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ABSTRACT


Prostaglandin E2 receptors (EP-Rs), like most prostanoid receptors, belong to the class of G protein-coupled ectoreceptors (GPCR) with seven transmembrane domains (Negishi, 1994). There are four subtypes of E-prostaglandin receptors (EP-Rs) that differ in their affinity to synthetic ligands and their G protein coupling specificity. EP1-Rs are linked to Gαq, EP2 and EP4 to Gαs, and EP3 to Gγ. The EP4-R, in contrast to the EP3β-R, shows rapid agonist-induced desensitization. The agonist-induced desensitization depends on the presence of the EP4-R carboxyl-terminal domain, which also confers desensitization in a Gq-coupled rEP3hEP4 carboxyl-terminal domain receptor hybrid (rEP3hEP4-Ct-R). To elucidate the possible mechanism of this desensitization, in vivo phosphorylation stimulated by activators of second messenger kinases, by prostaglandin E2, or by the EP3-R agonist M&B28767 was investigated in COS-7 cells expressing FLAG-epitope-tagged rat EP3β-R (rEP3β-R), hEP4-R, or rEP3hEP4-Ct-R. Stimulation of protein kinase C with phorbol-12-myristate-13-acetate led to a slight phosphorylation of the FLAG-rEP3β-R but to a strong phosphorylation of the FLAG-hEP4-R and the FLAG-rEP3hEP4-Ct-R, which was suppressed by the protein kinase A and protein kinase C inhibitor staurosporine. Prostaglandin E2 stimulated phosphorylation of the FLAG-hEP4-R in its carboxyl-terminal receptor domain. The EP3-R agonist M&B28767 induced a time- and dose-dependent phosphorylation of the FLAG-rEP3hEP4-Ct-R but not of the FLAG-rEP3β-R. Agonist-induced phosphorylation of the FLAG-hEP4-R and the FLAG-rEP3hEP4-Ct-R were not inhibited by staurosporine, which implies a role of G protein-coupled receptor kinases (GRKs) in agonist-induced receptor phosphorylation. Overexpression of GRKs in FLAG-rEP3hEP4-Ct-R-expressing COS-7 cells augmented the M&B28767-induced receptor phosphorylation and receptor sequestration. These findings indicate that phosphorylation of the carboxyl-terminal hEP4-R domain possibly by GRKs but not by second messenger kinases may be involved in rapid agonist-induced desensitization of the hEP4-R and the rEP3hEP4-Ct-R.

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ABBREVIATIONS: EP-R, E-prostaglandin receptor; GPCR, G protein-coupled receptors; InsP3, inositol trisphosphate; r, rat; h, human; PG, prostaglandin; PKA, cAMP-dependent protein kinase; PKC, Ca2+/phospholipid-dependent protein kinase; GRK, G protein-coupled receptor kinase; IBMX, 3-isobutyl-1-methylxanthine; PMA, phorbol-12-myristate-13-acetate; mAb, monoclonal antibody; PCR, polymerase chain reaction; bp, base pair; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PBS-T, PBS/Tween 20.
ity (Negishi, 1993a), and agonist-induced receptor desensitization (Negishi, 1993b; Nishigaki, 1996). Splice variants of the mouse or the bovine EP3-Rs that differ only in their carboxyl-terminal portions couple to different G proteins (Negishi, 1993a). Truncation of the murine EP3-R carboxyl-terminal domain leads to constitutive activation (Irie, 1994). Only one of the mouse EP3-R carboxyl-terminal splice variants, the EP3o-R, showed agonist-dependent desensitization (Negishi, 1993b). Similarly, of the two Gs-linked EP-Rs (EP2-R and EP4-R), only the EP4-R, which has a long serine- and threonine-rich carboxyl-terminal domain, shows rapid agonist-induced desensitization (Nishigaki, 1996). Agonist-induced desensitization but not G protein coupling was lost by truncation of the carboxyl-terminal domain of the human EP4-R (Bastepe and Ashby, 1997).

In a previous study, to elucidate the role of the EP-R carboxyl-terminal domain in G protein coupling and agonist-induced receptor desensitization, a receptor hybrid was generated consisting of the amino-terminal main portion of the Gs-coupled, nondesensitizable rat EP3β-R (rEP3β-R) up to the end of the seventh transmembrane domain and the carboxyl-terminal portion of the Gs-coupled, desensitizer-human EP4-R (hEP4-R) (Neuschafer-Rube, 1997a,b). This receptor hybrid retained the binding specificity of the EP3-R (i.e., it bound the EP3-R-specific agonist M&B28767 with higher affinity than prostaglandin (PG) E2 (PGE2). When stably expressed in HepG2 cells, the hybrid receptor exclusively coupled to Gs (Neuschafer-Rube, 1997a). The carboxyl-terminal domain of the EP4-R in the receptor hybrid was necessary and sufficient to confer agonist-induced receptor desensitization (Fig. 1) (Neuschafer-Rube, 1997b). The molecular events responsible for this effect are currently unknown. Rapid termination of signaling by GPCRs is typically initiated by receptor phosphorylation events catalyzed either by second messenger-activated kinases (such as cAMP-dependent protein kinase (PKA) or Ca2+/phospholipid-dependent protein kinase (PKC)) or by GPCR kinases (GRK) (Lohse, 1993; Premont, 1995). Currently, GRKs form a family of six such kinases (GRK1–6) that can phosphorylate serine and/or threonine residues in the carboxyl-terminal domains of ligand-occupied GPCRs (Premont, 1995).

To test the hypothesis that GRK-mediated phosphorylation of the hEP4-R carboxyl-terminal domain is responsible for receptor desensitization, hEP4-R (which may activate PKA), rEP3β-R, and the chimeric rEP3hEP4-Ct-R (Ct indicates the carboxyl-terminal domain), which do not activate second messenger-dependent kinases, were tagged with a FLAG-epitope and expressed in COS-7 cells. Receptor phosphorylation and sequestration promoted by specific agonists or second messenger kinase activators, such as phorbol-12-myristate-13-acetate (PMA) or forskolin, were then investigated.

The FLAG-hEP4-R and the FLAG-rEP3hEP4-Ct-R but not the FLAG-rEP3β-R were phosphorylated by agonist stimulation. The FLAG-rEP3hEP4-Ct-R phosphorylation was augmented by GRK overexpression. In addition, GRK overexpression enhanced agonist-induced FLAG-rEP3hEP4-Ct-R sequestration. These results indicate that GRK-mediated phosphorylation of the carboxyl-terminal domain of the hEP4-R in the FLAG-rEP3hEP4-Ct-R is possibly involved in the sequestration of this receptor.

### Experimental Procedures

#### Materials

All materials were of analytical grade and from commercial sources. M&B28767 was a generous gift from Rhone-Poulenc Rorer (Dagenham, United Kingdom). [3H]PGE2 was obtained from Amersham (Braunschweig, Germany); unlabeled PGE2, forskolin, 3-isobutyl-1-methylxanthine (IBMX), and PMA were purchased from Calbiochem-Novabiochem (Bad Soden, Germany). Cell culture media were obtained from Gibco-BRL (Eggenstein, Germany) and 32P from ICN (Meckenheim, Germany). Primers (Table 1) were synthesized by NAPS (Göttingen, Germany). Sepharose Cl-4B, protein-G Sepharose FF, glutathione agarose, and BrCN-activated Sepharose were purchased from Pharmacia (Freiburg, Germany). Trypsin (10,800 U/mg) and monoclonal antibody (mAb) factor FLAG-M2 were from Sigma Chemical Co. (Heidelberg, Germany). The sources of other materials are given in the text.


Cloning of the rEP3β-R (Neuschafer-Rube, 1994) and hEP4-R (Blaschke, 1996) cDNAs was carried out as described previously. The cDNA for the chimeric rEP3hEP4-Ct-R was constructed by recombinant polymerase chain reaction (PCR) technology. The protocol for the construction of the rEP3hEP4-Ct-R cDNA has been described in detail elsewhere (Neuschafer-Rube, 1997a). A FLAG-octapeptide sequence (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp)
Asp-Lys-C) recognized by the mAb FLAG-M2 was inserted after the
by PCR using Silver Star Taq-polymerase (Eurogentec, Seraing,
Belgium). The forward primers were FLAG-EP4 for the hEP4-R
cDNA and FLAG-EP3 for the two other cDNAs (Table 1) The reverse
primers were P2 for the rEP3β-R, P3 for the rEP3hEP4-Ct-R, and P4
for the hEP4-R (Table 1). All PCRs were performed with 10-ng
template (pcDNAI/EP3β-R, pcDNAII/EP4-Ct-R, and pcDNA/
AMP hEP4-R) and 35 cycles of the following temperature profile: 1
min at 95°C, 1 min at 60°C, and 2 min at 72°C. The resultant cDNA
fragments for the FLAG-rEP3β-R and the FLAG-rEP3hEP4-Ct-R
were cloned with the T/A cloning method into PUC57/T (MBI-Fermentas,
Vilnius, Lithuania) and verified by DNA sequencing. The
1100-base-pair (bp) NotI/FLAG-rEP3β-R fragment and the
1600-bp NotI FLAG-rEP3hEP-Ct-R fragment were further sub-
cloned in the eukaryotic expression vector pcDNAI (Invitrogen, de
Schelp, The Netherlands). The cDNA fragment for the FLAG-
hEP4-R was cleaved with HindIII and XbaI and cloned into pcDNAI/AMP.

Cell Culture and Transfection. COS-7 cells were cultured in
Dubucoc’s modified Eagle’s medium (DMEM) containing 10% (v/v)
fetal calf serum (FCS) and penicillin (100 units/ml) and streptomycin
(100 μg/ml) as antibiotics. COS-7 cells were seeded at a density of
1 × 10⁶ cells/10-cm-diameter plate and transiently transfected after
24 h using the DEAE-dextran method (Levesque, 1991) with pcD-
NAI/FLAG-rEP3β-R, pcDNA/AMP/FLAG-hEP4-R, or pcDNAI/
FLAG-rEP3hEP4-Ct-R (5 μg/plate) alone or together with pcDNAI or
cDNA expression vectors for GRK2, GRK3, GRK5, or GRK6 (5
μg/plate) (Oppermann, 1996a). Twelve hours after transfection, cells
were split into 6-well plates at a density of 2 × 10⁶ cells/well and
assays were performed 72 h after transfection.

Membrane Isolation and PGE₂ Binding Assay. For mem-
brane preparations, transfected cells were scraped into a homogeni-
ization buffer containing 25 mM Tris-HCl (pH 7.5), 250 mM sucrose,
10 mM MgCl₂, 1 mM EDTA and stored at −70°C. For PGE₂ binding,
membranes (20–50 μM PGE₂) were incubated with 5 nM
[^3H]PGE₂ in 100 μl of binding buffer for 1 h at 20°C. Nonspecific
binding was determined in the presence of 25 μM PGE₂. Bound
and unbound ligands were separated by rapid vacuum filtration
through GF 52 filters (Schleicher & Schuell, Dassel, Germany). Filters were
washed five times with 4 ml of ice-cold binding buffer. Radioactivity
retained on the filter was counted in 5 ml of Hydroluma (Baker,
Deventer, Netherlands). Binding constants were calculated by non-
linear regression analysis (LIGAND; Rovati, 1988).

Generation of a glutathione S-transferase (GST)/hEP4-R-Ct
Fusion Protein. A cDNA fragment encoding the carboxyl-terminal
domain of the hEP4-R from the end of the seventh transmembrane
domain was amplified by PCR using Silver Star Taq-polymerase.
The forward primer was EP4-Ct F (Table 1) and the reverse primer
was P4. PCR was performed with 10-ng template (pcDNAI/AMP/
FLAG-hEP4-R) and 35 cycles of the following temperature profile: 1
min at 95°C, 1 min at 60°C, and 2 min at 72°C. The resultant 521-bp
cDNA fragment was cloned blunt-end into PUC18 (Pharmacia) and
sequenced. The cDNA fragment was further subcloned into the
BamHI/EcoRI site of the prokaryotic expression vector pGEX-5X-3
(Pharmacia) to fuse the hEP4-R carboxyl-terminal domain to the
carboxyl terminus of the isopropyl-β-D-thiogalactopyranoside (IPTG)-
detectable GST that was encoded by the vector. Escherichia coli
strain BL-21 was transformed with this fusion plasmid. Synthe-
sis of the fusion protein was induced by 1 mM isopropyl-
β-D-thiogalactopyranoside for 5 h. The fusion protein was enriched from
crude cell extracts by glutathione-agarose affinity chromatography
according to the manufacturer’s instructions and purified to apparent ho-
mogeneity by preparative SDS-polyacrylamide gel electrophoresis
(PAGE) using the PrepCell 491 (Bio-Rad, Munich, Germany). The
purified GST-hEP4-R-Ct fusion protein was concentrated using cen-
tricon 10 (Amicon, Beverly, MA), dialyzed against PBS, and used as
antigen to raise polyclonal antibodies in rabbits.

<table>
<thead>
<tr>
<th>Receptor and Position</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>FLAG-EP3</td>
<td>5’-gcaagccgccaccctagacctcaagcggacgacgcaagGCCGGCGTGTTGGCGGCGGAGCAC</td>
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<td>5’-ggcgcggaagccctacacagacctcaagcggacgacgcaagTCCACTCCCCGGGTCATCGTCC</td>
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<tr>
<td>P2</td>
<td>5’-gggctcgctgctaggATATATCTCCTXGTGGGGAAGAAAT</td>
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<tr>
<td>P3</td>
<td>5’-tcgctgcgcctgctcAGGAGTTAAAGAGGTCACAAACAG</td>
</tr>
<tr>
<td>P4</td>
<td>5’-gggctcgctgctaggATATATCTCCTXGTGGGGAAGAAAT</td>
</tr>
<tr>
<td>EP4-Ct F</td>
<td>5’-AGAAAGACACGTGCTGTAACAAAGCAC</td>
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TABLE 1

Sequence and location of the PCR primers used to incorporate the FLAG-epitope into the rEP3β, hEP4-R and the hybrid rEP3hEP4-Ct receptors and to amplify the cDNA for the hEP4-R carboxyl-terminal domain. The locations given are the sequence positions in the data files retrieved from GenBank under the accession numbers indicated. The reverse primers are the sequences complementary to the indicated positions. Primers are shown in 5’ to 3’ direction.
Purification of Polyclonal Anti-hEP4-R-Ct IgG. Four weeks after the last immunization, the rabbit was bled. IgG was purified on protein G Sepharose FF-beads. IgGs were eluted with 50 mM glycine/HCl (pH 2.7) neutralized and dialyzed against PBS. Antibodies against GST were absorbed on GST-Sepharose, which was prepared by coupling recombinant GST to BrCN-activated Sepharose (10 mg of GST/ml of Sepharose).

Western Blotting. Membrane proteins (20–50 μg) of transfected or control cells were solubilized in Laemmli sample buffer under reducing or nonreducing conditions for 60 min at 37°C and 10 min at 60°C with vigorous shaking, separated on 10% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) by semidyblotting. The PVDF membrane was first blocked in 5% (w/v) skim milk in PBS, 0.1% (v/v) Tween 20 (PBS-T), and FLAG-tagged receptor proteins were detected by treating immunoblots overnight with 0.3 μg/ml rabbit anti-FLAG-R-Ct IgG in 1% (w/v) skim milk in PBS-T at 4°C followed by horseradish peroxidase-conjugated antirabbit IgG (1:20,000 dilution; Bio-Rad). FLAG-hEP4-R was also detected by overnight incubation with 0.5 μg/ml rabbit anti-hEP4-R-Ct IgG in 1% (w/v) skim milk in PBS-T at 4°C followed by horseradish peroxidase-conjugated antirabbit IgG (1:10,000 dilution; Amersham) for 60 min at room temperature. Anti-γ-antibody complexes were visualized with the enhanced chemiluminescence system (Amersham) according to the instructions of the company.

Intact Cell Phosphorylation. Transfected COS-7 cells in 6-well plates were washed two times with phosphate-free DMEM and prelabeled for 60 min with 150 μCi/ml [32P]orthophosphate in 500 μl of the same medium containing 10% (v/v) FCS that had been dialyzed extensively against 10 mM HEPES buffer (pH 7.5) containing 150 mM sodium chloride. After cell-labeling, various agents, in a volume of 500 μl in phosphate-free, 10% (v/v) dialyzed FCS containing DMEM, were added for 10 min at 37°C unless otherwise indicated. Where indicated, cells were treated with 400 nM staurosporine 20 min before stimulation. After stimulation, the medium was removed and the cells were washed twice with ice-cold PBS before immunoprecipitation.

Tryptic Receptor Cleavage in Intact Cells. Intact naive or PGE2-stimulated cells expressing FLAG-hEP4-R were treated for 5 min at 37°C with 400 nM staurosporine 20 min before receptor solubilization.

Immunoprecipitation. For immunoprecipitation, cells expressing FLAG-EP3β-R or the FLAG-rEP3hEP4-Ct-R were treated in lysis buffer (50 mM HEPES, pH 7.5, 5 mM EDTA, 10 mM sodium fluoride and 10 mM sodium pyrophosphate, with 0.2 mM Pefabloc SC), 10 μg/ml leupeptin, and 10 μg/ml soybean trypsin-inhibitor as protease inhibitors, transferred to microfuge tubes, and lysed by vigorous vortexing. Membranes were collected by centrifugation (10 min at 14,000g) and solubilized in 40 μl of lysis buffer containing 1% (w/v) SDS for 15 min at 65°C. After chilling to 4°C, 760 μl of concentrated detergent/salt solution was added to yield a final concentration of 1% (v/v) Triton X-100, 0.05% (w/v) SDS and 150 mM sodium chloride in lysis buffer (IP buffer). Cells expressing FLAG-hEP4-R were directly scraped in 800 μl of IP buffer and lysed by vigorous vortexing. Insoluble material was removed by centrifugation (30 min at 20,000g), and the supernatant was preclarified with 100 μl of 10% (v/v) Sepharose 4B in IP buffer containing 0.1% (w/v) bovine serum albumin for 60 min at 4°C. The preclarified supernatant was incubated for 2 h at 4°C with an immune complex of 15 μg of mAb FLAG-M2 or anti-hEP4-R-Ct IgG and 100 μl of 10% (v/v) protein-G Sepharose FF in IP buffer containing 0.1% (w/v) bovine serum albumin, which was performed by incubation for 60 min at 4°C. Immune complexes were collected by centrifugation and washed five times with ice-cold IP buffer. Samples were suspended in Laemmli sample buffer and prepared for SDS-PAGE as outlined above.

Proteins were either transferred to PVDF membrane and detected using biotin-labeled FLAG-M2 antibody and horseradish peroxidase-conjugated streptavidin (1:10,000 dilution; Jackson Immuno Research Laboratories, Inc., West Grove, PA) or gels were stained with Coomassie-blue and dried for PhosphorImager analysis if radioactive samples were loaded. To quantify receptor phosphorylation, the intensity of PhosphorImager bands observed was calculated with ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Sequestration Assay. For receptor sequestration studies, transfected cells were washed three times with 5 ml of HEPES buffer (pH 7.4) containing 140 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 1.2 mM KH2PO4, 11 mM glucose, and 15 mM HEPES (incubation buffer) and then preincubated for 10 min in the same buffer with or without 100 nM M&B28767. The agonist was removed by two washes with incubation buffer, an acid wash with 5 ml of 50 mM glycine and 150 mM NaCl (pH 3) for 1 min, and an additional two washes with incubation buffer. Cells were then detached from the tissue culture plates with 250 μl of ice-cold Ca2+-free incubation buffer containing 1 mM EDTA. Of this cell suspension, 50 μl were incubated in a total volume of 100 μl with 5 nM [3H]PGE2 for 30 min at 37°C. The reaction was stopped and cell-bound radioactivity was measured as outlined above in Membrane Isolation and PGE2 Binding Assay.

Results

Expression of FLAG-EP3β-R, FLAG-hEP4-R, and FLAG-rEP3hEP4-Ct-R in COS-7 cells. COS-7 cells were transiently transfected with the cDNAs for the FLAG-EP3β-R, FLAG-hEP4-R, and FLAG-rEP3hEP4-Ct-R cloned in the eukaryotic expression vectors pcDNAI or pcDNA/AMP. Competition binding studies with membrane preparations of transfected cells showed that all receptors had a similar affinity for PGE2 (FLAG-EP3β-R, Kd = 11 ± 1 nM; FLAG-hEP4-R, Kd = 5.9 ± 3 nM; FLAG-rEP3hEP4-Ct-R, Kd = 12 ± 4 nM; not shown). The Kd values were in the same range as those of the untagged wild-type receptors (Neušchäfer-Rube, 1994, 1997a), which indicates that the amino-terminal FLAG-tag did not alter the receptor binding properties. FLAG-EP3β-R and FLAG-rEP3hEP4-Ct-R were expressed to a comparable very high level (FLAG-EP3β-R, Bmax = 7.2 ± 0.3 pmol/mg of protein; FLAG-rEP3hEP4-Ct-R, Bmax = 5 ± 1 pmol/mg of protein) whereas FLAG-hEP4-R expression was somewhat lower (Bmax 0.9 ± 0.2 pmol/mg of protein).

Membrane proteins of transfected cells were separated by SDS-PAGE and blotted. Receptor proteins were detected with mAb FLAG M2. Under reducing conditions, the FLAG-EP3β-R protein appeared as four distinct bands with molecular masses of 80 kDa, 43 kDa, 41 kDa, and 39 kDa (Fig. 2A), whereas the protein mass of the receptor. This is supported by the observation that after receptor treatment with N-glycosidase F, only the 39-kDa band was visible (Boer, 1998). The 80-kDa band was assumed to represent a receptor aggregate, because aggregation of GPCRs is a frequently observed phenomenon. The FLAG-rEP3hEP4-Ct-R migrated as a single broader band with an apparent molecular mass around 55 kDa representing nonresolved di-, mono-, and nonglycosylated forms of the receptor. This is supported by the observation that after receptor treatment with N-glycosidase F, only the 39-kDa band was visible (Boer, 1998). The 80-kDa band was assumed to represent a receptor aggregate, because aggregation of GPCRs is a frequently observed phenomenon.
hEP4-R carboxyl-terminal domain. The FLAG-hEP4-R appeared as three broad bands with molecular masses of 110 kDa, 55 kDa, and 30 kDa. The 55-kDa band was assumed to represent the di-, mono-, and unglycosylated forms of the FLAG-hEP4-R protein, which has a predicted molecular weight of 52 kDa. This band was also detected with polyclonal anti-hEP4-R-Ct IgG, which did not stain membranes of untransfected cells (Fig. 2b). The 110-kDa band may represent a receptor aggregate as observed with the FLAG-rEP3-R. The 30-kDa band was supposed to represent an amino-terminal receptor fragment; this protein was only detected with the FLAG-M2 antibody but not with the anti-hEP4-R-Ct IgG (Fig. 2b).

SDS-PAGE mobility of FLAG-rEP3β-R and FLAG-rEP3hEP4-Ct-R under nonreducing conditions differed from electrophoresis under reducing conditions. Both receptors migrated as broad complexes with apparent molecular masses ranging from 100 to 150 kDa, which was not observed in nontransfected COS-7 cells and may represent receptor aggregates (Fig. 2A). By contrast, there was no difference in the migration profile of the FLAG-hEP4-R under reducing- or nonreducing conditions. Identical results for all three receptors were obtained by immunoprecipitation followed by detection of receptor proteins with a biotinylated FLAG-M2 antibody and peroxidase conjugated avidin (not shown). In most experiments, the FLAG-rEP3hEP4-Ct-R was difficult to detect after immunoprecipitation and separation under reducing conditions because it comigrated with the heavy-chain of the FLAG-M2 antibody, which masked the receptor protein during detection. Therefore, immunocomplexes with the FLAG-M2 antibody were routinely resolved under nonreducing conditions after in vivo phosphorylation and receptor immunoprecipitation.

Agonist-Induced Phosphorylation of the FLAG-hEP4-R and the FLAG-rEP3hEP4-Ct-R. To study agonist-induced phosphorylation of the FLAG-rEP3β-R, FLAG-hEP4-R, and FLAG-rEP3hEP4-Ct-R, COS-7 cells expressing FLAG-tagged receptors were labeled with 32P, and stimulated either with the EP3-R agonist M&B28767 (100 nM; FLAG-rEP3β-R and FLAG-rEP3hEP4-Ct-R) or PGE2 (1 μM; FLAG-hEP4-R) for 10 min. A phosphoprotein was immunoprecipitated with the FLAG-M2 antibody from solubilized proteins of cells transfected with the FLAG-rEP3hEP4-Ct-R (Fig. 3C) and FLAG-hEP4-R (Fig. 3E). These proteins comigrated with the FLAG-rEP3hEP4-Ct-R and the FLAG-hEP4-R detected by Western blotting. Phosphorylation of the FLAG-rEP3hEP4-Ct-R and the FLAG-hEP4-R were significantly enhanced upon agonist-stimulation (i.e., after pretreatment with M&B28767 or PGE2). In contrast, no phosphoprotein was immunoprecipitated from cells transfected with FLAG-rEP3β-R either with or without agonist-stimulation (Fig. 3A).

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Detection of FLAG-rEP3β-R, FLAG-rEP3hEP4-Ct-R, and FLAG-hEP4-R by immunoblotting. Membrane proteins (20–30 μg/lane) of control and transfected cells were resolved by SDS-PAGE under reducing or nonreducing conditions and transferred to PVDF membrane. Receptor proteins were detected with a sandwich of the mAb FLAG-M2 (A) or polyclonal anti-hEP4-R-Ct IgG (B) and horseradish-peroxidase-conjugated antimouse or antirabbit antiserum visualized with enhanced chemiluminescence as described in *Experimental Procedures*.

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Phosphorylation of the FLAG-rEP3β-R, FLAG-rEP3hEP4-Ct-R, and FLAG-hEP4-R by PGE2 or M&B28767 and activators and inhibitors of second-messenger-dependent kinases. 32P-labeled COS-7 cells expressing the FLAG-rEP3β-R, FLAG-rEP3hEP4-Ct-R, or the FLAG-hEP4-R were preincubated without or with 400 nM staurosporine for 20 min at 37°C before cellular stimulation. Cells were then stimulated for 10 min at 37°C with 1 μM PGE2, 100 nM M&B28767, 2 mM PMA, or 50 μM forskolin + 1 mM IBMX. Receptors were immunoprecipitated with FLAG-M2 antibody, resolved under nonreducing conditions by SDS-PAGE, and phosphorylation was determined by PhosphorImager analysis after 2 days. Top, a representative PhosphorImager analysis; bottom, the quantitative analysis from pooled data from three independent experiments. Receptor phosphorylation is expressed as a percentage of phosphorylation of untreated cells in absence of staurosporine. Data are means ± S.E.; *p < .05 compared with basal levels.
The M&B28767-induced phosphorylation of the FLAG-rEP3hEP4-Ct-R was dose-dependent and had an apparent EC₅₀ value between 1 and 10 nM and a maximum at 100 nM M&B28767 (Fig. 4). Phosphorylation of FLAG-rEP3hEP4-Ct-R thus occurred in an agonist-concentration range similar to the Kᵅ value of the receptor, revealing that only agonist-occupied receptor was a kinase substrate. FLAG-rEP3hEP4-Ct-R phosphorylation was rapid, starting at 1 min. It reached a maximum at 30 min and was sustained for at least 60 min in the presence of agonist (Fig. 5).

Because the FLAG-hEP4-R and the FLAG-rEP3hEP4-R proteins had only their carboxyl-terminal domains in common, it seems that the carboxyl-terminal domain conferred agonist-induced phosphorylation. To investigate whether agonist-induced receptor phosphorylation occurred in the carboxyl-terminal domain, FLAG-hEP4-R expressing COS-7 cells were stimulated with 1 µM PGE₂ for 10 min. Then intact cells were treated with trypsin to cleave the receptor exclusively in the extracellular loops. The third extracellular loop contains two potential cleavage sites for trypsin (R304 and K308) that allow to liberate the carboxyl-terminal domain attached to the seventh transmembrane domain. After protein solubilization, receptor and receptor fragments were immunoprecipitated with anti-hEP4-R-Ct IgG. Without trypsin treatment, anti-hEP4-R-Ct IgG immunoprecipitated a 55-kDa phosphoprotein from cells stimulated with PGE₂ that was absent in unstimulated cells (Fig. 6). After treatment of PGE₂-stimulated cells with trypsin, the 55-kDa phosphoprotein band was largely attenuated, whereas the majority of the immunoprecipitated radioactivity now appeared in a 20-kDa band; an additional weaker band at 33-kDa was also observed. The 20-kDa phosphoprotein most likely corresponded to a tryptic fragment ranging from the end of the third extracellular loop to the end of the carboxyl-terminal domain of the hEP4-R, which has a predicted molecular weight of 19 kDa. The 20-kDa phosphoprotein was not detected in nonstimulated cells. These results indicate that the PGE₂-induced phosphorylation of the FLAG-hEP4-R occurs largely in the carboxyl-terminal domain.

**Inhibition by Staurosporine of Second Messenger Kinase but Not Agonist-Induced Phosphorylation of the FLAG-rEP3hEP4-Ct-R and FLAG-hEP4-R.** To investigate which kinases effect agonist-induced FLAG-rEP3hEP4-Ct-R and FLAG-hEP4-R phosphorylation or are capable of phosphorylating the FLAG-rEP3β-R, cells were treated with agonist or with both specific activators and inhibitors of the second messenger-dependent kinases PKC or PKA. Forskolin, an activator that increases intracellular cAMP concentration and activates PKA, had no significant effect on either FLAG-rEP3β-R, FLAG-rEP3hEP4-Ct-R, or FLAG-hEP4-R phosphorylation (Fig. 3, A, C, and E). In contrast, the phorbol ester PMA, a potent PKC activator, led to a slight increase in FLAG-rEP3β-R phosphorylation (Fig. 3A) and induced a strong phosphorylation of the FLAG-rEP3hEP4-Ct-R and the FLAG-hEP4-R (Fig. 3, C and E). PMA induced FLAG-rEP3hEP4-Ct-R and FLAG-hEP4-R phosphorylation to a comparable extent as the agonists M&B28767 and PGE₂.

![Fig. 4](image-url) Concentration dependence of M&B28767-stimulated FLAG-rEP3hEP4-Ct-R phosphorylation. ³²P-labeled COS-7 cells expressing the FLAG-rEP3hEP4-Ct-R were stimulated for 10 min at 37°C with the concentrations of M&B28767 indicated. Receptor phosphorylation was analyzed as described in the legend to Fig. 3, untransfected COS-7 cells. Top, a representative PhosphorImager analysis. Bottom, the quantitative analysis from pooled data from three independent experiments. Receptor phosphorylation is expressed as a percentage of phosphorylation in unstimulated FLAG-rEP3hEP4-Ct-R cells. Data are means ± S.E.

![Fig. 5](image-url) Time-dependence of M&B28767-stimulated FLAG-rEP3hEP4-Ct-R phosphorylation. ³²P-labeled COS-7 cells expressing the FLAG-rEP3hEP4-Ct-R were stimulated for the times indicated at 37°C with 100 nM M&B28767. Receptors phosphorylation was analyzed as described in the legend to Fig. 3, untransfected COS-7 cells. Top, a representative PhosphorImager analysis. Bottom, the quantitative analysis from pooled data from three independent experiments. Receptor phosphorylation is expressed as a percentage of phosphorylation in unstimulated FLAG-rEP3hEP4-Ct-R cells. Data are means ± S.E.
Staurosporine at a concentration of 400 nM, which blocks PKA and PKC activity, inhibited PMA-induced FLAG-rEP3β-R, FLAG-rEP3hEP4-Ct-R, and FLAG-hEP4-R phosphorylation and decreased basal receptor phosphorylation (Fig. 3, B, D, and F). In contrast, staurosporine had no effect on M&B28767- or PGE2-induced phosphorylation, which indicates that neither PKC nor PKA are involved in agonist-induced FLAG-rEP3hEP4-Ct-R and FLAG-hEP4-R phosphorylation (Fig. 3, D and F).

Enhanced Agonist-Promoted Phosphorylation of the FLAG-rEP3hEP4-Ct-R by GRK Overexpression. As second messenger-activated kinases seemed not to be involved in agonist-induced FLAG-hEP4-R and FLAG-rEP3hEP4-Ct-R phosphorylation, it was assumed that the activated receptor serves as a substrate for GRK. To test this hypothesis, GRKs 2, 3, 5, and 6 were co-overexpressed with the FLAG-rEP3hEP4-Ct-R and agonist-induced receptor phosphorylation was determined. Western-blotting with antibodies specific for GRKs 2 or 3, or GRKs 5 or 6 (Oppermann, 1996a) revealed that COS-7 cells contained little intrinsic GRKs and that all GRKs were over-expressed to a comparably high level (not shown). GRK over-expression did not influence maximal [3H]PGE2 binding by the FLAG-rEP3hEP4-Ct-R (not shown). Overexpression of GRKs 2, 3, and 5 augmented basal and agonist-stimulated FLAG-rEP3hEP4-Ct-R phosphorylation by about 2-fold, whereas the ratio between basal and agonist-stimulated receptor phosphorylation remained constant (Fig. 7). GRK 6 was less effective. There was no significant difference in the capacity of GRKs 2, 3, and 5 to phosphorylate the receptor. These results show that under the conditions used in this cellular assay, the FLAG-rEP3hEP4-Ct-R seemed susceptible to agonist-promoted phosphorylation mediated by GRKs.

Enhanced Sequestration of the FLAG-rEP3hEP4-Ct-R by GRK Overexpression. To correlate GRK-mediated FLAG-rEP3hEP4-Ct-R phosphorylation with agonist-induced desensitization of the receptor previously observed in HepG2/rEP3hEP4-Ct-R cells (Neuschäfer-Rube, 1997b), GRK2 was coexpressed with the FLAG-rEP3hEP4-Ct-R or the nondesensitizable FLAG-EP3β-R as an internal control and sequestration of receptors as a response to agonist-exposure was examined. Receptor sequestration was measured as a loss of [3H]PGE2 binding sites on the plasma membrane of transfected COS-7 cells after a 10-min preincubation period with 100 nM M&B28767. In cells transfected with the FLAG-rEP3β-R, agonist exposure did not cause significant receptor sequestration either in the absence or in the presence of overexpressed GRK2 (Fig. 8). In contrast, preincubation of FLAG-rEP3hEP4-Ct-R-expressing cells with the agonist M&B28767 significantly decreased the number of [3H]PGE2 binding sites on the cell surface by about 15%. This agonist-induced receptor sequestration was augmented to about 30% when GRK2 was co-overexpressed with the FLAG-rEP3hEP4-Ct-R (Fig. 8). These results indicate that there may be a direct link between GRK-mediated phosphorylation and sequestration of the FLAG-rEP3hEP4-Ct-R induced by the EP3-R agonist M&B28767.

Discussion

The biological effects elicited by prostanoid receptors, like those of many other GPCRs, are regulated by an attenuation...
of their intracellular signal transduction in response to short- or long-term ligand exposure (Nordstedt, 1988; Sakai, 1996). Recent studies showed that the carboxyl-terminal domain of prostaglandin receptors plays an important role in this desensitization process. Variation of the EP3-R carboxyl-terminal domain created by alternative splicing resulted in different rapid agonist-induced receptor desensitization (Negishi, 1993b). The role of the carboxyl-terminal domain in desensitization is also underscored by the recent finding of a loss of desensitization in a carboxyl-terminally truncated EP4-R (Bastepe and Ashby, 1997). As previously shown, the carboxyl-terminal domain of the EP4-R is not only necessary but also sufficient to confer rapid agonist-induced desensitization in a hybrid receptor with the nondesensitizable rEP3-β-R (Fig. 1) (Neuschafer-Rube, 1997a). By contrast, the third intracellular loop of the EP4-R was neither necessary nor sufficient to mediate agonist-induced desensitization (Neuschafer-Rube, 1997a).

**Desensitization by Phosphorylation.** Agonist-induced receptor desensitization of GPCRs may be mediated by receptor phosphorylation. Several lines of evidence support this postulated link between agonist-stimulated desensitization and phosphorylation also for the hEP4-R and the rEP3hEP4-Ct-R. First, PGE2 induced both the desensitization of the hEP4-R (Bastepe, 1997) and phosphorylation of the FLAG-hEP4-R expressed in COS-7 cells (Fig. 3E). The EP3-R agonist M&B28767 did not induce desensitization of the rEP3-β-R, stably expressed in HepG2 cells (Neuschafer-Rube, 1997b), and was also inefficient in the induction of FLAG-rEP3-β-R phosphorylation (Fig. 3A). By contrast, the agonist stimulated both the desensitization of the rEP3hEP4-Ct-R in HepG2 cells (Neuschafer-Rube, 1997b) and phosphorylation of the FLAG-rEP3hEP4-Ct-R transiently expressed in COS-7 cells (Fig. 3C). Second, the time course of the M&B28767-induced FLAG-rEP3hEP4-Ct-R phosphorylation (Fig. 5) fit well with the kinetics of agonist-induced rEP3hEP4-Ct-R desensitization, measured as a reduced inhibition of forskolin-stimulated cAMP formation (Neuschafer-Rube, 1997b). Both effects were rapid, with an onset after 1 min and a maximum at 10 to 30 min.

Agonist-induced phosphorylation in GPCRs usually occurs in their carboxyl-terminal domains (Lohse, 1993). This is supported by studies with hybrid receptors, containing the carboxyl-terminal domain of the one and the remainder of another receptor. In a chimeric β3/β2-adrenergic receptor desensitization and phosphorylation after exposure to agonist was induced by the β2-adrenergic receptor carboxy-terminal domain (Liggett, 1993). The carboxyl-terminal tail of the thrombin receptor conferred rapid agonist-induced phosphorylation and desensitization in a chimeric 5-HT1/ thrombin receptor (Vouret-Craviari, 1995).

**Phosphorylation Sites.** Because the only structural difference between the rEP3-β-R and the rEP3hEP4-Ct-R is the carboxyl-terminal domain, agonist-induced phosphorylation in the latter receptor most likely occurred in the hEP4-R carboxyl-terminal domain. This assumption was supported by the finding that a tryptic fragment of the hEP4-R, which had an apparent molecular weight that corresponded to the predicted molecular mass of a tryptic fragment ranging from the third extracellular loop to the end of the carboxyl-terminal domain, was phosphorylated by PGE2-stimulation (Fig. 6). However, additional phosphorylation, which depends on the presence of the hEP4-R carboxyl-terminal domain, of other parts of the receptor (i.e., Ser or Thr residues in the first, second, or third intracellular loops), cannot be ruled out. rEP3-β-R and hEP4-R carboxyl-terminal domains contain different numbers of target sequences for protein kinases. The rEP3-β-R carboxyl-terminal domain contains only five serines, two of which are potential PKC-phosphorylation sites (consensus sequence S/TXR (Pearson and Kemp, 1991)) and no threonine. By contrast, the carboxyl-terminal domain of the hEP4-R contains 27 serine and 10 threonine residues, five of which are potential PKC-phosphorylation sites and nine of which might be defined as potential phosphorylation sites for GRKs; however, GRKs have no strict recognition sequence, but they seem to prefer Ser or Thr that are preceded by an Asp or Glu at a distance of three amino acids (Onorato, 1991; Fig. 1).

**Phosphorylation by GRKs.** Agonist-dependent receptor phosphorylation could be mediated either through second messenger-dependent kinases or GRKs. There are examples for both mechanisms. The human prostacyclin receptor, which increases cAMP-formation at low iloprost concentrations and InsP3-formation at high iloprost concentrations, was phosphorylated and desensitized by high iloprost concentrations only (Smyth, 1996). Phosphorylation and desensitization were inhibited by staurosporine, which implies a PKC-dependent mechanism. Conversely, GRK-dependent phosphorylation of Ser and Thr residues in the carboxyl-terminal domain has been shown to mediate receptor desensitization of the β1-adrenergic receptor (Freedman, 1995), the endothelin receptor (Freedman, 1997), the δ-opioid receptor (Pei, 1995), the thrombin receptor (Ishii, 1994), the type-1A angiotensin II receptor (Oppermann, 1996b), and the adenosine A3 receptor (Palmer, 1995), to name but a few. Several lines of evidence support the involvement of GRKs in...
agonist-stimulated hEP4-R and rEP3hEP4-Ct-R phosphorylation and desensitization: First, PKC- or PKA-dependent phosphorylation of the rEP3hEP4-Ct-R as the sole mechanism of desensitization seems to be unlikely, because in HepG2 cells stably transfected with the rEP3hEP4-Ct-R, which exclusively coupled to a Gs protein, agonist exposure increased neither cAMP nor InsP3, nor DAG formation (Neuschafer-Rube, 1997a,b) and thus, by inference, also did not activate PKA or PKC.

Second, PKA activation by forskolin, which led to a massive increase in cAMP formation in COS-7 cells (not shown), had no effect either on FLAG-rEP3hEP4-Ct-R phosphorylation. On the other hand, activation of PKC by a high dose (2 mM) of the phosphor ester PMA led to a slight (FLAG-rEP3hEP4-Ct-R or massive (FLAG-hEP4-R and FLAG-rEP3hEP4-Ct-R) receptor phosphorylation. On the other hand, the C-terminal domain of the Gs-coupled EP4 receptor confers agonist-dependent phosphorylation and desensitization of the human EP3 receptor (Tsuga, 1996, 1998).

In conclusion, the hEP4-R carboxyl-terminal domain in the FLAG-hEP4-R and the chimeric FLAG-rEP3hEP4-Ct-R seems to be a substrate for the PKC-inhibitor staurosporine, which, however, had no effect on the agonist-induced phosphorylation. Thus, in contrast to the regulation of the human prostacyclin receptor, PKC was apparently not involved in PGE2-regulation. Thus, in contrast to the regulation of the human prostacyclin receptor, PKC was apparently not involved in PGE2-regulation. A low-cost serum-free method compared to lipofection. Biotechniques 11:313–318.


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