Nonuniform Response in the Squid Axon Membrane Under 'Voltage-Clamp'[1]

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ABSTRACT

TASAKI, I. AND C. S. SPYROPOULOS. *Nonuniform response in the squid axon membrane under 'voltage-clamp.'* Am. J. Physiol. 193(2): 309–317. 1958.—By cooling a portion of the squid giant axon at a time, it was shown that the repetitive membrane currents observed under the so-called voltage-clamp conditions derived from the main portion of the 'clamped' axon membrane and not from the lateral unclamped portion. An improvement of the guard system by the use of the method of 'double voltage-clamp' also supported this conclusion. Using a microelectrode introduced through the axon surface, it was found that, when the clamping level was between 20 and 35 mv, some part of the membrane became fully active while other parts remained at rest. In this range of membrane depolarization, the active area was found to increase with increasing depolarization. It was stressed that spatial nonuniformity of the squid axon membrane has to be taken into consideration in the interpretation of the results of so-called voltage-clamp experiments.

IT HAS BEEN REPORTED in a preceding paper (1) that a repetition of discrete inward membrane currents can be observed in the squid axon under the so-called voltage-clamp conditions. The present investigation is an extension of our study on the discrete phenomena under 'voltage-clamp' by the use of several new techniques. Evidence will be presented indicating that the excitable membrane of the squid axon is spatially nonuniform. As a consequence of this nonuniformity, a mixed state of the membrane (a state in which only some parts of the membrane are responding) can be produced when an attempt is made to fix the potential difference across the membrane at a constant level.

METHODS

Preparation of Axons. The technique of dissecting squid giant axons and of inserting metal wire electrodes into the axons is essentially the same as that used in the preceding experiments (1). In most of the experiments described in this paper, axons were used after extensive cleaning of the surrounding fibers and tissues. This cleaning was carried out under the type of dark-field illumination used for dissection of vertebrate single nerve fibers.

Double Voltage-Clamp. The method of double voltage clamping was devised to improve the guard system in voltage-clamping. The experimental arrangement used is illustrated in figure 1. An axon of approximately 50 mm length was mounted horizontally on a lucite platform which consisted of a middle chamber surrounded by a lateral chamber. The partition between the chambers was made of a Lucite plate of approximately 2 mm thickness and 5 mm height. The width of the middle chamber was 7 mm and that of the lateral chamber was about 15 mm on either side of the middle chamber. The chambers were filled with sea water.

A set of internal electrodes consisting of two silver wires each 50 μ in diameter was thrust into the axon. The current-electrode, E_c in the diagram, had a completely bare surface of approximately 15 mm length. The potential-

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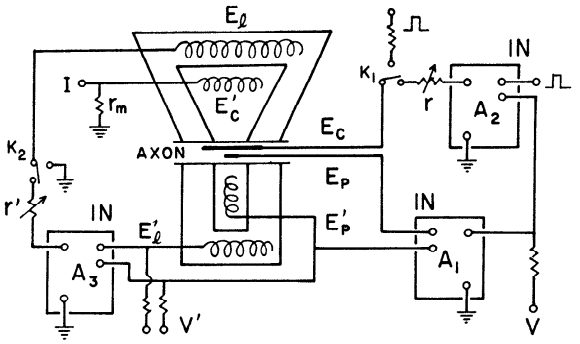

FIG. 1. Diagram of the arrangement used in 'double voltage-clamp' experiments (fig. 3). E_c and E_p internal current- and potential-electrodes respectively; E_l, E'_l, E'_p and E'_c, large external electrodes; A_1, a unity-gain differential preamplifier; A_2, a high-gain differential amplifier; A_3, another high-gain differential amplifier. V, potential difference across the membrane; V', potential difference between the middle and lateral pools; I, current required to vary the membrane potential along rectangular time courses. Further detail, in text.

electrode, E_p, was wound around the current-electrode, and its surface enamel layer was scraped off for a length of about 4 mm in the middle of the exposed surface of the current-electrode. These silver wire electrodes were used both with and without chloriding the surface electrolytically prior to each experiment.

All the external electrodes were made of large silver wire covered with a layer of gauze and agar-gel. As shown in the diagram, the potential-electrode in the middle pool, E'_p, was placed on the side opposite to the position of the external current electrode, E'_c. The potential difference between the two potential-electrodes, E_p and E'_p, recorded with a unity-gain differential amplifier, A_1 in the diagram, was taken as the measure of the membrane potential. In the lateral pool were immersed also two large electrodes, one on each side of the axon. One of these electrodes, E'_l, was used to measure the potential difference, V', between the two pools; the other electrode, E_l, was used to control the current flowing into the lateral pool.

The function of the circuit illustrated on the right-hand side of the nerve chamber was to maintain the potential difference between E_p and E'_p at a constant level by an automatic control of the membrane current flowing into the internal current-electrode, E_c. The details of the high-gain differential amplifier, A_2, and the resistance, r, between the axon and A_2 were described in the preceding paper (1). The circuit shown on the left-hand side of the axon was used to clamp the potential difference, V', between the two pools at zero. This was accomplished by amplifying the potential difference, V', between the pools 1000 times with a Tektronix amplifier (No. 122) and connecting the output of the amplifier to the current-electrode, E_l, in the lateral pool.

The procedure of a double voltage-clamp experiment was as follows: after a cleaned axon was mounted in the nerve chamber, the partitions between the middle and lateral pools were carefully sealed with vaseline. By filling the lateral pool with sea water stained with chlorphenol red and varying the fluid levels in the pools, it was shown that the sealing was water-tight. After raising the level of the sea water in the middle pool to approximately 5 mm, the action potential of the axon was recorded by connecting the source of rectangular voltage pulses (0.15 msec. in duration and up to 150 v. in strength) to the internal current-electrode, E_c, through a 2-megohm resistor. The two pools were directly grounded at this moment.

Then, by switching K_1 in the diagram for a period of 0.5–1 second in every 10 seconds, E_c was connected with resistor r and the amount of feed-back necessary to clamp the potential difference between E_p and E'_p was determined. Finally, keeping r at the highest practical value and switching K_2 toward r' as K_1 was connected to r, resistance r' was reduced step by step until the potential difference (V') between the two pools was completely suppressed by the automatic adjustment of the current flowing into the lateral pool. In practice, the potential difference between the pools was suppressed to the order of 1 mv when r' was reduced to 6–7 kilohms with the gain of A_3 kept at 1000. In this series of experiments, the resistance, r_m, for measuring the membrane current was 5 ohms. The experiments were carried out at room temperature (21–23°C).

Preparation of Microelectrodes. Hyperfine glass pipette electrodes were prepared by the alcohol method; glass pipettes were boiled in 95% ethanol under reduced pressure, and, after replacing alcohol in the pipettes once with distilled water, the pipettes were filled with a 3 M KCl solution. The resistance of the elec-

trodes used in the present experiments was in the range between 7 and 15 megohms (measured with the tip immersed in sea water). The cathode-followers used in conjunction with these microelectrodes were made of British tubes, Z729, in triode connection. The input capacity of these cathode followers was of the order of 10 $\mu\mu$f; when the resistance of the electrode was about 10 megohms, there was a slight distortion in the recorded potential.

RESULTS

Effect of Local Cooling Upon the Repetitive Inward Surges Under 'Voltage-Clamp.' The discrete and repetitive phenomena under 'voltage-clamp' could be demonstrated at low temperature (4–5°C) as well as at room temperature (20–22°C). By a fall in temperature, both the duration of the individual inward surge and the interval between surges were markedly prolonged. A comparison of the temperature coefficient of the discrete inward surge with that of the action potential recorded by the conventional method will be discussed by one of us (C.S.S.) in a subsequent paper. The purpose of the present series of experiments was to compare the effect upon the configuration of the repetitive inward surges of cooling the portion of the axon in the middle pool with that of cooling the portion of the axon in the lateral pool.

The arrangement used was similar to that in figure 1, except that the lateral pool was always grounded. The membrane current under voltage-clamp was measured by recording the potential drop across a 2-ohm resistor (r_m) connected between the external current-electrode (E_c') in the middle pool and ground. The length of the axon was approximately 50 mm, the length of the internal current-electrode was 22 (or 28) mm, and the width of the middle pool was 10 mm. The partitions between the pools were approximately 1.8 mm wide. The temperature of the fluid in the pools was measured with a thermister thermometer which had a response time of about 1 second.

In figure 2 are presented two examples of experiments in which the portion of the axon in the middle pool was cooled for a period of about 1 minute. This was done by filling the lateral pools with a large amount of sea water at room temperature and replacing the fluid in the middle pool quickly with sea water cooled

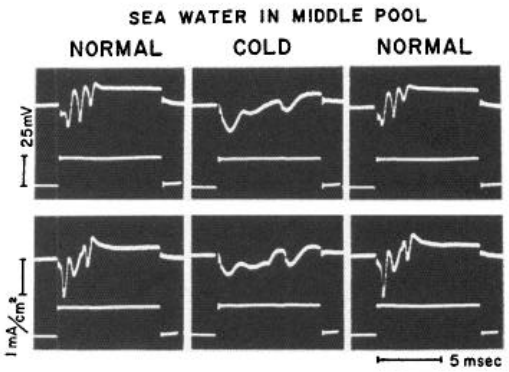

FIG. 2. Effect of cooling the axon in the middle pool upon the repetitive inward surges recorded from the middle portion of the axon. The lateral pools, in which the edges of the clamping electrode were immersed, were maintained at room temperature (22°C) throughout. The upper and lower rows were taken from 2 different axons.

down to about 5°C. Since there was some fluid remaining in the middle pool when the cold sea water was introduced, the temperature of the fluid in the middle pool at the time of recording was higher than that of the fluid poured into the pool. It was about 10°C in the experiment illustrated in the upper row of the figure and about 13°C in the experiment in the lower row. The temperature of the fluid in the lateral pool, measured at the edge of the internal current-electrode, at the moment of recording, was still within 1° below the room temperature (22°C). It was found that this local cooling affected the duration and the rhythm of the repetitive inward surges promptly and reversibly.

By the converse procedure, namely when the cold sea water was introduced into the lateral pool keeping the temperature of the middle pool approximately constant, no clear effect was observed upon the configuration and the rhythm of the repetitive surges. The action potential recorded in the middle pool (under current-clamp) remained also unaffected by cooling the portions of the axon in the lateral pool.

These observations can be regarded as an additional piece of evidence that discrete and repetitive inward surges of membrane current under 'voltage-clamp' derive from the portion of the axon in the middle pool and not from the portion of axon near the edges of the current-electrode. In a recent paper, Frankenhaeuser and Hodgkin (2) discussed the method

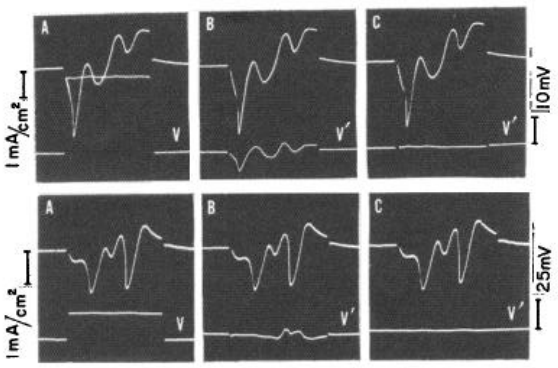

FIG. 3. Two examples of 'double-voltage-clamp' experiments. *Upper trace* represents the membrane current flowing through the portion of the axon in the middle pool in fig. 1. *Trace V* shows the time course of the membrane potential, and V' the potential difference between the middle and lateral pools. 22°C.

of eliminating 'propagation artifacts' arising from impulses in the lateral pool. We suspect that some of these 'artifacts' might have been a form of the repetitive surges which have concerned us in the present series of experiments. They could eliminate these inward surges by reducing the length of the axon in the lateral pools to the order of the length constant of the axon. This procedure, however, may affect not only the responsiveness of the portion of the axon in the lateral pools but also that of the axon in the middle pool. A less drastic attempt at eliminating the activity in the lateral pool was described in the preceding paper. It was found that application of a urethane solution (which eliminated the action potential without affecting the resting potential) to the portion of the axon in the lateral pool did not materially affect the repetitiveness of the axon under voltage-clamp.

Effect of Improvement of the Guard System by the Double Voltage-Clamp Technique. With the arrangement of figure 1, if there is no flow of current across the partitions between the two pools through the leakage resistance outside the axon, the current that flows through r_m can be regarded as deriving purely from the axon membrane in the middle pool. In this series of experiments, axons used were cleaned extensively and sealing of the partitions was water-tight. But, since there is still a thin conducting layer outside the excitable membrane, a potential difference between the pools may cause a flow of current between the pools. This leakage current tends to mix the membrane current from the portion of the axon in the middle pool with that in the lateral pool.

The flow of current through the leakage resistance outside the axon can be reduced to zero, if the potential difference between the two pools is eliminated completely by the second voltage-clamp device. The purpose of the present experiment is to examine whether or not the perfection of the guard system by this method affects the pattern of repetitive inward membrane currents observable under the 'voltage-clamp' conditions.

In figure 3 are presented two sets of records taken by this method (from 2 different axons). Records labeled *A* in the figure show the time course of the potential difference, V, across the membrane and that of the membrane current observed when the lateral pool was directly grounded. The repetitive character of the membrane current induced by the clamping pulse is evident. When the input of the lower oscillograph beam was switched at this moment to display the potential difference, V', between the pools, it was found that there was a small potential variation between the pools associated with some of the large inward surges in the membrane current (record *B*). Finally when the second voltage-clamp device was put into operation, the potential variation, V', between the pools was practically perfectly suppressed (records *C*). As can be seen in the records, the time course of the membrane current recorded was not affected to any measurable extent by the suppression of the potential variation.

Cole and Hodgkin (3) estimated the thickness of the conducting layer outside a cleaned axon to be equivalent to a layer of sea water of about 15 μ in thickness. If this value be adopted, then the leakage resistance between the two partitions of 2 mm width should be of the order of 10 kilohms. A potential difference of about 5 mv between the pools is expected to cause a leakage current of the order of 0.5 μa. When the potential difference is reduced to a level of about 1 mv, the leakage current should be of the order of 0.1 μa. The amplitude of the membrane current of a repeating inward surge observed in these experiments is approximately 100 μa. (Note that the area of the axon membrane in the middle pool is about 0.1 cm^2.)

It is therefore evident that the leakage of the current across the partitions is too small to alter the total membrane current by any appreciable amount.

This experiment, together with several other experiments stated in this and the preceding paper (1), can be regarded as convincing proof that the repetitive inward currents under 'voltage-clamp' arise in the portion of the axon membrane in the middle pool and do not derive from the membrane in the lateral pool.

Direct Measurement of the Membrane Potential of the Axon Clamped by Means of a Capillary Potential-Electrode. In the preceding experiments, the potential difference between the two potential-electrodes, one inside the axon and the other in the external fluid medium, was taken as the measure of the 'membrane potential.' When the membrane is capable of developing a strong inward current, however, the potential in the middle of the axon can be very different from the potential of the axoplasm in the immediate neighborhood of the membrane, due to the IR-drop in the axoplasm. In order to determine the amount of discrepancy between our measure of the 'membrane potential' and the actual potential drop across the membrane, we started exploring the potential near the membrane by introducing a hyperfine glass pipette electrode into the axon.

It was found by this method that the potential drop across the surface membrane of the axon was by no means held constant in voltage-clamp experiments. When the clamping pulse was in the range between 20 and 35 mv, the potential of the microelectrode inserted immediately underneath the membrane in the 'clamped' region of the axon showed a potential variation as large as 70–90 mv. The pattern of this potential variation recorded by means of a microelectrode was found to vary from spot to spot along the 'clamped' portion of the axon membrane and to change markedly with the potential level at which the membrane was 'clamped.' An example of such microelectrode experiments is reproduced in figure 4.

The experimental conditions under which these records were taken are illustrated by the diagram above the figure. The internal current-electrode, E_c, was in this case a 100-μ lightly

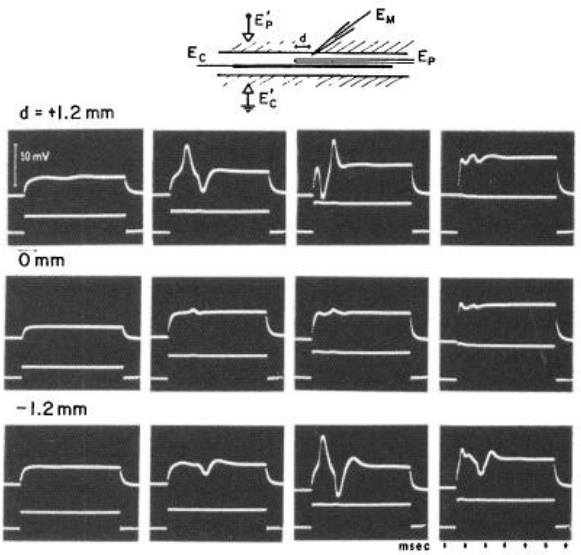

FIG. 4. Direct exploration with a microelectrode of the membrane potential of an axon under 'voltage-clamp.' A longitudinal glass capillary electrode, E_p, was used to monitor the clamping level of the 'membrane potential.' The distances (d) between the tip of the monitoring potential-electrode and the tip of the exploring microelectrode (E_m) are given. *Upper trace* represents the potential difference between E_m and E'_p. The *lower trace* shows the potential difference between E_p and E'_p. 20°C.

chlorided silver wire of approximately 14 mm length. The internal potential-electrode, E_p, was a glass capillary of 60–80 μ in diameter filled with 0.5 M KCl solution. In order to reduce the resistance of this electrode, the silver wire immersed in the saline was pushed almost to the tip of the glass pipette. The internal electrodes, E_p and E_c, were introduced into the axon by means of two separate micropositioners through two separate holes in the axon membrane, one at each end of a 50-mm-long axon. The advantage of using a separate internal electrode was to enable us to move the potential-electrode with the current-electrode fixed in the axon. Voltage-clamping was carried out by the feed-back amplifiers shown by the diagram in figure 1, right. The microelectrode was held by a third micro-positioner and was pushed into the axon in most experiments at a point near the middle of the internal current-electrode.

The records in the middle row in figure 4 show the results obtained when both the longitudinal potential-electrode and the microelectrode were at the same point along the axon. When the clamping pulse was less than

15 mv in amplitude, the potential at the tip of the microelectrode referred to the external potential electrode, E'_p, showed a rectangular time course. (Note that the rounding-off of the potential trace at the onset of rectangular pulses is due to the capacity of the input of the recording system.) For clamping pulses greater than about 20 mv, small potential variations (less than about 10 mv) were observed at the tip of the microelectrode. The potential at the tip of the longitudinal potential-electrode was held always at a constant clamping level. Evidently, this potential variation represents the IR-drop in the axoplasm between the tips of the microelectrode and the longitudinal potential-electrode.

When there was a distance of about 1 mm or greater between the tips of the two potential recording electrodes, E_p and E_m, and when the level of depolarization was between 20 and 35 mv, the potential recorded by the microelectrode showed variations which were often as large as 100 mv peak to peak. Effectively then, in this range of depolarizations, the membrane potential in the 'clamped' region of the axon was not clamped. For pulse intensities lower than 15 mv, the potential of the microelectrode followed the potential of the longitudinal potential-electrode; in other words, the membrane potential was clamped satisfactorily. When the clamping pulse was increased to levels above 50 mv, the divergence of the membrane potential from the clamping level became smaller again (see also fig. 5).

In starting our voltage-clamp experiments, we had been tacitly assuming that the surface membrane of the squid giant axon was physiologically uniform. Evidently, the observation described in this section proves that this assumption is completely wrong. When the threshold membrane potential (for a discrete inward current) is reached in one region of the axon membrane, there is a strong membrane current limited in this particular region of the axon membrane. This inward current is expected to bring about a potential variation along the axon, because of polarization of the current-electrode.

In this extremely complicated network consisting of the axon membrane, the axoplasm and the polarizable current-electrode, the potential at only one point in the axon, namely the potential at the tip of E'_p is held constant by the voltage-clamp arrangement. In the range of depolarizations between 20 and 35 mv, this particular point in the axon behaves like a 'node of a standing wave'; when the potential on one side of the electrode rises, the potential on the other side goes down, thus keeping the potential at the tip at a constant level which is determined by the amplitude of the clamping pulse. When the clamping pulse rises above 50 mv, the whole surface membrane is excited more or less uniformly; therefore, the membrane current, as well as the polarization of the current-electrode, becomes spatially uniform (cf. DISCUSSION). The results obtained by moving the longitudinal potential-electrode while keeping the hyperfine microelectrode fixed were comparable to those obtained by the reverse procedure in which the microelectrode was moved.

Membrane Potential of the Axon Clamped by Means of a Long Potential-Electrode. In the experiments described in the preceding section, the potential-electrode used to clamp the 'membrane potential' was a glass capillary. In the present section, we describe the results of observations in which the internal potential-electrode had a long recording surface instead of a point.

The arrangement of the internal electrodes and the feedback system used in this series of experiments was the one illustrated in diagram *A* of figure 1 in the preceding paper (1). Both the main current-electrode and the internal potential-electrode had an exposed surface of 5 mm length. The effective length of the lateral current-electrode on either side of the main electrodes was either 4.5 mm or 7.5 mm. This set of internal electrodes was made from three silver wires. Each wire was 50μ in diameter and coated with insulating enamel; the internal electrodes occupied a space of slightly greater than 100 μ in diameter along the axis of axons of approximately 500 μ. The microelectrode was introduced into the axon at various points in the middle section of the electrode set. The potential difference between the microelectrode and the external potential-electrode was recorded by the second beam of the dual-beam oscillograph together with either the current carried by the main current-electrode or the potential difference

between the internal and external potential-electrodes.

When the tip of the microelectrode was pressing the surface of the axon membrane (before penetration), very often it recorded small potential variations. These variations were up to about 5 mv in amplitude and usually negative in sign. Since such negative potential variations appeared always at the peaks of the repetitive inward membrane currents recorded by the current trace, it is evident that they represent the IR-drop in the medium caused by the current flowing inwards through the membrane at the point where the microelectrode is making contact.

Upon penetration of the microelectrode, there was a shift of approximately 60 mv in the potential of the microelectrode, representing the appearance of the resting potential at the electrode tip. When the clamping pulse was in the range between 20 and 35 mv, such a microelectrode revealed large potential variations, the pattern of which varied markedly from point to point along the 'clamped' region of the axon. No point along the 'clamped' region could be found where these large potential variations were absent. The potential recorded by the long internal potential-electrode appeared to correspond to the average of the potential along the 'clamped' portion of the axon.

An example of such observations is reproduced in figure 5. When the size of the clamping pulse approached the threshold for discrete inward surges, the potential at the tip of the microelectrode beneath the axon membrane showed a slight wavy variation (see also the record in figure 4, top, left). When the strength of the clamping pulse was increased slightly above this level, there was always a repetition of large diphasic potential variations. The potential at the peak of these potential variations, measured from the level of the resting potential, was frequently 70–90 mv. Undoubtedly, the potential changes represent the appearance of full-sized response in the region of the membrane where the microelectrode was inserted. The time interval between successive responses was usually twice as long as the interval between the peaks of discrete membrane currents recorded by the main current-electrodes. In some cases, however,

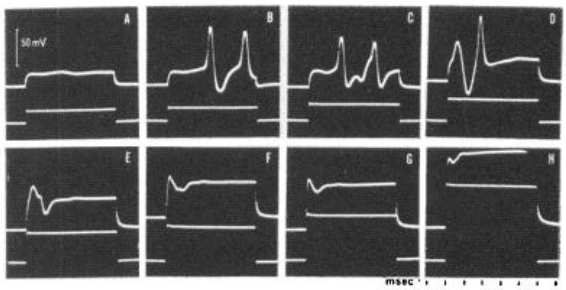

FIG. 5. Direct measurement with a microelectrode of the membrane potential of an axon under 'voltage-clamp.' The monitoring potential-electrode was a 5-mm long silver wire. The microelectrode was introduced in the middle of the potential-electrode. *Upper trace* represents the potential difference across the membrane recorded with the microelectrode. *Lower trace* shows the time course of the potential difference between the internal and external potential-electrodes. Note that the potential variation decreases in the lower row with increasing pulse intensity. 20°C.

the responses recorded by the microelectrode had the same frequency as the repetitive inward surges in the membrane current.

The pattern of potential variations recorded by the microelectrode varied markedly with the strength of the clamping pulse. When the pulse strength was above 50 mv, the repetitiveness in the membrane current disappeared, and correspondingly the membrane potential recorded by the microelectrode showed a single, diphasic variation. The time course of such a potential variation can easily be accounted for on the basis of the known properties of a response of the squid axon.

The upper potential trace in record *G* in the figure, for example, shows an initial peak of about 7 mv above the clamping level which was in this case approximately 62 mv. At the peak of activity, therefore, the membrane should be traversed by a strong inward current, and this current tends to lower the observed membrane potential due to the IR-drop within the membrane. As the activity in the membrane comes to an end, the membrane potential should fall rapidly. When the membrane potential falls below the clamping level, there should be an outward current through the membrane. The final, steady level of the membrane potential is very close to the level of the clamping pulse; this can be explained as due to the fact that the emf of the membrane stays well above the resting

potential during maintained depolarization (see fig. 3 in the subsequent paper (4)).

The time courses of the membrane potential in the range of depolarizing clamping pulses between 20 and 35 mv are complex but it is not very difficult to explain them, at least qualitatively. The peak values of the large repetitive responses (70–90 mv) are very close to the membrane action potential recorded by the microelectrode. (Note that, because of the input capacity of the cathode-follower, the action potential recorded by the microelectrode was slightly smaller than 100 mv). The difference between the amplitude of a full-sized action potential and the size of the recorded repetitive responses can be accounted for in terms of a potential drop across the internal resistance of the active membrane (see DISCUSSION).

The marked undershoot following individual responses can be explained as a reflection of *a*) the undershoot following an ordinary action potential and *b*) the polarization of the internal electrodes. When a limited area of the axon membrane becomes active, the polarization of the internal electrodes tends to lower the membrane potential in other, resting regions, thus maintaining the average potential in the clamped portion of the axon at a constant level (cf. also p. 219–220 in ref. 2). Some of the large responses may be attributed to the effect of polarization of the internal electrodes which tends to raise the potential of the axoplasm and consequently to reduce the membrane current. Smaller responses may be obtained if the recording microelectrode is situated near the boundary between the active and resting areas of the membrane.

DISCUSSION

In most of our fresh preparations, the active membrane had an emf of about 110 mv above the resting potential and an internal resistance of 6–12 ohm·cm^2 (cf. also p. 873 in ref. 5). To this value of the internal resistance, not only the resistance of the membrane proper but also those of other structures between the internal and external potential-electrodes are contributing. In the range of the membrane resistance mentioned above, smaller values were obtained at room temperature (20–22°C) and larger values were observed at low temperatures (5–7°C). In old preparations after repeated voltage-clamping, the resistance of the membrane during activity was higher than the values mentioned above.

The maximum inward membrane current observed under 'voltage-clamp' with rectangular pulses was, in most of our fresh preparations, in the range between 7 and 10 ma/cm^2 at room temperature (20–22°C); and at low temperatures (5–7°C) it was around 5 ma/cm^2. These currents were observed when the membrane was clamped at a level 50–60 mv above the resting potential. When the clamping pulse was terminated at the peak of this membrane current (the membrane being clamped at the resting level), a sudden, transient increase of about 30% in the membrane current could be demonstrated.

The specific resistance of the axoplasm of the squid axon is known to be of the order of 40 ohm·cm at room temperature and to increase by about 30% for a fall in temperature of 10° (6). The resistance of the axoplasm between the internal electrode of 50μ in diameter and the membrane of an axon of 500μ in diameter is therefore about 15 ohms for a unit length of the axon. Since the membrane of an axon of a length of 6.3 cm and 500 μ in diameter occupies an area of 1 cm^2, the contribution of the axoplasm resistance to the total internal resistance of the active membrane (6–12 ohm·cm^2) is of the order of 2–3 ohm·cm^2. When the membrane is traversed by a current of 8 ma/cm^2, the potential drop across the layer of axoplasm between the membrane and the internal electrode should be around 20 mv. This is considered to be the cause of the difference between the two potential traces in the middle row of figure 4.

Subtracting the axoplasm resistance just mentioned from the total internal resistance of the active membrane, we have a figure of 4–9 ohm·cm^2 as the resistance of the whole layer of the axon membrane during activity. When this layer is traversed by a current of 5–10 ma/cm^2, the drop in the membrane potential due to the internal resistance amounts to 40–50 mv. Therefore, a microelectrode introduced beneath such a fully responding area of the axon membrane should record a peak potential of only 60–70 mv. This argument justifies our statement that, when the

axon membrane was 'clamped' at a level between 20 and 35 mv above the resting potential, some part of the membrane becomes fully 'active' while other parts remain resting.

The lightly chlorided silver wire used as internal electrodes is highly polarizable and is inadequate to pass strong currents needed to clamp the potential of the squid axon. The polarization resistance of a 50μ silver wire of 6.3 cm length, measured with a constant current of about 1 ma, was generally between 50 and 70 ohms. The time constant of a roughly exponential change across the chloride layer of the electrode was of the order of one-third millisecond. We made an atempt to reduce electrode polarization by platinizing the surface instead of chloriding it. We found, however, that a platinized wire electrode is almost as polarizable as a chlorided silver wire electrode when it was tested after a series of voltage-clamp experiments.

It is possible that the rhythm of the voltage-clamp repetition in a normal axon at room temperature is influenced to a considerable extent by polarization of the internal electrodes. However, the marked effect of lowering the temperature (fig. 2) and of an injection of TEA (fig. 7 in ref. 1) upon the rhythm of repetition strongly suggests that polarization of the electrodes is not an essential factor for occurrence of the repetitive responses under 'voltage-clamp.' In the voltage-clamp experiments on single node preparations of the toad nerve fiber (7), the current-electrode used was undoubtedly nonpolarizable; repetitive responses under 'voltage-clamp' has been demonstrated in these preparations. The essential factor that causes a voltage-clamp repetition is evidently the strong tendency of the excitable membrane to take either one of the two stable potential states, active or resting.

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