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Different types of Ca^{2+} channels in mammalian skeletal muscle cells in culture

(electrophysiology/excitation–contraction coupling/excitable membranes)

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ABSTRACT **This paper describes the existence of two pharmacologically distinct types of Ca^{2+} channels in rat skeletal muscle cells (myoballs) in culture. The first class of Ca^{2+} channels is insensitive to the dihydropyridine (DHP) (+)-PN 200-110; the second class of Ca^{2+} channels is blocked by low concentrations of (+)-PN 200-110. The two pharmacologically different Ca^{2+} channels are also different in their voltage and time dependence. The threshold for activation of the DHP-insensitive Ca^{2+} channel is near −65 mV, whereas the threshold for activation of the DHP-sensitive Ca^{2+} channel is near −30 mV. Current flowing through the DHP-insensitive Ca^{2+} channel is transient with relatively fast kinetics. Half-maximal inactivation for the DHP-insensitive Ca^{2+} channel is observed at a holding potential $V_{h0.5}$ = −78 mV and the channel is completely inactivated at −60 mV. Two different behaviors have been found for DHP-sensitive channels with two different kinetics of inactivation (one being about 16 times faster than the other at −2 mV) and two different voltage dependencies. These two different behaviors are often observed in the same myoball and may correspond to two different subtypes of DHP-sensitive Ca^{2+} channels or to two different modes of expression of one single Ca^{2+} channel protein.**

Voltage-dependent Ca^{2+} channels are essential membrane transport systems that provide Ca^{2+} to the internal cellular medium for contraction and secretion. Voltage-sensitive Ca^{2+} channels in cardiac and smooth muscle have been known to be sensitive to a number of pharmacological agents (1, 2), which include dihydropyridines (DHPs) such as nitrendipine, nifedipine, or PN 200-110, phenylalkylamines such as verapamil, D600, or D888, benzothiazepines such as diltiazem, and a number of other types of compounds such as bepridil.

Voltage-dependent Ca^{2+} channels are present in skeletal muscle. They are situated in the transverse (T) tubular system (3–5). Their physiological role is not yet absolutely clear. Some investigators feel that they have no direct role in contraction, whereas others have an opposite opinion (see, for example, refs. 6 and 7). These voltage-sensitive Ca^{2+} channels are blocked by inhibitors of the DHP series (7–9). Skeletal muscle T-tubules have been found to be the richest source of DHP receptors (10) and are currently used for the biochemical isolation of the putative Ca^{2+} channel protein (11, 12). Our results obtained with the whole-cell patch-clamp technique using rat skeletal muscle cells (myoballs) in culture demonstrate the existence of different classes of Ca^{2+} channels in skeletal muscle.

MATERIALS AND METHODS

Rat myoballs were obtained by addition of 10 nM colchicine to primary cultures of thigh muscle of newborn rat (13). Rounded cells (myoballs) were voltage-clamped by the whole-cell variant of the patch-clamp technique (14) after the replacement of the culture medium by an external solution that contained (in mM): tetraethylammonium chloride, 150; $BaCl_2$, 2.5; $MgCl_2$, 1; glucose, 10; Hepes, 10 (titrated to pH 7.5 with KOH or Tris). The internal solution contained (in mM): CsCl, 150; EGTA, 5; glucose, 10; Hepes, 10 (pH 7.2 with KOH or Tris). The absence of both external and internal Na^+ prevented any Na^+ current. K^+ currents were blocked by external tetraethylammonium$^+$ and internal Cs^+. Ba^{2+} was chosen in place of Ca^{2+} to suppress residual outward currents due to Ca^{2+}-mediated permeabilities and Ca^{2+}-induced inactivation of Ca^{2+} channels. The osmolarity of solutions was adjusted to 300 mosM with sucrose.

Patch pipettes (2–6 MΩ) were connected to the headstage (10-GΩ feedback resistor) of the recording apparatus (RK 300, Biologic, Grenoble, France). The signal was filtered (1 kHz), sampled at 2 kHz, stored, and analyzed with a minicomputer system (Plurimat S, Intertechnique, France). Depolarizing pulses were generated by a microprocessor-controlled square-wave generator. Linear leak currents were subtracted by linear extrapolation from leak currents obtained for small depolarizing pulses. All experiments reported herein were performed at 20 ± 2°C and with a holding potential V_h = −90 mV. Statistical data are expressed as mean ± SD × $t_{0.05}$ ($t_{0.05}$ = Student–Froher variable with a confidence coefficient of 0.95).

RESULTS

Existence of Two Pharmacologically Distinct Ca^{2+} Channels and Properties of the DHP-Insensitive Ca^{2+} Channel. Fig. 1*A* shows that depolarizing pulses (500 ms) from a holding potential V_h = −90 mV induced the development of two kinds of inward currents. A rapidly activating inward current occurred for small depolarizing steps (until −30 mV). The peak amplitude of this current increased and the time to peak decreased with increasing step depolarizations. This inward current fully and rapidly inactivated in <100 ms at −30 mV. Progressively larger depolarizing pulses induced the development of a second inward current and the appearance, with a step to −18 mV, of a second peak of current. Depolarization steps to −10 mV and higher increased the second inward current, which progressively masked the early activating current. The presence of the two distinct inward currents is clearly seen in Fig. 1*B*, where peak current amplitudes are plotted against the membrane potential.

The early activating inward current was observed in 75% of the myoballs studied and its amplitude varied from cell to cell. This early activating conductance could be easily studied in 50% of the patch-clamped cells. The properties of

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Abbreviations: DHP, dihydropyridine; TTX, tetrodotoxin.

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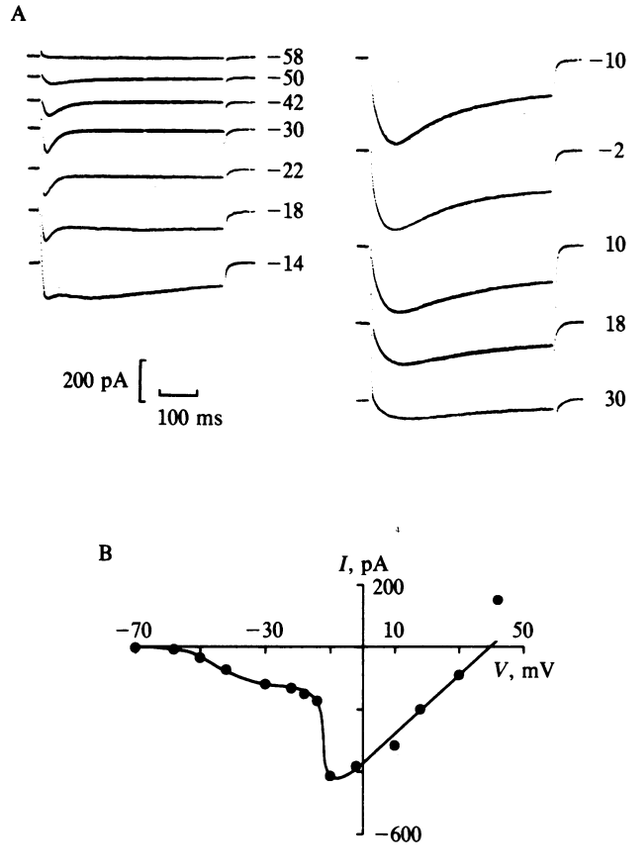

FIG. 1. Voltage dependence of inward currents in 2.5 mM Ba^{2+}. (*A*) Inward membrane currents, recorded with the whole-cell patch-clamp technique, in response to pulses from a holding potential of −90 mV to the indicated membrane potential (in mV) in the presence of 20 μM tetrodotoxin (TTX). (*B*) Peak inward current–membrane potential relationship for the experiments observed in *A*.

this voltage-dependent current are typical of Ca^{2+} channel currents: (*i*) application of a high concentration of TTX (20 μM) did not block or reduce the early activating current (Fig. 2*A*); (*ii*) addition to the bath solution of millimolar concentrations of Co^{2+} (Fig. 2*B*) or other divalent cations such as Mn^{2+} or Cd^{2+} (not shown) induced a rapid and reversible blocking of the early activating inward current; (*iii*) replacement of the external 2.5 mM Ba^{2+} solution by a solution containing 25 mM Ba^{2+} increased the current amplitude (Fig. 2*C*); (*iv*) the amplitude, voltage dependence, and kinetics of this early activating current are nearly identical in 25 mM Ba^{2+} and in 25 mM Ca^{2+} (not shown).

An important observation made in this work was that the early activating low-threshold Ca^{2+} channel was not sensitive to the most potent DHP inhibitor of slow Ca^{2+} channels, (+)-PN 200-110 (9, 11, 12), even at relatively high concentrations (2 μM) of the drug. This property permitted an easy separation of the two kinds of Ca^{2+} channel currents (Fig. 2*D*). After application of 2 μM (+)-PN 200-110 only the early inward current subsisted. The peak current amplitude–potential relationship (*I–V* curve) (Fig. 3*A*) for the DHP-insensitive Ca^{2+} channel indicates an activation threshold near −65 mV and a potential for maximal current amplitude around −30 mV.

The time-to-peak (16 ± 4 ms, $n = 9$, for a pulse to −30 mV) for the DHP-insensitive Ca^{2+} channel is short as compared to the time-to-peak observed for the DHP-sensitive Ca^{2+} channel (70 ms, for a pulse to −2 mV, not shown). Inactivation of the DHP-insensitive Ca^{2+} channel follows first-order kinetics (not shown) with a fast time constant (21 ± 3 ms, $n = 7$, for a test pulse to −30 mV). The steady-state inactivation curve of the DHP-insensitive Ca^{2+} channel (Fig. 3*B*) shows a half-maximal inactivation at a very low membrane potential ($V_{h0.5}$) of −78 mV.

Two Different Behaviors of the DHP-Sensitive Ca^{2+} Channels. Two different types of behavior have been observed for the inactivation phase of the DHP-sensitive Ca^{2+} channel. The first one is illustrated in Fig. 4*A*. In this particular myoball the inward current decayed very slowly (steps to −10, −2, and +10 mV). The second type of behavior is illustrated in Fig. 4*B*. In this myoball, a rapid phase of inactivation occurred before a slower phase was observed. *I–V* relationships for the two types of myoballs are represented in Fig. 4*C*.

Inactivation occurred with a single exponential time course (first-order kinetics) in experiments presented in Fig. 4*A* and with a double exponential time course in experiments shown in Figs. 4*B* and 5*A*. Among 18 different myoballs, a double exponential time course was observed in 14 myoballs and a single exponential time course was observed in only 4 myoballs. Interestingly, the slow time constant found in these

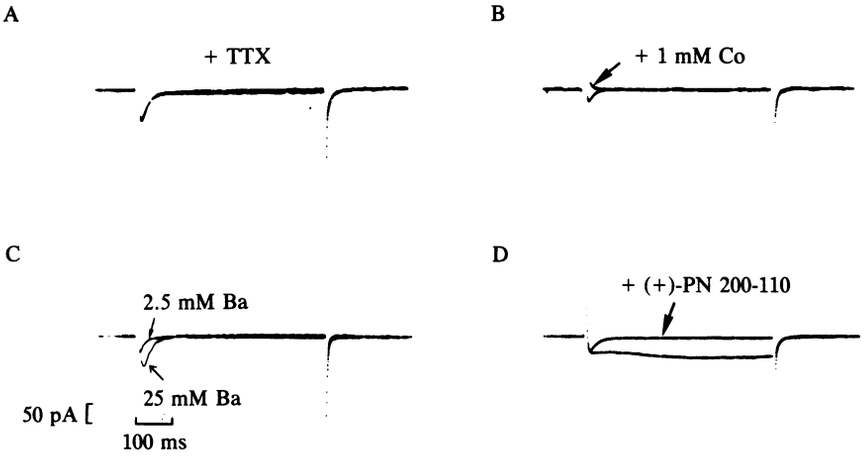

FIG. 2. Effects of TTX, Ba^{2+}, Co^{2+}, and (+)-PN 200-110 on the early inward current. (*A*) The early inward current induced by a pulse to −34 mV was not reduced by 20 μM TTX for 5 min. Control trace and trace in the presence of TTX are superimposable. (*B*) Blocking effect of 1 mM Co^{2+} (after 2 min) on the inward current elicited by a depolarizing step to −34 mV. (*C*) Effect of replacement of the 2.5 mM Ba^{2+} solution by a medium containing 25 mM Ba^{2+}. Time after solution exchange: 2 min. (*D*) Discriminating effect of (+)-PN 200-110 (2 μM) on the two types of inward currents elicited by a step depolarization to −18 mV. Experiments with (+)-PN 200-110 were performed in semidarkness. Time exposure to (+)-PN 200-110: 10 min.

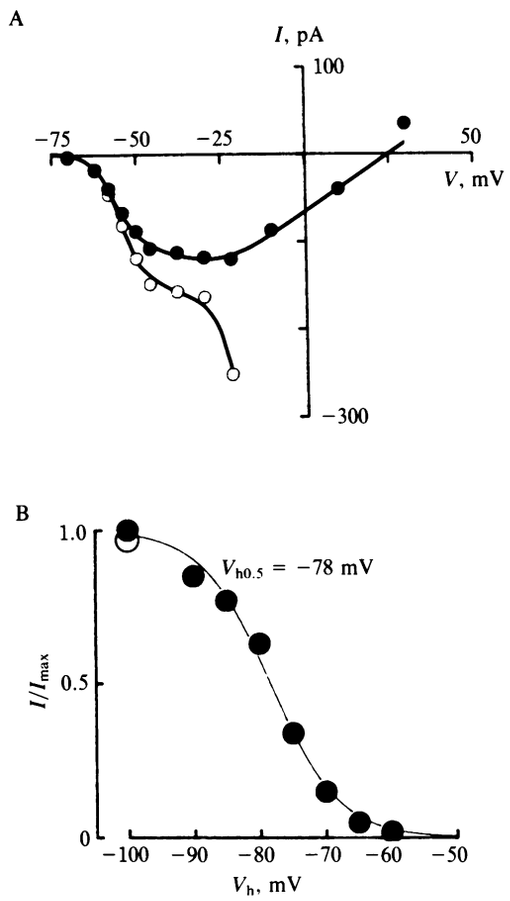

FIG. 3. *I–V* curve and voltage-dependent inactivation of the DHP-insensitive Ca^{2+} channel. (*A*) Peak current–membrane potential relationship in absence (○) and presence of 2 μM (+)-PN 200-110 (●). Time exposure to the drug: 10 min. TTX was also present at 20 μM. (*B*) Steady-state inactivation of the early inward current in 2.5 mM Ba^{2+} with 20 μM TTX. The peak inward current induced by a test pulse to −30 mV is plotted against the holding potential. Experimental points were fitted by the curve (solid line) corresponding to the following equation: $I = I_{max}[1 + \exp(V - V_{h0.5})/k]^{-1}$, with a potential for half-inactivation $V_{h0.5}$ = −78 mV and k = 5.2 mV. Minimal duration of the holding potential (V_h) shift: 30 s. ○, Control current amplitude with V_h at −100 mV after a potential jump from −100 to −60 mV.

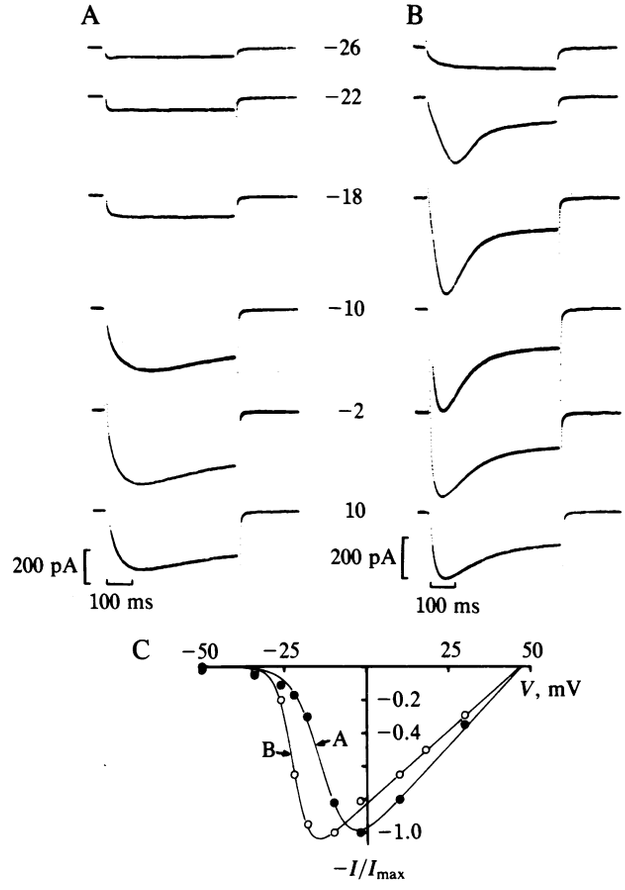

FIG. 4. Two different expressions of DHP-sensitive Ca^{2+} channels. (*A* and *B*) Time course of Ca^{2+} channel current in response to depolarizations at the potential indicated (mV) in two different myoballs. Note in *A* the presence of the early low-threshold inward current for depolarizing pulses to −26 and −22 mV. External solution: 2.5 mM Ba^{2+}. (*C*) Inward current peak amplitude–potential relationship for the experiments in *A* (●) and in *B* (○).

4 myoballs corresponded to the slow time constant found in myoballs that exhibited a double exponential decaying phase for the DHP-sensitive inward current. This is clearly seen in Fig. 5*B*, which shows the values of the time constants in the two types of myoballs at different membrane potential values. Statistical analysis of all of these data indicated a mean of 1.32 ± 0.39 s (n = 18) for the slow time constant and of 81 ± 25 ms (n = 14) for the fast one for a test pulse to −2 mV.

DISCUSSION

The first salient result presented in this paper is the demonstration that there exists in rat skeletal muscle cells in culture two pharmacologically distinct classes of voltage-sensitive Ca^{2+} channels: (*i*) the DHP-sensitive channels, which are blocked by classical Ca^{2+} channel inhibitors such as (+)-PN 200-110, and (*ii*) the DHP-insensitive channels, which are completely resistant to high concentrations of (+)-PN 200-110. These two types of channels are not only pharmacologically different but also different in their voltage and time dependence.

The newly found Ca^{2+} channel type in skeletal muscle is the DHP-insensitive channel. The main properties of this channel are the following. (*i*) It is activated at very low potentials beginning at −65 mV (Figs. 1*B* and 3*A*); its threshold for activation is much lower than that of the DHP-sensitive Ca^{2+} channels (Fig. 4*C*), which is near −30 mV. (*ii*) TheDHP-insensitive Ca^{2+} channel generates a transient inward current with relatively fast kinetics of opening and closing (time to peak of 16 ms for a pulse to −30 mV and a first-order time constant for inactivation of 21 ms for the same pulse). (*iii*) The DHP-insensitive Ca^{2+} channel is largely inactivated near the normal resting potential of rat muscle cells in culture (about −60 mV) and a conditioning hyperpolarization is necessary for its activation.

Interestingly, electrophysiological data on several types of neurones and neuronal cell lines recently have clearly shown the existence of several types of Ca^{2+} conductances (15–24). One of the Ca^{2+} conductances identified in neurones has a low threshold, is activated at relatively negative membrane potentials (−70 to −60 mV), and is transient in nature (refs. 18, 19, 22, 23; reviewed in ref. 24). It appears to be very similar in its properties to the DHP-insensitive Ca^{2+} conductance identified in this paper in muscle cells. A second type of Ca^{2+} conductance has been identified in neurones, which has similar properties to the well-known Ca^{2+} conductance identified in cardiac cells (for a review, see ref. 24). This Ca^{2+} conductance becomes activated at more depolarized potentials and shows a much less marked tendency to inactivate.

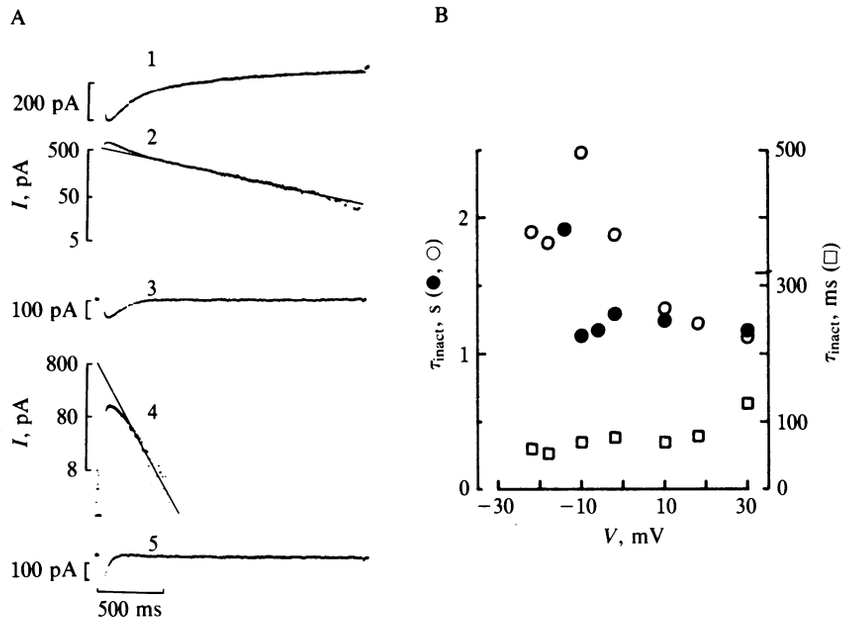

FIG. 5. Inactivation kinetics of DHP-sensitive Ca^{2+} channels. (*A*) Inward current evoked by a test pulse to −2 mV from a holding potential of −90 mV (trace 1). The semilogarithmic representation of trace 1 is shown in trace 2. The straight line provides the time constant (1.25 s) of the slow component of current decay. Subtraction of this slow current component to the initial current, trace 1, provides a new calculated current, trace 3. The same logarithmic treatment was applied to the current in trace 3 and gave a second (rapid) time constant (78 ms) (trace 4). Trace 5 was obtained after subtraction of the sum of the slow and fast current components. (*B*) Voltage dependence of time constants of inward current decays (τ_{inact}) from experiments in Fig. 4*A* (●) and 4*B* (○) correspond to the slow decaying phase and □ corresponds to the rapid decaying phase.

This type of conductance has many similarities with the DHP-sensitive Ca^{2+} channels identified in this paper with rat skeletal muscle cells in culture.

DHP-sensitive Ca^{2+} channels have in fact been shown to display two different behaviors characterized by (*i*) two different kinetics of inactivation and (*ii*) two different voltage dependencies. The existence of two different behaviors for DHP-sensitive Ca^{2+} channels could reflect either the presence of two different subtypes of DHP-sensitive Ca^{2+} channels or two different modes of expression of one single type of DHP-sensitive Ca^{2+} channel. Different subtypes of TTX-inhibitable Na^{+} channels are known to exist (see, for example, ref. 25). Therefore, it may be that different subtypes of DHP-sensitive Ca^{2+} channels also exist. They may correspond to slightly different proteins coded by slightly different genes. Such a situation is well known for isoenzymes. Different modes of opening and closing of DHP-sensitive Ca^{2+} channels are known to exist in cardiac and neuronal cells and have been particularly well revealed in the presence of Ca^{2+} channel agonists such as Bay K8644 (26, 27). Different functional modes could also be created, in principle, by covalent modification of the DHP-sensitive Ca^{2+} channel protein such as by phosphorylation produced by one of the many kinases that are known to regulate ion transport systems.

One important question is what is the physiological significance of the different populations of voltage-sensitive Ca^{2+} channels in mammalian skeletal muscle. DHP-sensitive Ca^{2+} channels have been shown recently to have a role in excitation–contraction coupling (7). DHP-insensitive Ca^{2+} channels may have a role in pacemaker activity linked to spontaneous contractions in noninnervated muscle and in action potential initiation, which both require activation of inward currents at relatively negative potentials.

Future work will be necessary to learn more about the low-threshold DHP-insensitive Ca^{2+} conductance in skeletal muscle. Important problems to solve include (*i*) discovery of a specific pharmacological agent for these channels (this is always an essential step in obtaining a better understanding of a voltage-sensitive channel) (28) and (*ii*) analysis of the developmental properties of these channels and of the role of innervation in their regulation. It is well known that different types of voltage-sensitive channels are expressed at different times of development (29) and that the expression of several of them in skeletal muscle is modulated by the state of innervation (30–34).

After this paper was submitted, two papers appeared describing the existence of subtypes of Ca^{2+} channels in neuronal and cardiac cells (35, 36). In both cases, a DHP-insensitive channel was described that is very similar to the one described here for skeletal muscle. Therefore, this channel seems to be present in most excitable cells.

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