Phosphorylation of C-protein, troponin I and phospholamban in isolated rabbit hearts

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Phosphorylation of myofibrillar and sarcoplasmic-reticulum (SR) proteins was studied in Langendorff-perfused rabbit hearts subjected to various inotropic interventions. Stimulation of hearts with isoprenaline resulted in the phosphorylation of both troponin I (TnI) and C-protein in myofibrils and phospholamban in SR. Phosphorylation of phospholamban could be reversed by a 15 min perfusion with drug-free buffer, after a 1 minute pulse perfusion with isoprenaline, at which time the mechanical effects of isoprenaline stimulation had also been reversed. However, both TnI and C-protein remained phosphorylated at this time. Moreover, the inhibition of Ca^{2+} activation of the Mg^{2+} -dependent ATPase (Mg-ATPase) activity associated with myofibrillar phosphorylation persisted in myofibrils prepared from hearts frozen after 15 min of washout of isoprenaline. To assess the contribution of C-protein phosphorylation in the decrease of Ca^{2+} activation of the myofibrillar Mg-ATPase activity, we reconstituted a regulated actomyosin system in which only C-protein was phosphorylated. In this system, C-protein phosphorylation did not contribute to the decrease in Ca^{2+} activation of Mg-ATPase activity, indicating that TnI phosphorylation is responsible for the diminished sensitivity of the myofibrils to Ca^{2+} . These observations support the hypothesis that phospholamban phosphorylation plays a more dominant role than TnI or C-protein phosphorylation in the mechanical response of the mammalian heart to β -adrenergic stimulation.

INTRODUCTION

There is ample evidence that proteins of the sarcoplasmic reticulum (SR) and of the myofilaments undergo phosphorylation during the response of the heart to adrenergic stimulation (Katz, 1979; Kranias *et al.*, 1985). In the case of the SR, the site of phosphorylation is on phospholamban, an oligomeric proteolipid which, when phosphorylated, stimulates the rate of SR Ca^{2+} transport (Tada & Inui, 1983). This effect is due to an increase in the affinity of the SR Ca^{2+} pump for Ca^{2+} (Kranias *et al.*, 1980; Tada *et al.*, 1980). In the case of the myofilaments, the sites of phosphorylation are on TnI (Solaro *et al.*, 1976), the inhibitory protein of the thin-filament troponin complex, and C-protein (Jeacocke & England, 1980), a constituent of the thick filament (Starr & Offer, 1971), located in the area of thick- and thin-filament overlap (Craig & Offer, 1976). Phosphorylation of cardiac myofilaments by cyclic AMP-dependent protein kinase results in a rightward shift of the myofilament force-pCa and Mg-ATPase-pCa relations (Ray & England, 1976; Holroyde *et al.*, 1979a; Mope *et al.*, 1980). This effect is associated with a decrease in the affinity of the Ca^{2+} -regulatory site on TnC for Ca^{2+} when TnI is phosphorylated (Holroyde *et al.*, 1979a; Robertson *et al.*, 1982). However, the role of C-protein in the regulation of myofilament structure-function relations in the desensitization of the myofilaments to Ca^{2+} is uncertain.

Evidence from studies *in vivo* and *in vitro* can be interpreted in a straightforward way to indicate that

phosphorylation of phospholamban and its effect on SR Ca^{2+} transport are related to mechanical responses, such as enhanced rate of relaxation, associated with adrenergic stimulation of the heart. Yet the relative contribution of myofilament phosphorylation to these mechanical responses is more difficult to assess. In the case of TnI phosphorylation, a functional role has been proposed on the basis of the altered myofilament sensitivity to Ca^{2+} . In some cases, though, the levels of phosphorylation of TnI have not been correlated with changes in inotropic state. For example, TnI remains phosphorylated during the decrease in force that follows termination of perfusion with isoprenaline (England, 1976; Stull *et al.*, 1981). In contrast, phosphorylation of C-protein has been well correlated with relaxation parameters measured in frog hearts responding to adrenergic and cholinergic agents (Hartzell, 1984). However, these studies of C-protein phosphorylation were performed in amphibian hearts, which contain little SR, and TnI phosphorylation was not simultaneously measured.

In order to understand better the role of C-protein phosphorylation in the response of the mammalian heart to adrenergic stimulation, we measured the dynamics of phosphorylation of C-protein in relation to phosphorylation of TnI and phospholamban and determined whether the effects of myofilament phosphorylation on the Ca^{2+} -sensitivity of the myofibrillar ATPase are due to TnI or C-protein phosphorylation. Our findings suggest that phosphorylation of phospholamban may play a more dominant role in the mechanical response of the mammalian heart to catecholamines than phosphoryl-

Abbreviations used: SR, sarcoplasmic reticulum; TnI, troponin I; TnC, troponin C; DMF, desensitized myofibrils; TnTm, troponin-tropomyosin; pCa, $-\log [\text{Ca}^{2+}]$; Mg-ATPase, Mg^{2+} -dependent ATPase.

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ation of TnI or C-protein. Furthermore, studies *in vitro* reported here suggest that C-protein phosphorylation does not contribute to the decreased sensitivity of the Mg-ATPase activity to Ca^{2+} observed upon myofibrillar phosphorylation.

MATERIALS AND METHODS

Perfusions

Hearts from male New Zealand White rabbits (1.8–3.0 kg) were perfused in a Langendorff-type apparatus using a modified Krebs–Henseleit buffer [containing (mM): NaCl, 118 or 108; KCl, 4.7; MgSO_4 , 1.2; KH_2PO_4 , 0.1; Na-EDTA, 0.5; glucose, 5.5; CaCl_2 , 2.5 or 9.0; NaHCO_3 , 25]. The perfusate was continuously aerated with O_2/CO_2 (19:1). Heart rate and left-ventricular pressure were monitored via a catheter introduced through the apex to the lumen of the left ventricle.

Intracellular phosphate pools were labelled by including [^{32}P]P_i (20 $\mu\text{Ci}/\text{ml}$; New England Nuclear, Boston, MA, U.S.A.) in the recirculating perfusion medium. All hearts were perfused for 60 min with [^{32}P]P_i-labelled Krebs–Henseleit buffer and for an additional total of 16 min with radiolabel-free buffer. Hearts were frozen at that time ('Control') or after perfusion with isoprenaline for 1 min ('Isoprenaline'), forskolin for 1 min ('Forskolin') or after 1 min pulse perfusion with isoprenaline followed by 15 min of drug-free perfusion buffer. In all cases the total time of perfusion in radiolabel-free buffer was 16 min. There was no significant change in the specific radioactivity of [γ - ^{32}P]ATP among hearts perfused on a particular day with the same stock solution of [^{32}P]P_i (the ratio of the low and high values in a typical group was about 0.8). The specific radioactivities (means $\pm$ S.E.M.) determined in c.p.m./pmol of ATP were 9.2 ± 2.1 ($n = 6$) for controls, 9.2 ± 1.7 ($n = 12$) for hearts frozen at the peak of the inotropic response to isoprenaline and 8.4 ± 1.0 ($n = 7$) for hearts frozen after washout of isoprenaline.

To terminate the perfusion experiments, the hearts were quickly frozen with clamps cooled to the temperature of liquid nitrogen. The frozen hearts were powdered in a stainless-steel percussion mortar and stored at -70°C .

Preparations

Myofibrils and microsomal fractions enriched in SR vesicles were prepared from individual frozen rabbit hearts as described previously (Kranias & Solaro, 1982). Myofibrils were prepared from the first pellet generated in the fractionation of microsomal vesicles. These methods have been shown to 'freeze' chemically phosphate-group-transfer reactions and prevent protein dephosphorylation during isolation procedures (Holroyde *et al.*, 1979b; Kranias *et al.*, 1985). Sarcolemmal contamination of these SR-enriched microsomal preparations has been shown to be limited to less than 10% (Kranias & Solaro, 1982).

Pig cardiac myofibrils were prepared by using a slight modification of the procedure described previously for dog hearts by Solaro *et al.* (1971). Myofibrils free of troponin and tropomyosin ('desensitized myofibrils') were prepared as described previously (Blanchard & Solaro, 1984) by the method of Lehman (1978). Myofibrils were extensively washed and dialysed in

buffer containing 1 mM-dithiothreitol, 0.1 mM- NaN_3 and 5 mM-Tris, pH 8.0. This resulted in the selective loss of troponin and tropomyosin and the loss of Ca^{2+} -sensitivity of the actomyosin Mg-ATPase. Troponin–tropomyosin (TnTm) complexes were purified from a powder containing diethyl ether-extracted myofibrils prepared from rabbit skeletal (erector spinae group) or pig cardiac muscle by the method of Stull & Buss (1977).

Reconstituted 'myofibrils' were prepared by incubating pig cardiac 'desensitized myofibrils' with either rabbit skeletal TnTm or pig cardiac TnTm. Reconstitution was accomplished by incubating the 'desensitized myofibrils' with TnTm complex (2:1, w/w) for 15 min at 21°C in a solution consisting of 250 mM-KCl, 2 mM- MgCl_2 , and 30 mM-imidazole, pH 7.0. The protein mixture was diluted to 60 mM-KCl, and reconstituted 'myofibrils' were collected and washed twice by resuspension and centrifugation (5000 g, 10 min).

Reconstituted 'myofibrils' were phosphorylated by using bovine heart cyclic AMP-dependent protein kinase (Sigma Chemical Co., St. Louis, MO, U.S.A.). Phosphorylation was carried out for 30 min at 21°C in a reaction mixture containing reconstituted 'myofibrils' (3 mg/ml), cyclic AMP-dependent protein kinase (0.1 mg/ml), 10 μM -cyclic AMP, 2 mM-ATP, 3 mM- MgCl_2 , 1 mM-EGTA, 5 mM-theophylline, 20 mM-NaF and 30 mM-imidazole, pH 7.0. In parallel experiments [γ - ^{32}P]ATP (50 Ci/mol) was included in the incubation mixture to permit phosphorylated proteins to be revealed, after electrophoresis, by autoradiography as described below. Both cyclic AMP and the cyclic AMP-dependent protein kinase were absent in incubation mixtures for non-phosphorylated control preparations. Phosphorylated reconstituted 'myofibrils' were washed and resuspended in 60 mM-KCl/2 mM- MgCl_2 /10 mM-NaF/30 mM-imidazole, pH 7.0, and used immediately for determination of myofibrillar Ca^{2+} -sensitive Mg-ATPase.

Assays

The specific radioactivity of [γ - ^{32}P]ATP in hearts perfused with [^{32}P]P_i was determined from the specific radioactivity of [^{32}P]phosphocreatine extracted and assayed as described by Kopp & Bárány (1979).

Cyclic AMP content in samples of myocardium from perfused hearts was determined by using a radioimmunoassay kit (du Pont Specialty Diagnostics, North Billerica, MA 01862, U.S.A.). Samples of frozen powdered myocardium from perfused rabbit hearts were homogenized in 6% (w/v) trichloroacetic acid, extracted with diethyl ether, acetylated and assayed in duplicate.

Myofibrillar Ca^{2+} -sensitive Mg-ATPase activity was measured in a reaction mixture containing 60 mM-KCl, 4 mM- MgCl_2 , 1 mM-EGTA, 10 mM-NaF and 30 mM-imidazole, pH 7.0. Ionized- Ca^{2+} concentrations in the reaction were varied between 10^{-8}M and 10^{-4}M by additions of CaCl_2 ; conditions were calculated by the method of Fabiato & Fabiato (1979), using a K_a value of $2.51 \times 10^6\text{M}^{-1}$ for the reaction of Ca^{2+} with EGTA at pH 7.0. Reactions were initiated with 2 mM-ATP, allowed to proceed for 10 min at 30°C and terminated with ice-cold 10% (w/v) trichloroacetic acid. The P_i concentration in the supernatant fraction was determined by the method of Carter & Karl (1982). The extent of myofibrillar protein phosphorylation was maintained throughout the incubation, as judged by autoradiography.

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Microsomal samples were prepared for protein determination as described by Nakamura *et al.* (1983).

Electrophoresis

Polyacrylamide-gel electrophoresis of myofibrillar and microsomal proteins was performed as described by Laemmli (1970), using 7 and 15 %-(w/v)-polyacrylamide gels for myofibrils and 15 % and 5–30 % gradient gels for SR. ^{32}P -labelled microsomes (microsomal fractions) or myofibrils were dialysed in buffer containing 1 % (w/v) SDS, 2 % (v/v) β -mercaptoethanol and 10 mM-Tris/glycine, pH 7.5. SR samples were not boiled before electrophoresis. Gels were stained with Coomassie Blue and destained with methanol/acetic acid/water (1:1:8,

by vol.). M_r markers were phosphorylase *b* (94 000), human serum albumin (66 000), ovalbumin (43 000), DNAase I (31 000), soya-bean trypsin inhibitor (21 500), and egg-white lysozyme (14 300). Autoradiography using X-ray-sensitive film (Kodak X-Omat XRP-1) and intensifying cassettes permitted the detection of ^{32}P -labelled proteins. Autoradiograms were analysed by densitometric scanning (Biomed Instruments, Chicago, IL, U.S.A.). Levels of ^{32}P were in a range that was proportional to the densitometric reading.

RESULTS

We have previously shown that β -adrenergic stimulation of perfused rabbit hearts was associated with phosphorylation of phospholamban in SR and TnI and C-protein in myofibrils (Kranias & Solaro, 1982; Kranias

Table 1. ^{32}P incorporation into phospholamban, TnI and C-protein

Data are expressed relative to the control values (= 1.00) and are presented as means $\pm$ S.E.M. for *n* hearts in each group. * Indicates $P < 0.05$ and ‡ indicates $P > 0.1$ for washout compared with isoprenaline. Abbreviations used: PLB, phospholamban; n.d., not determined.

Condition	Protein ...	^{32}P incorporation (relative to control)		
		PLB	TnI	C-protein
Control		1.00 (6)	1.00 (7)	1.00 (7)
Isoprenaline		5.44 ± 1.09 (6)	8.30 ± 1.90 (5)	1.77 ± 0.25 (5)
Washout of isoprenaline		1.26 ± 0.30 (6)*	5.98 ± 0.56 (5)‡	1.59 ± 0.25 (6)‡
Forskolin		n.d.	10.35 (2)	1.86 (2)

Fig. 1. SDS/15-(w/v)-polyacrylamide-gel electrophoresis and autoradiograms of myofibrils (50 μg) isolated from [^{32}P] P_i -perfused rabbit hearts

Photographs are shown of a Coomassie Blue-stained gel (Coo. Blue) and autoradiograms of myofibrils isolated from rabbit hearts frozen after perfusion with Krebs–Henseleit buffer (Ctrl); 1.0 μM -isoprenaline for 1 min (Iso); after 1 min pulse exposure to 1.0 μM -isoprenaline, followed by 15 min perfusion with drug-free Krebs–Henseleit buffer (Krebs wash), or 20 μM -sotalol (Sotalol wash).

Table 2. Cyclic AMP levels in myocardium from rabbit hearts perfused with various inotropic agents

Hearts were perfused as described in the Materials and methods section with $1\text{ }\mu\text{M}$ -isoprenaline, $10\text{ }\mu\text{M}$ -forskolin or washed out for 15 min with Krebs–Henseleit buffer. Data are expressed as pmol of cyclic AMP/mg of homogenate protein and presented as means $\pm$ S.E.M. for n hearts in each group (except forskolin; mean for two hearts). * $P < 0.05$ compared with control hearts.

Agent	n	[Cyclic AMP] (pmol/mg)
None (control)	7	6.01 ± 1.25
Isoprenaline	7	$12.93 \pm 1.09^*$
Forskolin	2	15.40
Washout	3	6.55 ± 2.23

et al., 1985). In experiments reported here, we further characterized phosphorylation of C-protein occurring through the cyclic AMP-dependent pathway. Table 1 summarizes results of experiments in which we measured levels of myofibrillar protein phosphorylation in hearts subjected to various inotropic interventions. Isoprenaline stimulation of the hearts was associated with increased phosphate incorporation into C-protein and TnI (Fig. 1), and phosphorylation of both of these proteins was blocked when hearts were perfused with a β -adrenergic blocking agent ($10\text{ }\mu\text{M}$ -propranolol or $20\text{ }\mu\text{M}$ -sotalol) for 5 min before and during exposure to isoprenaline (results not shown). The pattern of myofilament phosphorylation was the same in control hearts and those perfused with β -blocking agent alone. The effects of isoprenaline could be mimicked by perfusion with $10\text{ }\mu\text{M}$ -forskolin (Table 1), which directly stimulates membrane-associated adenylate cyclase (Metzger & Lindner, 1981).

Table 2 summarizes cyclic AMP levels measured in samples of frozen myocardium from hearts perfused with the various interventions described above. Stimulation with isoprenaline or forskolin resulted in a 2–3-fold increase in the cyclic AMP levels. The cyclic AMP level in the myocardium returned to control values by 15 min after termination of a 1 min pulse perfusion with isoprenaline. By this time, peak left-intraventricular pressure had also returned to the control level. Associated with this decrease in cyclic AMP level, there was no significant dephosphorylation of either C-protein or TnI (Table 1, Fig. 1). However, there was dephosphorylation of SR preparations (Table 1, Fig. 2) isolated from the same hearts. Similar results were obtained when sotalol ($20\text{ }\mu\text{M}$) was included in the washout medium (Fig. 1). In addition, the decrease in Ca^{2+} activation of myofibrillar Mg-ATPase activity shown to occur at the peak inotropic response to isoprenaline (Kranias *et al.*, 1985) also persisted in preparations from hearts frozen 15 min after isoprenaline-pulse-stimulus termination. The Mg-ATPase activities measured at pCa 6.2 and pH 7.0 and expressed as percentage maximal Mg-ATPase activity (pCa 4.75) were: 53.3 ± 2.9 , 39.7 ± 2.5 and 35.3 ± 2.1 (means $\pm$ S.E.M., $n = 4$) for myofibrils prepared from hearts frozen under control conditions, at the peak of the inotropic response and 15 min after pulse-stimulus termination respectively.

Fig. 2. Autoradiography of SDS/15-(w/v)-polyacrylamide gels of SR (101 μg) prepared from $[^{32}\text{P}]\text{P}_i$ -perfused rabbit hearts

SR was isolated from hearts frozen after perfusion with Krebs–Henseleit buffer (Control), $1.0\text{ }\mu\text{M}$ -isoprenaline (Iso) or 15 min perfusion with Krebs–Henseleit buffer after 1 min pulse perfusion with $1.0\text{ }\mu\text{M}$ -isoprenaline (Washout). PLB_H, phospholamban, migrating with an apparent M_r of 27 000 (non-boiled sample). When the samples were boiled in the presence of SDS, PLB migrated with an apparent M_r of 11 000 (PLB_L). Abbreviations: DF, dye front; O, origin.

Inasmuch as this diminution in Ca^{2+} activation of myofibrillar ATPase is associated with concurrent phosphorylation of both C-protein and TnI, it is important to assess the relative contribution of each of these phosphoproteins to the altered activity. Therefore we developed a system in which the effect of C-protein phosphorylation on myofilament Mg-ATPase activity

Fig. 3. Relation between pCa and percentage maximal Mg-ATPase activity in reconstituted regulated actomyosin system with or without cyclic AMP-dependent protein kinase phosphorylation

(a) Regulated actomyosin system reconstituted from DMF and skeletal TnTm complex in control ($\square$) and phosphorylated ($\blacksquare$) preparations. Inset: autoradiogram of reconstituted preparation phosphorylated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. (b) Regulated actomyosin system reconstituted from DMF and cardiac TnTm complex in control and phosphorylated preparations. Results are means $\pm$ S.E.M. for three preparations and are expressed as percentage maximal ATPase activity. Basal ATPase activity (pCa 8.0) was subtracted as each pCa point before normalization. See the Materials and methods section for details. Inset: autoradiogram of reconstituted preparation phosphorylated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

could be assessed independent of TnI phosphorylation. This was accomplished by reconstituting a regulated actomyosin system in which only C-protein became phosphorylated. Cardiac myofibrils devoid of the TnTm complex (and thus insensitive to Ca^{2+} regulation), were reconstituted with purified skeletal TnTm. This reconstitution scheme was devised to study phosphorylation in a relatively intact 'myofibril' in which the TnI is not a substrate for cyclic AMP-dependent protein kinase. Autoradiograms of the reconstituted hybrid preparations phosphorylated with cyclic AMP-dependent protein kinase in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ showed that C-

protein was the predominant phosphoprotein (Fig. 3a, inset). Minimal contamination with cardiac TnI was observed in these preparations. The reconstituted regulated actomyosin showed a sigmoidal relationship between free- Ca^{2+} concentration and Mg-ATPase activity. At low free Ca^{2+} (pCa < 8.0), ATPase activity was less than 25 % of maximal ATPase activity (measured at pCa 4.5), indicating incorporation of the regulatory protein complex. Phosphorylation of this cardiac-skeletal hybrid myofibrillar system was not associated with a significant shift of the Ca^{2+} -sensitivity of the Mg-ATPase (Fig. 3a). Phosphorylation of a regulated actomyosin system reconstituted from cardiac desensitized myofibrils and cardiac TnTm resulted in ^{32}P incorporation into both TnI and C-protein, and this was associated with a decrease in the sensitivity of the Mg-ATPase to Ca^{2+} (Fig. 3b). These findings suggest that phosphorylation of TnI is primarily responsible for the decrease in Ca^{2+} activation of myofibrillar ATPase.

DISCUSSION

The results of experiments reported here further characterize phosphorylation of C-protein *in situ* and report a differential dephosphorylation of phospholamban as compared with myofibrillar proteins measured in the same hearts after removal of adrenergic stimulation.

In mammalian hearts studied here, both C-protein and TnI were phosphorylated in response to isoprenaline stimulation and remained phosphorylated when left-ventricular pressure development had returned to control levels. This result is in agreement with previous studies in which TnI phosphorylation was observed to persist after reversal of the mechanical effects of catecholamine stimulation in perfused rabbit (Stull *et al.*, 1981) and rat (England, 1976) hearts. However, in another study of perfused rat hearts, Resink & Gevers (1982) reported that TnI dephosphorylation after β -agonist withdrawal was correlated with changes in force development. The reason for this discrepancy is unclear and may be related to species differences or methodology. In perfused amphibian hearts, C-protein phosphorylation has been correlated with an increased rate of twitch relaxation, suggesting an important role for C-protein in the regulation of the contraction cycle of these hearts (Hartzell, 1985). The finding of persisting C-protein and TnI phosphorylation reported here suggests that additional factors may be primarily responsible for the reversal of mechanical effects after β -agonist withdrawal in the mammalian heart.

Of the phosphoproteins examined in the present study, only phospholamban phosphorylation returned to basal levels when the effect of isoprenaline on left-ventricular pressure development had been reversed. The dissociation between dephosphorylation of SR and myofibrillar proteins measured in the same hearts suggests the possibility of intracellular compartmentation or activation of protein kinases and/or substrate-specific phosphatases. Whereas the understanding of kinase systems involved in phosphorylation is incomplete, even less is known regarding cardiac contractile and regulatory protein dephosphorylation by phosphatases. Dephosphorylation of C-protein, TnI and phospholamban appears to be regulated by cholinergic stimulation, and the phosphatase may be regulated by cyclic GMP

(England, 1976; Hartzell & Titus, 1982; Hartzell, 1984; Lindemann & Watanabe, 1985). However, direct regulation of protein phosphatases by cyclic GMP has not been documented. Recently, we have shown that an intrinsic protein phosphatase activity, associated with SR, can dephosphorylate phospholamban and reverse the stimulatory effects of protein kinases on the Ca^{2+} pump in cardiac SR (Kranias, 1985). Thus compartmentalization of regulatory enzymes and associated substrates may be important in reactions mediating the response of the heart to adrenergic stimulation.

In intact cardiac myofibrillar systems phosphorylated with cyclic AMP-dependent protein kinase, both TnI and C-protein become phosphorylated, and the relative contribution each phosphorylation makes to the alteration in biochemical function is not clear. It has been suggested that phosphorylation of avian C-protein may regulate actin-activated myosin ATPase activity measured at pH 8.0 (Hartzell, 1985), but the effect of C-protein phosphorylation on Ca^{2+} activation of the myofibrillar ATPase activity has not been previously studied. To address this question we have developed a reconstituted regulated actomyosin system in which only C-protein becomes phosphorylated. In this system there is no decrease in Ca^{2+} activation of the ATPase (measured at pH 7.0) when C-protein alone is phosphorylated. This finding corroborates earlier studies that demonstrated decreased Ca^{2+} activation of MgATPase activity in reconstituted actomyosin systems containing regulatory proteins with phosphorylated TnI (Solaro *et al.*, 1976; Bailin, 1979).

Our findings indicate that, under the conditions of our experiments, phospholamban phosphorylation is better correlated than is TnI or C-protein phosphorylation with the mechanical response of the mammalian heart to adrenergic stimulation. Yet in the intact animal, in which regulation of cardiac contraction involves well-controlled interactions between the sympathetic and parasympathetic nervous systems, relations among inotropic state, SR and myofilament protein phosphorylation may be different from those in isolated perfused preparations.

We are grateful to Mr. Mark Rocklin, Ms. Ursula McDonnell and Ms. Patricia Walker for excellent technical assistance, to Ms. Betsy Solaro for drawing the Figures, and to Ms. Anita Tolle for typing the manuscript. This research was supported in part by U.S. Public Health Service Grants RO1 HL22231, RO1 HL26057, PO1 HL22619. J.L.G. was the recipient of a University Dean's Distinguished Dissertation Fellowship.

REFERENCES

- Bailin, G. (1979) *Am. J. Physiol.* **236**, C41–C46
- Blanchard, E. M. & Solaro, R. J. (1984) *Circ. Res.* **55**, 382–391
- Carter, S. G. & Karl, D. W. (1982) *J. Biochem. Biophys. Methods* **7**, 7–13
- Craig, R. & Offer, G. (1976) *Proc. R. Soc. London Ser. B* **192**, 451–461
- England, P. J. (1976) *FEBS Lett.* **50**, 57–60
- Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505
- Hartzell, H. C. (1984) *J. Gen. Physiol.* **83**, 563–588
- Hartzell, H. C. (1985) *J. Mol. Biol.* **186**, 185–195
- Hartzell, H. C. & Titus, L. (1982) *J. Biol. Chem.* **257**, 2111–2120
- Holroyde, M. J., Howe, E. & Solaro, R. J. (1979a) *Biochim. Biophys. Acta* **586**, 63–69
- Holroyde, M. J., Small, D. A. P., Howe, E., Solaro, R. J. (1979b) *Biochim. Biophys. Acta* **587**, 629–637
- Jeacocke, S. A. & England, P. J. (1980) *FEBS Lett.* **122**, 129–132
- Katz, A. M. (1979) *Adv. Cyclic Nucleotide Res.* **11**, 303–343
- Kopp, S. J. & Bárány, M. (1979) *J. Biol. Chem.* **254**, 12007–12012
- Kranias, E. G. (1985) *J. Biol. Chem.* **260**, 11006–11010
- Kranias, E. G. & Solaro, R. J. (1982) *Nature (London)* **298**, 182–184
- Kranias, E. G., Mandel, F., Wang, T. & Schwartz, A. (1980) *Biochemistry* **19**, 5434–5439
- Kranias, E. G., Garvey, J. L., Srivastava, R. D., Solaro, R. J. (1985) *Biochem. J.* **226**, 113–121
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lehman, W. (1978) *Nature (London)* **274**, 80–81
- Lindemann, J. P. & Watanabe, A. M. (1985) *J. Biol. Chem.* **260**, 13122–13129
- Lowry, O. H., Rosenbrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 165–175
- Metzger, H. & Lindner, E. (1981) *Arzneim.-Forsch.* **31**, 1248–1250
- Mope, L., McClellan, G. B. & Winegrad, S. (1980) *J. Gen. Physiol.* **75**, 271–282
- Nakamura, J., Wang, T., Tsai, L. I. & Schwartz, A. (1983) *J. Biol. Chem.* **258**, 5079–5083
- Ray, K. P. & England, P. J. (1976) *FEBS Lett.* **70**, 11–16
- Resink, T. J. & Gevers, W. (1982) *J. Mol. Cell. Cardiol.* **14**, 329–337
- Robertson, S. P., Johnson, J. D., Holroyde, M. J., Kranias, E. G., Potter, J. D. & Solaro, R. J. (1982) *J. Biol. Chem.* **257**, 260–263
- Solaro, R. J., Pang, D. C. & Briggs, N. (1971) *Biochim. Biophys. Acta* **245**, 259–262
- Solaro, R. J., Moir, A. J. G. & Perry, S. V. (1976) *Nature (London)* **262**, 615–616
- Starr, R. & Offer, G. (1971) *FEBS Lett.* **15**, 40–44
- Stull, J. T. & Buss, J. E. (1977) *J. Biol. Chem.* **252**, 851–857
- Stull, J. T., Sanford, C. F., Manning, D. R., Blumenthal, D. K. & High, S. W. (1981) *Cold Spring Harbor Conf. Cell Prolif.* **8**, 823–839
- Tada, M. & Inui, M. (1983) *J. Mol. Cell. Cardiol.* **15**, 565–575
- Tada, M., Yamada, M., Ohmori, F., Kuzuya, T., Inui, M. & Abe, H. (1980) *J. Biol. Chem.* **255**, 1985–1992