Regulation of Phosphorylase Kinase in Rat Ventricular Myocardium

Role of Calmodulin

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SUMMARY. Conversion of phosphorylase b to a which is catalyzed by the enzyme phosphorylase kinase is known to require Ca++. Trifluoperazine, an inhibitor of calmodulin-dependent enzymes, was utilized in the present study to clarify the role in vivo of calcium-calmodulin regulation of phosphorylase kinase. Twenty-minute preperfusion of isolated rat ventricles with 10^{-5} M trifluoperazine had no effect on basal levels of phosphorylase a but significantly attenuated phosphorylase activation induced by either calcium (3.75 mM) or isoproterenol (3 \times 10^{-3} M, 3 \times 10^{-7} M). The positive inotropic effect of both agents and cyclic adenosine 3',5'-monophosphate (cAMP) levels were not altered by trifluoperazine in the perfused hearts. In addition, no effects of 10^{-4} M trifluoperazine were noted on \beta-adrenergic receptor binding of [3H](+)-Icarazolol or on adenylate cyclase activity. In vitro studies with partially purified rat cardiac phosphorylase kinase demonstrated 1.5- to 3-fold stimulation by exogenous calmodulin. The addition of 10^{-4} M trifluoperazine prevented calmodulin stimulation but had little effect on activity in the absence of exogenous calmodulin. The present results suggest that reversible binding of calcium-calmodulin may represent a physiological means for regulating phosphorylase kinase activity in rat cardiac muscle. (Circ Res 51:448–456, 1982)

PHOSPHORYLASE KINASE plays a central role in the regulation of glycogen metabolism in muscle. This enzyme catalyzes the phosphorylation and attendant activation of phosphorylase b, and also has recently been reported to phosphorylate and thereby inactivate skeletal muscle glycogen synthase (Roach et al., 1978). Despite intensive study of phosphorylase kinase in vitro, many questions remain unanswered or controversial in regard to the regulation of the enzyme in vivo.

Phosphorylase kinase can be activated by phosphorylation catalyzed by cAMP-dependent protein kinase (Hayakawa et al., 1973; Cohen, 1973). The demonstration of parallel increases in cAMP levels and phosphorylase b to a conversion in perfused hearts following catecholamine stimulation provides indirect evidence supporting the physiological importance of this means of regulation (Gross and Mayer, 1974). Recently, more direct confirmation of activation of phosphorylase kinase by phosphorylation has been obtained utilizing radiolabeled inorganic phosphate (\(^{32}P\)) perfusion of isolated rat hearts. A linear correlation between an increase in \(^{32}P\) content of phosphorylase kinase and an increase in the pH 6.8/8.2 activity ratio of the enzyme was demonstrated following epinephrine stimulation (McCullough and Walsh, 1979). This activity ratio has classically been used to determine the activation state of phosphorylase kinase. An increase in the pH 6.8/8.2 activity ratio occurs following phosphorylation (Gross and Mayer, 1974).

In vitro studies utilizing both skeletal and cardiac muscle phosphorylase kinase reveal that calcium is required for enzyme activity, and concentration-dependent increases in activity occur with calcium addition (Brostrom et al., 1971, Cooper et al., 1980). This calcium dependence is afforded by a unique subunit structure of skeletal muscle phosphorylase kinase (Shenolikar et al., 1979). The enzyme is composed of four tightly bound subunits designated as \(\alpha, \beta, \gamma, \delta\). The \(\delta\) subunit is calmodulin, the ubiquitous calcium-binding protein, which probably mediates the calcium dependence of the enzyme.

The importance of calcium as a physiological regulator of phosphorylase kinase has been suggested by several investigators. The administration of calcium to intact hearts has been shown to result in rapid phosphorylase b to a conversion (Friesen et al., 1966). Studies done in open-chest rats and isolated guinea pig papillary muscles reveal rapid phosphorylase b to a conversion induced by anoxia without concomitant changes in cAMP levels or increases in the phosphorylase kinase activity ratio (Dobson and Mayer, 1973; Dobson et al., 1976). The conversion of phosphorylase b to a could be prevented by removal of calcium in the buffers.

Despite the relatively detailed biochemical information regarding the enzyme that has recently been generated, there remain a number of questions regarding its in vivo regulation by calcium. What is the relative role of calcium vs. cAMP-dependent phosphorylation in regulation? What is the mechanism or mechanisms through which calcium regulation may occur in vivo?

Phenothiazine antipsychotic agents such as trifluoperazine can be utilized to study calmodulin-depen-
dent regulation of enzymes. In the presence of calcium, trifluoperazine binds to the calmodulin, inhibiting binding and, hence, activation of calmodulin-dependent enzymes such as myosin light chain kinase, cyclic nucleotide phosphodiesterase, or adenylate cyclase (Levin and Weiss, 1977; Weiss et al., 1980). Phenothiazines are reported to have no significant effect on the tightly bound calmodulin subunit of phosphorylase kinase (Shenolikar et al., 1979). The purpose of the present study was to examine the effects of trifluoperazine on the inotropic and glycogenolytic actions of isoproterenol and calcium in intact rat ventricles, and thereby clarify the role in vivo of calcium regulation of phosphorylase kinase. Partially purified rat cardiac phosphorylase kinase was studied in vitro and the findings correlated with results obtained in intact ventricles. The results of the present study suggest that calmodulin plays a role as a physiological regulator of rat cardiac phosphorylase kinase.

Methods

Heart Perfusion

Wistar rats of either sex weighing 300-350 g were injected intraperitoneally with heparin sulfate (500 units) 30 minutes prior to use. Animals were stunned with a blow to the head and the heart rapidly removed and placed in ice cold oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer (mm): NaHCO_3, 27.3; NaCl, 118; KCl, 4.8; KH_2PO_4, 1; MgSO_4, 1.2; CaCl_2, 1.25; and glucose, 11.1. The hearts were attached to a cannula and perfused by the Langendorff method with the same buffer at 37°C. Flow was maintained at 8 ml/min by a Buchler roller pump. The atria were trimmed off the heart and a silk suture placed in the ventricular apex for attachment to a Grass strain gauge transducer (FT03-C). Diastolic tension was set at 5 g. All hearts were paced at a rate of 210 stimuli/min. Perfusion pressure, developed tension, and the first derivative of developed tension (d/dt) were recorded continuously throughout the experiment. After a 5-minute period for stabilization, perfusion was either continued with the same Krebs Henseleit buffer or switched to buffer containing 10^{-5} m trifluoperazine for an additional 20 minutes. Then, for 30 seconds, hearts were administered either isoproterenol or Krebs-Henseleit buffer containing 3.75 mm CaCl_2. Drugs were administered continuously through a side arm which entered the perfusion cannula approximately 2 cm above the aorta. Preliminary time course studies demonstrated that the maximum changes in contractility, cAMP levels, and phosphorylase a had occurred within 30 seconds. Therefore, hearts were freeze-clamped with Wollenberger clamps after 30 seconds of isoproterenol or calcium administration for the biochemical studies unless otherwise stated. The pulverized ventricular tissue was stored in liquid nitrogen.

Biochemical Studies

Rat cardiac membrane vesicles were prepared essentially as previously described for canine myocardium (Jones et al., 1979). Membrane vesicles were stored frozen at -20°C in 0.25 m sucrose, 30 mm histidine. Assays of adenylate cyclase activity and β-adrenergic receptor binding were performed within 1 week. Activities remained stable for at least 1 month. Phosphorylase a activity was assayed by the method of Gilboe et al. (1972). Duplicate determinations were performed for each heart within 3 days of the perfusion studies. cAMP levels were measured as previously described (Steiner, et al., 1972; Watanabe and Besch, 1974). All determinations were done in duplicate with two tissue dilutions. Adenylate cyclase activity was assayed in rat cardiac membrane vesicles by measuring the conversion of [α-32P]ATP to cyclic [βγ]AMP as previously reported (Salomon et al., 1974). Isoproterenol stimulation of adenylate cyclase activity was measured in the presence of 10^{-4} m GTP (guanosine 5'-triphosphate). Recovery of cAMP was determined with [3H]cAMP and found to be routinely between 60 and 80%. Triplicate determinations were performed with values corrected for recovery.

β-Adrenergic receptor binding in the membrane vesicles was determined utilizing the [3H]±carazolol binding assay (Manalan et al., 1981). Rat cardiac membrane vesicle protein (50-200 μg) was incubated in 5 ml of medium containing 50 mm Tris buffer (pH 7.5 at 22°C), 9 mm MgCl_2, 1 mm ascorbic acid, [3H]±carazolol, and trifluoperazine in varying concentrations at 37°C for 60 minutes, a reaction time which allowed complete equilibration of [3H]±carazolol binding. Bound [3H]±carazolol was separated from free [3H]±carazolol by filtration through Whatman GF/C filters. Radioactivity was counted on a liquid scintillation counter with a counting efficiency of 56%. Specific binding was taken as that binding which was displacable by 20 μM (±) propranolol. All determinations were performed in triplicate.

Phosphorylase kinase activity was assayed by measuring the incorporation of 32P from [γ-32P]ATP into phosphorylase b. The reaction mixture contained final concentrations of 30 mM β-glycerophosphate, 30 mM Tris, 0.2 mM [γ-32P]ATP (500-1000 cpm/pmml), 6 mM Mg acetate, 2 mg/ml phosphorylase b, 0.2 mM DTT, and, where indicated, 0.6 mM CaCl_2, 1 mM EGTA, calmodulin, and trifluoperazine in varying concentrations. The resultant pH of the assay solutions was 6.8 or 8.2 as indicated. Activity was measured at 30°C. The reaction was initiated by the addition of ATP and magnesium acetate. Under the conditions utilized, the rate of 32P incorporation was linear with respect to protein concentration and time for at least 30 minutes. Unless otherwise stated, aliquots were removed at 20 minutes to determine the quantity of protein bound 32P by the filter paper method (Cooper et al., 1980). Under the above conditions, autophosphorylation of phosphorylase kinase was minimal (<10%).

Pure bovine cardiac protein kinase catalytic subunit was used to phosphorylate the partially purified phosphorylase kinase. The incubation mixture contained 1 μg catalytic subunit, phosphorylase kinase (0.77 mg/ml), 50 mM β-glycerophosphate, 2 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.25 mM ATP, and 6 mM Mg acetate. The reaction was terminated by addition of Norit A in 1 mg/ml bovine serum albumin to remove ATP. Phosphorylase kinase without the addition of catalytic subunit was processed identically. Aliquots of the supernatant then were assayed for phosphorylase kinase activity as described above.

Protein Preparations

Phosphorylase kinase was isolated from rat myocardium by the method of Cohen (1973) except that 0.5 mM phenylmethylsulfonyl fluoride, 0.05 mM N-acetyl-L-lysine chloromethyl ketone and 1 mM EGTA were included in the buffers. The most active fractions from the Sepharose 4B chromatography column were pooled, concentrated by vacuum dialysis, and stored under liquid nitrogen. There was no loss of activity for at least 1 month.
Calmodulin was prepared from bovine testicles using slight modifications of the method of Klee (1977). An extract was prepared by homogenizing bovine testicles in 0.1 M Tris-HCl buffer containing 0.5 mM phenylmethylsulfonyl fluoride. This extract was then heated at 90°C for 5 minutes, and calmodulin was purified from the supernatant fraction obtained by procedures described previously (Klee, 1977).

Pure bovine cardiac cAMP dependent protein kinase catalytic subunit was prepared by the method of Beavo et al. (1974).

Protein determinations were performed by the method of Lowry using bovine serum albumin as a standard (Lowry et al., 1951).

Analysis of Data

Contractile response was similar whether assessed by developed tension or dT/dt. All reported changes in contractility reported were made on the basis of changes in developed tension. Developed tension immediately prior to experimental interventions was designated 100% (10.1 g ± 0.5, n = 48).

Statistical significance of differences were determined by analysis of variance followed by modified t-tests where indicated (Wallenstein et al., 1980).

Materials

Pure rabbit skeletal muscle phosphorylase b was generously supplied by Drs. A. Depaoli-Roach and P. Roach. Trifluoperazine was provided by Smith, Kline and French. L-Isoproterenol, (±) propranolol, and ATP were purchased from Sigma Chemical Company. [γ³²P]ATP was purchased from ICN. [³H]cAMP, [¹⁴C]cAMP, [α³²P]ATP, [¹⁴C]α-d glucose-1-phosphate, and [³H]±carazolol were obtained from New England Nuclear. All other chemicals were at least reagent grade.

Results

Effects of Trifluoperazine on Phosphorylase Activation and Inotropic State of the Perfused Ventricles

Basal phosphorylase a in the perfused ventricles was 10.1% (Fig. 1). Both calcium and isoproterenol resulted in significant activation of phosphorylase (Fig. 1) with the isoproterenol activation being concentration dependent. Calcium activation of phosphorylase was not blocked by perfusion with 3 × 10⁻⁶ M propranolol (data not shown).

Perfusion with 10⁻⁵ M trifluoperazine had no effect on basal levels of phosphorylase a (Fig. 1) or on total phosphorylase activity (data not shown). Following perfusion with 10⁻⁵ M trifluoperazine, significant attenuation of phosphorylase activation was seen in the hearts stimulated by either calcium or isoproterenol (Fig. 1). Notably, trifluoperazine (10⁻⁵ M) perfusion did not completely inhibit the activation induced by either agent. Phosphorylase a levels in all stimulated hearts following trifluoperazine preperfusion were above basal levels.

In contrast to the inhibition of phosphorylase activation by trifluoperazine in the perfused ventricles, the positive inotropic effect of calcium and isoproterenol was not altered (Table 1). Calcium induced increases in contractile state were rapid. Maximal increases occurred in 15 seconds, after which developed tension decreased but remained significantly elevated. Maximal activation of phosphorylase occurred approximately 15 seconds after the peak inotropic response. These results parallel those published by

![Figure 1: Effect of trifluoperazine on phosphorylase a formation in the perfused rat ventricles following stimulation with calcium or isoproterenol for 30 seconds. Data shown as mean ± SEM (n = 5–7) 20-minute preperfusion with 10⁻⁵ M trifluoperazine (solid bars). * P < 0.05 as compared to control. ** P < 0.05 as compared to absence of trifluoperazine.](http://circres.ahajournals.org/figimg/10.1161/01.RES.51.4.450)
other investigators (Freisen et al., 1966). Isoproterenol-induced changes in contractility were not as rapid as those induced by calcium. The peak inotropic response occurred at 30 seconds with phosphorylase b to a conversion following the same time course as the mechanical changes.

A small (<10%) decrease in developed tension was noted after the 20-minute perfusion with trifluoperazine. However, trifluoperazine produced no alteration in the time of onset or the maximum developed tension induced by calcium or by either concentration of isoproterenol (Table 1).

### Effects of Trifluoperazine on the β-Adrenergic Receptor:Adenylate Cyclase System

In view of the complex regulation of phosphorylase kinase, it was imperative to exclude trifluoperazine interactions at regulatory sites other than calmodulin. Accordingly, additional studies were undertaken to examine possible trifluoperazine effects on the β-adrenergic receptor:adenylate cyclase system.

The concentration of trifluoperazine (10^{-5} M) utilized in the perfusion studies had no significant effect on [3H]±carazolol binding to the β-receptor (Fig. 2). Trifluoperazine at high concentrations (>10^{-5} M) did interfere with [3H]±carazolol binding to the β-receptor. Analysis of trifluoperazine inhibition of [3H]±carazolol binding revealed a Hill coefficient greater than two, a finding which is not consistent with a simple model of competition between trifluoperazine and carazolol. However, as the question of β-blocking properties of phenothiazines has been raised (Samet and Surawicz, 1974) we further assessed the effect of trifluoperazine on β-adrenergic regulation of adenylate cyclase in rat cardiac membrane vesicles.

Consistent with the observations of Potter et al. (1980), we found no effect of trifluoperazine (10^{-5} M) on either basal adenylate cyclase activity or the activity stimulated by guanine nucleotides and isoproterenol (Fig. 3). Further evidence against trifluoperazine induced changes in the cAMP system was obtained in vivo by determining cAMP levels in the perfused ventricles (Fig. 4). Basal cAMP was 0.39 pmol/mg wet weight. High calcium (3.75 mM) did not result in any significant changes in cAMP levels. No significant changes in cAMP were noted in either basal or cal-

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of maximum contractility (sec)</th>
<th>Contractility % of control</th>
</tr>
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<tbody>
<tr>
<td>Calcium 3.75 mM</td>
<td>15</td>
<td>167 ± 4 NS</td>
</tr>
<tr>
<td>+ trifluoperazine 10^{-5} M</td>
<td>15</td>
<td>168 ± 16 NS</td>
</tr>
<tr>
<td>Isoproterenol 3 X 10^{-5} M</td>
<td>30</td>
<td>126 ± 8 NS</td>
</tr>
<tr>
<td>+ trifluoperazine 10^{-5} M</td>
<td>30</td>
<td>113 ± 4 NS</td>
</tr>
<tr>
<td>Isoproterenol 3 X 10^{-4} M</td>
<td>30</td>
<td>193 ± 23 NS</td>
</tr>
<tr>
<td>+ trifluoperazine 10^{-4} M</td>
<td>30</td>
<td>196 ± 26 NS</td>
</tr>
</tbody>
</table>

Results expressed as mean ± SEM (n = 5-7). Trifluoperazine perfusion was for 20 minutes. NS = not significant.

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## Figure 2

**Effect of trifluoperazine on β-adrenergic receptor binding** as measured with [3H]±carazolol in rat cardiac membrane vesicles. Each point is the mean of triplicate determinations with se < 5%. [3H]±carazolol concentration was approximately 330 pm. Specific binding was taken as that binding which was displaceable by 20 μM (±) propranolol. Specific [3H]±carazolol binding, expressed as a percentage of binding in the absence of trifluoperazine, is plotted as a function of trifluoperazine concentration.

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## Figure 3

**Effect of trifluoperazine on isoproterenol activation of adenylate cyclase in rat cardiac membrane vesicles.** All determinations done in the presence of 10^{-4} M GTP. Data shown as mean ± SE (n = 3). ○, without trifluoperazine. ◦, with 10^{-6} M trifluoperazine. There are no significant differences between isoproterenol activation in the presence or absence of trifluoperazine.
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1.2
1.0
0.8
0.6
0.4
0.2
0

BASAL CALCIUM ISOPROTERENOL ISOPROTERENOL

$3 \times 10^{-9}$

$3 \times 10^{-8}$

**Figure 4.** Effect of trifluoperazine on cyclic AMP levels in the perfused rat ventricles following administration of calcium or isoproterenol for 30 seconds. Data shown as mean ± SEM (n = 5–7), 20-minute preperfusion with $10^{-9}$ M trifluoperazine (solid bars). *P < 0.05 as compared to control.

Calcium treated hearts which had been preperfused with trifluoperazine. As expected, isoproterenol perfusion significantly increased cAMP levels but hearts pre-perfused with trifluoperazine had levels comparable to their respective controls.

**In Vitro Studies of Phosphorylase Kinase**

Partially purified phosphorylase kinase was prepared from rat myocardium to determine whether added calmodulin would stimulate rat cardiac phosphorylase kinase. In the presence of calcium, the pH 6.8/8.2 activity ratio of the partially purified enzyme was 0.11. Activity in the absence of calcium was consistently low (<10%). The characteristics of the partially purified enzyme were similar to that of the skeletal muscle enzyme (Cohen, 1973). Notably, purification schemes similar to those utilized for skeletal muscle did not result in an entirely pure cardiac enzyme as shown by SDS gel electrophoresis. However, the relative 30 to 50-fold lower phosphorylase kinase content of rat myocardium precluded further purification of the rat cardiac enzyme (Shenolikar et al., 1979).

Phosphorylase kinase activity was stimulated 1.5- to 3-fold by the addition of calmodulin. Activation was concentration dependent with maximal stimulation observed at approximately $10^{-6}$ M calmodulin. Maximal activation at a similar calmodulin concentration was recently reported for the skeletal muscle enzyme (Cohen et al., 1979). The left panel in Figure 5 shows the results of a typical experiment with $10^{-6}$ M calmodulin. In the presence of EGTA, phosphorylase kinase activity remains minimal, even in the presence of additional calmodulin. The experiment illustrated in Figure 5 was done at pH 8.2, but similar stimulation was obtained at pH 6.8 (Table 2). Trifluoperazine in concentrations as low as one micromolar markedly inhibited stimulation by free calmodulin without any effect on the activity in the absence of additional calmodulin (Fig. 5). Higher concentrations of trifluoperazine partially inhibited the unstimulated enzyme.

Phosphorylation of the partially purified enzyme by cAMP-dependent protein kinase catalytic subunit also resulted in increased activity (Table 2). The effect of phosphorylation was greatest at pH 6.8. At pH 8.2 the stimulation following the addition of calmodulin was comparable to that of phosphorylation. Following phosphorylation, only slight activation was observed with exogenous calmodulin.

The limited quantity of enzyme obtained from rat hearts precluded accurate stoichiometric estimation of the subunit composition. However, if the $\delta$ subunit was not tightly bound, partial dissociation would be likely to occur with dilution. Serial dilutions of the enzyme 100-fold still resulted in identical stimulation of calmodulin of any one fraction (data not shown), thus providing indirect evidence that the calmodulin stimulation was not due to loss of the calmodulin subunit.

**Discussion**

Studies conducted in skeletal muscle suggest at least three potential mechanisms for activation of phosphorylase kinase (Shenolikar et al., 1979). First, the enzyme can be activated by cAMP-dependent protein kinase (i.e., phosphorylation). Second, calcium alone can stimulate activity, presumably by binding to the $\delta$ subunit (calmodulin) of the enzyme. Third, it has been shown in skeletal muscle that additional activation of phosphorylase kinase may result from the reversible binding of a second mole of calcium-calmodulin per mole of phosphorylase kinase. In cardiac muscle, the activation of phosphorylase kinase appears to involve the first two mechanisms described above (Gross and Mayer, 1975;
McCullough and Walsh, 1979). However, it has not yet been established whether the third mechanism operates physiologically in hearts.

In the present study, both isoproterenol and calcium resulted in rapid phosphorylase a formation in intact ventricles as expected (Fig. 1) (Friesen et al., 1966; McCullough and Walsh, 1979). Isoproterenol leads to activation of phosphorylase by stimulating formation of cAMP which in turn activates cAMP-dependent protein kinase which phosphorylates phosphorylase kinase (Gross and Mayer, 1975; McCullough and Walsh, 1979), and by increasing intracellular calcium concentrations (Stull and Mayer, 1979). The increased intracellular calcium associated with β-receptor activation could theoretically activate phosphorylase both by interacting with the δ subunit of phosphorylase kinase and by interacting with unbound calmodulin, the resulting calcium-calmodulin complex then further activating the enzyme. Phosphorylase activation in response to high calcium perfusion seen in the present study also presumably resulted from interaction with the δ subunit of phosphorylase kinase and unbound calmodulin. Trifluoperazine, an inhibitor of calmodulin-regulated processes, partially inhibited activation of phosphorylase by either calcium or isoproterenol (Fig. 1).

The inhibition of phosphorylase activation seen in the present study was dissociated from inotropic effects. Conflicting reports exist in the literature regarding the effects of phenothiazine drugs on contractility. In isolated rat atria, a concentration-dependent decrease in contractility was seen with promazine and thioridazine. However, the decrease in developed tension was accompanied by significant rate decreases and, thus, the negative inotropic effect may have reflected force-frequency relationships (Landmark, 1971). In the present study, all hearts were paced thus negating rate influenced changes. Chloropromazine at concentrations greater than 10^{-5} M also was reported to produce concentration-dependent decreases in contractility in isolated rat ventricles, which were paced (Langslet, 1971). The magnitude of the decreases was not stated, making direct comparison between those results and ours difficult. In the same study, no effect on positive inotropy induced by isoproterenol was noted. We did note a small but consistent decrease in developed tension after the 20-minute perfusion with trifluoperazine. However, there were no changes in the positive inotropic effects produced by either calcium or isoproterenol.

Because the regulation of phosphorylase kinase is complex, systematic studies were undertaken to evaluate effects of the trifluoperazine on the various steps involved in β-adrenergic activation of the enzyme.

### Table 2

**Effects of Phosphorylation by cAMP-Dependent Protein Kinase Catalytic Subunit and Calmodulin on Phosphorylase Kinase Activity**

<table>
<thead>
<tr>
<th>Method of activation</th>
<th>Phosphorylase kinase activity</th>
<th>pH 6.8</th>
<th>pH 8.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol (^{32})P incorp*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.5 ± 0.1</td>
<td>11.8 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Calmodulin (10^{-6} M)</td>
<td>9.4 ± 0.4†</td>
<td>25.4 ± 1.9†</td>
<td></td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>16.7 ± 0.5†</td>
<td>28.3 ± 1.4†</td>
<td></td>
</tr>
<tr>
<td>Phosphorylation + calmodulin (10^{-6} M)</td>
<td>24.3 ± 1.5†</td>
<td>31.8 ± 0.4†</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± se (n = 4).
† P < 0.01 as compared to control.
The attenuation of phosphorylase activation produced by trifluoperazine appeared to be independent of the \( \beta \)-adrenergic receptor:adenylate cyclase system. At concentrations of trifluoperazine greater than that used in the perfusion studies (10\(^{-5}\) M), multiple interactions were noted. The mechanism of these interactions is not well understood; however, interaction with phospholipids has been hypothesized (Mori et al., 1980).

The role of calcium in the regulation of cardiac adenylyte cyclase remains undefined. Some studies have suggested that calcium inhibits adenylyte cyclase from cardiac muscle (Potter et al., 1980). In an in vivo system such as the perfused ventricles, predicting the effect of trifluoperazine on cAMP levels is further complicated by the fact that phosphodiesterase, the enzyme which converts cAMP to 5'-AMP, requires calcium-calmodulin for activity. Nevertheless, trifluoperazine had no effect on basal or stimulated cAMP levels in the perfused ventricles (Fig. 4). Further, in agreement with other investigators (Mori et al., 1980), we found no effect of trifluoperazine on cAMP-dependent protein kinase (data not shown). The effect of trifluoperazine in the present study therefore was not mediated through inhibition of cAMP-induced phosphorylation of phosphorylase kinase.

The foregoing results exclude an effect of trifluoperazine on the regulation of phosphorylase kinase by phosphorylation. The remaining most likely site of action of trifluoperazine is on reversibly bound calmodulin, involved in the accessory calcium-dependent activation of phosphorylase kinase. Trifluoperazine has been reported to bind specifically to calmodulin in the presence of calcium (Levin and Weiss, 1977). In vitro binding studies have shown that trifluoperazine has a \( K_d \) for calmodulin of approximately 1.0 \( \mu \)M (Levin and Weiss, 1977). Our preliminary studies revealed that 10 \( \mu \)M trifluoperazine had no significant effect on contractility in rat hearts. This concentration should theoretically result in an intracellular concentration sufficient to inhibit calmodulin activation of phosphorylase kinase.

In vitro studies with the partially purified phosphorylase kinase provide explanations for the calcium-induced activation of phosphorylase in the intact hearts and the inhibition of this activation by trifluoperazine. Addition of calmodulin stimulated the activity of the partially purified enzyme in vitro (Fig. 5). Trifluoperazine inhibited, in a concentration-dependent manner, the calmodulin activation of phosphorylase kinase (Fig. 5). These results are consistent with recent in vitro studies of skeletal muscle phosphorylase kinase which showed that binding of a second molecule of calmodulin stimulates the enzyme (Shenolikar et al., 1979).

The results in the perfused ventricles suggest that calcium may regulate phosphorylase kinase in vivo both by directly interacting with the enzyme and by facilitating binding of a second mole of calmodulin to the enzyme. If calcium stimulation occurred only through the binding of calmodulin, trifluoperazine would be expected to abolish completely calcium activation of phosphorylase. Notably, in the perfused ventricles, trifluoperazine inhibited but did not completely abolish calcium-induced phosphorylase activation (Fig. 1). By analogy to in vitro data, the fraction of phosphorylase activation that was not blocked by trifluoperazine could be reasonably accounted for on the basis of calcium binding to a tightly bound calmodulin subunit of phosphorylase kinase in vivo. The possibility of direct interaction between trifluoperazine and a tightly bound calmodulin subunit of phosphorylase kinase must also be considered in interpreting the present results. Minimal inhibition of the partially purified enzyme by trifluoperazine was noted. Therefore, it is not possible to exclude entirely an effect of trifluoperazine on a tightly bound calmodulin subunit of phosphorylase kinase in the perfused ventricles. However, several lines of evidence suggest that the inhibition caused by trifluoperazine was not due solely to interaction with the calmodulin subunit. Similar to results with the skeletal muscle enzyme (Shenolikar et al., 1979), the magnitude of inhibition of cardiac phosphorylase kinase seen, even at the highest concentrations of trifluoperazine tested, was only 20–30% in vitro. Second, purification of skeletal muscle phosphorylase kinase utilizing techniques similar to those used in the present study results in an enzyme which can partially activate calmodulin-dependent phosphodiesterase and myosin light chain kinase (Wang et al., 1981). These results suggest that the phosphorylase kinase preparation contains small amounts of reversibly bound calmodulin which can activate other enzymes.

The results of the present study suggest that calcium-calmodulin also has a significant effect on phosphorylase activation by \( \beta \)-adrenergic agents. In vitro, addition of calcium-calmodulin results in only slight activation of phosphorylated phosphorylase kinase (Table 2). However, under some conditions, the activation by calmodulin is approximately equal to that seen with phosphorylation (Table 2). In vivo attenuation of phosphorylase activation by trifluoperazine was also noted in the isoproterenol-treated hearts (Fig. 1). Again, this inhibition of phosphorylase activation by trifluoperazine was not complete. Since our results excluded an effect of trifluoperazine on the \( \beta \)-adrenergic receptor-cAMP regulatory system, the partial inhibition of phosphorylase activation in the isoproterenol-treated hearts is most reasonably explained by trifluoperazine interference with the binding of free calcium-calmodulin to phosphorylase kinase. Thus, in vivo activation of phosphorylase kinase appears to be the net result of regulation by cAMP-dependent phosphorylation, calcium, and reversibly bound calcium-calmodulin.

Consistent with these conclusions, it has previously been shown that reducing the extracellular calcium...
concentration can inhibit β-adrenergic-stimulated
phosphorylase a formation (Namm et al., 1968; Bar-
Gross and Johnson (1980), in studies of murine ske-
etal muscle, showed that calcium was necessary for
maximal isoproterenol-induced phosphorylase acti-
vation. These investigators conclude that reducing the
extracellular buffer concentration of calcium in some
way changes the intracellular concentration of a factor
that regulates phosphorylase kinase activity. Based on
the results of the present study, it is highly probable
that the intracellular factor is calcium-calmodulin.
Additional support for calcium-calmodulin as this
regulator are their findings that the divalent cation
requirement for maximum isoproterenol-stimulated
phosphorylase a formation could be satisfied by
strontium but not by barium. These findings correlate
with the in vitro binding studies of Levin and Weiss
(1977). Strontium was almost as effective as calcium in
promoting binding of calmodulin to trifluopera-
zine. Barium, in contrast, had no effect on calmodulin
binding.

The magnitude of the inhibition of phosphorylase
a formation produced by trifluoperazine in the per-
fused ventricles supports the hypothesis that calcium is
a limiting factor in the regulation of both the
phosphorylated and nonphosphorylated enzyme. It
would appear that binding free calmodulin may en-
hance the calcium sensitivity of phosphorylase kinase.
Wang et al. (1981) have shown in vitro a 10-fold
decrease in activating concentrations of calcium re-
quired when exogenous calmodulin concentrations
were increased from 20 nM to 10 μM consistent with
this hypothesis. Myosin light chain kinase behaved
similarly in their studies in that the effective concen-
tration of calcium required for activation varies with
varying calmodulin concentrations.

The present study demonstrated trifluoperazine
inhibition of phosphorylase activation in perfused ven-
tricles. In addition, phosphorylase kinase stimulation
was observed in vitro by exogenous calmodulin.
These results suggest that reversibly bound calmo-
dulin might well represent a physiological means of
phosphorylase kinase regulation in rat cardiac muscle.

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References
Barovsky K, Gross SR (1981) Rate dependence of isoproterenol
stimulated phosphorylase a formation in mouse heart. J Phar-
macol Exp Ther 217: 326–332
cyclic AMP-dependent protein kinase(s) and its subunits from
Brostrom CO, Hunkeler EL, Krebs EG (1971) The regulation of skeletal
muscle phosphorylase kinase by Ca++. J Biol Chem 246:
1961–1967
Cohen P (1973) The subunit structure of rabbit-skeletal-muscle
phosphorylase kinase and the molecular basis of its activation
reactions. Eur J Biochem 34: 1–14
Cooper RH, Sul HS, McCullough TE, Walsh DA (1980) Purification
and properties of the cardiac isoenzyme of phosphorylase kinase.
J Biol Chem 255: 11794–11801
Daegelen-Proux D, Pierres M, Alexandre Y, Dreyfus J (1976)
Molecular heterogeneity of rabbit heart phosphorylase kinase.
Biochim Biophys Acta 452: 398–405
Dobson JG, Mayer SE (1973) Mechanisms of activation of cardiac
glycogen phosphorylase in ischemia and anoxia. Circ Res 33:
412–420
Dobson JG, Ross Jr, Mayer SE (1976) The role of cyclic adenosine
3',5'-monophosphate and calcium in the regulation of contractil-
ity and glycogen phosphorylase activity in guinea pig papillary
Friesen AD, Allen G, Valadares JRE (1966) Calcium-induced activa-
tion of phosphorylase in rat hearts. Science 155: 1108–1109
the assay of glycerone phosphorylases. Anal Biochem 47: 20–27
Gross SR, Johnson RM (1980) Role of extracellular Ca²⁺ in regu-
lating isoproterenol-stimulated phosphorylase a formation
in murine skeletal muscle. J Pharmacol Exp Ther 214: 37–44
Gross SR, Mayer SE (1974) Regulation of phosphorylase b to a in
conversion in muscle. Life Sci 14: 401–414
Hayakawa T, Perkins JP, Krebs EG (1973) Studies on the subunit
structure of rabbit skeletal muscle phosphorylase kinase. Bio-
chemistry 12: 574–580
Hayes JS, Mayer SE (1981) Regulation of guinea pig heart phos-
phorylase kinase by Ca²⁺, protein kinase, and calcium. Am J
Physiol 240: 340–349
Jones LR, Besch HR Jr, Fleming JW, McConnaughey MM, Watan-
abe AM (1979) Separation of vesicles of cardiac sarcosome from
vesicles of cardiac sarcoplasmic reticulum. J Biol Chem 254:
520–539
Klein CB (1977) Conformational transition accompanying the bind-
ing of Ca²⁺ to the protein activator of 3',5'-cyclic adenosine
monophosphate phosphodiesterase. Biochemistry 16: 1017–1024
Landmark K (1972) The action of promazine and thioridazine in
isolated rat atrium I. Effects on automaticity, mechanical per-
formance, refractoriness and excitability. Eur J Pharmacol 16:
1–7
Langlet A (1971) Effects of chlorpromazine, d,l-propranolol and
d-propranolol in the isolated rat heart: Modification of the
responses to isoproterenol and glucagon. Eur J Pharmacol 15:
164–170
Levin RM, Weiss B (1977) Binding of trifluoperazine to the calcium-
dependent activator of cyclic nucleotide phosphodiesterase. Mol
Pharmacol 13: 690–697
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein
measurement with the Folin phenol reagent. J Biol Chem 193:
265–275
Manalan AS, Besch HR Jr, Watanabe AM (1981) Characterization of
[3H]diazepam binding to beta-adrenergic receptors: Appli-
cation to study of beta-adrenergic receptor subtypes in canine
McCullough, TE, Walsh, DA (1979) Phosphorylation and dephos-
phorylation of phosphorylase kinase in the perfused rat heart. J
Biol Chem 254: 7345–7352
action of chlorpromazine, dibucaine and other phospholipid-
interacting drugs on calcium-activated, phospholipid-dependent
Nathan DH, Mayer SE, Maltbie M (1980) The role of potassium and
calcium ions in the effect of epinephrine on cardiac adenose
3',5'-monophosphate phosphorylase kinase and phosphor-
ylase. Mol Pharmacol 4: 522–530
Potter JD, Flisack MT, Wisler PL, Robertson SP, Johnson CL (1980)
Calcium dependent regulation of brain and cardiac muscle ade-
...
phosphorylation of muscle glycogen synthase by phosphorylase b kinase. J Cyclic Nucleotide Res 4: 245-257
Steiner AL, Parker CW, Kipnis DM (1972) Radioimmunoassay for nucleotides II Adenosine 3',5'-monophosphate in mammalian tissues and body fluids. J Biol Chem 247: 1114-1120

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