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Periodic Responses in Squid Axon Membrane Exposed Intracellularly and Extracellularly to Solutions Containing a Single Species of Salt

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Summary. A periodic membrane potential change was found to occur in squid giant axons which were internally and externally perfused with solutions of an identical composition and were hyperpolarized by passing a sustained inward current. The solution contained Co^{2+} or Mn^{2+} as the sole cation species at a concentration of 1–10 mM. The amplitude of the response was roughly 100 mV. The current intensity and the ion concentration had large effects on the response. The voltage-clamp technique revealed an N-shaped *I-V* characteristic of the membrane system. The membrane emf of the resting and excited states was almost the same but the membrane conductance was increased in the excited states. The response was suppressed with 4-aminopyridine reversibly but unchanged with tetrodotoxin or D-600. Those unusual ionic conditions did not deprive axons of their ability to produce ordinary action potentials in physiological solutions. The experimental conditions employed and the results obtained were very close to those for some of the artificial membrane models. Applicability of the physico-chemical theories developed for these models is discussed.

Key words: cobalt, manganese, 4-aminopyridine, squid axon, K-channel, artificial membrane

Various artificial membranes have been devised as the model for the nerve membrane. Lipid bilayer membranes doped with EIM (proteinaceous compounds from bacteria) mimic nerve excitation under ionic conditions similar to those of the biological system (Mueller & Rudin, 1967, 1968; Ehrenstein, 1971). However, the precise mechanism of the excitability is still unclear. An artificial membrane constructed from a porous glass membrane or a Sephadex gel membrane exhibits excitability under certain experimental conditions (Teorell 1955, 1959*a*, 1961). In this model, the membrane was exposed to solutions containing only one species of salt. The extreme simplicity of ionic conditions employed promoted clear understanding of physico-chemical aspects of the nerve-like activity of the model, but at the same time, obscured the significance of the membrane as a model for real biological excitable membranes. The aim of the present study is to demonstrate excitability of a biological membrane under the ionic conditions as simple as that of the model of Teorell. These conditions were realized by using intracellular and extracellular media containing isotonic glycerol, a few mM cobalt (or manganese) salt, and nothing else. [The reason for the use of this salt is that the extracellular divalent cation is indispensable for maintaining excitability (Frankenhaeuser & Hodgkin, 1957; Tasaki, Watanabe & Lerman, 1967), whereas intracellular Ca^{2+} has adverse effects on excitability (Tasaki, Watanabe & Takenaka, 1962; Tasaki et al., 1967)]. Studies of nerve membrane under such ionic conditions would help explain the nerve excitation in more precise physico-chemical terms (as those used for the models).

Materials and Methods

Giant axons of squid (*Loligo pealei*) available at the Marine Biological Laboratory, Woods Hole, Mass. were used. The diameter of axons used was 400–650 μm. The axon was excised and mounted in a Lucite chamber containing natural sea water. The entire part of the axon (28 mm) in the chamber was 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. Next, natural sea water in the chamber was replaced with a continuously flowing solution which contained 1–10 mM Mn- or Co-salt. Subsequently, the internal per-

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perfusion solution was switched from KF solution to the Mn- or Co-salt solution which was the same as that used externally. The flow of the internal perfusion solution was maintained at the rate of 30–50 µl/min.

The solution was prepared by adding a small amount of a concentrated salt solution of manganese or cobalt to a 12% (vol/vol) glycerol solution with a micropipette. When solubility of the salt was low, a weighed powder of the salt was added directly to the glycerol solution. No pH buffer was added, but the pH of the solution was usually 6.5–7.5.

The electrode used for recording the internal potential was a glass pipette (50 µm in inside diameter) filled with 0.6 M KCl-agar. A piece of silver-silver chloride wire connected the electrode to the amplifier input. The internal potential was referred to the potential measured by a calomel electrode immersed in the external solution at the corner of the chamber where the external solution was drained. The intracellular current electrode (used for passing stimulating or voltage-clamping current) was a piece of platinized platinum wire (100 µm in diameter) inserted into the axon at the perfusion zone. The length of the uninsulated portion at the tip was 20 mm. The external current electrode was a large platinized platinum coil placed along the axon for a length of 18 mm. Usually current was delivered to the membrane through a resistance of 10 MΩ. In a voltage-clamp study, the circuitry described by Moore (1971) was employed. A guard system similar to that described in the preceding paper (Terakawa, 1981) was used. The series resistance was not compensated. All observations were performed at room temperature (22–24° C).

Results

Resting Potential

Axons immersed in natural sea water and perfused intracellularly with a 400 mM K-salt solution had a resting membrane potential of −50 to −60 mV (inside negative). When both the external and internal media were replaced with solutions containing a single species of salt at an identical concentration, the resting membrane potential quickly fell. A typical example obtained by using $CoSO_4$ as a sole electrolyte species is shown in Fig. 1. In the course of 5–10 min, the membrane potential often became once positive and finally reached a level near zero. The following observations were performed after the membrane potential reached this steady level.

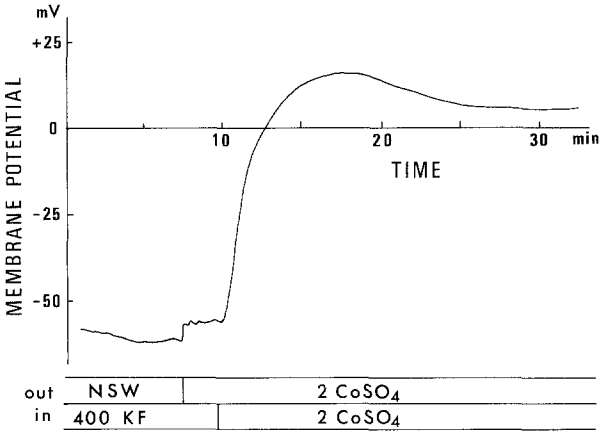

Fig. 1. Typical time course of the membrane potential change after replacement of the internal and external media with solutions containing 2 mM $CoSO_4$ and 12% glycerol. *Ordinate:* the level of the intracellular potential referred to the extracellular potential. *Abscissa:* (the experimental) time

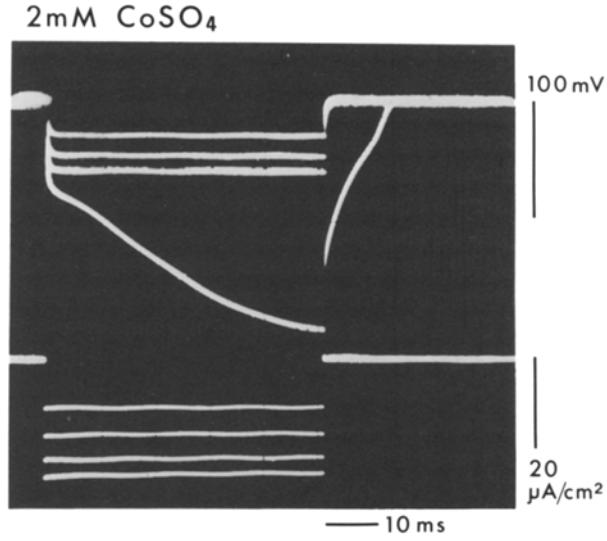

Fig. 2. Hyperpolarizing response obtained from an axon perfused intracellularly and extracellularly with solutions containing 2 mM $CoSO_4$ and 12% glycerol. Four inward current pulses of different amplitudes (lower traces) were applied to the membrane with an interval of 3 sec. Four responses of the membrane potential (upper traces) were recorded by photographic superposition. The membrane potential measured before application of the current pulse was −3.3 mV

Hyperpolarizing Response

Under the intracellular and extracellular perfusion with the same solution containing $CoCl_2$ (or $MnCl_2$), the membrane potential stayed in a quiescent state. Hyperpolarizing responses could be elicited by passing a current pulse through the membrane in an inward direction (*see* Fig. 2). The hyperpolarizing response was induced only by the current pulse whose amplitude exceeded a certain threshold. Above the threshold, the larger the amplitude of the pulse, the quicker the response. The hyperpolarizing response was similar in shape to the falling phase of an action potential. In Fig. 2, it is also evident that another type of physiological response could be induced at the end of the pulse. This response was similar to the rising phase of an action potential. The rising time of this response was about 10 msec.

Periodic Responses in Membrane Potential

The axons perfused intracellularly and extracellularly with identical Co^{2+} (or Mn^{2+}) solutions generated oscillatory responses in membrane potential when a sustained inward current was applied through the

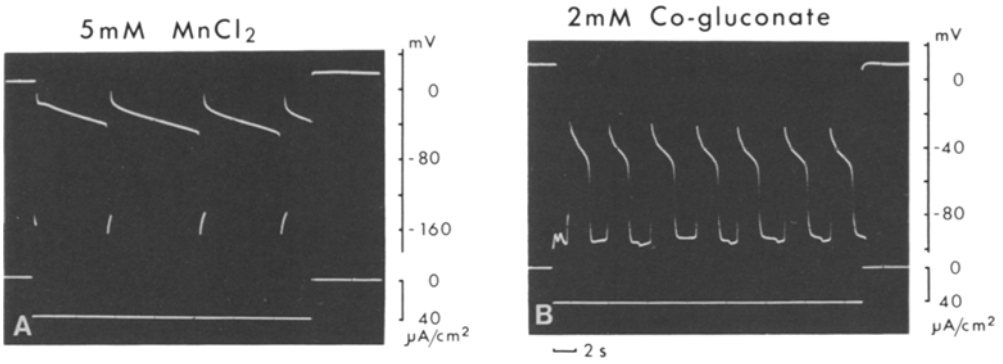

Fig. 3. Periodic variations of the membrane potential in response to passage of constant inward current. *A* The axon was perfused internally and externally with a solution containing 5 mM $MnCl_2$ and 12% glycerol. *B* The axon was perfused internally and externally with a solution containing 2 mM Co-gluconate and 12% glycerol. Upper traces show the membrane potential recorded intracellularly. Lower traces show membrane current; downward deflection indicates inward current

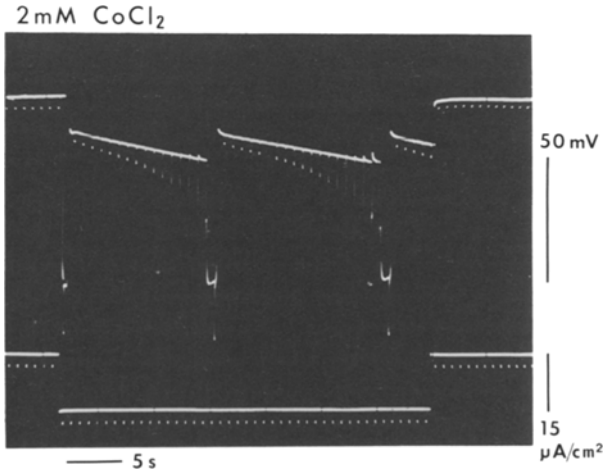

Fig. 4. Periodic variations of the membrane conductance. Both internal and external media contained 2 mM $CoCl_2$ and 12% glycerol. Small current pulses were superposed on the sustained inward current. The membrane potential level before application of sustained inward current was +6.6 mV

membrane. Two examples of such oscillatory responses are shown in Fig. 3. In Fig. 3*A* the axon was perfused internally and externally with 5 mM $MnCl_2$ solutions. At the onset of the inward current there was a hyperpolarizing response. This was followed by a gradual decrease in negativity of the membrane potential and by an abrupt depolarization to a level near zero. Then the membrane potential gradually increased its negativity and formed another hyperpolarizing response. These processes appeared periodically. The amplitude of the responses was usually 50–100 mV; the largest amplitude encountered was 175 mV. Fig. 3*B* shows another example of the oscillatory response obtained from an axon perfused with solutions containing 2 mM cobalt gluconate.

The oscillatory response could be repeated on more than 50 axons. The observed response was not an artifact generated by electrodes or by other measuring devices because of the following reasons. First, the periodic response could not be observed if the axon was treated intracellularly with Pronase (1 mg/ml) for more than 10 min before the observation. Second, the periodic response could not be observed if the axon was treated extracellularly with phospholipase A_2 (1 mg/ml) for a few minutes before the observation. The possibility that the observed excitability was maintained by ions other than those in the perfusion fluid was excluded by the following observations: (*i*) Oscillatory response could be demonstrated even after a prolonged (more than 1 hr) perfusion with the divalent cation solution. (*ii*) The response could be demonstrated in axons in which internal perfusion was initiated without using 400 mM KF solution (*iii*) The response could be demonstrated in axons from which the axoplasm was removed by the protease treatment of the entire length (about 30 mm). (*iv*) To record the responses, platinum wire electrodes could be used instead of KCl-filled electrodes.

The absence of the salt-concentration gradient across the membrane did not alter the membrane irreversibly. After observing the periodic response, action potentials of about 100 mV in amplitude and 1 msec in duration could be observed when the external medium was replaced with a solution containing both Na- and Ca-salts and the internal medium with a solution containing K-salt.

Periodic Changes in Membrane Conductance

The oscillatory variation in membrane potential was associated with an oscillatory variation in membrane conductance. In Fig. 4, short and small pulses of current were superposed to a large sustained inward current to estimate the membrane conductance. The membrane conductance was about 0.1 mS cm^{-2} when the membrane potential was in the low level (high

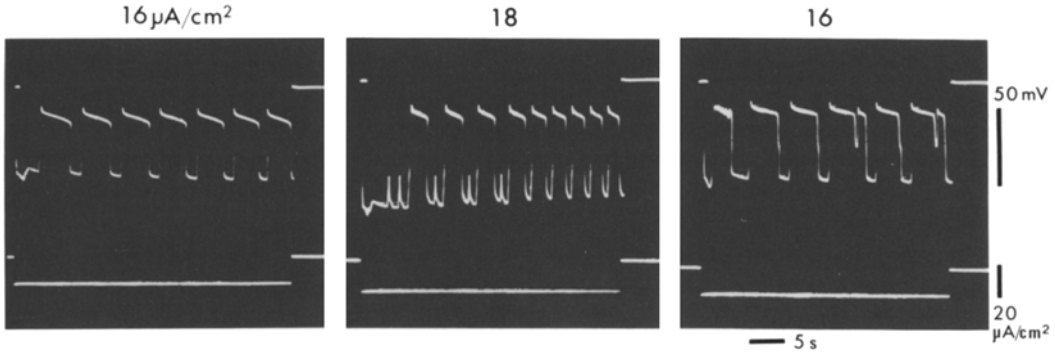

Fig. 5. Effect of current intensity on the periodic response. Internal and external media contained 2 mM $CoCl_2$ and 12% glycerol. The intensity of current was 16 μA cm^{-2} (left), 18 μA cm^{-2} (middle), and 16 μA cm^{-2} again (right)

negativity). The abrupt rise of the membrane potential (decrease in negativity) was accompanied by a sudden increase in membrane conductance to about 1 mS cm^{-2}. The voltage obtained by dividing the intensity of sustained inward current with the conductance was close to the membrane potential observed. In other words, the electromotive force of the membrane changed very little when the periodic responses appeared. The membrane conductance observed before application of the sustained inward current was about 0.7 mS cm^{-2} and that observed immediately after termination of the current was larger than 1 mS cm^{-2}. These observations indicated that the membrane was in the excited state when no current was flowing through the membrane.

Effect of Current on Periodic Responses

The amplitude and the duration of the oscillatory response varied in a wide range depending on various factors. One such factor was the strength of the sustained hyperpolarizing current applied to the membrane. The effect of changing the current on the oscillatory potential variation was examined in the axon perfused intracellularly and extracellularly with the same 2 mM $CoCl_2$ solution (Fig. 5). A small increase in current intensity shortened the duration of the oscillatory response and increased the amplitude of the response. A strong current tended to keep the membrane potential in the lower state. Abortive responses shown in Fig. 5 were one of the signs for this tendency. However, an attempt to suppress the response completely by applying a strong current was unsuccessful, because of the electrolysis taking place around the internal current electrode.

Effect of Salt Concentration on Periodic Responses

Concentration of the salt in media also affected the oscillatory response. This was studied by using $MnCl_2$ solutions as the internal and external media. In the range of 1–10 mM, the internal and external salt concentration could be varied independently without eliminating the response. When the $MnCl_2$ concentration in the external medium was increased from 2 to 10 mM, the period in which the membrane potential was in the high level became shorter (Fig. 6*A*). This period was shortened also by decreasing the salt concentration in the internal medium from 5 to 1 mM (Fig. 6*B*). Decreasing the internal salt concentration enhanced the amplitude of oscillation also. Conversely, increasing the internal salt concentration from 5 to 10 mM made the amplitude smaller and the duration longer; it was therefore difficult to maintain the oscillation at the internal salt concentration higher than 10 mM. The effects of increasing the external salt concentration and of decreasing the internal salt concentration were similar to those of increasing the intensity of the hyperpolarizing current. A large concentration gradient across the membrane built by perfusing solutions or by applying current is, perhaps, preferable for achievement of the low conductance state.

Relationship between Salt Concentration and Membrane Potential

In the absence of the current, the membrane was in the excited state since the membrane conductance was in a high level. In order to estimate the ion selectivity of the membrane in its excited state, the membrane potential in the absence of current was examined with varying the salt concentration in internal and external media. The result obtained by using $CoSO_4$ is shown in Fig. 7. Raising the salt concentration of the external medium did not change the membrane potential appreciably, whereas raising the salt concentration of the internal medium changed the membrane potential greatly. The membrane potential fell by about 16 mV with a decade increase in the internal salt concentration. These findings indicated that under the present ionic condition only the axoplasmic side of the membrane maintained a significant permselectivity for cation. The meaning of this curious fact remained unclear.

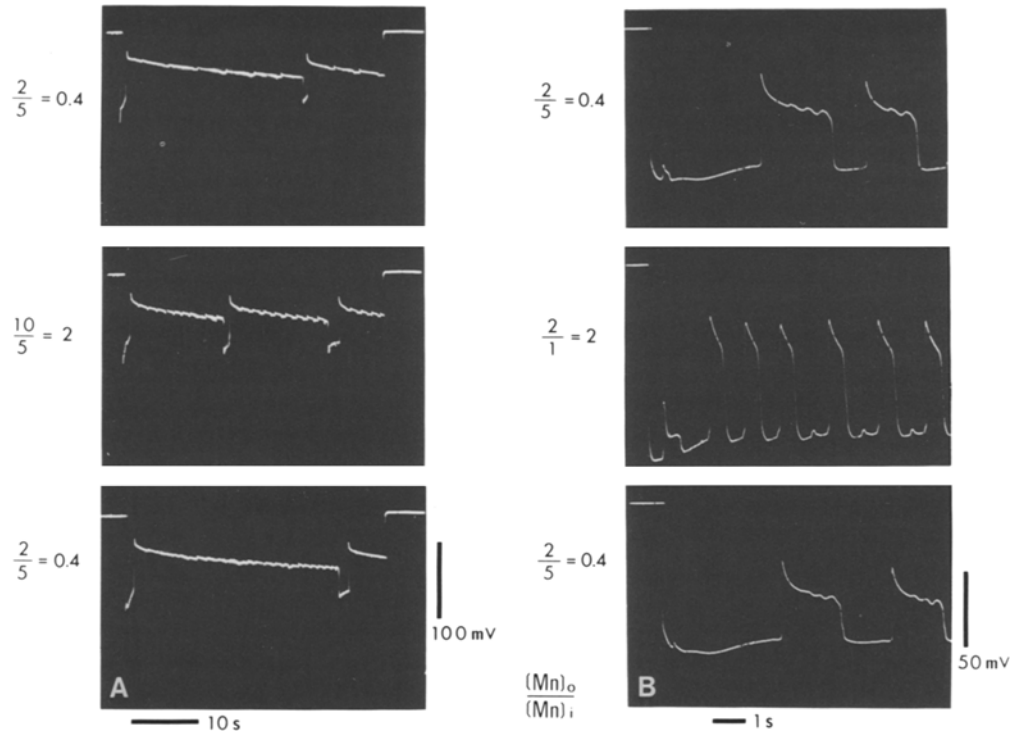

Fig. 6. Effect of salt concentration on the periodic response. Internal and external media contained $MnCl_2$ and 12% glycerol. The numerator of fractions represents the external concentration and the denominator represents the internal concentration (both expressed in mM). *A*, A series of records obtained from an axon with the internal concentration of $MnCl_2$ kept constant at 5 mM. The top record was obtained when the external concentration of $MnCl_2$ was 2 mM. The middle record was obtained 3 min after increasing the external $MnCl_2$ concentration from 2 mM to 10 mM. The bottom record was obtained 2 min after reducing the external $MnCl_2$ concentration from 10 mM to 2 mM. The intensity of current applied was 34 μA cm^{-2}. *B*, A series of records obtained from an axon with the external concentration kept constant at 2 mM. The top record was obtained when the internal $MnCl_2$ concentration was kept at 5 mM. The middle record was obtained 3 min after reducing the external $MnCl_2$ concentration from 5 mM to 1 mM. The bottom record was obtained 3 min after increasing the concentration from 1 mM to 5 mM. The intensity of hyperpolarizing current was 20 mA cm^{-2}

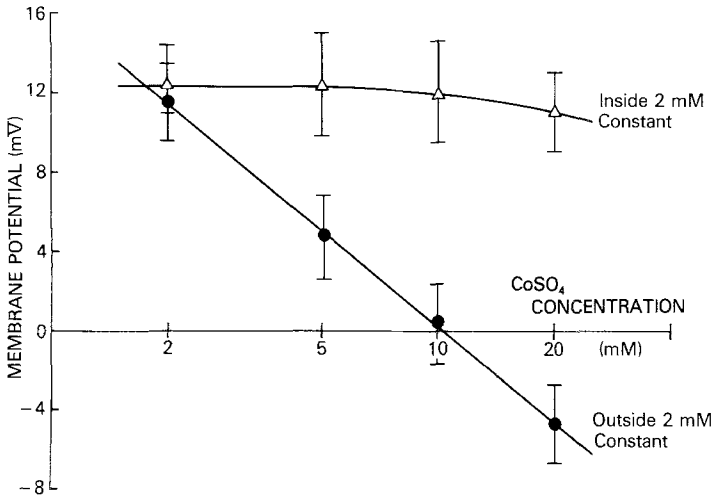

Fig. 7. Membrane potential measured in the absence of membrane current with varying internal and external salt concentrations. Solutions used contained $CoSO_4$ and 12% glycerol only. Means and standard errors are indicated. Triangles represent data obtained from seven axons with the external $CoSO_4$ concentration varied while the internal concentration kept constant at 2 mM. Filled circles represent data obtained from six axons with the internal $CoSO_4$ concentration varied while the external concentration kept constant at 2 mM

Membrane Currents Observed Under Voltage-Clamp

The electrical properties of the axon membrane exposed intracellularly and extracellularly to solutions containing single species of salt could be revealed by the voltage-clamp method also. With the holding potential set at about −130 mV, depolarizing and hyperpolarizing clamping pulses of 330 msec in duration were applied to the membrane. The resulting currents are shown in Fig. 8*A*. When the pulse was applied in depolarizing direction with an amplitude of 20 to 70 mV, a transient increase in inward current was observed. At the end of the pulse of 30 mV depolarization the increase in the inward current was still

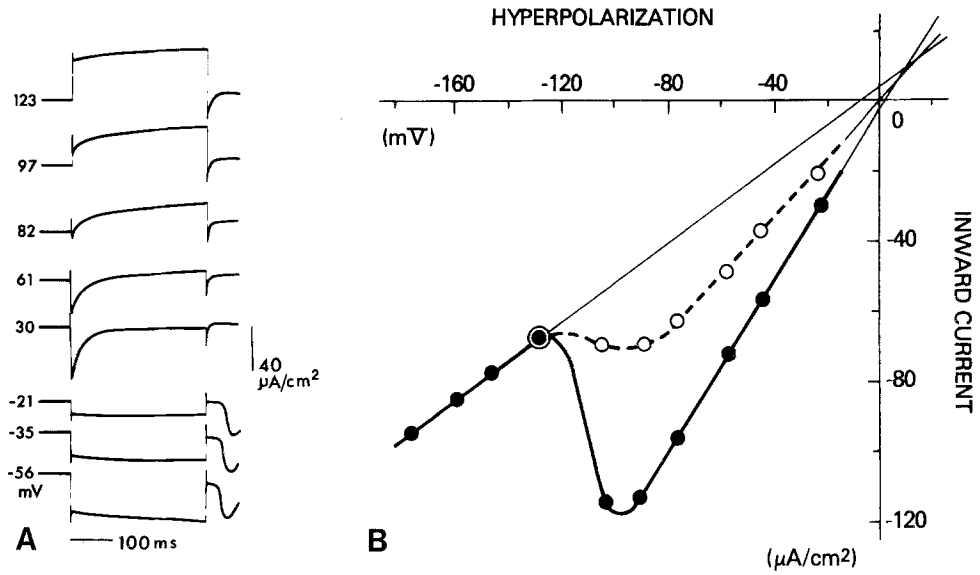

Fig. 8. Membrane currents observed under the voltage-clamp condition. Manganese chloride was used as a sole electrolyte species in media. The internal $MnCl_2$ concentration was 2 mM and the external $MnCl_2$ concentration was 5 mM. (*A*) Time courses of membrane current (*I*) in response to various voltage pulse (*V*). The holding potential was −132 mV. Numbers on the left represent amplitude of voltage-clamping pulse (in mV) measured from the holding potential; numbers without sign indicate depolarizing pulses and with minus sign indicate hyperpolarizing pulses. (*B*), *I–V* relation obtained from data in *A*. Filled circles represent membrane currents measured at the peak of the transient inward current or at the corresponding time when there was no peak other than capacitative surge. Open circles represent the membrane currents measured at the end of depolarizing pulses

maintained. When the pulse was applied in the hyperpolarizing direction, the current changed roughly in a rectangular form. In this case, a transient increase in inward current was observed after the termination of the voltage pulse. This response seemed to correspond to a "break-off response" which was also observed under the current-clamp condition, although this could be an invasion of action potentials from nonspace-clamped regions of the membrane. The current measured at the peak of transient and at the end of the pulse was plotted against the amplitude of the voltage pulse. The *I–V* relation thus obtained is shown in Fig. 8*B*. It was obvious that the membrane had two conductance states: high (1.3 mS cm^{-2}) and low (0.5 mS cm^{-2}). The electromotive force of the membrane system was estimated by extrapolating positive-slope portions of the *I–V* curve to zero current (*see* thin lines in Fig. 8*B*). It was again observed that the change in electromotive force during the transition between two conductance states was very small. The oscillatory response in membrane potential arose almost completely from the membrane conductance variation only.

Effects of Various Chemicals and Ions on Periodic Responses

The repetitive response observed with 2 mM $CoCl_2$ in the internal and external solution could not be suppressed by addition of TTX to the external medium at the concentration of 2 μM (Fig. 9, left). The response could not be suppressed either by D-600 (0.1 mM applied externally) (Fig. 9, middle), which is supposed to block the Ca-channel (Kohlhardt, Bauer, Krause & Fleckenstein, 1972). Dinitrostylbene disulfonic acid (DNDS) is believed to block the transport of anions in red blood cell membrane (Cabantchik, Knauf & Rothstein, 1978). This chemical added to internal and external solutions at the concentration of 1 mM was also ineffective in suppressing the response (Fig. 9, right). Procaine (5 mM) added to both solutions did not suppress the response either.

4-Aminopyridine (4-AP) is supposed to block the K-channel (Yeh, Oxford, Wu & Narahashi, 1976; Meves & Pichon, 1977). In Fig. 10 the effect of this chemical is shown. When 4-AP was added to the internal perfusion solution at the concentration of 0.1–0.5 mM the response was completely suppressed in 1–2 min. Eleven axons examined in a similar manner showed no exception. The suppressing effect of 4-AP was fairly reversible. The addition of KCl to the internal perfusion solution at the concentration of 0.5 mM had no detectable effect on the response. Therefore, it is not the monovalent charge but a specific structure of 4-AP that is responsible for the suppressing effect.

The pH of the solution had little effect on the responses; they appeared equally well when the pH of the solution varied in the range of 4.6–8.3 depending on the salt used. The responses were observed,

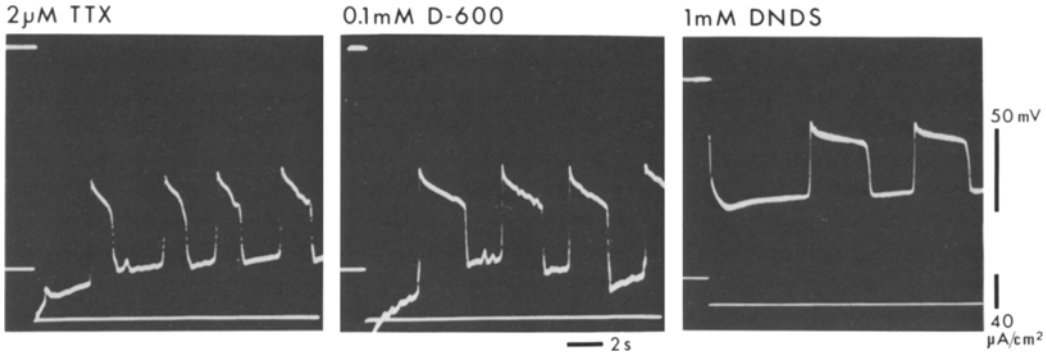

Fig. 9. Lack of effects of TTX, D-600, and DNDS on the periodic response observed with 2 mM $CoCl_2$ intracellularly and extracellularly. The left record was obtained 20 min after external application of 2 μM TTX. The middle record was obtained 20 min after external application of 0.1 mM D-600. The right record was obtained 20 min after external and internal application of 1 mM DNDS

as well, when the solution contained 1 mM Tris-HCl buffer (pH 7.3 ± 0.1) in addition to the divalent-cation salt.

The effect of varying the anion in the Co-salt was examined by using sulfate, fluoride, chloride, acetate, citrate and gluconate salts. There was no qualitative difference in electrophysiological behavior of the responses obtained with these anions.

Discussion

The experimental results indicate that excitability of squid axon membrane can be maintained in both internal and external media containing 12% glycerol, a few mM cobalt (or manganese) salt, and nothing else. Passage of inward current through the membrane induces periodic changes in membrane conductance without changing the membrane emf. The present ionic condition is symmetrical on both sides of the membrane and is the simplest one ever created experimentally in excitable cells. Although the condition is not physiological, the membrane maintains a specific sensitivity to 4-AP and ability to produce normal action potentials. This implies that studies of excitability under this condition can contribute to elucidation of general nature of ion-membrane interaction. The present ionic condition is well-suited, for example, for the gating current experiment because asymmetry in ion distribution can be ignored as a possible source of the asymmetry current.

The internal layer of the membrane has the permselectivity for cation (Fig. 7). The periodic response can be obtained equally well with various anions used as cobalt salts. Negatively charged DNDS does not suppress the response, but 4-AP which carries positive charge at neutral pH suppresses it (Figs. 9 and 10). These results suggest that Co^{2+} or Mn^{2+} is the major current carrier in the membrane under the present ionic condition.

According to the independent channel hypothesis,

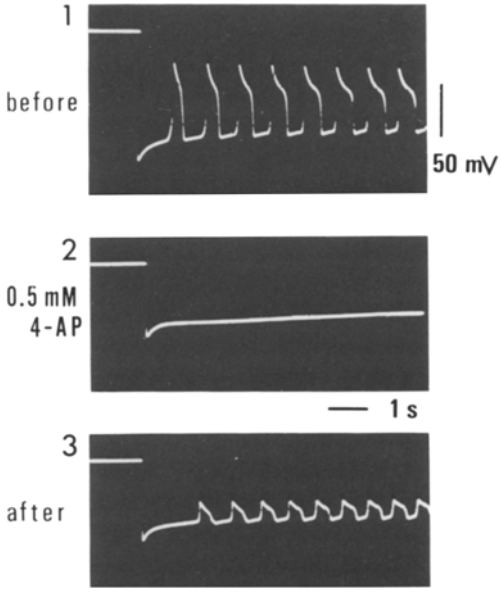

Fig. 10. Effect of 4-AP on the periodic response observed with 2 mM $CoCl_2$ intracellularly and extracellularly. Record 1 was obtained before application of 4-AP. Record 2 was obtained 2 min after internal application of 0.5 mM 4-AP. Record 3 was obtained 4 min after removal of 4-AP. The current applied was 36 μA cm^{-2} in all cases

the response observed may be attributable to the activity of the K-channel. Slow rising time (Fig. 1) and sensitivity to 4-AP (Fig. 10) support this idea. The repetition of responses can be explained as alternate activation and inactivation of the K-channel, though its kinetics must require complicated potential dependence. The response described in the present paper is very similar to some of the hyperpolarizing responses which were also attributed to the activity of the K-channel (Reuben, Werman & Grundfest, 1961; Nakajima, 1966; Nakajima & Kusano, 1966). A squid axon with its axoplasm left intact also exhibits the hyperpolarizing response when immersed in a K-rich solution (Segal, 1958; Moore, 1959; Tasaki,

1959) however, this response could not be observed when the axon is perfused intracellularly and extracellularly with K-solutions (Rojas & Ehrenstein, 1965). Probably, the K-channel requires a divalent cation for its full activity. More complete voltage-clamp studies – detailed examination of the ionic currents with the use of pharmacological agents – may be necessary to verify the involvement of the K-channel in the periodic response. At this moment, it is apropriate to call the responsible channel, if any, a 4-AP-sensitive divalent cation channel instead of the K-channel.

Oscillatory variations of the membrane potential and the membrane conductance during a constant flow of current were described on various artificial membranes placed between solutions containing only one species of salt. These membranes, proposed as nerve models, include porous glass membrane (Teorell, 1955, 1959*a*), Sephadex gel membrane (Teorell, 1961), polyelectrolyte membrane (Shashoua 1967, 1969), lipidic membrane (Monnier, Monnier, Goudeau & Rebuffel-Reynier, 1965; Monnier, 1968), and dioleylphosphate-Millipore filter membrane (Kamo, Yoshioka, Yoshida & Sugita, 1973). A common feature of these membranes is the possession of porous spaces surrounded by fixed charges. The physicochemical mechanisms operating in these models were analyzed by many investigators (Teorell, 1959*b*, 1962; Aranow, 1963; Franck, 1963; Kobatake & Fujita, 1964*a, b*; Katchalsky & Spangler, 1968; Mears & Page, 1972). The diffusion of ion and the flow of water through these porous spaces are considered to be important factors determining the time course of the responses. There are marked similarities between these models and squid axon membranes. Ionic conditions are very simple. Application of sustained current through the membrane elicits oscillation of the membrane potential. The oscillation of the membrane potential is solely attributable to the membrane conductance variation (not to the membrane emf change). Besides, a preliminary study on squid axon (not mentioned in Results) showed some relationship between the periodic responses and the hydrostatic pressure of perfusion solution, which is an important factor in Teorell's glass membrane model. Because of these similarities, it is highly possible that the diffusion process and the water flow are relating to at least some part of the excitation process of axon membrane. Direct measurements of the water flow across the axon membrane would give useful information.

Electrochemical processes taking place in the porous charged membranes are generally treated by irreversible thermodynamics (Katchalsky & Kedem, 1962; Katchalsky & Spangler, 1968; Kobatake, 1970). In fact, the oscillatory response of the 4-aminopyridine-sensitive channel can be observed when there are concentration gradients of Ca^{2+} and K^+ across the membrane, (*see*, the preceding paper, Terakawa, 1981) or when the ion concentration gradient is absent but the electric current is applied through the membrane (this paper). These observations strongly suggest that the oscillation of membrane state can not occur without dissipation of energy. In other words, high and low conductance states of the 4-aminopyridine-sensitive channel may be treated as a dissipative structure (Glansdorff & Prigogine, 1971). It is believed that detailed comparison between the artificial membrane and the rhythmical activity of the axon under the single-salt perfusion will provide more precise physico-chemical description of some of the excitation process in the biological membrane.

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