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Modulation of Sarcoplasmic Reticulum Ca^{2+}-Pump Activity by Membrane Fluidity†

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ABSTRACT: Intramolecular excimerization of 1,3-di-1-pyrenylpropane [Py(3)Py] was used to assess the fluidity of sarcoplasmic reticulum membranes (SR); on the basis of the spectral data, the probe incorporates completely inside the membrane probably somewhere close to the polar head groups of phospholipid molecules, however not in the very hydrophobic core. The excimerization rate is very sensitive to lipid phase transitions, as revealed by thermal profiles of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) bilayers. Cholesterol abolishes pretransitions and broadens the thermal profiles of the main transitions which vanish completely at 50 mol % sterol. Excimer formation in liposomes of SR total lipid extracts does not show any sharp transitions, as in the case of DMPC and DPPC. However, the plots display discontinuities at about 20 °C which are broadened by cholesterol and not observed at 50 mol % sterol. Also cholesterol has been incorporated in native SR membranes by an exchange technique allowing progressive enrichment without changing the phospholipid/protein molar ratio. As in liposomes, discontinuities of excimer formation at 20 °C are broadened by cholesterol enrichment. The full activity of uncoupled Ca^{2+}-ATPase is only affected by cholesterol above a molar ratio to phospholipid of 0.4. However, a significant decrease in activity (about 20%) is only noticed at a ratio of 0.6 (the highest technically achieved); at this ratio, about 28 lipid molecules per Ca^{2+}-ATPase are expected to be relatively free from cholesterol interaction. The vesicle structure is still intact at this high ratio, as judged from the absence of basal activity (not Ca^{2+} stimulated). However, the sterol significantly decreases to about 60% the energetic efficiency of Ca^{2+} pumping (Ca^{2+}/ATP ratio).

Sarcoplasmic reticulum membranes (SR)[1] have been extensively characterized in their structural and functional aspects (Weber et al., 1973; Hasselbach, 1979; Tada et al., 1978). The major protein, the Ca^{++}-pump enzyme, is intrinsically associated with membrane lipids which greatly influence the enzyme activity (Martonosi et al., 1971; Bennett et al., 1980; Johannsson et al., 1981a). Lipids in contact with the ATPase enzyme modulate its function through physical interactions (Bennett et al., 1980; Johannsson et al., 1981a), including changing membrane fluidity. Thus, Ca^{2+} translocation across SR membrane and molecular mechanisms associated to energy transductions between the electroosmotic energy of Ca^{2+} gradients and chemical energy of ATP may be modulated by lipid–protein interactions, presumably affected by membrane fluidity. Cholesterol has a condensing effect on the acyl chains of bilayers in the fluid state (Houslay & Stanley, 1982). When forced to interact with some membrane proteins, it has a strong inhibitory effect on their function (Warren et al., 1975). Cholesterol is normally excluded from direct contact with the Ca^{2+}-ATPase enzyme (Bennett et al., 1975; Johannsson et al., 1981a; Simmonds et al., 1982) and excluded from other membrane enzymes as well (Houslay & Stanley, 1982). Thus, cholesterol in moderate concentrations does not affect significantly the overall Ca^{2+}-ATPase activity. This work deals with an attempt to characterize the effect of cholesterol on membrane fluidity and the energetic coupling of Ca^{2+} pumping.

Materials and Methods

Fragmented sarcoplasmic reticulum from rabbit white muscles was prepared according to Madeira & Antunes-Madeira (1976), although isolation and ressuspension media always contained 2.5 mM DTT and 10 μM PMS. Protein was normally determined by the biuret method (Gornall et al., 1951). Some preparations were assayed by the Lowry procedure (Lowry et al., 1951) calibrated against the biuret analysis.

Lipids of SR were extracted by the method of Madeira & Antunes-Madeira (1976). Phospholipids were quantitated by measuring the inorganic phosphate (Bartlett, 1959), after hydrolysis of extracts at 180 °C in 70% $HClO_4$ (Böttcher et al., 1961). Cholesterol in lipid extracts was assayed by the

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[1] Abbreviations: ATP, adenosine triphosphate; Ca^{2+}-ATPase, (Ca^{2+} + Mg^{2+})-dependent ATP phosphohydrolase; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DTT, dithiothreitol; PMS, phenylmethanesulfonyl fluoride; Py, pyrene; $PyCH_2OCH_3$, (1-pyrenylmethyl) methyl ether; Py(3)Py, 1,3-di-1-pyrenylpropane; SR, sarcoplasmic reticulum; Tris, tris(hydroxymethyl)-aminomethane.

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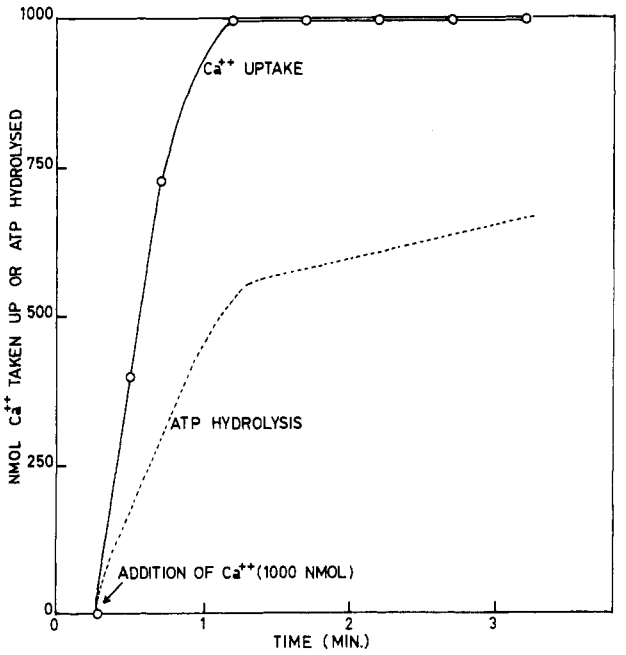

FIGURE 1: Profile of ATP hydrolysis during Ca^{2+} uptake in the presence of oxalate. This represents a typical experiment carried out at 30 °C, pH 6.9, in a medium (5 mL) containing 50 mM KCl, 5 mM $MgCl_2$, 5 mM oxalate, 5 mM Tris, and 1 mM MgATP. ATP hydrolysis was monitored by proton production. When the released organic phosphate is assayed, a similar profile is obtained. Ca^{2+} uptake was monitored by Millipore filtration of aliquots and measurements of calcium in filters by atomic absorption spectrometry. Note that the slope of ATP hydrolysis sharply decreases when the added Ca^{2+} has been taken up.

Lieberman–Bürchard reaction (Huang et al., 1961).

Liposomes were prepared by vortexing a mixture of 2.7 mg of phospholipid supplemented with the required amount of cholesterol in 5 mL of a buffer solution containing 0.1 M KCl and 5 mM Tris-HCl, pH 7.0, for 2 min, at a temperature above the main phase transition of lipid species. The mixtures were then briefly sonicated in a water bath to disperse lipid aggregates.

Incorporation of cholesterol into SR membranes was carried out by an exchange process taking place during the incubation of SR vesicles with small cholesterol-carrier liposomes (Madden et al., 1979). Phospholipids of SR (40 mg) and cholesterol (40 mg) were suspended in 9 mL of deoxygenated water, and the mixture was sonicated to clarity, under N_2, in brief bursts to avoid excess heating. The suspension was then centrifuged at 50000*g* for 60 min to remove undispersed lipid, excess cholesterol, and titanium particles from the sonicator probe. The clear supernatant was mixed with SR vesicles (concentrations described in legend to Figure 5) in media containing 1 M KCl, 0.25 M sucrose, 2.5 mM DTT, 10 μM PMS, and 10 mM Tris–maleate, pH 8.0. Incubation followed at 20 °C, under N_2, with gentle stirring. Sterol incorporation in SR was terminated by withdrawal of aliquots diluted in 2 volumes of ice-cold buffer containing 0.1 M KCl, 2.5 mM DTT, 10 μM PMS, and 10 mM Tris–maleate, pH 7.0. Vesicles of SR were collected by centrifugation and further purified from free liposomes by density gradient centrifugation (Madden et al., 1979). The pellets were rinsed with 0.1 M KCl buffer (as described above) and finally resuspended in the same buffer. Controls were similarly prepared except cholesterol was omitted in carrier liposomes.

Ca^{2+} uptake was estimated from recordings of ATP hydrolysis [see Madeira (1982)], as briefly documented by the typical experiment of Figure 1. After the added Ca^{2+} has been taken up, the slope of ATP hydrolysis changes sharply, thus permitting a precise measurement of hydrolyzed ATP. With oxalate present the amount of Ca^{2+} taken up virtually equals the total added, since this amount remains well below the maximal storage capacity of the vesicles [about 10 μmol/mg of protein (cf. Hasselbach, 1978, 1979)].

The maximal ATP splitting capacity of preparations was measured in the presence of the ionophore lasalocid (X-537A) to uncouple ATP hydrolysis from Ca^{2+} accumulation. The activity was determined by monitoring proton production due to ATP splitting (Madeira, 1978).

Incorporation of Py(3)Py and analogues in SR membranes and liposomes was carried out as before (Almeida et al., 1982). Liposomes of DMPC and DPPC were incubated at 35 and 50 °C, respectively. Blanks prepared under identical conditions served as controls for fluorometric measurements. These measurements were carried out with a Perkin-Elmer Model MPF-3 spectrofluorometer. The excimer to monomer fluorescence intensity ratio, I'/I, was evaluated as previously described (Almeida et al., 1982).

Results and Discussion

Spectral Data and Probe Location in Membranes. Intramolecular excimer formation under the conditions used in this study can be described by the excimer to monomer fluorescence intensity ratio I'/I, according to the following equation (Zachariasse et al., 1980).

$$\frac{I'}{I} \approx \frac{k_f'}{k_f} k_a \tau_0'$$

k_f', k_f, and k_a are the rate constants for excimer fluorescence, monomer fluorescence, and excimer formation, respectively, and τ_0' is the excimer lifetime in the absence of dissociation. Therefore, the ratio I'/I is primarily determined by k_a, since the radiative constants and τ_0' have been found to be essentially independent of temperature (Zachariasse et al., 1980). The fluorescence intensity ratio changes with the fluidity of viscous media and has been successfully used to monitor the fluidity of synthetic (Zachariasse et al., 1980) and native membranes (Melnick et al., 1981; Zachariasse et al., 1982; Almeida et al., 1982).

In order to determine the probe location in the membrane, we measured spectral parameters of the probe, Py(3)Py, and probe analogues, Py and $PyCH_2OCH_3$, to establish polarity and polarizability parameters. The results of polarizability were rationalized according to Zachariasse et al. (1982) and show a good linear correlation of the first vibrational peak of the ^{1}La band (Figure 2) and the refractive index function $(n^2 - 1)/(2n^2 + 1)$. The calculated refractive index for native membranes and lipid membranes reconstituted with SR lipids was close to 1.50, therefore, values higher than those of liquid paraffin. These findings demonstrate that the probes are located well inside the membrane away from the aqueous phase in an environment with a refractive index similar to that of liquid paraffin. Since the values found for native membranes are similar to those found with native lipids, we conclude that the membrane protein (mostly Ca^{2+}-ATPase enzyme) does not significantly perturb the probe location. Also similar results were obtained for the probes Py(3)Py, $PyCH_2OCH_3$, and pyrene. However, in the case of pyrene, liquid paraffin is slightly displaced from the linear correlation. Thus, the calculated refractive indexes for membranes are more uncertain than for the other probes.

The probe location was also determined by using a polarity parameter $(f - {}^1/_2 f')$. Here, $f = (\epsilon - 1)/(2\epsilon + 1)$ is the

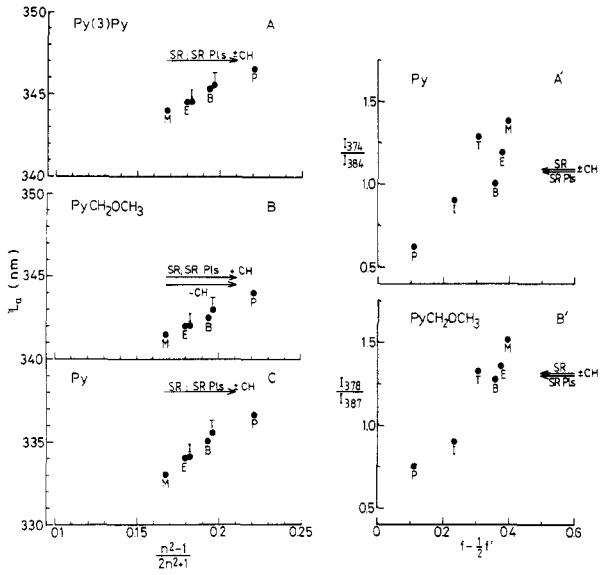

FIGURE 2: Spectral parameters of pyrene analogues as function of polarizability and polarity of solvents. (A), (B), and (C) describe the wavelength of the first absorption peak at the ^{1}La transition as a function of polarizability. (A') and (B') describe the ratio of indicated fluorescence peaks as a function of polarity. Probes incorporated in native membranes (SR), lipid membranes of SR lipids (SRPls) with or without cholesterol (CH), are indicated by arrows. Solvent symbols: M, methanol; E, ethanol; I, isopropyl ether; B, butanol; T, tetrahydrofuran; P, liquid paraffin.

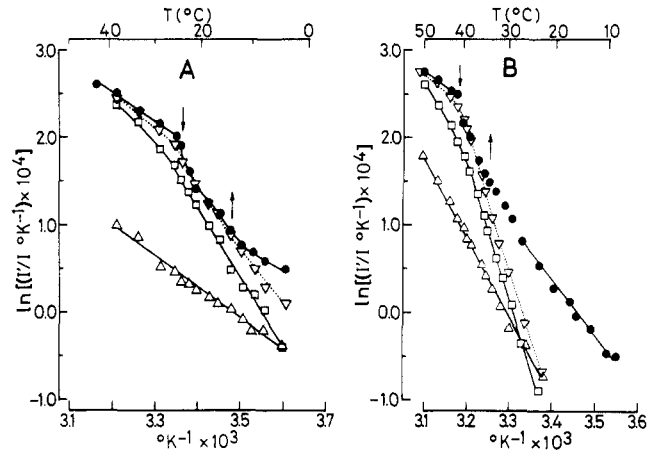

FIGURE 3: Excimer to monomer fluorescence intensity ratio, I'/I, of Py(3)Py incorporated in DMPC (A) and DPPC (B) bilayers enriched with cholesterol. The cholesterol molar ratio is 0 (●), 20 (▽), 33 (□), and 50 mol % (△). The downward and upward arrows indicate the main phase transition and pretransition temperatures, respectively, as reported by Houslay & Stanley (1982).

dielectric constant function and f', the polarizability function. This method was previously used by Zachariasse et al. (1982) on studies of erythrocyte ghosts. The intensity ratio of interesting fluorescence peaks (I_{378}/I_{387} for $PyCH_2OCH_3$ and I_{374}/I_{384} for pyrene) were plotted vs. polarity (Figure 2). These chosen analogues do not form any excimer at the experimental concentrations. Therefore, the measurement of peak intensities can be accurately done without the interference of excimer bands. The probes incorporated in native membranes or native lipids report a polarity value close to that of 1-butanol. Therefore, the probe is located away from the aqueous phase in regions different from pure paraffinic environments with polarities approaching that of 1-butanol. Therefore, the probes are apparently located completely inside the membrane, but not in the very hydrophobic core. They are displaced toward the polar head groups of phospholipid molecules. This hypothesis is consistent with the evidence provided by NMR experiments on ring current effects demonstrating that pyrene is predominantly located in the central methylene region of aliphatic chains of phosphatidylcholine bilayers, away from the polar head groups (Podo & Blasie, 1977). Furthermore, Zachariasse et al. (1982) examined erythrocyte membrane ghosts and reached similar conclusions.

The polarity of the solvent determines the peak magnitude in the fluorescence spectrum of Py(3)Py to a much smaller extent than for the analogues (Py and $PyCH_2OCH_3$). Therefore, Py(3)Py, almost insensitive to polarity, is preferred for fluidity measurements, since I'/I ratios are not affected by the possible differences in polarity of different systems, recombinants, and model solvents.

Effect of Cholesterol on Membrane Fluidity. (1) Synthetic Lipids. The temperature profiles of fluorescence intensity ratio, I'/I, in DMPC and DPPC bilayers, either pure or containing cholesterol, are described in Figure 3. The excimer to monomer fluorescence intensity ratio increases with increasing temperature. The pretransitions and the main phase transitions of DMPC and DPPC bilayers could be detected as sharp breaks in excimer formation, occurring at temperatures close to those observed with other techniques (Houslay & Stanley, 1982).

Under the assumption that Py(3)Py partitions similarly in free lipid and cholesterol-rich domains, the results obtained in DMPC and DPPC bilayers clearly show that the addition of cholesterol abolishes the pretransition and broadens the thermal profile of the main phase transition which is not detected at 50 mol % cholesterol. These results are compatible with the model presented by Presti et al. (1982), since at this cholesterol molar ratio there is a strong interaction between all cholesterol and phospholipid molecules (1:1 binding stoichiometry), thereby excluding phospholipid from participation in the transition. At 33 mol % cholesterol, a broad transition is observed which may be assigned to the "uncooperative melting" of loosely associated phospholipid with 1:1 cholesterol–phospholipid complexes. At 20 mol % cholesterol, this transition becomes sharper and probably results from the interfacial boundary phospholipid occurring between the complexes. Copeland & McConnell (1980) found that, above this molar ratio, free phospholipid domains disappear and the cooperative calorimetric sharp transition is replaced by a broad uncooperative transition. Disappearance of pure phospholipid domains at 20 mol % cholesterol is also evident from the sudden decrease of the coefficient for lateral diffusion of fluorescent phospholipids in phosphatidylcholine–cholesterol mixtures (Rubenstein et al., 1979; Owicki & McConnell, 1980).

Our results confirm that cholesterol has a condensing effect in the bilayer above the phase transition [see review of Demel & Kruyff (1976)]. However, as opposed to other observations (Marsh, 1974; Kawato et al., 1978; Delmelle et al., 1980; Presti & Chan, 1982), we did not observe the disordering effect in the gel phase ("dual effect"). Our results clearly show that cholesterol induces a progressive decrease in I'/I over the entire temperature range, either below or above the phase transition. A similar effect was described for phospholipid–cholesterol dispersions investigated by means of fluorescence polarization of perylene (Cogan et al., 1973). Studies of ^{2}H NMR in dimyristoyllecithin–cholesterol mixtures (Jacobs & Oldfield, 1979) indicate that, below the phase transition, the apparent effect of cholesterol depends on the depth where the label is located. At the methyl terminal (location of aliphatic tail), cholesterol seems to disorder the gel phase, whereas at the C_3-glycerol region (location of sterol nucleus), the sterol has

Table I: Activation Enthalpies (kJ/mol) for Excimer Formation of Py(3)Py in Liposomes of SR Lipid Extracts and Membrane Vesicles Enriched with Cholesterol

cholesterol/phospholipid molar ratio	ΔH^* (above break temp)	ΔH^* (below break temp)
lipid extract		
0.12[a]	21.3	28.6
0.25	22.7	31.0
0.50	23.5	31.7
1.00	27.8	27.8
membranes		
0.12[a]	24.2	29.0
0.28	25.3	29.0
0.40	26.8	29.6
0.46	27.2	30.8
0.60	27.8	31.3

[a] Intrinsic cholesterol.

an ordering effect. Our results suggest that the condensing effect of cholesterol is restricted to a particular domain of the hydrophobic region where Py(3)Py probably incorporates. Therefore, the cholesterol effect on phosphatidylcolines determined by Py(3)Py excimer formation reflects the local condensing effect upon the upper part of the hydrocarbon acyl chains, according to the prediction for probe location across the membrane thickness.

(2) Lipids Isolated from Sarcoplasmic Reticulum. The effect of cholesterol on fluorescence intensity ratio, I'/I, of Py(3)Py in bilayers prepared from total SR membrane lipids is shown in Figure 4A. The total lipid extract of SR membrane does not undergo any phase transition but remains in the fluid state over the temperature range 0–40 °C. Cholesterol decreases the fluidity over this temperature range, and a drastic effect is noticed when the molar cholesterol ratio changes from 33 to 50%.

The plots show a slight but discernible break which has been reported previously (Almeida et al., 1982). The break occurs around 20 °C, a temperature close to that observed for the discontinuity of SR Ca^{2+}-ATPase activity (Inesi et al., 1973; Madeira et al., 1974; Anzai et al., 1978). When cholesterol is increased to 20 and 33%, the break is still detected. The activation enthalpies derived from the slopes below and above the break temperature are not appreciably affected (Table I), indicating that the sterol does not perturb extensively the structural organization of the hydrocarbon core. The discontinuity disappears only when cholesterol is increased to 50 mol %. The discontinuity is probably related to a change in the overall structure of SR phospholipids, not involving a first-order phase transition (Martonosi, 1974). This change is abolished by cholesterol at the 50 mol % ratio, when each phospholipid interacts strongly with each cholesterol molecule in a stoichiometry of 1:1 (Presti et al., 1982).

(3) Membranes of Sarcoplasmic Reticulum. Cholesterol incorporation in native membranes was achieved by incubating SR vesicles with cholesterol-rich liposomes (Figure 5A). The phospholipid/protein molar ratio remains constant over the

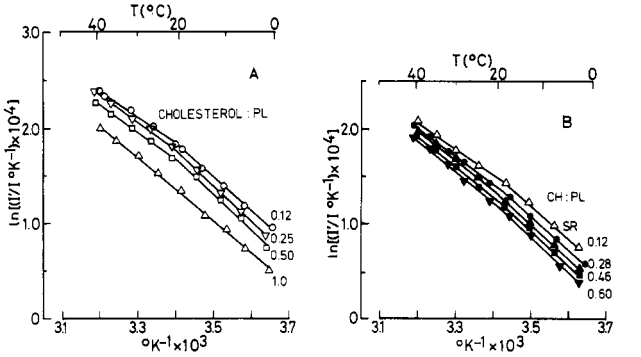

FIGURE 4: Excimer to monomer fluorescence intensity ratio, I'/I, of Py(3)Py in bilayers of SR lipids (A) or native membranes (B) containing different amounts of cholesterol as defined by cholesterol/phospholipid molar ratios.

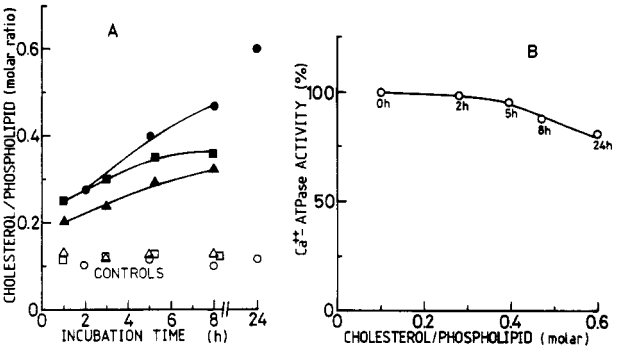

FIGURE 5: Time dependence of cholesterol incorporation in SR membranes (A) and uncoupled Ca^{2+}-ATPase activities of recombinants (B). SR vesicles (2 mg/mL) were incubated with liposomes of SR lipids enriched with cholesterol at the following lipid concentrations (mg/mL): (▲) 1.4; (■) 1.8; (●) 2.0 (here SR was 2.6 mg/mL). Incubation medium contained 1 M KCl, 0.25 M sucrose, 2.5 mM DTT, 10 μM PMS, and 10 mM Tris–maleate, pH 8.0, at 20 °C. Open symbols in (A) refer to controls of SR vesicles treated in similar conditions but in the absence of cholesterol. Part B describes the activities of recombinantes resulting from (●) in (A). These were determined at 37 °C in 2.5 mL of medium (50 mM KCl, 5 mM $MgCl_2$, 40 μM $CaCl_2$, and 5 mM Tris-HCl, pH 6.9) containing 0.25 mg of protein and 25 μM of ionophore X-537A. The reaction initiated by adding 1 mM MgATP was monitored by recording the proton production from ATP splitting.

incubation time (Table II). Therefore, the effects observed in membrane fluidity can be solely assigned to incorporated cholesterol, since any contribution of the lipid/protein ratio for the fluidity is avoided (Moore et al., 1978; Almeida et al., 1982).

In recombinants of SR membranes with cholesterol the ratio I'/I and consequently the fluidity around the probe become smaller with the increasing cholesterol/phospholipid molar ratio (Figure 4B). This effect is very similar to that observed in liposomes of SR membrane lipids. Therefore, over the temperature range from 2 to 40 °C, cholesterol has a con-

Table II: Ca^{2+}-Pump Activity and Ca^{2+}/ATP Ratio As Affected by Cholesterol Incorporated in Membranes of Sarcoplasmic Reticulum Vesicles[a]

incubation time with cholesterol-enriched liposomes (h)	phospholipid/cholesterol (molar)	phospholipid/protein (molar)	uncoupled ATPase activity (% of controls)	Ca^{2+}/ATP (% of controls)
0	9.1	70	100	100
2	3.6	78	98	74
5	2.5	76	95	80
8	2.1	73	87	64
24	1.7	71	80	58

[a] The values were calculated from experiments similar to those referred to Figure 5.

densing effect on membrane lipids.

The plots of the Py(3)Py excimer formation in SR membrane display a discontinuity around 20 °C which is related with a change in the lipid portion of the membrane (Almeida et al., 1982). The break displayed by the control membranes (Figure 4B) is attenuated as the cholesterol/phospholipid molar ratio increases. Also the break is still detected at a cholesterol/phospholipid molar ratio of 0.6 as described for liposomes of SR lipids. Probably the break would also disappear as the ratio approached 1.0, but this could not be shown, since the method used permitted a maximal sterol incorporation corresponding to a ratio of 0.6. Other methods for sterol incorporation have not been successful or produced damaged membranes, as discussed later. Furthermore, the presence of the Ca^{2+}-ATPase, the main protein in SR membrane, does not perturb the effect of cholesterol in membrane fluidity. Therefore, it is suggested that cholesterol at a molar ratio of up to 0.6 is excluded from the direct contact with the Ca^{2+}-ATPase enzyme, according to the conclusions of other investigators (Warren et al., 1975) based on functional activity parameters. As observed for SR lipid membranes, cholesterol only slightly affects the enthalpies, suggesting that the structural organization of the hydrophobic core is not appreciably perturbed (Table I).

Effect of Cholesterol on the Activity of Sarcoplasmic Reticulum. Several procedures were attempted to incorporate high cholesterol concentrations in recombinants of Ca^{2+}-ATPase and native lipids, namely, solubilization of lipid–cholesterol mixtures with detergents (octyl β-glucoside) and utilization of cholesteryl hemisuccinate as substitute for cholesterol. Unfortunately, these recombinants showed no Ca^{2+} accumulation capacity and, therefore, were not suitable for our studies. These could be successfully carried out in recombinants obtained from lipid exchange between SR vesicles and small liposomes of native lipids enriched with cholesterol at 1/1 molar ratio. Allowing lipid exchange for 24 h, at 20 °C, a maximal incorporation corresponding to a sterol/phospholipid molar ratio of 0.6 was achieved. Figure 5A and Table II summarize the composition of recombinants in typical experiments. All recombinants maintain a similar phospholipid to protein ratio ranging from 70 to 78, but a progressive cholesterol enrichment occurs over incubation. The recombinant of 8 h reached a phospholipid/cholesterol ratio of about 2.0. According to Presti et al. (1982), this stoichiometry would produce a combination of one sterol interacting strongly with one phospholipid molecule and a second phospholipid molecule loosely associated with the complex. It follows that about 35 lipid molecules per enzyme molecule are relatively free from interacting with cholesterol and a similar amount is strongly immobilized by the sterol. Ratios with less cholesterol favor the coexistence in the membrane of free lipid domains.

Since a minimum of about 30–40 lipid molecules is required to support a maximal ATPase activity (Hesketh et al., 1976; Metcalfe et al., 1976; Bennett et al., 1980), a decrease in activity would be observed when a phospholipid/cholesterol ratio of about 2.0 is approached. However, a significant decrease in activity (about 20%) was only noticed at a ratio of 1.7 for which about 28 lipid molecules are expected to be relatively free (Figure 5B). Therefore, these results concur well with the conclusions that cholesterol is excluded from direct contact with the ATPase enzyme (Warren et al., 1975) which probably segregates in cholesterol-poor or -deficient lipid domains (Kleeman & McConnell, 1976).

However, our results as well those of Johannsson et al. (1981) do not agree with those reported by Madden et al.

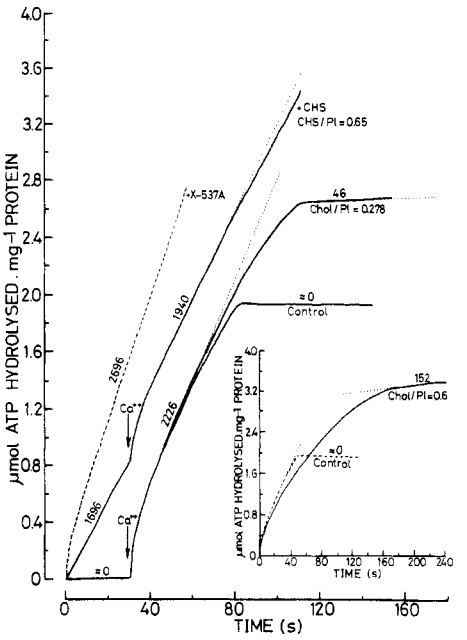

FIGURE 6: Activity of Ca^{2+} pump as affected by several treatments and cholesterol incorporation in membranes. The activities were determined at 37 °C in media (2.5 mL) containing 50 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 1 mM MgATP, and 5 mM Tris, pH 6.9. Stimulation by Ca^{2+} was achieved by adding 0.2 mM $CaCl_2$. Dashed line is the activity of membranes uncoupled by the presence of 25 μM X-537A. CHS states for a cholesteryl hemisuccinate recombinant in which the ratio sterol/phospholipid was about 0.6. The other curves in the main figure describe the behavior for a cholesterol recombinant (cholesterol/phospholipid = 0.3) and its respective control. The inset depicts the activity of a recombinant with maximal cholesterol incorporation (sterol/phospholipid = 0.6) and respective control. The residual ATPase activity (before Ca^{2+} addition) was almost nil either in controls or in recombinants. The steady-state ATPase (after Ca^{2+} uptake, upper portion of curves) is low in all cases, even for the recombinant 0.6. Figures over the traces refer to the specific activities in nanomoles of ATP hydrolyzed per minute per milligram. Note also the high residual activity of uncoupled membranes and cholesteryl hemisuccinate recombinant.

(1979). The apparent strong inhibition of ATPase activity reported by Madden et al. (1979) may be explained by an experimental artifact due to the inhibition by cholesterol of spontaneous uncoupling of thermally unstable sarcoplasmic reticulum isolated in the absence of dithiothreitol (Johannsson et al., 1981b).

Often, sarcoplasmic reticulum preparations contain a Mg^{2+}-ATPase activity in addition to the Ca^{2+}-stimulated ATPase (Makinose & Hasselbach, 1965; Makinose & The, 1965). The ATP splitting activity in the absence of added Ca^{2+}, termed basal activity (Makinose & Hasselbach, 1965), has been assigned to several sources including contamination by other membrane systems, e.g., plasma membranes (Flaherty et al., 1975; Malouf & Meissner, 1979), transverse tubular membranes, and mitochondria (Malouf & Meissner, 1979).

Recently Rosemblatt et al. (1981) demonstrated that purified SR preparations have no basal ATPase activity. They found that it is mostly localized in the transverse tubular system. Since our preparations have basal ATP splitting activities close to zero (Figure 6), we conclude that contamination with ATPases from sources other than sarcoplasmic reticulum was avoided. Thus, any activation of the ATPase before addition of Ca^{2+} can be solely assigned to residual free Ca^{2+} (Duggan, 1977) which may be of the order of 1 or 2 μM in our experimental conditions.

Native membranes and cholesterol recombinants exhibited an initial limited ATP hydrolysis which is related to the uptake

of residual Ca^{2+} in the medium (not shown). Subsequently, the hydrolysis rate remains close to zero and high rates are only observed after Ca^{2+} addition (Figure 6). Conversely, when the activity is followed in the presence of either a detergent or a Ca^{2+} ionophore (X-537A), a very high rate stimulated by residual Ca^{2+} (abolished by EGTA) is exhibited (Figure 6). This persists until all the added ATP is hydrolyzed, since the vesicles are uncoupled from Ca^{2+} accumulation. Therefore, activated levels of "residual" Ca^{2+}-ATPase usually represent increased membrane permeability. Intact vesicles only show stimulated activity upon Ca^{2+} addition during the phase of Ca^{2+} uptake. At the end of uptake, the activity returns to a low steady state approaching zero. Incorporation of cholesterol in SR membranes does not affect their intactness, since the activity before Ca^{2+} addition is almost zero even after maximal cholesterol incorporation (molar ratio cholesterol/phospholipid = 0.6).

Energetic Efficiency of Ca^{2+} Pumping As Affected by Cholesterol. Cholesterol extends the time interval required for the uptake of Ca^{2+} (Figure 6), although the Ca^{2+}-stimulated ATPase rate is only slightly decreased. When the maximal cholesterol has been incorporated, the Ca^{2+}-ATPase activity still remains high compared to the control (membranes incubated in the absence of cholesterol-enriched liposomes). However, the activity deviates from a quasi-linear pattern exhibited by the control (inset of Figure 6). Furthermore, a steady-state activity of about 152 $nmol \cdot mg^{-1} \cdot min^{-1}$ could be measured after the uptake of Ca^{2+}. This rate may appear significant compared to the negligible rate shown by control, but it is still low if compared to the activity of uncoupled membranes.

Unlike cholesterol, the analogue cholesteryl hemisuccinate induces extensive damage to membranes, based on the high "residual" ATPase activity and uncoupling of Ca^{2+}-ATPase from Ca^{2+} accumulation (Figure 6). This effect may result from the net negative charge of the analogue which may disturb the membrane structure by inducing disruption as a surfactant-like compound. Therefore, we wonder whether its use as a cholesterol substitute is reasonable despite the advantage of its high solubility in detergent solutions (Criado et al., 1982). Thus, when used in reconstitution systems, it should be ascertained that the membrane intactness is preserved.

The main effect of cholesterol on the activity of the Ca^{2+} pump is the increase of the relative amount of split ATP. In principle, this could be expected if cholesterol should induce membrane disruption and, therefore, partial uncoupling of the Ca^{2+} pump. This possibility may be ruled out, since cholesterol does not induce membrane disruption, as judged from the very low "residual" ATPase and also the steady-state activity after Ca^{2+} has been taken up. Therefore, it appears that membrane cholesterol decreases the energetic efficiency of the Ca^{2+}-pump enzyme in a specific fashion. Cholesterol decreases significantly the Ca^{2+}/ATP ratio to about 58% of control (Table II) when the cholesterol/phospholipid ratio is 0.6. This reflects a decreased activity of the pump as Ca^{2+} translocator, since membrane damage and significant inhibition of the pump potential activity (uncoupled) are not involved. The effect of cholesterol on membrane fluidity appears to contribute to the regulation of the energetic coupling of Ca^{2+} transport. However, cholesterol-mediated withdrawal of lipid molecules from the contact with the ATPase enzyme may contribute as well to the observed findings. Both processes can interfere with the conformation of the Ca^{2+}-pump system, either as a monomer or oligomer; this interference is reflected in activity parameters. Whatever the effect, it should result in Ca^{2+} extrusion by the pump after it has been bound in an energy-dependent process. It appears that Ca^{2+} bound to the phosphorylated enzyme intermediate is released to the external medium, rather than to the vesicular lumen. This loss of efficiency could presumably reflect changes at the ionophoretic channel of the pump system as a result of physical constraints imposed by the membrane under the influence of cholesterol.

Registry No. ATP, 56-65-5; ATPase, 9000-83-3; DMPC, 13699-48-4; DPPC, 2644-64-6; Py, 129-00-0; $PyCH_2OCH_3$, 91385-15-8; Py(3)Py, 61549-24-4; Ca, 7440-70-2; cholesterol, 57-88-5; cholesterol hemisuccinate, 1510-21-0.

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Immunochemical Studies of Conformational Alterations in Bone γ-Carboxyglutamic Acid Containing Protein†

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ABSTRACT: The Ca^{2+}-dependent transition of the vitamin K dependent bone protein bone Gla-containing protein (BGP) was investigated by use of anti-BGP antibody that reacts with the Ca^{2+}-dependent conformation of BGP. Antibody binding occurred in the presence of Ca^{2+} or Mg^{2+} with a K_d(app) of 1.75 mM for Ca^{2+}. Upon removal of Ca^{2+} with ethylenediaminetetraacetic acid, antibody binding was eliminated. Upon thermal acid decarboxylation of BGP, Ca^{2+}-independent binding of the antibody was restored. Thus, the epitope not expressed by fully carboxylated BGP in the absence of calcium ion was restored either by addition of Ca^{2+} or by decarboxylation of the protein. Circular dichroic studies of fully carboxylated and fully decarboxylated BGP indicated that addition of Ca^{2+} to the fully carboxylated protein or decarboxylation to produce the glutamic acid containing equivalent of BGP resulted in increased order structure (apparent α-helix) in the protein, and this alteration was coinicident with antibody binding. These data suggest that carboxylation of this vitamin K dependent protein may lead to increased disorder in the protein as compared to the glutamic acid containing equivalent. Upon Ca^{2+} binding a structure more equivalent to the Glu-containing protein is obtained.

Bone Gla-containing protein (BGP)[1] is a noncollagenous protein specific for bone tissue that contains three residues of the unique vitamin K dependent amino acid γ-carboxyglutamic acid (Gla) (Hauschka et al., 1975; Price et al., 1976). BGP binds to Ca^{2+} and more strongly to hydroxyapatite crystals (Hauschka et al., 1975; Price et al., 1976; Hauschka & Gallop, 1977; Poser & Price, 1979), and the presence of the three Gla residues is generally thought to be responsible for this Ca^{2+} interaction. The biological function of BGP in bone physiology is still unknown but might be related to its interaction with Ca^{2+} and hydroxyapatite.

Ca^{2+} mediates a structural transition in some of the vitamin K dependent blood-clotting proteins, including prothrombin (Nelsestuen, 1976; Stenflo, 1977; Bloom & Mann, 1978; Tuhy et al., 1979; Furie et al., 1979; Madar et al., 1980) and factor X (Keyt et al., 1982). Recently, a Ca^{2+}-dependent confor-

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[1] Abbreviations: BGP, bone Gla-containing protein (also called osteocalcin); Gla, γ-carboxyglutamic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin.

0006-2960/84/0423-4720$01.50/0