Stoichiometry and Voltage Dependence of the Sodium Pump in Voltage-clamped, Internally Dialyzed Squid Giant Axon

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ABSTRACT   The stoichiometry and voltage dependence of the Na/K pump were studied in internally dialyzed, voltage-clamped squid giant axons by simultaneously measuring, at various membrane potentials, the changes in Na efflux ($\Delta\Phi_{Na}$) and holding current (ΔI) induced by dihydrodigitoxigenin (H_2DTG). H_2DTG stops the Na/K pump without directly affecting other current pathways: (a) it causes no ΔI when the pump lacks Na, K, Mg, or ATP, and (b) ouabain causes no ΔI or $\Delta\Phi_{Na}$ in the presence of saturating H_2DTG. External K (K_o) activates Na efflux with Michaelis-Menten kinetics ($K_m = 0.45 \pm 0.06$ mM [SEM]) in Na-free seawater (SW), but with sigmoid kinetics in ~400 mM Na SW (Hill coefficient = 1.53 ± 0.08, $K_{1/2} = 3.92 \pm 0.29$ mM). H_2DTG inhibits less strongly ($K_i = 6.1 \pm 0.3$ μM) in 1 or 10 mM K Na-free SW than in 10 mM K, 390 mM Na SW (1.8 ± 0.2 μM). Dialysis with 5 mM each ATP, phosphoenolpyruvate, and phosphoarginine reduced Na/Na exchange to at most 2% of the H_2DTG-sensitive Na efflux. H_2DTG sensitive but nonpump current caused by periaxonal K accumulation upon stopping the pump, was minimized by the K channel blockers 3,4-diaminopyridine (1 mM), tetraethylammonium (~200 mM), and phenylpropyltriethylammonium (20–25 mM) whose adequacy was tested by varying [K]$_o$ (0–10 mM) with H_2DTG present. Two ancillary clamp circuits suppressed stray current from the axon ends. Current and flux measured from the center pool derive from the same membrane area since, over the voltage range −60 to +20 mV, tetrodotoxin-sensitive current and Na efflux into Na-free SW, under K-free conditions, were equal. The stoichiometry and voltage dependence of pump Na/K exchange were examined at near-saturating [ATP], [K]$_o$ and [Na]$_i$ in both Na-free and 390 mM Na SW. The H_2DTG-sensitive $F\Delta\Phi_{Na}/\Delta I$ ratio (F is Faraday's constant) of paired measurements corrected for membrane area match, was 2.86 ± 0.09 ($n = 8$) at 0 mV and 3.05 ± 0.13 ($n = 6$) at −60 to −90 mV in Na-free SW, and 2.72 ± 0.09 ($n = 7$) at 0 mV and 2.91 ± 0.21 ($n = 4$) at −60 mV in 390 mM Na SW. Its overall mean value was 2.87 ± 0.07 ($n = 25$), which was not significantly

different from the 3.0 expected of a 3 Na/2 K pump. H_2DTG-induced $\Delta\Phi_{Na}$ and ΔI declined together with hyperpolarization, $13 \pm 5\%$ and $14 \pm 6\%$ over $\sim$75 mV in Na-free SW and $34 \pm 5\%$ and $39 \pm 8\%$ over 60 mV in 390 mM Na SW. Steady-state pump current-voltage (I-V) relationships, computed as the difference (corrected for drift if necessary) between I-Vs with and without H_2DTG, confirmed that pump rate slowed with hyperpolarization; pump current declined monotonically $28 \pm 3\%$ ($n = 10$) over $\sim$70 mV in Na-free SW, and $42 \pm 4\%$ ($n = 6$) over 60 mV in high-Na SW. In five axons yielding pump I-Vs we also measured H_2DTG-sensitive Na efflux at 5 voltages between -60 and $+20$ mV. Hyperpolarization to -60 mV slowed H_2DTG-sensitive Na efflux monotonically, $11 \pm 2.5\%$ ($n = 3$) in Na-free SW, and $30 \pm 2.5\%$ ($n = 2$) in high-Na SW. The slightly steeper voltage dependence of H_2DTG-sensitive current than of Na efflux is probably due to a 5–10% contamination with nonpump current at depolarized potentials despite the presence of K channel blockers. We conclude that, under our conditions, Na/K pump-mediated Na efflux and current are voltage dependent, slightly in Na-free SW and more steeply in high-Na SW, and that this dependence reflects an effect of voltage and Na_o on forward Na translocation rather than on reverse Na translocation or on stoichiometry.

INTRODUCTION

The Na/K pump transports more Na ions in one direction across the cell membrane than K ions in the other, and so generates net membrane current (for reviews see Thomas, 1972b; De Weer, 1975; Gadsby, 1984; Glynn, 1984). For both thermodynamic and kinetic reasons the current produced by an electrogenic pump should vary with membrane potential, at least over some voltage range (for references see De Weer et al., 1988a).

If the transport stoichiometry is fixed and known, the equilibrium potential for the Na/K pump reaction, $E_{Na/K}$, at which pump current must vanish, can be estimated (e.g., Caldwell, 1973; Chapman and Johnson, 1978; De Weer et al., 1988a):

$$E_{Na/K} = [(\Delta G_{ATP}/F) + mE_{Na} - nE_K]/(m - n) \tag{1}$$

for an mNa/nK/1ATP stoichiometry, where ΔG_{ATP} is the free energy of hydrolysis of ATP, F is Faraday's constant, and E_{Na} and E_K are the electrochemical equilibrium potentials for Na and K, respectively. Insertion of values appropriate for respiring animal cells yield roughly -200 mV for $E_{Na/K}$ (e.g., De Weer, 1984; De Weer et al., 1988a, b). Obviously, Na/K pump current amplitude must decline towards zero at large negative membrane potentials.

Aware of the likelihood that Na/K pumping rate might decline on membrane hyperpolarization, a number of investigators over the past thirty or so years have sought relevant experimental evidence in a variety of tissues. Early results were largely inconclusive because of the limited voltage ranges explored and/or inappropriate methods for identifying Na/K pump fluxes or currents (reviewed in De Weer et al., 1988a). In particular, stopping or starting the pump is likely to have resulted in changes in [K] and in [Na] just outside and inside, respectively, the cell membrane (e.g., Gadsby, 1982). Those concentration shifts, in turn, probably caused alterations of currents in other pathways, which would mistakenly have been included in the estimates of Na/K pump current.

More recent studies, using preparations in which other current pathways were naturally sparse, as in oocytes (Turin, 1984; Lafaire and Schwarz, 1986; Eisner et al., 1987; Rakowski and Paxson, 1988), or could be diminished with blockers, as in cardiac myocytes (Gadsby et al., 1985b; Hasuo and Koketsu, 1985; Glitsch and Krahn, 1986), have demonstrated unequivocally that Na/K pump current, defined as cardiotonic steroid-sensitive voltage-clamp current, declines with membrane hyperpolarization over the physiological voltage range. Measurements limited to pump current, however, do not allow a distinction between voltage sensitivity of pump turnover rate and that of the stoichiometry of Na/K transport (although available preliminary data seem consistent with a stoichiometry that remains fixed over a wide range of conditions: see, e.g., Gadsby, 1984; De Weer et al., 1988b). To resolve this question directly, it is necessary to make simultaneous determinations of pump current and pump flux over a range of membrane potentials.

Thomas (1972a), using snail neurons, made the first attempt to correlate increments in net Na efflux, determined from the rate of extrusion of iontophoretically injected Na, with increments in voltage-clamp holding current. Similar measurements were later made by Eisner et al. (1981) and Glitsch et al. (1982) on sheep cardiac Purkinje strands that were transiently Na loaded by briefly exposing them to K-free solution. An alternative approach to determining pump stoichiometry, applicable in steady-state conditions, is to compare the shift in holding current caused by cardiotonic steroid application (De Weer, 1974) with the simultaneously measured reduction of radiotracer Na efflux. This approach was first applied by Cooke et al. (1974) to *Aplysia* neurons, and subsequently to perfused muscle cells of the giant barnacle by Lederer and Nelson (1984), and to *Xenopus* oocytes by Eisner et al. (1987). While these experiments all yielded stoichiometry values more or less consistent with the ratio 3 Na/2 K found in earlier work on red blood cells (Post and Jolly, 1957), none included simultaneous determinations of pump current and flux at more than one membrane potential, which is hardly surprising in view of the technical difficulties involved.

Ideally, cells should be internally dialyzed or perfused so that (a) steady-state conditions can be maintained with the specific activity of the tracer accurately known, and (b) the [ATP]/[ADP] ratio can be kept high to minimize the contribution of pump-mediated Na/Na exchange to the steroid-sensitive tracer Na efflux (Garrahan and Glynn, 1967b; De Weer, 1970; Glynn and Hoffman, 1971; Kennedy et al., 1986). Control experiments should demonstrate that, (c) if Na/K pump activity has already been abolished by some other means, application of cardiotonic steroid then has no effect on membrane current, and (d) if the pump is first fully inhibited by steroid, deliberate alterations of extracellular [K] or intracellular [Na], of a magnitude comparable to those that might accompany sudden arrest of the Na/K pump, do not alter membrane current (see Gadsby et al., 1985b; De Weer et al., 1988a, b). Two further technical requirements are that (e) measured changes in membrane current and tracer flux derive from the same area of cell membrane (not a problem in experiments with intact, essentially spherical, cells), and (f) membrane conductance be sufficiently low to permit the membrane potential to be held at desired levels for long periods of time with currents small enough to avoid cell damage or changes in internal pH.

We describe here experiments on voltage-clamped, internally dialyzed squid giant axons to test the effectiveness of procedures to overcome these difficulties. We find that at near-saturating concentrations of Na_i, K_o, and ATP, both in 390 mM Na and in Na-free seawater, Na/K pump current and ^{22}Na efflux decline in parallel as the membrane is hyperpolarized, but that this voltage dependence is weaker in Na-free seawater. The results show that the Na/K transport stoichiometry is independent of membrane potential and, hence, that the observed voltage dependence of pump current and flux reflects an influence of membrane potential on pump turnover rate and, more specifically, on the forward rate of Na translocation.

Some of the results have been briefly presented at meetings (Rakowski and De Weer, 1982a–c; Gadsby et al., 1986; De Weer et al., 1986, 1987).

METHODS

Internal Dialysis and Voltage Clamp

Axons from the hindmost stellar nerves of the squid, Loligo pealei, were freed of small fibers and connective tissue; branches were kept ≥1 axon diameter long. The lucite chamber for internal dialysis, modified from the design of Brinley and Mullins (1967), is shown schematically in Fig. 1, A and B. The horizontally mounted axon was drawn over, and tied with 7/0 silk to beveled and fire-polished glass cannulae (diameter, 350–400 μm) protruding through silicone rubber seals at both ends of the chamber (Fig. 1 A). The axon was positioned to minimize the number of branches (usually one to three) in the central pool. A 140–160-μm cellulose acetate capillary (Fabric Research Corp., Dedham, MA) made porous to small (≤1 kD) molecules by 20 h soaking in 0.1 N NaOH, was introduced for internal dialysis. A 75-μm sharpened tungsten wire was inserted into the capillary to stiffen it and to facilitate threading it through the axon from one cannula to the other. The wire was withdrawn before starting internal dialysis (1.4 μl/min). After insertion of the porous capillary and then an electrode assembly (described below), the axon was circled by two rings of stopcock grease (Thomas Scientific Co., Philadelphia, PA), usually 12 mm apart, to separate the central (experimental) pool of bathing fluid from the lateral pools holding the cannulated axon ends.

The electrode assembly consisted of a 75-μm-diameter blackened 90% platinum/10% iridium wire for passing current that was glued piggy-back to and extended 13 mm beyond a 3 M KCl-filled glass microelectrode (resistance 0.4–1.2 MΩ) whose tip was positioned, within the axon, approximately in the middle of the central pool. Membrane potential was measured between this and a flowing 3-M-KCl reference electrode (0.3–0.8 MΩ) (Fig. 1 B) dipped in the central pool, via a pair of matched calomel half-cells used because of their high stability. The platinum wire delivered the output of a custom-designed stable, low-noise voltage-clamp circuit (Rakowski, 1989). A blackened platinum plate in the central pool was held at virtual ground by a stable, low-noise operational amplifier, which also monitored the current flowing from the central pool at a gain of 1–8 V/μA.

A simplified diagram of the conducting pathways between the various compartments is shown in Fig. 1 C. Because of the series resistance, R_{iw}, between the tip of the internal microelectrode and the current-passing wire, any change in clamp current brought about by a change in the elements representing axon membrane in the central pool results in a corresponding change in current flow from the axon into the end pools and, via the grease seals, to the current monitor in the central pool. The recorded current signal thus overestimates the change in central-pool current by the amount of concomitant change in end-pool current. To avoid this problem, as depicted in Fig. 1 B, two additional voltage-clamp circuits held the voltage difference between each end pool and the central pool close to zero, thereby minimiz-

FIGURE 1. Schematic diagram of dialysis chamber (not to scale) and voltage-clamp apparatus. (A) Chamber. The axon (length, 32–36 mm) is mounted between two glass cannulae. The center (experimental) pool, holding ~0.3 ml, is isolated from the end pools by two 5-mm wide rings of stopcock grease. Artificial seawater enters (2 ml/min) at both ends of the center pool, and is aspirated from the middle. (B) Main and end-pool voltage-clamp circuits. Black circles represent calomel half-cells which, in the case of the end-pool electrodes, contact the end pools via agar 0 Na, 0 K seawater bridges. End-pool clamp current is delivered via two blackened platinum plates. The command voltage (V_c) drives a stable, low-noise feedback amplifier that holds the axon membrane potential at the desired value by passing current through an internal blackened platinum wire. (C) Simplified equivalent circuit of the experimental set-up. R_L and R_R, resistance of the axon membrane in the end pools; R_{LS} and R_{RS}, resistance of the grease seals; V_i, voltage sensed by the internal electrode; V_o, voltage sensed by the reference electrode in the center pool; $V_m = V_i - V_o$; V_L and V_R, voltage sensed in the end pools; R_M, resistance of the axon membrane in the center pool; E_M, resting potential when the Na/K pump is blocked; $R_{Na/K}$, equivalent chord resistance of the active Na/K pump; $E_{Na/K}$, pump reversal potential; V_W, voltage applied to the platinum wire; R_{iW}, resistance between the wire and the internal voltage measurement point.

ing longitudinal current flow across the grease seals. Tests of effectiveness of these end-pool voltage clamps are described below in connection with Fig. 8, and a more complete technical description of the problem and its solution are given by Rakowski (1989).

Flow System

External solution flowed to the chamber from a two-position valve (HV-4-4, 86729; Hamilton Co., Reno, NV). The second output from the valve flowed to waste, and the two inputs were fed by peristaltic pumps (MicroPerpex, LKB Instruments, Inc., Gaithersburg, MD). This arrangement allowed the second inflow line to be rinsed, up to the valve, with the new solution before switching (valve switcher 0138980; Hamilton Co.). To minimize fluid level fluctuations in the chamber (which caused small variations in recorded holding current), a mechanical compliance and resistance were interposed in the inflow tubing between valve and chamber. The compliance was a short section of thin-walled silastic tubing (602-235; Dow Corning Corp., Midland, MI) and the resistance was a longer section of narrow-bore tubing (602-135, Dow Corning). Solutions were filtered through a 2–3 cm length of Tygon tubing filled with aquarium filter material, inserted in each inflow line. Before entering the central pool, solutions were cooled as they passed through coils immersed in a water jacket that surrounded the chamber. Solution temperature (17–18°C) was measured by a thermistor embedded in the lucite chamber, and was controlled via the water circulating through the jacket. Fluid was drawn from the chamber by a third pump, turning ~5% faster than the inflow pumps, through a beveled, tapered glass capillary placed in the central pool so that fluid and air both entered the bevel, yielding an outflow broken into regularly sized and spaced bubbles which formed uniform drops at the fraction collector. Attention to such details was essential for generating flux and current records relatively free of fluctuations.

Solutions

For convenience we use the following shorthand: the concentrations of the principal components important for understanding a given experiment are stated (in millimolar) for the external then internal solution separated by two slashes (e.g., 0 Na//50 Na). The solutions' osmolalities were checked with a dewpoint osmometer (Wescor, Inc., Logan, UT) and adjusted to 930 mosmol kg^{-1}.

The normal artifical seawater had the following composition (in millimolar): 415 NaCl, 10 KCl, 10 CaCl$_2$, 25 MgCl$_2$, 25 MgSO$_4$, 0.05 EDTA, 5 Tris (tris-hydroxymethyl-aminomethane)-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate), pH 7.7. For experiments in K-free conditions K was replaced with Na, and in Na-free solutions Na was replaced with N-methyl-D-glucamine (NMG). In many experiments Mg salts were omitted, CaCl$_2$ was raised to 75 mM, NaCl was lowered to 380 mM, and 1 mM of the K channel blocker 3,4-diaminopyridine (DAP) was added. When required, 0.2 μM tetrodotoxin (TTX, Sigma Chemical Co, St. Louis, MO) was added from a 1-mM aqueous stock solution. A 10-mM stock solution of ouabain (Sigma Chemical Co.) in the appropriate artificial seawater was used to achieve a final concentration of 100 μM. H$_2$DTG (Boehringer-Mannheim Diagnostics, Inc., Indianapolis, IN) was prepared either as a 10-mM solution in ethanol and used at 10 μM or as a 100 mM solution in dimethyl sulfoxide (DMSO) and used at 100 μM. DMSO at the resulting concentration was without effect on membrane current (see Fig. 5), but 0.1% ethanol induced current changes of ~0.1 μA cm^{-2}, and was therefore also added to the appropriate control seawater.

The composition of the internal dialysis fluid was varied over the years in search of optimal conditions. In most (recent) experiments the approximate composition was (in millimolar): 50 Na, 200 tetraethylammonium (TEA), 20 phenylpropyltriethylammonium (PPTEA, as the bromide salt, a gift of Dr. C. M. Armstrong, University of Pennsylvania), 15 Mg, 5 Tris, 5 dithio-

threitol (DTT), 55 Cl, 2.5 EGTA, 5 ATP, 5 phosphoarginine (PA), 5 phosphoenolpyruvate (PEP), and ~0.4 M HEPES (pH 7.4). Whenever TTX-sensitive current and ^{22}Na efflux into Na-free seawater were to be measured, NMG replaced TEA and PPTEA (which we found to block Na channels at high concentrations) in the internal solution, which also contained 250 μM veratridine to increase the magnitude of the TTX-sensitive current. Variations in the composition of these two standard internal solutions are specified in the figure legends. The expression "blockers present" means that ~200 mM TEA and 20–25 mM PPTEA were present internally, and 0.2 μM TTX and 1 mM DAP externally.

Measurement of Drug-induced Changes in Holding Current

Programs for data acquisition and analysis were written in Basic by Ms. Margaret C. Jost. Current and voltage signals, low-pass filtered at 10 Hz by 6-pole Bessel filters (721L6L; Frequency Devices, Haverhill, MA), were alternately digitized at 20 Hz each with 12-bit resolution via an Apple IIe computer interface, averaged 15 data points at a time, and stored. This results in a temporal resolution of 0.75 s per saved point. Records of current and voltage shown in the figures were generated by connecting successive points. Straight lines were least-squares fitted to the linear portions of the current trace before and during exposure to H$_2$DTG, ouabain, or TTX. The magnitude of the elicited shift in holding current (ΔI) was then computed as the vertical distance between the two extrapolated lines at the time when the change was half complete. Because the operator positioned the cursors delimiting the segments of the data to be fitted, the procedure was, in part, subjective. In general, cursor positions were selected to yield approximately parallel lines before and after drug addition. There was about a 5% variability in the calculated value of ΔI owing to uncertainty in the position of these baselines.

Determination of Steady-State I-V Relationships

Steady-state I-V relationships were determined using voltage-clamp staircases of 5-mV steps each 1 s long. The command waveforms were generated by computer. The digitizing rate was 25 Hz per channel, and membrane current was obtained by averaging 12 points (over a period of 0.48 s) near the end of every step. Each complete run went from the holding potential (V_H) in the hyperpolarizing direction to a specified extreme voltage, then in the depolarizing direction to a specified extreme voltage, then back to the holding potential, so that current was measured twice at each voltage. If the range was kept between -120 and $+20$ mV, the resulting I-Vs usually showed little or no hysteresis (e.g., Fig. 7, *B* and *E*), our criterion for steady state.

Measurement of Tracer Efflux

Sodium efflux was measured with carrier-free ^{22}Na added to the dialysis fluid (~100 μCi ml^{-1}) usually at a [Na] of 50 mM. Approximately 30 min of dialysis were required for ^{22}Na efflux to reach steady state. The effluent from the central pool was collected in 1.5-min samples and assayed for radioactivity with either a gamma counter or preferably, since backgrounds were lower and counting efficiency slightly greater, a liquid scintillation counter. The samples were generally counted for 10 min, a time sufficient for a statistical error of $\leq 3\%$. There was some arbitrari-

ness in the selection of samples to be grouped to compute a mean efflux value before or during exposure to a drug. We generally took as many consecutive samples as possible, compatible with the requirement of steady state, but never fewer than three. We estimate that the statistical counting error and variability due to subjective selection of the baseline sample groups produced about a 5% uncertainty in the calculated change in Na efflux ($\Delta\Phi_{Na}$) caused by the drug.

Statistics and Errors

Mean values are given ± SEM unless otherwise stated. Since ratios are lognormally distributed, we give geometric rather than arithmetic means for the TTX- or H_2DTG-induced $\Delta\Phi_{Na}/\Delta I$ ratios, and calculate the SEM appropriate for lognormal distributions. Our estimates of reproducibility in measurements of $\Delta\Phi_{Na}$ and ΔI (to ~5% in each case, as discussed above) lead to an expected coefficient of variation (SD/mean) of ~7% for their ratio, assuming uncorrelated measurement errors (Kelley, 1947). The variation we found in TTX-induced and H_2DTG-induced $\Delta\Phi_{Na}/\Delta I$ ratios was slightly larger (~12%). Other errors that would affect the estimation of such ratios include day-to-day variations in calibration of isotope specific activity.

RESULTS

Specificity of H_2DTG Action

Our experiments require that the agent used to define Na/K pump activity be specific, that is, have as sole action arrest of the pump without additional direct or indirect effect on passive or active ionic currents. The current records of Fig. 2, A–C, demonstrate that 10 μM H_2DTG caused little change in holding current when Na/K pump turnover had been prevented by removal from internal and external solutions of any of three essential substrates: K, Na, or Mg. In each case, the upper and lower records show the control responses to 10 μM H_2DTG before and after the intervention. The very small response (0.05 μA cm^{-2}) in nominally K-free conditions in A possibly reflects a small amount of electrogenic Na/Na exchange (Lee and Blostein, 1980; Forgac and Chin, 1981; Goldschleger et al., 1987) as discussed below (see Activation of ^{22}Na efflux by external K). The upward drift in holding current in the absence of internal Mg (C) was probably caused by a slowly increasing (Mg)ATP-sensitive K conductance (Noma, 1983; Spruce et al., 1985; Gadsby et al., 1985a; Bezanilla et al., 1985, 1986). But application of H_2DTG caused no change in the rate of the outward current drift. This lack of response to H_2DTG after elimination of Na or Mg from the internal and external solutions demonstrates that 10 μM H_2DTG has no direct effect on the passive conductance of any ion present in the normal experimental solution since, for example, the records in B rule out changes in all ionic currents except those carried by Na itself, and a change in Na-mediated current is ruled out by the results in C. (A change in ionic conductance that mediates a current that fortuitously reverses at the chosen holding potential cannot be detected. However, the experiments of A–C in Fig. 2 [V_H, −60 to −66 mV], those of Fig. 3 [V_H, −62] and Fig. 4 [V_H, −20 mV] described below, and others not illustrated, were carried out at sufficiently diverse holding potentials to render this possibility unlikely.) The Q_{10} calculated from the current responses in D, 2.56, is

similar to the value of 2.46 reported for the Q_{10} of strophanthidin-sensitive Na efflux (De Weer, 1970).

The specificity of H_2DTG was tested further by assessing its effects on membrane current and Na efflux before, during, and after pump inhibition brought about by
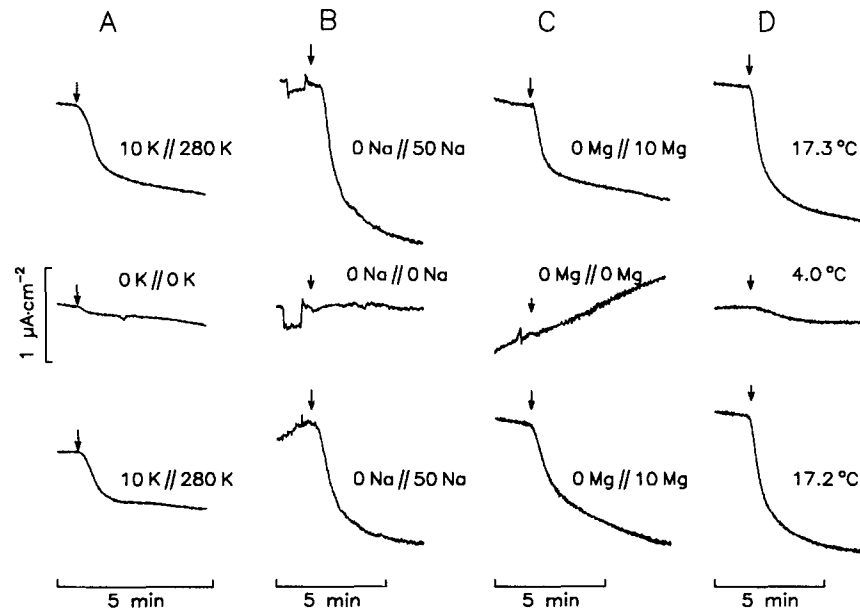

FIGURE 2. Reduction or elimination of 10 μM H_2DTG-induced ΔI when the Na/K pump is stopped by removal of K, Na, or Mg, or cooled. Top and bottom rows are controls; middle row shows tests. All seawaters contained 0.2 μM TTX. (*A*) K removal. *Top:* conditions, 10 K, 420 Na//50 Na, 280 K; ΔI 0.73 μA cm^{-2}. *Middle:* small ΔI 25 min after removal of external and all but 5 mM internal K. *Bottom:* 43 min after restoring original solutions; ΔI 0.51 μA cm^{-2} (axon diameter, 575 μm; V_H, −60 mV). (*B*) Na removal. *Top:* conditions, 10 K, 0 Na//50 Na, 306 K; ΔI 1.35 μA cm^{-2}. *Middle:* negligible ΔI 25 min after starting nominally Na-free, 315 mM K internal solution. *Bottom:* 27 min after restoring control solution; ΔI 1.19 μA cm^{-2} (diameter, 470 μm; V_H, −65 mV). (*C*) Mg removal. *Top:* in 10 K, 425 Na, 0 Mg, 60 Ca//50 Na, 306 K, 10 Mg; ΔI 0.72 μA cm^{-2}. *Middle:* negligible ΔI 40 min after starting Mg-free (10 mM *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid) internal solution. *Bottom:* 30 min after returning to control; ΔI 0.81 μA cm^{-2} (diameter, 465 μm; V_H, −66 mV). (*D*) Effect of cooling. Conditions: 10 K, 420 Na//50 Na, 306 K. *Top:* ΔI 1.28 μA cm^{-2}. *Middle:* after 37 min at 4°C; ΔI 0.17 μA cm^{-2}. *Bottom:* 23 min after returning to 17°C; ΔI 1.31 μA cm^{-2} (diameter, 480 μm; V_H, −67 mV). In *A, C,* and *D,* parallel changes in $\Delta\Phi_{Na}$ accompanied the changes in ΔI. End-pool clamps not in use.

energy depletion. Fig. 3 shows results from an axon internally dialyzed with fluid lacking ATP, PA, and PEP, but containing 2 mM diadenosine pentaphosphate to block adenylate kinase, 10 μM atractyloside to inhibit mitochondrial ATP production, and 25 mM L-arginine as substrate for endogenous arginine phosphokinase.

On removal of internal ATP the magnitudes of H_2DTG-induced ΔI and $\Delta \Phi_{Na}$ declined together over ~90 min, yet both quickly recovered when 5 mM ATP was restored to the dialysis fluid. The holding current drifts seen in episodes d, e, and f again presumably reflect changes in ATP-sensitive K permeability.

Results from a second experiment testing the effect of ATP removal are shown in Fig. 4. Before washout of high energy phosphate compounds, steady-state membrane I-Vs were determined with and without 100 μM H_2DTG present (A), and the difference is shown in B. The magnitude of the H_2DTG difference current at -20
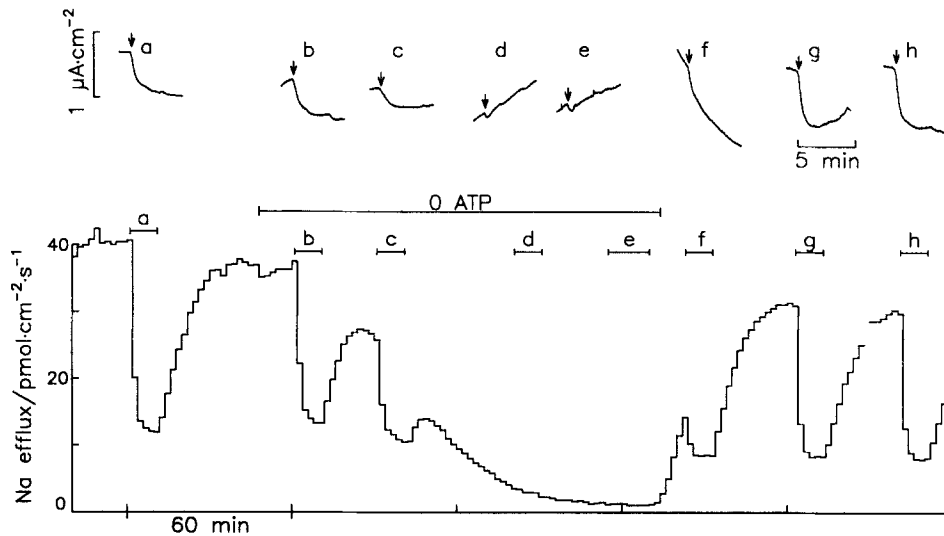
FIGURE 3.   Absence of response to H_2DTG after removal of ATP. ΔI (*top*) and $\Delta \Phi_{Na}$ (*bottom*) during removal and restoration of high-energy phosphates. External solution was 10 K, 420 Na throughout, with 0.2 μM TTX. No K channel blockers; axon diameter, 600 μm; V_H, -62 mV; end-pool clamps not in use. The first H_2DTG (10 μM) application caused a 1.2 μA cm^{-2} ΔI and a 28.2 pmol cm^{-2}s^{-1} $\Delta \Phi_{Na}$. The internal solution (50 Na, 280 K, 10 Mg, 5 ATP/PA) was then replaced with a similar one free of ATP and PA, and containing 25 mM L-arginine. Total ^{22}Na efflux, ΔI, and $\Delta \Phi_{Na}$ declined progressively during ATP washout; after 2.5 h, ΔI vanished and $\Delta \Phi_{Na}$ was 0.3 pmol cm^{-2}s^{-1}. The holding current rose from $+0.5$ μA cm^{-2} initially to $+6.5$ μA cm^{-2}. 90 min after restoring 5 ATP/PA internally, ΔI recovered to 0.9 μA cm^{-2} and $\Delta \Phi_{Na}$ to 22 pmol cm^{-2}s^{-1}; holding current returned to -1.6 μA cm^{-2}.

mV was 1.6 μA cm^{-2}. After 100 min of nucleotide-free dialysis, H_2DTG caused no change in holding current at -20 mV (C) but, 40 min after readmission of 5 mM ATP, the response to H_2DTG was fully restored (1.8 μA cm^{-2}; D). We conclude that when pump activity is stopped by removal of ATP, there is no response to 100 μM H_2DTG despite the presence of all the ions required for forward pump operation.

The results in Figs. 2–4 demonstrate that, at the concentrations tested, H_2DTG has a single action: it stops the Na/K pump, and has no effect on membrane current when the pump is prevented from carrying out Na/K exchange through lack of any

of its substrates. Nonetheless, an H_2DTG-induced ΔI might still include nonpump components, which arise as indirect consequences of stopping the Na/K pump, through accumulation of ions in restricted-diffusion spaces. Efforts to eliminate these components are discussed below in connection with Fig. 7.
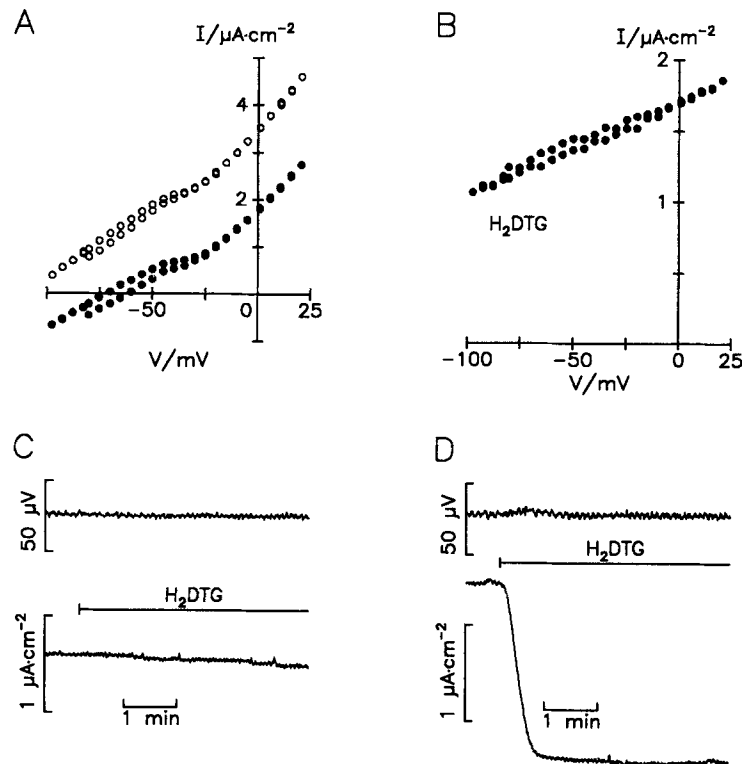

FIGURE 4. Lack of H_2DTG response in the absence of ATP. (*A*) Membrane I-V relationships between -100 and $+20$ mV recorded without (open symbols) and with (filled symbols) 100 μM H_2DTG present in 10 K, 0 Na, 75 Ca//100 Na, 0 K, 5 ATP/PA/PEP. (*B*) H_2DTG difference I-V relationship, from *A*; note the change in vertical scale. (*C*) Holding voltage and current records 100 min after starting dialysis with fluid free of high-energy phosphates. (*D*) Same, 40 min after restoring 5 ATP/PA/PEP; ΔI 1.8 μA cm^{-2}. Axon diameter, 480 μm; end-pool clamps in use; blockers present; V_H in *A* and *B*, -80 mV; in *C* and *D*, -20 mV.

Estimate of the Residual Voltage-Clamp Current Error

It is impossible to clamp the axon membrane at an absolutely constant voltage when the holding current is varying since a small error signal must remain to drive the required change in voltage-clamp current. In Fig. 4 *D* for example, the holding potential changed by <5 μV upon addition of H_2DTG. The membrane slope conductance, measured ~ 2 min after that recording, was 1.5 mS cm^{-2} (rather higher than the more usual conductances which fell in the 0.05–0.2 mS cm^{-2} range, with a
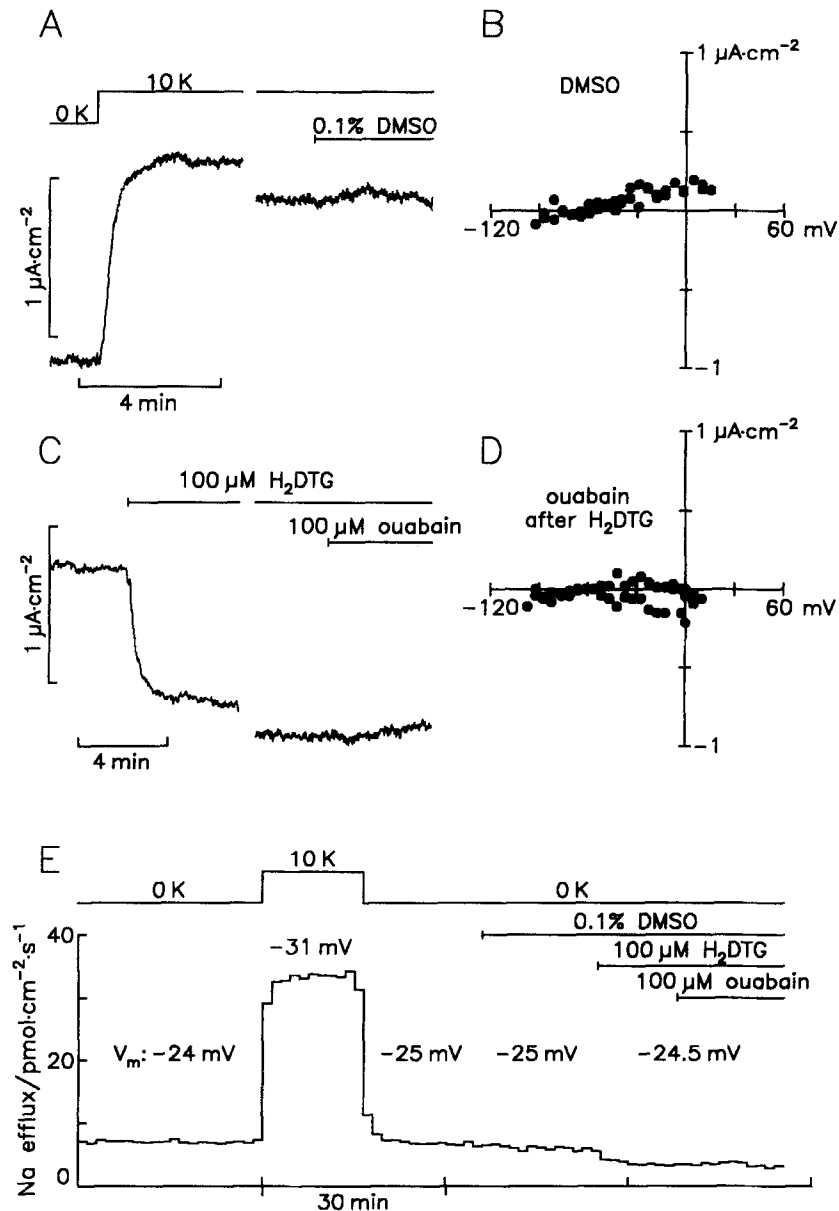
FIGURE 5. Lack of effect of DMSO on either current or ^{22}Na efflux, and of ouabain when saturating H_2DTG is already present. (A) ΔI (1.28 μA cm^{-2}) upon activation of Na/K pump by 10 K; DMSO (0.1%) elicited no further change. Diameter, 560 μm; V_H, -40 mV; conditions: 0 Na, 75 Ca//50 Na, 0 K, 5 ATP/PA/PEP; blockers present. (B) DMSO-sensitive I-V relationship, from records before and 5 min after addition of 0.1% DMSO; same axon as in A. (C) Lack of response to ouabain after H_2DTG. At 0 mV, ΔI caused by 100 μM H_2DTG was 0.8 μA cm^{-2}, but very small upon ouabain (100 μM) addition 24 min later. Axon 9 of Table I; diameter, 450 μm; conditions: 10 K, 390 Na, 75 Ca//52 Na, 0 K, 5 ATP/PA/PEP; blockers present. (D) Ouabain-sensitive I-V relationship, from records before and during exposure to

comparable degree of voltage control), giving an upper-range estimate of 0.007 μA cm^{-2} for the residual current error, or 0.4% of the H$_2$DTG-induced ΔI of Fig. 4 D.

Lack of Effect of DMSO

Many of our experiments demanded a high concentration (100 μM) of H$_2$DTG to inhibit the Na/K pump (see, e.g., Fig. 6) and it was then added from a 100 mM stock solution in DMSO. We therefore tested the effect of 0.1% DMSO alone on membrane current and Na efflux. Fig. 5 A shows ΔI (1.3 μA cm^{-2}) upon Na/K pump activation by a 10-mM-K external solution, in an axon dialyzed with 50 mM Na and 5 mM ATP, and held at -40 mV. (ΔI elicited by the addition of K will equal Na/K pump current only if the pump is completely stopped in the nominally K-free fluid and if adding external K causes no change in passive membrane current, conditions which are shown below to be not completely met in our experiments.) Subsequent addition of 0.1% DMSO caused no ΔI, demonstrating that it affected neither the Na/K pump nor any other current source unless, fortuitously, multiple effects canceled one another and/or the holding potential equaled the reversal potential of the putative DMSO-sensitive nonpump current. The first possibility is rendered unlikely by Fig. 4 C above which shows that 0.1% DMSO (added with 100 μM H$_2$DTG) has no effect on any current component surviving ATP withdrawal; the second is ruled out by the demonstration in Fig. 5 B that the DMSO difference I-V is very small, and comparable to "time only" differences (e.g., Fig. 7 D). In the experiment of Fig. 5 E, 10 mM external K reversibly activated a large ^{22}Na efflux, but 0.1% DMSO had no effect on the K-insensitive ^{22}Na efflux, corroborating the results from current measurements in Fig. 5, A and B.

Ouabain Has No Effect in Axons Already Exposed to 100 μM H$_2$DTG

The results shown in Fig. 5, C and E confirm that 100 μM H$_2$DTG fully inhibits the Na/K pump (see also Fig. 6 B, below). In axons already bathed in 100 μM H$_2$DTG, a saturating concentration of ouabain caused no further change in (a) the holding current at 0 mV (Fig. 5 C), or (b) the membrane current at any potential between -100 and $+10$ mV as shown by the ouabain difference I-V relationship (Fig. 5 D), or (c) ^{22}Na efflux (Fig. 5 E).

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100 μM ouabain, in the presence of 100 μM H$_2$DTG. Diameter, 530 μm; V_H, 0 mV; 10 K, 390 Na, 75 Ca//52 Na, 0 K, 5 ATP/PA/PEP; blockers present. (E) ^{22}Na efflux from unclamped axon treated with K channel blockers. Upon exposure to 10 mM K, the axon hyperpolarized reversibly from -24 to -31 mV, and ^{22}Na efflux reversibly rose by 26.1 pmol cm^{-2}s^{-1}. In the absence of external K, 0.1% DMSO produced no measurable $\Delta\Phi_{Na}$ or change in membrane potential, and 100 μM H$_2$DTG caused only a 2.4 pmol cm^{-2}s^{-1} $\Delta\Phi_{Na}$ (accompanied by a 0.5 mV depolarization) probably attributable to residual electrogenic Na/Na exchange. Further addition of 100 μM ouabain had no effect on ^{22}Na efflux or membrane potential. Diameter, 510 μm; conditions: 400 Na, 75 Ca//64 Na, 0 K, 5 ATP/PA/PEP; blockers present.

K_o-independent, H_2DTG-sensitive Sodium Efflux

In K-free solution (Fig. 5 E, after addition of DMSO) 100 μM H_2DTG caused a drop in ^{22}Na efflux that was 9% as large as the rise seen upon adding 10 mM K. This small further reduction of ^{22}Na efflux into nominally K-free solution sets an upper limit to the amount of pump-mediated Na/Na exchange occurring in 0 mM K fluid, and illustrates the effectiveness of the ATP-regenerating system in our dialysis fluid. Na/Na exchange is known to be enhanced by internal ADP (De Weer, 1970; Glynn and Hoffman, 1971) and inhibited by increasing the [ATP]/[ADP] ratio (Kennedy et al., 1986). The role of PEP in maintaining that ratio is demonstrated by the influence of dialysis fluid [PEP] on the size of the residual H_2DTG sensitive Na efflux in K-free seawater relative to that in 10 mM K seawater: in axons dialyzed with 0, 3, or 5 mM PEP (in addition to 5 mM ATP and 3–5 mM phosphoarginine), the residual efflux amounted to 30 ± 2% (n = 4), 15 ± 3% (n = 4), and 9 ± 3% (n = 6), respectively. Experiments with 5 mM PEP were conducted both at 0 mV and at −60 mV, with no perceptible difference. These residues represent upper limits for the contribution of Na/Na exchange to the H_2DTG-sensitive Na efflux in 10 mM K solution because, when [K]$_o$ is raised, Na/Na exchange is suppressed as Na/K exchange is activated, with the same kinetics (Garrahan and Glynn, 1967b). Thus, with 5 mM internal PEP and 10 mM [K]$_o$, when Na/K transport is 80% activated (Fig. 6 A, below) Na efflux via Na/Na exchange should amount to, at most, ~(1–0.8) × 9% = 2% of the H_2DTG-sensitive Na efflux. Whether this K_o independent, H_2DTG-sensitive ^{22}Na efflux into high Na seawater reflects electroneutral or electrogenic Na/Na exchange, or "uncoupled" Na efflux, will be discussed below.

Activition of ^{22}Na Efflux by External K

Several experiments described in this paper were carried out in Na-free seawater, where the stimulating effect of K on the Na/K pump has long been known to be much enhanced (Post et al., 1960). Thus, Baker et al. (1969) reported that efflux of ^{24}Na injected into intact *Loligo forbesi* axons was stimulated along a sigmoid curve, half maximally by 15 mM K, in 430 mM Na seawater but along a rectangular hyperbola, by 0.6 mM K, when choline chloride or sucrose replaced NaCl in the seawater. Fig. 6 A shows similar behavior in *Loligo pealei* axons internally dialyzed with K-free, 50 mM Na solution. In ~400 mM Na seawater, external K activated ^{22}Na efflux with sigmoid kinetics (Hill coefficient = 1.53 ± 0.08) and $K_{1/2}$ (K) = 3.92 ± 0.29 mM, whereas in Na-free seawater the activation by K was well fit by Michaelis-Menten kinetics, and was halfmaximal at 0.45 ± 0.06 mM K. Our internal dialysis with K-free medium facilitated examination of the low [K$_o$] range otherwise difficult to explore (Baker et al., 1969; De Weer, 1970) because of leakage of intracellular K into the pericellular restricted-diffusion space. But, even though the axons were dialyzed with K-free solutions, K channel blockers were still required since a high K permeability (especially at low membrane potentials) would cause a large K influx into the axon, leading to a depletion of K from the periaxonal space.

In the nominal absence of both external K and Na (open symbol at 0 mM K, Fig. 6 A), there was a small H_2DTG-sensitive ^{22}Na efflux whose magnitude was ~7% of that seen in 1 K, 0 Na. Since Na/Na exchange in squid axon is activated by Na$_o$ with

relatively low affinity (Baker et al., 1969), traces of Na in the nominally Na-free solution are unlikely to be responsible for this efflux. On the other hand, since 0.45 mM K stimulates ^{22}Na efflux half maximally in the absence of external Na, even a trace (22 μM) of K in the nominally K-free medium can account for the residual
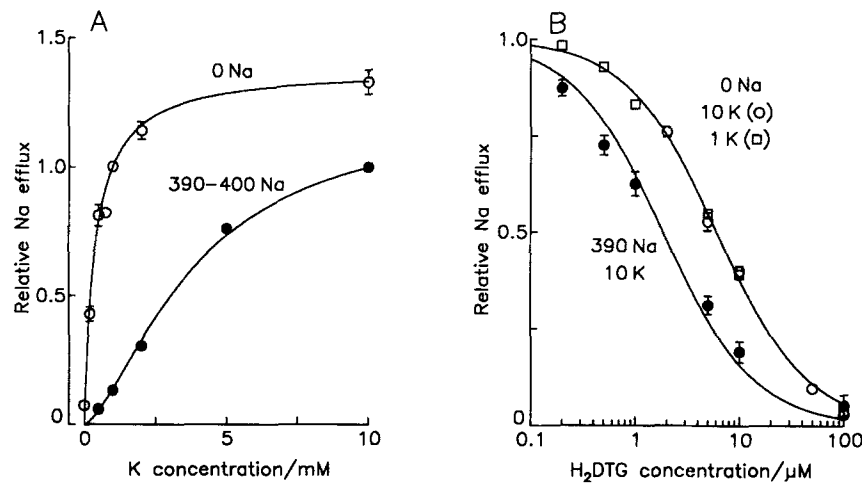

FIGURE 6. Sodium pump stimulation by K, and inhibition by H_2DTG, in unclamped axons. Points are means of three to seven determinations except for three lone points: 0.75 mM K in 0 Na (*A*), 0.2 μM H_2DTG in 1 K, 0 Na (*B*), and 100 μM H_2DTG in 10 K, 0 Na (*B*). SEM bars not shown when smaller than the symbol. (*A*) Activation by external K of ^{22}Na efflux from axons internally dialyzed with K-free solution containing 50 Na, 5 ATP/PA/PEP; blockers present. *Open symbols:* K activation in Na-free, NMG seawater, relative to activation by 1 mM K (zero level: efflux in the presence of 100 μM ouabain); the curve is a Michaelis-Menten equation through the 1 mM K reference point, with least-squares $K_{1/2}$ (K) = 0.45 ± 0.06 mM (26 points from 4 axons; V_m, −10 to −52 mV). *Filled symbols:* K activation in 390–400 mM Na seawater, relative to efflux at 10 mM K (zero level: efflux into 0 K, 400 Na medium). The curve is constrained through the 10 mM K reference point and obeys the Hill equation: flux = (maximal flux)/[1 + $(K_{1/2}/[K])^n$], with least-squares $K_{1/2}$ (K) = 3.92 ± 0.29 mM and n = 1.53 ± 0.08 (14 points from 4 axons; V_m, 0 to −23 mV). (*B*) Inhibition by H_2DTG of ^{22}Na efflux into Na-free or 390 mM Na seawater; internal solution and blockers as in *A*. All data relative to efflux in the absence of H_2DTG (zero level: with 100 μM ouabain present). The curves are Michaelis-Menten inhibition equations, constrained to go through the reference value. *Top:* Inhibition by H_2DTG in Na-free seawater, in the presence of 1 mM K (open squares; 17 points from 3 axons; V_m, −24 to −35 mV) or 10 mM K (open circles; 16 points from 5 axons; V_m, −10 to −34 mV). Least-squares K_i values were indistinguishable (6.10 ± 0.27 μM and 6.03 ± 0.25 μM, respectively); curve drawn for K_i = 6.1 μM. *Bottom:* Inhibition by H_2DTG in 10 K, 390 Na seawater; least-squares K_i = 1.76 ± 0.18 μM (25 points from 6 axons; V_m, −1 to −20 mV). Mean diameter of all 22 axons = 495 ± 10 μm.

^{22}Na efflux seen here. In fact, by atomic absorption spectrometry we found 27 μM K in that bathing solution, whence we conclude that the residual pump-mediated Na efflux in nominally K-free, Na-free seawater reflects mainly Na/K exchange. As a corollary, there is no need to invoke here any ^{22}Na efflux that is "uncoupled," i.e.,

not in exchange for K or Na (Garrahan and Glynn, 1967a; Dissing and Hoffman, 1983).

As already described (Fig. 5 E), nominally K-free, but high-Na solutions also sustained a small steroid-sensitive ^{22}Na efflux, on average ~9% the size of that into 10 K, high-Na medium. Here, "uncoupled" Na efflux is ruled out by the high external [Na] (Garrahan and Glynn, 1967a), nor can Na/K exchange be responsible, sustained by traces of K in the bathing medium, because of the much lower K affinity under high-Na conditions [$K_{1/2}$ (K) = 3.9 mM; see Fig. 6 A]. Most likely then, this residual H_2DTG-sensitive Na efflux (during dialysis with 5 ATP/PA/PEP) reflects mainly Na/Na exchange, presumably of the electrogenic, ATP-dependent sort (Lee and Blostein, 1980; Forgac and Chin, 1981; Cornelius and Skou, 1985; Goldschleger et al., 1987) rather than the electroneutral (Abercrombie and De Weer, 1978), ADP-stimulated sort (De Weer, 1970; Glynn and Hoffman, 1971; Kennedy et al., 1986) since the small H_2DTG-induced drop in Na efflux was usually accompanied by a similarly small change in membrane current (Fig. 2 A) or voltage (Fig. 5 E), but we did not attempt a detailed analysis of those small signals.

Inhibition of ^{22}Na Efflux by H_2DTG

The affinity of cardiotonic steroids for the sodium pump depends in a complicated way on extracellular [Na] and [K] (e.g., Glynn, 1957; Baker and Manil, 1968; Baker and Willis, 1972). Since our experiments required a reversible (hence low affinity) cardiotonic steroid (H_2DTG) and we were limited, for solubility and cost reasons, to a concentration of 100 μM, we determined inhibition constants in our experimental seawaters so as to know the extent of inhibition achievable under various conditions. Fig. 6 B shows kinetics of ^{22}Na efflux inhibition by H_2DTG in high-Na (10 K) or Na-free (1 or 10 K) seawater (relative to ouabain inhibitable efflux). H_2DTG affinity was indistinguishable in 1 and 10 K, Na-free seawaters (K_i = 6.10 ± 0.27 and 6.03 ± 0.25 μM, respectively) but about 3.5-fold higher (K_i = 1.76 ± 0.18 μM) in 10 K, 390 Na seawater. Consequently, at our highest [H_2DTG] (100 μM), 94% or 98% complete inhibition was attained in the absence or presence, respectively, of external Na.

Diminution of Influence of Changes in [K] on Membrane Current

Although, as shown above, the Na/K pump is the sole target for H_2DTG, the signal associated with its direct action must be separated from those caused by secondary effects of stopping the pump. Paramount among these, in voltage-clamped axons (and other cells; for review see De Weer et al., 1988a), is an inward K current due to the rise (≤1 mM, see Discussion) in periaxonal [K] expected to accompany cessation of pumped K influx. Even at V_H = −60 mV, where K conductance is only minimally activated, deliberate alternation between 9 and 10 mM [K]$_o$ (after inhibiting the pump with 10 μM ouabain) caused an average ΔI of 0.51 ± 0.08 μA cm^{-2} (12 tests on 5 axons), which was only ~90% suppressed by 1 mM DAP in the seawater. Obviously, at depolarized potentials where potassium conductance is vastly enhanced (Hodgkin and Huxley, 1952a), the current error reflecting periaxonal K accumulation upon stopping the pump would become intolerable. We attempted to minimize such K currents by including, besides 1 mM DAP in the seawater, TEA
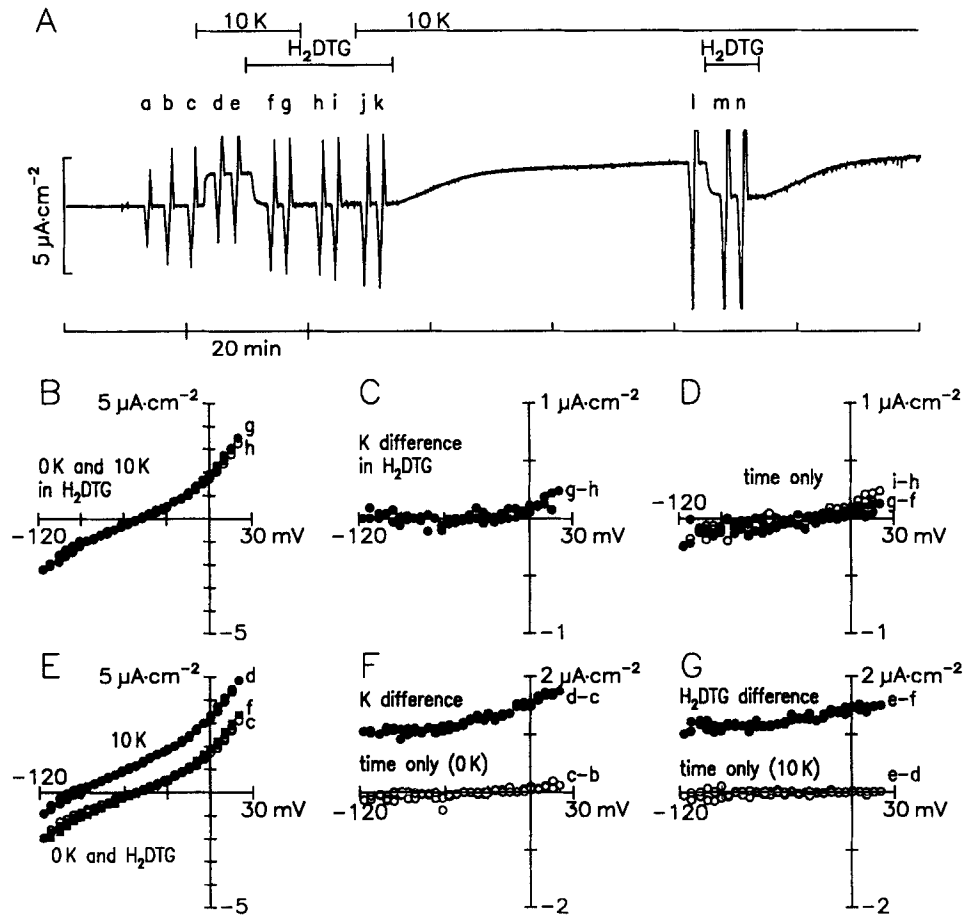
FIGURE 7. Effective K channel block by 205 mM internal TEA, 20 mM PPTEA, and 1 mM external DAP. (A) Effects of 10 mM external K and/or 100 μM H_2DTG on holding current (V_H, −30 mV). Sawtooth excursions a–n signify collection of I-V data with 1-s down-up-down voltage staircases; the first (a) spanned a narrower voltage range (−100 to +10 mV) than b–k (−120 to +20 mV) and l–n (−150 to +50 mV). (B) Superimposed membrane I-V relationships in H_2DTG when [K] was either 10 mM (g; filled circles) or 0 mM (h; open circles). (C) K difference I-V relationship in the presence of H_2DTG (g–h). (D) Differences (with time) between two pairs of successive I-V relationships in H_2DTG at 10 mM [K] (g–f; filled circles) and at 0 mM [K] (i–h; open circles). (E) I-V relationships in 0 mM [K] (c; open circles), 10 mM [K] (d; filled circles), and 10 mM [K] + H_2DTG (f; filled squares). (F) K difference I-V relationship without H_2DTG (d–c; filled circles) and "time only" difference I-V at 0 mM [K] (c–b; open circles). (G) H_2DTG difference I-V relationship (e–f; filled circles), and time only difference I-V plot in 10 mM [K] before H_2DTG (e–d; open circles). Axon diameter, 520 μm; conditions: 0 Na, 75 Ca//64 Na, 0 K, 5 ATP/PA/PEP; blockers present. For this and all subsequent figures the end-pool clamps were in use.

(~200 mM) and PPTEA (20–25 mM) in the dialysis fluid. The effectiveness of these K channel blockers was tested by examining the [K] sensitivity of membrane current after inhibiting the pump with H_2DTG.

Fig. 7 A shows shifts in holding current (at -30 mV) caused by switching external [K] between 0 and 10 mM (admittedly a far greater change than could ever occur upon stopping the pump) with and without 100 μM H_2DTG present, in Na-free seawater. (The sawtooth current excursions, labeled a through n, indicate I-V measurements which are discussed below. The increase in peak current amplitude with time, under constant conditions, e.g., between f and k, signifies a slow rise in membrane conductance.) Without H_2DTG, raising $[K]_o$ from 0 to 10 mM (Fig. 7 A between c and d) elicited a 1.36 μA cm^{-2} outward ΔI, corresponding to the $\Delta\Phi_{Na}$ seen in Fig. 5 E and, since subsequent addition of the almost saturating $[H_2DTG]$ (between e and f) caused an opposite ΔI of nearly equal magnitude (1.32 μA cm^{-2}), largely attributable to activation of the Na/K pump. In the continued presence of H_2DTG, alternation between 10 and 0 mM K no longer affected the holding current. On withdrawal of H_2DTG the holding current returned to its initial level at 10 mM K, revealing the rate of dissociation of H_2DTG from the pump (see also Figs. 9 and 10 below). A second application of H_2DTG (between l and m) caused a ΔI (1.24 μA cm^{-2}) similar to the first.

B and C show that, in the presence of H_2DTG, the steady-state membrane I-V relationships at 0 and 10 mM $[K]_o$, determined 5 min apart, were nearly identical between -120 and $+20$ mV. (Note that the ordinate is expanded fivefold in the difference record of C, and that the K_o dependent current is, if anything, outward, as if a permeability were induced by external K.) To assess the extent of the slow rise of conductance with time, which could obscure a more significant difference between the I-V curves in 10 K and 0 K seawater, its progress was monitored by making duplicate recordings in each solution. D shows the difference between a pair of I-Vs determined 2.5 min apart in 10 K (filled circles), and the difference between a subsequent pair taken 2.5 min apart in 0 K (open circles). These difference I-Vs (along with those in F and G discussed below) indicate that, under our conditions, only small conductance drifts occurred over the time needed to change the external solution and make a set of I-V measurements.

The "time only" difference curves of Fig. 7 D (as well as those in F) suggest a reversal potential for the drift current near -30 mV, the holding potential chosen for the present experiment. This explains the virtual lack of holding current drift, over a 2-h period, in A. Holding currents generally drifted outward at more positive V_H, and inward at more negative V_H. If the difference I-V curve (in H_2DTG) of C were corrected for time drift (from D), the membrane current still would not exceed ± 0.3 μA cm^{-2} between -120 and $+10$ mV. Considering that pericellular [K] changes of at most 1 mM are expected upon stopping the pump (see Discussion), our stringent 0–10 mM K test demonstrates that H_2DTG difference currents measured in the presence of these K channel blockers, will not be grossly contaminated with nonpump, K-sensitive currents.

The I-Vs in Fig. 7 E show that the outward current activated by external K, and that abolished by H_2DTG, are roughly equal not only at the holding potential but over the entire voltage range examined. This is seen more clearly when comparing

the higher-gain K difference and H_2DTG difference I-V plots of F and G, respectively, which diverge only at positive potentials, and only in the direction and by the approximate size of the K-dependent current in C. This is expected if the 0–10 mM K difference I-V of F included, besides the component due to pump activation, a small K-sensitive nonpump current (C) which is absent from (or at least much smaller in) the H_2DTG difference current (G). Though the current drift during episodes b–f was rather small (see the "time only" difference records in F and G), drift corrections, if applied to the K difference and H_2DTG difference curves, would slightly level the former (F) but steepen the latter (G). Such minor corrections might, if anything, reinforce the similarity between K-dependent current and H_2DTG-sensitive current evident from a direct comparison of F and G. The important point is that H_2DTG difference curves obtained in the presence of the K channel blockers are nearly free of contaminating nonpump (yet H_2DTG-induced) current components. Temporal drift corrections to the H_2DTG difference curves will be discussed again later.

Simultaneous Measurement of Current and Flux from a Given Membrane Area

To evaluate the stoichiometry of the Na/K pump from simultaneous determinations of pumped Na efflux and pump current, both measurements must derive from the same membrane area. This requirement, which should hold over the full potential range to be explored, is easily met in intact, essentially spherical cells, but difficult to satisfy in cannulated squid giant axons because currents can flow from the end pools whether or not they are grounded via platinum "guard" electrodes. Whereas grease seals between center and end pools are adequate barriers to bulk flow and diffusion, their electrical resistance (20–60 kΩ) is insufficient to prevent unwanted current flow when a potential difference of even a few millivolts exists across them. Moreover, any event that alters current flow across the central voltage-clamped axon membrane will cause additional current to flow from the internal wire, through the end pools and grease seals, to the center pool (see Fig. 1 C). This will cause an overestimation of TTX- and H_2DTG-induced holding current shifts. To eliminate potential differences between the end and center pools and thus prevent current flow between them, we have added a dual end-pool clamp system (see Rakowski, 1989). This end-pool clamp and grease seal arrangement was routinely tested on K-free, Na-containing axons bathed in Na-free, K-free seawater, where the simultaneously measured TTX-induced ΔI (in μA cm^{-2}) and $F\Delta\Phi_{Na}$ (in μC cm^{-2} s^{-1}) should be equal. Veratridine (250 μM) was put in the dialysis fluid to make steady-state, TTX-sensitive outward current at least as large as maximal pump current. We performed such "area match" tests, technical mishaps apart, in all experiments designed to examine pump stoichiometry. Because we found that PPTEA (and TEA at high concentrations) blocks current and flux through TTX-sensitive channels, it was necessary, after verifying the adequacy of the grease seals and end-pool clamps, to replace the internal (and, often, external) solution with that required for study of the Na/K pump, containing the channel blockers TEA and PPTEA (and DAP and TTX).

Examples of area match tests on two axons are shown in Fig. 8, A and C. Both axons were initially exposed to Na- and K-free seawater and internally dialyzed with
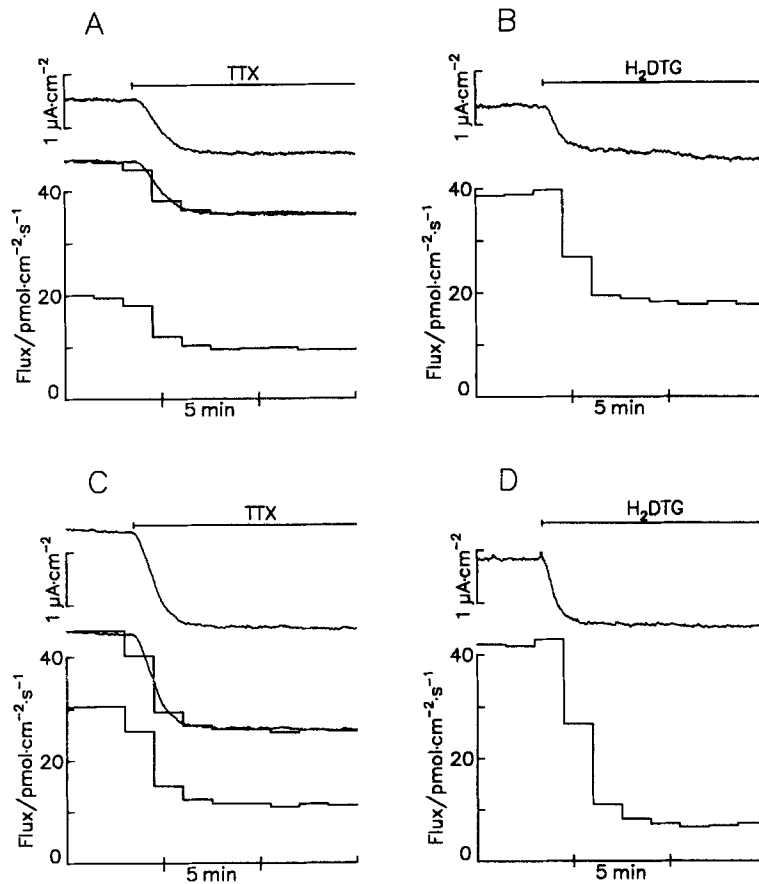
FIGURE 8. Flux/current membrane area match verification, and determination of Na/K pump stoichiometry in 390 mM Na and Na-free seawater. (A) ΔI (top; 1.03 μA cm^{-2}) and $\Delta\Phi_{Na}$ (bottom; 10.4 pmol cm^{-2}s^{-1}) elicited by 0.2 μM TTX; middle, traces superimposed to show equality; conditions: 0 K, 0 Na, 75 Ca//51 Na, 0 K, 0.25 veratridine, 5 ATP/PA/PEP; V_H, −30 mV. (B) ΔI (top; 0.76 μA cm^{-2}) and $\Delta\Phi_{Na}$ (bottom; 21.4 pmol cm^{-2} s^{-1}) caused by 10 μM H$_2$DTG; same axon as in A, but with conditions appropriate for measurement of Na/K pump activity (10 K, 390 Na, 75 Ca//52 Na, 0 K, 5 ATP/PA/PEP; blockers present), and V_H = 0 mV. Axon 11 of Table I; diameter, 450 μm. (C) As in panel A, but different axon. ΔI (top; 1.74 μA cm^{-2}) and $\Delta\Phi_{Na}$ (bottom; 18.9 pmol cm^{-2}s^{-1}) induced by TTX. Middle: data replotted to show equality. (D) H$_2$DTG-induced ΔI (top; 1.20 μA cm^{-2}) and $\Delta\Phi_{Na}$ (bottom; 39.6 pmol cm^{-2}s^{-1}) in 10 K, 0 Na, recorded after C on the same axon, but with V_H = 0 mV and internal solution as in B. Axon 7 of Table I; diameter, 430 μm.

K-free solution containing veratridine and ~50 mM Na. Current and flux scales have been adjusted to allow direct comparison and the data have been replotted and superimposed to demonstrate equality. TTX-sensitive flux and current are indeed approximately equal: their ratio was 0.98 in A and 1.05 in C. In 58 tests on 48 axons, this $F\Delta\Phi_{Na}/\Delta I$ ratio averaged 0.996 ± 0.015. Such correspondence was not generally found when the end pools were either simply grounded, or filled with an

insulator such as mineral oil. This problem affected our initial estimates of Na/K pump stoichiometry (Rakowski and De Weer, 1982b). The ratio's coefficient of variation (12%) was about that expected from the estimated errors in individual flux and current measurements (see Methods). The ratio showed no systematic dependence on voltage: 0.950 ± 0.011 (SEM, $n = 5$) at -60 mV, 0.974 ± 0.012 ($n = 8$) at -40 mV, 1.042 ± 0.048 ($n = 10$) at -30 mV, 0.934 ± 0.021 ($n = 16$) at -20 mV, 1.047 ± 0.033 ($n = 17$) at 0 mV, and 1.028 ± 0.001 ($n = 2$) at $+20$ mV. On the other hand, there was a significant ($P < 0.02$) correlation between the ratio of TTX-induced flux and current changes, and that of H_2DTG-induced changes: for 21 pairs of determinations on 12 axons (Table I, below) the correlation coefficient was 0.54. This is expected since systematic errors in, for example, calibration of membrane current or of isotope specific activity, should affect both ratios similarly.

Review of Control Experiments

The data thus far demonstrate that (a) the specific activity of the intracellular radio-tracer can be precisely controlled (Fig. 8 A and C) and relatively stable conditions maintained for appreciable times (Fig. 7); (b) pump-mediated Na/Na exchange under our conditions of forward pumping is at most ~2% of the H_2DTG-sensitive Na efflux (Fig. 5 E); (c) H_2DTG has as sole action arrest of the Na/K pump (Fig. 5) without direct effect on any other source of ionic current (Figs. 2–4); (d) alterations in membrane current, caused by K reaccumulation in the periaxonal space upon stopping the pump with H_2DTG, have been minimized with K channel blockers (Fig. 7); (e) membrane current and flux derive from the same surface area when the end-pool clamp system is used (Fig. 8, A and C); and (f) the membrane conductance has been reduced sufficiently (to typically <100 μS cm^{-2} between -80 and $+20$ mV) to permit large and prolonged excursions in membrane potential without axon deterioration (Fig. 7). These controls demonstrate the reliability and accuracy of the simultaneous measurements of Na/K pump-mediated current and flux required for the following examination of the stoichiometry of Na/K exchange at near-saturating $[Na]_i$, $[K]_o$, and $[ATP]$, at various membrane potentials, and in Na-free and high-Na seawaters.

Stoichiometry of the Na/K Pump Determined from Simultaneous Measurements of Pump Current and ^{22}Na Efflux

Representative results from two experiments to determine the stoichiometry of the sodium pump at different external [Na] are shown in Fig. 8, B and D. After verification of the area match, the axon of Fig. 8 A was bathed in 10 K, 390 Na, dialyzed with K-free solution containing 52 mM ^{22}Na and 5 mM ATP/PA/PEP, allowed to reach a new steady state (90 min), and exposed (B) to 10 μM H_2DTG. The H_2DTG-induced $\Delta\Phi_{Na}$ was 2.7 times larger than ΔI, about as expected for a 3 Na/2 K pump coupling ratio. We have already reviewed the evidence that, under our conditions, at most 2% of ^{22}Na efflux into 10 K, 390 Na represents Na/Na exchange that could falsify stoichiometry calculations. Nevertheless, we also assessed pump stoichiometry in Na-free seawater, where Na/Na exchange cannot take place (Fig. 8 D). After verification of the area match (C), 100 μM H_2DTG-sensitive current and flux were measured (D). Here the H_2DTG-induced $\Delta\Phi_{Na}$ was 3.2 times larger than ΔI.

Voltage Dependence of Na/K Pump Current and Flux

Since our technique allows the simultaneous measurement of H_2DTG-sensitive current and flux across a given membrane area at various holding potentials, we could investigate the voltage dependence not only of pump current or flux, but of the pump stoichiometry as well. This is, however, a demanding procedure. The ideal protocol includes determination of TTX-sensitive current and flux for area match verification, followed by replacement of internal and external solutions, and then by three reversible exposures to H_2DTG, two of them at the reference holding potential bracketing the third at the test potential, over 5 h of continuous internal dialysis
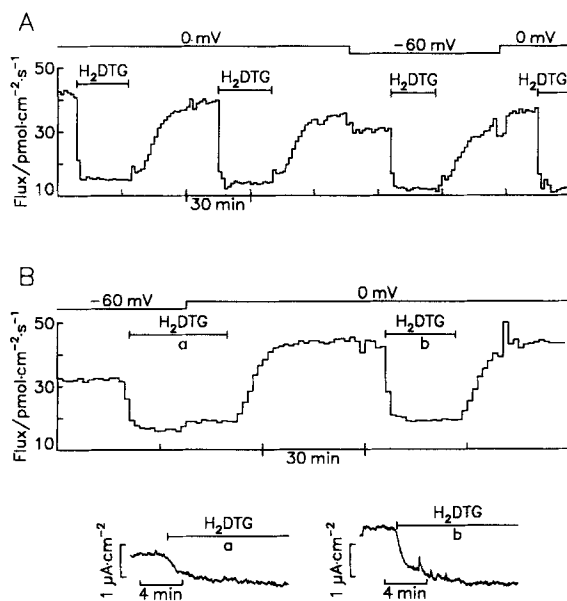

FIGURE 9. Voltage dependence of H_2DTG-sensitive current and ^{22}Na efflux in 390 mM Na seawater. (A) Effect of hyperpolarization (0 to −60 mV) on ^{22}Na efflux and H_2DTG-induced $\Delta\Phi_{Na}$ from an internally dialyzed axon bathed in 390 mM Na. Overall, ^{22}Na efflux at 0 mV declined 1.7 pmol cm^{-2}s^{-1} per h, from 42.5 to 36.2 pmol cm^{-2}s^{-1}. Four successive applications of 100 μM H_2DTG, separated by three recovery periods, caused $\Delta\Phi_{Na}$s of 27.3, 25.2, 18.6, and 24.3 pmol cm^{-2}s^{-1} at 0, 0, −60, and 0 mV, respectively. Area match test was not performed. Axon 9 of Table I; diameter, 450 μm; conditions: 10 K, 390 Na, 75 Ca//52 Na,

0 K, 5 ATP/PA/PEP; blockers present. (B) Effect of depolarization (−60 to 0 mV) on pump-mediated ^{22}Na efflux and current. 10 μM H_2DTG caused a $\Delta\Phi_{Na}$ and ΔI of 15.8 pmol cm^{-2}s^{-1} and 0.45 μA cm^{-2}, respectively at −60 mV (episode a) but 24.8 pmol cm^{-2}s^{-1} and 0.94 μA cm^{-2} at 0 mV (episode b). The TTX-sensitive $F\Delta\Phi_{Na}/\Delta I$ ratio determined on this axon (not shown) was 1.01. Axon 10 of Table I; diameter, 480 μm; conditions: 10 K, 390 Na, 75 Ca//52 Na, 0 K, 5 ATP/PA/PEP; blockers present.

under voltage clamp. It was seldom possible to complete such a fully bracketed set of measurements. In Fig. 9 A we show bracketed flux measurements on an axon in which area match was not checked, in Fig. 9 B, results from an experiment in which an area match test was performed before H_2DTG-induced $\Delta\Phi_{Na}$ and ΔI were measured at −60 mV and 0 mV, and in Fig. 10 we show a complete bracketed protocol to measure both H_2DTG induced ΔI and $\Delta\Phi_{Na}$ at 0, −80, and again 0 mV.

Fig. 9 A is a continuous record of ^{22}Na efflux over a 4-h period during which H_2DTG was applied four times, first twice at 0 mV, then at −60 mV, and again at 0

mV. Although H_2DTG-induced $\Delta\Phi_{Na}$ at 0 mV declines with time (~4%/h), hyperpolarization to -60 mV reversibly suppresses $\Delta\Phi_{Na}$ by ~25%. The voltage sensitivity of both H_2DTG sensitive ^{22}Na efflux and current is shown in Fig. 9 *B*. Since in this experiment the smaller response (*a*) preceded the larger (*b*), a slow run-down with time as seen in *A* cannot be responsible for the apparent voltage dependence. A complete experiment is shown in Fig. 10. In this case, however, the experiment was performed in Na-free seawater, where Na transport is energetically downhill. Hyperpolarization from 0 to -80 mV again clearly inhibited, reversibly, both H_2DTG-sensitive current and flux (by ~20%).

Table I lists results from experiments on 17 axons, that followed all or part of the protocols just described (Figs. 9 and 10): some were bracketed with respect to V_H, most included an area match test, and all were carried out with end-pool clamps in use and blockers present. They were performed in either Na-free or 390 mM Na
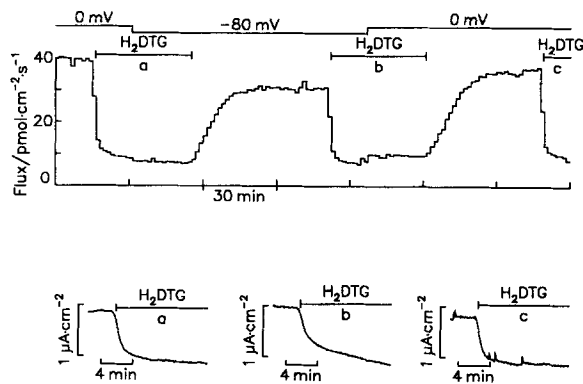

FIGURE 10. Effect of hyperpolarization (0 to -80 mV; bracketed data) on 100 μM H_2DTG-sensitive ^{22}Na efflux and current, in Na-free seawater. H_2DTG-induced $\Delta\Phi_{Na}$ and ΔI were, respectively, 29.7 pmol $cm^{-2}s^{-1}$ and 0.925 μA cm^{-2} at 0 mV (episode *a*), 23.0 pmol $cm^{-2}s^{-1}$ and 0.698 μA cm^{-2} at -80 mV (episode *b*), and 27.2 pmol $cm^{-2}s^{-1}$ and 0.848 μA cm^{-2} at 0 mV again (episode *c*). The TTX-sensitive $F\Delta\Phi_{Na}/\Delta I$ ratio (data not shown) was 0.985. Axon 3 of Table I; diameter, 660 μm; conditions 10 K, 0 Na, 75 Ca//52 Na, 0 K, 5 ATP/PA/PEP; blockers present.

seawater, and at either depolarized (generally 0 mV) or hyperpolarized (-60 to -90 mV) holding potentials. Fig. 11 graphically summarizes the average H_2DTG-induced $\Delta\Phi_{Na}$ (*A*; from columns *3* and *8* of Table I) and average H_2DTG-induced ΔI (*B*; from columns *4* and *9*) at depolarized (column 2) or hyperpolarized (column 7) potentials. Hyperpolarization between ~0 and -60 to -90 mV reduced both $\Delta\Phi_{Na}$ and ΔI, slightly in Na-free seawater (by 13 ± 5% and 14 ± 6%, respectively, over ~75 mV) and more steeply in 390 mM Na seawater (by 34 ± 5% and 39 ±8%, respectively, over 60 mV). Note that the current scale in Fig. 11 *B* is expanded threefold relative to the flux scale in *A*, so that identical slopes in the two panels mean a fixed stoichiometry of 3 Na per elementary charge transported. The average current and flux data clearly behave as if pump stoichiometry remained approximately constant at 3 Na/2 K, and hyperpolarization slowed the pump more in

TABLE I

Voltage Dependence of H₂DTG-sensitive Na Efflux and Current in Na-free and 390 mM Na External Solution

Axon	TTX $\frac{F\Delta\Phi_{Na}*}{\Delta I}$	V_H mv	$\Delta\Phi_{Na}$ pmol $\overline{cm^2 s}$	ΔI µA $\overline{cm^2}$	$\frac{F\Delta\Phi_{Na}}{\Delta I}$ Uncorr	Corr	V_H mv	$\Delta\Phi_{Na}$ pmol $\overline{cm^2 s}$	ΔI µA $\overline{cm^2}$	$\frac{F\Delta\Phi_{Na}}{\Delta I}$ Uncorr	Corr
								H₂DTG			
					[Na]ₒ — 0 mM						
1	—	−30	33.6	1.16	2.80	—	−90	28.5	0.93	2.96	(2.96)
2	1.06	0	33.6	1.07	3.03	2.86	−80‡	29.5	1.05	2.71	2.56
		0	28.1	1.02	2.66	2.51					
3	0.99	0	29.7	0.93	3.09	3.14	−80‡	23.0	0.70	3.18	3.23
		0	27.2	0.85	3.10	3.15					
4	0.88	0	29.5	—	—	—	−80	29.0	0.92	3.04	3.45
5	0.89	0	30.0	1.14	2.54	2.87	−60	31.1	1.06	2.83	3.20
6	0.88	0	31.8	1.16	2.64	3.01	−60	25.3	0.94	2.59	2.96
7	1.05	0	39.6	1.20	3.18	3.03					
		0	34.8	1.19	2.83	2.55					
Mean		−3	31.8	1.08	2.87	2.86	−75	27.8	0.93	2.88	3.05
SEM		3	1.2	0.04	0.08	0.09	5	1.2	0.05	0.09	0.13
n		10	10	9	8§	8	6	6	6	6	6ᴵ
					[Na]ₒ — 390 mM						
8	1.06	0	27.5	0.95	2.80	2.64					
9	—	0	27.3	0.87	2.81	(2.81)					
		0	25.2	—	—	—	−60‡	18.6	—	—	—
		0	24.3	0.83	3.04	(3.04)					
10	1.01	0	24.8	0.94	2.54	2.49	−60	15.8	0.45	3.41	3.38
11	0.98	0	21.4	0.76	2.73	2.79					
12	1.05	0	21.7	0.82	2.55	2.43	−60	12.3	—	—	—
13	1.40	0	23.2	—	—	—	−60	14.5	0.42	3.36	2.40
14	0.82	0	24.7	0.99	2.41	2.95	−60	17.9	0.69	2.52	3.08
15	—	0	—	0.96	—	—	−60	18.3	0.61	2.88	(2.88)
16	—	0	24.3	—	—	—					
17	—	0	26.8	—	—	—					
Mean	1.00	0	24.7	0.89	2.69	2.72	−60	16.2	0.54	3.02	2.91
SEM	0.04	—	0.6	0.03	0.08	0.09	—	1.0	0.06	0.22	0.21
n	12	12	11	8	7	7¶	6	6	4	4	4
Column	1**	2	3	4	5**	6**	7	8	9	10**	11ᴵ,**

The corrected data in columns 6 and 11 were obtained by dividing column 5 and 10, respectively, by the area match ratio given in column 1.

*Determined at either −20 or −30 mV.

‡Bracketed result.

§Excluding the point at −30 mV.

ᴵIncluding one uncorrected point enclosed in parentheses.

¶Including two uncorrected points enclosed in parentheses.

**Mean and SEM calculated for lognormal distribution. See Methods.

high-Na than in Na-free seawater. The slight difference in slope, in 390 mM Na seawater, between A and B is not statistically significant for these averaged data ($P > 0.5$).

Many entries in Table I, however, are paired measurements of ΔI and $\Delta\Phi_{Na}$ on the same axon, affording a more accurate estimation of pump stoichiometry and a more rigorous test of its voltage sensitivity. For most of these pairs, an area match test was

performed as well, allowing correction for mismatch if desired. Table I lists both uncorrected (columns *5* and *10*) and corrected (columns *6* and *11*) H_2DTG-induced $F\Delta\Phi_{Na}/\Delta I$ ratios. We will describe here only the area match-corrected data, since the correction should eliminate correlated systematic errors. It is obvious from the table, however, that the correction is small and does not affect our conclusions. The ratio of Na ions translocated per electronic charge moved was 2.86 ± 0.09 ($n = 8$) at 0 mV and 3.05 ± 0.13 ($n = 6$) at -60 to -90 mV in axons bathed in Na-free seawater, and 2.72 ± 0.09 ($n = 7$) at 0 mV and 2.91 ± 0.21 ($n = 4$) at -60 mV in axons bathed in high-Na seawater. Only one of these ratios, at 0 mV in high-Na seawater, is significantly ($P \sim 0.02$) different from 3. The most likely explanation (see Discus-
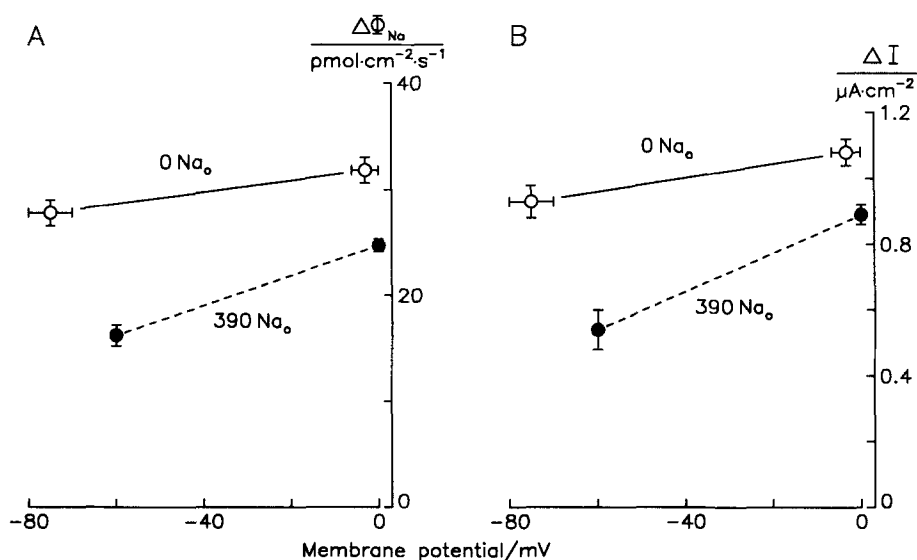
FIGURE 11. Summary of H_2DTG-sensitive currents and fluxes at low and high membrane potential in either 390 or 0 mM Na seawater. (*A*) Mean values $\pm$ SEM of H_2DTG-induced $\Delta\Phi_{Na}$, from columns *3* and *8* in Table I. (*B*) Mean values $\pm$ SEM of H_2DTG-induced ΔI, from columns *4* and *9* in Table I. The ordinate in *A* is three times that in *B* when scaled by Faraday's number, so that lines that have the same slope in *A* and *B* imply a fixed stoichiometry of 3 Na/2 K, independent of membrane potential.

sion) is that ΔI at 0 mV still includes a very small component of H_2DTG-induced change in nonpump current. Leaving that possibility open for now we calculate, from all paired flux and current measurements listed in Table I, an average $F\Delta\Phi_{Na}/\Delta I$ ratio of 2.87 ± 0.07 ($n = 25$), which is not significantly ($P \sim 0.1$) different from 3. Note also that its coefficient of variation ($\sim 12\%$) is not much larger than would be expected for a ratio of two measurements each subject to small ($\sim 5\%$) errors.

Voltage Dependence of Na/K Pump Current Determined from Steady-State Difference I-V Relationships

While repeated applications of H_2DTG at chosen holding potentials (Figs. 9–11, Table I) demonstrated that pump current and Na efflux are affected in parallel by

voltage, more detailed information on the form of that dependence over a wider voltage range was gained from Na/K pump I-V relationships computed as the difference between steady-state membrane I-Vs obtained immediately before, and during, application of H_2DTG. This method presupposes validation of the assumption that the steroid-induced change in membrane current is equal to the Na/K pump current. As described above (Fig. 7), contamination of our H_2DTG difference I-Vs with K-dependent nonpump currents that result indirectly from pump inhibition was minimized through the use of K-free internal dialysis and of the K channel
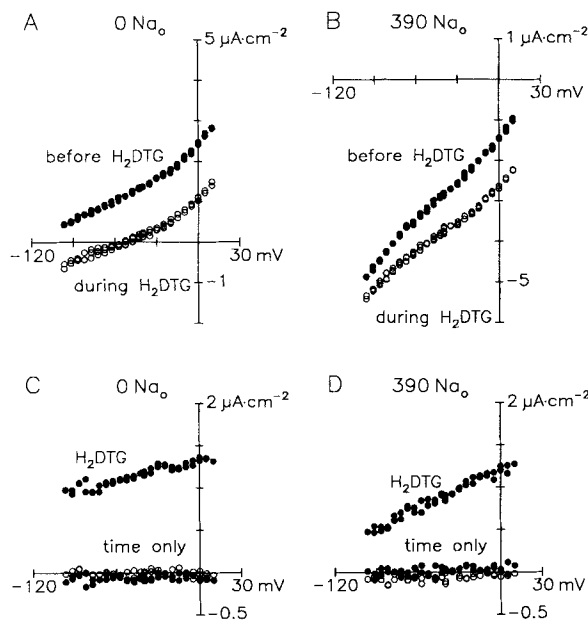
FIGURE 12. Na/K pump current-voltage relationships in Na-free and 390 mM Na solutions, obtained as difference I-V relationships from a single axon, using down-up-down staircases. (A) Four I-Vs recorded in Na-free solution, 4 min apart: two before (filled symbols) and two during (open symbols) exposure to 100 μM H_2DTG (temporal drift was sufficiently small to make each pair of records overlap). Axon diameter, 570 μm; V_H, -40 mV; conditions: 0 Na, 10 K, 75 Ca//64 Na, 0 K, 5 ATP/PA/ PEP; blockers present. (B) As in A but in 390 mM Na seawater. (C) H_2DTG difference I-V relationship in Na-free seawater from the records, 4 min apart, before and during exposure to H_2DTG, from A. The differences between the pairs of successive I-Vs, subtracted as in Fig. 7, before and during H_2DTG application are also shown and labeled "time only." (D) As in C but obtained from B.

blockers DAP, TEA, and PPTEA. Another source of error, slow drifts of membrane conductance, was monitored by repeating the I-V measurements at suitably spaced intervals, both before and after H_2DTG addition, and then examining the resulting "time only" difference I-V records.

In the experiment of Fig. 12, steady-state I-Vs were obtained from a single axon in the absence and presence of 100 μM H_2DTG, in both Na-free and 390 Na seawater. A shows membrane I-Vs recorded, twice each, with and without H_2DTG pres-

ent, while the axon was bathed in Na-free solution; the resulting difference I-Vs are plotted in C. After removing H_2DTG and switching to 390 mM external Na, a similar protocol was carried out (B; difference I-Vs plotted in D). Note the more negative holding current in B, an expected result of replacing external NMG with more permeant Na. The Na_o-dependent inward current in this axon was larger than usual, signifying a somewhat higher (TTX resistant) sodium permeability. In addition to the H_2DTG difference I-V obtained in Na-free seawater, C shows the differences between pairs of successive I-Vs taken 4 min apart in the absence (filled symbols) and in the presence (open symbols) of H_2DTG. These very small "time only" differences confirm that the membrane conductance was quite stable during the time (also 4 min) required to obtain the I-V pair before and after addition of H_2DTG. This justifies the conclusion that the H_2DTG difference I-V in C closely reflects the voltage sensitivity of the Na/K pump over this range of potentials in Na-free seawater. An entirely analogous conclusion is reached for D, where the H_2DTG difference I-V reveals the pump's voltage dependence in high Na seawater. The difference I-V in high Na is clearly steeper than that obtained on the same axon in Na-free solution, which confirms and extends the conclusion already drawn from the flux/current data summarized in Fig. 11.

The down-up-down voltage staircase technique, by sampling membrane current twice at each voltage, also provides a means of ascertaining that the membrane I-V records obtained before and during H_2DTG application are true steady-state measurements. None of the records in Fig. 12 show significant hysteresis, including the H_2DTG difference I-V curves of C and D, which represent Na/K pump current characteristics.

Demonstration of Na/K Pump Voltage Dependence Using Both the Flux Method and the I-V Method in the Same Axon

A protocol was designed to compare the voltage dependence of H_2DTG-sensitive [22]Na efflux with the pump I-V relationship in a single axon. Figs. 13 and 14 show two such experiments. In Fig. 13 the axon was bathed in 10 K, 390 Na seawater throughout. Efflux of [22]Na was measured at five membrane voltages (10.5 min each) before and after addition of 100 μM H_2DTG. The repeated flux measurements at 0 mV, before and after steroid addition, show that there was little drift with time of either H_2DTG-sensitive or insensitive [22]Na efflux. The ΔI caused (at 0 mV) by H_2DTG addition is shown in the inset of A. Since the voltage sensitivity of [22]Na efflux in the presence of H_2DTG is slight, the obvious voltage dependence of total [22]Na efflux without H_2DTG reflects mainly that of pump-mediated efflux. Steady-state I-Vs (B) were determined just before, and 14 min after, H_2DTG application; their difference, an estimate of the pump I-V relationship, is shown in C (filled symbols). Furthermore, there was very little temporal drift in background conductance, as shown by the small difference between a pair of I-Vs taken 6 min apart in H_2DTG ("time only" data in C).

Fig. 14 is from a second axon subjected to the protocol just described, except that it was bathed in 10 K, 0 Na, and no "time only" pair of I-Vs was taken to assess temporal drift in the background conductance. However, the holding current at 0 mV in 10 K was very stable: 4.17 μA cm^{-2} shortly after switching from 0 to 10 K,

and 4.08 μA cm^{-2} shortly before addition of H$_2$DTG, a time span of ~80 min; drift correction, therefore, would probably have had very little effect on the shape of the H$_2$DTG difference curve in C.

The experiments of Figs. 13 and 14 thus confirm that the Na/K pump rate is indeed more sensitive to voltage in 390 mM Na seawater than in Na-free seawater. Five experiments of this kind were carried out, three in Na-free and two in high-Na seawater. In Fig. 15 A we plot relative H$_2$DTG-sensitive ^{22}Na efflux at potentials between −60 and +20 mV, from both Na-free (open circles) and high-Na (filled

FIGURE 13. Voltage dependence of H$_2$DTG-sensitive current and ^{22}Na efflux in a voltage-clamped axon bathed in 390 mM Na seawater. (A) ^{22}Na efflux into 390 mM Na seawater, as affected by 10 mM K, various membrane potentials, and 100 μM H$_2$DTG. (Inset) H$_2$DTG-induced ΔI (1.27 μA cm^{-2}). Axon diameter, 470 μm; conditions: 10 K, 390 Na, 75 Ca//51 Na, 5 ATP/PA/PEP; blockers present. (B) Membrane I-V relationships determined 20 min apart from V_H = 0 mV, before (filled symbols) and during (open symbols) exposure to 100 μM H$_2$DTG; there is some hysteresis. (C) H$_2$DTG difference I-V (filled circles) from B. A third I-V was obtained during H$_2$DTG exposure (not shown); the difference between these successive I-Vs in H$_2$DTG 6 min apart, subtracted as in Fig. 7, is also plotted here and labeled "time only." Note that the recording of the first I-V is reflected as a dip in the ^{22}Na efflux record of A, just before application of H$_2$DTG, but that the corresponding dip in Fig. 14 A is much smaller.
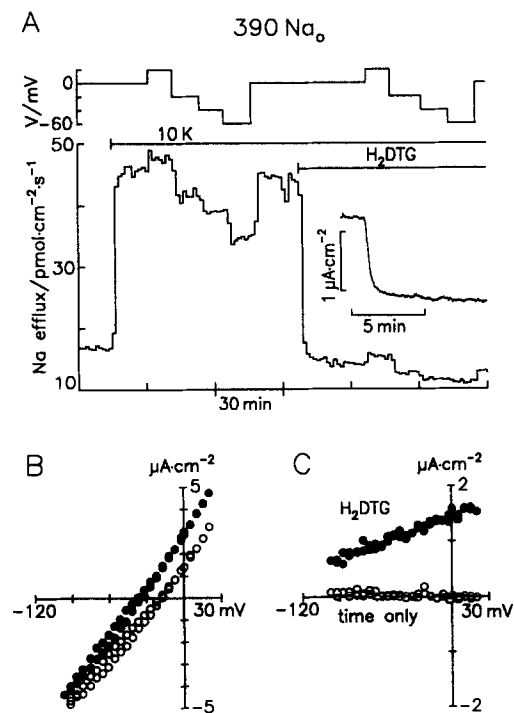
circles) experiments. Hyperpolarization inhibits ^{22}Na efflux more strongly in high-Na seawater (by 30 ± 3% between 0 and −60 mV; compare with 34 ± 5% in Fig. 11 A) than in Na-free seawater (by 11 ± 2% between 0 and −60 mV; compare with 13 ± 5% over ~75 mV in Fig. 11 A).

As for the voltage dependence of H$_2$DTG-sensitive current, we have already mentioned the unavoidable problem of temporal drift in background conductance when taking the difference between I-Vs obtained before and after application of H$_2$DTG; the examples shown so far (Figs. 4 B, 7 G, 12 C and D, 13 C, and 14 C) were chosen

for their relative lack of temporal drift. Properly spaced "time only" records, however, can be used to correct difference records for temporal drift, provided the drift is not large. This was done here by fitting arbitrary second- or third-degree polynomials to the "time only" difference records, and subtracting the resulting curves, appropriately scaled and weighted, from the "raw" H_2DTG difference data. Excluding experiments in which temporal current drift (at -60 mV) exceeded ~25% of the H_2DTG difference current (at 0 mV), we show in Fig. 15 B the average of all our H_2DTG difference I-Vs, scaled to the current amplitude at 0 mV, obtained in either Na-free (10 axons, open circles) or high-Na (6 axons, closed circles) seawater. Again, hyperpolarization inhibited pump current more steeply in high-Na seawater (by
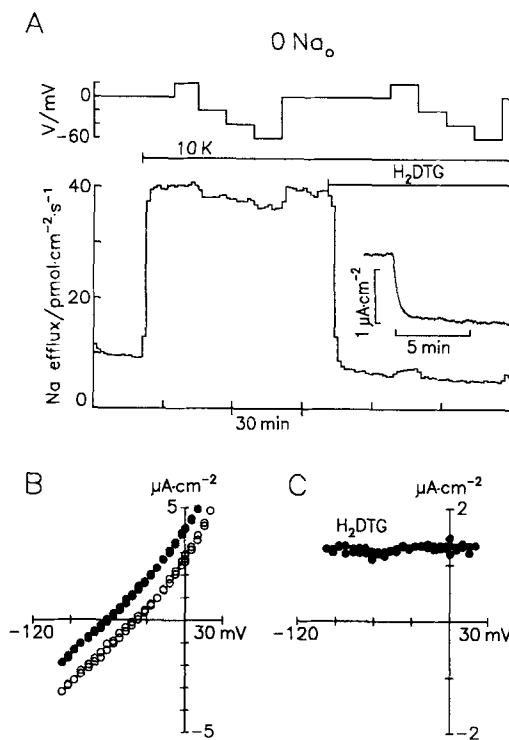
FIGURE 14. Voltage dependence of H_2DTG-sensitive current and ^{22}Na efflux into Na-free seawater. (*A*) Same protocol as in Fig. 13 *A* except that the axon was bathed in Na-free seawater. H_2DTG-induced ΔI (*inset*) 1.24 μA cm^{-2}. (*B*) Membrane I-Vs determined 20 min apart from $V_H =$ 0 mV before (filled symbols) and during (open symbols) exposure to 100 μM H_2DTG. (*C*) H_2DTG difference I-V obtained from *B*. Axon diameter, 480 μm; conditions: 10 K, 0 Na, 75 Ca//51 Na, 5 ATP/PA/PEP; blockers present.

$42 \pm 4\%$ between 0 and -60 mV; compare with $39 \pm 8\%$ in Fig. 11 *B*) than in Na-free seawater (by $28 \pm 3\%$ between 0 and -60 mV; compare with $14 \pm 6\%$ over ~75 mV in Fig. 11 *B*). Here, as in Fig. 11 (where the data were obtained as simultaneous transients at a chosen voltage) the tendency is for the H_2DTG-sensitive current to appear slightly more voltage dependent than ^{22}Na efflux at depolarized potentials. We believe that this reflects a slight (5–10%) residual contamination of our H_2DTG-sensitive current measurements with changes in nonpump currents resulting from periaxonal K reaccumulation upon stopping the pump, despite the presence of high concentrations of K channel blockers.

DISCUSSION

An electrogenic pump must be voltage dependent for thermodynamic and kinetic reasons. One argument is as follows: given that the Na/K pump produces a finite current at, say, 0 mV and that we may write a valid expression for the equilibrium potential $(E_{Na/K})$ of a pump of given Na/K/ATP stoichiometry the current it generates, $I_{pump} = (V_i - V_o - E_{Na/K})/R_{Na/K}$ (see Fig. 1 C), must vary with membrane voltage $(V_i - V_o)$ to vanish at the equilibrium potential. The voltage dependence of pump current need not be strong or uniform, however, and may be absent over a wide potential range, or even negative (Hansen et al., 1981; De Weer, 1984, 1986;
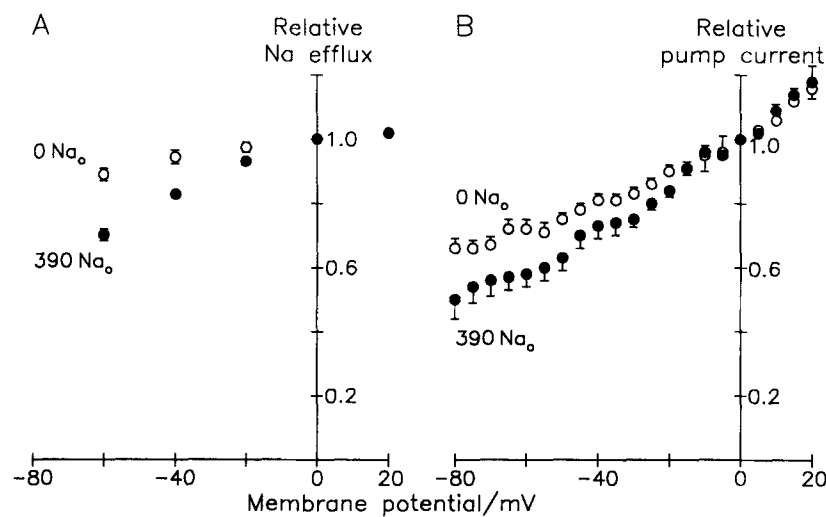
FIGURE 15.   Mean values of H_2DTG-sensitive ^{22}Na efflux and membrane current as functions of membrane potential in 390 mM Na and Na-free solution, relative to their respective values at 0 mV. (A) Relative H_2DTG-sensitive ^{22}Na efflux from two experiments in 390 mM Na solution (filled symbols) and three experiments in Na-free solution (open symbols) similar to, and including, those of Figs. 13 and 14. (B) Relative H_2DTG-sensitive membrane current from six axons in 390 mM Na (filled symbols) and 10 axons in Na-free solution (open symbols), determined by subtraction of I-Vs as in Fig. 12 and corrected for drift with time as described in the text. Voltages rounded to the nearest 5 mV. Standard error bars are shown when larger than the symbol.

Läuger and Apell, 1986). Nevertheless, on the assumption of linearity, a minimum estimate for the pump slope conductance (in that case, $1/R_{Na/K}$; Fig. 1 C) can be obtained. Assuming an Na/K pump whose current magnitude is 1.5 μA cm^{-2} at 0 mV and whose reversal potential (at nonzero values of internal K and external Na) is -200 to -240 mV (De Weer et al., 1988a) we obtain 6–8 μS cm^{-2} for the average slope conductance.

Attempts to establish voltage dependence of the Na/K pump have been reviewed (De Weer et al., 1988a). The early findings were largely inconclusive because either the voltage range explored was narrow, the measured currents and fluxes included

unknown contributions from sources other than the Na/K pump, or the experiments were done in Na-free solutions where the voltage dependence is slight. Recent experiments have been more successful, in particular those on isolated cardiac myocytes (Gadsby et al., 1985b; Hasuo and Koketsu, 1985; Glitsch and Krahn, 1986; Gadsby and Nakao, 1987; Bahinski et al., 1988) and on *Xenopus* oocytes (Béhé and Turin, 1984; Turin, 1984; Lafaire and Schwarz, 1986; Eisner et al., 1987; Rakowski and Paxson, 1988) where intrinsic ionic conductances are low or can be suppressed with channel-blocking drugs. From these investigations the Na/K pump current appears to be a saturating function of membrane potential at high external [Na], but only weakly voltage dependent in Na-free solutions, which is in general agreement with our results. We are able, however, to measure simultaneously both current and Na efflux generated by the pump, as well as their voltage dependence, allowing determination of pump stoichiometry and its dependence, or lack of it, on membrane potential. One previous attempt to make such measurements in *Xenopus* oocytes (Eisner et al., 1987) was inconclusive, as the contribution of Na/Na exchange to the measured flux remained unknown, leading the authors to suggest an increasing proportion of Na/Na exchange as the net forward Na/K pump rate is slowed by hyperpolarization.

One aim of this paper has been to validate our H_2DTG-sensitive current and flux measurements as accurate reflections of the Na/K exchange ("forward") mode of operation of the sodium pump, by systematic reduction of errors in both types of measurement. We have demonstrated that (a) the cardiotonic steroid H_2DTG specifically stops the Na/K pump and has no effect (nor does its vehicle DMSO) on the passive conductance of any ion present (Figs. 2–5); (b) the membrane conductance has been lowered sufficiently so that the holding currents required to clamp the axon membrane at widely varying potentials remain small (typically $< 10 \ \mu A \ cm^{-2}$) and relatively stable for hours, indicating little membrane deterioration and/or intracellular pH change, and allowing reliable measurements of H_2DTG-sensitive currents; (c) the membrane area from which the measured flux and current derive is the same (and remains so at various voltages) when ancillary voltage-clamp circuits (Fig. 1 B) are used to suppress stray currents from the cannulated axon ends; (d) the Na/Na exchanging mode of pump operation contributes only ~2% to the pumped ^{22}Na efflux measured under our conditions of near saturation with respect to ATP, Na_i, and K_o, and membrane potentials near resting; and (e) the use of 1 mM DAP externally and 200 mM TEA and 20–25 mM PPTEA internally renders the axon membrane nearly insensitive to changes in external [K] (in the presence of H_2DTG to block the pump), thus minimizing H_2DTG-induced nonpump currents caused by the rise in [K] in the periaxonal restricted-diffusion space that occurs on stopping the pump. The third point has been discussed by Rakowski (1989); the last two merit elaboration.

The only likely modes of sodium pump operation under the conditions of our measurements of stoichiometry and voltage dependence are (a) the forward Na/K exchanging, and (b) the electroneutral (Abercrombie and De Weer, 1978) and (c) electrogenic (Cornelius and Skou, 1985; Goldschleger et al., 1987) Na/Na exchanging modes. The backward mode is unlikely on energetic grounds, the K/K exchanging mode is suppressed by Na_i (Simons, 1974), and the "uncoupled" mode requires

the absence of both Na_o and K_o (Garrahan and Glynn, 1967a). We have argued above on kinetic grounds that only ~2% of our measured [22]Na efflux represents Na/Na exchange. Admittedly, the kinetics on which the argument was based were not determined over a wide range of membrane potentials; the reciprocal activation of Na/K exchange and inhibition of Na/Na exchange by K_o (Garrahan and Glynn, 1967b) was examined in fresh red blood cells whose membrane potentials are near 0 mV (Hoffman and Laris, 1974), while our K_o activation curve (Fig. 6 A) was obtained between 0 and −23 mV. However, an additional reason for believing that Na/Na exchange contributes negligibly to our flux measurements is the finding (Table I) that the H_2DTG-sensitive $F\Delta\Phi_{Na}/\Delta I$ ratios are indistinguishable in high-Na and in Na-free seawater (where, obviously, no Na/Na exchange can take place), both at −60 to −90 mV (2.91 ± 0.21 and 3.05 ± 0.13, respectively; $P > 0.6$) and at 0 mV (2.86 ± 0.09 and 2.72 ± 0.09, respectively; $P > 0.4$). Moreover, the flux/current ratio generally deviates from 3 (signifying a 3 Na/2 K stoichiometry) in the direction of, if anything, excess current, not Na efflux. The nearest plausible (as opposed to 8/5, 7/4, or 5/3) alternative stoichiometry, namely 2 Na/1 K (predicting $F\Delta\Phi_{Na}/\Delta I = 2$) is highly unlikely since it would require that, of our total H_2DTG-sensitive [22]Na efflux into 10 K high-Na seawater (~25 pmol $cm^{-2}s^{-1}$ on the average; Table I) almost 6 pmol $cm^{-2}s^{-1}$ be Na/Na exchange. This would predict, in K-free seawater (where the 80% suppression by K_o is lifted) an H_2DTG-sensitive [22]Na efflux (i.e., Na/Na exchange) of ~30 pmol $cm^{-2}s^{-1}$, which is more than ten times the 2–3 pmol $cm^{-2}s^{-1}$ we usually found.

Regarding cardiotonic steroid sensitive current measurements, the most obvious and most difficult to control (for review see De Weer et al., 1988a) source of error is the inward increment of (electrodiffusive) K current caused by the rise in periaxonal [K] which accompanies stopping of the forward Na/K pump. Frankenhaeuser and Hodgkin (1956) concluded that the periaxonal space of *Loligo* behaved as if it were limited by an outer barrier of (nonspecific) permeability $P = 6 \times 10^{-5}$ cm s^{-1}. A bath-to-cytoplasm active K transport rate of, say, 30 pmol $cm^{-2}s^{-1}$ will reversibly establish across that barrier a steady-state K concentration drop of 0.5 mM. Our tests with deliberate switches between 9 and 10 mM $[K]_o$, in the presence of ouabain but without K channel blockers, caused ΔIs of ~±0.5 μA cm^{-2} at −60 mV. Assuming constant-field behavior (Goldman, 1943) the membrane potassium permeability under those conditions is estimated to be:

$$P_K = \frac{\Delta I}{\Delta[K]} \frac{RT}{V_m F^2} (1 - e^{V_m F/RT}) \simeq 2 \cdot 10^{-6} \text{ cm s}^{-1}. \tag{2}$$

Converting this to K (chord) conductance (g_K) at −60 mV yields, for a 300 mM K_i axon bathed in 10 mM K seawater,

$$g_K = \frac{P_K}{V_m - E_K} \frac{V_m F^2}{RT} \frac{[K]_o - [K]_i e^{V_m F/RT}}{1 - e^{V_m F/RT}} \simeq 350 \ \mu S \text{ cm}^{-2}, \tag{3}$$

within the range (120–390 μS cm^{-2}) of estimates by Hodgkin and Huxley (1952b) for resting squid axons. The problem of such nonpump currents is most serious at depolarized potentials, however. Potassium conductance, assayed a few milliseconds after a voltage step, increases very steeply with depolarization (Hodgkin and Huxley,

1952a): near the resting potential, g_K rises e-fold per 5 mV depolarization to reach a plateau of ~35 mS cm^{-2} at positive potentials, a 100-fold enhancement over its resting value. The conductance is subject to slow (several seconds) inactivation (Ehrenstein and Gilbert, 1966) in a voltage-dependent manner. Slow, voltage-dependent inactivation of g_K explains why "true" steady-state K permeability is somewhat less steeply voltage dependent (e-fold per 7.4 mV, De Weer and Geduldig, 1978; compare also with Hodgkin and Keynes' [1955] value of e-fold per ~7.5 mV in *Sepia* axons) than g_K reached during brief voltage steps. From the equations of Chabala (1984) describing slow g_K inactivation, it can be estimated that steady-state g_K at $V_H \geq 0$ mV is ~75% inactivated compared with values at -60 mV. Combining these two effects of strong depolarization, 100-fold enhancement followed by 75% inactivation, we are still left with a 25-fold greater steady-state g_K at 0 mV than at -60 mV, or ~9 mS cm^{-2}. If we can tolerate, at most, a contamination by nonpump current of 0.1 μA cm^{-2} (five times smaller than we found at -60 mV in the absence of K channel blockers) when periaxonal [K] rises 1 mM, it is clear that we must suppress g_K at least (5 × 25 =) 125-fold, or 99.2%, by means of blockers.

Using net Na and K flux measurements, Post and Jolly (1957) established a transport stoichiometry of 3 Na/2 K for the sodium pump in erythrocytes. By means of paired measurements of H_2DTG-sensitive ^{22}Na efflux and current on *Loligo pealei* axons, we find here an average flux/current ratio of 2.87 ± 0.07 ($n = 25$), which is not significantly different from the value of 3 expected for 3 Na/2 K transport stoichiometry, and independent of membrane potential and [Na]$_o$ (with the possible exception, discussed below, of measurements at 0 mV in high-Na seawater). We now consider what m Na/n K stoichiometries, other than 3 Na/2 K, might be compatible with our data. Besides the 3/2, 6/4, 9/6, and 12/8 stoichiometries, which all predict a value of 3.0 for the ratio $F\Delta\Phi_{Na}/\Delta I$ calculated as $m/(m-n)$, no stoichiometry with m or n <14, except 11 Na/7 K (predicted ratio 2.75), is compatible with our experimental $F\Delta\Phi_{Na}/\Delta I$ ratio at the $P \geq 0.05$ level. (The next compatible ratio is 14 Na/9 K.) One stoichiometry, 8 Na/5 K (predicted ratio 2.67), is remotely (0.01 > P > 0.001) compatible; but for all others with m or n <14, the probability is vanishing ($P < 0.001$). Although no true Na/K pump reversal potential has been clearly demonstrated yet (but see Béhé and Turin, 1984; Turin, 1984), our results and those of others (e.g., Gadsby et al., 1985; Lafaire and Schwarz, 1986) strongly suggest that Na/K pump reversal potentials are negative to -100 mV. To satisfy this requirement, a pump with 6/4, 9/6, 11/7, or 12/8 Na/K stoichiometry would need to hydrolyze 2–3 ATP molecules per cycle—all mechanistically implausible schemes. This leaves 3 Na/2 K as the sole transport stoichiometry compatible with our findings.

The calculated $m/(m-n)$ stoichiometric ratios can also be compared with the slightly lower $F\Delta\Phi_{Na}/\Delta I$ ratio of 2.72 ± 0.09 ($n = 7$) we found at 0 mV in high Na seawater. Here the only stoichiometries with m or n <14 statistically compatible (at $P > 0.05$) with the data are 8 Na/5 K and 11 Na/7 K but, again, both are unlikely on thermodynamic and mechanistic grounds. A more plausible explanation for this slight deviation from what appears to be the true Na/K transport stoichiometry, 3 Na/2 K, is a small systematic overestimation of pump current at 0 mV, due to the inclusion in H_2DTG-sensitive current of a nonpump component caused by periax-

onal K reaccumulation, despite the presence of the K channel blockers. A 0.1 μA cm^{-2} error at 0 mV would fully account for the 9% deviation of our $F\Delta\Phi_{Na}/\Delta I$ from 3.0. We have just seen that such an error could result from a 1 mM [K] change if as little as $\sim 1\%$ of g_K remained unblocked, a reasonable possibility. Comparison of B (current) with A (flux) in Figs. 11 and 15 confirms that, if fixed stoichiometry is assumed, the current record (B) includes an extra component that becomes larger as the cell is depolarized, as expected for K channel-mediated current. If efforts to block the remaining K channels prove successful, we expect this small current contaminant to disappear altogether. We conclude, then, that the pump transport stoichiometry is indeed 3 Na/2 K, independent of voltage over the range explored and of whether the bathing seawater contains sodium. Since we have not determined the reversal potential of the pump nor the rate of ATP hydrolysis, our data give no information on the stoichiometric quantity of ATP used. However, hydrolysis of one ATP molecule per pump cycle provides sufficient free energy for stoichiometric export of 3 Na ions and import of 2 K ions over the entire voltage range explored here. There is no necessity, therefore, to postulate a pump stoichiometry other than 3 Na/2 K/1 ATP.

Our data (Figs. 4 B, 7 G, and 9–15) show that hyperpolarization inhibits pump turnover rate. This voltage dependence of H_2DTG-sensitive current and flux is unlikely to be an artifact caused by voltage dependence of H_2DTG action since the steroid is uncharged and present at a near-saturating concentration. In addition, Figs. 9 and 10 show that H_2DTG dissociation from the pump is too slow ($t_{1/2} \sim 7$ min), and approximately independent of V_H or the presence of sodium (Fig. 9, 0 mV and -60 mV in 390 mM Na seawater; Fig.10, -80 mV and 0 mV in Na-free seawater), to allow a significant alteration in H_2DTG block during the time (~ 1 min) required to record an I-V. Furthermore, when allowance is made for residual, H_2DTG-insensitive but K_o-dependent current (e.g., Fig. 7 C), the voltage dependence of the current elicited by switching from K-free to 10 mM K seawater is closely similar to that of the H_2DTG-sensitive current, both in Na-free solution (compare F and G of Fig. 7) and in 390 mM Na seawater (data not shown).

The main new finding, then, is that pumped unidirectional [22]Na efflux and current remain in fixed ratio and show the same monotonic voltage dependence. Pumped [22]Na efflux reports the voltage sensitivity of the forward cycle rate, independent of any postulated voltage effect on the backward rate. But since the [22]Na efflux/current ratio remains fixed, it follows that the observed effect of negative voltage on pump current cannot be mediated through an increase in reverse transport unless a simultaneous increase in electrogenicity (i.e., net charge per extruded Na) of the forward Na/K exchange were to compensate exactly for the loss of Na efflux, which is a farfetched hypothesis. In other words, under the conditions used here, hyperpolarization exerts its effect by slowing the forward pump rate, not by speeding the backward. An analogous reasoning applies to the effects of external Na. Figs. 11 and 15 confirm the finding of others (Béhé and Turin, 1984; Gadsby and Nakao, 1987; Rakowski and Paxson, 1988) that the voltage dependence of pump current is steeper in high-Na than in Na-free solution. But our figures show, in addition, that the voltage dependence of pumped unidirectional [22]Na efflux is identically affected by Na_o. While measurements limited to pump current cannot distinguish between reduction of forward or enhancement of backward rate, our

combined observations on the effects of external Na prove that under these forward-pumping conditions, Na_o inhibits the pump mainly or exclusively by slowing the forward reaction and it appears to do so in a voltage-dependent manner; i.e., Table I, columns *3* and *8*, indicates that 390 mM Na_o causes 22% and > 42% slowing, at ~0 and −60 to −75 mV, respectively. Our finding that both hyperpolarization and external sodium inhibit the Na/K pump mainly by slowing the forward rate, puts more severe mechanistic constraints on pump models than would be required by the current measurements alone.

Because the voltage dependence of pump rate is monotonic over the range explored here, there is no need to invoke more than one voltage-sensitive step in the pump cycle (Gradmann et al., 1982). Indeed, recent evidence from a number of laboratories (reviewed in De Weer et al., 1988a, b) suggests that the major (only?) voltage-sensitive step in the overall pump cycle is likely to occur after Na occlusion and before K translocation. Thus, Karlish and co-workers (Karlish et al., 1985; Rephaeli et al., 1986a, b; Goldshlegger et al., 1987) working on reconstituted pumps in phospholipid vesicles, provided some of the first evidence that Na translocation is voltage dependent, but that K translocation is not. Using isolated cardiac myocytes, Nakao and Gadsby (1986) have measured strophanthidin-sensitive pre–steady-state (transient) membrane charge movements (and the voltage dependence of their rate constants) elicited by voltage steps under K-free conditions that presumably restricted the pump to Na translocation, whereas Bahinski et al. (1988) found no such charge movements under Na-free conditions that should have been able to support K/K exchange. A possible corollary of these findings is that the "empty" pump carries two equivalent negative charges. Evidence that pump cycle steps involving Na translocation, though charge carrying (hence voltage sensitive), are not rate limiting during Na/K exchange comes from ATP-jump studies (using caged-ATP photolysis) of pre–steady-state pumped ^{22}Na efflux from membrane vesicles (Forbush, 1984) and pre–steady-state pump currents generated on plane bilayers (Fendler et al., 1985, 1987; Apell et al., 1987; Borlinghaus et al., 1987), and from voltage-jump studies of pre–steady-state pump currents in myocytes (Bahinski et al., 1988). The most likely major voltage-dependent step is either the $E_1 \rightarrow E_2$ transition of the Na-loaded enzyme or the Na-releasing step itself. It can be shown (Hansen et al., 1981) that a voltage-dependent, non–rate-limiting step (here, Na translocation) can nevertheless determine pump cycling rate by controlling the level of enzyme intermediate that enters the rate-limiting, voltage-independent step (here, K translocation). Our finding that both hyperpolarization and external Na ions inhibit cycling principally by slowing the forward rate raises the question whether the external [Na] controls the level of the enzyme intermediate entering the K translocation step in a voltage-dependent manner.

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