Biochimica et Biophysica Acta, 980 (1989) 348–356
Elsevier

BBA 74390

The effects of calcium, temperature and phospholamban phosphorylation on the dynamics of the calcium-stimulated ATPase of canine cardiac sarcoplasmic reticulum

Caroline Fowler [1,*], John P. Huggins [2], Christine Hall [1], Colin J. Restall [3] and Dennis Chapman [1]

[1] Department of Protein and Molecular Biology, Royal Free Hospital School of Medicine, London, [2] Department of Cellular Pharmacology, Smith Kline and French Research Ltd. Welwyn, Herts., and [3] Biochemistry Research Group, School of Biological Sciences, University College of Swansea, Swansea (U.K.)

(Received 4 August 1988)
(Revised manuscript received 17 January 1989)

Key words: ATPase; Sarcoplasmic reticulum; Phospholamban; Rotational diffusion; Phosphorescence polarization; Protein phosphorylation

Highly purified sarcoplasmic reticulum (SR) has been prepared from dog hearts and has been incubated with the triplet probe erythrosinyl isothiocyanate to specifically label the Ca^{2+}-stimulated ATPase (Ca^{2+}-ATPase) of the SR. The rotational mobility of the Ca^{2+}-ATPase has been studied in this erythrosin-labelled SR using time-resolved phosphorescence polarization. Qualitatively, the mobility of the cardiac Ca^{2+}-ATPase resembles that of skeletal muscle SR Ca^{2+}-ATPase. Addition of Ca^{2+} to SR affects the mobility of the Ca^{2+}-ATPase in a way consistent with a segment of the ATPase altering its orientation relative to the plane of the membrane. Phosphorylation of phospholamban in cardiac SR by the purified catalytic subunit of cAMP-dependent protein kinase, which is known to increase the activity of the Ca^{2+}-ATPase by deinhibition, also alters measured anisotropy. The changes observed are not compatible with dissociation of the Ca^{2+}-ATPase from phospholamban after the latter is phosphorylated. The data are more consistent with phospholamban associating with the Ca^{2+}-ATPase following phosphorylation, or more complex models in which only the hydrophilic domain of phospholamban binds with and dissociates from the Ca^{2+}-ATPase.

Introduction

The sarcoplasmic reticulum (SR) is a membrane system found within muscle fibres which is responsible for controlling the intracellular calcium concentration and is therefore intimately involved in the process of excitation-contraction coupling. The enzyme responsible for active calcium transport into the lumen of the SR is the calcium-dependent ATPase (Ca^{2+}-ATPase). The Ca^{2+}-ATPase in cardiac and slow twitch skeletal muscle is homologous with the enzyme found in fast twitch skeletal muscle [1]. However, biochemical studies [2] have shown that the cardiac SR Ca^{2+}-ATPase is regulated by an associated membrane protein called phospholamban, which exists as a pentamer of approx. 30000 [3]. Phosphorylation of phospholamban by the catalytic subunit of cAMP-dependent protein kinase causes a conformational change in phospholamban [4] which stimulates the activity of the Ca^{2+}-ATPase by increasing its sensitivity to Ca^{2+} [5]. This stimulation of the cardiac Ca^{2+}-ATPase following cAMP-dependent phospholamban phosphorylation partially explains the physiological observation that agents which increase cAMP in the heart enhance the rate of muscle relaxation [6].

Very little is known concerning the molecular details of how phospholamban interacts with the cardiac Ca^{2+}-ATPase. However, Inui et al. [7] showed that solubilization of cardiac Ca^{2+}-ATPase from SR using Triton X-100, followed by removal of the detergent using SM2 beads, gave an ATPase preparation with a K_m for Ca^{2+} similar to that observed in intact SR after

* Present address: Beechams Pharmaceuticals, Irvine, U.K.

Abbreviations: Ca^{2+}-ATPase, calcium-dependent ATPase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; SDS, sodium dodecylsulphate; SR, sarcoplasmic reticulum.

Correspondence: D. Chapman, Department of Protein and Molecular Biology, Royal Free Hospital School of Medicine, Rowland Hill Street, London, NW3 2PF, U.K.

phospholamban phosphorylation. This led to the proposal that phosphorylation of phospholamban causes its dissociation from the Ca^{2+}-ATPase. In this model phospholamban is depicted as an inhibitor of the Ca^{2+}-ATPase, with phosphorylation of phospholamban deinhibiting the enzyme [5,8].

This model can potentially be tested by using the fast reaction technique of time-resolved phosphorescence polarization. Time-resolved phosphorescence polarization has previously been used to study the mobility of the Ca^{2+}-ATPase in SR vesicles prepared from rabbit skeletal muscle [9]. Analysis of the time-dependent phosphorescence emission anisotropy can be used to obtain information about the molecular dynamics of the protein under study. Earlier work has shown that addition of Ca^{2+} during phosphorescence polarization measurements alters the anisotropy kinetics consistent with a change in the angle made by the axis of rotation and the plane of the membrane [10]. Hence it can be deduced that binding of Ca^{2+} causes a conformational change in the Ca^{2+}-ATPase which is thought to be associated with its catalytic activity.

In this study we have used phosphorescence polarization to investigate the mobility of the Ca^{2+}-ATPase in cardiac SR vesicles in order to study how its dynamics compare with those of the analogous enzyme in fast twitch skeletal muscle. In addition, we have explored the interaction of the Ca^{2+}-ATPase with its regulatory protein phospholamban. By measuring the rotational diffusion of the Ca^{2+}-ATPase, both before and after phosphorylation of phospholamban, information can be obtained about the size of the rotating particle. If, after phosphorylation, the Ca^{2+}-ATPase is observed to move slowly, this would be good evidence for phospholamban binding to the enzyme. Conversely, a decrease in the relaxation time of the Ca^{2+}-ATPase would suggest phospholamban is dissociating from the enzyme.

Methods

Membrane preparation

13–15 kg Beagle dogs of either sex from the SK&F Dog Unit were anaesthetised by intravenous injection of euthetal (1.3 ml/kg). The heart was rapidly removed from each animal and immersed in 0.154 M NaCl at room temperature for 5 min. Subsequently all procedures were performed at 4°C.

SR was prepared from dog hearts as described by Chamberlain et al. [11] with the following modifications: all buffers in the preparation contained Hepes rather than imidazole. Each trimmed heart was divided into three portions (approx. 25 g each) and, after slicing into 2–3 mm^2 cubes, each portion was homogenized in 120 ml of buffer (0.29 M sucrose, 0.5 mM dithiothreitol, 3 mM NaN_3, 10 mM Hepes (pH 6.9), 50 μM phenylmethanesulphonyl fluoride, 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) in a Kenwood Chef liquidiser at setting 1 for two bursts of 15 s each, setting 3 for 30 s and setting 5 for 30 s. The inclusion of proteinase inhibitors in this buffer, the subsequent resuspension buffer ('buffer A') and the buffers used in density gradient centrifugation were found to markedly improve the ATP-dependent Ca^{2+} uptake into the isolated SR vesicles. Salt-washed microsomes were prepared from the homogenate as described by Chamberlain et al. [11], except that glass wool was used to filter supernatants instead of cheesecloth and the final centrifugation at 250000 × g was performed in a Beckman Type 50 Ti rotor. These microsomes were frozen in liquid nitrogen and stored overnight at −70°C. Sucrose/dextran gradient centrifugation was performed for 6.5 h at 90000 × g (3200 rpm in a Beckman SW41 Ti rotor or 37000 rpm in a MSE MST 60.3 rotor). The density of fractions from these gradients was determined using a Bellingham and Stanley refractometer, calibrated with the solutions used in the density gradients. The band with a refractive index corresponding to 17.5–27% sucrose was diluted and centrifuged to a pellet (see Ref. 11). This pellet was resuspended in a total volume of 2.5 ml of 0.29 M sucrose, 0.2 M KCl, 10 mM Hepes (pH 6.7) using a Potter homogenizer (15 strokes), divided into aliquots, frozen in liquid nitrogen and stored at −70°C. Protein in these fractions was determined by the method of Lowry et al. [12].

Calcium uptake and ATPase measurements

ATP-dependent Ca^{2+} uptake into SR vesicles was measured essentially according to Ref. 13. SR (5–15 μg protein/ml) was incubated at 37°C in 20 μM $^{45}CaCl_2$ (specific activity 400 GBq/mol), 5 mM NaN_3, 100 mM KCl, 5 mM potassium oxalate, 10 mM ATPMg, 25 mM Hepes-NaOH (pH 6.9). At appropriate times, 1-ml aliquots were removed, rapidly passed through 0.45 μm HA Millipore filters and washed with two aliquots of 1 ml 5 mM NaN_3, 100 mM KCl, 2 mM $ATPNa_2$, 10 mM $MgCl_2$, 25 mM Hepes-NaOH (pH 6.9). The ^{45}Ca retained by the filters was determined by scintillation counting after dissolving the filters in 10 ml Picofluor 15 (Packard). The oxalate dependence of uptake is frequently used as a means of demonstrating that uptake is being measured into SR, as opposed to sarcolemma or mitochondria. The rate of ATP-dependent Ca^{2+} uptake in the absence of oxalate was negligible in all our fractions examined after sucrose/dextran density centrifugation.

The Ca^{2+}-independent ATPase in our SR preparation was measured in a buffer containing 4 mM EGTA, 2 mM phosphoenolpyruvate, 5 mM NaN_3, 3.5 mM $MgCl_2$, 2.5 mM potassium oxalate, 3 mM $ATPNa_2$, 0.1 mM NADH, 1 unit/ml lactate dehydrogenase (BCL), 1 unit/ml pyruvate kinase (BCL), 50 mM Hepes-NaOH (pH 7.5). ATP hydrolysis was determined from the rate

of decrease in absorbance at 340 nm after addition of SR. The Ca^{2+}-independent ATPase in our SR was 0.147 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$.

Labelling of SR and phosphorescence anisotropy measurements

SR was labelled with the tripiet probe, erythrosinyl 5-isothiocyanate (Molecular Probes Inc., Eugene, OR 97402), or with fluorescein isothiocyanate, as follows. Sucrose was removed from the SR by centrifugation at $100\,000 \times g$ for 30 min at 4°C. The SR was resuspended with probe dissolved in 50 mM sodium phosphate (pH 8.0) at a concentration of probe and SR protein which gave an equimolar concentration of probe to Ca^{2+}-ATPase, assuming the content of Ca^{2+}-ATPase in the SR to be 50% of protein present. The concentration of erythrosinyl isothiocyanate in solution was calculated from the absorbance at 530 nm. The absorption coefficient of erythrosinyl isothiocyanate was assumed to be the same as that of eosinyl isothiocyanate at their respective maxima at 530 and 522 nm ($8.3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) [14,15]. The concentration of fluorescein isothiocyanate in solution was calculated from the absorbance at 495 nm using an absorption coefficient of $8.3 \cdot 10^4\ M^{-1} \cdot cm^{-1}$. All manipulations were made under dim red illumination (Kodak Safelight filter No. 1) in order to prevent photo-oxidative damage to the SR proteins or lipids [16]. The SR was incubated with probe for 1 h at room temperature in the dark and then SR sedimented by centrifugation at $100\,000 \times g$ for 30 min at 4°C.

Erythrosin-labelled SR was prepared for phosphorescence anisotropy measurements by resuspending the membrane pellet at 0.3–0.5 mg protein/ml in 50 mM sodium phosphate, 66% glycerol (pH 8). The SR was then transferred to a phosphorescence cuvette and flushed with nitrogen to prevent oxygen quenching of the phosphorescence. The sample of erythrosin-labelled SR was excited by a 5 ns plane-polarised laser flash at 532 nm generated by a J.K. mini-Q neodymium-YAG laser system, fitted with a harmonic frequency-doubling crystal operating at 10 Hz. The electric vector of the laser excitation was oriented in the vertical direction. The laser-induced phosphorescence was collected from both sides of the sample cuvette, perpendicular to the direction of the laser beam, and focussed through polarizers (type 03 FPG 001, Melles Griot, Arnhem, The Netherlands) aligned parallel on one side and perpendicular on the other side to the electric vector of the laser excitation, yielding signals $I_{VV}(t)$ and $I_{VH}(t)$, respectively. The polarized phosphorescence components were detected with photomultipliers (EMI type D554, Thorn-EMI Electron tubes Ltd., Ruislip, Middlesex) after filtering with red-pass filters (3 mm Schott glass RG645 and 3 mm RG655, Barr & Stroud, Ltd., Glasgow). After amplification, the phosphorescence signals were captured in a DL1080 transient recorder and averaged in a DL4000 signal averager (Data Laboratories Ltd., Mitcham, Surrey).

After a suitable number of signals (typically 1024) was collected, the averaged data were transferred to a Sharp M280B desktop computer. Collection of these data sets was alternated with that of the same two emission components excited by horizontally polarized light produced by reorientation of the laser half-wave plate accessory. The collection of these two components ($I_{HV}(t)$ and $I_{HH}(t)$) allowed computation of fully corrected anisotropy, as given by the equation

$$R(t) = \frac{I_{VV}(t) \cdot G(t) - I_{VH}(t)}{I_{VV}(t) \cdot G(t) + 2I_{VH}(t)} \quad (1)$$

where

$$G(t) = I_{HH}(t)/I_{HV}(t).$$

Analysis of the anisotropy curves was performed using an iterative, non-linear least-squares program on a Gould PN6032 computer at the Royal Free Hospital School of Medicine.

Phosphorylation of SR in phosphorescence experiments

The catalytic subunit of cAMP-dependent protein kinase, purified to homogeneity, essentially according to Reimann and Beham [17], was provided by Dr. K. Murray of Dept. Cellular Pharmacology, SK&F. The catalytic subunit was stored at −70°C in 50% (v/v) glycerol, 100 mM sodium phosphate, 1 mM EGTA (pH 7). The specific activity of the kinase towards histone II-A was 0.25 μmol/min per mg C-subunit protein.

To determine the substrates in cardiac SR for the catalytic subunit of cAMP-dependent protein kinase, 12.5 μg SR protein was incubated in a total volume of 80 μl of 50 mM Hepes-Tris (pH 7), 100 mM $MgCl_2$, 2.5 mM EGTA, 0.5 mM dithiothreitol, 0.1 mM [γ-^{32}P]ATPNa_2 (specific activity 55 TBq/mol) and 0.6 μg/ml of the catalytic subunit of cAMP-dependent protein kinase for 20 min at room temperature. After centrifugation at $350\,000 \times g$ (at r_{av}) for 15 min in the TL-100.2 rotor of a TL-100 Beckman centrifuge at 4°C, the SR was dissolved by incubation at 30°C for 1 h in 2% SDS, 125 mM Tris-HCl, 20% glycerol, 40 mM dithiothreitol (pH 6.8). The SR was then examined by SDS-polyacrylamide gel electrophoresis and autoradiography. When phospholamban dephosphorylation was studied, phosphorylated SR was centrifuged to a pellet at $350\,000 \times g$ for 15 min at 4°C, resuspended in 50 mM sodium phosphate, 66% (v/v) glycerol (pH 8), and then incubated for the times and at the temperatures to which the SR was subjected in phosphorescence experiments. At various times during the incubation aliquots were removed and mixed with SDS sample buffer (10%

SDS, 125 mM Tris-HCl, 40 mM dithiothreitol (pH 6.8)) to give a final SDS concentration of 3.3% to stop any dephosphorylation reaction, and the membranes were solubilized at 100 °C for 4 min. Samples were subjected to SDS-polyacrylamide gel electrophoresis, the gel autoradiographed and phospholamban phosphorylation determined by video densitometry.

In experiments where SR proteins were phosphorylated prior to the determination of phosphorescence anisotropy, two identical samples of SR (approx. 0.7 mg protein each) were labelled with erythrosinyl isothiocyanate and centrifuged as described above. Each pellet was resuspended in 5 ml 50 mM Hepes-Tris (pH 7), 100 mM $MgCl_2$, 2.5 mM EGTA, 0.5 mM dithiothreitol, 1 mM $ATPNa_2$ and in addition 12.5 μg of the catalytic subunit of cAMP-dependent protein kinase was added to one sample. Both samples were then incubated for 20 min at room temperature, recentrifuged to sediment the membranes, and the pellets resuspended and degassed ready for phosphorescence measurement as above.

Gels and autoradiographs

15% SDS polyacrylamide gels were run according to Laemmli [18]. Bio-Rad SDS-polyacrylamide gel electrophoresis molecular weight standards were used. Autoradiographs were exposed within the linear range of absorbance [19], and were scanned using a Bio-Rad model 620 video densitometer. Peak areas were determined using Bio-Rad software for an IBM PC.

Results and Discussion

Development of a method for preparing high purity cardiac SR

Initial studies using cardiac SR obtained by conventional purification procedures (see, for example, Ref. 4) from rabbit, canine or pigeon hearts gave poor signal-to-noise ratios in phosphorescence polarization experiments. We therefore turned to the published method of Chamberlain et al. [11], which reports the preparation of cardiac SR with high purity and yield. Homogenization conditions were optimised in a Kenwood Chef liquidiser on the basis of both purity and yield of SR as judged by oxalate-supported, ATP-dependent Ca^{2+} uptake. The conditions that were chosen are described in Methods.

After centrifugation of membranes in a sucrose/dextran gradient (see Methods), three visible fractions were obtained at the densities indicated in Table I. All fractions obtained gave rates of oxalate-supported, ATP-dependent Ca^{2+} uptake which compared favourably to conventional purification methods (see, for example, Ref. 20), indicating substantial purification of SR. The greatest rate of Ca^{2+} uptake was measured in the 'top' gradient fraction, but the low yield made the use of these membranes in phosphorescence experiments impracticable. The 'middle' band from the gradient was therefore used in these experiments, as a compromise between purity and yield. The subcellular identity of the three bands was not further investigated, but it is possible that the fractions represented separation of junctional and longitudinal SR [21].

Fluorescein-labelling and phosphorylation of SR

The Ca^{2+}-ATPase of skeletal SR is labelled by fluorescein isothiocyanate, or its phosphorescent analogue erythrosin isothiocyanate, on a specific lysine residue (Lys-515) within the nucleotide-binding domain of the ATPase [1]. Fig. 1 shows the Coomassie blue-staining pattern of cardiac SR after SDS-polyacrylamide gel electrophoresis. The very predominant band with a M_r of 100 000 was identified as the Ca^{2+}-ATPase on the basis of its known M_r and predominance in purified SR [7]. SR was incubated with 1 mol/mol fluorescein isothiocyanate, as described in Methods, and then centrifuged in a TLA-100.2 rotor of a Beckman TL-100 centrifuge for 15 min at $350000 \times g$ (at r_{av}) at 4°C. The membrane pellet was dissolved in 10% (w/v) SDS, 125 mM Tris-HCl, 20% glycerol, 40 mM dithiothreitol and proteins separated using SDS-polyacrylamide gel electrophoresis. Fluorescein-labelled proteins were located by illuminating the gel with ultraviolet light. Only one fluorescent band was observed, which comigrated with the 100 000 M_r protein in Coomassie-blue stained gels (see Fig. 1). It was therefore concluded that under these conditions the Ca^{2+}-ATPase was the only labelled protein, and that we would expect to be able to measure phosphorescence from the Ca^{2+}-ATPase in cardiac SR labelled with the analogous erythrosin isothiocyanate molecule, as in skeletal muscle SR [10].

The substrates in cardiac SR for the catalytic subunit of cAMP-dependent protein kinase were identified as described in Methods. Fig. 1 indicates that the predominant band radiolabelled with ^{32}P was a protein of M_r 28 000. Boiling the sample prior to electrophoresis changed the migration in polyacrylamide gels so that the phosphorylated protein migrated with $M_r \approx 6000$

TABLE I

The densities, purity and yield of the three membrane fractions observed after sucrose/dextran centrifugation

Purity was assessed by measuring ATP-dependent Ca^{2+} uptake into membrane vesicles in the presence of 5 mM potassium oxalate by Millipore filtration, as described in Methods.

Fraction	Density (% sucrose)	Initial rate (nmol Ca^{2+}/min per mg protein)	Capacity after 5 min (μmol Ca^{2+}/mg protein)	Protein yield (mg protein/dog heart)
Top	10–12	517	1.55	0.56
Middle	17.5–27	211	1.11	2.95
Bottom	(loose pellet)	91	0.61	2.46

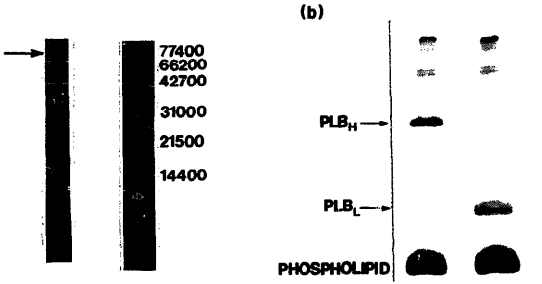

Fig. 1. (a) Coomassie blue-stained SDS-polyacrylamide gel of cardiac SR, showing the migration of molecular weight markers (Bio-Rad). The arrow shows the location of the fluorescein-labelled protein band. (b) An autoradiograph of an SDS-polyacrylamide gel on which phosphorylated cardiac SR has been separated. In the left-hand track, the sample was solubilized by incubation for 1 h at 30 °C, whilst in the right-hand track, the sample was additionally incubated at 100 °C for 4 min. PLB_H and PLB_L refer to the high and low M_r forms of phospholamban, respectiveiy.

(Fig. 1). This behaviour is characteristic of phospholamban [22,23] and indicates that the major substrate for catalytic subunit of cAMP-dependent protein kinase present in our preparation of cardiac SR is phospholamban. (A number of higher molecular weight bands were also phosphorylated to a lesser degree. This is not unusual for in vitro incubations of kinases with isolated SR, but these proteins are not thought to be involved in the regulation of the Ca^{2+}-ATPase.) Control experiments were also performed in which fluorescein-labelled SR was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. No effect of fluorescein-labelling on the ability of the catalytic subunit to phosphorylate phospholamban was observed.

Time-dependent anisotropy of erythrosin-labelled cardiac Ca^{2+}-ATPase

Excitation of erythrosinyl isothiocyanate with a brief pulse of light of suitable wavelength results in a transient emission of phosphorescence at wavelengths greater than 650 nm [15,24]. If the excitation light is plane polarized the emission will also be polarized and this can be quantified by calculation of the emission anisotropy.

In a situation where the probe is free to rotate isotropically, such as when it is in solution, the observed anisotropy will decay to zero within a few nanoseconds. However, if the probe is attached to a relatively slow moving substrate, such as an intrinsic membrane protein, then the decay of anisotropy will be much slower. In the latter case, the anisotropy will decay on a timescale of a few hundred microseconds and will show much more complex kinetics [9,25,26]. By studying the time dependence of the emission anisotropy information can be derived about both the dynamics and the degree of orientational constraint on protein movement.

Earlier studies of the closely related Ca^{2+}-ATPase from fast twitch skeletal muscle have shown that the protein dynamics are complex and show the presence of both a fast-moving polypeptide segment as well as a slower movement characteristic of the whole protein in the membrane [9,27]. The data obtained for the erythrosin-labelled cardiac SR shows much similarity to that seen with the enzyme derived from fast twitch skeletal muscle (Figs. 2 and 4). There is an initial rapid decrease in the anisotropy followed by a slower decay to a constant, time-independent term (see also Table I). Such a result is suggestive of segmental movement of a polypeptide combined with rotation of the whole protein. It is also evident from the curves shown in Figs. 2 and 4 that the initial anisotropy, r_o, is somewhat less than would be expected from the fundamental zero-point anisotropy of erythrosin which has been measured as 0.18 [15]. This is again in agreement with what has been observed with the enzyme from skeletal muscle and reflects the presence of a rapid, sub-microsecond movement which is beyond the time-resolution of the present apparatus.

Effect of temperature on time-dependent anisotropy

Fig. 2a shows the time dependency of the emission anisotropy for erythrosin-labelled cardiac SR, measured at 15, 20 and 25 °C. It can be seen that raising the temperature of the sample results in a characteristic increase in the rate at which anisotropy decays and simultaneously there is a reduction in the level of resid-

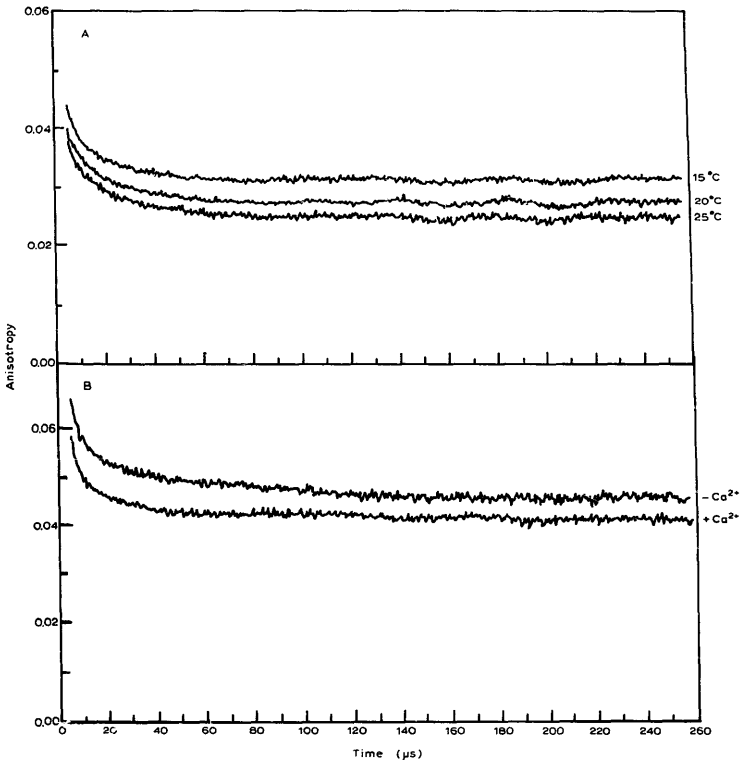

Fig. 2. (a) Measurement of the time dependency of phosphorescence anisotropy from erythrosin-labelled cardiac SR at 15, 20 and 25°C. (b) Phosphorescence anisotropy curves measured in the presence (+) and absence (−) of excess Ca^{2+}.

ual anisotropy. These effects are consistent with the protein increasing in mobility and possibly becoming less aggregated. Similar effects have been observed with a wide variety of other membrane proteins such as the band 3 anion transport system in human erythrocytes [28], rhodopsin in the visual receptor membrane [29] and the Ca^{2+}-ATPase from fast twitch skeletal muscle [30].

Analysis of the time-dependent anisotropy curves in terms of a theoretically sound mathematical model is a difficult procedure which is not always successful, particularly with complex experimental systems. However, it is sometimes possible to quantify the changes occurring in the anisotropy decays by fitting an empirical equation to the experimental data [28,29]. In the case of the present study, the data are well described by an

TABLE II

Typical values of parameters fitted to Equation 2 describing the decay of anisotropy measured after photo-excitation of erythrosin-labelled cardiac SR measured at various temperatures

	15°C	20°C	25°C
α	0.0441	0.0428	0.0436
ϕ_1 (μs)	3.8	3.8	3.4
β	0.0081	0.0092	0.0296
ϕ_2 (μs)	49.1	45.7	42.1
γ	0.0321	0.0296	0.0282

equation comprising two exponential terms together with a constant:

$$R(t) = \alpha \exp(-t/\phi_1) + \beta \exp(-t/\phi_2) + \gamma \qquad (2)$$

Typical parameters derived from fitting this expression to curves as shown in Fig. 2 are given in Table II. In agreement with the above qualitative description, raising temperature causes an increase in β and a reduction in ϕ_2, compatible with an increase in mobility of and within the Ca^{2+}-ATPase. The reduction in γ, which was consistently observed in these experiments on raising temperature, may indicate that the Ca^{2+}-ATPase becomes less aggregated in the SR membrane at high temperatures.

Effect of Ca^{2+} on time-dependent anisotropy

The effect of Ca^{2+} on the observed anisotropy curves is shown in Fig. 2b. The effect is qualitatively similar to that seen in skeletal muscle SR when Ca^{2+} was added during measurement of anisotropy [10]. In SR from both skeletal and cardiac muscle, Ca^{2+} was observed to change the kinetics of the time-dependent anisotropy. The interpretation of this result has been discussed in detail for the skeletal muscle Ca^{2+}-ATPase as being indicative of a change in the degree of motional constraint imposed on the probe relative to the rotational diffusion axis (see also Ref. 9). The resemblance of the results obtained here for the homologous Ca^{2+}-ATPase of cardiac SR are compatible with the idea that the binding of Ca^{2+} induces similar conformational changes in the structure of the cardiac ATPase protein.

Effect of phospholamban phosphorylation on time-dependent anisotropy

In order to examine the effects of phospholamban phosphorylation on Ca^{2+}-ATPase mobility, cardiac SR was incubated in parallel with ATP, and with or without the catalytic subunit of cAMP-dependent protein kinase. A sufficient concentration of ATP was included in the incubations in order that ATP hydrolysis by Ca^{2+}-independent ATPases present in the SR membrane preparation would not prevent phospholamban phosphorylation.

Early experiments using [γ-^{32}P]ATP, and analysing phospholamban phosphorylation using SDS-polyacrylamide gel electrophoresis and autoradiography, showed that at 30°C phosphorylated phospholamban was rapidly dephosphorylated (in approx. 30 min) by a phosphatase endogenous to the SR preparation. The presence of this phosphatase in SR membrane vesicles has previously been demonstrated by others [31].

Phosphate and fluoride are commonly used as inhibitors of phosphatases [32]. 50 mM phosphate was used as the buffer in phosphorescence experiments, but we wished to avoid using fluoride in buffers because this was known to inhibit the Ca^{2+}-ATPase (Huggins, J., unpublished data). We therefore decided to examine whether the use of low temperatures would reduce the rate of phospholamban dephosphorylation. SR with phosphorylated phospholamban was prepared and then incubated for the times and at the temperatures to which the SR was subjected in phosphorescence experiments. At various times during the incubation phospholamban phosphorylation was determined and expressed relative to that at the start of the incubation. The results of this experiment, displayed in Fig. 3, show that less than 15% of the initially phosphorylated phospholamban had become dephosphorylated. Hence dephosphorylation would not be expected to be a problem during phosphoresence anisotropy measurements when these experiments were performed at 15°C. Fig. 4 shows the effect on the Ca^{2+}-ATPase mobility of incubating cardiac SR under conditions in which phospholamban was phosphorylated (curve a) or under conditions where phosphorylation did not occur (curve b). Phosphoryla-

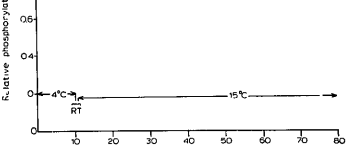

Fig. 3. The extent of phospholamban dephosphorylation after incubation at the temperatures and for the times to which SR was subjected during phosphorescence experiments. Phosphorylation is expressed relative to that observed at time zero. The conditions used to phosphorylate SR and study dephosphorylation are described in Methods. RT, room temperature.

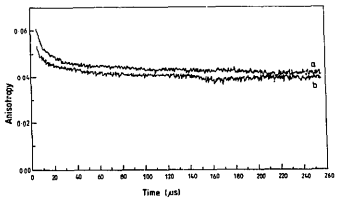

Fig. 4. Curve a, the measurement of phosphorescence anisotropy in cardiac SR after phosphorylating phospholamban with exogenously added catalytic subunit of cAMP-dependent protein kinase. Curve b, control measurement in which the catalytic subunit was omitted from the incubation so that phospholamban was not phosphorylated.

tion of phospholamban caused an upward shift in the anisotropy curve. It can be seen that the effect on the decay curve was quite small. However, the effect shown in the figure was reproducible when examined in a number of independent experiments.

Three hypothetical models of the interaction of phospholamban with the Ca^{2+}-ATPase are shown in Fig. 5. The figure indicates possible effects of phosphorylating phospholamban on its association with the Ca^{2+}-ATPase. Model I is the simplest model which fits the data of Inui et al. [7] and indicates that phospholamban phosphorylation causes its dissociation from the Ca^{2+}-ATPase. This would allow the Ca^{2+}-ATPase a greater

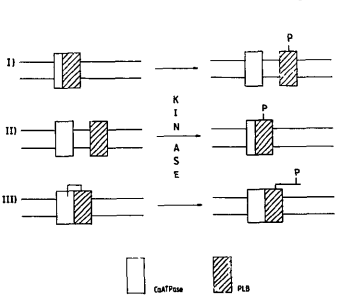

Fig. 5. Theoretical models by which the cardiac Ca^{2+}-ATPase may interact with phospholamban (PLB) when the latter is unphosphorylated (left) or phosphorylated (right) by cAMP-dependent protein kinase. The Ca^{2+}-ATPase is represented by an open rectangle and phospholamban by a hatched rectangle. The representation is purely diagramatic and is not meant to indicate relative masses or mobilities of the Ca^{2+}-ATPase and phospholamban. In model III, phospholamban is drawn as a hatched rectangle, represented the hydrophobic domain, and an arm (hydrophilic domain).

degree of mobility and increase the rate of anisotropy decay. Too little is known of the molecular structure of phospholamban to be able to make accurate quantitative predictions of the change in rotational diffusion of the Ca^{2+}-ATPase when phospholamban dissociates from it. However, the rotational diffusion coefficient of the Ca^{2+}-ATPase within the SR membrane would be expected to be inversely proportional to the square of the radius of the rotating particle [10]. Hence, if model I were correct, the dissociation of phospholamban (pentameric molecular weight 28 000) from the Ca^{2+}-ATPase (molecular weight 100 000) would be expected to cause a substantial increase in the rate of anisotropy decay. The results shown in Fig. 4, however, show that experimentally the opposite effect of phospholamban phosphorylation on anisotropy decay was observed and that phosphorylation of phospholamban decreases the rate at which anisotropy decays. The simplest interpretation of our data might therefore be that phospholamban phosphorylation causes its association with the Ca^{2+}-ATPase, as shown in model II of Fig. 5.

However, there are other possible interpretations of the spectroscopic data which are more consistent with biochemical studies of the structure of phospholamban. Sequencing studies of purified phospholamban [33] or of a cDNA clone encoding phospholamban [34] have shown that it is composed of a hydrophobic domain, which probably spans the phospholipid bilayer of the SR, and a hydrophilic, cytoplasmic domain. In addition, it has been shown that phosphorylation of phospholamban in SR vesicles by cAMP-dependent protein kinase causes a conformational change in the protein which is responsible for relieving inhibition of Ca^{2+}-ATPase activity [4,8]. Close examination of Fig. 4 shows that the principal effect of phosphorylation in reducing the rate at which anisotropy decays, occurs within the first 30 μs of measurement. The anisotropy curves are essentially parallel after this period. This might be interpreted to suggest that phospholamban phosphorylation affects a very mobile segment of the Ca^{2+}-ATPase, but does not alter the rotational diffusion of the Ca^{2+}-ATPase protein in the phospholipid bilayer. This idea is represented diagramatically in model III of Fig. 5. Here phospholamban is depicted with a hydrophobic domain and a hydrophilic arm. The Ca^{2+}-ATPase is shown associated with phospholamban via its hydrophobic domain, whether or not phospholamban is phosphorylated. The figure shows phospholamban phosphorylation causing its hydrophobic arm to dissociate from the Ca^{2+}-ATPase, thereby changing the mobility of a highly mobile segment of the Ca^{2+}-ATPase. This model would explain why the early part of the anisotropy curve is affected by the phosphorylation of phospholamban and also why the shape of the latter part of the curve is relatively unaffected. It is also compatible with the results of Inui et al. [7].

This is the first report of the use of phosphorescence polarization to study the mobility of the cardiac Ca^{2+}-ATPase in SR membranes. The mobility of this enzyme resembles that of the skeletal muscle SR Ca^{2+}-ATPase. However, cardiac Ca^{2+}-ATPase is also regulated by the phosphorylation of phospholamban by cAMP-dependent protein kinase. In this study we have observed changes in the Ca^{2+}-ATPase mobility after phosphorylation of phospholamban by the catalytic subunit of cAMP-dependent protein kinase. These changes are not consistent with a simple molecular mechanism of complete dissociation of the Ca^{2+}-ATPase and phospholamban when the latter is phosphorylated.

Acknowledgements

We wish to thank Dr. K. Murray for his generous provision of cAMP-dependent protein kinase catalytic subunit. We thank the British Heart Foundation for financial support contributing to this work.

References

1 Brandl, C.J., Green, N.M., Korczak, B. and MacLennan, D.H. (1986) Cell 44, 587–607.
2 Tada, M., Kirchberger, M.A., Repke, D.I. and Katz, A.M. (1974) J. Biol. Chem. 249, 6174–6180.
3 Wegener, A.D., Simmerman, H.K.B., Liepniks, J. and Jones, L.R. (1986) J. Biol. Chem. 261, 5154–5159.
4 Huggins, J.P. and England, P.J. (1987) FEBS Lett. 217, 32–36.
5 Hicks, M.J., Shigekawa, M. and Katz, A.M. (1979) Circ. Res. 44, 384–391.
6 Huggins, J.P. and England, P.J. (1985) in Molecular Mechanisms of Transmembrane Signalling (Cohen, P. and Houslay, M.D., eds.), pp. 57–87, Elsevier, Amsterdam.
7 Inui, M., Chamberlain, B.K., Saito, A. and Fleischer, S. (1986) J. Biol. Chem. 261, 1794–1800.
8 Kirchberger, M.A., Borchman, D. and Kasinathan, C. (1986) Biochemistry 25, 5484–5492.
9 Restall, C.J., Dale, R.E., Murray, E.K., Gilbert, C.W. and Chapman, D. (1984) Biochemistry 23, 6765–6776.
10 Restall, C.J., Coke, M., Murray, E.K. and Chapman, D. (1985) Biochim. Biophys. Acta 813, 96–102.
11 Chamberlain, B.K., Levitsky, D.O. and Fleisher, S. (1983) J. Biol. Chem. 258, 6602–6609.
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
13 Harigaya, S. and Schwartz, A. (1969) Circ. Res. 25, 781–794.
14 Cherry, R.J., Cogoli, A., Oppliger, M., Schneidder, G. and Semenza, G. (1976) Biochemistry 15, 3653–3656.
15 Garland, P.B. and Moore, C.H. (1979) Biochem. J. 183, 561–572.
16 Restall, C.J., Arrondo, J.L.R., Elliot, D.A., Jaskowka, A., Weber, W.V. and Chapman, D. (1981) Biochim. Biophys. Acta 670, 433–440.
17 Reimann, E.M. and Beham, R.A. (1983) Methods Enzymol. 99, 51–55.
18 Laemmli, U.K. (1970) Nature 227, 680–685.
19 Bonner, W.M. and Laskey, R.A. (1977) FEBS Lett. 82, 314–316.
20 Hollinworth, D.N. and England, P.J. (1978) Biochem. Soc. Trans. 6, 573–576.
21 Oeken, H.J., Von Nettlebladt, E., Zimmer, M., Flockerzi, V., Ruth, P. and Hoffmann, F. (1986) Eur. J. Biochem. 156, 661–667.
22 Kirchberger, M.A. and Antonetz, T. (1982) J. Biol. Chem. 257, 5685–5691.
23 Wegener, A.D. and Jones, L.R. (1984) J. Biol. Chem. 259, 1834–1841.
24 Murray, E.K., Restall, C.J. and Chapman, D. (1983) Biochim. Biophys. Acta 732, 347–351.
25 Cherry, R.J. (1979) Biochem. Biophys. Acta 559, 289–327.
26 Dale, R.E. and Eisinger, J. (1975) in Polarized Excitation Energy Transfer in Biochemical Fluorescence; Concepts (Chen, R.F. and Edelhoch, H., eds.), Vol. 1, pp. 275–278, Marcel Dekker, New York.
27 Burkli, A. and Cherry, R.J. (1981) Biochemistry 20, 138–145.
28 Nigg, E.A. and Cherry, R.J. (1979) Biochemistry 18, 3457–3465.
29 Coke, M., Restall, C.J., Kemp, C.M. and Chapman, D. (1986) Biochemistry 25, 513–518.
30 Spiers, A., Moore, C.H., Boxer, D.H. and Garland, P.B. (1983) Biochem. J. 213, 67–74.
31 Kranias, E.G. and Disalvo, J. (1986) J. Biol. Chem. 261, 10029–10032.
32 Huggins, J.P. and England, P.J. (1983) FEBS Lett. 163, 297–302.
33 Simmerman, H.K.B., Collins, J.H., Theibert, J.L., Wegener, A.D. and Jones, L.R. (1986) J. Biol. Chem. 261, 13333–13341.
34 Fujii, J., Ueno, A., Kitano, K., Tanaka, S., Kadoma, M. and Tada, M. (1987) J. Clin. Invest. 79, 301–304.