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Ca-K Bi-ionic Action Potential in Squid Giant Axons

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Summary. Under intracellular perfusion with a solution containing K^+ as the sole cation species, squid giant axons were found to be capable of developing all-or-none action potentials when immersed in a medium in which $CaCl_2$ was the only electrolyte. The adequate range of ion concentration for demonstrating this capability was mentioned. The reversal potential level measured by the voltage-clamp technique varied directly with the logarithm of the concentration of extracellular Ca-ion; the proportionality constant was close to $RT/2F$. The action potential observed under this Ca-K bi-ionic condition could not be suppressed by addition of tetrodotoxin or saxitoxin to the external medium. The external Ca-ion could be replaced with Co- or Mn-ion without eliminating the capability of the axons to develop action potentials. D-600 could not suppress the inward current observed under the voltage-clamp condition, but 4-aminopyridine could suppress it. The experimental findings were interpreted based on the current channel hypothesis and on the macromolecular theory.

Key words: bi-ionic action potential, Ca-dependent action potential, K-channel, squid axon, 4-aminopyridine

It was found that squid giant axons internally perfused with a solution containing salts of Na, Cs, or tetraethylammonium (TEA) ions remain excitable when immersed in a medium containing only the salt of Ca-ions (Tasaki, Watanabe & Singer, 1966*b*; Tasaki, Watanabe & Lerman, 1967; Watanabe, Tasaki & Lerman, 1967*a*; Tasaki, Takenaka & Yamagishi, 1968). In such a Ca-solution, all-or-none action potentials have been demonstrated with a variety of intracellular univalent cations but with the notable exception of K- and Rb-ions (Tasaki, Lerman & Watanabe, 1969). Prior to this finding, action potentials in the barnacle muscle fiber were found to be highly dependent on the Ca^{2+} concentration in external media (Hagiwara & Naka, 1964). In this case, the presence of intracellular K-salts did not disturb generation of the Ca-dependent action potentials. In unperfused squid axons (whose axoplasm contains about 400 mM K-salts), an increase in influx of Ca-ions through the membrane during membrane depolarization was shown by the use of radioactive calcium (Hodgkin & Keynes, 1957) or by the technique of aequorin injection (Baker, Hodgkin & Ridgway, 1971). Under the light of these experimental findings, it was not easy to explain why electrical excitability of the squid axon could not be demonstrated with Ca-ions externally and K-ions internally.

Katz and Miledi (1969) found that intracellular injection of TEA renders the synaptic membrane of the squid capable of generating the Ca-dependent action potential. This observation together with an early finding (Fatt & Ginsborg, 1958) led them to the hypothesis that blockage of the K-channel was necessary for generating the Ca-dependent action potential. This hypothesis became widely accepted, as a similar method – the application of TEA – was found to be useful in demonstrating Ca-dependent action potentials in various excitable tissues (Washio, 1972; Kleinhaus & Prichard, 1975; Beaty & Stefani, 1976; Stefani & Uchitel, 1976; Fukuda, Furuyama & Kawa, 1977; Horn, 1977; Horn & Miller, 1977; Ross & Stuart, 1978; Sanchez & Stefani, 1978). This hypothesis gave a reasonable explanation for the difficulty of demonstrating electrical excitability of squid axon with Ca-ions externally and K-ions internally.

The present paper describes a series of experiments demonstrating all-or-none action potentials in squid giant axons which are intracellularly perfused with

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a K-salt solution and immersed in a medium containing only Ca-salt. Electrophysiological properties of the axon membrane under such Ca-K bi-ionic conditions are described in detail. Furthermore, the reason why previous investigators failed in demonstrating "Ca-K bi-ionic action potentials" is clarified. It is evident that the hypothesis developed by Katz and Miledi (1969) is not capable of explaining the properties of the squid axon membrane. The abstract was published elsewhere (Terakawa, 1978).

Materials and Methods

Giant axons were obtained from *Loligo pealei* available at the Marine Biological Laboratory, Woods Hole, Mass. The diameter of the axons used was 350–700 μm. Following excision, axons were mounted in a Lucite chamber containing natural sea water and were perfused intracellularly using the double cannulation method (Tasaki, 1968). At the onset of internal perfusion, a 400 mM KF solution containing 0.05 mg/ml Protease VII (Sigma) was used for a period of about 1 min to remove axoplasm from the interior of the axon. Then, the proteolytic enzyme was washed out by continuous internal perfusion with an enzyme-free solution. The perfusion zone was 16–20 mm in length.

The external solutions were prepared by diluting a 400 mM $CaCl_2$ solution with a 12% (vol/vol) glycerol solution to a level between 0.2 and 100 mM. The pH of the external solution was adjusted to 8.1 ± 0.2 by using 0.2–1 mM Tris-HCl. In some experiments a known quantity of a concentrated solution of $CoCl_2$, tetrodotoxin (TTX), or 4-aminopyridine was added to the $CaCl_2$ solution with micropipettes (Finnpipette). In some cases, a known quantity of D-600 powder was added to the $CaCl_2$ solution. The internal solution was prepared by diluting a 9:1 mixture of 600 mM KF solution and 600 mEq/liter K-phosphate buffer with a 12% (vol/vol) glycerol solution to a level of 10–400 mM in the K^+ concentration. The pH of the K-phosphate buffer was 7.3 ± 0.1. In Table 1 the compositions of most of the solutions used are shown.

A piece of enameled platinum wire (100 μm in diameter) with a 20-mm long uninsulated and platinized portion at the end was used as the current electrode for supplying stimulation or voltage-clamp currents. Large coils of platinized platinum wire immersed in the external medium served as the ground electrode with a guard system with which a spatially uniform current distribution was attained. The length of the current measuring electrode was 4.5 mm, and that of the guard electrodes was 5.0 mm. These electrodes were separated from each other at a distance of 0.5 mm. The internal potential-recording electrode was made with a glass pipette (50 μm in inside diameter) filled with 0.6 M KCl-agar connected to chloridized silver wire. A Hg-HgCl half-cell placed in the external medium was used as the reference electrode for potential measurements. Both the potential-recording and the current-supplying electrodes were introduced into the axon interior through the outlet cannula.

The change in membrane impedance during electrical responses was measured by a bridge method similar to that used by Tasaki, Singer and Watanabe (1966*a*). In this case, a piece of platinum wire (100 μm in diameter) having a 1 mm-long platinized portion near the tip was used as the recording electrode. Alternating current of 3 kHz in frequency and of 5 mV in peak-peak amplitude was applied to the membrane. In voltage-clamp experiments, the circuitry described by Moore (1971) was used. The series resistance was not compensated since the study was directed mainly to the reversal potential. All observations were performed at room temperature (22–24 °C).

Table 1. Composition of solutions

External solutions Name	$CaCl_2$ (mM)	NaCl (mM)	Tris-HCl (mM)	Glycerol (%)[a]
470 Na	100	470	1	0
100 Ca	100	0	1	9.4
20 Ca	20	0	1	11.4
10 Ca	10	0	1	11.7
5 Ca	5	0	0.5	11.9
2 Ca	2	0	0.5	12.0
0.5 Ca	0.5	0	0.2	12.0

Internal solutions Name	KF (mM)	K-phosphate (mEq/liter)	Glycerol (%)[a]
400 K	360	40	4.0
100 K	90	10	10.0
50 K	45	5	11.0
25 K	22	3	11.5
10 K	9	1	11.8

[a] Expressed in volume percent.

Results

Generation of Ca-K Bi-ionic Action Potential

When an axon was intracellularly perfused with a solution containing 50 mM K^+ (solution 50 K in Table 1) and bathed in a solution containing 470 mM NaCl and 100 mM $CaCl_2$ (solution 470 Na in Table 1), electrical stimulation to the axon produced all-or-none action potentials of about 130 mV in amplitude (*see* Fig. 1–1). The action potential of such an axon was suppressed when sodium salt in the external solution was replaced with glycerol (Fig. 1–2). However, when $CaCl_2$ concentration in the bathing solution was reduced to a level less than 20 mM, the axon regained the ability to produce action potentials in response to electrical stimulation as seen in Fig. 1–3. Reduction of the external $CaCl_2$ concentration from 100 to 2 mM in the course of 2–4 min induced this ability most reproducibly. Among 60 axons examined, 53 axons were found to regain excitability by this procedure.

The action potential obtained from the axon internally perfused with a solution containing K^+ and bathed in a solution containing Ca^{2+} as the sole cation species (Ca-K bi-ionic action potential) had the following properties. The amplitude of the action potential was approximately 25 mV or slightly less, and the duration was between 50 and 300 msec (40 axons). The action potential behaved almost in an all-or-none manner when the intensity of the stimulating current pulse was increased by equal steps (Fig. 2–1).

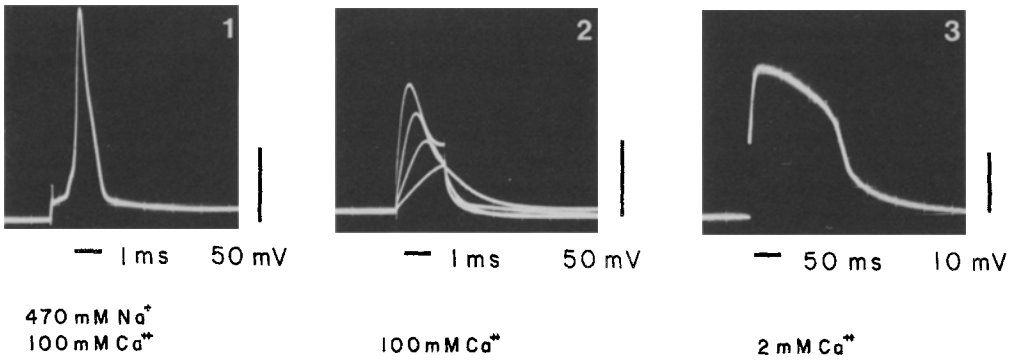

Fig. 1. Oscillograph records demonstrating bi-ionic action potential of an axon immersed in 2 mM $CaCl_2$ solution. The axon interior was perfused continuously with a solution containing 50 mM K^+. Record (1) was obtained when the axon was immersed in a 470 mM NaCl and 100 mM $CaCl_2$ solution. Stimulus duration was 0.06 msec. The resting potential was −54 mV. Record (2) was obtained shortly after removal of NaCl from the external medium. Four responses to electrical stimuli of different strengths were superposed, showing a lack of the all-or-none property. Stimulus duration was 1.9 msec. The resting potential was −50 mV. Record (3) was obtained 14 min after replacement of the 100 mM $CaCl_2$ solution with a 2 mM $CaCl_2$ solution. Stimulus duration was 1.9 msec. The resting potential was −53 mV

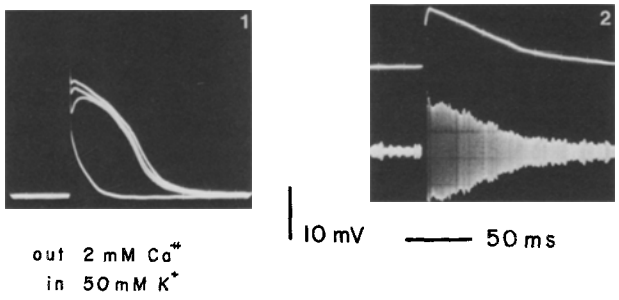

Fig. 2. All-or-none property and membrane impedance reduction. The axon was immersed in a 2 mM $CaCl_2$ solution and internally perfused with a solution containing 50 mM K^+. (1) Four responses to stimuli of different strengths were superposed, showing the all-or-none property of the electrical response. Stimulus duration was 2 msec. The resting potential was −58 mV. (2) The upper trace represents the potential variation and the lower trace represents the impedance variation recorded simultaneously. The impedance bridge was balanced initially for the resting state. Stimulus duration was 2 msec

Fig. 2–2 demonstrates that the action potential is associated with a reduction of membrane impedance. The impedance bridge used to obtain this result was balanced before stimulation. (The membrane resistance measured by this method was about 15 KΩ cm^2.) When an electrical stimulus was applied to the membrane, the bridge became unbalanced indicating that there was a change in the membrane impedance. By balancing the impedance bridge at the peak of action potential, this change was shown to represent a fall of the membrane impedance. The time course of the impedance change was quite similar to that of the potential variation.

At a given Ca^{2+} concentration, there was a gradual reduction in the action potential amplitude associated with a prolongation of the duration. These changes were accompanied by a decrease in resting potential, which shifted from about −55 mV to about −35 mV during a period of 20–40 min. In a medium containing 2 mM $CaCl_2$, axons were able to produce action potentials for up to 40 min, although in most axons, the ability to produce action potentials was lost in 20–30 min. Even after complete disappearance of the electrical activity, many axons regained the large resting potential (about −55 mV) and ability to produce action potentials when the Ca^{2+} concentration was once raised to around 100 mM and then reduced back to around 2 mM. This procedure could be repeated 3 or 4 times.

A reduction of the external Ca^{2+} concentration to 0.5 mM or less induced spontaneous repetitive firing of action potentials. The amplitude of these action potentials became progressively smaller and the interval between them became gradually shorter as illustrated in Figs. 3-1, 3-2, 3-3 and 3–4. The repetitive firing usually lasted for about 10 min, and afterwards the amplitude usually became very small. However, after the spontaneous action potentials disappeared, they could be restored by replacing the external solution with a $CaCl_2$ solution of a high concentration (higher than 20 mM) and then with a 0.5 mM (or lower) $CaCl_2$ solution (Fig. 3–5).

The results described above may be summarized as follows. The axon membrane stayed in its resting state when measured in a 100 mM $CaCl_2$ solution. A reduction of $CaCl_2$ concentration from 100 mM to a level below 20 mM induced the ability to produce action potential occasionally. A reduction of $CaCl_2$ concentration from 100 mM to 2 mM invariably restored the axon excitability. The membrane did not

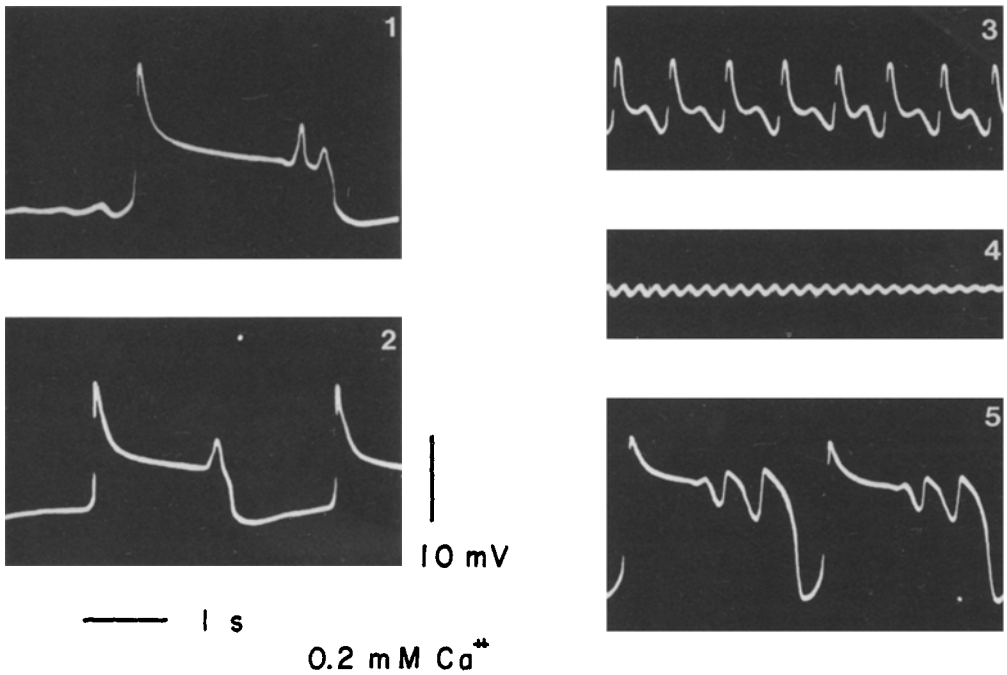

Fig. 3. Spontaneous firing of Ca-K bi-ionic action potentials induced by immersion of the axon in a low Ca^{2+} solution. The internal solution contained 50 mM K^+. Records (1), (2), (3) and (4) were obtained in 7, 8, 10 and 11 min, respectively, after replacement of an external 20 mM $CaCl_2$ solution with a 0.2 mM $CaCl_2$ solution. In record (1), note the subthreshold oscillation before the generation of full-sized action potentials. After taking record (4), the axon was treated with a 20-mM $CaCl_2$ solution for 15 min. Record (5) was obtained 5 min after replacement of the 20-mM $CaCl_2$ solution with a 0.2-mM $CaCl_2$ solution. The resting potential was about −53 mV in Record (1), −51 mV in (2), −46 mV in (3), −39 mV in (4), and −58 mV in (5)

Table 2. Resting membrane potential measured at various concentrations of external Ca^{2+} and Na^+ [a]

External solution [b]	470 Na	100 Ca	20 Ca	2 Ca
Mean value (mV)	−45.2	−45.7	−45.0	−47.8
Standard deviation (mV)	7.9	8.1	7.8	10.6
Standard error of the mean (mV)	1.8	1.8	1.7	2.3

[a] Data obtained from 22 axons.
[b] Names of solutions in Table 1 were used.

stay in the resting state for a long time in $CaCl_2$ solution of the concentration lower than 20 mM.

It was possible to demonstrate Ca-K bi-ionic action potentials at a variety of internal K-ion concentrations. With a 10, 25 or 100 mM K-salt solution (10 K-100 K in Table 1) used internally in conjunction with a 2 mM $CaCl_2$ solution externally, action potentials of about 20 mV in amplitude were obtained following electrical stimulation of the axon. However, in these cases, the ability of the axon to produce action potential was lost within a relatively short time. With a 400 mM K-salt solution (400 K in Table 1) inside, it was not possible to obtain any distinct action potential from an axon bathed in solutions whose Ca^{2+} concentrations were varied over a wide range.

Bi-ionic action potentials could also be obtained when the external medium contained $CaCl_2$ only and the internal medium contained Rb-salt as the sole electrolyte species. (Fluoride and phosphate were used as internal anions.) There was no significant difference in the behavior of the axon between 50 mM K^+ and 50 mM Rb^+ when used internally.

After demonstrating the bi-ionic action potentials in an axon, ordinary action potentials such as those shown in Fig. 1-1 could be obtained from the same axon by switching the external medium to a mixture of NaCl and $CaCl_2$ (solution 470 Na in Table 1).

The Resting Potential under Ca-K Bi-ionic Conditions

The resting potential was measured in axons immersed in solutions containing various concentrations of Ca^{2+} and perfused intracellularly with a solution containing 50 mM K^+. When the concentration of external Ca^{2+} was varied, the membrane potential varied transiently and then reached a steady level. Values of the membrane potential at this level were treated statistically and is shown in Table 2. When averaged, the membrane potential seemed to be rather constant at about −45 mV irrespective of external media. The standard deviation of the data was large at a Ca^{2+} concentration of 2 mM. A factor relating to this was

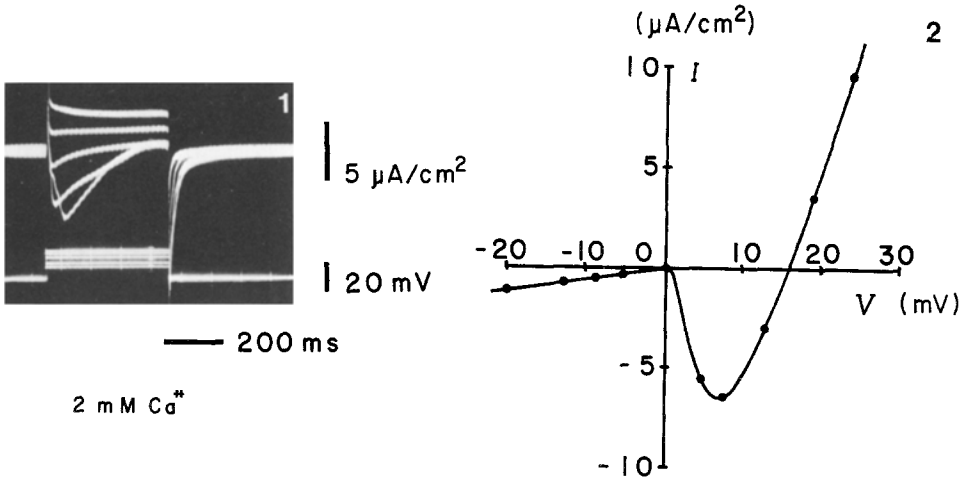

Fig. 4. Current-voltage relationship obtained from an axon immersed in a solution containing 2 mM $CaCl_2$. The internal solution contained 50 mM K^+. (1) The upper trace shows time course of the membrane currents which were required to produce rectangular membrane potential changes shown by the lower trace. (2) Relationship between the peak current (I) and the clamping voltage (V); the inward current is expressed as negative, $V=0$ corresponds to the holding potential level which is equal to the resting potential level before the onset of the voltage clamp (−60 mV)

an inaccuracy of the potential measurement at such a low ionic strength. Another factor derived from membrane properties. Some axons showed small resting potentials (−30 mV). These axons did not produce an action potential, but hyperpolarizing responses. Generally, in a 2 mM Ca-solution, axons which showed a large resting potential (about −55 mV) produced large action potentials.

Membrane Currents Observed under Voltage-Clamp

The membrane current was examined by the voltage-clamp method. When the external Ca^{2+} concentration was higher than 20 mM, the transient increase in inward current could not be demonstrated upon application of a positive voltage pulse. In such axons, plot of the observed current against the voltage of the clamping pulse produced a smooth, monotonically increasing curve. When the external Ca^{2+} concentration was reduced to a level below 20 mM, application of a positive voltage pulse induced a transient increase in inward current, reflecting the ability of the axon to produce action potentials at a low Ca^{2+} concentration. One example of the records of such inward currents obtained at the external Ca^{2+} concentration of 2 mM is shown in Fig. 4-1. The intensity of the inward current reached its peak 10–50 msec after the onset of the pulse. It decreased to the level observed before the application of the pulse at about 200 msec after the onset of the pulse. A plot of the peak current against the voltage of clamping pulse revealed an N-shaped I–V relationship (Fig. 4–2). This curve consisted of three portions with different slopes: a portion with a positive slope (about 0.1 mS cm^{-2}) on the left-hand side of the Figure, a portion with a steep positive slope (about 1 mS cm^{-2}) on the right-hand side, and a portion with a negative slope in the middle. The existence of two positive slopes clearly indicates that the membrane has two different conductance states.

The clamping voltage at which the inward current vanishes (reversal potential) represents the electromotive force (emf) of the membrane system at the peak of excitation. In order to examine the dependence of the emf on the Ca^{2+} concentration of the external solution, the reversal potential was measured at various Ca^{2+} concentrations. Fig. 5 shows the results obtained from six axons. The level of the reversal potential was roughly proportional to the logarithm of the external Ca^{2+} concentration with a proportionality constant $RT/2F$, where R is the gas constant, F the Faraday constant and T the absolute temperature.

Effect of TTX Added to the External Medium

Tetrodotoxin (TTX) is believed to be a Na-channel blocker (Narahashi, Moore & Scott, 1964; Nakamura, Nakajima & Grundfest, 1965). Previously, bi-ionic action potentials obtained with Ca^{2+} externally and Cs^+ internally were found to be completely suppressed by this toxin added to the external medium at the concentration of 130 nM (Watanabe, Tasaki & Singer, 1967*b*). This finding was confirmed by using the voltage-clamp technique also (Meves & Vogel, 1973). In the present study, the effect of TTX was examined in the following manner. Axons were internally perfused with a solution containing 50 mM K-salt and immersed in a solution containing 470 mM

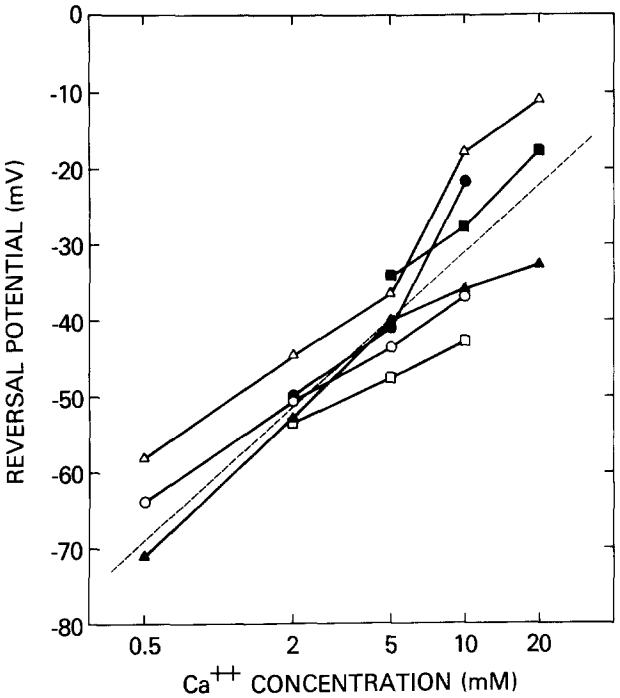

Fig. 5. The relationship between the reversal potential and the Ca^{2+} concentration of the external medium. The solutions 100 K–0.5 K in Table 1 were used. The reversal potential was measured on the leakage-corrected *I–V* curve. Different symbols represent data obtained from different axons. The slope of the dashed line is 29 mV per a decade change in the Ca^{2+} concentration. Abscissa is expressed in logarithmic scale

NaCl and 100 mM $CaCl_2$. On these axons, all-or-none action potentials of about 130 mV in amplitude were observed (Fig. 6-1). Addition of TTX to the external medium at the concentration of 150 nM eliminated these action potentials (Fig. 6-2). Next, with the TTX concentration maintained at a constant level, the external medium was replaced with a Na-free solution containing 2 mM $CaCl_2$. In response to stimulating current pulses, typical Ca-K bi-ionic action potentials were observed in the presence of TTX (Fig. 6-3). TTX did not suppress the Ca-K bi-ionic action potentials even at a concentration of 2 μM (for more than 20 min). In addition, saxitoxin, at a concentration of 170 nM, was shown not to have any effect on the Ca-K bi-ionic action potential. The effect of TTX added to the external medium was examined by the voltage-clamp method also. TTX had no effect on the maximum inward current observed under the Ca-K bi-ionic conditions. It was observed that addition of NaCl to the external $CaCl_2$ solution enhanced the maximum inward current greatly. TTX used at the concentration of 100 nM eliminated this enhancing effect of NaCl completely.

Effect of D-600 Added to the External Medium

D-600, a methoxyderivative of verapamil, is regarded as a selective blocker of the Ca-channel (Kohlhardt, Bauer, Krause & Fleckenstein, 1972). The effect of this chemical on excitability of squid axons under the Ca-K bi-ionic conditions was examined by the voltage-clamp method. The axons used were immersed in 2 mM $CaCl_2$ solution and perfused intracellularly with a solution containing 50 mM K^+. Addition of D-600 to the external solution at the concentration of 0.1 mM affected neither the inward current nor the reversal potential of these axons. The effect of D-600 was very small even at 1 mM (*see* Fig. 7); this concentration is about 1000 times as high as that employed by Kohlhardt et al. (1972) for the heart muscle.

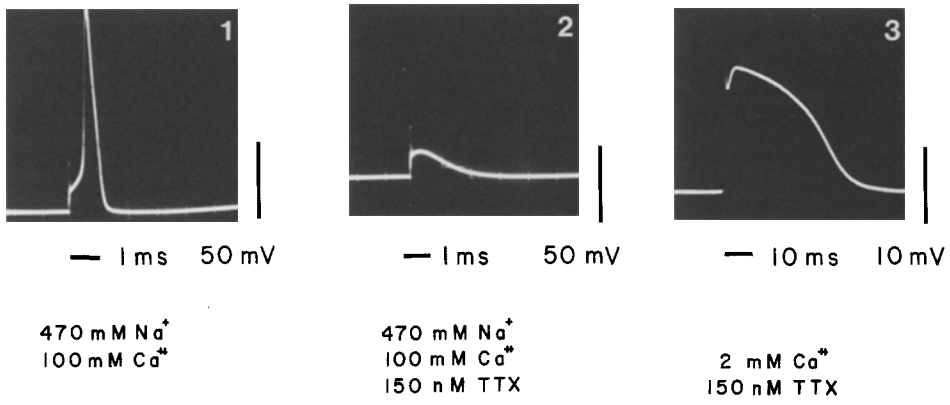

Fig. 6. Effect of TTX on the Ca-K bi-ionic action potential. The axon under study was internally perfused with a solution containing 50 mM K^+. Record (1) was obtained in the external medium containing 470 mM NaCl and 100 mM $CaCl_2$. Stimulus duration was 0.05 msec. The resting potential was −45 mV. Record (2) was obtained 90 sec after addition of 150 nM TTX to the external medium. Note that the ability of the axon to develop action potentials was suppressed by TTX. Stimulus duration was 0.05 msec. The resting potential was −47 mV. Record (3) was obtained 4 min after removal of NaCl and reduction of $CaCl_2$ to 2 mM; the external TTX concentration was kept unaltered. Stimulus duration was 2 msec. The resting potential was −52 mV

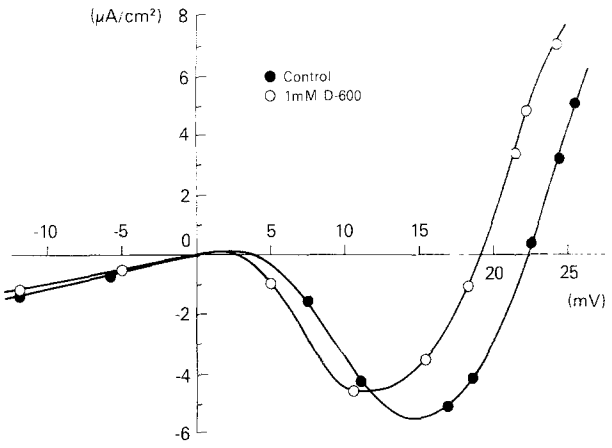

Fig. 7. Effect of D-600 added to the external medium on the *I–V* curve observed under the voltage-clamp condition. Filled circles represent data obtained before addition of D-600. Open circles represent data obtained 15 min after the addition of D-600. The concentration of D-600 was 1 mM. The holding potential was −57 mV

Effect of Co^{2+} or Mn^{2+} in the External Medium

Co^{2+} or Mn^{2+} is supposed to be a selective blocker of the Ca-channel (Hagiwara & Nakajima, 1966). When 2 mM $CoCl_2$ was added to the external medium containing 2 mM $CaCl_2$, the amplitude of action potential decreased and its duration increased (Figs. 8-1, 8-2). This effect of Co^{2+} was not reversible. A very similar effect to that of Co^{2+} was observed when the concentration of $CaCl_2$ in the external solution was raised from 2 to 20 mM. Addition of $MnCl_2$ to the external medium had an effect similar to that of addition of $CoCl_2$. Partial replacement of $CaCl_2$ with $CoCl_2$ affected neither the maximum inward current nor the slope conductance measured under the voltage-clamp condition (Fig. 8-3). Total replacement of 2 mM $CaCl_2$ with 2 mM $CoCl_2$ or 2 mM $MnCl_2$ did not suppress excitability (Figs. 8-4, 8-5). These observations can be summarized by stating qualitatively that the external layer of the squid axon membrane does not distinguish Co^{2+} or Mn^{2+} from Ca^{2+}.

Effect of 4-Aminopyridine Added to the Internal Medium

4-aminopyridine (4-AP) is regarded as a blocker of the K-channel on axon membranes of squid (Yeh, Oxford, Wu & Narahashi, 1976; Meves & Pichon, 1977) as well as cockroach (Pelhate, Hue & Chanelet, 1972; Pelhate & Pichon, 1974). Effects of 4-AP on excitability under the Ca-K bi-ionic condition were examined by the voltage-clamp method. The axons used were perfused internally with a 50 mM K^+ solution and immersed in 2 mM $CaCl_2$ solution. This chemical was applied internally at the concentration of 0.1–0.5 mM. In five axons examined, the increase of inward current observed upon application of positive clamping pulse was suppressed completely in 1–2 min. Examples are shown in Fig. 9. In the presence of 4-AP, the inward current could not be restored

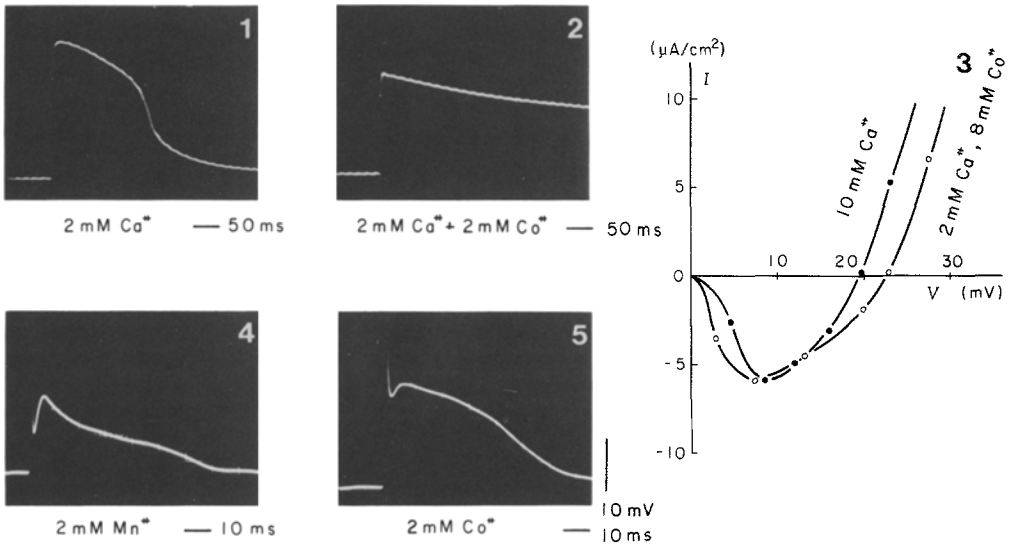

Fig. 8. Effects of external Co^{2+} and Mn^{2+}. All axons were internally perfused with a solution containing 50 mM K^+. (1) Action potential obtained from an axon immersed in a solution containing 2 mM $CaCl_2$. The resting potential was −53 mV. (2) Action potential obtained from the same axon approximately 3 min after addition of 2 mM $CoCl_2$ to the external solution. The resting potential was −48 mV. (3) *I–V* curve obtained by the voltage-clamp method. Filled circles represent a control obtained from an axon in a 10-mM $CaCl_2$ solution. Open circles represent the data obtained from the axon in a test solution containing 2 mM $CaCl_2$ and 8 mM $CoCl_2$. The holding potential was −58 mV. (4) and (5) Bi-ionic action potentials generated in axons immersed in a solution containing 2 mM $MnCl_2$ or 2 mM $CoCl_2$ as the sole electrolyte species. The resting potentials were −48 mV in (4) and −52 mV in (5)

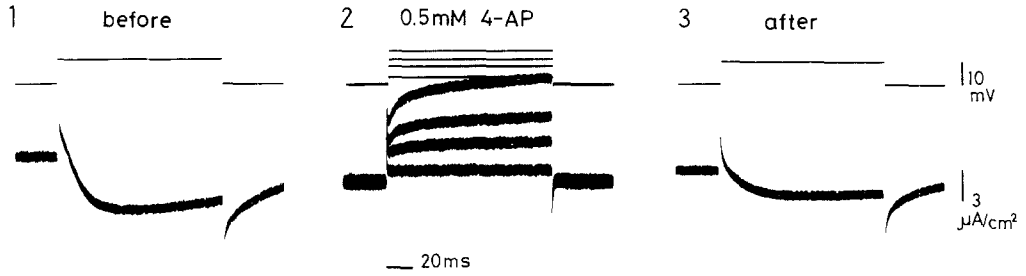

Fig. 9. Effects of 4-AP on the membrane current observed under the voltage-clamp condition. The axon was immersed in 2 mM $CaCl_2$ solution and intracellularly perfused with a solution containing 50 mM K^+. Upper (thin) traces represent the membrane potential and lower (thick) traces represent the membrane current. (1) The membrane current observed before application of 4-AP. (2) Membrane currents in response to four voltage clamping pulses observed 4 min after the internal application of 0.5 mM 4-AP. (3) The membrane current observed 8 min after the removal of 4-AP. The holding potential was −48 mV

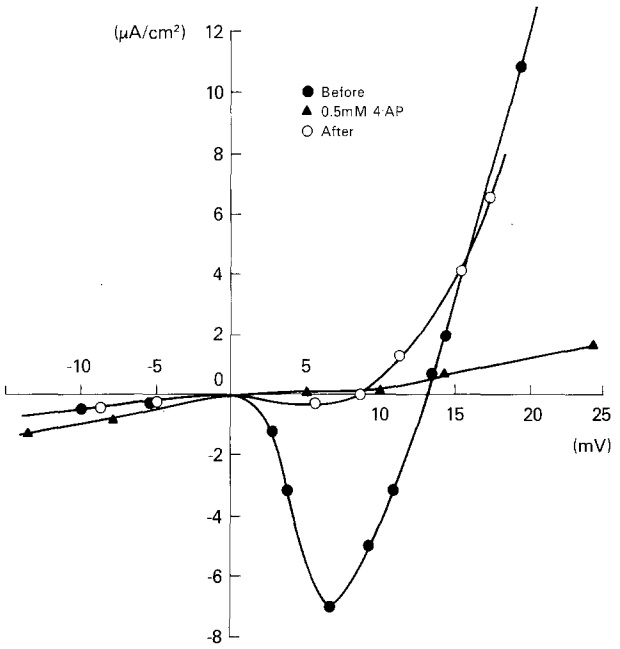

Fig. 10. Effect of 4-AP on the *I–V* curve observed under the voltage-clamp condition. The axon was immersed in 2 mM $CaCl_2$ solution and intracellularly perfused with a solution containing 50 mM K^+. Filled circles represent data obtained before the addition of 4-AP. Triangles represent data obtained 2 min after the addition of 0.5 mM 4-AP to the internal solution. Open circles represent data obtained 7 min after the removal of 4-AP. The holding potential was −50 mV

by changing the level of the holding potential. It is clear from the *I–V* curve shown in Fig. 10 that the membrane conductance measured by the positive voltage pulse decreased dramatically whereas the conductance measured by the negative voltage pulse increased slightly. These suppressing effects of 4-AP were partially reversible.

Discussion

Previous investigators believed that it was impossible to demonstrate Ca-dependent action potentials in squid giant axons perfused intracellularly with K-salt solution. Now it is possible. Following are the reasons why previous investigators failed to demonstrate Ca-K bi-ionic action potentials. (1) The axons exhibit excitability only when the concentration of Ca-salt in the external medium is as low as 2 mM. (2) The amplitude of the action potential is small. (3) The ability of the membrane to produce action potentials can be maintained for a relatively short time. In spite of these properties, the observed potential variations are proved to be action potentials by the presence of all-or-none properties and the impedance loss (Fig. 2).

The experimental results may be interpreted based on the widely accepted independent channel hypothesis. This hypothesis was originally developed by Hodgkin and Huxley (1952*a*, *b*) and supported by many investigators (Narahashi et al., 1964; Hagiwara & Nakajima, 1966; Hille, 1967; Armstrong, 1969). The excitability observed under the present ionic condition could not be suppressed by TTX, D-600, or Co-salt added to the external medium, but it was suppressed by 4-AP. These pharmacological observations suggest that the K-channel is responsible for the excitability described here. Consequently, the following are suggested. The mobility or the selectivity of Ca-ion in the K-channel increases during the process of excitation. The repetitive responses observed at low Ca^{2+} concentrations (Fig. 3) are ascribed to a cyclic change of conformation of the K-channel alone. The blockage of the K-channel is never a necessary condition for the maintenance of the Ca-dependent excitability in the squid axon. There is a point slightly difficult to understand; in spite of the intracellular presence of K^+, the activated K-channel becomes permeant to Ca^{2+} (not to K^+). A possible explanation would be that the K-channel acquires very special kinetics for the mobility of (or for the selectivity to) K-ion when the external Ca^{2+} concentration is low. At this stage of investigation, it would be more appropriate to call this channel simply a 4-AP sensitive channel than to call it the K-channel.

Alternatively, the present results may be interpret-

ed on the macromolecular theory in which transmembranous proteins are regarded as a proteinaceous ion exchanger (Tasaki, 1968). The dependence of the reversal potential on the external Ca^{2+} concentration can be explained from the theoretical calculation of the potential difference across the ion exchanger membrane under the bi-ionic conditions (Helfferich & Ocker, 1957; Helfferich, 1962; Tasaki, 1968). The stabilizing effect of the external Ca^{2+} arises from its capability of forming electrostatic cross-linkages in macromolecules (*see* e.g., Katchalsky, 1954; Katchalsky & Michaeli, 1955). An exchange of Ca^{2+} for K^+ (caused, e.g., by outward current) removes the linkages and therefore induces a conformational change of the membrane macromolecules to the excited state. In this theory, it is not fully explained how some chemicals (TTX, D-600, or 4-AP) suppress certain types of action potentials selectively.

The Ca-Cs bi-ionic action potential is highly sensitive to TTX (Watanabe et al., 1967*b*). This was confirmed by the voltage-clamp technique (Meves & Vogel, 1973). Now, it is widely believed that Ca-ion passes through the Na-channel, and that Cs-ion is a K-channel blocker (Adelman, 1971). On the contrary, the present study showed that the Ca-K bi-ionic action potential is not sensitive to TTX. Here arise questions: Why is the TTX sensitivity lost in the axon perfused with K-solutions? (It should be pointed out that normal axons produce TTX-sensitive action potentials with high intracellular K^+.) Is the insensitivity of Ca-K bi-ionic action potential to TTX really evidence against involvement of the sodium channel in this action potential? Answers for these questions await further studies, e.g. a biochemical study of TTX-binding to nerve membranes in low ionic strength solutions.

The present finding can be interpreted based on two theories – the independent channel theory and the macromolecular theory. In fact, these two theories may not be mutually exclusive. Taking advantage of the simplicity of the ionic condition, further studies on the Ca-K bi-ionic action potential may greatly contribute to an advancement of these theories into a unified and powerful form.

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Note Added in Proof

The Ca-Na bi-ionic action potential is insensitive to TTX (Watanabe & Tasaki, 1972). This insensitivity has recently been analyzed by Inoue (1980) in detail. According to his study, the generation of Ca-Na bi-ionic action potential is to be partly ascribed to the activity of the K-channel.