**α-Adrenergic Reduction of Cyclic Adenosine Monophosphate Concentrations in Rat Myocardium**

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**SUMMARY** We determined the effect of α-adrenergic receptor stimulation on cyclic adenosine monophosphate (cyclic AMP) concentrations in isolated myocytes from adult rat hearts and in isolated perfused rat hearts. Activation of α-adrenergic receptors with either phenylephrine (10^-8 M to 10^-6 M) or epinephrine (10^-8 M to 10^-6 M) plus propranolol (10^-6 M) resulted in a reduction in cyclic AMP levels in isolated myocytes. The action of phenylephrine was antagonized by phentolamine (10^-6 M). Phenylephrine (10^-5 M) attenuated cyclic AMP generation in response to isoproterenol (10^-6 M and 10^-5 M). However, this effect of phenylephrine was not antagonized by phentolamine. Elevation of cyclic AMP concentrations produced by glucagon and by theophylline in isolated myocytes was attenuated by phenylephrine and by epinephrine plus propranolol and the attenuation was antagonized by phentolamine. In isolated perfused rat hearts epinephrine (10^-6 M), when given with propranolol, diminished the rate of development of tension and also reduced tissue levels of cyclic AMP. Epinephrine alone, as well as isoproterenol, increased contractility and myocardial cyclic AMP concentrations as expected. These results indicate that catecholamines may increase or decrease cyclic AMP levels in rat myocardium, depending on the intensity of stimulation of receptor types. Increases are mediated by β-adrenergic receptors, whereas decreases appear to be mediated by α-adrenergic receptors.

IN 1967, Robison et al. hypothesized that activation of either α-adrenergic or β-adrenergic receptors may modify the activity of adenylate cyclase and result in changes in the rate of conversion of ATP to cyclic adenosine monophosphate (cyclic AMP). They further predicted that α-sympathomimetic agents could induce a decrease in the activity of the enzyme in some tissues. This original hypothesis has since been corroborated by studies which indicate that some α-adrenergic effects are mediated by a reduction in the tissue level of cyclic AMP.

Although there is an abundance of evidence that activation of α-adrenergic receptors can lead to alterations in the electrophysiological and mechanical functions of the heart, no intracellular mediator or biochemical basis for these effects has been identified. It has been fairly convincingly shown that these α-adrenergic effects are not mediated by an increase in myocardial cyclic AMP concentrations. The question remains, however, whether any of the α-adrenergic effects on the heart might be mediated by a decrease in tissue cyclic AMP levels. The purpose of the present study was to attempt to demonstrate whether stimulation of myocardial α-adrenergic receptors is associated with a fall in cyclic AMP concentrations. The studies were carried out using isolated adult rat heart cells. To extend the findings to the intact heart, we also performed a limited parallel study of α-adrenergic effects on contractility and cyclic AMP levels of isolated, perfused rat hearts.

**Methods**

**ISOLATION OF CELLS**

Our method for isolating cells is a modification of that described by Berry et al. Briefly, this involves Langendorff perfusion of rat hearts at 37°C with a well oxygenated, nominally Ca^2+- and Mg^2+-free buffer solution containing (mM): NaCl, 134; KCl, 5; sodium phosphate (pH 7.4), 6; and glucose, 10. After about 5 minutes, collagenase (80 U/ml) and hyaluronidase (82 U/ml) were added to the perfusion fluid and the perfusion was continued at 37°C for an additional 20 minutes. The hearts then were removed from the perfusion apparatus, sliced, and incubated in a shaking bath for a further 10 minutes in fresh perfusion fluid. The disaggregated tissue was quickly cooled and then filtered through nylon mesh. The cells were collected from the filtrate by centrifugation at a force of 37 g, washed twice by resuspension and recentrifugation in an incubation medium that contained (mM): NaCl, 120; KCl, 5; MgCl₂, 1.2; sodium phosphate (pH 7.4), 10; and glucose, 5. Further details of the method are described by Farmer et al.

Cell viability was determined by the criterion of trypan blue exclusion: the percentage of cells in three high power fields which were able to exclude 0.3% trypan blue was determined under a microscope using a hemocytometer counting chamber and a hand tally counter.

**EXPERIMENTS WITH ISOLATED CELLS**

The concentration of cells harvested by the isolation procedure was determined by quantifying the protein content per unit volume of suspended cells. For experiments, the isolated cells were resuspended to a final concentration of 4–6 mg dry weight of cells per ml. The cells (1-ml
samples) then were pipetted into 25-ml siliconized Erlen-
meyer flasks containing 2 ml of phosphate HEPES buffer
which had been oxygenated with 100% oxygen for 60
minutes prior to use. The flasks then were placed in a
Dubnoff shaking water bath heated to 37°C. All flasks
were allowed to preincubate in the water bath for 5 min-
utes to bring the temperature of the cells to 37°C. After
the 5-minute preincubation period drugs were added for
the actual experiment.

PERFUSION OF WHOLE HEARTS

Hearts from rats of either sex weighing 300–500 g were
perfused by the Langendorff method as previously de-
scribed. The modified Krebs-Henseleit perfusion buffer
contained (mm): NaCl, 118; KCl, 4.8; MgSO
4, 1.2;
KH
2PO
4, 1.0; NaHCO
3, 27.2; CaCl
2, 2.0 or 2.5; and
glucose, 11.1. The temperature was maintained at 37°C.
Hearts were paced with an electronic simulator at a rate of
210 stimuli/min. Diastolic tension was set at 5 g before
beginning the experiment. Developed tension, its first
derivative (dT/dt), and perfusion pressure were recorded
continually throughout the experiment. The contractile
responses were similar whether they were assessed from
developed tension or dT/dt. All reported analyses of
changes in contractility were made on the basis of changes
in dT/dt.

The hearts were perfused for at least 30 minutes before
starting any experimental intervention. Drugs were ad-
ministered through a side arm that entered the main perfu-
sion stream about 2 cm above the coronary arteries of
the heart. This arrangement allowed mixing and diluting of
the drugs immediately prior to use and also did not neces-
sitate prolonged oxygenation of the drugs. All of the drugs
were administered by continuous infusions.

ASSAY OF CYCLIC AMP

Hearts in which cyclic nucleotide levels were to be
measured were frozen quickly with metal tongs that previ-
siously had been cooled to the temperature of liquid nitro-
gen (~196°C). Freeze-clamped hearts were pulverized and
the powdered tissue was prepared for assay by homog-
genization in 5% trichloroacetic acid (TCA), which was
then removed by extraction with ether. The experiments
with isolated cells were terminated by the addition of 0.5
ml of 35% TCA to the cell suspension (final concentration
of TCA = 5%) followed by homogenization of the cells.
The TCA was removed by extraction with ether.

The extracted homogenates of both the whole hearts
and isolated cells were then introduced into an ion-ex-
change column (Dowex AG 1 x 8, 200–400 mesh, for-
mate form) to remove substances that might interfere with
the assay of cyclic AMP. Cyclic AMP concentrations were
measured by a competitive protein-binding assay using
binding protein derived from beef adrenal glands as
previously described.

CHEMICALS

L-Isoproterenol bitartrate, L-epinephrine bitartrate, L-
norepinephrine HCl, L-phenylephrine HCl, cyclic AMP,
theophylline, and hyaluronidase (purified from bovine
testes) were obtained from Sigma; purified collagenase
(derived from Clostridium histolyticum) from Worthington
Biochemicals; DL-propranolol HCl from Ayerst; crystal-
line glucagon from Lilly; phentolamine HCl from Ciba;
³H-cyclic AMP (specific activity, 37.7 Ci/mmol) from New
England Nuclear.

STATISTICAL ANALYSIS

The statistical significance of differences between means
was determined by unpaired t-tests.²⁷

RESULTS

VIABILITY OF ISOLATED CELLS

A detailed description of the morphology and biochemi-
cal properties of isolated rat heart cells is presented else-
where.²¹ Only those features of the cells which are directly
relevant to this study are considered in this paper. As-
essed within 5 minutes after preparing the cells, the per-
cent of the total number of cells which were viable, as
judged by their ability to exclude 0.3% trypan blue, was
72 ± 1% (mean ± SEM) for eight separate preparations.
At Ca
2+ concentrations of 10⁻⁵ m or less, the cells re-
mained viable for at least 30 minutes when incubated at
37°C.

The isolated cells were sensitive to the Ca
2+ concen-
tration in the incubation medium. When the Ca
2+ concen-
tration was increased to 10⁻⁴ m, viability of the cells de-
creased to about 45% in 5 minutes and about 30% in 12
minutes. Therefore, all cells were incubated in a medium
containing about 10⁻⁶ m Ca²⁺.

EFFECT OF CATECHOLAMINES ON CYCLIC AMP
CONCENTRATIONS IN ISOLATED CELLS

Cyclic AMP levels rapidly increase when cells are ex-
posed to 10⁻⁷ m isoproterenol (Fig. 1, inset). Further-
more, the steady state level of cyclic AMP appears to be
stable, during continuous exposure to isoproterenol, for at
least 5 minutes. We obtained similar results with kinetic
studies on the effect of norepinephrine (10⁻⁷ m) on cyclic
AMP levels. Thus, most of the experiments with catechol-
amines were terminated after 2 minutes of incubation of
cell with drugs.

The isolated cells responded to catecholamines in the
expected manner (Fig. 1). Isoproterenol was the most
potent catecholamine that increased cyclic AMP levels:
the threshold concentration for stimulation was between
10⁻⁶ m and 10⁻⁵ m, and 10⁻⁵ m produced a 250% increase
in cyclic AMP concentrations. To further support the β-
adrenergic receptor mediation of increased cyclic AMP
levels, cells were incubated with varying concentrations
of isoproterenol simultaneously with 10⁻⁶ m propranolol
(Fig. 2). Propranolol attenuated the response of the cells
to isoproterenol in a competitive manner.

EFFECT OF α-ADRENERGIC RECEPTOR AGONISTS ON
CYCLIC AMP CONCENTRATIONS IN ISOLATED CELLS

As in our studies on β-adrenergic receptor agonists, we
performed preliminary experiments to determine the time
course of the effects of α-adrenergic receptor agonists on
cyclic AMP levels (Fig. 3, inset). Phenylephrine (10⁻⁷ m)
appeared to reduce cellular cyclic AMP levels, but this effect developed much more slowly than the β-adrenergic receptor-mediated stimulation of cyclic AMP levels. In the presence of phenylephrine, cyclic AMP levels remained unchanged for 1 minute. After 90 seconds of continuous exposure to phenylephrine, cyclic AMP levels began to fall and reached a minimum at 2 minutes, after which the steady state levels appeared to stabilize.

The effect of phenylephrine, after 2 minutes of incubation, on cyclic AMP levels in isolated cells is shown in Figure 3. At 10^-8 M to 10^-6 M concentrations of phenylephrine there was a small (21% at 10^-6 M) reduction in cellular cyclic AMP levels. With 10^-5 M and 10^-4 M phenylephrine there was a small increase in cyclic AMP concentration. The competitive α-adrenergic receptor blocker phentolamine (10^-6 M) abolished the reduction in cyclic AMP concentration caused by phenylephrine but did not significantly attenuate its ability to elevate the cyclic AMP levels. Phentolamine alone (10^-7 M to 10^-5 M) did not affect cyclic AMP concentrations.

Epinephrine is a mixed-adrenergic receptor agonist. Of the naturally occurring catecholamines, it is the most potent agonist of both β- and α-adrenergic receptors. However, if its effect on one receptor type predominates, effects of its interaction with other receptors may be obscured. In an attempt to unmask obscured effects we performed a series of experiments using epinephrine plus propranolol (10^-6 M) to see whether we could elicit an α-adrenergic effect of the catecholamine. When β-adrenergic receptors were blocked, epinephrine reduced cyclic AMP levels, an effect opposite to that found when the catecholamine was given alone (Fig. 4). Between the concentrations of 10^-8 M and 10^-6 M, epinephrine given in the presence of propranolol reduced cyclic AMP levels, the maximal effect occurring at 10^-6 M. At this concentration, epinephrine reduced the nucleotide level 28% below basal values. Propranolol alone (10^-7 M to 10^-5 M) had no effect on cellular cyclic AMP levels.

**EFFECT OF PHENYLEPHRINE ON ISOPROTERENOL-INDUCED INCREASES IN CYCLIC AMP CONCENTRATIONS OF ISOLATED CELLS**

In other tissues it has been found that the reduction of cyclic AMP levels induced by α-adrenergic agents is more pronounced when cyclic AMP generation has been simultaneously stimulated. Consequently, we performed experiments to determine the effect of activating α-adrenergic
receptors during isoproterenol-induced stimulation of cyclic AMP levels. Phenylephrine (10^{-7} M) was without effect on the increase in cyclic AMP concentrations induced by isoproterenol (Fig. 5). However, 10^{-5} M phenylephrine attenuated the generation of cyclic AMP in response to two concentrations of isoproterenol, 10^{-8} M and 10^{-5} M. The α-adrenergic receptor antagonist phentolamine did not significantly alter this effect of phenylephrine.

EFFECT OF α-ADRENERGIC AGONISTS ON NONADRENERGIC AUGMENTATION OF CYCLIC AMP CONCENTRATIONS IN ISOLATED CELLS

The experiments described in the previous section were suggestive but did not prove that α-adrenergic agonists could attenuate cyclic AMP generation induced by isoproterenol: phentolamine did not reverse the effect of phenylephrine. Propranolol could not be used in these experiments, therefore we were limited to the use of phenylephrine as the α-adrenergic receptor agonist. In the series of experiments described in this section we augmented cyclic AMP levels independently of β-adrenergic receptor stimulation, by activating adenylate cyclase with glucagon and by inhibiting the breakdown of cyclic AMP with the phosphodiesterase inhibitor theophylline.

Phenylephrine in two concentrations (10^{-7} M and 10^{-6} M) significantly attenuated cyclic AMP generation in response to glucagon (Fig. 6). Phentolamine (10^{-6} M) abolished this effect of 10^{-7} M phenylephrine.

Similar results were obtained when cyclic AMP levels were increased by administration of theophylline. Both phenylephrine (10^{-7} M) and epinephrine (10^{-7} M) plus propranolol (10^{-6} M) attenuated the theophylline-induced stimulation of cyclic AMP levels (Fig. 7). Phentolamine (10^{-6} M) tended to reverse this effect of the α-adrenergic agonists.

EFFECT OF α-ADRENERGIC RECEPTOR BLOCKADE ON EPINEPHRINE-INDUCED AUGMENTATION OF CYCLIC AMP CONCENTRATIONS IN ISOLATED CELLS

If activation of α-adrenergic receptors reduces cyclic AMP levels or attenuates the generation of the nucleotide in response to stimulating agents, it is possible that the mixed α- and β-receptor agonist epinephrine is inhibiting its own ability to stimulate cyclic AMP levels. To test this possibility, we performed experiments to determine whether blockade of α-adrenergic receptors would potentiate epinephrine-induced elevation of cellular cyclic AMP levels. There appeared to be a slight increase in the response of cells of epinephrine in the presence of phentolamine. However, at no point was the change statistically significant.
EFFECT OF $\alpha$-ADRENERGIC RECEPTOR
STIMULATION ON MECHANICAL FUNCTION AND ON CYCLIC AMP LEVELS IN WHOLE HEARTS

Experiments were performed on isolated perfused rat hearts at several different Ca$^{2+}$ concentrations. A negative inotropic effect which might be expected to accompany a reduction in cyclic AMP levels could be seen only when hearts were perfused with 2.0 or 2.5 mM Ca$^{2+}$. At lower, more physiological Ca$^{2+}$ concentrations, no negative inotropy was observed. When the hearts were perfused with the high Ca$^{2+}$ concentrations, $10^{-6}$ M epinephrine in the presence of propranolol (3 x $10^{-6}$ M) produced a slowly developing negative inotropic effect which became maximal after about 7 minutes of perfusion with this drug combination (Fig. 8). In these experiments, propranolol alone sometimes produced a small negative inotropic effect. By 12–15 minutes of its infusion, however, contractility stabilized and then the epinephrine infusion was begun. Epinephrine ($10^{-6}$ M) alone always produced the expected increase in contractility (Fig. 8).

In a series of hearts preperfused with $10^{-5}$ M propranolol, $10^{-6}$ M epinephrine reduced tissue cyclic AMP levels below basal values (Fig. 9). Epinephrine alone and isoproterenol increased tissue cyclic AMP concentrations, as expected. Propranolol, by itself, did not alter the levels.

Discussion

During the past decade it has been demonstrated that catecholamines can reduce cyclic AMP levels in several different tissues derived from a variety of species. $2,5,29,30$ This effect appeared in each case to be mediated by the $\alpha$-adrenergic receptor because it was elicited by $\alpha$-receptor agonists (norepinephrine or epinephrine) and/or blocked by $\alpha$-antagonists (phenotolamine, phenoxybenzamine, dihydroergotamine, or ergotamine). Furthermore, in several of these studies the functional response associated with the fall in cyclic AMP level was opposite that associated with a rise in cyclic AMP concentration. $4,5,29$ From these data, it appears that in some tissues catecholamines can either increase or decrease cyclic AMP concentrations, depending on whether they interact with $\beta$- or $\alpha$-adrenergic receptors, and these changes in nucleotide levels may be associated with reciprocal functional effects.

It is generally accepted that cyclic AMP mediates the metabolic effects of catecholamines on the heart, $31$ and a large body of data suggests that this second messenger mediates the inotropic $26$ and chronotropic $32,33$ effects of catecholamines as well, although some controversy persists on this latter point. $34$ The present investigation was specifically designed to attempt to answer whether catecholamines can decrease, as well as increase, cyclic AMP levels in the myocardium. Our data indicate that $\alpha$-adrenergic stimulation decreased cyclic AMP levels.
α-ADRENERGIC REDUCTION OF CYCLIC AMP IN HEART/Watanabe et al.

ergic receptor stimulation can indeed result in a reduction in cyclic AMP levels. When α-receptors were stimulated in presumably basal or "resting" cardiac cells, the fall in cyclic AMP levels was very small. However, when the cyclic AMP levels in the cells were increased, the α-adrenergic reduction of cyclic AMP was magnified, an interaction that might be described as accentuated antagonism. Similar results have been found in other tissues. In fact, in some tissues, e.g., human platelets, and α-adrenergic receptor-mediated reduction in cyclic AMP level becomes apparent only after the concentration of the nucleotide has been increased by another agent.6

α-Adrenergic receptor stimulation attenuated the generation of cyclic AMP in response to glucagon, which interacts with putative glucagon receptors coupled to adenylate cyclase. It also reduced cyclic AMP elevation induced by theophylline, an agent which is thought to act by inhibiting phosphodiesterase. Such a generalized effect is compatible with α-adrenergic receptor inhibition of adenylate cyclase. If the mechanism by which α-adrenergic receptor stimulation reduced cyclic AMP levels were an increase in phosphodiesterase activity, it seems unlikely that the effect would persist in the face of maximal inhibition of phosphodiesterase by a high concentration of theophylline. Our data from cardiac tissue support the conclusions of other investigators using other tissues that α-adrenergic agonists may act by inhibiting the enzyme adenylate cyclase.2,3,38

The mecha...nism by which phenylephrine attenuated the effect of isoproterenol is unclear. If phenylephrine acted in this instance by stimulating α-adrenergic receptors, its effect should have been abolished by phentolamine. Phenylephrine is a weak β-adrenergic receptor agonist, as described by other investigators50 and confirmed by our own studies, and thus it could have acted as a β-adrenergic receptor antagonist when given with isoproterenol. We cannot explain the different action of phenylephrine when given with isoproterenol than when given with glucagon or theophylline. It has been suggested that the β- and α-adrenergic receptors are very closely related and may even represent two allosteric configurations of a single active site.39 In line with this notion, results of some studies have been interpreted as showing transformation of β- to α-adrenergic receptors.39 Perhaps, in our study, when β-adrenergic receptors were stimulated by isoproterenol, α-adrenergic receptors became inaccessible to phenylephrine. Such a phenomenon could also explain the lack of potentiation of epinephrine augmentation of cyclic AMP levels by phentolamine. If the mixed-adrenergic agonist epinephrine was limiting its own ability to stimulate adenylate cyclase, α-adrenergic receptor blockade should have enhanced the epinephrine effect, as has been observed in several other studies. However, such was not the case in our study.

A number of investigators have demonstrated that α-adrenergic receptor stimulation produces both electrophysiological and inotropic effects on the heart. A principle electrophysiological effect is a prolongation of the total duration of the monophasic action potential and is functionally manifested as a prolongation of the refractory period.9,11,15 With regard to the inotropic effects, the majority of the studies show that α-adrenergic receptor stimulation increases myocardial contractility.10,12,15-18 The mechanism by which α-adrenergic receptor stimulation increases contractility appears to be quite different from the mechanism for the β-adrenergic receptor stimulation. During α-adrenergic receptor stimulation, cyclic AMP levels are not increased,10,20 and many of the mechanical effects associated with α-adrenergic stimulation are different from those of β-adrenergic receptor stimulation.

In addition to the well known positive inotropic effects, several investigators have demonstrated a cardiodepressant effect of α-adrenergic receptor stimulation. Imai et al.7 in 1961 and James and colleagues13 in 1968 showed that the α-adrenergic receptor agonist methoxamine slowed heart rate and antagonized the cardioaccelerating effect of exogenously administered or endogenously released catecholamines. The former group also showed that methoxamine produced a negative inotropic effect on dog hearts and abolished the positive inotropic and chronotropic actions of epinephrine.6 A negative inotropic effect of α-adrenergic receptor stimulation has since been observed by several other investigators.8-16,17 Thus, α-adrenergic receptor stimulation may result in either an increase or a decrease in the inotropic state of the heart. These observations prompted Wenzel and Su8 to postulate that there are α- and β-stimulatory receptors and α-inhibitory receptors in the myocardium.

It appears that the negative inotropic effect of α-inhibitory receptors become manifest only under certain conditions, perhaps associated with a high cellular Ca2+ level. For example, Endoh and Schümann17 showed that methoxamine produced a negative inotropic effect in rabbit papillary muscle only during relatively rapid (1.5 Hz) stimulation, and we found that epinephrine plus propranolol produced a negative inotropic effect only if the hearts were perfused with buffer containing 2.0 or 2.5 mM Ca2+, a level about twice the physiological concentration in rats.

The physiological significance of our findings in isolated cells remains to be established. In the isolated cells it was not possible to determine the functional significance of the observed reduction in nucleotide levels. Our correlative study in whole hearts was performed to attempt to answer the question of whether α-adrenergic reduction of cyclic AMP levels occurs in intact cardiac tissue and to determine what functional significance lowering cyclic AMP levels might have. We found that when activation of α-adrenergic receptors depressed contractility, there was an associated reduction in tissue cyclic AMP levels in the hearts. As previously mentioned, several other investigators have also demonstrated a negative inotropic and chronotropic effect of α-adrenergic receptor stimulation on whole hearts.6-8,13,16,17 Recently, Posner and co-workers27 and Danilo and colleagues38 showed that activation of α-adrenergic receptors decreased spontaneous rates of isolated dog Purkinje fibers. Taken together, these diverse studies indicate that activation of α-adrenergic receptors can, under certain conditions, lead to a functional depression of cardiac tissue. If an increase in cyclic AMP levels...
does mediate the cardiac stimulatory effects of catecholamines, a reduction in cyclic AMP levels could logically be expected to produce the opposite effect. It thus appears possible that α-inhibitory receptors produce their cardiodepressant effects by reducing tissue cyclic AMP levels.

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