Excitation of squid giant axons in sodium-free external media

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TASAKI, ICHIJI, IRWIN SINGER, AND AKIRA WATANABE. *Excitation of squid giant axons in sodium-free external media.* Am. J. Physiol. 211(3): 746–754. 1966.—Studies of membrane impedance and potential variation were made on squid giant axons under sodium-free external conditions. Intracellular perfusion by the double-cannulation method was employed. It was found that with favorable internal conditions, excitability could be maintained with several organic and inorganic sodium substitutes, and that the excitation process in sodium-free media is not dissimilar from that in sodium-containing media. Tetrodotoxin was found to suppress excitability in all cases. The implications of these findings for the concept of sodium-specificity are discussed.

tetrodotoxin; sodium-free excitation; membrane impedance; internal perfusion

IN SEVERAL RECENT ARTICLES it has been shown that sodium ion is not absolutely essential for the maintenance of excitability. For example, the sodium ion in the external medium of frog nerve fibers can be replaced by various nitrogenous cations without eliminating the ability of the axon to produce action potentials (5). A number of investigators have extended sodium-substitution experiments of this type to other biological systems, including dorsal root ganglia of the frog (4), frog heart muscle (18), and single frog nerve-fiber preparations (6). This laboratory has recently reported that many of the nitrogenous cations (e.g., hydrazinium) used by Lorente de Nó (5) for frog nerve excitation are very favorable sodium substitutes in squid giant axons intracellularly perfused with solutions of RbF or CsF; under these conditions excitability can be maintained for hours (16). References to other sodium-free systems may be found in earlier articles (13, 16)

The present report deals with several extensions of the sodium-substitution experiments in the internally perfused squid giant axon. In one series of experiments variations in the a-c impedance of the membrane were recorded together with the action potential. In addition, it was shown that not only can ammonium and other nitrogenous cations replace external sodium, but that potassium and rubidium may also substitute for external sodium when internal conditions are favorable. Furthermore, tetrodotoxin was found to be effective in blocking the excitation process in these sodium-free media.

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METHODS

Giant axons were obtained from *Loligo pealii*, available at the Marine Biological Laboratory, Woods Hole, Massachusetts. The major portion of the small nerve fibers around the giant axon was removed under a dissecting microscope (Zeiss otoscope) in conjunction with dark-field illumination. Such cleaning was necessary for subsequent manipulation of the intracellular perfusion cannulas under direct visual observation. The double-cannulation method developed in this laboratory was employed for intracellular perfusion (9, 15). A giant axon (350–600 μ diam.) was mounted in a Lucite chamber filled with natural sea water. A glass cannula for intracellular perfusion was inserted into each end of the axon. The tip of the smaller (inlet, 150–200 μ diam.) cannula was placed concentrically within the larger (outlet, 250–300 μ diam.) cannula. The length of the perfusion zone, i.e., the distance between the tips of the two cannulas during perfusion, was 15–20 mm.

Internal perfusion solutions were prepared by mixing 0.6 M CsF, RbF, or NaF solutions with 12 vol % glycerol solutions. The pH of the perfusion fluid was adjusted between 7.2 and 7.4 with an isotonic phosphate salt solution of the corresponding alkali cation. After adjustment of the pH, the ratio of phosphate to fluoride salts in the perfusion solution was approximately 1:9.

Typical sodium-free external fluid media were prepared by mixing 0.6 M solutions of (e.g.) hydrazinium or guanidinium chloride (both obtained from Eastman Organic Chemical Co.) with 0.4 M $CaCl_2$ solution. Hy-

drazinium chloride was prepared by neutralizing a 64% hydrazine solution with hydrochloric acid (less than 5 mM) by flame photometry (Coleman Instrument Co., model 22) (see also DISCUSSION).

In general, rather high concentrations of external divalent cations were used in these experiments. These concentrations were required in order to suppress spontaneous repetitive firing of action potentials. An extensive discussion of this problem, particularly when cesium and rubidium salts are used for internal perfusion, has been presented in an earlier article from this laboratory (15). There is no doubt that calcium plays a vital role in the process of excitation, both in sodium-containing and sodium-free media (14), and that the precise mechanism by which this role is played will have to be investigated directly.

Generally, electric stimuli (1 shock/sec) were applied to the axon near the proximal end of the perfusion zone. In later experiments, when action potentials were recorded without simultaneous recording of the membrane impedance, stimuli were delivered through an intracellular platinum electrode located in the middle of the perfusion zone. A coil of platinum wire was immersed in the fluid medium in the perfusion chamber to serve as the ground electrode. Neither agar-gel nor cotton was placed around the ground electrode, so that rapid and complete exchange of the external fluid medium was ensured.

The method of measuring a-c impedance losses during excitation was slightly different from the method originally developed by Cole and Curtis (1) and was essentially the same as that used in this laboratory previously (10). A pair of platinum wire electrodes (one wire for measurement of a-c impedance and the other for recording action potentials) was introduced through the outlet cannula into the middle of the perfusion zone of the axon. The impedance electrode had a platinized surface, approximately 4 mm in length, near its tip; the remainder of the wire surface was left completely insulated with enamel. The potential electrode had an uninsulated portion, approximately 2 mm in length, which was positioned adjacent to the middle of the platinized area of the impedance electrode (Fig. 1, top).

The impedance bridge used in these experiments is shown diagrammatically in Fig. 1, top. The fixed resistance and capacitance, R and C, were 100 k-ohms and 0.001 μf, respectively. The output of the oscillator (Western Electric Co.) was attenuated so that the a-c amplitude across the axon membrane did not usually exceed 10 mv peak-to-peak. The output of the impedance bridge was led through a pair of high-pass filters (consisting of capacitors of 200 $\mu\mu$f, and resistors of 100 k-ohms, respectively) to a low-level preamplifier (Tektronix, model 122). The output of the preamplifier was brought through a band-pass filter (Allison Laboratories) tuned to the bridge ac, and displayed on a dual-beam oscilloscope (Tektronix, model 502). The a-c frequency used was 16 kc/sec. Simultaneous recordings of action potentials and impedance losses were obtained with this experimental arrangement. However, precise quantitative treatment of the impedance data was not possible, largely due to the stray capacitances present in various parts of the bridge. Therefore, the impedance records obtained in the present series of experiments were treated simply as qualitative measurements, reflecting the time course of the increase in the membrane conductance.

All the experiments were carried out at room temperature (19–20 C).

RESULTS

Replacement of External Sodium Ion With Hydrazinium or Guanidinium Ions

This series of experiments was carried out in the following manner. After insertion of the glass cannulas into a mounted giant axon (see METHODS), the natural sea water in the chamber was replaced with a sodium-free solution containing 0.3 M tetramethylammonium chloride (TMA-Cl) and 0.2 M $CaCl_2$. This replacement blocked the conduction of nerve impulses within 1–5 min. In order to wash out residual sodium ions as completely as possible, the sodium-free mixture in the chamber was replenished with fresh sodium-free solution several times during the following 5-min period. The fluid in the chamber was then replaced with the test solution, containing, for example, 0.3 M hydrazinium chloride and 0.2 M $CaCl_2$. No restoration of nervous conduction along the axon was observed following this replacement. (Further details of this procedure may be found in a previous article (16).) The next step in this experimental procedure was to initiate intracellular perfusion by separating the two cannulas and to observe the a-c impedance losses associated with the electric responses to stimulation.

Two exemplary sets of records obtained by this procedure are shown in Fig. 1, bottom. When intracellularly perfused with 0.1 M RbF (Fig. 1, bottom, left), a sodium-free external medium containing 0.3 M hydrazinium chloride and 0.2 M $CaCl_2$ was found to effectively restore the ability of the axon to develop large, all-or-none action potentials. As in natural sea water, these sodium-free action potentials were invariably associated with a distinct change in the balance of the impedance bridge. Initially, the impedance bridge was balanced for the membrane in the resting state. At the time of the action potential there was a transient unbalance in the bridge, where the maximum bridge unbalance roughly coincided with the peak of the action potential. The amplitude of the bridge unbalance increased directly with the a-c intensity applied to the bridge. A perfect bridge balance at or near the peak of the action potential could be produced by properly unbalancing the bridge in the resting state. These findings indicate that the observed changes in the bridge balance are signs of the impedance-loss associated with the action potential.

With these chemically defined conditions (50–100

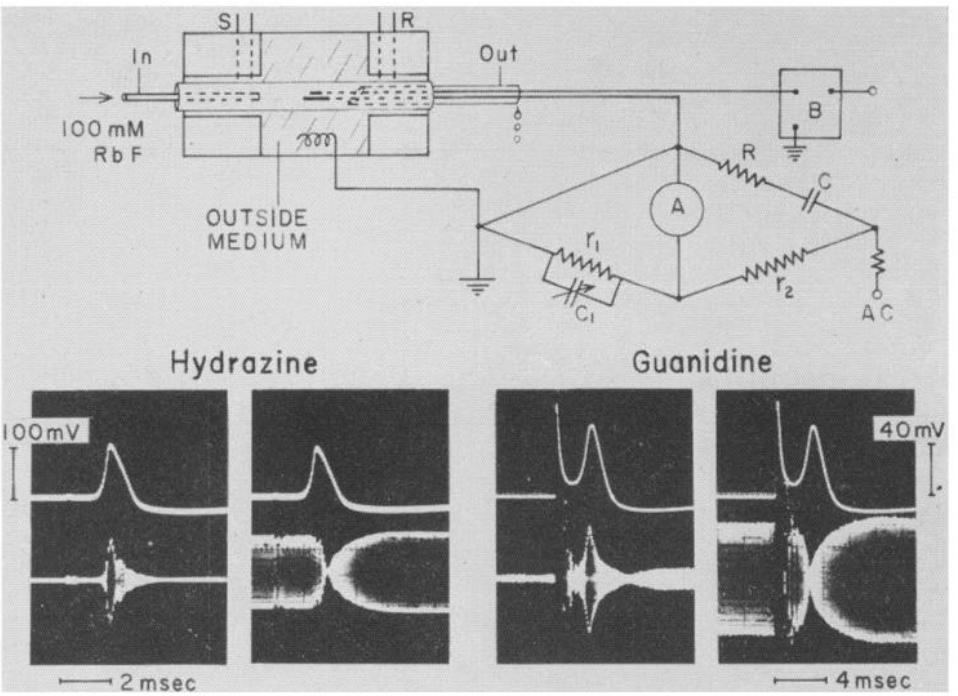

FIG. 1. Top. Experimental arrangement for impedance measurement. The Lucite chamber is indicated at left; cross-hatched area represents external medium. Positions of the external paired platinum stimulating (S) and recording (R) electrodes are indicated. The platinum ground electrode is represented by coil in outside medium. Positions of inlet (In) and outlet (Out) glass cannulas are indicated after separation. The internal perfusion medium was a mixture of 100 mM RbF and glycerol. The paired platinized impedance electrodes are positioned midway between inlet and outlet cannulas, concentrically within the outlet cannula and the axon. The smaller electrode (upper, in diagram) was led to the recording system (B); the larger electrode (lower, in diagram) received the output of the a-c oscillator (AC). Bottom. Impedance and potential variation in sodium-free external media. In each record, potential variations are indicated by upper trace; simultaneous impedance variations are indicated by lower trace. In each pair of records, the record at left was obtained when the impedance bridge was balanced initially for the resting state. Record at right was obtained when the bridge was unbalanced in the resting state, so that best balance could be obtained in the excited state. Left. The external medium contained 0.3 M hydrazinium chloride and 0.2 M $CaCl_2$; the internal perfusion solution contained 0.1 M RbF. Right. The external medium contained 0.1 M guanidinium chloride, 0.2 M TMA-Cl and 0.2 M $CaCl_2$; the internal solution contained 0.1 M RbF. (Note differences in voltage and time scales.)

mM RbF internally; 0.3 M hydrazinium chloride and 0.2 M $CaCl_2$ externally), the observed action potential amplitude was 70–100 mv; this is in good agreement with values reported previously (16). When 50–100 mM CsF was used instead of RbF in this type of experiment, the action-potential amplitude was between 80 and 120 mv (observations on 7 axons); in this case, both the period of impedance loss and the duration of the action potential were definitely increased.

The responses of an axon internally perfused with 0.1 M RbF and immersed in a solution containing 0.1 M guanidinium chloride, 0.2 M TMA-Cl, and 0.2 M $CaCl_2$ are shown in Fig. 1, bottom, right. The amplitude of the action potential was roughly 60–80 mv under these experimental conditions, which confirms results previously reported from this laboratory (16). Both the period of impedance loss and the duration of the action potential were clearly longer in axons with external media containing guanidinium chloride than with external media containing hydrazinium or sodium chloride.

Replacement of External Sodium Ion With Ammonium Ion

Lorente de Nó and his associates (5) have demonstrated that with frog nerve fibers ammonium ion can substitute for external sodium ion without a loss of excitability; similar observations have been made more recently with squid axons (13, 16). In the present series of experiments impedance studies were combined with observation of the action potential, to confirm and extend these early findings.

The method employed was somewhat different from that used in the previous section. After introducing the two perfusion cannulas into the axon, the external natural sea water was replaced with a mixture of 0.3 TMA-Cl and 0.2 M $CaCl_2$. Repeated washing with this sodium-free solution brought about a complete loss of excitability within 5 min. Intracellular perfusion was then instituted with 0.1 M CsF (glycerol) solution by separating the glass cannulas. No distinct action potentials could be observed at this time. Finally, a small amount of a mixture of, for example, 0.54 M NH_4Cl and 0.14 M $CaCl_2$, was added to the external medium, and the medium was very gently stirred. If no action potentials could be produced by stimulation at this stage, small quantities of the NH_4Cl-$CaCl_2$ mixture were successively added to the external medium. When clear all-or-none responses could be obtained by electric stimulation under these conditions, the impedance bridge was connected to the internal platinum wire electrode and the bridge was balanced in the resting state. The axon was then stimulated, and the impedance change (bridge unbalance) which accompanied the action potential of the axon was observed.

Examples of the records obtained under these experimental conditions from seven different axons are shown in Fig. 2, top (see also Fig. 3, bottom.) In these cases,

the amplitudes of the action potential reached a maximum when the ammonium ion concentration in the external medium reached approximately 60–80 mM. When more ammonium ion was added to the medium the action potential amplitude fell rapidly. Since it is well known that ammonium is a strongly depolarizing cation, it is not surprising that high concentrations of ammonium in the medium can lead to rapid membrane depolarization and subsequent loss of excitability. Whenever all-or-none action potentials were produced under these experimental conditions, there was a concomitant reduction in the membrane impedance, although the time course of the impedance loss was considerably longer than that of the positive phase of the action potential.

Replacement of External Sodium With Rubidium or Potassium Ion

The effect of substituting rubidium for sodium in the external medium was investigated by a procedure similar to that used in the preceding experiments with ammonium replacement (Fig. 3, top). The internal perfusion fluid was 0.1 M CsF (glycerol) solution; 0.6 M RbCl solution was successively added in small quantities to the sodium-free external medium (containing 0.3 M TMA-Cl and 0.2 M $CaCl_2$). In most of these cases the

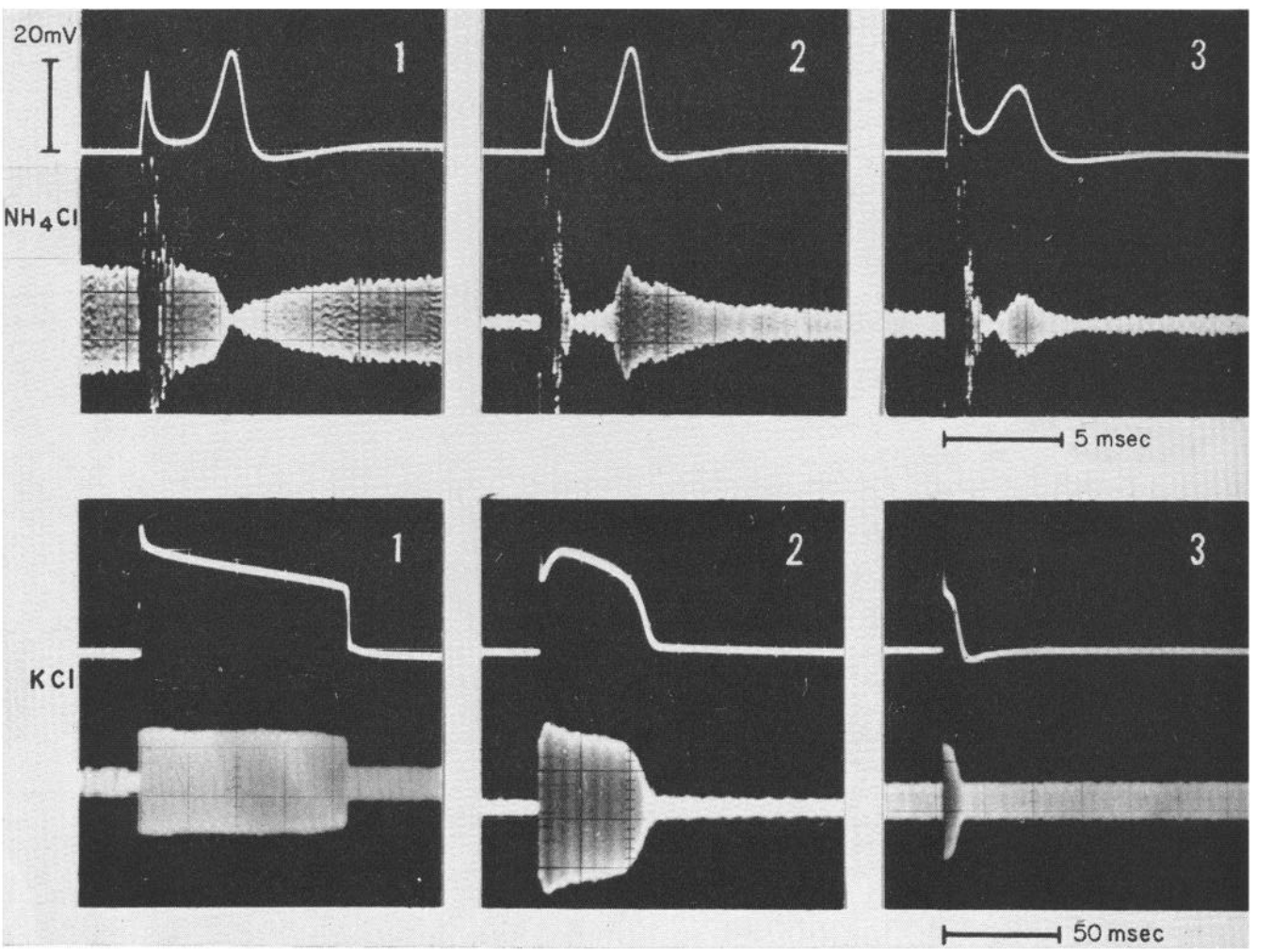

FIG. 2. Effects of replacement of sodium ion with ammonium or potassium ion. In each record, upper trace represents the potential variation and lower trace represents the simultaneous impedance variation. Top. Ammonium replacement (see also Fig. 3, bottom). The external medium was a mixture of NH_4Cl, TMA-Cl, and $CaCl_2$; the ammonium ion concentration was approximately 80 mM. The internal solution contained 0.1 M RbF. Record at left was obtained when the impedance bridge was at best balance during excitation; record in the center was obtained when the bridge was at best balance when the membrane was at rest; record at right was obtained with the electrodes at the distal end of the perfusion zone. Bottom. Potassium replacement (see also Fig. 4). The external medium was a mixture of KCl, TMA-Cl, and $CaCl_2$; the potassium ion concentration was approximately 7.5 mM. The internal solution contained 0.1 M CsF. These records were obtained when the impedance bridge was at best balance when the membrane was in the resting state. The impedance variation followed the time course of the potential variation throughout the plateau period following the peak. Records 2 and 3 were obtained from one axon at nearly the same stimulus intensity; note did ference in configuration of the responses to supra- and subthresholf-stimulation. (Note the 10-fold difference in time scale.)

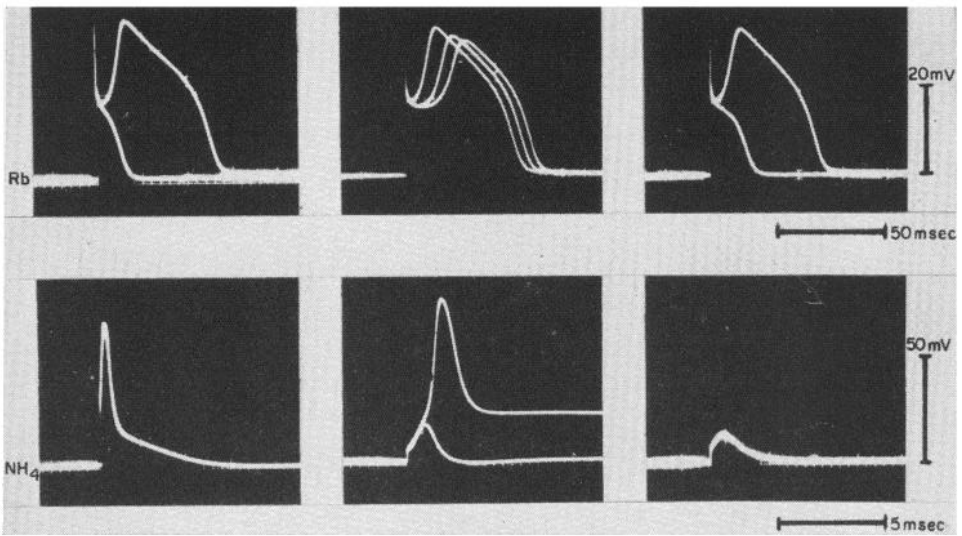

FIG. 3. All-or-none excitation in sodium-free external media. Top. Rubidium ion substitution. The external medium contained a mixture of RbCl, TMA-Cl, and $CaCl_2$; the rubidium-ion concentration was approximately 50 mM. The internal solution contained 0.07 M CsF and 0.007 M Cs-phosphate. First and third records show superposed subthreshold and suprathreshold responses. Second record shows three suprathreshold responses. Bottom. (See also Fig. 2, top). Ammonium ion substitution. The external medium contained a mixture of NH_4Cl, TMA-Cl, and $CaCl_2$; the ammonium ion concentration was approximately 80 mM. The internal solution contained 0.09 M CsF and 0.01 M Cs-phosphate. First record shows a suprathreshold response; third record shows a subthreshold response. Second record shows superposed subthreshold and suprathreshold responses, and a plateau following the peak of the suprathreshold response, similar to those observed with external potassium ion in Figs. 2 (bottom) and 4.

long, internal electrode (Fig. 1, top) was connected to the source of the stimulating currents rather than to the impedance bridge.

It was found that all-or-none action potentials appeared when the rubidium concentration in the external medium reached approximately 25–50 mM. When the rubidium concentration was significantly below this level, there was no clear sign of membrane excitability. With rubidium concentrations much above this level the membrane became depolarized, as described in the previous section for ammonium. The maximum value of the action-potential amplitude obtained under these conditions was between 30 and 60 mv. The configuration of the action potential was roughly triangular (Fig. 3), resembling those seen in single frog nerve fibers (10). However, the duration of the action potential was far longer than that observed in frog nerve fibers, ranging from 20 to 100 msec. The responses obtained under these conditions were somewhat labile, and the membrane showed a tendency toward depolarization with repeated stimulation.

Similar observations were made by using potassium (instead of ammonium or rubidium) as a substitute for external sodium ion (Fig. 4). As in previous experiments, giant axons were immersed in a sodium-free external medium containing 0.3 M TMA-Cl and 0.2 M $CaCl_2$; the internal solution was 0.1 M CsF (glycerol). A solution containing 0.01 M KCl, 0.29 M TMA-Cl, and 0.2 M $CaCl_2$ was gradually added. When the potassium concentration reached approximately 8–10 mM in the external fluid medium, relatively small, but definite all-or-none action potentials could be elicited from the axons. The amplitude of the action potential obtained when potassium ion replaced the external sodium ion was usually between 40 and 80 mv. The action potential was relatively long in duration (often greater than 10 msec), but was always accompanied by a corresponding impedance change (7 axons; Fig. 2, bottom).

This finding concerning external potassium is quite interesting since it is known that in many experimental systems in colloid and polymer chemistry, as well as in several biological systems, the physicochemical effects of potassium are intermediate between those of ammonium and rubidium. For example, it has been shown that the effect of internal potassium on the excitability of intracellularly perfused squid giant axons is also intermediate between ammonium and rubidium (15).

One significant finding obtained with rubidium or potassium ion added to the external fluid medium was that a gradual increase in the concentration of these cations often brought about a sudden, sharp rise in the membrane potential, in the absence of any applied electric current. These sudden rises in potential were observed most frequently during intracellular perfusion with 0.1 M CsF (glycerol) solution. This finding indicates that there is close similarity in the excitation processes which occur in isolated giant axons of the squid, single nodes of Ranvier of the frog, and preparations of *Nitella*. It has been shown by Hill and Osterhout (3) that a gradual increase in the external potassium ion concentration can initiate an action potential which has no repolarization phase. Similar phenomena were observed in single nodes of Ranvier of the frog nerve fiber treated with transition metal ions (11). The similarity of the phenomena observed in at least three different excitable tissues suggests that the same interpretation might apply to the excitation process in each of these excitable tissues. A detailed macromolecular model of the excitation process may be found elsewhere (14).

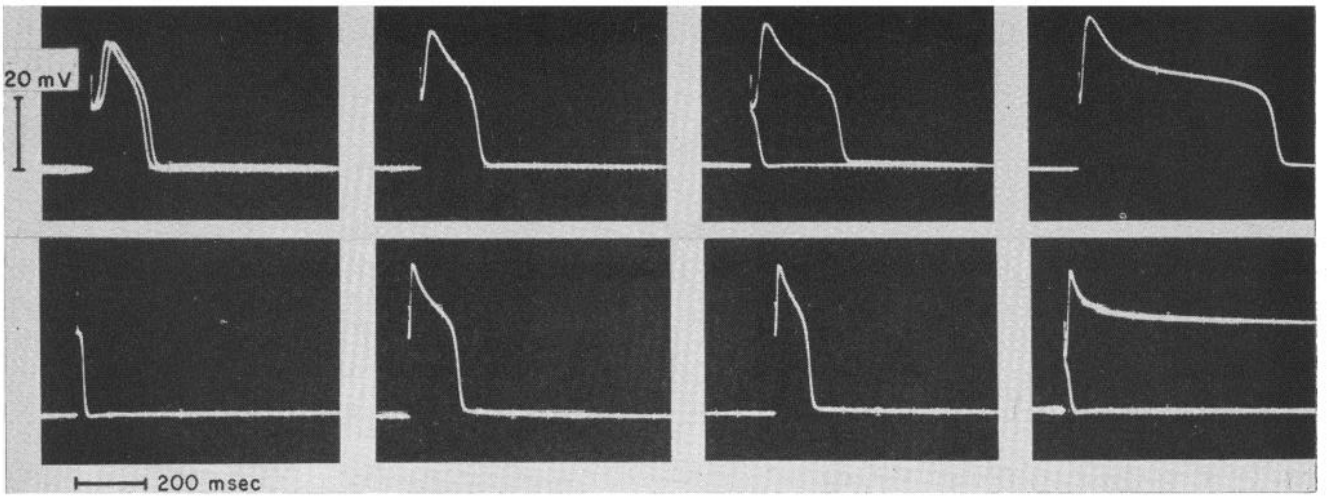

FIG. 4. Effects of replacement of external sodium ion with potassium ion. The external medium was a mixture of KCl, TMA-Cl, and $CaCl_2$; the potassium ion concentration was approximately 8–10 mM. The internal solution was a mixture of 0.09 M CsF and 0.01 M Cs-phosphate (pH 7.2). Upper and lower sets of records were taken from two different axons. Subthreshold responses are shown in 3rd, 5th, and 8th records. Third and eighth records also show superposed suprathreshold responses. All other records show suprathreshold responses, with a typical plateau following the peak (see also Fig. 2, bottom). Eighth record shows a marked plateau period; such plateaus may last more than 1 sec.

Intracellular Perfusion With Dilute NaF Solutions

As has been stated earlier, replacement of the natural sea water outside an unperfused squid giant axon with an isotonic mixture of TMA-Cl and $CaCl_2$ rapidly leads to a virtually complete loss of axon excitability. Addition of hydrazinium chloride to such a sodium-free medium (in the manner described for the alkali metal cations) usually resulted in the production of small, graded responses. In such unperfused axons application of a strong cathodal pulse often produced a graded response, whereas an anodal pulse of the same current intensity evoked no distinct response (Fig. 5, record 3). (Rarely, all-or-none action potentials were observed.)

In an axon immersed in a mixture of hydrazinium chloride and $CaCl_2$, the ability of the unperfused axon to produce all-or-none action potentials was restored by intracellular perfusion with a dilute solution of NaF. An example of experiments of this type is shown in Fig. 5. Record 1 in this figure was obtained from an unperfused axon immersed in natural sea water, with a glass-covered, steel wire electrode (30 μ diam.), discussed in a previous article (12). When the external natural sea water was replaced with a sodium-free mixture of 0.3 M choline chloride and 0.2 M $CaCl_2$ (see record 2), strong electric stimuli applied to the axon produced roughly exponentially decaying potential variations; when the polarity of the applied current was reversed, no significant change was observed in the time course of the potential variation. Record 3 was obtained approximately 1 min after replacement of the external fluid medium with a solution containing 0.3 M hydrazinium chloride and 0.15 M $CaCl_2$. At this stage, with no internal perfusion, there was a small graded response to cathodal stimuli. Records 4–8 show the successive changes in the electrophysiological properties of the axon membrane following institution of intracellular perfusion with a solution containing 90 mM NaF, 10 mM cesium phosphate buffer (pH 7.3), and glycerol. Initially, a graded response of relatively short duration was observed (before the onset of perfusion, record 3), which rapidly changed (after perfusion), first to a prolonged graded response, and then to a slow all-or-none response. (Note that the sweep speed of the oscilloscope was altered between records 5 and 6.) Occasional repetitive firing of impulses, following single-shock stimulation, may also be seen in the figure. In this experiment, flow of the perfusion fluid was maintained during the entire period of observation. Furthermore, in order to eliminate any possible spread of electrical activity from the unperfused (distal) portion of the axon, the outlet cannula was withdrawn to the cut end of the axon, so that the entire distal portion of the axon was perfused with the NaF solution. The excitability of such axons could be maintained for more than 30 min.

Action of Tetrodotoxin on Excitability in Sodium-Free Media

The puffer-fish poison, tetrodotoxin, is known to suppress action potentials in various excitable tissues (2). Recently, some investigators have speculated that tetrodotoxin specifically suppresses the "sodium carrier system" in the membrane (7, 8). In the present series of investigations, the effect of tetrodotoxin was examined in axons in which excitability was maintained in various sodium-free media.

Hydrazinium ion substitution. The effect of tetrodotoxin on

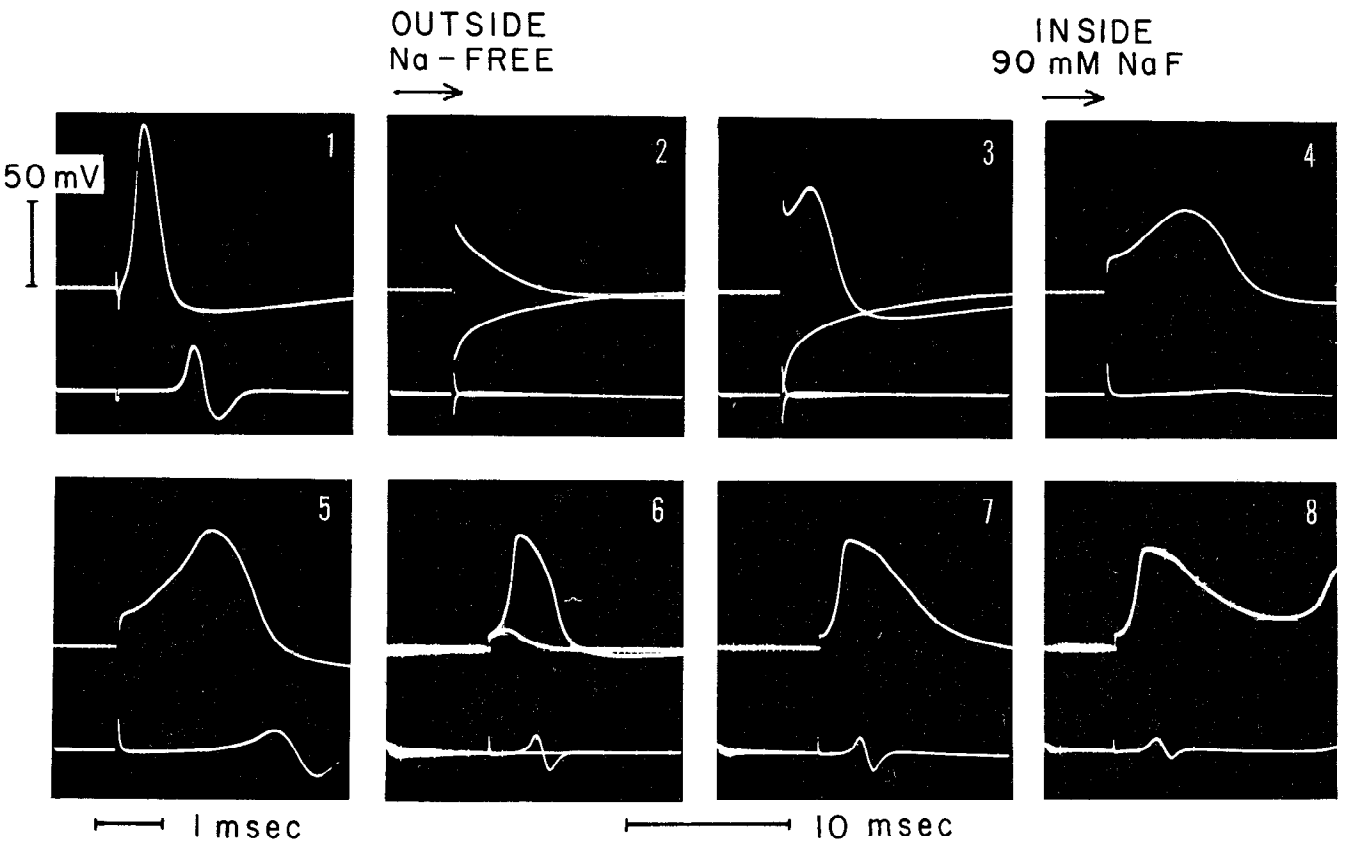

FIG. 5. Excitation of an axon with internal NaF solutions. Upper tracing in each record represents the potential variation recorded with a glass-covered steel wire internal microelectrode. Lower tracing represents the conducted potential variation recorded with the external pair of platinum electrodes (see Fig. 1, top). Record 1 was obtained in external natural sea water prior to perfusion. All subsequent records were obtained after removal of Na ion from the external medium. Record 2 was obtained after the natural sea water was changed to a mixture containing 0.3 M choline chloride and 0.2 M $CaCl_2$. No differences are observed in the superposed upper tracing between cathodal and anodal stimulation; i.e., there is no sign of excitation. Record 3 was obtained after the external solution was changed to a mixture containing 0.3 M HzCl and 0.15 M $CaCl_2$. There is no conducted action potential (lower tracing), but there is a clear sign of excitation in the superposed upper tracing. (The cathodal stimulus evokes a small peak, but there is no comparable response to anodal stimulation.) Records 4–8 were obtained after internal perfusion was instituted with a solution containing 0.09 M NaF and 0.01 M cesium-phosphate (pH 7.3). In record 4 there was only a graded response, which was not conducted. In record 5 a delayed, but definitely conducted impulse is observed. The all-or-none nature of this response is shown in record 6, where responses to subthreshold and suprathreshold stimuli are superposed on the upper tracing. (Note difference in time scale for this and subsequent records.) Records 7 and 8, obtained approximately 30 min after the onset of internal perfusion, show a gradual broadening of the potential variation, with a second spontaneous potential variation barely visible to the right of the record.

the action potential produced by axons in a solution of hydrazinium chloride and $CaCl_2$ was investigated on four axons. All axons were internally perfused with 0.1 M CsF or RbF (glycerol) solution. As described above, when immersed in an external fluid medium initially containing 0.3 M TMA-Cl and 0.2 M $CaCl_2$, the axon lost the ability to develop distinct action potentials; however, replacement of this external medium with a mixture of 0.3 M hydrazinium chloride and 0.2 M $CaCl_2$ restored excitability. When approximately 30 μl of a 10^{-5} g/ml tetrodotoxin solution (dissolved in the initial mixture of TMA-Cl and $CaCl_2$) was added to the external medium (total volume 1.5 ml), the amplitude of the action potential rapidly declined; simultaneously, the threshold for excitation rose rapidly. Frequently the excitability of the axon was completely suppressed in 1–2 min. When tetrodotoxin was removed from the medium there was a tendency toward recovery of excitability; however, recovery was rarely, if ever, complete.

Guanidinium ion substitution. The effects of tetrodotoxin on excitability maintained by external guanidinium ion were investigated in two axons. Either 0.1 M CsF or RbF was used for intracellular perfusion. The external medium contained 0.1 M guanidinium chloride, 0.2 M TMA-Cl, and 0.2 M $CaCl_2$. Tetrodotoxin (at a level of approximately 10^{-6} g/ml) was found to strongly suppress the process of excitation. The effect of tetrodotoxin was similar to that observed when 0.5% procaine was added to the external medium; but the effect of procaine was more reversible than the effect of tetrodotoxin.

Potassium ion substitution. Axons were immersed in a mixture of 0.3 M TMA-Cl and 0.2 M $CaCl_2$ and were internally perfused with 0.1 M CsF (glycerol) solution. As in the experiments described in a previous section,

excitability was restored by gradually introducing a solution containing 0.01 M KCl, 0.29 M TMA-Cl, and 0.2 M $CaCl_2$ into the external medium. When reproducible action potentials were obtained in response to cathodal stimulating currents, tetrodotoxin (at a concentration less than 10^{-6} g/ml) was added to the external medium. Following tetrodotoxin application, the threshold for excitation was raised, the duration of the action potential was distinctly decreased, and the amplitude of the action potential was reduced. It has been noted that the action potentials observed under these conditions have a rather slow rising phase; tetrodotoxin appeared to be less effective in suppressing these slow responses than in suppressing responses of short duration.

Other substitutions. Tetrodotoxin was also found to block excitability in sodium-free media when hydroxylamine (2 axons) or ammonium (3 axons) replaced the external sodium.

DISCUSSION

The experimental results described in this article indicate that the action potentials produced in axons immersed in sodium-free media are invariably associated with reduction of the membrane impedance. Since the angular frequency of the ac used for these impedance measurements (10^5 sec^{-1}) is far greater than the reciprocal of the membrane time constant (approximately 10^3 sec^{-1}), there is little or no spread of the bridge ac along the axons. Therefore, the impedance losses observed by this method reflect the properties of the restricted portion of the axon membrane near the impedance electrode.

There seems little doubt that the action potential records presented in this article also reflect the membrane properties of the axons. It might be argued that there were some sodium ions bound at or near the critical layer of the excitable membrane in these experiments. Since many biocolloids are known to possess cation-exchanger properties, it is quite likely that some fraction of the alkali metal and alkaline earth metal ions is tightly bound to various parts of the membrane structure. However, it is important to note that tightly bound or immobile ions in the membrane do not contribute either to the membrane potential or to the membrane impedance; consequently, they should be regarded as a part of the rigid structure of the membrane.

As far as mobile sodium ions remaining in the membrane are concerned, there is good evidence indicating that the time required for complete exchange is less than 1 min under the conditions of the present experiments. For example, the fluxes of radioactive tracers of the alkali metal ions introduced into the bulk solution on one side of the membrane are known to reach a stationary level within 1 min (17).

In a preceding article, we have shown that extensively cleaned axons immersed in a hydrazinium chloride-$CaCl_2$ mixture maintain their excitability for more than 1 hr (16). Repeated renewal of the external fluid medium does not affect the excitability of the axon. Since excitability can be eliminated within 1 min by replacing the hydrazinium ion in the fluid medium with TMA or choline ion and can be restored within 1 min by replacement with hydrazinium again, it does not appear possible to attribute the excitability of the axon in a hydrazinium-containing medium to a trace amount of sodium which might be released from various structures near the membrane. This argument is also supported by the fact that large all-or-none action potentials can be elicited from axons in sodium-free media only during intracellular perfusion with very favorable perfusion solutions such as 0.1 M CsF or RbF.

Since there is little doubt that under favorable conditions sodium ion is not essential for the excitation process in squid giant axons, as well as in many other biological systems, the property of sodium specificity does not appear to be necessary for excitable systems in general. There is no doubt that sodium is one of the most favorable of the external univalent cations for the maintenance of excitability, and that in the natural environment of the axon sodium ion must play an important role in excitation. However, the fact that under favorable internal conditions external sodium may be replaced by various nitrogenous cations and other alkali metals implies that the difference between sodium and other cations is quantitative rather than qualitative.

On this basis, it is not surprising that external application of tetrodotoxin is able to block the process responsible for excitation, regardless of which external cations are involved. Voltage clamp experiments also demonstrate that the inward current associated with excitation is abolished by tetrodotoxin, regardless of which cations may be responsible for that current (14 and manuscript in preparation).

The absence of any absolute specificity for sodium is also consistent with observations of the lyotropic series effects in macromolecular systems in general, as well as in squid giant axons (13, 15). It would seem that a more general mechanism than a "sodium-specific carrier" or a "sodium-specific pore" is necessary to account for the nonspecificity of the excitation process and the existence of the lyotropic series. The macromolecular model for the excitation process is consistent with both of these observations.

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