Sarcolemmal phospholamban is phosphorylated in isolated rat hearts perfused with isoprenaline

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Phosphorylation of phospholamban in cardiac sarcolemma is implicated in the increased influx of Ca^{2+} through the slow calcium channel induced by catecholamines. A method is described for the preparation of highly purified sarcolemmal vesicles from rat heart, and this has been used to examine the phosphorylation of phospholamban in $^{32}P_i$-perfused rat hearts. Phospholamban phosphorylation is increased 3-fold after 30 s of perfusion with 0.1 μM isoprenaline. The time course of this increase precedes the inotropic response by 5–10 s.

Phospholamban *Sarcolemma* *Calcium channel* *Cardiac muscle* *Phosphorylation* *Cyclic AMP*

1. INTRODUCTION

Phospholamban is an intrinsic membrane protein of cardiac muscle membranes which is phosphorylated by cyclic AMP-dependent [1] and Ca^{2+}/calmodulin-dependent [2] protein kinases. It was originally reported to have an M_r of 23 000 [3], but has subsequently been dissociated into smaller subunits [4–8]. It was first described in sarcoplasmic reticulum vesicles, where phosphorylation caused a stimulation of Ca^{2+} uptake. It was suggested that phosphorylation of phospholamban was implicated in the enhancement of the rate of relaxation of the heart caused by β-adrenoceptor agonists [9]. More recently, phospholamban has been shown to be present in sarcolemma [10]. Phosphorylation of sarcolemmal vesicles by endogenous protein kinases enhanced the activity of the sarcolemmal Ca^{2+} transport ATPase [11,12]. However, the significance of this in the regulation of Ca^{2+} fluxes in vivo is unclear, as the ATPase transports only a very small proportion of the total cytoplasmic Ca^{2+} during relaxation [13]. Microinjection of the catalytic subunit of cyclic AMP-dependent protein kinase (C-subunit) into single cardiac myocytes enhanced the slow inward calcium current during depolarisation [14]. Patch-clamp experiments [15] have also shown that this current is increased by cyclic AMP. Enhancement of the calcium current is largely responsible for elongation of the action potential after exposure of hearts to β-agonists [16]. Although in these experiments the target proteins for phosphorylation were not identified, it seems reasonable to propose that phosphorylation of phospholamban may be implicated in this response.

We report that phosphorylation of phospholamban in sarcolemma is stimulated in intact hearts perfused with isoprenaline. This was measured in sarcolemmal vesicles prepared free of sarcoplasmic reticulum contamination. The time course of phosphorylation during isoprenaline perfusion was very rapid, and preceded the inotropic response. This supports the hypothesis that phosphorylation in the sarcolemma mediates the increase in the slow calcium current induced by catecholamines.

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2. MATERIALS AND METHODS

2.1. *Preparation and characterisation of sarcolemmal vesicles*

Sarcolemmal vesicles were prepared from frozen rat hearts by an adaptation of the method of [12]. Four hearts were powdered in the frozen state, 15 ml of 0.75 M KCl, 0.2 mM dithiothreitol or 30 mM 2-mercaptoethanol, 5 mM histidine (pH 7.4) (buffer A) at 0°C added to the powder, and homogenisation carried out for 1 s in a small-volume blender. All subsequent steps were performed at 0–4°C. The homogenate was degassed under vacuum and centrifuged at $3000 \times g$ for 15 min. The pellet was re-homogenised for 3 s in 15 ml of buffer A and re-centrifuged. The pellet was stirred by hand with a glass rod for 1–2 min with 25 ml of 10 mM $NaHCO_3$, 0.2 mM dithiothreitol or 30 mM 2-mercaptoethanol, 5 mM histidine (pH 7.4) (buffer B). Unhomogenised material was removed and the suspension re-centrifuged as above. The pellet was homogenised in 20 ml buffer B using a Polytron (PT10 probe) at setting 6 with 3 bursts of 25 s each. The homogenate was centrifuged at $8000 \times g$ for 20 min, and the supernatant re-centrifuged at $36000 \times g$ for 45 min. The pellet was resuspended in 1 ml of 0.25 M sucrose, 5 mM histidine (pH 7.2) and layered onto a 10 ml gradient of sucrose (0.52–1.02 M) containing 0.6 M KCl, 10 mM histidine (pH 7.2). This was centrifuged in a swing-out rotor at $110000 \times g$ for 60 min. A tight, very pale band was formed just below the top of the gradient. This was removed, diluted with 8 vols of 0.6 M KCl, and centrifuged at $50000 \times g$ for 30 min. This pellet is referred to as the sarcolemmal vesicle preparation. A yield of 10 μg protein per g heart was routinely obtained. $(Na^+ + K^+)$-ATPase, K^+-stimulated *p*-nitrophenylphosphatase activity, and Ca^{2+}-stimulated Mg^{2+}-ATPase activity were measured in the presence of alamethicin as in [12]. ATP-dependent Ca^{2+} uptake was measured as in [12], using Tris-ATP to initiate uptake. Succinate dehydrogenase was assayed essentially as in [17], and 5′-nucleotidase in the presence of Triton X-100 as in [18];

2.2. *Perfusion and analysis of phosphorylated proteins*

Hearts were perfused with $^{32}P_i$ (0.25 mCi per heart) for 20–25 min as in [19], and then with 0.1 μM DL-isoprenaline for various times up to 30 s when the hearts were freeze-clamped at −186°C. Sarcolemmal vesicles were isolated as described above, but with the inclusion of 15 mM phosphate and 15 mM F^- in all buffers except the final 0.6 M KCl step. These were included to inhibit dephosphorylation of proteins during the isolation procedure. The vesicles were dissolved in sample buffer and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) as in [20]. The standard sampe buffer (unless indicated otherwise) contained 10% SDS, 0.6 M sucrose, 125 mM Tris-Cl, 0.45 M 2-mercaptoethanol (pH 6.8). Samples were heated at 100°C for 5 min immediately before electrophoresis unless otherwise stated. Autoradiography of the dried gells was performed as in [21]. ^{32}P incorporation into proteins was determined by densitometry using a Joyce-Loebl Chromscan 3 connected to a Hewlett-Packard desk-top computer. Peak areas were corrected for the [γ-^{32}P]ATP specific radioactivity of the hearts, measured as in [22].

For in vitro phosphorylation experiments, sarcolemmal vesicles were incubated with 0.175 mM [γ-^{32}P]ATP (spec. act. 0.6–1 $Bq \cdot pmol^{-1}$), 10 mM NaF, 30 mM Na phosphate, 1 mM $MgCl_2$, 0.5 mM EGTA, ±0.6 mg/ml C-subunit for 2 min at 37°C. The reaction was stopped by addition of trichloroacetic acid (final concentration 0.6 M) and the protein pellet washed with 25 mM Na phosphate before being dissolved in SDS-PAGE sample buffer.

2.3. *Materials*

Radiochemicals were obtained from Amersham. Alamethicin was a gift of Professor L. Will-Shalab. All other reagents were Analar grade, or the purest grade obtainable.

3. RESULTS

3.1. *Characterisation of the sarcolemmal vesicle preparation*

The specific activities (μmol product formed $\cdot h^{-1} \cdot$ mg protein^{-1}) of the following enzymes in the sarcolemmal vesicles were: $(Na^+ + K^+)$-ATPase, 38.1; K^+-stimulated *p*-nitrophenylphosphatase, 11.9; 5′-nucleotidase, 5.6. Succinate

dehydrogenase activity was undetectable. Because of the high level of Mg^{2+}-dependent ATPase activity (210 μmol · h^{-1} · mg^{-1}), it was not possible to measure accurately the Ca^{2+}-stimulated Mg^{2+}-ATPase activity. The values for the sarcolemmal marker enzymes were approximately 50% of those reported in preparations of sarcolemmal vesicles from pig heart using this method [12]. However, these values indicate a high degree of sarcolemmal purity when compared to other preparations [23–25].

Fig. 1 shows the time course of ATP-dependent Ca^{2+} uptake into the sarcolemmal vesicles. A number of characteristics of this uptake indicate that it is all due to sarcolemma, and that there is no contamination by sarcoplasmic reticulum in this preparation. Thus there is no stimulation of Ca^{2+} uptake by oxalate, and uptake is still taking place in the absence of oxalate after several minutes [26]. Ca^{2+} uptake was rapid and linear during the first 30 s. The accumulated Ca^{2+} could be released from the vesicles by the addition of 40 mM Na^+ but not by 40 mM K^+ (fig.2), indicating the presence of a Na^+/Ca^{2+} antiporter. The two

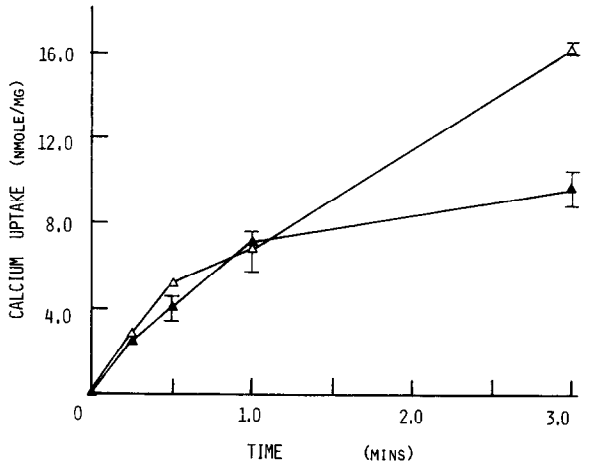

Fig. 1. The ATP-dependent uptake of calcium by sarcolemmal vesicles. Calcium uptake was measured by incubation of vesicles with 1 mM ATP, 75 μM $^{45}CaCl_2$ as in [12]. Separation of bound ^{45}Ca was by Millipore filtration. The assays were performed in the presence (▲) or absence (△) of 2.5 mM K oxalate. All points were corrected for passive calcium binding, which was 8.90 or 7.67 nmol · mg protein^{-1} in the presence or absence of oxalate, respectively. Each point is the mean ± SEM of 3 determinations.

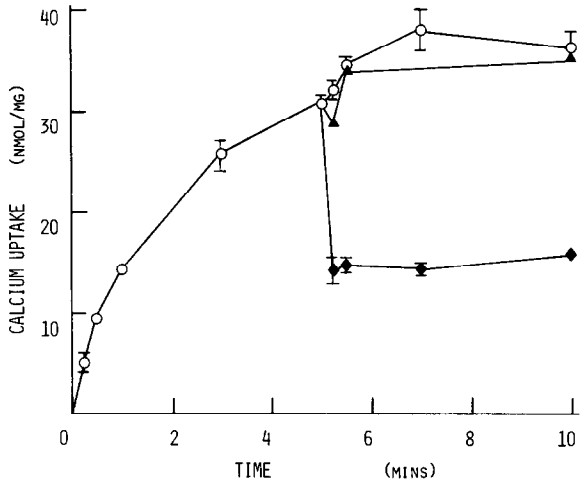

Fig. 2. The sodium-induced release of calcium from sarcolemmal vesicles. Vesicles were incubated with ATP and $^{45}CaCl_2$ as in fig.1, and ^{45}Ca uptake measured (○). After 5 min NaCl (◆) or KCl (▲) were added to a final concentration of 40 mM. Error bars represent the SEM of 3 determinations.

latter observations are characteristic of vesicles of sarcolemmal origin, whereas the first two results indicate no sarcoplasmic reticulum contamination.

3.2. *Characteristics of sarcolemmal phosphorylation*

Fig. 3a shows the autoradiograph scan of sarcolemmal vesicles phosphorylated in vitro by C-subunit. There are three major phosphorylated proteins of high mobility, with apparent M_r values of 22 000, 15 000 and 11 000. A minor phosphorylated protein of M_r 6000, and several high-M_r proteins were also present. The four phosphorylated proteins of $M_r \leqslant 22\,000$ probably represent different states of aggregation of phospholamban subunits [7,25].

When sarcolemmal vesicles were prepared from hearts perfused with $^{32}P_i$ and stimulated for 30 s with isoprenaline, three proteins only were phosphorylated. These had M_r values of 15 000, 11 000 and 6000 (fig.3b). If prior to electrophoresis the vesicles were dissolved in sample buffer containing 2% SDS and 5 mM $MgCl_2$ [8], there was generally only one phosphorylated protein with an M_r of 15 000 (fig.3c), although occasionally a minor phosphorylated component with M_r 6000 was also seen. If either 10 mM $MgCl_2$ was present

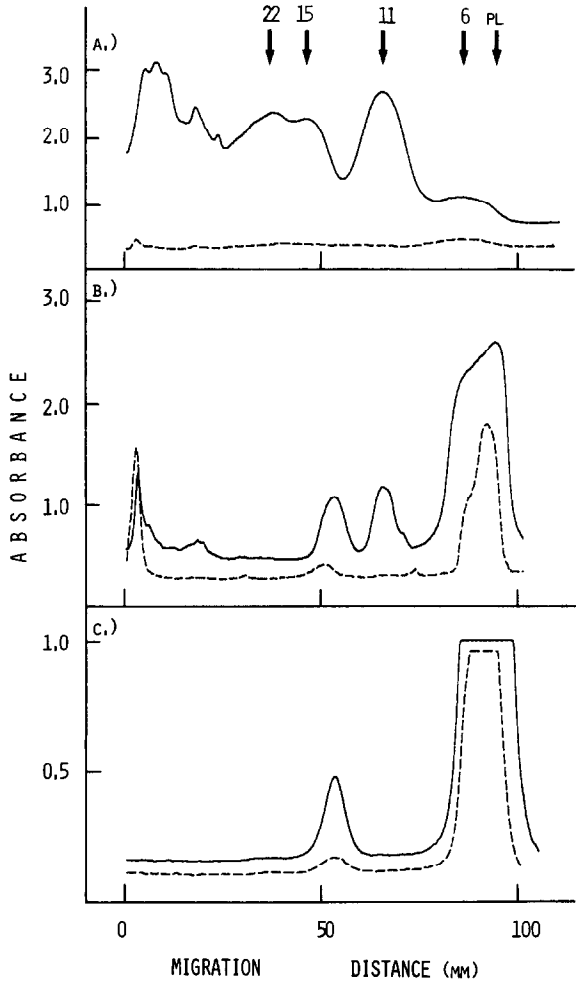

Fig. 3. Densitometric traces of autoradiographs of phosphorylated sarcolemmal vesicles. Vesicle preparations were dissolved in sample buffer as indicated, and subjected to SDS-PAGE with 15% polyacrylamide. (a) Vesicles were phosphorylated in vitro either with (——) or without (– – –) added C-subunit as described in section 2.2, and dissolved in sample buffer containing 10% SDS. (b and c) Vesicles were prepared from hearts perfused with $^{32}P_i$, and dissolved in sample buffer containing either 10% SDS (panel b) or 2% SDS, 5 mM $MgCl_2$ (panel c). (– – –) Control perfused hearts; (——) hearts perfused for 30 s with 0.1 μM isoprenaline. The arrows indicate the apparent M_r values (÷1000) of phospholamban subunits. PL indicates the position of phospholipid.

in the sample buffer, or if the samples were dissolved in the absence of $MgCl_2$ and then frozen before electrophoresis, the major phosphorylated protein had an M_r of 22000 (not shown).

Phospholamban has been shown to behave in an anomalous manner on SDS-PAGE. Thus heating and detergent [5–7] cause the protein to migrate with M_r values of about 11000 and 6000, whereas Mg^{2+} [8] and freezing [7] give M_r values of 22000 and 15000. The behaviour of the phosphorylated bands under various conditions in SDS-PAGE described above for sarcolemmal vesicles strongly suggests that all the bands represent different aggregation states of phospholamban. The absence of phosphorylated proteins of M_r 19000 and 27000, which would correspond to myosin P-light chain [27] and troponin-I [19,27] respectively, indicate that this preparation is not contaminated by myofibrils.

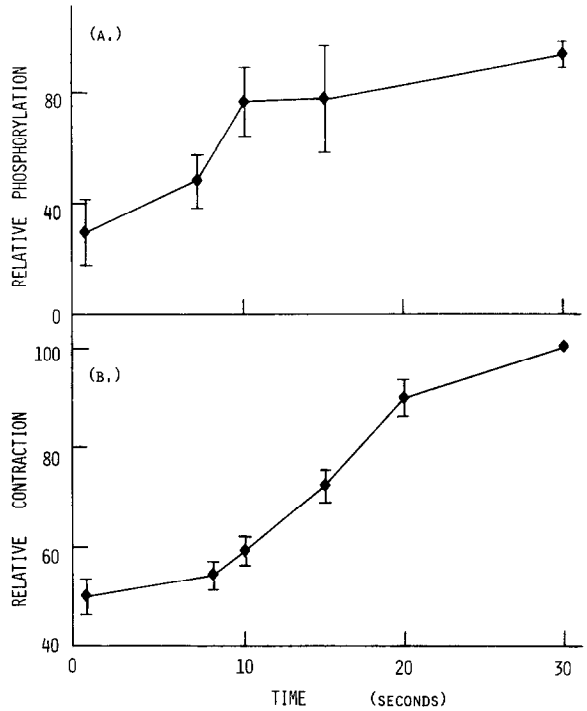

Fig. 4. Time course of phosphorylation of sarcolemmal phospholamban in $^{32}P_i$-perfused rat hearts in response to 0.1 μM isoprenaline (added at time = 0). (a) Vesicles were prepared from groups of 4 hearts frozen at the times indicated, and ^{32}P incorporation into phospholamban measured by densitometry of autoradiographs of gels run under the conditions of fig.3c. Results are expressed as % incorporation of ^{32}P into phospholamban relative to that after 30 s of perfusion with isoprenaline. Each point is the mean ± SEM of 4–7 groups of hearts. (b) Systolic peak tension, expressed relative to tension measured after 30 s of perfusion with isoprenaline.

The phosphorylation pattern obtained by incubation with C-subunit (fig.3a) clearly shows that several higher M_r proteins which can be phosphorylated in vitro are not phosphorylated in vivo. This may indicate denaturation of proteins during the vesicle preparation, exposing phosphorylation sites not available in vivo [28]. Alternatively, proteins not present on the cytoplasmic surface of the membranes will be available to the C-subunit if a proportion of the vesicles are prepared right-side-out.

3.3. *Time course of phospholamban phosphorylation in vivo*

Fig. 3 shows that the phosphorylation of phospholamban in sarcolemma is stimulated by perfusion of rat hearts with isoprenaline. The time course of this phosphorylation is shown in fig. 4 along with the contractile response. Phospholamban phosphorylation preceded the increase in contraction by 5–10 s. Thus phosphorylation was nearly maximal after 10 s of perfusion with isoprenaline, whereas maximal contractile force was only developed after 20–25 s. There was an approximately 3-fold increase of phospholamban phosphorylation with isoprenaline perfusion. The basal phosphorylation level may be due to the activity of the Ca^{2+}-dependent phospholamban kinase [12], rather than cyclic AMP-dependent protein kinase, since both troponin-I [19] and C-protein [27], two major substrates for the latter enzyme, are only weakly phosphorylated in the absence of catecholamine.

4. GENERAL DISCUSSION

These experiments show that phospholamban is present in cardiac sarcolemma, and that its phosphorylation is increased by catecholamines. It has been suggested [7] that phospholamban present in sarcolemmal vesicle preparations occurs either with the phosphorylation site on the extracellular surface of the membrane, or in some contaminating membrane structures (not sarcoplasmic reticulum). Our results showing the phosphorylation of phospholamban in vivo make the first of these suggestions very unlikely. The second suggestion cannot be disproved directly by our results, but a number of lines of evidence, discussed below, indicate a very close association between phosphorylation of phospholamban and changes in sarcolemmal calcium fluxes.

It has been reported previously that sarcolemma contained a protein of M_r 23 000, called calciductin [29], phosphorylation of which caused an apparent increase in the inward calcium current in a vesicular preparation. However, the calcium uptake measured in these experiments was probably not related to the inward current [30]. In addition, calciductin and phospholamban are probably identical [31], and the preparation was probably contaminated with sarcoplasmic reticulum [7].

Two earlier studies have also examined phosphorylation of proteins in sarcolemma in vivo. One of these [32] reported the phosphorylation of proteins of M_r 36 000 and 27 000, but no protein corresponding to phospholamban. There was no evidence for phosphorylation of these higher M_r proteins in our preparations in vivo, and we find it difficult to relate this study to the present results. The second study [33] showed an increase in phosphorylation of phospholamban with catecholamines in chick heart, but the sarcolemma preparations used were not free of other cell components.

The studies involving microinjection of C-subunit [14], and the effects of cyclic AMP in patch-clamp experiments [15], both strongly suggest that phosphorylation of a sarcolemmal protein is involved in the increased inward calcium current caused by catecholamines. Our experiments show that in vivo one protein only is phosphorylated in response to catecholamine, and that this protein appears to be identical with phospholamban. This is good evidence for the hypothesis that phosphorylation of phospholamban leads directly to an increased inward calcium current. The time course showing phosphorylation of phospholamban just preceding the increase in contraction also supports this argument.

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