The C-terminal domain of the G_s-coupled EP_4 receptor confers agonist-dependent coupling control to G_i but no coupling to G_s in a receptor hybrid with the G_i-coupled EP_3 receptor

Frank Neuschäfer-Rube, Kristina Hänecke, Volker Blaschke, Kurt Jungermann, Gerhard P. Püschel*

Institut für Biochemie und Molekulare Zellbiologie, Humboldtallee 23, Georg-August-Universität, 37073 Göttingen, Germany

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Abstract Prostaglandin E_2 receptors (EPR) belong to the family of G-protein-coupled receptors with 7 transmembrane domains. They form a family of four subtypes, which are linked to different G-proteins. EP_1R are coupled to G_q, EP_2 and EP_4R to G_s and EP_3R to G_i. Different C-terminal splice variants of the bovine EP_3R are coupled to different G-proteins. A mouse EP_3R whose C-terminal domain had been partially truncated no longer showed agonist-induced G_i-protein activation and was constitutively active. In order to test the hypothesis that the C-terminal domain confers coupling specificity of the receptors on the respective G-proteins, a cDNA for a hybrid rEP_3hEP_4R, containing the N-terminal main portion of the G_i-coupled rat $EP_{3\beta}R$ including the 7th transmembrane domain and the intracellular C-terminal domain of the G_s-coupled human EP_4R, was generated by PCR. HEK293 cells transiently transfected with the chimeric rEP_3hEP_4R cDNA expressed a plasma membrane PGE_2 binding site with a slightly lower K_d value for PGE_2 but an identical binding profile for receptor-specific ligands as cells transfected with the native rat $EP_{3\beta}R$. In $HepG_2$ cells stably transfected with the chimeric rEP_3hEP_4R cDNA PGE_2 did not increase cAMP formation characteristic of G_s coupling but attenuated the forskolin-stimulated cAMP synthesis characteristic of G_i coupling. This effect was inhibited by pre-treatment of the cells with pertussis toxin. Thus, the hybrid receptor behaved both in binding and in functional coupling characteristics as the native rat $EP_{3\beta}R$. Apparently, the intracellular C-terminal domain did not confer coupling specificity but coupling control, i.e. allowed a signalling state of the receptor only with agonist binding.

Key words: Prostaglandin receptor; Chimeric receptor; G-protein coupling

1. Introduction

Prostaglandin E2 (PGE_2) is a potent mediator of physiological and pathophysiological events in the body, for example stimulation of neurotransmitter release, regulation of the immune system and uterus contraction [1–3]. Its actions are mediated by binding to specific PGE_2 ectoreceptors (EPR), which belong to the family of G-protein-coupled receptors with 7 transmembrane domains. According to their affinity to receptor-specific agonists and antagonists and to the intracellular signal chains to which they are coupled, these receptors can be divided into 4 subclasses [4]: EP_1R are coupled to G_q, EP_2R and EP_4R to G_s and EP_3R receptors to G_i. Binding of PGE_2 to EP_3R on rat hepatocytes decreased glucagon-stimulated cAMP formation and glucose output [5] while binding of PGE_2 to EP_4R on human T-lymphocytes increased cAMP formation involved in differentiation and proliferation processes [6].

The PGE_2 receptors of the different subclasses display an overall homology of $< 50\%$ with the transmembrane regions being most conserved. However, the C-terminal domains of the G_i-coupled rat $EP_{3\beta}R$ ($rEP_{3\beta}R$), which consists of 36 amino acids, and the G_s-coupled human EP_4R (hEP_4R), which consists of 156 amino acids, have no homology [7,8].

The observation that different C-terminal splice variants of the bovine EP_3R were coupled to different G-proteins [9] led to the hypothesis that the C-terminal domain of the EPR might play an important role in G-protein coupling specificity. In order to test this hypothesis a cDNA for a receptor hybrid rEP_3hEP_4R containing the N-terminal region of the $rEP_{3\beta}R$ including the 7th transmembrane domain and the C-terminal domain of the hEP_4R was generated by PCR. After expression of the hybrid receptor by transient or stable transfection ligand binding and cAMP formation were measured to analyze binding behavior and the signal chain to which the hybrid receptor was coupled in comparison to the two parental receptors. The hybrid receptor behaved like the native $rEP_{3\beta}R$ indicating that the C-terminal domain did not confer G-protein coupling specificity.

2. Materials and methods

2.1. Materials

All materials were of analytical grade and from commercial sources. M&B 28767 and AH 23848B were generous gifts from Rhone-Poulenc Rorer (Dagenham, UK) and Glaxo (Hertfordshire, UK), respectively. [^{3}H]PGE_2 was obtained from Amersham (Braunschweig, Germany), unlabeled prostaglandins were purchased from Serva (Heidelberg, Germany) or Cascade (Berkshire, UK). Geneticin (G-418 sulfate) and cell culture media were obtained from Gibco-BRL (Eggenstein, Germany), forskolin was from ICN (Meckenheim, Germany) and pertussis toxin from Calbiochem-Novabiochem (Bad Soden, Germany). Primers (Table 1) were synthesized by Pharmacia (Freiburg, Germany). The sources of other materials are given in the text.

2.2. Cloning of rat $EP_{3\beta}$ and human EP_4 receptor cDNAs

Cloning of the $rEP_{3\beta}R$ cDNA was carried out as described [7]. For cloning of the hEP_4R total RNA was isolated from human T-lym-

*Corresponding author. Fax: (49) (551) 395960.

Abbreviations: CHO cells, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle medium; EPR, E-prostaglandin receptor; FCS, fetal calf serum; HAM-F12, nutrient mixture Ham's F-12; HEK cells, human embryonal kidney cells; IBMX, 3-isobutyl-1-methylxanthine; MBS, modified bovine serum; MEM, minimal essential medium; MES, 4-morpholine-ethanesulfonic acid; PCR, polymerase chain reaction; PG, prostaglandin; PTX, pertussis toxin

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phocytes by CsCl gradient centrifugation [10]. Poly(A)$^+$ mRNA was prepared by affinity purification using oligo-(dT) beads from Qiagen (Rathingen, Germany) according to the manufacturer's instructions. First strand cDNA was synthesized by oligo-$(dT)_{12-18}$-primed reverse transcription. PCR was carried out using 10 ng first strand cDNA as a template and oligonucleotide primers P1 and P2 (Table 1) corresponding to positions 261–287 and 1905–1879 of the hEP_4 receptor [6]. Thirty-five cycles of PCR using proof-reading Pwo polymerase were performed with the following temperature profile: 1 min 95°C, 1 min 60°C and 3 min 72°C. A 1643 bp fragment was amplified and cloned blunt end into the *Sma*I site of pBluescript (Stratagene, La Jolla, CA, USA). The insert was sequenced in both directions by cycle sequencing with dye-dideoxy terminator NTPs on the automatic DNA sequencer 373A (Applied Biosystems, Weiterstadt, Germany).

2.3. Construction of the chimeric rEP_3hEP_4 receptor cDNA

The cDNA for the chimeric rEP_3hEP_4R was constructed by recombinant PCR technology [11]. Using the cDNAs of the $rEP_{3\beta}R$ and the hEP_4R cloned into PUC 18 as templates, the N-terminal portion of the $rEP_{3\beta}R$ up to the end of the 7th transmembrane domain and the C-terminal domain of the hEP_4R were amplified by PCR in separate reactions using primer pairs P3/P5 for the $rEP_{3\beta}R$ and P4/P6 for the hEP_4R (Table 1). Primers P4 and P5 hybridized with their 3′-part to the respective template and were complementary to the other cDNA with their overhanging 5′-part. The 1161 bp (N-terminal $rEP_{3\beta}R$ fragment and 521 bp (C-terminal hEP_4R fragment) PCR products were isolated, mixed and fused in a third PCR using the primer pair P3/P6. All PCRs were performed with 10 ng template and 35 cycles of the following temperature profile: 1 min 95°C, 1 min 55°C and 2 min 72°C. The resultant 1660 bp cDNA fragment was cloned into PUC18 and verified by DNA sequencing.

2.4. Transient expression of receptor cDNAs

The cDNAs for $rEP_{3\beta}R$ and the chimeric rEP_3hEP_4R were subcloned into the *Not*I site of the eukaryotic expression vector pcDNA I (Invitrogen, San Diego, CA, USA). The resultant plasmids were transfected into HEK293 cells by a calcium phosphate method using 5% (v/v) modified bovine serum from Stratagene. The *Eco*RI/*Bam*HI fragment of the hEP_4R was subcloned into pcDNA/AMP (Invitrogen) and the resultant plasmid was transfected into COS-7 cells or $HepG_2$ cells using the DEAE-dextran method including chloroquine [12]. Transfected cells were cultured for 72 h in DMEM with 10% FCS for HEK293 and COS-7 cells, or MEM with 10% FCS for $HepG_2$ cells. For membrane preparations, the cells were scraped into a homogenization buffer containing 25 mM Tris-HCl pH 7.5, 250 mM sucrose, 10 mM $MgCl_2$, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. After homogenization of the cells in a Dounce homogenizer a crude membrane fraction was prepared by centrifugation of the homogenate at $100\,000 \times g$. The resulting pellet was suspended in binding buffer containing 25 mM MES/NaOH pH 6.2, 10 mM $MgCl_2$ and 1 mM EDTA and stored at −20°C.

2.5. PGE_2 binding assays with membranes of transfected HEK293 or COS-7 cells

For ligand binding membranes (20–50 μg protein) were incubated with 5 nM [^{3}H]PGE_2 and various concentrations of unlabelled PGE_2, the EP_3R agonist M&B 28767 and the EP_4R antagonist AH23848B in 100 μl binding buffer for 1 h at 20°C. Non-specific binding was determined in the presence of 10 μM PGE_2. Bound and unbound ligand were separated by rapid vacuum filtration through GF 52 filters (Schleicher & Schuell, Dassel, Germany). Filters were washed 5 times with 4 ml ice-cold binding buffer. Radioactivity retained on the filter was counted in 5 ml Hydroluma (Baker, Deventer, The Netherlands). Binding constants were calculated by non-linear regression analysis (LIGAND [13]).

2.6. Stable expression of rat $EP_{3\beta}$ receptor in CHO and rEP_3hEP_4 receptor in $HepG_2$ cells

Stable expression of the $rEP_{3\beta}R$ in CHO cells was carried out as described previously [7]. The 1.66 kb *Not*I cDNA fragment for the rEP_3hEP_4R was subcloned into the eukaryotic expression vector pRc/CMV (Invitrogen). 20 μg of the resultant plasmid was linearized and transfected into 10^7 $HepG_2$ cells by a calcium phosphate method using 5% (v/v) MBS. Transfectants were isolated in MEM containing 10% (v/v) FCS and 0.5 mg/ml G-418 as substrate of the selection marker aminoglycoside phosphotransferase (NEO). Clonal cell lines were isolated by single cell cloning and tested for expression by PGE_2 binding.

2.7. cAMP formation in transfected CHO or $HepG_2$ cells

CHO cells stably expressing the $rEP_{3\beta}R$ and $HepG_2$ cells stably expressing the chimeric rEP_3hEP_4R were cultured in 3.5 cm diameter plates to a density of 5×10^5 in HAM-F12 medium containing 10% (v/v) FCS for CHO cells and 1.2 mg/ml G-418 or MEM containing 10% FCS and 0.5 mg/ml G-418 for $HepG_2$ cells. cAMP assays with $HepG_2$ cells transiently transfected with the hEP_4R were performed 72 h after transfection. Where indicated, cells were pretreated with PTX (100 ng/ml) for 16 h. Cells were washed 3 times with 1 ml HEPES buffer pH 7.4 containing 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose and 15 mM HEPES and then pre-incubated in 1 ml of the same buffer with 1 mM IBMX at 37°C for 10 min. Then PGE_2, M&B 28767, AH23848B and forskolin (100 μM) were added in a volume of 10 μl buffer to the final concentration indicated. After incubation for 10 min the reaction was stopped by removing the buffer and freezing the cells in liquid nitrogen. Cells were lysed in 500 μl 10 mM HCl containing 1 mM IBMX for 1 h at 4°C. The lysate was centrifuged and cAMP was quantified in the supernatant with a ^{125}I-cAMP assay kit of Amersham (Braunschweig, Germany).

3. Results and discussion

3.1. Construction of the cDNA for the chimeric rEP_3hEP_4R

A cDNA for a chimeric receptor consisting of the N-terminal main portion of the G_i-coupled $rEP_{3\beta}R$ including the 7th transmembrane domain and the C-terminal intracellular domain of the G_s-coupled hEP_4R (cf. Fig. 1) was constructed by recombinant PCR technology. The junction between the 7th transmembrane domain and the C-terminal domain is marked both in the $rEP_{3\beta}R$ and the hEP_4R by an arginine/lysine (R/K) pair and these two amino acids are conserved in most prostaglandin receptors [14]. The C-terminal domains of the two receptors are different in size (Fig. 1). The hEP_4R C-terminal domain is 4.5-fold larger and contains no partial sequence with significant homology to the C-terminal domain of the $rEP_{3\beta}R$. The chimeric rEP_3hEP_4R cDNA was cloned into PUC18 and sequenced. The two receptor parts were fused in the correct position maintaining the original reading frame.

Table 1
Primers used to amplify receptor cDNAs

	Sequence	Receptor and position
P1	5′-AAAGCAGGTTGGAGGCGGGTCCAG-3′	hEP_4R (Genbank accession number: L28175) pos. 261–287 (forward)
P2	5′-CAGGATTTTATAAGGGTCCAGAAACAG-3′	hEP4R, pos. 1905–1879 (reverse)
P3	5′-AGCGACCGGCGCTCAGCTGG-3′	sequence flanking the *Eco*RI site of the vector λgt11 (short arm), originally used to amplify the $rEP_{3\beta}R$ cDNA cloned in λgt11 [7] (forward)
P4	5′-GGATCCCTGGGTTTATCTGCTGCTA/**AGAAAGACAGTGCTCAGTAAAGC***A*-3	$rEP_{3\beta}R$ (Genbank accession number: X80133), pos. 1056–1080/**hEP_4R**, pos. 1385–1408 (forward)
P5	inverted complementary sequence of **P4**	(reverse)
P6	5′-tcgcggccgctCAGGATTTTATAAGGGTCCAGAAACAG-3′	Primer P2+recognition site for *Not*I at the 5′-end (lower case) (reverse)

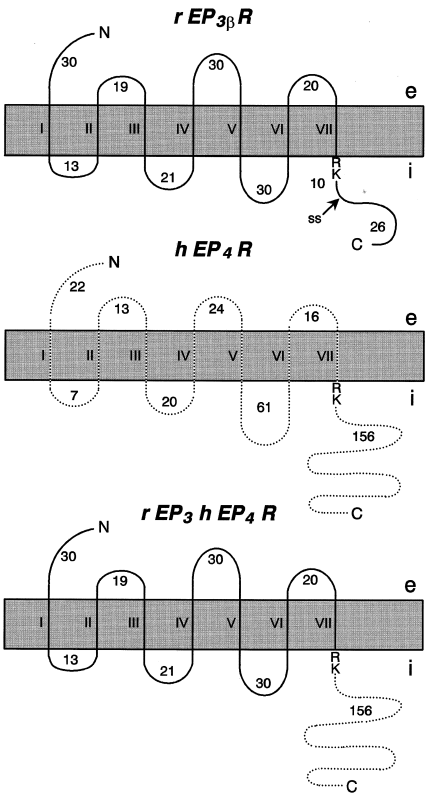

Fig. 1. Hypothetical structure of the G_i-coupled rat PGE_2 receptor ($rEP_{3\beta}R$), the G_s-coupled human PGE_2 receptor (hEP_4R) and a chimeric rEP_3hEP_4 receptor (rEP_3hEP_4R). Glycosylation sites in the N-terminal domains and the second extracellular loops as well as potential phosphorylation sites for PKA, PKC and β-adrenergic receptor kinases (βARKs) in the intracellular loops and the C-terminal domains have been omitted for clarity. SS: location of the splice site in the EP_3R.

The sequences of the respective receptor parts were 100% identical to the parental receptors domains.

3.2. *Binding characteristics of the chimeric rEP_3hEP_4 receptor*

Parental receptors and the rEP_3hEP_4R hybrid were expressed transiently in order to compare the binding characteristics of the receptors. While the $rEP_{3\beta}R$ and the rEP_3hEP_4R were expressed efficiently in HEK293 cells, high level expression of the hEP_4R was achieved in COS-7 cells only. The receptors were expressed to yield a similar maximal binding in the respective membrane preparations.

Membranes of cells transfected with either of these receptors displayed a single binding site for PGE_2. Apparent K_d values were 15 nM for the $rEP_{3\beta}R$, 3 nM for the rEP_3hEP_4R hybrid and 3 nM for the hEP_4R (Fig. 2). Thus, the K_d value of the hybrid receptor was 5-fold lower than the K_d value of the parental $rEP_{3\beta}R$ and identical to the K_d value of the hEP_4R. However, a K_d value of 3 nM has been reported for the mouse $EP_{3\beta}R$ that has 97% homology to the $rEP_{3\beta}R$ and 89% homology in the C-terminal domain [15].

$rEP_{3\beta}R$ and hEP_4R bound PGE_2 with similar affinity but hEP_4R had an almost 100-fold lower affinity for the EP_3R-specific ligand M&B 28767. The rEP_3hEP_4R hybrid bound M&B 28767 with the same K_d as the $rEP_{3\beta}R$ (Fig. 2).

AH23848B has been reported to be a specific antagonist of EP_4R, however, it proved not to be useful as a receptor-specific ligand in competition binding studies because it bound to $rEP_{3\beta}R$, the hybrid rEP_3hEP_4R and hEP_4R with similar low affinity (Fig. 2). This is in line with the finding that AH23848B besides being a weak EP_4R antagonist is also a weak agonist at EP_3R [16].

The hEP_4R C-terminal domain in the hybrid rEP_3hEP_4R thus did not shift the ligand binding characteristics towards the hEP_4R profile. The binding behavior of the hybrid rEP_3hEP_4 receptor was similar to the $rEP_{3\beta}$ receptor except that the apparent K_d for PGE_2 binding was about 5-fold lower in the chimeric receptor. This could be a reaction of small conformational changes in the receptor caused by the introduced foreign C-terminal domain which was in direct contact to the 7th transmembrane domain. The 7th transmembrane domain was postulated to be involved in forming the ligand binding site [14]. A change in receptor binding affinity which was caused by intracellular sequence mutations could be shown for the M1 muscarinic receptor, where mutations in the second intracellular loop led to a higher ligand affinity in mutant receptors [17].

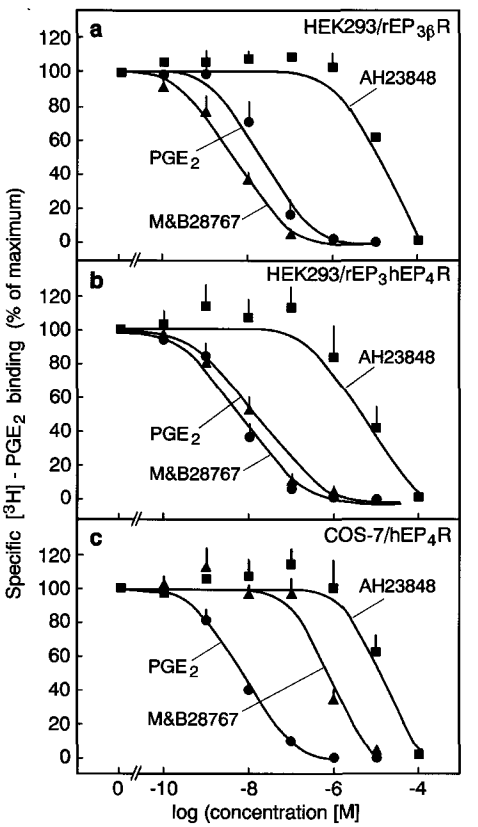

Fig. 2. Competition by PGE_2, M&B 28767 and AH23848B of $[^3H]PGE_2$ binding to membranes of HEK293 cells transfected with pcDNA I/$rEP_{3\beta}R$, pcDNA I/rEP_3hEP_4R or of COS-7 cells transfected with pcDNA I/AMP/hEP_4R. Cells were transfected as described in Section 2. Binding of 5 nM $[^3H]PGE_2$ to membranes of transfected cells was measured after 1 h at 20°C in the presence of the indicated concentrations of unlabelled PGE_2 or its analogs. M&B 28767 is an EP_3R agonists and AH23848B is an EP_4R antagonist. $[^3H]PGE_2$ binding in the presence of 10 μM PGE_2 was defined as unspecific binding. Maximal specific binding was set equal to 100%. Values are means ± S.E. of 3 different experiments performed in duplicate.

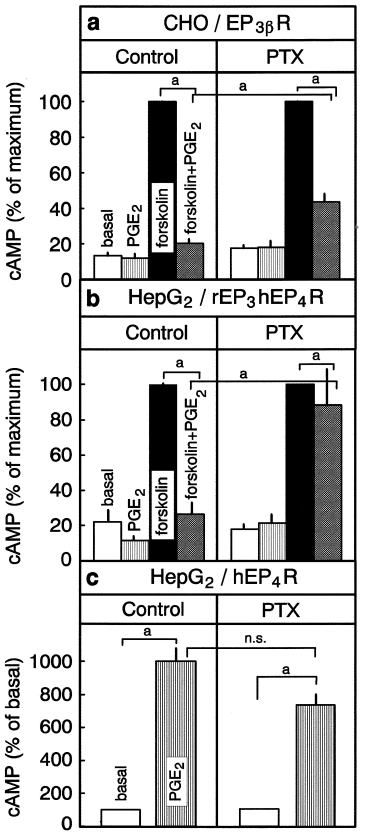

Fig. 3. Modulation of cAMP formation in CHO/rEP$_{3\beta}$R, HepG$_2$/rEP$_3$hEP$_4$R and HepG$_2$/hEP$_4$R cells by PGE$_2$. CHO cells were stably transfected with the pRc/CMV/rEP$_{3\beta}$R construct. HepG$_2$ cells were stably transfected with the pRc/CMV/rEP$_3$hEP$_4$R construct or transiently transfected with the pcDNA I/AMP/hEP$_4$R construct. cAMP formation induced by 1 μM forskolin, 1 μM PGE$_2$ or 1 μM forskolin+1 μM PGE$_2$ after 10 min at 37°C was determined by radioimmunoassay. cAMP formation in forskolin-stimulated (a, b) or unstimulated cells (c) was set equal to 100%. Values are means ± S.E. of 3 different experiments performed in duplicate. Statistics: Student's *t*-test for unpaired samples: a, $P < 0.01$; n.s., not significant.

3.3. *Functional properties of the stably expressed rEP$_3$hEP$_4$ receptor*

To analyze the intracellular signal chain of the chimeric receptor, the rEP$_3$hEP$_4$R cDNA was cloned into pRc/CMV and stably expressed in HepG$_2$ cells. HepG$_2$ cells were used because they lack intrinsic EP$_3$R, EP$_2$R or EP$_4$R. In contrast HEK293, COS-7 and CHO cells possess EP$_2$R and/or EP$_4$R and, as a consequence, showed a strong (HEK 293 and COS-7 cells) or slight (CHO cells) increase in cAMP formation upon PGE$_2$ stimulation in the untransfected state (data not shown).

A clonal cell line, HepG$_2$/rEP$_3$hEP$_4$R, expressing a PGE$_2$ binding site was isolated. In these cells 1 μM PGE$_2$ inhibited the forskolin-induced cAMP formation by 80% (Fig. 3b). The PGE$_2$-mediated inhibition of the forskolin-stimulated cAMP production was suppressed by only 10%, if the cells were pretreated with PTX. PGE$_2$ did not increase cAMP formation in either control or PTX-treated HepG$_2$/rEP$_3$hEP$_4$R cells. Thus, the rEP$_3$hEP$_4$R hybrid coupled to a PTX-sensitive G$_i$ protein but not to G$_s$. Its properties were nearly identical to stably expressed rEP$_{3\beta}$R in CHO/rEP$_{3\beta}$R cells (Fig. 3a). However, in these cells PTX was less effective and suppressed the PGE$_2$-mediated inhibition of forskolin-stimulated cAMP formation by only 60%. In HepG$_2$ cells which were transiently transfected with the cDNA for the hEP$_4$R 1 μM PGE$_2$ increased cAMP levels by 9-fold (Fig. 3c). This PGE$_2$-mediated cAMP increase was not affected by PTX pretreatment. Thus, the hEP$_4$R was exclusively coupled to a G$_s$ and not to G$_i$ protein. (Fig. 3c).

In dose-response curves with HepG$_2$/rEP$_3$hEP$_4$R cells PGE$_2$ and the EP$_3$R agonist M&B 28767 inhibited forskolin-stimulated cAMP formation half-maximally with an apparent IC$_{50}$ of about 1 nM which was in the same concentration range as needed for 50% competition of [^{3}H]PGE$_2$ binding (Fig. 4). M&B 28767 inhibited forskolin-stimulated cAMP formation with a similar IC$_{50}$ as in CHO/rEP$_{3\beta}$R cells but a 10-fold lower PGE$_2$ concentration was needed for half-maximal inhibition, which is in line with the lower K_d for PGE$_2$ observed in the binding studies with the rEP$_3$hEP$_4$R (Fig. 2b). These data show that the functional properties of the chimeric rEP$_3$hEP$_4$R were similar to those of the G$_i$-coupled rEP$_{3\beta}$R and that the C-terminal domain of the hEP$_4$R led to a slightly higher affinity for PGE$_2$ in the chimeric rEP$_3$hEP$_4$R.

3.4. *Influence of different receptor domains on receptor G-protein coupling*

Receptor domains involved in G-protein coupling have been analyzed in several R7G receptors. A unifying principle has not emerged yet due to sometimes rather contradictory results.

3.4.1. Role of the second and third intracellular loops. Experiments with chimeric receptors showed that the third intracellular loop plays a critical role in G-protein coupling of many receptors [17–23]. This was the case for the uncoupled dopamine D3-receptor in which the substitution of the third

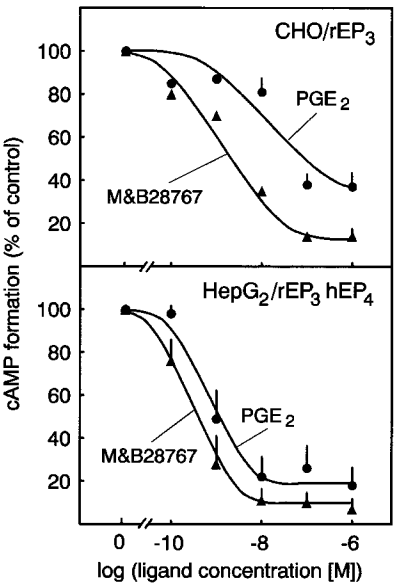

Fig. 4. Inhibition of forskolin-stimulated cAMP formation in CHO/rEP$_{3\beta}$R and HepG$_2$/rEP$_3$hEP$_4$R cells by PGE$_2$ and M&B 28767 cAMP formation in CHO/rEP$_{3\beta}$R and HepG$_2$/rEP$_3$hEP$_4$R cells was induced by 1 μM forskolin for 10 min at 37°C in the presence of the indicated concentrations of PGE$_2$ or the EP$_3$R agonists M&B 28767. cAMP content in the cells was determined by radioimmunoassay. cAMP formation in the absence of PGE$_2$ or M&B 28767 was set equal to 100%. Values are means ± S.E. of 3 different experiments performed in duplicate.

intracellular loop with that of the G_i-coupled dopamine D2-receptor led to functional G_i coupling, and for the G_q-coupled muscarinic M1 receptor, in which the substitution of this domain with the loop of the G_s-coupled β-adrenergic receptor led to an additional G_s coupling [17,21]. Experiments in which only parts of this intracellular loop were exchanged showed that a dodecapeptide near the N-terminus of this domain could mediate G_s coupling [22]. However, the G_s-coupled β-adrenergic receptor substitutions in the second and third loops with sequences of the G_i-coupled α_2-adrenergic receptor caused only decreased G_s but conferred no G_i coupling [22].

3.4.2. Role of the C-terminal domain. The normally G_s-coupled β-adrenergic receptor with parts of the third intracellular loop and the C-terminal domain of the G_i-coupled α_2-adrenergic receptor showed an additional coupling to G_i [22]. Additional coupling to G_i and G_q was also observed after truncation of the main portion of the C-terminal domain of the G_s-coupled PTH receptor [24]. A direct participation of the C-terminal domain in determining G-protein specificity and G-protein activation could be shown for the EP_3R. Four splice variants of the bovine EP_3R, which differed only in their C-terminal sequence, coupled to different G-proteins [9]. Two of these isoforms coupled to G_s protein, one to G_i and one to G_i, G_s and G_q. The splice variant which coupled to all three G-proteins was also found for the mouse EP_3R and showed similar promiscuous G-protein coupling [25]. However, seven C-terminal splice variants of the human EP_3R [26] which are in part highly homologous to the sequence of the bovine EP_3R C-terminal domains were all exclusively coupled to G_i protein. This discrepancy in G-protein coupling specificity could be due to the high expression level of the bovine EP_3R variants in a heterologous expression system or due to species specific sequence differences.

In the current study the C-terminal domain of the G_s-coupled hEP_4R was not able to shift the G_i-coupled $rEP_{3\beta}R$ to a G_s-coupled receptor or to confer an additional G_s coupling. In contrast, the chimeric rEP_3hEP_4R not only retained its $rEP_{3\beta}R$ binding profile but also its G_i coupling properties. Thus, the hEP_4R C-terminal domain seemed not to contain sufficient information for coupling the receptor to the G_s protein. This is in contrast to the different coupling profiles of the bovine EP_3R splice variants but in line with the coupling properties of the human EP_3R splice variants.

3.5. Influence of the C-terminal domain on G-protein activity

When the C-terminal domain of the mouse EP_3R was truncated directly after the EP_3R splice junction which was common for all splice variants and shortened the C-terminal domain to 10 amino acids (Fig. 1), the receptor no longer showed agonist-induced G_i activation but was constitutively active [27,28]. Apparently, the C-terminal domain had the role of keeping the G_i protein inactive as long as the receptor had not bound an agonist.

In the present study the C-terminal domain of the hEP_4R was able to take over this role of the $rEP_{3\beta}R$ C-terminal domain to prevent the hybrid rEP_3hEP_4R from being constitutively active. A reason for this behavior could be the presence of negative regulatory elements located in both the $rEP_{3\beta}R$ and hEP_4R C-terminal domain, which keep the $rEP_{3\beta}R$ and the rEP_3hEP_4R in an inactive conformation and prevent ligand-independent G_i protein activation. This is surprising because the $rEP_{3\beta}R$ and hEP_4R C-terminal domains are totally different in size and share no significant sequence homology. Potential candidates which can modulate protein coupling and activation are post-translational modifications like phosphorylation [29]. It is possible that the $rEP_{3\beta}R$ and hEP_4R C-terminal domains show a similar modification pattern, because both C-terminal domains contain potential phosphorylation sites which can possibly arrest the receptor/G_i protein complex in an inactive state in the absence of a ligand.

In summary, the C-terminal domain of the $EP_{3\beta}R$ does not seem to be the critical intracellular domain conferring G-protein coupling specificity; rather it appears to confer agonist-dependent coupling control: it may function as a negative regulator involved in the maintenance of a nonsignalling state of the ligand-free receptor. This effect can be mimicked by substitution with the C-terminal domain of the hEP_4R.

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