

Unitary Behavior of Skeletal, Cardiac, and Chimeric L-Type Ca^{2+} Channels Expressed in Dysgenic Myotubes

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ABSTRACT Skeletal and cardiac dihydropyridine receptors function both as voltage-dependent L-type calcium channels (L-channels) and as critical proteins that trigger calcium release from the sarcoplasmic reticulum in muscle. In spite of these similarities, skeletal L-channels exhibit a markedly slower activation rate than cardiac L-channels. We investigated the mechanisms underlying this difference by comparing the unitary behavior of L-channels in cell-attached patches of dysgenic myotubes expressing skeletal, cardiac, or chimeric dihydropyridine receptors. Our results demonstrate that ensemble averages activate rapidly for the purely cardiac dihydropyridine receptor and approximately five times more slowly for L-channels attributable to the purely skeletal dihydropyridine receptor or a chimeric dihydropyridine receptor in which only the first internal repeat and all of the putative intracellular loops are of skeletal origin. All of the constructs studied similarly exhibit a brief (2-ms) and a long (≥ 15 -ms) open time in the presence of Bay K 8644, neither of which depend significantly on voltage. In the absence of Bay K 8644, the fraction of total open events is markedly shifted to the briefer open time without altering the rate of ensemble activation. Closed time analysis of L-channels with cardiac-like, rapid activation (recorded in the presence of dihydropyridine agonist) reveals both a brief (~ 1 -ms) closed time and a second, voltage-dependent, long-lasting closed time. The time until first opening after depolarization is three to six times faster for rapidly activating L-channels than for slowly activating L-channels and depends strongly on voltage for both types of channels. The results suggest that a voltage-dependent, closed-closed transition that is fast in cardiac L-channels and slow in skeletal L-channels can account for the difference in activation rate between these two channels. **Key words:** single-channel recording • dihydropyridine • skeletal muscle

INTRODUCTION

The sarcolemma of skeletal and cardiac muscle contains proteins that bind derivatives of 1,4-dihydropyridines with high affinity (Norman et al., 1983; Fosset et al., 1983). In both cell types, these dihydropyridine receptors (DHPRs)¹ act as voltage-dependent L-type calcium channels (L-channels) and provide a functional

link between membrane excitation and intracellular calcium release from the sarcoplasmic reticulum. Nevertheless, the skeletal and cardiac DHPRs differ greatly with regard to their biophysical channel properties and in the mechanisms by which they control calcium release from the sarcoplasmic reticulum. For example, skeletal L-channels activate >20 times more slowly than cardiac L-channels (Beam et al., 1986; Tanabe et al., 1991). The primary sequence of repeat I (Tanabe et al., 1991), and more specifically the sequence within repeat I of the S3 segment and the linker connecting the IS3 and IS4 segments, is critical for this difference in L-channel activation (Nakai et al., 1994). However, any structural interpretation of how this region controls the rate of activation requires knowing whether the transition(s) it governs is voltage dependent or voltage independent.

Establishing the biophysical basis for the difference in activation kinetics of cardiac and skeletal L-channels requires the ability to compare unitary activity of these

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¹Abbreviations used in this paper: CAC6, rabbit skeletal dihydropyridine receptor; CARD1, rabbit cardiac dihydropyridine receptor; CSk9, chimeric dihydropyridine receptor with only putative intracellular loops of skeletal sequence; DHPR, dihydropyridine receptor; I_{dys} , dysgenic L-type current; L-channel, L-type calcium channel; SkC15, chimeric DHPR with repeat I and all putative intracellular loops of skeletal sequence.

channels, and of constructs derived from them, in a single system. An obstacle to such a comparison is that skeletal L-channels appear not to be easily expressed in nonmuscle cells, as only a few reports of such expression have appeared (Perez-Reyes et al., 1989; Lacerda et al., 1991). Additionally, L-channels in adult muscle are preferentially localized in transverse tubules (Fosset et al., 1983), a site inaccessible to cell-attached patch recordings. However, single-channel activity attributable to skeletal L-channels has been recorded from the surface membrane of cultured skeletal myotubes at a stage of development before the formation of transverse tubules (Dirksen and Beam, 1995). Moreover, myotubes from mice homozygous for the muscular dysgenesis mutation provide a useful system for the physiological expression of cDNAs encoding skeletal, cardiac, and chimeric L-channels (e.g., Tanabe et al., 1988, 1990, 1991; García et al., 1994; Nakai et al., 1994). These dysgenic myotubes lack the endogenous, skeletal L-type calcium current (Beam et al., 1986) as a consequence of a mutation causing premature termination of the skeletal muscle DHPR (Chaudhari, 1992).

The experiments described here are the first to compare directly in an intact muscle expression system the unitary properties of L-channels attributable to either the purely skeletal DHPR, the purely cardiac DHPR, or chimeric constructs of these two DHPRs. Our results demonstrate that, in the presence of Bay K 8644, slowly and rapidly activating L-channels exhibit two distinct, voltage-independent open times in approximately equal proportion. In the absence of agonist, the difference in skeletal and cardiac ensemble activation persists, in spite of the fact that the proportion of open events with a brief open time is dramatically increased. Slowly activating L-channels display a much longer latency to first opening ($\sim 5\times$) than rapidly activating L-channels. In both channel types, the first latency decreases markedly with depolarization and is slower than macroscopic activation. Our data are consistent with the idea that skeletal L-channel macroscopic activation involves a slow voltage-dependent transition before channel opening.

METHODS

Primary cultures of myotubes were prepared from skeletal muscle of newborn and dysgenic mice, as described previously (Beam and Knudson, 1988). All experiments were performed 7–11 d after the initial plating of myoblasts and were carried out at room temperature (20–22°C). Numerical figures are presented in the text and figures as mean \pm SEM.

Injection of Expression Plasmids

On the sixth or seventh day after the initial plating of myoblasts into primary culture, nuclei of dysgenic myotubes within demar-

cated regions of 35-mm culture dishes were microinjected (Tanabe et al., 1988) with expression plasmids (0.2–1.0 $\mu\text{g}/\mu\text{l}$) carrying cDNA inserts encoding either the skeletal DHPR, CAC6 (Tanabe et al., 1988), the cardiac DHPR, CARD1 (Mikami et al., 1989), or the chimeric DHPRs, SkC15 and CSK9 (Tanabe et al., 1991). Myotubes expressing cDNA were identified 1–4 d after injection on the basis of electrically evoked contractions (Tanabe et al., 1988; Tanabe et al., 1990).

Single-Channel Measurements

Unitary calcium channel currents were measured using the cell-attached mode of the patch-clamp technique (Hamill et al., 1981) as described previously (Dirksen and Beam, 1995). Fire-polished borosilicate pipettes were coated with Sylgard (Dow-Corning, Midland, MI) and had resistances of 2–4 M Ω when filled with the pipette solution. Currents were acquired with an EPC-7 (Medical Systems, Greenvale, NY) or 3900A (Dagan Corp., Minneapolis, MN) patch-clamp amplifier, filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA), and acquired at 10 kHz using an IDA Interface (INDEC Systems, Capitola, CA). Data were collected using the Basic-Fastlab (INDEC Systems) software and analyzed using a combination of pCLAMP (Axon Instruments, Foster City, CA) and an analysis program kindly provided by Dr. Don Campbell (Hatfield Marine Science Center).

Records of channel activity were digitally corrected for leak and capacitive currents by subtracting from each record either the average of multiple sweeps without channel openings (null sweeps) or a multiexponential curve that had been fitted to a null sweep. Ensemble averages were compiled by averaging all leak-subtracted current records in a series. To calculate the potential across the patch membrane (cell resting potential minus pipette potential), resting potentials were measured at the end of experiments by rupturing the cell-attached patch while in current clamp mode. Resting potentials averaged -59.0 ± 0.7 mV ($n = 28$). Thus, a value of -60 mV was assumed for the resting membrane potential in calculations of absolute patch potentials. All patches were either held at their resting potential (-60 mV) or 30 mV hyperpolarized to the resting potential (-90 mV). Single-channel openings and closings were determined with a 50% threshold crossing method. The duration of an open event was taken as the elapsed time between adjacent openings and closings. In patches containing more than one channel (the majority of experiments), open time durations were calculated only for excursions that went from the zero-current level to the single-current level and returned back to the zero-current level without interruption by openings to higher current levels. Openings that persisted at the end of the test depolarization were excluded from the analysis. Histograms of open time durations (binned at 0.5 ms) were constructed from data digitally filtered at 500 Hz. The open time histograms were subsequently fitted by the sum of one or two exponentials. All data presented in this paper come only from those cell-attached patches containing ≤ 4 L-channels. Histograms of closed time durations (binned at 0.5 ms) were constructed from data digitally filtered at 1 kHz only for patches containing a single channel. True single-channel patches were extremely rare and were only found for dysgenic myotubes injected with low concentrations of cDNA encoding CARD1 and CSK9. The presence of a single channel was judged by the presence of only a single open level in >200 sweeps to strong depo-

larizations (where P_o for many of the sweeps exceeded 0.5; see Fig. 5, *right*).

First latency distributions were measured for multiple 200-ms sweeps to a given potential by calculating the time after depolarization to the first open event detected by the 50% threshold crossing criterion. All of the individual first latencies for a given potential were then binned (0.5 ms), and the probabilities were calculated from the following expression:

$$L_n(t) = \frac{(\text{No. of sweeps without an opening before } t)}{(\text{total No. sweeps})}, \quad (1)$$

where $L_n(t)$ is the probability that a channel opens after time t , and t is the center value for a given bin in a patch possessing n channels. For n identical and independent channels (Aldrich et al., 1983), the first latency of a single channel can be calculated as follows:

$$L_1(t) = [L_n(t)]^{1/n}. \quad (2)$$

Dysgenic myotubes endogenously express a low level of rapidly activating L-type current, I_{dys} (Adams and Beam, 1989). In uninjected dysgenic myotubes, we detected the channels that evidently produce I_{dys} , but only very rarely (<1/100 patches and never more than a single channel in a patch). These channels exhibited a large unitary conductance (>25 pS), produced ensembles that activated rapidly, and had a brief latency to first opening. Because of their rare occurrence, these channels were not systematically studied. In a few experiments on dysgenic myotubes injected with CAC6 cDNA, an I_{dys} channel was apparently present in the same patch as expressed channels, based on the presence of large (cardiac-like; ~25-pS) unitary events intermingled with smaller (skeletal-like; ~14-pS) unitary events. Data from these experiments were not included in this study. The extremely low occurrence of I_{dys} would tend to limit its contamination of multichannel patches of cardiac-like channels (CARD1 and CSK9).

Solutions

Myotubes were bathed in a normal rodent Ringer solution consisting of the following (mM): 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES (pH = 7.40 with NaOH). Except where otherwise indicated, cell-attached patch pipettes were filled with a solution consisting of the following (mM): 110 BaCl₂, 0.003 tetrodotoxin, 0.005 (\pm) Bay K 8644 (kindly supplied by Dr. A. Scriabine, Miles Laboratories, Inc., New Haven, CT), and 10 HEPES (pH = 7.40 with TEA-OH).

RESULTS

Unitary Activity of Slowly and Rapidly Activating L-Channels

Fig. 1 shows unitary and ensemble activity of slowly and rapidly activating L-channels obtained from three different, representative, cell-attached patches. Data are shown for dysgenic myotubes injected with cDNA plasmids encoding the skeletal (CAC6, *left*), chimeric (SkC15, *middle*), or cardiac (CARD1, *right*) DHPR. A schematic representation of each of the expressed channels is shown above the corresponding current traces. Each

patch in Fig. 1 was depolarized from the holding potential to 0 mV for 200 ms. Representative leak-subtracted sweeps are shown in the middle panels. In this and all subsequent figures, channel openings are represented by downward current deflections. For all three constructs, open durations varied from <1 to tens of milliseconds at 0 mV in the presence of (\pm) Bay K 8644 (5 μ M). Occasionally, large single-channel tail currents of variable duration were observed when channels remained open upon repolarization.

The time to first channel opening was usually longer for CAC6 and SkC15 than for CARD1, which was also reflected in ensembles constructed for each patch (Fig. 1, *bottom*) by averaging multiple sweeps similar to those shown in the middle panels. As for L-channels in normal myotubes (Dirksen and Beam, 1995), ensembles of activity attributable to CAC6 and SkC15 displayed slow activation with time constants of tens of milliseconds. Upon repolarization, ensembles typically deactivated rapidly and in a biexponential manner. Ensembles of CARD1 unitary activity activated approximately five times faster than ensembles attributable to CAC6 or SkC15. Thus, our results on ensemble activation are in agreement with observations that slow skeletal activation of whole cell current is determined by repeat I (Tanabe et al., 1991; Nakai et al., 1994).

Slowly and Rapidly Activating L-Channels Exhibit Similar Open Times

Fig. 2 shows open time histograms (obtained from 200-ms depolarizations to 0 mV) for dysgenic myotubes expressing CAC6 (*left*), SkC15 (*middle*), or CARD1 (*right*). For all three constructs, open time histograms required the sum of two exponential functions to achieve an adequate fit. Each fit consisted of a brief (2–4 ms) open time and a second longer open time (15–50 ms), which tended to vary from experiment to experiment. Over a wide range of voltages, no consistent differences were observed between slowly and rapidly activating L-channels with regard to either the fitted time constants (τ) or the relative fraction of events (θ) associated with the brief and long openings (Table I). The average voltage dependence of the two calculated open times for CAC6 (*solid squares*), SkC15 (*solid triangles*), and CARD1 (*open squares*) are shown in Fig. 3. The two open times for both slowly and rapidly activating L-channels did not vary significantly with voltage over the range tested (–20 mV to +20 mV). Similar results have been reported in normal myotubes (Dirksen and Beam, 1995) and in a cell line expressing slowly activating L-channels (Caffrey, 1994). These results support the conclusions of others (Chen and Hess, 1990; Marks and Jones, 1992) that exit from the open state in voltage-dependent calcium channels is voltage independent.

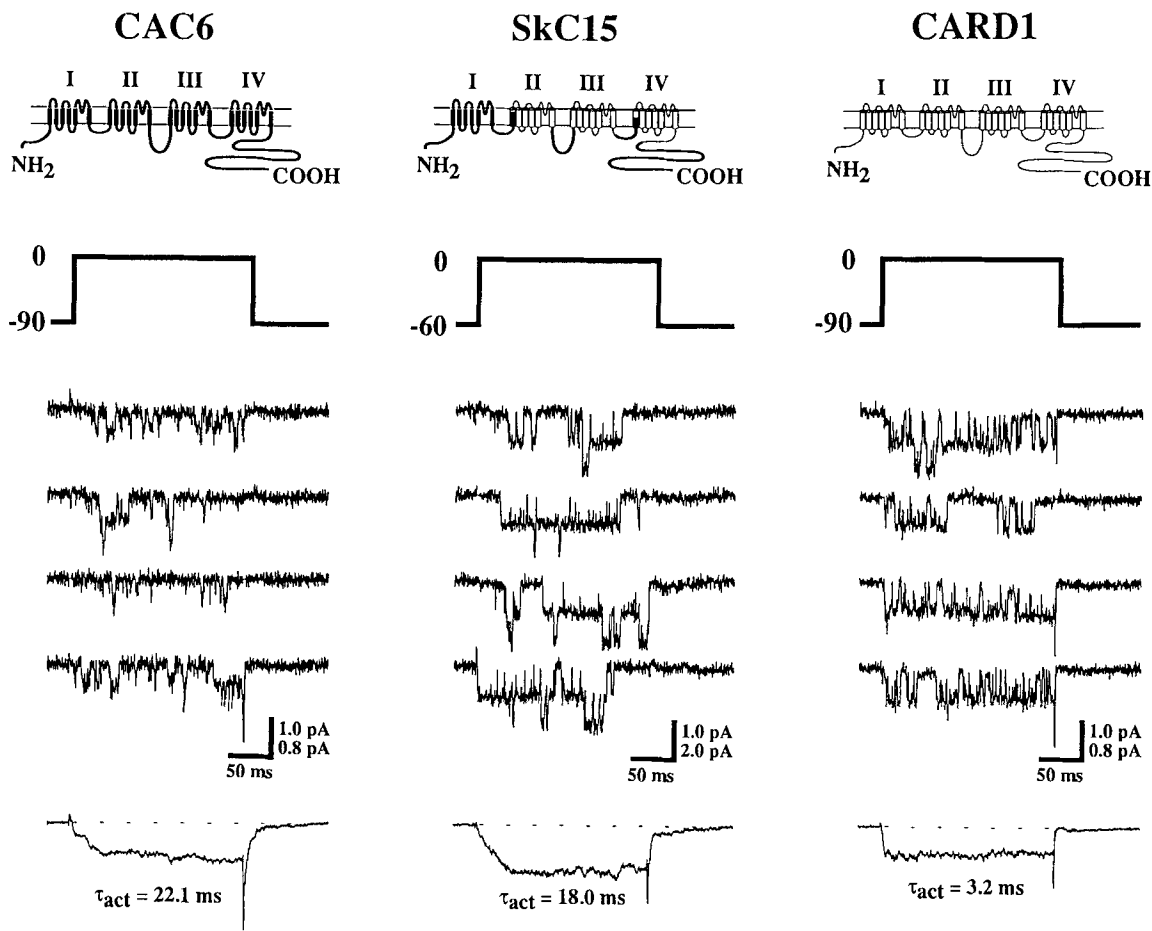


FIGURE 1. Unitary and ensemble activity of slowly and rapidly activating L-channels in cell-attached patches obtained from dysgenic myotubes expressing CAC6 (*left*, three-channel patch), SkC15 (*center*, four-channel patch), or CARD1 (*right*, three-channel patch). (*Top*) Schematic representations are shown for the voltage protocols and the constructs, with thick lines and filled cylinders representing regions of skeletal origin and thin lines and hollow cylinders representing regions of cardiac origin. (*Middle*) Representative leak-subtracted sweeps of unitary activity with downward deflections representing channel openings. For these experiments, 110 mM Ba^{2+} was the charge carrier and 5 μM (\pm) Bay K 8644 was present in the pipette solution. (*Bottom*) Ensemble averages of 62 (*left*), 40 (*middle*), or 40 (*right*) sweeps, with the zero current level indicated by the dashed line. The activation time constant (τ_{act}) for each experiment is shown below the corresponding ensemble. The upper and lower vertical calibrations apply to the unitary and ensemble data, respectively.

Rapidly and Slowly Activating L-Channels Exhibit Predominantly Brief Openings in the Absence of Bay K 8644

As described above, both slowly and rapidly activating L-channels in the presence of Bay K 8644 display brief and long openings, each constituting about half of the total number of open events ($\theta_1 \approx \theta_2 \approx 0.5$ in Table I). Since Bay K 8644 promotes long openings of cardiac L-channels (Hess et al., 1984), the high percentage of long openings is likely attributable to the presence of this DHP agonist. To determine the importance of long openings for the slower activation of SkC15 than CARD1, we also measured open times in the absence of Bay K 8644.

Representative sweeps of unitary and ensemble activity recorded from multichannel patches of SkC15 or

CARD1 without DHP agonist are illustrated in Fig. 4. To allow a direct comparison, sampling rate and filter frequency were the same as for the experiments with Bay K 8644. A dashed line is superimposed on the uppermost traces to indicate the estimated single-channel current amplitude. Although long openings should have been easily observable, the great majority of openings were very brief and typically not well resolved. For the data used to generate the ensembles shown in Fig. 4, there were only three openings longer than 12 ms both for SkC15 (2,744 total open events) and for CARD1 (1,851 total open events). Nonetheless, ensemble activation for SkC15 was similar whether or not the DHP agonist was present, and considerably slower than for CARD1.

In the absence of DHP agonist, the fraction of total

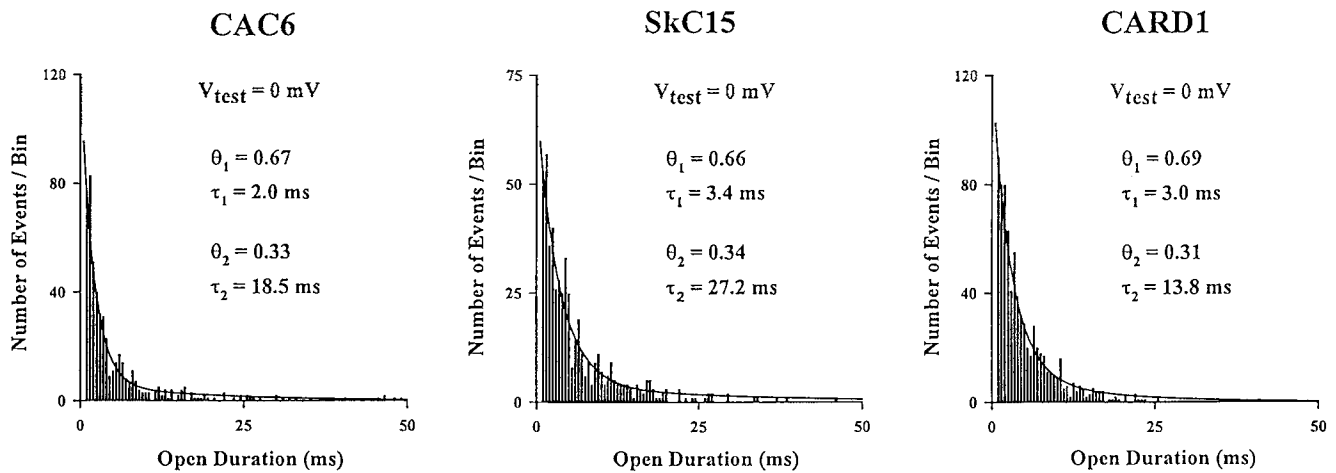


FIGURE 2. Open time histograms of slowly and rapidly activating L-channels. Unitary channel open events were elicited by 200-ms depolarizations from the holding potential to 0 mV applied to cell-attached patches of dysgenic myotubes expressing CAC6 (left), SkC15 (middle), or CARD1 (right). For each of these constructs, both brief and long open events were present. The smooth curves represent best fits of two summed exponentials (see Table I). The values of the time constant (τ) and the fraction of total events (θ) associated with each time constant are shown in the insets.

open events is shifted to a very brief open time (~ 1 ms) for both SkC15 and CARD1. Specifically, θ_1 determined from exponential fits of open time histograms obtained in the absence of DHP agonist (data not shown) was

0.92 ± 0.02 ($n = 3$) for SkC15 and 1.0 ($n = 3$) for CARD1 (data averaged from measurements at -10 and 0 mV). Since many apparent openings failed to satisfy the 50% threshold crossing criterion, the actual value

TABLE I
Open Time Parameters for Slowly and Rapidly Activating L-Channels

Construct	-10 mV				0 mV			
	θ_1	τ_1	θ_2	τ_2	θ_1	τ_1	θ_2	τ_2
		ms		ms		ms		ms
Normal	0.48 ± 0.04	3.4 ± 1.2	0.52 ± 0.04	21 ± 6	0.47 ± 0.04	2.2 ± 0.4	0.53 ± 0.04	29 ± 8
		(6)				(9)		
CAC6	0.57 ± 0.06	1.5 ± 0.3	0.43 ± 0.08	34 ± 8	0.65 ± 0.06	1.6 ± 0.2	0.35 ± 0.06	30 ± 13
		(5)				(6)		
SkC15	0.57 ± 0.08	3.4 ± 0.3	0.43 ± 0.08	30 ± 6	0.56 ± 0.17	3.1 ± 0.7	0.44 ± 0.17	18 ± 6
		(4)				(4)		
CARD1	0.43 ± 0.09	2.6 ± 0.6	0.57 ± 0.09	18 ± 6	0.47 ± 0.07	4.1 ± 0.5	0.53 ± 0.07	14 ± 3
		(6)				(6)		
		10 mV				20 mV		
Construct	θ_1	τ_1	θ_2	τ_2	θ_1	τ_1	θ_2	τ_2
		ms		ms		ms		ms
Normal	0.30 ± 0.07	2.1 ± 0.4	0.70 ± 0.07	50 ± 25	0.51 ± 0.07	2.8 ± 0.4	0.49 ± 0.07	33 ± 9
		(6)				(6)		
CAC6	0.60 ± 0.05	1.5 ± 0.3	0.40 ± 0.05	44 ± 20	0.66 ± 0.13	1.7 ± 0.65	0.34 ± 0.13	24 ± 2
		(4)				(2)		
SkC15	0.59 ± 0.09	3.4 ± 1.6	0.41 ± 0.09	15 ± 1	—	—	—	—
		(4)						
CARD1	0.32 ± 0.08	3.7 ± 1.2	0.68 ± 0.08	14 ± 2	0.45 ± 0.06	5.1 ± 0.2	0.55 ± 0.06	48 ± 20
		(4)				(2)		

Open time histograms were fit according to: $Y = (\omega_1/\tau_1)\exp[-t/\tau_1] + (\omega_2/\tau_2)\exp[-t/\tau_2]$, where Y is the number of counts in a given bin with a center time value t . Thus, $\omega_x/(\text{bin width})$ is the number of events associated with time constant τ_x , and the relative fraction (θ_x) of total events associated with τ_x is given as $\theta_x = \omega_x/(\omega_1 + \omega_2)$. Data are for patches (number of experiments shown in parentheses) with ≤ 4 channels. Data for normal myotubes are taken from Dirksen and Beam (1995).

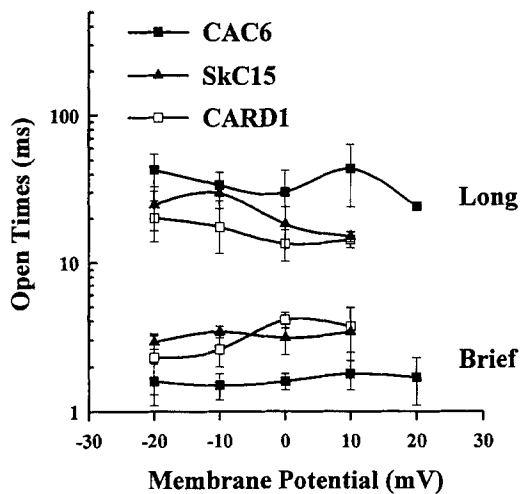


FIGURE 3. Voltage dependence of average open times (\pm SEM) for slowly (CAC6, *solid squares*, and SkC15, *solid triangles*) and rapidly (CARD1, *open squares*) activating L-channels expressed in dysgenic myotubes. Neither the long duration openings (which had values of similar magnitude for the three constructs) nor the brief duration openings (also having similar values for the three constructs) varied appreciably with voltage. Open times represent the average time constants obtained from two exponential fits as described in Table I.

of θ_1 would be expected to be greater than or equal to these values. Thus, in the absence of DHP agonist, long openings are obviously rare for both slowly and rapidly activating L-channels.

Rapidly Activating L-Channels Exhibit a Voltage-dependent Closed Time

We also attempted to measure the voltage dependence of closed times for the various constructs expressed in dysgenic myotubes. Unfortunately, it was not possible to obtain cell-attached patches containing only one L-channel (an essential requirement for meaningful closed time analysis) from either normal myotubes or the constructs CAC6 and SkC15. However, we occasionally obtained true single-channel patches from dysgenic myotubes injected with low concentrations of CARD1 or CSk9 cDNA. The construct CSk9 (Tanabe et al., 1991) is like SkC15 (see schematic in Fig. 1) in that all of the putative intracellular loops are of skeletal origin, but CSk9 differs in that all four repeats are of cardiac origin and CSk9 exhibits rapid activation. Fig. 5 shows consecutive leak-subtracted single-channel sweeps at three different voltages observed in a cell-attached patch containing a single CSk9 chimeric L-channel. At 0 mV, unitary activity was characterized by relatively infrequent openings separated by both brief and long closures. Upon stronger depolarization, openings were more frequent and were separated by predominantly

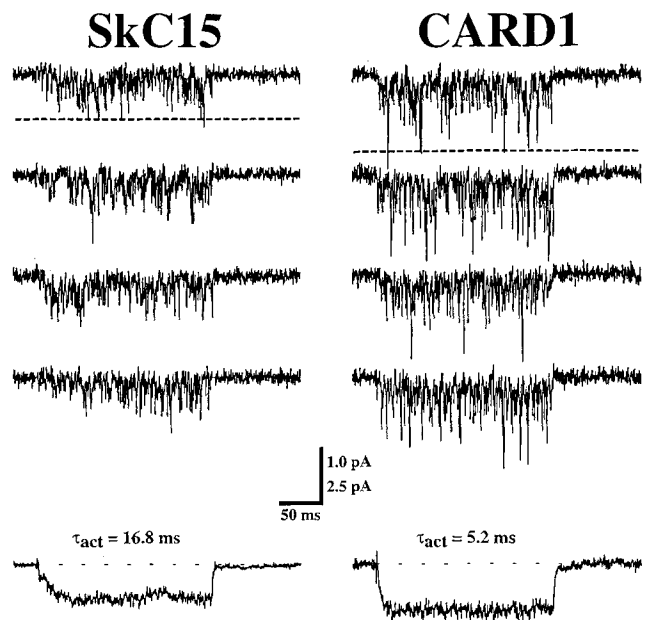


FIGURE 4. Unitary and ensemble activity of slowly and rapidly activating L-channels in the absence of DHP agonist. (*Top*) Selected leak-subtracted sweeps of unitary activity obtained from cell-attached patches of dysgenic myotubes expressing either SkC15 (*left*, 0 mV) or CARD1 (*right*, -10 mV). The dashed line superimposed on the first sweep in each series represents the averaged open-channel current level (-0.70 ± 0.09 pA and -1.26 ± 0.09 pA for SkC15 and CARD1, respectively) for experiments performed in the presence of DHP agonist. Note the absence of large single-channel tail currents upon repolarization, in contrast to the data of Fig. 1. (*Bottom*) Ensemble averages of 78 (*left*) or 114 (*right*) sweeps with the activation time constant (τ_{act}) shown for each ensemble. The upper and lower vertical calibrations apply to the unitary and ensemble data, respectively.

brief closings. Closed time histograms at all three potentials were fitted by the sum of two exponentials (Fig. 5, *bottom*). Although the brief closed time (~ 1 ms) is poorly resolved under these conditions (filtering at 1 kHz and binning at 0.5 ms), its presence is apparent. The duration of the longer closed time depended strongly on voltage. In Fig. 5, as the test potential was increased from 0 mV to +20 mV, the longer closed time was reduced almost sixfold, while the brief closed time changed little. Moreover, the fraction of events associated with the briefer closed time increased substantially with greater depolarization. Similar results were obtained from single-channel patches of CARD1 and in some multichannel patches in which all but one L-channel was inactivated by holding the patch at depolarized potentials for prolonged times (data not shown). These data confirm that rapidly activating L-channels exhibit two closed times (Cavalié et al., 1983; Hess et al., 1984): a brief closed time and a second, longer, voltage-dependent closed time.

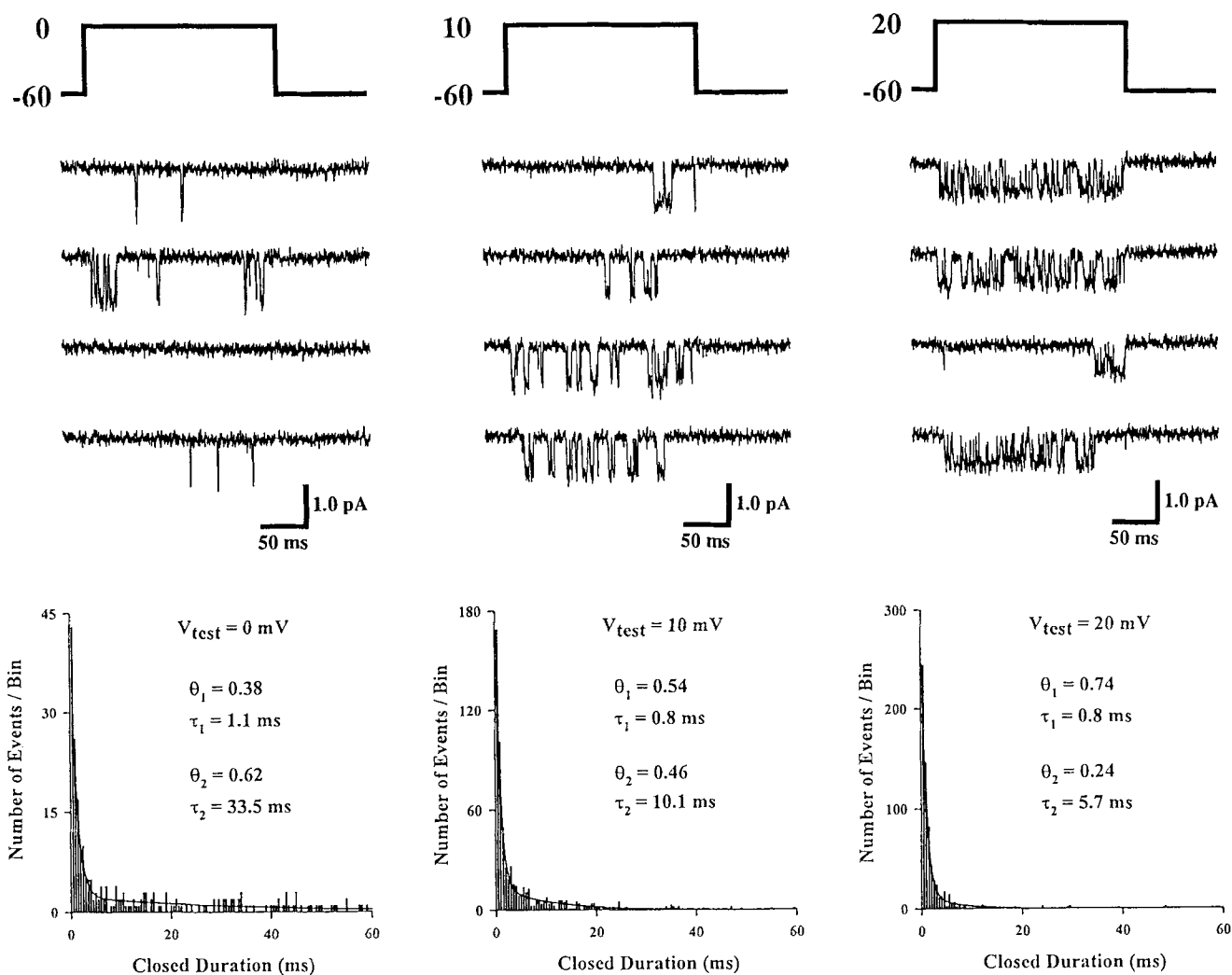


FIGURE 5. Closed time analysis of CSK9, a chimeric DHPR with rapid, cardiac-like activation (Tanabe et al., 1991). (Top) Consecutive leak-subtracted single-channel sweeps elicited by 200-ms depolarizations to either 0 mV (left), 10 mV (middle), or 20 mV (right) obtained from a single-channel, cell-attached patch of a dysgenic myotube expressing CSK9. Data were obtained in the presence of 5 μM Bay K 8644. (Bottom) Closed time histograms obtained from a total of 200 (0 mV), 80 (10 mV), or 40 (20 mV) sweeps and the best fits of two summed exponentials. To construct the histograms, the current traces were filtered at 1 kHz and event durations were binned at 0.5 ms. The values of the time constants and the fraction of total events associated with each time constant are shown in the insets.

First Latency Distributions of Slowly and Rapidly Activating L-Channels

To get additional information regarding the rate of transitions between closed states before channel opening, we measured the latencies to the first channel opening for slowly and rapidly activating L-channels (Figs. 6 and 7). Multiple sweeps were elicited by 200-ms depolarizations to various potentials, and the time from depolarization to the first channel opening (as determined by a 50% threshold crossing criterion) was calculated. Latencies were binned (0.5 ms), corrected for the number of channels in the patch, and fitted with a single exponential equation. Fig. 6 shows first latency

distributions at 0 mV for cell-attached patches obtained from a normal myotube (upper left) and dysgenic myotubes expressing either CAC6 (upper right), SkC15 (lower left), or CARD1 (lower right). The first latency distributions of each construct exhibited a slight sigmoidicity but were adequately described by a single exponential. Slowly activating L-channels (normal, CAC6, and SkC15) displayed a latency to first opening markedly slower than rapidly activating L-channels (CARD1).

The voltage dependencies of the time constants of L-channel activation and latency to first opening for the slowly and rapidly activating L-channels are compared in Fig. 7. Activation and latencies of normal myotubes and dysgenic myotubes expressing CAC6 and

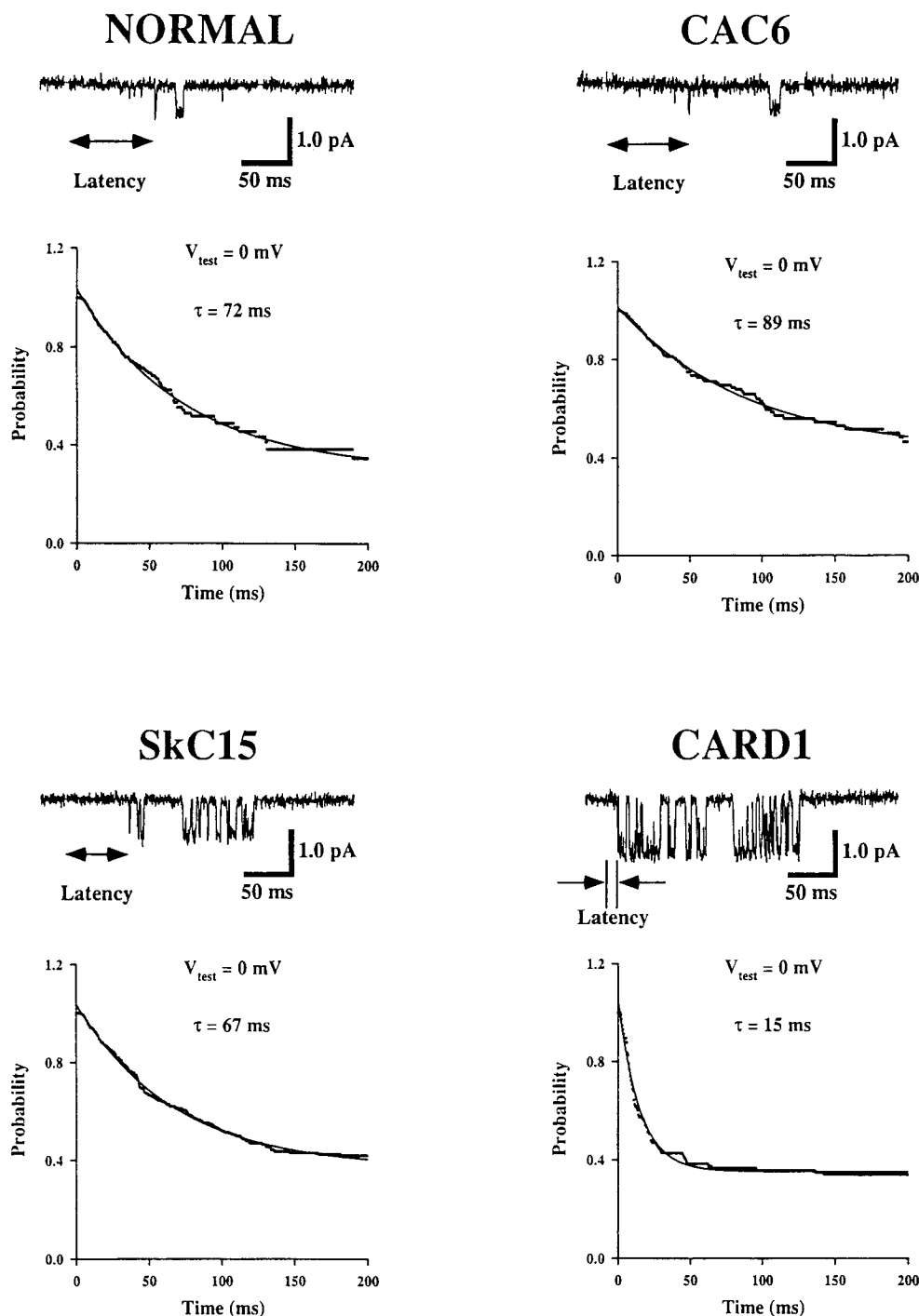


FIGURE 6. First latency analysis of slowly and rapidly activating L-channels in the presence of Bay K 8644 (5 μ M). Representative traces illustrating the latency between onset of depolarization from the holding potential to 0 mV and the first channel opening (defined as crossing a threshold 50% of the single open channel current level) for cell-attached patches obtained from a normal myotube (*upper left*, four-channel patch, 140 sweeps, 2 nulls) and dysgenic myotubes expressing CAC6 (*upper right*, three-channel patch, 81 sweeps, 8 nulls), SkC15 (*lower left*, two-channel patch, 200 sweeps, 34 nulls), or CARD1 (*lower right*, one-channel patch, 115 sweeps, 39 nulls). To produce first latency distributions, the measured first latencies were binned (0.5 ms) and corrected for the number of channels in the patch as described in Methods. The time constant obtained from a single exponential fit (see Table II) is shown in the inset to each first latency distribution. The first latency distribution of the rapidly activating L-channel (CARD1) decays approximately five times faster than those of the slowly activating L-channels (normal, CAC6, and SkC15).

SkC15 were pooled together as exhibiting skeletal-like kinetics and compared with those of CARD1. At all potentials tested, skeletal-like L-channels activated approximately five times slower than the cardiac L-channels. Moreover, the ensemble activation of both skeletal-like and cardiac L-channels became faster at stronger depolarizations. Thus, the voltage dependence of skeletal-like ensemble activation is like that

observed for activation of whole-cell currents recorded in normal myotubes (Dirksen and Beam, 1995) under comparable conditions (110 mM BaCl₂ + 5 μ M Bay K 8644). For both the skeletal-like and cardiac L-channels, ensemble activation was more rapid than the corresponding first latency at all potentials tested. Moreover, the pooled skeletal and cardiac first latencies were both found to be voltage dependent ($\sim e$ -fold/20

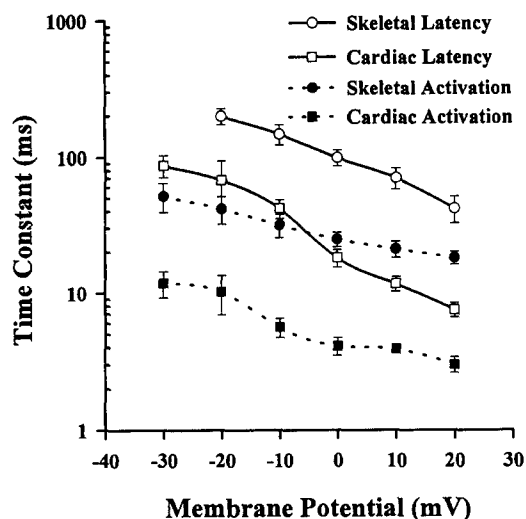


FIGURE 7. Voltage dependence of the average (\pm SEM) time constants of ensemble activation and first latency distribution for slowly and rapidly activating L-channels. Data for L-channels exhibiting slow activation (skeletal-like: normal, CAC6, and SkC15) were pooled. Skeletal L-channels (solid circles) activated more slowly than cardiac L-channels (solid square) at every voltage. In addition, skeletal L-channels exhibited a markedly slower latency to first opening (open circles) than cardiac L-channels (open squares).

mV). In addition, the skeletal-like L-channels exhibited first latency distributions that were three to six times slower at every potential than the cardiac first latencies.

DISCUSSION

Comparison of the Unitary Properties of Slowly and Rapidly Activating L-Channels

We have used the cell-attached patch-clamp technique to compare directly the unitary properties of skeletal, cardiac, and chimeric L-channels expressed in dysgenic myotubes. These properties were evaluated under identical conditions in a muscle system in which the expressed channels participated in functional excitation-contraction coupling (either skeletal or cardiac) like that in the native tissue, allowing investigation of the molecular and unitary mechanisms that underlie differences in skeletal and cardiac L-channel activation. Our results demonstrate that, in the presence of Bay K 8644, open times are neither voltage dependent nor appreciably different between the slowly and rapidly activating channels. However, rate of activation and first latency are markedly different for the two classes of L-channels (Table II). Thus, CAC6 and SkC15 exhibit first latency distributions similar to L-channels found in normal myotubes, approximately five times slower than that attributable to CARD1. Moreover, the ensemble activation of each construct parallels that of the first latency distribution but is faster at all voltages tested.

TABLE II
Kinetic Properties of Slowly and Rapidly Activating L-Channels

Construct	Activation (0 mV)	Latency (0 mV)
	ms	ms
Normal	27.4 \pm 2.6 (5)	147.9 \pm 30.9 (4)
CAC6	26.2 \pm 6.1 (5)	87.5 \pm 8.9 (6)
SkC15	17.2 \pm 1.4* (4)	67.4 \pm 17.1 (4)
CARD1	3.7 \pm 0.5† (10)	17.7 \pm 2.7* (6)

Data are for cell-attached patches with \leq 4 channels. Activation of ensemble currents was fitted according to $I(t) = I_{\infty} (1 - \exp(-t/\tau_{act}))$, where $I(t)$ is the current at time t after the depolarization, I_{∞} is steady-state current, and τ_{act} is the time constant of activation. First latency distributions were constructed as described in Methods and fitted according to $F(t) = A_0 + (A_1) \exp(-t/\tau_f)$. The numbers of experiments are shown in parentheses. Significance at * $P < 0.05$ or † $P < 0.001$ relative to normal myotubes using a two-sample t test (two-tail) assuming unequal variances.

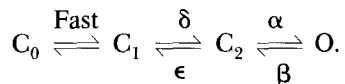
The present results support earlier work showing that, in the presence of DHP agonists, both slowly (Ma et al., 1991; Caffrey, 1994; Dirksen and Beam, 1995) and rapidly activating (Hoshi and Smith, 1987) L-channels exhibit two different open times. The existence of two open times implies the presence of two distinct open states, as has been suggested in the model of interconverting modes of L-channel gating (Hess et al., 1984). Alternatively, there could be a longer-lived open state reached only via the brief-lived open state. In any event, our experiments demonstrate that the long openings are rare for both slowly and rapidly activating L-channels in the absence of Bay K 8644.

Since open durations were measured only for adjacent opening and closing transitions between closed and single open states, long duration openings would tend to be underrepresented. This would be particularly true for patches exposed to Bay K 8644, since long duration events would have a higher probability of being interrupted by additional channel openings than would brief duration events. Further tending to cause an underestimate of long duration events was the exclusion from analysis of openings that persisted to the end of the test depolarization. Of course, brief openings are also likely to have been underrepresented to the extent that they were too brief to be resolved. In the absence of Bay K 8644, it seems likely that the predominant effect was an underrepresentation of brief duration events (since many apparent events failed to satisfy the 50% threshold crossing criterion). Particularly for the data obtained without DHP agonist, studies using fluctuation (Neher and Stevens, 1977), beta distribution (Yellen, 1984), or mean-variance (Patlak, 1993) analy-

ses would help to limit biases introduced by filtration and the use of a threshold crossing criterion.

Skeletal L-Channel Activation Appears to Involve a Slow Closed-to-Closed Transition

Although there is insufficient information to produce a well-constrained model to account for the difference in activation of skeletal- and cardiac-like L-channels, we can consider the predictions of the simple, sequential scheme given below:



In the above model, fast closed–closed transitions (here shown by a single $C_0 \rightleftharpoons C_1$ transition) produce the gating current (charge movement) that precedes channel opening (García et al., 1994). Because it has only a single open state, the model cannot account for the two distinct open times that are prominent when Bay K 8644 is present. However, the model could be applicable to the predominant gating behavior of L-channels in the absence of Bay K 8644 since the vast majority of openings are then brief, yet the difference between skeletal and cardiac activation persists. We wish first to consider the importance of the $C_2 \rightleftharpoons O$ transition for this difference in activation. In the absence of agonist, virtually all open events are brief (~ 1 ms), implying that $\beta \approx 1.0 \text{ ms}^{-1}$. Accordingly, if the $C_2 \rightleftharpoons O$ transition were rate limiting, then $\tau_{\text{act}} \approx (\alpha + \beta)^{-1}$ would be ≤ 1 ms, which argues that this transition is not rate limiting for skeletal activation (where $\tau_{\text{act}} > 20$ ms). By similar reasoning, as long as there is only a single transition into and out of the open state, this transition could not be rate limiting even for models that permit nonsequential paths for activation.

An alternative is to suppose that slow activation of skeletal L-channels is a consequence of the forward and reverse rates being slow for an obligatory, closed–closed transition before channel opening (in the model: $C_1 \rightleftharpoons C_2$). The forward rate for this transition (i.e., δ in the model) cannot be strongly voltage dependent. Otherwise, the differences in latency and activation rate between skeletal and cardiac L-channels would disappear with very strong depolarization. However, skeletal activation remains slow even at +60 mV (Dirksen and Beam, 1995). Although both the forward (δ) and reverse (ϵ) rates of the rate-limiting transition in skeletal L-channels must be small at potentials causing activation of current, the reverse rate must be large at negative potentials since skeletal L-channels deactivate rapidly upon repolarization (Figs. 1 and 4). Therefore, ϵ must have a strong voltage dependence to be slow at positive and fast at negative voltages. In summary, if channel closing reverses the path of channel

activation, then slow activation, slow first latency, and rapid deactivation of skeletal L-channels can be accounted for if the forward and reverse rates of the rate-limiting transition have an asymmetric voltage dependence such that both rates are slow at depolarized potentials and the reverse rate is rapid at negative potentials. Transitions having strongly asymmetric voltage dependence have been previously proposed for *Shaker* K^+ channel gating (Zagotta et al., 1994).

Skeletal L-Channels Exhibit a Slower Latency to First Opening than Cardiac L-Channels

Although open times are quite similar, the first latency is much slower for skeletal-like channels than cardiac-like channels. Moreover, for both types of channels, the first latency is slower than ensemble activation. First latency distributions that are slower than ensemble activation, particularly at negative voltages, have been observed previously in A7r5 smooth muscle cells (Marks and Jones, 1992), differentiated BC3H1 myocytes (Cafrey, 1994), guinea pig ventricular myocytes (Hess et al., 1984), and bovine adrenal chromaffin cells (Hoshi and Smith, 1987).

For both skeletal-like and cardiac-like L-channels, the first latency is strongly voltage dependent. At voltages that produce only partial activation, a long first latency would occur because the forward/reverse rates connecting closed states are similar enough that the channel can recycle through a set of closed states before opening. For stronger depolarizations, where one would expect only closed states near the open state to be occupied, the first latency approximates the sum of the inverse of the forward rate constants leading from the resting state to the open state. One of these forward rates must be relatively small in skeletal muscle to account for the slow first latency; moreover, activation would also be slow if the rate of the corresponding reverse transition is also small. As argued above, it seems unlikely that the transition with small forward and reverse rates is that from the final closed state to the open state. Thus, it seems attractive to assign the difference in first latency to an earlier transition (e.g., the $C_1 \rightleftharpoons C_2$ transition in the model above) that is fast for cardiac L-channels and slow for skeletal L-channels.

Measurements of closed times for the cardiac-like L-channels revealed both a brief dwell time (with undeterminable voltage dependence due to its brevity) and a longer-lived, voltage-dependent dwell time. The brief dwell time could represent the rate of exit from either the final closed state into the open state (α in the model above) or from a blocked state that is not traversed during activation (Zagotta et al., 1994). If such a blocked state exists, it would obviously alter the interpretation given to open times. If the longer closed time reflects a transition that occurs during activation, then

it can account for much of the measured first latency. It is this step that we argue to be different between skeletal and cardiac L-channels.

If one were able to make measurements of closed times for skeletal L-channels in native muscle, it would help to test the hypothesis that the final closed–open transition is not the one primarily responsible for the slow activation of skeletal-like L-channels. Unfortunately, out of the ~600 patches obtained on myotubes expressing skeletal L-channels, about half displayed no channels and the rest displayed multiple channels. None of these patches exhibited only a single channel as required for meaningful closed time analysis. The difficulty of finding patches containing only a single skeletal-like channel may be a consequence of the unique morphological arrangement of skeletal L-channels. Specifically, arrays of junctional tetrads (Block et al., 1988), intramembrane particles which appear to be DHPRs arranged in groups of four, are present in the sarcolemma of skeletal myotubes (Takekura et al., 1994) but not in cardiac muscle (Sun et al., 1995). An

individual tetrad fits within an ~30-nm square, and neighboring tetrads are separated by 40–50 nm. This structural arrangement may account for the difficulty in obtaining a single skeletal L-channel in cell-attached patches of cultured myotubes.

Above, it was argued that a slow closed–closed transition with an asymmetric voltage dependence can explain slow activation of skeletal-like L-channels. In voltage-gated ion channels, activation is thought to be driven by the redistribution within the membrane of charged residues within the S4 segments. Perhaps, a slow voltage-dependent conformational change of an S4 segment explains slow activation of skeletal L-channels. Nakai et al. (1994) demonstrated that, when IS3 and the IS3-IS4 linker have skeletal sequence in an otherwise cardiac L-channel, activation is slow. Thus, it is tempting to speculate that the rate of a voltage-dependent closed–closed transition is controlled by a physicochemical interaction of IS4 with the adjacent IS3 segment and the IS3-IS4 linker.

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