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Ryanodine Receptor of Skeletal Muscle Is a Gap Junction-Type Channel

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In the sarcoplasmic reticulum membrane of skeletal muscle, the ryanodine receptor forms an aqueous pore identified as the calcium-release pathway that operates during excitation-contraction coupling. The purified ryanodine receptor channel has now been shown to have four properties usually associated with gap junction channels: (i) a large nonspecific voltage-dependent conductance consisting of several open states; (ii) an inhibition of open probability by low *p*H; (iii) an inhibition of open probability by calcium; and (iv) a sensitivity to blockade by heptanol and octanol but not other alcohols. This functional homology may provide an insight into the mechanism of how muscle cells transduce depolarizadon into an intracellular release of calcium.

COUPLING OF MUSCLE CELL EXCITATION to mechanical contraction occurs at the junction between the transverse tubular (TT) foldings of the plasma membrane and the membrane of the sarcoplasmic reticulum (SR), the intracellular organelle involved in cellular Ca^{2+} homeostasis. In skeletal musde, the 450-kD ryanodine receptor has been identified as the major component of the "feet" structures that span the 100 Å cleft separating TT and SR (*1-3*). Using planar bilayer recording, we (*4, 5*) and others (*3, 6*) recently identified the purified 450-kD ryanodine receptor (Fig. 1A) as the Ca^{2+}-release channel of skeletal muscle. The purified channel displays a weak selectivity between Ca^{2+} and K^+ ions ($P_{Ca}/P_K = 6$; $P_{Cl} = 0$; where P is permeability) and a saturable conductance of 1 nS in K^+, 170 pS in Ca^{2+}, and about 100 pS in tris or choline (*5, 7*). Two characteristics of release channels, activation by ryanodine (*4-6*) and blockade by ruthenium red (*7*), arc shown in Fig. 1C. As in native release channels (*4*), submicromolar ryanodine decreases the conductance and increases mean open time, driving the purified channel into state of quasi-permanent activation. Further addition of cis-intemal 1 μ*M* ruthenium red produces a flickery block and a marked decrease in the fraction of time spent open or the open probability (p_o). In this experiment (Fig. 1C) $p_o = 0.16$ in control, $p_o = 0.85$ after treatment with ryanodine, and $p_o = 0.18$ after addition of ruthenium red. Activation by adenine nucleotides (*7*), another characteristic of release channels, has been described elsewhere (*5*). Thus, the purified 450-kD receptor is a functional SR release channel.

Previous studies in SR vesicles have shown that molecules up to the size of glucose could permeate through the release channel (*8*). This broad permeability to electrolytes and nonelectrolytes and the fact that the open channels have several levels of conductance of large unit value (Fig. 1B) suggested a similarity to gap junctions. Channels formed by purified gap junction proteins have relatively large conductances that in physiological saline vary from 400 pS (2 nS in 1*M* KCl) in the bovine lens MIP-26 protein (*9*) to 140 and 280 pS in the rat liver 27 kD protein (*10*). Similar values have been reported in situ, in heart and lacrimal cells (*11*). In the case of the ryanodine receptor there appears to be at least three open states (Fig. 1B) with approximate conductances in 0.25*M* KCl of 800 pS, 400 pS (260 pS in 0.15*M* KCl), and 200 pS. The largest conductance (800 pS) is associated with the briefest lifetime (60 μs), whereas the lowest conductance (200 pS) is associated with the longest lifetime (8 ms). The 400-pS channel is most frequently observed and is thus the main contributor to the p_o. Thus, like junctional channel openings recorded with purified proteins (*9, 10*), the ryanodine receptor has one or more conductance states that pass large nonspecific currents.

The strongest homology to junctional channels was found in the effects of protons, Ca^{2+} and 1-octanol, the well-known uncouplers of gap junctions (*12*). Over a narrow range, low *p*H doses the ryanodine receptor channel (Fig. 2A). We focused exclusively on the behavior of the most common 400-pS channel, which was present alone without other conductances in 78% of all recordings ($n = 48$). At *p*H 7.4 and *p*Ca 5, the average p_o is between 0.25 and 0.10 (average $p_o = 0.2$, SD = 0.05, $n = 10$). In this particular recording, $p_o = 0.14$. Reducing *p*H on either side of the channel results in a decrease in the frequency of openings and a shortening of lifetimes. Both parameters contribute to a decrease in p_o (Fig. 2D). Open probability drops from its near maximum value at *p*H 7.4 (normalized to 1) to almost null at *p*H 6.5. The absolute p_o at *p*H 6.5 was in the range $0.05 > p_o > 0.001$ (average $p_o = 0.03$, SD = 0.02, $n = 8$). The effect of *p*H was fully reversible within the range tested, and p_o reaches a maximum ($p_o = 0.35$) at *p*H values >7.6. The dose-response curve for *p*H titrations from either side (Fig. 2D) could be fitted with a cis *p*K. value for H^+ of 7.2 (SD = 0.12,$n = 4$), a trans value of 7.1 (SD = 0.05, $n = 4$), and Hill coefficients for H^+ of (cis) 4 (SD = 1.04, $n = 4$) and

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(trans) 4 (SD = 0.74), which indicates a steep relationship between H^+ concentration and channel opening. That solution *p*H actually modifies gating of ryanodine receptors is shown in Fig. 2, B and C, and in a plot of p_o versus voltage in Fig. 2E. At *p*H 7.4 channels remain open over a wide range of voltage (-100 mV to +120 mV). However, at *p*H 7.15 most channels are closed below +60 mV and they begin to open in the range of +60 to +100 mV. Transition from closed to open had a midpoint at +85.7 mV (SD = 9.3 mV, n = 8) and there was an e-fold increase in p_o every 3.5 mV. Thus, the receptor channel is steeply dependent on both membrane potential and *p*H, with the switching from closed to open occurring over an unusually narrow range.

At constant *p*H (7.4) and holding potential (+50 mV), p_o can be controlled by Ca^{2+} (Fig. 3). Open probability in symmetrical *p*Ca 5 is halved by cis 250-μM Ca^{2+} (SD = 50 μM, n = 10) with a Hill coefficient of 1.3 (SD = 0.l4, n = 10) or halved by trans 800-μ*M* Ca^{2+} (SD = 100 μ*M*, n = 4) with a Hill coefficient of 0.8 (SD = 0.12, n = 4). Activation of the channel by Ca^{2+} in the range of *p*Ca 7 to 5 is shown in the inset of Fig. 3, bottom. The latter demonstrates that, similar to Ca^{2+} fluxes in SR vesicles and native ryanodine-rcceptor release channels, the effect of Ca^{2+} is biphasic, consisting of an activation phase at high *p*Ca and an inhibitory phase at low *p*Ca (*5, 7, 8*). Inhibition by Ca^{2+} was reversible and produced a decrease in the mean open time and in the number of events per unit time. As in the case of protons, the lack of a marked sidedness of cis versus trans Ca^{2+} blockade suggested that the Ca^{2+} and proton sites are actually located in the aqueous pore, hence rendered accessible from both solutions. This is different from proton and Ca^{2+} blockade in gap junctions where sites are accessible from the cytoplasm only (*13*). However, this likely reflects the fact that in gap junctions the extracellular entryway of the pore is inaccessible from the extracellular solution. Finally, the ryanodine receptor was selectively inhibited by 1-heptanol and 1-octanol (Fig. 4). Each of six alcohols was tested separately after a control period with a constant $p_o > 0.2$ (Fig. 4, top traces). Over several minutes, the addition of 0.1% v/v (~6 m*M*) heptanol or octanol produced a complete closure of channels at all potentials from –80 mV to +80 mV. No effects on p_o were observed with hexanol, nonanol, or decanol. We also tested pentanol and found voltage-dependent effects. Pentanol had no consequence at a holding potential of –80 mV but inactivated the channel at +80 mV within 20 to 40 s. Reactivation required negative holding po-

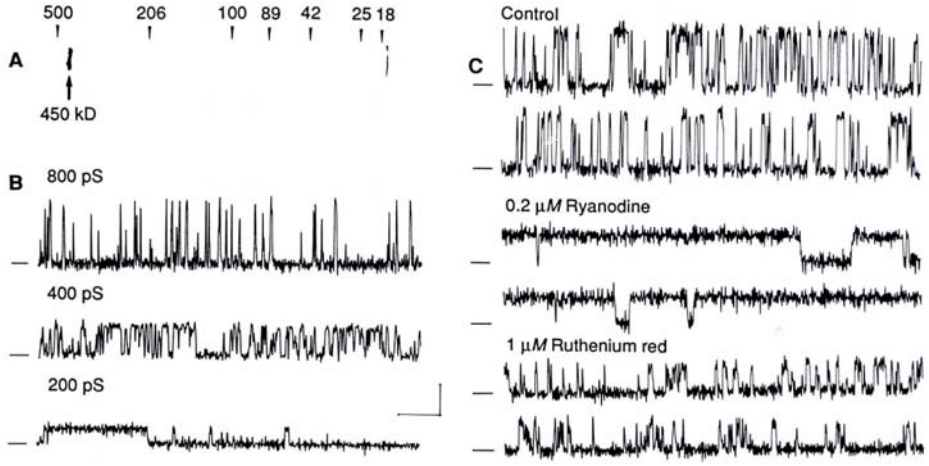

Fig. 1. (**A**) SDS-polyacrylamide gel electrophorcsis analysis of purified ryanodine receptor (3 μg) on a 3 to 12% gradient gel stained with Coomassie blue. The 450-kD receptor, indicated by an arrow (450 kD), migrated as a single band. Standards are indicated on top and are the same as in (*4*). (**B**) Multiple conductances of the purified ryanodine receptor channel. All records are at a holding potential of +100 mV (scale bars, 50 pA and 10 ms), 3-kHz low-pass filtering. Recording solution was symmetrical 0.25*M* KCl, 10 μ*M* $CaCl_2$, 10 m*M* tris-Hepes, *p*H 7.4. Traces were selected from eight separate experiments and represent the most frequently observed conductance levels. Linear fit of current-voltage characteristics resulted in slope conductances of 825, 420, and 210 pS (labeled 800,400, and 200 pS, respectively). (**C**) Traces from the same 400-pS channel at +100 mV (scale bars, 25 pA and 10 ms) before (control) and after the addition of 200 n*M* ryanodine and 1 μ*M* ruthenium red. Baseline is indicated next to each record. Ryanodine receptor was purified from CHAPS-solubilized triads (*4, 5*). Purity of the 450-kD protein determined by gel scans was greater than 95%. Scatchard analysis of [^{3}H] ryanodine binding to the purified receptor (*4, 5*) yielded a straight line with an apparent B_{max} and K_d of 490 pmol/mg and 7 n*M*, respectively. Unless specified, all recording solutions on both sides of the planar bilayer were composed of 0.25*M* KCl,10 μ*M* $CaCl_2$, and 10 m*M* Hepes-tris, pH 7.4. Chambers are denned as cis (side of protein addition, connected to head stage amplifier) and trans (protein-free side connected to ground). Receptor channels were incorporated into bilayers as described (*5*). Sidedness of incorporated channels is ds, intracellular and trans, extracellular (*7*). Receptor protein concentration in cis solution was 0.5 to 1.0 μg/ml. Phospholipid composition and recording procedures are described in (*4, 5, 7*). Total recording time in all figures was >100,000 s of which 11,400 s (with an average of 300 open events per second) were low-pass filtered on an 8-pole Bessel, digitized, and analyzed by computer. The total number of separate recordings was 48, from four preparations of purified receptor.

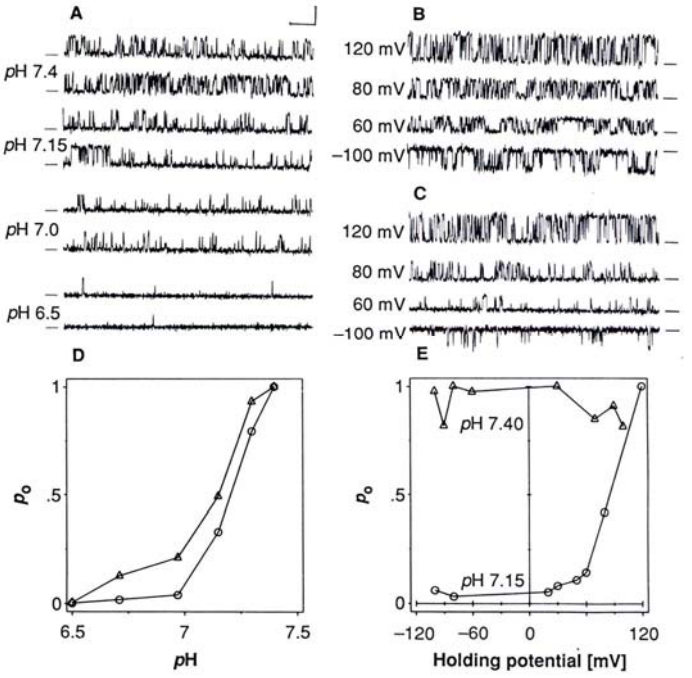

Fig. 2. The *p*H and voltage dependence of the ryanodine receptor channel. (**A**) Records from the same channel at a holding potential of +80 mV. Calibrated volumes of HCl or KOH were added to the cis or trans chambers to bring the solution *p*H to the desired final value. Single channel records are from a trans-side titration at a constant cis *p*H of 7.4. (**D**) p_o versus *p*H for a cis-side titrarion at a constant trans *p*H of 7.4 (triangles, n = 4 experiments) or a trans-side titration at a constant cis *p*H of 7.4 (circles, n = 4 experiments). p_o at pH 7.4 on both sides was taken as unity. (**B**) Voltage dependence at cis and trans *p*H 7.4. (**C**) Voltage dependence at cis and trans *p*H 7.15. (B) and (C) are from separate experiments. (**E**) p_o versus voltage at *p*H 7.4 (triangles, n = 10 experiments) and separately at *p*H 7.15 (circles, n = 4 experiments). Scales are 40 pA, 10 ms for all traces. A baseline trace is indicated for each record.

tendals. Voltage age dependence similar to that of pentanol was not observed for the rest of the alcohols tested.

In many tissues, including cardiac muscle (*12*), 0.1 to 0.2% 1-octanol lowers cell-cell electrical coupling and, when investigated in single channel recordings, leads to channel closure (*11*). The same result was reported in purified gap junction-channel proteins (*9*). In all preparations investigated (*12*), protons increase junctional resistance with *p*Ks in the range 7.3 to 6.5 and Hill coefficients ranging from 4.5 in embryonic cells to 1.0 in Purkinje fibers. However, the Ca^{2+} dependence and voltage dependence are highly variable. In general, it appears that when Ca^{2+} sensitivity is present, the Ca^{2+} levels that uncouple cells are much higher than the resting intracellular Ca^{2+}, in mammalian heart and salivary glands, on the order of 40 to 80 μ*M* Ca^{2+} (*13*). Our results show that dosing of ryanodine receptor channels by gap junction uncouplers followed quantitatively the results found in many of the cell pairs mentioned above. Proton blockade has an average (cis plus trans) *p*K of 7.2 and Hill coefficient of 4 (Fig. 2); Ca^{2+} blockade occurs from either side with an average (cis plus trans) pK_{Ca} of 3.3 and Hill coefficient of 1.3 (Fig. 3); and the alcohol specificity is for heptanol and octanol in the millimolar range (Fig. 4). The proton and Ca^{2+} parameters above are in good agreement with those of Spray *et al.* (*13*) in vertebrate embryonic gap junctions.

We consider this overall parallel between the ryanodine receptor and gap junctions significant for two reasons. First, even in the absence of structural homology between gap and ryanodine receptor proteins (*19*), the principles of subunit assembly implemented for gap junctions (*14*) may be applicable to ryanodine receptors. Most of the evidence to date (*2*, *3*) indicates that the ryanodine receptor unit is a homotetramer of the 450-kD protein (Fig. 1A). Indeed this is a large mulri-subunit complex that could conceivably make use of the subunit tilting model of Unwin and Zamphigi (*14*) to explain the various open states described in Fig. 1B. Second and most important, the mechanism of electrical signal transmission across gap junctions by means of two voltage-dependent gates in series (*15*) may be applicable to excitation-contraction coupling. Biochemical evidence suggests that the dihydropyridine receptor is present at the anchoring point of the ryanodine receptor to the TT membrane (*16*). The dihydropyridine receptor is a TT membrane protein presumed to be the voltage sensor of depolarization that opens the release channel (*17*). The dihydropyridine receptor may thus represent one of the two voltage-dependent gates necessary to open the release channel. We have recently characterized the ability of the ryanodine receptor to move charge and its dependence on voltage (*18*). The gating of gap junctions could thus help us to understand how ryanodine and dihydropyridine receptors are coupled.

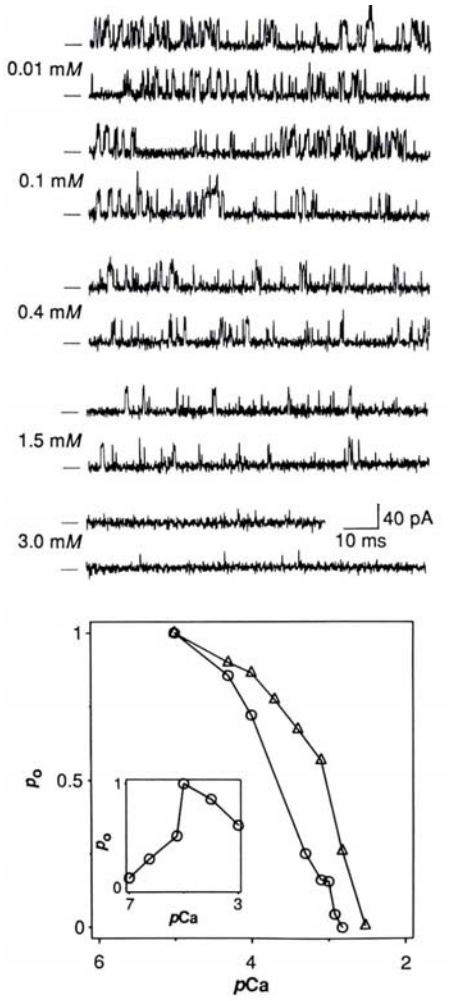

Fig. 3. Ca^{2+}-dependent inactivarion of ryanodine receptor channel. All records are at a holding potential of +50 mV, 3-kHz filtering. Control activity was recorded in 10 μ*M* $CaCl_2$, *p*H. 7.4. Records are for a trans-side titration at constant 10-μ*M* cis Ca^{2+}. Separate titrations for cis and trans side are shown by the circles (n = 10 experiments) and triangles (n = 4 experiments), respectively. A value of unity was given to the p_o at 10 μ*M* Ca^{2+}, both sides. Ca^{2+} concentrations were verified with a Ca^{2+} electrode. The Ca^{2+} concentration at which p_o has dropped to 50% of the control value was 250 μ*M* (cis) and 800 μ*M* (trans). The inset shows Ca^{2+}-dependent activation by cis Ca^{2+} at a constant trans *p*Ca of 7 (*5*, *6*).

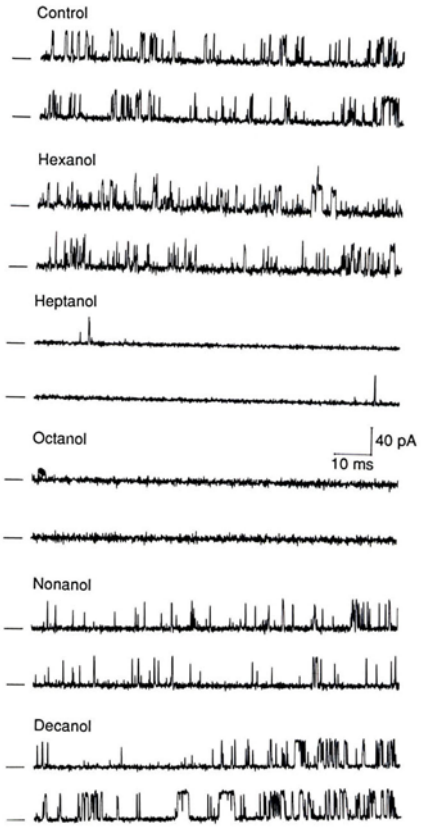

Fig. 4. Block of ryanodine receptor channels by heptanol and octanol. Records are from different experiments, each composed of a control period followed by trans addition of 0.1% (v/v) alcohol (Aldrich). A decrease in activity in heptanol (n = 3 experiments) or octanol (n = 6 experiments) developed within 5 min; n = 3 experiments for all other alcohols tested. Records labeled control and octanol are from the same experiment. All records are at +80 mV, 3 kHz filtering.

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