

Calcium Transients Associated with the T Type Calcium Current in Myotubes

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ABSTRACT Immature skeletal muscle cells, both in vivo and in vitro, express a high density of T type calcium current and a relatively low density of the dihydropyridine receptor, the protein thought to function as the I_{slow} calcium channel and as the voltage sensor for excitation-contraction coupling. Although the role of the voltage sensor in eliciting elevations of myoplasmic, free calcium (calcium transients) has been examined, the role of the T type current has not. In this study we examined calcium transients associated with the T type current in cultured myotubes from normal and dysgenic mice, using the whole cell configuration of the patch clamp technique in conjunction with the calcium indicator dye Fluo-3. In both normal and dysgenic myotubes, the T type current was activated by weak depolarizations and was maximal for test pulses to ~ -20 mV. In normal myotubes that displayed T type calcium current, the calcium transient followed the amplitude and the integral of the current at low membrane potentials (-40 to -20 mV) but not at high potentials, where the calcium transient is caused by SR calcium release. The amplitude of the calcium transient for a pulse to -20 mV measured at 15 ms after depolarization represented, on average, $4.26 \pm 0.68\%$ ($n = 19$) of the maximum amplitude of the calcium transient elicited by strong, 15-ms test depolarizations. In dysgenic myotubes, the calcium transient followed the integral of the calcium current at all test potentials, in cells expressing only T type current as well as in cells possessing both T type current and the L type current I_{dys} . Moreover, the calcium transient also followed the amplitude and time course of current in dysgenic myotubes expressing the cardiac, DHP-sensitive calcium channel. Thus, in those cases where the transient appears to be a consequence of calcium entry, it has the same time course as the integral of the calcium current. Inactivation of the T type calcium current with 1-s prepulses, or block of the current by the addition of amiloride (0.3–1.0 mM) caused a reduction in the calcium transient which was similar in normal and dysgenic myotubes. To allow calculation of expected changes of intracellular calcium in response to influx, myotubes were converted to a roughly spherical shape (myoballs) by adding 0.5 μM colchicine to culture dishes of normal cells. Calcium currents and calcium transients recorded from myoballs were similar to those in normal myotubes. The calculated change in calcium due to influx through the T type channel disagreed with the measured calcium concentration, suggesting that additional calcium might be released from the SR or that calcium buffers in the pipette had not equilibrated with the interior of the cell.

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INTRODUCTION

The skeletal muscle dihydropyridine (DHP) receptor functions as both a voltage sensor controlling sarcoplasmic reticulum (SR) calcium release and as a slowly activating calcium channel that gives rise to L type calcium current, I_{slow} (Tanabe, Beam, Powell, and Numa, 1988; Beam, Adams, Niidome, Numa, and Tanabe, 1992). Embryonic and neonatal skeletal muscle from mice and rats express a low level of I_{slow} and a much higher density of T type calcium current, also termed I_{fast} (Beam and Knudson, 1988a). The density of I_{slow} increases in magnitude as a function of postnatal development and it constitutes the main calcium current of adult muscle. In contrast, the T type calcium current becomes progressively smaller until it disappears after the third postnatal week (Beam and Knudson, 1988b). Before its disappearance, the T type channel may be important in developing muscle cells because it contributes a large fraction of calcium current and could represent a means of providing cytoplasmic calcium needed for diverse cellular functions, such as control of gene expression or enzymatic reactions. In dorsal root ganglion cells from chick embryos, it has been shown that the T type calcium current constitutes a disproportionately large fraction of the total calcium influx during a brief action potential (McCobb and Beam, 1991). Moreover, in cultured *Xenopus* neurons at early stages of differentiation, T type calcium current appears able to depolarize cells and trigger action potentials and contributes to spontaneous elevations of intracellular calcium (Gu and Spitzer, 1993). However, the contribution of the T type calcium current to cytoplasmic calcium in embryonic muscle is not known.

In this paper we studied the contribution of the T type calcium current to transient increases of cytoplasmic calcium in cultured muscle cells (myotubes) from normal and dysgenic mice and found that, with square voltage pulses, this channel can permit the influx of calcium sufficient to cause micromolar increases in cellular calcium content, and the influx may induce additional release from internal stores. A preliminary communication of some of the results has been made (García and Beam, 1994a).

MATERIALS AND METHODS

Experiments were performed on cultured cells obtained from skeletal muscle of normal and dysgenic mice. The procedure for the primary culture is described in detail in Beam and Knudson (1988a). Myotubes were studied 7–10 d after initial plating. For some experiments, myotubes were converted to a roughly spherical shape (myoballs) by addition of 0.5 μM colchicine to the primary cultures on the sixth day; the cells were examined on the following day.

Optical Measurements

For simultaneous measurement of calcium transients and membrane current, cells were loaded with Fluo-3 by adding the pentapotassium salt form to the solution contained in the whole-cell patch pipette. Cells in 35-mm culture dishes were mounted on the stage of a Nikon inverted microscope (Diaphot TMD) equipped for epifluorescence with a 75 W Xenon bulb. After rupture of the cell membrane and entry into the whole-cell mode (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981), a period of ~ 5 min was required to achieve adequate loading of the cell with indicator dye. A variable, rectangular slit in the epifluorescence excitation

pathway was adjusted (30–70 μm wide, 100–150 μm long) so that the fluorescent excitation was restricted to a longitudinal portion of the myotube, which excluded the patch pipette. Fluorescent illumination was begun 1 s before the onset of each voltage clamp command sequence by means of a computer-controlled shutter. The set of filters used for Fluo-3 was as follows: band-pass excitation filter centered at 470 nm (half band width 20 nm); dichroic long-pass mirror centered at 510 nm; long-pass emission filter centered at 520 nm. The background fluorescence was measured from each myotube before rupture of the patch and canceled by analogue subtraction. After rupture of the patch and dye entry into the cell, the baseline fluorescence (F_{base}) was monitored. The fluorescence records are expressed as $\Delta F/F$, where ΔF represents an increment in fluorescence from the baseline fluorescence ($\Delta F = F_{\text{transient}} - F_{\text{base}}$), and F is F_{base} (García and Beam, 1994b).

Calcium Concentration Calculations

Calibration of Fluo-3 was performed in our set-up with 20- μm wide capillary glass using “internal solution” and different concentrations of calcium. Values of changes in fluorescence due to increasing calcium concentrations were fitted to a 1:1 calcium:Fluo-3 reaction giving a dissociation constant (K_d) of 350 nM. This value of K_d is very similar to the $K_d = 400$ nM found by Kao, Harootunian, and Tsien (1989) and $K_d = 404$ nM found by Eberhard and Erne (1989). The maximum fluorescence, F_{max} , was obtained at the end of the experiment for each cell by disrupting the membrane with the patch pipette and allowing rapid entry of extracellular calcium into the cell. Similar values of F_{max} were obtained when we used the calcium ionophore A23187. The value for the minimum fluorescence, F_{min} , was calculated from F_{max} (corrected for the background fluorescence) under the assumption that Fluo-3 undergoes a 40-fold increase in fluorescence when bound to calcium (Kao et al., 1989). With these values, we used the equation:

$$[\text{Ca}^{2+}] = K_d^* \frac{F - F_{\text{min}}}{F_{\text{max}} - F}$$

to calculate the free calcium concentration inside the cells. To calculate intracellular buffering, a K_d of 5.6×10^{-3} M was used for magnesium binding to Fluo-3 as reported by Lattanzio and Bartschat (1991) at pH 7.4 and 21°C. For EGTA, the K_d was taken to be: 6.45×10^{-8} M and 1.17×10^{-12} M for calcium and magnesium, respectively (Fabiato, 1981, 1985; pH 7.4 and 21°C). The myofibrillar space was considered to be 80% of the total volume of the cells, based on ultrastructural parameters reported by Eisenberg and Kuda (1976) for fast-twitch fibers from adult guinea pigs. Calcium was considered to freely diffuse and react with Fluo-3 in this space.

Electrical Measurements

Transmembrane currents were recorded using the whole-cell configuration of the patch clamp technique (Hamill et al., 1981). Analogue compensation was used to reduce the effective series resistance, usually to < 1 M Ω . Cell capacitance, which was determined by integration of the control current elicited by a 30-mV hyperpolarization from the holding potential, was used to calculate the density of calcium currents (pA/pF). Test currents were corrected for remaining components of linear capacitative and resistive current by digital scaling and subtraction of the average of 10 control currents.

In the majority of the experiments, the voltage clamp command started from a holding potential of -80 mV to measure calcium currents and calcium transients. In the rest of the

experiments, a prepulse protocol (Adams, Tanabe, Mikami, Numa, and Beam, 1990) was used instead. In this protocol, voltage is first stepped from the holding potential (-80 mV) to -30 or -40 mV for 1 s and then to -50 mV for 25–30 ms, to varying test potentials for 15 ms, to -50 mV for 25–30 ms, and finally back to the holding potential again. Voltage clamp command sequences were applied once every 20 s.

Electrical and optical signals were sampled simultaneously at either 1, 5, or 10 kHz. Analogue filtering was set at 1–2 kHz for the electrical signals (8-pole Bessel filter) and at 0.1 kHz for the optical recordings (2-pole Bessel filter). All the records shown are single traces for both calcium currents and calcium transients.

Injection and Expression of pCARD1

On the sixth to seventh day after initial plating of dysgenic myoblasts into primary culture, myotube nuclei were microinjected with the expression plasmid pCARD1, which carries a cDNA insert encoding the rabbit cardiac muscle DHP receptor (Mikami, Imoto, Tanabe, Niidome, Mori, Takeshima, Narumiya, and Numa, 1989). Both the procedures for microinjection and identification of the cells expressing the plasmid are described in Tanabe, Mikami, Numa, and Beam (1990). The concentration of the cDNA in the injection pipette was 0.5–1 $\mu\text{g}/\mu\text{l}$. Myotubes were examined 2–4 d after the injection.

Solutions

Patch pipettes were made from borosilicate glass and had resistances of 1.6–2.1 M Ω when filled with internal solution, which contained (in millimolar): 145 Cs-aspartate, 10 HEPES, 5 Mg₂Cl, 0.1 Cs₂EGTA, and 0.2 K₅Fluo-3. The external solution contained (in millimolar): 145 TetraethylammoniumCl, 10 HEPES, 10 CaCl₂, and 0.003 TTX. The pH of the internal and external solutions was adjusted to 7.4 with CsOH.

Fluo-3 was obtained from Molecular Probes Inc. (Eugene, OR). Amiloride and colchicine were obtained from Sigma Chemical Co. (St. Louis, MO). Amiloride was prepared daily and dissolved in the external solution. Colchicine was prepared as a 200- μM stock solution in 35% ethanol and used at a concentration of 0.5 μM in medium. The bath volume of the recording chamber was ~ 0.5 ml. The bath was exchanged with at least 20 ml of any new solution. Temperature (22–24°C) was monitored with a temperature probe placed within the recording chamber. Data are presented as mean \pm SEM, with the number of experiments in parentheses.

RESULTS

Calcium Currents and Calcium Transients at Low and High Membrane Potentials in Normal Myotubes

Fig. 1 shows calcium currents and calcium transients elicited at different membrane potentials in a normal myotube. Test pulses to -30 mV (lasting 200 ms) and -20 mV (lasting 100 ms) elicited a large calcium current with the characteristics of a T type current (Nowycky, Fox, and Tsien, 1985): fast activation kinetics and time-dependent inactivation. In addition, calcium transients (noisier traces) were detected at the same membrane potentials. The calcium transients showed a slow rising phase and (in most of the myotubes) reached a steady level by the end of the 100–200-ms pulses and decayed very slowly after repolarization. The threshold for activation of both the

T type calcium current and the calcium transient was near -40 mV with 200-ms pulses. The peak amplitude of the T type calcium current, measured with 100–200 ms pulses at -20 mV, averaged -1.91 ± 0.39 pA/pF ($n = 22$), while the amplitude of the calcium transient at the same potential averaged 0.52 ± 0.14 $\Delta F/F$ ($n = 17$), measured at the end of the pulses. Myotubes that did not have T type calcium current did not display calcium transients in response to 200-ms test pulses to low membrane potentials. However, even in myotubes lacking T-type current, a small calcium transient was detectable towards the end of very long (500–1,000 ms) test pulses to -30 mV, presumably as a result of weak activation of the normal process of excitation-contraction coupling.

Because the internal solution contained 0.1 mM EGTA and 0.2 mM Fluo-3 as the only calcium buffers, myotubes were tested at stronger depolarizations with com-

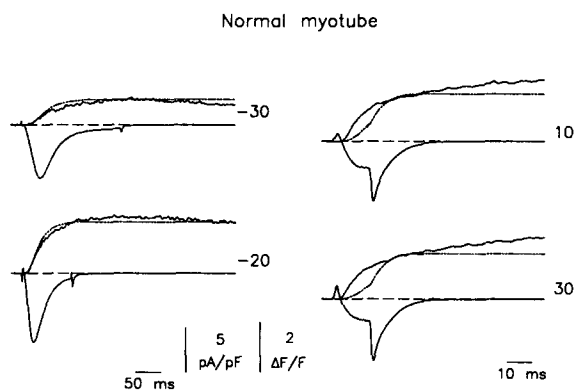


FIGURE 1. Calcium transients and calcium currents recorded from a normal myotube displaying T type current and I_{slow} . In this and all subsequent figures, the calcium transient is the noisier trace which remains at or above the dashed baseline. The membrane potential during the test pulse is indicated at the right. Test pulses to -30 mV (200 ms) and -20 mV (100 ms) elicited T type calcium current. Pulses to posi-

tive potentials were 15 ms in duration and elicited mainly I_{slow} . The dashed-dotted line at each potential corresponds to the integral of the current. Note that the calcium transient followed the time course of the T type current (-30 and -20 mV) but not of I_{slow} (10 and 30 mV). The maximum value of the integrals for the different potentials was: 270 pC at -30 mV, 225 pC at -20 mV, 111 pC at 10 mV, and 103 pC at 30 mV. 2 U $\Delta F/F$ is equivalent to an increase in calcium concentration of 50 nM.

mand pulses lasting only 15 ms to prevent dislodgement of the patch pipette due to movement. For these stronger depolarizations, the current is mainly carried by I_{slow} , with a small contribution from the T type calcium current. Thus, an average decrease of $2 \pm 0.2\%$ ($n = 7$) in the current was observed at stronger depolarizations when the prepulse protocol was applied to inactivate T type current, selectively. The main effect of inactivating T type current is to cause activation to be slower than when the T type calcium current was present. For the cell illustrated in Fig. 1, the contribution of T type current, especially at 10 mV, is larger than in most normal myotubes because the cell had a particularly large T type current (-7.68 pA/pF at -20 mV). On average, the maximum amplitude of the calcium current at positive potentials (20–30 mV), which corresponds mainly to I_{slow} , was -3.76 ± 0.30 pA/pF ($n = 20$) and the maximum amplitude of the calcium transient was 1.6 ± 0.48 $\Delta F/F$ ($n = 19$),

measured at the end of the 15-ms test pulses. For comparison, the amplitude of the T type calcium current at -20 mV, measured after 15-ms depolarization, had a mean amplitude of -1.26 ± 0.22 pA/pF ($n = 24$), while the mean amplitude of the calcium transient measured at the same time was 0.07 ± 0.02 $\Delta F/F$ ($n = 24$). Thus, the amplitude of the calcium transient at 15 ms after depolarization and at -20 mV (potential at which the T type calcium current has its peak amplitude) represented $4.26 \pm 0.68\%$ ($n = 19$) of the maximum amplitude of the calcium transient at strong test depolarizations. For these strong depolarizations, the transient is almost entirely due to calcium release from the sarcoplasmic reticulum under control of the voltage sensor. This idea is supported by experiments using application of amiloride to block, or prepulses to inactivate, T type calcium current (see below).

The presence of calcium transients at low membrane potentials only in myotubes with T type calcium current, suggests that calcium entry through T type channels can appreciably elevate intracellular calcium. If this were the case, then the calcium transient should follow the integral of the calcium current at low membrane potentials. Fig. 1 shows the integral of the calcium currents (*discontinuous lines*) superimposed on the traces corresponding to the calcium transients at the four indicated potentials. Note that the calcium transient follows the integral of the current at -30 and -20 mV very closely, but not at 10 or 30 mV. At high potentials, the calcium transient precedes the integral of the current, indicating that the transient is not the result of calcium influx through the slowly activating calcium channel. In normal myotubes, the calcium transient measured with weak command pulses might contain a contribution from calcium released from the SR under control of the voltage sensor. Thus, the experiments described below were designed to examine further the contribution of calcium influx through the T type calcium channel to intracellular calcium transients.

Calcium Transients Related to Calcium Currents in Dysgenic Myotubes

Because skeletal muscle from dysgenic mice expresses T type calcium current but does not express the DHP receptor that constitutes the voltage sensor and the slowly-activating calcium channel from normal muscle, it provides an excellent system to study the relationship of the calcium transient with the T-type calcium current in isolation over a wide range of membrane potentials. Fig. 2A shows the T type calcium current and the calcium transients obtained from a dysgenic myotube stimulated with 200-ms test pulses to varying test potentials. The T-type calcium current appeared at -40 mV, reached a maximum at -20 mV and then decreased in amplitude for stronger depolarizations. The calcium transient also appeared at -40 mV but was very delayed in relation to the start of the pulse. The transient initially increased in amplitude for increasing test depolarizations, reached a maximum at -20 mV and decreased in amplitude thereafter. Thus, the amplitude of the calcium transient mirrored the amplitude of the T type calcium current. Fig. 2B illustrates the amplitude of the T type calcium current, the amplitude of the calcium transient, and the value of the integral of the calcium current as a function of test potential. The smooth curve through the data points for the current was fitted as described in the figure legend. The same curve was inverted and normalized to the maximum

amplitude of the calcium transient and plotted in the upper part of Fig. 2 *B*. Clearly, in dysgenic myotubes, the amplitude of the calcium transient has the same voltage dependence as the amplitude of the T type calcium current and has a close relationship with the integral of the current. The average amplitude of the peak T type calcium current at -20 mV in dysgenic myotubes was -2.09 ± 0.36 pA/pF ($n = 17$) with 100–300 ms test pulses. The average amplitude of the maximum calcium transient measured at the end of the test pulse, was 0.12 ± 0.02 $\Delta F/F$ for the same myotubes. Under the same conditions in normal myotubes, the maximum

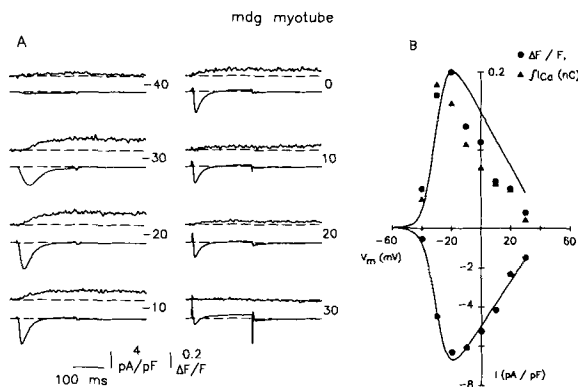


FIGURE 2. T type calcium current and calcium transients in dysgenic myotubes. (A) At each of the indicated test potentials, the calcium transient is illustrated on the upper baseline and the membrane current on the lower baseline. A calcium current was detected at -40 mV, reached a maximum amplitude at -20 mV and then decreased again for stronger test pulses. This dysgenic myotube did not have I_{dys} , as evi-

denced from the records obtained at positive potentials. The calcium transient mirrored the amplitude of the T type calcium current. (B) The peak amplitude of the calcium current and the calcium transient, measured as the average of the final 10 points (transient) or 5 points (current) at the end of the test pulse, is plotted as a function of test potential. Peak currents have been normalized by linear cell capacitance. Same cell as in A. The smooth curve for the calcium current data was obtained by fitting of the equation:

$$I = G'_{\max}(V - V_{\text{rev}})/[1 + \exp \{(V'_{G1/2} - V)/k'_G\}],$$

which gave values of $G'_{\max} = 114$ nS/nF, $V_{\text{rev}} = 43.3$ mV, $V'_{G1/2} = -30.2$ mV, and $k'_G = 4.1$ mV. The smooth curve through the calcium transient data was obtained by inverting and scaling the smooth curve through the current data.

calcium transient was slightly larger, 0.52 ± 0.14 ($n = 17$), presumably because voltage sensor-triggered calcium release contributed to the transient.

In addition to T type calcium current, some dysgenic myotubes express a low density of I_{dys} , an L type calcium current that differs from I_{slow} in having faster activation kinetics and a higher sensitivity to dihydropyridines (Adams and Beam, 1989). Fig. 3 shows the results obtained from a dysgenic myotube that expressed both T type calcium current and I_{dys} . The command pulses were 200 ms in duration for the voltages from -50 to -30 mV and 100 ms for the rest. As for the dysgenic myotube of Fig. 2, the T type current appeared at -40 mV (Fig. 3 A) and increased in amplitude with stronger depolarizations. However, unlike the cell of Fig. 2 A, the total amplitude of the inward current did not decrease at potentials positive to -20

mV because of the increasing contribution of I_{dys} which activates over a more positive range than T type channels. Also, unlike the myotube of Fig. 2, the amplitude of the calcium transient continued to increase beyond -20 mV. The most likely explanation for this behavior is that the transient at higher potentials is due to the entry of calcium via I_{dys} , because dysgenic myotubes do not have voltage sensors capable of eliciting release of calcium from the sarcoplasmic reticulum (Tanabe et al., 1988). Fig. 3 B compares the calcium transient and the integral of the calcium current for test potentials to -30 , -20 , and 30 mV. In contrast with normal myotubes, the calcium transient follows the integral of the inward current, even at strong test potentials.

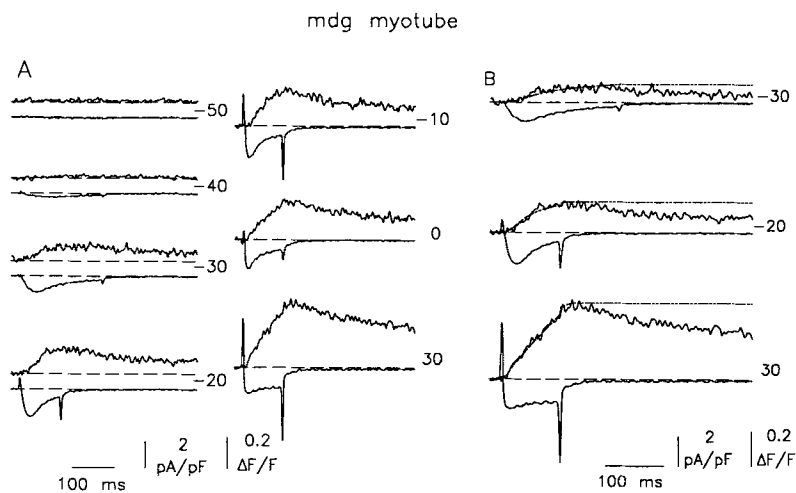


FIGURE 3. Calcium transients and calcium currents recorded from a dysgenic myotube displaying T type current and I_{dys} . (A) Numbers at the right indicate the membrane potential during the test pulse. Test pulses of -50 to -30 mV (200 ms) elicited only T type calcium current. Test pulses of -20 to 30 mV (100 ms) elicited T type calcium current and I_{dys} . (B) Comparison of integrated calcium current (*dashed-dotted line*) and calcium transient at test potentials of -30 , -20 , and 30 mV. The calcium transient followed the integral of the current at all membrane potentials.

Inactivation of T Type Calcium Current and the Calcium Transient

Another experiment to further test the possibility that the calcium transient is due to calcium influx through the T type calcium channel is presented in Fig. 4. Both normal and dysgenic myotubes displaying T type calcium current were stimulated with a 200-ms test pulse to -30 mV to elicit T type current and a calcium transient. After a rest period, application of the same test pulse was preceded by a 1-s prepulse to inactivate the T type current partially or completely. The pulse protocols are represented schematically at the bottom of Fig. 4. From holding potential of -80 mV (a), both the T type current and calcium transient were largest. After a prepulse to -40 mV (b), both the T type calcium current and calcium transient were reduced in

amplitude and both were completely suppressed after a prepulse to -30 mV (c). This result further strengthens the idea that the calcium transient at low membrane potentials in both normal and dysgenic myotubes is caused by the T type calcium current.

Blockade of the T Type Calcium Current Decreases the Amplitude of the Calcium Transient

As another approach for examining the contribution of T type calcium current to myotube calcium transients, we used 0.3–1.0 mM amiloride, which has been shown to block the T type calcium channel in mouse neuroblastoma cells (Tang, Presser, and Morad, 1988) and in chick dorsal root ganglion cells (McCobb and Beam, 1991) with little effect on the high-threshold calcium current. The effect of 0.5 mM amiloride on

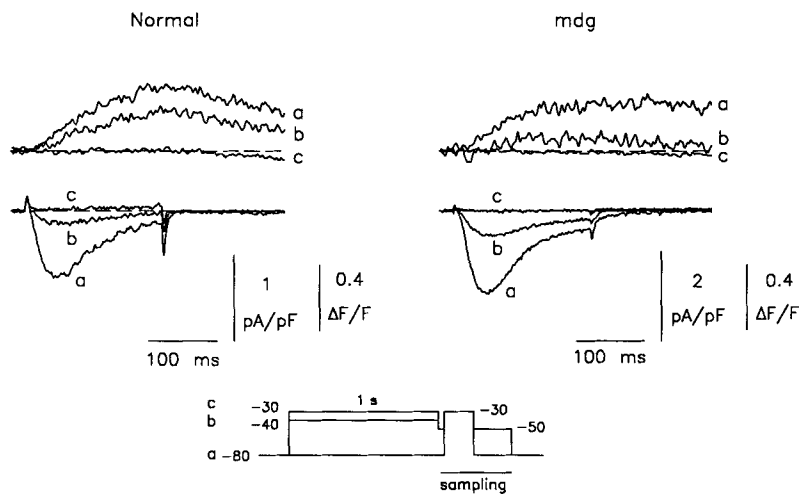


FIGURE 4. Inactivation of the T type calcium current and calcium transient with conditioning prepulses. Calcium currents and calcium transients were recorded with 200-ms test pulses to -30 mV from a normal (left) and a dysgenic (right) myotube without a conditioning prepulse (a) and after a 1-s prepulse delivered to -40 mV (b) or to -30 mV (c). The command voltage steps are depicted schematically at the bottom of the figure. The T type calcium current was reduced or completely inactivated by the prepulses and the calcium transient showed a parallel reduction.

the calcium currents and transients in normal and dysgenic myotubes is illustrated in Fig. 5. In normal myotubes under control conditions, a test pulse to -20 mV elicited a T-type calcium current and a slowly activating calcium transient, while a test pulse to 20 mV elicited a calcium current largely due to I_{slow} and a larger and more rapidly activating calcium transient. In dysgenic myotubes under control conditions, both the T type calcium current and calcium transient were smaller for a test pulse to 20 mV than for a test pulse to -20 mV. For 100–200 ms test pulses to -20 mV, amiloride had a similar effect on normal and dysgenic myotubes, causing an average reduction in the T type calcium current of $74.9 \pm 5.1\%$ (normal, $n = 8$) and $70.1 \pm 2.6\%$

(dysgenic, $n = 7$), and an average reduction in the calcium transient of $55.8 \pm 9.4\%$ (normal, $n = 8$) and $60.7 \pm 7.4\%$ (dysgenic, $n = 6$). At positive test potentials (15 ms pulse duration), amiloride had only little effect on normal myotubes, causing an average reduction of just $2.4 \pm 5.6\%$ ($n = 9$) in the calcium current and $26.4 \pm 17.4\%$ ($n = 9$) in the transient. This reduction in the transient can probably be accounted for by ongoing rundown. In control experiments with sham changes of solution, the transient was reduced by $19.3 \pm 10.6\%$ ($n = 7$). Amiloride was found to be without effect on charge movement in normal myotubes for either weak or strong test

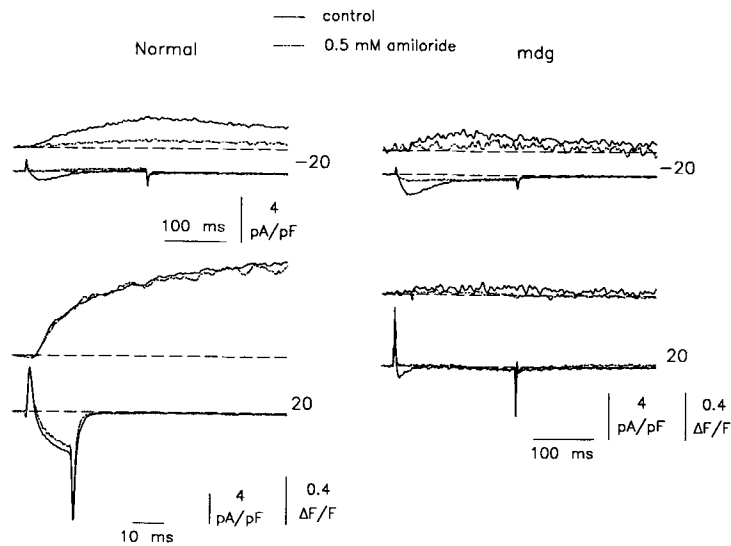


FIGURE 5. Effect of amiloride on calcium currents and calcium transients recorded from a normal (*left*) and a dysgenic (*right*) myotube. The test pulses were 200 ms in duration for the dysgenic myotube and for the normal myotube at -20 mV. The test pulse to 20 mV for the normal myotube was 15 ms in duration. Addition of 0.5 mM amiloride greatly reduced the amplitude of the T type calcium current with a consequent decrease in the calcium transient (dysgenic myotube at -20 and 20 mV, normal myotube at -20 mV). In the normal myotube, amiloride had little effect at 20 mV, indicating that T channels contributed little to either the total current or calcium transient at this potential.

depolarizations (data not shown). As in normal myotubes, amiloride had a smaller effect on dysgenic myotubes at strong depolarizations (measured at 15 ms after the onset of the test pulses to 20 – 30 mV), causing average reductions of $20.8 \pm 18.7\%$ ($n = 4$) for the current and $55.0 \pm 23.6\%$ ($n = 3$) for the transient, than at weak depolarizations. This smaller effect of amiloride can be explained by a significant contribution (in the cells studied) of I_{dys} to the total current and the transient at these potentials. Amiloride did not have any effect on the amplitude of I_{dys} (data not shown).

Calcium Transients Elicited by Cardiaclike Calcium Current in Dysgenic Myotubes

In another set of experiments we compared calcium transients produced by T type calcium current with calcium transients in dysgenic myotubes injected with pCARD1,

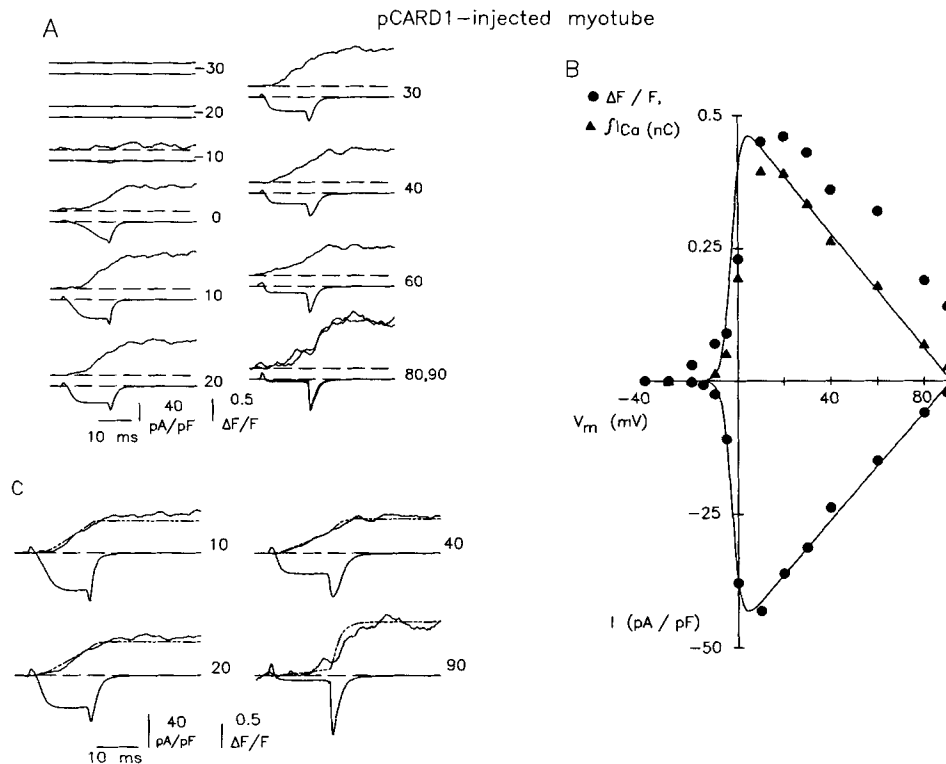


FIGURE 6. Calcium transient and calcium current expressed in dysgenic myotubes injected 2 d earlier with a plasmid encoding the cardiac DHP receptor. (A) Cardiaclike, rapidly activating calcium current and calcium transient recorded at different membrane potentials with 15-ms test pulses using the prepulse protocol. Note that the amplitude of the transient and current increase and then decrease in parallel with increasing test depolarization. At any given test pulse, the transient shows an additional increase (particularly noticeable at +80 and +90 mV) that coincides with the large, inward tail of calcium current that occurs when potential is repolarized at the end of the test pulse. (B) The peak amplitude of the calcium currents and the calcium transients shown in A are plotted as a function of test potential. The current was measured as the average of five points at the end of the 15-ms test pulses and the transient as the average of the final 10 points. The smooth curve through the current data was obtained by fitting the equation described in the legend to Fig. 2, which yielded values of $G'_{\max} = 506$ nS/nF, $V'_{\text{rev}} = 91.6$ mV, $V'_{G1/2} = -2.8$ mV, and $k'_C = 1.9$ mV. The smooth curve through the calcium transient data was obtained by inverting and scaling the curve fitted to the current data. (C) Comparison of integrated current (dashed-dotted line) and calcium transients at test potentials of 10, 20, 40, and 90 mV. The calcium transient followed the integral of the expressed cardiac current at all the potentials in the same manner as the transient followed the integral of the endogenous T type current and I_{dys} (cf. Fig. 3 B).

an expression plasmid encoding the DHP-sensitive calcium channel of rabbit cardiac muscle (Mikami et al., 1989). Fig. 6 illustrates calcium currents and transients in a pCARD1-injected dysgenic myotube, which was stimulated with 15-ms test pulses using the prepulse protocol in order to study in isolation the transient associated with the expressed, cardiac channel. As reported previously (Tanabe et al., 1990; García et

al., 1994), injection of pCARD1 resulted in expression of a rapidly activating calcium current and a calcium transient which was first detected at the same test potential (or sometimes ~ 10 mV larger) as that required to elicit detectable calcium current (Fig. 6A). The amplitude of both current and transient (measured at the end of the 15-ms test pulses) initially increased with increasing depolarization, reached a maximum, and then decreased for still stronger depolarizations (Fig. 6B). Thus, like transients associated with T type calcium current (compare with Fig. 2B), the transient in pCARD1-injected cells mirrored the amplitude of the calcium current. Note that for very strong depolarizations, close to the reversal potential for the calcium current (80 or 90 mV in Fig. 6A), both the restored current and the calcium transient were small during the pulse. However, upon repolarization, there was a large increase in calcium influx that caused a sizeable calcium transient. Fig. 6C compares the time course of the transient with that of the integral of the pCARD1 current for several membrane potentials. Similar to the dysgenic myotubes that express T type current and/or I_{dys} , the calcium transient followed the integral of the current at all the membrane potentials.

Calcium Currents and Calcium Transients in Myoballs

Calculating the change in intracellular calcium expected to result from the T type calcium current requires knowing cell volume, which is difficult for myotubes owing to their irregular shape and nonuniform thickness. Thus, we measured calcium currents and transients in spherically shaped cells (myoballs) produced by the addition of colchicine to cultures of normal myotubes (Fig. 7). As in normal myotubes, weak depolarizations elicited mainly T type calcium current and strong depolarizations mainly I_{slow} . Peak current amplitudes in the myoballs were -1.04 ± 0.22 pA/pF ($n = 11$) for 100–200-ms test pulses to -20 mV and -2.07 ± 0.31 pA/pF ($n = 11$) for 15-ms pulses to 20 or 30 mV, values about half those of normal myotubes. As in normal myotubes, the calcium transients in myoballs first appeared at the same test potential as the T type calcium current and then increased in amplitude with increasing test depolarizations to a saturating value at positive potentials. The average amplitude of the calcium transient was $0.71 \pm 0.14 \Delta F/F$ ($n = 11$) for 200-ms pulses to -20 mV and 1.07 ± 0.24 ($n = 11$) for 15-ms pulses to 20 or 30 mV, values similar to those of normal myotubes.

In the same way as in normal myotubes, the calcium transient in myoballs followed the integral of the T type calcium current at low test potentials (Fig. 7B). This result suggests that calcium transients at low potentials in myotubes are little influenced by longitudinal diffusion of calcium away from sites of entry through T type channels. Although the time course of the transient in myoballs was in good agreement with the integral of the T type current, its magnitude was not. Integrating the T type current, under the assumption that myofibrillar space was 80% of total cell volume (average diameter of myoballs = 42.7 ± 2.6 μm ; range of 32 to 57 μm), yielded a predicted increase in intracellular calcium of 3.35 ± 0.78 μM ($n = 11$, range of 1.4 to 8.0 μM). If the calcium buffers present in the pipette (0.1 mM Fluo-3, 0.1 mM EGTA) equilibrate with the inside of the cell, this increase in intracellular calcium should have raised free calcium by ~ 12 nM, whereas the actual increase of 199.64 ± 37.24 nM ($n = 11$, range 80 to 412 nM), calculated from the fluorescence signal, was much

larger. A possible explanation for this discrepancy could be that calcium influx through the T type channel elicits calcium release from an internal store, giving rise to a bigger calcium transient than expected from entry alone. Alternatively, the concentration of the calcium buffers may be much less inside the cells than in the pipette.

DISCUSSION

We have used the voltage clamp technique to study transient changes of intracellular calcium associated with T type calcium current in skeletal muscle in culture. In normal myotubes which displayed T type calcium current, the calcium transient

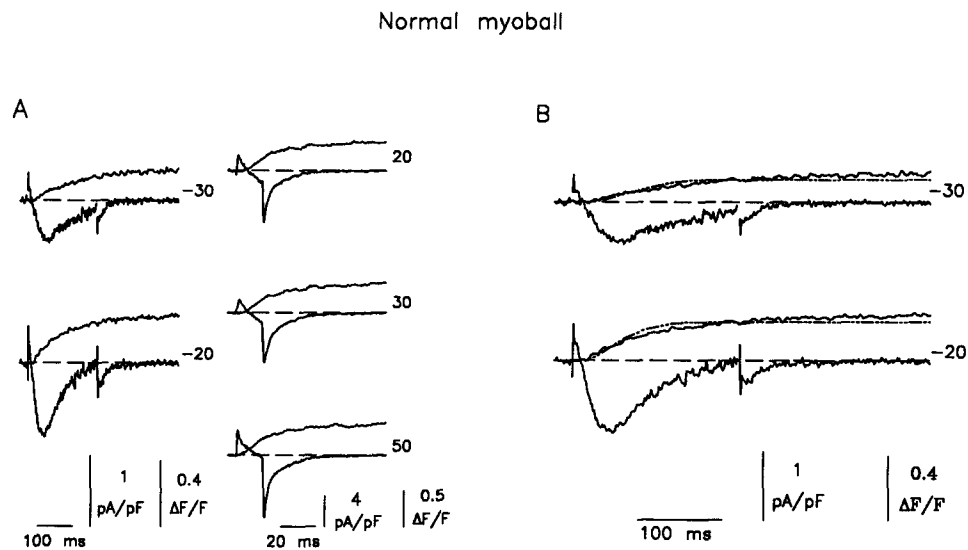


FIGURE 7. Calcium transients and calcium currents recorded from a myoball with T type current and I_{slow} . (A) Test pulses to -30 and -20 mV (200 ms) elicited only T type calcium current, while the pulses to positive potentials (20 ms) elicited mainly I_{slow} . (B) The integral of the calcium current and the calcium transient are compared for test potentials of -30 and -20 mV. The calcium transient followed the integral of the current at these potentials.

followed the integral of the current at low test potentials which preferentially elicited T type current (-40 to -20 mV), but not at high test potentials where the calcium transient is thought to be caused by calcium released from SR under the control of the voltage sensor. In dysgenic myotubes, which lack the normal voltage sensor, the calcium transient followed the integral of current at all test potentials, both in cells possessing only T type current and also in cells possessing both T type current and I_{dys} . Moreover, the calcium transient also followed the time course of current in dysgenic myotubes injected with pCARD1 (Mikami et al., 1989), an expression plasmid encoding the cardiac, DHP-sensitive calcium channel. Thus, in those cases where the transient appears to be a consequence of calcium entry, it has the same time course as the integral of calcium current.

In myoballs, where it was possible to calculate the expected change in intracellular calcium due to calcium influx, the change in intracellular calcium concentration was found to be ~16-fold larger than expected, based on the assumption that calcium buffering power of the myoplasm would be at least that of the calcium buffers present in the patch pipette. One obvious explanation for this discrepancy is that the calcium buffering power is much less than expected because the calcium buffers in the pipette had not equilibrated with the myoplasm. A second explanation is that the dye behaves differently in the cell than in the calibrating capillary tube, perhaps due to binding to cytoplasmic components. A third possible explanation is that calcium entering via T channels triggered additional calcium release from the sarcoplasmic reticulum (SR). In the case of pCARD1-injected dysgenic myotubes, measurements of calcium current and contraction after treatments to deplete calcium from the SR have provided support for the idea that calcium entering via the expressed, cardiac, calcium channel triggers calcium release from the SR (Tanabe et al., 1990). More recent experiments on pCARD1-injected myotubes (García et al., 1994) provide additional support for calcium entry-triggered release of calcium from the SR. Specifically, the majority of pCARD1-injected myotubes that display a high density of cardiac, L type calcium current also display electrically evoked contractions and large calcium transients, although a small proportion of pCARD1-injected myotubes express a high density of calcium current but do not display electrically evoked contractions or calcium transients. This result suggests that calcium entry alone is insufficient to cause an appreciable change in intracellular calcium and that in pCARD1-injected cells which display both calcium current and an appreciable calcium transient, calcium released from the SR must contribute significantly to the transient. Although it is unclear why some pCARD1-injected myotubes have large currents but no transients, one possibility is that those cells have lower than normal levels of SR calcium release channels (ryanodine receptors).

An interesting difference between normal and dysgenic myotubes is that the amplitude of the calcium transient, measured at -20 mV, where the amplitude of the T type calcium current is maximal, was about fourfold lower in the dysgenic cells even though the maximum amplitude of the T type calcium current was very similar to that of normal myotubes. One possible explanation for this discrepancy is that, in normal myotubes, voltage sensor-triggered release of calcium from the SR contributes to the calcium transient at -20 mV. However, in normal myotubes lacking T type calcium current, depolarizations to -20 mV elicited a very small calcium transient only when the duration of the depolarization was made much longer (500–1,000 ms) than those ordinarily used (100–300 ms). Moreover, with the standardly used depolarizations to -20 mV, the block of the T-type calcium current and the calcium transient by amiloride were comparable in normal and dysgenic myotubes. This is not what would be expected if calcium release triggered by the voltage sensor were important in normal myotubes but not in dysgenic ones. An observation that may be important for understanding the differences in the magnitude of calcium transients associated with T type calcium current is that levels of the SR calcium release channel appear to be several-fold lower in dysgenic myotubes than in normal myotubes (cf. Fig. 4 of Knudson, Chaudhari, Sharp, Powell, Beam, and Campbell, 1989). Different levels of the release channel could explain the

different sized calcium transients in dysgenic and normal myotubes, if, as suggested above, calcium entering via T type channels triggers release of calcium from the SR. The hypothesis that calcium entry via T type channels triggers calcium release from the SR requires that the T type channels be localized in proximity to SR calcium release channels. At present, we do not have a straightforward method for testing this prediction.

This work was supported by NIH research grants, NS24444 and NS28323 to K. G. Beam, and by a Postdoctoral Fellowship to J. García from the American Heart Association of Colorado.

Original version received 15 June 1994 and accepted version received 31 August 1994.

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