Permeation of Manganese, Cadmium, Zinc, and Beryllium Through Calcium Channels of an Insect Muscle Membrane

Abstract. *Larval muscle fibers of a beetle,* Xylotrupes dichotomus, *produce calcium spikes that are maintained when the fibers are bathed in saline solutions containing manganese, cadmium, zinc, or beryllium instead of calcium. This indicates that these cations permeate the calcium channels of the muscle fiber. By contrast, cobalt, nickel, and magnesium are nonpermeating and behave as competitive inhibitors of the permeation of the other divalent cations. Some of the permeating cations suppress delayed rectification.*

Manganese ion, which has been widely used as a competitive inhibitor of calcium permeation through excitable membranes (*1*), was recently considered a charge carrier during action potentials in squid giant axons (*2*) and mammalian cardiac muscles (*3*). However, since there are both Na and Ca channels in these membranes (*4*), it is not clear which channels Mn^{2+} permeates. We demonstrate here that a Mn^{2+}-dependent, all-or-none action potential can be elicited in a larval insect muscle fiber which generates virtually pure Ca spikes (*5*). The permeation of Mn^{2+} through the Ca channels in this insect muscle fiber raised the question of whether other divalent cations that have been considered nonpermeating might also permeate the Ca channels. Our experiments reveal that these muscle fibers also generate all-or-none action potentials when they are bathed in a saline solution containing Cd^{2+}, Zn^{2+}, or Be^{2+}; these divalent cations seem to permeate the Ca channels. By contrast, Co^{2+}, Ni^{2+}, and Mg^{2+} do not appear to permeate the membrane, although some of them interact with the Ca channels and act as inhibitors of action potential generation.

Ventral longitudinal muscle fibers (50 to 70 μm thick and 5 to 8 mm long) were dissected from a larva of the beetle *Xylotrupes dichotomus* Linné and impaled by two glass microelectrodes filled with 3*M* KCl (10 to 20 megohms). The fibers were bathed in a beetle saline solution [127 m*M* NaCl, 3.2 m*M* KCl, 40 m*M* $CaCl_2$, 2.5 m*M* $MgCl_2$, buffered at *p*H 7.4 with 5.0 m*M* tris-Cl or Na-Hepes (*6*)]; the composition of the solution was changed with the osmolarity held constant. In this muscle fiber, as in muscle fibers of various invertebrates such as crayfish (*7*), barnacle (*8*), and some insects (*9*), a depolarizing current pulse injected intracellularly elicited a graded response instead of an all-or-none action potential. When tetraethylammonium$^+$ (TEA^+), an inhibitor of potassium permeation (*7–9*), was added externally (37 m*M*, substituted for Na^+), the larval beetle muscle fiber became capable of generating an all-or-none action potential associated with an overshoot of 23.0 ± 6.9 mv (mean ± standard deviation) and a duration of 0.2 to 5 seconds (not illustrated). The action potential was considered to be a Ca spike (*5*) for the following reasons. (i) Its overshoot and its maximum rate of rise (MRR) were increased by increasing the external Ca^{2+} concentration. Between 2.5 and 10 m*M* Ca^{2+}, in particular, the increment in the overshoot was approximated by the Nernst relationship for a Ca electrode. In Ca^{2+}-free saline containing 90 m*M* Na^+, 40 m*M* Mg^{2+}, and 0.8 m*M* $EGTA^{4-}$, no action potential was elicited. (ii) Divalent cobalt, which competitively inhibits Ca spikes in various muscle preparations (*8–10*), reduced the MRR of the action potential (in 40 m*M* Ca^{2+}) by about one-half at 3 m*M* and almost completely blocked regenerative potential changes at 10 m*M*. (iii) Neither Na^+-free saline (replaced with $tris^+$ or TEA^+) nor 3 μ*M* tetrodotoxin interfered with action potential generation. (iv) When Sr^{2+} or Ba^{2+} was added instead of Ca^{2+}, similar action potentials of longer duration were elicited even in the absence of TEA^+.

In a saline solution containing Mn^{2+} (40 m*M* $MnCl_2$, Ca^{2+}- and TEA^+-free), an all-or-none action potential was elicited in the larval beetle muscle fiber (Fig. 1A). The action potential had a threshold (−19.9 ± 3.7 mv) slightly larger than that of the action potential found in 40 m*M* Ca^{2+} (−26.2 ± 2.6 mv), and the average MRR (1.8 ± 0.4 volt/sec) was smaller than that in 40 m*M* Ca^{2+} (3.8 ± 0.7 volt/sec). The membrane potential initially formed a small peak followed by a sag and then reached a maximum overshoot of 15.1 ± 3.5 mv in 15 to 30 seconds. During the prolonged plateau-like potential, the input conductance of the fiber, measured by small current pulses, increased to 15 to 20 times that of the resting fiber (not illustrated). This action potential lasted 30 to 120 seconds (Fig. 1C). No visible contraction could be observed in the muscle

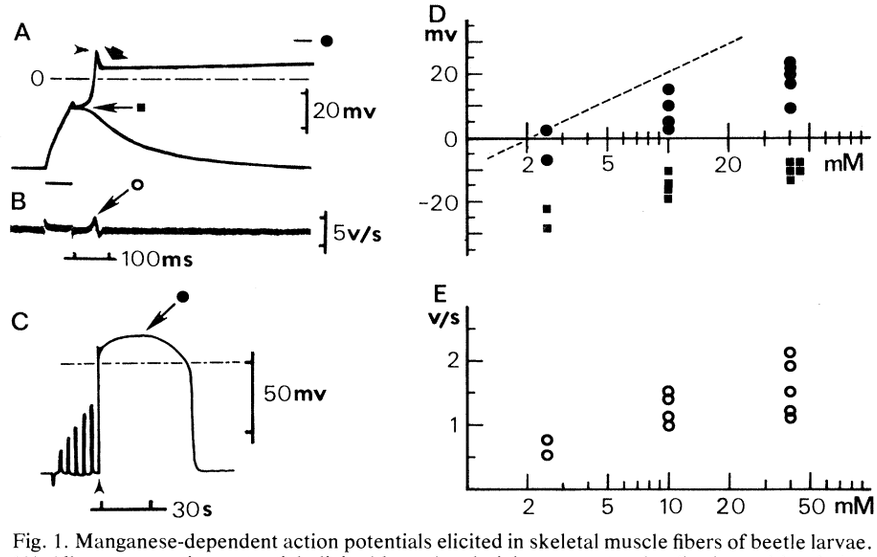

Fig. 1. Manganese-dependent action potentials elicited in skeletal muscle fibers of beetle larvae. (A) All-or-none action potential elicited by a depolarizing current pulse (horizontal bar below the record) when the membrane potential exceeded the threshold potential (■); two traces were superimposed. The wedge and the thick arrow point to the peak and sag of the overshoot, respectively. (—●) Maximum value of the overshoot of the action potential (21 mv). The broken line shows the reference potential. (B) Maximum rate of rise (arrow and ○) obtained by electronically differentiating the action potential from (A). (C) Manganese potential recorded with a slow time base. The amplitude of the current pulse was increased until the threshold was reached; the wedge shows the onset of the Mn potential; the arrow and ● indicate the maximum value of the overshoot. (D) Maximum value of the overshoot (●) and threshold membrane potential (■) for the action potential obtained from different fibers in the same preparation are plotted against the logarithm of the Mn^{2+} concentration. The dashed line indicates the Nernst slope expected if the membrane behaves as an Mn electrode, that is, 29 mv per tenfold change in the Mn^{2+} concentration. (E) Maximum rate of rise (○) of the Mn potential plotted against Mn^{2+} concentration. The ionic composition of the saline solution in (A) to (C) was 40 m*M* $MnCl_2$, 62.5 m*M* $MgCl_2$, 37 m*M* NaCl, 3.2 m*M* KCl, and 5.0 m*M* tris-Cl at *p*H 7.4. In (D) and (E) the composition of the solution was changed by replacing Mn^{2+} with equimolar Mg^{2+}.

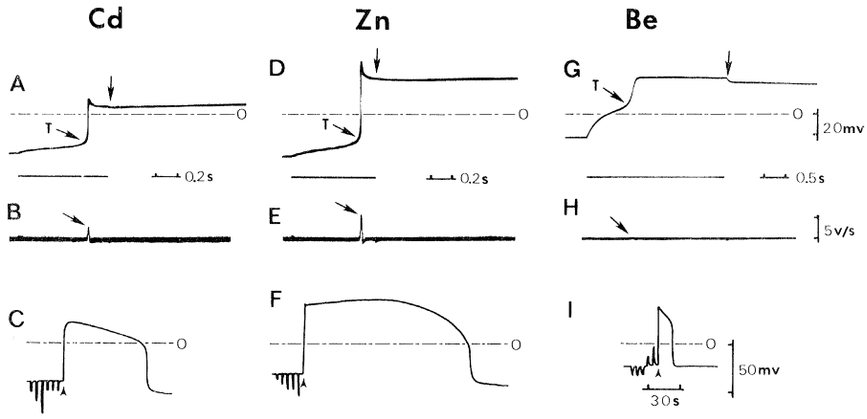

Fig. 2. All-or-none action potentials elicited in skeletal muscle fibers of beetle larvae bathed in saline solution containing Cd^{2+}, Zn^{2+}, or Be^{2+}. (A) Cadmium potential, an all-or-none action potential elicited in a saline solution containing 40 m*M* $CdCl_2$, 127 m*M* NaCl, 2.5 m*M* $MgCl_2$, 3.2 m*M* KCl, and 5.0 m*M* tris-Cl at *p*H 7.4. The arrow marked *T* indicates the threshold potential. The bar below the record shows the duration of the depolarizing current applied. The vertical arrow at the top indicates a small change of the membrane potential associated with cessation of the pulse current; this indicates that the input conductance of the fiber is increased during the overshoot. (B) Trace obtained by differentiating the action potential of (A) with an electronic circuit. An arrow indicates the MRR. (C) Slow time base trace of a Cd potential. The wedge shows the onset of the action potential; before its generation, the membrane was hyperpolarized by a series of current pulses. The broken line in (A) and (C) indicates the reference potential. (D to F) Zinc potential, similar to (A) to (C) but in 40 m*M* $ZnCl_2$, 127 m*M* NaCl, 3.2 m*M* KCl, 2.5 m*M* $MgCl_2$, and 5.0 m*M* Na-Hepes at *p*H 6.75. (G to I) Beryllium potential, similar to (A) to (C) or (D) to (F), but in 2.5 m*M* $BeSO_4$, 100 m*M* $MgCl_2$, 37 m*M* TEA-Cl, 0.8 m*M* K_4-EGTA, and 32 m*M* Na-MES at *p*H 5.0. Calibrations: (A, D, and G) 20 mv; (B, E, and H) 5 volt/sec; (C, F, and I) 50 mv and 30 seconds; (A, B, D, and E) 0.2 second; and (G and H) 0.5 second.

fiber under the binocular microscope (× 60).

The dependence of the action potentials on Mn^{2+} is demonstrated in Fig. 1, D and E, where their maximum overshoots, threshold potentials, and MRR's are plotted against the external Mn^{2+} concentration. The maximum overshoot increased with a 20-mv slope for a tenfold elevation of the Mn^{2+} concentration (Fig. 1D), while the threshold potential increased with a slope of about 8 mv. An increase in the membrane conductance to Mn^{2+} was thus responsible for the action potential generation. The MRR, which presumably reflected the amplitude of the transient inward current associated with the action potential (*8, 10*), increased with the increase in Mn^{2+} concentration (Fig. 1E). Addition of 10 m*M* Co^{2+} reversibly reduced the MRR of the Mn potential (in 40 m*M* Mn^{2+}) by about one-half; although a higher dose was required than for the Ca potential, Co^{2+} also acted as a competitive inhibitor of the Mn potential. Divalent manganese thus seemed to utilize the Ca channels in permeating the muscle membrane. Neither the maximum overshoot nor the MRR was altered significantly by addition of 3 μM tetrodotoxin or reduction of the external Na^+ concentration from 132 m*M* to 5 to 10 m*M* (*11*). Therefore, the possibility that Mn^{2+} acted by changing the permeability of the membrane to the other ions, especially Na^+, was ruled out.

One difference between the Mn potential and the Ca potential of this muscle fiber is that TEA^+ was not required to obtain regenerative potential change in Mn^{2+} saline solution. Also, TEA^+ was not required for generating the Sr and Ba potentials; Sr^{2+} and Ba^{2+} themselves reduced the potassium current in the muscle membrane (*7, 8*). By analogy, we assumed that Mn^{2+} also inhibits the potassium current; this could satisfactorily explain the regenerative occurrence and long duration of the Mn potential, even though its MRR was smaller than that of the Ca potential.

In a saline solution containing Cd^{2+} (Ca^{2+}- and TEA^+-free), an all-or-none action potential could be elicited in the beetle muscle membrane. In 40 m*M* Cd^{2+}, the threshold potential was -27.6 ± 5.0 mv, the maximum overshoot was 11.7 ± 7.0 mv, and the MRR was 2.7 ± 0.7 volt/sec for nine fibers (Fig. 2, A and B). The Cd potential lasted for 15 to 90 seconds (Fig. 2C). Another all-or-none action potential (Fig. 2, D to F) could be induced in muscle fibers bathed in saline solution containing 40 m*M* Zn^{2+} (Ca^{2+}- and TEA^+-free). The threshold, overshoot, and MRR were -17.0 ± 7.0 mv, 54.3 ± 11.4 mv, and 6.5 ± 0.9 volt/sec, respectively, for 12 fibers. The Zn potential lasted 120 to 300 seconds (Fig. 2F). It should be noted that the *p*H of the Zn^{2+}-containing solution was adjusted to 6.7 to 6.8 with either Na-Pipes, Na-Hepes, or tris-Cl buffer (5.0 m*M*) to avoid precipitation, which occurs at a higher *p*H. Furthermore, in a Be^{2+}-containing saline solution, the muscle membrane generated all-or-none action potentials (Fig. 2, G to I); the threshold, MRR, overshoot, and duration were $+10.4 \pm 5.9$ mv, 0.12 ± 0.04 volt/sec, 32.5 ± 8.7 mv, and 5 to 30 seconds, respectively, for ten Be potentials elicited with 2.5 m*M* $BeSO_4$ or $BeCl_2$. To prevent precipitation, the *p*H was adjusted to 5.0 with 32 m*M* Na-MES, to keep the membrane excitable at low *p*H, TEA^+ (37 m*M*), $EGTA^{4-}$ (0.8 m*M*), and a high Mg^{2+} concentration (40 to 100 m*M*) were required externally.

By contrast, no action potential could be elicited in these muscle fibers when they were immersed in saline solution containing Co^{2+} or Ni^{2+} (10 to 40 m*M* with 37 to 100 m*M* TEA^+ at *p*H 7.4). These divalent cations also acted as inhibitors of the Ca spike (*10, 12*). Magnesium was shown to be practically inert for the genesis of the action potential by experiments in which the muscle fibers became inexcitable when bathed in a saline solution free of Ca^{2+} (with 0.8 m*M* $EGTA^{4-}$), rich in Mg^{2+} (40 to 100 m*M*), and containing TEA^+ (37 to 100 m*M*). Inhibition of the Ca spike by Mg^{2+} was weak; for example, increasing the Mg^{2+} concentration from 10 to 100 m*M* caused no detectable change in the MRR of the action potentials obtained at 2.5 m*M* Ca^{2+}. We conclude that Co^{2+}, Ni^{2+}, and Mg^{2+} do not permeate the Ca channels of the beetle muscle membrane.

It may be interesting to note that the nonpermeating ions Co^{2+} and Ni^{2+} are transition metal ions of group VIII in the periodic table, while the permeating ones are from group IIb (Cd^{2+} and Zn^{2+}) and group VIIa (Mn^{2+}). Also, Be^{2+} belongs to group IIa. Since Ca^{2+}, Sr^{2+}, and Ba^{2+} are permeating, Mg^{2+} is an exception among the alkaline earth cations.

The divalent cations which are the charge carriers responsible for the generation of the action potentials described above probably utilize the Ca channels of the muscle membrane. Various parameters of the action potential such as threshold, overshoot, and MRR were related to the way these cations interacted with and passed through the molecular structures of the Ca channels. These interactions partly reflect the physicochemical properties of the cations in the aqueous solution (*13*) and the mem-

brane, such as ionic size, activity, affinity to membrane sites, and mobility in the membrane. For example, permeation of Ca^{2+} through the Ca channels in invertebrate muscle membranes is presumed to occur in two steps (*10, 12*): (i) reversible binding of Ca^{2+} to the sites at the membrane, and (ii) passage of Ca^{2+} through the Ca channels, whose conductance is time- and potential-dependent. A comparison of these parameters among the ionic species would provide a new basis for further investigation of the molecular mechanisms of calcium entry through the membrane.

Besides interacting with the Ca channels, some of the permeating cations, such as Sr^{2+}, Ba^{2+}, Mn^{2+}, Cd^{2+}, and Zn^{2+}, seemed to suppress the generation of a potassium current in the muscle membrane. This pharmacological action of the divalent cations also modifies the generation of action potentials in beetle muscle fibers.

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6. The buffers used were tris-Cl, tris(hydroxymethyl)methylamine chloride; Na-Hepes, sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EGTA, [ethylene-bis-(oxyethylenenitrilo)]tetraacetic acid; Na-Pipes, sodium 1,4-piperazinediethanesulfonate; and Na-MES, sodium 2-(*N*-morpholino)ethanesulfonate.
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11. In this experiment, NaCl was replaced by either tris-Cl, TEA-Cl, or $MgCl_2$ in the presence of 5.0 m*M* Na-Hepes buffer at *p*H 7.4.
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Early Chemical Evolution of Nucleic Acids: A Theoretical Model

Abstract. *Recent experimental work suggests a possible cyclical pathway for early prebiotic oligonucleotide formation that involves (i) dry-state (nontemplate) synthesis of random copolymers with mixed 2',5' and 3',5' bonds, (ii) passage of these oligomers into solution at low temperatures, and (iii) a preferential hydrolysis of the 2',5' bond in any short helices that have formed. This early system could have selected for complementary sequences that were largely 3',5'-linked, but may not have selected efficiently for a single enantiomer of ribose.*

In a recent review (*1*) of theories concerning the origin of life, the assumption was made that the oligonucleotides formed in prebiotic times would have been better able to resist hydrolytic degradation if they could have adopted a helical conformation. A further assumption was made that there existed a nonenzymic mechanism by which a single-stranded RNA molecule could be replicated with a fidelity such that one error was made per 100 bases. Unfortunately, actual attempts to demonstrate template-directed formation of oligoribonucleotides under simulated prebiotic conditions have invariably led (*2, 3*) to the production of a large excess of the unnatural or 2',5' internucleotide bond (*4*). Further, the rate of hydrolysis of this 2',5' bond actually increases when the oligonucleotide of which it is a part forms a right-handed helix (*5*). By contrast, a modest excess of the natural 3',5' bond is formed (*6*) in dry-state polymerization of adenosine 2',3'-phosphate catalyzed by ethylenediamine, but this reaction occurs in the absence of a template. We now report that these recent experimental observations combined with the earlier theory (*1*) show how, under the influence of a daily heating and cooling cycle, a system of oligonucleotide formation could have arisen that selected for (i) the natural 3',5' bond, (ii) longer oligomers, and (iii) complementary sequences. The possible importance in prebiotic chemistry of the natural cycles in temperature and humidity (day and night, tides, seasons) has been explored by others, both in theory (*1*) and through experiment (*7*). In addition, it has been suggested that simulation experiments are more convincing if they are carried out at temperatures that resemble those found on the earth today (*2*), and a recent calculation of the average temperature on the prebiotic earth (about 3.5 to 4 billion years ago) lends support to this suggestion (*8*). The conditions given in Fig. 1 may be taken to represent a semidesert locale that has a reasonably large swing in surface temperature and humidity between day and night. The actual temperatures shown were measured in the Namib Desert (South-West Africa) by Buskirk over a 26-hour period in August 1973 (*9*).

The cyclical scheme is shown in Fig. 1, and assumes the existence of ribonucleoside 2'- and 3'-monophosphates (*2*), which, in turn, are converted into the nucleoside 2',3'-phosphates—for example, by reaction with a condensing agent (*10*), or by heating with a catalyst. The cycle starts with the dry-state synthesis of random copolymer oligomers. This occurs at slightly elevated temperatures in the presence of a catalyst (such as imidazole) (*6*) but is not directed by a template, and the ratio of natural to unnatural bonds formed is about 2 : 1. As the sun sets, the surface temperature decreases, and the oligomers go into solution in the dew which forms. At these low temperatures, some of the oligomers will find complementary partners with which they can form short helices (*11*). As the sun rises on the following morning, the solution warms up, and a preferential degradation of the helical 2',5' bonds will occur (*5*). Helical 3',5' bonds will be protected, while nonhelical 2',5' or 3',5' bonds will degrade at an intermediate rate. The slow rise in temperature is important in that a sudden increase would merely melt the helices and not allow the selective degradation of the "unnatural" bonds to occur. The most stable species under these circumstances are long, complementary oligomers that are entirely 3',5'-linked (higher melting temperature, T_m). Helices that contain a number of gaps or nicks from hydrolysis of 2',5' bonds will have lowered thermal stability and a greater tendency to denature as the temperature increases. Eventually, the solution will dry out, and the residue will consist of a mixture of random copolymers of shorter chain length but with an increased fraction of 3',5' bonds than previously. In some of these oligomers, the terminal phosphate will be in the form of the 2' or 3' monoester, and in others as the 2',3'-phosphate (*12*). In order to ensure further coupling between these oligomers, the terminal phosphates should be in the 2',3' form. Reactivation of the 2' and 3' monoesters