Autonomic Control of Cardiac C-AMP

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ABSTRACT

Although catecholamines are known to increase cyclic adenosine 3'5'-monophosphate (C-AMP) in heart muscle, the relations of adrenergic and cholinergic mechanisms to inotropy and myocardial C-AMP levels and adenyl cyclase have not been defined. Accordingly, atrial and ventricular muscle preparations were studied in a myograph with a tension recorder. Adenyl cyclase, phosphodiesterase, and C-AMP were measured by chromatographic and isotopic methods. In atrial preparations, norepinephrine ($10^{-3}$ M) increased tension by 80 ± 10% (mean ± SE), adenyl cyclase by 40 ± 10% and C-AMP by 75 ± 12%, while carbamylcholine chloride ($10^{-4}$ M) reduced tension by 65 ± 10%, adenyl cyclase by 30 ± 12% and C-AMP by 50 ± 14%. The effects of carbamylcholine chloride on tension, adenyl cyclase, and C-AMP were blocked by atropine $10^{-5}$M. In paced ventricular (papillary) muscles, norepinephrine ($10^{-3}$M) increased tension by 70 ± 10%, adenyl cyclase by 50 ± 11% and C-AMP by 90 ± 17%. In contrast, carbamylcholine chloride ($10^{-4}$ M) reduced tension by only 7 ± 5%, adenyl cyclase by 12 ± 8%, and C-AMP by 10 ± 7%. These results demonstrate parallel changes in tension, adenyl cyclase, and C-AMP in atrium and ventricle. The failure of ventricular muscle to respond to carbamylcholine is mirrored by the adenyl cyclase system and C-AMP. Furthermore, the cholinergically mediated tension and adenyl cyclase changes in atria were specifically blocked by atropine. Thus, these results relate the different effects of cholinergic agents on atrium and ventricle to adenyl cyclase and C-AMP, and suggest specific receptor localization in these two types of myocardia.

KEY WORDS

- muscle tension
- adenyl cyclase
- carbamylcholine
- norepinephrine
- atria
- atropine
- ventricles
- rabbit
- cat
- dog

- The role of cyclic adenosine 3’5’-monophosphate (C-AMP) as the mediator of the metabolic effects of catecholamine has been investigated by a number of laboratories (1-3). In addition, several groups have demonstrated an increase in myocardial C-AMP closely associated in time with the characteristic inotropic effect of catecholamines (4-7). However, there is little information regarding the relation of cholinergic agents to myocardial C-AMP (8).

- Studies in isolated heart muscle preparations have shown that ventricular muscle in comparison to atrial myocardium is relatively insensitive to the negative inotropic actions of acetylcholine. The studies of Hollenberg et al. (9) and Vincent and Ellis (10) indicating that there is an antagonism between catecholamines and cholinergic agents, suggest some physiological competition between these two hormones. The studies of Vincent and Ellis (10), which demonstrated an antagonism by acetylcholine of the glycogenolytic effect of catecholamines in guinea pig atrium, coupled with the role of C-AMP in glycogenolytic effect of catecholamines, prompted the present study to compare the effect of adrenergic and cholinergic agents on changes in contractility and the C-AMP system in various intact muscles and subcellular preparation of atrial and ventricular myocardium from a number of species.
Materials and Methods

**MUSCLE PREPARATIONS**

Animals were anesthetized with either 50% O₂-50% CO₂ mixture or sodium pentobarbital and the hearts were quickly removed and prepared for study.

Heart slices from the atria and ventricles of rabbits were prepared with a Stadie-Riggs tissue slicer and then incubated for 45 minutes in 2 ml of Krebs-Ringer bicarbonate buffer with 1 μCi/ml of (1⁴C) adenine (New England Nuclear) as previously described (11).

Right ventricular papillary muscles and atrial muscle strips from cats were rapidly removed and placed in a muscle bath filled with Krebs-Ringer bicarbonate buffer at a constant temperature of 29° C and aerated with 95% O₂, 5% CO₂. The muscle was stimulated at 12 contractions/min by mass electrodes and developed tension was recorded as described previously (12).

Enzyme preparations were obtained by homogenizing dog, cat, and rabbit atrial and ventricular muscle in 0.25M sucrose and then assaying the 2000 X g pellet for adenyl cyclase activity and the whole homogenate for phosphodiesterase activity.

**BIOCHEMICAL METHODS**

C-AMP was measured by a double isotope technique as previously described (11). Heart slices or tissue fragments were incubated in 2 ml of modified Krebs-Ringer bicarbonate buffer containing 0.86 mg/ml calcium, 1 mg/ml glucose, 1 μCi/ml of ¹²⁵I adenine (New England Nuclear). Incubation was carried out in an atmosphere of O₂-CO₂ (95:5, v/v) at 37° C for the slice preparations. Papillary muscles and atrial muscle strips were incubated in the same buffer, but the total volume was 6 ml and the temperature was 37° C. Incubation was then carried out for 1 hour in all systems. The buffer was removed and fresh buffer without isotopes was then added. After 5 minutes of temperature equilibration, the agents to be tested were injected into the medium in a volume of 0.1 ml of buffer, and the incubation was carried out for the specified time of each experiment.

The tissue was then removed and placed in 0.5 to 1.0 ml of boiling distilled water containing 50 μg carrier C-AMP and 0.1 μc ¹³H C-AMP. The samples were boiled at 100° C for 5 minutes and then assayed for C-AMP as described previously (12).

C-AMP was purified by chromatography on a Dowex 1X8 anion exchange column and eluted with 0.01N HCl. The eluate was evaporated to dryness and then applied to a Whatman chromatography paper. After a 1-hour equilibration period, descending chromatography was carried out in the system ethanol-0.1M borate (3:5:1 v/v; pH 4.0) for 18 hours. Following chromatography, the paper was dried and examined under ultraviolet light for the C-AMP spot. This spot was cut out and placed in a vial with 20 ml of toluene containing 3 mg of 2,5-diphenyl oxazole and 0.6 mg p-bis-(5-phenyloxazolyl) benzene/ml. Double isotope counting was carried out in a scintillation counter. All ¹⁴C counts were corrected to 100% on the basis of tritium recovered. Variation in labeling of C-AMP between different series of experiments is

![Accumulation of ¹⁴C C-AMP by slices of rabbit atrial and ventricular myocardium 5 minutes after addition of 10⁻⁵M NE (left) and after 10⁻⁴M carbachol chloride (CCC), (right). After NE, differences between control and experimental values were significant by Student's t-test (P < 0.001); after carbachol chloride they were significant for the atrium (P < 0.01) but not for the ventricle.](http://circres.ahajournals.org/Download/10.1161/01.RES.28.3.378)

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Due to differences in specific activity of precursor, previous reported data demonstrated that the results obtained with this method corresponded closely to data obtained utilizing a double isotope dilution derivative-analysis method for measuring C-AMP and allows for measurement of pmole quantities of C-AMP (11).

In this study, the total C-AMP was not measured. The changes in 3H C-AMP were measured after tissue was washed and placed in fresh isotope-free buffer. These changes were then evaluated relative to control as reported elsewhere (11,13).

Adenyl cyclase activity was measured in the enzyme preparation material by the method of Streeto and Reddy (14), with the following modifications. The concentration of phosphoenolpyruvate (Sigma) was 6.35 μM and the concentration of pyruvate kinase (Sigma) was 0.08 mg protein/ml. All samples were incubated for 10 minutes at 37° C before addition of the 14C ATP (Schwartz BioResearch). The final incubation was 4 minutes unless otherwise stated in the text. All samples were chromatographed in ethanol 0.1M boric acid (3.5:1; pH 4.0) systems and isopropanol ammonia water (7:2:1) systems.

C-AMP phosphodiesterase was determined by measuring the disappearance of 3H C-AMP. A 0.5-ml aliquot of 0.25M sucrose homogenate was incubated at pH 7.8 in tris buffer with 0.003M magnesium sulphate, 0.1 μC 3H C-AMP (S.A. 50 μc/m mole) and 1 mg 14C C-AMP for 4 minutes. The mixture was then boiled for 3 minutes and the C-AMP chromatographically isolated as described above. Protein was determined by the Lowry method (15). All values for adenyl cyclase, phosphodiesterase and C-AMP are reported as counts per minute per milligram of protein of either 14C or 3H C-AMP unless otherwise stated. Results are expressed as means with standard error. The bars in figures represent the standard error. Norepinephrine was purchased from Sigma, carbamylcholine from Aldrich Chemical.

Results

In Figure 1, the effects of norepinephrine and carbamylcholine chloride on C-AMP in slices of atrial and ventricular rabbit myocardium are shown. Norepinephrine 10^-5M increased C-AMP by 70 ± 10% in atrium and by 65 ± 10% in ventricular myocardium (top). In contrast, carbamylcholine chloride 10^-5M reduced C-AMP by 45 ± 10% in atrium but only by 10 ± 8% in the ventricle (bottom).

In an attempt to relate these changes to physiological events, cat atrial strips and papillary muscles were studied in a myograph.

![Figure 1](image1.png)

*Figure 1* Effect of 10^-5M NE and 10^-5M carbamylcholine chloride (CCC) on accumulation of 14C C-AMP and tension development in cat atrial strips and right ventricular papillary muscle. NE effects were significant (P < 0.001), as were the effects of carbamylcholine chloride in the atrium (P < 0.001), although not for the ventricle.

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![Figure 2](image2.png)

*Figure 2* Effect of 10^-5M NE and 10^-5M carbamylcholine chloride (CCC) on accumulation of 14C C-AMP and tension development in cat atrial strips and right ventricular papillary muscle. NE effects were significant (P < 0.001), as were the effects of carbamylcholine chloride in the atrium (P < 0.001), although not for the ventricle.

![Figure 3](image3.png)

*Figure 3* Effect of 10^-5M carbamylcholine chloride (CCC) on the disappearance of 3H C-AMP from phosphodiesterase preparations of rabbit atrial and ventricular myocardium. No significant difference was noted between control and experimental values by Student's t-test.
Accumulation of \(^{14}\)C C-AMP in adenyl cyclase preparations from rabbit atrial and ventricular myocardium treated with either \(10^{-5}\)M NE or \(10^{-5}\)M carbamylcholine chloride (CCC). NE induced significant increments in adenyl cyclase activity in both atrial and ventricular preparations \((P < 0.01)\), and carbamylcholine chloride significant decrements \((P < 0.01)\) in atrial preparations.

Figure 2 shows the effect of carbamylcholine chloride and norepinephrine on both isometric tension development and C-AMP. It can be seen that the characteristic positive inotropic effect of norepinephrine on both the atrium and ventricle was associated with a similar and significant increment in C-AMP, measuring 71 ± 10% and 75 ± 14% respectively. Moreover, the characteristic negative inotropic effect of carbamylcholine in the atrium was associated with a 50 ± 15% decrease in atrial C-AMP. However, carbamylcholine chloride produced only a small decrease in tension in the papillary muscle and this was associated with only an 11 ± 6% decrement in C-AMP.

Phosphodiesterase and adenyl cyclase preparations were then studied to further evaluate the mechanisms by which C-AMP was altered. The effect of carbamylcholine chloride on phosphodiesterase activity in the rabbit atrium and ventricle is shown in Figure 3. There was no significant difference between control and treated enzyme preparations after 2, 4, and 6 minutes of incubation in both the atrial and the ventricular preparations.

The effect of norepinephrine \((10^{-5}\)M) and carbamylcholine chloride \((10^{-5}\)M) on adenyl cyclase activity from rabbit atrial and ventricular subcellular preparations \((2000 \times g\) pel-
Accumulation of $^{14}C$ C-AMP in adenyl cyclase preparations from rabbit atrial and ventricular myocardium treated with $10^{-5}$M carbamylcholine chloride (CCC) alone or with $10^{-5}$M atropine. Atrial adenyl cyclase activity was decreased significantly ($P < 0.01$) by carbamylcholine chloride, and this effect was blocked by atropine. No significant differences were noted in the ventricular preparation.

To evaluate the specificity of this cholinergic response, the enzyme and slice preparations from the rabbit were then studied with the addition of atropine ($10^{-5}$M). The cholinergic effect on both C-AMP (Fig. 5) and adenyl cyclase (Fig. 6) was blocked with equimolar concentrations of atropine both in the slice and in the 2000 x g pellet adenyl cyclase preparation. Similar effects were noted in the cat preparations as well. Figure 7 shows a dose-response curve of carbamylcholine for the cat atrial muscle adenyl cyclase preparation. A significant effect was present at $10^{-6}$M and above, but not at $10^{-7}$M.

Since there is a known competition (9, 16) between cholinergic and adrenergic compounds in mammalian myocardium, the combined effect of norepinephrine and carbamylcholine chloride on isometric tension development was studied in the cat atrium and cat right ventricular papillary muscle. Figure 8 shows the effect of carbamylcholine chloride $10^{-5}$M and norepinephrine $10^{-5}$M alone and norepinephrine $10^{-5}$M and carbamylcholine chloride $10^{-5}$M in combination on tension development in the cat atrial and ventricular
Changes in tension development produced by $10^{-5}$M NE alone or with $10^{-5}$M carbamylcholine chloride (CCC) in cat atrial and right ventricular papillary muscle. NE produced significant increments in tension in both atrial and ventricular muscle ($P < 0.001$). Carbamylcholine chloride decreased tension slightly though significantly ($P < 0.05$) in the ventricular preparation. In the atrial preparation it decreased developed tension significantly ($P < 0.01$) but did not alter the increments in tension produced by NE in ventricular muscle. However, in the atrium, carbamylcholine chloride significantly ($P < 0.01$) decreased the NE effect on tension.

Discussion

Cyclic AMP has been implicated in the control of numerous metabolic functions in various organs (13, 17-19). Cyclic AMP is synthesized from ATP by the actions of adenyl cyclase, which is membrane bound and magnesium dependent; it is broken down by a specific phosphodiesterase with the production of adenosine-5-monophosphate. In heart, skeletal muscle, and liver, the activation of phosphorylase produced by catecholamine has been shown to be mediated by cyclic AMP, which is effected by activation of adenyl cyclase (17). Whether the increase in cyclic AMP mediated by catecholamines is directly related to the observed enhancement of contractility as well as yet to be proved. Furthermore, whether negative hormonal inotropic influences are mediated by this system has not been explored.

In the present study, the positive inotropic effects of norepinephrine were associated with positive changes, while the negative inotropic effects of cholinergic agents were associated with negative changes in adenyl cyclase and $^{14}$C cyclic AMP. Moreover, the effects of norepinephrine were the same for both atrial and ventricular myocardium. However, while cholinergic agents were shown to decrease both adenyl cyclase and cyclic AMP as well as contractility in atrial tissue, they had little effect on mechanical activity or the cyclic AMP system in ventricular myocardium.

The different sensitivity of atrial and ventricular myocardium to acetylcholine has been noted previously (16), and the lack of response of the ventricular cyclic AMP system to cholinergic agents parallels this finding. Whether this lack of ventricular response to cholinergic substances is due to its limited vagal innervation (20) and possibly to a paucity of associated specific receptor sites or whether there are primary differences in the membranes of atrium and the ventricle is not known. Vincent and Ellis (10) have shown that catecholamines increase glycogenolysis in the atrium and that acetylcholine attenuates this response. In a more recent publication from their laboratory they reported that cholinergic stimulation did not antagonize the catecholamine inotropy on ventricle but under certain circumstances did reduce glycogenolysis (21). The present studies on the cyclic AMP system in the heart are compatible with these findings.

The role of C-AMP in mediating hormonal
changes in contractility is still a matter of debate. While the activation of phosphorylase is delayed relative to the changes in contractility induced by catecholamines, C-AMP is increased early both in this and other studies (3, 4, 28). Although exogenously administered C-AMP has not been shown to increase contractility in isolated cardiac preparations (6), the possibility that C-AMP does not penetrate myocardial cells has not been excluded. Recently, Skelton et al. have shown that the dibutyryl form of C-AMP, which may have greater transmembrane permeability, induced augmentation of contractility of isolated papillary muscles but only at very high concentrations (23). Moreover, the effect of dibutyryl adenosine-5-monophosphate has not been evaluated. Thus it is still not certain whether these relationships indicate that cyclic AMP is only associated with changes in intermediary metabolism which occur pari passu with the alterations in inotropy produced by autonomic agents or whether cyclic AMP directly influences contractility.

A unified hypothesis relating the various effects of C-AMP to changes in the kinetics of calcium has been proposed by Rasmussen and Tenenhouse (24). This hypothesis can be extended to the findings in cardiac muscle. Both adrenergic and cholinergic agents have been shown to affect calcium fluxes in tissue, although the site and precise mechanism is unknown (25). The interrelations of these latter findings relative to the effects of C-AMP have yet to be fully elucidated.

The ability of cyclic AMP to activate phosphorylase B kinase requires Ca$^{2+}$ (26-28). Furthermore, Ca$^{2+}$ itself can activate this enzyme system (29). Thus, the findings by Vincent and Ellis that cholinergic agents blocked the catecholamine-mediated glycogenolysis in atrium could be explained by either a reduction in C-AMP or calcium or both in this tissue (10). A similar relationship may hold true for the effects of autonomic agents on the chemical-mechanical coupling system in muscle. Indeed, Grossman and Furchgott showed that acetylcholine decreased calcium exchange in the guinea pig atrium, that this was associated with the decrement in tension development, and that both these effects were blocked by atropine (35). Thus two possible situations obtained in the myocardium treated with acetylcholine. This possible decrement in calcium exchange may be a direct effect of acetylcholine or may be mediated secondarily by C-AMP. Also, if C-AMP may have no direct effect on the calcium system then the decreased glycogenolysis observed by Vincent and Ellis (10) may be related more to the altered calcium state than to the altered C-AMP system.

In summary, both adrenergic and cholinergic mediation of changes in cardiac contractility has been shown to occur parallel with changes in adenyl cyclase and C-AMP. Whether C-AMP is directly associated with excitation-contraction coupling or only associated with changes in intermediary metabolism that occur pari passu with the alterations in inotropy is yet to be established.

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