Fast Activation of Dihydropyridine-sensitive Calcium Channels of Skeletal Muscle

Multiple Pathways of Channel Gating

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ABSTRACT Dihydropyridine (DHP) receptors of the transverse tubule membrane play two roles in excitation-contraction coupling in skeletal muscle: (a) they function as the voltage sensor which undergoes fast transition to control release of calcium from sarcoplasmic reticulum, and (b) they provide the conducting unit of a slowly activating L-type calcium channel. To understand this dual function of the DHP receptor, we studied the effect of depolarizing conditioning pulse on the activation kinetics of the skeletal muscle DHP-sensitive calcium channels reconstituted into lipid bilayer membranes. Activation of the incorporated calcium channel was imposed by depolarizing test pulses from a holding potential of -80 mV. The gating kinetics of the channel was studied with ensemble averages of repeated episodes. Based on a first latency analysis, two distinct classes of channel openings occurred after depolarization: most had delayed latencies, distributed with a mode at 70 ms (slow gating); a small number of openings had short first latencies, <12 ms (fast gating). A depolarizing conditioning pulse to +20 mV placed 200 ms before the test pulse (-10 mV), led to a significant increase in the activation rate of the ensemble averaged-current; the time constant of activation went from $\tau_m = 110$ ms (reference) to $\tau_m = 45$ ms after conditioning. This enhanced activation by the conditioning pulse was due to the increase in frequency of fast open events, which was a steep function of the intermediate voltage and the interval between the conditioning pulse and the test pulse. Additional analysis demonstrated that fast gating is the property of the same individual channels that normally gate slowly and that the channels adopt this property after a sojourn in the open state. The rapid secondary activation seen after depolarizing prepulses is not compatible with a linear activation model for the calcium channel, but is highly consistent with a cyclical model. A six-state cyclical model is proposed for the DHP-sensitive Ca channel, which pictures the normal pathway of activation of the calcium channel as two voltage-dependent steps in sequence, plus a voltage-independent step which is rate limiting. The model reproduced well the fast and slow gating modes of the calcium channel, and the effects of conditioning pulses. It is possible that the voltage-sensitive gating transitions of the DHP receptor, which occur early in the calcium channel activation sequence, could underlie the role of the voltage sensor and yield the rapid excitation-contraction coupling in skeletal muscle, through either electrostatic or allosteric linkage to the ryanodine receptors/calcium release channels. Key words: excitation-contraction coupling • signal transduction • sarcoplasmic reticulum

INTRODUCTION

In skeletal muscle, the products of a single gene perform two different roles. The dihydropyridine (DHP) receptor polypeptide of the transverse tubule (TT) membrane constitutes the pore forming $\alpha$ subunit of the voltage-dependent Ca channel (Tanabe et al., 1987). However, the DHP receptors work mainly as sensors of TT membrane voltage, controlling opening of the Ca release channels in the sarcoplasmic reticulum (SR) (Ríos and Brum, 1987; Tanabe et al., 1988). These two functions have strikingly different kinetics. In response to membrane depolarization the voltage sensors generate intramembrane charge movement (Schneider and Chandler, 1973), which is followed by release of calcium from the SR. The whole process from excitation (depolarization of TT membrane and movement of intramembrane charge) to opening of Ca release channels occurs in <5 ms (Simon and Schneider, 1988), while activation of the TT membrane Ca current ($I_{Ca}$) is generally very slow, having a time constant of >100 ms at -10 mV in frog skeletal muscle (Avila-Sakar et al., 1986). In addition, the movement of intramembrane charge associated with the voltage sensor is centered at $-40$ mV, while activation of $I_{Ca}$ by depolarization is centered at $-0$ mV (Pizarro et al., 1988).
The DHP-sensitive Ca channel and the voltage sensor of excitation-contraction (E-C) coupling are encoded by the same cDNA (Tanabe et al., 1988; Adams et al., 1990). The same DHP receptor molecule could have both functions. Alternatively, the two functions could be the role of two different molecules encoded by the same gene. A form of this hypothesis, proposed by De Jongh et al. (1989), was ruled out by Beam et al. (1992) who demonstrated that the COOH-terminal truncated DHP receptor could function as both voltage sensor and Ca channel.

To serve as an E-C coupling voltage sensor, the DHP receptor must be capable of fast conformational changes in response to membrane depolarization. Feldmeyer et al. (1990) and García et al. (1990) showed that it is possible to achieve fast activation of the "slow" \( I_{Ca} \) in frog skeletal muscle, by applying a depolarizing conditioning pulse before the test pulse. The conditioning-induced fast gating had a steep dependence on the intermediate potential between the conditioning and test pulses (Feldmeyer et al. 1992). Working on myoballs from rat skeletal muscle, Sculptoreanu et al. (1993) showed that the L-type Ca current could be potentiated by trains of depolarizing prepulses. The potentiation required rapid phosphorylation by cAMP-dependent protein kinase in a voltage-dependent manner, which shifted to the left the voltage-dependence of, and accelerated the rate of, channel activation. Melzer and co-workers (Feldmeyer et al. 1990) suggested that the fast transition underlying fast activation of \( I_{Ca} \) is involved in the rapid control of Ca release from the SR.

In the present paper, single channel reconstitution techniques are combined with macroscopic measurements in muscle fibers to decide whether the dual kinetics are a property of individual molecules. Using DHP receptors incorporated as Ca channels in planar bilayers, it is demonstrated that the fast transition is a property of the individual channels that normally gate slowly, thus confirming the interpretation of Feldmeyer et al. (1990). Recording \( I_{Ca} \) in frog muscle fibers, it is found that the transformation to fast gating is accompanied by changes in voltage dependence and in rate and extent of decay after the peak. We propose a molecular diagram that incorporates the two functions of the DHP receptor: as fast E-C coupling voltage sensor and slow voltage-dependent Ca channel.

**MATERIALS AND METHODS**

**Isolation of Transverse Tubule Membranes from Rabbit Skeletal Muscle**

Transverse tubule membranes were isolated from frozen rabbit skeletal muscle tissue following the procedures of Galizzi et al. (1984). These membrane vesicles contained 20-60 pmol DHP receptor/mg protein in a [\(^{3}H\)]PN200-110 binding assay. The vesicles were stored at \(-80^\circ C\), at a protein concentration of 5-7 mg/ml.

**Planar Bilayer Reconstitution of the DHP-sensitive Ca Channels**

Planar bilayer membranes were formed across an aperture of \(~200 \mu m\) diameter with a lipid mixture of phosphatidylethanolamine/phosphatidylserine/cholesterol in a ratio of 1:1:0.2 dissolved in decane at a concentration of 50 mg/ml. The cis (intracellular) solution contained 200 mM KCl, 3 mM Mg-ATP, 10 mM HEPES-Tris, pH 7.4; the trans (extracellular) solution contained 50 mM NaCl, 100 mM BaCl\(_2\), 10 mM Hepes-Tris, pH 7.4. To have consistently measurable Ca channel activities, a DHP agonist, Bay K 8644 was always present in the cis solution at a concentration of 0.3 \(\mu M\) (see Figs. 1 and 2) or 1 \(\mu M\) (see Figs. 4 and 5).

To incorporate the DHP-sensitive Ca channels, TT vesicles (1-3 \(\mu l\)) were added to the cis solution. The bilayer was initially held at \(-80 mV\) (holding potential). The Ca channel activity was measured with depolarizing pulses of various amplitudes and durations. Single channel currents were recorded with either an Axopatch-1C or an Axopatch 200A unit (Axon Instruments, Foster City, CA), and filtered at 100 Hz using an 8 pole Bessel filter. The experiments were carried out at room temperature (22-24°C).

The data presented were done at two different stages: from 1990 to 1992 at Rush Medical College (see Figs. 1, 2, 3, and 8) and from 1992 to 1994 at Case Western Reserve University (see Figs. 4 and 5).

**Analysis of Single Channel Data**

Processing of the current episodes started with construction of controls, by averaging the null sweeps. The controls, which contain only the linear capacitative and electrostrictive components, were subtracted from the test currents, yielding asymmetric current records. Conventional single channel analysis was done with pCLAMP software (Axon Instruments) and custom programs. Different pulse protocols were used to study the kinetics of activation and inactivation. Ensembles of ionic currents obtained with pulses to the same voltage were averaged; the averaged currents were fitted with the \( m^b \) time dependence. The procedures have been described in detail elsewhere (Ma et al., 1991).

**Measurements of Macroscopic Ca Current**

Macroscopic Ca currents \( (I_{Ca}) \) were recorded in segments of single fibers from the semitendinosus muscle of the frog *Rana pipiens*, mounted at slack length and voltage clamped in a double Vaseline gap chamber. The methods have been described in detail elsewhere (Gonzalez and Rios, 1993). The fibers were held polarized at a potential of \(-80 mV\). The currents shown are asymmetric, obtained as differences of currents during test pulses and control pulses, usually between \(-110 \) and \(-90 mV\). The internal solution contained \( (\text{in mM}): 10 \text{ BaF}, 1 \text{ Ca}, 5 \text{ ATP}, 135 \text{ Cs}, 88 \text{ glutamate}, 5.5 \text{ MgCl}_2, 5 \text{ creatine phosphate, 5 glucose, 10 HEPES}, \text{pH 7.0.} \) The high concentration of BaF was designed to prevent fiber contraction and to eliminate potential feedback of SR Ca release on the DHP-sensitive Ca channel (Gonzalez and Rios, 1993). 3 mM Ba in the external solution was the main carrier for current through the DHP-sensitive Ca channel. The external solutions contained, in addition, 135 CH\(_3\)SO\(_4\), 124 TEA, 0.001 TTX, 1.4 diaminopyridine, 5 Tris maleate, pH 7.0. The temperature was 10-12°C.

**RESULTS**

**Reconstituted Ca Channels Are Strictly Voltage- and Time-dependent**

Isolated TT membrane vesicles from rabbit skeletal muscle were fused with planar lipid bilayers. To study
Figure 1. Gating kinetics of the DHP-sensitive Ca channels in bilayers. (A) After fusion of vesicles, the bilayer was held polarized at -80 mV. Channel activity was elicited by depolarizing pulses to -10 mV. Represented are consecutive episodes, obtained at 7 s intervals. Channel openings were completely blocked by 20 μM nitrendipine added to the extracellular solution (data not shown). (B) 21 separate bilayer experiments were used in the ensemble average. A total of 478 episodes as in A were included. Blanks, which constituted 25.6% (2.5% SEM) of sweeps, were excluded from the average. The “smooth” line is the best fit curve $I = I_{\text{max}} \times m^3 \times h$, where $m = 1 - \exp(-t/\tau_m)$ and $h = h_0 + (h_0 - h_e) \times \exp(-t/\tau_h)$. $h_e$ was set to 0, since the channel inactivates completely at -10 mV with longer pulses (Ma et al., 1991). The best fit parameters were $I_{\text{max}} = -0.245 \text{ pA (0.006)}$, $\tau_m = 118 \text{ ms (4)}$, and $\tau_h = 1.24 \text{ s (0.04)}$. (C) First latency histogram of channel openings at -10 mV. The histogram was generated from eight experiments with a total of 280 episodes. The first two blank bins (4 ms) in the histogram correspond to the dead time of the system (due to Bessel filtering at 100 Hz). The two populations, fast and delayed open events, are clearly represented.

The present experiments are different from previous bilayer studies, where the Ca channel activities were measured at constant holding potentials (Affolter and Coronado, 1985; Ma and Coronado, 1988; Mejia-Alvarez et al., 1991). Under the present conditions, no persistent channel activity could have been measured at maintained depolarizing potentials because of voltage-dependent inactivation. In a few instances (18 out of 180 experiments), we were able to record channels that did not inactivate at maintained depolarizing potentials; interestingly, these channels did not show any voltage-dependence at all, since channel activities could be measured at -80 mV (data not shown).

A Depolarizing Conditioning Pulse Accelerates the Rate of Channel Activation

As observed by Feldmeyer et al. (1990) on macroscopic Ca currents, the activation of the single Ca channels in the voltage-dependent gating kinetics of the DHP-sensitive Ca channel, the bilayer was kept polarized at -80 mV, and the current carrier (100 mM Ba$^{2+}$) was present only in the extracellular solution (Ma et al., 1991). Inward Ba currents through the channel were recorded under stimulation by depolarizing test pulses. Records of consecutive single channel episodes are shown in Fig. 1A. The channels were closed at -80 mV and opened when the membrane was depolarized (by pulses of 2 s duration to -10 mV). The identity of the channels was certain because opening was highly dependent on the presence of Bay K 8644 and was prevented by the addition of nitrendipine (Ma et al., 1991). The average of a large number of episodes at test potential -10 mV displays a slow time course of activation followed by inactivation (Fig. 1B), that can be adequately fitted by the equation:

$$I = I_{\text{max}} \times m^3 \times h,$$

where $m$ and $h$ are first order time- and voltage-dependent activation and inactivation variables. The best fit activation time constant $\tau_m$ was 118 ms (SE of estimate, 4 ms) and the inactivation time constant $\tau_h$ was 1.24 s (0.04 s). The open probability of the reconstituted Ca channel increased steeply with voltage, following a Boltzmann distribution centered at -25 mV with a steepness factor of 7 mV and a maximum open probability of ~10% (Ma et al., 1991). These properties are similar to those of $I_{\text{Ca}}$ in the skeletal muscle of the frog at 15°C (Avila-Sakar et al., 1986). The kinetics of activation in rat muscle fibers, however, follow a single exponential function of time (Mejia-Alvarez et al., 1991). This can be reconciled since the ensemble average currents in the present experiments (illustrated in Fig. 1B) can be fitted almost equally well by the dependence $I_{\text{max}} \times m \times h$ (see Fig. 4). To examine the kinetics of openings at the single channel level, a histogram of first latency was constructed (Fig. 1C). The figure plots the absolute frequency (occurrences) of the first detected open event vs. time of the first transition (first latency). There are two well-defined groups of openings: a small number of fast open events, whose short latencies are contained in the first five bins, and a large group of openings, with a broad range of latency values, distributed with a peak at ~70 ms. The present experiments are different from previous bilayer studies, where the Ca channel activities were measured at constant holding potentials (Affolter and Coronado, 1985; Ma and Coronado, 1988; Mejia-Alvarez et al., 1991). Under the present conditions, no persistent channel activity could have been measured at maintained depolarizing potentials because of voltage-dependent inactivation. In a few instances (18 out of 180 experiments), we were able to record channels that did not inactivate at maintained depolarizing potentials; interestingly, these channels did not show any voltage-dependence at all, since channel activities could be measured at -80 mV (data not shown).
bilayers was made faster by a depolarizing conditioning pulse. A double pulse protocol (Fig. 2 A), was applied to the bilayer system. Consecutive single channel current episodes were obtained with a test pulse to -10 mV preceded by a conditioning pulse to +20 mV and are represented in the figure. The corresponding ensemble average is shown in B. The activation of the channel was much faster after the conditioning pulse (compare Figs. 1 B and 2 B). The conditioning depolarization reduced $\tau_0$ almost threefold to 44 ms (SE = 2 ms, standard error of estimate). In contrast, inactivation was not changed greatly ($\tau_1$ = 1.5 s, SE = 0.1 s).

The enhanced rate of channel activation strictly required the large conditioning pulse, as a subthreshold conditioning depolarization was not sufficient. The ensemble average shown in Fig. 2 D was elicited by a protocol that included a prepulse depolarization to -40 mV instead of a pulse to +20 mV; clearly, the channels subjected to this protocol did not activate more rapidly than the reference (Fig. 1 B).

Examination of the individual records in Fig. 2 A suggests that the increase in the overall rate of activation is largely due to the appearance of a group of episodes in which channel openings occurred almost instantaneously upon depolarization. This group is illustrated by the third, the tenth, and perhaps the fourth current trace in Fig. 2 A.

The effect of the conditioning pulse on the distribution of first latencies was illustrated in Fig. 3. The histogram in Fig. 3 A was derived from records subjected to a subthreshold conditioning pulse to -40 mV. This histogram was not very different from the one shown in Fig. 1 C; in both cases, the proportion of fast open events was approximately 7%. The histogram in Fig. 3 B, of openings after a conditioning pulse to +20 mV, is sharply different. The fast open events became more frequent, and a boundary between short and long latencies could be clearly located at 12 ms. The openings with latencies under 12 ms, contained in the first five bins of the histogram, went from 7 to 24%.

**Fast and Slow Gating Are Properties of the Same Channel**

The group of fast openings associated with the conditioning pulse might represent a fast gating mode of an individual molecule, capable of slow gating at other times, or the recruitment by the conditioning pulse of a different channel that is silent in reference conditions. That ensemble averages in Figs. 1 B and 2 B reached similar peak values and were almost superimposable in their late time course was consistent with a change in properties of the same channel. The following two additional tests confirmed that the two gating rates are properties of the same individual molecule.

First, the inactivation kinetics of the fast activating events was determined. This was done by collecting all episodes with first latencies under 12 ms and computing their ensemble average (the "conditional" average, shown in Fig. 2 C). By selecting all sweeps with fast openings, we forced the initial open probability to be close to unity. If these sweeps represented currents through a newly recruited rapid channel, then the reg-
ular, slow-gating channel would contribute little to the conditional average (the average open probability during the reference episodes is only 10%). Therefore, the kinetics of inactivation in the conditional average should be dominated by the fast gating channels. The conditional average (Fig. 2 C) has two interesting properties: first, it inactivates somewhat more rapidly, the best fit exponential decay shown has a time constant of 0.85 s (SE = 0.03 s), significantly less than in reference ($\tau_h = 1.2$ s). This indicates that the fast opening channels cannot be a newly recruited population, as such a group would contribute additively to the late current and increase it significantly, contrary to the observations. The small increase in inactivation rate will be shown later to agree with measurements of macroscopic $I_{ca}$ and predictions from a specific model. The other interesting aspect of Fig. 2 C is that the amplitude of the conditional ensemble current during the conditioning pulse (at +20 mV) is more than twofold greater than that of the ensemble average in Fig. 2 B, in which all sweeps were included. This observation indicates that the change in gating rate requires a sojourn into the open state.

The second test involved calculating the frequency of blank sweeps at $-10$ mV. The fraction of blanks ($N_b/N_t$) is expected to change substantially if a new population of channels is recruited. $N_b/N_t$ was 25.6% (SEM = 2.3%) in reference, and 24.7% (SEM = 2.5%) after conditioning (+20 mV). This lack of effect of the condi-

Figure 3. First latency histograms of the channel after conditioning pulses. (A) First latency histogram of openings conditioned by a subthreshold pulse ($-40$ mV). The collected frequency of the first 12 ms was ~7% of the 174 episodes in the histogram. (B) First latency histogram of openings conditioned by a pulse to +20 mV (inset). The early peak in the first five bins corresponds to ~24% of 198 episodes in the histogram.

Figure 4. Time-dependent potentiation of the Ca channel. (A) Ensemble averages with double pulse protocols at different $\tau_D$. The unconditioned channels had slow kinetics of activation ($a$, $n = 35$). The channel responded with fast activation, when a depolarizing conditioning pulse (+30 mV) was applied. The degree of conditioning effect decreased as the time interval was increased from $\tau_D = 50$ ms ($b$, $n = 17$), 100 ms ($c$, $n = 26$), 200 ms ($d$, $n = 7$), to 500 ms ($e$, $n = 6$). $n$, the number of experiments used in the ensemble average. Solid lines represent the best fit according to: $I = I_0 [1 - \exp(-t/\tau_m) \exp(-t/\tau_w)]$. The corresponding activation time constants were $\tau_m = 393.9 \pm 16.9$ ms ($a$), 16.1 $\pm$ 1.4 ms ($b$), 19.0 $\pm$ 1.1 ms ($c$), 60.7 $\pm$ 3.0 ms ($d$), and 199.6 $\pm$ 11.1 ms ($e$). (B) The ensemble averages were constructed by excluding those episodes with large tail currents at the intermediate pulse ($-50$ mV), thus, eliminating contribution of tail currents to the channel activation at the test potential. The best fit activation time constants were $34.0 \pm 1.6$ ms ($\tau_0 = 50$ ms) and $42.5 \pm 2.2$ ms ($\tau_0 = 100$ ms). (C) Plot of $\tau_m$ as a function of $\tau_D$. 

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conditioning pulse on \( N_p/N_t \) indicates that the number of active channels did not change.

Taken together, the results support the interpretation of Feldmeyer et al. (1990) that the fast gating is a property of the same molecules that normally gate slowly and show that the channels adopt this property after dwelling in the open state.

**Voltage and Time-dependent Potentiation of the Ca Channel**

Fast gating of the slow \( I_{Ca} \), or potentiation of the L-type Ca current in skeletal muscle, is a steep function of the intermediate voltage between the conditioning and test pulses, as shown in the studies of Feldmeyer et al. (1992) and Sculptoreanu et al. (1992, 1993). The recovery process, which underlines transition from fast to slow gating, became faster as the intermediate potential was more negative. To further understand the fast gating kinetics of the DHP-sensitive Ca channel, the following two series of experiments were performed.

First, the interval between the conditioning and test pulses (\( \tau_D \)) was varied, to study the time dependence of the conditioning effect. The pulse protocol is shown in Fig. 4 A. Here, the channel was conditioned to +30 mV for 250 ms, followed by an intermediate potential at -50 mV for different durations (\( \tau_D \)), then tested to a potential of -20 mV. The ensemble average of the control channels had slow kinetics of activation (\( \tau_A = 393.9 \pm 16.9 \) ms), which became faster after the conditioning pulse. Clearly, the degree of fast activation decreased as \( \tau_D \) was increased from 50 to 500 ms.

Due to the slow deactivation kinetics of the channel at -50 mV, probably as a result of the higher concentration of Bay K 8644 being used (1 \( \mu \)M, instead of 0.3 \( \mu \)M as in Figs. 1 and 2), the actual activation kinetics of the current during the test pulse could not be determined directly from Fig. 4 A, at \( \tau_D = 50 \) and 100 ms. Thus, new ensemble averages were constructed by excluding those episodes with large tail currents at the intermediate pulse (-50 mV) (Fig. 4 B). By doing so, we eliminated the contribution of tail currents to the channel current at the test potential. This selected ensemble average also activated with fast kinetics, suggesting that most of the fast open events were not due to incomplete deactivation, rather they were generated in response to the test pulse (-20 mV).

The ensemble averages of the test currents at -20 mV were fitted with first order kinetics of activation and inactivation, from which the time constants of activation (\( \tau_A \)) were obtained. From the plot of \( \tau_A \) vs. \( \tau_D \) (Fig. 4 C), it can be seen clearly that the changes in gating rate of the Ca channel had a steep dependence on the interval between the conditioning and test pulses. Essentially, the conditioning effect became negligible when \( \tau_D \) was >300 ms.

Second, the amplitude of the intermediate pulse was changed from -50 to -120 mV, to study the dependence of the conditioning effect on the intervening voltage (Fig. 5). With this hyperpolarization, the conditioning effect was essentially eliminated. Rarely, the channels opened with short first latencies, judging from the individual episodes shown in Fig. 5 A, and the conditioned channel activated as slowly as the unconditioned channel. The time constant of activation after conditioning, 215.2 ± 4.6 ms (Fig. 5 B), was not very different from the control channel (Fig. 4 A).

These results show that fast gating of the DHP-sensitive Ca channel is determined by both the conditioning pulse and the intervening voltage between the conditioning and test pulses.

**Properties of the Fast-activating Ca Current in Frog Muscle**

As will be seen in the discussion, when quantitative simulations are attempted with a model presented for
the DHP-sensitive Ca channel, reasonable assignments of parameter values inevitably lead to the prediction that the conversion of the channel from slow to fast kinetics of activation should be accompanied by a leftward shift in the activation voltage by several tens of millivolts. Since such a shift was not obvious in previous experiments (Feldmeyer et al., 1990), we reexamined the issue in frog muscle fibers.

Since several laboratories have recently found evidence of feedback between calcium release from the SR and the voltage sensor of E-C coupling (Pizarro et al., 1991), we did a series of experiments (illustrated in Fig. 6) using Ba$^{2+}$ as current carrier, and a high concentration of the fast Ca$^{2+}$ buffer BAPTA internally. This prevented movement, made intracellular Ca$^{2+}$ transients undetectably small, and presumably reduced to a minimum the possible feedback by Ca$^{2+}$ on the voltage sensor/Ca channel.

A conditioning pulse to +70 mV, 500 ms in duration, placed 200 ms before the test pulse, caused conversion to fast gating kinetics (Fig. 6, right). Since the extracellular solution was 3 mM Ba, this result shows that the kinetic conversion is not related to the increase in intracellular [Ca$^{2+}$] that accompanies the conditioning pulse or to the nature of the ion entering through the activated channel.

Peak I$_{Ba}$ from the experiment illustrated in Fig. 6 is plotted vs. test voltage in Fig. 7. As shown, after conversion to fast gating by the conditioning pulse (filled symbols) the current activated at more negative voltages. Since the descending branch of the curve was also shifted after conditioning, part of the shift may have been due to activation of the outward current at lower voltages, to depletion of Ba$^{2+}$ by the conditioning pulse, or both. In spite of these problems, it is clear that the Ba$^{2+}$ current started to activate at lower voltage after conversion to fast gating. A voltage shift was defined as the difference between the abscissas of the vertex of 2nd order polynomials fitted to I$_{Ba}$ (V) with and without conditioning (method described in figure legend). The shift was 6 mV in the experiment illustrated, and ranged between 5 and 9 mV in 17 other experiments.

One other difference was found in the kinetics after conditioning. As shown in Fig. 6, the decay of Ba$^{2+}$ cur-

![FIGURE 6. Measurement of macroscopic Ca currents (I$_{Ca}$) in frog muscle. A cut fiber from frog fast twitch muscle was held at -80 mV in a double Vaseline gap chamber, with internal solution 10 BAPTA and external 3 Ba. Shown are asymmetric currents (after subtraction of control currents obtained with negative-going pulses). The pulse protocols, leading to slow (left) or fast activation of current, are indicated at top. V is the variable test pulse voltage, listed next to the records. Fiber #925, linear capacitance = 18 nF. Temperature = 12°C.](image)

![FIGURE 7. Voltage-dependence of I$_{Ca}$ and the effects of conditioning pulse. Plot of peak Ba$^{2+}$ current as a function of test pulse voltage in reference (open symbols) and after a conditioning pulse that caused gating conversion (closed symbols, pulse protocols in Fig. 6). The voltages of maximum current were determined as the abscissa of the vertex of second order polynomials fitted to all data points of non-zero current, and are indicated by vertical lines that intersect the horizontal axis. Fiber # 925 (same experiment illustrated in Fig. 6).](image)
rent at 0 mV was very slow and became faster after conditioning (Fig. 6, right). This effect was clear at voltages more negative than those that activate outward current. It is unlikely to reflect different rates of Ba\(^{2+}\) depletion, since the kinetics are similar in records of very different size. It corresponds well with the increase in decay rate observed in bilayers ("conditional average" in Fig. 2 C).

Note that we have not used the term "inactivation" to identify this decay of current after the peak. Even though there is a slow voltage-dependent inactivation in reference conditions, it will be shown in the DISCUSSION that the increased rate of decay can be explained as a "deactivation," operating on top of the normal inactivation process. In this view, deactivation is a consequence of conversion to fast gating.

**DISCUSSION**

The present studies demonstrate that individual DHP-sensitive Ca channels, which normally gate slowly under membrane depolarization, are capable of opening very rapidly. The conversion from slow to fast gating requires a pretransition of the channel to the open state, with steep voltage- and time-dependent transition steps. Since the molecule can undergo both slow and rapid transitions driven by voltage, it is conceivable that the same molecule may perform slow Ca channel activation and fast control of Ca release from the SR.

Studies with other voltage-dependent ion channels demonstrate that opening of the channel pore is usually coupled to voltage-sensitive gating transitions via an additional process that is voltage independent (Oxford, 1981; Zagotta and Aldrich, 1990). The voltage-independent transition step involves a measurable increase in the volume available to water and is therefore termed the solvent-sensitive transition (Zimmerberg et al., 1990). Based on the experiments with substitution of deuterium oxide for water, Alicata et al. (1990) concluded that the sodium channels could activate via two alternative routes, a solvent-sensitive pathway or a solvent-insensitive pathway, depending on initial conditions. The multiple parallel activation mechanisms observed in the sodium channel are inconsistent with a linear sequential model for channel gating, and cyclic gating models have been proposed for activation of the sodium channel (Rayner et al., 1992). Similar cyclical activation mechanism has also been proposed for the gating of the Shaker K channel (Zagotta et al., 1994; Starkus et al., 1995).

The rapid secondary activation of the DHP-sensitive Ca channel seen after depolarizing prepulses is not compatible with a linear activation model, but is highly consistent with a cyclic activation mechanism. In the following, we consider possible models for activation of the DHP-sensitive Ca channel.

**A State Diagram for DHP Receptor Gating**

Feldmeyer et al. (1990) interpreted the fast activation of skeletal muscle I\(_{Ca}\) induced by the depolarizing conditioning pulse as due to the existence of at least two pathways, one fast and one slow, for the channels to pass from a closed into an open state. The state diagram suggested is represented in Fig. 8 A. This model assumes that the channel normally opens via C\(_1\) → C\(_2\) → O\(_3\), including a fast and a slow transition. The fast horizontal transitions (C\(_1\) → C\(_2\) and C\(_2\) → O\(_3\)) generate the measurable intramembrane charge movement (Q). The slow vertical transition C\(_2\) → O\(_3\) is the rate limiting step for the increase in the membrane Ca channel conductance (G). The conditioning depolarization drives the channel into state C\(_4\), and if the open channel closes via the fast route O\(_3\) → C\(_2\) rather than the slow route O\(_3\) → C\(_2\), then the reopening will be fast (through C\(_4\) → O\(_3\)) for as long as the channel dwells in state C\(_4\). Thus, the model explains qualitatively both slow and fast gating of the DHP-sensitive Ca channel.

The midpoint potential between C\(_1\) and C\(_2\) (V\(_{12}\)) should be close to −40 mV, as the experimental Q vs. V is centered at ~−40 mV, and that between C\(_2\) and O\(_3\) (V\(_{23}\)) should be close to 0 mV since G vs. V is centered at ~0 mV (Pizarro et al., 1988). Since Q vs. V is hardly affected by the conditioning pulse (Feldmeyer et al., 1990), the transition voltage V\(_{13}\) should be close to V\(_{12}\). Also to justify the small change in Q(V) upon conversion, the total charge involved in the horizontal transitions should be about the same, which in turn requires apparent valences to be equal. These considerations constrain the four state model to having approximately the same central potentials for its two horizontal transitions. Then, to satisfy microscopic reversibility, V\(_{14}\) should be approximately equal to V\(_{98}\). With these constraints, quantitative problems with the model presented in Fig. 8 A become apparent. Since the fast activating I\(_{Ca}\) that occurs after a conditioning pulse corresponds to transition C\(_1\) → O\(_3\), the activation of which should be centered at −40 mV instead of the usual 0 mV at which slow I\(_{Ca}\) activates. Instead of the predicted 40 mV shift, only a small shift of 5–10 mV was found in actual measurements (Fig. 7).

To account for the experimental result, more closed states must be added, in a manner that separates the high voltage-dependent transition from actual channel opening. Fig. 8 B illustrates our six-state cyclic model for the activation of the DHP receptor. The main difference from the four-state model is the assumption of two classes of steps or conformational changes in the pathway to channel opening, namely voltage-dependent (horizontal) and voltage-independent (vertical) transitions. Two voltage-dependent steps (C\(_1\) → C\(_2\) and C\(_2\) → C\(_3\) ), with different transition voltages (V\(_{12}\), V\(_{23}\) ) are the minimum necessary to account for the activa-
FIGURE 8. A state diagram of DHP receptor gating. (A) State model of Feldmeyer et al. (1990). See explanation in the text. (B) Six-state model for skeletal muscle Ca channel. The horizontal transitions are fast and voltage independent, while the vertical transitions are slow and voltage independent. A resting channel normally opens through C1 → C2 → O6, with the last step being rate limiting (slow). Upon repolarization the channel closes through C5 & C4 (rather than C3 & C2). Thus, a conditioned channel would open from C6, which is fast, as the rate limiting transition step is skipped. (C) Molecular representation of DHP receptor. The picture had three assumptions: (a) the channel as a whole can exist in two different configurations (□ and ○; ■ and ●); (b) repeat I of DHP receptor differs from the other three in two aspects, its equilibrium transition potential is more positive and its forward transition rate is slower, and (c) the transitions from squares to circles (□ → ○ and ■ → ●) in the vertical direction are voltage independent, which proceed in a cooperative way, i.e., the rates become progressively faster as more repeats are moved to their activated states. The midpotential potentials of the horizontal transitions could be determined by the voltage-dependent distribution of charge movement and channel conductance (V12 = ~40 mV and V23 = ~0 mV). The low open probability of the skeletal muscle Ca channel sets a limit on the final equilibrium constant (K□i ~0.25). α6 should be small, to account for slow activation of I\textsubscript{Ca} (α6 ~0.01 ms\(^{-1}\)). According to Feldmeyer et al. (1990), recovery of fast gating was slower near threshold potential of channel activation (~2 s, C3 → C4), and faster at resting potential (~200 ms, C3 → C1). Thus, α41 ~2.5 s\(^{-1}\) and α62 ~0.5 s\(^{-1}\).
voltage-dependent transitions will be fully activated, and no such closing will take place. This property was actually observed in the measurements of \( I_{Ca} \) in frog skeletal muscle (Fig. 6). As can be seen, the fast activating \( I_{Ca} \) decayed significantly faster than the control current at test potential of +15 mV.

Comparison with Measurements of \( I_{Ca} \) in Skeletal Muscle

The L-type Ca channels had different gating kinetics in amphibian and mammalian skeletal muscle fibers. It is well known that the kinetics of \( I_{Ca} \) activation in mammalian muscle is faster than that in amphibian muscle (\( \tau_n \sim 100 \) ms in frog; \( \tau_n \sim 20 \) ms in rat) (Avila-Sakar et al., 1986; Mejia-Alvarez et al., 1991). In frog skeletal muscle, the depolarizing conditioning pulse induced nearly 100% conversion of slow \( I_{Ca} \) to fast \( I_{Ca} \) (Feldmeyer et al., 1990, 1992; García et al., 1990), whereas a negative, or less obvious, result was measured in rodent muscle fiber: the conditioning pulse fails to increase the rate of \( I_{Ca} \) activation (personal communication with Drs. E. Stefani and K.G. Beam, and unpublished data of Shirokova and Rios).

Several factors could account for the apparent differences of single Ca channel and macroscopic \( I_{Ca} \) in mammalian and amphibian skeletal muscles. The DHP receptors in amphibian and mammalian skeletal muscle may differ from each other in their different stages of development and adaptation. As has been shown, the conditioning pulse-induced potentiation of \( I_{Ca} \) is a steep function of the intermediate state between conditioning and test pulses. In frog muscle, the return of fast gating back to slow gating had a time constant of \( \sim 250 \) ms at \(-20\) mV (a subthreshold potential for \( I_{Ca} \) activation) (Feldmeyer et al., 1990). In rat myoballs, the conditioning pulse-induced potentiation of \( I_{Ca} \) decayed significantly faster upon hyperpolarization, with a time constant of \( \sim 40 \) ms (Scultoreanu et al., 1992, 1993). In the bilayer experiments, significant changes in gating rate of the Ca channel required the duration between test and conditioning pulses to be \(<300\) ms (Fig. 4). Thus, one possible explanation is that measurements of \( I_{Ca} \) in rat muscle fibers are limited to a time interval of longer than 150 ms to allow for complete termination of the tail current, which may obscure the conditioning effect on fast gating.

Adams and Beam (1992) showed that the rate of \( I_{Ca} \) activation became faster with increased density of DHP receptor expression in dysgenic myoballs. In the present studies, activation of single Ca channels in bilayer had a time constant of 110 ms, which is significantly slower than that of \( I_{Ca} \) in mammalian muscle (\( \sim 20-40 \) ms) (Dirksen and Beam, 1995). It is possible that interactions between the DHP receptors may result in faster activation of the Ca channel, which may also alter the conditioning pulse effect.

The single channel measurements were all performed with Bay K 8644 present in the intracellular solution. This agonist increased the open lifetime of the L-type Ca channel (Hess et al., 1984), accompanied with significant changes in the voltage-dependent gating kinetics of the Ca channel in cardiac muscle (Bechem and Schramm, 1988). It is conceivable that part of the conditioning-induced fast gating of the Ca channel was due to the voltage-dependent binding of Bay K 8644 to the DHP receptor.

DHP Receptors as Fast E-C Coupling Voltage Sensor and Slow Ca Channel

The main arguments for the existence of separate voltage sensors of E-C coupling and slow Ca channels are the differences in kinetics and voltage dependence of activation of both functions. The model discussed in Fig. 8 shows how both functions, with adequate kinetics and voltage dependence, can be carried out by the
same molecule. Assuming that partially activated DHP receptors, C2 and Cα, which can be reached at fast speed and at lower voltages, could signal opening of the ryanodine receptor Ca release channel (personal communications with K.G. Beam, E. Stefani, and E. Rios), this model puts together the two functions of the DHP receptor: explaining slow activation of the Ca current and fast control of SR Ca release.

Two other observations have been taken as evidence of separate voltage sensors and Ca channels. One is that the magnitude of I_cα is less than expected given the density of DHP receptors in the TT membrane (Schwartz et al., 1985), which suggests that only a small fraction of DHP receptors function as Ca channels. It has been pointed out, however, that the small current may also be a consequence of low open probability of the Ca channel (Lamb and Walsh, 1987), which was found to be the case for TT membrane Ca channels in bilayers, even when they were fully activated by voltage and in the presence of the agonist Bay K 8644 (Ma et al. 1991).

The other observation that led Pizarro et al. (1988) to suggest that different DHP receptors are responsible for the voltage sensor of E-C coupling and the conducting unit of the L-type Ca channel was the remarkable difference in time course of recovery of I_cα and SR Ca release flux after inactivation by prolonged depolarization. The recovery of I_cα occurred without appreciable delay, following an exponential function of time, while repriming of Ca release followed a sigmoidal time course. The difference in time course of repriming can be explained if opening of one release channel is determined by the joint activation of multiple DHP receptors. This interpretation seems natural since DHP receptors appear in groups of four, the junctional tetrad identified in electron microscopy of freeze fractured transverse tubules by Block et al. (1988). Simon and Hill (1992) reported a 4th power relationship between Ca release flux and charge movement, while Rios et al. (1993) also found a power relationship, of exponent between 2 and 3. These results suggest that multiple DHP receptors are involved in the control of Ca release from the SR membrane. This interpretation makes the pharmacological differences between E-C coupling and Ca currents noted by Romey et al. (1988) easier to understand. A drug will be more effective in blocking Ca release than I_cα, for the same reason that I_cα recovers more rapidly than Ca release from voltage-induced inactivation, provided that it blocks early steps in the activation of I_cα (Feldmeyer et al., 1992).

Given that the cyclical model shown in Fig. 8 applies to activation of the DHP-sensitive Ca channels as well as the Na channel (Alicata et al., 1990; Starkus and Rayner, 1991) and K channel (Zagotta et al., 1994; and Starkus et al., 1995), it is reasonable to propose that voltage-sensitive gating transitions occur early in the Ca channel activation sequence and that it is these processes which could underlie the role of the voltage sensor and yield the rapid E-C coupling in skeletal muscle, through either electrostatic or allosteric linkage to the ryanodine receptor Ca release channel.

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