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Glial cells isolated from dorsal root ganglia express prostaglandin E_2 (EP_4) and prostacyclin (IP) receptors

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ABSTRACT

Isolated cells from adult rat dorsal root ganglia (DRG) are frequently used as a model system to study responses of primary sensory neurons to nociceptor sensitizing agents such as prostaglandin E_2 and prostacyclin, which are presumed to act only on the neurons in typical mixed cell cultures. In the present study, we evaluated the expression of prostaglandin E_2 (EP_4) and prostacyclin (IP) receptors in cultures of mixed DRG cells and in purified DRG glia. We show here that EP_4 and IP receptor agonists stimulated adenylyl cyclase activity in both mixed DRG cells and in purified DRG glia, and that these responses were specifically inhibited by EP_4 and IP receptor antagonists, respectively. The presence of EP_4 and IP receptors in DRG glia was further confirmed by the expression of EP_4 and IP receptor immunoreactivity and mRNA. With the increasing awareness of neuron-glial interactions within intact DRG and the use of isolated DRG cells in the study of mechanisms underlying nociception, it will be essential to consider the role played by EP_4 and IP receptor-expressing glial cells when evaluating prostanoid-induced sensitization of DRG neurons.

1. Introduction

Cultures of rat dorsal root ganglion (DRG) cells are commonly used as a model system to study the mechanisms underlying nociception and hyperalgesia (Gold et al., 1996). From such studies, prostaglandin E_2 (PGE_2) and prostacyclin have been shown to potentiate electrical currents (Lopshire and Nicol, 1998; Vaughan, 1998), to potentiate capsaicin-induced release of calcitonin gene related peptide and substance P (Southall and Vasko, 2001), and to prevent desensitization of the vanilloid receptor TRPV1 (Schnizler et al., 2008). While some of the effects of PGE_2 are protein kinase C-dependent (Sachs et al., 2009), the majority of potentiating effects of PGE_2 and prostacyclin are protein kinase A-dependent (Cui and Nicol, 1995; Ferreira and Nakamura, 1979; Hingtgen et al., 1995; Lopshire and Nicol, 1998).

In DRG cell cultures, the PGE_2 receptor subtype EP_4 and the prostacyclin (IP) receptor have been identified as the principal Gs-coupled prostanoid receptors likely to mediate the protein kinase A-dependent effects of these prostanoids (Bley et al., 1998; Rowlands et al., 2001; Smith et al., 1998; Wise, 2006). In DRG slices, IP receptor mRNA is predominantly expressed in peptidergic DRG neurons, and 24% of these neurons co-express EP_4 receptor mRNA (Oida et al., 1995). With the availability of the EP_4 receptor specific antagonist ONO-AE3-208 (Kabashima et al., 2002) and the IP receptor specific antagonist RO1138452 (also known as CAY10441; (Bley et al. (2006)), we decided to further investigate EP_4 and IP-dependent signalling in DRG cells.

DRG cell cultures from adult rats comprise a heterogeneous population of cells, morphologically distinguishable as phase-bright neurons and phase-dark non-neuronal cells. The non-neuronal cell population are principally Schwann cells and satellite glial cells, along with fibroblasts. The majority of non-neuronal cells in our DRG cell cultures express glial fibrillary acidic protein (GFAP) (Ng et al., 2010) and will therefore be referred to herein as glial cells. It is clear from *in vitro* studies and in animal models of inflammatory and neuropathic pain that neuron-glial interactions are crucial in pain processing (Ren and Dubner, 2010). Therefore, we have compared EP_4 and IP receptor responses in classical mixed DRG cell cultures with responses of purified DRG glial cells.

Herein, we provide further evidence that PGE_2 and the prostacyclin mimetic cicaprost activate adenylyl cyclase activity in DRG cell cultures via EP_4 and IP receptors, respectively. In addition, we demonstrate that DRG glial cells *in vitro* also express both EP_4 and IP receptors. This apparent up-regulation of EP_4 and IP receptors on DRG glial cells *in vitro* may subserve a similar anti-inflammatory role as EP_2 receptors in spinal cord microglial cells (Brenneis et al., 2011).

In conclusion, when cultures of adult rat DRG cells are used in any assessment of the nociceptor sensitization properties of PGE_2 and prostacyclin, it is essential to consider that EP_4 and IP receptor agonists/antagonists may also affect the activity of the associated glial cells, and to consider that interactions between DRG neurons and glial cells may affect the functional responses under investigation.

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2. Materials and methods

2.1. Reagents

Cicaprost was a gift from Schering AG (Berlin, Germany); ONO-AE1-259 [16S-deoxy-9β-chloro-15-deoxy-16-hydroxy-17,17-trimethylene-19,20-didehydro-PGF_2], ONO-AE1-329 [16-(3-methoxymethyl)phenyl-ω-tetranor-3,7-dithia-PGE_1] and ONO-AE3-208 [4-{4-cyano-2-[2-(4-fluoronaphthalen-1-yl) propionylamino] phenyl} butyric acid] were gifts from Dr. T. Maruyama of ONO Pharmaceutical Co. Ltd. (Osaka, Japan); AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid) and RO1138452 (CAY10441: 4,5-dihydro-N-[4-[[4-(1-methylethoxy) phenyl]methyl]phenyl]-1H-imadazol-2-amine) were purchased from Cayman Chemical (Ann Arbor, MI, USA) and PGE_2 was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of mixed DRG cell cultures

All experiments were performed under licence 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. The dorsal root ganglia were removed from all levels of the spinal cord of male Sprague-Dawley rats (150–200 g) and cultures were prepared as described previously (Ng et al., 2010). The cell pellet was resuspended in Ham's F14 medium and the morphologically distinct phase-bright neurons were counted using a haemocytometer slide. Cells were seeded at 5000 neurons/well onto 24-well tissue culture plates precoated with poly-DL-ornithine (500 μg/ml) and laminin (5 μg/ml), and assayed after 2 days in culture in an atmosphere of 5% CO_2 at 37 °C.

2.3. Preparation of glial cell cultures

The mixed cells were plated on poly-DL-ornithine coated tissue culture dishes (2 rats per 10 cm dish) and loosely attached neuronal cells were gently removed from the more firmly attached glial cells after overnight incubation. The remaining glial cells were harvested using trypsin (0.05% in phosphate-buffered saline (PBS)), and seeded onto poly-DL-ornithine/laminin-coated 24-well tissue culture plates to mimic conditions in mixed DRG cell cultures. DRG glial cells (small phase-dark cells) and any contaminating neurons (larger phase-bright cells) were counted using a haemocytometer slide and plated at 10000 glial cells per well, and assayed after 1 day in culture so that cell age (days *in vitro*) and cell numbers are equivalent to those in the mixed cell cultures on the day of assay.

2.4. Measurement of [^{3}H]cAMP production

Adenylyl cyclase activity was assayed as described previously (Wise, 2006). DRG cells were incubated with antagonists for 15 min prior to addition of agonist for a further 30 min at 37 °C in assay buffer (Hepes-buffered saline (HBS: 15 mM Hepes, pH 7.5; 140 mM NaCl; 4.7 mM KCl; 2.2 mM$CaCl_2.2H_2O$; 1.2 mM $MgCl_2.6H_2O$; 1.2 mM KH_2PO_4; 3.3 mM glucose) containing 1 mM 3-isobutyl-1-methyl xanthine (IBMX) to inhibit cyclic nucleotide phosphodiesterase activity). The production of [^{3}H]cAMP from cellular [^{3}H]ATP was estimated as the ratio of radiolabeled cAMP to total AXP (i.e. adenosine, ADP, ATP and cAMP), and is expressed as [cAMP]/[total AXP]×100 (i.e. % conversion), according to Maurice and Haslam (1990) and Salomon (1991). All assays were performed in duplicate.

2.5. cAMP ELISA assay

Cells were washed twice with HBS and incubated, in duplicate, for 30 min at 37 °C in HBS containing IBMX (1 mM). The reaction was stopped by addition of dodecyltrimethylammonium bromide, at a final concentration of 0.25%, and the concentration of cAMP was determined using a cAMP Biotrak Enzymeimmunoassay System, following the manufacturer's protocol.

2.6. Immunostaining

DRG cells were cultured on glass coverslips and were fixed using paraformaldehyde (4% in PBS) for 15 min, permeabilized with Triton X-100 (0.01% in PBS) for 15 min, then blocked with donkey serum (3%) in PBS for 30 min. Primary antibodies used recognised EP_4 receptor (1:500) and IP receptor (1:500) (Cayman Chemical, Ann Arbor, MI, USA). Specificity of immunostaining was demonstrated by loss of signal after preincubating primary antibodies with EP_4 or IP receptor antibody blocking peptides, respectively, (data not shown). Neurons were identified with antibodies recognising neuron-specific class III β-tubulin (TUJ-1,1:500; Abcam, Cambridge, MA, USA) and glial cells with antibodies recognising glial fibrillary acidic protein (Cy3-conjugated GFAP, 1:500; Sigma, St. Louis, MO, USA). Cells were incubated with primary antibodies at 4 °C overnight, followed by FITC- and Cy3-conjugated secondary antibodies (1:500; Jackson Immunoresearch Laboratories, West Grove, PA, USA), as appropriate. Hoechst 33342 stain (0.2 μg/ml; Invitrogen, Carlsbad, CA, USA) was used to identify cell nuclei. The coverslips were mounted with glycerol (90%) on glass slides and imaged using an Olympus FV1000-ZCD laser scanning confocal system fitted with an IX81 inverted microscope (Center Valley, PA, USA). Fluorescent images were merged using Adobe Photoshop CS3 (San Jose, CA, USA).

2.7. RT-PCR

Total RNA was extracted with TRIZOL (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Invitrogen, Carlsbad, CA, USA) to eliminate any contaminating genomic DNA. First-strand complementary DNA was synthesized from 2 μg RNA using SuperScript™ III First-Strand Synthesis SuperMix system (Invitrogen, Carlsbad, CA, USA). Negative control samples were generated in the absence of reverse transcriptase. An aliquot of each reaction was subsequently used as the template for PCR using FastStart High Fidelity PCR System with Taq DNA Polymerase (Roche Diagnostics, Hong Kong). The primers for rat EP_4 and IP were as described by Southall and Vasko (2001) and GAPDH was from Wu et al. (2009). PCR was initiated by a 2 min denaturation at 94 °C, followed by 42 cycles (for EP_4 and IP) of 45 s at 94 °C, 45 s at 60 °C, and 45 s at 72 °C, or 36 cycles (for GAPDH) of 45 s at 94 °C, 45 s at 53 °C, and 45 s at 72 °C and terminated by a final extension for 10 min at 72 °C. The PCR products were resolved using a 1.5% agarose gel (Invitrogen, Carlsbad, CA, USA) containing GelRed™ (Invitrogen, Carlsbad, CA, USA) and the DNA bands were visualized by UV illumination. The relationship between the amount of PCR products and the number of amplification cycles (32–42 for EP_4 and IP, and 36–42 for GAPDH) was linear (data not shown).

2.8. Data analysis

Values for EC_{50} and E_{max}, the maximum effect, were obtained by fitting the log concentration-response curves to the standard four-parameter logistic equation using GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, USA). pA_2 values were calculated using the Schild equation: $DR-1=[B]/K_B$. Values reported are means ± S.E.M. Comparisons between groups were made using ANOVA (analysis of variance) with Bonferroni's post-tests, or the extra sum-of-squares F-test, as appropriate. Statistical significance was taken as $P<0.05$.

2. Results

3.1. Distinct EP_4 and IP receptor-mediated activation of adenylyl cyclase in DRG cell cultures

Agonist-based analysis of receptor profiles in mixed DRG cell cultures confirmed our previous work (Wise, 2006), with pEC_{50} values of 7.38 ± 0.06 for cicaprost, 6.86 ± 0.19 for PGE_2, and 7.02 ± 0.10 for ONO-AE1-329 (EP_4 receptor agonist (Cao et al., 2002)) (Fig. 1A). Maximal responses to PGE_2 and ONO-AE1-329 were identical, and significantly lower than that of cicaprost ($P<0.01$; one-way ANOVA).

The IP receptor antagonist RO1138452 has a pK_i value of 9.0 for inhibition of cAMP accumulation in CHO-K1 cells stably expressing the human IP receptor (Bley et al., 2006) and a pA_2 of 8.2 in human pulmonary artery (Jones et al., 2006), therefore we used a single concentration of 100 nM to selectively inhibit IP receptors in rat DRG cells. Pretreatment of mixed DRG cells with RO1138452 shifted the cicaprost log concentration-response curve to the right, with an estimated pA_2 of 8.3 (Fig. 1B; pEC_{50} 5.98 ± 0.12, $P<0.001$, one-way ANOVA). According to the curve fitting analysis, the maximum response to cicaprost was significantly inhibited by RO1138452 ($P<0.001$; extra sum-of-squares F-test). RO1138452 was selective for inhibiting IP receptors as it showed no effect on responses to PGE_2 (Fig. 1C; pEC_{50} 6.96 ± 0.04) or ONO-AE1-329 (Fig. 1D; pEC_{50} 7.27 ± 0.15).

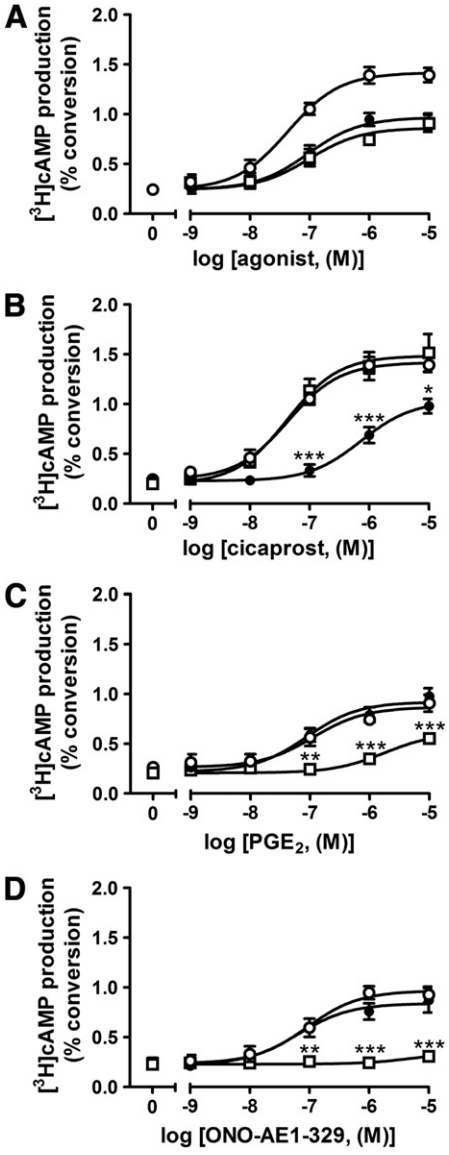

Fig. 1. PGE_2 and cicaprost specifically stimulate EP_4 and IP receptors, respectively, in mixed cultures of DRG cells. (A) Cells were incubated with increasing concentrations of the IP receptor agonist cicaprost [○], the EP receptor agonist PGE_2 [□], or the EP_4 receptor agonist ONO-AE1-329 [●]. (B,C and D) The effect of the IP receptor antagonist RO1138452 (100 nM, [●]) or the EP_4 receptor antagonist ONO-AE3-208 (100 nM, [□]) on agonist concentration-response curves [○]. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with control responses in absence of antagonist; two-way ANOVA. Data are presented as mean ± S.E.M., from 3 independent experiments. Error bars smaller than the symbol size are not shown.

ONO-AE3-208 is a potent EP_4 antagonist with a pK_i of 8.9 (Kabashima et al., 2002), therefore it was also used at 100 nM. ONO-AE3-208 shifted the PGE_2 log concentration-response curve to the right producing an estimated pA_2 of 8.3 (Fig. 1C; pEC_{50} 5.42 ± 0.19, $P<0.01$, one-way ANOVA), and completely antagonised ONO-AE1-329 (Fig. 1D). ONO-AE3-208 was selective for inhibiting EP_4 receptors as it did not affect cicaprost-stimulated responses (Fig. 1B; pEC_{50} 7.39 ± 0.09). A comparison of the non-linear curve fits for PGE_2 in the presence and absence of ONO-AE3-208 showed no significant effect on the maximum response to PGE_2, although higher concentrations of PGE_2 would be necessary to prove this. However, given that the pEC_{50} values for both PGE_2 and ONO-AE1-329 were similar, the remaining response to PGE_2 in the presence of ONO-AE3-208 could be due to activation of Gs-coupled EP_2 receptors. To resolve this issue, we compared the effect of ONO-AE3-208 with that of the EP1/EP2 antagonist AH6809 (10 μM) on maximal responses to PGE_2 (10 μM). PGE_2-stimulated [^{3}H]cAMP production was 1.24 ± 0.11 and 1.18 ± 0.08% conversion in the absence and presence of AH6809, respectively (means ± S.E.M., n = 3). ONO-AE3-208 decreased PGE_2 responses to 0.71 ± 0.10% conversion ($P<0.001$; two-way ANOVA) which was unaffected by co-treatment with AH6809 (0.58 ± 0.04 % conversion). The EP_2 agonist ONO-AE1-259 (100 nM) failed to significantly increase [^{3}H]cAMP production in mixed cell cultures (0.34 ± 0.03 and 0.49 ± 0.07% conversion for basal and ONO-AE1-259 stimulated cells, respectively).

3.2. Evidence for EP_4 and IP receptor-dependent responses in DRG glial cells: adenylyl cyclase activity

The EP_4 and IP receptor-mediated signalling in DRG cell cultures is expected to arise from activity in neurons, as EP_4 and IP receptor mRNA has been identified exclusively on neurons of rodent DRG slices (Kopp et al., 2004; Lin et al., 2006; Oida et al., 1995). The proportion of neurons in our DRG cell preparation after 2 days in culture is reduced from 50 ± 1% to 16 ± 1% (mean ± S.E.M., n = 5), due to the proliferation of glial cells, and even addition of arabinoside C, 5-fluoro-2′-deoxyuridine (50 μM) and uridine (150 μM) failed to have any marked effect on glial cell numbers at this time point (data not shown). By using the differential adhesion properties of neurons and glial cells (Lindsay, 1988) we generated a pure population of glial cells (98 ± 1% purity; mean ± S.E.M., n = 5), as shown in Fig. 2D. DRG neurons were readily identifiable under phase-contrast microscopy by their typical phase-bright spherical neuronal cell bodies (see Fig. 2C) which were absent in the glial cell preparation shown in Fig. 2D which comprises of phase-dark and flattened cells.

To compare adenylyl cyclase activity in mixed DRG cell cultures and cultures of glial cells, single concentrations of agonists were selected to produce equi-effective responses in the mixed DRG cell preparation. Therefore, we used the EC_{50} concentration of cicaprost (40 nM) and near-maximally effective concentrations of PGE_2 and ONO-AE1-329 (1 μM), which all significantly increased [^{3}H]cAMP production (Fig. 2A and B). As expected, pretreatment of mixed DRG cells with ONO-AE3-208 or RO1138452 significantly inhibited EP_4 and IP receptor-dependent signalling, respectively (Fig. 2A). Surprisingly, EP_4 and IP receptor-dependent signalling was also clearly evident in the glial cell fraction (Fig. 2B).

Basal adenylyl cyclase activity in glial cells was significantly lower than in mixed DRG cell cultures (0.09 ± 0.01 and 0.28 ± 0.03% conversion, respectively; $P<0.001$, one-way ANOVA). When assayed

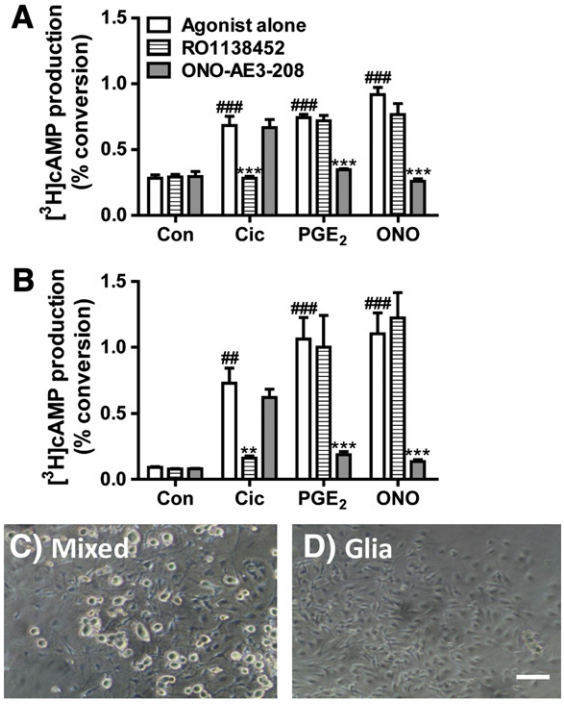

Fig. 2. Evidence for EP_4 and IP-dependent responses in mixed DRG cell cultures (A) and glial cell cultures (B). Adenylyl cyclase activity was determined in DRG cells incubated with Control solution (assay buffer; Con), PGE_2 (1 μM; PGE_2), ONO-AE1-329 (1 μM; ONO) or cicaprost (40 nM; Cic) either alone or following 15 min incubation with RO1138452 (100 nM) or ONO-AE3-208 (100 nM). Data are presented as mean ± S.E.M., from at least 3 independent experiments. ## $P<0.01$ and ### $P<0.001$ compared with Control untreated group; ** $P<0.01$ and *** $P<0.001$ compared with agonist alone group; two-way ANOVA. Representative phase contrast microscopic images of mixed DRG cell cultures (C), glial cell cultures (D). Note the absence of neurons (phase bright cells) in purified glial cell cultures (D). Scale bar = 100 μm.

by an ELISA method, cAMP levels were also lower in glial cell cultures (24 ± 3 and 39 ± 10 fmol/well for glial cells and mixed cell cultures, respectively, mean ± S.E.M., n = 3), suggesting that the lower basal [^{3}H] cAMP production in glial cells was not an artefact of the assay technique. When comparing data in the two different groups of cells by calculating fold-basal activity, all agonist-stimulated responses were significantly higher in glial cells ($P<0.01$; two-way ANOVA). For example, cicaprost (40 nM)-stimulated activity in mixed and glial cells was 2.47 ± 0.49 and 8.07 ± 1.29 fold basal, respectively, and PGE_2 (1 μM)-stimulated activity in mixed and glial cells was 2.99 ± 0.50 and 11.84 ± 1.89 fold basal, respectively.

To more fully characterise cicaprost and PGE_2-stimulated adenylyl cyclase activity in glial cells, full log agonist concentration-response curves were generated and compared with parent mixed DRG cell cultures (Fig. 3). pEC_{50} values for cicaprost were similar in mixed and glial cell cultures (7.50 ± 0.46 and 7.23 ± 0.28, respectively; mean ± S.E.M., n = 3). In addition, pEC_{50} values for PGE_2 were also similar in mixed and glial cell cultures (6.72 ± 0.49 and 6.42 ± 0.26). As observed previously in mixed cell cultures, maximal responses to PGE_2 in glial cells were significantly lower than responses to cicaprost ($P<0.001$; one-way ANOVA). Furthermore, maximal responses of mixed DRG cells were 23% ($P<0.001$; one-way ANOVA) and 50% of cicaprost and PGE_2 responses in glial cells, despite comparable numbers of glial cells in both cell groups. Although both the mixed cell cultures and the glial cell cultures represent cells after 2 days *in vitro*, the glial cells were replated after 1 day and then assayed after another day in culture. Nevertheless, when an equivalent number of glial cells were assayed after a further day in culture (i.e., 2 days undisturbed), their responses to cicaprost, PGE_2 and forskolin were unchanged and still greater than seen for the mixed cell cultures (data not shown).

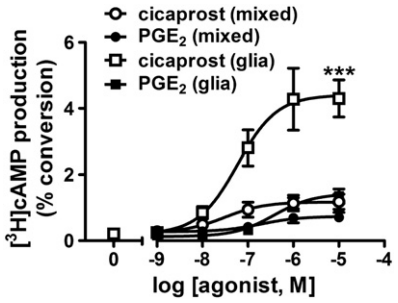

Fig. 3. A comparison of EP_4 and IP-stimulated adenylyl cyclase activity in DRG cell cultures. Mixed DRG cells (○,●) and glial cells (□, ■) were incubated with increasing concentrations of the IP receptor agonist cicaprost (○,□), or the EP receptor agonist PGE_2 (●,■). Data are presented as mean ± S.E.M., from 3 independent experiments. Error bars smaller than the symbol size are not shown. *** $P<0.001$ compared with maximum response of mixed DRG cell cultures to cicaprost; one-way ANOVA.

3.3. Evidence for EP_4 and IP receptors in DRG glial cells: immunocytochemistry and mRNA expression

Specific EP_4 receptor-immunoreactivity (EP_4-IR) and IP receptor-immunoreactivity (IP-IR) were readily detected in the plasma membrane and cytoplasm of DRG neurons (Fig. 4). Furthermore, both EP_4-IR and IP-IR were detected in the plasma membrane and cytoplasm of glial cells, with no obvious difference in staining intensity for glial cells in mixed DRG cell cultures and glial cell cultures (Fig. 5). For still further confirmation of EP_4 and IP receptors in glial cells, semi-quantitative RT-PCR was performed which showed EP_4 and IP receptor mRNA expression in both mixed cell cultures and pure glial cell cultures (Fig. 6). Expression of both EP_4 and IP receptors relative to GAPDH mRNA appeared greater in the mixed DRG cell population.

3. Discussion

Using EP and IP receptor specific agonists, we have previously demonstrated that DRG cells of adult rats express EP_4 and IP receptors *in vitro* (Rowlands et al., 2001; Wise, 2006). Herein we confirm this conclusion using the EP_4 receptor antagonist ONO-AE3-208 (Kabashima et al., 2002) and the IP receptor antagonist RO1138452 (Bley et al., 2006). ONO-AE3-208 competitively inhibited PGE_2-stimulated [^{3}H] cAMP production by mixed DRG cell cultures, with an estimated pA_2 value of 8.3 which is comparable with data from Kabashima et al. (2002) and helps confirm that PGE_2 is acting on EP_4 receptors to stimulate adenylyl cyclase activity. Both PGE_2 and the EP_4 receptor agonist ONO-AE1-329 produced comparable maximal stimulation of adenylyl cyclase activity, further suggesting that PGE_2 acts only on the EP_4 receptor subtype in rat DRG cells. However, despite the comparable responses to PGE_2 and ONO-AE1-329, a residual response remained for PGE_2 even in the presence of ONO-AE3-208 at a concentration which completely inhibited adenylyl cyclase activity stimulated by the EP_4 receptor specific agonist. It would appear unlikely though that PGE_2 is stimulating EP_2 receptors in these mixed cell cultures as the EP_2 receptor specific agonist (ONO-AE1-259) failed to significantly increase [^{3}H] cAMP production, the EP_1/EP_2 antagonist (AH6809) failed to inhibit PGE_2-stimulated responses, and EP_2 receptor immunoreactivity is reportedly low in DRG neurons (Zhao et al., 2007).

RO1138452 is highly selective for IP receptors and gave an estimated pA_2 value of 8.3 in mixed DRG cell cultures, which is comparable to reported values in other tissues (Bley et al., 2006; Jones et al., 2006). Although higher concentrations of cicaprost may have surmounted the inhibitory effect of RO1138452, curve fitting analysis suggested that the maximum response to cicaprost was significantly inhibited by

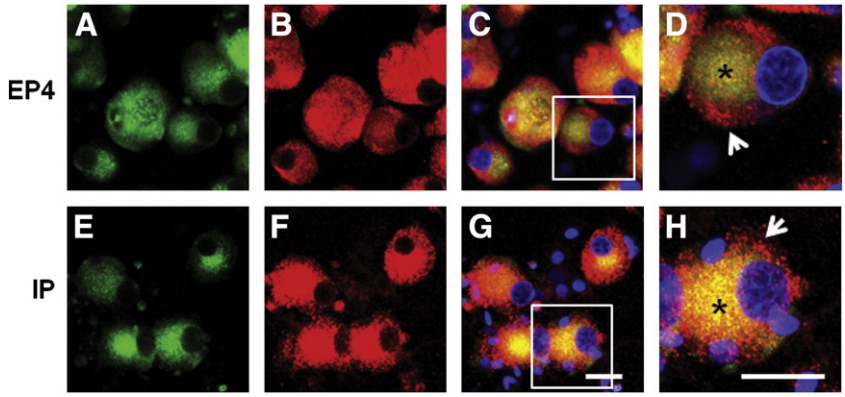

Fig. 4. Localization of EP_4 and IP receptor immunoreactivity in mixed DRG cell cultures. Fixed and permeabilized DRG cells were incubated with antibodies against EP_4 or IP receptors (Cy3 secondary antibodies, red; B, F), and neurons were detected using anti-TUJ-1 antibodies (FITC secondary antibodies, green; A, E). Cell nuclei were detected by Hoechst 33342 staining (blue). Merged images are shown in (C, G) and the insets were magnified to demonstrate subcellular localization of receptors (D, H). A–D: Confocal microscopy images for EP_4 receptors in neurons of mixed DRG cell cultures. E – H: Confocal microscopy images for IP receptors in neurons of mixed DRG cell cultures. Note the red ring on the surface of neurons (arrows) and yellow colocalization in the cytoplasm (asterisk). Images are representative of 2 independent experiments. Scale bars = 25 μm.

RO1138452. Such insurmountable inhibition has been observed previously in other systems, either due to agonist-dependent functional antagonism (Jones et al., 2006) or ascribed to pseudo-irreversible orthostatic antagonism (Ayer et al., 2008). Our previous failure to detect EP_3 receptor-dependent inhibition of adenylyl cyclase activity in DRG cells (Wise, 2006) suggests that the response profile of RO1138452 shown herein is more likely due to pseudo-irreversible orthostatic antagonism.

Both PGE_2-stimulated EP receptors and prostacyclin-stimulated IP receptors have been well characterised in isolated DRG cells as both are nociceptor sensitizing agents (Bley et al., 1998; Smith et al., 1998), but the presumption has been that only the neuronal cells are involved. This is presumably because the early studies of prostanoid receptor mRNA expression in DRG slices from normal mice found EP and IP receptors in DRG neurons, but not in glial cells (Oida et al., 1995). But DRG glial cells are not passive bystanders and can modulate functional responses of

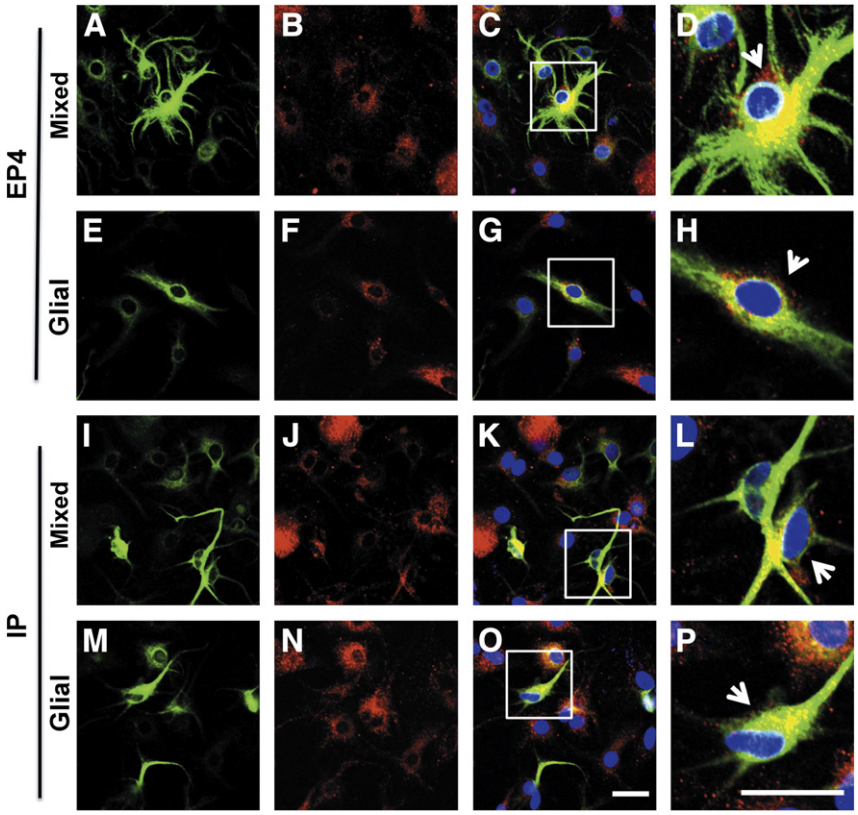

Fig. 5. Localization of EP_4 and IP receptor-immunoreactivity in glial cells in mixed DRG cell cultures and purified glial cell cultures. Fixed and permeabilized DRG cells were incubated with antibodies against EP_4 or IP receptors (FITC secondary antibodies, red; B, F, J and N), and glial cells were detected using Cy3-labeled anti-GFAP antibodies (green; A, E, I and M). Cell nuclei were detected by Hoechst 33342 staining (blue). Merged images are shown in C, G, K and O and the insets were magnified to demonstrated subcellular localization of receptors (D, H, L and P). A–H: Confocal microscopy images for EP_4 receptors in glial cells of mixed DRG cell cultures (A–D) and purified glial cell cultures (E–H). I–P: Confocal microscopy images for IP receptors in glial cells of mixed DRG cell cultures (I–L) and purified glial cell cultures (M–P). Note the red dots on the surface of glial cells (arrows) and yellow colocalization in the cytoplasm. Images are representative of 2 independent experiments. Scale bars = 25 μm.

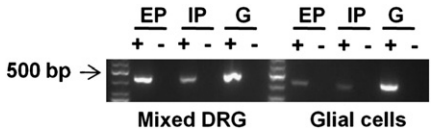

Fig. 6. EP_4 and IP receptor mRNA expression in DRG cell preparations. EP_4 and IP receptor mRNA was detected in both mixed DRG cell cultures and glial cell cultures by the presence of PCR products on an agarose gel. Expected product sizes are EP_4 423 bp, IP 431 bp, and GAPDH 452 bp. Lack of PCR products in the samples without reverse transcriptase (−) indicates a lack of contamination by genomic DNA. Data is representative of three independent experiments.

DRG neurons in slices (Thippeswamy et al., 2005; Zhang et al., 2007) and in isolated cell cultures (Heblich et al., 2001; Ng et al., 2010; Suadicani et al., 2010; Tang et al., 2007). Because typical DRG cell cultures from adult rats tend to be composed of more glial cells than neurons, we chose to check for EP_4 and IP receptor expression in isolated DRG glial cells. We were therefore surprised to discover both EP_4 and IP receptors in isolated DRG glial cells using EP_4 and IP receptor-specific agonists and antagonists; PGE_2 and ONO-AE1-329-stimulated [^{3}H]cAMP production was inhibited by the EP_4 receptor antagonist ONO-AE3-208 but not by the IP receptor antagonist RO1138452, and cicaprost-stimulated [^{3}H] cAMP production was inhibited by RO1138452 but not by ONO-AE3-208. Furthermore, agonist affinities were similar in both cell groups. In addition to the strong pharmacological evidence for the functional expression of EP_4 and IP receptors on DRG glial cells, we provide evidence of EP_4 and IP receptor immunoreactivity and mRNA expression in purified DRG glial cells. Cyclooxygenase-1/2-dependent PGE_2 production and cyclooxygenase-2-dependent prostacyclin production have been identified in mixed DRG cell cultures (Rowlands et al., 2001). COX-1-immunoreactivity, but not COX-2 immunoreactivity, has been localized in adult rat DRG neurons (Chopra et al., 2000), suggesting that DRG neurons may be the source of PGE_2 while cyclooxygenase-2-dependent prostacyclin (McAdam et al., 1999) may come from glial cells.

The process of dissociation and culturing is well known to produce neuronal responses different from those obtained in whole ganglia (Buschmann et al., 1998; Schoenen et al., 1989; Stebbing et al., 1998), leading to the proposal that the dissociated DRG neuron is an injured neuron with 'neuropathic' properties (Zheng et al., 2007). In whole DRG, the sensory neurons are surrounded by a tight sheath of satellite glial cells which help control the neuronal environment (Hanani, 2005), while myelin-expressing S100β-positive Schwann cells and GFAP-expressing Schwann cells ensheath the neurons (Campana, 2007). Schwann cells in rat sciatic nerve segments do not express EP receptors (Ma and Eisenach, 2003), but isolated Schwann cells from rat sciatic nerve are reported to express IP receptors (Muja et al., 2007). At the present time we are unable to distinguish satellite glial cells from Schwann cells in our DRG glial cell cultures as both cell types express GFAP in culture (Scholz and Woolf, 2007).

Satellite glial cells are similar to microglia in having properties of antigen presenting cells (van Velzen et al., 2009). Studies have shown that EP_2 receptors are not expressed in microglia of naïve or injured animals *in vivo* (Zhao et al., 2007), but that EP_2 and EP_4 receptor mRNA is increased in microglia following 24 h incubation with lipopolysaccharide (Noda et al., 2007). Activation of microglial EP_2 or EP_4 receptors inhibited lipopolysaccharide-induced cytokine release (Brenneis et al., 2011; Caggiano and Kraig, 1999) and up-regulation of EP_2 receptors in particular appeared to limit the synthesis of inflammatory mediators during chronic inflammation (Brenneis et al., 2011). Therefore, EP_4 and IP receptor expression by satellite glial cells and/or Schwann cells may be quiescent in intact ganglia, and only becomes evident on axotomy and subsequent dissociation of cells which serves to activate these cells.

In DRG *in vivo*, recent studies have demonstrated EP_4-IR in DRG neurons but not glial cells, but again in normal rats (Kopp et al., 2004; Lin et al., 2006). In a model of peripheral inflammation, Lin et al. (2006) reported an increase in EP_4-IR in DRG neurons with no reported expression in glial cells. However, injury to a peripheral nerve may produce a different profile of responses with glial cell hypertrophy (Lu and Richardson, 1991) and up-regulation of GFAP expression in activated satellite glial cells (Hanani, 2005) resulting in increased reactivity within the DRG (Davies et al., 2005; McMahon et al., 2005). In the partial sciatic nerve ligation model of neuropathic pain, EP_4-IR is increased in macrophages infiltrating the injured nerve and in unidentified resident cells, presumed to be fibroblasts (Ma and Eisenach, 2003), but EP_4-IR in associated DRG was not investigated. Therefore, despite the current lack of evidence for EP_4 and IP receptor expression by glial cells *in vivo*, studies of DRG in neuropathic pain models remain to be performed and the changes we describe herein could be characteristic responses of DRG cells following nerve injury.

With our novel findings, it is too early to fully understand the relative roles of EP_4 and IP receptors on isolated DRG neurons and glial cells, but there are already clues in the current study of a complex interaction between these cells. For example, the total number of glial cells in the mixed DRG cell culture was similar to that in the glial cell cultures on the day of assay, yet agonist-stimulated activity in the glial cell preparation was significantly greater than in the mixed DRG cell cultures. Both mixed cells and glial cells were assayed after a total of two days *in vitro*, therefore were comparable in relation to the original axotomy. When glial cells were left for a further day *in vitro*, responses were unchanged and remained greater than observed in mixed cell cultures. These results therefore suggest that the presence of neurons is inhibiting agonist-stimulated responses in glial cells. Because EP_4 and IP receptor mRNA expression was greater in relation to GAPDH mRNA in mixed cell cultures compared with glial cell cultures, it is unlikely that neuronal cells are inhibiting EP_4 and IP receptor mRNA expression by glial cells. As lipopolysaccharide increases the expression of EP_2 and EP_4 receptors on spinal microglia to provide a negative feedback system regulating neuroinflammation (Brenneis et al., 2011; Noda et al., 2007), we hypothesize that neuronal cells may be affecting the cell signalling activity of EP_4 and IP receptors to control the activity of the immune-like satellite glial cells. These intriguing conclusions are currently under investigation.

In the past, when cultures of adult rat DRG cells were used to assess the nociceptor sensitization properties of PGE_2 and prostacyclin, the presence of EP_4 and IP receptors on the associated glial cells was not recognised. In the future, it will be essential to consider that EP_4 and IP receptor agonists/antagonists may also affect the activity of the contaminating glial cells, and to consider that interactions between DRG neurons and glial cells may affect the functional responses under investigation.

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