

Calcium Currents in a Fast-Twitch Skeletal Muscle of the Rat

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ABSTRACT Slow ionic currents were measured in the rat omohyoid muscle with the three-microelectrode voltage-clamp technique. Sodium and delayed rectifier potassium currents were blocked pharmacologically. Under these conditions, depolarizing test pulses elicited an early outward current, followed by a transient slow inward current, followed in turn by a late outward current. The early outward current appeared to be a residual delayed rectifier current. The slow inward current was identified as a calcium current on the basis that (a) its magnitude depended on extracellular calcium concentration, (b) it was blocked by the addition of the divalent cations cadmium or nickel, and reduced in magnitude by the addition of manganese or cobalt, and (c) barium was able to replace calcium as an inward current carrier. The threshold potential for inward calcium current was around -20 mV in 10 mM extracellular calcium and about -35 mV in 2 mM calcium. Currents were net inward over part of their time course for potentials up to at least $+30$ mV. At temperatures of $20-26^{\circ}\text{C}$, the peak inward current (at ~ 0 mV) was $139 \pm 14 \mu\text{A}/\text{cm}^2$ (mean \pm SD), increasing to $226 \pm 28 \mu\text{A}/\text{cm}^2$ at temperatures of $27-37^{\circ}\text{C}$. The late outward current exhibited considerable fiber-to-fiber variability. In some fibers it was primarily a time-independent, nonlinear leakage current. In other fibers it appeared to be the sum of both leak and a slowly activated outward current. The rate of activation of inward calcium current was strongly temperature dependent. For example, in a representative fiber, the time-to-peak inward current for a $+10$ -mV test pulse decreased from ~ 250 ms at 20°C to 100 ms at 30°C . At 37°C , the time-to-peak current was typically ~ 25 ms. The earliest phase of activation was difficult to quantify because the ionic current was partially obscured by nonlinear charge movement. Nonetheless, at physiological temperatures, the rate of calcium channel activation in rat skeletal muscle is about five times faster than activation of calcium channels in frog muscle. This pathway may be an important source of calcium entry in mammalian muscle.

INTRODUCTION

Calcium currents have been described in a great variety of tissues, including invertebrate muscle, egg cells, neurons, secretory cells, and frog skeletal

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muscle (see Hagiwara and Byerly, 1981, for a recent review). The presence of calcium channels in frog skeletal muscle was first inferred from tetrodotoxin (TTX)-insensitive, calcium-dependent regenerative responses (Beatz and Stefani, 1976). Subsequently, the behavior of calcium channels in frog muscle was analyzed with voltage-clamp methods (Stanfield, 1977; Sanchez and Stefani, 1978; Almers and Palade, 1981; Almers et al., 1981). Recently, Chiarandini and Stefani (1983) described calcium-dependent regenerative responses in rat leg muscles. The purpose of the present experiments was to document the properties of calcium currents in rat muscle under voltage-clamp conditions. One motive for the study was to compare calcium channels in rat skeletal muscle with calcium channels in skeletal muscle of frogs, in other muscle cells (smooth and cardiac), and in unrelated cell types (e.g., neurons, *Paramecium*). In addition, a variety of evidence indicates that calcium channels and calcium-activated potassium channels have a prominent role in the physiology of developing skeletal muscle (Land et al., 1973; Barrett et al., 1981, 1982). Thus, it is important to know whether the properties and relative numbers of calcium channels and calcium-activated potassium channels in muscle fibers remain constant or change during development.

We have measured slow ionic currents in the rat omohyoid muscle, a fast-twitch muscle (Müntener et al., 1980) with a centrally located tendon that makes the muscle well suited for the three-microelectrode method of Adrian et al. (1970). After the larger and faster currents carried by sodium and potassium are blocked, the largest component of the residual current was a slow, inward current carried by calcium. Based on the ionic selectivity of the current and the agents that blocked it, calcium channels in rat muscle are similar to those described previously in frog muscle.

Although the presence of calcium currents in frog muscle has been documented, the function of these currents remains unknown. For example, Gonzalez-Serratos et al. (1982) demonstrated that concentrations of diltiazem sufficient to block calcium currents in frog muscle do not significantly affect the magnitude of the twitch, tetanus, or potassium contracture, and therefore concluded that the calcium currents have no obvious role in excitation-contraction coupling. Moreover, Sanchez and Stefani (1978) concluded that the kinetics of calcium currents in frog muscle are so slow that it is unlikely that an action potential activates enough channels to cause significant calcium entry. In contrast to frog muscle, we find that the activation of calcium currents is sufficiently fast at physiological temperatures in rat skeletal muscle to suggest that this pathway may represent an entry mechanism for calcium during one or more action potentials.

A preliminary account of this work has appeared (Donaldson and Beam, 1982).

METHODS

Omohyoid muscles were removed from male Sprague-Dawley rats (400–500 g) and the surface connective tissue was carefully dissected away from the region of the centrally located tendon that partially traverses the muscle. During the dissection,

the muscle was continuously superfused with oxygenated solution containing (in mM): 146 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 11 glucose, 10 Hepes, pH adjusted to 7.4 with NaOH. The muscle was then transferred to an experimental chamber (volume \cong 5 ml) on the stage of a compound microscope. During the measurement of ionic currents this chamber was perfused with oxygenated test solution. (The compositions of the standard solutions used are given in Table I.) Ordinarily the muscle was maintained in 10 mM Ca solution and only transiently exposed to the other solutions because an elevated calcium concentration improved fiber survival. In earlier experiments (muscle numbers \leq 88), the solution flow rate was 5 ml/min, and a complete change of solution, including the flushing of \sim 5 ml of dead space, took \sim 2 min. In later experiments (muscle numbers \geq 89), the flow rate was increased to 25 ml/min and the bathing solution could be changed in \sim 30 s. The times cited in the text and figure legends have been corrected for flushing of the dead space.

Ionic currents were measured by the three-microelectrode technique of Adrian et al. (1970). Two voltage-measuring electrodes, designated V₁ and V₂, were inserted

TABLE I
Test Solutions

Divalent ions	Designation
<i>mM</i>	
10 Ca 1 Mg	10 Ca
2 Ca 1 Mg	2 Ca
10 Ba 1 Mg	10 Ba
0 Ca 11 Mg	0 Ca

In addition to these divalent cations, solutions contained (mM): 146 TEA-Br, 5 CsBr, 400 sucrose, 11 glucose, 10 Hepes, (solution pH adjusted to 7.4 with NaOH), and in most experiments 1 μ M TTX. In some experiments, the test solution contained 5 mM 3,4-diaminopyridine (3,4-DAP) or 4-aminopyridine. Divalent ions were present as the acetate salt.

into the fiber at distances l and $2l$ from the central tendon, and a current-passing electrode was inserted a distance l' from the V₂ electrode; the controlled potential was that at V₁. In the experiments described here, l was 240–280 μ m and l' was 70–140 μ m. In the three-microelectrode method, membrane current density is measured as proportional to V₂ – V₁ (Adrian et al., 1970), with a proportionality constant that depends on a fiber's linear cable properties. The measurement of cable properties has been previously described (Beam and Donaldson, 1983). Test currents were elicited by depolarizing pulses, usually 0.5–2.0 s in duration and separated by an interpulse interval of 5–10 s. Data were acquired and analyzed with the aid of a PDP 11/03 laboratory computer (Digital Equipment Corp., Maynard, MA). The test currents were corrected for linear components of capacitive and ionic leakage currents by digital scaling and subtraction of a control current that was elicited by a 30-mV depolarization from the holding potential. Unless otherwise stated, the holding potential was –90 mV.

Microelectrodes for voltage measurement were pulled from either thin-walled aluminosilicate capillary tubing (F. Haer & Co., Brunswick, ME) or thick-walled borosilicate capillary glass (A-M Systems Inc., Everett, WA). The pipettes were filled with 3 M KCl and had resistances of 8–15 M Ω (thin-walled) or 10–40 M Ω (thick-walled). The higher-resistance, thick-walled electrodes were generally less damaging

to the muscle fibers. Current-passing electrodes were fabricated from thin-walled glass and filled with 2 M K-citrate, and had resistances of 8–20 M Ω .

RESULTS

Fig. 1 illustrates slow ionic currents measured in fibers of rat omohyoid muscles after pharmacological block of sodium and delayed rectifier currents. For small depolarizations, at early times after the onset of the test pulse, an

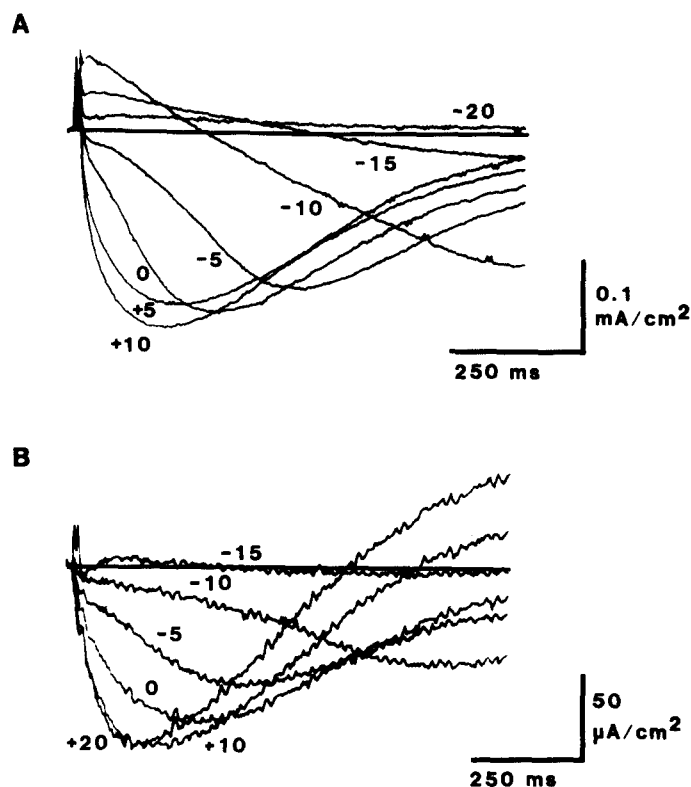


FIGURE 1. Slow ionic currents in rat omohyoid muscle. Fibers were bathed in 10 Ca solution (Table I), which contains 146 mM TEA⁺ to block K currents; for the records shown in *B*, K currents were further blocked by the addition of 5 mM 3,4-DAP. (A) Muscle 95-3, $T = 21.9^{\circ}\text{C}$. (B) Muscle 101-2, $T = 22.0^{\circ}\text{C}$, $E_h = -80$ mV.

“early outward current” is present; for stronger depolarizations, a “slow inward current” appears; and for the strongest depolarizations, at late times after onset of the test pulse, a “late outward current” is seen. The early outward current is prominent in the records shown in Fig. 1A: a depolarization to -20 mV produced a current that was outward for the duration of the test pulse, whereas a depolarization to -15 or -10 mV resulted in currents that were outward at early times and inward at later times. Such early outward currents were present to a variable extent in most fibers bathed in

10 mM Ca solution. The amplitude and kinetics of the early outward current suggest that it is carried by delayed rectifier channels that are not blocked by the 146 mM tetraethylammonium ion (TEA^+) present in the bath. In the absence of TEA^+ , \bar{g}_K in omohyoid muscle fibers at $\sim 20^\circ\text{C}$ averages ~ 21 mS/cm², V_K averages about -80 mV, and a depolarization to -10 mV activates a conductance of $\sim 0.7\bar{g}_K$ (Beam and Donaldson, 1983). Thus, at -10 mV in the absence of TEA^+ , delayed rectifier channels would be expected to produce a current of 1.03 mA/cm². In Fig. 1A the peak early outward current at -10 mV is ~ 75 $\mu\text{A}/\text{cm}^2$, or 7.3% of the current expected in the absence of block. This corresponds to a K_d of 11.5 mM, which is close to the value of 8 mM that has been determined in frog sartorius muscle (Stanfield, 1970).

In later experiments, 5 mM 3,4-diaminopyridine (3,4-DAP) largely eliminated the early outward current (Fig. 1B). For depolarizations beyond -5 mV, the kinetics of the currents in Fig. 1, A and B, are similar, which suggests that even without 3,4-DAP, contamination by delayed rectifier current is slight. Two factors probably account for the relative absence of contamination for moderately strong depolarizations. First, the delayed rectifier current in the omohyoid inactivates during a maintained depolarization and the rate of inactivation is faster for larger depolarizations (K. G. Beam and P. L. Donaldson, unpublished observations). Second, the delayed rectifier in the omohyoid shows cumulative inactivation, which increases with successive test pulses (Beam and Donaldson, 1983), and the larger depolarizations were delivered later in the test-pulse sequence.

A slow inward current was elicited when fibers were depolarized beyond a threshold level (-15 mV for the fiber in Fig. 1A and -10 mV for the fiber in Fig. 1B). For a test pulse just above threshold, the inward current rose very slowly to its peak (taking ~ 750 ms for currents like those in Fig. 1, which were measured at $\sim 20^\circ\text{C}$). The time-to-peak current decreased with increasing depolarization, but even for strong depolarizations the activation of current was quite slow (~ 150 ms for the currents in Fig. 1). In addition to increasing the speed of activation, increasing depolarization caused the inward current to become larger. For fibers bathed in 10 mM Ca, as in Fig. 1, both the amplitude and rate of activation reached a maximum for test pulses around $+10$ mV. For depolarizations beyond this level, the rate of activation remained roughly constant, whereas the peak inward current became smaller. The time course and maximum amplitude of the inward current showed considerable variability from fiber to fiber. The majority of measurements were made on fibers bathed in 10 mM Ca at temperatures of 20 – 26°C . In these fibers, the maximum inward current was 139 ± 14 $\mu\text{A}/\text{cm}^2$ (mean \pm SD, $N = 32$, range 46 – 376 $\mu\text{A}/\text{cm}^2$). At temperatures closer to 37°C , the maximum inward current was larger. The average maximum inward current measured in 16 fibers at temperatures of 26 – 37°C was 226 ± 28 $\mu\text{A}/\text{cm}^2$ (range 57 – 388 $\mu\text{A}/\text{cm}^2$). A part of the variability in the magnitude of the inward current may derive from variability in the measurement of linear cable properties since these measurements are used to calculate

the current density from the $V_2 - V_1$ signal (see Discussion in Beam and Donaldson, 1983). Nonetheless, much of the variability in peak inward current probably reflects true differences in the physiological state of individual fibers at the time of measurement.

In response to a prolonged depolarization, the inward current was not maintained but rather declined in a time-dependent fashion. The term "decay" will be used to describe this decline of inward current because it is mechanistically neutral and several processes may contribute to this decline, including voltage-dependent (Sanchez and Stefani, 1978) or calcium-dependent inactivation (Brehm and Eckert, 1978), depletion of calcium from a restricted extracellular space (Almers et al., 1981), and the activation of time-dependent outward currents (Connor, 1979). The contribution of a time-dependent outward current was suggested in some fibers by the presence of a late outward current (e.g., traces at +10 and +20 mV in Fig. 1B). In some fibers, the late outward current appeared to be primarily a nonlinear (time-independent) leak current temporarily masked by the slow inward current, but in other fibers it may have been, in part, a time-dependent current activated by calcium and/or voltage. The late outward current did not seem to be related to the early outward current because the former was frequently seen in fibers that lacked the latter (e.g., Fig. 1B). The source of the late outward current will be considered in more detail later.

The Inward Current Is Carried by Calcium

It is unlikely that the slow inward current arises from sodium channels because it was typically measured in media containing $1 \mu\text{M}$ TTX and in which the bulk of extracellular sodium had been replaced by TEA ion. Based on its kinetic similarity to the slow inward current in frog skeletal muscle (Sanchez and Stefani, 1978; Almers and Palade, 1981), an obvious hypothesis for the current in rat muscle is that it is a calcium current. As one test of this hypothesis, the extracellular calcium concentration was reduced from 10 to 2 mM (Fig. 2). For a test pulse of 0 mV, the current recorded in 2 Ca (Fig. 2, trace 2) was about half the size of the current in 10 Ca (trace 1). The current measured after the return to 10 Ca solution (Fig. 2, trace 3) was very similar to the control. Reducing extracellular calcium from 10 to 2 mM shifts the peak $I-V$ relationship of the slow inward current 10–20 mV in the hyperpolarizing direction (see below), but this shift does not account for the reduction in inward current amplitude illustrated in Fig. 2. Moreover, results similar to those shown in Fig. 2 were obtained when the reduction of extracellular calcium concentration was compensated by an increase in extracellular Mg.

As another test of the hypothesis that the slow inward current is a calcium current, we measured currents before and after the total replacement of extracellular calcium by magnesium. These experiments were difficult because the complete removal of calcium caused fibers to develop large leaks. Fig. 3 presents results from one experiment in which the fiber survived for several minutes after the solution change. The illustrated currents were elicited by a test pulse to -10 mV applied to the fiber before (trace 1) and at

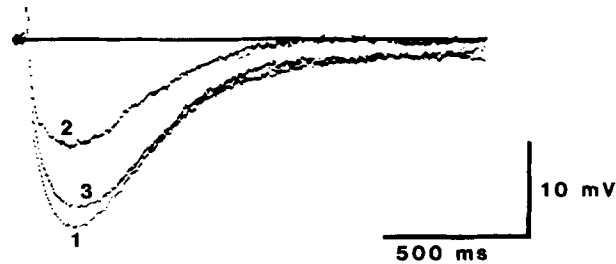


FIGURE 2. Peak inward current varies with external Ca. Current trace 1 was obtained in 10 Ca solution, current trace 2 after changing the solution to 2 Ca (total duration of exposure to 2 mM calcium was 5 min), and current trace 3 \sim 1 min after returning to 10 Ca. 5 mM 3,4-DAP was present throughout. Cable properties were not measured in this fiber so that currents are expressed in units of $V_2 - V_1$. Muscle 98-3, test pulse = 0 mV, $T = 26.6^\circ\text{C}$.

two times after changing the bathing solution from 10 Ca:1 Mg to 11 Mg (traces 2 and 3). As expected for a calcium current, the inward currents after the solution change are smaller than the control current. In addition, the presence of an inward current 4 min after the solution change (Fig. 3, trace 3), when extracellular calcium should have been reduced to a low level, suggests that Mg can carry inward current.

Peak Current-Voltage Relationship

The peak current-voltage relationship of the slow inward current is illustrated in Fig. 4 for a fiber under conditions that approximately mimic those in vivo (2 mM Ca, temperature of $\sim 33^\circ\text{C}$). In this fiber, test pulses to -45 and -40 mV elicited small currents that were outward for the duration of the test pulse. The maximum value of these outward currents has been plotted. Test pulses to -35 mV and above resulted in currents that were inward over at least part of their time course and for these potentials the peak amplitude of the inward current is plotted. The peak I - V relationship illustrated in Fig. 4 demonstrates that in 2 mM Ca both the threshold for inward current and the potential at which the maximum inward current is activated are shifted

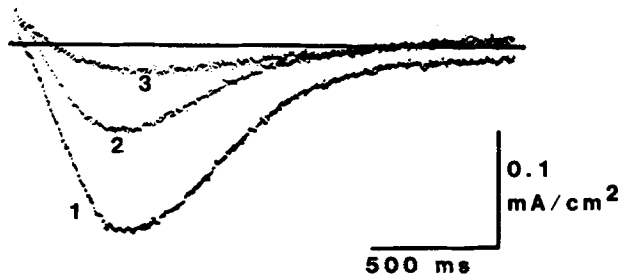


FIGURE 3. Amplitude of inward current is reduced by substitution of Mg for Ca. The trace labeled 1 is a control current in 10 Ca:1 Mg solution. Traces labeled 2 and 3 were obtained 2 and 4 min after changing to 11 Mg:0 Ca. Muscle 88-3, test pulse = -10 mV, $T = 24.4^\circ\text{C}$.

~20 mV in the hyperpolarizing direction compared with 10 mM Ca (cf. Fig. 1 and inset in Fig. 8).

Several factors complicate the interpretation of peak I - V curves for Ca currents in the omohyoid muscle. First, in many fibers, the slow inward current became regenerative over the negative slope region of the current-voltage relation. In these fibers, a just-suprathreshold test pulse produced a small, slow inward current, whereas a slightly larger test pulse produced a current that followed a similar initial trajectory, but after a latency that could be several hundred milliseconds, it developed a spike-like transient of inward current. Further increases in test-pulse amplitude reduced the latency without

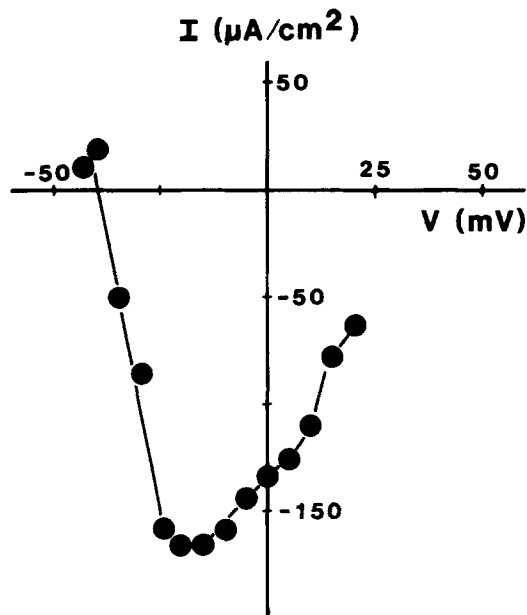


FIGURE 4. Peak current vs. voltage relation of slow ionic currents in 2 Ca. The data points represent the peak inward current at all test voltages, except at -45 and -40 mV where the peak outward current is plotted; for these test pulses the current was outward at all times. Test pulse duration, 1 s. Muscle 90-7, $T = 32.6^{\circ}\text{C}$.

much effect on the amplitude or shape of the inward current transient. Regenerative behavior will obviously distort the measured kinetics of activation as well as the negative slope region of the peak I - V relationship. Similar regenerative behavior has been described for calcium currents in frog skeletal muscle (Almers and Palade, 1981). It has been ascribed to a failure to control membrane potential within the transverse tubular system, where the calcium channels are hypothesized to be located (Nicola-Siri et al., 1980). Fig. 4 illustrates the peak I - V relationship of currents that did not show obvious signs of escape from potential control.

A second complication in the interpretation of the peak I - V relation is that the peak inward current represents the summation of inward and outward currents. (For the fiber in Fig. 4, a late outward current was present for test pulses to -10 mV and above.) For this reason, it is difficult to determine an accurate reversal potential for the inward current. The determination of an extrapolated reversal potential is further complicated by the observation in a variety of cells that the peak I - V relationship for Ca currents becomes nonlinear as the Ca reversal potential is approached (Hagiwara and Byerly, 1981). Nonetheless, the apparent reversal potential for the inward current is sufficiently positive to be consistent with the hypothesis that it is carried by calcium.

Blockers of Calcium Current

We have examined the effect on the slow inward current of the divalent cations Cd^{2+} , Ni^{2+} , Mn^{2+} , and Co^{2+} , all of which have been demonstrated to block calcium currents in a variety of other preparations (Hagiwara and Byerly, 1981). Fig. 5A illustrates currents from a fiber initially bathed in 10 Ca solution (Cont), and after the addition of 0.5 mM Cd to this solution (trace labeled Cd). In the presence of Cd, the test pulse no longer elicited an inward current. This blockade of inward current by Cd was partially reversed within 1 min (R1) after removing Cd, and more fully reversed after 3 min (R2).

The residual current observed in the presence of Cd, or "Cd-insensitive current," was usually outward and relatively time independent. A simple explanation for this Cd-insensitive current is that it represents a voltage-dependent leak current. The presence of a nonlinear leak makes it difficult to determine the true magnitude of the inward currents. For example, it would appear from the control record in Fig. 5A that a 1-s test pulse to $+20$ mV caused Ca channels to activate and then inactivate completely so that the current through calcium channels is zero by the end of the pulse. However, if the only effect of Cd is to block Ca channels, then the control record in Fig. 5A does not represent the true calcium current, which would instead be the difference between the inward current measured before the addition of Cd and the Cd-insensitive current. Accordingly, the 1-s test pulse to $+20$ mV evidently did not cause the control calcium current in Fig. 5A to inactivate completely, but rather caused it to decay to a level equal but opposite that of the residual outward current.

Because Cd blocked calcium current at a lower concentration and had a more easily reversed effect than the other divalent cations tested, it was used to probe the source of the late outward current. In Fig. 5B, a test pulse to $+30$ mV applied to a fiber bathed in 10 Ca (Cont) resulted in a current that was inward initially and outward at later times. The steady state level reached by this late outward current was the same as the steady state level reached by the Cd-insensitive current recorded in the same fiber (Cd). Therefore, the late outward current in this fiber can be explained as a nonlinear leak current that was masked at early times by inward calcium current. A different result

is illustrated in Fig. 5C. In this fiber, the late outward current measured before exposure to Cd was larger in amplitude than the Cd-insensitive outward current. One possible explanation for this result is that a calcium-activated K current (Meech and Standen, 1975) contributed to the late outward current in this fiber. The presence of calcium-activated K channels in adult rat muscle seems reasonable because these channels have been

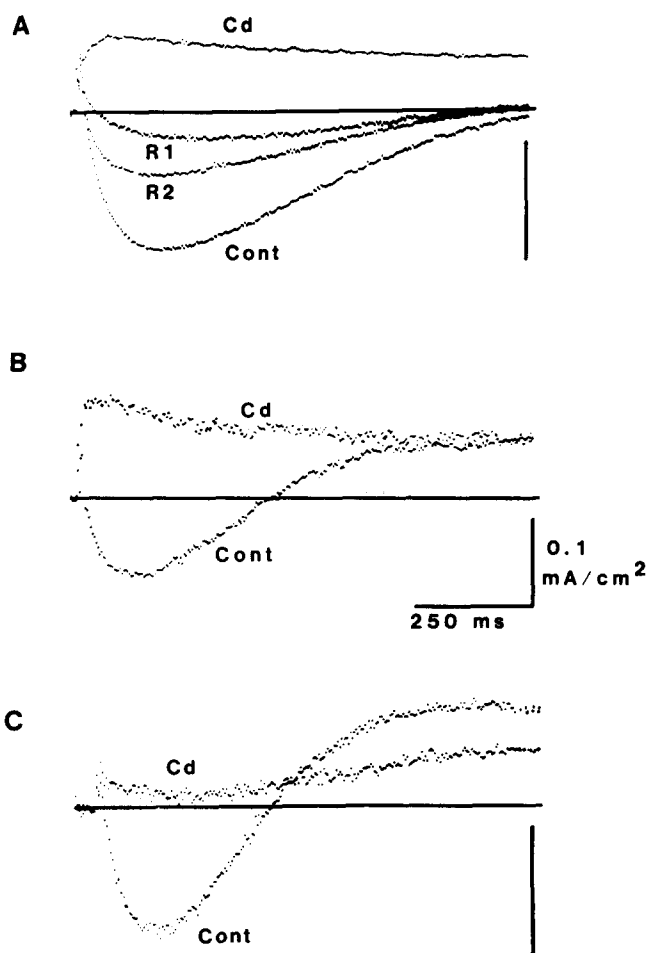


FIGURE 5. Cadmium reversibly blocks inward current. (A) Currents from a fiber in 10 Ca (Cont), 2 min after the addition of 0.5 mM Cd to the bathing solution (Cd), and 1 (R1) and 3 (R2) min after return to the control (10 Ca) solution. Muscle 84-7, test pulse = +20 mV, $T = 22.1^{\circ}\text{C}$. (B) Currents measured in 10 Ca before (Cont) and about 1 min after (Cd) the addition of 1 mM Cd. Muscle 83-1, test pulse = +30 mV, $T = 26^{\circ}\text{C}$. (C) Currents in 2 Ca solution (Cont) and 2 min after the addition of 0.5 mM Cd to this solution. Muscle 97-4, test pulse = +10 mV, $T = 27.0^{\circ}\text{C}$. In A-C, the vertical calibration equals 0.1 mA/cm².

described in embryonic rat skeletal muscle grown in tissue culture (Barrett et al., 1981, 1982). Additionally, the presence of such channels in adult rabbit skeletal muscle has been inferred from reconstitution experiments (Latorre et al., 1982). However, we cannot exclude the possibility that the result of Fig. 5C is due to Cd blocking a calcium-independent outward current. When the results in Fig. 5, B and C, are compared, it should be noted that the bathing solution in Fig. 5B contained 10 mM Ca, whereas that in Fig. 5C contained only 2 mM Ca. As described above (Fig. 2), one effect

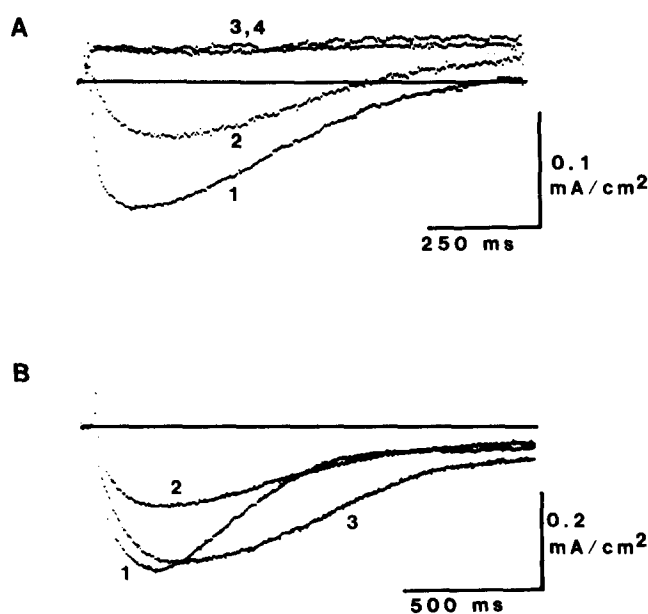


FIGURE 6. Nickel and manganese block inward current. (A) Trace 1 is a control current in 10 Ca, trace 2 was recorded ~ 1 min after the addition of 10 mM Ni^{2+} (total duration of exposure 2 min), and traces 3 and 4 were recorded ~ 2 and 5 min after return to the control (10 Ca) solution. Muscle 84-7, test pulse = +10 mV, $T = 26.3^\circ\text{C}$. (B) Currents obtained in 10 Ca (trace 1), ~ 4 min after the addition of 5 mM Mn^{2+} to this solution (trace 2), and ~ 4 min after restoration of the Mn-free control solution. Total duration of exposure to Mn was 5 min. 5 mM 3,4-DAP was present throughout. Muscle 105-5, test pulse = +10 mV, $T = 25.9^\circ\text{C}$.

of a lower calcium concentration was to reduce the magnitude of the slow inward current. An additional effect was to increase the magnitude of the late outward current, which suggests that extracellular calcium blocks a component of outward current. Perhaps the elevated calcium in Fig. 5B blocked the component of outward current that Cd would have otherwise blocked.

Fig. 6A illustrates the block of slow inward current by Ni. Compared with the control current (trace 1), the current measured just after the addition of

10 mM Ni to the bath (trace 2) was reduced in size and had a slower rate of activation. At the time when current trace 2 was obtained, the wash-in of Ni was not yet complete, and ~1 min later, both this wash-in and the block of inward current were complete. The block of calcium current by Ni was not readily reversed. 2 (trace 3) and 5 min (trace 4) after removal of Ni from the bath, the inward current was still absent and only a residual outward current was observed. This fiber deteriorated before inward current recovered, but another fiber of the muscle studied ~15 min later in the experiment had normal calcium currents.

The addition of 5 mM Mn caused both a reduction in the amplitude and a slowing of the activation and decay of the slow inward current. This is illustrated in Fig. 6B, which compares currents recorded before (trace 1) and after the addition of Mn to the bath (trace 2). Within 4 min after the removal of Mn (trace 3), the amplitude of the current had recovered, although its activation and decay were both slower than control. One possible explanation of why the alteration of kinetics outlasts the exposure to Mn is that internal Mn affects Ca channel gating and Mn had accumulated inside the fiber. Entry of Mn might be expected because this ion has been shown to carry inward current through calcium channels in frog skeletal muscle (Almers and Palade, 1981).

A fourth divalent ion tested for its ability to block calcium current in rat muscle was Co. The addition of 10 mM Co to the control 10 Ca solution reduced the amplitude of the slow inward current (not shown) but to a lesser extent than 5 mM Mn. Thus, Co was a less effective blocker than Cd, Ni, or Mn.

Barium Can Carry Inward Current

In addition to testing the effects of several divalent ions added to a calcium-containing solution, we examined the effects of replacing calcium by the ions Mg or Ba. These experiments were difficult because the preparation was much less stable after the complete withdrawal of extracellular calcium. (The preparation survived better when calcium was replaced by Ba than by Mg.) The effects of magnesium substitution on the slow inward current have already been described. The results of an experiment in which barium was substituted for calcium are illustrated in Fig. 7, which compares currents in 10 Ca (top set of traces), during replacement of calcium by 10 Ba (center set of traces), and after the return to 10 Ca (bottom set of traces). Because the maximum inward current in 10 Ba is slightly larger than that recorded in 10 Ca before exposure to barium, we conclude that the permeability of Ba is approximately the same as that of Ca. A more precise statement is difficult because (a) Ba caused a hyperpolarizing shift in the voltage dependence of the inward current, and (b) the maximum inward current measured after the return from barium to calcium was larger than that measured during exposure to barium. An increase in inward current magnitude after the return from barium to calcium was observed not only in the experiment illustrated in Fig. 7, but also in three other similar experiments.

In Fig. 7, the prominent early outward current present during the control run (top traces: steps to -20 and -10 mV) was eliminated by the substitution of Ba. This effect of barium is consistent with the notion that the early outward current is a delayed rectifier current, because barium applied either internally (Eaton and Brodwick, 1980; Armstrong and Taylor, 1980) or externally (Armstrong et al., 1982) blocks delayed rectifier K channels in squid axons.

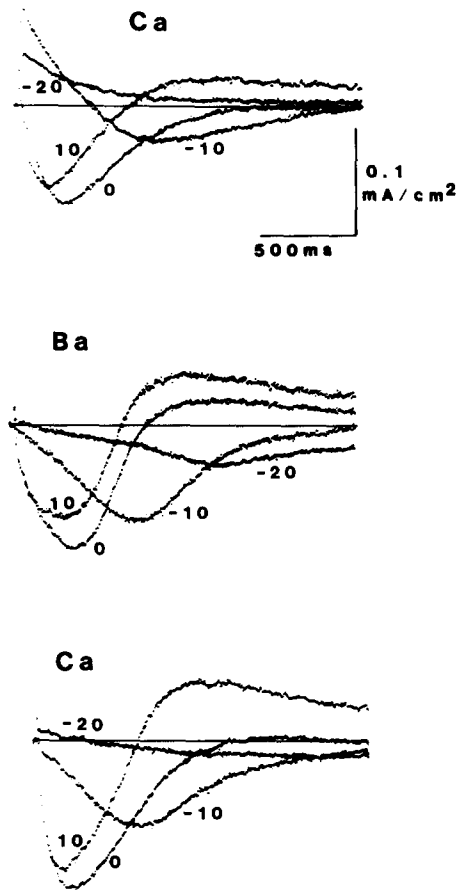


FIGURE 7. Barium is an effective carrier of inward current. Currents evoked by test pulses to the indicated potentials were recorded in 10 Ca (top set of traces), 2.5 min after replacement of extracellular calcium by 10 Ba (center set of traces), and $\sim 1 \text{ min}$ after returning to 10 Ca (bottom set of traces). Muscle 89-4, $T = 24.5^\circ \text{C}$.

Fig. 7 also illustrates that a late outward current was present with barium bathing the fiber (center traces: steps to 0 and $+10$ mV). Altogether, four fibers were stable enough to allow comparison of slow ionic currents before and after replacement of calcium by barium. In two of these, no late outward current was present with either calcium or barium bathing the fiber. In the

other two (including the one in Fig. 7), a late outward current was present both before and during the exposure to barium. In these two fibers it would seem that calcium-activated K channels were not the source of the late outward current since Ba does not activate such channels in molluscan neurones (Gorman and Hermann, 1979).

Calcium Currents at Physiological Temperature

In general, our measurements of calcium currents in the omohyoid muscle were made at temperatures from 20 to 26°C. However, because the currents measured at subphysiological temperatures may not accurately reflect the behavior of calcium channels under more physiological conditions, experiments were also carried out at higher temperatures. Fig. 8 compares currents measured in a single muscle fiber at temperatures of 22, 25, and 31°C for

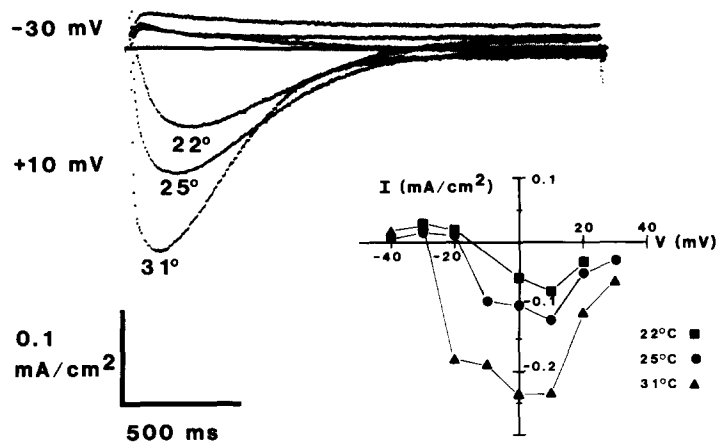


FIGURE 8. The kinetics and amplitude of calcium current are strongly temperature dependent. Currents from a single fiber at 22, 25, and 31°C are compared. The currents shown were for a test pulse to either -30 or +10 mV. The complete peak I - V relationships for this same fiber are illustrated in the inset. Muscle 81-3, 10 Ca solution.

test pulses to -30 or +10 mV. The elevation in temperature had little effect on the small outward currents elicited by the -30-mV pulse but caused an increase in both the amplitude and the speed of activation of the inward currents elicited by the +10-mV pulse. The peak I - V relationships illustrated in the inset to Fig. 8 demonstrate that the increase in the inward current was seen not only for the +10-mV pulses but at other potentials as well. Thus, except at -20 mV, the peak amplitude of the inward calcium currents at 31°C was ~2.5-fold larger than that of the inward currents at 22°C. The -20-mV test pulse evoked a large inward current at 31°C but not at the lower temperatures. Whether this represents a true shift in the potential dependence of calcium channel activation cannot be easily determined since the response to the -20-mV pulse at 31°C was clearly regenerative. At higher temperatures, the presence of a large inward current for a smaller depolari-

zation may be primarily a consequence of more rapid channel activation, since rapidly activating channels within the transverse tubular system would more readily escape from potential control than would slowly activating ones. The failure to control potential adequately will also affect the measured time course of currents, thus complicating the comparison of current kinetics at different temperatures. Because escape from control is expected to be serious only for test potentials in the negative slope region of the $I-V$ relationship, kinetic comparisons of currents at these test potentials were avoided. For the fiber illustrated in Fig. 8, the current elicited by a test pulse to +10 mV at

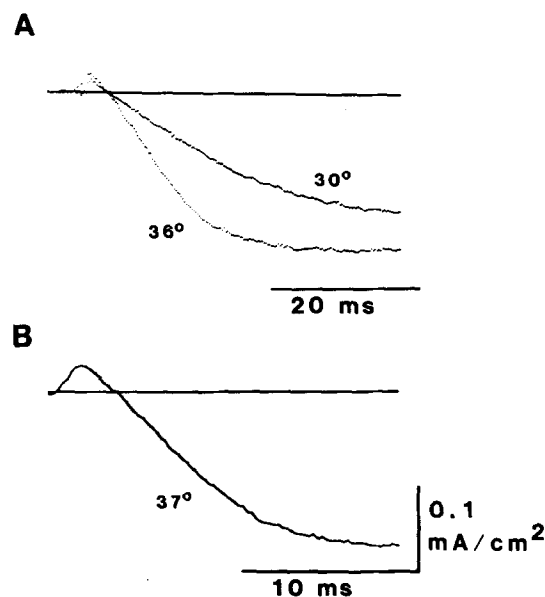


FIGURE 9. Ca current activates rapidly at physiological temperatures. Currents from a single fiber for a test pulse to 0 mV at 29.7 and 36.2°C (A), and for a test pulse to +10 mV at 36.8°C (B). In B, the leftmost portion of the trace corresponds to the onset of the test pulse. The currents in A were obtained in a modified 10 Ca solution which contained 5 mM 4-aminopyridine and in which the concentration of TEA-Br was reduced to 70 mM by equimolar replacement with choline-Br. The currents in B were obtained shortly after switching from the solution just described to one in which the TEA-Br was totally replaced by choline-Br. Muscle 125-4.

22°C required ~250 ms to reach its peak, whereas the current elicited at 31°C reached its peak within 100 ms.

Further increases in temperature caused a large further increase in the speed of activation. This is illustrated in Fig. 9A, which compares two currents recorded at temperatures of 30 and 36°C. The initial rate of rise at 36°C is nearly twice that at 30°C and the current at 36°C reached its peak in ~25 ms. Fig. 9B illustrates on an expanded time scale the rapid time course of calcium current activation at physiological temperature. The actual rate of

activation may be faster than it appears because the initial phase of activation is obscured by the presence of an outward current. This outward current probably represents voltage-dependent charge movement (cf. Simon and Beam [1983] for a description of charge movement in the omohyoid). Although it is partially obscured, the activation of inward current is quite rapid since the current illustrated in Fig. 9B reached half its maximal value within 8 ms of the onset of the test depolarization. The rapid rate of activation at physiological temperatures was not an artifactual consequence of the elevated calcium concentration employed in the experiment illustrated in Fig. 9 because a similar, rapid rate of activation was observed for currents recorded in physiological extracellular calcium (2 mM).

DISCUSSION

We report here that an inward current carried by calcium ions can be measured in the rat omohyoid muscle after the blockade of sodium and potassium channels. The presence of such a current is concordant with the recent report by Chiarandini and Stefani (1983) of calcium-dependent regenerative responses in rat leg muscles. They found that the regenerative responses are unaffected by TTX, are blocked when extracellular calcium was replaced by 10 mM Mg or 5 mM Co, and are blocked by the addition of 0.1–1.0 mM Cd or 5–6 μ M nifedipine. These results are consistent with ours for the agents tested in both sets of experiments.

A comparison of calcium channels in the rat omohyoid muscle and in frog muscle reveals both similarities and differences. Calcium channels in the omohyoid appear to be about equally permeable to Ca and Ba, whereas in frog muscle the channels are more permeable to Ba than Ca (Almers and Palade, 1981). In the omohyoid, Mg appears to be weakly permeant. In frog, Mg has been reported to be impermeant (Sanchez and Stefani, 1978) or weakly permeant (Almers and Palade, 1981). The inward current in the omohyoid is blocked, in order of effectiveness, by the addition of Cd, Ni, Mn, and Co. Of these ions, both Ni (Almers et al., 1981) and Co (Sanchez and Stefani, 1978) have been shown to block calcium currents in frog muscle. By completely replacing extracellular Ca with Mn, Almers and Palade (1981) demonstrated in frog muscle that Mn can carry inward current, although less effectively than Ca. We did not test directly whether Mn can permeate calcium channels in rat muscle because the fibers survived poorly when Ca was replaced by foreign ions. Thus, our results with the omohyoid do not differentiate between the possibility that Mn blocks the channel without permeating, and the possibility that Mn, as a less permeant ion, interferes with the passage of calcium ions through the channel.

Calcium currents measured in the omohyoid can be compared with currents measured in frog muscle bathed in solutions of roughly matching composition. Thus, Almers and Palade (1981) routinely used a solution containing 10 mM Ca with methanesulfonate as the predominant anion, Sanchez and Stefani (1978) used a solution containing 9 mM Ca with sulfate as the predominant anion, and we used a solution containing 10 mM Ca with

bromide as the predominant anion. Using a calcium-selective electrode, Almers and Palade (1981) determined the calcium activity of their 10 Ca solution to be 3.6 mM, and the calcium activity of the solution used by Sanchez and Stefani to be 2 mM. Because of its higher ionic strength, our 10 Ca solution would be expected to have a somewhat lower calcium activity (cf. Butler, 1968) than the 10 Ca solution of Almers and Palade. If these solution differences are taken into account, it can be said that when measured at comparable temperatures (20–26°C), calcium currents in the omohyoid are similar in amplitude, voltage dependence, and kinetics to calcium currents in frog muscle. For example, average values of 81 (Sanchez and Stefani, 1978) and 74 $\mu\text{A}/\text{cm}^2$ (Almers and Palade, 1981) were found for the maximum inward current in frog muscle, as compared with the somewhat larger value of 139 $\mu\text{A}/\text{cm}^2$ that we found for the omohyoid. The peak current-voltage relationships measured in rat (Fig. 8, inset) and frog muscles (Fig. 4, Sanchez and Stefani, 1978; Fig. 6, Almers and Palade, 1981) are also roughly alike, with the difference that the threshold for inward current in frog is somewhat more hyperpolarized (ca. -35 mV) than in rat (ca. -20 mV). The time-to-peak current is similar in rat and frog muscle. The times of 100–300 ms (at +15 mV) found by Almers and Palade (1981) compare well with our values of 100–250 ms (at +10 mV).

When measured at temperatures expected to be approximately physiological for both animals, the rate of activation of calcium channels in the rat omohyoid is faster than that in frog muscle. For a pulse to +10 mV at 37°C, the half-maximal activation time in the omohyoid was ~ 8 ms (Fig. 9B). This value can be compared with a half-maximal time of 40 ms in frog muscle at room temperature, 20–24°C (current at +15 mV illustrated in Fig. 11 of Almers and Palade, 1981). The duration of the action potential in amphibian muscle at 20–24°C is, at most, twice the duration of the action potential at 37°C in mammalian fast-twitch muscle (compare Table III of Nakajima and Bastian [1974] with Yonemura [1967] and Lewis [1972]). Thus, even if the rate of calcium channel activation is referenced to the duration of the action potential, this rate of activation is still considerably faster in rats than in frogs. It would appear, therefore, that calcium currents are more likely to represent an important source of calcium entry in mammalian muscle.

Although they are more rapid in rat muscle than in frog muscle, the calcium currents in rat muscle nevertheless activate rather slowly compared with calcium currents in many other cell types. For example, in single ventricular cells isolated from guinea pig hearts, the time to peak is ~ 2.5 ms for a calcium current at +20 mV and 21°C (Lee and Tsien, 1982), nearly 100-fold faster than the time to peak for a calcium current in rat skeletal muscle at the same temperature. The rapid activation of calcium currents in heart muscle is appropriate to the role of these currents in the genesis of the action potential and activation of contraction. By contrast, in skeletal muscle, calcium currents activate sufficiently slowly compared with the action potential to suggest that they are not an important source of the calcium that triggers contraction (Sanchez and Stefani, 1978). Other more direct experi-

mental tests also support the conclusion that calcium currents are not involved in excitation-contraction coupling in vertebrate skeletal muscle (Chiarandini et al., 1980; Gonzalez-Serratos et al., 1982).

In light of their relatively slow activation, the question remains whether calcium channels in the omohyoid are activated to any extent during an action potential. Answering this question is difficult because the initial phase of activation of calcium current is obscured by a transient outward current (Fig. 9B). The time course of this initial phase of activation must be known in order to determine whether an action potential activates calcium channels. An additional complication is that in frog muscle (and by inference, in rat muscle as well), calcium channels appear to reside in the transverse tubular system (Nicola-Siri et al., 1980). Thus, it is the time course of the tubular action potential, rather than the action potential recorded at the surface, that will determine whether calcium channels are opened. At physiological temperatures, the calcium current appears to activate on a time scale that is comparable to that of voltage-dependent charge movement. For the fiber illustrated in Fig. 9B, a depolarizing step to -30 mV (not illustrated) produced a charge movement that was uncontaminated by ionic current and was typical of the omohyoid muscle with respect to kinetics and quantity of charge moved (Simon and Beam, 1983). Thus, the larger test pulse to $+10$ mV should have caused the movement of ~ 30 nC/ μ F. Instead, the area under the early transient of outward current of the record illustrated in Fig. 9B corresponds to a charge of ~ 6 nC/ μ F. A simple explanation for this discrepancy is that the inward calcium current begins to activate earlier than the time at which the total current first becomes net inward, and that this rapidly activating calcium current cancels much of the outward current caused by charge movement. Horowicz and Schneider (1981) have demonstrated in frog muscle that depolarizing a fiber to the threshold for contraction moves $>40\%$ of the total amount of mobile charge. If it is assumed that the movement of this charge is required for excitation-contraction coupling (Schneider and Chandler, 1973), the tubular action potential must move an even larger fraction of the charge in order to produce a muscle twitch. It seems likely that a tubular action potential that moves sufficient charge to cause a twitch will also activate calcium channels, because the activation of calcium current in the omohyoid considerably overlaps the movement of charge.

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