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ACETYLCHOLINE MODULATION OF PHOSPHORYLASE AND CONTRACTILITY IN RAT HEARTS EXPOSED TO ANOXIA OR ISOPROTERENOL*

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Abstract—The effects of acetylcholine (ACh) on glycogen phosphorylase activated by either a cyclic AMP-dependent (isoproterenol) or a cyclic AMP-independent (anoxia) mechanism were examined. Cyclic adenosine 3′ : 5′-monophosphate and cyclic guanosine 3′ : 5′-monophosphate content, protein kinase and glycogen phosphorylase activities, and the contractile force of isolated perfused rat hearts exposed to either isoproterenol or anoxia were determined. Isoproterenol (1.6×10^{-7} M) produced an increase in cyclic AMP, activated protein kinase and glycogen phosphorylase, and increased intraventricular pressure developed by the myocardium. Acetylcholine did not alter basal phosphorylase activity or contractility. Acetylcholine, infused concurrently with isoproterenol, produced an increase in cyclic GMP and a decrease in cyclic AMP as well as in protein kinase and phosphorylase activity. The effects of ACh on cyclic GMP and phosphorylase were observed at 10^{-5} M. Exposure of isolated perfused hearts to anoxia decreased the intraventricular pressure developed. This negative inotropic effect was accompanied by an activation of glycogen phosphorylase that was independent of alterations in cyclic AMP or cyclic GMP. A high concentration of acetylcholine (10^{-4} M) further diminished the contractile activity of the heart and abolished the activation of phosphorylase. These effects also occurred in the absence of alterations in cyclic AMP but were coincident with an elevation of cyclic GMP. A lower concentration of ACh (10^{-5} M) infused during anoxia, however, elevated cyclic GMP without concurrent effects on cyclic AMP, protein kinase, phosphorylase or contractility. Thus, phosphorylase activated by a cyclic AMP-independent mechanism was not affected by doses of acetylcholine that were capable of suppressing phosphorylase activated by a cyclic AMP-dependent mechanism. These data support the concept that a reduction in cyclic AMP may be involved in mediating the effects of ACh on catecholamine-stimulated phosphorylase in myocardial tissue. The data do not support a role for cyclic AMP in the inhibition of anoxia-stimulated phosphorylase activation and, thus, some other modulating factor may be operative under these conditions. The role of cyclic GMP in this response remains in question.

Current evidence suggests that a number of cardiac actions of catecholamines, such as the positive inotropic effect and activation of phosphorylase, occur via alterations in the tissue content of cyclic AMP [1–4]. Antagonism between the adrenergic and cholinergic systems in ventricular myocardium has been described by a number of investigators [5–12]. George and his coworkers [11, 12] have suggested an antagonistic action of acetylcholine on the inotropic effect of catecholamines. In addition, Gardner and Allen [8, 9] reported that acetylcholine significantly reduced epinephrine-stimulated phosphorylase activity. In these studies, the actions of acetylcholine occurred concurrently with a decrease in cyclic AMP and an increase in cyclic GMP content of the myocardial tissue. George *et al.* [11] suggested that the negative inotropic effect of ACh in perfused rat heart was mediated by cyclic GMP. Watanabe and Besch [7] further demonstrated that ACh elevated cyclic GMP and decreased the inotropic response of guinea pig ventricular myocardium to isoproterenol but did not change basal myocardial contractility. It was suggested by these authors that cyclic GMP mediates the antiadrenergic effects of ACh since ACh attenuated the inotropic response to isoproterenol in the absence of alterations in cyclic AMP. More recently, Brooker [13] and Diamond *et al.* [14] have presented evidence which suggests that cyclic GMP is not responsible for the negative inotropic effects of acetylcholine. In both of these reports, atrial rather than ventricular preparations were used. In addition, neither group addressed the question of the antiadrenergic effects of ACh.

Gardner and Allen [10] have suggested that an inhibitory action of ACh on epinephrine-stimulated phosphorylase activity was a result of a decreased cyclic AMP level. These investigators demonstrated a close correlation between tissue cyclic AMP content and phosphorylase activity in both control and ACh-treated rat hearts. They did not, however, examine the effect of ACh on the inotropic state of the ventricular myocardium. The following study, therefore, was undertaken to test further the hypothesis that the negative inotropic effect of ACh as well as its ability to antagonize isoproterenol-stimulated phosphorylase is mediated through its ability to

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decrease the intracellular level of cyclic AMP. Since it has been demonstrated that anoxia increases phosphorylase by a cyclic AMP-independent mechanism [15], it was reasoned that if acetylcholine reduces phosphorylase solely by reducing cyclic AMP levels, it should have no effect on anoxia-elevated phosphorylase. In addition, since a number of investigators have suggested a possible role of cyclic GMP in the action of ACh, the role of this nucleotide was also investigated. The experiments reported here indicate that ACh can decrease phosphorylase activated by isoproterenol at a dose which is unable to decrease anoxia-stimulated phosphorylase. This suggests that ACh does not diminish phosphorylase activity directly but by an effect on cyclic AMP production. Higher doses of acetylcholine, however, did decrease anoxia-stimulated phosphorylase activity, suggesting an additional effect of ACh on this process. A preliminary report of this work has been presented [16].

METHODS

Rat heart perfusion. Male rats (Shapley Labs, Atlanta, GA), weighing between 250 and 350 g, were anesthetized with an i.p. injection of pentobarbital (60 mg/kg). The hearts were quickly removed and perfused through the aorta as described previously [9]. The perfusion buffer was a Krebs–Henseleit bicarbonate solution (pH 7.4) containing 120 mM NaCl, 5.6 mM KCl, 1.22 mM $CaCl_2$, 1.34 mM $MgSO_4$, 25.0 mM Na_2HCO_3, and 5.5 mM glucose. Perfusate was equilibrated either with 95% O_2–5% CO_2 for normal hearts or 95% N_2–5% CO_2 for anoxic hearts. All hearts were perfused at a constant flow rate of 10 ml/min at 37°. Hearts were electrically paced at 6 Hz at a voltage set at approximately twice threshold with a pulse of 10 msec duration. Mechanical function was determined by measuring the rate of ventricular pressure development (dP/dt) and peak ventricular pressure with a cannula inserted into the left ventricle. Since there were no differences between the effects of various treatments on dP/dt and peak left ventricular pressure, the latter measurement is reported here. Pressure was measured using a Statham pressure transducer (model P23 Gb) connected to a Gould–Brush recorder.

All hearts were perfused for a minimum of 15 min with a buffer equilibrated with 95% O_2–5% CO_2 before treatment. After equilibration, hearts were exposed to the appropriate drug treatment or to combined anoxia plus drug or anoxia alone. All drug solutions were infused at a rate of 0.2 ml/min. Anoxia was produced by switching to perfusate which had been equilibrated with 95% N_2–5% CO_2.

At appropriate times hearts were rapidly frozen using Wollenberger clamps cooled to the temperature of liquid nitrogen. Tissue was scraped free of excess media, pulverized in a percussion motor that had been precooled in liquid nitrogen, and stored at −60° until assayed.

Phosphorylase assay. Phosphorylase was determined in the direction of glucose-1-phosphate production by the method of Hardman *et al.* [17]. The results are expressed as the ratio of phosphorylase activity in the absence to that in the presence of 2 mM adenosine monophosphate. An increase in the ratio indicates an increase in the conversion of phosphorylase *b* to *a*, which is the more active form of the enzyme.

Cyclic nucleotide assays. Cyclic AMP content of the perfused heart muscle was assayed either by the radioimmunoassay as described by Steiner *et al.* [18] or by the activation of rabbit skeletal muscle protein kinase as determined by the cyclic AMP-dependent incorporation of ^{32}P-gamma label from ATP into purified casein [19]. Cyclic AMP levels determined by the radioimmunoassay were not significantly different from those obtained using the protein kinase activation assay. Cyclic GMP was assayed by radioimmunoassay as modified by Frandsen and Krishna [20].

Protein kinase. The activity state of protein kinase was determined by following the incorporation of labeled phosphate from the gamma position of ATP into histone as outlined by Keely *et al.* [21]. The results are expressed as the ratio of protein kinase activity in the absence to that in the presence of 1.67 μM cyclic AMP. An increase in the ratio indicates an increase in the activity state of the enzyme.

Statistics. All data were analyzed by Student's *t*-test. All results are expressed as mean ± S.E.M. In all cases, a P value of less than 0.05 was considered significant.

Materials. Stock solutions of isoproterenol and acetylcholine chloride (Sigma) were prepared in 0.1% sodium metabisulphite and were diluted with 0.9% NaCl just prior to use. Adenosine 5′-monophosphate, disodium EDTA, Type II-A histone, β-glycerophosphate, β-mercaptoethanol, adenosine 3′ : 5′-cyclic monophosphoric acid (sodium salt), guanosine 3′ : 5′-cyclic monophosphoric acid and trichloroacetic acid were purchased from the Sigma Chemical Co. (St. Louis, MO). 3-Isobutyl-methylxanthine was obtained from the Aldrich Chemical Co. (Milwaukee, WI). All nucleotides, sugar phosphates and enzymes used for the phosphorylase assay and the [^{32}P] ATP synthesis [22] were from the Boehringer–Mannheim Corp. (New York, NY). Carrier-free [^{32}P] inorganic phosphate was purchased from ICN (Irvine, CA). Guanosine 3′ : 5′-cyclic phosphoric acid [^{125}I]-2-*O*-succinyl (iodotyrosine methylester) and adenosine 3′ : 5′-cyclic phosphoric acid [^{125}I]-2-*O*-succinyl (iodotyrosine methylester) were purchased from New England Nuclear (Boston, MA). Antisera to cyclic AMP and to cyclic GMP were purchased from Janus Labs (El Cajon, CA). All other reagents were certified grade from the Fisher Scientific Co. (Pittsburgh, PA).

RESULTS

Temporal responses to isoproterenol in the presence and absence of acetylcholine. The time courses of the effects of isoproterenol on cyclic AMP and cyclic GMP levels, protein kinase and phosphorylase activities and ventricular pressure are shown in Fig. 1. Isoproterenol was infused into perfused rat hearts at a final concentration of 1.6×10^{-7} M for 1 min to allow maximal increases in cyclic AMP levels and phosphorylase activity to occur. Acetylcholine (10^{-5} M) or saline was then infused simultaneously

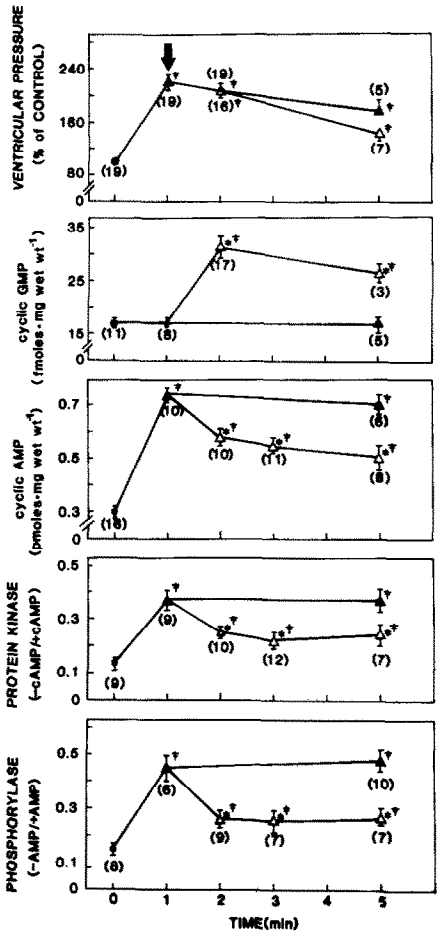

Fig. 1. Temporal responses to isoproterenol in the presence or absence of acetylcholine. Infusion of isoproterenol (1.6×10^{-7} M) was started at 0 time and continued for the duration of the experiment. Acetylcholine (10^{-5} M) or saline was started 1 min later (arrow). Hearts were freeze-clamped at times indicated and assayed as described in Methods. Each point represents the mean ± S.E.M. of the results from the number of hearts in parentheses. Isoproterenol plus saline-treated tissue is shown by (▲) and isoproterenol plus acetylcholine-treated tissues by (△). (●) indicates controls. A (‡) denotes $P < 0.05$ when comparing experimental to control and (*) denotes $P < 0.05$ when comparing acetylcholine–isoproterenol treated hearts to those treated with isoproterenol alone.

with isoproterenol for an additional 2 or 4 min. Cyclic AMP levels increased from a base of 0.32 ± 0.02 to 0.75 ± 0.06 pmole/mg wet weight after a 1 min isoproterenol infusion (Fig. 1). At 5 min of infusion of isoproterenol, cyclic AMP levels and protein kinase fell slightly but were still elevated significantly above control. Phosphorylase activity was elevated significantly (3-fold stimulation) at 1 min of isoproterenol and remained elevated for the duration of the experiment (5 min). In response to a 1 min isoproterenol infusion, ventricular pressure was increased to 213 ± 12 per cent of control. Pressure fell slightly by 2 min and remained elevated for the duration of the infusion.

After 1 min of ACh (10^{-5} M) infusion, isoproterenol-stimulated phosphorylase activity was depressed significantly. At this time, cyclic AMP levels were also depressed significantly when compared to those observed in the presence of isoproterenol alone. This dose of ACh also produced a 2-fold increase in the level of cyclic GMP. The isoproterenol-stimulated increase in ventricular pressure was unaltered after 1 or 5 min of ACh infusion. An infusion of acetylcholine (10^{-5} M) alone (i.e. in the absence of isoproterenol) also increased cyclic GMP at 1 min, but did not affect any of the other variables (Table 1).

Temporal responses to anoxia. The time courses of the effects of anoxia on ventricular pressure, cyclic GMP, cyclic AMP, protein kinase and phosphorylase activities are shown in Fig. 2. Anoxia had no effect on cyclic AMP or cyclic GMP tissue levels or protein kinase activity, but did stimulate phosphorylase activity. Maximum phosphorylase activity occurred 30 sec after the exposure to anoxia and remained elevated for 5 min. Anoxia decreased ventricular pressure significantly by 30 sec and continued to do so for the duration of the experiment.

The infusion of ACh (10^{-6} M) during anoxia significantly increased cyclic GMP content at 1 min, but had no effect on phosphorylase activity or ventricular pressure at any time interval studied. In addition, ACh (10^{-6} M) did not alter cyclic AMP levels or protein kinase activity (data not shown).

Dose responses to acetylcholine in the presence of isoproterenol or anoxia. Since 10^{-6} M ACh produced minimal effects both in the presence of isoproterenol or during anoxia, higher doses of ACh were used to attempt to define its action more clearly. Hearts were exposed to saline, isoproterenol (3.3×10^{-8} M), or anoxia for 1 min, at which time a simultaneous infusion of either saline or ACh, 10^{-5} or 10^{-4} M, was started. After an additional minute of treatment, the hearts were frozen and assayed. The results of these experiments are illustrated in Table 1. Acetylcholine in the presence of saline increased cyclic GMP in a dose-related fashion with no alteration in basal cyclic AMP, protein kinase, phosphorylase or ventricular pressure. In the presence of isoproterenol, ACh (10^{-5} M) increased cyclic GMP and attenuated the isoproterenol-stimulated phosphorylase activity but did not alter ventricular pressure. Cyclic AMP content and protein kinase activity, which were elevated by isoproterenol, were depressed significantly by ACh (10^{-5} M). In the presence of anoxia, 10^{-5} M ACh increased cyclic GMP levels with no effect on ventricular pressure, cyclic AMP, protein kinase or the anoxia-stimulated phosphorylase. At 10^{-4} M, however, ACh depressed the anoxia-stimulated phosphorylase and further decreased the ventricular pressure developed by the hearts. These changes in response to this high concentration of ACh were not accompanied by alterations in cyclic AMP but were coincident with an increase in tissue cyclic GMP content.

DISCUSSION

Role of cyclic AMP in the action of acetylcholine. In the present study, ACh antagonized the phosphorylase activating effects of isoproterenol (cyclic AMP-dependent) at doses which were lower than

Table 1. Effects of acetylcholine on contractile and biochemical responses to isoproterenol or anoxia*

Condition	Left ventricular pressure (% control)	Cyclic AMP (pmoles · mg wet wt^{-1})	Cyclic GMP (fmoles · mg wet wt^{-1})	Protein kinase (−cAMP/+cAMP)	Phosphorylase (−AMP/+AMP)
Saline	100 ± 4 (17)	0.32 ± 0.02 (18)	20 ± 2 (9)	0.15 ± 0.02 (20)	0.20 ± 0.02 (18)
+ACh 10^{-5} M	118 ± 13 (4)	0.44 ± 0.08 (4)	30 ± 5† (4)	0.11 ± 0.02 (4)	0.19 ± 0.02 (4)
+ACh 10^{-4} M	95 ± 16 (5)	0.38 ± 0.05 (6)	40 ± 5† (5)	0.12 ± 0.02 (6)	0.20 ± 0.02 (6)
Isoproterenol	182 ± 14‡ (18)	0.75 ± 0.06‡ (10)	20 ± 2 (14)	0.35 ± 0.02‡ (9)	0.43 ± 0.05‡ (6)
+ACh 10^{-5} M	200 ± 15‡ (19)	0.58 ± 0.04†‡ (10)	30 ± 4† (5)	0.24 ± 0.02†‡ (10)	0.26 ± 0.01†‡ (9)
Anoxia	53 ± 4‡ (18)	0.36 ± 0.03 (13)	18 ± 3 (13)	0.15 ± 0.01 (14)	0.34 ± 0.02‡ (14)
+ACh 10^{-5} M	42 ± 9‡ (4)	0.37 ± 0.02 (5)	34 ± 6†‡(5)	0.14 ± 0.01 (5)	0.28 ± 0.03‡ (5)
+ACh 10^{-4} M	30 ± 4†‡ (5)	0.37 ± 0.02 (5)	26 ± 2†‡(5)	0.16 ± 0.01 (5)	0.19 ± 0.02† (5)

* Hearts were exposed to saline, isoproterenol (3.3×10^{-8} M) or anoxia for 1 min, at which time a simultaneous infusion of either saline or ACh was started. After an additional minute hearts were frozen and assayed as described in Methods. Values are expressed as the mean ± S.E.M. for the number of hearts in parentheses.

† Indicates $P < 0.05$ when compared to respective control.

‡ Indicates $P < 0.05$ when compared to saline value.

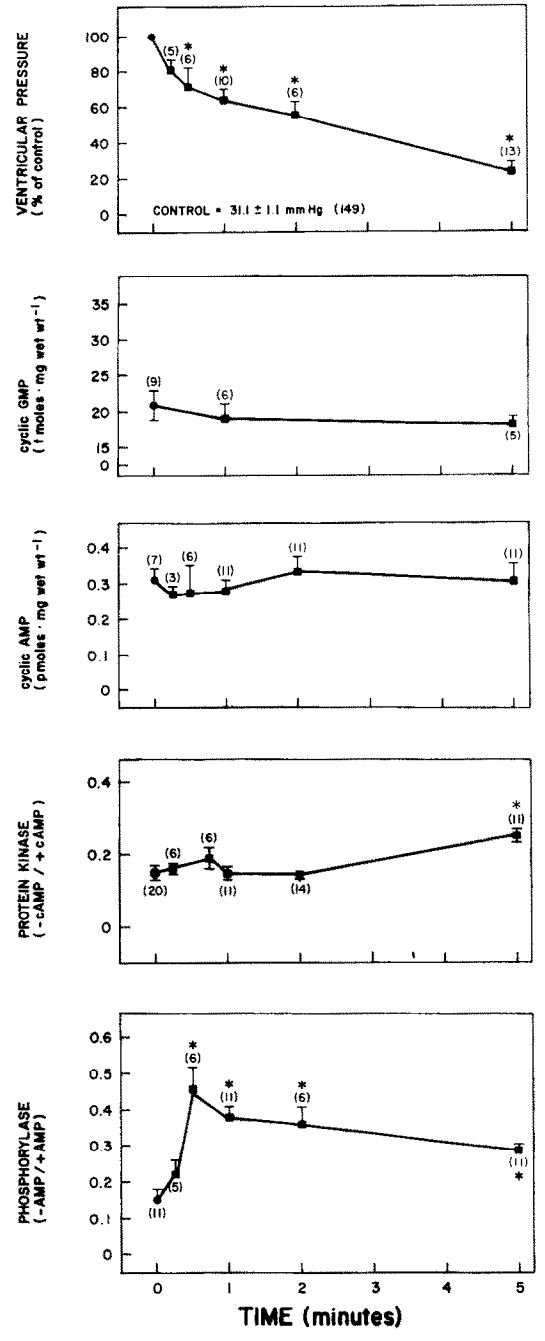

Fig. 2. Temporal responses to anoxia. Anoxia was produced at 0 time by switching to perfusate equilibrated with 95% N_2–5% CO_2, and was continued for the duration of the experiment. Each point represents the mean ± S.E.M. for the number of hearts in parentheses. (●) indicates controls. (*) Denotes $P < 0.05$ when compared with controls.

that required to affect phosphorylase stimulated by anoxia (cyclic AMP-independent). These data suggest that ACh may inhibit catecholamine stimulation of phosphorylase by reducing cyclic AMP levels. This hypothesis was first suggested by Murad *et al.* [5], who reported that adenylate cyclase activity in particulate preparations from dog liver and myocardium was depressed by acetylcholine. They suggested that this effect may explain the observation of Vincent and Ellis [6] that acetylcholine prevented increased glycogenolysis and contractile force in guinea pig hearts perfused with epinephrine, and the observations of Hess *et al.* [23] that acetylcholine decreased phosphorylase activity in rat heart. However, Murad *et al.* [5] were unable to demonstrate the effect of acetylcholine on a particulate preparation obtained from rat myocardium. Watanabe *et al.* [24] also demonstrated a cholinergic inhibition of catecholamine-stimulated adenylate cyclase which was dependent upon the presence of GTP. This may explain the inability of Murad *et al.* [5] to observe the effect of cholinergic agents on rat myocardial adenylate cyclase, since GTP was not included in the experiments of these investigators.

Gardner and Allen [10] subsequently demonstrated a correlation between the antagonism of epinephrine-stimulated phosphorylase by ACh and its ability to lower cyclic AMP in isolated perfused rat hearts. Keely *et al.* [25] demonstrated that ACh not only reduced cyclic AMP, but also attenuated the epinephrine activation of cyclic AMP-dependent protein kinase. In this study, we observed that ACh (10^{-5} M) essentially abolished the isoproterenol-stimulated phosphorylase activity but only partially antagonized the catecholamine effect on cyclic AMP levels and protein kinase activity. In addition, a larger concentration of ACh (10^{-4} M) attenuated the elevation of phosphorylase during anoxia, independently of a lowering of cyclic AMP. Activation of phosphorylase by anoxia is known to occur by a process which does not involve alterations in cyclic AMP [15, 26]. In this study, we have also demonstrated that anoxia does not stimulate phosphorylase via a cyclic AMP-independent activation of protein kinase. Thus, from the studies reported here, as well as those of Gardner and Allen [10] and Keely *et al.* [25], one must conclude that there are at least two components involved in the action of ACh on catecholamine-stimulated phosphorylase. One of these components involves the reduction of cyclic AMP-dependent protein kinase activity by attenuating the elevation of cyclic AMP, while the other must occur distal to the activation of cyclic AMP-dependent protein kinase.

An antiadrenergic action of ACh on contractility was not observed at times and with concentrations of ACh that reduced cyclic AMP levels. In addition, ACh (10^{-4} M) potentiated the decrease in contractility of the myocardium produced by anoxia. This occurred in the absence of alterations in cyclic AMP levels. These observations are consistent with those of a number of investigators who have demonstrated a dissociation between cyclic AMP lowering and negative inotropic effects of cholinergic agents [7, 25, 27]. Thus, either the intracellular pool of cyclic AMP involved in this action is sufficiently small that such changes are undetectable, or there exists other cyclic AMP-independent mechanism(s) by which ACh exerts its action. In this study, as well as those cited here, total cellular cyclic nucleotide levels and soluble protein kinase activities were measured. An increasing body of knowledge suggests that protein kinase and cyclic nucleotides may be compartmentalized [28, 29], and measurements of soluble activities may not reflect the active pools of these materials involved in the control of myocardial phosphorylase and contractility.

Role of cyclic GMP in the action of acetylcholine. Acetylcholine was capable of producing an elevation of cyclic GMP without decreasing contractility in the ventricular myocardium. Thus, there is a dissociation between the action of ACh on cyclic GMP and its effect on ventricular myocardial contractility. These results are consistent with those reported by Diamond *et al.* [14] using a cat atrial preparation and by Brooker [13] using guinea pig atria. Both of these groups reported a dissociation between the negative inotropic effects of cholinergic agents and their effects on cyclic GMP production in atrial tissue. We have also observed an elevation in cyclic GMP by ACh without an effect on the inotropic state of ventricular tissue, either in the presence or absence of isoproterenol. These data suggest that cyclic GMP does not mediate the action of ACh on the inotropic state.

The role of cyclic GMP in the action of ACh on catecholamine-stimulated phosphorylase, however, is still in question. The results reported here and those reported by Gardner and Allen [9] demonstrate that ACh infusion in the presence of catecholamine causes a rapid increase in cyclic GMP, which is accompanied by a decrease in phosphorylase activity. In that study, as well as the present study, ACh also produced a dose-dependent increase in cyclic GMP in the absence of catecholamine but did not affect basal myocardial phosphorylase activity. In addition, elevations in cyclic GMP in response to ACh are not accompanied by changes in phosphorylase activated by anoxia. Keely and Lincoln [30] have also demonstrated that nitroprusside, which increases cyclic GMP, does not antagonize the action of epinephrine on phosphorylase activity. Thus, an increase in cyclic GMP alone is not sufficient to account for the action of ACh on epinephrine-stimulated phosphorylase activity; other unknown factors must be involved.

The physiological significance of the inhibitory effect of ACh on myocardial glycogen phosphorylase is questionable. The anoxia-stimulated phosphorylase was reduced by ACh, but only at an extremely high concentration. In this study, ACh reduced isoproterenol-stimulated phosphorylase to 60 per cent of its stimulated level, whereas in the studies of Gardner and Allen [9] ACh reduced phosphorylase to 65 per cent. Whether this reduction occurs *in vivo* and whether it is sufficient to alter glycogen catabolism are uncertain. Hess *et al.* [23] were able to demonstrate a reduction in the amount of active phosphorylase in an *in situ* rat heart preparation exposed to ACh. Vincent and Ellis [6] demonstrated that ACh did antagonize glycogenolysis induced by epinephrine in guinea pig atrial tissue but not in

ventricular tissue. The studies reported here support the suggestion of Vincent and Ellis [6] that ACh does not alter basal glycogen metabolism but may do so under conditions which lead to rapid utilization of glycogen.

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