Phosphorylation of the 19,000-dalton Light Chain of Myosin in Perfused Rat Heart Under the Influence of Negative and Positive Inotropic Agents*

Stephen J. Kopp‡ and Michael Bárány
From the Department of Biological Chemistry, University of Illinois at the Medical Center, Chicago, Illinois 60612

The phosphorylation of myofibrillar proteins was studied in rat hearts perfused with $^{32}$Porthophosphate under the influence of negative (Mg$^{2+}$, Cd$^{2+}$, Pb$^{2+}$, low Ca$^{2+}$) and positive (isoproterenol, high Ca$^{2+}$) inotropic agents. The following physiological parameters were recorded: active tension, spontaneous heart rate, perfusion flow rate, and electrocardiogram. Of these parameters, only the active tension was found to be correlated with protein phosphorylation. The incorporation of $^{32}$Pphosphate into the 19,000-dalton light chain of myosin was correlated with both negative and positive inotropy.

The incorporation of $^{32}$Pphosphate into a 29,000-dalton protein with similar electrophoretic mobility to the inhibitory subunit of troponin was correlated with negative inotropy only. A specific Ca$^{2+}$-sensitive phosphorylation of the 48,000-dalton protein zone was detected. This myofibrillar protein comigrated with cardiac tropomyosin. The phosphorylation of the other myofibrillar proteins was virtually unaffected by the inotropic agents studied.

The results support the contention that changes in the phosphorylation of the 19,000-dalton light chain are associated with parallel changes in the contractile state of the myocardium. The present findings and those of previous investigators in conjunction with recent reports on phosphorylation of sarcallem and sarcoplasmic reticulum proteins (Walsh, D. A., Clippinger, M. S., Sivaramakrishnan, S., and McCullough, T. E. (1978) Biochemistry 18, 871-877; Lindemann, J. P., Jones, L. R., Besch, H. R., Jr., and Watanabe, A. M. (1978) Circulation 58(2), 21) induced by β-adrenergic stimulation in intact hearts suggest that the regulation of positive inotropy in the heart is through a multiple protein phosphorylation mechanism.

The increasing emergence of experimental results correlating altered protein phosphorylation induced by diverse stimuli (hormone, neural transmitter, and membrane depolariization) with specific biological responses has suggested a regulatory role for protein phosphorylation in the mediation of physiological responses (1, 2). In the mammalian myocardium physiological effectors which regulate the contractile state of the heart through extrinsic (e.g. hormonal) and intrinsic (e.g. myogenic) mechanisms may mediate or modulate this activity through a protein phosphorylation mechanism. The phosphorylation of myofibrillar proteins with purported regulatory actions in the control of cardiac contraction are the myofibrillar proteins TN-I (3-6), and myosin light chain (7), a sarcoplasmic membrane protein, phospholamban (8, 9), and sarcolemma membrane proteins (9-11).

Concerning the myofibrillar proteins, isoproterenol and epinephrine stimulation of perfused hearts by England initially demonstrated a relationship between TN-I phosphorylation and positive inotropism (3). This conclusion, however, had to be modified when later results demonstrated the lack of TN-I phosphorylation in a positive inotropy induced by glucagon (4). Similarly, Solaro et al. (5) and Ezrailson et al. (6) have shown a direct relationship between positive cardiac inotropic intervention induced by catecholamines and the phosphorylation of TN-I. Contrary to these findings, ouabain, increased frequency of stimulation, or an increased Ca$^{2+}$ concentration, all of which significantly augmented contractility, did not produce any increase in phosphorylation of TN-I (6).

In a further attempt to elucidate the role of phosphorylation of myofibrillar proteins in heart function, Freeman et al. (7) observed an inverse relationship between TN-I and myosin light chain phosphorylation upon adrenaline treatment, i.e., as TN-I phosphorylation increased, the light chain phosphorylation decreased. However, in a subsequent series of experiments no change in the phosphorylation of myosin light chain was found (12). In contrast, an increase in the $^{32}$P content of the light chain of papillary muscle stimulated by norepinephrine was noted (13).

We investigated the phosphorylation of several myofibrillar proteins in rat hearts perfused with $^{32}$Porthophosphate and exposed to various positive and negative inotropic agents. The phosphorylation of the 19,000-dalton myosin light chain correlated with all the changes in the contractile state of the heart.

EXPERIMENTAL PROCEDURES

Heart Perfusion—The heart perfusions were performed essentially as described previously (14). Briefly, heparinized Sprague-Dawley rats (200 to 300 g) of random sex were sacrificed by cervical dislocation and the hearts ($N = 45$) were removed and chilled in precooled perfusion solution (10°C), stopping the heart. While in this medium, the aorta was cannulated and tied for retrograde perfusion, and a single open loop was saturated through the heart, 5 mm from the apex, for recording systolic tension. The cannulated heart was then perfused for a 30-min equilibrium period to assure stability of each heart preparation. The standard control solution, as reported previously, was a modified Hartmann's solution containing 150 mm NaCl, 5.4 mm KCl, 1.05 mm MgCl$_2$, 1.9 mm CaCl$_2$, 5.6 mm glucose, 1734

\* This work was supported by Grants NS 12173 from the United States National Institutes of Health and the Muscular Dystrophy Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of National Institutes of Health National Research Service Award 1-F32 ES 05112 Tox.

‡ The abbreviations used are: TN-I, the inhibitory subunit of troponin; TN-T, the tropomyosin-binding subunit of troponin.
and 10 mM Tris-HCl buffer. The pH of the perfusate was 7.3 at 35°C throughout each experiment. The hearts were perfused at constant temperature (35°C) under constant hydrostatic pressure (82 to 95 mm Hg) maintained by regulating the oxygen gas pressure to the perfusion apparatus so as to maintain the internal reference standard for the determination of changes induced by the experimental perfusates within each heart preparation. Only stable preparations characterized by a rhythmic spontaneous heart rate and stable mechanical activity were studied.

Following the 30-min equilibration period, the hearts were equilibrated in the experimental perfusate: control, (N = 9); 20 mM MgCl₂, (N = 3); 6 x 10⁻⁴ M Pb₃(ClO₄)₂, (N = 3); 6 x 10⁻⁴ M CdCl₂, (N = 3); 0.9 mM CaCl₂, (N = 3); 1.25 mM CaCl₂, (N = 3); 2.8 mM CaCl₂, (N = 3); 6.5 mM CaCl₂, (N = 4); 7 x 10⁻⁴ M isotroporenate hydrochloride, (N = 4); or 7 x 10⁻⁴ M isotroporenate hydrochloride, (N = 4) at the identical temperature, pressure, and pH. This time interval was sufficient to determine basal heart rate and is shown in the figure. The recovery of the measured physiological parameters. At the conclusion of this perfusion period the radioactive experimental perfusate containing 1 mcCi of carrier-free ³²P per liter at the identical temperature, pressure, and pH was turned on and the nonradioactive experimental perfusate turned off. This radioactive perfusion continued for 40 min and was followed by an 18-min washout perfusion with the nonradioactive experimental perfusate to remove radioactive substances from the extracellular space. This 18-min time period was within the plateau phase of the ³²P-extracellular washout curve determined for these experimental conditions. Less than 0.4% of the total heart radioactivity was attributable to extracellular space radioactivity. Since the perfusion apparatus was a closed system, the flow rate during the radioactive perfusion was calculated as the difference between the initial perfusate volume minus the remaining volume plus the dead space in the system. The per cent of ³²P-uptake from the perfusate was determined as the ratio of total heart radioactivity to the total radioactivity exposed to the heart. Upon completion of the wash perfusion period the hearts were removed from the cannula and immediately immersed in iced buffer solution for myofibril extraction. The hearts were observed to stop in diastole after a maximum of a single contraction. This methodology yielded consistent reproducible results concerning the phosphorylation of myofibrillar proteins during myocardial diastole. Since the phase of the cardiac contraction cycle could not be correlated with a fast-clamping procedure, the hearts were not frozen in these studies.

**Temporal Control Perfusion**—Additional control perfusions using the identical conditions described were analyzed to assess the time dependence of ³²P-perfusion on ³²P-phosphocreatine incorporation and the 19,000-dalton light chain. These studies were undertaken to establish the time domain during which the incorporation of ³²P into phosphocreatine and the proteins is linear (cf. Fig. 2). Radioactive control perfusions lasting 10 min (N = 2), 20 min (N = 2), and 60 min (N = 2) were compared with the 40-min standard.

**Cardiac Myofibril Preparation**—Immediately after immersion of each heart in a solution containing 1.0% Triton X-100, 0.02% Tris-HCl, 0.05% deoxycholate, ATP, and iodoacetate, the myofibril pellet was washed twice with a solution containing 0.1% Triton X-100, 0.05% deoxycholate (sodium form), 0.1 mM NaF, 10 mM iodoacetamide, 5 mM EDTA, 0.02% Tris-HCl, and 0.01 M KCl, pH 7.2, and centrifuged at 600 x g for 30 min. To remove the Triton and deoxycholate the myofibrils were washed twice with the same solution minus the Triton X-100 and deoxycholate and centrifuged as before. The final myofibril pellet was then solubilized in sodium dodecyl sulfate (final concentration of 6%) in 0.05 mM sodium phosphate, pH 7.0, by high speed homogenization with a PCU-2 Brinkmann Polytron. The dissolved myofibril proteins were dialyzed against 0.1% sodium dodecyl sulfate in 0.1 M sodium phosphate, pH 7.0, overnight. The small amount of insoluble connective tissue was removed by centrifugation at 100,000 x g in a Spinco ultracentrifuge for 40 min and resuspended using protein precipitation by the biuret method (17). Appropriate dilutions were made to achieve a final concentration of 6 mg of protein/ml.

Fig. 1 illustrates the protein staining profile of our purified myofibrillar preparation on 10% polyacrylamide gels containing 1% sodium dodecyl sulfate and 8 M urea in 0.1 M sodium phosphate buffer, pH 7.0. Purified myosin, troponin, and tropomyosin were electrophoresed simultaneously with the rat heart myofibrils to identify the comigratory myofibrillar protein bands. The fastest moving band in the myofibrils (F) which has an apparent molecular weight of 19,000 comigrates with the myosin light chain in purified bovine heart myosin (Ger 2). Similarly, the 27,000-dalton protein zone in myofibrils comigrates with the 19,000-dalton light chain of phosphocreatine and the 19,000-dalton light chain. Bands C and D with apparent molecular weights of 30,000 and 29,000, respectively, have mobilities similar but not equivalent to purified bovine heart TN-1 (Ger 3), molecular weight 29,500. Leger et al. (18) have described cardiac TN-1 migration as two bands with molecular weights of 28,000 and 30,000. Myofibril band B, apparent molecular weight 38,000 comigrates with TN-T (Ger 5). Myofibril band A had the same TN-T migration as rat tropomyosin, molecular weight 48,000 (Ger 4). Cummins and Perry (19) were the first to describe that tropomyosin migrates on sodium dodecyl sulfate gels containing urea with a molecular weight around 50,000, thereby enabling the separation of tropomyosin from TN-T.

As described previously (20) approximately 100 μm (range 90 to 110 μm) of boiled dithiothreitol-reduced myofibrillar proteins were applied to each disc gel (5 x 131 mm), and electrophoresis was carried out at a current of 3 mA/tube for 21 h. The gels were stained, destained, and the entire lengths of six gels containing the myofibrillar sample were sliced into equal sections (approximately 3- to 4-mm widths), digested, and counted (30). As an example, approximately 10,000 counts per 20 min above background were detected in the 19,000-dalton light chain zone from control untreated heart myofibrils. The recovery of the counts applied to the gels varied between 70 to 80%, due primarily to losses on the cutting surfaces.

The radioactivities reported correspond to covalently bound (³²P)phosphate incorporation. No change in the counts per min per mg of perchloric acid supernatant and pellet was detectable under our conditions with the proteins with trichloroacetic acid, and washing with this acid, or after exhaustive dialysis against 3 x 100 volumes of 0.1 M phosphate, pH 7.0, solution. Furthermore, we have shown that under our conditions of gel electrophoresis any noncovalently bound phosphate is eluted from the gel.

**The Assay of Specific Activity of (³²P)Phosphocreatine**—The permeation of phosphate through excitable cell membranes is known to be extremely slow (21, 22). We have confirmed this observation in our (³²P)-experiments as well. From the specific activity data provided by Amersham/Seear for phosphorus-32, 30 to 100 Ci/mg, we have calculated that the 1 mCi of phosphorus-32 (used in our perfusion) contains 0.33 ng of phosphorus in a 1.6 μg/mCi sample weight (10 μg/mg). The calculated phosphorus uptake (7.25%) for perfused hearts corresponds (under these experimental conditions) to an uptake of 0.2 nmol of phosphate per 40 min.

Caldwell and Walster (23) showed that carrier-free (³²P) in the intracellular water of crab muscle fibers is rapidly equilibrated among (γ) orthophosphate, ATP, and arginine phosphate. We have found the existence of similar equilibration in rat heart muscle.

(³²P)orthophosphate = [γ-³²P]ATP = [(³²P)phosphocreatine (1)]
precipitate, it was chromatographed on a Dowex 1-X8 column (25). The ATP fraction was hydrolyzed with 10 times precipitated myosin, then treated with charcoal to isolate the γ-phosphate group of ATP in the supernatant. The three phosphate fractions, 1) inorganic phosphate, 2) phosphocreatine, 3) the γ-phosphate of ATP, were analyzed for phosphate content and radioactivity. No difference was found in specific radioactivities.

Since the γ-32P phosphate group of ATP is in equilibrium with either the [32P]-phosphate group of phosphocreatine or [32P]-orthophosphate, one can substitute the time-consuming determination of the specific radioactivity of the γ-32P phosphate from ATP with that of [32P]-phosphocreatine. (31P nmr reveals that the orthophosphate concentration in fresh rat heart is low.) The following procedure was adopted for the assay of specific activity of [32P]-phosphocreatine.

The [32P]-phosphocreatinine present in the perchloric acid-extracted supernatant was hydrolyzed by a 35-min incubation at 40°C in the presence of 1.0 N HClO, and 0.04 M NaMoO4. The phosphomolybdate complex was extracted in isopropyl acetate by vigorous mixing for 60 s. The isopropyl acetate phase was removed and the phosphate concentration determined at 310 nm; the extinction coefficient for P1, was determined to be 23.8 mM−1 cm−1 (26). An aliquot from the cuvette was taken for counting, and the specific activity of [32P]-phosphate in phosphocreatine was calculated. This specific activity was applied as the standard reference within each heart for the determination of incorporation (mols of [32P]-phosphate/mol of protein) into the 19,000-dalton light chain.

Calculations and Statistical Methods—Since the active tension of the control hearts was stable throughout the duration of the heart perfusions, the mean tension of the control hearts was used as the reference for normalization of the hearts exposed to the experimental agents. The percentage change in contractility induced by the experimental perfusate was calculated from the 30-min equilibrium control value for each heart. The mean change for each group of hearts was transposed into grams of active tension using the control as the standard reference. These calculations were necessary to normalize the variability from heart to heart in the absolute tension output, measured after the initial 30-min equilibration period. By normalizing the active tension induced by each experimental agent to values relative to control, meaningful correlations with changes in myofibril protein phosphorylation were facilitated.

The heart rate was calculated from the R-R interval of the electrocardiogram. The flow rate during the 40-min 32P-perfusion was measured as milliliters per min perfused through the heart. To normalize the variability caused by the differences in spontaneous heart rates between different heart preparations, the flow rate is expressed as milliliters per min perfused through the heart. To normalize the variability from heart to heart in the absolute tension output,
Phosphorylation of Myosin Light Chain in Perfused Heart

Table I
Light chain phosphorylation and physiological parameters during 32P perfusion of rat hearts under the influence of negative and positive inotropic agents

<table>
<thead>
<tr>
<th>Inotropic agent</th>
<th>N</th>
<th>32P phosphate/light chain</th>
<th>Active tension</th>
<th>Spontaneous heart rate</th>
<th>Perfusion flow rate</th>
<th>PR interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>0.116 ± 0.006</td>
<td>12.4 ± 0.08</td>
<td>163 ± 7</td>
<td>50 ± 5</td>
<td>51 ± 1</td>
</tr>
<tr>
<td>Negative inotropic agents</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mM MgCl₂</td>
<td>3</td>
<td>0.036 ± 0.008</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6 × 10⁻⁴ mM Pb₃(C₁₂H₁₇O₇)₂</td>
<td>3</td>
<td>0.042 ± 0.002</td>
<td>0.6 ± 0.2°</td>
<td>147 ± 5</td>
<td>81 ± 2°</td>
<td>156 ± 32°</td>
</tr>
<tr>
<td>6 × 10⁻³ mM CdCl₂</td>
<td>3</td>
<td>0.087 ± 0.007</td>
<td>4.4 ± 0.5°</td>
<td>120 ± 10</td>
<td>48 ± 7</td>
<td>97 ± 4°</td>
</tr>
<tr>
<td>9.0 × 10⁻³ mM CaCl₂</td>
<td>3</td>
<td>0.095 ± 0.003</td>
<td>9.0 ± 0.4°</td>
<td>150 ± 12</td>
<td>46 ± 1</td>
<td>68 ± 3°</td>
</tr>
<tr>
<td>1.25 mM CaCl₂</td>
<td>3</td>
<td>0.119 ± 0.002</td>
<td>9.4 ± 0.5°</td>
<td>162 ± 8</td>
<td>57 ± 3</td>
<td>70 ± 1°</td>
</tr>
<tr>
<td>Positive inotropic agents</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.8 mM CaCl₂</td>
<td>3</td>
<td>0.161 ± 0.006</td>
<td>15.7 ± 0.6°</td>
<td>179 ± 13</td>
<td>55 ± 8</td>
<td>55 ± 1</td>
</tr>
<tr>
<td>6.5 mM CaCl₂</td>
<td>4</td>
<td>0.177 ± 0.021</td>
<td>21.4 ± 0.2°</td>
<td>186 ± 15</td>
<td>48 ± 7</td>
<td>51 ± 2</td>
</tr>
<tr>
<td>7 × 10⁻⁴ mM Isoproterenol</td>
<td>4</td>
<td>0.167 ± 0.014</td>
<td>18.8 ± 0.7°</td>
<td>315 ± 26</td>
<td>46 ± 4</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>7 × 10⁻³ mM Isoproterenol</td>
<td>4</td>
<td>0.172 ± 0.028</td>
<td>19.1 ± 1.8°</td>
<td>336 ± 15</td>
<td>50 ± 3</td>
<td>48 ± 1</td>
</tr>
</tbody>
</table>

*p < 0.01.  
** p < 0.001.  
*** p < 0.001.  
**** p < 0.02.  
PR interval refers to electrocardiogram interval representative of atrioventricular nodal conduction time.

Fig. 2. The incorporation of 32P phosphate into phosphocreatine (P-creatine) and the 19,000-dalton light chain of myosin (LC-2) of rat heart as a function of time.

positive inotropic agents

Electrophoretic analysis of the light chain of myosin from control and negative inotropic agents showed a single band at 19,000 daltons. This band was used as an internal standard for quantitation of phosphorylation. There was no change in the relative phosphorylation of the light chain in hearts perfused with MgCl₂ (p < 0.01) andCdCl₂ (p < 0.001) compared to control (p < 0.05). The effect of these agents was dependent on the concentration of the agent used. For example, MgCl₂ at 10⁻⁴ M caused a 30% decrease in phosphorylation, while at 10⁻³ M it caused a 50% decrease.

Inotropic agents

The incorporation of P-creatine and the 19,000-dalton light chain of myosin as a function of time.

Effect of Negative Inotropic Agents on Protein Phosphorylation—Changes in myofibrillar protein phosphorylation induced by 20 mM MgCl₂, 6 × 10⁻⁴ mM Pb₃(C₁₂H₁₇O₇)₂, 6 × 10⁻³ mM CdCl₂, 0.9 mM CaCl₂, and 1.25 mM CaCl₂ were assessed. As compared to the control phosphorylation, 0.116 mol of 32P phosphate per mol of light chain, significantly depressed phosphorylation of the 19,000-dalton light chain was observed in hearts exposed to MgCl₂, Pb₃(C₁₂H₁₇O₇)₂, and CdCl₂ (Table I). Although not statistically significant at p < 0.02, depressed phosphorylation of the light chain was observed in hearts exposed to 0.9 mM CaCl₂. With the exception that exposure to 0.9 mM CaCl₂ contributed to erroneous phosphorylation results. Thus, in the experimental perfusions altered cardiac contractility was directly and solely attributable to the influence of the inotropic agents.

Effect of Positive Inotropic Agents on Protein Phosphorylation—Various concentrations of CaCl₂ (2.8 mM and 6.5 mM) and isoproterenol (7 × 10⁻⁴ mM and 7 × 10⁻³ mM) were studied to elucidate the influence of increased cardiac contractility on myofibrillar protein phosphorylation. Significant progressive increases in phosphorylation of the 19,000-dalton light chain were observed which correlated directly with the magnitude...
of positive inotropy. A complete correlation of contractile state measured as active tension with light chain $[^{32}P]$-phosphate incorporation is shown in Fig. 3. The least squares linear regression equation describing this relationship was determined to be $y = 0.0182X - 0.167$ with a regression coefficient of 0.96. These results suggest that the magnitude of the light chain phosphorylation during diastole may be a critical factor which predetermines or establishes the intrinsic contractile state of cardiac muscle.

These positive inotropic agents also stimulated a significant increase in the phosphorylation of the 29,000-dalton protein which was correlated positively to the induced augmentation for this relationship was calculated to be $y = 0.0182X - 0.167$. In addition to the elevated light chain, 29,000-dalton protein phosphorylation stimulated by 2.8 and 6.5 mM CaCl$_2$ phosphorylation of the 48,000-dalton protein which comigrates with tropomyosin was very significantly increased ($p < 0.001$), whereas 6.5 mM CaCl$_2$ alone increased the incorporation into the 42,000-dalton and the 38,000-dalton proteins ($p < 0.02$).

The mean heart rates and perfusion flow rates during $^{32}P$-perfusion are shown in Table I. The increased heart rate observed with isoproterenol is characteristic of adrenergic agonist stimulation of cardiac $\beta$-receptors. The heart rates of Ca$^{2+}$-stimulated hearts demonstrated a moderate increase in heart rates. The PR interval of the electrocardiogram showed slight decreases in response to isoproterenol which reflects enhanced conduction cell conductivity. The percentage uptake of $^{32}P$ from the perfusate (control mean, 7.2 ± 0.4) was decreased only in isoproterenol-treated hearts, as a result of the isoproterenol-induced positive chronotropy and the corresponding decrease in diastolic interval. However, as a result of the accelerated heart rate, a greater total volume of perfusate passed through these hearts during the 40-min period; thus the absolute amount of $^{32}P$ which entered the heart tissue was comparable to that of the control hearts.

**DISCUSSION**

Evidence was presented recently that the radioactivity observed in the light chain zone of myofibrils is associated with the light chain itself (20). The results reported herein demonstrate that changes in phosphorylation of the 19,000-dalton light chain are associated with parallel changes in the contractile state of the myocardium. The incorporation of $[^{32}P]$-phosphate into myofibrillar proteins induced by various inotropic agents demonstrated that only the phosphorylation of light chain exhibited consistent changes which correspond with both negative and positive inotropy in the heart. For instance, the linear regression coefficient of 0.96 (Fig. 3) indicates a significant direct correlation between increased $[^{32}P]$-phosphate incorporation into light chain and increased force of myocardial contraction. Since these hearts were stopped in diastole by rapid immersion in iced buffer, the magnitude of light chain phosphorylation likely reflects the phosphorylated state during diastole. Thus, a plausible interpretation would infer that phosphorylation of light chain may predetermine the force of active tension generated in the ensuing contraction. Recent studies showed that arterial smooth muscle contraction is associated with light chain phosphorylation (33). Furthermore, studies on the physiological role of light chain phosphorylation in skeletal muscle suggest that light chain phosphorylation enhances the interaction between cross-bridges and actin-containing filaments (34). The observed phosphorylation of light chain in heart could serve the same purpose.

Our data detecting an increased and decreased light chain phosphorylation in response to positive and negative inotropic interventions, respectively, leave open the possibility for a light chain phosphorylation-dephosphorylation during the heart cycle. Such a study would require the automatic freezing of the heart in systole and diastole, a technical problem which has to be solved. Until such an experiment is performed the observed differences in phosphorylation may reflect changes in the turnover of the light chain phosphate influenced by various agents through a direct or indirect control on the enzymes involved in phosphorylation-dephosphorylation of the light chain.

The observed effects of isoproterenol-induced positive inotropy on the phosphorylation of the 29,000-dalton protein (electrophoretic mobility similar to TN-1) are consistent with the effects described by previous investigators for TN-1 (3–6). Although the effects of positive inotropic agents on the phosphorylation of the 29,000-dalton protein show a positive correlation, these results support only a molecular model in which increased phosphorylation of this protein is associated with an increased contractile state greater than control. An association is not evident between altered phosphorylation of this protein and depressed myocardial contractility.

Phosphorylation of cardiac tropomyosin was reported recently (35). An apparent finding of this work is the specific Ca$^{2+}$-controlled phosphorylation of the 48,000-dalton protein in rat heart which comigrates electrophoretically with tropomyosin. Thus, altering the perfusate calcium concentration consistently induced parallel changes in the phosphorylation of this protein. This effect was not noted for the other negative and positive inotropic agents studied, with the exception that 20 mM MgCl$_2$ caused marginally significant reduction in the phosphorylation of this 48,000-dalton protein.

The modified physiological and phosphorylation activities induced by most of the agents studied are consistent with the hypothesis that the intracellular concentration of Ca$^{2+}$ is a significant mediator which contributes to the regulation of the cardiac contractile state. A plausible explanation for the mechanism through which the negative inotropic agents influence cardiac contractility would be to suggest that the transport of Ca$^{2+}$ from the extracellular space is depressed thereby reducing intracellular Ca$^{2+}$ levels, inhibiting the activity of the Ca$^{2+}$-
and calmodulin-dependent light chain kinase (36) and depressing light chain phosphorylation. Evidence reported by other investigators demonstrating altered Ca$^{2+}$ metabolism induced by elevated Mg$^{2+}$ (30–32) or the presence of Cd$^{2+}$ or Pb$^{2+}$ (14, 37–39) support this premise. Our results demonstrate that changes in the extracellular Ca$^{2+}$ concentrations do induce qualitatively similar changes in cardiac function and light chain, 48,000-dalton and 29,000-dalton protein phosphorylations.

Our results, as well as those of other investigators, detecting increased light chain and 29,000-dalton protein phosphorylation in positive inotropism induced by β-adrenergic stimulation may be extended by recent observations of Lindemann et al. (9) and Walsi et al. (11) which show increased [$^{32}$P]phosphate incorporations into the proteins of sarcolemma and sarcoplasmic reticulum of perfused guinea pig heart stimulated by isoproterenol and perfused rat heart stimulated by epinephrine, respectively. These findings clearly demonstrate that the regulation of positive inotropy involves the phosphorylation of several proteins either simultaneously or sequentially. The elucidation of the factors which coordinate such multiprotein phosphorylation effects is a challenging problem for future research.

Acknowledgments—We wish to thank Judith A. Fulton and Michael G. Lindholm for their expert assistance. We are indebted to Dr. Thomas Glonek for the 31P nmr analyses. We are grateful to Dr. Michael G. Lindholm for their expert assistance. We are indebted to Dr. Perry, S. V., Cole, H. A., Frearson, N., Molr, A. J. G., Nairn, A. J. G., and Perry, S. V. (1976) Biochem. J. 160, 295–304 and Frearson, N., Solaro, R. J., and Perry, S. V. (1976) Nature 262, 615–617 for future research.

REFERENCES
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