British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.02177.x
www.brjpharmacol.org

RESEARCH PAPER

Isolated dorsal root ganglion neurones inhibit receptor-dependent adenylyl cyclase activity in associated glial cells

KY Ng[1], BHS Yeung[1], YH Wong[2] and H Wise[1]*

[1]*School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, and* [2]*Division of Life Science, Hong Kong University of Science and Technology, Hong Kong, China*

Correspondence
Helen Wise, School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China. E-mail: helenwise@cuhk.edu.hk

Keywords
cAMP; CGRP receptors; dorsal root ganglion cells; EP_4 receptors; IP receptors; neurone–glial interactions; primary sensory neurones; β-adrenoceptors

Received
10 November 2011
Revised
6 July 2012
Accepted
15 August 2012

BACKGROUND AND PURPOSE

Hyper-nociceptive PGE_2 EP_4 receptors and prostacyclin (IP) receptors are present in adult rat dorsal root ganglion (DRG) neurones and glial cells in culture. The present study has investigated the cell-specific expression of two other G_s-protein coupled hyper-nociceptive receptor systems: β-adrenoceptors and calcitonin gene-related peptide (CGRP) receptors in isolated DRG cells and has examined the influence of neurone–glial cell interactions in regulating adenylyl cyclase (AC) activity.

EXPERIMENTAL APPROACH

Agonist-stimulated AC activity was determined in mixed DRG cell cultures from adult rats and compared with activity in DRG neurone-enriched cell cultures and pure DRG glial cell cultures.

KEY RESULTS

Pharmacological analysis showed the presence of G_s-coupled β_2-adrenoceptors and CGRP receptors, but not β_1-adrenoceptors, in all three DRG cell preparations. Agonist-stimulated AC activity was weakest in DRG neurone-enriched cell cultures. DRG neurones inhibited IP receptor-stimulated glial cell AC activity by a process dependent on both cell–cell contact and neurone-derived soluble factors, but this is unlikely to involve purine or glutamine receptor activation.

CONCLUSIONS AND IMPLICATIONS

G_s-coupled hyper-nociceptive receptors are readily expressed on DRG glial cells in isolated cell cultures and the activity of CGRP, EP_4 and IP receptors, but not β_2-adrenoceptors, in glial cells is inhibited by DRG neurones. Studies using isolated DRG cells should be aware that hyper-nociceptive ligands may stimulate receptors on glial cells in addition to neurones, and that variable numbers of neurones and glial cells will influence absolute measures of AC activity and affect downstream functional responses.

Abbreviations

CGRP, calcitonin gene-related peptide; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPPG, (RS)-α-cyclopropyl-4-phosphonophenyl-glycine; DRG, dorsal root ganglia; E4CPG, (RS)-α-ethyl-4-carboxyphenylglycine; EP_2, PGE_2 EP_2 receptor subtype; EP_4, PGE_2 EP_4 receptor subtype; GFAP, glial fibrillary acidic protein; HBS, HEPES-buffered saline; ICI118551, (+/–)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride; IP receptor, prostacyclin receptor; MK801, (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NGF, nerve growth factor; ONO-AE1-329, 16-(3-methoxymethyl)phenyl-ω-tetranor-3,7-dithia-PGE_1; ONO-AE3-208, 2-(2-(2-methyl-2-naphth-1-ylacetylamino)-phenylmethyl)-benzoic acid; RO1138452, 4,5-dihydro-1H-imidazol-Z-yl)-[4-(4-isopropoxybenzyl)phenyl]amine; SGC, satellite glial cell

Introduction

In intact sensory ganglia, the primary sensory neurones are ensheathed by satellite glial cells (SGCs) (Hanani, 2005). Dorsal root ganglia (DRG) cell cultures prepared from adult rats typically contain significantly more glial cells (SGCs, Schwann cells and some fibroblasts) than neurones and it is difficult to generate neurone-containing cultures of high purity (Ahmed *et al.*, 2006; Ng *et al.*, 2011). However, as SGCs may remain associated with neurones in dissociated cell cultures (Ceruti *et al.*, 2011), this a useful model system to examine neurone–glial communication underlying responses to nerve injury (for reviews, see McMahon *et al.*, 2005; Scholz and Woolf, 2007; Austin and Moalem-Taylor, 2010; Ren and Dubner, 2010). For example, in dissociated trigeminal ganglion cell cultures, bradykinin stimulates the release of calcitonin gene-related peptide (CGRP) from sensory neurones which activates CGRP receptors on SGCs, ultimately increasing the expression of purine P2Y receptors and affecting cytokine production (Ceruti *et al.*, 2011); cytokines such as TNF-α then increase neuronal excitability (Zhang *et al.*, 2007a).

Isolated cell cultures of DRG and trigeminal ganglia are frequently used as model systems to study neuronal responses relating to pain and migraine respectively (LaMotte, 2007; Ceruti *et al.*, 2008). It has generally been assumed that if a GPCR has been identified on primary sensory neurones in intact DRG, then these receptors will similarly be expressed only on neurones in isolated cell cultures. However, although the PGE_2 receptor (EP_4) subtype and the prostacyclin (IP) receptor are localized to neurones in intact DRG (Oida *et al.*, 1995), we have shown that the non-neuronal cell population in mixed DRG cell cultures also express these receptors (Ng *et al.*, 2011). Because the majority of non-neuronal cells in our DRG cell cultures express glial fibrillary-acidic protein (GFAP) (Ng *et al.*, 2010), we refer to these cells as DRG glial cells. What causes the up-regulation of EP_4 and IP receptors in isolated DRG glial cells is presently unknown, but up-regulation of EP_2 and EP_4 receptors in spinal cord microglial cells *in vivo* occurs in response to chronic, but not acute, inflammatory conditions to provide a negative feedback system regulating neuroinflammation (Noda *et al.*, 2007; Brenneis *et al.*, 2011).

During our work to identify EP_4 and IP receptors on glial cells of adult rat DRG cells *in vitro* (Ng *et al.*, 2011), we noted that AC responses in pure glial cell preparations were significantly greater than in mixed DRG neurone–glial cell preparations, despite similar numbers of glial cells. These observations suggested that DRG neurones were inhibiting agonist-stimulated AC responses by DRG glial cells, so we have examined this interaction in more detail, focusing on EP_4 receptors and IP receptors to determine if cell–cell contact is essential for neuronal inhibition of G_s-coupled GPCR responses in DRG glial cells. Activation of G_s-coupled prostanoid receptors is associated with hyper-nociceptive responses (Sachs *et al.*, 2009), and we have previously shown that AC activity in mixed DRG cell cultures is also stimulated by CGRP, isoprenaline and salbutamol (Rowlands *et al.*, 2001). Therefore, our goal here was also to pharmacologically characterize CGRP and β-adrenoceptor responses in mixed DRG cell cultures and to determine if these receptors were also present in pure glial cell cultures. We demonstrate here that in addition to G_s-coupled EP_4 and IP receptors, CGRP receptors and β_2-adrenoceptors are also functionally expressed in DRG glial cells. Furthermore, the activity of CGRP, EP_4 and IP receptors, but not β_2-adrenoceptors, appears to be subject to inhibition by DRG neurones.

Methods

Reagents

Cicaprost was a gift from Schering AG (Berlin, Germany). ONO-AE1-329 and ONO-AE3-208 were gifts from Dr T Maruyama of ONO Pharmaceutical Co. Ltd (Osaka, Japan). RO1138452 (CAY10441) was purchased from Cayman Chemical (Ann Arbor, MI, USA), and nerve growth factor (NGF) mouse 2.5 s was from Alomone Laboratories (Israel). Forskolin, MK801, PGE_2 and suramin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). S-(–)-Atenolol, CGRP, $CGRP_{8-37}$, CNQX, CPPG, E4CPG, formoterol, ICI118551 and isoprenaline were purchased from Tocris Bioscience (Bristol, UK).

Preparation of primary cultures of adult rat DRG cells

All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All experiments were performed under license from the Government of the Hong Kong SAR and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. The Chinese University of Hong Kong is in full compliance with standards for humane care and use of laboratory animals as reviewed and accepted by the US Office of Laboratory Animal Welfare. Male Sprague–Dawley rats (150–200 g) were deeply anaesthetized with pentobarbitone (135 mg kg^{-1}, i.p.) and the spinal cords were rapidly removed (the total number of rats used was estimated to be 180). The dorsal root ganglia were removed from all levels of the spinal cord of and cultures were prepared as described previously (Ng *et al.*, 2010). The cell pellet was resuspended in Ham's F14 medium containing Ultroser G (4%), penicillin (100 U·mL^{-1}) and streptomycin (100 μg·mL^{-1}), and the morphologically distinct phase-bright neurones and phase-dark glial cells were counted using a haemocytometer slide. The mixed DRG cell preparation typically contained 45 ± 2% neurones and 55 ± 2% glial cells (n = 7) on the day of preparation. Unless otherwise noted, mixed DRG cells were seeded at 5000 neurones·per well onto 24-well tissue culture plates precoated with poly-DL-ornithine (500 μg·mL^{-1}) and laminin (5 μg·mL^{-1}), and assayed after 2 days in culture in an atmosphere of 5% CO_2 at 37°C.

Preparation of neurone-enriched and pure glial cell cultures

For neurone-enriched preparations, the mixed DRG cells were plated on poly-DL-ornithine-coated tissue culture dishes (cells from two rats per 10 cm dish), as described previously (Ng *et al.*, 2010). After overnight incubation, the loosely attached neuronal cells were gently removed from the more firmly attached glial cells. Neurone-enriched cultures were plated at

5000 neurones per well, as described for the mixed DRG cell preparation, and assayed after another 24 h in culture. Neurone-enriched cell cultures typically contained 70 ± 6% neurones and 30 ± 6% glial cells (n = 7) on the day of preparation. Subpopulations of IB4-positive and IB4-negative DRG neurones were selected using magnetic bead assisted cell sorting, as described previously (Ng *et al.*, 2010). Cells were assayed after 1 day (IB4-negative) or 2 days (IB4-positive) to allow time to establish neurite outgrowth comparable with neurones in the mixed DRG cell preparation.

The remaining glial cells were harvested using trypsin (0.05% in PBS) and seeded onto poly-DL-ornithine/laminin-coated 24-well tissue culture plates to mimic conditions in mixed DRG cell cultures. By counting cell numbers after 2 days *in vitro* (DIV), we estimated that plating 10 000 glial cells per well and assaying after a further day in culture would provide glial cell numbers similar to those in the mixed DRG cell preparation. Glial cell preparations were counted using a haemocytometer slide and typically contained 99.4 ± 0.3% glial cells and 0.6 ± 0.3% neurones on the day of preparation (n = 7).

Counting proportions of cell types in culture

To determine the number of neurones and glial cells in culture, phase-contrast images of DRG cell preparations were captured using a stereoscopic microscope TMS-F with digital sight camera system TDS-L1 (Nikon). Large phase-bright neurones and small phase-dark glial cells were readily identifiable, and a minimum of 100 cells per well were counted from representative images of DRG cell cultures, in three independent preparations.

Measurement of [^{3}H]-cAMP production

AC activity was assayed as described previously (Wise, 2006; Ng *et al.*, 2011) using the [^{3}H]-adenine pre-labelling method. Unless otherwise stated, DRG cell cultures were incubated with [^{3}H]-adenine (2 μCi·mL^{-1}) overnight, on poly-DL-ornithine/laminin-coated tissue culture plates. The DRG cells containing [^{3}H]-ATP were washed to remove free [^{3}H]-adenine, then incubated with or without antagonists for 15 min prior to addition of agonist for a further 30 min at 37°C in 500 μL assay buffer (HEPES-buffered saline (HBS [mmol·L^{-1}]: HEPES [15], pH 7.5; NaCl [140]; KCl [4.7]; $CaCl_2.2H_2O$ [2.2]; $MgCl_2.6H_2O$ [1.2]; 1.2 mM KH_2PO_4; glucose [3.3]) containing 1 mmol·L^{-1} IBMX to inhibit cyclic nucleotide phosphodiesterase activity. The reaction was stopped by addition of ice-cold trichloroacetic acid and ATP, at a final concentration of 5% and 1 mmol·L^{-1} respectively. The plates were left for at least 60 min on ice before separating the [^{3}H]-cAMP from [^{3}H]-ATP by column chromatography (Barber *et al.*, 1980). Cell samples were loaded onto Dowex AG50W-X4 (200–400 mesh) columns and [^{3}H]-ATP eluted with 3 mL distilled water. A further 10 mL distilled water was added, and the eluant was loaded directly onto a neutral alumina column, which was eluted with 6 mL 0.1 mol·L^{-1} imidazole buffer, pH 7.5, to give a fraction containing [^{3}H]-cAMP. Scintillator (OptiPhase 'HiSafe' 3) was added for scintillation counting. The production of [^{3}H]-cAMP from cellular [^{3}H]-ATP was estimated as the ratio of radiolabelled cAMP to total AXP (i.e. adenosine, ADP, ATP and cAMP) and is expressed as [cAMP]/[total AXP] x 100 (i.e. % conversion). All assays were performed in duplicate.

Preparation of cell-specific [^{3}H]-adenine-labelled cells

Freshly isolated neurone-enriched fractions and glial cell fractions were incubated with [^{3}H]-adenine at 10-fold the usual concentration (20 μCi·mL^{-1}) overnight, on poly-DL-ornithine-coated tissue culture plates. To minimize glial cell contamination of the final neurone-enriched population of cells, the neurones then underwent a second round of differential adhesion and [^{3}H]-adenine-loaded neurones or [^{3}H]-adenine-loaded glial cells were incubated with unlabelled glia and neurones, respectively, on poly-DL-ornithine/laminin-coated tissue culture plates. To mimic the neurone–glial cell contact time in the majority of experiments, AC activity was measured after 1 day in co-culture (i.e. 3 DIV).

Preparation and testing of conditioned medium

Conditioned medium was harvested from neurone-enriched cultures after 24 h in culture, centrifuged (1000× g for 5 min) to eliminate cell debris then used to replace the cell culture medium of glial cell cultures (10 000 glial cells per well). Glial cell cultures were incubated for a further 15 min or overnight with conditioned medium before [^{3}H]-cAMP production was determined.

Use of transwell plates to assess role of cell–cell contact

Inserts and wells of transwell culture plates (0.4 μm polyester membrane, 12 mm insert, 12-well plate; Corning Inc., Wilkes-barre, PA, USA) were coated with poly-DL-ornithine and laminin as described above. To determine a role for soluble factors, neurone-enriched cells (5000 neurones and approximately 2000 glial cells) and purified glial cells (10 000 glial cells) were plated onto inserts and wells, respectively, and 500 μL medium added to each compartment. After overnight incubation with [^{3}H]-adenine (2 μCi·mL^{-1}), the inserts were removed and placed in empty 12-well plates. Cells in inserts and wells were washed, and AC activity was assayed in response to cicaprost and forskolin in 500 μL assay buffer as described above. Receptor antagonists were added throughout the overnight pre-labelling period and included in the assay buffer for 15 min before addition of cicaprost or forskolin. A schematic representing the culture and assay configurations is given in Figure 8C.

Immunocytochemistry

DRG cell preparations were cultured on poly-DL-ornithine and laminin-coated glass coverslips, washed twice with PBS and fixed in ice-cold acetone : methanol (1:1) for 20 min. After being washed with PBS, cells were permeabilized with Triton X-100 (0.3%) and blocked with donkey serum (3%) in PBS for 30 min. Antibodies against TUJ-1 (1:1000; Abcam, Cambridge, MA, USA) were used to identify neurones. Cells were incubated with anti-TUJ-1 antibodies at 4°C overnight, followed by AF647-conjugated secondary antibodies (1:500; Jackson Immunoresearch Laboratories, West Grove, PA, USA) at room temperature for 1 h. All samples were incubated for 4 min with Hoechst 33342 stain (0.2 μg·mL^{-1}; Invitrogen, Carlsbad, CA, USA) to identify cell nuclei. The coverslips were

mounted with glycerol (90%) on glass slides and imaged using an Olympus IX51 inverted microscope (Center Valley, PA, USA) with Qimaging Retiga-2000R CCD camera (Surrey, BC, Canada). Fluorescent images were merged using Adobe Photoshop CS4 (San Jose, CA, USA).

Data analysis

Unless otherwise stated, values reported are means ± SEM. Drug affinities are expressed as pEC_{50} values, which are the negative log of the EC_{50} value, and receptor nomenclature follows GRAC Guidelines (Alexander *et al.*, 2011). Comparisons between groups were made using ANOVA with Bonferroni's or Dunnett's *post hoc* tests, as appropriate, using GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was taken as $P < 0.05$.

Results

Characterization of EP_4 and IP-receptors in DRG neuronal cell cultures

To characterize EP_4 and IP receptor-stimulated AC activity in neurone-enriched cell cultures, log agonist concentration–response curves were generated and compared with responses in the parent, mixed DRG cell cultures (Figure 1A). As shown previously (Wise, 2006; Ng *et al.*, 2011), the efficacy of the IP receptor agonist cicaprost was greater than that of PGE_2 in mixed DRG cells, and neuronal responses to cicaprost were significantly smaller than responses in mixed DRG cell cultures. pEC_{50} values for cicaprost were similar in both cell groups (mixed: 7.45 ± 0.11; neurone-enriched: 7.88 ± 0.32; n = 3). pEC_{50} values for PGE_2 were also similar in both cell groups (mixed: 6.84 ± 0.42; neurone-enriched: 6.41 ± 0.89; n = 3). Forskolin-stimulated [^{3}H]-cAMP production was significantly less in neurone-enriched cultures compared with that in mixed DRG and glial cell cultures (Figure 1B).

DRG neurones consist of three main subpopulations with different biochemical and functional properties (Gavazzi *et al.*, 1999; Julius and Basbaum, 2001). To determine if the AC responses are also different in these subpopulations, agonist-stimulated AC responses were determined in IB4-negative cells (nerve growth factor-responsive, small and large diameter neurones) and IB4-positive cells (glial cell line-derived neurotrophic factor-responsive, small diameter neurones). EP_4 receptor agonists and cicaprost induced relatively small increases in [^{3}H]-cAMP production in cultures of IB4-negative (Figure 1C) and IB4-positive (Figure 1D) DRG cells. Despite the small response sizes, in both preparations cic-

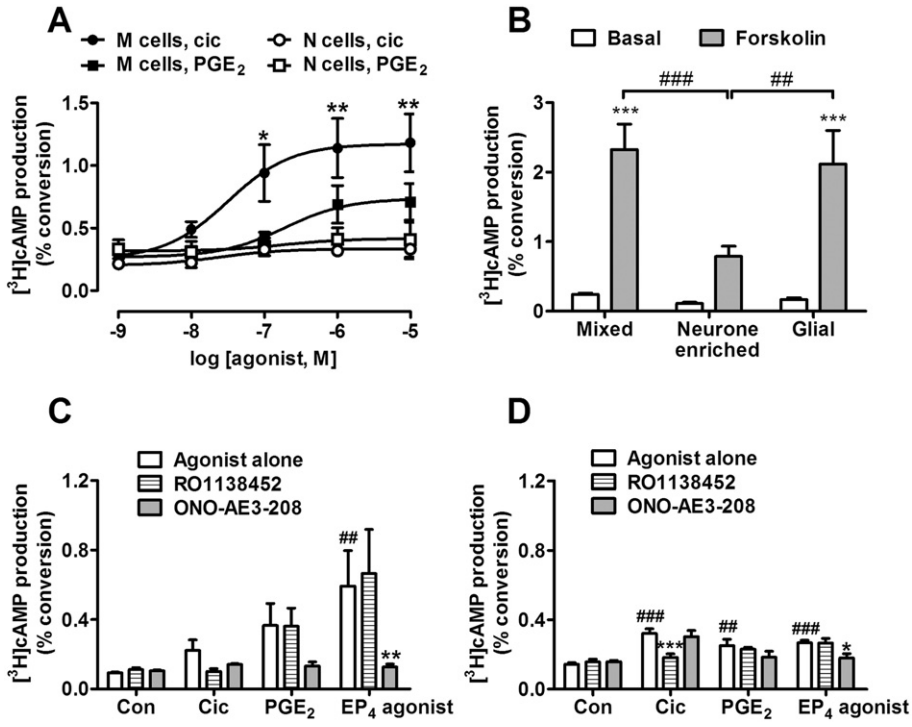

Figure 1

Evidence for EP_4 and IP-dependent responses in DRG neurone-enriched cultures. (A) AC activity was determined in neurone-enriched cultures and mixed DRG cells in response to cicaprost and PGE_2. *$P < 0.05$ and **$P < 0.01$ comparing responses in mixed DRG cells and neurone-enriched cultures; two-way ANOVA. (B) A comparison of basal and forskolin (1 μmol·L^{-1})-induced responses in mixed DRG cells, neurone-enriched cells and glial cells. ***$P < 0.001$ compared with basal activity, and ## $P < 0.01$ and ### $P < 0.001$ compared with neurone-enriched cultures; two-way ANOVA. (C) IB4-negative and (D) IB4-positive neurones were incubated with control solution (assay buffer; Con), PGE_2 (1 μmol·L^{-1}), ONO-AE1-329 (1 μmol·L^{-1}; EP_4 agonist) or cicaprost (40 nmol·L^{-1}; Cic) either alone or following 15 min incubation with RO1138452 (100 nmol·L^{-1}) or ONO-AE3-208 (100 nmol·L^{-1}). ## $P < 0.01$ and ### $P < 0.001$ compared with control untreated group; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with agonist alone group; one-way ANOVA. Data are presented as mean ± SEM, from at least three independent experiments.

aprost (40 nmol·L^{-1})-induced responses were inhibited by the IP receptor antagonist RO1138452 (100 nmol·L^{-1}), and responses to PGE_2 (1 μmol·L^{-1}) and the EP_4 receptor agonist ONO-AE1-329 (1 μm·L^{-1}) were inhibited by the EP_4 receptor antagonist ONO-AE3-208 (100 nmol·L^{-1}). Therefore, although cicaprost might be expected to stimulate EP_4 receptors (Abramovitz *et al.*, 2000), its actions in rat DRG cells are again specific for IP receptors (Ng *et al.*, 2011).

The responsiveness of DRG neurones is not enhanced by NGF

According to the literature, it is the cAMP generated in DRG neurones that is responsible for the hyperalgesic effect of PGE_2, yet data in Figure 1 showed that the neurone-enriched cultures were the least responsive to both agonist-dependent and agonist-independent activation of AC. Therefore, we looked for differences in assay conditions that might account for the relatively poor responses of neurone-enriched cell cultures.

Mixed DRG cells and neurone-enriched cell cultures were both assayed after 2 days *in vitro*, when neurite extension was similar (Figure 3B,G). But at this time point, the neurone-enriched cells had only been plated as enriched cultures for 1 day; therefore, because the small cAMP response size in neurone-enriched cultures was unexpected, we determined whether this resulted from the shorter period of incubation in their enriched form. Maintaining neurone-enriched preparations for several days in culture yielded cultures that were no longer enriched in neurones (Figure 2A), although the proportion of neurones was significantly higher than in mixed DRG cell cultures at all time points ($P < 0.001$, two-way ANOVA). Maintaining neurone-enriched preparations for an extra day increased [^{3}H]-cAMP production in all treatment groups (Figure 2B: $P < 0.001$, two-way ANOVA). The significantly increased response to forskolin after an extra day *in vitro* suggests that the overall increase in AC activity might result from the increased number of glial cells present rather than any change in responsiveness of neurones.

The relatively small cAMP response in neurone-enriched cultures might be due to a lack of neurotrophic factors normally generated by the glial cells; therefore, we incubated cells with NGF (50 ng·mL^{-1}) and determined AC activity in response to cicaprost (40 nmol·L^{-1}), ONO-AE1-329 (1 μmol·L^{-1}), PGE_2 (1 μmol·L^{-1}) and forskolin (1 μmol·L^{-1}). NGF increased neurite outgrowth (Figure 3C,H) but had no effect on the cAMP response in neurone-containing cultures (Figure 3A,F), or pure glial cell cultures (Figure 3K) ($P > 0.05$, two-way ANOVA).

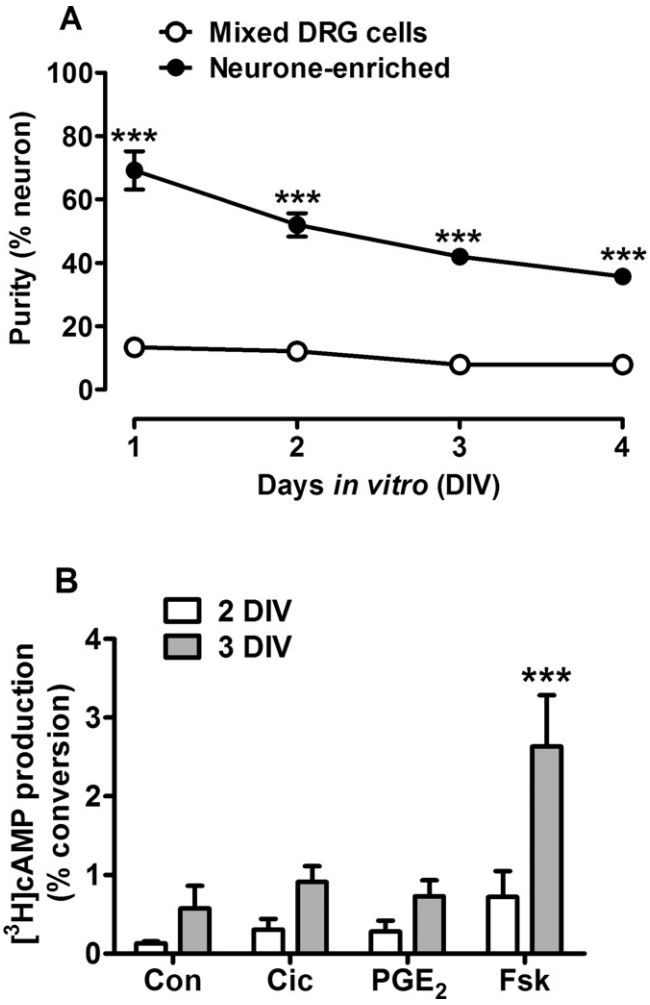

Figure 2

The poor response of neurone-enriched cultures is not improved by extending the time in culture. (A) The proportion of neurones in mixed DRG cultures and neurone-enriched cultures were calculated by counting a minimum of 100 cells per well. Data are presented as means ± SEM, $n = 6$ wells from three independent experiments. *** $P < 0.001$ compared with mixed DRG cell cultures (two-way ANOVA). (B) Neurone-enriched cells were cultured for 1 day (2 DIV) or 2 days (3 DIV) and [^{3}H]-cAMP production measured in response to control solution (assay buffer; Con), cicaprost (40 nmol·L^{-1}; Cic), PGE_2 (1 μmol·L^{-1}) or forskolin (1 μmol·L^{-1}; Fsk). Data are presented as means + SD, from two to three independent experiments. *** $P <$ 0.001 compared with response at 2 DIV (two-way ANOVA).

DRG neurones inhibit prostanoid-stimulated AC activity in glial cells

The AC activity data presented so far reflect the combination of [^{3}H]-adenine uptake and AC turnover properties of the different cell types. Therefore, to test our hypothesis that DRG neurones inhibit cicaprost and PGE_2-stimulated [^{3}H]-cAMP production by glial cells, each cell type was individually labelled before they were combined for co-culture, using cell numbers expected to match those of mixed DRG cells and pure glial cell cultures. Absolute [^{3}H]-cAMP production (% conversion) values in Figure 4 are therefore not directly comparable to those in previous figures because of the different [^{3}H]-adenine-labelling protocol required to maintain sufficient [^{3}H]-ATP inside the neurone-enriched cells after two days in culture. Cicaprost, PGE_2 and forskolin all significantly increased [^{3}H]-cAMP production in [^{3}H]-adenine-labelled glial cells (estimated as 20 000 cells per well on assay day) (Figure 4A; two-way ANOVA). In the presence of 10 000 unlabelled DRG neurones, both cicaprost- and PGE_2-stimulated responses were decreased (from 4.39 ± 0.15 to 2.53 ± 0.51% conversion for cicaprost, $P < 0.01$, two-way ANOVA; and from 1.98 ± 0.24 to 1.13 ± 0.16% conversion for

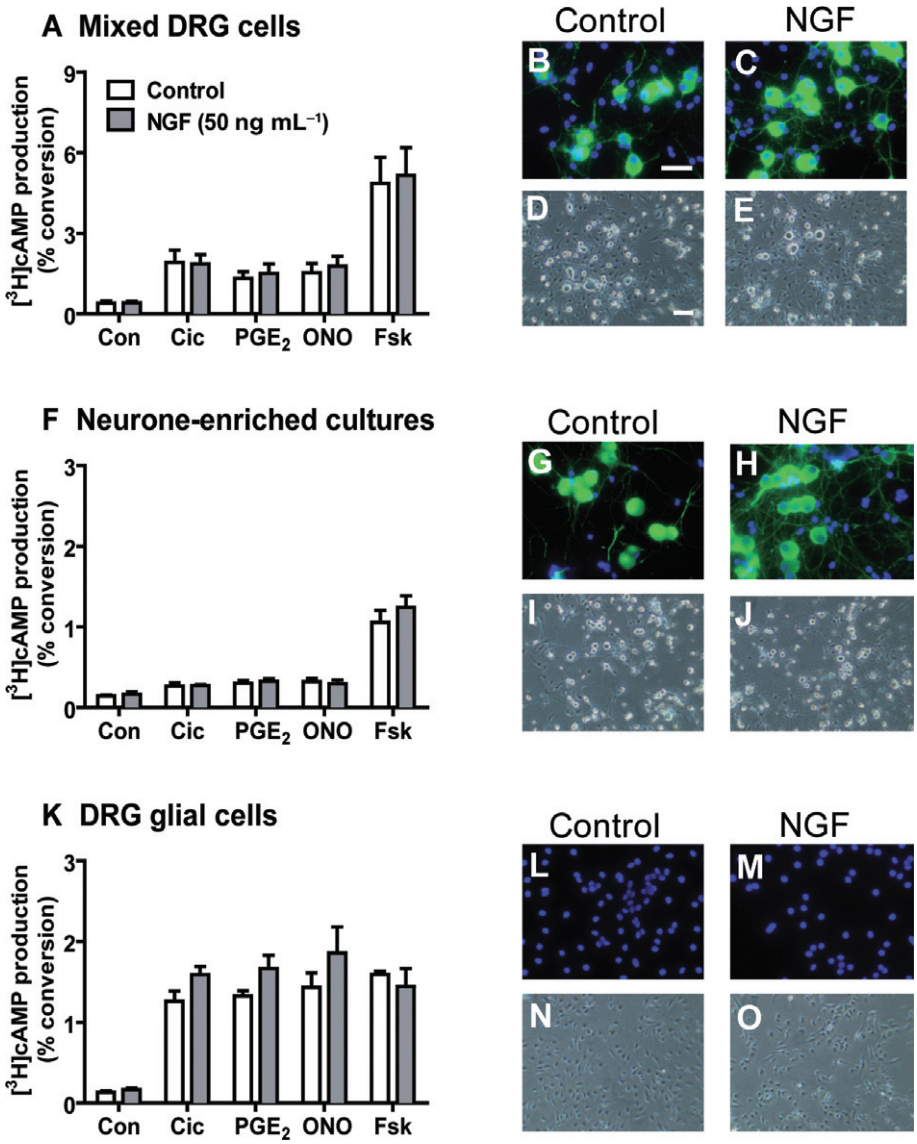

Figure 3

The presence of NGF does not enhance responses in neurone-enriched cell cultures. Immunocytochemistry showing similar neuronal morphology (TUJ-1) in both mixed DRG cell cultures (B–E) and neurone-enriched cultures (G–J). Cell nuclei are stained blue with Hoechst stain and indicate the presence of glial cells. Scale bar 100 μm. AC activity was determined in mixed DRG cells (A), neurone-enriched cell cultures (F) and glial cells (K) cultured with or without NGF (50 ng·mL^{-1}). Responses to control solution (assay buffer; Con), cicaprost (40 nmol·L^{-1}; Cic), PGE_2 (1 μmol·L^{-1}), ONO-AE1-329 (1 μmol·L^{-1}; ONO) and forskolin (1 μmol·L^{-1}). Data are presented as means ± SEM, from three independent experiments.

PGE_2), while responses to forskolin were unchanged (3.00 ± 0.50 to 3.80 ± 0.58% conversion) (Figure 4A). Conversely, only forskolin significantly increased [^{3}H]-cAMP production in [^{3}H]-adenine-labelled neurones (10 000 cells per well), and these responses were not affected by the presence of 20 000 unlabelled glial cells (Figure 4B; two-way ANOVA).

Characterization of CGRP receptors and β-adrenoceptors in DRG neurones and glial cells

The apparent neuronal inhibition of glial cell responses to cicaprost and PGE_2, but not to forskolin, mirror our earlier findings with mixed DRG cells where iloprost (IP receptor agonist)- and PGE_2-stimulated [^{3}H]-cAMP production increased as the number of neuronal cells decreased (Rowlands *et al.*, 2001). In that study, we showed that responses to CGRP were similarly affected, but that responses to β-adrenoceptor agonists (isoprenaline and salbutamol) behaved more like those to forskolin. We therefore examined the profile of CGRP and β-adrenoceptor responses in mixed DRG cells, neurone-enriched cell cultures and pure glial cell cultures; our hypothesis being that if glial cell responses were larger than those of mixed DRG cells, then a neurone-mediated inhibitory effect was likely to be present in the mixed DRG cell cultures. As an internal control, responses to cicaprost (40 nmol·L^{-1}) were assayed alongside our agonists of

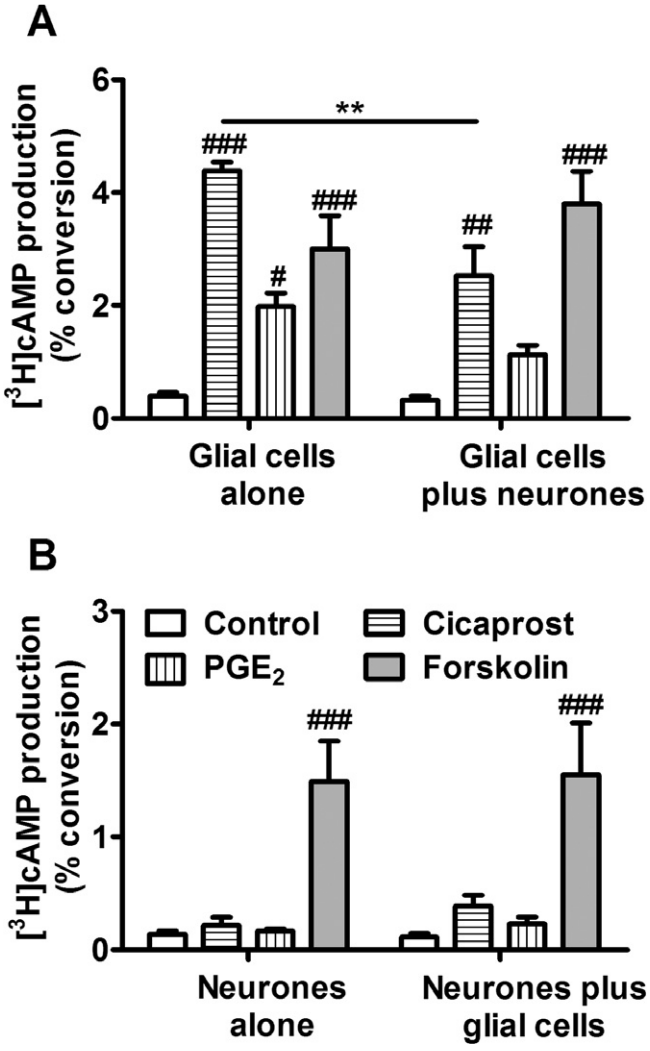

Figure 4

DRG glial cells are the major source of [^{3}H]-cAMP and its production is inhibited by the presence of neurones. DRG neurones and glial cells were individually labelled with [^{3}H]-adenine prior to co-culture with unlabelled glial cells and neurones respectively. (A) [^{3}H]-adenine-loaded glial cells (n = 20 000) were incubated alone or co-cultured with unlabelled DRG neurones (n = 10 000). (B) [^{3}H]-adenine-loaded DRG neurones (n = 10 000) were incubated alone or co-cultured with unlabelled glial cells (n = 20 000). After 1 day in co-culture, cell-specific [^{3}H]-cAMP production was determined in response to assay buffer, 40 nmol·L^{-1} cicaprost, 1 μmol·L^{-1} PGE_2 or 1 μmol·L^{-1} forskolin. Each data point is the mean ± SEM, from three independent experiments. **P < 0.01 compared with glial cells alone, and # P < 0.05, ## P < 0.01 and ### P < 0.001 compared with own control group; two-way ANOVA.

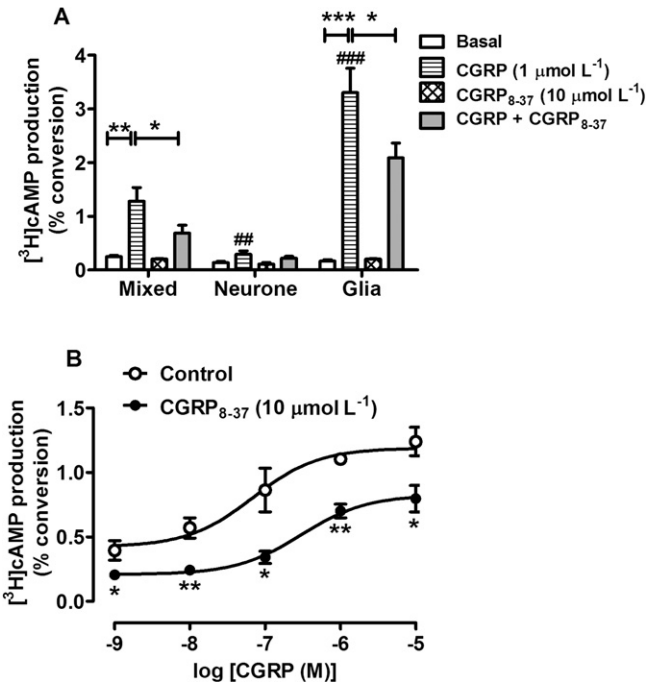

Figure 5

Comparison of CGRP-stimulated responses in DRG cell groups. AC activity was determined in mixed DRG cells (Mixed), neurone-enriched cell cultures (Neurone) and glial cells (Glia). (A) CGRP (1 μmol·L^{-1})-stimulated responses were inhibited by 15 min pretreatment of cells with the CGRP receptor antagonist $CGRP_{8-37}$ (10 μmol·L^{-1}). *P < 0.05, **P < 0.01 and ***P < 0.001 for comparison within cell group; ## P < 0.01 and ### P < 0.001 compared with agonist response in mixed DRG cell group; one-way ANOVA. (B) Log agonist concentration–response curves for CGRP-stimulated AC activity in mixed DRG cells in the presence or absence of $CGRP_{8-37}$ (10 μmol·L^{-1}). Data are presented as means ± SEM, from three independent experiments.

interest. In these experiments, cicaprost-stimulated AC activity was 0.81 ± 0.09% conversion for mixed DRG cells, 0.27 ± 0.04% conversion for neurone-enriched cells and 1.48 ± 0.19% conversion for glial cells (n = 3). Therefore, as shown previously, cicaprost-stimulated responses in glial cells were significantly higher than those in mixed DRG cells (P < 0.05) or neurone-enriched cells (P < 0.001; one-way ANOVA).

CGRP (1 μmol·L^{-1}) significantly increased [^{3}H]-cAMP production in mixed DRG cells and in glial cell cultures, but not in neurone-enriched cell cultures (Figure 5A). The CGRP responses in glial cells were significantly larger than those in mixed DRG cells (P < 0.001) and neurone-enriched cell cultures (P < 0.01), indicative of an inhibitory effect of neurones on the mixed DRG cells. Pretreatment of cells with the CGRP receptor antagonist $CGRP_{8-37}$ (10 μmol·L^{-1}) (Li *et al.*, (2008) had no effect alone, but significantly inhibited CGRP-stimulated [^{3}H]-cAMP production by 60 ± 6% (P < 0.001) in mixed DRG cells, by 40 ± 15% (P < 0.01) in neurone-enriched cells, and by 39 ± 3% (P < 0.001) in glial cells.

Although these CGRP receptor agonist and antagonist concentrations are widely used for *in vitro* studies (Li *et al.*, 2008), the failure of $CGRP_{8-37}$ to completely antagonize CGRP-stimulated [^{3}H]-cAMP production warranted further investigation. We therefore examined the effect of $CGRP_{8-37}$ (10 μmol·L^{-1}) on log agonist concentration–response curves in mixed DRG cell cultures (Figure 5B). CGRP stimulated AC activity with a pEC_{50} value of 7.40 ± 0.25 (n = 3), indicating that the agonist concentration of 1 μmol·L^{-1} used above was near maximally effective and may have accounted for the

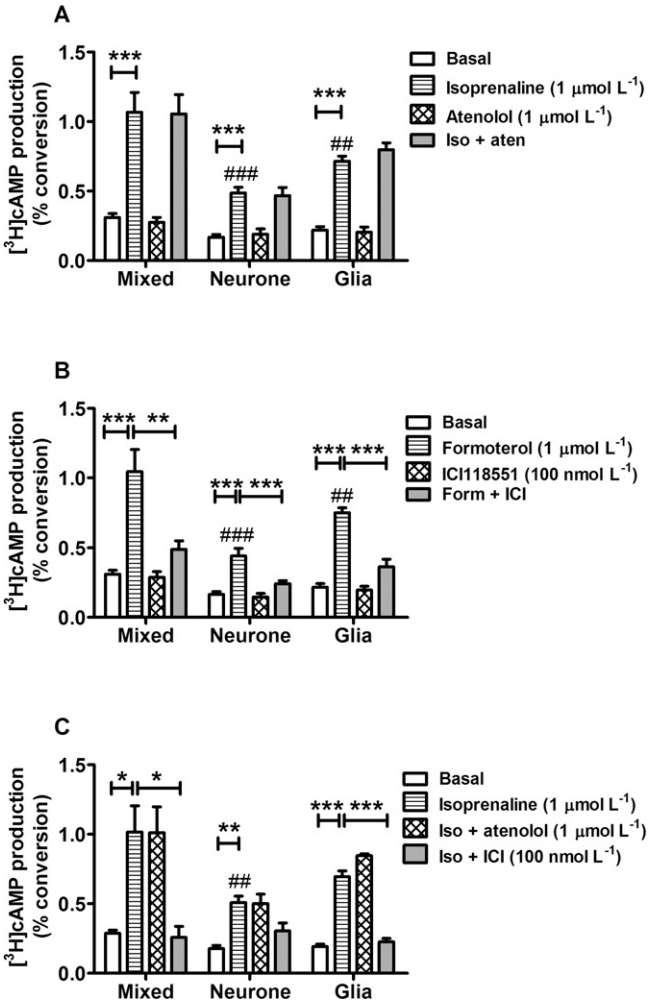

Figure 6

Comparison of β-adrenoceptor-stimulated responses in DRG cell groups. AC activity was determined in mixed DRG cells (Mixed), neurone-enriched cell cultures (Neurone) and glial cells (Glia). (A) Isoprenaline (1 μmol·L^{-1}; β_1/β_2-adrenoceptor agonist)-stimulated responses were not inhibited by the β_1-adrenoceptor antagonist atenolol (1 μmol·L^{-1}). (B) Formoterol (1 μmol·L^{-1}; β_2-adrenoceptor agonist)-stimulated responses were inhibited by the β_2-adrenoceptor antagonist ICI118551 (100 nmol·L^{-1}). (C) Isoprenaline (1 μmol·L^{-1}; β_1/β_2-adrenoceptor agonist)-stimulated responses were inhibited by the β_2-adrenoceptor antagonist ICI118551 (100 nmol·L^{-1}). *P < 0.05, **P < 0.01 and ***P < 0.001 for comparison within cell groups; ## P < 0.01 and ### P < 0.001 compared with agonist response in mixed DRG cell group; one-way ANOVA. Data are presented as means ± SEM, from four independent experiments.

relatively weak inhibitory activity of $CGRP_{8-37}$. In the presence of $CGRP_{8-37}$, the pEC_{50} value for CGRP was significantly decreased to 6.56 ± 0.19 (P < 0.05).

The mixed β_1/β_2-adrenoceptor agonist isoprenaline (1 μmol·L^{-1}) significantly increased [^{3}H]-cAMP production in all three DRG cell groups, with response sizes in neurone-enriched cell cultures and glial cells being significantly smaller than in mixed DRG cells (Figure 6A). The β_1-adrenoceptor antagonist atenolol (1 μmol·L^{-1}) had no effect alone and failed to inhibit the AC stimulating activity of isoprenaline (Figure 6A), suggesting that isoprenaline was acting via the β_2-adrenoceptor rather than the β_1-adrenoceptor subtype. To confirm this hypothesis, we showed that the specific β_2-adrenoceptor agonist formoterol (1 μmol·L^{-1}) also significantly increased [^{3}H]-cAMP production in all three cell groups, with mixed DRG cells again having a significantly larger response (Figure 6B). The response profiles to isoprenaline and formoterol suggest that the neurones do not inhibit the β_2-adrenoceptor-mediated responses of glial cells in the mixed DRG cell cultures.

Pretreatment of cells with the β_2-adrenoceptor antagonist ICI18551 (1 μmol·L^{-1}) had no effect alone, but significantly inhibited formoterol-stimulated [^{3}H]-cAMP production by 77 ± 2% (P < 0.001) in mixed DRG cells, by 78 ± 5% (P < 0.001) in neurone-enriched cells and by 76 ± 9% (P < 0.001) in glial cells (Figure 6B), further demonstrating the presence of β_2-adrenoceptors. In contrast to the lack of effect of atenolol, ICI118551 inhibited isoprenaline-stimulated [^{3}H]-cAMP production by 100 ± 10% (P < 0.001) in mixed DRG cells, by 58 ± 24% (P < 0.01) in neurone-enriched cells and by 93 ± 1% (P < 0.001) in glial cells (Figure 6C), confirming that isoprenaline was acting via β_2-adrenoceptors in DRG cells.

The inhibitory effect of neurones on glial cell AC activity depends on both cell–cell contact and neurone-derived soluble factors

We have shown that co-culturing glial cells with neurones resulted in inhibition of prostanoid-stimulated AC activity in glial cells and propose that CGRP-stimulated responses are similarly affected. This interaction between neurones and glial cells in culture could be direct through cell–cell contact or indirect by the release of soluble factors. In order to elucidate the mode of interaction in mixed DRG cells, glial cells were treated with conditioned medium generated from neurone-enriched cultures, and we looked for any inhibitory effect due to soluble factors in the conditioned medium. Conditioned medium was harvested from neurone-enriched cultures after 24 h then centrifuged to eliminate cell debris before addition to glial cell cultures. In this experiment, the number of glial cells and neurones in culture were equivalent to those in mixed DRG cultures on the assay day (estimated at 5000 neurones and 20 000 glial cells). As we have shown that neurones in cell cultures had a similar inhibitory effect regardless of whether either or both cell types had been labelled with [^{3}H]-adenine, we tested the effect of conditioned medium in cultures where all cells were radiolabelled and thus avoided the need to perform two steps of differential adhesion. We found that co-culturing glial cells with neurones significantly inhibited cicaprost-stimulated [^{3}H]-cAMP production (2.57 ± 0.46 to 1.21 ± 0.22% conversion, n = 4; P < 0.01, two-way ANOVA) and inhibited PGE_2-stimulated [^{3}H]-cAMP production (1.36 ± 0.21 to 0.59 ± 0.08% conversion, n = 4), without affecting basal or forskolin-stimulated [^{3}H]-cAMP production (Figure 7). However, pretreatment of glial cells with neurone-conditioned medium had no significant effect on AC activity.

This lack of effect of conditioned medium does not exclude the possibility that a highly unstable factor mediates this inhibitory activity. To test this hypothesis, we cultured

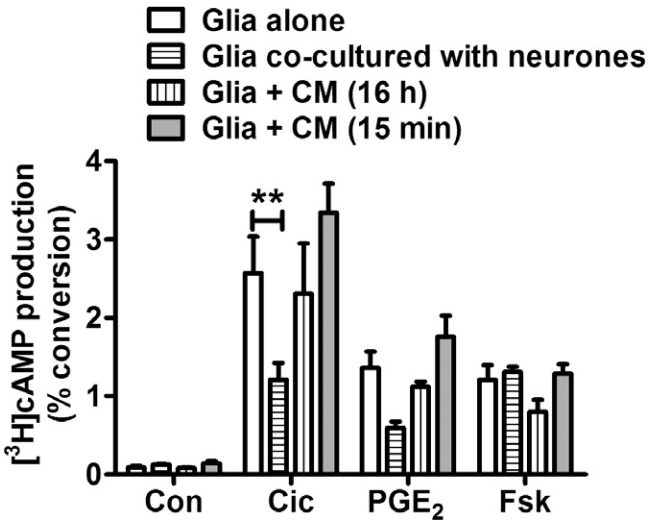

Figure 7

No effect of neurone-conditioned medium on EP_4 and IP receptor-mediated [^{3}H]-cAMP production by glial cells. Glial cells, glial cells co-cultured with neurones to mimic mixed DRG cell preparations, glial cells incubated with conditional medium overnight and glial cells incubated with conditioned medium for 15 min were incubated with control solution (assay buffer; Con), cicaprost (40 nmol·L^{-1}; Cic), PGE_2 (1 μmol·L^{-1}) or forskolin (1 μmol·L^{-1}; Fsk). Data are presented as means ± SEM, from four independent experiments. $^{**}P < 0.01$; two-way ANOVA.

glial cells in the bottom well of a transwell plate and put neurone-containing cultures in the insert. After an overnight incubation with [^{3}H]-adenine, we determined AC activity separately in cells cultured in inserts and wells. As shown previously, cicaprost-stimulated [^{3}H]-cAMP production was significantly higher in glial cell cultures (group A) compared with neurone and glial co-cultures (group B; Figure 8A). To test the hypothesis that soluble factors were being released by neurones, neurone-enriched cells were cultured in the inserts and glial cells in the wells (group C). When [^{3}H]-cAMP production was then determined separately in the neurone and glial cell cultures, glial cell responses to cicaprost (group C1) were significantly lower than responses of glial cells cultured in the absence of neurones (group A), suggesting that a soluble factor contributed to the overall inhibitory effect of neurones observed in group B. Neuronal responses to cicaprost were relatively small (group C2), as expected. Taken together these results suggest that both cell–cell contact and soluble factors are important for neuronal inhibition of cicaprost-stimulated glial cell responses.

Because all the experiments so far had shown that forskolin-stimulated responses in glial cells were not affected by the presence of neurones, we tested forskolin-stimulated responses alongside those to cicaprost in these transwell assays (Figure 8B). As shown previously, forskolin-stimulated [^{3}H]-cAMP production in neurone–glial co-cultures (group B, equivalent to mixed DRG cells) was significantly greater than in glial cells alone (group A). Furthermore, there was no

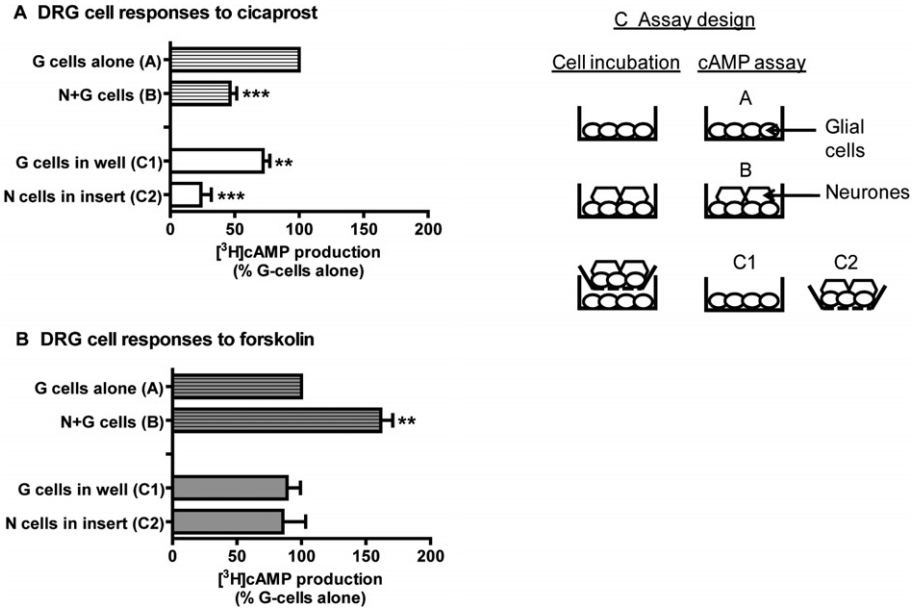

Figure 8

Neuronal inhibition of glial cell AC activity depends on soluble factor and cell–cell contact. DRG cell preparations were cultured in transwell dishes and assayed for AC activity as shown. (A) DRG glial cells (G cells) were cultured alone or (B) with neurones (N cells) to mimic mixed DRG cells to look for effects of cell–cell contact. To detect any effect of soluble factors, DRG glial cells were cultured in a transwell system, separated from neurone-containing cultures in the inserts (C), then AC activity was assayed in the separate compartments (C1 and C2, respectively) as summarized in (C). AC activity was determined in response to (A) cicaprost (40 nmol·L^{-1}) or (B) forskolin (1 μmol·L^{-1}), and AC activity compared with responses of glial cells alone (group A) in each experiment. Control responses (group A) to cicaprost were 2.59 ± 0.44% conversion, and to forskolin were 2.40 ± 0.18% conversion. Data are presented as means ± SEM, from five independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ for comparison with glial cells alone (group A); one-way ANOVA.

strong evidence here that neurones inhibit glial cell responses to forskolin (compare group A and C1).

Neuronal inhibition of glial cell AC activity is independent of purine and glutamine receptors

DRG neuronal somata release neurotransmitters such as ATP and glutamate (Zhang *et al.*, 2007a; Gu *et al.*, 2010); therefore, these ligands represent candidates for the soluble factors released by neurones to inhibit DRG glial cells. We observed again that cicaprost-stimulated AC activity in DRG glial cells was inhibited by 64% following direct contact with DRG neurones ($P < 0.001$) and by 38% due to the release of soluble factors ($P < 0.01$) (Figure 9). To investigate the role of ATP, DRG cells were pretreated with the non-specific purine receptor antagonist suramin (100 $\mu mol \cdot L^{-1}$), but no evidence for attenuation of neuronal inhibition of cicaprost-stimulated glial cell responses was observed using the transwell assay system (Figure 9A and B: $P > 0.05$, two-way ANOVA). Glutamate can stimulate both ionotropic and metabotropic glutamate receptors; therefore, DRG cells were pretreated with the AMPA and kainate receptor antagonist CNQX (20 $\mu mol \cdot L^{-1}$) plus the NMDA receptor antagonist MK801 (10 $\mu mol \cdot L^{-1}$) to inhibit ionotropic glutamate receptors. To inhibit metabotropic glutamate receptors, we used the $mGlu_2$ and $mGlu_3$ receptor antagonist CPPG (10 $\mu mol \cdot L^{-1}$) plus the $mGlu_1$ and $mGlu_2$ receptor antagonist E4CPG (10 $\mu mol \cdot L^{-1}$). No evidence for attenuation of neuronal inhibition of cicaprost-stimulated glial cell responses by glutamate receptor antagonists was observed using the transwell assay system (Figure 9C–F: $P > 0.05$, two-way ANOVA).

Again, there was no strong evidence here that neurones inhibit forskolin-stimulated [^{3}H]-cAMP production in DRG glial cells (Figure 9) as the response in group B was equivalent to the sum of the responses in groups C1 and C2. However, we observed a consistent 26% inhibition ($P > 0.05$) of forskolin-stimulated AC activity in glial cells that had been exposed to neurones (group C1), suggesting that there might be an interaction that is masked by the relatively large contribution of forskolin-stimulated [^{3}H]-cAMP generated by neurones in the mixed cell cultures (group B). To investigate this further, we compared agonist-dependent and agonist-independent AC activity in mixed DRG cell cultures, neurone-enriched cell cultures and pure glial cell cultures (Figure 10). Single concentrations of AC activators were chosen in this study based on the EC_{50} value for cicaprost-stimulated [^{3}H]-cAMP production in mixed DRG cells, and concentrations of PGE_2 and forskolin chosen to generate similar responses (Ng *et al.*, 2011). Like PGE_2, the single concentration of CGRP chosen was also a near-maximally effective concentration (Figure 5), but full log agonist concentration–response curves were not obtained for the β_2-adrenoceptor agonists. Nevertheless, the agonist response profiles suggest that the expression of CGRP receptors and/or their coupling to AC in DRG glial cells was greater than IP receptors, which was greater than EP_4 receptors (Figure 10H). In contrast, the CGRP and IP receptor-stimulated responses in neurone-enriched cell cultures were relatively weak compared with responses to agonists for other G_s-coupled GPCRs.

Discussion

Damage to the peripheral nervous system often results in hyperexcitability of DRG neurones, and the enhanced sensitivity of these primary sensory neurones to cAMP is maintained *in vitro* (Zheng *et al.*, 2007). Nevertheless, much remains to be understood about the role of cAMP in pain-related states (Inceoglu *et al.*, 2011), and a more complete understanding of the factors regulating cAMP production in dorsal root ganglia is essential. Our discovery of EP_4 and IP receptor expression in adult rat DRG glial cells *in vitro* in typical DRG cell preparations was unexpected (Ng *et al.*, 2011). In the current study, we further demonstrated that EP_4 and IP receptor-dependent AC responses in DRG neurone-enriched cell cultures were much weaker than those in typical mixed DRG cell cultures containing similar numbers of neurones but many more glial cells. In adult mouse DRG cell preparations, the expression of other pain-related signalling components falls in the first 2 days in culture and remains low unless NGF is added (Franklin *et al.*, 2009). Both PGE_2 and prostacyclin mediate their hyper-nociceptive responses in part by stimulating receptors on peptidergic neurones and potentiating neuropeptide release (Hingtgen *et al.*, 1995). Even though the majority of peptidergic DRG neurones are responsive to NGF (Averill *et al.*, 1995), we found that the addition of NGF did not enhance EP_4 and IP receptor agonist-stimulated responses in neurone-enriched DRG cell cultures. The neurone-enriched DRG cell preparation was similarly least responsive to the other G_s-coupled GPCR agonists studied herein, i.e. CGRP, formoterol and isoprenaline. When IP receptor-stimulated responses in mixed DRG cells were previously measured over a 5 day period, the weak response after 16 h in culture was assumed to result from a lack of time for neurones to adopt the typical phenotype of primary sensory neurones (Wise, 2006). The results from the current study challenge this assumption and suggest that the bulk of cAMP generated in typical DRG cell cultures derives from glial cells rather than neurones, and time is needed for these glial cells to express EP_4 and IP receptors.

CGRP responses are expected to be present in DRG glial cells, because CGRP receptor components have been identified in rat trigeminal ganglia in both neurones and SGCs *in vivo* and *in vitro* (Zhang *et al.*, 2007b; Li *et al.*, 2008). We found that the responses to CGRP in neurone-enriched cultures were relatively weak, but readily detectable in glial cell-containing DRG cell cultures, where they were significantly inhibited by $CGRP_{8-37}$, suggesting the presence of classical $CGRP_1$ receptors. The lack of complete inhibition of 1 $\mu mol \cdot L^{-1}$ CGRP-stimulated [^{3}H]-cAMP production by $CGRP_{8-37}$ (10 $\mu mol \cdot L^{-1}$) does not necessarily implicate a novel CGRP receptor in DRG cells, but results from using a near maximally effective concentration of CGRP. Nevertheless, the insurmountable antagonism displayed by $CGRP_{8-37}$ against a complete log concentration–response curve for CGRP was unexpected and might be due to a slow dissociating property of the antagonist (Lew *et al.*, 2000). The predominant second messenger system for CGRP receptors in neurones is reported to be cAMP (Ceruti *et al.*, 2011), and our data suggest this might also be the case for glial cells.

Adrenaline produces β_2-adrenoceptor-mediated mechanical hyperalgesia and sensitization of rat nociceptors *in vitro*

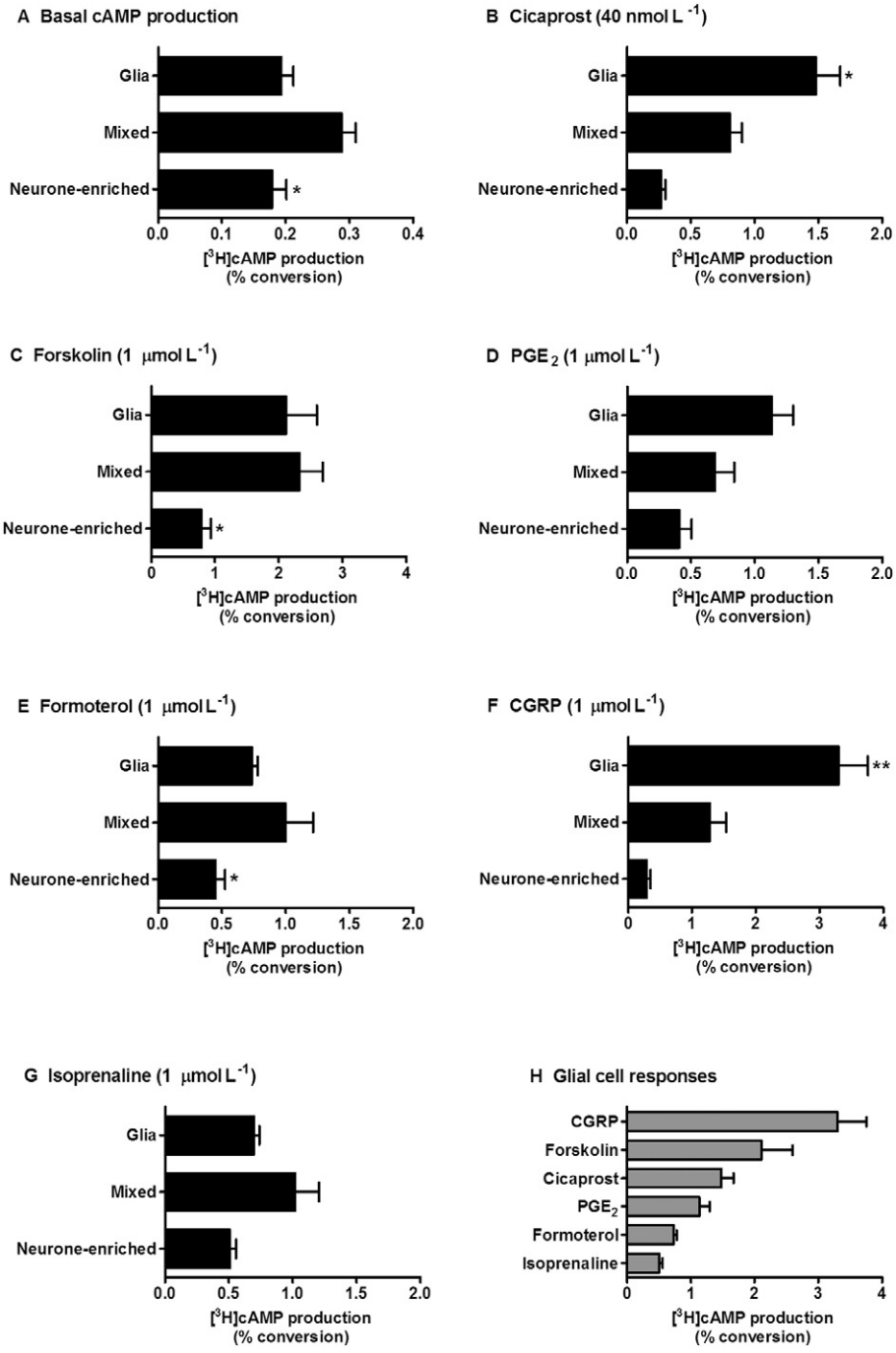

Figure 9

Summary of AC activity in mixed DRG cells, neurone-enriched cell cultures and glial cell cultures. Treatment groups A, C, E and G show similar responses in mixed DRG cell cultures and pure glial cell cultures, presumably because of the relatively large response from neurones. In contrast, treatments B, D and F show an apparent inhibitory effect of neurones in mixed DRG cell cultures. *$P < 0.05$, **$P < 0.01$ compared with mixed DRG cell responses; one-way ANOVA. A direct comparison of glial cell responses to the seven treatments is shown in (H). Data taken from Figures 1, 5 and 6.

(Khasar *et al.*, 1999; Aley *et al.*, 2001). Although we detected β_2-adrenoceptors in our neurone-enriched cell cultures and pure glial cell cultures, evidence for the *in vivo* expression of β_2-adrenoceptors in DRG is lacking (Nicholson *et al.*, 2005; Leon *et al.*, 2008). β-adrenoceptor-mediated responses in isolated rat DRG cells may be dependent on time in culture, with hyper-nociceptive β_2-adrenoceptor responses dominating in short-term cultures (Pluteanu *et al.*, 2002). The hypothesis that isolated DRG cells represent cells responding to axotomy is widely supported (LaMotte, 2007; Zheng *et al.*, 2007), so

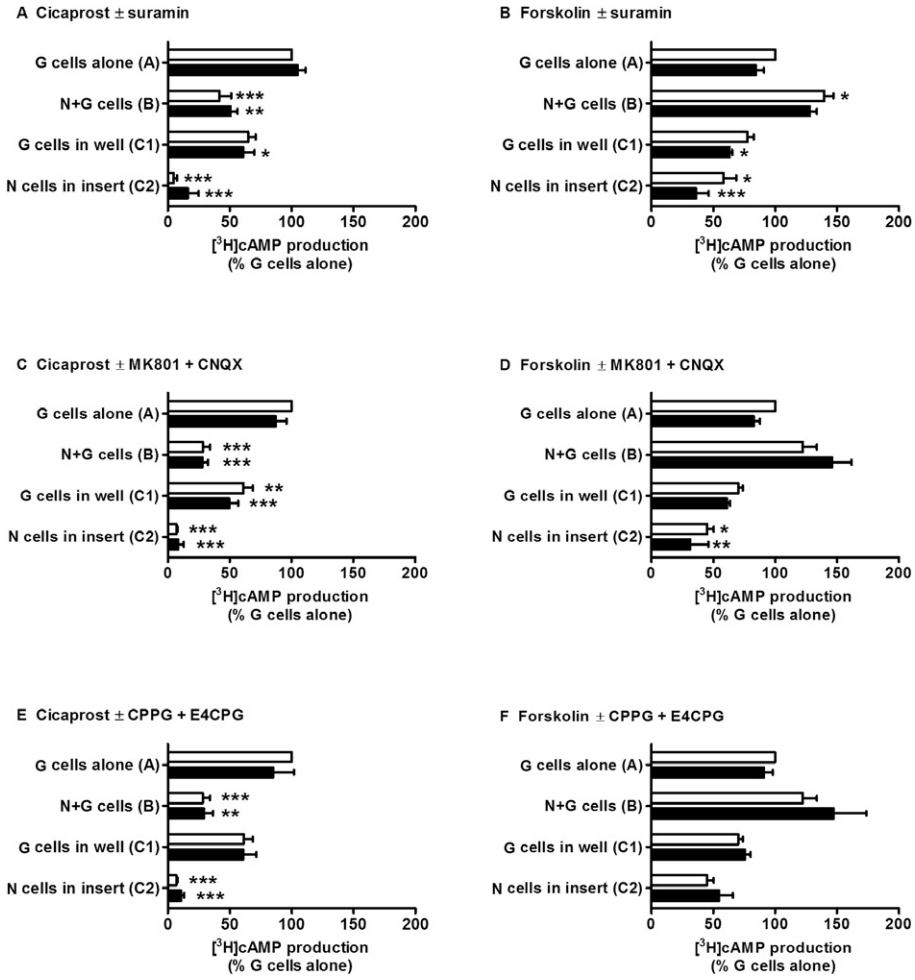

Figure 10

Neuronal inhibition of glial cell AC activity is independent of purine and glutamine receptors. DRG cell preparations were cultured in transwell dishes and assayed as described in Figure 8. AC activity was determined in response to (A) cicaprost (40 nmol·L^{-1}) ± suramin (100 μmol·L^{-1}); (B) forskolin (1 μmol·L^{-1}) ± suramin (100 μmol·L^{-1}); (C) cicaprost (40 nmol·L^{-1}) ± MK801 (10 μmol·L^{-1}) plus CNQX (20 μmol·L^{-1}); (D) forskolin (1 μmol·L^{-1}) ± MK801 (10 μmol·L^{-1}) plus CNQX (20 μmol·L^{-1}); (E) cicaprost (40 nmol·L^{-1}) ± CPPG (10 μmol·L^{-1}) plus E4CPG (10 μmol·L^{-1}); (F) forskolin (1 μmol·L^{-1}) ± CPPG (10 μmol·L^{-1}) plus E4CPG (10 μmol·L^{-1}). Control responses (group A) to cicaprost were 1.48 ± 0.28% conversion and to forskolin were 1.39 ± 0.26% conversion (n = 4). Data are presented as means ± SEM, with each inhibitor group tested in three independent experiments. AC activity compared with control responses of glial cells alone (group A) in each experiment. *P < 0.05, **P < 0.01 and ***P < 0.001 for comparison with glial cells alone (group A); one-way ANOVA.

the up-regulation (Ceruti *et al.*, 2011; Ng *et al.*, 2011) or down-regulation of GPCRs (Franklin *et al.*, 2009) relative to expression in whole DRG may reflect responses to nerve injury. Therefore, both time-dependent and cell type-dependent receptor expression needs to be clarified in mixed DRG cell cultures to allow for accurate interpretation of experimental paradigms.

Not only were AC responses in neurone-enriched cell preparations lower than anticipated, the relative size of the EP_4 and IP receptor-dependent responses in glial cells was also higher than responses of mixed DRG cells containing comparable numbers of glial cells. Furthermore, even though the IB4-positive and IB4-negative cell fractions contained similar numbers of neurones, the AC responses in the IB4-positive cell cultures were the smallest (Figure 1D), and this fraction contained the lowest proportion of glial cells (23% in IB4-positive cells vs. 37% in IB4-negative cells). The proportion of glial cells in a population of mixed DRG cells on the day of assay was >80% (Figure 2A). We therefore propose that most of the [^{3}H]-cAMP response determined in mixed DRG cell cultures derived not from neurones but from glial cells. Furthermore, because [^{3}H]-cAMP production decreased in neurone-containing cultures, we hypothesize that this glial response is inhibited by the presence of DRG neurones.

By selectively radiolabelling either neurones or glial cells with [^{3}H]-adenine, we were able to confirm that EP_4 and IP receptor-stimulated [^{3}H]-cAMP production by DRG glial cells was indeed inhibited by the presence of DRG neurones, but responses to forskolin were unaffected. We hypothesized that if AC responses to any G_s-coupled GPCR were higher in glial cells compared with mixed DRG cells, then neurones were inhibiting glial cell responses. Thus, CGRP receptor responses

in glial cells were inhibited by DRG neurones but β_2-adrenoceptor-mediated responses were not. This pattern of responses with CGRP, EP_4 and IP receptors having a different profile from those of β_2-adrenoceptors and forskolin was reminiscent of our earlier study, where we had mistakenly presumed that these G_s-GPCRs were expressed only on DRG neurones (Rowlands *et al.*, 2001).

The lack of effect of neurone-conditioned medium on glial cell responses to EP_4 and IP receptor agonists suggested that cell–cell contact was required to observe this inhibitory phenomenon, and/or a highly unstable soluble factor was involved. But when glial cells were co-cultured with neurone-enriched cells, but separated in a transwell culture system, IP receptor responses in glial cells were still inhibited, but to a lesser extent than when the two cell groups were in direct contact. Thus, the inhibitory effect of DRG neurones on DRG glial cell responses to IP receptor agonists is most likely mediated through a combination of cell–cell interactions and the release of unstable soluble factors. In contrast, the forskolin-stimulated response of glial cells in the transwell system was not significantly inhibited by neurone-dependent factors; therefore, it is unlikely that this inhibitory effect on IP receptor signalling is mediated via activation of $G_{i/o}$-protein coupled receptors. By comparing the profile of DRG cell responses to agonist-dependent and agonist-independent activation of AC, we see that the ligands fall into two distinct groups (Figure 10). We propose that the AC stimulating properties of CGRP, EP_4 and IP receptors in DRG glial cells are inhibited by the presence of DRG neurones, but we obtained no direct evidence for a similar inhibition of β_2-adrenoceptors and forskolin-stimulated responses.

Potential candidates for the inhibitory factor derived from neurones are ATP and glutamate (Zhang *et al.*, 2007a; Gu *et al.*, 2010), but inhibition of purine receptors or glutamine receptors did not attenuate the inhibitory effect of neurones on cicaprost responses in glial cells, nor did we observe any effect of these antagonists on forskolin-stimulated responses. Rat SGCs express $P2Y_2$ and $P2Y_4$ receptors (Villa *et al.*, 2010), and when a higher concentration of suramin was used to more effectively block $P2Y_4$ receptors (Wildman *et al.*, 2003) direct inhibition of cicaprost and forskolin-stimulated [^{3}H]-cAMP production by glial cells was observed (data not shown). Therefore, at the present time, we cannot completely exclude a role for $P2Y_4$ receptors because the classical antagonist Reactive Blue-2 (Wildman *et al.*, 2003) is no longer commercially available.

The precise identity of our glial cells targeted by the inhibitory action of neurones currently remains uncertain. Glutamine synthetase is the classical marker of SGCs in DRG sections, but since both SGCs and Schwann cells express glutamine synthetase (Procacci *et al.*, 2008), we cannot use this marker to distinguish SGCs from Schwann cells in the glial cell cultures. The SGCs in our glial cell preparation have properties similar to astrocytes and microglial cells (Hanani, 2005; van Velzen *et al.*, 2009), and neurone–microglial cell inhibitory signalling is well established (for references, see Ransohoff and Cardona, 2010). Therefore, SGCs may similarly be restrained by inhibitory influences generated by DRG neurones. For the Schwann cell component of our glial cell preparation, axonal contact would be expected to increase cAMP production and drive Schwann cell differentiation into a myelinating phenotype (Morgan *et al.*, 1991; Yoon *et al.*, 2008). Our hypothesis that neurones might be inhibiting Schwann cell cAMP production is apparently contradictory to expectations. According to Yoon *et al.* (2008), the effect of cAMP on Schwann cell differentiation is very much concentration-dependent and is heavily influenced by the presence of growth factors. Our hypothesis is that two days in culture might be too early to observe axonal contact-induced increases in cAMP in Schwann cells in the conditions used for our mixed DRG cell cultures. Therefore, any neuronal inhibition of Schwann cell cAMP production in these short-term cultures could lead to inhibition of Schwann cell differentiation and contribute to the high proportion of GFAP-positive cells observed in our mixed DRG cell cultures.

In conclusion, the presence of CGRP receptors and the up-regulation of β_2-adrenoceptors, EP_4 receptors and IP receptors on DRG glial cells *in vitro* significantly contribute to the overall assessment of AC activity in mixed DRG cell cultures. In future, the role of GPCR-stimulated cAMP-dependent hyper-nociceptive responses in mixed DRG cell cultures can no longer be assumed to result solely from a direct effect of agonists on DRG neurones. Furthermore, receptor-specific inhibition of glial cell AC activity by DRG neurones adds a further complexity to the analysis of DRG cell responses. Because DRG neurones and SGCs form unique functional complexes *in vivo*, the physical relationship between these cell types in culture should be carefully controlled in studies of neurone–glia communication. The inhibitory action of neurones described herein depends on both neurone–glial cell contact and neurone-derived soluble factors, and is unlikely to involve activation of either purine or glutamine receptors. This inhibitory activity of DRG neurones on DRG glial cells may be an important issue to consider for studies of neurone–glial cell communication within intact dorsal root ganglia.

Acknowledgements

We gratefully acknowledge the gifts of ONO-AE1-329 and ONO-AE3-208 from Dr T Maruyama of ONO Pharmaceutical Co. Ltd, Osaka, Japan, and of cicaprost from Schering AG, Berlin, Germany. KYN and BY were supported by studentships from the Chinese University of Hong Kong. This work was supported by a grant from the Research Grants Council of the Hong Kong Special Administrative Region (CUHK4516/06 M) and a Direct Grant from The Chinese University of Hong Kong (CUHK2041446).

Conflict of interest

The authors state no conflict of interest.

References

Abramovitz M, Adam M, Boie Y, Carriere M, Denis D, Godbout C *et al.* (2000). The utilization of recombinant prostanoid receptors to

determine the affinities and selectivities of prostaglandins and related analogs. Biochim Biophys Acta 1483: 285–293.

Ahmed Z, Mazibrada G, Seabright RJ, Dent RG, Berry M, Logan A (2006). TACE-induced cleavage of NgR and p75NTR in dorsal root ganglion cultures disinhibits outgrowth and promotes branching of neurites in the presence of inhibitory CNS myelin. FASEB J 20: 1939–1941.

Alexander SPH, Mathie A, Peters JA (2011). Guide to receptors and channels (GRAC), 5th edition. Br J Pharmacol 164: S1–S324.

Aley KO, Martin A, McMahon T, Mok J, Levine JD, Messing RO (2001). Nociceptor sensitization by extracellular signal-regulated kinases. J Neurosci 21: 6933–6939.

Austin PJ, Moalem-Taylor G (2010). The neuro-immune balance in neuropathic pain: involvement of inflammatory immune cells, immune-like glial cells and cytokines. J Neuroimmunol 229: 26–50.

Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV (1995). Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. Eur J Neurosci 7: 1484–1494.

Barber R, Ray KP, Butcher RW (1980). Turnover of adenosine 3′,5′-monophosphate in WI-38 cultured fibroblasts. Biochemistry 19: 2560–2567.

Brenneis C, Coste O, Altenrath K, Angioni C, Schmidt H, Schuh CD *et al*. (2011). Anti-inflammatory role of microsomal prostaglandin E synthase-1 in a model of neuroinflammation. J Biol Chem 286: 2331–2342.

Ceruti S, Fumagalli M, Villa G, Verderio C, Abbracchio MP (2008). Purinoceptor-mediated calcium signaling in primary neuron-glia trigeminal cultures. Cell Calcium 43: 576–590.

Ceruti S, Villa G, Fumagalli M, Colombo L, Magni G, Zanardelli M *et al*. (2011). Calcitonin gene-related peptide-mediated enhancement of purinergic neuron/glia communication by the algogenic factor bradykinin in mouse trigeminal ganglia from wild-type and R192Q Cav2.1 knock-in mice: implications for basic mechanisms of migraine pain. J Neurosci 31: 3638–3649.

Franklin SL, Davies AM, Wyatt S (2009). Macrophage stimulating protein is a neurotrophic factor for a sub-population of nociceptive sensory neurons. Mol Cell Neurosci 41: 175–185.

Gavazzi, I, Kumar, RDC, McMahon, SB, Cohen, J (1999). Growth responses of different subpopulations of adult sensory neurons to neurotrophic factors *in vitro*. Eur J Neurosci 11: 3405–3414.

Gu Y, Chen Y, Zhang X, Li GW, Wang C, Huang LYM (2010). Neuronal soma-satellite glial cell interactions in sensory ganglia and the participation of purinergic receptors. Neuron Glia Biol 6: 53–62.

Hanani M (2005). Satellite glial cells in sensory ganglia: from form to function. Brain Res Rev 48: 457–476.

Hingtgen CM, Waite KJ, Vasko MR (1995). Prostaglandins facilitate peptide release from rat sensory neurons by activating the adenosine 3′,5′-cyclic monophosphate transduction cascade. J Neurosci 15: 5411–5419.

Inceoglu B, Wagner K, Schebb NH, Morisseau C, Jinks SL, Ulu A *et al*. (2011). Analgesia mediated by soluble epoxide hydrolase inhibitors is dependent on cAMP. Proc Natl Acad Sci U S A 108: 5093–5097.

Julius, D, Basbaum, AI (2001). Molecular mechanisms of nociception. Nature 413: 203–210.

Khasar SG, McCarter G, Levine JD (1999). Epinephrine produces a β-adrenergic receptor-mediated mechanical hyperalgesia and in vitro sensitization of rat nociceptors. J Neurophysiol 81: 1104–1112.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: Reporting *in vivo* experiments: The ARRIVE Guidelines. Br J Pharmacol 160: 1577–1579.

LaMotte RH (2007). Acutely dissociated sensory neurons: normal or neuropathic? J Neurophysiol 97: 1–2.

Leon LA, Hoffman BE, Gardner SD, Laping NJ, Evans C, Lashinger ESR *et al*. (2008). Effects of the β3-adrenergic receptor agonist disodium 5-[(2R)-2-[[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL-316243) on bladder micturition reflex in spontaneously hypertensive rats. J Pharmacol Exp Ther 326: 178–185.

Lew MJ, Ziogas J, Christopoulos A (2000). Dynamic mechanisms of non-classical antagonism by competitive AT_1 receptor antagonists. Trends Pharmacol Sci 21: 376–381.

Li J, Vause CV, Durham PL (2008). Calcitonin gene-related peptide stimulation of nitric oxide synthesis and release from trigeminal ganglion glial cells. Brain Res 1196: 22–32.

McGrath J, Drummond G, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

McMahon SB, Cafferty WBJ, Marchand F (2005). Immune and glial cell factors as pain mediators and modulators. Exp Neurol 192: 444–462.

Morgan L, Jessen KR, Mirsky R (1991). The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (04^+) to a myelin phenotype (P_o^+, $GFAP^-$, $N\text{-}CAM^-$, NGF-receptor$^-$) depends on growth inhibition. J Cell Biol 112: 457–467.

Ng KY, Wong YH, Wise H (2010). The role of glial cells in influencing neurite extension by dorsal root ganglion cells. Neuron Glia Biol 6: 19–29.

Ng KY, Wong YH, Wise H (2011). Glial cells isolated from dorsal root ganglia express prostaglandin E_2 (EP_4) and prostacyclin (IP) receptors. Eur J Pharmacol 661: 42–48.

Nicholson R, Dixon AK, Spanswick D, Lee K (2005). Noradrenergic receptor mRNA expression in adult rat superficial dorsal horn and dorsal root ganglion neurons. Neurosci Lett 380: 316–321.

Noda M, Kariura Y, Pannasch U, Nishikawa K, Wang L, Seike T *et al*. (2007). Neuroprotective role of bradykinin because of the attenuation of pro-inflammatory cytokine release from activated microglia. J Neurochem 101: 397–410.

Oida H, Namba T, Sugimoto Y, Ushikubi F, Ohishi H, Ichikawa A *et al*. (1995). *In situ* hybridization studies of prostacyclin receptor mRNA expression in various mouse organs. Br J Pharmacol 116: 2828–2837.

Pluteanu F, Ristoiu V, Flonta ML, Reid G (2002). α_1-adrenoceptor-mediated depolarization and β-mediated hyperpolarization in cultured rat dorsal root ganglion neurones. Neurosci Lett 329: 277–280.

Procacci P, Magnaghi V, Pannese E (2008). Perineuronal satellite cells in mouse spinal ganglia express the gap junction protein connexin43 throughout life with decline in old age. Brain Res Bull 75: 562–569.

Ransohoff RM, Cardona AE (2010). The myeloid cells of the central nervous system parenchyma. Nature 468: 253–262.

Ren K, Dubner R (2010). Interactions between the immune and nervous systems in pain. Nat Med 16: 1267–1276.

Rowlands DK, Kao CL, Wise H (2001). Regulation of prostacyclin and prostaglandin E_2 receptor mediated responses in adult rat dorsal root ganglion cells, *in vitro*. Br J Pharmacol 133: 13–22.

Sachs D, Villarreal CF, Cunha FQ, Parada CA, Ferreira SH (2009). The role of PKA and PKCε pathways in prostaglandin E_2-mediated hypernociception. Br J Pharmacol 156: 826–834.

Scholz J, Woolf CJ (2007). The neuropathic pain triad: neurons, immune cells and glia. Nat Neurosci 10: 1361–1368.

van Velzen M, Laman JD, KleinJan A, Poot A, Osterhaus ADME, Verjans GMGM (2009). Neuron-interacting satellite glial cells in human trigeminal ganglia have an APC phenotype. J Immunol 183: 2456–2461.

Villa G, Fumagalli M, Verderio C, Abbracchio MP, Ceruti S (2010). Expression and contribution of satellite glial cells purinoceptors to pain transmission in sensory ganglia: an update. Neuron Glia Biol 6: 31–42.

Wildman SS, Unwin RJ, King BF (2003). Extended pharmacological profiles of rat $P2Y_2$ and rat $P2Y_4$ receptors and their sensitivity to extracellular H^+ and Zn^{2+} ions. Br J Pharmacol 140: 1177–1186.

Wise H (2006). Lack of interaction between prostaglandin E_2 receptor subtypes in regulating adenylyl cyclase activity in cultured rat dorsal root ganglion cells. Eur J Pharmacol 535: 69–77.

Yoon C, Korade Z, Carter BD (2008). Protein kinase A-induced phosphorylation of the p65 subunit of nuclear factor-κB promotes Schwann cell differentiation into a myelinating phenotype. J Neurosci 28: 3738–3746.

Zhang X, Chen Y, Wang C, Huang L-YM (2007a). Neuronal somatic ATP release triggers neuron-satellite glial cell communication in dorsal root ganglia. Proc Natl Acad Sci USA 104: 9864–9869.

Zhang Z, Winborn CS, Marquez de Prado B, Russo AF (2007b). Sensitization of calcitonin gene-related peptide receptors by receptor activity-modifying protein-1 in the trigeminal ganglion. J Neurosci 27: 2693–2703.

Zheng JH, Walters ET, Song XJ (2007). Dissociation of dorsal root ganglion neurons induces hyperexcitability that is maintained by increased responsiveness to cAMP and cGMP. J Neurophysiol 97: 15–25.