Effect of Diethyl Ether on the Adenosine Triphosphatase Activity and the Calcium Uptake of Fragmented Sarcoplasmic Reticulum of Rabbit Skeletal Muscle*

(Received for publication, October 10, 1960)

GIUSEPPE INESI,‡ Joseph J. Goodman, and Shizuo Watanabe
From the Cardiovascular Research Institute, University of California School of Medicine, and Veterans Administration Hospital, San Francisco, California 94129

SUMMARY

Treatment of fragmented preparations of sarcoplasmic reticulum with diethyl ether decreases the calcium uptake capacity of reticulum, while the ATP-binding capacity as well as the ATP-splitting activity of reticulum were increased. Diethyl ether-treated reticulum preparations retained both the “basal” and the “extra” splitting activities of Hasselbach and Makinose, although the latter activity was uncoupled from the calcium uptake activity. Taking advantage of the fact that calcium-dependent ATP splitting, namely, the extra splitting of ether-treated reticulum, is not confined in a short period of calcium uptake process, the following kinetic characteristics at 25° were obtained. In the presence of an optimal concentration of calcium, the half-maximal activation of the extra splitting by magnesium was obtained with 0.1 mM added MgCl₂. Overoptimal concentrations of calcium exerted a competitive inhibition. The plot of the extra splitting activity as a function of ATP concentration showed two discrete steps, which suggest two different calcium-dependent activities; their Michaelis-Menten constants are 10 μM and >1 mM, and their maximal activities are 1.7 and >0.6 μmoles of phosphate liberated per min per mg of reticulum protein. The ATP splitting by ether-treated preparations of reticulum in the presence of 1,2-bis-(2-dicarboxy methylaminoethoxy)ethane with no added calcium, namely, the basal splitting, occurred at ATP concentrations about the same as those for the second step in the extra splitting, but the activity was too small to account for the activity in the second step. On the other hand, the calcium uptake by nontreated preparations of reticulum also showed two steps as the ATP concentration increased; the first step occurred at ATP concentrations about one-tenth of those for the ATP-splitting activity mentioned above, but the second step occurred at about the same concentrations as those for the ATP splitting.

* This research was supported by National Science Foundation Research Grants G-10442 and GB-4754, United States Public Health Service Research Grants HE-06285 and HE-08578, and American Heart Association Grant 66-749
‡ Established Investigator of the American Heart Association.
This paper reports that not only ATP splitting but also ATP binding are uncoupled from calcium uptake when fragmented sarcoplasmic reticulum is treated with diethyl ether. Also reported is a morphological change of fragmented sarcoplasmic reticulum produced by diethyl ether treatment. A partial account of these observations was previously communicated (17).

EXPERIMENTAL PROCEDURE

Preparation of Microsomes—Muscle microsomes identified as fragmented sarcoplasmic reticulum were prepared essentially by the method described by Ebashi and Lipmann (3). Rabbit skeletal muscle (150 g) was homogenized in 5 mM histidine buffer of pH 6.8 (450 ml) with the aid of a Waring Blendor. A microsomal fraction was obtained by differential centrifugation of the homogenate; the fraction sedimented was collected between 8,000 and 40,000 × g. The differential centrifugation was repeated twice, and the reticulum thus isolated were suspended in 5 mM Tris-maleate buffer (pH 6.8).

Treatment with Diethyl Ether—Reticulum suspensions containing 1 to 3 mg of reticulum protein per ml in 5 mM Tris-maleate buffer (pH 6.8) were treated for 10 min at 25° with diethyl ether (2.5 to 25% in volume) by simply inverting the centrifuge tubes four or five times. Up to 5% (by volume) ether was completely mixed in aqueous solution. When ether was added in larger amounts, the insoluble excess was allowed to collect on the upper layer by standing still and was removed by gentle aspiration. Reticulum was then recovered by subsequent centrifugation and resuspended in 5 mM Tris-maleate (pH 6.8). Essentially a full recovery of reticulum protein and phospholipids was obtained; in other words, no appreciable loss of either protein or phospholipids (organic phosphate) was detected as a consequence of the ether treatment.

Calcium Uptake—Reticulum preparations were incubated in a medium containing 1 to 5 mM ATP, 0 to 100 mM added CaCl₂, 20 mM Tris-maleate (pH 6.8), 80 mM KCl, and 1 to 5 mM MgCl₂. Sometimes the medium also contained 0.1 mM EGTA or P-enolpyruvate, or both, and pyruvate kinase. ⁴⁰Ca was added to the incubation medium so as to obtain approximately 6000 cpm per ml. The concentration of reticulum in the incubation medium was 0.1 to 0.3 mg of reticulum protein per ml. After 1- to 5-min incubation at 25°, the incubation mixture was filtered through Millipore filter (HA 0.45 μ), and the residual ⁴⁰Ca in the filtrate was measured by a gas flow counter.

ATPase Activity—The reaction medium was identical with that used for the calcium uptake measurement, except that low concentrations of reticulum (20 to 100 μg of protein per ml) were used. The reaction was started by adding reticulum suspensions. Samples were taken every minute and added to an equal volume of 10% trichloracetic acid. The protein precipitated was removed by a brief centrifugation, and the ammonium molybdate-ammonium vanadate reaction (18) was used to estimate the inorganic orthophosphate in the supernatant (19). The initial slope, while the orthophosphate liberation is still linear with time, was taken to express the ATPase activity in micromoles of phosphate liberated per min per mg of reticulum protein.

Other ATPase Activity Measurements—In addition to the measurements described above, ATPase activity was also measured under the same conditions as above except that the incubation medium contained no added CaCl₂. The ATPase activity was measured either in the absence of CaCl₂ (ATPase without Ca) or in the presence of CaCl₂ (ATPase with Ca).

ATP-Binding—Reticulum preparations were incubated at 25° for 5 sec with ATP containing uniformly labeled ³²P-ATP, and the mixture was filtered through a Millipore filter (HA 0.45 μ). The radioactivity remaining in the filtrate was measured. P-enolpyruvate (2.5 mM) and 50 μg of pyruvate kinase per ml were used to maintain the concentration of ATP.

Electronmicroscopy—Reticulum preparations were centrifuged at 10,000 × g for 30 min, and the pellet thus obtained was fixed for 2 hours with glutaraldehyde-p-alddehyde solution (20) in 0.2 M cacodylate buffer (pH 7.4) containing 0.05% CaCl₂. The sample pellets were then washed with 0.2 M cacodylate buffer, postfixed in 1% osmium tetroxide in 2.67% (by volume) trimethylpyridine (pH 7.4), and embedded in Maraglas plastic. The thin sections of the embedded pellets were cut on a Porter-Blum MT-2 microtome with glass knives, mounted on grids with carbon support membranes, stained with uranyl acetate and lead citrate, and examined with a RCA EMU 3F.

RESULTS

1. General Properties of Reticulum Preparations—In the absence of oxalate, fragmented sarcoplasmic reticulum prepared in our laboratory was capable of taking up calcium in the amount ranging between 120 and 170 μmoles per reticulum protein (Fig. 1). These capacities are in agreement with those reported by Weber, Herz, and Reis (33). Calcium uptake was ATP-dependent, and a control run in the absence of ATP showed no appreciable calcium uptake. The half-maximal calcium uptake was obtained when the concentration of added CaCl₂ was approximately 16 μM. Since we have 0.1 mM EGTA in the system and the calcium contamination in the system is estimated by atomic absorption spectroscopy to be about 10 μM, the so-called "free Ca" concentration at the half-maximal capacity (in 5 min) is approximately 1 × 10⁻⁷ M, which is about equal to that reported by other investigators (9, 11). The presence of an ATP-regenerating system had no appreciable effect on the calcium uptake measured at a 5-min incubation (Fig. 1).

Our preparations of sarcoplasmic reticulum were able to catalyze hydrolysis of ATP at initial rates varying between 0.25 and 0.35 μmole of ATP split per min per mg of reticulum protein at 25° in a reaction medium containing 0.08 M KCl, 20 mM Tris-maleate buffer (pH 6.8), 3 mM ATP, 3 mM MgCl₂, and no added CaCl₂ (plus 10 to 20 μM Ca contaminated). The concentration

1 The abbreviation used is: EGTA, 1,2-bis-(2-dicarboxy methylaminoethoxy)ethylene.
of inorganic orthophosphate liberated upon completion of the reaction exceeded the concentration of ATP initially added to the reaction mixture. Therefore, our preparation of reticulum may have been contaminated with enzymes other than ATPase, for example, myokinase. The contamination with myosin (24), however, is negligible in our preparations; when reticulum preparations were washed (for 30 min) with Weber-Edsall solution (0.6 mM KCl, 0.94 mM NaHCO$_3$, 0.01 mM Na$_2$CO$_3$), no appreciable loss was detected either in the ATPase activity or in the protein content of reticulum preparations.

Our preparations of reticulum were also able to bind ATP in amounts between 0.35 and 0.91 μmole per g of reticulum protein (in the presence of an ATP-regenerating system). These amounts are about the same as those reported by Ebashi and his associates (3, 10), and confirmed by Weber et al. (23).

2. Effect of Diethyl Ether Treatment on Calcium Uptake, ATP Splitting, and ATP Binding—Fig. 2 shows that diethyl ether treatment of reticulum preparations reduces calcium uptake, while ATP binding and ATP splitting are both increased. It should be noted in the first place that diethyl ether is different from 1-butanol or deoxycholate (3, 10) in that it uncouples ATP binding from calcium uptake. Second, the increase in ATP splitting took place in the concentration range of diethyl ether (between 5 and 10% in volume) where the decrease in calcium uptake took place. Also, in this second aspect, diethyl ether is different from other reagents such as oleate (15), under the influence of which the activation of ATP splitting and the inhibition of calcium uptake took place in separate concentration ranges of reagents; a closer relation with the inhibition of calcium uptake, therefore, appears to exist in the activation of ATP splitting from calcium uptake activity. Further studies have thus been carried out with the ether-treated reticulum preparations.

3. Effect of EGTA on ATP Splitting—Hasselbach (6), Hasselbach and Makinose (7, 16), Makinose and Hasselbach (8), and Makinose and The (9) found that reticulum preparations contain two different activities for hydrolyzing ATP: the basal and the extra splitting of ATP. The basal splitting does not, but the extra splitting depends on calcium. A calcium chelator, EGTA, has thus been reported to inhibit the extra splitting by reducing free calcium added or contaminated in the reaction mixture (13, 25). Fig. 3 shows that the ATP-splitting activity of ether-treated preparations is greatly inhibited, whereas that of nontreated preparations is very little affected by EGTA. The contaminant calcium can therefore stay in the external medium and keep the extra splitting going. On the other hand, nontreated preparations can take up calcium, and, ATPase activity from the calcium uptake activity. Further studies have thus been carried out with the ether-treated reticulum preparations.

Nagai (27) reported that microsomal ATPase is stimulated by a careful treatment of microsomes with deoxycholate, and that the ‘stimulated’ ATPase activity is inhibited by EGTA to the level of the ‘latent’ ATPase activity which is not inhibited by EGTA. These ‘stimulated’ and ‘latent’ ATPase activities, although the effects of salyrgan and of ADP were not studied, are probably identical with the ‘extra’ and the ‘basal’ splitting of ATP, respectively.
therefore, the contaminant calcium can be taken up by reticulum preparations before the first aliquot of the reaction mixture is sampled for the activity measurement. As described in "Experimental Procedure" the first aliquot was taken at 1 min of incubation, and the activity was estimated from a slope of subsequent phosphate liberation. The activity of nontreated preparations, shown in Fig. 3, is therefore the activity after calcium uptake, and accordingly the one in the absence of the external free calcium. Fig. 4 shows experiments in which ATP splitting was measured by quick sampling after addition of CaCl₂. The ATP splitting by ether-treated preparations proceeds as fast as the extra splitting by nontreated preparations in the presence of oxalate. However, the former maintains its fast rate, whereas the latter, being coupled with calcium uptake, is of a brief duration. This situation makes a study of calcium-dependent ATP splitting much easier with ether-treated preparations than with nontreated preparations.

4. Effects of ADP and of Salyrgan on ATP Splitting—Makinose and The (9) showed that the basal splitting is insensitive to salyrgan and ADP; whereas the extra splitting is poisoned by salyrgan and strongly inhibited by ADP. Fig. 5 shows that in response to salyrgan and ADP, ATP splitting by nontreated preparations of reticulum and that by ether-treated preparations (without added calcium) behave essentially like the basal and the extra splitting, respectively. The inhibitory effect of ADP on the ATP splitting by ether-treated preparations is very strong, although like the EGTA inhibition, it does not seem to be complete. The half-maximal inhibition is obtained by approximately 0.32 mM ADP in the presence of 1 mM ATP; this suggests that the affinity of ADP (1/K₁ADP) is 3 times greater than that of ATP (1/K₁ATP). The sulfhydryl content measured after prolonged incubation with salyrgan was about the same for both ether-treated and nontreated preparations, 7.9 and 7.6 moles of sulfhydryl per 10⁵ g of reticulum protein, respectively.
values are comparable with those recently reported by Hasselbach and Seraydarian (26). When salyrgan is added to the medium just before the ATP hydrolysis run is started, ATP splitting by nontreated preparations is slightly activated by salyrgan in concentrations lower than about 7.6 moles per 10^6 g of reticulum protein (a concentration which would have blocked all of the —SH groups of reticulum protein). A further increase in the concentration of salyrgan produces inhibition, although incomplete. On the other hand, ATP splitting by ether-treated preparations is decreased by low concentrations of salyrgan and completely inhibited when the concentration of salyrgan exceeds the sulfhydryl content of reticulum preparations.

5. Effect of Calcium and Magnesium on ATP-splitting Activities—In Fig. 6, the ATP-splitting activity of ether-treated reticulum preparations is plotted as a function of magnesium concentration. In the absence of added calcium and in the presence of EGTA (0.1 mM), the ATP splitting is supposed to be purely the basal splitting. A result obtained under such a condition indicates that the basal splitting requires magnesium: the maximal activity is about 0.15 umole of phosphate per 10^6 g per mg of reticulum protein, and the half-maximal activity obtained with about 0.5 mM added MgCl₂.

Addition of increasing concentrations of CaCl₂ up to 50 or 100 μM (in the presence of 0.1 mM EGTA) raises the activity at all concentrations of added MgCl₂. Thus magnesium and calcium seem to cooperate with each other in activating the extra splitting activity of ether-treated reticulum preparations. However, a further increase in the concentration of added CaCl₂ greatly shifts the activation curve toward higher concentrations of MgCl₂. Therefore, an overoptimal concentration of calcium appears to inhibit the extra splitting by competing with magnesium. If we plot the difference between the activity in the absence of calcium and that in the presence of an optimal concentration of CaCl₂ (0.1 mM added calcium plus 0.1 mM EGTA) against the concentration of added MgCl₂, the resultant plot can be considered to represent the magnesium requirement of purely the calcium-dependent portion of ATP splitting, namely, the extra splitting. The maximal activity on such a difference plot is about 1.4 umoles of phosphate per min per mg of reticulum protein, and the half-maximal activity is obtained with about 0.1 mM added MgCl₂.

6. ATPase Activity as Function of ATP Concentration—Fig. 7A shows that in the absence of an added ATP-regenerating system (and in the absence of added calcium), the half-maximal activity is obtained with about 0.4 mM ATP for both nontreated and ether-treated reticulum preparations, whereas the maximum activity of 0.1 mM EGTA. Ether-treated (20%) preparations of sarcoplasmic reticulum in the concentration of 57 μg of protein per ml were used to measure ATP splitting in the presence (○) and in the absence (△) of 0.1 mM added CaCl₂. Nontreated preparations in the concentration of 290 μg of protein per ml were used to measure the calcium uptake in 10 sec (●) in the presence of 0.1 mM added CaCl₂. Arrows indicate approximately the half-maximal points.
(V_max) of ether-treated preparations is much higher than that of nontreated preparations; it appears that the Michaelis-Menten constant (K_m or K_ATP) remains unchanged and V_max is increased by diethyl ether treatment. However, it was found that in the presence of an ATP-regenerating system and of an optimal concentration of calcium, the ATP-splitting activity of ether-treated preparations was greatly increased at low concentrations of ATP (open circles in Fig. 7B). The result is reproducible when the double or the half-concentration of the ATP-regenerating system is used. Since ADP strongly inhibits the extra splitting (Fig. 5B), this observation seems to indicate that ADP, as soon as it is produced, inhibits the extra splitting unless the concentration of ATP is sufficiently high so as to partially overcome the ADP inhibition, and that the pyruvate kinase system has removed the ADP inhibition at low concentrations of ATP. Furthermore, the fact that a plot of activity versus ATP concentration with ether-treated reticulum preparations now shows two steps of increasing activity may reflect two different activities; one is saturated with relatively low concentrations of ATP and another with much higher concentrations of ATP. In fact, the activities in the first step can reasonably be fitted with a curve (a solid line in Fig. 7B) calculated on the assumption that the Michaelis-Menten constant (K_m) is 10 μM and the maximal activity (V_max) is 1.7 μmoles of phosphate liberated per min per mg of reticulum protein. If this is accepted, K_m and V_max for the second step must be >1 μM and >0.6 μmole per min per mg, respectively.

On the other hand, the basal splitting, i.e. the ATP splitting in the presence of EGTA with no added calcium (a curve at the bottom in Fig. 7B), is detectable only at relatively high concentrations of ATP where the second step mentioned above was observed. It should, however, be noted that the activity of the basal splitting is at most 0.15 μmole per min per mg, which is less than one-fourth that of the extra splitting in the second step (>0.6 μmole per min per mg).

Also shown in Fig. 7B (solid circles) is the initial calcium uptake in 10 sec by nontreated preparations of reticulum. This plot of calcium uptake as a function of ATP concentration also shows two consecutive steps; one reaches its half-maximal uptake at approximately 1 μM ATP, and the other step occurs at ATP concentrations of the order of ~10 μM. Occurrence of the two steps in such a plot of calcium uptake has been reported by Weber et al. (23), although, compared with our results, their second step was smaller in height (relative to the first step), and their first step occurred at even lower concentrations of ATP.

7. Electronmicrographs of Fragmented Sarcomplasmic Reticulum—The electronmicroscopic appearance of reticulum prepared in our laboratory is similar to that reported by Ebashi and Lipmann (3). Nontreated reticulum preparations contain vesicular elements and tubular elements (Fig. 8A). The vesicles, appearing in various tangential cuts, average approximately 0.2 μm in diameter and are delimited by a double layer membrane. The tubules are of different length and shape, and are often bent in a loop-like configuration. On the other hand, treatment with diethyl ether markedly changed the morphological appearance of reticulum preparations. In the sections obtained from pellets of diethyl ether-treated samples (Fig. 8B), the elements described above are almost totally replaced by round or ovoid (or both) vesicles (0.1 ~ 0.3 μm in diameter), delimited by a much thinner wall. These morphological observations are in harmony with the bi-
chemical evidence that diethyl ether-treated preparations have lost calcium uptake capacity (Fig. 2).

**DISCUSSION**

The result presented in Fig. 2 suggests that, under the influence of diethyl ether, ATP binding to reticulum preparations reflects formation of an intermediate for a process of ATP hydrolysis, and it can be uncoupled from calcium uptake. The contrary was suggested from the result obtained under the influence of 1-butanol or deoxycholate (3, 10). If there is only one kind of site in the reticulum preparations to which ATP attaches, the two results mentioned above seem to be flatly contradictory. However, the two results may be reconciled to each other if there are two kinds of sites and if both sites are somehow involved in the process of calcium uptake.

We have shown in Figs. 3, 4, and 5 that diethyl ether-treated preparations of reticulum contain two different types of ATP-splitting activity which are well identified with the basal and the extra splitting of ATP described by Hasselbach (6), Hasselbach and Makinose (7), Makinose and Hasselbach (8), and Makinose and The (9). The identification is based on all the criteria available: (a) calcium dependence (Fig. 3), (b) rates (Fig. 4), and (c), effects of ADP and of salyrgan (Fig. 5). Therefore, fragmented preparations of sarcoplasmic reticulum could have two different kinds of sites for ATP attachment corresponding to two different ATP-splitting activities. However, it seems to be well established that one of the two activities, the basal splitting, can never be coupled to calcium uptake. In support of this view Weber et al. (23) reported that the affinity for ATP of the calcium uptake system is much higher than that of the basal splitting activity. In other words, in low concentrations of ATP, the basal splitting is nil but essentially a full extent of calcium uptake can take place. We have also shown in Fig. 7B that the half-saturation of the extra splitting activity occurs with approximately 10 μM ATP, whereas that of the basal splitting activity occurs with ATP in a concentration higher than 1 mM.

On the other hand, we have shown in Fig. 7B that, as the ATP concentration increases, both the extra splitting of ATP and calcium uptake increase in a similar manner, forming two discrete steps. Specifically, the second step occurred at the same concentration range of ATP for both ATP splitting and calcium uptake. Therefore, one could consider the possibility that there is a second enzyme participating in the second step of calcium uptake in addition to the extra splitting enzyme which participates in the first step of calcium uptake. A further study is in progress to find out if there are in fact two different sites for ATP attachment, both of which are involved in the calcium uptake process.

Apart from the problem so far discussed, it should be pointed out that in the first step ATP splitting occurred at ATP concentrations about 10 times higher than those for calcium uptake. This difference is even larger if compared with the dependence of calcium uptake on ATP concentration reported by other investigators (23, 25). Since concurrence of the extra splitting with the calcium uptake seems to be well supported with nontreated preparations of reticulum (23), the above difference could be due to the fact that the extra splitting studied here is not that of nontreated preparations but that of ether-treated preparations. If so, this is the only difference that we have observed between the extra splitting coupled and that uncoupled with calcium uptake.

In this connection, it is interesting to mention that the data reported by Ebashi and Yamanouchi (25) may be interpreted as indicating that in the presence of oxalate, calcium uptake occurs at ATP concentrations much lower than those for the extra splitting, whereas in the absence of oxalate, both calcium uptake and the extra splitting occur in the same concentration range of ATP. In regard to the basal splitting, there is also a difference observed between nontreated and ether-treated preparations of reticulum; the inhibition by salyrgan was complete with ether-treated preparations, whereas it was incomplete with nontreated preparations (Fig. 5A). It is therefore possible that the basal splitting is also sensitive to salyrgan, but the —SH groups for the basal splitting in nontreated preparations are not readily accessible to salyrgan. These differences may have something to do with the drastic change in morphology induced by ether treatment (Fig. 8).

Acknowledgments—We are greatly indebted to Professors Manuel Morales and Jean Botts for their valuable discussion of this work.

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