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SODIUM CONDUCTANCE SHIFT IN AN AXON INTERNALLY PERFUSED WITH A SUCROSE AND LOW-POTASSIUM SOLUTION

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Internal perfusion of giant squid axons is a most valuable new method recently developed by Baker, Hodgkin & Shaw (1961, 1962*a*) and by Tasaki and co-workers (Oikawa, Spyropoulos, Tasaki & Teorell, 1961; Tasaki, Watanabe & Takenaka, 1962; Tasaki & Shimamura, 1962). This technique adds a new dimension to experimental investigations of the electrical characteristics of membranes of single nerve cells by making it possible to control the internal medium (as well as the outside medium).

One of the most interesting observations made by Baker, Hodgkin & Shaw (1962*b*), Tasaki *et al.* (1962) and Tasaki & Shimamura (1962) and also reported in a paper by Narahashi (1963) is the maintenance of full-sized action potentials when the internal potassium is reduced to low levels by substitution of sucrose or glucose. This occurs in the face of a reduction of the absolute membrane potential from -65 to -35 mV when the internal potassium is lowered from 530 to 11 mM. On the other hand, when the potassium is replaced by sodium, calcium, or choline, the action potential failed when the resting potential had fallen from -60 mV to about -40 mV (Baker, Hodgkin & Meves, 1963). In an intact axon this amount of depolarization would completely inactivate the sodium-carrying system as measured in a voltage clamp. Hodgkin presumed (personal communication) that there must have been a shift in the inactivation curve for sodium with sucrose replacement of internal potassium. Narahashi (1963) observed a shift in the effect of steady currents on the overshoot of the action potential giving further presumptive evidence of an inactivation change.

We therefore decided to study the membrane conductances and inactivation of sodium in a perfused axon by means of the voltage-clamp technique.

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METHODS

Perfusion and chamber. The method of extrusion and perfusion is the same as that described by Narahashi (1963), which is an adaptation of the method of Baker *et al.* (1962*a*). After the axoplasm had been extruded and the membrane reinflated with the standard internal solution containing 530 mM of potassium (as K_2SO_4) and 12 mM of NaCl, the end of the axon away from the cannula was tied off. The axon was then transferred to a chamber which was an adaptation of that used by Julian, Moore & Goldman (1962) for the voltage clamp of lobster axons. The tied-off end of the axon was pulled through horizontal holes (600 μ diameter) in the partitions separating the three compartments of the chamber in which natural sea water was flowing. The flow of isotonic sucrose solution was then started. It moved up through vertical holes to irrigate the two segments of the axon in the partitions and then it flowed along the axon into the central and lateral pools, as shown in Fig. 1. In order to obtain good insulation between the three pools the horizontal holes were somewhat gouged out, thus allowing the sucrose solution to flow around the axon to the top.

The rate of flow of sea water in the central pool was adjusted so that a very short length of axon, an artificial 'node', was exposed to sea water. Next, the solution in the lateral pools was changed to 500 mM-KCl with 25 mM-$CaCl_2$. The axon membrane was then cut so that the internal solution could flow out and go down the drainage channel of the potential pool and the perfusion flow was started by means of a hydrostatic pressure of 6–9 cm H_2O in a syringe connected to the cannulated axon in the left or current pool.

TABLE 1. Composition of internal solutions (mM)

	K^+	Na^+	Cl^-	SO_4^{2-}	Phosphate* (as $H_2PO_4^-$)	Sucrose	Conductivity (m-mho/cm)
High-potassium (HK)	530	12·4	12·4	250	15·6	446·4	32
Low-potassium (LK)	10·6	12·4	12·4	5	0·9	902·1	1

* KOH added to KH_2PO_4 or NaOH to NaH_2PO_4 to adjust pH to 7·7.

The solutions used for internal perfusion are given in Table 1. All of the externally applied solutions were passed through an ice bath en route to the axon chamber and the temperature of the solutions and axons was measured with a thermocouple. Although the temperature was not monitored continuously, it did not appear to vary from 7° C by more than $\pm 2°$ C.

Voltage clamp. The sucrose-gap voltage-clamp system developed by Julian *et al.* (1962) was adapted for the squid axon as shown in schematic and diagramatic form in Fig. 1. Connexions with the three pools were made by means of KCl-agar bridges to large stable Ag-AgCl electrodes.

The potential across the sucrose gap (between the central and right pool) was measured by an electrometer-transistor pre-amplifier. The output of this amplifier (1) was forced to follow holding and pulse command signals by the clamp amplifier (2) whose output changed the potential in the left-hand pools so as to inject or withdraw current as required. The current passing through the 'nodal' membrane was measured by an operational amplifier (3) whose summing point was connected to an electrode in the central pool which was maintained at virtual 'earth' level by means of feed-back from this amplifier. The membrane area (as a fraction of 10^{-3} cm^2) was entered as a setting on the membrane area potentiometer, so that the output I_m of amplifier (3) was directly proportional to the *current density*. When the axon was perfused with the low-conductivity LK solution, a small amount of the measured current (about 1·5 %) must have passed through the sucrose insulation between the current and the central pool.

The resistance through the sucrose surrounding the axon between the centre and either

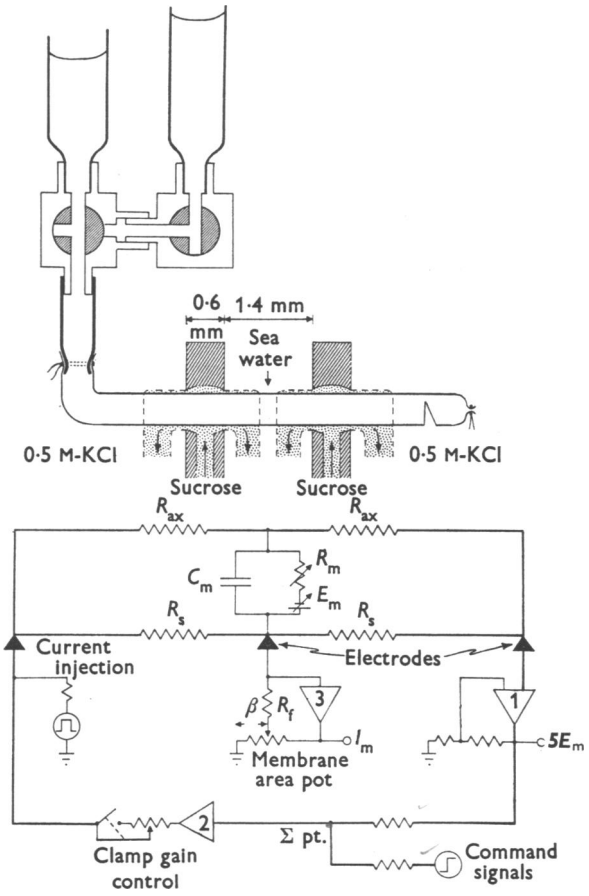

Fig. 1. Schematic diagrams of the experimental arrangement. The upper part represents a section through the axon chamber parallel to the fibre axis. The lucite partitions are striped and the flowing sucrose solution is stippled. A short segment (or artificial 'node') of the exterior of the axon remains in contact with the sea water flowing in the centre pool. The ends of the axon are in side pools containing isosmotic KCl solution. Internal perfusing solutions enter the axon via the cannula from the syringes (containing the standard and test solutions) and flow out of the cut in the axon in the right-hand pool.

The lower part of the figure is the electrical equivalent circuit. R_{ax} is the resistance through the axoplasm, R_s the resistance through the sucrose solution. C_m, R_m, and E_m^0 are the capacity, resistance, and open-circuit potential of the 'nodal' membrane respectively. Segments of the axon in KCl solution are shown as short circuits because of the low membrane resistance and zero membrane potential under these conditions. Electrodes are represented by small filled triangles. E_m is the potential difference between the right-hand pool and virtual earth as measured by electrometer amplifier 1 whose output is 5 E_m. In order to clamp, the switch of the clamp-gain control arrangement is closed and the resistance decreased to zero. Operational amplifier 2 will then inject a current pool to match E_m with the command signals at the summing point, Σpt. Currents through the 'node' membrane cause an IR drop across the known feedback resistor R_f of operational amplifier 3. If the area potentiometer is set to a value β calculated from microscopic measurement of the axon diameter and calculation of the membrane area, the output voltage I_m of amplifier 3 is a known and linear function of the membrane current density.

lateral pool was about 3 MΩ, and is indicated by R_s. The membrane resistance of the length of axon in the lateral pools was reduced to a very low value by the 500 mM-KCl solution and the membrane potential was also near zero for the standard high internal potassium. The longitudinal internal resistance of the perfusing fluid between the exposed node and either lateral KCl pool was in the neighbourhood of 1·5 kΩ (1 mm length) for the HK perfusate and 50 kΩ for the LK solution. The length of the 'node' was made as short as one half to one quarter of the diameter of the axon so as to have nearly uniform potential over this length. K. S. Cole (personal communication) has made calculations and resistor analogue model measurements and concludes that in an intact axon the membrane potential and current density will vary less than ±5% of the average value over a 150 μ 'nodal' length or ±10% over a 210 μ length of node. The same uniformity should exist when the axon is perfused with the HK solution which had approximately the same conductivity as axoplasm. The nodal membrane potential must have been less uniform with the LK perfusate, but we did not observe oscillations in the current records. In most of our experiments we were able to use a gap in the order of 200 μ in length. Such a nodal length gives a $3{\cdot}4 \times 10^{-3}$ cm² of membrane area in a 500 μ diameter axon. The resting resistance of such a nodal membrane would be in the order of 10 kΩ (note high conductance of I_0 curve in Results) and the voltage measured across the sucrose gap would be approximately 99% of the membrane potential E_m^0 for the HK perfusate (97·5% for the LK perfusate).

No correction of the measured membrane potential was made when the axon was perfused with the HK solution (630 mg K ions/l.) because the potential across the membrane in the right pool (containing 500 mM-KCl) was assumed to be zero or very close to it. This assumption is based on measurements under very similar conditions in perfused axons (Baker *et al.* 1962*b*, Fig. 2, curve *C*) and in intact axons (Curtis & Cole, 1942; Moore, 1959). When the central pool as well as the potential pool contained 500 mM-KCl the membrane potential was zero within ±3 mV. This means that the hyperpolarizing effect of the sucrose solution, which will be described later, was virtually absent under these conditions.

However, when the perfusate was the LK or low potassium solution (11 mM) the interior potential in the right-hand or potential pool is some 40 mV more positive than the outside bathed in 0·5 m-KCl, as reported by Narahashi (1963). The high resistivity of the low-potassium solution, the bulk of which is sucrose, makes the length constant very short and the injury current (through the cut for solution outflow in the right pool) small. Therefore a 40 mV junction correction to the observed difference between the central and right-hand pools was made in estimating the potential of the interior of the node. This correction appeared to be fairly satisfactory because, for the same concentrations of sodium inside and outside, about the same sodium equilibrium potential, E_{Na}, was obtained after this correction. The 7° C thermodynamic equilibrium potential expected from sodium activity measurements was 85 mV.

Because the resting potential observed by the sucrose-gap method is considerably more negative (by 20–40 mV) than that observed by a micro-electrode in the absence of sucrose flow, we usually made the holding potential equal to this 'resting potential'. (Although we do not yet know the way in which the sucrose flow brings a potential change about, it seems that a choice can be made between an assumption of a current through the membrane or a constant liquid junction potential in series with the membrane potential. Observations suggesting a current source are: (1) the amount of hyperpolarization is variable but often increases when the 'node' area is decreased; and (2) the shape of an action potential with sucrose hyperpolarization is similar to one with current hyperpolarization.) We also found that this amount of hyperpolarization was adequate to activate the sodium-carrying system completely. The membrane was given abrupt potential steps to carry the interior of the membrane over the potential range from −140 to +100 mV and the corresponding current flow through the membrane was observed.

This type of voltage control is somewhat faster than the system in which a micro-

electrode and axial wire (Cole & Moore, 1960*a*; Moore & Cole, 1963) are used and, in experiments on intact axons, was found to give a rise time of less than 50 μsec. A typical set of dual records of a potential step and the associated current is shown in Fig. 2. The current pattern (lower record) has the classical shape observed in other voltage-clamp techniques. Speed and accuracy of the present type of voltage clamp depends on the longitudinal interior conductivity in the axon. When the perfusing solution is too low in ionic content, inadequate clamping results. The inclusion of 12 mM-NaCl was therefore necessary for adequate clamping in the LK solution. It was also desirable in order to have a well defined sodium equilibrium potential.

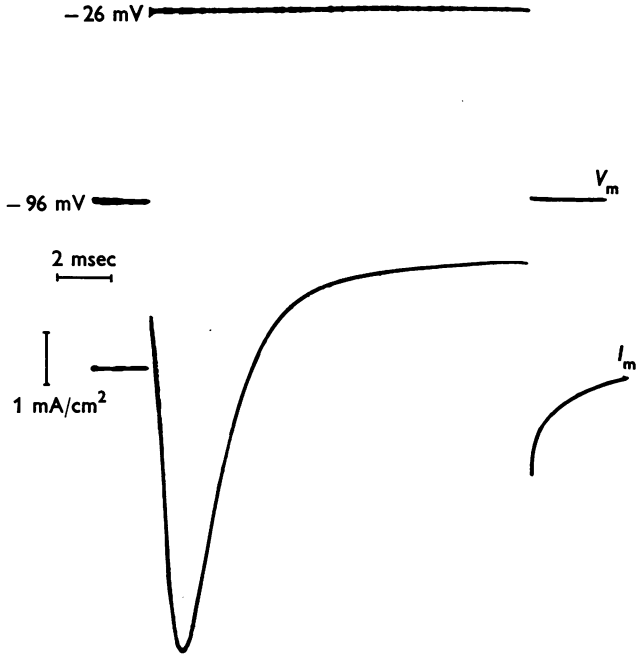

Fig. 2. Time course of membrane potential and current during the clamping pulse. Upper part shows the sudden displacement of membrane potential V_m from −96 to −25 mV. Lower part shows the time course of membrane current I_m during this pulse. Intact axon; 4° C.

When large currents flowed during potential steps, an error of a few millivolts was introduced in the measurement of the potential because of the IR drop in the exterior solution between the outside of the membrane and the earth electrode. Because we were looking for shifts in the activation and inactivation of the sodium currents rather than the absolute magnitude of the currents this would not affect our results appreciably, and we did not use a compensating circuit.

RESULTS

Figure 3 summarizes the currents observed in a typical experiment. The peak sodium current and the late or steady-state current are plotted as a function of voltage for both the HK solution (530 mM) and the LK solution (11 mM). In addition, we have plotted the initial jump in current

upon the application of the voltage step marked I_0 in the insert. The peak inward current or sodium current reverses at a potential of +90 mV (inside positive) which is, within the experimental error, equal to the expected thermodynamic equilibrium potential for 12 mM of sodium inside and 450 mM on the exterior.

Upon perfusion with the low-potassium solution, the most striking change in the curve is the shift of the voltage at which the sodium current turned on (or was 'activated') by some 45 mV in the inside-positive

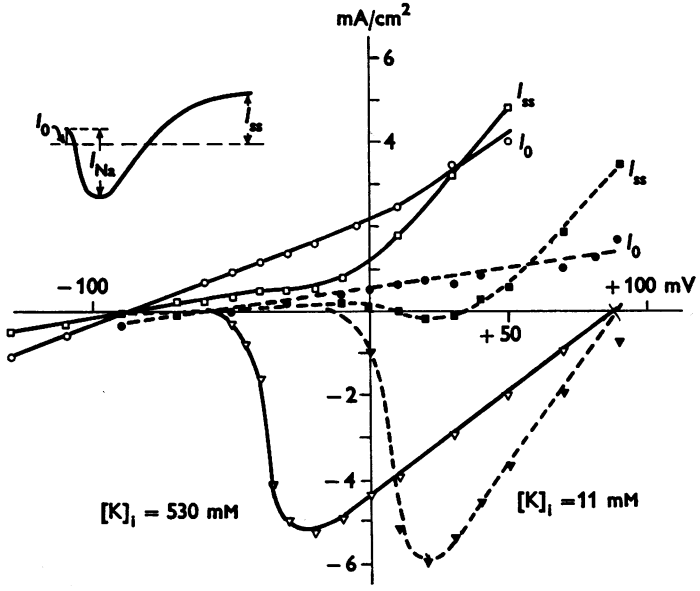

Fig. 3. Plot of ionic current characteristics as a function of membrane potential during a pulse; the parameters plotted are indicated in upper left-hand inset. Currents obtained with the LK internal perfusate are shown in the interrupted lines and the solid lines give the average of the preceding and following currents with the HK perfusate.

direction. The maximum sodium conductance (slope of the sodium current, I_{Na} against potential E curve) appears to be increased from 60 m-mho/cm² for high internal potassium to 85 m-mho/cm² for the low potassium.

In this experiment, in which there was good recovery on return to 530 mM inside potassium, the slope of the I_0 curve (initial jump of current) was reduced from about 30 m-mho/cm² in the HK solution to about 10 in the LK solution. After continued perfusion with the 11 mM-K solution for nearly 0·5 hr, the initial current in this axon no longer had a constant conductance but showed a negative slope in the +30 mV region and actually was inward (negative current on this plot) in the +50 mV region. However, in most of our studies the time of exposure to the low internal

potassium was only about 15 min and in these cases the initial current jump gave a linear current-voltage relation.

Inactivation measurements were made in the manner of Hodgkin & Huxley (1952*a*), the test pulse was chosen to produce a maximum peak sodium current and the preceding conditioning pulse was made about 20 msec in duration, which in control experiments proved to be a long enough period for the attainment of a steady state. The results of such a study of the sodium conductance are shown in Fig. 4. The ratio of the peak inward sodium current to the maximum sodium current is plotted

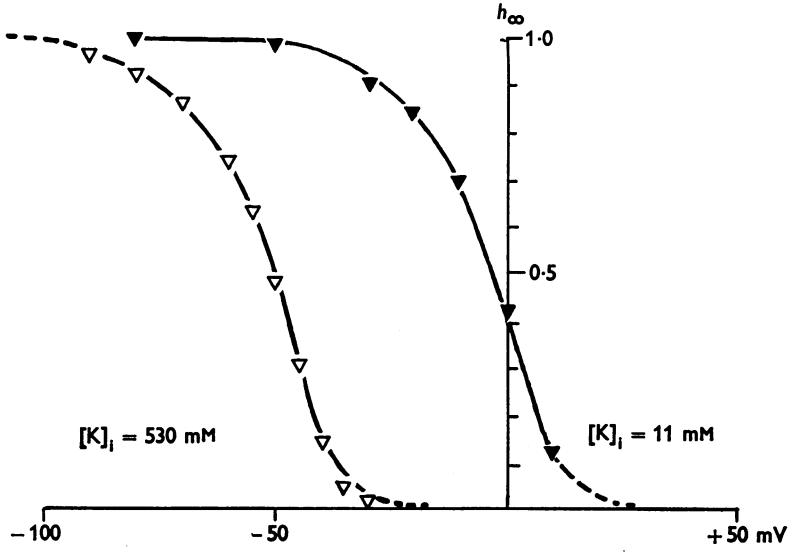

Fig. 4. Sodium inactivation curves (i.e. the peak sodium current during a pulse to fixed potential as a function of the steady potential preceding pulse) for HK and LK internal perfusates.

as a function of the steady potential preceding the step. When the axon was perfused with the standard high-potassium solution, the value of the steady potential for half-maximum currents (E_h) and the slope factor (k in the Frankenhaeuser & Hodgkin (1957) notation) were similar to those observed under similar experiments with intact axons. However, internal perfusion with the 11 mM-K solution caused a shift in E_h of about 45 mV (in the direction of inside more positive). The slope of the h_∞ curve, taken after the axon had been perfused with the LK solution for 25–30 min, was somewhat lower. From Narahashi's (1963) data, there is reason to think that the slope factor k was initially the same in the LK as in the HK solution and gradually increased (causing a decreased slope) as the perfusion continued (see Discussion).

The membrane action potentials in unclamped axons perfused with HK solutions were similar to those in intact axons. The hyperpolarization of the resting membrane produced by the flowing sucrose caused the amplitude of the spike to be large (130 mV or more). There is no undershoot during the recovery phase when the conductance to potassium is high, probably because this resting level is equal to, or more negative than, the potassium equilibrium potential. When the axon was perfused with the LK solution, the height of the action potential remained about the same but a plateau appeared in the recovery phase and its duration was prolonged to tens of milliseconds. After a long application (20–30 min) of LK solution the duration of the plateau became as long as 100 or 200 msec.

DISCUSSION

The absolute membrane potential at which the sodium is activated and inactivated has been rather invariant in the face of addition of anaesthetic agents or changes in the external ionic medium, with the exception of the divalent ion, calcium (and/or magnesium). Frankenhaeuser & Hodgkin (1957) showed that the sodium conductance characteristics shifted along the voltage axis about 9 mV for an e-fold change of external Ca^{2+}.

The potential axis shift of the sodium conductance activation and inactivation by alteration of the internal medium is rather similar to that brought about by a large increase in the external Ca^{2+} concentration. We did not have an extensive series of experiments and were not able to study the time constants of the sodium 'on' and 'off' processes systematically. However, there did not appear to be any dramatic difference between the time course of the sodium component in a current record during a pulse for a HK perfusate and a comparable record with a LK perfusion (the inside of the membrane during the pulse was 40 mV more positive in the LK than in the HK perfusate). We interpret this to mean that all the rate constants (in the Hodgkin–Huxley formulation) which determine the magnitude of the sodium conductance as well as the 'on' and 'off' dynamics were shifted along the voltage axis together with relatively minor, if any, other changes. We did not check the speed of the sodium 'shut-off' (upon repolarization after brief pulses to turn the sodium conductance on) and therefore cannot compare this aspect with the alterations observed by Frankenhaeuser & Hodgkin (1957) upon variation of the external Ca^{2+}.

Narahashi (1963) found that the overshoot of the action potential in the LK solution could be reduced by additional depolarization brought about by the application of current. The shape of this current-depolarization against overshoot curve was similar to that in a HK perfused axon

but shifted along the voltage axis (see Narahashi, 1963, Fig. 6). Although the overshoot does not give a direct measure of the sodium inactivation, it is related and this result clearly implies a shift in the inactivation curve without appreciable slope change. However, as the LK perfusion continued, the amount of current depolarization required to reduce the height of the overshoot of the action potential increased with time. This suggests a decreasing slope in the sodium inactivation curve.

It is not certain which of a number of factors is responsible for the observed shift in the sodium conductance system. It is probably not brought about by the reduction of sulphate, because Baker *et al.* (1962*b*) showed that the axon appeared to be rather indifferent to the anion and could be successfully perfused with chloride, methylsulphate, or isethionate. Baker *et al.* (1963) found that when the internal potassium is replaced by choline, caesium, or sodium, the resting potential is also reduced, but when it becomes less than about -40 mV excitability is lost. However, they also report that activity is maintained in spite of a resting potential of zero when the axon contains sucrose and a low sodium concentration. They tentatively conclude that it is the low ionic strength which is responsible for the shift in the sodium inactivation.

At the peak of the action potential, dV/dt will be zero and the net ionic current will also be zero. In this case one might expect the peak to coincide with the potential at which the sodium current is equal to (and in the opposite direction from) the initial current. This was generally true in our experiments. The reduced initial current with the low internal K perfusates caused the equality with the sodium current to occur at potentials closer to the sodium equilibrium potential ($+85$ mV). This accounts for the increased overshoot at the peak of the action potential which has been generally observed (Baker *et al.* 1962*a*; Tasaki *et al.* 1962; Narahashi, 1963). Baker *et al.* (1962*b*) account for the increased overshoot in the low-K solution by the equation

$$E = \frac{RT}{F} \ln \frac{[\mathrm{K}]_o + b[\mathrm{Na}]_o}{[\mathrm{K}]_i + b[\mathrm{Na}]_i},$$

where [ ] are activities, R, T and F have their usual significance and b is the ratio of the sodium to potassium permeability. At the peak of the action potential, b is in the neighbourhood of 10. This equation implies that sodium and potassium are the only major ions involved in the determination of the peak of the action potential.

The expected potassium equilibrium potential values for E_K (from potassium activities measured with a potassium electrode) are -90 mV and -2 mV for the HK and LK perfusates respectively. It is rather difficult to measure E_K experimentally because it changes rapidly with

the large and maintained potassium currents which flow in a voltage-clamped membrane (Frankenhaeuser & Hodgkin, 1956). However, a reduction in the steady-state current upon reduction of the internal potassium is to be expected on a qualitative basis. Both the steady-state currents in Fig. 3 appear to have an appreciable sodium current component which causes the slope conductance to be negative over a portion of the potential region. In fact, at some potentials, the steady-state current is actually inward for the LK perfusate. For these reasons we cannot say conclusively that the shift in E_K with altered internal potassium follows the Nernst relations.

The identification of the ion carrying the leakage current, or initial jump in current upon a step potential change, has so far not been made. From studies in which the external medium was varied, Adelman & Taylor (1961) suggest that it is carried by the outward movement of internal ions. It appears to be much too large to be chloride, because Caldwell & Keynes (1960) report very low chloride permeabilities by tracer measurement. In the Hodgkin–Huxley (1952*b*) equations the flow of potassium ions is the major contributor to the initial current jump when the membrane potential is pulsed to values between the leakage potential, V_L and the sodium equilibrium potential V_{Na}. In more recent experiments larger values for both the initial jump and potassium currents have been found in intact axons (Cole & Moore, 1960*b*) than those given in these equations. It is possible that the increased initial current is carried by potassium through the voltage-time dependent channel, but this appears unlikely from consideration of the constancy of the initial current jump upon strong hyperpolarizations and the delay of the onset of the potassium current (Cole & Moore, 1960*b*).

The initial current in Fig. 3 is larger still in the HK perfused condition. It is possible that mechanical trauma to the membrane during the process of axoplasm extrusion increased the leakage conductance. There is also some possibility that the initial jump was increased because of a brief transient artifact caused by an over-compensation in adjustment of the dynamic response of the control system. It appears unlikely that this effect is very appreciable, because the initial current jump became non-linear after a long exposure to an LK perfusate.

SUMMARY

1. The internally perfused squid giant axon has been voltage clamped by means of the sucrose-gap method.

2. Reduction of the internal potassium from 530 to 11 mM by substitution of sucrose resulted in:

(*a*) a shift in the activation and inactivation of the sodium conductance in the membrane by 45 mV, and

(*b*) reduction of 'leakage' current.

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