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Calcium Channel Activity in a Purified Dihydropyridine–Receptor Preparation of Skeletal Muscle†

Jeffrey S. Smith,‡ Edward J. McKenna,§ Jianjie Ma,‡ Janeen Vilven,‡ Pal L. Vaghy,§ Arnold Schwartz,§ and Roberto Coronado*,‡

Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, Texas 77030, and Department of Pharmacology and Cell Biophysics, University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0575

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ABSTRACT: A purified dihydropyridine–receptor complex (DHPR) of skeletal muscle consisting of a major polypeptide of M_r 150K under reducing conditions induces divalent cation selective channels when incorporated into planar lipid bilayers. Channels were inserted into preformed planar bilayers by two techniques: (i) direct dilution of detergent-solubilized DHPR into the aqueous chambers adjacent to the bilayer membrane or (ii) reconstitution of DHPR into phospholipid vesicles followed by fusion of the preformed vesicles to the planar bilayer membrane. Unlike native membrane preparations of t-tubules, which only have one major Ca channel type of slope conductance of 12 pS in symmetrical 100 mM Ba, the purified DHPR complex induced at least two channel types with conductances of 12–14 and 22 pS. Some recordings suggest that these two channels are statistically coupled in time, i.e., that they may correspond to substates of the same DHPR channel. Activity was found to occur spontaneously in the absence of the Ca channel agonist Bay k 8644. The 12–14-pS channel from DHPR exhibits voltage-dependent kinetics, is highly selective for barium ions, and was inhibited by micromolar nitrendipine. The 12–14-pS DHPR channel appears to be identical with functional Ca channels previously described in native t-tubules.

1,4-Dihydropyridines (DHP), phenylalkylamines, and benzothiazepines are a diverse group of compounds that can increase or decrease Ca channel activity, by inducing long-term opening or closing of Ca channels in several tissues (Schramm et al., 1983; Janis et al., 1984; Schwartz & Triggle, 1984; Brown et al., 1984; Hess et al., 1984; Kokubun & Reuter, 1984; Schwartz et al., 1985; Affolter & Coronado, 1985; Rosenberg et al., 1986; Caffrey et al., 1986; Vaghy et al., 1987a). The finding that transverse tubular membranes of skeletal muscle contain a large number of receptors that bind 1,4-dihydropyridines with high affinity (DHPR) has led to the solubilization and purification of the DHPR complex. A large peptide (M_r 135K–170K) appears to be a major subunit though many discrepancies exist about the presence of other possible subunits (Curtis & Catterall, 1984; Ferry et al., 1984; Galizzi et al., 1986; Flockerzi et al., 1986; Nakayama et al., 1986a,b, 1987; Cooper et al., 1987; Leung et al., 1987).

Skeletal muscle Ca channels, in vivo and in planar bilayers, are modulated by dihydropyridine agonists and antagonists (Charandini & Stefani, 1983; Schwartz et al., 1985; Affolter & Coronado, 1985). Thus, it has been of interest to investigate whether the purified DHPR can induce Ca flux or single-channel currents in reconstituted systems. Curtis and Catterall (1986) showed that a small fraction of DHPR promotes Ca flux in liposomes that can be increased by the dihydropyridine agonist Bay k 8644 and inhibited by several antagonists. Recordings of Ca single-channel activity present in DHPR has been recently described (Flockerzi et al., 1986). In this paper we show for the first time that a highly purified preparation of DHPR (Nakayama et al., 1986a,b, 1987) induces, among others, a 12–14-pS voltage-dependent, dihydropyridine-sensitive channel that is similar, on the basis of channel conductance, divalent ion selectivity, and drug sensitivity, to those identified in native t-tubule vesicles as bona fide DHP-sensitive Ca channels (Affolter & Coronado, 1985, 1986; Coronado & Affolter, 1986; Coronado & Smith, 1987). However, it is unclear whether Ca channels copurify with DHPR or DHPR per se constitutes a Ca channel. A preliminary report has been presented elsewhere (McKenna et al., 1987).

Materials and Methods

Purification of DHPR. t-Tubule membranes were prepared from rabbit skeletal fast (white) muscle as described by Nakayama et al. (1987). Briefly, these procedures employ freeze-thawed muscle and a French press treatment. Purification of DHPR from t-tubule membranes was performed as described by Curtis and Catterall (1984) and Nakayama et al. (1987), with minor modifications. t-Tubule membranes were not prelabeled with [^{3}H]-(+)-PN200-110 prior to solubilization with digitonin. Initial studies using prelabeled DHPR, as well as postlabeling experiments on each purifi-

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‡Baylor College of Medicine.
§University of Cincinnati Medical Center.

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cation step, indicated that peak protein fractions and peak binding was coincidental. Therefore, the protein peaks from each chromatography step were pooled, and the peak protein fractions from the sucrose density gradient were used for single-channel recording, radioligand binding studies, and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

Saturation Binding Assay. DHPR (0.1–0.3 μg) was incubated in a total volume of 0.25 mL of 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 0.2 mg/mL bovine serum albumin (BSA), 0.1% digitonin, 5% glycerol, and varying concentrations of [^{3}H]-(+)-PN200-110 (sp act. 82 Ci/mmol) at 25 °C for 60 min. Nonspecific binding was determined by including 3 μM cold (+)-PN200-110 in the incubation medium. After incubation, carrier protein (100 μL of 5 mg/mL BSA and 5 mg/mL γ-globulin mixture) was added, and protein was precipitated by addition of 3.5 mL of PEG buffer [10% poly(ethylene glycol) 10000, 10 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$]. Bound ligand was separated by rapid filtration through GF/C filters previously soaked in 0.3% poly(ethylenimine) solution for 60 min. The filters were washed twice with PEG buffer and put into plastic vials; 8 mL of Budget-Solve complete counting cocktail (Research Products International) was added, and the radioactivity was counted. The data were analyzed by a nonlinear least-squares Ligand program (Munson & Rodbard, 1980).

Reconstitution of DHPR into Phospholipid Vesicles. DHPR was reconstituted in phospholipid vesicles by detergent removal with Bio-Beads SM-2 adsorbent, essentially as described by Talvenheimo et al. (1982). Briefly, purified DHPR (6 pmol/100 μL) in 0.37 M sucrose, 5 mM sodium 4-morpholinepropanesulfonate (NaMOPS), pH 7.4, 0.1% digitonin, 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1 mM PMSF, 1 μM pepstatin A, 0.5 mM iodoacetamide, and 0.5 μg/mL antipain was added to 0.9 mL of 1.1% w/v TX-100, 0.35% w/v phosphatidylcholine (egg, Avanti Biochemicals, Birmingham, AL) and phosphatidylethanolamine (brain, Avanti) (1:1), 0.33 M sucrose, 600 μM $CaCl_2$, 550 mM NaCl, and 25 mM Tris–Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] pH 7.4, and incubated on ice for 30 min. Bio-Beads SM-2 adsorbent (Bio-Rad Inc., Richmond, CA), 0.2 g, was added and the mixture stirred overnight at 4 °C. Following overnight incubation, the solution was removed by aspiration with a 1-mL Eppendorf pipet and incubated an additional 2 h at 4 °C with a fresh 0.2-g portion of SM-2 adsorbent. Reconstituted vesicles were tested for channel activity in planar bilayers immediately following the second incubation with SM-2 adsorbent.

Planar Bilayers. Lipid bilayers were cast from 20–50 mg/mL phospholipid or phospholipid–cholesterol solutions dissolved in decane. Bovine brain phosphatidylethanolamine (PE) and phosphatidylserine (PS) (Avanti Polar Lipids, Birmingham, AL) were used in all experiments. Cholesterol (99.5% minimum) was purchased from Eastman Kodak (Rochester, NY). Low-pass filter settings (0.05–0.2-kHz corner frequency on 8-pole Bessel filter) and handling of single-channel data have been described in detail elsewhere (Coronado & Affolter, 1986). Incorporation of native t tubule membranes into planar bilayers was achieved under the ionic conditions described previously (Affolter & Coronado, 1985). Cis corresponds to the voltage-control side and side of DHPR addition while trans is the ground protein-free side. Native

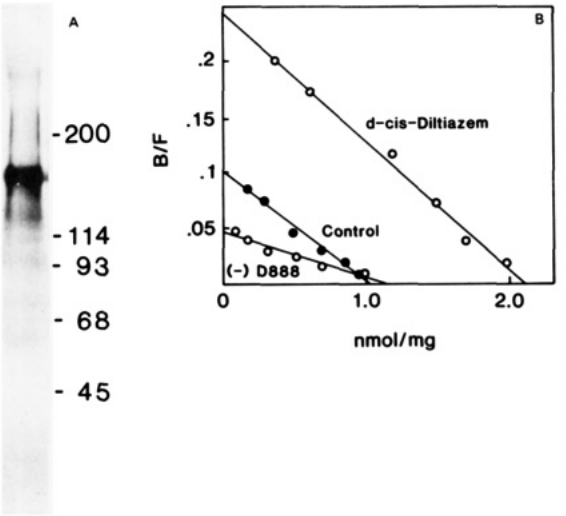

FIGURE 1: (Top) Analysis of the purified DHPR on a 5–15% linear gradient polyacrylamide gel with silver staining. Purified protein (4 μg) was heated to 60 °C for 10 min in sample buffer (3% SDS, 12 mM EDTA, 10% glycerol, 30 mM Tris-HCl, pH 7.6) containing 10 mM dithiothreitol (DTT). Location of molecular weight markers is shown on the left-hand side. (Bottom) Saturation [^{3}H]-(+)-PN200-110 binding in the absence (control) and the presence of either 50 μM *d-cis*-diltiazem or 10 μM (−)-D888. The K_d and B_{max} values are 4.35 nM and 1.0 nmol/mg for control, 3.73 nM and 2.1 nmol/mg for the diltiazem-treated receptors, and 10.5 nM and 1.1 nmol/mg for the (−)–D888-treated receptors.

t-tubules used in single-channel recordings were prepared from frozen back and leg muscle of rabbit essentially as described by Rosemblatt et al. (1981). The light membrane fraction recovered from the 10–20% sucrose interface was used in all experiments. Membranes were stored in 0.3 M sucrose, 100 mM KCl, 10 mM potassium 1,4-piperazinediethanesulfonate (K-PIPES), pH 7.4. All experiments were done in cis 50 mM NaCl, 100 mM $BaCl_2$, and 10 mM HEPES–Tris, pH 7.4, and trans 50 mM NaCl and 10 mM HEPES–Tris, pH 7.4. Reported results were collected from seven preparations of purified DHPR and from a total recording time of 6.3 h. The number of planar bilayers in which channel activity was present was 45 (8.4-min average recording time per bilayer). With the detergent dilution technique, channels were recorded in practically all attempts, provided that DHPR and cholesterol-containing bilayers were incubated together for 2 min or more. When this failed to occur, bilayers were broken and re-formed in the presence of DHPR. Reconstitution of DHPR into phospholipid vesicles greatly inhibited the insertion of channels into planar bilayer membranes as compared to the detergent dilution technique. With this technique, channels were recorded in only 40% of the attempts for a total recording time of 29.3 min.

RESULTS

Figure 1A shows the purified rabbit skeletal muscle DHPR on a silver-stained SDS–polyacrylamide gel. Under reducing conditions (i.e., 10 mM dithiothreitol), there is a major polypeptide of 150 kDa. Additional components of approximately M_r 170K, 100K, 50K, and 33K are apparent when gels are overloaded. The major polypeptide had an apparent molecular weight of 191K under nonreducing conditions (Nakayama et al., 1987). Densitometric scans of silver-stained SDS–polyacrylamide gels of the purified DHPR indicate 80% or greater purity of the 191/150-kDa polypeptide (data not shown). The B_{max} of [^{3}H]-(+)-PN200-110 binding to the purified protein (pooled fractions from sucrose density gradients) varied between 300 and 2100 pmol/mg of protein

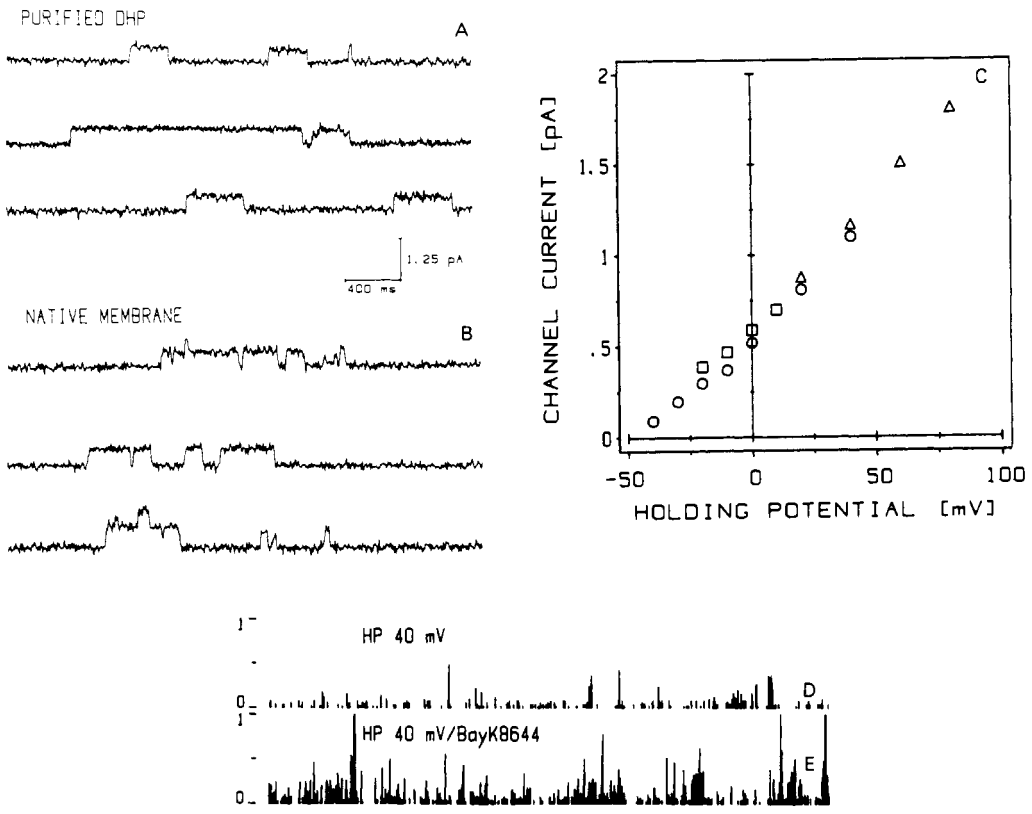

FIGURE 2: Comparison of Ca channels from native t-tubules and purified DHPR. Panels A and B correspond to Ca single channels recorded in 0.1 $BaCl_2$, HP 0 mV, after cis addition of 130 ng/mL (final concentration) purified DHPR (traces labeled purified DHP) or cis addition of 5 μg/mL (final concentration) purified native t-tubules (traces labeled native membrane). Time and current mark are 400 ms and 1.25 pA. (C) Single-channel current–voltage curve of mean unitary amplitudes recorded from native t-tubules (circles), purified DHPR (squares), or purified DHPR reconstituted from liposomes (triangles) in cis 100 mM $BaCl_2$–50 mM NaCl and trans 50 mM NaCl. Slope conductance of all data points was 13 ± 2 pS. (D and E) Scores of DHPR channel activity from the same experiment under four consecutive conditions: (D) 12-pS channels at HP 40 mV without agonist; (E) after cis addition of 5 μM Bay k 8644. (D) and (E) describe the fraction of time that a single channel spent open (p_o) as a vertical line of amplitude 0 (p_o = 0) to 1 (p_o = 1.0). Details are given in the text.

depending on the presence or absence of divalent cations, *d-cis*-diltiazem, and digitonin in the assay buffer and the time elapsed between purification and the binding assay. Similar ranges of B_{max} values have been reported by Streissnig et al. (1986) and Flockerzi et al. (1986). Allosteric regulation of [^{3}H]-(+)-PN200-110 binding to the purified protein by *d-cis*-diltiazem and (−)-D888 is shown in Figure 1B. The data represent Scatchard plots of three saturation binding experiments each performed with duplicate samples. *d-cis*-Diltiazem (50 μM) stimulated [^{3}H]-(+)-PN200-110 binding by increasing the B_{max}, whereas (−)-D888 (10 μM) inhibited binding by decreasing the affinity. These results are similar to those obtained with t-tubules (Vaghy et al., 1987a).

Channels Derived from DHPR–Digitonin Micelles. A straightforward procedure to record DHPR channels was to dilute detergent-extracted proteins directly into one of the two aqueous phases bathing the planar bilayer. Insertion of DHPR into planar bilayers was obtained after incubation of freshly purified or lyophilized DHPR protein–digitonin micelles with preformed planar membranes containing cholesterol, at a molar ratio of PE:PS:cholesterol = 2:2:1. Detergent and protein concentrations in the aqueous phase that yielded channels were 0.1–0.5 μg/mL (0.001–0.005%, w/v) digitonin and 30–150 ng/mL protein, respectively. Although higher concentrations of digitonin (>1 μg/mL) induced membrane breakage, extensive control experiments showed that in the concentration range used to record channels this detergent is electrically silent. Figures 2 and3 show single-channel records of purified DHPR with Ba as the current carrier. For simplicity of description, records that contain no more than one or two channels open simultaneously were selected. Panels A and B of figure 2 compare a 12-pS channel from DHPR and functional Ca channels from native t tubule membranes under similar conditions in mixed Na–Ba solutions. At 0-mV holding potential and with 100 mM Ba as current carrier, channels are approximately 0.5–0.6 pA in amplitude, and the current–voltage curve in Figure 2C shows that the slope conductance of the DHPR channels (squares) or native channels (circles) at 0 mV is approximately the same, 12 pS. In t-tubule Ca channels of native vesicles, the slope conductance in symmetrical 100 mM Ba is 12 pS (Ma & Coronado, 1987). Thus, this value seems to reflect a true conductive property of the t-tubule Ca channel and does not arise from the asymmetry of solutions used in Figure 2. In this respect, the t tubule and cardiac L-type Ca channels studied in planar bilayers (Rosenberg et al., 1986) are inherently different, given that the latter has a slope conductance of 23 pS under similar conditions.

The use of asymmetric solutions in Figure 2C was a critical step to verify if the 12-pS channel from DHPR was selective for divalent ions and excluded monovalent ions as previously reported for the native t-tubule channel (Affolter & Coronado, 1985). With the ionic gradients described for Figure 2C, equilibrium potentials for each of the ions present in solution are $E_{Ba} < -100$ mV (nominally negative infinite), $E_{Na} = 0$ mV, and $E_{Cl} = +40$ mV. Hence, Ba-selective open channels can be unambiguously identified as positive current (shown upward in records A and B of Figure 2), which become smaller at negative potentials, as the holding potential becomes closer to E_{Ba}. Even though the current–voltage curve of the t-tubule Ca channel is nonlinear at voltages close to the reversal potential (Coronado & Affolter, 1986), by linear extrapolation

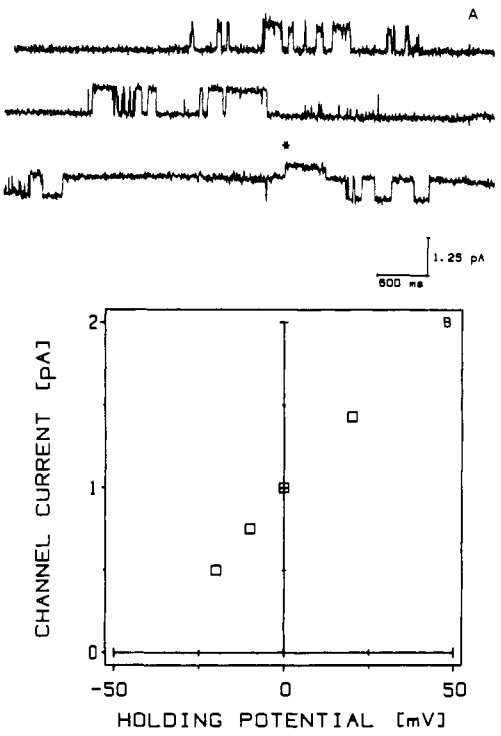

FIGURE 3: DHPR channels of large unitary conductance. (A) corresponds to tracings of 22-pS DHPR channels at HP 0 mV in 0.1 M $BaCl_2$. Asterisk indicates superposition of 12-pS and 22-pS DHPR channels; (B) current–voltage curve of mean unitary amplitudes in cis 100 mM $BaCl_2$–50 mM NaCl and trans 50 mM NaCl. Time and current mark are 600 ms and 1.25 pA.

of current amplitudes between −40 and 0 mV, we find that the DHPR channel has a zero-current reversal potential at approximately −50 mV. This result indicates that the purified channel reverses approximately 100 mV away from the chloride equilibrium potential and 50 mV away from the sodium equilibrium potential. True reversal is likely to be more negative given that currents approach reversal asymptotically in highly asymmetric solutions. A value of −50 mV nevertheless permits setting of a minimum permeability ratio: $P(Ba)/P(Na) = 20$, calculated from the constant-field equation with the ionic concentrations described above. Thus, the purified DHPR 12-pS channel reconstitutes the divalent ion permeation characteristics described for functional skeletal muscle Ca channels in in vivo and in planar bilayers (Cota & Stefani, 1984; McCleskey & Almers, 1985; Coronado & Smith, 1987).

Activation of the 12-pS DHPR channel by the dihydropyridine agonist Bay k 8644 and voltage is shown in the diary of channel openings given in Figure 2D,E. Each trace corresponds to a recording of 120 s, which was divided into consecutive segments of 500 ms. The fraction of time that a channel spent open (p_o) in each segment appears as a line with a vertical amplitude equal to p_o. Blank spaces correspond to $p_o = 0$, meaning the channel remained closed. Likewise, $p_o = 1$ means the channel was constantly open. In the absence of Bay k 8644, channels are brief, and the average p_o for the entire 10-s segment was approximately 0.1, corresponding to a mean open time of about 50 ms (Figure 2D). Addition of 2 μM Bay k 8644 (Figure 2E) resulted in an increase in the average p_o to about 0.3. Furthermore, under this condition there are numerous long-term openings in which $p_o = 1$, i.e., durations longer than the 500-ms sampling segment. Events of this nature are characteristic of dihydropyridine agonist induced activity and were never present without agonist. Records showing this type of activity are given in Figure 2B.

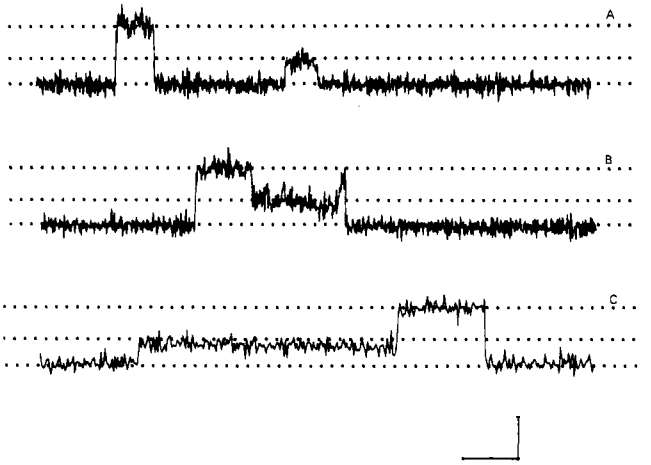

FIGURE 4: Substate conductances in DHPR channels. Dotted lines in each trace correspond to base line (low), 12 pS (middle), and 22 pS (high). All records are from different experiments, in cis 0.1 $BaCl_2$ as current carrier. (A) HP 10 mV, current mark = 1 pA; (B) HP 20 mV, current mark = 1.25 pA; (C) HP 0 mV, current mark = 0.77 pA. Time mark is 400 ms for all records.

Unlike in recordings from native t-tubule membranes (Affolter & Coronado, 1985), DHPR channels open spontaneously in either the presence or absence of 1–5 μM Bay k 8644. Direct action of agonist as shown in Figure 2D was seen in four out of six separate experiments. Inhibition by DHP antagonists nitrendipine and (−)-209-791 was far less successful for reasons not entirely clear. In 2 out of 10 recordings, 10 μM nitrendipine produced a statistically significant decrease in frequency and duration of spontaneous activity (not shown). Consistent action of antagonists was seen, however, when channels were recorded by the liposome fusion procedure (Figures 5 and 6). Thus, it appears that the detergent dilution procedure uncouples significant drug binding (Figure 2) from electrophysiological action. Divalent ion selectivity, however, as given in Figures 2 and 3, was observed routinely.

A second type of channel with a much larger unitary conductance, also present in DHPR, is shown in Figure 3A. In 100 mM Ba at a holding potential (HP) of 0 mV, current amplitudes for this channel are 1–1.2 pA, and the slope conductance is 22 pS. In an ionic regime similar to that described for the 12-pS channel, the reversal of currents occurs at potentials probably more negative than −40 mV (Figure 3B), a property that identifies this species as having a high divalent over monovalent ion selectivity. The asterisk in the bottom record of Figure 3A indicates a clear case of overlap in current of the 12-pS and 22-pS channels. In many cases, however, the activity of these two channels gives rise to overlapping currents that do not correspond to the sum of the two independently measured single-channel currents. This is shown in Figure 4. Figure 4A shows the average current levels (dotted lines) when the 12-pS and 22-pS channels open separately in time. Figure 4B indicates a case in which an open 22-pS channel closes momentarily to the 12-pS level, and Figure 4C indicates a separate instance in which a 12-pS channel opens to a 22-pS level before closing to base-line level. These are clear cases in which the activity of these two species is coupled in time and strongly suggest that the 12-pS and 22-pS conductances are kinetically linked, most probably by both being substate conductances of the same channel. Alternatively, it is possible that the two activities are derived from separate macromolecule channels, which in the process of purification and reconstitution have become kinetically coupled.

Channels Derived from Liposome Fusion. An alternative

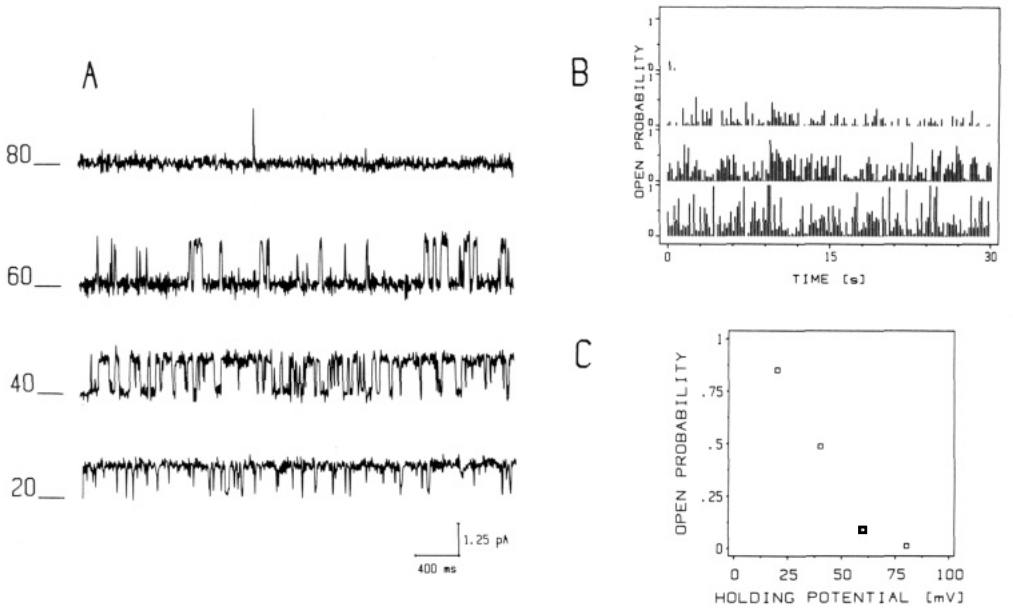

FIGURE 5: Voltage-dependent calcium channels from reconstituted phospholipid vesicles. (A) Channels were recorded in 100 mM $BaCl_2$, 50 mM NaCl, and 10 mM Tris–Hepes, pH 7.4 (cis) and 50 mM NaCl and 10 mM Tris–Hepes, pH 7.4 (trans). Bay K 8644 (1 μM) was present in the cis chamber. Horizontal bars to the left of each record indicate base-line current. Recordings were filtered at 150 Hz and digitized at 500 Hz. Slope conductance was 14 ± 1 pS, and the extrapolated reversal was more negative than −40 mV. (B) Diary of open probability for channels recorded at 20, 40, 60, and 80 mV, from bottom to top. Each window represents open probability during 200 ms of recorded time. Empty segments correspond to periods without open events. A total of 150 consecutive segments (30-s recorded time) is shown at each holding potential. (C) Open probability versus holding potential relation from single-channel records in (A).

method to record DHPR channels was to form proteoliposomes containing DHPR protein. Single channels incorporated from reconstituted phospholipid vesicles are shown in Figures 5 and 6. In Figure 5, channels were recorded as in Figures 2–4 in symmetrical 50 mM NaCl, 10 mM Tris–Hepes, pH 7.4, and 100 mM cis $BaCl_2$. Bay K 8644 (1 μM) was present in the cis chamber. The slope conductance was 14 pS, and the extrapolated reversal potential was more negative than −40 mV, indicating a high degree of selectivity for divalent cations (Figure 2C, triangles). Channels displayed a continuous bursting type kinetics. This behavior was typically not seen in single-channel recordings from native membranes or in channels reconstituted with the detergent dilution technique. Bay K 8644 activated single channels reconstituted from native membranes appear normally as clusters of long openings or bursts, which may be several seconds in duration [see Figure 2B and Affolter and Coronado (1985)]. Single-channel recordings in Figure 5A demonstrated a clear voltage dependence. This is further illustrated in Figure 5B, where diaries of single-channel openings are shown as 30-s segments recorded at 20, 40, 60, and 80 mV (bottom to top). Single-channel open probability was plotted versus holding potential in Figure 5C. Channel opening was found to decrease e-fold with a 23-mV increase in holding potential. This weak voltage dependence is in good agreement with the value of e-fold/25 mV previously reported for channels recorded from native t-tubule membranes (Affolter & Coronado, 1985). It should be noted that the sign of the voltage dependence for channels shown in Figure 5 is opposite that seen for channels recorded from native membranes. This is most likely due to a difference in orientation of receptor/channel proteins in the reconstituted vesicles.

Channels incorporated from preformed vesicles are inhibited by the dihydropyridine antagonist nitrendipine. Figure 6A shows channels recorded at 40 mV in the presence of 1 μM Bay K 8644. Open probability in this recording was $p_o = 0.5$. The addition of 10 μM nitrendipine cis caused a marked decrease in open probability ($p_o = 0.14$) as well as a decrease in the mean duration of open events. Nitrendipine had no

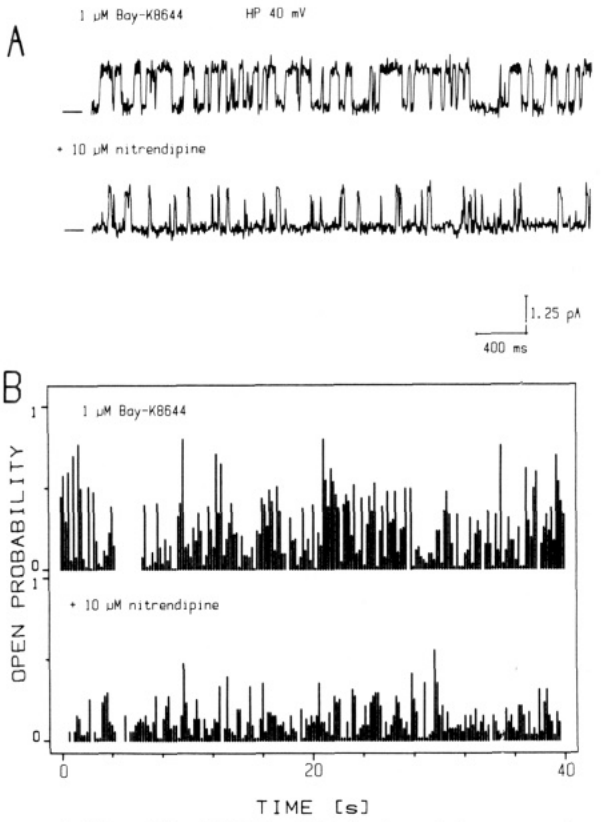

FIGURE 6: Nitrendipine inhibition of single channels from reconstituted phospholipid vesicles. (A) Channels were recorded at 40 mV as in Figure 5 in the presence of 1 μM Bay K 8644 plus 10 μM nitrendipine (lower recording). p_o in upper trace is 0.5; p_o in lower record is 0.14. Horizontal bars to the left of each record indicate base-line current. Records were filtered at 150 Hz and digitized at 500 Hz. (B) Diary plot of open probability vs time before and 30 s after 10 μM nitrendipine. Each segment represents p_o during 200 ms of recorded time.

apparent effect on unit amplitudes of single-channel currents. Figure 6B is a diary of channel openings constructed as in

Figures 2 and 5. Nitrendipine reduced channel opening throughout the entire 40-s segment. In every instance where incorporation of liposomes was successful ($n > 10$), channels were found to be voltage-dependent. Sensitivity to antagonist drugs (nitrendipine) was tested and observed routinely.

Discussion

Channels induced by DHPR can be successfully recorded after partitioning of micelles of DHPR protein and detergent into planar phospholipid bilayers or by reconstitution of DHPR into phospholipid vesicles followed by fusion of the liposomes to planar bilayer membranes. However, many of the variables that control the single-channel behavior of DHPR-induced channels as well as those that determine DHP–receptor binding and functional effects of DHP's are still poorly understood. We found little, if any, proportionality between the amount of purified DHP–receptors added to the planar bilayer solutions and the number of incorporated channels. Thus, no specific activities can be presently derived, and consequently, no attempt was presently made to identify the specific peptide components that give rise to the observed Ca channels. In other words, it is unclear at present if Ca channels copurify with DHPR or whether DHPR per se constitutes a Ca channel. In skeletal and cardiac muscle (Schwartz et al., 1985; Green et al., 1985) there are standing discrepancies between numbers of DHPR and functional Ca channels per cell. This, and the report of Curtis and Catterall (1986) that only 2–3.3% of the purified rabbit skeletal muscle DHPR can mediate drug-sensitive $^{45}Ca^{2+}$ fluxes in liposomes, raises the possibility that DHPR may be coupled to, but without forming an integral part of, the Ca channel. That the action of DHP's and Ca channel activity have been uncoupled in our preparation of purified receptor is evident in Figures 3 and 4. In these recordings, channels were spontaneously open in the absence of Bay k 8644. This type of activity is almost absent when the same experiments are performed with channels derived from native t tubule membranes (Affolter & Coronado, 1985). A large number of negative experiments with antagonists (discussed under Results) point in the same direction.

Only one of the channels induced by DHPR, a 12–14-pS channel (slope conductance at HP 0 mV in 0.1 M Ba), matches the conductive and gating properties previously described for the Ca channel of t-tubule membranes. Four criteria were used to identify the purified channel as a t-tubule Ca channel: single-channel conductance, voltage dependence of open channel formation, high selectivity for Ba over Na, and sensitivity to DHP agonist Bay K 8644. Clear-cut blockade by DHP antagonists could be demonstrated only in channels incorporated from preformed phospholipid vesicles. Although under identical recording conditions to those used in Figure 2 we have previously reported a conductance of 20 pS for the native t-tubule Ca channel (Affolter & Coronado, 1985), this value corresponds to the slope conductance at large positive potentials (more positive than HP +50 mV) where currents rectify strongly. At HP 0 mV, the slope conductance of the native channel is 12 pS (Coronado & Affolter, 1986), a value that we consider similar to the 12–14-pS Bay K 8644 sensitive channel presently reported.

Flockerzi et al. (1986) recently suggested that purified DHPR from skeletal muscle induced 20-pS (slope conductance HP 0 mV, 0.1 M Ba) cardiac L-type Ca channels. We consider this result puzzling for two reasons. First, we are not aware of any single-channel study made in skeletal muscle that describes L-type cardiac Ca channels. Second, the channel reported by Flockerzi et al. (1986) lacks the voltage dependence characteristic of cardiac L-type Ca channels recorded in planar bilayers (Rosenberg et al., 1986). Although in skeletal muscle several types of Ca channels seem to be present at different stages of development and differentiation, the electrophysiological evidence in vivo indicates that L-type Ca currents as such are not present in this tissue (Cota & Stefani, 1986; Toro et al., 1987). However, it may be entirely possible that the large conductance reported by Flockerzi et al. (1986) may be the 22-pS substate reported here. Since they did not report on the low-conductance 12-pS state, we take it to imply that in their preparation this conductance state was infrequent. If this is the case, it would appear that some domain in the purified protein, presently not under experimental control, has considerable influence on the conductive properties of the Ca channel protein.

Added in Proof

The preparation used in these reconstitution studies contains the receptor for 1,4-dihydropyridine and phenylalkylamine calcium channel modulators as determined by photoaffinity labeling with tritiated azidopine and tritiated LU49888 (Vaghy et al., 1987b).

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Opposite Responses of Rabbit and Human Globin mRNAs to Translational Inhibition by Cap Analogues†

Susan H. Shakin and Stephen A. Liebhaber*

Howard Hughes Medical Institute and Departments of Human Genetics and Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6072

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ABSTRACT: The translational efficiency of an mRNA may be determined at the step of translational initiation by the efficiency of its interaction with the cap binding protein complex. To further investigate the role of these interactions in translational control, we compare in vitro the relative sensitivities of rabbit and human α- and β-globin mRNAs to translational inhibition by cap analogues. We find that rabbit β-globin mRNA is more resistant to translational inhibition by cap analogues than rabbit α-globin mRNA, while in contrast, human β-globin mRNA is more sensitive to cap analogue inhibition than human α-globin mRNA. This opposite pattern of translational inhibition by cap analogues of the rabbit and human α- and β-globin mRNAs is unexpected as direct in vivo and in vitro comparisons of polysome profiles reveal parallel translational handling of the α- and β-globin mRNAs from these two species. This discordance between the relative translational sensitivities of these mRNAs to cap analogues and their relative ribosome loading activities suggests that cap-dependent events may not be rate limiting in steady-state globin translation.

Regulation of eukaryotic gene expression can be exerted in both the nucleus and the cytoplasm. While nuclear events, including transcription, processing, and transport, determine the pattern of mRNAs delivered to the cytoplasm, the final pattern of gene expression depends on the relative abilities of these mRNAs to direct protein synthesis. Studies in several systems now demonstrate that eukaryotic gene expression can be strongly influenced at the translational level [see, for example, Rosenthal et al. (1982), Babich et al. (1983), McGarry and Lindquist (1985), Reichel et al. (1985), and Warner et al. (1985); for a review, see Moldave (1985)]. One of the best-studied examples of translational regulation involves the balanced synthesis of α- and β-globin proteins in reticulocytes (Lodish, 1971, 1974). In both human and rabbit reticulocytes, the balanced synthesis of these proteins, which is critical to the normal development and function of red blood cells, occurs despite an excess of α-globin mRNA and appears to require more efficient translation of the less abundant β-globin mRNA [for a review, see Bunn et al. (1977)]. The differences in the translational activities of α- and β-globin mRNAs are reflected by differences in the distributions of these mRNAs in reticulocyte polysomes. In both rabbit and human reticulocytes, there is more efficient ribosome loading of β-globin mRNA compared to α-globin mRNA [rabbit (Hunt et al., 1968; Lodish, 1971); human (Clegg et al., 1971; Nathan et al., 1971; Boyer et al., 1974; Cividalli et al., 1974; Shakin & Liebhaber, 1986a)] as well as preferential sequestration of α-globin mRNA into translationally inactive complexes (Jacobs-Lorena & Baglioni, 1972; Shakin & Liebhaber, 1986a). Studies in rabbit reticulocytes suggest that the higher translational rate of β-globin mRNA results from a higher rate of β-globin translational initiation (Lodish, 1971).

The factors which control the efficiency of translational initiation in eukaryotic cells are still largely undefined. Re-

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* Address correspondence to this author at the Department of Human Genetics, University of Pennsylvania School of Medicine.

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