Mechanism of cicaprost-induced desensitization in rat pulmonary artery smooth muscle cells involves a PKA-mediated inhibition of adenylyl cyclase

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Sobolewski, Anastasia, Karen B. Jourdan, Paul D. Upton, Lu Long, and Nicholas W. Morrell. Mechanism of cicaprost-induced desensitization in rat pulmonary artery smooth muscle cells involves a PKA-mediated inhibition of adenylyl cyclase. Am J Physiol Lung Cell Mol Physiol 287: L352–L359, 2004.—Long-term infusion of prostacyclin, or its analogs, is an effective treatment for severe pulmonary arterial hypertension. However, dose escalation is often required to maintain efficacy. The aim of this study was to investigate the mechanisms of prostacyclin receptor desensitization using the prostacyclin analog cicaprost in rat pulmonary artery smooth muscle cells (PASMCs). Desensitization of the cAMP response occurred in 63 nM cicaprost after a 6-h preincubation with agonist. This desensitization was reversed 12 h after agonist removal, and resensitization was inhibited by 10 μg/ml of cycloheximide. Desensitization was heterogeneous since desensitization to other Gα-adenyl cyclase (AC)-coupled agonists, isoproterenol (1 μM), adrenomedullin (100 nM), or bradykinin (1 μM), was also reduced by preincubation with cicaprost. The reduced cAMP response to prolonged cicaprost exposure appeared to be due to inhibition of AC activity since the responses to the directly acting AC agonist forskolin (3 μM) and the selective AC5 activator NKH-477 were similarly reduced. Expression of AC2 and AC5/6 protein levels transiently decreased after 1 h of cicaprost exposure. The PKA inhibitor H-89 (1 μM) added 1 h before cicaprost preincubation (6 h, 63 nM) completely reversed cicaprost-induced desensitization, whereas the PKC inhibitor bisindolylmaleimide (100 nM) was only partly effective. Desensitization was not prevented by the Gβγ inhibitor pertussis toxin. In conclusion, chronic treatment of PASMCs with cicaprost induced heterologous, reversible desensitization by inhibition of AC activity. Our data suggest that heterologous Gα desensitization by cicaprost is mediated predominantly by a PKA-inhibitable isoform of AC, most likely AC5/6.

prostacyclin; cyclic adenosine 5’-monophosphate; heterologous desensitization; cAMP-dependent protein kinase

PROSTACYCLIN ANALOGS are proving effective treatments for severe pulmonary arterial hypertension (PAH) causing pulmonary vasodilation and, possibly, inhibition of smooth muscle cell proliferation (33). However, increasing doses are often required to maintain efficacy, an effect that may involve desensitization of the agonist response (25). Understanding the mechanism of prostacyclin (IP) receptor desensitization to sustained agonist exposure may be important for improving on existing therapeutic interventions for PAH.

Prostacyclin acts by binding to a G protein-coupled receptor (GPCR), the IP receptor, which directly stimulates the enzyme adenylyl cyclase (AC) via Gα that converts ATP to the second messenger cAMP. cAMP has been widely implicated in the control of pulmonary vascular tone (26) and inhibition of mito-
genetic pathways in vascular cells (6, 8, 33, 43). Equally, the coupling of other G proteins, such as Gq, to agonist receptors leads to the inhibition of AC activity (27). cAMP can also antagonize the effects of vasoconstrictor or growth signals acting via Gq-coupled receptors and receptor tyrosine kinases (15). Therefore, ACs play a central role in the regulation of vascular tone and proliferation after cell surface GPCR activation.

AC activity, therefore, is a major controller of intracellular cAMP, and to date, 10 isoforms of this enzyme have been demonstrated. AC isoforms display different tissue localizations (7, 19), and each AC isoform has distinct patterns of regulation, allowing ACs to serve as key integrators of relevant stimulatory and inhibitory signals (36a). For example, AC5/6 is negatively regulated by PKA (17, 21, 28). PKC generally stimulates AC isoforms (45) but has been shown to negatively regulate AC6 (23). ACs may also be distinguished by sensitivity to inhibitory Gα-subunits, notably AC5 and AC6, and to calcium/calmodulin, which can either positively or negatively regulate AC activity (11, 41, 42, 44).

Desensitization of the cAMP response after chronic agonist stimulation has been demonstrated in a variety of systems. In the classic model of GPCR regulation, agonist-induced receptor phosphorylation is mediated by PKA, PKC, or GPCR kinases (13, 30, 31). In addition, desensitization can occur by receptor internalization (short term) or reduced receptor expression (long term) (1); for example, IP receptor internalization in transfected HEK-293 cells (35). Heterologous receptor desensitization may occur at the level of reduced activity of Gα (12) or may involve changes in AC activity; for example, in the heterologous desensitization in peripheral blood mononuclear cells, chick hepatocytes (9, 32).

To date, no study has determined the mechanism of desensitization of the cAMP response to IP analogs in pulmonary artery smooth muscle cells (PASMCs), despite the potential clinical importance. We thus determined the time course of this desensitization and demonstrated the heterologous nature of this effect as well as the time course of resensitization. In addition, we show that desensitization is due to reduced activity of AC, an effect mediated predominantly by a PKA-inhibitable isoform of AC, thereby implicating AC5/6 in this response.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. NKH-477 was a gift.
from Makato Hosono (Nippon Kayaku, Tokyo, Japan). Polyclonal antibodies to AC2 and AC5/6 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cicaprost was a gift from Schering-Plough (UK). Bisindolylmaleimide, H-89, and bradykinin were purchased from Calbiochem (Beeston, Nottingham, UK). Adrenomedullin was purchased from Bachem (Germany). DMEM and FBS were purchased from Gibco (Invitrogen, Paisley, UK).

Isolation of peripheral PASMCs. Rat PASMCs were isolated from precapillary pulmonary arteries with a modification of a previously described method (18). These experiments were approved by the local animal care committee and were covered by Home Office Project Licence PPL/80/1567. Briefly, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg), and midline and lateral thoracotomies were performed to expose the trachea. The pulmonary artery was then cannulated through the right ventricle, the left atrium was incised, and the lungs were flushed with 10 ml of PBS at 37°C over 30 s. Fifteen milliliters of 0.5% iron oxide-0.5% agarose in DMEM at 45°C were then immediately infused into the pulmonary artery over 30 s, and the pulmonary artery was clamped. The trachea was cannulated, and the entire lung block was removed. The lungs were irrigated by tracheal instillation of 1% (wt/vol) agarose in DMEM (40 ml/kg). The preparation was then transferred to ice-cold DMEM for 10 min until the agarose had set. Subsequent sections (1–2 mm thick) were sliced from the bronchial tree of the lungs and minced with a razor blade. The tissue was then partially digested with collagenase (80 U/ml of culture medium) for 60 min at 37°C and sheared by five strokes through progressively smaller needles (18–25 gauge) to remove the surrounding parenchyma. The peripheral arteries were isolated with a magnetic separator (Promega), rinsed with 4°C DMEM, resuspended in 1 ml of DMEM-20% FBS, and plated in 25-cm² tissue culture flasks. The flasks were incubated in humidified air with 5% CO₂ at 37°C. The nonselective inhibitor of AC2, AC3, and AC5/6 overnight. For studies to determine the time course of resensitization to cicaprost-induced cAMP accumulation, after chronic incubation with cicaprost (63 nM), PASMCs were washed three times and placed in serum-free DMEM for 1–24 h and stimulated with cicaprost for 15 min (63 nM). To determine whether G proteins were involved in desensitization, pertussis toxin (50 ng/ml) was added to PASMCs for 16 h before and during chronic cicaprost exposure (6 h, 63 nM) and a 15-min stimulation with cicaprost (63 nM) (17). For experiments to determine the time course of resensitization to cicaprost incubations (63 nM) ranging from 15 min to 24 h. A 3-h course of cicaprost desensitization was conducted with the period of desensitization continued during cicaprost preincubation. The nonselective inhibitor of AC5/6, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg), and midline and lateral thoracotomies were performed to expose the trachea. The pulmonary artery was then cannulated through the right ventricle, the left atrium was incised, and the lungs were flushed with 10 ml of PBS at 37°C over 30 s. Fifteen milliliters of 0.5% iron oxide-0.5% agarose in DMEM at 45°C were then immediately infused into the pulmonary artery over 30 s, and the pulmonary artery was clamped. The trachea was cannulated, and the entire lung block was removed. The lungs were irrigated by tracheal instillation of 1% (wt/vol) agarose in DMEM (40 ml/kg). The preparation was then transferred to ice-cold DMEM for 10 min until the agarose had set. Subsequent sections (1–2 mm thick) were sliced from the bronchial tree of the lungs and minced with a razor blade. The tissue was then partially digested with collagenase (80 U/ml of culture medium) for 60 min at 37°C and sheared by five strokes through progressively smaller needles (18–25 gauge) to remove the surrounding parenchyma. The peripheral arteries were isolated with a magnetic separator (Promega), rinsed with 4°C DMEM, resuspended in 1 ml of DMEM-20% FBS, and plated in 25-cm² tissue culture flasks. The flasks were incubated in humidified air with 5% CO₂ at 37°C. After adherence, 4 ml of culture medium were added to the flasks 24 h later. After 10–14 days, a confluent monolayer of cells had grown from explanted arteries. The cells were trypsinized and passaged into 25-cm² flasks (passage 1) and grown to confluence in DMEM-10% FBS. Subsequent passages were carried out by splitting the flasks 1:3. Cells between passages 2 and 6 were used for experiments.

Measurement of cAMP. PASMCs were seeded into 24-well plates at 15,000 cells/well, grown to confluence and, serum starved in DMEM for a 48 h. Initial experiments were performed to determine the concentration response of PASMCs to cicaprost (1 nM–10 μM for 15 min). An EC₅₀ value of 63 nM was demonstrated, and this value was used in subsequent experiments for all cicaprost chronic and acute stimulations. To optimize conditions for studying desensitization, a time course of cicaprost desensitization was conducted with the period of cicaprost preincubations (63 nM) ranging from 15 min to 24 h. A 3-h desensitization period showed significant desensitization of the cAMP response. This preincubation of at least 3 h was used for future experiments. For experiments using other conventional Gα-coupled agonists, a 6-h chronic incubation with cicaprost was followed by a 15-min stimulation with isoproterenol (10–100,000 nM), or the AC5-selective forskolin derivative NKH-477 (10 nM–100 μM), was used after cicaprost preincubation in some experiments. The role of PKA- and PKC-regulated AC activity was investigated using bisindolylmaleimide, a potent inhibitor of PKC (100 nM) (37), and H-89, an inhibitor of PKA (1 μM) (5), for 1 h before and continuing during cicaprost preincubation. The nonselective inhibitor of cyclic nucleotide phosphodiesterases, IBMX, was added (50 μM) for 15 min before all acute agonist stimulations. At the end of each experiment, cells were lysed with 0.1 N HCl and assayed for cAMP activity using an ELISA kit (R&D Systems) according to the manufacturer’s instructions. Cell viability was assessed using the trypan blue exclusion assay (viability >98%) as well as cell number per well at the termination of each experiment. Cell number following the maximal 24-h cicaprost stimulation was unchanged as confirmed by cell proliferation studies (data not shown); therefore, data in the cAMP ELISAs were expressed as picomoles/milliliter.

Concentration response of cicaprost on intracellular cAMP. Before comprehensive desensitization studies, a full cicaprost concentration-response curve was conducted. Cicaprost caused a concentration-dependent increase in cAMP levels after a 15-min stimulation, with a characteristic sigmoidal curve and an EC₅₀
The EC\(_{50}\) value of 63 nM was the standard concentration used in subsequent experiments to determine the effects of chronic cicaprost exposure on PASMCs.

**Time course of cicaprost-induced desensitization of the cAMP response.**

To investigate the temporal characteristics of cicaprost desensitization, a time-course study was conducted. Significant cicaprost-induced desensitization occurred by 3 h with maximal desensitization occurring by 24 h (Fig. 1B). The presence of the nonselective phosphodiesterase inhibitor IBMX during the final 15-min stimulation by agonist increased absolute levels of cAMP but had no impact on the relative magnitude of the desensitization (Fig. 1C).

Cicaprost preincubation causes heterologous desensitization of the cAMP response. The cAMP response to acute (15 min) cicaprost exposure was measured with and without prior cicaprost preincubation to determine the concentration-response curve of the desensitization process. Desensitization did not significantly change the EC\(_{50}\) of the cAMP response to acute cicaprost challenge, suggesting that the affinity of the IP receptor for cicaprost was unaltered (Fig. 2). To determine the homologous/heterologous nature of this response, PASMCs were pretreated with cicaprost, washed, and stimulated with adrenomedullin, isoproterenol, or bradykinin (Fig. 2B). PASMCs preexposed to cicaprost showed a significant reduction in the agonist response when stimulated with all of the conventional G\(_{i/o}\) agonists used, compared with PASMCs, in the absence of cicaprost preincubation (\(n = 3, P < 0.05\)). A chronic stimulation of AC with the plant diterpine forskolin also induced a similar heterologous desensitization of the cAMP response after acute stimulation with adrenomedullin, bradykinin, isoproterenol, and cicaprost (data not shown).

**Cicaprost preincubation results in downregulation of AC activity.**

The possibility that AC may be involved in desensitization of the cAMP response was further addressed by using directly acting agonists of AC. Cicaprost time-course studies showed that after 3 h, significant desensitization of the cAMP response to forskolin occurred (\(n = 3, P < 0.05\)), with maximal desensitization occurring by 6 h (Fig. 3A). Detailed studies of the concentration-response curve to forskolin showed a downward shift in the curve after cicaprost preincubation (Fig. 3B) without a significant change in EC\(_{50}\) (\(n = 3, P < 0.05\)). Further studies on PASMCs using the selective

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**Fig. 1.** Effect of cicaprost preincubation on cicaprost-induced cAMP levels in pulmonary artery smooth muscle cells (PASMCs). A: PASMCs were stimulated with cicaprost (CIC) for 15 min (0.001–100 \(\mu\)M) in the presence of IBMX (50 \(\mu\)M). B: PASMCs were preincubated with cicaprost (63 nM, 15 min, 6 h), washed, and stimulated with cicaprost for 15 min (in the presence of IBMX). C: after a 6-h incubation with cicaprost (63 nM), acute 15-min stimulations were performed with 63 nM cicaprost in the presence or absence of IBMX (50 \(\mu\)M) \(*P < 0.05, n = 3\) compared with respective cicaprost response in nonpretreated cells by Student’s t-test. cAMP levels were measured using an ELISA. CON, control.

**Fig. 2.** Chronic cicaprost treatment causes desensitization of cAMP in response to cicaprost and other G\(_{i/o}\)-coupled agonists. \(*P < 0.05, n = 3\) compared with respective agonist control by Student’s t-test. Cells were incubated with cicaprost (6 h, 63 nM) and washed. Cicaprost was added for 15 min (A; 0.001–10 \(\mu\)M) or adrenomedullin (ADM; 0.1 \(\mu\)M), bradykinin (BRD; 10 \(\mu\)M), isoproterenol (ISO; 10 \(\mu\)M), and CIC (63 nM) were added (B) for 15 min. cAMP levels were determined by ELISA.
Student compared with respective forskolin response of noncicaprost-treated cells by cAMP levels were measured using an ELISA. The magnitude of desensitization was comparable to that achieved with acute forskolin stimulation. Further studies (Fig. 5B) showed that resensitization had again occurred at the level of AC. AC5/6 levels returned to at least baseline. The ability of PASMCs to resensitize to subsequent stimulation was investigated by introducing a wash step of 3–24 h after a 3-h preincubation with cicaprost. The cAMP level after each of the wash periods was measured after treatment of the membrane with N-glycosidase F (Fig. 4B), indicating at least one N-linked glycosylation site is present in PASMCs in these AC isoforms.

A time course of cicaprost stimulation showed that AC2 membrane-associated protein decreased significantly after a 1-h incubation with cicaprost compared with time-matched controls ($P < 0.05, n = 3$). By 3, 6, or 24 h of cicaprost exposure, AC2 protein levels returned to baseline (Fig. 4C). The expression of AC3 was unchanged throughout (Fig. 4C). Like AC2, AC5/6 membrane-associated protein decreased significantly compared with control cells after a 1-h cicaprost incubation (Fig. 4C; $P < 0.05, n = 5$). After 3 and 6 h of cicaprost incubation, AC5/6 levels returned to at least baseline. At the 6-h time point, the changes in AC2 and AC5/6 were inconsistent. Although there was a suggestion that AC5/6 was increased in some experiments at 6 h, this did not achieve statistical significance when the mean results from five experiments were analyzed. By 24 h, cicaprost-treated and -untreated PASMCs showed comparable levels of AC5/6.

**Time course of cicaprost resensitization of the cAMP response.** The ability of PASMCs to resensitize to subsequent cicaprost stimulation was investigated by introducing a wash step of 3–24 h after a 3-h preincubation with cicaprost. The cAMP level after each of the wash periods was measured after a 15-min stimulation with forskolin (Fig. 5A). A resensitization of the agonist response occurred 12 h after removal of the agonist ($n = 3, P < 0.05$). Resensitization of the cAMP response also occurred in response to acute forskolin stimulation (Fig. 5B), demonstrating that resensitization had again occurred at the level of AC. Further studies (Fig. 5C) showed that this reversal was prevented by the protein synthesis inhibitor cycloheximide ($n = 3, P < 0.05$). These data suggest that new protein synthesis is required for the process of resensitization.

**Role of PKC and PKA in cicaprost-induced desensitization.** Major signaling pathways shown to be involved in AC regulation, as well as receptor downregulation, are the PKA and PKC pathways. To provide evidence for the involvement of PKA or PKC in cicaprost-induced desensitization, inhibitors of these protein kinases were employed. After a 1-h preincubation with either bisindolylmaleimide (PKC) or H-89 (PKA) and a coincubation with cicaprost for 6 h, a reduction in desensitization was observed (Fig. 6). H-89 completely prevented the cicaprost-induced desensitization, whereas bisindolylmaleimide only partially prevented this effect. An increase in bisindolylmaleimide concentration up to 1 μM showed no further inhibition of cicaprost-induced desensitization (data not shown).

**Effect of $G_i$ inhibition on cicaprost-induced desensitization.** Previous studies on the mouse IP receptor suggested that $G_i$ stimulation may also result in $G_i$ activation through a PKA-dependent mechanism and subsequent inhibition of AC activity.
To determine whether G<i>ᵢ</i> was involved in the desensitization of AC in our system, the G<i>ᵢ</i> inhibitor pertussis toxin was used. After a 16-h preincubation with pertussis toxin and chronic cicaprost exposure, acute stimulation with cicaprost still displayed desensitization of the cAMP response (Fig. 7). In rat PASMCs, therefore, the desensitization seen with chronic cicaprost exposure appears to be Gi independent.

**DISCUSSION**

The present study demonstrates that the IP analog cicaprost causes heterologous desensitization of the cAMP response in PASMCs primarily through PKA-regulated AC isoforms, most likely AC5/6. This is the first study to implicate the specific AC isoforms AC5/6 in IP-induced desensitization in PASMCs. Desensitization appeared to be due to reduced expression of AC5/6 and possibly AC2 protein, and was independent of Gi proteins. Furthermore, resensitization of the acute response to cicaprost occurred more slowly than desensitization and required de novo protein synthesis.

Studies of the time course of cicaprost desensitization demonstrated that after 3 h of cicaprost preincubation, a significant desensitization of acute cicaprost-induced and forskolin-induced cAMP accumulation occurred. Rapid GPCR desensitization (within seconds or minutes) would suggest mechanisms, including receptor phosphorylation, such as that seen with the β-adrenoceptor phosphorylation by β-adrenoceptor kinase (22, 40). However, the longer time course of desensitization observed in our studies suggested either receptor internalization or downregulation of pathways downstream of the receptor (14, 35, 40). Desensitization of the cicaprost concentration-response curve showed a significant downward shift without a change in the EC5₀ value after cicaprost preincubation, suggesting that the affinity of cicaprost for the IP receptor was unchanged.

Further experiments using other G<i>ᵢ</i>α-coupled agonists, namely adrenomedullin, bradykinin, and isoproterenol, also showed desensitization of the agonist response with cicaprost preincubation, suggesting that the desensitization was heterol-
Heterologous desensitization of the $G_s$ signaling pathway has previously been described at the level of AC in chick hepatocytes (32). However, in rat airways, heterologous desensitization was found to be due to downregulation of $G_s$, and the response to forskolin in these cells remained intact (12). In contrast, our data show that the predominant effect of prolonged cicaprost exposure in rat PASMCs is reduced activity of AC.

NKH-477 is a forskolin derivative that has been shown to stimulate AC5 more potently than other AC isoforms (39). The finding that chronic cicaprost exposure led to a similar reduction in both acute forskolin- and NKH-477-stimulated cAMP accumulation suggested a predominant role for AC5 in cicaprost-induced desensitization.

We investigated the possibility that the desensitization to prolonged cicaprost exposure was due to reduced expression of AC isoforms, previously shown to be functionally important in rat PASMCs (19). Studies of AC isoform expression by Western blotting showed that there was no consistent change in AC3 levels after any period of cicaprost exposure. In contrast, there was an initial decrease in both AC2 and AC5/6 protein levels after a 1-h exposure to cicaprost, but following a further incubation of up to 24 h, protein levels returned to at least baseline. Interestingly, AC5/6 levels at 6 h following cicaprost incubation appeared to increase compared with time-matched controls, but the effect was inconsistent and did not achieve significance.

Fig. 5. Mechanisms of resensitization of the cAMP response. PASMCs were preincubated with cicaprost for 3 h (63 nM), washed, and incubated in serum-free media alone (for 0–24 h), and cAMP levels were measured after the addition of cicaprost for 15 min (A), forskolin (B), or cycloheximide (CHX; 10 μg/ml) for 24 h (C). cAMP levels were measured after a 15-min stimulation with cicaprost (63 nM). Data are shown as means ± SE; *$P < 0.05$, $n = 3$ compared with respective cicaprost response in the absence of cicaprost pretreatment by Student’s $t$-test.

Fig. 6. Effect of PKA and PKC inhibition on cicaprost-induced desensitization. Cicaprost preincubation decreased the cicaprost response to 31 ± 9.022%. Bisindolylmaleimide (BIS) and H-89 partially reversed this desensitization to 66 ± 12.5% and 90 ± 1.556%, respectively. *$P < 0.05$, $n = 3$ compared with the respective cicaprost response in the absence of cicaprost pretreatment. PASMCs were preincubated for 1 h with BIS or H-89 before and continuing cicaprost preincubation (63 nM) for 6 h, and cAMP levels were measured after the addition of cicaprost for 15 min (63 nM).

Fig. 7. Effect of the $G_i$ inhibitor pertussis toxin (PTX) on desensitization of the cicaprost response. PASMCs were preincubated for 16 h with pertussis toxin (50 ng/ml) before and continuing 6-h exposure to cicaprost (63 nM). cAMP levels were measured after a 15-min stimulation with cicaprost (63 nM). *$P < 0.05$, $n = 2$ compared with respective cicaprost response in the absence of cicaprost pretreatment.

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statistical significance. Time-course experiments in this study demonstrate a significant desensitization of the cAMP response after a 3-h exposure to cicaprost, whereas protein levels decreased 2 h earlier. This difference in time course of AC protein expression and effects on cAMP accumulation suggest that the major effect is likely to be on the activity of AC isoforms rather than a reduction in AC protein levels. In addition, these findings may also suggest that prolonged cicaprost exposure could result in increased activity of pathways inhibiting AC as well as an early reduction in the amount of AC protein.

Recently El-Haroun and colleagues (10) demonstrated that interleukin-1β, transforming growth factor-β1, and bradykinin caused a reduction in cAMP production in response to prostacyclin and demonstrated a decrease in the mRNA of specific AC isoforms, namely 1, 2, and 4 via a cyclooxygenase-2-dependent pathway after 24 h. Further comparison of that study with ours is difficult since El-Haroun and colleagues (10) relied solely on mRNA studies and did not demonstrate protein expression or attempt allosteric regulation of the cyclases involved. However, in our studies, cicaprost-induced desensitization is more rapid (3 as opposed to 24 h) and so suggests a more direct upstream regulation of AC, possibly via PKA (see below).

To determine the mechanism of reduced AC activity, functional studies using the recognized sensitivities of different AC isoforms to regulation by PKA and PKC were employed. Studies using PKA and PKC inhibitors indicated the involvement of PKA-dependent, and to a lesser extent, PKC-dependent AC isoforms. The primary signaling pathway involved in desensitization appeared to be mediated by PKA with almost complete reversal of the desensitization with PKA inhibition. Previous studies have shown that AC5 catalytic activity can be inactivated by phosphorylation by PKA (17). PKA interaction has also been suggested with AC6, which like AC5 contains a region that is regulated by PKA. Treatment of recombinant AC6, in insect cell membranes, with PKA showed loss of stimulation by high Gsα, whereas treatment of recombinant AC2 with Gsα had no effect (4). It is, therefore, likely that AC2 is not the major AC isoform involved in heterologous desensitization and that AC5/6 plays the predominant role. PKC inhibition also showed a reduction in desensitization after cicaprost exposure, although significant desensitization remained. PKC has been shown to inhibit specific AC isoforms, such as AC6 activity, through phosphorylation of Ser10 of the NH2 terminus and two cytosolic domains, C1 and C2 (20, 23). Conversely, AC2 possesses a PKC binding site within its sequence and has even been shown to activate AC2 (44), so it is possible that in this study, PKC inhibition with bisindoylmaleimide may have affected AC2 function in a negative manner. The interplay between PKA and PKC in the regulation of AC2 and other AC isoforms in PASMCs remains to be elucidated and will form the basis of future work.

PKA has been shown to possess a more complex role than originally thought in IP receptor-mediated signaling effects. Lawler and colleagues (21) have demonstrated that receptor switching from Gsα to Gi may be involved in mouse IP receptor signaling. After Gsα activation, activated PKA has been shown to phosphorylate the mouse IP receptor at Ser357, which in turn favors its coupling to Giα, leading to inhibition of AC. However, Giα does not appear to be involved in the cicaprost desensitization in rat PASMC as the presence of pertussis toxin had no effect on this response. In other systems, Gi has been shown to be a major regulator of AC5/6; however, in the present study, addition of carbachol as a Gs activator failed to demonstrate any effect on either acute or chronic cicaprost stimulation (data not shown). Jourdan and colleagues (19) were also unable to demonstrate inhibition of cicaprost-induced cAMP response with a panel of Gi activators. Rat PASMCs, therefore, appear to signal and so regulate AC through Gi-independent mechanisms in response to cicaprost stimulation.

The involvement of AC in the desensitization of physiological responses is of prime importance. Desensitization of AC activity in peripheral blood mononuclear cells from asthmatic patients after allergen challenge has also been demonstrated (9). Desensitization of β-adrenergic receptor stimulation is well characterized in chronic heart failure (CHF), and in both clinical and experimental observations, there is also desensitization at the postreceptor level. In CHF, AC is also desensitized by a reduction in catalytic activity of the enzyme (2, 29, 34) in addition to isoform composition (16) where, notably, in a model of cardiomyopathy, AC activity was decreased in conjunction with a decrease in AC5 and AC6 mRNA levels. This further emphasizes the role of specific isoforms in the regulation of physiological responses.

Other potential mechanisms of AC regulation are through calveolae and their principal component calveolin, which have been implicated in G protein-mediated transmembrane signaling. Toya and coworkers (38) demonstrated that calveolin peptides are able to directly inhibit specific AC isoforms in a calveolin subtype-dependent manner. Future work will investigate whether calveolin plays a role in the regulation of AC after cicaprost exposure.

In conclusion, our findings support a potential role for AC in the heterologous desensitization observed with cicaprost exposure. This desensitization appears to be regulated by a specific AC isoform (AC5/6) that is inhibited by PKA through a Gi-independent pathway. Manipulation of the signaling pathways involved in cicaprost-induced desensitization in the future may have implications for the treatment of pulmonary hypertension. The identification of the specific AC isoform(s) and the mechanisms involved in AC desensitization, as suggested by this study, may provide new therapeutic targets.

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GRANTS

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