MOLECULAR PHARMACOLOGY, 16, 841-850

Depolarization-Induced Inhibition of Cyclic AMP Accumulation: Cholinergic-Adrenergic Antagonism in Murine Atria

JOAN HELLER BROWN

Division of Pharmacology, Department of Medicine, M-013, University of California, San Diego, La Jolla, California 92093

(Received March 19, 1979)
(Accepted June 5, 1979)

SUMMARY

BROWN, JOAN HELLER: Depolarization-Induced inhibition of cyclic AMP accumulation: Cholinergic-adrenergic antagonism in murine atria. *Mol. Pharmacol.* **16**, 841-850 (1979).

Isolated mouse heart atria incubated in medium containing 57 mM potassium chloride (K^+) lose their capacity to make cyclic AMP when stimulated with isoproterenol. With lower concentrations of medium K^+ (22 and 40 mM) there is partial blockade of catecholamine sensitive cyclic AMP accumulation. Inhibition is apparent within minutes of exposure to high K^+ medium and is reversed in atria returned to medium of normal (6 mM) K^+ concentration. K^+ induced inhibition requires external calcium, and is blocked by atropine. The cholinergic, agonist, carbachol, also blocks isoproterenol-responsive cyclic AMP accumulation in murine atria, with an IC_{50} of 0.1 μM. Inhibition by carbachol is reversed by atropine, but in contrast to the effect of K^+, carbachol-induced inhibition does not require external calcium. Both K^+ and carbachol produce noncompetitive inhibition of isoproterenol stimulated cyclic AMP accumulation, with 85-90% inhibition at maximal concentrations. Neither manipulation lowers the basal cyclic AMP concentration or the increase in cyclic AMP produced by cholera toxin. Catecholamine-sensitive cyclic AMP accumulation is also markedly depressed in atria incubated with the cholinesterase inhibitor, physostigmine, and in atria exposed to millimolar concentrations of choline (to replace sodium). Atropine abolishes the inhibition produced by these interventions. The findings presented here demonstrate muscarinic antagonism of isoproterenol-sensitive cyclic AMP accumulation in murine atria and suggest that acetylcholine released from cholinergic nerve endings by K^+ depolarization or released spontaneously while cholinesterase is inhibited, can regulate cyclic AMP responses to adrenergic stimulation. The marked noncompetitive inhibition of atrial cyclic AMP accumulation by locally released acetylcholine may be basic to parasympathetic antagonism of sympathetic responses in the myocardium.

INTRODUCTION

Catecholamines cannot effect increases in cyclic AMP in hearts perfused with buffer containing 56 mM potassium chloride (1, 2). This block in cyclic AMP accumulation is associated with diminished activation of phosphorylase kinase and phosphorylase by catecholamines (1, 2). Uterine smooth muscle depolarized by elevated potassium is also rendered insensitive to the cyclic AMP elevating effect of catecholamines (3). In many tissues, however, exposure to elevated K^+ can increase the in-

This work was supported by N.I.H. Grant HLB 12373.

0026-895X/79/060841-10$02.00/0
Copyright © 1979 by Academic Press, Inc.
All rights of reproduction in any form reserved.

tracellular concentration of cyclic AMP (3–6), and catecholamine responses can be superimposed upon the cyclic AMP response to high K^+ (4, 5). The stimulatory effects of elevated K^+ are best explained by K^+ depolarization-induced release of neurotransmitters such as norepinephrine and adenosine, which then activate adenylate cyclase (3, 5, 6). No explanation has yet been provided for the inhibitory effect of K^+ seen in the heart and uterus.

In the present study I have used isolated mouse atria to explore the mechanism for K^+-induced inhibition of catecholamine responsive cyclic AMP accumulation. The data indicate that the effect of elevated K^+ is indirect and is mediated by depolarization-induced release of acetylcholine from myocardial nerve endings, with subsequent muscarinic inhibition of catecholamine-sensitive cyclic AMP accumulation.

METHODS

Adult male Swiss-Webster mice were sacrificed by cervical dislocation and the right and left atrial appendages excised from the *in situ* heart within 30 sec after sacrifice. Atria were rinsed in oxygenated Krebs-Henseleit buffer of the following composition (mM); NaCl, 118; KCl, 4.7; $CaCl_2$, 3.0; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; Na_2EDTA, 0.5; and glucose, 10, pH 7.4 at 37°. After rinsing, the individual atria were transferred to capped vials containing 2 ml of buffer equilibrated with 95% O_2–5% CO_2, and incubated for approximately 40 min prior to initiation of the experimental protocol. The medium was then removed and 2 ml of experimental medium (e.g., medium of altered ionic composition) containing 100 μM isobutylmethylxanthine (IBMX) was added for 20 minutes unless otherwise noted. When medium KCl was increased there was an equimolar decrease in NaCl to maintain isotonicity. When calcium was omitted, EGTA was substituted for EDTA. (±)-Isoproterenol, sodium ascorbate (.01 M, as vehicle for isoproterenol solutions), carbachol (carbamylcholine) and other drugs or combinations thereof were added in a volume of 20 μl for one minute. The tissue was removed, blotted, and frozen in freon cooled in liquid nitrogen and stored frozen at −70° until assayed.

Frozen tissues were homogenized in 0.5 ml ice-cold 10% TCA containing 0.25 pmol (~7000 cpm) ^{3}H-cyclic AMP to monitor for recovery. The TCA homogenates were centrifuged and the supernatants applied to Dowex 50 AG WX4 (200–400 mesh) columns of 1 g bed volume. The columns were washed twice with 1.2 ml H_2O and cyclic AMP eluted with 2.5 ml H_2O. Cyclic AMP recoveries were 60–80%. The column eluates were dried in a Savant concentrator, resuspended in 0.2 ml of 50 mM NaAc pH 4.5 and assayed by the protein binding assay of Gilman (7). The TCA precipitate was dissolved in NaOH and protein content determined by the method of Bradford (8).

RESULTS

In preliminary experiments basal and catecholamine-stimulated cyclic AMP concentrations in right and left atria were compared. No differences between the two atria were evident under any of the experimental conditions tested. In all subsequent experiments both atria were used, with approximately equal numbers of right and left atria in each experimental group. The cyclic AMP response to 30 μM isoproterenol was maximal between 30 sec and 1 minute and the intracellular concentration of cyclic AMP remained constant under the conditions utilized (in the presence of 100 μM IBMX) for at least 10 minutes. A one-minute time point was chosen to measure isoproterenol-stimulated cyclic AMP accumulation. The concentrations of cyclic AMP reported here are elevated due to the presence of the phosphodiesterase inhibitor. In one experiment in which IBMX was omitted the cyclic AMP concentrations were: basal, 9 pmol/mg protein; 30 μM isoproterenol, 23 pmol/mg protein, in accord with literature values.

Dose response curves for isoproterenol-stimulated cyclic AMP accumulation in atria incubated in control medium (6 mM K^+, including KH_2PO_4) and in medium containing 57 mM K^+ are shown in Fig. 1. Responses to all effective concentrations of isoproterenol were markedly decreased in 57 mM K^+ medium. Cyclic AMP accumulation was also inhibited in atria incubated

in 57 mM K^+ medium and exposed to isoproterenol for 0.5 min or for 5 min. Basal cyclic AMP concentrations were not diminished in high K^+ medium. The approximate ED_{50} for isoproterenol was the same (~0.1 μM) in atria incubated in normal or in 57 mM K^+ medium.

High concentrations of extracellular K^+ can cause morphological damage to cardiac muscle (9). It was therefore necessary to demonstrate that the impairment in the cyclic AMP response did not result from irreversible cell damage. Figure 2 shows the time course and reversibility of K^+ inhibition of the response to isoproterenol. The onset of K^+ inhibition was rapid, with maximal blockade of the cyclic AMP response evident after a two-minute incubation of atria in 57 mM K^+ medium. The response to isoproterenol increased significantly between two and forty minutes of exposure to 57 mM K^+, but was completely restored only when atria were returned to normal medium.

The K^+ concentration dependence for inhibition of catecholamine stimulable cyclic AMP accumulation is shown in Fig. 3. Significant attenuation of the response to isoproterenol was evident in atria incubated in medium containing 22 mM K^+ and inhibition was nearly maximal by 40 mM K^+. Equimolar decreases in the concentration of NaCl accompanied the increases in KCl in the bathing medium. Linden and Brooker (10) observed that catecholamine-stimulable cyclic AMP accumulation was depressed in guinea pig atria incubated in sodium-free medium. The effects of decreased sodium were tested by incubating atria in media containing normal KCl, but with a reduction in NaCl equivalent to that in 57 mM K^+ medium (from 118 mM to 67 mM). Sodium was replaced by either choline chloride or sucrose. Decreased sodium caused no depression of isoproterenol-stimulated cyclic AMP accumulation when sucrose replaced sodium (Table 1). However, when sodium was replaced by choline, isoproterenol-stimulated cyclic AMP accumulation was reduced to 26% of control values. The inhibitory effect of choline was prevented by inclusion of the muscarinic receptor antagonist, atropine, in the incubation medium.

Since the loss of catecholamine responsiveness produced by choline could be overcome by atropine, the effect of the latter agent on K^+-induced inhibition was examined. When atropine was present during incubation in 57 mM K^+ medium, there was no inhibition of the cyclic AMP response to isoproterenol (Fig. 4). The inhibition pro-

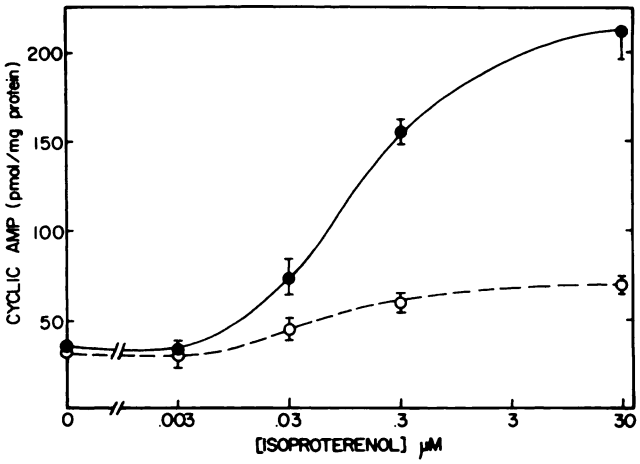

FIG. 1. *Isoproterenol-stimulated cyclic AMP accumulation in atria incubated in control or 57 mM K^+ medium*

Tissues were incubated for 20 minutes in 6 mM (●——●) or 57 mM K^+ (○- - -○) medium, and frozen one minute after addition of isoproterenol to the incubation medium. Values are means ± SEM of at least eight individual atria. Cyclic AMP accumulation was significantly depressed ($p < .05$, Student t-test) at all effective concentrations of isoproterenol (.03 μM and greater).

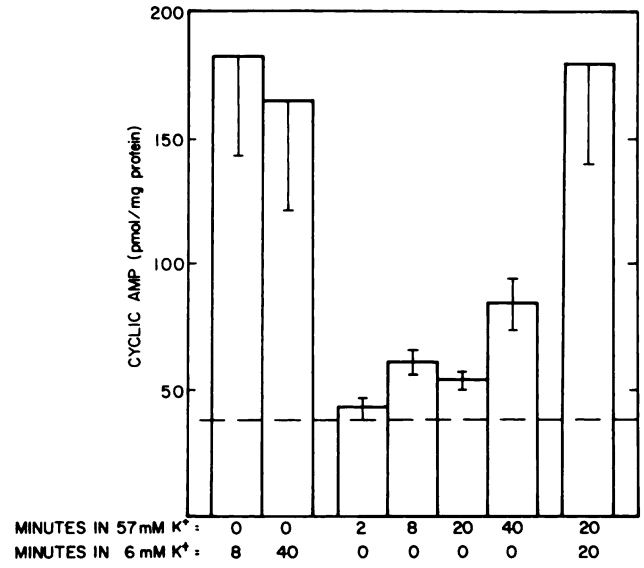

FIG. 2. *Time course and reversibility of K^+-induced inhibition of isoproterenol-stimulated cyclic AMP accumulation*

Tissues were incubated in control medium (6 mM K^+) or 57 mM K^+ medium for the times indicated prior to the addition of 30 μM isoproterenol. The response to isoproterenol was partially restored between 2 and 40 min in 57 mM K^+ ($p < .001$, one way analysis of variance) but reached control levels only when atria incubated in 57 mM K^+ were returned to control medium (last column). The broken line indicates the basal concentration of cyclic AMP, which was unaffected by K^+. Values are means ± SEM of five atria.

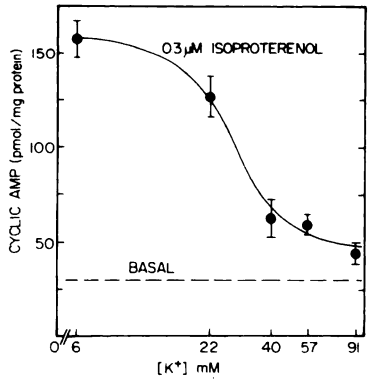

FIG. 3. *Dose response relationship for K^+-induced inhibition of isoproterenol-stimulated cyclic AMP accumulation*

Atria were incubated in media containing the indicated concentrations of K^+ for 20 min prior to the addition of isoproterenol (0.3 μM) for one minute. NaCl in the medium was decreased equimolar to the increase in KCl. Values given are means ± SEM of 20 atria at 6, 22 and 57 mM K^+ and of 5 atria at 40 mM and 91 mM K^+. The basal cyclic AMP concentration, indicated by the broken line, was the same at 6, 22 or 57 mM K^+. Cyclic AMP accumulation was significantly decreased at 22 mM K^+ ($p < .05$) and above.

TABLE 1

Effect of sodium substitution on isoproterenol sensitive cyclic AMP accumulation

Atria were incubated in Krebs Henseleit buffer of normal sodium chloride concentration (118 mM), or in medium in which NaCl was decreased to 67 mM and isotonicity maintained by the addition of either sucrose or choline chloride. Isoproterenol or ascorbate (basal) were added for one minute following a 20 min incubation in the indicated medium. Values are means ± SEM of 6–20 atria.

Medium	Cyclic AMP (pmol/mg protein)	
	Basal	ISO (30 μM)
Control (118 mM NaCl)	34 ± 2	252 ± 36
Low sodium (67 mM NaCl, 102 mM sucrose)	—	266 ± 32
Low sodium (67 mM NaCl, 51 mM choline)	34 ± 2	69 ± 5
Plus 30 μM atropine	—	232 ± 29

duced by 57 mM K^+ was also largely reversed when atropine was added along with isoproterenol for 1 minute rather than by inclusion in the incubation medium (Fig.

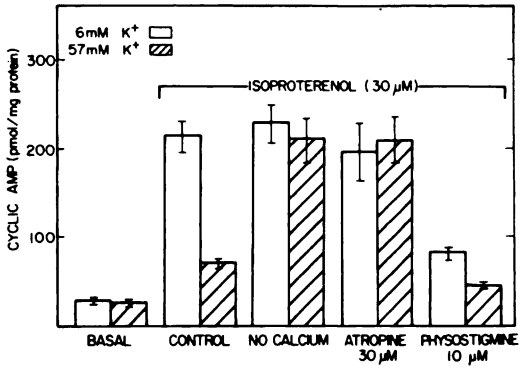

FIG. 4. *Ionic and pharmacological characteristics of K^+-induced inhibition of isoproterenol stimulated cyclic AMP accumulation*

Tissues were incubated for 20 minutes in 6 mM or 57 mM K^+ medium, or in the same media modified by the omission of calcium or by the addition of atropine or physostigmine. Isoproterenol (30 μM) or ascorbate (basal) was then added for one minute and the tissues frozen for cyclic AMP determinations. Values are means ± SEM.

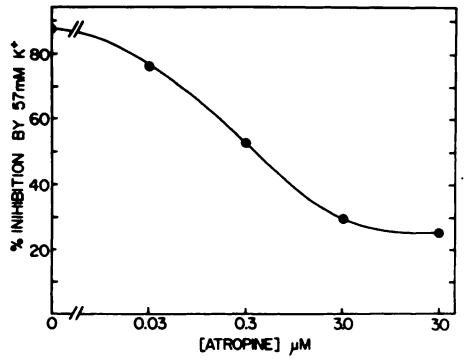

FIG. 5. *Reversal of K^+-induced inhibition by atropine*

Atria were incubated in 57 mM K^+ medium for 15 min and then challenged with 0.3 μM isoproterenol ± atropine for one minute. The cyclic AMP response to isoproterenol was inhibited by 88% in 57 mM K^+ medium (compared to the 4.7 fold increase above basal seen in control medium). Atropine caused a concentration dependent reversal of K^+-induced inhibition. The percent inhibition at each atropine concentration is calculated from values from 5 atria; standard errors are ± 5–15 %.

5). The ED_{50} for atropine-induced restoration of the response to isoproterenol was ~0.2 μM (Fig. 5). Neither hexamethonium (10 μM) nor phentolamine (1 μM) prevented K^+ induced inhibition (data not shown).

The most likely mechanism by which elevated K^+ would produce atropine-sensitive inhibition of cyclic AMP accumulation is through release of acetylcholine. Such an effect should be abolished in calcium-free medium since K^+ depolarization-induced transmitter release requires external calcium (11). As shown in Fig. 4, isoproterenol elicited a normal cyclic AMP response in atria incubated in 57 mM K^+ medium from which calcium was omitted (and 0.5 mM EGTA added).

If K^+-induced inhibition is caused by released acetylcholine, the inhibition should be potentiated when acetylcholine breakdown by acetylcholinesterase is prevented. Since potentiation would be most apparent with submaximal concentrations of K^+, the effect of the cholinesterase inhibitor physostigmine (10 μM), was assessed on atria incubated in 22 mM K^+ medium. The response to 0.3 μM isoproterenol was inhibited by 17% in the absence, and 66% in the presence, of physostigmine. However, physostigmine not only potentiated inhibition by K^+, but also decreased the cyclic AMP response to isoproterenol in medium of normal K^+ concentration. The inhibitory effect of physostigmine in normal and 57 mM K^+ medium is shown in Fig. 4. Atropine (30 μM) completely antagonized the inhibitory effect of physostigmine (data not shown).

The hypothesis that K^+-induced inhibition might result from an indirect activation of cholinergic receptors is supported by studies with the cholinergic agonist, carbachol (carbamylcholine). Carbachol, like 57 mM K^+, markedly depressed catecholamine-stimulable cyclic AMP accumulation in murine atria without decreasing basal cyclic AMP accumulation (Table 2). Inhibition of cyclic AMP accumulation by carbachol was prevented by atropine but, in contrast to inhibition by 57 mM K^+, did not require extracellular calcium (Table 2). The IC_{50} for carbachol was determined to be approximately 0.1 μM (Fig. 6). In dose response studies with 0.1 μM carbachol, cyclic AMP accumulation was inhibited to an equivalent extent (40–50%) at all concentrations of isoproterenol and the ED_{50} for isoproterenol (0.2–0.3 μM) was unchanged by carbachol (Fig. 7).

Neither histamine (100 μM) nor glucagon

TABLE 2

Effects of calcium omission and cholinergic agonists and antagonists on cyclic AMP accumulation in murine atria

Atria were incubated in control (6 mM K^+) medium, ± calcium for 20 min and then challenged with isoproterenol ± carbachol for one minute or atria were incubated in control medium containing carbachol (± 30 μM atropine) for 20 minutes prior to exposure to isoproterenol. Values are means ± SEM.

Medium	Cyclic AMP (pmol/mg protein)			
	Basal	ISO (30 μM)	ISO (0.3 μM)	ISO (0.3 μM) + CARB (30 μM)
Control	28 ± 3	203 ± 43	141 ± 16	39 ± 4
No calcium	21 ± 2	—	156 ± 20	34 ± 3
Carbachol	29 ± 1	62 ± 6	—	—
Carbachol + Atropine	24 ± 2	183 ± 36	—	—

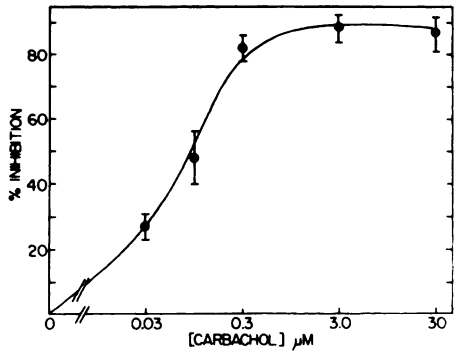

FIG. 6. *Cholinergic inhibition of isoproterenol-stimulated cyclic AMP accumulation*

Atria incubated in control medium were challenged with 0.3 μM isoproterenol ± carbachol for one minute. The values given are means ± SEM of 16–20 individual atria, and are expressed as a percent inhibition (by carbachol) of isoproterenol stimulated cyclic AMP accumulation.

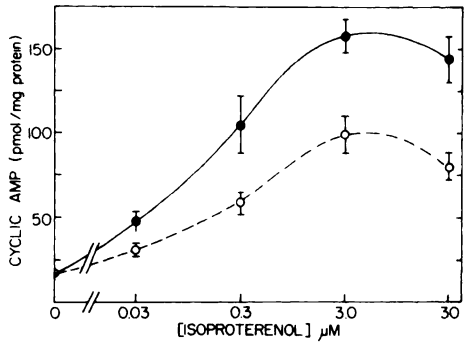

FIG. 7. *Insurmountable cholinergic antagonism of isoproterenol-stimulated cyclic AMP accumulation*

Isoproterenol alone (●——●) or in combination with 0.1 μM carbachol (○- - -○) was added to atria for one minute at the concentrations indicated. The cyclic AMP values given are means ± SEM of 5 atria.

(3 μM) increased the intracellular concentration of cyclic AMP in mouse atria; prostaglandin E_1 (10 μM) produced only a small increase in cyclic AMP. It was therefore not possible to determine whether elevated K^+ or carbachol antagonized the cyclic AMP response to other hormones. However when mouse atria were exposed to cholera toxin (5 μg/ml) in the presence of 100 μM IBMX for 90 min, the concentration of cyclic AMP doubled. Cyclic AMP remained essentially constant for at least 30 minutes after removal of the cholera toxin-containing medium and addition of fresh medium. Neither 57 mM K^+ nor 30 μM carbachol reduced the intracellular cyclic AMP accumulated in response to cholera toxin (Fig. 8).

DISCUSSION

Exogenous choline esters have been shown to inhibit cyclic AMP accumulation through a muscarinic receptor mechanism in a variety of preparations of mammalian myocardium (12–16). It is likely that the decrease in cyclic AMP produced by muscarinic receptor activation is responsible for cholinergic antagonism of catecholamine-induced glycogenolysis in the myocardium (15, 16) and perhaps also for the negative inotropic and chronotropic effects of exogenous acetylcholine. It has not been demonstrated, however, that cyclic AMP accumulation can be inhibited by activation of

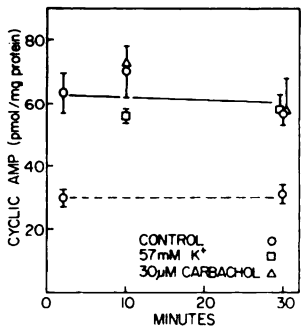

FIG. 8. *Failure of carbachol or 57 mM K^+ to decrease cyclic AMP accumulated in response to cholera toxin*

Atria were incubated in control (6 mM K^+) medium containing 5 μg/ml cholera toxin (or vehicle for basal) and 100 μM IBMX for 90 min. The media was then replaced by either fresh control medium, medium with 30 μM carbachol or 57 mM K^+ medium all with 100 μM IBMX. Atria were removed at 2 min, 10 min and 30 min after the media change. The solid line represents cyclic AMP concentrations in atria exposed to cholera toxin; the broken line is the basal cyclic AMP concentration.

the parasympathetic nerves innervating the myocardium. The atria are densely innervated by cholinergic nerve fibers of the parasympathetic nervous system as well as by sympathetic adrenergic fibers (17, 18). The findings presented here suggest that acetylcholine released from parasympathetic neurons either spontaneously or by K^+ depolarization can effectively suppress the atrial cyclic AMP response to catecholamines.

Acetylcholine release has not been determined directly in these studies on mouse atria. However, K^+ depolarization-induced release of acetylcholine from parasympathetic fibers in the guinea pig ileum has been studied by Paton and colleagues (19), and Lindmar *et al.* have recently described K^+-induced overflow of acetylcholine in the perfused avian heart (20). The characteristics of K^+-induced release described in these systems are consistent with those of K^+-induced inhibition of cyclic AMP accumulation in mouse atria. Both release and inhibition follow a similar K^+ dose-dependence, increasing sharply between 25 and 50 mM K^+ (ref. 19, Fig. 3); both processes require external calcium (ref. 20, Fig. 4); and both reach maxima after very short exposure of the tissue to elevated K^+ (ref. 20, Fig. 2). In addition, the observation that K^+-induced release of acetylcholine in the perfused heart is not maintained (20) could explain the partial recovery of the cyclic AMP response during prolonged incubation in 57 mM K^+ (Fig. 2).

If K^+-induced inhibition in the mouse atrium is mediated through released acetylcholine, the characteristics of K^+-induced inhibition should be mimicked by exogenous cholinergic agonists. Indeed, elevated K^+ and carbachol produced very similar patterns of inhibition. Both agents caused concentration-dependent antagonism of the response to isoproterenol (Fig. 3, Fig. 6) with 80–90% inhibition at maximal concentrations, and no decrease in basal cyclic AMP accumulation. Inhibition produced by either 57 mM K^+ or by carbachol was noncompetitive with respect to the concentration of isoproterenol (Fig. 1, Fig. 7). Both K^+ and carbachol were ineffective in the presence of atropine (Fig. 4, Table 2) and neither intervention disrupted cholera toxin-sensitive cyclic AMP accumulation (Fig. 8). The only feature that distinguished the effects of K^+ and carbachol was the requirement of K^+-induced inhibition for calcium. This finding is consistent with the proposal that calcium is necessary for K^+ depolarization-induced release, rather than for blockade of cyclic AMP accumulation.

The hypothesis that K^+-induced inhibition results from release of acetylcholine is supported by the observations that inhibition is blocked by atropine, mimicked by carbachol and potentiated by cholinesterase inhibitors. Nonetheless, several alternative explanations for K^+-induced inhibition should be considered. The possibility that high concentrations of K^+ interfere with cyclic AMP accumulation by causing morphological damage to cardiac cell structure can be eliminated on the basis that permanent structural disruption would not be reversed by removal of K^+ or by atropine. The possibility that depolarization of the cardiac cell membrane *per se* disrupts hormone-sensitive adenylate cyclase activity is also incompatible with the pharmacological characteristics of K^+-induced inhibition. Depolarization of the cardic mem-

brane by elevated external K^+ is due to a change in the relative concentrations of K^+ outside and inside the cell and should occur despite the presence of atropine or the omission of calcium from the incubation medium. Finally, cholinergic receptor agonists, which inhibit catecholamine-sensitive cyclic AMP accumulation in the atrium, are known to hyperpolarize atrial muscle cells (21, 22). This further suggests that depolarization of the cardiac cell membrane is not critical for blockade of isoproterenol stimulable cyclic AMP accumulation.

Acetylcholine released spontaneously (23) or by stimulation (24) of isolated mammalian hearts is normally rapidly degradated by acetylcholinesterase. The observations made here on atria incubated in the presence of physostigmine (Fig. 4) suggest that the spontaneous release of acetylcholine is sufficient to suppress catecholamine-stimulable cyclic AMP accumulation if acetylcholinesterase is inhibited. This interpretation is supported by the finding that atropine reverses the inhibitory effect of physostigmine, although studies with other structurally different cholinesterase inhibitors will be necessary to prove this mechanism of action. It appears that there is either a very active spontaneous release of acetylcholine in the mouse atrium or that the cholinergic receptor system is highly sensitive to locally released acetylcholine. On this basis, cholinesterase inhibitors should effectively inhibit sympathetic responses and elicit parasympathetic dominance in the atrium.

The cholinergic receptor that mediates the inhibitory effect of 57 mM K^+, carbachol, and cholinesterase inhibitors is apparently a muscarinic receptor, since atropine reverses the cholinergic inhibition while hexamethonium (a nicotinic ganglionic blocking agent) does not. It should be noted, however, that the concentration of atropine required for reversal is higher than would be predicted from the K_i of atropine for cardiac muscarinic receptors (25). In addition, another biochemical event associated with muscarinic receptor stimulation, i.e., the generation of cyclic GMP, can be dissociated from cholinergic inhibition of cyclic AMP accumulation in the atrium.[1] Further pharmacological characterization will be necessary to determine if the cholinergic receptor that mediates decreased cyclic AMP accumulation in the atrium is a classical postsynaptic muscarinic receptor.

Several observations made here may help to elucidate the molecular basis for muscarinic inhibition of cyclic AMP accumulation. The finding that muscarinic inhibition is noncompetitive indicates that carbachol does not compete with isoproterenol for beta receptor binding sites. While this conclusion may seem predictable, methacholine can alter the affinity of beta adrenergic agonists for cardiac beta adrenergic receptors under appropriate conditions (26). The cyclic AMP concentration in murine atria exposed to cholera toxin was not decreased by addition of either carbachol or 57 mM K^+ for 30 min (Fig. 8). This and other findings[1] suggest that neither K^+ nor carbachol act by increasing cyclic AMP degradation. The intracellular concentration of cyclic AMP seen after cholera toxin pretreatment should represent a steady state between continued synthesis and metabolism. Thus the results presented in Fig. 8 further suggest that cholera toxin-activated adenylate cyclase activity in intact mouse atria is not inhibited by K^+ or carbachol. The observations presented above, and the finding that the basal cyclic AMP content was unaffected by carbachol or elevated K^+, suggest that muscarinic agonists affect hormone receptor-cyclase coupling rather than the catalytic activity of adenylate cyclase or phosphodiesterase.

The biochemical mechanisms by which the sympathetic and parasympathetic nervous systems exert their opposing influences on myocardial function is not known. There is still controversy over the role of cyclic AMP as a mediator of the physiological responses of cardiac muscle to catecholamines (27) and there is even less resolution on the relationship between cyclic nucleotide concentrations and the negative inotropic or chronotropic effects of acetylcho-

[1] Brown, J. H., manuscript in preparation.

line (16, 27). It is noteworthy, however, that the features of sympathetic-parasympathetic antagonism derived from physiological studies are observed features of the cholinergic-adrenergic antagonism reported here. Negative chronotropic and inotropic responses to acetylcholine are best observed under conditions of high sympathetic tone (28), just as only catecholamine-stimulated (and not basal) cyclic AMP accumulation is inhibited by carbachol, K^+ or physostigmine. In addition, physiological effects of parasympathomimetic interventions are dominant over sympathomimetic responses (28, 29, 30) just as muscarinic inhibition of cyclic AMP accumulation cannot be surmounted by increasing concentrations of isoproterenol. This biochemical measure of cholinergic-adrenergic interactions should therefore be regarded as a feasible model, if not a potential molecular basis, for parasympathetic-sympathetic interactions in the myocardium. The model would predict that changes in parasympathetic activity and in the availability of released acetylcholine can regulate the expression of sympathetic responses mediated via cyclic AMP.

ACKNOWLEDGMENT

The technical assistance of Mr. Michael Branks is gratefully acknowledged. This work would not have been possible without the support and encouragement of Dr. Steven E. Mayer.

REFERENCES

1. Namm, D, S. E. Mayer and M. Maltbie: The role of potassium and calcium ions in the effect of epinephrine on cardiac cyclic adenosine 3'5'-monophosphate, phosphorylase kinase and phosphorylase. *Mol. Pharmacol.* **4:** 522–530, 1968.
2. Largis, E. E., J. Ashmore and R. E. Toomey: Effect of varying K^+ concentrations on phosphorylase activity, lipolysis and cAMP levels in perfused rat heart. *Proc. Soc. Exp. Biol. Med.* **141:** 31–37, 1972.
3. Verma, S. C. and J. H. McNeil: Isoproterenol induced relaxation, phosphorylase activation and cyclic adenosine monophosphate levels in the polarized and depolarized rat uterus. *J. Pharmacol. Exp. Ther.* **198:** 539–547, 1976.
4. Lundholm, L., T. Rall and N. Vamos: Influence of K-ions and adrenaline on the adenosine 3',5'-monophosphate content in rat diaphragm. *Acta. Physiol. Scand.* **70:** 127–128, 1967.
5. Shimizu, H., C. R. Creveling and J. Daly: Stimulated formation of adenosine 3',5'-cyclic monophosphate in cerebral cortex: synergism between electrical activity and biogenic amines. *Proc. Nat. Acad. Sci. USA* **65:** 1033–1040, 1970.
6. Zatz, M. and R. F. O'Dea: Efflux of cyclic nucleotides from rat pineal: release of guanosine 3',5'-monophosphate from sympathetic nerve endings. *Science* **197:** 174–176, 1977.
7. Gilman, A. G.: A protein binding assay for adenosine 3',5'-cyclic monophosphate. *Proc. Nat. Acad. Sci. USA* **67:** 305–312, 1970.
8. Bradford, M.: Quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254, 1976.
9. Emberson, J. W. and A. R. Muir: Changes in the ultrastructure of rat myocardium induced by hyperkalemia. *J. Anat* **104:** 411–421, 1969.
10. Linden, J. and G. Brooker: Sodium requirement for the positive inotropic action of isoproterenol on guinea pig atria. *Science* **199:** 539–541, 1978.
11. Cooper, J. R., F. E. Bloom and R. H. Roth: *The Biochemical Basis of Neuropharmacology*, Oxford Press, New York, 1978.
12. Murad, F., Y.-M. Chi, T. W. Rall and E. W. Sutherland: The effect of catecholamine and choline esters on the formation of adenosine 3',5' monophosphate by preparations from cardiac muscle and liver. *J. Biol. Chem.* **237:** 1233–1238, 1962.
13. La Raia, P. J. and E. H. Sonnenblick: Autonomic control of cardiac cAMP. *Circ. Res.* **28:** 377–384, 1971.
14. Lee, T. P., J. F. Kuo and P. Greengard: Regulation of myocardial cyclic AMP by isoproterenol, glucagon and acetylcholine. *Biochem. Biophys. Res. Commun.* **45:** 991–997, 1971.
15. Gardner, R. M. and D. O. Allen: The relationship between cyclic nucleotide levels and glycogen phosphorylase activity in isolated rat hearts perfused with epinephrine and acetylcholine. *J. Pharmacol. Exp. Ther.* **202:** 346–353, 1977.
16. Keely, S. L. Jr., T. M. Lincoln and J. D. Corbin: Interaction of acetylcholine and epinephrine on heart cyclic AMP-dependent protein kinase. *Am. J. Physiol.* **234(4):** H432–438, 1978.
17. Ehringer, B., B. Falck, H. Persson and B. Sporrong: Adrenergic and cholinesterase-containing neurons of the heart. *Histochemie* **16:** 197–205, 1968.
18. Jacobowitz, D. J., T. Cooper and H. B. Barner: Histochemical and chemical studies of the localization of adrenergic and cholinergic nerves in normal and denervated cat hearts. *Circ. Res.* **20:** 289–298, 1967.
19. Paton, W. D. M., E. S. Vizi and M. Aboo Zar: The

mechanism of acetylcholine release from parasympathetic nerves. *J. Physiol.* **215:** 819–848, 1971.

20. Lindmar, R., F. Loffelholz and H. Pompetzki: Acetylcholine overflow during infusion of a high potassium-low sodium solution into the perfused chicken heart in the absence and presence of physostigmine. *Nauyn Schmied. Arch. Pharmacol.* **299:** 17–21, 1977.
21. Trautwein, W.: Generation and conduction of impulses in the heart as affected by drugs. *Pharmac. Rev.* **15:** 277–332, 1963.
22. Glitsch, H. G. and L. Pott: Effect of divalent cations on acetylcholine release from cardiac parasympathetic nerve endings. *J. Physiol.* **279:** 655–668, 1978.
23. Day, M.: The release of substances like acetylcholine and adrenaline by the isolated rabbit heart. *J. Physiol.* **134:** 558–568, 1956.
24. Dieterich, H. A., R. Lindmar and K. Loffelholz: The role of choline in the release of acetylcholine in isolated hearts. *Nauyn Schmied. Arch. Pharmacol.* **301:** 207–215, 1978.
25. Fields, J. Z., W. A. Roeske, E. Morkin and H. I. Yamamura: Cardiac muscarinic cholinergic receptors. *J. Biol. Chem.* **253:** 3251–3258, 1978.
26. Watanabe, A. M., M. M. McConnaughey, R. A. Strawbridge, J. W. Fleming, L. R. Jones and H. R. Besch, Jr.: Muscarinic cholinergic receptor modulation of adrenergic receptor affinity for catecholamines. *J. Biol. Chem.* **253:** 4833–4836, 1978.
27. Stull, J. W. and S. E. Mayer: Biochemical mechanisms of adrenergic and cholinergic regulation of myocardial contractility, in *Handbook of Physiology*, Section on Circulation: The Heart. In press, 1979.
28. Levy, M. N.: Sympathetic-parasympathetic interactions in the heart. *Circ. Res.* **29:** 437–445, 1971.
29. Grodner, A. S., H.-G. Lahrtz, P. E. Pool and E. Braunwald: Neurotransmitter control of sinoatrial pacemaker frequency in isolated rat atria and in intact rabbits. *Circ. Res.* **27:** 867–873, 1970.
30. Carrier, G. O. and V. S. Bishop: The interaction of acetylcholine and norepinephrine or heart rate. *J. Pharmacol. Exp. Ther.* **180:** 31–37, 1972.