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EXCITABILITY, INSTABILITY AND PHASE TRANSITIONS IN SQUID AXON MEMBRANE UNDER INTERNAL PERFUSION WITH DILUTE SALT SOLUTIONS

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SUMMARY

Using giant axons of squid, *Doryteuthis*, available in Hokkaido, Japan, it was shown that axons internally perfused with a dilute sodium salt solution undergo an abrupt transition from a resting to a depolarized state on addition of KCl to an external medium containing $CaCl_2$. Under internal perfusion with a dilute solution of sodium or cesium salt, it was possible to induce abrupt transitions between the two (*i.e.*, resting and depolarized) states of the membrane by changing the temperature. "Giant fluctuations" in the state of the axon membrane were demonstrated at and near the critical points of the axon membrane. These findings are interpreted as supporting the view that an abrupt change in the membrane potential and conductance is an electrochemical manifestation of a phase transition of the membrane macromolecules.

INTRODUCTION

Under internal perfusion with various dilute salt solutions, squid giant axons are known to maintain their electrical excitability in a medium containing only a divalent cation salt (*e.g.* $CaCl_2$) and a nonelectrolyte (*i.e.* glycerol or sucrose)[1,2] .Under these conditions, addition of a univalent cation salt (*e.g.* KCl or NaCl) to the external medium frequently produces an abrupt depolarization, namely a sudden rise in the intracellular potential associated with a simultaneous fall in the membrane resistance[4,5]. In the present studies, such abrupt changes in the state of the membrane are inducted by various means including gradual cooling. These studies are conduced with a view toward obtaining further information as to the properties of membrane macromolecules.

METHODS

In all the experiments described in this article, giant axons of squid, *Doryteuthis bleekeri*, caught in the nothern part of the Sea of Japan near the city of Otaru, were used. The diameter of these axons ranged between 400 and 700 μm. The major portion of the small nerve fibers surrounding the giant axon was removed under a dissecting microscope. The axon was then tranferred to a Lucite chamber in which internal perfusion with two glass cannulae was performed[2,4]. The outside diameter of the inlet cannula was approx. 200 μm and that of outlet cannula was 350 μm. The tip of the outlet

cannula was cut at an angle of about 45° and rounded off in the manner described previously. The major portion of the axoplasm in the perfusion zone was removed with a dilute solution of pronase (0.1 mg/ml) administered internally for about 30 s. The flow rate of the internal perfusion fluid was between 20 and 30 μl/min.

The potential difference across the axon membrane was measured with a glass pipette Ag–AgCl electrode filled with 0.6 M KCl solution. To reduce outflow of the KCl solution, the pipette was filled with KCl–agar gel near the tip. This recording electrode was introduced into the center of the 15 mm long perfused zone of the axon through the outlet cannula. The external fluid medium was grounded with a large coil of Ag–AgCl wire embedded in agar gel (enclosed in a glass tube). A high-input impedance preamplifier (Nihon Kohden, Type MZ-3B), a dual beam oscilloscope (Iwasaki Elec., Type SS-5517), and an oscillograph camera (Nihon Kohden, Type PC-2B) were used for recording. When internal application of electric current pulses was required, a silver or platinum wire (50 μm in diameter) was inserted into the perfusion zone of the axon through the outlet cannula. Current pulses were obtained from pulse generators (Nihon Kohden, MSE-3) through a 1 MΩ resistor.

When rapid circulation of the external fluid medium was required, a device similar to that described previously[5] was employed. This device consisted of large reservoirs of solution, polyethylene tubing for delivering the solution to the perfusion chamber, a baffle (made of two perforated sheets of Lucite) for achieving uniform application of the solution, and a suction pipe for maintaining a constant level of the fluid in the chamber. The fluid in the suction pipe was electrically isolated from the suction pump by means of a trap. The flow rate of the external fluid was in the range between 20 and 30 ml/min. The temperature of the circulating fluid was measured with a copper-constantan thermocouple.

The temperature of the axon was regulated by circulating the medium maintained at either low (about 4°C) or high (about 20 °C) temperature. To achieve uniformity of the temperature along the internally perfused zone of the axon, a baffle was constructed in the axon chamber between the inlet of the external medium and the axon. Since the polyethylene tubing connecting the reservoir of the fluid and the nerve chamber was relatively long (approx. 40 cm), the change in the temperature near the axon was very smooth and gradual. It took approx. 90 s to lower the temperature from 20 °C to 5 °C.

To prepare salt solutions double glass-distilled water was used. Cesium salts were purchased from K and K Laboratories of California, Inc., in the form of CsF and CsOH The sodium salts were obtained from Nakarai Chemicals Co., Kyoto. Internal perfusion fluid was prepared by mixing a 0.6 M salt solution with a 12% (by vol.) glycerol solution and by adjusting the pH to 7.3 $\pm$ 0.1 with phosphate buffer. External fluid media were prepared by mixing a 0.55 M NaCl, 0.4 M $CaCl_2$ and 12% (by vol.) glycerol; the pH was adjusted to 7.8–8.0 with Tris buffer.

RESULTS

(1) Excitability and instability of axons internally perfused with dilute sodium or cesium salt solution

Using *Doryteuthis* axons under internal perfusion with a dilute salt solution, it was relatively easy to demonstrate electrical excitability of axons after the total elimi-

nation of the salts of univalent cations in the external medium. The amplitude of the action potential observed under internal perfusion with a 15–30 mM NaF or CsF solution varied to some extent the external Ca^{2+} concentration; it was usually between 50 and 100 mV. The resting potential was between −5 and −30 mV. It was also relatively simple to induce an abrupt rise in the intracelluar potential by addition af a univalent-cation salt to the external medium under these conditions.

Fig. 1 shows an example of oscillograph records obtained from axons internally perfused with a dilute solution of NaF and immersed in a $CaCl_2$ solution. Although axons under these conditions are electrically excitable, no stimulating current was applied to the axon membrane during these observations. Note that there is a threshold, or critical concentration of KCl for production of an abrupt change in the membrane potential. Similar abrupt changes were encountered when NaCl was used instead of KCl. However, the critical concentration for NaCl was more than 10 times as high as that for KCl.

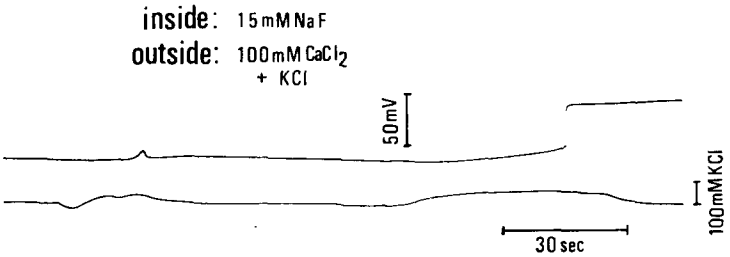

Fig. 1. Demonstration of instability of the squid axon membrane created by addition of potassium-salt to the external medium containing $CaCl_2$. The electrolyte compositions of the solutions used are given. The upper trace in each record represents changes in the membrane potential; the lower trace shows the concentrations of the univalent cation salt measured by means of a reversible chloride electrode (see ref. 5). No stimulating electrode was introduced in the axon. Temperature, 18 °C.

(2) Abrupt transitions induced by temperature changes

The following observations show that abrupt transitions between the resting and depolarized states of the membrane could be induced by changing the temperature of the external fluid medium. The diagram on the top of Fig.2 shows the experimental arrangement used. The axons were internally perfused with a dilute solution of either cesium- or sodium salt. The external medium contained both $CaCl_2$ and NaCl. The temperature of the medium was changed slowly and uniformly along the axon by the technique described under Methods.

Two examples of records presented in Fig. 2 show the typical results obtained. In these records, the horizontal deflections of the oscillograph beam represent the output of the thermocouple immersed in the surrounding fluid medium: the vertical deflection displays the membrane potential of the axon under study. When the axon was cooled gradually, the membrane potential changed only slightly at the onset of cooling. However, at about 5 °C under the present experimental conditions, the potential in the axon interior (referred to the potential of the medium) was found to jump upwards by more than 70 mV (see the arrows directed upwards). Further lowering of the temperature did not produce any abrupt change in the membrane potential. (A small, gradual fall in the potential following an abrupt transition is not related to the temperature change.)

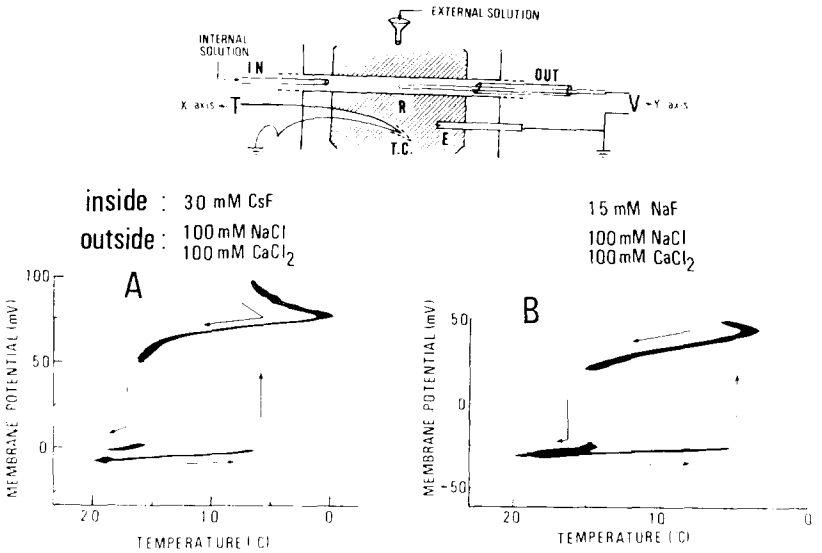

Fig. 2. Oscillograph records showing hysteresis in the membrane potential associated with a cyclic change in the temperature. The electrolyte compositions of the internal and external solutions used are given. The arrangement of the inlet (IN) and outlet (OUT) cannulae, the recording (R) and ground (E) electrodes and the tip of a thermocouple (TC) are illustrated on the top. A period of approx. 3 min was required to complete one cycle of temperature change. For further details, see text.

When the temperature of the surrounding fluid medium was raised gradually, the membrane potential changed very slowly at the outset. Again, at a critical temperature, the potential was found to change abruptly. (This abrupt change is indicated by the arrows directed downward in Fig. 2.) It is important to note that the critical temperature for this downward transition was much higher than that for the upward transition. In other words, there was a pronounced hysteresis in the state of the axon membrane brought about by cyclic temperature change. A similar pronounced hysteresis loop has been demonstrated in transitions induced by changing the external electrolyte composition[5].

(3) Fluctuation of the membrane potential near critical points

In both equilibrium and non-equilibrium thermodynamics, it is well known that the fluctuation in various measurable quantities of the system becomes very large and reaches a macroscopic level when the system becomes unstable at or near its critical point (see *e.g.* ref. 6, p. 104). The following observation shows that such a macroscopic fluctuation can actually be observed near transition points in the squid axon membrane.

Axons used in these observations were internally perfused with a dilute solution of CsF and were immersed in a slowly circulating external fluid medium containing $CaCl_2$ and NaCl. Initially, the Ca^{2+} : Na^+ concentration ratio in the medium was far above the critical level for an abrupt transition (see Trace A in Fig. 3). Then, the concentration ratio was gradually shifted toward the critical point as in the experiment

shown in Fig. 1. Fluctuations in the membrane potential, measured at the center of the perfused zone, are seen to increase gradually (see Traces B and C). Finally, the membrane underwent an abrupt transition to its depolarized state.

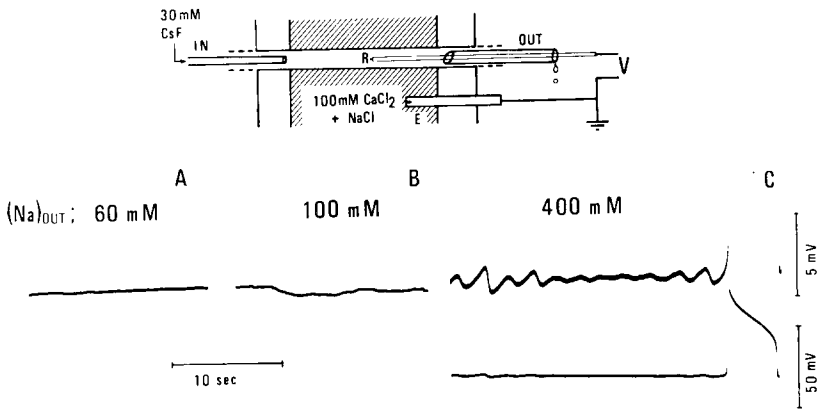

Fig. 3. Oscillograph records showing fluctuations in the membrane potential produced by addition of NaCl to the external medium. The electrolyte composition of the internal and external solutions are given. In C, a second oscillograph trace at a low voltage sensitivity (recorded simultaneously) is shown. Temperature, 19 °C.

As can be seen in Trace C, the amplitude of the fluctuation in the membrane potential was quite large. Since these large potential fluctuations appeared only when the external $Ca^{2+}:Na^{+}$ ratio was very close to the critical value, they come under the category of "giant fluctuations" in critical phenomena (see ref.7, p. 237). Near the critical point, the membrane potential waxed and waned spontaneously and the large potential jump took off from one of the peaks of the fluctuating membrane potential. There was a certain degree of periodicity in this potential variation. In axons internally perfused with a dilute solution of CsF, the period was of the order of 1–2 s. It is interesting to note that these giant fluctuations vanished completely following a large potential jump. As the membrane was brought close to the other transition point (*i.e.* the the critical point for repolarization) by raising the $Ca^{2+}:Na^{+}$ ratio in the external medium, giant fluctuations were found to appear again.

In intact axons as well as in those internally perfused with a dilute solution of potassium salt, it was difficult to demonstrate giant fluctuations in the membrane potential. This difficulty appears to be related to the shortness of the period of fluctuation and, hence, to the rapidity of the relaxation process following a transition. This was demonstrated by the following observation: An axon immersed in a solution containing both $CaCl_2$ and NaCl was internally perfused with a dilute solution of KF. Maintaining the external Na^{+} concentration at a constant level, the Ca^{2+} concentration was gradually reduced until repetitive firing of action potentials was finally observed. During the course of this gradual reduction in the $Ca^{2+}:Na^{+}$ ratio in the medium, the axon membrane was periodically subjected to brief pulses of weak inwardly directed current.

In a medium with relatively high Ca^{2+} concentration, a pulse of weak inward current produced a small change (hyperpolarization) in the membrane potential, which decayed roughly exponentially after the end of the pulse. When the $Ca^{2+}:Na^{+}$ ratio

was reduced, a pulse with the same intensity produced an oscillatory variation in the membrane potential. The oscillation was highly damped at the outset. However, as the external Ca^{2+} concentration was reduced further, the degree of damping was gradually diminished. Finally, immediately before the initiation of repetitive firing, a sustained (and often augmented) oscillation in the membrane potential was observed. The period of oscillation observed under these conditions was of the order of 10 ms, namely, shorter than about 1/100 of the corresponding period observed in axons perfused internally with CsF. This short period is considered to be a major factor which tends to desynchronize physiological activities at different sites of the membrane and to obscure fluctuations near the critical points.

DISCUSSION

In this study, the axon membrane was shown to undergo an abrupt transition from its resting to the depolarized state under a variety of experimental conditions. Besides electric stimuli, a rise in the extracellular univalent cation concentration and fall in the ambient temperature were found to be effective means of inducing such transitions. These findings can easily be understood on the assumption that a marked change in the conformational state of the axonal membrane is produced by a change in the univalent:divalent cation concentration ratio at the external surface layer of the membrane[4]. It is known that this layer possesses cation-exchange properties and that exchange of univalent cations for divalent cations in various artficial cation exchangers is exothermic. Therefore, it is expected that a change in the temperature creates a shift in the univalent:divalent cation concentration ratio in the membrane. A reverse process, namely, a transition from the depolarized to the resting state is known to be produced by a transient increase in the calcium salt concentration or by a heat pulse (see ref.8). The abrupt and discrete conformational changes are considered to represent phase transitions in the membrane macromolecules.

Additional evidence indicating the importance of the conformational states of membrane macromolecules may be found in a number of recent articles (see, *e.g.* refs 8–10). The relationship between the membrane potential and the state of the membrane has been discussed recently by one of us[11]. The present study of the behavior of the axonal membrane near the critical point may throw new light on the problem of oscillation of the membrane potential known since Arvanitaki's classical work[12].

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