Cholesterol in sarcoplasmic reticulum and the physiological significance of membrane fluidity

Axel JOHANSSON, Christopher A. KEIGHTLEY, Gerry A. SMITH and James C. METCALFE
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

(Received 10 November 1980/Accepted 19 January 1981)

Vesicles of sarcoplasmic reticulum from rabbit muscle can be loaded with cholesterol to at least 20 mol% with respect to endogenous sarcoplasmic-reticulum phospholipid without effect on the ATPase activity at 32°C. This applies both to sarcoplasmic-reticulum vesicles in which the ATPase activity is stably coupled to Ca^{2+} accumulation, and to sarcoplasmic-reticulum vesicles in which the sarcoplasmic-reticulum ATPase is activated severalfold by fully uncoupling the enzyme from net Ca^{2+} accumulation. Since the incorporation of cholesterol causes a large decrease in fluidity of sarcoplasmic-reticulum phospholipid bilayer, these results for sarcoplasmic reticulum raise the more general question of whether bilayer fluidity is important in modulating the function of membrane proteins under physiological conditions as is widely assumed, or whether the function of membrane proteins may be effectively buffered under normal operating conditions against changes in bilayer fluidity due to extraneous agents.

Native vesicles of sarcoplasmic reticulum have a ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase activity that is coupled to the active accumulation of Ca^{2+} into the vesicles (2 Ca^{2+} per ATP molecule under optimal conditions) (Martonosi & Feretos, 1975). If the native sarcoplasmic-reticulum vesicles are treated with uncoupling agents (e.g. the detergent cholate or the Ca^{2+} ionophore A23187), the ATPase is activated severalfold, but at the same time the enzymic activity is uncoupled from net accumulation of Ca^{2+} into the sarcoplasmic-reticulum vesicles (Scarpa *et al.*, 1972; Bennett, 1978). We have shown previously that cholesterol can be incorporated into fully uncoupled complexes of the purified ATPase with 1,2-dioleoylglycerophosphocholine (dioleoyl phosphatidylcholine) at up to equimolar proportions with the phospholipid without effect on the ATPase activity ($\pm 5\%$) at 37°C (Warren *et al.*, 1975). This observation is significant in showing that the large decrease in bilayer fluidity produced by cholesterol (Richards *et al.*, 1978) has no detectable effect in modulating the activity of the uncoupled enzyme in the dioleoyl phosphatidylcholine bilayer at physiological temperatures.

In this paper we confirm and extend the previous observation for the effect of cholesterol on this enzyme to show that both coupled and uncoupled sarcoplasmic-reticulum vesicles with stable ATPase activities can be loaded with cholesterol to at least 20 mol% with respect to endogenous sarcoplasmic-

reticulum phospholipid without effect on their respective ATPase activities at 32°C. We consider these results in relation to the general assumption that bilayer fluidity is important in determining membrane protein function at physiological temperatures.

A recent report by Madden *et al.* (1979), which apparently conflicts with our previous experimental data, showed a decrease in ATPase activity by about 40% at 32°C when loaded with cholesterol to about 20 mol%. We have reproduced this observation using their experimental protocol, but show that sarcoplasmic reticulum prepared in the absence of a thiol-reducing agent shows spontaneous uncoupling and activation of the ATPase under the experimental conditions used. The effect of cholesterol in decreasing this thermal instability of the sarcoplasmic reticulum accounts for the apparent inhibition of ATPase activity, which is not observed in stable sarcoplasmic-reticulum preparations.

Materials and methods

Sarcoplasmic reticulum was prepared by the protocol we have described previously (Warren *et al.*, 1974a), which was found to differ significantly from that of Madden *et al.* (1979) in that 1 mM-dithiothreitol was included during the preparation and is designated sarcoplasmic reticulum (+dithiothreitol). The sarcoplasmic-reticulum preparation

made in the same way but omitting dithiothreitol is termed sarcoplasmic reticulum (–dithiothreitol). The final suspension of sarcoplasmic reticulum (+dithiothreitol) (10 ml at 30–50 mg of protein/ml) was dialysed at 4°C for 16 h against 1 litre of sucrose buffer (300 mM-sucrose/1 M-KCl/50 mM-K₂HPO₄/KH₂PO₄, pH 8.0) before freezing in liquid N₂ and storing at –20°C.

Dipalmitoylglycerophosphocholine (dipalmitoyl phosphatidylcholine)/cholesterol liposomes (1:2 molar ratio), including [³H]dipalmitoyl phosphatidylcholine (Smith *et al.*, 1978) and [¹⁴C]-cholesterol (The Radiochemical Centre), were prepared as described by Madden *et al.* (1979), except that sonication was performed in 100 mM-KCl and 5 mM-histidine, pH 7.4. The purity of both radiochemical markers and lipids was checked by t.l.c. Liquid-scintillation counting was used to quantify dipalmitoyl phosphatidylcholine and/or cholesterol.

ATPase activities were measured by a coupled enzyme assay, and Ca²⁺ uptake and protein and lipid compositions were measured as described in Warren *et al.* (1974a). Sarcoplasmic reticulum was separated from dipalmitoyl phosphatidylcholine/cholesterol liposomes by sucrose-density-gradient centrifugation (Madden *et al.*, 1979). Protein recoveries were >85%. Details of the experimental protocols are given in the Figure legends.

Results

Effect of cholesterol on sarcoplasmic reticulum (+dithiothreitol) and sarcoplasmic reticulum (–dithiothreitol)

Sarcoplasmic reticulum (+dithiothreitol) vesicles were incubated with dipalmitoyl phosphatidylcholine/cholesterol liposomes (1:2 molar ratio) for up to 10 h at 20°C and portions were assayed at 32°C after 30 min pre-incubation in the assay medium in the absence of free Ca²⁺ (see the legend to Fig. 1). These conditions were chosen to resemble as closely as possible those used by Madden *et al.* (1979) to assay the ATPase activity of sarcoplasmic reticulum (–dithiothreitol). It can be seen from Fig. 1 that there was no significant change in the ATPase activity of sarcoplasmic reticulum (+dithiothreitol) for up to 10 h. After 8 h incubation the sarcoplasmic-reticulum (+dithiothreitol) vesicles were separated from the dipalmitoyl phosphatidylcholine/cholesterol liposomes. Lipid analysis of the sarcoplasmic reticulum (+dithiothreitol) vesicles showed that they had taken up 22 mol% cholesterol and less than 4 mol% dipalmitoyl phosphatidylcholine relative to the endogenous sarcoplasmic-reticulum phospholipid. The data for the corresponding experiment with sarcoplasmic reticulum (–dithiothreitol), replotted from the results of Madden *et al.* (1979) in Fig. 1, show, in marked contrast with sarcoplasmic reticulum

Fig. 1. ATPase activity of sarcoplasmic reticulum prepared in the presence or absence of dithiothreitol and the effect of cholesterol on the thermally unstable form.

The sarcoplasmic reticulum (+dithiothreitol) (1.5 mg/ml) was incubated at 20°C in the presence (■) or absence (□) of dipalmitoyl phosphatidylcholine/cholesterol liposomes (2.5 mg of phosphatidylcholine/ml and 2.5 mg of cholesterol/ml, molar ratio 1:2) in a solution containing 100 mM-KCl, 100 µg of streptomycin sulphate/ml, 100 units of penicillin G/ml and 5 mM-histidine, pH 7.4. Portions (10 µl) were removed and added to 2.0 ml of assay medium containing 100 mM-triethanolamine hydrochloride, 100 mM-KCl, 5 mM-MgSO₄, 2 mM-ATP, 0.5 mM-phosphoenolpyruvate, 0.15 mM-NADH, 50 µg of pyruvate kinase/ml (Boehringer), 50 µg of lactate dehydrogenase/ml (Boehringer) and 50 µM-EGTA, pH 7.4. The assay was started after 30 min incubation in the assay medium at 32°C by the addition of CaCl₂ to 100 µM and the ATPase activity was measured from the rate of decrease in A₃₄₀ in a spectrophotometer. The data for sarcoplasmic reticulum (–dithiothreitol) + cholesterol (●) are derived from Fig. 2 in Madden *et al.* (1979), Fig. 1 of which allows the conversion of these data into ATPase activity as a function of incubation time. The activity of sarcoplasmic reticulum (–dithiothreitol) incubated in the absence of liposomes (O) is from Fig. 3 in Madden *et al.* (1979).

(+dithiothreitol), the progressive reduction in ATPase activity they observed after incubation of sarcoplasmic reticulum (–dithiothreitol) with dipalmitoyl phosphatidylcholine/cholesterol liposomes at 20°C, when portions were assayed at 32°C after pre-incubation 'in the assay medium for at least 10 min' in the absence of free Ca²⁺.

Table 1. *ATPase activity and Ca²⁺ uptake of coupled or uncoupled sarcoplasmic reticulum*

Sarcoplasmic-reticulum prepared in the presence or absence of dithiothreitol was assayed for ATPase activity at 32°C after pre-incubation in the assay medium for 30 min without Ca²⁺ as described in the legend to Fig. 1, or immediately after transfer to the assay medium at 25°C. In experiments with ionophore A23187, the ionophore was added to 5 μM in the assay medium for the pre-incubation period at 32°C. Samples of the sarcoplasmic-reticulum preparations (25 mg/ml) were also treated with potassium cholate (28 mM) for 30 min at 0°C, diluted 10-fold into sucrose buffer for a further 60 min at 0°C before assaying 5 μl portions after 30 min incubation at 32°C without Ca²⁺ in 2.0 ml of the assay medium. Ca²⁺ uptake was measured using the same assay medium and conditions as for the ATPase assay but for the addition of potassium oxalate to 50 mM 1 min before the addition of Ca²⁺ (Warren *et al.*, 1974a). It should be noted that the coupling ratios (Ca²⁺/ATP) at 32°C are substantially lower than those that can be obtained from these sarcoplasmic-reticulum preparations under optimal conditions for the assay of Ca²⁺ uptake, where the coupling ratio is more than 1.5, compared with the limiting value of 2.0.

Addition	Temperature (°C)	Sarcoplasmic-reticulum (+dithiothreitol)		Sarcoplasmic-reticulum (–dithiothreitol)	
		ATPase activity (i.u.)	Ca ²⁺ /ATP ratio	ATPase activity (i.u.)	Ca ²⁺ /ATP ratio
None	32	2.0	0.80	7.1	0.01
Ionophore A23187 (5 μM)	32	7.4	0.01	6.8	0.01
Cholate (28 mM)	32	6.6	0.01	6.6	0.01
None	25	0.7	1.4	0.9	0.86

Ca²⁺ uptake and coupling in sarcoplasmic reticulum (+dithiothreitol) and sarcoplasmic reticulum (–dithiothreitol)

It is immediately apparent from Fig. 1 that the activity of sarcoplasmic reticulum (–dithiothreitol) is about 5-fold higher than sarcoplasmic reticulum (+dithiothreitol) when assayed under similar conditions at 32°C. Since the sarcoplasmic-reticulum (+dithiothreitol) preparation is highly purified [70–80% of the protein is (Ca²⁺ + Mg²⁺)-dependent ATPase (Warren *et al.*, 1974a)] and was only activated to values similar to sarcoplasmic reticulum (–dithiothreitol) when uncoupled, this suggested that the ATPase in sarcoplasmic reticulum (–dithiothreitol) might be uncoupled from Ca²⁺ accumulation into the vesicles under the experimental conditions used. This is confirmed in Table 1, which shows the normal pattern of response of sarcoplasmic reticulum (+dithiothreitol) to uncoupling agents, for example the detergent cholate or the Ca²⁺ ionophore A23187. These agents cause about a 4-fold increase in the sarcoplasmic-reticulum (+dithiothreitol) ATPase activity at 32°C, but completely uncouple the activity from Ca²⁺ accumulation (Ca²⁺/ATP = 0.01, compared with 0.80 in the absence of uncoupling agents under these conditions). However, sarcoplasmic reticulum (–dithiothreitol) showed similar ATPase activity (2.7 i.u.) and coupling ratio (0.55) to sarcoplasmic reticulum (+dithiothreitol) when the sarcoplasmic reticulum (–dithiothreitol) samples were assayed immediately after transfer from the incubation at 20°C to the assay medium at 32°C (i.e. omitting the pre-incubation for 30 min at 32°C in the absence of Ca²⁺ before assay).

The thermal stability of sarcoplasmic reticulum (+dithiothreitol) and sarcoplasmic reticulum (–dithiothreitol)

The above results clearly suggested that the sarcoplasmic reticulum (–dithiothreitol) preparation is thermally labile on incubation at 32°C in the absence of Ca²⁺, in contrast with sarcoplasmic reticulum (+dithiothreitol). This is confirmed in Figs. 2(a) and 2(b), which show the ATPase activities of sarcoplasmic reticulum (+dithiothreitol) and sarcoplasmic reticulum (–dithiothreitol) as a function of time in the assay medium without Ca²⁺ at 20, 32 and 37°C. The assays of ATPase activity were initiated by the addition of Ca²⁺ to the assay medium at the appropriate times (see the legend to Fig. 2). The sarcoplasmic reticulum (–dithiothreitol) preparation shows marked increases in ATPase activity with time, which become larger and faster at higher temperatures. Thus, at 32°C after 10 min incubation there was an 85% increase in activity compared with the activity at zero time. We note that further studies of the thermal activation of sarcoplasmic reticulum (–dithiothreitol) show that it is potentiated by low membrane concentration and the absence of free Ca²⁺, and that appropriate Ca²⁺ concentrations can substantially protect sarcoplasmic reticulum (–dithiothreitol) from thermal activation (Griffiths, 1975; Keightley, 1979).

The effect of cholesterol on the thermal activation and Ca²⁺ accumulation of sarcoplasmic reticulum (–dithiothreitol)

The assay conditions used by Madden *et al.* (1979) [sarcoplasmic reticulum (–dithiothreitol), elevated temperature and pre-incubation of dilute

Fig. 2. The effect of incubation at 20, 32 or 37°C on the ATPase activity of sarcoplasmic reticulum prepared in the presence or absence of dithiothreitol

Sarcoplasmic reticulum prepared in the presence (a) or absence (b) of dithiothreitol was incubated in the assay medium at 20, 32 or 37°C at 7.5 μg of protein/ml in the absence of Ca^{2+} before starting the ATPase assays at the times indicated by the addition of CaCl_2 to 100 μM , as described in the legend to Fig. 1.

membrane suspensions in Ca^{2+} -free medium] promote thermal activation of ATPase. We therefore tested the possibility that cholesterol might decrease the rate and extent of thermal activation of sarcoplasmic reticulum (–dithiothreitol) under these conditions, to account for the differences in response to cholesterol between sarcoplasmic reticulum (+dithiothreitol) and sarcoplasmic reticulum (–dithiothreitol) in Fig. 1.

In Fig. 3(a), the rate of thermal activation of sarcoplasmic reticulum (–dithiothreitol) loaded for 8 h at 20°C with cholesterol to 22 mol% from dipalmitoyl phosphatidylcholine/cholesterol liposomes is compared with the sarcoplasmic reticulum (–dithiothreitol) treated in the same way without the liposomes. There is a substantial decrease in the rate and extent of thermal activation at 32°C of the sarcoplasmic-reticulum (–dithiothreitol) vesicles loaded with cholesterol compared with sarcoplasmic-reticulum (–dithiothreitol) vesicles without cholesterol. There is no change in activity in sarcoplasmic-reticulum (+dithiothreitol) vesicles with or without cholesterol on incubation at 32°C under the same conditions (Fig. 3a), since these vesicles are thermally stable over the periods shown (see also Fig. 1).

We note that when cholesterol partially protects the sarcoplasmic-reticulum (–dithiothreitol) vesicles from thermal activation, there is also some protec-

tion against the uncoupling of the ATPase activity from Ca^{2+} accumulation. The uptake of Ca^{2+} by sarcoplasmic-reticulum (–dithiothreitol) vesicles with or without cholesterol after 12 min incubation at 32°C in the absence of free Ca^{2+} is compared in Fig. 3(b). The coupling ratio of $\text{Ca}^{2+}/\text{ATP}$ for vesicles with cholesterol is 0.20 and without cholesterol is 0.015. It should be emphasized that both the activities and coupling ratios of sarcoplasmic reticulum (–dithiothreitol) with or without cholesterol are similar when assayed immediately after transfer from 20°C to 32°C, before significant thermal activation has occurred. We conclude that thermal activation closely parallels the uncoupling of sarcoplasmic reticulum (–dithiothreitol).

The effect of cholesterol on ATPase activities in fully uncoupled sarcoplasmic reticulum (+dithiothreitol) and sarcoplasmic reticulum (–dithiothreitol)

Taken together the above data demonstrate that under conditions where Ca^{2+} accumulation remains coupled to ATPase activity in stable sarcoplasmic reticulum which is not undergoing spontaneous uncoupling, there is no effect of cholesterol on ATPase activity. This prompts the further question of whether the ATPase activity in fully uncoupled sarcoplasmic-reticulum vesicles is inhibited by cholesterol, contrary to extensive previous data for dioleoyl phosphatidylcholine–ATPase complexes

Fig. 3. The effect of cholesterol on thermal activation and Ca^{2+} uptake at 32°C in sarcoplasmic reticulum prepared in the absence of dithiothreitol

(a) Sarcoplasmic reticulum prepared in the presence (■ and □) or absence (● and ○) of dithiothreitol and incubated with (■ and ●) or without (□ and ○) dipalmitoyl phosphatidylcholine/cholesterol liposomes for 8 h at 20°C , as described in the legend to Fig. 1, was assayed for ATPase activity after incubation in the Ca^{2+} -free assay medium at 32°C as described in the legend to Fig. 2. Separation of the sarcoplasmic-reticulum samples from the dipalmitoyl phosphatidylcholine/cholesterol liposomes was as described by Madden *et al.* (1979), and the incorporation of cholesterol into sarcoplasmic reticulum was 22 mol% (see the text). (b) Sarcoplasmic reticulum (—dithiothreitol) after

(Warren *et al.*, 1975). We have re-examined this, using three different methods to activate the ATPase and uncouple its activity from Ca^{2+} accumulation.

In the first series of experiments sarcoplasmic reticulum (—dithiothreitol) was fully uncoupled by thermal activation before loading with cholesterol to about 20 mol% from dipalmitoyl phosphatidylcholine/cholesterol liposomes. It can be seen from Fig. 4(a) that the presence of cholesterol had no effect on the ATPase activity at 32°C . Thus cholesterol has no inhibitory action on the ATPase activity of fully uncoupled sarcoplasmic reticulum (—dithiothreitol).

In a second set of experiments, sarcoplasmic reticulum (+dithiothreitol) was fully uncoupled from Ca^{2+} accumulation by the addition of $5\mu\text{M}$ -ionophore A23187, before loading with cholesterol to 25 mol% (22 mol% for control samples without ionophore) from dipalmitoyl phosphatidylcholine/cholesterol liposomes. It can be seen from Fig. 4(b) that the ATPase activity of the ionophore-treated samples is constant during cholesterol loading, and the vesicles also remained fully uncoupled by the ionophore ($\text{Ca}^{2+}/\text{ATP} < 0.01$). The ATPase activities and coupling of the control sarcoplasmic-reticulum (+dithiothreitol) samples also remained unaltered. Very similar results were obtained using another ionophore (ionomycin) to uncouple sarcoplasmic reticulum (+dithiothreitol) before cholesterol loading.

Finally we used our original protocol to form fully activated complexes of ATPase with dioleoyl phosphatidylcholine $\pm$ cholesterol, using cholate to equilibrate the added lipid with the endogenous sarcoplasmic reticulum phospholipid, as described previously (Warren *et al.*, 1975) (see the legend to Fig. 4c). Under these conditions the complexes are fully uncoupled by exposure to cholate and show no detectable ATP-dependent Ca^{2+} accumulation (Warren *et al.*, 1974a, 1975). For these experiments we used sarcoplasmic reticulum (—dithiothreitol) with or without loaded cholesterol to determine whether the apparent inhibition of sarcoplasmic reticulum (—dithiothreitol) by cholesterol demonstrated in Fig. 3(b) is maintained after the complexes are fully activated by cholate. Samples of sarcoplasmic reticulum (—dithiothreitol) $\pm$ cholesterol were treated with a large excess of dioleoyl phosphatidylcholine containing various proportions of cholesterol in the presence of cholate. It can be seen from Fig. 4(c) that all of the complexes show the

loading with 22 mol% cholesterol as in (a) (●) or without cholesterol (○) was assayed for Ca^{2+} uptake at a protein concentration of $7.5\mu\text{g}/\text{ml}$ as described in the legend to Table 1, after pre-incubation for 12 min at 32°C in the assay medium in the absence of Ca^{2+} .

Fig. 4. The effect of cholesterol on the ATPase activity of sarcoplasmic reticulum fully uncoupled by incubation at 37°C, by ionophore A23187 or by cholate

(a) Sarcoplasmic reticulum (—dithiothreitol) (2 mg of protein/ml) was activated and fully uncoupled by incubation at 37°C for 20 min in a solution containing 100 mM-KCl, 5 mM-histidine and 0.050 mM-EGTA, pH 7.4. CaCl_2 was then added to 0.10 mM and the incubation was divided into two samples. The samples were incubated at 20°C in the presence (●) or absence (○) of dipalmitoyl phosphatidylcholine/cholesterol liposomes and 10 μl portions were subsequently assayed for ATPase activity at 32°C as described in the legend to Fig. 1. After 7 h incubation sarcoplasmic reticulum was separated from dipalmitoyl phosphatidylcholine/cholesterol liposomes (Fig. 3a) and analysed for protein and lipid. The sarcoplasmic reticulum lipid contained 25 mol% exogenous cholesterol but only 4 mol% exogenous dipalmitoyl phosphatidylcholine. (b) Sarcoplasmic reticulum (+dithiothreitol) (1.5 mg of protein/ml) in the presence (■) or absence (□) of cholesterol/dipalmitoyl phosphatidylcholine liposomes as in Fig. 1 was activated and fully uncoupled by addition of the Ca^{2+} ionophore A23187 to 5 μM . The samples were incubated at 20°C and 10 μl portions were subsequently assayed for ATPase activity at 32°C in the presence of 5 μM -ionophore A23187 as described in the legend to Fig. 1. After 7 h incubation sarcoplasmic reticulum was separated from the liposomes and analysed for protein and lipid; 25 mol% cholesterol had been incorporated into the samples with respect to sarcoplasmic-reticulum phospholipid. (c) Sarcoplasmic reticulum (—dithiothreitol) after loading with 22 mol% cholesterol as in (a) (●) or without cholesterol (○) was added to sonicated suspensions of dioleoyl phosphatidylcholine [di(18:1)PC] with cholesterol and cholate (Warren *et al.*, 1975), to give final concentrations of 20 mg of dioleoyl phosphatidylcholine/ml, 10 mg of cholate/ml and 0–8 mg of cholesterol/ml, and a final protein concentration of 0.9 mg/ml. The samples were incubated for 16 h at 0°C to allow complete equilibration of the lipids, then 5 μl portions were assayed for ATPase activity in 2.0 ml of assay medium at 37°C. Under these assay conditions, most of the cholate (>95%) dissociates from the dioleoyl phosphatidylcholine/cholesterol-ATPase complexes on dilution in the assay medium, and the residual bound cholate has no effect on ATPase activity (Warren *et al.*, 1974b).

same ATPase activity ($\pm 6\%$) at 37°C irrespective of the proportion of cholesterol in the dioleoyl phosphatidylcholine bilayer, or of the prior loading of the sarcoplasmic reticulum (—dithiothreitol) with cholesterol. This confirms our original observations for fully activated dioleoyl phosphatidylcholine-ATPase complexes containing cholesterol (Warren *et al.*, 1975).

Discussion

The data show that under conditions where the ATPase activity in sarcoplasmic reticulum vesicles is fully uncoupled or where the activity is coupled stably to Ca^{2+} accumulation, cholesterol has no effect on ATPase activity. The apparent inhibition of ATPase activity by cholesterol (Fig. 1) may be explained by an experimental artefact, due to the

inhibition by cholesterol of the spontaneous uncoupling of thermally unstable sarcoplasmic reticulum (—dithiothreitol). The magnitude of the apparent inhibition in Fig. 3(a) is similar to the 40% inhibition of ATPase activity reported by Madden *et al.* (1979) (Fig. 1). In addition, removal of the loaded cholesterol in sarcoplasmic reticulum (—dithiothreitol) would reverse the partial protection against thermal activation conferred by cholesterol, in agreement with the finding of Madden *et al.* (1979) that the effect of cholesterol under their experimental conditions is reversible. We conclude therefore that this effect of cholesterol on the thermal stability of sarcoplasmic reticulum (—dithiothreitol) is sufficient to account for all of the experimental observations of Madden *et al.* (1979).

The origin of the thermal activation process, and whether it arises, for example, from the oxidation of

protein thiol groups, or of unsaturated sarcoplasmic reticulum lipids is not clear, although the protection from thermal activation by dithiothreitol and by 10mM-Ca²⁺, but not 10mM-Mg²⁺, may argue for a protein rather than a lipid effect (Griffiths, 1975). It is worth emphasizing that dithiothreitol must be present throughout the preparation of sarcoplasmic reticulum until the final dialysis into sucrose buffer without dithiothreitol (see the legend to Fig. 1) to obtain a sarcoplasmic-reticulum preparation that is thermally stable. The residual dithiothreitol concentration in sarcoplasmic-reticulum (+dithiothreitol) preparations under assay conditions was less than 10nM in all experiments. On the other hand addition of dithiothreitol to sarcoplasmic-reticulum (-dithiothreitol) preparations for 1h before assay does not prevent thermal activation and uncoupling under the conditions described.

The most interesting implication of the present data is that the large and well-characterized decrease in bilayer fluidity produced by cholesterol (Hubbell & McConnell, 1968) does not inhibit the function of the ATPase either as an enzyme or as a Ca²⁺ pump. It remains possible that cholesterol has an equal and opposite activating effect on the ATPase that exactly balances any inhibitory action due to the decrease in bilayer fluidity, but this seems very improbable in view of the evidence that cholesterol-ATPase complexes without phospholipid have negligible activity, but can be fully reactivated by phospholipid (Warren *et al.*, 1975). We have previously suggested a molecular mechanism by which the sarcoplasmic-reticulum ATPase activity may be unaffected by the presence of high proportions of cholesterol in the phospholipid bilayer. In this model, cholesterol molecules are excluded from direct interaction with the ATPase by the selective interaction of the enzyme with a shell of phospholipid molecules that effectively determine its activity (Warren *et al.*, 1975).

We emphasize as strongly as possible that the absence of a significant effect of cholesterol and therefore of bilayer fluidity on sarcoplasmic reticulum ATPase function applies only to temperatures between 32 and 37°C and to the endogenous sarcoplasmic reticulum lipid bilayer or dioleoyl phosphatidylcholine bilayer in the experiments reported here. Under non-physiological conditions, at much lower temperatures, or in phosphatidylcholine bilayers that are substantially thicker or thinner than the endogenous sarcoplasmic reticulum lipid bilayer, very large effects of cholesterol on ATPase activity can be demonstrated (A. Johansson, unpublished work). More generally there is extensive evidence (Warren *et al.*, 1974b) that phospholipid phase transitions, which can

produce even larger changes in bilayer fluidity than cholesterol, can grossly affect the activity of sarcoplasmic reticulum ATPase. However, physical studies have shown that there is no evidence for a phospholipid phase transition in the sarcoplasmic reticulum lipid bilayer at 37°C, and we conclude that modulation of sarcoplasmic reticulum ATPase activity by changes in bilayer fluidity only occurs under conditions remote from those operating physiologically.

Under physiological conditions, the function of the sarcoplasmic reticulum ATPase appears to be effectively buffered against wide changes in fluidity. It is possible that the sarcoplasmic reticulum ATPase is highly atypical in this respect, perhaps because of its specialized function. However, in view of the widely-held assumption that the function of membrane proteins under physiological conditions is modulated by membrane fluidity, it is important to determine whether the absence of any effect of cholesterol on membrane protein activity under physiological conditions as reported here is a general phenomenon. The possibility that the function of proteins in plasma membranes, which normally contain high proportions of cholesterol, may be buffered against changes in bilayer fluidity under physiological conditions is worth considering. Specifically, does the removal of cholesterol from plasma membrane preparations generally affect the function of the membrane proteins within the bilayer?

References

- Bennett, J. P. (1978) Ph.D. Thesis, University of Cambridge
- Griffiths, P. A. (1975) Ph.D. Thesis, C.N.A.A.
- Hubbell, W. L. & McConnell, H. M. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 12-16
- Keightley, C. A. (1979) Ph.D. Thesis, University of Cambridge
- Madden, T. D., Chapman, D. & Quinn, P. J. (1979) *Nature (London)* **279**, 538-541
- Martonosi, A. & Feretos, R. (1975) *J. Biol. Chem.* **250**, 659-668
- Richards, C. D., Martin, K., Gregory, S., Keightley, C. A., Hesketh, T. R., Smith, G. A., Warren, G. B. & Metcalfe, J. C. (1978) *Nature (London)* **276**, 775-779
- Scarpa, A., Baldassare, J. & Inesi, G. (1972) *J. Gen. Physiol.* **60**, 735-749
- Smith, G. A., Montecucco, C. & Bennett, J. P. (1978) *Lipids* **13**, 92-94
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1974a) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 622-626
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1974b) *Biochemistry* **13**, 5501-5507
- Warren, G. B., Houslay, M. D., Birdsall, N. J. M. & Metcalfe, J. C. (1975) *Nature (London)* **255**, 684-687