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# Fast Kinetics of Adenosine Triphosphate Dependent $Ca^{2+}$ Uptake by Fragmented Sarcoplasmic Reticulum*

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ABSTRACT: ATP-dependent $Ca^{2+}$ uptake by fragmented sarcoplasmic reticulum was studied by measuring light absorbancy changes undergone by murexide, a metallochromic indicator. Rapid mixing by a stopped-flow apparatus and continuous monitoring by storage oscilloscopes permitted time resolution of the initial phase. $Ca^{2+}$ uptake proceeded linearly for the first 400–600 msec at a rate of 60–70 nmoles/mg of protein per sec (24–25°) and then declined to reach steady-state levels in 10–15 sec. Steady-state levels, but not the initial rates, were increased by changing the ATP concentration from 0.05 to 1.25 mM. No instantaneous binding of a measurable amount of $Ca^{2+}$ to sarcoplasmic reticulum was observed on addition of ATP. The experiments are consistent with a mechanism of active transport for the initial phase of ATP-dependent $Ca^{2+}$ uptake by sarcoplasmic reticulum and for induction of tension decay in muscle.

Vesicular fragments of sarcoplasmic reticulum can be isolated from muscle homogenates and shown to accumulate $Ca^{2+}$ in the presence of ATP (Hasselbach and Makinose, 1961, 1963; Ebashi and Lipmann, 1962). Measurements of the distribution of radioactive $^{45}Ca^{2+}$, in membrane fragments and reaction mediums separated by filtration (Martonosi and Feretos, 1964), produced the following information: (a) the yield of sarcoplasmic reticulum from weight unit of muscle tissue and the maximal capacity of sarcoplasmic reticulum for $Ca^{2+}$ accumulation *in vitro* are consistent with the amount of $Ca^{2+}$ to be sequestered in muscle to induce relaxation; (b) the high affinity of sarcoplasmic reticulum for $Ca^{2+}$ (Weber *et al.*, 1964, 1966) permits reduction of cytoplasmatic $Ca^{2+}$ below the concentration required for relaxation of myofibrils ($\sim 10^{-7}$ M) (Weber *et al.*, 1963). It was therefore proposed that, *in vivo*, sarcoplasmic reticulum controls the state of contraction or relaxation of myofibrils by regulating the intracellular $Ca^{2+}$ concentration (Hasselbach, 1964; Weber, 1966; Ebashi, 1965).

Measurements of radioactive $^{45}Ca^{2+}$ distribution are, however, too slow to allow the determination of the initial rapid rates of $Ca^{2+}$ uptake by sarcoplasmic reticulum. An alternative method is based on the spectrophotometric detection of changes in absorbance of the dye murexide (Ohnishi and Ebashi, 1963). This metallochromic indicator is specifically sensitive to the $Ca^{2+}$ concentration in the medium, and its use for the measurements of $Ca^{2+}$ transients in the presence of biological systems has been established (Mela and Chance, 1968; Jobsis and O'Connor, 1966; Geier, 1968).

Using a continuous-flow mixing device and the murexide method of detection, Ohnishi and Ebashi (1964) measured $Ca^{2+}$ uptake by sarcoplasmic reticulum at finite times and estimated that approximately 40 nmoles of $Ca^{2+}$ was taken up by 1 mg of sarcoplasmic reticulum within the first 30 msec of reaction. This figure, which is more than one order of magnitude higher than the initial rates of uptake extrapolated from the measurements of $^{45}Ca^{2+}$ distribution (Weber, 1966), was attributed to ATP-induced $Ca^{2+}$ binding to sarcoplasmic reticulum membrane (Ohnishi and Ebashi, 1964). It was proposed that this instantaneous binding is the first event in the ATP-dependent reaction of sarcoplasmic reticulum with $Ca^{2+}$, and the one relevant to the regulation of muscle contraction.

We have obtained continuous oscilloscopic tracings of murexide absorbancy changes in reactions initiated in a stopped flow apparatus. The rapid kinetics of $Ca^{2+}$ uptake by sarcoplasmic reticulum were compared to the data obtained by measurements of radioactive calcium and analyzed to determine whether (a) the initial uptake occurs as instantaneous binding or displays a resolvable time dependence; (b) the initial rates of uptake may be related to those of coupled hexergonic reactions, consistent with energy-dependent ion transport; (c) an accurate quantitative relation can be established in the appropriate time scale between $Ca^{2+}$ uptake by sarcoplasmic reticulum and its regulatory role in muscle contraction.

## Methods

Sarcoplasmic reticulum was prepared from white muscle of rabbit hind leg (McFarland and Inesi, 1971). Protein was estimated by the Folin method.

$Ca^{2+}$ uptake by sarcoplasmic reticulum was measured in reaction mixtures containing: 20 mM Tris-maleate (pH 6.8), 50 mM KCl, 10 mM $MgCl_2$, 140 $\mu$M total $Ca^{2+}$, 100 $\mu$M murexide (ammonium purpurate), and 0.4–1.6 mg of sarcoplasmic reticulum protein/ml. The reaction was initiated by the addi-

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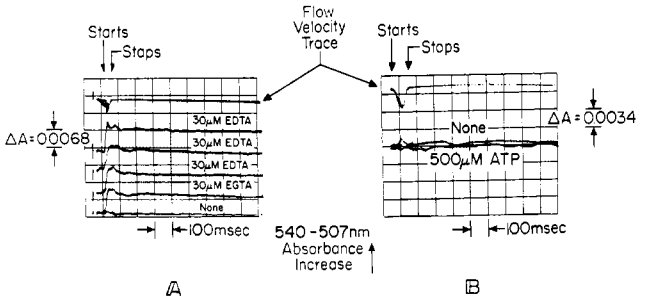


FIGURE 1: Oscilloscope traces of light absorbancy changes, on addition of ethylene glycol(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (A) and ATP (B), to a medium containing: 20 mM Tris-maleate (pH 6.8), 50 mM KCl, 140 μM $CaCl_2$, 10 mM $MgCl_2$, and 100 μM murexide. None refers to a control experiment obtained under the same conditions without the addition reported in the figures.

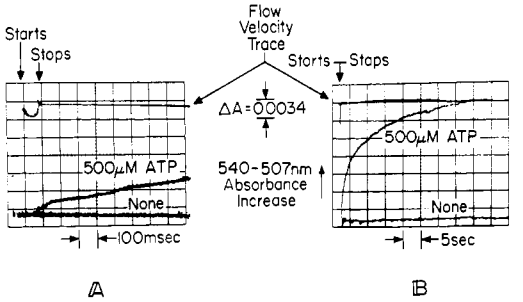


FIGURE 2: Addition of 500 μM ATP to a medium containing: 20 mM Tris-maleate (pH 6.8), 50 mM KCl, 140 μM $CaCl_2$, 100 μM murexide, 10 mM $MgCl_2$, and 0.7 mg of sarcoplasmic reticulum protein/ml. The oscilloscopic tracings were obtained at different time scales by using two different oscilloscopes.

tion of ATP (0.05–1.25 mM). The same medium without sarcoplasmic reticulum was used for control experiments. The temperature of the reaction medium was between 24 and 25°.

The Ca–murexide complex, as compared to murexide, exhibits a lower light absorbance at 540 nm and higher absorbance at 470-nm wavelength with an isosbestic point at 507 nm (Ohnishi and Ebashi, 1963). For this reason our experiments were carried out with the aid of a dual-wavelengths spectrophotometer (Chance, 1951) using 540 nm as the measuring wavelength. A reference wavelength of 507 nm (rather than the more sensitive 470 nm) was chosen to minimize the interference of light-scattering changes (Mela and Chance, 1968). The proper wavelengths were obtained employing narrow bandwidth interference filters. In the presence of murexide, $Ca^{2+}$ uptake by sarcoplasmic reticulum (or $Ca^{2+}$ binding by chelating agents) produces an increase in light absorbance at 540 nm by reducing the concentration of $Ca^{2+}$ available for formation of Ca–murexide complex.

ATP or other reagents were added to the reaction mixture using a regenerative stopped-flow apparatus (Chance *et al.*, 1967). The volume ratio of added reagents to reaction mixture was 1:80. Mixing time and absorbance changes were displayed on a storage oscilloscope and continuous tracings of absorbance changes were obtained.

In control experiments, radioactive $Ca^{2+}$ uptake by sarcoplasmic reticulum was measured as previously described (Inesi and Watanabe, 1967).

Crystalline NaATP (lot 100C-7620) and ethylene glycol-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid were purchased from Sigma, St. Louis, Mo.; murexide from K & K Chemicals, Plainview, N. Y. Ca contamination in ATP was 24 ppm, which is below the level of detection in our system.

## Results

In a reaction medium identical with those used to study $Ca^{2+}$ uptake by sarcoplasmic reticulum, $Ca^{2+}$ chelation by ethylene glycol(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid produced an increase in light absorbance (Figure 1A). This effect was completed within the mixing time and could be quantitatively reproduced by successive addition of ethylene glycol(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, within the limits of $Ca^{2+}$ availability in the medium. On the other hand, Figure 1B shows that addition of 500 μM ATP to the same medium did not produce any detectable change in absorbance. This was due to the presence of 10 mM $Mg^{2+}$ which effectively prevents formation of Ca–ATP complex in sizable amounts.

In the presence of sarcoplasmic reticulum, the addition of ATP was followed by an increase in light absorbance, related to ATP-dependent $Ca^{2+}$ uptake by sarcoplasmic reticulum. In fact, if sarcoplasmic reticulum was previously treated with diethyl ether (Inesi *et al.*, 1967; Fiehn and Hasselbach, 1969) to render the membrane leaky and unable to retain $Ca^{2+}$, no changes in absorbance were detected on addition of ATP.

Continuous oscilloscope recordings of murexide absorbance changes, recorded with different time scales, are reported in Figure 2. It is apparent that ATP-dependent $Ca^{2+}$ uptake by sarcoplasmic reticulum was rapid, but still much slower than the instantaneous binding by ethylene glycol(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

The shape of these activity curves is similar to that of curves obtained in experimental conditions where activity rates were sufficiently low to allow measurements of radioactive $^{45}Ca^{2+}$ distribution during the initial part of the reaction. Figure 3A shows the results of an experiment in which $Ca^{2+}$ uptake by

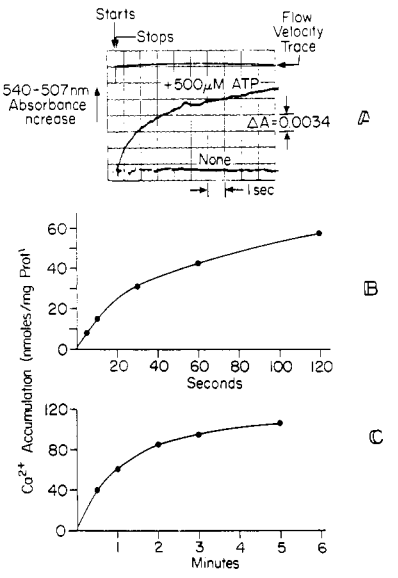


FIGURE 3: Time curves of $Ca^{2+}$ uptake by sarcoplasmic reticulum. (A) Addition of 500 μM ATP at 25°; reaction mixture as in Figure 2, monitored by murexide method. (B) Addition of 1 mM ATP at 5°; reaction mixture as in Figure 2, monitored by determination of radioactive $^{45}Ca$ distribution. (C) Addition of 5 mM *p*-nitrophenyl phosphate at 25°; reaction mixture as in Figure 2, monitored by determination of $^{45}Ca$ distribution. Sarcoplasmic reticulum was 0.65 mg of protein/ml in all the experiments.

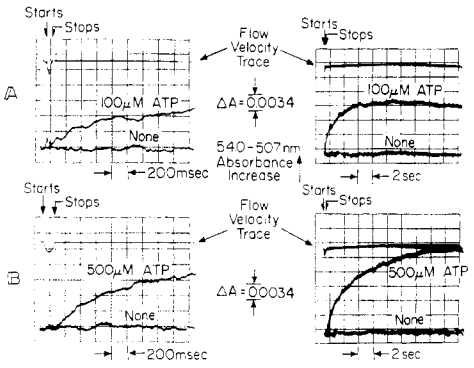


FIGURE 4: Effect of different concentration of ATP on the initial rate and steady-state levels of $Ca^{2+}$ uptake by sarcoplasmic reticulum. Reaction mixture as in Figure 2 except that the SR was 0.8 mg of protein/ml.

sarcoplasmic reticulum was induced by the addition of ATP at 25° and followed by recording the changes in absorbance by murexide. This trace is compared to time-dependent curves of radioactive $Ca^{2+}$ uptake by sarcoplasmic reticulum obtained after the addition of ATP at 5° (Figure 3B) or *p*-nitrophenyl phosphate at 25° (Figure 3C). Two observations apparent in these curves are their close similarity and the lack of any instantaneous binding.

The initial rate of uptake (at 24–25°) varied between 60 and 70 nmoles per sec per mg of protein, indicating a carrier turnover of approximately $10/sec^{-1}$, based on the highest reported number of phosphorylated ATPase sites in sarcoplasmic reticulum (Makinose, 1969; Inesi *et al.*, 1970).

Figure 4 shows that in two experiments where the ATP concentrations were 100 and 500 μM, respectively, identical initial rates of $Ca^{2+}$ uptake were obtained. In either case, it may be assumed that the $Ca^{2+}$ transport system was saturated both with respect to ATP and $Ca^{2+}$. In fact, experimental evidence indicates that half-maximal activation is obtained in the presence of $1 \times 15^{-6}$ M ATP (Ebashi and Lipman, 1962; Martonosi and Feretos, 1964; Weber *et al.*, 1964, 1966) and $1 \times 15^{-7}$ M $Ca^{2+}$ (Weber *et al.*, 1964; Hasselbach, 1964). Based on these values it can be calculated that, in the presence of 0.5 mM ATP and 140 μM $Ca^{2+}$, our system should have exhibited a zero-order behavior for a substantial part of the reaction. On the contrary, the initial rates remained constant only for 400–600 msec, then progressively declined to reach steady state levels in 10–15 sec. This decrease may be understood considering that (a) the measurements reflect net accumulation, rather than total transport (Johnson and Inesi, 1969); and (b) high intravesicular $Ca^{2+}$ inhibits further transport, independent of outside carrier saturation (Weber, 1971).

As opposed to the initial rates, the steady-state levels of $Ca^{2+}$ accumulated were sensitive to variations in ATP concentration (Figure 4) such that they could be raised by further addition of ATP. Maximal values of 120–140 nmoles of $Ca^{2+}$/mg of protein were obtained in the presence of 1.25 mM ATP. This effect of the ATP concentration may reflect the thermodynamic requirements for a $Ca^{2+}$ gradient across the membrane, as well as enzyme unsaturation consequent to substrate hydrolysis.

## Discussion

Based on our experiments and the reported values for activation energy in sarcoplasmic reticulum (Inesi and Watanabe, 1967; Sreter, 1969), we estimated that, at 37°, 120–140 nmoles of $Ca^{2+}$ can be removed by 4 mg of sarcoplasmic reticulum protein contained in 1 g of muscle, within 100 msec. This estimate is within the range of requirements for relaxation of average mammalian muscle. In this respect, our results are in agreement with those reported by Ohnishi and Ebashi (1964), in as much as sarcoplasmic reticulum activity can be demonstrated within an appropriate time scale for muscle relaxation.

It should be pointed out that the murexide method requires $Ca^{2+}$ concentrations of the order of $10^{-4}$ M in the reaction medium. These concentrations are higher than those found in muscle cells (0.5–10 μM) and for this reason, some reservation may be raised as to the physiological relevance of the rates determined in our experiments. However, measurements of $Ca^{2+}$ accumulation in the presence of oxalate (Hasselbach and Makinose, 1963), approximate determinations of initial rates in the absence of oxalate (Weber *et al.*, 1966), and studies on $Ca^{2+}$ activation of sarcoplasmic reticulum ATPase (Inesi and Almendares, 1968), strongly suggest that $Ca^{2+}$ transport follows Michaelis–Menten kinetics, with half-maximal activation at pCa = 7. Consequently, the transport system should be saturated in the presence of physiological $Ca^{2+}$ concentrations, as well as in our experimental conditions.

During our work, we failed to observe an instantaneous absorbancy change, that could be attributed to ATP-dependent $Ca^{2+}$ binding (Ohnishi and Ebashi, 1964), in amounts relevant to muscle relaxation. Rather the initial $Ca^{2+}$ uptake, continually recorded in our experiments, was well resolved in time, and the initial linear rates were similar to the highest obtained by measuring radioactive $^{45}Ca$ distribution and ATP hydrolysis (Hasselbach and Makinose, 1961, 1963).

Variations of sarcoplasmic reticulum activity have been attributed to aggregation of the vesicles, and to the extent of dispersion induced by different mixing procedures (Weber, 1966). This may be true, and it is possible that the activity of sarcoplasmic reticulum *in vivo* is greater than that which we obtained *in vitro*. However, a more effective mixing is unlikely to be the reason for the higher initial uptake obtained by Ohnishi and Ebashi (1964), relative to ours. The high efficiency of mixing in our experiments is demonstrated by complete $Ca^{2+}$ titration by ethylene glycol(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid within the mixing time (Figure 1A), as compared to 70% titration obtained in their experiments.

It is possible that part of the initial $Ca^{2+}$ uptake obtained by Ohnishi and Ebashi (1964) is due to ATP-independent $Ca^{2+}$ binding to sarcoplasmic reticulum. In fact, two syringes of equal volume were used in their mixing apparatus and, to avoid large changes in turbidity, both syringes contained sarcoplasmic reticulum: Syringe A contained sarcoplasmic reticulum and 400 μM $CaCl_2$; Syringe B contained sarcoplasmic reticulum and ATP. On mixing these components, it is likely that sarcoplasmic reticulum derived from syringe B bound sizable amounts of $Ca^{2+}$ derived from syringe A, independent of ATP. It is clearly shown by Fiehn and Migala (1971) and Chevallier and Butow (1971) that in mediums containing 500 μM $CaCl_2$ and 5–10 mM $MgCl_2$, sarcoplasmic reticulum binds 30–50 nmoles of $Ca^{2+}$/mg of protein in the absence of ATP. In this connection, it should be understood that the extent of ATP-independent $Ca^{2+}$ binding is much lower at $Ca^{2+}$ concentrations corresponding to the sarcoplasmic $Ca^{2+}$ levels ($\sim 1 \times 10^{-7}$ M).

The mixing apparatus used in our experiments permitted additions of small volumes of a solution containing only ATP to much larger volumes (volume ratios 1:80) of a medium

containing sarcoplasmic reticulum previously incubated with $Ca^{2+}$ and other reagents. Therefore, the measured $Ca^{2+}$ uptake was strictly ATP dependent.

In conclusion, our results are consistent with a mechanism of active transport for the rapid $Ca^{2+}$ uptake by sarcoplasmic reticulum, relevant to induction of relaxation in muscle. It should be pointed out that a small amount of $Ca^{2+}$ binding to the sarcoplasmic reticulum ATPase must occur, to cause enzyme activation (Inesi *et al.*, 1970). Our failure to detect it, however, indicates that this binding occurs either before the addition of ATP (Fiehn and Migala, 1971; Chevallier and Butow, 1971), or to an extent which is below the sensitivity of detection of our method ($\sim$3 $\mu$M). On the other hand, binding of $Ca^{2+}$ to low affinity sites at the inner side of the sarcoplasmic reticulum membrane (Fiehn and Migala, 1971; Chevallier and Butow, 1971; MacLennan and Wong, 1971; Carvalho, 1966) evidently occurs as a secondary process, when the $Ca^{2+}$ concentration in the sarcoplasmic reticulum vesicles is raised as a consequence of active transport.

## Acknowledgment

This work was carried out in the laboratory of Dr. Britton Chance, to whom the authors are very much indebted for his generous support and continuous help and advice.

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