

Distinct and complementary roles for α and β isoenzymes of protein kinase C in mediating vasoconstrictor responses to acutely elevated glucose

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Abstract:**BACKGROUND AND PURPOSE**

We investigate the hypothesis that elevated glucose increases contractile responses in vascular smooth muscle, and that this enhanced constriction occurs due to the glucose-induced protein kinase C (PKC)-dependent inhibition of voltage-gated potassium channels.

EXPERIMENTAL APPROACH

Patch-clamp electrophysiology in rat isolated mesenteric arterial myocytes was performed to investigate the glucose-induced inhibition of voltage-gated potassium (Kv) current. To determine the effects of glucose in whole vessel wire myography was performed in rat mesenteric, porcine coronary and human internal mammary arteries.

KEY RESULTS

Glucose-induced inhibition of Kv was PKC-dependent and could be pharmacologically dissected using PKC isoenzyme-specific inhibitors to reveal a PKC β -dependent component of Kv inhibition dominating between 0 and 10 mM glucose with an additional PKC α -dependent component becoming evident at concentrations greater than 10 mM. These findings were supported using wire myography in all artery types used, where contractile responses to vessel depolarization and vasoconstrictors were enhanced by increasing bathing glucose concentration, again with evidence for distinct and complementary PKC α /PKC β -mediated components.

CONCLUSIONS AND IMPLICATIONS

Our study provides compelling evidence that glucose-induced PKC α /PKC β -mediated inhibition of Kv current in vascular smooth muscle causes an enhanced constrictor response. Inhibition of Kv current causes a significant depolarization of vascular myocytes leading to marked vasoconstriction. The PKC-dependence of this enhanced constrictor response may present a potential therapeutic target for improving microvascular perfusion following percutaneous coronary intervention following myocardial infarction in hyperglycaemic patients.

Abbreviations

[Ca²⁺]_i, intracellular calcium ion concentration; DAG, diacylglycerol; Kv, voltage-gated potassium channel; PKC, protein kinase C

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements:

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Introduction:

In arterial and arteriolar vessels hyperglycaemia can mediate changes in both endothelial and smooth muscle cells, leading to changes in blood vessel diameter, blood pressure and vascular dysfunction (Hink et al., 2001; Arun et al., 2004; Zamami et al., 2008). Significant fluctuations in plasma glucose concentration also occur physiologically through the diurnal cycle of feeding and fasting; and such changes can be exaggerated under certain pathophysiological circumstances (e.g. type 1 or type 2 diabetes). According to NICE guidelines, diabetes is often associated with cardiovascular complications, including: coronary artery disease (leading to myocardial infarction and angina), peripheral artery disease (leg claudication, gangrene), and carotid artery disease (strokes, dementia). There are also microvascular complications caused by diabetes, such as diabetic retinopathy, kidney and nerve damage (NICE guidelines, 2014). Recent evidence suggests that the plasma concentration of blood glucose may also play a significant role in enhancing vasoconstriction and so have a deleterious effect on microvascular re-perfusion following percutaneous coronary intervention (Iwakura et al., 2003). The risk associated with these complications can be minimized by tight glycaemic control, although, there is a need for therapies to reduce the risk further. Acute hyperglycaemia (15 mmol/L), in healthy human subjects, increases systolic and diastolic blood pressure, heart rate and decreases leg blood flow and blood viscosity (Giugliano et al., 1997). Such acute glycaemic changes can cause rapid changes in vascular physiology, including alterations to 'resting' intracellular calcium concentration ($[Ca^{2+}]_i$) (Dunn and Nelson, 2010; Velmurugan and White, 2012), diacylglycerol (DAG) levels (Lee et al., 1989; Miele et al., 2007; Descorbeth and Anand-Srivastava, 2008), protein kinase C (PKC) localization/activity (Das Evcimen and King, 2007; Geraldles and King, 2010) and the generation of reactive oxygen species (Teshfamarian and Cohen, 1992; Inoguchi et al., 2000; Hink et al., 2001).

In arterial smooth muscle increases in glucose concentration often lead to vasoconstriction (Das Evcimen and King, 2007). We (Rainbow et al., 2006) and others (Li et al., 2003; Straub et al., 2009) have previously shown that this acute hyperglycaemic effect involves modulation of a voltage-gated potassium channel (Kv) conductance, which is a key determinant of membrane potential and vascular tone (Chen et al., 2006b; Rainbow et al., 2006). This change in vascular tone occurred in vessels, or cells, isolated from a normoglycaemic animal with no reported diabetes. These findings demonstrate an effect of the elevated glucose concentration per se rather than a diabetes-related change in contractile behaviour.

Vasoconstrictors modulate Kv through PKC isoenzymes (Rainbow et al., 2009) and there is accruing experimental evidence to support the proposition that glucose can also utilize this signal transduction mechanism (Lee et al., 1989; Miele et al., 2007; Descorbeth and Anand-Srivastava, 2008). Such constriction may occur via a number of locally, and somatically released constrictor mediators, such as endothelin-1 (ET1) (Tamarelle et al., 2013), neuropeptide Y (Clarke et al., 1987), 5-HT (McFadden et al., 1991) and angiotensin II (Arun et al., 2004).

In the present study we have addressed this hypothesis by investigating the link between acute elevations in glucose concentration and changes in vascular function. We present data demonstrating for the first time that a glucose concentration-dependent increase in vessel contraction is mediated by the DAG- and Ca^{2+} -sensitive PKC α and PKC β isoenzymes, as

also recently shown in cardiomyocytes (Sims et al., 2014). This mechanism appears to be common to rat mesentery, porcine coronary and human internal mammary arterial tissues. Crucially, we have shown that increases in glucose concentration within, or just beyond the normal physiological range, can significantly inhibit Kv current amplitude (Rainbow et al., 2006) and also alter the modulatory actions of vasoconstrictors, such as thromboxane A₂, UTP and angiotensin II (Nobe et al., 2003, 2008; Arun et al., 2004; Ghatta and Ramarao, 2004). A glucose concentration-dependent, PKC-mediated modulation of Kv current provides a novel mechanism by which glucose can acutely modulate vasoconstriction within the physiological and pathophysiological glycaemic range. Such glucose-induced constriction of vessels may provide a mechanism to help explain the worsened prognosis after acute myocardial infarction in patients with varying degrees of hyperglycaemia. Finally, inhibition of DAG- and Ca²⁺-dependent PKC isoenzymes may pinpoint a therapeutic target for improving coronary microvascular perfusion in the heart after percutaneous coronary intervention and so potentially improve prognosis after acute myocardial infarction, improve perfusion in retinopathy and also improve systemic perfusion of the extremities.

Methods

Tissue dissection

Small rat mesenteric arteries were dissected as described previously (Rainbow et al., 2006, 2009). Rats were culled by stunning and cervical dislocation. The care and Schedule 1 killing of animals conformed to the requirements of the United Kingdom Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039). The ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010; McGrath et al., 2010) have been followed in this study. Coronary arteries were dissected from porcine hearts obtained from a local abattoir. Human internal mammary arteries (IMA) were obtained from patients undergoing coronary artery surgery, immediately bathed in 5 mM glucose solution and transported on ice. The vessel was cleared of surrounding connective tissue and cut into rings. All vascular tissue was maintained on ice in 5 mM glucose solution until use. Human participants provided written, informed consent prior to tissue collection covered by the National Research Ethics Service (NHS) ethics committee approved study for University of Leicester Hospitals entitled, "Sample and Data Collection For Cardiovascular Research". All was carried out in accordance with the declaration of Helsinki.

Myography

Rat mesenteric (200–450 μm), porcine coronary (500–1000 μm) or human IMA (600–1500 μm) were cut into 1.4 mm length rings and mounted in a Mulvany-Halpern wire myograph (Danish Myo-Technology (DMT), Aarhus, Denmark) and maintained at a constant temperature of 37°C. After normalization, vessels were maintained at a tension equivalent to 90 mmHg and left to equilibrate in the test glucose solution for 30 min prior to experimentation. Solutions and vasoconstrictors were applied as indicated in the example traces provided in the figures. N ω -Nitro-L-arginine methyl ester hydrochloride (20 μM ; L-NAME) was included in all myography solutions unless stated otherwise. Data were normalized to tension after calibration. All vessels were incubated in the test glucose concentration for 30 min prior to experimentation unless otherwise indicated.

Patch clamp electrophysiology

Mesenteric arterial smooth muscle cells (MASMC) were isolated as described previously (Rainbow et al., 2006, 2009) and maintained on ice in 5 mM glucose (0.1 mM Ca²⁺) solution until use. Currents were recorded in the conventional whole-cell recording configuration. Isolated MASMC were allowed to adhere to a glass coverslip mounted on a heated perfusion system for 15 min in a 0 mM glucose extracellular solution at $32 \pm 2^\circ\text{C}$ also containing penitrem A (100 nM) to limit BKCa channel interference. MASMC were voltage-clamped at -65 mV in the whole-cell ruptured patch configuration and an initial current-voltage relationship (I/V(1)) recorded. After 9 min of perfusion with the test glucose concentration a second I/V was recorded (I/V(2)) and expressed as a fraction of that in 0 mM glucose ((I/V(2))/(I/V(1))). For experiments with cell-permeant Tat peptide-linked PKC isoenzyme-specific inhibitor peptides, cells were pre-incubated for 15 min in 0 mM glucose solution with 100 nM Tat-PKC inhibitor. Pharmacological inhibitors Gö 6976 (PKC α/β inhibitor) and LY379196 (PKC β -selective inhibitor, a gift from Eli Lilly & Co, Indianapolis, USA) (Wen et al., 2003) were used at 300 nM and pre-incubated with the tissue for 10 min prior to other experimental manipulations. Concentration-response data were calculated from the fraction of current remaining after 9 min of perfusion with the test glucose at +60 mV (from I/V(2)) compared to current at +60 mV in the control I/V (0 mM glucose). In all electrophysiological recordings the current was taken from the final 50 msec of the depolarizing step (protocol shown in Figure 1).

Solutions

All myography solutions were based on the following solution; in mM, 135 NaCl, 6 KCl, 10 HEPES, 1.2 MgCl₂, 1.8 CaCl₂, 20 total monosaccharide (e.g. 5 glucose + 15 mannitol), pH 7.4 (adjusted with NaOH). Zero-Ca²⁺ solution was as above with the omission of CaCl₂. High K⁺ solution (60 mM; 60K) was as above except with 81 NaCl and 60 KCl. Bathing solutions for electrophysiology were as above with 30 mM total monosaccharide (e.g. 5 glucose + 25 mannitol) and 0.1 CaCl₂. Pipette solution contained, in mM, 140 K⁺ (30 KOH and 110 KCl), 10 EGTA, 10 HEPES, 1 ATP, 0.5 GTP, 1 MgCl₂ and 3.92 CaCl₂ (equivalent to 100 nM free-Ca²⁺). All chemicals obtained from Sigma-Aldrich (Poole, UK) unless stated otherwise.

Pharmacological agents and vasoconstrictors

Stock solutions of Gö 6976 and LY379196 (1 mM in dimethylsulphoxide (DMSO)) were diluted to the test concentration in the appropriate bathing solutions. DMSO concentration was never greater than 0.1%, which had no effect on the intact vessels, or in isolated MASMC patch clamp, experiments when applied (data not shown). L-NAME, angiotensin II (ATII), endothelin-1 (ET1), Uridine-5'-triphosphate (UTP), and the thromboxane-mimetic U46619 (Tocris Bioscience, UK) were dissolved in the appropriate bathing solution as stock solutions and diluted to the appropriate concentration indicated in the example traces seen in the figures. PKC isoenzyme-selective, cell-permeant, Tat peptide-linked inhibitor peptides were used as previously described, pre-incubated for 30 min prior to use at a concentration of 100 nM (Rainbow et al., 2009; Sims et al., 2014; Brennan et al., 2015).

Data analysis

All electrophysiology data were analysed using pCLAMP10 software, Microsoft Excel 2010 and GraphPad Prism 6. Myography data were analysed using either pCLAMP10 or Lab Chart 7.2.5, Microsoft Excel 2010 and GraphPad Prism 6. All statistical analysis were performed in GraphPad Prism 6, the statistical test used for each dataset is reported in figure legends.

6a. Results

Kv current inhibited by extracellular glucose in a biphasic manner

We (Rainbow *et al.*, 2006), and others (Li *et al.*, 2003; Straub *et al.*, 2009), have reported that glucose inhibits the Kv current in freshly isolated smooth muscle cells. Initial experiments in this study were performed to further characterise the glucose-induced inhibition of the Kv current. Extracellular glucose inhibited Kv current in freshly isolated rat MASMC in a concentration-dependent manner (Figure 1Aii, Aiii and B). Further to this, there was a glucose concentration rightward shift in the voltage-dependence of activation calculated from the tail current (Figure 1C, -11.5 ± 0.8 vs -5.6 ± 0.6 mV in 0 and 20 mM glucose respectively, $P < 0.001$). This inhibitory response of increasing extracellular glucose was most accurately modelled by a two-site analysis (IC_{50}^H 4.9 ± 0.7 mM; IC_{50}^L 16.2 ± 1.1 mM), indicative of a biphasic response, with a maximal inhibitory effect on Kv current of around 50% (Figure 1D). Kv inhibition by increasing extracellular glucose concentration resulted in graded depolarization of membrane potential (Figure 1E, $P < 0.001$ at 20 mM glucose).

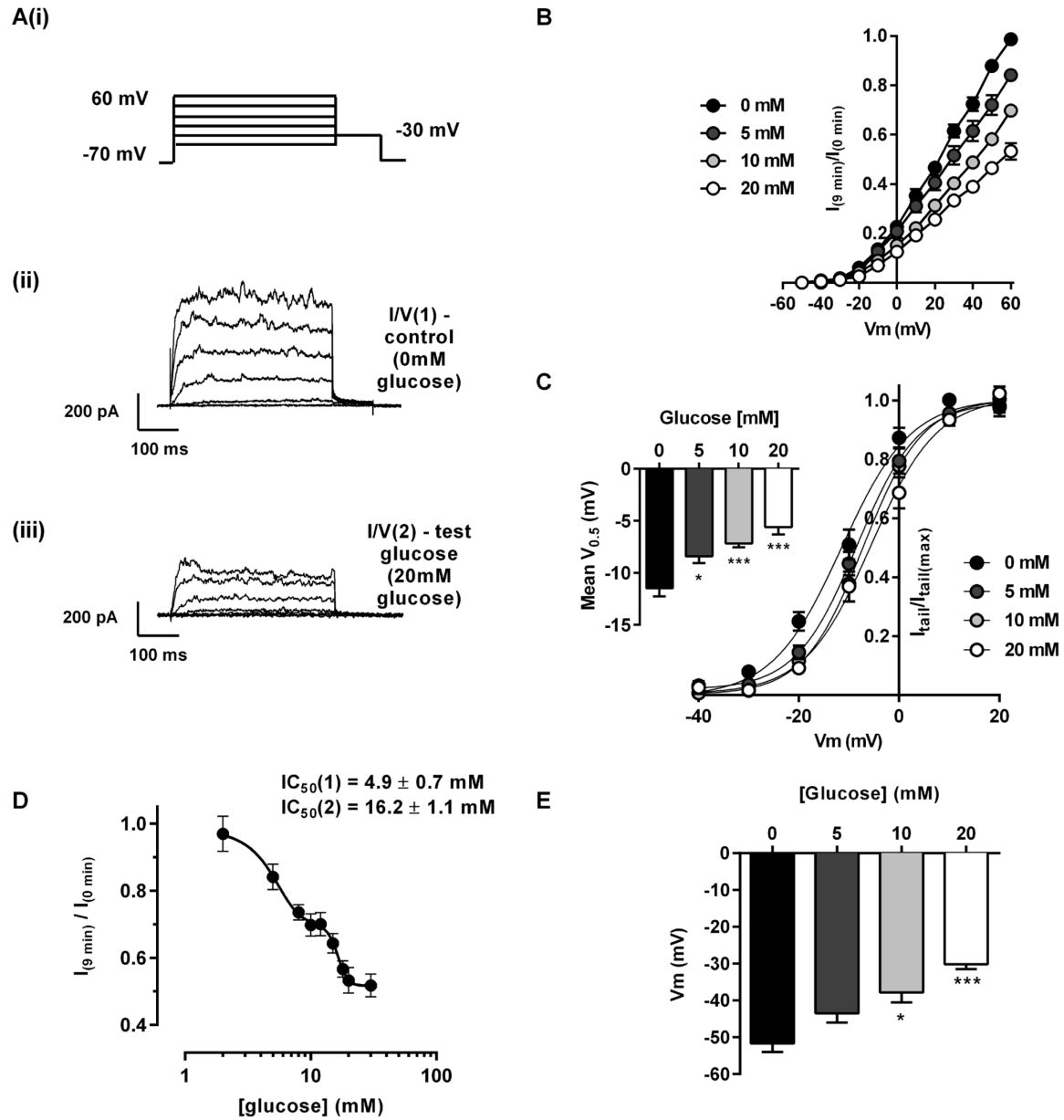


Figure 1: Kv currents are inhibited by glucose in a biphasic concentration-dependent manner in rat mesenteric arterial smooth muscle cells. (Ai-ii) Kv current measured from isolated MASMCS in 0 mM glucose using a series of depolarizing steps from -70 mV to +60 mV in 10 mV increments, 20 mV steps are shown for clarity. The solution was exchanged for one containing the test glucose (e.g. 20 mM (A(iii))), and a second I/V recorded (I/V(2)) after 9 min perfusion. Mean data showing current-voltage relationships (B) and activation curves (C; $V_{0.5}$ shown as insert) recorded from MASMCS cells incubated with 0, 5, 10 or 20 mM glucose. Data are expressed as I/V(2) normalized to the peak current in I/V(1) (at +60 mV). (D) Concentration-dependent inhibitory effect of increasing glucose concentration on Kv current; two-site analysis of the inhibition curve revealed IC_{50} values of 4.9 ± 0.7 and 16.2 ± 1.1 mM. (E) Histogram showing mean membrane potential after 9 min in solution containing the glucose concentration indicated (* $P < 0.05$; *** $P < 0.001$, ANOVA with Bonferroni's *post test*).

Glucose-mediated inhibition of Kv current is attenuated by inhibition of Ca²⁺/DAG-sensitive PKC isoenzymes

We have recently reported that glucose causes action potential prolongation, presumably via Kv inhibition, via conventional PKCs in freshly isolated cardiomyocytes (Sims *et al.*, 2014). To determine if a similar mechanism occurs in vascular smooth muscle we investigated the role of PKC in glycaemic modulation of the Kv current in MASMCs. Initial experiments used the PKC inhibitor, Tat-PKC 20-28 peptide which was able to prevent glucose-induced suppression of Kv current (Figure 2A-C). These data suggest that PKC played a role in inhibiting the amplitude of Kv current in MASMCs.

The PKC isoenzyme selectivity of Kv inhibition by elevated glucose was investigated using a selective-inhibitor of conventional, DAG/Ca²⁺-dependent PKCs, Gö 6976 (300 nM), or a combination of two isoenzyme-specific peptide inhibitors Tat-PKC α and Tat-PKC β (Figure 2D-F). Both small molecule and peptide inhibitors caused a complete reversal of current suppression by high (20 mM) glucose, and reversed the shift in the voltage-dependence of activation (Figure 2E). In contrast, treatment with Tat-PKC δ or Tat-PKC ϵ inhibitory peptides was without effect (Figure 2D). High glucose-induced membrane depolarization was also abolished by combined inhibition by Tat-PKC α -plus-Tat-PKC β , or Gö 6976 (Figure 2F). These data suggest that the PKC α and β isoenzymes are responsible for the inhibition of the Kv current imparted by high glucose in MASMCs.

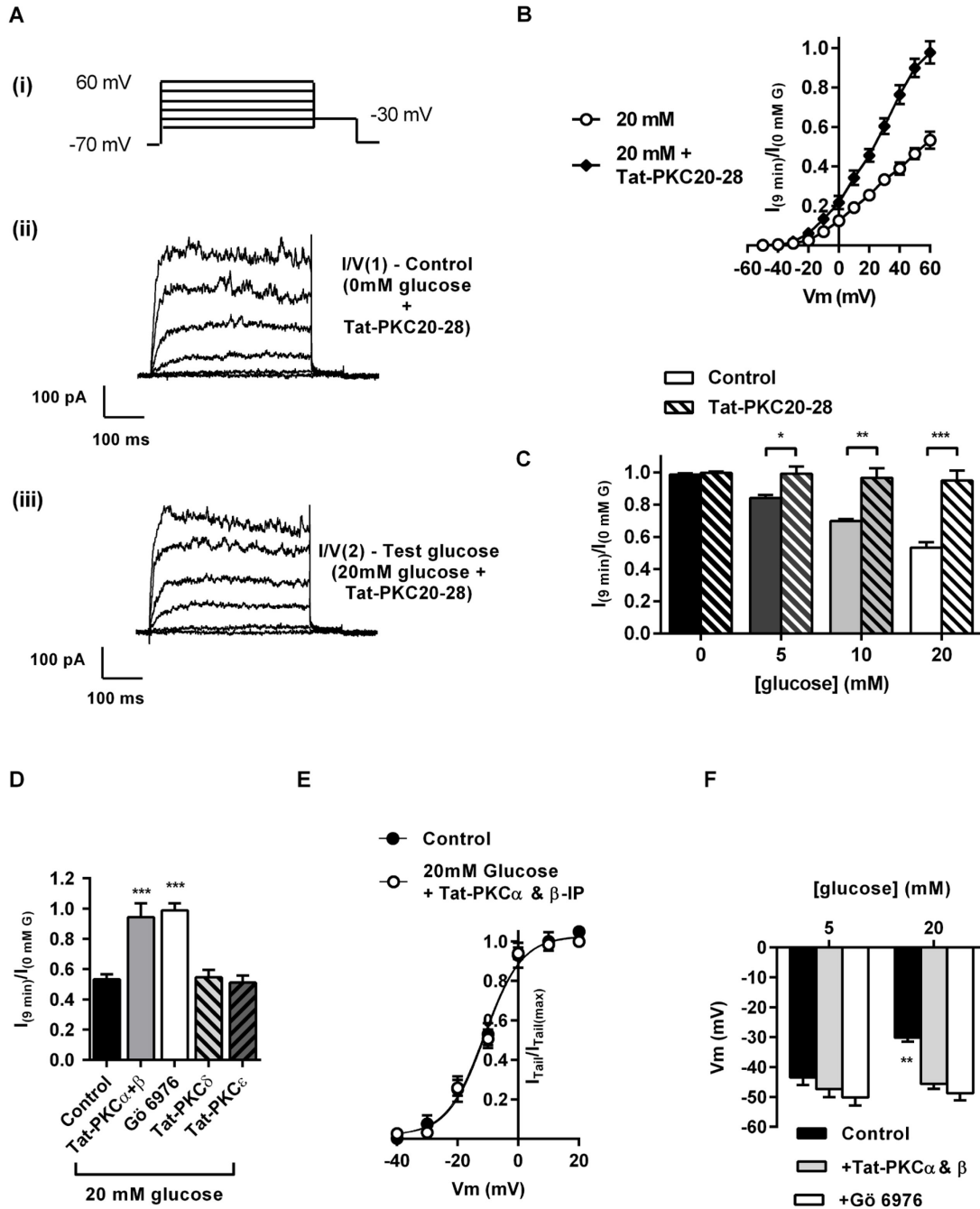
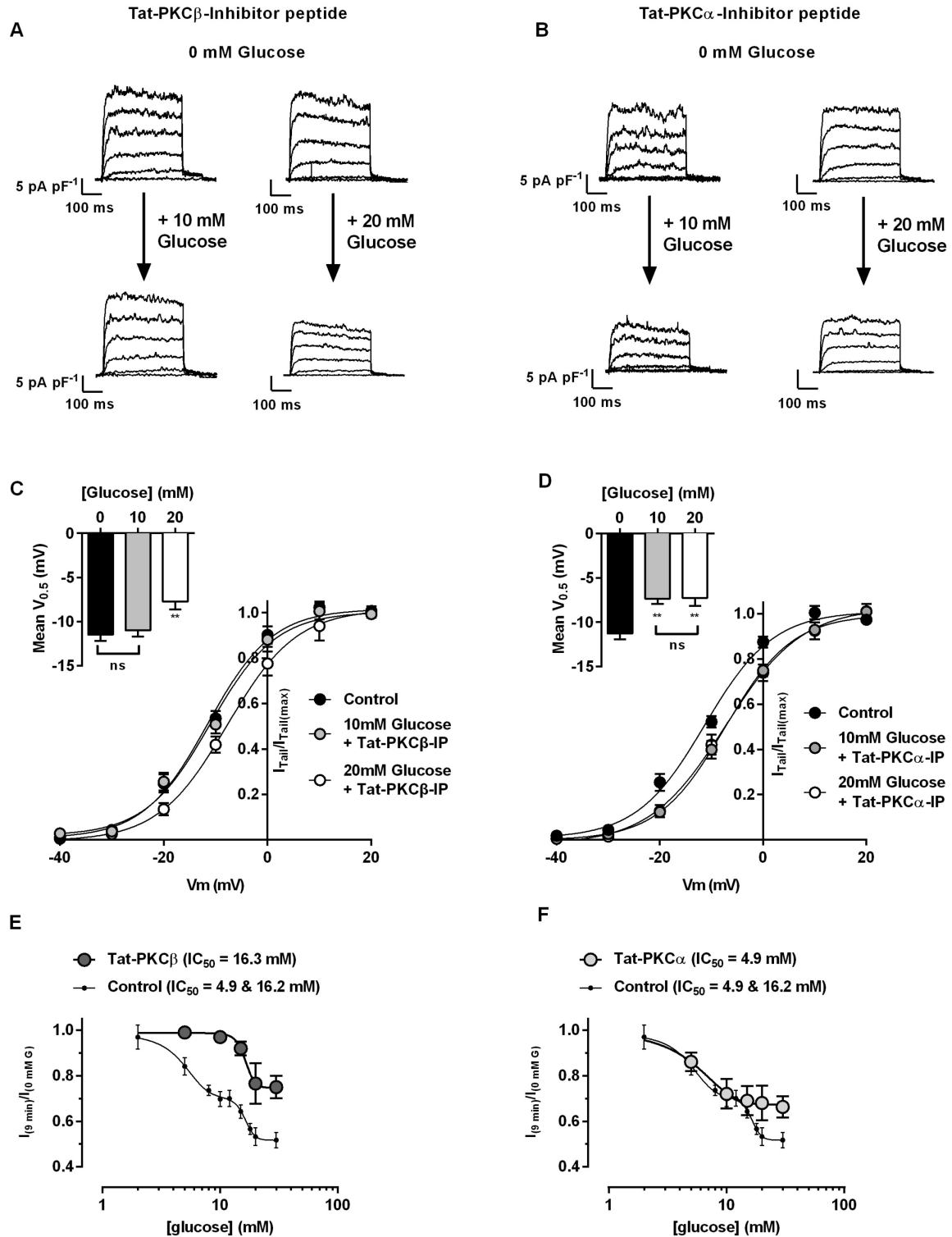


Figure 2: Tat-PKC20-28 attenuates inhibition of voltage-dependent potassium (Kv) current by glucose in rat mesenteric arterial smooth muscle cells. (Ai-ii) Kv current measured from isolated MASMCS in 0 mM glucose+ Tat-PKC20-28 using a series of depolarising steps from -70 mV to +60 mV in 10 mV increments. The solution was exchanged for one containing the test glucose Tat-PKC20-28 (e.g. 20 mM (A(iii))), and a second I/V recorded (I/V(2)) after 9 min perfusion. (B) Mean data I/V plots recorded from cells incubated with 20 mM glucose with and without Tat-PKC20-28. Data expressed as I/V(2) normalized to the peak current in I/V(1) (at +60 mV). (C) Histogram showing attenuation by Tat-PKC20-28 of Kv current inhibition via 0, 5, 10 or 20 mM glucose (* $P < 0.05$; ** $P < 0.05$; *** $P < 0.001$, ANOVA with Bonferroni's *post test*). (D) Histogram showing effects of Tat-PKC isoenzyme-specific inhibitor peptides (PKC $\alpha+\beta$, PKC δ or PKC ϵ ,

each at 100 nM), or pharmacological inhibition of PKC α + PKC β with Gö 6976 (300 nM) on the Kv current in the presence of 20 mM glucose ($***P < 0.001$, one-way ANOVA with Bonferroni's *post* test). (E) Mean data showing activation curves from MASMCs incubated with 0 or 20 mM glucose in the presence of Tat-PKC20-28. Data are expressed as I/V(2) normalized to the peak current in I/V(1) (at +60 mV). (F) Gö 6976 or Tat-PKC α + β inhibition prevented the depolarized membrane potential observed in the presence of 20 mM compared to 5 mM glucose ($**P < 0.01$, two-way ANOVA with Bonferroni's *post* test). Data in the absence of Tat-PKC or pharmacological inhibitors ('control' B to F) are taken from Figure 1 to allow direct comparison.

Dissecting the contribution of PKC α and β

To determine the relative contributions of PKC α and β to the Kv current inhibition caused by glucose, electrophysiological experiments were performed using Tat-PKC α or Tat-PKC β . The Tat-PKC β inhibitor peptide completely prevented the suppression of Kv current by glucose concentrations below 10 mM, but not by higher glucose concentrations (Figure 3A, C and E). In the presence of Tat-PKC β , increasing extracellular glucose caused a monophasic inhibition of Kv current (IC_{50} 16.3 ± 1.8 mM; Figure 3E). The Tat-PKC α peptide also caused a monophasic inhibition of current. Conversely to Tat-PKC β , Tat-PKC α only prevented the current suppression at glucose concentrations above 10 mM (IC_{50} 4.9 ± 1.3 mM; Figure 3F). These data suggest that PKC α and β play distinct and complementary roles in the actions of glucose on Kv current with PKC β modulating activity between 0 and 10 mM, and PKC α modulating the current between 10 and ~20 mM.



I/V(1) (at +60 mV). Tat-PKC β (E) and Tat-PKC α (F) each altered the maximal inhibitory response by glucose to Kv current and converted the biphasic effect into a monophasic curves. Each data point was obtained from >6 cells from >3 animals. In panels (E) and (F) concentration-response curves are repeated from Figure 1 to allow direct comparison with control.

Effect of extracellular glucose concentration on arterial contractility

Myography experiments were performed to determine the effect of high glucose on contraction. The contractile response of rat mesenteric arteries to addition of 60 mM K⁺ (60K) increased (by $\geq 30\%$) when extracellular glucose concentration was raised from 5 to 20 mM (Figure 4A-B). Additionally, there was a significant increase in the Ca²⁺ response to 60K concomitantly with tension in isolated vessels loaded with Fura-2 (Supporting information Figure S1). Consistent with our previous findings of the reversible nature of the Kv current inhibition by elevated glucose (Rainbow *et al.*, 2006), an initial application of 60K in 20 mM glucose, followed by washout with 5 mM glucose, yielded a smaller second 60K response (Figure 4C).

Using 125 mM K⁺ (125K) to abolish the K⁺ gradient completely, the effects of 20 mM glucose were investigated using the same protocol. 125K abolished the potentiation of contraction in 20 mM glucose, demonstrating that in 60K there remains a small hyperpolarizing K⁺ flux that can be modulated by glucose, presumably with PKC-dependence (Supporting Figure S2). In the absence of a complete molecular characterization of Kv expression in MASMC, the Kv1-family channel blocker Psora-4 (Marzian *et al.*, 2013) was used to probe the contribution of Kv channels. Pre-incubation with the Kv1-inhibitor Psora-4 enhanced the vasoconstrictor response to 60K in 5 mM glucose and no further increase was observed when glucose concentration was raised from 5 to 20 mM. Furthermore, Psora-4 (100 nM) elicited a marked inhibition of Kv and membrane depolarization, confirming a substantial role for Kv1 subunits as components of the voltage-gated K⁺ current seen in MASMC (control, -49 ± 2 mV; +Psora-4, -25 ± 2 mV). Together, these findings suggest that inhibition of channels containing Kv1-family subunits play a role in the enhanced vasoconstrictor response to depolarization after acute treatment with high glucose.

Consistent with our previous findings (Rainbow *et al.*, 2006), replacement of D-glucose with non-metabolizable L-glucose did not yield a potentiation of contractile response after incubation with 20 mM L-glucose. Furthermore, replacement of extracellular glucose with fructose (20 mM) yielded a potentiated second 60K response (Supporting Figure S3), consistent with a glycolytic metabolism-dependent mechanism.

The enhanced vasoconstriction and Ca²⁺ response to 60K in rat mesenteric arteries exposed to 20 mM glucose was abolished by the DAG/Ca²⁺-dependent PKC inhibitor Gö 6976 (Figure 4D-E, Supporting Figure S1). These data suggest the response imparted by high extracellular glucose is mediated via conventional PKC isoenzymes.

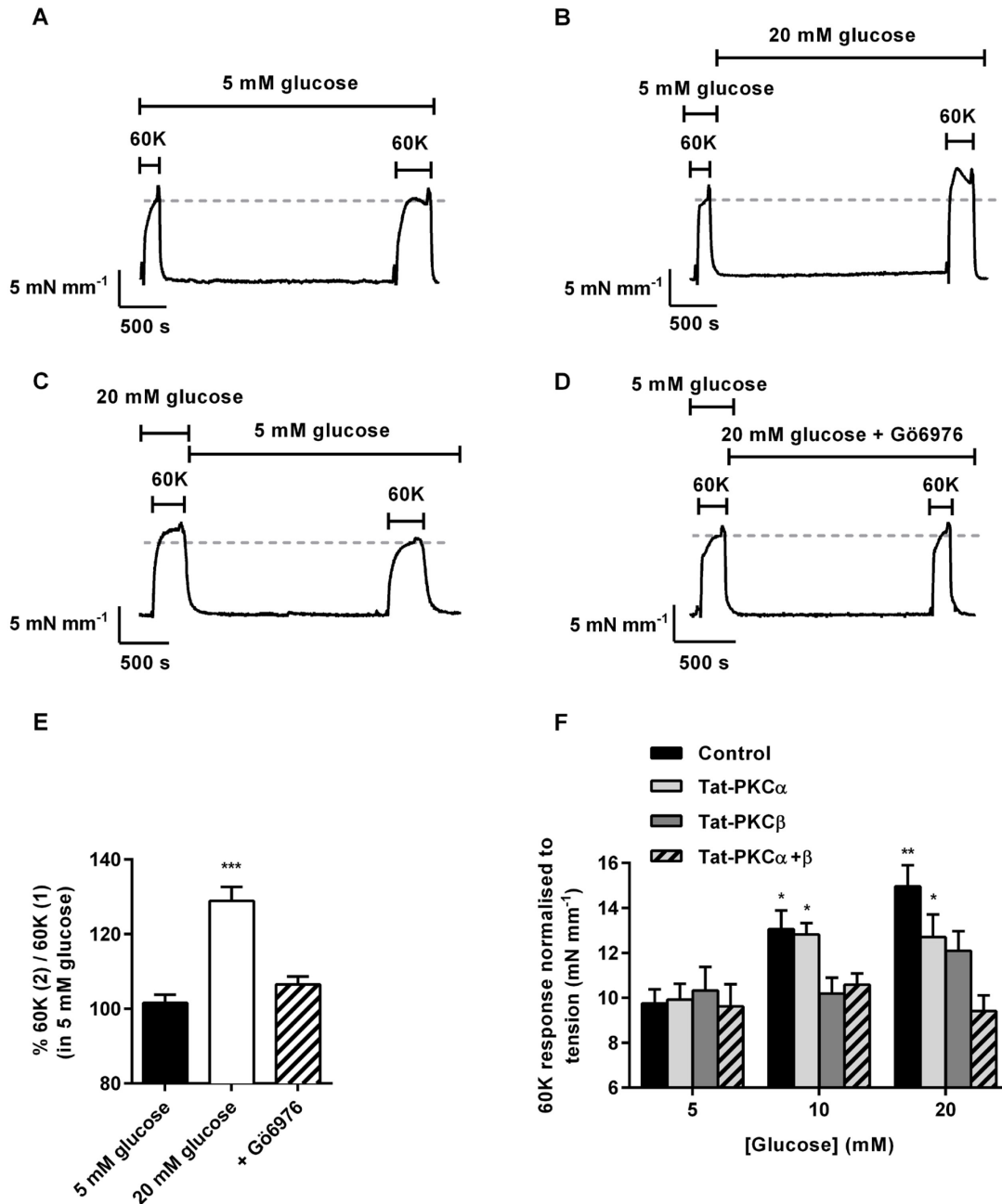


Figure 4: K⁺-induced vasoconstriction of rat mesenteric artery is enhanced by high extracellular glucose and reversed by PKC α + β inhibition. Example traces of tension produced to two applications of 60K (60K₍₁₎ & 60K₍₂₎) with 30 min incubation in (A) 5 mM glucose and (B) 20 mM glucose before 60K₍₂₎. (C) Example trace shows enhancement of 60K contraction by 20 mM glucose is reversed by 30 min incubation in 5 mM glucose. (D) Experiment identical to panel B except for addition of Gö 6976 (300 nM) after 60K₍₁₎. (E) Mean data showing increases in tension for 60K(2) relative to 60K(1) (%) and reversal of the high glucose potentiation by 300 nM Gö 6976 (** P <0.01; *** P <0.001, repeated measures ANOVA with Bonferroni's *post test*, or unpaired *t*-test). (F) Histogram showing the mean contractile response to 60K (mN mm⁻¹) for control vessels and vessels pre-incubated with the

indicated Tat-PKC isoenzyme inhibitors before switching to solution containing 5, 10 or 20 mM glucose (* $P < 0.05$; ** $P < 0.01$, two-way ANOVA with Bonferroni's *post test*).

Glucose-mediated enhancement of depolarization-induced vasoconstriction is reversed by selective PKC α and PKC β inhibition

Tat-PKC α and β peptides were used to determine the relative contributions of PKC α and β respectively to the enhanced contraction caused by high glucose in the presence of 60K. At an intermediate glucose concentration (10 mM), isoenzyme-selective PKC α inhibition had no effect, while PKC β inhibition completely abolished the enhanced contraction (Figure 4F). At a higher glucose concentration (20 mM), selective PKC α or PKC β inhibition each partially prevented the enhanced constriction, which was abolished in the presence of both inhibitors (Figure 4F). These contractile data are in accordance with the electrophysiological data shown in Figure 3. Combined, these findings suggest that DAG/Ca²⁺-dependent PKC isoenzymes play distinct and complementary roles in the actions of glucose on rat mesenteric artery contraction with PKC β modulating activity between 0 and 10 mM, and PKC α modulating the contraction between 10 and ~20 mM.

Contractile response to angiotensin II, but not endothelin-1, is enhanced by elevated glucose and attenuated by selective PKC α or β inhibition

To investigate if elevated glucose concentrations could alter the contractile response to vasoconstrictors, angiotensin II (ATII) and endothelin-1 (ET-1) were applied to rat mesenteric arteries in the presence on 5 mM and 20 mM glucose. In rat mesenteric arteries, increasing extracellular glucose concentration to 20 mM markedly enhanced (by ~2.5 mN mm⁻¹) the vasoconstrictor response to ATII (100 nM; Figure 5Ai-ii). This effect was abolished by pre-incubation with Tat-PKC α -plus-Tat-PKC β inhibitor peptides (Figure 5Aiii), or Gö 6976 (300 nM; Figure 5B). The constrictor response to ATII was completely abolished by selective inhibition of PKC ϵ by a Tat-conjugated inhibitor peptide, consistent with the known dependency of ATII vasoconstrictor activity on this novel PKC isoenzyme (Figure 5Aiv) (Rainbow *et al.*, 2009). Consistent with the glucose concentration-dependent inhibition of Kv, selective inhibition of PKC β , but not PKC α , abolished the hyperglycaemia-mediated increase in ATII-induced vasoconstriction at 10 mM glucose (Figure 5C); at higher glucose concentrations (20 mM), the enhanced response was fully attenuated by combined inhibition of PKC α and PKC β (Figure 5C).

Contractile responses to ET1 were abolished in small rat mesenteric arteries by PKC inhibition with Gö 6976 (Figure 5Diii and E), consistent with the predominantly PKC α -mediated mechanism of action reported for this vasoconstrictor (Rainbow *et al.*, 2009). ET1-mediated vasoconstriction was increased by increasing extracellular glucose to 10 mM, and largely prevented by selective PKC β inhibition (Tat-PKC β inhibitor peptide). No further enhancement was observed by increasing glucose concentration from 10 to 20 mM (Figure 5F). These data are consistent with high glucose and ET-1 both exerting their contractile effects through PKC α and so showing no further potentiation of contraction at glucose concentrations greater than 10 mM. This was supported by experiments investigating Kv

current inhibition by ATII and ET-1 in rat MASMC in 20 mM glucose, where ATII was able to further inhibit the current, whereas ET-1 was ineffective (Supporting information Figure S4). Additionally, the enhanced contractile responses in the presence of 20 mM glucose of rat mesenteric arteries to UTP and U46619 were also prevented by Gö 6976 (Supporting information Figure S5). Despite there being a common glucose-induced PKC-mediated enhancement of vasoconstrictor activity, the PKA-dependent dilatory pathway, via β -adrenoceptor stimulation, was unaffected by elevated glucose (Supporting Figure S6). Collectively, these data suggest there is a common mechanism by which enhanced concentrations of glucose causes inhibition of Kv current, membrane depolarisation, calcium influx and contraction.

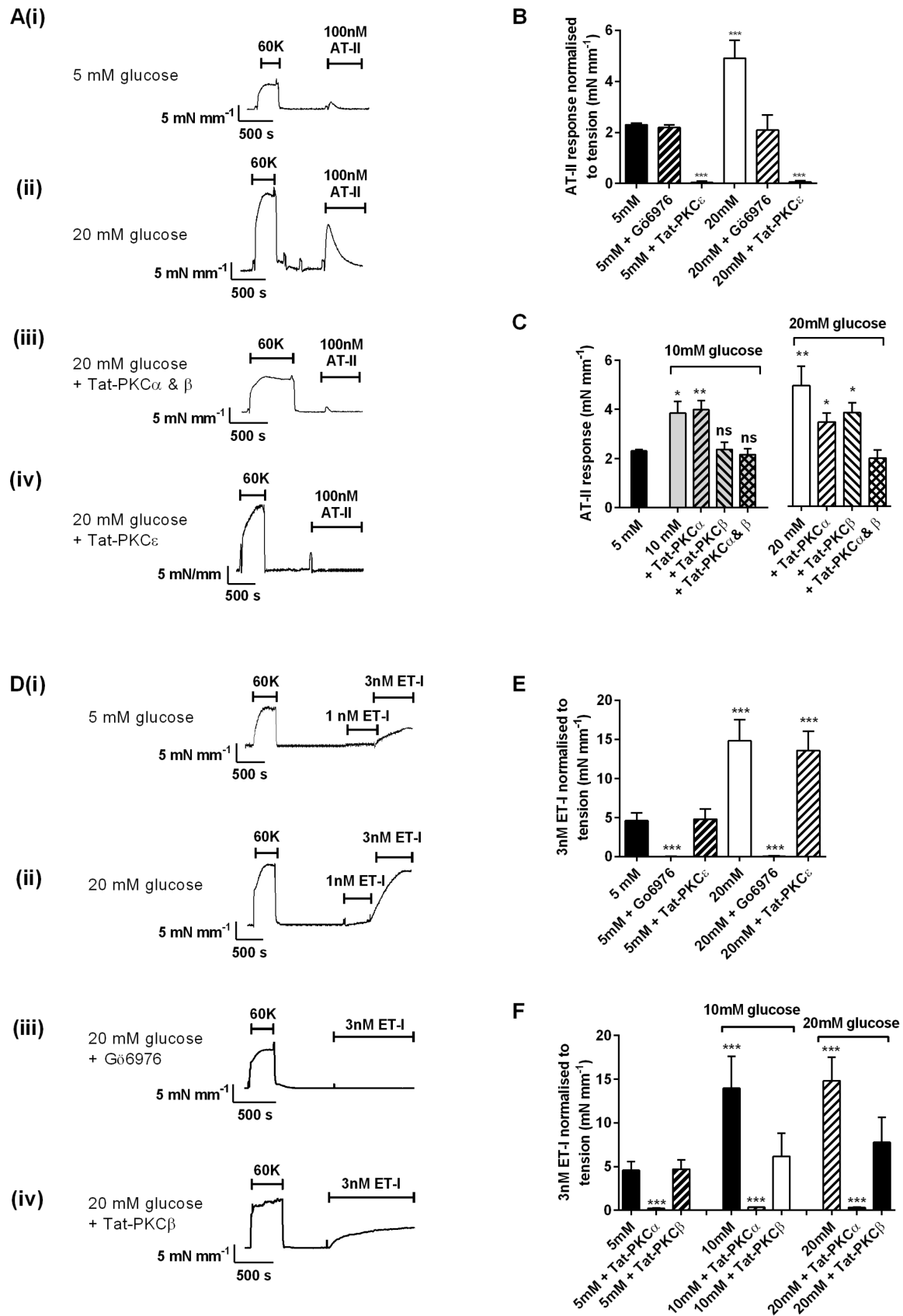


Figure 5: Agonist-induced constriction of rat mesenteric artery is enhanced by high extracellular glucose in a conventional PKC isoenzyme manner. Example traces of 60K

and AT-II (100 nM)-induced constrictor responses for rat mesenteric arteries in 5 (Ai) or 20 (Aii) mM glucose, 20 mM glucose + TatPKC α + β (Aiii) or Tat-PKC ϵ (Aiv). (B) Mean contractile responses to AT-II in 5 or 20 mM glucose with Gö 6976 or Tat-PKC ϵ . (C) Mean contractile responses to AT-II in 10 or 20 mM glucose with Tat- PKC α , Tat- PKC β and Tat-PKC α + β (≥ 12 vessels per condition from ≥ 4 animals: * $P < 0.05$; ** $P < 0.01$, one-way ANOVA with Bonferroni's *post-hoc* test). (D) Example traces of 60K and ET-1 (1 and 3 nM)-induced constrictor responses for rat mesenteric arteries in 5 (Di) or 20 (Dii) mM glucose, 20 mM glucose + TatPKC α + β (Diii) or Tat-PKC ϵ (Div). (E) Mean contractile responses to ET-1 (3 nM) in 5 and 20 mM glucose normalized to tension (mN mm $^{-1}$) in control vessels and vessels pre-treated with Gö 6976 or Tat-PKC ϵ inhibitor. (F), Mean contractile responses to ET-1 (3 nM) with 10 or 20 mM glucose in the absence and presence of Tat-PKC α or Tat-PKC β inhibitory peptides (≥ 10 vessels per condition from ≥ 5 animals: *** $P < 0.001$, ANOVA with Bonferroni's *post* test).

Glucose-mediated PKC-dependent enhancement of vasoconstriction in porcine coronary and human internal mammary arteries

To assess the generality of these findings, similar experiments were performed in porcine coronary arteries (Figure 6) and human internal mammary arteries (Figure 7). These data from porcine coronary and human internal mammary arteries were analogous to the findings in rat mesenteric arteries. In both vascular beds, the contractile response to 60K (Figure 6A-B and Figure 7A-B) were increased in the presence of elevated glucose. There was no further enhancement of ET1 porcine coronary vasoconstrictor activity above 10 mM extracellular glucose (Figure 6C). ET-1 contractions were abolished in porcine coronary arteries by PKC inhibition with Gö 6976, but not with the selective PKC β inhibitor LY379196 (300 nM) (Figure 6Aiii-iv, C). This further supports the predominantly PKC α -mediated mechanism of action reported for this vasoconstrictor (Rainbow *et al.*, 2009). Contractions of porcine coronary arteries and human internal mammary arterioles induced by U46619 (Figure 6D-E and Figure 7D-E) were markedly enhanced in high extracellular glucose. For each contractile stimulus tested on porcine coronary and human internal mammary arterioles; the presence of Gö 6976 prevented the enhanced contraction caused by high glucose (Figure 6B-C, F and Figure 7B-C, F). Selective PKC β inhibition (LY379196; 300 nM) also decreased the magnitude of contraction to each contractile stimulus tested. The data collected from these three different vascular beds (across three different species) suggest that PKC α - and β -mediated enhancement of contractions by high extracellular glucose is a common mechanism throughout the cardiovascular system.

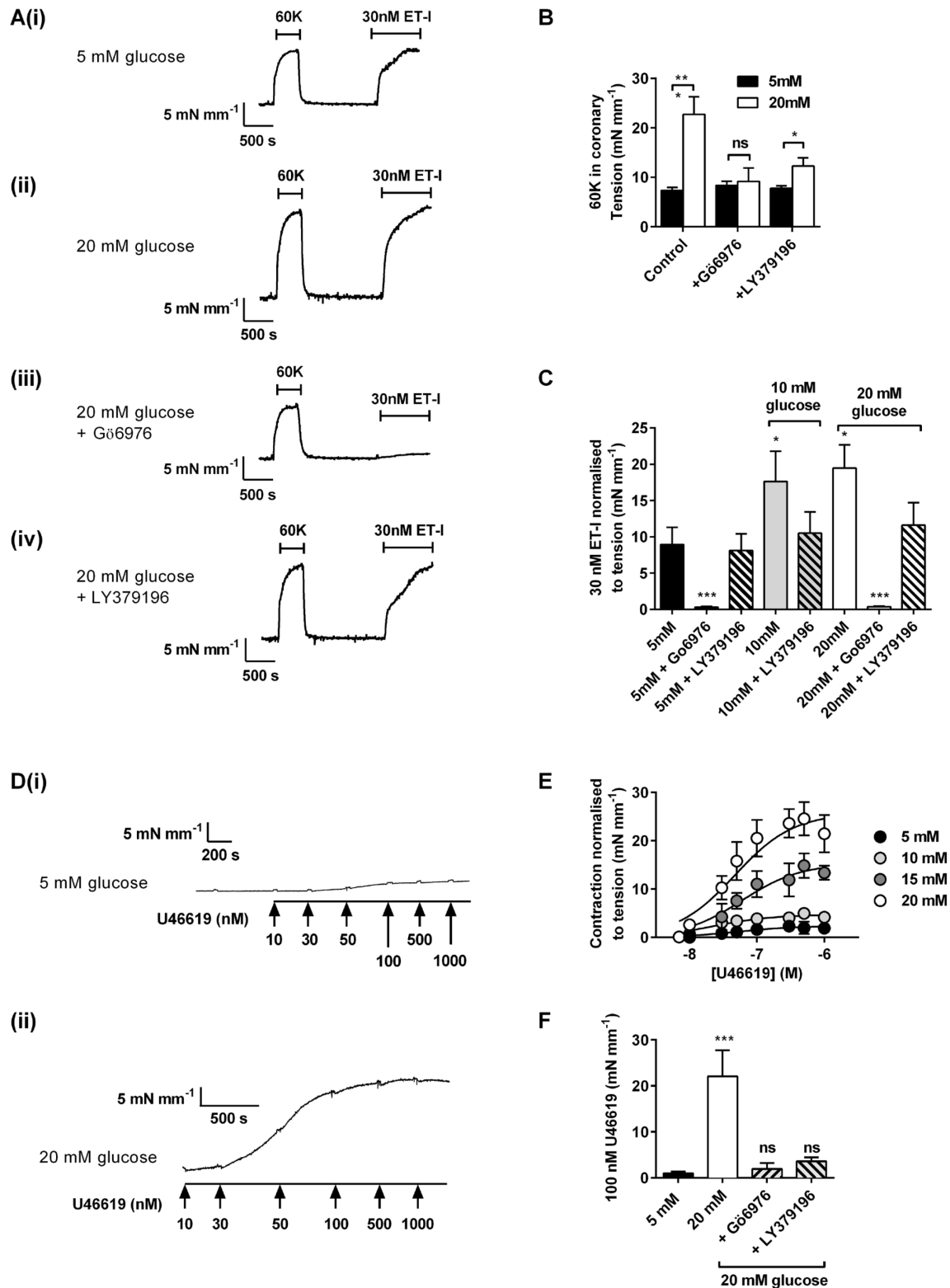


Figure 6: Agonist-induced constriction of porcine coronary artery is enhanced by high extracellular glucose in a conventional PKC isoenzyme manner. (A) Example traces of 60K and ET-1 (30 nM)-induced constrictor responses from porcine coronary arterial rings in 5 (Ai) or 20 (Aii) mM glucose, and in 20 mM glucose with Gö 6976 (Aiii), or PKC β -selective inhibitor LY379196 (Aiv). (B) Mean contractile responses to ET-1 in 5 or 20 mM glucose in

the absence or presence of Gö 6976 or LY379196. (C) Mean contractile responses to KCl in 5, 10 or 20 mM glucose in the presence or absence of PKC α and/or PKC β inhibitors (≥ 9 vessels per condition from ≥ 4 pigs: $*P < 0.05$; $***P < 0.001$, two-way ANOVA with Bonferroni's *post test*). (D) Example traces of U46619 (10-1000 nM)-induced constrictor responses from porcine coronary arterial rings in 5 (Di) or 20 (Dii) mM glucose. (E) Concentration-response curve to U46619 in the presence of 5, 10, 15, 20 mM glucose. (F) Mean contractile responses to U46619 (100 nM) in 5 and 20 mM glucose in the absence or presence of Gö 6976 or LY379196.

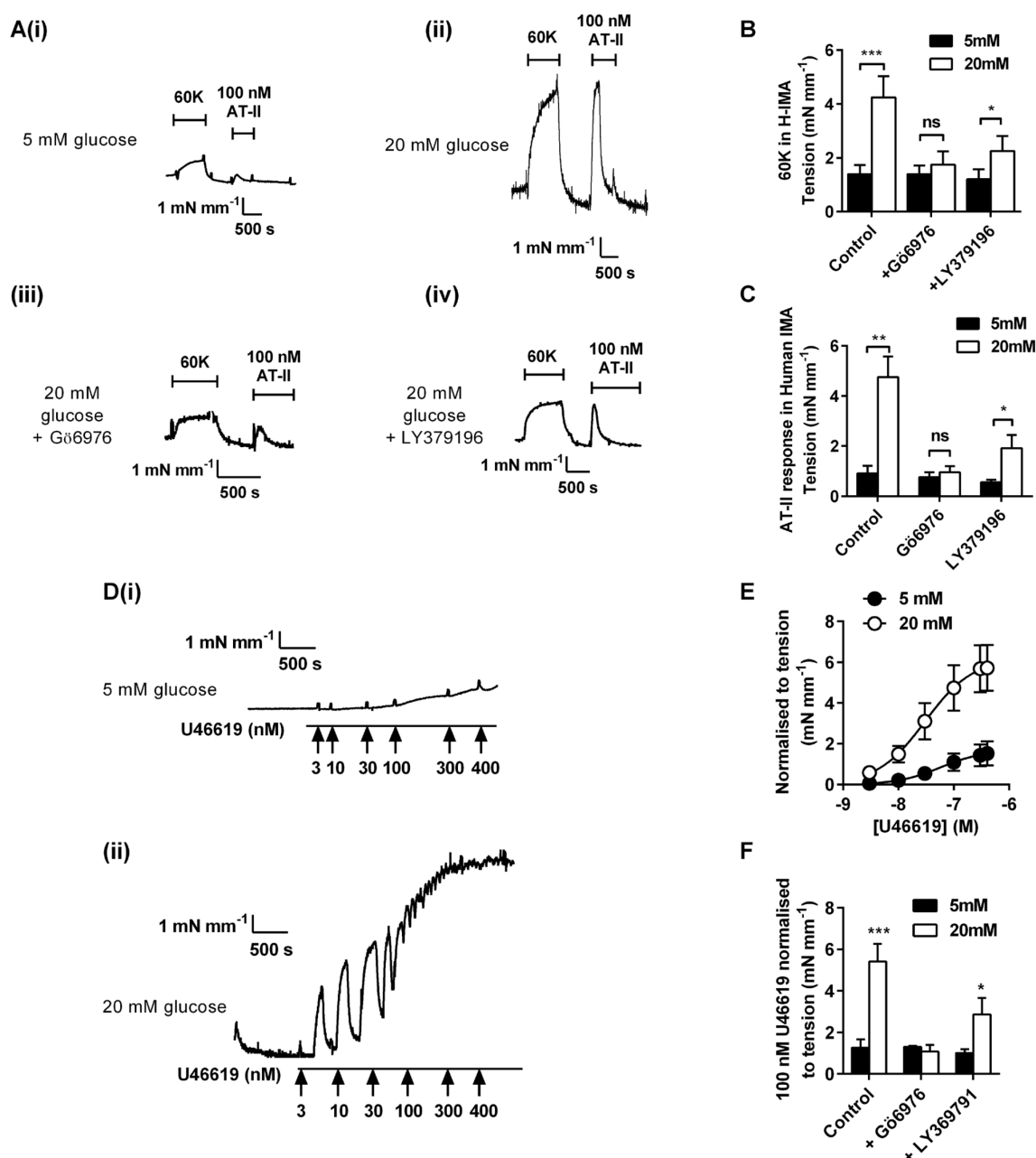


Figure 7: Agonist-induced constriction of human mammary artery is enhanced by high extracellular glucose in a conventional PKC isoenzyme manner. Example traces of 60K and ATII (100 nM)-induced constrictor responses human mammary artery in 5 (Ai) or 20 (Aii) mM glucose, 20 mM glucose + Gö 6976 (Aiii) or LY379196 (Aiv). (B) Mean contractile responses to 60K (B) and ATII (C) in 5 or 20 mM glucose with Gö 6976 or LY379196 (≥ 4 vessels per condition from ≥ 4 patients, $*P < 0.05$; $***P < 0.001$, two-way ANOVA with Bonferroni's *post* test). (D) Example traces of U46619 (3-400 nM)-induced constrictor responses from human mammary artery in 5 (i) or 20 (ii) mM glucose. (E) Concentration-response curve to U46619 in the presence of 5 and 20 mM glucose. (F) Mean contractile responses to U46619 (100 nM) in 5 and 20 mM glucose in the absence or presence of Gö 6976 or LY379196 (≥ 4 vessels per condition from ≥ 4 patients, $*P < 0.05$; $***P < 0.001$, two-way ANOVA with Bonferroni's *post* test).

6b. Discussion

We have presented novel experimental data indicating that acute changes in extracellular glucose concentration within the physiological/pathophysiological range increase basal tone and vasoconstrictor responses in small arteries from three different species. This occurs through glucose concentration-dependent recruitment of conventional PKC activities and modulation of specific K^+ currents. In particular, we report that acutely increasing extracellular glucose concentration leads to a biphasic inhibition of the voltage-gated K^+ current and membrane depolarization. In addition, this inhibitory effect on Kv is dependent on at least two DAG/ Ca^{2+} -dependent PKC isoenzymes, with PKC β playing a key role at lower glucose concentrations (≤ 10 mM) and the PKC α isoenzyme contributing at higher glucose concentrations. Furthermore, we demonstrate that this glucose-mediated PKC isoenzyme-dependent inhibition of Kv results in increased vascular tone and increased vasoconstrictor responsiveness through a mechanism that is conserved across a number of different vasoconstrictors, species and vascular beds.

Previous reports have shown that inhibition of Kv current occurs within minutes of elevating extracellular glucose (Li *et al.*, 2003; Rainbow *et al.*, 2006; Straub *et al.*, 2009). We hypothesized that increasing glucose concentration causes activation of PKC isoenzymes leading to inhibition of the Kv current, presumably by phosphorylation of Kv channel subunits. The exact molecular identity of the Kv current in arterial smooth muscle is complex and currently unclear making direct measurement of subunit phosphorylation difficult, however a number of subunits have been identified, with particular importance being placed on the Kv1 family (Knot and Nelson, 1995; Cole *et al.*, 1996; Albarwani *et al.*, 2003). Indeed, our data using Psora-4 (Marzian *et al.*, 2013; Wulff and Yarov-Yarovoy, 2013), and previous findings by others using Kv1.5 dominant-negative channel subunits (Chen *et al.*, 2006a), indicate an important role for this Kv family sub-group in vasoregulation. A number of other Kv subtypes have been described in systemic vascular smooth muscle, including Kv2, 4 and 7 (Ko *et al.*, 2008; Stott *et al.*, 2014). Given the hetero-multimerization possible within expressed Kv channel complexes, a role for other Kv sub-families cannot be ruled out.

Elevated glucose concentration is known to increase cellular levels of the endogenous PKC activator, DAG in a variety of tissues, including arterial smooth muscle (Inoguchi *et al.*, 1994; Nobe *et al.*, 2003, 2004). The increase in DAG concentration provides a potential mechanism for glucose-induced PKC activation, specifically activation of the DAG/ Ca^{2+} -dependent isoenzymes PKC α and PKC β . Basal cytoplasmic Ca^{2+} concentrations have also been reported to increase in rat tail artery smooth muscle cells across a similar range of extracellular glucose concentrations (5-20 mM) to that used in the present study (Barbagallo *et al.*, 1995). However, we did not observe an increase in basal Ca^{2+} in whole vessels during 30 min incubation with solution containing 20 mM glucose. However, we did not observe an increase in basal Ca^{2+} in whole vessels during 30 min incubation with solution containing 20 mM glucose. Despite this, there was a small constriction in response to the increased glucose during this time (0.95 ± 0.13 mN mm $^{-1}$). It is plausible that any changes in intracellular basal Ca^{2+} were too small to be detected by the recording system used. Additionally, there may be a glucose-induced PKC-dependent sensitization of contractile machinery giving a small

constrictor response for little or no apparent increase in $[Ca^{2+}]_i$. Further to this, Kv channels are one component of the hyperpolarizing response to depolarization that limits Ca^{2+} influx. BK_{Ca} channels, sensitive to both intracellular Ca^{2+} and voltage, together with K_{ATP} channel current could act to limit constrictor responses in the short-term with a subtle depolarization of basal membrane potential. Furthermore, the changes in membrane potential seen in whole-cell recordings of isolated cells may not occur as rapidly in the intact tissue, where cells are electrically coupled to adjacent cells. Basal intracellular Ca^{2+} increases in the whole vessel would not be as large in this scenario and so increases in $[Ca^{2+}]_i$ may not be detected. An enhanced response to 60K-induced depolarization under hyperglycaemic conditions was observed, suggesting an increase in Ca^{2+} influx, and/or enhanced Ca^{2+} release. It would seem likely that an enhanced Ca^{2+} influx can account for this elevated Ca^{2+} response, consistent with our hypothesis of a reduced Kv current. Reducing the Kv-induced hyperpolarizing current, resulting in further depolarization, would enhance L-type voltage-gated Ca^{2+} channel activity.

Collectively, our electrophysiology and myography data demonstrate that extracellular glucose increases in the 5-10 mM range lead to an increased vascular tone and vasoconstrictor responsiveness mediated by $PKC\beta$ /Kv current inhibition, while a $PKC\alpha$ /Kv-dependent component is evident at higher glucose concentrations. The importance of these signal transduction mechanisms is further emphasized by assessment of the response to specific vasoconstrictors with known mechanisms of action. The potentiation of vasoconstrictor responsiveness in the presence of increased extracellular glucose occurs via additive $PKC\alpha$ plus $PKC\beta$ -dependent mechanisms (see Figure 8). For example, the glucose-induced $PKC\beta$ - and $PKC\alpha$ -mediated mechanism leads to further vasoconstriction of ATII, a primarily $PKC\epsilon$ -dependent pathway, revealing the additive effect of the different PKC isoenzymes on Kv current inhibition and vasoconstriction. These findings of enhanced $PKC\alpha$ - and $PKC\beta$ -dependent vasoconstriction in high glucose are seen consistently using other vasoconstrictors, such as U46619 in porcine coronary and UTP in rat mesenteric arteries. Hence, it appears that glucose-mediated PKC-dependent inhibition of the Kv current not only increases basal tone, but also potentiates vasoconstrictor response to G-protein-activated signalling pathways, as well as (60K) depolarization-induced vasoconstriction. In contrast, the glucose-mediated potentiation of ET-1 induced vasoconstriction, a predominantly $PKC\alpha$ -dependent pathway (Rainbow *et al.*, 2009), is only observed in the 5-10 mM ($PKC\beta$ -dependent) range, with no increase in vasoconstrictor activity seen in the 10-20 mM ($PKC\alpha$ -dependent) range. In summary, the glucose-mediated potentiation of constriction is greater for agonists that do not act via $PKC\beta$ or $PKC\alpha$ (e.g. ATII) compared to agonist that constrict via $PKC\beta$ or $PKC\alpha$ (e.g. ET-1).

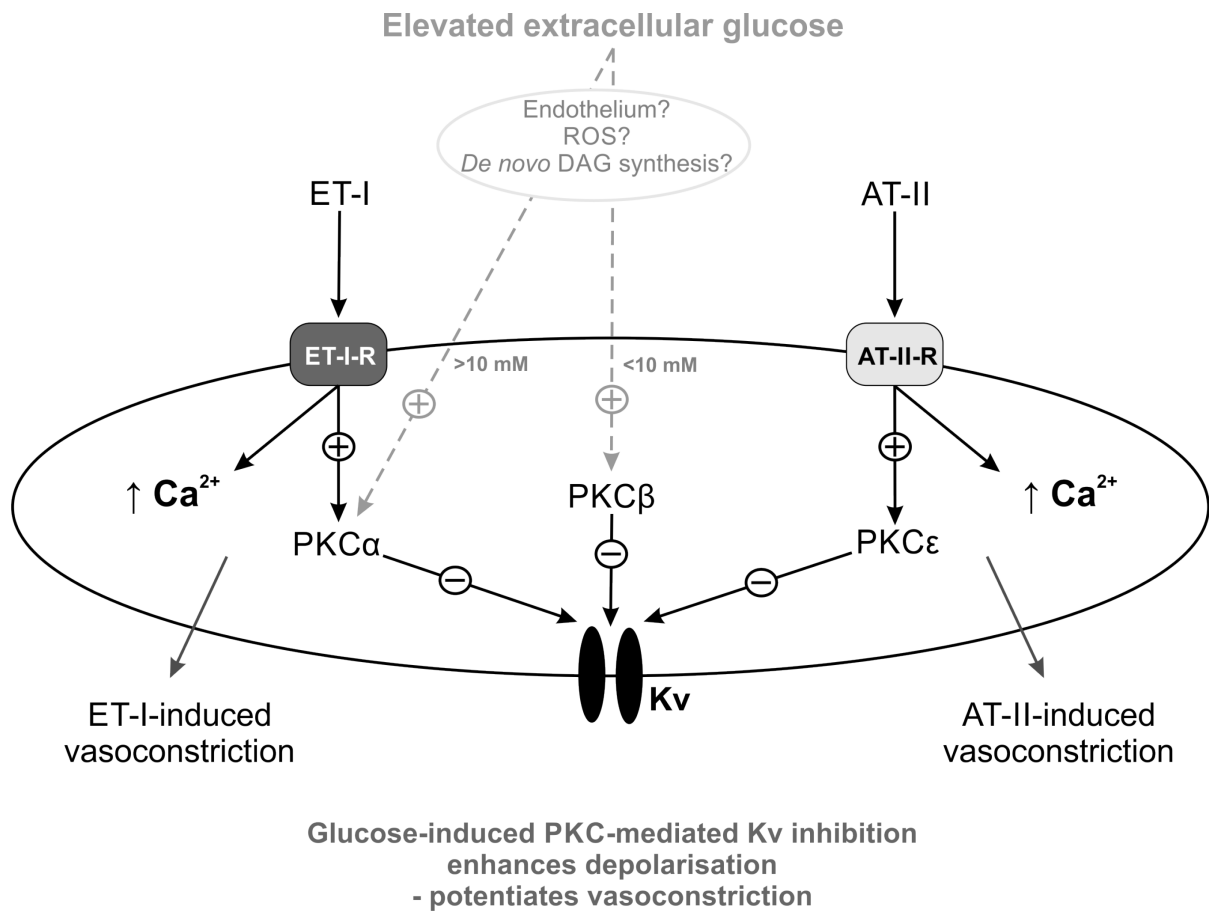


Figure 8: A representation of the proposed mechanism of glycaemia-enhanced vasoconstrictor signalling in vascular smooth muscle. Activation of PKCα and PKCβ with increasing glucose has a cumulative inhibitory effect on Kv current so enhancing vasoconstriction by depolarizing stimuli (such as 60K), or enhancing contraction to ATII, which activates an alternate PKC isoenzyme. ET1-induced vasoconstriction is only potentiated by glucose concentrations up to 10 mM, due to it acting via a PKCα-dependent pathway. The mechanism by which glucose activates PKC is currently unclear with ROS generation, *de novo* DAG synthesis, or a presently unidentified endothelially-derived modulator being potential candidates.

In contrast to a previous study in rat coronary arteries (Li *et al.*, 2003), we also provide experimental evidence that high glucose-induced DAG/ Ca^{2+} -dependent PKC-Kv-mediated potentiation of vasoconstriction does not significantly alter the vasodilatory activity of the β-adrenoceptor agonist, isoprenaline (Supporting Figure S6).

Our previous findings, and our current data in isolated MASMC, give added credence to our hypothesis that there is a direct effect of glucose, or a glucose metabolite(s), on PKC activation in vascular smooth muscle independent of the endothelium. The electrophysiology data in this current study and others (Li *et al.*, 2003; Rainbow *et al.*, 2006; Straub *et al.*, 2009) show that elevated glucose decreases Kv current in isolated vascular smooth muscle cells.

Furthermore, enhanced arterial contractility in elevated glucose was also maintained in the presence of the nitric oxide synthase inhibitor, L-NAME. This is a potentially important finding, as in hyperglycaemia endothelial dysfunction has been hypothesized to reduce the activity of NOS-dependent vasodilator pathways (Bohlen and Nase, 2001; Beckman *et al.*, 2002). Our data suggests that the effects of acute hyperglycaemia occur independently of any vascular endothelial dysfunction.

The involvement of reactive oxygen species, and enhanced generation in hyperglycaemic conditions, is a further potential mechanism for altered signalling. In our previous study, however, use of a free radical scavenger (N-(2-mercaptopropionyl)glycine) did not have a significant effect on the glucose-induced inhibition of Kv current in isolated MASMCs (Rainbow *et al.*, 2006). However, in another study, the use of superoxide dismutase and catalase partially reversed a high glucose-induced inhibition of Kv current, suggesting a role for superoxide radicals (Liu *et al.*, 2001). In addition, ROS has been suggested to have effects on other channels involved in vasoconstriction, including L-type voltage-gated Ca^{2+} channels (Shaifita *et al.*, 2015). In this recently published study the authors demonstrated an enhanced vasoconstriction, and an enhanced Ca^{2+} current, in response to ROS generation in a number of arterial beds, including mesenteric. Given that elevated glucose has been demonstrated to increase ROS in vascular tissue, further investigations will be required to characterize their effects on the enhanced constrictor responses seen in the present study, and whether other currents are modulated by this pathway.

Acute elevations of extracellular glucose have also been shown to have deleterious effects on the electrical properties of isolated cardiomyocytes, causing a marked prolongation of action potential duration (Hreiche *et al.*, 2009) and subsequent QT-prolongation, which is suggested to be pro-arrhythmic (Marfella *et al.*, 2001; Gordin *et al.*, 2008). Our own recent findings suggest a similar DAG/ Ca^{2+} -PKC isoenzyme-dependent dysregulation of electrical signalling in cardiac tissue (Sims *et al.*, 2014). The findings of the current study provide evidence for a common potential mechanism through which elevated glucose may exert direct deleterious effects on cardiovascular function with similar DAG/ Ca^{2+} -PKC isoenzyme-dependent mechanisms being evident in both heart and vasculature. The present study also demonstrates that hyperglycaemia exacerbates vasoconstriction both directly, and by exacerbating the response to circulating vasoconstrictors.

Hyperglycaemia has been previously shown to cause adverse effects on leg bloodflow (Giugliano *et al.*, 1997). Additionally, there is ample evidence that acute hyperglycaemia is a predictor of poor outcome in several vascular diseases, such as acute myocardial infarction, ischaemic stroke (Parsons *et al.*, 2002) and vascular (mesenteric) ischaemia (Schaffler *et al.*, 1998). It is presently unclear whether acute hyperglycaemia, observed under these conditions, has a cause-and-effect relationship. The need for further research into potential underlying mechanisms is clear, however, here we provide compelling evidence that specific PKC isoenzymes are implicated in a process that could contribute to this phenomenon. Our findings suggest the selective inhibition of PKC α and PKC β isoenzymes as a potential therapeutic target in patients with hyperglycaemia-complicating acute syndromes, such as myocardial infarction and ischaemic stroke. In clinical practice, inhibition of both PKC α and

PKC β may not be well tolerated, due to the ubiquitous expression and multiple roles of these PKC isoenzymes (Serova *et al.*, 2006; Rosse *et al.*, 2010). However, our demonstration of primarily PKC β isoenzymic involvement in Kv current modulation at 5-10 mM glucose, a level of hyperglycaemia associated with adverse prognosis following acute myocardial infarction (Goyal *et al.*, 2006; Kosiborod *et al.*, 2008; Squire *et al.*, 2010), suggests PKC β inhibition alone should have significant therapeutic potential in this context. The PKC β inhibitor ruboxistaurin has been shown to be well-tolerated in clinical trials for other indications, but has not to date been investigated in the context of such cardiovascular indications (Idris and Donnelly, 2006; Geraldles and King, 2010). Therefore, isoenzyme-specific PKC inhibition may represent a potential therapeutic target in the management of acute syndromes complicated by hyperglycaemia-potentiated vasoconstriction.

6d. References

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6e. Figure Legends

Figure 1: Kv currents are inhibited by glucose in a biphasic concentration-dependent manner in rat mesenteric arterial smooth muscle cells. (Ai-ii) Kv current measured from isolated MASMCs in 0 mM glucose using a series of depolarizing steps from -70 mV to +60 mV in 10 mV increments, 20 mV steps are shown for clarity. The solution was exchanged for one containing the test glucose (e.g. 20 mM (A(iii))), and a second I/V recorded (I/V(2)) after 9 min perfusion. Mean data showing current-voltage relationships (B) and activation curves (C; $V_{0.5}$ shown as insert) recorded from MASMCs cells incubated with 0, 5, 10 or 20 mM glucose. Data are expressed as I/V(2) normalized to the peak current in I/V(1) (at +60 mV). (D) Concentration-dependent inhibitory effect of increasing glucose concentration on Kv current; two-site analysis of the inhibition curve revealed IC_{50} values of 4.9 ± 0.7 and 16.2 ± 1.1 mM. (E) Histogram showing mean membrane potential after 9 min in solution containing the glucose concentration indicated (* $P < 0.05$; *** $P < 0.001$, ANOVA with Bonferroni's *post* test).

Figure 2: Tat-PKC20-28 attenuates inhibition of voltage-dependent potassium (Kv) current by glucose in rat mesenteric arterial smooth muscle cells. (Ai-ii) Kv current measured from isolated MASMCs in 0 mM glucose+ Tat-PKC20-28 using a series of depolarising steps from -70 mV to +60 mV in 10 mV increments. The solution was exchanged for one containing the test glucose Tat-PKC20-28 (e.g. 20 mM (A(iii))), and a second I/V recorded (I/V(2)) after 9 min perfusion. (B) Mean data I/V plots recorded from cells incubated with 20 mM glucose with and without Tat-PKC20-28. Data expressed as I/V(2) normalized to the peak current in I/V(1) (at +60 mV). (C) Histogram showing attenuation by Tat-PKC20-28 of Kv current inhibition via 0, 5, 10 or 20 mM glucose (* $P < 0.05$; ** $P < 0.05$; *** $P < 0.001$, ANOVA with Bonferroni's *post* test). (D) Histogram showing effects of Tat-PKC isoenzyme-specific inhibitor peptides (PKC α + β , PKC δ or PKC ϵ , each at 100 nM), or pharmacological inhibition of PKC α + PKC β with Gö 6976 (300 nM) on the Kv current in the presence of 20 mM glucose (*** $P < 0.001$, one-way ANOVA with Bonferroni's *post* test). (E) Mean data showing activation curves from MASMCs incubated with 0 or 20 mM glucose in the presence of Tat-PKC20-28. Data are expressed as I/V(2) normalized to the peak current in I/V(1) (at +60 mV). (F) Gö 6976 or Tat-PKC α + β inhibition prevented the depolarized membrane potential observed in the presence of 20 mM compared to 5 mM glucose (** $P < 0.01$, two-way ANOVA with Bonferroni's *post* test). Data in the absence of Tat-PKC or pharmacological inhibitors ('control' B to F) are taken from Figure 1 to allow direct comparison.

Figure 3: Kv current inhibited by glucose in a monophasic manner in rat mesenteric arterial smooth muscle cells in the presence of Tat-PKC β and Tat-PKC α inhibitor peptides. Kv current measured from isolated MASMCs in 10 or 20 mM glucose in the presence of (A) Tat-PKC β or (B) Tat-PKC α in a series of depolarising steps from -70 mV to +60 mV in 10 mV increments. Mean data showing activation curves ($V_{0.5}$ shown as insert) recorded from MASMCs cells incubated with 0, 10 or 20 mM glucose in the presence of (C) Tat-PKC β or (D) Tat-PKC α . Data are expressed as I/V(2) normalized to the peak current in I/V(1) (at +60 mV). Tat-PKC β (E) and Tat-PKC α (F) each altered the maximal inhibitory response by glucose to Kv current and converted the biphasic effect into a monophasic curves. Each data point was obtained from >6 cells from >3 animals. In panels (E) and (F)

concentration-response curves are repeated from Figure 1 to allow direct comparison with control.

Figure 4: K⁺-induced vasoconstriction of rat mesenteric artery is enhanced by high extracellular glucose and reversed by PKC α + β inhibition. Example traces of tension produced to two applications of 60K (60K₍₁₎ & 60K₍₂₎) with 30 min incubation in (A) 5 mM glucose and (B) 20 mM glucose before 60K₍₂₎. (C) Example trace shows enhancement of 60K contraction by 20 mM glucose is reversed by 30 min incubation in 5 mM glucose. (D) Experiment identical to panel B except for addition of Gö 6976 (300 nM) after 60K₍₁₎. (E) Mean data showing increases in tension for 60K(2) relative to 60K(1) (%) and reversal of the high glucose potentiation by 300 nM Gö 6976 (***P*<0.01; ****P*<0.001, repeated measures ANOVA with Bonferroni's *post* test, or unpaired *t*-test). (F) Histogram showing the mean contractile response to 60K (mN mm⁻¹) for control vessels and vessels pre-incubated with the indicated Tat-PKC isoenzyme inhibitors before switching to solution containing 5, 10 or 20 mM glucose (**P*<0.05; ***P*<0.01, two-way ANOVA with Bonferroni's *post* test).

Figure 5: Agonist-induced constriction of rat mesenteric artery is enhanced by high extracellular glucose in a conventional PKC isoenzyme manner. Example traces of 60K and AT-II (100 nM)-induced constrictor responses for rat mesenteric arteries in 5 (Ai) or 20 (Aii) mM glucose, 20 mM glucose + TatPKC α + β (Aiii) or Tat-PKC ϵ (Aiv). (B) Mean contractile responses to AT-II in 5 or 20 mM glucose with Gö 6976 or Tat-PKC ϵ . (C) Mean contractile responses to AT-II in 10 or 20 mM glucose with Tat- PKC α , Tat- PKC β and Tat-PKC α + β (≥ 12 vessels per condition from ≥ 4 animals: **P*<0.05; ***P*<0.01, one-way ANOVA with Bonferroni's *post-hoc* test). (D) Example traces of 60K and ET-1 (1 and 3 nM)-induced constrictor responses for rat mesenteric arteries in 5 (Di) or 20 (Dii) mM glucose, 20 mM glucose + TatPKC α + β (Diii) or Tat-PKC ϵ (Div). (E) Mean contractile responses to ET-1 (3 nM) in 5 and 20 mM glucose normalized to tension (mN mm⁻¹) in control vessels and vessels pre-treated with Gö 6976 or Tat-PKC ϵ inhibitor. (F), Mean contractile responses to ET-1 (3 nM) with 10 or 20 mM glucose in the absence and presence of Tat-PKC α or Tat-PKC β inhibitory peptides (≥ 10 vessels per condition from ≥ 5 animals: ****P*<0.001, ANOVA with Bonferroni's *post* test).

Figure 6: Agonist-induced constriction of porcine coronary artery is enhanced by high extracellular glucose in a conventional PKC isoenzyme manner. (A) Example traces of 60K and ET-1 (30 nM)-induced constrictor responses from porcine coronary arterial rings in 5 (Ai) or 20 (Aii) mM glucose, and in 20 mM glucose with Gö 6976 (Aiii), or PKC β -selective inhibitor LY379196 (Aiv). (B) Mean contractile responses to ET-1 in 5 or 20 mM glucose in the absence or presence of Gö 6976 or LY379196. (C) Mean contractile responses to KCl in 5, 10 or 20 mM glucose in the presence or absence of PKC α and/or PKC β inhibitors (≥ 9 vessels per condition from ≥ 4 pigs: **P*<0.05; ****P*<0.001, two-way ANOVA with Bonferroni's *post* test). (D) Example traces of U46619 (10-1000 nM)-induced constrictor responses from porcine coronary arterial rings in 5 (Di) or 20 (Dii) mM glucose. (E)

Concentration-response curve to U46619 in the presence of 5, 10, 15, 20 mM glucose. (F) Mean contractile responses to U46619 (100 nM) in 5 and 20 mM glucose in the absence or presence of Gö 6976 or LY379196.

Figure 7: Agonist-induced constriction of human mammary artery is enhanced by high extracellular glucose in a conventional PKC isoenzyme manner. Example traces of 60K and ATII (100 nM)-induced constrictor responses human mammary artery in 5 (Ai) or 20 (Aii) mM glucose, 20 mM glucose + Gö 6976 (Aiii) or LY379196 (Aiv). (B) Mean contractile responses to 60K (B) and ATII (C) in 5 or 20 mM glucose with Gö 6976 or LY379196 (≥ 4 vessels per condition from ≥ 4 patients, $*P < 0.05$; $***P < 0.001$, two-way ANOVA with Bonferroni's *post test*). (D) Example traces of U46619 (3-400 nM)-induced constrictor responses from human mammary artery in 5 (i) or 20 (ii) mM glucose. (E) Concentration-response curve to U46619 in the presence of 5 and 20 mM glucose. (F) Mean contractile responses to U46619 (100 nM) in 5 and 20 mM glucose in the absence or presence of Gö 6976 or LY379196 (≥ 4 vessels per condition from ≥ 4 patients, $*P < 0.05$; $***P < 0.001$, two-way ANOVA with Bonferroni's *post test*).

Figure 8: A representation of the proposed mechanism of glycaemia-enhanced vasoconstrictor signalling in vascular smooth muscle. Activation of PKC α and PKC β with increasing glucose has a cumulative inhibitory effect on Kv current so enhancing vasoconstriction by depolarizing stimuli (such as 60K), or enhancing contraction to ATII, which activates an alternate PKC isoenzyme. ET1-induced vasoconstriction is only potentiated by glucose concentrations up to 10 mM, due to it acting via a PKC α -dependent pathway. The mechanism by which glucose activates PKC is currently unclear with ROS generation, *de novo* DAG synthesis, or a presently unidentified endothelially-derived modulator being potential candidates.

On-line supporting information

Additional methods

Myography with Ca^{2+} fluorescence recording

Rat mesenteric arteries were mounted on a wire myograph as described above. Vessels were incubated for 90 min with 5 μ M Fura-2-AM (at 37°C). To limit exposure to ambient light, the myograph was covered and the vessel continuously perfused with a HEPES-buffered salt solution (composition (in mM): 135 NaCl, 6 KCl, 10 HEPES, 1.2 MgCl₂, 1.8 CaCl₂, 20 total monosaccharide (5 glucose + 15 mannitol) and 0.02 N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME), pH 7.4) throughout the protocol. Images were acquired at 0.1 Hz to minimize photobleaching. Acquisition rate was increased to 10 Hz during application of solutions containing 60 mM K⁺ (60K). Fluorescence was excited at 340 and 380 nm using a monochromator (PTI, Stanmore, UK) with emissions collected above 520 nm using a Cascade 512 camera (Roper Scientific, UK) and acquired using EasyRatioPro software (PTI). Data were analysed using WinFluor 3.5.9 software (kindly provided by Dr John Dempster, University of Strathclyde). Simultaneous tension data were acquired using pCLAMP8.

Figure S1

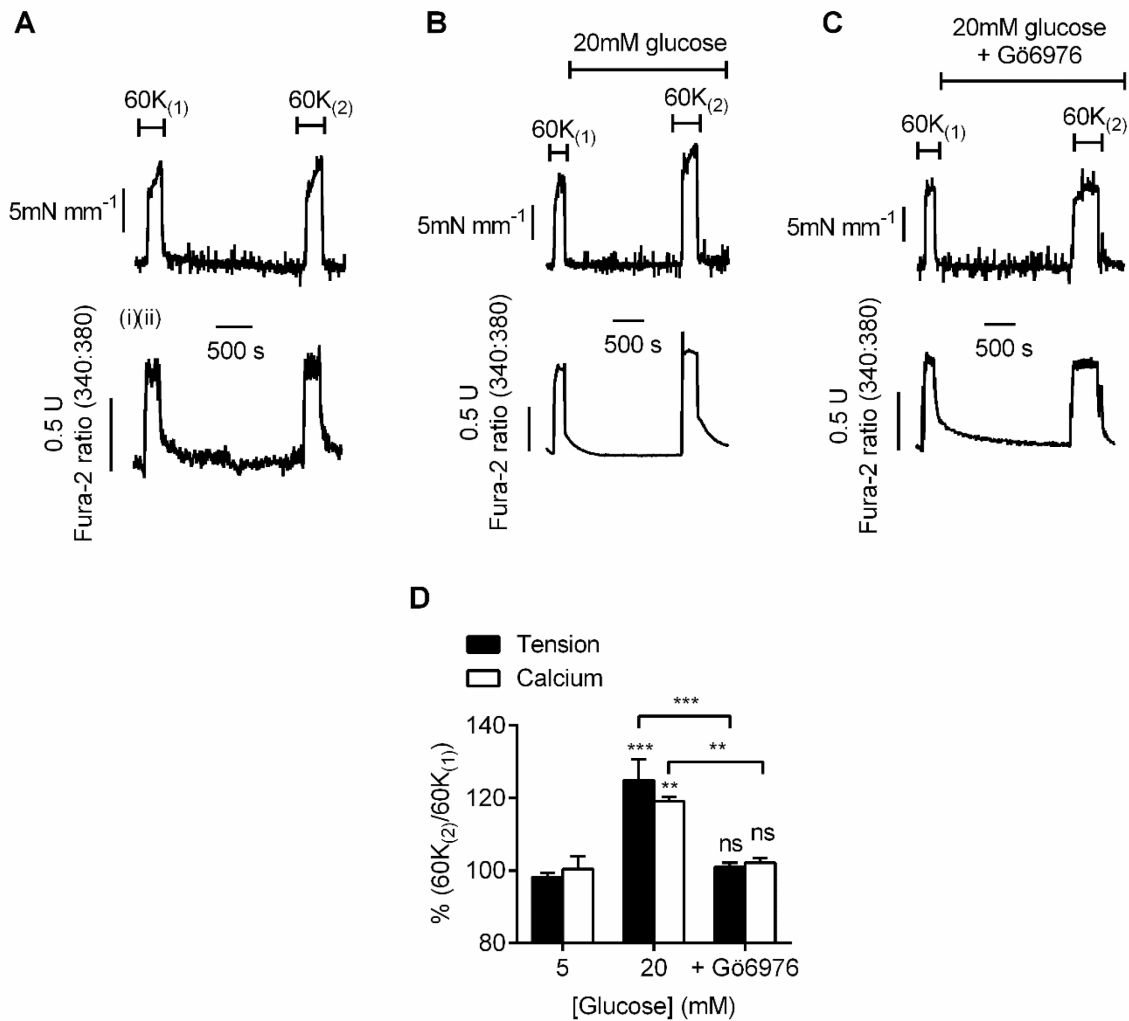


Figure S1 K⁺-induced increases in [Ca²⁺]_i and vasoconstriction are increased in the presence of high glucose and reversed by Gö6976. Isolated rat mesenteric arteries were stimulated to contract by two applications of 60 mM KCl (60K) separated by a 30 min washout period. Example traces showing 60K₍₁₎ and a second response to 60K (60K₍₂₎) after 30 min incubation in (A) 5 mM glucose, (B) 20 mM glucose and (C) 20 mM glucose in the presence of the cPKC inhibitor Gö6976 (300 nM) immediately after washout of the first 60K addition. (D) Mean data showing increases in tension (mN/mm) and Fura-2 ratio for 60K₍₂₎ relative to 60K₍₁₎ (%) and the reversal of the high glucose potentiation in the presence of

Gö6976 (** $P < 0.01$; *** $P < 0.001$, repeated measures ANOVA with Bonferroni's *post-hoc* test, or unpaired *t*-test).

Figure S2

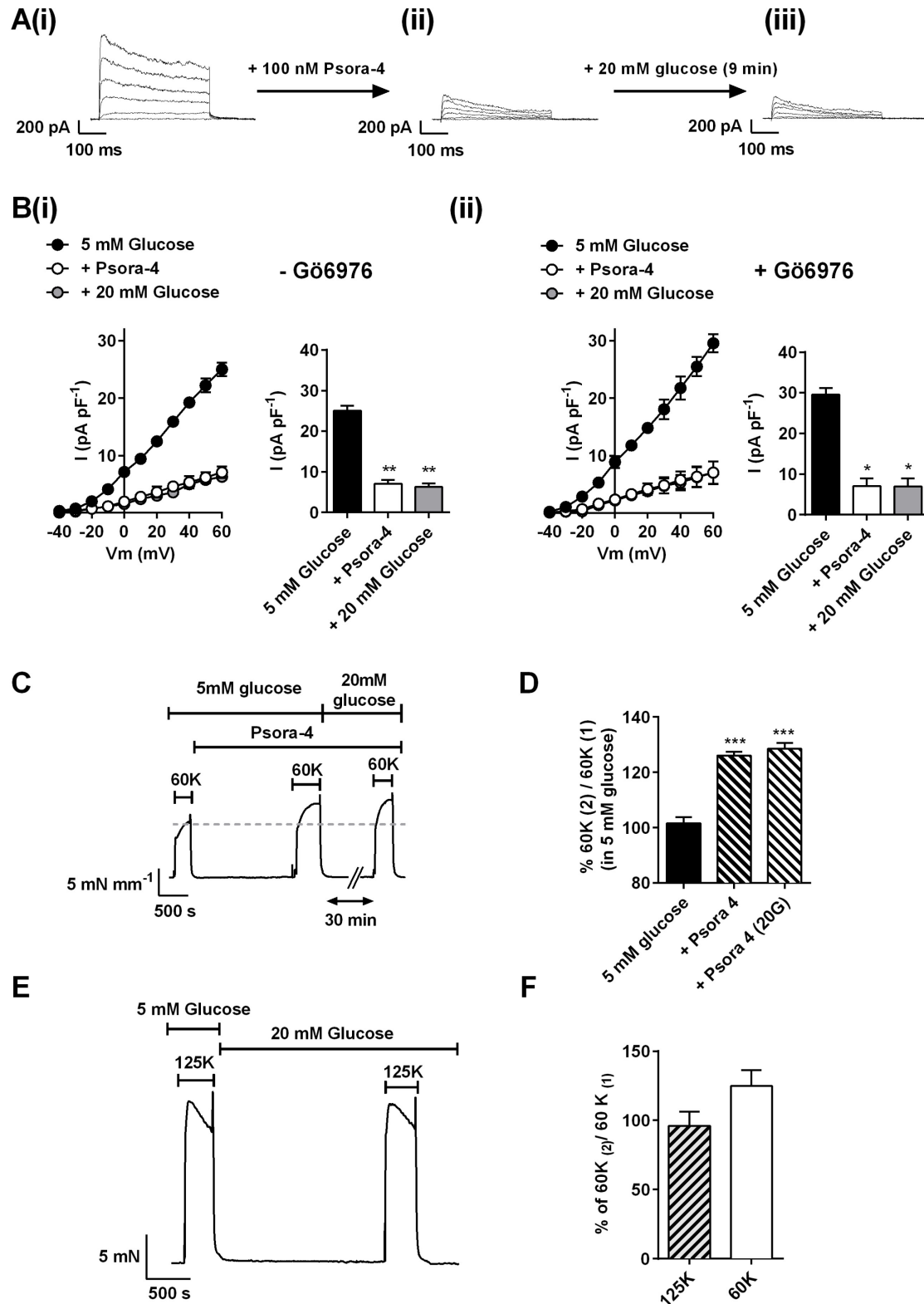


Figure S2 Glucose-mediated increases in high extracellular $[K^+]$ -induced vasoconstriction of mesenteric smooth muscle are mimicked by Kv1 channel blockade. (A) Example I/V relationships from MASMCS in 5 mM glucose before (i) and after (ii) perfusion with the Kv1 family channel inhibitor Psora-4 (100 nM) and (iii) after 9 min of perfusion with

20 mM glucose. **(B)** Mean I/V relationship and mean histogram of peak current from MASMCs before and after Psora-4 in 5 mM glucose and after 9 minutes of perfusion with 20 mM glucose in control **(i)** and after pre-treatment with 300 nM Gö976 **(ii)**. Mean data for membrane potential in the absence and presence of Psora-4 (* $P < 0.05$, ** $P < 0.01$, Repeated measures ANOVA with Bonferroni's *post* test: >4 cells from ≥ 3 animals). **(C)** Example trace showing 60K₍₁₎ and second 60K response (60K₍₂₎) after 30 min incubation with Psora-4 and 5 mM glucose with a third 60K addition in the presence of Psora-4 and 20 mM glucose. **(D)** Mean data showing increases in 60K₍₂₎ relative to 60K₍₁₎ (%) in the presence of Psora-4 (*** $P < 0.001$, repeated measures ANOVA with Bonferroni's *post-hoc* test, ≥ 6 vessels from ≥ 3 animals). **(E)** Example trace showing the response of mesenteric arteries to 125K-induced contraction in 5 and 20 mM glucose. **(F)** Mean data showing a lack of potentiation of the 125K-response in 20 mM glucose suggesting that the small hyperpolarizing effect from K⁺ currents seen in 60K is abolished by further decreasing the K⁺ gradient (≥ 8 vessels from ≥ 3 animals for each dataset).

Figure S3

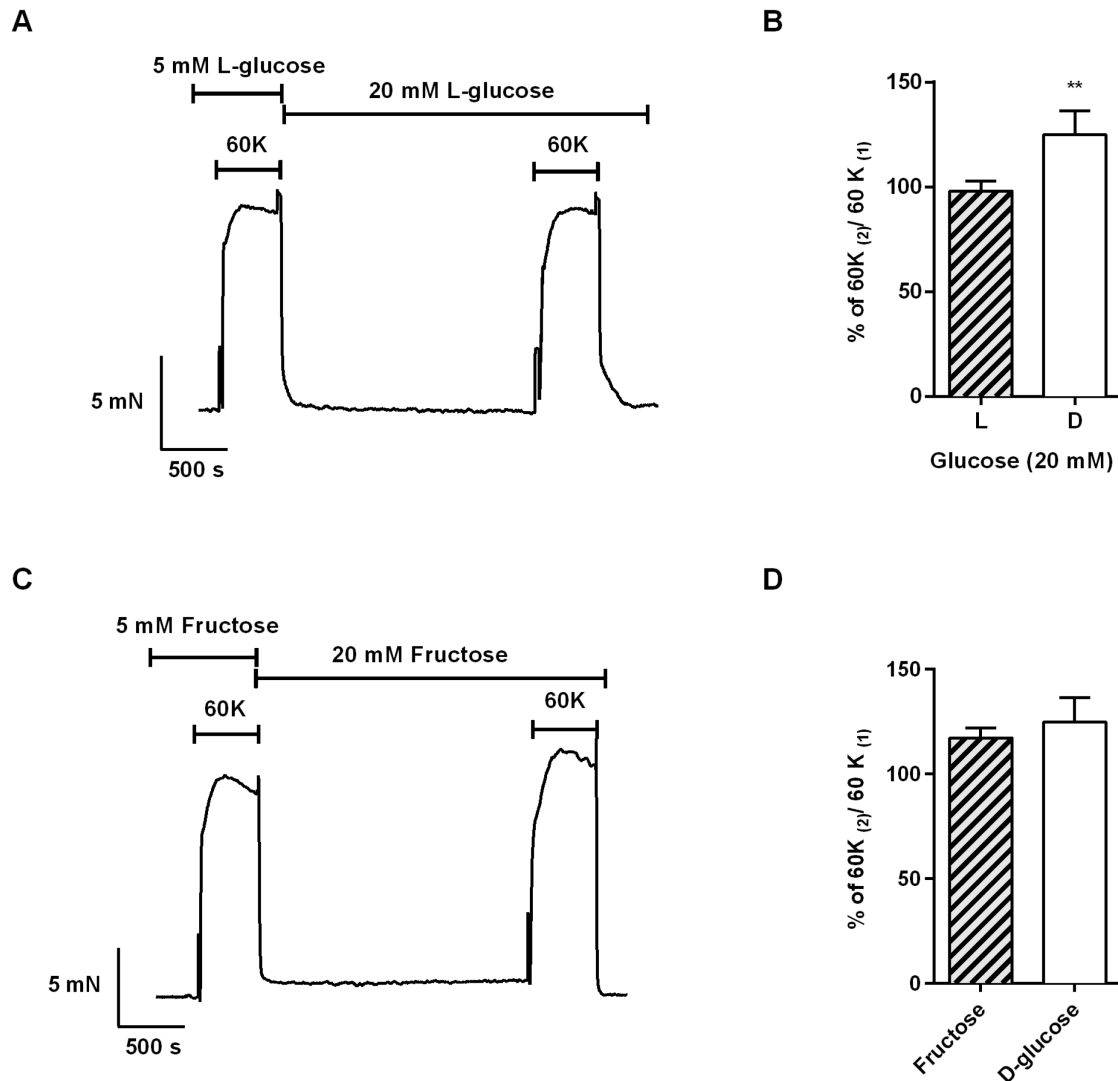


Figure S3 Enhanced K⁺-induced contraction by monosaccharides is dependent of glycolytic metabolism. Isolated rat mesenteric arteries were stimulated to contract by two applications of 60 mM KCl (60K₍₁₎ and 60K₍₂₎) separated by a period of 30 min. **(A)** 60K₍₁₎ contraction was in solution containing the non-metabolized monosaccharide L-glucose (5 mM) with 60K₍₂₎ added after 30 min incubation with the L-glucose (20 mM). **(B)** Mean data showing the % of 60K₍₂₎ in L- and D-glucose for comparison. **(C)** 60K₍₁₎ contraction was in solution containing fructose (5 mM) with 60K₍₂₎ added after 30 min incubation with 20 mM fructose. **(D)** Mean data showing the % of 60K₍₂₎ in 20 mM fructose to 20 mM D-glucose (≥ 6 vessels from ≥ 3 animals for each dataset).

Figure S4

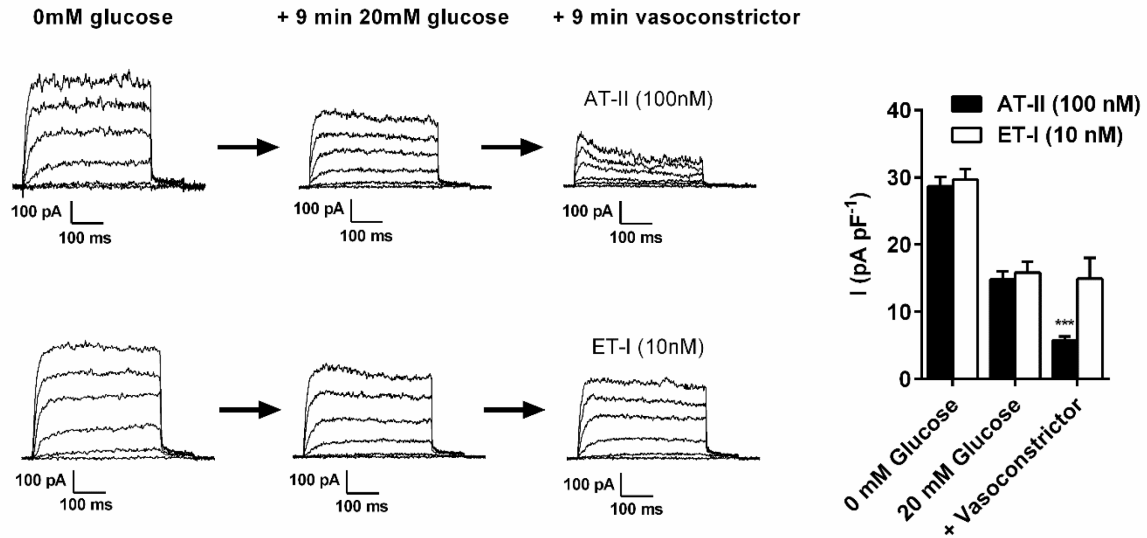


Figure S4 Additive and non-additive effects of high glucose for ATII and ET1 on Kv current inhibition in rat mesenteric artery smooth muscle cells. Representative whole-cell current recordings of Kv current from MASMC recorded in 0 mM glucose, after 9 min of perfusion with 20 mM glucose, and after a further 9 min of perfusion with ATII (100 nM) or ET1 (10 nM). Mean current (normalized to cell capacitance) in 0 mM glucose, after 9 min of perfusion with 20 mM glucose and after a further 9 min of perfusion with either ATII or ET1. Kv current was reduced on switching to 20 mM glucose ($P < 0.001$, repeated measures ANOVA with Bonferroni's *post hoc* test) with a further inhibition on co-addition of ATII ($***P < 0.001$), but not ET1 ($P > 0.05$, repeated measures ANOVA with Bonferroni's *post hoc* test, $n = 3$ animals (6 cells) for each dataset).

Figure S5

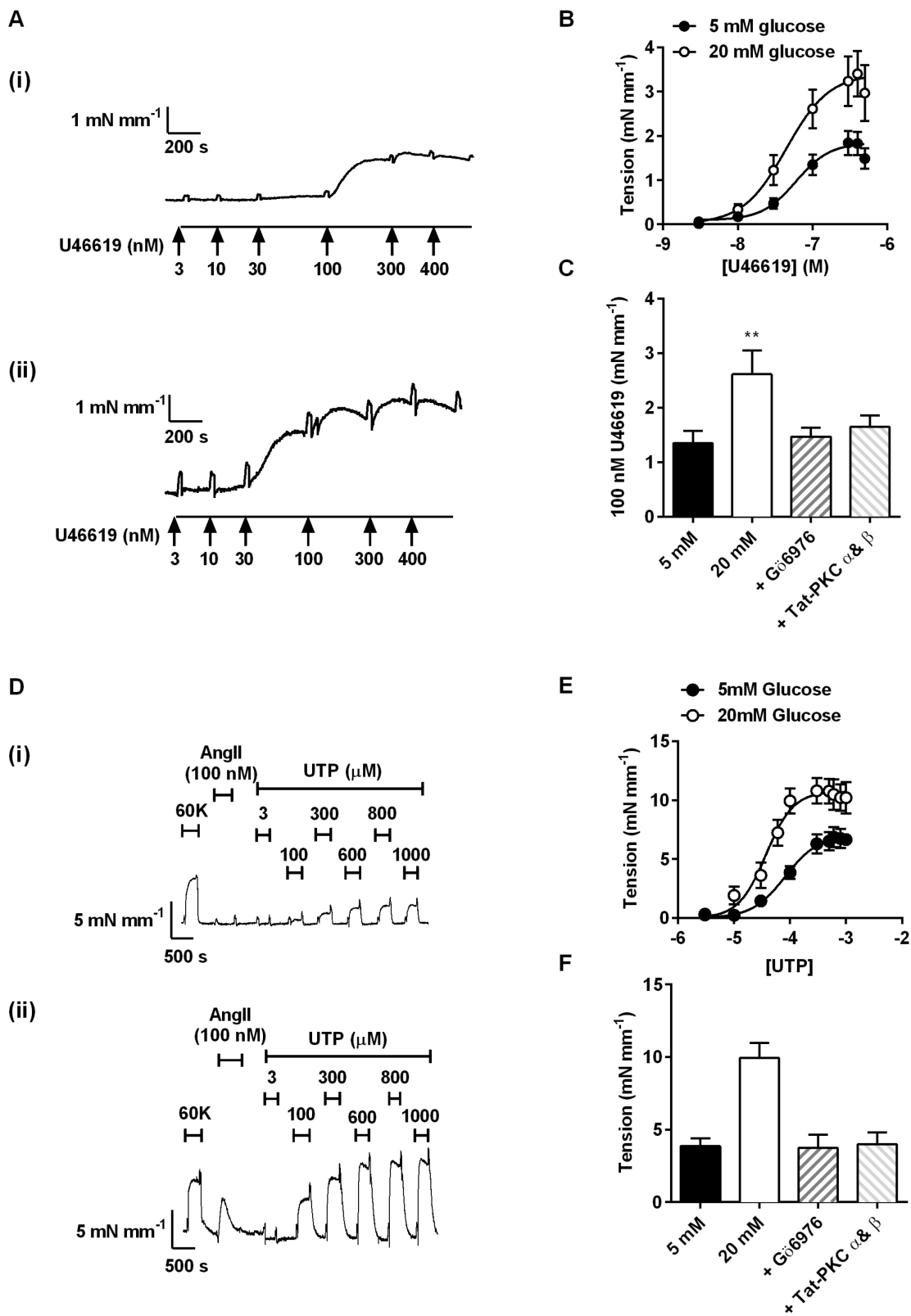


Figure S5 U46619- and UTP-induced constrictions of rat mesenteric artery are enhanced by high extracellular glucose in a conventional PKC isoenzyme-dependent manner. **(A)** Example traces of U46619 (3-1000 nM)-induced constrictor responses from rat mesenteric arterial rings in 5 **(i)** or 20 **(ii)** mM glucose. **(B)** U46619 concentration-response curves in the presence of 5 and 20 mM glucose. **(C)** Mean contractile responses to U46619 (100 nM) in 5 and 20 mM glucose in the absence or presence of Gö6976 or Tat-PKC $\alpha+\beta$. **(D)** Example traces of UTP (3-1000 nM)-induced constrictor responses from rat mesenteric arterial rings in 5 **(i)** or 20 **(ii)** mM glucose. Addition of 100 nM AngII to rat mesenteric arterial rings caused contraction in both 5 mM **(i)** and 20 mM **(ii)** glucose but was substantially enhanced in 20 mM as shown in Figure 5. **(E)** UTP concentration-response curves in the presence of 5 and 20 mM glucose. **(F)** Mean contractile responses to UTP (100 μ M) in 5 and 20 mM glucose in the absence or presence of Gö6976 or Tat-PKC $\alpha+\beta$.

Figure S6

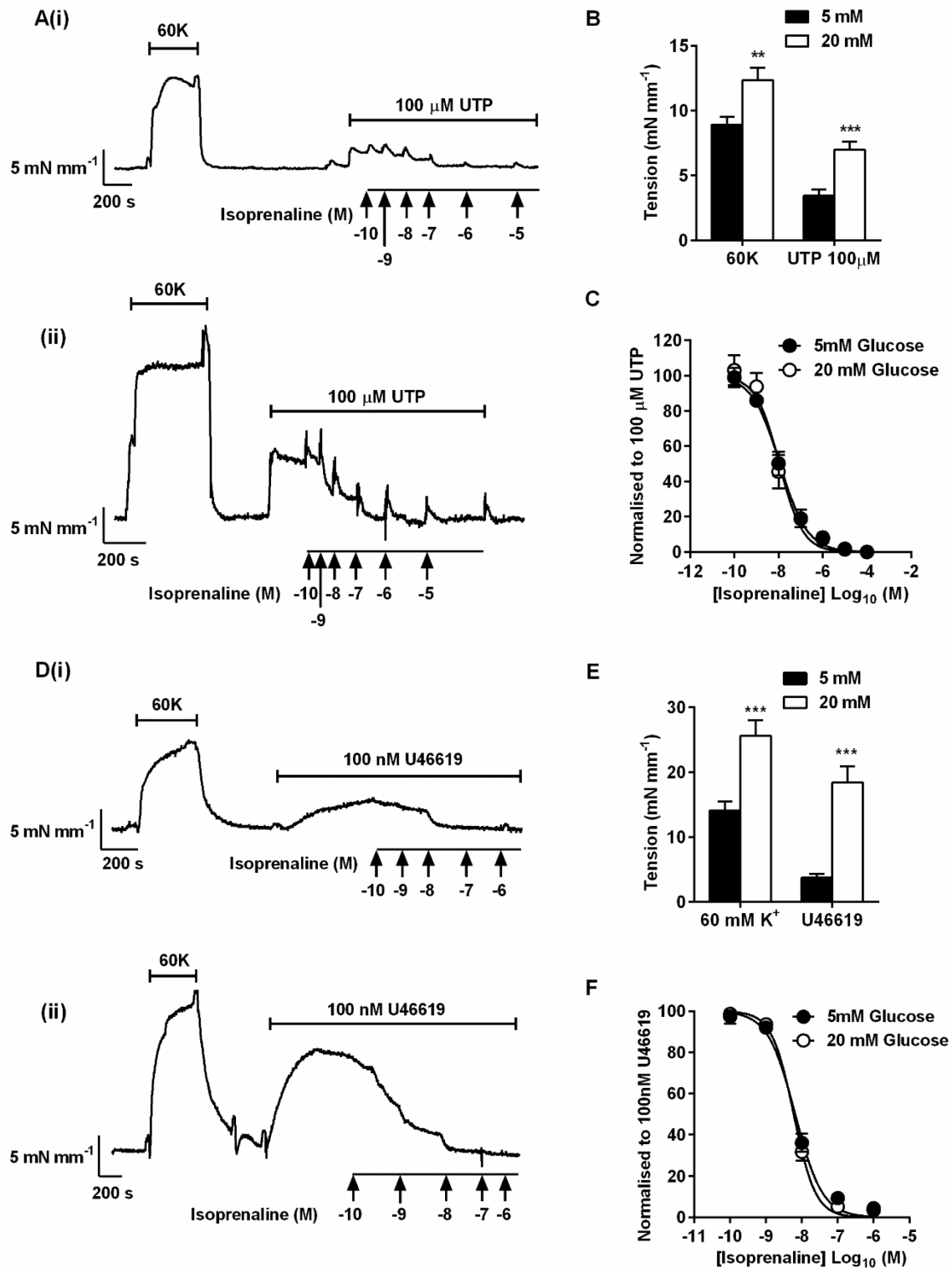


Figure S6 Vasodilator responses to a β -adrenoceptor agonist are unaffected by elevated extracellular glucose concentrations in isolated rat mesenteric artery. (A) Representative traces showing vasodilator responses of agonist (100 μ M UTP) pre-constricted small rat

mesenteric arteries by cumulative additions of isoprenaline in either 5 mM **(i)** and 20 mM **(ii)** glucose. **(B)** Mean contractile response to 60K and UTP in 5 and 20 mM glucose. Consistent with previous figures both vasoconstrictor activities are potentiated in elevated glucose (** $P < 0.01$, *** $P < 0.001$, unpaired Student's t -test). **(C)** Isoprenaline concentration-response curves normalized to the peak contraction induced by UTP in 5 or 20 mM glucose (mean IC_{50} values 11.5 ± 2.6 and 12.3 ± 1.9 nM at 5 and 20 mM glucose, respectively; $P > 0.05$, $n \geq 3$ animals (8 vessels) for each data-point). **(D)** Representative traces of vasodilator responses of agonist (U46619, 100 nM) pre-constricted porcine coronary arterioles by cumulative additions of isoprenaline in 5 **(i)** and 20 mM **(ii)** glucose. **(E)** Mean contractile response to 60K and 100 nM U46619 in 5 and 20 mM glucose demonstrating an enhanced vasoconstrictor response in elevated glucose (*** $P < 0.001$, unpaired Student's t -test). **(F)** Isoprenaline concentration-response curves normalized to the peak contraction induced by U46619 in 5 or 20 mM glucose (mean IC_{50} values 6.3 ± 2.1 and 6.1 ± 1.9 nM in 5 and 20 mM glucose, respectively; $P > 0.05$, $n \geq 4$ animals (16 vessels) for each data-point).