Interaction between Cyclic Adenosine Monophosphate and Cyclic Guanosine Monophosphate in Guinea Pig Ventricular Myocardium

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Abstract
The purpose of this study was to examine the mechanisms underlying adrenergic-cholinergic antagonism in ventricular myocardium. Myocardial contractility, cyclic adenosine monophosphate (AMP) levels, and cyclic guanosine monophosphate (GMP) levels were measured in isolated guinea pig ventricles after treatment with various inotropic agents given alone and simultaneously with acetylcholine. Acetylcholine alone markedly elevated cyclic GMP levels but did not substantially change myocardial contractility. However, the same concentration of acetylcholine significantly attenuated the inotropic effect of isoproterenol and histamine, two drugs that may act by increasing myocardial levels of cyclic AMP. The decrease in the inotropic response to isoproterenol did not appear to be due to a decrease in the generation of cyclic AMP, because cyclic AMP levels were similar in hearts receiving isoproterenol alone and those receiving isoproterenol with acetylcholine. Dibutyryl cyclic GMP also antagonized the inotropic action of isoproterenol. Acetylcholine did not alter the inotropic effects of ouabain, an agent that increases myocardial contractility without changing cyclic AMP levels. These results suggest that cyclic GMP mediates the antiadrenergic effects of acetylcholine by specifically antagonizing the inotropic actions of cyclic AMP.

Methods

Perfusion of Hearts
Guinea pigs of either sex weighing 400–600 g were injected intraperitoneally with heparin sulfate (500 units) about 30 minutes prior to use. Each guinea pig was killed by a blow to the head, after which the heart was rapidly removed and placed in ice-cold oxygenated...
Krebs-Henseleit buffer to remove the blood. The hearts were then attached to a cannula for perfusion by the Langendorff technique at a rate of 4.2-4.5 ml/min. The millimolar composition of the oxygenated (95% O₂-5% CO₂) Krebs-Henseleit buffer used to perfuse the hearts was: NaHCO₃ 27.2, NaCl 118.0, KCl 4.8, KH₂PO₄ 1.0, MgSO₄ 1.2, CaCl₂ 2.5, and glucose 11.1. Immediately after attaching the heart to the cannula, both atria and their appendages were carefully removed so that only the ventricles remained. The apex of the heart was attached with a silk suture to a strain-gauge transducer for measurement of developed tension. Diastolic tension was set at 5 g prior to beginning the experiment. Hearts were paced with an electronic stimulator at a rate of 210 stimuli/min except during the ouabain experiments when they were paced at a rate of 150 stimuli/min. In the latter experiments, the atrioventricular node was crushed to allow ventricular capture at a slower rate. The voltage used to pace the hearts was usually 2.5 v (10-25% greater than threshold), and the duration of each stimulus was 10 msec. The entire perfusion apparatus was enclosed in an organ perfusion box with a maintained temperature of 30°C and humidity of 100%. Developed tension, perfusion pressure, and the first derivative of tension development (dT/dt) were continually recorded and measured throughout the experiment. All analyses of changes in contractility were made on the basis of changes in dT/dt.

The hearts were perfused for at least 20 minutes prior to starting any experimental intervention. Drugs were administered through a side arm which entered the main perfusion 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 necessitate prolonged oxygenation of the drugs. All of the drugs were administered by continuous infusion.

The time course of the effects of acetylcholine, isoproterenol, and histamine were such that the inotropic effects of these drugs had stabilized and dT/dt had reached steady-state levels by 2 minutes of drug infusion. Moreover, in previous studies (in the case of cyclic AMP) (18) and in preliminary experiments (in the case of cyclic GMP), we had determined that cyclic nucleotide levels had reached a steady-state level after 2 minutes of drug infusion. Therefore, in biochemical studies with these drugs, most hearts were freeze-clamped after 2 minutes of drug infusion.

Cyclic Nucleotide Assays

Hearts in which cyclic nucleotide levels were to be measured were quickly frozen with metal tongs that had previously been cooled to the temperature of liquid nitrogen. Freeze-clamped hearts were immediately pulverized under liquid nitrogen with a mortar and pestle that had previously been cooled to the temperature of liquid nitrogen.

The powdered tissue was prepared for assay by homogenization in 5% trichloroacetic acid, which was then removed by extraction with ether. The extracted homogenate was then introduced into a 5 × 25-mm ion-exchange column (Dowex AG 1 × 8, 200-400 mesh, formate form) that had previously been equilibrated with 0.1v formic acid. Cyclic AMP was eluted with 2v formic acid and cyclic GMP with 4v formic acid. After freeze-drying of the eluates to concentrate the nucleotides and remove the formic acid, the lyophilized nucleotides were resuspended in distilled water for assay. Prior to the homogenization of the tissue, tracer amounts of tritiated cyclic AMP (approximately 3600 dpm or 0.04 pmol) and cyclic GMP (approximately 4400 dpm or 0.13 pmol) were added to the powdered hearts to check for recoveries. A portion of the resuspended nucleotides (after lyophilization) was counted to quantify recoveries, which usually were in the range of 60 to 75% for both cyclic nucleotides. The values reported for cyclic AMP and cyclic GMP have been corrected for recoveries.

Cyclic AMP levels were measured by a competitive protein-binding assay (19) using binding protein derived from beef adrenal glands (20), as previously described (18). Cyclic GMP levels were measured by a modification of a radioimmunoassay first described by Steiner et al. (21). The antisera and 125I-succinyl cyclic GMP (125I-Sc-cyclic GMP) were purchased from Collaborative Research, Waltham, Massachusetts. Only 125I-Sc-cyclic GMP that had been synthesized within the previous 6 weeks was used in these assays. The assay reaction mixture, in a final volume of 0.5 ml, contained 50 mM sodium acetate buffer (pH 6.2), antisera, 125I-Sc-cyclic GMP, and various amounts of unlabeled cyclic GMP or tissue extract. The concentrations of 125I-Sc-cyclic GMP and antisera were adjusted so that the amount bound in the absence of any added unlabeled cyclic GMP (Bₒ) was 35-40%. The limit of sensitivity of the assay was 0.03 pmol cyclic GMP/tube. Fifty percent displacement of Bₒ occurred at an added cyclic GMP level of 0.3 pmol/tube. The reaction was allowed to equilibrate over a period of at least 16 hours. Free 125I-Sc-cyclic GMP was separated from bound labeled nucleotide by the addition of an equal volume of a charcoal solution (300 mg of charcoal and 500 mg of bovine serum albumin in 100 ml of 50 mM acetate buffer) to the assay tube. The charcoal-assy mixture was allowed to equilibrate for 20 minutes. In preliminary experiments we found that Bₒ was unchanged during exposure to charcoal for time intervals ranging from 10 to 30 minutes. In the absence of antisera, the charcoal removed more than 97% of the 125I-Sc-cyclic GMP. The bound 125I-Sc-cyclic GMP was quantified by counting in a liquid scintillation spectrometer (efficiency 50%). All unknowns were assayed in duplicates of two tissue dilutions.

Drugs

L-Isoproterenol HCl, acetylcholine chloride, histamine HCl, dibutyryl cyclic GMP, ouabain, cyclic AMP, and cyclic GMP were purchased from Sigma Chemical Company. 3H-cyclic AMP (specific activity 37.7 c/mmole) was purchased from New England Nuclear Corporation. 3H-cyclic GMP (specific activity 15 c/mmole) was purchased from Amersham-Searle. 125I-Sc-cyclic GMP (specific activity 600 c/mmole) was purchased from Collaborative Research.

Results

Effect of Acetylcholine on Cyclic Nucleotide Levels and on Contractility of Isolated Guinea Pig Ventricles

The mean basal level of cyclic GMP in hearts freeze-clamped after about 30 minutes of perfusion was 0.015 ± 0.02 (SE) pmol/mg wet weight and that of cyclic AMP was 0.45 ± 0.02 pmol/mg wet weight.
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weight; both values are similar to those reported by other investigators who have measured cyclic nucleotide levels in the myocardium (21, 22). Acetylcholine increased cyclic GMP levels in the guinea pig ventricles (Fig. 1). The threshold concentration for increasing cyclic GMP levels appeared to be about $10^{-8}$ M, and there was a concentration-related increase in cyclic GMP levels: $10^{-7}$ M acetylcholine increased levels about 2.6-fold and $10^{-6}$ M increased levels about 4.3-fold. Cyclic AMP levels in the same hearts were unchanged by acetylcholine except at the highest concentration of acetylcholine studied ($10^{-6}$ M) for which the levels were slightly increased above the basal level (Fig. 1). This latter increase was probably due to acetylcholine-induced release of endogenous norepinephrine which in turn increased cyclic AMP levels in the hearts (23).

Acetylcholine produced only a slight negative inotropic effect that did not show marked concentration dependence. At a concentration of $10^{-8}$ M dT/dt was 94% of control, and at $10^{-7}$ M and $10^{-6}$ M dT/dt was 93% of control (Fig. 1). Furthermore, there did not appear to be any direct relationship between contractility and cyclic GMP levels. For example, at $10^{-6}$ M acetylcholine contractility was depressed 6% and cyclic GMP levels were increased two times over the basal level, and at $10^{-5}$ M acetylcholine contractility was 7% below control and cyclic GMP levels were increased 4.3 times above control (Fig. 1). In some hearts, a few seconds after the beginning of the drug infusion, acetylcholine produced a fall in perfusion pressure, presumably by causing dilation of coronary arteries (Fig. 2B) (24). The small negative inotropic effect seen in most hearts did not appear to be related to the occurrence or the magnitude of this fall in coronary vascular resistance.

INTERACTION BETWEEN INOTROPIC AGENTS THAT INCREASE CYCLIC AMP LEVELS AND ACETYLCHOLINE

Myocardial Contractility.—Given simultaneously with isoproterenol, acetylcholine markedly attenuated the positive inotropic effect of the catecholamine. A typical example of such an interaction is shown in Figure 2. In A, isoproterenol was given alone in a concentration of $10^{-9}$ M; contractility, as assessed by developed tension and the rate of tension development, was increased about 35% over control levels after 2 minutes of continuous infusion of the drug. In the same heart about 5 minutes after discontinuing isoproterenol infusion, the catecholamine was again infused in the same concentration, this time simultaneously with acetylcholine ($10^{-7}$ M). Most of the interaction studies were done with this concentration of acetylcholine, which was the middle of those concentrations at which both cyclic AMP and cyclic GMP were studied (Fig. 1). Acetylcholine virtually abolished the positive inotropic effect of this concentration of isoproterenol (Fig. 2B). Approximately 5

![Figure 1](http://circres.ahajournals.org/)

**Figure 1**

Effect of acetylcholine (ACh) on cyclic nucleotide levels and on contractility (dT/dt) of isolated perfused guinea pig hearts. Values are means ± SE. The number of hearts studied is given in parentheses under the histograms.
Recordings of developed tension (top tracing of each record), perfusion pressure (middle tracing), and $dT/dt$ (bottom tracing) in a guinea pig heart. Time marks denote 1-second intervals. Infusion of drug(s) was begun at the first arrow on each recording and stopped at the second arrow. In A and C, isoproterenol (ISO) was given alone in a concentration of $10^{-9}$M. In B, isoproterenol ($10^{-7}$M) was given simultaneously with acetylcholine (ACH) ($10^{-7}$M).

minutes after discontinuing the isoproterenol-acetylcholine combination, isoproterenol was again infused alone. At this time the heart had regained its ability to respond to isoproterenol (Fig. 2C).

The mean values of a series of such studies are presented in Figure 3. As can be seen, acetylcholine ($10^{-7}$M) significantly attenuated the inotropic effect of all concentrations of isoproterenol studied. A tenfold higher concentration of isoproterenol was required to produce the same inotropic effect in the presence of acetylcholine as that seen with the catecholamine alone. In these studies each heart received only one treatment, isoproterenol alone at one of the three concentrations or isoproterenol and acetylcholine ($10^{-7}$M). The hearts were freeze-clamped 2 minutes after the beginning of the drug infusion, and $dT/dt$ was measured immediately prior to clamping. All hearts had reached a steady state of developed tension by 2 minutes of drug infusion.

The inotropic effect of histamine, another drug thought to increase contractility by stimulating myocardial adenylate cyclase (25), was also attenuated by the simultaneous administration of acetylcholine. Acetylcholine ($10^{-7}$M) abolished the inotropic effect of $3 \times 10^{-7}$M histamine and significantly diminished the inotropic action of $10^{-6}$M histamine (Fig. 4).

Cyclic Nucleotide Levels.—Although it markedly attenuated the positive inotropic effect of isoproterenol, acetylcholine did not alter the amount of cyclic AMP generated in response to stimulation by the catecholamine. As shown in Figure 5, isoproterenol produced a concentration-dependent increase in cyclic AMP levels; the threshold for stimulation was about $10^{-9}$M. Acetylcholine decreased the mean cyclic AMP levels slightly in hearts receiving $10^{-7}$M isoproterenol, but the effect was not statistically significant. Similarly, in hearts receiving acetylcholine ($10^{-7}$M) with $10^{-6}$M or $10^{-5}$M isoproterenol, the mean values of cyclic AMP were unchanged from those of hearts receiving isoproterenol alone. The other noteworthy finding in this series of experiments was
that the amount of cyclic GMP formed in response to acetylcholine (10^{-7} M) given simultaneously with isoproterenol was more than twice as great as that formed in response to the same concentration of acetylcholine given alone (compare Fig. 5 with Fig. 1).

Augmented levels of cyclic GMP were not associated with depressed levels of cyclic AMP. However, the contractile response was lower than that expected for the levels of cyclic AMP found. In Figure 6, dT/dt immediately prior to freezing of the heart (after 2 minutes of drug infusion) is plotted against the cyclic AMP level in each of a series of hearts given isoproterenol alone (in various concentrations) or simultaneously with 10^{-7} M acetylcholine. There was a significant positive correlation between contractility and tissue cyclic AMP levels for hearts receiving isoproterenol alone (r = 0.68, P < 0.001) or simultaneously with 10^{-7} M acetylcholine (r = 0.40, P < 0.05). Furthermore, it appeared that the inotropic response to isoproterenol was blunted in the presence of acetylcholine, a given level of cyclic AMP being associated with a lower level of contractility. With the number of hearts studied (30 for isoproterenol alone and 26 for isoproterenol plus acetylcholine) the difference between the two slopes did not, however, attain statistical significance.

EFFECT OF DIBUTYRYL CYCLIC GMP ON ISOPROTERENOL-INDUCED INCREASES IN MYOCARDIAL CONTRACTILITY

To test whether the antagonistic effect of acetylcholine was mediated by cyclic GMP, a series of experiments was performed to examine the effects
Correlation between cyclic AMP levels and contractility (dT/dt) of the same hearts. Circles represent hearts receiving various concentrations of isoproterenol alone, and triangles represent the values for hearts receiving various concentrations of isoproterenol plus acetylcholine (10⁻⁷M). The slopes were estimated by the method of least squares (solid line for isoproterenol alone, r = 0.68, P < 0.001; broken line for isoproterenol plus acetylcholine, r = 0.40, P < 0.05).

Effect of dibutyryl cyclic GMP on the inotropic action of isoproterenol. Preperfusion of dibutyryl cyclic GMP in a concentration of 10⁻⁶M for 10 minutes or 10⁻⁵M for 5 minutes was without effect on the positive inotropic action of isoproterenol.

Antagonism of the inotropic action of isoproterenol was demonstrable only after perfusion of the drug at either of these concentrations for at least 15 minutes. This time course is analogous to that for the positive inotropic action of dibutryryl cyclic AMP: 15-20 minutes are required for the expression of the positive inotropic effects of this analogue of cyclic AMP (26). With 15 minutes of preperfusion of dibutryryl cyclic GMP, there was a concentration-related antagonism of the positive inotropic effect of 10⁻⁶M isoproterenol (Fig. 7).

Effect of acetylcholine on ouabain-induced increases in myocardial contractility

The time course of the actions of acetylcholine and ouabain were sufficiently different to allow separate analyses of their effects even when the two drugs were given simultaneously. Acetylcholine (10⁻⁷M) produced a slight (7%) reduction in contractility a few seconds after the beginning of its infusion together with ouabain (3 x 10⁻⁷M), just as it did when it was infused by itself (Fig. 1). Ouabain (3 x 10⁻⁷M) began to increase contractility after about 5 minutes of infusion when it was infused by itself or simultaneously with acetylcholine. When the changes in contractility due to
ouabain were expressed as percents of dT/dt prior to the beginning of infusion (percent of control), there appeared to be a slight delay in the onset of the effect of the cardiac glycoside when it was given with acetylcholine (Fig. 8). However, the contractile state of the hearts at 5 minutes of ouabain infusion was actually 5-7% greater than the level (previously depressed below control by acetylcholine) immediately prior to the onset of the glycoside effect. Except at the 5-minute point when the difference was apparent rather than real, the choline ester produced no alteration in the positive inotropic effect of ouabain (Fig. 8). If the contractile effect of ouabain were expressed in terms of dT/dt immediately prior to the onset of the glycoside effect, the inotropic action of ouabain would actually be slightly greater in the presence of acetylcholine than it is in its absence.

Discussion

Acetylcholine caused an increase in cyclic GMP levels in guinea pig hearts, presumably by interacting with muscarinic cholinergic receptors and stimulating the enzyme guanylate cyclase. These results thus confirm the work of other investigators who have studied tissue slices and isolated perfused rat hearts (16, 17). Unlike George and his colleagues (17), however, we found that acetylcholine by itself had very little effect on myocardial contractility. The small (7%) reduction that we observed in myocardial contractility did not appear to be related to the concentration of acetylcholine used or to the levels of cyclic GMP found in the hearts. The reasons for the difference in our results compared with those of George and his colleagues (17) are not readily apparent but may lie in differences in species used or in differences in experimental technique. It also seems possible that a higher background level of adrenergic tone may have been present in the studies of George et al. (17), perhaps due to release of endogenous norepinephrine by electrical stimulation. In any case, our results are consistent with those of many other studies which have shown very small decreases in myocardial contractility due to vagal stimulation. In isolated guinea pig ventricles, the contractile state of the autonomically isolated heart was not substantially changed by the drug.

The accentuated antagonism between the adrenergic and cholinergic systems, well-described by many investigators primarily in whole animal studies (8-13), was also observed in our studies using isolated guinea pig ventricles. The contractile effect of catecholamines was substantially attenuated by a concentration of acetylcholine that by itself left contractility virtually unchanged. The antagonism by acetylcholine was thus accentuated in the presence of increased adrenergic tone. This antagonism does not, however, appear to be specific for the adrenergic system, because acetylcholine also attenuated the positive inotropic effect of histamine, which is generally thought to interact with specific histaminergic receptors to stimulate myocardial adenylate cyclase (25). Meester and Hardman (10) have previously shown that acetylcholine diminishes the positive inotropic effects of theophylline, as well as catecholamines, in rabbit and turtle hearts. Thus, acetylcholine antagonizes the effects of members of several classes of drugs whose common end point is elevation of cyclic AMP levels.

In the present study, acetylcholine antagonized the inotropic effects of isoproterenol without attenuating the drug-induced generation of cyclic AMP. We were somewhat surprised by these results because of the observation from two other laboratories that acetylcholine reduces the catecholamine-induced elevation of cyclic AMP in cardiac tissues (15, 16). Furthermore, Vincent and Ellis (27) and Blukoo-Allotey et al. (28) have shown that acetylcholine antagonizes the glycogenolytic and phosphorylase-activating effects of epinephrine, two metabolic sequelae of catecholamine administration that are thought to be mediated by cyclic AMP. Finally, in preliminary studies using isolated rat heart cells, we detected a small attenuation of the isoproterenol-induced increase in cyclic AMP levels by acetylcholine (Hathaway, Watanabe and Besch, unpublished observations). The differences between the studies described in the present paper and those just cited include the following: species (guinea pig vs. rat or calf), preparation (intact isolated perfused heart vs. tissue slices or broken cell enzyme preparations), assay systems, and concentration of acetylcholine studied. Whatever the reason for the difference in results, the present studies suggest that acetylcholine antagonizes the effects of cyclic AMP rather than the generation of cyclic AMP. Our data do not exclude the possibility that acetylcholine may have marginally decreased cyclic AMP levels, but they do suggest that the primary effect of acetylcholine is on the expression of the actions of a given level of cyclic AMP.

One of the criteria originally set for implicating
cyclic AMP as a mediator of the effects of a hormone is whether the nucleotide or analogues of the nucleotide can mimic the actions of the hormone (29). Accordingly, analogues of cyclic AMP have been used in many studies in attempts to mimic actions of hormones such as epinephrine and glucagon. Because of the relatively recent discovery that cyclic GMP levels are increased in cardiac tissue exposed to acetylcholine and the rapidly accumulating evidence that cyclic GMP may be an intracellular second messenger for cholinergic receptor stimulation, it was of interest to see if the antagonistic effects of acetylcholine could be mimicked by an analogue of cyclic GMP. Based on a similar rationale, Krause and co-workers (30) have recently shown that dibutyryl cyclic GMP, a more lipid-soluble form of cyclic GMP, can mimic the negative chronotropic effect of carbachol in spontaneously beating cultured heart cells. In the present study, we were able to demonstrate that dibutyryl cyclic GMP antagonizes the inotropic action of isoproterenol. As in similar studies done with dibutyryl cyclic AMP (26), the results must be interpreted with caution, because relatively large amounts of the derivatives must be administered to mimic the effects of drugs that increase levels of the nucleotides. Additionally, in both cases a considerable amount of time is required for the pharmacological effects of the analogue to become manifest, presumably because the rates of penetration of the derivative into the cell and its conversion to the monobutyl or the native nucleotide are slow. Although our results do not prove that cyclic GMP mediates the acetylcholine antagonism of the actions of cyclic AMP, they are compatible with such an hypothesis. Thus, the well-known interaction between the adrenergic and cholinergic systems may, in fact, be an antagonism between cyclic AMP and cyclic GMP within the myocardial cell.

The antagonism by acetylcholine of the inotropic effect of drugs that increase cyclic AMP levels in the myocardium does not appear to merely be a nonspecific blunting action by the choline ester of any positive inotropic intervention. Rather, this action of acetylcholine appears to be specific for positive inotropic drugs whose actions are thought to be mediated via cyclic AMP. Acetylcholine did not alter the inotropic effect of ouabain, a cardiac glycoside that increases myocardial contractility without changing cyclic AMP levels. Other investigators, studying adrenergic-cholinergic interactions, have made similar observations. Vagal nerve stimulation or administration of acetylcholine does not alter the inotropic effects of a variety of agents or maneuvers that do not act through cyclic AMP, including cardiac glycosides, calcium ions, and paired pacing (10, 13).

The physiological importance of cholinergic regulation of atrial and pacemaker tissues is generally accepted (31), and interaction between the adrenergic and cholinergic systems in regulating these components of the heart is well recognized (32–37). The mechanism underlying such adrenergic-cholinergic interaction in the atria and the sinoatrial node may involve cyclic AMP–cyclic GMP antagonism. The physiological relevance of the present findings is uncertain, because the studies were conducted on ventricular myocardium, which has traditionally been thought to be relatively uninfluenced by the cholinergic system. However, in view of the recent studies which indicate that mammalian ventricles are innervated by cholinergic fibers and that vagal nerve stimulation can exert a direct negative inotropic effect on ventricular contractility, it is reasonable to suggest that our observations of cyclic AMP–cyclic GMP interactions in the ventricles may be physiologically relevant. The level of cyclic GMP may be controlled by cholinergic tone and in turn may modulate the effects of cyclic AMP within the ventricular myocardial cell.

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References

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22. Robison GA, Butcher RW, Oye I, Morgan HE, Sutherland EW: Effect of epinephrine on adenosine 3',5'-phosphate levels in the isolated perfused rat heart. Mol Pharmacol 1:168-177, 1965


27. Vincent NH, Ellis S: Inhibitory effect of acetylcholine on glycogenolysis in the isolated guinea pig heart. J Pharmacol Exp Ther 139:69-68, 1963


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