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Characterization of prostanoid receptor-evoked responses in rat sensory neurones

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1 Prostanoid receptor-mediated sensitization, or excitation, of sensory nerve fibres contributes to the generation of hyperalgesia. To characterize the prostanoid receptors present on sensory neurones, biochemical assays were performed on primary cultures of adult rat dorsal root ganglia (DRG) and the F-11 (embryonic rat DRG × neuroblastoma hybrid) cell line.

2 In DRG cultures, the IP receptor agonists, cicaprost and carbaprostacyclin ($cPGI_2$) stimulated cyclic AMP accumulation. Prostaglandin E_2 (PGE_2) also increased cyclic AMP levels, but to a lesser extent, while carbocyclic thromboxane A_2 ($cTxA_2$), PGD_2 and $PGF_{2\alpha}$ had negligible effects. The rank order of agonist potency was cicaprost $> PGE_2 = BMY45778 = cPGI_2 = PGI_2$. In the F-11 cells, the rank order of agonist potency for the stimulation of cyclic AMP accumulation was: cicaprost > iloprost = $cPGI_2$ = P-GI_2 = BMY45778 > PGE_2 = $cTXA_2$. In DRG cultures, cicaprost induced significantly more accumulation of inositol phosphates than PGE_2.

3 To examine the effects of prostanoids on C-fibre activity, extracellular recordings of d.c. potentials from the rat isolated vagus nerve were made with the 'grease-gap' technique. PGI_2 (0.1 nM – 10 μM) produced the largest depolarizations of the nerve. The rank order of agonist potency was: $PGI_2 = cPGI_2 = PGE_1 > cTXA_2 > PGE_2 = PGD_2 = TXB_2 > PGF_{2\alpha}$.

4 Prior depolarization of nerves with either forskolin (10 μM) or phorbol dibutyrate (1 μM) alone significantly reduced the response to PGI_2 (10 μM), while simultaneous application of both forskolin and phorbol dibutyrate attenuated PGI_2 responses almost completely.

5 Putative EP_1 and/or TP receptor-selective antagonists had no effect on the responses to PGI_2, $cPGI_2$ or PGE_2 in the three preparations studied.

6 Collectively, these data are consistent with a positive coupling of IP receptors to both adenylyl cyclase and phospholipase C in sensory neurones. These findings suggest that IP receptors play a major role in the sensitization of rat sensory neurones.

Keywords: Prostacyclin; prostanoid receptors; sensory neurones; cyclic AMP; dorsal root ganglia; vagus nerve; grease-gap; F-11

Introduction

Prostanoids activate different second messenger pathways through interaction with several G-protein coupled receptors (see Coleman *et al*., 1994; Pierce *et al*., 1995 for reviews). The current classification of these prostanoid receptors (i.e. DP, EP, FP, IP, TP) is based upon the differential rank order of potency for prostaglandin D_2 (PGD_2; DP), prostaglandin E_2 (PGE_2; EP), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; FP), prostaglandin I_2 (PGI_2; IP) and thromboxane (TxA_2; TP), as well as the selective activity of synthetic agonists and antagonists (for reviews, see Coleman *et al*., 1994; Ushikubi *et al*., 1995). Differences in either biochemical or tissue responses to PGE_2, together with the use of available selective agonists and antagonists, has led to the subdivision of EP receptors into EP_1, EP_2, EP_3 and EP_4 subtypes. cDNAs encoding prostanoid receptors that correspond to each of these eight subtypes have been cloned from human and mouse tissues (see Pierce *et al*., 1995; Ushikubi *et al*., 1995).

Prostanoids, notably PGI_2 and PGE_2, are generated in response to injury or inflammation and can sensitize, or directly activate, sensory nerve endings in several animal models of nociception (reviewed by Ferreira, 1979). PGE_2 has been used most frequently as the sensitizing agent in animal studies (e.g. Schaible & Schmidt, 1988; Tonussi & Ferreira, 1992) probably for several reasons. Firstly, PGE_2 was isolated, and became commercially available, many years before PGI_2 and, secondly, PGE_2 is inherently more stable than PGI_2. Also, PGE_2 was more potent as a nociceptive agent than $PGF_{2\alpha}$, PGD_2 or TxA_2 in early clinical studies (Horton, 1963; Ferreira, 1972). Consequently, PGE_2 has attained wide recognition as a principle, pain-producing, inflammatory mediator, although the receptor(s) mediating its effects are undefined. However, most studies directly comparing the algesic effects of these two compounds suggest that PGI_2, in spite of its short half-life (Dusting *et al*., 1978), has more potent *in vivo* hyperalgesic properties than PGE_2 (Ferreira *et al*., 1978; Higgs *et al*., 1978; Juan, 1979; Doherty *et al*., 1987; Berkenkopf & Weichman, 1988; Birrell *et al*., 1991; Schepelmann *et al*., 1992). These observations thus suggest a primary role for IP, rather than EP, receptors in the induction of hyperalgesia. However, the unequivocal characterization of the prosanoid receptor(s) mediating sensitization of sensory neurones *in vivo* is complicated not only by metabolic and/or pharmacokinetic differences between different endogenous prostanoids, but by a lack of selective antagonists for EP, IP and FP receptors.

Sensitizing effects of endogenous prostanoids have been demonstrated *in vitro* on different indicators of sensory neurone function, including ion channel activation, neuropeptide release and second messenger levels (Pitchford & Levine, 1991; Devor *et al*., 1992; Rueff & Dray, 1993; Cui & Nicol, 1995; Hingtgen *et al*., 1995; England *et al*., 1996; Gold *et al*., 1996; Nicol *et al*., 1997). Most of these studies have concentrated on PGE_2, but in general, those studies which have utilized both PGI_2 and PGE_2 suggest that the response to

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PGI_2 is of greater magnitude (Pitchford & Levine, 1991; Birrell & McQueen, 1993; Undem & Weinreich, 1993; Hingtgen *et al*., 1995; Nicol *et al*., 1997). However, to date, there has not been a rigorous pharmacological characterization of these responses with regards to the relative contributions of EP and IP receptors.

The aim of the present study was to utilize simple *in vitro* models to characterize the prostanoid receptor types on sensory neurones, thus clarifying the role of these receptors in sensitization. Specifically, primary tissue cultures of adult rat dorsal root ganglion (DRG) and F-11 (embryonic rat DRG × neuroblastoma hybrid) cells were used to examine the coupling of prostanoid receptors to both adenylyl cyclase and phosphoinositide hydrolysis. The use of primary cultures of adult rat DRG for pharmacological studies is limited by the time required to establish such cultures and the small total number of neurones which can be obtained from mature ganglia. Therefore, F-11 cells, which exhibit several of the properties of authentic DRG neurones (Platika *et al*., 1985; Francel *et al*., 1987), were used to permit a more comprehensive pharmacological analysis.

Exogenously applied PGE_2 and PGI_2 have been shown to excite the terminals of the vagus nerve *in vivo* (Roberts *et al*., 1985). However, neurohormonal receptors are localized not only on the terminals or cell bodies of sensory neurones but also on the axons. Indeed, the axons of the vagus nerve, the majority of which are C-fibres (Paintal, 1963), possess both ligand-gated and guanine nucleotide binding protein-linked receptors for several neurotransmitters which have been localized definitively to C-fibre cell bodies (Brown & Marsh, 1978, Ireland & Tyers, 1987; Trezise *et al*., 1993; Bley *et al*., 1994). Therefore, the rat isolated vagus nerve preparation also was used to study the role of prostanoids in activation of sensory neurones.

Methods

Preparation of primary culture of adult rat DRG neurones

Rats were killed by CO_2 inhalation in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Roche Bioscience.

DRG were removed from all spinal levels of male Sprague-Dawley rats (200–250 g) under sterile conditions. Cultures were prepared by use of a modification of the method described by Lindsay *et al*. (1991). Briefly, DRG were dissected into Ca^{2+} and Mg^{2+}-free HBSS containing 10 mM HEPES pH 7.4. The DRG were incubated twice for 90 min each in 0.125% collagenase in HAM's F-12/DME containing 6% glucose (final concentration), 5 mM HEPES, 4% Ultroser G, 1% penicillin-streptomycin (HAM's F-12/DME plus USG) at 37°C. They were rinsed with HAM's F-12/DME before incubation for 30 min at 37°C in 0.125% trypsin in HAM's F-12/DME. The media was aspirated and the DRG dispersed by trituration with a small bore, fire-polished pipette in 0.01% DNAse in HAM's F-12/DME plus USG. The cell suspension was centrifuged for 5 min at 1000 r.p.m., the supernatant discarded and the pellet resuspended in the appropriate volume of HAM's F-12/DME plus USG, containing 100 ng ml^{-1} nerve growth factor (NGF) and 100 μM 5-fluorodeoxyuridine (5-FDU) (to inhibit non-neuronal cell proliferation). Cells were plated onto Nunc 24-well tissue culture plates precoated with poly-D-lysine (10 μg ml^{-1}) and laminin (5 μg/well) and grown for 3–5 days *in vitro* in an atmosphere with 5% CO_2 at 37°C. Cells were plated at a concentration of 15×10^3 cells/well for [^{3}H]-inositol phosphates and cyclic AMP accumulation studies.

Preparation of F-11 cells

F-11 (embryonic rat DRG × mouse N18TG neuroblastoma) (Platika *et al*., 1985) cells were grown as monolayers in 162 cm^2 tissue culture flasks in HAM's F-12 containing hypoxanthine with 15% foetal bovine serum in an atmosphere with 5% CO_2 at 37°C. Flasks were maintained until approximately 80% confluent and then passed at a 1 : 4 dilution. Before use, cells were mechanically detached from the flasks, centrifuged for 5 min at 1000 r.p.m., rinsed once in 25 mM HEPES-buffered DMEM (pH 7.4), recentrifuged and then resuspended in buffer containing 200 μM isobutylmethylxanthine (IBMX) at a concentration of 2×10^6 cells ml^{-1}.

Measurement of cyclic AMP formation

Adenylyl cyclase activity in DRG cultures and F-11 cells was determined by measuring the accumulation of cyclic AMP in the presence of a phosphodiesterase inhibitor. DRG cultures at 3–4 days *in vitro* were rinsed, then incubated in 10 mM HEPES-buffered DMEM, pH 7.4, containing 10 μM indomethacin (to inhibit tonic prostanoid synthesis) and 10 μM rolipram, at 37°C for the duration of the experiment. Cells were preincubated for 15 min in the absence or presence of antagonist, before incubation for 15 min with agonist. The reactions were terminated by addition of ice-cold, 20% perchloric acid with subsequent incubation for 20 min on ice. Samples were neutralized by addition of 2 M KOH containing 25 mM HEPES, then aliquots of the supernatant were taken, acetylated and assayed with an adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) Flashplate kit. All experiments were performed in duplicate.

F-11 cells (400×10^3 cells) were incubated in 25 mM HEPES-buffered DMEM, pH 7.4, containing 200 μM IBMX at 30°C for the duration of the experiment. Cells were preincubated for 10 min in the absence or presence of antagonist before incubation for 20 min with agonist. The reactions were terminated as described above and aliquots of the supernatant were taken and assayed with a cyclic AMP SPA kit. All experiments were performed in triplicate.

The cyclic AMP content of individual samples was determined by interpolation from the appropriate Flashplate or SPA kit cyclic AMP standard curve, constructed with the Origin 4.1 (Microcal Software Inc., Northampton, MA) graphics programme.

[^{3}H]-inositol phosphates formation

Phospholipase C (PLC) activity in DRG cultures was determined by measuring the accumulation of total [^{3}H]-inositol phosphates, in the presence of LiCl, in cells that had been preincubated with *myo*-2-[^{3}H]-inositol. Briefly, DRG cultures at 3–4 days *in vitro* were incubated overnight at 37°C in inositol-free HAM's F-12 media containing 50 μCi ml^{-1} *myo*-2-[^{3}H]-inositol. Cells were rinsed then preincubated for 10 min at 37°C in inositol-free HAM's F-12 containing 10 μM indomethacin, before incubation with agonist for 10 min, in the presence of 10 mM LiCl, at 37°C. The reactions were terminated by addition of ice-cold 20% perchloric acid, with subsequent incubation for 20 min on ice. Samples were neutralized by addition of 1 M KOH and 100 mM Tris buffer, before separation of the [^{3}H]-inositol

phosphates on Dowex columns as described previously (Brown *et al*., 1984). The radioactivity in the [^{3}H]-inositol phosphate fraction was expressed as a percentage of the total lipid radioactivity. All experiments were performed in triplicate.

Rat isolated vagus nerve

Extracellular recordings of d.c. potentials in the desheathed rat vagus were made according to standard 'grease-gap' techniques (Marsh *et al*., 1987; Newberry, 1988), with the slight modifications described by Bley *et al*. (1994). Modified Marsh ganglion baths (Hugo Sachs Electronics, Type #858) were used for all recordings; Ag-AgCl electrodes were fabricated from 1 ml syringes and filled with agarose containing 1.5 M NaCl. Male Sprague-Dawley rats (200–300 g; Charles River, MA) were killed by halothane inhalation and 2–3 cm lengths of vagus nerve (caudal to the nodose ganglia) were removed, desheathed, and placed in cold, oxygenated Krebs solution that contained the following (in mM): NaCl 119, KCl 2.5, $MgSO_4$ 1.3, $NaHCO_3$ 26.2, NaH_2PO_4, $CaCl_2$ 2.0 and glucose 11.0. After incubation, for approximately 30 min, in cold (4°C) Krebs solution (in the absence or presence of antagonist) the nerves were transferred to the Hugo Sachs chambers and sealed with high-vacuum grease (Dow-Corning Silicone). A circulating water heater was used to set the temperature of the static portion of the Hugo Sachs chambers and to heat the superfusate, via a glass condenser, to 30°C. The portion of the nerve in the centre section of each bath was continuously superfused with Krebs solution in the absence or presence of drug, gassed with 5% CO_2 in O_2. The flow rate to each chamber was approximately 6 ml min^{-1}, driven by a peristaltic pump. To confirm the integrity of the nerve and adequacy of superfusion, all experiments began with depolarizations induced by exposure to Krebs solution augmented with 3 mM KCl for 1 min. Initial experiments demonstrated that this concentration of potassium ions caused depolarizations similar in amplitude to the largest prostanoid receptor-mediated events. The results include data from only those nerves that showed trans-partition resistances of greater than 25 kΩ and potassium-mediated depolarizations greater than 300 μV.

Prostanoids were added to the oxygenated Krebs solution reservoirs approximately 30 s before perfusion over the nerve. When experiments required relatively long (approximately 25 min) prostanoid applications, small quantities of freshly prepared PGI_2- or $cPGI_2$-containing Krebs solution were repeatedly added to the larger oxygenated reservoir. For generation of non-cumulative concentration-response curves, agonist exposures were for 1 min, with 15 min between addition of agonists. The nerves were exposed to receptor antagonists for at least 45 min before subsequent agonist application. Only one concentration-response curve was obtained from each nerve.

D.C. voltages were amplified 1000 times, filtered at 1000 Hz, and sampled at 3 Hz. A dedicated computer data acquisition system, using an analog-digital converter (Axon Instruments, Foster City, CA) and an Axobasic programme (Keith Bley) displayed, stored and analysed the voltage records. The acquisition programme also directed a motorized valve (Hamilton Company, Reno, NV) to switch between solutions at set intervals. During each experiment, simultaneous recordings were made from four nerves.

Data analysis

To account for the variability in the levels of cyclic AMP accumulation between individual DRG culture preparations, the fold increase over basal cyclic AMP accumulation was calculated, where fold increase = (cyclic AMP accumulation-basal)/basal. Concentration-response curves for DRG cultures (fold increase over basal versus concentration) and F-11 cells (pmol mg^{-1} protein versus concentration) were fitted individually to a sigmoidal concentration-response model by non-linear regression analysis within the Origin 4.1 graphics programme (Microcal). For DRG cultures the minimum response was constrained to zero, with the exception of BMY45778 curves, where the slope was constrained to 1 and the minimum value was not fixed. The mean pEC_{50} was calculated by taking the average of the individual estimates. Agonist potenticies (expressed as the pEC_{50}) in the isolated vagus nerve preparation were calculated by the relationship of Parker & Waud (1971), by use of iterative curve fitting techniques (Leung *et al*., 1992). These calculations were undertaken in the Department of Biomathematics, Roche Bioscience. Independent agonist concentration-response curves were fitted simultaneously to a sigmoidal model and a mean $pEC_{50} \pm$ s.e.mean value determined. Statistical levels of significance ($P < 0.05$) were calculated by use of parametric *t* tests, or analysis of variance (ANOVA) with *post-hoc* Fisher's LSD test for pairwise comparisons.

Compounds

The following chemicals and materials were obtained from the sources indicated: bradykinin, phorbol dibutyrate (Research Biochemicals Inc., Natick, MA); PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, carbaprostacyclin (see Coleman *et al*., 1994), carbocyclic thromboxane A_2, thromboxane B_2, SQ 29,548 ([15(1α,2β(5Z), 3β,4α)]-7-[3-[2(phenylamino)carbonyl]hydrazino]methyl]-7-oxobicylo[2,2,1]-hept-2-yl]-5-heptenoic acid; Sprague *et al*., 1980) (Cayman Chemical Company, Ann Arbor, MI); cicaprost was a generous gift from Dr Fiona McDonald (Schering AG., Germany); SC-19220 (8-chorodibenz[B,F][1,4]oxazepine-10 (11H)-carboxylic acid, 2-acetylhydrazide), SC-25469 (8-chorodibenx[B,F][1,4]oxazepine-10(11H)-carboxylic acid, 2-(5-chloro-1-oxopentyl)hydrazide) and SC-51332 (8-chorodibenx[B,F] [1,4]oxazepine-10(11H)-carboxylic acid, 2-[3-[2-(furanylme-thyl)thio]-1-oxopropyl]hydrazide; Hallinan *et al*., 1994) were kindly provided by Dr E. Ann Hallinan of Searle (Skokie, IL); rolipram, BMY45778 ([3-[4-(4,5-diphenyl-2-oxazolyl)-5-oxazolyl]phenoxy]acetic acid; Seiler *et al*., 1997) and AH6809 (7-isopropoxy-9-oxoxanthene-2-carboxylic acid) were synthesized by the Roche Bioscience Institute of Organic Chemistry; Ham's F-12 medium, DMEM, penicillin-streptomycin, Hanks Balanced Salt Solution (HBSS), mouse laminin (Gibco BRL, Life Technologies Ltd., Grand Island, NY); foetal bovine serum (Hyclone Labs, Logan, UT); Ultroser G (Life Technologies Ltd., Paisley, U.K.); HAM's F-12/DME high glucose powdered media (Irvine Scientific, Irvine, CA.); nerve growth factor 2.5 s (Promega, Madison, WI); trypsin (Worthington Biochemical Corporation, Freehold, NJ); collagenase, hypoxanthine (Boehringer-Mannheim, Indianapolis, IN); forskolin, (Calbiochem, San Diego, CA); iloprost, *myo*-2-[^{3}H]-inositol (17.1 Ci mmol^{-1}), cyclic AMP Scintillation Proximity Assay kit (Amersham, U.K.); cyclic AMP Flashplate Assay kit (NEN, Boston, MA); DNAse I, isobutylmethylxanthine, 5-fluorodeoxyuridine, poly-D-lysine (70–100 kDa) (Sigma, St. Louis, MO). F-11 neuroblastoma cells were a kind gift from Dr Mark Fishman (Massachusetts General Hospital, MA).

PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 carbaprostacyclin ($cPGI_2$), SC-51322, SC-25469, SC-19220, AH6809, indomethacin and rolipram were dissolved with dimethylsulphoxide (DMSO)

into 10 mM stock solutions, and immediately stored at −20°C. Carbocyclic thromboxane A_2 ($cTXA_2$) was stored as a 10 mM stock solution in ethanol. Due to the chemical instability of many prostanoids, care was taken to minimize the time that compounds spent in an aqueous state. Thus, ampules of the DMSO stock solutions were thawed only immediately before use.

Results

Effect of prostanoids on cyclic AMP levels in DRG cultures

The effects of prototypical agonists (at 1 μM) for the five prostanoid receptors on cyclic AMP accumulation in adult rat DRG cultures at 3–4 days *in vitro*, are shown in Figure 1a. The basal cyclic AMP accumulation in DRG cultures in the presence of indomethacin (10 μM) and rolipram (10 μM) was 1.65±0.10 pmol/well ($n=37$ independent culture preparations). A stable analogue of prostacyclin, $cPGI_2$, and PGE_2 at concentrations of 1 μM, produced an accumulation of cyclic AMP of 4.85±0.43 pmol well ($n=13$) and 3.82±0.26 pmol/well ($n=14$), respectively. The stable thromboxane analogue $cTXA_2$ evoked a smaller response and both PGD_2 and $PGF_{2\alpha}$ had negligible effects. In comparison, forskolin, which directly activates adenylyl cyclase, stimulated accumulation of 6.49±0.55 pmol cyclic AMP/well ($n=12$) and 26.66±4.63 pmol cyclic AMP/well ($n=3$), at concentrations of 0.1 and 1 μM, respectively, which corresponded to 2.67±0.21 and 13.38±0.84 fold increases over basal.

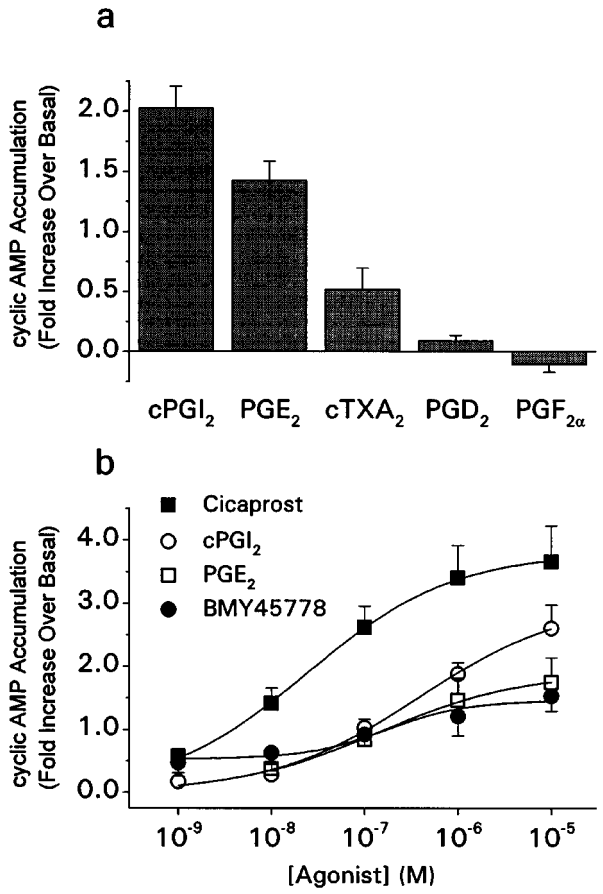

Figure 1 Effect of prostanoids on cyclic AMP accumulation in adult rat DRG cultures. Cells were preincubated for 15 min in HBSS containing 10 mM HEPES, 10 μM rolipram and 10 μM indomethacin at pH 7.4 and 37°C, before incubation with agonist at the indicated concentrations for 15 min in the same buffer. (a) Effect of $cPGI_2$, PGE_2, $cTXA_2$, PGD_2 and $PGF_{2\alpha}$ (each at 1 μM). (b) Effect of increasing concentrations of cicaprost, $cPGI_2$, PGE_2 and BMY45778. Data represent mean of 3–14 independent experiments, each performed in duplicate; vertical lines indicate s.e.mean.

To characterize further the distinction between EP and IP reeptor-mediated increases in cyclic AMP levels in DRG cultures, concentration-response curves to $cPGI_2$, PGI_2, PGE_2 and the IP agonists, cicaprost and BMY45778, were constructed (Figure 1b; Table 1). The selective IP receptor agonist, cicaprost, and $cPGI_2$ evoked similar maximum responses but cicaprost was more potent than $cPGI_2$ (Table 1). In comparison, the maximum responses to PGI_2, BMY45778 and PGE_2 were lower (Table 1). The rank order of agonist potency was cicaprost > PGE_2 = BMY45778 = $cPGI_2$ = PGI_2.

At a concentration of 1 μM, the mixed IP/EP_1 receptor agonist, iloprost, evoked an accumulation of 4.62±0.37 pmol cyclic AMP/well ($n=3$). In contrast, in the presence of the mixed EP_1/EP_3 receptor agonist, sulprostone (1 μM), and the mixed EP_2/EP_3 receptor agonist, misoprostol (1 μM), cyclic AMP accumulation in adult DRG cultures was 1.66±0.13 ($n=3$) and 1.29±0.17 pmol cyclic AMP/well ($n=3$), respectively, which was not significantly different from basal accumulation ($P>0.05$). To investigate further the role of EP_3 receptors in sensory neurones, the effect of sulprostone on both forskolin- (100 nM) and cicaprost- (100 nM) stimulated cyclic AMP accumulation was examined. Following pretreatment with sulprostone (15 min, 1 μM), cyclic AMP accumulation was 2.02±0.63 pmol/well ($n=3$), which was not significantly different from 2.01±0.52 pmol/well ($n=3$) in control cultures ($P>0.01$). Both forskolin (100 nM) and cicaprost (100 nM) evoked a significant increase in cyclic AMP accumulation in control and sulprostone-pretreated cells. Cyclic AMP accumulation was respectively, 7.54±1.92 pmol/well ($n=3$) and 6.33±0.35 pmol/well ($n=3$), in control cells ($P<0.05$ compared to basal, Fisher's LSD test), and 7.07±1.87 pmol/well ($n=3$) and 5.65±0.34 pmol/well ($n=3$), after sulprostone pretreatment ($P<0.05$, compared to basal; Fisher's LSD test). There was no significant difference in the magnitude of response to either cicaprost or forskolin between control and sulprostone pretreated cells ($P>0.05$).

Table 1 Prostanoid-mediated cyclic AMP accumulation in adult rat DRG cultures

	pEC_{50}	*Maximum fold increase over basal*	*Slope*	n
Cicaprost	7.86±0.28	3.74±0.56	0.78±0.09	6
$cPGI_2$	6.53±0.17	2.94±0.43	0.61±0.03	5
BMY45778	6.82±0.22	1.41±0.22	1.0*	4
PGI_2	6.01±0.47	1.81±0.08	0.89±0.07	3
PGE_2	6.92±0.18	1.90±0.42	0.59±0.08	4

Data represent mean±s.e.mean for pEC_{50}, maximum response and slope for the number of independent experiments indicated (n). Each experiment was performed in duplicate. Concentration-response (fold increase over basal) curves were analysed as described in Methods. *For BMY45778 the minimum response was consistently greater than vehicle control, therefore the minimum fold increase over basal was not constrained to zero, but rather, sigmoidal concentration-response curves were fitted to a model where the slope was constrained to 1.

The putative EP_1 receptor antagonist SC-51322 (1 μM) had no effect on cicaprost (100 nM)-, $cPGI_2$ (1 μM)- or PGE_2 (1 μM)-evoked cyclic AMP accumulation in DRG cultures (data not shown).

Effect of prostanoids on cyclic AMP accumulation in F-11 cells

In F-11 cells, prostanoid receptor agonists produced a concentration-dependent increase in cyclic AMP accumulation (Figure 2; Table 2) over a basal cyclic AMP accumulation of 94.9 ± 14.2 pmol mg^{-1} protein ($n=11$). Cicaprost was the most potent agonist tested, while PGI_2, $cPGI_2$ and iloprost were somewhat less potent, although each agonist evoked a similar maximum response (Table 2). In comparison, PGE_2 was significantly less potent than each of the IP agonists. The maximal response to PGE_2 could not be determined accurately, since at concentrations greater than 10 μM, there was a dramatic increase in cyclic AMP accumulation, the nature of which was not investigated. The responses to $PGF_{2\alpha}$, PGD_2 and TxA_2 were significantly smaller than those to cicaprost, even at 10 μM. Therefore, the rank order of potency was: cicaprost > iloprost = $cPGI_2$ = PGI_2 = BMY45778 > PGE_2 = $cTXA_2$.

In the presence of the putative EP_1 receptor-selective antagonist SC-19220 (100 nM), the response to $cPGI_2$ (10 nM) and PGE_2 (1 μM), respectively, was $95.2 \pm 3.9\%$ ($n=3$) and $92.0 \pm 12.7\%$ ($n=3$) of the control response in the absence of antagonist.

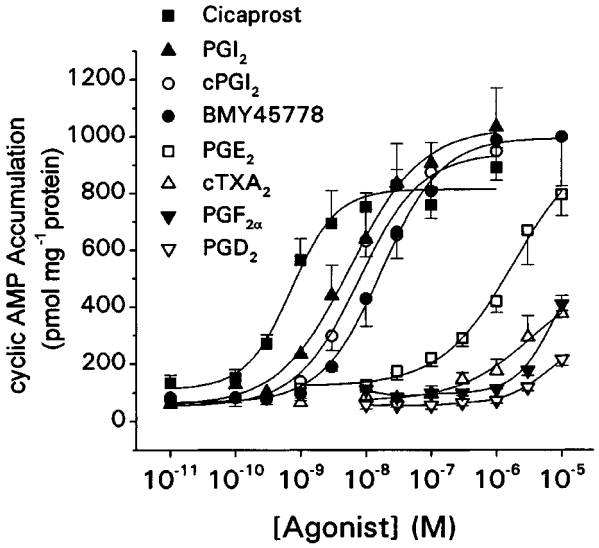

Figure 2 Effect of prostanoids on cyclic AMP accumulation in F-11 cells. Cells were incubated with increasing concentrations of agonist for 20 min in DMEM containing 25 mM HEPES, 200 μM IBMX at 30°C. Data represent mean of 3–7 independent experiments, each performed in triplicate; vertical lines indicate s.e.mean.

Table 2 Prostanoid-mediated cyclic AMP accumulation in F-11 cells

	pEC_{50}	*Maximum* (pmol mg^{-1} protein)	*Slope*	n
Cicaprost	9.13 ± 0.07	824 ± 54	1.16 ± 0.20	4
Iloprost	8.38 ± 0.24	958 ± 51	1.04 ± 0.16	4
$cPGI_2$	8.21 ± 0.09	930 ± 49	1.28 ± 0.15	6
PGI_2	8.16 ± 0.22	1037 ± 132	0.90 ± 0.15	3
BMY45778	7.72 ± 0.14	995 ± 113	1.00 ± 0.12	3
PGE_2	5.66 ± 0.11	1042 ± 133*	0.90 ± 0.13	7
$cTXA_2$	5.48 ± 0.39	526 ± 161	1.06 ± 0.26	3
PGD_2	< 5	–	–	3
$PGF_{2\alpha}$	< 5	–	–	3

Data represent mean $\pm$ s.e.mean for the pEC_{50}, maximum response and slope for 3–7 independent experiments (each performed in triplicate). Concentration-response curves were analysed as described in Methods. *At concentrations of PGE_2 greater than 10 μM, a dramatic increase in cyclic AMP accumulation was observed. The nature of this response was not investigated further, and for concentration-response curve analysis, data for more than 10 μM PGE_2 were not included.

Effect of prostanoids on [^{3}H]-inositol phosphates accumulation in DRG neurones

To examine the effect of prostanoids on phosphoinositide hydrolysis in adult DRG cultures, agonist concentrations were chosen which evoked near-maximal responses in the cyclic AMP accumulation studies. $cPGI_2$ and PGE_2 at a concentration of 1 μM, stimulated accumulation of [^{3}H]-inositol phosphates over a basal level of $0.63 \pm 0.05\%$ total [^{3}H]-PI (phosphatidyl inositols) ($n=15$; Figure 3). In contrast, $cTXA_2$, $PGF_{2\alpha}$ and forskolin (100 nM), at a concentration which evoked a similar increase in cyclic AMP accumulation to cicaprost (1 μM) (see above), had neglibible effects on [^{3}H]-inositol phosphates levels (Figure 4). PGD_2 (1 μM)-evoked [^{3}H]-inositol phosphates accumulation was $0.68 \pm 0.14\%$ total [^{3}H]-PI ($n=4$), which also was not significantly different from basal ($P>0.05$). The greatest effect was observed with the IP agonists $cPGI_2$ (1 μM) and cicaprost (100 nM) (Figure 4). However, the response magnitude was less than that seen with bradykinin (10 nM), which evoked a significant accumulation of [^{3}H]-inositol phosphates to $2.47 \pm 0.26\%$ total [^{3}H]-PI ($n=8$) ($P<0.01$).

Since prostanoids stimulated both cyclic AMP and inositol phosphates accumulation, potential interactions between these

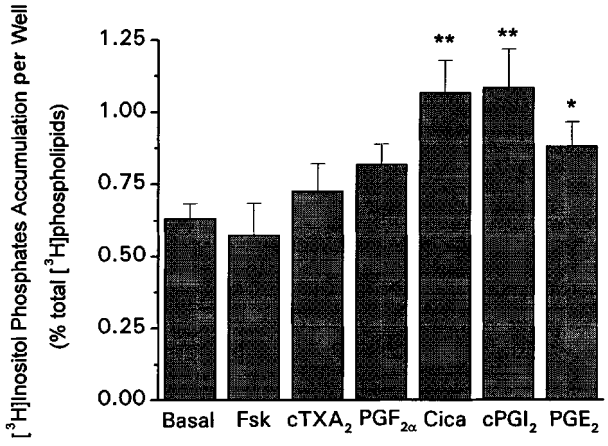

Figure 3 Effect of prostanoids and forskolin on [^{3}H]-inositol phosphates accumulation in DRG cultures. Adult DRG cultures were loaded overnight with *myo*-2-[^{3}H]-inositol. Cells were preincubated for 10 min in inositol-free HAM's F-12 medium at 37°C, containing 10 μM indomethacin, before incubation with forskolin (100 nM; Fsk); $cTXA_2$ (1 μM), $PGF_{2\alpha}$ (1 μM); cicaprost (100 nM; Cica); $cPGI_2$ (1 μM) and PGE_2 (1 μM) for 10 min in the same medium with 10 mM LiCl added. Total [^{3}H]-inositol phosphates were extracted and separated. Data represented mean $\pm$ s.e.mean for 4–13 independent experiments, each performed in triplicate. *$P<0.05$, **$P<0.01$, significantly different from basal [^{3}H]-inositol phosphates accumulation.

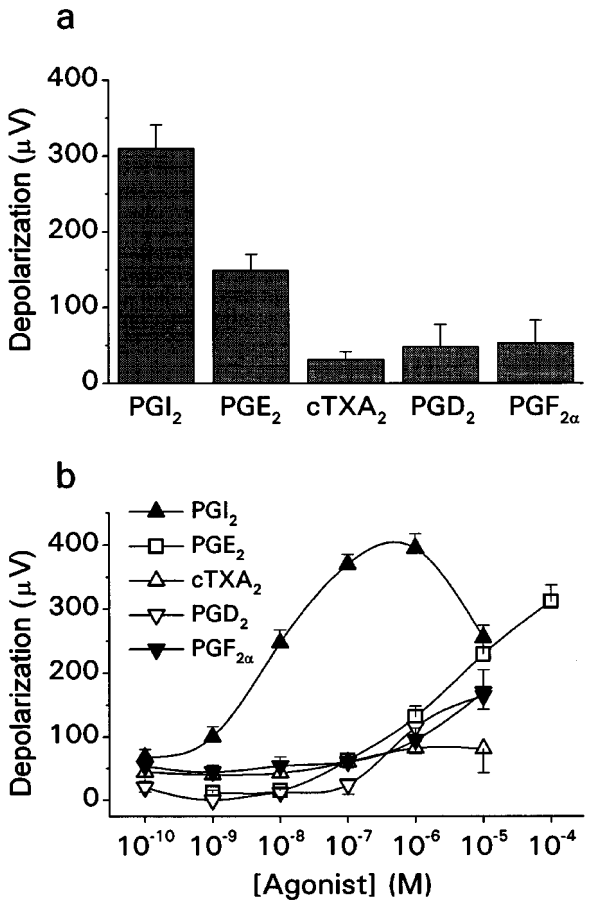

Figure 4 Prostanoid receptor-mediated depolarizations in the rat isolated vagus nerve. (a) Mean voltage deflections caused by 1 min applications of PGI_2, PGE_2, $cTXA_2$, PGD_2 and $PGF_{2\alpha}$ at 1 μM at 30°C. (b) Non-cumulative concentration-effect curves. Mean voltage deflections caused by 1 min applications of increasing concentrations of prostanoid at 30°C with 15 min inter-application intervals.

two signalling pathways were examined. Forskolin alone had no effect on [^{3}H]-inositol phosphates accumulation in DRG cultures (see above). To determine whether activation of the phosphoinositide pathway, and hence protein kinase C (PKC), influenced cyclic AMP accumulation the PKC-activating phorbol ester, phorbol dibutyrate (PDBU) was used. PDBU (1 μM) significantly increased basal cyclic AMP accumulation (Table 3). Furthermore, following pretreatment with PDBU (1 μM, 15 min) there was a significant increase in both cicaprost (100 nM)- and forskolin (100 nM)-evoked cyclic AMP accumulation (Table 3). Thus, cicaprost and forskolin, respectively, evoked an accumulation of cyclic AMP over basal of 4.25 ± 0.34 ($n=4$) pmol/well and 5.26 ± 1.14 ($n=4$) pmol/well in control cells, in comparison to a $8.50 \pm 0.51 \pm$ ($n=4$) pmol/well and 12.42 ± 2.01 ($n=3$) pmol/well in PDBU-pretreated cells.

Effect of prostanoids on rat isolated vagus nerve preparation

Single concentrations of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and $cTxA_2$ (1 μM) depolarized the vagus nerve (Figure 4a). The greatest depolarization was observed with PGI_2 followed by PGE_2, while PGD_2, $PGF_{2\alpha}$ and $cTxA_2$ had negligible effects. The endogenous prostanoids (0.1 nM – 10 μM) produced concentration-dependent increases in depolarizations of the vagus nerve (Figure 4b). PGI_2 was the most potent agonist studied and also exhibited the greatest maximal response (Table 4). Maximal responses to PGE_2, $PGF_{2\alpha}$, PGD_2 and $cTxA_2$ from non-cumulative concentration-response curves were significantly smaller than those to PGI_2 in the tested concentration range (Figure 4b, Table 4). $cPGI_2$ yielded complex concentration-response curves, since depolarizations at 0.1 nM were greater than observed at 1 nM. The potency of $cPGI_2$ was similar to that of PGI_2, but the maximum response magnitude was smaller. The non-sigmoidal shape of the $cPGI_2$ concentration-response curve may have been a result of receptor desensitization. Indeed, when nerves were exposed to a single, maximal concentration of $cPGI_2$ (10 μM) the resulting average depolarization (472 ± 44 μV, $n=8$) was significantly larger than that evoked by $cPGI_2$ (10 μM) during measurement of the non-cumulative concentration-response curve (98 ± 4 μV, $n=4$). Depolarizations induced by PGI_2 also exhibited desensitization, thus the response to PGI_2 (10 μM) during concentration-response experiments was 235 ± 19 μV ($n=11$), in comparison to 542 ± 34 μV following a single exposure to PGI_2 ($n=4$). The rank order of all agonists studied was, therefore: $PGI_2 = cPGI_2 = PGE_1 > cTXA_2 > PGE_2 = PGD_2 = TXB_2 > PGF_{2\alpha}$ (Table 4).

Table 3 Effect of PDBU (1 μM) on cicaprost- and forskolin-mediated cyclic AMP accumulation in adult DRG cultures

	Control (pmol/well)	*PDBU pretreatment* (pmol/well)
Basal	1.93 ± 0.38	3.64 ± 0.47#
Cicaprost (100 nM)	6.17 ± 0.29*	12.15 ± 0.34*
Forskolin (100 nM)	7.19 ± 1.41*	14.66 ± 2.18*

Data represent mean ± s.e.mean for 3–4 independent experiments (each performed in duplicate). Cells were pretreated PDBU (1 μM) for 15 min before incubation in the absence or presence of cicaprost or forskolin at the concentrations indicated. #$P<0.05$, significantly different from control basal; *$P<0.05$, significantly different from appropriate basal, in the absence or presence of PDBU (Fisher's LSD test).

Table 4 Potency and maximal reponses of various prostanoid receptor ligands in the rat isolated vagus nerve

Agonist	pEC_{50}	*Maximum depolarization* (μV)	n
PGI_2	8.42 ± 0.19	393 ± 21	11
PGE_2	6.24 ± 0.12	279 ± 26	12
PGD_2	6.22 ± 0.12	164 ± 21	4
TxA_2	6.96 ± 0.23	81 ± 38	4
$PGF_{2\alpha}$	5.82 ± 0.46	170 ± 35	8
$cPGI_2$	7.92 ± 0.77	181 ± 8	4
PGE_1	7.52 ± 0.05	331 ± 42	4
TxB_2	6.20 ± 0.70	61 ± 23	4
PGI_2 + 1 μM AH6809	8.79 ± 0.34	423 ± 12	4
PGI_2 + 10 μM SC-19220	8.55 ± 0.14	442 ± 40	4
PGI_2 + 1 μM SC-25469	8.51 ± 0.23	402 ± 35	4
PGI_2 + 1 μM SC-51322	8.12 ± 0.10	445 ± 38	4
PGI_2 + 1 μM SQ-29548	8.88 ± 0.19	322 ± 15	4
PGI_2 + 10 μM indomethacin	8.65 ± 0.15	403 ± 24	4
PGE_2 + 1 μM SQ-25469	6.24 ± 0.09	349 ± 21	8

Values are expressed as means ± s.e.mean. All maximum depolarizations were determined at 10 μM, except those for PGI_2, PGE_1 and $cPGI_2$, which were measured at 1 μM.

The present findings suggest that sustained applications of either $cPGI_2$ or PGI_2 cause receptor desensitization. To investigate whether PGI_2- and PGE_2-evoked depolarizations were mediated through activation of a single prostanoid receptor, $cPGI_2$ (10 μM; $n=8$) or PGI_2 (10 μM; $n=4$) were superfused over the nerves for 20 min before the simultaneous application of PGE_2 (10 μM) for one minute. The rate of desensitization was significantly greater for $cPGI_2$ (Figure 5a) than PGI_2. The addition of PGE_2 (10 μM) to the superfusate, in the continued presence of $cPGI_2$ or PGI_2, produced depolarizations of 19 ± 4 μV and 54 ± 7 μV, respectively, which were significantly ($P<0.01$) smaller than those recorded for PGE_2 (10 μM) alone (279 ± 26 μV) (Figure 5b).

The onset of prostanoid receptor-mediated depolarizations was slower than that for responses induced by ligand-gated ion channel receptors, such as capsaicin or nicotine (Bevan & Geppetti, 1994). Thus, the average rate of depolarization induced by single applications of PGI_2 (10 μM) was 4 ± 1 μV s^{-1} ($n=4$), which was significantly different from the rates of depolarization of 18 ± 3 μV s^{-1} ($n=8$) for capsaicin (10 μM) and 12 ± 1 μV s^{-1} ($n=4$) for nicotine (30 μM) ($P<0.01$).

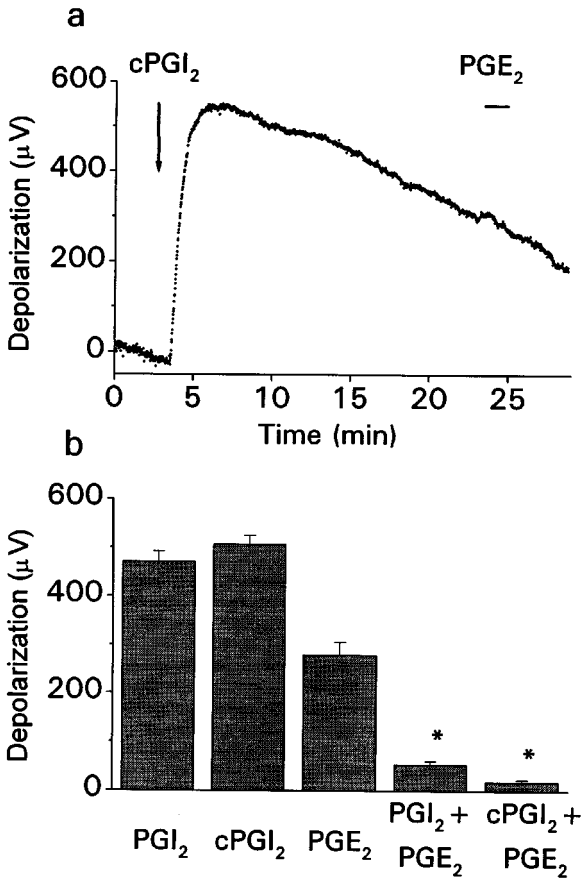

Figure 5 Desensitization of IP receptors attenuated responses to PGE_2. (a) D.c. voltage trace from a representative isolated nerve recording. Superfusion with 10 μM $cPGI_2$ commenced at the time indicated by the downward arrow (and continued for the duration of the experiment). The 1 min application of 10 μM PGE_2 is indicated by the horizontal bar. (b) A comparison of the maximum mean responses generated by the following conditions: 20 min applications of 10 μM PGI_2 (PGI_2) or 10 μM $cPGI_2$ ($cPGI_2$); 1 min applications of 10 μM PGE_2 (PGE_2) during construction of PGE_2 concentration-response curves; 1 min applications of 10 μM PGE_2 on top of the sustained depolarizations produced by PGI_2 (PGI_2+PGE_2) or $cPGI_2$ ($cPGI_2+PGE_2$). *$P<0.01$, significantly smaller depolarization for PGI_2+PGE_2 or $cPGI_2+PGE_2$ compared to PGE_2 alone.

Prostanoid receptors on the rat isolated vagus nerve were characterized further by use of available antagonists. The putative EP_1 receptor-selective antagonists, SC-51322 (1 μM) and other related dibenzoxapines such as SC-19220 (1 or 10 μM) or SC-25469 (1 or 10 μM) did not significantly reduce the potency or maximal response of PGI_2 (see Table 4). Similarly, neither the EP_1/DP receptor antagonist, AH6809 (1 μM) nor the TP receptor antagonist SQ-29548 (1 μM) affected responses to PGI_2 (Table 4). Finally, SC-25469 (1 μM, $n=4$) failed to antagonize responses to PGE_2 (Table 4).

Role of adenylyl cyclase and phosphoinositide hydrolysis in PGI_2-mediated depolarizations of the vagus nerve

The present study has demonstrated that in adult rat DRG cultures, IP receptors are positively coupled to both adenylyl cyclase and PLC. Therefore, forskolin and PDBU were used in an attempt to mimic the depolarization kinetics of PGI_2 receptor activation. Exposure to forskolin (10 μM; $n=4$) for 1 min evoked depolarizations that were significantly smaller (342 ± 54 μV) and slower (2.7 ± 0.4 μV s^{-1}) than those caused by PGI_2 (10 μM). Likewise, PDBU (1 μM; $n=3$) for 1 min evoked depolarizations (439 ± 9 μV at a rate of 1.6 ± 0.3 μV s^{-1}) that were significantly larger and slower. The inactive analogues 1,9-dideoxy-forskolin (10 μM) and α-phorbol 12,13-didecanoate (1 μM) have been shown previously to produce negligible effects (Rang & Ritchie, 1988; Bley *et al.*, 1994).

The effect of prior activation of adenylyl cyclase and PKC on PGI_2-mediated depolarizations was examined next. Preparations were treated with forskolin (10 μM) or PDBU (1 μM), for 15 min, and once stable depolarizations were attained the nerves were exposed to PGI_2 (10 μM) for 1 min (Figure 6a). Under these conditions, PGI_2-induced responses were significantly ($P<0.01$) smaller in nerves exposed to forskolin than to PDBU alone, even though the stable depolarizations caused by forskolin were smaller (Figure 6b). When nerves were superfused with both forskolin (10 μM) and PDBU (1 μM) for 15 min ($n=8$), the stable depolarizations were significantly ($P<0.01$) larger than those obtained with either agent alone (Figure 6). Under these conditions, the effect of PGI_2 (10 μM) was extremely small and significantly different ($P<0.01$) from the PGI_2 response observed when nerves were pretreated with either forskolin or PDBU alone (Figure 6b).

Discussion

Pharmacology of prostanoid responses in adult rat DRG cultures, F-11 cells and rat isolated vagus nerve

In DRG cultures, both the IP and EP receptor agonists, $cPGI_2$ and PGE_2, respectively, increased cyclic AMP levels, while $cTxA_2$, PGD_2 and $PGF_{2\alpha}$ had negligible effect. The highly selective IP receptor agonist cicaprost was the most potent agent tested and, analogous to $cPGI_2$, evoked a greater maximal increase in cyclic AMP accumulation than PGE_2. The IP receptor agonist BMY45778 also increased cyclic AMP levels but the response magnitude was smaller, which may reflect its partial agonist activity at the IP receptor (Wise & Jones, 1996).

In DRG cells the small response to PGE_2 may result from simultaneous activation of EP receptors which are positively-

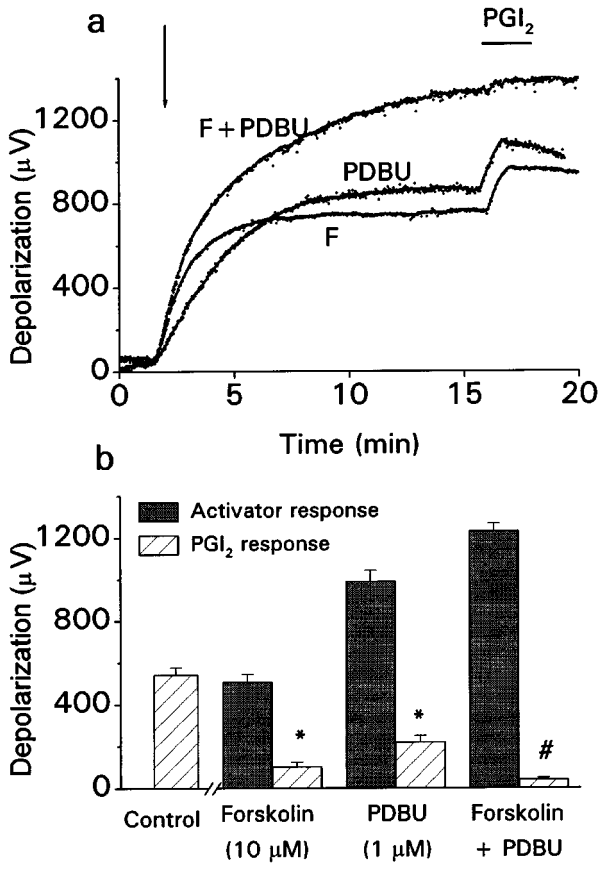

Figure 6 Effect of forskolin and phorbol dibutyrate on PGI_2-mediated depolarization in the rat isolated vagus nerve. (a) Superimposed voltage traces from 3 experiments where 10 μM forskolin (F), 1 μM phorbol dibutyrate (PDBU) or 10 μM forskolin and 1 μM phorbol dibutyrate (F+PDBU) were applied to nerves at the time indicated by the downward arrow and remained present for the duration of the experiment. The concomitant application of 10 μM PGI_2 for 1 min is indicated by the bar. (b) Maximum responses to 10 μM forskolin and/or 1 μM PDBU, in addition to maximum responses to a 1 min application of 10 μM PGI_2 to naive nerves (control) or nerves preincubated for 15 min with enzyme activator (10 μM forskolin and/or 1 μM PDBU). Data represent mean$\pm$ s.e.mean ($n=4-12$). *$P<0.01$, significantly different from control PGI_2 response; #$P<0.01$, significantly different from PGI_2 response in the presence of forskolin or PDBU alone.

($EP_{2/4}$) or negatively- (EP_3) coupled to adenylyl cyclase (Mao *et al*., 1996). EP_3 and, to a lesser extent EP_1 and EP_4, receptor mRNA has been identified in mouse DRG neurones (Oida *et al*., 1995). The localization of EP_2 mRNA in DRG has not been determined. The role of $EP_{2/4}$ receptors in PGE_2-mediated stimulation of adenylyl cyclase in DRG cultures cannot be determined at present, due to lack of receptor subtype selective agonists or antagonists. However, the mixed EP_1/EP_3 and EP_2/EP_3 receptor agonists, sulprostone and misoprostol (Coleman *et al*., 1994), respectively, had no effect on basal cyclic AMP accumulation. Furthermore, sulprostone had no effect on either forskolin- or cicaprost-stimulated cyclic AMP accumulation. These data, therefore, provide no evidence for functional EP_3 receptors in adult rat DRG cultures. However, a significant degree of tonic inhibitory activity was observed in DRG cultures, as suggested by the ability of pertussis toxin pretreatment to significantly enhance basal cyclic AMP accumulation (Jacqueline Smith, unpublished observations.). This high level of basal inhibition may have obscured detection of EP_3 receptor-mediated inhibition of adenylyl cyclase.

Pharmacological characterization of prostanoid receptor-mediated activation of adenylyl cyclase in the F-11 cells demonstrated that the rank order of agonist potency was: cicaprost > iloprost = $cPGI_2$ = PGI_2 = BMY45778 > PGE_2 = $cTXA_2$. Therefore, in both DRG cultures and F-11 cells the response to an IP anonist(s) was greater than that to other endogenous prostanoids, consistent with IP receptor activation (Kiriyama *et al*., 1997). The absolute potencies of the IP agonists tested were higher in F-11 than DRG cells, which may be a function of differences in receptor-coupling efficacy or stability of compounds in the various incubation conditions. In addition, the potency of PGE_2 was greater in DRG cultures, which may reflect a greater complement of EP receptors in these cells.

The rank order of agonist potency for depolarization of the isolated vagus nerve preparation was: $PGI_2 = cPGI_2 = PGE_1 > cTXA_2 > PGE_2 = PGD_2 = TXB_2 > PGF_{2\alpha}$. This rank order is also consistent with IP receptor activation (Kiriyama *et al*., 1997) and, to our knowledge, is the first extensive characterization of IP receptor-mediated depolarizations of the isolated vagus nerve. These data support *in vivo* studies, suggesting a physiological role for vagal IP receptors. Thus, PGI_2 and PGE_2 are known to stimulate reflex coughing and bronchoconstriction, probably via stimulation of afferent vagal C-fibres which innervate the lung (Roberts *et al*., 1985).

The endogenous prostanoids exhibit a considerable degree of receptor cross-reactivity (Coleman *et al*., 1994; Kiriyama *et al*., 1997), therefore it is conceivable that the effects of PGE_2 in these *in vitro* preparations may, in part, be mediated via the IP receptor. Several putative EP antagonists, including SC-19220 and SC-51322, had no effect on the responses to either PGI_2/$cPGI_2$ or PGE_2. However, it should be noted that these compounds are known as EP_1 antagonists (Coleman *et al*., 1994) and as such would not be expected to inhibit EP_2 and/or EP_4 receptors, which are coupled to activation of adenylyl cyclase. The EP_1 receptor-selectivity of these compounds is based upon their antagonist activity against PGE_2-evoked responses in guinea-pig isolated ileum. However, the results from this bioassay must be interpreted with caution, since additional EP receptors, other than the EP_1 subtype, are involved in the response (Lawrence *et al*., 1992). Moreover, SC-19220 exhibits negligible affinity for any one of the four stably-expressed mouse EP receptors (Kiriyama *et al*., 1997) or human EP_1 and EP_3 receptors (Keith Bley, unpublished observations).

In the isolated vagus nerve preparation, pretreatment with $cPGI_2$ and PGI_2 attenuated the response to a subsequent application of PGE_2. One interpretation of this finding is that PGI_2, $cPGI_2$ and PGE_2 depolarize vagus nerves through action at a single receptor. Vagal nerves still exhibited a response to high K^+ after agonist pretreatment, indicating that nerve depolarization could occur. However, the possibility that $cPGI_2$ and PGI_2 had caused heterologous receptor desensitization cannot be excluded.

IP receptor activation also stimulated phosphoinositide hydrolysis in adult rat DRG cultures, although the response to both 1 μM cicaprost and $cPGI_2$ was less than half that to 10 nM bradykinin. The potency of cicaprost for activation of PLC was not determined. Activation of IP receptors stably-expressed in CHO cells increases both cyclic AMP and IP_3 levels, suggesting that IP receptors can couple to multiple signal transduction pathways, although with different efficacies (Namba *et al*., 1994). Furthermore, in a number of different

cell types endogenous IP receptor-mediated increases in $[Ca^{2+}]_i$ have been observed (Watanabe *et al*., 1991; Vassaux *et al*., 1992; Schwaner *et al*., 1992).

The present findings suggest that PGI_2-evoked depolarization of the vagus nerves may be mediated through a concomitant activation of cyclic AMP-dependent protein kinase (PKA) and PKC. Both forskolin and PDBU (Rang & Ritchie, 1988) produce depolarizations similar to those following exposure to PGI_2. In addition, pretreatment of nerves with forskolin or PDBU alone reduced subsequent PGI_2-evoked depolarizations, and when added in combination, virtually abolished them. In DRG cultures, PDBU elevated cyclic AMP levels and potentiated the response to both cicaprost and forskolin. PKC can modulate the adenylyl cyclase system at several sites (see Houslay, 1991 for review) and the mechanism(s) underlying the effects of PDBU on cyclic AMP levels is unclear. PKC may act directly on adenylyl cyclase to increase enzyme sensitivity or indirectly via phosphorylation of $G_{i/o}$-coupled receptors to attenuate tonic inhibitory activity.

IP receptors on sensory neurones

The present findings support autoradiographic and *in situ* hybridization studies which have demonstrated IP receptors on sensory neurones. A high density of binding sites for [^{3}H]-iloprost was observed in rat DRG and the dorsal horn of the spinal cord (Matsumura *et al*., 1995). The latter sites are thought to represent IP receptors on the terminals of primary afferents, since binding site density decreased following dorsal rhizotomy. Furthermore, [^{3}H]-iloprost binding sites accumulated following ligation of the vagus nerve, consistent with axonal transport of IP receptors to the central and/or peripheral terminals of primary afferents. In mouse DRG, $\sim$40% of neurones were strongly labelled with IP mRNA, the majority of these were small diameter neurones, although both large and small neurones were labelled (Oida *et al*., 1995). The latter cells are thought to correspond to the unmyelinated C-fibre and thinly-myelinated Aδ-fibre nociceptive neurones (Lawson, 1992). In the present study, it is unclear if only a subset of DRG neurones responded to prostanoids, since the biochemical responses of the total population of DRG neurones were measured. To resolve this question it would be necessary to conduct experiments directed at single cells, by use of either dynamic imaging or electrophysiological techniques.

PGI_2 was a potent agonist in F-11 cells and the isolated vagus nerve preparation, but was consistently less potent when tested in DRG cultures. PGI_2 is a very labile compound (Dusting *et al*., 1978) and therefore may undergo differential degradation in the various incubation conditions. An alternative explanation is that the IP receptor in spinal DRG is different from that in F-11 cells and nodose ganglion cells. However, this seems unlikely, given the similarity in responses to both the stable PGI_2 analogue, $cPGI_2$, and to the highly selective IP receptor agonist, cicaprost.

Pharmacological evidence has been presented recently which suggests the existence of two subtypes of the IP receptor in neuronal tissue (Takechi *et al*., 1996). The 'IP_1' receptor, which is equivalent to the platelet and smooth muscle form of the IP receptor, exhibits high affinity for $cPGI_2$, cicaprost and iloprost. In contrast, the 'IP_2' subtype has high affinity for isocarbacyclin and $cPGI_2$, but low/negligible affinity for iloprost and cicaprost. In F-11 and DRG cells, cicaprost was the most potent agonist tested, suggesting that the IP receptor in these cells corresponds to the putative IP_1 subtype. Furthermore, preliminary studies with reverse-transcriptase polymerase chain reaction techniques have identified molecular species in rat DRG and F-11 cells which are completely homologous to the rat and mouse platelet IP receptors, respectively (R. Salazar, personal communication).

IP receptors and sensitization of sensory neurones

In sensory neurones, activation of PKA and PKC induces neuronal sensitization (reviewed by Ferreira & Nakamura, 1979; Coderre, 1993). A number of recent electrophysiological studies have demonstrated that several types of ion channel can be regulated by PGI_2 or PGE_2 or agents which elevate intracellular cyclic AMP levels. Thus cyclic AMP- or PGI_2/PGE_2-mediated inhibition of potassium channel currents in sensory neurones has been demonstrated (Fowler *et al*., 1985; Nicol *et al*., 1997). In guinea-pig nodose and trigeminal ganglion cells, PGE_2 and forskolin enhance I_h, an inward, hyperpolarization-activated cation current that plays a role in the control of repetitive firing (Ingram & Williams, 1994). In small DRG neurones the activation threshold of a tetrodotoxin-resistant sodium current is lowered by PGE_2, forskolin or other neurotransmitters which activate adenylyl cyclase (Gold *et al*., 1996; England *et al*., 1996; Cardenas *et al*., 1997). Persistently active tetrodotoxin-sensitive Na^+ channels may contribute to the resting potential of peripheral nerves, as both in isolated vagus (S. Amagasu, personal communication) and optic (Stys *et al*., 1993) nerves, superfusion of tetrodotoxin produces a rapid hyperpolarization.

A key question is which endogenous prostanoid, and associated receptor, is important with respect to activation and/or sensitization of sensory neruones *in vivo?* Evidence suggests that PGI_2 is at least as important as PGE_2 for the induction of hyperalgesia associated with inflammation or injury. The major metabolite of PGI_2, 6-keto-$PGF_{1\alpha}$, is found in higher concentrations than PGE_2 in inflammatory exudates (Brodie *et al*., 1980), human arthritic knee joint synovial fluid (Bombardieri *et al*., 1981) or the peritoneal cavity fluid from mice injected with irritants (Berkenkopf & Weichman, 1988). With two exceptions (Tyers & Haywood, 1979; Whelan *et al*., 1991); which may reflect species and/or tissue differences or PGI_2 instability, most studies have found that PGI_2 is at least equipotent with PGE_2 at exciting sensory neurones (see review by Ferreira, 1979; Juan, 1979; Birrell *et al*., 1991; Mizumura *et al*., 1991; Devor *et al*., 1992; Schepelmann *et al*., 1992; Birrell & McQueen, 1993) and causing algesia (Ferreira *et al*., 1978; Higgs *et al*., 1978; Doherty *et al*., 1987; Taiwo & Levine, 1988). The most convincing evidence for a role of IP receptors in inflammatory hyperalgesia has been obtained from recent studies with transgenic mice lacking the IP receptor (Murata *et al*., 1997). In these animals, the acetic acid-induced writhing response and paw oedema, resulting from carrageenan injection, were reduced to the same levels as those observed in indomethacin-treated wild-type mice. The modest writhing response induced by PGE_2 was unchanged in the transgenic animals. Direct evidence for a role of PGE_2 in inflammatory pain comes from studies in which a selective, neutralizing anti-PGE_2 monoclonal antibody was used. This antibody (2B5) inhibits, to the same extent as indomethacin, phenylbenzoquinone-induced writhing in mice (Mnich *et al*., 1995), or carrageenan-induced paw hyperalgesia and oedema in rats (Portanova *et al*., 1996).

Conclusion

IP receptor agonists potently stimulate both adenylyl cyclase and phosphoinositide hydrolysis in primary cultures of sensory

neurones and neuroblastoma × sensory neurone hybrid cells, and evoke excitatory responses in the rat isolated vagus nerve. Unequivocal characterization of the receptor type(s) responsible for the physiologically relevant prostanoid-mediated responses in sensory neurones awaits the development of selective antagonists for the IP receptor and the different EP receptor subtypes. However, the rank orders of both endogenous and synthetic agonist potency, and the lack of effect of the available EP or TP receptor antagonists in each *in vitro* preparation, are consistent with IP receptor activation. These findings suggest that IP receptors may play an important role in the sensitization of sensory neurones.

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