

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

## II. *Inhibition of outflux in calcium-free media*

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**ABSTRACT** The outflux of calcium from vesicles of sarcoplasmic reticulum is controlled by the concentration of ionized calcium in the medium. In a calcium-free medium calcium outflux is at a minimum provided ATP and Mg are present. If they are removed calcium outflux becomes rapid and has a rate constant similar to that of outflux during steady-state  $^{40}\text{Ca}$ - $^{46}\text{Ca}$  exchange with saturating calcium concentrations in the medium. In order to maintain the low permeability state of the membrane only the presence but not the continued hydrolysis of ATP is necessary.

### INTRODUCTION

Calcium flux across reticulum membranes depends on the saturation of the transport system with calcium and ATP (Hasselbach, 1964) and, in addition, is subject to at least two kinds of control. The first control mechanism depends on the concentration of free calcium in the interior of the vesicles and was described in the preceding paper (Weber, 1971). The second mechanism depends on the concentration of ionized calcium in the medium which regulates the rate of calcium outflux. If the concentration of ionized calcium is lowered to about  $10^{-9}$  M outflux is at a minimum (Weber et al., 1966). In this paper some experiments are described which further characterize this control mechanism. Retention of accumulated transport substrate in a substrate-free medium is not restricted to the reticulum but occurs also in other transport systems (Drahota et al., 1965; Osborn et al., 1961).

### METHODS

Reticulum from fast skeletal muscle of the rabbit was used as in earlier studies (Weber et al., 1966). The methods for the determination of calcium uptake and release and of ATP hydrolysis have been described previously (Weber et al., 1966). The standard

medium for calcium uptake, that was used here, had the following composition: 0.3–0.6 mg reticulum protein/ml; 40 mM KCl; 10 mM imidazole buffer, pH 7.0; 30 or 60  $\mu\text{M}$   $^{45}\text{CaCl}_2$ ; 2.5 mM MgATP.

#### RESULTS

In order to measure the rate of calcium outflux into a calcium-free medium, reticulum was first allowed to accumulate calcium from a standard medium. Following that it was placed into a calcium-free medium simply by removing the remaining calcium from the medium through chelation to EGTA (ethyleneglycolbis( $\beta$ -aminoethyl-ether)- $N,N'$ -tetraacetic acid). (EGTA has been shown not to penetrate into the vesicles (Weber et al. [1966].) Thereupon the accumulated calcium was released into the medium at a very slow rate as described previously (Weber et al., 1966) and as can be seen here from the control curve in Fig. 2. This rate of net outflow was much slower than the rate of outflux for the same amount of intravesicular calcium before calcium had been removed (compare Figs. 4 and 7 in the preceding paper). This inhibition of outflow into a calcium-free medium was not accompanied by increased ATP hydrolysis because vesicles, containing accumulated calcium, hydrolyzed ATP at the same rate as did empty vesicles (Fig. 1). It is assumed that ATP hydrolysis by empty vesicles in a calcium-free medium is not caused by the transport enzyme but is due to a contaminating ATPase because no phosphorylprotein is formed during the activity of this ATPase (Makinose, 1969) and because this ATPase has a much lower affinity for ATP than the transport enzyme (Weber et al., 1966). Although the activity of the contaminating enzyme is measured by necessity in the absence of calcium and that of the transport enzyme in its presence the apparent  $K_m$  for ATP can be compared because the  $K_m$  of the transport enzyme is independent of the calcium concentration (Yamamoto and Tonomura, 1967). In addition this ATPase responds to inhibitors in a different manner than does the transport enzyme (Hasselbach, 1964).

Although the inhibition of calcium outflux does not depend on the continued hydrolysis of ATP it does require its presence. That was shown by the experiment described in Fig. 2 A. After 30 sec preincubation of the vesicles in a standard medium, apyrase and AMP-deaminase were added together with EGTA to rapidly hydrolyze the ATP present. The amount of apyrase added was probably sufficient to remove all the ATP within a few seconds because, when added together with ATP before calcium accumulation, it prevented calcium uptake completely (Fig. 2 A, bottom curve). As a result of the simultaneous removal of ATP and calcium about two-thirds of the accumulated calcium was released very rapidly. The outflux of the remaining calcium was considerably slower with a first-order rate constant of about  $0.47 \text{ min}^{-1}$  as compared to the initial one of about  $3 \text{ min}^{-1}$ . However, the second-rate con-

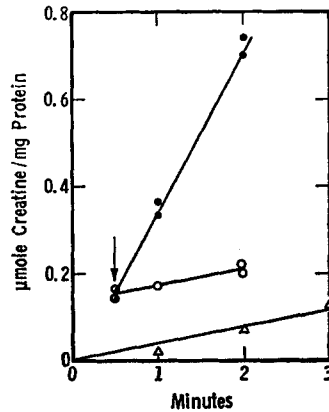


FIGURE 1. No activation of ATP hydrolysis during retention of accumulated calcium in vesicles suspended in a calcium-free medium. Ordinate indicates ATP hydrolyzed as measured by creatine liberated from creatine phosphate in a coupled enzyme system. Open symbols, in the presence of 1 mM free EGTA. Open circles, after 30 sec incubation in a standard medium (enriched by 2.0 mM creatine phosphate + 0.1 mg/ml kinase) to obtain filling of the vesicles with calcium, 10 mM EGTA was added to remove all remaining calcium from the medium. Open triangles, empty vesicles were incubated in the presence of EGTA. Solid circles, for a comparison the rate of ATP hydrolysis is shown that accompanied the retention of accumulated calcium when the remaining calcium had not been removed from the medium and  $\text{Ca}^{2+}$  was saturating. This activation of ATP hydrolysis presumably is coupled to the back transport of calcium lost by outflux (i.e. it accompanies exchange).

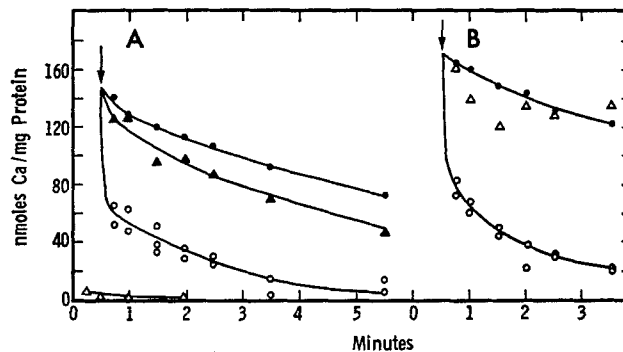


FIGURE 2. The effect of the removal of either ATP or magnesium on the rate of calcium outflux into a calcium-free medium. Ordinate, calcium outflux in nanomoles calcium per milligram vesicle protein. After 30 sec incubation in a standard medium A, EGTA alone was added to give a final concentration of 8 mM (solid circles); or it was added together with AMP-deaminase and the boiled supernatant of *apyrase* (solid triangles) to evaluate the effect of any agents present in the apyrase preparation; or it was added together with native apyrase and deaminase (open circles). As a control apyrase was added together with ATP at the start of the incubation (open triangles). B, EGTA only was added as in A (solid circles); or together with MgCHTA (open triangles); or just CHTA was added to a final concentration of 6 mM (open circles). Plotting outflows as first-order reactions gave straight lines for the second slope.

stant was still higher than that of the control, which had a value of  $0.22 \text{ min}^{-1}$ .

The inhibition of calcium outflux also requires magnesium in addition to ATP. The removal of magnesium together with calcium by the addition of CHTA (1,2-diaminocyclohexane-*N,N'*-tetraacetic acid,  $pK_{Mg}$  10.3 [Chaberek and Martell, 1959]) had an effect similar to that caused by the removal of ATP (Fig. 2 B). A large part of the calcium was released with a rate constant of about  $3 \text{ min}^{-1}$  and the remainder more slowly with a constant of about  $0.5 \text{ min}^{-1}$ . CHTA seemed to act only through the chelation of magnesium; it had no effect if added as the chelate of Mg or Mn.

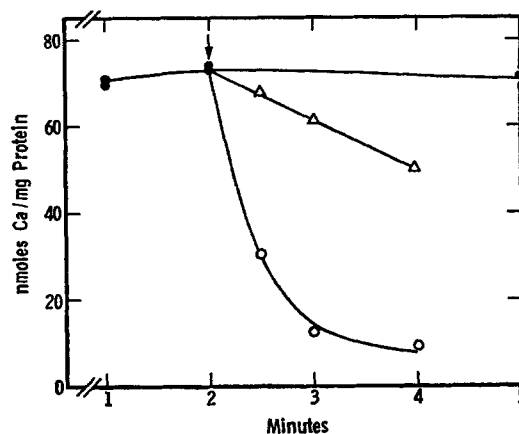


FIGURE 3. The effect of the addition of salyrgan on the outflow of calcium into a calcium-free medium. Ordinate, outflow of calcium. Solid circles, control = outflow of calcium from vesicles left in the preincubation medium; open triangles, EGTA only added after preincubation as in Fig. 2; open circles, EGTA + salyrgan (0.1 mM) added after preincubation.

Magnesium and ATP became ineffective and rapid calcium release ensued, when SH groups were blocked by high concentrations of salyrgan (0.5 mM) (Fig. 3). Such release has been demonstrated previously (Martonosi and Feretos, 1964; Hassalbach and Seraydarian, 1966).

#### DISCUSSION

It is not known whether the outflow of calcium into a calcium-free medium occurs by free diffusion, is carrier-mediated, or uses both mechanisms. However, even if outflow were entirely due to free diffusion the membrane permeability for free diffusion must be very low since the rate constant is only about  $0.003 \text{ sec}^{-1}$ .

One may question whether the rate of outflux has not been underestimated. Is it possible that most of the calcium that reached the outer surface of the membranes was immediately transported back into the interior? Two argu-

ments may be raised against such an assumption. First, calcium reaching the outside would be immediately chelated by EGTA. Even with the accumulated calcium returned to the medium the pCa would be too high for the transport system to retain bound calcium. Second, if there were back transport one would expect it to be coupled to the hydrolysis of ATP since strict coupling has been demonstrated under nearly all conditions in which careful simultaneous measurements of ATP hydrolysis and calcium uptake were made. We observed, however, that no acceleration of ATP hydrolysis accompanied the retention of accumulated calcium inside vesicles suspended in a calcium-free medium. In a calcium-free medium vesicles containing accumulated calcium hydrolyzed ATP at the same speed as did empty vesicles.

This state of low calcium permeability requires the continued presence of magnesium and ATP but apparently not the continued hydrolysis of ATP. Consistent with this conclusion one finds that calcium outflow is not increased at very low ATP concentrations (about  $10\ \mu\text{M}$  when the rate of ATP hydrolysis in a calcium-free medium is greatly reduced compared with ATP concentrations of about  $0.5\ \text{mM}$  (Weber et al. [1966].) Quite the contrary, the outflux rate into a calcium-free medium is always lower at  $10\ \mu\text{M}$  ATP than at higher concentrations. This is possibly due to the second control mechanism described in the accompanying paper whereby flux is modulated by the concentration of free; i.e., not magnesium-chelated ATP: the higher its concentration the greater the flux rate.

The reduction of calcium outflux on removal of calcium from the medium is considerable, with the rate constant reduced more than 10-fold, from  $0.05\ \text{sec}^{-1}$  for exchange during steady state to  $0.003\ \text{sec}^{-1}$ .

Retention of accumulated substrate in a substrate-free medium has been reported for other transport systems such as the galactose permease of *E. coli* (Osborn et al., 1961). With this system it was demonstrated that the sugar was rapidly released on treatment with dinitrophenol, presumably because of the resultant removal of ATP. It would be interesting to know whether here also only the presence but not the turnover was needed for the sugar retention.

A rationalization of these findings is given by the scheme developed by Kepes (1960). It is based on the assumption that outflux is completely or nearly completely carrier-mediated and that any component of free diffusion is small if it exists at all. Carrier mobility in the activated system (i.e. coupled to an energy source) depends on the presence of transport substrate on the side where it is picked up. Without the transport substrate the carrier is immobilized on the outside of the membrane. This scheme is especially attractive because the range of calcium concentrations that activate outflux is identical with the range over which calcium saturation of the transport system increases (Weber et al., 1966, Fig. 20).

What would be the function of ATP and magnesium? Although it cannot be excluded that they simply maintain the membrane in a state where it is relatively impermeable to free diffusion of calcium, they may also act directly on the transport system. In the framework of the scheme by Kepes they may be required to immobilize the carrier on the external membrane surface. The small amount of bound ATP (Ebashi and Lipmann, 1962; Weber et al., 1966; Inesi and Almendares, 1968) is consistent with an action of ATP on the transport units. It is not certain how magnesium functions: as the free ion or as a component of the chelate MgATP. Although it has been reported (Inesi and Almendares, 1968) that ATP binding occurs also in the presence of EDTA, i.e. when it does not exist as MgATP, it cannot be ruled out that this ATP is bound to sites other than the transport enzyme. It is possible that magnesium is required for calcium uptake and the formation of phosphorylated protein (Hasselbach and Makinose, 1961; Makinose, 1969) because MgATP is the substrate for the transport enzyme.

In conclusion one may state that the transport system for calcium possesses two control mechanisms. Both serve to reduce the energy required for the maintenance of a concentration gradient across the membranes during steady state. To this end, each control acts to inhibit calcium outflux thereby minimizing the hydrolysis of ATP which is associated with the transport of calcium back into the vesicles. In the first case accumulated calcium inhibits calcium flux across the membrane. Evidence for this mechanism and its modulation by free ATP has been described in the preceding paper. Second, outflux is reduced further on lowering the concentration of calcium ions in the medium to levels below saturation of the transport system. This dependence of calcium flux on calcium in the medium applies only to the activated transport system; i.e., both ATP and magnesium must be present possibly because MgATP is required. For the first case, i.e. inhibition by accumulated calcium, it is not known whether accumulated calcium can inhibit only the activated transport system (i.e. when it contains magnesium and ATP) or whether it would slow flux under all conditions.

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