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RESTING AND ACTION POTENTIAL OF SQUID GIANT AXONS INTRACELLULARLY PERFUSED WITH SODIUM-RICH SOLUTIONS

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The axoplasm of freshly excised squid giant axons contains a relatively low concentration of sodium (see, e.g., Steinbach and Spiegelman[1]). It is generally believed that a low sodium and a high potassium concentration in the axoplasm is the condition necessary for the maintenance of normal excitability of the axon. Until quite recently, however, there was no direct means of varying the intracellular ionic composition to test whether this condition is actually necessary. Owing to the development of the methods of intracellular perfusion in recent years,[2–6] it is now possible to examine the effects of high sodium in the interior of the axon upon the resting and action potentials.

In a previous paper,[3] we have shown that both the resting and action potentials of the squid axon can be maintained for considerable time under continuous intracellular perfusion with pure sodium salt solutions. The present investigation is an expansion of those reported in the previous paper, using different sodium salts. We have found that, when the sodium concentration in the perfusing fluid is high, the difference in chemical species of anions in the fluid can markedly influence the excitability. Using glutamate and other types of sodium salts, we could maintain all-or-none action potentials with a fairly large overshoot under continuous perfusion with sodium-rich solutions.

Materials and Methods.—Experiments were carried out on giant axons of *Loligo pealii*. The perfusion techniques developed in this laboratory[2, 3] were used. In most of the experiments, a mixture of monosodium and monopotassium salts of L-glutamic acid was used to prepare perfusing solutions. The tonicity of the solutions was maintained by adding glycerol. We used 12 volume per cent aqueous solution of glycerol instead of isotonic sucrose solution as in previous experiments. The pH was adjusted to 7.2–7.3 by adding a small amount of phosphate buffer.

In order to prevent impairment of the resting and action potentials by sodium-rich perfusing fluids,[3] the Na-ion concentration was lowered and the Ca-ion concentration was raised in the outside medium. Our standard external medium contained 300 mM NaCl, 45 mM $MgCl_2$, 22 mM $CaCl_2$, and 400 mM sucrose, and its pH was adjusted to 8 with a trace amount of tris(hydroxymethyl)aminomethane. (Note that Woods Hole sea water contains 534 mM Na, 56 mM Mg, 6 mM Ca, and 18 mM K.) At the onset of perfusion, however, the Mg- and Ca-concentrations in the medium were temporarily increased to 67 and 33 mM, respectively. Ordinarily, no repetitive firing of impulses was observed under these conditions.

The perfusing fluid was forced into an inlet pipette of about 170 μ in outside diameter (see the left-hand side of the diagram in Fig. 1) by a hydrostatic pressure of 10–50 cm H_2O. The outflow pipette (on the right in the diagram) was 300–350 μ. The distance between the tips of the two pipettes was, in the experiments of Figures 1 and 2, approximately 12 mm. A large portion of the axoplasm in the perfusion zone was removed beforehand. The rate of flow of the fluid through the

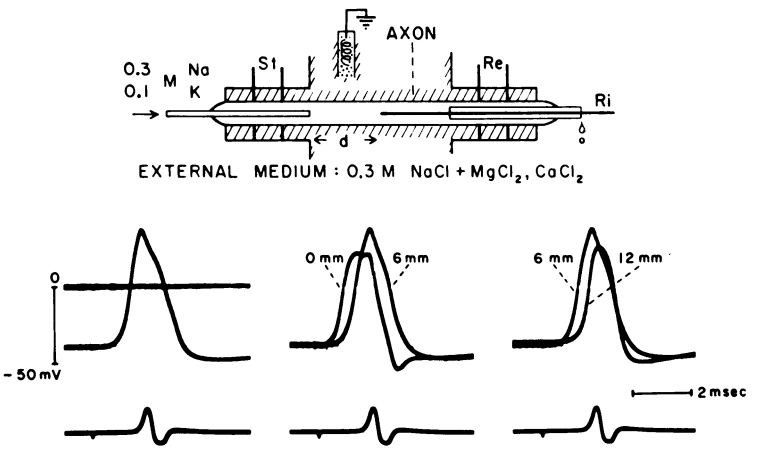

FIG. 1.—*Top:* Schematic illustration (not to scale) of the experimental device used to measure the resting and action potentials of squid giant axons under perfusion with equal concentration of sodium inside and outside the membrane. *St*, stimulating electrodes; *Re*, extracellular recording electrodes; *Ri*, intracellular recording electrode (movable). *Bottom:* Oscillograph records obtained under the conditions illustrated above. In the middle and right records, the distance from the tip of the inlet pipette to that of the recording pipette (*d* in the diagram) was varied. The lower oscillograph trace shows action potentials recorded simultaneously from electrodes *Re*.

interior of the axon was 15–25 mm^3 per min. The diameter of the axons used was usually 500–600 μ.

Stimulating shocks from a Grass stimulus isolation unit were delivered through a pair of platinum electrodes (St in the diagram) to the unperfused portion of the axon. The intracellular recording electrode (Ri) was filled with 0.6 *M* ammonium chloride and was inserted into the axon through the open end of the outlet pipette with a micromanipulator. The resistance of the electrode was 5–15 megohms. A Bak unity-gain electrometer and a Tektronix oscillograph (Type 502) were used for recording the intracellular potentials. Propagation of impulses across the perfused zone was monitored by simultaneously recording the extracellular potential with another pair of platinum electrodes (Re). Most of the experiments were carried out at room temperature (21–23°C).

Results.—Figure 1 shows an example of the resting and action potentials observed under continuous intracellular perfusion with sodium salts of the same concentration on both sides of the axonal membrane. The perfusing fluid contained 100 mM K-glutamate in addition to 300 mM Na-glutamate. The outside medium was the standard solution (see *Materials and Methods*) containing 300 mM NaCl. The flow of the sodium-rich perfusion fluid was maintained for about 40 min before these records were taken. The amplitude of the action potential reached a stationary value of approximately 83 mV (75–90 mV in most cases) within 1 min after the onset of perfusion; it remained practically unchanged for approximately 80 min in this example. (At the end of this period the observation was discontinued because the flow of perfusing fluid ceased.) The oscillograph trace marked "O" mV was obtained with the internal recording electrode (Ri) removed from the axon and immersed in the surrounding fluid medium. The resting potential measured in this fashion was usually 38–45 mV under these experimental conditions (see Fig. 2). The overshoot, i.e., the difference in amplitude between the resting potential and the action potential was 35–43 mV.

The superimposed records in the middle and right frame of Figure 1 show the distribution of the resting and action potentials within the 12-mm perfusion zone. There was hardly any shift in the potential (dc) level when the internal recording electrode was moved from one extreme end (0 mm) to the other (12 mm); this indicates that no appreciable current was flowing through the axon membrane in the perfused zone. The amplitude of the action potential was largest in the middle part of the perfused zone. Although the reason for the reduction in amplitude near the ends of the zone is not altogether clear, essentially the same type of potential distribution was observed in all of our perfused axons. Evidently there was a strong inward-directed membrane current in the middle of the perfused zone at the peak of activity. The velocity of the nerve impulse in the perfused zone could be calculated from the time difference between the rising phases of the action potential recorded at different points; a velocity of about 15 m per sec was obtained under these experimental conditions.

In order to determine the time course of the membrane conductance during activity, impedance measurements were carried out using a straightforward ac Wheatstone bridge and an electronic filter. An 11 kc per sec sinusoidal current was applied through a platinized platinum electrode (100 μ in diameter and enclosed in glass tubing) to a cleaned surface of the axon in the middle of the perfused zone. The ac voltage between the impedance electrode and the large ground electrode was about 50 mV, peak to peak. When the impedance electrode was pushed against the cleaned axon surface, there was a large increase in the impedance of the system.

At the time of arrival of an impulse at the center of the perfused zone, there was a distinct fall in the impedance. The time course of the impedance loss was very similar to, but slightly longer in duration than, that in normal axons described by Cole and Curtis.[7]

In the experiment of Figure 1 the perfusing fluid contained 100 mM potassium glutamate in addition to sodium. (In this figure the duration of the action potential is slightly longer than that of unperfused axons.) Complete elimination of potassium from the perfusing fluid prolonged the falling phase of the action potential further. The duration of such prolonged action potentials was sensitive to the Mg- and Ca-ion concentration in the medium; an increase in the divalent cations shortened the duration. Neither the resting potential nor the overshoot was strongly affected by the substitution of glycerol for the K-glutamate in the perfusing fluid. A complete substitution of bromide- or sulfate-ion for the chloride-ion in the surrounding fluid medium had no significant effect upon the resting potential, the duration, or the amplitude of the action potential.

By using a special valve described previously, it was easy to change from one kind of perfusing fluid to another and to determine the resting and action potentials under different internal conditions. The effect of varying the ratio of the internal concentration of sodium to that of potassium was studied by this method. Figure 2 (data collected from 17 different axons) shows the dependence of the resting and action potentials upon the internal sodium-potassium ratio under the condition of a

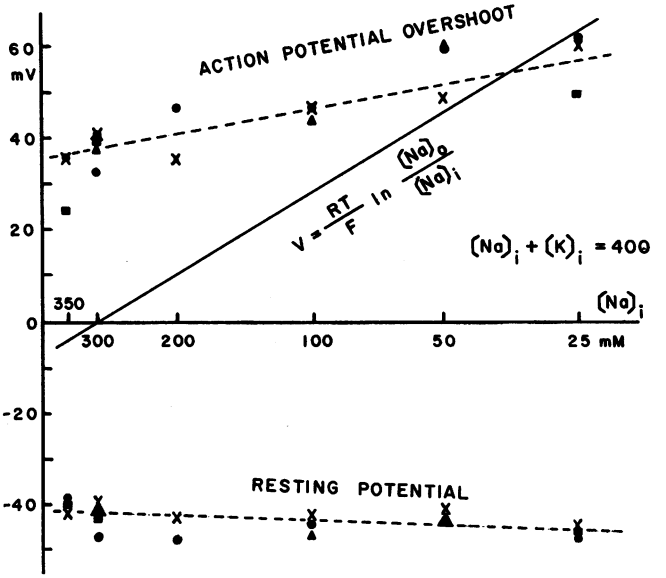

FIG. 2.—Resting potential and overshoot of action potential of intracellularly perfused squid giant axons plotted as a function of Na-ion concentration in the perfusing fluid, $[Na]_i$. The sum of the internal Na- and K-ion concentrations was held at a constant level of 400 mEq per l throughout. The perfusing fluid contained 470 mM glycerol besides Na- and K-salts in the glutamate form. The external medium contained 300 mM NaCl, 45 mM $MgSO_4$, and 22 mM $CaCl_2$. A 50% increase in the Mg- and Ca-ion concentrations outside did not alter the results significantly.

constant total concentration (400 mM). The resting potential was practically unaffected by a large change in the concentration ratio. The overshoot was reduced by a large increase in the internal sodium, but the observed reduction was far smaller than is predicted by the Nernst equation applied to the Na-ion concentrations across the membrane (see the continuous straight line in the figure).

The approximate constancy of the resting potential over a wide range of internal sodium-potassium ratio is apparently inconsistent with the results reported by Shaw *et al.*[5], who demonstrated a large change in the membrane potential resulting from the replacement of K-ion with Na-ion. Previously, we also reported that an increase of the internal Na-ion in the sulfate or chloride form to a level higher than 200 mEq per l produced a rapid deterioration of the resting and action potential.[2] The difference between the findings described in this paper and those reported previously is due to the difference in the experimental conditions. In the present series of experiments, solutions of Na- and K-salts as glutamate (instead of sulfate) and mixed with glycerol solution (instead of sucrose) were used. In the experiment of Figure 2 the excitability of the axon was always preserved, and the reversibility of the concentration effect was maintained in the entire range of concentrations examined.

Figure 3 illustrates our finding that the difference in anion species does produce a significant influence upon the resting and action potential of the axon when the

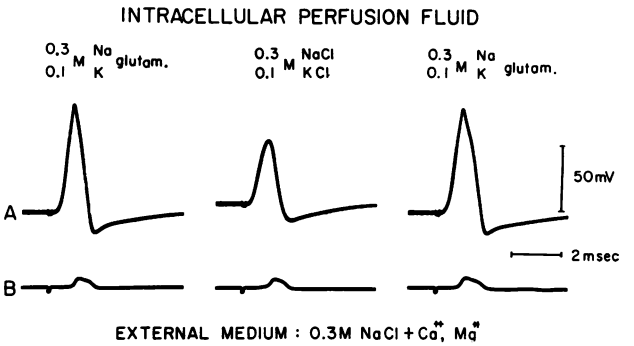

FIG. 3.—Oscillograph records showing the effect of different anions in the perfusing fluid upon the resting and action potentials of squid giant axon. The compositions of the internal and external solutions are given. Trace A, intracellular potential (dc) recorded in the middle of 9 mm long perfused zone. Trace B, extracellularly recorded action potential. The first (left) record was taken 14 min after the onset of perfusion; the second record, approximately 1 min after the replacement of the perfusion fluid; the third record, 6 min after the original perfusion fluid was readmitted.

ionic strength of the perfusing fluid is relatively high. The first (left) record in this figure was obtained under the conditions similar to those for Figure 1. When the glutamate in the perfusing fluid was completely replaced with chloride (middle record), there was a marked fall in the action potential and to some extent in the resting potential. Frequently, conduction across the perfused zone was blocked under these circumstances. When the original perfusing solution was readmitted (right record), both the resting and action potentials regained their original values.

The anion effect mentioned above was markedly reduced when the total salt concentration in the perfusing fluid was decreased to a low level (less than 100 mEq per l). Thus, many anions which show a depressing effect upon the action potential at high concentrations (such as tartrate, iodide, etc.) were found to be harmless at low concentration. We sought other anions which might be more effective than glutamate in preserving excitability. We could maintain the resting and action potentials by using the sodium salt of aspartic acid and of EDTA (ethylenediaminetetraacetic acid) in the perfusing fluid, but no accurate comparative study was made of the survival time of the perfused axons with these anions.

Discussion.—The electric responses recorded from axons perfused with sodium-rich solutions (Fig. 1) are actually generated in the perfused zone and do not represent a spread of electricity from the lateral unperfused zones. As is well known, the space constant for electrotonic spread of dc is determined by the membrane resistance and the axoplasmic resistance. The membrane resistance of the perfused axons was measured with a silver wire electrode 11 mm long inserted in a perfused zone 18 mm long, and was found to be close to that in the normal axon. The resistivity of the perfusing fluid was measured with an ac Wheatstone bridge at 1 kc and was found to be about 80 ohms-cm, which is slightly higher than twice the resistivity of the normal axoplasm.[8] Therefore, the space constant cannot be longer in perfused axons than in unperfused ones. It is obviously impossible to interpret the results furnished in Figure 1 in terms of spread of electricity from the lateral, unperfused zones.

Spread of electric current along the axon is greatly reduced when high-frequency ac is used instead of dc. The apparent space constant is reduced by a factor of $1/\sqrt{\pi f \tau}$, where f is the frequency of ac and τ the time constant (see equation 2 in Tasaki and Hagiwara[9]). Introducing 1.1×10^4 sec^{-1} for f and 10^{-3} sec for τ, the reduction factor is found to be about $1/6$. Since our impedance electrode was making contact with the axonal surface at one spot, the spread of ac from the bridge in our impedance measurement is limited to a zone which is approximately of the order of the axon diameter. Therefore, the impedance loss observed by this method can be safely taken as a sign of excitation in the perfused portion of the membrane.

The experimental data presented in Figure 2 indicates that, as long as the excitability in the perfused zone is maintained, replacement of K-ion in the perfusing fluid with Na-ion does not produce any significant change in the resting potential. This finding is not surprising for us, since we have known that the K-permeability as measured by the radioisotope method is not very different from the Na-permeability in the resting (excitable) state of the perfused axon.[6] In the depolarized (inexcitable) state which is often reached in perfusion with sodium-rich solutions, the permeability properties are very different from those in the resting (excitable) state. According to our concept of "two stable states" in the nerve membrane,[6] it is improper to treat an axon depolarized by excessive Na-ion (or other univalent cation) as being in the resting state.

Figure 2 also shows that a large overshoot of action potential can be obtained under the condition of equal Na-ion concentrations inside and outside the membrane. A sizable overshoot is observed even when the internal Na-concentration is slightly higher than the external value (see 350 mM Na in the figure). This clearly indicates that the peak value of the action potential cannot be calculated from the

Na-ion concentrations on the two sides of the membrane. The observed dependence of the overshoot on the Na-ion was approximately 20 mV for a 10-fold change in concentration, namely, about $^1/_3$ of the Nernst slope.

According to our basic idea[6] as to the nature of nerve excitation, the action potential is produced by an exchange reaction between divalent and univalent cations in a critical layer in the membrane. Since divalent ions are present only in the external medium, the site where such a process takes place must be located at and near the external surface of the major diffusion barrier in the membrane. If one assumes that the mobility and selectivity for Na-ion are roughly the same as those for K-ion in the excited state of the membrane, substitution of internal K-ion with Na-ion should not influence the action potential. Actually, however, the permeability for Na-ion (as measured by the tracer technique) is somewhat greater than that for K-ion.[6] The Na-dependence of the amplitude of the action potential can reasonably be related to the difference in permeability of the two ions. However, the difference between Na- and K-ion is, according to the interpretation stated above, far smaller than what was postulated in the theory of Hodgkin and Huxley.[10]

The observation of Figure 3 has demonstrated the importance of selecting proper chemical species of anions in order to maintain excitability under perfusion with sodium-rich solutions. Also, the difference in anion species does not influence the experimental results, if the intracellular concentration of the sodium salt is very low. These findings are consistent with the view that the process of nerve excitation takes place in the layer of the membrane with negative fixed charges.[6] When the salt concentration in the perfusing fluid is low, the anions should be strongly excluded from this critical layer because of the Donnan effect. The Donnan exclusion becomes weaker with the increasing salt concentration in the medium (see, e.g., Helfferich, p. 136[11]); therefore, more anions tend to invade the critical layers at a higher salt concentration. Anions in this layer can exert specific effects upon the ion-exchange properties by virtue of their charge, ion size, polarizability, etc. In general, a rise in the anion concentration in the critical layer should reduce the perm-selectivity, tending ordinarily to lower the membrane potential.

Summary.—Excised giant axons of the squid were perfused intracellularly with Na-glutamate solutions, with or without adding K-glutamate. Immersing the axons in media containing the same Na-concentration as the perfusing fluid, all-or-none action potentials with a large overshoot were observed for more than one hr. Replacement of Na-ion in the perfusing fluid with K-ion did not bring about any significant change in the resting potential. This finding is consistent with the view that the K- and Na-permeabilities are not very different in the resting, excitable state of the membrane. At high concentrations of Na-ion in the perfusing fluid, the difference in chemical species of anions in the perfusing fluid changed significantly the electrophysiological behavior of the axon.

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*DISTURBANCES OF NUCLEIC ACID METABOLISM PRECEDING DELAYED RADIONECROSIS OF NERVOUS TISSUE**

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Delayed radionecrosis of mammalian central nervous tissue is characterized by dose-dependent, unusually long latencies, extending perhaps over $^1/_4$ of the life expectancy of the irradiated individual.[1] This pathologic response has been observed in several mammalian species and regularly follows local exposures in the therapeutic range, i.e., a total dose equivalent to a single exposure in the order of 10^3 rads, a rate in the order of 10^2 rads min^{-1}, an LET in the order of KeV μ^{-1}, and a field size measuring in the order of cm^2. No experimental or other data on the pathogenesis of delayed radionecrosis of nervous tissue are available, and the morphologic and biologic changes of the exposed tissues during the interval, if any, are unknown. It has been suggested that the extreme length of the latency period might be related to the low rate of molting turnover of interstitial, i.e., mesodermal and neuroglial, elements.[1]

Material and Methods.—Female Carworth rats, aged 90–110 days, were exposed under light nembutal anesthesia to a single dose of 3,500 rads of 250 KVP X rays, HVL 2.1 mm copper, at a rate of 320 ± 10 rads min^{-1}. The animals were lead-shielded except for a 1.5 cm slit, exposing the second, third, and fourth thoracic vertebrae and the immediately adjacent tissues.[2] Animals so treated develop clinical signs of paraplegia after a mean latency of 200 days with a standard deviation of ±20 days (N = 22) and a range of 166–227 days. At 3, 7, 14 days, 1, 2, 3, 4, 5, 6, and 7 months following the exposure, four animals—two irradiated rats and two sham-irradiated controls matched for age—were injected with H^3-labeled thymidine. The first injection was given at 10 A.M. under light ether anesthesia into the retro-orbital sinus, and consisted of 250 μc H^3-thymidine, with a specific activity of 800 mc/Mol in 0.25 cc. Identical injections were given 8 hr and 16 hr later, respectively. One irradiated animal with its control was sacrificed 3 hr after the last injection and the other pair three days later. Under deep anesthesia, the animals were perfused, using a modification of Cammermeyer's technique.[3] The irradiated part of the spinal cord and the corresponding segment from the control animals were removed from the fixed cadavers, and the four specimens comprising one series were always embedded in the same paraffin block. Sections were cut at 4 μ; after deparaffination they were coated with Kodak NTB-2 emulsion, stored