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Sulfhydryl Alkylating Agents Induce Calcium Current in Skeletal Muscle Fibers of a Crustacean (*Atya lanipes*)

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Summary. Voltage-clamp experiments using the three-microelectrode voltage clamp technique were performed on ventroabdominal flexor muscles of the crustacean *Atya lanipes*. Potassium and chloride currents were found to underlie the normal, passive response of the muscle. Blocking potassium currents with tetraethylammonium and replacing chloride ions with methanesulfonate did not unmask an inward current. By treating the muscle with the sulfhydryl-alkylating agent 4-cyclopentene-1,3-dione an inward current was detected. The current induced by the agent is carried by Ca^{2+}, since it is abolished in Ca^{2+}-free solutions. The induced Ca^{2+} current is detected at about -40 mV and reaches a mean maximum value of -78 $\mu A/cm^2$ at *ca.* -10 mV. At this potential the time to peak is close to 15 msec. The induced Ca^{2+} current inactivated with 1-sec prepulses which did not elicit detectable Ca^{2+} current; the fitted h_∞ curve had a midpoint of -38 mV and a steepness of 5.0 mV. Measurements of isometric tension were performed in small bundles of fibers, and the effects of the sulfhydryl-alkylating agents 4-cyclopentene-1,3-dione and N-ethylmaleimide were investigated. Tetanic tension was enhanced in a strictly Ca^{2+}-dependent manner by 4-cyclopentene-1,3-dione. The amplitude of K^+ contractures increased after treatment with N-ethylmaleimide. It is concluded that Ca^{2+} channels are made functional by the sulfhydryl-specific reagents and that the increase in tension is probably mediated by an increase in Ca^{2+} influx through the chemically induced Ca^{2+} channels.

Key Words calcium current · skeletal muscle · sulfhydryl · invertebrate muscle

Introduction

Ca^{2+} channels are responsible for the regenerative electrical excitability in arthropod skeletal muscle fibers (Fatt & Ginsborg, 1958; Hagiwara & Naka, 1964; Hagiwara, Hayashi & Takahashi, 1969; Keynes et al., 1973; Mounier & Vassort, 1975; Hencek & Zachar, 1977; Ashcroft & Stanfield, 1982; Gilly & Scheuer, 1984; for reviews *see* Hagiwara & Byerly, 1981; Hagiwara, 1983). The proposed physiological role of the channels is to control the flow of extracellular Ca^{2+} required, either directly or indirectly, to regulate Ca^{2+} release by the sarcoplasmic reticulum, for contractile activation (Zacharova & Zachar, 1967; Ashley & Ridgway, 1970; Atwater, Rojas & Vergara, 1974; Caputo & DiPolo, 1978; Gilly & Scheuer, 1984; Scheuer & Gilly, 1986).

This proposal is based mostly on studies performed on *Crustacea*. The electrical and mechanical behavior of crustacean muscle is widely diversified, ranging from graded responses associated with weak, local contractions to regenerative Ca^{2+} spikes accompanied by strong mechanical responses (for review *see* Hoyle, 1983). Early experiments showed that by exposing crustacean muscles to agents that block the outward K^+ current which shunts the inward Ca^{2+} current, the electrical and mechanical responses could be converted from weak to strong (Fatt & Katz, 1953).

Skeletal muscle fibers of the crustacean *Atya lanipes* have been found to be electrically inexcitable; however, following exposure to sulfhydryl-alkylating agents such as N-ethylmaleimide (NEM), the fibers are able to generate Ca^{2+} action potentials (Zuazaga & del Castillo, 1978*a,b*, 1985). This conversion cannot be brought about by blockers of K^+ currents (Zuazaga & del Castillo, 1985; Zuazaga et al., 1991).

The present experiments were performed to provide additional information on this phenomenon by studying the underlying membrane currents and tension generation. We found that sulfhydryl-alkylating agents induced Ca^{2+} current and enhanced mechanical activity in these crustacean muscle fibers. Preliminary results have been reported (Lizardi et al., 1989).

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Table. Composition of solutions (in mM)

Chloride solutions[a]	NaCl	KCl	TEACl	$NaHCO_3$	$CaCl_2$	$MgCl_2$
A	205.0	5.4	—	2.0	13.6	2.4
B	55.0	5.4	150.0	2.0	13.6	2.4
C	205.0	5.4	—	2.0	—	16.0
D	130.0	5.4	—	2.0	60.0	2.4
E[b]	205.0	5.4	—	2.0	—	—
F	200.0	10.0	—	2.0	13.6	2.4
G	190.0	20.0	—	2.0	13.6	2.4
H	180.0	30.0	—	2.0	13.6	2.4
I	150.0	60.0	—	2.0	13.6	2.4
J	120.0	90.0	—	2.0	13.6	2.4
K	90.0	120.0	—	2.0	13.6	2.4
L	60.0	150.0	—	2.0	13.6	2.4

Methanesulfonate solutions[c]	$NaCH_3SO_3$	KCH_3SO_3	$TEACH_3SO_3$	$Ca(CH_3SO_3)_2$	$Ba(CH_3SO_3)_2$	$Mg(CH_3SO_3)_2$
M	55.0	5.4	150.0	13.6	—	2.4
N	130.0	5.4	150.0	60.0	—	2.4
O	130.0	5.4	150.0	—	60.0	2.4

[a] pH = 7.4 (Tris).
[b] Solution E also contained 5 mM EGTA.
[c] pH = 7.4 (MOPS).

Materials and Methods

Current- and voltage-clamp experiments with microelectrodes were performed on ventroabdominal flexor muscles of the freshwater crustacean *A. lanipes*, collected in streams at El Yunque rain forest, Puerto Rico, and kept in aquaria at room temperature (20–22°C). Microelectrode experiments were done on fibers in whole muscles from the second or third segment. Small bundles of 15–20 fibers from the muscles were used for mechanical experiments.

Current-Clamp Recordings

Muscles were dissected in normal (van Harreveld, 1936) solution (solution A, the Table). Fibers were impaled with two microelectrodes positioned with an interelectrode distance of about 40 μm. The membrane potential recording electrode 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 current-clamp or bridge mode which compensates electrode voltage drop during current passing. A microcomputer (Dell System 310, Dell Computer, TX) was used to trigger the internal command of the amplifier for injecting current pulses. Membrane potentials were filtered at 500 Hz (−3 dB, 8-pole Bessel) and sampled at 12-bit resolution by the microcomputer every 1–2 msec. Experiments were performed either at room temperature (20–22°C) or at 10–13°C.

Voltage-Clamp Experiments

The three-microelectrode voltage-clamp technique near the end of the fiber (Adrian, Chandler & Hodgkin, 1970) was used. Two microelectrodes filled with 3 M KCl were inserted at distances $x = l$ and $x = 2l$ from the end of the fiber to record membrane potentials V_1 and V_2, respectively. A third electrode filled with 2 M K-citrate, inserted at $x = 2l + l'$, was used to deliver current intracellularly. The current electrode was screened to earth to within 2–3 mm from its tip. Electrode resistances ranged from 3–25 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_o) through the third microelectrode. Thus, the membrane potential was controlled at $x = l$; however, to measure membrane capacity per unit surface, C_m, the potential was controlled at $x = 2l$ as described by Adrian et al. (1970).

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. This allowed 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.

Membrane current density (I_m, μA/cm^2) was calculated according to the equation (Adrian et al., 1970):

$$I_m = \frac{a(V_2 - V_1)}{3l^2 R_i} \tag{1}$$

where a is the fiber radius, V_1 and V_2 are the membrane potentials recorded at $x = l$ and $x = 2l$, respectively, and R_i is the resistivity of the myoplasm. R_i was assumed to be 125 Ω · cm at 25°C as in other crustacean muscle fibers (Fatt & Katz, 1953; Fatt & Ginsborg, 1958). It was also assumed that R_i did not change with the tonicity of the external solution but that it varied with temperature having 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 and the

electrical constants were calculated following the equations by Adrian et al. (1970).

The approximation for the membrane current density is correct to within 5% is l is less than 2 space constants (λ); this occurs when $(V_2 - V_1)/V_1$ is less than 6 (Adrian et al., 1970). In these experiments we were well within the 5% error limit. Furthermore, the voltage clamp gave reasonable values for the electrical constants: in normal solution, the space constant (λ) was 0.24 ± 0.02 mm (10), the specific membrane resistance (R_m) was 170 ± 28 $\Omega \cdot cm^2$ (10) and the total capacity per unit surface (C_m) was 55 ± 8 $\mu F/cm^2$ (7) (mean ± SE (number of observations)). These values agree with those reported for other crustacean muscle fibers (Fatt & Katz, 1953).

Linear membrane currents were subtracted digitally on-line 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 −70 or −75 mV which is close to the resting potential of these fibers (Zuazaga & del Castillo, 1978*a*, 1985). All stimulus pulses were generated by the microcomputer, and data were stored on hard disk for further analysis. Experiments were performed at 10–13°C.

Mechanical Experiments

Isometric tension was measured by a mechanoelectronic transducer (Cambridge Technology, Series 400A) whose output was stored on a video cassette recorder (Bezanilla, 1985) and displayed on a strip chart recorder (Gould). Tetanic stimulation (20–30 Hz) was used to assess the condition of the fibers. Fiber rest length was adjusted to produce maximum tension in response to single suprathreshold stimuli. Experiments were carried out at room temperature (20–22°C).

Solutions

The composition of the different solutions is shown in the Table. Nominally Ca^{2+}-free solution (solution C) contained Mg^{2+} to replace Ca^{2+}. Solutions containing a high concentration of Ca^{2+} or Ba^{2+} (solutions D, N and O) were prepared by replacing 3 Na^+ with two divalent cations. In Cl^--free solutions, Cl^- was replaced with the impermeant anion methanesulfonate ($CH_3SO_3^-$). K^+ contractures were elicited by elevation of extracellular K^+ which isotonically replaced Na^+ (solutions F–L).

The sulfhydryl alkylating agents NEM and 4-cyclopentene-1,3-dione (4-CPD; *see* Toro-Goyco, Zuazaga and del Castillo, 1978) were used at a concentration of 2–4 mM. In some of the mechanical experiments, lower concentrations (50–100 μM) of NEM were used. The alkylating agents were prepared in normal solution (solution A) shortly before use.

The membrane permeant Ca^{2+} chelator 5,5′-difluoro BAPTA, AM was prepared in a divalent cation-free solution to which 5 mM EGTA was added (solution E).

Chloride solutions were buffered with tris(hydroxymethyl) aminomethane (Tris, 0.05 M) at pH 7.4; Cl^--free solutions were buffered with 3-(N-morpholino) propanesulfonic acid (MOPS, 4 mM) at the same pH. In some voltage-clamp experiments 300–400 mM sucrose was added to prevent mechanical artifacts.

NEM was purchased from Aldrich, 4-CPD from Fluka, and 5,5′-difluoro BAPTA, AM from Molecular Probes. All other chemicals were from Sigma or Aldrich.

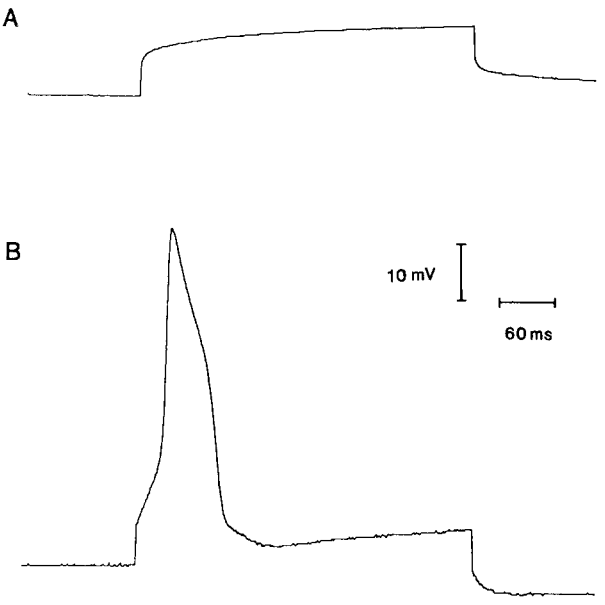

Fig. 1. Effect of the sulfhydryl-specific reagent 4-CPD on current-clamped *Atya* muscle fibers. (*A*) Completely passive response evoked in an untreated fiber by a 40-nA depolarizing current step. (*B*) Ca^{2+} spike elicited by a 20-nA depolarizing step in another fiber following treatment with the reagent. Depolarizing current steps were applied from the fibers' resting potentials which were −75 mV (*A*) and −74 mV (*B*).

Protocol and Analysis

The experiments were designed to analyze the normal electrical and mechanical responses of the muscle fibers and the responses following brief (5–10 min) exposure to the sulfhydryl-alkylating agents; after this period of time the agents were routinely washed out.

The voltage dependence of the Ca^{2+} current was studied by applying depolarizing pulses of 500–1000 msec duration. Standard double-pulse techniques were used to determine the steady-state inactivation curve.

The fitting procedure to experimental data was done by a nonlinear, least-square method. Averages of data values which appear in figures are expressed as mean ± SEM.

Results

Induction of Ca^{2+} Spikes and Ca^{2+} Current

Figure 1 shows the action of the sulfhydryl alkylating agent 4-CPD on current-clamped *Atya* muscle fibers. Figure 1*A* shows that the response which is normally evoked by depolarizing current is completely passive. In contrast, when fibers are exposed to 4-CPD, a Ca^{2+} spike is elicited by depolarizing current (Fig. 1*B*). Other sulfhydryl-alkylating agents such as NEM and maleimide are similarly able to convert

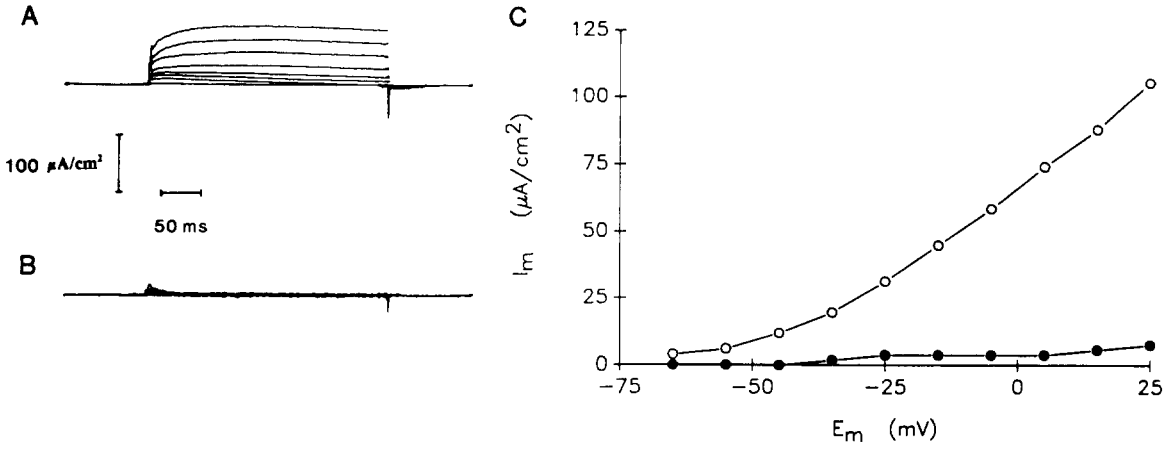

Fig. 2. Membrane currents in untreated fibers. (*A*) Superimposed current records elicited by step depolarizations to several potentials from a fiber bathed in normal solution (solution A). (*B*) Current records from the same fiber when the normal bathing solution was replaced with a Cl^--free, TEA-containing solution (solution M). (*C*) Current-voltage relation of the fiber; open circles, fiber bathed in solution A and closed circles, fiber bathed in solution M. Note the absence of inward current after outward currents were abolished. E_h, −75 mV.

the inexcitable *Atya* muscle membrane into one capable of firing Ca^{2+} spikes when depolarized (Zuazaga & del Castillo, 1978*a*, 1985).

The ionic currents underlying these dramatically different current-clamp responses were investigated. Figure 2*A* shows superimposed membrane current records elicited by step depolarizations to several potentials from a voltage-clamp experiment on an untreated fiber bathed in normal solution (solution A). Sustained outward currents were seen. When the normal solution bathing the fiber was replaced with a Cl^--free, TEA-containing solution (solution M), the outward currents were almost completely abolished, as shown in Fig. 2*B*. Figure 2*C* shows the current-voltage relation of this fiber. Open symbols represent outward currents elicited by depolarizing pulses in control experiments and filled symbols, after outward currents were eliminated by TEA and methanesulfonate ions. These results indicate that the ionic currents underlying the normal, passive response of the fibers are carried by K^+ and Cl^-.

More significantly, the results illustrated in Fig. 2 show that when the K^+ current is blocked by TEA and Cl^- ions are replaced by the impermeant anion methanesulfonate, no inward currents can be detected. In 34 fibers tested under these ionic conditions, inward cationic currents could not be recorded.This is in sharp contrast not only to other crustacean muscles (Hagiwara et al., 1969; Keynes et al., 1973; Mounier & Vassort, 1975; Hencek & Zachar, 1977) but also to both twitch and tonic fibers of the frog where the use of similar solutions unmasks an inward Ca^{2+} current (Sanchez & Stefani, 1978, 1983; Huerta & Stefani, 1986).

Attempts to unmask a Ca^{2+} current by other means were without success. Inward currents could not be detected in fibers bathed in TEA-methanesulfonate solutions containing high (60 mM) concentrations of Ca^{2+} or Ba^{2+} (solutions N and O, respectively). Since the graded responses of barnacle muscle fibers can be converted to Ca^{2+} action potentials by internal perfusion with Ca^{2+} chelators (Hagiwara & Naka, 1964), *Atya* muscle fibers were exposed for periods of up to 20 hr to the membrane-permeant Ca^{2+} chelator 5,5′-difluoro BAPTA, AM at a concentration of 50 μM. These procedures also failed to unmask Ca^{2+} spikes or inward Ca^{2+} currents.

The membrane currents recorded from fibers previously exposed to sulfhydryl-alkylating agents differ markedly from those of control fibers. The muscle fiber in Fig. 3 was exposed to 4-CPD for 10 min; the agent was then washed out and voltage-clamp recordings performed. Figure 3*A* shows the currents recorded in normal solution (solution A) at different potentials. A slow transitory inward current, which increased in amplitude and became faster as the command pulse increased, was detected. The current was maximal near 0 mV. With the command pulses driving the membrane potential to positive values, the inward current decreased in amplitude. Figure 3*B* shows records of membrane current from the same fiber when Ca^{2+} was replaced by Mg^{2+} (solution C). No inward current could be recorded in the absence of Ca^{2+} and only an outward current was seen. Furthermore, subtraction of records in Fig. 3*B* from those in 3*A* generated the records in 3*C*, the component of current due to the inward flow of Ca^{2+} ions. Thus, by exposing the

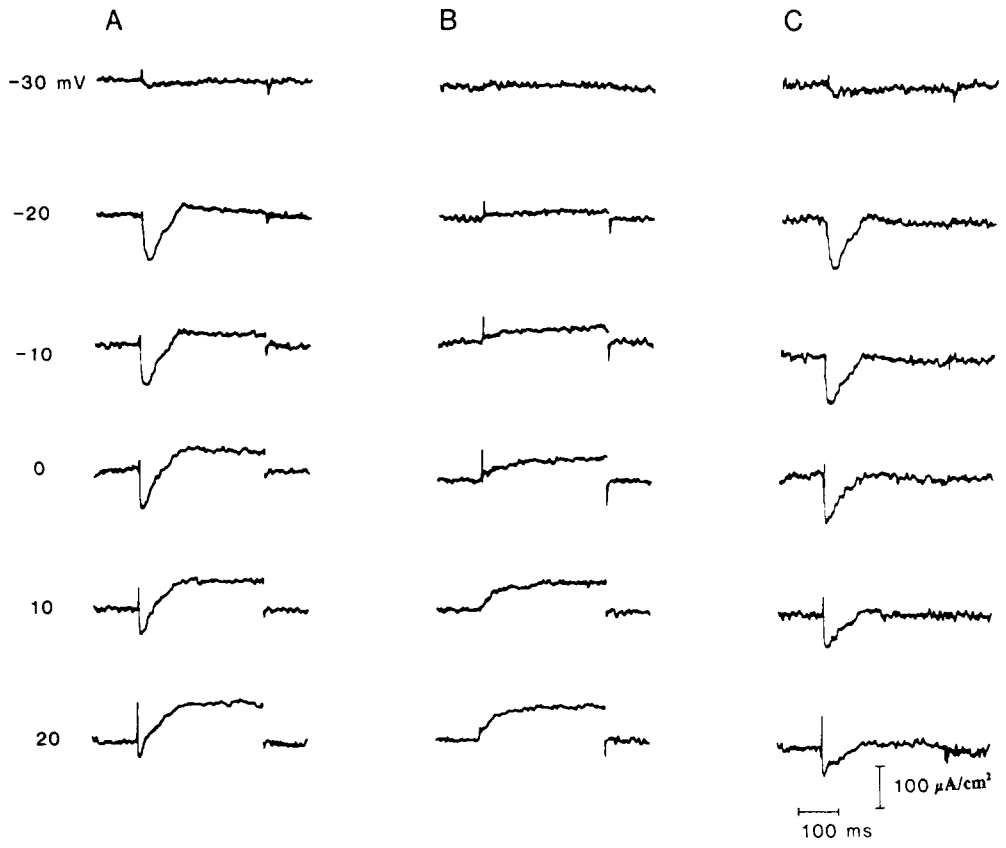

Fig. 3. Membrane currents in fibers treated with the sulfhydryl-alkylating agent 4-CPD. (*A*) Records of total membrane currents during command pulses to different potentials (numbers at left); fiber bathed in normal solution (solution A). (*B*) Membrane currents during command pulses to the potentials shown at left; fiber bathed in nominally Ca^{2+}-free solution (solution C). (*C*) Component of current carried by Ca^{2+}, generated by the subtraction of records in *B* from those in *A*. Same experiment throughout. E_h, −70 mV.

fibers to 4-CPD, a slow inward current that is carried by Ca^{2+} is induced.

Properties of the Ca^{2+} Current

The inward Ca^{2+} current induced by 4-CPD and shown in Fig. 3*C* was not maintained during the pulse, suggesting that the current inactivates. This and other properties of the induced Ca^{2+} current were explored by performing voltage clamp recordings in TEA solution (solution B) to block most of the outward current. Figure 4 shows records of I_{Ca} during steps from a holding potential of −75 mV. I_{Ca} increased in amplitude as the command pulse was increased. With depolarizations larger than −10 mV, I_{Ca} decreased in amplitude. The outward currents seen during the depolarizing step to +5 mV are carried by Cl^-, since solution B contains this anion, and probably also by K^+, since, even in the absence of Cl^- and in the presence of TEA, a remaining outward current was recorded (*see* Fig. 2).

The current associated with the depolarizing pulse to −15 mV in Fig. 4 shows notches. Because of the complex intracellular membrane system of clefts and transverse tubules present in these fibers (Bonilla et al., 1992), these notches are probably due to lack of potential control, especially if the Ca^{2+} currents are generated at the level of the tubular and cleft membranes.

The inward Ca^{2+} currents shown in Figs. 3*C* and 4 were not maintained during the pulse. They decayed spontaneously after reaching a maximum, indicating that I_{Ca} inactivates. This will be further explored below (*see* Fig. 6).

The current-voltage relationship of the fiber shown in Fig. 4 is shown in Fig. 5*A*. I_{Ca} was detected at about −40 mV and was maximal at −10 mV. The maximum peak I_{Ca} ranged from −25 to −130 $\mu A/cm^2$ in fibers with 30–60 μm radius, averaging −78 ± 10 $\mu A/cm^2$ (n = 10). As will be shown below, the amplitude of the Ca^{2+} current induced by 4-CPD decreases as the time after exposure to the agent increases (*see* Fig. 7). Peak I_{Ca} was, therefore, measured during the first 10–15 min after its induction.

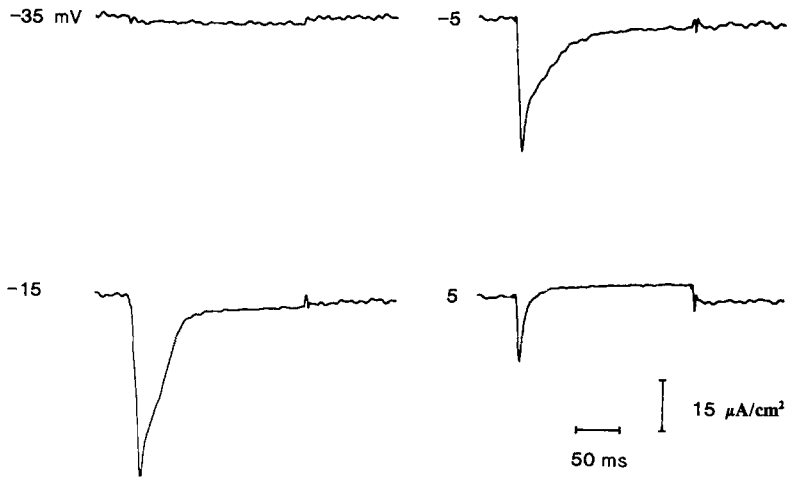

Figure 4. Ca^{2+} current induced by 4-CPD. Membrane current records during command pulses to different potentials (numbers at left); treated fiber bathed in TEA-containing solution (solution B). E_h, −75 mV.

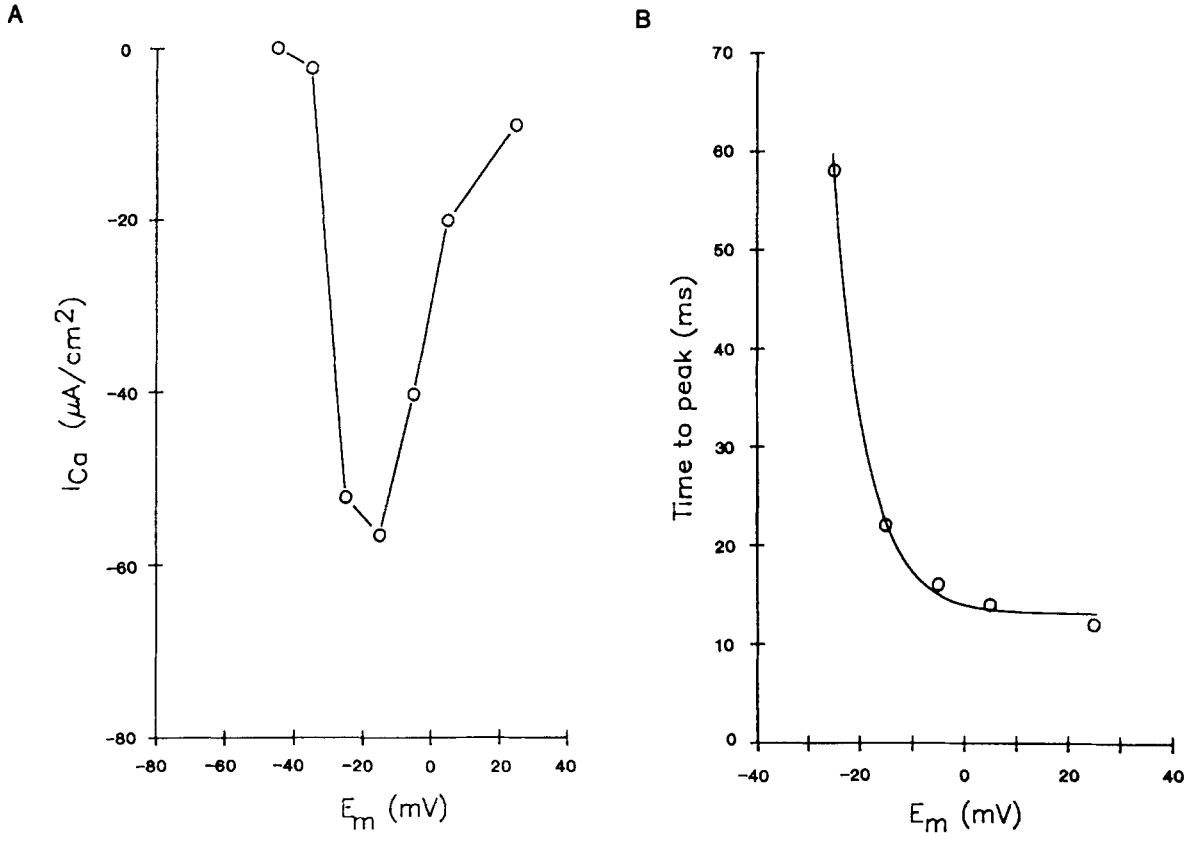

Fig. 5. (*A*) Relation between membrane current and voltage for fiber shown in Fig. 4. (*B*) Relation between time to peak and voltage for same fiber.

Figure 5*B* shows the voltage dependence of the time to peak for the fiber in Fig. 4. At membrane potentials near −10 mV, the time to peak was close to 15 msec.

The experiment shown in Fig. 6 was designed to determine whether the induced Ca^{2+} channels have a voltage-dependent inactivation mechanism. Steady-state inactivation of peak I_{Ca} (h_∞) during a test pulse to −15 mV was investigated with 1 sec prepulses to different potentials (inset in Fig. 6*C*). The records in Fig. 6*A* are membrane currents that show the effect of increasing the amplitude of the

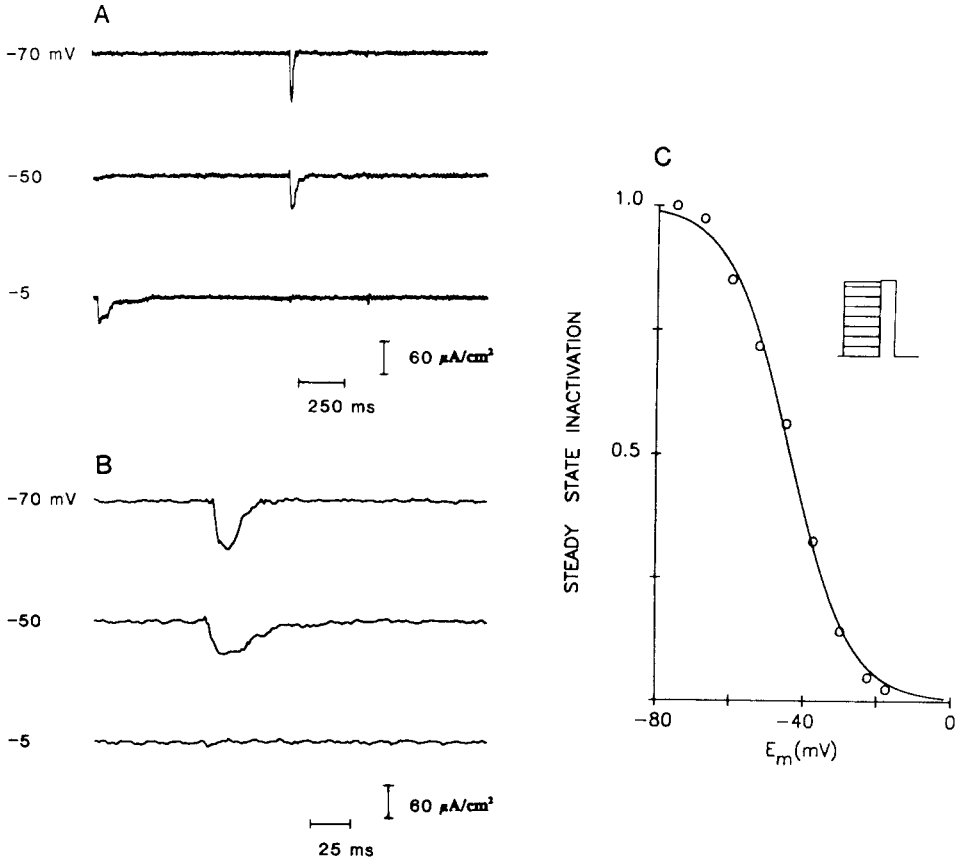

Fig. 6. Inactivation of the induced Ca^{2+} current. (*A*) membrane currents during a 0.4-sec test pulse to −15 mV following a 1-sec prepulse to different potentials (numbers at left). Fiber bathed in TEA-containing solution (solution B). (*B*) Membrane currents during the test pulse to −15 mV on an expanded time scale. Numbers at left indicate prepulse potential. (*C*) Relation between Ca^{2+} current amplitude during the test pulse *vs.* the membrane potential during the prepulse. Pulse protocol shown in inset. E_h, −75 mV.

prepulse on I_{Ca} during the test pulse. Those in Fig. 6*B* show I_{Ca} during the test pulse on an expanded time scale. In both Fig. 6*A* and *B* the top record shows the I_{Ca} during the test pulse following a prepulse to −70 mV; in the middle record a prepulse to −50 mV produced a decrease of I_{Ca} during the test pulse; this reduction became more evident as the amplitude of the prepulse increased (bottom record).

The results of this experiment are plotted in Fig. 6*C*. The smooth curve was drawn according to the equation:

$$h_\infty = [1 + \exp((E - E_{h_{1/2}})/k_h)]^{-1} \quad (2)$$

where E is the prepulse potential, $E_{h_{1/2}}$ the mid-point potential and k_h is a measure of the steepness of the curve. In this experiment, $E_{h_{1/2}} = -43.9$ mV and $k_h = 8.1$ mV. In a total of four fibers, the best fitted parameters were $E_{h_{1/2}} = -37.5 \pm 3.4$ mV and $k_h = 5.0 \pm 0.4$ mV. At the holding potential of −75 mV, the induced Ca^{2+} channels are less than 4% inactivated.

The results of the two-pulse protocol experiments suggest that inactivation of the chemically induced I_{Ca} is voltage dependent and does not require Ca^{2+} entry into the cell. In addition, the top two records in Fig. 6*B* show that, during the test pulse, the larger amplitude I_{Ca} decays faster than the smaller amplitude I_{Ca}. If the Ca^{2+} channels in this muscle are mainly located in clefts and tubules, Ca^{2+} depletion in this restricted extracellular space could be another factor contributing to the decay of I_{Ca}, as demonstrated in cut frog muscle fibers (Almers, Fink & Palade, 1981). The relative contribution of these two mechanisms to the decay of I_{Ca} during maintained depolarizations was not further analyzed.

The excitability-inducing effect of NEM and other sulfhydryl-alkylating agents is transient, lasting 30–60 min; thereafter, the fibers gradually lose their ability to generate Ca^{2+} spikes and become inexcitable again (Zuazaga & del Castillo, 1978*a*, 1985). The experiment of Fig. 7 shows that peak I_{Ca} decreased as the time after exposure to the alkylating

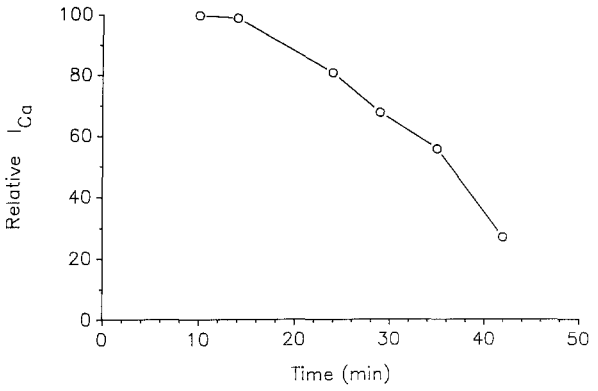

Fig. 7. Decay of the induced Ca^{2+} current as a function of time. 4-CPD was added at $t = 0$ min, and measurements started at $t = 10$ min.

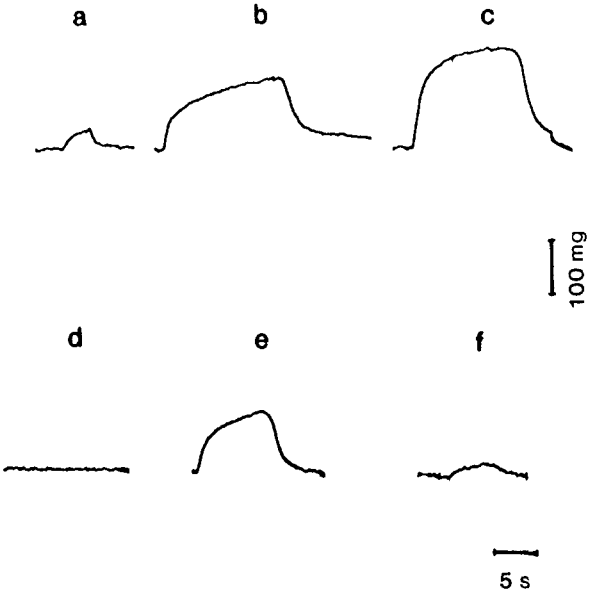

Fig. 8. Effect of 4-CPD on mechanical activity. Isometric tension was measured at 20 Hz in a bundle of about 20 fibers before (trace *a*) and at different times following exposure to 4-CPD (traces *b–f*). Times and solutions for each record are: (*a*) untreated fiber in normal solution (solution A); (*b*) 9 min of treatment, normal solution; (*c*) 26 min, high Ca^{2+} solution (solution D); (*d*) 30 min, Ca^{2+}-free solution (solution C); (*e*) 37 min, normal solution; (*f*) 46 min, normal solution.

agent increased. In this fiber, peak I_{Ca} decreased by 50% *ca.* 25 min after it was induced; only 25% of peak I_{Ca} remained 35 min after its induction.

These times are variable from fiber to fiber but usually the induced I_{Ca} disappears in 30–60 min. The run-down of I_{Ca} occurs even if the agent is allowed to remain in the bath or if the fiber is not current or voltage clamped.

Enhancement of Mechanical Activity by Sulfhydryl-Alkylating Agents

Mechanical activation in arthropod muscle depends on extracellular Ca^{2+}, and the contractile threshold is highly correlated with the occurrence of an inward Ca^{2+} current (Zacharova & Zachar, 1967; Gilly & Scheuer, 1984). The following mechanical experiments were designed to test whether the I_{Ca} induced by the sulfhydryl-alkylating agents might have an effect on contractile activation in *Atya* muscle.

Figure 8 shows an experiment which illustrates that 4-CPD enhanced tetanic tension in a strictly Ca^{2+}-dependent manner; the time course of the effect is also shown. In record *a*, tetanic tension before exposure to the agent was recorded in normal solution (solution A). The tension records *b–f* were obtained from the same bundle of fibers in different solutions and at different times following exposure to 4-CPD. In record *b*, 9 min of exposure had elapsed and tension, recorded in normal solution, was greatly enhanced. Record *c* was obtained in a high Ca^{2+} solution (solution D) 26 min after exposure to the agent; an even larger mechanical response was seen. Record *d* was obtained in a nominally Ca^{2+}-free solution (solution C) 30 min following 4-CPD exposure; contractile activation was abolished in Ca^{2+}-free media. In records *e* and *f*, tension was again recorded in normal solution (solution A). After 37 min tetanic tension was still larger than control (record *e*). Tension declined to values close to control after 46 min (record *f*). The time course of the effect of 4-CPD on tension, therefore, was very similar to that of I_{Ca} (Fig. 7). These results strongly suggest that the enhancement of tension in muscle fibers treated with the sulfhydryl-alkylating agent is probably due to an increase in Ca^{2+} influx mediated by the induced Ca^{2+} currents.

The effect of the sulfhydryl-alkylating agent NEM on contractures evoked by high extracellular K^+ concentrations, $[K^+]_o$, was also investigated. The tension records in Fig. 9*A* were obtained by elevation of $[K^+]_o$ to 90 mM (top) and to 120 mM $[K^+]_o$ (bottom) at different times (min) following NEM treatment (numbers below records). Peak contracture tension increased markedly shortly after NEM treatment. Eventually, tension returned to values close to control. The time course of the effect of NEM on K^+ contractures was similar to that of 4-CPD on I_{Ca} and tetanic tension. Peak tension generated during K^+ contractures is plotted in Fig. 9*B* as a function of $[K^+]_o$. Contracture threshold was *ca.* 20 mM $[K^+]_o$ for both control and NEM-treated muscles.

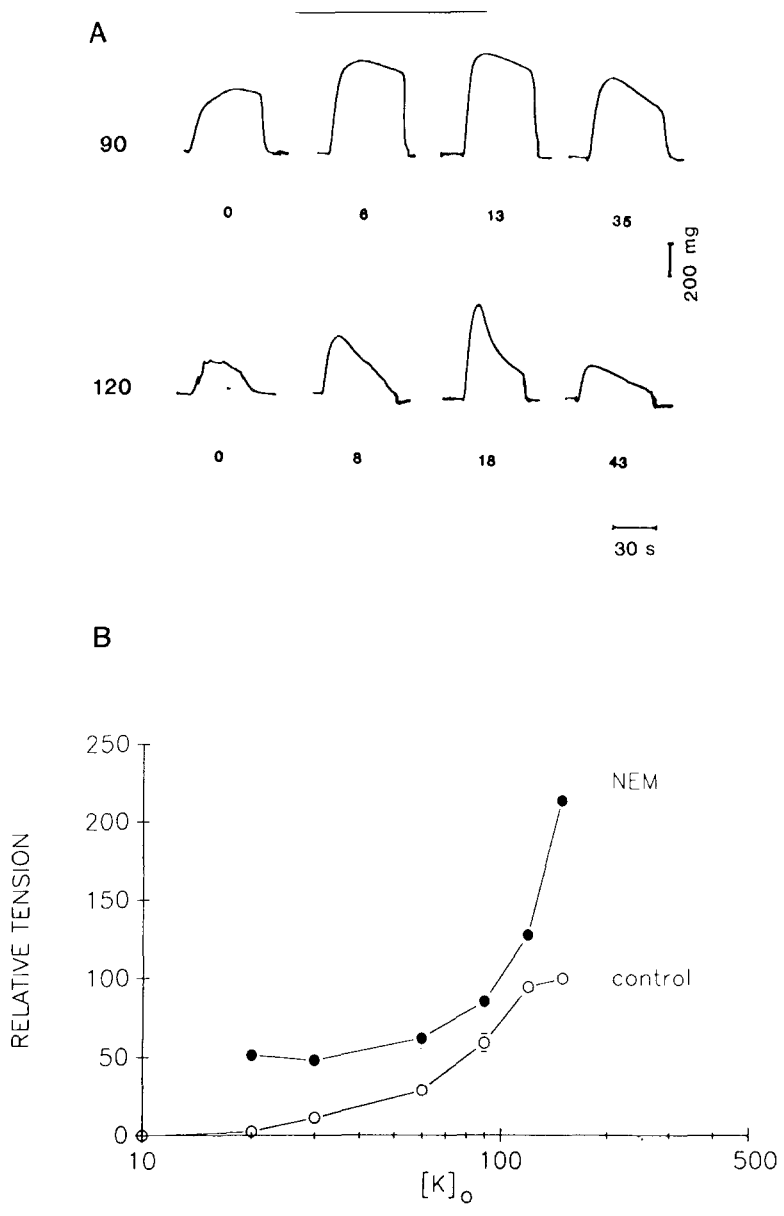

Figure 9. Effect of NEM on K^+ contractures. (*A*) Isometric tension records obtained in two different experiments by elevation of $[K^+]_o$ to 90 mM (top) and 120 mM (bottom) at different times (min, numbers below records) following NEM (50 μM) treatment. (*B*) Peak contracture tension as a function of $[K^+]_o$; open circles are untreated fibers and filled circles are fibers treated with NEM. Each point is mean ± SE of 7–9 determinations.

Discussion

Comparison with Previous Work

In the present paper we confirm and extend the previous work performed on *Atya* skeletal muscle. Using the voltage-clamp technique we found that K^+ and Cl^- currents underlie the passive electrical responses of untreated muscle fibers and, furthermore, that when these currents are blocked, Ca^{2+} channels are not unmasked. We also found that the sulfhydryl-alkylating agent 4-CPD induces an inward, Ca^{2+}-dependent current (I_{Ca}) in *Atya* muscle fibers. Therefore, the chemically-induced I_{Ca} underlies the Ca^{2+} spike observed, following exposure to this agent, by Zuazaga and del Castillo (1978*b*, 1985).

I_{Ca} is detected at about -40 mV and reaches a maximum value of about $-80\,\mu A/cm^2$ near -10 mV. Thus, the voltage dependence and maximum density of the chemically induced I_{Ca} in *Atya* muscle is similar to the calcium current present in normally Ca^{2+}-spiking arthropod muscle. For example, peak inward Ca^{2+} current density is $-140\,\mu A/cm^2$ in crayfish muscle fibers (Hencek & Zachar, 1977) and $-113\,\mu A/cm^2$ in stick insect fibers (Ashcroft & Stanfield, 1982). Also, Ca^{2+} current in these muscles is detected near -40 mV and peaks near 0 mV (Hencek & Zachar, 1977; Ashcroft & Stanfield, 1982). The voltage dependence and maximum density of I_{Ca} in *Atya* are also

similar to those of the slow Ca^{2+} current ($I_{Ca,s}$) in frog twitch muscle fibers (Sanchez & Stefani, 1978).

The chemically-induced Ca^{2+} current in *Atya* reaches peak values within 15–55 msec and then inactivates. The time course of its activation is, thus, comparable to that of the Ca^{2+} current in other arthropods (Keynes et al. 1973; Hagiwara, Fukuda & Eaton, 1974; Hencek & Zachar, 1977; Ashcroft & Stanfield, 1982) and to that of the fast Ca^{2+} current ($I_{Ca,f}$) in frog skeletal muscle (Cota & Stefani, 1986; Arreola et al. 1987). It is, however, about 10 times faster than the activation time course of $I_{Ca,s}$ in twitch muscle fibers of the frog (Sanchez & Stefani, 1978, 1983).

As the Ca^{2+} current in crab (Mounier & Vassort, 1975) and crayfish muscle (Hencek & Zachar, 1977) and the $I_{Ca,s}$ in frog muscle (Sanchez & Stefani, 1983), I_{Ca} in *Atya* undergoes voltage-dependent inactivation. The midpoint and steepness of the fitted h_∞ curve are close to those of the Ca^{2+} current in crab (Mounier & Vassort, 1975) and of $I_{Ca,s}$ in frog muscle (Sanchez & Stefani, 1983). The h_∞ curve of the Ca^{2+} current in crayfish muscle is steeper and the midpoint lies at a more negative membrane potential (Hencek & Zachar, 1977).

It is possible that the Ca^{2+} channels in *Atya* muscle fibers are mainly located in clefts and/or tubules, as they do in barnacle and frog muscle (Keynes et al. 1973; Nicola-Siri, Sanchez & Stefani, 1980; Almers et al. 1981). This would limit voltage control and explain the observed notches in the inward current records. Further, if Ca^{2+} channels reside in tubular membranes, Ca^{2+} depletion in this limited extracellular space may contribute to the decay of I_{Ca} during maintained depolarizations, as suggested by the observation that, at the same voltage, lower amplitude Ca^{2+} currents decay slower than larger amplitude currents (Fig. 6*B*). Additional studies are needed to determine the localization of the Ca^{2+} channels and the relative contribution of voltage-dependent decrease in channel conductance, and Ca^{2+} depletion in tubules to I_{Ca} inactivation.

Mechanical Activation

Our results show, in addition, that the sulfhydryl-alkylating agents NEM and 4-CPD enhance contractile tension in *Atya* muscle. The enhancement of tetanic tension is strictly dependent on extracellular Ca^{2+}. In bullfrog atrial muscle, which like *Atya* muscle requires extracellular Ca^{2+} for mechanical activation, NEM has been shown to transiently increase both Ca^{2+} current and tension (Aomine & Abe, 1978). Furthermore, the time course of the electrical and mechanical effects of the sulfhydryl-specific reagents on *Atya* muscle closely parallel those of NEM on bullfrog atrial muscle. In addition, the fast activation time course of the chemically induced I_{Ca} in *Atya* muscle is consistent with a central role in depolarization-contraction coupling. Thus, our results strongly suggest that in *Atya* muscle the increase in tension is due to an increase in Ca^{2+} influx mediated by the chemically induced I_{Ca}.

However, it is unlikely that Ca^{2+} influx through the chemically induced Ca^{2+} channels is the sole source of activator Ca^{2+}, since in untreated muscles tetanic tension is also strictly dependent on extracellular Ca^{2+} (Bonilla et al., 1992). *Atya* muscle fibers are sensitive to caffeine and caffeine contractures are produced even in Ca^{2+}-free solutions (Bonilla et al., 1992). This indicates that the sarcoplasmic reticulum of *Atya* muscle can release Ca^{2+} in response to this drug. If we assume that the sarcoplasmic reticulum of *Atya* muscle can also release Ca^{2+} in response to a depolarization, then the absolute requirement for extracellular Ca^{2+} for mechanical activation might be explained by postulating that, similar to skeletal muscle fibers from vertebrates, the voltage sensor involved in excitation-contraction coupling (Schneider & Chandler, 1973) has a site that must be occupied by a Ca^{2+} cation for normal function. This has been proposed to account for the observation that extracellular Ca^{2+} plays a key role in excitation-contraction coupling in frog skeletal muscle fibers (Brum et al., 1988). Although in frog muscle other divalent and monovalent extracellular metal ions substituted for Ca^{2+} bring back excitation-contraction coupling (Pizarro et al., 1988), in *Atya,* ions other than Ca^{2+} might bind with much lower affinity to the site at the voltage sensor. In Ca^{2+}-free solutions containing 30 mM Mg^{2+}, contractile tension failed to develop (M.C. Garcia, J.A. Sanchez and C. Zuazaga, *unpublished observations*).

Significance of the Findings

The list of membrane proteins susceptible to modification by sulfhydryl-specific reagents is very extensive, and includes ion channels, receptors for neurotransmitters and hormones, and ion transport proteins (Narahashi, 1974; Zuazaga, Steinacker & del Castillo, 1984). The activity of some of these transmembrane proteins is regulated by membrane-bound and cytosolic proteins, which in turn can be modified by sulfhydryl reagents. For example, NEM can block the hormone-induced inhibition of adenylate cyclase by alkylating sulfhydryl groups on the *a*-subunits of the GTP-binding proteins G_i and/or G_o (Aktories, Schultz & Jacobs, 1982; Asano & Ogasawara, 1986; Winslow et al., 1987). Since G proteins

play an essential role in modulation of several types of ion channels, either directly or through the involvement of second messengers (Brown & Birnbaumer, 1990), it is conceivable that G proteins also play a role in the chemically induced Ca^{2+} currents that we have described. In agreement with this possibility, recent whole-cell voltage-clamp experiments on bullfrog atrial muscle cells have shown that NEM substantially increases the Ca^{2+} current in these cells probably by alkylating the GTP-binding proteins associated with inhibition of Ca^{2+} currents (Nakajima, Irisawa & Giles, 1990). It cannot be ruled out, however, that NEM and related compounds can also affect the Ca^{2+} channel directly. Regardless of the biochemical pathway involved, it appears that in *Atya* skeletal muscle fibers, the sulfhydryl-specific reagents either increase the probability that the channels will open, provided that they are available for voltage activation, or increase the probability that the channels are available. This, and the molecular and biochemical basis of the effect, await further investigation.

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