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Characterization of chimeric prostacyclin/prostaglandin D_2 receptors

Helen Wise *

Department of Pharmacology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR, People's Republic of China

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Abstract

The functional activity of two chimeric mouse prostacyclin/prostaglandin D_2 (IP/DP) receptors, in which the carboxyl-terminal region of the IP receptor was progressively replaced by that of the DP receptor, was examined in Chinese hamster ovary (CHO) cells. The order of potency of prostaglandin D_2, prostaglandin E_2 and the IP receptor agonists cicaprost, iloprost and BMY 45778 (3-[4-(4,5-diphenyl-2-oxazolyl)-5-oxazolyl]phenoxy]acetic acid) to stimulate cyclic AMP production was identical for the $IP_{N\text{-}VII}/DP_C$, $IP_{N\text{-}V}/DP_{VI\text{-}C}$ and wild-type IP receptors. $IP_{N\text{-}VII}/DP_C$ receptor-expressing cells showed increases in basal adenylate cyclase activity, agonist potency and coupling efficiency. In addition, the intrinsic activity of the partial IP receptor agonists BMY 45778 and PGE_2 was significantly increased in $IP_{N\text{-}VII}/DP_C$ receptor-expressing cells. Therefore, substitution of just the carboxyl-terminal tail of the IP receptor by that of the DP receptor appears to result in a chimeric IP/DP receptor with all the properties of a constitutively-active receptor.

Keywords: Constitutively-active receptor; Chimeric receptor; IP receptor; Partial agonist; Prostacyclin

1. Introduction

Prostanoid receptors form a distinct group within the superfamily of G protein-coupled receptors (Pierce et al., 1995), and are classified into the following five types: prostaglandin D_2 (DP) receptor, prostaglandin E_2 (EP) receptor, prostaglandin $F_{2\alpha}$ (FP) receptor, prostacyclin (IP) receptor and thromboxane A_2 (TP) receptor (Coleman et al., 1994). Among the presently cloned eight types and subtypes of prostanoid receptors, four mediate increases in cyclic AMP by coupling to G_s; these are DP, EP_2, EP_4 and IP receptors. The sequence homology among these functionally related receptors is higher than among the other subgroups with the amino acid sequences of the mouse DP and mouse IP receptors showing 58% identity in the transmembrane domains (Pierce et al., 1995).

Chimeric receptors have been frequently used to determine the regions involved in various functions of receptors and to determine the regions involved in selective agonist and antagonist binding. Because of the similarity between mouse IP and mouse DP receptors, Kobayashi et al. (1997) prepared chimeric receptors where the carboxyl-terminal tail of the mouse IP receptor was successively replaced by the corresponding region of the mouse DP receptor, and were able to identify the domains of these receptors which confer ligand binding specificities to each receptor. For example, the sixth and seventh transmembrane domains of the mouse IP receptor confers the specificity of this receptor to bind selectively to prostaglandin E_1 and not to prostaglandin E_2, and the third transmembrane domain of the mouse DP receptor confers the selective binding of prostaglandin D_2 to this receptor.

In addition, the chimeric $IP_{N\text{-}VII}/DP_C$ receptor, in which the carboxyl-terminal tail of the mouse IP receptor was replaced by that of the mouse DP receptor (Fig. 1), showed a 12- and 16-fold increased affinity for [^{3}H]iloprost and [^{3}H]prostaglandin E_1 (Kobayashi et al., 1997). As with other G protein-coupled receptors, the carboxyl tails of prostanoid receptors are known to affect the specificity and efficacy of G protein coupling (Namba et al., 1993; Sugimoto et al., 1993; Irie et al., 1994), as well as the sensitivity to agonist-induced desensitization (Negishi et al., 1993; Harazono et al., 1994; Nishigaki et al., 1996; Bastepe and Ashby, 1997). The seventh transmembrane domain is highly conserved among prostanoid receptors (Pierce et al., 1995), and the non-variable residues are maintained in these mouse IP/DP chimers.

* Tel.: +852-2609-6849; fax: +852-2603-5139.
E-mail address: helenwise@cuhk.edu.hk (H. Wise)

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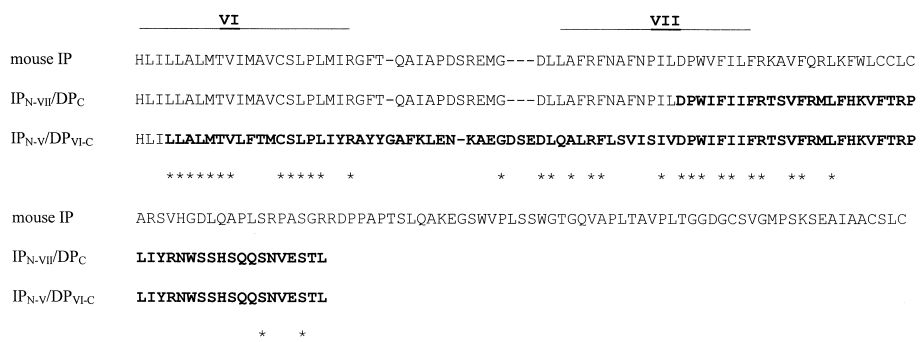

Fig. 1. Comparison of amino acid sequence of mouse IP, IP$_{N-VII}$/DP$_C$ and IP$_{N-V}$/DP$_{VI-C}$ receptors. Sequences from mouse DP receptor are given in bold type. Identical amino acid residues in mouse IP and mouse DP receptors are indicated by asterisks. Positions of the putative transmembrane domains VI and VII are indicated above the sequences. Sequence data from Ushikubi et al. (1995).

Therefore, I decided to compare the ability of DP, EP and IP receptor agonists to stimulate cyclic AMP production in Chinese hamster ovary (CHO) cells transiently expressing the mouse IP receptor or two of the chimeric mouse IP/DP receptors, IP$_{N-VII}$/DP$_C$ and IP$_{N-V}$/DP$_{VI-C}$. Predicting that the increased affinity of the IP$_{N-VII}$/DP$_C$ receptor for the IP receptor agonists iloprost and prostaglandin E$_1$ might influence coupling to G$_s$, I included the partial IP receptor agonist BMY 45778 (3-[4-(4,5-diphenyl-2-oxazolyl)-5-oxazolyl]phenoxy]acetic acid) (Seiler et al., 1997) in this study.

2. Materials and methods

2.1. Cell culture

CHO cells were cultured in Ham's F-12 medium supplemented with 10% foetal bovine serum, 100 i.u./ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were passaged twice weekly by trypsinization when near confluent.

2.2. Transient expression of receptors in CHO cells

Cells were grown to approximately 80% confluency in 175 cm^2 culture flasks, then transfected with receptor cDNAs in pCMX expression vector using LipofectAMINE liposome reagent and Opti-mem I reduced serum medium for 5 h, according to the manufacturer's instructions. Total cDNA per flask was 7.5 μg for mouse IP (0.5 μg/ml), 20 μg for IP$_{N-VII}$/DP$_C$ (1.33 μg/ml) and 6.0 μg for IP$_{N-V}$/DP$_{VI-C}$ (0.4 μg/ml). Full details of the preparation of these chimeric receptors can be found in Kobayashi et al. (1997).

2.3. [^{3}H]cyclic AMP accumulation assay

At 24 h after transfection, cells were harvested by trypsinization and seeded at 2×10^5 cells in 12-well culture plates containing 1 ml Ham's F-12 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 stopped after aspiration of the buffer by addition of 1 ml ice-cold 5% trichloroacetic acid containing 1 mM ATP. The plates were left for at least 30 min on ice before separating the [^{3}H]cyclic AMP from [^{3}H]ATP by column chromatography (Barber et al., 1980). The accumulation of intracellular [^{3}H]cyclic AMP was calculated as the ratio of radiolabeled cyclic AMP to total AXP (i.e., cyclic AMP, ADP and ATP), and is expressed as [cyclic AMP]/[total AXP] × 1000. At least 6 wells were used for the determination of basal cyclic AMP accumulation in each experiment, and where appropriate, [^{3}H]cyclic AMP accumulation was normalised against the maximum response to cicaprost obtained within the same experiment. Solvent controls were run as appropriate, but neither dimethylsulfoxide nor ethanol interfered with the assay at the concentrations used.

2.4. Radioligand binding

At 48 h after transfection, cells were washed with phosphate-buffered saline (PBS) containing 10 mM ethylenediaminetetraacetic acid and then removed from the 175 cm^2 flask with a cell scraper. The cell pellet was resuspended in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, 1 mM EGTA, 1 mM benzamide HCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride) and homogenised using a Polytron homogeniser. Cell membranes were pelleted by centrifugation of the cell homogenate at 48,000 × g for 30 min. The membrane pellet was washed once by resuspension in fresh lysis buffer and centrifugation. The final crude membrane pellet

was resuspended in a small volume of lysis buffer and stored at −80°C until used for assay. Protein content of cell membrane preparations was determined according to the Bradford method using a kit purchased from Bio-Rad (Richmond, CA) and albumin as the standard.

To determine the density of receptors in CHO cell membranes, 20 μl membranes (50–150 μg) were incubated for 60 min at 30°C with 0.2–50 nM [^{3}H]iloprost in 100 μl of 20 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethylsuphonyl fluoride and 3 μM indomethacin. Nonspecific binding was defined as [^{3}H]iloprost bound in the presence of 20 μM iloprost. Incubations were terminated by vacuum filtration over glass fibre filters (Whatman GF/C), using a Brandel cell harvester. Filters were washed three times with ice-cold 20 mM HEPES, pH 7.0, 10 mM $MgCl_2$ and 1 mM ethylenediaminetetraacetic acid to remove unbound radioactivity, and filters were left overnight in 3 ml HiSafe 3 scintillant before counting. Binding constants were determined by nonlinear regression analysis of at least five experiments, each performed in duplicate.

2.5. Data analysis

Ligand binding and adenylate cyclase data were analyzed using the computer program GraphPad Prism. pEC_{50} is the negative logarithm of the EC_{50} value for stimulation of adenylate cyclase activity. Fold basal activity is stimulated cyclic AMP accumulation divided by basal cyclic AMP production. Values reported are mean ± S.E.M.

The coupling efficiency of the different receptors was calculated according to the following equation taken from Whaley et al. (1994):

$$\frac{(EC_{50})r}{K_d - EC_{50}} = \frac{k_{-1}}{k_1} \quad (1)$$

Where EC_{50} is the concentration of iloprost required to produce half of the maximal adenylate cyclase activity, K_d is the dissociation constant for iloprost and the receptor, and r is the receptor concentration. k_1 is the agonist-dependent parameter, which is the rate constant for activation of adenylate cyclase as catalysed by the complex between the receptor and iloprost. k_{-1} is set to one because it is a measure of the intrinsic GTPase activity of $G_{s\alpha}$ and should be the same for identical cell lines (i.e., the host CHO cells).

2.6. Reagents

8-[^{3}H]adenine (specific activity 27 Ci/mmol) and [^{3}H]iloprost (specific activity 15.3 Ci/mmol) were purchased from Amersham (Far East) Trading Ltd. Dowex AG 50W-XA (200–400 mesh) was purchased from Bio-Rad (USA), and HiSafe 3 scintillant from Pharmacia Biotech Far East Ltd. All other compounds were supplied by Gibco (USA) or Sigma (USA). CHO cells were a gift from Dr. Y.H. Wong (Department of Biology, Hong Kong University of Science and Technology). The following gifts are also gratefully acknowledged: cDNAs for mouse IP, $IP_{N\text{-}VII}/DP_C$ and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptors from T. Kobayashi (Department of Pharmacology, Kyoto University); cicaprost from Schering AG (Germany), and BMY 45778 from Bristol-Myers Squibb (USA).

3. Results

3.1. Choice of target cell for transfection studies

To assess the functional activity of mouse IP and two chimeric mouse IP/DP receptors, cells were transfected with receptor cDNA and their ability to produce cyclic AMP in response to agonists was determined at 48 h post-transfection. A preliminary study using COS-7 (African green monkey kidney) cells confirmed that agonists showed increased potency, and surprisingly, that partial agonists showed increased efficacy in cells expressing $IP_{N\text{-}VII}/DP_C$ receptors (Wise et al., 1998). However, in contrast with CHO cells, wild-type COS-7 cells showed a small cyclic AMP response to the IP receptor agonist cicaprost (data not shown), therefore more detailed investigations were continued using CHO cells.

3.2. [^{3}H]iloprost binding studies

It is essential to consider receptor density in any analysis of functional activity of mutant receptors, because receptor levels play an important role in the responsiveness of cells to partial and full agonists (Samama et al., 1993; Yuan et al., 1994; January et al., 1998). In order to achieve a similar level of expression of each of the receptor cDNAs, CHO cells were transfected with different concentrations of cDNA (0.5 μg/ml, 1.33 μg/ml and 0.4 μg/ml for mouse IP, $IP_{N\text{-}VII}/DP_C$ and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptors, respectively). Preparations of membranes from each of these groups of cells showed that there was no significant difference between the maximal binding (B_{max}) values, with the mean density of specific binding sites for [^{3}H]iloprost ranging from 103–181 fmol/mg membrane protein (Table 1). The dissociation constant for [^{3}H]iloprost was significantly lower in CHO cells expressing $IP_{N\text{-}VII}/DP_C$ receptors compared with mouse IP receptor-expressing cells (Table 1), confirming the observations of Kobayashi et al. (1997) in COS-7 cells. In addition, receptor-independent stimulation of adenylate cyclase by forskolin was similar in these three cell groups (Table 2) indicating that the increased amount of cDNA used for expressing the $IP_{N\text{-}VII}/DP_C$ receptor did not influence the ability of CHO cells to generate cyclic AMP.

Table 1
[^{3}H]Iloprost binding properties of mouse IP and chimeric mouse IP/DP receptors expressed in CHO cells
Values are means ± S.E.M. from 7–8 experiments.

Receptor	K_d (nM)	B_{max} (fmol/mg membrane protein)
mouse IP	9.3 ± 1.4	121 ± 15
$IP_{N\text{-}VII}/DP_C$	3.5 ± 0.5[a]	181 ± 35
$IP_{N\text{-}V}/DP_{VI\text{-}C}$	10.5 ± 4.8	102 ± 20

[a] Significantly lower than mouse IP, $P < 0.01$ by Student's t test.

3.3. Activation of adenylate cyclase

3.3.1. Effects on affinity and efficacy

The basal level of adenylate cyclase activity was significantly greater in cells expressing $IP_{N\text{-}VII}/DP_C$ receptors compared with mouse IP and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptor-expressing cells (Table 2). If the response to a maximally effective concentration of cicaprost was calculated relative to the altered basal level, there appeared to be no difference between the three groups of cells (Table 2). Because the decreased K_d and increased basal adenylate cyclase activity of cells expressing $IP_{N\text{-}VII}/DP_C$ receptors is indicative of a constitutively-active receptor (Parker and Ross, 1991; Samama et al., 1993), I also examined the absolute levels of cicaprost-stimulated adenylate cyclase activity in cells expressing mouse IP, $IP_{N\text{-}VII}/DP_C$ and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptors (Fig. 2A). Cicaprost tended to produce a greater maximal increase in adenylate cyclase activity in cells expressing the $IP_{N\text{-}VII}/DP_C$ receptor, but this difference was not statistically significant (increase in [^{3}H]cyclic AMP accumulation was 5.63 ± 0.77, 7.49 ± 0.94 and 5.63 ± 1.31 for mouse IP, $IP_{N\text{-}VII}/DP_C$ and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptors respectively, $n = 6–7$).

Because the absolute amount of cyclic AMP produced by cells transfected on different occasions tended to be variable, all subsequent results have been normalised against the maximal response to cicaprost obtained in the same experiment (Figs. 2B and 3). Although this normalization process would tend to mask any constitutive behaviour of receptors, it helped clarify the data obtained with the agonists being tested. Both cicaprost and iloprost are considered full agonists for IP receptors (Coleman et al., 1994), and both showed a similar statistically-significant increase in pEC_{50} values for stimulating cyclic AMP

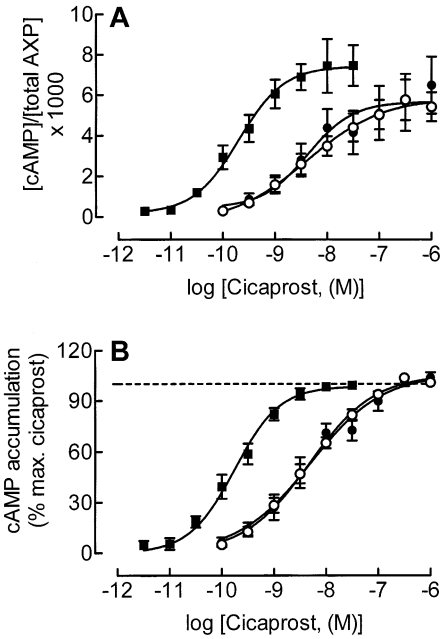

Fig. 2. The effect of cicaprost on cyclic AMP accumulation by cells expressing mouse IP and chimeric mouse IP/DP receptors. CHO cells were transfected with cDNA for mouse IP (○), $IP_{N\text{-}VII}/DP_C$ (■) and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ (●) receptors, and assayed for cyclic AMP accumulation in response to cicaprost 48 h post-transfection, as described in Materials and Methods. Values are means ± S.E.M. for at least 6 experiments. (A) Cyclic AMP accumulation expressed as stimulated minus basal level for each treatment group (see Table 2 for basal activity values). (B) Cyclic AMP accumulation normalised against the maximum response to cicaprost in each experiment.

production in cells expressing $IP_{N\text{-}VII}/DP_C$ receptors compared with mouse IP and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptor-expressing cells (Table 3). The maximal response to iloprost was similar to that of cicaprost in each of the three groups of cells.

The intrinsic activity of agonists was determined by comparing their maximal ability to elevate cyclic AMP with that of the full agonist cicaprost. The nonprostanoid prostacyclin mimetic BMY 45778 showed partial agonist activity in both mouse IP and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptor-expressing cells (Fig. 3); the intrinsic activity of BMY 45778 being only 73–77% that of cicaprost (Table 3). However, in cells expressing $IP_{N\text{-}VII}/DP_C$ receptors, the intrinsic activity of BMY 45778 was significantly increased to 100% that of cicaprost. Furthermore, prostaglandin E_2 also

Table 2
General properties of CHO cells transfected to express mouse IP and chimeric mouse IP/DP receptors
Values are means ± S.E.M. from at least 6 experiments.

Receptor	Adenylate cyclase activity		
	Basal activity ([cAMP]/[total AXP] × 1000)	10 μM forskolin (fold basal activity)	1 μM cicaprost (fold basal activity)
mouse IP	0.82 ± 0.05	18.81 ± 3.37	8.30 ± 1.21
$IP_{N\text{-}VII}/DP_C$	1.12 ± 0.07[a]	16.99 ± 1.77	7.22 ± 0.82
$IP_{N\text{-}V}/DP_{VI\text{-}C}$	0.76 ± 0.08	22.29 ± 3.00	8.48 ± 1.85

[a] Significantly greater than mouse IP and $IP_{N\text{-}V}DP_{VI\text{-}C}$ receptors, $P < 0.01$ by Student's t test.

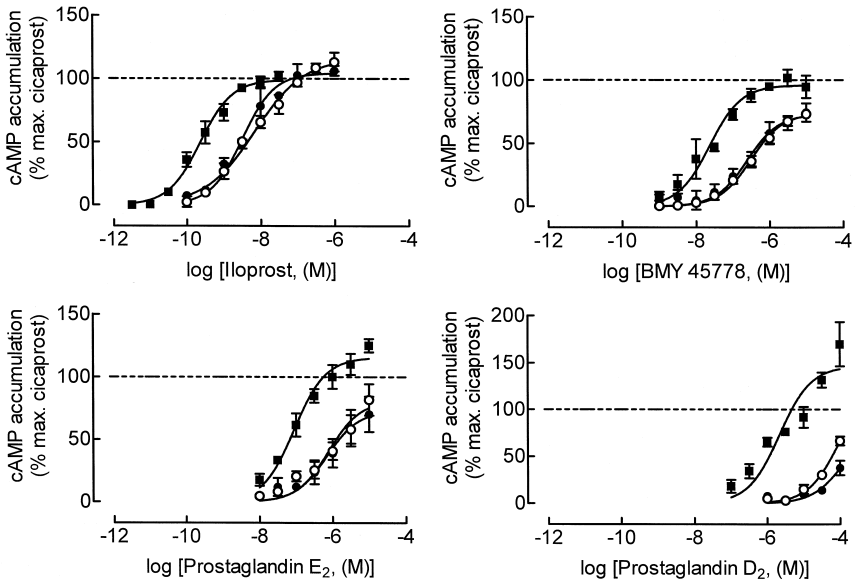

Fig. 3. The effect of DP, EP and IP receptor agonists on cyclic AMP accumulation by cells expressing mouse IP and chimeric mouse IP/DP receptors. CHO cells were transfected with cDNA for mouse IP (○), $IP_{N\text{-}VII}/DP_C$ (■) and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ (●) receptors, and assayed for cyclic AMP accumulation in response to agonists 48 h post-transfection, as described in Materials and Methods. Values are means ± S.E.M. for at least 3 experiments.

has low potency and low efficacy for stimulating adenylate cyclase in cells expressing mouse IP and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptors, yet both these properties were significantly increased in cells expressing $IP_{N\text{-}VII}/DP_C$ receptors (Table 3). Prostaglandin D_2 showed very low potency in stimulating cyclic AMP production in both mouse IP and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptor-expressing cells, yet its potency was also significantly increased in cells expressing $IP_{N\text{-}VII}/DP_C$ receptors (Table 3). The maximal response to prostaglandin D_2 in $IP_{N\text{-}VII}/DP_C$ receptor-expressing cells was highly variable and tended to be considerably higher than the maximal response to cicaprost. The log concentration–response curve for prostaglandin D_2 in $IP_{N\text{-}VII}/DP_C$ receptor-expressing cells may indeed resolve into two distinct components.

3.3.2. Effects on coupling efficiency

In an attempt to quantify the different behaviour of these three receptors, I have used an equation from Whaley et al. (1994) which allows one to calculate the receptor coupling efficiency for G_s-coupled receptors. Using Eq. (1) (see Materials and Methods), the coupling efficiency values for mouse IP, $IP_{N\text{-}VII}/DP_C$ and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptors were 3.9, 84.9 and 14.5 respectively. The $IP_{N\text{-}VII}/DP_C$ receptor was therefore 21.6-fold more efficient in activating adenylate cyclase than the wild-type mouse IP receptor.

4. Discussion

In 1997, Kobayashi et al. described a series of chimeric receptors in which the carboxyl-terminal tail of the mouse IP receptor was successively replaced by the corresponding region of the mouse DP receptor. One of these chimeric receptors, $IP_{N\text{-}VII}/DP_C$, showed a marked increase in affinity for IP receptor agonists. I have shown here that, when expressed in CHO cells, all three receptors studied (mouse

Table 3
Adenylate cyclase activating properties of mouse IP and chimeric mouse IP/DP receptors expressed in CHO cells
Values are means ± S.E.M. from at least 3 experiments.

Agonist	mouse IP		$IP_{N\text{-}VII}/DP_C$		$IP_{N\text{-}V}/DP_{VI\text{-}C}$	
	pEC_{50}	Intrinsic activity	pEC_{50}	Intrinsic activity	pEC_{50}	Intrinsic activity
cicaprost	8.38 ± 0.09	1.00	9.75 ± 0.12[a]	1.00	8.33 ± 0.27	1.00
iloprost	8.20 ± 0.22	1.10 ± 0.08	9.63 ± 0.19[a]	0.99 ± 0.02	8.37 ± 0.10	1.04 ± 0.04
BMY 45778	6.40 ± 0.10	0.77 ± 0.01	7.59 ± 0.18[a]	1.00 ± 0.08[a]	6.59 ± 0.05	0.73 ± 0.07
prostaglandin E_2	6.11 ± 0.08	0.81 ± 0.13	7.05 ± 0.08[a]	1.16 ± 0.06[a]	6.10 ± 0.13	0.74 ± 0.13
prostaglandin D_2	4.17 ± 0.26[b]	1.29 ± 0.35[b]	5.60 ± 0.22[a]	1.57 ± 0.37	4.23 ± 0.24[b]	0.70 ± 0.24[b]

[a] Significantly greater than mouse IP and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptors, $P < 0.05$ by Student's t test.
[b] Estimated values due to limited range of prostaglandin D_2 concentrations.

IP, $IP_{N\text{-}VII}/DP_C$ and $IP_{N\text{-}V}/DP_{VI\text{-}C}$) coupled to adenylate cyclase and all responded to agonists with an order of potency expected of an IP receptor (Coleman et al., 1994). In contrast to the radioligand binding data of Kobayashi et al. (1997) obtained using COS-7 cells, neither prostaglandin E_2 nor prostaglandin D_2 displayed increased potency in CHO cells expressing the $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptor, but increased potency was observed in $IP_{N\text{-}VII}/DP_C$ receptor-expressing cells. Furthermore, the ability of prostaglandin E_2 to stimulate adenylate cyclase activity in the wild-type and chimeric receptors is identical in CHO and COS-7 cells (unpublished observations), and follows the same pattern for all agonists tested. These differences cannot therefore be explained simply in terms of the higher receptor expression levels in Kobayashi et al. (1997) compared with the present report, nor to the use of a different host cell for transfection, and this issue remains unresolved.

When looking at the functional coupling of these chimeric receptors expressed in CHO cells, the basal level of cyclic AMP accumulation was significantly increased in cells expressing the $IP_{N\text{-}VII}/DP_C$ receptor, as were both the potency and efficacy of IP receptor agonists for stimulating adenylate cyclase. Because the $IP_{N\text{-}VII}/DP_C$ receptor was expressed at a similar density as the mouse IP and $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptors, we can be confident that the observed changes in potency and efficacy are not simply the result of different receptor densities (Samama et al., 1993).

The trio of properties of increased affinity, increased efficacy and increased basal activity are characteristics of agonist-independent, constitutively-active receptors (Parker and Ross, 1991; Samama et al., 1993). Studies of constitutively-active receptors have resulted in a reassessment of the ternary complex model of agonist action on G protein-coupled receptors, resulting in the two-state model of receptor activation which incorporates an explicit isomerization of the receptor (R) to an active state (R*) (Samama et al., 1993; Leff, 1995). In the absence of agonist, the equilibrium between R and R* defines the basal level of the receptor-mediated response. Thus, high levels of receptor expression are anticipated to increase basal activity as the portion of the expressed G protein-coupled receptor in the active state R* increases with increasing receptor level (Scheer and Cotecchia, 1997). The extent to which basal adenylate cyclase activity of constitutively-active receptors is increased can be variable, but 2–3-fold increases are common (Parker and Ross, 1991; Samama et al., 1993; Stevens and Milligan, 1998). I observed a mere 1.4-fold increase in basal adenylate cyclase activity in CHO cells expressing the $IP_{N\text{-}VII}/DP_C$ receptor. It is possible that if the $IP_{N\text{-}VII}/DP_C$ receptor were expressed at a higher density (Samama et al., 1993), or if CHO cells expressed greater levels of adenylate cyclase enzyme (Stevens and Milligan, 1998), then a more marked effect on basal activity would be noticeable.

Evidence that the $IP_{N\text{-}VII}/DP_C$ receptor is a constitutively-active receptor is perhaps stronger when one looks at the change in intrinsic activity of partial agonists relative to full agonists. In the two-state model of receptor activation, agonists produce their effects by preferentially binding to and thereby increasing the proportion of R*. In terms of such models, the intrinsic activity of a ligand reflects the ratio of its affinities for the two states, and partial agonists are less selective for R* compared with full agonists. For constitutively-active receptors in which the equilibrium already favours formation of R*, then the apparent intrinsic activity of partial agonists increases to a greater extent than that of full agonists. Hence we observe that, although the efficacy of cicaprost, iloprost, BMY 45778, prostaglandin E_2 and prostaglandin D_2 tended to increase with the $IP_{N\text{-}VII}/DP_C$ receptor, the increase relative to cicaprost was greater for the partial IP receptor agonists BMY 45778 and prostaglandin E_2.

BMY 45778 was chosen for this study because in all studies reported to date, it consistently behaves as a partial IP receptor agonist for stimulation of adenylate cyclase. However, the extent of its intrinsic activity compared to the full agonist iloprost is highly tissue-dependent; 80–100% in human platelets (Seiler et al., 1997), 56% in rat peritoneal neutrophils (Wise, 1996), 43% in human neuroblastoma SK–N–SH cells (Wise and Chow, 1996), and 51% in COS-7 cells expressing mouse IP receptors (Wise et al., 1998). But in addition to the influence of tissue-dependent factors, we also need to consider the receptor-dependent properties which govern coupling to G_s proteins. Calculation of coupling efficiencies helps give a quantitative measure of the ability of the agonist-bound receptor to stimulate adenylate cyclase, independent of tissue-dependent factors (Whaley et al., 1994). I have calculated that, when expressed in the same cell type, the $IP_{N\text{-}VII}/DP_C$ receptor is 21.6-fold more efficient in activating adenylate cyclase than is the mouse IP receptor.

Analysis of the constitutively-active G protein-coupled receptors has provided important information about the molecular mechanisms underlying receptor activation and drug action, but there is little consistency regarding the site of mutations capable of generating constitutively-active receptors. A conserved cysteine residue is frequently located among the first 15 amino acids of the carboxyl-terminal tail distal to the seventh transmembrane domain of many G protein-coupled receptors, and this has been implicated in high affinity binding and/or G protein coupling (O'Dowd et al., 1988, 1989). Indeed, the mouse IP receptor has three cysteine residues in this region of the carboxyl-terminal tail, but since they are lacking in the mouse DP receptor sequence (Fig. 1), they cannot account for the increased affinity and coupling efficiency of the $IP_{N\text{-}VII}/DP_C$ receptor.

When a series of mutant avian β-adrenoceptors were prepared in which the carboxyl-terminal tail was progressively truncated, it was noted that these were constitu-

tively-active receptors, and the authors speculated that the cytoplasmic tail of the avian β-adrenoceptor might participate in a constraining effect on the native receptor (Parker and Ross, 1991). Sequences in the parathyroid hormone (PTH)/PTH-related peptide (PTH/PTHrP) receptor carboxyl-terminal tail appear to lower the affinity of the wild-type receptor for agonists, and decrease the efficacy with which the receptor interacts with G_s (Iida-Klein et al., 1995). Although these modifications to the PTH/PTHrP receptor failed to produce a constitutively-active form of receptor, they also implicate the carboxyl-terminal tail as a constraining element in the function of this G protein-coupled receptor. This concept of a constraining function of part of the G protein-coupled receptor is an attractive theory to explain the generation of constitutively-active receptors. From a structural point of view, Samama et al. (1993) proposed that these constitutively-active receptors can be envisioned as being impaired in their key constraining function, thus spontaneously 'relaxing' into their active conformation in the absence of agonist.

Both the mouse EP_3 and human EP_3 receptors have several isoforms differing in the carboxyl-terminal tail, and truncation at the alternative splicing site produces a constitutively-active receptor (Hasegawa et al., 1996; Jin et al., 1997). Jin et al. (1997) proposed that the core of the EP_3 receptor, comprising the seven transmembrane domains, couples constitutively to G_i (i.e., the receptor is stabilised in an active conformation) and that the carboxyl-terminal tail blocks this interaction. Agonist binding to the EP_3 receptor causes a conformation change that moves the carboxyl-terminal tail away from the G protein interaction region (presumably within the seven transmembrane domains) and allows receptor-G protein coupling to occur. The EP_3 receptor though is not a G_s-coupled receptor, but the EP_4 receptor is, and truncation of its carboxyl-terminal tail does not produce a constitutively-active receptor (Bastepe and Ashby, 1997). By making a chimeric EP_3/EP_4 receptor where the seventh transmembrane domain and carboxyl-terminal tail of the EP_3 receptor was replaced by the corresponding region of the EP_4 receptor, it was noted that the carboxyl-terminal tail of the EP_4 receptor does not confer coupling specificity, and the chimeric receptor remains able to couple to G_i (Neuschaferrube et al., 1997). The addition of the carboxyl-terminal tail of the EP_4 receptor appeared to provide coupling control, in that it prevented coupling of the agonist-free receptor to G_i, possibly in the same manner as proposed for the carboxyl-terminal tail of the EP_3 receptor.

If the loss or change in identity of the carboxyl-terminal tail of the mouse IP receptor was sufficient to account for the constitutive activity of the $IP_{N\text{-}VII}/DP_C$ receptor, then we would also expect the $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptor to display constitutive activity, and it clearly does not. It is possible that the sixth and seventh transmembrane domains of the mouse DP receptor normally restrains the carboxyl-terminal tail of the mouse DP receptor, preventing its constitutive activity. The sixth and seventh transmembrane domains of the mouse IP receptor cannot serve this same function and therefore in $IP_{N\text{-}VII}/DP_C$ receptors, the restraint imposed on the carboxyl-terminal tail of the mouse DP receptor is lost, resulting in the constitutive activity seen in the $IP_{N\text{-}VII}/DP_C$ receptor. In contrast for the $IP_{N\text{-}V}/DP_{VI\text{-}C}$ receptor, the restraining nature of the sixth and seventh transmembrane domains from the mouse DP receptor is restored, and the carboxyl-terminal tail of the mouse DP receptor is held in check, unable to allow agonist-independent coupling to G_s. Unfortunately, the mouse DP receptor containing the sixth and seventh transmembrane domains of the mouse IP receptor failed to be expressed in COS-7 cells (Kobayashi, T., personal communication), therefore the proposed constraining nature of the sixth and seventh transmembrane domains of the mouse DP receptor cannot be fully proven.

In conclusion, the marked increase in binding affinity of the $IP_{N\text{-}VII}/DP_C$ receptor for [^{3}H]iloprost and [^{3}H]prostaglandin E_1 is reflected in the functional activity of this chimeric mouse IP/DP receptor. Thus, CHO cells expressing the $IP_{N\text{-}VII}/DP_C$ receptor showed increased basal adenylate cyclase activity, and both agonist potency and the intrinsic activity of partial agonists were significantly increased. Together with the observed increase in coupling efficiency of this $IP_{N\text{-}VII}/DP_C$ receptor, one can conclude that substitution of the carboxyl-terminal region of the mouse IP receptor by that of the mouse DP receptor results in a chimeric IP/DP receptor with all the properties of a constitutively-active receptor. Further studies will be required to determine the important amino acids in the $IP_{N\text{-}VII}/DP_C$ receptor which are responsible for these observations.

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