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Protein kinase A-dependent coupling of mouse prostacyclin receptors to G_i is cell-type dependent

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Abstract

The ability of the prostacyclin (IP) receptor agonist cicaprost to activate G_s-, $G_{q/11}$- and G_i-mediated cell signalling pathways has been examined in Chinese hamster ovary (CHO) cells and human embryonic kidney 293 (HEK 293) cells expressing the cloned human (hIP) or mouse (mIP) prostacyclin receptor, and compared with data from NG108-15 and SK-N-SH cells that endogenously express rat/mouse and human IP receptors, respectively. Cicaprost stimulated [^{3}H]cyclic AMP production with EC_{50} values of 1.5–22 nM, and stimulated [^{3}H]inositol phosphate production (EC_{50} values 49–457 nM) in all but the SK-N-SH cells. Cicaprost failed to inhibit forskolin-stimulated [^{3}H]cyclic AMP production in any of these cell lines. Therefore, although both human and mouse IP receptors couple to G_s and $G_{q/11}$-mediated signalling pathways in a cell type-dependent manner, we could find no evidence for IP receptor coupling to G_i.

Keywords: Prostacyclin receptor; Adenylyl cyclase; Phospholipase C; G protein coupling; Receptor switching

1. Introduction

The prostacyclin (IP) receptor is a G protein-coupled receptor belonging to the family of prostanoid receptors (Narumiya et al., 1999). IP receptors couple primarily to activation of adenylyl cyclase via Gs, and can also couple to activation of phospholipase C leading to phosphatidyl inositol turnover and mobilization of intracellular calcium ($[Ca^{2+}]_i$) (see Wise and Jones, 2000). Whether or not IP receptor coupling to $G_{q/11}$ is physiologically relevant remains uncertain, since to date, activation of this pathway in native cells has only been demonstrated using the non-specific IP receptor agonist iloprost in piglet cerebral microvascular smooth muscle cells (Parkinson et al., 2000) and in isolated rat dorsal root ganglion cells in vitro (Smith et al., 1998). Activation of multiple signalling pathways by IP receptor agonists (observed as increases in cyclic AMP, inositol trisphosphate or $[Ca^{2+}]_i$) has been reported in transformed cell lines such as BNu2cl3 mouse mast cells, Ob1771 mouse pre-adipocytes, human erythroleukaemia (HEL) cells and human megakaryoblastic leukaemia (MEG-01) cells (for references, see Wise and Jones, 1996).

A recent report has shown that the IP receptor endogenously expressed in mouse erythroleukaemia (MEL) cells and the cloned mouse (mIP) prostacyclin receptor stably overexpressed in human embryonic kidney 293 (HEK 293) cells can couple to G_i (Lawler et al., 2001). Furthermore, the mIP receptor in HEK 293 cells couples to both $G_{q/11}$ and G_i in a protein kinase A (PKA)-dependent manner (Lawler et al., 2001). In contrast, the cloned human (hIP) prostacyclin receptor stably overexpressed in HEK 293 cells couples independently to G_s and $G_{q/11}$ and does not couple to G_i (Miggin and Kinsella, 2002). We have previously demonstrated that IP receptor agonist-stimulated [^{3}H]cyclic AMP production in Chinese hamster ovary (CHO) cells transiently expressing mIP receptors and in human neuroblastoma SK-N-SH cells was not affected by pertussis toxin treatment, suggesting a lack of coupling of both mIP and human IP receptors to G_i (Kam et al., 2001). Therefore, we have extended our studies here to examine the G protein coupling capacity of human and rodent IP receptors in a variety of cell lines stably or transiently expressing IP receptors. Our studies fail to support the hypothesis that mouse IP receptor switching from G_s to G_i is a universal phenomenon.

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2. Materials and methods

2.1. Cell culture

CHO cells were cultured in Ham's F-12 medium supplemented with 10% foetal bovine serum. HEK 293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) medium supplemented with 10% foetal bovine serum, and assays were performed in cell culture dishes treated with poly-D-lysine. Rat/mouse neuroblastoma-glioma (NG108-15) cells were cultured in DMEM medium supplemented with 6% foetal bovine serum, 0.1 mM sodium hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine and 2 mM L-glutamine. SK-N-SH cells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum. All culture media were supplemented with 100 i.u./ml penicillin and 100 μg/ml streptomycin, and cells were maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C.

2.2. Transient expression of receptors in CHO and HEK 293 cells

For [^{3}H]cyclic AMP assays, CHO and HEK 293 cells were grown to approximately 80% confluency in a cell culture flask, then transfected with hIP (1.0 μg/ml for CHO cells and 0.5 μg/ml for HEK 293 cells) or mIP (0.5 μg/ml) using LipofectAMINE liposome reagent and Optimem I reduced serum medium for 5 h, according to the manufacturer's instructions. At 24 h after transfection, the transfected cells were harvested by trypsinization and seeded at 1×10^5 cells in 24-well culture plates containing 1 ml medium plus antibiotics and 1% foetal bovine serum. For [^{3}H]inositol phosphate assays, cells were transfected at approximately 80% confluency in 12-well plates and maintained in 1 ml medium plus antibiotics and 10% foetal bovine serum. All transfected cells were assayed 48 h post-transfection, when cells were 80% to 90% confluent.

2.3. Preparation of CHO cells stably expressing hIP receptors

The complete cDNA of the human IP receptor (hIP), cloned as a 2 kb fragment into pBluescript (humanPGIR; Katsuyama et al., 1994) was a generous gift of Dr. T. Kobayashi (Department of Pharmacology, Kyoto University). For stable transfection of CHO cells, the cDNA of the hIP receptor was subcloned as a 2 kb *Eco*RI-fragment into the expression plasmid pcDNA3.1. CHO cells were transfected with 1.32 μg/ml hIP in a 90-mm culture dish, and 48 h post-transfection, G418 (300 μg/ml) selection was applied. After approximately 14 days, G418-resistant colonies were selected by dilution-cloning and screened by adenylyl cyclase assays. Clone CHO-hIP-16 was selected for use and was maintained in culture medium containing 150 μg/ml G418 until the prelabelling step of the adenylyl cyclase assay.

2.4. [^{3}H]cyclic AMP accumulation assay

[^{3}H]cyclic AMP accumulation was assayed following overnight incubation of cells with [^{3}H]adenine, as described previously (Chow et al., 2001). Briefly, when cells were at approximately 80% confluency (i.e. 24 h after transfection for CHO and HEK 293 cells), they were incubated in culture medium plus antibiotics and 1% foetal bovine serum. After an overnight incubation (16–20 h) with [^{3}H]adenine (1 μCi/ml), the medium was aspirated and the cells washed twice with 1 ml HEPES-buffered saline (HBS: 15 mM HEPES, pH 7.4, 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose). Cells were challenged, in duplicate, with test compounds for 30 min at 37 °C in HBS containing 1 mM 3-isobutyl-1-methyl xanthine and 3 μM indomethacin. The reaction was terminated by aspiration and addition of 1 ml ice-cold 5% trichloroacetic acid containing 1 mM ATP. [^{3}H]cyclic AMP was separated from [^{3}H]ATP by column chromatography (Barber et al., 1980). Adenylyl cyclase activity was estimated as the ratio of radiolabelled cyclic AMP to total AXP (i.e. adenine, cyclic AMP, ADP and ATP), and is expressed as % conversion ([cyclic AMP]/[total AXP] × 100%).

2.5. [^{3}H]inositol phosphate accumulation assay

[^{3}H]inositol phosphate accumulation was assayed in 12-well plates following 42 h incubation of cells with [^{3}H]*myo*-inositol, as described previously (Chow et al., 2001). Briefly, cells were washed twice with 1 ml HBS followed by 10 min incubation at 37 °C in HBS containing 20 mM LiCl and 3 μM indomethacin. The buffer was removed and replaced with fresh buffer containing agonists for a further 60 min. The reaction was terminated by aspiration and addition of 0.75 ml ice-cold 20 mM formic acid. [^{3}H]inositol phosphates were separated from the [^{3}H]total inositol fraction by column chromatography (Conklin et al., 1992). The production of [^{3}H]inositol phosphates was estimated as the ratio of radiolabelled inositol phosphates to total inositol and is expressed as % conversion ([inositol phosphates]/[total inositol] × 100%). Assays were performed in duplicate.

2.6. Data analysis

pEC_{50} values (negative logarithm of the EC_{50} value) and E_{max} values (maximum response) from log concentration–response curves were obtained by fitting the log concentration–response curves to the standard four-parameter logistic equation using GraphPad Prism software version 3.0 (GraphPad Software, USA). Values reported are mean ± S.E.M. of at least three independent experiments. Statistical

Table 1
Activation of adenylyl cyclase by cicaprost in different cell lines

Cell line	Adenylyl cyclase				
	Cicaprost-stimulated activity			Basal activity (% conversion)	*n*
	pEC_{50}	Hill coefficient	Maximum response (fold over basal)		
hIP-CHO	8.82 ± 0.10	0.62 ± 0.05[a]	40 ± 6	0.12 ± 0.03	3
hIP-HEK 293	8.44 ± 0.11	0.34 ± 0.03[a]	32 ± 20	0.10 ± 0.05	3
SK-N-SH (human)	7.65 ± 0.19	0.92 ± 0.38	4 ± 1	0.14 ± 0.01	3
mIP-CHO	7.95 ± 0.08	0.61 ± 0.02[a]	37 ± 17	0.11 ± 0.06	3
mIP-HEK 293	8.57 ± 0.18	0.55 ± 0.04[a]	35 ± 13	0.04 ± 0.01	3
NG108-15 (rat/mouse)	8.12 ± 0.06	1.32 ± 0.15	110 ± 26	0.15 ± 0.00	3

CHO and HEK 293 cells were assayed for agonist-stimulated [^{3}H]cyclic AMP accumulation 48 h post-transfection, as described in Section 2. Values are means ± S.E.M. from experiments performed in duplicate.
[a] Hill coefficient significantly different from unity.

significance is taken when $P < 0.05$, using Student's *t*-test or one-factor analysis of variance, as appropriate.

2.7. Reagents

8-[^{3}H]adenine (specific activity 27 Ci/mmol) was purchased from Amersham Biosciences (UK) and *myo*-[2-^{3}H]inositol (20 Ci/mmol) from NEN Life Science Products (USA). All other compounds were supplied by Bio-Rad (USA), Gibco (USA) or Sigma (USA). CHO and SK-N-SH cells, and cDNA for the rat μ-opioid receptor were gifts from Dr. Y.H. Wong (Department of Biochemistry, Hong Kong University of Science and Technology). HEK 293 cells were a gift from Dr. C.H.K. Cheng (Department of Biochemistry, The Chinese University of Hong Kong), and NG108-15 cells were a gift from Dr. R.R. Fiscus (Department of Physiology, The Chinese University of Hong Kong). The following gifts are also gratefully acknowledged: cDNAs for hIP and mIP receptors from Dr. T. Kobayashi (Department of Pharmacology, Kyoto University); cicaprost and sulprostone from Schering (Germany).

3. Results

3.1. Coupling of IP receptors to G_s-mediated signalling pathways

Cicaprost stimulated [^{3}H]cyclic AMP production in a concentration-dependent manner in CHO and HEK 293 cells transiently expressing hIP and mIP receptors, and in NG108-15 and SK-N-SH cells which endogenously express IP receptors (Table 1). The pEC_{50} values for cicaprost were similar for hIP and mIP receptors when transiently expressed in HEK 293 cells, but were significantly higher for hIP compared with mIP receptors transiently expressed in CHO cells ($P < 0.01$). The affinity of human IP receptors for cicaprost in SK-N-SH cells was significantly lower than for hIP receptors transiently expressed in CHO cells or HEK 293 cells ($P < 0.05$). Hill coefficients were significantly less than unity for cells transiently expressing IP receptors, but were no different from unity for the two neuronal cell lines (Table 1).

Table 2
Activation of phospholipase C by cicaprost in different cell lines

Cell line	Phospholipase C				
	Cicaprost-stimulated activity			Basal activity (% conversion)	*n*
	pEC_{50}	Hill coefficient	Maximum response (fold over basal)		
hIP-CHO	7.00 ± 0.11	0.97 ± 0.15	2.5 ± 0.3	6.65 ± 0.61	3
hIP-HEK 293	7.28 ± 0.01	1.78 ± 0.09	26 ± 4	0.72 ± 0.08	3
SK-N-SH (human)	< 4	ND	ND	9.86 ± 4.53	3
mIP-CHO	6.88 ± 0.04	1.07 ± 0.16	4.8 ± 1.0	5.00 ± 0.87	3
mIP-HEK 293	7.31 ± 0.10	1.66 ± 0.17	23 ± 2	0.67 ± 0.06	3
NG108-15 (rat/mouse)	6.34 ± 0.13	1.58 ± 0.42	6.5 ± 0.2	6.66 ± 1.35	3

CHO and HEK 293 cells were assayed for agonist-stimulated [^{3}H]inositol phosphate accumulation 48 h post-transfection, as described in Section 2. Values are means ± S.E.M. from experiments performed in duplicate.
ND: Not determined.

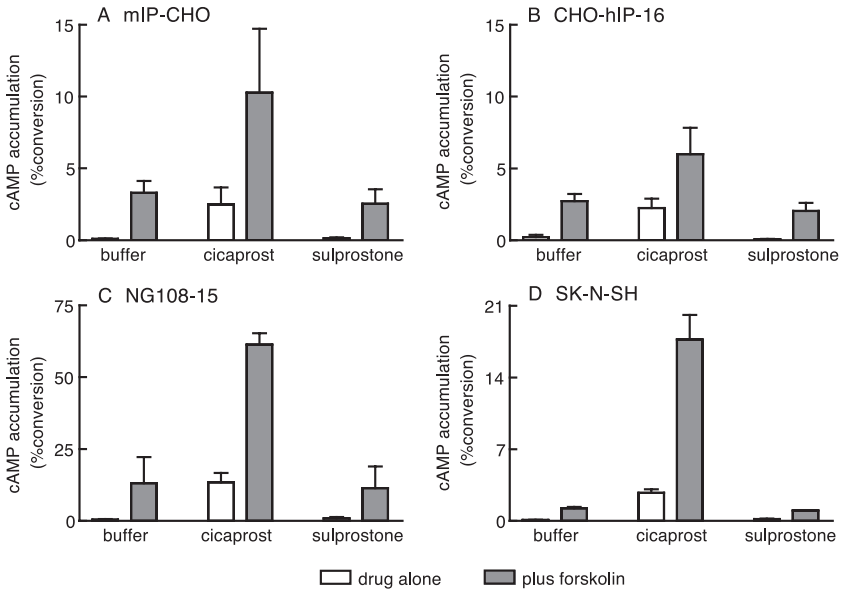

Fig. 1. The effect of cicaprost and sulprostone on forskolin-stimulated [^{3}H]cyclic AMP production in CHO cells expressing IP receptors, and in cell lines endogenously expressing IP receptors. Cells were incubated for 30 min with control buffer, cicaprost (1 μM) or sulprostone (1 μM) alone, or in combination with forskolin (10 μM). Values are means ± S.E.M., $n = 3$.

3.2. Coupling of IP receptors to $G_{q/11}$-mediated signalling pathways

Cicaprost stimulated [^{3}H]inositol phosphate production in a concentration-dependent manner in CHO and HEK 293 cells transiently expressing hIP and mIP receptors, and in NG108-15 cells which endogenously express IP receptors (Table 2). There was no significant difference between the affinity of hIP and mIP receptors when transiently expressed in CHO cells or HEK 293 cells. Cicaprost failed to stimulate [^{3}H]inositol phosphate production in SK-N-SH cells when tested at concentrations from 1 nM to 100 μM.

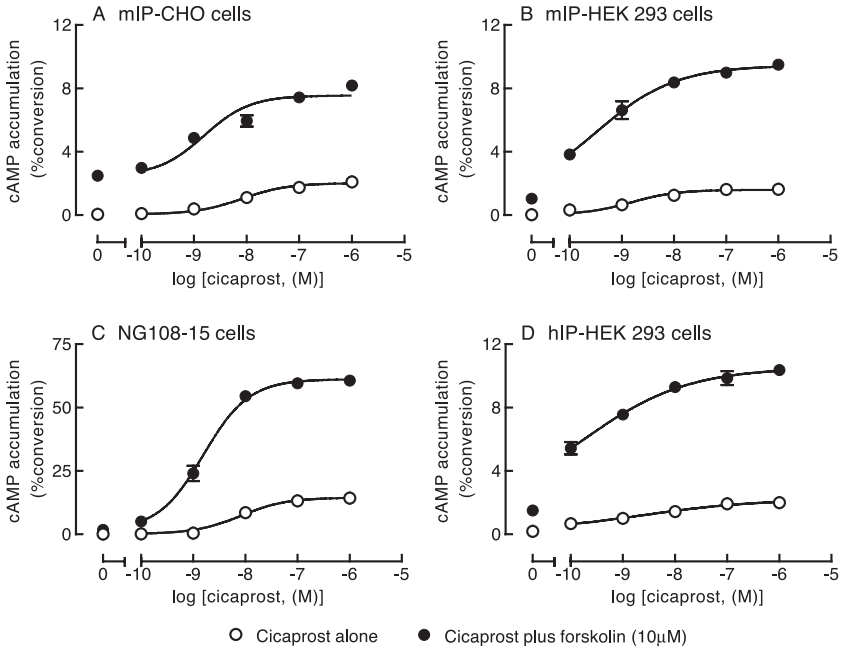

Fig. 2. The effect of different cicaprost concentrations on forskolin-stimulated [^{3}H]cyclic AMP production. CHO cells transiently expressing mIP receptors (A), HEK 293 cells transiently expressing mIP (B) or hIP receptors (D), and NG108-15 cells endogenously expressing rat/mouse IP receptors (C) were incubated for 30 min with cicaprost in the absence and presence of forskolin (10 μM). Data shown are from single experiments (means ± S.D., $n = 2$) representative of three independent experiments. High concentrations of cicaprost did not produce a decrease in the [^{3}H]cyclic AMP response to forskolin in any of these experiments. Error bars within the size of the symbols are not shown.

3.3. Lack of coupling of IP receptors to G_i-mediated signalling pathways

In our preliminary study to determine if IP receptors could couple to G_i proteins, we incubated CHO-hIP-16 cells, human SK-N-SH cells, mIP-CHO cells, and rat/mouse NG108-15 cells with the IP receptor agonist cicaprost (1 μM), or the EP_3/EP_1 agonist sulprostone (1 μM), with and without the adenylyl cyclase activator forskolin (10 μM) (Fig. 1). In contrast to the data of Lawler et al. (2001), stimulation of IP receptors with cicaprost did not result in inhibition of forskolin-stimulated [^{3}H]cyclic AMP accumulation (Fig. 1), but produced a significant potentiation of forskolin responses in the two neuronal cell lines ($P<0.05$; Fig. 1C,D). Sulprostone failed to inhibit forskolin-stimulated [^{3}H]cyclic AMP accumulation in CHO cells or in the neuronal cell lines.

To confirm the apparent lack of both human and rodent IP receptor coupling to G_i, we tested cicaprost over an extensive concentration range (100 pM–1 μM) for its ability to inhibit forskolin-stimulated [^{3}H]cyclic AMP accumulation (Fig. 2). The combination of cicaprost and forskolin failed to produce an inhibitory effect on [^{3}H]cyclic AMP accumulation at any concentration of cicaprost tested (Fig. 2). Maximum responses (curve-fitting of fold over basal values) for cicaprost (110 ± 26) and forskolin (18 ± 26) were significantly increased ($P<0.01$) to 409 ± 31 fold over basal for the combination treatment in NG108-15 cells. Although the combined effect of cicaprost and forskolin in mIP-CHO cells (157 ± 80 fold basal) was greater than the sum of the individual responses (cicaprost 37 ± 17; forskolin 41 ± 7), this effect did not reach a level of statistical significance. A similar effect was seen in hIP-HEK 293 and mIP-HEK 293 cells where maximum responses to cicaprost (hIP 32 ± 20; mIP 35 ± 13) and to forskolin (hIP 24 ± 15; mIP 23 ± 8) were increased to 128 ± 62 and 180 ± 76 fold over basal for the combination treatment in hIP-HEK 293 and mIP-HEK 293 cells, respectively. The presence of forskolin significantly ($P<0.05$) increased the cicaprost pEC_{50} in mIP-HEK 293 (9.63 ± 0.22), NG108-15 (9.18 ± 0.23), and hIP-HEK 293 (9.76 ± 0.37) cells, without altering the Hill coefficients. The pEC_{50} value for cicaprost in the presence of forskolin remained unchanged for mIP-CHO cells (7.94 ± 0.50).

To demonstrate that mIP receptors did not couple to G_i to activate the phospholipase C pathway, we incubated mIP-HEK 293 cells with pertussis toxin (100 ng/ml, 18 h) before assaying [^{3}H]inositol phosphate production (Fig. 3). Pertussis toxin significantly ($P<0.01$) decreased basal levels of [^{3}H]inositol phosphate production ($0.63 \pm 0.03\%$ and $0.42 \pm 0.01\%$ conversion for control and pertussis toxin-treated cells, respectively), but did not affect the potency of cicaprost (pEC_{50} values: 6.90 ± 0.18 and 7.13 ± 0.18, for control and pertussis toxin-treated cells, respectively), nor the efficacy of cicaprost to increase [^{3}H]inositol phosphate production (fold over basal values: 9.13 ± 1.76 and 10.92 ± 2.06, for control and pertussis toxin-treated cells, respectively). The lack of effect of pertussis toxin on cicaprost-stimulated [^{3}H]inositol phosphate production was not due to a failure to ADP-ribosylate the G_i proteins since the inhibitory activity of the μ-opioid receptor agonist [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) on the μ-opioid receptor similarly transfected into HEK 293 cells was lost following pertussis toxin treatment (data not shown).

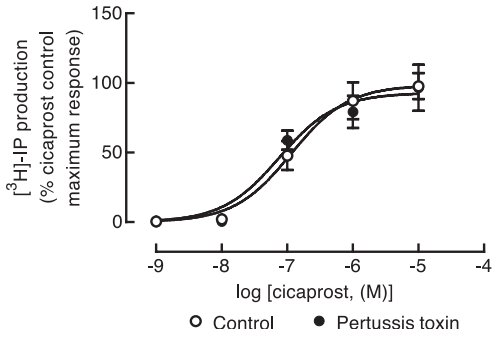

Fig. 3. The effect of pertussis toxin on cicaprost-stimulated [^{3}H]inositol phosphate production. HEK 293 cells were transfected with mIP (0.5 μg/ml) and assayed for [^{3}H]inositol phosphate accumulation as described in Section 2. Cells were treated with pertussis toxin (100 ng/ml) for 18 h before assay. Basal activity was $0.63 \pm 0.03\%$ and $0.42 \pm 0.01\%$ conversion for control and pertussis toxin-treated groups, respectively. Data has been normalised against the maximum fitted control response to cicaprost in each experiment (9.3 ± 1.8 fold over basal). Values are means ± S.E.M., $n=3$.

4. Discussion

When iloprost was first demonstrated to increase inositol phosphate production in CHO cells stably expressing the mIP receptor, it was also demonstrated that this process was pertussis toxin-insensitive and was not evoked by dibutyryl cyclic AMP (Namba et al., 1994). Furthermore, while pretreatment of these mIP receptor-expressing CHO cells with cholera toxin to degrade G_s resulted in a loss of iloprost-induced cyclic AMP production, it did not affect the inositol phosphate response. Together these results suggested that the mIP receptor couples independently to G_s and $G_{q/11}$.

Recently, contradictory evidence has been presented demonstrating that the mIP receptor, stably overexpressed in HEK 293 cells, couples to $G_{q/11}$ only as a consequence of prior coupling to G_s and PKA-mediated phosphorylation of the mIP receptor on Ser^{357} (Lawler et al., 2001). Yet the PKA inhibitor H-89 (*N*-[-2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) enhanced both cicaprost-stimulated [^{3}H]cyclic AMP and [^{3}H]inositol phosphate production in mIP-CHO cells (Kam et al., 2001), suggesting an autoinhibitory role, rather than a facilitatory role, for cyclic AMP/PKA in CHO cells. These results suggest that, at least in CHO cells, mIP receptor coupling to $G_{q/11}$ is not G_s/PKA-dependent. Furthermore, while the protein kinase C (PKC)

inhibitor staurosporine had no significant effect on cicaprost-stimulated [^{3}H]cyclic AMP production, it significantly increased cicaprost-stimulated [^{3}H]inositol phosphate production in mIP-CHO cells (Kam et al., 2001), supporting a role for PKC-dependent phosphorylation of IP receptors in the process of homologous desensitization (Smyth et al., 1998). Lawler et al. (2001), however, found no effect of the PKC inhibitor GF109203X (3-(1-[dimethylaminopropyl]indol-3-yl]-4-[indol-3-yl]maleimide) on mIP receptor coupling to G_s, $G_{q/11}$ or to G_i in HEK 293 cells, although Ser^{357} in the mIP receptor is recognised as a predicted target residue for both PKA and PKC phosphorylation by computational analysis (Blom et al., 1999).

The ability of IP receptors to couple to both G_s and $G_{q/11}$ is not restricted to transfection experiments using CHO and HEK 293 cells, as it is readily seen in rat/mouse neuroblastoma-glioma NG108-15 cells (Tables 1 and 2). Nevertheless, IP receptor coupling to $G_{q/11}$ is still a cell type-dependent phenomenon, being absent in the human neuroblastoma SK-N-SH cell line (Table 2). The human IP receptor endogenously expressed in SK-N-SH cells displayed significantly lower affinity than the hIP receptor expressed in CHO and HEK 293 cells, and failed to activate phospholipase C, as observed previously (Kam et al., 2001). Our SK-N-SH cells display an age-dependent loss of adenylyl cyclase response to IP receptor agonists, without a change in agonist affinity. However, the lack of human IP receptor coupling to $G_{q/11}$ in SK-N-SH cells in the present study is unlikely to result from a low receptor density (as reflected by the relatively weak adenylyl cyclase response) as a similar lack of coupling to $G_{q/11}$ was observed previously when cicaprost stimulated a 34-fold over basal increase in [^{3}H]cyclic AMP production (Kam et al., 2001). Other endogenously expressed $G_{q/11}$-coupled receptors, e.g. bradykinin receptor, are clearly responsive in these SK-N-SH cells (Kam et al., 2001). Dual signalling is common among G_s-coupled receptors, and is often dependent on receptor density (Zhu et al., 1994). However, mIP receptor coupling to $G_{q/11}$ in CHO cells in the present study is unlikely to be due to overexpression of receptors, as B_{max} values for [^{3}H]iloprost binding were 100–200 fmol/mg protein (Wise, 1999; and unpublished observations), which is approximately one-fifth the density of IP receptors in platelets (Jones et al., 1997), and platelet IP receptors do not couple to $G_{q/11}$ (Wise et al., 2002). We can also conclude that cicaprost-mediated activation of phospholipase C is independent of G_i, since pertussis toxin failed to influence both the potency and efficacy of cicaprost in mIP-HEK 293 cells (Fig. 3).

The mIP receptors stably overexpressed in HEK 293 cells were, however, reported to couple to G_i, with $G_{q/11}$ and G_i coupling occurring in a PKA-dependent manner (Lawler et al., 2001). In contrast, the hIP receptor stably overexpressed in HEK 293 cells couples independently to G_s and $G_{q/11}$ and does not couple to G_i (Miggin and Kinsella, 2002). PKA-dependent switching of receptor coupling from G_s to G_i accounts for the ability of vasoactive intestinal peptide and β-adrenoceptor agonists to elevate intracellular levels of Ca^{2+} in mouse pancreatic and submandibular gland cells (Luo et al., 1999), and for epinephrine to activate extracellular signal regulated kinase (ERK) in CHO and HEK 293 cells overexpressing β_2-adrenoceptors (Daaka et al., 1997; Zamah et al., 2002).

Our results presented herein (Figs. 1 and 2) fail to confirm that mouse IP receptor coupling to G_i is a general property of mouse IP receptors, because no inhibition of forskolin-stimulated [^{3}H]cyclic AMP accumulation was detected in either mIP-CHO, mIP-HEK 293 or NG108-15 cells, and pertussis toxin did not enhance cicaprost-stimulated [^{3}H]cyclic AMP production in mIP-CHO cells (Kam et al., 2001). Cicaprost could, however, activate adenylyl cyclase so strongly in these cell lines that any inhibitory effect of mIP receptor coupling to G_i would be undetectable, and therefore may be of little functional relevance. In HEK 293 cells stably overexpressing mIP receptors, cicaprost and forskolin were reported to interact synergistically at low cicaprost concentrations (e.g. 1 nM), with higher concentrations of cicaprost (10 nM–1 μM) inhibiting forskolin-stimulated cyclic AMP production (Lawler et al., 2001). In the present study, however, in mIP-CHO cells and mIP-HEK 293 cells, and especially in NG108-15 cells, a synergistic interaction occurred between cicaprost and forskolin at all cicaprost concentrations tested (100 pM–1 μM) (Fig. 2). Therefore, while the mIP receptor may switch to G_i coupling in a PKA-dependent manner when overexpressed in HEK 293 cells, such a sequence of events does not take place universally in all cell types, or in all isolates of HEK 293 cells. Discrepancies have also been noted on the generality of β_2-adrenoceptor switching from G_s to G_i as an important factor in initiating specific cell signalling pathways. For example, β_2-adrenoceptors deficient in PKA consensus sites were still able to activate ERK in another study using HEK 293 cells (Friedman et al., 2002). Clearly, transformed cell lines are not uniform in their properties. To rationalise these contrary results, Lefkowitz's group took nine isolates of HEK 293 cells, and demonstrated a high variablity (0–100%) in the sensitivity of isoprenaline-stimulated ERK to inhibition by pertussis toxin (Lefkowitz et al., 2002). Therefore, activation of ERK by G_s-coupled receptors may proceed through G_s or G_i proteins depending on the HEK 293 cell isolate being studied.

We can, however, confirm that the hIP receptor does not appear to couple to G_i proteins. As reported previously (Fong et al., 1998) and more recently for hIP receptors stably overexpressed in HEK 293 cells (Miggin and Kinsella, 2002), and for human IP receptors endogenously expressed in platelets and HEL cells (Miggin and Kinsella, 2002), we also show that hIP receptors in hIP-CHO and hIP-HEK 293 cells, and in CHO-hIP-16 and SK-N-SH cells, fail to couple to G_i (Figs. 1 and 2). Indeed, cicaprost and forskolin were shown herein to interact synergistically in all cells expressing human IP receptors, presumably as a result of an increase in high affinity binding of forskolin to

the catalytic subunit of adenylyl cyclase in the presence of agonist stimulation of G_s-coupled receptors (Barber, 1988).

In all cases where cicaprost and iloprost have been shown to increase $[Ca^{2+}]_i$ in platelet-like cells (e.g. MEG-01, CMK, HEL), the response has been independent of cyclic AMP production (Adachi et al., 1989; Watanabe et al., 1991; Schwaner et al., 1992; Feoktistov et al., 1997), suggesting independent coupling of human IP receptors to G_s and $G_{q/11}$. Since MEG-01, CMK and HEL cells are all forms of human megakaryocytic cells, then the overwhelming conclusion is that the human IP receptors do not require PKA-dependent phosphorylation to couple to $G_{q/11}$, and cannot couple to G_i.

In conclusion, contrary to reports of mouse IP receptor coupling to G_i in MEL cells and in HEK 293 cells stably overexpressing the mIP receptor (Lawler et al., 2001), we could find no such evidence for mIP receptors transiently expressed in CHO cells or HEK 293 cells, or in NG108-15 cells which endogenously express rat/mouse IP receptors. Taken together with our previous studies, we conclude that PKA-dependent switching of mouse IP receptor coupling from G_s to $G_{q/11}$ and G_i is not a universal phenomenon.

Acknowledgements

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