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THE MODULATION OF Ca^{2+}-ATPase ACTIVITY OF SARCOPLASMIC RETICULUM BY MEMBRANE CHOLESTEROL

THE EFFECT OF ENZYME COUPLING

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Summary

The Ca^{2+}-ATPase activity of sarcoplasmic reticulum is relatively low (less than 2 I.U.) in vesicles where enzyme activity is geared to calcium accumulation. Modulation of membrane fluidity by enriching the membrane with cholesterol has no significant effect on enzyme activity. Collapsing the Ca^{2+} gradient with the calcium ionophore, A23187, unmasks the inhibitory effect of membrane cholesterol on enzyme activity.

Sarcoplasmic reticulum has served as a useful biomembrane system for investigating the interaction between bilayer lipids and intrinsic membrane proteins. Ca^{2+}-ATPase (EC 3.6.1.3) is the major intrinsic protein of the membrane and it is known to depend for its activity on the surrounding phospholipids, although these can be replaced by suitable detergents without loss of enzyme activity [1]. On the basis of a series of studies of the Ca^{2+}-ATPase reconstituted with a number of defined lipids, Metcalfe and coworkers [2,3] have put forward a general theory concerning the biochemical consequences of the interaction of membrane lipids with intrinsic membrane proteins. In the context of the Ca^{2+}-ATPase of sarcoplasmic reticulum the theory postulates that the protein interacts directly with a slowly exchanging annulus of about

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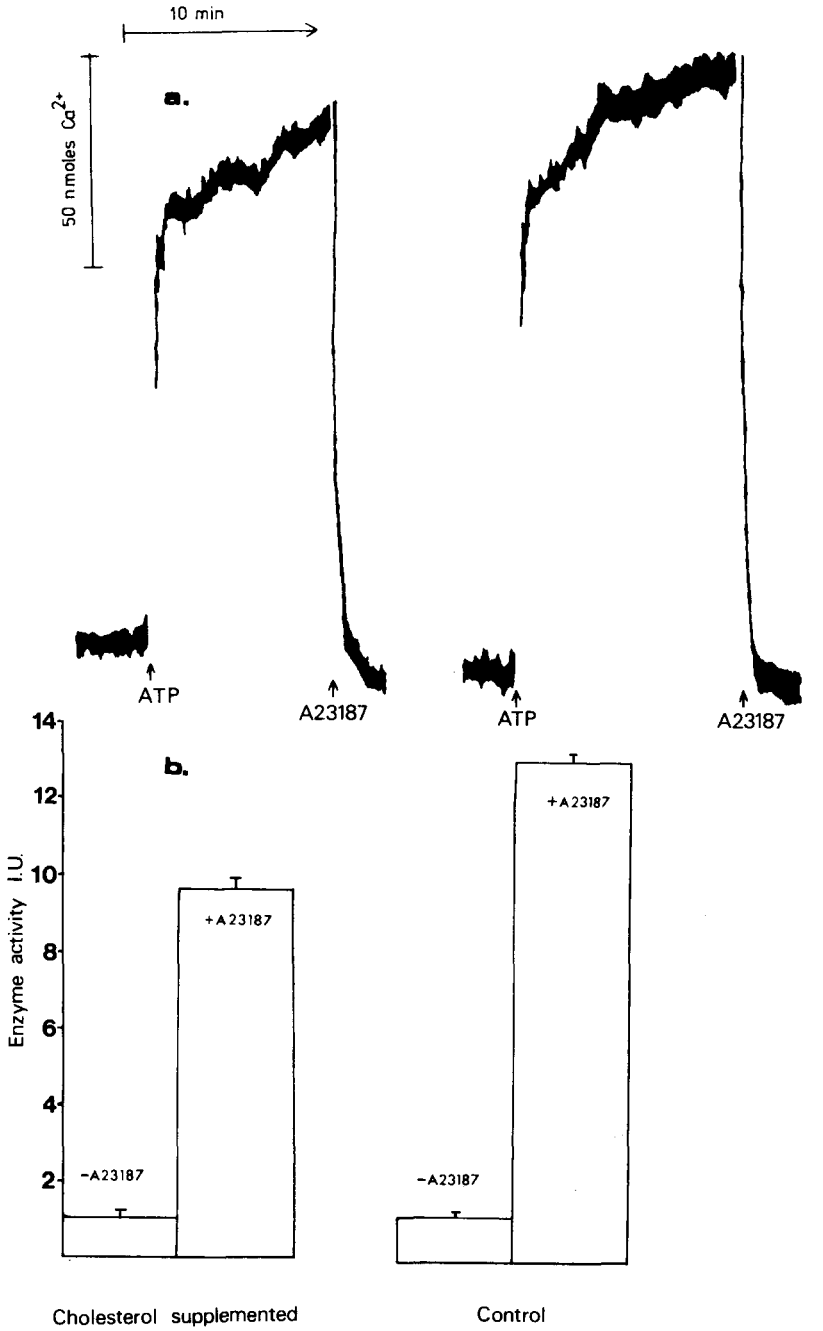

Fig. 1. Sarcoplasmic reticulum vesicles (2 mg protein/ml), prepared according to the method of Warren et al. [5], were incubated with cholesterol-rich liposomes (2 mg dipalmitoyl phosphatidylcholine; 2 mg cholesterol/ml) at 20°C for 5 h. The preparation of the liposomes, the incubation medium and the conditions were as described previously [4]. To terminate the incoporation of cholesterol, 2 vol. of ice-cold buffer consisting of 0.1 M KCl, 5 mM histidine, pH 7.4, was added to the suspension which was then centrifuged at 50 000 × *g* for 30 min. The supernatant containing the liposomes was decanted off and the pellet of sarcoplasmic reticulum resuspended in 0.1 M KCl buffered with 5 mM histidine, pH 7.4. Samples of sarcoplasmic reticulum vesicles for which the level of incorporated cholesterol was determined were further purified on a sucrose gradient as in Ref. 4. The control preparations were incu-

30 phospholipid molecules. Cholesterol is said to be excluded from the phospholipid annulus [2]. Moreover, it has been suggested that the annulus protects the enzyme from any changes in bulk membrane fluidity caused by the incorporation of cholesterol into the membrane [3].

We have examined the biochemical evidence used to support the annulus hypothesis by incorporating cholesterol into sarcoplasmic reticulum vesicles and observing the effects on enzyme activity. It was found that increasing amounts of cholesterol caused a proportionate decrease in enzyme activity [4]. This result apparently contrasts with the observation reported by Warren et al. [2], who found that cholesterol had no significant effect on the activity of Ca^{2+}-ATPase reconstituted with phosphatidylcholine/cholesterol mixtures provided the phospholipid : ATPase mole ratio was greater than 30 : 1 and the assays were performed at temperatures greater than about 30°C. Cholesterol apparently inhibits enzyme activity in these complexes at temperatures less than 30°C (see Fig. 7b of Ref. 3).

Apart from the method of reconstituting the enzyme system with cholesterol, the other major difference in enzyme preparations is whether or not membranes are prepared in the presence of protease inhibitor and the reducing agent, dithiothreitol. We have therefore compared the properties of sarcoplasmic reticulum vesicles prepared as described previously [4] with those prepared by the method of Warren et al. [5] who included 5 μM phenylmethylsulphonyl fluoride and 1 mM dithiothreitol in their isolation medium. It was found that the Ca^{2+}-ATPase activity of the native membranes prepared in the absence of protease inhibitor and reducing agent was considerably greater (11.6 I.U.) than in membranes prepared in the presence of these reagents (1.2 I.U.). To determine whether differences in the relationship between enzyme activity and calcium transport in membranes prepared by the two methods were responsible for the observed differences in enzyme activities the effect of the calcium ionophore, A23187, was studied. Addition of the ionophore caused only a relatively small increase in Ca^{2+}-ATPase activity for the preparation made in the absence of protease inhibitor and reducing agent (11.6 to 14.2 I.U.) but produced more than a 10-fold increase in activity (1.2 to 13.0 I.U.) for the enzyme prepared by the method of Warren et al. [5].

These observations are consistent with the idea that membranes prepared in the presence of phenylmethylsulphonyl fluoride and dithiothreitol are in a coupled state in that enzyme activity is geared to the transport of calcium into the vesicles. This was confirmed by measuring the capacity of vesicles to accumulate calcium. Membranes prepared in the absence of protease inhibitor and reducing agent and preincubated in the presence of ATP showed no detectable uptake of calcium on subsequent addition of the divalent ion.

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bated in the absence of liposomes. The molar ratio of cholesterol to phospholipid was increased from approx. 1 : 20 in the native membrane to 1 : 5 in those membranes incubated with cholesterol-rich liposomes. Ca^{2+}-ATPase activities (shown in the lower part of the figure) were determined at 32°C using an ATP-regenerating enzyme system as in Ref. 4. The extravesicular calcium concentrations determined spectroscopically [6] are shown as a function of time after additions of ATP and A23187 in the upper part of the figure. The sarcoplasmic reticulum protein concentration used in this assay was 250 μg/ml. The concentration of A23187 used to uncouple the vesicles was 6.4 μM. The values of enzyme activity shown are the means of six samples. The standard error of the means is indicated.

Membranes prepared by the method of Warren et al. [5], on the other hand, were found to retain the capacity to accumulate calcium (see control preparation of Fig. 1a).

The low ATPase activity of the coupled vesicle preparation, therefore, is due to the high intravesicular calcium concentration generated by the pumping process which serves to limit the steady-state enzyme activity to the rate of efflux of calcium from the vesicles [9]. The addition of A23187 renders the membranes permeable to calcium, thereby abolishing the concentration gradient of the cation and allowing the enzyme to operate at maximal rates.

In view of the differences in properties of sarcoplasmic reticulum prepared by the two methods we decided to study the effect of cholesterol on Ca^{2+}-ATPase activity in vesicles prepared by the method of Warren et al. [5]. As shown in Fig. 1 tightly coupled preparations of the enzyme show only a slight decrease in Ca^{2+}-ATPase activity when cholesterol is incorporated into the membrane. This could be ascribed to either an inhibition of Ca^{2+}-ATPase activity by cholesterol or a reduction in the permeability of the membrane to calcium which regulates the steady-state activity of the enzyme. When the vesicles are rendered permeable to calcium by addition of A23187, however, it can be seen that the presence of cholesterol causes a marked inhibition of Ca^{2+}-ATPase activity. It is not possible to determine whether this inhibition is caused by an effect of cholesterol on the bulk lipid bilayer fluidity or by direct interaction with the hydrophobic domain of the protein. An effect of cholesterol on membrane permeability to calcium can nevertheless be excluded by the demonstration that A23187 completely collapses the calcium gradient in both the cholesterol-loaded preparation and in the control (see Fig. 1a).

The present results clearly show that vesicles of sarcoplasmic reticulum, whether or not they are supplemented with cholesterol, that are prepared in the presence of protease inhibitor and dithiothreitol are tightly coupled. It has been reported previously that Ca^{2+}-ATPase prepared in this way and reconstituted with defined phospholipids behaves in a similar way [5]. The Ca^{2+}-ATPase activity in these preparations, where the membrane remains tightly sealed to Ca^{2+}, is restricted by the rate of dissipation of the concentration gradient of calcium created by the pumping process. The effect of membrane cholesterol on enzyme activity in these preparations is obscured by the fact that the rate limiting step of the process is likely to be the efflux of calcium from the vesicles. Collapsing the calcium gradient in coupled preparations with calcium ionophore allows expression of maximum catalytic activity and unmasks the inhibitory effect of cholesterol on the Ca^{2+}-ATPase.

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