# EVIDENCE FOR A CALCIUM-GATED CATION CHANNEL IN SARCOPLASMIC RETICULUM VESICLES

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## 1. Introduction

The existence of monovalent cation selective channels has been described in sarcoplasmic reticulum membranes incorporated into artificial bilayers [1,2] or in native vesicles [3,4]. These channels may provide an electrical shunt which prevents large transmembrane potentials developing during calcium release or uptake; they may therefore play an important role in the mechanism of the excitation–contraction coupling, and in the regulation of the calcium transport by the $Ca^{2+}$-ATPase.

We have studied the permeability of SR vesicles to cations using a fluorescent probe of membrane potential; diS-$C_3$-(5) [5] and with radioactive tracers ($^{22}Na$, $^{86}Rb$, and $^{45}Ca$). SR vesicles are found to contain a monovalent cation-selective conductance channel, which is strongly pH-dependent ($pK \simeq 7.4$ at 0°C), and which possesses a high affinity specific calcium site ($K_d \simeq 1\ \mu M$) on the cytoplasmic side of the vesicles. Acid pH and binding of calcium block the conductance of this channel.

## 2. Materials and methods

### 2.1. *Sample preparation*

SR vesicles were prepared from rabbit skeletal muscle as in [6] with an additional 2 h incubation in 0.6 M KCl before the final washing steps. The final suspension contained 20 mg/ml vesicles in 10 mM Mops, 0.1 M KCl and 0.3 M sucrose. Samples (1.5 ml) were rapidly frozen and stored in liquid nitrogen.

### 2.2. *Fluorescence measurements*

Fluorescence changes are measured with a Durrum D 117 fluorimeter modified to use a standard 1 × 1 cm cuvette. The suspensions are continuously stirred from the top of the cuvette and reagents are injected with Hamilton syringes (1–10 μl). The fluorescence of the dye is excited at 622 nm and the emitted light measured at 90° through a 671 nm Balzers interference filter. The other experimental conditions are described in the figure legends.

### 2.3. *Filtration experiments*

The filtrations are performed on Millipore filters HAWP (0.45 μm). The thermostated filtration equipment used has been described in [7]. The radioactivity of the filters is measured in a PPO–POPOP dioxane mixture with an Intertechnique SL 3000 counter.

## 3. Results

The permeability of SR vesicles to various ions had been investigated by light-scattering measurements [8]. This study revealed a relatively high permeability for $K^+$, $Na^+$ or $Cl^-$. $K^+$ diffusion potentials can therefore be generated across the membrane by imposing a $[K^+]$ gradient in the presence of weakly permeable ions such as $Tris^+$ and $gluconate^-$ [3,4,9]. Another way of generating a transmembrane potential is to increase the $K^+$ permeability relatively to the $Na^+$ permeability by addition of valinomycin: a large negative inside potential is obtained when valinomycin is added to $K^+$-loaded

*Abbreviations:* SR, sarcoplasmic reticulum; DiS-C3-(5), 3,3'-dipropylthiodicarbocyanine iodide; Mops, morpholinopropane sulfonic acid; EGTA, ethylene glycol-*bis*-(amino-2 ethylether) *N,N,N'N'*-tetraacetic acid

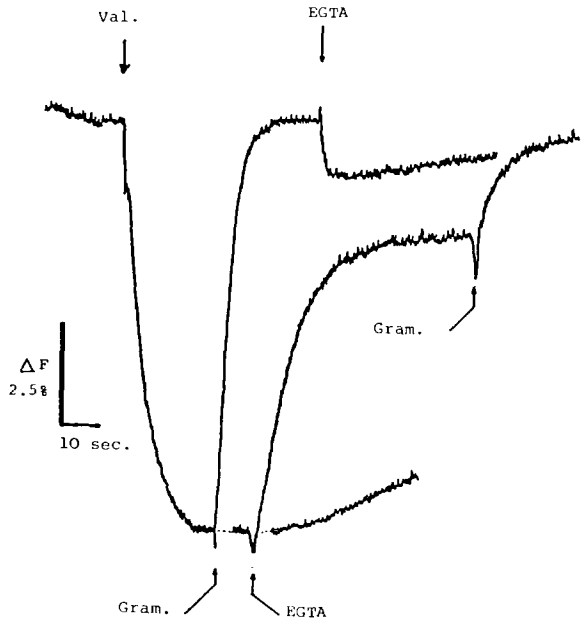


Fig.1. Opening of a conductance channel by chelation of $Ca^{2+}$. Membrane potentials are monitored with the fluorimetric dye, diS-$C_3$-(5) [5]. Vesicles loaded with 20 mM K-Mops and 5 mM Mg-glutamate by overnight dialysis are suspended in 20 mM Na-Mops, 5 mM glutamate and 1 $\mu$M diS-$C_3$-(5) under continuous stirring. An inside negative potential is generated by injection of 25 nM valinomycin. Fast dissipation of the $K^+$ potential is induced: (1) by addition of 10 mM of the non-specific ionophore gramicidin; or (2) by chelation of contaminant $Ca^{2+}$ ($\simeq$10 $\mu$M) with 250 $\mu$M EGTA (resulting free $[Ca^{2+}] < 10^{-8}$ M). Gramicidin induces a much smaller fluorescence change when it is added after EGTA than when it is added before, indicating that the large fluorescence increase induced by EGTA injection can be safely interpreted as a depolarization of the membrane. The occurence of a small fluorescence decrease upon injection of EGTA after depolarization by gramicidin shows that the dye is also slightly sensitive to the conformation of the $Ca^{2+}$-ATPase; this signal is reversed by addition of $Ca^{2+}$ (fig.2) inset. Experimental conditions: 0°C (pH 7.8) 30 $\mu$g membrane/ml.

vesicles suspended in a $Na^+$ medium (fig.1). This $K^+$ diffusion potential is slowly dissipated by passive $Na^+$ influx; it is rapidly suppressed by a non-specific ionophore such as gramicidin which can accelerate the $Na^+$ influx. We have found however that $K^+$ diffusion potentials can only be observed in the presence of $Ca^{2+}$: addition of EGTA (fig.1) rapidly neutralizes the transmembrane potential generated by valinomycin, suggesting that chelation of $Ca^{2+}$ outside the vesicles increases the $Na^+$ conductance of the membrane. Similarly, if EGTA is added to a suspension of $K^+$-loaded vesicles ($Na^+$ out) prior to valinomycin, the $K^+$ diffusion potential generated upon addition of valinomycin rapidly decreases with increasing duration of EGTA treatment (fig.2). These experiments indicate that $K^+$ has been exchanged for $Na^+$ during incubation in low $Ca^{2+}$: both $Na^+$ and $K^+$ conductances are therefore increased in the presence of EGTA. Fig.2 also shows that the EGTA-induced conductance is strongly pH dependent ($pK \simeq 7.4$ at 0°C).

The $[Ca^{2+}]$-dependence of the conductance has been studied by measuring the rate of the EGTA-induced dissipation of the potential in experiments similar to that in fig.1: varying pCa were obtained by adding varying amounts of EGTA. Fig.3 shows that the conductance channel possesses a blocking site of very high apparent affinity for calcium ($K_d \simeq 1$ $\mu$M at 0°C in the presence of 5 mM $Mg^{2+}$).

The existence of a $Ca^{2+}$-gated $Na^+$–$K^+$-channel has been confirmed with the use of radioactive tracers and Millipore filtration in the absence of concentration gradients. Chelation of $Ca^{2+}$ with EGTA induces an important acceleration of $^{22}Na^+$ or $^{86}Rb^+$ efflux, whereas it has no effect on the release of trapped $^{45}Ca^{2+}$ (fig.4). These experiments therefore demonstrate that the EGTA-induced conductance is specific for monovalent cations such as $Na^+$, $K^+$ or $Rb^+$.

Fig.2. Time course and pH-dependence of the EGTA-induced discharge of the $K^+$ gradient. $K^+$-loaded vesicles are diluted in a $Na^+$ medium as in fig.1. The recording of one experiment is schematically drawn in the inset: EGTA is injected in the cuvette 90 s after dilution of the membranes and excess $Ca^{2+}$ is added after various incubation times to stop the action of EGTA. After this EGTA treatment valinomycin (25 nM) is injected to measure the remaining $K^+$ gradient. The valinomycin-induced potential is measured by the fluorescence change of diS-$C_3$-(5): (•) no EGTA treatment ($Ca^{2+} \simeq 10$ $\mu$M); (○) incubation with 250 $\mu$M EGTA (resulting free $[Ca^{2+}] < 10^{-8}$ M) for various time duration.

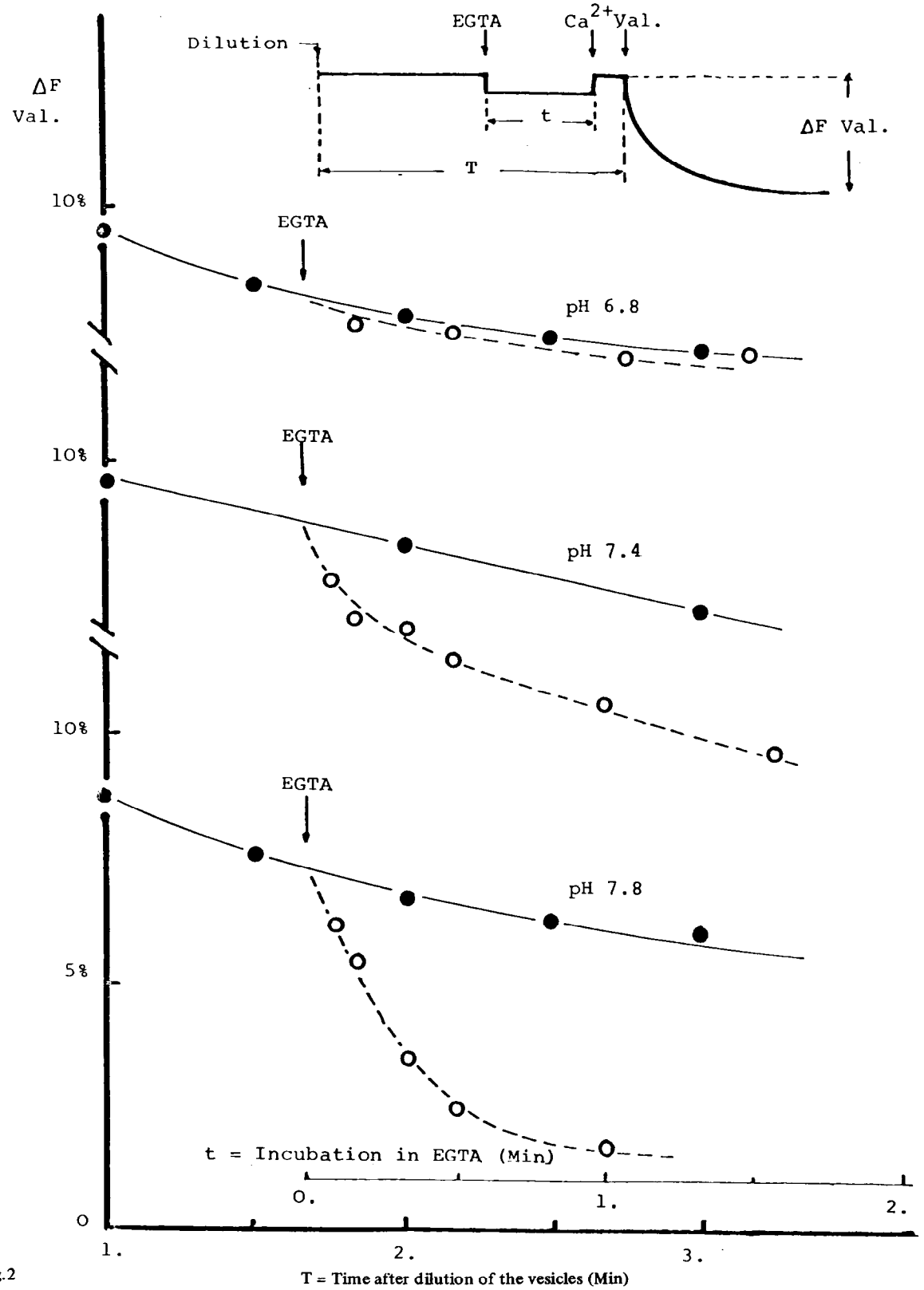


Fig. 2

T = Time after dilution of the vesicles (Min)

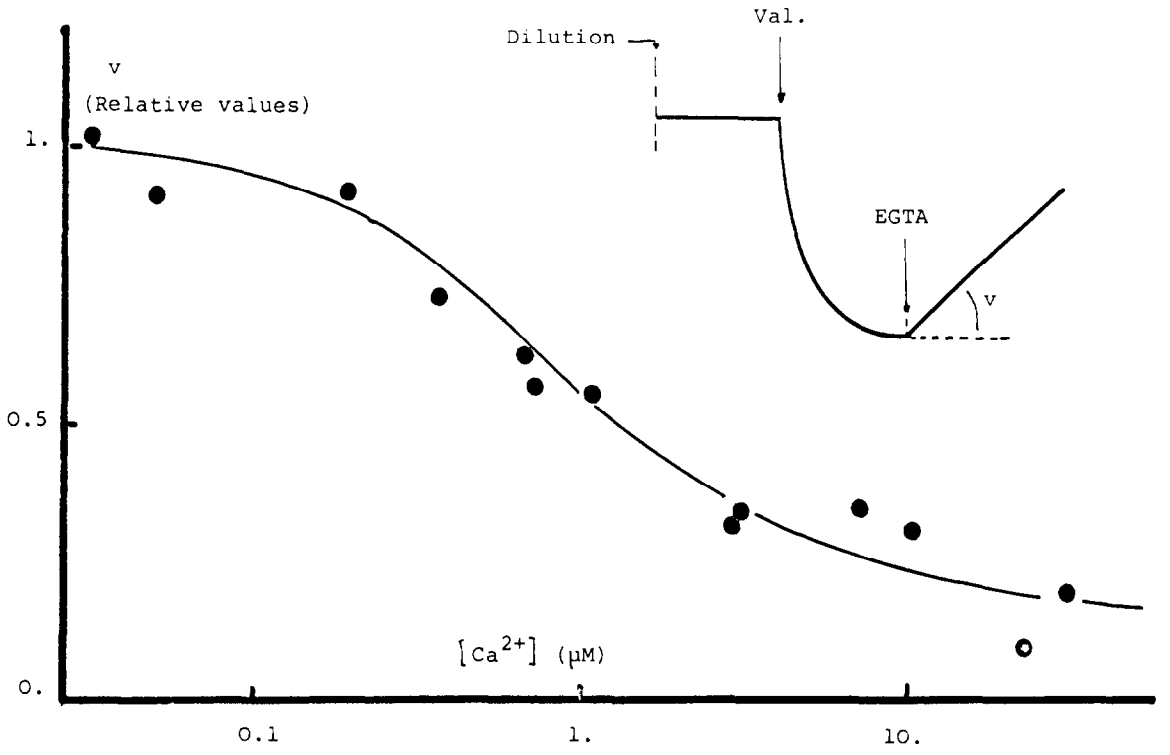


Fig.3. Titration of the calcium concentration dependence of the channel opening. The rate of dissipation of the potential upon addition of varying amounts of EGTA (cf. fig.1) is plotted as a function of the free $[Ca^{2+}]$, calculated using tabulated stability constants [10] ($K_d$ (EGTA-Ca) = 0.02 μM at pH 7.8 in the presence of 5 mM $mg^{2+}$). Experimental conditions: 0°C (pH 7.8); total [calcium] 300 μM; EGTA, 270–600 μM.

## 4. Discussion

The existence of $K^+$ or $Na^+$ channels in SR membranes was suggested from two different kinds of experiments: the conclusion [3,4] that SR vesicles are composed of two types which differ in their permeability to $K^+$ and $Na^+$: ~2/3rd, of the vesicles, which present a high permeability (type I), are suggested to contain monovalent cation channels. The rest of the vesicles (type II) are able to retain cations for a few minutes. In these experiments, only those vesicles which are able to maintain a $K^+$ gradient have been studied, i.e., the relatively impermeable vesicles (type II) which according to [3,4] do not contain cation channels. Since the experiments [in 3,4] were done at pH 7 and in the presence of $Ca^{2+}$ the channels that we observe were blocked. If type I vesicles were present in our preparation, we have to assume that they have completely exchanged their $^{86}Rb^+$ or $^{22}Na^+$ content in <5 s (first point in fig.4). The channels described here are therefore distinct from those observed in [3,4].

The other report on the existence of monovalent cation channels in SR membranes comes from the conductance measurements [1,2] on artificial bilayer systems. The pH dependence of the channels that we observe in SR vesicles is very similar to that in [1,2]. Although [1,2] also reported an asymmetrical gating by $Ca^{2+}$, the effect occured at a much higher concentration ($K_d$ = 0.8 mM) in their experiments. It could be suggested that the discrepancy with our results might arise from modifications due to the incorporation of the proteins into the bilayer membrane.

The affinity of the blocking site for calcium in our experiments ($K_d \simeq 1$ μM) is of the same order as that of the high affinity site of the $Ca^{2+}$-ATPase [7]. This suggests as an alternative hypothesis that the ATPase itself could perform, in the absence of $Ca^{2+}$, an electrically silent monovalent cation exchange when no potential is imposed across the membrane, or an electrogenic monovalent cation transport in response to a transmembrane potential. The only indication so far against this hypothesis is that we have failed to observe

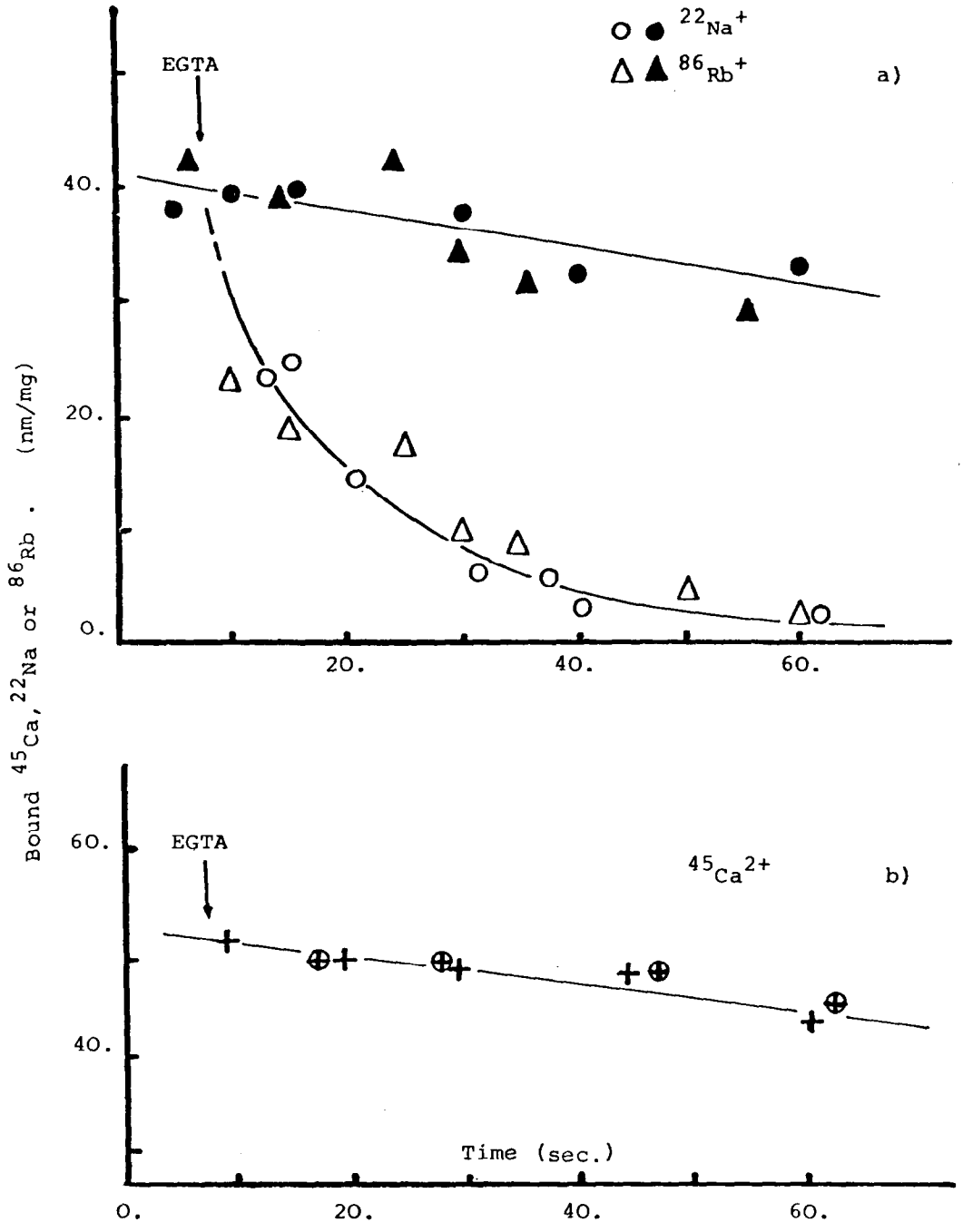


Fig.4. Measurement of $^{22}Na^+$, $^{86}Rb^+$ and $^{45}Ca^{2+}$ efflux by Millipore filtration. Vesicles are incubated for 1 h at 0°C (pH 7.8) in: (a) 20 mM Na ($^{22}Na$)-Mops or K ($^{86}Rb$)-Mops, 5 mM Mg-glutamate and 100 μM Ca-glutamate; (b) 20 mM K-Mops, 5 mM Ca ($^{45}Ca$)-glutamate. Each point corresponds to the filtration of a 1 ml suspension sample (0.3 mg protein) on Millipore filter (HAWP 0.45 μm). The filter is washed for varying periods of time with a few ml on non-radioactive buffer: (●,▲,+) same ionic composition; (○,△,⊕) same ionic composition for 6–8 s (washing off the radioactivity trapped in the filter), then changed for the same buffer except that Ca-glutamate is replaced by 250 μM EGTA (resulting free $[Ca^{2+}] < 10^{-8}$ M). The presence of 5 mM Ca-glutamate inside the vesicles does not modify the EGTA-induced $Na^+$ and $Rb^+$ conductance increase. Experimental conditions: 0°C (pH 7.8).

any inhibitory effects of ADP or internal calcium.

That the gating of the channels is achieved at $\mu M$ $Ca^{2+}$ levels provides a new basis for understanding the mechanism of the calcium release [11,12]. Indeed, it implies that the conductance properties of the SR membrane can be regulated by levels of $Ca^{2+}$ which physiologically occur in the fiber. It suggests in particular that a small increase of $Ca^{2+}$ in the fiber might allow a depolarisation of the SR membrane to take place.

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