The term excitation-coupled Ca$^{2+}$ entry (ECCE) designates the entry of extracellular Ca$^{2+}$ into skeletal muscle cells, which occurs in response to prolonged depolarization or pulse trains and depends on the presence of both the 1,4-dihydropyridine receptor (DHPR) in the plasma membrane and the type 1 ryanodine receptor in the sarcoplasmic reticulum (SR) membrane. The ECCE pathway is blocked by pharmacological agents that also block store-operated Ca$^{2+}$ entry, is inhibited by dantrolene, is relatively insensitive to the DHP antagonist nifedipine (1 μM), and is permeable to Mn$^{2+}$. Here, we have examined the effects of these agents on the L-type Ca$^{2+}$ current conducted via the DHPR. We found that the nonspecific cation channel antagonists (2-APB, SKF 96356, La$^{3+}$, and Gd$^{3+}$) and dantrolene all inhibited the L-type Ca$^{2+}$ current. In addition, complete (>97%) block of the L-type current required concentrations of nifedipine >10 μM. Like ECCE, the L-type Ca$^{2+}$ channel displays permeability to Mn$^{2+}$ in the absence of external Ca$^{2+}$ and produces a Ca$^{2+}$ current that persists during prolonged (~10-second) depolarization. This current appears to contribute to the Ca$^{2+}$ transient observed during prolonged KCl depolarization of intact myotubes because (1) the transients in normal myotubes decayed more rapidly in the absence of external Ca$^{2+}$; (2) the transients in dysgenic myotubes expressing SkEIIIK (a DHPR α$_{1S}$ pore mutant thought to conduct only monovalent cations) had a time course like that of normal myotubes in Ca$^{2+}$-free solution and were unaffected by Ca$^{2+}$ removal; and (3) after block of SR Ca$^{2+}$ release by 200 μM ryanodine, normal myotubes still displayed a large Ca$^{2+}$ transient, whereas no transient was detectable in SkEIIIK-expressing dysgenic myotubes. Collectively, these results indicate that the skeletal muscle L-type channel is a major contributor to the Ca$^{2+}$ entry attributed to ECCE.

INTRODUCTION

The skeletal muscle L-type Ca$^{2+}$ channel (1,4-dihydropyridine receptor [DHPR]) serves as the voltage sensor for excitation–contraction (EC) coupling (Tanabe et al., 1988). Conformational changes in the DHPR in response to membrane depolarization are coupled to gating of the type 1 RYR (RYR1) of the SR (Beam and Horowicz, 2004). The resultant Ca$^{2+}$ influx via RYR1 into the myoplasm initiates contraction (Beam and Horowicz, 2004). It has also been reported that the interaction between the DHPR and RYR1 activates entry of extracellular Ca$^{2+}$ into the myoplasm via an as-yet-identified pathway (Cherednichenko et al., 2004, 2008; Hurne et al., 2005; Yang et al., 2007; Gach et al., 2008; Lyfenko and Dirksen, 2008). This form of Ca$^{2+}$ entry, termed excitation-coupled Ca$^{2+}$ entry (ECCE), requires expression of both the DHPR and RYR1, as ECCE is absent in dysgenic (DHPR α$_{1S}$ subunit–null) and dyspedic (RYR1-null) myotubes, respectively. Cherednichenko et al. (2004) proposed that ECCE is independent of L-type Ca$^{2+}$ current via the DHPR because ECCE (as assessed by Mn$^{2+}$ quench of the ratiometric Ca$^{2+}$ indicator Fura-2) persists in dysgenic myotubes transfected with an α$_{1S}$ pore mutant (SkEIIIK) thought to conduct only monovalent cations (Dirksen and Beam, 1999). Thus, it was proposed that ECCE involved an as-yet-identified channel activated by conformational coupling to both the DHPR and RYR1.

When myotubes are stimulated either by maintained depolarization with KCl or by repetitive, brief electrical stimuli, myoplasmic Ca$^{2+}$ levels initially rise and then decay as a consequence of declining release from the SR, although this decay is slowed by the entry of external Ca$^{2+}$ associated with ECCE (Yang et al., 2007; Cherednichenko et al., 2008; Gach et al., 2008). Furthermore, ECCE is accentuated in myotubes expressing RYR1 constructs that carry mutations causing malignant hyperthermia in humans (Yang et al., 2007; Cherednichenko et al., 2008). Thus, it appears that ECCE may be important in normal skeletal muscle for helping to maintain force generation during tetanic contraction.

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Abbreviations used in this paper: 2-APB, 2-aminoethoxy diphenylborate; DHPR, 1,4-dihydropyridine receptor; EC, excitation–contraction; ECCE, excitation-coupled Ca$^{2+}$ entry; SOCE, store-operated Ca$^{2+}$ entry.
stimulation, and that accentuated ECCE may contribute to the pathophysiological increase in myoplasmic Ca\(^{2+}\) that occurs during episodes of malignant hyperthermia.

ECCE is blocked by 2-aminoethyl diphenylborate (2-APB), SKF 96356, La\(^{3+}\), and Gd\(^{3+}\) (Cherednichenko et al., 2004; Hurne et al., 2005) and thus has a pharmacological profile similar to that of store-operated Ca\(^{2+}\) entry (SOCE) and transient receptor potential–like channels. However, ECCE can occur without any store depletion (Cherednichenko et al., 2004, 2008; Hurne et al., 2005; Yang et al., 2007; Gach et al., 2008; Lyfenko and Dirksen, 2008) or in cells in which stores are fully depleted (Cherednichenko et al., 2004; Gach et al., 2008). Furthermore, all attempts to identify transient receptor potential or SOCE channels as the molecular basis of ECCE have failed (Lee et al., 2006; Lyfenko and Dirksen, 2008; Woo et al., 2008).

While attempting to identify the Ca\(^{2+}\) current associated with ECCE, we noticed that 2-APB, SKF 96356, La\(^{3+}\), and Gd\(^{3+}\) each block the skeletal muscle L-type current, as has been reported for ECCE (Cherednichenko et al., 2004; Hurne et al., 2005; Yang et al., 2007). These results prompted us to reexamine the similarities and differences between the skeletal muscle L-type current and ECCE. We found that the L-type current is incompletely (~50%) blocked by 1 μM nifedipine and partially inhibited by the anti-malignant hyperthermia drug dantrolene, as described previously for ECCE (Yang et al., 2007; Cherednichenko et al., 2008). Also like ECCE (Cherednichenko et al., 2004, 2008; Hurne et al., 2005; Gach et al., 2008; Lyfenko and Dirksen, 2008), we found that the L-type channel can conduct Mn\(^{2+}\) in the absence of external Ca\(^{2+}\).

In addition to examining the pharmacology of the DHPR, we evaluated the contribution of the L-type current to myoplasmic Ca\(^{2+}\) transients. In normal myotubes, the Ca\(^{2+}\) transient decays during maintained depolarization due to inactivation of EC coupling (Beam and Horowicz, 2004). This decay is accelerated by the removal of external Ca\(^{2+}\) or by the addition of Gd\(^{3+}\), consistent with the idea that Ca\(^{2+}\) entry contributes to the transient (Cherednichenko et al., 2004; Yang et al., 2007). In the present study, a substantial contribution of L-type current to this entry was revealed by the observation that the decay of the myoplasmic Ca\(^{2+}\) transient in dysgenic cells expressing SkEIIIK was essentially unchanged by the removal of extracellular Ca\(^{2+}\) and resembled that of normal myotubes in the absence of external Ca\(^{2+}\) or in the presence of Gd\(^{3+}\). Furthermore, when intracellular Ca\(^{2+}\) release was blocked by ryanodine, no Ca\(^{2+}\) transients were detectable in SkEIIIK-expressing dysgenic myotubes. Collectively, our results indicate that the skeletal muscle L-type Ca\(^{2+}\) current is a major contributor to the Ca\(^{2+}\) entry that occurs during depolarization of myotubes.
Ca\textsuperscript{2+} Imaging
Myotubes were washed with Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free Ringer’s solution (in mM: 146 NaCl, 5 KCl, 10 HEPES, and 11 glucose, pH 7.4, with NaOH) twice and loaded with 5 μM Fluo-3-AM (Invitrogen) dissolved in Rodent Ringer’s solution (in mM: 146 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, and 10 HEPES, pH 7.4, with NaOH) for 20 min. Myotubes were then washed three times in Rodent Ringer’s solution with gentle agitation. Fluo-3-AM–loaded myotubes bathed in either Rodent Ringer’s solution or 0 Ca\textsuperscript{2+} Rodent Ringer’s solution (in mM: 146 NaCl, 5 KCl, 3 MgSO\textsubscript{4}, and 10 HEPES, pH 7.4, with NaOH) were then placed on the stage of an LSM META scanning laser confocal microscope (Carl Zeiss, Inc.) and viewed under either 10 or 40× magnification. 10–100 μM 2-APB (Sigma-Aldrich) or LaCl\textsubscript{3} (Sigma-Aldrich) was dissolved in 40% EtOH and diluted to various concentrations just before experiments.

Pharmacology
Ryanodine (Sigma-Aldrich) was reconstituted in 40% EtOH and diluted to 200 μM in differentiation medium. GdCl\textsubscript{3} (Sigma-Aldrich) or LaCl\textsubscript{3} (Sigma-Aldrich) was dissolved in water to make 1-M stock solutions and was then added to standard external recording solution for a final trivalent cation concentration of 100 μM. 2-APB (Sigma-Aldrich) and SKF 96356 (provided by O. Delbono, Wake Forest University, Winston-Salem, NC) were dissolved in DMSO (Sigma-Aldrich) to make 1-M and 50-mM stock solutions, respectively. Nifedipine (EMD) was dissolved in DMSO (Sigma-Aldrich) to make 1-M and 50-mM stock solutions and was then added to standard external recording solution (in mM: 71 NaCl, 80 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, and 10 HEPES, pH 7.4, with NaOH) twice and loaded with 5 μM Fluo-3-AM (Invitrogen) dissolved in Rodent Ringer’s solution (in mM: 71 NaCl, 80 KCl, 3 MgSO\textsubscript{4}, and 10 HEPES, pH 7.4, with NaOH) for 20 min. Myotubes were then washed three times in Rodent Ringer’s solution and allowed the sampling of responses from many myotubes simultaneously, which was especially useful for myotubes expressing SkEIIIK in the presence and absence of a 20-s KCl depolarization /peak F/F, the following parameters were assessed: (1) \(t_{\text{peak}}\) = [time at peak F/F], (2) \(t\text{_{1/2} decay}\) = time of decay from peak to 50% of peak F/F, and (3) \(r_{20}\) = \([F\text{ at the end of a 20-s KCl depolarization}] / [\text{peak F/F}] \times 100\).

Analysis
Figures were made using the software program SigmaPlot (version 7.0; SSPS Inc.). All data are presented as mean ± SEM. Statistical comparisons were by ANOVA or by unpaired two-tailed t test (as appropriate), with \(P < 0.05\) considered significant.

Online Supplemental Material
Fig. S1 shows that SKF 96356 accelerated the inactivation kinetics of the L-type current sufficiently so that block was essentially complete at test pulses slightly longer than the test pulse shown in Fig. 1 D. Fig. S2 shows the I-V relationships for dysgenic myotubes expressing SkEIIIK in the presence and absence of 5 μM ± Bay K 8644. Fig. S3 shows that the anti-malignant hyperthermia drug dantrolene partially inhibits Ca\textsuperscript{2+} entry during long exposure to high KCl that was more uniform for entire myotubes slower, the latter method had the advantage of producing an exposure to high KCl that was more uniform for entire myotubes and allowed the sampling of responses from many myotubes simultaneously, which was especially useful for myotubes expressing the SkEIIIK construct. Fluorescence amplitude data are expressed as \(\Delta F/F\), where \(F\) represents the baseline fluorescence before the application of high KCl Ringer’s solution, and \(\Delta F\) represents the change in peak fluorescence during the application of high KCl Ringer’s solution. In addition to peak \(\Delta F/F\), the following parameters were assessed: (1) \(t_{\text{peak}}\) = [time at peak \(\Delta F\)] – [time at onset of the upswing of the transient], (2) \(t\text{_{1/2} decay}\) = time of decay from peak to 50% of peak \(\Delta F/2\), and (3) \(r_{20}\) = \([\text{F at the end of a 20-s KCl depolarization}] / [\text{peak \(\Delta F\)}] \times 100\).
The Skeletal L-type Current Contributes to ECCE

The inactivation kinetics of the L-type current sufficiently so that block was essentially complete (98 ± 2% block; n = 3) at 500 ms (Fig. S1). The L-type current was not appreciably affected by exposure to DMSO vehicle (7 ± 5%; n = 3; P < 0.0001, ANOVA; Fig. 1 E).

Complete Block of Skeletal L-type Current Requires a High Concentration of Nifedipine

A small component of ECCE is blocked by the 1,4-dihydropyridine L-type Ca²⁺ channel antagonist, nifedipine (1 μM) (Yang et al., 2007), whereas ECCE is completely blocked by 50 μM nifedipine (see Fig. 7 C). Block of the L-type current showed a similar dependence on nifedipine dose; only partial block was produced by either 1 or 10 μM nifedipine (Fig. 2, A and B), whereas nearly complete block required 50 μM nifedipine (Fig. 2 C). The dose–response curve for nifedipine is shown in Fig. 2 D.

## Table I

<table>
<thead>
<tr>
<th>Condition</th>
<th>G_max (nS/nF)</th>
<th>V_1/2 (mV)</th>
<th>k_G (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal (10 mM Ca²⁺ external)</td>
<td>244 ± 12</td>
<td>18.1 ± 1.4</td>
<td>4.4 ± 0.2</td>
</tr>
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</tr>
<tr>
<td>normal (2 mM Ca²⁺ external)</td>
<td>115 ± 16a</td>
<td>7.8 ± 2.4b</td>
<td>4.7 ± 0.5</td>
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<td>normal (10 mM Ca²⁺ external) + 10 μM dantrolene</td>
<td>228 ± 10</td>
<td>22.4 ± 0.8a</td>
<td>5.2 ± 0.2b</td>
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<td>(12)</td>
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<tr>
<td>normal (10 mM Mn²⁺ external)</td>
<td>135 ± 6b</td>
<td>16.7 ± 1.9</td>
<td>5.6 ± 0.2b</td>
</tr>
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<td>(4)</td>
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</tbody>
</table>

Data are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested. Data were fit according to Eq. 1 (see Materials and Methods). Asterisks indicate significant differences compared to L-type currents recorded from normal, untreated myotubes in 10 mM external Ca²⁺.

*P < 10⁻⁷, t test.
*P < 0.01, t test.
L-type Channels Inactivate Slowly and Conduct Substantial Current at Weak Test Potentials in Physiological Ca²⁺

The pharmacological data described above were obtained using 200-ms test pulses and 10 mM Ca²⁺ in the external recording solution to allow comparison with previous data from our laboratory. Under these conditions, no L-type current was observable at test potentials below +10 mV. In contrast, ECCE can be strongly evoked by ~80 mM KCl (Hurne et al., 2005), which results in depolarizations to between −10 and 0 mV (Allen, P.D., and J.R. López, personal communication). To determine whether L-type current can be observed in the same range of potentials as ECCE, we reduced the external Ca²⁺ to the more physiological 2 mM used for the ECCE measurements (Cherednichenko et al., 2004, 2008; Hurne et al., 2005; Yang et al., 2007; Gach et al., 2008; Lyfenko and Dirksen, 2008). This maneuver reduced L-type current amplitude and caused an ~11-mV depolarizing shift in the I-V relationship measured with 200-ms test pulses (P = 0.0011; Fig. 4 A and Table I). However, the currents were much larger with ~10 s depolarizations.

Dantrolene Inhibits L-type Currents

The anti-malignant hyperthermia drug dantrolene at 10 μM blocks ~65% of ECCE as measured by Mn²⁺ quench (Cherednichenko et al., 2008). We found that 10 μM dantrolene also inhibited the L-type current in myotubes (Fig. 3 and Table I). At +30 mV (near the peak of the I-V relationship), dantrolene inhibited L-type current by ~19% compared with untreated control myotubes (−9.7 ± 0.6 pA/pF; n = 12 vs. −11.9 ± 0.5 pA/pF; n = 10, respectively; P < 0.05). Dantrolene also caused an ~5-mV depolarizing shift in the I-V relationship (Fig. 3 C). Collectively, the data presented in Figs. 1–3 indicate that the skeletal L-type Ca²⁺ current and ECCE have very similar pharmacological profiles.

Figure 3. Dantrolene inhibits skeletal L-type Ca²⁺ currents. Recordings of L-type currents elicited by 200-ms depolarizations from −50 mV to the indicated test potentials are shown for an untreated normal myotube (A) or a normal myotube exposed to 10 μM dantrolene for >10 min at 25 °C (B). (C) Comparison of I-V relationships for untreated (●; n = 10) and dantrolene-treated myotubes (○; n = 12). Currents were evoked at 0.1 Hz in 10-mV increments after a prepulse protocol (see Materials and methods). Current amplitudes were normalized by linear cell capacitance (pA/pF). The smooth curves are plotted according to Eq. 1, with best-fit parameters presented in Table I.

Figure 4. In 2 mM of external Ca²⁺, L-type currents are activated by weak depolarizations and inactivate very slowly. (A) Comparison of I-V relationships for normal myotubes in 10 mM of external Ca²⁺ (●; n = 10) or 2 mM of external Ca²⁺ (○; n = 4) elicited at 0.1 Hz by test potentials ranging from −20 through +80 mV in 10-mV increments after a prepulse protocol. The smooth curves are plotted according to Eq. 1. The best-fit parameters for each plot are presented in Table I. (B) Recordings of L-type currents in 2 mM of external Ca²⁺ elicited by 9,800-ms depolarizations from −80 mV to the indicated test potentials.

Bannister et al. 83

Published December 29, 2008
large L-type currents were observed at depolarizations to +30 mV (Fig. 5 B, bottom trace). A substantial current remained upon the replacement of 10 mM Ca\(^{2+}\) by 10 mM Mn\(^{2+}\) (Fig. 5 B, middle trace). This Mn\(^{2+}\) current was blocked nearly completely by 10 μM nifedipine (95 ± 2% channel block; \(n = 4\)), confirming that the Mn\(^{2+}\) entry was indeed via L-type channels (Fig. 5 B, top trace).

To approximate the conditions that have been previously used to measure ECCE via Mn\(^{2+}\) quench, we also recorded L-type currents in the presence of 0.5 mM Mn\(^{2+}\) (Fig. 5 C). Under these conditions, the average current amplitude was reduced (Fig. 5 D), and there was a substantial hyperpolarizing shift in the activation of the Mn\(^{2+}\) current (i.e., peaking at \(-11601\) mV; Fig. 5, C and D).

**Skeletal Muscle L-type Channels Pass Mn\(^{2+}\)**

In addition to producing Ca\(^{2+}\) entry measurable as a component of myoplasmic Ca\(^{2+}\) transients (compare with Yang et al., 2007), ECCE can be assayed by quench of Fura-2 fluorescence resulting from Mn\(^{2+}\) influx in the absence of extracellular Ca\(^{2+}\) (Cherednichenko et al., 2004, 2008; Hurne et al., 2005; Gach et al., 2008; Lyfenko and Dirksen, 2008). Fig. 5 A shows that the L-type channel also conducts Mn\(^{2+}\) ions in the absence of external Ca\(^{2+}\) (peak current of −4.1 ± 0.3 pA/pF at +30 mV; \(n = 4\)). Under standard recording conditions (10 mM external Ca\(^{2+}\)), large L-type Ca\(^{2+}\) currents were observed at depolarizations to +30 mV (Fig. 5 B, bottom trace). A substantial current remained upon the replacement of 10 mM Ca\(^{2+}\) by 10 mM Mn\(^{2+}\) (Fig. 5 B, middle trace). This Mn\(^{2+}\) current was blocked nearly completely by 10 μM nifedipine (95 ± 2% channel block; \(n = 4\)), confirming that the Mn\(^{2+}\) entry was indeed via L-type channels (Fig. 5 B, top trace).

To approximate the conditions that have been previously used to measure ECCE via Mn\(^{2+}\) quench, we also recorded L-type currents in the presence of 0.5 mM Mn\(^{2+}\) (Fig. 5 C). Under these conditions, the average current amplitude was reduced (Fig. 5 D), and there was a substantial hyperpolarizing shift in the activation of the Mn\(^{2+}\) current (i.e., peaking at −40 mV; Fig. 5, C and D).

**Ca\(^{2+}\) Influx via the L-type Channel Accounts for the Majority of Ca\(^{2+}\) Influx in Response to Extended Depolarization**

Cherednichenko et al. (2004) found that ECCE could be detected in dysgenic myotubes after the expression like those used to elicit ECCE (Cherednichenko et al., 2004; Hurne et al., 2005) because activation was so slow (Fig. 4 B). In particular, L-type currents evoked at −10 mV in 2 mM Ca\(^{2+}\) had a time-to-peak of 4.02 ± 1.02 s (\(n = 6\); Table II). Furthermore, the current inactivated very little under these conditions (36% in 9 s; Table II). Collectively, the data in Fig. 4 indicate that long, weak depolarizations elicit substantial, sustained L-type current in the presence of physiological Ca\(^{2+}\).

<table>
<thead>
<tr>
<th>(I_{\text{peak}}) (pA/pF)</th>
<th>(t_{\text{peak}}) (s)</th>
<th>(t_{1/2,\text{decay}}) (s)</th>
<th>(r_9) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal (−10 mV)</td>
<td>−0.7 ± 0.2</td>
<td>4.02 ± 1.02</td>
<td>ND</td>
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<td>(5)</td>
<td>(6)</td>
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</tr>
<tr>
<td>normal (0 mV)</td>
<td>−2.3 ± 0.6</td>
<td>2.54 ± 1.14</td>
<td>2.65 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(6)</td>
<td>0.52 ± 0.10</td>
</tr>
</tbody>
</table>

Data presented here were obtained from 9.8 s test potentials from −80 to −10 and 0 mV with 2 mM Ca\(^{2+}\) in the external recording solution. \(r_9\) is ratio of the current remaining at 9 s to the peak current. \(t_{1/2,\text{decay}}\) at −10 mV was not determined (ND) because the test potential was too short to accurately assess this parameter in three of six cells. Data are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested.

**Figure 5.** The skeletal muscle L-type channel conducts Mn\(^{2+}\). (A) I-V relationship for normal myotubes in 10 mM of external Mn\(^{2+}\) (○; \(n = 4\)) for currents elicited at 0.1 Hz by test potentials ranging from −20 through +80 mV in 10-mV increments after a prepulse protocol. The smooth curves are plotted according to Eq. 1. The best-fit parameters for each plot are presented in Table I. (B) Representative currents recorded at +30 mV from a normal myotube in 10 mM of external Mn\(^{2+}\) (bottom trace), in 10 mM Mn\(^{2+}\) (middle trace), and in 10 mM Mn\(^{2+}\) after acute application of 10 μM nifedipine (top trace). (C) L-type current evoked by steps from −50 to 0 mV (top) and to +30 mV (bottom) in 0.5 mM Mn\(^{2+}\). (D) Average peak currents in 10 mM of divalent cation at a test potential of +30 mV (left) and for 0.5 mM Mn\(^{2+}\) at the indicated test potentials (right).
of a DHPR α_{1S} subunit in which a conserved glutamate in the pore-forming region of repeat III was mutated to lysine (SkEIIIK) (Dirksen and Beam, 1999). Because SkEIIIK produces no obvious inward Ca^{2+} current during 200-ms depolarizations or subsequent repolarization (Dirksen and Beam, 1999) (Fig. S2), it was concluded that ECCE was independent of cation flux via the L-type channel. To assess a potential contribution of the L-type current to ECCE, we compared myoplasmic Ca^{2+} transients that were elicited locally by 20-s exposures to elevated (80 mM) KCl in both untransfected normal myotubes and dysgenic myotubes expressing SkEIIIK. Ca^{2+} transients of normal myotubes elicited by locally applied 80 mM KCl (Fig. 6 A) rose to a peak and decayed slowly (t_{1/2 decay} = 10.64 ± 0.98 s; n = 24) and incompletely (r_{20} = 31.92 ± 5.03%). As shown in Fig. 6 B, transients of normal myotubes obtained in nominally 0 mM Ca^{2+} decayed much faster (t_{1/2 decay} = 4.98 ± 0.45 s; n = 12; P < 0.0005, t test) and more completely (r_{20} = 4.36 ± 2.38%; P < 0.001, t test) than transients measured in 2 mM Ca^{2+} (Fig. 6, B–D, superimposed gray trace). An accelerated rate of decay was also observed in experiments in which 100 μM Gd^{3+} was added to the 80 KCl Ringer’s solution in the micropipette (t_{1/2 decay} = 3.49 ± 0.47 s; r_{20} = 2.23 ± 0.74%; n = 14; Table III), supporting the idea that the acceleration of decay in 0 mM Ca^{2+} resulted from a loss of Ca^{2+} entry rather than from some other effect of removing Ca^{2+} from the bath.

In 2 mM Ca^{2+}, high KCl-evoked Ca^{2+} transients of dysgenic myotubes expressing SkEIIIK also decayed more rapidly than Ca^{2+} transients of normal myotubes (Fig. 6 C), having decay kinetics (t_{1/2 decay} = 4.47 ± 0.52 s; r_{20} = 10.41 ± 2.70 s; n = 9; Fig. 6, E and F) more similar to those of normal myotubes in nominally 0 mM Ca^{2+} than in 2 mM Ca^{2+} (Table III). Furthermore, the decay kinetics of Ca^{2+} transients in myotubes expressing SkEIIIK were little affected by the removal of extracellular Ca^{2+} (t_{1/2 decay} = 5.68 ± 1.16 s; r_{20} = 4.97 ± 2.17%; n = 5; Fig. 6, D–F, and Table III). The fact that Ca^{2+} removal accelerated the decay of Ca^{2+} transients in normal myotubes but not in dysgenic myotubes expressing SkEIIIK is consistent with the idea that Ca^{2+} entry slows the decay of transients in normal myotubes bathed in 2 mM Ca^{2+}, and that this entry does not occur for SkEIIIK.

In control experiments, untransfected dysgenic and dyspedic myotubes failed to respond to locally applied 80 mM KCl (n = 12 and 14, respectively; Table III). Myotubes harvested from mice null for the DHPR β_{1S} subunit also failed to respond to locally applied 80 mM KCl Ringer’s solution (n = 10; Table III).

SkEIIIK Supports Minimal Ca^{2+} Entry during Long, Global Depolarizations

To assess changes in myoplasmic Ca^{2+} attributable solely to Ca^{2+} influx, we applied 80 mM KCl globally to myotubes after block of intracellular Ca^{2+} release by ryanodine pretreatment (200 μM; > 1 h; 37 °C). Under these conditions, high KCl-evoked Ca^{2+} transients of normal myotubes rose to a peak and decayed slowly (t_{1/2 decay} = 10.64 ± 0.98 s; n = 24) and incompletely (r_{20} = 31.92 ± 5.03%). As shown in Fig. 6 B, transients of normal myotubes obtained in nominally 0 mM Ca^{2+} decayed much faster (t_{1/2 decay} = 4.98 ± 0.45 s; n = 12; P < 0.0005, t test) and more completely (r_{20} = 4.36 ± 2.38%; P < 0.001, t test) than transients measured in 2 mM Ca^{2+} (Fig. 6, B–D, superimposed gray trace). An accelerated rate of decay was also observed in experiments in which 100 μM Gd^{3+} was added to the 80 KCl Ringer’s solution in the micropipette (t_{1/2 decay} = 3.49 ± 0.47 s; r_{20} = 2.23 ± 0.74%; n = 14; Table III), supporting the idea that the acceleration of decay in 0 mM Ca^{2+} resulted from a loss of Ca^{2+} entry rather than from some other effect of removing Ca^{2+} from the bath.

In 2 mM Ca^{2+}, high KCl-evoked Ca^{2+} transients of dysgenic myotubes expressing SkEIIIK also decayed more rapidly than Ca^{2+} transients of normal myotubes (Fig. 6 C), having decay kinetics (t_{1/2 decay} = 4.47 ± 0.52 s; r_{20} = 10.41 ± 2.70 s; n = 9; Fig. 6, E and F) more similar to those of normal myotubes in nominally 0 mM Ca^{2+} than in 2 mM Ca^{2+} (Table III). Furthermore, the decay kinetics of Ca^{2+} transients in myotubes expressing SkEIIIK were little affected by the removal of extracellular Ca^{2+} (t_{1/2 decay} = 5.68 ± 1.16 s; r_{20} = 4.97 ± 2.17%; n = 5; Fig. 6, D–F, and Table III). The fact that Ca^{2+} removal accelerated the decay of Ca^{2+} transients in normal myotubes but not in dysgenic myotubes expressing SkEIIIK is consistent with the idea that Ca^{2+} entry slows the decay of transients in normal myotubes bathed in 2 mM Ca^{2+}, and that this entry does not occur for SkEIIIK.

In control experiments, untransfected dysgenic and dyspedic myotubes failed to respond to locally applied 80 mM KCl (n = 12 and 14, respectively; Table III). Myotubes harvested from mice null for the DHPR β_{1S} subunit also failed to respond to locally applied 80 mM KCl Ringer’s solution (n = 10; Table III).

SkEIIIK Supports Minimal Ca^{2+} Entry during Long, Global Depolarizations

To assess changes in myoplasmic Ca^{2+} attributable solely to Ca^{2+} influx, we applied 80 mM KCl globally to myotubes after block of intracellular Ca^{2+} release by ryanodine pretreatment (200 μM; > 1 h; 37 °C). Under these conditions, high KCl-evoked Ca^{2+} transients of normal myotubes rose to a peak and decayed slowly (t_{1/2 decay} = 10.64 ± 0.98 s; n = 24) and incompletely (r_{20} = 31.92 ± 5.03%). As shown in Fig. 6 B, transients of normal myotubes obtained in nominally 0 mM Ca^{2+} decayed much faster (t_{1/2 decay} = 4.98 ± 0.45 s; n = 12; P < 0.0005, t test) and more completely (r_{20} = 4.36 ± 2.38%; P < 0.001, t test) than transients measured in 2 mM Ca^{2+} (Fig. 6, B–D, superimposed gray trace). An accelerated rate of decay was also observed in experiments in which 100 μM Gd^{3+} was added to the 80 KCl Ringer’s solution in the micropipette (t_{1/2 decay} = 3.49 ± 0.47 s; r_{20} = 2.23 ± 0.74%; n = 14; Table III), supporting the idea that the acceleration of decay in 0 mM Ca^{2+} resulted from a loss of Ca^{2+} entry rather than from some other effect of removing Ca^{2+} from the bath.
TABLE III

<table>
<thead>
<tr>
<th>Condition</th>
<th>ΔF/F (ΔF/F)</th>
<th>t_peak (s)</th>
<th>t_1/2 decay (s)</th>
<th>r_20 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal (2 mM Ca(^{2+}))</td>
<td>3.25 ± 0.35</td>
<td>3.76 ± 0.34</td>
<td>10.64 ± 0.98</td>
<td>31.92 ± 5.03</td>
</tr>
<tr>
<td>normal (0 mM Ca(^{2+}))</td>
<td>1.31 ± 0.15a</td>
<td>1.97 ± 0.22b</td>
<td>4.98 ± 0.45a</td>
<td>4.36 ± 2.38b</td>
</tr>
<tr>
<td>normal + 100 μM Gd(^{3+})(2 mM Ca(^{2+}))</td>
<td>1.77 ± 0.18c</td>
<td>1.94 ± 0.19c</td>
<td>3.49 ± 0.47c</td>
<td>2.23 ± 0.74c</td>
</tr>
<tr>
<td>dysgenic + SkEIIIK (2 mM Ca(^{2+}))</td>
<td>0.90 ± 0.18a</td>
<td>3.51 ± 0.50a</td>
<td>4.47 ± 0.52a</td>
<td>10.41 ± 2.70a</td>
</tr>
<tr>
<td>dysgenic + SkEIIIK (0 mM Ca(^{2+}))</td>
<td>0.25 ± 0.09a</td>
<td>2.70 ± 0.45a</td>
<td>5.68 ± 1.12a</td>
<td>4.97 ± 2.17a</td>
</tr>
<tr>
<td>dysgenic (2 mM Ca(^{2+}))</td>
<td>no response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dyspedic (2 mM Ca(^{2+}))</td>
<td>no response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β(1) null (2 mM Ca(^{2+}))</td>
<td>no response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dysgenic + SkEIIIK + ryanodine</td>
<td>no response</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data presented here were obtained with depolarizations evoked by 20-s applications of 80 mM KCl Ringer’s solution via a micropipette placed near the cell of interest. Where indicated, 100 μM Gd\(^{3+}\) was added to the 80 mM KCl Ringer’s solution in the micropipette. See Materials and methods for definitions of t_peak, t_1/2 decay, and r_20. Data are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested. Footnotes indicate significant differences compared to normal myotubes in 2 mM Ca\(^{2+}\) by unpaired t test.

\*P < 0.001.
\*P < 0.005.
\*P < 0.05.

Experimental conditions, 40 of 42 normal myotubes showed a large, slowly activating Ca\(^{2+}\) entry from the extracellular space in response to global application of 80 mM KCl (ΔF/F = 2.11 ± 0.16, n = 40; Fig. 7A). The presence of either 100 μM Gd\(^{3+}\) or 50 μM nifedipine, which both block the L-type current (Figs. 1 and 2), completely eliminated this slow Ca\(^{2+}\) entry (n = 6 and n = 10, respectively; Fig. 7, B and C). In contrast to the robust Ca\(^{2+}\) transients observed in ryanodine-treated normal myotubes, no Ca\(^{2+}\) transients were detectable (ΔF/F < 0.05) in ryanodine-treated dysgenic myotubes expressing SkEIIIK (n = 31; Fig. 7 D). Collectively, these results support the idea that L-type Ca\(^{2+}\) current is a major contributor to Ca\(^{2+}\) entry in response to prolonged, weak depolarization.

The SkEIIIK Pore Mutant Allows Inward Divalent Current

Previously, it was reported that in dysgenic myotubes expressing SkEIIIK, entry of divalent cations in response to depolarization could be measured with the indicator Fura-2 (Cherednichenko et al., 2004), whereas we were unable to detect such entry with the indicator Fluo-3 (Figs. 6 and 7). Thus, we sought to determine if SkEIIIK could permit a small influx of divalent cations that might have accounted for the signal measured with Fura-2 by Cherednichenko et al. (2004). In this regard, it is important to note that L-type Ca\(^{2+}\) channels display at least two distinct open states of either brief or long duration (i.e., Mode 1 and Mode 2 gating, respectively) (Hess et al., 1984; Pietrobon and Hess, 1990; Dirksen and Beam, 1996). Compared with the short-lived open state, the long-duration open state is relatively unpopulated during 200-ms depolarizations to potentials below about +50 mV (Dirksen and Beam, 1996). However, entry into the long-duration open state does occur during longer depolarizations (e.g., as during extended exposure to elevated KCl) and is accelerated by stronger depolarizations as well as by exposure to dihydropyridine agonists (Hess et al., 1984; Pietrobon and Hess, 1990; Dirksen and Beam, 1996). Importantly, the long duration open state of the wild-type channel preferentially allows inward divalent current (Leuranguer et al., 2003). Thus, we tested whether strong depolarization in the presence of ±Bay K 8644 could cause SkEIIIK to enter a long-lived open state that would produce inward Ca\(^{2+}\) tail current upon repolarization.

Fig. 8A compares currents for depolarizations to either 0 or +80 mV recorded from a dysgenic myotube expressing SkEIIIK and bathed in 5 μM ±Bay K 8644. The depolarization to 0 mV, which is near threshold for activation of ionic current, evoked little outward current and resulted in only a small inward current upon the subsequent repolarization to −50 mV. However, the depolarization to 80 mV evoked a robust, slowly activating outward current (see peak I-V in Fig. S2) and resulted in an appreciable, slowly decaying inward current upon repolarization to −50 mV. As shown in Fig. 8B, this slowly decaying inward current did not appear to be a consequence of the potentiation by ±Bay K 8644 of the endogenous L-type current that can be present at low levels in dysgenic myotubes (n = 14; I_{0m}) (Adams and Beam, 1989).
cated the presence of inward Ca\textsuperscript{2+} current via the mutant L-type channel. Thus, these results strongly support the hypothesis that SkEIIIK can enter into a long-duration

In dysgenic cells expressing SkEIIIK, the presence of ± Bay K 8644 caused the inward current elicited by repolarization from +80 to −50 mV to be both larger and more slowly decaying than in the absence of the DHP agonist (Fig. 8 C), consistent with the idea that the inward current represents Ca\textsuperscript{2+} influx through channels relaxing from the long open state to closed. The total charge transported during the inward current elicited by repolarization to −50 mV was obtained by integration and is plotted in Fig. 8 D. The total charge for repolarization from 0 to −50 mV (3.6 ± 0.7 nC/μF; \(n = 9\)) was similar to that previously measured (see Fig. 4 of Dirksen and Beam, 1999). This charge was likely to have been nonionic and to have arisen from closed–closed gating transitions of the DHPR because a 200-ms depolarization to 0 mV activates little ionic conductance via SkEIIIK (Dirksen and Beam, 1999). Compared with 0 mV, the total charge was substantially larger for repolarization from +80 to −50 mV (10.5 ± 1.1 nC/μF; \(n = 9\)). A fraction of this additional charge movement (−66% of the total 300% increase) was also likely to have been nonionic and to have arisen from gating transitions that would have occurred for repolarization to −50 mV after a 200-ms depolarization to +80 mV, but not after a 200-ms depolarization to 0 mV. Possibly, the remaining portion (−33% of the 300% increase) of additional charge movement was a consequence of inward Ca\textsuperscript{2+} current that resulted from the entry of a small fraction of the SkEIIIK channels into the long-duration open state that supports such current. In the presence of ± Bay K 8644 (Fig. 8 D, gray bars), the total charge after repolarization from +80 to −50 mV was so large (33.5 ± 8.0 nC/μF; \(n = 10\)) compared with that after repolarization from 0 to −50 mV (4.4 ± 1.0 nC/μF; \(n = 10\)) that it clearly indicated the presence of inward Ca\textsuperscript{2+} current via the mutant L-type channel. Thus, these results strongly support the hypothesis that SkEIIIK can enter into a long-duration

Figure 7. SkEIIIK supports minimal Ca\textsuperscript{2+} entry during long global depolarizations. Representative Ca\textsuperscript{2+} transients evoked by global perfusion of 80 mM KCl Ringer’s solution for normal myotubes (A), normal myotubes exposed to 100 μM Gd\textsuperscript{3+} (B), normal myotubes exposed to 50 μM nifedipine (C), and dysgenic myotubes expressing SkEIIIK (D). In each case, myotubes were exposed to 200 μM ryanodine for >1 h at 37 °C before experiments to block the contribution of RYR1 to the Ca\textsuperscript{2+} transient. In experiments with SkEIIIK, a slower sampling rate was used to minimize any bleaching of the Fluo-3 dye. Expression of SkEIIIK was confirmed by electrically evoked (100 V, 5 ms) contractions (17 of 20 myotubes tested) before ryanodine treatment (see Papadopoulos et al., 2004).

Figure 8. Potentiated SkEIIIK conducts inward Ca\textsuperscript{2+} current. Representative currents are shown for a ± Bay K 8644–treated (5 μM) dysgenic myotube expressing SkEIIIK (A) and a ± Bay K 8644–treated, uninjected dysgenic myotube (B). The inset illustrates the tail currents shown in A on an expanded time base. (C) Summary of amplitudes (left) and deactivation time constants (right) of SkEIIIK tail currents recorded in the absence (\(n = 9\)) and presence (\(n = 10\)) of ± Bay K 8644. (D) Total charge moved obtained by integration of tail currents recorded in the absence (\(n = 9\)) and presence (\(n = 10\)) of ± Bay K 8644 from dysgenic myotubes expressing SkEIIIK. Asterisks indicate significant differences between untreated and ± Bay K 8644–treated cells (*, \(P < 0.05\); **, \(P < 0.01\), \(t\) test).
open state that permits inward divalent cation flux. However, the similarity of Ca\textsuperscript{2+} transient decay in SkEIIIK-expressing dysgenic myotubes in 2 mM Ca\textsuperscript{2+} and normal myotubes in 0 mM Ca\textsuperscript{2+} (Fig. 6 and Table III) and the lack of detectable SkEIIIK-mediated transients in the presence of ryanodine (Fig. 7 D) argues that such a mode of Ca\textsuperscript{2+} permeation via SkEIIIK must be quite small compared with Ca\textsuperscript{2+} entry via the native L-type channel.

**DISCUSSION**

Here, we demonstrate that the skeletal muscle L-type Ca\textsuperscript{2+} current and ECCE share many common attributes (summarized in Table IV). Thus, the skeletal muscle L-type channel is blocked by nonspecific cation channel antagonists (Fig. 1), requires nearly 50 μM nifedipine for complete channel block (Fig. 2), and is inhibited by dantrolene (Fig. 3). We also show that in 2 mM of external Ca\textsuperscript{2+}, the L-type Ca\textsuperscript{2+} current, like ECCE, is persistently activated by weak voltage steps (Fig. 4). In addition, we demonstrate that the L-type channel is permeable to Mn\textsuperscript{2+} in the absence of bath Ca\textsuperscript{2+} (Fig. 5). During prolonged (20-s) KCl depolarization, Ca\textsuperscript{2+} transients in intact, normal myotubes rise to a peak and then decay, and this decay is more rapid after the removal of external Ca\textsuperscript{2+} or in the presence of Gd\textsuperscript{3+}; the decay of transients in dysgenic myotubes expressing the DHPR α\textsubscript{S} subunit pore mutant (SkEIIIK), which has a low permeability to Ca\textsuperscript{2+}, is similar to that of transients in normal myotubes in a Ca\textsuperscript{2+}-free external medium and is little affected by the removal of extracellular Ca\textsuperscript{2+} (Fig. 6 and Table III). Collectively, these results suggest that Ca\textsuperscript{2+} entry via the L-type channel slows the decay of Ca\textsuperscript{2+} transients during prolonged depolarization of normal myotubes. After block of SR Ca\textsuperscript{2+} release by pretreatment with 200 μM ryanodine, depolarization of normal myotubes still resulted in large myoplasmic Ca\textsuperscript{2+} transients, whereas depolarization did not elicit a detectable transient in dysgenic myotubes expressing SkEIIIK (Fig. 7), providing strong support to the idea that the L-type channel is the predominant source of Ca\textsuperscript{2+} entry. Although SkEIIIK has very low permeability to Ca\textsuperscript{2+}, we

<table>
<thead>
<tr>
<th>TABLE IV</th>
<th>Common attributes of ECCE and the skeletal muscle L-type current</th>
</tr>
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<tbody>
<tr>
<td><strong>ECCE</strong></td>
<td><strong>L</strong></td>
</tr>
<tr>
<td>Is voltage-dependent</td>
<td>yes\textsuperscript{a,b}</td>
</tr>
<tr>
<td>Conducts Ca\textsuperscript{2+}</td>
<td>yes\textsuperscript{a,b,c}</td>
</tr>
<tr>
<td>Requires α\textsubscript{1S} subunit</td>
<td>yes\textsuperscript{a,b,c}</td>
</tr>
<tr>
<td>Requires β\textsubscript{1a} subunit</td>
<td>yes\textsuperscript{a,b,c}</td>
</tr>
<tr>
<td>Reduced by siRNA against α\textsubscript{2β} subunit</td>
<td>yes\textsuperscript{a,b,c}</td>
</tr>
<tr>
<td>Requires RYR1</td>
<td>yes\textsuperscript{a,b,c}</td>
</tr>
<tr>
<td>Blocked by 100 μM La\textsuperscript{3+}</td>
<td>increased expression\textsuperscript{b,c,j}</td>
</tr>
<tr>
<td>Blocked by 100 μM Gd\textsuperscript{3+}</td>
<td>greater potentiation\textsuperscript{a,n}</td>
</tr>
<tr>
<td>Blocked by 20 μM SKF 96356</td>
<td>complete\textsuperscript{a,e}</td>
</tr>
<tr>
<td>Blocked by 100 μM 2-APB</td>
<td>complete\textsuperscript{a,e}</td>
</tr>
<tr>
<td>Blocked by 1 μM nifedipine</td>
<td>complete\textsuperscript{a,e}</td>
</tr>
<tr>
<td>Blocked by 50 μM nifedipine</td>
<td>complete\textsuperscript{a,e}</td>
</tr>
<tr>
<td>Reduced by ≥10 μM dantrolene</td>
<td>complete\textsuperscript{a,e}</td>
</tr>
<tr>
<td>Conducts Mn\textsuperscript{2+}</td>
<td>yes\textsuperscript{a,b,k,l}</td>
</tr>
<tr>
<td>Divalent entry via SkEIIIK</td>
<td>yes\textsuperscript{a,b,k,l}</td>
</tr>
<tr>
<td>Mediated by Orai1/STIM1</td>
<td>yes\textsuperscript{a,b}</td>
</tr>
</tbody>
</table>

References:
\textsuperscript{a}Cherednichenko et al., 2004.
\textsuperscript{b}Hurne et al., 2005.
\textsuperscript{c}Beam et al., 1986.
\textsuperscript{d}Tanabe et al., 1988.
\textsuperscript{e}This paper.
\textsuperscript{f}Lyfenko and Dirksen, 2008.
\textsuperscript{g}Gregg et al., 1995.
\textsuperscript{h}Strube et al., 1996.
\textsuperscript{i}Papadopoulos et al., 2004.
\textsuperscript{j}Sheridan et al., 2004.
\textsuperscript{k}Gach et al., 2008.
\textsuperscript{l}Obermair et al., 2005.
\textsuperscript{m}Nakai et al., 1996.
\textsuperscript{n}Avila and Dirksen, 2000.
\textsuperscript{o}Yang et al., 2007.
\textsuperscript{p}Cherednichenko et al., 2004, no concentration reported.
\textsuperscript{q}Cherednichenko et al., 2008.
\textsuperscript{r}Szentesi et al., 2001.
did find that after strong depolarization in the presence of 0.5 mM dantrolene, the channel could conduct inward Ca\textsuperscript{2+} tail currents upon repolarization (Fig. 8).

One very clear conclusion from our results is that any of the manipulations that have been used to evaluate the magnitude of ECCE will necessarily produce changes in current via the L-type Ca\textsuperscript{2+} channel (Table IV) and thus the contribution of the L-type current to the myoplasmic Ca\textsuperscript{2+} transient. For example, the addition of Gd\textsuperscript{3+}, 20 μM SKF 96356, and 100 μM 2-APB and the removal of external Ca\textsuperscript{2+} block 80–100% of ECCE (Cherednichenko et al., 2004; Hurne et al., 2005), and we find they also block L-type Ca\textsuperscript{2+} current to a similar extent. Conversely, the relative insensitivity of ECCE to 1 μM nifedipine has been taken as an indication that it is independent of L-type current (Yang et al., 2007). In particular, Yang et al. (2007) reported that 1 μM nifedipine causes only a 5% decrease in the decay of the sustained phase of the KCl-evoked transient for wild-type RYR1 (Fig. 6 B of Yang et al., 2007), whereas 100 μM La\textsuperscript{3+} causes a 14% decrease (Fig. 4 B of Yang et al., 2007). If it is assumed that the altered decay rate for 100 μM La\textsuperscript{3+} reflected a complete block of ECCE, then 1 μM nifedipine caused a 36% block, close to the ~50% block of L-type current that we found for 1 μM nifedipine (Fig. 2 D).

We found that maximal L-type Ca\textsuperscript{2+} currents in myotubes were reduced ~15% by 10 μM dantrolene (Fig. 3), a similar finding to that of Szentesi et al. (2001), who observed that L-type currents are reduced ~25% in adult rodent fibers by 25 μM dantrolene. These electrophysiological results are in good agreement with the observation that Ca\textsuperscript{2+} transients evoked by 40 mM KCl were reduced ~20% in 10 μM of dantrolene-pretreated myotubes compared with matched control myotubes (Fig. S3). However, an earlier report indicated that 10 μM dantrolene caused a more substantial (~65%) reduction in quench of Fura-2 fluorescence by Mn\textsuperscript{2+} entry in response to 40 mM KCl or electrical stimulation (Cherednichenko et al., 2008). In evaluating the difference in dantrolene sensitivity found in the two sets of experiments, it should be noted that the response of the fluorescent indicator (Fluo-3 to Ca\textsuperscript{2+}, Fura-2 to Mn\textsuperscript{2+}) depends not only on the magnitude of the entry flux, but also on the divergent affinity of the indicator and on the buffering and removal of the divalent cations that have entered the myoplasm. Consequently, the change in fluorescence might well not depend linearly on the entry flux, making it difficult to directly compare these measurements.

The dependence of ECCE on the presence of both the DHPR α\textsubscript{1a} subunit and RYR1 (Cherednichenko et al., 2004; Hurne et al., 2005; Lyfenko and Dirksen, 2008) is also a feature of the slow L-type Ca\textsuperscript{2+} current, which is totally absent in dysgenic muscle cells lacking α\textsubscript{1a} (Beam et al., 1986; Tanabe et al., 1988), and greatly reduced in dyspedic muscle cells lacking RYR1 (Nakai et al., 1996; Avila and Dirksen, 2000). This distinguishes ECCE and the slow L-type current from SOCE, which functions normally in dysgenic and dyspedic myotubes (Cherednichenko et al., 2004; Hurne et al., 2005; but see Lyfenko and Dirksen, 2008). Another parallel between ECCE and the slow L-type Ca\textsuperscript{2+} current is that myotubes null for the DHPR β\textsubscript{1a} subunit lack ECCE (Table III) and have greatly reduced expression of L-type current (Gregg et al., 1996; Strube et al., 1996; Papadopoulos et al., 2004; Sheridan et al., 2004). Interestingly, knockdown of the DHPR α\textsubscript{1a}β\textsubscript{1a} subunit in myotubes by siRNA causes a reduction in ECCE (Gach et al., 2008) and an accelerated inactivation of the L-type Ca\textsuperscript{2+} current measured with 200-ms depolarizations (Obermair et al., 2005; Gach et al., 2008; Obermair et al., 2008). During prolonged depolarizations, the accelerated inactivation might greatly reduce Ca\textsuperscript{2+} entry via the L-type channel.

Previously, Hurne et al. (2005) attempted unsuccessfully to measure a current associated with ECCE by the application of 6-s test pulses to −20 mV. In retrospect, this result is consistent with the hypothesis that the L-type Ca\textsuperscript{2+} current is a major contributor to the ECCE signal because depolarizations to −20 mV would not have activated L-type current in 10 mM of extracellular Ca\textsuperscript{2+} (Fig. 4 A), as used in the measurements of Hurne et al. (2005). In 2 mM Ca\textsuperscript{2+}, however, the leftward shift of activation has the result that long (~10-s) depolarizations to −10 or 0 mV evoke sustained L-type currents (Fig. 4 B) that would support substantial Ca\textsuperscript{2+} entry under the conditions used for measuring ECCE. In addition, relatively weak depolarizations activate currents carried by Mn\textsuperscript{2+} in the absence of the normal Ca\textsuperscript{2+} (Fig. 5), which means that the L-type channel would produce Mn\textsuperscript{2+} entry under the conditions used to measure Mn\textsuperscript{2+} quench attributed to ECCE (Table IV).

During prolonged depolarization of normal myotubes, the entry of Ca\textsuperscript{2+} appears to slow the decay of the myoplasmic Ca\textsuperscript{2+} transient inasmuch as removal of extracellular Ca\textsuperscript{2+} causes the decay to become more rapid (Fig. 6). This same manipulation does not greatly affect the time course of transients in dysgenic myotubes expressing SkEIIIK, which decay much like those of normal myotubes in a Ca\textsuperscript{2+}-free medium. In principle, removing extracellular Ca\textsuperscript{2+} could not only have eliminated Ca\textsuperscript{2+} entry, but also increased inactivation of the voltage sensor for EC coupling (Brum et al., 1988a,b). Indeed, the amplitude of the Ca\textsuperscript{2+} transients in Ca\textsuperscript{2+}-free medium was smaller for both normal myotubes and dysgenic myotubes expressing SkEIIIK (Table III), consistent with the possibility that removal of Ca\textsuperscript{2+} increased steady-state inactivation of voltage sensors in resting myotubes. Cherednichenko et al. (2004) also observed a similar reduction of the KCl-evoked Ca\textsuperscript{2+} transient in a Ca\textsuperscript{2+}-free medium. However, altered inactivation during the KCl application does not seem to have been a major factor in causing more rapid decay because: (1) an acceleration of decay like that seen with Ca\textsuperscript{2+} removal was
caused by the administration of Gd³⁺ concurrently with the high KCl, and (2) removal of external Ca²⁺ had little effect on the time course of Ca²⁺ transients for SkEIIIK.

Our measurements of Ca²⁺ transients in intact myotubes strongly support the idea that the L channel is a major contributor to ECCE. These transients were characterized both in myotubes where intracellular release occurred via RYR1 (Fig. 6) and in myotubes in which this release had been blocked by prior exposure to ryanodine (Fig. 7). Under the latter situation, depolarization of normal myotubes, but not dysgenic myotubes expressing SkEIIIK, elicits large Ca²⁺ entry transients. Thus, any Ca²⁺ entry via a pathway other than the L-type channel must be too small to be detected by our experiments (myotubes loaded with Fluo-3-AM). This result raises the obvious question of why previous work found expression of SkEIIIK in dysgenic myotubes restored ECCE as indicated by the entry of either Mn²⁺ or Ca²⁺ (Cherednichenko et al., 2004; their Fig. 4 and not depicted, respectively). One possibility is that SkEIIIK has sufficient permeability to divalent cations to produce a signal detectable by Fura-2 as used by Cherednichenko et al. (2004), but not by Fluo-3 as used in our current experiments. Indeed, our electrophysiological data (Fig. 8) demonstrate that after a brief (200-ms), strong (+80 mV) depolarization in the presence of the DHP agonist ±Bay K 8644, repolarization of cells expressing SkEIIIK results in an inward tail current carried by Ca²⁺ (the only external cation other than TEA). This inward tail current seems likely to be a consequence of the entry of SkEIIIK into a long-duration open state, which has been shown to be promoted in L-type channels by both strong depolarization and DHP agonists (see, for example, Wilkens et al., 2001). Entry into this long-duration open state also occurs for much weaker depolarizations in the absence of DHP agonist as long as these depolarizations are sustained (Pietrobon and Hess, 1990). The long-duration open state of both the wild-type cardiac L-channel and the pore mutant “CEIIIK” appears to be rectifying and to permit the flow of inward divalent current, but not that of outward current (Leuranguer et al., 2003). Thus, it seems a reasonable possibility that SkEIIIK could support a very small inward divalent current during long depolarizations and that this small current might account for the detection of Mn²⁺ entry by means of Fura-2 in myotubes expressing SkEIIIK (Cherednichenko et al., 2004).

In skeletal muscle, the DHPR has dual functions as an L-type Ca²⁺ channel and as the voltage sensor for EC coupling. The physiological role of the L-type Ca²⁺ current has remained obscure because the voltage sensor function of the DHPR does not require the entry of external Ca²⁺ (Fig. 6) (Armstrong et al., 1972; Tanabe et al., 1990; Dirksen and Beam, 1999). However, the results described here support the hypothesis that the L-type Ca²⁺ current mediated by the DHPR is the major (and perhaps sole) component of ECCE. Previously, the Ca²⁺ entry ascribed to ECCE was shown to help maintain myoplasmic Ca²⁺ levels during either sustained depolarizations or during trains of repetitive brief stimuli (Cherednichenko et al., 2008; Gach et al., 2008). It now appears that this role can be reassigned to the L-type Ca²⁺ current and perhaps represents one of its physiological functions in skeletal muscle.

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