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EFFECTS OF VARIOUS POTASSIUM SALTS AND PROTEASES UPON EXCITABILITY OF INTRACELLULARLY PERFUSED SQUID GIANT AXONS

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The ability of the squid giant axon to develop action potentials can be preserved for hours under continuous intracellular perfusion if solutions of the proper salts are chosen for perfusion.[1,2]   In the early stage of the development of the perfusion technique, a great emphasis was placed on the importance of the proper choice of intracellular cations for maintenance of axonal excitability.   Recently, however, it became increasingly clear that intracellular anions exert a great influence upon the resting and action potentials of the axon.

The first half of the present article deals with the results of a systematic study of the effects of various anions in the intracellular perfusion fluid on the bioelectric potentials of the axons.   It is shown that the anions examined can be arranged according to their effects in an order similar to the classical lyotropic series in protein chemistry[3] and in muscle physiology.[4]

The second half of this article deals with the effects of various proteolytic (and other) enzymes in the perfusion fluid upon the resting and action potentials.   As recently observed by Rojas and Luxoro,[5] intracellularly administered proteases produce strong injurious effects upon giant axons while these enzymes are totally ineffective when applied externally.   These and other findings described in this article suggest the existence of a close relationship between the polypeptides in the membrane and the excitability of the giant axon.

Methods.—Partially cleaned giant axons of *Loligo pealii* were used in the present study.   The details of our technique of intracellular perfusion were described in a previous article of this series.[1]   The glass pipette for introducing the perfusion fluid into the axon was approximately 160 μ in outside diameter, and the glass drainage pipette was roughly 300 μ.   The length of the perfusion zone varied between 12 and 25 mm.   The resting and action potentials of the axons were determined under continuous perfusion with a movable glass pipette electrode which was introduced into the axon through the drainage pipette.   The electrode was approximately 100 μ in diameter, and was filled with isotonic KCl solution.   Stimulating current pulses were delivered to the axon near the end of its perfused zone.   A Bak unity-gain electrometer (Electronics for Life Sciences), a Tektronix oscillograph (type 502), and a Grass camera were used for recording bioelectric potentials.

Perfusion fluid was prepared by mixing 12.5 vol per cent glycerol solution with various isotonic salt solutions.   The pH of the solution was adjusted to 7.2–7.3
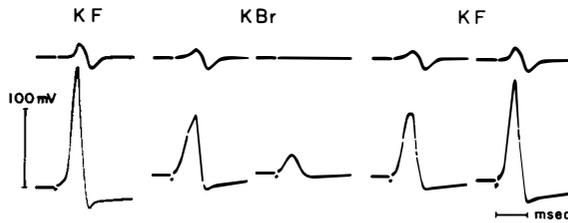
FIG. 1.—Intracellular perfusion of a squid giant axon first with 400 mM KF (*left*), next with 400 mM KBr (*middle*), and then again with the original KF solution (*right*). The lower oscillograph tracing shows intracellularly recorded resting and action potentials in the middle of the perfusion zone. The upper tracing represents extracellular recording of impulses propagated across the perfusion zone. Natural sea water was used as the extracellular fluid medium. The second and third records (from left) were taken 0.5 and 1.5 min, respectively, after substitution of KBr for KF. The fourth and fifth records were obtained 0.5 and 1 min, respectively, after replacement of KBr with KF. The diameter of the axon used was 650 μ; the length of the perfusion zone was 16 mm (temperature, 22°C).

with a small amount of phosphate buffer. Enzyme solutions were prepared by dissolving 1 mg of the commercially available material in 1 ml of a mixture of two parts of 600 mM K-aspartate and one part of the glycerol solution. On several occasions, a 400 mM K-fluoride or a 50 mM Na-glutamate solution was used for dissolving enzymes. The rate of flow of the perfusion fluid through the interior of the axon was roughly 10–20 mm³ per min. The room temperature was 20–23°C.

Results.—(1) *Influences of inorganic and organic anions:* For comparison of the effects of various anions in the axon interior, a large number of axons (approximately 70) were intracellularly perfused with potassium salts of various anions. As a rule, the concentration of the perfusion fluid was kept at the level of 400 mM. Examination of four halides, F, Cl, Br, and I, immediately showed that potassium fluoride was most suitable for maintenance of large bioelectric potentials. There was a rapid increase in the amplitude of the action potential when the interior of an axon was perfused with KF. With axons immersed in natural sea water and perfused with a 400 mM KF solution, a resting potential of approximately 60 mv and an action potential of 150–165 mv in amplitude were observed.

By the use of a simple valve described previously,[1] it is easy to switch one perfusion fluid to another and compare the effects of two different anions. The oscillograph records in Figure 1 show the difference in effect between bromide and fluoride ions. When 400 mM KF solution in the axon was switched to 400 mM KBr solution, there was a rapid fall in the amplitude of the action potential accompanied by a slight reduction in the resting potential. Neural conduction across the perfusion zone was suspended within 1–2 min after substitution of Br for F. When the perfusion fluid was switched back to the KF solution, conduction was immediately restored and the amplitude of the action potential returned almost to the original level within approximately 1 min.

Similar tests on other halides indicated that KI was more, and KCl was less, effective than KBr in depressing the resting and action potentials. The depressive effect of heavy halide ions was diminished when the ionic strength was decreased by diluting the solution with glycerol or sucrose solution.[6] The effect of various anions could be compared on a quantitative basis by determining the lowest concentrations of potassium halides required for conduction block. We found that the critical concentration of KI was roughly 100 mM and that of KBr approximately
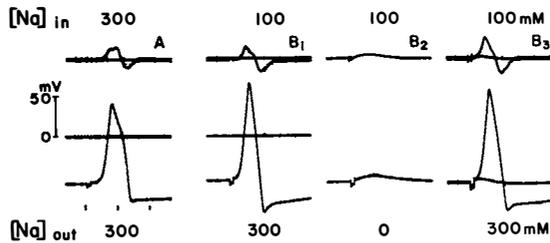
FIG. 2.—(*A*) Intracellular perfusion of a squid giant axon (550 μ) with a solution containing 300mM NaF and 180 mM KF. (*B*₁), (*B*₂), and (*B*₃): perfusion with a solution of 100 mM NaF and 380 mM KF. The external medium contained 300 mM NaCl, 50 mM MgCl₂, and 10 mM CaCl₂ in (*A*), (*B*₁), and (*B*₃). *Record B*₂ was taken approximately 2 min after replacement of NaCl in the medium with sucrose. The length of the perfusion zone was 12 mm. The zero level of the potential trace was obtained by withdrawing the recording electrode from the axon and placing it in the surrounding fluid medium. The time markers are 1 msec apart (22°C).

200 mM. The order of halogen ions arranged according to the magnitude of their depressive action was as follows:

$$F < Cl < Br < I.$$

The comparison between F and Cl was based simply on the difference in the size of the action potential in two solutions at 400 mM concentration. By comparing the present findings with our previous findings with sulfate ion, we infer that the proper position for SO₄ is between F and Cl.

Similar tests on various polyatomic anions gave the following order:

aspartate, glutamate < citrate < tartrate < acetate < nitrate < thiocyanate.

Aspartate and glutamate ions were comparable to fluoride ion in their effect upon the action potential; the resting membrane potential, however, was about 10 mv smaller with K-aspartate and glutamate than with fluoride. The effect of thiocyanate was similar to that of Br and I; perfusion with 400 mM KCNS solution brought about conduction block within 1 or 2 min. Recovery from the effect of perfusion with KCNS was imperfect generally.

(2) *Substitution of NaF for KF in perfusion fluid:* Since fluoride ion in the perfusion fluid was found to exert a favorable influence upon the axonal membrane, it appeared worth while to study the effect of substitution of NaF for KF in the axon upon bioelectric potentials. Axons used in this study were immersed in a medium containing 300 mM NaCl, 50 mM MgCl₂, 10 mM CaCl₂, and 220 mM sucrose. Such external media containing low Na and/or high Mg or Ca tend to prevent repetitive firing of impulses which frequently results from perfusion with Na-rich solutions.[7] Intracellular perfusion fluid was prepared by mixing 12.5 vol per cent glycerol with solutions of 600 mM K- and Na-fluoride.

Record A in Figure 2 was obtained with a perfusion fluid containing 300 mM NaF and 180 mM KF. In this case the Na-concentrations, as well as the ionic strengths, were equal inside and outside the axon. As can be seen in the figure, large action potentials were observed under these experimental conditions. No significant variation in the resting potential was encountered within the 12–17-mm long perfusion zone. The amplitude of the action potential was largest at or near the center of the perfusion zone. Our measurements on 12 axons under these

conditions indicated that the resting membrane potential was approximately 60 (58–63) mv and the amplitude of the action potential 90–110 mv. The overshoot of the action potential was therefore 30–50 mv.

The question may be raised as to whether or not the Na-ion in the perfusion fluid is chemically free. It is known that the activity coefficients of most Na- and K-halides are 0.65–0.70 at 400 mM (see Table 8.10 in Robinson and Stokes[8]). We determined the activities of the Na-ion on both sides of the membrane by the use of a sodium-ion electrode (Beckman 39278). As can be expected from the ionic strengths of the two solutions, there was no significant difference in the sodium-ion activity on the two sides of the membrane. The "sodium equilibrium potential" determined by this method was only 2–3 mv, namely, less than one tenth of the observed value of the overshoot. This situation is very similar to that in previous experiments using aspartate and glutamate as intracellular anions.[6, 9]

Record B_1 in Figure 2 was obtained with 100 mM NaF and 380 mM KF in the perfusion fluid. The external fluid medium contained, as in the preceding observation, 300 mM NaCl together with 50 mM $MgCl_2$ and 10 mM $CaCl_2$; hence, the sodium equilibrium potential calculated from the Nernst equation applied to Na-ion is 28 mv. The observed value of the overshoot under these conditions was 64–70 mv. It is evident, therefore, that the overshoot of the action potential is not determined simply by the Na-concentration ratio inside and outside the membrane.

Record B_2 was taken approximately 2 min after replacing NaCl in the outside medium with an osmotically equivalent amount of sucrose. The rapid loss of excitability in this Na-free medium indicates that the external surface of the axon membrane exhibits the normal sodium sensitivity. *Record* B_3 shows that recovery from the Na-deficiency in the outside medium is prompt and nearly perfect.

The substitution experiments mentioned above also demonstrate that the resting membrane potential remains practically uninfluenced by the replacement of K in the perfusion fluid by Na. A similar finding was described in a previous article.[6] If either bromide or chloride forms of salts are used in these substitution experiments, very different results are obtained. [6, 9, 10]

(3) *Effects of proteases and other enzymes in perfusion fluid:* In the present study, various enzymes were administered intracellularly by the perfusion technique. We examined the effects of 7 proteases, 5 lipases, and 4 other enzymes. The effects of enzymatic destruction of the axon interior upon bioelectric potentials are described below.

(*a*) Trypsin (Worthington, 1× and 2× crystallized). The effect of this endopeptidase was examined on 7 different axons. In all cases, conduction across the perfused zone was blocked within 2.5–6 min after the onset of perfusion with this enzyme (1 mg/ml). A reduction of both the resting and action potentials preceded conduction block. No repetitive firing of impulses was observed during perfusion with this enzyme. The effect upon bioelectric potentials vanished after boiling the enzyme for 60 min.

(*b*) Alpha-chymotrypsin (Worthington, 3× crystallized and lyophilized) and gamma-chymotrypsin (Worthington, 2× crystallized and lyophilized). Six axons were used to test the effects of these endopeptidases, 3 for each of the enzyme preparations. These enzymes (1 mg/ml) caused depolarization and repetitive firing of
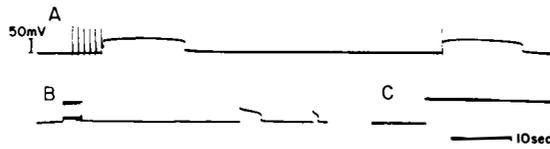
FIG. 3.—Demonstration of "flip-flop phenomena" in squid giant axons under intracellular perfusion with solutions containing ficin. In (*A*), the protease was dissolved in 400 mM K-aspartate solution; in (*B*) and (*C*), 50 mM Na-glutamate solution was used as solvent. The outside medium was natural sea water in (*A*), and a 300 mM NaCl and 50 mM MgCl₂ solution in (*B*) and (*C*).

impulses, followed by conduction block within 3–6 min. After heating at 100°C for 60 min, the enzyme preparations became harmless to the axon.

(*c*) Papain (Worthington, 2× crystallized). Perfusion with K-aspartate solution containing papain (1 mg/ml) brought about depolarization and repetitive firing of impulses followed by conduction block within 6–30 min. In 8 out of 16 axons examined, an unexpected phenomenon, "spontaneous flip-flop," was observed. This phenomenon was more consistently observed with ficin dissolved in K-free Na-glutamate solution (see below).

(*d*) Ficin (Worthington, crystallized; Sigma, 2× crystallized). The effect of ficin was examined on approximately 30 axons; the result was very similar to that of papain. In the course of continuous perfusion with this peptidase, a sudden rise in the intracellular potential (30–85 mv depolarization) followed by an abrupt return to the original potential level was frequently observed (see Fig. 3). This "flip-flop" phenomenon was often triggered by repetitive stimulation of the axon (see *Record A*). It was sometimes preceded by a spontaneous discharge of high frequency impulses (*Record B*). Conduction across the perfused zone was completely suspended during the depolarized phase. On sudden and spontaneous repolarization of the membrane, conduction was immediately restored. The duration of the depolarized phase was extremely variable, ranging from several sec to 1 min. Sudden depolarization with no subsequent recovery was encountered on several occasions (see *Record C*). An increase in the Ca- or Mg-concentration in the outside medium during the depolarized phase frequently induced sudden repolarization. A decrease in the divalent cation concentration or lowering of the temperature of the outside medium often triggered sudden depolarization.

(*e*) Carboxypeptidase A (Worthington, crystallized and DFP-treated) and carboxypeptidase B (Worthington). These exopeptidases were examined on 6 axons, 3 axons for each preparation. Gradual depolarization, followed by conduction block, was produced within 7–15 min after the onset of perfusion with this enzyme. No repetitive firing of impulses was observed.

(*f*) Leucine aminopeptidase (Worthington). Perfusion of three different axons with this exopeptidase (1 mg/ml) brought about a decrease in the resting membrane potential and subsequent conduction block in approximately 30 min. No repetitive firing of impulses was seen.

(*g*) L-Amino acid oxidase (Worthington). Gradual depolarization and subsequent conduction block without repetitive firing resulted in 24–30 min (observations on 3 axons).

(*f*) Pancreatic lipase (Worthington, lyophilized). Gradual depolarization and

subsequent conduction block without repetitive firing occurred in 10–20 min (6 axons examined).

(*g*) Phospholipase C (Worthington). Gradual depolarization, followed by conduction block was observed in 10–40 min (5 axons examined). Phospholipase D (Sigma; Mann) also was examined on 3 axons; the effect of this enzyme appeared to be similar to (but slightly slower in action than) phospholipase C.

(*h*) Other enzyme preparations. DPNase, RNase, DNase, and neuraminidase were found to have no clear effect upon bioelectric potentials of the perfused axon within 40 min.

In a separate series of experiments, we examined the effect of several proteases (trypsin, chymotrypsin, and ficin), lipase, phospholipase C, and collagenase applied to the external surface of the axon. These enzymes were dissolved in the natural sea water (1 mg/ml) in which the axons were immersed. We could not detect any injurious effect upon the axon under these experimental conditions.

Discussion.—The experimental findings described under *Results* show that the inner surface of the squid axon membrane behaves very differently from the outer surface in response to the action of various salts and enzymes. On the outer surface the membrane potential is insensitive to complete replacement of the major anion species (i.e., Cl in artificial sea water) by other anions (bromide or glutamate, for example). Lowering of the salt concentration in the outside medium (by dilution with isotonic sucrose) increases the resting membrane potential logarithmically (Teorell, T., and C. S. Spyropoulos, unpublished). These facts strongly suggest that the outer layer of the axon membrane possesses negative fixed charge. Measurements of fluxes of various radio-tracers in intracellularly perfused axons[11] indicate that the membrane as a whole is more permeable to cations (Na, K, Rb, Cs, Ca, guanidine) than to anions (Cl, Br, SO_4, glutamate).

The pronounced "anion effect" shown under *Results* indicates that the inner layer of the axon membrane behaves, to some extent, like an anion-exchanger rather than a cation-exchanger. The resting membrane potential often increases when the intracellular potassium-salt concentration is lowered;[10] this fact also indicates that the inner layer cannot be treated as a simple cation-exchanger.

We have seen under *Results* that enzymatic destruction of both protein and lipid components of the membrane leads to reduction of the resting potential and loss of excitability. Since Beutner's classical work,[12] the lipid layer has been considered by many investigators (cf. Tobias[13]) as the site of production of bioelectric potentials. Now it seems evident that the protein component in the membrane also plays an important role in the maintenance of the resting potential and production of action potentials. The protein layer can provide the membrane with negative and/or positive fixed charges, depending upon the pH, the chemical species, and concentration of the salt in the perfusion fluid. According to Bungenberg de Jong (see pp. 259, 287, and 299 in Kruyt[14]), the electric charge at the phase boundary of a complex colloid system is affected by the valence, radius, and polarizing power of the ions in the medium in the order similar to that in the lyotropic series. There seems little doubt that the cation and anion effects seen in the present electrophysiological study arise from the interaction between the fixed charges in the inner layer of the axon membrane and the ions in the perfusion fluid.

In the first article of this series,[1] we have stressed that, for analysis of bioelectric

potentials in the squid axon, the membrane has to be treated as a system consisting of layers with different properties. We stress here again that the sign and magnitude of the fixed charge, the ion-selectivity, the mobilities of various ions, the susceptibility to various enzymes, etc., of the membrane should be considered as functions of the coordinate normal to the surface.

A further analysis of the "flip-flop phenomenon" described under *Results* is in progress.

Summary.—By the method of intracellular perfusion, the effects of various anions upon the resting and action potentials of the squid giant axon were studied. Among the anions examined, fluoride, aspartate, and glutamate ions were found to be most favorable for maintaining large action potentials. Anions could be arranged according to the magnitude of their intracellular effect in an order similar to the well-known lyotropic series. By using a mixture of NaF and KF in the perfusion fluid, a large overshoot of action potential was obtained with approximately equal Na-ion activities inside and outside the axon. Various endopeptidases, exopeptidases, and lipases were shown to destroy the axon membrane functionally. A close relationship between the protein layer of the membrane and electrical excitability is suggested.

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