DETECTION OF AN INITIAL BURST OF Ca^{2+} TRANSLOCATION IN SARCOPLASMIC RETICULUM

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SUMMARY: Rapid quench methods were used to determine Ca^{2+} uptake, ATPase phosphorylation and Pi production in the transient state of Sarcoplasmic Reticulum. It was found that within 20 milliseconds of the addition of ATP maximal levels of phosphorylated enzyme intermediate are reached and an initial burst of Ca^{2+} uptake is completed. This burst, kinetically distinct from the following transport activity, is related to the phosphorylated intermediate with a molar ratio of two.

The early phase of ATP dependent Ca^{2+} uptake by SR vesicles was first resolved by the use of metallochromic indicators (1) and stopped flow mixing (2). This method, however, limits the choice of Ca^{2+} concentrations and the use of Ca^{2+} buffering systems. Alternatively, initial Ca^{2+} uptake can be measured with the aid of radioactive tracer and a rapid mixing apparatus for addition of ATP and EGTA at sequential time intervals (3). Using the EGTA quench method we have been able to obtain measurements of initial transport velocities at Ca^{2+} concentrations of physiological interest. Furthermore, we have demonstrated that phosphorylation of one mole of ATPase is accompanied by translocation of two moles of Ca^{2+} across the membrane. This phenomenon is kinetically distinct from subsequent Pi production and Ca^{2+} transport.

METHODS: SR vesicles were obtained from white muscle of rabbit hind legs as previously described (4).

^{45}Ca-Ca^{2+} uptake by SR vesicles was initiated by addition of ATP to appropriate reaction mixtures, and quenched either by Millipore (HA 0.45μ) filtration, or by addition of an equal volume

Abbreviations: SR, sarcoplasmic reticulum; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid; MOPS, morpholinopropane sulfonic acid.

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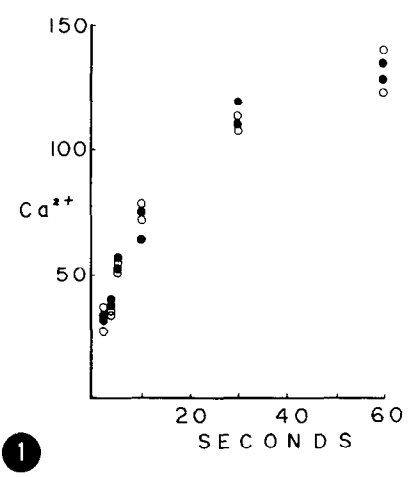
of quenching mixture (10 mM EGTA, 80 mM KCl, 20 mM MOPS, pH 6.8) followed by Millipore filtration to remove the SR vesicles. The filtered medium was then collected for determination of residual ^{45}Ca-Ca^{2+}. When necessary, time resolution within the milliseconds time scale was obtained by starting and quenching the reaction with the aid of a Durrum D-133 multimixing apparatus.

ATPase phosphorylation and Pi production were measured as described by Froehlich and Taylor (5).

RESULTS AND DISCUSSION: In reaction mixtures permitting Ca^{2+} transport by SR vesicles, complete quenching of transport activity is obtained upon addition of sufficient EGTA to reduce the Ca^{2+} concentration in the medium below 1×10^{-8}M. Following quenching, the SR vesicles retain unchanged levels of accumulated Ca^{2+} for at least 30 seconds, thereby allowing sufficient time for filtrations and separation of the loaded vesicles from the reaction mixture.

Measurement of Ca^{2+} uptake with the EGTA method yields time curves identical to those obtained by filtering the reaction mixture without previous EGTA quench. This is demonstrated in experiments in which Ca^{2+} uptake is coupled to hydrolysis of a substrate (e.g. acetylphosphate) that is utilized at slow rates (Fig. 1). On the other hand, a useful feature of the EGTA quench method is its suitability for fast kinetic experiments with the aid of rapid mixing devices. In this manner, time resolution of Ca^{2+} uptake can be obtained even when ATP is used as a substrate (Fig. 2).

As compared to methods involving Ca^{2+} detection with metallochromic indicators, the EGTA quench method has the advantage of permitting direct measurements of radioactive tracer and great flexibility in the use of Ca·EGTA buffers. Consequently, the Ca^{2+} concentration in the medium can be varied within a wide range, while total Ca^{2+} is maintained sufficiently high to sustain transport activity. Therefore, we have been able to measure velocities of Ca^{2+} transport in the presence of Ca^{2+} concentrations of physiological interest. In fact, velocities obtained at pCa 5



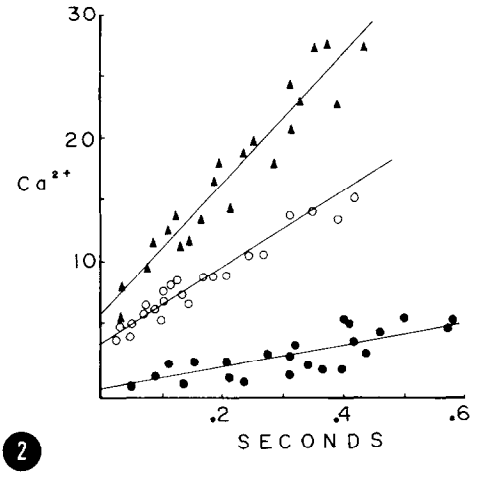


Fig. 1 Acetylphosphate dependent Ca^{2+} uptake (nanomoles/mg protein) by SR vesicles. Comparison of experiments in which the uptake was stopped by straight filtration (○), as opposed to EGTA quench followed by filtration (●). Reaction mixture: 20 mM MOPS (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 0.26 mg SR protein/ml and 0.1 mM ^{45}Ca-$CaCl_2$. Reaction started by the addition of 1.0 mM acetylphosphate. Temp.: 25°C.

Fig. 2 ATP dependent Ca^{2+} uptake (nanomoles/mg protein) by SR vesicles. Reaction mixture: 20 mM MOPS (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 0.1-0.2 mg SR protein/ml, 0.06 mM ^{45}Ca-$CaCl_2$ and EGTA adjusted to yield 2.2×10^{-5}M (▲), 9×10^{-7}M (○), and 1×10^{-7} (●) free Ca^{2+} based on the Ca·EGTA $K_a = 2 \times 10^6$. The reaction was started by the addition of ATP to a final concentration of 0.12 mM and quenched with an equal volume of a solution containing 10 mM EGTA, 80 mM KCl and 20 mM MOPS. Temp.: 25°C.

and saturating ATP concentrations vary between 55 and 60 nmoles/mg protein/second, corresponding to a rate constant of 15 sec^{-1} (25°C), based on a maximal level of 4 nanomoles phosphoenzyme/mg protein. These values are quite similar to those obtained at pCa 4 (2) and validate previous inferences on the adequacy of SR with regard to sequestration of myoplasmic Ca^{2+} and relaxation of myofibrils.

An interesting feature of the Ca^{2+} uptake data obtained by the EGTA quench method is their extrapolation to intercepts higher than 0 (Fig. 2), indicating the occurrence of a burst within 20 milli-

seconds of the addition of ATP. This phenomenon is kinetically distinct from the following transport activity.

It should be pointed out that an initial burst was not noticed when the activity was monitored by the use of metallochromic indicators (2). This difference is due to baselines (absence of ATP) which are not comparable in the two methods. In fact, while in the quench method 10 mM EGTA is used to effectively remove the Ca^{2+} bound to SR in the absence of ATP, in the optical method Ca^{2+} concentrations permitting binding to SR are present.

The initial burst obtained with the quench method implies translocation of bound Ca^{2+} to a position not accessible to EGTA. This phenomenon does not occur if ATP, which phosphorylates the ATPase protein (6,7), is substituted by an inactive analogue such as AMP-PNP. Therefore, utilization of the nucleotide and formation of phosphoenzyme are required for its occurrence.

The levels of the Ca^{2+} uptake intercepts vary with the concentration of Ca^{2+} and reach maximal values of 6-8 nmoles/mg protein at saturating Ca^{2+} concentrations. Since in identical experimental conditions 3.5-4.0 nmoles of phosphoenzyme are formed within the first 20 milliseconds of the addition of ATP (Fig. 3), the ratio of Ca^{2+} intercept to phosphoenzyme levels is approximately two. It is noteworthy that Pi production does not show a burst simultaneous to that observed for Ca^{2+}.

In conclusion, Ca^{2+} binding to SR is necessary for ATPase phosphorylation and the latter is a prerequisite for the initial burst of Ca^{2+} uptake. Therefore, it is apparent that upon phosphorylation of one mole of ATPase, two moles of bound Ca^{2+} are transferred from the outer to the inner surface of the vesicles. It is clear that the observed Ca^{2+} movement is an intermediate step in the active transport cycle and is consistent with mechanisms

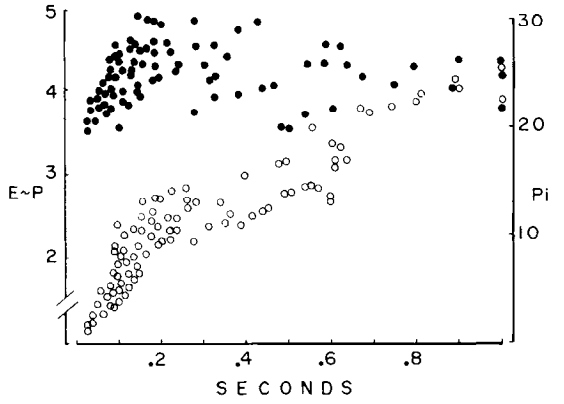


Fig. 3 Formation of phosphoenzyme (nanomoles E~P/mg protein: ●) and production of Pi (nanomoles/mg protein: ○). Reaction mixture: 20 mM MOPS (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 0.35-0.40 mg SR protein/ml, 0.1 mM $CaCl_2$ and 0.1 mM EGTA. The reaction was started by the addition of [γ-^{32}P]ATP to a final concentration of 0.1 mM, and was quenched with an equal volume of 1 N HCl. Temp.: 25°C.

previously suggested by Makinose (8), Sumida and Tonomura (9) and Ikemoto (10). The coupling of Ca^{2+} movements to ATPase phosphorylation indicates that this step plays a central role in energy transduction by the SR membrane.

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