Biochem. J. (1985) **226**, 113–121
Printed in Great Britain

Phosphorylation and functional modifications of sarcoplasmic reticulum and myofibrils in isolated rabbit hearts stimulated with isoprenaline

Evangelia G. KRANIAS,* J. Lee GARVEY,† R. Dayal SRIVASTAVA† and R. John SOLARO*†
**Department of Pharmacology and Cell Biophysics and †Department of Physiology and Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267, U.S.A.*

(*Received 18 July 1984/Accepted 16 October 1984*)

Isoprenaline stimulation of perfused rabbit hearts was associated with simultaneous phosphorylation of proteins in the myofilaments and phospholamban in the sarcoplasmic reticulum (SR). Hearts were perfused with Krebs–Henseleit buffer containing [^{32}P]P_i, freeze-clamped in a control condition or at the peak of the inotropic response to isoprenaline, and myofibrils and SR were prepared from the same hearts. Stimulation of ^{32}P incorporation in troponin I (TnI) and C-protein by isoprenaline was associated with a decrease in Ca^{2+}-sensitivity of the myofibrillar Mg^{2+}-dependent ATPase activity. Stimulation of ^{32}P incorporation in SR by isoprenaline was associated with an increase in the initial rates of oxalate-facilitated Ca^{2+} transport, assayed with SR vesicles in either microsomal fractions or homogenates from the perfused hearts. These findings provide evidence that phosphorylation of TnI, C-protein and phospholamban in the intact cell is associated with functional alterations of the myofibrils and SR which may be responsible in part for the effects of catecholamines on the mammalian myocardium.

The response of heart cells to β-adrenergic stimulation is likely to involve co-ordinated changes in the extents of covalent phosphorylation of regulatory proteins of SR and myofilaments. For SR, extensive experiments *in vitro* (LaRaia & Morkin, 1974; Kirchberger *et al.*, 1974; Wray & Gray, 1977; Hicks *et al.*, 1979) and subsequent studies *in vivo* (LePeuch *et al.*, 1980; Kranias & Solaro, 1982; Lindemann *et al.*, 1983) suggest that during β-adrenergic stimulation the SR should become phosphorylated and that this phosphorylation should result in a stimulation of the initial rate of Ca^{2+} transport. The studies *in vitro* have mimicked the increase in cyclic AMP and activation of cyclic AMP-dependent protein kinases, shown to occur in hearts stimulated with catecholamines (Tsien, 1977; Katz, 1979), by adding cyclic AMP to incubation media containing cardiac SR vesicles and endogenous (LaRaia & Morkin, 1974; Wray & Gray, 1977; Kranias *et al.*, 1982) or exogenous cyclic AMP-dependent protein kinases (Tada *et al.*, 1975; Schwartz *et al.*, 1976; Manalan & Jones, 1982). The results of these studies have shown that (1) the SR becomes phosphorylated (Tada *et al.*, 1975), (2) the site of phosphorylation is a polymeric proteolipid named phospholamban (Lamers & Stinis, 1980; Kirchberger & Antonetz, 1982; Louis *et al.*, 1982), and (3) the extent of phosphorylation of phospholamban is directly correlated with the initial rate of Ca^{2+} transport by the SR vesicles (Kirchberger *et al.*, 1974; LaRaia & Morkin, 1974) and the formation and decomposition of the phosphoenzyme (E ~ P) intermediate of the Ca^{2+}-dependent ATPase (Kranias *et al.*, 1980*a*; Tada *et al.*, 1980).

Experiments by several groups (LePeuch *et al.*, 1980; Kranias & Solaro, 1982; Lindemann *et al.*, 1983), have provided evidence consistent with an increase in phospholamban phosphorylation in SR preparations derived from hearts freeze-clamped during stimulation with catecholamines. In these studies the ATP pool of the heart was labelled by perfusion with buffers containing [^{32}P]P_i. Microsomal fractions enriched in SR vesicles were prepared and analysed by gel electrophoresis. Autoradiography of the gels showed incorporation of label into protein bands which have been identified as phospholamban in preparations of SR phosphorylated *in vitro*. These studies require that the procedures for preparation of the SR fraction ensure an inhibition of phosphate-group transfer,

Abbreviations used: SR, sarcoplasmic reticulum; TnI, troponin I; SDS, sodium dodecyl sulphate.

that there be a net change in the amount of covalently bound phosphate and that the microsomal fractions be virtually free of contaminant proteins that are phosphorylated in the heart and are of similar mobility to phospholamban. Such requirements are difficult to meet. For example, preparations of SR vesicles are contaminated with sarcolemmal vesicles that appear to contain phosphorylated proteins of the same relative mobility as phospholamban on analytical polyacrylamide gels (Manalan & Jones, 1982; Rinaldi *et al.*, 1982). In fact, more recent evidence suggests that phospholamban and calciductin, a phosphoprotein apparently localized in sarcolemma, may be the same protein (Capony *et al.*, 1983). In the study by Kranias & Solaro (1982), sarcolemmal contamination was limited to 10% of the total protein, but the possibility that even this low percentage contamination provided a significant portion of the radioactivity assayed in the gels could not be absolutely ruled out. Because of these difficulties in interpreting data obtained from measurements of the extents of phosphorylation in SR fractions, prepared from [^{32}P]P_i perfused hearts, it becomes important to show not only that perfusion of hearts with catecholamines results in phosphorylation of the SR, but also that the Ca^{2+}-transport activity of the SR is altered.

For myofibrils, there is general agreement that phosphorylation of TnI and C-protein occurs in response to β-adrenergic stimulation of the heart (England, 1976; Solaro *et al.*, 1976; Jeacock & England, 1980). Studies *in vitro* have shown that these phosphorylations are associated with a rightward shift in the plots of myofibrillar ATPase activity and tension versus free Ca^{2+} (Ray & England, 1976; Mope *et al.*, 1980). The mechanism for this decreased sensitivity appears to be a decrease in the affinity of troponin C for Ca^{2+} (Holroyde *et al.*, 1979*a*). However, it is not yet clear whether the phosphorylation *in situ* affects myofibrillar activity in the same way as phosphorylation *in vitro* (Holroyde *et al.*, 1979*a*; Resink & Gevers, 1981; Sistare *et al.*, 1981).

In the present study we report that the positive inotropic effect of isoprenaline, which we have previously shown to be accompanied by simultaneous phosphorylation of phospholamban and TnI, is associated with an increase in the initial velocity of Ca^{2+} transport by SR vesicles and a decrease in the sensitivity of the Mg^{2+}-dependent ATPase activity to Ca^{2+} with no change in maximum activity.

Materials and methods

Heart perfusions and preparations

Rabbit hearts were perfused retrogradely with modified Krebs–Henseleit buffer in a Langendorff-type apparatus in which force and heart rate were recorded (Solaro *et al.*, 1976; Kranias & Solaro, 1982). Myofibrils and micosomal fractions enriched in SR vesicles were prepared from the same heart as previously described (Kranias & Solaro, 1982).

Control experiments were performed to verify that dephosphorylation of SR proteins was prevented during isolation of the vesicles. ^{32}P-labelled SR vesicles, isolated from hearts perfused with [^{32}P]P_i, were added (86μg/ml) to the supernatant fraction obtained after centrifugation of homogenates from frozen powdered hearts that had been perfused with non-labelled phosphate. The amount of SR protein added was determined from estimates of the amount of SR protein present in the supernatant fraction. Samples were then taken at various times of incubation at 0°C, and ^{32}P incorporation into acid-precipitable protein was measured. The data in Table 1 show that no significant dephosphorylation occurred over the first 1h at 0°C.

For experiments in which we measured Ca^{2+} transport by unfractionated SR vesicles, 300–500mg of frozen heart powder was homogenized in 10vol. of either 50mM-KH_2PO_4/0.3M-sucrose, pH7.0, or 10mM-imidazole, pH7.0. Homogenization was done with a Teflon/glass Thomas tissue grinder with the pestle driven at 500rev./min for 20–40 passes. The rates of Ca^{2+} transport, measured in the homogenates, were the same regardless of the homogenization medium. Pilot experiments showed that Ca^{2+} transport was the same whether measured with frozen heart powder or fresh tissue immediately taken from the control perfused hearts.

Table 1. *Stability of* [^{32}*P*]*phosphorylated proteins in SR isolation buffer*

SR vesicles were prepared from [^{32}P]P_i-perfused rabbit hearts as described in the Materials and methods section. The ^{32}P-labelled membrane vesicles were added (86μg of SR/ml) to the supernatant of the first centrifugation (4300*g* for 10min) and incubated at 0°C. At the indicated times, the acid-precipitable ^{32}P radioactivity (c.p.m.) was determined. The zero-time point was obtained immediately after the addition of ^{32}P-labelled SR to the supernatant. Values shown are the arithmetic means (±S.E.M.) for three determinations.

Time (min)	Acid-stable [^{32}P]P_i (c.p.m./0.5ml)	(%)
0	933±36	100±4
30	1003±27	107±3
45	975±31	104±3
60	858±26	92±3

Cardiac myofibrils were prepared by slight modification of the procedures described previously by Solaro *et al.* (1971) and Holroyde *et al.* (1979*b*) in which contaminant membranes are removed by extraction in Triton X-100 and phosphate-group transfer reactions are chemically 'frozen'. The starting material for the preparation of myofibrils was the pellet obtained after the first centrifugation of heart homogenate. Pure TnI was prepared from the myofibrillar fraction by affinity chromatography on troponin C–Sepharose columns as previously described (Solaro *et al.*, 1976; Moir *et al.*, 1980).

ATPase activity and Ca^{2+} transport

Myofibrillar Mg^{2+}-dependent ATPase activity was measured at pH 7.0, 30°C in 1 ml reaction mixtures containing 60 mM-KCl, 30 mM-imidazole, 2 mM-Mg^{2+}, 2 mM-$MgATP^{2-}$, 1 mM-EGTA and 10 mM-NaF as previously described (Holroyde *et al.*, 1979*a*). The extents of myofibrillar phosphorylation were maintained throughout the incubation, as judged from autoradiography and determination of acid-precipitable $[^{32}P]P_i$ at the beginning and end of the incubation.

Ca^{2+} transport in fractions enriched in SR vesicles was measured by a filtration technique modified from previous techniques (Harigaya & Schwartz, 1969; Davis *et al.*, 1983). SR vesicles (0.1 mg/ml) were preincubated for 2 min at 30°C in a reaction medium consisting of 30 mM-histidine/HCl (pH 7.0), 0.1 M-KCl, 6 mM-$MgCl_2$, 5 mM-NaN_3, 2.5 mM-oxalate and 0.5 mM-EGTA, with $^{45}CaCl_2$ added to give the desired free Ca^{2+} concentration, and the transport reaction was initiated by addition of ATP (5 mM final concn.).

Ca^{2+} transport by unfractionated SR vesicles in ventricular homogenates was assayed within 30 min of homogenization. The assay was carried out at 37°C in reaction mixtures containing 0.3–0.5 mg of homogenate protein/ml, 50 mM-KH_2PO_4, 5 mM-potassium oxalate, 50 mM-KCl, 10 mM-NaN_3, 7.5 mM-$MgCl_2$, 5.0 mM-Na_2ATP, 100 μM-EGTA and different amounts (20–104 μM) of $^{45}CaCl_2$ (0.1 μCi/ml). Ca^{2+} contributed by the homogenate and the reagents was determined by atomic absorption spectroscopy and amounted to about 5 nmol/ml. Components of the reaction mixture except Ca^{2+} were preincubated at 37° for 2 min, and uptake was initiated by adding $^{45}CaCl_2$ to the final desired free Ca^{2+} concentration. At various times, samples of the reaction mixture were rapidly filtered through a 0.45 μm Millipore filter, and 0.1 ml samples of the filtrate were assayed for radioactivity.

Phosphorylation assays

The specific radioactivity of $[^{32}P]$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 & Barany (1979).

SR vesicles (1 mg/ml), prepared from hearts perfused with non-radioactive or with $[^{32}P]P_i$-containing media, were phosphorylated for 5 min at 30°C in 50 mM-potassium phosphate (pH 7.0), containing 10 mM-$MgCl_2$, 10 mM-NaF, 0.5 mM-EGTA, 0.5 mM-[γ-^{32}P]ATP, 1 μM-cyclic AMP and 10 μg of cyclic AMP-dependent protein kinase/ml.

Soluble protein kinase activity was determined with a portion (25 μl) of the first supernatant fraction (F_1) obtained during the SR isolation procedure. Assay conditions were the same as those for SR phosphorylation, except that cyclic AMP-dependent protein kinase was omitted and histone (10 mg/ml) was present. $[^{32}P]P_i$ incorporation and hydroxylamine sensitivity of the phosphate bonds was determined as previously described (Kranias *et al.*, 1980*b*). For the assays of soluble protein kinase activity, the acid-stable $[^{32}P]P_i$ present in the sample (25 μl) used for assays was subtracted in order to obtain net incorporation into histone. The protein kinase activity is expressed as the ratio of activity in the absence of cyclic AMP to that in the presence of 1 μM-cyclic AMP.

Gel electrophoresis

Gel electrophoresis under denaturing conditions was performed by the procedures described by Laemmli (1970). ^{32}P-labelled SR or ^{32}P-labelled myofibrils were dialysed in buffer containing 10 mM-Tris/histidine buffer (pH 7.5), 2% (v/v) β-mercaptoethanol and 1% SDS. Some of the samples were heated at 90°C for 5 min before SDS (0.1%)/polyacrylamide-gel electrophoresis. M_r markers were phosphorylase *b* (94000), human serum albumin (66000), ovalbumin (43000), deoxyribonuclease I (31000), soya-bean trypsin inhibitor (21500) and egg-white lysozyme (14300).

Protein determination

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. For determination of protein concentrations in samples containing β-mercaptoethanol and SDS, sodium deoxycholate was added and the protein was subsequently separated from the interfering reagents by precipitation with trichloroacetic acid as previously described (Nakamura *et al.*, 1983).

Results

Treatment of rabbit hearts with isoprenaline was associated with a significant ($P = 0.03$, $n = 7$)

increase in the [^{32}P]P_i incorporated into SR vesicles (Table 2), in agreement with previous observations (Kranias & Solaro, 1982; Lindemann *et al.*, 1983). Furthermore, treatment with hydroxylamine (Kranias *et al.*, 1980*b*) did not significantly alter the amounts of [^{32}P]P_i remaining associated with SR vesicles, suggesting that the ^{32}P incorporated represented phosphoester bonds.

We have previously reported that the phosphorylation of SR associated with β-adrenergic stimulation of rabbit hearts occurs in an 11000-M_r protein, most probably phospholamban (Kranias & Solaro, 1982). However, in some experiments, ^{32}P incorporation also occurred in a 24000-M_r protein with a mobility that could be altered by the solubilization conditions used. When the ^{32}P-labelled SR samples were boiled in the presence of 1% SDS for 5min, before electrophoresis, all the radioactivity was associated with the 11000-M_r protein (Fig. 1). To determine the similarity of the phosphoproteins in SR isolated from ^{32}P-perfused hearts with those obtained during phosphorylation *in vitro* of SR, rabbit hearts were perfused with [^{32}P]P_i and a portion of the isolated ^{32}P-labelled vesicles (Fig. 1*a*) were additionally phosphorylated *in vitro* by cyclic AMP-dependent protein kinase (Fig. 1*b*). As a control in this study, SR vesicles were isolated in parallel from rabbit hearts perfused with non-radioactive phosphate, and these vesicles were then phosphorylated *in vitro* by cyclic AMP-dependent protein kinase, under the same conditions as for the SR vesicles isolated from hearts perfused with [^{32}P]P_i (Fig. 1*c*). Gel electrophoresis of ^{32}P-labelled SR labelled under various conditions revealed the presence of the same phosphoproteins, indicating that identical proteins may be phosphorylated *in vitro* as in the beating heart (Fig. 1).

To determine the functional significance of SR phosphorylation, cardiac SR vesicles isolated from pairs of control and stimulated hearts were assayed for Ca^{2+} transport in the presence of oxalate. Stimulation of the hearts by isoprenaline was associated with a significant ($P = 0.05$, $n = 5$) increase in the initial rates of Ca^{2+} transport by SR vesicles (Table 2), as expected from studies *in vitro* on the effect of cyclic AMP-dependent phosphorylation on the Ca^{2+} pump (Kirchberger *et al.*, 1974; LaRaia & Morkin, 1974; Tada *et al.*, 1974; Hicks *et al.*, 1979). Since stimulation of Ca^{2+} transport *in vitro* was associated with phosphorylation of phospholamban by cyclic AMP-dependent protein kinase(s), it was decided to determine the effects of isoprenaline on cyclic AMP accumulation or on the activation state of the cyclic AMP-dependent protein kinase, the enzyme that mediates the actions of cyclic AMP (Corbin & Keely, 1977; Tsien, 1977). Data summarized in Table 2 show the cyclic AMP-dependent protein kinase activity ratio for control and isoprenaline-stimulated hearts. The activity ratio in control hearts was higher than that previously reported for rabbit hearts (Corbin *et al.*, 1977; Hayes *et al.*, 1980), but this may be attributed to the presence of high endogenous cyclic AMP concentrations, since perfusion and isolation media included 3-isobutyl-1-methylxanthine and extraction media did not contain Norit for rapid absorption of cyclic AMP (Palmer *et al.*, 1980). However, stimulation of the hearts by isoprenaline was associated with a significant ($P < 0.001$, $n = 7$) increase in the soluble protein kinase activity compared with control hearts (Table 2). Although this finding must be interpreted with caution (Palmer *et al.*, 1980), it suggests that the increased ^{32}P incorporation into phospholamban in the intact hearts is mediated by

Table 2. *Isoprenaline stimulation of phospholamban phosphorylation, Ca^{2+} transport and soluble protein kinase activity in beating hearts*

SR vesicles were isolated from control and isoprenaline-stimulated rabbit hearts as described in the Materials and methods section. Samples of each preparation were used to determine the ^{32}P incorporated in triplicate. Samples of SR vesicles were also used to determine the initial rates of Ca^{2+} transport at 3μM free Ca^{2+}. Samples of the first supernatant fraction obtained during isolation of SR vesicles (F_1) were used for determination of protein kinase activity assayed in the absence (−) or presence (+) of 1μM-cyclic AMP. The values shown represent the means ± S.E.M. for n pairs of control and stimulated hearts. Experimental details are described in the Materials and methods section.

	Control	Stimulated
[^{32}P]P_i incorporation (pmol/mg of SR protein) ($n = 7$)	288 ± 28	410 ± 43
Ca^{2+} transport (nmol/min per mg of SR protein) ($n = 5$)	8.9 ± 1.7	14.6 ± 1.9
Protein kinase (−/+) (pmol of [^{32}P]P_i/mg of F_1 protein ($n = 7$)	0.56 ± 0.03	0.82 ± 0.03

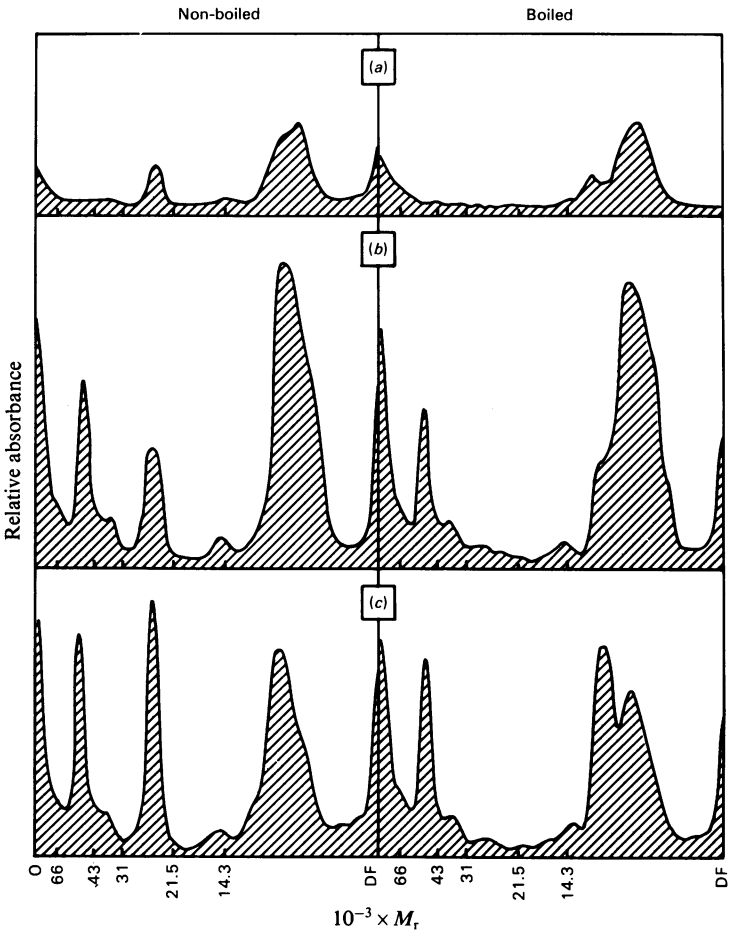

Fig. 1. *Autoradiography of SDS (0.1%)/polyacrylamide (15%) gels of rabbit cardiac SR (62 μg) phosphorylated under various conditions*

(*a*) SR isolated from isoprenaline-stimulated rabbit heart perfused with [^{32}P]P_i; (*b*) same SR as in (*a*) and additionally phosphorylated *in vitro* by cyclic AMP-dependent protein kinase (Sigma) in the presence of [γ-^{32}P]-ATP; (*c*) SR isolated from rabbit hearts perfused with non-radioactive phosphate and phosphorylated *in vitro* as in (*b*). The 55000-M_r phosphoprotein (*b* and *c*) was due to autophosphorylation of the cyclic AMP-dependent protein kinase, and it was the only band observed in samples of the protein kinase alone. 'Boiled': samples boiled for 5 min before electrophoresis; 'Non-boiled': samples kept at room temperature. O, Origin; DF, dye front.

cyclic AMP-dependent protein kinases, as expected from several studies *in vitro* (Kranias & Solaro, 1983).

Thus SR vesicles isolated from rabbit hearts stimulated with isoprenaline demonstrate an increase in ^{32}P incorporation associated with increased rates of Ca^{2+} transport. Yet, compared with conventionally prepared SR vesicles, with rates of transport of 50–100 nmol of Ca^{2+}/min per mg (Suko, 1973), the velocity of Ca^{2+} transport is relatively slow (9–15 nmol/min per mg) in these preparations, most probably because of the conditions (freezing of tissue and use of NaF and phosphate in isolation buffers) required to isolate the vesicles without changing the state of phosphorylation. Therefore, in addition to the studies on isolated SR vesicles, we also measured Ca^{2+} transport by unfractionated SR vesicles in a homogenate of the left ventricle. We have previously defined incubation conditions under which Ca^{2+} uptake is restricted to SR vesicles in the homogenate (Solaro & Briggs, 1974; Briggs *et al.*, 1977), and have discussed the validity and advantages of this approach (Pagani & Solaro, 1984). The time course of SR Ca^{2+} uptake from control and isoprenaline-stimulated hearts measured at saturating concentrations of free Ca^{2+} (pCa 5) is shown in Fig. 2. The uptake was linear with time

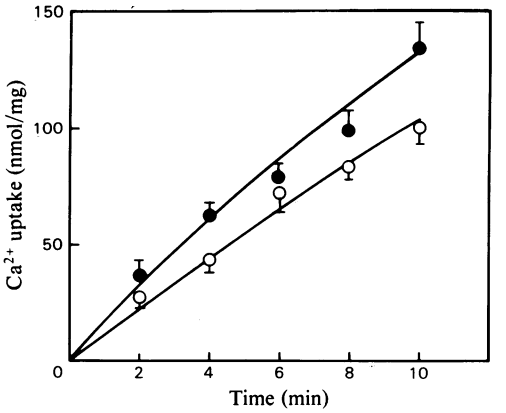

Fig. 2. *Time course of Ca^{2+} uptake by SR vesicles in homogenates of left-ventricular samples from control (○) and isoprenaline-stimulated (●) rabbit hearts measured at pCa 5.0*

Data are expressed per mg of homogenate protein and are presented as means ± S.E.M. for preparations from 8–14 controls and 6 stimulated hearts. See the Materials and methods section for incubation conditions and other details.

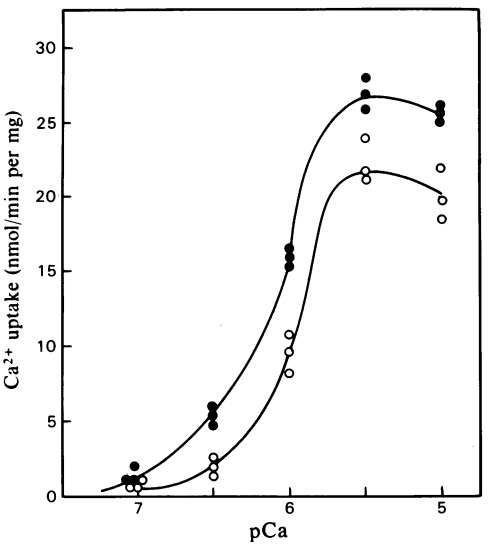

Fig. 3. *Relation between pCa and the velocity of Ca^{2+} uptake by SR vesicles in homogenates prepared from samples of left ventricle of control (○) and isoprenaline-stimulated (●) rabbit hearts*

Velocity data are expressed per mg of homogenate protein for three control and three isoprenaline-stimulated preparations. See the Materials and methods section for incubation conditions and other details.

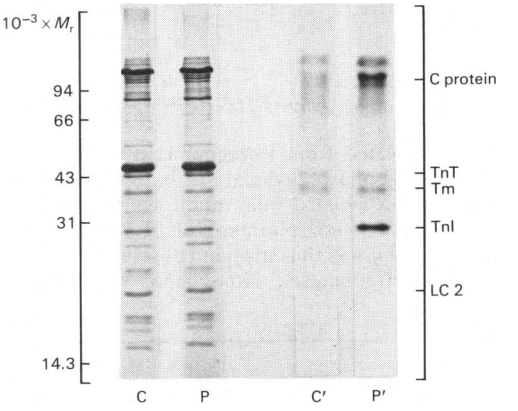

Fig. 4. *SDS (1.0%)/polyacrylamide (15%)-gel electrophoresis and autoradiography of myofibrils (60 μg) isolated from rabbit hearts perfused with $[^{32}P]P_i$*

Photographs of Coomassie Blue-stained gel and autoradiogram of myofibrils isolated from control (C, C′) and isoprenaline-stimulated (P, P′) hearts. Key: TnT, troponin T; Tm, tropomyosin; LC 2, myosin light chain 2.

for at least the first 6 min of the reaction, and there was a significant increase in the rate of Ca^{2+} transport by SR vesicles in the homogenates prepared from hearts stimulated with isoprenaline (Fig. 2). This increase was more clearly evident at lower free Ca^{2+} concentrations, as shown in Fig. 3, which depicts results of three experiments in which we measured the velocity of Ca^{2+} transport by SR vesicles over a broad range of pCa. Furthermore, the pCa_{50} (pCa giving half-maximal velocity) was about 0.2 unit higher in the stimulated (6.13) than in the control (5.96) preparations, indicating that the increase in velocity of Ca^{2+} transport was also associated with an increase in the sensitivity of the transport system to free Ca^{2+}.

Myofibrils, prepared from the same isoprenaline-stimulated hearts, in which we measured SR function and phosphorylation, showed a net increase in the extent of phosphorylation of TnI and a higher-M_r protein (Fig. 4), most probably C-protein (Jeacock & England, 1980; Hartzell, 1984). In control hearts, TnI contained 1.22 ± 0.07 ($n = 9$) mol of P_i/mol and in hearts stimulated with isoprenaline TnI contained 1.72 ± 0.07 ($n = 7$) mol of P_i/mol. Associated with this increase in TnI and C-protein phosphorylation was a rightward shift of the plot of myofibrillar Mg^{2+}-dependent ATPase activity versus pCa (Fig. 5).

Discussion

Evidence from studies *in vitro* and *in vivo* strongly suggests that cyclic AMP-dependent phosphorylation of phospholamban in SR and of TnI and C-protein in the myofibrils may be responsible for at least part of the catecholamine effects on the mammalian myocardium (Tsien, 1977; Katz,

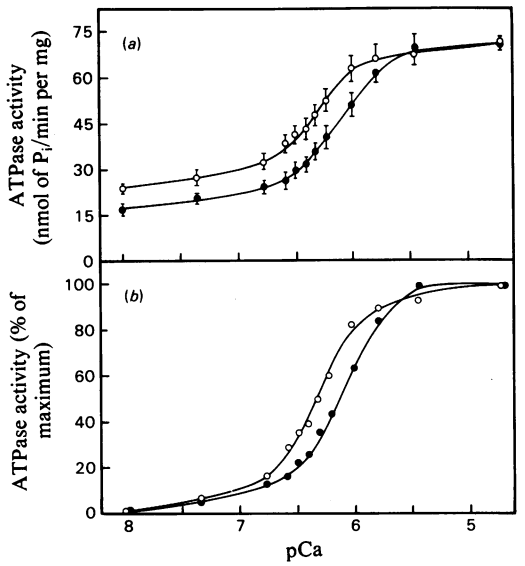

Fig. 5. *The pCa–Mg^{2+}-dependent ATPase activity relation for myofibrils prepared from rabbit hearts freeze-clamped during perfusions under control conditions* (○) *and at the peak of the inotropic response to 1 μM-isoprenaline* (●)
(*a*) Data are presented as means ± S.E.M. for nine control and seven isoprenaline-stimulated hearts. See the Materials and methods section for details. (*b*) Relative ATPase activity of myofibrils from control and isoprenaline-stimulated hearts. Basal ATPase activity, at pCa 8, was subtracted from the mean total ATPase activity, at each pCa shown in (*a*) before normalization.

1979; Kranias & Solaro, 1983; Hartzell, 1984). We have previously shown that an 11000-M_r protein in SR and TnI in the myofibrils are phosphorylated in beating hearts in response to β-adrenergic stimulation (Kranias & Solaro, 1982). In the present study, we report that a 24000-M_r protein was sometimes additionally phosphorylated in SR, and this phosphoprotein appeared to be interconvertible with the 11000-M_r protein. As such these phosphoproteins were identical with cardiac SR proteins from other species (Bidlack & Shamoo, 1980; Lamers & Stinis, 1980; Jones *et al.*, 1981; Louis *et al.*, 1982). In addition, we also show evidence for phosphorylation of C-protein, in addition to TnI in myofibrils prepared from isoprenaline-stimulated rabbit hearts. However, to establish the significance of SR, TnI and C-protein phosphorylation in the intact cell, it was necessary to demonstrate that changes in phosphorylation *in vivo* were correlated with functional alterations in SR and myofibrils.

Lindemann *et al* (1983) reported stimulation of the Ca^{2+}-dependent ATPase activity in SR vesicles isolated from isoprenaline-stimulated guinea-pig ventricles. The Ca^{2+}-dependent ATPase activities were very low in that study, and, as pointed out by those authors, the activity may not have been coupled to the transport of Ca^{2+}. Thus it was important to show clearer evidence for changes in the function of SR vesicles upon isoprenaline stimulation of the hearts, and for this reason we measured Ca^{2+} transport by SR vesicles in microsomal fractions and in homogenates prepared from control and stimulated hearts.

Our findings indicate that, in both microsomal and unfractionated SR vesicles, Ca^{2+} transport at low [Ca^{2+}] (<1 μM) was about 2–3 times higher in vesicles from isoprenaline-stimulated hearts than in those from controls. This relative increase in activity is of the same order as that obtained by several laboratories with phosphorylation of SR vesicles *in vitro* by cyclic AMP-dependent protein kinase (LaRaia & Morkin, 1974; Tada *et al.*, 1974; Hicks *et al.*, 1979). Studies *in vitro* have also previously demonstrated that stimulation of SR Ca^{2+} transport by cyclic AMP-dependent phosphorylation is associated with an increase in the rates of individual steps of the Ca^{2+}-dependent ATPase reaction sequence, resulting in an overall enhancement of the apparent affinity of the pump for Ca^{2+} (Kranias *et al.*, 1980*a*). In the present study we also showed that the plot of velocity of Ca^{2+} transport versus pCa, in unfractionated SR, was shifted to the left, as expected from findings *in vitro*.

Perfusion of rabbit hearts with isoprenaline resulted in net phosphorylation of TnI as well as phosphorylation of a protein of higher M_r, most probably C-protein, which is associated with myosin and may be important in regulation of actomyosin ATPase activity (Yamamoto & Moos, 1983) and/or thick-filament assembly (Offer, 1972). There was no evidence for a change in myosin P-light-chain phosphorylation with isoprenaline, and this is in agreement with previous studies using rabbit hearts (Holroyde *et al.*, 1979*a*; Stull *et al.*, 1981). Myofibrils prepared from rabbit hearts stimulated with isoprenaline, and thus phosphorylated by the endogenous cyclic AMP pathway, showed a lower sensitivity to Ca^{2+} of the myofibrillar ATPase activity. This shift in Ca^{2+} affinity was qualitatively the same as in myofibrils phosphorylated *in vitro* by incubation with exogenous cyclic AMP and cyclic AMP-dependent protein kinase (Ray & England, 1976; Holroyde *et al.*, 1979*b*; Yamamoto & Ohtsuki, 1982). Furthermore, there was no change in maximal activity, and this finding is in agreement with previous studies *in vitro* by several investigators, including ourselves (Ray & England, 1976; Holroyde *et al.*, 1979*a*; Yamamoto & Ohtsuki, 1982). Our findings

also agree with reports on chemically skinned preparations of rabbit cardiac muscle, in which it was shown that phosphorylation was associated with shifts in half-maximal tension development to higher free Ca^{2+} concentrations, with no change in maximal tension (Mope *et al.*, 1980; Herzig *et al.*, 1981). However, there have been conflicting reports on the effect of phosphorylation *in vivo* on the ATPase activity of myofibrils, in preparations from rat hearts perfused with isoprenaline. In one study (Resink & Gevers, 1981) it was reported that both $V_{max.}$ and Ca^{2+}-sensitivity of the actomyosin ATPase activity were significantly depressed, whereas in a similar study (Sistare *et al.*, 1981) it was reported that $V_{max.}$ was greatly elevated with no change in sensitivity of the myofibrillar ATPase activity to Ca^{2+}. Further experiments are probably necessary to elucidate the reasons for these differences in results, which may be related to species differences (rat versus rabbit) or differences in the isoenzymic form of myosin or other proteins in the same species (Martin *et al.*, 1982).

Previous schemes (Katz, 1979; Kranias & Solaro, 1983), based mainly on findings *in vitro*, have suggested a role for SR and myofibrillar phosphorylation in mediating some of the effects of catecholamines on cardiac function. The present work shows that SR, TnI and C-protein are phosphorylated at the peak of the inotropic response to isoprenaline, and that these phosphorylations, which occurred by mechanisms *in vivo*, are associated with an increased rate of Ca^{2+} transport by SR vesicles and a decreased sensitivity of the myofibrillar Mg^{2+}-dependent ATPase activity to Ca^{2+}. Exactly how these phosphorylations are related to the altered mechanical properties of the heart during catecholamine stimulation is not clear. Extents of SR phosphorylation appear to parallel the temporal change in relaxation rate with pulse perfusion of guinea-pig hearts with catecholamines (Lindemann *et al.*, 1983), but extents of TnI phosphorylation in rabbit heart do not (Stull *et al.*, 1981). For C-protein, a correlation of extent of phosphorylation with relaxation rate has been shown in frog heart (Hartzell, 1984), but not in mammalian hearts (Jeacock & England, 1980). Thus there may be complex interrelations between SR and myofibrillar phosphorylation, with the effect of phospholamban phosphorylation on intracellular Ca^{2+} movements playing a dominant role in the mammalian heart and with myofibrillar phosphorylation being more important in the amphibian heart.

We are grateful to Denise Reeves, Mark Rocklin and Elizabeth Howe-Cornwell for providing excellent technical assistance, to Ms. Gwen Kraft 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 RO1HL22231, RO1HL26057, PO1HL22619 (III B, IV A), by a Grant-in-Aid from the S.W. Ohio Chapter of the American Heart Association and by Research Career Development Awards HL00464 (R. J. S.) and HL00775 (E. G. K.).

References

Bidlack, J. M. & Shamoo, A. E. (1980) *Biochim. Biophys. Acta* **632**, 310–325

Briggs, F. N., Poland, J. L. & Solaro, R. J. (1977) *J. Physiol. (London)* **266**, 587–594

Capony, J.-P., Rinaldi, M. L., Guilleux, F. & Demaille, J. (1983) *Biochim. Biophys. Acta* **728**, 83–91

Corbin, J. D. & Keely, S. L. (1977) *J. Biol. Chem.* **252**, 910–918

Corbin, J. D., Sugden, P. H., Lincoln, T. M. & Keely, S. L. (1977) *J. Biol. Chem.* **252**, 3854–3861

Davis, B. A., Schwartz, A., Samaha, F. J. & Kranias, E. G. (1983) *J. Biol. Chem.* **258**, 13587–13591

England, P. J. (1976) *Biochem. J.* **160**, 295–304

Harigaya, S. & Schwartz, A. (1969) *Circ. Res.* **25**, 781–794

Hartzell, C. F. (1984) *J. Gen. Physiol.* **83**, 563–588

Hayes, J. S., Brunton, L. L. & Mayer, S. E. (1980) *J. Biol. Chem.* **255**, 5113–5119

Herzig, J. W., Köhler, G., Pfitzer, G., Rüegg, J. C. & Wölffle, G. (1981) *Pflügers Arch.* **391**, 208–212

Hicks, M. J., Shigekawa, M. & Katz, A. M. (1979) *Circ. Res.* **44**, 384–391

Holroyde, M. J., Howe, E. & Solaro, R. J. (1979*a*) *Biochim. Biophys. Acta* **586**, 63–69

Holroyde, M. J., Small, D. A. P., Howe, E. & Solaro, R. J. (1979*b*) *Biochim. Biophys. Acta* **587**, 629–637

Jeacock, S. A. & England, P. J. (1980) *FEBS Lett.* **122**, 129–132

Jones, L. R., Maddock, S. W. & Hathaway, D. R. (1981) *Biochim. Biophys. Acta* **641**, 242–253

Katz, A. M. (1979) *Adv. Cyclic Nucleotide Res.* **11**, 303–343

Kirchberger, M. A. & Antonetz, T. (1982) *Biochem. Biophys. Res. Commun.* **105**, 152–156

Kirchberger, M. A., Tada, M. & Katz, A. M. (1974) *J. Biol. Chem.* **249**, 6166–6173

Kopp, J. S. & Barany, M. (1979) *J. Biol. Chem.* **254**, 12007–12012

Kranias, E. G. & Solaro, R. J. (1982) *Nature (London)* **298**, 182–184

Kranias, E. G. & Solaro, R. J. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 33–38

Kranias, E. G., Mandel, F., Wang, T. & Schwartz, A. (1980*a*) *Biochemistry* **19**, 5434–5439

Kranias, E. G., Bick, R. & Schwartz, A. (1980*b*) *Biochim. Biophys. Acta* **628**, 438–450

Kranias, E. G., Schwartz, A. & Jungmann, R. A. (1982) *Biochim. Biophys. Acta* **709**, 28–37

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Lamers, J. M. J. & Stinis, J. T. (1980) *Biochim. Biophys. Acta* **624**, 443–459

LaRaia, P. J. & Morkin, E. (1974) *Circ. Res.* **35**, 298–305

LePeuch, C. J., Guilleux, J. C. & Demaille, J. G. (1980) *FEBS Lett.* **114**, 165–168
Lindemann, J. P., Jones, L. R., Hathaway, D. R., Henry, B. G. & Watanabe, A. M. (1983) *J. Biol. Chem.* **258**, 464–471
Louis, C. F., Maffitt, M. & Jarvis, B. (1982) *J. Biol. Chem.* **257**, 15182–15186
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Manalan, A. S. & Jones, L. R. (1982) *J. Biol. Chem.* **257**, 10052–10062
Martin, A. F., Pagani, E. D. & Solaro, R. J. (1982) *Circ. Res.* **50**, 117–124
Moir, A. J. G., Solaro, R. J. & Perry, S. V. (1980) *Biochem. J.* **185**, 505–513
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
Offer, G. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 87–93
Pagani, E. D. & Solaro, R. J. (1984) *Am. J. Physiol.* **247**, 909–915
Palmer, W. K., McPherson, J. M. & Walsh, D. A. (1980) *J. Biol. Chem.* **255**, 2663–2666
Ray, K. P. & England, P. J. (1976) *FEBS Lett.* **70**, 11–16
Rinaldi, M. L., Capony, J. P. & Demaille, J. G. (1982) *J. Mol. Cell. Cardiol.* **14**, 279–289
Resink, T. J. & Gevers, W. (1981) *Cell Calcium* **2**, 105–123
Schwartz, A., Entman, M. L., Kaniike, K., Lane, L. K., Van Winkle, W. B. & Bornet, E. P. (1976) *Biochim. Biophys. Acta* **426**, 57–72
Sistare, F. D., Lichtenberg, L., Sugg, R. G., McFarland, S. A. & Villar-Palasi, C. (1981) *J. Cyclic Nucleotide Res.* **7**, 85–93
Solaro, R. J. & Briggs, F. N. (1974) *Circ. Res.* **34**, 531–540
Solaro, R. J., Pang, D. C. & Briggs, F. N. (1971) *Biochim. Biophys. Acta* **245**, 259–262
Solaro, R. J., Moir, A. J. G. & Perry, S. V. (1976) *Nature (London)* **246**, 615–617
Stull, J. T., Sanford, C. G., Manning, D. R., Blumenthal, D. K. & High, C. W. (1981) *Cold Spring Harbor Conf. Cell Proliferation. Protein Phosphorylation* **8**, 823–839
Suko, J. (1973) *J. Physiol. (London)* **228**, 563–582
Tada, M., Kirchberger, M. A., Repke, D. I. & Katz, A. (1974) *J. Biol. Chem.* **249**, 6174–6180
Tada, M., Kirchberger, M. A. & Katz, A. M. (1975) *J. Biol. Chem.* **250**, 2640–2647
Tada, M., Yamada, M., Ohmori, F., Kuzuya, T., Inui, M. & Abe, H. (1980) *J. Biol. Chem.* **255**, 1985–1992
Tsien, R. W. (1977) *Adv. Cyclic Nucleotide Res.* **8**, 363–420
Wray, H. L. & Gray, R. R. (1977) *Biochim. Biophys. Acta* **461**, 441–459
Yamamoto, K. & Ohtsuki, I. (1982) *J. Biochem. (Tokyo)* **91**, 1669–1677
Yamamoto, K. & Moos, C. (1983) *J. Biol. Chem.* **258**, 8395–8401