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Biochimica et Biophysica Acta 1509 (2000) 195–202

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Desensitization, surface expression, and glycosylation of a functional, epitope-tagged type I PACAP (PAC_1) receptor

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Received 15 February 2000; received in revised form 18 July 2000; accepted 20 July 2000

Abstract

To study desensitization and glycosylation of the type I pituitary adenylate cyclase-activating polypeptide (PACAP) receptor (PAC_1R), a hemagglutinin (HA) epitope was inserted within the N-terminal extracellular domain, allowing immunological detection of PAC_1R both in intact and permeabilized cells. PAC_1R was tagged without loss of functions in ligand binding and ligand-stimulated cAMP production. In transiently transfected COS-7 cells, PAC_1R was localized both in the plasma membrane and the cytoplasm around the nucleus. By immunoblot analysis, the immunoreactive bands with relative molecular masses ranging from 45 to 70 kDa were detected in the membrane fractions of PAC_1R-expressing COS-7 cells. Digestion of the membranes with endoglycosidase F or treatment of the cells with tunicamycin decreased the size of the receptor to major bands of smaller size (approximately 45 and 48 kDa), suggesting that these two forms of PAC_1R represent core proteins. Flow cytometric analysis indicated that the agonist promoted a disappearance of cell surface receptor. In accordance with this observation, preexposure of cells to PACAP38 induced a desensitization of PAC_1R to the agonist response, although it did not cause a reduction in PAC_1R mRNA or protein level and even slightly elevated them. These results suggest that agonist-induced desensitization of PAC_1R involves the receptor sequestration.

Keywords: PACAP receptor; Hemagglutinin epitope; Cell surface expression; Glycosylation; Desensitization; Internalization

1. Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) has an amino acid sequence identity of 68% with vasoactive intestinal polypeptide (VIP), indicating that it is a member of the VIP/secretin/glucagon/growth hormone-releasing hormone family. PACAP exists in two amidated forms, PACAP38 and PACAP27, sharing the same N-terminal 27 amino acids [1,2]. PACAP regulates a wide range of physiological functions in the central and peripheral nervous systems as a hypophysiotropic hormone, neurotransmitter, neuromodulator, and vasoregulator [1–3]. Molecular cloning studies have shown that these actions of PACAP are mediated by receptors encoded by at least three different genes. VIP_1

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0005-2736/00/$ – see front matter
PII: S0005-2736(00)00295-9

($VPAC_1$) and VIP_2 ($VPAC_2$) receptors binds VIP and PACAP with similar affinities, whereas PACAP (PAC_1) receptor specifically binds PACAP [4–6].

Previously, we cloned cDNA for PAC_1 receptor (PAC_1R) [5,7], isolated genes for PAC_1R, $VPAC_1$ receptor and the PACAP ligand and determined their structural organization [8–10]. In order to understand the functional meanings of PAC_1R, we have initially revealed its localization in the rat brain, adrenal gland and superior cervical ganglion by in situ hybridization histochemistry [11–13], and more recently, we have generated PAC_1R exon 2-deficient mice (submitted elsewhere).

Aimura and coworkers generated a polyclonal rabbit antibody directed against a 25-residue polypeptide corresponding to the C-terminal intracellular domain of the rat PAC_1R, which have proven invaluable in the detection and localization of the receptor protein [14]. At present, however, the successful production of antibody to the ectodomain of PAC_1R, which can detect the receptor protein on the intact cells, has not yet been reported. Previously, we tried to make antibodies in rabbits by immunizing with a fusion protein consisting of glutathione-*S*-transferase and the N-terminal extracellular domain of the rat PAC_1R which was produced in *Escherichia coli*. The resultant sera, however, contained very low-titer antibodies which reacted only poorly with PAC_1R expressed in mammalian cells, probably because of a high degree of glycosylation of the receptor protein (unpublished observation).

In the present study, we have generated hemagglutinin (HA) epitope-tagged PAC_1R (HA-PAC_1R), in which the epitope tag was placed in the N-terminal extracellular domain, to facilitate immunological detection of PAC_1R both in intact and permeabilized cells, and flow cytometric analysis, and studied subcellular localization, posttranslational processing (especially glycosylation), and desensitization of PAC_1R.

2. Materials and methods

2.1. Construction of the hemagglutinin (HA) epitope-tagged PAC_1R (HA-PAC_1R)

The HA-PAC_1R was created by converting the sequence WMTETIGDS (residues 70–78) within the N-terminal extracellular domain of the mouse PAC_1R to the 9-amino-acid HA epitope (sequence YPYDVPDYA) recognized by the monoclonal antibody 12CA5 [15]. The HA sequence was introduced into the mouse PAC_1R cDNA clone pCAPM17 [7] by oligonucleotide-directed mutagenesis using TAA GAC CAA GTC TAC CCC TAC GAT GTC CCC GAC TAC GCT GGC TTT GCT GAT AGT AAT TCC oligonucleotide. The mutated and adjacent regions were confirmed by restriction enzyme mapping and DNA sequencing analyses. The resultant plasmid, designated pHA-CAPM17, as well as pCAPM17 were transiently or stably expressed in COS-7 and Chinese hamster ovary (CHO-K1) cells, respectively.

2.2. Cells and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, and were transiently transfected with plasmids expressing pHA-CAPM17 or pCAPM17 by the DEAE–dextran method as described previously [5]. CHO-K1 cells were maintained in Ham's F12 medium containing 10% fetal calf serum. Stably transfected cells were established by cotransfection of plasmids expressing pHA-CAPM17 or pCAPM17 together with pSTneoB [16] by electroporation and selecting with 0.6 mg/ml G418. Clones were screened for expression of the recombinant receptors by PACAP38-stimulated cAMP production (both pHA-CAPM17 and pCAPM17) and immunofluorescent staining (pHA-CAPM17).

2.3. Radioligand binding and cAMP assays

Crude membrane fractions were prepared from the transiently transfected COS-7 cells 48 h after transfection essentially as described previously [5]. Binding of [^{125}I]PACAP27 to the membranes was assayed as described [5]. To determine the nonspecific binding, a large excess (1 μM) of unlabeled peptide was included in the assay mixture. The membranes from COS-7 cells transfected with the vector alone did not show any significant binding of [^{125}I]PACAP27. cAMP production in transfected COS-7 cells was

quantified with a [^{125}I]cAMP assay kit as described [17].

2.4. Immunoblot analysis of membrane fractions

Immunoblot analysis was performed as described [18] with several modifications. Briefly, crude membranes or cellular lysates prepared from the transfected cells were reduced with dithiothreitol, resolved by electrophoresis through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to Immobilon-P filters (Millipore); the filters were blocked with 5% nonfat dry milk in TBS-T and incubated with the first monoclonal antibody, 12CA5 [15], at a 1:1000 dilution followed by a peroxidase-labeled antimouse second antibody. Chemiluminescence was used for detection. Endoglycosidase F digestion of the membranes was performed according to the manufacturer's instruction (New England Biolabs), except that the reaction was performed at room temperature. Immunofluorescence study was performed using fluorescein (FITC)-labeled second antibody. Intact and NP40-permeabilized (0.2% NP40, 30 min at 37°C) cells were blocked with DMEM containing 10% FCS and 1% BSA, incubated with the first antibody at a 1:750 dilution followed by a FITC-labeled antimouse second antibody, and examined using confocal microscopy. Flow cytometric analysis was performed by FACS Calibrator (Molecular Dynamics) as described [19].

2.5. Northern blot analysis and reverse transcription–polymerase chain reaction (RT–PCR)

After exposure to agonist, total cellular RNAs were extracted from the transfected cells. As probes, a 990 bp *Pst*I DNA fragment of pCAPM17 [7] was used. Northern hybridization was carried out under high stringency essentially as described [5]. RT–PCR was performed as described [20]. For PCR of mouse PAC_1R mRNA, the following oligonucleotides were designed as primers: F10, 5′-AGC TGC ACT GTA CCC GTA AC-3′ (sense, corresponding to bases 692–711 [7]); R33, 5′-GCT GTG CTG TCA TTC ATA TCC-3′ (antisense, corresponding to bases 1044–1064 [7]). The PCR products were separated by 1.5% agarose gel electrophoresis and stained with fluorescent DNA binding dye, Vistra Green (Amersham Pharmacia Biotech), then their fluorescence was measured with fluorescent image analyser, FluorImager595 (Molecular Dynamics, Tokyo, Japan). The numbers of amplification and the amounts of cDNAs used for the reaction were optimized for quantitation of RNAs. The β-actin housekeeping

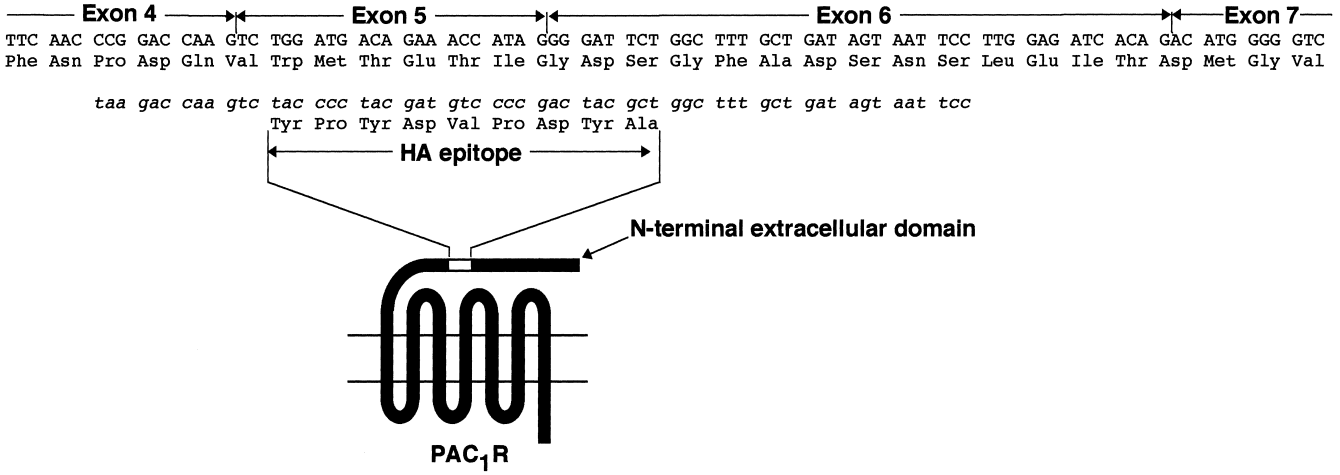

Fig. 1. Construction of hemagglutinin (HA) epitope-tagged PAC_1R (HA-PAC_1R). The nucleotide sequence and amino acid sequence of the mouse PAC_1R around exons 5 and 6 of the PAC_1R gene, and the PCR primer used for oligonucleotide-directed mutagenesis (italic letters) are shown. The HA-PAC_1R was created by converting the sequence WMTETIGDS (residues 70–78) within the N-terminal extracellular domain of the mouse PAC_1R to the 9-amino-acid HA epitope (sequence YPYDVPDYA) recognized by the monoclonal antibody 12CA5.

gene was simultaneously reverse transcribed and amplified as the internal reference standard.

3. Results

3.1. Functional properties of HA-PAC$_1$R

The mouse PAC$_1$R [7] was tagged with the HA antigen epitope at amino acids 70–78, a part of the 21-amino-acid sequence (amino acids 69–89) in the N-terminal extracellular domain, which has no homologous domain in any other member of the VIP/secretin/glucagon receptor family [5,7] and was encoded by exons 5 and 6 of the mouse PAC$_1$R gene [8] (Fig. 1). To study the functional properties of HA-PAC$_1$R, HA-PAC$_1$R cDNA (pHA-CAPM17) as well as PAC$_1$R cDNA (pCAPM17) was transfected into COS-7 cells and tested for the ability to bind PACAP and to activate cAMP production. As shown in Fig. 2, introduction of the HA nonapeptide sequence did not alter the binding property and the receptor activity, as compared with the wild-type mouse PAC$_1$R: HA-PAC$_1$R displayed similar high-affinity binding properties for PACAP38 (for HA-PAC$_1$R, $K_d = 0.92$ nM and for PAC$_1$R, $K_d = 0.60$ nM) and responded well to PACAP with increases in cAMP generation. As PAC$_1$R is also coupled to activation of phospholipase C, and then it increases the intracellular calcium concentration ($[Ca^{2+}]_i$) [1], we next examined PACAP-induced changes of $[Ca^{2+}]_i$ in CHO-K1 cells stably transfected with the wild-type PAC$_1$R or HA-PAC$_1$R by using single-cell calcium imaging [18]. PACAP38 evoked $[Ca^{2+}]_i$ rise through both receptors with pEC_{50} ($-\log EC_{50}$) of 7.6 (wild-type PAC$_1$R) and 7.8 (HA-PAC$_1$R) for the peak $[Ca^{2+}]_i$ response and with pEC_{50} of 7.6 (wild-type PAC$_1$R) and 7.7 (HA-PAC$_1$R) for the number of responsive cells. PACAP27 also evoked $[Ca^{2+}]_i$ rise with pEC_{50} of 7.8 (wild-type PAC$_1$R) and 7.0 (HA-PAC$_1$R) for the peak $[Ca^{2+}]_i$ response, while VIP had no effect on the calcium response.

3.2. Immunolocalization of HA-PAC$_1$R in transiently transfected COS-7 cells

Next, we studied the subcellular localization of HA-PAC$_1$R by immunofluorescence microscopy. As shown in Fig. 3a, nonpermeabilized COS-7 cells transfected with HA-PAC$_1$R showed intense staining

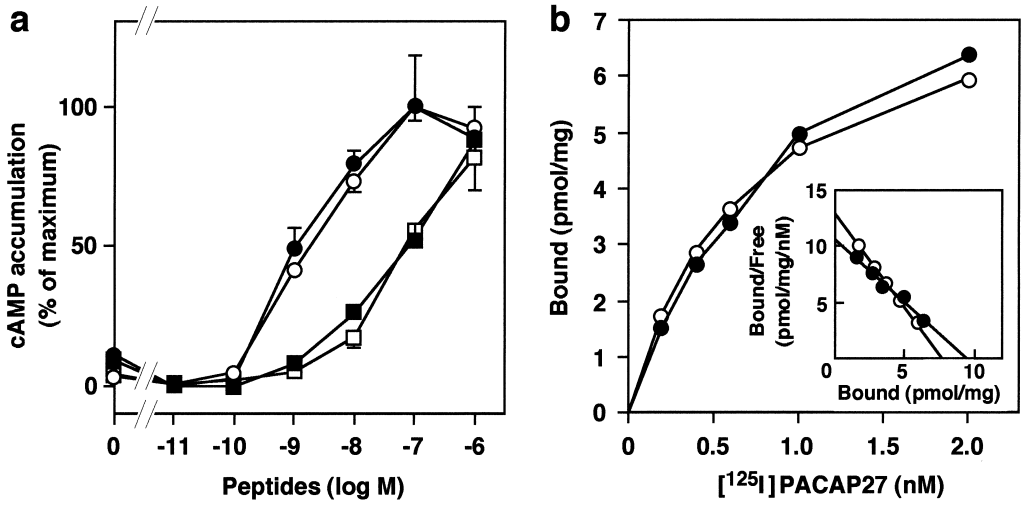

Fig. 2. Pharmacological characteristics of HA-PAC$_1$R. (a) Accumulation of intracellular cAMP in COS7 cells transfected with the HA-PAC$_1$R (filled symbols) or PAC$_1$R (open symbols) expression plasmid. The cells were incubated with various concentrations of PACAP38 (circles) and VIP (squares), and the cAMP accumulated in the cells was quantified. Values are expressed as mean ± standard error of triplicate experiments. The results shown are representative of three independent experiments. (b) [^{125}I]PACAP27 binding to membranes from COS-7 cells transfected with PAC$_1$R and HA-PAC$_1$R cDNAs. COS-7 cells were transfected with the HA-PAC$_1$R (closed circles) or PAC$_1$R (open circles) expression plasmid, and the membranes from the transfected cells were incubated with various concentrations of [^{125}I]PACAP27. Inset: Scatchard plot analysis of [^{125}I]PACAP27 binding. Values are expressed as mean of duplicate experiments. The results shown are representative of three independent experiments.

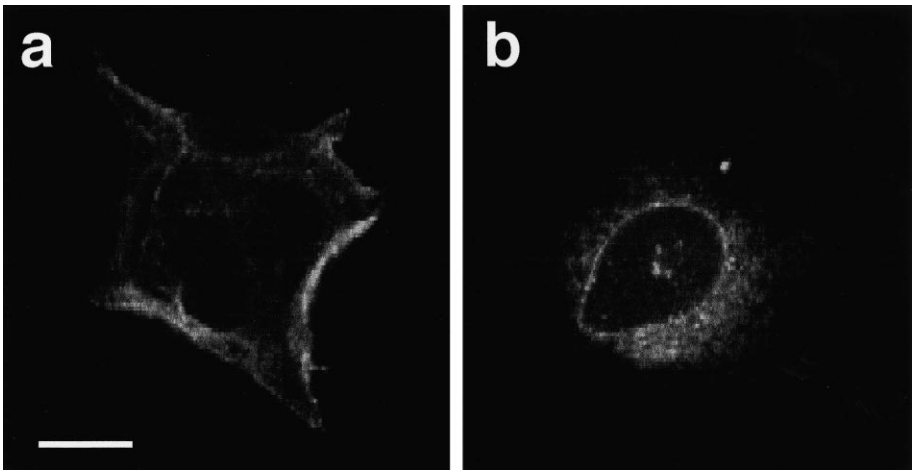

Fig. 3. Cell surface and perinuclear distribution of HA-PAC_1R studied by confocal immunofluorescence microscopy. COS-7 cells were transfected with the HA-PAC_1R expression plasmid. Immunofluorescence experiments were carried out with nonpermeabilized (a) or permeabilized (b) cells. Each panel is representative of six independent experiments. Bar = 25 μm.

of the plasma membrane, which was frequently observed to be both diffuse and clustered at the plasma membrane. Studies with permeabilized HA-PAC_1R-expressing cells revealed additional staining of intracellular membrane structures with strong staining of the nuclear perimeter (most likely the endoplasmic reticulum/Golgi complex) (Fig. 3b). This staining pattern of PAC_1R immunofluorescence was the same over a wide range of receptor expression in transiently transfected COS-7 cells. A similar pattern was also obtained in CHO-K1 cells stably transfected with HA-PAC_1R (data not shown).

3.3. Immunoblot analysis of HA-PAC_1R glycosylation

To examine the contribution of carbohydrate moieties to the apparent molecular mass of PAC_1R, we used an inhibitor of *N*-glycosylation, tunicamycin, as well as an endoglycosidase, endoglycosidase F/peptide-*N*-glycosidase F, and performed immunoblot analysis of HA-PAC_1R expressed in COS-7 cells. As shown in Fig. 4, the immunoreactive bands with relative molecular masses ranging from 45 to 70 kDa were detected in the crude membrane fractions of HA-PAC_1R-expressing COS-7 cells. A similar result was obtained in the plasma membrane fractions (data not shown). Treatment of the membranes with endoglycosidase F decreased the size of the receptor to major bands of smaller size (approximately 45 and 48 kDa) with a faint broad band of 50–55 kDa. Treatment of the cells with tunicamycin decreased the size of PAC_1R to two compact bands of 45 and 48 kDa, suggesting that these two forms of PAC_1R represent core proteins.

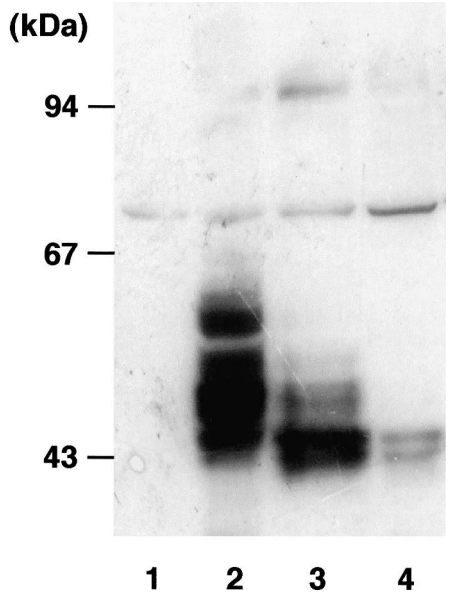

Fig. 4. Immunoblot analysis of HA-PAC_1R expressed in COS-7 cells. The crude membrane fractions of COS-7 cells transfected with the PAC_1R (lane 1) or HA-PAC_1R (lanes 2–4) expression plasmid were subjected to immunoblot analysis: lane 1, negative control; lane 2, nontreatment; lane 3, membranes treated with 0.5 units/μl endoglycosidase F; lane 4, membranes from the cells cultured in a medium containing 1 μg/ml tunicamycin for 40 h. The results shown are representative of three independent experiments.

3.4. Agonist-dependent desensitization and internalization of PAC_1R

It has been reported that prolonged incubation of CATH.a cells with PACAP attenuates agonist-stimulated cAMP accumulation [21]. To determine whether CHO cells transfected with PAC_1R desensitize in response to PACAP, PAC_1R-expressing CHO-K1 cells were pre-exposed to 100 nM PACAP38 and then subsequently challenged by the same concentration of PACAP. As shown in Fig. 5a, the cAMP response to PACAP38 added in the second incubation gradually declined and maximal desensitization was observed at 8 h (to about 40% of the initial response to PACAP38). A similar result was obtained in the cells pre-exposed to PACAP38 as low as 1 nM, and also in CHO cells stably transfected with HA-PAC_1R (data not shown). In accordance with this observation, flow cytometric analysis revealed that the agonist promoted a disappearance of cell surface receptor (Fig. 5b). However, PACAP38 exposure did not cause a reduction in PAC_1R mRNA or protein level and even slightly elevated them at 2–4 h (Fig. 5c). The results suggest that agonist-dependent desensitization of the cAMP response occurred via sequestration of PAC_1R away

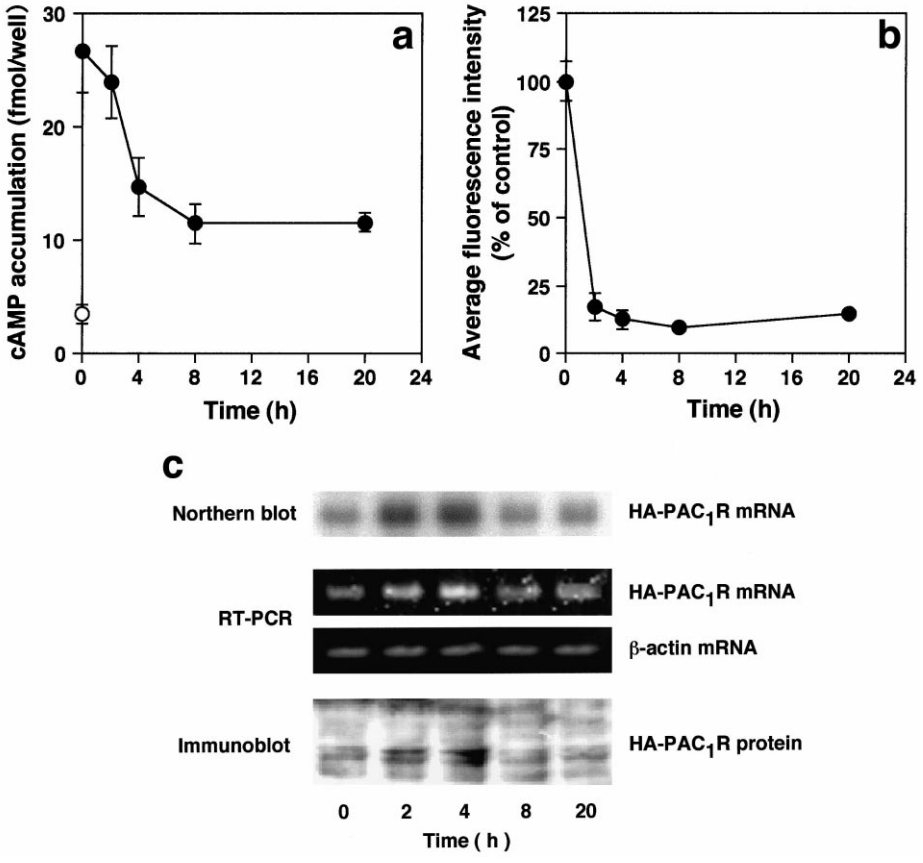

Fig. 5. Agonist-induced desensitization and internalization of HA-PAC_1R in stably transfected CHO-K1 cells. (a) CHO-K1 cells expressing PAC_1R were incubated for indicated time periods with 100 nM PACAP38, washed, and then the ability of a second PACAP38 (100 nM) exposure to increase intracellular cAMP levels (closed circles), and basal cAMP level (open circle) were assayed. (b) CHO-K1 cells expressing HA-PAC_1R were incubated for indicated time periods with 100 nM PACAP38, and the cell surface expression was assessed by average fluorescence intensity per fluorescence-positive and propidium iodide-negative cells, which was analyzed by flow cytometry. (c) HA-PAC_1R mRNA and protein levels. CHO-K1 cells expressing HA-PAC_1R were incubated for indicated time periods with 100 nM PACAP38. The cellular lysates (30 μg) were subjected to immunoblot analysis, and 10 μg and 5 ng of the total cellular RNA were subjected to Northern blot analysis and RT–PCR analysis, respectively, as described in Section 2.

from the cell surface rather than downregulation of the receptors.

4. Discussion

The HA-PAC_1R was created by converting the sequence within the 21 amino acid sequence in the N-terminal extracellular domain to HA epitope. Mutation of this region was selected for the following reasons: (1) a suitable antibody which can recognize the extracellular domain present on the cell surface was not available; (2) the 21-amino-acid sequence has no homologous domain in any other member of the VIP/secretin/glucagon receptor family, and is encoded by two alternative exons 5 and 6 of the mouse PAC_1R gene, which can be spliced out; (3) the naturally occurring splicing variant missing the 21 amino acids has almost normal receptor function (even slightly higher affinity for PACAP27 than the wild-type receptor) [22], suggesting that there is no amino acid residue essential for the receptor function in the 21-amino-acid sequence; and (4) the hydropathy profile of PAC_1R suggests that the 21-amino-acid sequence is hydrophilic and is, therefore, likely to be on the surface of the molecule. The rationale for choosing the HA epitope was that the Chou–Fasman [23] and Garnier–Robson [24] predictions both demonstrated only a small change in the secondary structure of the receptor protein when tagged with HA epitope. Fortunately, this approach was successful: introduction of the HA nonapeptide sequence did not alter the binding property and the receptor activity, as compared with the native PAC_1R.

In immunoblot studies, treatment with endoglycosidase F or tunicamycin decreased the size of HA-PAC_1R to compact bands of 45 and 48 kDa, suggesting that these two forms of HA-PAC_1R represent core proteins. These apparent molecular masses of 45 and 48 kDa are the same as or close to that reported for the human PAC_1R (48 kDa) purified from Sf9 insect cells, in which incomplete glycosylation seems occur [25]. However, these molecular masses are slightly lower than the expected mass (54.5 kDa) calculated from the amino acid sequence [7], suggesting that they may represent truncated receptor forms, although another possibility for proteolytic degradation of the receptor protein cannot be excluded.

Studies with permeabilized HA-PAC_1R-expressing cells revealed strong staining of the nuclear perimeter, most likely the endoplasmic reticulum/Golgi complex. The finding that this staining pattern was the same over a wide range of receptor expression in transiently transfected COS-7 cells, and in stably transfected CHO-K1 cells suggests that intracellular retention of PAC_1R is not simply the result of overexpression. In pheochromocytoma PC12 cells, which endogenously express PAC_1R [13], HA-PAC_1R was localized both in the plasma membrane and the cytoplasm (data not shown). Intracellular retention has also been reported for other G protein-coupled receptors, including the calcitonin receptor [26], parathyroid hormone (PTH)/PTH-related peptide receptor [27], and thyrotropin-releasing hormone receptor [28], although cell type-specific differences in receptor localization has been demonstrated in thyrotropin-releasing hormone receptor [28].

In desensitization studies, pretreatment of CHO-K1 cells expressing PAC_1R with PACAP38 caused a reduction of PACAP-stimulated cAMP accumulation and a disappearance of cell surface receptor in a parallel manner. These results showed for the first time that agonist-induced PAC_1R desensitization at least partly involves mechanisms underlying the sequestration of the receptors away from the cell surface. Although the receptor sequestration may not entirely explain the observed desensitization, downregulation of the receptor protein or mRNA levels is unlikely to be involved. Possible mechanisms of PAC_1R sequestration include clathrin-mediated endocytosis and a caveolin-dependent mechanism [29].

To investigate the desensitization of PAC_1R under natural physiological conditions, PACAP-induced desensitization of cAMP signaling was examined in rat cultured astrocytes, which endogenously express a relatively low level of PAC_1R ([5] and our unpublished observation). Pre-exposure of the cells to PACAP38 reduced the cAMP response, similarly to what was seen in CHO cells (data not shown), although we could not examine the sequestration of PAC_1R. A future study is necessary to investigate the sequestration of the native PAC_1R into the intracellular compartment by using an antibody recognizing the native PAC_1R.

In summary, PAC_1R was tagged with HA epitope at the N-terminal extracellular domain without loss of functions in ligand binding, cAMP production, and $[Ca^{2+}]_i$ elevation. By using HA-PAC_1R, we performed immunocytochemical, immunoblot, and flow cytometric analyses to study subcellular localization, posttranslational processing (especially glycosylation), and desensitization of PAC_1R. HA-PAC_1R was expressed both in the plasma membrane and the cytoplasm with relative molecular masses ranging from 45 to 70 kDa and core proteins being 45 and 48 kDa. Agonist-induced desensitization of PAC_1R-mediated cAMP accumulation paralleled a disappearance of cell surface receptor. This epitope-tagged PAC_1R will be valuable for further studies on the posttranslational processing, especially glycosylation, plasma membrane sorting, and sequestration/internalization of PAC_1R.

Acknowledgements

This research was supported, in part, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and by grants from Uehara Memorial Foundation and Takeda Science Foundation.

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