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# Protein Dephosphorylation Rates in Myocytes after Isoproterenol Withdrawal

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**ABSTRACT.** Dephosphorylation of substrates for cyclic AMP-dependent protein kinase is essential for reversing the effects of this enzyme. It has been proposed that the relevant phosphatase(s) is stimulated by muscarinic cholinergic agonists, thereby accentuating cholinergic antagonism of β-adrenergic agonists in the heart. To test this hypothesis, dephosphorylation of the three major substrates of cardiac cyclic AMP-dependent protein kinase (phospholamban, troponin-I, and C-protein) was examined. In isolated myocytes, isoproterenol caused concentration-dependent phosphorylation of these three proteins. Simultaneous exposure to acetylcholine with the isoproterenol caused a rightward shift in the concentration–response curve that was similar for protein phosphorylation in myocytes and for the inotropic response of the intact heart. The addition of propranolol after exposure to isoproterenol resulted in protein dephosphorylation, the onset of which was accelerated by acetylcholine. However, acetylcholine did not affect the rate of dephosphorylation for any of the proteins, indicating that phosphatase activity in cardiac muscle is not enhanced by acetylcholine. BIOCHEM PHARMACOL **59**;12:1513–1519, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** myocytes; phospholamban; phosphatase; cholinergic; adrenergic; phosphorylation

It is well accepted that phosphorylations catalyzed by cyclic AMP-dependent protein kinase cause the inotropic and biochemical changes seen in cardiac muscle after an increase in cyclic AMP [1]. Increased cyclic AMP can result from stimulation of adenylate cyclase by β-adrenergic agonists or other receptors coupled to the cyclase via G-proteins, or from inhibition of phosphodiesterase. Inhibition by muscarinic cholinergic agonists of the effects caused by β-adrenergic agonists is a classic system of physiological antagonism [2]. The mechanisms by which muscarinic agonists inhibit the actions of β-adrenergic agonists are known to include inhibition of adenylate cyclase via $G_i$ [3–5] and are proposed to include activation of protein phosphatase [6, 7]. Although there is evidence for acetylcholine-induced inhibition of inhibitor-1 phosphorylation [6], which should produce a disinhibition of protein phosphatase type 1, an effect of cholinergic agonists on phosphatase activity, which should be detectable as an increase in the rate of protein dephosphorylation, has not been demonstrated.

Regulation of phosphatases in various tissues has been the focus of much interest [8–10]. Characterization of the phosphatases in regard to distribution, cofactors, and regulatory mechanisms has progressed rapidly [11–14]. In the heart, type 1 phosphatase has been shown to be inhibited secondary to inhibitor-1 phosphorylation induced by an increase in cyclic AMP [6, 15–17]. Substrates for type 1 phosphatase in the heart include phospholamban and the $Ca^{2+}$ channel [18–20]. Inactivation of the main phosphatase opposing the actions of cyclic AMP-dependent kinase potentially contributes to both the effects of the kinases and their duration. Other phosphatases that are important in the heart are type 2A phosphatase [21] and calcineurin [22–25]. Calcineurin is stimulated by $Ca^{2+}$ and calmodulin and is the only phosphatase known to be controlled by second messengers in a manner analogous to kinases [26]. The substrate specificity of calcineurin is narrow, unlike other phosphatases, and includes inhibitor-1 and the Nuclear Factor of Activated T-cell (NFAT) family of transcription factors [27–29].

The biochemical effects following activation or inactivation of adenylate cyclase by β-adrenergic or muscarinic cholinergic agonists, respectively, are difficult to follow in intact muscle preparations because of error inherent in assays for cyclic nucleotides and phosphoproteins. Large errors are introduced when several dozen different muscles are labeled independently to examine a time course or dose–response curve. This error is due partially to nonuniform specific activity of [$^{32}$P]ATP, which occurs when muscles are labeled individually but also is increased by the

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manipulations that each sample must undergo in preparation for analysis. Using preparations of $Ca^{2+}$-tolerant isolated myocytes, we have examined entire time courses and concentration–response curves in single preparations with uniform specific activity of label.

In the present study we examined dephosphorylation of phospholamban, troponin-I, and C-protein in the absence and presence of acetylcholine following a brief exposure of isolated myocytes or the intact heart to isoproterenol. Inhibition of phosphorylation in the myocytes and of force of contraction in the intact heart was evident with acetylcholine, and acetylcholine accelerated the onset of dephosphorylation. However, the rate of dephosphorylation for phospholamban, troponin-I, and C-protein was not accelerated by acetylcholine in either preparation.

## MATERIALS AND METHODS

### *Determination of Phosphate Incorporation Using the Intact Heart*

Hearts were obtained from heparinized (500 U heparin, administered 60 min prior to killing) guinea pigs of random sex weighing 400–450 g. Hearts were removed following cervical dislocation, perfused at a flow rate of 6.0 mL/min via the aorta as previously described [30] with Krebs–Henseleit solution of the following composition: NaCl, 118.0 mM; $NaHCO_3$, 27.2 mM; KCl, 4.8 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 1.8 mM; sodium pyruvate, 2.5 mM; dextrose, 10.0 mM; saturated with 95% $O_2$/5% $CO_2$, resulting in a pH of 7.4. The temperature of the perfusate was maintained at 32°, and resting tension was adjusted to 5.0 g. Hearts were stimulated electrically at 3.0 Hz during the course of the experiment. Effluent from the hearts was discarded for the first 15 min of perfusion, after which the perfusion circuit was changed to a recirculating system containing 50 mL of the same buffer to which 3.0 mCi of [$^{32}$P]orthophosphate had been added. Following 40 min of recirculating perfusion, the circuit was switched back to the flow-through branch for 5 min to allow washout of unincorporated $^{32}$P prior to the addition of test drugs by continuous infusion. At the indicated times, hearts were freeze-clamped, pulverized under liquid nitrogen, and stored at −70° until used.

### *Heart Perfusion for Determination of Force of Contraction*

Hearts were obtained and perfused as described above, except that perfusate was never recirculated and no radioactive material was added. Force of contraction was monitored continuously using a force transducer and recorded on a Gould strip chart recorder. Drugs were diluted from stocks immediately before use into Krebs–Henseleit solution containing 0.5 mM sodium bisulfite and were administered by continuous infusion. Each concentration of isoproterenol was infused for 2 min and was followed immediately by infusion of the next higher concentration.

### *Myocyte Isolation*

Myocytes were isolated from guinea pig hearts as previously described [30]. Myocyte preparations were used when they contained at least 80% viable cells. Viability was assessed by the rod-shaped criterion, using cells that had been preserved in 10% buffered formalin at the time of the experiment. Prior to initiation of experiments, myocytes were suspended in a buffer of the following composition: NaCl, 132.0 mM; dextrose, 10.0 mM; KCl, 4.8 mM; $MgSO_4$, 1.2 mM; HEPES, 10.0 mM; $CaCl_2$, 1.8 mM; bubbled with 100% $O_2$ and with pH adjusted to 7.4 using NaOH. Cells were incubated with [$^{32}$P]orthophosphate (3 mCi/100 mg cell protein) for 40 min, and unincorporated $^{32}$P was washed from the cells prior to addition of the drug.

### *Sample Preparation and SDS–PAGE*

Myocytes were sampled for separation of proteins by SDS–PAGE by dispersing cells in a solution composed of 2% SDS, 5 mM EGTA, 5 mM EDTA, 30 mM KF, and 10% glycerol. Cell protein in samples varied between 0.5 and 2.0 mg/mL. For quantitative analysis of $^{32}$P incorporation, samples were placed in boiling water for 2 min prior to electrophoresis on 12% polyacrylamide slab gels according to Porzio and Pearson [31] for resolution of phospholamban and troponin-I or according to Laemmli [32] for resolution of C-protein. Fifty to one hundred micrograms of cell protein was applied to each lane, gels were stained and dried, and $^{32}$P-labeled proteins were identified by autoradiography. Protein bands of interest were cut from the gels, and $^{32}$P content was determined by liquid scintillation spectroscopy.

### *Analysis of Cyclic Nucleotides*

Cell and tissue cyclic AMP and cyclic GMP contents were determined by radioimmunoassay as previously described [33].

## RESULTS

Identifications of phospholamban, troponin-I, and C-protein were made by measuring their mobility during SDS–PAGE and by assessing their ability to be phosphorylated in the intact cells and hearts exposed to isoproterenol. Phospholamban was identified further by the characteristic shift in mobility of this protein during SDS–PAGE after being boiled in solution containing SDS. In addition to the three proteins examined in this study, a protein of approximately 34 kDa was phosphorylated in response to isoproterenol.

To use isolated myocytes as a model for studying the biochemical response of the cells to interaction of cholinergic and β-adrenergic agonists, it was necessary to establish that the myocytes respond to these agents as the intact heart does. Therefore, the cholinergic antagonism of β-adrenergic agonists was examined in myocytes as an inhibi-

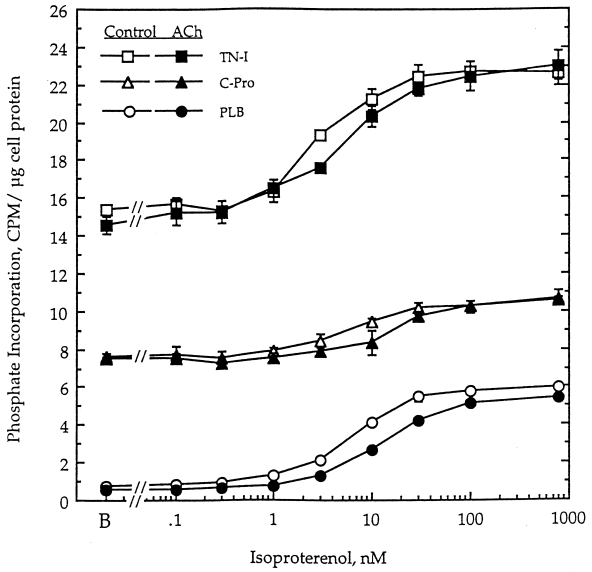


**FIG. 1. Phosphorylation of phospholamban (PLB), troponin-I (TN-I), and C-protein (C-Pro) in isolated myocytes exposed to isoproterenol in the presence (filled symbols) or absence (open symbols) of 1.0 μM acetylcholine. The shift in the concentration dependence of isoproterenol-induced phosphorylation was significant ($P < 0.05$) for each protein, as determined by ANOVA. B indicates cells that were not exposed to isoproterenol. Data are means ± SEM, N = 4, for all points. Where error bars are not shown, they lie within the symbol.**

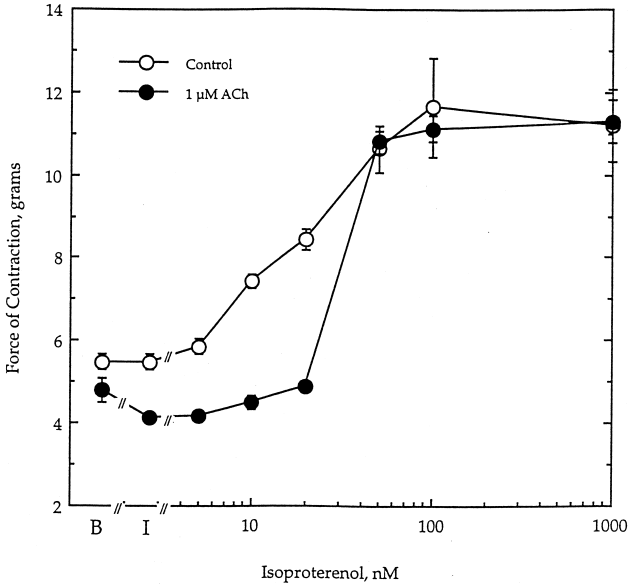


**FIG. 2. Acetylcholine-induced shift in the concentration–dependence for the positive inotropic effect of isoproterenol, shown in a cumulative concentration–response curve for isoproterenol in the absence and presence of 1.0 μM acetylcholine in guinea pig Langendorff preparations. B represents the value after equilibration and I the value after exposure to acetylcholine or vehicle control. Data are means ± SEM, N = 4, for all points.**

tion of protein phosphorylation and in the intact heart as an inhibition of the inotropic response to isoproterenol. In myocytes, isoproterenol elicited a concentration-dependent increase in phosphorylation of phospholamban, troponin-I, and C-protein. The concentration dependence for isoproterenol-induced protein phosphorylation in the isolated myocytes was shifted approximately 2-fold to the right by 1 μM acetylcholine (Fig. 1). Troponin-I was approximately 2-fold more sensitive to the isoproterenol than phospholamban, with C-protein being of intermediate sensitivity (Table 1). The inhibition of phosphorylation produced by acetylcholine was observed as a shift in the concentration–response curves to the right without a decrease in maximal phosphorylation of any of the proteins (Fig. 1). The potency of isoproterenol to increase the force of contraction in intact cardiac muscle and the shift to the right in isoproterenol potency caused by acetylcholine in the intact heart were similar to the potency and shift in potency seen in myocytes for protein phosphorylation (Fig. 2). Also, like the effect of acetylcholine in the myocytes, the inhibition of isoproterenol stimulation of contraction was observed as a shift in the concentration–response curve without a decrease in the maximum response (Fig. 2).

Dephosphorylation of phospholamban after a brief exposure of either the isolated myocytes or the intact heart to isoproterenol occurred much faster than dephosphorylation of either troponin-I or C-protein. Incubation of myocytes for 2 min with 0.1 μM isoproterenol resulted in 3.5-, 1.3-, and 0.6-fold increases in $^{32}P$ incorporation in phospholamban, troponin-I, and C-protein, respectively (N = 12–18). Removal of β-adrenergic stimulation by the addition of 1 μM propranolol resulted in dephosphorylation of each of these proteins. Dephosphorylation of phospholamban after the addition of propranolol occurred as a single exponential process after a 15-sec delay (Fig. 3). The half-life of phosphorylated phospholamban in the intact myocytes was 0.72 min (N = 6, 95% confidence limits 0.54 to 1.05 min). The addition of 1 μM acetylcholine with the propranolol did not have a significant effect on the rate of dephosphorylation. The half-life with propranolol plus acetylcholine was 0.52 min (N = 6, 95% confidence limits 0.36 to 0.93 min). The addition of acetylcholine in the continued presence of β-adrenergic stimulation resulted in incomplete dephosphorylation (Fig. 3). Although the rate of dephosphorylation was not affected by acetylcholine, the onset of

**TABLE 1. Concentration dependence of isoproterenol for phosphorylation of phospholamban, troponin-I, and C-protein**

| | Isoproterenol stimulation $EC_{50}$ (nM) | |
|---|---|---|
| **Protein** | **Control** | **ACh (1 μM)** |
| Phospholamban | 7.1 ± 0.1* | 12.9 ± 0.2*† |
| Troponin-I | 3.1 ± 0.2‡ | 6.6 ± 0.3†‡ |
| C-protein | 5.6 ± 0.7* | 13.9 ± 4.7*† |

Data presented in Fig. 1 were analyzed to determine the $EC_{50}$ for isoproterenol. Values are means ± SEM, N = 4. Significant differences were determined by ANOVA.

*Significantly different ($P < 0.05$) from troponin-I.

†Significantly different ($P < 0.05$) from control.

‡Significantly different ($P < 0.05$) from phospholamban.

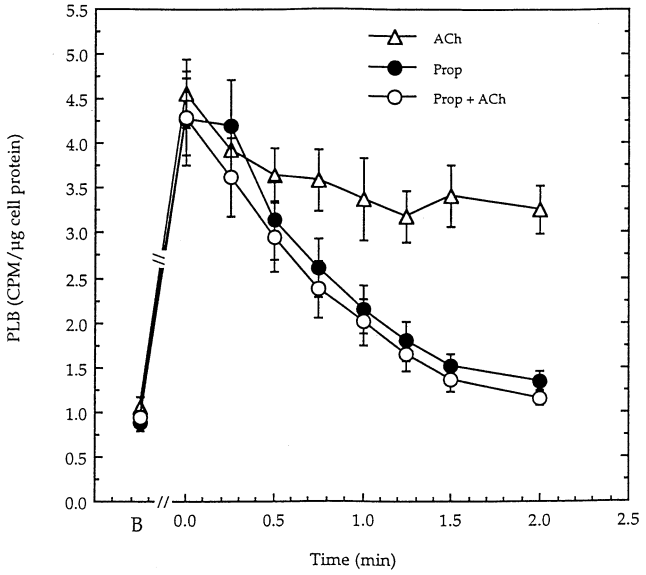


**FIG. 3. Dephosphorylation of phospholamban in isolated myocytes after a 2-min exposure to 0.1 μM isoproterenol. Dephosphorylation was initiated at time zero by the addition of 1.0 μM acetylcholine (ACh), 1.0 μM propranolol (Prop), or both (Prop + ACh). B indicates cells that were not exposed to isoproterenol. Data are means ± SEM, N = 6.**

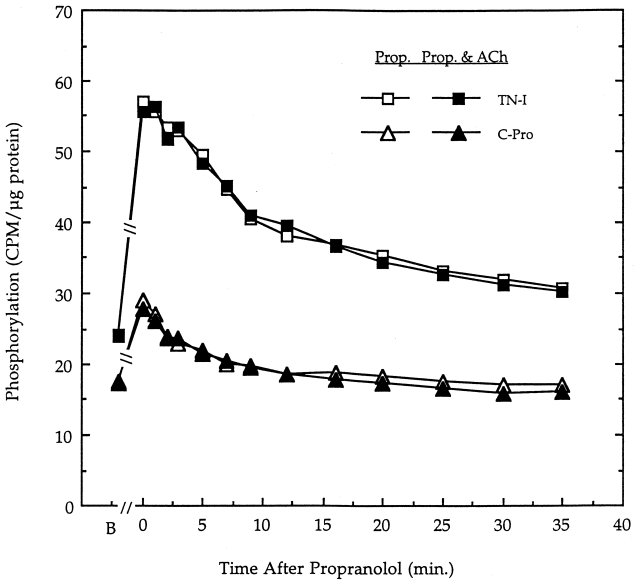


**FIG. 5. Dephosphorylation of isolated myocyte troponin-I (TN-I) and C-protein (C-Pro) after a 2-min exposure to 0.1 μM isoproterenol. Dephosphorylation was initiated at time zero by the addition of 1.0 μM propranolol (open symbols) or 1.0 μM acetylcholine plus 1.0 μM propranolol (filled symbols). B indicates cells that were not exposed to isoproterenol. Data are means ± SEM, N = 6.**

the dephosphorylation was accelerated by the cholinergic agonist irrespective of the addition of propranolol (Fig. 4).

Dephosphorylations of troponin-I and C-protein in myocytes were also described as single exponential processes (Fig. 5). Estimates for half-lives of phosphorylated troponin-I and C-protein in the isolated myocytes are 7.1 and 3.5 min, respectively. Time courses for the dephosphorylation of these proteins were not affected by acetylcholine applied simultaneously with propranolol (Fig. 5).

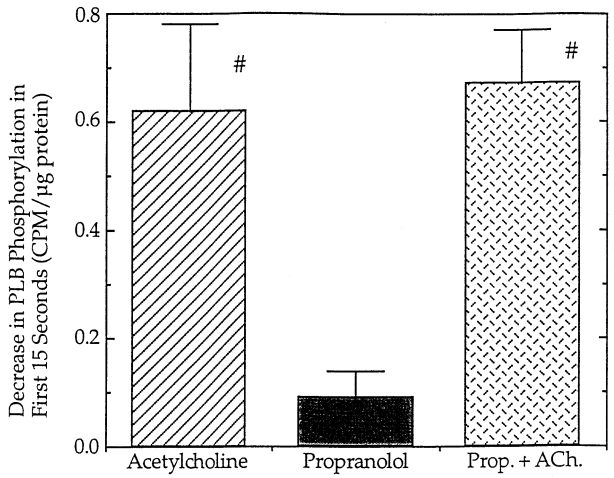


**FIG. 4. Decrease in phosphorylation of phospholamban 15 sec after initiating the dephosphorylation reaction. Data for the experiment shown in Fig. 3 were used to calculate the effect of propranolol and acetylcholine during the first 15 sec after they were added to the myocytes stimulated with isoproterenol. Myocytes treated with acetylcholine alone or acetylcholine plus propranolol had a significant decrease in phospholamban phosphorylation within 15 sec of initiating the dephosphorylation (#, $P < 0.05$). Phospholamban phosphorylation in myocytes treated with propranolol was not decreased significantly after 15 sec. Data are means ± SEM, N = 6.**

Because the preparations of isolated myocytes used in these studies are quiescent, it is possible that a contraction-dependent event such as $Ca^{2+}$ influx is essential for phosphatase activation by acetylcholine. If so, phosphatase activation would not occur in quiescent tissues such as the isolated myocytes. To confirm that acetylcholine does not accelerate dephosphorylation in intact muscle, the experiments were repeated using intact guinea pig hearts. The intact hearts were exposed to isoproterenol for 5 min before initiating dephosphorylation with propranolol alone or propranolol plus acetylcholine. Acetylcholine did not affect dephosphorylation time courses of any of the proteins examined (Fig. 6). The effectiveness of acetylcholine in these preparations was demonstrated by the increase in cyclic GMP in the hearts treated with acetylcholine (Fig. 7).

## DISCUSSION

The ability of cholinergic agonists to antagonize the actions of β-adrenergic agonists is well established. One mechanism proposed to contribute to this physiological antagonism is an activation of phosphatase(s) by cholinergic agonists. Experimentally, the best evidence in support of cholinergic-dependent phosphatase activation comes from reports showing changes in the time courses for dephosphorylation of troponin-I [34] and phospholamban [35] after isoproterenol is withdrawn and acetylcholine is in-

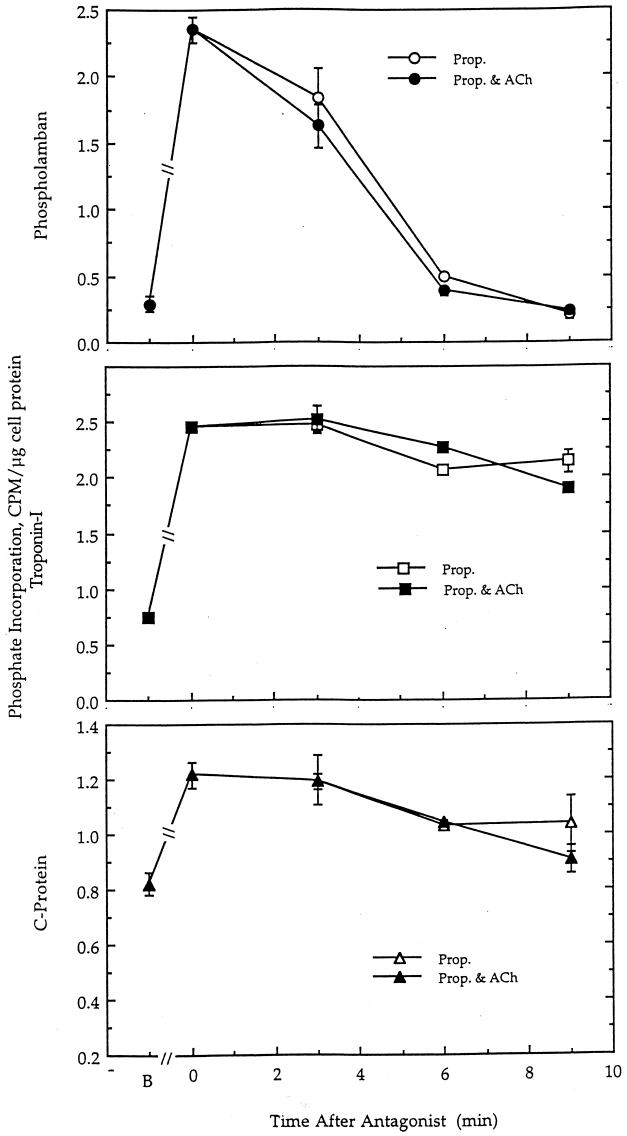


**FIG. 6. Dephosphorylation of phospholamban, troponin-I, and C-protein after a 5-min exposure of isolated guinea pig hearts to 0.1 μM isoproterenol. Dephosphorylation was initiated at time zero by the addition of 1.0 μM propranolol (open symbols) or 1.0 μM acetylcholine plus 1.0 μM propranolol (filled symbols). B indicates hearts that were not exposed to isoproterenol. Data are means ± SEM, N = 3.**

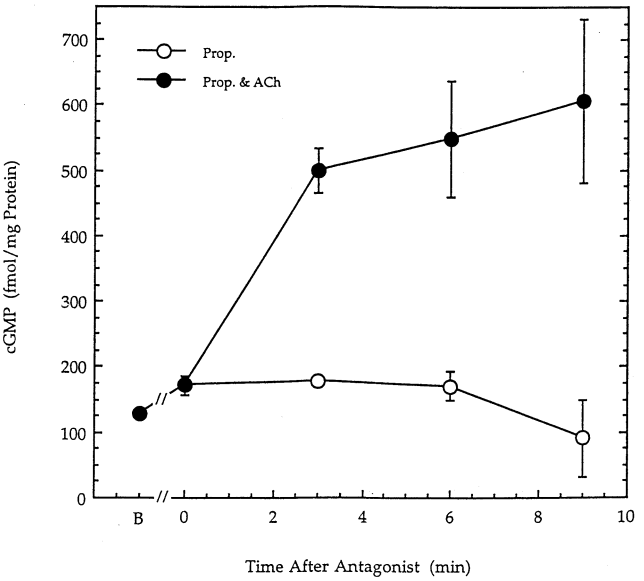


**FIG. 7. Cyclic GMP in guinea pig hearts used to determine the dephosphorylation time course. Freeze-clamped tissue was sampled for determination of cyclic GMP. B indicates hearts that were not exposed to isoproterenol. Data represent means ± SEM for three hearts assayed in triplicate.**

fused into intact hearts. In the work reported here, the effect of acetylcholine on rates of protein dephosphorylation in isolated myocytes or intact hearts was examined. By using myocyte preparations that have a uniform specific activity of cellular [$^{32}$P]ATP, it was possible to obtain very accurate time courses for even the very rapid dephosphorylation of phospholamban. These time courses were used to calculate the rates of protein dephosphorylation as they occur in the intact cell. This dephosphorylation rate is the most direct measure of the phosphatase activation state as it relates to the substrates that are important in cardiac muscle contraction. It was found that acetylcholine does not change the rate of protein dephosphorylation for phospholamban, troponin-I, or C-protein, and it is concluded that cholinergic agonists do not activate phosphatases in the heart that act on these substrates.

In contrast to the data presented here showing that acetylcholine did not increase the rates of protein dephosphorylation, there is good evidence that type 1 phosphatase activity in the heart is inhibited following an increase in cyclic AMP and is increased by cholinergic agonists [16, 17]. Because type 1 phosphatase is important for phospholamban dephosphorylation [19], acetylcholine was expected to increase the rate of dephosphorylation for that protein. This did not occur in the myocytes or in the intact heart, suggesting that any effect of acetylcholine on type 1 phosphatase activity is limited to a pool of enzyme that does not contribute to phospholamban dephosphorylation. Data showing the dephosphorylation time courses, presented in Figs. 3 and 6, support the hypothesis that prolonged exposure to β-agonists causes an inhibition of type 1 phosphatase. In the isolated myocytes, the exposure to isoproterenol was limited to 2 min. With this brief exposure, the dephosphorylation of phospholamban was rapid and essentially complete in 2 min. This time course agrees very well with previous data obtained with intact hearts, which show a very rapid reversal of phospholamban phosphorylation when isoproterenol is withdrawn after 2 min [35]. As shown in Fig. 6, however, when the β-adrenergic stimulation in the heart was continued for 5 min, the dephosphorylation of phospholamban was slowed significantly and was incomplete even 6 min after isoproterenol was withdrawn. The decrease in the rate of phospholamban dephosphorylation can result from sustained activity of cyclic AMP-dependent kinase, from inhibition of phosphatase, or from a combination of both. From previous work in

this area, it is likely that inhibition of type 1 phosphatase secondary to phosphorylation of inhibitor-1 does contribute to the observed effect [6, 16, 17].

A mechanism by which cholinergic agonists would stimulate the type 1 phosphatase is not apparent, but could involve activation of calcineurin phosphatase activity and a subsequent dephosphorylation of inhibitor-1 resulting in reactivation of type 1 phosphatase [10]. However, irrespective of the phosphatase involved or mechanism of activation, an increase in phosphatase activity by a cholinergic agonist should result in an increase in the rate of dephosphorylation of the major substrates for cyclic AMP-dependent kinase. This clearly did not occur in either the isolated myocytes or the intact heart.

The proteins examined in this study had a 2-fold difference in sensitivity to isoproterenol, with troponin-I being the most sensitive and phospholamban the least sensitive. The difference in sensitivity of these proteins to cyclic AMP-dependent protein kinase was of the same magnitude with different agonists. Isoproterenol, isobutylmethylxanthine, and forskolin each were more potent in causing phosphorylation of troponin-I than phospholamban, with C-protein being intermediate (data not shown). Inhibition of protein phosphorylation by acetylcholine was observed as a 2- to 3-fold shift in the concentration–response curve to isoproterenol without a decrease in the maximum effect of the β-agonist. Similar inhibition of the positive inotropic effect of isoproterenol occurs in the intact heart, suggesting that the relevant mechanisms for cholinergic inhibition of β-adrenergic effects are maintained in the isolated myocyte preparations. The dephosphorylation rate for proteins after removal of β-adrenergic stimulation was not increased by acetylcholine in either the isolated myocytes or the intact heart. The differences between the present results and those presented earlier [34, 35] may be due to differences in experimental design. In the experiments examining troponin-I phosphorylation [34], isoproterenol stimulation was not blocked by a β-adrenergic antagonist at the time acetylcholine was administered, and the perfusion was done at constant pressure. The vasodilatation produced by acetylcholine could have accelerated the washout of isoproterenol and increased the apparent rate of dephosphorylation. However, this seems unlikely because of the very slow rate of dephosphorylation of troponin-I shown in Figs. 5 and 6 and reported previously [34]. In the experiments in which phospholamban dephosphorylation was examined [35], it is possible that hemodynamic effects resulting from acetylcholine accelerated the onset of dephosphorylation of phospholamban. In that study, dephosphorylation was too rapid to make any distinction between an effect of acetylcholine on the rate of dephosphorylation and on the onset of dephosphorylation. As shown in this study, acetylcholine did shorten the time to onset of dephosphorylation without acceleration of the dephosphorylation rate.

The experiments described in this paper were intended to give a direct examination of the question of cholinergic activation of phosphatases in the heart. Previous work has indicated that in the presence of acetylcholine, cardiac proteins are dephosphorylated at a faster rate [34, 35]. The present results do not support an activation of phosphatase by stimulation of cholinergic receptors, but they are consistent with an inhibition of phosphatase by prolonged β-adrenergic stimulation.

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