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Effect of the biochemical state of the Ca-ATPase protein of scallop sarcoplasmic reticulum on its interaction with *trans*-parinaric acid

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The polyene fluorescent probe *trans*-parinaric acid (tPA) was used to compare lipid–protein interactions in the scallop fragmented sarcoplasmic reticulum (FSR) between biochemical states where the Ca-ATPase molecules were arranged differently in the membrane and had different tertiary conformations. The state of the bulk lipid phase was examined over the temperature range −3 to +32°C by exciting the tPA directly at 320 nm. The state of the system close to the Ca-ATPase protein was followed over the same temperature range by indirectly exciting the tPA through resonance energy transfer from the Ca-ATPase protein, with approximately one twentyfifth the quantum yield of the directly excited probe. Raising the tPA/lipid ratio in the membrane to high levels (approx. 1:9), caused the quantum yield of indirectly excited tPA to reach a maximum, which may reflect saturation of the annular lipid phase with the probe, or contribution to the fluorescence from indirectly excited tPA bound directly to the protein. In the presence of 0.1 M KCl, a thermal perturbation was observed at approx. 7°C using indirect excitation when the Ca^{2+}-binding sites on the Ca-ATPase were occupied, and the subunits were disorganized. This transition was not detected in the presence of 0.1 M KCl and EGTA, when the Ca^{2+}-binding sites were empty, and the Ca-ATPase subunits were organized in dimeric arrays. The transition seen with the $E_1(Ca^{2+})_2$ form of the membrane may involve an event at the protein/lipid interface, or a change in the environment of tPA bound to the Ca-ATPase. The temperature at which the perturbation occurs is close to that of a discontinuity in the Arrhenius plot of the Ca-ATPase enzyme activity determined in the presence of 0.1 M KCl (Kalabokis, V.N. and Hardwicke, P.M.D. (1988) J. Biol. Chem. 263, 15184–15188). No perturbation was observed in the bulk properties of the lipid component of the membrane in either the $E_1(Ca^{2+})_2$ or E_2 states.

Introduction

The lipid phase of the sarcoplasmic reticulum has been extensively investigated (for review, see Ref. 1). However, there is very little information on comparative differences in the interaction of the lipid phase with the Ca-ATPase protein between well-defined biochemical states associated with the catalytic cycle [2]. The subunits of the Ca-ATPase in the scallop SR are organized differently in the membrane in the $E_1(Ca^{2+})_2$, stabilized E_2 and phosphorylated states. When the membranous enzyme is stabilized in the E_2 (Ca^{2+}-free) state, the Ca-ATPase subunits form a quasi-crystalline lattice in which dimers of the Ca-ATPase are arranged into helical ribbons [3–6]. This type of structure may function to stabilize the scallop SR membrane during the relaxed state of the muscle [7]. The dimeric arrangement of Ca-ATPase subunits has also been described in detail in the E_2 state of the rabbit SR in the presence of decavanadate (see, for example, Ref. 8). In the phosphorylated states (E_2-P and $E_1 \sim P$), the pump molecules occupy a lattice in which there is only a single subunit per unit cell [9], and Cr(III)ATP induces a similar structure in rabbit SR [10]. The subunits are largely disorganized in the $E_1(Ca^{2+})_2$ state of the scallop SR [9], unlike the rabbit SR [10]. There are also differences in the tertiary

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Abbreviations: FSR, fragmented sarcoplasmic reticulum; tPA, *trans*-parinaric acid; Mops, 3-(*N*-morphilino)propanesufonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AMP-PCP, adenosine 5′-[β,γ-methylene]triphosphate.

structure of both the rabbit and scallop Ca-ATPase between the states, as shown by the tryptic cleavage patterns [11,12]. These different structures and arrangements of the Ca-ATPase molecules among the biochemical forms of the enzyme are likely to be reflected in the organization of the lipid component of the membrane, since this lies in the interstices between the Ca-ATPase molecules and their contacts with one another. The protein environment of the lipid will differ among the different states. Different pools of lipid may be restricted by the protein-protein contacts between the subunits in the different states, and thus possess different fluidities and mobilities. Moreover, the surface of the protein interacting with the lipid is likely to vary in the various biochemical states of the membrane. Thus, it is of interest to see whether the properties of the lipid phase and its interaction with the Ca-ATPase is affected by placing the enzyme in different biochemical states related to the physiological function of the enzyme.

In this report, we have attempted to correlate changes in the structure of the lipid phase with changes in the biochemical and structural state of the Ca-ATPase subunits in the scallop FSR membrane. This has been done by following the temperature dependence of the quantum yield of the polyene fluorescent probe *trans*-parinaric acid (tPA) incorporated in scallop FSR membranes. The quantum yield of tPA is enhanced in the gel relative to the liquid crystalline state of the lipid, which allows changes in the fluidity of the membrane to be detected [13,14]. In addition, if the same membrane contains areas having different degrees of order, tPA will partition into the more ordered areas of the lipid bilayer, when its quantum yield is increased [15,16]. It is also possible to distinguish between changes in the bulk lipid phase and at the lipid/protein interface, since the probe may be excited either directly or via energy transfer from tryptophan side chains [17]. tPA has been regarded as a good probe [18], since the chromophoric reporter group is provided by the fatty acid chain itself. However, Lee and co-workers [19–21] have observed binding of fatty acids to the Ca-ATPase component of the rabbit SR, and the possibility that some of the tPA probe fluorescing by energy transfer from Trp and Tyr may be bound to the Ca-ATPase protein has to be considered.

Methods

Deep sea scallops (*Placopecten magellnici*) were obtained from the Marine Biology Laboratory, Woods Hole, MA. Native scallop FSR vesicles were prepared as described previously [22], with the modification that the discontinuous sucrose gradient consisted of three steps containing 0.8, 1.0 and 1.25 M sucrose. The band at the top of the 0.8 M layer was enriched in sarcolemma, as judged by the high ouabain-inhibitable Na,K-ATPase activity and the presence of many sheet-like structures on examination by negative staining in the electron microscope. The band layering at the 1.0/1.25 M interface was used in the studies reported here. tPA (Molecular Probes, Eugene, OR) was dissolved in deoxygenated ethanol to give a 4 mM solution, which was stored under argon at −20°C. Butylated hydroxytoluene was added in a 1:500 molar ratio to tPA.

Fluorescence measurememts were made with an SLM 8000c spectrofluorometer, fitted with a thermostated cell holder. All solutions were flushed with argon prior to use, and the sample compartment flushed with argon throughout the experiments. All intensity measurements were made in the ratio mode relative to rhodamine B in ethanol. The sample was magnetically stirred, and its temperature directly monitored with a YSI-400 probe.

Results

The interaction of the Ca-ATPase with phospholipids was examined with the fluorescent fluidity probe, tPA. tPA can be excited either (i) directly in its absorption band at 320 nm; or (ii) indirectly by energy trans-

TABLE I

Composition of the media used to place the scallop FSR into different biochemical states

Medium	Glycerol (% v/v)	EGTA (mM)	$CaCl_2$ (mM)	$MgCl_2$ (mM)	AMP-PCP (mM)	P_i (mM)	KCl (M)	pH 20°C	Biochemical state
Ia	20	4	0.04	0	1	0	0	7.4	E_2 dimer lattice
Ib	20	4	0.04	0	0.12	0	0	7.4	E_2 dimer lattice
II	20	4	0.04	0	0	0	0.1	7.4	E_2 dimer lattice
III	20	1	1.02	0	0	0	0	7.4	$E_1(Ca^{2+})_2$
IV	20	1	1.02	0	0	0	0.1	7.4	$E_1(Ca^{2+})_2$
V	20	0	1	0	0	0	0.1	7.4	$E_1(Ca^{2+})_2$
VI	20	0	10	0	0	0	0.1	7.4	$E_1(Ca^{2+})_2$
VII	40	10	0.04	20	0	8	0	7.0	E_2-P monomer
VIII	40	10	0.04	12	0	0	0	7.0	E_2 dimer

fer from tryptophan side chains. Direct excitation provides information about the entire lipid bilayer, whilst indirect excitation allows the protein/lipid interface to be studied. The absorption bands for tPA and tryptophan overlap, and excitation at 295 nm, often used for studies of tryptophan fluorescence, will produce some direct excitation of the tPA, in addition to indirect excitation by energy transfer from tryptophan. Therefore, following Sklar et al. [14] and Blazyck et al. [17], in order to avoid any direct excitation of the tPA and obtain excitation purely by the indirect mechanism, tryptophan side chains in the Ca-ATPase were excited at 250 nm, a wavelength at which tPA does not absorb. (Resonance energy transfer from tyrosine residues excited at 250 nm will also excite tryptophan side chains [23].) Fluorescence data were obtained at different temperatures, and plotted on a logarithmic scale versus the reciprocal of the absolute temperature. In this presentation of the data, phase changes are detected by changes in the slope of the plot [15,24]. Data were acquired in the photon counting mode for the sample channel, with the gain adjusted to give 20 000–70 000 cpm according to the sample and mode of excitation, so that the fluorescence intensity scales in the figures are not directly comparable. Under otherwise identical conditions (same sample, slit widths, gain), with a 1:75 molar ratio of tPA to phospholipid, indirect excitation gave 0.04 of the quantum yield obtained with direct excitation.

Temperature dependence of the fluorescence of tPA incorporated in scallop FSR

The effect of changing the biochemical state of the Ca-ATPase protein on the temperature dependence of tPA incorporated in the FSR membrane was studied. Membranes were suspended in one of the media described in Table I. Media Ia, Ib, II and VIII cause the Ca-ATPase subunits to organize into a quasi-crystalline quaternary structure in which the molecules form ribbons of dimers (stabilized E_2, Ref. 7), and in which the T_2 trypsin cleavage site is protected [12]. Medium VII places the the Ca-ATPase molecules in the E_2-P state, in which the enzyme subunits are arranged in parallel strands, with a single subunit in each unit cell [9]. Media III and IV yield a largely random distribution of Ca-ATPase in the membrane ($E_1(Ca^{2+})_2$). Media Ib and II, which promote dimer ribbon formation, were used to test the effect of KCl on the temperature dependance of tPA fluorescence in the stabilized E_2 form of the membrane, and III–VI were used to determine the effect of KCl on tPA fluorescence when the Ca-ATPase was in the $E_1(Ca^{2+})_2$ state.

(i) Experiments using direct excitation of tPA. The temperature dependence of the intensity of fluorescence produced by direct excitation of tPA at 320 nm indicated that phase separations do not occur in the

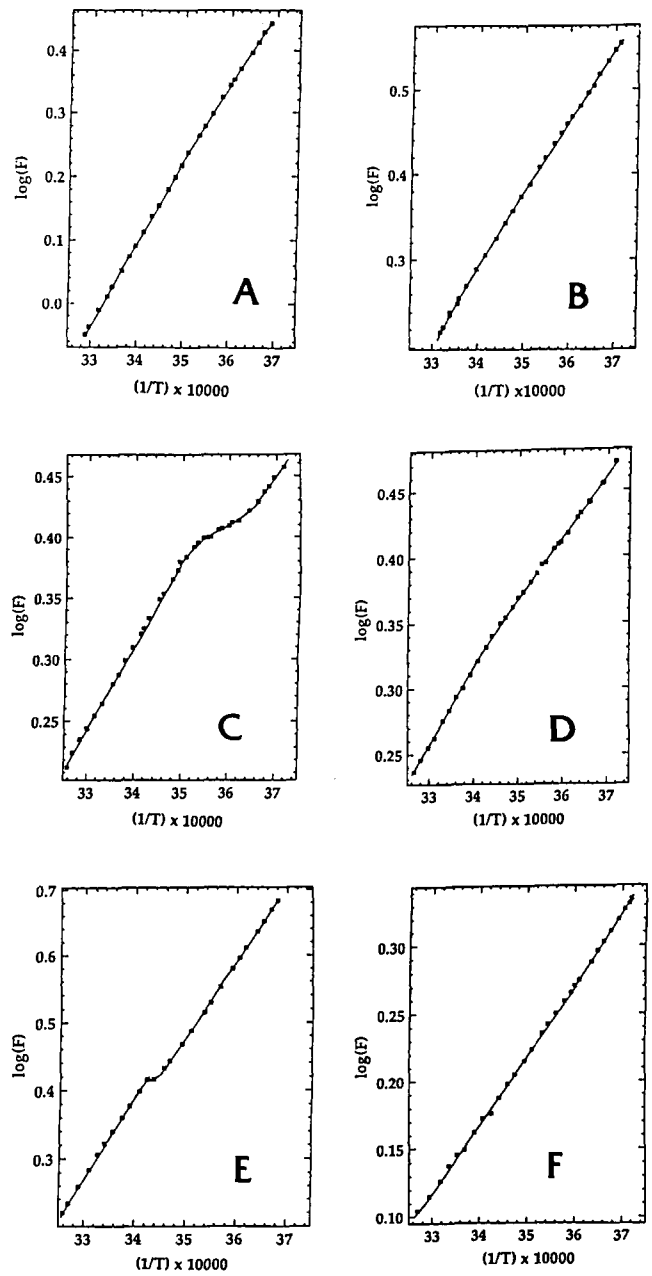

Fig. 1. Effect of Ca^{2+} on the temperature dependence of the fluorescence intensity of tPA incorporated in scallop FSR. Scallop FSR was present at 0.25 mg ml^{-1}, and tPA at 2 μM, to give a 1:75 ratio of tPA to SR phospholipid. tPA was excited directly in its absorption band at 320 nm, and the fluorescence intensity monitored at 410 nm. For indirect excitation, tPA was excited at 250 nm to avoid the direct excitation which would occur if Trp excitation at 295 nm were used (because of the appreciable absorption at 295 nm by tPA), and tPA fluorescence intensity followed at 410 nm. The concentration of FSR protein and tPA were 0.25 mg ml^{-1} and 2 μM, respectively. (A) Direct excitation, 25 μM Ca^{2+} + 0.1 M KCl (Medium IV). (B) Indirect excitation, 25 μM Ca^{2+}, no KCl (Medium III). (C) Indirect, 25 μM Ca^{2+} + 0.1 M KCl (Medium IV). (D) Indirect excitation, 1 mM Ca^{2+} + 0.1 M KCl (Medium V). (E) Direct excitation, 10 mM Ca^{2+} + 0.1 M KCl (MediumVI). (F) Indirect excitation, 10 mM Ca^{2+} + 0.1 M KCl Medium VI).

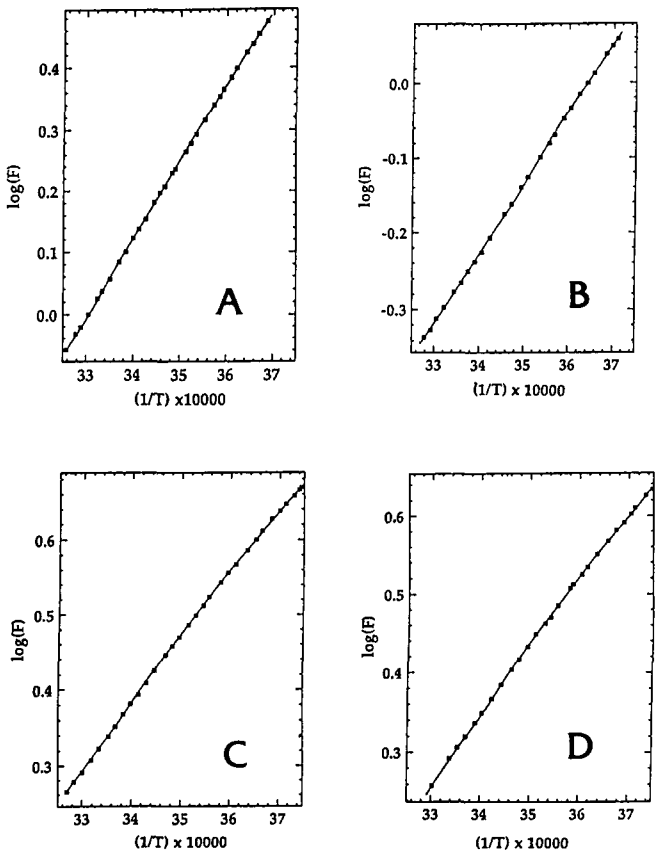

Fig. 2. Effect of dimerization on the temperature dependence of the fluorescence intensity of tPA incorporated in scallop FSR. The experiments were conducted exactly as for those reported in Fig. 1, except for the composition of the media. (A) Direct excitation, +0.1 M KCl (Medium II). (B) Indirect excitation, +0.1 M KCl (Medium II). (C) Indirect excitation, +AMP-PCP (Medium Ib). (D) Indirect excitation, ethylene glycol (Medium VIII).

bulk of the lipid bilayer in the temperature range −3 to +32°C in the $E_1(Ca^{2+})_2$, E_2 dimer and E_2P states (Fig. 1A,B, and Figs. 2A and 3A), implying that most of the lipid phase is largely unaffected by the state of the Ca-ATPase. However, in the presence of very high Ca^{2+} concentrations (10 mM, medium VI, Fig. 1E), a thermal perturbation was repeatedly observed at 20°C. This effect of extremely high Ca^{2+} concentrations may be related to its binding to low affinity sites on membrane phospholipids, as suggested by Ca^{2+} binding studies [5,7,25,26], and probably does not result from physiologically relevant alterations in the structure of the protein.

(ii) Experiments using indirect excitation of tPA. When tPA was added in increasing amounts to scallop FSR membranes in the presence of K^+, and the probe excited indirectly by energy transfer from the protein, the fluorescent intensity of the sample reached a maximum value (Fig. 4) at total tPA/phospholipid ratios of $> 1/10$. As discussed below, this is consistent either with the attainment of some saturating level of tPA in the annular lipid zone, or/and occupation of a limited number of fatty acid binding sites on the Ca-ATPase protein. In the absence of K^+, a different dependence was observed, with a significantly lower quantum yield (Fig. 4). The effect of temperature on these signals was monitored at a tPA/phospholipid ratio of 1:75. In contrast to the effects observed with the bulk lipid phase, tPA molecules within energy transfer distance of the Ca-ATPase were affected by the state of the Ca-ATPase. While no thermal perturbation could be detected when the membrane was in the $E_1(Ca^{2+})_2$

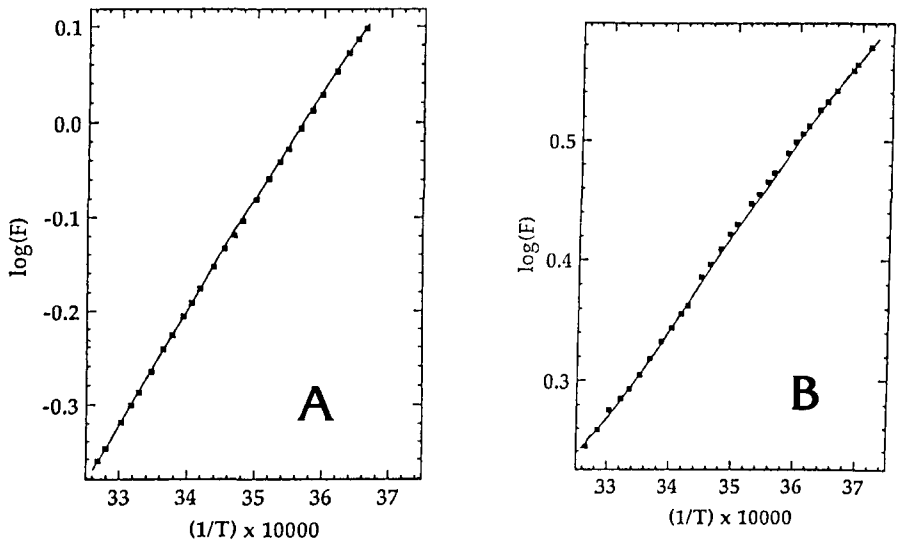

Fig. 3. Effect of phosphorylation to the E_2P state on the temperature dependence of the fluorescence intensity of tPA incorporated in scallop FSR. The experiments were carried out exactly as for those described in Figs. 1 and 3, except for the composition of the medium. (A) Direct excitation, Medium VII. (B) Indirect excitation, Medium VII.

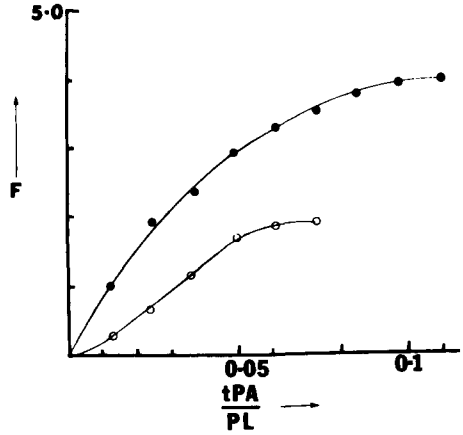

Fig. 4. Change in the fluorescence intensity by indirect excitation with increasing tPA content. Increasing amounts of tPA dissolved in deoxygenated ethanol were added to 0.1 mg ml^{-1} scallop FSR at 20°C. ○, 20% (v/v) glycerol, 1 mM $CaCl_2$, 50 mM Mops-Tris (pH 7.0); ●, 20% (v/v) glycerol, 0.1 M KCl, 1 mM $CaCl_2$, 50 mM Mops-Tris (pH 7.0).

form in the absence of K^+ (Fig. 1B), on raising the K^+ concentration to the approximately physiological value of 0.1 M, a well-defined and reproducible thermal perturbation occurred at approx. 7°C (Fig. 1C). Raising the concentration of free Ca^{2+} well above that needed to completely fill the Ca^{2+}-binding sites on the protein led to the disappearance of the 7°C transition at the protein/lipid interface (Fig. 1D,F). As with the appearance of the thermal perturbation at 20°C in the bulk lipid phase at very high Ca^{2+} (10 mM) concentrations, the latter effect is probably due to changes in the overall structure of the lipid phase produced by binding of Ca^{2+} to membrane phospholipids, which mask effects involving the Ca-ATPase protein. At the intermediate Ca^{2+} concentration of 1 mM, a change of slope was detected at approx. 17°C using the criteria of Bigelow et al. (1986), when indirect excitation was used

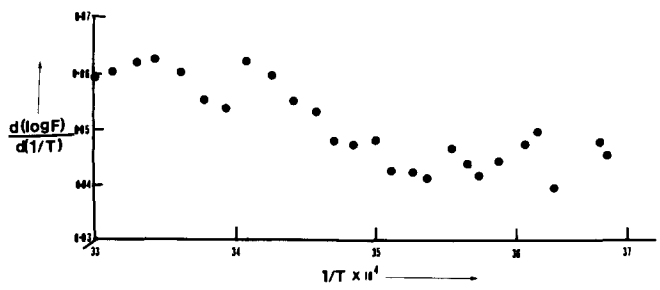

Fig. 5. Detection of discontinuity in the temperature dependence of indirectly excited tPA in the presence of 1 mM $CaCl_2$. The data in Fig. 1D were differentiated by the method of Savitzky and Golay [54]. A change in slope is apparent at a $1/T$ value correponding to approx. 17°C. This was confirmed by using the criteria of Bigelow et al. [55] for the significance of changes in slope of Arrhenius plots, when the difference in the slopes of the linear least mean squares fitted lines from $1/T = 32$ to $1/T = 34$ and from $1/T = 35$ to $1/T = 37$ was found to be greater than the sum of the standard deviations of those slopes.

(Figs. 1D and 5). At that Ca^{2+} concentration, the membrane is presumably in a transiitonal state between that observed with 25 μM and 10 mM Ca^{2+}.

When the membrane was in the stabilized E_2 state, with the Ca-ATPase subunits organized into dimer ribbons, no thermal perturbations were observed at the protein/lipid interface over the temperature range −3 to +32°C in the absence of 0.1 M K^+ (Fig. 2B,C). In marked contrast to the effect of 0.1 M KCl on the $E_1(Ca^{2+})_2$ membranes, addition of 0.1 M K^+ to the dimer state did not lead to the appearance of any discontinuity in the log$F$ vs. $1/T$ plot (Fig. 2D). Thus, in 0.1 M K^+, a clear difference exists between these two biochemical forms of the scallop FSR, which may reflect alterations at the protein/lipid interface. When the membranes were in the E_2-P state (which cannot be studied in the presence of K^+), again no clear perturbation was seen (Fig. 3B), with no statistically significant changes in slope.

Discussion

In the work reported here we have used the fluorescent membrane probe *trans*-parinaric acid to determine if structural changes in the Ca-ATPase protein of scallop FSR associated with different biochemical states of the protein affect its interaction with the lipid phase of the membrane. Addition of tPA to very high ratios of tPA to phospholipid produced a maximum in the fluorescence intensity observed when the probe was indirectly excited by energy transfer from Trp side chains on the protein. Interaction of fatty acid with lipid bilayers is complex [27]. Lee and co-workers (see, for example, Refs. 18–20) have reported binding of fatty acid directly to the Ca-ATPase protein at both annular and non-annular sites, and one interpretation of the experiment is that saturation of a limited number of binding sites for tPA (a fatty acid) on the protein is occuring. The saturation effect may also be explicable in terms of a limited solubility of tPA in the annular region of the bilayer, which ordinarily consists of approx. 30 molecules of phospholipid surrounding the transmembrane domain of the protein (see, for example, Refs. 28–30). The annulus is unlikely to be able to accomodate an indefinite increase in its content of tPA. The finding that the fluorescence intensity of indirectly excited tPA is substantially higher in the presence of 0.1 M K^+, is consistent with suggestions that K^+ immobilizes annular phospholipid in rabbit SR [31], since tPA partitions between gel phase and liquid-crystalline phase lipid in an approx. 5-fold ratio, with an approx. 3-fold increase in quantum yield [13,14]. However, effects of K^+ on tPA binding sites on the Ca-ATPase are also possible. Both tPA molecules within the lipid annulus and unbound to the Ca-ATPase, and/or tPA bound to the Ca-ATPase may in

fact contribute to the effects seen in the indirect excitation experiments.

The presence of a discontinuity in the $\log F$ vs. $1/T$ plot observed at physiological K^+ concentration (0.1 M) was dependent on occupancy of the Ca^{2+} binding sites on the Ca-ATPase, produced by changes in the free Ca^{2+} concentration from 10 nM to 25 μM. Over this range, the scallop SR changes from the E_2 to the E_1 state, with major changes in both the tertiary [12] and quaternary structure of the Ca-ATPase protein [5,7,9]. Parts of the Ca-ATPase molecule that are interfaced with other Ca-ATPase molecules in the dimer (E_2) form of the membrane will contact lipid in the E_1 state, leading to both modification of the protein/lipid interface and changes in the pools of lipids sequestered between the Ca-ATPase molecules. Assuming that the fluorescence signal from indirectly excited tPA represents energy transfer from tryptophan side chains to tPA partitioned into the annular region, the thermal perurbation at approx. 7°C represents a Ca^{2+}-dependent effect on the protein/lipid interface. Several studies point to the importance of the protein/lipid interface in the normal functioning of the enzyme, e.g., there is evidence that a lipid-dependent rotational motion of the Ca-ATPase is associated with the rate-limiting catalytic step [32,33]. No differences were seen between the bulk lipid phase in the E_1 and E_2 states, so that changes in the biochemical and structural state of the Ca-ATPase protein have little effect on most of the surrounding lipid molecules. A change in activation free energy for the Ca-ATPase activity occurs at 11°C in scallop SR [21], relatively close to the effect reported here.

The dependence of the transition at approx. 7°C seen with the E_1 form of the enzyme on the presence of K^+ and the higher quantum yield of indirectly excited tPA in the presence of K^+ fit the observations of Selinsky and Yeagle [31], who found that detection of a motionally resticted subpopulation of phospholipid molecules in rabbit FSR by NMR required 0.1 M K^+: the results reported here also suggest that the interaction between the Ca-ATPase and the adjacent lipid molecules is sensitive to the K^+ concentration. K^+ has long been known to activate the Ca^{2+}-activated ATPase activity of the SR [34], and K^+ is now thought to modulate several enzymatic steps in the Ca-ATPase reaction sequence [35], e.g., K^+ activates the hydrolysis of the E_2-P form of the rabbit enzyme by binding to the cytoplasmic aspect of the membrane [36]. K^+ is also known to interact with the Ca^{2+} binding domain on the enzyme [37,38]. In the case of the scallop SR, K^+ renders thiol groups on the protein less accessible to the thiol reagent 5,5′-dithiobis(2-nitrobenzoate), and modifies the tryptic digestion pattern [12], as well as activating the enzyme activity and inducing dimer ribbons of Ca-ATPase subunits in the E_2 state [7]. The K^+ concentration inside living muscle cells is approx. 100–140 mM [39,40], so that the effects seen in 100 mM K^+ are physiologically relevant.

Effects at extremely high Ca^{2+} concentrations (10 mM), when a thermal transition is manifested in the bulk phase of the lipid, and the K^+-dependent ~7°C transition at the protein/lipid interface in the $E_1(Ca^{2+})_2$ state disappears, are likely to involve changes in the lipid phase of the membrane produced by binding of Ca^{2+} to relatively low affinity sites on the phospholipid. Many studies have indicated interactions between phospholipids and Ca^{2+} [e.g., 41]. Although bridging Ca^{2+} can be bound with high affinity to pure PS lamellae that have been brought into close proximity [42], normally high concentrations of Ca^{2+} are usually required for significant effects in mixtures of phospholipids [e.g., 42], with the affinity of the mixed phospholipid membranes for Ca^{2+} decreasing as the proportion of PS in the mixture falls [43]. Typically, phospholipid mixtures become saturated with Ca^{2+} over the mM concentration range [e.g., 44], and low affinity binding of Ca^{2+} to both rabbit and scallop FSR has been ascribed to the ion interacting with the phospholipid component of the membrane [5,7,24,25]. London and Feigenson [45] have in fact reported a very clear lateral phase separation induced by 10 mM Ca^{2+} in recombinant membranes of the rabbit Ca-ATPase combined with synthetic lipids. Their observations were not due to direct interaction between the Ca-ATPase and Ca^{2+}. Thus, the effects that were observed in the work reported here with native scallop FSR vesicles at high Ca^{2+} concentrations are probably unrelated to the biochemical state of the Ca-ATPase, and result from changes in the state of the lipid phase produced by occupation of low affinity Ca^{2+} binding sites on phospholipid. Although Ca^{2+} concentrations in the mM range can occur within the lumen of the SR when it is loaded with Ca^{2+} and the muscle is relaxed [46], it is not clear whether such Ca^{2+}-dependent effects on the lipid phase could play any physiological role in SR function.

The transition observed at the protein/lipid interface at approx. 7°C in 100 mM K^+ with the E_1 form of the scallop FSR but not in the E_2 form at 100 mM K^+ reflects a difference in the relationship between the Ca-ATPase and the neighboring lipid molecules between the two states of the enzyme. Of the 13 tryptophan residues of the rabbit Ca-ATPase, 12 are predicted to lie in the transmembrane helices [47] near the cytoplasmic and luminal surfaces of the bilayer (only Trp-552 in the nucleotide binding domain is external to the bilayer). Presumably, it is from similarly distributed Trp residues that resonance energy transfer to tPA in the lipid phase occurs in the scallop FSR in the experiments using indirect excitation. There is evidence that Ca^{2+} binding sites in the Ca-ATPase involve residues

in transmembrane helices M4, M5, M6 and M8 [48,49], and binding of Ca^{2+} to the Ca-ATPase causes an increase in the quantum yield of the Trp residues in the transmembrane part of the enzyme, with the Trp in the extramembrane nucleotide binding domain contributing little to the effect [50]. This change in the environment of the transmembrane Trp residues must reflect changes in the structure of that domain of the Ca-ATPase due to occupancy of the Ca^{2+} binding sites. Thus, any difference in the lipid phase close to the protein/lipid interface between $E_1(Ca^{2+})_2$ and E_2 at physiological K^+ concentrations may derive from the same structural changes in the transmembrane domain of the Ca-ATPase that give rise to the enhancement of Trp fluorescence on Ca^{2+} binding. However, a Ca^{2+}-binding site on the stalk region of the Ca-ATPase has been reported by several laboratories [51–53], and the transmembrane region of the Ca-ATPase may not be the only part of the Ca-ATPase affected by Ca^{2+} binding. Such a location could be important if tPA bound at non-annular sites plays a role in the effects reported here.

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