Regulatory Mechanisms of the Calcium Transport System of Fragmented Rabbit Sarcoplasmic Reticulum

I. *The effect of accumulated calcium on transport and adenosine triphosphate hydrolysis*

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ABSTRACT The rate of ATP hydrolysis decreases very rapidly during the first 2 sec of calcium uptake. It changes with time in a manner similar to that described for calcium net uptake by other workers, suggesting that the two activities are coupled. The decline in both rates may be ascribed to an inhibitory effect of accumulated calcium on calcium influx and ATPase activity for the following reasons. During the steady state, Ca-Ca and Sr-Ca exchange and the rate of ATP hydrolysis are much slower than the initial rate of net calcium uptake and the associated ATP hydrolysis. If the accumulation of free calcium is prevented by calcium-oxalate precipitation the initial rate of net calcium uptake does not decay during prolonged periods of transport. Furthermore, passive preloading of vesicles with calcium inhibits the rate of hydrolysis in proportion to the extent of preloading. The inhibition of steady-state flux is alleviated by free ATP; i.e., not chelated with magnesium, but not by free ITP.

INTRODUCTION

This paper describes a mechanism that attenuates the flux of calcium across reticulum membranes. Calcium present in the interior of the vesicles appears to be capable of inhibiting calcium flux and the coupled hydrolysis of ATP. This effect of accumulated calcium was noted previously (Weber et al., 1966, Table 7 and Fig. 22) and has been further characterized by the experiments described in this paper.

As a result of the inhibitory action of accumulated calcium one would expect the rate of ATP hydrolysis to decline rapidly with time during the initial

phase of calcium accumulation until a steady state is reached. We observed such a rapid fall of the rate of ATP hydrolysis during the first few seconds of calcium accumulation. Its time course was similar to that of the fall of the rate of calcium uptake as described by Ohnishi and Ebashi (1964) and Harigaya et al. (1968). This parallel behavior provides additional support for the view that calcium uptake under all conditions depends upon ATP hydrolysis.

METHODS

Preparation of Reticulum This was prepared as described previously (Weber et al., 1966). Before separating the reticulum from mitochondria the mixture was stored as a pellet until all of the endogenous ATP was hydrolyzed (about 1 hr). Reticulum was then isolated by differential centrifugation. Since the experiments described here required large amounts of reticulum, fresh reticulum was prepared every few days.

Strontium Uptake and Sr-Ca Exchange These were measured and calculated as previously described (Weber et al., 1966). In all these experiments vesicles were preincubated with ATP and either EGTA or calcium for 1 min prior to the addition of strontium. After preincubation with calcium the accumulated calcium exchanged with the added labeled ^{89}Sr which was buffered with EGTA (EGTA:SrEGTA = 10:1). For the measurement of Sr uptake into empty vesicles the vesicles were preincubated for 1 min with ATP and EGTA in order to cancel out, in the comparison between uptake under the two conditions, any modification of the transport mechanism caused by ATP during the preincubation period. When ATP hydrolysis was measured under the same conditions in parallel experiments, Sr was not labeled.

Hydrolysis of ATP and ITP during Steady State or in the Presence of Oxalate This was measured as described previously (Weber et al., 1966; Weber, 1969). The amount hydrolyzed was calculated from the amount of inorganic phosphate released as determined by the method of Taussky and Schorr (1953). In some experiments feeder systems for ATP were present: either phosphoenolpyruvate and kinase or creatine phosphate and kinase. In the latter case the amount of creatine liberated was determined (Eggleton et al., 1943).

Initial Rates of Hydrolysis The reticulum was preincubated with a small volume (1.3–2.5 ml) of the desired medium (containing EGTA or calcium or simply KCl + imidazole buffer, pH 7.0) in a 10 ml beaker. Just before the start of the reaction this mixture was set to stir at maximal rate on a magnetic stirrer. The reaction was started by pouring from another 10 ml beaker the desired ATP-containing mixture of about 3–4.5 ml. This mixture consisted of (*a*) MgATP and Mg (in the amounts indicated), (*b*) either EGTA or Ca or CaEGTA to adjust the pCa to the desired value, and (*c*) sufficient imidazole base to maintain the pH at 7.0. (The required amount was determined for each experiment by titration.) Control weighings of the beaker, after the ATP-containing solution had been poured out, determined the precise amount of the added solution (on the average about 95 % of the total). The addition of the ATP-containing mixture was immediately followed by the addition of the solution for termination of the reaction: either 2 ml 20 % trichloroacetic acid or 2 ml 10 mM para-

chloromercuric benzoate. Each solution was delivered by a different person. A third person determined with a stopwatch the interval between the moment when the first and the second solution hit the surface of the reticulum-containing mixture. These intervals measured 0.7–1.1 sec. After some practice quite reproducible performances were obtained.

With such very brief intervals between the addition of ATP and denaturing agents one may be concerned about the rate of denaturation of the enzyme. If the enzyme were capable of continuing its function—at least partially—for a significant fraction of a second after the addition of the denaturing agent a precise measurement of ATP hydrolysis during incubation periods of 1 to a few seconds would not be possible. Control experiments with two people adding simultaneously ATP and the denaturing agent established that the error due to a delay in the denaturation of the enzyme amounted to not more than about 10% of the total amount hydrolyzed in 1 sec.

The blanks for phosphate and creatine, respectively, were determined separately for ATP and creatine phosphate, respectively, and for the reticulum preparations. The reticulum preparations contributed the major part of the phosphate as well as the creatine blank.

Calculations

Concentrations of free ions were calculated using the binding constants for EGTA as given in Chaberek and Martell (1959) and for ATP as determined by Burton (1959).

Reagents

We used crystalline sodium ATP and sodium ITP (98–100% purity) from Sigma Chemical Co., St. Louis, Mo.; creatine phosphate and phosphoenolpyruvate from Calbiochem (Los Angeles, Calif.); creatinephosphokinase and phosphoenolpyruvate kinase from Boehringer Mannheim Corp., N.Y. All other chemicals were analytical grade. Distilled water was further purified by ion exchange resin.

RESULTS

It had been observed previously that the rate of calcium exchange in vesicles during steady state (influx = efflux) is considerably lower than the rate of initial calcium net uptake at the same pCa (Weber et al., 1966, Table 7, Fig. 22). This applied to exchange between ^{40}Ca and ^{45}Ca or between Sr and Ca. Similarly, the calcium-dependent hydrolysis of ATP was slower during the ensuing steady state than during the first 15–30 sec after ATP addition; i.e., during net calcium uptake.

Fig. 1 shows that even during the first second after ATP addition, ATP is hydrolyzed more slowly if the vesicles are passively preloaded with calcium. The passive preloading with calcium was achieved in the following way. First, reticulum was incubated in the absence of ATP with high concentrations of calcium under the assumption that in the absence of ATP vesicles would be permeable to calcium. After 10–15 min preincubation ATP hydrolysis was started and the pCa of the medium adjusted to a value between 5 and 6 by the

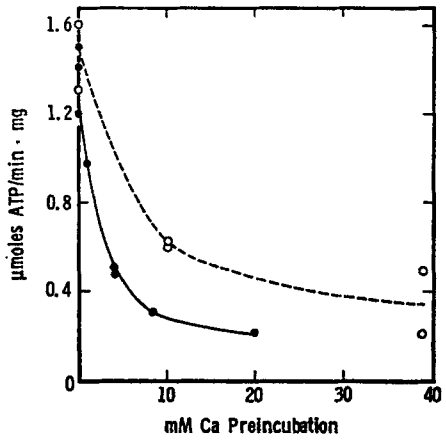

FIGURE 1. ATP hydrolysis by isolated vesicles during 1 sec incubations before and after preloading with various calcium concentrations. Comparison of two different experiments to show the variability of the results. Ordinate, rate of ATP hydrolysis by the transport enzyme only (i.e. Ca-independent ATP hydrolysis not included) during 1 sec incubations with ATP after preloading with various calcium concentrations. Abscissa, concentrations of calcium ions present during the preloading period; i.e., concentrations presumably present inside the preloaded vesicles during ATP hydrolysis. Experiment 1 Open circles, during preincubation with high calcium: reticulum 15 mg/ml; 20 mM imidazole, pH 7.0; 80 mM KCl; Ca as indicated on abscissa. Total volume 1.3 ml. During ATP hydrolysis following preloading: 36 mM imidazole, pH 7.0; 32 mM KCl; 10 mM CaEGTA, 0.4 mM EGTA; 1.3 mM MgATP; 3.3 mM $MgCl_2$; total volume 5.3 ml; duration of incubation, 0.7-1.0 sec, 24.5°C. Experiment 2 Solid circles, during preincubation with high calcium reticulum 10 mg/ml; 20 mM imidazole, pH 7.0; 80 mM KCl; Ca as indicated on abscissa; total volume 2.5 ml. During ATP hydrolysis following preloading: 36 mM imidazole, pH 7.0; 32 mM KCl; 10 mM CaEGTA, 0.4 mM EGTA; 1.3 mM MgATP, 3.3 mM $MgCl_2$; total volume 6.1 ml; duration of incubation 0.7-1.0 sec; 24.5°C. In all experiments with incubation periods of about 1 sec the accuracy of the data is much poorer than after more conventional incubation periods because the signal:noise ratio is quite low. The amount of phosphate liberated by the transport enzyme that is indicated on the ordinate represents about 5–40% of the total phosphate. The high background level of total phosphate (constant throughout the experiment) is derived from two sources: (*a*) Pi present in the reticulum which was used in very high concentrations, (*b*) Pi liberated by the calcium-independent ATPase that, with a rate of 0.3 and 0.5 μmole/min·mg, respectively, was especially high in this series of experiments. An indication of the accuracy is given by the divergence of points for the same value on the abscissa; i.e., it is about 25% at no preloading when ATPase activity was highest and 100% at the most inhibited rates after preloading.

simultaneous addition of ATP and EGTA. EGTA does not penetrate the vesicle membranes (Weber et al., 1966) and therefore may chelate only the external calcium. Acidification was prevented by combining EGTA with an adequate amount of imidazole base. Hydrolysis was measured after incubation with ATP for only 1 sec in order to compare the rate of ATP hydrolysis after

passive calcium preloading with that of vesicles whose calcium content remained low over a significant fraction of the incubation period. Fig. 1 shows that the inhibition of the rate of hydrolysis increased with increasing concentrations of calcium during passive preequilibration. Only the calcium-dependent rate was plotted; i.e., the rate of hydrolysis by the calcium-independent ATPase was subtracted from the measured rates of hydrolysis.

The extent of inhibition following passive preloading of the vesicles is somewhat variable (Table I). There may be two different reasons for this. First, the

TABLE I

THE EFFECT OF CALCIUM PRELOADING ON THE INITIAL RATE OF ATP HYDROLYSIS

Experiment No.	Ionized calcium		Total amounts hydrolyzed
	Inside	Outside	
			μmoles/min per mg protein
1.	0.01 μM	0.23 mM	1.6
	0.01 μM	10.0 mM	0.7
	21.0 mM	0.23 mM	0.4
	21.0 mM	10.0 mM	0.2
2.	20.0 μM	20.0 μM	1.3
	20.0 μM	10.0 mM	0.49
	10.0 mM	20.0 μM	0.52
	10.0 mM	10.0 mM	0.38
3.	6.0 mM	10.0 μM	1.4
	0.01 μM	6.0 mM	0.7
	6.0 mM	6.0 mM	0.57

About 10 mg protein/ml reticulum that were present during preincubation in 1.6–1.4 ml incubation mixture were diluted to a final concentration of about 4 mg/ml; 0.8–1.4 mM MgATP; 3.5–4.0 mM Mg excess; imidazole, pH 7.0: (1) 39, (2) 18, (3) 49 mM; KCl: (1) 32, (2) 16, (3) 54 mM; total EGTA: (1) 9, (2) 4, (3) 1.8 mM; total Ca: (1) 9, (2) 4–10, (3) 8.0 mM; creatine phosphate: (1) 0, (2) 4, (3) 3.5 mM; kinase: (1) 0, (2) 0.4, (3) 0.35 mg/ml. 1 sec incubations for (1) and (2) and 2 sec for (3); room temperature.

control rate of ATP hydrolysis may vary depending upon the accumulated internal calcium which in turn would be expected to influence the extent of inhibition during the first second of calcium accumulation. Second, variable amounts of the passively introduced calcium may be lost during the mixing period. Slight inadequacies of mixing may allow some calcium to diffuse out in response to lowering its concentration in the medium before ATP has fully activated the transport system, thereby preventing efflux of calcium. Even with adequate mixing there may be a lag before activation is complete. With the transport system fully activated one would expect only that amount of

calcium to leave that is in excess of the steady-state concentration of accumulated calcium.

The inhibition of ATPase activity by calcium on the inside of vesicle membranes is comparable to that caused by calcium on the outside (Table I). One may wonder whether external calcium, i.e. high calcium added together with ATP, actually exerts its effect on the outside of the membrane or whether sufficient calcium enters passively during the mixing period to affect the inner side of the membrane. (In the reverse experiment calcium can affect only the inside because calcium leaking out would be chelated by EGTA.) The observation that raising calcium simultaneously on both sides of the membrane has a slightly additive effect (Table I) may argue against this. However, calcium on both sides may elevate the effective inside concentration by preventing outflow during the period of mixing.

Since the foregoing experiment showed that increasing concentrations of calcium on the inside of the vesicles increasingly inhibited ATPase activity, one may expect a considerable drop in the rate of ATP hydrolysis during calcium accumulation. Figs. 2 and 3 B show that the rate of ATP hydrolysis rapidly declined with time until a steady state was reached after a few seconds' incubation. In contrast, if vesicles had been passively preloaded with calcium, the hydrolysis of ATP would be slow right from the beginning (Fig. 3 A). The calcium-independent ATPase activity which was measured in the absence of calcium (in the presence of 4.0 mM EGTA) was linear with time (Fig. 2 B).

It was not possible to find by extrapolation of the rate to zero time the true initial rate of ATP hydrolysis by the transport enzyme (i.e. total ATP hydrolyzed minus the amount of ATP hydrolyzed during the same time interval in the absence of calcium). The fall in the rate of ATP hydrolysis during the first few seconds was too steep for extrapolation. The true initial rate is of interest because one would like to know whether it may indicate a rate of calcium removal sufficiently fast to account for relaxation (assuming a strict coupling between ATP hydrolysis and calcium transport, with 2 calcium accumulated per ATP hydrolyzed as first demonstrated by Hasselbach and Makinose (1963) in the presence of oxalate, and confirmed later in its absence by Weber et al. [1966].) Our highest measured rates, during an interval somewhat shorter than 1 sec, are nearly 2 μmoles/min.mg indicating a rate of calcium uptake of 4 μmoles/min.mg. These hydrolysis rates, however, are much lower than, under some conditions, the rates with reticulum whose membranes are so leaky that no calcium can be accumulated on the inside, e.g. after solubilization by oleic acid (Martonosi, 1964) or other detergents (Fig. 4). With Triton X-100 we sometimes found rates of hydrolysis higher than those in Fig. 4, i.e. as high as 5 μmoles/min.mg, which is 2.5 times higher than the initial rate for intact vesicles during calcium accumulation.

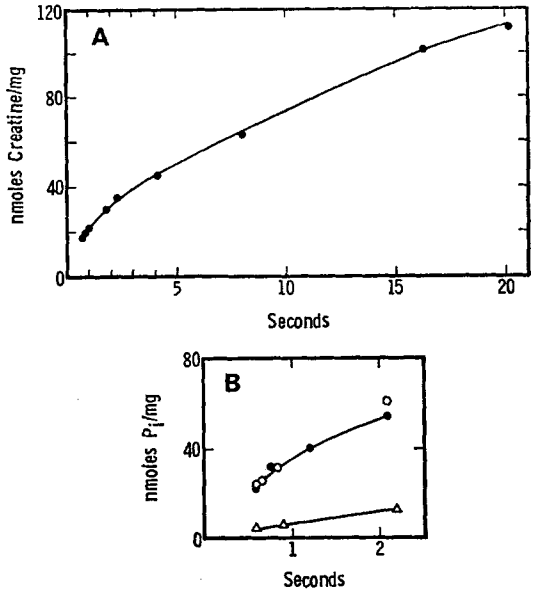

FIGURE 2. Hydrolysis of ATP during the first few seconds after ATP addition. Large changes of the rate in the absence (B) and in the presence (A) of a phosphoryl donor. A, ordinate, total ATP hydrolysis (i.e. by transport enzyme + Ca-independent enzyme) expressed as creatine liberated in a coupled enzyme system. During preincubation: reticulum 7 mg/ml; 5 mM imidazole, pH 7.0; 50 mM KCl; 10 mM creatine phosphate and 1 mg/ml kinase; total volume 2.0 ml. During subsequent ATP hydrolysis: 0.8 mM MgATP; 4.0 mM $MgCl_2$; 18 mM imidazole, pH 7.0; 60 mM KCl; 4.0 mM CaEGTA; total volume 5.0 ml. The creatine blank contributed by creatine present in the reticulum and that in the creatine phosphate was constant in all experiments. Since the amount of creatine liberated from creatine phosphate increased with increasing periods of incubation the noise:signal ratio was lowest for the shortest incubation period of 0.7 sec. Creatine contributed by the blank was nearly as high as that liberated from creatine phosphate. B, ordinate, total ATP hydrolysis expressed as phosphate liberated. Circles, Ca^{2+} in medium saturating during ATP hydrolysis, 10 mM CaEGTA, 0.4 mM EGTA; solid circles, during accumulation of calcium in the interior of the vesicles; open circles, when the accumulation of free calcium is limited by Ca-oxalate precipitation because 5 mM oxalate was present. Triangles, time course of Ca-independent ATP hydrolysis, 5.2 mM EGTA. During preincubation: reticulum 7.0 mg/ml; 20 mM imidazole, pH 7.0; 110 mM KCl; 21 mM EGTA; total volume 2.45 ml. During subsequent ATP hydrolysis: 1.6 mM MgATP; 4.0 mM $MgCl_2$; 40 mM imidazole, pH 7.0; 56 mM KCl; total volume 5.4 ml. The lowest signal:noise ratio after 0.5 sec incubation was about 0.9 (phosphate blank was the same for all experiments).

Although these agents may have activated the ATPase activity, one may also consider the possibility that these high rates result, because calcium on both sides of the membranes remains too low to be inhibitory. The following observations may be cited in support. First, under conditions when calcium accumulation by intact vesicles is *less* than maximal, because the transport sys-

tem is not saturated with calcium, the difference in the rate of ATP hydrolysis between intact and solubilized vesicles is relatively small. The difference is greatest when calcium filling of intact vesicles is highest (Fig. 4); i.e., when the inhibition by accumulated calcium is maximal. Second, the rate of ATP hydrolysis by solubilized membranes depends on the calcium concentration of the medium in a manner similar to that of intact vesicles (Fig. 4). Third, con-

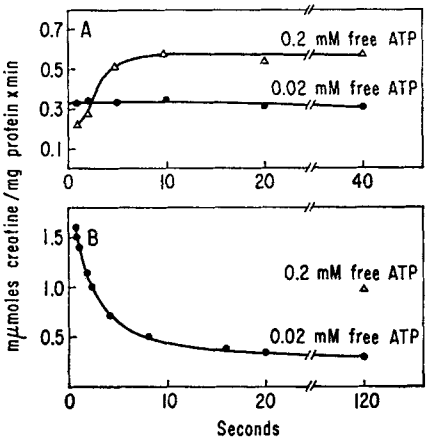

FIGURE 3. The change of rate of hydrolysis with time without (B) and with (A) calcium preloading. A, triangles, high free ATP = 0.2 mM in excess of MgATP; solid circles, low free ATP = 4 mM Mg in excess of MgATP. During preincubation: reticulum 16 mg protein/ml; 8.0 mM imidazole, pH 7.0; 54 mM KCl; 39 mM $CaCl_2$; total volume 1.3 ml. During subsequent ATP hydrolysis: 1.6 mM MgATP; 36 mM imidazole, pH 7.0; 40 mM KCl; 4.0 mM creatine phosphate, 0.2 mg/ml kinase; 10 mM CaEGTA, 0.2 mM EGTA = 5 μM Ca^{2+}. The creatine blank which was the same in all experiments was four times higher than the creatine liberated after 0.85 sec incubation, but after 20 sec incubation it was only one-fourth of that liberated. B, same experiment as in A but without calcium during preincubation and with low free ATP (i.e. 4.0 mM Mg excess) except for one point (open triangle), that had high free ATP; i.e., 0.2 mM free ATP.

tact with calcium above 0.1 mM is also inhibitory to solubilized membranes (Fig. 4, Martonosi, 1968). In other words, apart from having a much higher turnover, the ATPase activity is not changed by solubilization. Therefore, it may be considered possible that the increase in the apparent turnover is simply due to a lack of inhibition by accumulated calcium. Furthermore, even these rates may not be maximal due to an inhibitory effect of the solubilizing reagents. Such inhibition becomes very marked at higher concentrations as we observed for Triton X-100 (cf. also Martonosi et al., 1968, for oleic acid).

We had previously observed that the extent of inhibition by accumulated calcium during steady state was modified by altering either the concentration

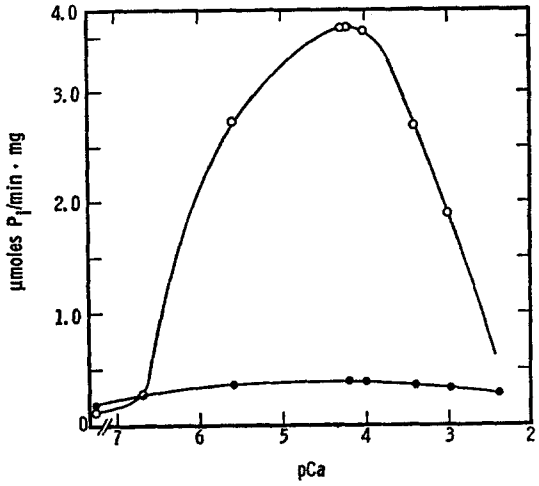

FIGURE 4. Comparison of steady-state rates of ATP hydrolysis in the presence and absence of Triton X-100. Reticulum 0.66 mg protein/ml; 20 mM imidazole, pH 7.0; 40 mM KCl; 4.0 mM MgATP; 4.0 mM Mg excess; 2.0 mM EGTA + CaEGTA when present; open circles, Triton X-100 2.2 mg/ml protein; solid circles, no Triton = intact vesicles; 1 min incubations at 24.5°C.

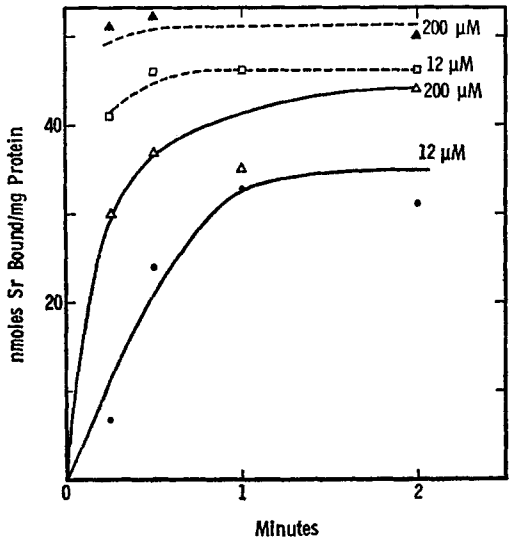

FIGURE 5. The effect of free ATP on Ca-Sr exchange in maximally Ca-filled vesicles (open triangles, solid circles) and on Sr uptake into empty vesicles (open squares, solid triangles). Broken lines indicate net uptake of Sr into empty vesicles, solid lines Sr entry in exchange for previously accumulated Ca. Numbers on curves indicate the concentration of free ATP; 200 μM when 200 μM ATP was present in addition to MgATP; 12 μM when Mg was 4 mM in excess of MgATP. During preincubation (see Methods): 0.9 mg reticulum/ml; 30 mM imidazole, pH 7.0; 70 mM KCl; 10 mM creatine phosphate, 1 mg/ml kinase; 1.0 mM $MgCl_2$; 0.1 mM MgATP. 130 μM Ca^{2+} = 150 nmoles/mg reticulum protein (open triangles, solid circles); 1.0 mM EGTA (solid triangles, open squares). During preincubation with calcium 125 nmoles calcium per mg reticulum protein were taken up. During Sr uptake: 0.53 mg reticulum/ml; 20 mM imidazole, pH 7.0; 40 mM KCl; 6.0 mM creatine phosphate, 0.8 mg/ml kinase; 0.6 mM free EGTA, 60 μM SrEGTA (= 110 nmoles/mg reticulum). During Ca-Sr exchange Ca^{2+} initially was 0.004 μM. This value could have risen to 0.03 μM when all accumulated calcium had exchanged for Sr.

of total ATP (Weber et al., 1966) or the ratio of free magnesium to MgATP (Weber, 1968). Fig. 5 shows how increasing the concentration of free, i.e. unchelated, ATP (or lowering the concentration of ionized magnesium, but see below) increased the rate of Ca-Sr exchange although unchelated ATP had only a slight influence on the steady-state concentration of accumulated Ca or Sr. (It had been shown previously that Ca and Sr seem to enter by the same transport system (Weber et al. [1966].) The effect of free ATP on the *rate* of net uptake could not be measured since it was nearly complete at the first point of measurement. Raising the free ATP also increased the rate of ATP hydrolysis

TABLE II

THE EFFECT OF EXCESS NUCLEOSIDE TRIPHOSPHATE ON HYDROLYSIS DURING 1 MIN INCUBATIONS

Excess Mg	Free nucleoside triphosphate *mM*	Rate of hydrolysis
		μmole/min per mg protein
	1.2 mM MgITP	
4.0	0	0.23
0	0.2	0.23
0	0.4	0.23
2.0 + 6.0 mM oxalate	0	0.60
	1.2 mM MgATP	
4.0	0	0.32
0	0.2	0.56
0	0.4	0.62
2.0 + 6.0 mM oxalate	0	0.98

3.0 mM phosphoenol pyruvate + 20 μg/ml kinase; 10 mM imidazole, pH 7.0; 4.0 mM CaEGTA (4 μmoles Ca/mg reticulum protein), 40 mM KCl. 1 min incubations at 24.5°C.

as the exchange rate (Figs. 3 and 5) was increased. Raising free ATP from 12 to 200 μM in the experiment described in Fig. 5 increased the amount of ATP hydrolyzed during a 1 min incubation from 56 to 100 nmoles/mg protein and the rate of exchange from about 42 to 130 nmoles/min.mg protein. (The exchange rate was estimated from the initial slope of the Sr uptake in Fig. 5.) It cannot be stated whether it is significant that the exchange rate increased more than ATP hydrolysis since the correction factor for ATP hydrolysis (blank + Ca-independent hydrolysis) was rather high. It should also be noted that the coupling ratio of one in this experiment was rather low.

The experiment described in Fig. 5 does not indicate whether the activation of the flux rate and ATP hydrolysis was caused by the increase in the concentration of unchelated ATP or the reduction of the magnesium concentration since both are interrelated. The data presented in Table II, however, suggest that lowering the concentration of ionized magnesium was not responsible for the activation. When calcium transport was supported by ITP instead of ATP, the hydrolysis of ITP seemed also to be inhibited by accumulated cal-

cium as indicated by a comparison of steady-state conditions with continued net uptake in the presence of oxalate (Table II). In the presence of oxalate the concentration of free calcium in the interior of the vesicles is reduced by calcium oxalate precipitation. (It was not possible to compare the rate of ITP

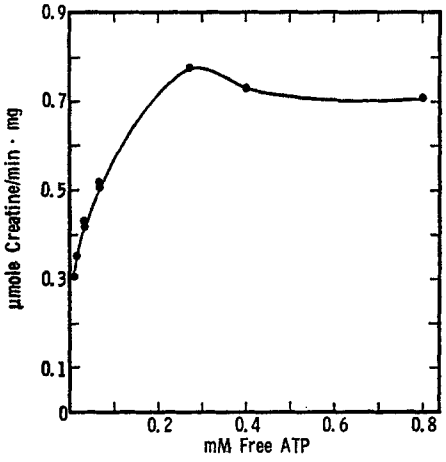

FIGURE 6. ATP hydrolysis during 1 min incubations as a function of free ATP. 0.6 mg/ml reticulum protein; 20 mM imidazole, pH 7.0; 40 mM KCl; 4.0 mM creatine phosphate and 0.1 mg/ml kinase; 2.0 mM Ca-EGTA; 0.4–6.0 mM MgATP; maximal excess Mg 2.0 mM; 24.5°C.

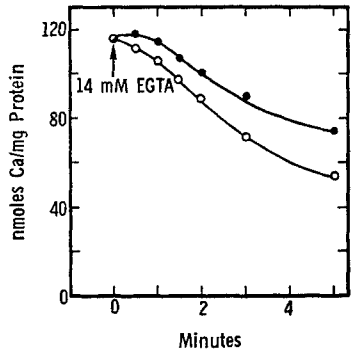

FIGURE 7. Calcium outflow into Ca-free medium with low and high free ATP. Preincubation medium for calcium uptake: 0.6 mg/ml reticulum protein; 20 mM imidazole, pH 7.0; 40 mM KCl; 2.0 mM creatine phosphate and 0.1 mg/ml kinase; 50 μM $^{45}Ca^{2+}$; 0.8 mM MgATP; 4.0 mM Mg excess. 1 min preincubation, 25 nmoles Ca/mg protein taken up. Then 2.1 ml of a mixture containing EGTA, ATP, and Mg were added to 5.0 ml incubation mixture to give a final concentration of 14 mM EGTA (5×10^{-11} M Ca^{2+}), 3.4 mM MgATP, and either 5.5 mM Mg excess (solid circles) or 0.28 mM free ATP excess (open circles); 24.5°C.

hydrolysis during 1 sec incubations with steady-state rates because the phosphate contamination of ITP preparations was too high.) The inhibition of the rate of ITP hydrolysis during steady state, however, was not alleviated by the lowering of the concentration of ionized magnesium that results from raising the concentration of free ITP (Table II). (Magnesium is chelated by ITP at

least as well as by ATP [Hotta et al., 1961].) Therefore it appears likely that the increase in the concentration of free ATP rather than the lowering of the concentration of ionized magnesium was responsible for the activation of hydrolysis and flux. Fig. 6 shows how the rate of steady-state ATP hydrolysis depends on the concentration of free ATP.

Free ATP does not seem to activate by creating a leak in the membrane. It is conceivable, for instance, that free ATP enters the vesicles in preference to MgATP and leaves as the calcium-chelate, thereby creating an additional channel for calcium efflux. However, if that were the case one would expect free ATP to raise the rate of calcium outflow into a calcium-free medium just as much as it raised the rate of exchange. Fig. 7 shows that free ATP only slightly enhances the rate of calcium outflow. The mechanism by which free ATP activates or prevents inhibition of ATP hydrolysis may be quite complex since activation occurred only after several seconds of incubation with free ATP (Fig. 3 A).

DISCUSSION

There are two reasons why one may consider that the attenuation of flux and ATP hydrolysis during steady state is due to accumulated calcium. First, calcium influx and ATP hydrolysis remained high when the accumulation of free calcium in the interior was reduced by calcium oxalate accumulation (Table II; Weber et al., 1966). Therefore other reactions related to the transport process seem to be ruled out. Second, passive calcium preloading also was inhibitory.

Hasselbach and Makinose (1963; Makinose and Hasselbach, 1965) observed that the rate of calcium oxalate uptake increased with increasing oxalate concentrations. Although this observation may indicate that the rate of Ca-oxalate accumulation is limited by the rate of oxalate influx, it is just as likely, in view of the findings described here, that the enhancement is due to the lowering of the internal calcium concentration, as has already been suggested by Makinose and Hasselbach (1965).

It should be stressed that hydrolysis of ATP is only *modified* by calcium accumulated in the interior; otherwise, it is strictly dependent on the saturation of the transport system with external calcium just as is calcium influx. (Yamamoto and Tonomura, 1968; Makinose, 1969).

The mechanism of the inhibition by accumulated calcium is not understood. We do not know whether the effect of calcium depends on its binding to phospholipids, or to low affinity binding sites on a carrier protein, or on the ATP enzyme. In view of thse uncertainties it is not profitable to even speculate as to how free ATP may reverse this inhibition. It should be stressed though that free ATP cannot cause a general permeability increase for calcium because it increased only very little calcium outflow into a calcium-free

medium. Its action on calcium flux requires the presence of calcium in the medium as well as in the interior of the vesicles.

It seems attractive to speculate that agents that destroy the integrity of membranes cause an increase in the rate of ATP breakdown by preventing the accumulation of any calcium so that the transport mechanism can function at maximal rate rather than by accelerating an unspecific; i.e., uncoupled hydrolytic process. Although there is no way to measure transport activity under these conditions, one may cite in support the observation that the ATPase activity retains the same dependence on the calcium concentration of the medium as did calcium uptake and ATP hydrolysis in intact vesicles.

The attenuation of calcium flux by internal calcium should be taken into account when considering the fall in the rate of calcium net uptake with time as observed by Ebashi (Ohnishi and Ebashi, 1964) and his colleagues (Harigaya et al., 1968). (We are not equipped to measure initial rates of calcium uptake over periods as short as those for the measurement of ATP hydrolysis.) Ebashi considers the reduction of the rate of calcium uptake with time as evidence for a second-order reaction as the rate-limiting step. In his opinion calcium uptake consists of the binding of calcium to sites on the outside of the membranes, so that the rate declines in proportion to the decrease in the number of free binding sites. Ebashi and Endo (1968) assume that this reaction is not associated with ATP hydrolysis and cite in support unpublished data indicating that the rate of ATP hydrolysis remains constant with time. Since the data on ATP hydrolysis have not been published, the discrepancy in experimental results cannot be explained. However, our observation that the calcium-dependent initial rate of hydrolysis rapidly falls with time in contrast to the linear calcium-independent breakdown suggests that the initial calcium uptake is also coupled to ATP hydrolysis.

If a coupling factor of 2 (Hasselbach and Makinose, 1963; Weber et al., 1966) is assumed, our highest rates with intact vesicles would allow for a rate of calcium uptake of 3–4 μmoles Ca/min per mg. If one considers the rates of hydrolysis in the presence of detergents representative of true initial rates, without calcium inhibition, these rates would suggest potential transport rates of 10–12 μmoles/min.mg. If such rates occurred during initial net calcium uptake 100 nmoles calcium could be removed from the cytoplasm of 1 g muscle by 5 mg reticulum protein within 100 msec. Such an interval is compatible with relaxation times at 24°C and a realistic amount of reticulum protein per gram muscle (compare Peachey, 1965).

Winegrad observed (1968) that after tetanic stimulation calcium returns only rather slowly to the area near the terminal cysternae and transverse tubules. It appeared as if it were kept for a period of time in the longitudinal tubules, increasing their calcium content. If our preparations had consisted mostly of longitudinal tubules and if the rate of relaxation in living muscle were mostly dependent on longitudinal tubules, then the rate of calcium re-

moval from myofibrils should be slowed when the calcium content of the longitudinal tubules is raised. As a result calcium would be given a longer time to act and the twitch might develop more tension. In this manner the inhibition of uptake by accumulated calcium may be responsible for post-tetanic potentiation as was first suggested by Winegrad (1968).

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