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Regulation of the cAMP-elevating effects of isoproterenol and forskolin in cardiac myocytes by treatments that cause increases in cAMP☆

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Abstract

Regulation of the cAMP-elevating effects of isoproterenol and forskolin in cardiac myocytes by treatments that cause increases in cAMP. We have found that elevations in cyclic AMP (cAMP) have long-term effects on both the β-adrenergic receptor and adenylyl cyclase in cultured chick ventricular myocytes. Pretreatment with isoproterenol for 15 min markedly reduced the cAMP-elevating effect of a subsequent treatment with isoproterenol 18 h later. Responses to isoproterenol were similarly reduced after overnight treatments with forskolin or phosphodiesterase inhibitors. Furthermore, these same treatments also markedly blunted the cAMP-elevating effect of forskolin, a direct activator of adenylyl cyclase. The blunting of the isoproterenol effect was greater than that of the forskolin effect, at least partially because the pretreatments caused a decrease in the number of β-adrenergic receptors as well as a net decrease in adenylyl cyclase activity. Experiments with a recombinant adenovirus to express luciferase under the control of cAMP responsive elements (CREs) showed that the same treatments elevated cAMP sufficiently to drive the transcription of a gene with CREs in its promoter. The blunting of both the isoproterenol and forskolin responses was blocked by the inhibition of protein synthesis or by infecting cells with a recombinant adenovirus that expresses rabbit muscle cAMP-dependent protein kinase inhibitor (PKI). It is hypothesized that one or more adenylyl cyclase isozymes responsible for the generation of cAMP in the myocytes, along with other proteins previously reported to regulate β-adrenergic receptors and perhaps adenylyl cyclase, are negatively regulated by cAMP, most likely at the level of gene expression, and that this regulation may have therapeutic consequences in the treatment of cardiac diseases.
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We have studied the regulation of cAMP metabolism by G-protein-coupled receptors in cultured embryonic chick myocytes in response to acute and long-term perturbations. Previously, we reported that the formation of cAMP in response to β-adrenergic receptor stimulation in embryonic chick cardiac myocytes is inversely related to the concentration of extracellular calcium and is potentiated by calcium channel blockers [1]. That is, elevations in $[Ca^{2+}]_i$ acutely inhibit, or blunt, the cAMP-elevating effect of β-receptor stimulation, and blockade of calcium channels can prevent the calcium-dependent effect. More long-term blunting of β-adrenergic receptor agonist-induced elevations in cAMP also has been reported in several types of cells including C_6 glioma, and DDT1-MF2 smooth muscle cells, and embryonic chick cardiac myocytes. This blunting, or desensitization, correlates with decreases in the concentrations of β-adrenergic receptors [2–5] and has been ascribed to decreases in the transcription of β-adrenergic receptor genes [2,5–7], to a decreased stability of β-adrenergic receptor mRNA [3,8,9], and to the up-regulation of muscarinic acetylcholine receptors and the α-subunits of a pertussis toxin-sensitive G-protein, most probably G_i [4]. However, it is unclear if modifications

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of other components of the cAMP signal transduction pathway also contribute to long-term desensitization. We report herein the results of studies on embryonic chick ventricular myocytes in which we have investigated the more long-term control of cAMP metabolism. We have found that any treatment that would be expected to elevate cAMP in the cardiac myocytes leads to a striking and long-term loss of the cAMP-elevating response to forskolin, a direct activator of adenylyl cyclase (AC) [10]. In addition, the same treatments caused almost a complete long-term loss of the cAMP-elevating effect of the β-adrenergic receptor agonist isoproterenol (ISO), as well as a decrease in receptor number. It is proposed that cardiac β-adrenergic receptors in embryonic chick myocytes are under the control of cAMP either at the transcriptional level and/or at the level of mRNA stability as suggested for β-adrenergic receptors in other cells [2,5–9]. The results of the experiments with forskolin clearly suggest that the cAMP signal transduction pathway is also under the control of cAMP at a level further downstream, namely at the level of adenylyl cyclase. That is, these results suggest that elevations in cAMP can have long-term, negative effects on adenylyl cyclase activity. While there is little known about transcriptional regulation or mRNA stability with regard to adenylyl cyclase, an acute cAMP-dependent effect on adenylyl cyclase to decrease its activity has been suggested to occur [11].

Materials and methods

Materials. Fertilized White Leghorn chicken eggs were purchased from Sharp Sales (West Chicago, IL). Tissue culture supplies were from either Gibco-BRL (Grand Island, NY) or Celox (Hopkins, MN). Reagents for constructing recombinant adenoviruses using the AdEasy system [13] were kindly provided by Drs. T.-C. He and B. Vogelstein (Johns Hopkins University, Baltimore, MD). Milrinone and rolipram were gifts from Sterling-Winthrop Research Institute (Rensselear, NY) and Berlex Laboratories (Cedar Knolls, NJ), respectively. [^{125}I]-cyanopindolol ([^{125}I]CYP) was purchased from NEN Life Science Products (Boston, MA). All other reagents and chemicals were from commercial sources, primarily Sigma (St. Louis, MO).

Primary cultures of embryonic chick ventricular myocytes. Ventricular cardiac myocytes were isolated as previously described [1], preplated to enrich cultures in myocytes (>90% myocytes), and cultured in a M199-based medium containing 0.5% chicken serum, 1% lipid concentrate, and 2% serum supplement TM-235 (Celox, Hopkins, MN) [1]. Cultures were studied 3–5 days after plating (~10^6 cells/well) in 24-well cluster dishes.

Construction and use of recombinant adenovirus to express luciferase under the control of CREs. The plasmid pADneo2 C6-BGL [12] that contains an insert to express luciferase under the control of six CREs was a gift from Dr. A. Himmler (Bender GmbH, Vienna, Austria). The insert was removed from the plasmid using *Not*I and *Bam*HI, ligated into pADTrack-CMV that was cut with *Not*I and *Bgl*II, and co-transfected with pADEasy-1 into electrocompetent BJ5183 bacteria as described [13]. Recombinant adenovirus (AdCRELuc) was isolated and purified from 911 cells as described [13]. A control adenovirus was constructed in a similar manner using the vector pADTrack-CMV expressing green fluorescent protein, but without the subcloned gene under the control of the CREs.

For viral infection, cells were incubated for 2 h (37 °C) in 250 μl of the M199-based medium containing the virus (MOI ~ 100) after which 750 μl of the complete growth medium was added. Cells were treated as described in individual experiments; luciferase was assayed 2 days post-infection using a luciferase assay system (Promega, Madison, WI).

Measurement of relative changes in cAMP using the [^{3}H]adenine-labeling technique. Relative levels of cAMP expressed as percent conversion of [^{3}H]cAMP from [^{3}H]adenine were determined essentially as previously described [1]. Briefly, cells in 24-well cluster plates were labeled by incubating cells with [^{3}H]adenine (2 μCi/ml, 300 μl/well) in a physiological salt solution (PSS) containing adenosine deaminase (2 U/ml) for 60–90 min. Afterwards the cells were rinsed with the PSS, incubated for 10 min in 250 μl of the PSS containing adenosine deaminase ± additions as specified, and incubated an additional 10 min with an additional 250 μl of the PSS containing ISO or forskolin (FK) + phosphodiesterase (PDE) inhibitors as indicated, and the incubation terminated. Unless otherwise specified, rolipram (Rol, 100 μM) + milrinone (Mil, 10 μM) were used to inhibit PDE activity [1]. Initially incubations were terminated by aspirating the incubation medium and adding 1 ml of 5% trichloroacetic acid (TCA); later reactions were terminated by the addition of an equal volume of 10% TCA. The two procedures gave the same results (data not shown) and the latter protocol was used in most of the experiments reported herein. Cyclic AMP was isolated using Dowex and alumina columns as described by Salomon [14]. Percent conversion of [^{3}H]ATP to [^{3}H]cAMP was calculated after using [^{14}C]cAMP to correct for cAMP recovery [1]. Unless otherwise specified data shown are from individual experiments (4–6 wells per group) which were repeated at least 2–3 times. This approach was taken because of the limited number of highly purified cultures that could be produced and by the necessity of using 24-well culture dishes to obtain a good signal for the cAMP assays. Statistics were performed on each culture dish (generally one group of 4 wells and four groups of 5 wells) and the results were accepted if they were the same in 2–3 successive experiments with no conflicting results. Figures show data as means ± standard errors. Standard error bars are not shown if they are too small to show in the figure. As preliminary experiments showed that none of the treatments studied affected basal cAMP levels, single (untreated) basal levels were performed in subsequent experiments and are shown in the figures. Statistics were performed using GraphPad Prism (Graph Pad Software, San Diego, CA) accepting $P < 0.05$ as significant. Student's t test was used for comparisons involving two groups; ANOVA followed by Tukey's test was used for experiments involving more than two groups.

Miscellaneous methods. Protein was assayed using a Coomassie protein assay reagent (Pierce, Rockford, IL). Crude membrane preparations for binding were made essentially as previously described [15]. Binding reactions ([^{125}I]CYP, 20 mM Hepes buffer, pH 7.5, 1 mM EDTA, 5 mM $MgSO_4$, 0.1% BSA, and membranes) were incubated for 75 min at 30 °C after which bound and free ligands were separated by filtration over GF/B filters. DL-Propranolol (10 μM) was used to establish nonspecific binding. [^{125}I]CYP binding was partitioned into binding to β_1- and β_2-adrenergic receptors using the β_1-receptor antagonist CGP-20712A as previously described [16].

Results

Isoproterenol pretreatment blunts the subsequent effects of isoproterenol and forskolin

In order to investigate the mechanisms involved in the long-term regulation of cAMP formation in cardiac myocytes, we assessed the effect of pretreating embry-

onic chick ventricular myocytes with ISO and measuring subsequent responses to ISO or forskolin. These experiments were precipitated by the initial observation that treatments of myocytes with ISO (10 μM) for 15 min approximately 18 h prior to a second addition of ISO resulted in a markedly reduced responsiveness of the cells to the cAMP-elevating effect of ISO. In order to test whether such an effect might be due to modifications at the level of the β-adrenergic receptor or adenylyl cyclases, we performed a similar experiment in which we tested the effect of ISO pretreatment on the subsequent responsiveness of the cells to ISO and forskolin, a direct activator of adenylyl cyclase (Fig. 1). Cells were pretreated with ISO (10 μM) for 15 min, rinsed, and then tested 18 h later for responsiveness to ISO or forskolin. Not only was the response to ISO (10 μM) markedly blunted (Fig. 1), but also the response to forskolin (3 μM) was strikingly decreased in the ISO-pretreated cells (Fig. 1).

Since we have previously reported that changes in intracellular Ca^{2+} can negatively regulate cAMP production in the cultured chick myocytes and that the calcium channel blocker D-600 markedly increases the cAMP-elevating effect of ISO in chick myocytes when $[Ca^{2+}]_o = 0.9$ mM [1], we tested if the Ca^{2+} sensitivity of adenylyl cyclase contributed to the blunting response by measuring cAMP formation in the presence and absence of D-600 (Fig. 1). While D-600 markedly potentiated the responses to both ISO and forskolin, the blunting effect

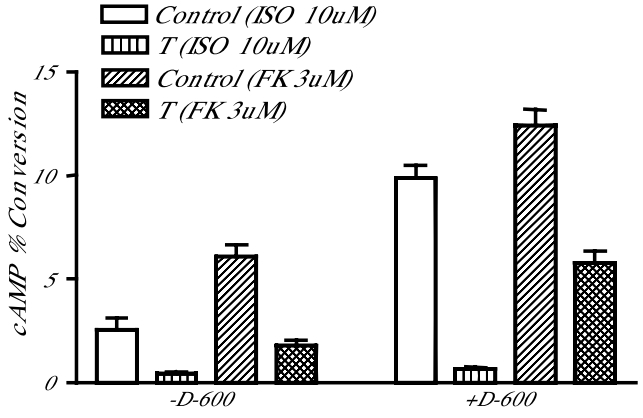

Fig. 1. Effect of pretreatment with ISO (10 μM for 15 min) on subsequent cAMP-elevating responses to ISO (10 μM for 10 min) or forskolin (3 μM for 10 min) ~18 h later. The experiments were performed in the absence (left) or presence (right) of the calcium channel blocker D-600 (10 μM), which was added 10 min prior to the addition of ISO or forskolin. The experiment was performed in medium containing 0.9 mM Ca^{2+}. T, ISO pretreated. Responses in all groups pretreated with ISO (T) were significantly ($P < 0.05$) decreased from their corresponding controls. The concentration of ISO tested (10 μM) was maximal for cAMP production (data not shown). While the concentration of forskolin tested (3 μM) was near the bottom of the forskolin dose–response curve (see Fig. 6B), this concentration of forskolin was chosen because 1–3 μM generally produced a response greater than, or similar to, the maximal ISO response.

of ISO pretreatment was evident whether or not the calcium channels were blocked (Fig. 1). Thus, the blunting effect of ISO pretreatment did not appear to be dependent on a variation in the sensitivity of a calcium-sensitive adenylyl cyclase to $[Ca^{2+}]_i$.

The blunting effect of pretreatments with cAMP-elevating agents is not restricted to specific PDE inhibitors

Previous reports have established that any treatment that elevates cAMP in DDT1-MF2 cells, or C_6 glioma cells, or embryonic chick myocytes can cause a blunting of the cAMP-elevating effects of β-receptor agonists [2,4–6]. Therefore we performed experiments to determine if pretreatments with other cAMP-elevating agents would cause a blunting of cAMP formation in response to subsequent treatments with ISO or forskolin in cardiac myocyte cultures we prepared. Cells were treated overnight (~18 h) with forskolin or PDE inhibitors, washed, and then labeled with [^{3}H]adenine for subsequent measurement of cAMP levels. (Note, the PDE inhibitors were present during all cAMP assays but only during some pretreatments as noted.) Approximately 100 min elapsed between the termination of the pretreatment and the subsequent exposure to ISO or forskolin. Pretreatment of chick ventricular myocytes with low concentrations of forskolin (Fig. 2A) markedly reduced the ability of ISO to stimulate increases in cAMP. This suggested that the effects on the β-adrenergic receptor did not require agonist occupancy of the receptor. Similarly, the overnight incubation of cells with a combination of PDE inhibitors, rolipram + milrinone, blunted the subsequent ISO response. Importantly, pretreatment with the PDE inhibitors rolipram + milrinone also caused a blunting of the ability of forskolin to stimulate increases in cAMP (Fig. 2B). Furthermore, pretreatment with another PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), also caused a significant blunting of the cAMP-elevating effect of forskolin (Fig. 2B). Thus, the blunting effect of pretreatments with PDE inhibitors was not restricted to specific PDE inhibitors. Since the abilities of either the β-adrenergic receptor agonist or forskolin to elevate cAMP were markedly reduced, the results suggested that the treatments that elevated cAMP might have affected more than one step in the cAMP signal transduction pathway.

PKI expression significantly increases the response to isoproterenol and forskolin in both control and rolipram + milrinone pretreated cells

Since any pretreatment that elevated cAMP blunted the subsequent effects of ISO and forskolin to elevate cAMP, we tested whether the activation of protein kinase A (PKA) was necessary for the blunting to occur. We used a recombinant adenovirus, AdPKI [17], to

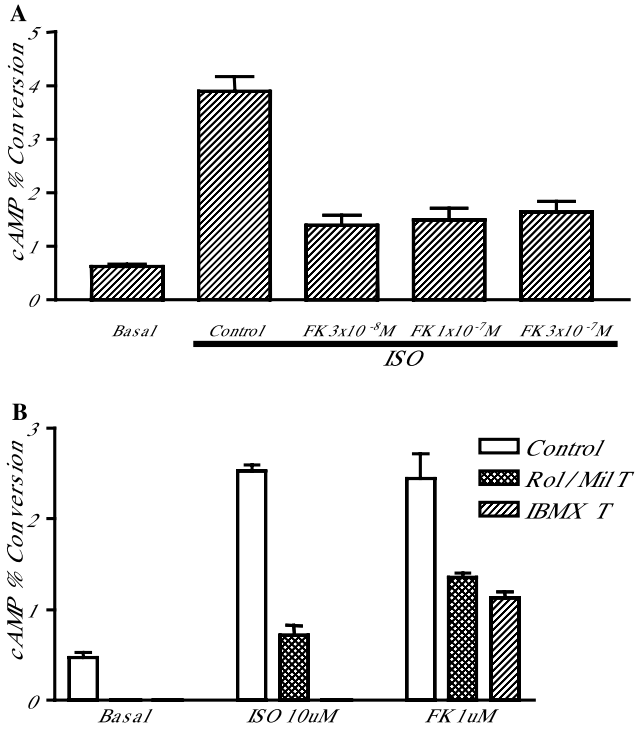

Fig. 2. Effects of pretreatments with cAMP-elevating agents on the subsequent cAMP-elevating responses to ISO or forskolin. (A) Effect of treatments with different concentrations of forskolin overnight on subsequent cAMP-elevating effect of ISO (10 μM). Responses to all groups pretreated with forskolin were significantly ($P < 0.05$) decreased from control. (B) Effect of overnight treatments with rolipram + milrinone (Rol/Mil T) on the cAMP-elevating effects of ISO or forskolin and the effect of an overnight treatment with IBMX (IBMX T) on the cAMP-elevating effect of forskolin. The responses in all treatment groups (T) were significantly decreased from their corresponding controls. Both experiments were performed in 0.9 mM Ca^{2+} in the presence of 10 μM D-600. The forskolin or PDE inhibitors used for the pretreatments were washed out prior to labeling the cells with [^{3}H]adenine, and approximately 100 min elapsed before the cells were treated with ISO or forskolin for cAMP measurements. Other conditions were as described in the legend to Fig. 1.

express PKI in the myocytes for this purpose. The infection of cells with the AdPKI at an MOI of 20 blocked or reversed the blunting effect of rolipram + milrinone pretreatment on the subsequent responses to forskolin and ISO (Fig. 3). This MOI was chosen because we had previously used a similar adenovirus that expresses β-gal to show that human microvascular endothelial cells express β-gal in <30% and >95% when infected with MOIs of 10 and 100, respectively [17]. Interestingly, infection with the PKI-expressing virus (MOI = 20) also potentiated the effects of ISO and forskolin in cells not subjected to rolipram + milrinone pretreatment. The infection of cells (same MOI = 20) with a control virus which only expressed green fluorescent protein had no effect on responses to ISO or forskolin (data not shown). This suggests that basal cAMP levels might also contribute to regulation of cAMP generation.

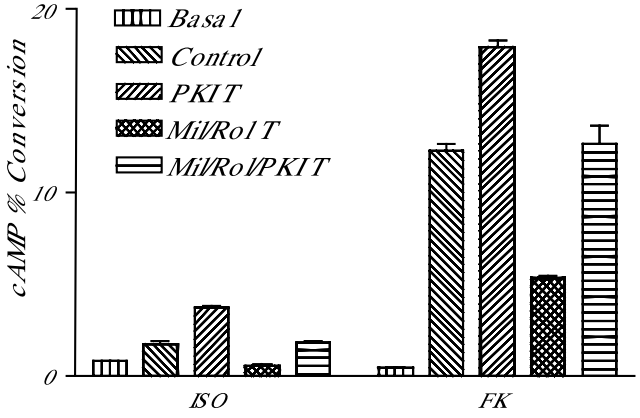

Fig. 3. Effect of expression of PKI on cAMP-elevating effects of ISO (10 μM) and forskolin (3 μM) in control and rolipram + milrinone pretreated cells. Cells were infected with AdPKI 48 h before the cAMP measurements and rolipram + milrinone were added approximately 18 h before the experiment. Other conditions were as in the legend to Fig. 2. PKI expression significantly increased the responses to ISO and forskolin in both control and rolipram + milrinone pretreated cells (all $P < 0.05$).

PDE inhibitor may affect the transcription of the genes containing CREs in their promoters

Since we thought it unlikely that the blunting effect of a 15 min treatment with ISO would cause a cAMP-dependent phosphorylation to occur that would remain 18 or so hours later, we hypothesized that the blunting effect was somehow dependent on the phosphorylation of a cAMP binding protein and the subsequent modulation of genes containing CREs in their promoters. In order to test this possibility we used a model system consisting of a recombinant adenovirus to express luciferase under the control of six CREs (AdCRELuc) to determine if the treatments that caused blunting also elevated cAMP sufficiently to drive the transcription of a gene containing CREs in its promoter. This plasmid used to make this virus and consequently the recombinant virus also codes for the expression of green fluorescent protein. The efficiency of gene transfer into myocytes using the Ad vectors (± the luciferase-CRE construct) was determined by visualization of cells with an inverted fluorescent microscope and showed that >90% myocytes expressed green fluorescent protein after 2 days post-infection with the recombinant adenoviruses at 100 MOI (data not shown). We then tested myocytes to determine if short-term ISO treatment would result in an increase in luciferase activity. Treatments with various concentrations of ISO for 15 min increased the amount of luciferase in the cells the next day in a dose-dependent manner (Fig. 4). The infection of cells with the related adenovirus which expressed only green fluorescent protein (MOI = 100) did not affect the cAMP-elevating effect of ISO (data not shown). Similar experiments on cells infected with AdCRELuc showed that the over-

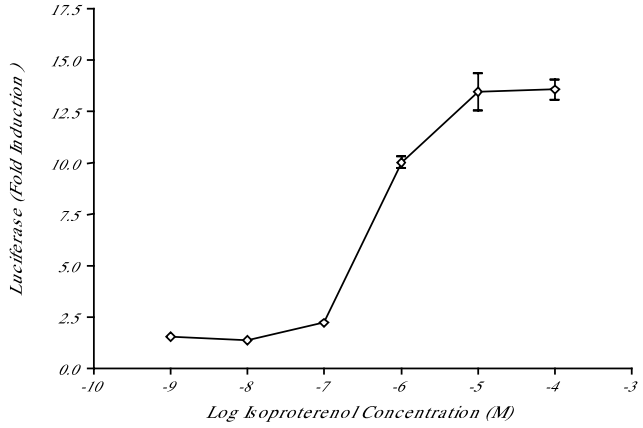

Fig. 4. Dose–response curve for ISO to induce the expression of luciferase in cardiac myocytes. Cells were infected with AdCRELuc (MOI 100) at time zero, treated with ISO at various concentrations for 15 min approximately 24 h later, and assayed for luciferase at approximately 48 h. Luciferase expression is depicted as fold increase over basal expression.

night treatment with milrinone or milrinone + rolipram caused 4- to 40-fold elevations in luciferase the next day (Fig. 5). Thus, it is clear that the pretreatments that blunted the cAMP-elevating effects of ISO and forskolin can affect the transcription of luciferase under the control of CREs. While this model system only directly demonstrates the regulation of this gene, the results imply that the treatments with the PDE inhibitors used here may well affect the transcription of other genes containing CREs in their promoters.

The maximal effects of isoproterenol, and perhaps forskolin, are depressed by pretreatments with PDE inhibitors

It is not evident from the data thus far presented whether the blunting effect of PDE inhibitor pretreatment involved decreases in the maximum responses to ISO and forskolin or a shift in their EC_{50}s. Fig. 6A shows that 10 μM ISO produced a maximal response in both control and rolipram + milrinone pretreated cells, clearly demonstrating that the maximal response to ISO was reduced by the PDE inhibitor pretreatment. Fig. 6B shows dose–response curves to forskolin in control and rolipram + milrinone pretreated cells. These results demonstrated that the responses were clearly depressed in the PDE inhibitor group at all concentrations of forskolin studied. (The blunting of the response to 1 μM forskolin is not clear in this figure because of the scaling to show the responses to the higher concentrations.) However, the highest concentration of forskolin tested (30 μM) did not clearly give a maximum response; the effects of higher concentrations of forskolin were not tested because of the large percent conversion of [^{3}H]adenine and depletion of ATP.

In all experiments in which we measured the blunting effect caused by pretreatment with PDE inhibitors on subsequent exposures to either ISO or forskolin it appeared that the response to ISO was blunted more than that of forskolin, and treatments that elevated cAMP

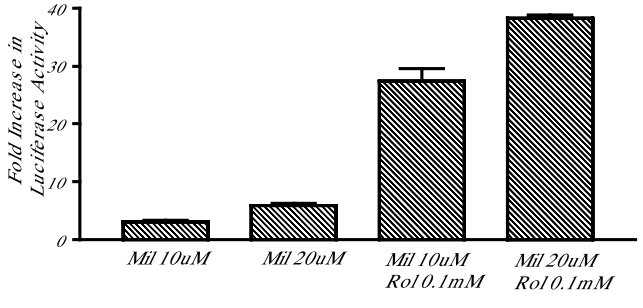

Fig. 5. Effects of milrinone or rolipram + milrinone pretreatments on luciferase expressed in myocytes. The experimental design is the same as detailed in the legend to Fig. 4 except that treatments with the PDE inhibitors were overnight. Luciferase was increased in all pretreated groups compared to basal expression ($P < 0.05$).

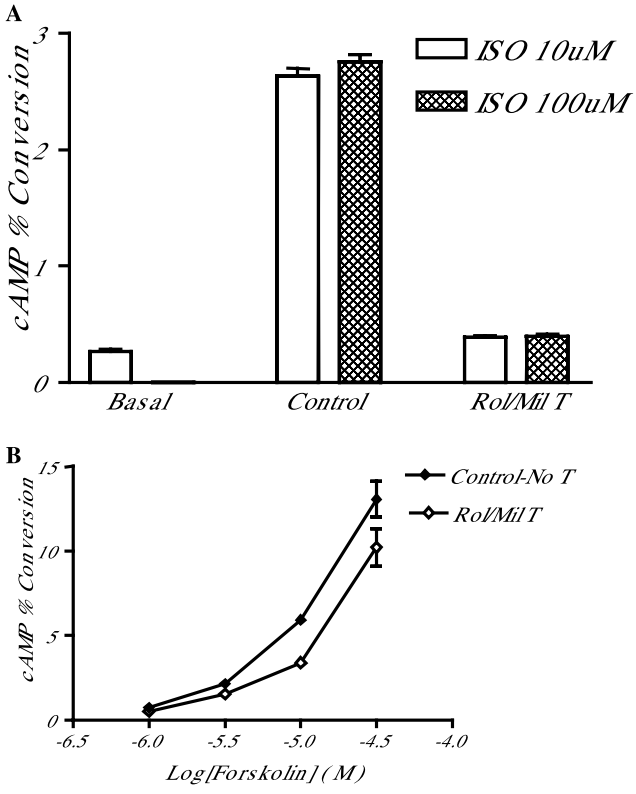

Fig. 6. Effects of rolipram + milrinone pretreatment on the cAMP-elevating effects of two high concentrations of ISO (A) and on the dose–response curve to forskolin (B). Conditions were as described in the legend to Fig. 2 except that the concentrations of ISO and forskolin varied as depicted. The response to 10 μM ISO was not significantly different from the response to 100 μM ISO (A) in either the control or the rolipram + milrinone pretreated groups ($P > 0.1$). The responses to forskolin (B) in the rolipram + milrinone pretreated cells were significantly less than their corresponding controls for all concentrations studied ($P < 0.05$).

have been reported to decrease the amount of β-receptor protein in other cells [2,4,5], we assayed the binding of [^{125}I]CYP to membranes prepared from cells pretreated overnight with rolipram + milrinone. In five experiments in which the binding of [^{125}I]CYP was determined at a concentration of 211–275 pM (~10 times the K_D determined in these cells, data not shown), the binding of the ligand to the membranes prepared from rolipram + milrinone treated cells was $56.5 \pm 3.7\%$ of control (significantly different, $P < 0.05$). These results suggested that a decrease in β-adrenergic receptor number, as well as a modification of AC activity, contributed to the blunting of the β-adrenergic receptor response caused by elevations in cAMP. As it has been reported that β_1-adrenergic receptors in embryonic chick cardiac myocytes couple to adenylyl cyclase, while β_2-receptors in the same cells couple to phospholipase A_2 [17], this ~50% loss of receptors does not necessarily reflect only a loss of receptors coupled to adenylyl cyclase. In three experiments in which the binding of [^{125}I]CYP was partitioned into β_1- and β_2-receptors using the β_1-receptor antagonist CGP-20712A, the relative amounts of β_1- and β_2-receptors were unaffected by rolipram + milrinone treatment (~60% β_1 in both cases, data not shown), suggesting that both β_1- and β_2-receptors may be down-regulated by the rolipram + milrinone treatment.

Discussion

The novel results presented here demonstrated that pretreatment of cardiac myocytes with agents that elevate cAMP could affect subsequent cAMP generation at multiple levels. Both the ability of a β-adrenergic receptor agonist and that of a direct activator of adenylyl cyclase to elevate cAMP were attenuated chronically by pretreatment of myocytes with cAMP-elevating agents. In addition, elevation of cAMP led to decreases in the number of β-adrenergic receptors. Taken together, these results suggested that both the β-adrenergic receptor and adenylyl cyclase were affected by pretreatment of cells with cAMP-elevating agents. The effect of such treatments on adenylyl cyclase has not been previously reported. Even though it has been known for some time that treatments that elevate cAMP decrease the amounts of both β_1- [5,7,8] and β_2- [2,6,9] adrenergic receptors expressed in various types of cells, most work on the regulation of signaling responses has focused on the acute control of β-adrenergic and other receptors via receptor phosphorylation [18]. Although receptor phosphorylation mechanisms are undoubtedly important for the control of a large number of G-protein coupled receptors, the potential for therapeutically useful agents such as β-adrenergic receptor agonists and PDE inhibitors to decrease the sensitivity of important transmitter systems via other mechanisms cannot be ignored. It has been reported [19] that the long-term (72 h) treatment of neonatal rat heart cells in culture with noradrenaline decreases the ability of forskolin to stimulate the adenylyl cyclase activity in subsequently prepared membranes. However, these workers ascribed this to an increase in a $G_i\alpha$ rather than a decrease in adenylyl cyclase. It is not possible to compare these and the present results as we find that the β-adrenergic receptors in embryonic chick myocytes rapidly desensitize, suggesting that the receptors would be unresponsive during a large part of a 72 h pretreatment. Interestingly, it should be noted that alterations in β-adrenergic receptor signaling have been implicated in congestive heart failure [20,21], a disease also associated with elevated levels of circulating norepinephrine [22], and that a recent report [23] suggests that severely failing myocardium from children contains a reduced level of adenylyl cyclase catalytic activity unrelated to changes in a G_i.

Previous results from other laboratories [2,4–6], as well as the present results, clearly establish the fact that blunting of the effects of β-receptor agonists occurs hours after exposure to agents that elevate cAMP, and that such affects are not specifically generated after treatments with β-receptor agonists. Part, at least, of this blunting has been shown to be associated with decreases in β-receptor protein in other cell types [2,4,5]. Similarly, we found that the number of β-adrenergic receptors (estimated by the binding of high concentrations of the β_1, β_2 antagonist, [^{125}I]CYP) in membranes prepared from cardiac myocytes treated overnight with rolipram + milrinone was reduced by approximately 50%. Thus, part of the blunting of the ISO response may have been due to a decreased number of β-receptors. (It should be mentioned that previous workers [2] reported that an increase in β_2-adrenergic receptor mRNA occurs at a time before a subsequent fall in receptor mRNA and receptor number. We have not measured mRNA or studied similar treatments to determine if β-receptor up-regulation precedes the decreases in β-receptor protein or responsiveness reported herein.)

Although an acute cAMP-dependent regulation of adenylyl cyclase has been observed in chick hepatocytes [11], a more long-term regulation such as that demonstrated herein has not been reported. This type of regulation has widespread implications in that the signaling by many G-protein coupled receptors could potentially be influenced by a negative modification of adenylyl cyclase. An illustration of this is evident in the present assessment of β-adrenergic receptor signaling in cardiac myocytes. Since adenylyl cyclase is downstream of the β-adrenergic receptor in the cAMP signal transduction pathway, and the blunting of the ISO response was greater than the blunting of the forskolin response, we hypothesize that the blunting of the ISO response was

due to changes both at the level of β-adrenergic receptors and that of adenylyl cyclase. That a modification of adenylyl cyclase is involved in the long-term blunting of the responsiveness of β-adrenergic receptors has not been demonstrated previously.

The blunting of the forskolin response was markedly attenuated by *Pseudomonas* toxin (data not shown), suggesting that protein synthesis was necessary for the blunting at the level of adenylyl cyclase to occur. Importantly, it was recently reported that CRE-mediated gene transcription in cultured embryonic chick cardiomyocytes is attenuated by forskolin pretreatment [24]. For a variety of reasons the authors speculated that this may be due to an up-regulation of the inhibitory cAMP-responsive early repressor (ICER). An interesting possibility is that the protein that needs to be synthesized to modulate adenylyl cyclase is ICER. This protein has been reported to be involved in the cAMP-mediated regulation of β_1-adrenergic receptors in C_6 glioma cells [7]; however, as yet there is no evidence to suggest that it is involved in the modulation of adenylyl cyclase. Alternately, an inducible protein that destabilizes mRNA could be involved [8]. Interestingly, it has been reported that human failing heart contains not only a decreased level of β_1-adrenergic receptors but also an increase in AUF1, a protein involved in mRNA destabilization [25]. Thus, increases in cAMP may induce an effect on adenylyl cyclase mRNA stability or on the stability of the mRNA of another protein that is involved in adenylyl cyclase regulation. Further experiments are needed to determine which of these mechanisms are involved. It is well established that type V and VI adenylyl cyclases are the primary isoforms of adenylyl cyclase expressed in cardiac myocytes [26–28]. We previously used degenerate PCR primers designed to amplify all adenylyl cyclase isoforms that amplify cDNAs derived from chick myocyte mRNA. We isolated and sequenced many (>20) clones and found all sequences to be homologous with either type V or type VI adenylyl cyclase [29]. RNA protection assays subsequently performed showed that the myocytes contained 4–5 times type V mRNA as compared to type VI. Thus, although we were able to isolate a type IX adenylyl cyclase from a chick heart library suggesting that at least one other adenylyl cyclase isozyme is expressed in myocytes [30], it is likely that the expression of either type V or VI adenylyl cyclase is modulated in the experiments reported herein, i.e., by elevations in cAMP.

The hypothesis that the blunting effect of PDE inhibitors on the ISO and forskolin responses is mediated by cAMP assumes that treatments with the PDE inhibitors caused physiologically relevant elevations in cAMP and implies that an activation of PKA is involved. The abilities of the various treatments that blunt the effects of ISO and forskolin to also markedly elevate luciferase under the control of CREs (Figs. 4 and 5) suggested that these treatments have the potential to affect the transcription of any genes with one or more CREs in their promoters. Similar experiments with luciferase under the control of the promoter of the relevant adenylyl cyclase isozyme(s) will be necessary to directly test if the transcription of an adenylyl cyclase gene is under the control of cAMP. In the meantime, the ability of PKI expressed by infection of a recombinant adenovirus (AdPKI) to block the blunting effect of rolipram + milrinone (Fig. 3) supported the hypothesis that the activation of PKA was necessary for the blunting effect. Interestingly, the cAMP-elevating effects of both ISO and forskolin were increased in control (unpretreated), PKI-adenovirus-infected cells (Fig. 3). While there are various explanations for this observation, the most straightforward explanation is that PKA exerts a tonic inhibitory effect on adenylyl cyclase either directly or indirectly by a yet to be defined mechanism.

To summarize, we have shown that treatments that cause significant elevations in cAMP as measured by a recombinant adenovirus expressing luciferase under the control of CREs cause a blunting of the cAMP-elevating effects of a β-adrenergic receptor agonist (ISO) and a direct stimulator of adenylyl cyclase (forskolin). The blunting of the effect of forskolin clearly implicates a change in the level of adenylyl cyclase, is dependent on protein synthesis, and involves PKA. It is hypothesized that such events could occur with the chronic use of drugs such as PDE inhibitors and could also be involved in the progression of disease states such as congestive heart failure. However, it should be noted that the present study was performed on a model system, cardiomyocytes in culture. While this model system has many advantages, other influences appear to be of importance in the intact human being. An excellent review of this and related areas has recently appeared [31].

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