Possible Effects of Membrane Polarization on Calcium Uptake by and Release from Sarcoplasmic Reticulum Vesicles

H. JDAÏAA, G. BRÛLÉ, F. FOURNIER and P. GUILBAULT

Laboratoire de Physiologie Cellulaire, Université des Sciences et Techniques de Lille Flandres-Artois, 59655 Villeneuve d'Ascq Cedex, France

Abstract. Rates of calcium uptake by and calcium release from sarcoplasmic reticulum vesicles isolated from skeletal muscle of the crab seem to depend on membrane potential generated by potassium (K) and chloride (Cl) gradients. This does not appear to be due to an effect of the ions themselves since media of different ionic compositions leading to the same membrane potential, also lead to the same ATP hydrolysis and the same Ca uptake by SR vesicles. From a large positive intravesicular potential (conditions termed "normal" in this paper), membrane depolarization of passively Ca loaded vesicles, produced by changes in K and Cl concentrations in the media, resulted in: i) decrease in rate of calcium uptake; ii) decrease in calcium loading; iii) increase in rate of calcium release despite a decrease in the driving force for calcium ions. Moreover, the addition of caffeine (5 mmol/l) to the different polarization media resulted in a increase in calcium release.

Key words: Calcium uptake — Calcium release — Membrane polarization — Isolated sarcoplasmic reticulum vesicles — Crab

Introduction

Calcium concentration in skeletal muscle cytoplasm is largely regulated by the calcium transport system of the sarcoplasmic reticulum (SR). Translocation of calcium across the SR membrane is coupled with ATP hydrolysis. This transport is assumed to generate a positive inside membrane potential. Moreover, it has been suggested that the membrane potential across the SR may play a role in the regulation of calcium uptake and calcium release (Martonosi 1984; Navarro and Essig 1984; Antoniu et al. 1985; Jona and Martonosi 1986): in particular, Smith et al. (1985) described voltage dependence and selectivity of

the nucleotide stimulated SR channel in rabbit skeletal muscle; this Ca channel presents a 125 pS conductance.

Different authors studying different preparations have explained the excitation-contraction coupling either by calcium induced calcium release and/or by depolarization induced calcium release. Using SR vesicles isolated from crab leg muscle fibres and by creating membrane potentials of different values generated by potassium (K) and chloride (Cl) gradients at a constant [K].[Cl] product, we could observe similar changes in calcium release and uptake as those reported by other authors (Martonosi 1984; Jona and Martonosi 1986; Antoniu et al. 1985; Navarro and Essig 1984). The changes in membrane potential were induced by modifying the concentrations of the different ionic species in the media. However, we shall show first that the extravesicular media of different ionic composition have no specific effect if they produce identical polarization. Indeed, it is well known that Ca uptake by Ca ATPase may, or may not, be influenced by extravesicular K concentration (see Martonosi 1984; Schuurmans Stekhoven and Bonting 1981). Under these conditions, we have shown that depolarization of isolated vesicle membranes from a large positive inside membrane potential reduced Ca loading capacity and increased Ca release which was further enhanced in the presence of caffeine.

Our results prompted us to postulate the possible existence of voltage dependent calcium channels in fragmented sarcoplasmic reticulum of the crab muscle fibre including several types of vesicles prepared from junctional and free sarcoplasmic reticulum. This hypothesis concerning voltage dependent calcium channels has been further supported by more recent reports (Kranias et al. 1985; Smith et al. 1985). Smith et al. (1985) described a voltage dependent calcium channel in SR membrane with an elementary conductance of 125 pS. Moreover, by incorporating several of these calcium channels into a phospholipids bilayer, these authors were able to show that the potential dependent channels open upon depolarization of the same type as that induced in our experiments with isolated vesicles. The Ca channels described by Smith et al. (1985) were stimulated by ATP and their opening time has been reported to increase with depolarization.

Materials and Methods

Preparation of sarcoplasmic reticulum vesicles

Crab muscle reticulum vesicles were prepared according to the procedure described by several authors (Carvalho and Léo 1967; Martonosi et al. 1968; Duggan and Martonosi 1970; Huddart et al. 1974). In brief, crab skeletal muscles were thoroughly washed in saline solution (100 mmol/l KCl, pH : 6.8) at 0 °C and homogenized in medium C or medium D containing Ca^{2+} ions (10 mmol/l) and no ATP, pH : 6.8 (see Table 1 for ionic composition of media C and D). The homogenate was

centrifuged at 1600 × *g* (3 °C) for 15 min. The supernatant was centrifuged at 11,000 × *g* for 15 min, and the resulting supernatant at 80,000 × *g* for 40 min. The final sediment containing different passively Ca loaded vesicles (heavy and light SR vesicles) was resuspended in 4 ml of medium A, C or D, pH : 6.8; the compositions of the media are listed in Table 1. Protein concentration was determined by the method of Lowry et al. (1951). In our samples, protein concentration ranged from 0.010 to 0.100 mg/ml.

The preparation employed corresponds to light and heavy sarcoplasmic reticulum vesicles. The SR membrane is composed of two morphologically distinct regions, longitudinal Sr and terminal cisternae; the terminal cisternae are partially junctionally associated with the transverse tubules. The purity of the isolated SR was examined by transmission electron microscopy. Mitochondrial contamination was very small: Na azide (5×10^{-4} mol/l) did not decrease the ATPase activity, similarly the sarcolemmal membrane contamination was also very small since the ATPase activity was not modified in the presence of ouabaine. The vesicles employed thus corresponded to the SR membrane fraction.

Generation of membrane potential

Vesicles suspended in medium A, C or D were diluted in the respective polarization media (see Table 1).

With *intravesicular medium C*, the equilibrium potential of the K^+ ion (E_K), identical with the equilibrium potential of the Cl^- ion, was 80 mV when diluted in medium A; $E_K = E_{Cl} = 40$ mV when diluted in medium B; $E_K = E_{Cl} = 0$ mV when diluted in medium C; and $E_K = E_{Cl} = 80$ mV when diluted in medium D.

With *intravesicular medium D*, $E_K = E_{Cl} = 0$ mV when diluted in medium D.

Potentials generated in this way were either zero or inside positive. When K and Cl concentration gradients were established, the membrane potential was inferior to E_K and E_{Cl} as the proton equilibrium potential (E_H) was 0 mV (intra and extravesicular pH = 6.8) and Ca^{2+} (E_{Ca}) was negative; the weaker the permeability Ca^{2+} ions and to protons was, the nearer the potential came to E_K and E_{Cl}.

Measurement of ATP hydrolysis

ATP hydrolysis was measured spectrophotometrically using a modification of the method of Fiske and Subbarow (1925) to allow measurements of small concentrations of P_i produced by ATP splitting (Brûlé et al. 1983).

Measurements of calcium uptake and release

Calcium uptake and release from SR were also measured spectrophotometrically using arsenazo III as an indicator of extravesicular calcium (Scarpa et al. 1978). The use of both arsenazo III and standard controls to demonstrate its specificity for calcium in SR experiments has been reported previously (Scarpa 1979). Changes in arsenazo absorption were measured at 660 nm. Sarcoplasmic reticulum vesicles were suspended in a 2 ml-cuvette containing polarization medium with arsenazo (100 μmol/l) at 18 °C. ATP and Mg^{2+} ions were added to initiate active calcium uptake. Once the net transport ceased (after $\simeq$ 30 min) the addition of vanadate (100 μmol/l) allowed inhibiton of Ca ATPase, with the half-inhibitory concentration of approximately 10 μmol/l vanadate (O'Neal et al. 1979).

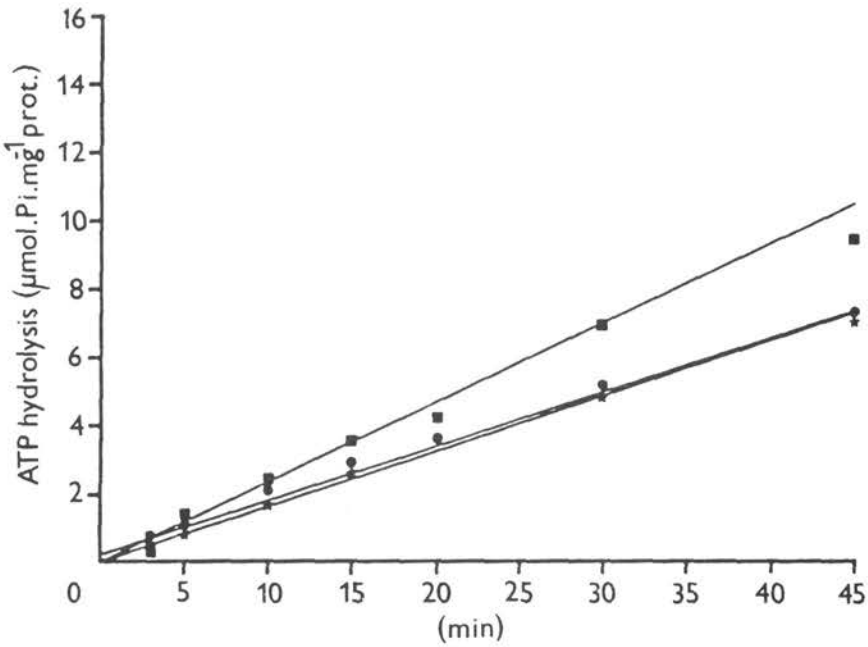

Fig. 1. ATP hydrolysis measured as a function of time for vesicles initially incubated for 20 min in medium A corresponding to membrane polarization of 80 mV (positive inside potential). After this time, steady state of Ca loading was obtained and the vesicles divided into 3 aliquots were incubated to measure ATP hydrolysis in medium A, B or C producing membrane polarization of about 80, 40 and 0 mV respectively. Vesicles were initially homogenized in medium C containing Ca (10 mmol/l) and no ATP.

Presentation of results

Each experimental series comprised 6 experiments, and a particular SR vesicle preparation was employed for each series. The individual series differed in SR protein concentrations (0,01 to 0,1 mg/ml). In these conditions, the absolute values of Ca release or uptake, determined from Ca concentration, are modified by the protein concentration. Actually, most of Ca initially contained in vesicles (for the medium used to prepare SR vesicles: see Table 1) was immediately released via the "Ca induced Ca release" mechanism (see Results, Effects of membrane polarization); thus the extravesicular Ca activity was modified and consequently also the electrochemical gradient for Ca^{2+} ions. Owing to this, the results could not be expressed as mean $\pm$ SE (standard error of the mean). However, results of the individual experimental series were similar as for the relative values.

Results

A: ATP hydrolysis after Ca loading of vesicles

As illustrated in Figs. 1 and 2, ATP hydrolysis expressed (in terms of μmol

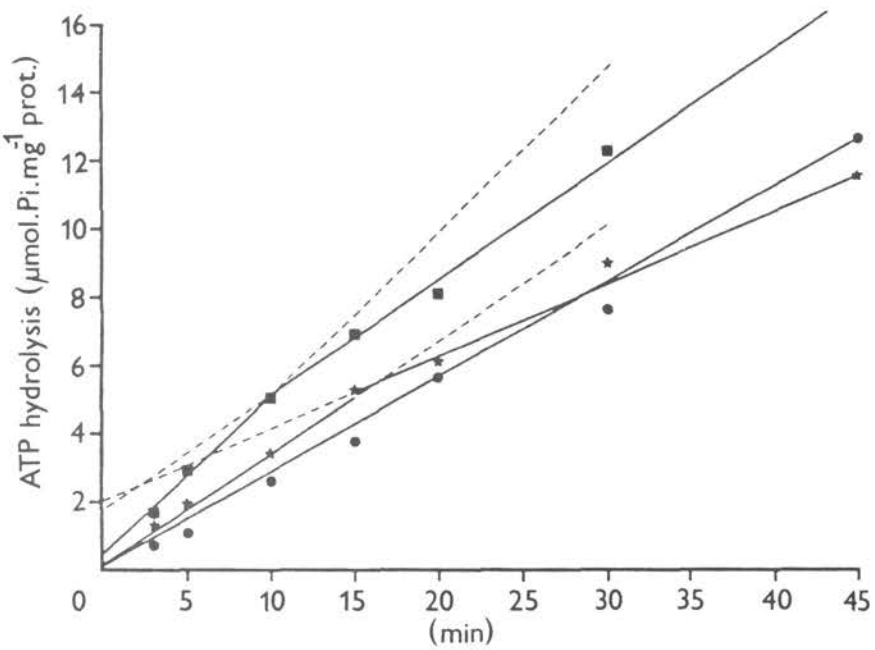

Fig. 2. ATP hydrolysis measured as a function of time for vesicles initially incubated for 20 min in medium C corresponding to membrane polarization close to 0 mV. After this time steady state of Ca loading was obtained and the vesicles divided into 3 aliquots were incubated to measure ATP hydrolysis in medium A, B or C producing membrane polarization of about 80, 40 and 0 mV respectively. Vesicles were initially homogenized in medium C containing Ca (10 mmol/l) and no ATP. Polarization of the SR membrane resulted in an increase in the ATP hydrolysis rate during the first minute after the replacement of medium C by A or B. Extrapolated value of ATP hydrolysis at the beginning of experiment (media A and B) gives a value of 2 μmol . mg^{-1} prot.

P_i . mg^{-1} prot.) in function of time is dependent on membrane polarization and/or ionic composition of the extravesicular medium. In one experimental series, the vesicles were first homogenized, and thus passively Ca loaded, in modified medium C (i.e. medium C supplemented with additional 10 mmol/l Ca and containing no ATP). They were then preincubated in medium A for 20 min in order to reach steady state active Ca loading. Subsequently the vesicles were diluted in different media, A, B or C, and ATP hydrolysis was found to vary linearly over time (Fig. 1); the slope of this linear relationship corresponded to the rate of ATP hydrolysis. On the other hand, as shown in Fig. 2, when vesicles were first homogenized in the same modified medium C and then preincubated in medium C, again for 20 min, the ATP hydrolysis at the time of incubation in polarization media varied linearly only when the vesicles were resuspended in

Table 1. Composition of media in mmol/l. pH adjusted with tris maleate buffer to 6.8

	A	B	C	D
KCH_3COO	107	24.5	5	—
KCH_3CH_2COO	—	—	—	107
KCl	5	—	—	5
Tris HCl	—	24.8	107	—
ATP Na_2	4	4	4	4
Mg $(CH_3COO)_2$	4	4	4	4
Sucrose	—	126	—	—

polarization medium C (see Table 1; polarization close to 0 mV). When vesicles were diluted in medium A or B, producing positive inside membrane potentials of close to 80 and 40 mV respectively, the ATP hydrolysis rate (Fig. 2) was not constant over time. Moreover, as also shown in Fig. 2, once the steady state has been reached, the extrapolated value of ATP hydrolysis at time $t = 0$ was about 2 μmol . P_i . mg^{-1} protein. This value suggests some extra Ca uptake, within the first minutes, by vesicles in medium A or B compared to those in medium C. These results, therefore, seem to show that membrane potential can regulate maximal Ca loading of SR. This is in agreement with reports by Jona and Martonosi (1986) and Navarro and Essig (1984) in so far as, in our experiments, the ions in the different media of varying composition had no direct effect on ATP hydrolysis, thus allowing membrane potential to be generated.

B: Calcium uptake by vesicles

Calcium uptake by SR vesicles was analysed by measuring (with arsenazo III) the decrease in Ca concentration in the media containing isolated vesicles. In our experimental conditions the extravesicular medium corresponded to the intracellular medium of the muscle fibres. In this work, extravesicular Ca concentration is denoted $(Ca^{2+})_i$. Prior to investigating the effects of variation in membrane potential on Ca uptake, it was first checked whether media of different ionic composition yet generating the same polarization, did not modify Ca uptake by SR.

1. Effects of different extravesicular media generating identical membrane potentials

For this analysis of Ca uptake, vesicles corresponding to 0.008 mg of protein were suspended in 1 ml volume of the testing media.

Vesicles were first homogenized in medium C with Ca^{2+} ions (10 mmol/l) and no ATP, and were thus passively Ca loaded. They were then incubated for

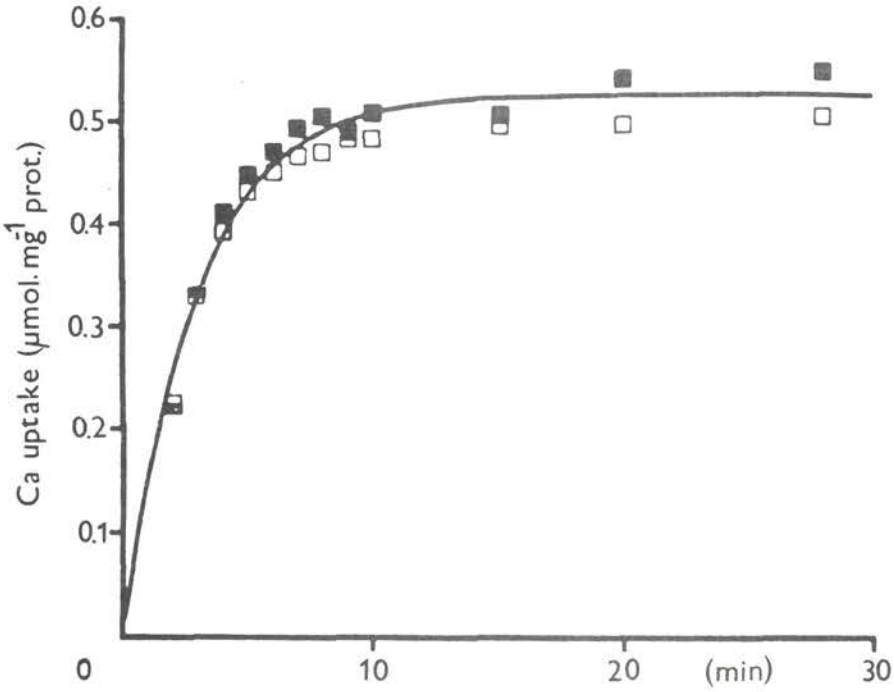

Fig. 3. Calcium uptake by SR vesicles dependent on ATP hydrolysis was monitored by measuring extravesicular calcium $(Ca^{2+})_i$, via absorption changes of arsenazo III at 660 nm. Two aliquots of SR vesicles with the same concentration (0.008 mg . ml^{-1}) were suspended in two media, producing the same membrane polarization of SR vesicles close to 80 mV and containing CH_3COO^- anions (■) (medium A) or $CH_3CH_2COO^-$ anions (□) (medium D); see Table 1. Vesicles were initially homogenized in medium C containing Ca (10 mmol/l) and no ATP.

only 30 s in medium C, again with no ATP. Two aliquots, each corresponding to 0.008 mg/ml protein were diluted in either medium D or medium A. For each of these two media the membrane potential generated, at least at the beginning of the experiment, was close to E_K and E_{Cl}, i.e. around 80 mV (positive inside membrane potential). Thus, upon resuspension in polarization medium vesicles which had been previously calcium loaded (during homogenization; see Materials and Methods), immediately released a significant proportion of their calcium via the well-known Ca induced Ca release mechanism. This mechanism is likely inactivated and is no more operative thereafter (Fabiato 1985). Indeed, it is well known that the greater the calcium concentration in the vesicles $(Ca^{2+})_e$ is, the less need there is for the extravesicular concentrations $(Ca^{2+})_i$ to be high in order to induce Ca release from SR (Endo et al. 1970; Ford and Podolsky 1970). Traces of Ca present in the extravesicular media (no EGTA can be used

in our experiments) are sufficient to trigger the Ca induced Ca release mechanism. This significant calcium release in the presence of ATP subsequently led to calcium uptake by SR (Fig. 3). The slow Ca uptake may have resulted from the inhibition, complete or not, of the Ca induced Ca release mechanism (Fabiato 1983, 1985) and from incomplete inactivation of Ca-ATPase activity, in spite of the high extravesicular Ca concentration $(Ca^{2+})_i$ at the outset of our experiment. In effect, it has been shown that for $(Ca^{2+})_i$ ranging from 0.1 to 1 μmol/l, calcium half maximally stimulates the ATPase activity whereas concentrations above 5—10 μmol/l are inhibitory (Inesi 1971; Inesi and al. 1967; Schuurmans Stekhoven and Bonting 1981).

As soon as ATP was introduced to medium A or D (Fig. 3) at a strong polarization ($E_K = E_{Cl} = 80$ mV) Ca uptake appeared and the rate of uptake decreased over time. Obviously the presence of $CH_3CH_2COO^-$ ions instead of CH_3COO^- ions changed neither Ca uptake nor the maximal calcium loading; moreover, vesicles previously homogenized in medium containing 10 mmol/l of Ca and either Tris Cl, medium C, or CH_3CH_2COOK, medium D, and incubated in the same media with ATP showed the same rate of Ca uptake as well as the same maximal loading value (not shown); in both cases the membrane potential was near zero.

The increase in ATP hydrolysis observed upon positive intravesicular membrane potential (Fig. 2) thus probably reflect an actual increase in Ca uptake which is dependent on the membrane potential: the latter is suggested by the fact that an identical loading of the vesicles can be brought about for the same initial membrane potential by replacing both K by Tris and CH_3COO^- by $CH_3CH_2COO^-$ or by Cl^-. Shuurmans Stekhoven and Bonting (1981) point out that certain monovalent cations have been reported to have no effect on ATPase, inhibitory or activatory depending on the type of membrane, the tightness and on protein-lipid interaction (The and Hasselbach 1972).

2. Effects of membrane polarization

Since the ionic species do not appear to be involved directly in Ca uptake, the potential dependent uptake was analysed on 3 lots of vesicles from the same homogenate, each correspondig to 0.02 mg of suspended protein per ml of polarization medium; A ($E_K = E_{Cl} = 80$ mV), B ($E_K = E_{Cl} = 40$ mV) or C ($E_K = E_{Cl} = 0$ mV). However, before separating the vesicles into the 3 lots for suspension in media A, B and C, they were put in medium A (for 30 s), with an initially low calcium concentration $(Ca^{2+})_i = 1—3$ μmol/l. After 30 s in medium A, $(Ca^{2+})_i$ still reached 200 μmol/l. When the previously passively calcium loaded vesicles (see Materials and Methods) were suspended in medium A, they immediately released a significant proportion of their Ca via the Ca induced Ca

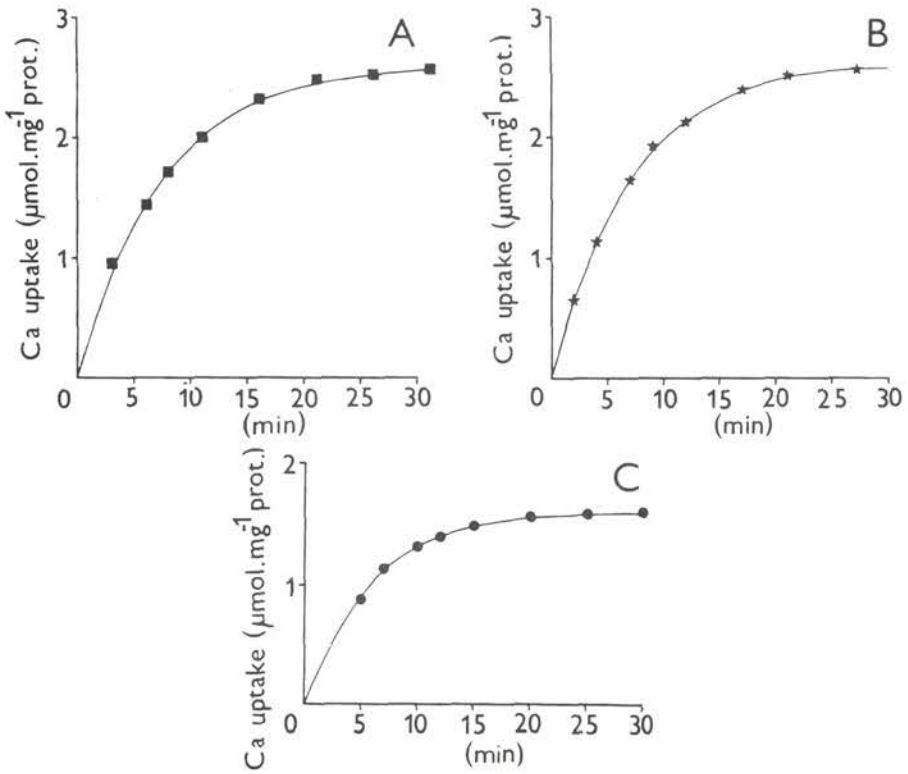

Fig. 4. Calcium uptake by SR dependent on ATP hydrolysis was monitored by measuring extravesicular calcium $(Ca^{2+})_i$ via absorption changes of arsenazo III at 660 nm. SR (0.02 mg . ml^{-1}) was suspended in the 3 tested media at 20 °C (*A*: $E_K = E_{Cl} = 80$ mV, ■; *B*: $E_K = E_{Cl} = 40$ mV, ★; *C*: $E_K = E_{Cl} = 0$ mV, ●; for more details, see Table 1). Vesicles were initially homogenized in medium C containing Ca (10 mmol/l) and no ATP. The rate of calcium uptake decreased exponentially with time. The time constant of loading seems the same for *A*, *B* and *C*, but the maximal loading capacity was modified by membrane potential. Parameters of equation giving absorption changes as a function of $(Ca^{2+})_i$ were determined for each medium.

release mechanism. Fig. 4 gives the results obtained from the 3 lots of vesicles. Curve A, which corresponds to polarization medium A, shows that the rate of Ca uptake decreased exponentially with time. The time dependence of Ca uptake (expressed in μmol Ca . mg^{-1} prot. per min) is given by $y = \bar{y}(1 - e^{-t \div \zeta})$, where y is the maximum loading value, ζ is the loading time constant. The initial rate of Ca uptake calculated by this equation is 0.35 μmol Ca . mg^{-1} prot. min^{-1}. If for one molecule of ATP hydrolysed two Ca ions are transferred across the SR membrane, an ATP hydrolysis rate of 0.17 μmol . P_i . mg^{-1} prot. min^{-1} is obtained. This value is very low compared to that given by Makinose (1969) in

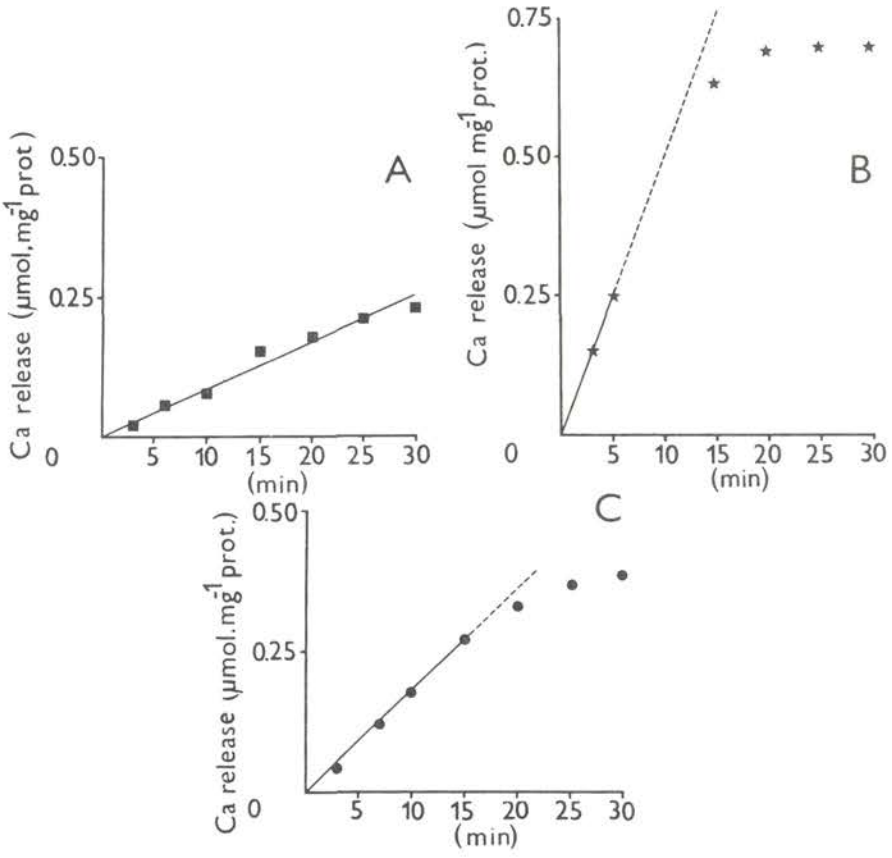

Fig. 5. Analysis of calcium release from loaded vesicles as a function of time, the same samples as employed to measure calcium uptake (Fig. 4). Once the maximal value of calcium uptake had been reached, 1 μl of a large concentration of vanadate (final concentration 100 μmol/l) was added. The initial rate of calcium release was dependent on membrane potential: 0.008 μmol . mg^{-1} prot. min^{-1} for a large inside positive membrane potential (medium A: $E_K = E_{Cl} = 80$ mV (■)); 0.05 μmol . mg^{-1} prot. min^{-1} for medium B: ($E_K = E_{Cl} = 40$ mV (★)); 0.017 μmol . mg^{-1} prot. min^{-1} for medium C: ($E_K = E_{Cl} = 0$ mV (●)). Parameters of equation giving absorption changes as a function of $(Ca^{2+})_i$ were determined for each medium.

conditions of maximum stimulation (8 μmol . mg^{-1} prot. min^{-1} at pH 6.8). Curve B (Fig. 4) corresponding to polarization medium B ($E_K = E_{Cl} = 40$ mV) shows that, compared with polarization medium A, neither the initial rate of calcium uptake nor the maximal value of loading capacity were modified. In contrast, changes were observed for medium C ($E_K = E_{Cl} = 0$ mV, curve C, Fig. 4). In medium C the maximal capacity of calcium uptake (1.60 μmol . mg^{-1}

prot.) was significantly reduced: for media A and B it was around 2.6 μmol . mg^{-1} prot. Moreover, the initial rate of calcium uptake for medium C seems also reduced (0.27 μmol . mg^{-1} prot. min^{-1}; mean value 0,35 μmol . . mg^{-1} . min^{-1} for media A and B).

C: Calcium release

Calcium release from SR was investigated using the same isolated vesicles employed for the study of calcium uptake (see precedent paragraph and Fig. 4). Thus, once the steady state of calcium uptake was reached, calcium release from the vesicles was studied after the inhibition of calcium ATPase by adding a small volume of concentrated vanadate solution to each of the 3 media (A, B, C). One μl of a 10^{-1} mol/l vanadate solution was diluted in 1 ml of the respective medium, to which vesicles were added. The final concentration (100 μmol/l) was large enough to completely inhibit the calcium ATPase (O'Neal et al. 1979).

Figure 5 shows the time dependence of calcium release (μmol. calcium . mg^{-1} protein) under 3 membrane polarizations (corresponding to media A: $E_K = E_{Cl} = 80$ mV; B: $E_K = E_{Cl} = 40$ mV and C: $E_K = E_{Cl} = 0$). In medium A (curve A) Ca release showed linear time dependence within 30 min. This suggests that the relatively strong driving force for Ca^{2+} ions (E_K and E_{Cl} act opposite to E_{Ca}) probably did not change very much; this may also point to a low permeability to Ca^{2+} ions. The slope of the linear relationship corresponding to the rate of calcium release is about 0.008 μmol . mg^{-1} prot. min^{-1}. This value is very small compared with that of calcium uptake (0.35 μmol . mg^{-1}).

Under conditions of reduced driving-force for calcium ions (curve B, vesicles in medium B), calcium release was no longer linear throughout the experiment (30 min). Calcium release was linear only for about the first 5 min, with a release rate of 0.05 μmol . mg^{-1} prot. min^{-1}. The large release at the beginning of the experiment probably results in a significant decrease in driving force for calcium ions and consequently in a decrease in the rate of calcium release although Ca^{2+} conductance is maintained. However the enhanced initial rate of calcium release compared with that in medium A, seems to indicate that depolarization of the SR membrane produced an increase in calcium membrane conductance; this is supported by the fact that the maximal Ca uptake by vesicles was the same as for medium A (see Fig. 4) and, on the other hand, the driving force for Ca^{2+} ions was considerably decreased. With a lower polarization, membrane conductance to Ca^{2+} ions also seemed to be significant (vesicles in medium C, $E_K = E_{Cl} = 0$ mV) because under these conditions, the Ca release rate was still relatively high (0.017 μmol . Ca . mg^{-1} prot. min^{-1}; see curve C, Fig. 5) despite a smaller Ca uptake for vesicles in medium C (see curve C, Fig. 4) and a weak driving force for Ca^{2+} ions.

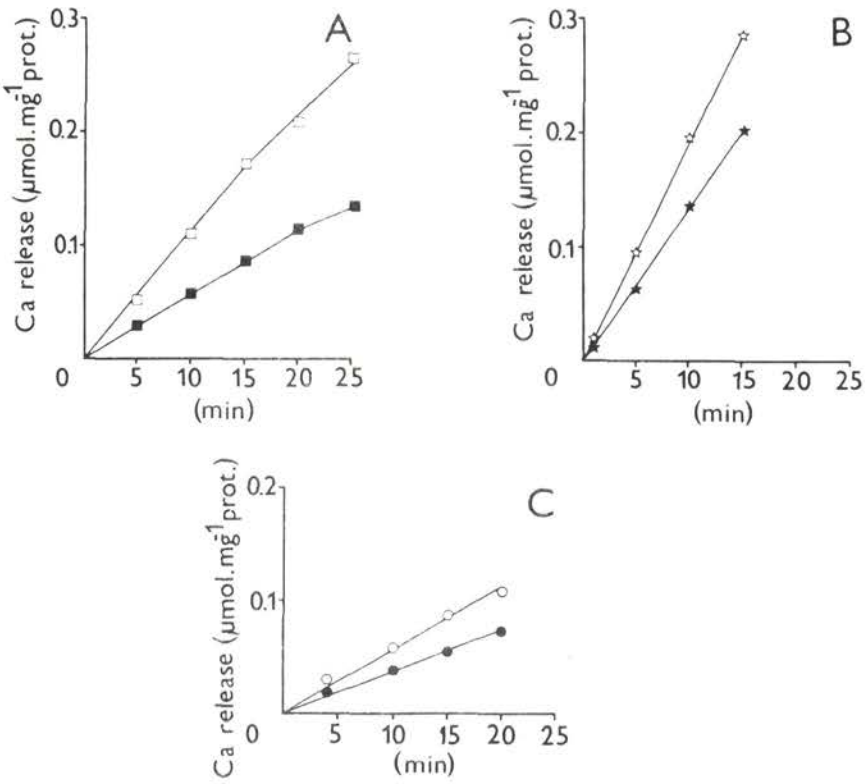

Fig. 6. Effect of caffeine on polarization induced calcium release. SR vesicles were previously calcium loaded in the presence of 4 mmol/l ATP. As before, calcium release was measured after the addition of vanadate (100 μmol/l) in absence (■, ★, ●) and in the presence of caffeine (5 mmol/l) (□, ☆, ○). The caffeine induced increase in calcium release was dependent on membrane potential. Parameters of equation giving absorption changes as a function of $(Ca^{2+})_i$ were determined for each polarization medium (A, B, C). Vesicles were initially homogenized in medium C containing Ca (10 mmol/l) and no ATP.

To summarize, in isolated SR vesicles from crab skeletal fibers, membrane potential seems to play a role in both Ca release and uptake. As far as release is concerned, and in agreement with recent data of Smith et al. (1985), membrane depolarization appears to increase membrane conductance to Ca^{2+} ions (see Smith et al. 1985, Fig. 3).

D: Caffeine induced calcium release

The effect of caffeine addition (5 mmol/l) on Ca release after maximal Ca loading was studied, under conditions of SR polarization using media A, B and

C, in presence of vanadate. This set of experiments, does not allow comparing rates of Ca release from SR in absence and in the presence of caffeine between the three polarization media used. The effect of each polarization medium in the presence or in absence of caffeine was studied on a special vesicle preparation because, for technical reasons, Ca could not be dosed simultaneously to 6 different vesicle suspensions.

In a strong polarization medium (medium A; $E_K = E_{Cl} = 80\,mV$) caffeine considerably increased the rate of Ca release from SR; the release rate in fact doubled under these conditions (see curves A, Fig. 6). On the other hand, in a weaker polarization medium (medium B; $E_K = E_{Cl} = 40\,mV$) there was only a 33 % increase. Caffeine had an even weaker effect upon a greater depolarization of SR membrane (medium C: $E_K = E_{Cl} = 0\,mV$) with an only 30 % increase (curve C, Fig. 6).

Discussion

Our results concerning the effects of membrane polarization on Ca uptake by and release from SR vesicles makes us to hypotheses that, at least in these isolated vesicles, the depolarization induced Ca release mechanism does actually exist; this does not imply the rejection of the Ca induced Ca release mechanism which, according to our results, appears to become inoperative both because of excessively high extravesicular Ca concentration and because of its inactivation (see Fabiato 1983, 1985). This calcium induced calcium release mechanism was operative at the beginning of experiments when isolated vesicles were prepared, filled with intravesicular calcium rich solution (10 mmol/l), thus being passively loaded, and suspended in the different polarization media. At the beginning of experiments, i.e. just before the addition of vesicle suspension, the media contained a small quantity of calcium $(Ca^{2+})_i = 1—3\,\mu mol/l$. Immediately after the addition of vesicle suspension, $(Ca^{2+})_i$ markedly increased since after 30 s, its concentration reached 200 μmol/l. Subsequently, $(Ca^{2+})_i$ started decreasing (Fig. 4) probably due to inactivation of the calcium induced calcium release mechanism (see Fig. 2 of Fabiato 1983; Fabiato 1985). This rapid Ca release may correspond to that occurring upon switching the medium as reported by different authors regardless of whether this leads to membrane depolarization or not (see Martonosi 1984; Nagasaki and Kasi 1983).

Moreover, our results for calcium uptake as summarized by curves in Fig. 4, seem to demonstrate that the maximal loading capacity varies with membrane potential: for media A and B ($E_K = E_{Cl} = 80$ or 40 mV respectively), it was on average 2.6 $\mu mol \cdot mg^{-1}$ protein whereas for medium C ($E_K = E_{Cl} = 0\,mV$) it was 1.6 $\mu mol \cdot mg^{-1}$ protein only. These two very different values indicate that SR membrane potential may play a role in calcium ATPase activity. Figs. 1, 2,

3 show that the nature of the ions did not modify either ATP hydrolysis or Ca uptake for the same initial membrane polarization. In agreement with this hypothesis, it has been shown that ATPase crystallization is induced by sodium vanadate through its interaction with the phosphate binding site of the enzyme; this crystallization depends on membrane polarization.

The vanadate enzyme form accumulates in parallel with the degree of inhibition of ATPase activity. At a concentration sufficient to saturate the high affinity calcium binding site of the enzyme, calcium prevents the formation of new crystals and cracks those formed previously (Dux and Martonosi 1983a). The inside positive membrane potential accelerates and the inside negative membrane potential retards the crystallization of calcium ATPase, indicating an effect of membrane potential on the conformation of Ca ATPase (Dux and Martonosi 1983b). The membrane potential of SR may thus play a significant role in the calcium release mechanism during excitation-contraction coupling of skeletal muscle fibers by reducing the maximal loading capacity of SR, this reduction being induced by membrane "depolarization".

The depolarization induced calcium release hypothesis is also supported by numerous experiments carried out by different authors (for a review see Martonosi 1984). In particular, caffeine induced calcium release from vesicles is partially inhibited by valinomycin, which increases membrane potential by increasing inside positivity. Miyamoto and Racker (1982) proposed the following sequence of events:

1) Calcium or caffeine induced calcium release through a calcium gate creates an inside negative potential; 2) this negative potential opens voltage sensitive calcium channels, leading to the release of calcium sufficient for contraction. Recently, Smith et al. (1985) showed the existence of a voltage dependent Ca channel in SR membrane that presents a large unitary conductance (125 pS); 3) movement of potassium in the opposite direction through separate potassium channels (Latorre and Miller 1983; Miller 1983) serve as the charge compensation mechanism.

The increase in the potassium content of terminal cisternae after calcium release (Somlyo et al. 1981a,b) provides support to this interpretation. Furthermore, in agreement with our results (Fig. 5) Miyamoto and Racker (1982) further suggest that generation of an inside negative potential by the application of chloride and potassium gradients to SR vesicles causes calcium release via the above steps 1 and 2. Finally, Endo et al. (1981) observed inhibition of caffeine induced calcium release in vesicles with negative potential, whereas other investigators found no correlation between the membrane potential imposed by ion gradients and the rate or extent of calcium release from SR vesicles (Beeler et al. 1979; Meissner and Mc Kinley 1976). However, although under our experimental conditions caffeine had an effect on Ca release, as also reported by Endo

et al. (1981), this effect is all the greater when the inside potential is more positive (Fig. 6).

Furthermore, experiments performed on skinned fibres by other authors show that changes in the SR membrane potential may also modify calcium release. Indeed during calcium accumulation in skinned cardiac muscle fibres, the fluorescence and absorbance changes of voltage sensitive dyes indicate hyperpolarization, i.e. SR may become inside positive (Fabiato 1981, 1982). This is in agreement with the electrogenic nature of the calcium transport (Beeler 1980; Beeler et al. 1984; Zimniak and Racker 1978). When the calcium concentration in the extravesicular medium, $(Ca^{2+})_i$, is raised to 3×10^{-7} mol/l, calcium induced calcium release ensues, accompanied by large fluorescence and absorbance changes of voltage sensitive dyes. The direction of these changes suggests SR depolarization, i.e. the development of an inside negative membrane potential (Fabiato 1981, 1982). The results obtained in our experiments are in agreement with those of most other authors to the extent that the membrane potential of isolated SR vesicles is shown to play a role in the rate of Ca uptake, maximal Ca loading and in Ca release. At the same time our results also differ because they lead us to assume that the ionic concentration gradients may be maintained for several minutes after a switch in the media, whereas generally this potential is reported to disappear rapidly (for a review see Martonosi 1984; Navarro and Essig 1984). It must also be pointed out that our experimental conditions are different. In our experiments Ca release and uptake are dependent on the membrane potential which is modified by the ion substitution method. We always work with a constant [K]. [Cl] product in order to minimise the effects of osmotic pressure which can induce swelling or shrinkage. Moreover, in order to avoid the rapid disappearance of the membrane potential generated by K and Cl gradients, the vesicles are formed in an intravesicular medium (modified C or D medium) with Ca concentration 10 mmol/l. Because of passive Ca loading, this gives an initial intravesicular/extravesicular concentration ratio of the order of 1000 to 10,000. This value corresponds to that reported in the literature for Ca gradient in in vivo muscles at rest. At the beginning of the experiments, the potential across the SR membrane should be such that the SR lumen is positive with respect to the outside (the ionic composition of the SR lumen is high in Ca^{2+} ions and low in K^+ ions, see Table 1). This ionic condition is similar to that occurring in vivo and consequently, we may assume that the mechanism of "depolarization induced Ca release" is operative. This hypothesis is in agreement with that proposed recently by Stephenson (1985 a, b; 1987); she obtained a similar Ca release in skinned skeletal muscle fibers. She writes: "Stimulation by imposed ion gradients at constant [K]. [Cl] product results in ^{45}Ca release with two components: a large Ca dependent efflux, responsible for contractile activation and a small Ca-insen-

sitive efflux. The Ca-insensitive stimulation is sustained, consistent with sustained depolarization". The sustained stimulation corresponding to a small release of Ca, linear with time as shown in Fig. 5, was consistent with the fact that the depolarizing diffusion potential dissipated relatively slowly at constant [K] . [Cl] product. Finally, linear change of Ca release over time observed in our conditions is in accordance with the maintenance of membrane polarization corresponding to passive distribution of K^+ and Cl^- ions according to Donnan equilibrium. The effect of Ca conductance on membrane potential is relatively low compared to Cl and K conductances since, during the first minutes of experiment, Ca release from SR vesicles was linear.

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