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Activation and Conductance Properties of Ryanodine-Sensitive Calcium Channels from Brain Microsomal Membranes Incorporated into Planar Lipid Bilayers

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Summary. Rat brain microsomal membranes were found to contain high-affinity binding sites for the alkaloid ryanodine (k_d 3 nM, B_{max} 0.6 pmol per mg protein). Exposure of planar lipid bilayers to microsomal membrane vesicles resulted in the incorporation, apparently by bilayer-vesicle fusion, of at least two types of ion channel. These were selective for Cl^- and Ca^{2+}, respectively. The reconstituted Ca^{2+} channels were functionally modified by 1 μM ryanodine, which induced a nearly permanently open subconductance state. Unmodified Ca^{2+} channels had a slope conductance of almost 100 pS in 54 mM CaHEPES and a $Ca^{2+}/TRIS^+$ permeability ratio of 11.0. They also conducted other divalent cations ($Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+}$) and were markedly activated by ATP and its nonhydrolysable derivative AMP-PCP (1 mM). Inositol 1,4,5-trisphosphate (1–10 μM) partially activated the same channels by increasing their opening rate. Brain microsomes therefore contain ryanodine-sensitive Ca^{2+} channels, sharing some of the characteristics of Ca^{2+} channels from striated but not smooth muscle sarcoplasmic reticulum. Evidence is presented to suggest they were incorporated into bilayers following the fusion of endoplasmic reticulum membrane vesicles, and their sensitivity to inositol trisphosphate may be consistent with a role in Ca^{2+} release from internal membrane stores.

Key Words ATP · calcium channel · endoplasmic reticulum · inositol trisphosphate · microsomes · planar bilayers · ryanodine

Introduction

Ca^{2+}-uptake by the endoplasmic reticulum is an important mechanism for controlling cell $[Ca^{2+}]$ (Blaustein, Ratzleff & Schweitzer, 1978). The equivalent but obviously more specialized sarcoplasmic reticulum (SR) of muscle cells also *releases* Ca^{2+} to initiate contraction (Costantin, 1975). This SR Ca^{2+} store was found to be sensitive to the alkaloid ryanodine (Sutko et al., 1979). Rabbit skeletal (Smith, Coronado & Meissner, 1985) and canine cardiac (Rousseau et al., 1986) SR membranes are now known to contain large-conductance (~100 pS) "Ca^{2+} release" channels which are modified by ryanodine (Rousseau, Smith & Meissner, 1987). High-affinity (k_d 1–10 nM) ryanodine binding sites from striated muscle SR have been purified and reconstituted to give functional Ca^{2+} channels (Lai et al., 1988; Smith et al., 1988). At the structural level revealed by electron microscopy aggregates of the ryanodine receptor/Ca^{2+} release channel complex appear to form the SR "foot process," projecting into the 100-Å cleft that separates the internal SR membrane system from the cell surface membrane (reviewed by Fill & Coronado, 1988).

The Ca^{2+} channels of skeletal and cardiac SR are activated by Ca^{2+} itself and by ATP (Smith et al., 1985, 1986; Rousseau et al., 1986), including nonhydrolysable ATP analogues. Inositol 1,4,5-trisphosphate ($InsP_3$) is also effective in some species (Suarez-Isla et al., 1988). Canine aortic smooth muscle SR contains Ca^{2+} channels (Erlich & Watras, 1988) that are sensitive to $InsP_3$ but have a Ca^{2+} conductance only a tenth of that of striated muscle channels. This study was undertaken to examine the hypothesis that the endoplasmic reticulum (ER) of noncontractile cells, specifically brain microsomes, might also contain Ca^{2+} channels, some of which could be activated by $InsP_3$ (Streb et al., 1983; Berridge & Irvine, 1984). To test these possibilities, procedures similar to those employed with SR membranes (Smith et al., 1985) were used to incorporate rat brain microsomal vesicles, which were also found to have high-affinity ryanodine binding sites, into voltage-clamped planar lipid bilayers. The microsomes contained at least two types of ion channel. One was an anion-selective channel with no measurable permeability for Ca^{2+}. In most experiments separate Ca^{2+} channels were co-incorporated. These were sensitive to ryanodine and partially activated by $InsP_3$.

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Considerable attention has been paid in these experiments, and their interpretation, to the fact that it is difficult to assign a specific physical origin to reconstituted ion channels. Nevertheless, circumstantial evidence suggests that the Ca^{2+} channels come from an internal membrane system or subsystem (Putney, 1986) in neuronal cells, probably part of the endoplasmic reticulum. This would have obvious implications, given their sensitivity to $InsP_3$, but their activation by the 2nd messenger seems to be incomplete. The discovery that brain microsomes bind ryanodine with high-affinity, and that low concentrations of ryanodine modify the reconstituted channel, may provide a means for localizing and purifying the channel protein.

Materials and Methods

Rat forebrains were homogenized in 0.32 M sucrose (pH 7.4) containing 1 mM PMSF and 10 μg per ml trypsin inhibitor (Sigma). Endoplasmic reticulum vesicles, isolated as a microsomal fraction using a standard differential centrifugation procedure (Gray & Whittaker, 1962) were suspended in 0.4 M sucrose, frozen in liquid N_2 and stored in small aliquots at −80°C. Protein concentrations were measured by the method of Lowry et al. (1951). [^{3}H]ryanodine binding (0.5–50 nM in duplicate, specific activity 54.7 Ci/mmol, New England Nuclear) was assayed (Lai et al., 1988) in a 1-M KCl, 20-mM PIPES-KOH (pH 7.4) buffer containing 1 mM ATP, 100 μM Ca^{2+}, 1 mM PMSF and 50–100 μg protein per ml, adding 50 μM unlabeled ryanodine (Calbiochem) to measure nonspecific binding. The mixtures were incubated at 37°C for 60 min before filtration (Whatman GF/C filters) and liquid scintillation counting.

Planar bilayers were cast at room temperature (20°C) from a decane suspension containing both phosphatidylethanolamine and phosphatidylserine (each 15 mg per ml, bovine brain or heart, Avanti), or just 30 mg per ml phosphatidylethanolamine, which was spread over a 300–500 μm hole in a polystyrene partition separating two solution-filled chambers and allowed to thin spontaneously. The choice of lipids made no difference to the characteristics of incorporated channels, and did not affect measured reversal potentials. The *cis* chamber (to which membrane vesicles were added) was voltage-clamped at various potentials relative to the *trans* chamber, which was grounded, and transmembrane currents were digitized and recorded on videotape using a modified audio processor. All voltages are quoted as *cis-trans* potentials.

Initially, bilayers were bathed in solutions containing 50–500 mM choline Cl with up to 2 mM $CaCl_2$ and 10 mM TRIS/HEPES (pH 7.4). Osmotic gradients (*cis* > *trans* by at least 5 : 1) and stirring promoted the incorporation of Cl^- channels (unit currents flowing *trans-cis* at zero mV, reversing at positive holding potentials). Both chambers were then perfused with Cl^--free solutions, normally 54 mM CaHEPES *trans* (250 mM HEPES neutralized by $CaOH_2$ to a pH of 7.4) and 100 μM Ca^{2+} in 125 mM TRIS/250 mM HEPES (pH 7.4) *cis* (Smith et al., 1985, 1986). Using high salt (3 M LiCl) agar bridges minimized liquid-junction pd's, which only became significant (2 mV) with Ca^{2+}-solutions. Assuming, as for SR (Miller & Rosenberg, 1979; Smith et al., 1986), that channels incorporated via the fusion of right-side-out vesicles, the cytoplasmic side of incorporated microsomal channels faced the *cis* chamber and the lumenal side *trans*. Any co-incorporated Ca^{2+} channels were therefore appropriately orientated and exposed to an approximation of the physiological Ca^{2+} gradient.

Recordings of transmembrane currents were reconverted to analogue format and low-pass filtered to reduce high-frequency noise (using a Krohn-Hite filter in "RC" mode with a front-panel setting of 0.3–0.6 kHz). Channel activity was analyzed by hand or currents were redigitized (at 2–5 kHz) for computer-aided measurements and illustrations. The Nernst equation was not applicable with relatively complex mixtures of permeant ions and relative cation *vs.* anion permeabilities were calculated to satisfy the Goldman-Hodgkin-Katz voltage equation

$$E = -RT/zF \cdot \ln \frac{[X^+]_{trans} + R[Y^-]_{cis}}{[X^+]_{cis} + R[Y^-]_{trans}} \quad (1)$$

where E is the reversal potential (mV) and R is the ratio of the permeabilities of the cation X^+ and anion Y^- (RT/F is 25 mV at 20°C and z represents valency). Divalent *vs.* monovalent cation permeability ratios ($R^{2+/+}$) were calculated following the same principles (Fatt & Ginsborg, 1954) so that, in standard Ca^{2+} solutions with negligable Ca^{2+} *cis* and no monovalent cation *trans*

$$R^{2+/+} = [X^+]_{cis}/[X^{2+}]_{trans} \cdot [(1 + e^{FE/RT}) \cdot e^{FE/RT}]/4. \quad (2)$$

For channel lifetime analysis the durations of only full channel openings were measured. The amplitudes of full openings always had a SD < 3% of the mean (>30 measurements from a given record), and suitable openings were therefore defined to be those that reached at least 90% of the mean maximum amplitude to single out all the relevant events while excluding any partial (substate) openings.

Results

Fusion of Microsomes with Planar Bilayers

Planar bilayer membranes were formed in 50 mM choline chloride and brain microsomal membranes were added to the *cis* chamber to a final protein concentration of about 10 μg per ml. Cl^--selective channels usually appeared within 15 min of the creation of an osmotic gradient of choline Cl (*cis* > *trans*), as described under Materials and Methods. Examples from three experiments are shown in Fig. 1. Similar channel-like activity was never seen over periods of 1–2 hr if bilayers were exposed to the same conditions without membranes. Several Cl^- channels were examined in more detail, and in 10 experiments which included five bilayers where it was subsequently shown that no Ca^{2+} channels had been co-incorporated (*see below*) the channels had a main slope conductance of 31 ± 6 pS (mean ± SD, n = 10) and often displayed subconductance states at 40–70% of the main conductance level. In the absence of co-incorporated Ca^{2+} channels these Cl^- channels showed no measurable permeability to

Ca^{2+}. They were not, however, ideally selective between Cl^- and choline$^+$. Reversal potentials varied between +25 and +35 mV with 10:1 *cis*:*trans* gradients, consistent with Cl^-:choline$^+$ permeability ratios of 3.6 to 6.7.

Subsequent perfusion of the bilayer chambers with Cl^--free Ca^{2+}-containing solutions often revealed co-incorporation of Ca^{2+} channels with Cl^- channels (Fig. 2), characteristic Ca^{2+} currents of 3–4 pA flowing *trans-cis* while the *cis* chamber was clamped at 0 mV. It should be noted that under these ionic conditions and at a holding potential of 0 mV, Ca^{2+} was the only ion for which there was an appropriate gradient to generate these currents. There was no electrochemical driving force for $HEPES^-$ (250 mM *cis* and *trans*) and $TRIS^+$ could only show a net flux in the *opposite* direction. In other experiments KCl was substituted for choline Cl and a large variety of K^+ channels was seen. These ranged in conductance from 10–20 pS to ~200 pS. These latter were Ca^{2+}-activated K^+ channels, similar to those reported from rat brain synaptosomes by Farley and Rudy (1988). Co-incorporation of Ca^{2+} and K^+ channels never occurred. Similar experiments with five preparations of whole synaptosomes and three preparations of synaptic plasma membrane vesicles (Cotman,

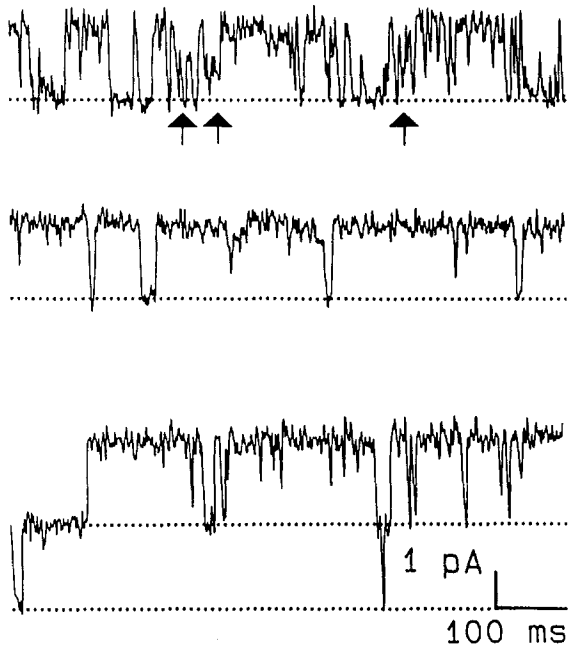

Fig. 1. Unit currents (shown upwards) through Cl^--selective channels exposed to 500 mM choline Cl *cis*/50 mM *trans*, holding potential −30 mV (*cis-trans*). Recordings from three different experiments are shown, with two channels in the last trace. Arrows indicate substates in top trace, and the closed levels are marked (.....)

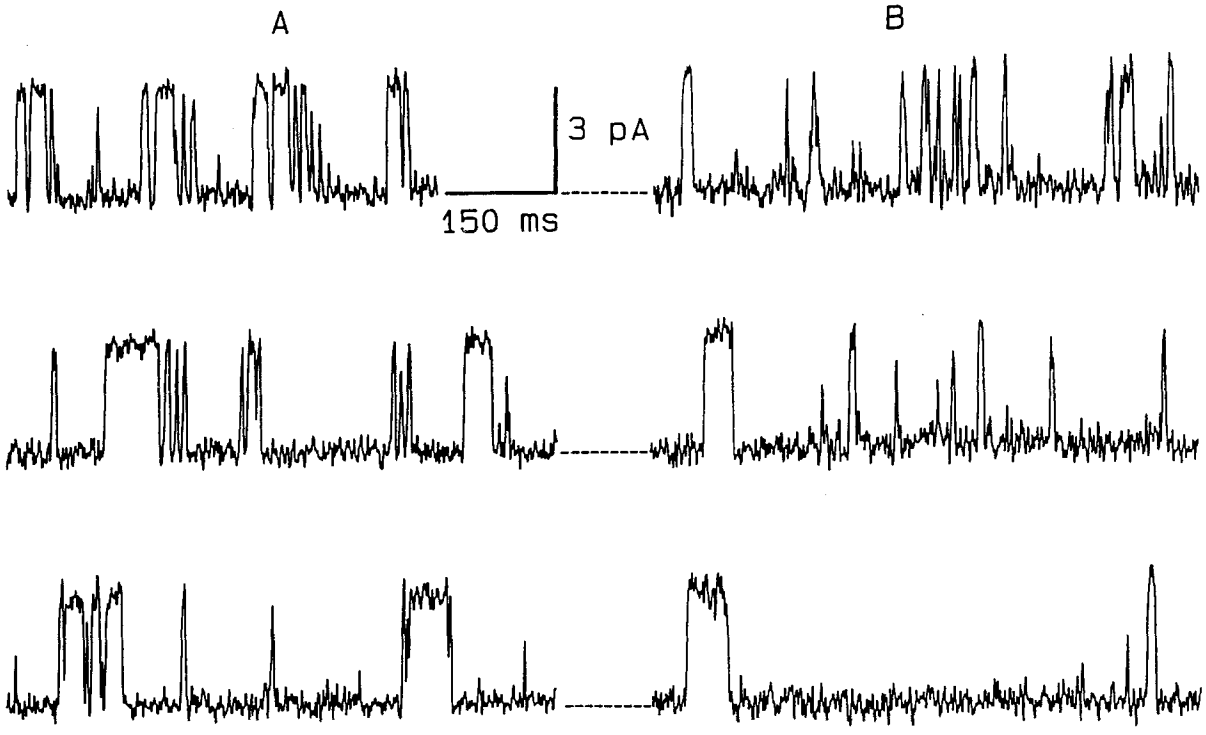

Fig. 2. Currents (shown upwards) through Ca^{2+}-selective channels reconstituted from two preparations of microsomes (*A* and *B*). Membranes were voltage clamped at 0 mV, and Ca^{2+} flowed from the *trans* (250 mM CaHEPES) to the *cis* (100 μM Ca^{2+}, 125 mM TRIS/250 mM HEPES) chamber

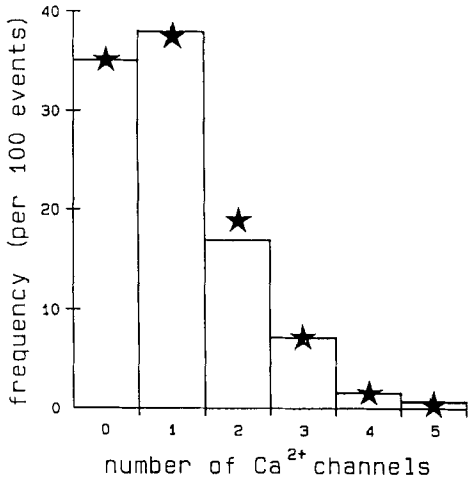

Fig. 3. Numbers of Ca^{2+} channels incorporated in 100 experiments (bars) compared to the expectation of a Poisson distribution (★), $\exp(-m) \cdot (m^x/x!)$, where m is the mean number of channels per "packet" and x represents 0, 1, 2, . . . , N channels per packet

1974), often in $BaCl_2$ or $CaCl_2$ solutions from the outset, produced no Cl^- channels or high-conductance Ca^{2+} channels in >50 experiments.

Attempts to incorporate only Ca^{2+} channels, using Ca^{2+}-containing Cl^--free solutions throughout, were unsuccessful. A possible reason for this is discussed in due course. The appearance of Cl^- channels (a single channel in >90% of experiments) was clearly an important marker for Ca^{2+} channels and strongly suggested that both were carried into the bilayer by the fusion of channel-containing membrane vesicles. This possibility was tested by counting the total number of Ca^{2+} channels co-incorporated with Cl^- channels in 100 experiments (Fig. 3). The results conformed to a Poisson distribution consistent with the random all-or-none fusion of "packets" of Ca^{2+} channels containing on average 1.06 channels per packet.

Ryanodine Binding and Block

In six experiments involving a total of 10 reconstituted Ca^{2+} channels 1–10 μM ryanodine added to the *cis* chamber caused a characteristic "partial block," illustrated in Fig. 4*A*. The modification usually occurred several minutes after adding low concentrations of ryanodine, and proceeded channel by channel in the case of multi-channel experiments. Modified channels became "locked" into a 40% subconductance state which only closed briefly and infrequently. The trace in Fig. 4*A* illustrates the occasional appearance of another substate within the ryanodine-bound subconductance state itself.

Ryanodine also bound with high affinity to brain microsomes, and a typical saturation isotherm is presented in Fig. 4*B* with its associated Scatchard plot in Fig. 4*C*. Nonspecific binding was always a linear function of added [^{3}H]ryanodine and never contributed >12% of total binding at the measured k_d. Four separate microsomal preparations analyzed in detail using saturation isotherms and Scatchard plots gave B_{max} and k_d values of 590 ± 70 fmol per mg protein and 2.7 ± 0.7 nM, respectively (means ± SD, n = four preparations). There was no evidence for more than one binding site at [^{3}H]ryanodine concentrations up to 50 nM.

Channel Conductance Properties

The reconstituted Ca^{2+} channels occasionally appeared to open (and close) to relatively short-lived intermediate conductance levels between the fully-open and the closed, baseline level. For some channels this behavior was especially obvious at high negative holding potentials. Various transitions from a 2-min recording of a single channel held at −40 mV have been collected in Fig. 5 to display two subconductance states in addition to the fully open state. These observations may be relevant to the origin and structure of the channel (as discussed later), but in practice most channels only appeared to open to one obvious fully-open level (bilayer noise limiting resolution to 2 msec).

The amplitudes of these maximal single-channel currents were measured at a range of holding potentials to examine current-voltage (I/V) relationships and to calculate single-channel slope conductances. Examples of traces from one experiment, and an overall I/V relationship combining measurements made in 18 bilayers using five separate vesicle preparations, are shown in Fig. 6, *A* and *B*, respectively. The traces in Fig. 6*A* illustrate true current reversal between +30 and +40 mV under the standard asymmetric [Ca^{2+}] conditions employed. The voltage-dependence of channel opening was not investigated in detail but fractional open times were noticeably increased at positive holding potentials. The mean equilibrium potential of +36 mV (Fig. 6*B*) gives a $Ca^{2+}/Tris^+$ permeability ratio of 11.0 (correcting for the junction potential). Currents increased disproportionately at negative holding potentials and the slope conductance varied from 75–125 pS.

It was often possible to obtain I/V relationships with 54 mM CaHEPES both *cis* and *trans* and these were ohmic (Fig. 7*A*). Under such conditions the single-channel slope conductance was 97 ± 7 pS (mean ± SD, n = 5). The single-channel fluxes for several divalent cations could be compared in the

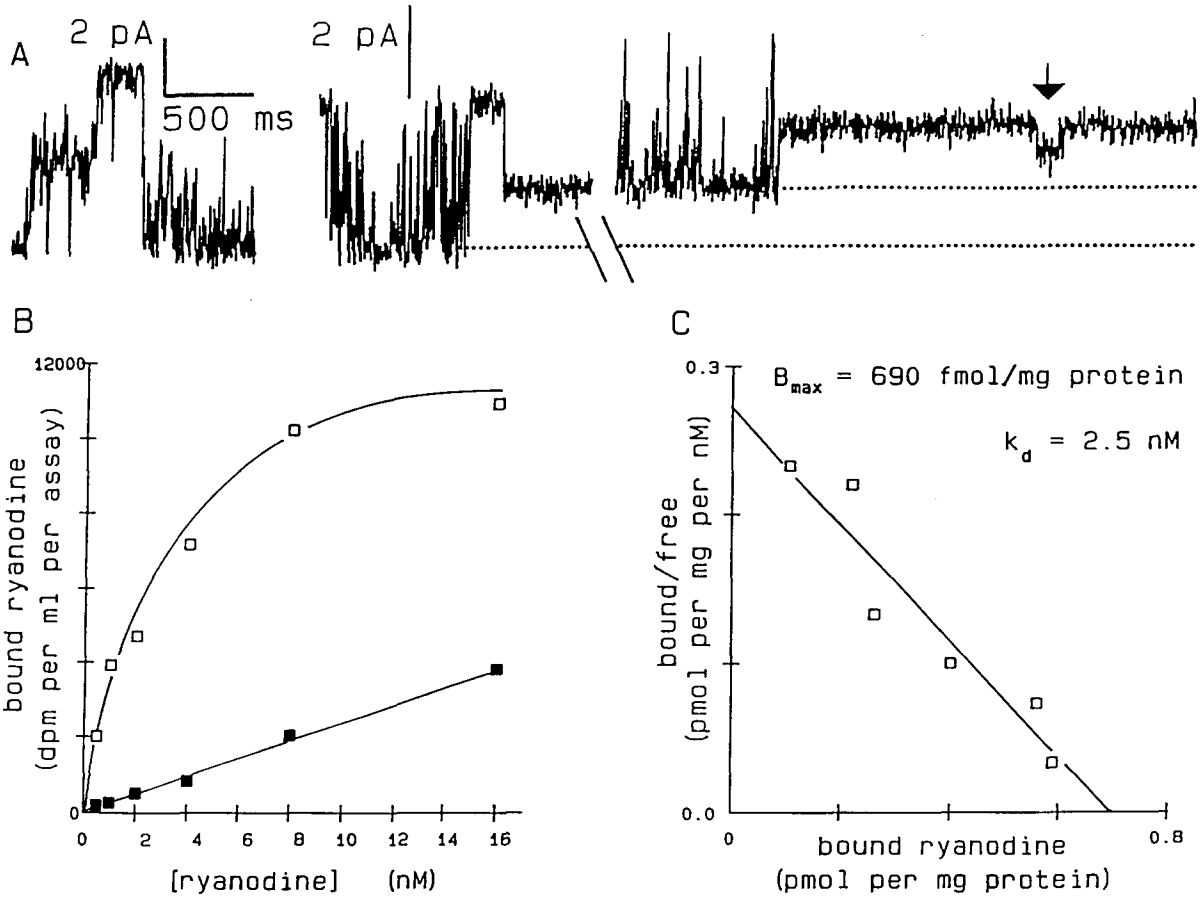

Fig. 4. Ryanodine binding and "block." (*A*) Two Ca^{2+} channels in the same bilayer successively modified by *cis* ryanodine, 203 sec after adding 1 μM then 15 sec after adding 10 μM. Note change in amplitude scale and arrowed sublevel. (*B*) Typical saturation binding assay for brain microsomes showing nonspecific (■) and specific (□) [^{3}H]ryanodine binding. Curve was fitted to parameters from Scatchard plot in *C*

same channel by adopting normal recording conditions (low *cis* [Ca^{2+}]) but perfusing the *trans* chamber successively with Ca, Ba, Sr and MgHEPES. Part of a typical experiment is shown in Fig. 7*B*. The slope conductance (g) always followed the sequence $gBa^{2+} > gCa^{2+} > gSr^{2+} > gMg^{2+}$, but reversal potentials could not be reliably separated. The unusually large Mg^{2+} conductance was noted, and suggested that the channel might be significantly permeant to monovalent cations. In experiments with 250 mM HEPES/180 mM KOH (pH 7.4) *cis*, and CaHEPES *trans*, reversal potentials close to +15 mV gave an approximate Ca^{2+}/K^+ permeability ratio of only 4.4. However, the presence of other (albeit relatively anion-selective) channels must complicate the interpretation of such experiments.

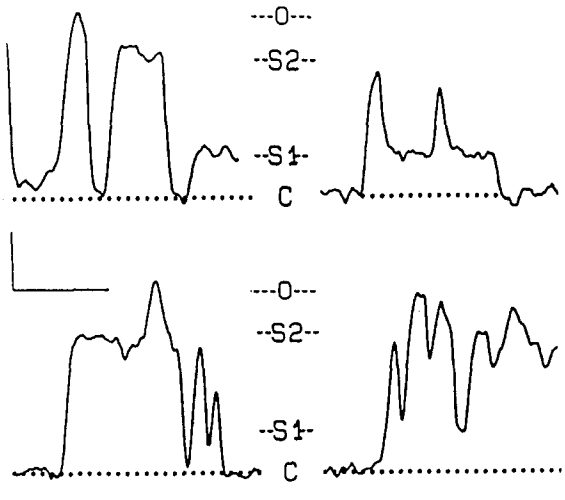

Fig. 5. Apparent substates of the same single channel recorded at −40 mV, showing transitions between closed (*C*), fully-open (*O*) and intermediate-conductance (*S1* and *S2*) states. Scale bars 2 pA, 20 msec

Activation by ATP and $InsP_3$

Most of the reconstituted Ca^{2+} channels were relatively insensitive to *cis* (cytoplasmic) [Ca^{2+}]. In

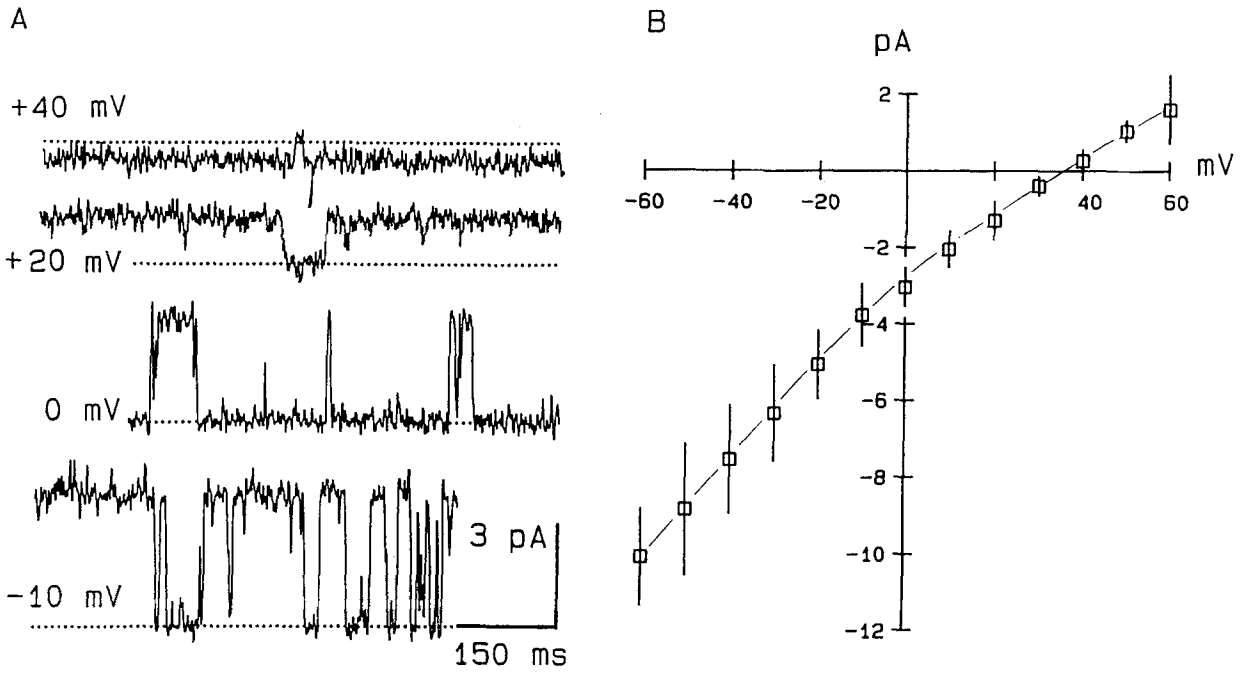

Fig. 6. (*A*) Ca^{2+} channel currents from one experiment at various (*cis-trans*) holding potentials. Closed levels are indicated. Note unit currents reverse between +40 and +20 mV. (*B*) Current-voltage (*I/V*) relationship for Ca^{2+} channels from 18 experiments involving five microsome preparations. Bars show ±SD. Negative currents, representing Ca^{2+} flowing *trans-cis*, reverse at +36 mV

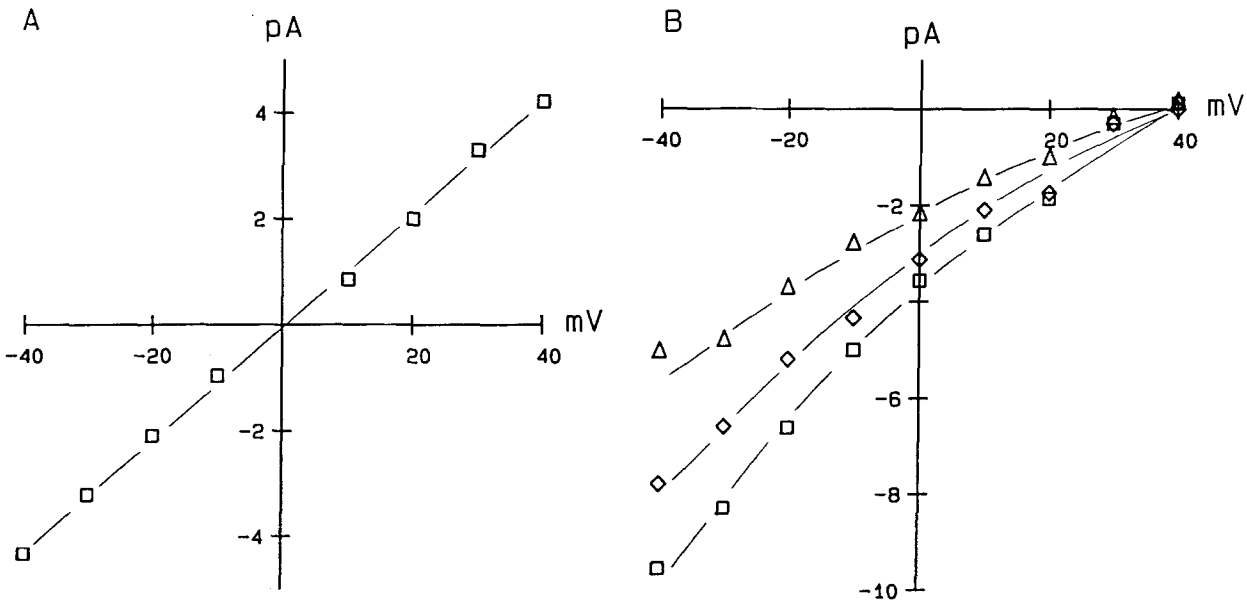

Fig. 7. (*A*) *I/V* relationship with 54 mM CaHEPES both *cis* and *trans*. Assuming $HEPES^-$ is impermeant, the slope conductance for Ca^{2+} is 106 pS. (*B*) *I/V* relationships for the same single channel comparing 54 mM Ca- (◇), Mg- (△) and Ba- (□) HEPES *trans*, standard recording conditions *cis*. The (maximum) slope conductances are 120, 75, and 150 pS, respectively

nine experiments the fractional open times of only three channels were altered (decreased) when *cis* free $[Ca^{2+}]$ was reduced from 100 μM to less than 1 nM by adding EGTA. In 10 experiments caffeine (1–40 mM) was added to the *cis* chamber. Concentrations of at least 5 mM consistently activated the channels, but activation was always incomplete even with 40 mM caffeine. In contrast, ATP (0.1–1 mM) markedly activated the channel. This only occurred from the *cis* side (*cis vs. trans* additions were compared in seven experiments). Typical recordings are shown in Fig. 8. The nonhydrolysable

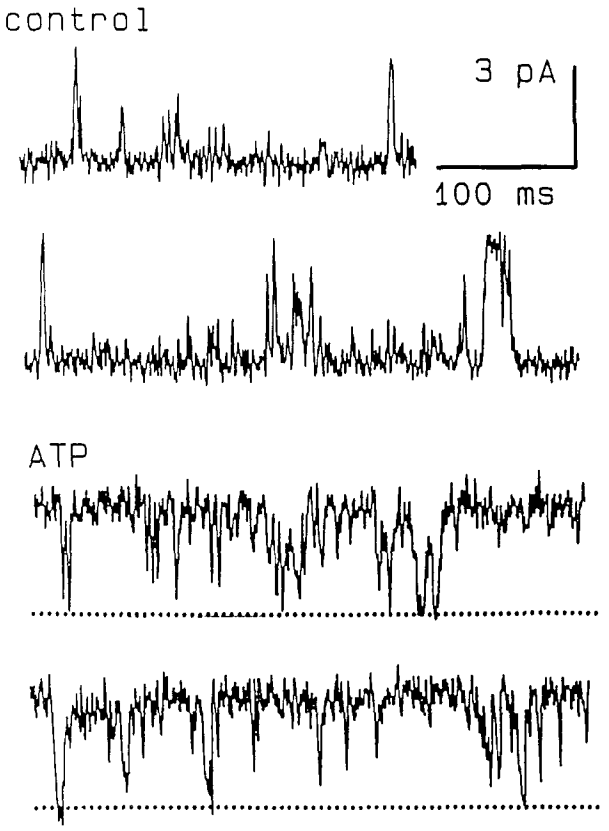

Fig. 8. Activation of a single Ca^{2+} channel by 1 mM ATP. Standard recording conditions, holding potential 0 mV. The closed levels are indicated for ATP

ATP-derivative AMP-PCP was equally effective at similar concentrations, eliminating activation mechanisms involving phosphorylation. Reperfusion with nucleotide-free solutions restored normal channel activity, suggesting reversible (noncovalent) binding. In 11 out of 12 experiments 1 mM ATP increased fractional open times from <0.5 (usually <0.2) to >0.9. Apart from ATP and AMP-PCP a range of adenine nucleotides and related compounds were able to activate the channel to some extent, and in three experiments the following consistent order of effectiveness emerged: ATP = AMP-PCP > AMP > cAMP > ADP > GTP = cGTP = adenosine.

Addition of 1–10 μM $Ins(1,4,5)P_3$ (Boehringher or Sigma), but not inositol 1-phosphate or inositol 1,4-diphosphate (Sigma), to the *cis* chamber partially activated 18 out of 20 microsomal Ca^{2+} channels from five preparations (e.g., Fig. 9). Control activity and $InsP_3$ activation were compared in six experiments which each appeared to involve only a single Ca^{2+} channel. Fractional open times increased from 0.06 ± 0.04 to 0.21 ± 0.09 (means ± SD, $n = 6$), and as expected the differences were significant ($0.01 > P > 0.001$, paired t test). A more detailed analysis of the lifetimes of the fully open state was undertaken for five single-channel experiments (*see* Materials and Methods). The results were all similar, and in each case $InsP_3$ increased the number of full channel openings but did not af-

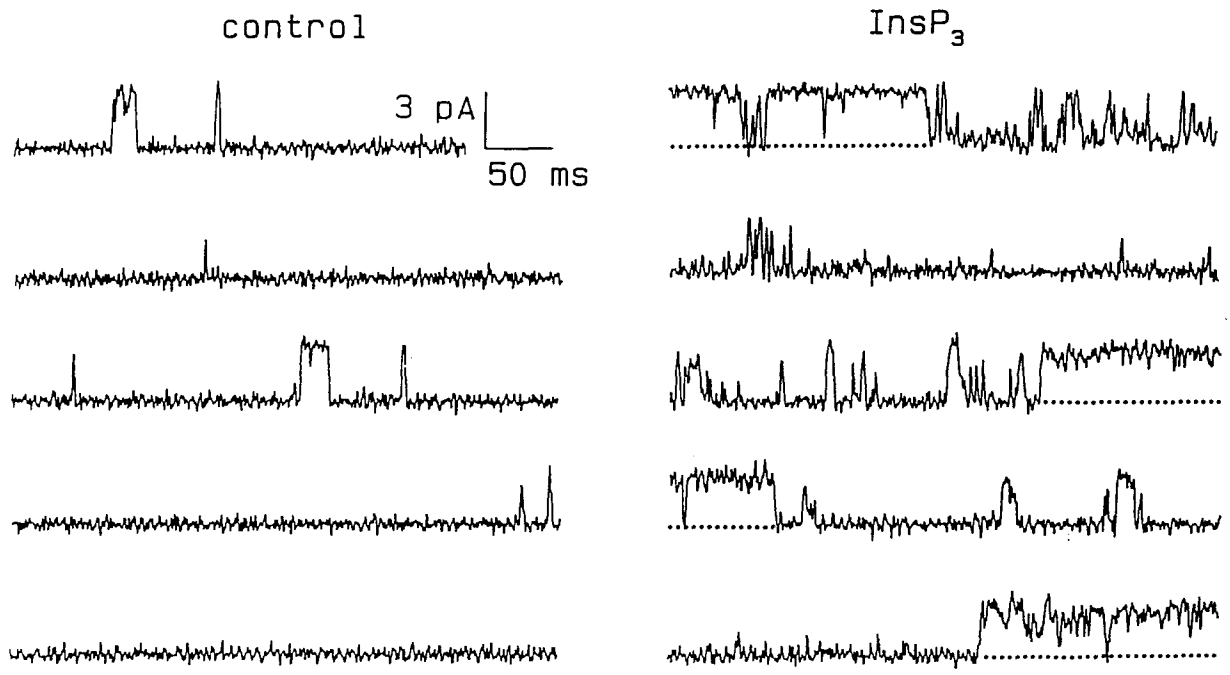

Fig. 9. Inositol trisphosphate activation of a single Ca^{2+} channel. Continuous 2-sec sections (adjoining 400-msec segments are split for viewing) from 3-min recordings before and after addition of 2 μM $InsP_3$ to the *cis* chamber. Standard ionic conditions, holding potential 0 mV

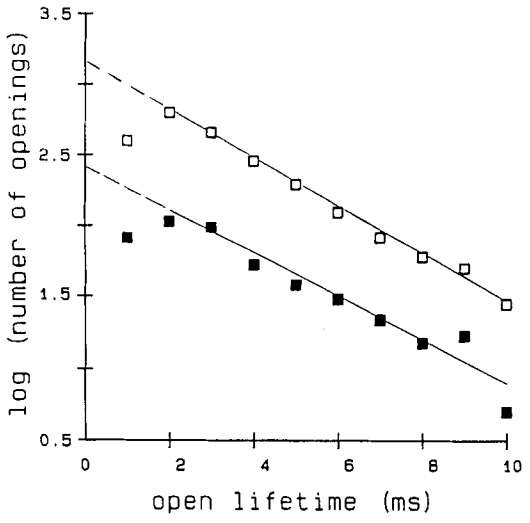

Fig. 10. Semilogarithmic frequency plots (bin width 1 msec) of the durations of *full* openings measured from 3-min recordings of the single channel shown in Fig. 9, before (■) and after (□) $InsP_3$ addition. Openings lasting <2 msec are incompletely resolved, and (least squares) fits from 2–10 msec give mean lifetimes of 2.7 and 2.8 msec, respectively. Only one lifetime component is obvious from these measurements

fect their duration (Fig. 10). $InsP_3$ seemed to be acting on the same channel as ATP: of six channels challenged with $InsP_3$ followed by ATP (or AMP-PCP), five responded to both ligands with an increase in open probability (one channel was unaffected by both). Neither ATP or GTP (each 100 μM), or reducing *cis* $[Ca^{2+}]$ to 10 μM, increased the sensitivity of the channels to $InsP_3$.

Discussion

Cellular Location of the Ca^{2+} Channels

Reconstituting otherwise inaccessible ion channels in planar lipid bilayers offers unique advantages for studying ion transport at the level of single protein molecules (Miller, 1986), but unless a very specific channel marker is available it may be impossible to determine the original location of the channel within a cell. A recent study (Stanley, Ehrenstein & Russel, 1988) attempted to answer this question by showing that the success rate for reconstituting a particular ion channel paralleled the relative purity of an identified membrane fraction. Even stronger evidence of this kind could be inferred from the present study where only microsomal, and not cell surface membranes, seemed to contain Cl^- channels or high-conductance Ca^{2+} channels. However, any sort of quantitative approach is undermined by the fact that only a very small proportion of perhaps a billion added channels actually appear in a given bilayer. It is impossible to test the underlying assumptions that channels fuse randomly and at a constant rate.

Ion channels do generally seem to become incorporated into bilayers following the osmotically-driven fusion of channel-containing membrane vesicles (Miller & Rosenberg, 1979; Woodbury & Hall, 1988). Just as Poisson statistics provided the basis for "quantal" transmitter release at the neuromuscular junction (Katz, 1969), their application to channel incorporation can provide strong evidence for the fusion of a population of membrane vesicles containing 0, 1, 2, . . . N channels, in this study an average of 1.06 Ca^{2+} channels per vesicle. By definition, these vesicles also contained distinctive Cl^- channels, not dissimilar to the Cl^- channels seen in SR and other intracellular membrane preparations (Tanifjui, Sokabe & Kasai, 1987; Stanley et al., 1988). These anion channels may provide an important clue to the location of nearby Ca^{2+} channels, and their presence together within the same membrane system could provide a route for charge compensation during Ca^{2+} (or other permeant cation) movements. Failure to observe Ca^{2+} channels (and presumably vesicle fusion) in Cl^--free conditions supports the idea that Cl^- channels acted as fusion promoters (Woodbury & Hall, 1988) during membrane reconstitution. There are other approaches to ion channel reconstitution, and encouragingly single-channel Ca^{2+} currents, apparently sensitive to $InsP_3$, have been reported after "tip-dipping" brain microsomal lipid and proteolipid extracts (Vassilev, Kanazirska & Tien, 1987). Those reconstitution experiments should have randomly sampled all (lipid-soluble) membrane components including microsomal channels (though presumably substantially modified by exposure to solvents like methanol and chloroform).

The sensitivity of the Ca^{2+} channels to low concentrations of ryanodine is very similar to that of striated muscle SR Ca^{2+} channels (Rousseau et al., 1987; Smith et al., 1988) and is consistent with the discovery of high-affinity ryanodine binding sites in brain microsomes. Are these binding sites located on the same molecules that function as Ca^{2+} channels? Ryanodine bound tightly to microsomal membranes (k_d about 3 nM) but was only effective on reconstituted channels at 1 μM. The same findings with striated muscle SR channels have been taken to reflect very slow binding. That is, if single channel molecules could have been observed for hours (or days) they would eventually have been modified even by nM concentrations of the alkaloid. Could

the high-affinity ryanodine binding sites (mean B_{max} 0.6 pmol per mg protein) have originated from vascular smooth muscle SR, given the rich blood supply of brain tissue? The only data concerning ryanodine binding to smooth muscle have been obtained with uterine microsomes (Toro et al., 1989). These bound relatively small amounts of ryanodine, only reaching a maximum of 1.5 pmol per mg protein in a highly purified subpopulation. Despite possible differences in assay conditions, this seems to be too low to support any significant (vascular) smooth muscle contribution to ryanodine binding in brain. Also, while Ca^{2+} channels have been reconstituted from arterial smooth muscle (Erlich & Watras, 1988) they only had a conductance of 10 pS, not the high conductance of 100 pS reported here. Ryanodine binding studies, extended to a wide range of purified brain membrane fractions, may well provide answers to questions concerning Ca^{2+} channel location, together with a marker for channel purification.

Characteristics of the Reconstituted Channels

The Ca^{2+} channels in this report resemble Ca^{2+} release channels from muscle SR (Smith et al., 1986). Activation by Ca^{2+} itself could not be reliably demonstrated for the microsomal channel, but both are activated by adenine nucleotides and caffeine. They also display a similar high conductance for Ca^{2+} and comparable relative cation permeabilities in bi-ionic conditions (Smith et al., 1988). For the microsomal channels $pCa^{2+}/pK^{+}/pTris^{+} = 1/0.2/0.08$. Both channels are very permeant to other divalent cations including Mg^{2+}, distinguishing them from surface membrane voltage-activated (Nelson, French & Krueger, 1984; Lansman, Hess & Tsien, 1986) and putative receptor-operated (Kuno & Gardner, 1987; Zschauer et al., 1988) Ca^{2+} channels. Perhaps the most interesting property of the microsomal Ca^{2+} channel is its activation by the second messenger $InsP_3$ (incidentally suggesting that sensitivity to $InsP_3$ and caffeine are not mutually exclusive at the molecular level, *cf.* Meldolesi, Pompeo & Pozzan, 1988). However, the action of $InsP_3$ is surprisingly mild compared to activation by ATP (at physiologically-relevant concentrations). Even if the channels are very close to sites of $InsP_3$ generation, some form of inhibition seems to be necessary to counteract continuous ATP activation. In this respect there may be a role for protons (*discussed below*). GTP was only marginally effective as a channel activator, even in combination with $InsP_3$ (Dawson, 1985), but because this was a minimal system there may be a role for guanine nucleotides in vivo (Gill et al., 1986). In future studies it will also be important to investigate the actions of other second messengers (Morris et al., 1987; Parker & Miledi, 1987) and protein kinase C (Nishizuka, 1984).

Ma et al. (1988) presented a variety of observations illustrating the functional (but not structural) similarity of purified skeletal SR Ca^{2+} release channels to gap junction channels. Many of those points also apply to the brain microsomal channel described here, specifically its high unit conductance, relatively nonselective general cation permeability and activation by ATP. There is also some evidence for substate behavior both with and without ryanodine (Figs. 4 and 5). Overall, the presence of substates would be consistent with the idea that such channels are formed by the assembly of several identical oligomers (Fill & Coronado, 1988) and could provide a physical basis for the cooperativity of $InsP_3$ activation observed by Meyer, Holowka and Stryer (1988) and the observation here that $InsP_3$ increases the number (but not the duration) of full openings (Fig. 10). There are several examples of other channels which also appear to consist of equivalent, or near-equivalent, subunits (Mannella, Colombini & Frank, 1983; Engel et al., 1985; Zampighi, Hall & Kreman, 1985). Experiments at much higher resolution might answer the question of whether the current rectification shown by the microsomal channel (Fig. 7), which is too modest to be consistent with a constant-field electrodiffusion model (Hille, 1984), could be due to voltage-dependent substate behavior and only partial openings appearing at positive holding potentials. These experiments could not be carried out in planar bilayers (with Ca^{2+} as the current carrier) because, unlike patch clamping, the large area of the membranes will always contribute too much noise to single-channel recordings.

One final point about the possibility of a structurally distinct but functionally related "superfamily" of gap-junction like proteins is that the SR channel at least is inhibited by protons (Ma et al., 1988). If this turns out to be a general property shared by microsomal channels, and comprehensive binding studies were to suggest that some brain microsomal Ca^{2+} channels are present in secretory vesicles which also have proton pumps (Pollard et al., 1977), more consideration will have to be given to the hypothesis that channels could play a direct part in vesicle-plasma membrane fusion and neurotransmitter release.

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Note Added in Proof

Takeshima, H., et al. (1989), *Nature (London)* **339:**439–445, have recently cloned and sequenced the cDNA for a rabbit skeletal muscle ryanodine receptor, with important implications for the location and purification of similar proteins from other tissues.