Simultaneous measurement of changes in current and tracer flux in voltage-clamped squid giant axon

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ABSTRACT A method is described for the simultaneous measurement of changes in membrane current and unidirectional radiotracer flux in internally dialyzed voltage-clamped squid giant axons. The small currents that are produced by electrogenic transport processes or steady-state ionic currents can be resolved using this method. Because the use of grounded guard electrodes in the end pools is not, by itself, an adequate means of eliminating end-effects, two ancillary end pool clamp circuits are described to eliminate extraneous current flow from the ends of the axon. The end pool voltage-clamp circuits serve to minimize net current flow between the end pools and center pool, and employ stable, low-impedance calomel electrodes to monitor the potentials of the end and center pools. The adequacy of the method is demonstrated by experiments in which unidirectional ^{22}Na efflux and current, flowing through tetrodotoxin (TTX)-sensitive Na channels into Na-free seawater, under K-free conditions, are shown to be equal. The equality of unidirectional TTX-sensitive flux and current is maintained over the entire range of membrane potentials examined (−60 to +20 mV). The method has been applied to a series of experiments in which the voltage dependence and stoichiometry of the Na/K pump have been measured (Rakowski et al., 1989), and can be applied in general to the simultaneous measurement of changes in current and flux of other electrogenic transport processes, and of currents through ionic channels that open under steady-state conditions.

INTRODUCTION

During the past few years several laboratories have become interested in making direct measurements of the small currents produced by electrogenic membrane transport processes. Extensive work has been done using the giant axon of the squid because it is possible to control both the external and internal ionic conditions and to measure radioisotopically labeled unidirectional fluxes by the technique of internal dialysis (Brinley and Mullins, 1967). The dialysis technique can be combined with the classical internal wire voltage-clamp technique (Hodgkin et al., 1952) to provide a very powerful method for analyzing the voltage dependence of membrane transport processes. Internal dialysis has proven to be more generally useful for studies on active transport processes, whereas internal perfusion (Baker et al., 1962) has been widely used in studies on passive ionic currents.

The development of techniques for the simultaneous measurement of pump-mediated current and flux was stimulated by the pioneering work of Thomas (1972) who used intracellular sodium selective microelectrodes to measure net fluxes and a voltage-clamp technique to monitor transient membrane current after injection of Na into snail neurons. De Weer (1974) measured the change in voltage-clamp holding current and flux produced by addition of a pump-specific toxin in a study of steady-state Na/K pump activity, and Cooke et al. (1974) used this pump clamp technique to measure strophanthidin-induced changes in Na efflux and current, and thus compute pump stoichiometry, in *Aplysia* neurons. The change in K current owing to reaccumulation of extracellular K on stopping the Na/K pump with ouabain or other drugs is a significant potential source of error in the estimation of Na/K pump current (Isenberg and Trautwein, 1974). Gadsby (1984) has discussed the necessity of minimizing this source of error in the measurement of Na/K pump current by employing K channel blocking agents. Work in perfused barnacle muscle fibers (Lederer and Nelson, 1984) illustrates the difficulty of obtaining accurate simultaneous measurements of current and flux over a wide voltage range without the use of K channel blocking agents or an end pool clamp system. We have used the combined pump clamp technique for measurement of both current and flux to investigate the voltage dependence of the Na/K pump in squid giant axons (Rakowski et al., 1989). A similar methodology for current measurement has been used by DiPolo et al. (1987) to investigate the voltage dependence of currents resulting from Na/Ca exchange in squid axons.

One of the major technical problems that arise in comparing simultaneous measurements of changes in current and flux in squid axons is the requirement that current and flux be measured from precisely the same

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membrane area. This is not a problem using whole-cell voltage-clamp techniques, because of the absence of cut ends. However, using whole cells, the flux measurements from small cells are inaccurate, and in the case of large (1.0–1.2 mm diam) amphibian eggs (Eisner et al., 1987) the intracellular solution composition is not under experimental control. The squid giant axon is an ideal preparation since both flux measurements and electrical current measurements are of comparable accuracy and have resolution that is adequate to detect small changes in current or flux (<0.1 μA cm^{-2} or 1 pmol cm^{-2} s^{-1}), and the intracellular fluid composition can be controlled by internal dialysis. We have developed a technique that is capable of eliminating extraneous current flow from the cannulated ends of the axon and have demonstrated that the TTX-sensitive efflux into the central experimental pool and the current change produced by TTX are equal under conditions of unidirectional current and flux.

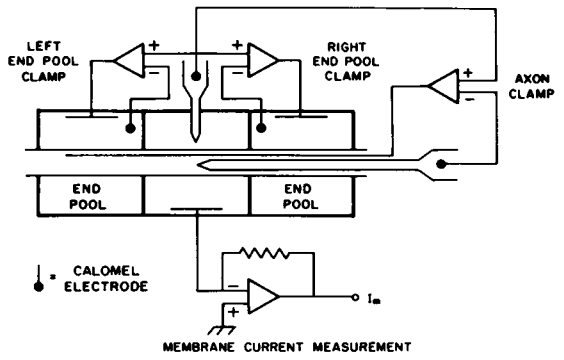

FIGURE 1 Schematic diagram of the experimental chamber for simultaneous measurement of current and flux in squid giant axons. A giant axon of the squid, *Loligo pealii*, is mounted on two glass cannulae and a dialysis capillary (not shown), internal platinum wire, and glass electrode are inserted. The membrane potential across the center experimental pool is measured as the difference between the internal glass electrode and a similar 3 M KCl-filled micropipette placed in the center pool. Calomel electrodes are used for stability at all solution/metal interfaces that are involved in a voltage measurement. The membrane potential difference is used as the input to the main axon clamp system shown in Fig. 2 *A*. The current from the center pool is measured by an operational amplifier in virtual ground configuration. This membrane current measurement system is shown in greater detail in Fig. 2 *B*. In addition to the main clamp amplifier, two ancillary end pool clamp amplifiers are shown. These are described in greater detail in Fig. 2 *C*. The end pool clamps maintain the extracellular potentials in the end and center pools equal and so prevent net current flow between them.

METHODS

Experimental chamber

Freshly dissected giant axons from the hind-most stellar nerve of the squid, *Loligo pealii*, were cleaned of smaller axons, cut and tied to glass cannulae that protruded from the end walls of the experimental chamber, and permitted the insertion of an internal blackened platinum wire (75 μm) and glass capillary electrode (resistance, 0.4–1.2 MΩ) as shown in Fig. 1. A cellulose acetate dialysis capillary (350–375 μm diam), made porous in its central region by 20-h treatment in 1 N NaOH, was inserted from the opposite end of the axon. The dialysis capillary is not shown in Fig. 1. After inserting the internal hardware, the floor of the chamber was raised so that the central experimental part of the axon could be mechanically isolated from the cannulated and presumably damaged ends using stopcock grease (Lubriseal, Thomas Scientific, Philadelphia, PA). This arrangement separated the axon into three regions, right and left end pools, and a central experimental pool. The center pool was superfused with artificial seawater at a rate of 2 ml/min. The width of the center pool was 12 mm and its volume ~0.5 ml. Additional experimental details are given in Rakowski et al. (1989).

Solutions

Experiments were conducted in a Na-free, K-free artificial seawater having the following composition (in millimolar): 395 *N*-methylglucamine (NMG), 75 Ca^{2+}, 545 Cl^-, 5 tris(hydroxymethyl)aminomethane (Tris), 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 5 ethylenediaminetetraacetic acid (EDTA), 1 3,4-diaminopyridine (DAP). The osmolality of the solution was 930 mOsm/Kg and the pH 7.7. The K-free internal solution composition was as follows (in millimolar): 50 Na^+, 310 NMG, 100 Cl^-, 250 Hepes, 15 Mg^{2+}, 10 ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 5 Tris, 5 adenosinetriphosphate (ATP), 5 phosphoarginine, 5 phosphoenolpyruvate, 5 dithiothreitol, 0.25 veratridine (pH = 7.4). Veratridine was present in the internal dialysate to increase the steady-state TTX-sensitive outward current into the Na-free seawater. Carrier-free ^{22}Na was added to the internal dialysate to achieve a level of radioactivity of 200,000 cpm/μl (specific activity 1.8 Ci/mol). The superfusing seawater was collected directly into scintillation vials using a fraction collector and ^{22}Na efflux measured by liquid scintillation counting.

Voltage-clamp electronics

Simplified schematic diagrams of the voltage-clamp electronics are shown in Fig. 2. Fig. 2 *A* shows the electrical circuit for the main axon clamp. It is of conventional design except that very stable low-noise amplifiers have been selected and the frequency response limited by a large value of feedback capacitance (0.012 μF) and the limited frequency response of the amplifiers. The various analog output signals were heavily filtered (10 Hz). A greater frequency response is not warranted in experiments of this type since the temporal resolution is limited by the radiotracer sample collection time. The reference (V_{ref}) and internal (V_{in}) voltages are led to operational amplifiers arranged in unity gain, noninverting configuration. The outputs from these amplifiers are compared and amplified by a second-stage amplifier. The combination of these three input amplifiers functions as an instrumentation amplifier with a gain of 10 having a 10 KΩ trimming potentiometer for adjustment of common mode rejection. The output from the first three amplifiers is available as a filtered output (10 V_m) and is also used as input to the summing junction of the voltage-clamp feedback amplifier (adjacent to C_f and R_f in Fig. 2 *A*). The summing junction of the control amplifier also receives input from an external voltage command signal (V_c) or from a stable offset voltage (holding potential) circuit provided by two 9-volt alkaline batteries and a unity gain operational amplifier. The values of the feedback capacitor (C_f), feedback resistor (R_f), and load resistor (R_L) are selectable by shorting rotary switches. The ranges are given in the legend to Fig. 2. Under

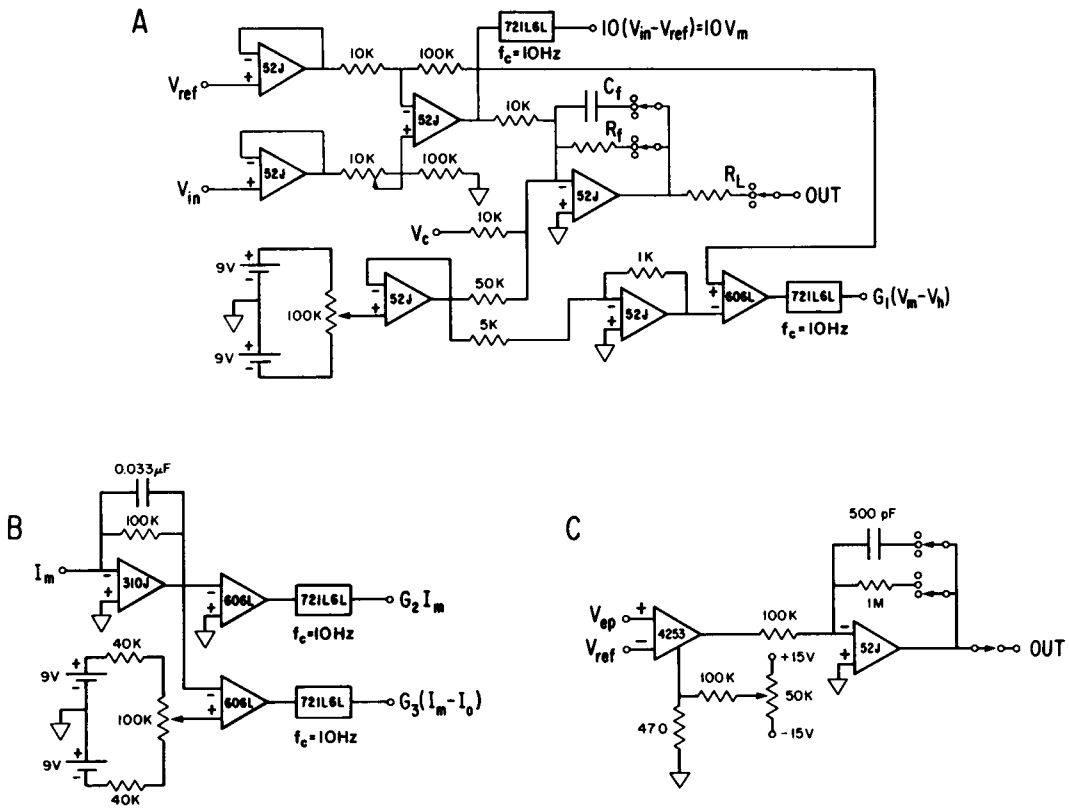

FIGURE 2 Simplified electrical schematic diagram of the voltage-clamp and current measurement circuits. (*A*) Main axon voltage clamp circuit. The principle of operation is described in the text. Operational amplifiers (52J) and the instrumentation amplifier (606L) were purchased from Analog Devices Inc., Norwood, MA. Eight pole Bessel filters with a 3 db cut-off frequency of 10 Hz (721L6L) were purchased from Frequency Devices, Haverhill, MA. The range of switch selectable feedback capacitors (C_f) was from 100 pF to 0.012 μF and the range of feedback resistors (R_f) was from 100 KΩ to open loop (infinite). The load resistance (R_L) ranged from 1 MΩ to 10 KΩ and was normally kept at 20 KΩ. (*B*) Current measurement circuit. A varactor bridge amplifier (310J; Analog Devices Inc.) was used in current-to-voltage configuration, and its analog current output was made available at low gain or at high gain with the provision of a D.C. offset voltage. (*C*) End pool clamp circuit. Two are required. One for each end pool. The voltage in the end pool (V_{ep}) is compared with the center pool voltage (V_{ref}) using an instrumentation amplifier (Teledyne-4253; Hastings-Raydist, Hampton, VA) having an offset capability. The control amplifier for the end pool clamp circuit was a 52J operational amplifier. The circuit was normally operated at full open loop gain (10^6) with no load resistance.

normal conditions the feedback resistor rotary switch was in the open position so that the clamp amplifier operated at its full open loop gain (10^6 at 10 Hz). This is necessary in order to reduce the magnitude of the residual voltage error to an acceptably low value (Rakowski and Paxson, 1988). The feedback capacitance was normally set at its highest value (0.012 μF). The load resistance (R_L) required to achieve stability under full open-loop gain (but limited frequency response) conditions was 20 KΩ. In addition to the low gain (10 V_m) voltage output, provision is made to observe the difference between the measured membrane potential (V_m) and the command holding potential (V_h) at high gain (G_1 = 750). Because the magnitude of steady-state holding current and the changes in holding current produced by TTX are small (1–10 μA cm^{-2}), no provision for series resistance compensation was required.

Current measurement circuit

The circuit used to measure the current collected from the center pool (I_m) is shown in Fig. 2 *B*. It is simply an operational amplifier connected in current-to-voltage configuration in which the platinum plate in the center pool is maintained at virtual ground. The amplifier chosen for this conventional task (model 310J; Analog Devices, Inc. Norwood, MA), however, is somewhat unconventional in design in that it uses a varactor bridge circuit to obtain stability and low noise. Other low-noise, stable amplifiers may be equally suitable. The current amplifier output is available either at a low gain (G_2) of 0.1 mV/nA or at a variable high gain (G_3) of up to 8 mV/nA. Both outputs were low pass filtered at 10 Hz.

The necessity of end pool clamps

Hodgkin, Huxley, and Katz (1952) used grounded guard electrodes in the end regions of their internal wire voltage-clamp system. While this method is satisfactory for measurement of the relatively large and brief voltage-dependent ionic currents through Na and K channels (~5 mA cm^{-2}), it is inadequate for measurement of steady-state currents such as Na/K pump current (~1 μA cm^{-2}) because measurement of electrogenic pump current requires passing steady-state current that produces

electrode polarization and small differences in the extracellular potentials in the center and end pools. A simplified schematic diagram that illustrates the problem and its resolution is shown in Fig. 3.

In order to measure radioisotopic efflux from the axon, the center pool must be physically isolated from the adjacent end pools. A convenient means of achieving this physical isolation is by surrounding a short length of the axon (2–4 mm) with stopcock grease. The grease seals produce a significant electrical resistance (R_{LS} and R_{RS}) between the center and end pools, so that the extracellular compartments (V_L, V_R, and V_O) are not necessarily isopotential. The main voltage clamp circuit (Fig. 2 *A*) passes current (I) through the internal longitudinal wire so as to maintain the measured membrane potential ($V_i - V_0$) at the command value. Upon introducing a toxin that changes one or more of the equivalent circuit elements of the axon membrane, a new value of clamp current is required to maintain $V_i - V_o$ at the desired holding potential. The internal longitudinal wire is isopotential (V_W) and drives current through three pathways: (*a*) the axon membrane in the center pool (I_M), (*b*) the cannulated end of the axon in the left (I_L) and (*c*) right (I_R) end pools. The resistor R_{iW} represents the impedance of the platinum wire/solution interface and the radial resistance of the axoplasm. The resistors R_L and R_R represent the sum of the various impedances between the internal wire and the external solution in the left and right end pools at potentials V_L and V_R, respectively.

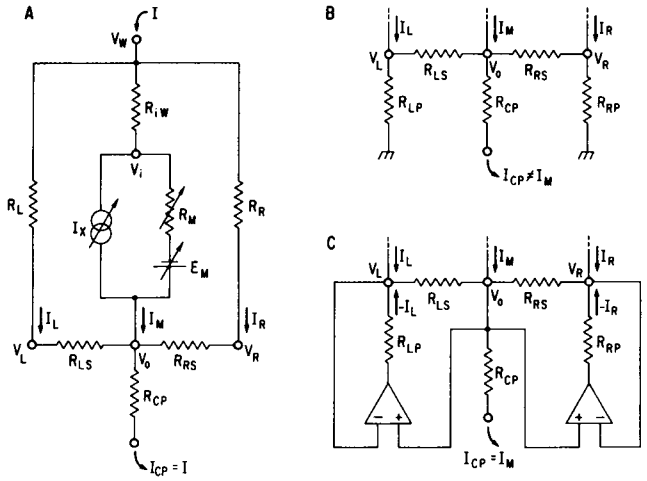

FIGURE 3 Electrical equivalent circuit of current pathways in the experimental chamber. (*A*) End pools isolated by grease seals and not grounded. (*B*) Grounded guard electrodes placed in the end pools. (*C*) Cancellation of end pool current by addition of end pool clamps. I = total clamp current; V_W = voltage applied to the internal platinum wire; R_{iW} = resistance between the internal wire and internal voltage measurement point; V_i = voltage measured by the internal 3M KCl electrode; I_X = current produced by the electrogenic transport process of interest; R_M = membrane resistance, E_M = membrane potential in the absence of I_X and clamp current; R_L and R_R = resistances of the current pathway from the internal wire to the left and right end pools, respectively; V_L and V_R = voltages measured in the left and right end pools, respectively; V_O = voltage measured in the center pool; R_{LS} and R_{RS} = resistances of the left and right grease seals, respectively; I_L and I_R = currents flowing in the left and right end pool pathways, respectively; I_M = membrane current; R_{CP} = resistance of the center pool platinum plate; I_{CP} = current measured from the center pool; R_{LP} and R_{RP} = resistances of the left and right end pool platinum plates, respectively. Note that only in *C* is the current measured from the center pool equal to the membrane current.

One possible method of measuring membrane current in which the end pools are electrically isolated is apparent from Fig. 3 *A*. If the seal resistances R_{LS} and R_{RS} can be made sufficiently large compared with the combined resistance of the current pathway through the axon membrane in the center pool, I_L and I_R will be small compared with I_M, and the current measured through the platinum plate in the center pool (I_{CP}) will be approximately equal to I_M. Such complete electrical isolation is difficult to achieve, however. Results from experiments in which isolation was attempted by the use of silicon oil, mineral oil, and air gaps are discussed in the experimental section. In general, leaving the left and right end pools floating results in the current measured from the center pool simply being equal to the total applied clamp current (I) rather than the membrane current (I_M).

A second possible solution is the classical one shown in Fig. 3 *B*(Hodgkin et al., 1952) in which the top portion of the complete equivalent circuit has been omitted for clarity, and only the changes that illustrate grounding the end pools through guard electrodes (blackened platinum plates placed in the end pools) are shown. If physical isolation of the three pools is not required as is the case where only current measurements are being made, the seal resistances R_{LS} and R_{RS} are essentially zero; the three pools are isopotential ($V_L = V_O = V_R$); and measurements of membrane current density can be made by maintaining strictly uniform radial geometry, measuring the area of axon membrane beneath the central plate, and by assuming that the impedance of the left and right guard electrodes (R_{LP}, R_{RP}) and the center platinum plate (R_{CP}) are equal to each other. These assumptions, however, are not warranted, in general. In the case where the seal resistances are finite and the end and center pools are not isopotential, the guard electrodes serve as a current divider network in which the current measured from the center platinum plate is not necessarily equal to the membrane current ($I_{CP} \neq I_M$). Indeed, when clean, freshly platinized guard electrodes were placed in the end pools of an existing chamber and grounded, most of the membrane current simply flowed to ground despite the resistance of the grease seals.

The solution of the problem is shown in Fig. 3 *C*. Two ancillary voltage clamp circuits have been added. These serve to prevent longitudinal current flow through the grease seals by keeping the extracellular potential across them close to zero. The extracellular potentials in the end pools (V_L and V_R) are measured using stable calomel electrodes connected to the pools by agar bridges, and are compared with the extracellular center pool potential (V_O) which is measured by a flowing 3M KCl pipette containing another calomel electrode. The measured voltage differences, $V_O - V_L$ and $V_O - V_R$ (always $<10\ \mu V$ with the end pool clamps switched on), drive currents through the guard electrodes equal in size and opposite in sign to the currents flowing into each end pool from the internal wire. The performance of this active guard electrode system is independent of changes in guard electrode impedance (R_{LP} and R_{RP}) that result from polarization of the platinum plates by passing steady current. Data are given below that demonstrate the validity of the end pool clamp system. Since the end pool clamps operate to match the current flow through the cut and cannulated axon ends, the current measured through the center platinum plate is equal to the membrane current emerging in the region of the axon between the grease seals ($I_{CP} = I_M$).

End pool clamp circuit

A simple circuit to provide this end pool clamp capability is shown in Fig. 2 *C*. A stable, low-noise instrumentation amplifier is used to compare the voltage in the end pool (V_{ep}) with the reference voltage measured in the center pool (V_{ref}). Before the experiment, the reference

electrode is placed in the end pool and the offset trim circuit is adjusted to null any electrode or diffusion potential difference. After zeroing the output when both electrodes are in the same pool, the reference electrode is moved to the opposite end pool where the second end pool clamp circuit is similarly zeroed. (Absolutely precise zeroing of the end pool clamp circuit is not critical for the performance of the system. Experiments were conducted in which deliberate D.C. potential differences of ±30 mV were maintained between end and center pools. This maneuver produced additional steady current that was unaffected by the addition of TTX to the center pool). After zeroing both end pool clamp circuits, the reference electrode is returned to the center pool and the resting difference between the center and end pools is recorded. Typically the end pools are found to be more positive than the center pool owing to depolarization of the cut and cannulated axon ends with respect to the undamaged central section of the axon.

It is also convenient to record the end-pool-to-end-pool electrical resistance through the grease seals. This resistance measurement provides a rough check on the mechanical integrity of the seals. It was not convenient to measure the resistance across each seal individually, but was satisfactory to measure the sum of the two seal resistances in series by measuring the resistance between the calomel electrodes in each end pool. The apparent seal resistance measured in this way also includes the parallel resistance pathway for current flow into, along, and then out of the axon between the two end pools. We routinely sought to provide 50–100 KΩ seal resistances and usually advanced mechanical pistons to tighten the seals and increase the resistance. This was done before turing on the end pool clamps. Tightening the seals led to increases in the center-to-end-pool potential difference as expected for an approximately constant injury current crossing an increased seal resistance.

The potential difference between the center pool and each end pool was monitored and led to the summing junction of an operational amplifier (52J) for each end pool clamp. Provision was made to adjust the feedback resistance and capacitance using shorting rotary switches. During routine operation the feedback capacitance was left at 500 pF and the feedback resistance setting was kept in the open position to achieve the full open loop gain of the control amplifier. Variable load resistance was also available (not shown); however, the circuit was found to be extremely stable and no load resistance was required. The frequency response characteristics of the end pool clamp circuit in Fig. 2 *C* make it considerably faster than the main clamp circuit in Fig. 2 *A* because of the smaller value of the feedback capacitance (500 pF). This provides stability in that the two ancillary end pool clamp circuits can readily follow, and properly compensate for, the changes that are produced, for example, during voltage-clamp pulses commanded in the center pool region.

An additional feature of the end pool clamp circuit is that we were able to measure the true zero current (resting) potential of the axon membrane in the center pool region free from the influence of injury currents originating from the depolarized, cannulated axon ends. In the absence of seals, for a 500 μm diam axon, with a specific membrane conductance of 50 μs cm^{-2}, typical for our experiments, and axoplasmic resistivity of 100 Ωcm, the calculated electrical length constant is >1 cm. This is sufficiently long for the axon in the center pool to be affected by damaged regions of the axon in the end pools and even by the cut and cannulated ends of the axon. The effective length constant and, hence, influence of end pool injury currents on the resting potential in the center pool is decreased by the presence of grease seals which increases the resistance of the extracellular current pathway. On activating both end pool clamps, the membrane potential measured between the intracellular KCl electrode and the reference electrode in the center pool typically became a few millivolts more negative, owing to the elimination of longitudinal depolarizing current from the injured axon ends. The main clamp circuit was turned on after the end pool clamps, and the holding potential of the main clamp was adjusted until the current output of the main clamp was zero, at which point the holding potential equalled the true resting membrane potential.

RESULTS

Equality of the membrane area from which current and flux are measured

Because we wished to demonstrate that the arrangement described above was capable of eliminating artifactual currents originating from the end pools, we established conditions in which experimentally induced changes of unidirectional flux and current carried by an ionic species should be identical. This condition is met for current flow through a highly selective ion channel that excludes all but one ionic species and which can be blocked specifically without affecting any other component of membrane current. Outward Na current into Na-free seawater through TTX-sensitive channels, in the total absence of K (which has been shown to be significantly permeant through voltage-dependent Na channels, Chandler and Meves, 1965), fulfills these requirements. Because we were interested in providing this demonstration for measurements of ^{22}Na efflux, it was convenient to conduct the experiments in Na-free and K-free seawater and to use an internal dialysate that contained 50 mM Na and was K-free so that there would be an outward gradient for Na. Because Na efflux through TTX-sensitive Na channels is normally small at a holding potential of −30 mV, it was also convenient to increase the number of Na channels that are open at the holding potential by means of 250 μM veratridine which inhibits inactivation of Na channels (Sutro, 1986) and produces a hyperpolarizing shift of Na channel gating (Liebowitz et al., 1986).

In the experiment shown in Fig. 4, the TTX-sensitive ^{22}Na efflux should equal the TTX-sensitive current in Na-free seawater ($[Na]_i$ = 50 mM). The membrane potential was held at −30 mV and was maintained to within ±20 μV during the current change produced by addition of 200 nM TTX. The inward current shift (Fig. 4 *A*) reflects TTX block of outwardly directed Na current. The magnitude of the holding current change was measured by positioning a pair of cursors on a computer display to select the segments to be used for a straight-line least squares fit to the data obtained before and during application of TTX. The magnitude of the response to TTX was then measured as the difference between the extrapolated baselines at the time when the response was one half of its final value. The fitting procedure is shown in Fig. 4 *A* and yielded a value of 1.35 μA cm^{-2} for the magnitude of the current change (ΔI) produced by

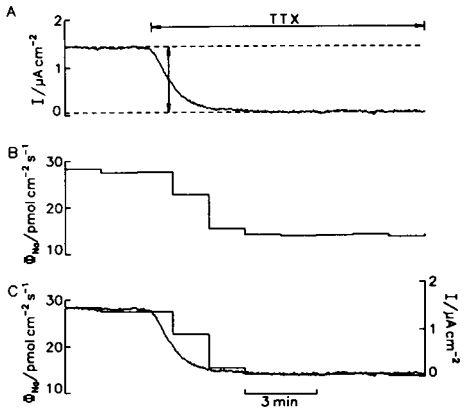

FIGURE 4 Demonstration of the adequacy of the end pool clamp system. (*A*) Membrane current change upon addition of 200 nM TTX to the bathing solution and method for the measurement of the amplitude of the TTX-sensitive current change by a least-squares linear fitting procedure described in the text. (*B*) Change in ^{22}Na efflux upon adding TTX. (*C*) The change in flux and current produced by addition of TTX are replotted to show overlap. Because current is measured into Na-free seawater, the equality of current and unidirectional efflux demonstrates that they derive from the same membrane area. Axon diameter 480 μm. Temperature 17°C. Since Faradays constant (F) is 96,487 Coul mol^{-1}, 10 pmol cm^{-2} s^{-1} = 0.965 μA cm^{-2}.

addition of TTX in this axon. The ordinate scalings in Fig. 4 have been chosen so that a given displacement on the current or flux axis represents the same number of ions per second crossing the membrane. The TTX-induced change in ^{22}Na efflux ($\Delta\Phi_{Na}$), estimated as the difference of mean flux values calculated from five samples before, and five samples after, the addition of TTX (allowing three sample intervals for the change to occur), was 14.0 pmol cm^{-2} s^{-1} which corresponds to a current magnitude ($F\Delta\Phi_{Na}$) of 1.35 μA cm^{-2} in agreement with the TTX-sensitive current measurement as confirmed by the overlapping traces in Fig. 4 *C*. Results of this kind were routinely observed, the average value of the flux to current ratio ($F\Delta\Phi_{Na}/\Delta I$) being 1.04 ± 0.05 ($n$ = 10, mean ± SEM) at a holding potential of −30 mV. Additional data at various holding potentials are discussed further in this paper.

Area match obtained at various holding potentials

It is possible, though unlikely, that −30 mV might be an optimal potential for obtaining a precise match of current and flux. For simultaneous measurements of changes in current and flux made at various membrane potentials to be useful, it is necessary to demonstrate that the accuracy of the area match is independent of the holding potential. The results from 58 measurements of TTX-sensitive current and flux made on 48 axons at six levels of holding potential between −60 and +20 mV are shown in Table 1. The mean ratios of TTX-sensitive flux to current ($F\Delta\Phi_{Na}/\Delta I$) were significantly different from unity at three of these potentials (value at −20 mV at the $p < 0.01$ and at −60 and +20 mV at the $p < 0.05$ level of confidence), suggesting that there were small residual systematic errors of up to 6.6% in these groups. The data were collected over a period of three years and measurements were not randomized with respect to membrane potential. Nevertheless, the overall mean value of $F\Delta\Phi_{Na}/\Delta I$ was 0.996 ± 0.015, not significantly different from 1.0 ($0.7 < p < 0.8$) as expected if changes in flux and current derive from the same membrane area. The slope of the linear regression line of $F\Delta\Phi_{Na}/\Delta I$ versus membrane potential was 0.0013 ± 0.0008 mV^{-1} which is not significantly different from zero ($0.1 < p < 0.2$), suggesting that there was no systematic dependence of the value of $F\Delta\Phi_{Na}/\Delta I$ on membrane potential. The coefficient of variation (SD/mean) of the overall average of $F\Delta\Phi_{Na}/\Delta I$ was 12%. This is slightly larger than the variability expected from a calculation of a ratio of values, each of which have a variability of 5%, but about as expected for the ratio of two differences in which each value has a coefficient of variation of 5%.

TABLE 1 Effect of membrane potential on the ratio of TTX-sensitive flux to current

Membrane potential	$F\Delta\Phi_{Na}/\Delta I$	Number of measurements	Number of axons
mV	mean ± SEM		
−60	0.950 ± 0.011	5	4
−40	0.974 ± 0.012	8	5
−30	1.042 ± 0.048	10	10
−20	0.934 ± 0.021	16	11
0	1.047 ± 0.033	17	16
+20	1.028 ± 0.001	2	2
Overall	0.996 ± 0.015	58	48

Area match results without end pool clamps

Before the development of the end pool clamp technique, several attempts were made to eliminate extraneous current flow from the ends by electrically isolating them. If the end pools contained artificial seawater and were left ungrounded but were isolated from the center pool by grease seals, as in Fig. 3 *A*, the mean value of the unidirectional TTX-sensitive flux to current ratio ($F\Delta\Phi_{Na}/\Delta I$) was 0.69 ± 0.05 (mean ± SEM, 14 observations on 10 axons). This is significantly different from 1.0 ($p < 0.001$) and suggests that extraneous current from

the axon ends accounts for an increase in the measured current change of ~30% over the actual membrane current, despite the presence of the grease seals and, hence, of a finite seal resistance.

If the artificial seawater was omitted from the end pools and the axon ends exposed to air, the measured value of $F\Delta\Phi_{Na}/\Delta I$ was 0.64 ± 0.07 (six measurements on two axons), suggesting that current flow from the ends still occurred in a moist layer of electrolyte adhering to the exterior of the axon. An apparent improvement in electrical isolation was achieved by bathing the axon ends in mineral oil or silicon oil (unpolymerized Sylgard, Dow-Corning Corp., Midland, MI). Values of $F\Delta\Phi_{Na}/\Delta I$ of 0.85 ± 0.04 (32 measurements on 14 axons), and 0.71 ± 0.04 (nine measurements on two axons) were obtained with mineral oil, or Sylgard, in the end pools, respectively. All of these area match ratios without end pool clamps are significantly different from 1.0 (largest $p < 0.002$), suggesting that none of the maneuvers produced adequate electrical isolation of the end pools.

Caution against blind application of the method

One application of the method is to determine whether the stoichiometry of Na/K pump operation is constant at 3Na/2K and independent of changes in membrane potential (Rakowski et al., 1989; for review see De Weer et al., 1988). Simultaneous measurements of Na/K pump current and flux, using the end-pool clamp system, are illustrated in Fig. 5. The external solution was Na-free and contained 10 mM K, and the internal solution (see Methods) was appropriate to support normal forward Na/K pump activity. Passive K conductance was partially blocked by use of 1 mM external 3,4 diaminopyridine. At a holding potential of −60 mV the specific pump inhibitor dihydrodigitoxigenin (H_2DTG) produced an inward shift of the holding current, of 1.15 $\mu A\ cm^{-2}$, owing to block of outwardly directed Na/K pump current. The simultaneously measured change in ^{22}Na efflux was 31.1 pmol $cm^{-2}\ s^{-1}$ or, expressed as current, 3.00 $\mu A\ cm^{-2}$. The flux and current data are plotted in Fig. 5 using a vertical scale for flux that is three times that for current when both are expressed in the same units. Assuming that the Na/K pump transport stoichiometry is 3Na/2K, and that there is no source of contaminating ionic current, the flux and current records should overlap. Clearly, they do not. This lack of agreement cannot be explained by contamination of the Na efflux by pump-mediated Na/Na exchange, because that would lead to overestimation, not underestimation, of pumped Na efflux, and in any case the experiment was done in Na-free seawater. The result suggests that there is contamination by additional

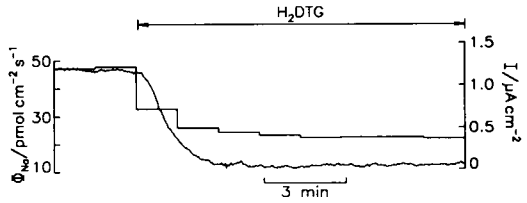

FIGURE 5 Simultaneous measurement of changes in H_2DTG-sensitive current and Na efflux. (*Lower trace*) membrane current change produced by the addition of 100 μM H_2DTG at a holding potential of −60 mV. (*Upper trace*) efflux of ^{22}Na before and after the application of H_2DTG. The current has been scaled so that it will appear to be three times the size of an equal magnitude flux when expressed in the same units. That is, the current and flux traces should overlap for an Na/K pump stoichiometry of 3Na/2K. Note that the change in current is greater than 1/3 of the change in Na efflux. Axon diameter 500 μm. Temperature 17°C.

H_2DTG-sensitive current. The source of this contaminating current, however, is not from the end pools since after the application of H_2DTG, TTX was washed out for 30 min and an area match test carried out by reapplication of TTX. The ratio of TTX-sensitive flux to current was 1.02, confirming that flux and current in this axon derived from the same membrane area. We conclude, therefore, that there is a source of contaminating H_2DTG-sensitive ionic current, probably K current, despite the use of 1 mM DAP.

Despite evidence that H_2DTG specifically stops the Na/K pump and has no direct effect on passive ionic conductances (Rakowski et al., 1989), H_2DTG-sensitive ionic current changes may occur as an indirect consequence of stopping the Na/K pump. When the pump is running, it can partially deplete the extracellular restricted-diffusion space (Frankenhaeuser and Hodgkin, 1956) of K. Reaccumulation of K on stopping the pump with H_2DTG will result in an inward ionic current that masquerades as H_2DTG-sensitive pump current unless passive K channel conductance is essentially completely blocked. The major point, then, is that the method described here cannot be blindly applied with even the most specific channel, pump or carrier blocking agents. Ionic current changes that are a secondary consequence of stopping transport activity must be prevented in order, for example, to equate H_2DTG-sensitive current with Na/K pump current.

Experiments similar to that shown in Fig. 5 were done at either 0 mV or −60 mV in Na-free seawater using either 100 μM H_2DTG or ouabain. Results are reported from only those axons which had an area match ratio between 0.9 and 1.1. The mean value of ouabain- or H_2DTG-sensitive flux to current, using the end pool clamp system and DAP as a K channel blocker, was

2.07 ± 0.10 (13 measurements on 12 axons) at 0 mV and 2.55 ± 0.18 (9 measurements on 8 axons) at −60 mV. Both of these mean values are significantly different from the expected ratio of 3.0 ($p < 0.05$) suggesting that both values have a systematic error owing to contamination by H_2DTG-sensitive ionic current. The two mean values are also significantly different from each other ($p < 0.05$). Because the ratio of H_2DTG-sensitive flux to current is closer to 3.0 at −60 than at 0 mV, this suggests that the magnitude of the contaminating ionic current is reduced by hyperpolarization, a characteristic expected of K channel current. Exhaustive experiments, described in Rakowski et al. (1989) showed that if the remaining sensitivity of the axon to changes in external [K] is further diminished by inclusion of 200 mM tetraethylammonium and 20–25 mM phenylpropyltriethylammonium in the dialysate, the H_2DTG-sensitive flux to current ratio is not significantly different from the value of 3.0 expected for an Na/K pump stoichiometry of 3Na/2K.

These results serve to illustrate the degree of caution required in interpreting simultaneous measurements of changes in current and flux in terms of the stoichiometry of an ion transport mechanism, even when a specific inhibitor is available, as in the case of the Na/K ATPase. The paper by Rakowski et al. (1989) should be consulted for a more complete discussion of the application of the end pool clamp method to accurate measurement of Na/K pump current, and of the minimization of other sources of error.

DISCUSSION

A method has been described for the direct measurement of changes in steady-state current and flux in voltage-clamped squid giant axons. This report gives details of the voltage-clamp electronics and describes the use of two ancillary end pool clamp circuits to eliminate longitudinal current flow from the cannulated end regions of the axon. Use of end pool clamps avoids problems caused by polarization of guard electrodes and permits the accurate resolution of the small magnitude currents mediated by electrogenic transport processes such as the Na/K pump. The method has been developed for use in conjunction with the techniques of internal dialysis and of radiotracer efflux measurement. We have demonstrated the accuracy of the method by experiments in which TTX-sensitive current and flux into Na-free seawater were measured in the total absence of K, conditions in which the unidirectional flux and current should be of equal magnitude. The agreement is excellent and is independent of the membrane potential at which the measurements are made (Table 1). Empirically we have found that apparent seal resistances of 50–100 KΩ give satisfactory results. It is possible to achieve even higher values of seal resistance. For example, Begenisich and Busath (1981) obtained seal resistances of 100–600 KΩ by removing the surface layer of seawater from the axon by gently blotting it and enclosing it in a device that allowed additional grease to be pressure injected at three points around the axon circumference. However, because increasing the seal resistance under end pool clamp conditions is of little consequence, provided that the seal is mechanically intact, we have not found it necessary to make a special effort to increase the seal resistance. Instead, we regard a measured value of apparent seal resistance in excess of 40 KΩ as an indication of having achieved a sufficiently mechanically tight seal. Ideally, optimum conditions would be achieved with mechanically and electrically tight, but very narrow seals. In practice, however, as the amount of stopcock grease is increased to further tighten the seals, the width of the seal regions increases, eventually creating an additional compartment in which differing fractions of the current and efflux across the cell membrane may be collected in the center experimental pool. It is useful, therefore, to establish an objective criterion such as the measured seal resistance in order to decide when the mechanical seal is adequate.

Although a great deal is known about the electrochemistry of the hydrogen electrode consisting of a blackened platinum plate in an aqueous solution saturated with hydrogen and free of oxygen (e.g., Hills and Ives, 1961), the electrochemistry of blackened platinum plates used in complex salt solutions containing dissolved oxygen and used as a pathway for current flow is only poorly understood. The passage of current through such an electrode/solution interface will result in the development of a counter e.m.f. (electrode polarization) that is a nonlinear function of the current magnitude and duration. The counter e.m.f. will eventually saturate at the interfacial potential which is sufficient to produce electrolysis of H_2O (~0.4V). This counter e.m.f. masquerades as an increase in electrode impedance of the guard and center pool plates (R_{LP}, R_{RP}, and R_{CP} in Fig. 3), and unless precautions are taken to ensure equal current density across the plates in the end and center pools, the assumption that these electrode impedances are equal is not warranted.

The importance of making control measurements for the possible presence of end effects was recognized by Gilly and Armstrong (1984) in experiments that characterized a small population of TTX-sensitive, threshold Na channels in squid axons and that required resolution of currents smaller than 1 $\mu A\ cm^{-2}$. These authors did not use an end pool clamp system, but instead used various

tests for the presence of end effects such as the application of TTX to the region of axon in the air gaps, the reduction of external [Na], and the comparison of currents measured from different regions of the same axon. It is not clear, however, whether these tests are adequate to demonstrate the absence of end effects, and future experiments on ionic currents of such small magnitude may benefit by the use of an end pool clamp system.

We have not attempted to use the end pool clamp system in high speed voltage-clamp measurements. However, since the impedances of the bath electrodes can be made quite small, an end pool clamp system should function adequately in high-speed applications provided that the capacitance of the electrodes is kept small or electrically neutralized. The requirement that the main clamp be made slower than the end pool clamps for stability places a slight restriction on the practical clamp speed that can be achieved. However, because the external electrode impedances can be made quite small compared with the impedance of the internal electrode, this requirement is of little consequence, and clamp speeds up to 10 KHz should be attainable if low-noise, high-speed amplifiers are chosen and low-pass filters are eliminated. It is not necessary to use end pool clamps, however, unless large membrane currents are being passed for prolonged times, or unusually small currents need to be resolved. That is, the use of an end pool clamp system should be considered whenever significant polarization of guard electrodes could affect the results.

The present method allows measurements of current and flux to be made over a range of membrane potentials, and is particularly well-suited to the determination of Na/K pump current and flux since highly specific drugs are available for stopping the Na/K pump. The technique should also prove useful for the investigation of other steady-state currents, for example, those mediated by TTX-sensitive Na channels, or those generated by electrogenic transport processes such as Na/Ca exchange. Of course, the method is by no means restricted to steady-state measurements and can be used to study the rates of relatively slow modulatory processes on various components of membrane current.

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