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COMMENTARY

THE QUESTIONABLE ROLE OF CYCLIC GUANOSINE 3′:5′-MONOPHOSPHATE IN HEART

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Proof that parasympathetic nerve impulses are mediated by chemicals was first obtained on the basis of the ability of electrical stimulation of the vagus nerve to release a substance capable of slowing the contractile rate of perfused frog hearts [1]; subsequently, the substance was found to be acetylcholine. Acetylcholine and related choline esters produce a negative inotropic as well as a negative chronotropic response when applied to cardiac preparations *in vitro* or *in vivo*. These inhibitory actions of choline esters are most pronounced in the presence of catecholamines or during other interventions which tend to increase myocardial cyclic adenosine 3′:5′-monophosphate (cyclic AMP) [2]. The phrases "antiadrenergic effects" [3] and "accentuated antagonism" [2] have been used to describe the increased effectiveness of choline esters in the presence of β-adrenergic agonists. These descriptions refer to the fact that choline esters produce a decrease in both the potency and the efficacy of catecholamines simultaneously applied to isolated cardiac preparations.

Cyclic AMP has been well established as a "second messenger" which mediates the cardiac contractile effects of the sympathetic nervous system and catecholamines [4]. Cyclic guanosine 3′:5′-monophosphate (cyclic GMP) has been proposed as a possible second messenger responsible for mediation of the contractile effects of the parasympathetic nervous system and choline esters. The basis for this proposition is: (1) the initial reports of a rise in cyclic GMP in myocardial preparations exposed to choline esters [5–8]; (2) the ability of lipophilic derivatives of cyclic GMP to mimic some of the contractile effects of choline esters [3, 9, 10]; (3) the ability of cyclic GMP to depress the magnitude of spontaneous tension oscillations in broken cardiac cell preparations [11]; (4) the ability of cyclic GMP-dependent protein kinase to phosphorylate troponin I *in vitro* [12]; and (5) the appeal of drawing a parallel between roles for cyclic GMP, in response to parasympathetic neurotransmission, and for cyclic AMP, in response to sympathetic neurotransmission. With regard to the latter point, Goldberg *et al.* [13] advanced the "yin yang" hypothesis which proposed that cyclic AMP and cyclic GMP have opposing actions in a variety of organ systems.

We will discuss evidence and present data which suggest that the contractile effects of choline esters on cardiac muscle are not mediated by cyclic GMP. We have employed isolated atria, which are more sensitive to the contractile effects of choline esters than are isolated ventricular or perfused whole heart preparations. In the rat atrium it is possible to dissociate the negative inotropic and antiadrenergic effects of choline esters from cyclic GMP elevation. In this tissue choline esters produce a marked decrease in developed tension without influencing tissue levels of cyclic GMP. Cyclic GMP can be elevated by treating atria with nitroprusside without producing choline ester-like contractile effects. To determine if nitroprusside can mimic the effects of choline esters during conditions when myocardial cyclic AMP is elevated, this agent can be studied in the presence of β-adrenergic agonists. The results of these experiments revealed that, if a chemical interaction between nitroprusside and catecholamines is prevented, nitroprusside has very little effect on developed tension, even during conditions when cyclic AMP is elevated. Nitroprusside also does not alter the ability of methacholine to decrease developed tension.

The other type of information used to support the proposition that cyclic GMP mediates the contractile actions of choline esters is that lipophilic derivatives of cyclic GMP mimic the actions of choline esters when applied to isolated cardiac preparations. An antiadrenergic action by cyclic GMP analogs (i.e. their ability to decrease the apparent potency of catecholamines) is the most provocative of these effects since cyclic GMP has been found to promote cyclic AMP degradation in broken cell systems [14,15]. This effect of cyclic GMP on cyclic AMP metabolism suggests a possible mechanism for the antiadrenergic actions of cyclic GMP analogs. However, we have demonstrated that two additional negative inotropic agents, verapamil and cyclic AMP (applied exogenously) in addition to decreasing developed tension in rat atria, produce a pronounced shift to the right in the potency of isoproterenol. These "antiadrenergic" effects by verapamil and exogenously applied cyclic AMP detract from the significance of the antiadrenergic effects of cyclic GMP analogs. Also, concentrations of 8-bromo-cyclic GMP (8-Br-cGMP) and methacholine, which produce comparable effects on developed tension, do not produce comparable antiadrenergic effects. We conclude that cyclic GMP does not mediate the actions of choline esters in rat and guinea pig atria, and that it is of questionable relevance in other

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cardiac preparations. Both the negative inotropic and antiadrenergic effects of choline esters may result primarily from their direct effects on the sarcolemma.

Diverse effects of choline esters

The effects of choline esters on cardiac contractility result from more than one action. Part of the negative inotropic response to choline esters *in vivo* is mediated by the ability of muscarinic agonists to inhibit norepinephrine release from sympathetic nerve terminals [16]. In addition to this antiadrenergic action at the level of the nerve, choline esters produce an antiadrenergic action at the level of the muscle, i.e. they can antagonize the effects of catecholamines applied directly to isolated cardiac preparations including mammalian ventricles [2,17]. Negative inotropic and chronotropic effects of choline esters can be observed in the absence of catecholamines or other agents which elevate cyclic AMP. The latter actions of choline esters are most pronounced in mammalian atria, amphibian ventricles [1], and avian ventricles, while they are very small or absent in mammalian ventricles [3,17]. In addressing the question of the role of cyclic GMP in cardiac muscle, one must examine both the antiadrenergic effect of choline esters and their effects in the absence of other drugs.

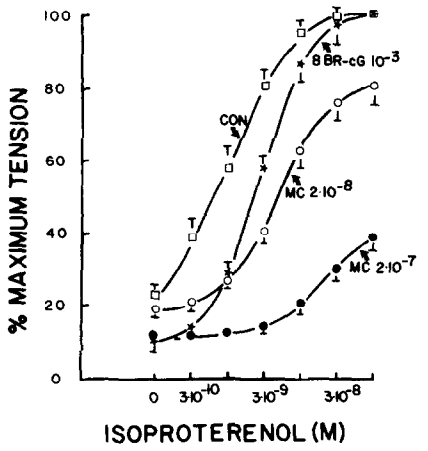

Fig. 1. Effects of 8-Br-cGMP and methacholine on the potency and efficacy of isoproterenol. Isolated left atria from 200 to 300 g male Wistar rats were placed in a buffered solution containing in mM: NaCl 118; KCl, 4.75; KH_2PO_4, 1.2; $MgSO_4$, 1.2; dextrose, 5.5; $NaHCO_3$, 25; and $CaCl_2$, 2. The solution was bubbled with 95% oxygen–5% CO_2 and maintained at 30°; the pH was 7.4. The atria were stimulated through platinum–iridium point electrodes with 10 msec monophasic pulses just above the threshold for contractions at a rate of 1 Hz. After a 60-min equilibration period, 10^{-7} M *l*-isoproterenol was added to establish maximal tension for each atrium. After five washes and 45 min basal tension was restored. Atria were then pretreated with 8-Br-cGMP, 1 μM physostigmine (all atria) and/or methacholine (MC) for 20, 10 and 5 min respectively, before cumulative additions of isoproterenol. Each point is the mean ± standard error of four atria. The negative inotropic effect of 8-Br-cGMP in the absence of *l*-isoproterenol was greater than the negative inotropic effect of 2×10^{-8} M methacholine at $P < 0.05$.

Contractile effects of methacholine in atria

The contractile effects of cumulative methacholine additions to atrial preparations stimulated at different rates reveal a marked frequency dependence. Methacholine (3 μM) decreased developed tension 9 ± 1, 62 ± 3, and 82 ± 4 per cent, respectively, in guinea pig atria stimulated at 0.1, 1 and 3 Hz (mean ± standard error, n = 4). A frequency dependence of the inotropic actions of choline esters was also observed in rat atria and has been reported previously in cat atria [18]. In light of this frequency dependence, it is unlikely that choline esters produce a direct inhibitory effect on contractile proteins. On the other hand, frequency dependence might result from an ability of choline esters to inhibit the movement of calcium across the sarcolemma. Verapamil is an example of another compound which decreases the movement of contractile calcium across the sarcolemma, decreases developed tension, and displays a marked frequency dependence [19].

The characteristic negative inotropic and antiadrenergic actions of methacholine in rat atria are illustrated in Fig. 1. In addition to decreasing developed tension in the absence of other drugs, methacholine decreases the potency and efficacy of the β-adrenergic agonist, isoproterenol, in a dose-dependent manner.

Contractile effects of cyclic GMP analogs in atria

8-Br-cGMP can decrease developed tension in rat atria, and can reduce the potency, but not the efficacy of isoproterenol (Fig. 1). The negative inotropic action of this agent develops over a period of 10–15 min. Neither negative inotropic nor antiadrenergic effects can be observed in response to comparable doses of guanosine, cyclic GMP, or 8-Br-cyclic inosine monophosphate. Exogenously applied cyclic AMP does depress contractility, but this effect is fully developed in less than a minute. It is notable that, while 1 mM 8-Br-cGMP is more effective than 20 nM methacholine as a negative inotropic agent, it is far less effective as an inhibitor of the positive inotropic response to isoproterenol (Fig. 1). These data indicate that methacholine acts by mechanisms other than those affected by 8-Br-cGMP.

Lack of correlation between cyclic GMP elevation and negative inotropy

Although cyclic GMP elevation has been associated with negative inotropy, some studies suggest that these responses are not causally related. In perfused guinea pig hearts, concentrations of acetylcholine which increased cyclic GMP in excess of 4-fold decreased developed tension less than 10 per cent [3]. On the other hand, in isolated guinea pig atria, a concentration of carbachol (0.3 μM) which had no significant effect on the tissue concentration of cyclic GMP decreased developed tension over 80 per cent (Fig. 2). The cat atrium also required relatively high concentrations of acetylcholine to elicit a small transient rise in cyclic GMP [21]. In general, a comparison of the atrial and ventricular actions of choline esters reveals that the atrial negative inotropic response is much more pronounced, while the cyclic GMP response is smaller, more transient, and

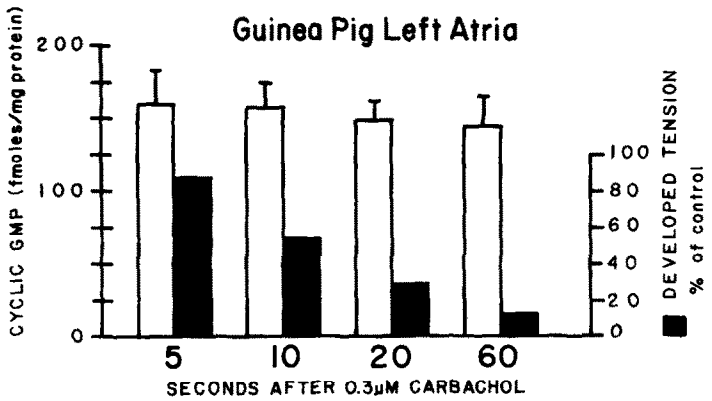

Fig. 2. Time course of cyclic GMP content and the onset of the negative inotropic action of 0.3 μM carbachol. Seven to eight atria, stimulated at a rate of 3 Hz, were used at each time point. (Reprinted from Brooker [20].)

requires higher concentrations of muscarinic agonists. An extreme case of this generalization is illustrated in Table 1. These data indicate that in the rat atrium no increase in cyclic GMP can be observed in response to very large additions of methacholine which virtually abolish developed tension. Thus, in perfused guinea pig hearts choline esters induce large increases in cyclic GMP associated with small changes in developed tension, while in rat atria large decreases in developed tension occur in the absence of an elevation of tissue cyclic GMP.

Effect of nitroprusside in the absence of β-adrenergic agonists

A useful means of evaluating the role of cyclic GMP in mediating the actions of choline esters is to observe the effect of elevating this nucleotide by a means other than by adding choline esters. Such a means has been provided by the discovery that nitric oxide can activate both particulate and soluble guanylate cyclase in intact as well as broken cell preparations [24]. Nitroprusside, which generates nitric oxide in solution [25], has been employed to generate large (> 10-fold) increases in guinea pig atrial [26] and cat atrial [21] cyclic GMP. These rises in cyclic GMP were not associated with any decline in developed tension. The effects of 1 mM nitroprusside on contractile tension and on cyclic GMP levels were determined in rat atria prepared as described in the legend for Table 1. Nitroprusside was added to some atria 15 min prior to freezing. Control and nitroprusside-treated atria contained 290 ± 26 and 1580 ± 542 fmoles/mg of protein, respectively, of cyclic GMP (mean ± standard error, n = 6) while cyclic AMP did not change. Developed tension in the atria treated with nitroprusside declined only 6 ± 3 per cent during the 15-min interval.

Table 1. Cyclic GMP levels in rat left atria*

Exp.	Methacholine concn (μM)	Exposure time (sec)	Cyclic GMP (fmoles/mg protein)
1	0		242 ± 23
	1	5	218 ± 20
	1	15	236 ± 38
	1	25	280 ± 38
2	0		284 ± 60
	10	30	207 ± 32
3	0		272 ± 13
	10	30	301 ± 65
4	0		291 ± 41
	10	30	245 ± 28
5	0		196 ± 31
	10	30	225 ± 37

* Isolated rat left atria were stimulated at a rate of 3 Hz. Control or methacholine-treated atria were frozen between tongs precooled in liquid nitrogen. Cyclic GMP was determined in acetylated extracts of tissues according to the method of Harper and Brooker [22]. Proteins were determined on an autoanalyzer by a modification of the method of Lowry *et al.* [23]. The results of experiments performed on four atria per condition on five different days are displayed. In similarly treated atria, 1 and 10 μM methacholine produced an 80 ± 2 and 89 ± 2 per cent decline in developed tension, respectively, within 1 min (n = 4). Ten μM Methacholine was maximally effective on tension development.

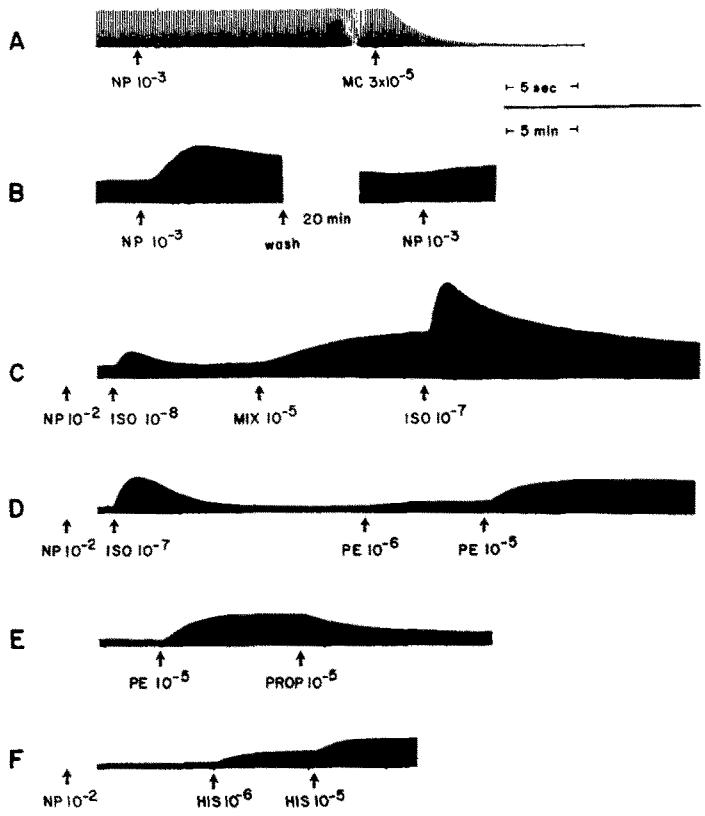

Fig. 3. Contractile effects of nitroprussides alone and in combination with agents which elevate myocardial cyclic AMP. Each panel shows a contractile record from a single rat (A) or guinea pig (B–F) left atrium. All atria except (B) were derived from animals treated with reserpine (5 mg/kg) 15–20 hr prior to being killed. (A) was stimulated at a rate of 6 Hz, and (B) through (F) at a rate of 3 Hz. In (C), (D) and (F) nitroprusside was added 10–15 min prior to the displayed records. Abbreviations: NP = nitroprusside; MC = methacholine; PE = *l*-phenylephrine hydrochloride; PROP = *l*-propanolol; and HIS = histamine dihydrochloride.

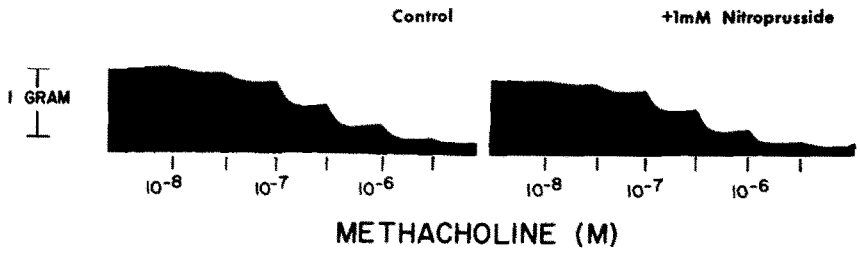

Fig. 4. Failure of nitroprusside to influence the contractile effects of methacholine in rat atria. A rat left atrium derived from an animal pretreated with 5 mg/kg of reserpine 18 hr prior to being killed was stimulated at a rate of 3 Hz. The atrium was exposed to cumulative additions of methacholine (10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6} and 3×10^{-6} M) at 2-min intervals. After two washes and a 20-min equilibration period, the atrium was treated with 1 mM nitroprusside, and the methacholine additions were repeated. In other experiments not shown, rat or guinea pig atria were exposed to cumulative additions of methacholine only once in the presence or absence of nitroprusside. The potency of methacholine was not significantly influenced by nitroprusside ($P > 0.1$ by Student's *T*-test, $n = 4$).

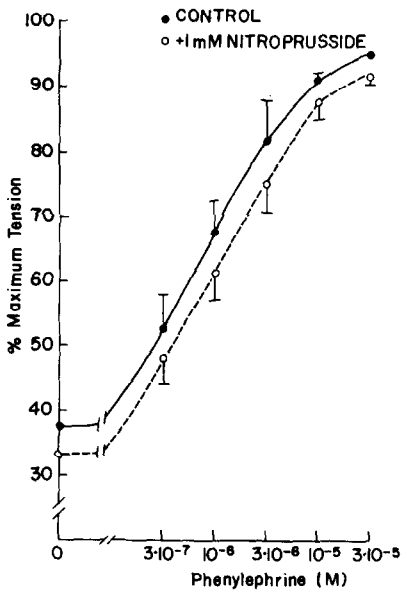

Fig. 5. Failure of nitroprusside to influence the potency of phenylephrine in the rat atria. Rat left atria derived from reserpinized animals were bathed in the buffer described in the legend of Fig. 1 except that the calcium content was reduced to 1 mM. Maximal tension was determined as in Fig. 1. Cumulative doses of *l*-phenylephrine hydrochloride were added at 5-min intervals in the presence or absence of nitroprusside (n = 4). In this figure and in Figs. 6 and 7, the calcium content of the buffer was reduced to magnify the increment in contractility produced by β-adrenergic agonists.

Although the addition of 1 mM nitroprusside to rat atria, which were derived from reserpinized animals (and thereby depleted of endogenous norepinephrine), had little or no effect on developed tension, the subsequent addition of a high concentration of methacholine could virtually abolish contractions within 10 sec (Fig. 3A). In atria which were not reserpinized, the addition of nitroprusside evoked a transient positive inotropic response which was reduced or absent when the drug was washed out and reapplied (Fig. 3B). Diamond *et al.* [21] also reported a small positive inotropic response after nitroprusside addition to isolated cat atria. We found that this reponse could be reduced either by reserpinizing animals or by pretreating atria with the β-adrenergic antagonist propranolol, suggesting that it results from the release of endogenous norepinephrine.

Effects of nitroprusside in combination with agents which elevate myocardial cyclic AMP

The addition of 1–10 mM nitroprusside to guinea pig or rat atria previously exposed to catecholamines (isoproterenol or norepinephrine) produced a negative inotropic action. These results suggest an antagonistic interaction which might be interpreted as occurring at the cyclic nucleotide level; however, further experiments revealed that there is a chemical interaction between nitroprusside and catecholamines leading to the destruction of the catecholamines. Destruction of isoproterenol in the medium of an atrium pretreated with nitroprusside is suggested in Fig. 3C. Isoproterenol produced only a transient positive inotropic response in the presence of nitroprusside. On the other hand, the phosphodiesterase inhibitor, 1-methyl-3-isobutyl xanthine (MIX), produced a stable positive inotropic response in the presence of nitroprusside. Even in the presence of MIX and nitroprusside, the action of isoproterenal was transient. The noncatecholamine, phenylephrine, produced a stable inotropic response in the presence of nitroprusside (Fig. 3D). Fig. 3E was added to illustrate that the action of phenylephrine is mediated by a β-adrenergic receptor. Also, a positive inotropic response of guinea pig atria to the noncatecholamine, histamine, is stable in the presence of nitroprusside (Fig. 3F). These data suggest that nitroprusside promotes the destruction of the easily oxidizable catechol moiety. The ability of 1 mM nitroprusside to reduce tension in the presence of norepinephrine or isoproterenol was completely blocked by the prior addition of the antioxidant ascorbic acid (10 μM). Proof that nitroprusside promotes the destruction of catecholamines was provided by incubating 1 μM norepinephrine or isoproterenol in the same container with 1 mM nitroprusside for 30 min. Under these circumstances an aliquot of these solutions (final catecholamine concentration, 10 nM) failed to evoke even a transient inotropic response when added to atria. We can conclude that nitroprusside, which is rapidly reduced in aqueous solutions [25], accelerates the oxidation of catecholamines, and that this process is blocked by antioxidants.

Figure 4 illustrates that pretreatment of reserpinized rat atria with 1 mM nitroprusside failed to influence the effect of methacholine on developed tension. This experiment demonstrates that choline esters produce their contractile effects even if cyclic GMP levels are already elevated. These results also eliminate the possibility that nitroprusside produces a secondary action which blocks the ability of cyclic GMP to depress contractility.

In the presence of 10 μM ascorbate, pretreatment of rat atria with 1 mM nitroprusside had no effect on the ability of isoproterenol to affect cyclic AMP or force generation ($P > 0.05$ by Student's *t*-test, n = 4). However, ascorbate, in addition to blocking catecholamine oxidation, may influence nitric oxide generation and activation of guanylate cyclase. Therefore, the effect of nitroprusside on the inotropic response to the noncatecholamine, phenylephrine, was studied in the absence of ascorbate. The results of this study are illustrated in Fig. 5. Although nitroprusside has a slight depressant effect on contractility, it has no effect on the potency of phenylephrine. Similarly, in reserpinized guinea pig left atria paced at 1 Hz, a high concentration of nitroprusside (20 mM) decreased developed tension in control and histamine (1 μM)-pretreated preparations 9 ± 3 and 7 ± 2 percent respectively. In contrast, methacholine (0.2 μM) decreased developed tension 33 ± 4 and 59 ± 5 percent in control and histamine (1 μM)-pretreated preparations. Thus, in contrast to both choline esters and 8-Br-cGMP, nitroprusside has little effect in the absence of other drugs, and cannot reverse the contractile effect of

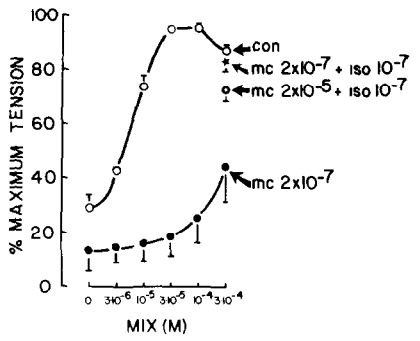

Fig. 6. Influence of methacholine on the inotropic response to 1-methyl-3-isobutyl xanthine (MIX). Rat left atria were prepared and maximal tension was determined as described in the legend for Fig. 1. Atria were pretreated with methacholine (MC) and/or 1 μM physostigmine for 5 and 10 min, respectively, prior to cumulative additions of MIX. *l*-Isoproterenol (ISO) and additional methacholine were added in the presence of 300 μM MIX. Each point is the mean ± standard error of four atria.

agents which elevate myocardial cyclic AMP in rat or guinea pig atria. The lack of similarity between nitroprusside and 8-Br-cGMP indicates one of the following: (1) nitroprusside increases cyclic GMP largely in a physiologically unimportant pool, (2) 8-Br-cGMP produces effects equivalent to very large levels of cyclic GMP, or (3) 8-Br-cGMP has a nonspecific depressant effect on contractility.

Effect of methacholine on cyclic AMP metabolism

The phosphodiesterase inhibitor MIX is as efficacious as isoproterenol at increasing developed tension in rat atria. Figure 6 illustrates that methacholine decreases the potency of MIX in a manner similar to its effect on isoproterenol. However, in the presence of a combination of high concentrations of MIX (300 μM) and isoproterenol (10^{-7} M), methacholine is not very effective at decreasing developed tension. These data suggest that methacholine may act in part by decreasing the ability of the tissue to generate cyclic AMP. As indicated in Table 2, 1 μM methacholine reduced cyclic AMP elevation in atria exposed to 0.1 μM isoproterenol, but this action alone is not sufficient to account for the resultant decline in developed tension. These results are consistent with those of Keely *et al.* [27] who demonstrated that the inotropic effect of epinephrine on perfused rat hearts could be completely blocked by a concentration of acetylcholine which did not abolish cyclic AMP elevation or protein kinase activation. Table 2 also shows that, in the presence of isoproterenol plus methacholine, cyclic GMP elevation is absent in rat atria, so the antiadrenergic effect of methacholine is apparently mediated by a direct effect of the choline ester, or by some factor not involving cyclic nucleotide metabolism. In similar experiments, spontaneously beating right atria were exposed to isoproterenol and MIX plus or minus methacholine. Again, altered cyclic GMP levels could not account for the contractile effects of methacholine.

It might be argued that the atria described in Table 2 failed to develop a full inotropic response during the 1-min interval in which they were exposed to isoproterenol prior to freezing. However, as shown in Fig. 7, the inability of isoproterenol to overcome the inhibitory action of 1 μM methacholine is not reversed by time or isoproterenol concentration. The muscarinic antagonist atropine rapidly and completely reversed the antiadrenergic actions of methacholine. The negative inotropic effect of methacholine (in the absence of isoproterenol) in rat atria was also rapidly (< 5 min) reversed by the addition of atropine or by washing. On the other hand, we confirmed the observation of Nawrath [28] that the negative inotropic effect of 8-Br-cGMP on rat atria could only be partially reversed even after prolonged (> 60 min) periods of washing. Furthermore, while

Table 2. Rat atrial cyclic nucleotide levels and contractile responses during exposure to combinations of methacholine, isoproterenol and MIX*

	Cyclic AMP (pmoles/mg protein)	Cyclic GMP (fmoles/mg protein)	% Control twitch tension	% Control time to peak tension	% Control rate
Left atria					
Control	3.5 ± 0.4	458 ± 53	100	100	
l-Isoproterenol	15.0 ± 1.4†	416 ± 38	228 ± 10	90 ± 2	
l-Isoproterenol + methacholine	7.1 ± 0.9‡	473 ± 69	41 ± 6‡	100 ± 5	
Right atria					
Control	4.9 ± 0.4	284 ± 18	100		100
MIX + *l*-isoproterenol	34.3 ± 3.7†	355 ± 45	213 ± 18		164 ± 10
MIX + *l*-isoproterenol + methacholine	13.0 ± 0.8‡	373 ± 29§	136 ± 9‡		157 ± 11

* Rat atria in buffer containing 2 mM calcium were exposed to MIX (10^{-5} M), isoproterenol (10^{-7} M) and methacholine (10^{-6} M) for 3, 2 and 1 min, respectively, before freezing. Left atria were paced 1 Hz, and developed an average tension of 0.54 ± 0.04 g (mean ± standard error) prior to drug additions. The average time to peak tension was 72.5 ± 1.2 msec. The right atria beat spontaneously at an average rate of 3.02 ± 0.1 Hz and an average tension of 0.63 ± 0.18 g. Cyclic nucleotide levels and contractile parameters determined just prior to freezing were obtained from the same tissues. N = 8–9.

† Different from control, atria $P < 0.01$.

‡ Different from Iso (left atria) or MIX + Iso (right atria), $P < 0.01$.

§ Different from control $P < 0.02$; not different from MIX + Iso, $P > 0.05$.

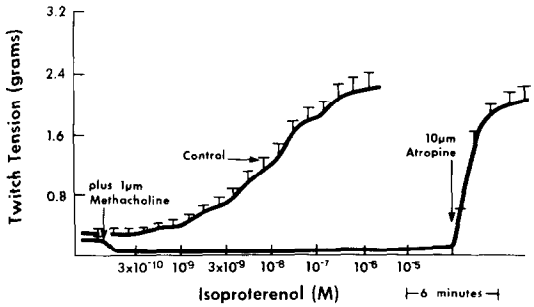

Fig. 7. Influence of methacholine on the ability of isoproterenol to increase developed tension in rat atria. Rat left atria were stimulated at a rate of 1 Hz. All atria were pretreated with 1 μM physostigmine and some atria were pretreated with 1 μM methacholine. *l*-Isoproterenol was added cumulatively at 3-min intervals as indicated on the abscissa. Each curve represents the mean tension ± standard error of four atria plotted as a function of time.

acetylcholine does not produce a negative inotropic effect on isolated mammalian ventricular preparations (including cat papillary muscle) in the absence of catecholamines [17], Nawrath [28] observed a 67 percent decrease in the developed tension of cat papillary muscle exposed to 1 mM 8-Br-cGMP alone. These data represent additional evidence suggesting that choline esters and cyclic GMP analogs decrease contractility in the absence of catecholamines by different mechanisms.

Actions of choline esters not mimicked by cyclic GMP analogs

It has been known since 1958 that acetylcholine increases potassium conductance in the myocardium [29]. A resultant decrease in the rate of phase 4 depolarization in pacemaker cells may account for the negative chronotropic effects of choline esters. Also, an increase in potassium conductance produces a shortening of the action potential duration during exposure to acetylcholine [30]. This action is probably responsible, at least in part, for reducing developed tension.

In addition to producing negative inotropic and chronotropic effects in atria, and antiadrenergic actions in atria and ventricles, choline esters have been reported to increase the magnitude of contracture development in depolarized cardiac preparations [31, 32], to increase ^{42}K efflux from rat atria [33], and to decrease adenylate cyclase activity from broken cell cardiac preparations [34–37]. Of these six effects, cyclic GMP analogs have been reported to mimic only two—the negative inotropic and antiadrenergic actions—and, as mentioned above, the antiadrenergic effect is weak relative to the effect of methacholine. Nitroprusside does not mimic any of the contractile effects of choline esters.

Choline esters appear to exert effects on the sarcolemma where they alter the conductance of potassium and calcium [32,38]. On the other hand, cyclic GMP analogs, in addition to not influencing potassium conductance [33], fail to mimic the ability of choline esters to decrease the frequency of spontaneously beating myocardial preparations in the absence of β-adrenergic agonists [28,39,40]. This lack of effect on basal rate, which is probably related to the inability of these agents to enhance potassium conductance, is significant in light of the fact that the negative inotropic effect of choline esters is thought to result at least partly from shortening of the cardiac action potential, an action which is also secondary to increased potassium conductance.

The very pronounced frequency dependence of the negative inotropic action of choline esters suggests that they do not directly influence contractile proteins. It is also unlikely that choline esters influence calcium mobilization from intracellular pools since the application of acetylcholine during a pause between beats decreases the duration of the first post-rest action potential but has only a slight effect on the first post-rest contraction [41]. A locus of action at the level of the sarcolemma is consistent with the possibility that some, or all of the contractile effects of choline esters are direct, and do not require the involvement of a "second messenger".

Trautwein and Trube [11] suggested that cyclic GMP might exert a direct inhibitory effect on the sarcoplasmic reticulum since spontaneously beating broken cardiac cell preparations displayed a decreased frequency and force of contraction during exposure to 20 or 33 μM cyclic GMP. In order to appraise the physiological significance of this response to the addition of cyclic GMP to broken cells, it is necessary to calculate the approximate intracellular concentration of this nucleotide. The ratio of the milligrams of tissue wet weight to milligrams of protein in the rat atrium is about 10 [42]. Measurements of basal levels of cyclic GMP in various cardiac preparations have ranged between 100 and 1500 fmoles/mg of protein; low levels (<500 fmoles/mg of protein) are consistently observed when cyclic GMP is determined by radioimmunoassay, while high levels (>1000 fmoles/mg of protein) have been reported when enzyme cycling assays have been employed [20]. Using these limits, the total amount of basal free cyclic GMP is only 1×10^{-8} to 1.5×10^{-7} M assuming that no cyclic GMP is bound. A 4-fold rise in these levels in response to choline esters would increase this concentration to 6×10^{-7} M at most. In single cardiac cells from which the sarcolemma has been stripped, the application of cyclic GMP produces a biphasic contractile effect. One μM cyclic GMP slightly enhances developed tension, while 100 μM cyclic GMP has a depressant contractile effect.* This biphasic effect of cyclic GMP, which is inhibitory only when cyclic GMP is elevated to nonphysiologically high levels, is mediated by direct actions on contractile proteins rather than on the sarcoplasmic reticulum.* Thus, the ability of cyclic GMP to depress contractility in broken cell cardiac preparations when applied in concentrations 30–1000 times higher than physiological levels is of dubious significance.

The observation that cyclic GMP-dependent protein kinase can phosphorylate troponin I [12] should also be interpreted with caution. The same protein is phosphorylated by cyclic AMP-dependent protein kinase [43] which is not consistent with opposing physiologic roles for the cyclic nucleotides. Also, it is more likely that physiological levels of cyclic GMP

* 32. A. Fabiato, personal communication, 1978.

($< 1\ \mu M$) enhance rather than inhibit contractility by their direct effects on contractile proteins.

Nonspecificity of antiadrenergic actions

A possible means by which cyclic GMP analogs influence contractility in cardiac muscle is by activating one type of cyclic AMP phosphodiesterase [14, 15]. This enzyme had an apparent K_a of 3×10^{-7}M for cyclic GMP and influences the affinity, but not the maximum velocity, of the phosphodiesterase. In addition to inhibiting the effectiveness of low concentrations of isoproterenol, cyclic GMP analogs decrease the contractile effects of low, but not high doses of dibutyryl cyclic AMP in cat papillary muscle [10], consistent with the hypothesis that cyclic GMP influences the affinity of phosphodiesterase for cyclic AMP. However, the contractile effects of choline esters cannot be accounted for entirely by a depression of cardiac cyclic AMP. Furthermore, at least part of the ability of choline esters to prevent cyclic AMP elevation is caused by an inhibition of adenylate cyclase activity which can be demonstrated in broken cells and is not mediated by cyclic GMP [34–37].

It is apparent in Fig. 1 that both methacholine and 8-Br-cGMP have inhibitory influence on the ability of isoproterenol to increase developed tension. Compared to control atria, 1 mM 8-Br-cGMP produced a significant ($P < 0.05$) 1.7-fold increase of the ED50 of isoproterenol. Similarly, dibutyryl cyclic GMP (2.5×10^{-4} M) produced a significant 1.8-fold increase in the ED50 for isoproterenol. Pretreatment with 20 and 200 nM methacholine, respectively, produced a 3.1- and 8.8-fold increase in the ED50 for isoproterenol compared to controls.

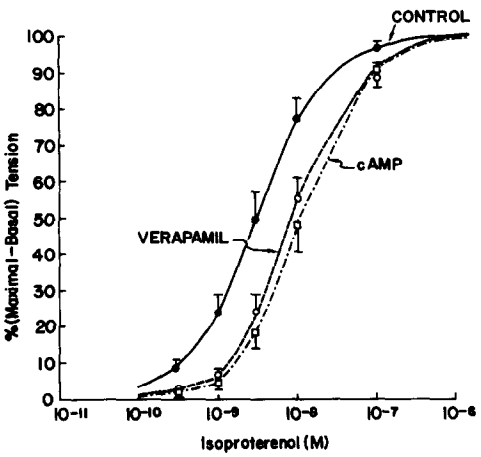

Fig. 8. Antiadrenergic actions of verapamil and cyclic AMP. Rat left atria from reserpinized animals were bathed in a buffer containing 1 mM calcium and paced at a rate of 1 Hz. Verapamil (1 μM) or cyclic AMP (1 mM) was added to some atria for 45 or 15 min, respectively, prior to cumulative additions of *l*-isoproterenol at 5-min intervals. Basal tension (prior to the addition of any isoproterenol) was subtracted from the response to each *l*-isoproterenol concentration and plotted as a percent of the maximal–basal (response to 1 μM *l*-isoproterenol–basal) tension. Elevating *l*-isoproterenol above 1 μM did not increase developed tension in the presence or absence of other drugs. The ED50 values (see text) were determined by fitting a least squares line to the log dose vs log (maximal–basal) tension relationship and extrapolating to the 50 per cent response value. Each curve represents the mean ± standard error of five atria.

A problem with the hypothesis that cyclic GMP analogs produce antiadrenergic effects by influencing cyclic AMP metabolism is that nitroprusside, which elevates myocardial cyclic GMP, fails to reproduce this effect. Consideration of this problem led us to suspect that negative inotropic agents in general might alter the influence of isoproterenol on contractility, i.e. produce "antiadrenergic" effects. Two agents which decrease rat atrial contractility were studied. Verapamil (1 μM) decreased developed tension 54 ± 3 per cent ($n = 5$) over a period of 40 min. Cyclic AMP (1 mM), applied exogenously, depressed contractility 47 ± 3 per cent within 1 min. The potency of isoproterenol in control atria and in the presence of verapamil or cyclic AMP is illustrated in Fig. 8. Verapamil and cyclic AMP, respectively, produced a 3.4- and 3.8-fold increase in the ED50 of isoproterenol. The significance of these observations is that they suggest that "antiadrenergic" actions are produced nonspecifically by agents which reduce calcium conductance at the level of the sarcolemma. Clearly, antiadrenergic effects are not limited to choline esters and cyclic GMP analogs. The inability of either 1 mM 8-bromo-cyclic inosine monophosphates or 1 mM cyclic GMP to depress contractility in rat atria supports the possibility that the effects of 8-Br-cGMP are the result of an action on phosphodiesterase. However, the inability of nitroprusside to mimic this response suggests that the effect could result from a nonspecific action at the level of the sarcolemma, or that cyclic GMP derivatives produce effects equivalent to massive rises in cyclic GMP which do not occur physiologically. In any event, both a verapamil-like antiadrenergic effect and an inhibitory effect on adenylate cyclase activity may be responsible for the antiadrenergic effects of choline esters in rat atria, which are not mediated by cyclic GMP.

Conclusions

Choline esters, in addition to modulating the activity of adrenergic nerves, produce several direct effects on cardiac muscle. In atrial muscle they decrease the rate and force of contraction. Although it is sometimes possible to correlate rises in myocardial cyclic GMP with the addition of choline esters, this observation does not necessarily indicate that cyclic GMP mediates the contractile effects of choline esters. In mammalian ventricular muscle it has been possible to elicit large rises in cyclic GMP with minimal contractile effects. We have demonstrated that, in guinea pig and rat atria, large negative inotropic effects elicited by choline esters are not accompanied by detectable changes in tissue cyclic GMP.

The contractile effects of choline esters are associated with an increase in potassium conductance. The inability of cyclic GMP analogs to mimic the negative chronotropic effect of choline esters in the absence of catecholamines is probably related to their inability to enhance potassium conductance which is of primary importance in mediating the rate of pace-

maker cells. An increase in potassium conductance not mediated by cyclic GMP is also likely to contribute to the negative inotropic response of choline esters by virtue of decreasing the duration of the cardiac action potential. Furthermore, although 8-Br-cGMP alone has been reported to produce a marked decrease in developed tension in cat papillary muscle, choline esters alone produce minimal contractile effects on all types of mammalian ventricular muscle, despite the fact that they promote cyclic GMP elevation. Thus, cyclic GMP derivatives can apparently depress developed tension by a means different than that used by choline esters. These considerations strongly suggest that the negative chronotropic and inotropic actions of choline esters in the absence of β-adrenergic stimulation are not mediated by cyclic GMP. The inability of nitroprusside to influence contractility, and the inability of methacholine to promote cyclic GMP formation in the rat atrium, where the negative intropic effect of choline esters is particularly pronounced, strengthen this argument.

The proposition that cyclic GMP elevation alone mediates the antiadrenergic actions of choline esters is also doubtful. These actions are not accompanied by cyclic GMP elevation in the rat atrium, and they are not mimicked by nitroprusside. In addition, there are three other pieces of information which detract from this proposition: (1) concentrations of methacholine and 8-Br-cGMP which produce a comparable decrease in developed tension do not produce comparable antiadrenergic actions; (2) it has been shown that choline esters have a direct inhibitory effect on adenylate cyclase activity in broken cell preparations, an action which is not mediated by cyclic GMP; and (3) antiadrenergic effects of choline esters, i.e. an ability to decrease the potency of isoproterenol, is mimicked by verapamil and cyclic AMP, suggesting that this effect is not necessarily the result of a specific cyclic GMP-dependent action. Since cyclic GMP has been reported to activate cyclic AMP phosphodiesterase at reasonable concentrations in broken cell preparations, it is possible that cyclic GMP analogs do depress cyclic AMP formation in cardiac muscle. However, nitroprusside elevates cyclic GMP and does not produce an antiadrenergic action. Also, choline esters produce very pronounced antiadrenergic effects in rat atria which are not associated with cyclic GMP elevation. These data suggest that factors other than cyclic GMP generation are involved in the antiadrenergic response.

The contractile effects of choline esters can be entirely accounted for without invoking the involvement of a "second messenger". The known effect of choline esters on potassium and calcium conductance, and the inability of physiological concentrations of cyclic GMP to influence the function of intracellular organelles are consistent with the proposition that choline esters act primarily at the level of the sarcolemma. This is also probably the locus of action for inhibitory effects of choline esters on adenylate cyclase activity. The similarity between verapamil and methacholine, both with regard to their frequency dependence and their ability to decrease the potency of isoproterenol, suggests a possible common locus of action at the level of the sarcolemma.

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