Biochemical Pharmacology, Vol. 58, pp. 471–476, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00119-7

SHORT COMMUNICATION

Preservation of G_i Coupling of a Chimeric EP_3/I-Type Prostaglandin (IP) Receptor

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ABSTRACT. For the EP_3 subtype of prostaglandin E receptors, different C-terminal splice variants are known, which are coupled to distinct heterotrimeric GTP-binding proteins (G-proteins). To test the hypothesis that the C-terminal domain is essential for the G-protein-coupling specificity of the EP_3 receptor, we exchanged the carboxyl-terminal tail of a porcine G_i-coupled EP_3 receptor isoform for the corresponding C-terminal part of a G_s-coupled prostaglandin receptor. The porcine EP_3 receptor was truncated at a lysine (K_{350}) residue at the end of the seventh transmembrane region, representing the splicing site of the different EP_3 receptor isoforms. The wild-type C-terminus (37 amino acids) was substituted by the C-terminal tail (89 amino acids) of the human I-type prostaglandin receptor (hIP-R). The G-protein coupling of the resulting chimeric receptor protein was studied in transfected Chinese hamster ovary (CHO) cells. Stimulation of the chimeric receptor protein with the EP_3 receptor-specific agonist M&B 28.767 did not increase adenosine 3′,5′-cyclic monophosphate (cAMP) formation but did reduce the forskolin-stimulated cAMP formation, indicating G_i coupling. Furthermore, the chimeric receptor did not show constitutive activity as demonstrated for the C-terminally truncated EP_3 receptor. Thus, coupling specificity of the EP_3 receptor is not exclusively mediated by the carboxyl-terminal tail, and constitutive activity of a C-terminally truncated EP_3 receptor can be suppressed by the hIP-R C-terminus. BIOCHEM PHARMACOL **58**;3:471–476, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. EP_3 receptor; IP receptor; chimeric receptor; G-protein coupling; CHO cells; cAMP

Prostaglandin E_2 (PGE_2) exhibits a broad range of biological actions through binding to specific transmembrane receptors of the G-protein-coupled receptor superfamily (GPCR) [1–3]. Pharmacologically, four different subtypes (EP_1–EP_4) have been defined according to their affinity to various agonists and antagonists, and their G-protein-coupling specificity [3]. Among the EP receptor family, the EP_3 subtype is unique due to the existence of multiple isoforms, distinguished by varying C-terminal amino acid sequences generated by alternative splicing [4–6]. The observation that different splice variants of the EP_3 receptor are coupled to different G-proteins† [7, 8] led to the hypothesis that the C-terminal domain of the receptor might determine G-protein-coupling specificity [9]. However, the precise molecular mechanisms for regulation of receptor–G-protein interaction are still incompletely understood. Truncation of the complete C-terminus of the mouse $EP_{3\beta}$ receptor leads to agonist-independent constitutive G_i coupling [10, 11]. Furthermore, different EP_3 receptor isoforms of the mouse and human show different degrees of agonist-independent constitutive G_i activity [12, 13]. These data suggest that the core of the EP_3 receptor is able to associate with and to activate G_i and that the carboxyl-terminal tail of the protein is responsible for suppression of G_i coupling of the unstimulated receptor.

Recently, we reported the cloning of a porcine G_i-coupled EP_3 receptor which was highly homologous to the constitutively active murine $EP_{3\gamma}$ receptor [14]. In order to further elucidate the role of the C-terminal domain of this EP_3 receptor for its G-protein coupling, we constructed a chimeric receptor in which the 37 amino acids of the original EP_3 receptor C-terminus were replaced by the 89 amino acids of the human IP receptor C-terminus, which is coupled to G_s [15, 16]. Sequence analysis revealed no obvious homology between the two C-termini. The chimeric receptor was expressed in CHO cells, and receptor-mediated cAMP formation was determined in order to analyze G-protein coupling in comparison to the wild-type receptor.

MATERIALS AND METHODS

Construction of a Chimeric EP_3/IP Receptor cDNA

Cloning of a complete porcine EP_3 receptor cDNA (accession number AJ001201) and expression in CHO cells has been described previously [14]. The complete cDNA of the

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† *Abbreviations:* cAMP, adenosine 3′,5′-cyclic monophosphate; CHO, Chinese hamster ovary; G-protein, heterotrimeric GTP-binding protein; IP, I-type prostaglandin; and hIP-R, human I-type prostaglandin receptor.

Received 28 August 1998; accepted 19 February 1999.

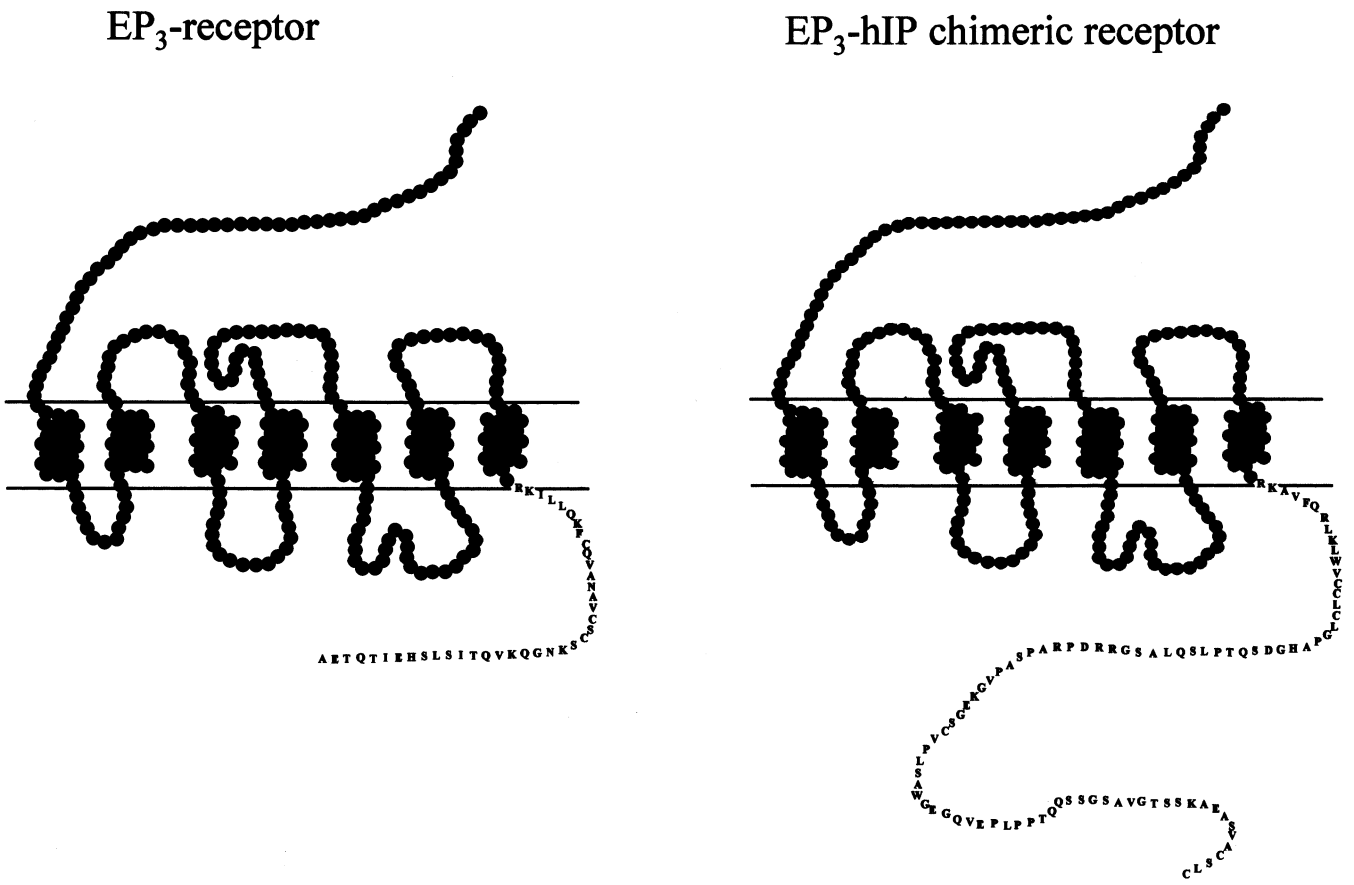

FIG. 1. Schematic depiction of the porcine EP_3 receptor and the EP_3/IP chimeric receptor protein. The C-terminal tails of the receptor proteins are shown in the single letter code.

human IP receptor (hIP), cloned as a 1.4 kb fragment into pcDNAIamp (hIP11/6.pcDNAIamp), was a generous gift of Dr. M. Abramowitz (Merck Frosst). The human IP receptor C-terminus was amplified with the oligonucleotides P1 (5′ CTTGGATCCCTGGGTTTATCTGCTGCTAAG-AAAGTCCGTCTTCCAGCGGCTGAAGC 3′, the first 34 nucleotides code for the porcine EP_3 receptor, followed by 22 nucleotides encoding the IP receptor cDNA sequence. A single *Bam*HI site which was used for further cloning is underlined) and P2 (5′ AGGGCCCTCTAG-ATGCATGC 3′, derived from the multiple cloning site of pcDNAIamp. A single *Xba*I site which was used for further cloning is underlined). The resulting PCR fragment of 0.6 kb was isolated, cut with *Bam*HI/*Xba*I and used for substitution of the C-terminal EP_3 receptor fragment. The chimeric receptor was cloned into the expression plasmid pcDNA3 (Invitrogen), resulting in $pcEP_3$-hIP.

Construction of a C-terminally Truncated Porcine EP_3 Receptor

A polymerase chain reaction (PCR) using the primers P_{stop} (TCTTGGATCCCTGGGTTTATCTGCTGCTAAGA-AAGTGACTCCTTCAAAAAGTTTTGCCAGG) and P2 (5′AGGGCCCTCTAGATGCATGC 3′) was performed on a pcDNA3-based plasmid carrying the complete porcine EP_3 receptor cDNA ($pcEP_3$). The original EP_3 receptor C-terminus in $pcEP_3$ was then replaced by the *Bam*HI/*Eco*RI cut PCR fragment resulting in $pcEP_{3\text{-stop}}$.

Transfection of COS-7 and CHO Cells

COS-7 cells were cultured in Dulbecco's modified Eagle's medium with Glutamax-I (GIBCO BRL, Life Technologies) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (0.1 mg/mL). Transient transfection was performed by means of the diethyl-aminoethyl-dextran methodology [17]. For expression in COS-7 cells, the cDNA of the human IP receptor was subcloned as a 1.4 kb *Eco*RI fragment into pcDNA3. Selection of transfectants was performed by culturing cells for 3 weeks in medium containing 500 μg/mL G418 (Calbiochem). CHO cells were cultured in Ham's F12 medium (GIBCO BRL, Life Technologies) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (0.1 mg/mL). Cells were transfected by means of a modified polybrene method with linearized plasmid DNA as described elsewhere [18]. Clonal selection of stable integrants was performed in a medium containing 250 μg/mL G418. For detection of chimeric receptor protein, membrane proteins of transfectants were prepared as described previously [14],

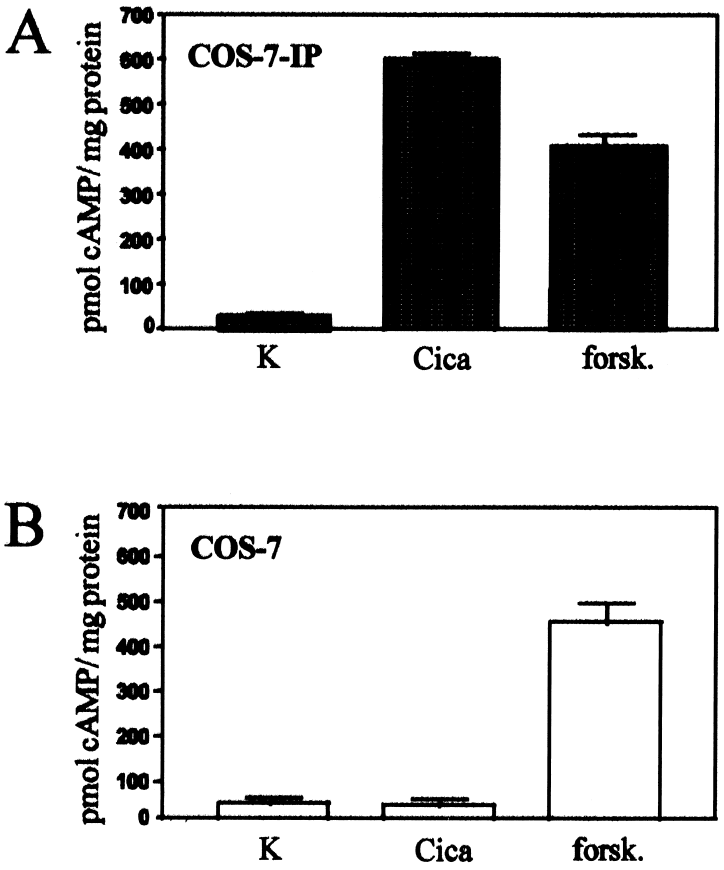

FIG. 2. Modulation of the cAMP formation in (A) COS-7-cells expressing the human IP receptor (COS-7-IP) and (B) mock-transfected COS-7 cells. The effect of forskolin (forsk; 3 μM) or cicaprost (cica; 100 nM) was monitored. Values are means (± SEM) of three independent experiments performed in triplicate.

separated by polyacrylamide gel electrophoresis and electrotransferred to a nylon membrane (Immobilon P; Millipore). For immunodetection, membranes were probed with antibodies against an N-terminal peptide of the porcine EP_3 receptor followed by incubation with a peroxidase-coupled secondary antibody (Dianova). Signals were detected by enhanced chemiluminescence (ECL kit, Amersham). Ligand binding studies were performed as described elsewhere [14].

Measurement of cAMP Formation

Cells were grown on 6-well plates to near confluency. Medium was removed by aspiration and cells were preincubated in Hanks' balanced salt solution containing 1 mg/mL BSA, 10 mmol/L HEPES (pH 7.3), and 1 mmol/L isobutylmethylxanthine (Calbiochem) for 10 min at 37°. The cells were then treated with 3 μmol/L forskolin or 100 nmol/L M&B 28.767 (Rhone-Poulenc Rorer) and 3 μmol/L forskolin for 10 min at 37°. The reaction was stopped by adding ice-cold ethanol. Ethanol was removed by aspiration and cells were overlayed with 1 mL of radioimmunoassay buffer (150 mmol/L NaCl, 8 mmol/L Na_2HPO_4, 2 mmol/L NaH_2PO_4, pH 7.4) and frozen overnight at −80°. cAMP in the supernatant was determined by radioimmunoassay [19] and protein content by the BioRad assay (BioRad).

RESULTS AND DISCUSSION

Construction and Expression of a Chimeric EP_3/IP Receptor

A chimeric receptor cDNA, consisting of the porcine EP_3 receptor sequence up to the end of the 7th transmembrane domain and the carboxyl-terminal tail of the human IP receptor, was generated by means of polymerase chain reaction technology. A conserved arginine/lysine (RK) pair at the end of the 7th transmembrane domain marked the junction site of the different receptor fragments. In contrast to the short EP_3 receptor C-terminus (37 amino acids), the carboxyl-terminal tail of the human IP receptor comprised 89 amino acids (Fig. 1). Correct fusion of the EP/IP fragments, maintaining the reading frame and exclusion of mutations due to the polymerase chain reaction, was proven by sequencing of the chimeric receptor cDNA. For characterization of ligand binding specificity of the chimeric receptor proteins, COS-7 cells were transiently transfected with the expression plasmid $pcEP_3$-hIP. Hybrid receptors revealed similar binding characteristics as the wild-type porcine EP_3 receptor (data not shown).

G-Protein Coupling of the Chimeric Receptor

G_s-protein coupling of the wild-type human IP receptor was monitored in transiently transfected COS-7 cells (COS-7-IP), which showed a significant increase in cAMP produc-

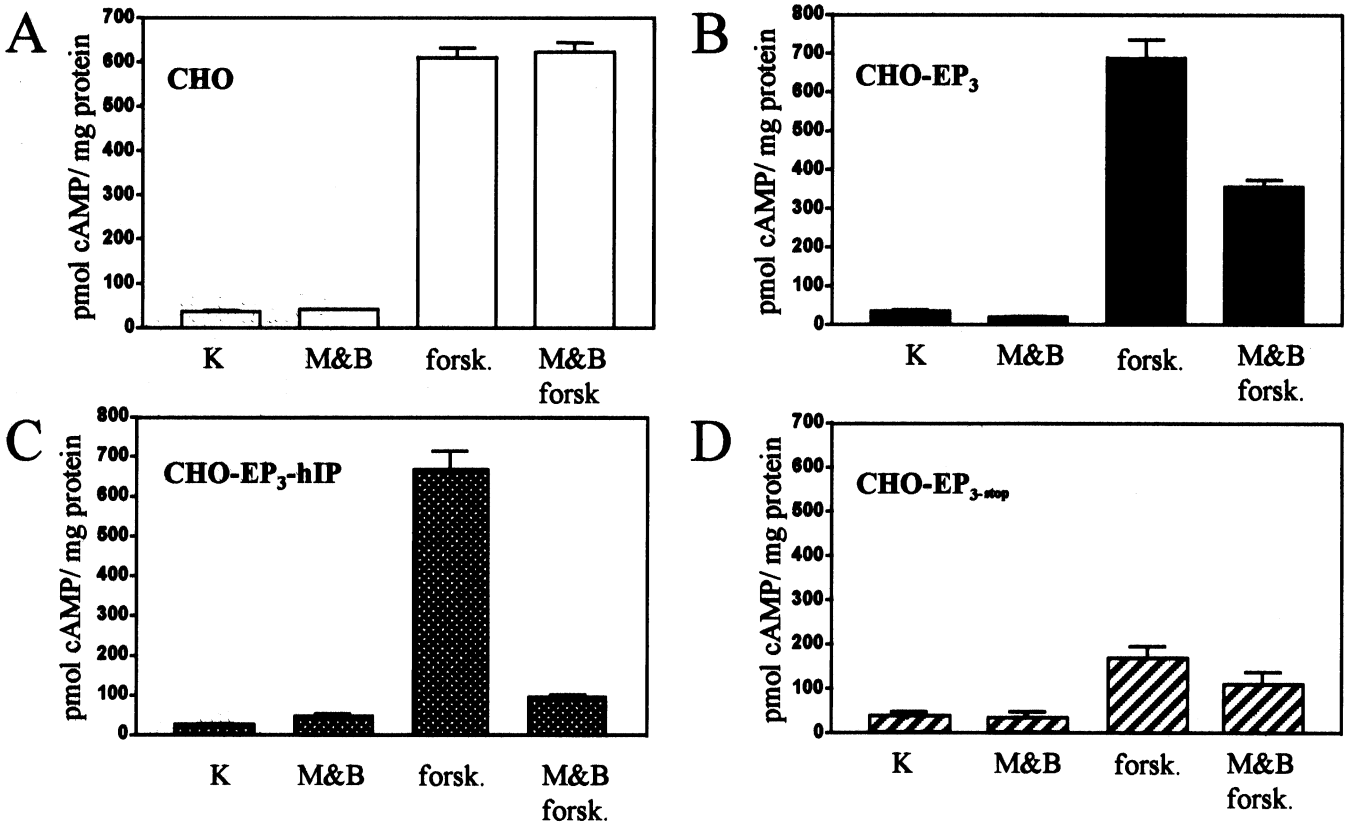

FIG. 3. Inhibition of forskolin (forsk; 3 μM)-induced cAMP formation in CHO cells expressing the chimeric EP_3/IP receptor: (A) mock-transfected CHO cells (CHO); (B) CHO cells expressing the wild-type porcine EP_3 receptor (CHO-EP_3); (C) CHO cells expressing the chimeric EP_3-hIP receptor (CHO-EP_3-hIP); and (D) CHO cells expressing the C-terminally truncated EP_3 receptor (CHO-$EP_{3\text{-stop}}$). M&B 28.767 had no effect in mock-transfected CHO cells (A) but reduced forskolin-induced cAMP formation in CHO-EP_3 cells (B) from 687 ± 46 to 355 ± 16.8 pmol cAMP/mg protein and CHO-EP_3-hIP-cells (C) from 677 ± 49 to 98 ± 1.9 pmol cAMP/mg protein. CHO-$EP_{3\text{-stop}}$ cells (D) revealed low levels of forskolin-induced cAMP formation in the absence of the agonist (189 ± 12 pmol cAMP/mg protein), indicating constitutive activity. Values are means (± SEM) of three independent experiments performed in triplicate.

tion after stimulation with the IP receptor-specific agonist cicaprost (100 nM) (Fig. 2A). No elevation of cAMP was observed in mock-transfected COS-7 cells treated with cicaprost (Fig. 2B).

For analysis of G-protein coupling of the chimeric receptor, the plasmid pcEP$_3$-hIP was linearized with *Pvu*I and transfected into CHO cells. A clonal cell line, expressing the chimeric EP$_3$/IP receptor (CHO-EP$_3$-hIP), was isolated. Since CHO cells were reported to express small amounts of EP$_4$ receptors [12] but no endogenous EP$_3$ receptors, we used the highly EP$_3$ receptor-specific agonist M&B 28.767 [20] for investigation of G-protein coupling instead of the general EP receptor agonist prostaglandin E_2. While M&B 28.767 (100 nM) had no effect on the forskolin-induced cAMP formation in mock-transfected CHO cells (Fig. 3A), it did inhibit this same cAMP formation in CHO-EP$_3$-hIP cells by 85% (Fig. 3C). Thus, the wild-type EP$_3$ receptor and the chimeric receptor protein are both coupled to an inhibitory G-protein and, in contrast to the IP receptor, not coupled to G_s. In CHO cells expressing the wild-type EP$_3$ receptor (CHO-EP$_3$), M&B 28.767 inhibited the forskolin-induced cAMP formation by 50% (Fig. 3B). The stronger inhibition of cAMP formation in the CHO-EP$_3$-hIP cells as compared to the CHO-EP$_3$ cells was consistent with a higher receptor expression as assessed by Western blot analysis (data not shown). Ligand binding studies revealed a B_{max} of 2.3 pmol/mg protein (CHO-EP$_3$) and B_{max} of 12 pmol/mg protein (CHO-EP$_3$-hIP). CHO cells, expressing a C-terminally truncated EP$_3$ receptor, revealed constitutive receptor activity as indicated by low levels of forskolin-induced cAMP formation in the absence of the agonist (Fig. 3D). This was consistent with the constitutive activity of a C-terminally truncated human and mouse EP$_3$ receptor, respectively [10, 13, 21]. Additionally, concentration–response curves were performed in CHO-EP$_3$ and CHO-EP$_3$-hIP cells. The effect of M&B 28.767 was comparable in both transfectants (Fig. 4). Since the exchange of the original EP$_3$ receptor C-terminus for the IP receptor C-terminus did not confer G_s coupling but retained G_i coupling of the chimeric receptor protein, the C-terminal domain alone did not determine G-protein-coupling specificity of the investigated EP$_3$ receptor. While the current study was underway, it was shown by other researchers that a chimeric EP$_3$/EP$_4$ receptor containing the

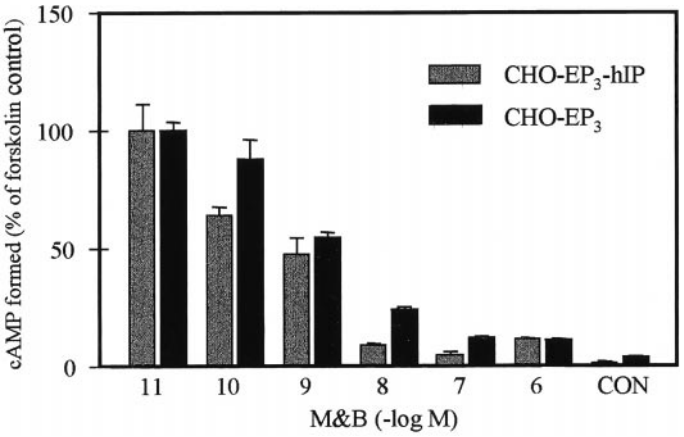

FIG. 4. Comparison of the effect of the EP_3 receptor-specific agonist M&B 28.767 on the inhibition of forskolin (3 μM)-induced cAMP formation. CHO cells expressing the wild type EP_3 receptor (CHO-EP_3) or the chimeric EP_3/IP receptor (CHO-EP_3-hIP) were incubated with M&B 28.767 at the indicated concentrations (10^{-11} M–10^{-6}M). CON = cells without forskolin or M&B 28.767 treatment. Values are means (± SEM) of three independent experiments performed in triplicate.

EP_4 receptor C-terminus was still coupled to G_i as the wild-type EP_3 receptor [22] and not to G_s as the wild-type EP_4 receptor. These data were consistent with our results.

Furthermore, forskolin-induced cAMP formation of CHO-EP_3-hIP cells (Fig. 3C, 677 ± 49 pmol/mg protein) was comparable to forskolin-induced cAMP formation in mock-transfected CHO cells (Fig. 3A, 621 ± 22 pmol cAMP/mg protein), indicating a lack of constitutive activity of the unstimulated chimeric receptor. These data were consistent with the findings of Neuschäfer-Rube *et al.* [22] who showed that an EP_3/EP_4 chimeric receptor lacked constitutive activity in contrast to a C-terminally truncated receptor. Therefore, the human IP receptor C-terminus, as well as the human EP_4 receptor C-terminus, conferred coupling control, i.e. allowed receptor-mediated signal transduction only upon agonist binding. However, the C-terminal domains of the human EP_4 receptor and the hIP receptor differ strongly in length and reveal no obvious sequence homologies.

Interestingly, forskolin-induced cAMP formation in CHO-EP_3 cells (687 ± 46 pmol/mg protein) was also comparable to mock-transfected cells, indicating a lack of constitutive activity of the used porcine EP_3 receptor isoform in spite of its high homology to the constitutive murine $EP_{3\gamma}$ receptor (90% identity of the C-termini) [11]. Jin *et al.* [13] reported recently that the human $EP_{3\text{-II}}$ receptor isoform, which is the human homolog of the constitutive murine $EP_{3\gamma}$ receptor, lacked constitutive activity as well. Therefore, slight variations in the receptor sequence were probably responsible for the difference in constitutive activity between murine receptor on the one hand and porcine and human receptor on the other and require further study.

In conclusion, this study may contribute to a better understanding of structural requirements for G-protein-coupling specificity and the repression of constitutive receptor activity.

The authors are grateful to Petra Kuger for excellent technical assistance and Erika Lohmann for secretarial help.

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