Agonist-induced long-term desensitization of the human prostacyclin receptor

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Abstract Phosphorylation of the human prostacyclin (PGI_2) receptor (hIP-R) by diacylglycerol-regulated protein kinase C (PKC) has been reported to be responsible for its rapid desensitization in HEK293 cells. In this study we demonstrate, that human fibroblasts reveal a much slower hIP-R desensitization kinetics, which was neither affected by stimulation nor inhibition of PKC by either phorbol 12-myristate-13-acetate or GF-109203X suggesting a different cellular mechanism. Although agonist-promoted sequestration of a C-terminally green fluorescent protein-tagged hIP-R was demonstrated, it did not account for the long-term desensitization. Concanavalin A did not abolish, but accelerated receptor desensitization kinetics. Resensitization of hIP-R involved receptor recycling and/or de novo synthesis of receptor protein, depending on the duration of prior desensitization. This is the first study investigating the mechanisms of hIP-R desensitization in intact human cells naturally expressing hIP-R. Our data suggest, that a hitherto unknown mechanism of hIP-R long-term desensitization, which is independent of receptor phosphorylation by conventional and novel type PKC isoforms or endocytosis, is a key event in regulating the cellular responsiveness to PGI_2. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Iloprost; Prostacyclin receptor; Desensitization; Human prostacyclin receptor–green fluorescent protein; Resensitization

1. Introduction

Prostacyclin (PGI_2), the major arachidonic acid metabolite produced in vascular cells, exerts its biological actions via a specific receptor (IP-R), which belongs to the family of G protein-coupled receptors (GPCRs). IP-R may couple to multiple G protein/effector systems, including cyclic adenosine monophosphate (cAMP) formation via G_s and IP_3 formation via G_i [1]. PGI_2 and several mimetics have been used for a number of clinical indications, including pulmonary hypertension [2], peripheral arterial occlusive disease and others [3]. 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. To understand the mechanism underlying this desensitization is of major interest and might result in significant improvement of therapeutical efficacy of PGI_2 mimetics.

Termination of the signal after agonist challenge is essential for GPCRs. The prototypical model for GPCR regulation involves three key mechanisms [4]. The first and most rapid phase of desensitization occurs within seconds to minutes after exposure to agonist and is due to agonist-induced receptor phosphorylation mediated by second messenger kinases, such as PKA and protein kinase C (PKC) or G protein-coupled receptor kinase (GRKs), eventually uncoupling the receptor from its G protein. This event, referred to as short-term desensitization, is followed by sequestration of the receptor away from the cell surface [5–7]. Finally, more prolonged receptor stimulation may cause a net decrease in total receptor number at the membrane surface, often accompanied by degradation of receptor protein, termed down-regulation.

The human IP-R, overexpressed in HEK293 cells, exhibits rapid agonist-induced desensitization occurring within minutes [8,9]. This process involves phosphorylation of the C-terminal tail by PKC and thus seems to follow the general paradigm of GPCR signal attenuation. In contrast to this short-term desensitization, cell systems naturally expressing IP-R reveal a much slower time course of desensitization requiring 3–10 h [10–13]. The mechanism involved in this agonist-induced long-term attenuation is still poorly understood and was investigated in the present study.

2. Material and methods

2.1. Materials

All cell culture reagents, TRIzol™ and LipofectAMINE PLUS™ were purchased from Gibco Life Technologies (Karlsruhe, Germany). Iloprost was kindly provided by Schering (Berlin, Bergkamen, Germany). Isoproterenol, cycloheximide, phorbol 12-myristate-13-acetate (PMA) and isobutylmethylxanthine (IBMX) were purchased from Sigma (Deisenhofen, Germany). GF-109203X was from Alexis (Grünenberg, Germany). All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The β_2AR cDNA (human), GRK 2 (bovine) and GRK 5 (bovine) cDNAs were a generous gift from Dr. N.J. Freedman (Duke University, Durham, NC, USA).

2.2. Cell culture and transfections

Human fibroblasts CRL1635 were obtained from ATCC (Manassas, VA, USA). Fibroblasts were grown in Dulbecco's modified Ea-

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Abbreviations: cAMP, cyclic adenosine monophosphate; c-, conventional; GFP, green fluorescent protein; GRK, G protein-coupled receptor kinase; GPCR, G protein-coupled receptor; hIP-R, human prostacyclin receptor; n-, novel; PGI_2, prostacyclin; PKC, protein kinase C; PMA, phorbol 12-myristate-13-acetate

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gle's high glucose medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin. All cDNAs were cloned into the mammalian expression vector pcDNA3 for transfection. For transient transfection cells were grown to 50–80% confluency and transfected with 1 μg DNA/6 well using 4 μl LipofectAMINE and 6 μl PLUS reagent. In case of GRK 2 and GRK 5 overexpression, cells were split the day after transfection into 24-well dishes and assayed the following day.

2.3. cAMP measurements

Fibroblasts were grown to 90% confluency in 24-well plates. After preincubation in HBSS containing 1 mg/ml BSA, 10 mM HEPES (pH 7.3) and 1 mM IBMX for 10 min at 37°C, cells were stimulated with 100 nM iloprost for the indicated times. Reactions were stopped by aspiration and addition of ice-cold 96% ethanol. Dried samples were overlaid with 300 μl RIA-buffer (150 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.4) and frozen overnight at −80°C. cAMP in the supernatant was determined by radioimmunoassay [14]. For desensitization assays cells were pretreated with 100 nM iloprost for the indicated times and washed three times with HBSS. cAMP formation was then measured as described [14]. Concanavalin A (0.25 μg/ml), PMA (2 μM) and GF-109203X (1 μM) were added 15 min prior to iloprost during the desensitization procedure. For resensitization studies, cells were desensitized by 6 h treatment with 100 nM iloprost. Iloprost was removed by three rapid washes with medium (w/o FCS), fresh medium was added, and the cells were placed in a CO_2 incubator for the indicated periods of time. Cycloheximide (50 μg/ml) was present during the resensitization procedure. Cells were then challenged with iloprost and cAMP measurements were performed as described above. Protein determination was performed according to the method of Bradford [15] and adenylyl cyclase activity was expressed as pmol cAMP mg^{-1} protein.

2.4. Generation of the human PGI_2 receptor (hIP-R)–green fluorescent protein (GFP) construct

The GFP was fused in-frame to the C-terminal end of hIP-R by means of PCR. Using the hIP-R cDNA as template, the first PCR was performed applying a sense oligonucleotide, containing an internal hIP sequence 5′-AGCAGTACTGCCCCGGCAGCTGGTGCTTCC-3′ and a receptor specific antisense oligonucleotide (5′-GTCAGCTT-GAAGATATCGCAGAGGGAG-3′), containing an *Eco*RV site (underlined) instead of the original TGA stop codon. The 0.7-kb PCR product was digested with *Eco*NI/*Eco*RV and ligated into pcDNA3. The resulting plasmid (pc3hIP-Rmut) contained hIP-R without the C-terminal *Eco*NI/*Eco*RV fragment. In a second PCR reaction the GFP sequence was generated from the plasmid pEGFP-C1 (Clontech, Heidelberg, Germany) using a sense oligonucleotide, containing an *Eco*RV site (underlined) upstream of the GFP start codon (bold letters): 5′-TGCGATATC**ATG**GTGAGCAAG-GGCGAG-3′ and an antisense oligonucleotide, containing a *Xho*I site (underlined) located 3′ of the GFP stop codon (bold letters): 5′-TCTCTCTGGAG**TTA**TCATCCGGACTTGTACAGCTC-3′. The second PCR product was digested with *Eco*RV/*Xho*I and ligated into pc3hIPmut resulting in pc3hIP-R–GFP.

2.5. Confocal laser 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 were cultured on 12-mm glass coverslips and GFP fluorescence was examined by confocal microscopy 48–72 h after transfection. For analysis of receptor sequestration, cells were transiently transfected with pc3hIP-R–GFP and stimulated with 100 nM iloprost.

2.6. Data analysis

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

3. Results and discussion

3.1. IP receptor expression and desensitization in human fibroblasts

Stimulation of human fibroblasts with iloprost (100 nM) resulted in a significant accumulation of cAMP (280 ± 20 pmol/(min × mg protein) vs. 2.9 ± 0.2 pmol/(min × mg protein), $n = 3$), indicating the presence of functionally active IP receptors. To examine whether IP-R undergoes agonist-dependent short-term desensitization in human fibroblasts, cells were challenged with iloprost for increasing time periods in the presence of 1 mM IBMX, a phosphodiesterase inhibitor, and the amount of cAMP after each stimulation period was measured. The kinetics of cAMP formation was comparable to that of forskolin, a direct activator of adenylyl cyclase, indicating that no significant short-term desensitization occurred (Fig. 1A). In contrast to this, challenge of endogenously expressed β_2-adrenergic receptors with 100 nM isoproterenol resulted in a plateau of cAMP formation after 10 min, reflecting the known short-term desensitization of β_2-adrenergic receptors. However, prolonged stimulation of fibroblasts with iloprost for 1–7 h led to a significant decrease in the cAMP response to agonist challenge. Agonist-induced

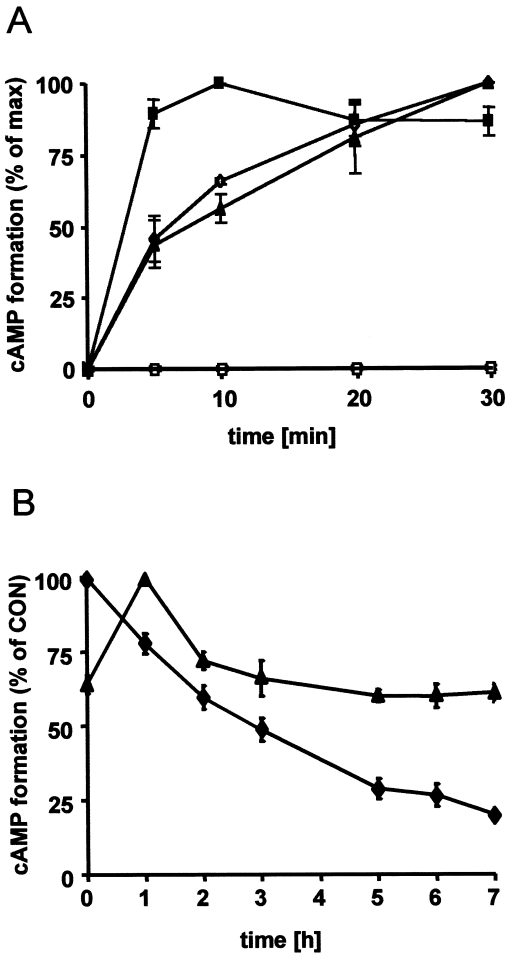


Fig. 1. IP receptor desensitization in human fibroblasts. A: Agonist-stimulated cAMP generation in untreated fibroblasts. Cells were either unstimulated (□) or continuously stimulated with 100 nM iloprost (♦), 10 μM forskolin (▲) or 100 nM isoproterenol (■), respectively, for the indicated times. cAMP values are expressed as percent of maximal cAMP formation during measurement. Data are means of three independent experiments performed in triplicate. B: Iloprost (♦) or forskolin (▲) stimulated cAMP generation in fibroblasts pretreated with iloprost for the indicated times. cAMP values are percent of non-desensitized control. Data are means of three independent experiments performed in triplicate.

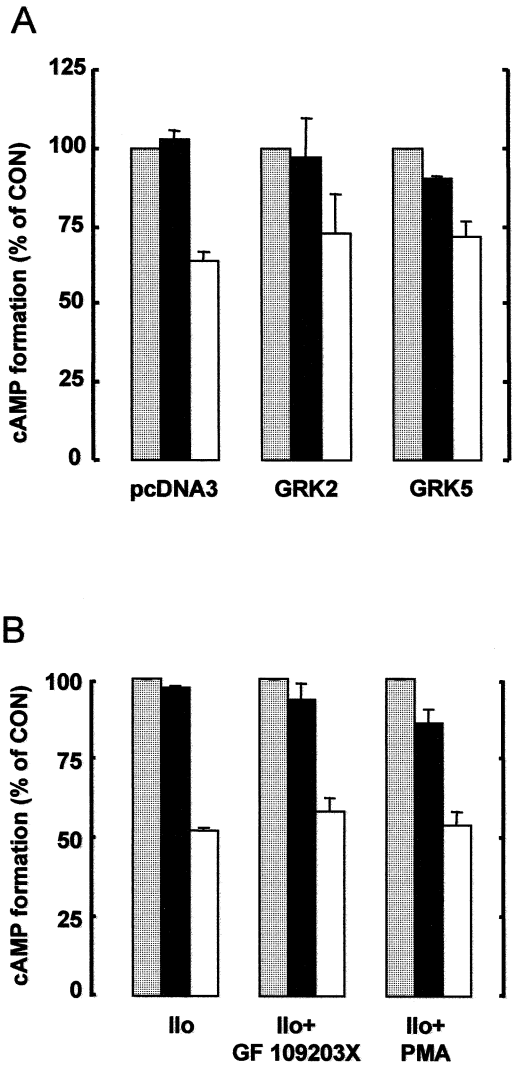


Fig. 2. Effect of GRK 2,5 overexpression, PKC stimulation, and PKC inhibition on hIP-R desensitization, Iloprost-stimulated cAMP response in fibroblasts either without prior agonist challenge (gray bars) or after pretreatment with 100 nM iloprost for 30 min (black bars) or 3 h (white bars). A: Cells were transfected with the expression vector pcDNA3 containing either GRK 2 or GRK 5 or pcDNA3 only. B: Cells were coincubated during desensitization with the PKC inhibitor GF-109203X (1 μM) or treated with the PKC activator PMA (2 μM), as indicated. cAMP values are expressed as percent of non-desensitized control. Data are means of three independent experiments performed in triplicate.

cAMP formation was decreased by about 50% upon preincubation with iloprost at 3 h, and by about 75% at 6 h (Fig. 1B). When cells were challenged with forskolin (10 μM) at any time point of iloprost pretreatment, the changes in cAMP response did not parallel the agonist-induced desensitization curve, rather a transient increase in cAMP formation occurred at 1 h. Therefore, down-regulation of adenylyl cyclase as a possible reason for reduced cAMP formation after continuous stimulation of cells with iloprost could be excluded.

3.2. *Effect of GRK overexpression or PKC activation on hIP-R desensitization kinetics*

In recent studies, GRKs have been shown to promote agonist-stimulated desensitization of various GPCRs [16,17] with GRK 2 and GRK 5 being the most potent enzymes [16,18]. Possible target sites for GRK-dependent phosphorylation are also present in the amino acid sequence of the hIP-R. To further define the role of GRKs in hIP-R desensitization, we analyzed the effect of GRK 2 and GRK 5 overexpression on hIP-R desensitization kinetics. However, although transfection of fibroblasts with GRK 2 and GRK 5 expression plasmids led to significant production of the proteins (data not shown) they did neither attenuate receptor signaling at the level of cAMP formation nor affected hIP-R desensitization kinetics (Fig. 2A).

Agonist-induced PKC phosphorylation of the C-terminal tail of hIP-R dictates rapid receptor desensitization in HEK293 cells [8]. In HEK293 cells receptor phosphorylation was also observed if cells were treated with PMA, indicating a role of conventional (c-) or novel (n-) type PKC isoforms. Therefore, we investigated the effect of prior c- and n-type PKC isotype kinase activation on receptor activity and desensitization kinetics in human fibroblasts. Pretreatment of cells for 30 min with 2 μM PMA did not result in any mitigation of

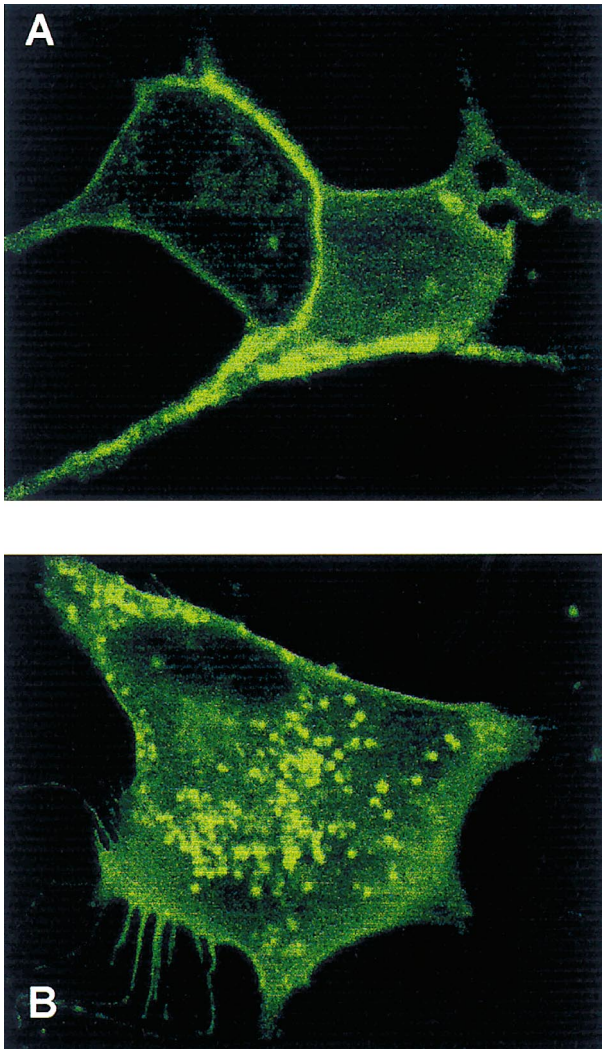


Fig. 3. Sequestration of GFP-tagged hIP-R (hIP-R–GFP) in human fibroblasts. Confocal imaging of iloprost-induced sequestration of hIP-R–GFP in human fibroblasts. Cells were either untreated (A) or stimulated with iloprost for 8 h (B). Data are derived from one representative experiment out of two with similar results.

subsequent stimulation of cAMP formation by the IP receptor agonist iloprost or significant acceleration of desensitization kinetics (Fig. 2B). Furthermore, treatment of cells with the PKC inhibitor GF-109203X (1 μM), a concentration inhibiting c- and n-type PKC isoforms [19], had also no effect on desensitization kinetics (Fig. 2B). These findings suggest that c- and n-type PKC isoforms as well as GRK 2 and GRK 5, respectively, play only a minor if any role in hIP-R desensitization. However, a role of atypical (diacylglycerol/PMA insensitive) PKC isoforms, as recently reported for the μ-opioid receptor [20], cannot be excluded in our experiments.

3.3. Sequestration of GFP-tagged hIP-R

Receptor sequestration was proved using a hIP-R–GFP fusion protein. This method was applied because the expression level of endogenously expressed hIP-R was too low to allow ligand binding studies. Moreover, most receptor GFP fusion proteins retain their biological activity and reveal the same trafficking pattern as the native protein [21]. Addition of GFP to the C-terminus resulted in a functionally active receptor as demonstrated by the agonist-induced cAMP formation in transiently transfected CHO cells (not shown). In immunoblots, performed on cell lysates from hIP-R–GFP-expressing fibroblasts with an antibody specific to GFP, the tagged receptor appeared as a single protein band with a molecular weight of about 70 kDa, representing the summation of the 44 kDa hIP-R plus the 27 kDa GFP-tag (not shown). Sequestration of hIP-R–GFP in fibroblasts was visualized by confocal microscopy 3 days after transfection. When resting cells were studied, hIP-R–GFP was localized to the plasma membranes (Fig. 3A). After stimulation with iloprost (100 nM, 8 h) significant receptor sequestration away from the membrane and redistribution into a distinct punctate pattern was seen (Fig. 3B).

3.4. Effect of inhibitors of endocytosis on agonist-induced desensitization kinetics

For several GPCRs, like the somatostatin receptor [22], desensitization seems to be predominantly a consequence of receptor sequestration away from the plasma membrane without prior receptor phosphorylation. We, therefore, assayed the effect of concanavalin A, which inhibits endocytosis, on agonist-stimulated long-term desensitization. Pretreatment of cells with concanavalin A (0.25 μg/ml) for 15 min prior to stimulation with iloprost did not abolish, but rather accelerate desensitization kinetics (Fig. 4). While continuous stimulation of cells with iloprost (1 h) led to a reduction of cAMP formation to 88% ($n=3$) of control, concanavalin A pretreatment reduced cAMP formation to 60% ($n=3$) at the same time point. Stimulation of cells for 3 h with iloprost led to a reduction of cAMP formation to 54% ($n=3$) of control value, while concanavalin A pretreatment reduced cAMP formation to 24% ($n=3$) at the same time point. Receptor endocytosis, therefore, seems not to account for the long-term desensitization of the cAMP response to iloprost. The acceleration of kinetics rather indicates that receptor endocytosis seems to be at least partly necessary for receptor resensitization by allowing receptor recycling.

3.5. Resensitization of hIP-R

Different mechanisms may contribute to receptor resensitization after removal of agonist, namely receptor recycling

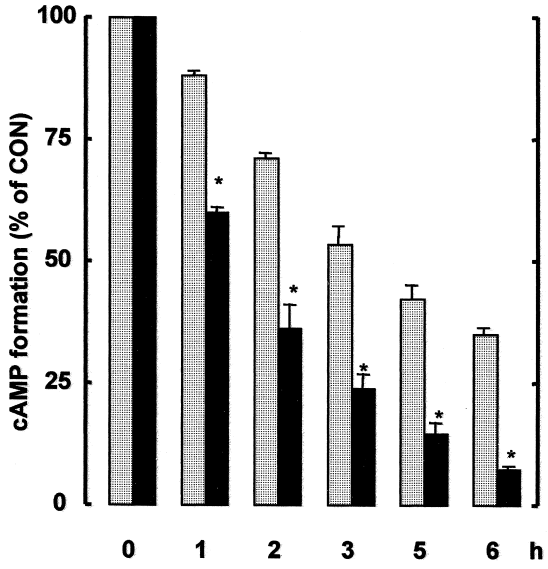


Fig. 4. Effect of concanavalin A on agonist-induced desensitization, Cells were subjected to desensitization as described in Fig. 1. cAMP response was assayed in cells either untreated (gray bars) or treated with concanavalin A (0.25 μg/ml) (black bars) during desensitization. Data are means of three independent experiments performed in triplicate. $*P \leq 0.05$.

from an endosomal compartment back to the membrane and/or de novo synthesis of receptor protein. Both mechanisms have been described for hIP-R. While hIP-R in platelets recycle, IP-R resensitization in NG 108-15 cells requires de novo synthesis [23,24]. Our experiments revealed that withdrawal of receptor agonist results in a slow resensitization of IP receptors in human fibroblasts. After treatment with iloprost for 6 h, fibroblasts were exposed to agonist-free conditions to allow receptor resensitization. While formation of cAMP was reduced significantly in desensitized cells and remained low in the continuous presence of agonist, cAMP formation was restored completely when cells were kept in agonist-free medium for 18 h. Resensitization in the presence of cycloheximide (50 μg/ml) was only slightly reduced (Fig. 5A). In contrast, resensitization following 16 h of desensitization was abolished by cycloheximide, suggesting that prolonged stimulation probably leads to receptor degradation and/or down-regulation, making de novo receptor synthesis a prerequisite of resensitization (Fig. 5B). It should be noted that cycloheximide had no significant effect by its own on cell viability. It is concluded that agonist-triggered internalization at first allows receptor recycling but prolonged stimulation may then lead to receptor degradation, making de novo protein synthesis a prerequisite of resensitization. The observation that the duration of desensitization determines whether recycling or degradation of receptor occurs, has been recently described for other GPCRs including the β_2-adrenergic [25,26] and opioid receptors [27].

Perturbations at the level of PGI_2 formation or at IP-R level have been related to various cardiovascular disorders [28–30]. Several therapeutic strategies aimed to enhance PGI_2 production and successfully reduced restenosis in different animal models [31–33]. However, available data from clinical trials are conflicting and currently do not support the concept that PGI_2 or stable mimetics may be successfully

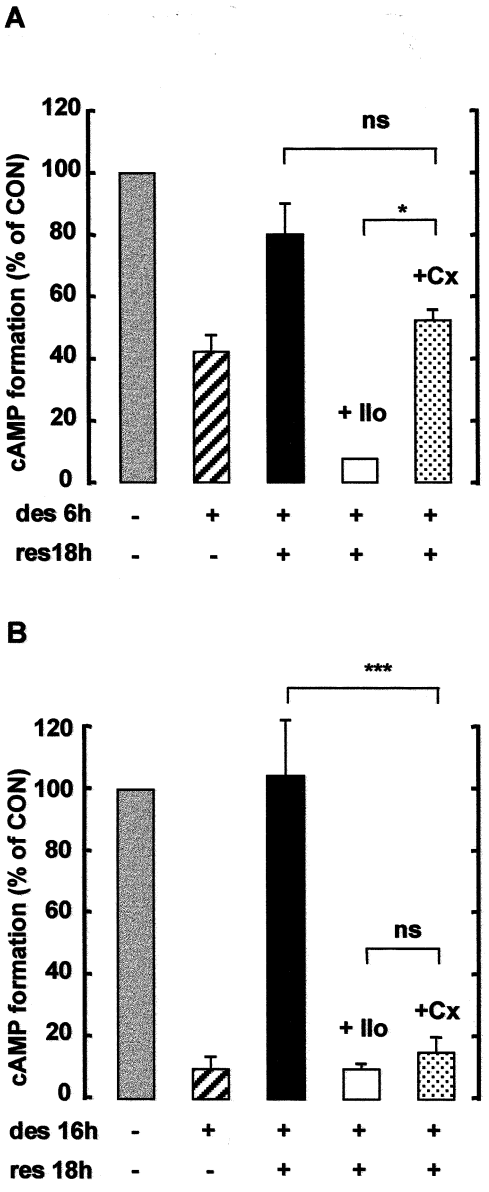


Fig. 5. Resensitization of hIP-R, cAMP formation was determined in fibroblasts desensitized with 100 nM iloprost for 6 h (A, des 6 h) or 16 h (B, des 16 h), respectively, followed by an agonist free time of 18 h (res 18 h) to allow resensitization. Cells were treated as follows: stimulated without prior desensitization and resensitization (gray bars), desensitized (hatched bars), desensitized and resensitized (black bars), desensitized and resensitized in the continuous presence of iloprost, (Ilo, white bars), desensitized and resensitized in the presence of cycloheximide (Cx, dotted bars). Data are means of three independent experiments performed in triplicate. $^{*}P \leq 0.05$, $^{***}P \leq 0.001$, ns: not significant.

used to suppress restenosis after PTCA [34,35]. These findings may be explained, at least partly, by IP-R long-term desensitization. Although short-term administration of iloprost may induce beneficial effects, long-lasting agonist stimulation will reduce efficacy, e.g. of antiplatelet [36] or antimitogenic [12] effects of iloprost. It has been demonstrated some time ago that inhibition of endogenous prostaglandin synthesis by indomethacin sensitized cells to prostaglandin administration [37]. Thus, endogenously synthesized prostaglandins may already contribute to receptor desensitization in vivo. These findings suggest that the actual state of receptor sensitivity in addition to the agonist concentration markedly determines cellular effects of prostaglandins.

Taken together, our data suggest that the signaling capacity of hIP-R is attenuated by desensitization processes involving several distinct, but overlapping mechanisms which do not require the action of GRKs or c-/n- PKC isoforms in human fibroblasts. Possible mechanisms may include receptor phosphorylation by tyrosine kinases as described for the μ-opioid receptor [38]. Moreover, as reported for the m3-muscarinic receptor, casein kinase 1α may represent as well an alternative pathway to GPCR phosphorylation [39]. Finally, accelerated receptor protein degradation may result in reduced total membrane receptor during long-term stimulation, thus reducing receptor signaling. Further experiments will be necessary to find out how specific desensitization of hIP-R is realized and which cellular components of the endocytotic machinery are involved in the internalization procedure.

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