Potentiation of the Cardiac L-Type Ca²⁺ Channel (α₁C) by Dihydropyridine Agonist and Strong Depolarization Occur via Distinct Mechanisms

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Abstract A defining property of L-type Ca²⁺ channels is their potentiation by both 1,4-dihydropyridine agonists and strong depolarization. In contrast, non–L-type channels are potentiated by neither agonist nor depolarization, suggesting that these two processes may be linked. In this study, we have tested whether the mechanisms of agonist- and depolarization-induced potentiation in the cardiac L-type channel (α₁C) are linked. We found that the mutant L-type channel GFP-α₁C(TQ→YM), bearing the mutations T1066Y and Q1070M, was able to undergo depolarization-induced potentiation but not potentiation by agonist. Conversely, the chimeric channel GFP-CACC was potentiated by agonist but not by strong depolarization. These data indicate that the mechanisms of agonist- and depolarization-induced potentiation of α₁C are distinct. Since neither GFP-CACC nor GFP-CCAA was potentiated significantly by depolarization, no single repeat of α₁C appears to be responsible for depolarization-induced potentiation. Surprisingly, GFP-CACC displayed a low estimated open probability similar to that of the α₁C, but could not support depolarization-induced potentiation, demonstrating that a relatively low open probability alone is not sufficient for depolarization-induced potentiation to occur. Thus, depolarization-induced potentiation may be a global channel property requiring participation from all four homologous repeats.

Key words: ion channel modulation • facilitation • muscle

Introduction Voltage-gated Ca²⁺ channels respond to membrane depolarization by allowing Ca²⁺ into the cell, and, thus, mediate a variety of cellular responses in neurons and muscle, including transmitter release, neurite outgrowth, altered gene expression, exocytosis, and muscle contraction (Reuter, 1983; Hoshi and Smith, 1987; Tsien et al., 1988; Tanabe et al., 1993). Thus, modification of Ca²⁺ influx provides an important mechanism for the regulation of many downstream Ca²⁺-dependent responses. One source of modification is potentiation, whereby a channel is stabilized in the open state, and intracellular Ca²⁺ levels are increased as a result.

L-type Ca²⁺ channels show a shift in gating mode in response to either strong depolarization or a 1,4-dihydropyridine (DHP)* agonist. After strong depolarization, the channel enters a state of higher open probability (Pₒ) and long open times that can be detected by a number of different pulse paradigms. For example, after a strong, conditioning depolarization (e.g., +120 mV) followed by a 50–150-ms return to the holding potential, a subsequent, moderate depolarization elicits a Ca²⁺ channel current that is about twofold larger than that measured without the conditioning depolarization (Bourinet et al., 1994; Cens et al., 1996, 1998). This effect, which implies an alteration of gating that persists after channel closing, will be designated “depolarization-induced facilitation.” Both L- and non–L-type channels are also able to undergo another form of prepulse facilitation (“Ca²⁺/CAM-dependent facilitation”; Lee et al., 2000; Zühlke et al., 2000; DeMaria et al., 2001), which differs from depolarization-induced facilitation in that it has a bell-shaped dependence on the prepulse potential that arises from a primary dependence upon Ca²⁺ entry. Ca²⁺/CAM-dependent facilitation appears to depend specifically (Lee et al., 2000) upon the β₂a subunit, and does not occur in cells expressing β₁, the isoform present in the dysgenic myotubes used in our study. Unlike Ca²⁺/CAM-dependent facilitation, depolarization-induced facilitation may be related to another form of altered gating observed when a strong depolarization is followed immediately by repolarization to an intermediate potential (Hoshi and Smith, 1987; Pietrobon and Hess, 1990; Kleppisch et al., 1994). This phenomenon, referred to here as de-
polarization-induced potentiation, results in a mode of gating characterized at the single-channel level by high $P_o$ and long open times, which is also referred to as “mode 2” gating (Pietrobon and Hess, 1990). As an indication of depolarization-induced entry into mode 2, we have measured whole-cell tail currents upon repolarization to −50 mV immediately after a strong, conditioning depolarization. With this protocol, high $P_o$ and long open times are reflected by an increased tail-current amplitude and slower rate of tail-current decay, respectively. Mode 2 gating of L-type channels is also promoted by DHP agonists (Hess et al., 1984; Nowycky et al., 1985; Hoshi and Smith, 1987). Given the similarities between agonist- and depolarization-induced potentiation, it is important to know the degree to which the two processes are related.

The cardiac L-type channel, $\alpha_{IC}$, normally exhibits a low $P_o < 0.05$ (Cachelin et al., 1983; Lew et al., 1991) and can be potentiated by both strong depolarization and DHP agonists. By contrast, the neuronal non-L-type channel, $\alpha_{IA}$, exhibits a high $P_o$ of ~0.6 (Llinas et al., 1989) and lacks both depolarization-induced facilitation (Bourinet et al., 1994), and DHP-induced potentiation (Sather et al., 1993). These observations suggest the possibility that depolarization- and agonist-induced potentiation can only occur in channels like $\alpha_{IC}$ that have an intrinsically low $P_o$.

In an attempt to determine whether potentiation of $\alpha_{IC}$ by DHP agonist and strong depolarization occurs via a common pathway, we have characterized wild-type and mutant $\alpha_{IC}$ channels and chimeric channels composed of $\alpha_{IC}$ and $\alpha_{IA}$ sequence. The channels were fused at their amino termini to green fluorescent protein (GFP), expressed in dysgenic myotubes and examined using whole-cell patch clamp. For an $\alpha_{IC}$ in which the agonist binding site was mutated (GFP-$\alpha_{IC}(TQ\rightarrow YM)$), 10 μM Bay K 8644 had no significant effect on whole-cell currents, whereas depolarization-induced potentiation remained intact. A chimeric channel containing repeat II and the I-II linker of $\alpha_{IA}$ sequence embedded in L-type background (GFP-CACC) could not support depolarization-induced potentiation but was potentiated by DHP agonist. Channels containing three (CACC), two (CCAA), or no ($\alpha_{IA}$) repeats of L-type sequence were not potentiated significantly by depolarization, suggesting that depolarization-induced potentiation cannot be localized to any single channel repeat. Interestingly, despite having a relatively low estimated $P_o$ comparable to that of $\alpha_{IC}$, GFP-CACC was not potentiated by depolarization, indicating that depolarization-induced potentiation must not be dependent solely on a low $P_o$. Our results demonstrate that the mechanisms of DHP agonist- and depolarization-induced potentiation of $\alpha_{IC}$ are distinct, and that depolarization-induced potentiation may be a global channel property requiring the participation of all four homology repeats.

**Materials and Methods**

**Construction of Chimeric and Mutant $\alpha_1$ cDNAs**

A and C denote sequence derived from $\alpha_{1C}$ (Mori et al., 1991) or $\alpha_{1A}$ (Mikami et al., 1989), respectively. An asterisk indicates a restriction site introduced by PCR. The wild-type clones GFP-$\alpha_{1C}$ and GFP-$\alpha_{1A}$ were produced by fusing the $\alpha_1$ subunit of either the cardiac L-type channel ($\alpha_{IC}$) or neuronal P/Q-type channel ($\alpha_{IA}$) at the amino terminus to GFP as previously described (Grabner et al., 1998). The GFP tag has been shown not to alter any of the functional properties of the $\alpha_{1C}$ and $\alpha_{1A}$ subunits (Grabner et al., 1998). The clone GFP-$\alpha_{1C}(TQ\rightarrow YM)$ was created using overlapping PCR mutagenesis (Horton et al., 1992) to replace two residues in the IIIS5 transmembrane domain of $\alpha_{IC}$ (Thr 1066 and Glu 1070), which were previously identified as essential components of the DHP binding site (Mitterdorfer et al., 1989; Nowycky et al., 1985; Hoshi and Smith, 1987). Given the similarities between agonist- and depolarization-induced potentiation, it is important to know the degree to which the two processes are related.

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Expression and Electrophysiological Analysis of Channels in Dysgenic Myotubes

1 wk after plating, primary cultures of mouse dysgenic myotubes (Adams and Beam, 1989), which lack an endogenous α1 subunit (Knudson et al., 1989), were microinjected in single nuclei with cDNAs (200–600 ng/μl) encoding GFP-tagged α1 subunits. 36–52 h after injection, expressing myotubes were identified by green fluorescence and used for electrophysiology. Macroscopic Ca\(^{2+}\) currents were measured using the whole-cell patch-clamp method (Hamill et al., 1981). Whole-cell patch pipettes of borosilicate glass had resistances of 1.5–2.0 MΩ when filled with an internal solution containing 140 mM cesium aspartate, 10 mM Cs\(_2\)EGTA, 5 mM MgCl\(_2\), and 10 mM HEPES, pH 7.4 with CsOH. The external bath solution contained 10 mM CaCl\(_2\), 145 mM TEA-Cl, and 10 mM HEPES, pH 7.4 with TEA-OH, plus 3 μM tetradotoxin. Test currents were obtained by stepping from a holding potential of −80 to −30 mV for 1 s (to inactivate endogenous T-type Ca\(^{2+}\) current; Adams et al., 1990), to −50 mV for 30–50 ms, to the test potential for 200 ms, to −50 mV for 125 ms, and back to −80 mV. Test currents were corrected for linear components of leak and capacitative currents by digitally scaling and subtracting the average of 10 preceding control currents elicited by hyperpolarizing steps (20–40 mV in amplitude) applied from

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**Figure 1.** Activation of currents produced by GFP-α1C or GFP-α1A expressed in dysgenic myotubes. (A) Representative Ca\(^{2+}\) currents elicited by 200-ms depolarizing steps to the indicated test potentials from a holding potential of −80 mV, followed by repolarization to −50 mV. Putative membrane topology for each GFP-tagged channel construct is indicated, where dark gray and light gray represent α1C and α1A sequence, respectively. (B) Average (± SEM) conductance versus voltage relationships for GFP-α1C (circles; n = 9) and GFP-α1A (inverted triangles; n = 10). The smooth curves represent best fits of the expression 1/[1 + exp (−(V − \(V_{1/2}\))/\(k_G\))], which yielded the following values: for GFP-α1C, \(V_{1/2}\) = 8 mV, \(k_G\) = 7.3 mV; and for GFP-α1A, \(V_{1/2}\) = 19 mV, \(k_G\) = 5.1 mV.
Maximum channel $P_o$ was calculated from the average, measured corded with fast sampling (10 kHz). Tail-current amplitude ($I_{\text{tail}}$) was measured with a single exponential function. Maximal Ca$^{2+}$ was measured by fitting tail currents with a single exponential function. The activation potential ($V_{1/2}$) was calculated by fitting peak inward current and compensated series resistance.) was measured, after the addition of 0.5 mM Cd$^{2+}$ and 0.1 mM La$^{3+}$ to the bath, by integration of $Q$ on for a 15-ms test pulse (except for tail currents, data were sampled at 1 kHz. Tail currents, elicited by repolarizing to $-50$ mV for 125 ms, were recorded with fast sampling (10 kHz). Tail-current amplitude ($I_{\text{tail}}$) was measured 0.5 ms after the onset of the repolarization from the test pulse to $-50$ mV. The rate of tail-current decay ($\tau_{\text{deact}}$) was measured by fitting tail currents with a single exponential function. Maximal Ca$^{2+}$ conductance ($G_{\text{max}}$) and half-maximal activation potential ($V_{1/2}$) were calculated by fitting peak inward current values with the equation:

$$I = G_{\text{max}} \cdot (V - V_{\text{rev}})/(1 + \exp[-(V - V_{1/2})/k_c]),$$  \hspace{1cm} (1)$$

where $I$ is the peak inward Ca$^{2+}$ current measured at the test potential ($V$), $V_{\text{rev}}$ is the reversal potential, and $k_c$ is a slope factor. The values of $G_{\text{max}}$ and $V_{\text{rev}}$ were used to calculate normalized conductance as a function of voltage (see Figs. 1 A and 4 C) according to the equation: $G(V) = I/[G_{\text{max}} \cdot (V - V_{\text{rev}})]$.

Maximum immobilization-resistant charge movement ($Q_{\text{max}}$) was measured, after the addition of 0.5 mM Cd$^{2+}$ and 0.1 mM La$^{3+}$ to the bath, by integration of $Q$ for a 15-ms test pulse (exponentially rounded with a time constant of 100 $\mu$s) $+ 40$ mV. Maximum channel $P_a$ was calculated from the average, measured values of $G_{\text{max}}$ and $Q_{\text{max}}$ according to the equation:

$$P_a = (q \cdot G_{\text{max}} \cdot F)/(\gamma \cdot Q_{\text{max}} \cdot A),$$  \hspace{1cm} (2)$$

where $Q_{\text{max}} = Q_{\text{max}}^\text{e}$ - average dysgenic charge (2.5 nC/µF; Adams et al., 1990), $q$ is the single-channel conductance in 10 mM Ca$^{2+}$, assumed to be 4 pS for $\alpha_{1A}$ (Adams et al., 1994), 5.8 pS for $\alpha_{1C}$ (Gollasch et al., 1992), or an average of the two (4.9 pS) for chimeras CACC and CCAA. $q$ is the assumed single-channel gating charge (9 e$^-$; Noceti et al., 1996), $F$ is Faraday’s constant (96,487 C/mol) and $A$ is Avogadro’s number (6.023 $\times 10^{23}$ e$^-$/mol).

Several different measures were used to quantify potentiation. For the DHP agonist (±) Bay K 8644, one measure was the ratio $I_{\text{tail}}^{\text{drug}}/I_{\text{tail}}^{\text{control}}$, where the numerator and denominator represent the peak currents elicited by depolarizing test pulses in the presence or absence of drug, respectively. $I_{\text{tail}}^{\text{drug}}$ was usually elicited by a $V_{\text{test}}$ of approximately $+20$–$30$ mV, and $I_{\text{tail}}^{\text{control}}$ for a $V_{\text{test}}$ of $+20$–$30$ mV more hyperpolarized. Agonist-induced potentiation was also measured by means of the ratios $I_{\text{tail}}^{\text{drug}}/I_{\text{tail}}^{\text{control}}$ and $\tau_{\text{deact}}^{\text{drug}}/\tau_{\text{deact}}^{\text{control}}$, where the tail current was produced by repolarizing to $-50$ mV from a $V_{\text{test}}$ of $+40$ mV. Depolarization-induced potentiation was quantified by the ratios $I_{\text{tail}}^{\text{drug}}/I_{\text{tail}}^{+40}$ and $\tau_{\text{deact}}^{\text{drug}}/\tau_{\text{deact}}^{+40}$, where the numerator and denominator were determined from tail currents produced after repolarization to $-50$ mV after a $V_{\text{test}}$ of $+90$–$110$ mV or +40 mV, respectively.

**Statistical Analysis**

Statistical significance was assessed using one-way analysis of variance (ANOVA) and SAS software (version 8). All data are presented as mean ± SEM.

**RESULTS**

$\alpha_{1C}$ Is Potentiated by DHP Agonist and Strong Depolarization while $\alpha_{1A}$ Is Potentiated by Neither

Fig. 1 A illustrates representative whole-cell Ca$^{2+}$ currents elicited by depolarizing dysgenic myotubes expressing either GFP-$\alpha_{1C}$ or GFP-$\alpha_{1A}$ to the indicated potentials, followed by repolarization to $-50$ mV. Based upon steady-state activation calculated from peak currents during the test depolarizations (Fig. 1 B), both channels were fully activated by test pulses to +40 mV.
and above. Consistent with this, the tail currents for GFP-α1C had a similar amplitude and time course after the depolarizations to either +40 or +60 mV (Fig. 1 A). However, for GFP-α1C, the tail current after the +60-mV depolarization was larger and decayed more slowly than the tail after the +40-mV step. This behavior is an indication that strong depolarization caused α1C channels to enter a mode of gating having longer open times and increased P_o.

Fig. 2 compares currents produced by GFP-α1C and GFP-α1A before and after exposure to 10 μM Bay K 8644. For GFP-α1A, application of Bay K 8644 had little effect on either the current elicited by a test depolarization to +20 mV or on the tail current after repolarization (Fig. 2 A). Likewise, the average, peak current versus voltage relationship for GFP-α1A was not significantly (P > 0.1) affected by the agonist (Fig. 2 B). By contrast, Bay K 8644 caused a hyperpolarizing shift in the test potential evoking maximum inward current for GFP-α1C, together with a substantial increase in the magnitude of this current. In addition to affecting the peak current, Bay K 8644 also caused an approximately threefold increase in tail-current amplitude (I_tail) and in the time constant of tail-current deactivation (τ_deact) for GFP-α1C (Fig. 2 A and Table I). Overall, the effects of Bay K 8644 on GFP-α1C tail currents qualitatively resemble those of strong depolarization (compare Figs. 1 A and 2 A).

Fig. 3 illustrates a more detailed characterization of tail currents in cells expressing GFP-α1C or GFP-α1A. Fig. 3 A shows the standard protocol for quantifying depolarization-induced potentiation, which was determined as the ratio of either I_tail or τ_deact for a tail current after a V_test of +90 mV, to the corresponding values for a tail current after a V_test of +40 mV. By both measures, GFP-α1C showed substantial depolarization-induced potentiation, whereas GFP-α1A did not (Fig. 3 A and Table I). Fig. 3 B illustrates the dependence of I_tail on prior test potentials ranging from −40 to +80 mV. For GFP-α1A, I_tail reached a maximum after a V_test of +30 mV, in good agreement with the conductance versus voltage curve calculated from peak currents (Fig. 1 B). For still stronger depolarizations, I_tail became smaller for GFP-α1A, as expected for a channel undergoing voltage-dependent inactivation that becomes faster with stronger depolarization. In contrast to GFP-α1A, I_tail for GFP-α1C increased monotonically over the entire range of test potentials. This monotonic increase differs from the saturating conductance versus voltage relationship (Fig. 1 B) and is consistent with entry into a potentiated state having high P_o. This monotonic voltage dependence also suggests that depolarization-induced potentiation is not dependent upon Ca^{2+} entry during the prepulse. The application of 10 μM Bay K 8644 caused a still further increase in P_o, which is indicated by a substantial increase in I_tail for GFP-α1C at any given test potential (Fig. 3 B). In the presence of agonist, I_tail was still increased by stronger test depolarizations, up to at least +70 mV. Table I summarizes the effects of DHP agonist and strong depolarization on tail currents for GFP-α1C and GFP-α1A.

**Mutation of T1066 and Q1070 of α1C Eliminates Agonist- but Not Depolarization-induced Potentiation**

The observation that GFP-α1C is potentiated by both agonist and strong depolarization, whereas GFP-α1A is potentiated by neither, raises the possibility that agonist- and depolarization-induced potentiation are linked. As one test of this hypothesis, we created GFP-
500 Distinct Mechanisms of Potentiation

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\alpha_{1C}(TQ\rightarrow YM), \text{ in which two residues of IIIS5 that are critical for the DHP sensitivity of } \alpha_{1C} \text{ (Mitterdorfer et al., 1996; He et al., 1997) were converted to the corresponding residues of } \alpha_{1A}. \text{ Currents produced by GFP-}\alpha_{1C}(TQ\rightarrow YM) \text{ were not affected by the addition of 10 } \mu\text{M Bay K 8644 (Fig. 4 A), whereas depolarization-induced potentiation was intact (Fig. 4 B). In particular, tail currents were larger and decayed more slowly after a } V_{\text{test}} \text{ of +90 mV compared with a } V_{\text{test}} \text{ of +40 mV (Fig. 4 B, top) and the tail-current amplitude increased monotonically as a function of test potential (Fig. 4 B, bottom). On average, GFP-}\alpha_{1C}(TQ\rightarrow YM) \text{ was quantitatively similar to GFP-}\alpha_{1C} \text{ with respect to depolarization-induced potentiation, but was indistinguishable from GFP-}\alpha_{1A} \text{ in the lack of agonist-induced potentiation (Table I). Interestingly, mutation of T1066 and Q1070 in the IIIS5 transmembrane segment of } \alpha_{1C} \text{ resulted in a decreased steepness, and positive shift, of the steady-state activation curve in comparison to GFP-}\alpha_{1C} \text{ (Fig. 4 C), indicating that these residues can affect activation gating. Taken together, the data of Fig. 4 demonstrate that depolarization-induced potentiation still occurs in a mutant } \alpha_{1C} \text{ lacking a response to DHP agonist.}

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\[\text{Agonist-induced Potentiation Persists in the Absence of Depolarization-induced Potentiation}\]

In an attempt to determine whether a single repeat of } \alpha_{1C} \text{ is sufficient to allow depolarization-induced potentiation, we constructed the chimeras GFP-CACC and GFP-CCAA and tested them for agonist- and depolarization-induced potentiation (Fig. 5). GFP-CACC consists of repeat II and the I-II linker of } \alpha_{1A} \text{ in an otherwise } \alpha_{1C} \text{ background (Fig. 5, A and C), and thus contains an intact DHP agonist binding site (Grabner et al., 1996; Hockerman et al., 1997; Ito et al., 1997; Sindegger et al., 1997). As shown in Fig. 5 A, 10 } \mu\text{M Bay K 8644 potentiated maximum inward current in cells expressing GFP-CACC and caused a leftward shift in the peak current versus voltage relationship. Quantitatively, both effects were similar to those of 10 } \mu\text{M Bay K 8644 on GFP-}\alpha_{1C} \text{ (Table I). However, this chimera failed to show the large depolarization-induced potentiation characteristic of either GFP-}\alpha_{1C} \text{ or GFP-}\alpha_{1C}(TQ\rightarrow YM) \text{ (Table I; also, compare Fig. 5 C with Figs. 3 B and 4 B). Thus agonist-induced potentiation can be present in a channel construct that lacks significant depolarization-induced potentiation. The chimera GFP-CCAA (Fig. 5, B and D) consists of the first two repeats and the II-III} \]

![Diagram](image)
linker of $\alpha_{1C}$ fused to repeats III and IV of $\alpha_{1A}$. GFP-CCAA lacked both agonist- and depolarization-induced potentiation (Fig. 5, B and D, and Table 1). Fig. 6 summarizes the effects of strong depolarization and DHP agonist on the constructs GFP-$\alpha_{1C}$, GFP-$\alpha_{1C}(TQ\rightarrowYM)$, GFP-CACC, and GFP-$\alpha_{1A}$. The asterisks indicate a significant ($P < 0.05$) difference from onefold (where onefold indicates a lack of potentiation). Fig. 6 demonstrates that depolarization-induced potentiation can persist in the absence of potentiation by agonist (i.e.,
GFP-αIC(TQ→YM), and potentiation by agonist can occur in the absence of depolarization-induced potentiation (i.e., GFP-CACC); therefore, the two processes likely occur via distinct mechanisms.

No Single Repeat of αIC Is Sufficient for Depolarization-induced Potentiation

Fig. 7 shows that in terms of both amplitude of tail currents (A) and \( \tau_{\text{decay}} \) (B), the chimeras GFP-CACC and GFP-CCAA, like α1A, lacked depolarization-induced potentiation. Because GFP-CACC lacked depolarization-induced potentiation, none of the three cardiac repeats contained in this construct (i.e., I, III, and IV) appears to be sufficient, individually or in concert, to mediate this process. In addition, because GFP-CCAA contains a cardiac repeat II and lacked depolarization-induced potentiation, a cardiac repeat II does not appear to be sufficient, either alone or in combination with repeat I. In conclusion, no single repeat of αIC seems to be sufficient for depolarization-induced potentiation, which may instead represent a more global property. Several combinations of multiple repeats are
expressed in dysgenic myotubes. The dashed lines at onefold indicate no potentiation. Asterisks indicate a significant difference (P < 0.05) from 1. The number of cells tested in each group ranged from five to eight.

DI S C U S S I O N
In the present study, we have examined DHP- and depolarization-induced potentiation of L-type Ca\(^{2+}\) channels by expressing GFP-tagged cardiac (α_1C) and neuronal (α_1A) \(\alpha_1\) subunits in dysgenic myotubes. For GFP-α_1C, both strong depolarization and agonist (10 μM Bay K 8644) caused tail currents to become larger and to decay more slowly, whereas tail currents for GFP-α_1A were not affected by either manipulation. Introduction of two point mutations (T1066Y and Q1070M) into GFP-α_1C abolished potentiation by agonist without any evident effect on potentiation by depolarization. Conversely, agonist but not depolarization caused potentiation of a chimera of α_1C and α_1A (GFP-CACC). Because depolarization-induced potentiation was absent for both GFP-CCCA and the chimera GFP-CCAA, it appears that no single repeat of α_1C can be responsible for this process. GFP-CACC displayed a relatively low estimated P_0, quite similar to that of GFP-α_1A, whereas the estimated P_0 for both GFP-CCCA and GFP-α_1A was much higher. Therefore, a channel that displays a low P_0 (and is potentiated by agonist) can fail to be potentiated by depolarization.

Independent Pathways for Potentiation by DHP
Agnost and Depolarization
Unitary records of L-type Ca\(^{2+}\) channels have been described as having three modes of gating upon depolarization: mode 0 (null sweeps); mode 1 characterized by brief openings (<1 ms) in bursts; and mode 2 defined by longer openings and high P_0 (Hess et al., 1984). Mode 1 is the predominant mode accessed during moderate depolarizations from the holding potential in the absence of DHP agonist, whereas mode 2 is promoted by the presence of agonist (Hess et al., 1984). Strong depolarization also promotes long openings of L-type channels in both chromaffin (Hoshi and Smith, 1987) and cardiac cells (Pietrobon and Hess, 1990). Because we have found that potentiation by either agonist or depolarization can be eliminated without a quantitative reduction in the effect of the other, it appears that these two processes occur via distinct pathways. In addition, we have used a concentration of agonist (10 μM Bay K 8644) that is supramaximal (Kokubun and Reu-
Distinct Mechanisms of Potentiation

ter, 1984); therefore, the additional potentiation of tail currents by depolarization in the presence of the agonist also strongly suggests the presence of two independent pathways leading to a potentiated open state. Several other labs have likewise concluded from the additivity of the effects of depolarization and agonist, that these two stimuli cause an increased Po by distinct pathways (Bourinet et al., 1994; Parri and Lansman, 1996). Moreover, single-channel measurements show both different open times and first latencies depending on whether potentiation is induced by depolarization or agonist (Hoshi and Smith, 1987). In combination, these data suggest not only that mode 2 gating can be accessed by multiple pathways, but also that mode 2 consists of more than one potentiated open state.

Bay K 8644 is well-known to shift activation in the hyperpolarizing direction (Fig. 2; Hess et al., 1984; Sanguinetti et al., 1986), indicating that it shifts equilibrium towards the open state of the channel. We have shown here that mutation of residues T1066 and Q1070 in the IIIS5 transmembrane domain of α1C not only ablates the response to agonist, but also shifts the voltage dependence of activation oppositely, in the depolarizing direction. On this basis, one could hypothesize that Bay K 8644 promotes a conformation of these two resi-

### Table 11

| Units | GFP-α1C | GFP-CACC | GFP-CCAA | GFP-α1A |
|-------|---------|----------|----------|---------|
| I<sub>max</sub> pA/pF | 35.2 ± 4.1 (19) | 7.0 ± 1.1 (16) | 4.8 ± 1.1 (18) | 22.3 ± 4.8 (16) |
| V<sub>1/2</sub> mV | +6.0 ± 0.6 (10) | +7.6 ± 2.2 (16) | +15.3 ± 1.5 (17) | +18.1 ± 1.7 (12) |
| G<sub>max</sub> nS/nF | 434.6 ± 76.1 (15) | 118.5 ± 13.8 (16) | 105.0 ± 20.7 (18) | 352.0 ± 61.9 (16) |
| Q<sub>max</sub> nC/μF | 20.9 ± 2.7 (6) | 3.0 ± 0.5 (11) | 2.9 ± 0.4 (10) | 3.2 ± 0.3 (7) |
| Po | 0.007 (5) | 0.028 (6) | 0.144 (10) | 0.212 (7) |

I<sub>max</sub> is the peak inward current determined by measuring Ca<sup>2+</sup> currents elicited with 200-ms test depolarizations ranging from −40 to +100 mV. Values for half-maximal activation potential (V<sub>1/2</sub>) and maximal conductance (G<sub>max</sub>) were determined by fitting peak Ca<sup>2+</sup> currents according to Eq. 1. Maximum immobilization-resistant charge movement (Q<sub>max</sub>) was measured by integration of the “On” gating current for a 15-ms step to +40 mV. Open channel probability (Po) was estimated using Eq. 2 and measured values of G<sub>max</sub> and Q<sub>max</sub> (as described in MATERIALS and METHODS). All data are presented as mean ± SEM, with numbers in parentheses indicating the number of cells tested.

Figure 7. Depolarization-induced potentiation cannot be localized to any single channel repeat. Average depolarization-induced potentiation of tail-current amplitude (A), or tail-current deactivation (B) for the indicated constructs. Channel open probability (Po) for the same constructs (C) was estimated according to Eq. 2 (as described in MATERIALS and METHODS). Values for Po were as follows: α<sub>1C</sub> = 0.01, CACC = 0.03, CCAA = 0.14, and α<sub>1A</sub> = 0.21. The dashed lines at onefold indicate no potentiation. The number of cells tested in each group ranged from five to eight. Asterisks indicate a significant difference (P < 0.05) from 1.
dues that stabilizes open states of the channel, and mutation of these residues destabilizes this conformation.

**Role of Accessory Subunits and of Phosphorylation in Depolarization-induced Potentiation**

The accessory β subunit has been shown to influence modal gating of αIC. In particular, comparison of αIC expressed with or without the β2a subunit showed that β2a increased both open times and the proportion of long openings (Costantin et al., 1998). β subunits also have been reported to affect depolarization-induced facilitation, which may be mechanistically related to depolarization-induced potentiation (Introduction). Specifically, depolarization-induced facilitation was found to occur when αIC was coexpressed with the β1, β3, or β4 subunits (Bourinet et al., 1994; Cens et al., 1998), but not with β2a (Cens et al., 1996), raising the possibility that the β subunit plays a direct role in depolarization-induced facilitation of αIC, and perhaps in potentiation as well. However, others have found that depolarization-induced potentiation of the smooth muscle α1C occurs in the absence of any β subunit (Kleppisch et al., 1994).

Whatever the exact role of the β subunit, our results demonstrate that depolarization-induced potentiation is strongly influenced by the α subunit itself, because all of the α1C constructs examined in this study have a conserved “alpha interaction domain” (site of β subunit binding; Pragnell et al., 1994) and were expressed with a common β subunit (β1a, which is endogenous to skeletal muscle; Ruth et al., 1989).

Evidence has been presented that PKA-dependent phosphorylation occurring during depolarizing pulses is necessary for depolarization-induced facilitation of α1S (Sculptoreanu et al., 1993b; Johnson et al., 1994), the cardiac α1C (Sculptoreanu et al., 1993a), and the neuronal α1C (Sculptoreanu et al., 1995). Evidence also has been presented that phosphorylation during depolarization is not involved in depolarization-induced facilitation of the neuronal α1C, although basal phosphorylation may be required (Bourinet et al., 1994). If phosphorylation is required (either basal or voltage-dependent), then it seems unlikely to involve phosphorylation of α1C directly because truncation of the consensus PKA sites (Gao et al., 1997) of the α1C carboxyl tail does not eliminate depolarization-induced facilitation (Cens et al., 1998). Consistent with this result, we found that depolarization-induced potentiation does not occur for GFP-CACC even though it contains all the consensus PKA sites of α1C.

**Structural Determinants of Depolarization-induced Potentiation and Low Po**

As discussed above, brief openings predominate during activation of α1C by modest depolarizations applied from a negative holding potential (mode 1 gating). The conformational changes responsible for activation of these brief openings occur rapidly (macroscopic activation occurs with a time constant of several ms at +30 mV; Tanabe et al., 1991). Depolarization-induced entry into mode 2 occurs on a significantly slower time scale (with a time constant of several hundred ms at +30 mV) and over a much more positive voltage range (Pietrobon and Hess, 1990). Despite these differences, depolarization-induced potentiation resembles mode 1 activation in being strongly voltage-dependent: based on two-state Boltzmann fits, the effective gating charge is 2.5 for depolarization-induced potentiation and 3.2 for mode 1 activation (Pietrobon and Hess, 1990). Thus, the question arises as to the identity of the voltage sensor for depolarization-induced potentiation. One possibility is that, after undergoing the relatively rapid movements leading to mode 1 openings, the S4 segments can undergo subsequent, slower movements in response to still stronger depolarization. It is equally possible that structures other than S4 serve as voltage sensors for depolarization-induced potentiation. Because we found that neither GFP-CACC nor GFP-CCAA undergo depolarization-induced potentiation, it seems unlikely that the voltage-sensing structures for depolarization-induced potentiation are localized within a single repeat. Rather, depolarization-induced potentiation of α1C appears to require large movements of charge distributed throughout the protein.

L-type channels like α1S and α1C differ from α1A channels in that the L-type channels display agonist- and depolarization-induced potentiation, and also have a much lower Po, raising the possibility that the structural determinants of potentiation and low Po reside in similar structures. However, the chimera GFP-CACC had a relatively low Po, yet did not display significant depolarization-induced potentiation. Because the chimera GFP-CCAA displayed a high Po, the amino-terminal half of α1C (repeats I and II) does not appear to be an important determinant of low Po; instead, structural requirements for low Po may reside in the carboxyl half of the protein. Certainly, it is attractive to hypothesize that repeats III and IV are important for the intrinsic, low Po of L-type channels since these same two repeats play an essential role in agonist binding, which increases Po. A role for the carboxyl tail in determining Po is suggested by previous work showing that Po of α1C is markedly increased by partial truncation of the carboxyl tail (Wei et al., 1994).

As stated earlier, the Po of the L-type channels containing α1C (<0.05; Cachelin et al., 1983; Lew et al., 1991) is much lower than that of the neuronal channels containing α1A (0.6; Llinas et al., 1989) or α1B (0.5; Delcour and Tsien, 1993). Because single-channel conductance varies less than twofold amongst these channels ([α1C] Kokobun and Reuter, 1984; [α1A] Zhang et al., 1993; [α1B] Rittenhouse and Hess, 1994), the produc-
tion of an equivalent macroscopic current would require a much higher density of the L-type channels. A primary role of L-type Ca\(^{2+}\) channels in muscle is to regulate Ca\(^{2+}\) movements through ryanodine receptors. For this control to be relatively tight, it may be useful to have an \(\sim 1:1\) correspondence between the plasmalemmal L-type channels and the intracellular ryanodine receptors. Perhaps this correspondence is best served by a relatively high density of low Po channels. Conversely, a high Po and relatively low channel density would be advantageous when it is critical that a cellular response be triggered by the activation of only a few channels. Important goals for future work will be to better define the structures determining the differences in Po between \(\alpha_{1C}\) and neuronal channels like \(\alpha_{1A}\) and \(\alpha_{1B}\), and to identify the conformational rearrangements that occur during potentiation of L-type channels.

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REFERENCES

Adams, B.A., and K.G. Beam. 1989. A novel Ca\(^{2+}\) current in dysgenic skeletal muscle. J. Gen. Physiol. 94:429–444.

Adams, B.A., T. Tanabe, A. Mikami, S. Numa, and K.G. Beam. 1990. Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs. Nature. 346:569–572.

Adams, B.A., Y. Mori, M.S. Kim, T. Tanabe, and K.G. Beam. 1994. Heterologous expression of BI Ca\(^{2+}\) channels in dysgenic skeletal muscle. J. Gen. Physiol. 104:985–996.

Bourinet, E., P. Charnet, W.J. Tomlinson, A. Stea, T.P. Snutch, and T.P. Snutch. 1994. Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs. Nature. 346:569–572.

Adams, B.A., Y. Mori, M.S. Kim, T. Tanabe, and K.G. Beam. 1994. Heterologous expression of BI Ca\(^{2+}\) channels in dysgenic skeletal muscle. J. Gen. Physiol. 104:985–996.

Bourinet, E., P. Charnet, W.J. Tomlinson, A. Stea, T.P. Snutch, and J. Nargeot. 1994. Voltage-dependent facilitation of a neuronal \(\alpha_{1C}\)-type calcium channel. EMBO J. 13:5032–5039.

Cachelin, A.B., J.E. de Peyer, S. Kobukun, and H. Reuter. 1983. Ca\(^{2+}\) channel modulation by 8-bromocyclic AMP in cultured heart cells. Nature. 304:462–464.

Cens, T., M.E. Mangoni, S. Richard, J. Nargeot, and P. Charnet. 1996. Coexpression of the \(\beta_2\) subunit does not induce voltage-dependent facilitation of the class C L-type Ca\(^{2+}\) channel. Pflügers Arch. 431:771–774.

Cens, T., S. Restituito, A. Vallentin, and P. Charnet. 1998. Promotion and inhibition of L-type Ca\(^{2+}\) channel facilitation by distinct domains of the subunit. J. Biol. Chem. 273:18308–18315.

Costantin, J., F. Noceti, N. Qin, X. Wei, L. Birnbaumer, and E. Stefani. 1998. Facilitation by the \(\beta_2\) subunit of pore openings in cardiac Ca\(^{2+}\) channels. J. Physiol. 507:93–103.

Delcour, A.H., and R.W. Tsien. 1993. Altered prevalence of gating modes in neurotransmitter inhibition of N-type Ca\(^{2+}\) channels. Science. 259:980–984.

DeMaria, C.D., T.W. Soong, B.A. Alseikhan, R.S. Alvania, and D.T. Yue. 2001. Calmodulin bifurcates the local Ca\(^{2+}\) signal that modulates P/Q-type Ca\(^{2+}\) channels. Nature. 411:484–489.

Gao, T., A. Yatani, M.L. Dell’Acqua, H. Sako, S.A. Green, N. Dascal, J.D. Scott, and M.M. Hosey. 1997. cAMP-dependent regulation of cardiac L-type Ca\(^{2+}\) channels requires membrane targeting of PKA and phosphorylation of channel subunits. Neuron. 19:185–196.

Gollasch, M., J. Hescheler, J.M. Quayle, J.B. Patlak, and M.T. Nelson. 1992. Single Ca\(^{2+}\) channel currents of arterial smooth muscle at physiological Ca\(^{2+}\) concentrations. Am. J. Physiol. 263: C948–C952.

Grabner, M., Z. Wang, S. Hering, J. Stiessenig, and H. Glossmann. 1996. Transfer of 1,4-dihydropyridine sensitivity from L-type to class A (BI) Ca\(^{2+}\) channels. Neuron. 16:207–218.

Grabner, M., R.T. Dirksen, and K.G. Beam. 1998. Tagging with green fluorescent protein reveals a distinct subcellular distribution of L-type and non-L-type Ca\(^{2+}\) channels expressed in dysgenic myotubes. Proc. Natl. Acad. Sci. USA. 95:1903–1908.

Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch. 391:85–100.

He, M., I. Bodis, G. Mikala, and A. Schwartz. 1997. Motif III S3 of L-type Ca\(^{2+}\) channels is involved in the dihydropyridine binding site: A combined radioligand binding and electrophysiological study. J. Biol. Chem. 272:2629–2633.

Hess, P., J.B. Lansman, and R.W. Tsien. 1984. Different modes of Ca\(^{2+}\) channel gating behaviour favoured by dihydropyridine Ca\(^{2+}\) agonists and antagonists. Nature. 311:538–544.

Hockerman, G.H., B.D. Johnson, M.R. Abbot, T. Scheuer, and W.A. Catterall. 1997. Molecular determinants of high-affinity phenylalkylamine block of L-type Ca\(^{2+}\) channels in transmembrane segment H5 and the pore region of the \(\alpha_1\) subunit. J. Biol. Chem. 272:18759–18765.

Horton, R.M., H.D. Hunt, S.N. Ho, J.K. Pullen, and L.R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Genie. 77:61–68.

Hoshi, T., and S.J. Smith. 1987. Large depolarization induces long openings of voltage-dependent Ca\(^{2+}\) channels in adrenal chromaffin cells. J. Neurosci. 7:571–580.

Ito, H., N. Klugbauer, and F. Hofmann. 1997. Transfer of the high affinity dihydropyridine sensitivity from L-type to non-L-type Ca\(^{2+}\) channel. Mol. Pharmacol. 52:735–740.

Johnson, B.D., T. Scheuer, and W.A. Catterall. 1994. Voltage-dependent potentiation of L-type Ca\(^{2+}\) channels in skeletal muscle cells requires anchored cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA. 91:11492–11496.

Kleppisch, T., K. Pedersen, C. Strobing, E. Bosse-Donecve, V. Flockezi, F. Hofmann, and J. Hescheler. 1994. Double-pulse facilitation of smooth muscle \(\alpha_1\) subunit Ca\(^{2+}\) channels expressed in CHO cells. EMBO J. 13:2502–2507.

Knudson, C.M., N. Chaudhari, A.H. Sharp, J.A. Powell, K.G. Beam, and K.P. Campbell. 1989. Specific absence of the \(\alpha_1\) subunit of the dihydropyridine receptor in mice with muscular dysgenesis. J. Biol. Chem. 264:1345–1348.

Kobukun, S., and H. Reuter. 1984. Dihydropyridine derivatives prolong the open state of Ca\(^{2+}\) channels in cultured cardiac cells. Proc. Natl. Acad. Sci. USA. 81:4824–4827.

Lee, A., T. Scheuer, and W.A. Catterall. 2000. Ca\(^{2+}\)/calmodulin-dependent facilitation and inactivation of P/Q-type Ca\(^{2+}\) channels. J. Neurosci. 20:6830–6838.

Lee, W.Y., L.V. Hryshko, and D.M. Bers. 1991. Dihydropyridine receptors are primarily functional L-type Ca\(^{2+}\) channels in rabbit ventricular myocytes. Circ. Res. 69:1139–1145.

Llinas, R.R., M. Sugimori, and B. Cherksey. 1989. Voltage-dependent Ca\(^{2+}\) conductances in mammalian neurons. The P channel. Ann. NY Acad. Sci. 560:103–111.

Mikami, A., K. Imoto, T. Tanabe, T. Niidome, Y. Morii, H. Takeshima, S. Narumiya, and S. Numa. 1989. Primary structure...
