β1-adrenoceptor-stimulated lactate production in cultured astrocytes is predominantly glycogen-independent.

Xianguo Jiang*, John Challiss, and Paul Glynn.

Department of Molecular and Cell Biology, University of Leicester, Leicester, LE1 9HN, UK.

* Present address: Department of Neurology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China.

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Corresponding author:
Paul Glynn
Department of Molecular and Cell Biology, University of Leicester, Leicester, LE1 9HN, UK.
pg8@leicester.ac.uk
+44-116 254 4656

Abbreviations.

DEX, dexmedetomidine; DMSO, dimethylsulfoxide; GP, glycogen phosphorylase; GS, glycogen synthase; ISO, isoprenaline; NA, noradrenaline.

Noradrenaline (NA) promotes breakdown of the glucose-polymer, glycogen, and hence enhances glycolytic production of lactate in astrocytes. Here, in cultured rat cerebrocortical astrocytes, we examined the contributions of different adrenoceptor subtypes to NA-modulated glucose metabolism, and the relationship of NA-induced glycogenolysis to lactate production. Stimulation of astrocytic glucose metabolism by NA was mediated predominantly via β1-adrenoceptors and cAMP. Constitutive β1-adrenoceptor activity – in the absence of exogenous NA - contributed to the basal rate of glycogen turnover. Although mRNAs encoding both β1- and β2-adrenoceptors were detected in these astrocytes, β2-adrenoceptors contributed little to NA-induced modulation of glucose metabolism. Activation of α2- and α1-adrenoceptors in these cells decreased cAMP and increased cytosolic Ca^{2+}, respectively, but did not modulate NA-induced glycogenolysis: α2-adrenoceptors because glycogenolysis was induced maximally by NA concentrations that only began to inhibit cAMP production; and α1-adrenoceptors possibly because of desensitisation and depletion of Ca^{2+} stores. Under basal conditions, astrocytes converted glucose to extracellular lactate in near stoichiometric manner. When glucose-starved astrocytes were given fresh glucose-containing medium, lactate accumulation displayed a brief lag period before attaining a steady-state rate. During this lag period NA, acting at β1-adrenoceptors, increased the rate of lactate accumulation both in the absence and presence of an inhibitor of glycogen turnover. At the steady-state, the rate of glucose incorporation into accumulated glycogen was ~5% of that into lactate, but NA enhanced lactate output by 20-50%: this further indicates that NA, via β1-adrenoceptors and cAMP, can enhance astrocytic lactate production independently of its effect on glycogen turnover.

1. Introduction

Seminal studies on hormonal regulation of metabolism of the glucose-polymer, glycogen, led to the discovery of the second messenger cAMP and this, in turn, to discovery of G protein-coupled receptors (GPCRs). Glycogen levels reflect a balance between synthesis catalysed by glycogen synthase (GS) and breakdown catalysed by glycogen phosphorylase (GP). The latter can be activated allosterically by AMP and this is a major mechanism during glucose (and hence energy) depletion when cellular AMP/ATP ratios increase. GP is also activated covalently via phosphorylation mediated by GP-kinase. This enzyme can be activated either by binding Ca^{2+} via its calmodulin subunit, or by being phosphorylated by cAMP-dependent protein kinase A (PKA). Moreover, the activity of GS is inhibited by phosphorylation by either PKA or by Ca^{2+}-dependent GS-kinase. Thus, glycogen synthesis and breakdown are regulated in a reciprocal fashion, and increased cytosolic Ca^{2+} and cAMP usually induce net glycogen breakdown [1].

Glycogen is present, not only in liver and skeletal muscle, but also in astrocytes in the brain [2]. Noradrenaline (NA), released throughout the brain from terminals of locus coeruleus neurons, activates GPCRs termed adrenoceptors. β- and α2-adrenoceptors, via Gs and Gi proteins, respectively, modulate cAMP levels; α1-adrenoceptors, via Gq proteins, increase cytosolic Ca^{2+}. Early pharmacological studies in cultured rodent astrocytes showed that NA promoted glycogen breakdown predominantly via β-adrenoceptors. However, Sorg & Magistretti [3] found evidence also for α1-adrenoceptor-induced glycogenolysis, and Subbarao & Hertz [4] surprisingly found that NA-induced glycogenolysis was inhibited by antagonists of either β- or α2-adrenoceptors. Subsequent experiments have shown that astrocytic glycogenolysis induced by the β-adrenoceptor agonist isoprenaline is mediated by the β1- and not the β2-subtype of adrenoceptor [5].

Glycogen turnover forms a branch pathway – or shunt [6] – of glucose metabolism (Fig 1). Glucose-6-phosphate derived either directly from glucose itself, or via glycogen turnover, is metabolized by glycolysis to generate pyruvate. In astrocytes much of this pyruvate is reduced to lactate that, in turn, is exported from the cell via monocarboxylate transporters. Cultured astrocytes have been used to investigate links

between glycogen turnover and lactate export. An early study showed that glucose withdrawal from cultured astrocytes led to rapid glycogen breakdown and concomitant appearance of a stoichiometric amount of lactate in the incubation medium [7]. More recently it has been reported that NA augments the output of lactate from cultured astrocytes under certain experimental conditions [6, 8]. However, these studies provided little detail and did not quantify the contribution of this extra lactate to that produced by basal metabolism of glucose.

Increased knowledge of the receptor subtypes that mediate noradrenergic regulation of astrocytic glucose metabolism is of fundamental neurochemical interest and also may facilitate understanding of unexpected effects of those adrenoceptor-targeted drugs widely-used in cardiovascular and respiratory medicine that can cross the blood-brain barrier. Here we present a detailed and quantitative pharmacological characterisation of the effects of NA on glycogen turnover and lactate production in cultured astrocytes.

2. Materials and Methods.

2.1 Isolation, culture, and experimental incubations of astrocytes.

Experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986 Home Office regulations. Two day-postnatal (P2) Wistar rats were culled using a Schedule 1 method, a procedure compliant with the University of Leicester Ethical Committee. Wistar rats were bred as a colony at the University of Leicester. This study was not pre-registered. No exclusion criteria were predetermined and no neonatal rats were excluded except when litter size exceeded 6 in which case a maximum of 6 were included at random. Both female and male neonates were used without pre-selection. Apart from this, no randomisation was performed to allocate individual neonates in the study. For each astrocyte isolation, 4 to 6 neonates from a single litter were used. All astrocyte isolations were carried out between June 2016 and August 2018 and, over this period, astrocyte preparations for the experiments described in this study were performed on approximately 60 occasions. Animals were culled between 9-10am on each occasion.

Astrocytes were isolated following the procedure of [9]. Cortical tissue was dissected from P2 rat brains, digested with trypsin, and cells were cultured in flasks containing

Dulbecco's Minimal Essential Medium (DMEM) with 4.5g/l glucose and 15% foetal bovine serum (FBS) and penicillin-streptomycin (PS) for 7 days. Flasks were shaken overnight in an orbital shaker and the resulting astrocyte-enriched monolayer was harvested and plated in DMEM with 4.5g/l glucose, 10% FBS and PS in 24-well plates at a density of 0.75-1.5 x 10^5 cells per well (equivalent to 4 to 8 24-well plates per astrocyte preparation). After a further 6-7 days the culture medium was aspirated and replaced with 1ml serum-free DMEM containing 1g/l glucose. After overnight incubation, experimental incubations were initiated by addition of NA or other drugs. (NA and all other catecholamines were diluted in 10mM sodium acetate, pH 4.0). All culture and experimental incubations were carried out in a humidified incubator in an atmosphere of 5% CO_2 at 37^o. Experimental incubations were terminated after varying periods up to 150min. Aliquots of media were saved at 4^o for assay of lactate and glucose. Medium was then aspirated, cells were washed with ice-cold Tris-buffered saline (150mM NaCl in 10mM Tris-HCl, pH7.4) and then lysed by addition of 100µl 30mM HCl/0.1% Triton X-100. Lysates were transferred to 0.6ml Eppendorf tubes and centrifuged (14,000rpm; 4min). Aliquots of the resulting supernatant were used for assay of glycogen, lactate, and total protein.

2.2 Assays of lactate, glucose, glycogen and protein.

All assays were colorimetric, used a 96-well plate format, and were run at room-temperature (~20^o): absorbances were read in a Tecan plate-reader.

Lactate in 10µl aliquots of media was assayed by addition of 100µl of a reaction mix containing lactate oxidase (1 munit), horseradish peroxidase (100 munits), Amplex Red substrate (1µg) in 0.25M sodium phosphate buffer, pH7.4. Absorbance at 560nm (A560) was read immediately after addition of the reaction mix to provide a zero-time value, and then again after 10 min at room-temperature. Zero-time values were subtracted from 10 min values and lactate content determined by comparison with a standard curve of lactate dilutions over the range 0 to 10nmol run in duplicate on each 96-well plate.

Glucose in appropriately diluted aliquots of media was determined in analogous manner to that described for lactate, except glucose oxidase (100munits) replaced lactate oxidase in the reaction mix, and each 96-well plate also ran a standard curve of

glucose dilutions (0 to 10nmol). Details are described together with those for glycogen assay.

Glycogen was determined as glucose after digestion with amyloglucosidase (AG) using conditions similar to those described by Sorg & Magistretti [3]. Aliquots (25µl) of cell lysates were incubated at room-temperature for 2h with 25µl 0.1M sodium acetate, pH4.7 with or without AG (10mg/ml). On each 96-well plate, a standard curve of bovine liver glycogen (0 to 800ng) was run in parallel to monitor efficiency of the AG digest. A glucose standard curve (0 to 10nmol glucose in 50µl of a buffer comprising a 1:1 mix of 0.1M sodium acetate, pH4.7: 30mM HCl/0.1% Triton X-100) was also run on each 96-well plate. After 2h, 50µl of reaction mix (composition as for glucose assay above) was added, and A560 was read. Glycogen in the lysates or standards was determined as the difference between A560 values obtained after incubations in the presence or absence of AG.

Protein was determined in 10µl aliquots of cell lysates by addition of 100µl diluted Bradford reagent and reading absorbance at 595nm after 5min. Protein (bovine serum albumin) standard curves (0 to 8µg) were run on each 96-well plate.

2.3 cAMP experiments and determinations.

After overnight incubation in serum-free DMEM (1g/l glucose) astrocytes were incubated (37^{o}) for 10min in Krebs-buffer with additions noted in the figures. Incubations were terminated and intracellular cAMP levels determined as described previously [10].

2.4 Incubations for determination of changes in cytosolic [Ca^{2+}].

Astrocytes were grown on poly-lysine-coated coverslips. Before each experiment cells were incubated overnight in serum-free DMEM (1g/l glucose). Cells were then transferred to Krebs-buffer, loaded with Fluo-4-AM, and cytosolic [Ca^{2+}] determined as described previously [11].

2.5 Detection of β-adrenoceptor mRNAs.

Standard molecular biology protocols were followed. RNA was extracted from at least 10^6 astrocytes using an Ambion RNA mini kit and then used as template for cDNA synthesis using the Superscript III reverse transcriptase system (Invitrogen)

according to the manufacturer's instructions. The cDNA was used as temple for PCR reactions using primers within the sequences of mRNAs for rat β1- and β2-adrenoceptors and GAPDH (as a positive control reaction). Primer sequences were: β1-adrenoceptor: Forward 5′-GCATCATCATGGGTGTGTTC-3′; Reverse 5′-CCAGTTGAAGACGAAGAGG-3′. β2-adrenoceptor: Forward 5′-ATTTCTGGTGCGAGTTCTGG-3′; Reverse 5′-AAGGGCGATGTGATAGCAAC-3′. GAPDH: Forward: 5'-GGCATTGCTCTCAATGACAA-3′; Reverse 5′-TGTGAGGGAGATGCTCAGTG-3′. PCR was performed for 30 cycles: 94-96^{o}C (1 min), 65^{o}C (1 min), 72^{o}C (3 min). PCR products were resolved by electrophoresis on 1% (w/w) agarose gels, stained with ethidium bromide, then visualised and photographed on a UV trans-illuminator.

2.6 Materials.

DMEM and other cell culture reagents were from Gibco: DMEM with 4.5g/l glucose (cat.no. 31966-021); DMEM with 1g/l glucose (cat.no. 31885-023); DMEM without glucose (cat.no. 11966-025); foetal bovine serum (cat.no. 10270-106); penicillin-streptomycin (cat.no. 15070-063). Enzymes and most other reagents were from Sigma: amyloglucosidase (cat.no. A1602); horseradish peroxidase (cat.no. P8375); glucose oxidase (cat.no. G2133); lactate oxidase (cat.no. L9795); glycogen (cat.no. G0885); noradrenaline (cat.no. N5785); isoprenaline (cat.no. I5627); phentolamine (cat.no. P7547); prazosin (cat.no. P7791). Four adrenoceptor ligands were from Tocris: A-61603 (cat.no. 1052); CGP-20712 (cat.no. 1024); ICI-118551 (cat.no. 0821); dexmedetomidine (cat.no. 2749); as was the GP-inhibitor, CP-316819 (cat.no. 3542). Fluo-4-AM (cat.no. F14217) was from Invitrogen. Amplex Red (10-acetyl-3,7,-dihydroxyphenoxazine; peroxidase substrate) was from Cayman Chemicals, cat.no. CAY-10010469.

2.7 Experimental design and statistical analysis.

All experiments described in this study used astrocytes sub-cultured in 24-well plates and were performed within less than 3 weeks of the isolation of that batch of astrocytes. Experiments were not blinded and no sample calculation was performed. The data presented in this study are means from three experiments with, in each experiment, determinations for either lactate, glycogen, or cAMP in 3 wells of a 24-

well plate for each experimental condition. Individual experiments were performed between 9 – 12am on different days, generally using astrocytes isolated on different occasions. Values for lactate and glycogen per well were expressed as glucose equivalents per mg of cell protein. Data were plotted and analysed using Prism-7 software. Data in figures are presented as mean ± standard deviation and, in histograms, the three mean values obtained in each of the three experiments are shown individually. Pairs of values were compared by unpaired, two-tailed Student's t-test, and P-values <0.05 were accepted as indicating significant difference. Data were not assessed for normality and no test for outliers was conducted.

3. Results.

3.1 NA induces glycogenolysis predominantly via β1-adrenoceptors in cultured astrocytes.

Under the conditions described in Methods for isolation, culture and incubation, the basal glycogen content of our rat cerebrocortical astrocyte preparations was 102 ± 26 nmol glucose-equivalents (Glc-Eq) per mg protein (mean and standard deviation of values obtained in the nine sub-figures in Figs 2-4). These values are comparable with those reported for mouse cortical astrocyte preparations (106 ± 12 nmol/mg, [4]; 135 ± 13 nmol/mg [12]; 90 ± 8 nmol/mg [5]). NA (1μM) induced rapid depletion of glycogen in cultured astrocytes: within 20 min glycogen was reduced by 50-60nmol Glc-Eq per mg protein and maintained at this level for a further 40min. Co-addition with NA of the GP inhibitor, CP-316819 (30μM), significantly diminished NA-induced glycogenolysis (Fig 2A).

In our astrocyte cultures, the β-adrenoceptor agonist isoprenaline (ISO) induced glycogen breakdown ~2-fold more potently than NA (pEC50 (-logM) 8.14 ± 0.08 and 7.77 ± 0.06, respectively) (Fig 2B). ISO-induced glycogenolysis was reversed by addition of the non-selective β-adrenoceptor antagonist, timolol, and basal glycogen levels were restored within 90min – equivalent to a re-synthesis rate of at least 0.6-0.7nmol Glc/mg protein/min (Fig 2C).

mRNAs encoding both β1- and β2-adrenoceptors were detected in our rat astrocyte preparations, although their respective levels were not quantified by the method used

(reverse transcriptase PCR) (Fig 2D). However, while the glycogenolytic effect of NA was blocked by low concentrations of the β1-antagonist, CGP-20712, it was barely affected by the β2-antagonist, ICI-116551 (Fig 2E). Glycogenolysis evoked by activation of β-adrenoceptors is mediated by cAMP as these receptors couple to Gs proteins that activate adenylyl cyclase. Astrocytic cAMP accumulation was also predominantly stimulated via β1-adrenoceptors: thus, while CGP-20712 potently inhibited ISO-induced cAMP accumulation (Ki = 0.54nM), ICI-116551, even at 1μM, caused <40% inhibition (Fig 2F). We conclude that in our rat astrocyte cultures NA induces glycogenolysis predominantly via β1-adrenoceptors.

3.2 α2-adrenoceptors do not modulate NA-induced astrocytic glycogenolysis.

In many cellular systems where cAMP mediates a physiological response, agonist dose-response curves for the latter are left-shifted relative to the former. This was also the case with our cultured astrocytes, where glycogenolysis was stimulated to 73% of maximum by ISO concentrations that induced <10% of the maximal cAMP response (Fig 3A). α2-adrenoceptors couple to Gi proteins that inhibit adenylyl cyclase activity. In the presence of the α2-adrenoceptor *agonist* dexmedetomidine (DEX) the cAMP response to ISO was right-shifted and the maximum reduced (Fig 3B). NA stimulated cAMP production to levels approximately half of those induced by ISO. However, in the presence of the general α-adrenoceptor *antagonist,* phentolamine, maximum cAMP levels evoked by NA were doubled (Fig 3C): this is consistent with phentolamine blocking NA-activation of α2-adrenoceptors. Thus, low concentrations (0.03 to 0.1μM) of NA primarily activate β-adrenoceptors and increase cAMP, while at concentrations ≥1μM, α2-adrenoceptor-mediated reduction of cAMP becomes significant. Consequently, at high NA concentrations, a stimulatory effect of phentolamine on cAMP levels is apparent. These observations indicate that our cultured astrocytes express functional β1- and α2-adrenoceptors .

To investigate whether α2-adrenoceptor activation modulates astrocytic glycogen metabolism we induced glycogenolysis by a sub-maximal concentration of ISO (10nM) and showed that this was reversed in a dose-dependent manner by DEX (Fig 3D). Thus, the small increase in cAMP evoked via β-adrenoceptors by 10nM ISO was reversed by DEX-induced Gi-activation via α2-adrenoceptors, and consequently

glycogenolysis was inhibited. Moreover, between a period of 1 - 2h, DEX (100nM) also stimulated *basal* levels of astrocytic glycogen in a time-dependent fashion (Fig 3E). This observation suggests that basal levels of cAMP contribute to ongoing glycogen turnover in cultured astrocytes. (Later in this study (Fig 6) we present evidence that these astrocytes have constitutively-active β1-adrenoceptors that may contribute to basal cAMP production).

The foregoing observations indicate that functional α2-adrenoceptors are present in cultured astrocytes and that, under particular experimental conditions, their activation can be observed to modulate glycogen turnover. However, blocking these receptors with phentolamine had no significant effect on NA-evoked glycogenolysis (Fig 3F). This is because glycogenolysis was maximally-induced by relatively low concentrations (0.1 – 1μM) of NA, lower than required for α2-adrenoceptor activation of Gi and inhibition of adenylyl cyclase. The effect of an α2-adrenoceptor *agonist* (DEX) that, by activating Gi, can reduce further the low levels of cAMP required to stimulate glycogenolysis, contrasts with the lack of effect of an α2-adrenoceptor *antagonist* (PA) that does not alter glycogenolysis, which is already is stimulated maximally by high concentrations of NA.

3.3 α1-adrenoceptor-induced increase in cytosolic Ca^{2+} does not mediate NA-induced glycogenolysis in cultured astrocytes.

α1-adrenoceptors couple to Gq proteins and increase cytosolic Ca^{2+} by inducing its release from the endoplasmic reticulum. In hepatocytes, α1-adrenoceptor activation induces glycogenolysis via increased cytosolic Ca^{2+} [13]. However, in our astrocyte cultures, while NA (1μM) and 25mM extracellular K^+ induced glycogenolysis, the α1-adrenoceptor agonist A61603 (1μM) did not (Fig 4A).

High extracellular K^+ induces cell depolarisation and Ca^{2+} influx via plasma membrane voltage-gated calcium-channels, but thapsigargin elevates cytosolic Ca^{2+} via inhibition of the endoplasmic reticulum membrane calcium pump. Muller et al [12] have shown that thapsigargin induces glycogenolysis in mouse astrocytes. We confirmed that in our rat astrocytes, glycogen breakdown could be induced, albeit relatively transiently, by thapsigargin (Fig 4B).

To determine whether functional α1-adrenoceptors were present in our cultured astrocytes we demonstrated increases in cytosolic Ca^{2+} induced both by NA (Fig 4C), (in a manner blocked by the α1-adrenoceptor antagonist prazosin (Fig 4D)), and by both A61603 and thapsigargin (Fig 4E). Thus, activation of α1-adrenoceptors in our astrocyte preparations induces increased cytosolic Ca^{2+} but not glycogenolysis.

Rapid desensitisation of astrocytic Ca^{2+} responses to α1-adrenoceptor activation has been reported in mouse neocortical slice preparations [14]. To investigate possible α1-adrenoceptor desensitisation in our astrocyte cultures, cells were perfused successively with A61603 at 10nM, then 1μM, and then 10nM (Fig 4F). Under these conditions the second response at 10nM A61603 was significantly less ($P = 0.012$) than the first, suggesting α1-adrenoceptor desensitisation (Fig 4F).

Taken together the foregoing observations indicate that glycogenolysis in our rat astrocyte cultures can be induced, as would be expected, by sustained increases in cytosolic Ca^{2+} by influx via plasma membrane voltage-gated calcium channels, but not by α1-adrenoceptor-induced calcium release from the endoplasmic reticulum. Depletion of calcium stores may contribute to the transient nature of thapsigargin-induced glycogen breakdown and calcium store depletion plus receptor desensitisation to the failure of α1-adrenoceptor activation to cause glycogenolysis.

3.4 Effect of NA on incorporation of glucose by cultured astrocytes into glycogen and extracellular lactate.

The experiments described above were done by adding NA or other agents to confluent astrocytes that had been incubated overnight (18-20h) in 1ml serum-free DMEM containing glucose at an initial concentration of 5.5mM. A metabolic consequence of this overnight incubation was conversion of ~20% of the original (5.5μmol) glucose into extracellular lactate. Thus, a disappearance of 1.15 $\pm$ 0.24μmol glucose was matched by formation of 2.56 $\pm$ 0.34μmol lactate (determinations from 4 independent astrocyte isolations). Since 1mol glucose generates 2mol lactate via glycolysis, this represents a near to stoichiometric conversion.

To compare the incorporation of glucose into lactate and glycogen and investigate the effect of NA on both processes, we incubated astrocytes overnight in glucose-free DMEM, then changed the medium to fresh 5.5mM-glucose-DMEM and followed accumulation of lactate and glycogen over the next 60 min (Fig 5). Under these conditions, basal extracellular lactate accumulation accelerated over a period of 60min, with values at 20, 40 and 60 min of 28 $\pm$ 9, 93 $\pm$ 10, and 295 $\pm$ 52 nmol glucose-equivalents (Glc-Eq)/mg protein, respectively (Fig 5A). In the presence of NA, the rate of lactate accumulation was both increased and more linear with corresponding values of 107 $\pm$ 14, 228 $\pm$ 29, and 432 $\pm$ 68 nmol Glc-Eq/mg protein, respectively (Fig 5A). In the presence of CP-316819, mean rates of lactate accumulation at 20 and 40 min were at least doubled compared to basal conditions although these increments were not statistically-significant (Fig 5B). In the presence of CP-316819, NA induced a further significant increase in lactate accumulation at 20 min from 56 $\pm$ 38 to 162 $\pm$ 19 nmol Glc-Eq/mg protein (Fig 5C).

Under these same experimental conditions, basal glucose incorporation into glycogen also accelerated over 60min: at 20, 40, and 60min, values were 6 $\pm$ 12, 28 $\pm$ 8, and 68 $\pm$ 10 nmol Glc-Eq/mg protein, respectively (Fig 4D). In the presence of either NA (Fig 5D) or CP-316819 (Fig 5E), incorporation of glucose into glycogen was prevented. In the presence of CP-316819, NA had no effect on the incorporation of glucose into glycogen (Fig 5F). Given CP-316819's properties as a GP inhibitor ([15]; and see Fig 2A), its inhibition of glucose incorporation into glycogen is perhaps surprising. However, CP-316819 is reported to be a potent GP inhibitor under normoglycaemic, but not hypoglycaemic, conditions [15] and we note that when CP-316819 was added in Fig5E, astrocytes had been incubated overnight without glucose. Moreover, another GP inhibitor, 1,4-di-deoxy-1,4-imino-D-arabinitol, has been reported to inhibit glycogen synthesis in rat hepatocytes albeit 10-fold less potently than its inhibition of GP [16]. We have not investigated the concentration-dependence of inhibition of glycogen accumulation by CP-316819: the significant point is that when 30μM CP-316819 is inhibiting glucose incorporation into glycogen, NA still enhances lactate production at 20min (Fig5C) implying a glycogen-independent mechanism.

To determine the subtype of β-adrenoceptor that mediates NA-induced increase in lactate output before a steady-state of glucose metabolism, astrocytes were glucose-starved overnight before addition of fresh 5.5mM glucose-containing DMEM without or with NA and β1- and β2-antagonists for an additional 40min (Fig 6): at this time-point NA increased lactate output by 37.7 ± 2.5 % over basal values. While even the highest concentration (1μM) of the β2-antagonist, ICI-118551, failed to reduce the NA-induced increase in lactate, the β1-antagonist, CGP-20712 was effective at 0.1 and 1μM, both in the absence (Fig 6A), and presence (Fig 6B) of CP-316819. Thus, all the observed effects of NA on glucose metabolism in our cultured astrocytes were mediated via β1-adrenoceptors.

At 1μM, the β1-antagonist reduced lactate output to less than basal levels (78 ± 6%: P = 0.032, unpaired, 2-tailed Student's t-test; Fig 6A). In anterior pituitary cells, constitutively-active β1-adrenoceptors have been proposed to contribute to basal cAMP levels, and CGP-20712 decreases these by acting as an inverse agonist [17]. Together with our observation that cAMP levels contribute to basal rates of glycogenolysis in cultured astrocytes (Fig 3E), it appears that β1-adrenoceptors in these cells have detectable constitutive activity that can be reduced by CGP-20712 acting as an inverse agonist. Moreover, since, in the presence of CP-316819, 1μM CGP-20712 reduces NA-induced lactate output only to basal level (103 ± 6%; Fig 6B) and not less, it appears that the effect of *constitutively-active* β1-adrenoceptors on lactate output is mediated solely via glycogen turnover.

The data in Fig 5 indicate that the most marked effects of NA on astrocytic lactate output are observed before a steady-state of glucose metabolism is attained. To accurately measure steady-state lactate production we incubated astrocytes overnight in glucose-free-DMEM, then changed the medium to fresh 5.5mM glucose-DMEM and incubated the cells for 60min before adding NA and following lactate output for the next 90min (Fig 7). Under these conditions, lactate accumulation was linear both in the absence and presence of NA with rates of 15.8 ± 0.5 and 21.8 ± 0.8 nmol Glc-Eq/mg protein/min, respectively and these slope values differed significantly (P=0.0032) (Fig 7A). Thus, at a steady-state of glucose metabolism, NA enhanced astrocytic lactate output by 36.7 ± 13.5% (mean and sd of 3 independent experiments;

range 20 – 50%). By contrast, under these same conditions, while NA inhibited glucose incorporation into glycogen, the basal rate of glycogen accumulation was 0.76 ± 0.08 nmol Glc-Eq/mg protein/min, – only 5% of the rate of incorporation of glucose into lactate (compare Fig 7B and 7A). The disparity between an increase in lactate output of ~6nmol (ie, 15.8 to 21.8) Glc-Eq/mg protein/min with a glycogen accumulation rate of ~0.8nmol Glc-Eq/mg protein/min suggests that the dominant mechanism for the NA-induced increase in lactate output is independent of glucose flux through glycogen.

4. Discussion.

The major finding of this study with cultured rat astrocytes is that NA via β1-adrenoceptors increases lactate output predominantly by mechanisms that do not involve glycogen turnover. This is despite the fact that NA also inhibits synthesis, and increases breakdown, of glycogen via β1-adrenoceptors, and that constitutive activity of the latter contributes to the basal rate of glycogen turnover. Although activation of α2-adrenoceptors in our cultured astrocytes diminished cAMP levels, these receptors do not modulate NA-induced glycogenolysis. These observations are consistent with earlier observations in cultured astrocytes showing firstly, by radio-ligand binding assays, that these cells express predominantly β1-adrenoceptors [18], and secondly, that α2-adrenoceptor agonists inhibit ISO-induced, and α2-antagonists enhance, NA-induced cAMP production [19]. Here we have extended these observations to a physiologically-relevant end-point - glycogenolysis. The α2-adrenoceptor agonist DEX evokes an increase in basal glycogen levels presumably by reducing basal cAMP production that, in turn, partially reflects constitutive β1-adrenoceptor activity. However, NA induces maximal glycogenolysis at concentrations below those required to cause inhibition of cAMP production and so, although they are present, α2-adrenoceptors do not influence NA-induced glycogen turnover.

Similarly we found that our astrocyte cultures have functional α1-adrenoceptors that induce increased cytosolic Ca^{2+} in response to NA over the same range of concentrations (maximal at 1μM) as those that induce glycogenolysis. However, although glycogen breakdown in these astrocytes is indeed caused by sustained

increases in cytosolic Ca^{2+}, α1-adrenoceptor activation does not do so, possibly because of receptor desensitisation and depletion of calcium stores.

Two published investigations are relevant to our present observations on the effects of NA on lactate output in cultured astrocytes. Walls et al [6] showed that 100μM NA induced a 3-fold increase in lactate output from mouse cerebellar astrocytes at 30min but not at 60 min. This transient enhancement by NA of glucose conversion to lactate echoes our own findings (Fig 5) even though it was observed under substantially different experimental conditions. More recently, Gutierrez et al [8], using rat cerebrocortical astrocytes reported that addition of NA to fresh medium increased lactate output at 20min by ~50%. Thus, both these reports show that NA can enhance astrocytic lactate output. We have extended these findings by quantifying basal and NA-induced glycogen and lactate turnover both before and after a steady-state of glucose metabolism.

We interpret our observations in terms of the scheme in Fig 1. After overnight glucose-starvation intracellular levels of all glucose-derived metabolites are relatively low. With re-addition of glucose, levels of Glc-6-P and Glc-1-P will rise and both glycolysis and the glycogen shunt pathway will move towards their steady-state activity levels. At times before establishment of the steady-state (~40min) the flux of glucose into glycogen may be relatively high at the expense of flux through glycolysis: at this stage NA, by inhibiting GS activity, will block flux of glucose to glycogen, allowing an increased flux through glycolysis, reflected in 2- to 4-fold increases in lactate output. Analogously, although the values did not reach statistical significance, CP-316819-mediated inhibition of glycogen turnover also showed a tendency to increase lactate production during this pre-steady state period. Subsequently, when glucose metabolism attains a steady-state (~60min), while NA continues to inhibit GS and activate GP, basal glucose incorporation into accumulated glycogen is only 5% of that into lactate: under these conditions the dominant mechanisms for NA-induced enhancement of lactate output are independent of glycogen turnover.

Activation of β-adrenoceptors has been shown to enhance uptake of glucose into rat and mouse astrocyte cultures [20, 21]. The increase in glucose uptake – 30 to 50% - would be sufficient to account for the glycogen-independent NA-induced increase in lactate output that we observed here. However, while Catus et al [21] concluded that increased glucose uptake into their mouse astrocytes was mediated by activation of either β1- or β2-adrenoceptors, NA-stimulated lactate output in our rat cultures was almost entirely via β1-adrenoceptors. Gutierrez et al [8] concluded that β2-adrenoceptor activation was responsible for stimulation of lactate production by their rat astrocytes, but this was based solely on the effect of a very high concentration (100μM) of the β-adrenoceptor agonist, clenbuterol.

The contributions of β1- versus β2-adrenoceptors to the physiological modulation of astrocytic glucose metabolism are still not resolved. In part this is because of differences between astrocytes in the adult brain *in vivo* and those cultured from neonatal brain, or even those in acute slices from adult brain. Both β1-and β2-adrenoceptor mRNAs are expressed in adult brain astrocytes [22], but while functional β1-adrenoceptor cAMP-signalling is evident in the cell bodies [23], β2-adrenoceptor-immunoreactivity is confined to fine processes [24]. This spatial segregation of β1- and β2-adrenoceptors in astrocytes echoes the finding that, in heart, the signalling ability of β2-adrenoceptors is discretely localized, whereas β1-adrenoceptors signal in a diffuse, global manner [25]. Likewise, glycogen granules are located in the fine processes, rather than in the cell bodies, of astrocytes *in vivo* [26, 27]. Astrocyte cellular processes and glycogen are substantially diminished in slices acutely isolated from adult brain [28]. The lack of astrocyte cellular processes in brain slice preparations and in cultured neonatal astrocytes may be accompanied by loss of functional β2-adrenoceptors (even though their mRNA is expressed): this may explain why NA-induced glycogenolysis in both brain slices [29] and in cultured astrocytes (present study) is mediated almost entirely via β1-adrenoceptors. Nevertheless, although caution is clearly required in extrapolating *in vitro* observations to events *in vivo*, our present findings attest to the importance of β1-adrenoceptors and cAMP as mediators of NA stimulation of glycogen-independent glucose metabolism in astrocytes.

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Figure Legends.

Fig 1: Noradrenergic regulation of glucose metabolism in cultured astrocytes. Glucose (Glc) is imported into astrocytes via a glucose transporter (*GLUT*) and phosphorylated to yield glucose-6-phosphate (Glc-6-P) that in turn is metabolized via glycolysis (indicated by a series of 5-arrows) to yield pyruvate (Pyr). The latter is reduced to lactate (Lac), the majority of which is rapidly exported from astrocytes via a monocarboxylate transporter (*MCT*). A fraction of Glc-6-P is isomerized to glucose-1-phosphate (Glc-1-P) and this can be incorporated into glycogen in a reaction catalysed by *Glycogen Synthase* (*GS*). Glycogen can be broken down to Glc-1-P by the action of *Glycogen Phosphorylase* (*GP*). Noradrenaline (NA) acting at *β1-adrenoceptors (AR*) induces formation of cAMP that, via *protein kinase A* (not shown), activates (+) *GP* and inhibits (-) *GS.* Evidence is presented in this study that NA enhances lactate output both by inhibiting glucose flux through glycogen, and through glycogen-independent mechanisms (presumably mediated by cAMP) with rate-limiting step(s) at some point in the pathway within the broken lines.

Fig 2: NA induces glycogenolysis via β1-adrenoceptor in cultured astrocytes. **(A)** NA (1μM) was added at zero-time with either DMSO (0.3% final) or CP-316819 (30μM in 0.3%DMSO) and the change in glycogen at the indicated times (min) was determined. *, $P < 0.05$ (unpaired, 2-tailed Student's t-test) indicates a significant reduction in glycogen versus the value at the same time-point in the presence of CP-316819. **(B)** NA or ISO at the indicated concentrations were added for 60min before determination of glycogen. **(C)** ISO (1μM) was added at zero-time and glycogen content was determined at 30, 60, 120, and 240min of incubation. At 30min, some wells also received the non-selective β-adrenoceptor antagonist, timolol (TIM; 10μM) and glycogen in these wells was determined at 60, 120, and 240min of incubation. *, ($P<0.05$) and ** ($P<0.01$), indicate values significantly different to zero-time (unpaired, 2-tailed Student's t-test). **(D)** Reverse-transcriptase PCR of RNA from cultured astrocytes was performed as in Methods. The expected sizes (bp) of the PCR products were: β1-adrenoceptor (-AR), 370; β2-adrenoceptor (-AR), 330; GAPDH, 92. **(E)** Cells were preincubated (30min) with the indicated concentrations of β-adrenoceptor antagonists before addition of NA (1μM) and incubation for a further

60min before glycogen determination. **(F)** Cells were preincubated (30min) with the indicated concentrations of β-adrenoceptor antagonists before addition of ISO (0.1μM) and incubation for a further 10min before cAMP was determined.

Fig 3: α2-adrenoceptors in cultured astrocytes mediate reduced cAMP but do not modulate NA-induced glycogenolysis.

(A).cAMP and glycogen were determined after incubation with ISO for 10 min and 60 min, respectively. **(B)** Cells were pre-incubated (10 min) without or with the α2-agonist dexmedetomidine (DEX; 300nM) before incubation for a further 10 min with the indicated concentrations of isoprenaline and subsequent determination of cAMP. **(C)** Cells were incubated (10min) with agonists at the indicated concentrations before determination of cAMP. When present, the α2-antagonist, phentolamine (PA) was 10μM. **(D).** Cells were incubated (60min) with a sub-maximal concentration (10nM) of ISO plus the indicated concentrations of the α2-agonist, DEX, before determination of glycogen. **(E)** Cells were incubated with 100nM DEX for the times indicated before determination of glycogen. **, P<0.01 (unpaired, 2-tailed Student's t-test) indicates significant increase over zero-time value. **(F).** Cells were incubated (60min) with NA at the indicated concentrations in the absence or presence of PA (10μM) before determination of glycogen.

Fig 4: NA-induced cytosolic Ca^{2+} increase via α1-adrenoceptors does not evoke glycogenolysis in cultured astrocytes.

(A): Cells were incubated with either the α1-adrenoceptor agonist A61603 (1μM) or with KCl (25mM) for the indicated times (10, 30, 60 min) or NA (1μM; for 60 min) before determination of glycogen. **(B):** Cells were incubated with thapsigargin (Tg, 2μM) for the indicated times (10, 30, 60, 90, 120 min) or with NA (1μM for 60 min) before determination of glycogen. **, P<0.01, ***, P < 0.001 (unpaired, 2-tailed Student's t-test) indicates significant difference from zero-time value (mean and sd from 3 (A) or 5 (B) experiments). **(C-F):** Cells, loaded with Fluo-4 for fluorescent indication of changes in cytosolic Ca^{2+} (see Methods), were perfused for the indicated periods with drugs at the indicated concentrations. **(F):** Areas under curves for fluorescence data for 15 individual cells were determined for the first (4.11 ± 1.35)

and second (2.66 $\pm$ 1.57) responses to 10nM A61603; these were significantly different P = 0.012 (two-tailed, unpaired t-test).

Fig 5: Pre-steady-state glucose metabolism in cultured astrocytes: effects of NA and CP-3168198.

Astrocytes were incubated overnight in glucose-free DMEM. Fresh 5.5mM-glucose-DMEM was then added with or without NA (1μM) or CP-316819 (30μM) and incubation was continued for up to 60min. Extracellular lactate **(A-C)** and intracellular glycogen **(D-F)** were determined at the times indicated (min) and expressed as glucose-equivalents (Glc-Eq). Note 10-fold different scale for **(A-C)** vs **(D-F).** *, P <0.05; **, P <0.01 (unpaired, 2-tailed Student's t-test) indicates significant effect of NA versus basal **(A and D)**, of CP316819 versus basal **(E),** and of NA + CP316819 versus CP316819 only **(C).**

Fig 6: Effects of NA on lactate output before glycolytic steady-state in cultured astrocytes are mediated via β1-adrenoceptors.

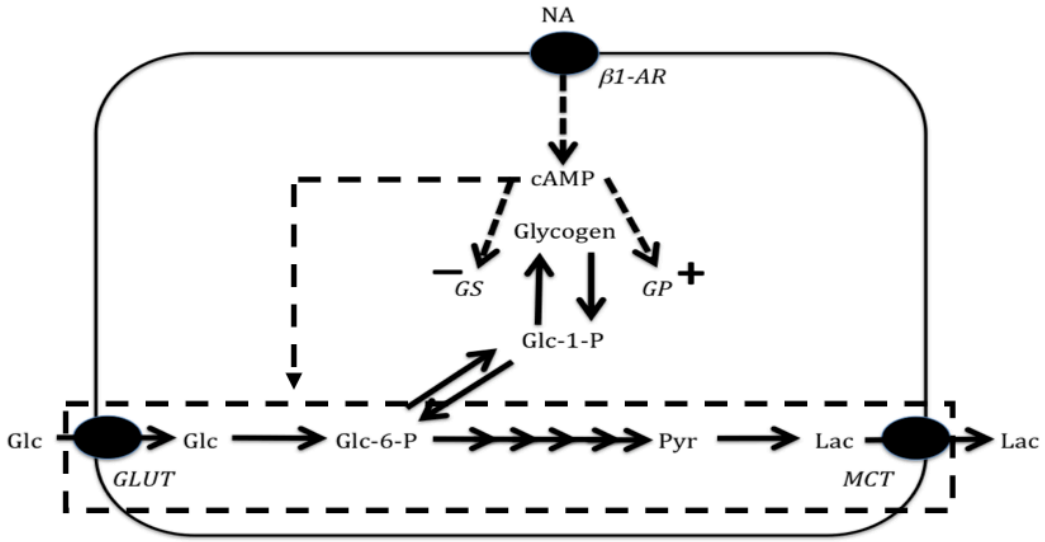
Astrocytes were glucose-starved overnight before addition of fresh 5.5mM glucose-DMEM with or without NA (1μM), and β1- and β2-antagonists (CGP-20712 and ICI-118551) at the concentrations indicated, and **(A, C)** DMSO (0.3%) or **(B, D)** CP-316819 (30μM). Incubations were terminated at 40min and extracellular lactate was determined. Data are expressed relative to values for (basal) lactate in the absence of NA (indicated by the dashed line). **, P <0.01; ***, P <0.001 (unpaired, 2-tailed Student's t-test) indicates significantly different to value in presence of NA without antagonist. *, P<0.05, indicates value significantly less than basal lactate level.

Fig 7: At glycolytic steady-state NA increases lactate output in cultured astrocytes.

Astrocytes were incubated overnight in glucose-free DMEM. Fresh 5.5mM-glucose-DMEM was then added and incubation continued for 60min. At that point (zero-time in Figs) NA (1μM) or vehicle was added and incubation continued for the times indicated. Extracellular lactate **(A)** and intracellular glycogen (**B**) were determined and expressed as glucose equivalents. Data are means and sd from 3 independent experiments Lines were fit to data-points by *linear regression* analysis (**A**, basal R^2=0.998, NA R^2=0.997: **B**, basal R^2=0.976, NA R^2=0.758). In **B**, while the basal

slope (0.756 $\pm$ 0.084) differs significantly (P = 0.012) from zero, the slope in the presence of NA (-0.549 $\pm$ 0.219) does not differ significantly (P = 0.12) from zero.

Jiang etal: FIG 1.



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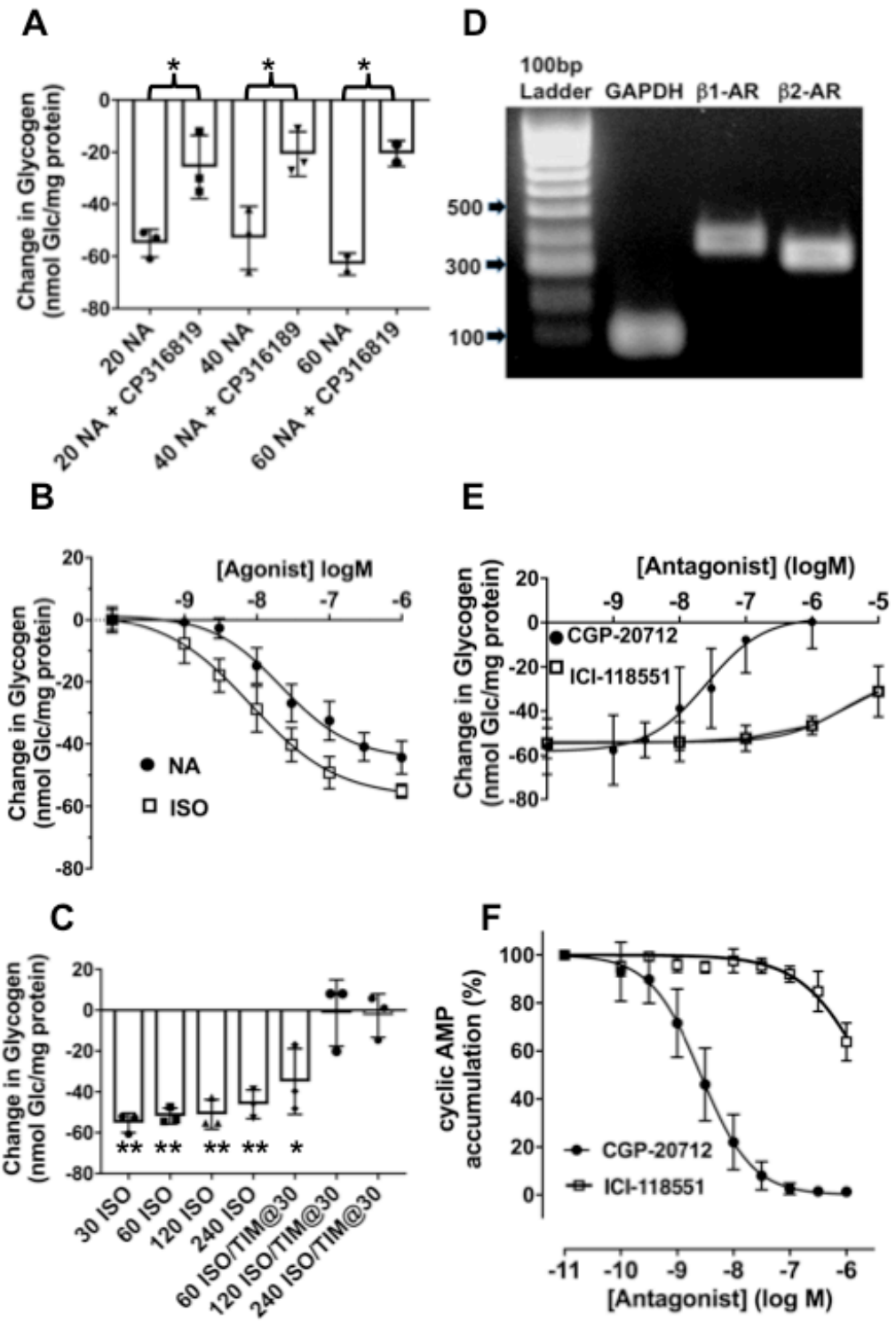

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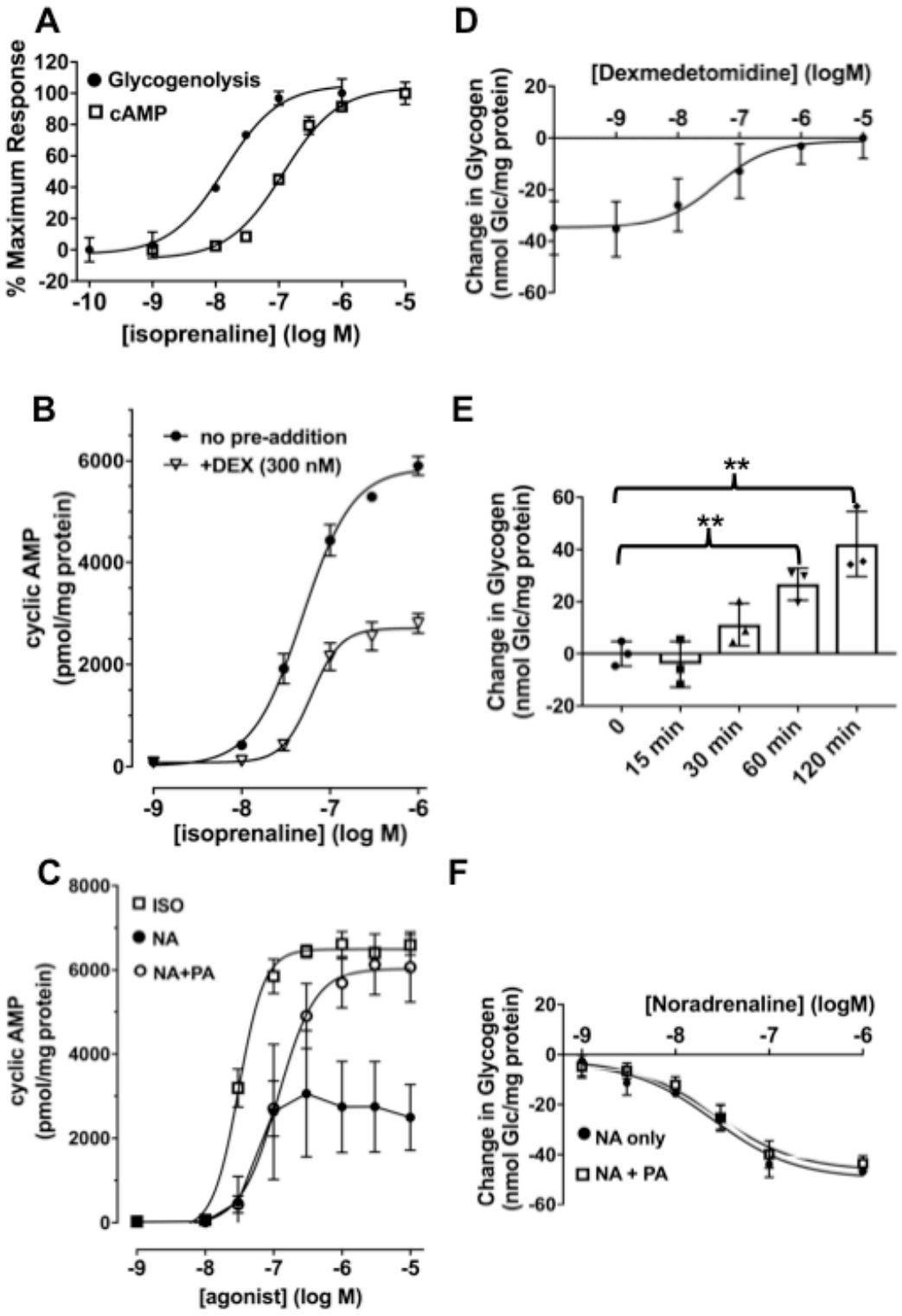

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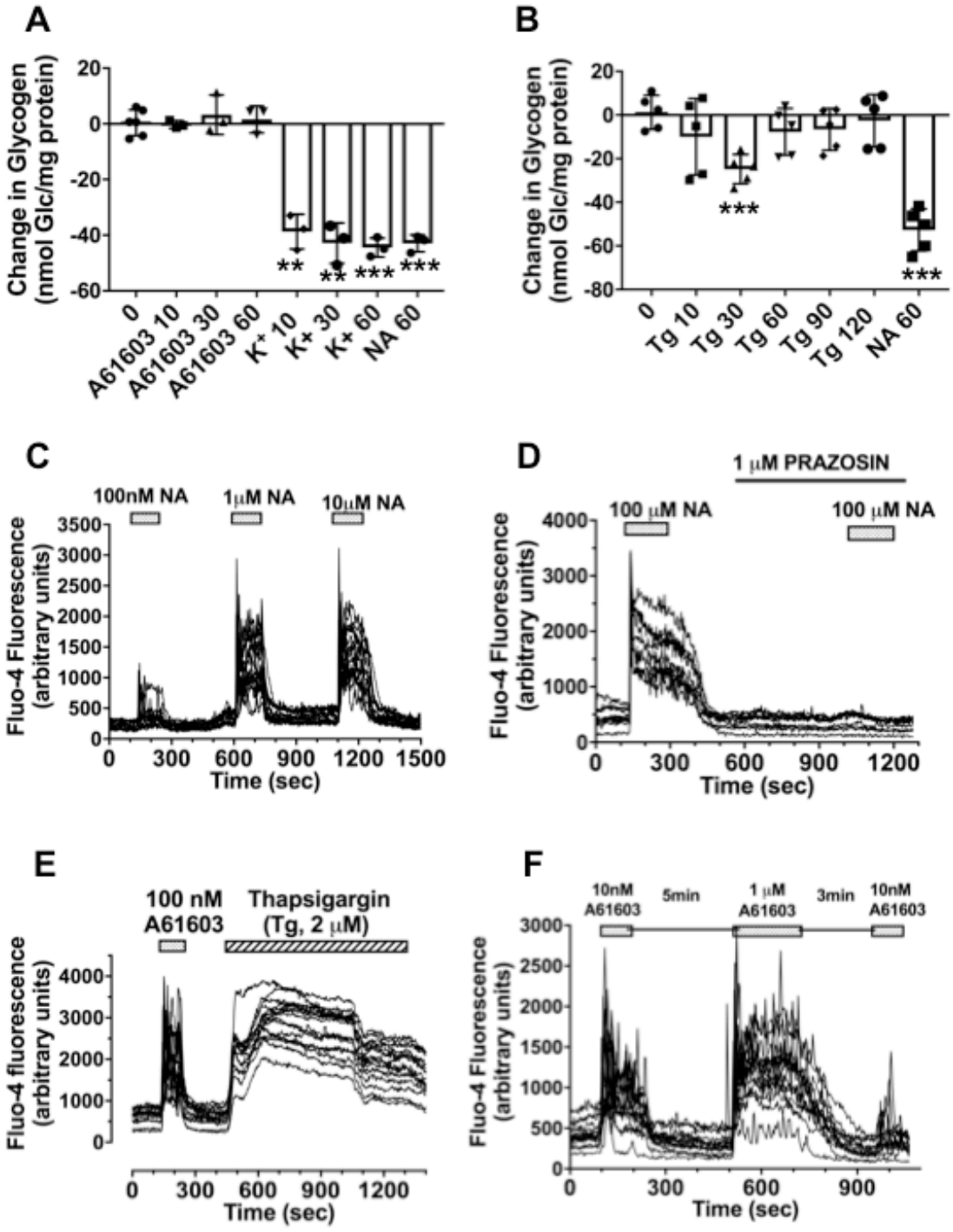

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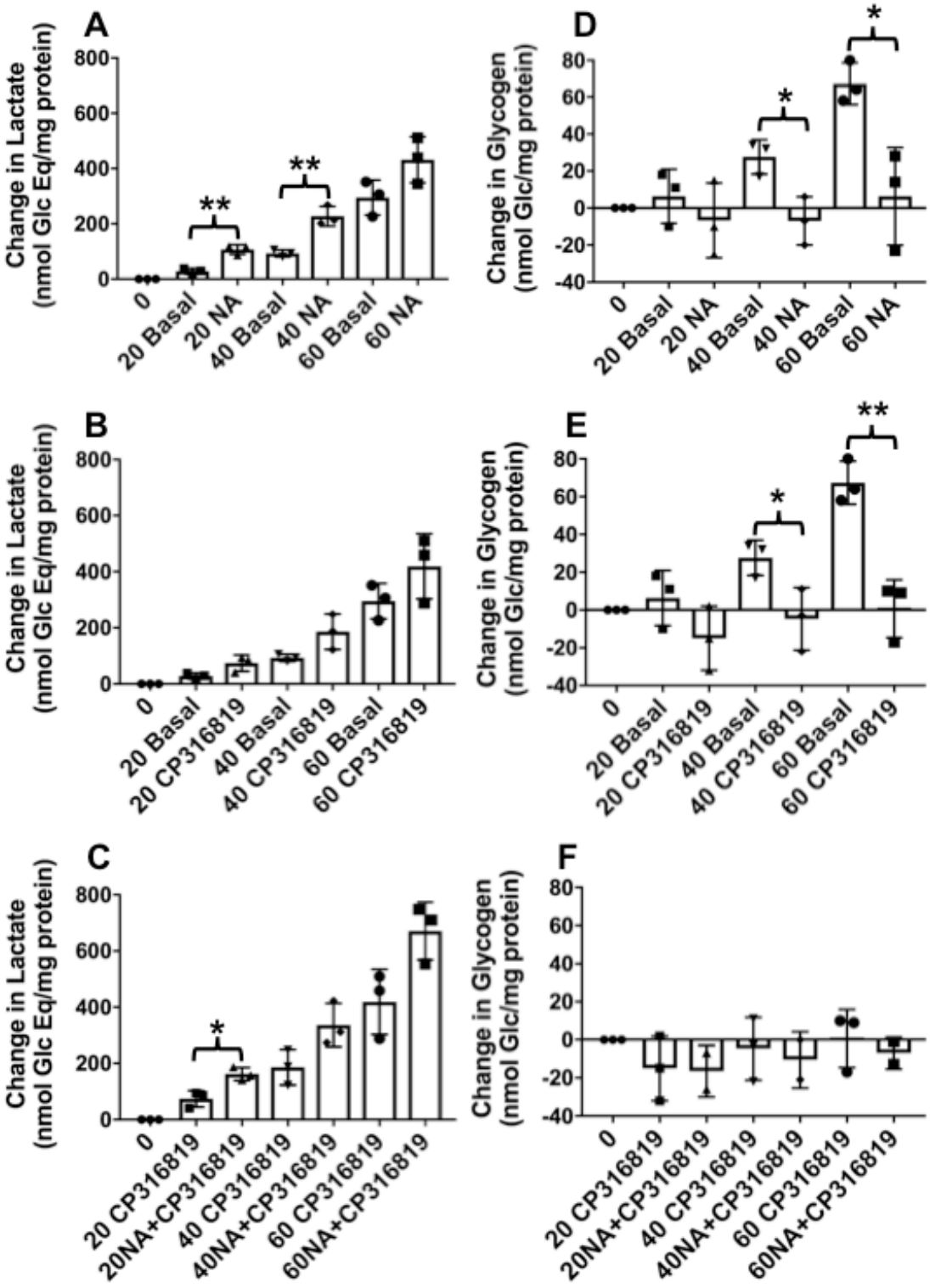

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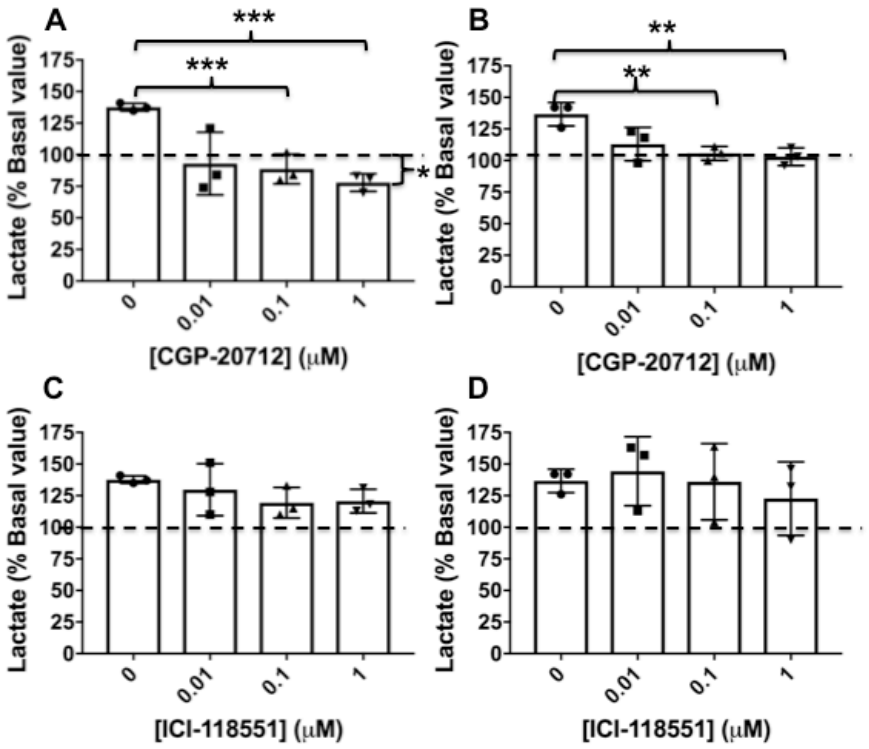

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