Regulation of prostacyclin and prostaglandin E_2 receptor mediated responses in adult rat dorsal root ganglion cells, *in vitro*

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1 Primary cultures of adult rat dorsal root ganglia (DRG) were prepared to examine the properties of prostacyclin (IP) receptors and prostaglandin E_2 (EP) receptors in sensory neurones.

2 IP receptor agonists, cicaprost and iloprost, stimulated adenylyl cyclase activity with EC_{50} values of 22 and 28 nM, respectively. Prostaglandin E_1 (PGE_1) and prostaglandin E_2 (PGE_2) were 7 fold less potent than cicaprost and iloprost, with PGE_2 displaying a lower maximal response.

3 Adenylyl cyclase activation by iloprost, PGE_1 and PGE_2, but not by forskolin, was highly dependent on DRG cell density. Although the potency of iloprost and PGE_2 for stimulating adenylyl cyclase was unchanged, their maximal responses were significantly increased at low cell density.

4 Both IP and $EP_{2/4}$ receptors could be down-regulated by agonist pretreatment, however the presence of cyclo-oxygenase (COX) inhibitors did not prevent this apparent down-regulation of IP and $EP_{2/4}$ receptors at high DRG cell densities.

5 Stimulation of adenylyl cyclase by the neuropeptide calcitonin gene-related peptide was also decreased at high DRG cell density, whereas the responses to β-adrenoceptor agonists were increased at high DRG cell density.

6 Addition of nerve growth factor (NGF), or the addition of anti-neurotrophin antibodies during the 5-day culture of DRG cells, had no effect on IP receptor-mediated responses.

7 These results indicate that G_s-coupled receptors involved in nociception are regulated in a variable manner in adult rat sensory neurones, and that this cell density-dependent regulation may be agonist-independent for IP and $EP_{2/4}$ receptors.

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Introduction

Prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2) both have well recognized roles as hyperalgesic agents due to their ability to sensitize sensory nerve fibres; see reviews by Bley *et al.* (1998) and Wise & Jones (2000). Until recently, PGE_2 has attracted most attention as the key prostaglandin involved in hyperalgesia, but PGI_2 has effects comparable with PGE_2 as a hyperalgesic or sensitizing agent (Bley *et al.*, 1998). In mice lacking the PGI_2 (IP) receptor, inflammatory pain responses are reduced to the level seen in indomethacin-treated animals, and PGI_2 administered by intraperitoneal injection, but not PGE_2, is a potent inducer of writhing behaviour in mice (Murata *et al.*, 1997). In addition, more recent studies of IP receptor-deficient mice indicates that bradykinin released following intraplantar injection of carrageenan mediates mouse paw oedema *via* induction of PGI_2 formation and activation of IP receptors

(Ueno *et al.*, 2000). Both autoradiographic studies of [³H]-iloprost binding in the rat nervous system (Matsumura *et al.*, 1995), and of IP receptor mRNA distribution in the mouse (Oida *et al.*, 1995), indicate a role for IP receptors in sensory neurones such as those of the dorsal root ganglia (DRG).

Sensory neurones from the DRG can be maintained in culture, and have been found to provide a useful *in vitro* model to study PGI_2 and PGE_2-mediated sensitization of responses to algesic agents (Gold *et al.*, 1996). The antigenic phenotype of adult rat DRG cells is largely maintained in culture, such that the neuronal subpopulations present *in vivo* are present in cultures in approximately the same proportions (Gavazzi *et al.*, 1999). However, changes in the peptidergic phenotype in primary sensory neurones does occur *in vivo* following axotomy (Hökfelt *et al.*, 1994). While the functional consequences of these changes remain undefined, their parallel in DRG cells maintained in culture is even less well defined. The conditions under which isolated DRG cells

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are maintained in culture are heavily influenced by the experimental test system. Thus, for electrophysiological experiments on ion channels, freshly prepared DRG cells are used (Ueno *et al.*, 1999). For studies on second messengers, 5 days in culture is a common period (Smith *et al.*, 1998) with the addition of nerve growth factor (NGF) to allow expression of bradykinin receptors (Kasai *et al.*, 1998). NGF is also added to maintain the concentration of sensory peptides for neuropeptide release studies (Lindsay *et al.*, 1989) where relatively high concentrations of DRG cells are used.

Prostanoids such as PGI_2 and PGE_2 activate G protein-coupled receptors (GPCRs) in a wide range of tissues (Narumiya *et al.*, 1999). IP receptors couple primarily to G_s, although they can couple to G_q (Namba *et al.*, 1994) and have been shown to stimulate phospholipase C activity in adult rat DRG cells *in vitro* (Smith *et al.*, 1998). Receptors for PGE_2 have been classified into four major subtypes according to Coleman *et al.* (1994): EP_1 which increases intracellular Ca^{2+} concentrations, possibly *via* G_q, EP_2 and EP_4 which couple to G_s, and EP_3 which couples primarily to G_i. Adult rat DRG cells possess EP receptors able to stimulate adenylyl cyclase (Smith *et al.*, 1998), and these are referred to herein as $EP_{2/4}$ since the lack of suitably selective agonists and antagonists has meant that precise functional classification is not yet possible. Approximately 20–40% of the IP receptor mRNA-positive neurones in mouse DRG co-express EP_1, EP_3 and EP_4 receptor mRNA (EP_2 mRNA was not examined) (Oida *et al.*, 1995).

GPCRs undergo rapid desensitization following agonist stimulation, with chronic agonist exposure resulting in decreased receptor expression known as down-regulation (Koenig & Edwardson, 1997). The anti-platelet aggregation properties of PGI_2 are down-regulated by agonist exposure (Giovanazzi *et al.*, 1997), as are the anti-proliferative effects of the PGI_2 analogue iloprost in cultured bovine coronary artery smooth muscle cells (Zucker *et al.*, 1998). For cells with endogenous production of PGI_2, such as endothelial cells, this agonist-induced desensitization of IP receptors may be sufficient to limit detection of IP receptor-mediated responses (Schrör, 1993). A similar effect is seen for EP receptors where their detection in the choroidal vasculature of newborn pigs, either by receptor binding or functional studies, is also hampered unless the animals are treated with cyclo-oxygenase (COX) inhibitors prior to experimentation (Abran *et al.*, 1997).

During the course of our studies on prostanoid receptor regulation of DRG cell activity, we noted a marked increase in cyclic AMP production by adult rat DRG cells in response to iloprost when DRG cells were cultured at lower cell densities (Rowlands & Wise, 1999). The aim of this current paper was to examine this phenomenon in more detail and to compare the responses of DRG cells to both IP and $EP_{2/4}$ agonists. This paper shows that inhibition of COX activity fails to reverse the apparent down-regulation of IP and $EP_{2/4}$ receptors at high cell densities, and that this down-regulation is not a consistent property of G_s-coupled receptors involved in nociception. In addition, we have attempted to identify the factors responsible for the agonist-independent regulation of IP and $EP_{2/4}$ receptors in DRG cells.

Methods

Preparation of primary culture of adult rat DRG neurones

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 based on the method described by Smith *et al.* (1998). Briefly, rats were anaesthetized with pentobarbitone (40 mg kg^{-1}, i.p.) and dissected following procedures approved by the Animal Research Ethics Committee of The Chinese University of Hong Kong. DRG were dissected into Ham's F14 medium (Ham's Nutrient Mixture F14 medium containing 4% Ultroser G, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin), followed by incubation with 0.125% collagenase (3 h), then 0.25% trypsin (30 min, 37°C, 5% CO_2) in serum-free Ham's F14 medium. After rinsing in Ham's F14 medium, the DRG were resuspended in 2 ml 90 μg ml^{-1} DNase I and 100 μg ml^{-1} soybean trypsin inhibitor in Ham's F14 medium and the DRG dispersed by trituration with a small-bore, fire-polished Pasteur pipette. The cell suspension was centrifuged at $96 \times g$ for 10 min at room temperature through a cushion of 15% bovine serum albumin to eliminate much of the cellular debris and produce a neuronally enriched pellet of dissociated neurones (Gavazzi *et al.*, 1999). The cell pellet was resuspended in Ham's F14 medium containing 50 ng ml^{-1} NGF and 10 μM arabinoside C to inhibit non-neuronal cell proliferation. Cells were plated onto 24-well tissue culture plates (Nunc) precoated with poly-DL-ornithine (500 μg ml^{-1}) and laminin (5 μg ml^{-1}) and maintained in culture for 5 days in an atmosphere of 5% CO_2 at 37°C.

Measurement of [^3H]-cyclic AMP production

After an overnight incubation (18 h) with [^3H]-adenine (2 μCi ml^{-1}; 1 μCi well^{-1}), the medium was aspirated and the cells washed twice with 1 ml HEPES-buffered saline (HBS (mM): HEPES 25, pH 7.4, NaCl 135, KCl 3.5, CaCl$_2$ 2.5, MgCl$_2$ 1.0, glucose 3.3). Cells were challenged with test compounds for 30 min at 37°C in assay buffer (HBS containing 1 mM 3-isobutyl-1-methyl xanthine (IBMX) to inhibit phosphodiesterase activity, and 3 μM indomethacin to inhibit tonic prostanoid synthesis). The reaction was stopped by addition of ice-cold trichloroacetic acid and ATP, at a final concentration of 5% and 1 mM, respectively. The plates were left for at least 30 min on ice before separating the [^3H]-cyclic AMP from [^3H]-ATP by column chromatography (Barber *et al.*, 1980). Cell samples were loaded onto Dowex AG50W-X4 (200–400 mesh) columns and [^3H]-ATP eluted with 3 ml distilled water. A further 10 ml distilled water was added and the eluant loaded directly onto a neutral alumina column, which was eluted with 6 ml 0.1 M imidazole buffer, pH 7.5, to give a fraction containing [^3H]-cyclic AMP. Scintillator (OptiPhase 'HiSafe' 3, 10 ml) was added for scintillation counting.

The production of [^3H]-cyclic AMP from cellular [^3H]-ATP in the presence of a phosphodiesterase inhibitor (i.e. adenylyl cyclase activity) was estimated as the ratio of radiolabelled cyclic AMP to total AXP (i.e. cyclic AMP, ADP and ATP), and is expressed as [cyclic AMP]/[total AXP] × 1000. The absolute basal values for [^3H]-cyclic AMP production at each DRG cell density are indicated in the Figure and Table

legends. All assays were performed in triplicate where s.e.mean values were typically less than 10% of the mean values. Solvent controls were run as appropriate, but neither dimethylsulphoxide nor ethanol interfered with the assay at the concentrations used.

Measurement of 6-keto $PGF_{1\alpha}$ and PGE_2

After 5 days in culture, the media was removed from the cultured DRG cells, lyophilized, and assayed for 6-keto $PGF_{1\alpha}$ and PGE_2 by enzyme immunoassay (EIA) according to the manufacturer's instructions.

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 Graph-Pad Prism software version 3.0 (GraphPad Software Inc., San Diego, U.S.A.):

$$E = E_{min} + (E_{max} - E_{min})/1 + \{EC_{50}/x\}^n$$

where E is the measured response, E_{min} and E_{max} are the minimum and maximum asymptotes, EC_{50} is obtained from the 'location parameter' of the curve, x is the concentration and n is the slope factor of the curve ('Hill slope'). pEC_{50} is the negative logarithm of the EC_{50} value for stimulation of adenylyl cyclase activity. Fold basal activity is stimulated [^{3}H]-cyclic AMP production divided by basal [^{3}H]-cyclic AMP production. Values reported are means ± standard error mean. Comparisons between groups were made using the Student's *t*-test or ANOVA with Bonferroni's post-tests, as appropriate. Statistical significance is taken as $P < 0.05$.

Drugs

Cicaprost and iloprost were gifts from Schering AG, Berlin, Germany. 8-[^{3}H]-adenine (specific activity 27 Ci mmol^{-1}) was purchased from Amersham (Far East) Trading Ltd. Dowex AG 50W-XA (200–400 mesh) was purchased from Bio-Rad (U.S.A.), and OptiPhase 'HiSafe' 3 scintillant from Pharmacia Biotech Far East Ltd. Collagenase B and trypsin were purchased from Roche Diagnostics (U.S.A.), Ham's Nutrient Mixture F14 from Imperial Laboratories (U.K.), and nerve growth factor (mouse 2.5s) from Alomone Labs. (Israel). Anti-neurotrophin antibodies were from Chemicon (U.S.A.), and 6-keto $PGF_{1\alpha}$ and PGE_2 EIA kits from Cayman Chemical (U.S.A.). All other compounds were supplied by Gibco (U.S.A.) or Sigma Chemical Co. (U.S.A.).

Results

Effect of prostanoids on [^{3}H]-cyclic AMP production

The prostanoid agonists cicaprost, iloprost, PGE_1 and PGE_2 stimulated [^{3}H]-cyclic AMP production with pEC_{50} values of 7.65 ± 0.34, 7.55 ± 0.22, 6.73 ± 0.21 and 6.78 ± 0.29 ($n = 4$–5), respectively (Figure 1). Maximum [^{3}H]-cyclic AMP production ([cAMP]/[total AXP] × 1000) was 8.51 ± 0.32,
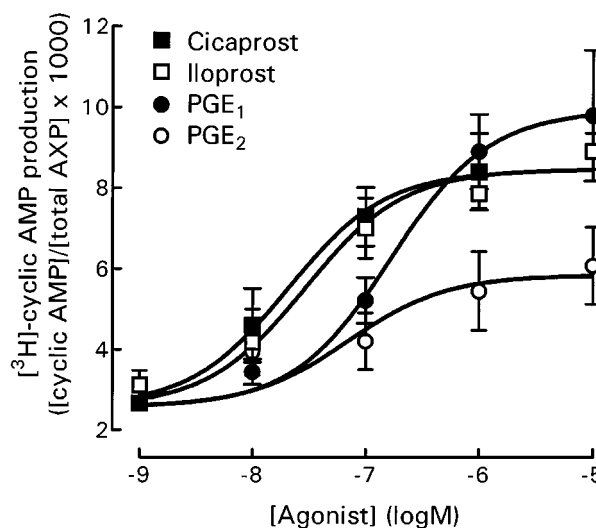

Figure 1 Effect of prostanoids on [^{3}H]-cyclic AMP production by DRG cells. Cells were incubated at 15,000 cells well^{-1} in HBS containing IBMX (1 mM) and indomethacin (3 μM) as described in Methods. Data represent mean ± s.e.mean of 4–5 experiments, each performed in triplicate. Basal [^{3}H]-cyclic AMP production ([cAMP]/[total AXP] × 1000) was 2.57 ± 0.12, $n = 6$.

8.90 ± 0.91, 10.31 ± 1.63 and 6.09 ± 0.92 for cicaprost, iloprost, PGE_1 and PGE_2, respectively. All log concentration-response curves were best fitted with a slope value of one. Maximal stimulation by the non-receptor activator of adenylyl cyclase, forskolin (Laurenza *et al.*, 1989), was at least 10 fold greater than that of the receptor agonists, with a pEC_{50} value of approximately 4.34 ($n = 5$). Concentrations of 50 or 75 nM forskolin were used in subsequent experiments to match the adenylyl cyclase activating responses of the receptor agonists.

Influence of cell density on [^{3}H]-cyclic AMP production

When DRG cells were plated out at different densities, decreasing the DRG cell density from 15,000 cells well^{-1} through 10,000 and 5000 cells well^{-1} to 500 cells well^{-1} produced a highly significant ($P < 0.001$, two-way ANOVA) increase in agonist-stimulated [^{3}H]-cyclic AMP production (Figure 2a). Although basal adenylyl cyclase activity in the 500 cells well^{-1} group was significantly lower than the 5000 and 10,000 cells well^{-1} groups ($P < 0.05$, one-way ANOVA with Bonferroni's post-tests, see Figure 2 legend), this does not entirely account for the change in fold basal values shown in Figure 2a, because forskolin-stimulated adenylyl cyclase activity was clearly unaffected under the same conditions (Figure 2b).

To determine if the increased responsiveness of DRG cells at low cell number represented a change in agonist potency and/or maximum response to agonists, concentration-response curves for iloprost and PGE_2 were obtained using DRG cells at 500 cells well^{-1} and compared with data from 15,000 cells well^{-1} (Figure 3). The decreased cell density did not affect the potency of iloprost with pEC_{50} values of 7.09 ± 0.01 ($n = 3$), and 7.55 ± 0.23 ($n = 4$) for 500 and 15,000 cells well^{-1}, respectively. Maximum responses to
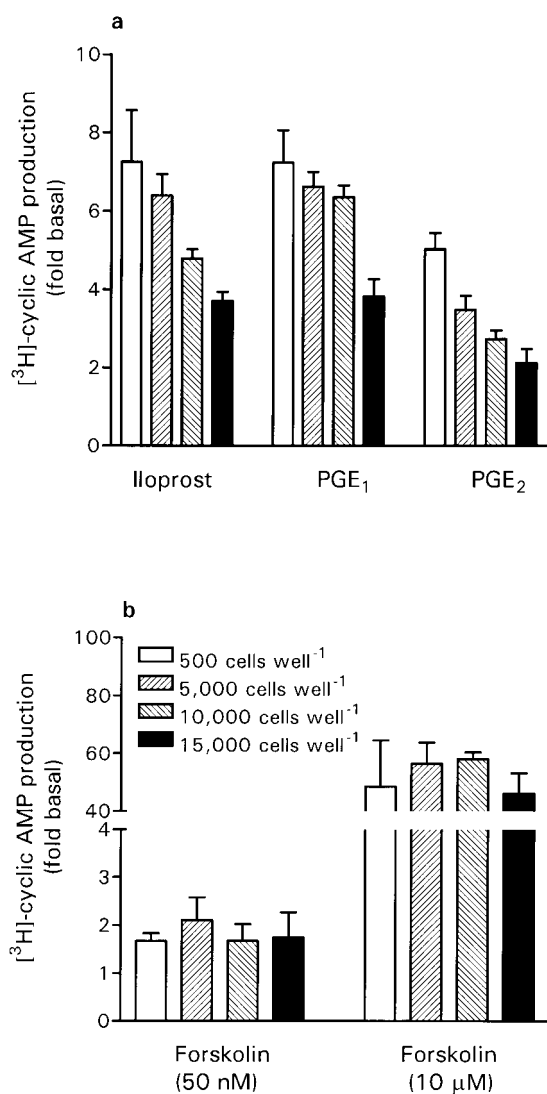
Figure 2 Effect of DRG cell density on responses to activators of adenylyl cyclase. DRG cells were plated at 500, 5000, 10,000 and 15,000 cells well^{-1}, and [^{3}H]-cyclic AMP production assayed in response to iloprost (10 μM), PGE$_1$ (10 μM), PGE$_2$ (10 μM) and forskolin (50 nM and 10 μM). Basal [^{3}H]-cyclic AMP production ([cAMP]/[total AXP] $\times$ 1000) for each condition was: 500 cells, 2.00$\pm$0.19*; 5000 cells, 3.51$\pm$0.35; 10,000 cells, 3.46$\pm$0.39 and 15,000 cells, 3.01$\pm$0.26. *$P<0.05$ compared with 5000 and 10,000 cells well^{-1} groups, one-way ANOVA with Bonferroni's post-tests. Data represent mean$\pm$s.e.mean of 3–6 experiments, each performed in triplicate.
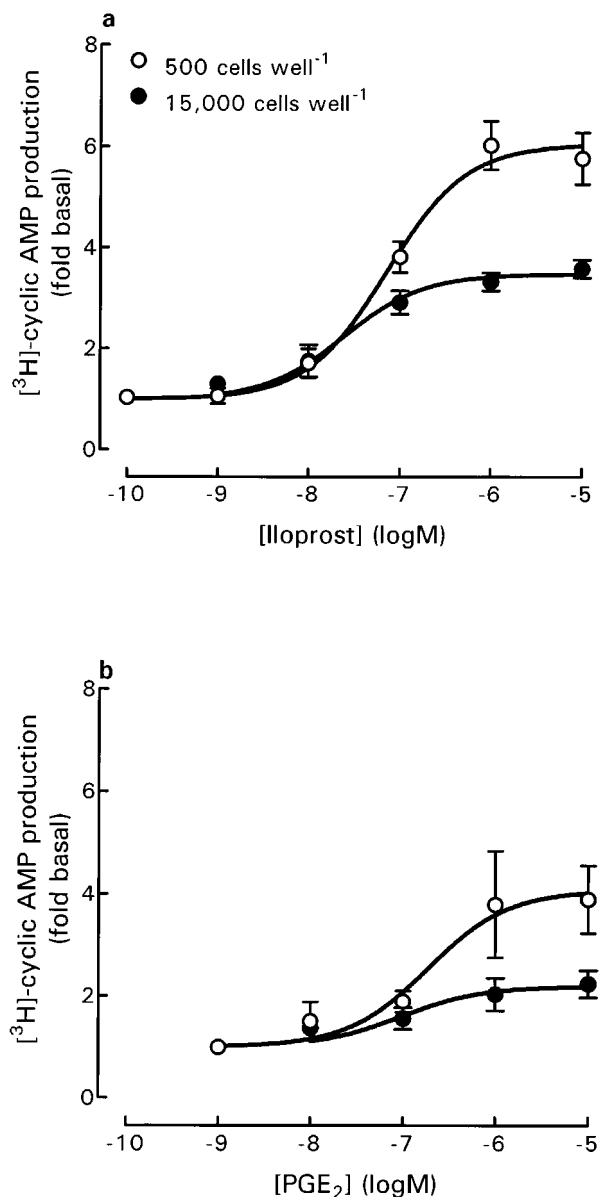
Figure 3 Effect of iloprost and PGE$_2$ on DRG cell responses at different cell densities. For iloprost concentration-response curves, basal [^{3}H]-cyclic AMP production ([cAMP]/[total AXP] $\times$ 1000) was 2.16$\pm$0.27 and 2.29$\pm$0.36 for 500 and 15,000 cells well^{-1}, respectively. For PGE$_2$ concentration-response curves, basal [^{3}H]-cyclic AMP production ([cAMP]/[total AXP] $\times$ 1000) was 1.62$\pm$0.16 and 2.69$\pm$0.23 for 500 and 15,000 cells well^{-1}, respectively ($P<0.05$, Student's *t*-test). Data represent mean$\pm$s.e.mean of 3–4 experiments, each performed in triplicate.

iloprost (calculated as fold basal values) were however significantly different ($P<0.001$, Student's *t*-test) at 6.03$\pm$0.38 and 3.36$\pm$0.14 for 500 and 15,000 cells well^{-1}, respectively.

The decreased cell density also did not affect the potency of PGE$_2$ with pEC$_{50}$ values of 6.59$\pm$0.17 ($n=4$), and 6.78$\pm$0.29 ($n=4$) for 500 and 15,000 cells well^{-1}, respectively. Maximum responses to PGE$_2$ (calculated as fold basal values) were also significantly different ($P<0.001$, Student's *t*-test) at 4.28$\pm$0.81 and 2.25$\pm$0.25 for 500 and 15,000 cells well^{-1}, respectively.

Investigation of factors which might influence adenylyl cyclase responses

Activation of prostanoid receptors coupled to G_i proteins DRG cells at 15,000 cells well^{-1} were incubated for 15 min with 1 μM sulprostone (EP$_1$/EP$_3$ agonist) before addition of cicaprost, PGE$_2$ or forskolin (Table 1). Sulprostone alone had no effect on basal [^{3}H]-cyclic AMP production, and failed to inhibit stimulated [^{3}H]-cyclic AMP production.

Agonist-induced down-regulation Pretreatment of DRG cells with high concentrations of IP and EP agonists for 18 h produced a significant down-regulation of their ability to respond further to stimulation by the same agonist (Table 2). Forskolin acts directly on adenylyl cyclase and therefore failed to produce homologous desensitization (Table 2). A similar pattern of responses was obtained for DRG cells at the lower cell density of 500 cells well^{-1}; pretreatment with the same adenylyl cyclase activator produced an inhibition of 46% for 10 μM iloprost, 56% for 10 μM PGE$_1$, 29% for 10 μM PGE$_2$ and 0% for 75 nM forskolin (data from two experiments, each performed in triplicate).

We therefore included the non-selective COX inhibitors indomethacin and ibuprofen for the 5 days of DRG cell culture to look for any influence from endogenously-produced prostanoids. The Ham's F14 medium was supplemented with 3 μM indomethacin, 10 μM ibuprofen or solvent control, and these agents were re-applied after 2 days. After a total of 5 days, the production of 6-keto PGF$_{1\alpha}$ (the stable metabolite of PGI$_2$) and PGE$_2$ was determined by EIA, and adenylyl cyclase activity in response to 10 μM iloprost and 10 μM PGE$_2$ was determined. Although the COX inhibitors produced a significant

reduction in 6-keto PGI$_{1\alpha}$ and PGE$_2$ production by DRG cells in culture, there was no significant effect on the ability of iloprost or PGE$_2$ to stimulate the production of [^{3}H]-cyclic AMP (Table 3).

Effect of protein kinase C activation DRG cells at 15,000 cells well^{-1} were incubated with 1 μM phorbol 12-myristate 13-acetate (PMA) for 15 min before the addition of cicaprost, PGE$_2$ or forskolin (Table 4). Although PMA appeared to increase the adenylyl cyclase response to cicaprost, this was not a statistically significant effect. There was however no evidence to suggest that PMA could decrease the response to PGE$_2$. As noted previously by Smith *et al.* (1998), phorbol esters significantly increased forskolin-stimulated adenylyl cyclase in DRG cells.

Effect of nerve growth factor DRG cell cultures (500 and 15,000 cells well^{-1}) were maintained in NGF-containing and NGF-free Ham's F14 medium for 5 days. Increasing the concentration of exogenous NGF from 0 to 200 ng ml^{-1},

Table 1 Effect of sulprostone on [^{3}H]-cyclic AMP production

Agonist	Control (fold basal activity)	Sulprostone (fold basal activity)
Cicaprost (100 nM)	2.01 ± 0.22	2.12 ± 0.33
PGE$_2$ (10 μM)	1.86 ± 0.13	2.49 ± 0.45
Forskolin (75 nM)	1.36 ± 0.09	1.89 ± 0.29

DRG cells (15,000 cells well^{-1}) were pretreated ± sulprostone (1 μM) for 15 min. Basal [^{3}H]-cyclic AMP production ([cAMP]/[total AXP] × 1000) was 3.62 ± 0.77 for controls, and 3.16 ± 0.16 for sulprostone-treated cells. Data represent mean ± s.e.mean of three experiments, each performed in triplicate.

Table 2 Homologous regulation of [^{3}H]-cyclic AMP production

Pretreatment drug	Response following pretreatment (% response of untreated cells)
Iloprost (10 μM)	55 ± 7*
PGE$_1$ (10 μM)	54 ± 5*
PGE$_2$ (10 μM)	79 ± 8
Forskolin (75 nM)	117 ± 6

10 μM iloprost, 10 μM PGE$_1$, 10 μM PGE$_2$, 75 nM forskolin or control buffer was added for 18 h to DRG cells (15,000) cells well^{-1}) maintained in Ham's F14 culture medium. The cells were then washed and stimulated with the same concentration of drug and [^{3}H]-cyclic AMP production assayed. Results are expressed as: (fold basal response following pretreatment) divided by (fold basal response in the absence of pretreatment) × 100. Basal [^{3}H]-cyclic AMP production ([cAMP]/[total AXP] × 1000) for the untreated cells was 3.22 ± 0.43. Data represent mean ± s.e.mean of four experiments, each performed in triplicate. *$P < 0.001$ compared with untreated test group, one-way ANOVA with Bonferroni's post-tests.

Table 3 Effect of COX inhibitors on 6-keto PFG$_{1\alpha}$ and PGE$_2$ production and IP or EP agonist-stimulated [^{3}H]-cyclic AMP production

	Control	Indomethacin	Ibuprofen
Cyclic AMP production			
Basal activity	2.03 ± 0.62	1.82 ± 0.15	1.59 ± 0.34
10 μM iloprost (fold basal activity)	4.53 ± 0.80	3.87 ± 0.38	5.52 ± 0.96
10 μM PGE$_2$ (fold basal activity)	4.65 ± 1.12	4.51 ± 0.68	5.54 ± 0.96
Prostanoid production			
6-keto PGF$_{1\alpha}$ (pg/ml)	118.0 ± 6.6	102.9 ± 5.7	75.2 ± 4.9*
PGE$_2$ (pg/ml)	36.1 ± 1.0	18.3 ± 0.9*	20.6 ± 0.9*

DRG cells were cultured at 15,000 cells well^{-1} in control medium, or medium containing indomethacin (3 μM) or ibuprofen (10 μM), for 5 days. For the adenylyl cyclase assay, data represent mean ± s.e.means of three experiments, each performed in triplicate. Basal [^{3}H]-cyclic AMP production is expressed as [cyclic AMP]/[total AXP] × 1000. For the measurement of prostanoids, data represent mean ± s.e.mean values from 6 wells. 6-Keto PGF$_{1\alpha}$ and PGE$_2$ were assayed in culture supernatants. *$P < 0.001$ compared with control group, one-way ANOVA with Bonferroni's post-tests.

Table 4 Effect of PMA on [^{3}H]-cyclic AMP production

Agonist	Control (fold basal activity)	PMA (fold basal activity)
Cicaprost (100 nM)	1.69 ± 0.26	2.17 ± 0.14
PGE$_2$ (10 μM)	1.65 ± 0.15	1.72 ± 0.15
Forskolin (75 nM)	1.33 ± 0.17	2.23 ± 0.15*

DRG cells (15,000 cells well^{-1}) were pretreated ± PMA (1 μM) for 15 min. Basal [^{3}H]-cyclic AMP production ([cAMP]/[total AXP] × 1000) was 4.75 ± 0.90 for controls, and 5.46 ± 0.20 for PMA-treated cells. Data represent mean ± s.e.mean of five experiments, each performed in triplicate. *$P < 0.01$ compared with control group, two-way ANOVA with Bonferroni's post-tests.

failed to modify the responsiveness of DRG cells, at either cell density, to 1 μM cicaprost (Figure 4).

Effect of anti-neurotrophin antibodies   To determine if DRG cells at 15,000 cells well^{-1} produce a neurotrophic factor capable of down-regulating IP and EP$_{2/4}$ receptors, we maintained DRG cell cultures (15,000 cells well^{-1}) in NGF-free Ham's F14 medium plus anti-neurotrophin antibodies for 5 days. Anti-mouse NGF, anti-human brain-derived neurotrophin (BDNF), anti-human neurotrophin-3 (NT-3) and anti-human neurotrophin-4/5 (NT-4/5) antibodies were used at the supplier's recommended dilutions to specifically neutralize any endogenously-produced neurotrophins. None of the anti-neurotrophin antibodies used significantly modified the adenylyl cyclase response to 1 μM cicaprost above the level of the control antibody (Table 5). Anti-NGF antibodies were also tested in DRG cells at 500 cells well^{-1} in comparison to the absence of exogenous NGF, but again there was a lack of effect on the response to cicaprost (Table 5).

Effect of DRG cell density on non-prostanoid receptor agonists

To determine if the responsiveness of DRG cells to other agonists involved in nociception was also influenced by DRG cell density, we examined the effect of calcitonin gene-related peptide (CGRP), isoprenaline and salbutamol which stimulate G$_s$-coupled receptors. While the response to CGRP was also increased by decreasing the cell density from 15,000 to 500 cells well^{-1}, the responses to the non-selective β-adrenoceptor agonist isoprenaline and the

selective β_2-adrenoceptor agonist salbutamol showed the opposite effect and were decreased as cell numbers decreased (Figure 5).

Discussion

Although cicaprost and iloprost are commonly used as IP agonists (Wise & Jones, 2000), their non-selectivity could result in stimulation of [^{3}H]-cyclic AMP production in DRG

Table 5   Effect of anti-neurotrophin antibodies on DRG cell responsiveness

| | [^{3}H]-cyclic AMP production | |
| | Basal activity | Cicaprost (1 μM) |
Antibody	([cyclic AMP]/ [total AXP] $\times$ 1000)	(fold basal activity)
15,000 cells well^{-1}		
None	1.57 $\pm$ 0.31	3.37 $\pm$ 0.24
Ig control	1.45 $\pm$ 0.26	4.45 $\pm$ 0.27
Anti-NGF (1:500 dilution)	1.40 $\pm$ 0.29	4.39 $\pm$ 0.57
Anti-BDNF (1:200 dilution)	1.40 $\pm$ 0.34	4.58 $\pm$ 0.41
Anti-NT-3 (1:200 dilution)	1.24 $\pm$ 0.15	3.94 $\pm$ 0.59
Anti-NT-4/5 (1:200 dilution)	1.24 $\pm$ 0.23	4.52 $\pm$ 0.31
500 cells well^{-1}		
None	1.64 $\pm$ 0.42	6.40 $\pm$ 1.38
Anti-NGF (1:500 dilution)	1.50 $\pm$ 0.13	6.66 $\pm$ 1.25

DRG cells were cultured for 5 days in NGF-free Ham's F14 medium containing anti-neurotrophin antibodies. Cells were washed and stimulated with 1 μM cicaprost and [^{3}H]-cyclic AMP production assayed according to Methods. Data represent mean $\pm$ s.e.mean of three experiments, each performed in triplicate.
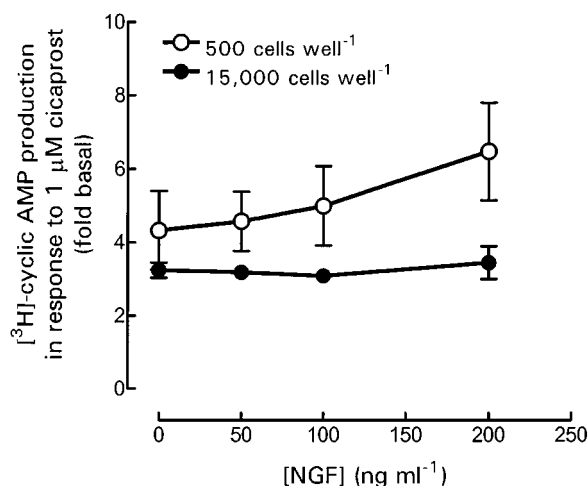

Figure 4   Effect of NGF on DRG cell responsiveness. DRG cells were cultured for 5 days in Ham's F14 medium with increasing concentration of NGF. Cells were washed and stimulated with 1 μM cicaprost, and [^{3}H]-cyclic AMP production assayed as described in Methods. Basal [^{3}H]-cyclic AMP production ([cAMP]/[total AXP] $\times$ 1000) was 1.78 $\pm$ 0.12, 1.81 $\pm$ 0.23, 1.60 $\pm$ 0.14 and 1.49 $\pm$ 0.20 for 0, 50, 100 and 200 ng ml^{-1} NGF, respectively, for 500 cells well^{-1}. Basal [^{3}H]-cyclic AMP production ([cAMP]/[total AXP] $\times$ 1000) was 1.41 $\pm$ 0.25, 1.54 $\pm$ 0.22, 1.66 $\pm$ 0.31 and 1.77 $\pm$ 0.35 for 0, 50, 100 and 200 ng ml^{-1} NGF, respectively, for 15,000 cells well^{-1}. Data represent mean $\pm$ s.e.mean of three experiments, each performed in triplicate.
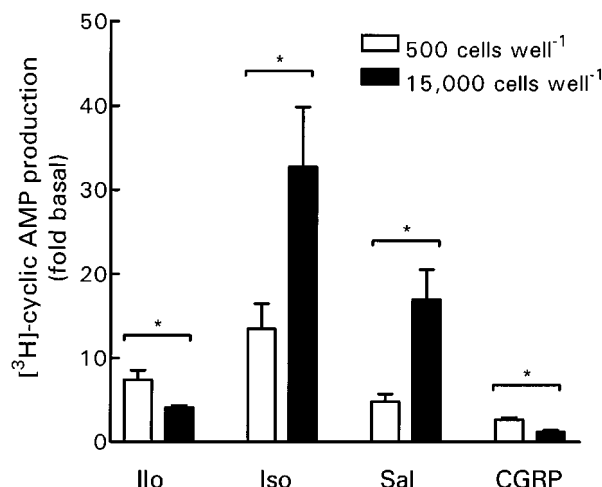

Figure 5   Effect of DRG cell density on [^{3}H]-cyclic AMP production in response to non-prostanoid receptor agonists. Cells were incubated in HBS containing IBMX (1 mM) and indomethacin (3 μM) as described in Methods. Data represent mean $\pm$ s.e.mean of 4–5 experiments, each performed in triplicate. Iloprost (Ilo, 10 μM), isoprenaline (Iso, 1 μM), salbutamol (Sal, 1 μM) and CGRP (1 μM). Basal [^{3}H]-cyclic AMP production ([cAMP]/[total AXP] $\times$ 1000) was 1.96 $\pm$ 0.28 and 2.58 $\pm$ 0.24 for 500 and 15,000 cells well^{-1}, respectively. *$P < 0.05$, Student's *t*-test.

cells *via* activation of rat EP_4 receptors (Boie *et al.*, 1997). However, the potency of cicaprost and iloprost for stimulating [³H]-cyclic AMP production in rat DRG cells is much more suggestive of an action at IP receptors, as reported previously by Smith *et al.* (1998), and as supported by observations on the extent of [³H]-iloprost binding (Matsumura *et al.*, 1995) and IP receptor mRNA distribution in dorsal root ganglia (Oida *et al.*, 1995). The potency of PGE_1 for stimulating adenylyl cyclase activity in DRG cells is only 10 fold lower than that of cicaprost and iloprost, and PGE_1 has comparable maximal effects to cicaprost and iloprost, as would be expected if PGE_1 is acting here on rat IP receptors (Sasaki *et al.*, 1994). Iloprost additionally is a potent agonist at EP_1 receptors (Dong *et al.*, 1986) where it would be expected to increase intracellular Ca^{2+} concentrations. However, the log concentration-response curves for cicaprost and iloprost are practically superimposable, indicating that the EP_1 agonist property of iloprost does not interfer with its ability to stimulate adenylyl cyclase in DRG cells.

In addition to stimulating $EP_{2/4}$ receptors, PGE_2 can also interact with rat IP receptors, at concentrations approximately 300 fold higher than its K_d for the rat EP_2 and EP_4 receptors (Sasaki *et al.*, 1994). Therefore, the relatively low potency and low maximal response of PGE_2 could suggest that it is acting at IP receptors and/or EP_2 or EP_4 receptors at low level expression, but the lack of specific antagonists for IP and EP receptors prevents full clarification of this issue at the present time.

GPCRs undergo agonist-induced desensitization (Koenig & Edwardson, 1997), and this is a well-established characteristic of IP receptors in cells such as NG108-15 neuroblastoma cells and platelets (Jaschonek *et al.*, 1988; Kelly *et al.*, 1990). Results presented in the current study using DRG cells would suggest that down-regulation of IP and $EP_{2/4}$ receptors is inversely related to the cell density. To account for the different extent of metabolic labelling of DRG cells plated at different densities, we have calculated [³H]-cyclic AMP production as [cAMP]/[AXP] × 1000, rather than simply use the d.p.m. values for the [³H]-cyclic AMP fractions collected. In the majority of experiments, the basal levels of [³H]-cyclic AMP production were not affected by cell density (see Figures 3a, 4, 5 and Table 5), presumably indicating that the metabolic activity of these cells was similar. However, for the data in Figure 2, even though there was no significant difference in the basal adenylyl cyclase activity between DRG cells plated at 500 and 15,000 cells well^{-1}, or between the 5000, 10,000 and 15,000 cells well^{-1} groups, basal adenylyl cyclase activity was significantly lower in the 500 compared with the 5000/10,000 cells well^{-1} groups. This latter result over emphasises the increased responses seen at 500 cells well^{-1} when results are expressed as fold basal; a necessary procedure to standardize for the variability between different batches of DRG cells. However, when expressing results as fold basal, significant differences in maximal responses to cicaprost and iloprost are still demonstrable in the absence of decreases in basal [³H]-cyclic AMP production in the 500 cells well^{-1} groups (see Figures 3a, 4, 5 and Table 5).

[³H]-cyclic AMP production in response to a low concentration of forskolin showed a slight cell density-dependent effect, but the pattern did not parallel that seen with the prostanoid agonists, and was absent when the results were expressed relative to the basal level of adenylyl cyclase activity. Moreover, [³H]-cyclic AMP production in response to a higher concentration of forskolin showed no obvious cell density-dependent properties. Therefore, we believe that the changes observed for iloprost, PGE_1 and PGE_2-mediated responses are due to altered receptor availability and/or altered receptor-G protein coupling, rather than changes in adenylyl cyclase function.

The lack of effect of cell density on agonist potency despite the altered maximal responses to iloprost and PGE_2 together suggest that we are looking at down-regulation of IP and $EP_{2/4}$ receptors in high-density cultures of DRG cells. Indeed, in our DRG cell cultures at both low and high cell density, IP and $EP_{2/4}$ receptors demonstrated homologous desensitization; this agonist-induced desensitization or down-regulation is frequently associated with phosphorylation of the receptor. Studies on HEK 293 cells transfected with the human IP receptor indicate that agonist-dependent phosphorylation of the human IP receptor occurs *via* protein kinase C (Smyth *et al.*, 1996), resulting in desensitization of both the cyclic AMP and inositol phosphate responses to iloprost (Smyth *et al.*, 1998). Rat IP receptors have an additional protein kinase A phosphorylation site (Sasaki *et al.*, 1994), but its role in IP receptor regulation has not been reported. The rat EP_2 and EP_4 receptors can be phosphorylated by both protein kinase A and protein kinase C (Boie *et al.*, 1997), and although they differ in their susceptibility to agonist-induced short-term desensitization, both EP receptors display similar patterns of down-regulation in response to prolonged exposure to PGE_2 (Nishigaki *et al.*, 1996).

We have confirmed the results of Smith *et al.* (1998) showing that activation of protein kinase C increases adenylyl cyclase responses to forskolin, but its effect on receptor agonists was rather weak. Part of the difficulty in interpreting the results here could lie in the presence of different isoforms of adenylyl cyclase in the various cell types comprising the DRG cell preparation. All these isoforms would be responsive to forskolin, but not all would respond in the same manner to protein kinase C activators (Tang & Hurley, 1998). Furthermore, activation of protein kinase C is known to produce variable effects on prostanoid receptor responses. Phorbol esters increase cicaprost-stimulated adenylyl cyclase activity in UMR-106 osteoblast cells (Khanin *et al.*, 1999) yet have no effect on NG108-15 neuroblastoma cells (Krane *et al.*, 1994). EP_2 receptors in both rat neonatal microglial cultures (Patrizio *et al.*, 2000), and in UMR-106 cells (Khanin *et al.*, 1999) are inhibited by phorbol esters. Clearly the situation of agonist-dependent receptor phosphorylation in DRG cells is likely to be complex, and further studies may reveal subtle differences here between rat IP and $EP_{2/4}$ receptors.

IP receptors possess an isoprenylation site in their C-terminal tails, and this is essential for coupling to adenylyl cyclase and phospholipase C pathways (Hayes *et al.*, 1999). However, it is also unlikely that the observed down-regulation of IP and $EP_{2/4}$ receptors in DRG cells is due to inhibition of isoprenylation because EP receptors, indeed all other prostanoid receptors, lack isoprenylation sites. Increased expression and activation of a prostanoid receptor with opposing effects, i.e. the EP_3 receptor, might account for the reduced maximum responses to IP agonists and PGE_2 at higher cell densities. By definition, PGE_2 would be expected to stimulate all subtypes of EP receptors, and indeed has

similar affinity for the rat EP_4 and EP_3 receptors (Boie *et al.*, 1997). In addition, concentrations of 1 μM cicaprost and iloprost are also likely to stimulate EP_3 receptors (Boie *et al.*, 1997; Kiriyama *et al.*, 1997). However, we have excluded the possible stimulation by IP agonists and PGE_2 of inhibitory EP_3 receptors at high DRG cell density because sulprostone failed to inhibit cicaprost, PGE_2 and forskolin-stimulated [^{3}H]-cyclic AMP production. These results confirm those of Smith *et al.* (1998) demonstrating a lack of functional EP_3 receptors in rat isolated DRG cell preparations. Furthermore, the prostanoid receptor data alone might suggest that high-density DRG cell preparations produce a factor/s capable of stimulating an alternative G_i-coupled pathway. However, such a factor should similarly affect the responses to forskolin, isoprenaline and salbutamol, and this is clearly not the case.

As suggested above, a typical DRG cell preparation contains not only neuronal cells, but also non-neuronal cells such as astrocytes and Schwann cells. Our current experimental design does not allow us to distinguish the cellular source of cyclic AMP, but all cell counts are based on the morphologically distinct neuronal cell component of our DRG cell preparation. Evidence for the presence of IP and $EP_{2/4}$ receptors in glial cells, rather than neuronal cells, is contradictory. For example, there is no evidence for IP receptor mRNA in mouse glial cells (Oida *et al.*, 1995), or human glioblastoma cell lines (Verity *et al.*, 1999), yet IP receptors, but not $EP_{2/4}$ receptors, have been identified in newborn rat astroglial cell cultures (Seregi *et al.*, 1988). Furthermore, there is minimal activation of adenylyl cyclase by iloprost in human astrocytoma cells (Ortmann, 1978), and in primary cultures of rat type-1 and type-2 astrocytes (Ito *et al.*, 1992).

In rat aorta there is a clear inverse relationship between PGI_2 synthase mRNA and 6-keto $PGF_{1\alpha}$ production, and the expression of IP receptor mRNA (Numaguchi *et al.*, 1999). Recent studies provide immunohistochemical evidence of PGI_2 synthase in the central nervous system, within neuronal rather than non-neuronal cells (Mehl *et al.*, 1999), however, preliminary data suggests a lack of PGI_2 synthase in neuronal cells of adult rat DRG (Hans-J. Bidmon, personal communication). IP and EP receptors are often down-regulated in normal cell systems, and detected clearly only after use of COX inhibitors to inhibit the endogenous production of prostanoids, as is the case for endothelial cells (Schrör, 1993), and the choroidal vasculature (Abran *et al.*, 1997). Pretreatment of rat DRG cells in culture with non-selective COX inhibitors failed to increase the response to either iloprost or PGE_2. Indeed, the concentration of prostanoids released into the culture medium of our DRG cell preparation was in the low picomolar range, which would be insufficient to stimulate IP and $EP_{2/4}$ receptors and therefore unlikely to cause receptor down-regulation. These results suggest that the cell density-dependent down-regulation of IP and $EP_{2/4}$ receptors is independent of endogenously-produced ligands.

Since endogenously-produced prostanoids do not appear to be responsible for receptor down-regulation at high cell densities, then perhaps some factor is responsible for up-regulation of prostanoid receptors at low cell densities. For example, IP receptor expression is induced by tumour necrosis factor-α (TNFα) in mouse osteoblastic cells (MC3T3-E1) (Wang *et al.*, 1999). Actually, this effect of TNFα

was suppressed by COX inhibition, suggesting that it is in part mediated by prostaglandins and is therefore unlikely to provide a mechanism to explain our results.

Neurones from adult DRG can survive independently of trophic factors (Lindsay, 1988; Gavazzi *et al.*, 1999), but NGF is routinely included in our DRG cell cultures because it is reported to maintain the concentration of sensory neuropeptides such as CGRP and substance P (Lindsay *et al.*, 1989). NGF can increase the sensitivity to bradykinin in capsaicin-sensitive DRG cells (Kasai *et al.*, 1998); an effect probably mediated *via* the low affinity neurotrophin receptor p75 (Petersen *et al.*, 1998). In addition, NGF enhances neurite outgrowth (Gavazzi *et al.*, 1999) and inhibiting the action of endogenously-produced NGF decreases capsaicin sensitivity of isolated DRG cells (Bevan & Winter, 1995; Nicholas *et al.*, 1999). NGF can differentially regulate the expression of a variety of GPCRs in pheochromocytoma (PC12) cells where it decreases adenosine A_{2A} (Lee *et al.*, 1995; Nie *et al.*, 1999), secretin (Lee *et al.*, 1995) and angiotensin II type 2 (AT2-R) (Kijima *et al.*, 1995) receptors, and up-regulates muscarinic m4 (Lee & Malek, 1998) and pituitary adenylate cyclase-activating polypeptide (PACAP) receptors (Cavallaro *et al.*, 1995). However, we have found no evidence for regulation of IP receptors in DRG cells by NGF. Furthermore, the ability of PGE_2 to increase cyclic AMP levels, and its ability to sensitize rat DRG cells to capsaicin, is also unaffected by NGF (Southall & Vasko, 2000).

Approximately 40% of DRG neurones express both the high affinity, NGF-selective tyrosine kinase receptor trkA and CGRP, and are therefore considered to be nociceptive neurones (Snider & Wright, 1996). Of the remaining small to medium-sized cells, most express trkB, the receptor for BDNF and NT-4/5, while the larger proprioceptive neurones express trkC, the receptor for NT-3. Neurotrophins can be produced by target cells, supportive cells, and by the neuronal cells themselves (Dray, 1996). Indeed, BDNF may have an autocrine role in the survival of a subpopulation of DRG cells since BDNF (and NT-3 or NT-4/5, but not NGF) can rescue adult rat DRG cells treated with antisense oligonucleotides to BDNF (Acheson *et al.*, 1995). In the present study, culture of DRG cells in the presence of antibodies capable of specifically neutralizing BDNF, NGF, NT-3 or NT-4/5, had no influence on the response of DRG cells to cicaprost. Thus, while neurotrophins may influence neurite outgrowth, neuropeptide content, or capsaicin-sensitivity of DRG cells, they do not appear to influence responsiveness to the hyperalgesic prostanoids, PGI_2 and PGE_2.

Although it is well recognized that the chemical environment in which DRG cells are maintained is likely to influence their behaviour in culture (Dray, 1996), the marked influence of cell density has been less well recognized. A recent report shows that cell density influences the expression of neuropeptide Y (NPY) in isolated adult rat DRG cells, where fewer neurones express NPY in high rather than low density cultures (Kerekes *et al.*, 2000). Predicting the influence of cell density on neuropeptide content and receptor expression levels of DRG cells *in vitro* is not yet possible. For example, we tested the neuropeptide CGRP because it acts as an autoreceptor on primary afferents (Levine *et al.*, 1993) and would be expected to increase cyclic AMP production

(Wimalawansa, 1992). As observed for IP and EP$_{2/4}$ agonists, adenylyl cyclase responses to CGRP were also inhibited at high cell density. In contrast, the responses to isoprenaline and salbutamol increased at high cell density. These β-agonists were tested because adrenaline can also produce cyclic AMP-dependent hyperalgesia and nociceptor sensitizing effects in the rat (Khasar *et al.*, 1999). Thus, we have shown here that GPCRs involved in nociception behave in an unpredictable cell density-dependent fashion.

Although the neuronal subpopulations present in DRG *in vivo* are thought to be present in isolated cultures in approximately the same proportions (Gavazzi *et al.*, 1999), it is difficult to compare the relative densities of cells in the two situations. Therefore, as isolated adult rat DRG cell preparations are becoming increasingly popular for the study of algesic and hyperalgesic agents, we should perhaps be more cautious when looking at interactions between different receptor systems when the assay systems require different densities of DRG cells.

In conclusion, the responsiveness of G$_s$ protein-coupled receptors in isolated DRG cell preparations is system-dependent. For IP and EP$_{2/4}$ receptors, this cell density-dependent regulation of GPCR responses seems to be agonist-independent, and does not depend on the presence of neurotrophins.

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