Neural Regulation of Cyclic AMP, Cyclic AMP-Dependent Protein Kinase, and Phosphorylase in Bullfrog Ventricular Myocardium

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SUMMARY. The roles of cyclic AMP and cyclic AMP-dependent protein kinase in the contractile and metabolic responses to neural stimulation were studied in the bullfrog heart perfused in situ. The interaction of parasympathetic and sympathetic nerves in the regulation of myocardial cyclic AMP, cyclic AMP-dependent protein kinase, and phosphorylase also was studied. Stimulation of the left vagosympathetic trunk in the presence of atropine (10 μM), i.e., sympathetic stimulation, caused frequency-dependent increases in ventricular contractility, cyclic AMP concentration, and activities of cyclic AMP-dependent protein kinase and phosphorylase. Maximal responses occurred with stimulation at frequencies of 5–20 Hz. Responses at 5 Hz persisted throughout 1 minute of continuous stimulation. In contrast, at 100 Hz, all responses except phosphorylase activation were transient during 1 minute of continuous stimulation. The frequency- and time-dependence of the cyclic AMP and cyclic AMP-dependent protein kinase responses were found to be very similar to those of the contractile response. These data are consistent with the hypothesis that cyclic AMP and cyclic AMP-dependent protein kinase serve as mediators of the sympathetic contractile response. All biochemical and mechanical responses to sympathetic neural stimulation were blocked by propranolol (1 μM), suggesting that only β-adrenergic receptors are involved in the sympathetic responses. In the absence of atropine, stimulation of the left vagosympathetic trunk had no effect on cyclic AMP concentration or the activities of cyclic AMP-dependent protein kinase and phosphorylase. Therefore, simultaneous parasympathetic stimulation totally blocked the sympathetic biochemical responses. This correlates with a total blockade of the sympathetic contractile response by simultaneous parasympathetic stimulation. Stimulation of the left vagosympathetic trunk in the presence of propranolol (without atropine), i.e., parasympathetic stimulation alone, had no significant effect on cyclic AMP concentration or the activities of cyclic AMP-dependent protein kinase and phosphorylase. (Circ Res 51: 551–559, 1982)
was correlated with an increase in contractility. However, as with many intact mammalian preparations, there are factors which complicate the interpretation of the data. Several such factors are (1) an intact vagal reflex, (2) the presence of circulating hormones, (3) probable change in the coronary perfusion rate, and (4) the presence of an anesthetic agent. Each of these factors may affect sympathetic neurotransmission or the postjunctional responses to catecholamines.

In an attempt to minimize these potential problems in our study, we have used a simple animal model, the bullfrog heart perfused in situ, to study the biochemical mechanisms of neural regulation of the myocardium. The bullfrog heart is particularly suitable for this study since it has a relatively simple autonomic innervation and lacks a coronary vascular system. We have studied the role of cyclic AMP and cA-PK in the regulation of myocardial contractility and phosphorylase activity by the autonomic nervous system. We have also investigated the interaction between parasympathetic and sympathetic nerves in regulating cyclic AMP concentrations and activities of cA-PK and phosphorylase in the ventricular myocardium.

Methods

Heart Preparation

Adult bullfrogs (male and female, 10–15 cm in body length) were obtained from College Biological. After storage at 4°C for 2–7 days, each frog was double-pithed, the chest cavity was opened, and the pericardium was removed. A frog-Ringer’s solution modified from that of McAfee et al. (1978) contained 73.2 mM NaCl, 3.2 mM KC1, 1.8 mM Na2HPO4, 0.59 mM NaH2PO4, 2.0 mM CaCl2, 25.0 mM NaHCO3, 16.2 mM glucose, and 0.050 mM EDTA (Na2) and was aerated with 95% O2-5% CO2. The solution was introduced through the posterior vena cava and the perfusion pressure was maintained at 6 mm Hg. (The frog heart lacks a coronary vascular system.) The right and left anterior vena cavae were ligated. The aorta was cannulated and attached to a pressure transducer (Statham, P23Dc) which, in turn, was connected to a Grass Polygraph (model 7B) for measurement of aortic pressure. The resistance to aortic outflow was adjusted to give a peak systolic pressure of 15–20 mm Hg. One end of a silk suture was tied to the apex of the heart while the other end was attached to a force-displacement transducer (Grass, FT 03C). This not only gave us another index of ventricular contractility, but also raised the ventricle from the chest cavity, thus facilitating the rapid freezing of the ventricles. The left vagus nerve (left vagosympathetic trunk) was isolated and placed in contact with bipolar platinum electrodes connected to a stimulator (Grass SD9). The nerve was severed at a point cranial to the electrodes. Hearts were allowed to beat at their own intrinsic rate of 40–50 beats/min and were equilibrated for 20 minutes before addition of drugs or stimulation of the nerves. In those experiments requiring vagal stimulation, the nerve was stimulated with square-wave pulses of 1 msec duration and a voltage 50% above that giving a maximal contractile response. At the indicated times following infusion of a drug or stimulation of the vagus nerves, the ventricles were frozen between silver clamps precooled in liquid nitrogen (Mayer et al., 1974). The frozen hearts then were powdered in a stainless steel percussion mortar and pestle (precooled in liquid nitrogen) and stored at −60°C until assayed. The concentration of cyclic AMP and the activities of cyclic AMP-dependent protein kinase and phosphorylase remain unchanged at −60°C for several months.

Measurements

Contractility

The maximum value of the first derivative of the aortic pressure wave, dP/dt, was used as one index of contractility. Another index of contractility was the strength of ventricular contraction as measured at the apex. Measurements were made at the peak of the response and at 1 minute following the beginning of nerve stimulation. Contractility was then expressed as the percent of the control value which was measured at a time just prior to the beginning of stimulation.

Cyclic AMP

Approximately 40 mg of frozen, powdered tissue was added to 0.5 ml of ice-cold 10% trichloroacetic acid (TCA) containing 7000 counts/min [3H]cyclic AMP as a tracer, and immediately homogenized in ground glass homogenizing tubes. After centrifugation, the TCA extracts of the samples were partially purified over Dowex 50 columns, then lyophilized and resuspended in 50 mM sodium acetate, pH 4.5 (Hayes and Mayer, 1981). Cyclic AMP in the resuspended sample was measured by the protein-binding assay of Gilman (1970). The amount of protein in the sample was measured by the method of Bradford (1976) with bovine serum albumin (Sigma, A4378) as the standard. The cyclic AMP content was adjusted for the percent of recovery of [3H]cyclic AMP and was expressed as pmol/mg protein.

Cyclic AMP-Dependent Protein Kinase (cA-PK)

A 10-mg sample of frozen, powdered heart was added to 400 μl of ice-cold homogenizing buffer containing 10 mM potassium phosphate, 10 mM EDTA (Na2), 0.5 mM isobutylmethylxanthine, 6 mM dihydrothreitol, and 100 mM NaCl, pH 6.8, and immediately homogenized in a ground glass homogenizing tube. The homogenate was centrifuged for 3 minutes at 4°C in a Beckman Microfuge. The assay was started by adding 10 μl of the supernatant fraction of the tissue homogenate to an assay tube containing 50 μl of a reaction mixture. Final concentration of each reagent from the reaction mixture was 1 mg/ml of histone (Sigma type VII or Worthington type Hf2b), 2.1 mM aminophylline, 6.7 mM Mg acetate, 15.8 mM NaF, 14.2 mM K2HPO4, 0.19 mM adenosine triphosphate (ATP), and 40 counts/min per pmol ATP-γ-32P. Kinase activity was calculated from the rate of 32P incorporation into histone using ATP-γ-32P as the phosphate donor (Corbin and Reimann, 1974). To determine the degree of intracellular activation of cA-PK we assayed the samples with (+) and without (−) cyclic AMP (5 μM) and with (+) and without (−) protein kinase inhibitor (PKI, 10 μg per assay tube). Preliminary experiments have shown that 5 μM cyclic AMP fully activates cA-PK and that 10 μg PKI per assay tube is sufficient to inhibit all of the cA-PK activity in the samples without inhibiting other kinase activity. Therefore, PKI was used to help determine the amount of kinase activity contributed by cA-PK. This is especially important when assaying tissue such as the bullfrog ventricular myocardium, where 15% of the kinase activity that is activatable by cyclic AMP under our assay conditions is activity contributed by cyclic GMP-dependent protein kinase (Fiscus et al., 1982). The activity ratio of cA-PK and phosphorylase in the ventricular myocardium.
PK was calculated from the equation:
\[
PK = \frac{\text{activity(–cyclic AMP – PKI)} - \text{activity(–cyclic AMP + PKI)}}{\text{activity(+cyclic AMP – PKI)} - \text{activity(+cyclic AMP + PKI)}}
\]

This value reflects the activation state of cA-PK in the myocardial tissue at the time of freezing.

**Phosphorylase**

Phosphorylase was assayed with and without the activator, 5'-AMP (2 mM), in the direction of glucose-1-phosphate production by a fluorescent coupled enzyme method (Hardman et al., 1965). The results are expressed as the ratio of the activity without AMP (phosphorylase a) to phosphorylase activity assayed with AMP (total of phosphorylase a plus phosphorylase b).

**Materials**

The heat-stable inhibitor of cA-PK (PKI) was prepared from frozen rabbit skeletal muscle by a modification of the method of Damaille et al. (1971). The PKI was purified up to the step of phosphocellulose chromatography. Further purification of PKI by affinity chromatography was found to be unnecessary for our purposes. The binding protein for the cyclic AMP assay (the holoenzyme of cyclic AMP-dependent protein kinase) was partially purified from rabbit skeletal muscle by the method of Wastila et al. (1971).

Histone was obtained from Sigma Chemical (type VII) or from Worthington Biochemical (type Hf2b). Other material and their suppliers were as follows: d, l-propranolol (Ayerst Laboratories, Inc.), diithiothreitol (Calbiochem), DE52 (Whatman), [3H]cyclic AMP (New England Nuclear), ATP-γ-<sup>32</sup>P (Amersham), isobutylmethylxanthine (Aldrich Chemical), d, l-isoproterenol (a gift from Sterling-Winthrop Laboratories), and phosphoglucomutase (Boehringer). Atropine sulfate, ATP (A6144), EDTA (Nao), bovine serum albumin (A4378), phosphocellulose, cyclic AMP, aminophylline, glycosylase, nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate dehydrogenase, and 5'-AMP (A1752) were obtained from Sigma Chemical Co.

**Statistics**

All statistical comparisons between biochemical values were made using an unpaired t-test. A P value less than 0.05 was considered statistically significant. Values obtained from hearts exposed to nerve stimulation in the presence of drugs were compared to values from control hearts (unstimulated) exposed to the same drugs.

**Results**

**Mechanical Responses**

The frequency-response relationship for two indices of ventricular contractility, force of contraction and +dP/dt (the maximum rate of rise of the aortic pressure wave), are illustrated in Figure 1. Measurements were made after 1 minute of continuous stimulation of the left vagosympathetic trunk at the indicated frequencies. Without the prior infusion of atropine, vagal stimulation produced a frequency-dependent decrease in contractility. The heart represented in Figure 1 showed a complete loss of contractility during vagal stimulation. Two other hearts behaved similarly under these conditions, while several other hearts showed a partial loss of contractility. Frequencies of 1 Hz or higher were required to produce these negative inotropic responses. Since both sympathetic and parasympathetic nerve fibers are present in the left vagosympathetic trunk in amphibians, the data indicate that the parasympathetic response predominates over the sympathetic response in this preparation.

When the left vagosympathetic trunk was stimulated after a 15-minute infusion of atropine (10 μM), a frequency-dependent increase in contractility was seen (Fig. 1). This typical sympathetic response indicates that the prior infusion of atropine (10 μM) effectively blocks the parasympathetic component of vagal stimulation. A maximal increase in contractility was observed with frequencies of 5–20 Hz. Vagal stimulation with frequencies above 20 Hz produced a smaller mechanical response. The maximum –dP/dt, an index of the rate of relaxation, was also increased with a similar frequency-response. Similar results were obtained with four other hearts.

The increase in contractility to sympathetic stimulation was transient. Maximal responses were reached at 34, 21, and 16 seconds after the beginning of continuous stimulation at 0.5, 5, and 100 Hz, respectively (Table 1). Therefore, the time required to reach the maximal contractile response was less for the higher frequencies. By 1 minute, the responses to stimulation at all frequencies had diminished, but contractility was still elevated above control values in ventricles exposed to vagal stimulation at 0.5 and 5 Hz. Contractile response to stimulation at 100 Hz had returned to control values by 1 minute. This partially explains the small contractile change observed with stimulation at the higher frequencies when contractile response is measured at 1 minute (Fig. 1).

It is important to note that heart rates were not altered by stimulation of the left vagosympathetic trunk following atropine infusion. This indicates that sympathetic fibers in this neural trunk do not innervate the pacemaker cells to any appreciable extent. Therefore, the heart rate was not a factor in the biochemical responses to sympathetic neural stimu-
TABLE 1
Average Mechanical Response and Time to Maximum Response after Vagal Stimulation at 0.5, 5, and 100 Hz

<table>
<thead>
<tr>
<th>Frequency of stimulation (Hz)</th>
<th>Time to maximum response (sec)</th>
<th>Maximum response at 1 min (% control dP/dt)</th>
<th>Response at 1 min (% control dP/dt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>34.2 ± 2.4 (4)</td>
<td>136 ± 8.3* (4)</td>
<td>125 ± 10* (4)</td>
</tr>
<tr>
<td>5</td>
<td>21.2 ± 2.8 (6)</td>
<td>176 ± 7.8* (6)</td>
<td>125 ± 14* (6)</td>
</tr>
<tr>
<td>100</td>
<td>15.8 ± 2.3 (5)</td>
<td>156 ± 23* (5)</td>
<td>107 ± 26* (5)</td>
</tr>
</tbody>
</table>

* P < 0.05 compared to control values. Atropine (10 µM) was infused for 15 minutes in all hearts prior to stimulation. Numbers in parentheses indicate the number of hearts used in each group.

lation. Parasympathetic neural stimulation, i.e., without atropine present, caused a slowing of the heart rate, along with a decrease in ventricular contractility. Although slowing of the heart rate may influence ventricular contractility, it should be emphasized that a similar negative inotropic effect was seen in hearts with direct electrical pacing of the ventricle.

Frequency-Dependence of the Sympathetic Biochemical Responses

The changes in cyclic AMP concentration and activities of cyclic AMP-dependent protein kinase (cA-PK) and phosphorylase in ventricular myocardium following sympathetic neural stimulation are shown in Figure 2. The left vagosympathetic trunk was stimulated at the indicated frequencies following a 15-minute infusion of atropine (10 µM). The ventricles were freeze-clamped after 1 minute of continuous stimulation. The changes in cyclic AMP concentration and cA-PK activity had a similar frequency-response relationship. Both responses reached a peak (3.1-fold increase for cyclic AMP concentration and 2.8-fold increase for cA-PK activity ratios) with stimulation at 5 Hz for 1 minute. At 100 Hz, cyclic AMP concentration was not significantly elevated above control values and cA-PK activity was elevated only 1.7-fold above control values after 1 minute of stimulation. Phosphorylase activity followed a similar frequency-response pattern at lower frequencies (0.5 and 5 Hz), but continued to rise to a peak response (3.2-fold increase above control) at 20 Hz and remained elevated (2.7-fold increase above control) at 100 Hz.

Time Course of the Sympathetic Biochemical Responses

The time course of the cyclic AMP and cA-PK responses with stimulation frequencies of 5 and 100 Hz are illustrated in Figure 3. At 5 Hz, the cyclic AMP concentration was elevated at all time points (5, 15, 60, and 180 seconds). Peak responses (3.3-fold increase) occurred at 60 seconds. After an initial delay, the activity ratio of cA-PK followed a similar time course.

At 100 Hz, cyclic AMP concentration was elevated at 5 and 15 seconds but not at 60 seconds. The peak cyclic AMP response (3.3-fold increase) occurred at 15 seconds. Again, the cA-PK response appeared to have an initial delay in its onset, but then followed a time course similar to that of cyclic AMP. Note that the time courses of the cyclic AMP and cA-PK responses (Fig. 3) were similar to that of contractility (Table 1) with 100 Hz stimulation; i.e., all three responses reached a maximum at approximately 15 seconds and then declined by 60 seconds. Phosphorylase activity, on the other hand, remained elevated at 60 seconds with 100 Hz stimulation (Fig. 2).
Correlation between Cyclic AMP and Cyclic AMP-Dependent Protein Kinase (cA-PK)

The concentrations of cyclic AMP and the activity ratios of cA-PK in myocardial samples were found to be closely correlated \((r = 0.89)\) (Fig. 4). The hearts were exposed to sympathetic neural stimulation \((0.5-100\) Hz) or to infused isoproterenol \((0.1 \text{ nM-1 \mu M})\). A linear relationship existed between the log of the cyclic AMP concentration and the activity ratio of cA-PK. Note that the relationship was very similar whether the ventricles were stimulated by the sympathetic nerves or by infused isoproterenol. However, one difference between biochemical responses to nerve stimulation and to infused isoproterenol was the maximal cyclic AMP and cA-PK response. With nerve stimulation, the highest concentration of cyclic AMP was \(11.5 \pm 2.4\) pmol/mg protein \((n = 4)\) and the highest cA-PK activity ratio was \(0.42 \pm 0.06\) \((n = 6)\). Isoproterenol, in contrast, caused an elevation of cyclic AMP to \(23.1 \pm 3.7\) \((n = 4)\) and an increase in cA-PK activity ratio to \(0.55 \pm 0.09\) \((n = 4)\).

Propranolol Blockade of Sympathetic Responses

The effects of propranolol on the biochemical responses to sympathetic neural stimulation are shown in Table 2. Atropine \((10 \mu M)\) was infused for 15 minutes in all hearts in this experiment to block parasympathetic responses. When added to unstimulated hearts, propranolol \((1 \mu M)\) caused a small decrease in cyclic AMP concentrations. Phosphorylase and cA-PK activities were not significantly altered under these conditions. Sympathetic stimulation at 5 Hz, which had caused a large increase in the concentration of cyclic AMP and in the activities of cA-PK and phosphorylase, was no longer effective in eliciting these responses when propranolol \((1 \mu M)\) was present. All responses to stimulation at 100 Hz were likewise blocked by propranolol \((1 \mu M)\). It should be noted that, although the phosphorylase value for 100 Hz stimulation plus propranolol appears to be elevated above control values, it was not significantly different \((P > 0.05)\) from the "control-plus-propranolol" value.

Parasympathetic Blockade of Sympathetic Responses

The effect of stimulating the left vagosympathetic trunk without prior infusion of any drugs is shown in Table 3. Under these conditions, both branches of the autonomic nervous system are allowed to act on the heart. In this experiment, two hearts stopped contracting, while the other three hearts showed a decrease in ventricular contractility of approximately 50%. The data from Table 3 show that simultaneous stimulation of both parasympathetic and sympathetic nerves has no effect on the concentration of cyclic AMP or on the activities of cA-PK and phosphorylase in the ventricular myocardium. This is in sharp contrast to the large increase in all three biochemical variables seen as a result of sympathetic stimulation, i.e., vagal stimulation in the presence of atropine (Table 2). Therefore, stimulation of the parasympathetic nerves totally blocks the sympathetic response. Atropine alone caused a small increase in the concentration of cyclic AMP and the activation state of cA-PK (compare Tables 2 and 3).

Effect of Parasympathetic Stimulation Alone

The effect of stimulating the left vagosympathetic trunk following a 15-minute infusion of propranolol \((1 \mu M)\) without atropine was investigated. Under these conditions, the sympathetic component of vagal stimulation was selectively blocked, thus allowing only parasympathetic nerves to affect the heart. Of the eight hearts tested, one heart stopped contracting and the others showed a decrease in contractility of approximately 50%. Parasympathetic neural stimulation alone had no significant effect on cyclic AMP concentration or cA-PK activity (data not shown).

Discussion

We have used a simple animal model for studying the biochemical mechanisms involved in autonomic neural regulation of the heart. We have selected the bullfrog heart perfused \textit{in situ} as our model and have thus avoided many of the problems often associated with preparation of mammalian hearts \textit{in situ}. These problems are listed in the Introduction. We have used this model to help elucidate the role of cyclic AMP and its target enzyme, cyclic AMP-dependent protein
### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Cyclic AMP (pmol/mg protein)</th>
<th>Cyclic AMP-dependent protein kinase (−cAMP/+cAMP)</th>
<th>Phosphorylase (−AMP/+AMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.56±0.30 (13)</td>
<td>0.15±0.01 (13)</td>
<td>0.14±0.03 (11)</td>
</tr>
<tr>
<td>5 Hz stimulation</td>
<td>5.52±1.17 (7)</td>
<td>0.25±0.04 (5)</td>
<td>0.31±0.08 (7)</td>
</tr>
<tr>
<td>Control plus propranolol</td>
<td>2.17±0.23 (5)</td>
<td>0.12±0.02 (4)</td>
<td>0.18±0.04 (5)</td>
</tr>
<tr>
<td>5 Hz stimulation plus propranolol</td>
<td>2.36±0.38 (5)</td>
<td>0.11±0.01 (4)</td>
<td>0.16±0.04 (5)</td>
</tr>
<tr>
<td>100 Hz stimulation plus propranolol</td>
<td>2.98±0.31 (5)</td>
<td>0.12±0.01 (5)</td>
<td>0.24±0.04 (5)</td>
</tr>
</tbody>
</table>

Atropine (10 μM) was infused for 15 minutes in all hearts prior to stimulation. Propranolol (1 μM) was infused for 15 minutes in the indicated hearts. The ventricular myocardium was freeze-clamped after 1 minute of stimulation of the left vagus nerve. The number of hearts used in each group is indicated in parentheses. All data from animals with vagal stimulation plus propranolol were compared to data from control animals plus propranolol for statistical analyses.

* P < 0.05.
† P < 0.01.

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Cyclic AMP (pmol/mg protein)</th>
<th>Cyclic AMP-dependent protein kinase (−cAMP/+cAMP)</th>
<th>Phosphorylase (−AMP/+AMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.68±0.17 (14)</td>
<td>0.10±0.005 (10)</td>
<td>0.11±0.02 (14)</td>
</tr>
<tr>
<td>5 Hz stimulation</td>
<td>3.28±0.43 (5)</td>
<td>0.11±0.01 (5)</td>
<td>0.095±0.02 (5)</td>
</tr>
</tbody>
</table>

No drugs were infused in this experiment. This allowed both parasympathetic and sympathetic components of vagal stimulation to be expressed. Stimulated hearts were frozen after 1 minute of continuous stimulation. The number of hearts used in each group is indicated in parentheses.
changes in cyclic AMP concentration and contractility.

A discrepancy therefore existed between phosphorylase activity and cyclic AMP concentration. The activity ratio of cA-PK was found to be slightly elevated (from 0.15 to 0.25) with 100 Hz stimulation. A discrepancy therefore existed between phosphorylase activity and changes in cyclic AMP concentration and contractility with stimulation at 100 Hz.

It should be emphasized that all measurements in the frequency-response study were taken after 1 minute of continuous neural stimulation. Under these conditions, the contractile responses at all frequencies were found to be transient, reaching a maximal response some time before 1 minute, and then diminishing by the end of the stimulation period (Table 1). We reasoned that the biochemical responses may also be transient during neural stimulation, and that this may be a reason for the discrepancy between cyclic AMP response and phosphorylase response at 100 Hz. We therefore measured the biochemical variables in samples frozen at various times during the stimulation period (Fig. 3). With stimulation at 5 Hz, cyclic AMP response had reached a maximum (3.3-fold increase) at 60 seconds and had declined by 3 minutes. In contrast to the response at 5 Hz, the cyclic AMP accumulation with 100 Hz stimulation reached a maximum (3.3-fold increase) at 15 seconds and then returned to a value not significantly different from the control value by 60 seconds. After an initial delay of a few seconds, activation of cA-PK followed a time course similar to that of the cyclic AMP response at both 5 and 100 Hz.

The data illustrate that the time course of the cyclic AMP response (and the cA-PK response) is compatible with the hypothesis that these biochemical alterations mediate the contractile response to sympathetic neural stimulation. For example, at 100 Hz, both cyclic AMP and the mechanical response reached their maximum in approximately 15 seconds and then diminished to near control values by 1 minute. Phosphorylase activity, on the other hand, remained elevated at 1 minute following 100 Hz stimulation (Fig. 2). It appears, therefore, that the inactivation of phosphorylase does not occur as rapidly as does the decline in cyclic AMP concentration or the decrease in contractility. A similar disparity between the time courses of cyclic AMP response and phosphorylase activation was observed in isolated perfused guinea pig hearts after a 10-second pulse of isoproterenol (Hayes and Mayer, 1981). In this experiment, there was an initial increase in cyclic AMP concentration, cA-PK activity, and contractility followed by a return to near control values by 1 minute. Phosphorylase activity, in contrast, remained elevated at 1 minute in the guinea pig hearts.

The reason for the transient nature of the cyclic AMP and contractile responses in our preparation is presently unclear. Several possible mechanisms for the short duration of the sympathetic responses are (1) depletion of the releasable catecholamine stores within the sympathetic nerves of the heart (Levy and Blattberg, 1976b), (2) feedback inhibition of neurotransmitter release (Westfall, 1977), (3) rapid desensitization of the β-adrenergic receptor (Lefkowitz and Williams, 1978), (4) calcium-induced inhibition of adenylyl cyclase and/or activation of cyclic nucleotide phosphodiesterase (Tsien, 1977), (5) an accumulation of a product, such as adenosine, which could inhibit the β-adrenergic response (Dobson, 1981), and (6) reversal of the atropine-antagonism at cholinergic receptors, thus allowing cholinergic inhibition of the β-adrenergic response (Brown, 1979). Levy and Blattberg (1976b) have shown that continuous stimulation at 8 Hz of the left ansa subclavia in open-chest, anesthetized dogs produces a transient increase in myocardial contractile force and in norepinephrine overflow into the coronary sinus. Both responses reached a peak by 1 or 2 min and then declined after 10 minutes of continuous stimulation. They suggested two possible mechanisms for the transience of the responses: (1) depletion of releasable norepinephrine and (2) an α-adrenergic receptor-mediated feedback inhibition of norepinephrine release. Similar mechanisms may be involved in our study. A desensitization of the β-adrenergic response (positive chronotropy, positive inotropy, and activation of adenylyl cyclase) has been observed in kitten atria after a 3-hour exposure to high concentrations (30 μM) of isoproterenol (Kaumann and Birnbaumer, 1976). The authors suggested that this desensitization was a result of a decrease in the availability of β-adrenergic receptors. However, this form of tachyphylaxis does not seem to be a probable explanation for the transience of the sympathetic response in our preparation, since we used a much shorter duration of exposure (1-minute stimulation). Furthermore, isoproterenol at a high concentration (1 μM) caused a very large increase in cyclic AMP concentration (from 2.67 to 23.1 pmol/mg protein) in bullfrog ventricular myocardium following 1 minute of continuous infusion. This increase in cyclic AMP was more than double that of the largest increase to sympathetic neural stimulation. Therefore, a rapid desensitization of the β-adrenergic receptor does not appear to have occurred following isoproterenol infusion. The cause of the transience of the response to sympathetic nerves is probably a combination of several of the mechanisms listed above.

We have shown that the activity ratio of cA-PK, as measured by our modified assay procedure (see Methods), was highly correlated (r = 0.89) with the log of the cyclic AMP concentration (Fig. 4). Although one would predict a close correlation under in vitro, cell-free conditions, the relationship between cyclic AMP and cA-PK under in vivo conditions probably is not as simple. It has been proposed that multiple
functional pools of cyclic AMP and cA-PK exist within the myocardial cell (Hayes et al., 1980). The activation of these different pools of cA-PK occurs in response to different stimuli and probably reflects the intracellular availability of cyclic AMP to the cA-PK. Our data show that, in bullfrog ventricular myocardium, a close correlation exists between the concentration of cyclic AMP in whole tissue homogenates and the activation state of cA-PK in the supernatant fraction of the tissue homogenates. In addition, our data show that the relationship between cyclic AMP and cA-PK is the same for infused isoproterenol as it is for sympathetic neural stimulation, thus illustrating the similarity in the biochemical responses to the two forms of stimulation.

All biochemical and contractile responses to sympathetic neural stimulation were blocked by propranolol (1 μM). This indicates that only β-adrenergic receptors are involved in these sympathetic responses. This is important, since it has been suggested that the stimulation of α-adrenergic receptors in mammalian heart leads to a positive inotropic response independent of cyclic AMP (Schümann, 1980). In addition, it has been hypothesized that α- and β-adrenergic receptors in frog myocardium are interconverted by changes in temperature (Kunos, 1978). However, the presence of α-adrenergic receptors and adrenergic receptor interconversion in frog myocardium remain a controversial issue (Kunos, 1978; Benfey, 1979).

When the parasympathetic component of left vagosympathetic stimulation is allowed to express itself by omitting atropine from the perfusing solution, the stimulation no longer produces a cyclic AMP response. In addition, the activities of cA-PK and phosphorylase are no longer changed by neural stimulation. This indicates that the parasympathetic nervous system is capable of totally blocking the sympathetic biochemical responses. This correlates with the total blockade of the sympathetic contractile response by simultaneous parasympathetic stimulation. In fact, under these conditions, the parasympathetic response (negative inotropism) predominates (Fig. 1). Based upon our understanding of autonomic effects in the mammalian heart, we can predict that the functional antagonism between the two branches of the autonomic nervous system occurs by at least two mechanisms. First, acetylcholine (ACh), the parasympathetic neurotransmitter, has been shown to inhibit release of norepinephrine from sympathetic nerves in isolated rabbit hearts by stimulating prejunctural muscarinic receptors (Löffelholz and Muscholl, 1969). Levy and Blattberg (1976a) have further shown that stimulation of parasympathetic nerves in anesthetized dogs causes a 30% reduction in the norepinephrine overflow into the coronary sinus elicited by stimulation of cardiac sympathetic nerves. This response was blocked by atropine, indicating that muscarinic receptors were involved. The second potential site of parasympathetic antagonism is postjunctional, i.e., at the myocardial cell. An example of this is the antagonism by ACh of the positive inotropic response caused by catecholamines infused into isolated perfused rat hearts (Keely et al., 1978). One mechanism of this antagonism is a muscarinic inhibition of the β-adrenergic agonist-induced activation of adenylate cyclase (Brown, 1979). However, other mechanisms have been proposed (Flitney and Singh, 1981). In our preparation, we observed a complete inhibition by parasympathetic stimulation of the cyclic AMP response to sympathetic stimulation. This parasympathetic effect occurred at the same time as a parasympathetic-induced antagonism of the sympathetic contractile response. Therefore, an inhibition of the cyclic AMP response may be a major mechanism in the parasympathetic antagonism of the sympathetic response in this intact, nerve-heart preparation. These results may help to explain why the response to parasympathetic stimulation is greater in hearts under a higher degree of sympathetic tone.

A small increase in the concentration of cyclic AMP and in the activation state of cA-PK was observed following infusion of atropine (10 μM) for 15 minutes (compare Tables 2 and 3). Although a direct action by atropine cannot be ruled out at this time, the most probable explanation for this effect of atropine is an inhibition of the action of endogenous acetylcholine in antagonizing the action of endogenous catecholamines. The fact that propranolol blocks the effects of atropine (Table 2) adds support to this hypothesis.

Parasympathetic stimulation alone, i.e., stimulation of the left vagosympathetic trunk following a 15-minute infusion of propranolol (1 μM), had no significant effect on myocardial cyclic AMP or on the activities of cA-PK and phosphorylase. Some investigators have observed a decrease in cyclic AMP concentrations in the frog myocardium following infusion of ACh (McAfee et al., 1978; Flitney and Singh, 1981). However, this response to ACh is not found consistently in all studies (Stull and Mayer, 1979) and may depend upon the species used or upon the influence of endogenous catecholamines.

Our data provide evidence for the involvement of cyclic AMP and of its target enzyme, cA-PK, in the regulation of myocardial contractility and metabolism by the autonomic nervous system. In addition, this simple animal model has now been characterized and will be useful in further elucidating the biochemical mechanisms of parasympathetic-sympathetic interactions in the regulation of the myocardium.

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Neural regulation of cyclic AMP, cyclic AMP-dependent protein kinase, and phosphorylase in bullfrog ventricular myocardium.

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