EXCITABILITY OF SQUID GIANT AXONS IN THE ABSENCE OF UNIVALENT CATIONS IN THE EXTERNAL MEDIUM

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Many excitable tissues are able to develop action potentials in the absence of any salt of univalent cation in the external medium. Osterhout and his associates[1] have shown that *Nitella* cells, which have been rendered inexcitable by prolonged immersion in distilled water, regain their excitability when calcium salts are added to the external medium. Fatt and Katz,[2] Hagiwara, Chichibu, and Naka,[3] and others have demonstrated that crustacean muscle fibers can produce large action potentials in media containing only salts of divalent cations. Frog ventricles[4] and nerve cells in frog dorsal root ganglia[5, 6] also are known to remain excitable in the absence of any univalent cations in the surrounding fluid medium.

In contrast to the excitable systems stated above, it is generally believed that the squid giant axon requires sodium salts or other univalent cation substitutes for sodium in the external medium to maintain excitability. This article describes a series of observations demonstrating that under intracellular perfusion,[7, 8] squid giant axons are capable of developing large action potentials in the absence of univalent cations in the external medium.

In previous articles from this laboratory,[8, 9] detailed description has been made of the order of "favorability" of various inorganic and organic salts in the intracellular perfusion fluid. It has been shown that the common univalent cations form the following order of (decreasing) favorability:

$$Cs > Rb > K > NH_4 > Na > \ldots$$

and that the anions form the following order:

$$F > \text{phosphate} > \text{glutamate, aspartate} > \ldots .$$

In order to demonstrate excitation in the absence of univalent cations in the external medium, a combination of the most favorable cations and anions was used in the internal fluid medium (cesium fluoride and/or phosphate, in most cases).

Methods.—The technique of intracellular perfusion used in this study is described in the preceding articles[7, 8] of the present series. A giant axon was obtained from *Loligo pealii* (available at the Marine Biological Laboratory, Woods Hole, Mass.) and mounted horizontally in a Lucite chamber (approximately 33 mm in length). Two glass cannulae were inserted into the ends of the axon. The smaller cannula (approximately 150 μ in outside diameter) was connected to the reservoir of intracellular perfusion fluid. The larger cannula (roughly 300 μ in diameter) was used for drainage of the fluid and for guiding the intracellular stimulating and recording electrodes into the perfusion zone of the axon (see Fig. 1, *top*).

In most of the present studies, the axoplasm in the perfusion zone was removed by the method of enzymatic digestion first described by Tasaki, Singer, and Takenaka[8] and later greatly improved by Takenaka and Yamagishi.[10] The enzyme-containing internal perfusion fluid was usually prepared by mixing 12 (vol) % glycerol solution with a 600 mM CsF solution, and subsequently adding a protease, "pronase" (Calbiochem), to a concentration level of 0.05–0.1 mg/ml. The pH of the solution was adjusted to 7.3 ± 0.1 by adding a small amount of Cs-phosphate buffer. The concentration ratio (expressed in equivalent/liter) of fluoride to phosphate was usually 10:1.

[Chlorphenol red (0.1 mg/ml) was frequently used for identification of the enzyme solution.]

In later experiments, the following experimental procedure was employed. Two perfusion cannulae were introduced in an axon immersed in natural sea water. With the two cannulae overlapping in the center of the axon, flow of the extracellular fluid (containing only a salt of divalent cation) was initiated; the rate of fluid flow was 10–30 ml/min. (The chemical compositions of the external fluid will be described later.) This procedure eliminated axon excitability within 1 min. Next, flow of the intracellular fluid from the inlet cannula through the overlapping outlet cannula was instituted. With the flow of both the external and internal solutions maintained, the two glass cannulae in the axon were separated so as to initiate perfusion of the axon with the protease-containing solution. The length of the internal perfusion zone was 12–20 mm. Approximately 1.5 min after separation of the two cannulae, the original intracellular perfusion fluid was switched to an enzyme-free solution of the same composition. Finally, a simulating and a recording electrode were introduced through the outlet cannula into the center of the perfusion zone of the axon.

The internal stimulating electrode was an enameled silver wire (50 μ in diameter) with a 20-mm-long uninsulated portion at the end. Stimulating currents were obtained from a Tektronix pulse generator through a 2- (or 20-) megohm resistor. The rectangular stimulating current pulses were applied between the internal electrode and a large Ag wire ground electrode immersed in the external fluid medium. The recording electrode was either an enameled Ag wire (50 μ in diameter) with a 1-mm-long uninsulated portion at the tip, or a glass capillary (approximately 100 μ in diameter) filled with a 600-mM KCl solution. The recording tip of the electrode was located in the center of the internal perfusion zone. The intracellular potential was recorded with a Tektronix oscilloscope.

Most of the experiments described in this article were carried out at room temperature (18–22°C). In one series of experiments, essentially the same results were obtained when temperature of the circulating external fluid medium was maintained between 10 and 16°C.

Results.—(1) *All-or-none action potentials in axons immersed in calcium chloride solutions:* In the first series of observations, the external salt solutions were prepared by mixing 400 mM $CaCl_2$ (or $CaBr_2$) solution with 12 (vol) per cent glycerol solution. In most cases, a small amount of tris-(hydroxymethyl)aminomethane (approximately 1 mM) was added to the external medium to maintain the pH at 8.0. (Several experiments in which no pH-buffer was added to the external medium yielded the same results as those described below.) The concentration of the Ca-salt was varied between 50 and 400 mM.

When the external medium (natural sea water) was replaced with the Ca-salt solution prior to initiation of intracellular perfusion, the axon invariably became completely inexcitable. Following removal of the axoplasm with pronase, most axons were found to regain their ability to respond to stimulating current pulses when intracellular perfusion with a dilute (25–100 mM) Cs-salt solution was instituted. An electric response to an outward-directed membrane current could be recognized at the beginning as a small, transient potential rise superposed on the exponentially rising potential variation caused by the stimulating current. Under continuous intracellular perfusion with a dilute CsF solution, there was, as a rule, a gradual augmentation in the amplitude and duration of the response. After 3–10 min of intracellular perfusion, stimulating current pulses evoked, in most axons, large and prolonged action potentials in an all-or-none manner.

An example of the records of action potentials obtained by this procedure is presented in Figure 1. In this example the threshold current intensity was approximately 2 μA for a stimulus duration of 10 msec. (The area of the membrane under study was roughly 0.3 cm^2.) For current pulses far below the threshold, the variation in the membrane potential caused by the pulse was approximately ex-

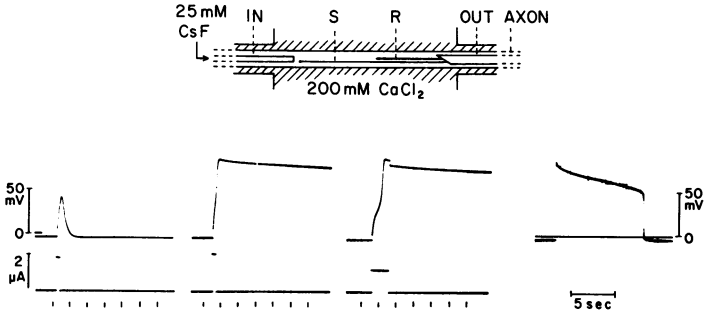

FIG. 1.—*Top:* Schematic diagram showing the experimental arrangement used to obtain the oscillograph records shown below. The inlet cannula (*IN*), outlet cannula (*OUT*), stimulating wire electrode (*S*), and recording glass capillary electrode (*R*) are shown. The distance between the tips of the two cannulae was approximately 20 mm. The diameter of the axon was approximately 500 μ. *Bottom:* Records of action potentials observed in the absence of univalent cation in the external fluid medium. In the first record (extreme left), the stimulating current pulse (indicated by the lower oscillograph trace) was subthreshold. The time markers are 50 msec apart. The last record was taken at a slower sweep speed. The zero level for the potential trace indicates the potential observed when the recording electrode (filled with 0.6 *M* KCl) was placed in the outside fluid medium. 21°C.

ponential. As the current intensity approached the threshold, a variable (graded) "subthreshold" response was generally observed. For current intensities greater than the threshold, the membrane potential attained a level which was nearly independent of the intensity (all-or-none).

The amplitude of the action potentials observed under these conditions was somewhat variable from preparation to preparation; in most cases, it was 60–90 mV. On several occasions, action potentials of about 100 mV in amplitude were observed. In a given preparation, the action potential amplitude increased with the external $CaCl_2$ concentration. (At a calcium concentration of 50 mM, there was frequently spontaneous firing of action potentials.)

The duration of an action potential observed under these conditions was very long; it was, in most cases, 0.1–20 sec. In the range of calcium concentrations between 100 and 400 mM, there was a definite tendency for the action potential duration to decrease as the Ca-concentration increased.

The configuration of the action potential was very similar to that of the axon treated internally with TEA (tetraethylammonium-ions).[11] As in TEA-treated axons, the phenomenon of "abolition" of action potentials could be demonstrated by application of brief pulses of inward-directed current pulses. When the duration of the action potential was very long, a brief pulse of inward membrane current applied during the plateau of the action potential produced an "all-or-none response with reversed polarity" similar to those observed in the frog dorsal root ganglion.[6]

In an axon immersed in 200 mM $CaCl_2$ solution and internally perfused with a 25–50 mM CsF (glycerol) solution, the effective membrane resistance was of the order of 10^4 ohm·cm^2 (at rest). When such an axon developed an action potential, there was a profound fall in the membrane resistance (determined by measurement of the changes in membrane potential produced by rectangular current pulses). The fall in the membrane resistance at the peak of action potential was estimated to be approximately one sixth of the resting value. During the period where the

action potential slowly declined, the membrane resistance gradually approached the value observed in the resting state.

The resting membrane potential was determined with a glass capillary electrode filled with 600 mM KCl solution; the resting potential was operationally defined as the difference in the potential levels observed with the tip of the electrode in- and outside the axon. With 25–50 mM CsF (glycerol) internal solution and 200 mM $CaCl_2$ external solution, the resting potential was found to be between 0 and -10 mV, the average being approximately -4 mV (negative inside).

The experiment described above was highly reproducible. The ability of a cleaned squid giant axon to produce large, all-or-none action potentials could be maintained for more than 1 hr in continuously and rapidly circulating $CaCl_2$ solution. During the initial 3–10-min period after initiation of intracellular perfusion of such an axon, there was a gradual increase in the amplitude of the action potential. The action potential could be suppressed immediately by replacing the $CaCl_2$ solution with circulating 400 mM $MgCl_2$ solution. When the external circulating fluid was switched back to the original $CaCl_2$ solution, the excitability could be restored immediately. All these findings indicate that the observed action potentials are manifestations of excitation of the squid giant axon in the absence of univalent cations in the external medium.

(2) *Effect of pronase on axon membrane:* In the observations described in the preceding section, a dilute (0.05–0.1 mg/ml) solution of a protease, pronase, was used for a brief (1.5-min) period of time to remove the axoplasm in the perfusion zone (see *Methods*). Therefore, tests were made to determine whether or not this enzyme had any discernible effect on the normal physiological properties of the axon membrane. It was found that axons immersed in natural sea water could survive for more than 20 min under continuous intracellular perfusion with a 400-mM KF solution containing 0.25 mg/ml of pronase. All of the characteristics of the observed action potential appeared to be normal until the stage immediately before enzyme action led to the loss of excitability. This finding is consistent with the result obtained by Takenaka and Yamagishi,[10] using "prozyme" (Kyowa Hakko) instead of pronase. ["Prozyme" and "nagarse" (Nagase) were also used in the present investigation with *Loligo pealii;* the results obtained were almost indistinguishable from those obtained with pronase. With trypsin (Worthington) used in place of pronase, responses observed in axons immersed in 200 mM $CaCl_2$ solution appeared to be slightly smaller than those described in the preceding section.]

Axons treated with pronase exhibit a strong dependence on the external Na-concentration. An example of these experiments is shown in Figure 2, where an axon immersed in a solution containing 450 mM NaCl and 100 mM $CaCl_2$ was internally perfused with a 400-mM KF (glycerol) solution containing 0.25 mg/ml pronase. The enzyme was allowed to digest the axoplasm for a period of 1.5 min; following this period, intracellular perfusion was continued with an enzyme-free solution of the same composition. Approximately 12 min after the onset of intracellular perfusion, the Na-ion in the external medium was replaced with TEA-ion. As can be seen in the figure, the ability of the axon to produce action potentials was immediately suppressed by this procedure. When Na-ion was reintroduced into the external medium, excitability was promptly restored. This observation supports

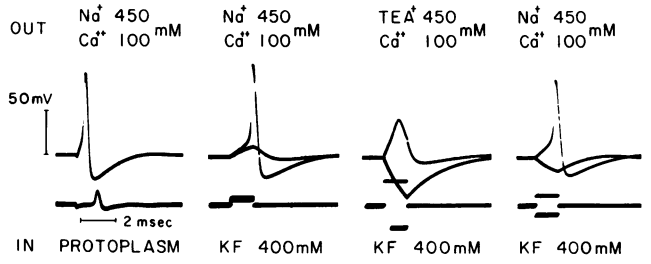

FIG. 2.—Oscillograph records showing the Na-dependence of the action potential after removal of the axoplasm with a protease, "pronase." The first record was taken before and the following three records after treatment with pronase. The external and internal media of the axon are indicated. The lower trace in the first record shows an extracellularly observed action potential. In the other records, the lower trace shows the time course of the stimulating current pulses applied through a wire electrode (20 mm in length). 22°C.

the view that pronase does not appreciably alter the normal properties of the axon.

In another series of experiments, attempts were made to demonstrate the excitation phenomena described in the preceding section without using pronase. When an enzyme-free dilute CsF solution was used as the internal perfusion fluid, it was extremely difficult to maintain intracellular flow. (This difficulty is due to swelling of the axoplasm in solutions of low ionic strength.) When flow stopped, perfusion could be resumed only by removal of the axoplasm by suction applied to the outlet cannula; this procedure frequently injured the axon membrane mechanically.

In a few cases, it was possible to maintain continuous flow of the dilute CsF solution without using the proteolytic enzyme. In these cases, all-or-none action potentials were obtained from axons in an external medium containing only $CaCl_2$ and glycerol. In other cases, flow of the internal fluid was maintained only for a period of several minutes; in these axons only graded responses were observed.

(3) *Action potentials observed with strontium- or barium-salts in the external medium:* In axons under continuous intracellular perfusion with a dilute CsF solution, an external medium containing $SrCl_2$, $SrBr_2$, or $BaCl_2$ could be used to maintain excitability. Examples of action potentials observed under these conditions are shown in Figure 3. Solutions were prepared as described in *Methods* and section 1. The electric responses observed under these conditions were very similar to those

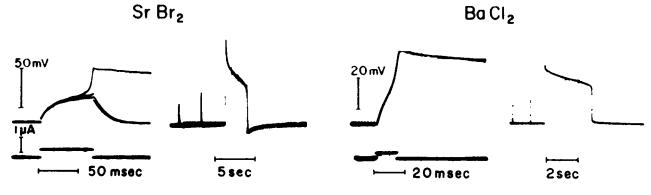

FIG. 3.—*Left:* Record of all-or-none action potentials observed in an axon immersed in a 100-mM $SrBr_2$ solution and internally perfused with a 25-mM CsF solution. The record at the left was obtained approximately 7 min and the record at the right (taken at a slower sweep speed) about 17 min after the onset of intracellular perfusion. Note that there was an increase in the action potential amplitude during the 10-min interval. 20°C. *Right:* Action potentials obtained form an axon immersed in 200 mM $BaCl_2$ solution and internally perfused with a 25-mM CsF solution. The experimental procedure used was similar to that in the experiment of Fig. 1. 21°C.

obtained in Ca-salt solutions. In axons immersed in $SrCl_2$ or $SrBr_2$ solutions, the average amplitude and the duration of the responses were similar to those in $CaCl_2$ solutions. However, in $BaCl_2$ solutions the amplitude of the response appeared to be smaller than the response obtained in Ca-salt solutions.

Attempts were made to demonstrate all-or-none action potentials in axons immersed in solutions containing only Mg-salts. It was not possible to maintain excitability in a solution of pure magnesium chloride or sulfate, under the conditions used for the preceding experiments. When an external $CaCl_2$ solution was replaced with $MgCl_2$ solution at the same concentration, there was a reversible suppression of excitability (see § 1).

Discussion.—The experimental findings described under *Results* indicate that the presence of univalent cations in the external fluid medium is not an essential condition for the maintenance of excitability, and that the external divalent cations are important in the production of action potentials. These results also demonstrate that the presence of the most favorable cation (cesium) in the axon interior is needed for the maintenance of excitability under these conditions. (It was found that replacement of about 5% of the internal Cs-ion with Rb-, K-, or Na-ion had little or no effect on the axon excitability; but replacement of a greater fraction frequently suppressed the action potential.)

The experimental results described in this article strongly support the macromolecular interpretation of the process of nerve excitation and the "two-stable-states" theory proposed in previous articles from this laboratory.[8, 11, 12] In this interpretation, external divalent cations and internal univalent cations play important roles. According to this interpretation, the outer layer of the axon membrane possesses a relatively high density of fixed negative charge. In the resting state of the membrane, these negative sites are occupied predominantly by divalent cations derived from the external medium. An outward-directed (stimulating) current through the membrane replaces a fraction of the divalent cations in the (resting) membrane with univalent cations (Cs-ions in the present case) derived from the internal medium. The replacement of intramembrane divalent cations with univalent cations produces a cooperative, macromolecular "phase transition" which transforms the membrane macromolecules into their excited state; in this macromolecular conformation the predominant counterions in the (excited) membrane are univalent cations. [In the field of colloid and polymer chemistry, there are several observations showing that changes in the mole-ratio between the divalent and univalent cations in the medium product "phase transitions" (see, e.g., Clowes[13] and Michaeli[14]).]

In the excited state of the membrane, there is an increased interdiffusion of cations across the membrane, associated with a reduction in the membrane resistance. The increase in interdiffusion slowly alters the ionic environment of the membrane and produces a gradual change in the membrane potential and resistance. When the alteration of the ionic environment reaches a certain critical level, the membrane undergoes a transition to the resting state, accompanied by a sudden fall in the membrane potential. Following this transition, the ionic environment of the membrane gradually returns to normal.

According to the macromolecular interpretation of the mechanism of excitation outlined above, the "favorability" of Cs-ion in the internal fluid medium derives

from its ability to displace the divalent cation in the membrane without disrupting the membrane structure. The difficulty of obtaining action potentials from axons immersed in pure Mg-salt solutions may be interpreted as indicating that in the (resting) state of the membrane Mg-ions are weakly bound to the membrane (compared to Ca-ions) and cannot be displaced cooperatively by Cs-ions.

In previous articles from this laboratory[8, 12] it has been stressed that the squid axon membrane has a multilayered structure. As examined with various enzymes and neutral salts, the properties of the outer layer of the membrane are very different from those of the internal layer. Therefore, calculation of the membrane potential, based on the theory of charged membrane,[15–17] is difficult even under relatively simple experimental conditions.

Summary.—Under continuous intracellular perfusion with a dilute CsF (or Cs-phosphate) solution, squid giant axons immersed in media containing only salts of divalent cations were found to maintain their ability to produce all-or-none action potentials. The experimental results are interpreted in terms of two stable conformations for the membrane macromolecules, and support the two-stable-states theory of excitation.

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