Multiple conductance states of the purified calcium release channel complex from skeletal sarcoplasmic reticulum

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ABSTRACT The CHAPS-solubilized and purified 30S ryanodine receptor protein complex from skeletal sarcoplasmic reticulum (SR) was incorporated into planar lipid bilayers. The resulting electrical activity displayed similar responses to agents such as C^{a2+}, ATP, ryanodine, or caffeine as the native Ca^{2+} release channel, confirming the identification of the 30S complex as the Ca^{2+} release channel. The purified channel was permeable to monovalent ions such as Na^+, with the permeability ratio $P_{Ca}/P_{Na} \sim 5$, and was highly selective for cations over anions. The purified channel also showed at least four distinct conductance levels for both Na^+ and Ca^{2+} conducting ions, with the major subconducting level in NaCl buffers possessing half the conductance value of the main conductance state. These levels may be produced by intrinsic subconductances present within the channel oligomer. Several of these conductances may be cooperatively coupled to produce the characteristic 100 ± 10 pS unitary Ca^{2+} conductance of the native channel.

INTRODUCTION

The physiological contraction of skeletal muscle is initiated by a transient depolarization of the surface membrane which then propagates to the transverse (T−) tubule membrane and triggers a rapid release of Ca^{2+} ions from its major intracellular storage site, the sarcoplasmic reticulum (Endo, 1977). The molecular mechanism through which coupling between surface membrane excitation and muscle contraction is achieved (excitation-contraction coupling), and more specifically, the mode of signal transmission between the T-tubules and SR, has been the subject of much recent interest, but has remained an elusive problem to solve (Martonosi, 1984; Somlyo, 1985). One approach towards elucidation of this complex phenomenon is to study the consequence of the process, i.e., the rapid Ca^{2+} release effected through SR Ca^{2+} channels.

Fractionation of fragmented SR results in enrichment of Ca^{2+} release activity in the "heavy" SR vesicle fraction (Meissner, 1984), which corresponds to the terminal cisternae region of SR that is involved in a junctional association with the T-tubules. Studies employing rapid mixing (Nagasaki and Kasai, 1983; Ikemoto et al., 1985; Meissner, et al., 1986, Meissner, 1986*a*), and single-channel recording techniques (Smith et al. 1985, 1986) have shown that Ca^{2+} release from isolated heavy SR vesicles can be (*a*) stimulated by micromolar Ca^{2+} and millimolar adenine nucleotides, (*b*) inhibited by micromolar calmodulin and ruthenium red and millimolar Mg^{2+}, and (*c*) is mediated by a high-conductance Ca^{2+} channel. Recently, demonstration of the plant alkaloid ryanodine as a Ca^{2+} release channel-specific marker (Fleischer et al., 1985; Pessah et al., 1986; Meissner, 1986*b*; Rousseau et al., 1987) has facilitated isolation of the channel as a complex of apparent sedimentation coefficient 30S (Lai et al., 1987), which has been shown by several laboratories to comprise polypeptides of apparent relative molecular mass ~400,000 (Inui et al., 1987; Campbell et al., 1987; Lai et al., 1988). Upon incorporation of the 30S complex into planar lipid bilayers, channels with properties similar to those observed for the native SR Ca^{2+} release channel were evident. Negative-stain electron microscopy further revealed the complex to exist as an apparent four-subunit structure encircling a central "pore," which appeared morphologically identical to the unique structure of the protein bridges ("feet") which span the T-SR junctional gap (Lai et al., 1988).

In this report, we describe novel observations of heterogeneous conductance states occurring in single Ca^{2+}- and Na^+-conducting channels derived from incorporation of the purified 30S complex into planar lipid bilayers. Comparison of results obtained in this study with known properties of the native SR Ca^{2+} release channel (Smith et al., 1985, 1986; Rousseau et al., 1987) indicates the identity of the two channels, and also suggests that the characteristic 100 ± 10 pS unitary Ca^{2+} conductance of the native channel may result from simultaneous activation of several intrinsic, cooperatively coupled, sublevel conductances present within the channel oligomer. These results have been communicated in part in abstract form (Liu et al., 1988).

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MATERIALS AND METHODS

Preparation of purified Ca^{2+} release channel complex

The 30S ryanodine receptor/Ca^{2+} release channel complex was isolated by solubilization of heavy SR vesicles (Meissner et al., 1986) in 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), followed by sucrose density gradient centrifugation in the presence of CHAPS and exogenous phospholipid, as previously described (Lai et al., 1988). Gradient fractions (~20 μg protein/ml) corresponding to the peak of [^{3}H]ryanodine-bound receptor obtained in a separate parallel-centrifuged sucrose gradient were collected and analyzed for purity by SDS-polyacrylamide gradient gel electrophoresis and silver staining (Lai et al., 1988), and either used immediately for bilayer incorporation or stored frozen at −135°C. No differences were found between channels obtained in fresh and frozen samples.

Planar lipid bilayer measurements

The planar lipid bilayer technique employed in these studies was essentially the same as that used to observe the single-channel behavior of the native Ca^{2+} release channel, first described by Smith et al. (1985, 1986), with minor modifications. All lipids were purchased from Avanti Polar Lipids Inc., Birmingham, AL. Mueller-Rudin lipid bilayers (bovine brain phosphatidylethanolamine, bovine brain phosphatidylserine and L-α-diphytanoyl lecithin in a 5:3:2 ratio in *n*-decane solution) were formed on a small circular aperture (300 μm in diameter) through the wall of a plastic cup (polyvinylidene difluoride) separating two chambers (3 ml volume each). To observe channel-induced current fluctuations, aliquots of purified sample (0.2–3.0 μl) were added to one side (defined as *cis*) of the two chambers containing symmetrical solutions of buffered NaCl (20 mM NaPipes, 100 μM $CaCl_2$, 100 μM EGTA, pH 7.0 with various NaCl concentrations, as specified in the figure legends). Channels were incorporated spontaneously into the lipid bilayer and were detected as steplike increases in current. To obtain Ca^{2+} currents after the initial channel incorporation in NaCl solutions, the chambers were perfused with a "Ca^{2+} solution" of 53 mM $Ca(OH)_2$/250 mM Hepes, pH 7.4 *trans*, and a "Tris/Hepes buffer" of 125 mM Tris/250 mM Hepes, 100 μM $CaCl_2$, 100 μM EGTA, pH 7.4 *cis*, as described in the figure legends.

Electrical signals were filtered at 300 Hz through an eight-pole low-pass Bessel filter and digitized at 2 KHz for storage on hard disk.

RESULTS

The CHAPS-solubilized ryanodine receptor can be readily separated from other SR proteins due to its rapid migration rate when sedimented through density gradients of sucrose. SDS-gel electrophoresis of the ryanodine receptor peak fractions revealed the presence of a single major polypeptide with apparent relative molecular mass ~400,000 (Lai et al., 1988).

Upon addition of small aliquots of the purified 30S ryanodine receptor complex into the *cis* chamber of the bilayer apparatus, a large dilution (1,000–15,000-fold) of the CHAPS detergent resulted in spontaneous fusion of the protein into the membrane which was manifested by appearance of electrical currents through the bilayer. As described in a recent report from this laboratory (Lai et al., 1988), the purified protein, which is permeable to monovalent cations, was incorporated into the bilayer in solutions of symmetrical NaCl initially, because large conductances were derived under these conditions. A very noticeable and frequently observed phenomenon upon channel incorporation was the appearance of multiple conductance levels after an apparent single fusion event. Fig. 1 shows a representative selection of single-channel recordings obtained in symmetrical 100 mM NaCl buffer

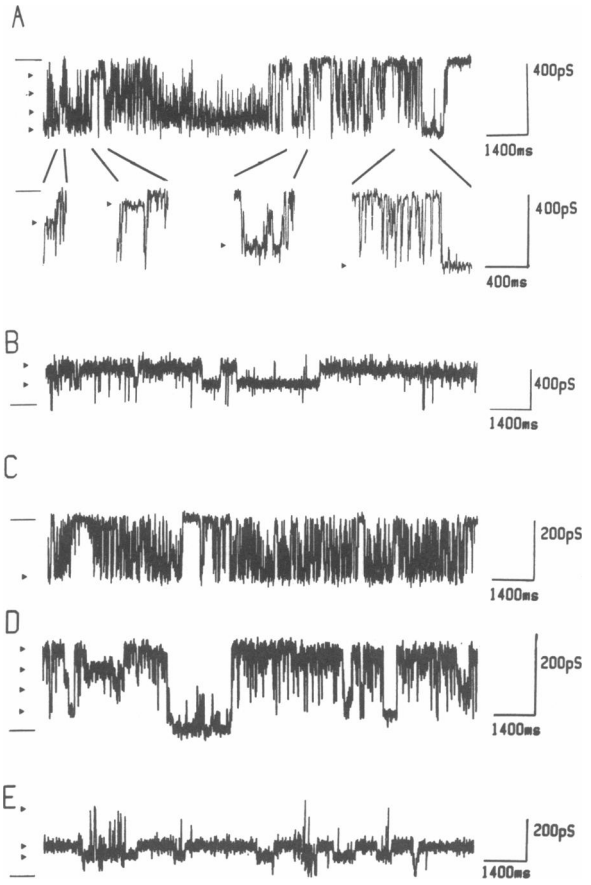

FIGURE 1 Multiple conductance levels of the Na^+-conducting Ca^{2+} release channel. Single-channel recordings of Na^+ current of purified ryanodine receptor incorporated into a planar lipid bilayer in symmetrical 100 mM NaCl buffer. The results of five separate experiments are shown (*A–E*). Baseline current denoted by bar, and conductance levels by arrows, on left of each trace. (*A*) Maximal conductance (γ_{max}) of 400 pS with additional conductances (γ) of 300, 200, and 100 pS. Each level shown on an expanded time scale in lower trace. Holding potential (HP) = 18 mV. (*B*) γ_{max} = 400 pS with additional 200 pS level. HP = −8 mV. (*C*) γ_{max} = 200 pS. HP = 30 mV. (*D*) γ_{max} = 200 pS with additional 150,100 and 50 pS levels. HP = −40 mV. (*E*) Slow-transition conductance levels of 150 and 100 pS with additional fast-transition 200 pS conductance occurring from 100 pS level. HP = −40 mV.

which illustrates a 400 pS major conductance and additional conductance levels of 300, 200, and 100 pS (*A*), and 400 pS main conductance with a second discrete level of 200 pS (*B*). In the majority of experiments in symmetrical 100 mM NaCl buffer (65%; Table 1), the main conductance level found was 200 pS (*C*), which was often resolved into four levels with successive 50-pS increments, resulting in conductances of 200, 150, 100, and 50 pS (*D*). The appearance of up to four clearly defined conductance levels was a consistent observation from experiment to experiment, from different preparations of purified protein, and with either fresh or frozen samples. An additional interesting, but rare, phenomenon observed was the disparate behavior of channel conductances within a single recording. Fig. 1 *E* shows a trace displaying a 200 pS conductance with fast open ↔ closed transitions which opened predominantly from a slow-transition 100 pS conducting level, but not from the main slow-transition 150 pS conductance. Because the rapid opening and closing events occurred from the 100 and not the 150 pS level and no 50 pS conductance was observed, this suggests that the various conductances described represent a single channel operating in different gating modes.

Although most single-channel recordings in NaCl buffers displayed multiple levels of conductance, the most frequent additional sublevel was always approximately half the magnitude of the maximal conductance (Table 1). Fig. 2 shows the effect of increasing NaCl concentration on the conductance of the two major conductance levels, which indicated a similar apparent saturation at ~500 mM NaCl. The NaCl concentration for half-maximal conductance (γ max/2) of ~75 mM was also similar for both conductance levels, suggesting that common channel permeation mechanisms for the conducting ion (Na^+ ions in these conditions; see below) was occurring for both conductances.

The typical pharmacological responses displayed by the native Ca^{2+} release channel were also exhibited by the

TABLE 1 Conductance states of the purified Ca^{2+} release channel complex in 100 and 500 mM NaCl

NaCl concentration	Maximum conductance	Number of appearances*	Additional conductances‡
mM	*pS*		*pS*
100	400	5	300, 200, 100
100	200	10	150, 100, 50
500	600	19	450, 300, 150
500	300	5	225, 150, 75

*Number of experiments in which single-channel recordings displaying clear subconductance states were obtained, the sum of which represents ~30% of the total number of recordings made. ‡The most frequently observed subconductance levels are underlined.

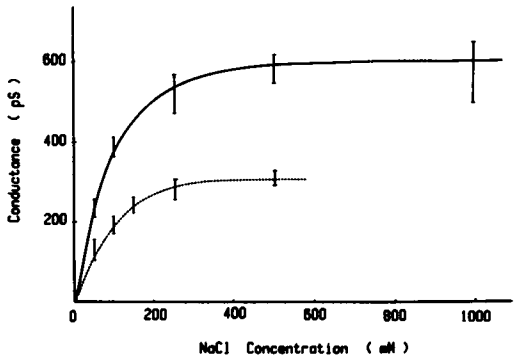

FIGURE 2 Conductance vs. [Na^+] curve. Relationship between major (*solid line*) and main sublevel (*dashed line*) conductance with increasing Na^+ concentration in symmetric NaCl buffers gives a rectangular hyperbola with γ_{max} of 600 and 300 pS, respectively, and identical K_m of ~75 mM.

Na^+-conducting reconstituted 30S ryanodine receptor complex. Fig. 3 *A* shows sequential traces from records obtained in symmetrical 150 mM NaCl in which the fraction of channel open time (P_0) was reduced from 0.36 to 0.001 by lowering the *cis*-free Ca^{2+} concentration from 6 μM to 40 nM (Fig. 3 *A*, *second trace*). When 2 mM ATP *cis* was added, the P_0 increased to 0.37 (Fig. 3 *A*, *third trace*), and on addition of 5 mM Mg^{2+} *cis*, the P_0 again decreased to 0.001 (Fig. 3 *A*, *lower trace*). The sequential traces of Fig. 3 *B* illustrate that a profound effect of ryanodine on the gating and conductance behavior of the reconstituted channel can be readily observed. Upon addition of ryanodine *cis* to 10 μM into symmetrical 500 mM NaCl buffer, the 600 pS main conductance (*upper trace*) closed briefly, then gated into an almost permanently open substate of 220 pS conductance (Fig. 3 *B*, *middle trace*, *arrow*), which then entered occasionally into a further, distinct, substate conductance of 150 pS (Fig. 3 *B*, *lower trace*). The transition from closed to ryanodine-modified substate after addition of micromolar ryanodine is a relatively rare event occurring in 10–20% of the experiments. The most commonly observed transition is from the open to ryanodine-modified state. The ryanodine-induced substates were very stable conditions of the channel and could be maintained, without responding to the addition of exogenous pharmacological agents, until the termination of the experiment by spontaneous breakage of the bilayer. These results were similar to those previously reported on the effect of ryanodine on the native Ca^{2+} release channel (Rousseau et al., 1987; Imagawa et al., 1987; Nagasaki and Fleischer, 1988). Caffeine has also been shown to modulate single native SR Ca^{2+} release channels by acting as an activator, with most pronounced enhancement of channel opening effected at low Ca^{2+} concentrations (Rousseau et al.,

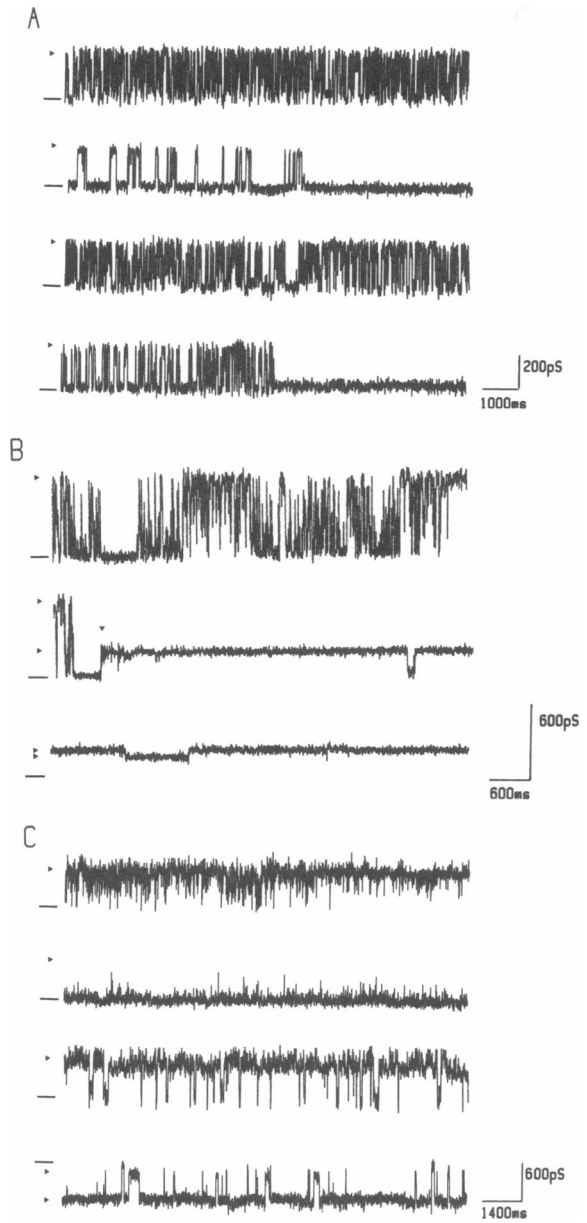

FIGURE 3 Pharmacological properties of the Na^+-conducting Ca^{2+} release channel. (*A*) Single-channel recording in 150 mM NaCl buffer, with 6 μM free Ca^{2+} *cis*, P_0 = 0.36 (*upper trace*), after adding EGTA *cis* to give 40 nM free Ca^{2+} *cis*, the channel inactivated to yield a steady-state P_0 = 0.001 (*second trace*), after adding ATP (sodium salt) *cis* to 2 mM, P_0 = 0.37 (*third trace*), and after adding Mg^{2+} *cis* to 5 mM, rapid inactivation again occurred to a steady-state P_0 = 0.001 (*lower trace*). HP = −10 mV. (*B*) Single-channel recording in 500 mM NaCl buffer, γ_{max} = 600 pS (*upper trace*), after adding ryanodine *cis* to 10 μM, γ = 220 pS (*second trace*), with an occasional γ = 150 pS level observed (*third trace*). HP = −20 mV. (*C*) Single-channel recording in 500 mM NaCl buffer, γ_{max} = 600 pS with 6 μM free Ca^{2+} *cis*, P_0 = 0.73 (*upper trace*), after adding EGTA *cis* to give 40 nM free Ca^{2+} *cis*, P_0 = 0.001 (*second trace*), and after adding caffeine *cis* to 10 mM, P_0 = 0.78 (*third trace*). HP = −5 mV. Lower trace, conditions as in third trace with HP = +5 mV.

1988). A similar activation by caffeine at low Ca^{2+} was also observed for the reconstituted ryanodine receptor channel. In Fig. 3 *C*, the P_0 of a 600 pS channel obtained in 500 mM NaCl was reduced from 0.73 to 0.001 by decreasing the *cis*-free Ca^{2+} from 6 μM to 40 nM (Fig. 3 *C*, *top* and *second trace*). When 10 mM caffeine *cis* was added, the channel reactivated within a few seconds and the P_0 increased accordingly to 0.78 without any change in the maximal current amplitude (Fig. 3 *C*, *third trace*). However, after caffeine activation, the additional conductance of one quarter (150 pS) of the maximum level of 600 pS, which was previously present but indistinct (Fig.

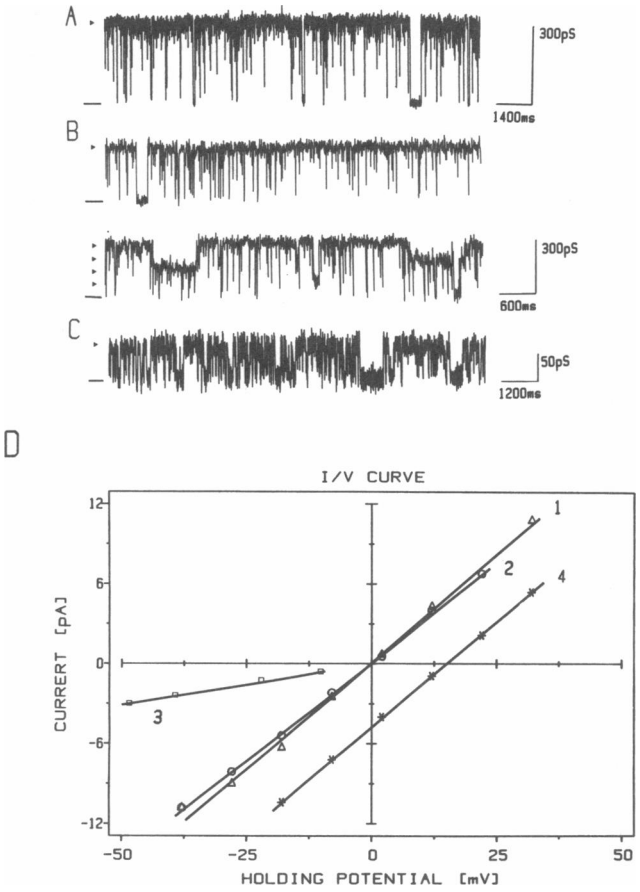

FIGURE 4 Monovalent and divalent cation permeability of the Ca^{2+} release channel. Single-channel recordings upon sequential perfusion of *cis* and/or *trans* chambers are illustrated (*A–C*). (*A*) Na^+ current in symmetric 500 mM NaCl buffer, γ_{max} = 310 pS. HP = −40 mV. (*B*) Na^+ current after *cis* and *trans* perfusion with 250 mM NaPipes buffer (100 μM $CaCl_2$, 100 μM EGTA, 250 mM NaPipes pH 7.0), γ_{max} = 290 pS (*upper trace*), and with additional conductance levels of 220, 145, and 75 pS (*lower trace*). HP = −30 mV. (*C*) Ca^{2+} current after *trans* perfusion with 24 mM $CaCl_2$, 10 mM NaPipes, 10% glycerol, γ_{max} = 70 pS. HP = −40 mV. (*D*) Current-voltage relationship in conditions *A*, *B* and *C* are given by curves 1, 2 and 3, respectively. Curve 4 was obtained in 250 mM NaPipes buffer *cis*, 500 mM NaCl buffer *trans*, γ_{max} = 310 pS (i.e., intermediate step between recordings in condition *A* and *B*).

3 *C*, *top trace*), could be more clearly discerned (Fig. 3 *C*, *bottom trace*, *arrow*).

Fig. 4 shows a series of single-channel recordings taken from one continuous experiment in which a main conductance of 310 pS observed in 500 mM NaCl (Fig. 4 *A*) was found to remain essentially unchanged, at 290 pS, after perfusion of the *cis* and *trans* chambers with 250 mM NaPipes buffer (Fig. 4 *B*). This result, together with other experiments in which the reversal potential shifted to $> +50$ mV after perfusion of the *cis* chamber with TrisHepes buffer, indicated that the major conducting ion under these conditions must be Na^+. The lower trace of Fig. 4 *B* further shows that four approximately equivalent increments of 75 pS, resulting in conductance levels of 290, 220, 145, 75 pS, could be displayed in NaPipes buffer. When the *trans* chamber was then perfused with a solution of 24 mM $CaCl_2$ a 70 pS conductance level resulted (Fig. 4 *C*), which corresponds to the 100 ± 10 pS unit conductance regularly found in 50 mM Ca^{2+} *trans*. In Fig. 4 *D* is plotted the current-voltage relationship for the above-mentioned experimental conditions and from which, by applying constant field theory, the reconstituted channel's permeability ratio for Ca^{2+} over Na^+ ions (P_{Ca}/P_{Na}) was calculated to be ~5. The permeability ratio for divalent over monovalent cations could not previously be determined in single-channel recordings of native SR Ca^{2+} release channels due to the presence of endogenous channels permeable to monovalent cations and anions in the native SR vesicles used for bilayer incorporation (Miller, 1978; Meissner, 1983).

To obtain pure Ca^{2+} currents after channel incorporation in 100 mM NaCl, the *cis* chamber was perfused with Tris-Hepes buffer and the *trans* chamber with Ca^{2+} solution. The reversal potential observed for these ionic conditions was +30 mV which gives a ratio P_{Ca}/P_{Tris} of ~8.7. This latter value is very similar to that reported previously for the native (Smith et al., 1985, 1986) and ryanodine receptor-derived channel (Imagawa et al., 1987). Fig. 5 illustrates a typical recording of a Ca^{2+} current recorded thus, which exhibited a unitary conductance of 90 pS (Fig. 5 *A*, *top trace*). When the *cis*-free Ca^{2+} concentration was lowered from 2.5 μM to 70 nM, the P_0 changed from 0.04 to 0.005 (Fig. 5 *A*, *second trace*), but was then increased to 0.07 when ATP *cis* was added to 2 mM (Fig. 5 *A*, *third trace*). After addition of Mg^{2+} *cis* to 2.5 mM the P_0 again decreased to 0.02 (Fig. 5 *A*, *fourth trace*), and was further reduced to ~0 after addition of 20 μM ruthenium red *cis* (Fig. 5 *A*, *lower trace*). The characteristic effect of ryanodine on the Ca^{2+} conducting channel was also readily observed (Fig. 5 *B*). Upon addition of 10 μM ryanodine *cis* to a channel of unit conductance 90 pS, a modified sublevel of conductance of 35 pS resulted, which could not be reversed by perfusion of the free ryanodine in the *cis* chamber. The reduction in

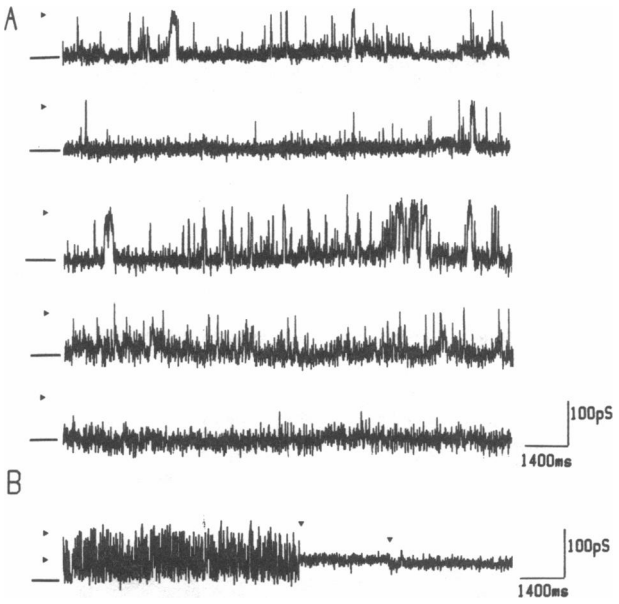

FIGURE 5 Pharmacological properties of the Ca^{2+}-conducting Ca^{2+} release channel. Single-channel recordings in 53 mM Ca^{2+} solution *trans* and TrisHepes buffer *cis*, γ = 90 pS (*A* and *B*). (*A*) With 2.5 μM free Ca^{2+} *cis*, P_0 = 0.04 (*upper trace*), after adding EGTA *cis* to give 70 nM free Ca^{2+} *cis*, P_0 = 0.005 (*second trace*), then ATP (sodium salt) *cis* to 2 mM, P_0 = 0.07 (*third trace*), then Mg^{2+} *cis* to 2.5 mM, P_0 = 0.02 (*fourth trace*), and finally ruthenium red *cis* to 20 μM, P_0 = 0 (*lower trace*). Before perfusion with Ca^{2+} solution *trans* and TrisHepes buffer *cis*, the channel's Na^+ ion unitary conductance was 400 pS with an additional 200 pS sublevel, in symmetric 100 mM NaCl buffer. (*B*) Upon addition of ryanodine *cis* to 10 μM, the γ_{max} = 90 pS abruptly decreased to γ = 35 pS, with a further decrease to γ = 27 pS also noticeable (*vertical arrows*).

unit conductance to ~40% of the fully open state induced by ryanodine for both the Na^+- (Fig. 3 *B*) and Ca^{2+}-conducting (Fig. 5 *B*) channel, is much the same value as that reported for the native Ca^{2+} release channel from heavy SR vesicles (Rousseau et al., 1987; Imagawa et al., 1987; Nagasaki and Fleischer, 1988). Moreover, the data of Figs. 3 and 5, which shows the presence of Ca^{2+}, caffeine, and adenine nucleotide-activating sites, Mg^{2+} and ruthenium red inhibitory sites, and the ryanodine interaction site, therefore indicate that the entire gamut of pharmacological responses so far observed for the native Ca^{2+} release channel in single channel recordings can be faithfully reproduced in Na^+- or Ca^{2+}-conducting channels derived from the reconstituted purified 30S ryanodine receptor complex.

The distinctive appearance of discrete sublevels of conductance observed for monovalent cations was also demonstrated in experiments where Na^+ was replaced by Ca^{2+} as the permeant ion. Fig. 6 shows representative pairs of recordings from a series of different experiments in which Na^+-conducting channels were perfused with

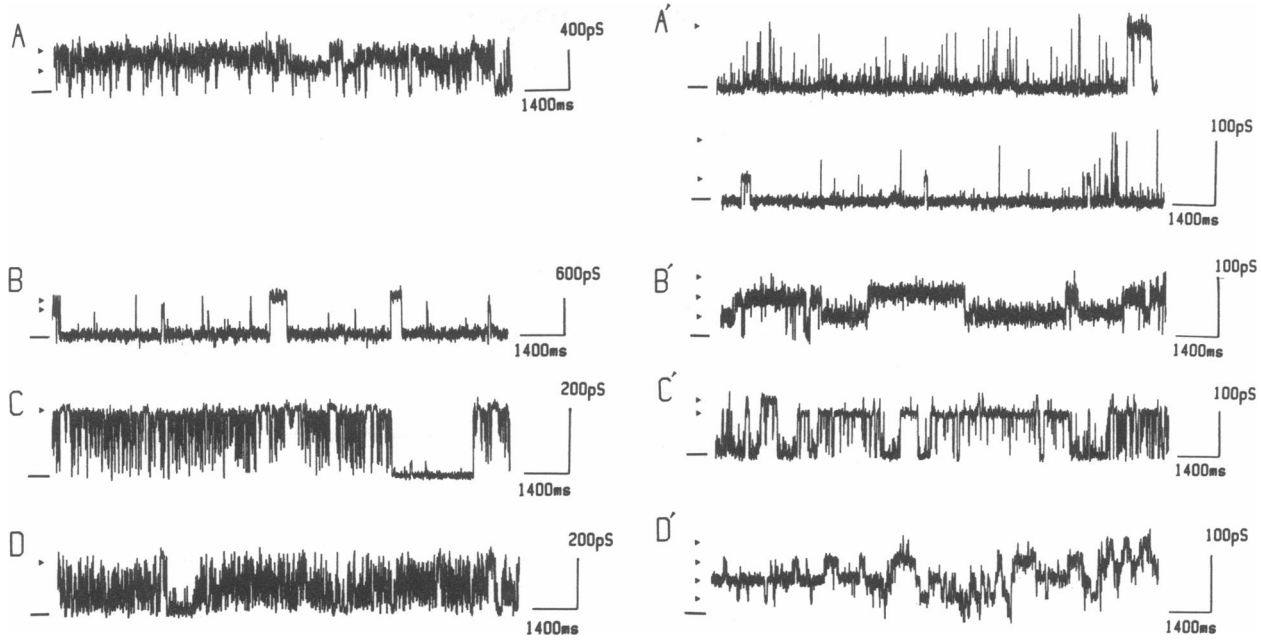

FIGURE 6 Relationship between Na^+- and Ca^{2+}-conductance behavior of the Ca^{2+} release channel. Single-channel recordings of Na^+ conductance in NaCl buffers (*left*) and subsequent Ca^{2+} conductance upon perfusion with Ca^{2+} solution *trans* and TrisHepes buffer *cis*(*right*). (*A*) In symmetric 100 mM NaCl buffer, γ_{max} = 400 pS with an additional 200 pS level. HP = −10 mV. (*A'*) In Ca^{2+} solution (*trans*) γ_{max} = 95 pS (*upper trace*) with a 33 pS conductance also observed after reduction of *cis* free Ca^{2+} to 6 nM (*lower trace*). HP = −30 mV. (*B*) In symmetric 500 mM NaCl buffer, γ_{max} = 600 pS with an additional 450 pS level. HP = −5 mV. (*B'*) In Ca^{2+} solution (*trans*) γ = 65 and 35 pS with occasional 95 ps level also observable. HP = −30 mV. (*C*) In symmetric 100 mM NaCl buffer, γ_{max} = 200 pS. HP = −30 mV. (*C'*) In Ca^{2+} solution (*trans*), γ_{max} = 90 pS and occasionally 120 pS. HP = −20 mV. (*D*) In symmetric 100 mM NaCl buffer, γ_{max} = 200 pS. HP = −20 mV. (*D'*) In Ca^{2+} solution (*trans*), with 6 nM free Ca^{2+} *cis*, γ = 30, 60, and 90 pS with occasional 120 pS level. HP = −20 mV.

Ca^{2+} solutions and the various levels of conductance for Na^+, initially, and Ca^{2+}, subsequently, are illustrated. Fig. 6 *A* shows a Na^+ current with a unit conductance of 400 pS (in 100 mM NaCl buffer) and a sublevel conductance of 200 pS, which upon perfusion resulted in a 95 pS Ca^{2+} channel. However, when the μM Ca^{2+} in the *cis* medium was reduced to 6 nM by addition of EGTA *cis*, a new conductance level of 35 pS was clearly distinguishable (Fig. 6 *A'*, *lower*). A similar conductance of 35 pS, and also a new level of 65 pS, was observed in another recording performed in μM Ca^{2+} *cis* (Fig. 6 *B'*). In this experiment, the 95 pS maximal level was reached only infrequently. The previous Na^+ unit conductance before perfusion with Ca^{2+} solution was of 600 pS (in 500 mM NaCl buffer) with an additional 450 pS sublevel (Fig. 6 *B*). In some instances, there appeared a distinct higher-level Ca^{2+} conductance of 120 pS, which was infrequently present but could be clearly discriminated from the major conductance level of 90 pS (Fig. 6 *C'*). The corresponding Na^+ unit conductance obtained for this channel before perfusion was 200 pS (in 100 mM NaCl buffer, Fig. 6 *C*). In Fig. 6 *D* an initial Na^+ current of 200 pS unit conductance (in 100 mM NaCl buffer) afforded a Ca^{2+} current which displayed four clear conductance levels of 30, 60, 90, and 120 pS (Fig. *D'*). The major conductance states were 60 and 90 pS, with the 30 and 120 pS levels observed less frequently. Thus, as well as illustrating the presence of sub-and higher-states of Ca^{2+} conductance, an additional, perhaps surprising, corollary of Fig. 6 and other similar experiments (not shown) is that there appears to be no strict correlation between the conductance size and number of conductance levels of the reconstituted ryanodine receptor channel when it switches from conducting Na^+ to conducting Ca^{2+} ions upon buffer perfusion.

DISCUSSION

The ryanodine receptor from skeletal muscle SR has been purified by several laboratories using column chromatography (Inui et al., 1987), immunoaffinity chromatography (Campbell et al., 1987; Imagawa et al., 1987) and density gradient centrifugation (Lai et al., 1987, 1988), and found to comprise high molecular weight polypeptides of M_r 350,000–450,000 which are assembled into a large oligomeric complex of apparent sedimentation coefficient 30S (Lai et al., 1987, 1988). Structural analysis of

the purified protein by negative-stain electron microscopy indicated morphological identity with the tetrameric feet structures (Ferguson et al., 1984) which span the T-SR junctional gap (Inui et al., 1987; Lai et al., 1987, 1988), and also revealed a central hole of 1–2 nm diameter within the complex, surrounded by an area of higher protein density (Lai et al., 1988).

Recently, successive reports from three laboratories indicated, in unison, that incorporation of the purified ryanodine receptor into planar lipid bilayers induced a Ca^{2+}-conducting pathway, suggesting its putative role as the Ca^{2+} release channel of SR (Imagawa et al., 1987; Lai et al., 1988; Hymel et al., 1988). However, there were markedly discrepant properties obtained for the channel activity described by the various investigators. Imagawa et al. (1987), using digitonin-purified ryanodine receptor, showed that two Ca^{2+} conductance states of 35 and 22 pS were present in their reconstituted preparation, which, significantly, lacked the characteristic pharmacological responses of the native Ca^{2+} release channel, although [^{3}H]ryanodine binding studies indicated retention of these sites in the purified protein. Lai et al. (1988) and Hymel et al. (1988) both found, with CHAPS-purified ryanodine receptor, that the native channel's pharmacological responses to Ca^{2+}, ATP, Mg^{2+}, and ruthenium red could be functionally retained; however, only the former group (Lai et al., 1988) described reconstitution of the large unitary Ca^{2+} conductance of ~90 pS reminiscent of the native channel, whereas the latter (Hymel et al., 1988) reported the occurrence of a wide variety of conductances ranging from 3.8 to 120 pS and larger. In congruity with Lai et al. (1988), Smith et al. (1988) reported in a recent abstract that, with CHAPS-purified receptor, they were able to observe the pharmacology and unitary conductance of the native release channel. It thus appears a consensus view is beginning to emerge that the CHAPS-purified ryanodine receptor complex consists of an oligomeric assembly, possibly tetrameric (Lai et al., 1988), of high M_r proteins of ~400,000 which comprises the high conductance pathway and relevant regulatory sites of the native SR Ca^{2+} release channel.

In this report we have confirmed and extended the above-mentioned "consensus" observations by demonstrating, in addition, the well-defined effects of ryanodine and caffeine on the purified Ca^{2+} release channel/ryanodine receptor. We further showed that the purified channel is capable of large monovalent cation (Na^+) conductances (γ_{max} = 600 pS in 500 mM NaCl buffer) and displays at least four distinct levels of conductance for both Na^+ and Ca^{2+} ions. The purified channel's unitary conductance for Na^+ ions of 600 pS in 500 mM NaCl buffer appears to be the maximum attainable level since the native Ca^{2+} release channel, when recorded under identical conditions, also displayed a unitary conductance of 600 pS (unpublished observations). The relative permeability of the Ca^{2+} release channel to other ions could not previously be analyzed in single-channel recordings using native heavy SR vesicles due to the presence of "contaminating" channels permeable to K^+ and Cl^- ions (Miller, 1978; Meissner, 1983). Isolation of the release channel has now made this study feasible and reveals that the channel is highly selective for cations over anions (Cl^-). However, the relatively low permeability ratio P_{Ca}/P_{Na} ~5 observed for the release channel suggests a less stringent divalent over monovalent selectivity as compared with the voltage-sensitive Ca^{2+} channels from T-tubules, which display a permeability ratio $P_{Ca}/P_{Na} > 25$ (Coronado and Affolter, 1986). These same T-tubule Ca^{2+} channels have also been shown to conduct monovalent cations in the absence of free divalent cations (Coronado and Smith, 1987). The high conductance observed for monovalent cations is a marked property of the purified ryanodine receptor channel and is in accord with the relatively large Ca^{2+} conductance of the release channel. Similarly high conductance (γ_{max} = 1 nS) through the reconstituted purified channel has been reported for K^+ ions (Smith et al., 1988).

A puzzling finding that we encountered using Na^+ as the permeant ion is the presence of two distinct levels of maximal unit conductance within recordings from separate experiments which were performed in identical Na^+ concentrations (Table 1). With 100 mM Na^+, a 400 pS maximal conductance is found which is less frequently observed (33%) than in the majority of experiments in which a maximal conductance of 200 pS is present (67%). In each case, up to four clearly identifiable conductance levels can be distinguished, with the major sublevel of conductance being half the maximal level, i.e., 100 and 200 pS for the 200- and 400-pS channels, respectively. A similar result is evident from recordings obtained in 500 mM Na^+ (Table 1). Two levels of maximal conductance, 600 and 300 pS, are found, again with each displaying four sublevel conductances and with the major substate being half the maximal conductance. However, in these higher Na^+ concentrations, the larger of the two maximal conductances (600 pS) was obtained more often (79%) than that of the lower 300 pS level (21%). Two rational explanations for this type of apparently inconsistent behavior are possible: (*a*) The lower maximal level, 200 and 300 pS in low and high Na^+, respectively, results from partial denaturation of the channel protein which, in a highly reproducible fashion, produces half the conductance that is seen normally for all (maximal and sub-) levels of conductance, i.e., each of the four conductances are equally reduced to produce 50% of their normal conductance. (*b*) There are not four but eight sublevels of conductance, with multiples of 50 and 75 pS in low and high Na^+, respectively, which, when all are fully coupled,

produce the highest conductances observable of 400 and 600 pS. The presence of "half" conductances of 200 and 300 pS must be attributed to irreversible alteration of the conducting pathway resulting in inactivation/denaturation of four of the eight conductances. A dependence of the channel conformation on Na^+ concentration must also be postulated to account for the change in proportion of higher and lower maximal conductances in the different Na^+ buffers.

An additional, almost provocative, observation is the presence, in Ca^{2+} buffers, of four approximately equivalently spaced conductance levels, with the highest 120 pS level distinct from the major conductance level of 90 pS (Fig. 6). Because the native Ca^{2+} release channel from heavy SR vesicles has been found to exhibit a unitary conductance of 100 ± 10 pS under identical recording conditions, this indicates that the presence of a higher-level conductance of 120 pS described here may not be a commonly occurring conductance state of the native release channel. Further, the sublevels of Ca^{2+} conductance observed for the reconstituted purified channel (30 and 60 pS) are generally not evident in single-channel recordings from native membranes. However, we have infrequently observed the appearance of subconductance states in the native Ca^{2+} release channel which are short-lived and at the limit of resolution of the planar bilayer recordings. In this regard, it is of interest to note that a recent report describing the patch-clamping of "sarcoball" membranes from frog skeletal muscle revealed that, in addition to the major conductance level of 90–100 pS obtained in 53 mM Ca^{2+}, the Ca^{2+} release channel also displayed bursts of higher conductance up to 140–150 pS (Stein and Palade, 1988). These findings, and ours, may indicate that four (or more) equivalent intrinsic conductance levels of ~30 pS are associated within the channel multimeric complex such that three of the four conductances are cooperatively coupled to produce a 90–100 pS "native" conductance, with the fourth level only loosely, or not at all, coupled to the other three. When, rarely, all conductances are simultaneously open, a "full" conductance of ~120 pS is produced. Hence, the presence of sublevels of conductance in the reconstituted, purified channel may be a consequence of modification of this coupling, upon its solubilization in detergent and subsequent isolation, to produce the variety of conductance levels observed. The possibility that detergent may be inducing the uncoupling of channel conductances via an interaction with the extramembranous portion of the channel protein appears unlikely because no such effect was observed when CHAPS, up to 160 μM, was present in single-channel recordings of the native Ca^{2+} release channel (unpublished observations).

The observations of multiple conductance states also pose the possibility that their presence may be of relevance during physiological Ca^{2+} release from SR, because the release channel is believed to be attached to the muscle surface membrane via a specific association with one, or more, T-tubule proteins. These putative Ca^{2+} release channel-specific proteins of the T-tubule membrane may be directly involved in regulation of the various conductance states of the SR Ca^{2+} release channel. The predominance of four conductance states may, or may not, be related to the apparent tetrameric structure of the receptor complex, as inferred from ryanodine binding stoichiometry, sedimentation, and structural data (Lai et al., 1988). Analysis of negatively-stained electron micrographs of the complex indicated that in addition to the central hole within the clover-leaf structure, each of the four leaves possessed a large, deep depression or hole (Lai et al., 1988). It has, therefore, to remain conjecture whether (*a*) each leaf constitutes an individual ion-conducting channel which contributes a sublevel conductance of ~30 pS to an oligomer which functions in a multibarreled fashion, as has been suggested for a chloride channel from *Torpedo* electroplax (Miller and White, 1984) and a renal tubule K^+ channel (Hunter and Giebisch, 1987), or (*b*) the central hole is the channel pore whose opening is regulated by separate, coupled subgates present within the channel complex. The former case appears the least likely of the two possibilities given that the diameter of the hole (or depression) in each leaf measures up to 4 nm, a size much larger than would be expected for a channel pore (Hille, 1984), and the frequently distinguishable appearance of single breaks in the loop of a leaf to produce two "arms" protruding from the central higher density area (unpublished observations). However, a more comprehensive understanding of the mechanism involved in the alternative suggestion above is clearly lacking and awaits further detailed analysis. One possible approach, currently under investigation in this laboratory, would be to identify conditions to dissociate the purified oligomeric complex into monomers, and then determine, by bilayer reconstitution, whether channels of smaller unitary conductance can be produced.

The presence of subconducting states is not a new phenomenon, having been reported in a variety of other ion channels which have been studied by patch-clamping techniques, such as the acetylcholine (Hamill and Sackmann, 1981), glycine and GABA (Hamill et al., 1983), and glutamate and aspartate (Cull-Candy and Usowicz, 1987) receptors, all of which are believed to comprise an oligomeric association of multiple subunits. In planar lipid bilayer studies of the multimeric dihydropyridine-sensitive Ca^{2+} channel, subconductances have also been observed (Ma and Coronado, 1988). It may, therefore, be

likely that all multisubunit ion channels possess subconductance states, and that the native Ca^{2+} release channel from heavy SR vesicles, when analyzed at better time resolution in patch clamp recordings, will also reveal distinct subconducting levels.

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