BI-IONIC ACTION POTENTIALS IN SQUID GIANT AXONS INTERNALLY PERFUSED WITH SODIUM SALTS

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Previous studies from this laboratory[1] have demonstrated that squid giant axons, internally perfused with dilute solutions of cesium phosphate or fluoride, maintain electrical excitability in an external medium containing only the salt of a divalent cation (calcium, strontium, or barium). It was emphasized that the presence of univalent cations (such as sodium) externally is not essential for production of all-or-none action potentials under these conditions. The term "bi-ionic" action potentials has been introduced[2] to designate electric responses produced under these conditions in which only two critical cations (a univalent ion internally and a divalent ion externally) are involved in the process of excitation.

The present report deals mainly with the electrophysiological properties of squid giant axons internally perfused with dilute solutions of a sodium salt. With sodium as the sole intracellular cation, squid giant axons were found to maintain excitability in external media containing a calcium salt as the sole electrolyte species.[2] It is to be noted that the gradient of the sodium ion concentration in these axons is opposite to that of unperfused axons immersed in sea water. These experimental findings may be explained in terms of the hypothesis postulating a conformational change (phase transition) of membrane macromolecules associated with a cooperative ion exchange process between uni- and divalent cations at negatively charged sites of the macromolecules.[3, 4]

Methods.—Giant axons from *Loligo pealii,* available at the Marine Biological Laboratory in Woods Hole, Massachusetts, were used. The major portion of small nerve fibers and other adherent tissue surrounding the giant axon were removed under a dissecting microscope during the process of dissection. The technique of internal perfusion used in the present study was substantially the same as that used in previous studies from this laboratory.[1] The giant axon was mounted horizontally in a Lucite chamber (approximately 33 mm in length) containing natural sea water, and a glass cannula was inserted into each end of the preparation. Initially, the tip of the smaller, inlet cannula (150 μ in diameter) was placed concentrically within the lumen of the larger, outlet cannula (250–300 μ in diameter). Internal perfusion was initiated by separating the two cannulae. The inlet cannula was connected to a reservoir of internal perfusion fluid by means of a small polyethylene tubing. In most of the present experiments, the length of the internal perfusion zone was between 14 and 21 mm.

Internal perfusion solutions were usually prepared by mixing a 12 (vol) % glycerol solution with a 600 mM sodium phosphate solution (pH 7.3 $\pm$ 0.1).[4a] In order to remove the axoplasm from the perfusion zone, perfusion was instituted with a sodium phosphate solution containing 0.05 mg/ml Pronase[1, 5] (Calbiochem) for a period of 1 to 1.5 min and then switched to enzyme-free solution. The flow rate of the internal perfusate, controlled by adjusting the height of the fluid reservoir, was maintained at 15–20 μl/min.

External fluid medium was, as a rule, a mixture of 400 mM $CaCl_2$ and 12 (vol) % glycerol; the pH was adjusted to 8.0 $\pm$ 0.1 with tris(hydroxymethyl)aminomethane buffer (less than 1 mM). (Note that the presence of a pH buffer in the external medium is not essential for the maintenance of bi-ionic action potentials.[1]) Continuous flow of the external medium was maintained at 10–30 ml/min throughout the course of the experiments. A trap served to electrically isolate the suction apparatus used to drain the chamber from the external fluid medium.

The internal stimulating electrode was an enameled silver wire (50 μ in diameter) with a 15-mm uninsulated portion at the end. Stimulating currents were obtained from a Tektronix pulse generator and delivered through a 10-megohm resistor. Prior to initiation of internal perfusion, extracellular stimulating electrodes (platinum), located near the site of recording, were employed.

The fluid in the chamber was grounded either with a calomel electrode (Beckman) or with a large coil of chloridized silver wire. The calomel electrode was placed in the continuously flowing external media in such a manner that KCl diffusing out of the electrode could not reach the axon. The intracellular potential was measured with a 100-μ glass pipette filled with 600 mM KCl solution used in conjunction with a calomel electrode; the connection between the KCl solution and the calomel electrode was made with KCl-agar gel. The electric resistance of the recording electrode was generally between 3 and 5 megohms. In several experiments, an internal silver wire electrode was used for recording bi-ionic action potentials; no detectable difference was observed between the action potential amplitudes determined with a silver wire electrode and those measured with a KCl-calomel electrode.

Results.—(1) *Demonstration of bi-ionic action potentials in axons internally perfused with sodium salt solution:* The experimental procedure used to demonstrate electrical excitability in intracellularly perfused axons was as follows: After inserting the two perfusion cannulae longitudinally into the axon (with the tip of the inlet cannula concentrically within the lumen of the outlet cannula), rapid flow of the extracellular perfusion fluid was initiated. When the external fluid contained only the salt of a divalent cation, the action potential of the axon with normal axoplasm (i.e., as yet unperfused internally) was suppressed. Then, intracellular perfusion with a dilute solution of sodium phosphate or fluoride was initiated, and the ability of the axon to produce action potentials was tested from time to time by the use of internal stimulating and recording electrodes. By this procedure, squid giant axons internally perfused with 5 to 50 mM sodium phosphate or fluoride were found to regain and then maintain excitability for up to two hours in external media containing 30 to 100 mM $CaCl_2$ as the sole electrolyte species. (Under these conditions, all-or-none action potentials were demonstrated in 55 out of 58 axons examined.) With concentrations of Ca-ion externally in excess of 100 mM, it was difficult to maintain excitability for a long period of time.

Figure 1 shows records from an experiment demonstrating excitation under these bi-ionic conditions. Record *A* was obtained from an axon (containing normal axoplasm) immersed in a solution containing 300 mM NaCl, 100 mM $CaCl_2$, and glycerol (to maintain tonicity). The action potentials observed under these conditions were large (approximately 100 mv) in amplitude and short (1 msec) in duration. Then, the rapidly flowing external medium was switched to a 100 mM $CaCl_2$ (glycerol) solution; in this Na-free external medium the action potential disappeared within approximately one minute (record *B*). Next, by separating the tips of the two perfusion cannulae, intracellular perfusion with a 10 mM sodium phosphate (glycerol) solution was initiated (see *Methods*). With the flow rates of the intra- and extracellular perfusion fluids maintained at constant levels, there was a gradual increase in the amplitude of potential changes produced by outward-directed (stimulating) current pulses. All-or-none action potentials (see record *C*) were observed about 12 minutes after the onset of internal perfusion.

The time required for axons to regain their ability to develop all-or-none action potentials varied widely from preparation to preparation. In general, the time was shorter when a longer portion of the axon was exposed to the internal perfusion fluid. With a 15-mm-long portion of the axon interior exposed to a 10–30 mM

sodium phosphate solution, and with a 100 mM $CaCl_2$ solution externally, an approximately steady state was usually reached between 5 and 15 minutes after the onset of intracellular perfusion. The amplitude of the observed action potentials was generally between 45 and 70 mv, and the duration of the action potentials between 0.2 and 20 seconds. The resting membrane potential in axons with 30 mM sodium phosphate internally and 100 mM $CaCl_2$ externally was usually 25 to 40 mv (inside negative) once a steady state was reached. The resting membrane resistance in the steady state was of the order of 10 kilohm·cm^2. At the peak of the action potential, the resistance was estimated to be between one-twentieth and one-tenth the resting value (determined by measurement of changes in membrane potential produced by rectangular current pulses).

Replacement of the external $CaCl_2$ solution with $MgCl_2$ solution of the same concentration always resulted in a reversible suppression of excitability. However, excitability could be maintained with external media containing a mixture of Mg and Ca (40 mM $MgCl_2$ plus 10 mM $CaCl_2$) in concentrations close to that of natural sea water. Substitution of calcium bromide or ethylsulfate for $CaCl_2$ had no significant effect on the excitation process.

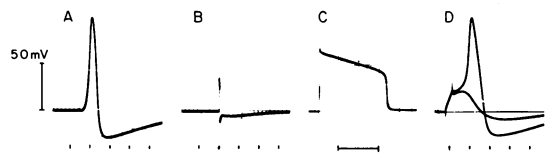

FIG. 1.—Oscillograph records demonstrating development of an all-or-none action potential in an axon perfused internally with sodium phosphate solution and externally with $CaCl_2$ solution. Records *A* and *B* were taken before initiation of internal perfusion; the external medium contained 300 mM NaCl and 100 mM $CaCl_2$ in *A* and only 100 mM $CaCl_2$ in *B*. The time markers are 1 msec apart. Record *C* was taken approximately 12 min after the onset of internal perfusion with 10 mM sodium phosphate; the external medium was 100 mM $CaCl_2$ (as in *B*). The time marker represents 10 sec. Record *D* was obtained from the same axon after switching the internal perfusion fluid to 400 mM KF and the external medium to the solution used in *A;* sub- and suprathreshold responses are superposed. Time markers are 1 msec apart. The stimulus duration was 0.1 msec for *A* and *B*, 100 msec for *C*, and about 0.3 msec for *D*. Axon diameter: approximately 400 μ. 21°C.

Record *D* in Figure 1 was taken to demonstrate that the bi-ionic experimental conditions produced no permanent alteration in the electrophysiological properties of the axon membrane. After typical, prolonged action potentials under the bi-ionic conditions were demonstrated, the internal perfusion fluid was switched to a 400 mM KF solution and the external medium to the initial solution containing 300 mM NaCl and 100 mM $CaCl_2$. Both the amplitude and the configuration of the recorded action potential were very similar to those of the action potential observed before initiation of internal perfusion (compare *A* and *D*). (Note that the method of stimulation for the last record was different from that for the first record.)

(2) *Action potential overshoot and cannula artifact:* It is generally believed that the peak level, or the overshoot, of the action potential in the squid giant axon is

determined by the ratio of the sodium-ion concentrations across the axon membrane. The following expression is generally used to describe this overshoot:

$$E = \frac{RT}{F} \ln \frac{[\mathrm{Na}]_e}{[\mathrm{Na}]_i},$$

where E is the peak value of the action potential referred to the potential level in the surrounding medium, RT/F has the usual thermodynamic meaning (about 25 mv at 20°C), and $[\mathrm{Na}]_e$ and $[\mathrm{Na}]_i$ are the extra- and intracellular sodium-ion concentrations, respectively. Under the experimental conditions of record C in Figure 1, the external medium was sodium-free, i.e., $[\mathrm{Na}]_e = 0$, while the internal sodium concentration, $[\mathrm{Na}]_i$, was finite. The peak of the action potential expected from the sodium-ion concentration ratio across the axon membrane is, therefore, a

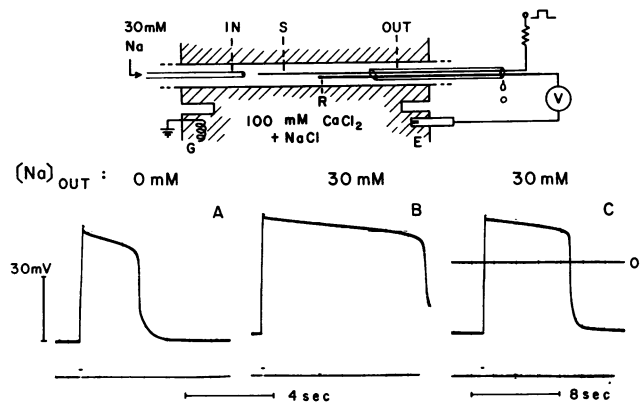

FIG. 2.—*Top:* Schematic diagram showing the experimental arrangement used to determine the resting and action potentials of axons internally perfused with sodium salt solutions. The inlet cannula (*IN*), outlet cannula (*OUT*), stimulating wire electrode (*S*), recording glass-pipette electrode (*R*), external calomel electrode (*E*), and Ag-AgCl ground electrode (*G*) are indicated. The recording electrodes (*R* and *E*) were connected to differential inputs of an oscilloscope via a high input-impedance differential amplifier (designed by A. Bak). The axon is represented by two horizontal lines enclosing the cannulae.

Bottom: Resting and action potentials recorded from an axon internally perfused with a 30 mM sodium salt solution. The external media contained 100 mM $CaCl_2$ for all the records. The external sodium concentrations were varied as indicated. The internal anion was a 1:1 mixture of fluoride and phosphate in this case. Stimuli used were 100 msec in duration and approximately 1.5 $\mu a/cm^2$ in intensity (indicated by the lower trace). Note that record *C* was taken at a slower sweep speed. In record *C*, the potential recorded when the internal recording electrode was withdrawn and placed in the external medium was superposed on the action potential trace. Axon diameter: approximately 450 μ. 20°C.

large negative value. Actually, however, the observed level at the peak of the bionic action potential was positive (inside) and was roughly 20 mv above the potential level of the external medium in most axons examined.

When the intracellular perfusion zone is relatively short, action potentials recorded with a high-resistance recording electrode may be contaminated by the so-

called "cannula artifact," which can give rise to a spurious estimate of the action potential overshoot.[6] Such an artifact can be avoided by the precautions described elsewhere,[7] and is negligible when the rate of potential change is very small. Under bi-ionic conditions, the fall in the membrane potential from the peak of the action potential is very slow. Consequently, it is safe to conclude that the cannula artifact makes no contribution to present measurements.

Figure 2 shows the effect of addition of a small amount of sodium salt to the external medium. Record *A* was taken approximately 13 minutes after the onset of internal perfusion with a 30 mM sodium salt (glycerol) solution; the external medium was a 100 mM $CaCl_2$ (glycerol) solution at this moment. (These experimental conditions are similar to those for record *C* in Figure 1.) Next, the rapidly flowing external fluid medium was switched to a solution containing 30 mM NaCl, 100 mM $CaCl_2$, and glycerol (thereby eliminating a concentration difference for sodium across the axon membrane). By this procedure, the resting potential was reduced by about 4 mv (less negative inside) and the action potential amplitude was increased by about 4 mv (see record *B*). Immediately after photographing an action potential under these conditions, the glass-pipette recording electrode was moved from the axon interior to the external fluid medium and the oscillograph trace representing the potential level in the external medium was superposed on the action potential record (Fig. 2*C*). Records obtained in this manner with 13 axons indicated that a sizeable action potential overshoot exists under these experimental conditions.

Resting potential decreased (inside became less negative) and overshoot of the action potential showed a tendency to increase gradually (with time) under continuous internal and external perfusion. When both the resting and action potential reached approximately steady levels, the overshoot observed with 30 mM Na-ion on both sides of the axon membrane (and 100 mM $CaCl_2$ outside) was usually between 20 and 25 mv. The existence of an action potential overshoot with equal sodium-ion concentrations across the membrane has been reported previously.[7]

(3) *Bi-ionic action potentials with intracellular cations other than sodium:* Further experiments have shown that it is possible to demonstrate bi-ionic action potentials, qualitatively similar to those described above, with a dilute internal solution of the phosphate salt of any of the following univalent cations: Li, tetramethylammonium, tetraethylammonium, tetrapropylammonium, choline, ethylamine, ethanolamine, guanidine, or methylguanidine.[2] Quantitative aspects of the excitation process (amplitude and shape of the action potential) were found to be profoundly influenced by variation of the intracellular cation as well as by the addition of univalent cation to the external medium. Analyses of these aspects of the excitation process are now in progress.

Discussion.—It was shown under *Results* that squid giant axons internally perfused with dilute (5–50 mM) sodium salt solutions are capable of producing action potentials in external media containing only the salt of a divalent cation (30–100 mM $CaCl_2$). Under these conditions, the total external divalent cation concentration is very close to that of natural sea water,[8] and the internal sodium ion concentration is of the same order as that found in normal squid axoplasm.[9] These experimental findings indicate:

(1) Axons maintain excitability with the usual concentration gradient for sodium across the axonal membrane reversed (Fig. 1*C*).

(2) The presence of sodium ion externally is not essential for the excitation process (Figs. 1*C* and 2*A*).

(3) Action potential overshoots are not determined solely by the sodium concentration ratio across the membrane (Fig. 2*B*, *C*).

It is generally believed that action potentials are generated by a specific increase of membrane permeability to sodium ions. If this were the case, action potentials evoked under the bi-ionic experimental conditions (Fig. 2*A*) would be expected to be opposite in polarity, and voltage clamping would be expected to reveal an outward-directed early membrane current in response to a depolarizing voltage pulse. In fact, voltage clamp experiments (unpublished) revealed early inward-directed membrane currents in these axons. It should be emphasized that action potentials with sizeable overshoots were observed without a sodium ion concentration difference across the membrane (Fig. 2*B*, *C*). It follows, then, that a specific increase of sodium permeability and influx of sodium ions into the cell cannot be the primary cause of the excitation process under the present experimental conditions.

The experimental findings may be satisfactorily explained by the "two-stable-states" hypothesis which postulates the existence of two stable conformational states of the membrane macromolecules.[3] According to this hypothesis, transitions between the two stable conformations are brought about by a cooperative process involving ligands and membrane macromolecules. A similar argument has been developed by Changeux *et al.*[10]

In the resting state, the external layer of the axon membrane is rich in divalent cations derived from the external medium. A strong outward-directed (stimulating) current through the membrane replaces a portion of the divalent cations with univalent cations derived from the axon interior, triggering a transition of the membrane macromolecules to their conformation in the excited state. This transition brings about an increase in ion mobilities and a change in the ion selectivity of the membrane which, in turn, alter the membrane potential and conductance. Termination of the action potential is attributed to a decrease in the univalent cation concentration within the membrane (as the consequence of diffusion into the external medium), resulting in a return of the membrane macromolecules to their resting conformational state.[3, 4]

Summary.—Squid giant axons, internally perfused with dilute sodium salt solutions, were found to maintain excitability in external media containing the salt of a divalent cation as the sole electrolyte species. Under these bi-ionic conditions, with the usual gradient for sodium across the cell membrane reversed, action potentials with overshoots of approximately 20 mv were observed. Elimination of the sodium concentration difference across the axon membrane did not affect the ability of the axons to produce all-or-none action potentials. These findings are consistent with the "two-stable-states" theory of excitation which proposes that a conformational change of membrane macromolecules, associated with a cooperative ion-exchange process at negatively charged sites of the macromolecules, is the primary event in excitation.

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