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The C-terminal domain of the human EP_4 receptor confers agonist-induced receptor desensitization in a receptor hybrid with the rat $EP_{3\beta}$ receptor

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Abstract Prostaglandin E_2 receptors (EPR), which belong to the family of heterotrimeric G protein-coupled ectoreceptors with seven transmembrane domains, can be classified into four subtypes according to their ligand binding and G protein coupling specificity. Of these, $EP_{3\beta}R$ is coupled to G_i, whereas EP_4R is coupled to G_s. EP_4R, in contrast to $EP_{3\beta}R$, shows agonist-induced desensitization. The C-terminal domain and the third intracellular loop of these receptors have been implicated in G protein coupling specificity and desensitization. Here, receptor hybrids consisting of the main portion of rat $EP_{3\beta}R$ and either the C-terminal domain or the third intracellular loop of human EP_4R were used to study the contribution of the respective receptor domains to G protein coupling and desensitization. Neither the EP_4R C-terminal domain nor the EP_4R third intracellular loop alone was sufficient to change the coupling specificity of the rEP_3hEP_4 receptor hybrids from G_i to G_s or to confer additional G_s coupling. However, the EP_4R C-terminal domain but not the third intracellular loop was necessary and sufficient to mediate rapid agonist-induced, second messenger-independent desensitization in the G_i-coupled hybrid receptors.

Key words: Prostaglandin receptor; Chimeric receptor; Receptor desensitization; G protein-coupled receptor kinase; G protein coupling

1. Introduction

Prostaglandin E_2 receptors (EPR), like most prostanoid receptors, belong to the class of G protein-coupled ectoreceptors with seven transmembrane domains [1]. There are four subtypes of EPR that differ in their affinity to synthetic ligands and their G protein coupling specificity: EP_1R are linked to G_q and increase $InsP_3$ and hence the cytosolic Ca^{2+} concentration, EP_2R and EP_4R are coupled to G_s and increase intracellular cAMP, while EP_3R are coupled to G_i and decrease hormone-stimulated cAMP formation [2]. These receptors display an overall sequence homology of about 50%, with the putative transmembrane domains being most conserved [3]. So far, little is known about the structure-function relationship of these receptors. A couple of highly conserved amino acids have been shown to be necessary for ligand binding, i.e. the Arg corresponding to Arg^{310} in the seventh transmembrane domain of the rat $EP_{3\beta}R$ [4], or signal transduction to the G protein, i.e. the amino acid corresponding to Asp^{319} in the seventh transmembrane domain of rat EP_3R [5]. The C-terminal domain has been implicated in control of agonist-dependent coupling to G proteins. A mutant receptor, which was truncated beginning 10 amino acids after the end of the seventh transmembrane domain, was constitutively active but showed no ligand-dependent coupling control [6,7]. In order to further elucidate the role of the C-terminal domain in G protein coupling, in a previous study a receptor hybrid was generated consisting of the N-terminal main portion of the G_i-coupled $EP_{3\beta}R$ up to the end of the seventh transmembrane domain and the C-terminal portion of the G_s-coupled EP_4R [8]. In this receptor hybrid which was coupled to G_i exclusively the C-terminal domain of the EP_4R restored the agonist-dependent coupling control that was lost in the truncated EP_3R. A second function that has been attributed to the C-terminal domain in EPRs is the agonist-induced receptor desensitization. $EP_{3\alpha}R$ and $EP_{3\beta}R$ are splice variants that differ in their C-terminal domain. Of these, only $EP_{3\alpha}R$ showed agonist-dependent desensitization [9]. Furthermore, of the two G_s-linked EPRs, EP_2R and EP_4R, only EP_4R has a long serine- and threonine-rich C-terminal domain and shows rapid agonist-induced desensitization [10]. While the current study was under way, it was shown by C-terminal truncation of the EP_4R that the C-terminal domain is necessary for agonist-induced desensitization but not for G protein coupling [11]. Here, the role of the C-terminal domain in receptor desensitization of EPRs was studied in receptor hybrids. It was found that the C-terminal domain (Ct) of EP_4R in a rEP_3hEP4-Ct receptor hybrid is not only necessary but sufficient to confer ligand-induced receptor desensitization while the third intracellular domain (IIIi) of the EP_4R did not confer desensitizability in a rEP_3hEP4-IIIi receptor hybrid.

2. Materials and methods

2.1. Materials

All materials were of analytical grade and from commercial sources. M&B 28767 was a generous gift from Rhone-Poulenc Rorer (Dagenham, UK). [^{3}H]PGE_2 was obtained from Amersham (Braunschweig, Germany), unlabeled prostaglandins were purchased from Serva (Heidelberg, Germany) or Calbiochem-Novabiochem (Bad Soden, Germany) who also provided pertussis toxin (PTX). Geneticin (G418 sulfate) and cell culture media were obtained from Gibco-BRL (Eggenstein, Germany), forskolin was from ICN (Meckenheim, Germany). Primers (Table 1) were synthesized by Pharmacia (Freiburg,

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

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Germany) or NAPS (Göttingen, Germany). The sources of other materials are given in the text.

2.2. Construction of the chimeric rEP_3hEP_4 receptor cDNAs

Cloning of the $rEP_{3\beta}R$ [12] and hEP_4R [8] cDNAs was carried out as described previously. The cDNA for the chimeric rEP_3hEP_4-CtR and rEP_3hEP_4-IIIiR were constructed by recombinant PCR technology [13]. The protocol for the construction of rEP_3hEP_4-CtR cDNA has been described in detail elsewhere [8]. The rEP_3hEP_4-IIIiR cDNA was generated using the cDNAs of $rEP_{3\beta}R$ and hEP_4R cloned into PUC 18 as templates. The N-terminal portion of $rEP_{3\beta}R$ up to the end of the 5th transmembrane domain, the third intracellular loop of hEP_4R and the C-terminal portion of $rEP_{3\beta}R$ starting at the 6th transmembrane domain were amplified by PCR in separate reactions using primer pairs P1/P2 and P5/P6 for the N-terminal and C-terminal portions of $rEP_{3\beta}R$ and P3/P4 for the hEP_4R third intracellular loop (Table 1). Primers P2 and P5 hybridized with their 3′-part to the rEP_3R template and were complementary to the hEP_4R cDNA with their overhanging 5′-part. Similarly, primers P3 and P4 hybridized with hEP_4R with their 3′-part and were complementary to sequences of $rEP_{3\beta}R$ with their 5′-overhanging ends. The 873 bp (N-terminal $rEP_{3\beta}R$ fragment), 231 bp (hEP_4R third intracellular loop) and 536 bp (C-terminal hEP_4R fragment) PCR products were isolated, mixed and fused in a third PCR using the primer pair P1/P6. All PCRs were performed with the proofreading PWO polymerase (Boehringer, Mannheim, Germany) 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 1592 bp cDNA fragment was cloned into PUC18 and verified by DNA sequencing.

2.3. Stable expression of rat $EP_{3\beta}$ receptor in CHO and rEP_3hEP_4-Ct receptor and rEP_3hEP_4-IIIi receptor in $HepG_2$ cells

Stable expression of $rEP_{3\beta}R$ in CHO cells was carried out as described previously [7]. The 1.66 kbp *Not*I cDNA fragment for rEP_3hEP_4-CtR and the 1.6 kbp *Hin*dIII cDNA fragment of rEP_3hEP_4-IIIiR were subcloned into the eukaryotic expression vector pRc/CMV (Invitrogen). 20 μg of the resultant plasmid was linearized and transfected into 10^7 cells by electroporation (CHO cells) or a calcium phosphate method using 5% (v/v) MBS ($HepG_2$ cells). Transfectants were isolated in HAM-F12 containing 10% FCS and 1.2 mg/ml G418 (CHO cells) or MEM containing 10% (v/v) FCS and 0.5 mg/ml G418 ($HepG_2$ cells) 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. hEP_4R was transfected transiently into $HepG_2$ cells by the DEAE-dextran method as described previously [8].

2.4. PGE_2 binding assays with transfected $HepG_2$ or CHO cells

CHO cells stably expressing $rEP_{3\beta}R$ and $HepG_2$ cells stably expressing the chimeric receptors were cultured in 6 cm diameter plates to a density of 1.5×10^6 in HAM-F12 medium containing 10% (v/v) FCS for CHO cells and 0.5 mg/ml G418 or MEM containing 10% FCS and 0.5 mg/ml G418 for $HepG_2$ cells. For ligand binding studies cells were washed three times with 5 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 (incubation buffer) and then preincubated for 5 min in the same buffer with or without 100 nM M&B 28767. The agonist was removed by two washes with incubation buffer, an acid wash with 5 ml of 50 mM glycine, 150 mM NaCl pH 3 for 1 min and two additional washes with incubation buffer. Cells were then detached from the tissue culture plates with 250 μl Ca^{2+}-free incubation buffer containing 1 mM EDTA. Of this cell suspension 50 μl was incubated in a total volume of 100 μl with 10 nM [^{3}H]PGE_2 for 30 min at 37°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&Schüll, Dassel, Germany). Filters were washed four times with 4 ml ice-cold binding buffer. Radioactivity retained on the filter was counted in 5 ml Hydroluma (Baker, Deventer, Netherlands).

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

CHO cells stably expressing $rEP_{3\beta}R$ and $HepG_2$ cells stably expressing the chimeric receptors 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 G418 or MEM containing 10% FCS and 0.5 mg/ml G418 for $HepG_2$ cells. cAMP assays with $HepG_2$ cells transiently transfected with hEP_4R were performed 72 h after transfection. Where indicated, cells were pretreated with PTX (100 ng/ml) for 16 h. Cells were washed three times with 1 ml incubation buffer and then preincubated in 1 ml of the same buffer with or without 100 nM M&B 28767 for 5 min. The agonist was removed as described for the binding studies. Cells were preincubated with 1 ml incubation buffer containing 1 mM IBMX at 37°C for 10 min. Then PGE_2, M&B 28767 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

3.1. G protein coupling specificity

Exchange of the C-terminal domain of the G_i-coupled $EP_{3\beta}$ receptor starting directly after the putative seventh transmembrane domain with the C-terminal domain of the G_s-coupled EP_4 receptor yielded a receptor hybrid (rEP_3hEP_4-Ct receptor) that had similar binding characteristics as the wild type receptor (not shown) and was coupled exclusively to G_i (Fig. 1c). As with the wild type receptor (Fig. 1a), PGE_2 reduced the forskolin-stimulated cAMP formation to about 20% of the maximum. This inhibition was attenuated by pretreatment of the cells with PTX (not shown). No G_s-mediated PGE_2-dependent increase in cAMP formation, which is typical for wild type EP_4R (Fig. 1b), was observed in cells expressing the

Table 1
Sequence and location of the PCR primers used to generate the hybrid receptors

	Sequence (5′–3′)	Receptor and position
P1	AGCGACCGGCGCTCAGCTGG	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)
P2	GGCGGTGCATGCGGAGCAGCGCGCC/ *GATGGTCGCCAGGTTGCAGGCAAA*	hEP_4R pos. 1034–1110, Acc. No. L28175/ $rEP_{3\beta}R$ pos. 792–769, Acc. No. X80133 (reverse)
P3	*TTTGCCTGCAACCTGGCGACCATC*/ GGCGCGCTGCTGCGCATGCACCGC-3′	$rEP_{3\beta}$ pos. 769–792/ hEP_4 pos. 1010–1033 (forward)
P4	*ACACACATGATCCCCATAAG*/ GATCTCGGCGCCCGCGATGCGGCG	$rEP_{3\beta}R$ pos. 902–883/ hEP_4R pos. 1192–1169 (reverse)
P5	inverted complementary sequence of P4 (forward)	
P6	*CTGAGGCTGGAGATATTTCTGCACTGAGTC*	located in the 3′-UTR of the $rEP_{3\beta}R$ cDNA 121 bp after the stop codon (reverse)

The locations given are the sequence positions in the data files retrieved from GenBank under the accession numbers indicated. The reverse primers are the complementary sequences to the indicated positions. Primers are shown in the 5′ to 3′ direction. The $EP_{3\beta}R$ sequences are shown in italics.

rEP$_3$hEP$_4$-Ct receptor hybrid. Similarly, the rEP$_3$hEP$_4$-IIIi receptor hybrid, containing the third intracellular loop of human EP$_4$R from the end of the fifth to the beginning of the sixth putative transmembrane domain, showed identical binding characteristics to wild type EP$_{3\beta}$R (not shown) and was coupled exclusively to a PTX-sensitive (not shown) G_i protein. In cells expressing the rEP$_3$hEP$_4$-IIIi receptor hybrid (Fig. 1d) PGE_2 reduced forskolin-stimulated cAMP formation to a similar extent as in cells expressing wild type EP$_{3\beta}$R (Fig. 1a). The rEP$_3$hEP$_4$-IIIi receptor hybrid did not couple to G_s. Thus, neither the C-terminal domain nor the third intracellular loop alone contained sufficient information to shift the coupling specificity of the hybrid receptors from G_i to G_s.

3.2. *Induction of desensitizability by the EP$_4$R C-terminal domain but not the EP$_4$R third intracellular loop*

Cells stably expressing either wild type EP$_{3\beta}$R, rEP$_3$hEP$_4$-Ct or the rEP$_3$hEP$_4$-IIIi receptor hybrids were preincubated with a saturating concentration of the EP$_3$R agonist M&B 28767 (100 nM) for 5 min. The agonist was then completely removed (see Section 2). In cells expressing the wild type receptor or the rEP$_3$hEP$_4$-IIIi receptor hybrid, containing the third intracellular loop of the hEP$_4$R, preincubation did not reduce specific PGE_2 binding of 10 nM [^{3}H]PGE_2. By contrast, preincubation with the EP$_3$ agonist of cells expressing the rEP$_3$hEP$_4$-Ct receptor hybrid, containing the hEP$_4$R C-terminal domain, reduced specific [^{3}H]PGE_2 binding to 60%

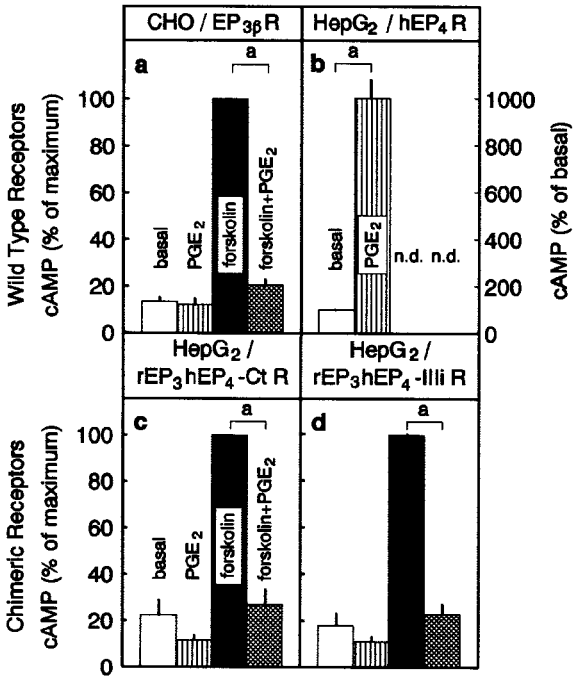

Fig. 1. G protein coupling specificity of wild type and hybrid receptors. CHO cells were stably transfected with the pRc/CMV/rEP$_{3\beta}$R construct (a). HepG$_2$ cells were stably transfected with the pRc/CMV/rEP$_3$hEP$_4$-Ct construct (c) or the pRc/CMV/rEP$_3$hEP$_4$-IIIi construct (d) or they were transfected transiently with the pcDNA I/AMP/hEP$_4$R construct (b). 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,c,d) or unstimulated cells (b), respectively, was set at 100%. Values are means ± S.E.M. of three different experiments performed in duplicate. n.d., not determined. Statistics: Student's *t*-test for unpaired samples: a, $P < 0.05$.

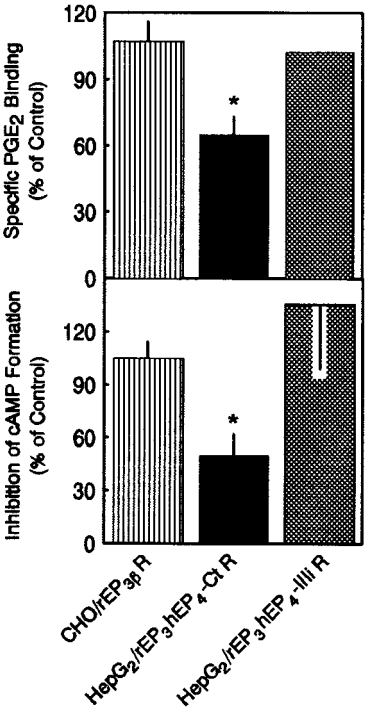

Fig. 2. Induction by the hEP$_4$R C-terminal domain of desensitizability in the rEP$_3$hEP$_4$-Ct receptor hybrid. Cells were transfected stably as described in the legend to Fig. 1 either with the wild type pRc/CMV/rEP$_{3\beta}$R or with one of the hybrid receptor pRc/CMV/rEP$_3$hEP$_4$-Ct or pRc/CMV/rEP$_3$hEP$_4$-IIIi constructs. In a preincubation period cells were exposed to a saturating concentration (100 nM) of the EP$_3$R agonist M&B 28767 for 5 min. The agonist was then removed by an acid wash followed by extensive washing with incubation medium (see Section 2). For binding assays cells were detached from the tissue culture plates with EDTA buffer. Binding was measured in the cell suspension in presence of 10 nM [^{3}H]PGE_2 with 10 μM unlabelled PGE_2 to determine unspecific binding after 30 min at 37°C. cAMP formation was determined by radioimmunoassay in cell cultures after stimulation for 10 min with 1 μM forskolin and 5 nM M&B 28767. Values are means ± S.E.M. percent of control cells tested in parallel that were not exposed to the agonist in the preincubation period. Student's *t*-test for paired samples: $^*P < 0.05$.

of the untreated control (Fig. 2). Likewise, the M&B 28767-dependent inhibition of forskolin-induced cAMP formation was not attenuated by agonist pretreatment in cells expressing either wild type EP$_{3\beta}$R or the rEP$_3$hEP$_4$-IIIi receptor hybrid, yet was reduced to about 50% in cells expressing the rEP$_3$hEP$_4$-Ct receptor hybrid (Fig. 2). Thus, the EP$_4$R C-terminal domain but not the third intracellular loop of the EP$_4$R conferred agonist-induced desensitization to the hybrid receptors.

3.3. *Time course of desensitization*

Cells expressing wild type EP$_{3\beta}$R, the rEP$_3$hEP$_4$-Ct receptor hybrid or the rEP$_3$hEP$_4$-IIIi receptor hybrid were preincubated for 1 h in incubation buffer. At different times prior to the end of the preincubation period a saturating concentration of the EP$_3$R agonist M&B 28767 (100 nM) was added. At the end of the preincubation period the agonist was removed by an acid wash and the inhibition of forskolin-induced cAMP formation by 5 nM M&B 28767 was determined as detailed above. In cells expressing either wild type EP$_{3\beta}$R or the rEP$_3$hEP$_4$-IIIi receptor hybrid pretreatment with

M&B28767 for different times did not affect the later M&B 28767-dependent inhibition of forskolin-induced cAMP formation (Fig. 3). Throughout the time course 5 nM M&B 28767 reduced forskolin-induced cAMP formation in control cells by between 4/5th and 9/10th (not shown). This was set at 100% inhibition. In cells expressing the rEP_3hEP_4-Ct receptor hybrid (Fig. 3) the inhibition of forskolin-stimulated cAMP formation was reduced almost to the minimum of 60% already after 2 min. The minimum was reached after 10 min of preincubation. With 1 h of preincubation desensitization was no longer observed (Fig. 3). Thus, the agonist-induced desensitization of the rEP_3hEP_4-Ct receptor hybrid was rapid and transient.

3.4. Modulation of the dose response curve by desensitization

In untreated cells expressing either wild type $EP_{3\beta}R$, the rEP_3hEP_4-Ct receptor hybrid or the rEP_3hEP_4-IIIi receptor hybrid M&B 28767 inhibited the forskolin-induced cAMP formation with an ED_{50} of 7×10^{-9} M, 10^{-9} M and 2×10^{-9} M, respectively. At saturating concentrations the forskolin-induced cAMP formation was reduced by 9/10th with all three receptors (Fig. 4). In cells expressing wild type $EP_{3\beta}R$ or the rEP_3hEP_4-IIIi receptor hybrid preincubation for 5 min with 100 nM M&B 28767 did not affect the ED_{50} or the maximal inhibition. By contrast, in cells expressing the rEP_3hEP_4-Ct receptor hybrid preincubation with M&B 28767 increased the ED_{50} by about half an order of magnitude. In addition, the maximal inhibition was attenuated: at saturating agonist concentrations forskolin-stimulated cAMP formation was reduced by only about 60%. Thus, the agonist-induced desensitization of the rEP_3hEP_4-Ct receptor hybrid appeared to reduce both receptor affinity and number of functionally coupled receptors.

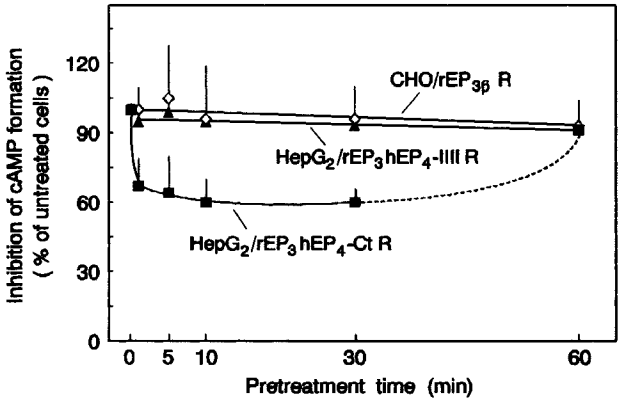

Fig. 3. Time dependence of desensitization of the EP_3hEP_4-Ct receptor hybrid. Cells were transfected stably as described in the legend to Fig. 1 either with the wild type pRc/CMV/$rEP_{3\beta}R$ or with one of the hybrid receptor pRc/CMV/rEP_3hEP_4-Ct or pRc/CMV/rEP_3hEP_4-IIIi constructs. In a preincubation period cells were exposed to a saturating concentration (100 nM) of the EP_3R agonist M&B 28767 for the time indicated. The agonist was then removed by an acid wash followed by extensive washing with incubation medium (see Section 2). cAMP formation was determined by radioimmunoassay in these cell cultures after stimulation for 10 min with 1 μM forskolin and 5 nM M&B 28767. Values are means ± S.E.M. percent of control cells tested in parallel that were not exposed to the agonist in the preincubation period ($n=4$).

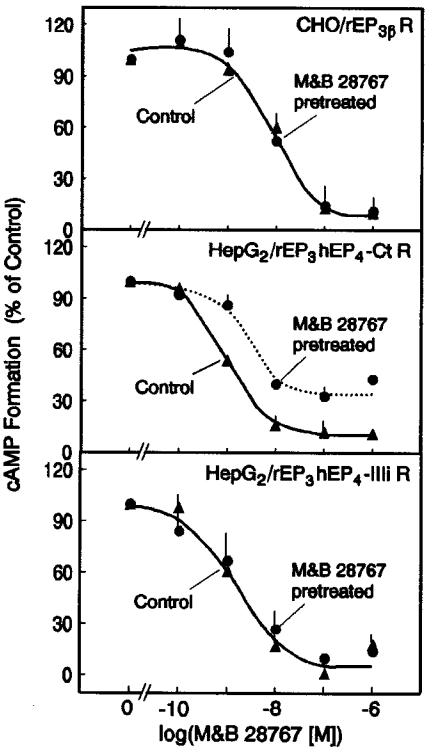

Fig. 4. Modulation by desensitization of the dose dependence of the M&B 28767-mediated inhibition of forskolin-induced cAMP formation by the EP_3hEP_4-Ct receptor hybrid. Cells were transfected stably as described in the legend to Fig. 1 either with the wild type pRc/CMV/$rEP_{3\beta}R$ or with one of the hybrid receptor pRc/CMV/rEP_3hEP_4-Ct or pRc/CMV/rEP_3hEP_4-IIIi constructs. Where indicated, cells were exposed to a saturating concentration (100 nM) of the EP_3R agonist M&B 28767 in a preincubation period of 5 min. The agonist was then removed by an acid wash followed by extensive washing with incubation medium (see Section 2). cAMP formation was determined by radioimmunoassay in cell cultures after stimulation for 10 min with 1 μM forskolin and the indicated concentration of M&B 28767. cAMP formation after forskolin stimulation was set at 100%. Values are means ± S.E.M. ($n=4$).

4. Discussion

4.1. G protein coupling specificity

Receptor domains involved in G protein coupling have been analyzed in several G protein-coupled receptors (GPCR). A unifying principle has not emerged. In different systems the second and third intracellular loops as well as the C-terminal domain have been shown to contribute to G protein coupling specificity. Thus, substitution of the third intracellular domain of the G_q-coupled muscarinic M1 receptor with the loop of the G_s-coupled β-adrenergic receptor led to an additional G_s coupling [14,15]. However, in 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 coupling but conferred no G_i coupling [16]. Four splice variants of bovine EP_3R, which differed only in their C-terminal sequence, coupled to different G proteins [17], implying that the C-terminal domain might contain sufficient information to determine G protein coupling specificity. However, seven C-terminal splice variants of human EP_3R [18], which are in part highly homologous to the sequence of the bovine EP_3R C-terminal domains, were all ex-

clusively coupled to G_i protein. In line with these latter results, the current study showed (Fig. 1) that neither the third intracellular loop nor the C-terminal domain of the G_s-coupled human EP_4R alone contained sufficient information to switch coupling specificity from G_i to G_s or to confer additional G_s coupling in the rEP_3hEP4-IIIi and rEP_3hEP_4-Ct receptor hybrids containing main portions of the G_i-coupled rat $EP_{3\beta}R$.

4.2. Site conferring agonist-induced desensitization of the rEP_3hEP_4-Ct receptor hybrid

Rapid agonist-induced desensitization is a common mechanism to interrupt the intracellular signal propagation of many GPCR, primarily those coupled to G_s but also those coupled to G_i and G_q [19]. It had previously been shown that of the two G_s-linked PGE_2Rs only EP_4R but not EP_2R underwent rapid agonist-induced desensitization [10]. The presence of potential phosphorylation sites in the large C-terminal domain and/or the third intracellular loop of EP_4R that are missing in EP_2R have been implicated in this process. The role of the C-terminal domain is underscored by the recent finding of a loss of desensitization in a C-terminally truncated EP_4R [11]. The current study showed that the C-terminal domain of the EP_4R is necessary and also sufficient to confer rapid agonist-induced desensitization in a hybrid receptor, while the third intracellular loop of the EP_4R was neither necessary nor sufficient to mediate agonist-induced desensitization (Fig. 2). This is in line with the results obtained with a chimeric β_3/β_2-adrenergic receptor, in which the C-terminal domain of the desensitizable β_2-adrenergic receptor conferred agonist-induced desensitization to the non-desensitizable β_3-adrenergic receptor [20].

4.3. Possible mechanism of agonist-induced desensitization of the rEP_3hEP_4-CtR

Agonist-induced receptor desensitization of GPCR has been shown to be mediated by receptor phosphorylation through either second messenger-dependent kinases or G protein-coupled receptor kinases (GRK).

4.3.1. PKA-mediated desensitization. Phosphorylation of PKA sites (RRXS) in the third intracellular loop and the C-terminal domain has been discussed as a possible mechanism of EP_4R desensitization [10]. From the current study a PKA-dependent phosphorylation as the only mechanism of receptor desensitization can be excluded, since the rEP_3hEP_4-Ct receptor hybrid did not mediate an agonist-dependent increase in cAMP formation (Fig. 1) and hence activation of protein kinase A.

4.3.2. PKC-mediated desensitization. In cells transfected with an epitope-tagged human IP receptor [21], iloprost increased cAMP and, at higher concentrations, $InsP_3$ formation. Receptor phosphorylation occurred only at iloprost concentrations that stimulated $InsP_3$ formation and was inhibited by PKC inhibitors but not by PKA inhibitors. Therefore, a PKC-dependent phosphorylation has been implicated in the desensitization and sequestration of the human prostacyclin receptor. The C-terminal domain of the EP_4R contains five potential phosphorylation sites for PKC (SXK/R). However, a PKC-dependent phosphorylation of rEP_3EP_4-CtR as the sole mechanism of desensitization seems to be unlikely since in transfected cells agonist exposure did not lead to an increase in $InsP_3$ formation (not shown) and thus, by inference, also not to DAG-dependent PKC activation.

4.3.3. GRK-mediated desensitization. The C-terminal domain of the EP_4R contains, in addition to potential PKA and PKC phosphorylation sites, a large number of potential phosphorylation sites for GRKs, which have no strict recognition sequence but seem to prefer Ser or Thr residues that are preceded by an Asp or Glu at a distance of 2–3 amino acids [21]. GRK-dependent phosphorylation of Ser and Thr residues in the C-terminal domain has been shown to mediate receptor desensitization of the β-adrenergic [22] receptor and of rhodopsin [23] and thus is a likely mechanism also for the agonist-induced desensitization of the rEP_3hEP_4-Ct receptor hybrid. While in most receptors phosphorylation of the C-terminal domain seems to mediate the agonist-induced desensitization that is independent of second messenger-activated kinases, a GRK-dependent phosphorylation of the third intracellular loop has been shown to mediate the agonist-induced desensitization of the α_2-adrenergic receptor [24]. Yet, the third intracellular loop of EP_4R can be excluded as the sole site mediating EP_4R desensitization since the third intracellular loop of EP_4R did not confer agonist-induced desensitization to the rEP_3hEP_4-IIIi receptor hybrid (Fig. 2).

4.4. Mode of desensitization

4.4.1. Modulation of affinity and maximal response. Rapid agonist-induced desensitization of the rEP_3hEP_4-Ct receptor hybrid reduced both its affinity towards the ligand and the maximal biological response (Fig. 4). This is similar to the desensitization of the G_s-coupled β_2-adrenergic receptor which was accompanied by both reduction of affinity and maximal response already after 2 min of agonist exposure [25] and the desensitization pattern of the parent EP_4R [10] whose C-terminus was incorporated into the receptor hybrid. However, rapid GRK-mediated desensitization of the G_i-coupled α_2-adrenergic receptor only decreased its affinity for the ligand and not the maximal response [24]. Similarly, brief exposure to PGE_2 reduced the ED_{50} but not the maximal response of the G_i-coupled $EP_{3\alpha}R$ [9]. Only after long-term exposure to their respective agonist (24 h) both receptors showed reduced maximal biological responsiveness [24,9]. Thus, rapid reduction of the maximal biological response may by typical of G_s-coupled receptors. This hypothesis is at variance with the mode of desensitization of a β_3/β_2-adrenergic receptor hybrid which showed only a reduced affinity but a normal maximal response in a cAMP assay after 30 min of agonist exposure [20]. However, the same paper reports a 20% reduction of maximal ligand binding after 15 min of agonist exposure. The discrepancy of full biological effect despite reduced maximal binding might be due to a large number of spare receptors in the receptor-overexpressing cells.

4.4.2. Resensitization. Surprisingly, the agonist-induced desensitization of the rEP_3hEP_4-Ct receptor hybrid was transient. This contrasts with the β_2-adrenergic receptor whose affinity and maximal response continued to decline over a period of 180 min of agonist exposure. This might reflect the fact that in the β-adrenergic receptor system GRKs and PKA can act in tandem [26]. The very early GRK-mediated desensitization was followed by a slower but sustained PKA-mediated desensitization. Since rEP_3hEP_4-CtR did not activate any second messenger system, this second phase of second messenger-activated kinase-dependent desensitization is missing in this system, explaining the transient nature of desensitization.

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References

[1] Negishi, M., Sugimoto, Y. and Ichikawa, A. (1995) J. Lipid Mediat. Cell Signal. 12, 379–391.
[2] Coleman, R.A., Smith, W.L. and Narumiya, S. (1994) Pharmacol. Rev. 46, 205–229.
[3] Ichikawa, A., Sugimoto, Y. and Negishi, M. (1996) J. Lipid Mediat. Cell Signal. 14, 83–87.
[4] Negishi, M., Harazono, A., Sugimoto, Y., Hazato, A., Kurozumi, S. and Ichikawa, A. (1995) Biochem. Biophys. Res. Commun. 212, 279–285.
[5] Audoly, L. and Breyer, R.M. (1997) Mol. Pharmacol. 51, 61–68.
[6] Irie, A., Sugimoto, Y., Namba, T., Asano, T., Ichikawa, A. and Negishi, M. (1994) Eur. J. Biochem. 224, 161–166.
[7] Hasegawa, H., Negishi, M. and Ichikawa, A. (1996) J. Biol. Chem. 271, 1857–1860.
[8] Neuschäfer-Rube, F., Hänecke, K., Blaschke, V., Jungermann, K. and Püschel, G.P. (1997) FEBS Lett. 401, 185–190.
[9] Negishi, M., Sugimoto, Y., Irie, A., Narumiya, S. and Ichikawa, A. (1993) J. Biol. Chem. 268, 9517–9521.
[10] Nishigaki, N., Negishi, M. and Ichikawa, A. (1996) Mol. Pharmacol. 50, 1031–1037.
[11] Bastepe, M. and Ashby, B. (1997) Mol. Pharmacol. 51, 343–349.
[12] Neuschäfer-Rube, F., DeVries, C., Hänecke, K., Jungermann, K. and Püschel, G.P. (1994) FEBS Lett. 351, 119–122.
[13] Higuchi, R. (1990) PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego, CA, pp. 177–183.
[14] Wong, S.K., Parker, E.M. and Ross, E.M. (1990) J. Biol. Chem. 265, 6219–6224.
[15] Van Leeuwen, D.H., Eisenstein, J., O'Malley, K. and MacKenzie, R.G. (1995) Mol. Pharmacol. 48, 344–351.
[16] Liggett, S.B., Caron, M.G., Lefkowitz, R.J. and Hnatowich, M. (1991) J. Biol. Chem. 266, 4816–4821.
[17] Negishi, M., Sugimoto, Y., Namba, T., Irie, A., Narumiya, S. and Ichikawa, A. (1995) Adv. Prostaglandin Thromboxane Leukotriene Res. 23, 255–257.
[18] Schmid, A., Thierauch, K.H., Schleuning, W.D. and Dinter, H. (1995) Eur. J. Biochem. 228, 23–30.
[19] Lohse, M.J. (1993) Biochim. Biophys. Acta 1179, 171–188.
[20] Ligget, S.B., Freedman, J.J., Schwinn, D.A. and Lefkowitz, R.J. (1993) Proc. Natl. Acad. Sci. USA 90, 3665–3669.
[21] Smyth, E.M., Nestor, P.V. and FitzGerald, G.A. (1996) J. Biol. Chem. 271, 33698–33704.
[22] Dohlman, H.G., Bouvier, M., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J. (1987) J. Biol. Chem. 262, 14282–14288.
[23] Thompson, P. and Findlay, J.B. (1984) Biochem. J. 220, 773–780.
[24] Liggett, S.B., Ostrowski, J., Chesnut, L.C., Kurose, H., Raymond, J.R., Caron, M.G. and Lefkowitz, R.J. (1992) J. Biol. Chem. 267, 4740–4746.
[25] Bouvier, M., Hausdorff, W.P., De-Blasi, A., O'Dowd, B.F., Kobilka, B.K., Caron, M.G. and Lefkowitz, R.J. (1988) Nature 333, 370–373.
[26] Freedman, N.J., Liggett, S.B., Drachman, D.E., Pei, G., Caron, M.G. and Lefkowitz, R.J. (1995) J. Biol. Chem. 270, 17953–17961.