where vascular regeneration without excessive inflammation or thrombosis is essential.

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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 pH; (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 depolarization into an intracellular release of calcium.

OUPLING 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 Ca²⁺ homeostasis. In skeletal muscle, 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 Ca²⁺-release channel of skeletal muscle. The purified channel displays a weak selectivity between Ca²⁺ 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 Ca²⁺, 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), are 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-internal 1 μM ruthenium red produces a flickery block and a marked decrease in the fraction of time spent open or the open probability (p_0) . In this experiment (Fig. 1C) $p_0 = 0.16$ in control, $p_0 = 0.85$ after treatment with ryanodine, and $p_0 = 0.18$ 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 1M 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 0.25M KCl of 800 pS, 400 pS (260 pS in 0.15M 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_0 . 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, Ca²⁺, and 1-octanol, the well-known uncouplers of gap junctions (12). Over a narrow range, low pH closes 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 pH 7.4 and pCa 5, the average p_0 is between 0.25 and 0.10 (average $p_0 = 0.2$, SD = 0.05, n = 10). In this particular recording, $p_{o} = 0.14$. Reducing pH 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_0 (Fig. 2D). Open probability drops from its near maximum value at pH7.4 (normalized to 1) to almost null at pH6.5. The absolute p_0 at pH 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 pHwas fully reversible within the range tested, and p_0 reaches a maximum ($p_0 = 0.35$) at pH values >7.6. The dose-response curve for pH titrations from either side (Fig. 2D) could be fitted with a cis pK 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 pHactually modifies gating of ryanodine receptors is shown in Fig. 2, B and C, and in a plot of p_0 versus voltage in Fig. 2E. At pH7.4 channels remain open over a wide range of voltage (-100 mV to + 120 mV). However, at pH 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_0 every 3.5 mV. Thus, the receptor channel is steeply dependent on both membrane potential and pH, with the switching from closed to open occurring over an unusually narrow range.

At constant pH(7.4) and holding potential (+50 mV), p_0 can be controlled by Ca²⁺ (Fig. 3). Open probability in symmetrical pCa 5 is halved by cis 250- μM Ca²⁺ $(SD = 50 \ \mu M, \ n = 10)$ with a Hill coefficient of 1.3 (SD = 0.14, n = 10) or halved by trans 800- μM Ca²⁺ (SD = 100 μM , 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 pCa 7 to 5 is shown in the inset of Fig. 3, bottom. The latter demonstrates that, similar to Ca²⁺ fluxes in SR vesicles and native ryanodinereceptor release channels, the effect of Ca²⁺ is biphasic, consisting of an activation phase at high pCa and an inhibitory phase at low pCa (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 Ca²⁺ 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 $p_0 > 0.2$ (Fig. 4, top traces). Over several minutes, the addition of 0.1% v/v (~6 mM) heptanol or octanol produced a complete closure of channels at all potentials from -80 mV to +80 mV. No effects on p_0 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 electrophoresis analysis of purified ryanodine receptor $(3 \mu g)$ 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 +100mV (scale bars, 50 pA and 10 ms), 3-kHz low-pass filtering. Recording solution was symmetrical 0.25M KCl, 10 µM CaCl₂, 10 mM tris-Hepes, pH 7.4. Traces were selected from eight separate experiments and represent the most frequently observed conductance levels. Linear fit of currentvoltage 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 \dot{M} ryanodine and 1 μM 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 [³H]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 nM, respectively. Unless specified, all recording solutions on both sides of the planar bilayer were composed of 0.25M KCl, 10 μ M CaCl₂, and 10 mM Hepes-tris, pH 7.4. Chambers are defined 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 cis, 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 pH to the desired final value. Single channel records are from a trans-side titration at a constant cis pH of 7.4. (**D**) p_o versus pH for a cis-side titration at a constant trans pH of 7.4 (triangles, n = 4 experiments) or a transside titration at a constant cis pH of 7.4 (circles, n = 4 experiments). p_0 at pH 7.4 on both sides was taken as unity. (B) Voltage dependence at cis and trans pH 7.4. (C) Voltage dependence at cis and trans pH 7.15.



(B) and (C) are from separate experiments. (E) p_0 versus voltage at pH 7.4 (triangles, n = 10 experiments) and separately at pH 7.15 (circles, n = 4 experiments). Scales are 40 pA, 10 ms for all traces. A baseline trace is indicated for each record.

tentials. 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 pKs 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 Ca²⁺ dependence and voltage dependence are

° 0.5 ż pCa 0 6 4 2 pCa

Fig. 3. Ca²⁺-dependent inactivation of ryanodine receptor channel. All records are at a holding potential of +50 mV, 3-kHz filtering. Control activity was recorded in 10 μM CaCl₂, pH 7.4. Records are for a trans-side titration at constant 10- μM cis Ca²⁺. 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_0 at 10 μM Ca²⁺, both sides. Ca²⁺ concentrations were $\mu M Ca^{2+}$ verified with a Ca²⁺ electrode. The Ca²⁺ concentration at which p_0 has dropped to 50% of the control value was 250 μM (cis) and 800 μM (trans). The inset shows Ca²⁺-dependent activation by cis Ca^{2+} at a constant trans pCa of 7 (5, 6).

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highly variable. In general, it appears that when Ca²⁺ sensitivity is present, the Ca²⁺ levels that uncouple cells are much higher than the resting intracellular Ca²⁺, in mammalian heart and salivary glands, on the order of 40 to 80 $\mu M \operatorname{Ca}^{2+}(13)$. Our results show that closing 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) pK 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 Ca²⁺ 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 rvanodine receptor and gap junctions significant for two reasons. First, even in the absence of structural homology between gap



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 $(n = 6 \text{ experi$ ments) 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.

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 450kD protein (Fig. 1A). Indeed this is a large multi-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.

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Olmec Settlement Data from La Venta, Tabasco, Mexico

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Research at La Venta, a major Olmec center on a low salt dome 12 kilometers from the Gulf Coast has revealed evidence of initial occupation (about 1750 to 1400 B.C.) along levees of the silted-in Río Barí, north of the site core in a transitional estuarine-riverine environment. Between 1400 and 1150 B.C. settlement expanded to nearby La Venta itself, which between 1150 and 800 B.C. developed into a major temple-town complex. Local development peaked at La Venta and along the river levees between 800 and 500 B.C. In this span La Venta headed a local three-tiered site hierarchy as social distinctions expanded to the peripheral Río Barí sites. New excavations show that growth in population size and density at La Venta preceded the development of sociopolitical complexity. These data contradict the traditional organizational reconstruction of Olmec society, the "vacant ceremonial center" model, and provide qualified support for a model that presents riverine resource concentration as a significant factor in the evolution of Olmec civilization.

REMAINS OF OLMEC SOCIETY, which flourished in Mexico's Gulf coastal lowlands between 1150 and 500 B.C., present an opportunity for archeological research to delineate the origins and evolution of complex human organizations. The Olmec are one of only a few known primary civilizations, having statelike organizations that evolved from less complex prototypes without input from other systems. As one of the world's first tropical lowland civilizations, the Olmec also provide a major antecedent to later Classic Maya civilization.

Although theories about the evolution of Olmec civilization have been prevalent, archeological data relevant to their evaluation have been sparse. New settlement data from research completed at one of the largest Olmec sites, La Venta, have begun to correct this disparity. Previous research at La Venta (1) and another major Olmec site, San Lorenzo (2), was aimed at monumental architectural and sculptural remains. The San Lorenzo investigations provided the first detailed cultural sequence for an Olmec site, but much was based on ceramics collected from redeposited construction fills. This highlights the lack of crucial settlement research that specifically targets evidence from undisturbed remains (primary contexts), representing the full range of ancient human activities (3).

Ancient occupation sites with primary contexts discovered by one of us (W.F.R.) at and adjacent to La Venta in 1986 and 1987 provide a basis for inferring patterns of population growth and density during both the Early Preclassic span (1400 to 1150 B.C.), which immediately preceded the emergence of Olmec civilization, and the Middle Preclassic era (1150 to 500 B.C.), corresponding to La Venta's major growth and occupation. These settlement data can be correlated with evidence of dynamically changing local environmental conditions and subsistence activities during the same time interval. The new findings indicate that growth in population size and density in resource-rich estuarine and riverine environments preceded local emergence of social ranking or stratification and other archeological hallmarks of civilization.

La Venta occupies a salt dome (4), now located 12 km south of the Gulf of Mexico amid freshwater swampland (Fig. 1). In the

environmentally static traditional model it functioned as an isolated "island" religious sanctuary, surrounded by uninhabited swamp, occupied by a small group of Middle Preclassic priestly elite who ruled a dispersed population of swidden farmers in the adjacent uplands; the people periodically assembled in the center to perform labor obligations and attend ceremonies and markets (1, 6, 8). Although recent theories have challenged this "vacant ceremonial center" model as inadequate to explain the origins and operation of Olmec society (5), lack of relevant archeological data and recent disturbance by oil exploitation in the Olmec area have combined to frustrate the evaluation of both the traditional and newer models.

Previous work in the core of La Venta ("Complex A") excavated monumental construction and found huge buried caches of worked serpentine and other imports (1). Fourteen new test excavations placed in 1986 disclosed functionally diverse settlement features between 100 and 500 m northeast of Complex A, in areas known as complexes E and G (Fig. 2). These include house floors and storage pits [Operations (Ops.) 28, 31, 33, and 34], urn burials and large pottery offerings (Op. 29), and a serpentine artifact workshop (Op. 30-1), associated with Middle Preclassic ceramics and other artifacts. Three of these features (Ops. 29, 30-1, and 33-2) produced carbon-14 dates clustered near 700 B.C. (uncalibrated; Table 1). These findings indicate substantial Middle Preclassic occupation in the site core, contradicting the conclusion that La



Fig. 1. Map of the La Venta region showing the nine identified levee occupational sites along the abandoned Río Barí channels. Sites marked by circles, including Isla Chicozapote (1), Isla Alor (2), and Isla Catalina (5) are moundless; sites marked by triangles, including Isla Yucateca (3) and San Andres (4) have central mounds.

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