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Long-term-desensitization of prostacyclin receptors is independent of the C-terminal tail

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Abstract

Persistent stimulation of the G_s protein-coupled prostacyclin receptor (IP-R) causes its slow desensitization in a variety of cell types, a significant desensitization requiring several hours. To evaluate the role of the human IP-R C-terminus in desensitization and agonist-induced internalization, a C-terminally truncated hIP-receptor was generated. The C-terminal 68 amino acid residues were deleted by introduction of a stop codon for exchange of the original S319 codon (termed D318 mutant). Wild-type (WT) and truncated receptor were expressed in COS1 cells. Pretreatment of cells with the stable prostacyclin mimetic cicaprost (200 nM) desensitized cAMP production via WT and D318 receptors to similar extents. The cAMP response of WT and D318, respectively, was reduced by approximately 50% of maximal cAMP formation after 8 hr of continuous agonist stimulation, indicating significant long-term desensitization. Moreover, agonist-promoted sequestration of WT and D318 C-terminally tagged with green fluorescent protein was demonstrated, indicating that receptor internalization was not prevented by truncation of the C-terminus. These results demonstrated that long-term desensitization and sequestration of hIP-R did not depend on structures located in the hIP-R C-terminus.

Keywords: cAMP; COS1 cells; G protein-coupled receptor; Internalization; Prostacyclin; Receptor desensitization

1. Introduction

PGI_2, the major arachidonic acid metabolite produced in vascular cells, exerts its multiple effects mostly by increased generation of cAMP subsequent to its binding to a specific IP-receptor (IP-R), which belongs to the family of GPCRs [1,2]. PGI_2 and several mimetics have been used for a number of clinical indications including pulmonary hypertension, peripheral arterial occlusive disease and others [3,4]. Although short-term administration of PGI_2 induced beneficial effects, they were less pronounced after more chronic use. These findings may be explained by a time-dependently reduced IP-R responsiveness to repeated agonist challenge, referred to as receptor desensitization.

Signal termination after repeated agonist challenge is a commonly observed phenomenon among GPCRs. The archetypical model for GPCR regulation involves a rapid phase of desensitization occurring within seconds to minutes after exposure to the agonist, due to agonist-induced receptor phosphorylation mediated by second messenger kinases or receptor kinases (GRKs), uncoupling the receptor from its G protein [5]. This event, referred to as short-term desensitization, is followed by sequestration of the receptor away from the cell surface [6,7]. In contrast to most GPCR, IP-R reveals a much slower time course, significant desensitization requiring 3–10 hr [8–10]. However, the mechanisms involved in this long-term attenuation of GPCR signal transduction are still incompletely understood. Recently, a number of studies focussing on GPCRs have examined the role of the C-terminus in agonist-induced internalization and desensitization, with varying results. C-terminal truncation of

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Abbreviations: AC, adenylyl cyclase; β-AR, beta-adrenergic receptor; EGFP, enhanced green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; IBMX, 3-isobutyl-1-methyl-xanthine; IP-R, prostacyclin receptor; PGI_2, prostacyclin.

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β_2-ARs impaired its rapid desensitization, but a slow agonist-induced loss of β_2-receptor activity was shown to be independent of the receptor C-terminal tail [11]. The present study was designed to analyze the relevance of the IP-R C-terminus in receptor long-term desensitization. Herein, we present evidence that the C-terminal tail of hIP-R is dispensable for receptor long-term desensitization and sequestration.

2. Materials and methods

2.1. Plasmids and site-directed mutagenesis

Complete hIP-R cDNA was kindly provided by Dr. M. Abramovitz (Merck Frosst Canada Inc.). To permit selection in mammalian cells, a 1.4 kb *Eco*RI fragment, containing the entire human IP-R coding sequence, was cloned into the *Eco*RI site of pCDNA3.1Amp (Invitrogen). The C-terminally truncated mutant was generated by introduction of a stop codon in the region coding for the intracellular tail domain using a QuikChange kit (Stratagene). The primers used were 5′-GCCCACGGAGACTGACAGACACCCCT-TTCC-3′ (sense) and 5′-GGAAAGGGGTGTCTGTCAG-TCTCCGTGGGC-3′ (antisense) for exchange of the S319 codon for a stop codon (mutant D318). Successful mutation was verified by sequencing. C-terminally EGFP-tagged versions of full-length IP-R and of the mutant D318 were generated by PCR-based replacement of the stop codon by a *Bam*HI site for the full-length coding region and by introduction of an *Apa*I site after the codon for amino acid 318. The resulting PCR products were introduced into pEGFP-N3 (Clontech).

2.2. Transfection and cell culture

COS1 cells were maintained in DMEM with Glutamax-I (Gibco-BRL Life Technologies) containing 15% fetal calf serum, penicillin (100 U/mL) and streptomycin (0.1 mg/mL). Transfection was performed by the calcium phosphate coprecipitation method following standard protocols. Selection of transfectants was done by growing cultures for 3 weeks in medium containing 500 μg/mL G418 (Calbiochem). For all experiments, subconfluent monolayers were grown in the presence of 3 μM indomethacin for 16 hr prior to incubation with agonists and inhibitors, respectively, to avoid desensitization of hIP-R by endogenously synthesized PGI_2. Preincubation with the selective IP-R agonist cicaprost (ZK96480) was performed prior to assaying as indicated in the results.

2.3. Determination of intracellular cAMP

Cells were grown to subconfluency in 6-well-plates and pretreated according to the experimental protocol. Growth medium was removed and monolayers were washed twice with Hank's buffered sodium saline (HBSS) containing 1 mM $CaCl_2$, 5.5 mM glucose, 1 g/L bovine serum albumin and 10 mM Hepes, pH 7.6. After incubation for 10 min with HBSS containing 1 mM IBMX from Sigma Chemical Co., cAMP formation was stimulated by addition of cicaprost (ZK96480), which was a gift from Schering AG. Agonists were removed by aspiration and the reaction was stopped with 750 μL 96% ethanol/well (4°), followed by evaporation and rehydratation in 1 mL/well 50 mM Tris–HCl, 4 mM EDTA, pH 7.5. Intracellular cAMP was released by freeze–thawing (−80°) and determined by a conventional radioimmuno assay. Protein content of extracts were determined by Bio-Rad assay (Bio-Rad Laboratories).

2.4. Confocal microscopy

Confocal microscopy was performed on a Leica DM IRB/E (inverse), DM TCS SP (confocal) laser scanning microscope using 40 × 1.3 or 63 × 1.4 numerical aperture oil immersion lenses. Cells expressing EGFP-tagged IP-R or mutant D318 were agonist-treated as indicated and fixed by 3% *para*-formaldehyde in PBS for 20 min.

2.5. Data analysis

Data were compared by Student's *t*-test, followed by Bonferroni's test for multiple comparisons. A P value of <0.05 was considered significant.

3. Results and discussion

3.1. Long-term desensitization of hIP-R in COS1 cells

COS1 cells, stably expressing human IP-R (hIP-RCOS1), were assayed for cAMP generation upon treatment with the selective IP-R agonist cicaprost. Mock-transfected cells were used as a control. Basal cAMP values were similar for both cell lines with 4.7 ± 1.5 pmol in mock-transfected cells and 5.8 ± 1.2 pmol cAMP/mg protein in hIP-RCOS1 cells, respectively. Direct stimulation of AC with 100 nM forskolin increased cAMP formation in mock-transfected cells (15.9 ± 2.2 pmol cAMP/mg protein) comparably to hIP-RCOS1 cells (17.8 ± 2.5 pmol cAMP/mg protein), whereas 200 nM cicaprost significantly increased cAMP values only in hIP-RCOS1 (75.6 ± 8.2 pmol cAMP/mg protein) cells but not in mock-transfected COS1 cells (5.1 ± 3.3 pmol cAMP/mg protein). Prolonged treatment with 200 nM cicaprost led to a marked long-term desensitization (Fig. 1A), the cAMP response was reduced to approximately 50% of maximal cAMP formation after 8 hr of continuous agonist stimulation. These data are consistent with receptor desensitization kinetics in naturally IP-R expressing cell types, like NG108-15 cells [8], smooth muscle cells [9] and fibroblasts [10]. Due to the similar

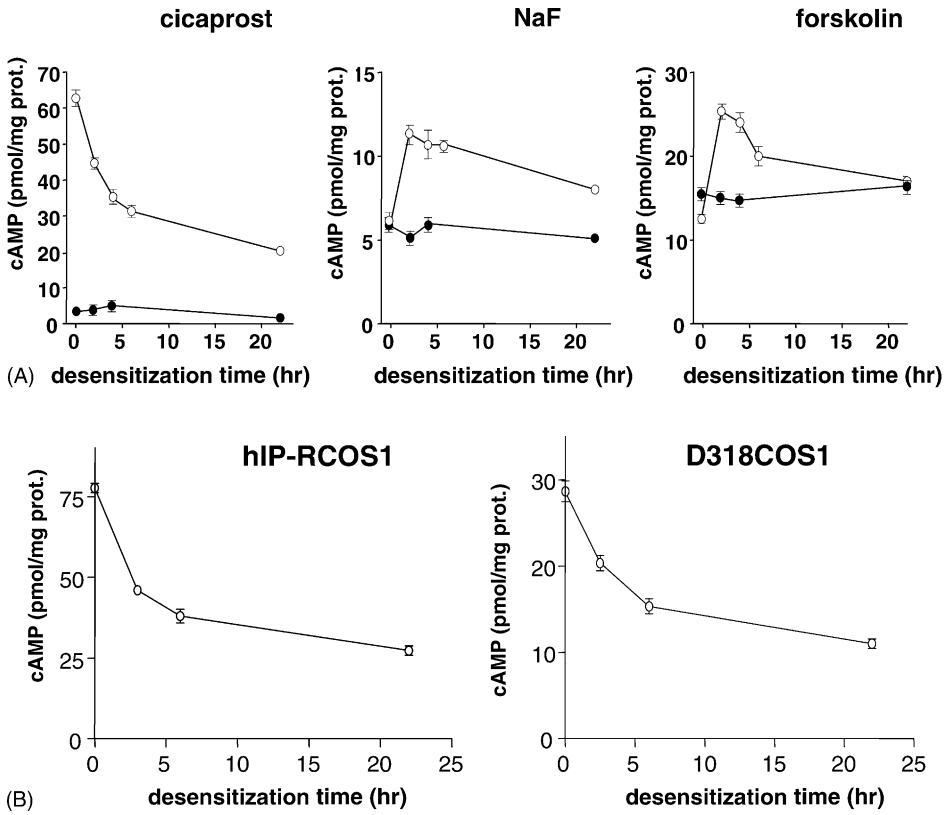

Fig. 1. Desensitization of hIP-R and C-terminally truncated hIP-R in COS-1 cells (A) cicaprost-, NaF- and forskolin-stimulated cAMP responses in hIP-RCOS1 (○) and mock-transfected COS1 cells (●) after pretreatment with 200 nM cicaprost for the indicated times. The results are mean ± SEM values of three experiments. (B) Long-term desensitization of D318 hIP-R deletion mutant. cAMP responses of COS1 cells, expressing either the WT (hIP-RCOS1) or truncated receptors (D318COS1), were determined after pretreatment with 200 nM cicaprost for the indicated times by rechallenge with 200 nM cicaprost. cAMP values are mean ± SEM values of three experiments.

time course in COS-1 cells and cells naturally expressing IP-R, it is likely that the mechanisms involved in this process might also be similar.

When hIP-RCOS1 cells were stimulated with NaF (10 mM) or forskolin (100 nM) at any time point of cicaprost pretreatment, the changes in cAMP response did not parallel the agonist-induced desensitization curve, rather a transient increase in cAMP formation occurred at 1 hr. Therefore, down-regulation of G_s protein or AC, respectively, as a possible explanation for reduced cAMP formation after continuous stimulation of cells with cicaprost could be excluded (Fig. 1A).

3.2. Long-term desensitization of C-terminally truncated hIP-R in COS1 cells

Determinants in the hIP-R C-terminus have been reported to be responsible for its rapid desensitization in HEK293 cells, occurring within seconds of agonist treatment [11,12]. To investigate the function of the intracellular C-terminal tail for long-term hIP-R desensitization, we have constructed a C-terminally truncated hIP-R mutant by exchange of the S319 codon for a stop codon (mutant D318). D318, like the WT-receptor, showed intact coupling to G_s in COS1 cells, as determined by cAMP formation, although the cAMP response in D318COS1 cells was considerably weaker (about 30% as compared to hIP-RCOS1 cells). This was in agreement with a significantly reduced number of cell surface receptors in D318COS1 cells as compared to in hIP-RCOS1 cells. [^{3}H]Iloprost-binding to the surface of intact cells was only about 10–20% of that of the WT (data not shown). This suggests that membrane localization, but not G_s coupling, is impaired in D318COS1 cells. In spite of this localization, the cAMP response was decreased upon persistent exposure to cicaprost similar to the WT (Fig. 1B), indicating that long-term desensitization does not depend on determinants located in the hIP-R C-terminus. These features match β_2-AR long-term desensitization, which, in contrast to β_2-AR short-term desensitization, is also independent of the receptor C-terminus [13].

3.3. Sequestration of surface receptors

To evaluate the role of the human IP-R C-terminus in agonist-induced internalization, agonist-promoted sequestration of WT and D318 C-terminally tagged with EGFP was analyzed. Addition of EGFP to the C-terminus resulted in functionally active receptors as determined by the agonist-induced cAMP formation in COS1 cells. The

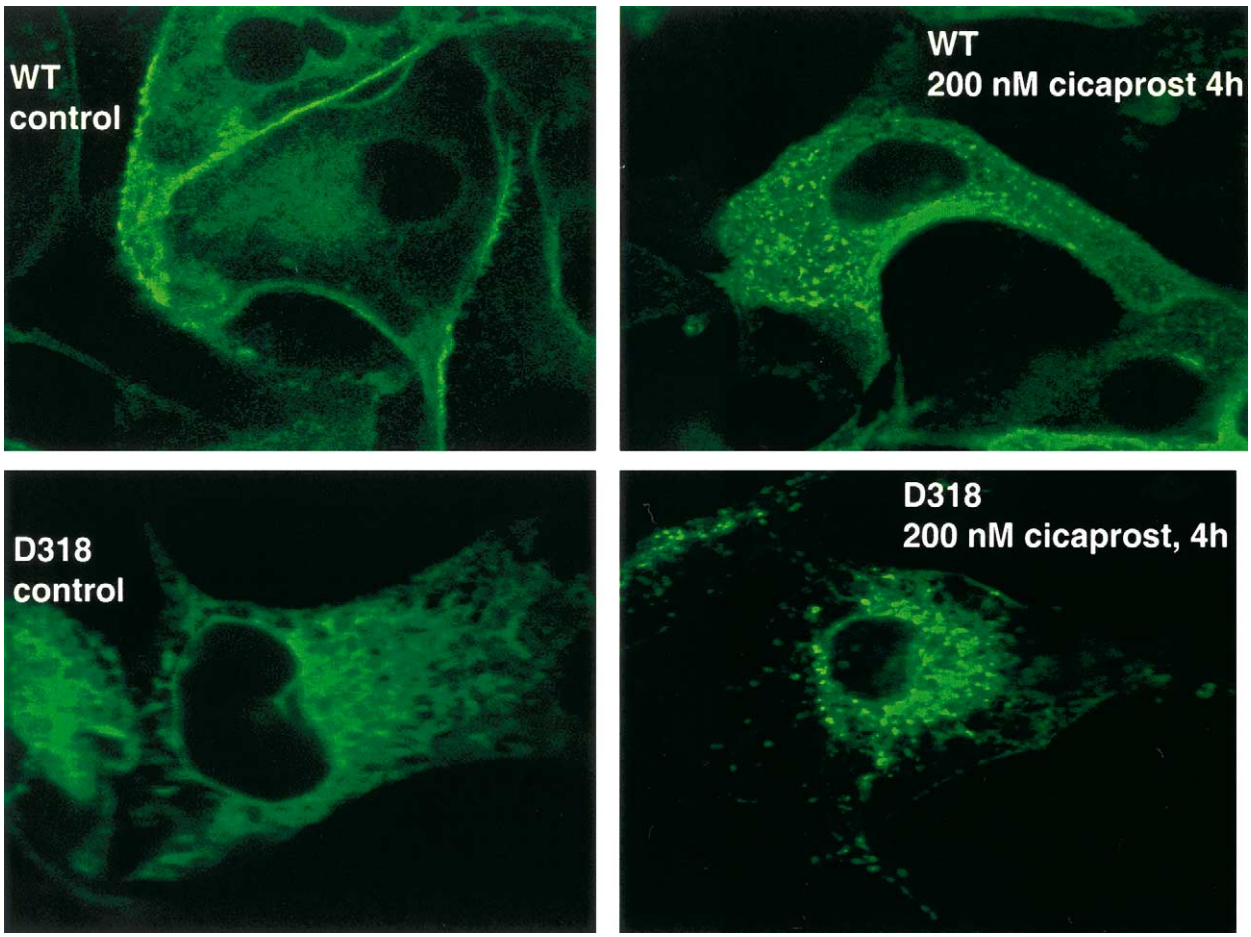

Fig. 2. Sequestration of full-length and truncated hIP-R–EGFP COS1 cells transiently transfected with IP-R–EGFP (A, B) or D318–EGFP (C, D) were either untreated (A, C) or stimulated with 200 nM cicaprost (B, D) for 4 hr, as indicated. Localization of EGFP fluorescence in *para*-formaldehyde-fixed cells was visualized by confocal microscopy.

amount of cAMP formation and desensitization kinetics resembled those of the WT or D318 mutant receptor, respectively (not shown). *Para*-formaldehyde-fixed cells showed that pretreatment with the IP-R agonist cicaprost drastically changed hIP-R–EGFP distribution (Fig. 2A and B). Whereas the receptor was predominantly found in the plasma membrane in untreated cells, it was translocated to cytoplasmic vesicular structures after pretreatment with cicaprost (200 nM) for 4 hr. Although D318–EGFP revealed a more diffuse distribution pattern in untreated cells, probably due to less efficient membrane anchoring [14], compared to hIP-R–EGFP, agonist-induced sequestration of D318–EGFP could be observed. Receptor redistribution into a distinct punctate pattern after 4 hr treatment with cicaprost (200 nM) could be detected (Fig. 2C and D). These data suggest that hIP-R sequestration does not require the C-terminal tail. This is in contrast to a recent report by Smyth *et al.* [12], who found that deletion of the C-terminus prevented iloprost-induced rapid hIP-R internalization in HEK293 cells. However, rapid hIP-R desensitization in HEK293 cells and long-term desensitization in COS-1 cells, are likely to be mediated by different mechanisms.

Furthermore, pretreatment of cells with concanavalin A (0.25 μg/mL), which inhibits endocytosis, prior to stimulation with cicaprost did not abolish desensitization kinetics (not shown). Receptor endocytosis, therefore, seems not to account for the long-term desensitization of the cAMP response to cicaprost.

To understand the mechanisms underlying desensitization of human IP-R is highly desirable and might result in significant improvement of therapeutical efficacy of PGI_2 mimetics. Our results provide evidence that long-term IP-R desensitization and agonist-induced sequestration do not require the intracellular C-terminal tail of the protein. Further work is required to elucidate relevant determinants in the receptor protein and to identify distinct cellular components involved in this process.

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