EVIDENCE OF ELECTROGENICITY OF THE SARCOPLASMIC RETICULUM Ca^{2+} PUMP AS MEASURED WITH FLOW DIALYSIS METHOD

Christophe GARRET, Daniel BRETHES and Jean CHEVALLIER*

Institut de Biochimie Cellulaire et Neurochimie du CNRS, Université de Bordeaux II, 1 rue Camille Saint-Saëns, 33077 Bordeaux cedex, France

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1. Introduction

Vesicular sarcoplasmic reticulum preparations (SR) isolated from skeletal muscle are able to accumulate calcium actively by an ATP-dependent transport system embedded in a lipid bilayer [1]. To better understand the molecular mechanism of the cation translocation, studies were performed to further characterize the calcium pump. The assumption that a proton gradient observed during the calcium translocation was the motive force which sustained the uptake [2,3] has been ruled out in [4] showing that this increase in $[H^+]$, which developed as a secondary effect, was actually due to a calcium binding inside the vesicles. The electrogenicity of the pump was studied mostly by use of optical probes [3,5,6]. The results showed that calcium uptake seemed to build up a transmembrane electrical potential having a positive internal polarity of ~50–60 mV [5]. This conclusion has been questioned [7].

Here, using a more direct experimental approach of these measurements, we demonstrate the creation of a positive internal polarity inside the SR vesicles during calcium uptake. The results were obtained by means of flow dialysis [8] a technique which allows continuous determination of changes in the concentration of solute in external medium. The electrical transmembrane potential ($\Delta\Psi$) was determined by measuring the accumulation of [^{14}C]thiocyanate (SCN^-). It was calculated from the Nernst equation:

$$\Delta\Psi = \frac{RT}{nF}\log\frac{(SCN^-)_{in}}{(SCN^-)_{out}}$$

using steady state concentration value determined during the calcium uptake process. We show in a set of experiments that thiocyanate ion can be used to follow a positive transmembrane potential created under artificial conditions. Therefore we are able to confirm the electrogenicity of the pump and to quantitate the value of the internal positive potential obtained during the calcium translocation. In addition we discuss the influence of permeant ions such as K^+ in the compensation process which should occur during the uptake.

2. Materials and methods

Sarcoplasmic reticulum vesicles (SR), 21 mg/ml were prepared as in [9] and stored at −20°C in a medium containing Hepes–NaOH 10 mM (pH 7.5) and sucrose 300 mM. In some experiments vesicles were incubated at 4°C overnight in a large volume of buffer (see figure legends) sedimented at 80 000 × *g* for 1 h and 4°C, resuspended at 16–20 mg/ml and used the same day. The amount of endogenous calcium was ~20 nmol/mg protein. The vesicular internal volume was 4 μl/ml protein [10]. The Ca^{2+}-dependent ATPase activity measured by P_i liberation according to [11] was 0.85 μmol ATP hydrolyzed . mg protein^{-1} . min^{-1} at 20°C. The rate of Ca^{2+} uptake (V_{Ca} = 0.1 μmol Ca^{2+} accumulated . mg protein^{-1} . min^{-1}) and the capacity of the vesicles to sequester the cation (C_{Ca} = 50–60 nmol/mg protein) were obtained in the absence of precipitating agent by the filtration technique [12].

Abbreviations: SR, sarcoplasmic reticulum; Hepes, hydroxypiperazine–ethanesulfonic acid; AMP–PNP, adenyl imidodiphosphate

* To whom correspondence should be addressed

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Artificial membrane potentials were generated by gradients of K^+ and Cl^- after a 100-fold dilution of SR in different buffers and were detected by [^{14}C]-SCN^- accumulation inside the vesicles (final conc. 0.16–0.20 mg/ml). Artefacts induced at low ionic strength by charged molecules such as ATP on the dialysis membrane were prevented by initiating the uptake by addition of SR in the upper chamber of the flow dialysis apparatus at a final concentration of 3–5 mg/ml. For each experiment controls were performed using either ADP or AMP–PNP instead of ATP. In these experimental conditions the error due to the radioactive countings is ~1%. Therefore any signal variation less than that value was considered as non-significant (figure legends). Potassium [^{14}C]thiocyanate (59–62 mCi/mmol) was purchased from Amersham. Sigma analytical grade reagents were used otherwise.

3. Results and discussion

As shown in fig.1 and in the conditions described in table 1, membrane potentials have been generated between the intravesicular space and the medium by gradient of K^+ or Cl^- permeant ions. Positive potential of +118 mV could be theoretically induced by dilution either of the Tris–methanesulfonate filled vesicles into K-methanesulfonate medium or Tris–HCl filled material into Tris–methanesulfonate buffer. The transitory thiocyanate accumulation allows the measurement of potentials of the order of +100 mV (table 1) whatever the ion (Cl^- or K^+). This phenomenon is not due to dilution artefacts since no SCN^- accumulation can be detected in control experiments when no or negative potentials (not shown) are artificially created between the intravesicular and external spaces. Therefore SCN^- accumulation can be used to monitor positive transmembrane potential in SR vesicles. The slow time response of the apparatus (~15 s in our conditions) is a limiting step in this kind of experiment. Consequently no fast kinetic measurements can be done.

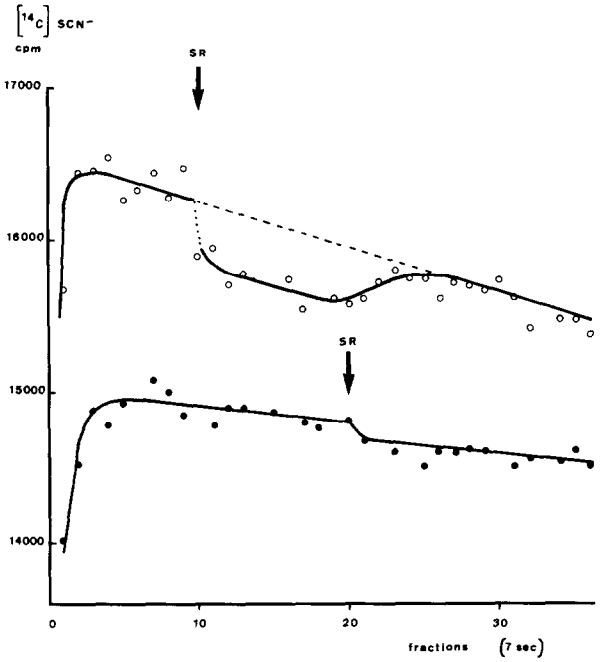

Fig.1. Effects of membrane potential induced by K^+ influx on thiocyanate repartition. SR vesicles incubated overnight at 4°C in 250 mM Tris–methanesulfonate buffer (pH 7.1) (16 mg/ml) were diluted 100-fold at 4°C into 250 mM K-methanesulfonate buffer (pH 7.1) (○). [^{14}C]SCN-K was 125 μM. Control experiments were performed by the same dilution of the vesicles in the incubation buffer (●) [^{14}C]-SCN-K at 117 μM. One fraction was taken every 7 s. The 2.7% signal variation observed upon creation of the membrane potential is significant compared to the error of the counting which is 0.8% in these experimental conditions.

Table 1
Measurement of membrane potential upon artificial conditions

Internal medium	External medium	$\Delta\Psi^a$	$\Delta\Psi^b$
Tris–methanesulfonate	K–methanesulfonate	118 mV	99 mV
Tris–HCl	Tris–methanesulfonate	118 mV	94 mV
Tris–methanesulfonate	Tris–methanesulfonate	0	0

[a] Theoretical value calculated from the Nernst equation
[b] Value obtained from the results in fig.1

The buffer concentration was 250 mM. A positive membrane potential was created either by K^+ influx or by Cl^- efflux after dilution as in the text and fig.1

When Ca^{2+} uptake is triggered by addition of SR an immediate SCN^- accumulation is observed, which is not obtained if, instead of ATP, ADP is added in the upper chamber (fig.2). In this case the decrease in radioactivity is the same as that observed upon SR addition in a medium containing no nucleotide (fig.2) or in a medium where AMP–PNP is added instead of ATP (not shown). Therefore it appears that the accumulation of thiocyanate ion is only promoted when calcium translocation occurs. When limiting amount of ATP and Ca^{2+} are used (A, table 2), the positive transmembrane potential value obtained is 41 ± 3 mV (fig.2,3). From 4 separate experiments the average value of this potential is 39 ± 7 mV (A, table 2). Since the detection limit value for the potential is 15 mV in these conditions, the results clearly show that the pump is electrogenic in nature and creates a positive transmembrane potential during the calcium uptake process.

When non-limiting amount of Ca^{2+} is used (0.3–0.35 mM instead of 0.1 mM) and when the rate of uptake is decreased by working at 4°C, a potential of ~40 mV is still measured (B, table 2). If 150 mM KCl is present in the medium no potential can be detected (C, table 2) suggesting that K^+ or K^+ and Cl^- are able to compensate the change displacement created during the calcium uptake. In fig.2,3 and table 2(A,B), the total concentrations of Na^+ and Cl^-

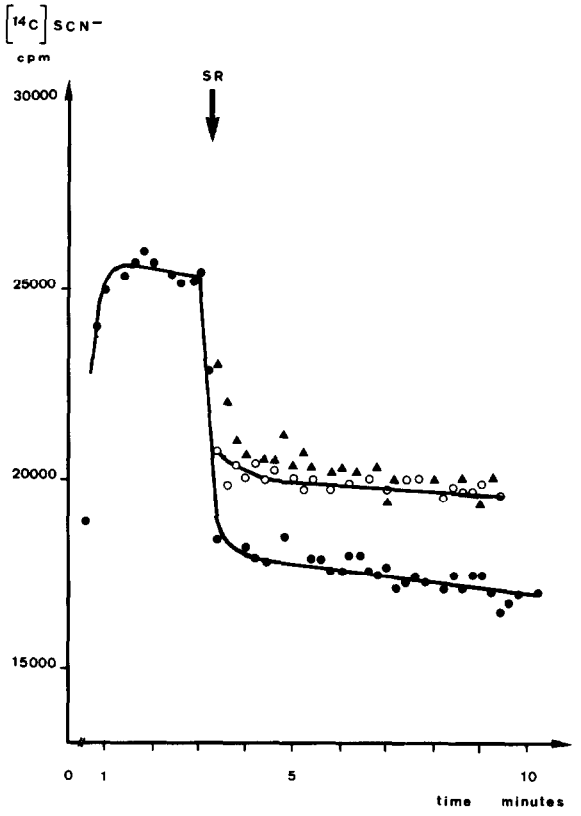

Fig.2. Creation of a membrane potential during calcium translocation as revealed by thiocyanate accumulation. In 0.8 ml 20 mM Hepes 5 mM $MgCl_2$ (pH 7.5) buffer 300 mM sucrose, ATP, $CaCl_2$ and [^{14}C]SCN-K were successively added at final conc. 4.8 mM, 0.1 mM and 57.4 μM, respectively. After 3 min the reaction was initiated by addition of SR (→) to reach 4.7 mg/ml final concentration (vesicular internal volume of 15.0 μl) in the upper chamber of the flow dialysis apparatus. Experiments were done with ATP (•), with ADP (4.8 mM) (○), and by addition of the same volume of vesicles without any nucleotides in the upper chamber (▲). The 10% signal variation observed upon creation of the membrane potential is significant compared to the error of the counting which is 1% in these experimental conditions. One fraction each 12 s at 20°C.

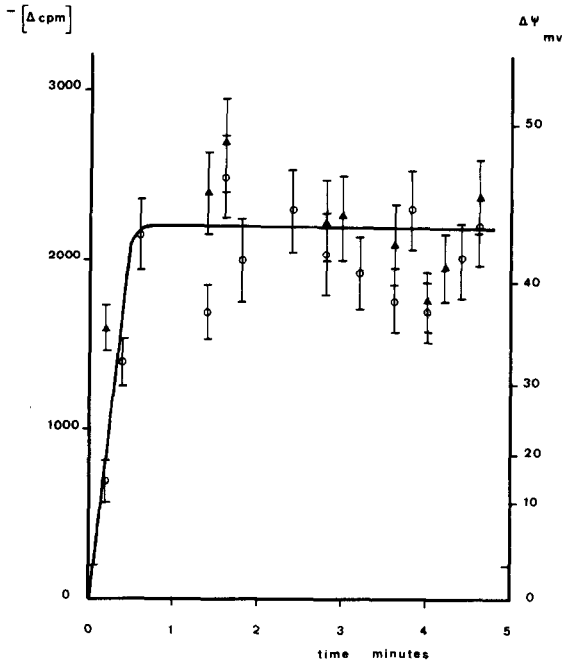

Fig.3. Time course of ΔΨ during calcium translocation. ΔΨ is calculated from the extent of SCN^- accumulation determined by flow dialysis experiment as in fig.2. Δcpm-Values are calculated by difference between the curves obtained with ATP and either ADP (○) or by dilution with SR in absence of nucleotide (▲). The standard error is represented by the vertical lines. Time zero refers to the addition of SR vesicles in the upper chamber. In our experimental conditions ATP is hydrolyzed 3 min after the addition of the vesicles and the calcium is almost completely sequestered 30 s after the beginning of the experiment.

Table 2
Values of the transmembrane potential measured in different experimental conditions

Conditions			$\frac{(SCN^-)_{in}}{(SCN^-)_{out}}$	$\Delta\Psi$ (mV)
A				
Hepes 10 mM (pH 7.5)	20°C	SR from 3.2–4.7 mg/ml		
sucrose 300 mM		$CaCl_2$ 0.1–0.12 mM		
$MgCl_2$ 5 mM,		K-SCN 27–100 μM	4.0–5.6	39 ± 7 (4)
ATP-Na 4.8 mM				
B				
Same buffer	4°C	SR from 5.4–4.7 mg/ml		
		$CaCl_2$ 0.31 and 0.35 mM	4.5	39 ± 3 (2)
C				
Same buffer	20°C	SR 3.2 and 3.4 mg/ml		
+150 mM KCl		$CaCl_2$ 0.1 and 0.1 mM		
		K-SCN 64 and 100 μM	1.0	Nothing (2)
D				
Hepes–Tris 10 mM (pH 7.2)	20°C	SR 5 mg/ml		
sucrose 300 mM		$Ca(OH)_2$ 0.2 mM		
ATP–Mg 10 mM		K-SCN 63 μM	3.3–4.6	35 ± 4 (1)

Each experiment was performed as described and analyzed in fig.2 and 3, respectively. The buffer compositions displayed in 'Conditions' are the final composition after addition of SR. Vesicles were resuspended in Hepes–NaOH 10 mM (A–C) or in Hepes–Tris 10 mM at 21 mg/ml. In all these buffer conditions the calcium uptake ability (C_{Ca}) was of the order of 50–60 nmol/mg protein (A,B,D) and120 nmol/mg protein (C), as determined in separate sets of experiments with 0.5 mg/ml of SR. Numbers of the experiments done were shown in the brackets

are 30 mM and 10 mM, respectively. Such concentrations may compensate partially the transmembrane positive potential. This hypothesis can be ruled out since a potential of 35 mV can be obtained in a medium containing Hepes–Tris buffer, ATP (Mg-salt) and $Ca(OH)_2$ neutralized with free salt Hepes solution (D, table 2).

To induce the release of the accumulated SCN^- and therefore provide further evidence that this phenomenon was due to calcium uptake, experiments were performed with A 23187 Ca^{2+} ionophore added in the medium during the translocation process. Unfortunately, at low ionic strength (KCl omitted in the buffer) an artifactual modification of the dialysis membrane permeability to the thiocyanate ion was observed which affected dramatically the results. Our attempts tend to indicate another technical approach (filter method) allowing us to perform this kind of experiment.

4. Conclusion

Obviously these results need some comments. Firstly we should confirm the electrogenicity of the sarcoplasmic reticulum Ca^{2+} pump (positive potential inside) and we should emphasize the possible importance of potassium ions in the compensation process. Secondly, the maximal value of the potential created during the uptake in our experiments is ~40 mV which is fairly low when compared to the 100 mV potential the membrane can sustain under artificial conditions. Even though a possible role of inorganic phosphate produced during ATP hydrolysis in chelation of free calcium inside the vesicles cannot be ruled out, we can assume that a mechanism acting on the Ca^{2+} pump itself takes place to control the charge translocation during the uptake. The exact nature and the importance of this regulation on the process of the Ca^{2+} uptake has to be studied in more detail.

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