Pathways of calcium release from heavy sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle

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The active uptake and efflux of Ca^{2+} from suspensions of vesicles from heavy rabbit muscle sarcoplasmic reticulum have been examined using the antipyrylazo III dye method in the presence of various nucleotide triphosphate substrates to support active Ca^{2+} accumulation. On addition of ATP, Ca^{2+} is rapidly accumulated and maintained at high internal concentrations until the substrate for pump protein is exhausted. Ca^{2+}-induced Ca^{2+} release which is inhibited by ruthenium red can be demonstrated. The kinetics of Ca^{2+} release via these channels is different from the Ca^{2+} efflux observed after substrate exhaustion. This rate was found to be dependent on the type of nucleotide triphosphate, decreasing in the order ATP > GTP > CTP > ITP UTP. It is suggested that different conformations of the Ca^{2+} pump protein induced by the different substrates may result in the creation of pathways for the facilitated diffusion of Ca^{2+}.

Ca^{2+} channel; Sarcoplasmic reticulum; Ca^{2+}-ATPase; Membrane transport

1. INTRODUCTION

The physiological mechanism of Ca^{2+} release from sarcoplasmic reticulum to initiate muscle contraction is presently unknown. The membrane of sarcoplasmic reticulum, however, contains one or more types of gated Ca^{2+} channels [1,2] through which the release of Ca^{2+} from the cisternae of the sarcoplasmic reticulum occurs upon the excitation of muscle cells [3]. The characteristics of Ca^{2+} release have been examined in detail in skinned muscle fibers and isolated vesicles from the sarcoplasmic reticulum. Such studies have shown that Ca^{2+} release depends on Ca^{2+} concentration, is potentiated by adenine nucleotides and caffeine and is inhibited by Mg^{2+}, ruthenium red and procaine [4–7].

The channels responsible for Ca^{2+}-induced Ca^{2+} release appear to be localised in the heavy fraction

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of the sarcoplasmic reticulum. The activation of these channels during ATP-dependent Ca^{2+} uptake is the primary factor in reducing Ca^{2+} uptake by fragmented heavy sarcoplasmic reticulum [8,9]. The gating of the channels appears to be biphasic during ATP-dependent Ca^{2+} uptake as judged by the kinetics of Ca^{2+} accumulation [7]. Accordingly, the initial net rate of Ca^{2+} uptake is slow due to the activation of Ca^{2+} channels and subsequently increases as the channels are inactivated when the external Ca^{2+} concentration is reduced to less than about 0.1 μM. The inactivation process is said to involve an ATP-dependent phosphorylation of the proteins of the sarcoplasmic reticulum membranes [10].

It has been suggested that Ca^{2+} may be released from the so-called light fraction of the sarcoplasmic reticulum by the creation of channels formed by the oligomeric association of Ca^{2+}-ATPase molecules [11,12]. The present study was undertaken to determine whether Ca^{2+} release from heavy sarcoplasmic reticulum was mediated exclusively through the Ca^{2+}-gated Ca^{2+} channels.

2. MATERIALS AND METHODS

The heavy fraction of fragmented sarcoplasmic reticulum was prepared from rabbit hind leg skeletal muscle as described elsewhere [7]. The final pellet was resuspended in a solution containing 0.1 M KCl and 5 mM Tris-maleate (pH 7.0) and stored at 0°C for use within a week of preparation.

Ca^{2+} uptake into the vesicles of the sarcoplasmic reticulum or its release from the vesicles was measured using the Ca^{2+}-indicator dye, antipyrylazo III [13]. Extravesicular free Ca^{2+} concentration was monitored continuously by measuring the differences in absorbance of 50 μM antipyrylazo III at 720 and 790 nm using a Hitachi-557 dual-wavelength spectrometer. Calibration was performed under the same conditions as those used for the measurement of Ca^{2+} uptake and release before substrate addition and/or after exhaustion of the substrate. The absorption changes were found to be linear with Ca^{2+} concentrations up to about 60 μM.

The uptake of calcium into a suspension of heavy sarcoplasmic reticulum vesicles (1 mg protein/ml) was initiated by addition of nucleotide triphosphate (0.5 mM) in a reaction mixture consisting of 40–50 μM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1 M KCl, 50 μM antipyrylazo III buffered to pH 7.0 with 50 mM Pipes/Tris. The Ca^{2+} release was initiated by addition of Ca^{2+} (2.5 or 5 μM) or caffeine (5 mM) in the presence of ATP or by addition of ADP (0.1 mM) or AMP (0.25–1 mM) in the presence of nonadenine nucleotide triphosphates. The addition of the same amounts (5–10 μl) of only buffer does not provide any visible effects. The addition of 50–100 μl of the buffer shifts the signal down as a result of dilution, not up as in the case of Ca^{2+} release initiation. Unless specified, an ATP regenerating system of creatine phosphate and creatine kinase was not present.

3. RESULTS

The characteristics of Ca^{2+} uptake into and efflux from a suspension of heavy sarcoplasmic reticulum vesicles are illustrated in fig.1. Addition of ATP to the system without regeneration of the substrate (fig.1a) results in a rapid uptake of Ca^{2+} into the vesicles in a process mediated by Ca^{2+}-ATPase. The amount of Ca^{2+} bound by ATP (and ADP) was determined in the system when net Ca^{2+} accumulation was prevented by the presence of the Ca^{2+} ionophore, A23187 (5 μM). It was found to be 15 nmol and 8.5 nmol, respectively, under conditions used. The same amount of Ca^{2+} was bound by other nucleotide tri- and diphosphates (not shown). After about 4 min all of the substrate is utilized (curve 3) and Ca^{2+} diffuses out of the vesicles until the gradient is abolished; the difference in concentration of Ca^{2+} between the initial and final states is due to the formation of a Ca^{2+}-ADP complex. If another aliquot of

ATP is added a further cycle of Ca^{2+} uptake and release is observed.

Addition of small amounts of Ca^{2+} (2.5 or 5 μM) during the steady-state accumulation phase (curves 1 and 2) provokes a fast release of Ca^{2+} from the vesicles. The amount of Ca^{2+} released by addition of 5 μM Ca^{2+} was 4.3 $\pm$ 1.5 nmol/mg protein irrespective of the concentration of Ca^{2+} accumulated inside the vesicles. A similar release of Ca^{2+} is potentiated by addition of caffeine (5 mM) to the system under the same conditions (not shown). The presence of ruthenium red (0.1–1 μM), a known inhibitor of Ca^{2+} release through the Ca^{2+} channels, was found to prevent Ca^{2+} release induced by either Ca^{2+} or caffeine and extended the steady-state phase of Ca^{2+} accumulation about 3-fold presumably by coupling the rate of ATP hydrolysis to the rate of Ca^{2+} release from the vesicles [14]. This seems to indicate that some part of the Ca^{2+} channels is open during the steady-state phase under the conditions used. It can be seen that when all the ATP is consumed by the Ca^{2+}-ATPase pump protein the characteristics of Ca^{2+} efflux from the vesicles are identical to preparations incubated in the absence of ruthenium red whether or not Ca^{2+} channels have been activated during the steady-state phase. When using an ATP regenerating system (creatine phosphate plus creatine kinase, fig.1b), the steady-state phase of Ca^{2+} accumulation is greatly extended and the amount of Ca^{2+} released by addition of 5 μM Ca^{2+} during the steady-state phase increases about 4–5-fold to 21.2 $\pm$ 4.1 nmol Ca^{2+}/mg protein. The same amount of Ca^{2+} is released by addition of 5 mM caffeine (not shown).

The rate of Ca^{2+} uptake decreases in the presence of the ATP regenerating system (fig.1b). There are at least two explanations for this phenomenon. First of all, the possible competition between creatine phosphate (5 mM) and ATP (0.5 mM) for the nucleotide-binding site of Ca^{2+}-ATPase can slow down the rate of Ca^{2+} accumulation. On the other hand the regenerating system maintains the ATP concentration at a relatively high and constant level (about 0.5 mM) during Ca^{2+} uptake. In the absence of the regenerating system, the ATP concentration decreases very quickly. It was shown that ATP activates the Ca^{2+} release channels and positive cooperativity for ATP was found [5,7]. So the fast
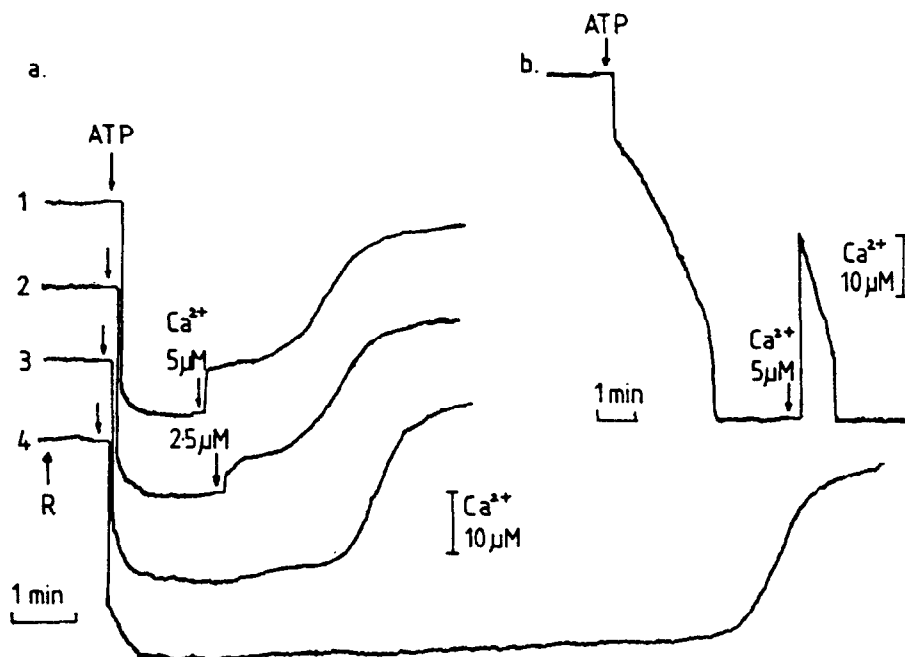
Fig.1. Characteristics of Ca^{2+} uptake mediated by ATP in the absence (a) and presence (b) of an ATP regenerating system consisting of 5 mM creatine phosphate and 10 IU creatine kinase. ATP (to a final concentration of 0.5 mM) was added at the arrows to initiate Ca^{2+} uptake. Ruthenium red (R) was added in a concentration of 40 nM.

decrease of the ATP concentration in the absence of the regenerating system provides the fast inactivation of the Ca^{2+} channels, whereas in the presence of the regenerating system ATP keeps these channels in the open state longer.

To investigate further the mode of Ca^{2+} efflux via Ca^{2+}-gated Ca^{2+} channels and the kinetics of Ca^{2+} efflux via channels not inhibited by ruthenium red, different nucleotide triphosphate substrates were employed to drive the Ca^{2+} pump [15,16]. Fig.2 shows that the rate of Ca^{2+} uptake in the presence of CTP (fig.2a) and ITP (fig.2b) into heavy sarcoplasmic reticulum vesicles is similar to that when ATP is the substrate. It can be seen, however, that the rate of passive efflux of Ca^{2+} when the substrate is exhausted is much slower than when ATP is the substrate. A summary of the efflux data for these and other nucleotide triphosphate substrates are presented in table 1 which shows considerable variation from one substrate to another. Furthermore, Ca^{2+}-induced Ca^{2+} release from vesicles accumulating Ca^{2+} with substrates of CTP or ITP (also GTP and UTP, not shown) is not observed which is consistent with

earlier reports that nucleotide triphosphates other than ATP do not convert Ca^{2+} channels from an inactive to an active state [17]. Nevertheless, addition of ADP (or AMP) in concentrations up to 1 mM are able to activate Ca^{2+} channels and potentiate Ca^{2+} release from the vesicles. The precise amount of Ca^{2+} released on addition of ADP is difficult to calculate because of the relatively high proportion of Ca^{2+} released.

Additional experiments were performed to examine adenosine nucleotide phosphate stimulated release of Ca^{2+} from vesicles accumulating calcium in the presence of GTP (figs 3 and 4). Preliminary experiments showed that the amount of calcium released on addition of 0.1 mM ADP was the same irrespective of whether it is added during the steady-state accumulation of Ca^{2+} or during the passive efflux stage when the GTP substrate is limiting (not shown). The amount of Ca^{2+} released on addition of AMP to vesicles accumulating Ca^{2+} under steady-state conditions was found to be directly related to the concentration of AMP over the range 0.25–1.0 mM (fig.3). Thus, the amount increased from 5.5 to 25.9 nmol Ca^{2+}/mg protein
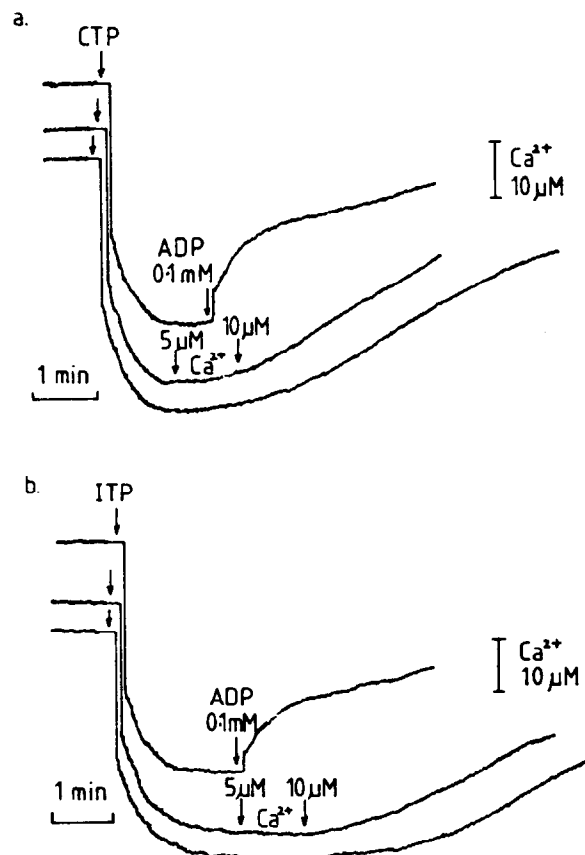
Fig.2. Time-course of uptake and efflux of Ca^{2+} mediated by (a) 0.5 mM CTP and (b) 0.5 mM ITP added to start the process (arrows). Where indicated ADP (0.1 mM) and Ca^{2+} $CaCl_2$ were added.

over this range of AMP concentrations. The effect of blocking by ruthenium red on the AMP-stimulated Ca^{2+} release in heavy sarcoplasmic

Table I

The rate of spontaneous Ca^{2+} release from heavy sarcoplasmic reticulum vesicles after substrate exhaustion in the presence of different nucleotide triphosphate substrates

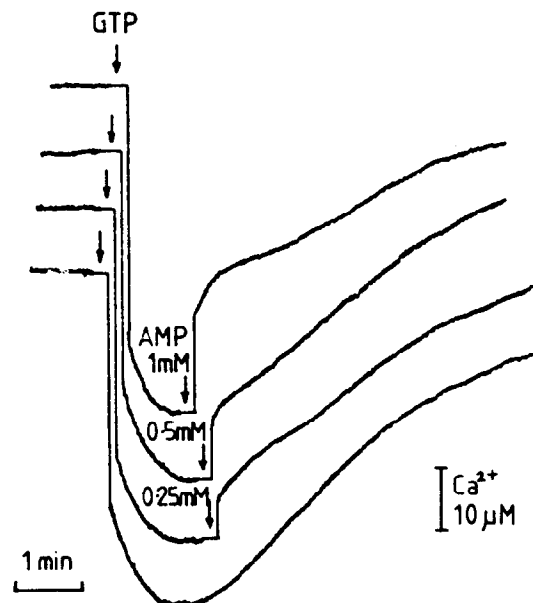| Nucleotide triphosphate substrate	Rate of Ca^{2+} release	
	nmol Ca^{2+}/mg protein in min	%
ATP	18.1	100
GTP	10.3	57
CTP	9.1	50
ITP	7.7	43
UTP	3.8	21

Fig.3. The effect of AMP concentration on Ca^{2+} release from heavy sarcoplasmic reticulum vesicles accumulating Ca^{2+} in the presence of GTP (0.5 mM) added at the start of the reaction (arrows). The concentration of AMP is indicated.
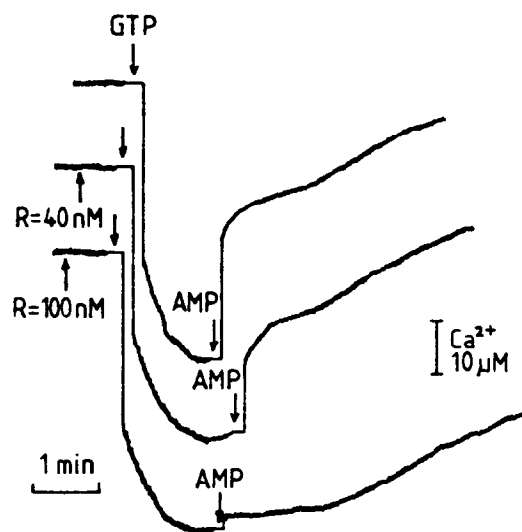

Fig.4. The effect of ruthenium red (R) on AMP-stimulated release of Ca^{2+} from heavy sarcoplasmic reticulum vesicles accumulating Ca^{2+} in the presence of GTP (0.5 mM) added at the start of the reaction (arrows). AMP (1 mM) was added to the reaction at the time-points indicated.

reticulum vesicles accumulating Ca^{2+} with GTP substrate (fig.4) shows that the Ca^{2+} channels activated by AMP are identical to those activated by Ca^{2+} and caffeine in the ATP-driven system. In the latter case addition of AMP (or ADP) does not cause stimulated Ca^{2+} release presumably because the channels are already in an activated state and may even provide access for the passive release of calcium.

4. DISCUSSION

The present results suggest the existence of at least two pathways of Ca^{2+} release from heavy sarcoplasmic reticulum, one via Ca^{2+} activated channels and another passive release process that is distinct from these Ca^{2+} channels. The rate of release through this process is dependent on the nucleotide triphosphate substrate used to drive the active transport of Ca^{2+} into the vesicles and is not sensitive to the ruthenium red. We have shown previously [18] that the conformational state of the Ca^{2+} pump protein is modulated by ATP concentrations greater than 0.1 mM: a range of other nucleotide triphosphates did not possess this ability even when present in considerably higher concentrations. In view of the effect of ATP and other nucleotide triphosphates on the rates of passive Ca^{2+} efflux (table 1) it could be argued that the pathway of Ca^{2+} release involves the particular configuration of the Ca^{2+} pump protein. Earlier studies of light sarcoplasmic reticulum membranes [11,12] led to the conclusion that the pump protein may form oligomeric complexes through lateral association in the membrane and create channels through which Ca^{2+} may leak out of the vesicles. The data about the relatively slow Ca^{2+} release from light sarcoplasmic reticulum vesicles were published recently [19]. Moreover, it was shown that the Ca^{2+}-ATPase protein itself provides Ca^{2+} efflux from the reconstituted vesicles [20]. Our preliminary experiments with light sarcoplasmic reticulum vesicles (Rubtsov, A.M.and Lushchak, V.I., unpublished) show that the passive Ca^{2+} release takes place in this system after the substrates are exhausted. Moreover, the rate of the

Ca^{2+} release from the light sarcoplasmic reticulum vesicles has the very same dependence on the nucleotide triphosphate substrate used as those from the heavy sarcoplasmic reticulum vesicles. Therefore, we suggest that different conformations of the Ca^{2+}-ATPase protein induced by the different substrates may result in the creation of pathways for the Ca^{2+} release both from the light and heavy sarcoplasmic reticulum vesicles.

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