Phospholamban is a good substrate for cyclic GMP-dependent protein kinase *in vitro*, but not in intact cardiac or smooth muscle

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1. Cyclic GMP-dependent protein kinase phosphorylates purified phospholamban. It also phosphorylates phospholamban present in vesicles of cardiac sarcoplasmic reticulum and smooth muscle microsomal fractions, and in transformants of *Escherichia coli* which contain a plasmid into which a gene encoding phospholamban has been inserted. 2. *In vitro* the phospholamban present in cardiac sarcoplasmic reticulum membranes is a better substrate for cyclic GMP-dependent protein kinase than for cyclic AMP-dependent protein kinase. 3. Studies using [³²P]P_i to label the cellular ATP in intact cardiac or smooth muscle failed to demonstrate that phosphorylation of phospholamban occurs in response to stimuli which increase intracellular cyclic GMP. Possible reasons for this functional separation between increased cyclic GMP and phosphorylation of phospholamban are discussed.

INTRODUCTION

Phospholamban is an integral membrane protein of the sarcoplasmic reticulum (SR) of cardiac muscle (Tada *et al.*, 1974) and some types of smooth muscle (Raeymaekers & Jones, 1986). In the heart the phosphorylation of phospholamban by cyclic AMP-dependent protein kinase stimulates the activity of the Ca²⁺-stimulated ATPase of the SR (Tada *et al.*, 1974). This effect has been used to explain the increased rate of relaxation observed when cardiac muscle is perfused with agents which increase the concentration of intracellular cyclic AMP (Huggins & England, 1985).

More recently, it has been shown that phospholamban may also be phosphorylated by cyclic GMP-dependent protein kinase on the same residue as that phosphorylated by cyclic AMP-dependent protein kinase (Raeymaekers *et al.*, 1988a). This phosphorylation stimulates ATP-dependent Ca²⁺ uptake into SR vesicles purified from cardiac (Raeymaekers *et al.*, 1988a) or smooth (Raeymaekers *et al.*, 1988b) muscle. This observation is of particular interest in smooth muscle, where a number of vasodilators, such as endothelium-derived relaxing factor and organic nitrates, are thought to act by increasing intracellular cyclic GMP (Rapoport *et al.*, 1983; Itoh *et al.*, 1985). Also, it has been found in aorta that the time course of the activation of cyclic GMP-dependent protein kinase parallels that of the increase in cyclic GMP and tissue relaxation (Fiscus *et al.*, 1985). Observations on smooth muscle tissue preparations and single cells have shown that, after inducing contraction, increasing intracellular cyclic GMP by incubation with sodium nitroprusside or 8-bromo cyclic GMP decreases the intracellular free Ca²⁺ concentration (Morgan & Morgan, 1984; Rashatwar *et al.*, 1987; Kai *et al.*, 1987). It is therefore possible that cyclic GMP-stimulated phosphorylation of phospholamban might mediate the relaxing effect of some vasodilators. Cardiac muscle also contains large amounts of cyclic GMP-dependent protein

kinase (Lincoln & Corbin, 1983), and cyclic GMP in this tissue increases after cholinergic stimulation (England, 1976), although the role of cyclic GMP in the heart remains unclear (Lincoln & Corbin, 1983). However, it might be expected that phospholamban would be phosphorylated in cardiac or smooth muscle if these tissues were treated with appropriate agents in order to increase intracellular cyclic GMP.

The work described in this paper confirms that phospholamban is a substrate for cyclic GMP-dependent protein kinase, when purified phospholamban and vesicles of cardiac SR or microsomes (microsomal fractions) prepared from pulmonary artery are used. In addition, phospholamban is shown to be phosphorylated by cyclic GMP-dependent protein kinase when expressed in transformants of *Escherichia coli* which contain a plasmid in which a synthetic gene encoding phospholamban is present. Analysis of the kinetics of phosphorylation show that phospholamban is a good substrate for cyclic GMP-dependent protein kinase compared with cyclic AMP-dependent protein kinase *in vitro*. However, perfusion studies have failed to demonstrate that cyclic GMP-dependent phosphorylation occurs in whole tissue. This indicates that phospholamban and cyclic GMP-dependent protein kinase and/or cyclic GMP are functionally separated in the intact cell.

Some of the work described here has been reported previously in short abstract form (Huggins, 1988; Mattinsley & Huggins, 1988).

EXPERIMENTAL

Purification of membranes and proteins

Guinea-pig cardiac SR was prepared as described by Huggins & England (1987). Where hearts were first perfused with [³²P]P_i (see below), they were rapidly removed from the perfusion needle and homogenized within 5 s. For these hearts, buffers additionally con-

Abbreviation used: SR, sarcoplasmic reticulum.

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tained 30 mM-sodium phosphate to inhibit dephosphorylation. Pellets were resuspended in 1.17 M-sucrose/10 mM-Hepes, pH 7.2, and frozen in liquid N₂ until SDS sample buffer was added. A sample was assayed for protein so that the protein loaded on to SDS gels could be standardized between tracks.

Sheep pulmonary arteries were obtained from a local abattoir, transported to the laboratory on ice and dissected free from connective, fatty and endothelial tissues. Smooth muscle microsomes from these arteries were prepared as described by Morel *et al.* (1981). Microsomes were prepared from de-endothelialized rabbit abdominal aortic rings as follows (all operations were performed at 4 °C). Each ring was homogenized by using the smallest probe of a Polytron homogenizer for two 5 s bursts at setting 5 in 4 ml of 20 mM-Hepes (pH 7.4), containing 0.25 M-sucrose, 1 μ M of pepstatin A/ml, 1 μ M of leupeptin/ml, 1 μ M of antipain/ml, and 15 mM-2-mercaptoethanol. The homogenate was centrifuged at 1500 g for 15 min and then at 27000 g for 15 min without intermediate deceleration. The supernatant was filtered through glass wool and centrifuged for 30 min at 75000 rev./min (200000 g_{av.}) in the TL100.2 rotor of a Beckman TL100 centrifuge. The final pellets were directly dissolved in 2% (w/v) SDS/62.5 mM-Tris/HCl/20% (w/v) glycerol/40 mM-dithiothreitol by incubation at 30 °C with frequent agitation.

Cyclic GMP-dependent protein kinase was purified up to the affinity-chromatography step on 8-(2-aminoethyl)-amino-cyclic AMP-Sepharose 4B (obtained from Pharmacia, Uppsala, Sweden) as described by Lincoln (1983). Eluted kinase was further purified as follows. Cyclic AMP was added to the kinase eluted from the affinity column to give a final concentration of 10 mM. The enzyme was dialysed for 12 h at 4 °C against 20 mM-sodium phosphate/2 mM-EDTA/25 mM-2-mercaptoethanol, pH 7. This material was then applied to a Mono Q f.p.l.c. column which was first washed with 20 mM-Tris/1 mM-EDTA/5% glycerol/7.5 mM-2-mercaptoethanol, pH 7.5, and then eluted with a gradient of 0–1 M-NaCl in the same buffer. The eluted kinase (at 0.23–0.28 M-NaCl) was concentrated by dialysis against solid sucrose, followed by 50% glycerol/20 mM-sodium phosphate/2 mM-EDTA/25 mM-2-mercaptoethanol, pH 7, and stored at –20 °C. Scanning of an SDS/polyacrylamide gel showed the kinase to be ~90% pure with respect to total protein. The preparation had a maximum specific activity with 100 μ M of the peptide Arg-Lys-Arg-Ser-Arg-Ala-Glu (obtained from Peninsula Laboratories, St. Helens, Lancs., U.K.) (Glass & Krebs, 1982) of 2.4 μ mol/min per mg. The maximum stimulation of activity by cyclic GMP was 13-fold.

The catalytic subunit of cyclic AMP-dependent protein kinase was purified to homogeneity as described by Reimann & Beham (1983). The specific kinase activity with Malentide (obtained from Ocean Biologicals, Edmonds, WA, U.S.A.), the peptide corresponding to the phosphorylation site on the β -subunit of phosphorylase kinase (Malencik & Anderson, 1983), was 0.7 μ mol/min per mg. The inhibitor protein of cyclic AMP-dependent protein kinase was purified from rabbit skeletal muscle as described by Schlender *et al.* (1983).

Phospholamban was purified from bovine hearts as follows. Eight lots of 250 g minced lean bovine ventricle were homogenized (see Jones *et al.*, 1985) and then centrifuged either at 5000 g for 10 min or at 3000 g for

3 h at 4 °C. The combined supernatants were filtered through glass wool and centrifuged at 22000 g for 1 h to pellet crude SR membranes. The remainder of the preparation was as described by Jones *et al.* (1985).

Phosphorylation conditions

Purified phospholamban was phosphorylated in a solution containing 50 mM-Hepes/Tris, 100 mM-MgCl₂, 2.5 mM-EGTA, 0.2% (w/v) Triton X-100, 0.5 mM-dithiothreitol, 60 μ g of phospholamban/ml, 0.8 μ M-cyclic GMP, 5 μ g of cyclic GMP-dependent protein kinase/ml and 0.1 mM- $[\gamma$ -³²P]ATP, sp. radioactivity 50 TBq/mol (total volume 40 μ l). The solution was incubated at 30 °C for 2 min in the absence of kinase and for 5 min after kinase addition. At this point 10 μ l of 15% SDS/125 mM-Tris/HCl/40 mM-dithiothreitol/20% glycerol, pH 6.8, was added and the sample heated to 100 °C for 4 min before SDS/polyacrylamide-gel electrophoresis.

Cardiac- and smooth-muscle membranes were phosphorylated in the same solution, except that phospholamban and Triton X-100 were excluded and membranes included (30 and 450 μ g of protein/ml for cardiac SR and sheep pulmonary-artery microsomes respectively). The phosphorylation reaction was performed for 10 min at 30 °C and stopped by addition of 0.5 ml of 0.15 M-sucrose/0.1 M-EDTA/10 mM-Hepes/Tris, pH 7. Membranes were centrifuged to a pellet at 350000 g_{av.} (100000 rev./min in the TL100.2 rotor of a Beckman TL100 centrifuge) and then solubilized in 2% SDS/62.5 mM-Tris/HCl/20% glycerol/40 mM-dithiothreitol, pH 6.8, by incubation with agitation at 30 °C. In some cases samples were additionally heated to 100 °C for 4 min.

In experiments where the kinetics of phospholamban phosphorylation were studied, cardiac SR was phosphorylated as above, but the concentration of SR was varied, and in some cases the catalytic subunit of cyclic AMP-dependent protein kinase was present instead of cyclic GMP and cyclic GMP-dependent protein kinase. The rate of phospholamban phosphorylation by either kinase was linear with time if a 2 min incubation at 30 °C was used (results not shown). There was no detectable phosphorylation in this period in the absence of kinase or SR.

E. coli pellets were phosphorylated by incubation for 10 min at 30 °C in 70 mM-Hepes/Tris containing 17.5 mM-MgCl₂, 14 mM-EGTA, 0.3% Triton X-100, 0.7 mM-dithiothreitol, 0.7 μ M-cyclic GMP, 0.5 μ g of cyclic GMP-dependent protein kinase/ml and 60 μ M- $[\gamma$ -³²P]ATP, sp. radioactivity 50 TBq/mol. The reaction was stopped as for purified phospholamban (see above), except that the final incubation was for 15 min at 30 °C and only on some occasions was an additional incubation at 100 °C for 4 min performed.

Methods with intact tissues

Guinea-pig hearts were perfused with [³²P]P_i (25 MBq/heart) as described by England (1975). Hearts were also perfused with Krebs medium alone for 2 min, with Krebs medium for 1½ min and 50 nM-L-isoprenaline for 30 s, or with 1 μ M-carbamoylcholine chloride for 2 min. In some cases 8-bromo cyclic GMP or 8-bromo GMP (Na⁺ salts) were added directly to the medium containing [³²P]P_i to give a nucleotide concentration of 0.1 mM for 15 min.

Similar experiments were performed with de-endo-

thelialized rings of rabbit abdominal aorta (chosen because it is relatively free from connective tissue); 5 ml water-jacketed organ baths were constructed and 5 mm-wide rings were bathed in low- P_i Krebs medium (England, 1975) for 15 min, in the same medium containing 7.5 MBq of [32 P] P_i for 2 h and then in non-radioactive medium again for 5 min. Contraction and relaxation were induced as described in the Results and discussion section, and then the rings were frozen by Wollenberger clamps at -186°C . Tension was measured in parallel experiments, but found to be very reproducible in terms of time courses and drug effects between preparations.

Other methods

The methods used in preparing transformants of *E. coli* were as follows. A synthetic gene encoding the primary protein sequence of phospholamban described by Fujii *et al.* (1987) was prepared by synthesizing six oligonucleotides using an ABI DNA synthesizer. These oligonucleotides were annealed and ligated by standard techniques (Maniatis *et al.*, 1982) and the synthetic gene was cloned in the vector pMGI, the crucial feature of which is that it permits the expression of cloned genes in *E. coli* under the control of the λ pL promoter (Young *et al.*, 1983). Expression driven by this promoter is temperature-sensitive in strain AR58, which carries the cI857ts mutation (Gross *et al.*, 1985). The resulting plasmid (pSK32) thus contains the structural gene for phospholamban under the control of the λ pL promoter. Cells of AR58 transformed with pSK32 were grown to an A_{600} of 0.6 in LB (Maniatis *et al.*, 1982), containing ampicillin at 100 $\mu\text{g}/\text{ml}$, at 32°C . Expression of phospholamban was induced by shifting the cultures to

42°C , and had attained a maximum 2 h after temperature shift. *E. coli* pellets were resuspended by vortex-mixing in 1% Triton X-100/10 mM-Hepes, pH 7.4.

SDS gels were run with a 15% (w/v)-acrylamide separating gel as described by Laemmli (1970). Bio-Rad protein standards were used. Autoradiographs were exposed within the linear range of absorbance (Bonner & Laskey, 1977) and were scanned with a Bio-Rad VD620 video densitometer. Peak areas were determined by using Bio-Rad software for an IBM-type personal computer.

Protein was measured as described by Lowry *et al.* (1951), or as described by Bradford (1976) for kinases, or by Flores (1978) for purified phospholamban.

RESULTS AND DISCUSSION

Experiments with purified kinase

Purified phospholamban was phosphorylated when incubated with cyclic GMP-dependent protein kinase in the presence of $0.8\ \mu\text{M}$ -cyclic GMP (Fig. 1a, track 1). Inclusion of enough of the heat-stable inhibitor protein of cyclic AMP-dependent protein kinase to inhibit this kinase completely (results not shown) did not decrease phosphate incorporation into phospholamban by cyclic GMP-dependent protein kinase (Fig. 1a, track 3). Hence the observed phosphorylation was not due to contamination of either of the protein preparations with cyclic AMP-dependent protein kinase or its catalytic subunit.

Some proteins are better substrates for kinases when denatured than when they possess their native conformation. Also, it is possible that the method used here to purify phospholamban (Jones *et al.*, 1985) purifies a major proteolytic product of phospholamban, namely

Fig. 1. Phosphorylation of phospholamban, either purified or present in various membranes, by cyclic GMP-dependent protein kinase and [γ - 32 P]ATP

Shown are autoradiographs of SDS/polyacrylamide gels on which samples have been separated after phosphorylation. The following substrates were used: (a) purified phospholamban (PLB); (b) cardiac SR; (c) pulmonary artery microsomes; (d) *E. coli* in which phospholamban had been expressed (see the text). Samples were solubilized in SDS by incubation at 30°C or for 4 min at 100°C ('Boil') as indicated. The high- (approx. 28000) and low- (5500) M_r forms of phosphorylated phospholamban are indicated by 'H' and 'L' respectively. The inhibitor protein of cyclic AMP-dependent protein kinase, PKI, was included as shown in the incubations with purified phospholamban.

with the first nine *N*-terminal amino acids absent (Fujii *et al.*, 1987). We therefore investigated whether phospholamban could be phosphorylated when present in membranes prepared from heart or smooth muscle, where the phospholamban would not be expected to be denatured or proteolysed. Phospholamban was identified by its property of migrating with various mobilities in SDS/polyacrylamide gels (Kirchberger & Antonetz, 1982; Wegener & Jones, 1984). Solubilizing samples at low temperatures (e.g. 30 °C) causes phospholamban to migrate as a pentamer with an apparent M_r of 27000–29000, whereas solubilization at 100 °C causes a monomeric form (M_r ~5500) to be observed. Fig. 1(b) shows that a protein present in cardiac SR was phosphorylated by cyclic GMP-dependent protein kinase. The phosphorylated protein migrated with a M_r of 28000 or 5500, depending on the conditions used to solubilize the SR before electrophoresis. This demonstrates that the phosphorylated protein was phospholamban.

Also, microsomes prepared from sheep pulmonary artery smooth muscle were incubated with cyclic GMP-dependent protein kinase and cyclic GMP. The results of a typical autoradiograph (Fig. 1c) show that in smooth muscle membranes phospholamban was not the predominant phosphoprotein. In these experiments phosphorylated cardiac SR was used as a standard to indicate the positions of the high- and low- M_r forms of phospholamban (indicated by 'H' and 'L' respectively in Fig. 1c). It can be seen that in smooth muscle microsomes a protein is present which is phosphorylated by cyclic GMP-dependent protein kinase and which may be identified as phospholamban on the basis that it has the same electrophoretic properties as cardiac phospholamban.

These results with muscle membranes support the very recent report by Raeymaekers *et al.* (1988a), who investigated phospholamban phosphorylation using a proteolytic fragment of cyclic GMP-dependent protein kinase that was catalytically active. In addition, results have been obtained with *E. coli* into which a plasmid had been inserted which contained a synthetic gene encoding the sequence of dog cardiac phospholamban under the control of a temperature-sensitive promoter (see the Experimental section). Fig. 1(d) shows that cyclic GMP-dependent protein kinase phosphorylates phospholamban present in the bacteria, and that the phosphorylated protein undergoes the same M_r transformations as are seen when phospholamban is present in mammalian membranes. These results confirm that phospholamban, and not a protein with similar properties, is phosphorylated by cyclic GMP-dependent protein kinase.

Comparison of phospholamban phosphorylation by cyclic AMP- and cyclic GMP-dependent protein kinases

The above results show that cyclic GMP-dependent protein kinase may phosphorylate phospholamban when this is presented to the enzyme in several forms. In an attempt to assess the physiological significance of this observation, we have used guinea-pig cardiac SR as a substrate for cyclic GMP-dependent protein kinase or the constitutively active catalytic subunit of cyclic AMP-dependent protein kinase. It is known that phospholamban is a substrate for cyclic AMP-dependent protein kinase in perfused hearts (Lindemann *et al.*,

Fig. 2. Example of the kinetics of the phosphorylation of phospholamban, present in vesicles of cardiac SR, by cyclic GMP-dependent protein kinase

The lines show the best fits of the data to Michaelis-Menten (---) or co-operative (—) kinetics

1983). Therefore, by comparing the kinetics of phospholamban phosphorylation by the two different kinases, it might be possible to make conclusions about whether activation of cyclic GMP-dependent protein kinase in whole tissue is likely to result in phospholamban phosphorylation.

Incubations were performed with various concentrations of SR protein, and phospholamban phosphorylation was determined as described in the Experimental section. Fig. 2 shows one example of the curve fitting obtained to the data for cyclic GMP-dependent protein kinase. Surprisingly, the data for each kinase fitted co-operative rather than Michaelis-Menten kinetics. The explanation for the observed co-operativity is not fully clear, but may represent a local compartmentation of phospholamban caused by either its subunit structure or its association with the SR membrane. Thus, after one subunit of phospholamban is phosphorylated by kinase and dissociation of the phospholamban-kinase complex occurs, the probability of kinase binding to another nearby phospholamban subunit may be greater than the probability that the kinase dissociates first into the bulk aqueous phase. An alternative explanation for the data is that phospholamban phosphorylation may be an ordered process, so that the phosphorylation of one subunit necessarily follows the phosphorylation of another subunit until any particular multimer of phospholamban is phosphorylated to 1 mol of phosphate/mol of subunit. Similar substrate-dependent mechanisms have been proposed to explain the negative co-operativity of myosin light-chain kinase activity (Persechini & Hartshorne, 1983).

Table 1 shows the kinetic parameters determined for the phosphorylation of cardiac SR phospholamban by cyclic AMP- or cyclic GMP-dependent protein kinases. The kinetics of histone phosphorylation, determined under the same conditions as for phospholamban phosphorylation, are also shown. Histone is known to be a relatively good substrate for cyclic GMP-dependent protein kinase (Lincoln & Corbin, 1983). Table 1 shows that cardiac SR phospholamban is as good a substrate for cyclic GMP-dependent protein kinase as for cyclic AMP-dependent protein kinase. The $K_{0.5}$ and co-operativity values for the two enzymes are very similar, but the V_{max} of the cyclic GMP-dependent protein kinase for phospholamban is approx. 4 times that observed for cyclic AMP-dependent protein kinase. It might therefore be expected that, just as increases in intracellular cyclic AMP can cause phospholamban phosphorylation in

Table 1. Relative kinetics of phospholamban phosphorylation by the catalytic subunit (C-SUB) of cyclic AMP-dependent protein kinase or by cyclic GMP-dependent protein kinase (cGK) in the presence of 1 μ M-cyclic GMP

Parameters for cardiac SR are means $\pm$ S.D. for three independent experiments. Values determined for Sigma histone 2a are shown for comparison.

	C-SUB	cGK
Cardiac SR		
$K_{0.5}$ (mg/ml)	0.59 ± 0.16	0.41 ± 0.14
Relative V_{max}	100	397 ± 37
h	2.04 ± 0.5	1.9 ± 0.65
Histone 2a		
K_m (μ M)	600	75
V_{max} (nmol/min per μ g)	0.226	2.25

whole tissue (Lindemann *et al.*, 1983), increases in intracellular cyclic GMP would do likewise.

Phosphorylation experiments with intact tissue

Guinea-pig hearts were perfused with [32 P]P_i to label intracellular ATP, as described in the Experimental section. Hearts were also perfused with carbamoylcholine or 8-bromo cyclic GMP, known to increase tissue cyclic GMP (Lincoln & Corbin, 1983), or isoprenaline to raise tissue cyclic AMP (England, 1976). Control perfusions were also performed with either 8-bromo GMP or Krebs medium alone. The negative inotropic response of hearts to carbamoylcholine or nucleotides was determined in two ways. Firstly, contraction was measured during addition of these drugs alone. Neither carbamoylcholine nor 8-bromo cyclic GMP affected tension development. In addition, the effect of these agents on the positive

inotropic response of the hearts to 60 s treatment with 1 nM-isoprenaline was determined. Carbamoylcholine (2 min, 1 μ M) and 8-bromo cyclic GMP (15 min, 0.2 mM) caused a 62 % and 66 % decrease in the inotropic response of isoprenaline respectively. 8-Bromo GMP (15 min, 0.2 mM) did not decrease the response of the hearts to isoprenaline.

Fig. 3(a) shows the phosphorylation pattern observed in an SR fraction prepared from the hearts. Isoprenaline resulted in an increased phosphorylation of phospholamban, which is identified by its two interconvertible M_r forms. The observed 32 P incorporation in phospholamban after various treatments was quantified by densitometric scanning of the high- M_r band of phospholamban, obtained when samples were solubilized in SDS at 30 °C. Treatment for 30 s with 50 nM-isoprenaline gave a 32 P incorporation of 1859 ± 107 % (mean $\pm$ S.E.M. for three hearts) of that in the absence of drug. However, no significant change in phospholamban phosphorylation could be observed after perfusing hearts with carbamoylcholine, 8-bromo cyclic GMP or 8-bromo GMP, within the error of experimental determination (average S.E.M. for the three groups = 61 % of control; $n = 3$ for each group). It was therefore concluded that the response of the guinea-pig hearts to carbamoylcholine and 8-bromo cyclic GMP (a decrease in their ability to respond to isoprenaline) was not mediated through the phosphorylation of phospholamban.

Similar experiments were performed with smooth muscle by using 5 mm-thick rings of de-endothelialized rabbit abdominal aorta. In this case contraction was induced by K⁺ depolarization by using 80 mM-KCl for 10 min or 8 μ M-L-phenylephrine for 10 min. Further addition of 150 nM-sodium nitroprusside for 10 min decreased tension by 9 % for K⁺-depolarized rings and 70 % for phenylephrine-precontracted rings. These effects of nitroprusside are well documented and known to be accompanied by large increases in intracellular cyclic

Fig. 3. Autoradiographs of SDS/polyacrylamide gels on which were separated samples of membranes prepared from (a) guinea-pig hearts or (b) rings of rabbit abdominal aorta

Tissues were perfused or bathed in [32 P]P_i, and with the following as shown and as described in the text: (a) Krebs medium alone (tracks 1 and 2), 1 μ M-carbamoylcholine (tracks 3 and 4), 0.2 mM-8-bromo GMP (tracks 5 and 6), 0.2 mM-8-bromo cyclic GMP (tracks 7 and 8) or 50 nM-isoprenaline (tracks 9 and 10); (b) Krebs medium alone (track 11), 80 mM-KCl with (track 12) or without (track 14) 150 nM-sodium nitroprusside, or 80 μ M-L-phenylephrine with (track 13) or without (track 15) 150 nM-sodium nitroprusside. In (a) samples were either solubilized in SDS by incubation at 30 °C, or for 4 min at 100 °C ('Boil') as indicated. The migration of M_r standards is shown.

GMP (Itoh *et al.*, 1985; Rapoport *et al.*, 1985). In parallel experiments, aortic rings were labelled by bathing in Krebs medium containing [32 P]P_i (see the Experimental section) and freeze-clamped after treatment with Krebs medium alone, with Krebs medium containing 80 mM-KCl or 8 μ M-L-phenylephrine, or with either of these in the presence of sodium nitroprusside. Microsomal membranes were prepared from the rings by a rapid method, and then the entire membrane preparation from each aortic ring was analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. The results of these experiments (Fig. 3b) show that we were unable to demonstrate phosphorylation of phospholamban under these conditions. In some experiments, samples were incubated at 30 °C or 100 °C before electrophoresis, but no phosphoprotein exhibited an M_r transformation typical of phospholamban. Preliminary experiments were also performed using strips of pig gastric smooth muscle, which are reported to contain especially large amounts of phospholamban (Raeymaekers & Jones, 1986). After incubation of this tissue as described above for aortic rings, no increase in phospholamban phosphorylation by nitroprusside was observed (results not shown).

General discussion

This paper reports that, although phospholamban is a relatively good substrate for cyclic GMP-dependent protein kinase *in vitro* (Table 1), under the conditions used here increases in intracellular cyclic GMP do not result in increased phospholamban phosphorylation in intact cardiac or smooth muscle (Fig. 3). The failure to observe an increased phosphorylation in intact heart is unlikely to be due to a phospholamban phosphatase (see, e.g., Kranias & Di Salvo, 1986) dephosphorylating the protein during membrane preparation. This is because membranes were prepared rapidly, at 4 °C, where dephosphorylation is slow (Fowler *et al.*, 1989), and in the presence of phosphatase inhibitors. Also, large increases in phospholamban phosphorylation were observed after raising intracellular cyclic AMP with isoprenaline (Fig. 3a). Cyclic AMP- and cyclic GMP-dependent protein kinases phosphorylate the same serine residue in phospholamban *in vitro* (Raeymaekers *et al.*, 1988a), and so dephosphorylation of phospholamban could only occur if carbamoylcholine and 8-bromo cyclic GMP caused activation of a phosphatase which was not inhibited by low temperatures or phosphate and fluoride. It is possible that, despite the efforts made here to inhibit dephosphorylation, if a very low level of phospholamban phosphorylation occurred after increased cyclic GMP, then phosphatase action might prevent its observation, whereas the large increases in phosphorylation after increased cyclic AMP would still be observed. However, Lindemann *et al.* (1983) found virtually no dephosphorylation when phosphorylated cardiac SR was incubated in homogenates in similar conditions to the membrane preparations used in the present experiments. Similarly, dephosphorylation is not likely to explain the absence of an effect of nitroprusside on phospholamban phosphorylation in smooth muscle, where a very rapid microsome preparation was used.

For a particular protein-phosphorylation reaction to be considered to be of physiological significance, it is necessary to demonstrate that the phosphorylation of this protein correlates with a physiological event in intact tissues or cells (Krebs & Beavo, 1979). We have examined

intact cardiac and smooth muscle at times when various drugs have just achieved a maximal relaxation, but, with agents which are known to increase cyclic GMP, we have failed to observe the phosphorylation of phospholamban. In aorta it is known that the activation of cyclic GMP-dependent protein kinase parallels relaxation when tissue cyclic GMP is increased (Fiscus *et al.*, 1985), and so it would be expected that, if phospholamban phosphorylation were the primary mechanism whereby smooth-muscle relaxation occurs, we should have detected this phosphorylation after the incubations used in these experiments. To our knowledge, activity ratios for cyclic GMP-dependent protein kinase have not been determined in cardiac muscle. However, the concentration of isoprenaline used in these experiments increased cyclic AMP from approx. 2 to 10 nmol/g dry wt., whereas concentrations of muscarinic agonist which give a near-maximal tissue response (as used here) caused cardiac cyclic GMP to increase approx. 3-fold from a basal value of ~ 1 nmol/g dry wt. (England, 1976).

Although the possibility cannot be excluded that phospholamban is phosphorylated by cyclic GMP-dependent protein kinase *in vivo* under different conditions from those used here, our results demonstrate that agents which increase intracellular cyclic GMP are able to have physiological actions on cardiac and smooth muscle without affecting phospholamban phosphorylation. Alternative suggestions have been made as to how activation of cyclic GMP-dependent protein kinase might induce relaxation in smooth muscle. A sarcolemmal Ca²⁺ pump may be stimulated by the action of cyclic GMP-dependent protein kinase (Suematsu *et al.*, 1984; Popescu *et al.*, 1985; Rashatwar *et al.*, 1987). Also, voltage-dependent Ca²⁺ channels may be inhibited (Ousterhout & Sperelakis, 1987) and receptor-operated channel gating affected by rises in intracellular cyclic GMP (Godfraind, 1986). Three membrane proteins have been described which are specifically phosphorylated by cyclic GMP-dependent protein kinase (Parks *et al.*, 1987), and these may be involved in some of the above processes, although the report that one of these substrates is the sarcolemmal Ca²⁺-ATPase itself (Furukawa & Nakamura, 1987), has not been substantiated (Baltensperger *et al.*, 1988). The role of cyclic GMP-related processes in cardiac muscle is still unclear (Lincoln & Corbin, 1983).

A large number of proteins have been shown to be phosphorylated by cyclic GMP-dependent protein kinase. Many of these are also phosphorylated by cyclic AMP-dependent protein kinase. Lincoln & Corbin (1983) have concluded that often in these cases a physiological role for cyclic GMP-dependent protein phosphorylation is unlikely. The phosphorylation of phospholamban by cyclic GMP-dependent protein kinase is an attractive mechanism to explain some of the effects of cyclic GMP, especially in smooth muscle. Our inability to demonstrate that this occurs in intact tissues indicates a functional separation between increases in cyclic GMP and phosphorylation of phospholamban in the cell. In cardiac muscle there is over 10 times more cyclic AMP-dependent protein kinase than cyclic GMP-dependent protein kinase (Lincoln *et al.*, 1976). This may explain why phospholamban is not phosphorylated after an increase in tissue cyclic GMP, but is phosphorylated when cyclic AMP is increased. However, tissue responses to 8-bromo cyclic GMP are normally taken to indicate the involvement of cyclic GMP-dependent protein kinase (Lincoln

& Corbin, 1983). Hence, even though there is a relatively small amount of cyclic GMP-dependent protein kinase present in the heart, at least some of the effects of increases in cyclic GMP would be expected to be mediated through protein phosphorylation. In addition to the relatively small amount of cyclic GMP-dependent protein kinase present in heart, the functional separation between cyclic GMP and phospholamban phosphorylation might be the result of phospholamban being inaccessible to cyclic GMP-dependent protein kinase, the presence of large amounts of phospholamban phosphatase relative to kinase in the subcellular vicinity of phospholamban, or the localization of phosphodiesterase in a way which prevents the activation by cyclic GMP of cyclic GMP-dependent protein kinase near phospholamban. Further research is needed to distinguish between these possibilities.

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