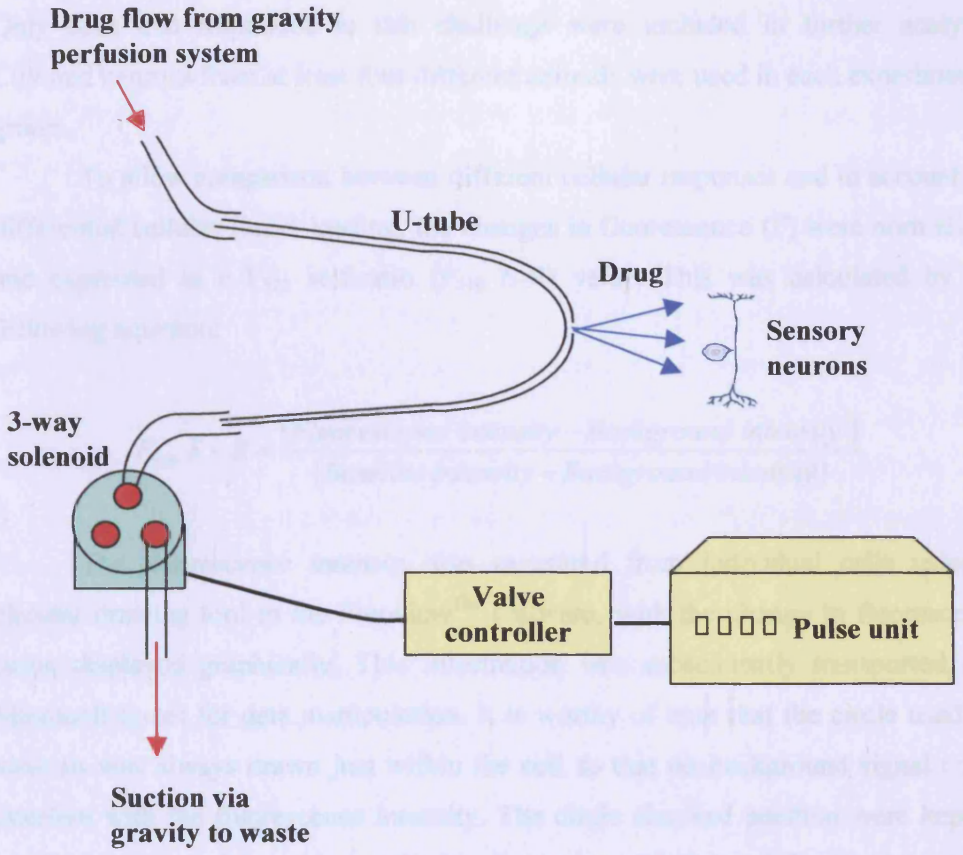


Figure 2.2: Schematic showing U-tube drug application method. Following priming, drugs continuously flow through the U-tube without being expelled onto the cells of interest. Activation of the pulse unit triggers closure of the solenoid valve so that drug is expelled out of the U-tube hole.

The cells containing the de-esterified fluo-4 were transferred to a perfusion chamber as detailed in section 2.7.2 and continuously perfused with experimental solutions. All experiments were performed in a darkened room, to ensure that the background signal was maintained at minimal levels. Cell fluorescence was visualised upon illumination with an argon laser at an excitation wavelength (λ_{ex}) of 488nm. Barrier filters were set to capture emission at wavelengths (λ_{em}) greater than 510nm. The cells were imaged at room temperature and real time fluorescence was captured by Olympus Fluoview™ 300 (version 4.2) software, at a frequency of 0.5Hz, for a continuous period of 200s. All experiments were performed at room temperature.

2.7.4 Data Analysis

Cell viability and integrity was confirmed by the Ca^{2+} response to a depolarisation produced by cellular perfusion with 60mM KCl Ringer's solution. Only cells that responded to this challenge were included in further analysis. Cultured neurons from at least four different animals were used in each experimental group.

To allow comparison between different cellular responses and to account for differential cellular fluo-4 loading, the changes in fluorescence (F) were normalised and expressed as a F_{510} self-ratio (F_{510} S-R) value. This was calculated by the following equation:

$$F_{510}\ S-R = \frac{(Fluorescence\ intensity - Background\ intensity)}{(Baseline\ intensity - Background\ intensity)}$$

The *fluorescence intensity* was measured from individual cells using a circular drawing tool in the Fluoview™ software, with the change in fluorescence being displayed graphically. This information was subsequently transported into Microsoft Excel for data manipulation. It is worthy of note that the circle used for analysis was always drawn just within the cell so that no background signal could interfere with the fluorescence intensity. The circle size and position were kept as consistent as possible when analysing the same cell from different time-series experiments. To obtain the *background intensity*, a circle was drawn around a cell-free area within the field of view and this value was then subtracted from each of the cellular fluorescence intensities. The *baseline intensity* measurements were calculated as a mean of the intensities obtained in the 5 seconds immediately prior to each drug application. This procedure ensured that any reduction in the fluorescence intensity due to photobleaching of the fluorescent dye was taken into account. As the *self-ratio* expresses the fluorescence trace as a ratio over the baseline, no change in fluorescence intensity gives a ratio of 1, whereas an increase in fluorescence shows a ratio >1.

Using this method of normalisation, comparisons were made between the normalised calcium transients under different experimental conditions. Peak heights and area under the peaks were calculated using Excel and Origin (Microcal Software

Inc., Northampton, MA) software respectively. Data are expressed as mean ± SEM of *n* cells.

Treatment effects within each experimental data set were statistically analysed using GraphPad Prism software (GraphPad Prism version 3.02; GraphPad software Inc., San Diego, CA, U.S.A.). Data passing the Kolmogorov-Smirnov normality test dictated whether parametric tests were chosen over non-parametric tests. As statistical comparisons were required for more than 3 groups, either parametric or non-parametric analysis of variance (ANOVA) tests were performed. For non-Gaussian data sets, Kruskal-Wallis tests were performed in all circumstances except where matching data sets were available. In these circumstances a Friedman's test was applied. A *post hoc* Dunn's test was performed subsequent to the above tests when $P<0.05$ in order to compare all pairs of groups. For data that followed a Gaussian distribution, one-way ANOVA tests were performed unless matching data sets were available, in which case a repeated measures ANOVA test was employed. When $P<0.05$, the tests were followed by a *post hoc* Tukey's test to compare all groups of data.

Concentration-response data were normalised to the maximal response and fitted in GraphPad Prism using the Hill equation:

$$y = \frac{V_{max}\, x^n}{EC_{50}^n + x^n}$$

Where V_{max} is the maximum response (100%), *n* the Hill coefficient, and EC_{50} the concentration required to evoke a half maximal response.

2.8 MEASUREMENT OF INTRACELLULAR cAMP

2.8.1 Extraction of cAMP from continuous cells

CHO-EP_2 and HEK-EP_4 cells were grown to approximately 70% confluency in poly-d-lysine ($0.1 mgml^{-1}$) coated 24-well plates (Scientific Laboratory Supplies) for 2 days prior to experimentation. Cells were treated with 3μM indomethacin (Sigma) 24 hours before experimentation to stop any cellular production of prostaglandins. Confluent 24-well plates were washed twice with 1ml of warm

(37°C), pre-gassed (5% CO_2 and 95% O_2) *Krebs Henseleit Buffer (KHB)* (10mM HEPES, 118mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 25mM $NaHCO_3$, 1.3mM $CaCl_2$, 11mM glucose, pH7.4). The cells were then left in a water bath for 15 minutes to allow them to settle. After this period, drug additions (50µl) were made to 0.4ml of KHB. In the case of the HEK EP_4 cells, it was necessary to add 500µM 3-isobutyl-1-methylxanthine (IBMX) for 30-minutes to inhibit phosphodiesterase enzymes from breaking down the cAMP. All of these additions were done in duplicate wells. The reaction was terminated by replacing the stimulating solution with 0.5ml of ice-cold, 0.5M trichloroacetic acid (TCA). Extraction of cAMP took place for a minimum of 15-minutes on ice. Samples were subsequently pipetted into 1.5ml eppendorf tubes and combined with 50µl of 10mM EDTA (pH 7.0). Blank buffer was also prepared in a similar way so that standards could be made up for the generation of a cAMP standard curve. For these, approximately 10X eppendorf tubes were prepared containing 500µl TCA (0.5M) and 50µl EDTA (10mM) (*Blank buffer*). To each of the eppendorf tubes (samples and blanks), 500µl of 50:50 1,1,2,-trichlorotrifluorothane and tri-n-octylamine oils were added to neutralise the TCA. The samples and blanks were then vortexed 3 times over a period of 15-minutes at room temperature. They were subsequently centrifuged at room temperature for 2 minutes at 1000rpm with 200µl of the supernatant being transferred to fresh eppendorf tubes. Finally, 50µl of 60mM $NaHCO_3$ was used to neutralise all the samples and blanks. These were then vortexed and stored at 4°C for up to a week.

To determine the concentration of cellular proteins, each of the wells were aspirated of any remaining TCA and treated with 250µl of 0.1M NaOH for 20-30 minutes. The NaOH was transferred from each of the wells into 5ml test tubes and combined with a further 250µl of 0.1M NaOH that was used to wash out the wells. The protein samples were then subject to a Lowry protein assay [Lowry *et al.*, 1951].

2.8.2 cAMP competitive binding Assay

The amount of cAMP was determined by a competitive binding assay using a cAMP adrenal binding protein. This involves the competition between cAMP present in samples or standards and [^{3}H] cAMP for association with the cAMP binding protein. 2µl of [^{3}H] cAMP (TRK498, Amersham) was diluted in 10ml of cAMP

buffer (50mM Tris, 4mM EDTA, pH 7.5), which provided between 50000-100000 d.p.m per 100µl. A 500µl stock aliquot of cAMP binding protein was diluted with 15ml cAMP buffer and kept on ice. To each tube of sample (50µl), 100µl of [^{3}H] cAMP and 150µl of cAMP binding protein was added. Each was then vortexed and incubated at 4°C for at least 90 minutes.

A charcoal buffer was used to separate bound cAMP and free cAMP. A 0.2% solution of BSA was prepared in cAMP buffer (0.1g BSA dissolved in 50ml buffer). To this, 0.5% Charcoal (BDH Norit, GSX) was subsequently added and stirred periodically to ensure that the charcoal remained in solution. To every sample, 250µl of ice-cold charcoal solution was added. The tubes were vortexed and allowed to stand on ice for 10 minutes before being centrifuged at 1000*g* for 4 minutes at room temperature. The supernatant (400µl) was then removed into scintillation vials, combined with Safefluor scintillant (4ml), and counted in a scintillation counter.

2.8.3 cAMP standard curve

In order to convert the counts obtained from the above procedure into amounts of cAMP, a standard curve was produced (figure 2.3). A stock solution of cAMP (5µM) (Sigma) was sequentially diluted in blank buffer to achieve concentrations of cAMP of 10, 8, 6, 4, 2, 1, 0.5, 0.25, and 0.125picomoles per 50µl. A tube containing blank buffer was used to define total binding and 50µl of 5µM cAMP was used to determine non-specific binding and defined the bottom of the curve. Each standard was produced in duplicate. The standards (50µl) were treated in exactly the same way as the samples (see section 2.4.2).

2.8.4 Data Analysis

Data from cAMP assays was the mean of two duplicate data points in each separate assay and is expressed as pmol cAMP.mg^{-1} protein. Concentration-response curves were constructed using GraphPad Prism and EC_{50} values were obtained using the software (section 2.7.4). Agonist potency is expressed as pEC_{50} values representing -log (EC_{50}).

Values of inhibitory binding constants were obtained from the Cheng-Prusoff equation [Cheng & Prusoff, 1973]:

$$K_i = \frac{[IC_{50}]}{1 + \frac{[L]}{K_D}}$$

Where L and K_D represent the concentration and equilibrium dissociation constant of the agonist respectively and IC_{50} denotes the concentration of the competing ligand, which reduces specific binding by 50%. These are expressed as pK_i values ($-\log K_i$).

In addition, pK_B ($-\log K_B$) values were obtained according to the equation:

$$K_B = \frac{[A]}{(Concentration\ ratio - 1)}$$

Where [A] is the concentration of the antagonist and the *concentration ratio* is the EC_{50} in the presence of the antagonist divided by the EC_{50} in the absence of antagonist.

All data is expressed as mean ± SEM for *n* experiments.

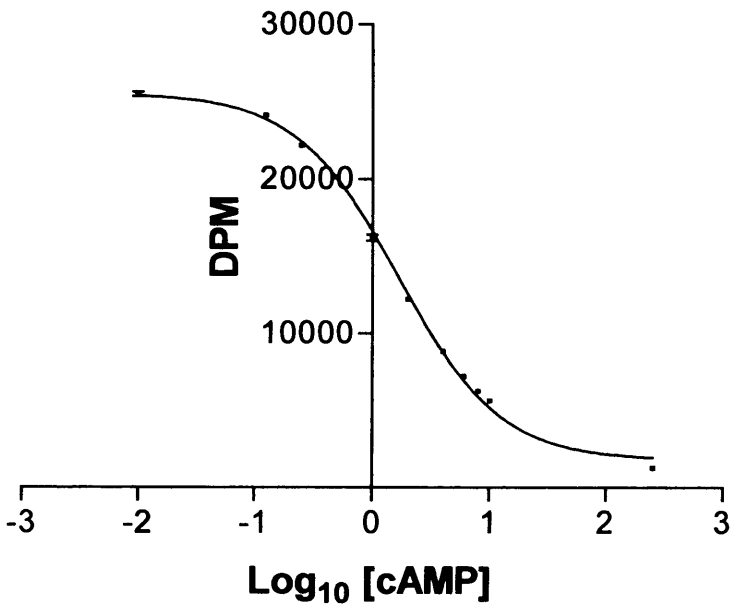


Figure 2.3: Example of a cAMP standard curve

Chapter Three

Characterisation of prostaglandin E receptor antibodies and the distribution of EP_4 in the rat sensory nervous system

3.1 INTRODUCTION

Prostaglandin E_2 is known to produce nociceptive behaviour in rodents following injection into the hindpaw [Ferreira *et al.*, 1978] or after direct spinal application [Taiwo & Levine, 1988], indicating a role for PGE_2 in both the peripheral and central nervous system (section 1.3.2.2 and 1.6.4). This inflammatory mediator produces its pro-nociceptive effects by activating at least one type of prostaglandin E (EP) receptor (section 1.3.2.3). To date, it has been impossible to elucidate, which EP receptor subtype(s) are important in PGE_2-mediated nociception, and whether the same receptor subtype(s) are responsible for mediating the actions of peripherally and spinally generated PGE_2.

Prostanoid compounds, of which Prostaglandin E_2 is an example, have common structural features such as an α-carboxylic acid, a hydroxyl group at position 15, and two aliphatic side chains (figure 1.2). These structures are believed to have a role in receptor binding and activation, and are thought to recognise specific conserved sequences present within prostanoid receptors. There is approximately 20-30% amino acid sequence homology shared between prostanoid receptors, but when grouped according to their functionality the EP_2, EP_4, IP, and DP receptors share 31-44% homology, and the EP_1, EP_3, FP, and TP receptors share 33-39% homology [Negishi *et al.*, 1995]. The highly conserved regions are present in the third and seventh transmembrane domains and in the second extracellular loop [Narumiya, 1994, Negishi *et al.*, 1995]. Among these sequences, the arginine residue in the seventh transmembrane domain is believed to be the binding site of the α-carboxylic acid of prostanoid molecules, and consistent with this, point mutations at this arginine residue in the human TP [Funk *et al.*, 1993] and rabbit EP_3 [Audoly & Breyer, 1997] receptors has been shown to result in a loss of ligand binding activity.

Non-conserved sequences such as those present within the N- and C-termini of prostanoid receptors have been exploited in the manufacture of subtype-specific PG receptor antibodies.

Selective antibodies are commonly used as probes to identify proteins that share similar structural and pharmacological characteristics. They are used in Western blotting to determine the apparent molecular mass of a protein and detect changes in expression under various conditions. Selective antibodies are also used to localise the protein of interest within tissues using immunocytochemical techniques.

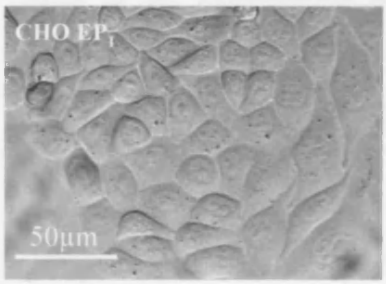
There is limited information in the literature regarding the expression of the various PG receptor subtypes in the peripheral and central nervous system (sections 1.3.2.3 and 1.6.4.1), largely due to a lack of selective PG receptor antibodies. This information is extremely important, as the expression of PG receptors within the peripheral and central nervous system should be indicative of the EP receptor subtype(s) that are important in mediating the effects of PGE_2. Any EP receptors involved in PGE_2–mediated nociception would be expressed in the DRG and spinal cord at sites consistent with an involvement in nociceptive processing (sections 1.2 and 1.6.1). Therefore, the aim of this section of work was to characterise a variety of EP prostaglandin receptor antibodies obtained from industrial collaborators Glaxo SmithKline (GSK) and also from commercial sources in an attempt to create a distribution map of EP prostaglandin receptors in the rat peripheral and central nervous system.

3.2 RESULTS

3.2.1 Characterisation of prostaglandin receptor antibodies

The various PG receptor antibodies (table 2.1, 2.2, 2.3) were profiled by screening lysates prepared from cell lines expressing EP_1, EP_2, EP_{3A}, and EP_4 receptor subtypes using Western blotting and immunocytochemistry techniques. In addition, the antibodies were tested against native proteins prepared from animal tissue known to express the various prostaglandin receptor subtypes using Western blotting.

A



B

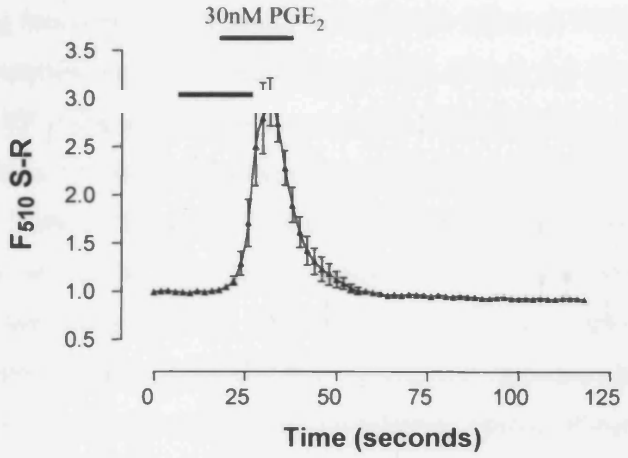


Figure 3.1: PGE_2-evoked intracellular calcium response in EP_1 expressing CHO K1 cells. A) Transmitted light image of EP1 CHO cells. B) Calcium response obtained in EP1-CHO cells loaded with 5µM fluo-4 during application of 30nM PGE2 for 30 seconds via U-tube. The trace is a population mean ±SEM of 12 randomly selected cells (3 cells from 4 coverslips).

3.2.1.1 Functionality of prostaglandin receptor expressing cell lines

Prior to characterisation of the prostaglandin receptor antibodies, it was necessary to determine the functionality of the PG receptor-expressing cell lines provided by industrial collaborators Glaxo SmithKline (GSK). This was achieved using confocal calcium imaging and cAMP accumulation assays.

3.2.1.1.1 CHO-EP_1 cell line

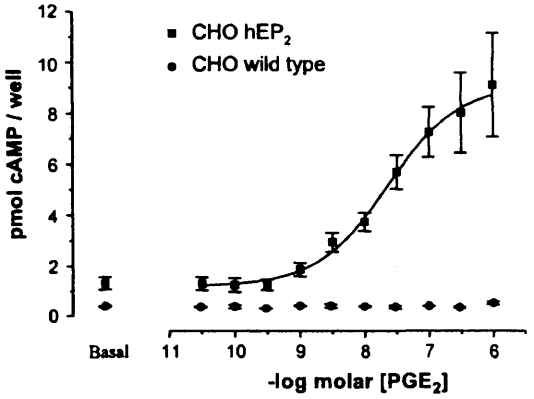
It is well established that the EP_1 receptor is coupled to a rise in intracellular calcium $[Ca^{2+}]_i$ when activated by the inflammatory mediator PGE_2 [Watabe *et al.*, 1993, Båtshake *et al.*, 1995]. Therefore, in order to test whether the EP_1-CHO cells were expressing functional EP_1 receptor proteins, the effect of PGE_2 on intracellular calcium concentration was examined in these cells. Figure 3.1 shows a transmitted light image of EP_1-CHO cells (figure 3.1A) and a representative calcium response (figure 3.1B) from 12 randomly selected cells (3 cells from 4 coverslips). U-tube application of 30nM PGE_2 for 30 seconds elicited a rapid rise in intracellular calcium, which returned to basal levels despite the continuous presence of agonist (figure 3.1B), indicating that the EP_1-CHO cell line was expressing functional receptors. To eliminate the possibility that the calcium response obtained during U-tube application was due to mechanical stimulation, normal Ringer's solution was applied for 30 seconds. No response was witnessed in the EP_1-CHO cells, thus indicating that the response to PGE_2 was not a mechanical artefact (data not shown). Wild type CHO cells were also devoid of responses to PGE_2, eliminating the possibility of a second receptor being responsible for the observed rises in $[Ca^{2+}]_i$.

3.2.1.1.2 CHO-EP_2 and HEK-EP_4 cell lines

As the prostaglandin EP_2 and EP_4 receptors are coupled to G_s [Regan *et al.*, 1994, Katsuyama *et al.*, 1995, Breyer *et al.*, 1996, Nishigaki *et al.*, 1996], cAMP accumulation assays were carried out by industrial collaborators Glaxo SmithKline (GSK) to determine whether the EP_2-CHO and EP_4-HEK cell lines were functionally expressing their respective receptors [Wilson *et al.*, 2004]. As shown in Figure 3.2, PGE_2 produced a concentration-related increase in cAMP accumulation in both EP_2-CHO cells (pEC_{50}=7.5±0.3; n=7; Figure 3.2A) and EP_4-HEK cells (pEC_{50}=10.3±0.1;

n=9; figure 3.2B), proving that the EP_2-CHO and EP_4-HEK cell lines were expressing functional receptors.

A



B

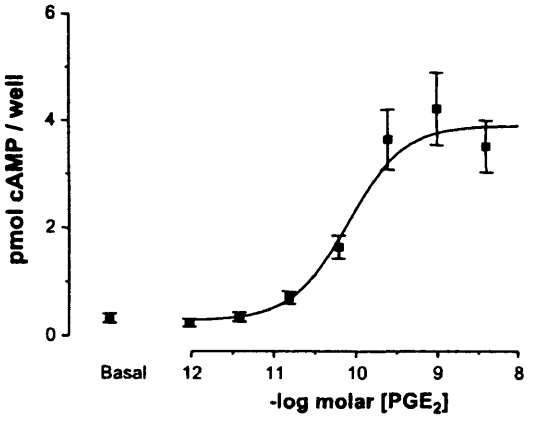


Figure 3.2: Accumulation of cAMP in response to PGE_2 in EP_2 CHO cells and EP_4-HEK cells. **A)** Concentration response curve for PGE_2-induced cAMP accumulation in EP_2-CHO cells (■) and PGE_2-induced cAMP accumulation in WT CHO cells (●). Data are the mean (±standard deviation) of 7 determinants, each performed in duplicate. **B)** Concentration response curve for PGE_2-induced cAMP accumulation in EP_4-HEK cells. Data are the mean of 9 determinants (±standard deviation), each performed as a single replicate [Wilson *et al.*, 2004].

3.2.1.2 Specificity of GSK and commercially available PG receptor antibodies

The GSK antibodies that were tested (table 2.1) against PG expressing cells by Western blotting all lacked specificity for the PG receptors against which they were raised. Figure 3.3 shows a representative Western blot of cell lysates prepared from EP_1, EP_2, EP_{3A}, and EP_4 expressing cell lines and a wild type HEK cell line,

using the GSK antibody R311 (anti-EP_4, 1:250) raised in the rabbit. The antibody recognised a single band in each of the cell lysates, indicating that the R311 antibody lacked specificity for the EP_4 protein. Similar results were obtained for the other rabbit GSK antibodies, the rabbit Cayman EP_2 and EP_3 antibodies (table 2.2), and the rabbit alpha diagnostics EP_2 and EP_3 antibodies.

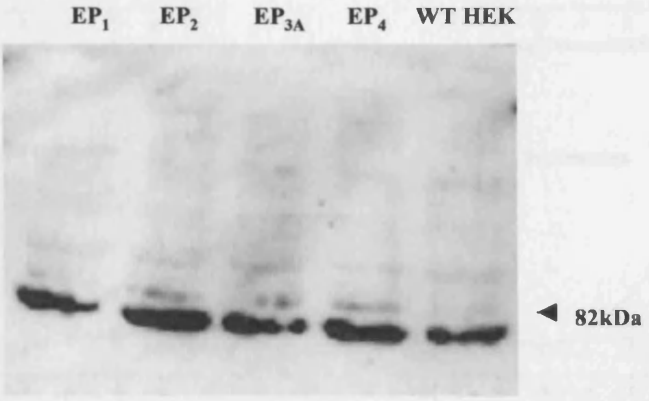


Figure 3.3: Western blot on PG receptor expressing cell lysates and WT HEK cell lysates using the R311 antibody (anti-EP_4). This figure demonstrates the lack of specificity of the GSK R311 antibody (1:250) for the EP_4 receptor.

3.2.1.3 Specificity of the Pepceuticals antibodies

Due to the lack of selective PG receptor antibodies, two sequences unique to the EP_1 (R13, R14) and EP_{3C} (R15, R16) receptors were submitted to Pepceutical's for antibody generation in rabbits (table 2.3). To characterise these antibodies, preliminary ELISAs and Western blotting experiments were performed.

The highest dilution for R13 detected by ELISA was 10^{-2}M (figure 3.4A). Although the titer of the antibody was low, the absorbance signal was blocked in the presence of the EP_1 peptide, indicating that the antibody recognised the peptide against which it was raised. Figure 3.4B shows the antibody titration curve for R13 together with the pre-immune absorbance readings. The background noise was of such a high level that it was unclear as to whether the pre-immune serum for R13 recognised the EP_1 peptide. The titration curve for R14 is shown in figure 3.4C. For this antibody, the highest dilution detected by ELISA was 10^{-3}M. The absorbance signals were reduced in the presence of the EP_1 peptide (figure 3.4C), indicating that the antibody recognised the peptide against which it was raised. No recognition of the peptide was witnessed with the pre-immune serum (figure 3.4D).

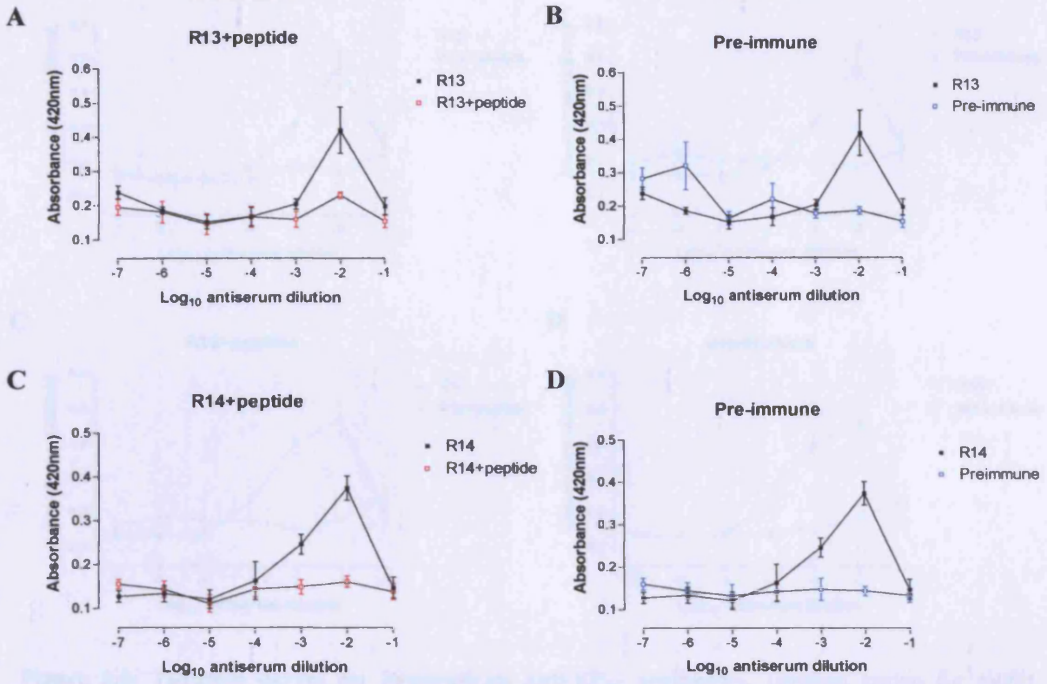


Figure 3.4: Titration curves for Pepceuticals anti-EP_1 antibodies. Titration curves for rabbit numbers 13 (R13) and 14 (R14), anti-EP_1 antibodies in the presence and absence of the EP_1 antigen ($10\mu gml^{-1}$) **(A and C)** and compared to the titration curves obtained with the pre-immune sera **(B and D)**. Data points represent 2 preliminary experiments (error bars show data range).

The ELISA results obtained for the anti-EP_{3C} antibodies R15 and R16 are shown in figure 3.5. The highest dilution of R15 antibody detected was 10^{-3}M. The absorbance signals were reduced in the presence of the EP_{3C} peptide (figure 3.5A), indicating that the antibody recognised the peptide against which it was raised. No recognition of the peptide was observed with the pre-immune serum (figure 3.5B). For the R16 antibody, the highest dilution detected by ELISA was 10^{-4}M (figure 3.5C). In the presence of the EP_{3C} peptide, the absorbance signals were reduced, indicating that the antibody recognised the EP_{3C} peptide (figure 3.5C). There was no recognition of the EP_{3C} peptide with the pre-immune serum (figure 3.5D).

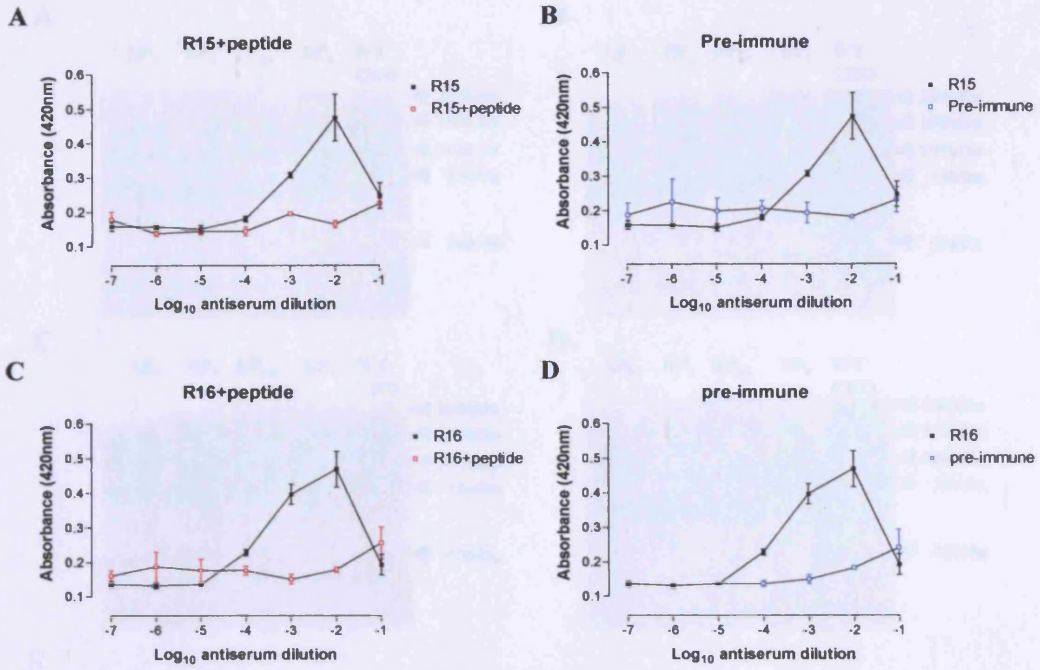


Figure 3.5: Titration curves for Pepceuticals anti-EP_{3C} antibodies. Titration curves for rabbit numbers 15 (R15) and 16 (R16), anti-EP_{3C} antibodies in the presence and absence of the EP_{3C} antigen ($10\mu gml^{-1}$) **(A and C)** and compared to the titration curves obtained with the pre-immune sera **(B and D)**. Data points represent 2 preliminary experiments (error bars show data range).

Figure 3.6 shows Western blots obtained using the Pepceutical's anti–EP_1 antibodies R13 (1:100) (figure 3.6A) and R14 (1:200) (figure 3.6B), and anti-EP_{3C} antibodies R15 (1:200) (figure 3.6C) and R16 (1:200) (figure 3.6D). Multiple immunoreactive bands were observed for each antibody in the PG receptor expressing cell lines, indicating that the antibodies were binding non-specifically.

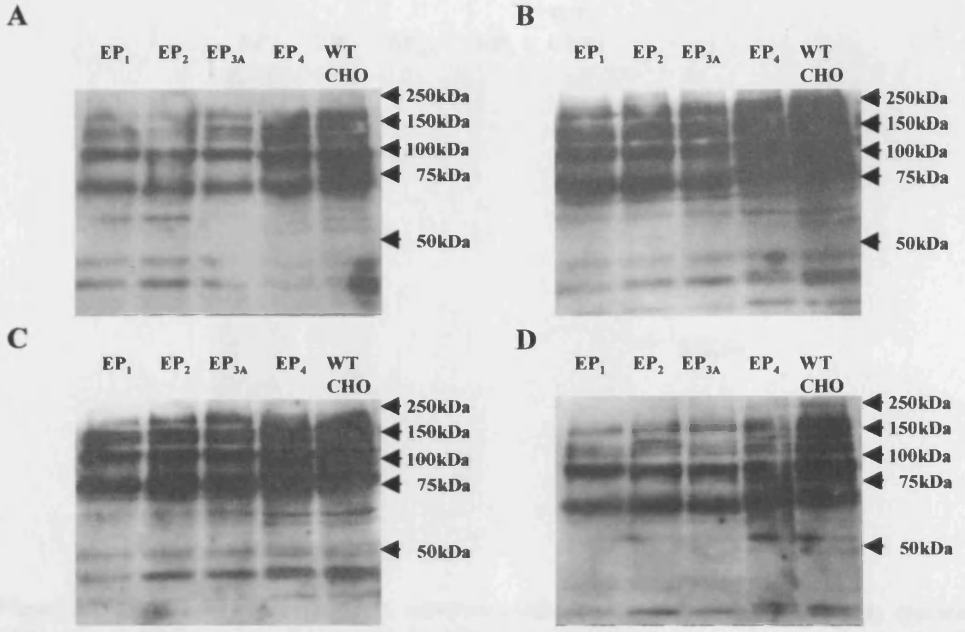


Figure 3.6: Western blot on PG receptor expressing cell lysates and WT CHO cell lysates using the Pepceuticals antibodies. Western blots using the anti-EP_1, R13 (1:100) **(A)** and R14 (1:200) **(B)** antibodies, and the anti-EP_{3C}, R15 (1:200) **(C)** and R16 (1:200) **(D)** antibodies show that the antibodies lack specificity for their respective proteins.

3.2.1.4 Specificity of the Cayman EP_1 antibody to the EP_1 protein

The results from a Western blotting experiment using the Cayman EP_1 receptor antibody (1:250) are shown in figure 3.7. A single band was detected at approximately 43kD in EP_1 CHO cells, which is the expected molecular weight of the rat EP_1 receptor protein (table 3.1). Immunoreactive bands were also observed at approximately 37kD and 50–75kD in the EP_1-CHO cells. No cross reactivity of the antibody was observed in any of the other PG expressing cell lines, indicating that the antibody showed specificity to the EP_1 receptor protein.

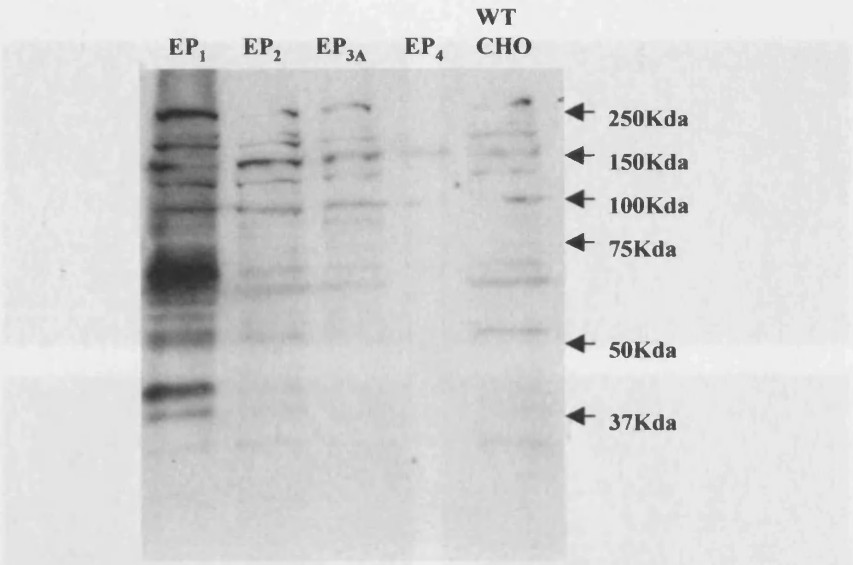


Figure 3.7: Specificity of Cayman EP_1 receptor antibody. Bands were observed in EP_1 expressing CHO cells at 40kDa and 50-72kD using the EP_1 antibody (1:250). No bands were observed in EP_2, EP_{3A} expressing CHO cells, EP_4 expressing HEK cells or WT CHO cells.

	EP_1 (P70597)	EP_2 (Q62928)	$EP_{3\alpha/A}$ (P34980)	EP_4 (P34980)
Molecular weight (kDa)	43.047	39.771	39.942	53.365
Length (amino acid residues)	405	357	369	488
N-Glycosylation sites	3	3	2	2
PKA sites	3	1	2	3
PKC sites	7	8	6	10
Casein kinase II sites	2	4	2	5
N-Myristoylation sites	11	7	4	8
Amidation site	0	0	0	0

Table 3.1: Molecular weights and different functional sites present in rat EP prostaglandin receptors.

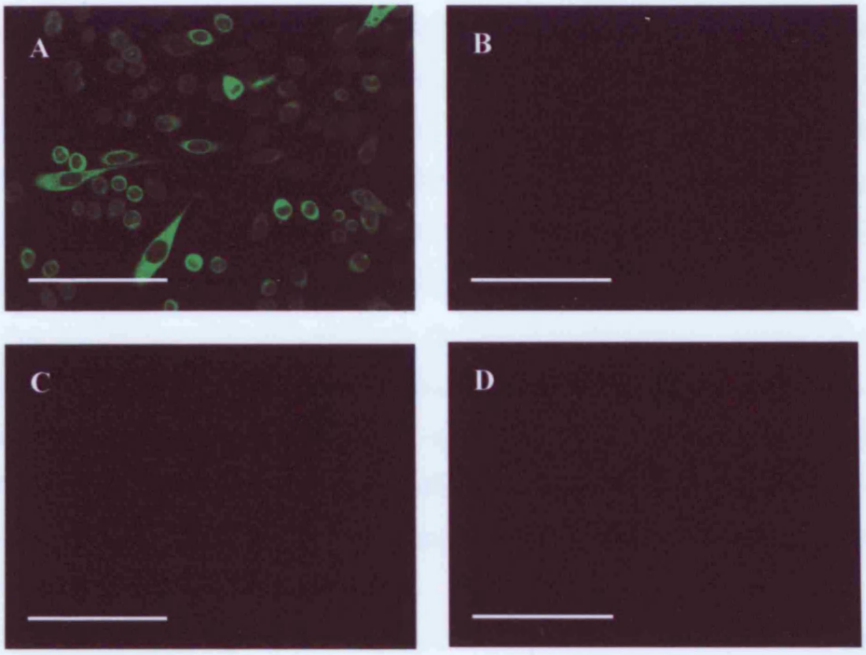


Figure 3.8: EP_1 immunolabelling in prostaglandin receptor expressing cell lines and wild type CHO cells. Labelling was observed in only the EP_1-CHO cells using the Cayman EP_1 antibody (1:250) and was localised to the cell membrane **(A)**. Pre-incubation of the EP_1 antibody (1:250) with the EP_1 control peptide ($20\mu gml^{-1}$) abolished labelling in EP_1-CHO cells **(B)**. No labelling was present in EP_2-CHO **(C)** or WT CHO **(D)** cells. Scale bars, 100μm

Figure 3.8 shows the results of immunocytochemistry performed on EP_1 and EP_2 expressing CHO cells, and WT CHO cells using the Cayman EP_1 receptor antibody (1:250). The immunofluorescence from the goat, anti-rabbit FITC-conjugated secondary antibody (1:200) is illustrated as green in these high magnification confocal images and is referred to as EP_1-like immunoreactivity (EP_1-li). EP_1-li was localised predominantly at the plasma membrane of EP_1-CHO cells (figure 3.8A) but was not observed in EP_2 (figure 3.8B) or WT (figure 3.8C) CHO cells. Pre-incubation of the EP_1 antibody (1:250) with the control peptide ($20\mu gml^{-1}$) (figure 3.8D) greatly reduced the intensity of labelling in the EP_1-CHO cells, indicating that the EP_1 antibody showed specificity to the EP_1 protein. Omission of the EP_1 antibody from the incubation buffer completely abolished FITC-conjugated secondary antibody labeling (data not shown), proving that no non-specific binding of the secondary antibody was occurring.

To determine whether the Cayman EP_1 antibody recognised the EP_1 protein in rat tissue, Western blotting experiments were performed using homogenates of DRG kidney and intestine, both of which, are known to express high levels of EP_1 [Båtshake *et al.*, 1995]. As shown in figure 3.9, the Cayman EP_1 antibody (1:100) recognised multiple bands throughout both tissues, indicating that the antibody binds non-specifically to multiple proteins. Therefore, no immunocytochemical distribution studies were performed in tissue using this antibody.

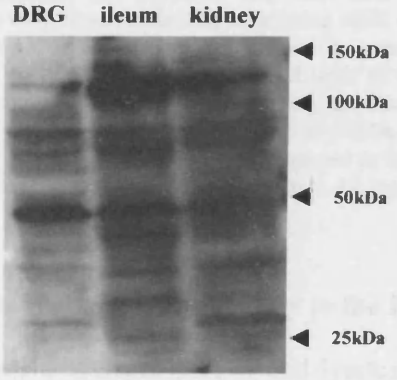


Figure 3.9: Western blot of Cayman EP_1 receptor antibody in native tissue. This figure demonstrates the lack of specificity of the Cayman EP_1 antibody for the EP_1 protein in native tissues known to express the EP_1 receptor.

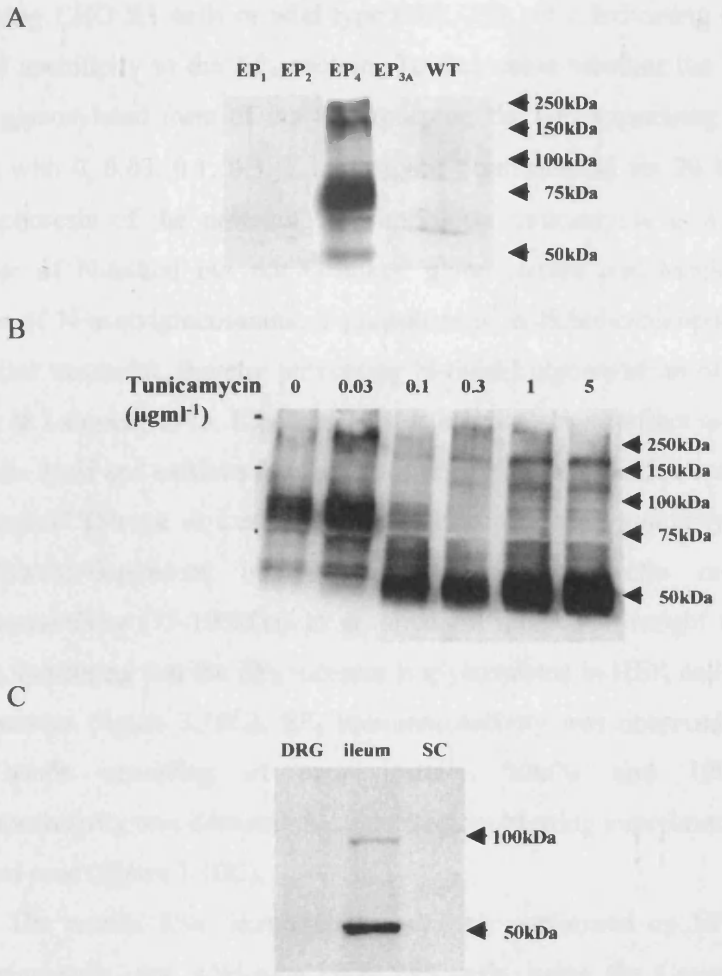


Figure 3.10: Specificity of Cayman EP_4 antibody and inhibition of EP_4 receptor glycosylation with tunicamycin. (A) Bands were observed in EP_4 expressing HEK cells at 50kDa, 75-100kDa, and 150-250kDa using the EP_4 antibody (1:1000). No bands were observed in EP_2, EP_{3A} expressing CHO cells or WT HEK cells. **(B)** EP_4-HEK cells were treated with 0, 0.03, 0.1, 0.3, 1, and 5μgml^{-1} tunicamycin. As the concentration of tunicamycin increased, the band at 75kDa (glycosylated EP_4 receptor) decreased in immunoreactivity whereas the band at 50kDa increased in immunoreactivity using the EP_4 antibody. **(C)** In native tissue, bands were observed in ileum at 50kDa and 100kDa. No bands were observed in DRG and spinal cord. The blots shown are representative of four independent experiments.

3.2.1.5 Specificity of the Cayman EP_4 antibody to the EP_4 protein

Figure 3.10A shows a Western blot of cell lysates prepared from EP_1, EP_2, EP_{3A}, and EP_4 expressing cell lines and a wild-type HEK-293 cell line, using the Cayman EP_4 antibody (1:1000). In EP_4-HEK cells, a minor immunoreactive band was observed at approximately 50kDa, (table 3.1; molecular weight of the EP_4 receptor is 53kDa) and major immunoreactive bands were observed at 75-100kDa and 150-250kDa. There was no cross-reactivity witnessed in EP_1, EP_2, and EP_{3A}

expressing CHO K1 cells or wild type HEK-293 cells, indicating that the antibody showed specificity to the EP_4 protein. To determine whether the 75-100kDa band was a glycosylated form of the EP_4 receptor, the EP_4 expressing HEK cells were treated with 0, 0.03, 0.1, 0.3, 1, and 5μgml^{-1} tunicamycin for 20 hours prior to gel electrophoresis of the proteins. The antibiotic tunicamycin is a highly selective inhibitor of N-linked but not O-linked glycosylation and blocks the enzymatic transfer of N-acetylglucosamine 1-phosphate to dolichol-mono-phosphate within 1 hour after treatment, thereby preventing N-linked glycosylation of various proteins [Tkacz & Lampen, 1975, Elbein, 1987]. It has little or no effect on *de novo* protein synthesis itself and exhibits low general toxicity in the effective concentration range 0.1-20μgml^{-1} [Struck & Lennarz, 1977]. Treatment with tunicamycin resulted in a concentration-dependent increase in the electrophoretic mobility of EP_4 immunoreactivity (75-100kDa) to an apparent molecular weight of 50kDa (figure 3.10B), indicating that the EP_4 receptor is glycosylated in HEK cells. In whole tissue homogenates (figure 3.10C), EP_4 immunoreactivity was observed in the rat ileum with bands occurring at approximately 50kDa and 100kDa. No EP_4 immunoreactivity was detected through Western blotting experiments in the rat DRG or spinal cord (figure 3.10C).

The results from immunocytochemistry performed on EP_2, EP_{3A} and EP_4 expressing cells, and wild-type HEK 293 cells, using the Cayman EP_4 antibody (1:1000) are shown in figure 3.11. The immunofluorescence from the goat, anti-rabbit FITC-conjugated secondary antibody (1:200) is illustrated as white in these digitised images and is referred to as EP_4-like immunoreactivity (EP_4-li). EP_4-li was localised predominantly at the plasma membrane of EP_4 expressing HEK cells (figure 3.11A). No immunoreactivity was observed in the EP_2 (figure 3.11C) and EP_{3A} (figure 3.11D) expressing cells, in the wild-type HEK cells (figure 3.11E), or in any of the cell types during the secondary antibody control experiments (data only shown for EP_4-HEK cells; figure 3.11F), confirming that the EP_4-li is EP_4 receptor-specific. Pre-incubation of the EP_4 antibody (1:1000) with the control peptide (40μgml^{-1}) (figure 3.11B) greatly reduced the intensity of labelling in EP_4 expressing HEK cells, indicating that the EP_4 antibody recognises the peptide against which it was raised.

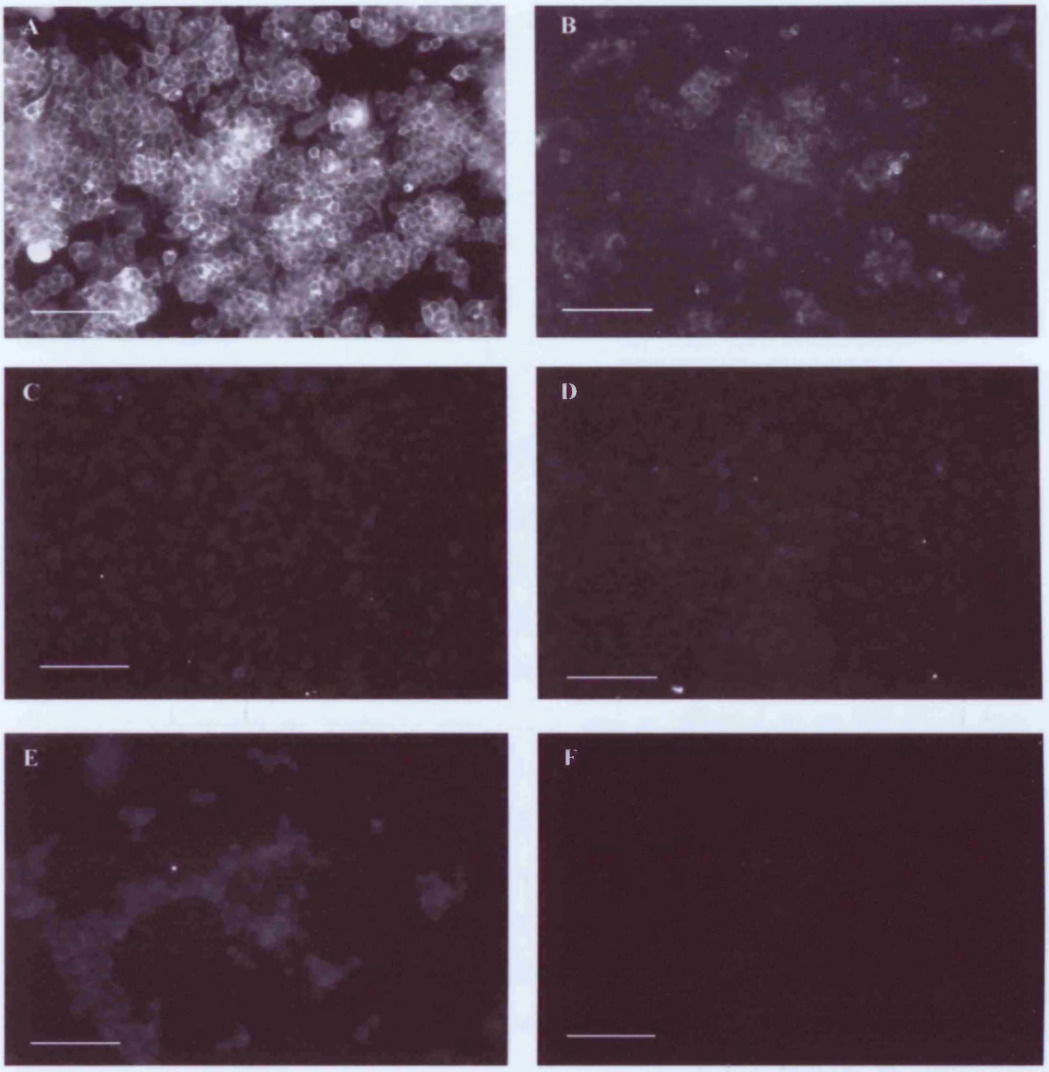


Figure 3.11: EP_4 immunolabelling of prostaglandin expressing cells and wild type HEK cells. EP_4 immunolabelling using the EP_4 antibody (1:1000) was observed in only the EP_4-HEK cells and was localised to the cell membrane **(A)**. Preincubation of the EP_4 antibody (1:1000) with the EP_4 control peptide ($40\mu gml^{-1}$) reduced the intensity of EP_4 labelling in EP_4 HEK cells **(B)**. No staining was observed in EP_2 **(C)**, EP_{3A} **(D)**, or WT HEK **(E)** cells or during the secondary antibody control **(F)**. Scale bars, 100μm.

3.2.2 Localisation of the EP_4 receptor in rat DRG and spinal cord by immunocytochemistry

Of all the antibodies tested, only the Cayman EP_1 and EP_4 receptor antibodies showed specificity to their respective proteins when screened using PG expressing cell lines. In native tissue however, only the Cayman EP_4 antibody showed specificity to the EP_4 protein. As a result, this antibody was used to create a distribution map of EP_4 receptor expression in the rat DRG and spinal cord.

3.2.2.1 Localisation of EP_4 in the rat DRG

Figure 3.12A illustrates a representative composite photomicrograph of a 12μm transverse section of a rat lumbar DRG, which has been immunolabelled for EP_4 using the Cayman EP_4 antibody (1:500) raised in the rabbit. The immunofluorescence from the goat anti-rabbit FITC-conjugated secondary antibody (1:200) is illustrated as white in this image and is referred to as EP_4-li. A very specific pattern of EP_4-li was observed in the DRG with preferential labelling occurring randomly in small neuronal cell bodies. Within the cell bodies the labelling was present in the cytoplasm and in some cells, staining of the nuclear membrane was apparent. In addition, distinct EP_4-li was present in the axonal tracts from small diameter neuronal cells, which suggests that the EP_4 receptor may be transported to peripheral and central axons from the neuronal cell bodies. These observations are illustrated more clearly in the higher magnification confocal images, in which the EP_4-li is shown in green (figure 3.12B and C). Pre-incubation of the EP_4 antibody (1:500) with the control peptide ($40\mu gml^{-1}$) greatly reduced the intensity of EP_4-li in the DRG (figure 3.13), indicating that the staining is specific to the EP_4 receptor. Omission of the EP_4 antibody from the incubation buffer completely abolished FITC-conjugated secondary antibody labeling (data not shown), indicating that no non-specific binding of the secondary antibody was occurring in the DRG.

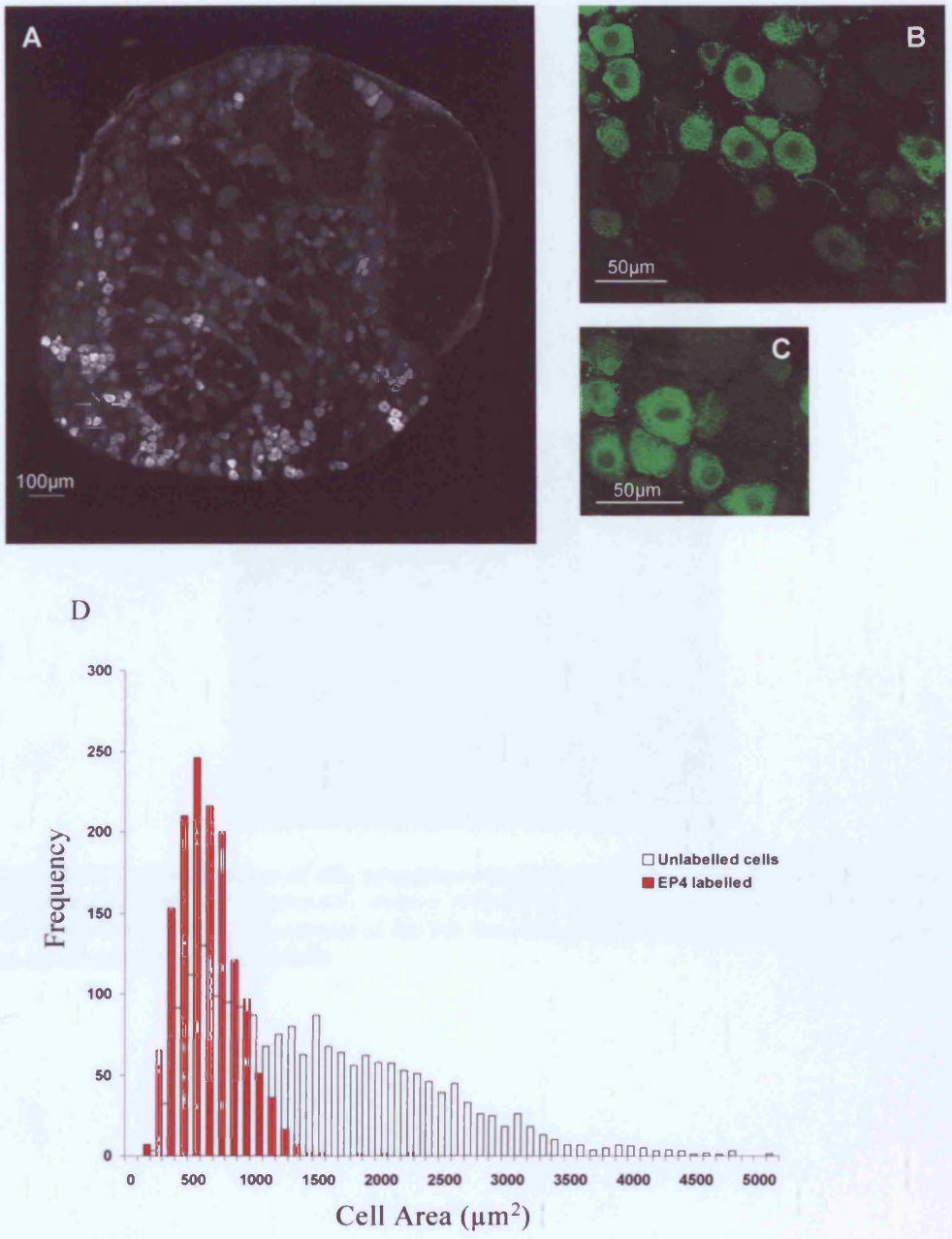


Figure 3.12: EP_4 immunolabelling in lumbar (L4-L6) rat DRG neurons. (A) Representative DRG section labelled for EP_4 (Cayman EP_4 antibody, 1:500). Staining occurred randomly throughout the ganglion in small sized neuronal cell bodies. Confocal images of EP_4-labelled DRG neurons show clear axonal **(B)** and nuclear membrane **(C)** staining. **(D)** The cell size-frequency distribution derived from 9 ganglia shows that most EP_4 labelled neurons were less than 1000µm^2 with a mean cell area of 545.45 ±6.6µm^2.

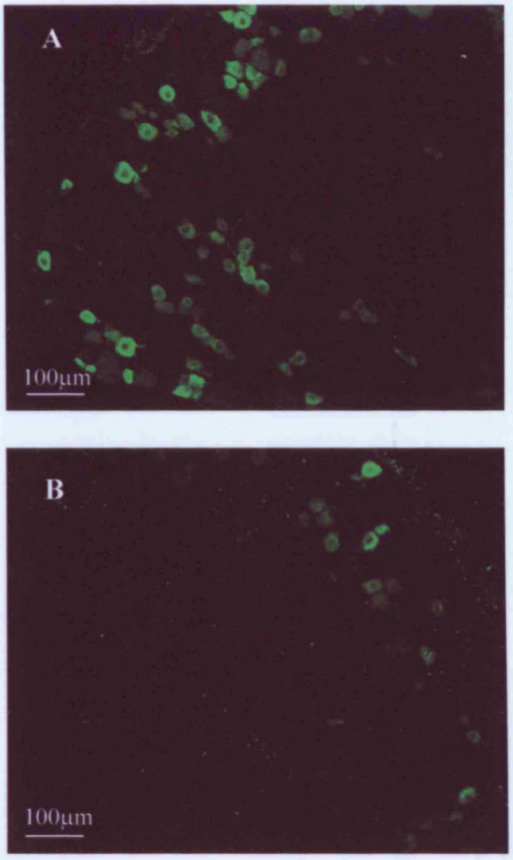


Figure 3.13: Confocal images of EP_4 immunoreactivity in the DRG in the absence and presence of blocking peptide. The Cayman EP_4 receptor antibody (1:500) produces labelling of small diameter cells in the DRG **(A)**. In the presence of the EP_4 blocking peptide ($40\mu gml^{-1}$) this immunoreactivity was almost completely abolished **(B)**.

	All Cells	Labelled		<1000μm² labelled		1000-2000μm² labelled		>2000μm² labelled	
	Count	Count	%	Count	%	Count	%	Count	%
EP_4	3374	1433	42.47	1315	91.77	116	8.09	2	0.14
CGRP	2232	691	30.96	509	73.66	153	22.14	29	4.19
IB4	2459	968	39.37	913	94.32	55	5.68	0	0

	All Cells	Unlabelled		<1000μm² unlabelled		1000-2000μm² unlabelled		>2000μm² unlabelled	
	Count	Count	%	Count	%	Count	%	Count	%
EP_4	3374	1941	57.53	654	33.69	768	39.57	519	26.74
CGRP	2232	1541	69.04	601	39.00	510	33.10	430	27.90
IB4	2459	1491	60.63	752	50.44	541	36.28	198	13.28

	All EP_4 labelled cells	Dual Labelled		<1000μm² labelled		1000-2000μm² labelled		>2000μm² labelled	
	Count	Count	%	Count	%	Count	%	Count	%
EP_4-CGRP	1029	412	40.04	370	89.81	40	9.71	2	0.49
EP_4-IB4	876	796	90.87	766	96.23	30	3.77	0	0

Table 3.2: Morphometric analysis of single and dual labelling in rat dorsal root ganglia.

A morphometric analysis revealed that 42.47% of DRG cell bodies exhibited distinct EP_4-li (table 3.2). The size-frequency histogram shown in figure 3.12D illustrates that EP_4-li occurred mainly in small neuronal cell bodies; 91.77% of neurons exhibiting EP_4-li were less than 1000μm^2 in cell area, 8.09% were 1000-2000μm^2 in cell area, and only 0.14% of neurons exhibiting EP_4-li were greater than 2000μm^2 in cell area (table 3.2). The mean cell area of an EP_4-li neuron was 545.45 ± 6.6μm^2, whereas the mean cell area of an unlabelled neuron was 1471.76 ± 20.37μm^2.

3.2.2.2 Co-localisation of the EP_4 receptor with CGRP and IB4 in the rat DRG

To further characterise the localisation of EP_4 within the rat lumbar DRG, dual labelling immunocytochemistry experiments were performed using CGRP and IB4, which are the markers of peptidergic and non-peptidergic C-fibre nociceptors respectively (section 1.2).

3.2.2.2.1 Localisation of EP_4 with CGRP

Figure 3.14A illustrates a representative photomicrograph of a 12μm transverse section of rat lumbar DRG, which has been immunolabelled for CGRP using the Acris CGRP antibody (1:1000) raised in the guinea pig. The immunofluorescence from the goat, anti-guinea pig TR-conjugated secondary antibody (1:400) is shown as white in this digitised image, and is described herein as CGRP-like immunoreactivity (CGRP-li). CGRP-li was observed as diffuse speckling throughout the cytoplasm of the DRG cell body, and was present in both small and intermediate sized cells. The higher magnification confocal image shown in figure 3.14B illustrates these observations more clearly (CGRP-li shown in red). Omission of the CGRP antibody from the incubation buffer completely abolished TR-conjugated secondary antibody labeling (data not shown), indicating that no non-specific binding of the secondary antibody was occurring.

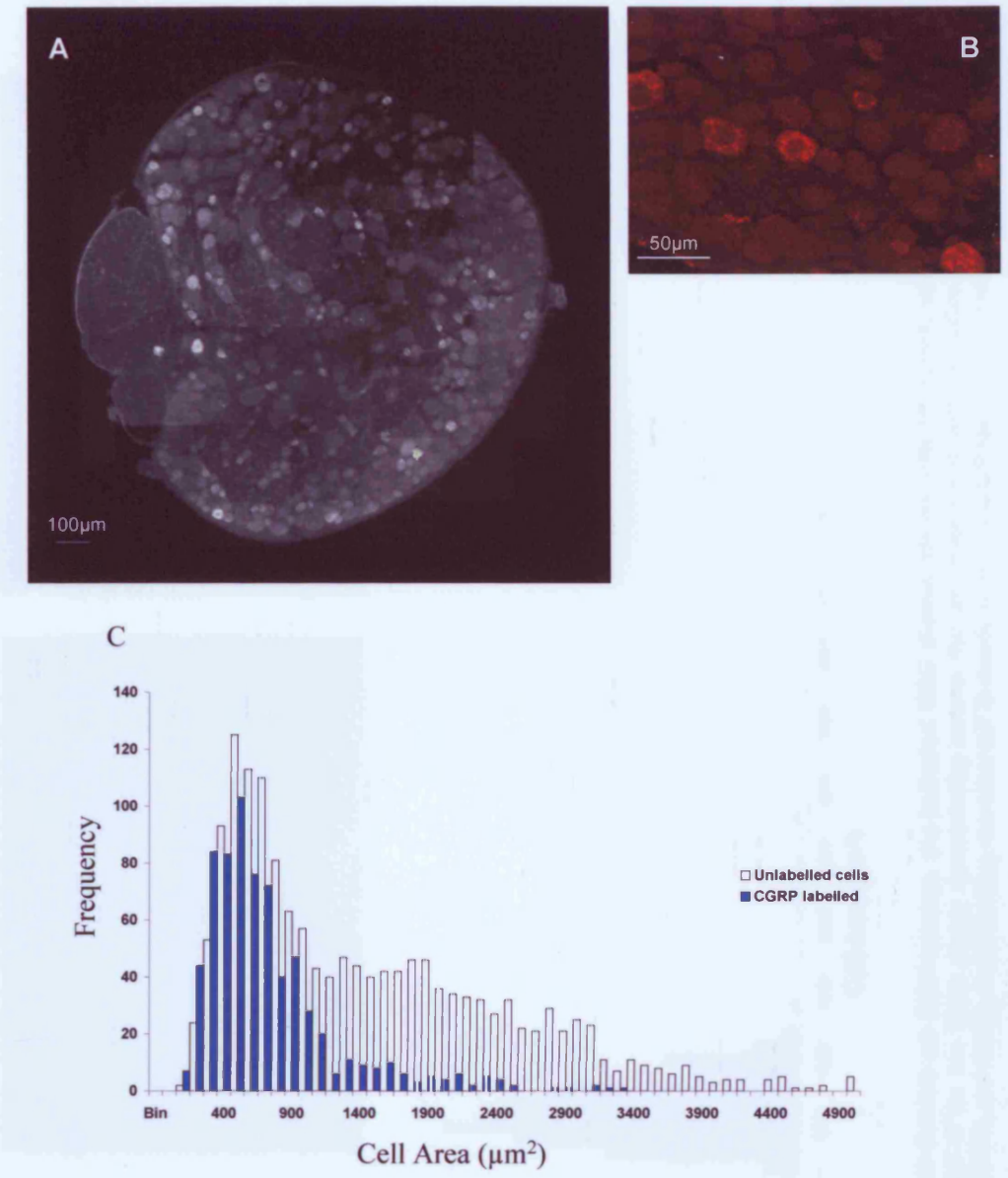


Figure 3.14: CGRP immunolabelling in lumbar (L4-L6) rat DRG neurons. (A) Representative DRG section labelled for CGRP (Acris CGRP antibody, 1:1000). Staining occurred randomly throughout the ganglion in small and medium sized neuronal cell bodies. **(B)** Confocal image showing the typical CGRP pattern of diffuse speckling within the cell cytoplasm. **(C)** Cell size-frequency distribution derived from 6 ganglia. Most CGRP labelled neurons were less than 1000µm^2 but labelling was also present in medium to large sized neurons (>1000µm^2). The mean cell area of a CGRP labelled cell was 768.82 ±19.11µm^2.

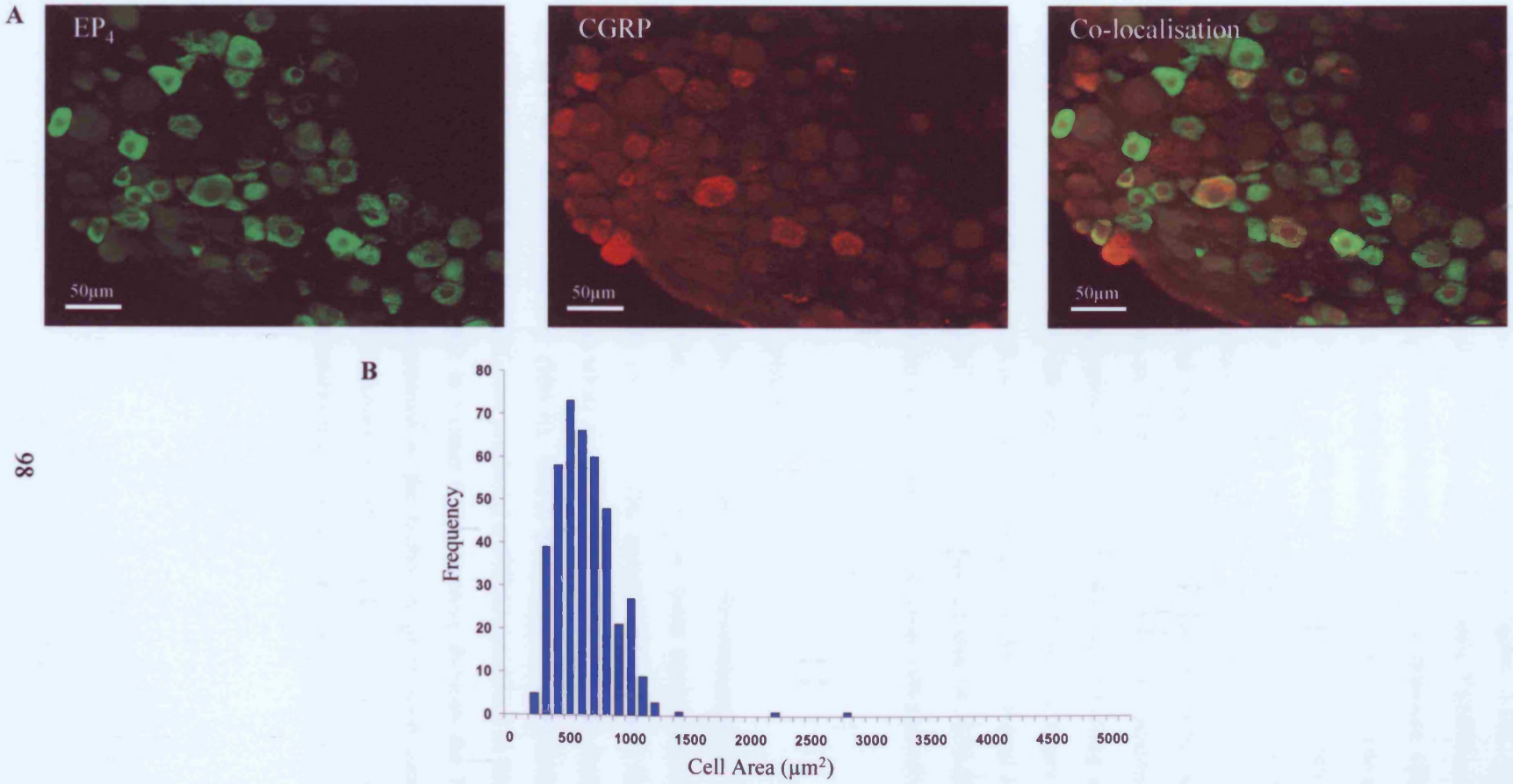


Figure 3.15: Co-localisation of EP_4 and CGRP in lumbar (L4-L6) rat DRG neurons. (A) Individual DRG neurons labelled with EP_4 (green) and CGRP (red) antibodies. The panel on the right shows a composite image of the two labels clearly demonstrating neurons that are labelled with both antibodies. **(B)** Cell size frequency distribution derived for 6 ganglia dual labelled with EP_4 and CGRP. Dual labelling was observed in mainly small cells (<1000µm^2) with a mean cell area of 576.22 ±12.52µm^2.

A morphometric analysis showed that CGRP-li was present in 30.96% cells (table 3.2). The size-frequency distribution shown in figure 3.14C illustrates that CGRP-li occurred in both small and intermediate sized cells; 73.66% cells had a cell area of less than 1000μm^2, 22.14% cells had a cell area of between 1000-2000μm^2, and 4.19% cells had a cell area of greater than 2000μm^2 (table 3.2). The mean area of a CGRP labelled cell was 768.82 ± 19.11μm^2, whereas the mean area of an unlabelled cell was 1495.88 ± 25.34μm^2. These findings correspond to published observations [McCarthy & Lawson, 1990].

When DRG sections were double labelled for CGRP and EP_4, morphometric analysis revealed that 40.04% of EP_4-labelled cells were also labelled by CGRP (table 3.2). Representative examples of EP_4 and CGRP dual labelling are shown in figure 3.15A. As illustrated by the size-frequency distribution in figure 3.15B, dual labelling was restricted to small neuronal cell bodies; 89.81% of dual labelled cells had a cell area less than 1000μm^2, 9.71% of cells had an area of 1000-2000μm^2, and 0.49% cells had a cell area greater than 2000μm^2. The mean cell area of dual labelled cells was 576.22 ± 12.52μm^2.

3.2.2.2.2 Localisation of EP_4 with IB4

Figure 3.16A illustrates a representative photomicrograph of a 12μm transverse section of rat lumbar DRG, which has been immunolabelled for TR-conjugated isolectin B4 (IB4) (3μgml^{-1}). The immunofluorescence from the TR-conjugated IB4 is illustrated as white in this digitized image, and is described herein as IB4-like immunoreactivity (IB4-li). IB4-li was randomly distributed in small neuronal cell bodies of the DRG and produced a diffuse speckling in the cytoplasm. In addition, IB4-li was present in axonal tracts running through the DRG. These observations are clearly demonstrated in the higher magnification confocal image shown in figure 3.16B (IB4-li shown in red). Incubation of the sections in the absence of IB4 reduced immunolabelling to background levels (data not shown).

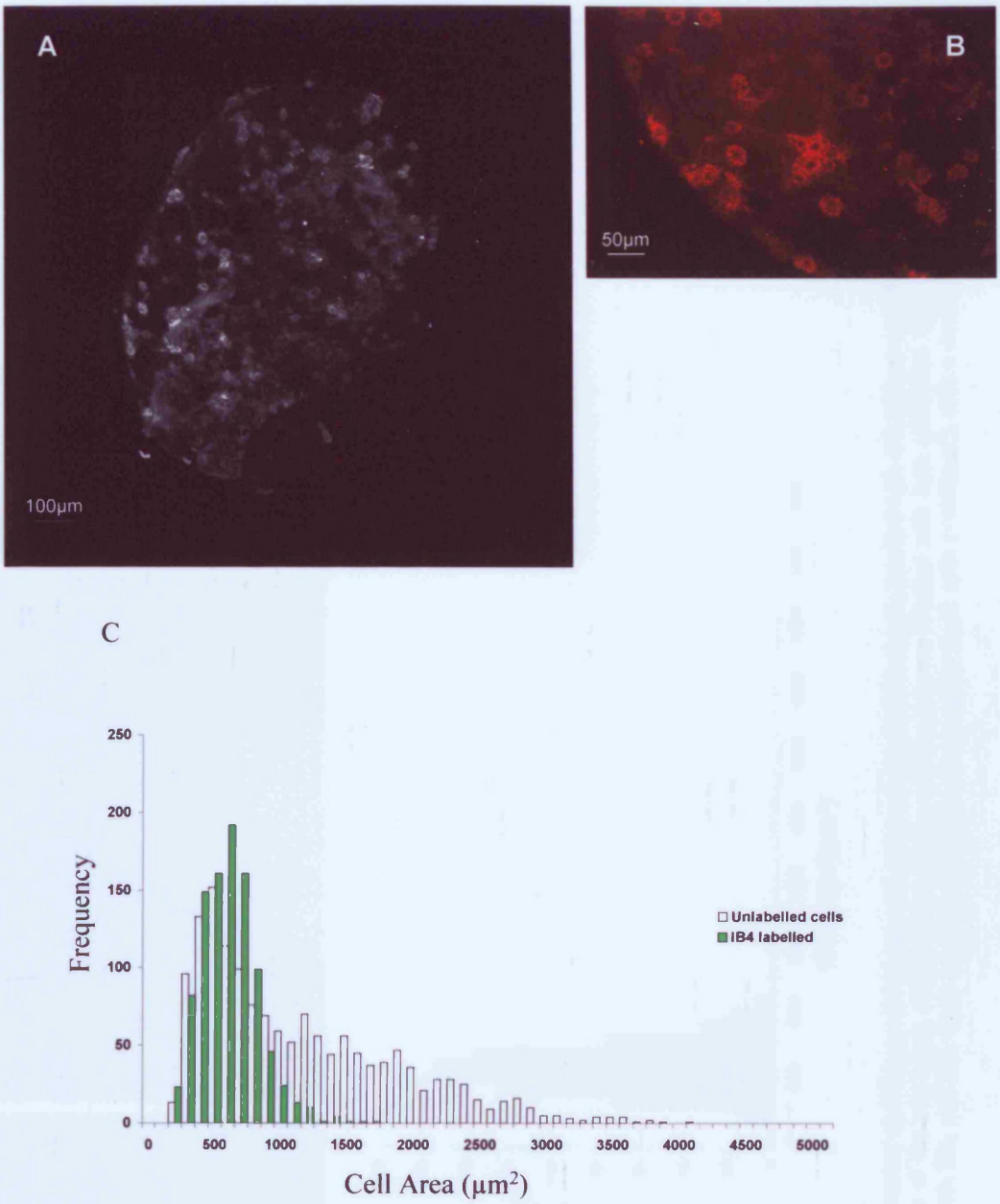


Figure 3.16: IB4 immunolabelling in lumbar (L4-L6) rat DRG neurons. (A) Representative DRG section labelled for IB4 ($3\mu gml^{-1}$). Staining occurred randomly throughout the ganglion in small sized neuronal cell bodies. **(B)** Confocal image showing the typical IB4 pattern of diffuse speckling within the cell cytoplasm. **(C)** Cell size-frequency distribution derived from 6 ganglia. Most IB4 labelled neurons were less than $1000\mu m^2$ with a mean cell area of $550.36 \pm 6.88\mu m^2$.

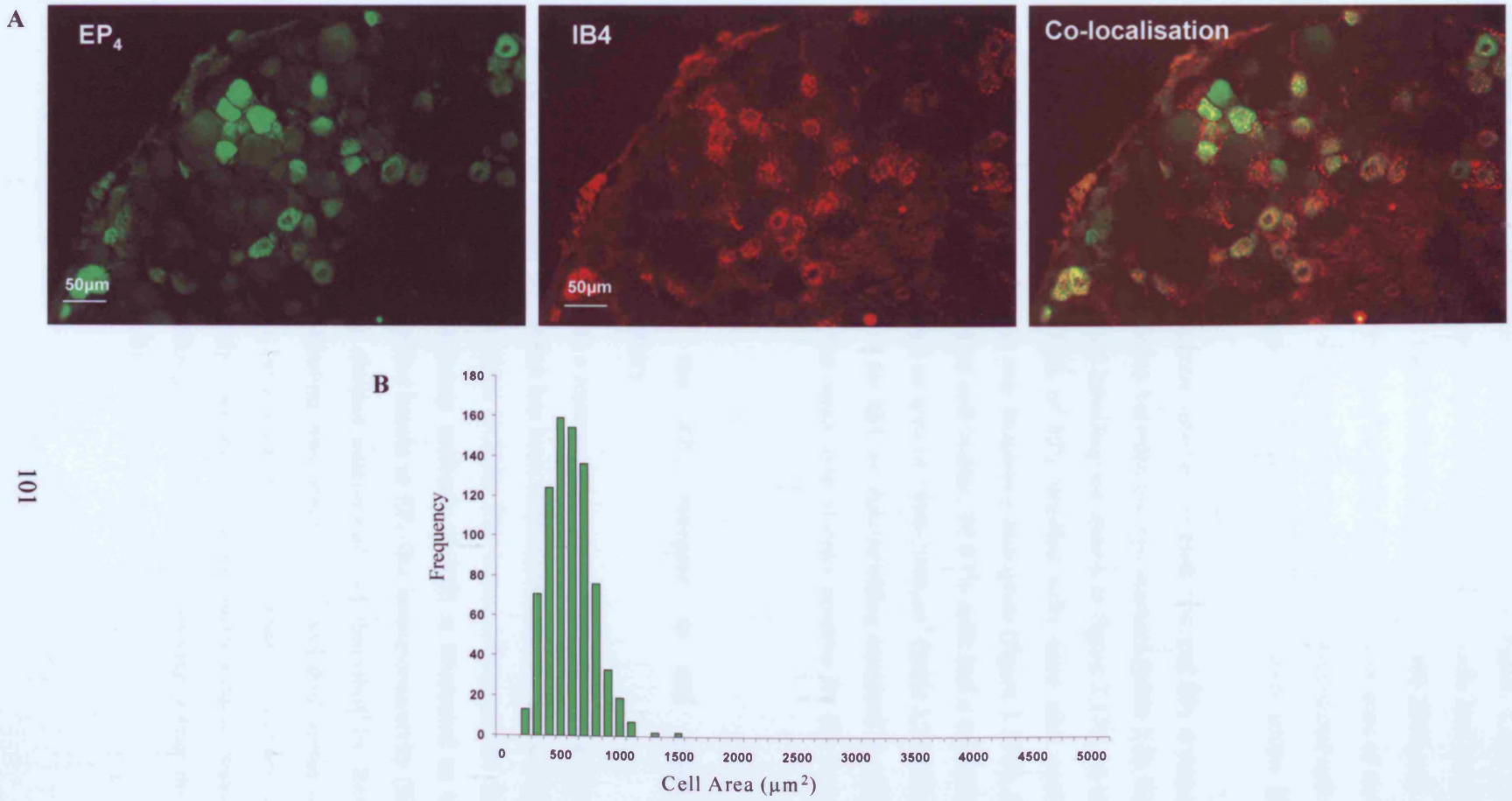


Figure 3.17: Co-localisation of EP_4 and IB4 in lumbar (L4-L6) rat DRG neurons. (A) Individual DRG neurons labelled with EP_4 (green) and IB4 (red) antibodies. The panel on the right shows a composite image of the two labels clearly demonstrating neurons that are labelled with both antibodies. **(B)** Cell size frequency distribution derived for 6 ganglia dual labelled with EP_4 and IB4. Dual labelling was observed in mainly small cells (<1000µm^2) with a mean cell area of 533.59 ±6.79µm^2.

A morphometric analysis revealed that IB4-li was present in 39.37% cells (table 3.2). The size-frequency distribution shown in figure 3.16C illustrates that IB4-li was restricted to small sized cell bodies; 94.32% cells had a cell area of less than 1000μm^2, 5.68% cells had a cell area of between 1000-2000μm^2, and no cells had a large cell area (i.e. >2000μm^2) (Table 3.2). The mean area of an IB4 labelled cell was 550.36 ± 6.88μm^2, whereas the mean area of an unlabelled cell was 1102.12 ± 19.28μm^2. These findings correspond to published observations [Silverman & Kruger, 1990].

When DRG sections were labelled for both IB4 and EP_4 it was apparent that there was a considerable overlap between the two markers (table 3.2). Representative examples of EP_4 and IB4 dual labelling are shown in figure 3.17A. A morphometric analysis revealed that 90.87% of EP_4 labelled cells were also positive for IB4 labelling and as shown in the size-frequency histogram (figure 3.17B), dual labelling was confined to small neuronal cell bodies; 90.87% cells had a cell area of less than 1000μm^2 and 3.77% cells had an area of 1000-2000μm^2 (table 3.2). Not surprisingly, given the data reported above for IB4, no dual labelling occurred in cells with a large cell area (i.e. >2000μm^2). The mean area of cells positive for the two markers was 533.59 ± 6.79μm^2.

3.2.2.3 Localisation of the EP_4 receptor in rat spinal cord by immunocytochemistry

Figure 3.18 illustrates a representative photomicrograph of a 12μm transverse section of rat lumbar SC, which has been immunolabelled for EP_4 using the Cayman EP_4 antibody raised in the rabbit (1:500). The immunofluorescence from the anti-rabbit FITC-conjugated secondary antibody (1:200) is illustrated as white in this digitized image, and is described herein as EP_4-like immunoreactivity (EP_4-li). EP_4-li did not correlate with any distinct anatomical loci described by Rexed in 1952 (section 1.6.1). However, labeling was observed in individual spinal cord neurons throughout the grey matter, indicating that EP_4 is widely expressed in the CNS. Omission of the EP_4 antibody from the incubation buffer reduced immunolabelling levels to background intensities, indicating that the staining within the spinal cord was not due to non-specific binding (data not shown).

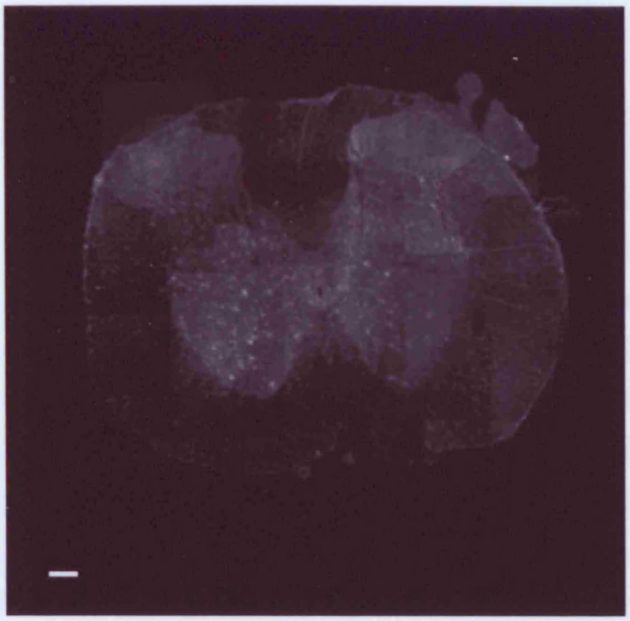


Figure 3.18: EP_4 immunolabelling in rat lumbar (L4-L6) spinal cord. Labelling was observed in individual spinal cord neurons but not in individual spinal laminae. Scale bar, 200μm.

3.2.2.4 Localisation of EP_4 in cultured DRG neurons

Cultured DRG neurons can be used as a model system for studying primary afferent function. Therefore, to assess whether EP_4 receptor expression persists under these conditions and whether such a system might be appropriate for studying neuronal EP_4 receptor function *in vitro*, immunocytochemistry was performed on rat primary DRG cultures using the Cayman EP_4 antibody (1:500) (figure 3.19). In figure 3.19A, the immunofluorescence from the goat, anti-rabbit FITC-conjugated secondary antibody (1:200) is illustrated as green and is referred to as EP_4-like immunoreactivity (EP_4-li). Many cells exhibited EP_4-li especially at the plasma membrane and the nuclear membrane. This is clearly illustrated in the higher magnification confocal image shown in figure 3.19B (EP_4-li shown in green). The size-frequency distribution was not established for EP_4 expression in these cells, however many of the neurons that survived culture conditions were generally small-sized and exhibited capsaicin sensitivity, indicating a nociceptor phenotype.

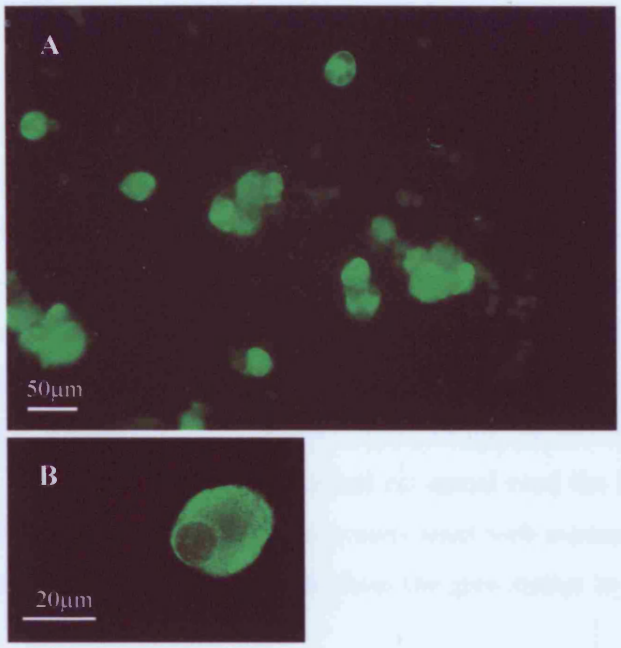


Figure 3.19: EP_4 immunolabelling in cultured rat lumbar (L4-L6) DRG neurons. (A) Many small-sized cultured DRG neurons exhibited labelling for EP_4 (1:500), indicating that EP_4 expression persists in primary tissue culture **(B)** Confocal image showing one small DRG neuron labelled for EP_4. A higher intensity of EP_4-li was observed at the plasma and nuclear membranes.

3.3 DISCUSSION

The work in this chapter has demonstrated using Western blotting and immunocytochemistry techniques on PG receptor expressing cells and native tissue that the Cayman EP_4 receptor antibody shows selectivity for the EP_4 protein. Furthermore, evidence has been provided for the N-linked glycosylation and oligomerisation of EP_4 receptors, which are both believed to be essential for correct PG receptor function and sorting to the plasma membrane. In normal rat DRG tissue, the EP_4 receptor was restricted to small diameter cells of a nociceptor phenotype and was present in distinct neuronal ultra-structures, including the nuclear membrane and neuronal axons. In contrast, within the normal rat spinal cord the EP_4 receptor was not concentrated in any of the spinal laminae associated with nociceptive processing, but was instead distributed uniformly throughout the grey matter in individual spinal neurons.

3.3.1 Prostaglandin receptor antibody characterisation

A wide range of PG receptor antibodies claimed to be subtype-specific were acquired from industrial collaborators Glaxo SmithKline (GSK) and various commercial sources. Western blotting and immunocytochemistry experiments on CHO K1 and HEK 293 cells stably expressing the various PG receptor subtypes assessed the selectivity and specificity of these antibodies. The functionality of the PG expressing cell lines was confirmed by performing specific assays, which assessed whether stimulation of the various receptors with PGE_2 evoked the expected second messenger system signal coupling response. For the EP_1 receptor, calcium imaging was utilised because stimulation of the EP_1 receptor with PGE_2 is known to promote a rise in intracellular calcium [Watabe *et al.*, 1993, Båtshake *et al.*, 1995]. As PGE_2 produced a transient rise in intracellular calcium in EP_1-CHO cells, it was concluded that the EP_1-CHO cell line was expressing functional EP_1 receptors. The functional pharmacology of the EP_2 and EP_4 cell lines has previously been characterised utilising cAMP accumulation assays [Wilson *et al.*, 2004], as both receptor subtypes are coupled to G_s and the activation of adenylate cyclase [Regan *et al.*, 1994, Katsuyama *et al.*, 1995]. Stimulation of both EP_2-CHO and EP_4-HEK cells with PGE_2 resulted in a concentration-dependent rise in cAMP accumulation, proving that both cell lines were expressing functional receptors.

When tested against the various PG receptor expressing cell lines, only the Cayman EP_1 and EP_4 receptor antibodies showed specificity to their corresponding proteins. The Cayman EP_1 receptor antibody was not however suitable for distribution studies as the antibody bound non-specifically to a variety of different cellular proteins when tested in native tissue. Therefore, the work described in this chapter has focused on the EP_4 receptor distribution in the rat DRG and SC.

3.3.1.1 Selectivity of the Cayman EP_4 antibody

In Western blotting experiments the Cayman EP_4 antibody recognised a single band at approximately 53kDa (the expected molecular weight for the EP_4 receptor) in only EP_4 expressing HEK cells, indicating that the antibody shows specificity to the EP_4 protein. However, immunoreactive bands were also observed at higher molecular weights in EP_4-HEK cell lysates. To determine whether any of these bands were due to glycosylation of the EP_4 receptor, the EP_4-HEK cells were treated with tunicamycin for 20 hours before the experiment. This treatment resulted in a decrease in immunoreactivity of the 75-100kDa band leaving an apparently single entity with a molecular weight of approximately 53kDa, indicating that the EP_4 receptor protein molecules are modified post-translationally with carbohydrate moieties, thereby producing glycoproteins. In line with this, the rat EP_4 receptor has two potential N-linked glycosylation sites (asparagine (Asn)-x-serine/threonine) at Asn^7 in the amino terminal segment and Asn^{177} in the second extracellular loop [Sando *et al.*, 1994]. Furthermore, there is substantial evidence to suggest that other prostaglandin receptor subtypes are also subject to N-linked glycosylation, including the EP_3 [Huang & Tai, 1998, Böer *et al.*, 2000], IP [Zhang *et al.*, 2001], and TP [Walsh *et al.*, 1998] receptor subtypes. It is believed that the glycosylated form of the prostaglandin receptor is necessary for correct sorting and trafficking of the protein to the plasma membrane [Walsh *et al.*, 1998, Böer *et al.*, 2000], normal ligand binding [Huang & Tai, 1998, Walsh *et al.*, 1998, Zhang *et al.*, 2001], and signal transduction [Walsh *et al.*, 1998, Zhang *et al.*, 2001]. Interestingly, the glycosylated form of the EP_4 receptor seen here prior to application of tunicamycin is approximately 20kDa heavier than the EP_4 receptor in its native form. Similar observations have previously been reported for the EP_3 [Nakamura *et al.*, 2000] and TP receptor subtypes [Mais *et al.*, 1992].

The EP_4 receptor antibody also recognised a tunicamycin-insensitive band of protein at approximately 150kDa in EP_4-HEK cells. It is hypothesised that this band represents an oligomer of the EP_4 receptor as there is substantial evidence to suggest that dimerisation / oligomerisation occurs in a variety of other GPCRs. The first evidence to support the existence of such structures came from the observation that the β_2-adrenoceptor could form oligomers that were dependent upon the presence of specific sequences found in transmembrane domain VI of the receptor [Hebert *et al.*, 1996]. Dimerisation / oligomerisation has since been reported for the IP [Giguère *et al.*, 2004] and TP [Laroche *et al.*, 2005] receptors. For the TP receptor (both α and β subtypes), the formation of oligomers has been shown to be highly important for successful second messenger system signal coupling [Laroche *et al.*, 2005]. Interestingly, intermolecular interactions have also been reported to occur between IP and TPα receptor subtypes, leading to the formation of heteroligomers [Wilson *et al.*, 2004]. This is an interesting finding as these prostanoid receptor subtypes are coupled to different second messenger systems and their respective ligands produce biologically opposite effects. PGI_2 activates the IP receptor, which is coupled predominantly to G_s (section 1.3.2.3.2), is a potent vasodilator, inhibits platelet aggregation [Moncada & Vane, 1981] and smooth muscle cell growth *in vitro* [Zucker *et al.*, 1998], and demonstrates anti-thrombotic and anti-platelet actions *in vivo* [Cheng *et al.*, 2002]. Both isoforms of the TP receptor (α and β) couple to PLC but TPα has been implicated in the activation of G_s and TPβ is also believed to activate G_i [Hirata *et al.*, 1996, Vezza *et al.*, 1999, Walsh *et al.*, 2000]. The ligand of the TP receptor, TxA_2 is an important mediator in vascular disease as it acts as a potent vasoconstrictor [Dorn *et al.*, 1987], stimulates platelet aggregation [FitzGerald, 1991], amplifies the activity of other platelet agonists [FitzGerald, 1991], and stimulates proliferation of smooth muscle cells [Pakala *et al.*, 1997]. Wilson and colleagues have shown that stimulation of TPα/IP heterodimers in HEK cells with TP agonists leads to a preferential stimulation of G_s, resulting in a PGI_2-like cellular response, an effect, which was not observed in HEK cells expressing either receptor alone [Wilson *et al.*, 2004b]. Therefore, the heterodimerisation of IP and TPα receptors potentially inhibits the deleterious effects of TxA_2. It is hypothesised that heteroligomerisation is not limited to IP and TPα receptor subtypes alone. Other prostaglandin receptors may also have the capability of forming

heterodimers / heteroligomers, thereby inhibiting or enhancing the effect of one another under pathophysiological conditions.

Oligomerisation of the EP_4 receptor was also observed in native tissue. EP_4 immunoreactive bands were observed in rat ileum at 50 and 100kDa. The former band is the approximate molecular weight for the EP_4 protein whereas the latter is approximately twice the expected molecular weight, indicating a dimerised form of the receptor. No bands were observed in DRG or spinal cord tissue. Although the EP_4 receptor has been detected by Northern blot in these tissues [Donaldson *et al.*, 2001], the expression levels are not expected to be as high as in the ileum where expression of EP_4 occurs abundantly [Honda *et al.*, 1993, Bastien *et al.*, 1994, Breyer *et al.*, 1996]. Therefore, in order to detect the EP_4 receptor protein in these crude tissues by Western blotting methods, it is envisaged that prior immunopurification of the tissue homogenates would be required to enrich the concentration of EP_4 receptor proteins. In support of this theory, Nakamura and colleagues have shown that this procedure was necessary for the detection of the EP_3 receptor in rat brain tissue by Western blotting [Nakamura *et al.*, 1999].

EP_4-related immunofluoresence in EP_4-HEK cells was localised predominantly at the plasma membrane. This staining was not present in the other prostaglandin expressing cells or in the wild type HEK cells, and was completely abolished in the presence of the Cayman EP_4 antigen, further clarifying the specificity of the Cayman EP_4 antibody. The localisation of the EP_4 receptor to the plasma membrane of EP_4-HEK cells is in agreement with results obtained from a previous immunocytochemical study on EP_4 expressing HEK cells, which utilised a different, custom-made selective EP_4 antibody [Slipetz *et al.*, 2001].

3.3.2 EP_4 localisation in the rat lumbar dorsal root ganglion

In the rat lumbar DRG (L_4-L_6), small diameter primary afferent fibres, which transmit noxious information from the periphery can be divided into two subpopulations; those that contain neuropeptides such as Substance P and CGRP, and those that bind the isolectin IB4 (section 1.2). In this chapter, evidence has been provided, which indicates that the EP_4 receptor has a role in nociception. Morphometric analysis of immunocytochemical localisation of EP_4 in the DRG has shown the preferential localisation to small diameter ($<1000\mu m^2$) neuronal cell

bodies. Further support for those cells expressing the EP_4 receptor as nociceptors has been provided by their co-localisation with the nociceptor markers CGRP and IB4. Whilst there was co-localisation with both markers, the EP_4 receptor was predominantly localised to IB4 labelled, non-peptidergic neurons, which are associated with a phenotype that includes expression of the TRPV1 receptor for noxious heat and the $P2X_3$ ATP receptor, which is also important in pain signalling [Guo *et al.*, 1999]. Although peptidergic and non-peptidergic neurons are polymodal, a functional distinction correlating with morphological characterisation is now apparent [Snider & McMahon, 1998, Caterina & Julius, 1999], whereby neuropeptide-containing neurons contribute to neurogenic inflammation and are thought to largely contribute to inflammatory pain [Mantyh *et al.*, 1997], whereas IB4 positive neurons have been implicated in neuropathic pain [Malmberg *et al.*, 1997].

The EP_4 receptor has previously been detected in the rat DRG [Donaldson *et al.*, 2001] but no information regarding the expression or distribution of this receptor within the DRG has been provided. A recent study however, has shown that the EP_4 receptor contributes to PGE_2-mediated stimulation of renal sensory neurons, and as part of the work, the Cayman EP_4 antibody was utilised to map the EP_4 receptor immunocytochemically to Th_9-L_1 DRGs, which contain the majority of cell bodies of afferent renal nerves [Kopp *et al.*, 2004]. The researchers have reported that EP_4-li was present within approximately 40% of DRG neurons, of which approximately 40% also contained CGRP-li. The results of Kopp and colleagues concur with the findings reported in this chapter, which implies that the EP_4 receptor is expressed at similar levels in both the Th_9-L_1 and L_4-L_6 DRGs, whose afferent projections predominantly innervate the renal pelvic wall and hindpaw of the rat respectively. It is worthy of note however, that the work presented in this chapter is the first to clarify the specificity of the Cayman EP_4 antibody and provide a detailed morphometric analysis of EP_4 receptor distribution within the rat L_4-L_6 DRGs.

Expression of the EP_4 receptor in the rat lumbar DRG was observed in distinct neuronal ultrastructures, including the axonal tracts and nuclear membrane (Figure 3.12). The sensory neurons of the rat lumbar DRG are predominantly psuedounipolar and have a bifuracated axon with central and peripheral projections. Therefore, the presence of EP_4-li within the axonal tracts running through the lumbar

DRG suggests that this receptor may be transported to peripheral and central terminals and could be involved in both peripheral and central pain processes.

The occurrence of the EP_4 receptor at the nuclear membrane is intriguing, as it has generally been assumed that signal transduction cascades are initiated at the plasma membrane and not the nuclear membrane. However, evidence suggests that the nuclear envelope plays a major role in signal transduction as heterotrimeric and low molecular weight G-proteins [Saffitz *et al.*, 1994, Baldassare *et al.*, 1997], phospholipase C [Divecha *et al.*, 1991], phospholipase D [Baldassare *et al.*, 1997], and adenylate cyclase [Yamamoto *et al.*, 1998] have all been shown to localise there. Furthermore, the detection of PGE_2, COX-1, and COX-2 in the perinuclear envelope [Spencer *et al.*, 1998, Chopra *et al.*, 2000], has led researchers to speculate that prostaglandins are not only synthesised, but also act at or near this site. In line with this, the EP_1 [Bhattacharya *et al.*, 1998], $EP_{3\alpha}$ [Bhattacharya *et al.*, 1999], and EP_4 [Bhattacharya *et al.*, 1999] receptors have all been localised to the nuclear membrane of porcine cerebral microvascular endothelial cells and HEK cells stably over-expressing the various receptors. In addition, it has been demonstrated that nuclear prostanoid receptors are functional as stimulation of nuclear EP_1 [Bhattacharya *et al.*, 1998] and $EP_{3\alpha}$ [Bhattacharya *et al.*, 1999] receptors using the respective receptor agonists leads to calcium uptake in intact isolated nuclei. In the case of the $EP_{3\alpha}$ receptor, this was observed to be dependent on the pertussis toxin sensitive G-protein G_i [Bhattacharya *et al.*, 1999].

Nuclear calcium is known to play a major role in the regulation of gene transcription [Santella & Carafoli, 1997, Rogue & Malviya, 1999, Bading, 2000]. Activation of both the EP_1 and $EP_{3\alpha}$ receptors leads to perinuclear calcium uptake, implying that these receptors could influence the transcription of various genes. In support of this, activation of nuclear EP_1 receptors leads to the induction of the immediate early gene *c-fos* in swiss 3T3 cells stably overexpressing EP_1 [Bhattacharya *et al.*, 1998], and activation of nuclear $EP_{3\alpha}$ receptors leads to an increased transcription of the gene for inducible nitric oxide synthase (*iNOS*) in porcine brain endothelial cells [Bhattacharya *et al.*, 1999]. Therefore, during nociceptive processes it is envisaged that enhanced PGE_2 synthesis and activation of EP receptors influences the expression of various proteins, leading to pro-nociceptive effects. In line with this, it has been shown that the proportion of cultured rat DRG

neurons that express NK_1 receptors for the inflammatory mediator substance P (section 1.3.7) increase following PGE_2 treatment [Segond von Banchet *et al.*, 2003].

The localisation of the EP_4 receptor to small diameter DRG cells makes the EP_4 receptor an attractive candidate for mediating the nociceptive effects of PGE_2. As the expression of the EP_4 receptor was maintained in cultured DRG neurons, this provides an ideal model system by which to further study the role of the EP_4 receptor in nociceptor function.

3.3.3 EP_4 in the rat lumbar spinal cord

In the rat lumbar spinal cord, EP_4-li was observed uniformly throughout the grey matter in individual spinal cord neurons, but was not confined to any of the superficial spinal laminae, which would be expected if the EP_4 receptor had a role in spinal nociceptive processing (section 1.6.1). One explanation for this is that the EP_4 receptor is not involved in mediating the effects of spinal PGE_2 during normal physiological functioning, but under pathophysiological conditions, is upregulated and contributes to spinal PGE_2-mediated nociception. Indeed, an *in situ* hybridisation study in rats has shown that following an inflammatory challenge (lipopolysacharide, interleukin-1β, and turpentine), the level of EP_4 transcription is dramatically up–regulated in the corticotrophin–releasing factor (CRF) neurons of the parvocellular paraventricular nucleus and in the catecholaminergic cells of the nucleus of the solitary tract, ventrolateral medulla, and locus coeruleus, indicating an important role for the EP_4 receptor in these regions during inflammation [Zhang and Rivest, 1999]. In order to prove whether this is true of EP_4 receptor expression in the spinal cord, *in situ* hybridisation and / or immunocytochemical studies would need to be conducted on spinal cord tissue from rat models of inflammation and chronic pain states. It is worthy of note however, that there is accumulating evidence to suggest a role for the EP_1 receptor as opposed to the EP_4 receptor in spinal nociceptive processing [Minami *et al.*, 2001, Mebane *et al.*, 2003, Nakayama *et al.*, 2002, 2004, Bär *et al.*, 2004].

In conclusion, it is hypothesised that in the normal rat, the EP_4 receptor has an important role in mediating the pro-nociceptive effects of peripherally released PGE_2. Centrally released PGE_2 however, either acts via a PG receptor distinct from

the EP_4 receptor or acts presynaptically on the central terminals of primary afferent fibres to produce pro-nociceptive effects.

Chapter Four

Sensitising effects of prostaglandin E_2 in rat cultured DRG neurons

4.1 INTRODUCTION

Sensitisation of primary afferent nociceptors (Aδ and C fibres) is one factor that contributes to the hyperalgesia, which accompanies inflammation and tissue injury. Characteristic traits of sensitised primary afferent fibres include a decreased threshold for a response to mechanical or thermal stimuli, an increased response to a supra-threshold stimulus, and in some cases, spontaneous activity [Millan, 1999].

Substances released in injured or inflamed tissue such as prostanoids, bradykinin, and ATP (section 1.3) can contribute to the sensitisation of primary afferent neurons. Such substances produce peripheral sensitisation by two different molecular mechanisms. Firstly, the threshold for firing or subsequent firing patterns may be changed by the modification of voltage-gated ion channel properties (section 1.5.1). Secondly, other ion channel (non voltage-gated) or chemical mediator-evoked responses, which underlie noxious thermal or mechanical transduction currents, may be enhanced by the action of various components of inflammation (section 1.4.1.2 and 1.5.2).

The work in this chapter is concerned with the latter mechanism and has focussed on the TRPV1 channel, which is a heat, proton, and capsaicin-gated ion channel, expressed on C-fibre nociceptors (section 1.4). It has a prominent role in thermal nociception as demonstrated by studies using TRPV1 knockout mice. In these studies, TRPV1 knockout mice exhibit pathological mechanical hyperalgesia but do not show thermal hyperalgesia after mustard oil or complete Freund's adjuvant-induced inflammation [Caterina *et al.*, 2000, Davies *et al.*, 2000]. This indicates that the TRPV1 protein is a prerequisite for the molecular changes responsible for inflammation-evoked thermal hypersensitivity, and infers that inflammatory mediators can sensitise TRPV1 function. This theory has been exploited in this chapter by investigating the effect of the inflammatory substance

PGE_2 on TRPV1-evoked responses. Studies have previously shown that PGE_2 can sensitise the TRPV1-evoked inward current in DRG neurons [Lopshire & Nicol, 1997, 1998]. However, due to the lack of selective PG receptor compounds, no previous attempts have been made to determine whether the sensitisation is PG receptor dependent. The work in this chapter has utilised a selection of selective PG receptor compounds, kindly provided by industrial collaborators GlaxoSmithKline (GSK) to address this question. Primary cultures of DRG neurons from adult rats were chosen as a model system for the experiments as primary afferent fibres have cell bodies in dorsal root ganglia (DRG), and are principally responsible for conveying signals for noxious, thermal and mechanical sensation to the spinal cord (section 1.2). Calcium imaging studies were performed on cultured rat DRG cells as the use of this technique has been shown to be a reliable indicator of TRPV1 function [Greffrath *et al.*, 2001, Savidge *et al.*, 2001].

4.2 RESULTS

4.2.1 Intracellular calcium responses in DRG neurons evoked by direct PGE_2 application

Before the sensitising effects of PGE_2 were investigated, experiments were performed to characterise the intracellular calcium responses evoked by direct PGE_2 application in cultured DRG cells. Figure 4.1 shows a transmitted light image of 2-day old cultured adult rat DRG cells. The DRG cells are easily identified as they have rounded cell bodies with fine processes extending from the neuronal soma and some visible nuclei apparent. In addition, elongated glial cells can be observed, which become established approximately 2 days after the culture procedure and appear more flattened as compared to the DRG cells.

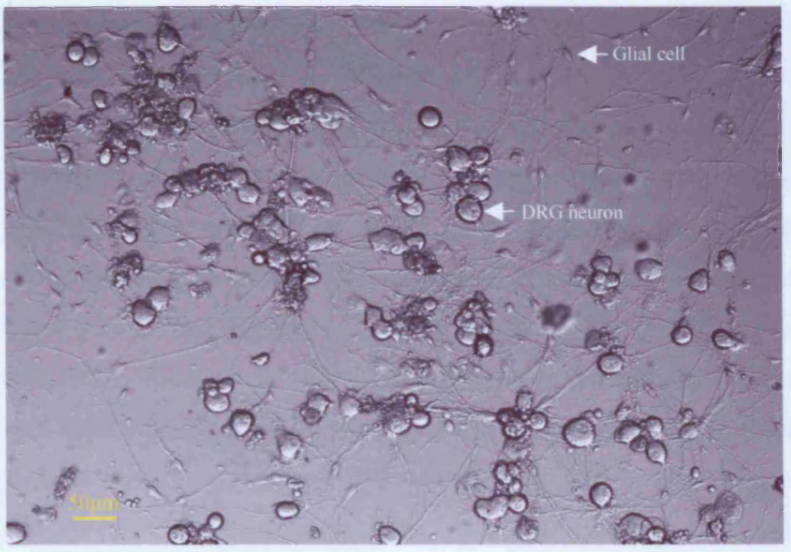


Figure 4.1: Transmitted light image of cultured adult rat DRG neurons. The annotations show examples of DRG and glial cells.

PGE_2 (500nM) was applied for 50 seconds via U-tube, and evoked a rise in intracellular calcium in only 7.8% (47/599) of cells. Figure 4.2 shows some examples of the PGE_2 responses obtained in different DRG cells. In 46.8% (22/47) of the responding cells, PGE_2 evoked a rapid rise in $[Ca^{2+}]_i$, which declined back to basal levels upon agonist removal (figure 4.2A). Two types of oscillatory responses were also apparent. The first, occurred in 10.6% (5/47) cells and was characterised by baseline erratic oscillations, which were sustained even after PGE_2 application had ceased (figure 4.2B). The second type occurred in 34.0% (16/47) cells and was characterised by an increase in $[Ca^{2+}]_i$, which was maintained in the presence of agonist, with erratic oscillations occurring above the elevated level (figure 4.2C). Finally, in 8.5% (4/47) cells a transient increase in $[Ca^{2+}]_i$ was observed, which rapidly returned to basal levels despite the continued presence of agonist (figure 4.2D).

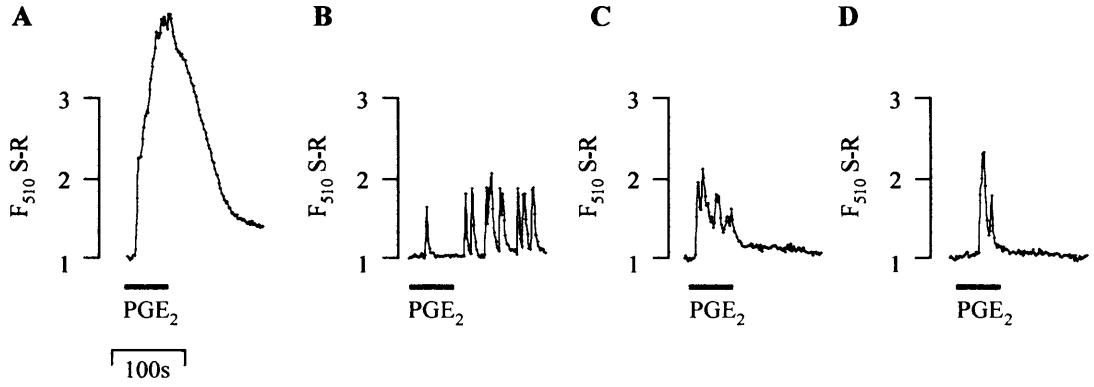


Figure 4.2: Effect of PGE_2 on intracellular calcium in capsaicin-sensitive DRG neurons. A to D) Traces showing examples of $[Ca^{2+}]_i$ responses obtained from four different DRG neurons after application of PGE_2 (500nM, 50 seconds).

To determine whether the PGE_2-evoked calcium responses were EP receptor mediated, an antagonist cocktail consisting of 500nM GW683868X (EP_1 antagonist), 500nM GW627368X (EP_4 antagonist), and 500nM GW671021X (EP_3 antagonist) was applied to cultured DRG cells. These compounds were kindly donated by industrial collaborators GlaxoSmithKline, and their properties are summarised in table 4.1. In this series of experiments, all of the aforementioned types of PGE_2-evoked calcium responses were pooled for analysis purposes. The experimental record shown in figure 4.3A shows calcium responses obtained upon repeated application of PGE_2 (500nM, 50 seconds) at 10-minute intervals in the presence and

Compound	Prostaglandin Receptor	Agonist / Antagonist	Molecular Weight	Solubility	Potency	Reference
GW683868X	EP_1	Antagonist	522	DMSO	pK_i = 8.5 (Functional assay)	A Michel, personal communication
GW627368X	EP_4	Antagonist	544	DMSO	pK_i = 7.1 (Binding assay) pK_B = 7.9 (Functional assay)	Wilson & Giles, 2005 Jones & Chan, 2005 Wilson *et al.*, 2006
GSK324202A	EP_4	Agonist (partial)	405	DMSO	pEC_{50} = 7.7 (Functional assay-68% of maximum PGE_2 response) pK_i = 7.1 (Binding assay)	Giblin *et al.*, 2002
GW671021X	EP_3	Antagonist	536	DMSO	pIC_{50} = 7.8 (Functional assay) pK_i = 9.5 (Binding assay)	Juteau *et al.*, 2001

Table 4.1:Properties of selective prostaglandin receptor compounds obtained from Glaxo SmithKline.

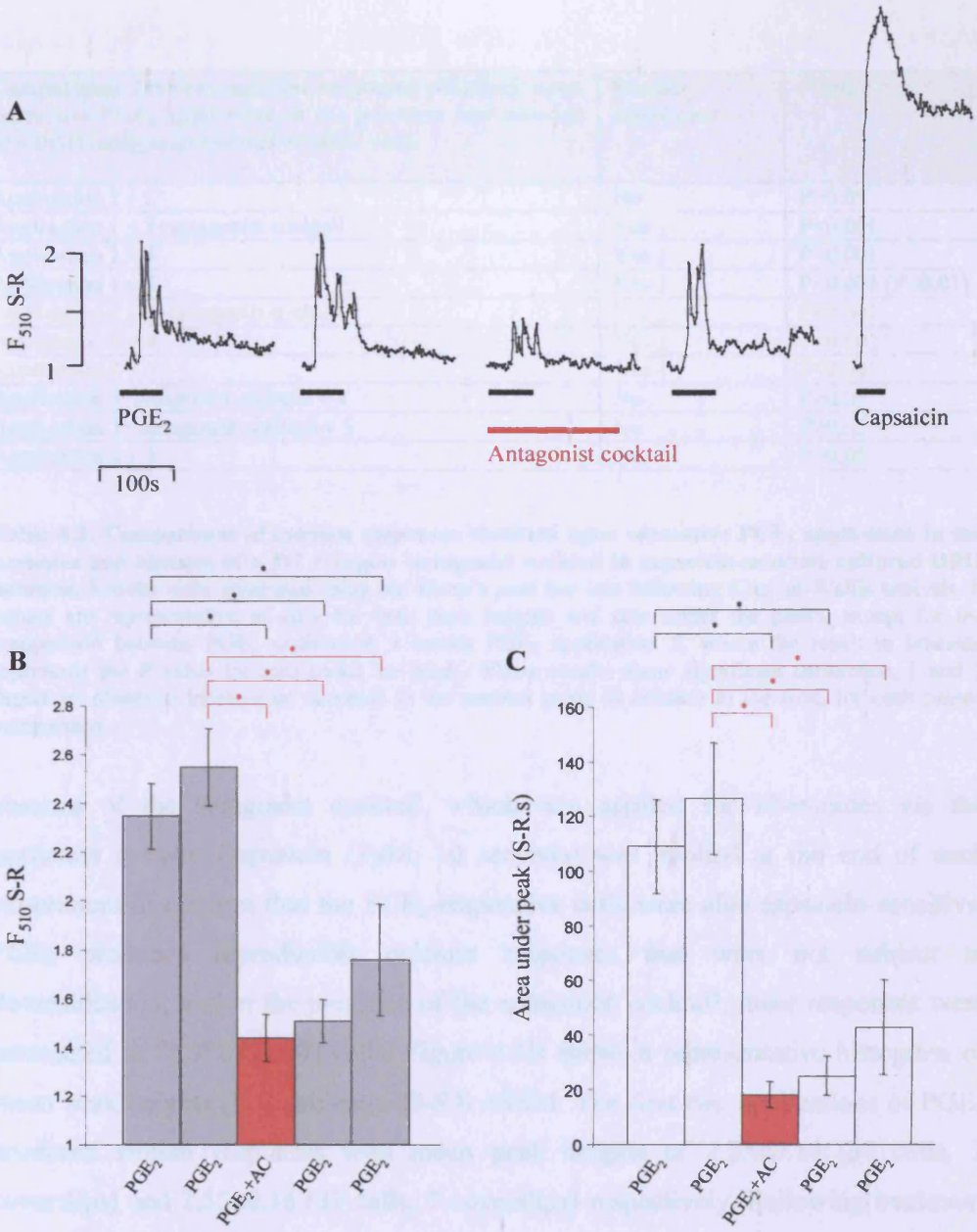


Figure 4.3: Effect of PGE_2 on intracellular calcium in the presence and absence of an EP_1, EP_3, and EP_4 receptor antagonist cocktail in capsaicin-sensitive DRG neurons. A) An experimental record illustrating calcium responses obtained upon repeated application of PGE_2 and blockade of the responses by a PG receptor antagonist cocktail (AC). PGE_2 (500nM, 50 seconds) was delivered 4-times (*bars*) with 10-minute periods between applications in the presence and absence of a PG receptor AC (500nM GW683868X, GW671021X, and GW627638X; 10-minutes) (*red bar*). Capsaicin (1μM) was applied at the end of each experiment to ascertain whether the PGE_2-responsive neurons were also capsaicin-sensitive. **B)** and **C)** Histograms showing mean peak heights (F_{510} S-R) ±SEM **(B)** and mean areas under the peaks (F_{510} S-R.s) ±SEM **(C)** of 37 (PGE_2 application 1; 7 coverslips), 37 (PGE_2 application 2; 7 coverslips), 37 (PGE_2 application 3+AC; 7 coverslips), 37 (PGE_2 application 4; 7 coverslips), and 23 (PGE_2 application 5; 5 coverslips) cells. For both peak heights and areas under the peaks, Kruskal-Wallis analysis confirmed significant difference ($P<0.0001$). Brackets denote significant comparisons (*$P<0.001$) from a *post hoc* Dunn's test (see table 4.2 for all comparisons).

Comparisons between calcium responses obtained upon successive PGE_2 application in the presence and absence of a PGR antagonist cocktail in DRG cells.	**Results significant**	***P value***
Application 1 v 2	No	P>0.05
Application 1 v 3+antagonist cocktail	Yes ↓	P<0.001
Application 1 v 4	Yes ↓	P<0.001
Application 1 v 5	Yes ↓	P<0.001 (P<0.01)
Application 2 v 3+antagonist cocktail	Yes ↓	P<0.001
Application 2 v 4	Yes ↓	P<0.001
Application 2 v 5	Yes ↓	P<0.001
Application 3+antagonist cocktail v 4	No	P>0.05
Application 3+ antagonist cocktail v 5	No	P>0.05
Application 4 v 5	No	P>0.05

Table 4.2: Comparisons of calcium responses obtained upon successive PGE_2 application in the presence and absence of a PG receptor antagonist cocktail in capsaicin-sensitive cultured DRG neurons. Results were generated using the Dunn's *post hoc* test following Kruskal-Wallis analysis. P values are representative of data for both peak heights and area under the peaks, except for the comparison between PGE_2 application 1 versus PGE_2 application 5, where the result in brackets represents the P value for area under the peak. Where results show significant difference, ↑ and ↓ depict an observed increase or decrease in the second group in relation to the first, for each paired comparison.

absence of the antagonist cocktail, which was applied for 10-minutes via the perfusion system. Capsaicin (1μM, 10 seconds) was applied at the end of each experiment to confirm that the PGE_2-responsive cells were also capsaicin-sensitive. PGE_2 produced reproducible calcium responses that were not subject to desensitisation, and in the presence of the antagonist cocktail, these responses were attenuated in 78.7% (37/47) cells. Figure 4.3B shows a representative histogram of mean peak heights (F_{510} self-ratio (S-R)) ±SEM. The first two applications of PGE_2 produced similar responses with mean peak heights of 2.35±0.14 (37 cells, 7 coverslips) and 2.55±0.16 (37 cells, 7 coverslips) respectively. Following treatment with the antagonist cocktail (500nM, 10-minutes), the mean peak height of the third PGE_2 response decreased to 1.44±0.10 (37 cells, 7 coverslips). After washout, the mean peak heights of the fourth and fifth PGE_2 responses were 1.51±0.09 (37 cells, 7 coverslips) and 1.76±0.23 (23 cells, 5 coverslips) respectively. Kruskal-Wallis analysis (section 2.7.4) of the mean peak height data showed that differences between the calcium responses were significant (*P<0.0001)*. The results of the *post-hoc* Dunn's test are summarised in table 4.2. Significant differences were found between calcium responses evoked by PGE_2 in naïve cells as compared to antagonist cocktail-treated cells; in the presence of the antagonist cocktail, the PGE_2-evoked

response was reduced to 32.88±4.84%. After antagonist cocktail washout, subsequent applications of PGE_2 were also reduced significantly as compared to the first and second PGE_2 applications, indicating that the antagonist cocktail was having a prolonged effect. It is reasonable to postulate therefore, that the PGE_2-evoked calcium responses are mediated by activation of one or more subtypes of EP receptor.

Due to the complex nature of some of the oscillatory calcium responses, it was deemed insufficient to analyse the peak height data alone. Therefore, the areas under the peaks were also investigated. The histogram shown in figure 4.3C shows the corresponding mean areas under the peaks (S-R.s) ±SEM of the PGE_2-evoked calcium responses; 109.21±17.16 (application 1), 126.74±20.51 (application 2), 18.63±4.75 (application 3+AC), 25.24±6.96 (application 4), and 43.04±17.17 (application 5). These were also found to be significantly different ($P<0.0001$; Kruskal-Wallis test), with a Dunn's *post hoc* test revealing similar P values to those obtained for the mean peak heights (Table 4.2).

4.2.2 Sensitisation of capsaicin-elicited calcium responses by PGE_2 in DRG neurons

To investigate the sensitising effect of PGE_2 on TRPV1, experiments were first performed to acquire baseline data on the effect of successive capsaicin application on intracellular calcium responses in cultured rat DRG neurons.

4.2.2.1 Two different types of calcium response are evoked by capsaicin

It is well established that TRPV1 is present in the DRG [Caterina *et al.*, 1997, Michael & Priestley, 1999, Guo *et al.*, 1999, Sanchez *et al.*, 2001] (section 1.4). Figure 4.4A shows a representative Western blot of DRG tissue homogenates and cultured DRG cell lysates, using $5\mu gml^{-1}$ rabbit TRPV1 antibody (Calbiochem). A band of protein was observed at 98kDa (expected molecular weight of the rat TRPV1 receptor) in each of the DRG sample preparations, which confirmed the presence of TRPV1 in the DRG. The presence of TRPV1 in the cultured DRG cell preparation demonstrated that TRPV1 survived primary cell culture procedures.

Figure 4.4 also shows examples of calcium responses obtained in isolated DRG neurons when 80nM capsaicin was applied directly to the cells for 10 seconds

via U-tube application. Most of the capsaicin-sensitive cells were small or medium diameter (15-35μm). Type-1 (transient) cells exhibited a rapid increase in intracellular calcium, which reached a peak before declining back to basal levels (figure 4.4B), whereas type-2 (prolonged) cells evoked a rapid increase in intracellular calcium, which did not recover back to basal levels (figure 4.4C). As the type-2 cells remained flooded with calcium, these cells could not be included in data analysis as their behaviour was extremely difficult to quantify.

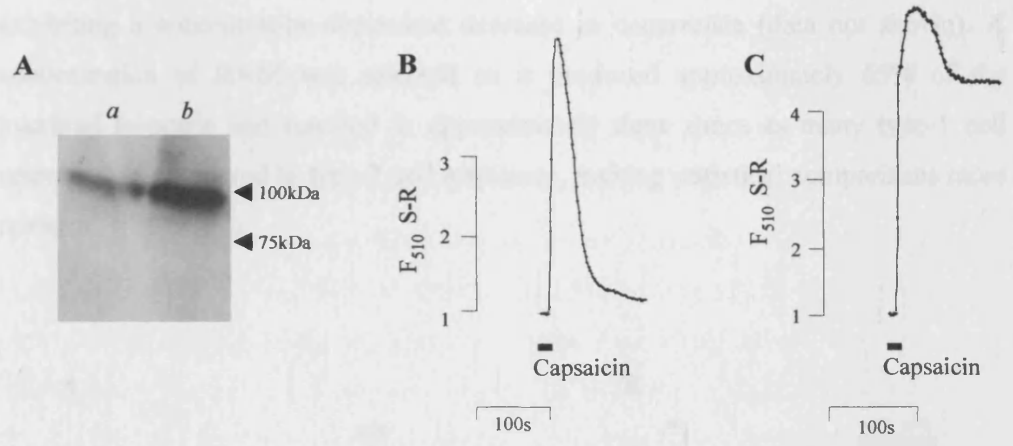


Figure 4.4: TRPV1 in cultured DRG neurons evokes two different types of calcium response upon application of capsaicin. A) Representative Western blot of cultured DRG cell lysates *(Lane a)* and DRG tissue homogenates *(Lane b)* using Calbiochem TRPV1 antibody ($5\mu gml^{-1}$). A single band was observed at approximately 98kDa (expected molecular weight of rat TRPV1) in both DRG preparations, indicating the presence of TRPV1. **B)** and **C)** Representative traces showing two different types of calcium response produced when 80nM capsaicin was delivered for 10 seconds onto cultured DRG neurons by U-tube application. Type 1 (transient) cells are characterised by a rapid rise in $[Ca^{2+}]_i$, which returns back to basal levels **(B)**, whereas type 2 (prolonged) cells are characterised by a rapid rise in $[Ca^{2+}]_i$, which does not recover back to basal levels **(C)**.

The 80nM concentration of capsaicin was chosen for calcium imaging experiments as a result of concentration / response experiments (figure 4.5). It proved impossible to construct a suitable concentration / response curve in the same cell due to the inherent problem of TRPV1 desensitisation (section 1.4.1.1). Therefore, various concentrations of capsaicin (10^{-9}, 10^{-8}, $4x10^{-8}$, $8x10^{-8}$, 10^{-6}, and 10^{-5}M) were applied to cells from different coverslips. Figure 4.5A shows the concentration-dependent effect of capsaicin with an EC_{50} occurring at approximately 45nM. This concurs with previously reported capsaicin EC_{50} values in the range of 35-72nM for intracellular calcium responses in DRG neurons [Cholewinski *et al.*, 1993, Jerman *et al.*, 2002, Stucky *et al.*, 1998]. In addition, there was a

concentration-dependent increase in the total number of cells that responded to capsaicin from 8.75% (7/80) at 1nM capsaicin to 76.1% (124/163) cells at 10μM capsaicin (figure 4.5B). When the total responsive cells were divided into type-1 and type-2 sub-populations a specific pattern emerged for each cell type. For cell type-2, a concentration-dependent increase was observed such that as the concentration of capsaicin increased towards a maximum, the proportion of type-2 cell responses prevailed more than type-1 cell responses (data not shown). Conversely, for cell type-1, the opposite was true, with the proportion of type-1 responsive cells exhibiting a concentration-dependent decrease in occurrence (data not shown). A concentration of 80nM was selected as it produced approximately 65% of the maximal response and resulted in approximately three times as many type-1 cell responses as compared to type-2 cell responses, making statistical comparisons more relevant.

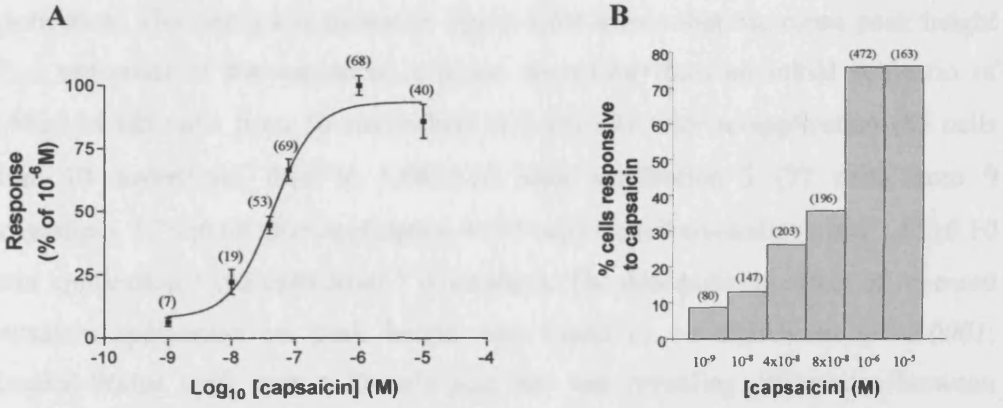


Figure 4.5: Concentration response effect of capsaicin in cultured rat DRG cells on intracellular calcium. A) Concentration-response curve for capsaicin, plotted as a percentage of the maximal response (10^{-6}M). Data are expressed as mean ±SEM with the numbers in parentheses representing the sample size for each capsaicin concentration. The EC_{50} value for capsaicin-induced increase in intracellular calcium was ~45nM. **B)** Histogram illustrating the concentration-dependent effect of capsaicin on the percentage of responsive cells. Numbers in parentheses represent the sample size.

4.2.2.2 Desensitisation of type-1 calcium responses upon successive applications of capsaicin

On initial application of capsaicin (80nM, 10 seconds), calcium responses were witnessed in 33.0% (138/418) (10 coverslips) of single 2-day-old cultured rat DRG neurons. Of these capsaicin-sensitive cells, 23.4% (98/418) were type-1 cells and 9.6% (40/418) were type-2 cells. Figure 4.6A shows an example of type-1 calcium responses obtained in isolated DRG neurons upon successive applications (10-minute intervals) of capsaicin (80nM, 10 seconds). The first response consisted of a rapid increase in intracellular calcium, which reached a peak before declining back to basal levels. Upon re-application of capsaicin, only 26.6% (111/418) of cells responded with an increase in intracellular calcium, indicating that desensitisation of the response had occurred. Of these cells, 19.4% (81/418) were type-1 cells and 7.2% (30/418) were type-2 cells. As shown in figure 4.6A, the calcium responses obtained in those cells that responded to subsequent applications of capsaicin (10-minute intervals) were attenuated compared to responses obtained during the initial application. The histogram shown in figure 4.6B shows that the mean peak height (F_{510} self-ratio) of the capsaicin response decreased from an initial self-ratio of 3.68±0.14 (85 cells from 10 coverslips) to 1.59±0.07 after re-application (85 cells from 10 coverslips), then to 1.88±0.10 after application 3 (77 cells from 9 coverslips), 1.73±0.08 after application 4 (77 cells from 9 coverslips), and 1.65±0.10 after application 5 (63 cells from 7 coverslips). The desensitising effect of repeated capsaicin application on peak height was found to be significant (*$P<0.0001$*; Kruskal-Wallis test), with a Dunn's *post hoc* test revealing differences between capsaicin applications 2, 3, 4, and 5 as compared to the first capsaicin response (table 4.3). To put this into perspective, the initial response was desensitised to 22.21±2.11% (capsaicin application 2). The histogram shown in figure 4.6C shows the corresponding mean areas under the peaks (self-ratio.s), which decreased from 150.16±12.45 (application 1) to 19.36±1.4 (application 2), to 36.09±5.59 (application 3), to 31.20±4.56 (application 4), and finally to 24.82±4.37 (application 5). Again, these mean areas under the peaks were found to be significantly different (*$P<0.0001$*; Kruskal-Wallis test), with a Dunn's *post hoc* test revealing the same differences that were found for the mean peak heights (table 4.3). The desensitisation of the calcium

response manifest by repeated capsaicin applications is often referred to as *tachyphylaxis* (section 1.4.1.1).

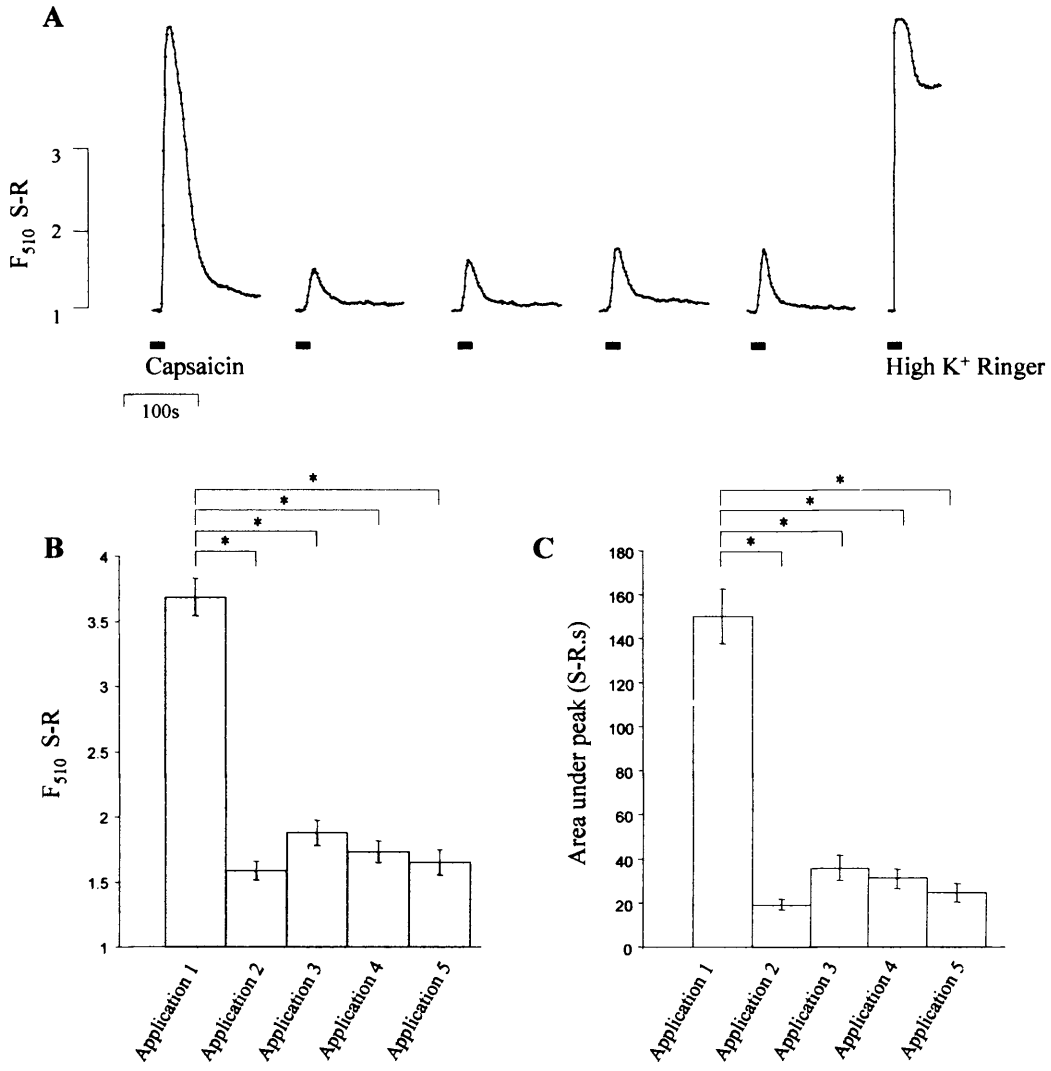


Figure 4.6: Desensitisation of capsaicin-elicited calcium responses. A) An experimental record illustrating the desensitising effect of repeated capsaicin application on the capsaicin-elicited calcium response. Capsaicin (80nM; 10 seconds) was delivered five times (*bars*) with a 10-minute period between applications. Application of 60mM KCl was used at the end of each experiment to check cell viability. **B)** and **C)** Histograms showing the mean peak heights (F_{510} S-R) ±SEM **(B)** and mean areas under the peaks (F_{510} S-R.s) ±SEM **(C)** for 85 (application 1; 10 coverslips), 85 (application 2; 10 coverslips), 77 (application 3; 9 coverslips), 77 (application 4; 9 coverslips), and 63 (application 5; 7 coverslips) cells. For both peak heights and area under the peaks, Kruskal-Wallis analysis confirmed significant difference within the data ($P<0.0001$). Brackets denote the significant comparisons (*$P<0.001$) from a *post hoc* Dunn's test (see table 4.3 for all comparisons).

Comparisons between calcium responses obtained upon successive capsaicin application in DRG cells.	**Results significant**	***P value***
Application 1 v 2	Yes ↓	P<0.001
Application 1 v 3	Yes ↓	P<0.001
Application 1 v 4	Yes ↓	P<0.001
Application 1 v 5	Yes ↓	P<0.001
Application 2 v 3	No	P>0.05
Application 2 v 4	No	P>0.05
Application 2 v 5	No	P>0.05
Application 3 v 4	No	P>0.05
Application 3 v 5	No	P>0.05
Application 4 v 5	No	P>0.05

Table 4.3: Comparisons of calcium responses obtained upon successive applications of capsaicin with 10-minute intervals in cultured DRG cells. Results were generated using the Dunn's *post hoc* test following Kruskal-Wallis analysis. P values are representative of data for both peak heights and area under the peaks. Where results show significant difference, ↑ and ↓ depict an observed increase or decrease in the second group in relation to the first, for each paired comparison.

A small proportion of type-1 cells (13.3%; 13/98) were witnessed during the course of the above work that did not exhibit tachyphylaxis upon repeated capsaicin application. These cells were included in the statistics if re-application of capsaicin produced calcium responses, which were >80% of the initial response (application 1) and are referred to as type-1b cells. Figure 4.7A shows an example of type-1b calcium responses obtained in isolated DRG neurons upon successive applications of capsaicin (80nM, 10 seconds). Similar calcium responses were observed following each successive capsaicin application, indicating that desensitisation had not prevailed. Figures 4.7B and C show representative histograms of mean peak heights (figure 4.7B) and areas under the peaks (figure 4.7C). No significant differences (P>0.05; ANOVA, section 2.7.4) were found between the mean peak heights of 3.44±0.36 (application 1, 13 cells from 7 coverslips), 3.25±0.33 (application 2, 13 cells from 7 coverslips), 3.43±0.41 (application 3, 12 cells from 6 coverslips), 3.19±0.41 (application 4, 12 cells from 6 coverslips), and 2.97±0.37 (application 5, 10 cells from 5 coverslips). The corresponding mean areas under the peaks of 170.75±34.98 (application 1), 119.04±25.37 (application 2), 132.68±31.20 (application 3), 126.18±31.50 (application 4), and 101.51±37.83 (application 5) were also not significantly different from one another (P>0.05, ANOVA). It is worthy of note however, that when the results from this group of cells were pooled with the rest

of the type-1 cells, the mean peak heights and areas under the peaks still showed significant desensitisation (*P<0.0001*; Kruskal-Wallis test followed by Dunn's test).

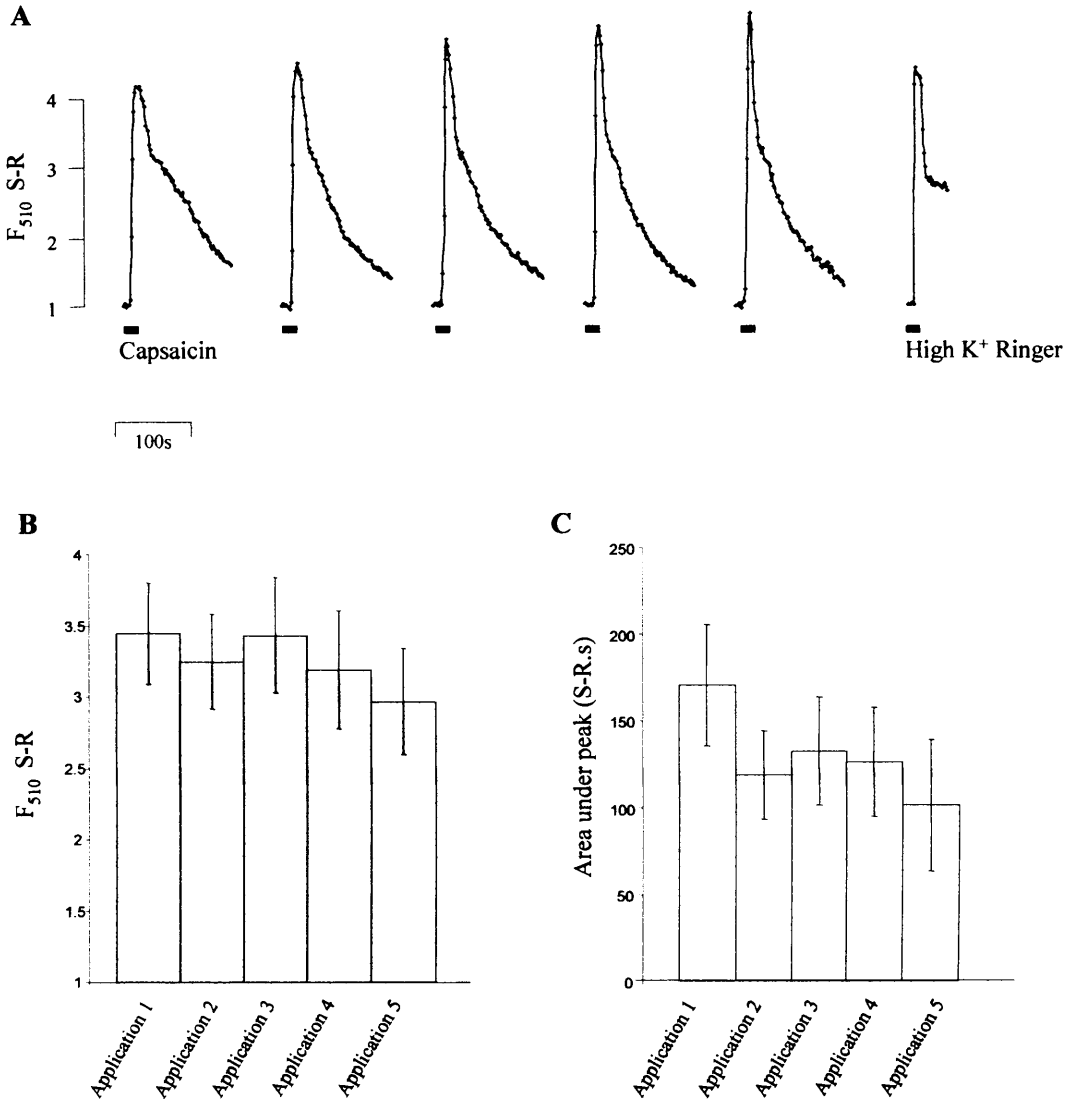


Figure 4.7: A subset of neurons not subject to capsaicin-elicited calcium response desensitisation. A) An experimental record illustrating a group of neurons (type 1b) not subject to the desensitising effect of repeated capsaicin application on intracellular calcium responses. Capsaicin (80nM; 10 seconds) was delivered five times (*bars*) with a 10-minute period between applications. Application of 60mM KCl was used at the end of each experiment to check cell viability. **B)** and **C)** Histograms showing the mean peak heights (F_{510} S-R) ±SEM **(B)** and mean areas under the peaks (F_{510} S-R.s) ±SEM **(C)** for 13 (application 1; 7 coverslips), 13 (application 2; 7 coverslips), 12 (application 3; 6 coverslips), 12 (application 4; 4 coverslips), and 10 (application 5; 5 coverslips) cells. For both peak heights and area under the peaks, no significant differences were found; *P>0.05*; ANOVA.

4.2.2.3 Sensitisation of type-1 capsaicin-evoked calcium responses by PGE_2 in DRG neurons

To examine the effect of PGE_2 (500nM) on capsaicin-evoked Ca^{2+} responses, cells were successively challenged with capsaicin (80nM, applied for 10s via U-tube) every 10-minutes, before and after the application of 500nM PGE_2 for 3-minutes via the perfusion system. Capsaicin–induced calcium responses were witnessed in 30.5% (167/547, 15 coverslips) of 2-day old cultured DRG cells. Of these capsaicin-sensitive cells, 26.0% (142/547) exhibited type-1 responses and 4.6% (25/547) exhibited type-2 responses. Following PGE_2 treatment, sensitisation of the capsaicin-evoked calcium response was observed in 61.27% (87/142) of type-1 capsaicin-responsive cells. In addition, PGE_2 influenced the response of some neurons that did not respond to an initial capsaicin application with a rise in intracellular calcium, referred to as non-responsive cells (figure 4.8A). In these non-responsive cells, a second application of capsaicin resulted in a detectable calcium response in only 0.4% (1/280) of cells. However, after treatment with PGE_2, the application of capsaicin evoked large responses in 5.8% (22/380) of these previously *silent* cells (figure 4.8B). Therefore, the number of capsaicin-sensitive type-1 cells and *silent* cells that were sensitised by PGE_2, as a proportion of the total cell population (capsaicin responsive and non-responsive) was 19.9% (109/547, 15 coverslips); 79.8% (87/109) of the sensitisation occurred in type-1 capsaicin-responsive neurons and 20.2% (22/109) in *silent* neurons. It is worthy of note that both the capsaicin-sensitive cells and *silent* cells were pooled during analysis. However, to avoid bias, statistical tests were also performed on each group separately. *P* values for the two separate groups were in agreement with those obtained when the cell types were pooled.

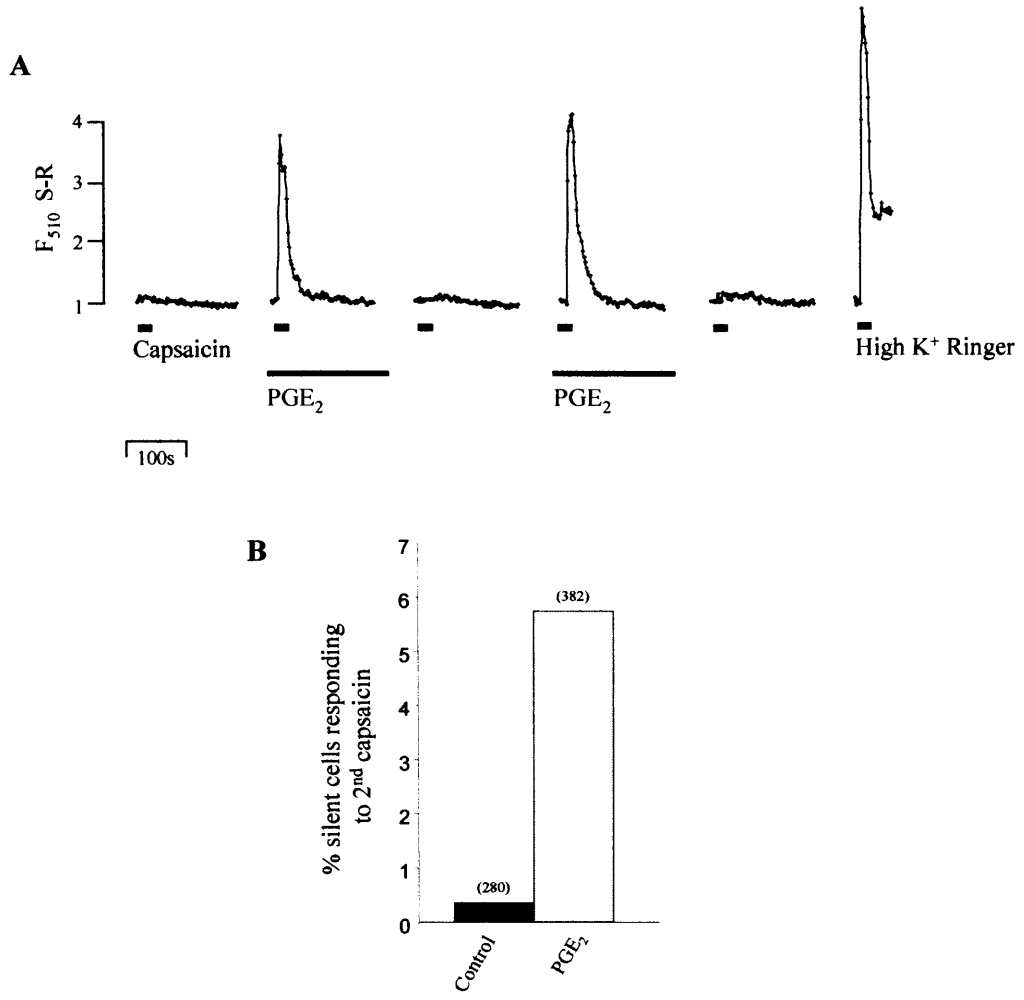


Figure 4.8: Induction of capsaicin-sensitivity in *silent* neurons by PGE_2 treatment. A) An experimental record illustrating the sensitising effect of PGE_2 on a capsaicin-insensitive (*silent*) neuron. Capsaicin (80nM; 10 seconds) was delivered five times (*bars*) with a 10-minute period between applications in the presence (*bars*) and absence of PGE_2 (500nM; 3-minutes). Application of 60mM KCl was used at the end of each experiment to check cell viability. **B)** Histogram showing the proportion of capsaicin-insensitive neurons that responded to re-application of capsaicin with a rise in intracellular calcium in the absence of PGE_2 (control) and following PGE_2 treatment. The numbers in parentheses denote the sample size.

The experimental record shown in figure 4.9A shows representative calcium responses obtained upon repeated applications of capsaicin (80nM, 10 seconds) in the presence and absence of PGE_2 (500nM, 3-minutes). Following PGE_2 treatment, capsaicin-evoked calcium responses were potentiated as compared to the responses produced when capsaicin was applied alone, indicating that sensitisation of the capsaicin response had occurred. The histogram shown in figure 4.9B reveals that the capsaicin-evoked calcium response increased from an initial peak height (F_{510} S-R) of 2.51±0.13 (application 1, 109 cells from 15 coverslips) to 3.50±0.10 after

application 2 in the presence of PGE_2 (109 cells from 15 coverslips), decreased to 2.09±0.10 upon application 3 after PGE_2 washout (109 cells from 15 coverslips), increased to 3.05±0.12 after application 4 following the re-application of PGE_2 (82 cells from 11 coverslips), and finally decreased to 1.92±0.11 upon application 5 after PGE_2 washout (82 cells from 11 coverslips). Kruskal-Wallis analysis of the mean peak height data showed that differences between the calcium responses were significant (*P<0.0001*). The results of the *post-hoc* Dunn's test are summarised in table 4.4. Statistically significant differences were found between calcium responses evoked by capsaicin in naïve cells as compared to those in cells pre-treated with PGE_2, showing that PGE_2 produces sensitisation and re-sensitisation of TRPV1. The initial capsaicin response (application 1) was sensitised to 217.44±18.17% following application of PGE_2 and subsequently re-sensitised to 199.28±20.91%, which when compared to the desensitisation witnessed upon repeated capsaicin application in the absence of PGE_2 (section 4.2.2.2), is potentiated approximately 9-fold. The histogram shown in figure 4.9C shows the corresponding mean areas under the peaks (F_{510} S-R.s) of 71.11±8.94 (application 1), 164.71±15.08 (application 2+PGE_2), 42.11±5.63 (application 3), 101.10±11.10 (application 4+PGE_2), and 31.75±5.72 (application 5). The areas were also found to be significantly different (*P<0.0001*; Kruskal-Wallis test), with a Dunn's *post hoc* test revealing similar P values to those obtained for the mean peak heights (table 4.4).

A small proportion of type-1 cells (19.7%; 28/142) exhibited capsaicin-evoked calcium responses that were unchanged in the presence of PGE_2 (data not shown). These cells were included in statistical comparisons if re-application of capsaicin produced calcium responses in both the presence and absence of PGE_2, which were >80% of the initial capsaicin-evoked response (application 1). It is proposed that these cells derive from the type-1b cell group reported previously (section 4.2.2.2). Statistical analysis of the mean peak heights and areas under the peaks of this cell group showed no significant differences between each of the capsaicin responses (P>0.05; ANOVA), proving that no significant sensitisation by PGE_2 had prevailed.

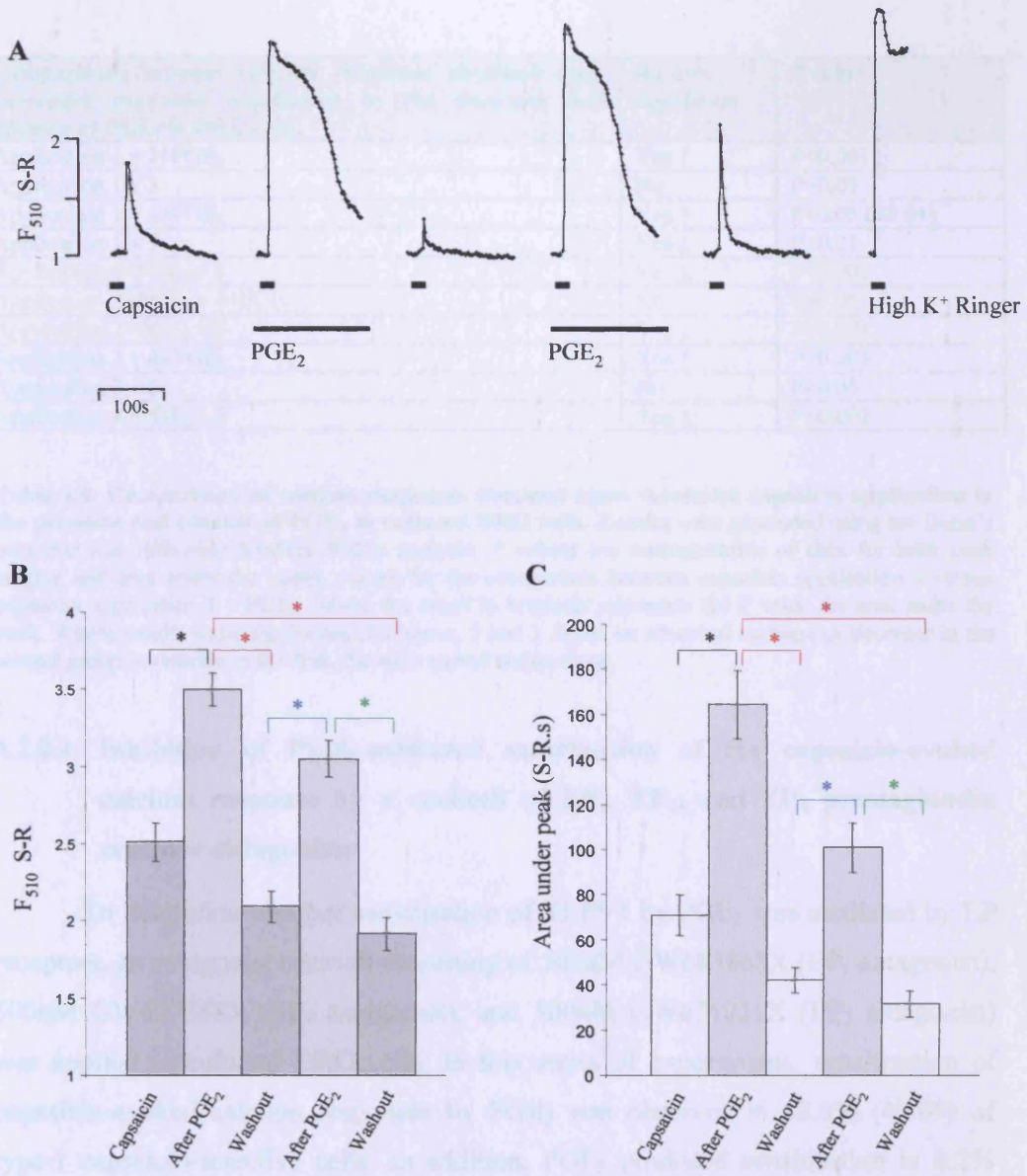


Figure 4.9: Sensitisation of the capsaicin-evoked calcium response by PGE_2. A) An experimental record illustrating the sensitising effect of PGE_2 on the capsaicin-elicited calcium response. Capsaicin (80nM; 10 seconds) was delivered five times (*bars*) with a 10-minute period between applications in the presence (*bars*) and absence of PGE_2 (500nM; 3-minutes). Application of 60mM KCl was used at the end of each experiment to check cell viability. **B** and **C)** Histograms showing the mean peak heights (F_{510} S-R) ±SEM **(B)** and mean areas under the peaks (F_{510} S-R.s) ±SEM **(C)** for 109 (capsaicin application 1; 15 coverslips), 109 (capsaicin application 2+PGE_2; 15 coverslips), 109 (capsaicin application 3; 15 coverslips), 82 (capsaicin application 4+PGE_2; 11 coverslips), and 82 (capsaicin application 5; 11 coverslips) cells. For both peak heights and area under the peaks, Kruskal-Wallis analysis confirmed significant difference ($P<0.0001$). Brackets denote significant comparisons (*$P<0.001$) from a *post hoc* Dunn's test (see table 4.4 for all comparisons).

Comparisons between calcium responses obtained upon successive capsaicin application in the presence and absence of PGE_2 in DRG cells.	**Results significant**	***P value***
Application 1 v 2+PGE_2	Yes ↑	P<0.001
Application 1 v 3	No	P>0.05
Application 1 v 4+PGE_2	Yes ↑	P<0.05 (<0.01)
Application 1 v 5	Yes ↓	P<0.01
Application 2+PGE_2 v 3	Yes ↓	P<0.001
Application 2+PGE_2 v 4+PGE_2	No	P>0.05
Application 2+PGE_2 v 5	Yes ↓	P<0.001
Application 3 v 4+PGE_2	Yes ↑	P<0.001
Application 3 v 5	No	P>0.05
Application 4+PGE_2 v 5	Yes ↓	P<0.001

Table 4.4: Comparisons of calcium responses obtained upon successive capsaicin application in the presence and absence of PGE_2 in cultured DRG cells. Results were generated using the Dunn's *post hoc* test following Kruskal-Wallis analysis. P values are representative of data for both peak heights and area under the peaks, except for the comparison between capsaicin application 1 versus capsaicin application 4 + PGE_2, where the result in brackets represents the P value for area under the peak. Where results show significant difference, ↑ and ↓ depict an observed increase or decrease in the second group in relation to the first, for each paired comparison.

4.2.2.4 Inhibition of PGE_2-mediated sensitisation of the capsaicin-evoked calcium response by a cocktail of EP_1, EP_3, and EP_4 prostaglandin receptor antagonists

To determine whether sensitisation of TRPV1 by PGE_2 was mediated by EP receptors, an antagonist cocktail consisting of 500nM GW683868X (EP_1 antagonist), 500nM GW627368X (EP_4 antagonist), and 500nM GW671021X (EP_3 antagonist) was applied to cultured DRG cells. In this series of experiments, sensitisation of capsaicin-evoked calcium responses by PGE_2 was observed in 58.0% (40/69) of type-1 capsaicin-sensitive cells. In addition, PGE_2 produced sensitisation in 6.2% (12/193) of *silent* cells. The number of cells that were sensitised by PGE_2, as a proportion of the total cell population (all capsaicin responsive and non-responsive) was 19.7% (52/264, 8 coverslips); 76.9% (40/52) of the sensitisation occurred in type-1 capsaicin-responsive cells and 23.1% (12/52) in *silent* cells. Again, the two cell groups were pooled for data analysis purposes.

Figure 4.10A shows a representative trace of a *silent* cell sensitised by PGE_2 (500nM, 3-minutes) to produce individual calcium responses upon application of capsaicin (80nM, 10 seconds). Following treatment with the antagonist cocktail (500nM, 10-minutes) however, capsaicin failed to evoke a significant calcium response despite treatment with PGE_2, indicating inhibition of sensitisation. The

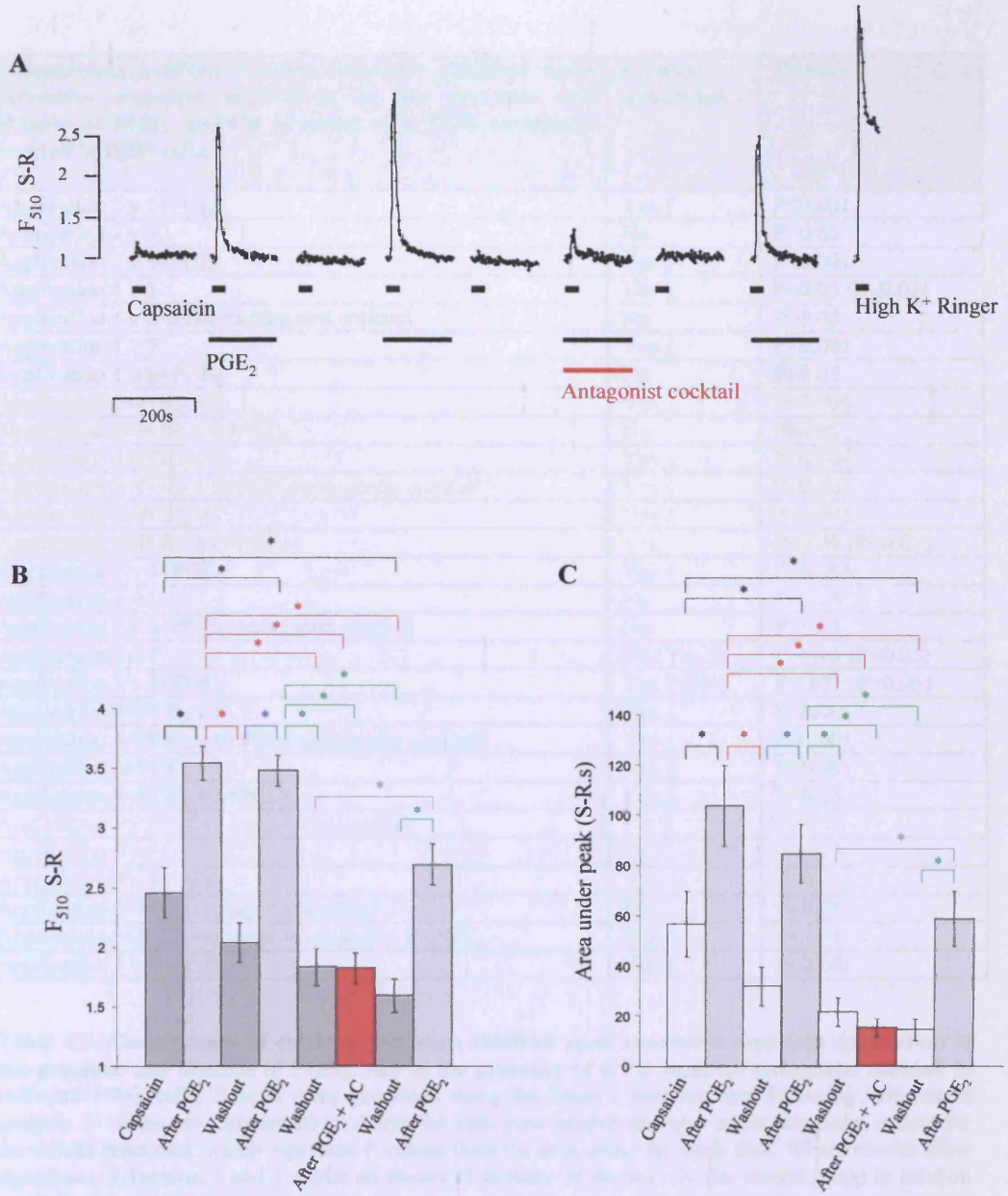


Figure 4.10: Inhibition of PGE_2-induced sensitisation of capsaicin-evoked calcium responses by a prostaglandin receptor (EP_1, EP_3, and EP_4) antagonist cocktail. A) An experimental record illustrating the sensitising effect of PGE_2 on capsaicin-evoked calcium responses from a *silent* neuron and inhibition of sensitisation following treatment with a PG receptor antagonist cocktail (AC). Capsaicin (80nM; 10 seconds) was delivered eight times (*bars*) with a 10-minute period between applications in the presence (*bars*) and absence of PGE_2 (500nM; 3-minutes), and in the presence of the antagonist cocktail (500nM GW683868X, GW671021X, and GW627638X; 10-minutes) (*red bar*). Application of 60mM KCl was used at the end of each experiment to check cell viability. **B** and **C)** Histograms showing the mean peak heights (F_{510} S-R) ±SEM **(B)** and mean areas under the peaks (F_{510} S-R.s) ±SEM **(C)** for 44 cells from 8 coverslips. For both peak heights and area under the peaks, Friedman analysis confirmed significant difference ($P<0.0001$). Brackets denote significant comparisons (*$P<0.001$) from a *post hoc* Dunn's test (see table 4.5 for all comparisons).

Comparisons between calcium responses obtained upon successive capsaicin application in the presence and absence of PGE_2, and the presence of a PGR antagonist cocktail in DRG cells.	**Results significant**	***P value***
Application 1 v 2+PGE_2	Yes ↑	P<0.001
Application 1 v 3	No	P>0.05
Application 1 v 4+PGE_2	Yes ↑	P<0.001
Application 1 v 5	Yes ↓	P<0.05 (P<0.01)
Application 1 v 6+PGE_2+antagonist cocktail	No	P>0.05
Application 1 v 7	Yes ↓	P<0.001
Application 1 v 8+PGE_2	No	P>0.05
Application 2+PGE_2 v 3	Yes ↓	P<0.001
Application 2+PGE_2 v 4+PGE_2	No	P>0.05
Application 2+PGE_2 v 5	Yes ↓	P<0.001
Application 2+PGE_2 v 6+PGE_2+antagonist cocktail	Yes ↓	P<0.001
Application 2+PGE_2 v 7	Yes ↓	P<0.001
Application 2+PGE_2 v 8+PGE_2	Yes ↓	P<0.05 (P<0.01)
Application 3 v 4+PGE_2	Yes ↑	P<0.001
Application 3 v 5	No	P>0.05
Application 3 v 6+PGE_2+antagonist cocktail	No	P>0.05
Application 3 v 7	No (Yes ↓)	P>0.05 (P<0.05)
Application 3 v 8+PGE_2	Yes ↑ (No)	P<0.05 (P>0.05)
Application 4+PGE_2 v 5	Yes ↓	P<0.001
Application 4+PGE_2 v 6+PGE_2+antagonist cocktail	Yes ↓	P<0.001
Application 4+PGE_2 v 7	Yes ↓	P<0.001
Application 4+PGE_2 v 8+PGE_2	Yes ↓	P<0.05
Application 5 v 6+PGE_2+antagonist cocktail	No	P>0.05
Application 5 v 7	No	P>0.05
Application 5 v 8+PGE_2	Yes ↑	P<0.001
Application 6+PGE_2+antagonist cocktail v 7	No	P>0.05
Application 6+PGE_2+antagonist cocktail v 8+PGE_2	Yes ↑	P<0.01
Application 7 v 8+PGE_2	Yes ↑	P<0.001

Table 4.5: Comparisons of calcium responses obtained upon successive capsaicin application in the presence and absence of PGE_2, and in the presence of a PG receptor antagonist cocktail in cultured DRG cells. Results were generated using the Dunn's *post hoc* test following Friedman's analysis. P values are representative of data for both peak heights and area under the peaks, except for the results bracketed, which represent P values from the area under the peak data. Where results show significant difference, ↑ and ↓ depict an observed increase or decrease in the second group in relation to the first, for each paired comparison.

histogram in figure 4.10B shows the mean peak height data ±SEM for 44 cells from 8 coverslips. The mean peak height of the capsaicin-evoked calcium response increased from 2.46±0.21 (application 1) to 3.54±0.14 (application 2) following treatment with PGE_2, decreased to 2.04±0.16 (application 3) after washout, and increased to 3.48±0.13 (application 4) following re-application of PGE_2. After a further washout, the peak height decreased to 1.83±0.16 (application 5), and remained unchanged at 1.82±0.13 (application 6) following treatment with PGE_2 and the antagonist cocktail. After washout of the drugs, the peak height increased from

1.59±0.14 (application 7) to 2.74±0.15 (application 8) following re-application of PGE_2. Statistical analysis using the Friedman's test (section 2.7.4) revealed that differences between the mean peak heights of the capsaicin-evoked calcium responses were significant (*P<0.0001*). The results of the *post-hoc* Dunn's test are summarised in table 4.5. Significant differences were found between calcium responses evoked by capsaicin in naïve cells as compared to those in cells pre-treated with PGE_2, indicating that PGE_2 produces significant sensitisation and re-sensitisation of TRPV1. Furthermore, the PGE_2-induced sensitisation of TRPV1 appears to be mediated by EP receptors, as the antagonist cocktail significantly inhibited PGE_2-induced sensitisation in 84.6% (44/52) cells. After antagonist cocktail washout, significant recovery from inhibition was apparent following re-application of PGE_2. However, the sensitisation was significantly reduced as compared to sensitisation prior to antagonist cocktail treatment, indicating that the antagonist cocktail was either having a prolonged effect on TRPV1 sensitisation or was not completely washout out. Summarising the data, the initial capsaicin-evoked response was sensitised to 205.22±25.12% (application 2+PGE_2, n=32) and 198.55±22.82% (application 4+PGE_2) before antagonist cocktail application, compared to 62.36±9.30% (application 6+PGE_2+ antagonist cocktail) after antagonist cocktail application. Therefore, pre-treatment with the antagonist cocktail inhibited the sensitisation of TRPV1 by PGE_2 approximately 3-fold. The histogram shown in figure 4.10C shows the corresponding mean areas under the peaks of 56.65±13.02 (application 1), 103.72±16.04 (application 2+PGE_2), 31.73±7.71(application 3), 84.54±11.63 (application 4+PGE_2), 21.52±5.52 (application 5), 15.36±3.58 (application 6+PGE_2+antagonist cocktail), 14.53±4.37 (application 7), and 58.68±10.88 (application 8+PGE_2) respectively. This data also showed statistically significant differences (*P<0.0001*; Friedman's test), with a Dunn's *post hoc* test revealing similar P values to those obtained for the mean peak heights (table 4.5).

In this series of experiments, type-1b cell responses occurred in 24.64% (17/69) cells. The capsaicin-evoked calcium responses in these cells were similar in both the presence and absence of PGE_2 (data not shown). Treatment of the cells with the antagonist cocktail (500nM, 10-minutes) failed to diminish the responses (data not shown). Statistical analysis of the mean peak heights and areas under the peaks revealed no significant differences between the responses (P>0.05; ANOVA), indicating that the cells were not sensitised by PGE_2, and that the reproducibility of

capsaicin-evoked calcium responses was not due to activation of EP_1, EP_3, or EP_4 receptors in these cells.

4.3 DISCUSSION

Calcium is an important intracellular messenger that participates in the regulation of enzymes, ion channels, and the release of neurotransmitters [Llinas *et al.*, 1992, Robitaille *et al.*, 1993]. Therefore, any change in intracellular calcium concentration may impact the excitability of primary afferent neurons [Kostyuk & Verkhratsky, 1994], and is often used as a relevant marker for measuring a response to a chemical when investigating processes of excitation and sensitisation in such cell types. The work in this chapter has utilised Ca^{2+} imaging and demonstrated that the inflammatory mediator PGE_2 has major sensitising effects, but only minor, direct effects on capsaicin-sensitive rat DRG neurons. These observations are in agreement with those made in previous studies [Birrell *et al.*, 1991, 1993, Stucky *et al.*, 1996] (section 1.3.2.2) and are discussed in the following sections.

4.3.1 Direct effects of PGE_2 on DRG cells

The direct application of PGE_2 resulted in reproducible increases in intracellular calcium in only a small proportion (~8%) of capsaicin-sensitive cultured rat DRG cells, which concurs with results obtained in previous studies [Stucky *et al.*, 1996, Linhart *et al.*, 2003]. However, an additional study by Smith and colleagues observed similar PGE_2-evoked calcium responses in approximately 50% of DRG cells, which is considerably higher than the findings reported here and those reported elsewhere [Smith *et al.*, 2000]. This discrepancy may reflect the higher recording temperature (37°C) used in the study by Smith and colleagues, as well as a relatively high concentration of PGE_2 (1μM). The latter seems unlikely however, as Linhart and colleagues only observed responses in 29% of cells with 10μM PGE_2. The concentration of PGE_2 (500nM) used in the experiments reported in this chapter was selected, as it is similar to concentrations reported for PGE_2 in inflammatory exudates [Bombardieri *et al.*, 1981, Higgs *et al.*, 1983, Cheng *et al.*, 1998].

The observation that PGE_2 evoked calcium responses in only a small proportion of DRG cells is consistent with *in vivo* studies, which have demonstrated weak excitatory effects of PGE_2 on mechanoceptors (filaments with group III and IV fibres; table 1.1) in the normal rat ankle joint after arterial injection [Grubb *et al.*, 1991, Birrell *et al.*, 1991, 1993]. In some of these studies, PGE_2 potentiated the excitatory effects of bradykinin on joint mechanoceptors [Grubb *et al.*, 1991, Birrell

et al., 1993], indicating that PGE_2 has more of a sensitising role rather than a direct excitatory role in the rat. Interestingly, similar experiments performed in the normal cat knee joint have shown substantial excitation of mechanoceptors by direct PGE_2 administration [Schaible & Schmidt, 1988, Schepelmann *et al.*, 1992], possibly reflecting species differences.

Several types of calcium response were witnessed following application of PGE_2 during the course of this study. These included small oscillatory responses and transient spikes, as well as much larger rises in intracellular calcium, all of which have been reported previously [Smith *et al.*, 2000, Linhart *et al.*, 2003]. Although, the mechanisms underlying the PGE_2-evoked increases in intracellular calcium were not investigated in detail during the course of the work described in this chapter, application of a prostaglandin receptor antagonist cocktail inhibited the majority of responses, indicating the involvement of EP prostaglandin receptors. Previous studies have shown that the PGE_2-evoked calcium responses are also dependent upon the activation of PKA [Smith *et al.*, 2000] and the presence of extracellular calcium [Smith *et al.*, 2000, Linhart *et al.*, 2003]. Therefore, it has been postulated that PGE_2 promotes calcium influx by phosphorylation of voltage-gated calcium channels [Smith *et al.*, 2000], which is supported by the observation that cadmium ions (non-selective calcium channel blocker) block PGE_2-evoked calcium responses in DRG cells [Linhart *et al.*, 2003]. In line with this hypothesis, PGE_2 has been reported to increase the HVA I_{Ca} in embryonic avian sensory neurons [Nicol *et al.*, 1992]. However, alternative mechanisms cannot be ruled out as there is substantial evidence to suggest that PGE_2 inhibits the HVA I_{Ca} in a variety of other cell types (section 1.5.1.3). Another possible explanation for the PGE_2-mediated influx of calcium through voltage-gated calcium channels is a hyperpolarizing shift in membrane potential, resulting from modulation of voltage-gated sodium and potassium currents by PGE_2 (sections 1.5.1.1 and 1.5.1.2). This would explain the erratic intracellular calcium oscillations often witnessed following PGE_2 application, whereby transient fluctuations in membrane potential could lead to transient openings of voltage-gated calcium channels. Alternatively, the depolarisation of DRG neurons by PGE_2 could activate calcium release from intracellular stores [Simpson *et al.*, 1995].

4.3.2 Sensitising effects of PGE_2 on DRG cells

Peripherally administered PGE_2 is known to evoke thermal hypersensitivity in animals [Negus *et al.*, 1993, 2004, Yang & Gereau, 2002]. As the TRPV1 channel is currently believed to be the principle molecular component mediating heat-activated currents in C-fibre nociceptors [Caterina *et al.*, 1997], this would indicate an interaction between TRPV1 and PGE_2. In line with this, evidence has shown that PGE_2 can sensitise the capsaicin-evoked inward current in rat DRG cells [Lopshire & Nicol, 1997, 1998]. It was therefore anticipated that the capsaicin-evoked calcium response might also be sensitised by PGE_2. If true, it was postulated that this would represent a good model system for studying PGE_2-mediated sensitisation of substantial numbers of nociceptors during a single experiment.

It is worthy of note that during the course of the work described in this chapter, the first published evidence appeared that supported the hypothesis that PGE_2 does indeed modulate the capsaicin-evoked calcium response in DRG [Hu *et al.*, 2002] and vagal neurons [Gu *et al.*, 2003].

4.3.2.1 Two different types of calcium response are evoked by capsaicin

There were two distinct types of calcium transient observed in DRG neurons following application of capsaicin. The first type (type-1) consisted of a transient rise in intracellular calcium, which returned to basal levels, whereas the second type (type-2) was prolonged and did not exhibit any recovery within the time frame of the experiment. These two types of capsaicin-evoked calcium response have been reported previously [Dedov & Roufogalis, 1998, Stucky *et al.*, 1998, Hu *et al.*, 2002] and appear to be associated with different rates of calcium influx [Dedov & Roufogalis, 1998]. However, the significance of them is not yet fully understood. Evidence has shown that TRPV1 channel subunits can assemble to form homo and heteromers [Kedei *et al.*, 2001], which indicates that there may be distinct multimeric TRPV1 channels with differing properties. Indeed, two distinct classes of TRPV1 channel have been described in rat DRG neurons [Acs *et al.*, 1996, 1997] (section 1.4.1.1), which may be related to two different capsaicin-evoked conductances [Peterson *et al.*, 1996].

4.3.2.2 Type-1 capsaicin-evoked calcium responses are subject to desensitisation

Of the two types of capsaicin-evoked calcium response described in this chapter, only the type-1 cells were included in experimental analysis. This was because the type-2 cells remained flooded with calcium making their behaviour extremely difficult to quantify. Furthermore, the majority of type-2 cells appeared to be damaged, as they often did not respond to a depolarising concentration of potassium.

The proportion of type-1 capsaicin-sensitive cells was seen to decrease following successive applications of capsaicin, with those cells responding with a rise in intracellular calcium being subject to *tachyphylaxis* (section 1.4.1.1). These observations reflect the occurrence of capsaicin–induced desensitisation, which has been well documented in the literature and has been shown to be dependent upon a variety of different factors, including calcium, calmodulin, and calcineurin (section 1.4.1.1). Desensitisation of inward currents evoked by noxious heat (natural stimulus of TRPV1) in sensory neurons has also been well documented (section 1.4.1.1). In relation to normal physiological functioning, the process of desensitisation represents an effective way for the prevention of excessive neuronal firing and transmission of nociceptive information.

It was apparent that a small proportion of type-1 cells did not exhibit tachyphylaxis, and these have been referred to throughout this chapter as type-1b cells. Interestingly, only one previous study in adult rat vagal sensory neurons has reported reproducible capsaicin-evoked calcium responses, utilising similar experimental parameters compared to those used in this study [Gu *et al.*, 2003]. The significance of the type-1b cells is not understood at present and further experiments are required to elucidate why they are not subject to tachyphylaxis. It is proposed that the concentration of capsaicin (80nM) used in this study is not implicated in the absence of tachyphylaxis in the type-1b cells, as a previous study utilising 5-7 week old mouse DRG cells, employing 20nM capsaicin, has reported that tachyphylaxis occurs in all capsaicin-sensitive cells [Hu *et al.*, 2002]. This discrepancy may reflect species variation between mice and rats, and / or a difference in response according to animal age. However, a more fundamental explanation may lie in the variation in primary cell culture conditions. Hu and colleagues maintained their cultures for 6-8

days before experimentation and did not supplement the culture medium with NGF. Conversely, the DRG cultures used throughout the course of this work, and the vagal sensory neuron cultures utilised by Gu and colleagues [Gu *et al.,* 2003] were maintained for 48 hours before experimentation and were supplemented with NGF (section 2.6).

It is known that the presence of NGF is necessary for the growth and development of small diameter capsaicin-sensitive neurons (sections 1.2 and 1.4.1.2.3). However, NGF can also promote sensitisation of both the capsaicin-evoked inward current and capsaicin-evoked calcium response in DRG neurons (section 1.4.1.2.3). Therefore, it is hypothesised that NGF present in the culture medium might have triggered sensitisation of TRPV1 in the small group of type-1b neurons, leading to reproducible capsaicin-evoked calcium responses. Indeed, DRG neurons grown in an NGF-free medium have been shown to respond routinely to capsaicin with a desensitised calcium response [Bonnington & McNaughton, 2003]. However, following application of NGF, intracellular calcium responses evoked by capsaicin were increased in ~37% cells and were reproducible in nature, indicating the occurrence of a prolonged sensitisation of TRPV1 function [Bonnington & McNaughton, 2003].

Another consideration for the existence of type-1b responses is that cultured DRG cells can release the neuropeptides CGRP and SP in response to capsaicin stimulation [Vedder & Otten, 1991, Southall & Vasko, 2000, Skoff *et al.,* 2003, Nakae *et al.,* 2005, Hingten *et al.,* 2006]. These neuropeptides are important inflammatory mediators, which cause vasodilation and produce neurogenic inflammation (section 1.3.7). Interestingly, the application of CGRP has been reported to evoke rises in intracellular calcium in cultured DRG cells, which are not subject to desensitisation [Segond von Banchet *et al.,* 2002]. Therefore, in light of such evidence, it is plausible that the type-1b intracellular calcium responses witnessed in the present study are the result of elevated levels of extracellular neuropeptides.

4.3.2.3 Type-1 capsaicin-evoked calcium responses are sensitised by PGE2

PGE_2 produced sensitisation of TRPV1 function in small diameter nociceptors. The sensitised neurons could be divided into two distinct groups, based

on initial capsaicin sensitivity. The first group included cells that responded to initial application of capsaicin with a rise in intracellular calcium. In these cells (~60%), PGE_2 treatment increased both the amplitude and area under the peak of the subsequent capsaicin-evoked calcium response, demonstrating that sensitisation of TRPV1 had occurred. This observation concurs with previous studies that have shown similar sensitising effects of PGE_2 on capsaicin-evoked calcium responses in DRG neurons [Hu *et al.*, 2002] and vagal neurons [Gu *et al.*, 2003].

The second group comprised cells (~6%) that were initially non-responsive when challenged with capsaicin, but showed large capsaicin-evoked calcium responses after PGE_2 treatment, providing further evidence of TRPV1 sensitisation. This cell group has been referred to throughout this chapter as the *"silent"* cell population and was not witnessed during successive applications of capsaicin in the absence of PGE_2. A similar recruitment of *"silent"* nociceptors by PGE_2 has been reported previously [Schaible & Grubb, 1993, Stucky *et al.*, 1996, Smith *et al.*, 2000]. Any increase in the number of responsive nociceptors would lead to spacial summation at the level of spinal neurons, and represents an important mechanism by which PGE_2 may contribute to hyperalgesia.

The mechanism underlying the modulation of TRPV1 sensitivity by PGE_2 in both cell groups was found to involve the activation of EP receptors, as a prostaglandin receptor antagonist cocktail, containing selective EP_1, EP_3, and EP_4 antagonists, abolished sensitisation of the capsaicin-evoked calcium response by PGE_2 in the majority of cells. Previously, TRPV1 sensitisation by PGE_2 has been shown to be dependent upon activation of the cAMP transduction cascade (section 1.5.2). Therefore, taken together, this indicates that the sensitising effects of PGE_2 are mediated through one or more G_s-coupled PG receptor(s), leading to PKA-evoked post-translational changes in TRPV1.

A novel finding in this chapter was that following initial sensitisation by PGE_2, subsequent applications of the prostanoid resulted in re-sensitisation of the capsaicin-evoked calcium response in both of the cell types described above. This is particularly interesting as it suggests that the PG receptor(s) involved in sensitising TRPV1 are not subject to desensitisation. It is worthy of note that during this series of experiments, treatment with PGE_2 sometimes caused a prolonged sensitisation of TRPV1, such that following washout of PGE_2, capsaicin-evoked calcium responses were of a similar magnitude to those witnessed in the presence of agonist (data not

shown). This suggests that the action of PGE_2 on the TRPV1 channel may extend beyond short-lived sensitisation mechanisms to encompass a more prolonged maintenance of TRPV1 in a sensitised state. Such an effect of PGE_2 on the TRPV1 channel could provide a potential mechanism for the well-documented ability of the prostanoid to produce thermal hypersensitivity in animals.

As reported earlier in this chapter (section 4.2.1), calcium responses evoked by direct PGE_2 application were also seen to be reproducible in DRG cells, which is consistent with previous studies [Linhart *et al.*, 2003, Smith *et al.*, 2000], and further supports the hypothesis that PG receptors are not subject to desensitisation in this cell type. In contrast, following PGE_2 stimulation of EP_4 receptor expressing recombinant cells, the EP_4 receptor has been shown to undergo rapid desensitisation kinetics due to receptor internalisation [Slipetz *et al.*, 2001]. The reason behind this discrepancy is unclear at present but is likely to reflect the different cellular systems utilised.

Although capsaicin was used in this study to activate TRPV1, it is an exogenous ligand of TRPV1 and is not likely to activate the channel during inflammation. Various endogenous ligands of TRPV1 do however exist, including the endocannabinoid, anandamide, which can also evoke TRPV1-mediated rises in intracellular calcium and activate TRPV1-evoked inward currents in DRG neurons [Hwang *et al.*, 2000, Jerman *et al.*, 2002, Ohta *et al.*, 2005, van der Stelt *et al.*, 2005] (section 1.4). Interestingly, a recent study has shown using the colbalt uptake technique in DRG cells that PGE_2 and BK can increase the efficacy of anandamide in evoking TRPV1 activity [Singh-Tahim *et al.*, 2005]. Anandamide acts at both the TRPV1 and CB1 receptors with differing affinities and thereby can concentration-dependently regulate nociceptors [Ahluwalia *et al.*, 2003]. At low concentrations, the molecule has been shown to reduce the activity and excitability of nociceptors via CB1 activation, whereas at higher concentrations, anandamide was seen to stimulate nociceptors by TRPV1-mediated excitation. Therefore, during an inflammatory challenge, it is conceivable that PGE_2 not only alters the sensitivity of the TRPV1 channel but also alters the potency of endogenous TRPV1 ligands to produce TRPV1-mediated excitation of nociceptors.

After witnessing the sensitising effects of PGE_2, it was anticipated that the prostanoid might also sensitise the capsaicin-evoked calcium responses of the type-1b subpopulation of neurons. A number of hypotheses have already been discussed in the preceding section (section 4.3.2.2) to explain the non-desensitising nature of

this cellular group, however there was one further consideration, that a background neuronal release of PGE_2 was having a sensitising effect on TRPV1 function. This hypothesis was subsequently proved flawed, as PGE_2 application had no effect on the capsaicin-evoked calcium responses of the type-1b cells, in either the presence or absence of the EP receptor antagonist cocktail. Moreover, inclusion of indomethacin in the culture medium and extracellular perfusion solution was without effect (data not shown). This shows that PGE_2-mediated activation of EP receptors does not underlie the occurrence of reproducible capsaicin-evoked calcium responses in type-1b cells.

In summary, PGE_2 produced intracellular calcium responses in only a small number of DRG cells via activation of EP receptors, indicating that it does not make a substantial contribution to directly activating rat nociceptors. Consistent with PGE_2 having a major sensitising role, the prostanoid enhanced the capsaicin-evoked calcium response in approximately two thirds of capsaicin-sensitive neurons, which would normally be subject to *tachyphylaxis*. In addition, PGE_2 was also seen to sensitise a small proportion of *silent* neurons to become capsaicin-responsive. The sensitising effect of PGE_2 on TRPV1 channel function was found to be dependent upon EP receptor activation. Interestingly, the EP receptors were not subject to desensitisation, as PGE_2 evoked repeatable calcium responses, and also re-sensitised capsaicin-evoked calcium responses reproducibly in both capsaicin-sensitive and *silent* DRG cells, following the re-exposure of the cells to the agonist. This is a very important finding as it means that during pathophysiological functioning, PGE_2 could continuously sensitise and re-sensitise TRPV1 function, and thereby contribute to or provide the basis for thermal hyperalgesia witnessed *in vivo*.

Chapter Five

Characterisation of prostaglandin E receptors involved in PGE_2-mediated sensitisation of sensory neurons

5.1 INTRODUCTION

Calcium imaging results presented in chapter four have demonstrated how TRPV1 channel desensitisation occurs in response to repeated capsaicin stimulation in cultured rat DRG neurons (section 4.2.2.2). Activation of the TRPV1 channel by capsaicin, as well as protons and noxious heat, leads to excitation of nociceptive primary afferent fibres (section 1.4). Therefore, desensitisation of the TRPV1 channel represents an important way in which the body might control thermal nociception during normal physiological functioning (section 1.4.1). Conversely, under pathophysiological conditions, it is known that TRPV1 enters a sensitised state (section 1.4.1). In line with this, evidence was also provided in the previous chapter to show that the inflammatory mediator, PGE_2, evoked reproducible sensitisation of capsaicin-elicited calcium responses in DRG neurons (section 4.2.2.3), which is consistent with peripherally administered PGE_2 producing thermal hypersensitivity in animals [Negus *et al.,* 1993, 2004, Yang & Gereau, 2002]. Furthermore, the sensitising effect of the prostanoid was found to be dependent upon PG receptor activation (section 4.2.2.4).

There has been much investigation within the literature into the mechanisms underlying the sensitivity of TRPV1 channel function (section 1.4.1). It is believed that phosphorylation is important for maintaining TRPV1 in a sensitised state and / or rescuing TRPV1 from a desensitised state (section 1.4.1.1). Indeed, mechanisms underlying the sensitisation of TRPV1 by various inflammatory mediators have been shown to involve either PKA or PKC dependent pathways (section 1.4.1.2 and 1.5.2). These findings suggest that any one of the EP receptor subtypes could underlie PGE_2-mediated sensitisation of TRPV1 as they couple to a variety of different G-proteins and signalling cascades (section 1.3.2.3).

Pharmacological agents with varying selectivities for the individual EP receptor subtypes have been used extensively to elucidate, which EP receptors mediate the differential effects of PGE_2. A study by Abramovitz and colleagues has summarised binding affinity data for a number of these compounds at each prostanoid receptor subtype [Abramovitz *et al.*, 2000]. The most commonly used EP receptor agonists include butaprost (EP_2), sulprostone (EP_3 but also has lower affinity for EP_1 and FP), misoprostol (EP_3 but also has similar affinity for EP_2 and EP_4), and 17-phenyl-PGE_2 (EP_1 but also has affinity for EP_3). Unfortunately these are not without their problems; butaprost can undergo slow de-esterification of its C1-methyl ester group to produce the biologically more active free acid, which may confound the discrimination of EP_2 and EP_4 receptors in some tissues [Jones, 2004], and the other agonists display additional affinity for other EP receptor subtypes. Such poor subtype selectivity means most of these agonists are used in combination with various EP receptor antagonists in order to block any unwanted EP receptor activation. However, the identification of selective EP receptor antagonist compounds has also proved difficult. Commonly used EP receptor antagonists include SC51089 (EP_1 but also has lower affinity for EP_3, FP, and TP), SC51322 (EP_1 but also has lower affinity for EP_3, and TP), AH6809 (EP_2 but has similar affinities for EP_1, and DP) and AH23848 (EP_4 but has a much higher affinity for TP), but again the selectivities and therefore, biological usefulness of these compounds remain questionable.

More recently, a series of EP receptor ligands have been developed including the agonists; ONO-DI-004 (EP_1), ONO-AE1-259 (EP_2), ONO-AE-248 (EP_3), and ONO-AE1-329 (EP_4) [Yamamoto *et al.*, 1999, Suzawa *et al.*, 2000], and the antagonists; ONO-8713 (EP_1) [Watanabe *et al.*, 2000], ONO-AE3-240 (EP_3) [Amano *et al.*, 2003], ONO-AE3-208 (EP_4) [Kabashima *et al.*, 2002], EP_4A (EP_4) [Machwate *et al.*, 2001], the diphenyloxazole "compound 8" (EP_4), and the Nδ-Z-ornithine "compound 11" (EP_4) [Hattori *et al.*, 2005]. Although the majority of these have additional low affinity for at least one other EP receptor subtype, these are believed to be amongst the most potent and selective ligands currently available.

Compound	Compound type	BINDING pK_i, and FUNCTIONAL pK_i[1], pIC_{50}[2], pK_B[3]							Reference
		EP_1	**EP_2**	**EP_{3I}**	**EP_4**	**FP**	**IP**	**TP**	
GW683868X	EP_1 antagonist	8.5[1]	<6.0[1]	5.8[1], 5.0[2]	<6.0[1]	—	—	6.6[1], 5.8[2]	A Michel, personal communication
GW671021X	EP_3 antagonist	<5.3	<5.3	9.5, 7.8[2]	<6.0	—	—	—	Juteau *et al.*, 2001
GW627368X	EP_4 antagonist	<5.1	<5.1	<5.2	7.1, 7.9[3]	<5.1	<5.3	6.9	Wilson & Giles, 2005 Wilson *et al.*, 2006
GSK324202A	EP_4 agonist	<5.4	<5.4	<5.4	7.1	<5.4	<5.4	<5.4	Giblin *et al.*, 2002

Table 5.1: The affinity of EP receptor compounds (GlaxoSmithKline) for cloned human prostanoid receptors.

The principle aim of the work described in this chapter was to determine, which EP receptors are important in mediating the sensitising effects of PGE_2 on the capsaicin-evoked calcium response in cultured DRG neurons. This was addressed using novel potent and selective EP receptor compounds (table 5.1) supplied by industrial collaborators GSK, utilising the protocol developed in chapter four (sections 4.2.2.3 and 4.2.2.4).

5.2 RESULTS

5.2.1 Selectivity of the EP_1 antagonist (GW683868X), EP_4 antagonist (GW627368X), and EP_4 agonist (GSK324202A) at respective prostanoid receptors

The selectivities of the EP receptor compounds have previously been characterised in detail by GSK, using radioligand binding experiments and functional assays, performed on prostanoid receptor expressing cell lines (table 5.1). However, separate experiments were also conducted during the course of the work described in this thesis, to demonstrate the functionality of some of these compounds at their respective cloned human prostanoid receptors. The EP_1 receptor is coupled to a rise in intracellular calcium [Watabe *et al.*, 1993, Båtshake *et al.*, 1995]. Therefore, the effect of the EP_1 antagonist on PGE_2-stimulated calcium responses in EP_1 expressing CHO cells was investigated. The second messenger pathway utilised by the EP_4 receptor involves the activation of adenylate cyclase [Breyer *et al.*, 1996, Nishigaki *et al.*, 1996]. Consequently, the two EP_4 receptor compounds were tested using cAMP accumulation assays performed in EP_4 expressing HEK cells.

5.2.1.1 Effect of EP_1 antagonist GW683868X on PGE_2-evoked calcium responses in EP_1 expressing CHO cells

Prior to the performance of the EP_1-CHO cell experiments, eight cells were chosen randomly from each coverslip to include in statistical analysis. Figure 5.1A shows an experimental record of calcium responses obtained in EP_1-CHO cells upon repeated application of PGE_2 (30nM, 20 seconds) at 5-minute intervals in the absence and presence of GW683868X (500nM, 5-minutes). The concentrations of compounds used in these experiments were selected according to their K_i values

obtained from the binding or functional studies in EP_1-CHO cells (table 5.1) [Wilson *et al.*, 2006]. PGE_2 evoked reproducible calcium responses that were not subject to desensitisation, and in the presence of GW683868X, these responses were almost abolished in all of the cells analysed. The histogram shown in figure 5.1B is representative of mean peak heights (F_{510} S-R) ±SEM. The first two applications of PGE_2 produced similar responses with mean peak heights of 3.38±0.18 (40 cells, 5 coverslips) and 3.05±0.12 (40 cells, 5 coverslips) respectively. Following treatment with the EP_1 antagonist, GW683868X (500nM, 5-minutes), the mean peak height of the third PGE_2 response decreased to 1.04±0.01 (40 cells, 5 coverslips), and after washout, the fourth PGE_2 response showed recovery with a mean peak height of 1.99±0.19 (24 cells, 3 coverslips). Kruskal-Wallis analysis of the mean peak height data showed that differences between the calcium responses were significant (*$P<0.0001$*). The results of the *post-hoc* Dunn's test are summarised in table 5.2. Significant differences were found between calcium responses evoked by PGE_2 in naïve cells as compared to GW683868X treated cells, indicating that the antagonist was inhibiting the cloned human EP_1 receptor; GW683868X caused an inhibition to 3.24±1.44% (~97% inhibition). After antagonist washout, the response was significantly increased following re-application of PGE_2, indicating that the effects of GW683868X were reversible.

The histogram shown in figure 5.1C shows the corresponding mean areas under the peaks (S-R.s) ±SEM of the PGE_2-evoked calcium responses; 24.11±1.91 (application 1), 21.36±1.22 (application 2), 0.81±0.19 (application 3+GW683868X), and 13.26±2.62 (application 4). These were also found to be significantly different (*$P<0.0001$*) with a Dunn's *post hoc* test revealing similar P values to those obtained for mean peak heights (table 5.2).

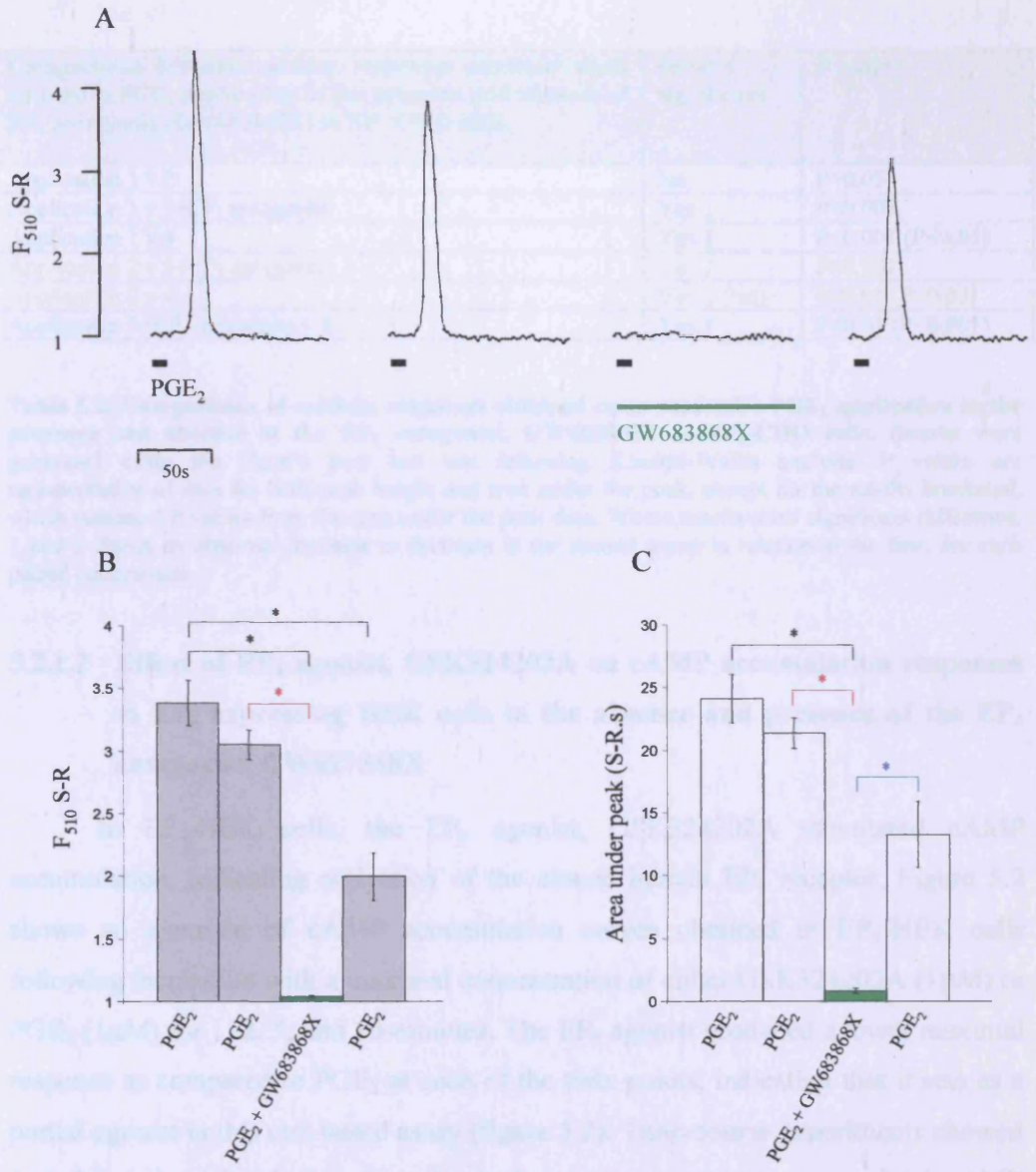


Figure 5.1: Inhibition of PGE_2-evoked rises in intracellular calcium by the EP_1 antagonist GW683868X in EP_1 expressing CHO K1 cells. A) An experimental record illustrating PGE_2-evoked calcium responses and their inhibition following treatment with the EP_1 antagonist, GW683868X. PGE_2 (30nM; 20 seconds) was delivered four times (*bars*) with a 5-minute period between applications in the presence (*green bar*) and absence of GW683868X (500nM; 3-minutes). **B** and **C)** Histograms showing the mean peak heights (F_{510} S-R) ±SEM **(B)** and mean areas under the peaks (F_{510} S-R.s) ±SEM **(C)** for 40 (PGE_2 applications 1-3; 6 coverslips) and 24 (PGE_2 application 4; 3 coverslips) cells. For both peak heights and area under the peaks, Kruskal-Wallis analysis confirmed significant difference ($P<0.0001$). Brackets denote significant comparisons (*$P<0.001$) from a *post hoc* Dunn's test (see table 5.2 for all comparisons).

Comparisons between calcium responses obtained upon successive PGE_2 application in the presence and absence of EP_1 antagonist (GW683868X) in EP_1-CHO cells.	**Results significant**	***P value***
Application 1 v 2	No	P>0.05
Application 1 v 3+EP_1 antagonist	Yes ↓	P<0.001
Application 1 v 4	Yes ↓	P<0.001 (P<0.05)
Application 2 v 3+EP_1 antagonist	Yes ↓	P<0.001
Application 2 v 4	Yes ↓ (No)	P<0.01 (P>0.05)
Application 3+EP_1 antagonist v 4	Yes ↑	P<0.01 (P<0.001)

Table 5.2: Comparisons of calcium responses obtained upon successive PGE_2 application in the presence and absence of the EP_1 antagonist, GW683868X in EP_1-CHO cells. Results were generated using the Dunn's *post hoc* test following Kruskal-Wallis analysis. P values are representative of data for both peak height and area under the peak, except for the results bracketed, which represent P values from the area under the peak data. Where results show significant difference, ↑ and ↓ depict an observed increase or decrease in the second group in relation to the first, for each paired comparison.

5.2.1.2 Effect of EP_4 agonist, GSK324202A on cAMP accumulation responses in EP_4 expressing HEK cells in the absence and presence of the EP_4 antagonist, GW627368X

In EP_4-HEK cells, the EP_4 agonist, GSK324202A stimulated cAMP accumulation, indicating activation of the cloned human EP_4 receptor. Figure 5.2 shows an example of cAMP accumulation curves obtained in EP_4-HEK cells following incubation with a maximal concentration of either GSK324202A (1μM) or PGE_2 (1μM) for 1, 2, 5, and 10-minutes. The EP_4 agonist produced a lower maximal response as compared to PGE_2 at each of the time points, indicating that it acts as a partial agonist in this cell-based assay (figure 5.2). Time-course experiments showed that the optimal incubation time for both agonists to produce a maximal cAMP accumulation in these cells was 5-minutes. As a result this exposure time was used for all further cAMP accumulation experiments.

Figure 5.3 shows the concentration-dependent increase in cAMP accumulation produced in EP_4-HEK cells in response to GSK324202A stimulation. The pEC_{50} obtained in 7 independent experiments was 7.14±0.12. Figure 5.4 shows that following a 30-minute incubation with 30nM GW627368X (selective EP_4 antagonist) the concentration response curve for GSK324202A was shifted to the right (pEC_{50}=6.67±0.21; n=4). The pK_B (section 2.8.4) for GW627368X obtained from the 4 independent experiments was 7.57±0.07.

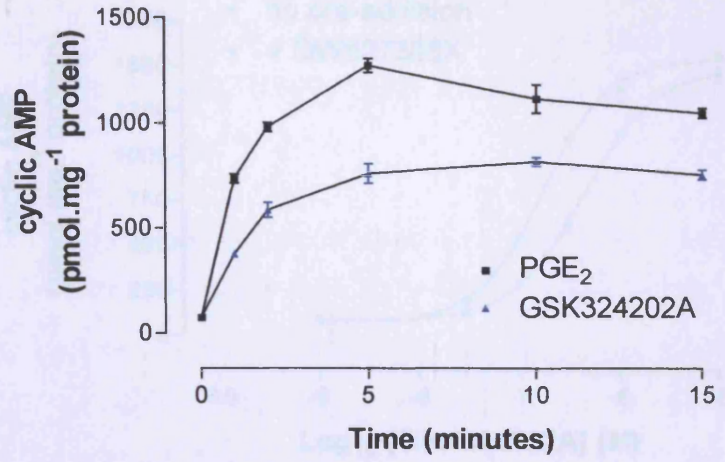


Figure 5.2: Time course of cyclic AMP accumulation in response to GSK324202A (1μM) and PGE_2 (1μM) in EP_4-HEK cells. The data are from one experiment that was replicated three times. Each point is the mean of two wells of transfected cells (error bars show data range).

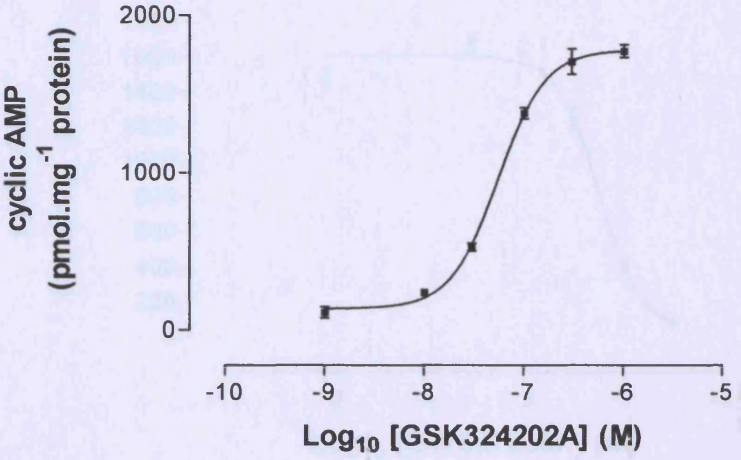


Figure 5.3: Concentration response effect of GSK324202A on cAMP accumulation in EP_4-HEK cells. The data are from one experiment that was replicated seven times. Each point is the mean of two wells of transfected cells (error bars show data range). The pEC_{50} value (mean ±SEM) for the seven experiments was 7.14±0.12.

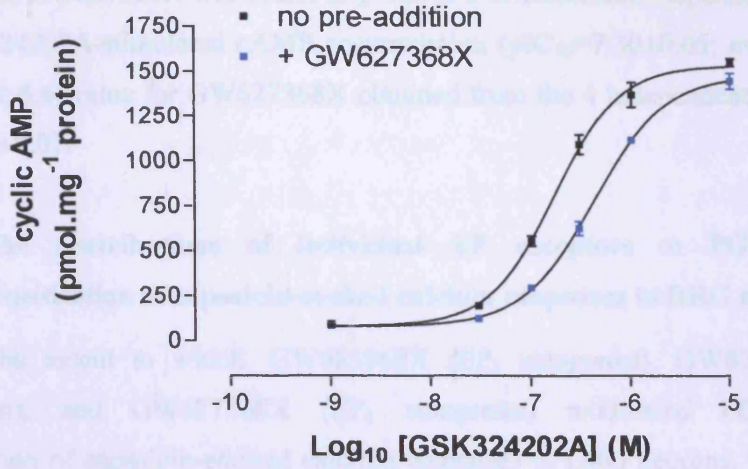


Figure 5.4: GSK324202A concentration response curves for cAMP accumulation in EP_4-HEK cells in the absence and presence of GW627368X. Cells were incubated with 30nM GW627368X for 30-minutes prior to exposure to GSK324202A for 5-minutes. The data are from one experiment that was replicated four times. Each point is the mean of two wells of transfected cells (error bars show data range). The pK_B value (mean ±SEM) for the four experiments was 7.57±0.07.

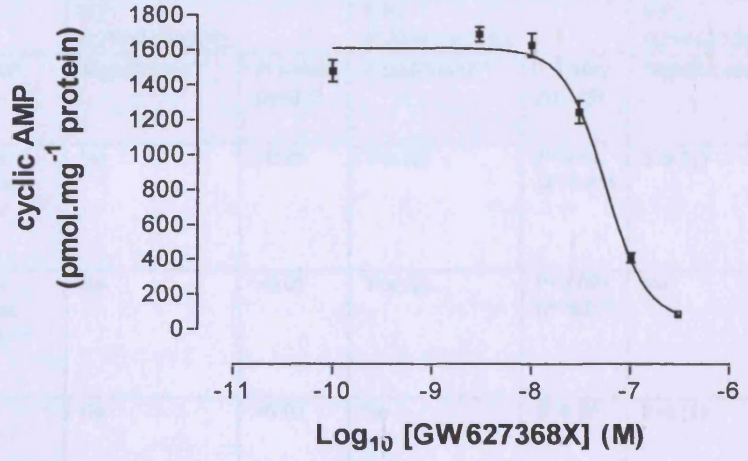


Figure 5.5: Concentration dependent effects of GW627368X on GSK324202A-stimulated cAMP accumulations in EP_4-HEK cells. Cells were pre-incubated with the indicated concentrations of GW627368X for 30-minutes before addition of GSK324202A (100nM; 5-minutes). The data are from one experiment that was replicated four times. Each point is the mean of two wells of transfected cells (error bars show data range). The pK_i value (mean ±SEM) for the four experiments was 7.67±0.07.

The effect of GW627368X on GSK324202A-stimulated cAMP accumulation was also investigated and is illustrated in figure 5.5. EP_4-HEK cells were pre-incubated for 30-minutes with increasing concentrations of GW627368X (3, 10, 30, 100, 300nM) and then stimulated with 100nM GSK324202A for 5-minutes. The EP_4

antagonist, GW627368X was found to produce a concentration-dependent inhibition of GSK324202A-stimulated cAMP accumulation (pIC_{50}=7.30±0.05; n=4). The pK_i (section 2.8.4) value for GW627368X obtained from the 4 independent experiments was 7.67±0.07.

5.2.2 The contributions of individual EP receptors to PGE_2-mediated sensitisation of capsaicin-evoked calcium responses in DRG neurons

The extent to which, GW683868X (EP_1 antagonist), GW671021X (EP_3 antagonist), and GW627368X (EP_4 antagonist) modulated PGE_2-mediated sensitisation of capsaicin-elicited calcium responses in DRG neurons, was assessed by analysing all cells sensitised by PGE_2 from each individual set of experiments. The Friedman's test was used to compare the sensitisation produced following 500nM PGE_2 application for 3-minutes, before, during, and after treatment with the

	PG receptor Antagonist					
	EP_1 (GW683868X)		**EP_3 (GW671021X)**		**EP_4 (GW627368X)**	
Comparisons	**Significant?**	***P value (n=24)***	**Significant?**	***P value (n=35)***	**Significant?**	***P value (n=30)***
Sensitisation before **versus** during antagonist application	No	>0.05	Yes (↓)	P<0.01 (P<0.05)	Yes (↓)	P<0.01 (P<0.001)
Sensitisation before **versus** after antagonist washout	No	>0.05	Yes (↓)	P<0.001 (P<0.05)	No	>0.05
Sensitisation during antagonist application **versus** after antagonist washout	No	>0.05	No	P>0.05	Yes (↑)	P<0.01 (P<0.001)

Table 5.3: Comparison of PGE_2 sensitised capsaicin-evoked calcium responses obtained before, during, and after individual PG receptor antagonist treatment in cultured DRG cells. Results were generated using the Dunn's *post hoc* test following Friedman's analysis. P values are representative of data for both peak heights and area under the peaks (*brackets*). Where results show significant difference, ↑ and ↓ depict an observed increase or decrease in the second group in relation to the first, for each paired comparison.

individual PG receptor antagonists for 10-minutes (all antagonists were used at 500nM) (table 5.3). Where $P<0.05$, a *post hoc* Dunn's test was used to distinguish

significant observations. It is worthy of note, that sensitisation in those cells not responsive to individual antagonist treatment, was abolished following antagonist cocktail treatment (data not shown).

For the EP_1 antagonist, GW683868X (n=24; 4 coverslips), Friedman's analysis (table 5.3) revealed no significant difference ($P>0.05$) between capsaicin-evoked calcium responses produced in the presence of PGE_2, before, during, and after antagonist treatment, for both mean peak height and area under the peak data, indicating that the EP_1 receptor does not make a significant contribution to PGE_2-mediated sensitisation of TRPV1 in DRG neurons. Of the PGE_2-sensitised cells, only 12.5% (3/24) cells were dependent upon EP_1 receptor activation.

For experiments utilising the EP_3 antagonist, GW671021X (n=35; 5 coverslips) and the EP_4 antagonist, GW627368X (n=30; 5 coverslips), Friedman's analysis (table 5.3) of mean peak height and area under the peak data revealed that of the cells sensitised by PGE_2, significant inhibition ($P<0.05$) of sensitisation had occurred in the presence of the two individual antagonists, indicating that EP_3 and EP_4 receptor subtypes make a significant contribution to the sensitisation of TRPV1. GW671021X (EP_3 antagonist) and GW627368X (EP_4 antagonist) inhibited sensitisation of TRPV1 by PGE_2 in 45.7% (16/35) and 60% (18/30) cells respectively. Following GW671021X washout, significant inhibition was still apparent, whereas following GW627368X washout, significant recovery of inhibition had prevailed (table 5.3).

In the following three sections, experiments involving each of the PG receptor antagonists have been discussed in more detail. Data obtained from the EP_3 and EP_4 antagonist experiments has been grouped to include only those cells, in which sensitisation of TRPV1 was inhibited.

5.2.2.1 Role of the EP_1 receptor in PGE_2-mediated sensitisation of Type-1 capsaicin-evoked calcium responses in DRG cells

As discussed in the preceding section, the EP_1 antagonist, GW683868X failed to produce significant inhibition of TRPV1 sensitisation when all PGE_2 sensitised cells were subject to analysis (table 5.3). In this series of experiments, sensitisation of capsaicin-evoked calcium responses by PGE_2 was observed in 54.5% (18/33) of type-1 capsaicin-sensitive cells. In addition, PGE_2 produced sensitisation in 5.3%

(6/114) of *silent* cells (section 4.2.2.3). The number of cells sensitised by PGE_2, as a proportion of the total cell population (all types of capsaicin responsive and non-responsive cells) was 16.3% (24/147, 4 coverslips), with 75.0% (18/24) of the sensitisation occurring in type-1 capsaicin-responsive cells and 25.0% (6/24) in *silent* cells. Type-1 capsaicin responsive cells and *silent* cells were grouped for data analysis.

Figure 5.6A shows representative calcium responses produced upon repeated applications of capsaicin (80nM, 10 seconds) in the absence and presence of PGE_2 (500nM, 3-minutes), as well as in the presence of PGE_2 after treatment with GW683868X (500nM, 10-minutes). Following PGE_2 treatment, it was apparent that TRPV1 sensitisation had occurred, as responses were either of a similar magnitude or potentiated as compared to the initial capsaicin response (application 1). The reader is referred back to chapter 4, section 4.2.2.2 for comparison with control capsaicin desensitisation experiments. In the presence of GW683868X, the sensitisation produced by PGE_2 was similar to previous sensitisations. The histogram in figure 5.6B shows the mean peak height data ±SEM for 24 cells from 4 coverslips. The mean peak height increased from 2.55±0.29 (application 1) to 3.32±0.26 (application 2) following treatment with PGE_2, decreased to 1.83±0.20 (application 3) after washout and increased to 3.29±0.30 (application 4) upon re-application of PGE_2. After a further washout, the peak height decreased to 2.01±0.24 (application 5) and increased to 2.89±0.27 (application 6) following treatment with PGE_2 and GW683868X. After washout of the drugs, the peak height increased from 2.35±0.29 (application 7) to 2.84±0.23 (application 8) after re-application of PGE_2. Statistical analysis using the Friedman's test revealed significant differences ($P<0.0001$) between the mean peak heights of the capsaicin-evoked calcium responses. The results of a *post hoc* Dunn's test are summarised in table 5.4. Comparisons between calcium responses evoked by capsaicin in naïve cells as compared to those in cells pre-treated with PGE_2 revealed that sensitisation and re-sensitisation of TRPV1 had occurred. In the presence of the EP_1 antagonist, PGE_2-mediated sensitisation of the capsaicin-evoked calcium response was not significantly attenuated ($P>0.05$, Friedman's analysis and *post hoc* Dunn's test), and remained at 91.52±4.70% of the previous sensitisation produced by PGE_2.

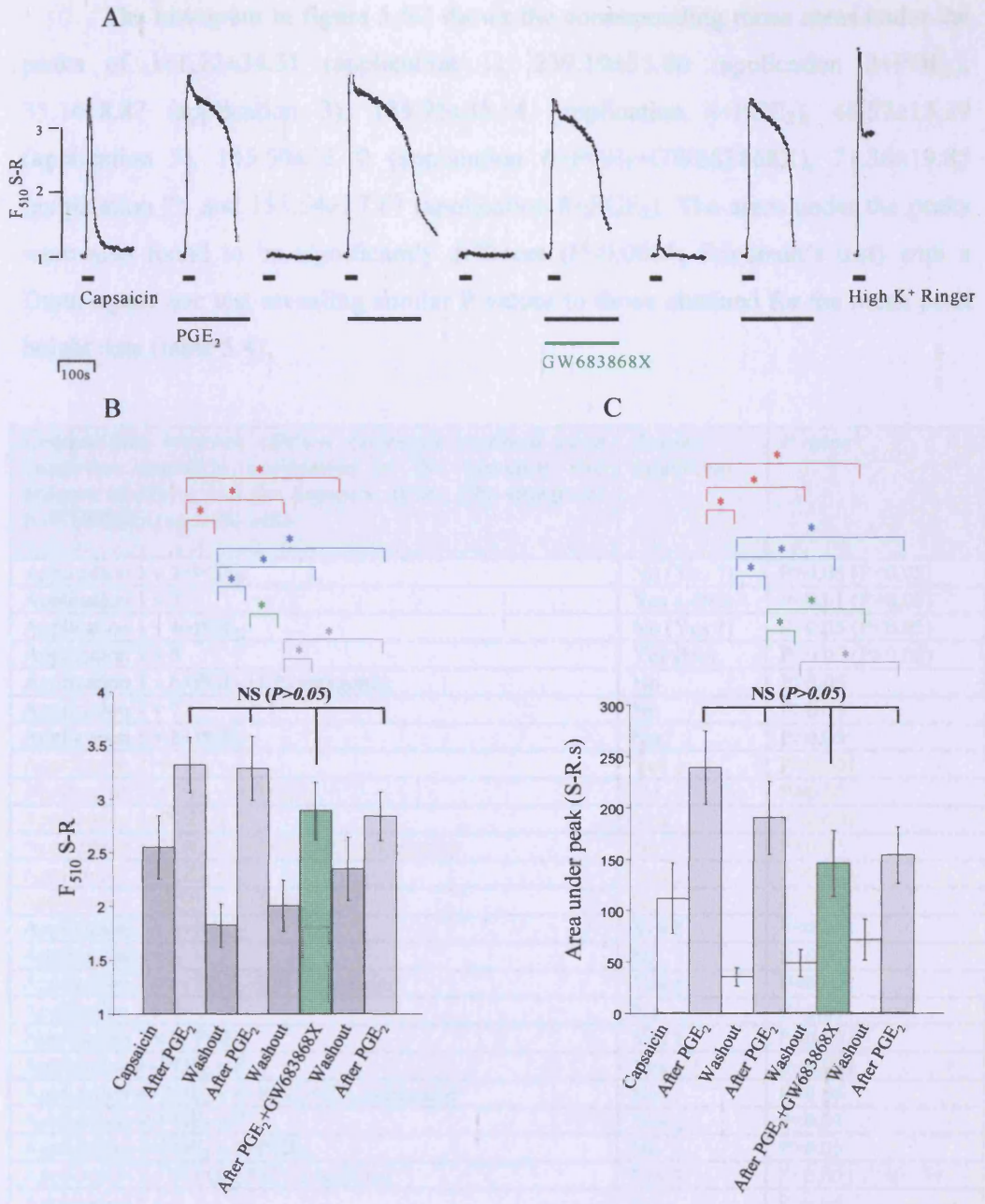


Figure 5.6: Contribution of the EP_1 receptor to PGE_2-induced sensitisation of capsaicin-evoked calcium responses in cultured DRG cells. A) An experimental record illustrating the sensitising effect of PGE_2 on capsaicin-evoked calcium responses, and the lack of effect on sensitisation following treatment with the EP_1 antagonist, GW683868X. Capsaicin (80nM; 10 seconds) was delivered eight times (*bars*) with a 10-minute period between applications in the presence (*bars*) and absence of PGE_2 (500nM; 3-minutes), and in the presence of GW683868X (500nM; 10-minutes) (*green bar*). Application of 60mM KCl was used at the end of each experiment to check cell viability. **B** and **C)** Histograms showing mean peak heights (F_{510} S-R) ±SEM **(B)** and mean areas under the peaks (F_{510} S-R.s) ±SEM **(C)** for 24 cells from 4 coverslips. For both peak heights and area under the peaks, Friedman's analysis confirmed significant difference ($P<0.0001$). Brackets denote significant (*$P<0.001$) and key non-significant comparisons from a *post hoc* Dunn's test (see table 5.4 for all comparisons).

The histogram in figure 5.6C shows the corresponding mean areas under the peaks of 111.72±34.31 (application 1), 239.19±35.80 (application 2+PGE_2), 35.14±8.87 (application 3), 189.95±35.54 (application 4+PGE_2), 48.57±13.39 (application 5), 145.50±32.10 (application 6+PGE_2+GW683868X), 71.36±19.85 (application 7), and 153.54±27.67 (application 8+PGE_2). The areas under the peaks were also found to be significantly different (P<0.0001; Friedman's test) with a Dunn's *post hoc* test revealing similar P values to those obtained for the mean peak height data (table 5.4).

Comparisons between calcium responses obtained upon successive capsaicin application in the presence and absence of PGE_2, and the presence of the EP_1 antagonist (GW683868X) in DRG cells.	**Results significant**	***P value***
Application 1 v 2+PGE_2	No (Yes ↑)	P>0.05 (P<0.05)
Application 1 v 3	Yes ↓ (No)	P<0.01 (P>0.05)
Application 1 v 4+PGE_2	No (Yes ↑)	P>0.05 (P<0.05)
Application 1 v 5	Yes (No)	P<0.05 (P>0.05)
Application 1 v 6+PGE_2+EP_1 antagonist	No	P>0.05
Application 1 v 7	No	P>0.05
Application 1 v 8+PGE_2	No	P>0.05
Application 2+PGE_2 v 3	Yes ↓	P<0.001
Application 2+PGE_2 v 4+PGE_2	No	P>0.05
Application 2+PGE_2 v 5	Yes ↓	P<0.001
Application 2+PGE_2 v 6+PGE_2+EP_1 antagonist	No	P>0.05
Application 2+PGE_2 v 7	Yes ↓	P<0.001
Application 2+PGE_2 v 8+PGE_2	No	P>0.05
Application 3 v 4+PGE_2	Yes ↑	P<0.001
Application 3 v 5	No	P>0.05
Application 3 v 6+PGE_2+EP_1 antagonist	Yes ↑	P<0.001
Application 3 v 7	No	P>0.05
Application 3 v 8+PGE_2	Yes ↑	P<0.001
Application 4+PGE_2 v 5	Yes ↓	P<0.001
Application 4+PGE_2 v 6+PGE_2+EP_1 antagonist	No	P>0.05
Application 4+PGE_2 v 7	Yes ↓	P<0.01
Application 4+PGE_2 v 8+PGE_2	No	P>0.05
Application 5 v 6+PGE_2+EP_1 antagonist	Yes ↑	P<0.001 (P<0.01)
Application 5 v 7	No	P>0.05
Application 5 v 8+PGE_2	Yes ↑	P<0.001
Application 6+PGE_2+EP_1 antagonist v 7	No (Yes ↓)	P>0.05 (P<0.05)
Application 6+PGE_2+ EP_1 antagonist v 8+PGE_2	No	P>0.05
Application 7 v 8+PGE_2	No (Yes ↑)	P>0.05 (P<0.01)

Table 5.4: Comparisons of calcium responses obtained upon successive capsaicin application in the presence and absence of PGE_2, and in the presence of the EP_1 antagonist, GW683868X in cultured DRG cells. Results were generated using the Dunn's *post hoc* test following Friedman's analysis. P values are representative of data for both peak heights and area under the peaks, except for the results bracketed, which represent P values from the area under the peak data. Where results show significant difference, ↑ and ↓ depict an observed increase or decrease in the second group in relation to the first, for each paired comparison.

5.2.2.2 Role of the EP_3 receptor in PGE_2-mediated sensitisation of Type-1 capsaicin-evoked calcium responses in DRG cells

The EP_3 antagonist, GW671021X was found to significantly reduce sensitisation of TRPV1 during analysis of all PGE_2 sensitised DRG cells (table 5.3). In this series of experiments, application of PGE_2 sensitised capsaicin-evoked calcium responses in 44.4% (24/54) of type-1 capsaicin-sensitive cells. In addition, PGE_2 produced sensitisation in 6.5% (11/169) of *silent* cells. The total number of cells (capsaicin responsive and non-responsive) sensitised by PGE_2, as a proportion of the total cell population was 15.7% (35/223, 5 coverslips); 68.6% (24/35) occurred in type-1 capsaicin sensitive cells and 31.4% (11/35) in *silent* cells. To illustrate the potency of the EP_3 antagonist, GW671021X, on PGE_2-mediated sensitisation of the capsaicin-evoked calcium response, only cells that were inhibited by the EP_3 antagonist (16/35) were considered in the following analysis (type-1 capsaicin-responsive and *silent* cells were pooled for data analysis).

The experimental trace shown in figure 5.7A shows representative calcium responses produced following repeated applications of capsaicin (80nM, 10 seconds) every 10-minutes in the absence and presence of PGE_2 (500nM, 3-minutes), as well as in the presence of PGE_2 (500nM, 3-minutes) after treatment with GW671021X (500nM, 10-minutes). PGE_2 produced potentiation of the capsaicin-evoked calcium response, but in the presence of GW671021X, failed to potentiate the capsaicin-evoked calcium response to the same extent. The histogram in figure 5.7B shows mean peak heights ±SEM for 16 cells from 5 coverslips. The values were 1.59±0.17 (application 1), 3.22±0.16 (application 2+PGE_2), 1.48±0.13 (application 3), 3.17±0.17 (application 4+PGE_2), 1.54±0.17 (application 5), 2.08±0.21 (application 6+ PGE_2+ GW671021X), 1.35±0.09 (application 7), and 2.47±0.26 (application 8+PGE_2). Statistical analysis using the Friedman's test revealed that differences between the mean peak heights of the capsaicin-evoked calcium responses were significant (*$P<0.0001$*). The results of a *post hoc* Dunn's test are summarised in table 5.5. Comparisons between calcium responses evoked by capsaicin in naïve cells as compared to those in cells pre-treated with PGE_2 revealed that sensitisation and re-sensitisation of TRPV1 had occurred. GW671021X significantly (*$P<0.001$*, Friedman's analysis and *post hoc* Dunn's test) inhibited to 49.79±7.51% PGE_2-induced sensitisation of TRPV1 in 45.7% (16/35) cells, indicating a role for the EP_3

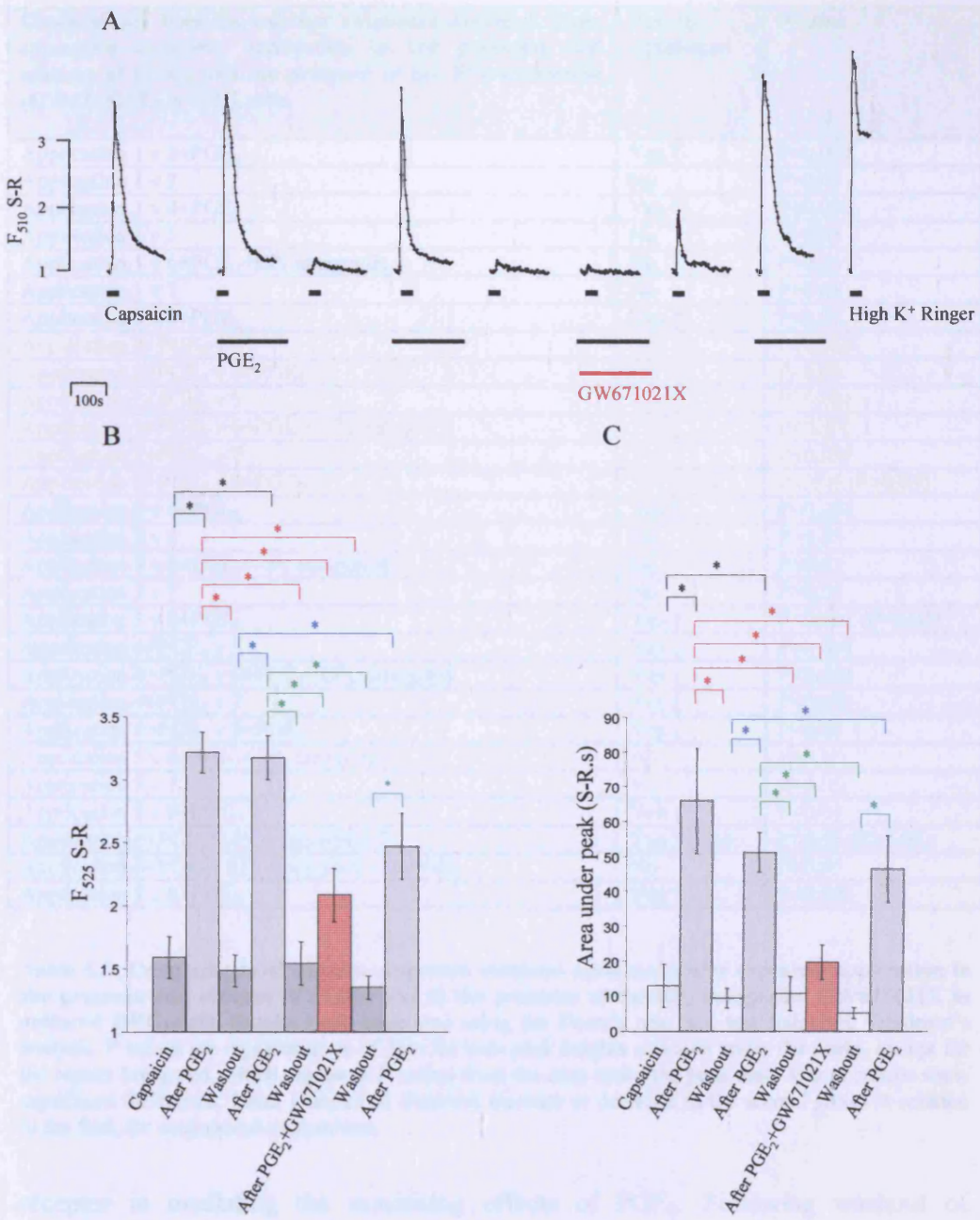


Figure 5.7: Contribution of the EP_3 receptor to PGE_2-induced sensitisation of capsaicin-evoked calcium responses in cultured DRG cells. A) An experimental record illustrating the sensitising effect of PGE_2 on capsaicin-evoked calcium responses and inhibition of sensitisation following treatment with the EP_3 antagonist, GW671021X. Capsaicin (80nM; 10 seconds) was delivered eight times (*bars*) with a 10-minute period between applications in the presence (*bars*) and absence of PGE_2 (500nM; 3-minutes), and in the presence of GW671021X (500nM; 10-minutes) (*red bar*). Application of 60mM KCl was used at the end of each experiment to check cell viability. **B** and **C)** Histograms showing the mean peak heights (F_{510} S-R) ±SEM **(B)** and mean areas under the peaks (F_{510} S-R.s) ±SEM **(C)** for 16 cells from 5 coverslips. For both peak heights and area under the peaks, Friedman's analysis confirmed significant difference ($P<0.0001$). Brackets denote significant comparisons (*$P<0.001$) from a *post hoc* Dunn's test (see table 5.5 for all comparisons).

Comparisons between calcium responses obtained upon successive capsaicin application in the presence and absence of PGE_2, and the presence of the EP_3 antagonist (GW671021X) in DRG cells.	**Results significant**	***P value***
Application 1 v 2+PGE_2	Yes ↑	P<0.001
Application 1 v 3	No	P>0.05
Application 1 v 4+PGE_2	Yes ↑	P<0.001
Application 1 v 5	No	P>0.05
Application 1 v 6+PGE_2+EP_3 antagonist	No	P>0.05
Application 1 v 7	No	P>0.05
Application 1 v 8+PGE_2	Yes ↑	P<0.01
Application 2+PGE_2 v 3	Yes ↓	P<0.001
Application 2+PGE_2 v 4+PGE_2	No	P>0.05
Application 2+PGE_2 v 5	Yes ↓	P<0.001
Application 2+PGE_2 v 6+PGE_2+EP_3 antagonist	Yes ↓	P<0.001
Application 2+PGE_2 v 7	Yes ↓	P<0.001
Application 2+PGE_2 v 8+PGE_2	Yes ↓ (No)	P<0.05 (P>0.05)
Application 3 v 4+PGE_2	Yes ↑	P<0.001
Application 3 v 5	No	P>0.05
Application 3 v 6+PGE_2+EP_3 antagonist	No	P>0.05
Application 3 v 7	No	P>0.05
Application 3 v 8+PGE_2	Yes ↑	P<0.001 (P<0.01)
Application 4+PGE_2 v 5	Yes ↓	P<0.001
Application 4+PGE_2 v 6+PGE_2+EP_3 antagonist	Yes ↓	P<0.001
Application 4+PGE_2 v 7	Yes ↓	P<0.001
Application 4+PGE_2 v 8+PGE_2	Yes ↓	P<0.05
Application 5 v 6+PGE_2+EP_3 antagonist	No	P>0.05
Application 5 v 7	No	P>0.05
Application 5 v 8+PGE_2	Yes ↑	P<0.01
Application 6+PGE_2+EP_3 antagonist v 7	Yes ↓ (No)	P<0.05 (P>0.05)
Application 6+PGE_2+ EP_3 antagonist v 8+PGE_2	No	P>0.05
Application 7 v 8+PGE_2	Yes ↑	P<0.001

Table 5.5: Comparisons of calcium responses obtained upon successive capsaicin application in the presence and absence of PGE_2, and in the presence of the EP_3 antagonist, GW671021X in cultured DRG cells. Results were generated using the Dunn's *post hoc* test following Friedman's analysis. P values are representative of data for both peak heights and area under the peaks, except for the results bracketed, which represent P values from the area under the peak data. Where results show significant difference, ↑ and ↓ depict an observed increase or decrease in the second group in relation to the first, for each paired comparison.

receptor in mediating the sensitising effects of PGE_2. Following washout of GW671021X, PGE_2 produced significant recovery from inhibition. However, the re-sensitisation produced by PGE_2 was significantly reduced as compared to that seen prior to GW671021X treatment, indicating that the antagonist was having a prolonged effect or was slow to wash out.

The histogram in figure 5.7C shows the corresponding mean areas under the peaks of 12.98±6.29 (application 1), 66.12±15.12 (application 2+PGE_2), 9.42±2.84 (application 3), 51.23±5.67 (application 4+PGE_2), 10.70±4.88 (application 5), 19.70±4.94 (application 6+PGE_2+GW671021X), 5.24±1.54 (application 7), and

46.44±9.48 (application 8+PGE_2) respectively. This data also showed statistically significant differences ($P<0.0001$; Friedman's test), with a Dunn's *post hoc* test revealing similar P values to those obtained for the mean peak heights (table 5.5).

5.2.2.3 Role of the EP_4 receptor in PGE_2-mediated sensitisation of Type-1 capsaicin-evoked calcium responses in DRG cells

Statistical analysis of all PGE_2 sensitised cells, obtained during studies utilising the EP_4 antagonist, GW627368X revealed that the EP_4 receptor was also important in mediating sensitisation of TRPV1 (table 5.3). In this series of experiments, PGE_2 produced sensitisation of the capsaicin-evoked calcium response in 58.7% (27/46) of type-1 cells and in 2.6% (3/115) of *silent* cells. The number of cells sensitised by PGE_2, as a proportion of the total cell population was 18.0% (30/167, 5 coverslips); 90% (27/30) of the sensitisation occurred in type-1 cells and 10% (3/30) in *silent* cells. The two populations were pooled and to illustrate the potency of the EP_4 antagonist, GW627368X, only cells subject to antagonist inhibition (18/30) were included in the following analysis.

The experimental trace in figure 5.8A shows representative capsaicin-evoked calcium responses (80nM, 10 seconds) obtained at 10-minute intervals in the absence and presence of PGE_2 (500nM, 3-minutes), as well as in the presence of PGE_2 (500nM 3-minutes) and GW627368X (500nM, 10-minutes). Following treatment with GW627368X, there was clear inhibition of PGE_2-mediated TRPV1 sensitisation. The histogram in figure 5.8B shows the mean peak heights ±SEM for 18 cells from 5 coverslips. The values obtained were 3.27±0.26 (application 1), 3.02±0.14 (application 2+PGE_2), 1.94±0.17 (application 3), 3.10±0.17 (application 4+PGE_2), 2.58±0.23 (application 5), 1.81±0.19 (application 6+PGE_2+GW627368X), 1.73±0.14 (application 7), and 2.92±0.16 (application 8+PGE_2). Significant differences were evident between comparisons ($P<0.0001$; Friedman's test). The results of the *post hoc* Dunn's test are shown in table 5.6. Comparisons between calcium responses evoked by capsaicin in naïve cells as compared to those in cells pre-treated with PGE_2 revealed that sensitisation and re-sensitisation of TRPV1 had occurred. The EP_4 antagonist GW627368X significantly reduced TRPV1 sensitisation to 38.49±7.67% in 60.0% (18/30) cells, indicating a role for the EP_4

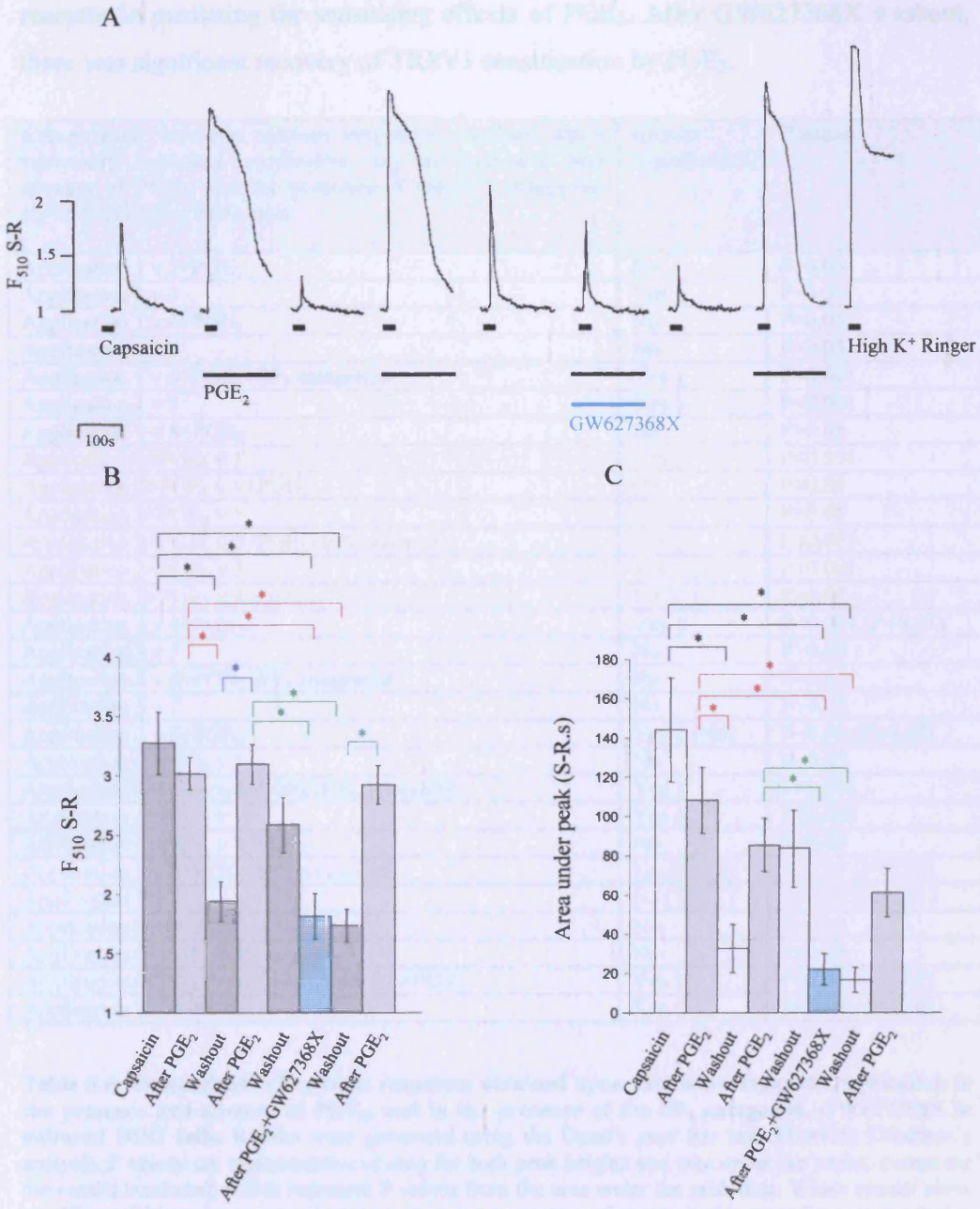


Figure 5.8: Contribution of the EP_4 receptor to PGE_2-induced sensitisation of capsaicin-evoked calcium responses in cultured DRG cells. A) An experimental record illustrating the sensitising effect of PGE_2 on capsaicin-evoked calcium responses and inhibition of sensitisation following treatment with the EP_4 antagonist, GW627368X. Capsaicin (80nM; 10 seconds) was delivered eight times (*bars*) with a 10-minute period between applications in the presence (*bars*) and absence of PGE_2 (500nM; 3-minutes), and in the presence of GW627368X (500nM; 10-minutes) (*aqua bar*). Application of 60mM KCl was used at the end of each experiment to check cell viability. **B)** and **C)** Histograms showing the mean peak heights (F_{510} S-R) ±SEM **(B)** and mean areas under the peaks (F_{510} S-R.s) ±SEM **(C)** for 18 cells from 5 coverslips. For both peak heights and area under the peaks, Friedman's analysis confirmed significant difference ($P<0.0001$). Brackets denote significant comparisons (*$P<0.001$) from a *post hoc* Dunn's test (see table 5.6 for all comparisons).

receptor in mediating the sensitising effects of PGE_2. After GW627368X washout, there was significant recovery of TRPV1 sensitisation by PGE_2.

Comparisons between calcium responses obtained upon successive capsaicin application in the presence and absence of PGE_2, and the presence of the EP_4 antagonist (GW627368X) in DRG cells.	**Results significant**	***P value***
Application 1 v 2+PGE_2	No	P>0.05
Application 1 v 3	Yes ↓	P<0.001
Application 1 v 4+PGE_2	No	P>0.05
Application 1 v 5	No	P>0.05
Application 1 v 6+PGE_2+EP_4 antagonist	Yes ↓	P<0.001
Application 1 v 7	Yes ↓	P<0.001
Application 1 v 8+PGE_2	No	P>0.05
Application 2+PGE_2 v 3	Yes ↓	P<0.001
Application 2+PGE_2 v 4+PGE_2	No	P>0.05
Application 2+PGE_2 v 5	No	P>0.05
Application 2+PGE_2 v 6+PGE_2+EP_4 antagonist	Yes ↓	P<0.001
Application 2+PGE_2 v 7	Yes ↓	P<0.001
Application 2+PGE_2 v 8+PGE_2	No	P>0.05
Application 3 v 4+PGE_2	Yes ↑	P<0.001 (P<0.01)
Application 3 v 5	No	P>0.05
Application 3 v 6+PGE_2+EP_4 antagonist	No	P>0.05
Application 3 v 7	No	P>0.05
Application 3 v 8+PGE_2	Yes ↑ (No)	P<0.01 (P>0.05)
Application 4+PGE_2 v 5	No	P>0.05
Application 4+PGE_2 v 6+PGE_2+EP_4 antagonist	Yes ↓	P<0.001
Application 4+PGE_2 v 7	Yes ↓	P<0.001
Application 4+PGE_2 v 8+PGE_2	No	P>0.05
Application 5 v 6+PGE_2+EP_4 antagonist	No (Yes ↓)	P>0.05 (P<0.05)
Application 5 v 7	No (Yes ↓)	P>0.05 (P<0.01)
Application 5 v 8+PGE_2	No	P>0.05
Application 6+PGE_2+EP_4 antagonist v 7	No	P>0.05
Application 6+PGE_2+ EP_4 antagonist v 8+PGE_2	Yes ↑	P<0.01 (P<0.05)
Application 7 v 8+PGE_2	Yes ↑	P<0.001 (P<0.01)

Table 5.6: Comparisons of calcium responses obtained upon successive capsaicin application in the presence and absence of PGE_2, and in the presence of the EP_4 antagonist, GW627368X in cultured DRG cells. Results were generated using the Dunn's *post hoc* test following Friedman's analysis. P values are representative of data for both peak heights and area under the peaks, except for the results bracketed, which represent P values from the area under the peak data. Where results show significant difference, ↑ and ↓ depict an observed increase or decrease in the second group in relation to the first, for each paired comparison.

The histogram in figure 5.8C shows the corresponding mean areas under the peaks ±SEM, which were 143.99±26.30 (application 1), 108.24±16.60 (application 2+PGE_2), 32.73±12.21 (application 3), 85.23±13.62 (application 4+PGE_2), 83.55±19.45 (application 5), 22.06±7.97 (application 6+PGE_2+GW627368X), 16.65±6.27 (application 7), and 60.94±12.39 (application 8+PGE_2) respectively. This data also showed statistically significant differences (P<0.0001; Friedman's test),

with a Dunn's *post hoc* test revealing similar P values to those obtained for the mean peak heights (table 5.6).

5.2.3 Sensitisation of type-1 capsaicin-evoked calcium responses by the EP_4 agonist, GSK324202A in DRG neurons

To further support a role for the EP_4 receptor in the sensitisation of TRPV1, experiments were performed on DRG cells to establish whether the selective EP_4 agonist, GSK324202A could sensitise the capsaicin-evoked calcium response in a similar manor to PGE_2. In this series of experiments, GSK324202A produced sensitisation of the capsaicin-evoked calcium response in 65.2% (107/164) of type-1 cells and 2.3% (10/436) of *silent* cells, whereas PGE_2, when applied following GSK324202A application, was only seen to produce sensitisation in 42.1% (69/164) of type-1 cells and none of the *silent* cells. The percentage of cells sensitised by GSK324202A as a proportion of the total cell population was 17.6% (117/665, 11 coverslips), with 91.5% (107/117) of the sensitisation being observed in type-1 cells and the remaining 8.5% (10/117) in *silent* cells. The type-1 capsaicin-sensitive and *silent* cells that were sensitised by GSK324202A were pooled in the following data analysis.

Figure 5.9A shows representative calcium responses produced upon repeated application of capsaicin (80nM, 10 seconds) at 10-minute intervals in the absence and presence of GSK324202A (250nM, 3-minutes) or PGE_2 (500nM, 3-minutes). In the presence of either agonist, the capsaicin-evoked calcium responses were sensitised. The histogram in figure 5.9B shows mean peak height data ±SEM for 117 (capsaicin applications 1 to 4, 11 coverslips), 105 (capsaicin application 5, 10 coverslips), and 89 (capsaicin application 6, 8 coverslips) cells. These were 3.05±0.12 (application 1), 3.51±0.11 (application 2+GSK324202A), 1.62±0.06 (application 3), 2.30±0.10 (application 4+PGE_2), 1.91±0.09 (application 5), and 3.19±0.12 (application 6+GSK324202A). Kruskal-Wallis analysis of mean peak height data showed that differences were significant ($P<0.0001$). The results of the *post hoc* Dunn's test are summarised in table 5.7. Statistical comparisons between calcium responses evoked by capsaicin in naïve cells as compared to those in cells treated with GSK324202A, revealed that sensitisation and re-sensitisation of TRPV1

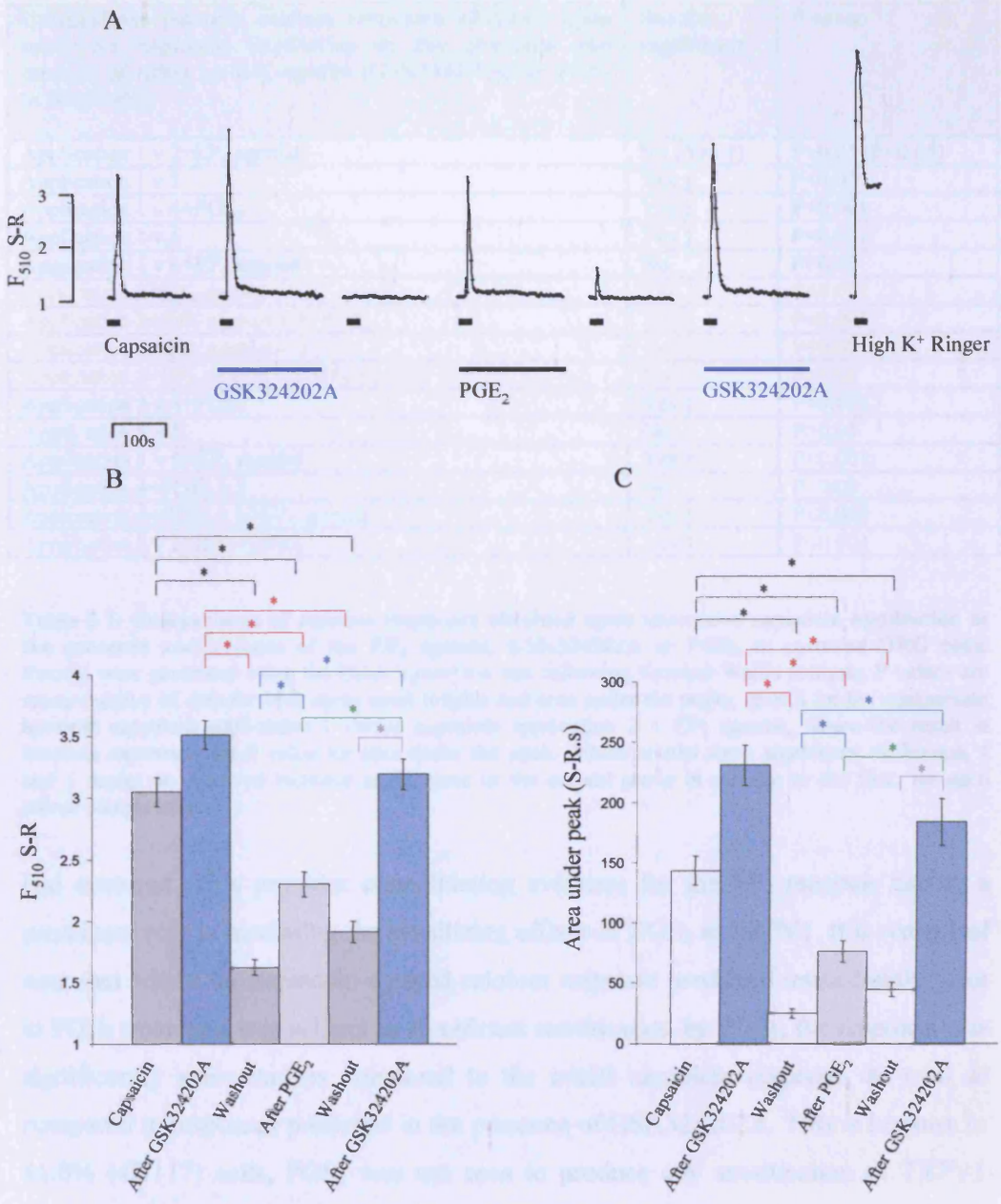


Figure 5.9: Sensitisation of the capsaicin-evoked calcium response by the EP_4 agonist GSK324202A in cultured DRG cells. A) An experimental record illustrating the effect of GSK324202A and PGE_2 treatment on the capsaicin-elicited calcium response. Capsaicin (80nM; 10 seconds) was delivered six times (*bars*) with a 10-minute period between applications in the presence and absence of either GSK324202A (250nM; 3-minutes, *blue bars*) or PGE_2 (500nM; 3-minutes, *black bars*). Application of 60mM KCl was used at the end of each experiment to check cell viability. **B)** and **C)** Histograms showing the mean peak heights (F_{510} S-R) ±SEM **(B)** and mean areas under the peaks (F_{510} S-R.s) ±SEM **(C)** for 117 (capsaicin applications 1 to 4; 11 coverslips), 105 (capsaicin application 5; 10 coverslips), and 89 (capsaicin application 6; 8 coverslips) cells. For both peak heights and area under the peaks, Kruskal-Wallis analysis confirmed significant difference ($P<0.0001$). Brackets denote significant comparisons (*$P<0.001$) from a *post hoc* Dunn's test (see table 5.7 for all comparisons).

Comparisons between calcium responses obtained upon successive capsaicin application in the presence and absence of either an EP_4 agonist (GSK324202A) or PGE_2 in DRG cells.	**Results significant**	***P value***
Application 1 v 2+EP_4 agonist	No (Yes ↑)	P>0.05 (P<0.05)
Application 1 v 3	Yes ↓	P<0.001
Application 1 v 4+PGE_2	Yes ↓	P<0.001
Application 1 v 5	Yes ↓	P<0.001
Application 1 v 6+EP_4 agonist	No	P>0.05
Application 2+EP_4 agonist v 3	Yes ↓	P<0.001
Application 2+ EP_4 agonist v 4+PGE_2	Yes ↓	P<0.001
Application 2+ EP_4 agonist v 5	Yes ↓	P<0.001
Application 2+EP_4 agonist v 6+EP_4 agonist	No	P>0.05
Application 3 v 4+PGE_2	Yes ↑	P<0.001
Application 3 v 5	No	P>0.05
Application 3 v 6+EP_4 agonist	Yes ↑	P<0.001
Application 4+PGE_2 v 5	No	P>0.05
Application 4+PGE_2 v 6+EP_4 agonist	Yes ↑	P<0.001
Application 5 v 6+EP_4 agonist	Yes ↑	P<0.001

Table 5.7: Comparisons of calcium responses obtained upon successive capsaicin application in the presence and absence of the EP_4 agonist, GSK324202A or PGE_2 in cultured DRG cells. Results were generated using the Dunn's *post hoc* test following Kruskal-Wallis analysis. P values are representative of data for both mean peak heights and area under the peaks, except for the comparison between capsaicin application 1 versus capsaicin application 2 + EP_4 agonist, where the result in brackets represents the P value for area under the peak. Where results show significant difference, ↑ and ↓ depict an observed increase or decrease in the second group in relation to the first, for each paired comparison.

had occurred. This provides consolidating evidence for the EP_4 receptor having a prominent role in mediating the sensitising effects of PGE_2 at TRPV1. It is worthy of note that whilst the capsaicin-evoked calcium response produced immediately prior to PGE_2 treatment was subject to significant sensitisation by PGE_2, the response was significantly attenuated as compared to the initial capsaicin response, as well as compared to responses produced in the presence of GSK324202A. This is because in 41.0% (48/117) cells, PGE_2 was not seen to produce any sensitisation of TRPV1 (figure 5.10). When these cells were removed from analysis, sensitisation produced by PGE_2 was not found to be significantly different from that produced by the EP_4 agonist (data not shown). Summarising the data, the initial capsaicin response (application 1) was sensitised to 125.20±6.65% (application 2+ GSK324202A) and 112.89±7.77% (application 6+GSK324202A) in the presence of GSK324202A and to 69.13±6.28% (application 4+PGE_2) after PGE_2 application.

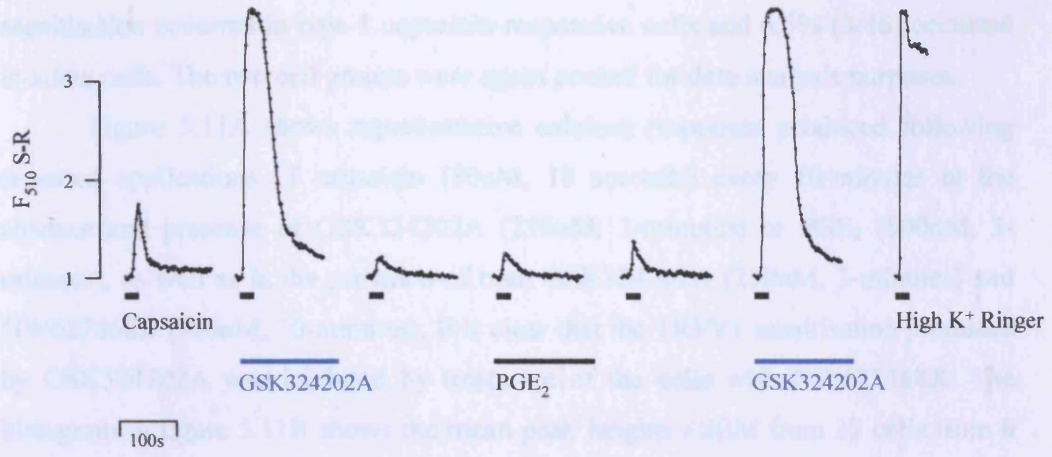


Figure 5.10: Experimental trace demonstrating the lack of PGE_2-evoked sensitisation of the capsaicin-evoked calcium response produced, following prior sensitisation using the EP_4 agonist, GSK324202A in DRG cells. Capsaicin (80nM; 10 seconds) was delivered six times (*bars*) with a 10-minute period between applications in the presence (*bars*) and absence of GSK324202A (250nM; 3-minutes, *blue bars*) and PGE_2 (500nM; 3-minutes, *black bars*). Application of 60mM KCl was used at the end of each experiment to check cell viability.

The histogram in figure 5.9C shows the corresponding mean areas under the peaks of 143.86±12.10 (application 1), 231.83±16.60 (application 2+GSK324202A), 25.27±4.01 (application 3), 77.00±8.88 (application 4+PGE_2), 45.14±6.00 (application 5), and 184.73±19.28 (application 6+GSK324202A) respectively. The areas under the peaks were also found to be significantly different (P<0.0001; Kruskal-Wallis test), with a Dunn's *post hoc* test revealing similar P values to those obtained for the mean peak heights (table 5.7).

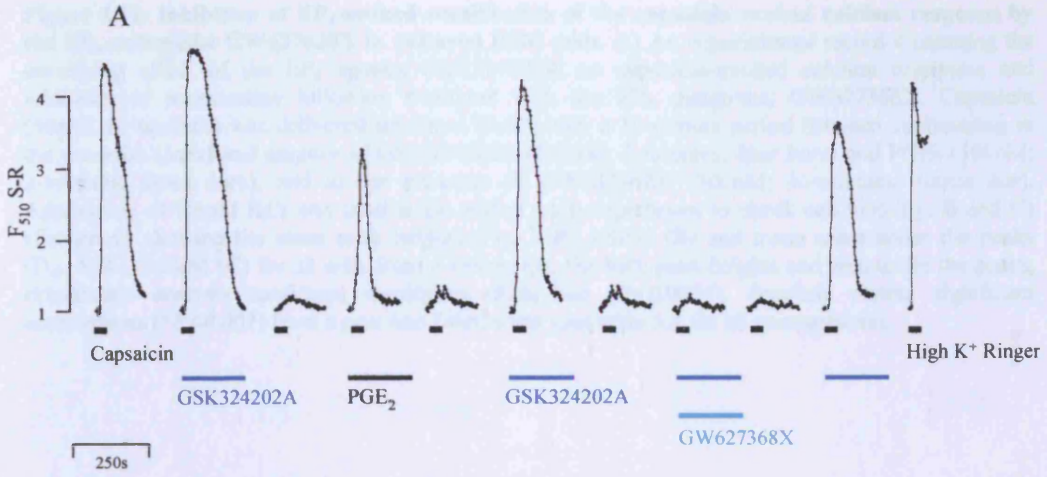
5.2.3.1 Effect of the EP_4 antagonist, GW327638X on EP_4 agonist, GSK324202A-induced sensitisation of the capsaicin-evoked calcium response in DRG cells

The effect of the EP_4 antagonist GW627368X was investigated on GSK324202A-mediated sensitisation of the capsaicin-evoked calcium response in DRG cells. In this series of experiments, sensitisation of capsaicin-evoked calcium responses by GSK324202A was observed in 52.4% (43/82) type-1 capsaicin-sensitive cells and 1.3% (3/236) of *silent* cells. The number of cells that were sensitised by GSK324202A, as a proportion of the total cell population (capsaicin-responsive and non-responsive) was 13.8% (46/334); 93.5% (43/46) of the

sensitisation occurred in type-1 capsaicin-responsive cells and 6.5% (3/46) occurred in *silent* cells. The two cell groups were again pooled for data analysis purposes.

Figure 5.11A shows representative calcium responses produced following repeated applications of capsaicin (80nM, 10 seconds) every 10-minutes in the absence and presence of GSK324202A (250nM, 3-minutes) or PGE_2 (500nM, 3-minutes), as well as in the presence of both GSK324202A (250nM, 3-minutes) and GW627368X (500nM, 10-minutes). It is clear that the TRPV1 sensitisation produced by GSK324202A was inhibited by treatment of the cells with GW627368X. The histogram in figure 5.11B shows the mean peak heights ±SEM from 32 cells from 6 coverslips. These were 2.65±0.14 (application 1), 3.03±0.16 (application 2+GSK324202A), 1.83±0.11 (application 3), 2.41±0.15 (application 4+PGE_2), 1.91±0.13 (application 5), 2.90±0.14 (application 6+GSK324202A), 1.66±0.11 (application 7), 2.01±0.14 (application 8+GSK324202A+GW627368X), 1.60±0.10 (application 9), and 2.44±0.15 (application 10+GSK324202A). Statistical analysis using the Friedman's test revealed that differences between the mean peak heights were significant ($P<0.0001$). The results of the *post hoc* Dunn's test are summarised in table 5.8. GSK324202A-mediated sensitisation of the capsaicin-evoked calcium response was reduced to 50.46±5.81% by GW627368X in 74.4% (32/43) of type-1 capsaicin-sensitive cells but in none of the *silent* cells. After GW627368X washout, significant re-sensitisation of TRPV1 by GSK324202A was apparent.

The histogram in figure 5.11C shows corresponding mean areas under the peaks ±SEM of 113.41±16.19 (application 1), 164.85±21.29 (application 2+GSK324202A), 31.60±5.34 (application 3), 68.12±9.16 (application 4+PGE_2), 40.15±7.57 (application 5), 139.74±18.29 (application 6+GSK324202A), 20.22±3.48 (application 7), 44.78±8.90 (application 8+GSK324202A+GW627368X), 18.68±4.12 (application 9), and 78.52±12.48 (application 10+GSK324202A) respectively. The areas under the peaks were also found to be significantly different ($P<0.0001$; Friedman's test), with a Dunn's *post hoc* test revealing similar P values to those obtained for the mean peak heights (table 5.8).


A
F_{510} S-R
4
3
2
1
Capsaicin
High K⁺ Ringer
GSK324202A
PGE_2
GSK324202A
GW627368X
250s

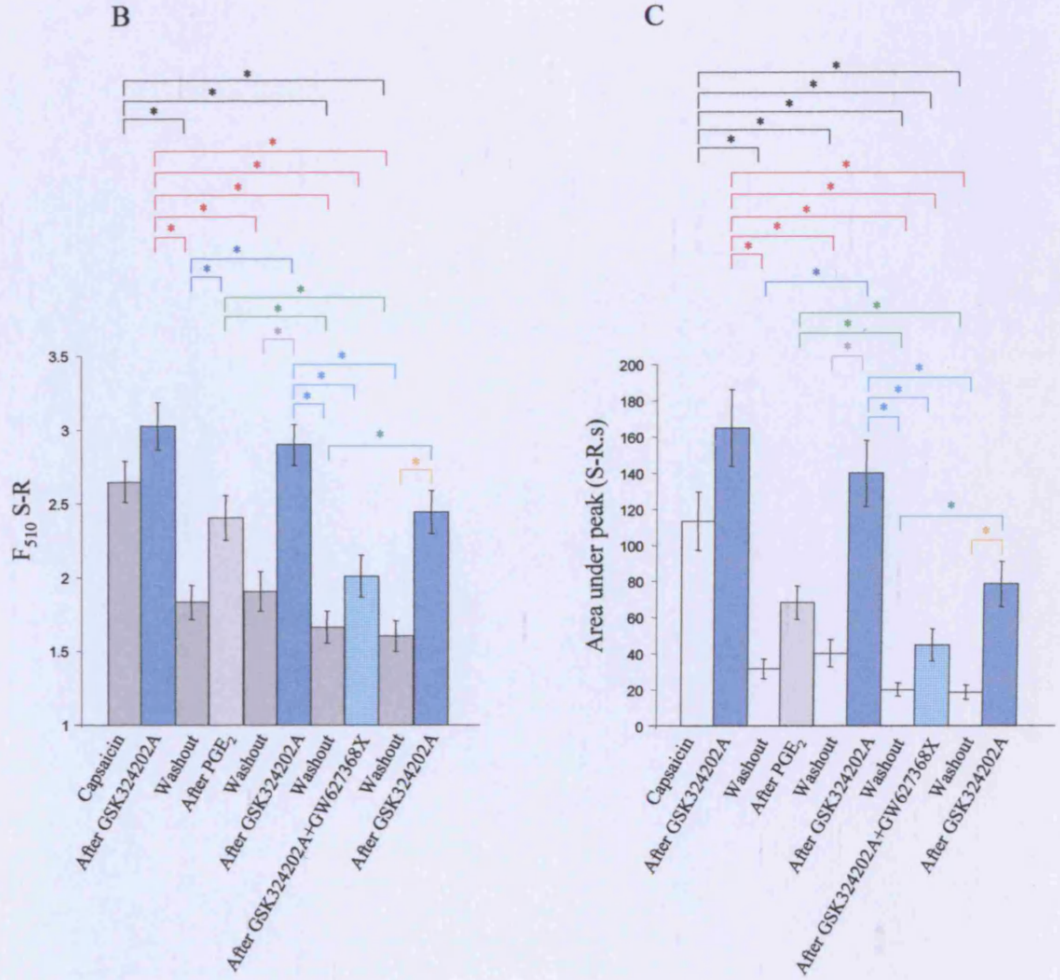

B
C
F_{510} S-R
Area under peak (S-R.s)
Capsaicin
After GSK324202A
Washout
After PGE_2
Washout
After GSK324202A
Washout
After GSK324202A+GW627368X
Washout
After GSK324202A

Figure 5.11: Inhibition of EP_4-evoked sensitisation of the capsaicin-evoked calcium response by the EP_4 antagonist GW627638X in cultured DRG cells. A) An experimental record illustrating the sensitising effect of the EP_4 agonist GSK324202A on capsaicin-evoked calcium responses and inhibition of sensitisation following treatment with the EP_4 antagonist, GW627368X. Capsaicin (80nM; 10 seconds) was delivered ten times (*bars*) with a 10-minute period between applications in the presence (*bars*) and absence of GSK324202A (250nM; 3-minutes, *blue bars*) and PGE_2 (500nM; 3-minutes, *black bars*), and in the presence of GW627368X (500nM; 10-minutes) (*aqua bar*). Application of 60mM KCl was used at the end of each experiment to check cell viability. **B** and **C)** Histograms showing the mean peak heights (F_{510} S-R) ±SEM **(B)** and mean areas under the peaks (F_{510} S-R.s) ±SEM **(C)** for 32 cells from 6 coverslips. For both peak heights and area under the peaks, Friedman's analysis confirmed significant difference ($P<0.0001$). Brackets denote significant comparisons (*$P<0.001$) from a ***post hoc*** Dunn's test (see table 5.8 for all comparisons).

Comparisons between calcium responses obtained upon successive capsaicin application in the presence and absence of EP_4 agonist (GSK324202A) or PGE_2, and in the presence of EP_4 agonist following EP_4 antagonist (GW627368X) treatment in DRG cells.	**Results significant**	***P value***
Application 1 v 2+EP_4 agonist	No	P>0.05
Application 1 v 3	Yes ↓	P<0.001
Application 1 v 4+PGE_2	No	P>0.05
Application 1 v 5	Yes ↓	P<0.01 (P<0.001)
Application 1 v 6+EP_4 agonist	No	P>0.05
Application 1 v 7	Yes ↓	P<0.001
Application 1 v 8+EP_4 agonist+EP_4 antagonist	Yes ↓	P<0.05 (P<0.001)
Application 1 v 9	Yes ↓	P<0.001
Application 1 v 10+EP_4 agonist	No	P>0.05
Application 2+EP_4 agonist v 3	Yes ↓	P<0.001
Application 2+EP_4 agonist v 4+PGE_2	No (Yes ↓)	P>0.05 (P<0.05)
Application 2+EP_4 agonist v 5	Yes ↓	P<0.001
Application 2+EP_4 agonist v 6+EP_4 agonist	No	P>0.05
Application 2+EP_4 agonist v 7	Yes ↓	P<0.001
Application 2+EP_4 agonist v 8+EP_4 agonist+EP_4 antagonist	Yes ↓	P<0.001
Application 2+EP_4 agonist v 9	Yes ↓	P<0.001
Application 2+EP_4 agonist v 10+EP_4 agonist	No (Yes ↓)	P>0.05 (P<0.01)
Application 3 v 4+PGE_2	Yes ↑ (No)	P<0.05 (P>0.05)
Application 3 v 5	No	P>0.05
Application 3 v 6+EP_4 agonist	Yes ↑	P<0.001
Application 3 v 7	No	P>0.05
Application 3 v 8+EP_4 agonist+EP_4 antagonist	No	P>0.05
Application 3 v 9	No	P>0.05
Application 3 v 10+EP_4 agonist	Yes ↑	P<0.05
Application 4+PGE_2 v 5	No	P>0.05
Application 4+PGE_2 v 6+EP_4 agonist	No	P>0.05
Application 4+PGE_2 v 7	Yes ↓	P<0.001
Application 4+PGE_2 v 8+EP_4 agonist+EP_4 antagonist	No	P>0.05
Application 4+PGE_2 v 9	Yes ↓	P<0.001
Application 4+PGE_2 v 10+EP_4 agonist	No	P>0.05
Application 5 v 6+EP_4 agonist	Yes ↑	P<0.001
Application 5 v 7	No	P>0.05
Application 5 v 8+EP_4 agonist+EP_4 antagonist	No	P>0.05
Application 5 v 9	No	P>0.05
Application 5 v 10+EP_4 agonist	No	P>0.05
Application 6+EP_4 agonist v 7	Yes ↓	P<0.001
Application 6+EP_4 agonist v 8+EP_4 agonist+EP_4 antagonist	Yes ↓	P<0.001
Application 6+EP_4 agonist v 9	Yes ↓	P<0.001
Application 6+EP_4 agonist v 10+EP_4 agonist	No (Yes ↓)	P>0.05 (P<0.05)
Application 7 v 8+EP_4 agonist+EP_4 antagonist	No	P>0.05
Application 7 v 9	No	P>0.05
Application 7 v 10+EP_4 agonist	Yes ↑	P<0.001
Application 8+EP_4 agonist+EP_4 antagonist v 9	No	P>0.05
Application 8+EP_4 agonist+EP_4 antagonist v 10+EP_4 agonist	No	P>0.05
Application 9 v 10+EP_4 agonist	Yes ↑	P<0.001

Table 5.8: Comparisons of calcium responses obtained upon successive capsaicin application in the presence and absence of the EP_4 agonist, GSK324202A or PGE_2, and in the presence of the EP_4 agonist following EP_4 antagonist (GW627368X) treatment in cultured DRG cells. Results were generated using the Dunn's *post hoc* test following Friedman's analysis. P values are representative of data for both peak heights and area under the peaks, except for the results bracketed, which represent P values from the area under the peak data. Where results show significant difference, ↑ and ↓ depict an observed increase or decrease in the second group in relation to the first, for each paired comparison.

5.3 Discussion

Sensitisation of sensory neurons is believed to be the mechanism that underlies PGE_2-mediated hyperalgesia (section 1.3.2.2). This prostanoid produces its effects by activating at least one prostaglandin E receptor (EP) subtype (section 1.3.2.3). To date, it has been impossible to elucidate, which EP receptors are important in mediating the pro-nociceptive effects of PGE_2, especially since all subtypes of EP receptor have been reported to be present in the DRG [Donaldson *et al.*, 2001]. With the aid of novel potent and selective PG receptor compounds, provided by industrial collaborators, Glaxo SmithKine (GSK), it has been possible to define the involvement of the various PG receptors in the sensitisation of sensory neurons. The results presented in this chapter have shown that both the EP_3 and EP_4 receptors, but not the EP_1 receptor have an important role in mediating the sensitising effects of PGE_2 on the capsaicin-elicited calcium response in adult rat DRG neurons.

5.3.1 Specificity of the PG receptor compounds

The specificity of each of the PG receptor compounds for their respective cloned human prostanoid receptors have been fully characterised (table 5.1), and this data was recently published in its entirety for the EP_4 receptor antagonist, GW627368X [Wilson *et al.*, 2006]. It is believed that these molecules are some of the most potent and selective developed to date, which means they will be invaluable for defining the function of the various PG receptors within different cellular systems in the future.

It was not the intention of this chapter to evaluate in detail the pharmacology of each of the EP receptor compounds utilised. Data has been included to demonstrate the functionality of some of the compounds against their respective cloned human prostanoid receptors. These experiments revealed that the compounds tested were functional at the prostanoid receptors for which they were developed, and also provided consolidating evidence that the EP_1-CHO and EP_4-HEK cell lines were expressing functional receptors.

5.3.2 Involvement of three different EP receptors in mediating the sensitising effects of PGE_2 on TRPV1 in sensory neurons

Results from chapter four showed that the sensitisation of capsaicin-evoked calcium responses by PGE_2 in rat sensory neurons is dependent upon the activation of EP receptors, as a prostaglandin receptor antagonist cocktail, containing the selective EP_1 (GW683868X), EP_3 (GW671021X), and EP_4 (GW627368X) receptor antagonists supplied by GSK, inhibited sensitisation of TRPV1 by PGE_2 in the majority of DRG cells tested (section 4.2.2.4). As a result, these antagonists were used individually to determine, which receptor(s) were responsible for mediating TRPV1 sensitisation.

5.3.2.1 Sensitisation of TRPV1 is independent of EP_1 receptor activation

When DRG cells were treated with the EP_1 receptor antagonist, GW683868X, PGE_2 was still capable of sensitising capsaicin-evoked calcium responses in the majority (87.5%) of cells (table 5.3) analysed, indicating that the EP_1 receptor does not have a major role in mediating the sensitising effects of PGE_2 at TRPV1.

It is believed that TRPV1 is highly phosphorylated in its resting state, becoming rapidly dephosphorylated following activation, which is one way desensitisation of channel function is thought to occur (section 1.4.1.1). The protein kinases A (PKA) and C (PKC) have been shown to rescue TRPV1 from desensitisation by re-phosphorylating the channel (section 1.4.1.1). In line with this, various inflammatory mediators have been shown to produce sensitisation of TRPV1 by activating one of these kinases (1.4.1.2). Studies in rat sensory neurons using the inflammatory mediator PGE_2 to sensitise capsaicin-evoked responses have demonstrated the involvement of PKA as opposed to PKC [Pichford & Levine, 1991, Lopshire & Nicol, 1998, Gu *et al.,* 2003]. This supports the observation that EP_1 does not have an important role in PGE_2-mediated sensitisation of TRPV1, as the second messenger system for the EP_1 receptor is believed to couple to $G_{q/11}$ and the downstream activation of PKC.

Moriyama and colleagues have provided contrasting evidence to support a role for the EP_1 receptor in mediating the sensitising effects of PGE_2 at TRPV1 [Moriyama *et al.,* 2005]. The authors have shown that short-term PGE_2 (1μM)

exposure (1.5-minutes) potentiated the capsaicin-elicited inward current in adult mouse DRG neurons, an effect, which was mimicked by the EP_1 agonist, ONO-DI-004, and inhibited by the EP_1 antagonist, ONO-8713. Furthermore, the effects of PGE_2 were found to be dependent upon PKC activation [Moriyama *et al.*, 2005]. The reasons for the discrepancy between the observations reported in this chapter as compared to those reported by Moriyama and colleagues are not clear at present. Species variation cannot be ruled out as Moriyama and colleagues utilised DRG cells from adult mice, whereas the work described in this chapter was performed on adult rat DRG cells. However, it is worthy of note that a previous study utilising similar experimental parameters to the present study, which was performed using cultured mouse DRG cells obtained from the same strain, and similar aged mice to those utilised by Moriyama and colleagues, has provided evidence to suggest that sensitisation of capsaicin-evoked calcium responses via mGlu5 receptor-evoked PGE_2 production is via a PKA and not a PKC-dependent mechanism [Hu *et al.*, 2002]. This again supports a non-EP_1 activated route.

Another consideration is that the EP_1 receptor compounds utilised by Moriyama and colleagues, were used at concentrations that could have produced activation of other EP receptors [Moriyama *et al.*, 2005]. However, the authors reported no PGE_2-mediated potentiation of the capsaicin-elicited inward current in DRG cells from EP_1 knockout mice, which supports their findings in normal mouse DRG cells. Interestingly, Moriyama and colleagues also observed that during long-term exposure (6.5-minutes) to either PKA or PKC stimulating compounds, only the compounds that stimulated PKA activation produced sensitisation of the capsaicin-evoked inward current, which led them to hypothesise that during longer PGE_2 applications, the sensitisation of TRPV1 could involve a receptor other than the EP_1 receptor. Indeed, their work on recombinant cells showed that following prolonged treatments with PGE_2, potentiation of the capsaicin-evoked inward current was only produced in HEK cells co-expressing TRPV1 and the EP_4 receptor, and not in HEK cells co-expressing TRPV1 and any other EP receptor [Moriyama *et al.*, 2005]. These results suggest that the acute effects of PGE_2 on TRPV1 could be mediated by the EP_1 receptor, but the more prolonged effects of PGE_2, for example those occurring during inflammation, involve the EP_4 receptor. Unfortunately, Moriyama and colleagues did not investigate the effects of PGE_2 on TRPV1 responses after longer exposure times in mouse DRG cells.

5.3.2.2 The EP_3 and EP_4 receptors make substantial contributions to sensitisation of TRPV1

In contrast to the EP_1 receptor, both the EP_3 and EP_4 receptors were found to have an involvement in mediating the sensitising effects of PGE_2 on sensory neurons. This is evident from the fact that the EP_3 antagonist, GW671021X, and the EP_4 antagonist, GW627368X, significantly inhibited PGE_2-mediated sensitisation of capsaicin-evoked calcium responses in substantial proportions (45.7% and 60.0% respectively) of rat DRG cells. Furthermore, the selective EP_4 agonist, GSK324202A was seen to produce sensitisation and re-sensitisation of capsaicin-elicited calcium responses, which were inhibited following GW627368X (EP_4 antagonist) application in the majority (74.4%) of the cells analysed.

The EP_3 and EP_4 receptors have previously been implicated in mediating the sensitising effects of PGE_2 in sensory neurons [Kumazawa *et al.*, 1993, 1996, Southall & Vasko, 2001, Kopp *et al.*, 2004]. However, the majority of these studies have again utilised EP receptor compounds that are claimed to be specific but are known to activate other EP receptor subtypes at the applied concentrations. Unlike most others, Southall & Vasko have taken a different approach, utilising antisense oligonucleotides against each EP receptor mRNA in order to "knock down" expression of the various receptors in cultured rat DRG neurons, and so determine, which receptors contribute to their sensitisation. The authors revealed PCR products for the EP_1, EP_2, EP_{3C} (same as rat EP_{3B} and mouse $EP_{3\gamma}$), and EP_4 receptors in the cultured DRG cells, and showed that treatment of the cultures for 48 hours with antisense oligonucleotides of EP_{3C} and EP_4 mRNA, diminished expression of these receptors and abolished PGE_2-stimulated cAMP production or PGE_2-mediated potentiation of capsaicin-evoked SP and CGRP release [Southall & Vasko, 2001].

The EP_3 antagonist, GW671021X, used in the present study does not discriminate between EP_3 receptor splice variants, but if the findings of Southall & Vasko are correct, then it is the EP_{3B} (aka EP_{3C}) receptor, which is mediating the sensitising effects of PGE_2 at TRPV1, as the authors claim that this is the only EP_3 receptor splice variant present in cultured rat DRG cells [Southall & Vasko, 2001]. However, it is worthy of note, that Donaldson and colleagues identified all EP receptor subtypes, including all EP_3 receptor splice variants using RT-PCR on adult rat DRG tissue homogenates [Donaldson *et al.*, 2001]. In any case, according to the

view that TRPV1 is sensitised as a result of phosphorylation by protein kinases, only the EP_{3B} ($EP_{3C}/EP_{3\gamma}$) receptor, which is believed to stimulate cAMP formation through its agonist-induced G_s activity [Irie *et al.*, 1993, Negishi *et al.*, 1996], would be expected to stimulate PKA activation and so sensitise TRPV1. The other rat EP_3 splice variants are coupled to G_i and should in all probability inhibit TRPV1 sensitisation. An intriguing consideration is that during normal physiological functioning, the constitutive G_i activity of the EP_{3B} receptor might contribute to keeping nociceptors in a quiescent state, whereas under pathophysiological conditions, the repetitive stimulation of EP_{3B} receptors by PGE_2 might contribute to nociceptor sensitisation. Further investigation is necessary to clarify which EP_3 receptor splice variant is involved in PGE_2-mediated sensitisation of the capsaicin-evoked calcium response.

One interesting observation made during the course of the work carried out in this chapter was that following sensitisation of the capsaicin-elicited calcium response using the EP_4 agonist, GSK324202A, subsequent PGE_2 treatment was only seen to produce further sensitisation of TRPV1 in a reduced proportion (59%) of the EP_4 agonist sensitive cells. It is unlikely that this phenomenon is as a result of EP_4 receptor desensitisation, as both agonists can produce reproducible sensitisation of capsaicin-elicited calcium responses in DRG neurons (sections 4.2.2.4 and 5.2.3.1). One possible explanation for these observations is that PGE_2 was binding to an EP receptor other than the EP_4 receptor, which has no involvement in mediating TRPV1 sensitisation. This is certainly a possibility, as a saturating concentration of PGE_2 (500nM) was used in the work, which could activate any one of the EP receptor subtypes, given that the affinity of PGE_2 for its receptors ranges between 0.33nM and 19nM [Abramovitz *et al.*, 2000, Wilson *et al.*, 2006].

An alternative explanation is that the selective EP_4 agonist interacts with the receptor in such a way as to modify the binding affinity of PGE_2. Prostanoid binding to the EP_4 receptor is known to be dependant upon two distinct regions within the molecule structure, namely the seventh transmembrane domain [Chang *et al.*, 1997], and the extracellular loops [Stillman *et al.*, 1998, 1999]. Given the results presented in this chapter, it is unlikely that application of the EP_4 agonist caused a significant change in the binding site(s) of the receptor, as this would not explain why re-application of the EP_4 agonist after PGE_2 treatment, led to a similar TRPV1 sensitisation as compared to the initial sensitisation produced by this ligand. Instead,

the EP_4 agonist might have caused subtle changes in receptor conformation, thereby reducing the binding affinity of PGE_2, without affecting its own binding affinity. Unfortunately, without the aid of molecular modelling simulations it is impossible to show whether the binding of the EP_4 agonist would elicit any conformational changes in the receptor that could have affected the binding of PGE_2 and the EP_4 agonist to different extents.

A final consideration is that the EP_4 agonist could be a more potent and selective ligand for EP_4 receptors in adult rat DRG neurons as compared to PGE_2. In support of this hypothesis, the EP_4 antagonist, GW627368X, utilised in the present study, which has a similar chemical structure to the EP_4 agonist (figure 2.1) has been shown to exhibit a greater potency at native porcine EP_4 receptors as compared to cloned human EP_4 receptors [Giblin *et al.*, 2002, Wilson *et al.*, 2005].

The above two sections have discussed the involvement of the EP_1, EP_3, and EP_4 receptors in PGE_2-mediated sensitisation of TRPV1. However, no attempt was made during the course of the work described in this thesis to investigate the role of the EP_2 receptor. This was due to the lack of truly selective EP_2 receptor compounds available and time constraints. One important observation made in the previous chapter was that PGE_2-mediated sensitisation of capsaicin-elicited calcium responses could be completely inhibited by treatment with a PG receptor antagonist cocktail (GSK EP_1, EP_3, and EP_4 antagonists) in almost all (~85%) DRG cells tested (section 4.2.2.4). Therefore, it is postulated that the EP_2 receptor does not have a significant role in mediating the sensitising effects of PGE_2 at TRPV1 in cultured rat DRG cells. Further work is needed to confirm this hypothesis, utilising either novel EP_2 antagonists (selective EP_2 antagonists are not yet available) and / or a combination approach using existing EP_2 agonists (butaprost or ONO-AE1-259) and other prostanoid receptor antagonists as necessary.

In summary, the EP_3 and EP_4 antagonists, but not the EP_1 antagonist significantly inhibited PGE_2-mediated sensitisation of capsaicin-evoked calcium responses in DRG neurons, indicating an involvement of these receptors in mediating the sensitising effects of PGE_2. Further evidence to support a role for the EP_4 receptor was provided by the observation that a selective EP_4 agonist could enhance the capsaicin-elicited calcium response in approximately two thirds of capsaicin-

sensitive neurons, which would normally be subject to *tachyphylaxis*. In addition, the EP_4 agonist was also seen to sensitise a small proportion of *silent* neurons to become capsaicin-responsive. Both of these observations represent TRPV1 sensitisation and are similar to those witnessed following PGE_2 treatment. This EP_4 agonist-dependent sensitisation of TRPV1 was abolished in the majority of DRG neurons following incubation with the EP_4 antagonist. Interestingly, in those cells sensitised by the EP_4 agonist, subsequent PGE_2 application was not always seen to produce re-sensitisation of the capsaicin-evoked calcium response. The reasons behind this require further clarification.

Chapter Six

Conclusions and future work

Aspirin and NSAIDs are among the most widely used pharmaceutical agents worldwide [Rocca, 2006]. These drugs produce their anti-inflammatory and analgesic effects by interfering with the synthesis of prostaglandins, which are important chemical mediators released during tissue injury (section 1.3.2). In particular PGE_2 and PGI_2 are implicated in the inflammatory response to injury and in sensitising peripheral nociceptors (section 1.3.2.2), whereas PGE_2, $PGF_{2\alpha}$, and PGD_2 are all believed to have an involvement in central sensitisation processes (section 1.6.4). The rate-limiting step in the production of endogenous prostaglandins is activation of an enzyme known as cyclo-oxygenase (COX) (section 1.3.2.1), and nearly 30 years ago, the therapeutic effects of NSAIDs were clearly shown to be the result of COX inhibition [Vane, 1971].

There are two isoforms of COX, which have similar actions in the prostaglandin synthetic pathway (section 1.3.2.1) but are encoded by two different genes. COX-1 is constitutively expressed in most tissues and produces PGs that serve a number of physiological "housekeeping" functions, such as modulation of platelet aggregation and cytoprotection in the gastrointestinal mucosa [Rocca & FitzGerald, 2002]. In contrast, COX–2, is constitutively expressed in discrete regions of the CNS and in the renal cortex [Seibert *et al.,* 1994]. However, its expression can be induced in macrophages, fibroblasts, vascular endothelial cells, and smooth muscle cells, by various cytokines, endotoxins, growth factors, or tumor promoters. Therefore, COX-2-dependent PGs play a major role in mediating pain, the inflammatory response to injury, and cell proliferation. Since the older NSAIDs inhibit both COX-1 and COX-2, their use results in side effects (e.g. stomach ulcers) attributable to the loss of COX-1 function and therapeutic effects attributable to the inhibition of COX-2. The discovery of COX-2 led to the idea that agents, which could specifically inhibit only this isoform might relieve pain and inflammation, without the unwanted side effects exhibited by traditional NSAIDs. Indeed, the late nineties saw the emergence of highly selective COX-2 inhibitors, colloquially referred to as Coxibs, which showed

comparable anti-inflammatory and analgesic activities with fewer gastrointestinal complications than traditional NSAIDs [Baigent & Patrono, 2003]. Unfortunately, these drugs have been associated with hazardous cardiovascular effects during long-term studies [Ray *et al.*, 2002, Jones *et al.*, 2005], which has led to the recent withdrawal of a number of them from the market. Consequently, the development of new therapeutic strategies, associated with fewer side effects is imperative for a more successful approach to the treatment of chronic pain states. One possible route towards better therapeutics is to target downstream PG receptors that are specifically involved in nociception. Therefore, the aim of the work in this thesis was to investigate, which PG receptor subtype(s) have an involvement in PG-mediated peripheral and central pain mechanisms.

In chapter three an array of PG receptor antibodies were characterised in order to attempt to create a distribution map of PG receptor expression within the rat peripheral and central nervous system. This work was performed because any PG receptors involved in PG mediated nociception should be expressed in the DRG and spinal cord at sites consistent with an involvement in nociceptive processing (sections 1.2 and 1.6.1). Of the antibodies tested, one EP_4 receptor antibody was found to show high selectivity towards its target protein. In Western blotting experiments on EP_4-HEK cells, the EP_4 protein was detected in its native form, glycosylated, and oligomerised forms, the latter two of which, are believed to be essential for correct PG receptor function and sorting to the plasma membrane (section 3.3.1.1). Interestingly, whilst the EP_4 protein was detected within the rat ileum in both its native and dimerised forms, it could not be detected using normal Western blotting procedures in rat DRG or SC. This was believed to be due to the lower levels of expression of EP_4 in these tissues, in contrast to the ileum where expression occurs abundantly [Honda *et al.*, 1993, Bastien *et al.*, 1994, Breyer *et al.*, 1996]. In order to detect the EP_4 receptor protein in crude DRG and SC tissues by Western blotting methods, it is envisaged that prior immunopurification of the tissue homogenates would be required to enrich the concentration of EP_4 receptor proteins, as discussed in chapter three. If this procedure proved successful then Western blotting experiments could be performed using DRG and SC tissue homogenates, extracted from rats subjected to either chronic constriction injury (CCI) or Freund's complete adjuvant (FCA), in order to determine whether EP_4 receptor expression changes following chronic or inflammatory pain states respectively.

The use of the EP_4 antibody in immunocytochemistry experiments identified constitutive expression of the EP_4 protein in a subpopulation of putatively defined nociceptors of the rat lumbar DRG. Furthermore, the EP_4 receptor was observed to be present in distinct neuronal ultra-structures, including the neuronal axons and nuclear membrane. The observation that EP_4-li was present within the axonal tracts running through the lumbar DRG suggests that the EP_4 protein may be transported to peripheral and central terminals and therefore could be involved in both peripheral and central pain processes.

The localisation of the EP_4 receptor to the nuclear membrane signifies that it may be able to influence the transcription of various genes, as reported for nuclear EP_1 and $EP_{3\alpha}$ receptors [Bhattacharya *et al.,* 1998, 1999]. Indeed, it has been shown that in addition to coupling to G_s, the EP_4 receptor can activate phosphatidylinositol 3-kinase (PI3K), leading to phosphorylation of extracellular signal related kinases (ERKs) and the induction of early growth response factor-1 (EGR-1) [Fujino *et al.,* 2003, Regan, 2003]. This induction of EGR-1 expression is significant since it regulates a number of genes, including PGE_2-synthase [Naraba *et al.,* 2002]. A second way in which EP_4 receptor signalling via PI3K could influence gene transcription is via the glycogen synthase kinase-3 (GSK-3) / β-catenin signalling pathway [Fujino *et al.,* 2002, Regan, 2003]. GSK-3 phosphorylates the transcriptional activator β-catenin, leading to its cytosolic sequestration and degradation. However, when the activity of GSK-3 becomes inhibited by PI3K-dependent phosphorylation, β-catenin translocates to the nucleus where it can alter gene expression via interactions with the Tcf / Lef family of transcription factors [Cadigan & Nusse, 1997]. Interestingly, nuclear β-catenin accumulation and Tcf-mediated transcriptional activation has been shown to upregulate COX-2 expression [Araki *et al.,* 2003]. Therefore, stimulation of the EP_4 receptor has the potential to induce expression of both PGE_2-synthase and COX-2, thereby setting up a positive feedback loop in which the increased synthesis of PGE_2 would further stimulate the receptor.

In the rat spinal cord, EP_4-li was observed uniformly throughout the grey matter in individual spinal neurons, but was not confined to any of the superficial laminae, which would be expected if the EP_4 receptor had a role in spinal nociceptive processing (section 1.6.1). This implies that centrally released PGE_2 either acts at a PG receptor distinct from the EP_4 receptor or acts presynaptically on the central

terminals of PAFs to produce pro-nociceptive effects. Further work should be carried out to determine whether the EP_4 receptor becomes upregulated in the spinal cord dorsal horn following the induction of chronic or inflammatory pain, in a similar way to that reported for the corticotrophin-releasing factor neurons of the parvocellular nucleus, and the catacholaminergic cells of the nucleus of the solitary tract, ventrolateral medulla, and locus coeruleus [Zhang and Rivest, 1999].

The lack of selective PG receptor antibodies meant that only the EP_4 receptor was successfully mapped within the DRG and spinal cord. Therefore, in order to map the distribution of the other PG receptors, *in situ* hybridisation experiments should be explored to evaluate whether this could represent a more successful approach.

In chapter four, primary cultures of DRG neurons from adult rats were utilised as a model system for studying the direct and sensitising effects of PGE_2 in the peripheral nervous system. This system was chosen because DRG neurons contain the cell bodies of primary afferent fibres, which are principally responsible for conveying signals for noxious, thermal and mechanical sensation to the spinal cord. Moreover, EP_4 receptor expression was shown to persist in the DRG following primary cell culture (section 3.2.2.4), indicating that this cellular system was suitable for studying neuronal EP receptor function *in vitro*. It was observed from these experiments that PGE_2 only produced reproducible intracellular calcium responses in a small number (~8%) of DRG neurons, indicating that the prostanoid does not make a substantial contribution to directly activating rat nociceptors. Evidence is now emerging to suggest that PGE_2 acts instead, to sensitise nociceptors either by modifying the properties of voltage-gated ion channels (section 1.5.1), or by enhancing other ion channel or chemical mediator-evoked responses that underlie noxious thermal or mechanical transduction currents (section 1.5.2). The work in chapter four was concerned with the latter mechanism and was focused on the TRPV1 channel, which is a heat, proton, and capsaicin-gated ion channel expressed on C-fibre nociceptors (section 1.4). PGE_2 was seen to reproducibly enhance the capsaicin-evoked calcium response in the majority of capsaicin-sensitive neurons (~60%), which would normally be subject to *tachyphylaxis* (desensitisation). Furthermore, PGE_2 also sensitised a small proportion of *silent* neurons to become capsaicin responsive (~6%). Taken together, these observations provide strong evidence to show that PGE_2 can successfully sensitise and re-sensitise TRPV1 channel function.

Both the direct and sensitising effects of PGE_2 were found to be dependent upon EP receptor activation, as a *cocktail* of novel potent and selective EP receptor antagonists (EP_1, EP_3, and EP_4 antagonists; table 5.1) was observed to abolish the majority of PGE_2-evoked calcium responses, and PGE_2-mediated sensitisation of capsaicin-elicited calcium responses in DRG neurons. As the effects of PGE_2 were observed to be reproducible, this provides strong evidence to suggest that the EP receptors involved were not subject to desensitisation in these neurons. This is significant as during pathophysiological functioning, the constant activation and re-activation of EP receptors may underlie the ability of PGE_2 to produce mechanical [Ferreira *et al.*, 1978, Khasar *et al.*, 1994, 1995, Yang & Gereau, 2003] and thermal [Negus *et al.*, 1993, 2004, Yang & Gereau, 2002] hyperalgesia in animals.

During the course of the work described in chapter four, it was apparent that a small population of DRG neurons (type-1b) were not subject to desensitisation following repeated capsaicin application. It was initially anticipated that PGE_2, spontaneously released from DRG neurons sensitised the TRPV1 channel, leading to this behaviour. However, this hypothesis was proved flawed when type-1b responses were not subject to further sensitisation following PGE_2 application, and persisted in the presence of the EP receptor antagonist cocktail, and following inclusion of indomethacin in the culture medium and the extracellular perfusion solution. It would be interesting to try and decipher the reasons behind why these neurons exhibit a non-desensitising nature. As discussed in chapter four, NGF present in the culture medium could have triggered sensitisation of TRPV1 in the type-1b neurons, leading to the observed reproducible capsaicin-evoked calcium responses. Growing the DRG neurons in an NGF-free medium to determine whether type-1b responses still prevail could be used to test this. An alternative hypothesis made in chapter four was that the type-1b calcium responses resulted from elevated levels of extracellular neuropeptides released following capsaicin stimulation. Therefore, it would also be prudent to repeat the experiments in the presence of various neuropeptide receptor antagonists to determine whether type-1b responses persist.

The work described in chapter five has demonstrated that both the EP_3 and EP_4 receptors have a role in mediating the sensitising effects of PGE_2 on the capsaicin-evoked calcium response in rat DRG neurons, by utilising novel potent and selective EP receptor compounds (table 5.1). A study by Southall and Vasko has previously reported similar findings in adult rat DRG neurons using a different

experimental approach. The authors observed that the ability of PGE_2 to augment capsaicin-evoked neuropeptide release, was only inhibited by reducing the expression of both EP_{3C} and EP_4 receptors simultaneously, using antisense oligonucleotides directed at the mRNA for these receptor subtypes [Southall & Vasko, 2001]. When the expression of only one of these receptor subtypes was abolished, PGE_2-mediated sensitisation still prevailed. These findings indicate that the EP_{3C} (aka EP_{3B}) and EP_4 receptors can work independently of each other to produce sensitisation of sensory neurons. In support of this, the work in chapter five has shown that the EP_4 receptor agonist produced similar sensitisation of TRPV1 (~65% type-1 capsaicin sensitive cells and ~3% *silent* cells) as compared to that described for PGE_2, and both the EP_3 and EP_4 receptor antagonists, when applied individually, significantly inhibited PGE_2-mediated potentiation of TRPV1-evoked responses in approximately 46% and 60% of cells respectively. Whilst the receptors appear to act independently of each other, it is hypothesised that they may also act co-operatively, because the EP receptor antagonist cocktail (EP_1, EP_3, and EP_4 receptor antagonists) abolished PGE_2-mediated sensitisation of TRPV1 in the majority of cells tested (~85%). As the EP_1 receptor antagonist had no significant effect on PGE_2-mediated sensitisation of TRPV1, it would appear that most of the inhibition observed was due to the combined effect of the EP_3 and EP_4 receptor antagonists. It is also postulated that the EP_3 and EP_4 receptors co-exist on the same DRG neurons, as PGE_2-mediated sensitisation of TRPV1 produced in those cells not responsive to individual EP_3 or EP_4 antagonist treatment, was abolished following antagonist cocktail treatment. The implications of these findings are exciting as during chronic pain states a co-operative or synergistic action of EP_3 and EP_4 receptors might produce maximal sensitisation of nociceptors, whereas during acute pain states, only one receptor subtype may be responsible for sensitising nociceptors, leading to a less marked sensitisation.

Further work needs to be carried out to ascertain the second messenger systems implicated in EP_3 and EP_4-mediated TRPV1 sensitisation in DRG neurons, which may also lead to the elucidation of the specific EP_3 receptor splice variant involved. In addition, the hypothesis made in chapter five, regarding the EP_2 receptor having a limited role in mediating the sensitising effects of PGE_2 on TRPV1 needs to be clarified. Due to the lack of availability of truly selective EP_2 receptor compounds, however, evaluation of existing EP_2 agonists (butaprost or ONO-AE1-

259) [Jones, 2004] in the presence of other selective prostanoid receptor antagonists (to block any non-selective binding of the EP_2 agonists) may clarify whether any sensitisation of the capsaicin-evoked calcium response can be induced. It is important to reinforce, at this point, that Southall and Vasko observed no role for the EP_2 receptor in sensitising sensory neurons using antisense oligonucleotides against the mRNA for this receptor subtype [Southall & Vasko, 2001].

Although capsaicin is a good indicator of TRPV1 function, it is an exogenous ligand of TRPV1 and would not be expected to activate the channel during chronic and inflammatory pain states. The natural stimulus of the TRPV1 channel is noxious heat (section 1.4), and several investigators have shown that PGE_2 can enhance heat-evoked responses (noxious range) in cultured rat DRG neurons [Reichling & Levine, 1997], as well as in C-fibre nociceptors in the canine testis-spermatic nerve preparation [Mizumura *et al.*, 1993, Kumazawa *et al.*, 1994, 1996]. Therefore, an important future direction would be to study whether the EP receptor subtypes involved in sensitising the capsaicin-evoked calcium response are also implicated in mediating sensitisation of noxious heat-evoked responses. Ideally, this would be done using a pharmacological approach in conjunction with a non-pharmacological approach to reinforce any findings. The use of antisense oligonucleotides directed against the specific EP receptor subtypes or the generation of EP receptor knockout animals both represent successful approaches to fill the latter criterion. Another future direction would be to determine whether calcium responses produced by anandamide [Jerman *et al.*, 2002, Ohta *et al.*, 2005, van der Stelt, *et al.*, 2005], 15(S)-HPETE [Ohta *et al.*, 2005] and NADA [Sagar *et al.*, 2004], which are endogenous activators of TRPV1, can be sensitised by PGE_2 in DRG neurons, in a manner similar to that described for capsaicin-evoked calcium responses (section 4.2.2).

The work in this thesis has focused on the involvement of PGE_2 in peripheral nociception. However, PGI_2 is also believed to have a pertinent role in this process, but was not investigated during the current study due to time constraints. It is envisaged that because PGI_2 is coupled to both G_s and $G_{q/11}$ and can thereby activate both PKA and PKC dependent pathways respectively, this prostanoid could sensitise capsaicin-elicited calcium responses in DRG neurons in a similar manner to PGE_2. In support of a role for PGI_2 in mediating TRPV1 sensitisation, previous studies have shown that PGI_2 can potentiate the capsaicin-evoked inward current in mouse [Moriyama *et al.*, 2005] and rat [Pitchford & Levine, 1991] DRG neurons. If PGI_2

was shown to sensitise the capsaicin-evoked calcium response, it would be interesting to see whether the same cells are sensitised by both prostanoids, and if the magnitude of sensitisation evoked by PGE_2 and PGI_2 is of a similar amplitude. It is anticipated that PGI_2 would produce stronger sensitising effects on TRPV1 compared to PGE_2 as it has been shown to be the more potent agonist in rat DRG neurons [Smith *et al.*, 1998, Rowlands *et al.*, 2001].

It is now widely accepted that prostaglandins can also facilitate nociceptive transmission in the spinal cord, and thereby contribute to central sensitisation (section 1.6.4). Therefore, an important future direction for this work would be to study the effects of PGE_2 in the spinal cord (cultured spinal cord neurons or spinal cord slice preparations) by utilising calcium imaging or electrophysiological techniques in order to determine whether the same EP receptors are involved in mediating the effects of PGE_2 centrally, as they do peripherally. Again, this would ideally be addressed utilising both pharmacological and non-pharmacological approaches in order to reinforce any findings. The general consensus in the literature to date is that the EP_1 receptor has a strong involvement in spinal nociception, but again the selectivity of some of the EP receptor compounds utilised in these studies remains an issue [Minami *et al.*, 2001, Mebane *et al.*, 2003, Bär *et al.*, 2004, Nakayama *et al.*, 2002, 2004].

Taken together the work in this thesis has demonstrated that both the EP_3 and EP_4 receptors contribute to mediating the sensitising effects of PGE_2 on primary afferent nociceptors in the rat periphery. Nociceptor sensitisation is believed to be the key mechanism by which, this important inflammatory mediator produces thermal [Negus *et al.*, 1993, 2004, Yang & Gereau, 2002] and mechanical [Ferreira *et al.*, 1978, Khasar *et al.*, 1994, 1995, Yang & Gereau, 2003] hypersensitivity in animals. Therefore, these results are important from a drug development perspective, as they may direct efforts to identify a more efficacious approach for novel prostanoid receptor based analgesic therapies.

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