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Rapid filtration measurements of Ca^{2+} release from cisternal sarcoplasmic reticulum vesicles

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A radioactive tracer and rapid filtration method was applied to the study of Ca^{2+} release from sarcoplasmic reticulum (SR) vesicles which were preloaded passively (equilibration with millimolar Ca^{2+}) or actively (in the presence of ATP or acetyl phosphate). The method allows complete substitution of the loading mixture with release medium in constant flow, and time resolution between 0.01 and 10.0 s. Net release can be clearly distinguished from isotope exchange. The latter is prominent in longitudinal SR vesicles. Net Ca^{2+} release is observed only from cisternal SR vesicles, is Ca^{2+} (micromolar) dependent, and is accelerated by inactive ATP analogues, or ATP itself, even in the presence of Mg^{2+}. Net release has a strong pH dependence (between 6 and 7), and very little temperature dependence (consistent with a passive channel). In media of physiological significance (1 mM ATP, 1 mM magnesium, and free Ca^{2+} in the micromolar range), net Ca^{2+} release proceeds with a rate constant of approx. 100 s^{-1}.

Ca^{2+} release; Sarcoplasmic reticulum; Excitation-concentration coupling; Rapid filtration

1. INTRODUCTION

The Ca^{2+} pump of sarcoplasmic reticulum (SR), and its role in removal of myoplasmic Ca^{2+} to induce relaxation of muscle fibers, are understood in considerable detail. In contrast, the mechanism of Ca^{2+} release from SR, which is pertinent to excitation-contraction coupling, is not well understood. In this regard, a Ca^{2+}-dependent Ca^{2+} release has been observed in skinned fibers [1–3] and isolated SR vesicles [4–12]. Further progress has been achieved with the separation of vesicles derived from cisternal ('heavy' fraction) and longitudinal ('light' fraction) SR, and the incorporation of a calcium channel into model bilayers [13]. The most direct measurements of calcium fluxes are those based on isotopic tracer. In this type of experiments, Ca^{2+} release from SR vesicles has been triggered by perturbations produced by large dilutions of loading media with release media; kinetic resolution was then obtained by stopping the release with quenching media at serial times, and filtering the vesicles out of the quenched reaction mixture [12]. We here describe experiments carried out with radioactive tracer and a fast filtration method, permitting changes of the reaction mixture by complete substitution of the loading medium, and yielding millisecond resolution of release kinetics with no need for quenching.

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2. MATERIALS AND METHODS

Light vesicles derived from longitudinal SR were prepared as described by Eletr and Inesi [14].

Heavy vesicles derived from cisternal membranes were prepared and purified as follows: rabbit hindleg skeletal (white) muscle was first cooled in ice-cold 0.1 mM EDTA (pH 7), minced with scissors, and homogenized (200 g/1000 ml of

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5 mM imidazole-HCl, pH 7.4) in a Waring blender for two periods of 30 s each, with a 30 s interval. The homogenate was then centrifuged at 5200 × g_{max} for 15 min. The supernatant was collected, filtered through four layers of gauze, and centrifuged again for 10000 × g_{max} for 30 min. The resulting pellet was rinsed with 0.3 M sucrose, 80 mM KCl, 10 mM Mops, pH 6.8, and 0.1 mM PMSF (medium 1), and resuspended in 12 ml medium 1 with the aid of a hand homogenizer. Aliquots (2 ml) of this suspension were placed on a discontinuous gradient consisting of an upper layer of 18 ml medium 1 containing 32% sucrose (n = 1.3845) and a lower layer of 18 ml medium 1 containing 38% sucrose (n = 1.3955). Following centrifugation at 136000 × g_{max} for 4 h in a swinging bucket rotor, the white membrane suspension at the 32–38% interface was collected, diluted with 1.5 vols medium 1 containing no sucrose, and subjected to a 130000 × g_{max} centrifugation for 45 min. The pellet was collected, resuspended in 2 ml medium 1 (to a concentration of approx. 10 mg/ml), frozen in dry ice/ethanol in small separate aliquots and stored at −70°C.

Electrophoretic analysis of this preparation revealed the presence of a high calsequestrin content and high-M_r proteins, as described for cisternal SR [15].

Passive loading of the vesicles with calcium was obtained by incubating 1 mg SR protein/ml of 1 mM $CaCl_2$ (supplemented with ^{45}Ca to yield about 10000 cpm per nmol), 80 mM KCl, and 10 mM Mops, pH 6.8, for 2–3 h at 22°C. Active loading was obtained by incubating 0.08 mg SR protein/ml of 20 mM Mops, pH 6.8, 80 mM KCl, 2 mM $MgCl_2$, 50 μM $CaCl_2$ (supplemented with ^{45}Ca to yield about 10000 cpm per nmol) and 0.5 mM ATP or 2 mM acetyl phosphate for 2 min at 22°C.

For the release experiments, aliquots (80 μg protein each) of passively loaded vesicles were diluted with 1 ml non-release medium (20 mM Mops, pH 6.8, 80 mM KCl, 5 mM $MgCl_2$ and 1 mM EGTA) and placed on Millipore (0.65 μm pore size) filters. Alternatively, 1 ml aliquots of actively loaded mixture (80 μg protein each) were placed directly on the filters, and the medium was filtered through. The loaded filters were then flushed with various volumes of release medium for variable time intervals, and the vesicles on the filter were finally washed with 2 ml non-release medium. The time and velocity of flow were controlled electronically with the aid of a Biologic (Pullman, Washington) fast filtration apparatus. The residual calcium on the filters was estimated by determination of radioactivity.

Free Ca^{2+} concentrations were calculated [16] from total calcium and total EGTA, based on the constants given by Schwartzenbach et al. [17] and the pK values given for EGTA by Blinks et al. [18], taking into account pH and magnesium concentrations.

3. RESULTS

Ca^{2+}-induced Ca^{2+} release has been previously observed from passively loaded vesicles, following perturbations produced by large dilutions of the loading medium in order to change its composition significantly. In these experiments, calcium release is quenched at serial times with EGTA-Mg^{2+} added by means of a fast mixing device, and then the reaction mixture is filtered and the residual calcium associated with the vesicles in the filters is determined [19]. In experiments carried out by this method, we observed kinetics of calcium release similar to those reported by Meissner et al. [20].

We tested our fast filtration method by placing aliquots of passively loaded vesicles on the filters, and then flushing the loaded vesicles with release medium for a controlled time, without any quenching. The resulting efflux is shown in fig.1, displaying kinetics identical to those obtained with the dilution method. An advantage of the filtration method is that the medium can be totally changed when the release is initiated. Therefore, experimental perturbations can be introduced more easily. One such perturbation is temperature. Fig.1A shows experiments in which the vesicles were passively loaded at 22°C, and then the efflux was initiated with a medium equilibrated at different temperatures. It is of interest that, contrary to the high temperature dependence of the calcium pump [20], calcium release from loaded vesicles is virtually temperature independent.

Since it was reported that the amount of calcium release in 10 s varies with pH [12], we carried out a set of experiments to resolve the kinetics of calcium efflux at various pH values. For the experiments shown in fig.1B we loaded the vesicles

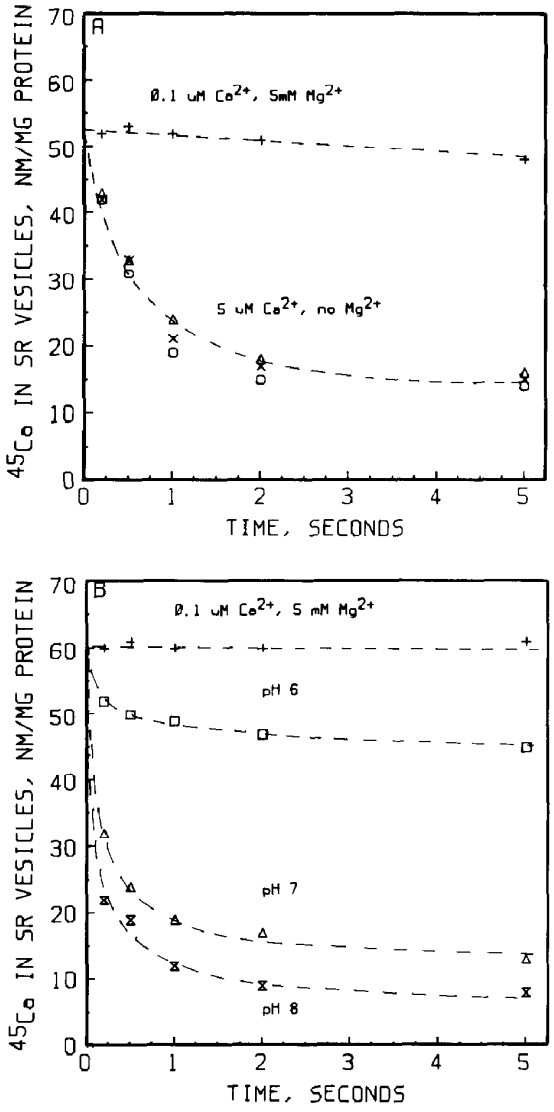

Fig.1. Temperature (A) and pH (B) dependence of Ca^{2+} release from passively loaded heavy vesicles. The vesicles were loaded passively (see section 2) at 22°C, pH 6.8. For each experimental point an aliquot (80 μg protein) of the loaded vesicles was diluted with 1 ml non-release medium (0.1 μM Ca^{2+}, 5 mM Mg^{2+}), placed on the Millipore filter and flushed with release media ($CaCl_2$ and EGTA to yield 5 μM free Ca^{2+}, 20 mM Mops, pH 6.8, and 80 mM KCl). For the temperature studies (A), the release media were maintained at 7 (×), 25 (Δ) or 32°C (○). For the pH studies (B), the release media were at pH 6 (□), 7 (Δ) or 8 (⧗). Non-release media at various pH and temperatures yielded identical results (+). Only one curve is shown for non-release media which represents the average for all temperature or pH conditions.

passively at pH 6.8, and then used a release medium of different pH. It is apparent that the efflux is much slower at acid than at neutral or alkaline pH.

Calcium release is significantly accelerated by ATP analogues [20] such as AMP-PNP (fig.2).

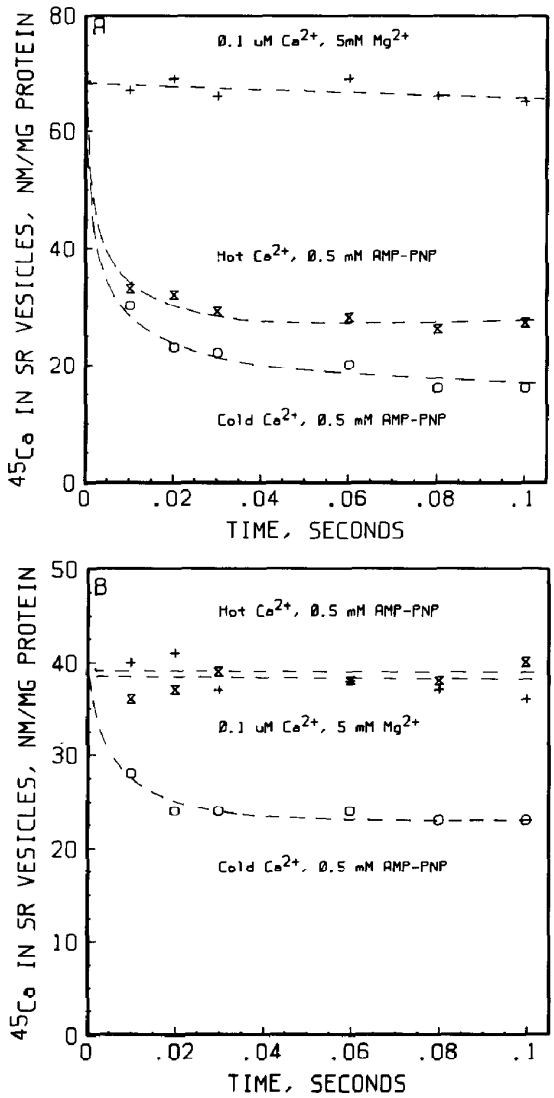

Fig.2. Comparison of net release (⧗) and exchange (○) from passively loaded heavy (A) and light (B) vesicles. The vesicles were passively loaded (see section 2) and the kinetics of Ca^{2+} efflux were studied at 22°C and pH 6.8. The release media contained either non-radioactive calcium (○) or calcium radiolabeled with a specific activity (~10000 cpm/nmol) identical to that of the calcium load (⧗), in addition to 20 mM Mops, 80 mM KCl and 0.5 mM AMP-PNP.

Under these conditions, we compared the efflux of radiolabeled calcium loads, using release media containing either non-radioactive calcium or calcium radiolabeled with a specific activity identical to that of the calcium load. In this manner we were able to distinguish net calcium release from isotope exchange. We found that release from heavy (cisternal) SR vesicles (fig.2A) resulted in a net reduction of their calcium load, while the release of radioactive calcium observed with light (longitudinal SR) vesicles (fig.2B) was prevalently an exchange phenomenon resulting in no change of the net calcium load. This suggests that calcium exits the heavy vesicles through a channel, while in light vesicles an isotope exchange is operated by the ATPase.

Another advantage of the filtration method is that the vesicles can be filled actively (in the presence of ATP), and then the active loading medium (including ATP) can be totally removed as the release medium is flushed through. Unless ruthenium red is included [12,22], calcium uptake by heavy vesicles is significantly lower than uptake by light vesicles, even in the presence of high Mg^{2+}. We found that net release from vesicles loaded with ATP involves only a small fraction of the load, while a larger exchange is observed (fig.3A). It is possible that loading with ATP selects for a small fraction of light vesicles present in heavy SR preparations, with consequent lack of release. On

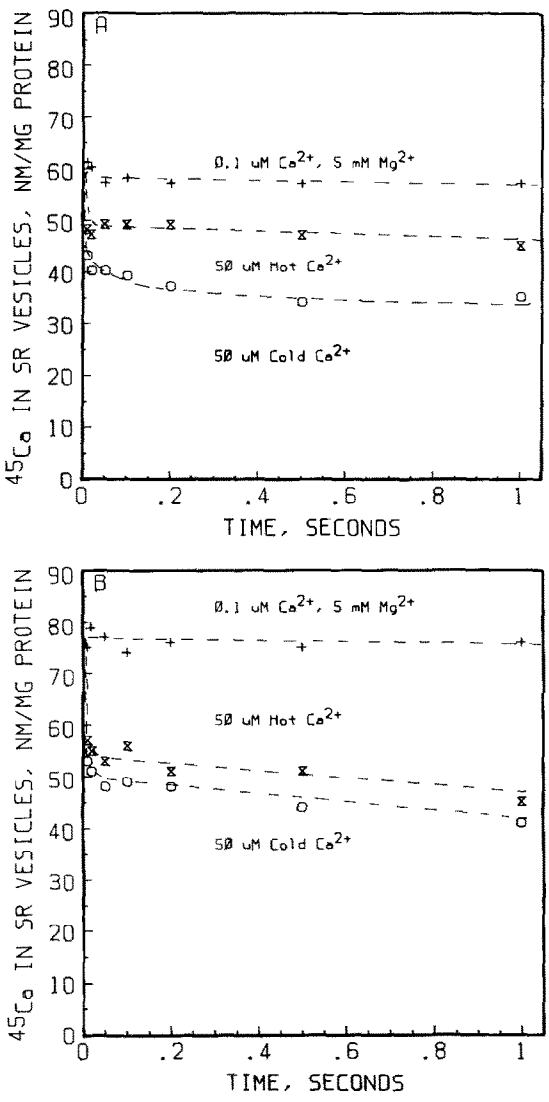

Fig.3. Net release (⧗) and exchange (○) from actively loaded heavy vesicles. The vesicles were loaded actively at 22°C and pH 6.8 with ATP (A) or acetyl phosphate (B), as described in section 2. For each experimental point, an aliquot of this suspension (80 μg/ml) was placed on the filter and flushed with release media containing 20 mM Mops, 80 mM KCl, 50 μM radioactive (⧗) or non-radioactive (○) calcium.

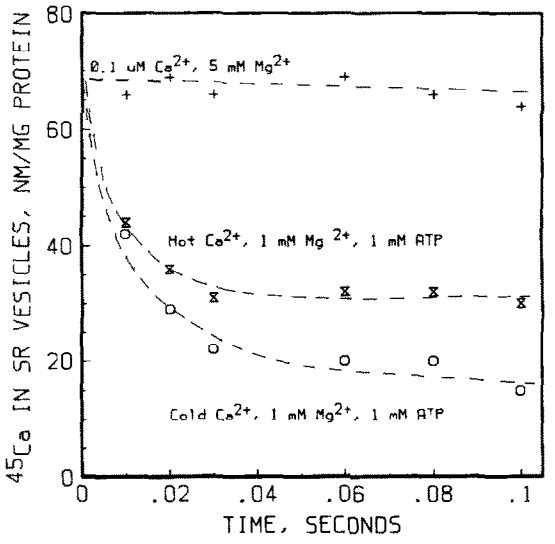

Fig.4. Net release (⧗) and exchange (○) from passively loaded heavy vesicles in the presence of Mg^{2+} and ATP. The vesicles were loaded passively at 22°C and pH 6.8 as described in section 2. The release media contained 20 mM Mops, 80 mM KCl, 100 μM radioactive (⧗) or non-radioactive (○) calcium, 1 mM Mg^{2+} and 1 mM ATP.

the other hand, it is also possible that the channel is closed by some gating mechanism during the preincubation with ATP [22], and the characteristics of release are consequently changed. It is important to note that a better calcium load is obtained when acetyl phosphate is used instead of ATP. A more prominent release is observed in this case as well (fig.3B).

Finally, we determined the kinetics of release triggered by physiologically relevant media. It is shown in fig.4 that in passively loaded vesicles, net calcium efflux is very prominent and occurs with a rate constant of approx. 100 s^{-1}, when release media containing ATP and magnesium are added.

4. DISCUSSION

Following a comparative survey of rapid kinetic methods and instrumentation (stopped flow, rapid quench and rapid filtration) in our laboratory, we find that the rapid filtration method is most convenient in release experiments with SR vesicles, since it allows handling of small samples (30–80 μg protein/sample), passive or active preloading, complete substitution of loading media with release media, use of small volume of release media, and time resolution between 10 ms and 10 s.

Our observations indicate that the Ca^{2+}-dependent Ca^{2+} release is a specific feature of cisternal SR vesicles, since it is not observed with longitudinal SR vesicles. The kinetics of release are in fact limited by the semipermeability properties of the cisternal membrane, since we found that passive or active loads are immediately released from the vesicles when a calcium ionophore is included in the release media. A permeability constant (P_{Ca}) for the cisternal SR membrane can then be estimated according to

$$P_{Ca} = k(V/A)$$

where k is the observed rate constant (100 s^{-1}, under the conditions for fig.4), V the average volume (6 cm^3/g) and A the average area (3×10^6 cm^2/g) of the vesicles. The resulting value is 2×10^{-4} $cm \cdot s^{-1}$, as opposed to 2×10^{-8} $cm \cdot s^{-1}$ in non-release media. The velocity of Ca^{2+} release (in the absence of a transmembrane electrical potential) can be approximated by

$$F = P\Delta C$$

Assuming 1×10^{-2} M Ca^{2+} inside the vesicles, and 1×10^{-8} M in the myoplasm, the velocity of Ca^{2+} release from cisternal SR should be approx. 1×10^{-9} $mol \cdot s^{-1} \cdot cm^{-2}$, which is in the range expected for contractile activation. It can be calculated that, independent of voltage gating, a 100 mV electrical potential would only change the velocity of release to less than one order of magnitude in the appropriate direction.

Although the physiological gating mechanism is not known, it is apparent that the Ca^{2+} channel of cisternal vesicles is subject to regulation, since changes of Ca^{2+} and/or H^+ concentrations can produce 3 or 4 orders of magnitude change in the permeability constant.

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