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Silent Calcium Channels in Skeletal Muscle Fibers of the Crustacean *Atya lanipes*

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Abstract. The superficial (tonic) abdominal flexor muscles of *Atya lanipes* do not generate Ca^{2+} action potentials when depolarized and have no detectable inward Ca^{2+} current. These fibers, however, are strictly dependent on Ca^{2+} influx for contraction, suggesting that they depend on Ca^{2+}-induced Ca^{2+} release for contractile activation. The nature of the communication between Ca^{2+} channels in the sarcolemmal/tubular membrane and Ca^{2+} release channels in the sarcoplasmic reticulum in this crustacean muscle was investigated. The effects of dihydropyridines on tension generation and the passive electrical response were examined in current-clamped fibers: Bay K 8644 enhanced tension about 100% but did not alter the passive electrical response; nifedipine inhibited tension by about 70%. Sr^{2+} and Ba^{2+} action potentials could be elicited in Ca^{2+}-free solutions. The spikes generated by these divalent cations were abolished by nifedipine. As the Sr^{2+} or Ba^{2+} concentrations were increased, the amplitudes of the action potentials and their maximum rate of rise, V_{max}, increased and tended towards saturation. Three-microelectrode voltage-clamp experiments showed that even at high (138 mM) extracellular Ca^{2+} concentration the channels were silent, i.e., no inward Ca^{2+} current was detected. In Ca^{2+}-free solutions, inward currents carried by 138 mM Sr^{2+} or Ba^{2+} were observed. The currents activated at voltages above −40 mV and peaked at about 0 mV. This voltage-activation profile and the sensitivity of the channels to dihydropyridines indicate that they resemble L-type Ca^{2+} channels. Peak inward current density values were low, *ca.* −33 $\mu A/cm^2$ for Sr^{2+} and −14 $\mu A/cm^2$ for Ba^{2+}, suggesting that Ca^{2+} channels are present at a very low density. It is concluded that Ca^{2+}-induced Ca^{2+} release in this crustacean muscle operates with an unusually high gain: Ca^{2+} influx through the silent Ca^{2+} channels is too low to generate a macroscopic inward current, but increases sufficiently the local concentration of Ca^{2+} in the immediate vicinity of the sarcoplasmic reticulum Ca^{2+} release channels to trigger the highly amplified release of Ca^{2+} required for tension generation.

Key words: Crustacean muscle — L-type calcium channels — Excitation-contraction coupling — Calcium-induced calcium release

Introduction

Studies of the electrical excitability of crustacean skeletal muscle led to the discovery of voltage-sensitive Ca^{2+} channels (Fatt & Katz, 1953; Fatt & Ginsborg, 1958; Hagiwara & Naka, 1964). The voltage-dependent inflow of Ca^{2+} through Ca^{2+} channels accounts entirely for the excitability of arthropod skeletal muscle, whose electrical responses range from weak, local depolarizations to overshooting, propagating Ca^{2+} action potentials (crab: Fatt & Katz, 1953; Fatt & Ginsborg, 1958; Mounier & Vassort, 1975; barnacle: Hagiwara & Naka, 1964; Keynes et al., 1973; crayfish: Hencek & Zachar, 1977; insect: Washio, 1972; Ashcroft & Stanfield, 1982; scorpion: Gilly & Scheuer, 1984, 1993; isopod: Erxleben & Rathmayer, 1997). As in vertebrate cardiac muscle, Ca^{2+} influx through voltage-dependent Ca^{2+} channels is required for tension generation in skeletal muscle of crustaceans (Zacharova & Zachar, 1967; Gainer, 1968; Ashley & Ridgway, 1970; Atwater, Rojas & Vergara, 1974; Caputo & Di Polo, 1978) and other arthropods (Gilly & Scheuer, 1984). Although some studies suggest that Ca^{2+} influx is sufficient to account for tension development in crustacean muscle (Atwater et al., 1974),

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others indicate that Ca^{2+} influx mainly serves to trigger the release of Ca^{2+} from the sarcoplasmic reticulum (SR) that activates contraction (Ashley & Lea, 1978; Caputo & Di Polo, 1978; Mounier & Goblet, 1987). Thus, as in vertebrate cardiac muscle (Fabiato, 1983), in crustacean skeletal muscle calcium-induced calcium release (CICR) has been proposed to be the mechanism of excitation-contraction (E-C) coupling: a small entry of extracellular Ca^{2+} is considerably amplified by this mechanism to provide the Ca^{2+} required for contraction (Vergara & Verdugo, 1988; Györke & Palade, 1992, 1993, 1994; Palade & Györke, 1993).

The superficial (tonic) abdominal flexor muscle fibers of the crustacean *Atya lanipes* are dependent on extracellular Ca^{2+} for the activation of contraction (Bonilla et al., 1992). These long-sarcomere striated fibers possess an ample and well-developed SR, with extensive regions of transverse (T) tubule/SR dyad contacts; the SR releases Ca^{2+} in response to the application of caffeine (Bonilla et al., 1992). However, in sharp contrast to other arthropod skeletal muscle fibers, these muscles are electrically inexcitable: they do not generate graded potentials or all-or-none Ca^{2+} spikes when depolarized (Zuazaga & del Castillo, 1985) and, under voltage-clamp conditions, inward currents carried by Ca^{2+} in response to depolarizing voltage steps are unmeasurably small, even when all outward currents are suppressed (Lizardi et al., 1992).

The present studies were carried out to suggest possible explanations to the paradoxical requirement of extracellular Ca^{2+} for contractile activation *vis-a-vis* the absence of measurable inward Ca^{2+} current in this crustacean muscle. We found that Ca^{2+} influx is an absolute requirement for tension generation. The voltage-dependent Ca^{2+} channels that mediate this influx were unmasked, in the complete absence of extracellular Ca^{2+}, using Sr^{2+} and Ba^{2+} as current carriers. The voltage-activation characteristics of the Sr^{2+} and Ba^{2+} currents, and the sensitivity of the channels to dihydropyridines (DHPs) indicate that they resemble L-type Ca^{2+} channels in vertebrate cardiac muscle. We have called them "silent Ca^{2+} channels" because, even when the driving force for Ca^{2+} was increased tenfold, Ca^{2+} current density remained unmeasurably low. Our findings suggest that E-C coupling in this electrically inexcitable muscle is mediated by a remarkably high gain CICR mechanism: a very small entry of Ca^{2+} through the silent Ca^{2+} channels results in the macroscopic release of Ca^{2+} from the SR required for contractile activation. Preliminary results of portions of this work have been reported (Monterrubio, Lizardi & Zuazaga, 1997).

Materials and Methods

Preparation

Adult specimens of the freshwater crustacean *Atya lanipes* were collected in streams at El Yunque rain forest, Puerto Rico, and kept in aquaria at room temperature (20–22°C). Mechanical and electrophysiological experiments were performed on intact fibers from the first or second segment of the superficial abdominal flexor muscle, dissected in control (van Harreveld, 1936) solution (solution A, Table). A detailed description of the preparation, the dissection and the arrangement of the muscles has been published previously (Bonilla et al., 1992).

Mechanical Recordings

Single muscle fibers or small monolayer bundles (4–7 fibers) were dissected, leaving only a small segment of cuticle at both ends for attachment to the bottom of the experimental chamber and to the level of the transducer, respectively. A single fiber was impaled near its middle with two microelectrodes positioned with an intraelectrode distance of 50–70 μm, and current clamped as described below. Tension was simultaneously measured using an isometric mechanoelectronic transducer (Cambridge Technology, Series 400A, MA), whose output was filtered at 500 Hz (−3 dB, 8-pole Bessel) and sampled at 12-bit resolution every 1–2 msec by a microcomputer. The fibers were stretched to about 1.25 resting length (sarcomere length *ca.* 10.5 μm), where maximum tension occurs (Bonilla et al., 1992). In some experiments, the muscle fibers were stimulated extracellularly. Single mechanical responses were evoked from bundles of 2–5 fibers by supramaximal electrical stimuli applied longitudinally via two Pt wires in the bath. Square pulses of 300 msec duration and 8–15 V intensity were applied using a Grass S11 (Grass Instruments, MA) stimulator. Mechanical recordings were carried out at room temperature (20–22°C).

Electrophysiological Recordings

The current- and voltage-clamp techniques used in the present studies are described in detail elsewhere (Lizardi et al., 1992). Briefly, in current-clamp experiments, the fibers were impaled with two microelectrodes positioned with an intraelectrode distance of 50–70 μm. The microelectrode for membrane potential recording was filled with 3 M KCl and its resistance was 15–25 MΩ. Constant current pulses were delivered via a 2-M K-citrate-filled electrode, bevelled to a resistance of 3–7 MΩ, using an Axoclamp-2A (Axon Instruments, CA) amplifier in the bridge-mode which compensates electrode voltage drop during current passing. The internal command of the amplifier was triggered by a microcomputer with pCLAMP software (Axon Instruments, CA), to inject current pulses. Membrane potentials were filtered at 500 Hz (−3 dB, 8-pole Bessel) and differentiated using an RC circuit with a time constant of 10 msec. The potentials and their time derivatives were sampled at 12-bit resolution every 1–2 msec. All experiments were performed at room temperature (20–22°C).

For voltage clamp experiments, the three-microelectrode technique developed by Adrian, Chandler and Hodgkin (1970) was used. The three microelectrodes impaled a muscle fiber near its end. Two electrodes positioned at distances l and 2 l from the fiber end were used to record the membrane potentials V_1 and V_2, respectively. They were filled with 3 M KCl. A third electrode filled with 2M K-citrate, inserted at a distance $2\,l + l'$, was used to deliver current intracellularly. Electrode resistances ranged from 3 to 25 MΩ. The Axoclamp-2A amplifier was used, in the two-electrode voltage-clamp mode, to display and record V_1 and to measure current flow (I_0) through the third microelectrode. The membrane potential was, therefore, controlled at l.

V_2 was measured by a unity gain voltage follower (W.P. Instruments, Model 750) whose output was fed to a digital voltmeter and to channel 1 of the dual-trace amplifier of an oscilloscope (Tektronix, Model 5A18N). V_1 was fed to channel 2 of the dual-trace amplifier, allowing scaling and subtraction of the membrane potentials V_1 and V_2. The output of the dual-trace amplifier, the amplified V_2-V_1 voltage

signal, was filtered at 500 Hz (−3 dB, 8-pole Bessel) and sampled at 12-bit resolution by the microcomputer every 1–2 msec.

To calculate membrane current density (I_m, $\mu A/cm^2$), Adrian et al. (1970) have shown that I_m is approximately equal to:

$$I_m = \frac{a(V_2 - V_1)}{3\, l^2\, R_i}$$

where a is the fiber radius, V_1 and V_2 are the membrane potentials recorded at distances l and 2 l, respectively, and R_i is the resistivity of the myoplasm. R_i was assumed to be 125 $\Omega \cdot$ cm at 25°C as in other crustacean muscle fibers (Fatt & Katz, 1953; Fatt & Ginsborg, 1958), and that it varied with temperature with a Q_{10} of 1.4, as in other arthropods (Ashcroft, 1980). l varied from 60 to 100 μm and l' from 20 to 30 μm; they were directly measured with the microscope. The fiber radius was calculated following the equations by Adrian et al. (1970).

Linear membrane currents were subtracted digitally online by a P/4 procedure (Armstrong & Bezanilla, 1974) from a subholding potential which was 15 mV more negative than the holding potential (E_h). E_h was held at −75 mV which is close to the resting potential of these fibers (Zuazaga & del Castillo, 1985). Pulse generation, data acquisition and analysis were done using the microcomputer and pCLAMP software.

Voltage-clamp experiments in Ca^{2+}-containing solutions were performed at room temperature (20–22°C). To slow the time course of the currents recorded in Sr^{2+}-and Ba^{2+}-containing solutions and improve current control, voltage-clamp experiments in these solutions were performed at 5–10°C.

Solutions and Drugs

The composition of the different solutions is shown in the Table. Ca^{2+}-free solutions (solutions C–M) contained 5 mM EGTA. Solutions containing a high concentration of Ca^{2+}, Sr^{2+} or Ba^{2+} were prepared by replacing 3 Na^+ with two divalent cations. In Cl^--free solution (solution N), Cl^- was replaced with the impermeant anion methanesulfonate ($CH_3\ SO_3$); Na^+ was replaced with the K^+ channel blocker TEA. Solutions were buffered with 3-(N-morpholino) propanesulfonic acid (MOPS, 4 mM) at pH 7.4. Nifedipine and S(−)- Bay K 8644 were dissolved in ethanol, and diluted in the bath solution to the specified concentration; the resulting solvent concentration was 0.1% or less. Similar concentrations of ethanol had no significant effect on the experimental results. The DHPs were prepared and applied under subdued light. All chemicals were purchased from Sigma.

Results

Ca^{2+} Influx is Required for Contraction

Figure 1 shows that tension generation in *A. lanipes* skeletal muscle fibers is absolutely dependent upon the presence of Ca^{2+} in the extracellular medium. The left panel shows the contraction evoked by a series of increasing depolarizing current steps when the fiber was bathed in control solution containing 13.6 mM Ca^{2+} (solution A, Table). Notice that the fiber did not generate an active response when depolarized, as previously reported (Zuazaga & del Castillo, 1985; Lizardi et al., 1992). In contrast, (*see* Fig. 1, right panel), in the absence of Ca^{2+}

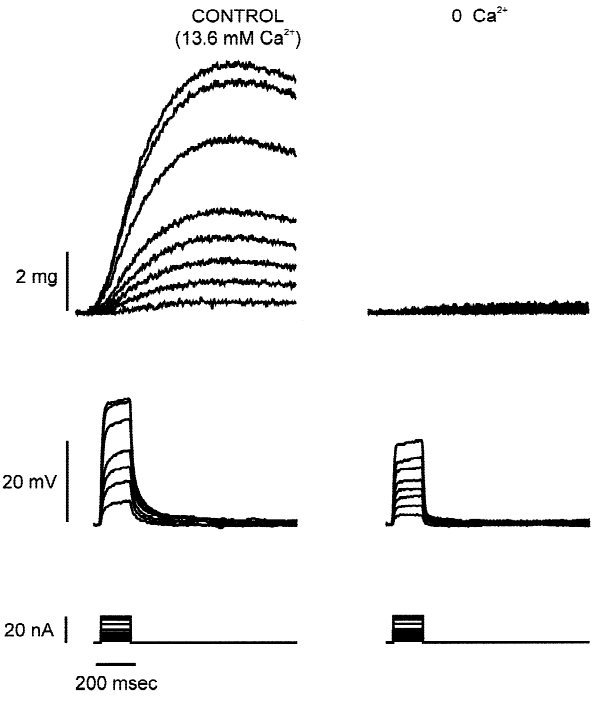

Fig. 1. Depolarization-evoked tension and the electrical response in a fiber bathed in control solution (solution A, left panel) or in Ca^{2+}-free solution (solution M, right panel). Left panel: top traces show tension measurements in response to a series of depolarizing current steps (bottom traces); middle traces show the passive electrical responses of the muscle fiber. Right panel: top traces show that tension fails in the absence of extracellular Ca^{2+} when depolarizing current steps (bottom traces) are applied; middle traces show the electrical responses of the fiber. Same experiment throughout; resting potential −75 mV (left panel) and −60 mV (right panel).

in the extracellular medium (solution M), the fiber was unable to generate tension when depolarized. Contractile failure was not due to depletion of Ca^{2+} from the SR because, in Ca^{2+}-free solution, caffeine releases Ca^{2+} from the SR evoking contractures in these fibers (Bonilla et al., 1992). Thus, as in barnacle and scorpion skeletal muscles (Caputo & Di Polo, 1978; Gilly & Scheuer, 1984), the SR of *A. lanipes* muscle does not become depleted of Ca^{2+} in the absence of extracellular Ca^{2+}. Therefore, these results suggest that in this muscle, contraction failed in Ca^{2+}-free solution because there was no Ca^{2+} influx to trigger CICR.

The absolute requirement of Ca^{2+} influx for contractile activation is further supported by experiments where Cd^{2+}, an inorganic Ca^{2+} channel blocker, was used to prevent the influx of Ca^{2+}, as illustrated in Fig. 2. In these experiments, extracellular electrical stimulation was used to evoke the mechanical responses. The left trace in Fig. 2*A* shows the response to a single stimulus evoked in control solution containing 13.6 mM Ca^{2+} (solution A). In the presence of 4 mM Cd^{2+}, tension generation was very rapidly (1–2 min) and completely abolished (middle trace); tension returned to control levels upon washing out the Ca^{2+} channel blocker (right trace).

Table. Composition of solutions (in mM)[a]

	NaCl	KCl	$CaCl_2$	$MgCl_2$	$BaCl_2$	$SrCl_2$	EGTA
A	205.0	5.4	13.6	2.4	—	—	—
B	22.0	5.4	138.0	—	—	—	—
C	206.1	5.4	—	—	—	13.6	5.0
D	196.5	5.4	—	—	—	20.0	5.0
E	166.5	5.4	—	—	—	40.0	5.0
F	136.5	5.4	—	—	—	60.0	5.0
G	19.5	5.4	—	—	—	138.0	5.0
H	206.1	5.4	—	—	13.6	—	5.0
I	196.5	5.4	—	—	20.0	—	5.0
J	166.5	5.4	—	—	40.0	—	5.0
K	136.5	5.4	—	—	60.0	—	5.0
L	19.5	5.4	—	—	138.0	—	5.0
M	229.0	5.4	—	—	—	—	5.0

Methanesulfonate solution			
	$TEACH_3SO_3$	KCH_3SO_3	$Ca(CH_3SO_3)_2$
N	22.0	5.4	138.0

[a] Solutions were buffered with 4 mM MOPS (pH = 7.4).

Caffeine contractures were evoked in control solution and in Cd^{2+}-containing solution to provide further support to our conclusion that contractile failure was not due to depletion of Ca^{2+} from the SR. Figure 2*B* illustrates the results of experiments where the fibers were first exposed to 2 mM caffeine in control solution (solution A) for 30–35 seconds (left bar) and then to control solution containing 2 mM caffeine and 4 mM Cd^{2+} to block the electrically generated mechanical responses (right bar). No statistical differences were observed in the peak tension of the contractures evoked by caffeine in control solution and in Cd^{2+}-containing solution, where the electrically generated mechanical response was abolished. These results indicate that contractile failure was not due to alteration of the SR Ca^{2+} load.

Sarcolemmal and tubular Ca^{2+} channels in crustacean skeletal muscles have been found to be sensitive to DHPs (Hurnak et al., 1990; Erxleben & Rathmayer, 1997). For example, the graded electrical response elicited by depolarization of twitch muscle fibers of the marine isopod *Idotea baltica* is converted into an action potential upon exposure to Bay K 8644, an agonist of L-type Ca^{2+} channels; nifedipine, an L-type Ca^{2+} channel antagonist, reduces the action potential back to a graded response (Erxleben & Rathmayer, 1997). These observations prompted us to test the effects of DPHs on the electrical response and tension generation in *A. lanipes* muscle fibers. We found that in the presence of 10 μM Bay K 8644, the tension evoked by a depolarizing current step is markedly larger than that evoked in control solution (Fig. 3*A*, top traces). Upon washing out the drug, tension returned to control levels. Interestingly, the passive electrical response was not converted into an action potential during exposure of the fiber to Bay K 8644 (Fig. 3*A*, bottom traces). The time course of the effect of the drug on contractile activation is shown in Fig. 3*B*. Depolarization-induced tension increased within 10 min, following application of Bay K 8644, and was maximally enhanced after about 35 min. In three different fibers, tension generation in response to depolarization was reversibly increased, with similar time course, by about 100 and 200%; changes in the passive electrical response of the fibers were not observed in these experiments.

The L-type Ca^{2+} channel blocker nifedipine, on the other hand, inhibited the contractile response in *A. lanipes* muscle fibers in a reversible manner (Fig. 4*A*, top traces). In the experiment shown, the tension generated by a depolarizing current step was reduced about 78% in the presence of 10 μM nifedipine. The electrical response was not significantly changed by the drug (Fig. 4*A*, bottom traces). The time course of the inhibitory action of nifedipine on depolarization-evoked tension (Fig. 4*B*) was similar to that of the Bay K 8644-induced enhancement of tension (Fig. 3*B*).

Measurement of Ionic Currents Through Ca^{2+} Channels

The abolition of contraction in Ca^{2+}-free solution, in Cd^{2+}-containing solution, and the effects of the DHPs on E-C coupling suggest that Ca^{2+} channels are present in the sarcolemmal/tubular membrane of *A. lanipes* muscle fibers. In an attempt to unmask the channels, we increased the driving force for Ca^{2+} tenfold (solution B), but, although depolarization-evoked tension increased considerably, the electrical response remained completely passive (*data not shown*).

However, when 13.6 mM Ca^{2+} was isotonically substituted for Sr^{2+} or Ba^{2+} in the presence of 5 mM EGTA in the bathing solutions (solutions C and H, respectively), action potentials could be elicited by depolarizing current pulses (Fig. 5). Under these conditions, mechanical activation did not occur, and we therefore measured action potential amplitudes and their time derivatives. We found that in Ca^{2+}-free, EGTA-containing solutions, as the concentration of the current-carrying divalent cation was increased, the amplitudes of the action potentials and their maximum rate of rise, V_{max}, increased (Fig. 5*A* and *B*, top and bottom traces, respectively). V_{max} was assumed to be proportional to the maximal inward current (Hagiwara & Takahashi, 1967) and is plotted as a function of Sr^{2+} or Ba^{2+} concentration in Fig. 5*C*. V_{max} tended towards saturation as the divalent cation concentration was increased. The experimental points were fitted assuming a Michaelis-Menten type kinetics:

$$V_{max} = \frac{[C^{2+}]_o}{[C^{2+}]_o + K_m} (V_{max\text{-}s})$$

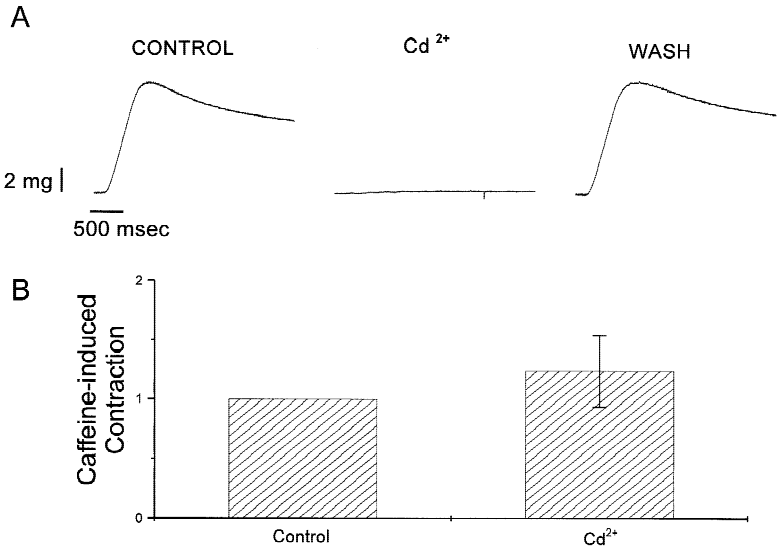

Fig. 2. Mechanical responses evoked by extracellular electrical stimulation are reversibly abolished by Cd^{2+}. (*A*) Responses to a single supramaximal electrical stimulus evoked in control solution (left trace); tension fails to develop in the presence of 4 mM Cd^{2+} (middle trace); upon washing out Cd^{2+}, tension returns to control levels (right trace). Same experiment throughout. (*B*) Peak tension of the contractures evoked by caffeine in control solution (left bar); peak tension of caffeine contractures in Cd^{2+}-containing solution (right bar). Each bar is mean ± SEM of 4 experiments; the same bundle of fibers was used for both control and test trials.

where $[C^{2+}]_o$ is the divalent cation concentration in the external solution, $V_{max\text{-}s}$ is the saturated V_{max} at an infinite $[C^{2+}]_o$, and K_m is the Michaelis constant. The curves of Fig. 5*C* were drawn according to the values of $V_{max\text{-}s}$ and K_m obtained which were 6.2 *V*/sec and 14.6 mM for Sr^{2+} and 3.8 *V*/sec and 9.8 mM for Ba^{2+}, respectively. The influx of these divalent cations is mediated by L-type Ca^{2+} channels because, as shown in Fig. 6, 10 μM nifedipine reduces the Sr^{2+} spike and its derivative.

To unmask the Ca^{2+} channels in *A. lanipes* muscle fibers using Sr^{2+} and Ba^{2+} as current carriers we had to take extreme care to keep the free Ca^{2+} concentration in the bathing solution very low, because it appeared that the influx of these divalent cations through the channels was reduced by extracellular Ca^{2+} (*data not shown*). Thus, all Ca^{2+}-free solutions contained 5 mM EGTA.

Biophysical Characterization of the Silent Ca^{2+} Channels

Voltage-clamp experiments with three microelectrodes (Adrian et al., 1970; *see* Materials and Methods) were carried out to characterize some of the properties of the currents through the sarcolemmal/tubular Ca^{2+} channels. To enhance inward current density, the divalent cation concentration was increased tenfold (*see* Table). The ionic currents in solutions containing the different divalent cations are shown in Fig. 7. Figure 7*A* illustrates a family of currents recorded at different potentials from a fiber superfused with a Cl^--free solution containing TEA and 138 mM Ca^{2+} (solution N). 3, 4-Diaminopyridine (0.5 mM) was added to this recording solution. Inward current was not observed, even though the driving force for Ca^{2+} was increased tenfold, Cl^- currents eliminated and K^+ currents inhibited ($n = 5$). Because inward cur-

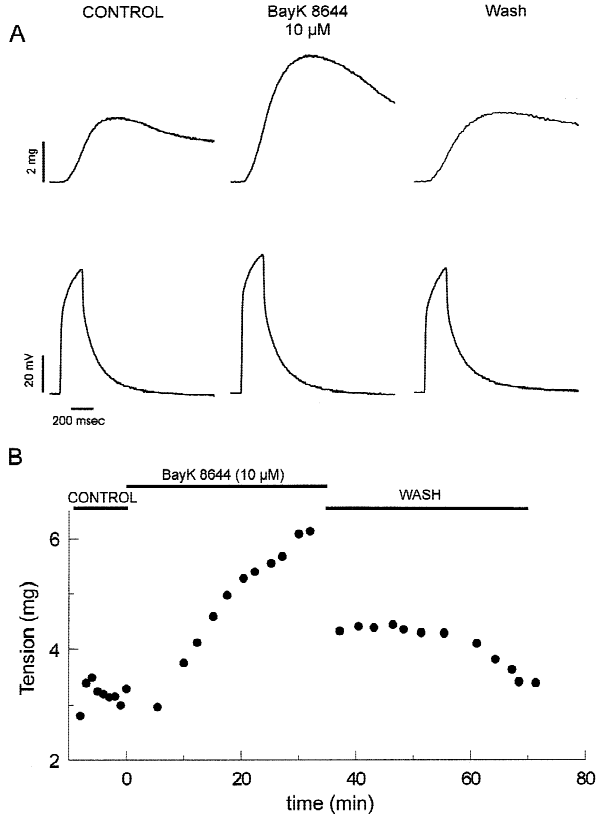

Fig. 3. Effects of Bay K 8644 on tension and the electrical response. (*A*) Depolarization-evoked tension (top traces) increases in the presence of 10 μM Bay K 8644; tension reverses to control levels upon washing out the drug. The passive electrical response of the fiber (bottom traces) is not converted into an action potential in the presence of Bay K 8644. Same experiment throughout; injected current 400 nA, resting potential −76 mV. (*B*) Time course of the effect of Bay K 8644 on depolarization-induced tension.

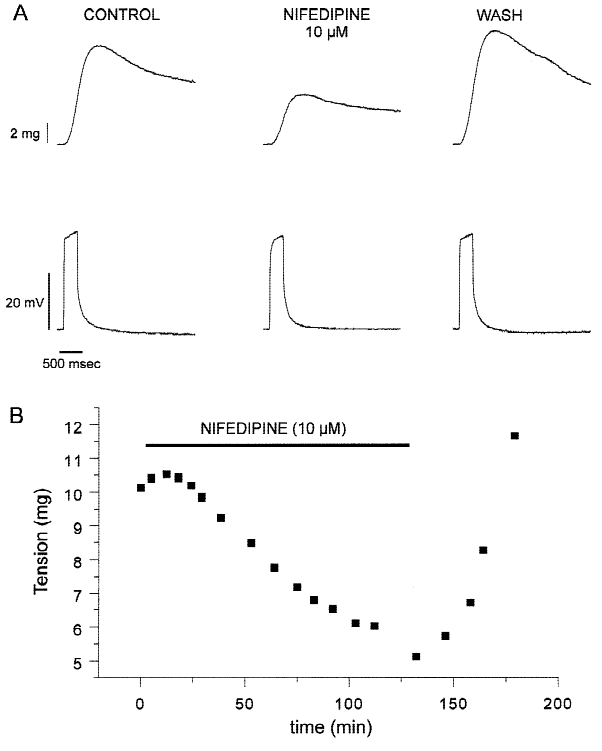

Fig. 4. Nifedipine inhibits depolarization-evoked tension. (*A*) Tension (top traces) is inhibited in a reversible manner by 10 μM nifedipine; the drug does not affect the electrical response (bottom traces). Same experiment throughout; injected current 100 nA, resting potential −52 mV. (*B*) Time course of the effect of nifedipine on depolarization-induced tension.

rent density was unmeasurably low under these ionic conditions, we have called the channels "silent Ca^{2+} channels." Notice that repolarization appears to elicit a slowly deactivating inward tail current, suggesting the activation of the silent Ca^{2+} channels.

The currents recorded from fibers bathed in Ca^{2+}-free (5 mM EGTA) Sr^{2+}- or Ba^{2+}-containing solutions differ markedly from those of fibers bathed in Ca^{2+}-containing solution. To slow the time course of the currents and improve current control, these recordings were performed at 5–10°C. Figure 7*B* shows the membrane currents recorded from a fiber superfused with Ca^{2+}-free, 138 mM Sr^{2+}-containing solution (solution G). A slow transitory inward current through the silent Ca^{2+} channels was observed. The current activated near −20 mV, was maximal near −5 mV and decreased in amplitude with depolarizations larger than 0 mV. With the command pulse driving the membrane potential to positive values, a small outward current was also observed. The maximum peak Sr^{2+} current ranged from −5 to −78 μA/cm^2 in fibers with 30 to 65 μm radius, averaging −33 ± 11 μA/cm^2 (mean ± SEM, $n = 7$) and was maximal near 0 mV.

Inward current through the silent Ca^{2+} channels was also observed when the fibers were bathed in Ca^{2+}-free, 138 mM Ba^{2+}-containing solution (solution L), as shown in Fig. 7*C*. The current activated near −20 mV, was maintained during the pulse and was maximal near +5 mV; outward current was not detected. In 8 fibers tested under these ionic conditions, inward Ba^{2+} current ranged from −3 to −30 μA/cm^2, averaging −14 ± 4 μA/cm^2 (mean ± SEM) near 15 mV.

The results described above indicate that Sr^{2+} passes through *A. lanipes* silent Ca^{2+} channels more easily than Ba^{2+}. This is further supported by experiments performed in the same fiber where the bathing solution was changed from Sr^{2+} to Ba^{2+}. Figure 8 shows current-voltage relations from a fiber obtained in Ca^{2+}-free 138 mM Sr^{2+} (solution G) and Ca^{2+}-free 138 mM Ba^{2+} (solution L). Peak inward current density decreased about fivefold when Sr^{2+} was replaced by Ba^{2+}, and the curve shifted by about 15 mV to more positive potentials. A similar reduction in peak current upon changing from 138 mM Sr^{2+} to 138 mM Ba^{2+} was found in two other fibers thus studied.

Discussion

The main finding of this work is that Ca^{2+} channels are present in the sarcolemmal/tubular membrane of skeletal muscle fibers of the crustacean *A. lanipes.* These channels are silent, i.e., Ca^{2+} current density is unmeasurably low, but inward current through them may be measured in Ca^{2+}-free solutions using Sr^{2+} and Ba^{2+} as current carriers. The significance of the findings for the E-C coupling process in this crustacean muscle will be discussed below.

Evidence for the Presence of Ca^{2+} Channels

Under physiological conditions, the presence of Ca^{2+} channels in this muscle, and their role in E-C coupling is revealed not only by the failure of contraction in the absence of extracellular Ca^{2+} and in the presence of Cd^{2+} but also by the enhancement of tension produced by the L-type Ca^{2+} channel agonist Bay K 8644 and its inhibition by nifedipine, an L-type Ca^{2+} channel antagonist. When the channels are unmasked in Ca^{2+}-free solutions containing other divalent cations, the abolition of the Sr^{2+} spikes by nifedipine provides additional evidence that these channels are similar to L-type Ca^{2+} channels (Fig. 6). These results indicate that the silent L-type Ca^{2+} channels mediate the influx of Ca^{2+} required for contractile activation and the influx of Sr^{2+} and Ba^{2+} required for the generation of an active electrical response.

Characteristics of the Inward Currents

The inward current carried by Ca^{2+} through the silent Ca^{2+} channels in this preparation is too small to be mea-

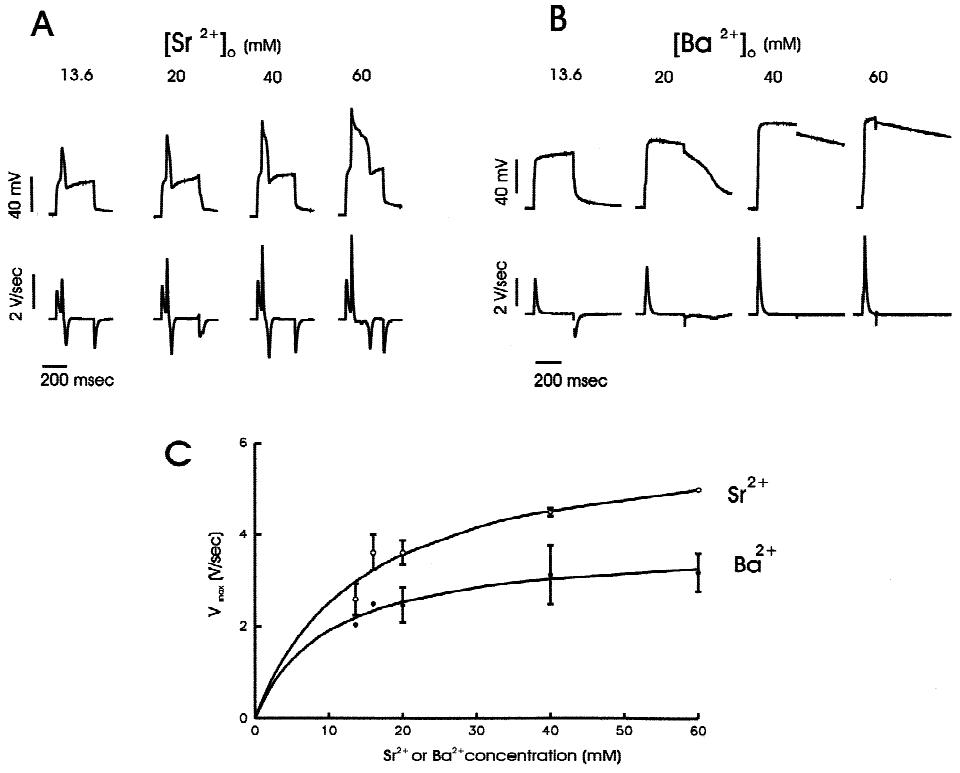

Fig. 5. Action potentials recorded in Ca^{2+}-free, Sr^{2+}- or Ba^{2+}-containing solutions in current-clamp experiments. (*A*) An action potential (top left trace) was generated in response to depolarizing current in 13.6 mM Sr^{2+}-containing solution (solution C). The amplitude of the action potentials and their derivatives (top and bottom traces, respectively) increased as the Sr^{2+} concentration in the bathing medium (shown above the traces) was increased. Same experiment throughout; injected current 40 nA. Resting potential of the fiber was −70 mV. (*B*) Ba^{2+} action potentials (top traces) and their derivatives (bottom traces) evoked by a 30 nA depolarizing step from a different fiber bathed in solutions containing the Ba^{2+} concentrations shown above the traces. Resting potential −80 mV. (*C*) Current (V_{max})-concentration relationships in Sr^{2+} and Ba^{2+} solutions. The curves are the best nonlinear fit of the experimental data to the Michaelis-Menten equation. The values of $V_{max\text{-}s}$ and K_m were 6.2 *V*/sec and 14.6 mM for Sr^{2+} and 3.8 *V*/sec and 9.8 mM for Ba^{2+}, respectively. Each point is mean ± SEM of 4 determinations.

sured, even when the driving force is increased tenfold; however, inward currents carried by high concentrations of Sr^{2+} and Ba^{2+}, in Ca^{2+} -free solutions, may be measured and described (Figs. 7 and 8). Although the Sr^{2+} and Ba^{2+} currents were not isolated by either ionic substitutions (i.e., using the impermeant anion methanesulfonate instead of Cl^-) or pharmacological interventions (blockage of K^+ currents by TEA and 3,4-diaminopyridine), our results show the following. First, the Sr^{2+} and Ba^{2+} currents activate at potentials above −40 mV and peak close to 0 mV, another indication that L-type Ca^{2+} channels mediate the influx of these divalent cations. Second, the peak inward current carried by Sr^{2+} is larger than that carried by Ba^{2+}, suggesting that the selectivity profile of the silent Ca^{2+} channels is $I_{Sr} > I_{Ba} > I_{Ca}$. This profile is distinct from that of vertebrate L-type Ca^{2+} channels ($I_{Ba} > I_{Sr} > I_{Ca}$). Third, Ba^{2+} currents appear to activate and inactivate much more slowly than Sr^{2+} currents. It is possible that, as in other arthropod muscles (Ashcroft & Stanfield, 1982), Ba^{2+}-carried currents activate more slowly than those carried by other divalent

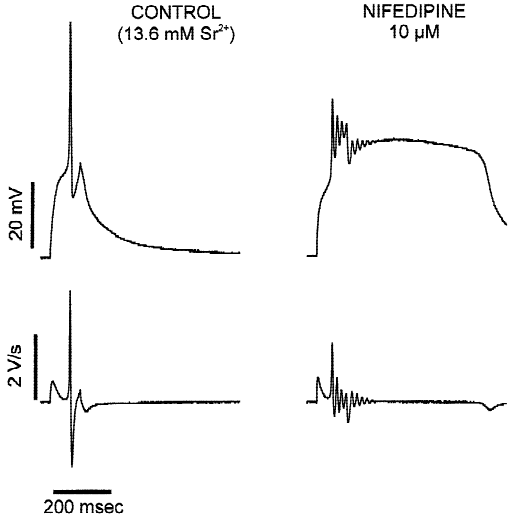

Fig. 6. Nifedipine reduces the spike recorded in Ca^{2+}-free 13.6 mM Sr^{2+}-containing solution (solution C) (top traces) and its time derivative (bottom traces). Resting potential of the fiber −77 mV.

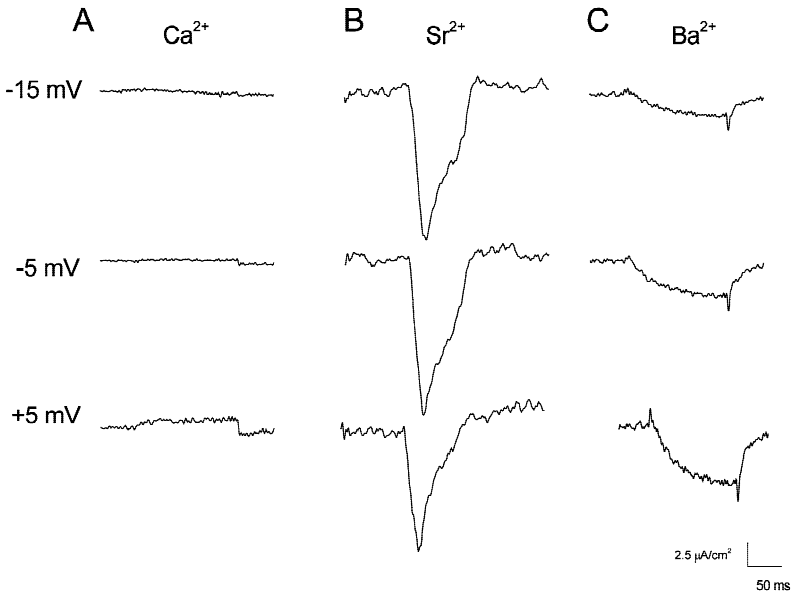

Fig. 7. Membrane currents recorded in 138 mM divalent cation solutions in voltage-clamp experiments. (*A*) Records of membrane currents during command pulses to different potentials (numbers at left); fiber bathed in Cl^--free solution containing TEA and 138 mM Ca^{2+} (solution N); 0.5 mM 3,4-diaminopyridine was added. T = 20–22°C. (*B*) Total membrane currents during command pulses to the potentials shown at left; fiber bathed in Ca^{2+}-free, 138 mM Sr^{2+}-containing solution (solution G). T = 5–10°C. (*C*) Records of total membrane currents during command pulses to the potentials shown at left; fiber bathed in Ca^{2+}-free, 138 mM Ba^{2+}-containing solution (solution L). T = 5–10°C. (*A*), (*B*) and (*C*) are records from three different fibers. Note differences in recording temperatures.

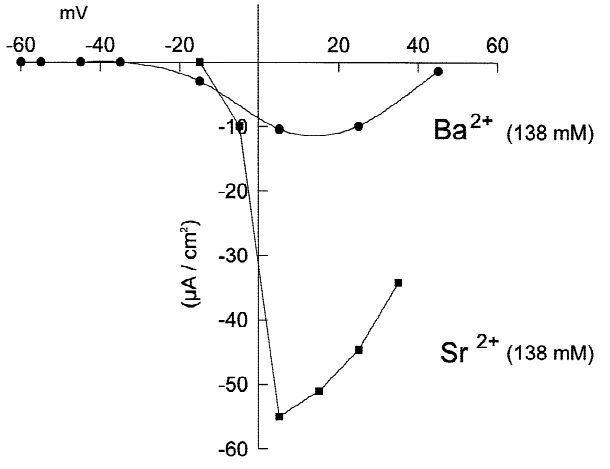

Fig. 8. Current-voltage curves in Sr^{2+}- *vs.* Ba^{2+}-containing solutions. Peak currents were measured during pulses to different voltages in a solution containing 138 mM Sr^{2+} (solution G). The solution was then changed to one containing 138 mM Ba^{2+} (solution L) and voltage steps were repeated. Same experiment throughout.

cations. The slower apparent inactivation kinetics of the Ba^{2+} currents is probably due to blockage of the outward K^+ current by Ba^{2+} but not by Sr^{2+}. Isolated Ba^{2+} and Sr^{2+} currents must be carefully studied to resolve these issues. The silent Ca^{2+} channels reside in tubular membranes (*see below*); this would limit voltage control and explain the observed notches in the inward current records.

The distinguishing characteristic of the inward membrane current in this muscle is its low density. When compared to Ca^{2+}-generated current in other arthropod muscles (Hencek & Zachar, 1977; Ashcroft & Stanfield, 1982; Gilly & Scheuer, 1993), current density in *A. lanipes* is 4- to 10-fold lower, even though high concentrations of permeant divalent cations were used to increase inward membrane current. Unlike *A. lanipes* muscle, which is electrically inexcitable, the muscles in these other arthropods generate either all-or-none Ca^{2+} spikes or graded potentials. Assuming that the single channel conductance of the Ca^{2+} channels in *A. lanipes* muscle is similar to that of Ca^{2+} channels in other crustacean muscles (Hurnak et al., 1990; Bishop, Krouse & Wine, 1991; Erxleben & Rathmayer, 1997), these results suggest that in *A. lanipes* muscle fibers Ca^{2+} channels occur in very low density. This would explain why, under physiological conditions, Ca^{2+} influx is not enough to generate a macroscopic inward current.

Implications for E-C Coupling

Recent work in our laboratory indicates that the silent Ca^{2+} channels in *A. lanipes* skeletal muscle are mainly located in the T-tubules (Monterrubio et al., 1999). Thus, the silent Ca^{2+} channels are in close proximity to the SR Ca^{2+} release channels in the dyad. In vertebrate cardiac muscle, CICR depends on the ability of tubular Ca^{2+} channels to increase the local concentration of Ca^{2+} in its immediate vicinity; the close juxtaposition of tubular Ca^{2+} channels and SR Ca^{2+} release channels in the dyad results in a larger amount of Ca^{2+} to flow from the SR to activate contraction (*see* Gomez et al., 1997 and references therein). There is evidence that CICR in crustacean muscle is also mediated by a local control mechanism (Györke & Palade, 1992, 1993). Our results suggest that CICR in *A. lanipes* muscle operates with an unusually high gain: microscopic Ca^{2+} influx through the

silent Ca^{2+} channels is sufficient to increase the local concentration of Ca^{2+} in the immediate vicinity of the SR Ca^{2+} release channels in the dyad and trigger the highly amplified release of Ca^{2+} required for contractile activation. Therefore, the local-control mechanism of E-C coupling might explain the paradoxical requirement of Ca^{2+} influx for the activation of contraction *vis-a-vis* the absence of measurable Ca^{2+} current in this crustacean muscle.

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