A glutamate-to-lysine substitution at position 1014 within the selectivity filter of the skeletal muscle L-type Ca\(^{2+}\) channel (Ca\(_V\)\(1.1\) or 1,4-dihydropyridine) abolishes Ca\(^{2+}\) flux through the channel pore. Mice engineered to exclusively express the mutant channel display accelerated muscle fatigue, changes in muscle composition, and altered metabolism relative to wildtype littermates. By contrast, mice expressing another mutant Ca\(_V\)\(1.1\) channel that is impermeable to Ca\(^{2+}\) (Ca\(_V\)\(1.1\) N617D) have shown no detectable phenotypic differences from wildtype mice to date. The major biological difference between the Ca\(_V\)\(1.1\) E1014K and Ca\(_V\)\(1.1\) N617D mutants elucidated thus far is that the former channel conducts robust Na\(^+\) and Ca\(^{2+}\) currents in patch-clamp experiments, but neither of these monovalent conductances seems to be of relevance in vivo. Thus, the basis for the different phenotypes of these mutants has remained enigmatic. We now show that Ca\(_V\)\(1.1\) E1014K readily conducts 1,4-dihydropyridine-sensitive K\(^+\) currents at depolarizing test potentials, whereas Ca\(_V\)\(1.1\) N617D does not. Our observations, coupled with a large body of work by others regarding the role of K\(^+\) accumulation in muscle fatigue, raise the possibility that the introduction of an additional K\(^+\) flux from the myoplasm into the transverse-tubule lumen accelerates the onset of fatigue and precipitates the metabolic changes observed in Ca\(_V\)\(1.1\) E1014K muscle. These results, highlighting an unexpected consequence of a channel mutation, may help define the complex mechanisms underlying skeletal muscle fatigue and related dysfunctions.

During excitation–contraction (EC)\(^2\) coupling in skeletal muscle, the L-type Ca\(^{2+}\) channel (Ca\(_V\)\(1.1\) or 1,4-dihydropyridine receptor) activates Ca\(^{2+}\) release from the sarcoplasmatic reticulum via the type 1 ryanodine receptor in response to depolarization of the plasma membrane (1–3). Because EC coupling is fast and does not appear to depend upon a soluble second messenger (e.g. Ca\(^{2+}\)), there is general agreement that there is direct or indirect conformational coupling between these two channels within a larger macromolecular signaling complex (3–5). In addition to its primary function as an EC coupling voltage sensor, Ca\(_V\)\(1.1\) also conducts L-type Ca\(^{2+}\) current (3, 6).

To address the importance of Ca\(^{2+}\) flux via Ca\(_V\)\(1.1\) for greater muscle function, two distinct mouse lines have been engineered. Both of these strains carried single amino acid substitutions that rendered the channel impermeable to Ca\(^{2+}\) while sparing EC coupling. In one model, Ca\(_V\)\(1.1\) had a targeted mutation within the selectively filter (E1014K) (7) known to eliminate nearly all divalent flux (5, 8–10). Although the amplitude of myoplasmic Ca\(^{2+}\) release evoked by low-frequency stimulation (LFS; 1 Hz) was virtually identical in flexor digitorum brevis (FDB) fibers obtained from wildtype and homozygous Ca\(_V\)\(1.1\) E1014K mice, Ca\(_V\)\(1.1\) E1014K FDB fibers displayed a pronounced fatigue phenotype because the amplitudes of successive Ca\(^{2+}\) transients decayed more rapidly than in wildtype fibers during high-frequency stimulation (HFS; 50 or 100 Hz) (7). In addition, homozygous expression of the Ca\(_V\)\(1.1\) E1014K channel caused a gain of glycolytic type IIB fibers at the expense of type IIX fibers in extensor digitorum longus and type IIx and type I fibers in soleus muscles (7). Ca\(_V\)\(1.1\) E1014K mice were later reported to develop multiple metabolic deficiencies leading to increased fat mass and overall body weight (11).

A mouse model based on the non-conducting zebrafish \(\alpha_{1S}\)-b isoform has also been generated (12). These mice expressed a Ca\(_V\)\(1.1\) channel having an aspartate for asparagine swap adjacent to the selectivity filter at position 617 (13, 14). In stark contrast to Ca\(_V\)\(1.1\) E1014K mice, Ca\(_V\)\(1.1\) N617D mice had no detectable phenotypic differences from wildtype littermates in a variety of assays. Notably, the authors found no significant effects on body weight, fertility, muscle mass/composition, EC coupling, SR Ca\(^{2+}\) store content, twitch or tetanic force, muscle fatigue following HFS, locomotor function or the expression levels of Ca\(_V\)\(1.1\), and other mediators of Ca\(^{2+}\) signaling in skeletal muscle (e.g. RyR1, Orai1, STIM1, SERCA1, CSQ1, CSQ2, etc.).

Although neither Ca\(_V\)\(1.1\) E1014K nor Ca\(_V\)\(1.1\) N617D conduct appreciable L-type Ca\(^{2+}\) current in either cultured or acutely dissociated muscle cells, the former channel is known to conduct Na\(^+\) and Ca\(^{2+}\) currents. Reduction of external Ca\(^{2+}\) in patch-clamp experiments has revealed inward Ca\(_V\)\(1.1\) E1014K-mediated Na\(^+\) currents (10), whereas the ability of Ca\(_V\)\(1.1\)
E1014K to conduct large-amplitude outward Cs\(^+\) currents was established during the initial characterization of the mutant (5). The selectivity of Ca\(_V\)1.1 N617D for monovalent cations has not yet been defined, but significant outward currents were not observed in experiments performed with 100–145 mM Cs\(^+\) present in the patch pipette (12–14).

The apparent impermeability of Ca\(_V\)1.1 N617D to Cs\(^+\) suggests that the altered selectivity of Ca\(_V\)1.1 E1014K underlies the phenotypic differences between Ca\(_V\)1.1 E1014K and Ca\(_V\)1.1 N617D mice. Obviously, the Cs\(^+\) permeability of Ca\(_V\)1.1 E1014K is inconsequential with regard to phenotype because Cs\(^+\) is not present in significant quantities in skeletal muscle fibers in vivo. Likewise, Na\(^+\) flux via Ca\(_V\)1.1 E1014K is unlikely to be the basis for these differences because the channel opens much too slowly to conduct Na\(^+\) during the upstroke of a single action potential (7). However, the ability of Ca\(_V\)1.1 E1014K to conduct the other physiologically relevant monovalent cation, K\(^+\), has not been determined.

In this work, we expressed YFP-tagged Ca\(_V\)1.1 E1014K and Ca\(_V\)1.1 N617D channels in tsA-201 cells to demonstrate that Ca\(_V\)1.1 E1014K functions as a non-inactivating K\(^+\) channel, whereas Ca\(_V\)1.1 N617D does not. We postulate, based on a large body of earlier work regarding fatigue (reviewed in detail in Ref. 15), that the conversion of Ca\(_V\)1.1 to a K\(^+\) channel within the transverse tubules (triad junctions) of homozygous Ca\(_V\)1.1 E1014K muscle results in enhanced K\(^+\) accumulation leading to accelerated fatigue during periods of high activity. Because changing the selectively of Ca\(_V\)1.1 via the E1014K mutation has not been investigated previously as the mechanism of the accelerated fatigue phenotype, our results may help clarify the impact of L-type Ca\(^{2+}\) flux into skeletal muscle.

**Results**

**Successful expression of YFP-Ca\(_V\)1.1 E1014K in tsA-201 cells**

We first examined whether the Ca\(_V\)1.1 E1014K mutant could be functionally expressed in tsA-201 cells with similar biophysical properties as previously reported for Ca\(_V\)1.1 E1014K expressed in dysgenic myotubes. For this purpose, we constructed a YFP-Ca\(_V\)1.1 E1014K fusion construct similar to the untagged Ca\(_V\)1.1 E1014K channel utilized by Dirksen and Beam (5). tsA-201 cells were then transfected with YFP-Ca\(_V\)1.1 E1014K or YFP-Ca\(_V\)1.1 and β\(_{1\alpha}\), α\(_{5\beta-1}\), and Stac3 auxiliary channel subunits (16); positively transfected cells were identified by YFP fluorescence (Fig. 1A, inset). With 2 mM Ca\(^{2+}\)/150 TEA\(^+\) in the bath (i.e. TEA-Tyrode's solution; see “Experimental procedures”) and ~160 mM Cs\(^+\) in the pipette solution, cells expressing YFP-Ca\(_V\)1.1 E1014K yielded no inward Ca\(^{2+}\) or Na\(^+\) current but supported sizable outward Cs\(^+\) currents \(I_{\text{dens}} = +83.4 \pm 11.6\) pA/pF at +80 mV, \(n = 18\); Fig. 1A) with an current–voltage \((I–V)\) relationship similar to that reported earlier for untagged Ca\(_V\)1.1 E1014K expressed in *dysgenic* myotubes (Fig. 1C) (5, 8). As expected, the cells expressing YFP-Ca\(_V\)1.1 displayed typical inward L-type Ca\(^{2+}\) currents peaking near +30 mV \(I_{\text{dens}} = −4.8 \pm 1.0\) pA/pF, \(n = 12\); Fig. 1B). Importantly, the L-type current mediated by YFP-Ca\(_V\)1.1 did...
**Ca\(_v\).1.1 E1014K conducts K\(^+\)**

![Graph showing charge movements for YFP-Ca\(_v\).1.1 and YFP-Ca\(_v\).1.1 E1014K](image)

**Figure 2. Similar charge movement for YFP-Ca\(_v\).1.1 and YFP-Ca\(_v\).1.1 E1014K.** Representative recordings of charge movements elicited by 20-ms depolarizations from −80 mV to −60, −40, −20, 0, +20, and +40 mV are shown for tsA-201 cells transfected with Ca\(_v\).1.1 E1014K (A) or YFP-Ca\(_v\).1.1 (B) with β\(_{1\alpha}\), β\(_{2\alpha}\), d\(_{1}\), and Stac3. The Q–V relationships for non-transfected tsA-201 cells (Δ\(\alpha\); n = 5) and for tsA-201 cells expressing YFP-Ca\(_v\).1.1 E1014K (n = 18) or YFP-Ca\(_v\).1.1 (n = 12) channels are shown in C. Charge movements were evoked at 0.1 Hz by test potentials ranging from −70 mV through +50 mV in 10-mV increments in the presence of 500 μM Cd\(^{2+}\) and 100 μM La\(^{3+}\). The smooth curves are plotted according to Equation 2 with the following parameters: \(Q_{\text{max}} = 9.1 \pm 1.7 \text{nC/μF}, Q_{\text{rev}} = 7.2 \pm 4.2 \text{mV} \) and −15.7 ± 3.6 mV, and \(k = 14.8 \pm 1.7 \text{mV} \) and 14.2 ± 1.4 mV for YFP-Ca\(_v\).1.1 E1014K and YFP-Ca\(_v\).1.1, respectively.

Not reverse until test potentials nearing +80 mV (\(V_{\text{rev}} = 79.2 ± 2.3 \text{mV}; \) Fig. 1, B and C). tsA-201 cells expressing YFP-Ca\(_v\).1.1 E1014K produced charge movement with similar magnitude and voltage dependence compared with cells expressing YFP-Cav\(_{\text{C}}\).1.1 (\(Q_{\text{max}} = 9.1 \pm 1.7 \text{nC/μF}, n = 18 \) versus 9.5 ± 1.3 nC/μF, \(n = 12\), respectively; \(p > 0.05\), unpaired \(t\) test; Fig. 2, A–C). Five non-transfected cells had no inward current, very little outward current, and no quantifiable charge movement (Figs. 1C and 2C).

**YFP-Ca\(_v\).1.1 E1014K conducts K\(^+\), but YFP-Cav\(_{\text{C}}\).1.1 N617D does not**

Fig. 3A shows a family of currents recorded from a tsA-201 cell expressing YFP-Cav\(_{\text{C}}\).1.1 E1014K with TEA-Tyrode’s solution in the bath and ~150 mM K\(^{+}\) in the pipette. No inward L-type Ca\(^{2+}\) or Na\(^{+}\) currents were detectable, but non-activating outward currents were evident at test potentials greater than 0 mV (\(I_{\text{dens}} = 92.9 ± 14.6 \text{pA/pF} \) at +80 mV; \(n = 16\); Fig. 3, A and D). By contrast, YFP-Ca\(_v\).1.1 N617D produced virtually no outward current above the average tsA-201 background

**Figure 3. Ca\(_v\).1.1 E1014K conducts outward K\(^+\) current, but Ca\(_v\).1.1 N617D does not.** Representative current families elicited by 100-ms depolarizations from −80 mV to −30, −10, +10, +30, +50, and +90 mV are shown for tsA-201 cells expressing YFP-Ca\(_v\).1.1 E1014K (A), YFP-Ca\(_v\).1.1 N617D (B), or YFP-Ca\(_v\).1.1 (C) with β\(_{1\alpha}\), β\(_{2\alpha}\), d\(_{1}\), and Stac3. Please note different scale in C. The red K\(^+\) denotes the outward current carrier. A confocal image confirming successful heterologous expression of YFP-Ca\(_v\).1.1 N617D in tsA-201 cells is shown in the inset of B. Scale bar, 10 μm. D shows a comparison of YFP-Ca\(_v\).1.1 E1014K (○; \(n = 9\)), YFP-Ca\(_v\).1.1 N617D (gray circles; \(n = 9\)), and YFP-Ca\(_v\).1.1 (□; \(n = 7\)) average peak \(I–V\) relationships. The \(I–V\) relationship for non-transfected tsA-201 cells in TEA-Tyrode’s solution (Δ\(\alpha\); \(n = 7\)) is shown. The black circle denotes the outward charge carrier. A confocal image confirming successful heterologous expression of YFP-Ca\(_v\).1.1 N617D in tsA-201 cells is shown in the inset of B. Scale bar, 10 μm. D shows a comparison of YFP-Ca\(_v\).1.1 E1014K (○; \(n = 9\)), YFP-Ca\(_v\).1.1 N617D (gray circles; \(n = 9\)), and YFP-Ca\(_v\).1.1 (□; \(n = 7\)) average peak \(I–V\) relationships. The \(I–V\) relationship for non-transfected tsA-201 cells in TEA-Tyrode’s solution (Δ\(\alpha\); \(n = 7\)) is shown. The current was evoked at 0.1 Hz by test potentials ranging from −50 mV through +90 mV in 10-mV increments. The smooth curve for YFP-Ca\(_v\).1.1 is plotted according to Equation 1 with the following parameters: \(G_{\text{max}} = 371 ± 81 \text{pS/pF}, V_{\text{rev}} = 21.5 ± 4.2 \text{mV}, V_{\text{rev}} = 70.7 ± 2.9 \text{mV}, \) and \(k = 13.9 ± 1.7 \text{mV}\).
The currents were evoked at 0.1 Hz. The exposures acutely to nifedipine. Fig. 4 particular experiment is depicted in Fig. 4A. normalized I–V relationships before (black circles) and at (gray circles) peak inhibition by 10 μM nifedipine (n = 6). The currents were evoked at 0.1 Hz. The numbers correspond to the traces shown in A, C, normalized I–V relationships before (black circles) and at (gray circles) peak inhibition by 10 μM nifedipine (n = 6). Values for individual experiments are represented by (C) and (O). The errors bars in this panel indicate S.D. Significant differences by two-tailed, unpaired t test are indicated.

Thus, the bulk of the K+ current arose primarily from the expressed CaV1.1 E1014K channel rather than via endogenous K+ channels.

**Bi-ionic current recordings from YFP-CaV1.1 E1014K**

Because CaV1.1 E1014K channels conduct Na+ (10) as well as K+, we recorded hybrid Na+ and K+ currents with regular Tyrode's solution in the bath. In these experiments, we observed both inward Na+ and outward K+ current with a bi-ionic reversal potential ~+20 mV (n = 10; Fig. 5A). No inward nor appreciable outward currents were observed in seven recordings made from cells expressing CaV1.1 N617D under identical conditions (Fig. 5B). The bionic current–voltage relationships for tsA-201 cells expressing either YFP-CaV1.1 E1014K or YFP-CaV1.1 N617D are shown in Fig. 5C.

**Discussion**

In this study, we used the newly developed tsA-201 cell heterologous expression system to demonstrate that the CaV1.1

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**Figure 4. K+ currents conducted by CaV1.1 E1014K are sensitive to inhibition by nifedipine.** A, K+ currents recorded before (trace 1) and during applications of 500 nM (trace 2) and 10 μM (trace 3) nifedipine to a tsA-201 cell expressing YFP-CaV1.1 E1014K, β1d, γ2, and Stac3 (holding potential = −80 mV, test potential = +80 mV). B, time course of current decline following nifedipine application. The currents were evoked at 0.1 Hz. The numbers correspond to the traces shown in A, C, normalized I–V relationships before (black circles) and at (gray circles) peak inhibition by 10 μM nifedipine (n = 6). The currents were evoked at 0.1 Hz. The numbers correspond to the traces shown in A, C, normalized I–V relationships before (black circles) and at (gray circles) peak inhibition by 10 μM nifedipine (n = 6). Values for individual experiments are represented by (C) and (O). The errors bars in this panel indicate S.D. Significant differences by two-tailed, unpaired t test are indicated.

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**Figure 5. Ionic currents conducted by CaV1.1 E1014K in the presence of both K+ and Na+.** Representative inward and outward currents recorded with 150 mM Na+ in the bath and 150 mM K+ in the pipette are shown for tsA-201 cells coexpressing either CaV1.1 E1014K (A) or CaV1.1 N617D (B) with β1d, α2δ-1, and Stac3. Bi-ionic current families shown were evoked by 100-ms steps from −80 mV to −30, −10, +10, +30, +50, +70, and +90-mV increments. The blue Na+ and red K+ denote the inward and outward charge carriers, respectively. Average bi-ionic I–V relationships for tsA-201 cells expressing either CaV1.1 E1014K (black circles; n = 10) or CaV1.1 N617D (gray circles; n = 7) are shown in C.
E1014K mutant channel will readily conduct K⁺ (Fig. 3). Although Caᵥ1.1 E1014K is known to conduct Na⁺ current (10), L-type Ca²⁺ channels are unlikely to be open during the upstroke of a single muscle action potential when the membrane potential favors the influx of Na⁺ (7). The same cannot be said unequivocally for the more depolarized stages of the action potential when the driving force for K⁺ is strong (21, 22). Thus, Caᵥ1.1 E1014K is likely to function as a K⁺ channel when knocked in to skeletal muscle, particularly during repetitive activity or prolonged depolarization when the channel enters high Pᵦ gating modes (17, 23).

We have also found that the outward K⁺ current mediated by Caᵥ1.1 E1014K has a similar sensitivity to the DHP antagonist nifedipine (Fig. 4) as has been reported earlier for native and heterologously expressed wildtype Caᵥ1.1 channels in cultured myotubes (8, 18). The sensitivity of Caᵥ1.1 E1014K to nifedipine in our voltage-clamp experiments contrasts with in vitro work showing that the mutation reduces the affinity of the channel for radiolabeled PN200-100 (isradipine) in membrane preparations obtained from tsA-201 cells expressing either wildtype or mutant Caᵥ1.1 α₁S with only β₁α₁ and α₃β₁-1 subunits (24). The results of our experiments indicate that E1014K mutation does not greatly affect DHP sensitivity of Caᵥ1.1 in a cellular context, as suggested by the binding assays of Peterson and Catterall (24).

To investigate the impact of the E1014K mutation on muscle activity, Lee et al. (7) loaded Caᵥ1.1 E1014K FDB fibers with Mag-Fluo4 AM dye and then recorded myoplasmic Ca²⁺ transients elicited by field stimulation. They found that amplitudes and durations of the Ca²⁺ transients remained constant for both wildtype and Caᵥ1.1 E1014K fibers in response to 1 Hz LFS, indicating that the basic mechanism of EC coupling was not likely affected by the mutation. During 50 or 100 Hz HFS, the amplitudes of successive Ca²⁺ transients in wildtype fibers decreased steadily, whereas the amplitudes of Ca²⁺ transients in Caᵥ1.1 E1014K fibers decayed more rapidly (see Fig. 2, A–C, of Ref. 7). In this regard, a role for K⁺ accumulation within the transverse-tubule lumen and other extracellular compartments has been described as a major contributor to action potential/EC coupling failure and subsequent declines in force during some HFS protocols (15, 25–30). During the repolarization phase of the skeletal muscle action potential, K⁺ exits the myoplasm via delayed rectifier K⁺ channels to restricted extracellular compartments before being returned actively to the fiber by the Na⁺/K⁺-ATPase pump, diffusion, etc.) and Cl⁻ removal routes (31) or passively via inward rectifier K⁺ channels when the membrane potential becomes more negative than Eₚ (32–35). During LFS, these clearance mechanisms are sufficient to maintain the K⁺ gradient (31). However, transport by the pump and inward rectifier channels cannot keep pace with the K⁺ efflux via delayed rectifier channels during prolonged depolarization or HFS protocols similar to those employed by Lee et al. (15, 30, 33–39). Even with the assistance of Cl⁻ flux via CIC-1 channels in stabilizing the resting potential, the result is net K⁺ accumulation sufficient to elevate the local membrane potential to a point at which Na⁺ channels inactivate (30, 40). Failure of EC coupling follows because of: 1) the inability of the fiber to propagate action potentials (30, 41) and 2) inactivation of Caᵥ1.1 itself (42).

Based on our observation that Caᵥ1.1 E1014K functions as a triad junction-targeted K⁺ channel (Figs. 3 and 5), we propose a relatively simple model that may explain why failure of EC coupling ensues more rapidly in response to repetitive HFS in Caᵥ1.1 E1014K muscle than in wildtype or Caᵥ1.1 N617D muscle in Fig. 6. As described above, endogenous delayed rectifier K⁺ channels provide an avenue for K⁺ efflux into the lumen during HFS or prolonged depolarization in wildtype muscle (Fig. 6A) (34, 35, 43). The introduction of a substantial K⁺ conductance via every single Caᵥ1.1 channel in Caᵥ1.1 E1014K muscle would accelerate the elevation of the local membrane potential, resulting in action potential and EC coupling failure leading to more rapid declines in force (Fig. 6B). In particular, the probability of EC coupling failure is high because of the high density of Caᵥ1.1 channels in the triad junction subcompartment (44, 45); the abundance of delayed rectifier channels at triad junctions has not been determined, but we have chosen arbitrarily to include these channels at low density relative to Caᵥ1.1 in our model (as opposed to omitting them altogether). Because Caᵥ1.1 N617D, like wildtype Caᵥ1.1, does not conduct appreciable K⁺ at physiological potentials (Fig. 3, B–D), K⁺...
accumulation proceeds at a rate similar to wildtype muscle (Fig. 6C).

The most direct way to test the idea that K⁺ flux via Caᵥ1.1 E1014K accentuates K⁺ accumulation in extracellular compartments would be to assess the reversal potentials of the inward rectifier currents in wildtype, Caᵥ1.1 E1014K, and Caᵥ1.1 N617D fibers before and immediately following HFS or long depolarizations (33–35). Specifically, a higher conductance and more profound depolarizing shift in the reversal potential for the inward rectifier current in Caᵥ1.1 E1014K fibers relative to wildtype and Caᵥ1.1 N617D fibers would be evident if elevated extracellular K⁺ levels underlie the accelerated decay of Ca²⁺ transient amplitudes during HFS. Because our data indicate that nifedipine inhibits K⁺ flux via Caᵥ1.1 E1014K (Fig. 4), the increase in the conductance and the shift in the reversal potential of the inward rectifier current would be reduced or eliminated by application of a DHP antagonist.

The assertion of Lee et al. (7) that Na⁺ influx is via Caᵥ1.1 E1014K is not likely during the upstroke of a single action potential is almost certainly correct, but it is important to note that our results do not completely exclude a role for Na⁺ flux via Caᵥ1.1 E1014K during the later stages of the action potential. However, it would seem that the depolarizing influence of a Na⁺ tail current in Caᵥ1.1 E1014K muscle would be similar to that of Ca²⁺ tail current in wildtype muscle (Fig. 3C). Likewise, the Goldman–Hodgkin–Katz relationship implies that an additional Na⁺ flux into the relatively large volume of the myoplasm would have nearly the same weight on resting membrane potential as K⁺ efflux into the confined space of the transverse tubules, which only account for < 0.5% of the total fiber volume (43, 46, 47). Related to this point, currents recorded in nearly equimolar Na⁺ external and K⁺ internal displayed a bi-ionic reversal potential of ~20 mV, which was depolarized ~15 mV relative to potentials where outward K⁺ currents became visible in the absence of external Na⁺ (compare Fig. 5C with Fig. 3D). Because the bi-ionic reversal potential is the voltage in which the fluxes of Na⁺ and K⁺ are in equilibrium, this parameter does not provide information regarding the absolute flux of either individual ion, other than that the fluxes are equal and opposite at that potential (47). In regard to the model presented in Fig. 6, a very negative Nernst potential (~ −98 mV in skeletal muscle) (47) dictates that K⁺ will exit the myoplasm in vivo at potentials hyperpolarized to the bi-ionic reversal potential if a K⁺ ion occupies the selectivity filter of the open channel.

If Caᵥ1.1 E1014K supports K⁺ efflux during repetitive activity in vivo, the implication is that there is physiological Ca²⁺ flux via the wildtype Caᵥ1.1 channel. Based on the observations of Dayal et al. (12) indicating that the Caᵥ1.1 N617D substitution has no detectable physiological consequences, the conclusion that L-type Ca²⁺ flux is vestigial in muscle of mice and, by extrapolation, other mammals, appears correct (but see Ref. 22). Although our findings support the idea that the N617D mutation is without effect because it simply eliminates an ionic flux that is of very little or no consequence, the fatigue phenotype of Caᵥ1.1 E1014K muscle can be linked reasonably to the additional K⁺ flux that occurs as a result of the altered selectivity of the latter channel. What is not so clear is how this additional K⁺ conductance could cause fiber atrophy, fiber-type switching, altered fatty acid metabolism, decreased energy expenditure, and gain of fat mass in mice over the course of weeks (7, 11). In view of the apparent relationship between aberrant membrane excitability in skeletal muscle and metabolic dysfunction, the need for further investigation is obvious and pressing; our findings provide a necessary first step toward making this connection.

### Experimental procedures

#### Molecular biology

YFP fused-Caᵥ1.1 E1014K and -Caᵥ1.1 N617D were derived from the plasmid YFP-Caᵥ1.1 (48). The generation of YFP-Caᵥ1.1 E1014K was undertaken by Genscript, Inc. Four point mutations (ttc gag to ttt aaa) spanning rabbit Caᵥ1.1 (GenBank™ accession number X05921) bp 3037−3042 were introduced into YFP-Caᵥ1.1 through two rounds of PCR mutagenesis using the 4125-bp template excised from YFP-Cav.1.1 with EcoRI–PmlI restriction enzyme sites. The sequence of the forward primers were 5'-cgcactcagctctgacagctatcaggtctt-3' and 5'-tcactctgtgcccttacag-3' and 5'-aatgtagtggcaggttgcctcagttacgaggg-3'. The sequence of the reverse primers were 5'-cgcactcagctctgacagctatcaggtctt-3' and 5'-aatgtagtggcaggttgcctcagttacgaggg-3'. These primers were used to generate a new 4125-bp fragment containing the four mutations that was then subcloned back into the original vector using EcoRI–PmlI restriction sites. Like the generation of the original untagged Caᵥ1.1 E1014K clone in pCAC6 generated by Dirksen and Beam (SKeIIK) (5), our strategy not only swapped in a lysine codon (aaa) in place of the glutamate codon (gag) at position 1014 but also modified the preceding phenylalanine codon (ttt for ttg) at position 1013 to introduce a DraI restriction site (ttt aaa). The completed construct was verified first by Xhol restriction digest and sequencing at Genscript and later by the presence of the introduced DraI restriction site upon arrival at the University of Colorado.

For YFP-Caᵥ1.1 N617D, a single a-to-g point mutation at bp 1849 of rabbit Caᵥ1.1 was introduced into YFP-Caᵥ1.1 using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies). The sequence of the forward primer was 5'-acaggtgaggtggagctgtgctgatgtgcatc-3' and 5'-gtgtgtgatctgatgtgcatc-3'. The sequence of the reverse primer was 5'-gtgtgtgatctgatgtgcatc-3'. The completed construct was verified by sequencing at the DNA Sequencing Core of the Barbara Davis Center at the University of Colorado. YFP-Caᵥ1.1 and YFP-Caᵥ1.1 N617D expression plasmids were both gifts from Dr. K. G. Beam (University of Colorado School of Medicine).

#### tsA-201 cell culture and transfection

tsA-201 cells (ATCC) were cultured as described previously (49, 50). The transfection mixture contained expression plasmids encoding YFP-Caᵥ1.1, YFP-Caᵥ1.1 E1014K, or YFP-Caᵥ1.1 N617D with β₂α₁, α₁δ₁, and Stac3 auxiliary subunits (16). With the exception of Stac3 (2 μg), 1 μg of each cDNA was used per well. The day after transfection, the cells were trypsinized and replated onto 35-mm Primaria-treated culture dishes (BD Falcon). Successfully transfected cells were used in recording experiments ~24 h later.
CaV1.1 E1014K conducts K+

Ionic current recordings

Pipettes were fabricated from borosilicate glass and had resistances of 2.0–4.0 MΩ when filled with internal solution. For Cs⁺ current recordings, the internal solution consisted of 140 mm cesium aspartate, 10 mm Cs₂-EGTA, 5 mm MgCl₂, and 10 mm HEPES, pH 7.4, with CsOH. For K⁺ current recordings, the internal solution consisted of 130 mm potassium aspartate, 20 mm KCl, 1 mm MgCl₂, 10 mm EGTA, and 10 mm HEPES, pH 7.4, with KOH. To record Na⁺/K⁺ bi-ionic current–voltage relationships, the external solution contained 137 mm NaCl, 4 mm KCl, 2 mm CaCl₂, 2 mm MgCl₂, 10 mm KCl, 5 mm HEPES, 10 mm glucose, and 1 mm 4-aminopyridine, pH 7.4, with NaOH. Otherwise, the external solution contained 145 mm TEA-Cl, 4 mm KCl, 2 mm CaCl₂, 2 mm MgCl₂, 10 mm HEPES, 10 mm glucose, and 1 mm 4-aminopyridine, pH 7.4, with TEA-OH. The latter two solutions are referred to as Tyrode’s solution and TEA-Tyrode’s solution, respectively, in the text of “Results.”

All ionic current recordings were corrected for linear cell capacitance and leakage currents using an on-line −P/4 subtraction protocol. K⁺ or Cs⁺ currents were filtered at 2 kHz and digitized at 5 kHz. Cell membrane capacitance (C_m) was determined by integration of a transient from −80 to −70 mV using Clampex 8.0 or 10.3 (Molecular Devices) and was used to normalize current amplitudes (pA/pF). The time constant for decay of the whole-cell capacity transient (τ_m) was reduced as much as possible using the analog compensation circuit of an Axon 200B amplifier (Molecular Devices). The average values of C_m, τ_m, and access resistance (R_a) for all recordings were 42.3 ± 3.1 pF, 374 ± 42 μs, and 9.3 ± 0.6 MΩ, respectively (n = 98 cells). Where applicable, L-type Ca²⁺ I–V relationships were fitted using the following equation:

\[ I = G_{\text{max}} \times (V - V_{\text{rev}})/[1 + \exp(-(V - V_{1/2})/k_G)] \]  

(Eq. 1)

where \( I \) is the current for the test potential \( V \), \( V_{\text{rev}} \) is the reversal potential, \( G_{\text{max}} \) is the maximum channel conductance, \( V_{1/2} \) is the half-maximal activation potential, and \( k_G \) is the slope factor. Nifedipine (TICI Chemicals) was dissolved in 100% EtOH to make a 10 mm stock solution and then diluted to 10 μM and 500 μM in TEA-Tyrode’s solution just prior to experiments. During experiments, nifedipine working solutions were applied through a manually operated, gravity-driven global perfusion system.

Charge movement recordings

To record charge movements from YFP-CaV1.1 and YFP-CaV1.1 E1014K, the Cs⁺-based internal recording solution was used (see “Ionic current recordings”). For YFP-CaV1.1 N617D, charge movements were recorded with the same internal solution used to record K⁺ currents. In all three cases, the bath solution contained 145 mm TEA-Cl, 4 mm KCl, 2 mm CaCl₂, 2 mm MgCl₂, 0.1 mm LaCl₃, 0.5 mm CdCl₂, 10 mm HEPES, 10 mm glucose, and 1 mm 4-aminopyridine, pH 7.4, with TEA-OH. Linear components of leak and capacitive current were corrected with −P/4 online subtraction protocols. Output filtering was at 5–10 kHz, and digitization was at 25 kHz. For analysis, the ON component of the charge transient (Q_ON) was normalized to C_m and plotted as a function of test potential (V), and the resultant Q–V relationships were fitted according to the following equation:

\[ Q_{\text{ON}} = Q_{\text{max}}/[1 + \exp((V - V)/k_Q)] \]  

(Eq. 2)

where \( Q_{\text{max}} \) is the maximal \( Q_{\text{ON}} \), \( V_Q \) is the potential causing movement of half the maximal charge, and \( k_Q \) is a slope parameter. All experiments were performed at room temperature (−23 °C).

Confocal imaging

Images of live tsA-201 cells expressing either YFP-CaV1.1 E1014K or YFP-CaV1.1 N617D were acquired as described previously (50).

Analysis

The figures were made using the software program SigmaPlot (version 11.0, Systat Software, Inc.). All data are presented as means ± S.E. All statistical comparisons were by unpaired, two-tailed t test, unless otherwise noted. p < 0.05 was considered significant.

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