HETEROGENEITY OF CONDUCTANCE STATES IN CALCIUM CHANNELS OF SKELETAL MUSCLE

JIANJIE MA AND ROBERTO CORONADO
Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, Texas 77030

ABSTRACT The single channel conductance of the dihydropyridine (DHP)-sensitive calcium channel from rabbit skeletal muscle transverse tubules was analyzed in detail using the planar bilayer recording technique. With 0.1 M $BaCl_2$ on both sides of the channel (symmetrical solutions), the most frequent conductance is 12 pS, which is independent of holding potential in the range of −80 to +80 mV. This conductance accounts for ~80% of all openings analyzed close to 0 mV. Two additional channels of conductance 9 and 3 pS are also present at all positive potentials, but their relative occurrence close to 0 mV is low. All channels depend on the presence of agonist Bay K 8644 and are inhibited by the antagonist nitrendipine. The relative occurrence of 9 and 3 pS can be increased, and that of 12 pS decreased, by several interventions such as external addition of cholesterol, lectin (wheat germ agglutinin), or calmodulin inhibitor R24571 (calmidazolium). The 9- and 3-pS channels are also conspicuous at positive potentials larger than +40 mV. We suggest that 9- and 3-pS channels are two elementary conductances of the same DHP-sensitive Ca channel. Under most circumstances, these two conductances are gated in a coupled way to generate a channel with a unitary conductance of 12 pS. Interventions tested, including large depolarizations, probably decompose or uncouple the 12-pS channel into 9 and 3 pS.

INTRODUCTION

Studies in planar lipid bilayers have shown that the single channel conductance of cardiac and skeletal muscle dihydropyridine (DHP)–sensitive calcium channels is inherently different. In symmetrical solutions of 0.1 M $BaCl_2$, the current-voltage relationship for both channels is ohmic, but the slope conductance of the cardiac channel is 23 pS whereas that of the skeletal channel is only 12 pS (Rosenberg et al., 1986; Ma and Coronado, 1987). In reports previous to these above, the use of asymmetric solutions and the presence of other ions such as Na (Affolter and Coronado, 1985; Coronado and Smith, 1987) or Mg (Ehrlich et al., 1986), although useful in defining ionic selectivity, made this difference in conductance less noticeable. Ehrlich et al. (1986) reported a unitary value of 8 pS for the cardiac Ca channel in 0.25 M Ba/0.1 M Mg (internal solution) and 0.1 M Ba/0.01 M Mg (external solution), and Coronado and Affolter (1986) reported a voltage-dependent slope of 20 pS (−50 mV), 13 pS (0 mV), 7 pS (+40 mV) in 0.1 M Ba (external solution), 0.1 M Cs (internal solution) for the skeletal Ca channel.

In contrast, purified dihydropyridine receptor complex (DHPR) of skeletal muscle has been shown to induce DHP-sensitive Ca channels of 20-pS slope conductance in symmetrical 0.1 M Ba (Flockerzi et al., 1986). Hence, the purified channel would appear to be similar in conductance to the DHP-sensitive long-lasting type Ca channel of cardiac membranes. The presence of this apparently new "cardiac-like" 20-pS channel in purified preparations of skeletal muscle prompted us to investigate in further detail the open channel properties of muscle Ca channels to determine if 20-pS channels are present in native t-tubule membranes. Native refers to membranes purified by conventional fractionation procedures without detergent extractions. We report here that ~80% of channel activity elicited by agonist Bay K 8644 and inhibited by antagonist nitrendipine is composed of a channel with a slope conductance of 12 pS in symmetrical 0.1 M $BaCl_2$. The presence of 20-pS channels described in the purified DHP receptor preparation (Flockerzi et al., 1986) could not be confirmed in our preparation of native membranes. However, inspection of large numbers of records shows two additional but less frequent conductances of unit values 9 and 3 pS. The occurrence of 9- and 3-pS levels could be increased by several manipulations that presumably affect the bilayer lipid phase or the channel protein itself. The results suggest that native skeletal muscle Ca channels have at least two elementary conductances, which can be uncoupled from the most common 12-pS channel by in situ biochemical interventions.

MATERIALS AND METHODS

Planar Bilayers and Measurements

Equimolar mixtures of phosphatidylethanolamine and phosphatidylserine from bovine brain were used in all experiments. Lipids were purchased from Avanti Polar Lipids (Birmingham, AL) and stored in chloroform at −80°C. Bilayers were formed by the Mueller-Rudin method in Lexan cups containing apertures of 300 μm. Lipids were dried under N_2 at the

moment of use and dissolved in decane (Aldrich Chemical Co., Milwaukee, WI) at a concentraton of 20 mg lipid/ml. The cup side (3-ml vol) was connected via an Ag/AgCl electrode and an agar/KCl bridge to the head-stage input of a List L/M EPC 7 amplifier (List-Electronic, DA-Eberstadt, FRG). The bath side (3-ml vol) was connected to ground using the same electrode arrangement. Potential differences between electrodes were kept within ±2 mV. Channels were incorporated into the bilayer from membranes added to the cup side at a final protein concentration of 10–50 μg/ml; 1–2 μM Bay K 8644 was present in all experiments in the protein-containing chamber.

Records were filtered at 0.05–0.1-KHz corner frequency using an eight-pole Bessel filter (Frequency Devices, Springfield, MA), digitized at 0.5–2.0 points/ms using a 12-bit resolution A/D converter (Keithley Instruments, Inc., Cleveland, OH), and fed into a PC/AT computer (IBM Instruments, Inc., Danbury, CT). Storage was done on 10-MB Bernoulli (Iomega, Roy, UT) disk cartridges. Mean amplitude of open channels was measured in two ways: (*a*) after separation of open and closed current levels using a dual threshold detector program described previously (Coronado and Affolter, 1986), or (*b*) by sorting all sampled current points into 256 bins at a gain of ~0.02 pA/bin and fitting baseline samples and open current samples as a sum of two or more Gaussian distributions.

Preparation of T-Tubule Membranes

T-tubule membranes were prepared from rabbit back and leg white muscle by a modification of the microsome fractionation procedure of Meissner (1984). Light muscle microsomes sedimenting at 10%/20% sucrose interface were used in all experiments. Portions of back and leg muscle are partially homogenized in buffer A (0.3 M sucrose, 20 mM Hepes-Tris pH 7.2) with four 15-s pulses in a food processor. Tissue is completely homogenized in 3 vol of buffer A at high speed (twice at 30 s) in a blender (Waring Products, New Hartford, CT). The total homogenate is centrifuged for 30 min at 2,600 *g* (4,000 rpm) in a GSA-Sorval rotor. The supernatant is reserved, and the pellet is rehomogenized in 3 vol of buffer A and centrifuged as before. The combined 2,600 *g* supernatants are centrifuged at 10,000 *g* (8,000 rpm) in the GSA-Sorvall and the resulting supernatant discarded. The 10,000 *g* pellets are resuspended and

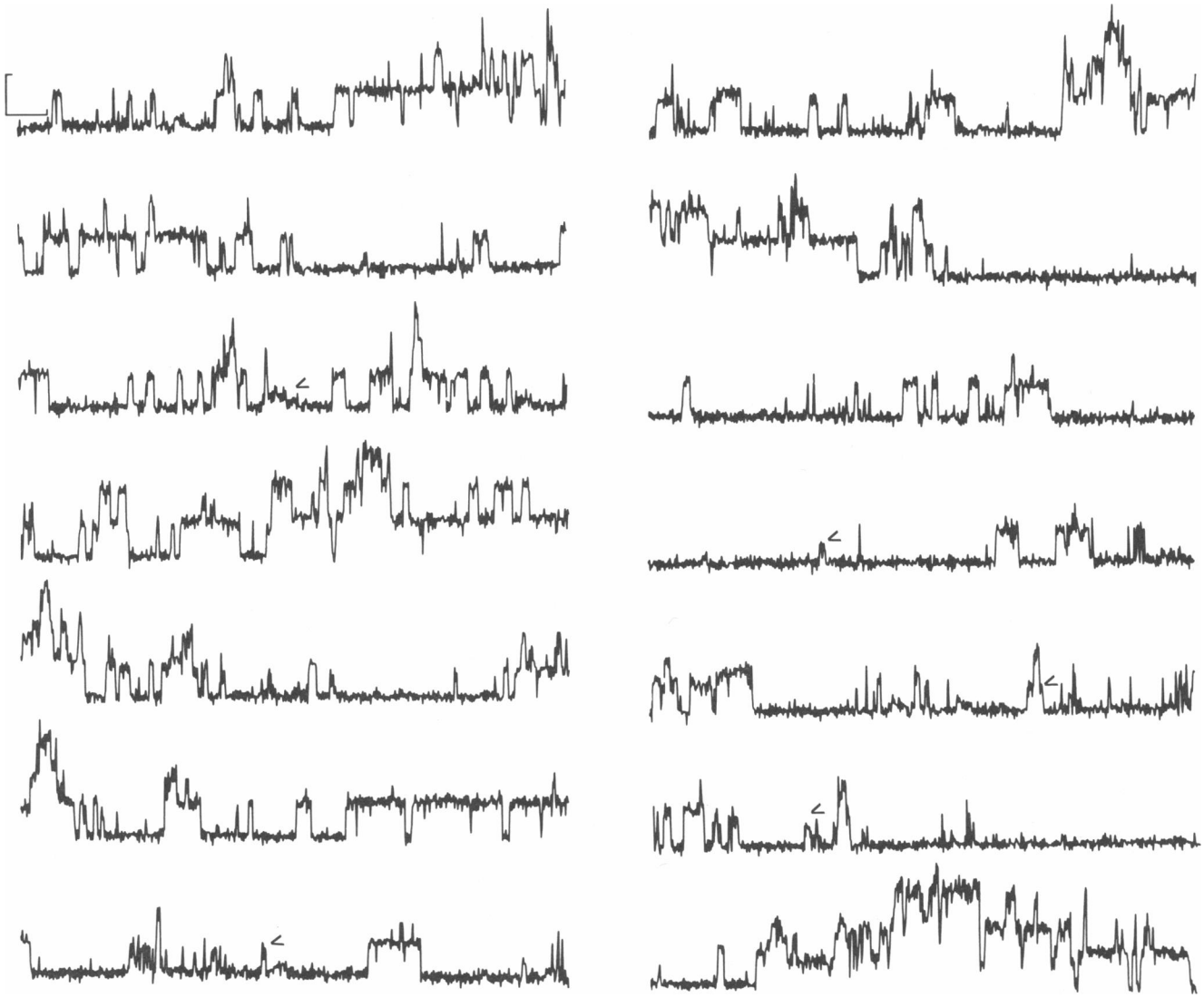

FIGURE 1 Barium current through t-tubule Ca channels. Continuous records (*from top to bottom, left first, right second*) taken at HP 0 mV, 0.1-KHz filter corner frequency, 1 μM Bay K 8644. Internal solution was 0.1 M $BaCl_2$, 0.05 M NaCl 10 mM Hepes-Tris, pH 7.0. External solution was 0.05 M NaCl, 10 mM Hepes-Tris, pH 7.0. Time and current scales are 750 ms and 0.65 pA. Duration of each segment is 10 s.

briefly homogenized in 0.6 M KCl, 5 mM Na-Pipes, pH 6.8 with two strokes of a motor driven Teflon/glass homogenizer followed by incubation on ice for 1 h. Salt-treated microsomes are sedimented at 90,000 *g* (32,000 rpm) in a model 35 rotor (Beckman Instruments, Inc., Palo Alto, CA) and resuspended in 10% wt/wt sucrose, 0.4 M KCl, 5 mM Na-Pipes, pH 6.8. This material is layered onto discontinuous sucrose gradients (8 ml of 20%, 8 ml of 25%, 6 ml of 30%, 6 ml of 35%, 4 ml of 40%) containing 0.4 M KCl, 5 mM Na-Pipes, pH 6.8, and centrifuged overnight (18 h) at 26,000 rpm in a Beckman SW.27 rotor. Fractions are collected from the sucrose interfaces by aspiration with a Pasteur pipette, diluted with ice-cold glass-distilled water, and pelleted at 90,000 *g* (32,000 rpm) in a Beckman 35 rotor. Pelleted membranes are suspended in 0.3 M sucrose, 0.1 M KCl, 5 mM Na-Pipes pH 6.8 and frozen in liquid N_2 until use. Membrane fractions from the 10%/20% and 20%/25% sucrose interfaces routinely showed the highest PN200-110 binding activity (20–40 pmol/mg protein).

Solutions and Chemicals

For recordings, solid salts of $BaCl_2$, $CaCl_2$, and NaCl were analytical grade (Johnson Matthey Co., Herefordshire, England). Buffer Hepes free acid (Calbiochem-Behring Corp., La Jolla, CA) and buffer Trizma free base (Tris[hydroxymethyl]aminomethane; Sigma Chemical Co., St. Louis, MO) were reagent grade. Solutions were prepared the same day of experiments using all-glass twice-distilled water (12–16 MΩ-cm resistivity). Unless otherwise specified, solutions contained 10 mM Hepes buffered to pH 7.0 with solid Trizma (~2 mM Tris final concentration). Cholesterol (Eastman Kodak Co., Rochester, NY) was added to the bath side aqueous phase from a concentrated stock solution in methanol. Compound R24571 (calmidazolium; Sigma Chemical Co.) was added to the bath side aqueous phase from a concentrated solution in ethanol. Lectin (prepared from wheat germ of *Triticum vulgaris;* Sigma Chemical Co.) was added to the bath side aqueous phase from a concentrated solution in 50 mM NaCl, 10 mM Hepes-Tris pH 7.0.

For preparative procedures used in the purification of t-tubules sucrose and salts of K and Na were reagent grade (Fisher Scientific Co., Pittsburgh, PA). Pipes (Behring Diagnostics, San Diego, CA); Hepes, Trizma (sources described above) were reagent grade.

RESULTS

Three Conductances in T-tubule Ca Channels

Fig. 1 shows a continuous record of barium current through Ca channels at HP 0 mV, 1 μM Bay K 8644 (10 s per displayed segment). The current carrier is internal 0.1 M Ba; thus it flows in the outward direction. Based on the superposition of open currents, there is contribution from at least three channels. Histograms representing the frequency of all encountered current values in these recordings are shown in Fig. 2 *A* and expanded twofold in Fig. 2 *B*. Subtraction of baseline current in Fig. 2 *B* reveals the approximate distribution of counts due to open channels. The shape of the distribution of single open channels (largest peak in Fig. 2 *B*) is slightly asymmetric. A gaussian curve (not shown) with mean = 0.61 pA and SD = 0.051 pA is adequate to fit ~80% of counts under this peak. In addition, Fig. 2 *B* shows two or more minor peaks due to simultaneous openings. Although not clearly represented in the histograms, there are numerous events which in Fig. 1 appear to have mean currents 2–3 SD below the mean open current of the population. Some of these low conductance events are indicated in Fig. 1 by arrowheads. The analysis of these "subunitary" conductances was approached by direct inspection of large numbers of records at high gain and large driving force. The latter was best achieved by setting up recordings in symmetrical solutions. Examples of the occurrence of three distinct well-resolved current levels are shown in Fig. 3 at HP + 80 mV (80 mV positive to the 0 mV reversal potential). These segments are clear cases of single long-lasting, non-overlapping events which were found to have significantly different sample means, 0.96 pA (H, high), 0.71 pA (I, intermediate), and 0.28 pA (L, low). A test of significance is given below. At all potentials, except 80 mV (Fig. 4), H channels were found to be the most frequent occurrence. H channels have a mean slope conductance of 12 pS, which was measured at 11 different HPs (Fig. 5, *left*). As shown in Fig. 3, *right* (nitrendipine), all conductance types are inhibited by 10 μM nitrendipine. The three conductances shown in Fig. 3 were the only types consistently observed after screening ~1,200 openings from five preparations of native rabbit t-tubules.

Amplitude distributions of H, I, and L channels were separated from the total distribution of open channels as shown in Fig. 4. In the construction of these histograms, only recordings containing single transitions were used to

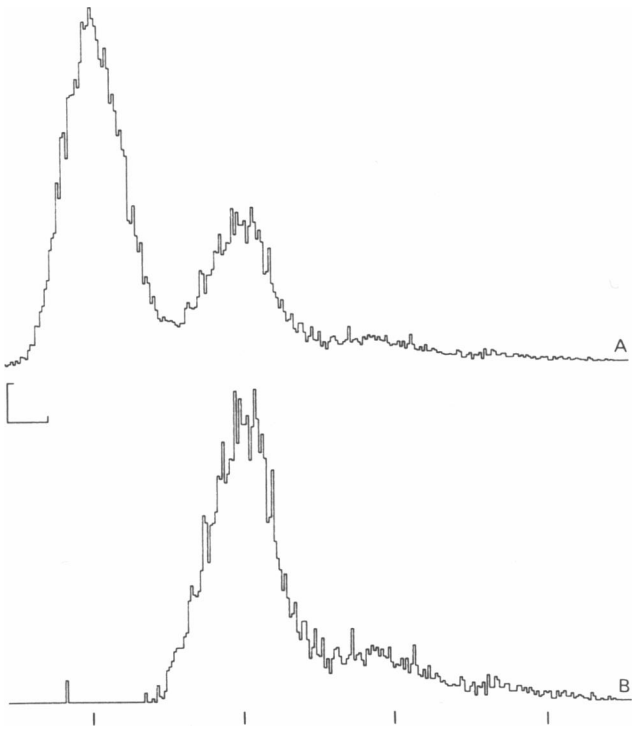

FIGURE 2 Histograms of sampled current. Records from Fig. 1 were sampled at 2 ms/point, and current samples (70,000 total) were sorted into 256 bins at a gain of 0.01 pA/bin. Total width of histograms is 2.5 pA. *A* corresponds to a histogram of the total samples. *B* is a twofold enlargement; baseline counts where subtracted. Spacing bars are 0.6-pA apart. *X*, *Y* scale corresponds to 160 counts, 0.16 pA in *A* and 80 counts, 0.16 pA in *B*.

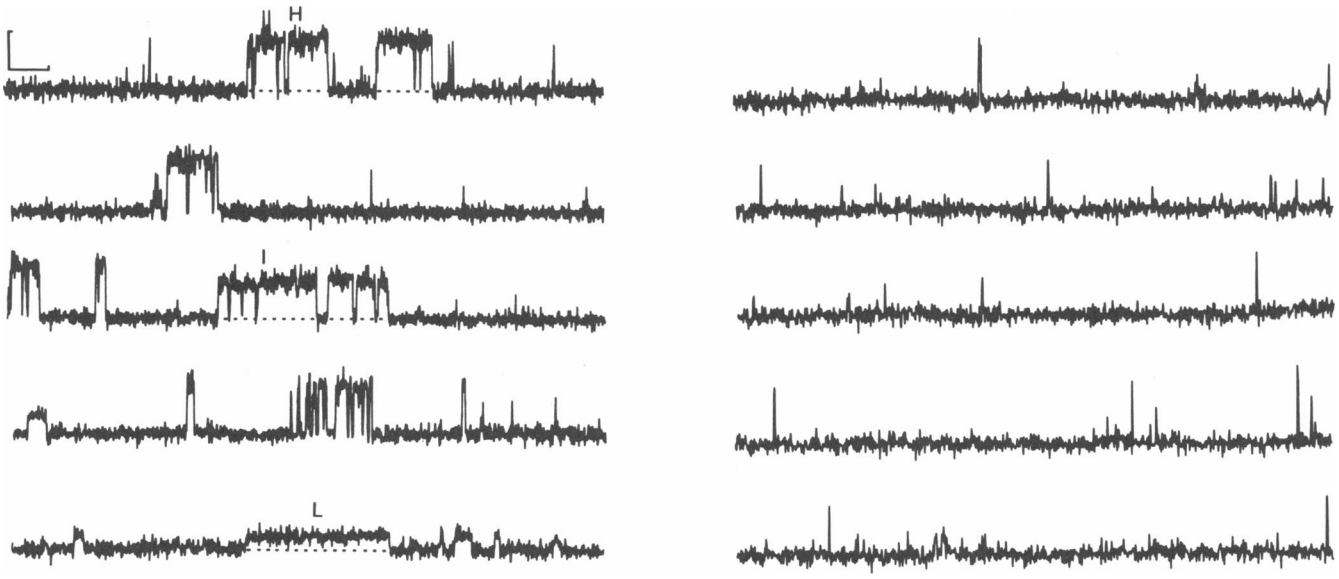

FIGURE 3 Three conductance states in t-tubule Ca channels. Barium current was recorded HP 80 mV in symmetrical (same solution on both sides) 0.1 M $BaCl_2$, 0.05 M NaCl, 0.01 M Hepes-Tris, pH 7.0. Time and current scales are the same for both panels, 750 ms, 0.65 pA. (*Left*) Selected records to demonstrate the occurrence of three current levels, H (0.96 pA), I (0.72 pA), and L (0.28 pA). (*Right*) Representative records after addition of 10 μM nitrendipine.

avoid overlapping currents from multiple channels. Starting with a histogram of sampled current (as in Fig. 2 *A*), the baseline was fitted with a single gaussian distribution. The theoretical curve was overlayed with the data points, and one was subtracted from the other. The top histograms in Fig. 4 show remaining counts of open channel current after baseline subtraction at two HPs, 40 and 80 mV. Distributions of open channels were found to have several peaks. This was particularly clear at large positive potentials such as in the histograms at 80 mV where open counts are composed of two distinct peaks and a shoulder at the high current end of the distribution. Consistent with the identification of three channels, we always found that a minimum number of three gaussian curves was necessary to fit the distributions. To separate H, I, and L out of the total counts shown in Fig. 4 (*top*), (*a*) the outer and inner edges of the histogram were each fit by a single gaussian

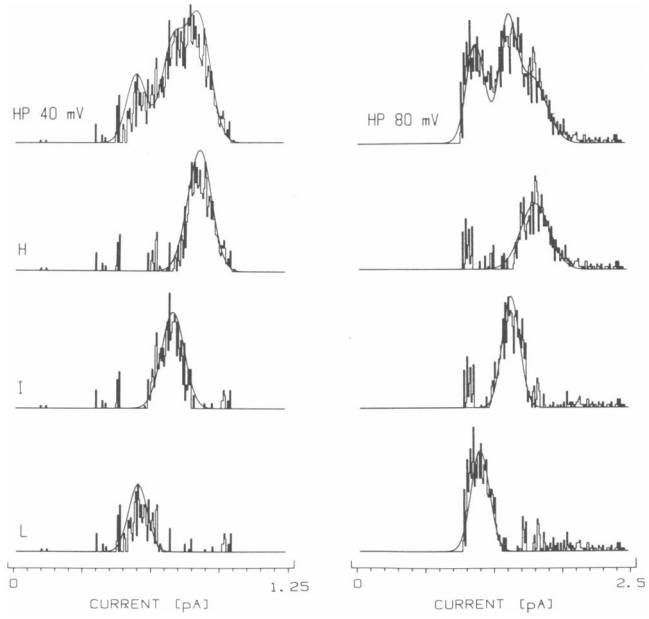

FIGURE 4 Separation of H, I, and L channels at two holding potentials. Current was sampled at 10 ms/point and sorted at a resolution of 0.005 pA/bin (HP 40 mV) or 0.01 pA/bin (HP 80 mV); 32,506 samples (325-s recording time) were sorted at HP 40 mV and 30,511 (305-s recording time) at HP 80 mV. After subtraction of baseline samples, top histograms show 2,873 samples of single open channels at HP 40 mV and 2,745 samples at HP 80 mV. The total histogram was decomposed into three populations denoted H, I, and L, from top to bottom, respectively. Solid lines are gaussian curves with parameters given in Table I.

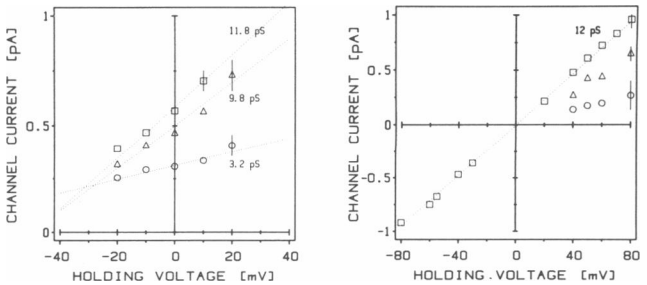

FIGURE 5 Current–voltage curves for H, I, and L channels. (*Left*) Currents in internal 0.1 M $BaCl_2$, 0.05 M NaCl; external 0.05 M NaCl. (*Right*) Currents in symmetrical (same solution on both sides) 0.1 M $BaCl_2$, 0.05 M NaCl. Bars indicate 2 SD of the data point with the largest error.

curve; (*b*) counts under the theoretical curves fitted at each edge were subtracted from total counts; (*c*) the center of the distribution revealed after subtraction of the edges was fitted separately (labeled I); and (*d*) subtraction of counts in I from total counts revealed H and L. Smooth lines in H, I, and L correspond to gaussian curves fitted to each population using a least squares difference program. The smooth line in Fig. 4 (*top*) corresponds to the sum of the three smooth curves below. At HPs up to 40 mV, H channels were found to be the most frequent; however, at 80 mV, I types are dominant. Relative occurrence and mean durations of sampled, H, I, and L events at the two test potentials are summarized in Table I. Entries in Table I show that isolation of events by peak current histograms as described above or by selecting current threshold detectors for the open and closed current levels as described previously (Coronado and Affolter, 1986) gave the same results. H, I, and L amplitudes derived by the two methods are indistinguishable (>80% overlap of means and SDs). Because H and I channels are so close in unit current with parameters at HP 80 mV, (i = 0.96 pA, SD = 0.06 pA, n = 102) vs. (i = 0.71 pA, SD = 0.08, n = 174), respectively, we subjected the data to a normal deviate test for the difference of two sample means. Under the assumption that H and I have equal population means, we asked whether the observed difference between sample means is significant. Deviations from the population mean equal to or larger than that observed between means of H and I channels had probability of occurrence $P < 10^{-4}$ at HP 80 mV and $P < 10^{-3}$ at HP 40 mV. Hence, we rejected the hypothesis that means of H and I channels are equal.

TABLE I
AMPLITUDE AND LIFETIME OF H, I, AND L CALCIUM CHANNELS OF SKELETAL MUSCLE

	No. of events	Mean current	Standard deviation	Fraction open time	Mean duration
	n	*pA*	*pA*	*%*	*ms*
HP 80 mV					
H	102	0.96/0.99	0.06/0.05	1.39/1.63	127
I	174	0.71/0.67	0.08/0.10	3.57/1.92	48
L	143	0.28/0.28	0.06/0.04	2.92/2.82	37
HP 40 mV					
H	102	0.48/0.49	0.09/0.03	3.90/4.32	173
I	79	0.36/0.36	0.08/0.03	2.93/0.94	47
L	61	0.18/0.21	0.07/0.02	1.77/1.72	30

Entries separated by a shill were obtained by two separate methods. First entry correspond to parameters fitted from histograms of sampled current. Samples under H, I, and L peaks were separated from the distribution of total samples by curve fitting and subtraction as described in text. Second entry correspond to parameters from histograms of H, I, and L events collected separately. Three current threshold values were used to reject baseline current and open currents from two additional levels. Both measurements were done on same recordings. Sampling rate was 10 ms/point; recording time was 353-s HP 80 mV, and 334-s HP 40 mV.

When I and L channels were compared, P was $<10^{-6}$. Current–voltage relationships separately for H, I, and L are shown in Fig. 5 under two ionic regimes, while carrying outward Ba (*left*) or under symmetrical solutions (*right*). With 0.1 M Ba on both sides, linear fit of slope conductance resulted in approximate values 12, 9, and 3 pS for H, I, and L, respectively. With 0.1 M Ba on the internal side and 0.05 M Na on the external side, there is outward current at 0 mV, which aims at a reversal at negative potentials (E_{Ba} is nominally minus infinity; $E_{Na} = 0_{mV}$). Linear extrapolation of currents suggest a reversal of currents at potentials more negative than −50 mV. Thus, all channels have high Ba over Na selectivity.

Ca Channel Conductances Induced by Calmodulin Inhibitor, Lectin, and Cholesterol

Apparently unrelated substances were found to promote large numbers of "subunitary" conductances. When analyzed in detail, induced conductances were found to be similar in amplitude and nitrendipine sensitivity to the H, I, and L channels that occur spontaneously and were described above. As shown in Fig. 6, micromolar concentrations of the calmodulin inhibitor calmidazolium (Compound R-24571), or wheat germ lectin protein, or cholesterol, added to the external solution results in the appearance of large numbers of lower conductance channels, which are infrequent in controls. These compounds are only effective when added to the external side and had no consequence when added to the internal solution (not shown). For testing compounds, activity was first collected under control and then continued after compound addition and after nitrendipine inhibition. In all three cases, channels were inhibited almost to completion by 10 μM nitrendipine added at the end of the compound test period. Spontaneously occurring H, I, and L channels are also inhibited by 10 μM nitrendipine to similar levels (see Fig. 3). The reduction in mean current inducted by these agents is qualitatively different from the flickery block described for Mg and other inorganic Ca channel blockers (Nelson, 1986; Lansman et al., 1986; Hess et al., 1986). In our case, amplitudes were found to be independent of test compound concentration, and the effects are not easily reversible after extensive perfusion of the bath solution. Amplitudes and relative occurrences of control and induced channels are given in Table II. The main effect of these agents is to change the relative frequency of H, I, and L. However, amplitudes before and after additions remain approximately constant. Compound R-24571 virtually eliminates H channels (10-fold reduction) with little change in the occurrence of I or L. On the other hand, wheat germ agglutinin (WGA) and cholesterol lower the occurrence of H channels 5–10-fold and increase L (2-fold) or I (10-fold), respectively. The constancy of H, I, and L channels under all tested conditions was further investigated in Fig.

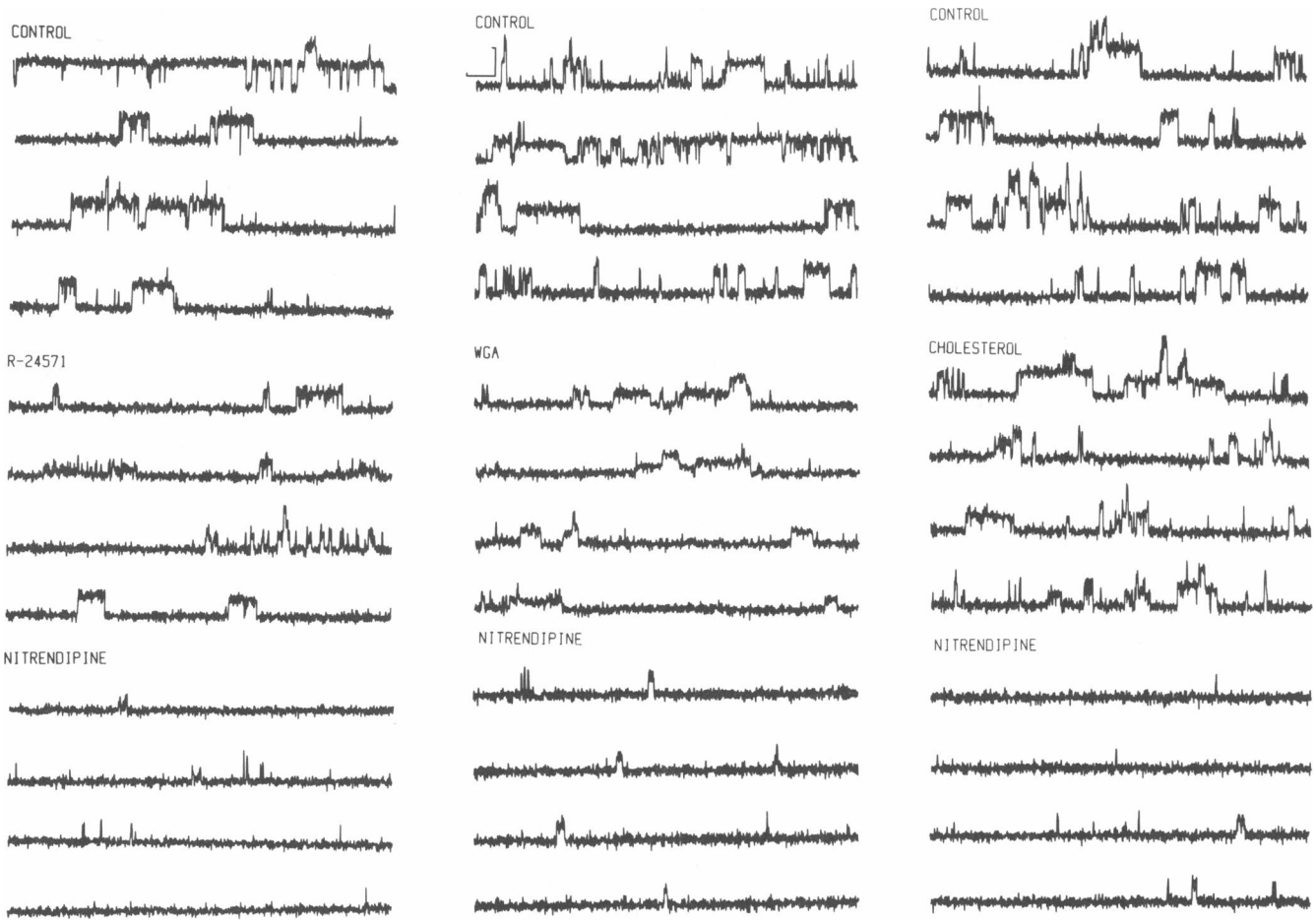

FIGURE 6 Ca channels modified by R-24571, WGA, and cholesterol. Recordings from three experiments are shown at right, center, and left. (*Top to bottom*) Traces correspond to control, after compound addition, and after 10 μM nitrendipine. R-24571 (0.5 μM), WGA (10 μM), cholesterol (0.5 mM) were added from methanol stock solutions to the external solution. Solutions were the same as in Fig. 1, HP 0 mV. Current and time scales correspond to 0.65 pA, 750 ms.

7 by plotting channel amplitudes as x, y pairs, where x is the amplitude control and y is the test amplitude after addition of either R-24571 (*squares*), WGA (*circles*), or cholesterol (*triangles*). Both control and test periods are from the same experiment. Horizontal bars indicate 2 SD of control whereas vertical bars indicate 2 SD during test period. Control events and events after additions are clustered into three populations (H, I, and L amplitudes), which are invariant with additives. The correlation coefficient for the nine data pairs was 0.98, and the slope ($\pm$SD) and intercept ($\pm$SD) of the best line (*dotted line*) was 0.97 ($\pm$0.075) and 0.027 (0.032), respectively. Thus, compounds tested did not create new channels.

That stationary probabilities of H, I, and L could be modified during a recording gave us an opportunity to test, under a variety of conditions, whether 12-pS H channels did in fact arise from a random superposition of 9-pS I channels and 3-pS L channels. The relative occurrence of H, I, and L was found to deviate from statistical independence in a significant manner. We reasoned that if I and L were independent from H channels, then the probability product $P(\mathrm{I}) \times P(\mathrm{L})$ should equal the probability $P(\mathrm{H})$. That is, a 12-pS channel would arise from the superposition of two independent 9- and 3-pS channels as governed by binomial statistics. This basic expectation was tested in Fig. 8 using the six sets of data in Table II as well as the separation of H, I, and L at different potentials (Table I). In Fig. 8, each of 12 points represents a recording where H, I, and L were separated and their respective probabilities computed. For independent channels, we expected an approximately linear correspondence between the probability of finding an H channel and the product of the probabilities of finding I or L channels, each computed separately. On the contrary, over about five orders of magnitude the two quantities appear unrelated.

DISCUSSION

The heterogeneity of conductances described here in the preparation of rabbit skeletal muscle calcium channels most probably arises from one channel type having three conductance states: H, I, and L. In favor of this hypothesis

TABLE II
EFFECTS OF CALMODULIN INHIBITOR, WGA, AND CHOLESTEROL ON DISTRIBUTIONS OF CONDUCTANCE LEVELS

	No. of samples	Mean current	Standard deviation	Relative occurrence
	n	*pA*	*pA*	*% total samples*
Control	(33,491)			
H	4,595	0.57	0.13	13.72
I	1,348	0.41	0.88	4.04
L	1.263	0.26	0.10	3.77
R-24571	(34,751)			
H	310	0.60	0.07	0.90
I	1,897	0.46	0.10	5.48
L	1,331	0.28	0.11	3.83
Control	(16,028)			
H	1,598	0.56	0.09	9.97
I	946	0.42	0.09	5.90
L	781	0.23	0.11	4.87
WGA	(17,270)			
H	154	0.56	0.05	0.89
I	1,100	0.42	0.08	6.37
L	2,050	0.29	0.09	11.87
Control	(23,933)			
H	2,331	0.58	0.12	13.50
I	261	0.44	0.06	1.25
L	732	0.29	0.10	4.24
Cholesterol	(17,264)			
H	505	0.60	0.09	2.92
I	2,184	0.44	0.15	12.65
L	787	0.26	0.10	4.56

Tabulated are three separate experiments where channels were first collected during a control period and after an addition of 0.5 μM Compound R-24571 (calmidazolium), 10 μM WGA (lectin), or 0.5 mM cholesterol to the bath side (external) aqueous phase. In parentheses next to each control and addition are given the total number of sampled points (baseline plus open current) from which amplitude histograms and relative occurrences were derived. Sampling rate was 10 ms/point. Current levels were separated as described in Fig. 4 and text.

are most of our results: (*a*) H, I, and L do not gate with statistical independence; (*b*) the relative frequency of H, I, and L can be modified in situ while amplitudes remain unchanged; and (*c*) all states, induced or spontaneous, are inhibited by micromolar concentrations of nitrendipine, hence related to the DHP-sensitive channel. All states were also found to be inhibited by 10 μM D600 or its quaternary derivative, D890 (not shown). Two of the test compounds, WGA and cholesterol, can block the appearance of H states and at the same time significantly increase the frequency of I or L states. This suggests that H, I, and L are in fact linked via precursor–product relationships. However, details of such kinetics scheme are presently unknown since only the long lasting transitions are adequately resolved (Fig. 3). Transformation of one channel into another, for example, upon occurrence of long-lasting transitions from H to I or L or vice versa, have been observed but these are rare. The relative occurrence of

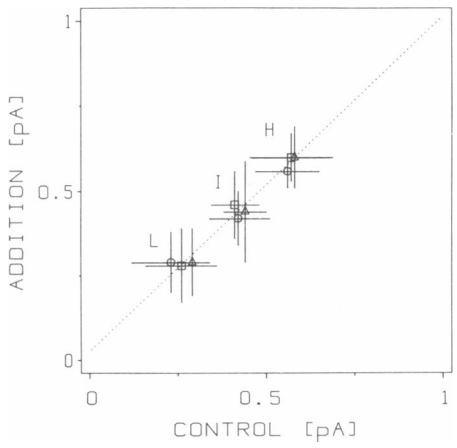

FIGURE 7 Correlation of H, I, and L amplitudes before and after addition of WGA, R-24571, or cholesterol. Horizontal and vertical bars indicate mean and 2 SD of single channel amplitude measurements before (*x* axis) and after (*y* axis) addition of WGA (*circles*), R-24571 (*squares*), cholesterol (*triangles*). Details are given in Fig. 6. The dotted line is the least squares regression line, slope = 0.978 (±0.072 SD), intercept = 0.027 (±0.032 SD).

observable transitions among H, I, and L levels (relative to all observable transitions) was <1%. Strong evidence against the alternative hypothesis, i.e., that H, I, and L represent three independent channels, is the fact that H states (12 pS) do not arise from an independent superposition of I states (9 pS) and L states (3 pS). This was shown in Fig. 8 where in 12 separate recordings under a variety of conditions, the long-term probability of H was found to be uncorrelated with the joint probabilities of I and L. This is a straightforward test for statistical independence which should have prevailed if the identified H, I, and L levels represented independent channels. Instead, the data suggest that 9 and 3 pS are two elementary conductances that are gated simultaneously most of the time. When this

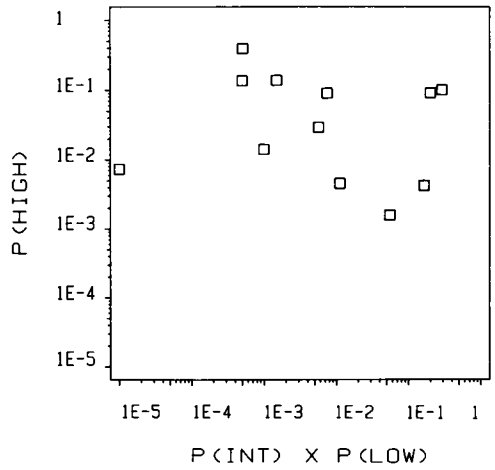

FIGURE 8 Lack of correlation of $P(H)$ and $P(I) \times P(L)$. Each point (12 total) is a data set where the relative occurrence (probability) of H, I, and L was separated either by sorting current samples or by amplitude threshold detectors. Nine of these data sets are given in Tables I and II.

happens, a 12-pS channel is formed. At two HPs where the data were analyzed in detail (Table I), the 12-pS channel was the most frequent and the longest-lived, hence the most stable.

The presence of more than one unit conductance per open channel has been documented extensively for many channels (see Sachs, 1983), but only recently for Ca channels (Carbone and Lux, 1987; Prod'hom et al., 1987). The Ca channel unit conductance, next to its kinetics and pharmacology, have been used as hallmarks to recognize different Ca channel types in cells (Nowycky et al., 1985; Nilius et al., 1985). Therefore, the existence of subsets of the same channel with significantly different mean conductance represents a new level of complexity in Ca channel analysis. Difficulties that subtypes or substates of the same channel may introduce in single channel analysis are that (*a*) different states may have different lifetimes and (*b*) different states may be induced at different voltages. Both types of phenomena seem to be present in our case. H channels appear to have 3–4-fold longer lifetime than I or L at the two voltages analyzed, and large depolarizations make H channels less frequent (Table I). There is some precedence at the whole tissue level for the heterogeneity that we observe at the single channel level. Toro et al (1987) recently described three types of Ca currents in mammalian skeletal muscle in culture. Evidently in neonatal rats, two fast inactivating-types are present in addition to the more classical slow-type. The slow-type has also been described in adult rat muscle (Donaldson and Beam, 1983), whereas both slow and fast types are present in frog (Cota and Stefani, 1986; Garcia and Stefani, 1987). If present in cells, H, I, and L channels may introduce kinetic heterogeneity, which is suggested in Table I by the voltage-dependent occurrence and lifetimes of these states. Large depolarizations make I and L more frequent (relative to H), and both states have lifetimes 3–4-fold briefer than H; H states have high occurrence at HP 0 mV and negative holding potentials. However, it is not immediately clear how this voltage-dependence may contribute to the macroscopic currents in skeletal muscle particularly since our channels are drug-modified and those studied by Toro and Stefani (1987) are elicited by voltage steps in the absence of agonists. For the same reason, it is unlikely that I or L channels reported here may be directly related to the low conductance transient Ca channel type described in many cell types other than skeletal muscle; the latter are insensitive to dihydropyridines (Nowycky et al., 1985; Nilius et al., 1985).

Besides the chemicals tested here, only a few ligands, i.e., alkaloids (Barnes and Hille, 1987; Imagawa et al., 1987), excitatory amino acids (Jahr and Stevens, 1987; Cull-Candy and Usowicz, 1987), and possibly protons (Hanke and Miller, 1983; Prod'hom et al., 1987), have been shown to promote conductance states that are otherwise infrequent. The effects of wheat germ lectin are significant in that the DHP receptor of skeletal muscle has but one major glycoprotein subunit of 135–170 kD (Curtis and Catterall, 1986; Galizzi et al., 1986; Flockerzi et al., 1986; Nakayama et al., 1987; Leung et al., 1987; Smith et al., 1987). As expected from the commonly found distribution of glycosilated residues in membrane proteins, effects of WGA are restricted to the external face of the Ca channel. Effects of calmidazolium and cholesterol are more difficult to track because of their high lipid solubility. The site of action for these compounds could be on the membrane phase but also in some hydrophobic domain of the channel or its DHP receptor. For example, it is known that some calmodulin inhibitors behave as general membrane perturbants while others, including R-24571, apparently have specific effects on DHP occupancy in the Ca channel (Johnson, 1984). Additional evidence that Ca channel conductance can be chemically altered arises from reconstitution studies using purified DHP receptor preparations. Smith et al (1987) showed that a purified preparation of DHPR consisting of a major polypeptide of 150 kD under disulfide-reducing conditions induced two types of Ca channels when incorporated into planar bilayers. In addition to the 12-pS H channel described here, there is a 20-pS channel, kinetically linked to the H channel, but apparently present only in purified DHPR preparations (see also Flockerzi et al., 1986). Our present work could not confirm the presence of 20-pS channels in native t-tubules. Hence, it would appear that either (*a*) some domain in the purified protein, presently not under experimental control, has considerable influence on the conductive properties of the Ca channel; or (*b*) the density of 20-pS channels is extremely low in our preparation of native skeletal muscle t-tubules.

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