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Some Electrical Properties of the Membrane of the Barnacle Muscle Fibers under Internal Perfusion

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Summary. Intracellular perfusion technique has been applied to the muscle fibers of the barnacle species, *Balanus nubilus*. In these fibers, generation and the form of the calcium spike was governed by the frequency of stimulation and intra- and extracellular calcium concentrations. Voltage-clamp experiments showed that the magnitude of the potassium outward current was controlled by the intracellular calcium concentration whose increase, nearly 10^3-fold, raised the resting membrane conductance and the outward potassium current. On the other hand, application of 10 mM zinc ions inside the muscle fiber had no effect on either the resting potential or the outward potassium current but suppressed the early inward calcium current. Similarly, the inward calcium current was decreased by low concentration of sodium ions in the extracellular fluid only when its ionic strength was made low by substituting sucrose for the sodium salt. Measurement of outward current with the muscle fiber in calcium-free ASW solution and intracellularly perfused with several cationic solutions established the selectivity sequence TEA < Cs < Li < Tris < Rb < Na < K for the potassium channel.

Hagiwara and co-workers have given a detailed description of the behavior of the barnacle muscle membrane when it is in a state of rest (Hagiwara, Chichibu & Naka, 1964; Hagiwara, Gruener, Hayashi, Sakata & Grinell, 1968) and also subject to electrical stimulation (Hagiwara *et al.*, 1964; Hagiwara & Naka, 1964; Hagiwara & Nakajima, 1966*a*, *b*; Hagiwara & Takahashi, 1967; Hagiwara, 1973) after microinjection of different compounds into the muscle fiber. The early inward current carried by Ca and other divalent cations (Hagiwara, Fukuda & Eaton, 1974) and the late outward current carried by K ions under voltage-clamp conditions have been described when the muscle fiber is microinjected (Hagiwara, Hayashi & Takahashi, 1969) and also when it

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is under internal perfusion (Keynes, Rojas, Taylor & Vergara, 1973). The internal perfusion technique was originally applied to the muscle fibers of the barnacle species, *Megabalanus psittacus*, and was later used to study the effects of several anions and cations on the resting membrane potential (Lakshminarayanaiah & Rojas, 1973; Lakshminarayanaiah, 1974) and of pH and ionic strength on the potassium system (Lakshminarayanaiah & Rojas, 1975). The intracellular perfusion method has not been applied to single muscle fibers of other barnacle species available on the west coast of the United States. This has been done using the *Balanus nubilus* species in the present study in which some electrical properties of the barnacle muscle membrane subject to voltage-clamp under different intra- and extracellular ionic environments are described.

Materials and Methods

Large specimens of barnacle species, *Balanus nubilus*, used in the early stages of this research were supplied by the Pacific Biomarine Supply Co., Venice, California; and in later stages they were received from Mr. David King, Friday Harbor, Washington.

Single giant fibers were isolated from the two muscles, Depressor scutorum lateralis and Depressor scutorum rostralis. The experimental procedures in general were similar to those described by Keynes *et al.* (1973).

Insertion of the perfusion pipette into the muscle fiber was performed in 0 Ca ASW (artificial sea water) in the bath to prevent the contractions of the fibers. But this procedure was inadequate for the purpose probably due to the presence of residual Ca in the fibers and so 3 mM tris-EGTA [tris(hydroxymethyl)aminomethane-ethyleneglycol-bis(β-aminoethylether)-N,N′-tetraacetic acid] was added to the internal perfusion fluid to eliminate muscle contractions. In the course of penetration of the muscle fiber, the internal solution was slowly infused manually from the pipette. The solution made a pathway in the muscle fiber for the advancement of the pipette. This procedure was repeated until the end of the pipette reached a few millimeters away from the tendon end of the muscle fiber after which the perfusion was carried out mechanically by the infusion pump (Braun Apparatebau, Melsungen, West Germany). In most of the experiments, the internal solutions were perfused at a rate of 2.5 μl/min. The optimal maximum rate was 6 μl/min. When rates exceeding this value were used, the resting membrane potential (inside negative) quickly became more positive (depolarization). Low resting potentials [$<|35|$ mV] were also observed when muscle fibers of diameter less than 1300 μm were used. So, muscle fibers of diameter 1500 μm and higher were always used.

Solutions

The compositions of both internal and external solutions used in the experiments are given in Tables 1 and 2, respectively. Osmolarity of all solutions was adjusted to 1000 (mosmoles/kg water) using sucrose or glycine. The pH of the solutions was maintained at 7.3 with 5 mM tris-HCl buffer.

Table 1. Composition of internal solutions in mmoles/liter: pH = 7.3

Solution	K acetate (Ac)	K isethionate (Ise)	TEA-Cl	Tris-Cl	Tris-EGTA	Sucrose (Suc)	Glycine (Gly)
A. Standard	180[a]			5	3	540–629	
B. Ise + Suc		180		5	3	540–629	
C. Ac + Gly[b]	180			5	3		740–800
D. Ise + Gly[c]		180		5	3		817
E. TEA	180		60	5	3	480	

[a] In the preparation of internal solutions containing Rb, Cs, Na, Li, Tris or NH_4 ions, KAc was replaced by the acetate of the ion concerned. In the case of TEA ion, 180 mM tetraethylammonium chloride was substituted for KAc. In the preparation of solution containing 5 or 10 mM Zn ion, the required amount of $ZnCl_2$ was added to solution A and the final pH was adjusted.[1]

[b] The pH was adjusted by adding small amounts of Tris or HCl solution.

[c] Small amounts or Tris base or dl-histidine were added to adjust the pH.

In the preparation of internal solutions containing known amounts of free Ca^{2+} ion, solutions A and B shown in Table 3 were used. Solutions A and B were mixed in the required proportion and the free calcium ion concentration $(Ca^{2+})_f$ was estimated from the formula (Portzehl, Caldwell & Rüegg, 1964; Hagiwara & Nakajima, 1966*b*)

$$(Ca^{2+})_f = \frac{(Ca)_t}{4.83 \times 10^6 [(EGTA)_t - (Ca)_t]}$$

where t stands for the total concentration. All experiments were carried out at room temperature (21–23 °C).

Generally, sucrose is used as the solute to make the internal solutions isosmotic with the external ASW and so the solutions so prepared would be relatively more viscous. Further, sucrose, unlike some other nonprotein amino acids (Schoffeniels, 1967), is not found in any crustacean system. Consequently an amino acid, glycine, was used in place of sucrose to make up the osmolarity of the internal solutions (*see* Table 1). These solutions were relatively less viscous than those in which sucrose has been used. A

[1] Isaacson (1961) has pointed out that at pH 7.0, the concentration of Zn ions in solution would be 0.7 mM. This is based on the value of 7×10^{-18} (moles/liter)^3 determined by Fulton and Swinehart (1954) for K_{sp}, the solubility product of $Zn(OH)_2$, the form of which was orthorhombic. When we prepared our internal solution containing Zn at a concentration of 10 mM by adding the required amount of 0.25 M $ZnCl_2$ (pH = 3.0) to a known volume of the internal solution A (Table 1), no precipitate, colloidal or coarse, was detected and a clear solution resulted when the final pH was adjusted to 7.3 by adding Tris. Consequently, we infer that $Zn(OH)_2$, if any formed, would have precipitated if it were in crystalline form. A literature search revealed that $Zn(OH)_2$ exists in seven different forms, amorphous, α, β_1, β_2, γ, δ and ε (Feitknecht & Schindler, 1963) and only the amorphous variety exists under normal conditions. The values for K_{sp} for six forms (except α) are in the range $2 \times 10^{-15} - 2.2 \times 10^{-16}$ at an ionic strength of 0.2 (Schindler, Althaus & Feitknecht, 1964). So we infer that the form of $Zn(OH)_2$ in our internal solution conforms probably to the amorphous state.

Table 2. Compositions of artificial sea water (ASW) in mmoles/liter: pH = 7.3

Solutions	K^+	Na^+	Ca^{2+}	Mg^{2+}	Cl^-	$Tris^+$	Sucrose
A. Standard	10	418	10	20	498	10	118
B. 0 Ca	10	418		30	498	10	118
C1. 60 Ca	10	430	60		565	5	75
C2. 0 Ca	10	430		60	565	5	75
D1. 430 Na[a] 60 Ca	10	430	60		565	5	75.3
D2. 200 Na 60 Ca	10	200	60		335	5	410
D3. 10 Na 60 Ca	10	10	60		145	5	756
D4. 430 Na 0 Ca	10	430		60	565	5	75.3

[a] In this solution Na was replaced by Li, choline or guanidine to prepare solutions that contained 200 Na + 230 Li (choline or guanidine) + 60 Ca and 10 Na + 420 Li (choline or guanidine) + 60 Ca.

Table 3. Ca buffer solutions: concentration in mmoles/liter

Solution	EGTA	Ca-acetate	KOH	Tris-Cl	Sucrose
A[a]	45		180	10	706
B[b]	45	45	180	10	616

[a] The pH was adjusted to 7.3 by adding small amounts of approximately 8 M acetic acid.
[b] The pH was adjusted to 7.3 by adding tiny crystals of tris(hydroxymethyl)amino methane. The final pH of all internal solutions prepared by mixing the above solutions A and B was also adjusted to 7.3.

number of exploratory perfusion experiments in which resting potential was monitored showed that solutions A to D shown in Table 1 were all equally effective in generating and maintaining the resting potentials which were usually between −45 and −52 mV at a perfusion rate of 2.5 µl/min. The usefulness or other effect if any of glycine in the internal solution was not explored any further. In all experiments, therefore, unless otherwise stated, only K-acetate solutions containing sucrose were used as the standard internal solutions.

Results

Stimulation and Generation of Action Potential

Under normal conditions, the membrane of the barnacle muscle fiber does not give an "all-or-none" action potential in response to electrical stimulation. Hagiwara and co-workers (Hagiwara & Naka, 1964; Hagiwara *et al.*, 1964; Hagiwara & Nakajima, 1966*a*, *b*) have shown that

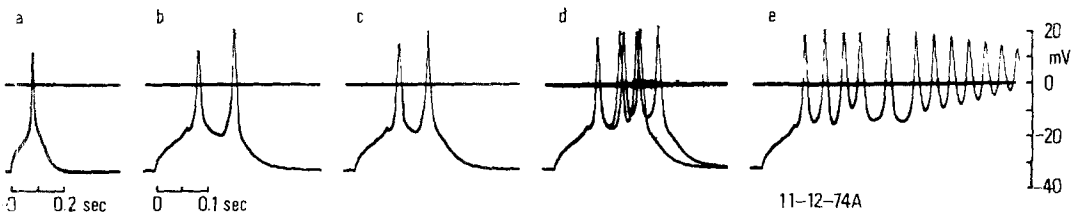

Fig. 1. A sequence of action potentials elicited in a barnacle muscle fiber subject to successive stimulation given every 3.3 sec. *a* to *e* are action potentials produced by first, second, third, fourth and fifth stimulation given in succession, respectively. The muscle fiber was stimulated by applying a brief pulse of current through the current wire. The external solution was a standard ASW containing 10 mM Ca^{2+} and 20 mM Mg^{2+}; the internal solution contained K acetate, glycine and 3 mM tris-EGTA

when the level of Ca concentration inside the muscle fiber was reduced ($<10^{-7}$ M), the fiber on stimulation responded with an all-or-none spike. The nature of this response is very complex and often times unpredictable. We pursued this aspect further with the internally perfused barnacle muscle fibers. Responses elicited in one of the fibers are shown in Fig. 1. The muscle fiber subject to a threshold stimulus of approximately 50 msec duration responded with a single spike, the overshoot being about 15 mV. With successive stimulation of the same magnitude applied every 3.3 sec, the initial response of a single spike changed to responses with multiple spikes, repetitive discharge and finally a plateau lasting from a few seconds to several minutes. With replacement of the external solution by a high Ca ASW (60 mM Ca and 0 Mg), the muscle fiber stabilized and gave on stimulation a single spike which was abolished when the high Ca ASW was replaced by one containing 0 Ca and 60 mM Mg (results not given). Thus, the nature of the membrane response to stimulation is dependent on the levels of intracellular calcium which in turn are regulated by the flow of calcium from outside during stimulation. These observations accord well with those of Hagiwara and Nakajima (1966*b*).

Zinc ions applied internally at a concentration of 5 mM had no effect on the resting potential. Due to the presence of 3 mM tris-EGTA in the internal fluid, it is likely that the effective zinc ion concentration is less than 5 mM, as its association constant with EGTA is larger than that of calcium ion (Holloway & Reilley, 1960). Consequently, 10 mM zinc ions were used to increase its free ion concentration. Even this showed no effect on the resting potential. However, 10 mM zinc in the internal solution eliminated the action potential (*see* Fig. 2*Ab*). This effect is more

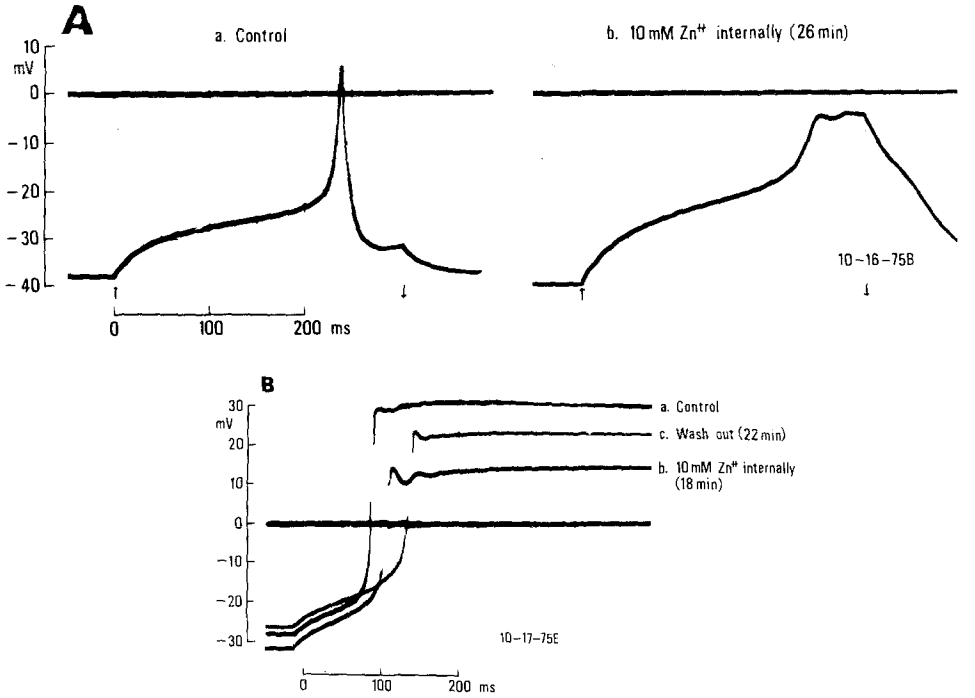

Fig. 2. Records of action potential from two muscle fibers. Fiber *A* was internally perfused with the standard solution A (Table 1) and bathed in normal ASW containing 10 mM Ca and 20 mM Mg before and during internal application of Zn ions. Action potential '*a*' is control and '*b*' is 26 min after start of application of 10 mM Zn ions. Arrows indicate the onset and termination of the stimulation pulse. Fiber *B* was perfused with the internal solution E (*see* Table 1) containing 60 mM TEA and immersed in ASW containing 60 mM Ca and 0 Mg before and during internal application of 10 mM Zn ions. Action potential '*a*' is control and '*b*' is 18 min after start of application of 10 mM Zn ions. '*c*' is 22 min after returning the fiber to control solution

clearly demonstrated in Fig. 2*B* in which the action potentials obtained with a muscle fiber internally perfused with solution E of Table 1 containing 60 mM TEA (tetraethylammonium ion), are shown. In these experiments, TEA ion is used in the internal solution to act on the potassium channels to depress and/or eliminate (*see* Fig. 4) the flow of potassium ions (Tasaki & Hagiwara, 1957; Armstrong & Binstock, 1965; Keynes *et al.*, 1973) and thereby prevent the appearance of considerable oscillations in the action potential. Because of the depression of potassium flow, the action potentials shown in Fig. 2*B* are not of the usual type (*see* Fig. 1) in that they have long plateaus showing no repolarization phase. The height of the action potential was suppressed by about 20 % exhibiting no obvious action on the shape or duration of its plateau

(compare *a* and *b* in Fig. 2*B*). However, the depression of the action potential was partially reversible (*see* Fig. 2*Bc*). These effects of zinc ions seem to be similar to the effects on the Ca spike of external zinc ions studied by Hagiwara and Takahashi (1967) who found the suppression of the Ca spike by Zn^{2+} and Fe^{2+} ions, unlike those of UO_2^{2+} and La^{3+} ions, to be reversible.

Potassium Channel Selectivity to Internal Monovalent Cations

In order to follow the selectivity of the steady-state channel to several monovalent cations, voltage-clamp experiments were performed using

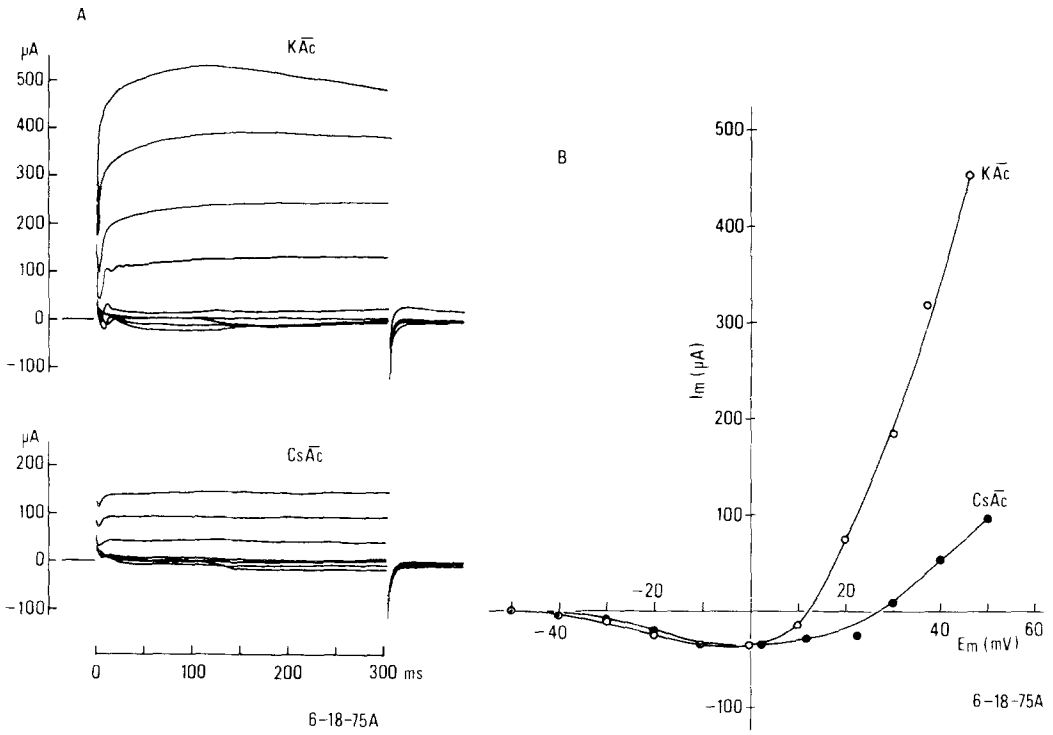

Fig. 3. Effect of replacement of internal K ion by Cs ion on the outward membrane currents. (*A*) Family of outward membrane currents associated with −10 mV step depolarization from the holding potential of −50 mV. The muscle fiber was immersed in 0 Ca and 30 mM Mg ASW. Upper panel: standard internal solution A (Table 1). Lower panel: K in the internal solution replaced by Cs. (*B*) Current-voltage curves for the steady-state outward current derived from the results in *A*. Open circles correspond to internal solution containing K acetate and closed circles correspond to internal solution containing Cs acetate. Leakage current has been subtracted. The small inward current noted is probably due to some residual Ca in the external solution

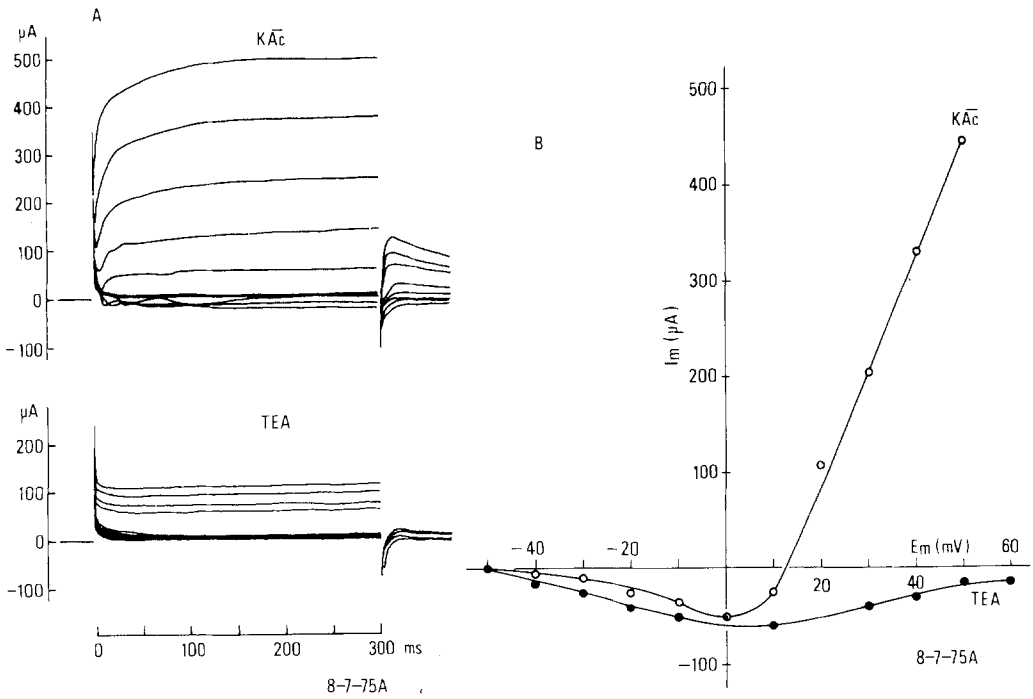

Fig. 4. Effect of replacement of internal K acetate by TEA-Cl on the outward currents produced in 0 Ca and 30 mM Mg ASW. (*A*) Family of outward currents associated with 10 mV step depolarization from the holding potential of −50 mV. Upper panel: standard internal solution A (Table 1). Lower panel: TEA-Cl was substituted for K acetate in the standard internal solution. (*B*) Current-voltage curves for the steady-state outward current derived from the results in *A* from which leakage currents have been subtracted. Open circles correspond to K acetate internal solution and filled circles represent TEA-Cl internal solution. The small inward current is probably due to some residual calcium in the external solution

the muscle fibers in 0 Ca and 30 mM Mg ASW. This procedure eliminated oscillations in the membrane currents (Keynes *et al.*, 1973). 180 mM potassium acetate in the internal solution were replaced completely by several test cations such as Rb, Cs, Na, Li, Tris or NH_4. Acetates of the respective cations were used in all cases except TEA which was used as its chloride.

In each case, families of membrane currents were taken about 30 min after exposure of the muscle fiber to the internal test solution. In the case of the test ion cesium or TEA, the magnitude of the delayed current was greatly reduced, the reduction being more in TEA than in Cs (*see* Figs. 3 and 4). This effect observed in a number of preliminary experiments at intervals of 10, 15, 20, 25, and 30 min after application of the test solution

showed that the optimum equilibration period was less than 15 min. We chose a period of 30 min for purposes of equilibration when the internal solution was changed. The ability of the potassium channel to carry outward current in the presence of TEA or Cs noted in Figs. 3 and 4 is in agreement with the results of Keynes *et al.* (1973). When voltage-clamp experiments were performed in 10 mM Ca ASW, the internal solutions containing 180 mM Cs or TEA suppressed the oscillations in the membrane currents. Similarly, Hagiwara *et al.* (1974) have shown that elimination of oscillations can also be brought about by the reduction of the current in the Ca or transient channel by the presence of Co^{2+} ions in the external ASW. These results taken together suggest, as proposed by Keynes *et al.* (1973), that the oscillatory behavior in the membrane current arises from an interaction through a series resistance of the potassium and calcium carrying systems.

Voltage-clamp experiments with the barnacle muscle fiber containing internal NH_4^+ ions could not be carried out successfully, as the muscle fibers showed contractions and broke.

The late currents carried by Li^+, $Tris^+$ and Rb^+ ions through the steady-state channel are about 28, 22 and 19%, respectively, less than those carried by K^+ ions (*see* Fig. 5). Nevertheless, these currents are larger than those carried by Cs^+ or TEA^+ ions. In the case of the Na^+ ion, the magnitude of its outward current relative to that of the K^+ ion was not very clear-cut (results not given), as no change in the magnitude of the current in some fibers and a reduction of about 24% in some other fibers were seen. Overall, the ability of the steady-state channel to carry currents due to several cations applied internally decreased in the order $K \geqq Na > Rb > Tris > Li > Cs > TEA$.

The current-voltage relations shown in Figs. 3*B*, 4*B* and 5 (experiments performed in 0 Ca and 30 mM MgASW) show, after correction for leakage, inward membrane currents. In the case of TEA (Fig. 4*B*), all the current is in the inward direction and relatively large. Also in this case the leakage current was found to be larger than those observed in the other cases. Atomic absorption spectrophotometry revealed that the quantity of Ca in our 0 Ca ASW solutions used was between 10^{-5} and 10^{-6} M and so the small inward currents seen roughly between -40 and 20 mV arise probably from residual calcium present in tubules and/or invaginations which presumably are entrenched deep within the body of the muscle fiber (Selverston, 1967). This type of inward current in 0 Ca ASW was not observed by either Keynes *et al.* (1973) or by one of us (N.L.) who used another barnacle species, *Megabalanus psittacus*, to

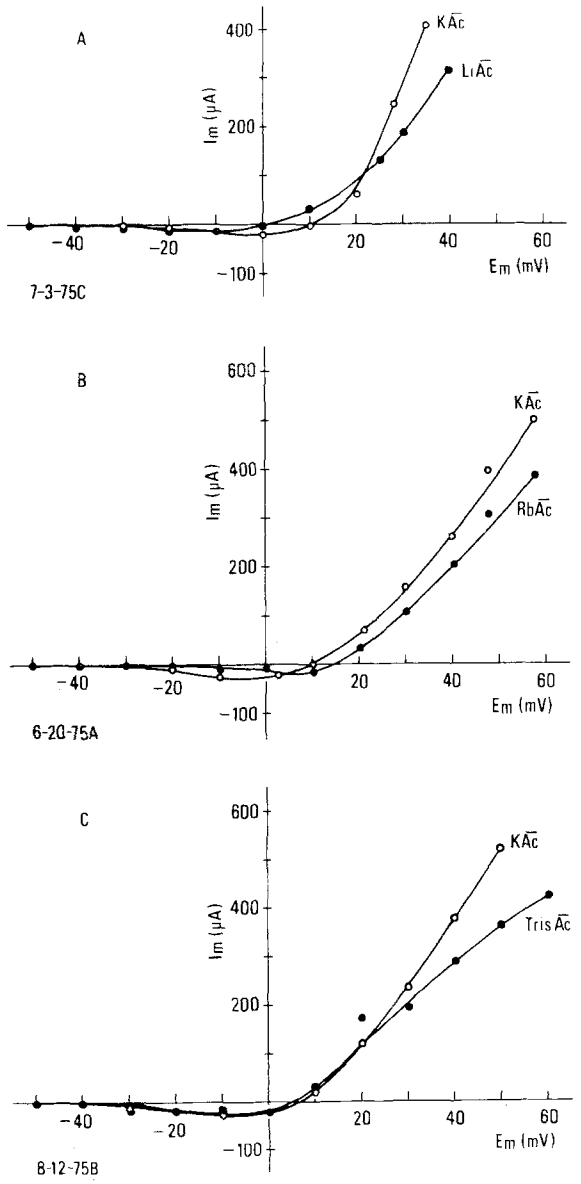

Fig. 5. Effects of replacement of K^+ in the internal solution by Li^+, Rb^+ and $Tris^+$ ions upon the relation between outward current and voltage. The barnacle muscle fiber was in 0 Ca and 30 mM Mg ASW. Open circles represent the control with 180 mM K acetate in the internal solution and filled circles correspond to test solutions with 180 mM Li^+ (*A*), Rb^+ (*B*), and $Tris^+$ (*C*). In all cases, leakage curents have been subtracted. Small inward currents noted are probably due to residual calcium in the 0 Ca ASW

measure only the outward currents as a function of internal ionic strength (Lakshminarayanaiah & Rojas, 1975; *also some unpublished work*). This difference in behavior (i.e. presence or absence of inward current in 0 Ca ASW) could arise possibly from the fact that fibers from *Megabalanus* inflate (almost twice the original size) more readily than those from *Balanus* upon initiation of internal perfusion. This increase in size of the fiber from *Megabalanus* should result in an increase in the size of the tubules and invaginations and thus lead to dilution of residual calcium present there. This type of reduction in Ca concentration cannot occur in the *Balanus* fibers as they inflate little upon initiation of internal perfusion. However, the fibers were inflated by application of pressure; but voltage-clamp experiments could not be carried out due to low resting potential and rapid deterioration of the fibers.

Effect of Intracellular Calcium Concentration on the K Current

Although the effects of intracellular Ca concentration upon excitability of the barnacle muscle fiber have been investigated by Hagiwara and Nakajima (1966*b*), there does not seem to be any study of the effects of the same on the K system. On the other hand, in *Aplysia* (Meech, 1972) and *Helix aspersa* (Meech, 1974) neurons, and in cat spinal motoneurons (Krnjevic & Lisiewicz, 1972; Krnjevic, Paul & Werman, 1975) increased concentration of intracellular Ca enhanced the K conductance. In addition, Barrett and Barrett (1976) have shown that, of the two types of afterhyperpolarization (one fast, lasting 5–10 msec and the other slow, lasting 60–200 msec) observed in frog motoneurons following an action potential, the slow afterhyperpolarization, which along with the fast afterhyperpolarization was brought about by an enhanced K conductance, was dependent upon the Ca concentration. At low Ca concentration ($\leqq 0.2$ mM), the slow afterhyperpolarization was reversibly inhibited; whereas at high Ca concentration it was enhanced. It has been suggested that the slow Ca influx following depolarization enhanced the K conductance underlying the slow afterhyperpolarization. Further it has been shown that both the slow Ca and K currents, as observed in the barnacle muscle membrane, were required for the generation and regulation of low frequency repetitive discharge in the frog motoneurons. Contrarily, Begenisich and Lynch (1974) found that as much as 10 mM of internal Ca had no effect on the ionic currents in the squid giant axon. It is important therefore to see if there are any effects of Ca on the K currents in the barnacle muscle fiber.

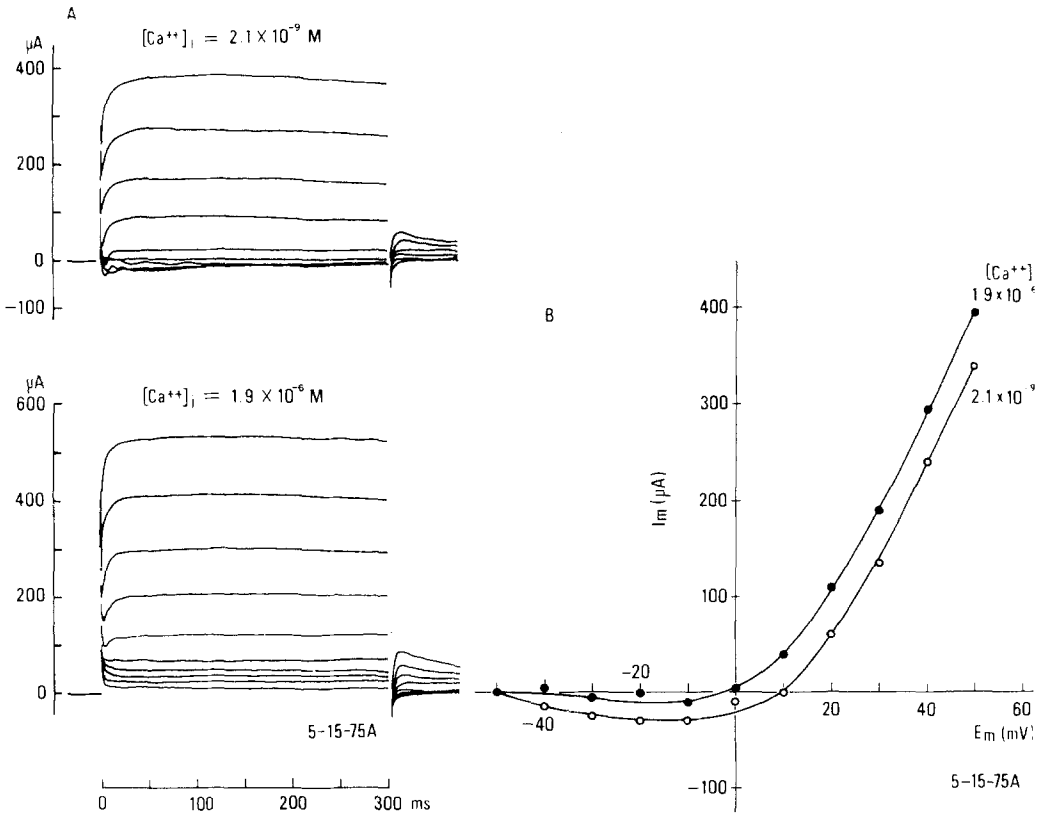

Fig. 6. Effect of internal Ca concentration on outward currents due to K in the voltage-clamped muscle fiber membrane. (*A*) Family of outward membrane currents associated with 10-mV step depolarizations from the holding potential of −50 mV obtained in a muscle fiber perfused internally with solutions containing 2.1×10^{-9} M (upper panel) and 1.9×10^{-6} M (lower panel) Ca^{2+} ions. The muscle fiber was immersed in 0 Ca and 30 mM Mg ASW. (*B*) Current-voltage curves obtained from the outward currents represented in *A*. Leakage currents have been subtracted. Open circles correspond to internal solution containing 2.1×10^{-9} M Ca^{2+} ion and filled circles represent the solution containing 1.9×10^{-6} M Ca^{2+} ion. The small inward currents are probably due to residual calcium in 0 Ca ASW

As oscillations in membrane currents can be abolished either by elimination of the early inward current by using 0 Ca and 60 mM Mg ASW or by depression of the late outward current by using 60 mM TEA in the internal solution (Keynes *et al.*, 1973), experiments were performed with muscle fibers immersed in 0 Ca ASW to observe the effects of intracellular Ca concentration on the K currents. For this purpose, internal solutions containing 1.9×10^{-6}, 2.1×10^{-7}, 2.3×10^{-8} and 2.1×10^{-9} M Ca^{2+} ions were prepared using the solutions given in Table 3.

Fig. 6 shows the tracings of membrane currents recorded from a muscle fiber that was first perfused with an internal solution of K-acetate containing low Ca^{2+} ions (2.1×10^{-9} M) and then a family of membrane currents at various depolarizations was recorded. Next the internal solution was switched to one of higher Ca^{2+} concentration and the second family of membrane currents was taken about an hour after the onset of perfusion. From the families of membrane currents shown in Fig. 6*A*, the current-voltage relations shown in Fig. 6*B* at 200 msec after the onset of depolarizing pulses were derived. In the construction of these curves, the leakage currents have been subtracted. Interestingly, the leakage currents were bigger when the muscle fiber contained higher levels of intracellular Ca^{2+} ion. In no case was any hyperpolarization of the resting membrane following an increase in internal Ca^{2+} ion concentration observed.

The kinetics of the turning on of the K currents do not seem to be affected by the internal Ca^{2+} ion concentration (*see* Fig. 6*A*). Current records similar to those shown in Fig. 6*A* were also obtained when the concentration of intracellular Ca^{2+} ion was 2.1×10^{-7} or 2.3×10^{-8} M. Comparison of currents shown in Fig. 6*A*, upper panel, with those shown in the lower panel indicate an augmentation of the current at high Ca concentration. The magnitude of increase in the K current (*see* Fig. 6*B*) often times varied from fiber to fiber ranging from 4 to 24 %. In some fibers when 2.1×10^{-7} or 2.3×10^{-8} M Ca^{2+} ion was used, the increase in the K current was on the lower side of the range. But in all the six fibers tested the tendency was to raise the K current when the intracellular Ca was increased. In tune with this increase in K current, there is a shift on the voltage axis of the current-voltage curve in the direction of hyperpolarization. This shift although in the right direction cannot be due to a change in the surface potential brought about by "screening" or binding of Ca^{2+} ion to the negative charges on the inner side of the membrane. If this mechanism were true, other divalent ions should also cause a shift in the hyperpolarizing direction, the magnitude being dependent on the nature of the ion (Blaustein & Goldman, 1968; Hille, 1968; Hille, Woodhull & Shapiro, 1975). Zn^{2+} ions as shown in the following section exert little or no effect on the K current and the slight change seen in the shift on the voltage axis of the current-voltage curve (*see* Fig. 8) is in the opposite direction. So the results of Fig. 6 lead us to conclude that elevated levels of Ca inside the muscle fiber increased resting membrane conductance and activated an increase in the K conductance of the membrane.

Effects of Intracellular Zn^{2+} Ions on Membrane Currents

Typical results derived from voltage-clamp experiments with muscle fibers immersed in normal ASW and perfused with standard internal solution containing 5 or 10 mM Zn^{2+} ions are shown in Fig. 7. It is difficult from these results to delineate the effects of Zn^{2+} ions on either the first peak of the transients or the steady-state current. In view of this, voltage-clamp experiments were performed using muscle fibers in which either the early inward (fiber in 0 Ca ASW) or the late outward (60 mM TEA in the internal solution) current was eliminated or depressed. The current-voltage relation shown in Fig. 8 indicates that intracellular Zn^{2+} ions exert little or no effect on the K currents. Fig. 9 shows two sets of records of membrane currents obtained with the muscle fiber in 60 mM Ca and 0 Mg ASW and perfused with solution E (*see* Table 1) containing zero (*A*) and 10 mM (*B*) Zn^{2+} ions. The current-voltage curves derived from these records are given in Fig. 10. The peak amplitude of the first downward deflection of the early membrane current and the magnitude of the steady-state current measured at 300 msec after onset of the depolarizing pulse, with leakage currents subtracted, are plotted in Fig. 10*A* and *B*, respectively. The peak amplitude of the first downward deflection in the membrane currents is greatly depressed, about 77 % at −20 mV and about 61 % at −10 mV (*see* Fig. 10*A*) by 10 mM Zn. This effect was less pronounced on the transient (*see* Fig. 10*A*) and the steady-state (Fig. 10*B*) currents when the membrane potential was in the range 20 to 60 mV. It is generally agreed that the membrane current in the first downward deflection is carried mainly by Ca^{2+} ions in the range of membrane potentials from −40 to 10 mV and therefore the transient is a reasonable measure of the Ca current. At membrane potentials greater than 20 mV, the current is contaminated with some residual current due to K^+ ions since it is known that 60 mM TEA inside the fiber will not completely block the K current (Keynes *et al.*, 1973). Consequently, the effect of Zn on the Ca current becomes less clear-cut at membrane potentials greater than 20 mV. The conclusion therefore that follows from the results of Figs. 8 and 10 is that 10 mM intracellular Zn blocks or rather depresses the action potential by suppressing preferentially the Ca current without affecting the K current that is seen when the fiber is in 0 Ca ASW. Probably a similar mechanism may operate to suppress the Ca spike when Zn is applied externally (Hagiwara & Takahashi, 1967). Besides, the results of Figs. 9 and 10 lead to an interesting inference which is based on the results of Fig. 6 (i.e. Ca mediated increase in K

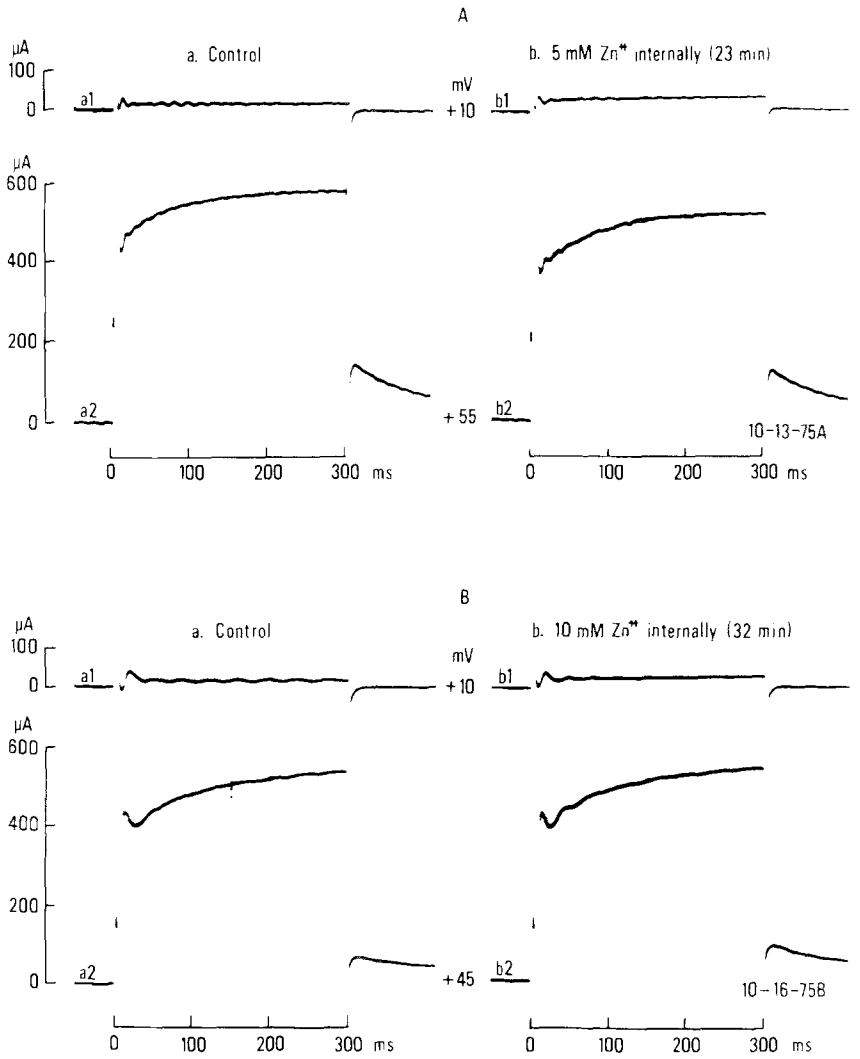

Fig. 7. Effect of intracellular Zn ions on the membrane currents in a muscle fiber subject to voltage-clamp. The membrane currents relate to step depolarizations to the levels of membrane potential indicated before (*a*'s) and during (*b*'s) application of 5 mM (*A*) and 10 mM (*B*) Zn ions internally. The tracings of records in *A* and *B* are from different muscle fibers wich were perfused with the standard internal solution and bathed in normal ASW. The holding potential was −50 mV

conductance). When the muscle fiber is in solution E (Table 1), Ca influx could trigger an additional K current (see Meech & Standen, 1975) and if so, this current may be blocked by Zn. This general inference may not have any validity (quantity of Ca influx under stimulation may be too small to activate the additional K current) and needs to be investigated,

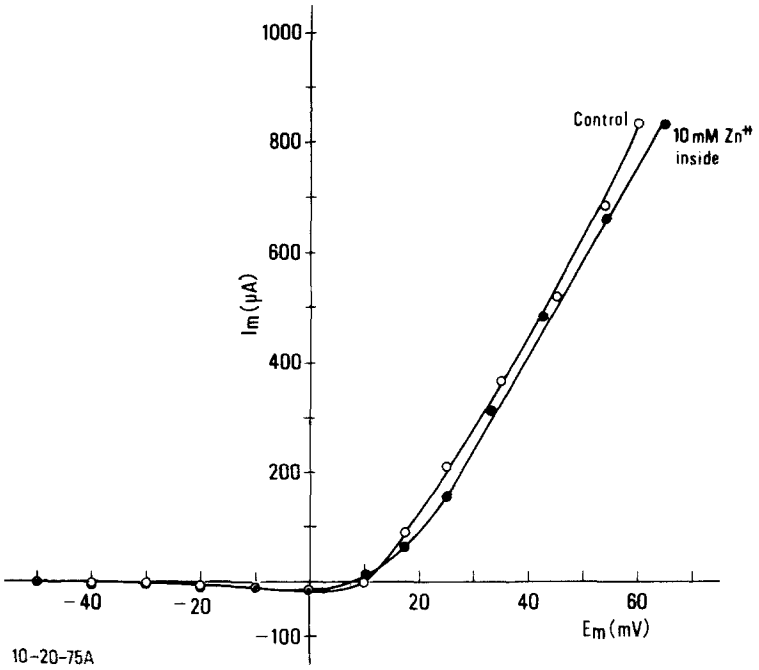

Fig. 8. Effect of 10 mM Zn ions on the outward K currents. Current-voltage curves for the steady-state currents before (open circles) and during (filled circles) application of 10 mM Zn ions inside a barnacle muscle fiber, internally perfused and immersed in 0 Ca and 60 mM Mg ASW. The holding membrane potential was −50 mV. Outward currents from which leakage currents have been subtracted were measured at 250 msec

since the results of Fig. 10 do not offer any evidence on this point due to the presence in the internal solution of 60 mM TEA which has been observed to block both the Ca dependent and independent K conductance in *Helix aspersa* neurons (Meech & Standen, 1975).

Effects of Na and Li Ions on the Inward Current through the Ca Channel

Voltage-clamp experiments were performed with the muscle fiber immersed in 10 mM CaASW and perfused with 180 mM Na or Li acetate solution. The records of currents following application of 40 mV (*A*.1 and *B*.1) and 80 mV (*A*.2 and *B*.2) depolarizing pulses to the membrane held at a potential of −50 mV are shown in Fig. 11*A* and *B*. Both Na and Li ions suppressed the oscillatory behavior in membrane currents (compare

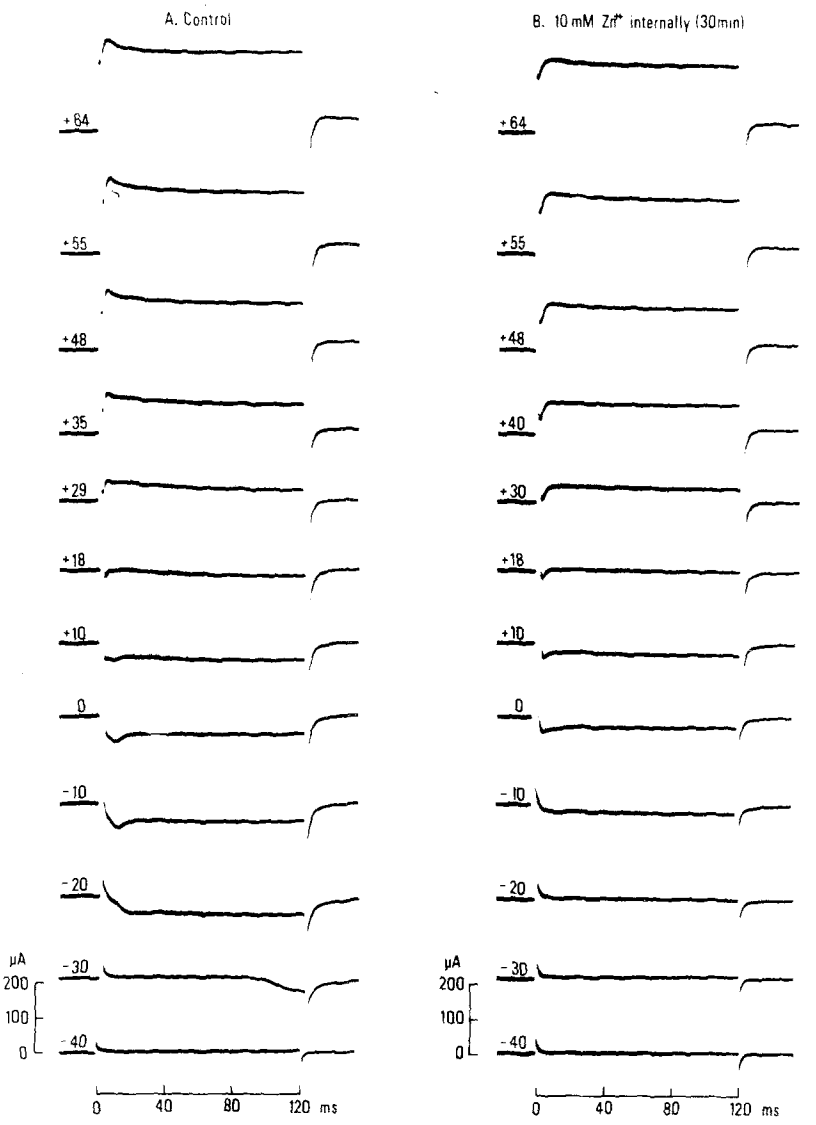

Fig. 9. Effect of 10 mM Zn ions on the membrane currents in a barnacle muscle fiber perfused with internal solution E (Table 1) containing 60 mM TEA and bathed in 60 mM Ca and 0 Mg ASW. (*A*) Family of membrane currents associated with step depolarizations of 10 mV from the holding membrane potential of −50 mV before application of 10 mM Zn ions. (*B*) Family of membrane currents 30 min after start of application of Zn ions. The muscle fiber used in these experiments was the same as the one used in experiments of Fig. 2*B*

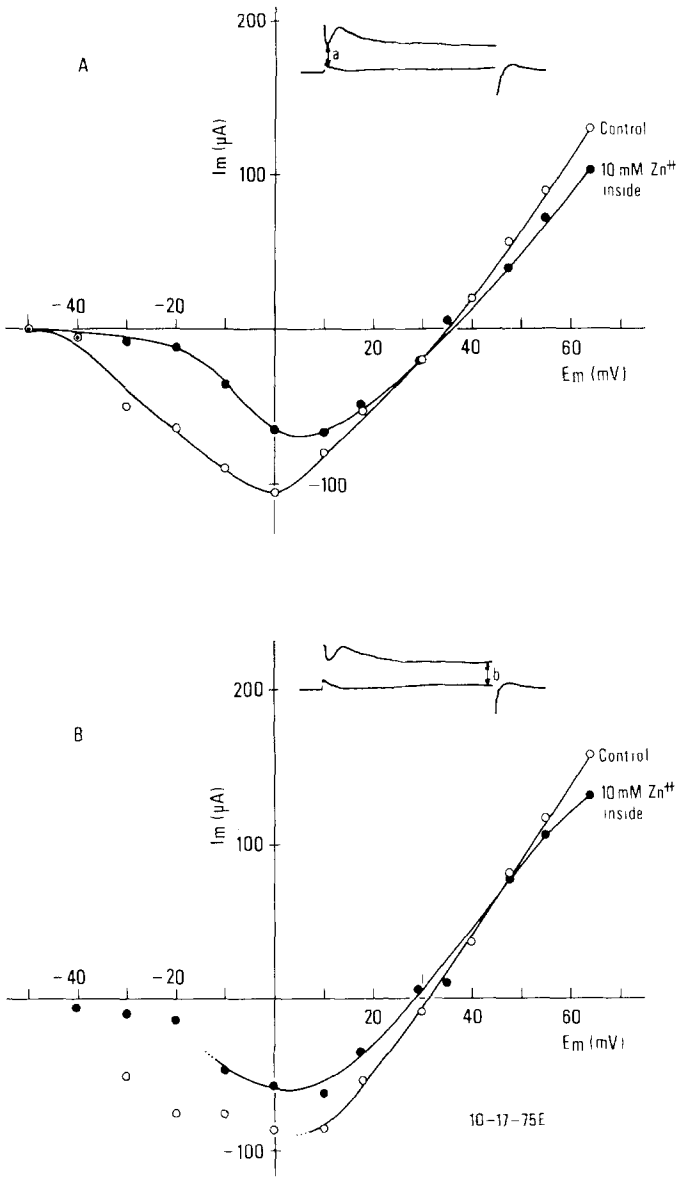

Fig. 10. Effect of Zn ions on membrane currents. (*A*) Current-voltage curves for the first downward peak transient current before (open circles) and during (filled circles) application of 10 mM Zn ions. (*B*) Current-voltage curves for the steady-state current before (open circles) and during (filled circles) application of 10 mM Zn ions. The current-voltage curves were derived from the families of membrane currents shown in Fig. 9 after subtraction of the currents due to leakage. See the insets for the measurements of the peak amplitude of the first downward deflection (*a*) and of the amplitude of the steady-state (*b*) membrane currents

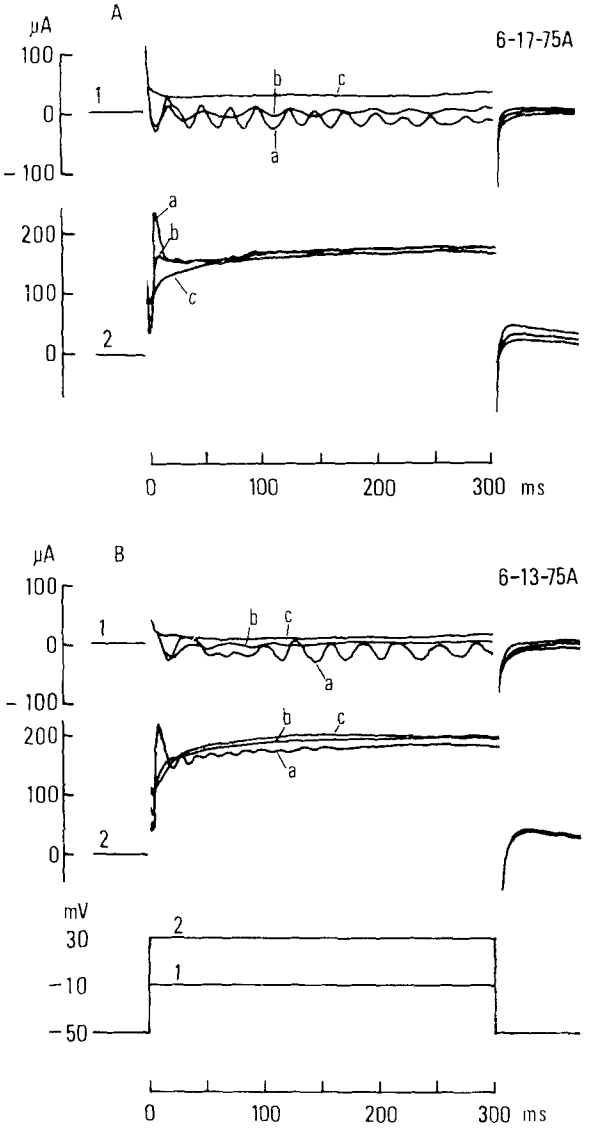

Fig. 11. Effects of replacement of K ions in the internal solution by Na(*A*) or Li(*B*) ions upon membrane currents associated with the application of 40 mV (1) and 80 mV (2) depolarization pulses. The holding potential was −50 mV. External solutions: *a* and *b* curves were obtained with the fiber in standard ASW and *c* curves obtained in 0 Ca and 30 mM Mg ASW. Internal solutions: *a* curves obtained in 180 mM K acetate solution and *b* and *c* curves obtained when K acetate was replaced by Na acetate (*A*) or Li acetate (*B*)

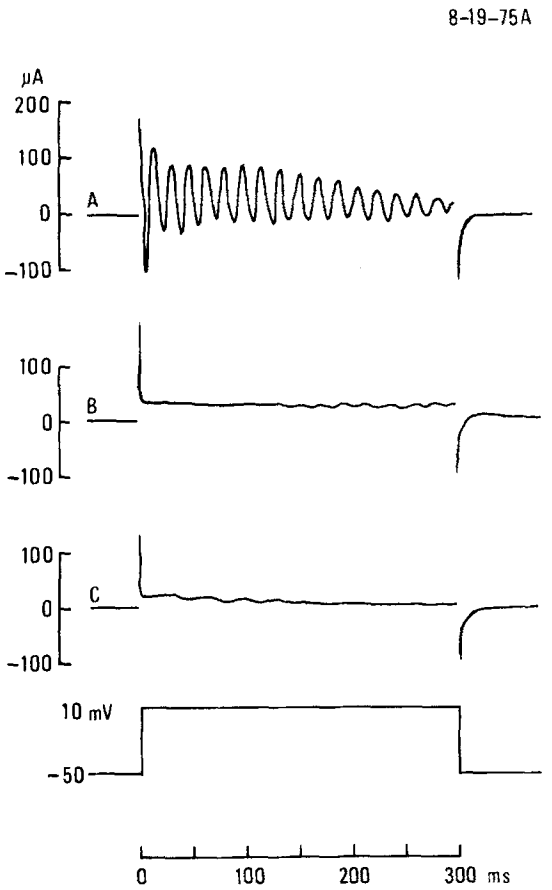

Fig. 12. Effects of extracellular Na ion concentration on membrane currents associated with a 60-mV depolarization pulse. The holding membrane potential was −50 mV. Na ions in the standard ASW were replaced progressively with sucrose. (*A*) Control; (*B*) 200 mM Na ASW; (*C*) 10 mM Na ASW. The internal solution contained 180 mM K acetate and sucrose (solution A of Table 1)

b curves with *a* curves and *a* and *b* curves with *c* curves obtained in 0 Ca ASW) although the first deflection in the current continued to exist (*see b* curves in Fig. 11). In experiments of this type and those described already where different internal cations were used, it is possible, in view of the high selectivity of the channel to K ions, that the observed outward currents are mixed with a small fraction due to residual K ions crossing the membrane. Outflux measurements using appropriate isotopes would probably provide answers to this problem and such an investigation is being planned.

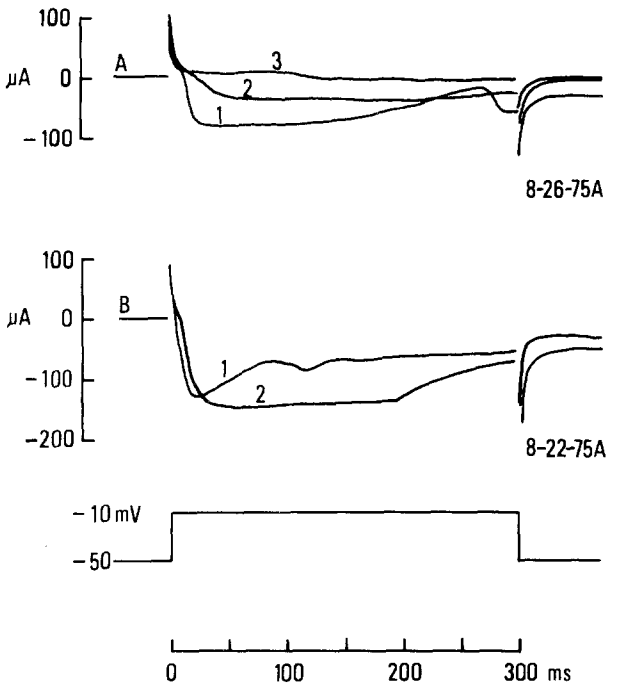

Fig. 13. Effects of replacement of external NaCl by sucrose (*A*) and choline chloride (*B*) on inward Ca currents associated with a 40-mV depolarization pulse. The holding membrane potential was −50 mV. The barnacle muscle fiber was perfused internally with 180 mM K acetate solution containing 60 mM TEA (solution E of Table 1) to depress the outward K currents. The external solutions were: (1) 430 Na ASW, (2) 200 Na ASW and (3) 10 Na ASW. These solutions are, respectively, D1, D2 and D3 of Table 2

Effects of External Na Concentration on the Inward Current

Hagiwara and Naka (1964) have shown that removal of Na from the external solution has no effect on the overshoot and the time course of the spike in the barnacle muscle fiber. When osmotically equivalent quantity of sucrose was substituted for NaCl in the ASW, they noted some reduction in the spike potential. However, we found the amplitude of the initial spike to remain constant when Na in ASW was 200 or 10 mM; but the height of the plateau following the initial spike was decreased. In Fig. 12 membrane currents under voltage-clamp recorded in low ionic strength ASW solutions are given. Fig. 12*A* shows the characteristic oscillations in membrane current normally seen in muscle fiber in ASW (D1, Table 2) and perfused with solution A (*see* Table 1). When the ASW is changed to D2 (*see* Table 2) oscillations are dampened

and there is reduction in current (*see* Fig. 12*B*). Further reduction in oscillations and outward membrane current (Fig. 12*C*) occurs when the external solution is changed to D3 (Table 2). These results indicate that the net membrane current (algebraic sum of inward and outward currents) which is outward is reduced. There is also reduction or possibly elimination of inward current, although such an effect is not seen directly. Confirmation of such an effect is shown in Fig. 13*A* which depicts the tracings of records of membrane current obtained when the muscle fiber is perfused with solution E (*see* Table 1) containing TEA. Curves 1, 2 and 3 of Fig. 13*A* clearly indicate progressive decrease in inward current following decrease in the extracellular Na concentration. Whether this inward current decrement is mediated through a reduction in ionic strength of the external solution is shown in Fig. 13*B*. Substitution of choline for Na in the outside solution had no effect on the inward current. Similarly, Li and guanidine were without any effect on the inward current. Consequently, the decrements in membrane currents noted in Figs. 12*B*, 12*C* and 13*A* become attributable to the low ionic strength of ASW.

Discussion

When the intracellular concentration of Ca was raised from 2.1×10^{-9} to 1.9×10^{-6} M, the steady-state K current in the barnacle muscle fiber was augmented. Such a facilitation by Ca of the membrane permeability to K has been observed in red blood cells (Whittam, 1968; Lew, 1970; Romero & Whittam, 1971), *Aplysia* neurons (Meech, 1972), *Helix aspersa* neurons (Meech, 1974; Meech & Standen, 1975) and cat motoneurons (Krnjevic and co-workers, 1972; 1975). On the contrary, Begenisich and Lynch (1974) found that 10 mM intracellular Ca had little effect on the ionic currents in the squid giant axon. Similarly, Meves and Meech (*unpublished*, quoted in Meech, 1974) found 10 mM EGTA perfused internally in the squid giant axons to be without significant effect on the K currents.

In his studies on *Aplysia* neurons, Meech (1972) suggested that Ca increased the K permeability of the membrane by combining with the phospholipid components of the cell membrane. This suggestion was based on the findings of Papahadjopoulos and Bangham (1966) who noted that the diffusion of K through liquid crystals of phosphatidyl serine increased when Ca was present in a concentration greater than

0.8 mM. Whether this mechanism underlies the Ca-mediated increase in K conductance observed in barnacle muscle fiber is difficult to assess. It may be that Ca somehow augments K conductance by (1) adsorption onto the membrane, (2) neutralization of negative charges on the membrane, or (3) chemical combination with a more specific membrane site. In any case, intracellularly applied Ca^{2+} ions increase resting membrane conductance, open K channels and probably make them more open or more selective to K^+ ions. The possibility that a new K channel may be created by the action of Ca is discredited on grounds that TEA in *Helix aspersa* neurons has similar action on both Ca dependent and independent K conductance (Meech & Standen, 1975). As opposed to this action of Ca, Zn^{2+} ions at a concentration of 10 mM were without any effect on the K current in the barnacle muscle fiber. However, they suppressed the peak transient current carried by Ca^{2+} ions. In skeletal muscle on the other hand, Zn depressed the delayed K conductance (Kao & Stanfield, 1970; Stanfield, 1975). Begenisich and Lynch (1974) found that Zn^{2+} ions, in addition to other ions such as Co^{2+}, Cd^{2+} and Ni^{2+}, reduced the peak Na current and slowed the kinetics of K current in the squid giant axon. Similarly, 10 mM Zn has been found to block the movement of charge within the membrane responsible for opening the Na channel (Armstrong & Bezanilla, 1974). These actions of divalent ions have been attributed to the differences in their chemical binding affinities to membrane phospholipids and/or proteins (Begenisich & Lynch, 1974; Stanfield, 1975).

The other main results of this study show that when the ionic strength of ASW was lowered, the Ca channel ability to carry current was reduced. This reduction may arise from an increase in the series resistance and/or change in the binding properties of Ca^{2+} to the negatively charged sites on the membrane. Although we did not use compensation for the series resistance, it can be shown that the decrease in the inward current due to increase in the series resistance following change of external solution to one of low ionic strength is very small.

The resistance of solutions D1, D2 and D3 (*see* Table 2) and of internal solution E (*see* Table 1) have been measured and found to be 18.24, 38.05, 83.33 and 48.11 Ωcm^2, respectively. These values become roughly 4.6, 9.5, 20.8 and 7.2 Ωcm^2 if we consider the distance of the current measuring electrode outside to the surface of the muscle fiber to be 0.25 cm and that of the inner current electrode from the "membrane surface" to be 0.15 cm. It is assumed that the inner current electrode is in the center of the fiber. These values for the distances were chosen from

the width of the groove (8 mm) in which the muscle fiber of 3 mm approximate diameter was placed. The values for the series resistance given above are too low in relation to a value of $2\text{–}3 \times 10^3\,\Omega\text{cm}^2$, the resistance of the barnacle muscle membrane measured by Hagiwara and Naka (1964), or about $1.5 \times 10^3\,\Omega\text{cm}^2$ estimated for the perfused fibers, to significantly affect the membrane current. Consequently, external solution of low ionic strength must be influencing some membrane parameter to reduce the inward Ca current.

The investigations of Hagiwara *et al.* (1974) have shown that Ca^{2+}, Sr^{2+} and Ba^{2+} ions passed through the early channel (or "Ca" channel) and gave the inward current which, however, was blocked by Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} ions. The latter phenomenon is considered to arise from competitive adsorption at the membrane sites (near and/or in the channel) which are normally occupied by Ca ions. The inward Ca current, $I_{Ca}(V)$, is assumed to be given at a given membrane potential (V) by a Langmuir type adsorption equation

$$I_{Ca}(V) = \frac{I_{Ca(max)}(V)\,Ca_0^{2+}}{Ca_0^{2+} + K_{Ca}(V)} \tag{1}$$

where K_{Ca} is the apparent dissociation constant of the site which reacts with Ca_0^{2+} ions in the external solution. $I_{Ca(max)}$ is the maximum value of I_{Ca} when Ca^{2+} ions are adsorbed to all the available sites. Both K_{Ca} and $I_{Ca(max)}$ are voltage dependent.

On the basis of Eq. (1) it is suggested that the parameter that is affected by the change in ionic strength is the apparent dissociation constant K_{Ca}. Both theory (Laitinen, 1960) and experiments in aqueous solutions of weak acids (Daniels & Alberty, 1955) indicate that an increase in apparent dissociation constant follows an increase in ionic strength of the solution. This means that I_{Ca} at a given voltage will decrease as the ionic strength is increased on the assumption that $I_{Ca(max)}$ remained constant. This prediction is contradicted by the experimental findings. Consequently, the concepts evolved for electrolyte solutions in which different species involved in the reaction retain their freedoms of rotation, vibration and translation become inapplicable to a reaction that takes place at a membrane-solution interface where two of the species involved in the interfacial reaction

$$Ca^{2+} + X^{2-} \rightleftharpoons CaX \tag{2}$$

(X^{2-}, the site and CaX, the reaction product) will have no translational freedom. In the above reaction the concentration of Ca refers to the

concentration in the vicinity of the site X and not to the bulk concentration. For reaction (2), the thermodynamic dissociation constant K'_{Ca} is given by

$$K'_{Ca} = \frac{a_{Ca} a_X}{a_{CaX}} = K_{Ca} \frac{f_{Ca} f_X}{f_{CaX}} \tag{3}$$

where a's and f's are activities and activity coefficients, respectively. Substituting Eq. (3) in Eq. (1) gives on rearrangement

$$I_{Ca} = \frac{I_{Ca(max)} Ca_0^{2+}}{Ca_0^{2+} + K'_{Ca} F} \tag{4}$$

where $F = (f_{CaX})/(f_{Ca} f_X)$ and may be called the activity factor which is unity for an ideal system. But our system is far from ideal and corresponds to a Donnan system due to the immobility of the sites fixed in the membrane. In such systems it is well established (Lakshminarayanaiah, 1969) that as the external solution is made more dilute (low ionic strength), the term $(f_{Ca} f_X)$, instead of tending towards unity as generally observed in aqueous electrolyte solutions, will move towards low values. In such a situation, the value of the term F will become high leading to a low value for I_{Ca}, as the other terms in Eq. (4) remain constant. What this means then is that when external solutions of low ionic strength are used, the inward membrane current I_{Ca} is reduced by changes in the activity coefficients of small ions in the membrane-solution interface in such a direction as to increase the value of the factor F. This happens when the reaction (2) is favored to occur more to the right than to the left.

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