

# SARCOBALLS: DIRECT ACCESS TO SARCOPLASMIC RETICULUM $\text{Ca}^{2+}$ CHANNELS IN SKINNED FROG MUSCLE FIBERS

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**ABSTRACT** In skeletal muscle, twitch contraction is caused by the rapid release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR) (Endo, M. 1977. *Physiol. Rev.* 57:71–108) via  $\text{Ca}^{2+}$  conducting channels in the SR membrane (Smith, J. S., R. Coronado, and G. Meissner, 1985. *Nature (Lond.)*. 316:446–449; Suarez-Isla, B. A., G. Orozco, P. F. Heller, and J. P. Froehlich. 1986. *Proc. Natl. Acad. Sci. USA*. 83:7741–7745). To facilitate study of these and other intracellular channels, we have developed a method which allows direct patch-clamp recording of currents through SR channels in native membrane. The  $\text{Ca}^{2+}$ -release channel studied using this method exhibits two predominant conductance levels (80–100 pS and 120–160 pS), conducts  $\text{Ca}^{2+}$  preferentially over  $\text{K}^+$  ( $P_{\text{Ca}}/P_{\text{K}} = 6.5$ ), is highly voltage sensitive, blocked on one side by ruthenium red (1  $\mu\text{M}$ ), and displays enhanced activity in the presence of caffeine (5 mM). Studied in skinned fibers, this channel appears fundamentally similar to homologous channels from isolated rabbit SR incorporated into bilayers, with some distinct differences.

## INTRODUCTION

In muscle, the sarcoplasmic reticulum (SR) acts as both source and sink for  $\text{Ca}^{2+}$  which either causes or contributes to the development of contractile tension. In skeletal muscle, SR is apparently the sole source of  $\text{Ca}^{2+}$  mediating twitch tension (1). Early studies, attempting to determine the physiological release mechanisms, were performed on skinned fibers and isolated SR vesicles and they produced evidence that a variety of candidate stimuli can elicit  $\text{Ca}^{2+}$  release.

More recently, the advent of single channel techniques (2) has made it possible to voltage clamp channels from SR vesicles incorporated into lipid bilayers (3). This approach has made it clear that  $\text{Ca}^{2+}$ -conducting channels are present in the SR membrane and that they are activated by a number of stimuli previously shown to cause  $\text{Ca}^{2+}$  release from isolated vesicles:  $\text{Ca}^{2+}$ , ATP, and caffeine (4, 5, 6). Additionally, these channels are blocked by release blockers, ruthenium red and  $\text{Mg}^{2+}$  (4, 5). The general agreement between these channel properties and the properties of the release mechanisms suggest that  $\text{Ca}^{2+}$  release from the SR is mediated by  $\text{Ca}^{2+}$  conducting channels.

Whereas bilayer experiments have been productive and enlightening, accumulating evidence indicates that channel properties may vary with lipid environment. Both current amplitude and gating kinetics of incorporated channels are influenced by the constituent phospholipids

through both surface charge (7, 8) and non-charge related lipid/protein interactions (9). At the same time, the SR membrane has been shown to contain an asymmetric distribution of several constituent phospholipids, the most prevalent of which is phosphatidylcholine (PC) (10). PC has been shown to inhibit vesicle fusion into bilayers (11) such that the PC content of bilayers is generally made very low compared with native SR membrane (4, 5, 12). For these reasons, channels incorporated into artificial lipid bilayers may not behave as they do in native SR membrane.

Given this possibility, it is clear that physiological properties of these channels might be better examined in native membrane, accessed using methods that perturb the membranes minimally. Ideally, one would hope to directly patch SR membrane. As approaches toward this ideal, Suarez-Isla and co-workers (13) have studied SR channels by applying "tip-dipping" methods to isolated native membranes, whereas Tang et al. (14) have found that lobster antennae muscle is unusually rich in SR that can be patch-clamped directly. At the same time, we have been developing another approach allowing direct study of SR channels with the combined advantages of rapid and simple access to the SR membrane of a widely studied cell type without using biochemical isolation procedures. The simplicity of the method also indicates that it may be more generally applicable to the study of other cell types.

We present here our finding that frog skeletal muscle

fibers skinned mechanically (15) in  $\text{Ca}^{2+}$ -containing saline solution exhibit blebs of membrane at their surfaces within seconds of skinning. These small blebs coalesce to form larger blebs which we have termed "sarcoballs." Results from single-channel patch-clamp studies indicate that sarcoballs contain  $\text{Ca}^{2+}$ -permeable channels which originate in the SR. We conclude that we are recording from what must be predominantly SR membrane rendered accessible by a very simple technique. Preliminary accounts of this work have been presented as abstracts (16, 17).

## METHODS

### Sarcoball Preparation

One end of an individual fiber is dissected from the semitendinosus muscle of *Rana catesbeiana* in either a physiological saline solution (PSS) of the following composition (in mM): NaCl, 117; KCl, 2.5;  $\text{CaCl}_2$ , 1.8; 3-(*N*-morpholino)-propanesulfonic acid (MOPS), 5; pH adjusted to  $7.20 \pm 0.01$ ; tonicity,  $240 \pm 3$  mOsm or in a relaxing solution (RS) containing (in mM): K Aspartate 120,  $\text{Na}_2$  creatine phosphate (CP) 5,  $\text{Na}_2$  adenosine triphosphate (ATP) 3,  $\text{MgSO}_4$  3,  $\text{CaCl}_2$  0.0281, tris maleate 3, and tris, EGTA 0.1, pH 7.1, tonicity  $240 \pm 3$  mOsm. This solution has a free  $\text{Ca}^{2+}$  concentration of  $0.1 \mu\text{M}$ . Fine forceps are used to rapidly peel the sarcolemma (SL) (15) while holding the free end of the fiber immobile. In PSS, sarcoballs form immediately after this mechanical skinning, and persist in a wide variety of electrolytes and tonicities. The skinned portion of the fiber is transferred to a 1-ml recording chamber containing the solution which is to be on the bath side of excised patches. The rapidity of formation allows electrical recording of channel activity in as little as 1 min.

### Electrical Recording

Micropipettes were fashioned from an intermediate hardness glass (7052; Corning Glass Works, Corning, NY) according to Hamill et al. (2) and fire polished to resistances between 5 and 15 M $\Omega$ . The signal was amplified with a List EPC-7 patch-clamp amplifier, filtered at 20 kHz (Wavetek, San Diego, CA), digitized (Sony PCM501), and the digitized signal recorded on VCR tape for later analysis. The membrane forms high-resistance (10–100 G $\Omega$ ) seals against conventional glass micropipettes used in patch-clamp voltage clamping (2). Because of the unknown composition of the sarcoball interior, we record mainly from excised inside/out (I/O) patches, with the outer surface of the sarcoball facing into the pipette, and the reference potential on the bath side. To avoid the large conductance  $\text{Cl}^-$  channels present in the SR membrane (18, 19), gluconate was used as a highly impermeant anion though all solutions contained 5 mM  $\text{Cl}^-$ . Records shown here were obtained from single-channel patches though patches contained zero to four channels.

### Electron Microscopy

Skinned fibers were fixed in a phosphate-buffered saline (PBS) containing 3% glutaraldehyde for 2 h at room temperature and then postfixed in 1% osmium tetroxide in the same buffer. Fixed fibers were dehydrated in alcohol, followed by three changes of propylene oxide, and finally embedded in Epon resin. Silver sections were cut with a diamond knife on an ultramicrotome (LKB Instruments, Inc., Gaithersburg, MD), mounted on copper grids, stained with uranyl acetate, and examined in a JEOL CX-100 electron microscope.

## RESULTS AND DISCUSSION

Skeletal muscle fibers from frogs, skinned mechanically (15) in the presence of  $\text{Ca}^{2+}$ , exhibit blebs of membrane at their surfaces, termed "sarcoballs" (Fig. 1). Sarcoballs,

which may be quite large ( $>100 \mu\text{m}$ ), appear to represent the coalescence of large numbers of smaller blebs of internal membrane which form at the fiber surface within seconds of removing the sarcolemma (SL) in PSS. Similarly, fibers skinned in a relaxing solution (RS, pCa 7) exhibit well defined striations and no evidence of membrane blebbing until  $\text{Ca}^{2+}$  is added to the bath (pCa 2.8). The sudden elevation in  $\text{Ca}^{2+}$  results in a maximal contraction of the fiber and the appearance of sarcoballs at the fiber surface within seconds. This result indicates that there is nothing unusual about our skinning method and that this blebbing phenomenon has heretofore gone unnoticed because studies using mechanically skinned fibers have, with good reason, not allowed unrestrained shortening in the presence of high  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]$ ). We have not yet defined the  $\text{Ca}^{2+}$  requirements or the degree of shortening necessary for sarcoball formation but we suspect that these are the two critical variables involved in the process.

Single-channel current records from an excised patch of sarcoball membrane are shown in Fig. 2. The fully opened channel slope conductance ( $\alpha$ ) in symmetrical 52.5 mM  $\text{Ca}^{2+}$  varies from 120 to 160 pS whereas the predominant substate ( $\beta$ ) is  $\sim 60\%$  of  $\alpha$ . The channel is open to the  $\beta$  level the majority of the time, whereas the fully open level ( $\alpha$ ) is generally seen in bursts on top of the  $\beta$  level (Fig. 2A). The bursting behavior implies that the  $\beta$  level either consists of two states of equal conductance or that the rate constant for the  $\beta$ -to- $\alpha$  transition is variable. Complete closures occur from either the  $\alpha$  or  $\beta$  levels. In addition to  $\alpha$  and  $\beta$  levels, we see a multiplicity of less stable conductance levels, in contrast with previous reports of muscle  $\text{Ca}^{2+}$  release channels which exhibit only one open level in bilayers (4, 5, 12). Fully opened, this channel is similar in size to the  $\text{Ca}^{2+}$ -release channel (125 pS) reported by others from SR vesicles incorporated into bilayers (4) and considerably larger than any surface  $\text{Ca}^{2+}$  channel described for muscle (7, 20).

The high open probability ( $P_0$ ) in symmetric 52.5 mM  $\text{Ca}^{2+}$  is contrary to expectations based upon previous studies which showed high  $\text{Ca}^{2+}$  to block release from SR vesicles (21) and skinned cardiac fibers (22), presumably by blocking this channel. Similarly, Smith et al. (5) found  $[\text{Ca}^{2+}]$  over 1 mM on the cytoplasmic face of the membrane to cause a reduction in  $P_0$  of the rabbit SR  $\text{Ca}$ -release channel in bilayers. We cannot yet determine the degree to which this apparent discrepancy may be attributed to differing methodologies, preparations, experimental conditions, or other uncontrolled variables.

In symmetric 52.5 mM Ca (Fig. 2), the channel inactivates at potentials ( $E_m$ ) more positive than +30 mV, and long duration closures appear at  $E_m$  more negative than -50. In contrast, substituting 100 mM K gluconate (with 25  $\mu\text{M}$  added Ca) in the pipette alters this relationship such that the steady-state  $P_0$  is zero at  $E_m \leq 0$  and is

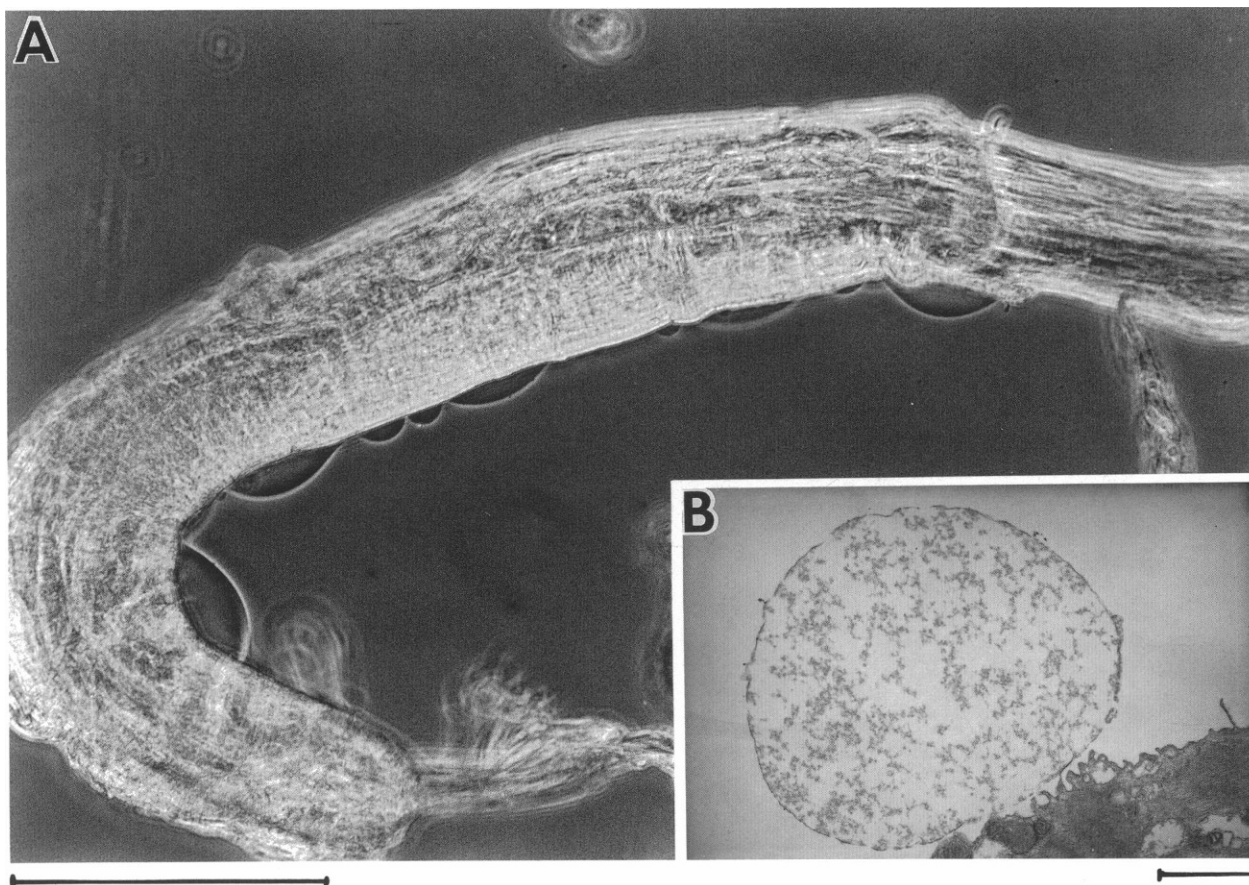


FIGURE 1 (A) Blebs of internal membrane protruding from the surface of a mechanically skinned muscle fiber photographed with phase contrast optics. (B) Photographs taken with the transmission electron microscope show both large and minute vesicles, bounded by a bilayer and containing varying amounts of flocculent material. Calibration bars, (A) 200  $\mu\text{m}$ ; (B) 2.5  $\mu\text{m}$ .

increased only at more positive potentials (Fig. 3 C). The difference in the records of Figs. 2 and 3 may be due, in part, to surface charge effects (23) arising from the differences in  $[\text{Ca}^{2+}]$  on the pipette side of the membrane. The sensitivity to  $E_m$  illustrated in Fig. 3 C is more marked than that reported for similar channels recorded in bilayers (4) and, like the subconductance states, must reflect differences in either technique or species. Additionally, the degree of sensitivity seen here suggests that small changes in SR membrane potential may be accompanied by large changes in  $\text{Ca}^{2+}$  permeability.

Lowering pipette  $[\text{Ca}^{2+}]$  to 0.3  $\mu\text{M}$  reduces  $P_0$  (Fig. 3 C, triangles).  $\text{Ca}$ -induced  $\text{Ca}$  release from the SR (24) occurs in both isolated SR vesicles (25) and skinned fibers (26) implying a  $\text{Ca}^{2+}$ -sensitive site on the cytoplasmic side of the channel. Up to 1 mM,  $\text{Ca}^{2+}$  on the cytoplasmic face of the large  $\text{Ca}^{2+}$  channel causes a concentration-dependent increase in  $P_0$  (5). Without attempting to quantify the relationship, we show that increased  $P_0$  accompanies increased  $\text{Ca}^{2+}$  over the physiological range of concentrations.

Records made with 52.5 mM  $\text{Ca}^{2+}$  in the bath and 100

mM K in the pipette show that the channel conducts  $\text{K}^+$  readily (Fig. 3). A selectivity equation allowing comparison of mono- vs. divalent cations (27) was used to determine that  $P_{\text{Ca}}/P_{\text{K}} = 6.5 \pm 3.0$ , ( $n = 5$ ). This value is identical to the  $P_{\text{Ca}}/P_{\text{K}}$  recently reported for the purified  $\text{Ca}^{2+}$ -release channel from rabbit skeletal muscle in bilayers (28). Net  $\text{K}^+$  movement into the SR during tetanic stimulation has been shown using electron probe analysis and has been suggested to reflect charge balancing during  $\text{Ca}^{2+}$  efflux (29). This SR  $\text{Ca}^{2+}$  channel and the SR  $\text{K}^+$  channel recorded in bilayers (30), if different entities, constitute two pathways by which  $\text{K}^+$  might move during  $\text{Ca}^{2+}$  release. We have not yet seen the  $\text{K}^+$ -selective SR conductance described by Labarca and Miller (30).

Caffeine potentiates twitch contractions in low concentrations ( $\leq 1$  mM) and causes contracture in higher concentrations (31). Others have shown that these effects are, at least in part, attributable to a direct action of caffeine on the SR  $\text{Ca}^{2+}$  release channel, causing an increase in  $P_0$  (6, 13, 32). To ascertain whether the sarcoball channel is similarly responsive, we applied 5 mM caffeine to the bath while recording from a single channel patch which showed

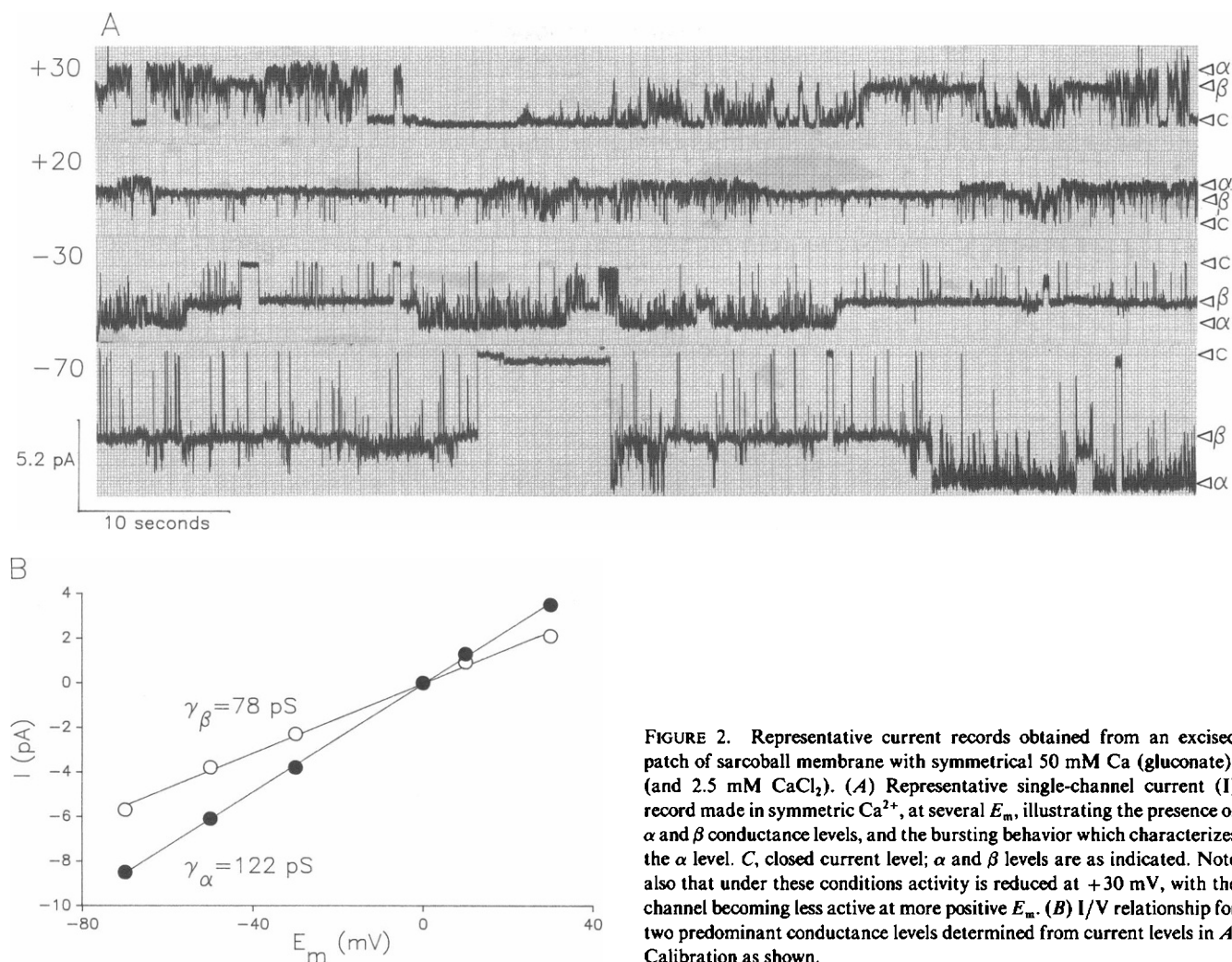


FIGURE 2. Representative current records obtained from an excised patch of sarcoball membrane with symmetrical 50 mM Ca (gluconate)<sub>2</sub> (and 2.5 mM CaCl<sub>2</sub>). (A) Representative single-channel current (I) record made in symmetric Ca<sup>2+</sup>, at several  $E_m$ , illustrating the presence of  $\alpha$  and  $\beta$  conductance levels, and the bursting behavior which characterizes the  $\alpha$  level. C, closed current level;  $\alpha$  and  $\beta$  levels are as indicated. Note also that under these conditions activity is reduced at +30 mV, with the channel becoming less active at more positive  $E_m$ . (B) I/V relationship for two predominant conductance levels determined from current levels in A. Calibration as shown.

little activity before caffeine addition (Fig. 4). In 2 min, the activity increased to long duration openings at zero and negative potentials where essentially no activity was previously observed. The dramatic caffeine-induced increase in  $P_0$  confirms that this is the SR Ca<sup>2+</sup>-release channel and that sarcoball formation does not eliminate caffeine sensitivity.

Similarly, the polyamine ruthenium red (RR) blocks Ca<sup>2+</sup> release from isolated SR vesicles (33) and has been shown to block Ca<sup>2+</sup> release channels incorporated into bilayers (4). RR (1  $\mu$ M) included in pipette solutions results in 100% (8/8) of patches devoid of channel activity compared with 35% (13/37) in its absence; 10  $\mu$ M RR added to the bath had no effect on channel activity ( $n = 5$ ). Outside/out patches (O/O, cytoplasmic membrane surface facing bath) were used to confirm that 2  $\mu$ M RR added to the cytoplasmic side of the membrane rapidly blocks existing channel activity. These results suggest both uniform orientation of channels and polarity of block with the RR-sensitive site on the cytoplasmic side of the membrane.

Finding this channel in the majority of patches formed

on sarcoballs, and an average of one to two such channels per patch ( $n > 40$ ), suggests a large number of these channels in sarcoball membranes. Insofar as the Ca<sup>2+</sup>-release channel is thought to be localized and confined to the face of the terminal cisternae (TC) abutting the transverse tubule, we considered whether the number of channels we find might reflect a mixing of SR membranes resulting in an even distribution of the TC channels in the sarcoball membrane. Based upon surface area measurements of SR components in frog muscle (34) and the size and distribution of "feet" structures, currently thought to correspond to channels (35), we calculate that channels we record are more than an order of magnitude too dilute to represent an even distribution.<sup>1</sup> Bleb formation in sarco-

<sup>1</sup>Channel density. Square "feet" proteins are confined to the region of the terminal cisternae (TC) membrane facing the triadic gap (35). Arranged in a "checkerboard" array of feet 222 Å on a side, defining spaces of the same dimension, 507 feet proteins will occupy 1  $\mu$ m<sup>2</sup>. This is the calculated density of Ca<sup>2+</sup> channels in the TC membrane. According to Peachey (34), transverse tubular (t-tubular) membrane constitutes 4.9% of the total intracellular membrane and 80% of the t-tubule is covered by

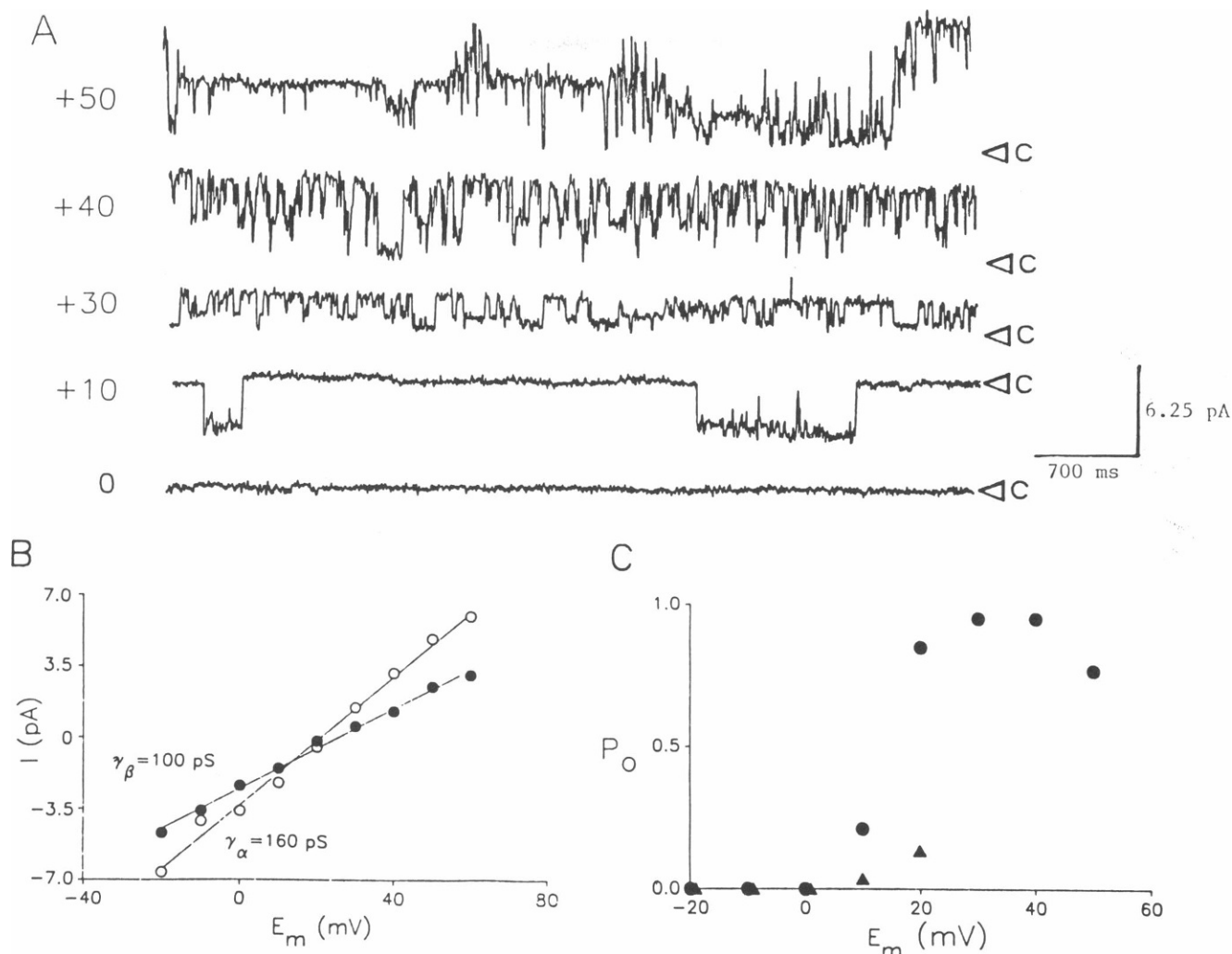


FIGURE 3 (A) Representative current records obtained from single-channel patch with 50 mM Ca (gluconate)<sub>2</sub> on bath side and 100 mM K gluconate in pipette (symmetric 5 mM Cl<sup>-</sup>), at several  $E_m$ . C, closed current level. At  $E_m$  above the reversal potential ( $E_{rev} = +18$  mV), K is the predominant carrier of current; at  $E_m$  negative to  $E_{rev}$ , Ca is the predominant current carrier. (B)  $I/V$  relationships for  $\alpha$  (open circles) and  $\beta$  (closed circles) current levels determined from the records illustrated in A. Points at negative potentials were obtained during brief periods of channel activity when  $E_m$  was first changed. (C) Steady state  $P_0$  to any open level for the channel illustrated in A recorded in the presence of 100 mM K<sup>+</sup> with 12.5  $\mu$ M Ca<sup>2+</sup> added on the pipette side of the membrane (closed circles).  $P_0$  values were determined by using the program Analysis applied to representative 45–60 s current records at the  $E_m$  shown, digitized at 1,500 Hz, and discriminators set at ~50% of the  $\beta$  current level. Triangles are  $P_0$  from a representative single channel recorded with 100 mM K<sup>+</sup> and 0.3  $\mu$ M Ca<sup>2+</sup> (EGTA buffered) on the cytoplasmic face of the membrane. At  $E_m \geq +30$  mV,  $P_0$  is considerably more variable ( $n = 3$ ). Calibration as shown.

lemmae of skeletal muscle is accompanied by increased lateral mobility of some of the integral proteins (36). This lower than expected density, therefore, might suggest that only a fraction of the TC channels are free to diffuse within the bilayer and the rest remain anchored in groups which cannot be patched. Alternatively, it is possible that only a fraction of the Ca<sup>2+</sup>-release channels present in the membrane are activated under our experimental conditions.

TC. Thus, we calculate that junctional TC constitutes 3.92% of total intracellular membrane and 4.2% of total SR. If junctional TC membranes were uniformly dispersed throughout the total SR membrane, we expect a 23.8-fold dilution of TC channels. Accordingly, we might expect to see about 20 channels/ $\mu$ m<sup>2</sup> or 60 channels in the typical 3  $\mu$ m<sup>2</sup> patch. Instead, we see an average of about two channels per patch.

The ubiquity of this channel in sarcoball membranes, its large conductance to Ca<sup>2+</sup>, limited selectivity for Ca<sup>2+</sup> over K<sup>+</sup>, sensitivity to Ca<sup>2+</sup> on the cytoplasmic face, to caffeine, and to ruthenium red all support the notion that this is the frog SR Ca<sup>2+</sup>-release channel, now rendered accessible simply by removing the SL. Finding SR channels in these vesicles is not surprising considering that SR constitutes the majority (74–85%) of internal muscle membranes, based on membrane surface areas calculated from measurements of Peachey (34) and Mobley and Eisenberg (37) on frog fibers. Because of possible contamination with transverse-tubule or mitochondrial membranes, we cannot yet assess whether sarcoballs are composed solely of SR although systematic study of the anion conductances present (18) has not found evidence of either the mitochondrial

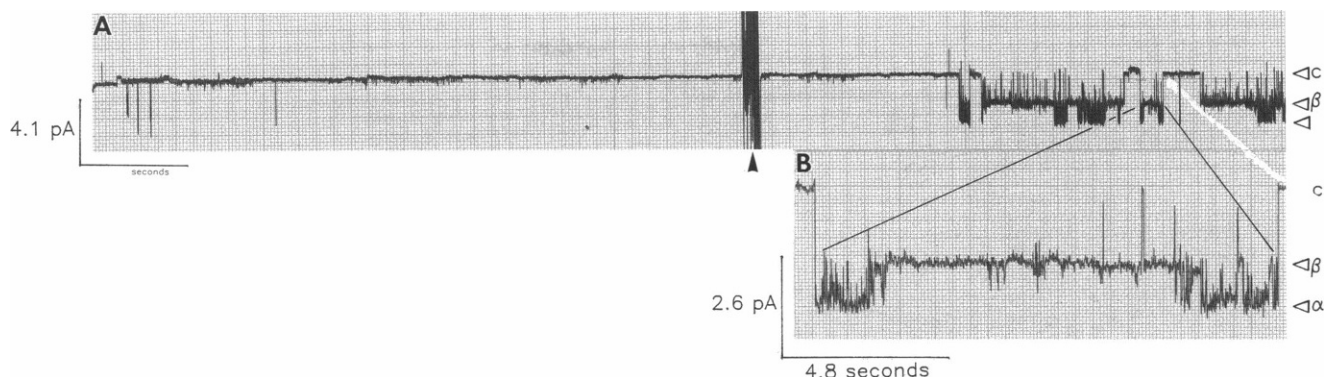


FIGURE 4 Effect of caffeine. Record *A* shows currents from an excised single-channel patch with 52.5 mM Ca in the bath and 102.5 mM K in the electrode (with nominally 12.5  $\mu$ M  $\text{Ca}^{2+}$  present, no EGTA, symmetric 5 mM  $\text{Cl}^-$ ) and  $E_m = 0$  mV. Upon seal formation, the channel opened transiently then remained quiescent for the next 5 min. Approximately 2 min after the introduction of caffeine (5 mM) to the bath (artifact and arrows), the channel began to conduct  $\text{Ca}^{2+}$  (downward openings). Record *B* is an expanded portion of *A*, showing the two major conductance levels ( $\alpha = 146$  and  $\beta = 86$  pS) and closing transitions from both levels. We have recorded caffeine activation under similar conditions in five patches. Calibration as shown.

voltage-dependent anion channel (VDAC) (38) or surface membrane  $\text{Cl}^-$  channels. (19, 22).

The only technical limitation inherent in this approach is that receptors on the cytoplasmic surface of the SR will be inside the pipette when making I/O or ball-attached patches. This is not an insurmountable problem as access to these receptors may be obtained a number of ways, including pipette perfusion, O/O patches, flash photolysis of caged compounds, or the use of lipid-soluble compounds. This impediment notwithstanding, sarcoballs represent a simple and fast means of accessing individual SR channels in frog skeletal muscle, the traditional proving ground for seminal ideas on excitation-contraction coupling in muscle. Considering the labile properties reported for other types of  $\text{Ca}^{2+}$  channel (39), the speed of the method may be its greatest asset. Additionally, the simple nature of the method suggests that it will be applicable to the study of internal membranes in other cell types.

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