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RESEARCH PAPER

Defining protein kinase/phosphatase isoenzymic regulation of $mGlu_5$ receptor-stimulated phospholipase C and Ca^{2+} responses in astrocytes

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BACKGROUND AND PURPOSE

Cyclical phosphorylation and dephosphorylation of a key residue within the C-terminal domain of the activated type 5 metabotropic glutamate ($mGlu_5$) receptor is believed to cause the synchronous, oscillatory changes in inositol 1,4,5-trisphosphate and Ca^{2+} levels observed in a variety of cell types. Here, we have attempted to better define the kinase and phosphatase enzymes involved in this modulation.

EXPERIMENTAL APPROACH

Ca^{2+} and [^{3}H]inositol phosphate ([^{3}H]IP_x) measurements in astrocyte preparations have been used to evaluate the effects of pharmacological inhibition of protein kinase C (PKC) and protein phosphatase activities and small interfering RNA-mediated specific PKC isoenzymic knock-down on $mGlu_5$ receptor signalling.

KEY RESULTS

Ca^{2+} oscillation frequency or [^{3}H]IP_x accumulation in astrocytes stimulated by $mGlu_5$ receptors, was concentration-dependently decreased by protein phosphatase-1/2A inhibition or by PKC activation. PKC inhibition also increased [^{3}H]IP_x accumulation two- to threefold and changed the Ca^{2+} response into a peak-plateau response. However, selective inhibition of conventional PKC isoenzymes or preventing changes in $[Ca^{2+}]_i$ concentration by BAPTA-AM loading was without effect on $mGlu_5$ receptor-stimulated [^{3}H]IP_x accumulation. Selective knock-down of PKCδ was without effect on glutamate-stimulated Ca^{2+} responses; however, selective PKCε knock-down in astrocytes changed Ca^{2+} responses from oscillatory into peak-plateau type.

CONCLUSION AND IMPLICATIONS

These data confirm the acute regulation of $mGlu_5$ receptor signalling by protein kinases and protein phosphatases and provide novel data pinpointing the isoenzymic dependence of this regulation in the native $mGlu_5$ receptor-expressing rat cortical astrocyte. These data also highlight a potential alternative mechanism by which $mGlu_5$ receptor signalling might be therapeutically manipulated.

Abbreviations

CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DAPI, 4′,6-diamidino-2-phenylindole; DHPG, (*S*)-3,5-dihydroxyphenylglycine; GFAP, glial fibrillary acidic protein; [^{3}H]IP_x, [^{3}H]inositol mono-, bis- and trisphosphate fraction; mGlu, metabotropic glutamate; PDBu, phorbol 12,13-dibutyrate; PP1/PP2A, protein phosphatase-1/2A; PP2B, protein phosphatase 2B

Introduction

Astroglia were originally considered to play a solely homeostatic role in the brain, providing neurones with the correct environment in which to fulfil their many roles. However, the idea that astrocytes play a crucial part in signalling within the CNS has now emerged, with evidence to show that this type of glial cell can communicate with other astrocytes and also with neurones, giving rise to the concept of the 'tripartite synapse' (Pasti *et al.*, 1997; Allen and Barres, 2009). Astrocytes express an array of G protein-coupled receptors (GPCRs), including adrenoceptors and muscarinic, 5-hydroxytryptaminergic, glutamatergic, purinergic and GABAergic receptors (Porter and McCarthy, 1997; Agulhon *et al.*, 2008).

Glutamate is the major excitatory neurotransmitter in the CNS, and its effects are mediated by activation of ionotropic and metabotropic glutamate (mGlu) receptors. Eight receptor subtypes constitute the $mGlu_{1-8}$ receptor family (Conn and Pin, 1997; receptor nomenclature follows Alexander *et al.*, 2009), with a number of studies providing evidence for $mGlu_3$ and $mGlu_5$ receptors being the major subtypes expressed in glial cells (Miller *et al.*, 1995; Cai *et al.*, 2000; Aronica *et al.*, 2003). $mGlu_5$ receptors couple primarily via $G_{q/11}$ proteins to stimulate increases in phospholipase C (PLC) activity leading to characteristic oscillatory changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via a mechanism dependent on protein kinase C (PKC) (Kawabata *et al.*, 1996; Nakahara *et al.*, 1997). The mechanism by which $mGlu_5$ receptor activation leads to the generation of Ca^{2+} oscillations has been investigated, predominantly in recombinant cell lines, and is termed 'dynamic uncoupling' (Nash *et al.*, 2002). The process of dynamic uncoupling involves rapid and repeated cycles of phosphorylation and dephosphorylation of Ser^{839} (Kim *et al.*, 2005). Phosphorylation of this residue by PKC is thought to uncouple the receptor from its G protein terminating PLC activation, with rapid reactivation occurring through Ser^{839} dephosphorylation by an as yet poorly defined protein phosphatase activity (Nakahara *et al.*, 1997). Thus, repeated cycles of phosphorylation and dephosphorylation of the $mGlu_5$ receptor following agonist activation result in the characteristic Ca^{2+} oscillatory responses observed (Nash *et al.*, 2001, 2002; Atkinson *et al.*, 2006).

Protein kinase C isoenzymes have been classified into three subfamilies: conventional PKCs (cPKC, e.g. α, β and γ) which are dependent for activation on Ca^{2+} and diacylglycerol (DAG), novel PKCs (nPKC, e.g. δ, ε, η and θ) which are independent of Ca^{2+}, but dependent on DAG, and atypical PKCs (aPKC, e.g. ζ and λ) which are independent of both Ca^{2+} and DAG (Parker and Murray-Rust, 2004). The precise PKC isoenzymes involved in $mGlu_5$ receptor–Ca^{2+} dynamic uncoupling are not yet specified and there is evidence for the expression in astrocytes of an array of PKC isoenzymes, including representatives of the conventional, novel and atypical subfamilies (Rudkouskaya *et al.*, 2008). Codazzi *et al.* (2001) have provided evidence for the involvement of conventional PKC isoenzymes in the generation of glutamate-induced Ca^{2+} oscillations. This group also showed that the oscillatory increases in intracellular Ca^{2+} and DAG led to conventional PKCs translocating from the cytoplasm to the plasma membrane. In contrast to these findings, in other studies, Ca^{2+}-independent isoenzymes of PKC have been reported to phosphorylate the $mGlu_5$ receptor to uncouple it from PLC activity (Uchino *et al.*, 2004). While the mechanism(s) underlying $mGlu_5$ receptor-induced Ca^{2+} oscillations has been investigated quite widely in recombinant systems without consensus, little has been carried out to elucidate this fundamental mechanism in a cell type endogenously expressing the $mGlu_5$ receptor. In the present study we provide new pharmacological and molecular genetic evidence for the involvement of dynamic uncoupling in the generation of Ca^{2+} oscillations in rat cortical astrocytes and define the relative isoenzymic kinase(s) and phosphatase(s) activities involved in this cycle.

Methods

Astrocyte preparation and culture

All animal care and experimental procedures complied with the UK Animal (Scientific Procedures) Act 1986 and conformed to the Guide for Care and Use of Laboratory Animals US (NIH Publication No. 85-23, revised 1996). Cortical astrocytes were prepared as described previously (Bradley *et al.*, 2009). In brief, Wistar rats (1–2 days of age) were decapitated and the cortices removed. Tissues were digested with trypsin [bovine pancreatic; 0.025% (w/v)] and dissociated by trituration with fire-polished glass pipettes. The cell suspension was centrifuged (170 xg; 8 min), and pellet resuspended in Dulbecco's minimum essential medium (DMEM) containing GlutaMAX-1 with sodium pyruvate, 4500 $mg \cdot L^{-1}$ glucose, 15% heat-inactivated FBS, 2.5 $\mu g \cdot mL^{-1}$ amphotericin B and 0.1 $\mu g \cdot mL^{-1}$ gentamicin. Cells were seeded into poly-D-lysine-coated cell culture flasks and incubated at 37°C in a humidified air: 5% CO_2 atmosphere for 7 days, with medium being replaced after 4 days. On the 7th day *in vitro* (DIV 7), medium was replaced again and flasks were transferred to a shaking incubator overnight (37°C; 320 r.p.m.). On the following day (DIV 8), cells were washed twice with phosphate-buffered saline (PBS without Ca^{2+}/Mg^{2+}) and harvested with 0.25% (w/v) trypsin, 0.02% (w/v) EDTA. Cells were subsequently seeded onto pre-coated poly-D-lysine tissue culture plates for experiments. After 24 h (DIV 9), medium was replaced with DMEM containing GlutaMAX-1 with sodium pyruvate, 4500 $mg \cdot L^{-1}$ glucose, 2.5 $\mu g \cdot mL^{-1}$ amphotericin B and 0.1 $\mu g \cdot mL^{-1}$ gentamicin and G5 supplement. Cells were used for experiments at DIV 11–13.

Immunocytochemistry

Rat cortical astrocytes were seeded at 3×10^5 cells per well onto 22 mm borosilicate coverslips in culture medium and the following day medium was changed to medium containing G5 supplement (where necessary). After 48 h medium was removed, cell monolayers were washed with PBS (without Ca^{2+}/Mg^{2+}) and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were briefly washed in PBS and permeabilized in PBS-Triton X-100 (0.01%) containing 1% BSA, for 1 h at room temperature. After three 5 min washes, cells were incubated with a 1:100 dilution of an $mGlu_5$ receptor antibody in PBS overnight at 4°C. The $mGlu_5$ receptor antibody was washed off with PBS

and cells were incubated with a 1:100 dilution of an antibody for glial fibrillary acidic protein (GFAP) in PBS at room temperature for 1 h. Following three further 5 min PBS wash steps, cells were incubated at room temperature for 1 h with a 1:400 dilution of secondary antibodies (Alexa Fluor® 488 and Alexa Fluor® 647). Coverslips were washed in PBS and mounted onto glass microscope using *Slowfade*® gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI). Coverslips were maintained at 4°C in darkness, until analysis using an Olympus FV500 confocal microscope (Olympus, Tokyo, Japan).

Western blot analysis

Rat cortical astrocytes were seeded in poly-D-lysine-coated 60 mm dishes in culture medium and the following day medium was changed to medium containing G5 supplement (where necessary). After the indicated time periods, plates were placed on ice and washed twice with ice-cold PBS and cells were solubilized by addition of 150 µL of lysis buffer [20 mM Tris/HCl, pH 7.4, 1% Triton X-100, 10% (v/v) glycerol, 137 mM NaCl, 2 mM EDTA and a 1:100 dilution of a protease inhibitor cocktail]. Samples were collected and centrifuged at 21 000× *g* for 10 min at 4°C. Aliquots of the supernatant (80 µL) were extracted and added to 20 µL of 5× Laemmli sample buffer [300 mM Tris/HCl, pH 6.8, 15% (w/v) SDS, 50% (v/v) glycerol and 50 mM dithiothreitol (DTT)]. Samples were separated by 12.5% SDS-PAGE using the Bio-Rad mini-gel system. Electrotransfer to nitrocellulose membranes was performed using the Bio-Rad semi-dry blotting apparatus in transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol). Non-specific binding sites were blocked by incubation of the nitrocellulose membrane in 5% non-fat milk powder in TBST buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature. Membranes were subsequently incubated overnight at 4°C with primary antibody (dilutions specified in figure legends). Blots were then washed three times for 10 min in TBST and incubated at room temperature for 60 min with the secondary antibody conjugated to horseradish peroxidase (1:1000 dilution in 5% milk/TBST). Following this, blots were washed three times for 10 min in TBST before chemiluminescence detection using ECL reagent and exposure to HyperFilm.

Total [^{3}H]inositol phosphate accumulation assay

Rat cortical astrocytes were seeded at 150 × 10^3 cells per well in poly-D-lysine-coated 24-well plates. The following day, cells were incubated in fresh medium containing G5 supplement and 2.5 µCi·mL^{-1} [^{3}H]inositol for 48 h. Cell monolayers were washed twice and incubated in Krebs-Henseleit buffer (KHB; composition – 118.6 mM NaCl, 4.7 mM KCl, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.7 mM glucose, 10 mM HEPES, pH 7.4) containing glutamic-pyruvic transaminase (GPT; 3 U·mL^{-1}) and pyruvate (5 mM) for 25 min at 37°C. LiCl (10 mM) was added for a further 20 min prior to agonist incubations for 20 min. Reactions were terminated by aspiration of buffer followed by rapid addition of 500 µL of ice-cold trichloroacetic acid (0.5 M). Samples were extracted from each well and mixed with 100 µL of EDTA (10 mM, pH 7.0) and 500 µL of a 1:1 mixture of tri-*n*-octylamine and 1,1,2-trichlorofluoroethane. Samples were centrifuged at 21 000× *g*. for 2 min and 400 µL of the upper aqueous phase was transferred into fresh microfuge tubes containing 100 µL of $NaHCO_3$ (62.5 mM). The [^{3}H]inositol mono-, bis- and trisphosphate fraction ([^{3}H]IP$_x$) was recovered by anion-exchange chromatography on Dowex AG1-X8 formate columns and radioactivity was determined by liquid scintillation counting.

Single-cell intracellular $[Ca^{2+}]_i$ assay

Rat cortical astrocytes were seeded onto 22 mm borosilicate coverslips at 3 × 10^5 cells per well and grown to approximately 80% confluency in medium containing G5 supplement. Cells were loaded with Fura-2 AM (4 µM) in KHB containing pluronic acid F127[2] (0.36 mg·mL^{-1}) for 45–60 min at room temperature. Coverslips were then transferred to the stage of a Nikon Diaphot inverted epifluorescence microscope, with an oil immersion objective (×40) and a SpectraMASTER II module (PerkinElmer Life Sciences). Cells were excited at wavelengths of 340 and 380 nm using a SpectraMASTER II monochromator and emission was recorded at wavelengths above 520 nm. The ratio of fluorescence intensities at these wavelengths is given as an index of $[Ca^{2+}]_i$. All experiments were performed at 37°C; drug additions were made via a perfusion line.

siRNA knock-down of endogenous PKCδ or PKCε levels

Expression of PKC isoenzymes was determined using standard immunoblotting protocols as described above using specific antibodies against PKCδ and PKCε. Rat cortical astrocytes (2 × 10^6) were transfected with 0.5 or 1.5 µg of negative-control, anti-PKCδ or anti-PKCε siRNAs. The anti-PKCδ siRNAs used for endogenous PKCδ knock-down were PKCδ siRNA 1 (#s139243; 5′-GAUUCAAGGUCUAUAACUAtt-3′) and PKCδ siRNA 2 (#s139244; 5′-GCAAGGUACUGCUU GCAGAtt-3′). Anti-PKCε siRNAs used for knock-down of endogenous PKCε were PKCε siRNA 1 (#s218083; 5′-AGCCGAGAAUUAAAACCAAtt-3′) and PKCε siRNA 3 (#s131201; 5′-CUCUAUUGCUGCUUCCAGAtt-3′). Following nucleofection, cells were plated onto poly-D-lysine-coated plates in astrocyte culture medium, which was replaced 24 h later by a similar medium containing G5 supplement. Cells were lysed 48 h later, and expression of PKCδ or PKCε was assessed by immunoblotting (see above). PKC expression was quantified using the GeneGnome image analysis system (Syngene, Cambridge, UK).

Data analysis

Concentration–response relationships were analysed by non-linear regression using GraphPad Prism 5.0 software (San Diego, CA, USA). For statistical tests, where only two datasets were being compared, an unpaired Student's *t*-test (two-tailed) was used, where $P < 0.05$ was deemed statistically significant. Where more than two datasets were compared, one-way or two-way analysis of variance (ANOVA) tests were used with $P < 0.05$ being accepted as significantly different. ANOVA tests were followed by the Bonferroni's or Dunnett's *post hoc* test. All statistical analyses were performed using GraphPad Prism 5.0 software.

Materials

Tissue culture reagents, G5 supplement and Fura-2 AM were from Invitrogen (Paisley, UK). *Myo*-[^{3}H]inositol was from GE Healthcare (Chalfont St. Giles, UK). Glutamate, staurosporine, calyculin A, cantharidin (*S*)-3,5-dihydroxyphenylglycine (DHPG) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were obtained from Tocris Cookson Ltd. (Bristol, UK). Phorbol 12,13-dibutyrate (PDBu) and the antibody raised against glial fibrillary acidic protein (GFAP) were from Sigma-Aldrich Chemical Co. (Poole, UK). Ro 31-8220, Gö6976 and myristoylated PKC 20-28 were obtained from Merck Biosciences (Nottingham, UK). Antibody raised against the C-terminus of $mGlu_5$ receptor was obtained from Millipore (Durham, UK). Antibodies raised against specific PKC isoenzymes were purchased from BD Biosciences (Oxford, UK). Silencer® pre-designed small interfering (si)RNA targeted to PKCδ and PKCε and control siRNA were purchased from Ambion (TX, USA). Rat astrocyte nucleofector® kit was from Lonza (Wokingham, UK). All other chemicals and reagents were purchased from Sigma Chemical Co. (Poole, Dorset, UK).

Results

$mGlu_5$ receptor expression and function in rat cerebrocortical astrocytes

Expression of the $mGlu_5$ receptor has been widely reported in cortical astrocytes (Miller *et al.*, 1995). In our experiments, expression of $mGlu_5$ receptors was detectable but low in DIV 7 glial preparations. Following overnight shaking to dissociate non-astrocyte cell types, G5 growth factor supplement was added and $mGlu_5$ receptor immunoreactivity increased over the subsequent 48–96 h; in contrast, $mGlu_1$ receptor immunoreactivity was not detectable (data not shown). Immunocytochemistry showed that astrocytes have a typically stellate morphology and $mGlu_5$ receptor expression was colocalized in astrocytic processes with GFAP (Figure 1A).

Stimulation of the $mGlu_5$ receptor is known to induce robust oscillatory changes in $[Ca^{2+}]_i$ in astrocytes (Nakahara *et al.*, 1997). At low concentrations of glutamate (3–10 μM) low frequency (~1 oscillation per minute), baseline Ca^{2+} oscillations were observed (Figure 1B,C), whereas at maximally effective glutamate concentrations (≥30 μM) Ca^{2+} oscillations on a raised baseline were observed with frequencies of approximately three oscillations per minute (Figure 1B,C). Neither single-cell Ca^{2+} responses to glutamate (Figure 1D), nor [^{3}H]IP_x responses to quisqualate (Figure 1E) were affected by the presence of the AMPA/kainate receptor antagonist, CNQX.

Protein phosphatase regulation of $mGlu_5$ receptor-mediated Ca^{2+} and [^{3}H]IP_x responses

We explored the effects of two protein phosphatase inhibitors, calyculin A and cantharidin, which are reported to inhibit PP1 and PP2A activities. Both calyculin A (100 nM; 5 min; Figure 2A,C) and cantharidin (100 μM; 15 min; Figure 2B,D) caused marked reductions in the Ca^{2+} oscillation frequency stimulated by glutamate (100 μM). Previously, we have undertaken analyses of Ca^{2+} signalling behaviours in CHO-*lac*-mGlu5a cells (Atkinson *et al.*, 2006; Bradley *et al.*, 2009) classifying responses into four categories [non-responders (NR); single peak (SP), oscillatory (OS), peak-and-plateau (PP)]. Here, we undertook similar analyses to assess the effects of protein phosphatase inhibition on the distribution of single-cell Ca^{2+} responses between these types of response. Following treatment with calyculin A and cantharidin, the number of cells that responded with an oscillatory (OS) Ca^{2+} pattern decreased, while the number of cells that either failed to respond (NR) or responded with only a single peak (SP) response increased (Figure 2E). In addition, Ca^{2+} oscillation frequency was markedly decreased in the subpopulation of astrocytes (≥80%) initially exhibiting this pattern of response (Figure 2F).

Pre-incubation of [^{3}H]inositol-labelled astrocytes with calyculin A (100 nM; 5 min) or cantharidin (100 μM; 15 min) caused concentration-dependent decreases in [^{3}H]IP_x accumulation stimulated by quisqualate (10 μM; Figure 2G,H). In contrast, pre-incubation with the calcineurin inhibitor, FK506 (10 μM; 30 min), had no significant effects on [^{3}H]IP_x accumulation stimulated by quisqualate (10 μM; data not shown).

Effects of altering PKC activity on $mGlu_5$ receptor-mediated responses

Addition of increasing concentrations of the PKC activator, PDBu (0.01–0.1 μM), caused concentration-dependent decreases in single-cell glutamate-evoked Ca^{2+} oscillation frequency and a shift in the distribution of Ca^{2+} responses between the different response categories (NR, SP, OS, PP), such that the number of cells that failed to respond (NR), or to respond with a single transient (SP) were increased (Figure 3A–E). Similarly, PDBu also caused a concentration-dependent inhibition [pIC_{50} (M), 7.65 ± 0.08] of [^{3}H]IP_x accumulation stimulated by quisqualate (10 μM; Figure 3F).

Inhibition of PKC activity using the broad-spectrum PKC inhibitors, staurosporine (3 μM; 15 min) or Ro 31-8220 (10 μM; 15 min), caused ≥2-fold increases in the maximal quisqualate-stimulated [^{3}H]IP_x accumulation, without significant change in the apparent potency of quisqualate to stimulate this response [Figure 4A; pEC_{50} (M) values: control, 6.98 ± 0.06; +staurosporine, 6.95 ± 0.07; +Ro 31-8220, 6.89 ± 0.10]. Time-course experiments showed that quisqualate-stimulated [^{3}H]IP_x accumulations were linear over the 20 min incubation period in the absence and presence of PKC inhibitors (data not shown). The effect of staurosporine or Ro 31-8220 on the maximal [^{3}H]IP_x accumulation stimulated by quisqualate was concentration-dependent (Figure 4B), yielding pEC_{50} (M) values of 6.47 ± 0.09 and 5.61 ± 0.16, for staurosporine and Ro 31-8220 respectively. While activation of PKC activity by PDBu (1 μM) could almost completely suppress quisqualate-stimulated [^{3}H]-IP_x accumulation under control conditions, in the presence of staurosporine or Ro 31-8220 the inhibitory effect of acute PKC stimulation was completely lost (Figure 4C). In addition, the potentiating effect of staurosporine pre-incubation (3 μM; 15 min) on agonist-stimulated [^{3}H]IP_x accumulation appeared to be selective for the $mGlu_5$ receptor in rat cortical astrocytes, as this inhibitor was without effect on the small, but significant,

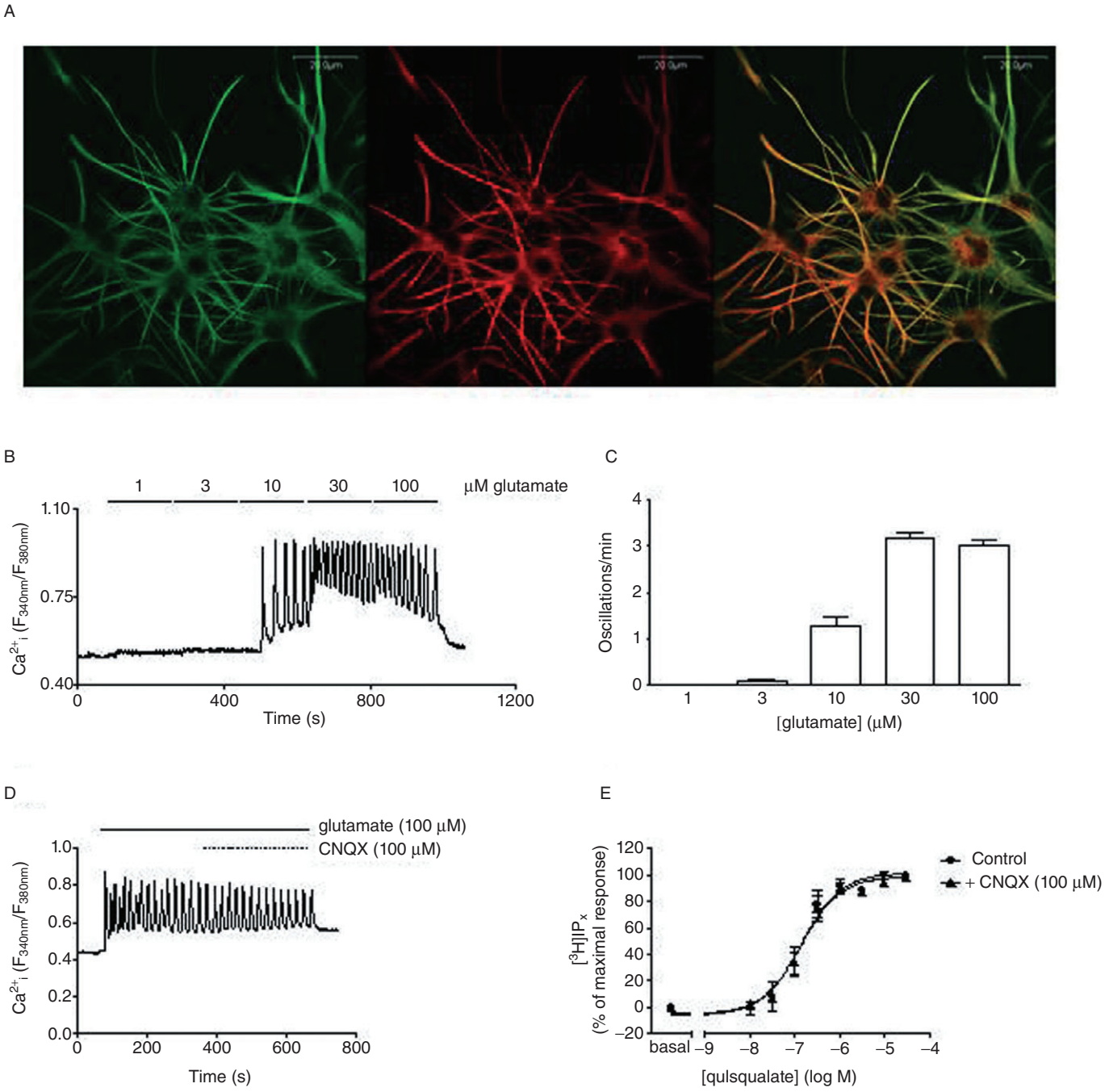
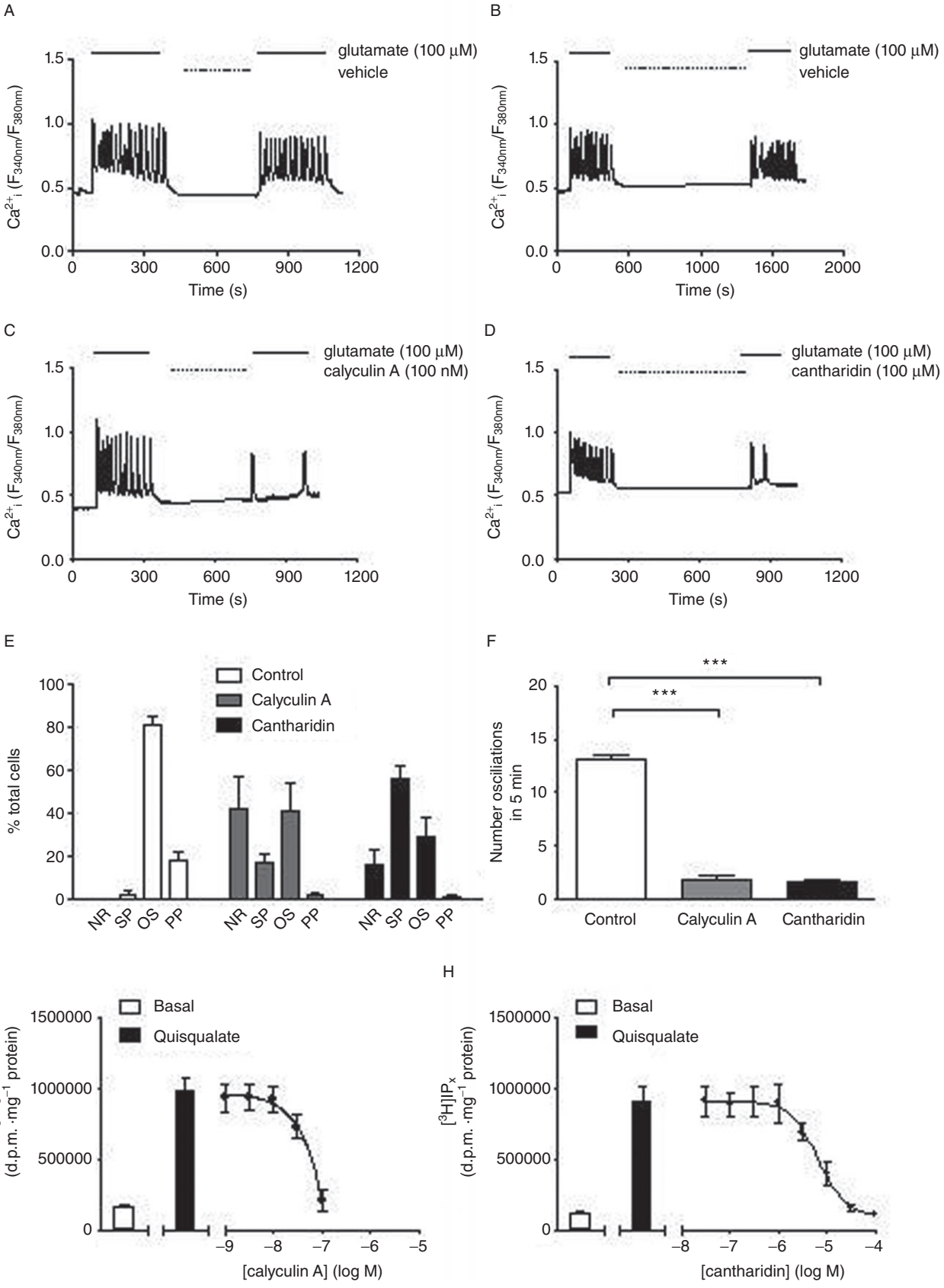


Figure 1

Characterization of $mGlu_5$ receptor expression and signalling in rat cortical astrocytes. Detection of $mGlu_5$ receptor expression in cultured rat cortical astrocytes by immunocytochemistry (A). Double immunostaining was performed, using antibodies raised against the $mGlu_5$ receptor (left panel) or GFAP (centre panel). Image overlay is shown in the right panel. (B) Representative trace showing the effects of increasing glutamate concentration on single-cell Ca^{2+} oscillation frequency in rat cortical astrocytes. Mean data (±SEM for at least 30 cells over at least three separate experiments), showing the number of oscillations in a 3 min period in astrocytes stimulated incrementally with increasing concentrations of glutamate, are shown in panel C. Panels D and E show the effects of the AMPA receptor inhibitor, CNQX, on agonist-stimulated Ca^{2+} responses and $[^3H]IP_x$ accumulations in rat cortical astrocytes. (D) Representative trace showing Ca^{2+} oscillations elicited by glutamate (100 μM) perfusion for 5 min followed by co-perfusion of glutamate (100 μM) with CNQX (100 μM). Data are representative of at least 50 cells over three separate experiments. (E) Concentration–response curves showing quisqualate-stimulated $[^3H]IP_x$ accumulations in cells pre-incubated in the absence or presence of CNQX (100 μM; 15 min): pEC_{50} (M) values (mean ± SEM; three separate experiments performed in duplicate) were 6.86 ± 0.13 and 6.84 ± 0.13 in the absence and presence of CNQX respectively.


A
glutamate (100 μM)
vehicle
Ca2+i (F340nm/F380nm)
Time (s)
B
glutamate (100 μM)
vehicle
Ca2+i (F340nm/F380nm)
Time (s)
C
glutamate (100 μM)
calyculin A (100 nM)
Ca2+i (F340nm/F380nm)
Time (s)
D
glutamate (100 μM)
cantharidin (100 μM)
Ca2+i (F340nm/F380nm)
Time (s)
E
Control
Calyculin A
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% total cells
NR SP OS PP
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***
Number oscillations in 5 min
Control
Calyculin A
Cantharidin
G
Basal
Quisqualate
[3H]IPx (d.p.m. ·mg−1 protein)
[calyculin A] (log M)
H
Basal
Quisqualate
[3H]IPx (d.p.m. ·mg−1 protein)
[cantharidin] (log M)

Figure 2

Effects of protein phosphatase inhibition on agonist-stimulated single-cell Ca^{2+} responses and [^{3}H]IP$_x$ accumulations in rat cortical astrocytes. Representative traces showing the response of single astrocytes to perfusion with glutamate (100 μM) for 5 min followed immediately by the indicated incubation period with vehicle (A, B), 100 nM calyculin A (C) or 100 μM cantharidin (D), followed by a second glutamate (100 μM) perfusion period for 5 min. Data were analysed to assess the effects of each inhibitor on the distribution of glutamate-stimulated Ca^{2+} responses between no-response (NR), single-peak (SP), oscillatory (OS) or peak-plateau (PP) behaviours (E), or for effects on Ca^{2+} oscillation frequency (F). Data are shown as means ± SEM for least 20 individual cells recorded over at least three separate experiments. ***$P < 0.001$, significantly different as shown (unpaired Student's *t*-test). Panels G and H show basal and quisqualate (10 μM)-stimulated [^{3}H]IP$_x$ accumulations in astrocytes (means ± SEM for three to four separate experiments performed in duplicate) following pre-incubation with various concentrations of calyculin A (for 5 min, G) or cantharidin (for 15 min, H). The mean pIC$_{50}$ (M) value for cantharidin inhibition of the quisqualate-stimulated response was 5.15 ± 0.09; a pIC$_{50}$ value was not determined for calyculin A; however, a concentration of 100 nM was sufficient to cause complete inhibition of the agonist-stimulated response.

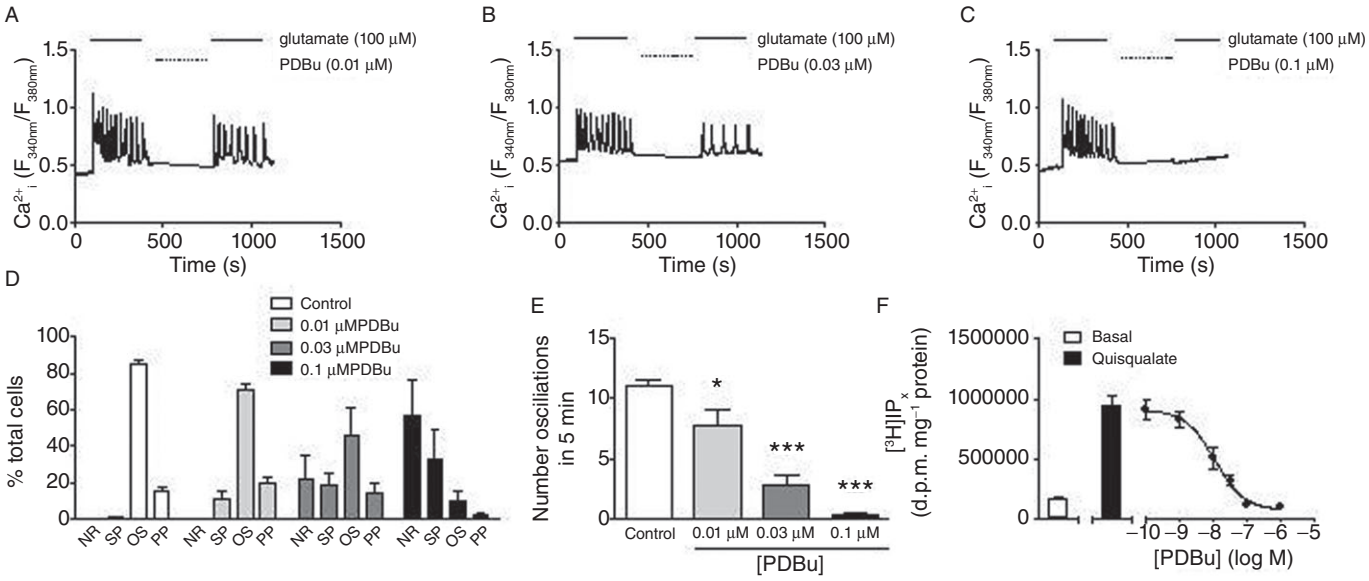

Figure 3

Effects of PKC stimulation on agonist-stimulated Ca^{2+} oscillations and [^{3}H]IP$_x$ accumulation in rat cortical astrocytes. Representative traces showing Ca^{2+} oscillations elicited by glutamate (100 μM) before and after a 5 min incubation period with 10 nM (A), 30 nM (B) and 100 nM (C) PDBu. Panel D shows the distribution of glutamate-stimulated Ca^{2+} responses between no-response (NR), single-peak (SP), oscillatory (OS) or peak-plateau (PP) behaviours in the absence or presence of increasing PDBu concentrations. Panel E shows the mean number of oscillations elicited by glutamate (100 μM) stimulation before and after incubation with 10 nM, 30 nM or 100 nM PDBu. Data are shown as means ± SEM for at least 24 individual cells over three to four separate experiments. *$P < 0.05$, ***$P < 0.001$ significantly different from control (one-way ANOVA; Bonferroni's multiple comparison test). Panel F shows basal and quisqualate (10 μM)-stimulated [^{3}H]IP$_x$ accumulation in astrocytes following pre-incubation for 5 min with the indicated concentrations of PDBu [pIC$_{50}$ (M) = 7.96 ± 0.21; $n = 4$].

[^{3}H]IP$_x$ accumulations stimulated by methacholine (1 mM), noradrenaline (300 μM) or ATP (300 μM) (Figure 4D).

At the single-cell level, glutamate (100 μM) perfusion initiated typical Ca^{2+} oscillatory patterns in 80% of astrocytes analysed; however, following a brief incubation period with staurosporine (3 μM; 15 min), subsequent glutamate addition produced a peak-plateau Ca^{2+} response in 100% of cells (Figure 5A,B).

PKC isoenzymes involved in dynamic uncoupling are independent of [Ca^{2+}]$_i$

To investigate the Ca^{2+} dependence of PKC isoenzymes involved in receptor phosphorylation and hence the generation of mGlu$_5$ receptor-mediated Ca^{2+} oscillations, we initially used a pharmacological approach to inhibit conventional PKC isoenzymes, that is, those that are activated by Ca^{2+} and DAG binding. Pre-incubation with Gö6976 (10 μM; 20 min) or myristoylated PKC 20-28 (10 μM; 30 min) had no effect on the Ca^{2+} oscillation frequency stimulated by glutamate (100 μM) addition (Figure 6A–C), with ≥80% of cells displaying robust Ca^{2+} oscillations following glutamate perfusion in both control and inhibitor-treated cells (data not shown). In addition, pre-incubation with either Gö6976 or myristoylated PKC 20-28 had no significant effect on [^{3}H]IP$_x$ accumulations stimulated by quisqualate (10 μM) addition (Figure 6D). To further explore the dependence of the PKC isoenzyme(s) involved in mGlu$_5$ receptor phosphorylation, we loaded astrocytes with BAPTA-AM (to chelate and prevent changes in [Ca^{2+}]$_i$) and assessed the effect of this manipulation on agonist-stimulated [^{3}H]IP$_x$ accumulation (Figure 6E). BAPTA-AM (100 μM; 30 min) had no significant effect on

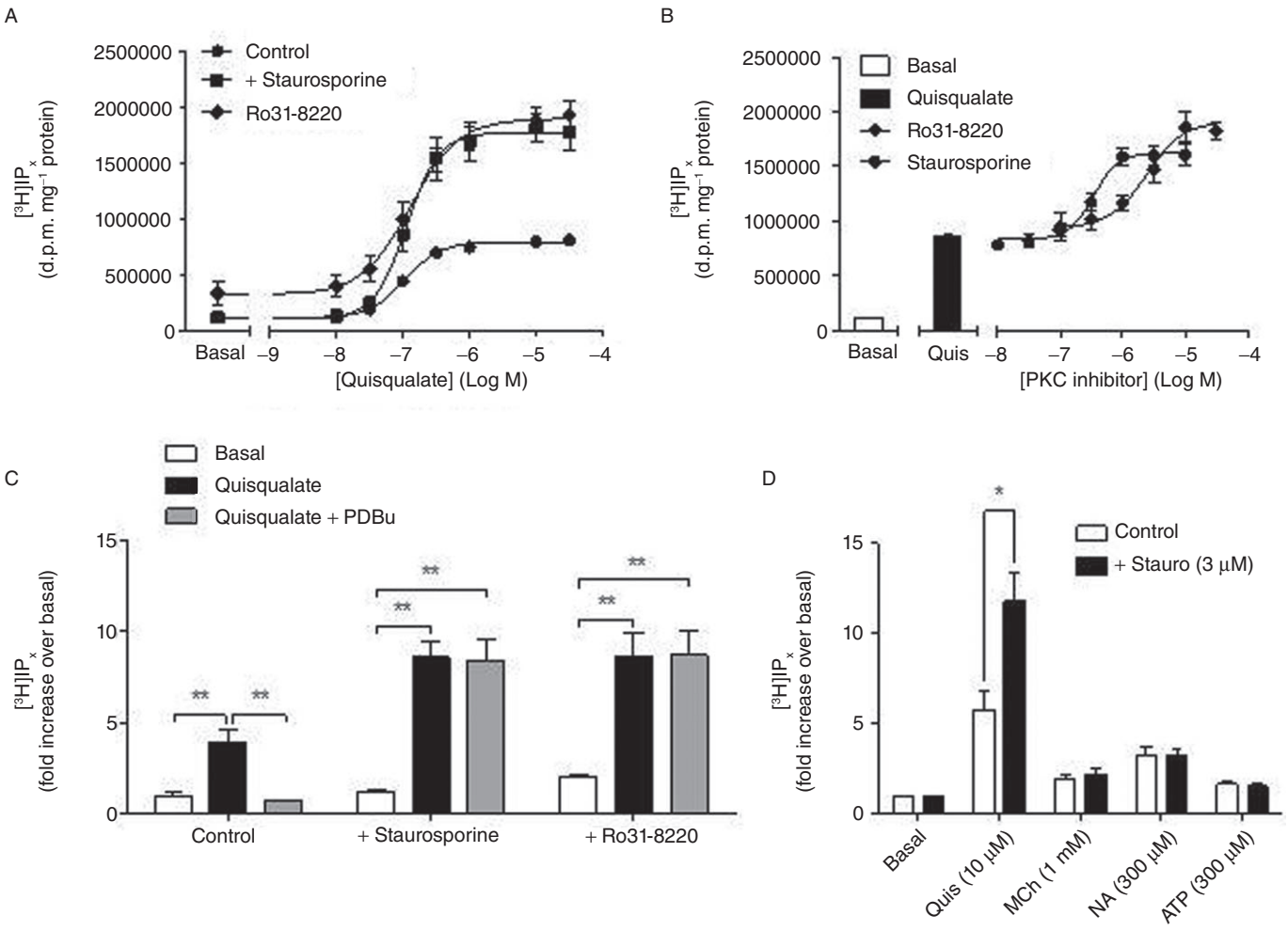

Figure 4

Effects of PKC inhibition on agonist-stimulated $[^3H]IP_x$ accumulation in rat cortical astrocytes. Concentration–response curves (A) for quisqualate-stimulated $[^3H]IP_x$ accumulation in cells pretreated with vehicle (control), staurosporine (3 μM; 15 min) or Ro 31-8220 (10 μM; 15 min): mean (±SEM) pEC_{50} (M) values were 6.97 ± 0.05, 6.93 ± 0.05 and 6.89 ± 0.13 respectively. Concentration-dependence (B) of $[^3H]IP_x$ accumulation for staurosporine (0.01–10 μM) or Ro 31-8220 (0.1–30 μM) pretreated astrocytes stimulated with quisqualate (10 μM): mean pEC_{50} (M) values for potentiation of the quisqualate response by staurosporine or Ro 31-8220 were 6.38 ± 0.10 and 5.33 ± 0.05 respectively. Panel C shows the effects of acute PDBu (1 μM; 5 min pre-incubation) treatment on $[^3H]IP_x$ accumulations stimulated by 10 μM quisqualate in the absence or presence of staurosporine (3 μM) or Ro 31-8220 (10 μM): data are shown as means ± SEM for three to four separate experiments performed in duplicate. $^{**}P < 0.005$ significantly different as shown (one-way ANOVA followed by Bonferroni's multiple comparison test). Panel D shows $[^3H]IP_x$ accumulations stimulated by quisqualate (10 μM), methacholine (MCh) (1 mM), noradrenaline (NA) (300 μM) or ATP (300 μM) in the absence or presence of the broad spectrum PKC inhibitor, staurosporine (3 μM; 15 min pre-incubation). Data shown are means ± SEM for three separate experiments performed in duplicate. Additions of quisqualate, MCh, NA or ATP significantly increased $[^3H]IP_x$ accumulations over basal, but only in the case of quisqualate was there a significant effect ($^*P < 0.05$; paired Student's *t*-test), of staurosporine.

quisqualate-stimulated $[^3H]IP_x$ accumulation [quisqualate concentration–response curve pEC_{50} (M) values: control, 7.02 ± 0.06; +BAPTA-AM, 6.92 ± 0.08]. To demonstrate that $[Ca^{2+}]_i$ is effectively clamped by the BAPTA-AM-loading protocol Ca^{2+} responses to glutamate (100 μM) control and BAPTA-AM-loaded (100 μM; 30 min) cells are also shown (Figure 6E).

Elucidating the PKC isoenzyme(s) involved in $mGlu_5$ receptor-mediated Ca^{2+} oscillations

We assessed the expression of different PKC isoenzymes in astrocyte cultures following differentiation with G5 supplement, using various antisera specific to PKCα, PKCβ, PKCγ, PKCδ, PKCε, PKCθ, PKCι and PKCζ (Figure 7). Of the conventional PKC isoenzymes, PKCα and PKCβ, but not PKCγ, were expressed in our astrocyte cultures. All novel PKC isoenzymes investigated, PKCδ, PKCε and PKCθ, could be detected in our cultures. In contrast, the atypical PKC isoenzymes, PKCι, but not PKCζ, immunoreactivity could be detected.

In the light of our evidence for the involvement of Ca^{2+}-independent PKC isoenzymes, we extended our study next by focusing on the novel PKCs. We transfected astrocytes with siRNAs targeting PKCδ or PKCε to deplete endogenous expression levels: optimal depletion of the relevant target was

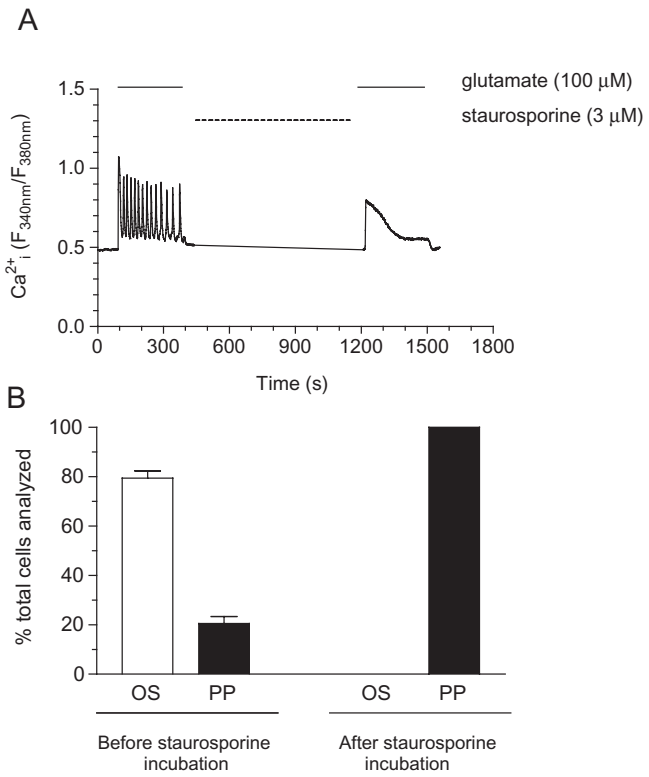

Figure 5

Effects of staurosporine on glutamate-stimulated Ca^{2+} oscillations in rat cortical astrocytes. (A) Representative trace showing Ca^{2+} oscillations elicited by glutamate (100 μM) before and after a 15 min incubation period with staurosporine (3 μM). Panel B shows % of cells displaying oscillatory (OS) or peak-plateau (PP) behaviours to glutamate (100 μM) stimulation, with and without staurosporine addition. Data are shown as means ± SEM from 51 individual cells over three separate experiments.

achieved 72 h after siRNA nucleofection, and this period also allowed sufficient time for differentiation of astrocytes in the G5 supplement. An isoenzyme-specific effect for each anti-PKC siRNA was supported by the lack of effect of anti-PKCε siRNA on PKCδ expression (Figure 8A) and *vice versa* (Figure 8B). A negative-control siRNA had no effect on either PKCδ or PKCε expression (Figure 8A,B). Quantification of the knock-down achieved by each siRNA was assessed densitometrically. The two anti-PKCδ siRNAs tested caused knock-down of their target by approximately 90% and 80%, respectively, and both anti-PKCε siRNAs achieved ≥80% depletion of PKCε (Figure 8A,B).

To examine the functional consequences of isoenzyme-specific depletion of PKCδ or PKCε, we utilized Ca^{2+} imaging to assess the effects of knock-down at a single-cell level. Astrocytes were transfected with programme-only (no siRNA present), negative-control siRNA (0.5 μg) or anti-PKCδ (0.5 μg) siRNAs; after 72 h cells were stimulated with glutamate (100 μM, 5 min; Figure 9A–D). Ca^{2+} responses here were subclassified as oscillatory (OS), oscillatory driving into a sustained peak (OS > PP) or a peak-plateau (PP) Ca^{2+} responses (see Figure 9E right-hand panels for examples of each). In astrocytes nucleofected using programme-only, or with control siRNA, an oscillatory Ca^{2+} response was seen in the majority (≥70%) of cells analysed. Following PKCδ knock-down, the distribution of Ca^{2+} responses between OS, OS > PP and PP on glutamate addition was not altered, with ~70% of all cells analysed displaying an oscillatory Ca^{2+} response (Figure 9E), suggesting that $mGlu_5$ receptor phosphorylation/dephosphorylation is unaffected by a marked (≥80%) loss of cellular PKCδ activity. Quantification of glutamate-stimulated Ca^{2+} oscillation frequencies showed no significant differences between vector-control and anti-PKCδ siRNA-transfected astrocytes (data not shown).

Astrocytes transfected with anti-PKCε siRNAs displayed a range of Ca^{2+} responses with a clear shift in the distribution of (OS, OS > PP and PP) Ca^{2+} responses from oscillatory towards peak-plateau (Figure 10A–G). Summary data (Figure 10G) show that the number of cells displaying an oscillatory Ca^{2+} response was significantly reduced in PKCε-depleted cells (to ≤40% of cells analysed). Co-nucleofection with anti-PKCδ and anti-PKCε siRNAs also reduced the proportion of astrocytes exhibiting peak-plateau responses to glutamate, but the shift from OS to PP was no greater than was seen with anti-PKCε siRNA nucleofection alone (data not shown). Ca^{2+} oscillation frequencies stimulated by glutamate were not significantly different between control siRNA and anti-PKCε siRNA-transfected cells in the respective subpopulations of astrocytes showing this response pattern. These data suggest that the PKCε, but not the PKCδ, isoenzyme was involved in the acute regulation of $mGlu_5$ receptor-mediated Ca^{2+} oscillations in astrocytes.

Discussion

This study was undertaken to elucidate further the mechanisms that underlie $mGlu_5$ receptor-stimulated Ca^{2+} oscillations in rat cerebrocortical astrocytes, initially using a pharmacological approach, and then extending our investigation to include molecular genetic methods. Our new data both confirm and extend previous studies, performed mostly in recombinant cell systems, to uncover the specific protein phosphatase and PKC isoenzymes involved in the regulation of astrocytic $mGlu_5$ receptor–Ca^{2+} signalling.

Although $mGlu_1$ and $mGlu_5$ receptors are structurally related and preferentially couple via $G_{q/11}$ to stimulate PLC activity, these receptors elicit contrasting Ca^{2+} responses at the single-cell level. In contrast to stimulation of $mGlu_1$ receptors, which has, in the main, been reported to cause peak-plateau Ca^{2+} responses (Hermans *et al.*, 1998; Kawabata *et al.*, 1996, 1998; Atkinson *et al.*, 2006; but see Dale *et al.*, 2001), stimulation of the $mGlu_5$ receptor initiates rapid and robust Ca^{2+} oscillations in $mGlu_5$ receptor-expressing recombinant cell lines (Kawabata *et al.*, 1996, 1998; Nash *et al.*, 2002; Atkinson *et al.*, 2006), as well as in native cells, such as astrocytes and neurones (Nakahara *et al.*, 1997; Flint *et al.*, 1999; Codazzi *et al.*, 2001). $mGlu_5$ receptor-driven Ca^{2+} oscillations occur in synchrony with cellular oscillations in IP_3 concentration, indicating that these oscillatory responses involve a cyclical receptor-mediated on/off switching of PLC activity (Nash *et al.*, 2001, 2002). Substantial evidence has now accumulated to support the notion that these IP_3/Ca^{2+} oscillations are brought about by a process termed 'dynamic

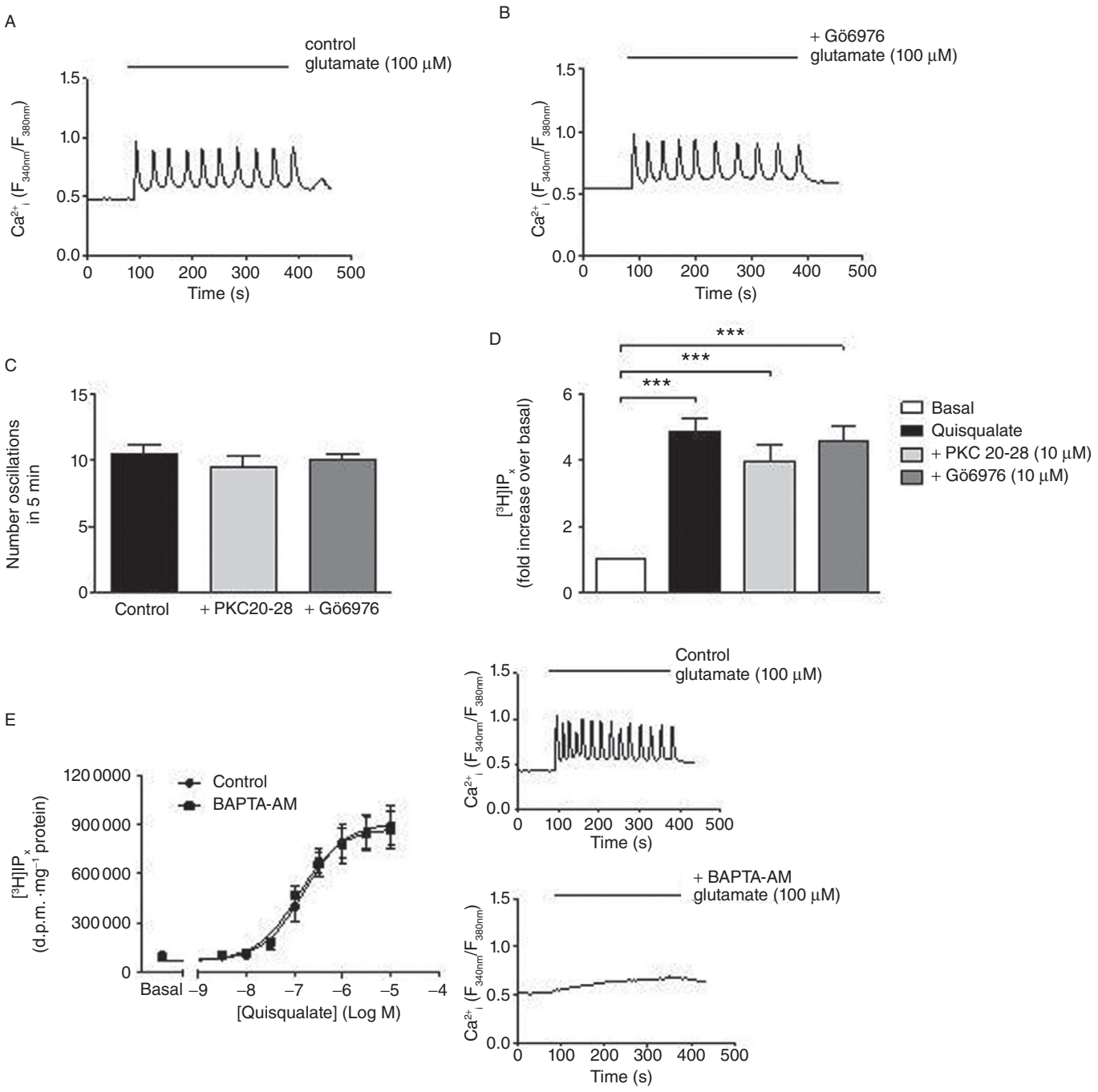

Figure 6

Assessing the Ca^{2+} dependence of the PKC isoenzyme(s) involved in $mGlu_5$ receptor regulation. Representative traces showing Ca^{2+} oscillations elicited by glutamate (100 μM) in control (A) and Gö6976 (10 μM; 20 min)-pretreated (B) cells. Panel C shows the number of oscillations elicited by glutamate (100 μM) stimulation in control, PKC 20-28- and Gö6976-pre-incubated cells: data are shown as means ± SEM from at least 30 individual cells over three separate experiments. Panel D shows $[^3H]IP_x$ accumulations in response to stimulation with 10 μM quisqualate when cells were pre-incubated with PKC 20-28 (10 μM; 30 min) or Gö6976 (10 μM; 20 min): data shown are means ± SEM for six separate experiments performed in duplicate. ***$P < 0.001$ significantly different as shown (one-way ANOVA followed by Bonferroni's multiple comparison test). Panel E shows concentration–response curves for $[^3H]IP_x$ accumulations after 20 min stimulation with various concentrations of quisqualate in control and BAPTA-AM (100 μM; 30 min)-loaded cells. Mean (±SEM) pEC_{50} (M) values for quisqualate-stimulated responses were 7.02 ± 0.06 in control and 6.92 ± 0.08 in BAPTA-loaded cells. Insets to panel E show representative single-cell Ca^{2+} responses to 5 min perfusion with glutamate (100 μM) in control (upper) and BAPTA-loaded (lower) cells.

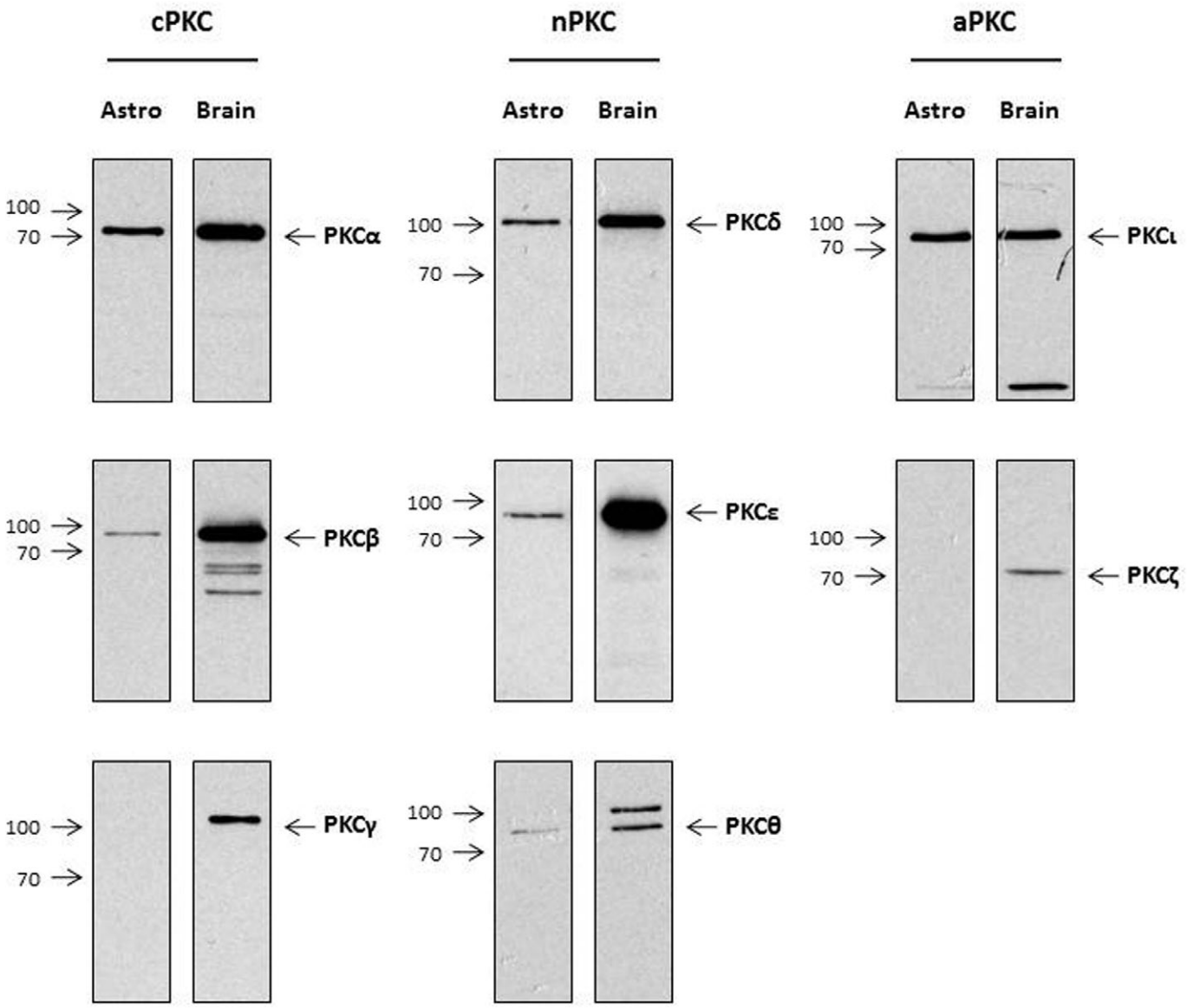

Figure 7

Analysis of PKC isoenzyme expression in rat cortical astrocytes. Rat brain tissue and astrocyte cell lysates were prepared and resolved by 12.5% SDS-PAGE as described in *Methods*. Protein levels were detected using antisera specific to PKCα, PKCβ, PKCγ, PKCδ, PKCε, PKCθ, PKCι and PKCζ. Representative blots from three independent experiments are shown for each PKC isoenzyme.

uncoupling' (Nash *et al.*, 2002), where mGlu$_5$ receptor-driven activation of PKC leads to receptor phosphorylation, specifically at Ser^{839}, to uncouple the receptor from G$_{q/11}$ and an as yet poorly defined protein phosphatase activity rapidly dephosphorylates the receptor to allow restoration of mGlu$_5$ receptor–G$_{q/11}$ coupling (Kawabata *et al.*, 1996; Nakahara *et al.*, 1997; Nash *et al.*, 2002; Kim *et al.*, 2005; but see Dale *et al.*, 2001).

Stimulation of the astrocyte mGlu$_5$ receptor initiates robust Ca^{2+} oscillations that can reach a frequency of approximately three oscillations per minute. Application of calyculin A or cantharidin, relatively selective inhibitors of the serine/threonine protein phosphatases PP1/PP2A (Ishihara *et al.*, 1989; Honkanen, 1993), caused significant reductions in glutamate-evoked Ca^{2+} oscillation frequency, in agreement with previous findings (Nakahara *et al.*, 1997; Nash *et al.*, 2002). These data support the involvement of specific protein phosphatase isoenzyme(s) in the cyclical phosphorylation/dephosphorylation of mGlu$_5$ receptors in astrocytes. Pharmacological inhibition of PP1/PP2A also caused concentration-dependent reductions in quisqualate-stimulated [^{3}H]IP$_x$ accumulation, providing corroborative evidence for the involvement of protein phosphatases in the cyclical restoration of signalling initiated by mGlu$_5$ receptors. The close proximity of GPCRs and protein phosphatases, as well as scaffolded protein kinase/phosphatase complexes, is now well documented (Pitcher *et al.*, 1995; Bauman and Scott, 2002; Dodge-Kafka *et al.*, 2010). Indeed, the existence of a

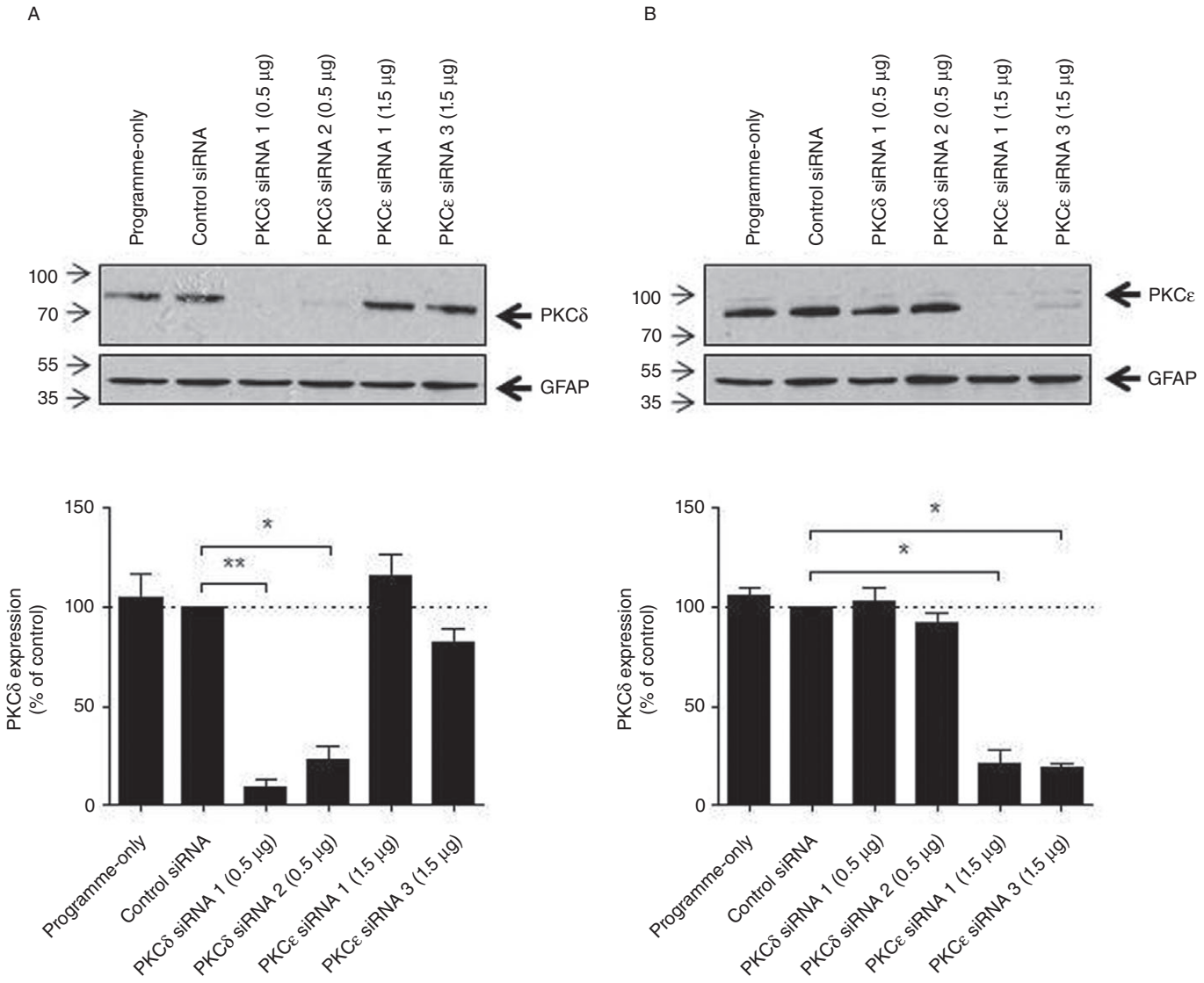

Figure 8

Isoenzyme-specific anti-PKC siRNAs can deplete PKC protein expression in astrocytes. Astrocytes were transfected with control, anti-PKCδ or anti-PKCε siRNAs (0.5 or 1.5 μg) by nucleofection as described in *Methods*. After 72 h, cells were lysed and protein levels were detected using antisera specific to PKCδ (A) and PKCε (B). Representative immunoblots from at least three separate experiments are shown with lower panels showing GFAP expression. Densitometric analyses of PKCδ and PKCε immunoreactivity for each immunoblot normalized to GFAP expression are shown in the lower panels, means ± SEM; $*P < 0.05$; $**P < 0.005$ significantly different as shown (one-way ANOVA followed by Dunnett's multiple comparison test).

complex of the $mGlu_5$ receptor with PP2A has been demonstrated in neurones *in vivo* (Mao *et al.*, 2005), while mGlu receptor–protein phosphatase interactions have also been reported for other mGlu receptor subtypes (Flajolet *et al.*, 2003).

The $mGlu_5$ receptor has also been reported to exist in a signalling complex with the protein phosphatase, calcineurin (PP2B). The C-terminal of $mGlu_5$ possesses two binding sites for this Ca^{2+}/calmodulin-dependent protein phosphatase that facilitate receptor dephosphorylation and recovery from PKC-mediated desensitization (Minakami *et al.*, 1997; Alagarsamy *et al.*, 2005). However, we found that preventing changes in $[Ca^{2+}]_i$ (by BAPTA-AM loading) in astrocytes had no effect on $mGlu_5$ receptor-stimulated $[^3H]IP_x$ accumulation, suggesting that a Ca^{2+}-dependent protein phosphatase was unlikely to be involved in the acute cyclical phosphorylation/dephosphorylation of the $mGlu_5$ receptor. This finding is also in agreement with the previously reported lack of effect of FK506 on (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylate-induced Ca^{2+} oscillations in astrocytes (Nakahara *et al.*, 1997).

Our study has also focused on the role of PKC(s) in the generation of $mGlu_5$ receptor-mediated Ca^{2+} oscillations, initially through pharmacological manipulation of cellular PKC activities. Phorbol ester stimulation of PKC activity caused a concentration-dependent decrease in $mGlu_5$ receptor-stimulated $[^3H]IP_x$ accumulation and Ca^{2+} oscillation

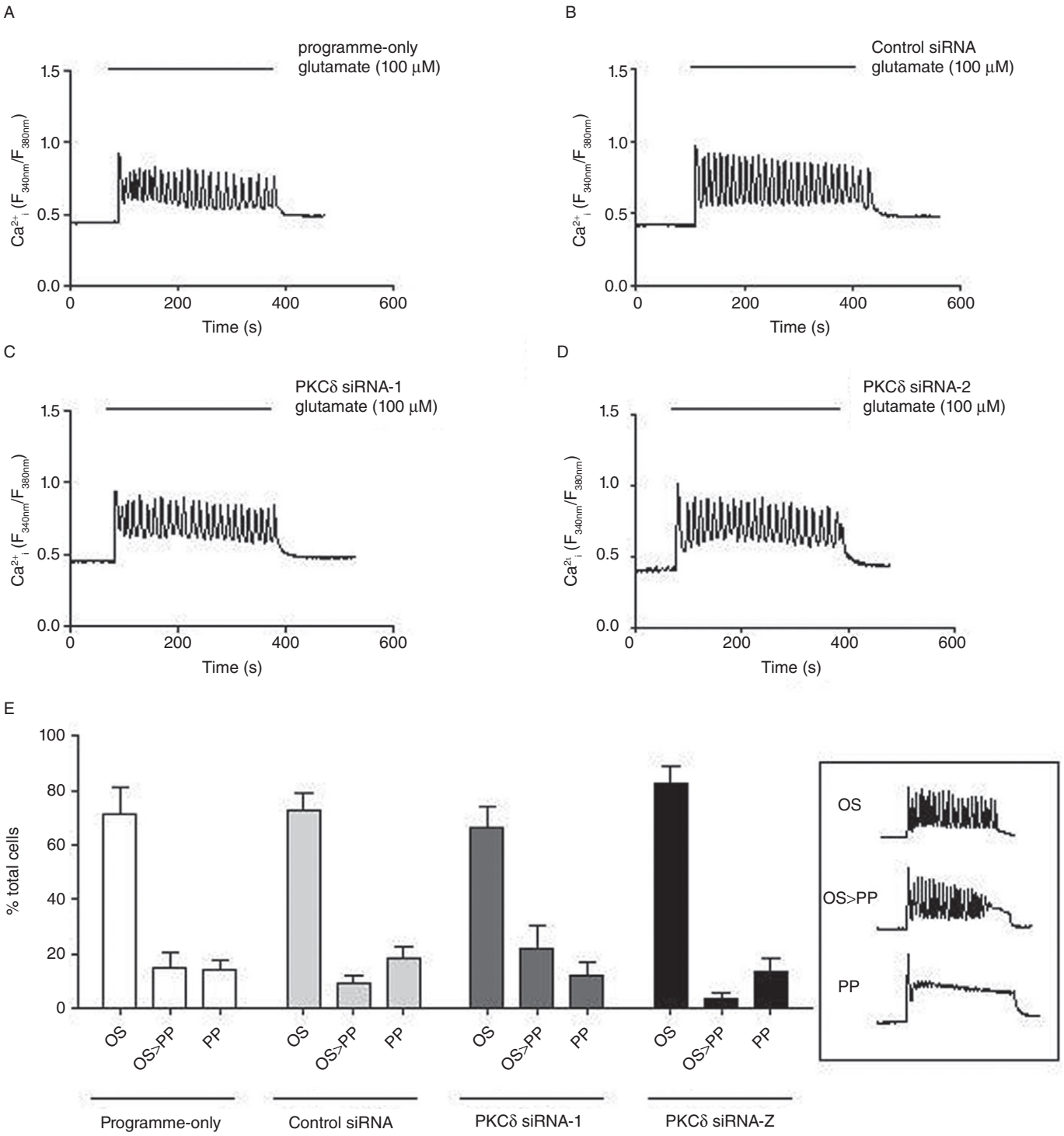

Figure 9

Effects of PKCδ siRNA knock-down on glutamate-stimulated single-cell Ca^{2+} responses in astrocytes. Representative traces showing Ca^{2+} responses elicited by glutamate (100 μM) stimulation in astrocytes nucleofected with no siRNA (programme-only; A), control siRNA (B), PKCδ siRNA-1 (0.5 μg) (C) or PKCδ siRNA-2 (0.5 μg) (D). Mean ± SEM data from at least 100 cells over at least four separate experiments are shown in panel E for % of the total number of cells analysed that gave an oscillatory (OS), oscillatory driving into sustained plateau (OS > PP) or a peak-plateau (PP) response when stimulated with glutamate (100 μM). Inset graphs (right) present typical Ca^{2+} responses defining each category.

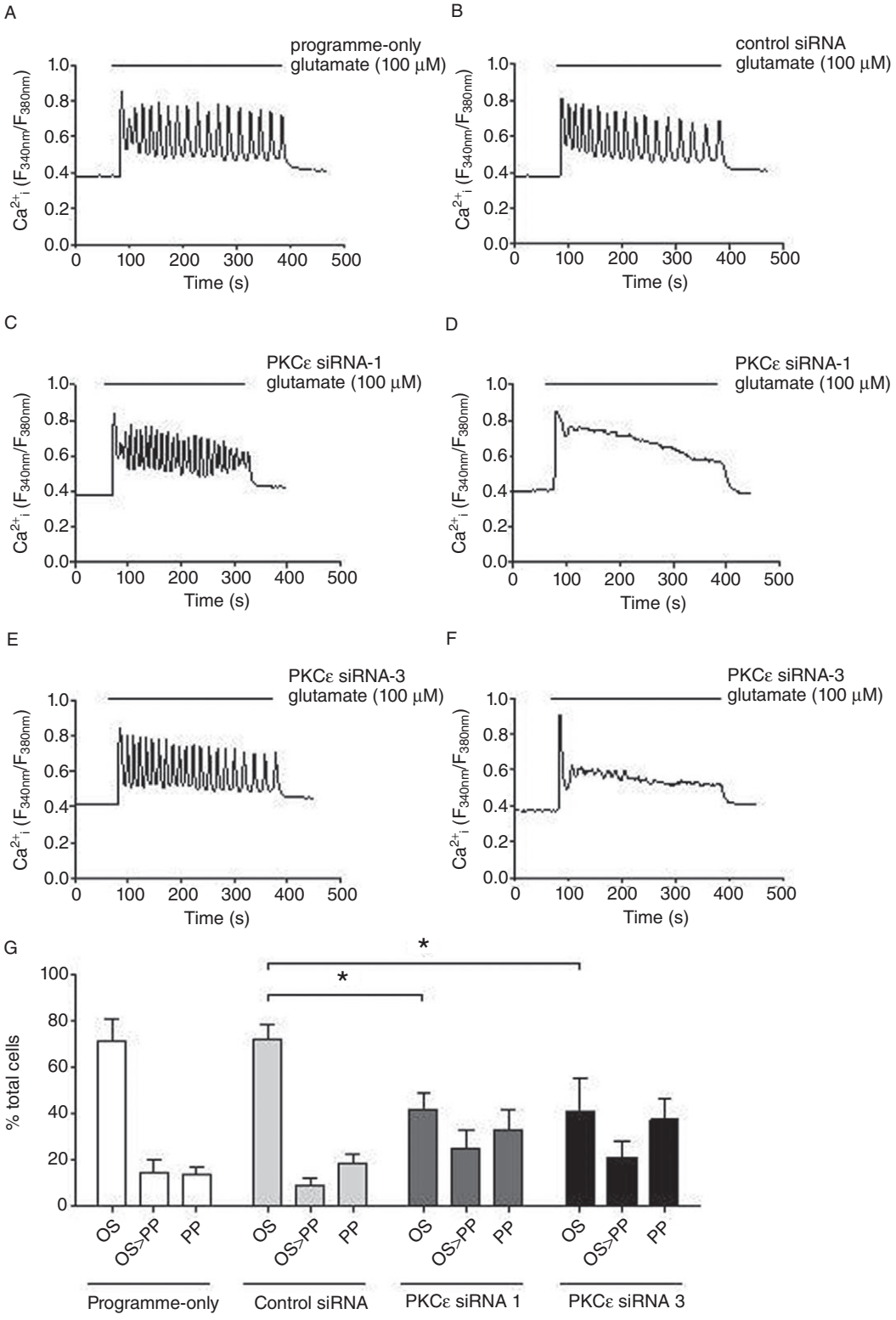

Figure 10

Effects of PKCε siRNA knock-down on glutamate-stimulated single-cell Ca^{2+} responses in astrocytes. Representative traces showing Ca^{2+} responses elicited by glutamate (100 μM) stimulation in astrocytes nucleofected with no siRNA (programme-only; A), control siRNA (B) PKCε siRNA-1 (1.5 μg) (C, D) or PKCε siRNA-3 (1.5 μg) (E, F). Mean ± SEM data from at least 100 cells over at least four separate experiments are shown in panel G for % of the total number of cells analysed that gave an oscillatory (OS), oscillatory driving into sustained plateau (OS > PP) or a peak-plateau (PP) response when stimulated with glutamate (100 μM). *$P < 0.05$ significantly different as shown (two-way ANOVA followed by Bonferroni's multiple comparison test).

frequency. It has previously been reported that the PKC inhibitor, staurosporine, can completely abolish $mGlu_5$ receptor-stimulated Ca^{2+} oscillations in recombinant cell systems and astrocytes (Kawabata *et al.*, 1996; Nakahara *et al.*, 1997). Here, we have not only investigated the effects of PKC inhibitors on single-cell Ca^{2+} responses evoked by $mGlu_5$ receptors, but also looked more directly at the effects of PKC inhibition on PLC activity. In cortical astrocytes PKC inhibition converted $mGlu_5$ receptor-driven Ca^{2+} oscillatory responses into sustained peak-plateau responses. The broad-spectrum PKC inhibitors, staurosporine and Ro 31-8220, each caused an approximately twofold increase in the maximum $[^3H]IP_x$ accumulation stimulated by quisqualate. These data are consistent with the $mGlu_5$ receptor existing for longer periods in a dephosphorylated, $G_{q/11}$-coupled and PLC stimulatory state in the presence of the PKC inhibitor. A similar increase in quisqualate-stimulated $[^3H]IP_x$ accumulation could be observed in CHO-*lac*-mGlu5a cells treated overnight with PDBu to down-regulate conventional and novel PKC isoenzymes; however, G5-differentiated cortical astrocytes did not tolerate chronic exposure to phorbol ester treatment, preventing us providing such complementary data in the native $mGlu_5$ receptor-expressing system (data not shown).

Previous attempts to define the specific PKC isoenzyme(s) involved in $mGlu_5$ receptor dynamic uncoupling have failed to produce a consensus. Stimulation of $mGlu_5$ receptor in HEK cells has been shown to lead to the translocation of PKCγ and δ isoenzymes from the cytoplasm to the membrane, although only PKCδ was reported to phosphorylate the $mGlu_5$ receptor (Uchino *et al.*, 2004). In contrast, Codazzi *et al.* (2001) reported in hippocampal astrocytes that $mGlu_5$ receptor-stimulated PKC activation is dependent on repetitive DAG and Ca^{2+} spikes and hypothesized a role for conventional PKCs in acute receptor regulation. In contrast to the latter finding, the work presented here showed that Gö6976 and PKC 20-28, inhibitors of conventional PKCs, had no effect on $mGlu_5$ receptor-stimulated responses in cortical astrocytes, at concentrations previously demonstrated to inhibit fully conventional (Ca^{2+}- and DAG-dependent) PKCs (Eichholtz *et al.*, 1993; Martiny-Baron *et al.*, 1993). Supportive evidence for the $mGlu_5$ receptor-regulating PKC being a Ca^{2+}-independent isoenzyme comes from the lack of effect that 'clamping' of astrocyte $[Ca^{2+}]_i$ (by BAPTA-AM loading) has on quisqualate-stimulated $[^3H]IP_x$ accumulation.

To take our study further, we decided that molecular genetic approaches would be needed to more precisely pinpoint the PKC isoenzyme(s) involved in $mGlu_5$ receptor regulation and decided to attempt isoenzyme-specific knock-down using RNAi. G5-differentiated cortical astrocyte cultures express an array of PKC isoenzymes including members of the conventional, novel and atypical subfamilies. Consistent with a previous study, we report the lack of expression of PKCγ in astrocytes, but show the expression of PKCα, β, δ, ε, θ and ι using isoenzyme-specific antibodies (Slepko *et al.*, 1999). However, in contrast to the findings of Slepko *et al.* (1999), we did not detect PKCζ, consistent with the findings of Asotra and Macklin (1994). Based on our findings suggesting the involvement of a Ca^{2+}-independent, but DAG (or at least phorbol ester)-regulated PKC activity in acute $mGlu_5$ receptor regulation, we focused on the isoenzyme-specific knock-down of novel PKCs using validated siRNAs. The most effective PKCδ siRNA utilized here caused >90% depletion of this isoenzyme 72 h after nucleofection into astrocytes, but had no effect on glutamate-stimulated single-cell Ca^{2+} responses; in contrast the most effective PKCε siRNA caused ~80% knock-down and resulted in a significant shift in the distribution of Ca^{2+} responses from oscillatory towards peak-plateau (see Figure 10). That glutamate-stimulated Ca^{2+} oscillations were abolished only in a proportion of astrocytes may have been a consequence of the incomplete knock-down of PKCε achieved. It is possible (and indeed likely) that cellular PKCε levels normally exceed those necessary to mediate $mGlu_5$ receptor phosphorylation and therefore a large depletion is needed before an effect is seen. It also remains a possibility that PKC isoenzymes additional to PKCε are involved in $mGlu_5$ receptor dynamic uncoupling.

Thus, our data provide new evidence to implicate PKCε, but not PKCδ in acute $mGlu_5$ receptor phosphorylation. However, more definitive evidence will require the effects of PKCε knock-down on the phosphorylation status of Ser^{839} within the $mGlu_5$ receptor C-terminal domain to be investigated. While these two closely related and similarly regulated novel PKCs often fulfil redundant, interchangeable roles, there are also examples of PKCε and PKCδ fulfilling distinct cellular functions (Pears *et al.*, 2008; Frangioudakis *et al.*, 2009; Sivaraman *et al.*, 2009). It is also noteworthy that PKCε is the most abundantly expressed novel PKC in the brain, where it is thought to fulfil numerous neuronal functions, including the regulation of ion channels and neurotransmitter release (Shirai *et al.*, 2008). Important roles for PKCε have also been highlighted in glial cells, including the differentiation of astrocytes from neural precursor cells (Steinhart *et al.*, 2007). An earlier study investigating effects of staurosporine on astrocytic phenotype and PKC isoenzymic expression showed that this inhibitor caused membrane-associated PKCε to be translocated to the cytoplasm, without altering the distribution of the other PKC isoenzymes studied (Kronfeld *et al.*, 1995). Such a staurosporine-induced redistribution of PKCε away from the plasma membrane may help to explain the effectiveness of this agent to ablate completely $mGlu_5$ receptor-stimulated Ca^{2+} oscillations. In addition, agonist stimulation of another family C GPCR, the calcium-sensing (CaS) receptor, can also generate robust Ca^{2+} oscillations, most likely through the cyclical phosphorylation/dephosphorylation of a key C-terminal residue (Thr^{888}) (Miedlich *et al.*, 2002; Young *et al.*, 2002; Davies *et al.*, 2007; McCormick *et al.*, 2010). It has also been reported that expression to constitutively active forms of PKCβ or PKCε can reduce or abolish Ca^{2+} oscillations, suggesting that PKCε (and perhaps other PKC isoenzymes) is an important regulator of the CaS receptor (Young *et al.*, 2002).

In conclusion, our data provide additional, supportive evidence for the involvement of specific PKCs and protein phosphatases in the rapid cyclical phosphorylation and dephosphorylation of the $mGlu_5$ receptor that define Ca^{2+} oscillation frequency generated by agonist stimulation in rat cerebrocortical astrocytes; in particular, we highlight the roles of specific Ca^{2+}-independent PKC and protein phosphatase isoenzymes in shaping receptor-driven Ca^{2+} oscillatory behaviours that may in turn determine an array of glial functions, including the release of gliotransmitters (Agulhon *et al.*, 2008; Fiacco *et al.*, 2009) and the programming of transcrip-

tional events (Gu and Spitzer, 1995; De Koninck and Schulman, 1998; Dolmetsch *et al.*, 1998).

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Conflict of interest

None.

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