

Molecular cloning and expression of a prostaglandin E₂ receptor of the EP_{3β} subtype from rat hepatocytes

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Abstract

Rat hepatocytes have previously been reported to possess prostaglandin E₂ receptors of the EP₃-type (EP₃-receptors) that inhibit glucagon-stimulated glycogenolysis by decreasing cAMP. Here, the isolation of a functional EP_{3β} 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_{3β} 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 PGE₂ with an apparent K_d of 15 nM. PGE₂ > PGF_{2α} > PGD₂ competed for [³H]PGE₂ binding sites as did the EP₃ receptor agonists M&B 28767 = sulprostone > misoprostol but not the EP₁ receptor antagonist SC 19220. In stably transfected CHO cells M&B 28767 > sulprostone = PGE₂ > misoprostol > PGF_{2α} inhibited the forskolin-elicited cAMP formation. Thus, the characteristics of the EP_{3β} receptor of rat hepatocytes closely resemble those of the EP_{3β} 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_{2α}, E₂ and D₂ increased InsP₃ formation, glycogen phosphorylase activity and glucose output apparently via PGF_{2α} receptors (FP receptors) and PGE₂ receptors of the subtype 1 (EP₁ receptors) that are linked to phospholipase C by a G_o protein [1–5]. In contrast, in rat hepatocytes stimulated with glucagon PGE₂ and to a lesser extent PGF_{2α} and PGD₂ inhibited the glucagon-induced increase in cAMP formation, glycogen phosphorylase activity and glucose output. These latter effects were mediated via PGE₂ receptors of the subtype 3 (EP₃ receptors) that inhibit adenylate cyclase via a pertussis toxin sensitive G_i protein [3,6–8]. Rat hepatocytes may also contain PGE₂ receptors of the subtype 2 (EP₂ 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 characterized

only pharmacologically, has been gained by molecular cloning and expression [9,10]. The sequence data available on the human thromboxane A₂ receptor [11] and the mouse mastocytoma PGE₂ receptor (subtype EP₃) [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 PGE₂ receptor of the EP_{3β} 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. [³H]PGE₂ 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 Ca²⁺-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 × g. Viable hepatocytes were further purified by centrifugation through a gradient containing 58% Percoll.

2.3. PCR-amplification of EP₃ 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.

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 EP₃ 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. EP₃ 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 λ gt11 (Gibco-BRL) DNA with *Eco*RI adaptors including an internal *Not*I site (Pharmacia). The 10⁶ clones derived from this library were screened by hybridisation with the cloned 640 and 730 bp EP₃ receptor probes labelled with digoxigenin by PCR replacing 5% of the dTTP by 11-digoxigenin-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 EP₃ 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 MgCl₂, 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 MgCl₂ and 1 mM EDTA and stored at -20°C.

2.6. PGE₂ binding assays with membranes of transfected HEK293 cells

For ligand binding membranes (20–50 µg protein) were incubated with 5 nM [³H]PGE₂ 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 PGE₂. 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 EP₃ 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 10⁷ 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 PGE₂ 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 × 10⁵ 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 CaCl₂, 1.2 mM KH₂PO₄, 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 [¹²⁵I]cAMP assay kit of Amersham.

3. Results and discussion

3.1. Sequence of the rat hepatocyte EP₃ 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 EP₃ 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 α 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 λ gt11. 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

-102	AGCAGAGCCCCGGGCGCCGCTCTCCAGCGCGTCCCGTG	-61
CAACCCCGCAGCTGAGCCAGAGGCTCCGGCCCTGTGCGCCCTACCGCGCCCGCCACT		-1
ATGGCCGGCGGTGTGGCGCGGAGCACTCGGTTGAAGCGCACAGCAACCACTCAAGTGCT	60	
M A G V W A P E H S V E A H S N Q S S A	20	
GCCGACGGCTGCGGCTCTGTGTCGGTGGCTTCCCATCATCAGTGGTCACTGGCTTC	120	
A D G C G S V S V A F P I T M M V T G F	40	
GTTGGCAACGCGGTGGCCATGTTGCTGTGTCTCGGCGAGTATAGACGCGCGAGAGCAA	180	
V G N A L A M L L V S R S Y R R R E S K	60	
CGCAAAAGTCTTCTCTGCTGTCATGCTGGCTGGCGCTCACCGACTTGGTGGGCGAG	240	
R K K S F L L C I G W L A L T D L V G Q	80	
CTCTGACAGTCGGTGGTCTCTCTGCTGACCTGTCCAGCGACGCTGGGAGCAACTC	300	
L L T S P V V I L V Y L S Q R R W E Q L	100	
GACCCATCGGGCGCTGTGACCTTCTTCGGGCTGACCATGACAGTGTTCGGACTGTCC	360	
D P S G R L C T F F G L T M T V F G L S	120	
TGCTCTTGGTGGCGCGCATGGCGGTGAGCGCGCTGGCTATCGTGGCGCGAC	420	
S L L V A S A M A V E R A L A I R A P H	140	
TGGTATGGCCACATGAAGACTCGCGCCACCGCGCGTACTGCTGGTGTGGCTG	480	
W Y A S H M K T R A T R A V L S T G V G L	160	
TCTGTGCTCGCTTCCGCGTGTGCTGTGCTGGCGTGGCGCGTACAGCGTGCAGTGG	540	
S V L A F A L L P V L G V G R Y S V Q W	180	
CCCGGCACGTGCTCTCATCAGCACCGCGCGCGCGGCAACGAGACGACTCTGCGCGG	600	
P G T F W C F I S T G P A G N E T D S R	200	
GAGCGCGGCGCGTGGCTTTGCTTCCGCTTCCGCTTCTAGGCTTGTGCTGTGCTG	660	
E P G S V A F A S A F A C L G L L A L V	220	
GTCACCTTTGGCTGCAACCTGGCGACCATCAAGCCCTGGTGTCCGCTGCGCGGCAAA	720	
V T F A C N L A T I K A L V S R C R A K	240	
GCCGCGCGCTCGCAGTCCAGCGCCAGTGGGCGCGGATCACACGAGACGGTATCCAG	780	
A A A S Q S S A Q W G R I T T E T A I Q	260	
CTTATGGGATCATGTGTACTGCTCGCTGTGCTGGTGGCGCTATTGATAATGATGCTG	840	
L M G I M C V L S V C W S P L L I M M L	280	
AAAATGATCTCAATCAGATGTCTAGAGCAATGCAAGACGAGATCGGAAAGGAGAAG	900	
K M I F N Q M S V E Q C K T Q M G K E K	300	
GAGTGAATTCCTCTAATCGCGCTTCCGCTGGCTTCCGCTGAACAGATCTTGGATCCC	960	
E C N S F L I A V R L A S L N Q I L D P	320	
TGGGTTTATCTGCTGCTAAGAAAGATCTTCTTCAAGTTCTTGGCAGATGATGAACAC	1020	
W V Y L L L R K I L L R K F C Q M M N N	340	
CTGAAGCGGATTTTCATGCAATACCTGCTTCCCTGAGTATGAGAATTCTTCCCCCAGG	1080	
L K R S F I A I P A S L S M R I S S P R	360	
GAAGGATACTGAATCATTTTGATTGTA	1109	
E G *	362	

Fig. 1. Nucleotide and deduced amino acid sequence of the rat hepatocyte EP₃ receptor. Amino acids differing from the mouse mastocytoma EP₃ 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_{3β} receptor [13]. While this work was in progress additional prostaglandin receptors have been cloned. The rat EP_{3β} receptor showed 85% homology to the human [19] and 83% to the rabbit [20] kidney EP_{3α} receptor. Sequence homologies to mouse mastocytoma EP₁, EP₂, 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_{3β} 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_{3β} receptor and the EP_{3α} 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_{3α} and EP_{3β} [13] receptor and the EP_{3α} receptor from rat kidney [24], yet were found in the EP_{3α} receptor from human [19] and rabbit [20] kidney. The C-terminal β-peptide of the mouse mastocytoma EP_{3β} 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_{3β} receptor. Furthermore, Gly-352 of the mouse mastocytoma EP_{3β} receptor is replaced by a Ser-353 of the rat hepatocyte EP_{3β} receptor. Thus, the rat EP_{3β} receptor C-terminal peptide contains an additional potential phosphorylation site.

3.2. Ligand binding properties of the transiently expressed EP_{3β} 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 PGE₂ binding site with an apparent K_d of 15 nM. About 0.6 pmol receptor per mg membrane protein were expressed. PGF_{2α} and PGD₂ competed with [³H]PGE₂ for its binding site with about 100- and 1000-fold lower affinity, respectively (Fig. 2). The EP₃ receptor subtype agonists M&B 28767 and sulprostone had a slightly higher affinity for the PGE₂ binding site than had PGE₂ (Fig. 2), whereas the EP₃ receptor agonist misoprostol had a roughly 10-fold lower affinity. The EP₁ receptor ligand SC 19920 did not compete with [³H]PGE₂. The ligand binding properties are similar to those described for the EP_{3β} receptor of mouse mastocytoma [13] except

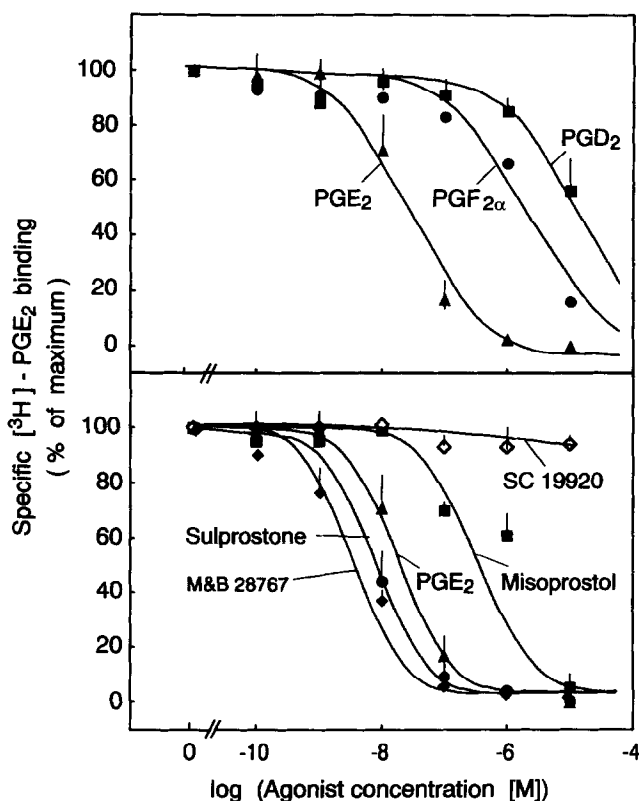


Fig. 2. Competition by PGE₂, PGF_{2α}, PGD₂, M&B 28767, sulprostone, misoprostol and SC 19920 of [³H]PGE₂ binding to membranes of pcDNA I 15/1 transfected HEK293 cells. HEK293 cells were transfected with the pcDNA I 15/1 construct. Binding of 5 nM [³H]PGE₂ was measured after 1 h at 20°C in presence of the concentrations of unlabelled prostaglandins and their analogs indicated. M&B 28767, sulprostone and misoprostol are EP₃ receptor agonists, SC 19920 is an EP₁ receptor antagonist. [³H]PGE₂ binding in presence of 10 μM PGE₂ was defined as unspecific binding.

that the apparent K_d for PGE₂ binding was about 5-fold higher.

3.3. Functional properties of the stably expressed EP₃ receptor

Clone 15/1 was subcloned into pRc/CMV and stably expressed in CHO cells. Expression of a PGE₂ binding site was about 10-fold lower than in HEK293 cells transiently transfected with pcDNA I 15/1 (not shown). In transfected CHO cells PGE₂ inhibited the forskolin-induced cAMP formation up to 60% (Fig. 3). About 10 nM PGE₂ caused a half-maximal inhibition. 50-fold higher concentrations of PGF_{2α} were needed for half-maximal inhibition. PGD₂, despite of its binding to the PGE₂ receptor, did not significantly reduce forskolin-induced cAMP formation in CHO cells. The EP₃ 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

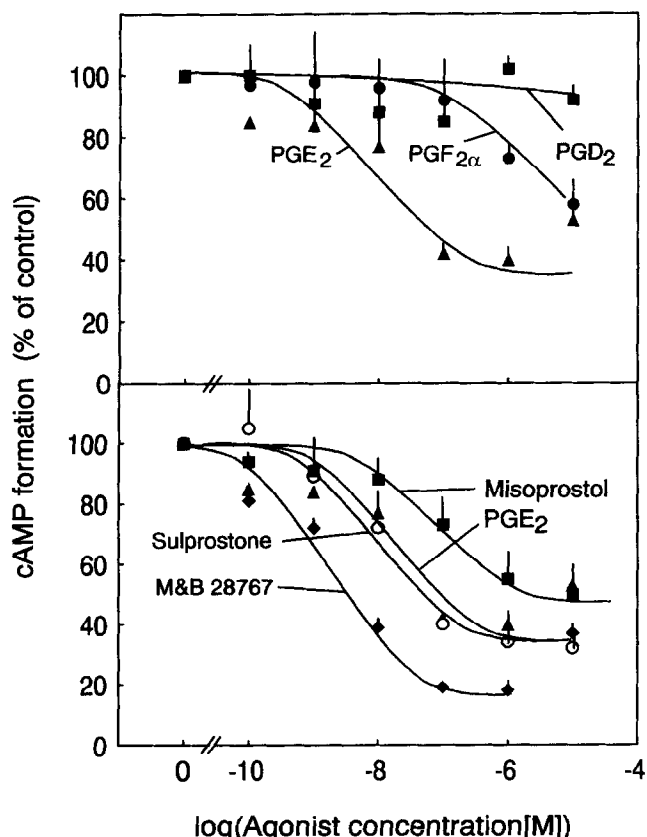


Fig. 3. Inhibition of forskolin-stimulated cAMP formation in pRc/CMV 15/1 transfected CHO cells by PGE₂, PGF_{2α}, PGD₂, M&B 28767, sulprostone and misoprostol. CHO cells were stably transfected with the pRc/CMV 15/1 construct. cAMP formation induced by 1 μM forskolin after 10 min at 37°C in presence of the concentrations indicated of PGE₂, PGF_{2α}, PGD₂ or the EP₃ receptor agonists M&B 28767, sulprostone and misoprostol was determined by radioimmuno assay. cAMP formation in absence of prostaglandins was set equal to 100%.

[³H]PGE₂ binding. Mock transfected CHO cells did neither express a PGE₂ binding site nor was forskolin-stimulated cAMP formation inhibited by PGE₂.

The functional properties of the cloned rat hepatocyte EP_{3β} receptor were very similar to those of the mouse mastocytoma EP_{3β} receptor [13], yet differed slightly from the properties of the G_i coupled PGE₂ receptor in isolated rat hepatocytes [3]. In hepatocytes glucagon-induced cAMP formation was inhibited by PGE₂. For half-maximal inhibition of glucagon-induced cAMP formation in hepatocytes about 30-fold higher concentrations of PGE₂ were needed than for half-maximal inhibition of forskolin-induced cAMP formation in transfected CHO cells. Furthermore, in hepatocytes misoprostol and sulprostone had a roughly identical ED₅₀ 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 E₂ and its analogs by hepatocytes and CHO cells. PGE₂ is very rapidly inactivated and

degraded in hepatocyte cultures and suspensions by peroxysomal β-oxidation, ω-oxidation and oxidation of the C-15 OH group [25]. It is, therefore, not clear at present whether the newly cloned EP_{3β}-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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