Proc. Natl. Acad. Sci. USA
Vol. 77, No. 12, pp. 7484–7486, December 1980
Neurobiology

Current recorded from a cut-open giant axon under voltage clamp

(axon sheet/ionic currents/gating currents)

ISABEL LLANO AND FRANCISCO BEZANILLA

Department of Physiology, Ahmanson Laboratory of Neurobiology, Brain Research Institute and Jerry Lewis Neuromuscular Research Center, University of California, School of Medicine, Los Angeles, California 90024; and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Communicated by Dr. Susumu Hagiwara, September 2, 1980

ABSTRACT **Squid giant axons were cut open and the resulting membrane sheet was positioned in a chamber separating two compartments. Under voltage clamp, normal sodium, potassium, and gating currents could be recorded for several hours. This preparation allows direct access to the internal side of the axonal membrane and should prove useful for recording of ionic currents, gating currents, and channel-induced current fluctuations in the same membrane sheet.**

In the search for a single-nerve preparation that allows recording of ionic currents, gating currents, and channel-induced fluctuations, we have found that the squid axon can be cut open and a sheet of membrane separating two compartments can be maintained under voltage clamp conditions for several hours. We report here the methods of this preparation and show that the cut-open axon exhibits membrane currents with the same voltage dependence and kinetics recorded from internally perfused axons.

METHODS

Segments of giant axon (400–600 μm in diameter) from the squid *Loligo pealei* obtained at the Marine Biological Laboratory, Woods Hole, MA were used in these experiments. The experimental chamber consisted of two compartments connected by a Plexiglas partition with a circular hole of 250-μm diameter in the center (Fig. 1). A hydrostatic pressure difference could be maintained between the two compartments by means of a manometer system connected to the lower compartment, similar to the procedure described by Kostyuk and Krishtal (1) for internal dialysis of isolated snail neurons. Solutions in each compartment were changed independently. The solutions used were as follows: artificial sea water, 440 mM NaCl/50 mM $MgCl_2$/10 mM $CaCl_2$/10 mM Tris·HCl; internal potassium fluoride solution, 400 mM KF/230 mM sucrose/10 mM Tris·HCl; and sucrose/Tris, 750 mM sucrose/5 mM Tris·HCl. All solutions were buffered to pH 7.2. The temperature in the chamber was regulated by an electronically controlled Peltier unit, bath temperature being held at 10–12°C.

A short segment of a cleaned axon (*ca.* 2 cm) was transferred to the chamber, in which both compartments were filled with artificial sea water. The axon was placed on the Plexiglas partition that previously had been covered with a thin layer of vaseline; the portion of the axon to be used was centered on top of the hole. A small amount of negative pressure (5–15 cm of water) was applied to the lower compartment to hold the axon in place. A cut was made at each end of the axon, leaving a 2- to 2.5-mm-long segment, which was then opened by cutting the membrane longitudinally with iridectomy scissors. After this procedure, the cut-open axon was held to the sides of the hole by the difference in pressure, invaginating slightly downwards. We did not attempt to press the axon sheet between two plates as was successfully done by Hagiwara *et al.* (2) for barnacle muscle fibers.

The solution in the upper compartment (now bathing the internal side of the membrane) was rapidly changed to an isosmotic sucrose/Tris solution and later to internal potassium fluoride solution. Artificial sea water was kept in the lower compartment. In most of the experiments, the solution in each compartment was kept running continuously. Membrane voltage was measured with two electrodes (P_{vi} and P_{ve}) made of glass pipettes with a tip of 30–40 μm internal diameter connected through KCl/agar bridges to Ag/AgCl pellets. A floating platinum wire was inserted in the pipettes to reduce the high-frequency impedance. The pipette P_{ve} that measured the potential (V_e) of the external side of the membrane (now facing down) was filled with artificial sea water and was positioned from the lower compartment (centered with the hole) a short distance below the axonal membrane (*ca.* 10 μm). The pipette P_{vi} that measured the potential (V_i) of the internal side of the membrane (now facing up) was filled with internal potassium fluoride solution and was mounted on a manipulator that allowed us to position it close to the membrane (*ca.* 10 μm). Membrane voltage was measured as $V_m = V_i - V_e$ and was compared to a dc holding potential V_H and command pulses V_c in a conventional voltage clamp circuit, which passed current through the cut-open axon with a platinized platinum plate E_e in the bottom compartment and a Ag/AgCl pellet E_i connected to the upper compartment by a KCl/agar bridge. Membrane currents were measured by one of two methods. (*i*) The total membrane current was monitored using the upper-compartment current electrode (E_i) connected to a current-to-voltage converter, which held the internal (upper) bath at virtual ground. (*ii*) In most experiments, the partial current I_p was measured through a smaller area of the cut-open axon by means of a glass pipette (P_{vi}) with a tip diameter of 10–30 μm connected to a Ag/AgCl pellet with a KCl/agar bridge. This electrode was connected to a current-to-voltage converter, which maintained the pipette at virtual ground. The current-measuring pipette was mounted on a hydraulic micromanipulator and was positioned close to the membrane (*ca.* 10 μm). In this second method, the internal bath was held directly at ground through the upper-compartment current electrode (E_i).

The voltage clamp circuitry was of conventional design, and it had provision for series-resistance compensation (Fig. 1). Settling time was about 5 μsec. The systems for pulse generation and for data collection were similar to those described by Bezanilla and Armstrong (3) and Bezanilla and Taylor (4).

RESULTS AND DISCUSSION

After the axon was opened, a large fraction of the axoplasm diffused away from the flattened sheet, the amount remaining being variable from axon to axon. Once the internal solution

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

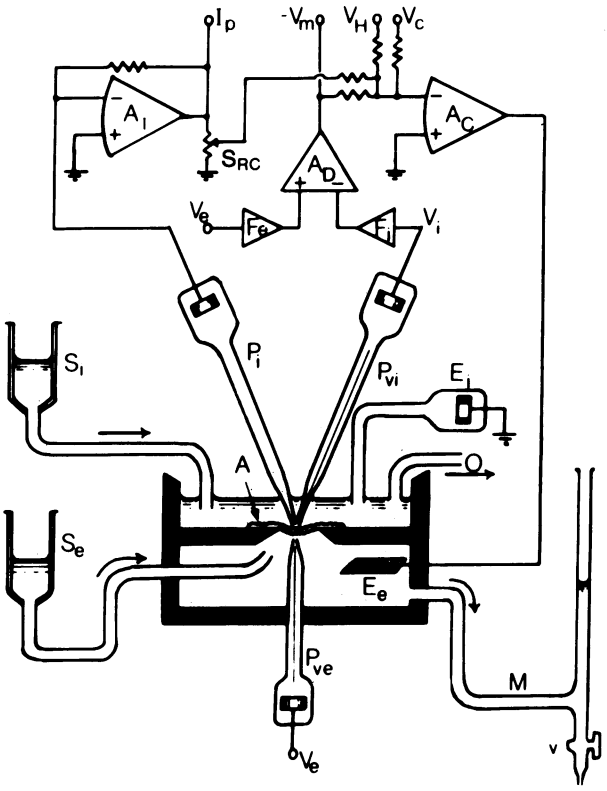

FIG. 1. Schematic diagram of the experimental setup (not drawn to scale). A, cut-open axon; A_C, voltage-clamp control amplifier; A_D, differential amplifier; A_I, current-to-voltage converter; E_e, external current electrode; E_i, internal current electrode; F_e, voltage follower for external voltage electrode; F_i, voltage follower for internal voltage electrode; I_p, current measured with the P_i; M, manometer used to control negative pressure in the bottom compartment; O, suction outlet of the upper compartment; P_i, current-measuring pipette; P_{ve}, external voltage pipette; P_{vi}, internal voltage pipette; S_{RC}, series-resistance compensation adjustment; S_e, external solution reservoir; S_i, internal solution reservoir; V_c, command pulses; V_e, external potential; V_H, holding potential; V_i, internal potential; V_m, membrane potential; V, valve used to adjust negative pressure in the lower compartment. Arrows indicate the circulation of solutions in the chamber compartments.

was changed to internal potassium fluoride solution, a potential difference of about 10–15 mV (inside negative) was recorded between the two compartments. The actual membrane potential of the axon sheet was unknown due to the shunt resistance introduced by the imperfect seal between the axon sheet and the Plexiglas wall. We found that if at this stage the axon was stable and did not move because of the negative pressure, it could be maintained excitable for several hours. When the sheet was voltage clamped, holding the inside at about 70 or 80 mV negative with respect to the outside, sodium, potassium, and leakage currents could be recorded for short, step depolarizations. Initially sodium currents were small, but they became larger after a few seconds as the sodium conductance recovered from slow inactivation.

To evaluate the leakage resistance around the edge of the hole (R_{sh}), we measured the total membrane current in response to hyperpolarizing pulses under voltage clamp. The resistance calculated from these measurements ranged from 300 KΩ to 600 KΩ. The input resistance of a 250-μm-diameter sheet of axonal membrane should be on the order of 10 MΩ; therefore, R_{sh} is practically equal to the measured resistance. The leakage currents are thus large enough to be comparable to the ionic currents; for this reason, leakage subtraction was used to study the kinetics and voltage dependence of the Na and K currents.

In early experiments the voltage electrodes were positioned far from the hole separating the chamber compartments, and most of the time it was impossible to achieve voltage control, as evidenced by a very steep voltage dependence of the sodium current. Under these conditions, recordings of the membrane current with a pipette positioned close to the center of the axon sheet showed less leakage, but the voltage dependence of the sodium current still was very steep.

However, when the voltage electrodes were placed very close to the center of the axon sheet (as described in the method section), the voltage dependence of the total membrane current became very similar to the currents recorded from internally perfused axons, although the kinetics were slightly distorted. Fig. 2*a* presents records of total membrane current for increasing depolarizing steps from a holding potential of −80 mV [leakage and capacity being subtracted with P/4 procedure (see Fig. 2 legend)]. Sodium and potassium currents show the typical voltage dependence, but sodium currents decay too fast for depolarizations to −30 mV and −20 mV. These results indicate that the failure in control observed with electrodes placed far away from the center of the sheet was mainly due to an inadequate measurement of membrane potential that is not homogenous due to the shunt at the periphery. In several experiments measuring total membrane current, the maximum inward current (I_{Na}) for the 250-μm-diameter axon sheet ranged from 0.6 to 2.0 mA/cm^2; that of the maximum outward current (I_K) for a 10-msec depolarization to 50 mV varied between 0.5 and 1.6 mA/cm^2. These amplitudes are slightly smaller than the peak currents recorded in perfused squid axons; the difference could be introduced by the shunt resistance of the periphery.

The final arrangement pictured in Fig. 1 was designed to measure the voltage and the current from the center of the cut-open axon, minimizing the contributions of the periphery. Membrane currents recorded under these conditions are shown in Fig. 2*b*. The current pipette was 30 μm in internal diameter, and it was positioned at the center of the hole and very close to the membrane. The P/4 procedure (see Fig. 2 legend) was used to subtract the capacitive transients and the leakage current, which resulted from the fact that no attempt was made in these experiments to achieve a seal between the membrane and the pipette (i.e., the pipette was not pushed against the membrane, but rather just positioned a short distance from it.) The holding potential was −90 mV, and depolarizing steps were applied to give the indicated membrane potentials. The kinetics of sodium and potassium currents looked normal, indicating that the region from which current was recorded was under accurate membrane potential control. A plot of peak inward (sodium) and maximum outward (potassium) currents as a function of membrane potential (Fig. 2*c*) shows normal voltage dependence for the ionic conductances.

When the internal potassium concentration was decreased by partial replacement of potassium ions by tetramethylammonium ions and the external sodium concentration was decreased by replacement of four-fifths of the sodium ions by Tris ions, it was possible to record sodium gating currents and sodium and potassium ionic currents simultaneously using the P/4 technique (Fig. 2*d*). This recording shows approximately the same signal-to-noise ratio attained in perfused axons.

The cut-open squid axon preparation is thus appropriate to record ionic and gating currents under voltage clamp conditions with free access to both sides of the membrane. When compared to the internally perfused squid axon, the main drawback of the opened axon is the shunt resistance that prevents mea-

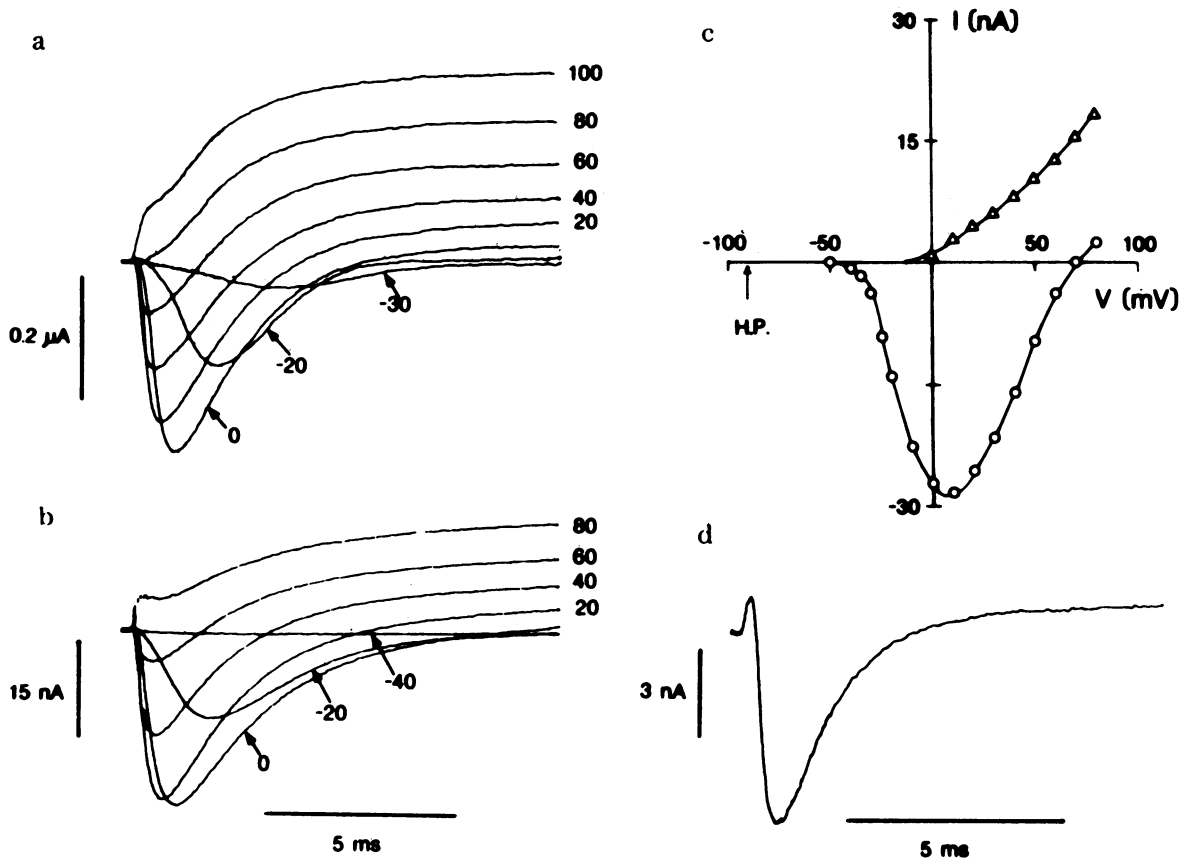

FIG. 2. (*a*) Recording of total membrane current. Holding potential, −80 mV; depolarizing steps bring membrane potential to values indicated on each trace. Each current trace is the result of the current produced by a depolarizing pulse, P, minus the current produced by four pulses of amplitude P/4 starting from a membrane potential of −110 mV. There was no series-resistance compensation; temperature was 12°C. (*b*) Recording of partial membrane currents with a 30-μm pipette. Holding potential, −90 mV; depolarizing steps to membrane potential were as indicated on each trace. The P/4 procedure was used, starting from a membrane potential of −120 mV. Time resolution at short times was decreased because the compensation for series resistance made the records oscillatory. Average of two sweeps; temperature was 10°C. (*c*) Current-voltage relation for experiment shown in Fig. 2*b*. Abscissa, membrane potential (V_m) in mV; ordinate, membrane current (I_P) in nA. ○, peak inward current; △, maximum outward current at the end of a 10-msec pulse. (*d*) Recording of membrane current with a 30-μm pipette under partial replacement with impermeant ions. Holding potential, −70 mV; depolarizing pulse to +20 mV. The P/4 procedure was used, starting from a membrane potential of −160 mV. Average of five sweeps; no series-resistance compensation; temperature was 12°C.

surements of the resting membrane potential and isotope fluxes. However, this preparation uses shorter axon segments, and it is simple to set up. Its main advantage is the fact that the internal side of the membrane is facing upwards, offering the possibility of noise and single-channel recording. In a recent paper, Conti and Neher (5) have been successful in recording single potassium-channel fluctuations by applying a small bent pipette to the inside of the axon. As these authors discussed, the serious disadvantage of the perfused axon preparation is the stray capacitance associated with the current-measuring pipette, which limits the recording bandwidth to 500 Hz. This limitation is not present in the cut-open axon because the level of the solution in the upper compartment can be maintained low, decreasing the stray capacitance of the current-measuring pipette and increasing the available bandwidth. This consideration and the possibility to study gating and ionic currents in the same preparation should prove the cut-open axon as a useful preparation for a better understanding of the mechanisms underlying gating phenomena in nerve membranes.

Note Added in Proof. Recently we have successfully implemented this technique with axons from *Loligo opalescens* (320–375 μm in diameter). Facilities and squid for these recent experiments were kindly provided by Alvin Siger at the University of Southern California Marine Science Center (Santa Catalina Island, CA).

We are indebted to Dr. Robert E. Taylor for providing space and help and to Mr. Julio Fernandez for invaluable discussions during the course of this research. We thank Mr. Donald Curtis for building the experimental chamber. This work was supported by a grant from E. I. du Pont de Nemours & Co., the Muscular Dystrophy Association, and U.S. Public Health Service Grant AM 25201-01.

1. Kostyuk, P. G. & Krishtal, O. A. (1977) *J. Physiol. (London)* **270**, 545–568.
2. Hagiwara, S., Naka, K. & Chichibu, S. (1964) *Science* **143**, 1446–1448.
3. Bezanilla, F. & Armstrong, C. M. (1977) *J. Gen. Physiol.* **70**, 549–566.
4. Bezanilla, F. & Taylor, R. E. (1978) *Biophys. J.* **23**, 479–484.
5. Conti, F. & Neher, E. (1980) *Nature (London)* **285**, 140–143.