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Effects of Divalent Cation Ionophores on the Neuron Membrane of the Crayfish

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Summary. The effects of divalent cation ionophores, A23187 and X-537A, on the electrical membrane properties were investigated by using the soma membrane of the X-organ of the crayfish. They reduced the amplitude and maximum rate of rise of Ca-action potential in lower concentration. As the concentration increased, a reduction of membrane resistance and hyperpolarization occurred simultaneously. Further increase resulted in membrane depolarization with a further decrease in resistance. The threshold concentration of X537A was 100 times higher than that of A23187. These effects were reversible only when the application period was relatively short, while a longer application resulted in an incomplete reversibility or in no reversibility at all. The ionophore effect was facilitated in high Ca medium and diminished in low Ca medium. In Sr medium, the same effects on the resistance and the membrane potential were barely observable. TEA reduced the effects of A23187 but did not completely inhibit the effects. The Na-action potential was also reduced by the higher concentration of the ionophore. From these results it is concluded that the divalent cation ionophores, A23187 and X537A, carry divalent cation, Ca ions in a physiological medium, into the neuron soma through the membrane and the consequent increase of the intracellular divalent cations induces K conductance increase and that higher concentration of the ionophore induces the increase in the conductance of the other ion species, such as Na.

Several antibiotics isolated from some actinomycetes have been shown to allow the transport of inorganic cations across the aqueous lipid phase boundary and the artificial lipid bilayer and to induce conductance in the lipid bilayer (Reed & Lardy, 1972*a-b*; Pressman, 1973; Case, Vanderkooi & Scarpa 1974; Célis, Estrada-O & Montal, 1974). Some of them, such as X537A and A23187, transport divalent cations (Pfeiffer, Reed & Lardy, 1974) and are called "divalent cation ionophores". They have been applied to various kinds of biological systems (reviewed by Pressman, 1976). Their effects were well explained by inference that these ionophores transport Ca ions through the plasma membrane or release Ca ion from the intracellular organella: e.g., (i) increased muscle tone and induced contracture, (ii) facilitated the secretion of intracellular materials, (iii) caused Ca release from mitochondria and sarcoplasmic reticulum (SR) fragments.

The intracellular Ca ion concentration, which is usually regulated to be very

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low, influences the activity of the excitable neural membranes. An increase in the intracellular Ca ion concentration was found to cause the membrane unexcitability in the giant axon of *Loligo* (Tasaki, Watanabe & Takenaka, 1962; Tasaki, Watenabe & Lerman, 1967) and the depression of the Ca and the Na inward currents in the molluscan neurons (Kostyuk & Krishtal, 1977). A moderate increase in the intracellular Ca ions gives rise to an increase in the membrane conductance in molluscan neurons (Meech & Strumwasser, 1970; Meech, 1972, 1974*a-b*) and in the motoneuron (Krnjevic & Lisiewicz, 1972). Furthermore, it has been found that the Ca ion inflow following a membrane potential change induces a K-conductance increase which may trigger the falling phase of the action potentials (Meech, 1974*b*; Meech & Standen, 1974, 1975; Clusin, Spray & Bennett, 1975; Isenberg, 1975; Mounier & Vassort, 1975; Barrett & Barrett, 1976). After-hyperpolarization following spike activities may in part be induced by the Ca ions flowing into the neuron during the spike potential (Minota, 1974).

The present paper is aimed at investigating the effect of divalent cation ionophores, X537A and A23187, on the excitable neural membrane that generates Ca and Na dependent action potentials and the effect of the consequent increase in the divalent cation concentration inside the membrane on the electrical properties of the membrane. It was inferred that divalent cation ionophores increase the intracellular Ca ion concentration of the X-organ of the crayfish. Consequent increase in membrane conductance was clearly observed.

Materials and Methods

Neuron somata of the isolated X-organ of the crayfish, *Procambarus clarkii*, were impaled with a glass microelectrode filled with 3 M KCl for recording the membrane activities. Arrangements for stimulating the membrane and recording the electrical activity of the neuron were almost the same as those reported previously (Iwasaki & Satow, 1971). The X-organ was mounted in a Lucite trough filled with a 0.7-ml saline, and perfused with a TTX-Harreveld solution or a test solution which flowed at a speed of 1-1.3 ml per min.

Stimulating currents were passed through the bridge circuit with a series resistance of 200 MΩ and monitored by a resistor inserted in one of the arms of the bridge. Electrode resistance ranged 15-30 MΩ and the membrane resistance was as high as 100 MΩ in some cases, so that the rectangular current was distorted, inevitably, by the conductance change during the action potential elicited by it. To calculate membrane resistance, membrane potential changes by hyperpolarizing currents with 500 msec duration were divided by the initial value of the current. The first derivatives of the membrane potential change were obtained through a C-R coupling circuit with a time constant of 100 μsec.

Tetrodotoxin, TTX (Sankyo Co.), was used to obtain the Ca-action potentials. TTX-Harreveld solution is denominated as a standard solution in the present paper: 10^{-7}g/ml TTX, 205 mM Na, 13.5 mM $CaCl_2$, 5.4 mM KCl, 2.6 mM $MgCl_2$, N, N-dimethyl-formamid (DMF) 1%, buffered by $NaHCO_3$ to pH 7.1. In order to change the divalent cation concentration of the medium, equimolar Sr or Mn ions were substituted for the Ca ion of the Harreveld solution. The K concentration was elevated in such a way that the product of K and Cl ion concentration was constant in some experiments. Propionate ion was adopted for substituting the Cl ion. In some experiments, 10^{-5}g/ml picrotoxin was added to the so-

lution to sooth the membrane fluctuations that resulted from spontaneous synaptic bombardments.

Ionophores were dissolved in DMF and stored in a freezer as a stock solution and was used within two months. This was diluted by a TTX-Harreveld solution or the test solution to 1% of the final concentration of DMF on the day of the experiments. No noticeable change in the electrical activities of the neuron membrane was observed in this concentration of DMF. Antibiotic divalent cation ionophore A23187 was generous gift of Eli Lilly and Co. (Indianapolis) and X537A was given by Nippon Roche K.K. (Kamakura).

Results

The Effects of A23187 on the Membrane Activities in a Standard Solution

The neuron soma of the X-organ of the crayfish develops both Na-dependent and Ca-dependent action potentials (Iwasaki & Satow, 1971). Tetrodotoxin (TTX) eliminates the Na-dependent component of the action potential elicited by the depolarizing current passed through the membrane. Ca-dependent action potentials obtained in the presence of TTX have been shown to have every characteristic of the so-called Ca action potentials. In order to analyze the membrane activities, the resting potential, peak level, and maximum rate of rise of the Ca action potentials and resistance calculated from the hyperpolarizing potential divided by the applied current of 500 msec duration were registered with time.

The soma was perfused with TTX DMF solution previously. An application of a solution containing 0.02 μM of the antibiotic divalent cation ionophore, A23187, resulted in a decrease in the spike potential height and the max. rate of rise at the beginning without having any effect on the membrane resistance (Fig. 1). A second application of a higher dose of A23187 caused a further decrease of the amplitude and the max. rate of rise of the action potential and caused a decrease in the resitance afterwards. The membrane potential was kept constant at the control resting membrane potential throughout this and following experiments by passing current, unless otherwise mentioned, because the activation of Ca-action potentials is dependent upon the membrane potential level previously conditioned (Geduldig & Gruener, 1970; Iwasaki, Satow & Kuroda, 1973; Standen, 1974, 1975).

When the resting potential was not controlled, the membrane hyperpolarized to $-60 \sim -70$ mV (Fig. 2*A-B*). The hyperpolarization coincided with a decrease in the resistance in every case, and the threshold concentration for both was always higher than the decrease in the spike potential (Fig. 1).

Extremely high doses of A23187 depolarized the membrane, while a previous application of lower doses hyperpolarized it (Fig. 2**A**). The resistance vigorously decreased during the depolarization. In many cases, the effects of A23187 was reversible if the application time was less than 2–4 min, even with the higher dose (Fig. 2*B*). The recovery tendency, however, was often followed by a complete

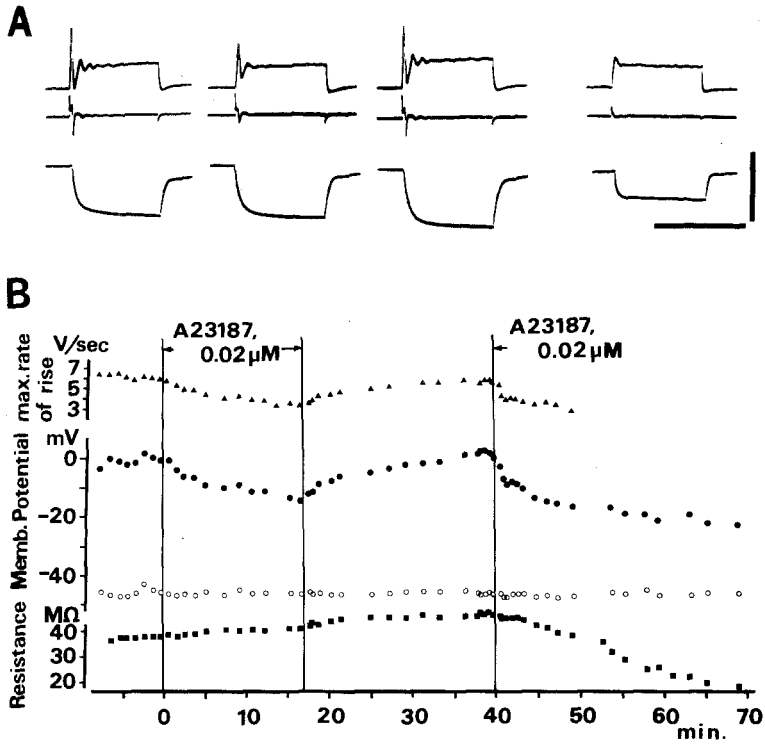

Fig. 1. The effects of A23187 on Ca-action potential, its max. rate of rise and membrane resistance. (*A*): Ca-action potential in TTX (10^{-7}g/ml) medium (upper), the first derivatives (middle), and membrane potential change by hyperpolarizing currents for 500 msec (lower). The experiments were performed in succession (from left to right): before, 15 min in A23187 (0.02 μM), 39 min in a standard solution, 69 min in A23187 (0.05 μM) after the first application of the ionophore. Calibration: 50 mV and 500 msec. (*B*): Time sequence of the effect of A23187 on the amplitude of the Ca-action potential (filled circles), its max. rate of rise (triangles), and membrane resistance (squares). Zerotime was taken at the first application of A23187 (0.02 μM). The membrane potential (open circles) was kept constant by passing current through the electrode

unexcitability of the membrane and by a further decrease in resistance when the application time was long, even with a low dose. Furthermore, successive application of the ionophore tended to facilitate the effects on the spike and resistance. This accumulative effect of the ionophore may be indicative of the intramembrane or the intracellular action of the ionophore which had moved into the cell during the previous application. The threshold concentration of A23187 to decrease the action potential and to decrease the resistance varied with neurons. For example, 0.02 μM was effective in one neuron to decrease the max. rate of rise of the Ca-action potential, while 0.5 μM was not enough to decrease it in another neuron. It appears that the cell posseses its own threshold concentration for an

ionophore. Accordingly, before examining the effect of ions or TEA, the threshold concentration of A23187 for that particular neuron in a TTX-Harreveld solution containing 13.5 mM Ca was determined for every neuron.

The Effects of X537A on Membrane Activities in a Standard Solution

X537A, also derived from *Streptomyces,* posseses the ability to transport cations including divalent cations. Although this ionophore combines with monovalent cations (Degani *et al.*, 1973; Cornelius, Gärtner & Haynes, 1974), similar effects to those of A23187 have been reported with regard to contraction, secre-

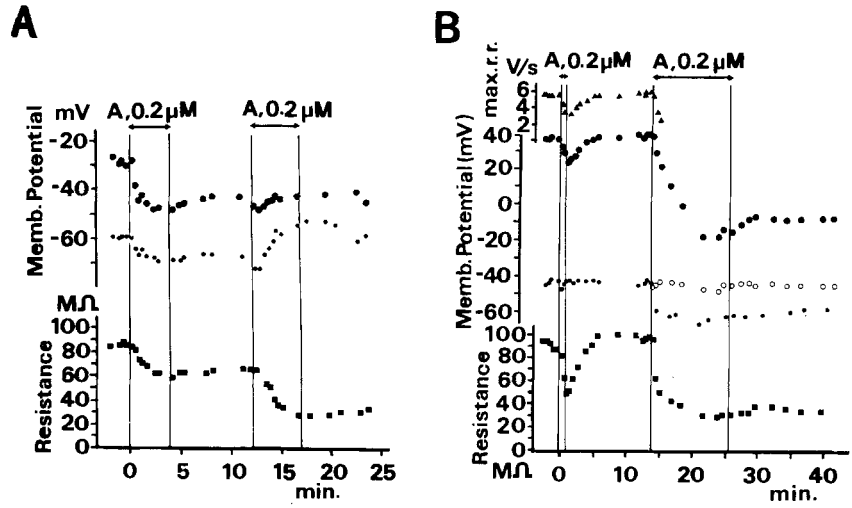

Fig. 2. (*A*): The effects of A23187 on the membrane potential. The symbols are the same as in Fig. 1, except the resting membrane potential (filled circles). The application of A23187 (0.3 μM) hyperpolarized the membrane by 10 mV with a simultaneous decrease of the spike potential and the membrane resistance. On the second application of A23187 (1.5 μM), the hyperpolarization (first two points after application) was immediately followed by depolarization, which recovered in the control solution. During depolarization, membrane resistance decreased further to 20% of the control. The solution contained picrotoxin (10^{-5}g/ml) throughout. (*B*) The effects of A23187 on the membrane potential and reversibility. The symbols used are the same as in Fig. 1. The resting membrane potential (filled circles) became hyperpolarized immediately after the second application, so that the membrane potential was kept at −45 mV by passing currents (open circles). The action potentials, max. rate of rise, and resistance were plotted with these controlled membrane potentials after the second application. The membrane hyperpolarized by 20 mV in this neuron after the application of A23187 for 12 min and gradually recovered in a control solution. The decrease in the Ca-action potential and resistance recovered almost completely after a 50-sec application, while it appeared to take a long time to recover after a 12-min application of the same concentration of A23187. The tendency to recover seems to be followed by an irreversible decrease in resistance and the spike potential. TEA (20 mM) and picrotoxin (10^{-5}g/ml) were present in the medium throughout this experiment

tion, Ca release and so forth, in a variety of cells. X537A showed essentially the same effects as A23187 on the X-organ neuron of the crayfish, i.e., a decrease in the amplitude and the max. rate of rise of the Ca-action potential and hyperpolarized the membrane with a reduction in membrane resistance. The threshold and the effective concentration for X537A was almost 100 times higher than that of A23187. Membrane depolarization was often observed following hyperpolarization which occurred at a time corresponding to resistance decrease as seen in Fig. 3. It differs from A23187 in that the threshold concentration for hyperpolarization by X537A was close to that for depolarization, so that, in some cases, a second application of X537A depolarized the membrane while the first one hyperpolarized it.

At a low concentration, a slight augmentation in the spike height and the max. rate of rise was often observed instead of a diminution, without any change in the resting potential and in the resistance. This augmentation had seldom been observed in the A23187 solution, except in the very early phase of the ionophore effect.

Divalent Cations and Ionophore Effects

Ca ion. Divalent cation ionophores have been reported to bind divalent cations and transport them across the lipid phase of an artificial or living cell membrane.

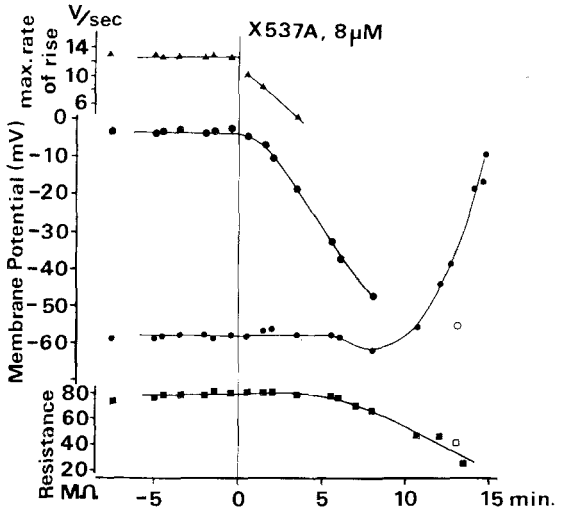

Fig. 3. The effect of X537A on the Ca-action potential, the membrane potential, and resistance. The symbols are the same as in Fig. 1. The amplitude of the Ca-action potential and its max. rate of rise decreased within 1 min after application of X537A (8 μM) without change in the membrane potential (filled circles) and membrane resistance until 5 min after application. Membrane hyperpolarization and the decrease of membrane resistance occurred coincidentally after 5 min. It was followed by an abrupt depolarization. The resistance at the membrane potential near the control level during hyperpolarizing current (open circle) was symbolized by the open square

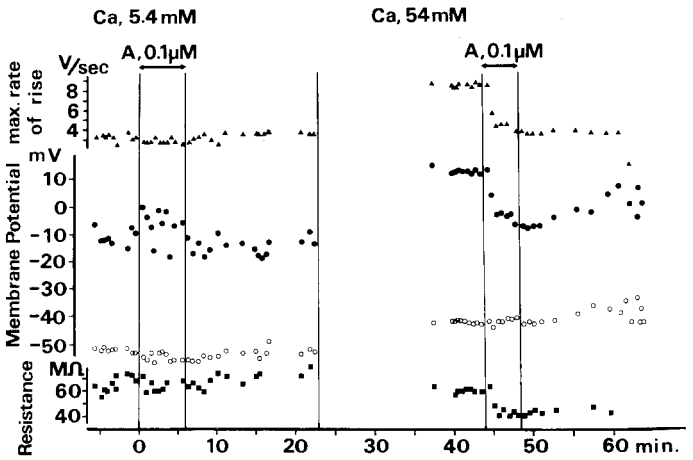

Fig. 4. The effects of A23187 and extracellular Ca concentration. The symbols are the same as in Fig. 1. Time zero was taken at the first application of A23187 (0.1 μM) in a low Ca medium (5.4 mM) in which no Ca-action potentials were elicited. The solution was switched to high Ca medium at 23 min. The effects of A23187 on membrane resistance in a low Ca medium was not clear, and the scattered potentials caused by the depolarizing currents might be due to the frequent appearance of spontaneous membrane fluctuations. In a high Ca medium, the ionophore brought about a noticeable decrease in the Ca-action potential and resistance

An increase in the Ca ion concentration of the medium should increase the amount of Ca^{++} ions transported and consequently increase the ionophore effect on the electrical activities of the membrane. In Fig. 4, the Ca concentration of the perfusing medium was increased from 5.4 to 54 mM. Although the inhibitory effect on the spike potential could not be evaluated because no action potentials were elicited in the low Ca medium, membrane resistance was maintained during and after application of the ionophore. The same concentration of the ionophore and shorter application time, but in a ten times higher Ca concentration, brought about strong inhibitory effects on the spike potential and membrane resistance. This tendency of the Ca ion concentration effect was always observed.

Sr ion. In many systems in which Ca ions play a role as a charge carrier, Sr ions can be used as a substitute for Ca ions (reviewed by Reuter, 1973). In the X-organ of the crayfish, action potentials were developed in a medium containing Sr ions instead of Ca ions. The amplitude of the Sr-action potential and the max. rate of rise are larger than that of the Ca-action potential in some cases. The application of the ionophore A23187 resulted in the inhibition of the Sr-spike and membrane resistance decreased with membrane hyperpolarization in the Sr medium with virtually no Ca ions (Fig. 5). This effect could not be due to the Ca ions remaining in the medium because almost the same effect was observed in the EGTA-Sr medium in which free Ca ions are expected to be reduced to one hundredth. In the Ca-free-Sr medium, free Ca ions were on the order of 10^{-5}M while in the EGTA-Sr medium free Ca ions were on the order of 10^{-7}M. Considering

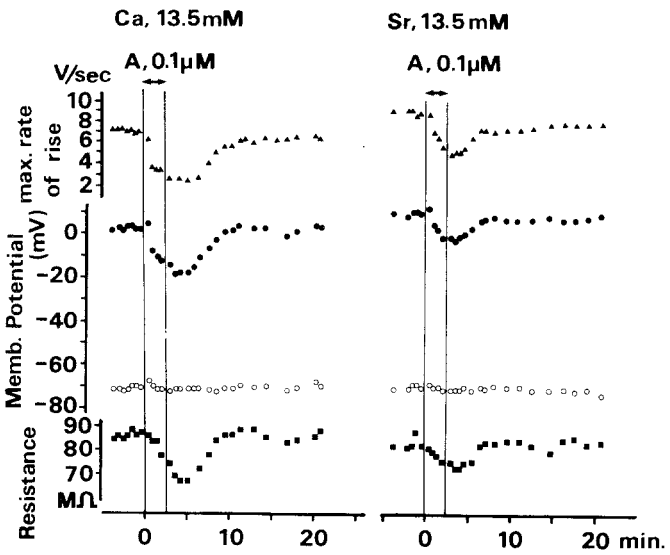

Fig. 5. The effects of A23187 on the Sr-action potential and membrane resistance. The effects of A23187 on the action potential and resistance in a Ca-free-Sr medium were similar to those in a TTX-Harreveld solution containing Ca ions. Time zero was taken at the respective applications of the ionophore. Symbols are the same as in Fig. 1

the accumulative effect and the incomplete recovery of A23187, it seems that the ionophore in the Sr medium was somewhat less effective than in the Ca medium.

Mn ion. The effects of the ionophore largely diminished with the addition of Mn ions to TTX-Harreveld saline as seen in Fig. 6. The first application of 0.3 μM A23187 for 3 min decreased the Ca-action potential as well as membrane resistance. During the second application of the ionophore in a solution containing 11 mM Mn, in which the Ca-action potential could not develop, the effect on membrane resistance was hardly observable. However, the resistance gradually reduced after the ionophore was removed and it did not recover. The same effect was also observed in a Ca-free Mn solution. Considering the much larger binding constant of the ionophore to Mn ion (Pfeiffer *et al.*, 1974), it is more likely that the most of the ionophore bound to Mn and transported it into the cell and a few Mn ions dissociated in the cell produced the slight resistance decrease. But the possibility that a trace of the Ca ions transported into the cell produced the resistance effect and that the Mn ions has little effect on the inside of the membrane still remains.

Mg ion. Subtraction of Mg ions from a TTX-Harreveld solution which contained 2.6 mM of the Mg ions did not bring about any change in the ionophore effect either on the spike potential or on resistance, so that it appears that the effects of the ionophore in a TTX-Harreveld solution cannot be due to the Mg ions transported by the ionophore. But this ineffectiveness of Mg ion may not mean that Mg ion is not transported or that it is transported but does not have any effect on the resistance change, because the binding or affinity of A23187 to Mg ion is weaker than it is to Ca (Reed & Lardy, 1972*a*).

TEA on the Effect of Ionophore

Tetraethylammonium (TEA), which is considered to block the K permeability increase, lengthened the action potential to induce a plateau in the X-organ neuron. It tended to reduce the inhibitory effects of the ionophore on membrane resistance (Fig. 7). The second and longer application of the ionophore in the TEA solution, however, brought about significant effects on both Ca-action potential and resistance, suggesting that either 20 mM of TEA was not sufficient to block the K-conductance mechanism or the effect of TEA was essentially incomplete. The reduction of the ionophore effect by TEA indicated that the K-conductance increase is involved in the resistance decrease caused by the ionophore. Alternatively, from the incomplete action of TEA, the possibility still remains that the decrease in the membrane resistance resulted, in part, from some mechanism other than the K-conductance increase induced by the increase of the intracellular Ca concentration.

The Effect of the Potassium Ion on the Resting Membrane Potential

The ionophores reduced the membrane resistance and simultaneously hyperpolarized the membrane. It strongly suggests the increase in the K conductance by the ionophore. The soma membrane of the X-organ of the crayfish is depolarized by elevating the external K concentration. The depolarization fits the Nernst equation well only when the K concentration was over 20 mM. Much less depolarization was observed when the K concentration was lower than 20 mM. Permeability mechanism for ion species other than K, possibly Na, may contribute to the membrane resting potential to some extent. In a solution containing the

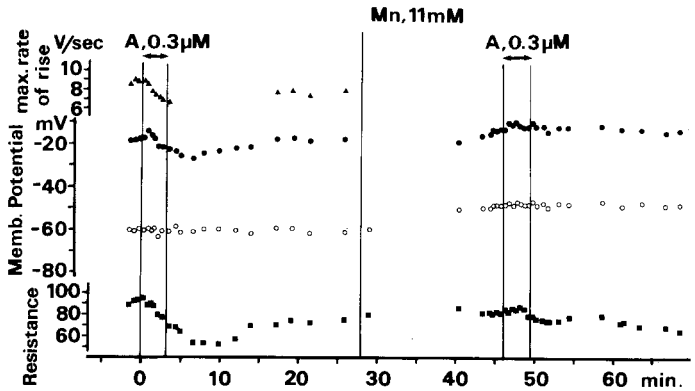

Fig. 6. The effects of A23187 and Mn ions. In a TTX-Harreveld solution, A23187 (0.3 μM) decreased the Ca-action potential and resistance. The addition of Mn ions (11 mM) to the TTX-Harreveld solution abolished the initiation of the Ca-action potentials. Membrane depolarized during the experiment for an unknown reason. The application of A23187 resulted in a faint decrease in resistance, although after subtraction of A23187 from the medium, a progressive decrease in resistance occurred

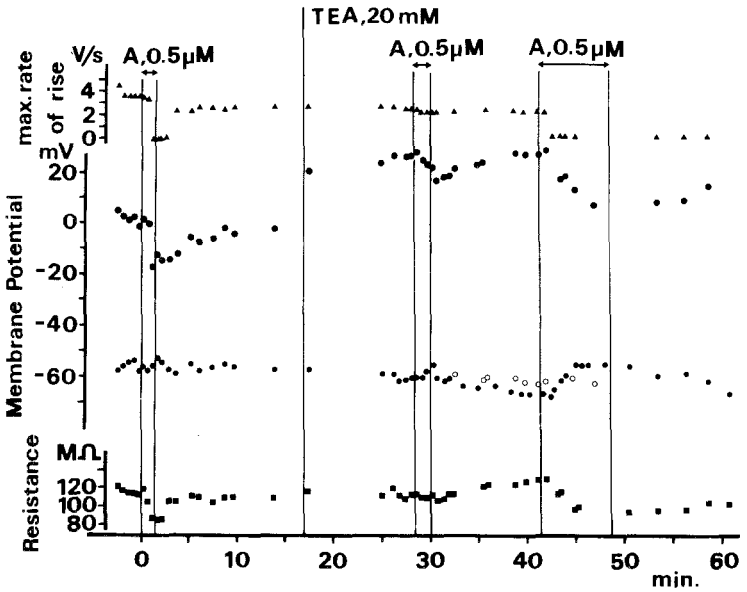

Fig. 7. The effects of A23187 in the TEA medium. TEA (20 mM) was added to a TTX-Harreveld solution at 17 min after the first application of A23187 (0.5 μM) for 1.5 min. In the TEA medium, a small reduction in the Ca-action potential was observable. However, second and longer application of the ionophore resulted in a decrease in the action potential and resistance. In the TEA medium, after the first application of A23187 the membrane potential gradually hyperpolarized. The action potential, max. rate of rise, and resistance were plotted only at the membrane potential level ranging from −57 to −63 mV. The membrane potentials, which are symbolized by the open circles, were kept to these levels by passing current

ionophore A23187, in which the membrane had hyperpolarized and resistance had decreased, elevation of K concentration of the medium induced greater depolarization than that in the control medium. In the control, a fourfold increase in the K concentration depolarized the membrane by 12 mV and an eightfold increase depolarized it by 23 mV on the average in a particular membrane. During the application of A23187 (0.5 μM) in which the membrane hyperpolarized by 8.5 mV, the same changes in the K concentration resulted in depolarization of 25 and 56 mV on the average, respectively. It is evident that the membrane behaves like more K electrode in the ionophore-containing solution than in the control one.

The Effects of Ionophore on the Na-Action Potential

In a normal Harreveld solution, the action potential of the X-organ of the crayfish consists of two components which are responsible for the permeability increase of Na and Ca ions, respectively (Iwasaki & Satow, 1971). Each component seems to be independent because the application of either TTX which blocks the Na component or Mn ions which block the Ca component leaves the other com-

ponent. The latter procedure shortened the action potential leaving its initial rising phase unchanged, suggesting that the initial phase of the action potential consists of a Na-component and the later one consists of a Ca-component (Yagi & Iwasaki, 1977).

The Ca-action potential is inhibited as the membrane potential becomes hyperpolarized, "hyperpolarizing inactivation" (Geduldig & Gruener, 1970; Iwasaki *et al.*, 1973; Iwasaki & Kuroda, 1974; Standen, 1974, 1975). In the TTX medium, the max. rate of rise of the Ca-action potential (filled circles) was plotted against the membrane potential at which the membrane was kept over 10 sec by passing current (Fig. 8). In a normal Harreveld solution in which the Na- and Ca-dependent action potentials were developed in the neuron, the max. rate of rise (open circles) and the half duration (triangles) of the action potentials were plotted (Fig. 8). The half duration was defined as the duration at the membrane potential level of 50% of the potential difference between the threshold and the peak of the action potential. The half duration became shorter at the membrane potential level at which the Ca-action potential had been inhibited. The change in the duration and the disappearance of the Ca-action potential were always coincidentally observed. In other words, there are two half durations in the action potential, one in the hyperpolarized membrane potential level and the other in the depolarized level. The one shifts to the other according to the membrane potential level at which the Ca-action potential disappears. The addition of Mn to a Harreveld solution decreased the half duration (Yagi & Iwasaki, 1977). These results provide the evidence that the early phase of the action potential composed of the Na and Ca components in the physiological solution corresponds to the Na

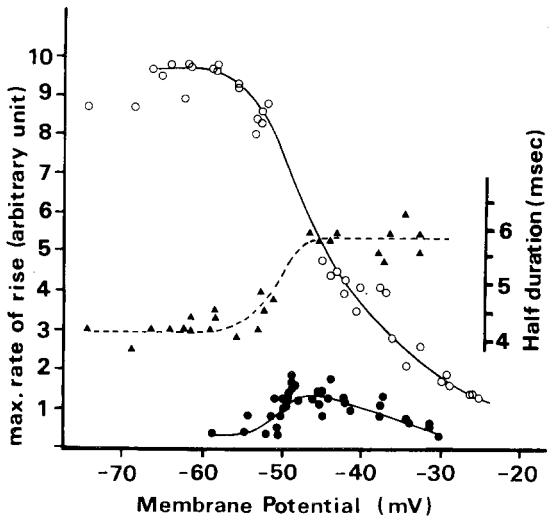

Fig. 8. The change of the duration of action potential with the membrane potential. The max. rate of rise (open circles) and the half duration (*see* text, triangles) in a normal Harreveld solution and the max. rate of rise of the action potential in the TTX medium (filled circles) were plotted against the membrane potential level conditioned for more than 10 sec. There seems to be two types of action potentials with different half durations

activation (Na spike) and the later phase corresponds to the Ca activation (Ca spike) and that the two components are activated independently.

The effect of A23187 on the action potential in a Harreveld solution appeared first in the duration of the action potential. The relationship between the half duration and the membrane potential was shown in Fig. 9, in a Harreveld solution (*a*) and after application of A23187 (*b*). The relation (*b*) was taken 18 min after application of the ionophore (0.2 μM) for 3 min. The change in the duration is exactly the same as that obtained with a solution containing Mn ions (Yagi & Iwasaki, 1977). The second and larger concentration of A23187 resulted in a further decrease in the half duration along the membrane potential axis (*c*) and the diminution of action potentials and the max. rate of rise (not illustrated), suggesting the inhibition of Na-action potential. In the Mn solution, X537A also reduced the action potential and membrane potential as well. It could be inferred from these facts that the divalent cation ionophores affect primarily the Ca component and later on the Na component. However, it is not clear whether the effect on the Na-permeability system is a direct one or an indirect one through the increased K permeability, because the decrease in membrane resistance always occurred when the Na-action potential was reduced by the ionophore.

Discussion

The present experiment disclosed the action of the divalent cation ionophore on the neural membrane of the crayfish. In the soma membrane of the X-organ of the crayfish, A23187 and X537A reduced the amplitude of the Ca-action potential and its max. rate of rise at the beginning and hyperpolarized the membrane and decreased membrane resistance afterwards. The hyperpolarization occurred in parallel with a reduction in membrane resistance. When the ionophore concentration was low, inhibition in the Ca-action potential took place without effect on the resting potential and the membrane resistance. These results lead us to conclude that the divalent cation ionophore carries Ca ions into the neuron to increase intracellular Ca ion concentration close to the membrane with a consequent reduction in the ratio of $[Ca^{++}]_o$ to $[Ca^{++}]_i$, which results in a reduction of the amplitude and the max. rate of rise of the Ca-action potentials, and that increased intracellular Ca concentration, in turn, brings about the increase in the K conductance as proposed in molluscan neurons (Meech & Strumwasser, 1970; Meech, 1972, 1974*a-b*; Meech & Standen, 1974, 1975) and in cat motoneurons (Krnjević & Lisiewicz, 1972). It could be considered that the divalent cation concentration inside the cell is harmful to the initiation of action potentials, as is the case in giant axons (Tasaki *et al.*, 1962, 1967) and molluscan neurons (Kostyuk & Krishtal., 1977). This would be an alternative explanation for the reduction of Ca-action potential by the ionophore. Ca-induced outward currents have been reported in crab muscle fibers (Mounier & Vassort, 1975), frog motoneurons (Barrett & Barrett, 1976), and Helix neurons (Meech & Standen, 1975). The duration of the action potential changes with the intracellular Ca ion concentration (Meech, 1974*c*; Isenberg, 1975), suggesting that the K-conductance increase was provoked by the increased Ca concentration during the action potential. The decrease in resistance and the hyperpolarization caused by the divalent cation iono-

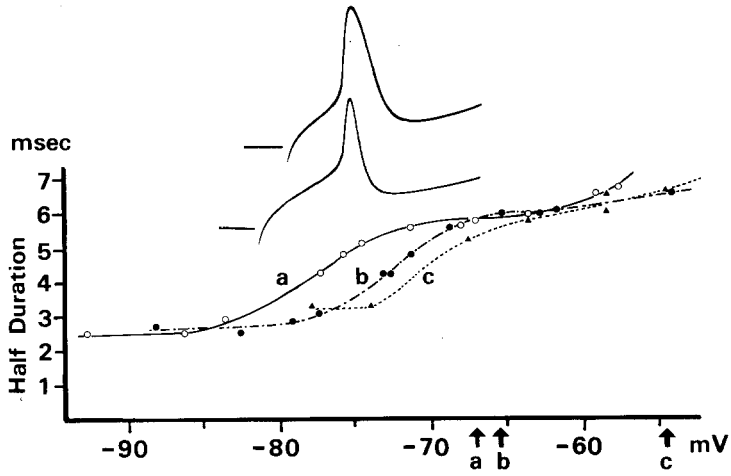

Fig. 9. The effect of A23187 on the Na-action potential. The half duration was plotted against the membrane potential as in Fig. 8, in a normal Harreveld solution (*a*, open circles), 18 min after application of A23187 (0.2 μM) for 3 min (*b*, filled circles), and 4 min in the A23187 (0.3 μM) (*c*, triangles). The experiments were performed consecutively. *Inset:* The action potentials in a Harreveld solution before (upper) and after A23187 (lower) at the membrane potential −77 mV were chosen from *a* and *b*, respectively. The reduction of the half duration was noticeable with little change in the max. rate of rise. The arrows in the membrane potential axis represent the resting potentials during the respective experiments

phore in the X-organ neuron soma is an indication of divalent cation-induced K conductance, since the membrane responded as a K electrode only when in the hyperpolarized condition by the ionophore.

In the concentration of the ionophores which caused the pronounced effects on the Ca-action potential and the membrane resistance, hyperpolarization always occurred. In the same preparation extremely high concentration of A23187 or a little higher concentration of X537A caused the depolarization with the great decrease of the membrane resistance and sometimes irreversible changes of the cell. The depolarizing effect of X537A has also been reported on the muscle membrane (Devore & Nastuk, 1975; Cochrane & Douglas, 1975). The threshold concentration of divalent cation ionophore for hyperpolarization used in the present experiment was much lower than that used in their experiment. For example, 0.02 μM of A23187 or 2 μM of X537A was effective in the present experiment. The concentration of X537A for depolarization was a little higher than that for hyperpolarization, while it was much higher in the case of A23187 as seen in Figs. 2 and 3. Thus it is considered that the primary effect of the ionophore on the membrane potential is the hyperpolarization, and resistance decrease resulted from the increase in intracellular Ca concentration. The depolarization with the great decrease of membrane resistance caused by the higher doses of A23187 and X537A could be due to the permeability increase for ion species other than K. Devore & Nastuk supposed that the Na-conductance channel was provided by

X537A. Therefore, it is likely that the effect of X537A is attributable to the depolarization which induces secondary effects on the cell: contraction, secretion, etc.. In this connection, a reconsideration of the action of the divalent cation ionophores, especially the action of X537A, must be useful. In some secretory machinery, A23187 has been found to be much less effective than X537A or to have no effect at all (Schwartz *et al.*, 1974; Nakazato & Douglas, 1974; Cochrane *et al.*, 1975; Kita & Van der Kloot, 1976; Thoa *et al.*, 1974). Some of these secretory effects might be due to the depolarizing effect of X537A rather than the effect of the divalent cations carried by the ionophore.

The effect of A23187 on the membrane resistance still remained after substitution of Sr or Mn for Ca in the TTX-Harreveld solution, indicating the potency of Sr and Mn for membrane resistance. The rank order of the action of A23187 on the decrease in the membrane resistance in solutions with different cation was Ca > Sr >> Mn in the X-organ neuron soma. It has been reported that the binding affinity sequence in aqueous media has the order; Mn > Ca > Sr (Reed & Lardy, 1972*a*; Pfeiffer *et al.*, 1974). From the inconsistent order in the divalent cations, it is deduced that the resistance decrease caused by A23187 is not due to the introduction of the A23187-divalent cation complex, but to the introduction of free divalent cations into the cell, because strong binding affinity may result in difficulty in dissociation of divalent cations after the complex entered the cell. From this point of view, the faint effect of the Mn ions on membrane resistance might not be due to the weak potency of Mn ions for the resistance mechanism, but to the slow dissociation of Mn-ionophore complex in the cell.

In a solution containing TEA, the resistance decreased gradually if the ionophore was applied repeatedly (Fig. 7). This may suggest the incomplete effect of TEA on the K-conductance mechanism. Alternatively, it may be possible that a part of the K conductance induced by Ca ions transported by the ionophore is not sensitive to TEA; otherwise, there exists another conductance mechanism than K. Two types of K conductance were reported, one of which was not blocked by TEA (Barrett & Barrett, 1976; Krnjević *et al.*, 1976).

When the ionophore was applied for a longer period, recovery in the spike potential and resistance was incomplete, and sometimes a trend towards recovery was followed by a further decrease in the spike and resistance in the control solution (Figs. 2*B* and 6). The release of Ca ions from the intracellular organella, such as mitochondria and endoplasmic reticulum (Otsuki, 1969; Carafoli & Lehninger, 1971), may be responsible for the long-term effects of the ionophore. The effect of the ionophore in the Ca-free medium have been reported regarding egg activation and secretion, and the release of Ca ion from the intracellular organella has been suggested (Schroeder & Strickland, 1974; Steinhardt & Epel, 1974; Steinhardt *et al.*, 1974; Nordman & Currell, 1975; Feinman & Detwiler, 1974; White, Rao & Gerrard, 1974).

In summary, the divalent cation ionophore, A23187 and X537A, decreased the Ca-action potential of the X-organ neuron soma at the outset and its membrane resistance afterwards. The membrane hyperpolarization occurred in parallel with the decrease in the membrane resistance. These results can be explained by the transportation of divalent cations by these ionophores through the neuron mem-

brane and consequent increase of the divalent cation concentration just inside the cell. With higher doses of the ionophore, depolarization took place. These ionophores are considered to be useful probes to alter the divalent cation distribution across cell membranes and to study the function of the divalent cations inside the cells, so far as moderate concentrations are used.

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