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Molecular cloning and expression of a prostaglandin E_2 receptor of the $EP_{3\beta}$ subtype from rat hepatocytes

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Abstract

Rat hepatocytes have previously been reported to possess prostaglandin E_2 receptors of the EP_3-type (EP_3-receptors) that inhibit glucagon-stimulated glycogenolysis by decreasing cAMP. Here, the isolation of a functional $EP_{3\beta}$ receptor cDNA clone from a rat hepatocyte cDNA library is reported. This clone can be translated into a 362-amino-acid protein, that displays over 95% homology to the $EP_{3\beta}$ receptor from mouse mastocytoma. The amino- and carboxy-terminal region of the protein are least conserved. Transiently transfected HEK 293 cells expressed a single binding site for PGE_2 with an apparent K_d of 15 nM. $PGE_2 > PGF_{2\alpha} > PGD_2$ competed for [^{3}H]PGE_2 binding sites as did the EP_3 receptor agonists M&B 28767 = sulprostone > misoprostol but not the EP_1 receptor antagonist SC 19220. In stably transfected CHO cells M&B 28767 > sulprostone = PGE_2 > misoprostol > $PGF_{2\alpha}$ inhibited the forskolin-elicited cAMP formation. Thus, the characteristics of the $EP_{3\beta}$ receptor of rat hepatocytes closely resemble those of the $EP_{3\beta}$ receptor of mouse mastocytoma.

Key words: Prostaglandin receptor; Hepatocyte (rat); Molecular cloning and expression

1. Introduction

Hepatocyte carbohydrate metabolism has been shown to be modulated by prostaglandins in two opposing ways: In unstimulated rat hepatocytes prostaglandins $F_{2\alpha}$, E_2 and D_2 increased $InsP_3$ formation, glycogen phosphorylase activity and glucose output apparently via $PGF_{2\alpha}$ receptors (FP receptors) and PGE_2 receptors of the subtype 1 (EP_1 receptors) that are linked to phospholipase C by a G_o protein [1–5]. In contrast, in rat hepatocytes stimulated with glucagon PGE_2 and to a lesser extent $PGF_{2\alpha}$ and PGD_2 inhibited the glucagon-induced increase in cAMP formation, glycogen phosphorylase activity and glucose output. These latter effects were mediated via PGE_2 receptors of the subtype 3 (EP_3 receptors) that inhibit adenylate cyclase via a pertussis toxin sensitive G_i protein [3,6–8]. Rat hepatocytes may also contain PGE_2 receptors of the subtype 2 (EP_2 receptors) which activate adenylate cyclase via a G_s protein [3,6].

In the last three years structural information about prostanoid receptors, that had so far been charaterized only pharmacologically, has been gained by molecular cloning and expression [9,10]. The sequence data available on the human thromboxane A_2 receptor [11] and the mouse mastocytoma PGE_2 receptor (subtype EP_3) [12, 13] made it possible to characterize the rat hepatic prostaglandin receptors on a molecular level. Here, as a first hepatic prostaglandin receptor, the molecular cloning, expression and characterization of the rat hepatocyte PGE_2 receptor of the $EP_{3\beta}$ subtype is reported.

2. Materials and methods

2.1. Materials

All materials were of analytical grade and from commercial sources. M&B 28767, sulprostone, misoprostol and SC-19920 were generous gifts from Rhone-Poulenc Rorer (Dagenham, UK), Schering Pharmaceutical (Berlin, Germany) and Searle (Skokie, USA), respectively. [^{3}H]PGE_2 was obtained from Amersham (Braunschweig, Germany), unlabeled prostaglandins were purchased from Paesel (Frankfurt, Germany) or Cascade (Berkshire, UK). Geneticin (G-418 sulphate) was obtained from Gibco-BRL (Eggenstein, Germany) and forskolin was from ICN (Meckenheim, Germany). The sources of other materials are given in the text.

2.2. Hepatocyte purification

Hepatocytes were isolated from male Wistar rats (200–260 g) according to Meredith [14] without the use of collagenase by perfusion with Ca^{2+}-free Krebs–Henseleit buffer containing 2 mM EDTA as described previously [3]. The bulk of detritus and non-parenchymal cells were removed by repeated sedimentation of hepatocytes at $50 \times g$. Viable hepatocytes were further purified by centrifugation through a gradient containing 58% Percoll.

2.3. PCR-amplification of EP_3 receptor cDNA fragments

Total RNA was isolated from purified hepatocytes by CsCl gradient centrifugation [15]. Poly(A)$^+$ mRNA was prepared by affinity purification using oligo-(dT) beads from Quiagen (Rathingen, Germany) according to the manufacturer's instructions. First strand cDNA was synthesized by reverse transcription using oligo-$(dT)_{12-18}$ (Pharmacia,

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Abbreviations: CHO cells, chinese hamster ovary cells; DMEM, Dulbecco's modified eagle medium; FCS, fetal calf serum; HEK cells, human embryonal kidney cells; IBMX, 3-isobutyl-1-methylxanthine; PCR, polymerase chain reaction; PG, prostaglandin.

The sequence has the EMBL Data Library accession number: X80133 *R. norvegicus* mRNA for hepatocyte EP3 beta receptor.

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Freiburg, Germany) as a primer. PCR was carried out using 1–10 ng first strand cDNA as a template and oligonucleotide primers corresponding to position 651–680 and 1264–1293 of the mouse EP_3 sequence [13]. Thirty-five cycles of PCR were performed with the following temperature profile: 40 s 95°C, 40 s 60°C and 1.5 min 72°C. A 640 bp and a 730 bp fragment were amplified and cloned into PUC18 (Pharmacia). Nucleotide sequence analysis was carried out on double stranded templates using the dideoxy chain termination method [16]

2.4. EP_3 receptor cDNA cloning

Rat hepatocyte cDNA was prepared from hepatocyte poly(A)+ RNA by an oligo-(dT) priming method using a cDNA synthesis kit (Pharmacia) and inserted into the *Eco*RI site of λgt11 (Gibco-BRL) DNA with *Eco*RI adaptors including an internal *Not*I site (Pharmacia). The 10^6 clones derived from this library were screened by hybridisation with the cloned 640 and 730 bp EP_3 receptor probes labelled with digoxigenin by PCR replacing 5% of the dTTP by 11-digoxygenin-dUTP. Positive plaques were isolated and analysed with restriction digestion and PCR. The cDNAs of three clones were amplified by PCR using primers flanking the *Eco*RI cloning site, subcloned into PUC18 and sequenced.

2.5. Transient expression of the EP_3 receptor cDNA in HEK293 cells

The full-length 2.0-kb *Not*I cDNA fragment of one clone (clone 15/1) was subcloned into the eucaryotic expression vector pcDNA I (Invitrogen, San Diego, USA). The resultant plasmid was transfected into HEK293 cells by a calcium phosphate method using 5% (v/v) modified bovine serum from Stratagene (La Jolla, USA). Cells were cultured for 72 h in DMEM with 10% FCS and then 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 phenyl-methyl-sulfonyl-fluoride (PMSF). After homogenization of the cells in a dounce homogenizer a crude membrane fraction was prepared by centrifugation of the homogenate at 100,000 × *g*. The resulting pellet was suspended in binding buffer containing 25 mM Tris-HCl pH 6.2, 10 mM $MgCl_2$ and 1 mM EDTA and stored at −20°C.

2.6. PGE_2 binding assays with membranes of transfected HEK293 cells

For ligand binding membranes (20–50 μg protein) were incubated with 5 nM [^{3}H]PGE_2 and various concentrations of unlabelled prostaglandins, receptor agonists and antagonists in 100 μl binding buffer for 1 h at 20°C. Non-specific binding was determined in presence of 10 μM PGE_2. Bound and unbound ligand were separated by rapid vacuum filtration through GF 52 filters (Schleicher and Schüll, Dassel, Germany) [17]. Filters were washed with 4 ml ice-cold binding buffer. Radioactivity retained on the filter was counted in 5 ml Hydroluma (Baker, Deventer, NL). Binding constants were calculated by non-linear regression analysis (LIGAND [18]).

2.7. Stable expression of EP_3 receptor cDNA in CHO cells

The full-length 2.0-kb *Not*I cDNA fragment of clone 15/1 was subcloned into the eucaryotic expression vector pRc/CMV (Invitrogen). 20 μg of the resultant plasmid were linearized and transfected into 10^7 CHO cells by electroporation. Transfectants were isolated by growing the cells in HAM F-12 medium containing 10% (v/v) FCS and 1.2 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 and inhibition of forskolin-induced cAMP formation.

2.8. cAMP-formation in transfected CHO cells

CHO cells expressing the cDNA of clone 15/1 were cultured in 3.5 cm diameter plates to a density of 5×10^5 in HAM-F12 medium containing 10%(v/v) FCS. 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 prostaglandins, receptor agonists and forskolin (1 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.

3. Results and discussion

3.1. Sequence of the rat hepatocyte EP_3 receptor

Hepatocyte cDNA was used as template for PCR. The primer corresponded to position 651–680 and 1264–1293 of the published sequence of the mouse mastocytoma EP_3 receptor [13]. PCR yielded two products of about 640 and 730 bp (not shown) as described for mouse kidney, uterus, stomach and lung [13]. The PCR products were cloned into PUC 18 and partially sequenced. They showed high homology to the a and β splice variants of the mouse mastocytoma EP3 receptor, which contain 365 and 361 amino acids, respectively and differ only in their C-terminal peptide [13]. Both PCR products were labelled with digoxigenin and used to screen a rat hepatocyte cDNA library in λgt11. Three cDNA clones were isolated.

Clone 15/1 was subcloned into PUC 18 and sequenced in both directions. An open reading frame of 1086 bp

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                -102 AGCAGAGCCCCGGGCCCCGCCGTCCTCCAGCGCGCTCCGCTG   -61
CAACCCCGCAGCTGAGCCCAGAGGCTCCGGCCCTGTGCGCCCTACCGCGGCCCCGCCACT    -1

ATGGCCGGCGTGTGGGCGCCGGAGCACTCGGTTGAAGCGCACAGCAACCAGTCAAGTGCT    60
M  A  G  V  W  A  P  E  H  S  V  E  A  H  S  N  Q  S  S  A     20
                                          ♥
GCCGACGGCTGCGGCTCTGTGTCCGTGGCCTTCCCCATCACCATGATGGTCACTGGCTTC   120
A  D  G  C  G  S  V  S  V  A  F  P  I  T  M  M  V  T  G  F     40

GTGGGCAACGCGCTGGCCATGTTGCTTGTCGCCGCAGCTATAGACGCCGGGAGAGCAAA   180
V  G  N  A  L  A  M  L  L  V  S  R  S  Y  R  R  R  E  S  K     60
      I                                   ♦
CGCAAAAAGTCTTTCCTGCTGTGCATTGGCTGGCTGGCGCTCACCGACTTGGTGGGGCAG   240
R  K  K  S  F  L  L  C  I  G  W  L  A  L  T  D  L  V  G  Q     80
         ♦                                                II
CTCCTGACCAGTCCGGTGGTCATCCTCGTGTACCTGTCGCAGCGACGCTGGGAGCAACTC   300
L  L  T  S  P  V  V  I  L  V  Y  L  S  Q  R  R  W  E  Q  L    100

GACCCATCGGGGCGCCTGTGCACCTTCTTCGGGCTGACCATGACAGTGTTCGGACTGTCC   360
D  P  S  G  R  L  C  T  F  F  G  L  T  M  T  V  F  G  L  S    120
                                                   III
TCGCTCTTGGTGGCCAGCGCCATGGCCGTGGAGCGCGCCCTGGCTATCCGTGCGCCGCAC   420
S  L  L  V  A  S  A  M  A  V  E  R  A  L  A  I  R  A  P  H    140

TGGTATGCCAGCCACATGAAGACTCGCGCCACGCGCGCGGTACTGCTGGGTGTGTGGCTG   480
W  Y  A  S  H  M  K  T  R  A  T  R  A  V  L  L  G  V  W  L    160

TCTGTGCTCGCCTTCGCGCTGCTGCCTGTGCTGGGCGTGGGCCGCTACAGCGTGCAGTGG   540
S  V  L  A  F  A  L  L  P  V  L  G  V  G  R  Y  S  V  Q  W    180
      IV
CCCGGCACGTGGTGCTTCATCAGCACCGGGCCGGCGGGCAACGAGACGGACTCTGCGCGG   600
P  G  T  W  C  F  I  S  T  G  P  A  G  N  E  T  D  S  A  R    200
                                          ♥
GAGCCGGGCAGCGTGGCCTTTGCCTCCGCCTTCGCCTGTCTAGGCTTGCTGGCTCTGGTG   660
E  P  G  S  V  A  F  A  S  A  F  A  C  L  G  L  L  A  L  V    220
                                                      V
GTGACCTTTGCCTGCAACCTGGCGACCATCAAAGCCCTGGTGTCCCGCTGCCGGGCCAAA   720
V  T  F  A  C  N  L  A  T  I  K  A  L  V  S  R  C  R  A  K    240

GCCGCCGCCTCGCAGTCCAGCGCCCAGTGGGGCCGGATCACCACGGAGACGGCTATCCAG   780
A  A  A  S  Q  S  S  A  Q  W  G  R  I  T  T  E  T  A  I  Q    260

CTTATGGGGATCATGTGTGTACTGTCCGTCTGCTGGTCGCCGCTATTGATAATGATGCTG   840
L  M  G  I  M  C  V  L  S  V  C  W  S  P  L  L  I  M  M  L    280
                                    VI
AAAATGATCTTCAATCAGATGTCAGTAGAGCAATGCAAGACGCAGATGGGAAAGGAGAAG   900
K  M  I  F  N  Q  M  S  V  E  Q  C  K  T  Q  M  G  K  E  K    300

GAGTGCAATTCCTTCCTAATCGCCGTTCGCCTGGCTTCGCTGAACCAGATCTTGGATCCC   960
E  C  N  S  F  L  I  A  V  R  L  A  S  L  N  Q  I  L  D  P    320
                                                VII
TGGGTTTATCTGCTGCTAAGAAAGATCCTTCTTCGAAAGTTCTGCCAGATGATGAACAAC  1020
W  V  Y  L  L  L  R  K  I  L  L  R  K  F  C  Q  M  M  N  N    340

CTGAAGCGGAGTTTCATTGCAATACCTGCTTCCCTGAGTATGAGAATTTCTTCCCCCAGG  1080
L  K  R  S  F  I  A  I  P  A  S  L  S  M  R  I  S  S  P  R    360

GAAGGATAACTGAATCATTTTGGATTGTA                                  1109
E  G  *                                                        362
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Fig. 1. Nucleotide and deduced amino acid sequence of the rat hepatocyte $EP_{3\beta}$ receptor. Amino acids differing from the mouse mastocytoma $EP_{3\beta}$ receptor are printed in bold face, putative transmembrane regions are underlined and numbered with roman numerals. Potential phosphorylation sites for protein kinase A and *N*-glycosylation sites are marked by diamonds and hearts, respectively.

could be translated into a 362 amino acid protein (Fig. 1). The protein displayed >95% sequence homology with the mouse mastocytoma $EP_{3\beta}$ receptor [13]. While this work was in progress additional prostaglandin receptors have been cloned. The rat $EP_{3\beta}$ receptor showed 85% homology to the human [19] and 83% to the rabbit [20] kidney $EP_{3\alpha}$ receptor. Sequence homologies to mouse mastocytoma EP_1, EP_2, and mouse ovary FP receptors [21–23] were below 50%. Non-conservative exchanges were clustered in the N- and C-terminal domain.

The sequence-deduced molecular mass was 39628 Da. Hydrophobicity analysis indicated the existence of 7 membrane spanning domains and a hydrophobic C-terminus similar to the mouse $EP_{3\beta}$ receptor. Protein kinase A phosphorylation sites (Ser-59 and Ser-64) and *N*-glycosylation sites (Asn-16 and Asn-194) described for the mouse mastocytoma $EP_{3\beta}$ receptor and the $EP_{3\alpha}$ receptor from rat kidney [24] were preserved. In the middle of the cDNA (position 453–459) an additional triplet was located. Thereby a proline was replaced by an Arg and Ala which are not present in the mouse $EP_{3\alpha}$ and $EP_{3\beta}$ [13] receptor and the $EP_{3\alpha}$ receptor from rat kidney [24], yet were found in the $EP_{3\alpha}$ receptor from human [19] and rabbit [20] kidney. The C-terminal β-peptide of the mouse mastocytoma $EP_{3\beta}$ receptor contains 3 Ser and 1 Thr, that are 4 potential phosphorylation sites which have been proposed to play a role in the regulation of the signalling activity of ligand-occupied receptors [13]. Notably, all these potential phosphorylation sites in the C-terminal β peptide are conserved. Yet Thr-343 of the mouse mastocytoma β peptide is replaced by Ser-344 in the rat hepatocyte $EP_{3\beta}$ receptor. Futhermore, Gly-352 of the mouse mastocytoma $EP_{3\beta}$ receptor is replaced by a Ser-353 of the rat hepatocyte $EP_{3\beta}$ receptor. Thus, the rat $EP_{3\beta}$ receptor C-terminal peptide contains an additional potential phosphorylation site.

3.2. Ligand binding properties of the transiently expressed $EP_{3\beta}$ receptor

The cDNA of clone 15/1 was subcloned into pcDNA I and transiently expressed in HEK293 cells. Membranes of the cells transfected with pcDNA I 15/1, in contrast to membranes of mock transfected cells, expressed a unique PGE_2 binding site with an apparent K_d of 15 nM. About 0.6 pmol receptor per mg membrane protein were expressed. $PGF_{2\alpha}$ and PGD_2 competed with [^{3}H]PGE_2 for its binding site with about 100- and 1000-fold lower affinity, respectively (Fig. 2). The EP_3 receptor subtype agonists M&B 28767 and sulprostone had a slightly higher affinity for the PGE_2 binding site than had PGE_2 (Fig. 2), whereas the EP_3 receptor agonist misoprostol had a roughly 10-fold lower affinity. The EP_1 receptor ligand SC 19920 did not compete with [^{3}H]PGE_2. The ligand binding properties are similar to those described for the $EP_{3\beta}$ receptor of mouse mastocytoma [13] except

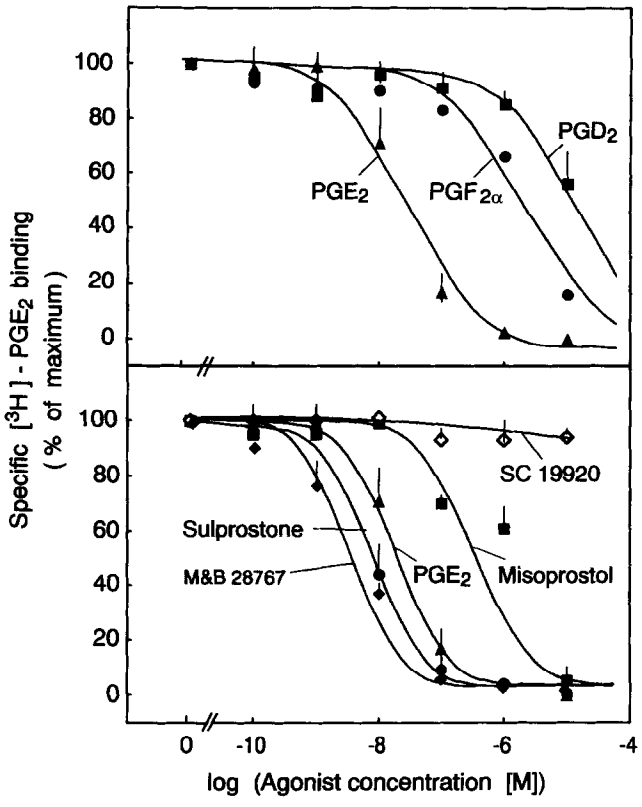

Fig. 2. Competition by PGE_2, $PGF_{2\alpha}$, PGD_2, M&B 28767, sulprostone, misoprostol and SC 19920 of [^{3}H]PGE_2 binding to membranes of pcDNA I 15/1 transfected HEK293 cells. HEK293 cells were transfected with the pcDNA I 15/1 construct. Binding of 5 nM [^{3}H]PGE_2 was measured after 1 h at 20°C in presence of the concentrations of unlabelled prostaglandins and their analogs indicated. MandB 28767, sulprostone and misoprostol are EP_3 receptor agonists, SC 19920 is an EP_1 receptor antagonist. [^{3}H]PGE_2 binding in presence of 10 μM PGE_2 was defined as unspecific binding.

that the apparent K_d for PGE_2 binding was about 5-fold higher.

3.3. Functional properties of the stably expressed EP_3 receptor

Clone 15/1 was subcloned into pRc/CMV and stably expressed in CHO cells. Expression of a PGE_2 binding site was about 10-fold lower than in HEK293 cells transiently transfected with pcDNA I 15/1 (not shown). In transfected CHO cells PGE_2 inhibited the forskolin-induced cAMP formation up to 60% (Fig. 3). About 10 nM PGE_2 caused a half-maximal inhibition. 50-fold higher concentrations of $PGF_{2\alpha}$ were needed for half-maximal inhibition. PGD_2, despite of its binding to the PGE_2 receptor, did not significantly reduce forskolin-induced cAMP formation in CHO cells. The EP_3 subtype agonists M&B 28767, sulprostone and misoprostol inhibited forskolin-induced cAMP formation with decreasing potency (Fig. 3). The concentrations of prostaglandins and their analogs that inhibited forskolin-stimulated cAMP formation half-maximally were in the same concentration range as needed for 50% competition of

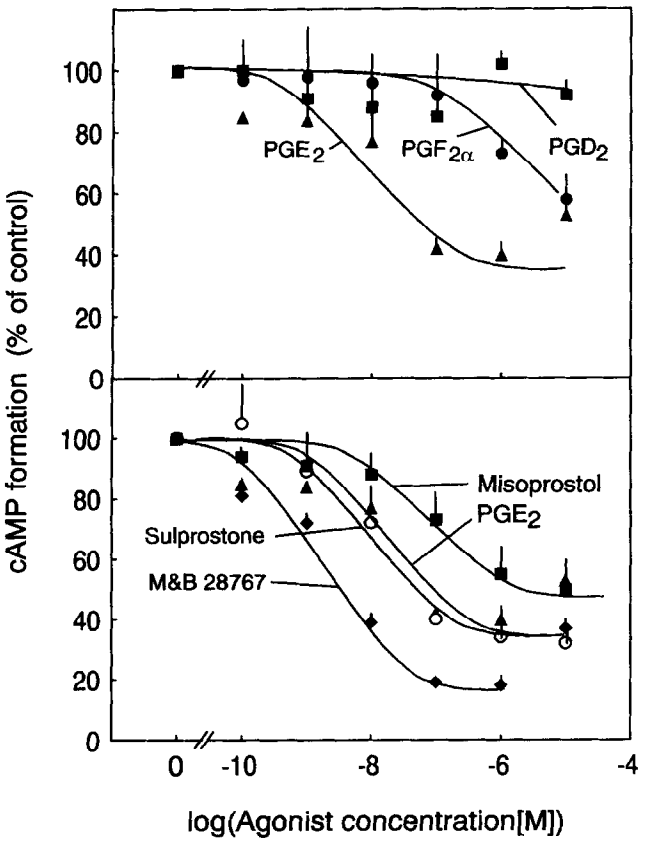

Fig. 3. Inhibition of forskolin-stimulated cAMP formation in pRc/CMV 15/1 transfected CHO cells by PGE_2, $PGF_{2\alpha}$, PGD_2, M&B 28767, sulprostone and misoprostol. CHO cells were stably transfected with the pRc/CMV 15/1 construct. cAMP formation induced by 1 μM forskolin after 10 min at 37°C in presence of the concentrations indicated of PGE_2, $PGF_{2\alpha}$, PGD_2 or the EP_3 receptor agonists M&B 28767, sulprostone and misoprostol was determined by radioimmuno assay. cAMP formation in absence of prostaglandins was set equal to 100%.

[^{3}H]PGE_2 binding. Mock transfected CHO cells did neither express a PGE_2 binding site nor was forskolin-stimulated cAMP formation inhibited by PGE_2.

The functional properties of the cloned rat hepatocyte $EP_{3\beta}$ receptor were very similar to those of the mouse mastocytoma $EP_{3\beta}$ receptor [13], yet differed slightly from the properties of the G_i coupled PGE_2 receptor in isolated rat hepatocytes [3]. In hepatocytes glucagon-induced cAMP formation was inhibited by PGE_2. For half-maximal inhibition of glucagon-induced cAMP formation in hepatocytes about 30-fold higher concentrations of PGE_2 were needed then for half-maximal inhibition of forskolin-induced cAMP formation in transfected CHO cells. Furthermore, in hepatocytes misoprostol and sulprostone had a roughly identical ED_{50} for the inhibition of glucagon-induced cAMP formation, whereas in transfected CHO-cells sulprostone was clearly more potent than misoprostol. This might be due to a different equipment with G_i proteins in hepatocytes and CHO cells. It might also be due to differences in the metabolism of prostaglandin E_2 and its analogs by hepatocytes and CHO cells. PGE_2 is very rapidly inactivated and degraded in hepatocyte cultures and suspensions by peroxysomal β-oxidation, ω-oxydation and oxidation of the C-15 OH group [25]. It is, therefore, not clear at present whether the newly cloned $EP_{3\beta}$-receptor from rat hepatocytes is involved in the inhibition of glucagon-induced cAMP formation, glycogen phosphorylase activation and glucose output from rat hepatocytes.

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