K^+- and HCO_3^--dependent Acid–Base Transport in Squid Giant Axons
I. Base Efflux

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ABSTRACT We used microelectrodes to monitor the recovery (i.e., decrease) of intracellular pH (pH_i) after using internal dialysis to load squid giant axons with alkali to pH_i values of 7.7, 8.0, or 8.3. The dialysis fluid (DF) contained 400 mM K^+ but was free of Na^+ and Cl^-. The artificial seawater (ASW) lacked Na^+, K^+, and Cl^-, thereby eliminating effects of known acid–base transporters on pH_i. Under these conditions, halting dialysis unmasked a slow pH_i decrease caused at least in part by acid–base transport we refer to as "base efflux." Replacing K^+ in the DF with either $NMDG^+$ or TEA^+ significantly reduced base efflux and made membrane voltage (V_m) more positive. Base efflux in K^+-dialyzed axons was stimulated by decreasing the pH of the ASW (pH_o) from 8 to 7, implicating transport of acid or base. Although postdialysis acidifications also occurred in axons in which we replaced the K^+ in the DF with Li^+, Na^+, Rb^+, or Cs^+, only with Rb^+ was base efflux stimulated by low pH_o. Thus, the base effluxes supported by K^+ and Rb^+ appear to be unrelated mechanistically to those observed with Li^+, Na^+, or Cs^+. The combination of 437 mM K^+ and 12 mM HCO_3^- in the ASW, which eliminates the gradient favoring a hypothetical K^+/HCO_3^- efflux, blocked pH_i recovery in K^+-dialyzed axons. However, the pH_i recovery was not blocked by the combination of 437 mM Na^+, veratridine, and CO_2/HCO_3^- in the ASW, a treatment that inverts electrochemical gradients for H^+ and HCO_3^- and would favor passive H^+ and HCO_3^- fluxes that would have alkalinized the axon. Similarly, the recovery was not blocked by K^+ alone or HCO_3^- alone in the ASW, nor was it inhibited by the K-H pump blocker Sch28080 nor by the Na–H exchange inhibitors amiloride and hexamethyleneamiloride. Our data suggest that a major component of base efflux in alkali-loaded axons cannot be explained by metabolism, a H^+ or HCO_3^- conductance, or by a K–H exchanger. However, this component could be mediated by a novel K/HCO_3^- cotransporter.

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INTRODUCTION

It has been known for some time that intracellular pH (pH_i) in squid giant axons is regulated, at least in part, by an ion transporter that appears to exchange extracellular Na^+ and HCO_3^- for intracellular Cl^- and H^+ (Boron and Russell, 1983). Similar transporters are present in snail neurons (Thomas, 1984), barnacle muscle (Boron, McCormick, and Roos, 1979, 1981), fibroblasts (L'Allemain, Paris, and Pouyssegur, 1985), rat renal mesangial cells (Boyarsky, Ganz, Sterzel, and Boron, 1988) and rat hippocampal CA1 neurons (Schwiening and Boron, 1994). These Na^+-dependent Cl-HCO_3 exchangers are blocked by stilbene derivatives such as DIDS, and respond to decreases in pH_i by extruding acid equivalents from the cell, thereby returning pH_i toward its initial value. Although the squid axon uses a Na^+-dependent Cl-HCO_3 exchanger to recover from intracellular acid loads, we questioned whether the axon also has a mechanism for recovering from intracellular alkali loads. A variety of vertebrate cells recover from alkali loads by means of a transporter that exchanges extracellular Cl^- for intracellular HCO_3^-, and is sensitive to disulfonic stilbene derivatives (Vaughan-Jones, 1982; Chaillet, Amsler, and Boron, 1986). In contrast to the Na^+-dependent Cl-HCO_3 exchanger, this Na^+-independent Cl-HCO_3 exchanger can function in the total absence of Na^+ and is stimulated by increases in pH_i. Certain epithelial cells, including renal proximal tubule cells (Boron and Boulpaep, 1983), possess an electrogenic Na/HCO_3 cotransporter that normally moves Na^+ and HCO_3^- out of cells and thereby decreases pH_i. Similar to the Na^+-dependent Cl-HCO_3 exchanger, the electrogenic Na/HCO_3 cotransporter is blocked by DIDS. However, it does not transport Cl^-. Finally, Leviel, Borensztein, Houillier, Paillard, and Bichara (1992) suggested that a DIDS-sensitive K/HCO_3 cotransporter can contribute to the recovery of pH_i from alkali loads in medullary thick ascending limbs from rat kidney.

The present study was initiated in an attempt to identify a Cl-HCO_3 exchanger in squid axons internally dialyzed to a pH_i of ~8.0 (initial pH_i = ~7.35). In control experiments in which we removed all $Na^+$and Cl^- from both the dialysis fluid and the artificial seawater and removed K^+ as well from the seawater, we were surprised to observe a pH_i recovery (i.e., decrease) that requires intracellular K^+ (or Rb^+). This pH_i decrease can be blocked by the simultaneous presence of K^+ and HCO_3^- in the seawater, but not by a combination of Na^+, veratridine, and HCO_3^- that produces similar pH_i and V_m changes, nor by either K^+ or HCO_3^- alone. Thus, a major component of this "base efflux" is most easily accounted for by a novel K/HCO_3^- cotransporter. In experiments described in an accompanying paper (Hogan, Cohen, and Boron, 1995), in which we dialyzed axons with a K^+-free fluid, we found that simultaneously introducing K^+ (or Rb^+) and CO_2/HCO_3^- to the seawater caused a rapid pH_i decrease (because of the influx of CO_2), followed by a sustained pH_i increase, at least part of which appears to be the result of "base influx." This pH_i increase is not inhibited by disulfonic stilbene derivatives, even at high doses. Because base influx cannot be produced by introducing either K^+ alone or CO_2/HCO_3^- alone, it is most likely mediated by the same novel K/HCO_3 cotransporter that is responsible for the pH_i decrease in axons dialyzed with K^+.

Portions of this work have been published in preliminary form (Boron and Hogan, 1991).

METHODS

General

Because our general approach in the experiments reported here was similar to that used in previous studies on squid axons from this laboratory (Boron, 1985; Boron and Knakal, 1989, 1992), we will only briefly outline our methods, except in cases where significant differences exist between previous and present work. The experiments were conducted at the Marine Biological Laboratory, Woods Hole, MA. We microdissected a 3–4-cm length of giant axon, 400–700 μm in diameter, from specimens of the squid *Loligo pealei*, and stored the axon in natural seawater at ~4°C. A single axon was cannulated horizontally at both ends in a chamber designed for internal dialysis (Brinley and Mullins, 1967). Cellulose acetate dialysis tubing (Fisher Research Laboratories, Dedham, MA) having an outer diameter of 140 μm was inserted through one cannula, threaded down the axon, and out the opposite cannula. An 18-mm length of this tubing, positioned in the central portion of the axon, had been permeabilized by hydrolysis in 0.1 N NaOH. The dialysis capillary was perfused with dialysis fluid (DF) at a rate of ~2.1 μl/min. A voltage-sensitive microelectrode and a pH-sensitive microelectrode also were inserted into the axon through opposite cannulas and arranged so that their tips were centered in the axon within ~500 μm of one another.

The open-tipped voltage electrode was filled with 3M KCl. Because we were concerned about the possible leakage of KCl out of KCl-filled electrodes, we experimented with filling the voltage electrodes with 1M $NMDG^+$/glutamate and 1M K^+/glutamate. However, we found that the tip potentials of these electrodes changed by >10 mV (which would produce an apparent pH_i shift of ~0.15) when $[K^+]$ was altered. We therefore adopted the strategy of using electrodes having relatively small tip diameters (outer diameter: ~5–10 μm). We found that the fluid in the electrode tips had, by the end of the experiments, spontaneously gelled, so that fluid could not easily be forced out of the tip under pressure. These electrodes usually had tip potentials <1 mV and resistances of 1–3 MΩ. Because we used the same voltage electrode in many experiments, we presume that the loss of KCl was minimal.

The pH-sensitive microelectrodes were made according to the design of Hinke (1967), with exposed pH-sensitive glass tips (Clark Electromedical Instruments, Pangbourne, UK) protruding from a shank fabricated from lead glass (model 0120; Corning Glass Works, Corning, NY). Descriptions of our use of high-impedance electrometers, the acquisition of data by computer, and the computer control of the experiments are provided elsewhere (Boron and Russell, 1983; Boron, 1985). We determined the slopes of the pH-sensitive microelectrodes as previously described, using high-ionic-strength buffers. We determined the offset of the microelectrode in each experiment by assuming that the pH_i achieved at the end of the period of dialysis was the same as the pH of the dialysis fluid. The axon was superfused continuously with artificial seawater (ASW). The temperature was maintained at 22°C.

Solutions

Artificial seawaters. Our standard extracellular fluid was a Na^+-, K^+-, Cl^--, and HCO_3^--free artificial seawater (0/0/0/0 ASW) buffered to pH 8.00, and having the following composition (in mM): 437.2 $NMDG^+$, 62.5 Mg^{2+}, 3.0 Ca^{2+}, 563 D-gluconate, 0.1 $EDTA^=$, 5 of the anionic form of *N*-[2-hydroxyethyl]piperazine-*N'*-[3-propanesulfonic acid] (EPPS), and 5 of the neutral form of EPPS (computed assuming that the pK is 8.0). The pH was adjusted to 8.00 by adding NMDG-free base or EPPS-free acid. We also made a variant of this 0/0/0/0 ASW in which we titrated the solution to pH 7.00. The osmolality, measured with a vapor-pressure osmometer (model 5100C; Wescor Inc., Logan, UT), was adjusted to 970 ± 5 mOsm/kg with either mannitol or water. This and all other nominally CO_2/HCO_3^--free solutions were gassed with 100% oxygen to minimize the concentration of dissolved CO_2. All artificial seawaters contained 10^{-5} M ouabain.

We made an ASW free of K^+, Na^+, and Cl^- but containing 12 mM HCO_3^- by replacing 12 mM gluconate in the 0/0/0/0 ASW with 12 mM HCO_3^-. This solution (0/0/0/12-HCO_3^- ASW) was made by (*a*) adding all the components except CO_2/HCO_3^- and the last 12 mM of NMDG free base, (*b*) bringing the solution to volume and titrating to pH 8.00, (*c*) adding 12 mM NMDG free base, and then (*d*) gassing with 0.5% CO_2 until the pH stabilized at 8.00. As discussed previously, the actual $[HCO_3^-]_o$ is less than the nominal $[HCO_3^-]_o$, because of the formation of CO_3^- ion pairs with Na^+, Mg^{2+}, and Ca^{2+} (Boron and Knakal, 1989).

We generated a 437-K^+ ASW by replacing 437 mM $NMDG^+$ in our 0/0/0/0 ASW with K^+. This solution also contained 7 mM less magnesium gluconate than did the 0/0/0/0 ASW. We made upward adjustments to pH with KOH. We generated a 437-K^+/12-HCO_3^- ASW by replacing 12 mM gluconate in the 437-K^+ ASW with 12 mM HCO_3^-.

In experiments in which we determined intracellular buffering power, we exposed cells to a variant of our standard 0/0/0/0 ASW in which 2.5–40 mM of the $NMDG^+$/gluconate had been replaced with NH_4NO_3.

Sch28080 was obtained from Schering Corporation (Kenilworth, NJ). HMA was purchased from E. Cragoe (Nacogdoches, TX). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

The ASWs were delivered to the chamber through CO_2-impermeable Saran tubing (Clarkson Equipment and Controls, Detroit, MI).

Dialysis fluids. Our standard internal dialysis fluid (DF) lacked Na^+, K^+, and Cl^- and was titrated to pH 8.00. This 0/0/0/pH-8.00 DF had the following composition (in millimolar): 417.2 $NMDG^+$, 7 Mg^{2+}, 16 $Tris^+$, 414 glutamate, 4 ATP^{4-}, 1 $EGTA^=$, 15.2 of the anionic form of *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), 4.8 of the neutral form of HEPES (computed assuming a pK of 7.50), 95 glycine, and 0.5 phenol red. NMDG free base or glutamic acid was used to titrate the pH to 8.00 at 22°C. Osmolality was adjusted to 965–970 mOsm/kg using either glycine or water. ATP was added to the DF from a 400 mM Tris/ATP stock (pH 7.0) stored at −20°C. In some experiments, a variation of this DF was titrated to pH values as high as 8.3 or as low as 7.75.

In some experiments, we used a 0/0/0/pH-8.00 DF in which the $NMDG^+$ was replaced with tetraethylammonium (TEA^+).

A pH-8.00 dialysis fluid containing 400 mM K^+, but no Na^+ or Cl^- (the 400-K^+ DF) was made by replacing 400 mM $NMDG^+$ with K^+. Similarly, we made DFs in which the $NMDG^+$ was replaced with 400 mM Li^+, Na^+, Rb^+, or Cs^+. For these DFs, upward adjustments to pH were made with the appropriate alkali-metal hydroxide.

Calculation of Acid–Base Transport Rates

As described previously (Boron and Knakal, 1989, 1992), pH_i data were acquired by computer. Rates of pH_i change (dpH_i/dt) were determined from linear curve fits, performed by computer, to the data. We define acid–base flux (J) in the same way we have previously defined acid extrusion rate: the net efflux of H^+ (or other acid) plus the net influx of HCO_3^- (or other base). J is thus positive for fluxes that produce a pH_i increase (base influx), and negative for fluxes that produce a pH_i decrease (base efflux). We computed J as the product of dpH_i/dt, total intracellular buffering power (β_T), and volume-to-surface ratio. β_T was taken as the sum of the intrinsic buffering power (β_I, measured as described below) and the open-system CO_2/HCO_3^- buffering power ($\beta_{HCO_3^-}$, which we assumed to be the theoretical value of $\ln 10 \times [HCO_3^-]_i$). More recent work, in which we actually measured β_T in experiments in which axons were exposed to CO_2/HCO_3^-, confirms the assumption that β_T is the sum of β_I and the computed $\beta_{HCO_3^-}$ (Zhao, Hogan, Bevensee, and Boron, 1995). We computed the volume-to-surface ratio from the axon diameter, assuming the axon to be a cylinder.

Intracellular Buffering Power

Using an approach described previously (see Roos and Boron, 1981), we computed intrinsic intracellular buffering power from the results of experiments in which we exposed axons to a pH 8.00 ASW containing NH_3/NH_4^+. Before the exposure to, NH_3/NH_4^+ the axons were internally dialyzed with either a 400 mM K^+ or a 400 mM $NMDG^+$ dialysis fluid. So that we could determine the pH_i dependence of β_I, the DFs had pH values ranging from 7.70 to 8.30. So that the NH_3/NH_4^+-induced pH_i increase was consistently ~0.2, we matched the total NH_3/NH_4^+ concentration to the pH of the DF: 2.5–5 mM for a pH_{DF} of 7.70 or 7.75, 5–10 mM for a pH_{DF} of 8.00, and 10–40 mM for a pH_{DF} of 8.30. Fig. 1 *A* shows a buffering-power experiment in which an axon was dialyzed to a pH_i of 8.30 with a K^+-free fluid. After dialysis was halted and pH_i drifted slowly downward (see Results), exposing the axon to an ASW containing 40 mM total NH_3/NH_4^+ caused a pH_i

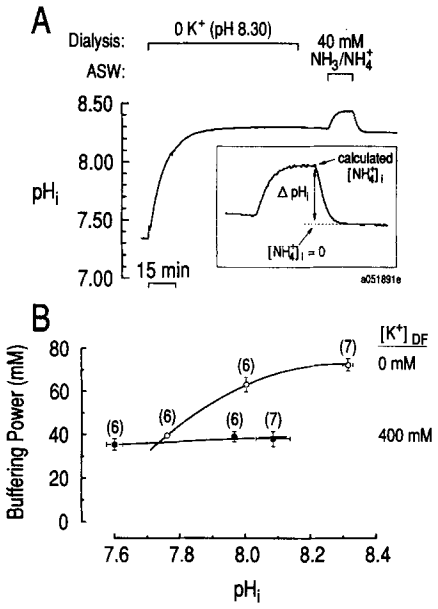

FIGURE 1. Intracellular buffering power in dialyzed axons. (*A*) Experiment showing the effect of exposing an axon, previously dialyzed to pH_i 8.30 with a DF containing no K^+ (i.e., 400 mM $NMDG^+$), to an ASW containing 40 mM NH_3/NH_4^+ pulse. (*B*) Summary of pH_i dependence of buffering power, both for axons dialyzed with 400 mM K^+ and those dialyzed with a K^+-free DF containing 400 mM $NMDG^+$.

increase owing to the influx of the weak base NH_3, whereas removing the NH_3/NH_4^+ had the opposite effect. We computed β_I from the equation $\beta_I = \Delta[NH_4^+]_i/\Delta pH_i$. To compensate for drift in the pH_i baseline after application of the NH_3/NH_4^+ ASW, we fitted the pH_i-vs.-time record after NH_3/NH_4^+ removal (after a time when all NH_3 was judged to have left the axon) with a line and back-extrapolated this line to a time late during the NH_3/NH_4^+ exposure (see inset of Fig. 1 *A*). The $[NH_4^+]_i$ at the end of the NH_3/NH_4^+ exposure was calculated from the final pH_i, the pH_o and $[NH_4^+]_o$, assuming that NH_3 was equilibrated across the axon membrane: $[NH_4^+]_i = [NH_4^+]_o \times 10^{(pH_i - pH_o)}$. ΔpH_i was taken as the difference in pH_i values between the extrapolated line and the final pH_i record during the NH_3/NH_4^+ exposure, as shown in the figure. In each experiment, the pH_i associated with the β_I value was the average of these two pH_i values.

Fig. 1 *B* summarizes the pH_i dependence of β_I for the two dialysis fluids noted above. β_I was consistently higher for axons dialyzed with a fluid containing 400 mM $NMDG^+$ than for one containing 400 mM K^+, as expected for the additional buffering power provided by the $NMDG^+$ itself. Also as expected, given that NMDG has a pK of ~9.5 , β_I increased with pH_i in the presence of

NMDG. In our calculations of acid–base fluxes (see above), we assumed that β_I was governed by the 400-K^+ relationship for all DFs except for the 400 mM $NMDG^+$ DF. More recent work (Zhao et al., 1995) confirms that β_I for axons dialyzed with 400 mM TEA^+ is approximately the same as for axons dialyzed with 400 mM K^+. Although the β_I-vs.-pH_i relationship for 400 mM K^+ extended to a pH_i of only ~8.1, we used the line of best fit for flux calculations at pH_i values as high as ~8.3, inasmuch as the slope of the regression line was nearly zero.

Statistics

Results are expressed as the mean ± SEM. Statistical comparisons were done using the paired or unpaired Student's *t* tests, as indicated in the text. *P* values less than 0.05 were considered statistically significant. The dependence of intrinsic buffering power on pH_i was determined by fitting a line or a second degree polynomial to the data.

RESULTS

Our experiments fall into two major groups. In the first, reported in this paper, axons usually were dialyzed with a fluid containing 400 mM K^+, and we studied the decrease in pH_i presumably caused in large part by a base-efflux mechanism. In the second, reported in the accompanying paper (Hogan et al., 1995), axons usually were dialyzed with a K^+-free fluid, and we studied the increase in pH_i presumably caused by base influx.

Dependence of Base Efflux on Internal K^+

Fig. 2, *A* and *B*, shows the results of two experiments in which we monitored the recovery of pH_i from acute intracellular alkali loads imposed by 80-min periods of internal dialysis (segment *ab*) with a fluid titrated to a pH of either 7.75 or 7.81. In both cases, the dialysis fluid (DF) was free of Na^+ and Cl^-. Also in both cases, the pH 8.00 ASW to which the exterior of axons were exposed was free of Na^+, K^+, Cl^-, and HCO_3^-. Under these conditions of ion replacement, all known acid-loading HCO_3^- transporters (i.e., Cl-HCO_3 exchangers and electrogenic Na/HCO_3 cotransporters) should have been blocked. After dialysis with a K^+-free DF was halted in the experiment shown in Fig. 2 *A*, pH_i decreased at a rather low rate (*bc*). Moreover, the rate of intracellular acidification was not affected appreciably by decreasing the pH of the ASW (pH_o) from 8.00 to 7.00 (*cd*), and then returning it to 8.00 (*de*). Fig. 2 *B* shows the results of an experiment that was similar to the first, except that the DF contained 400 mM K^+. In this second case, the rate of pH_i decrease after the cessation of dialysis (*bc*) was far higher than in the first case. In addition, the rate of intracellular acidification twice[1] was increased by reducing pH_o from 8.00 to

1. We found in other experiments that, even at a fixed pH_o of 8.00, the rate of intracellular acidification gradually decreased as pH_i declined. Thus, the first switch to a pH-7 ASW in Fig. 2 *B* caused only a small increase in the rate of acidification (*cd* vs. *bc*). Nevertheless, the rate of intracellular acidification decreased appreciably when pH_o was returned to 8.00 (*de* vs. *cd*). Thus, the flux during *cd* was greater than the average of the fluxes in *bc* and *de*. The intracellular acidification rate plainly increased when pH_o was lowered to 7.0 for a second time (*ef* vs. *de*), and slowed again when pH_o was returned to 8.00 for the final time (*fg* vs. *ef*).

7.00 (*cd* and *ef*). This observation is consistent with the hypothesis that the pH_i is mediated by an acid–base transporter in the plasma membrane. The mean data for experiments in which pH_o was decreased from 8.00 to 7.00 are discussed below in connection with Fig. 5.

The leftmost portion of Fig. 2 *C* summarizes the results of 28 experiments similar to segment *bc* of Fig. 2 *A* ($[K^+]_{DF} = 0$), whereas the leftmost portion of Fig. 2 *D* summarizes the comparable data from 23 experiments similar to those shown in Fig. 2

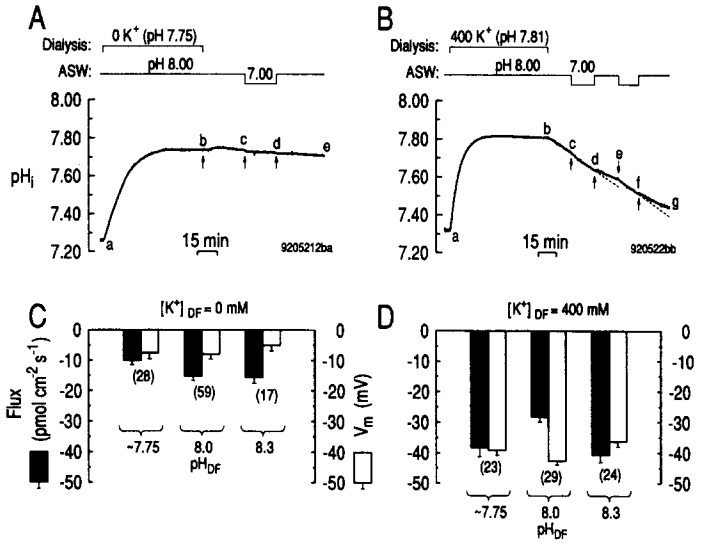

FIGURE 2. Intracellular K^+ dependence of base efflux. (*A*) Experiment on an axon dialyzed to pH_i 7.75 with a K^+-free DF. Dialysis, begun at point *a*, was halted at point *b*, returning control of pH_i to the axon. During segment *cd*, the pH of the ASW was decreased from 8.00 to 7.00. (*B*) Experiment on an axon dialyzed to pH_i 7.81 with a DF containing 400 mM K^+. During segments *cd* and *ef*, the pH of the ASW was decreased from 8.00 to 7.00. (*C*) Summary of data obtained in three pH_i ranges on axons dialyzed with a K^+-free DF. The filled bars represent net acid–base fluxes (a negative number indicates net base efflux), and the open bars, mean membrane potential during the period in which the flux was computed. The numbers of observations are given in parentheses. The vertical hash marks indicate SEM values. The mean pH_i values were 7.749 ± 0.004 (pH_{DF} 7.75 *bars*), 7.991 ± 0.006 (pH_{DF} 8.0 *bars*), and 8.287 ± 0.004 (pH_{DF} 8.3 *bars*). (*D*) Summary of data obtained in three pH_i ranges on axons dialyzed with a DF containing 400 mM K^+. The mean pH_i values were 7.742 ± 0.014 (pH_{DF} 7.75 *bars*), 7.948 ± 0.007 (pH_{DF} 8.0 *bars*), and 8.244 ± 0.007 (pH_{DF} 8.3 *bars*).

B ($[K^+]_{DF}$ = 400 mM). In these 51 experiments, axons were dialyzed with a DF that contained either 0 or 400 mM K^+ and was titrated to pH values between 7.75 and 7.81. Comparing the leftmost portions of Fig. 2, *C* and *D*, shows that, for comparable pH_i values, the mean base efflux was appreciably larger (~38 pmol cm^{-2} s^{-1}) when the DF contained K^+ than when the DF was K^+ free (~10 pmol cm^{-2} s^{-1}). The mean membrane potential (V_m) was also more negative in axons dialyzed with K^+ (~−39 vs. ~−8 mV). We observed that dialyzing with K^+-free vs. K^+-containing

DFs had similar effects on base efflux and V_m in axons dialyzed to a pH_i of 8.00 (n = 59 + 29 = 88, middle pair of bars in Fig. 2, *C* and *D*) and in axons dialyzed to pH_i 8.30 (n = 17 + 24 = 41, rightmost pair of bars in Fig. 2, *C* and *D*).

Effect of Other Intracellular Cations on Base Efflux

TEA^+ vs. $NMDG^+$. Because in the experiments shown in Fig. 2 we dialyzed with either $NMDG^+$ or K^+, these data cannot be used by themselves to ascertain whether the stimulation of base efflux observed in K^+-dialyzed axons was a result of the introduction of K^+ or the removal of $NMDG^+$. We therefore examined the effect on base efflux of dialyzing axons with 400 mM tetraethylammonium (TEA^+). As can be seen by comparing the leftmost two pairs of bars in Fig. 3, the choice of replacing K^+ with $NMDG^+$ or TEA^+ had no significant effect on either the mean base efflux or the mean V_m. We conclude that it is the presence of intracellular K^+, or perhaps a consequence of the presence of K^+ (e.g., hyperpolarization), that promotes base efflux in squid axons.

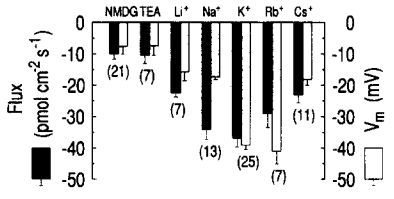

FIGURE 3. Effect on base efflux of dialyzing with various monovalent cations. Data were obtained from experiments similar to those shown in Fig. 2, *A* and *B*. The filled bars represent net acid–base fluxes (a negative number indicates net base efflux), and the open bars, mean membrane potential during the period in which the flux was computed. Numbers of observations are given in parentheses; vertical hash marks indicate SEM values. The mean pH_i values for the segments for which the flux and V_m data were obtained were 7.745 ± 0.005 ($NMDG^+$), 7.761 ± 0.004 (TEA^+), 7.763 ± 0.006 (Li^+), 7.768 ± 0.006 (Na^+), 7.735 ± 0.014 (K^+), 7.694 ± 0.045 (Rb^+), and 7.769 ± 0.007 (Cs^+).

Alkali-metal ions. We also determined whether four Group-IA elements (Li^+, Na^+, Rb^+, and Cs^+) can support a postdialysis intracellular acidification after an 80-min period of dialysis with 400 mM of the cation. Postdialysis flux and V_m data for experiments in which axons were dialyzed to pH_i 7.80 with one of the above four Group-IA cations, or K^+, are summarized by the five rightmost pairs of bars in Fig. 3. For each ion, the rate of pH_i decrease was greater than that observed after dialysis with $NMDG^+$ or TEA^+. For Li^+ and Cs^+, the apparent base efflux was 12–13 pmol cm^{-2} s^{-1} greater than the baseline flux in $NMDG^+$ or TEA^+. For Rb^+, the apparent base efflux was ~19 pmol cm^{-2} s^{-1} greater than the baseline flux, and for Na^+, ~24 pmol cm^{-2} s^{-1}.

Effect of decreasing pH_o to 7.00 in axons dialyzed with Li^+, Na^+, Rb^+, or Cs^+. Although each of the Group-IA cations tested supported a postdialysis pH_i decrease, the data of Fig. 3 do not address the issue of whether the mechanism of the pH_i decrease was the same in all cases. For example, loading an axon with a particular cation could lead to metabolic changes that produce a pH_i decrease. Loading an axon with 400 mM Na^+, particularly in the absence of extracellular Na^+, could lead to an accumulation of intracellular Ca^{2+}, which could in turn lead to a decrease in pH_i

because of the displacement of H^+ from Ca^{2+}/H^+ buffers. Such processes might not be affected by decreasing extracellular pH. Therefore, for each of the ions summarized in Fig. 3, we determined whether the apparent base efflux observed after halting dialysis was stimulated by decreasing pH_o from 8.00 to 7.00, following the same protocol used for axons dialyzed with $NMDG^+$ in Fig. 2 *A* (*cd*) and K^+ in Fig. 2 *B* (*cd* and *ef*). Examples for Li^+, Na^+, Rb^+, and Cs^+ are shown in Fig. 4, *A–D*, which shows portions of these experiments that correspond to segments *bcde* in Fig. 2 *A* or *bcdefg* in Fig. 2 *B*. As can be seen by comparing the rates of pH_i decrease at a pH_o of 7.00 with those immediately before and after (i.e., at a pH_o of 8.00), extracellular acidification increased the rate of pH_i decrease only when the cation was Rb^+. It is of interest that in the experiment shown with Na^+, we removed Ca^{2+} from the ASW; in this experiment, apparent base efflux was indistinguishable from that

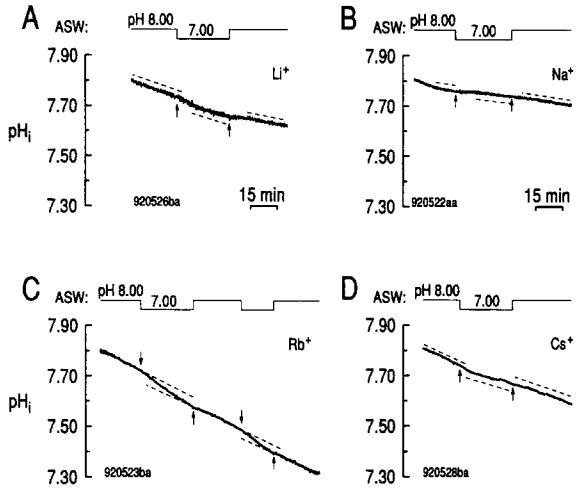

FIGURE 4. Effect of decreasing extracellular pH from 8.00 to 7.00 in axons dialyzed with various cations. The design of experiments is the same as shown in Fig. 2, *A* and *B*. (*A*) Li^+. (*B*) Na^+. (*C*) Rb^+. (*D*) Cs^+.

observed in axons dialyzed with $NMDG^+$ or TEA^+, and decreasing pH_o seems to have slowed the pH_i decrease.

The paired analyses (pH_o 7.00 vs. 8.00) of a larger group of data, obtained in experiments such as those in Fig. 4, *A–D*, are summarized in Fig. 5. In each experiment, we determined apparent base efflux under three conditions: (*a*) at pH_o 8.00 in the immediate postdialysis period, when pH_i was relatively high; (*b*) during the exposure to the pH_o 7.00 ASW, when pH_i was somewhat lower; and (*c*) after returning pH_o to 8.00, when pH_i was lowest. We averaged the fluxes as well as pH_i and V_m values in periods *a* and *c*, and compared these averages with the comparable values for period *b*. For each cation, average pH_i and V_m values from periods *a* and *c* were very similar to the values from period (*b*). However, as summarized in Fig. 5, only in the cases of K^+ and Rb^+ was apparent base efflux stimulated by low pH_o. Decreasing pH_o from 8.00 to 7.00 had no significant effect on apparent base efflux for axons dialyzed with 400 mM TEA^+, $NMDG^+$, Li^+, or Na^+. In the case of dialysis with Cs^+, decreasing pH_o actually caused a decrease in base efflux. We conclude that the pH_i decreases observed in axons dialyzed with Li^+, Na^+, or Cs^+ are unrelated mech-

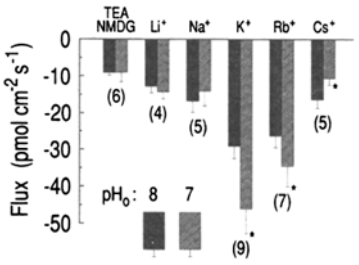

FIGURE 5. Summary of the effect of decreasing extracellular pH from 8.00 to 7.00 in axons dialyzed with various cations. The design of experiments is the same as shown in Fig. 2, *A* and *B*, and in Fig. 4. pH_i was ~7.75. The solid bars indicate fluxes computed when extracellular pH was 8.00 and the hashed bars, when pH_o was 7.00. The asterisks indicate statistical significance ($P < 0.05$).

anistically to the pH_i decrease supported by K^+. Inasmuch as Rb^+ substitutes for K^+ in a wide variety of ion transporters, it is reasonable to hypothesize that Rb^+ and K^+ produce pH_i decreases by similar mechanisms.

Models of Base Efflux

Fig. 6 illustrates several possible mechanisms for the pH_i decrease in K^+-loaded axons, such as that shown in Fig. 2 *B*. These models are not mutually exclusive. In principle, the pH_i decreases could have been caused by the metabolic production of acid. On the other hand, it is not clear why metabolism should have been augmented, either directly or indirectly, by the introduction of K^+ to the DF, or by acidification of the extracellular fluid.

A second possibility is that the pH_i decreases were produced by the passive influx of H^+ or the passive efflux of OH^- or HCO_3^-. According to this scenario, the stimulation of base efflux by intracellular K^+ or Rb^+ would be secondary to the more negative V_m (see mean V_m data in Fig. 3), which would favor H^+ influx and OH^- or HCO_3^- efflux. Indeed, for all three pH_i ranges summarized in Fig. 2 *C* for K^+-dialyzed axons, the electrochemical gradient for H^+ (computed from the mean pH_i data) favors a passive influx of this ion. For experiments conducted in the nominal absence of CO_2/HCO_3^-, we cannot compute a HCO_3^- gradient. However, it is likely that metabolism generates a small amount of intracellular HCO_3^- and thus establishes an in-to-out HCO_3^- chemical gradient and an even larger in-to-out electrochemical gradient. Thus, based only on the data presented in Fig. 2, passive movements of H^+, OH^-, or HCO_3^- cannot be ruled out as a possible mechanism for the pH_i decrease supported by a high $[K^+]_i$ or $[Rb^+]_i$.

A third possibility is that the pH_i decreases were a result of the exchange of K^+ for H^+ (Hofer and Machen, 1992). According to this model, the baseline base ef-

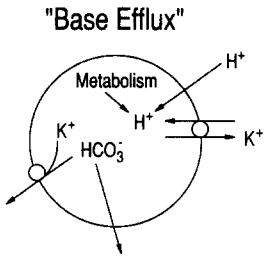

FIGURE 6. Possible mechanisms of base efflux at high pH_i.

flux in axons dialyzed with a K^+-free DF would be supported by the small amount of K^+ that is likely to remain inside the axon, either because of incomplete washout via dialysis, or because of leak of some K^+ from the KCl-filled voltage electrode. Increasing $[K^+]_{DF}$ to 400 mM would stimulate K–H exchange and speed the pH_i decrease. This model makes two important predictions: (*a*) base efflux should be blocked or even reversed (depending on the pH_i and pH_o values) by sufficiently increasing $[K^+]_o$. For example, if pH_i were the same as pH_o (so that $[H^+]_i = [H^+]_o$), then raising $[K^+]_i$ to match $[K^+]_o$ would halt K–H exchange. (*b*) Base efflux should not be affected by introducing CO_2/HCO_3^-.

A fourth possibility is that the pH_i decrease in Fig. 2 *B* was mediated by a novel K/HCO_3 cotransporter. Two predictions of this model are of interest: (*a*) unless the actual $[K^+]_o$ in the unstirred layer around the axon (Frankenhauser and Hodgkin, 1956) exceeds zero by a sufficiently large amount, base efflux should be stimulated by introducing CO_2/HCO_3^- into the ASW. (*b*) Base efflux should be blocked by simultaneously increasing both $[K^+]_o$ and $[HCO_3^-]_o$, but not by increasing $[K^+]_o$ alone.

Effect of Increasing Extracellular $[K^+]$

In the experiment shown in Fig. 7 *A*, an axon is dialyzed to a $[K^+]_i$ of 400 mM and a pH_i of 8.00. The figure shows the portion of the experiment after dialysis had been halted and pH_i began to fall (*ab*). Because the ASW had a pH of 8.00, pH_i was

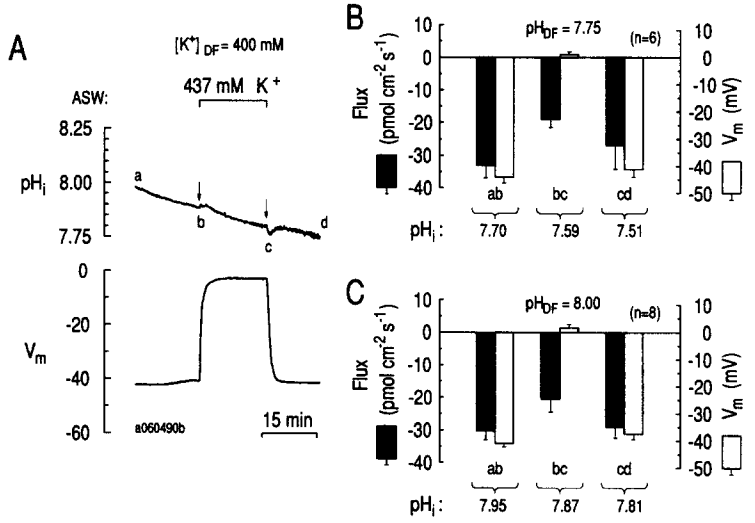

FIGURE 7. Effect of increasing only $[K^+]_o$ in axons dialyzed to a $[K^+]_i$ of 400 mM. (*A*) Experiment showing the time course of pH_i after dialysis was halted. During the indicated time, 437 mM extracellular $NMDG^+$ was replaced with K^+. (*B*) Summary of paired data, obtained in six experiments such as shown in *A*, in which the pH of the DF was 7.75. The left pair of bars indicates the mean flux and V_m before addition of K^+ (segment *ab* in *A*); the center pair, during the latter part of the K^+ exposure (*bc*); the right pair, after K^+ removal (*cd*). (*C*) Summary of similar paired data, obtained in nine experiments in which the pH of the DF was 8.00.

$\leq pH_o$ throughout this experiment. If the pH_i decrease in Fig. 2 were entirely the result of either H^+ influx or K–H exchange, it would be blocked or even reversed by making $[K^+]_o \geq [K^+]_i$. However, Fig. 7 *A* shows that, by itself, raising $[K^+]_o$ to 437 mM modestly slowed but did not abolish the pH_i decline (*bc* vs. *ab* and *cd*). Similar observations were made in axons dialyzed to a pH_i of 7.75. On average, the inhibition of base efflux was ~37% in paired experiments among axons dialyzed to a pH_i of 7.75 (Fig. 7 *B*), and ~30% for axons dialyzed to a pH_i of 8.00 (Fig. 7 *C*).

Analysis of passive H^+/OH^- flux hypothesis. The depolarization elicited by increasing $[K^+]_o$ from 0 to 437 could have inhibited a portion of the total base efflux as a result of the passive flux of H^+ or OH^-. Although we cannot rule out this possibility, the base efflux remaining in the presence of 437 extracellular K^+ (e.g., *bc* in Fig. 7 *A*) cannot be the result of a passive flux. For example, in axons dialyzed to a pH_i of ~8.0, the mean pH_i at which base efflux was computed (i.e., segment *bc*) was 7.87. Thus, the mean E_H was −7.4 mV, slightly more negative than the mean V_m of +1.6 mV. This difference is ~9 mV, which is probably larger than the cumulative errors in measuring pH and V_m, and suggests that a passive flux should have produced, if anything, a pH_i increase during *bc*, rather than the decrease that we consistently observed. The case against passive fluxes is stronger for axons dialyzed to a pH_i of 7.75. Because these cells had a mean pH_i of 7.59 during *bc*, their mean E_H was −24.1 mV. This is substantially more negative than the mean V_m of +1.0 mV. Thus, we can conclude that the pH_i decrease in segment *bc*, observed with substantial levels of K^+ present both inside and outside the axon, could not have been a result of the passive influx of H^+ or to the passive efflux of OH^-.

Analysis of K–H exchange hypothesis. Although K–H exchange could have been responsible for part of the pH_i decrease observed in K^+-loaded axons exposed to a K^+-free ASW (*ab* in Fig. 7 *A*), it did not contribute to the pH_i decrease observed when K^+ was added to the ASW (*bc* in Fig. 7 *A*). For axons dialyzed to a pH_i of 8.00, both the H^+ gradient and the K^+ gradient would cause a hypothetical K–H exchanger to function in the direction of net H^+ efflux during segment *bc*, not the net influx needed to explain our data. The conclusion concerning the H^+ gradient is reached by comparing the mean pH_i at time of analysis (i.e., 7.87) with the pH_o of 8.00; thus, the outward H^+ chemical gradient is 1.35:1. Because $[K^+]_i$ was 400 mM and $[K^+]_o$ was 437 mM, there would be an inward K^+ chemical gradient of 1.09:1.

For axons dialyzed to a pH_i of 7.75, the thermodynamic argument against K–H exchange is even stronger. Because the mean pH_i at the time of analysis was 7.59 (pH_o = 8.00), there would be an outward H^+ chemical gradient of ~2.6:1 to go along with the inward K^+ chemical gradient of 1.09:1. Thus, at both pH_i values, the pH_i decrease in segment *bc* could not have been caused by K–H exchange.

Analyses of hypotheses invoking HCO_3^- transport. Evaluating whether the data from Fig. 7 for segment *bc* are consistent with either a passive efflux of HCO_3^- or K/HCO_3 cotransport model is difficult because we cannot estimate the actual $[HCO_3^-]_i$, which was almost certainly greater than the nominal value of zero. If axonal metabolism generated a mild in-to-out HCO_3^- gradient, as is reasonable to suppose, the depolarization caused by increasing $[K^+]_o$ would slow but not eliminate a passive HCO_3^- efflux. Similarly, an increase in $[K^+]_o$ per se would slow but

not eliminate K/HCO_3 cotransport. Thus, the data from Fig. 7 are consistent with both the HCO_3^--efflux and the K/HCO_3-cotransport models.

Effect of Increasing Extracellular and Intracellular $[HCO_3^-]$

Fig. 8 *A* shows an experiment in which an axon, previously dialyzed with a pH 8.00/400-mM-K^+ DF, was exposed to an ASW containing 0.5% CO_2/12 mM HCO_3^-. The figure shows the part of the experiment after dialysis had been halted, and pH_i decreased at the fairly rapid pace (*ab*) characteristic of a 400-mM internal K^+. The

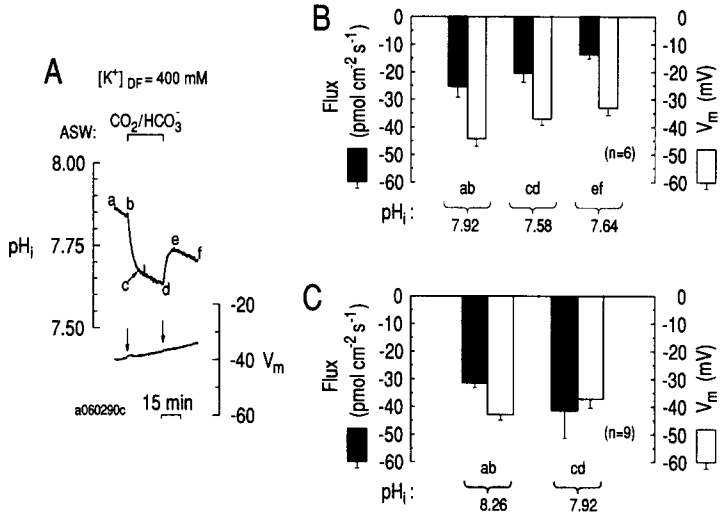

FIGURE 8. Effect of adding 0.5% CO_2/12 mM HCO_3^- to the ASW in axons dialyzed with a 400 mM K^+ fluid. (*A*) Experiment showing the time course of pH_i after dialysis was halted. During the indicated time, CO_2/HCO_3^- was added without changing pH_o. (*B*) Summary of paired data, obtained in six experiments such as shown in panel *A*, in which the pH of the DF was 8.00. The left pair of bars indicates the mean flux and V_m before addition of CO_2/HCO_3^- (segment *ab* in *A*); the center pair, during the latter part of the CO_2/HCO_3^- exposure (*cd*); the right pair, after CO_2/HCO_3^- removal (*ef*). The mean pH_i values for the periods in which the flux and V_m data were computed are given below the braces. Note the substantial difference in pH_i values corresponding to the absence and presence of CO_2/HCO_3^-. (*C*) Summary of similar paired data, obtained in nine experiments in which the pH of the DF was 8.30. In these experiments, the axons were not returned to a CO_2/HCO_3^--free ASW.

subsequent exposure to 0.5% CO_2/12 mM HCO_3^- elicited a rapid pH_i decrease (*bc*), because of the influx of CO_2, the subsequent hydration to form H_2CO_3, and the dissociation of H_2CO_3 to form intracellular HCO_3^- and H^+. However, pH_i continued to fall fairly rapidly in the presence of CO_2/HCO_3^- (*cd*). Removing the CO_2/HCO_3^- caused a rapid pH_i increase (*de*), because of CO_2 efflux, followed by a slower decline (*ef*) that presumably reflects the continuation of the same process evident in *ab*. The three pairs of bars in Fig. 8 *B* summarize the paired results from six similar experiments on axons dialyzed to pH_i 8.00 in which we measured the acid–base

flux before (*ab*), during (*cd*), and after (*ef*) an exposure to CO_2/HCO_3^-. Fig. 8 *C* summarizes results for experiments on nine axons dialyzed to pH_i 8.30 in which we measured the flux before (*ab*) and during (*cd*) an exposure to CO_2/HCO_3^-. Comparing the fluxes computed for segments *ab* and *cd* is difficult because the pH_i data were obtained at very different pH_i values, and acid–base transport can be very sensitive to differences in pH_i. One approach is to compare the *cd* and *ef* values for the axons dialyzed to pH_i 8.00 (Fig. 8 *B*), inasmuch as the mean pH_i values are rather similar. Although the net base efflux was somewhat greater in the presence of than in the absence of CO_2/HCO_3^-, the difference was not statistically significant (paired *t* test, two tail).

Another approach is to compare data obtained in the presence of CO_2/HCO_3^- (e.g., segment *cd* in Fig. 8 *A*) with those from other experiments in which we dialyzed axons to pH_i 7.75–7.81, halted dialysis, and then determined base efflux in

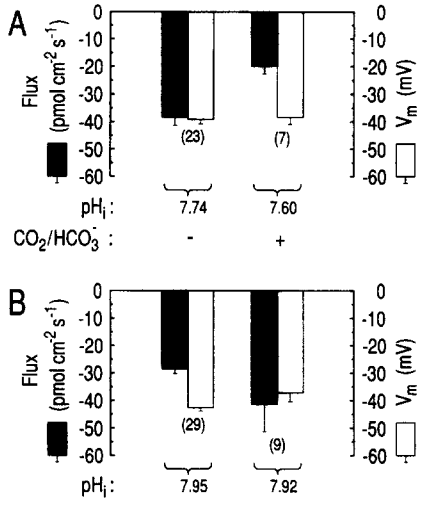

FIGURE 9. Effect of CO_2/HCO_3^-, comparing unpaired axons approximately matched to the same pH_i values. (*A*) The left pair of bars summarizes data for axons dialyzed to a pH_i of ~7.75, in which flux and V_m values were obtained immediately after dialysis was halted. These are the same data summarized by the left pair of bars in Fig. 2 *D*. The right pair of bars summarizes the segment-*cd* data from seven experiments similar to that shown in Fig. 8 *A* in which the pH_{DF} was 8.00. (*B*) The left pair of bars summarizes data for axons dialyzed to a pH_i of 8.00, in which flux and V_m values were obtained immediately after dialysis was halted. These are the same data summarized by the middle pair of bars in Fig. 2 *D*. The right pair of bars summarizes the segment-*cd* data from nine experiments similar to that shown in Fig. 8 *A* in which the pH_{DF} was 8.30.

the absence of CO_2/HCO_3 (e.g., segment *bc* in Fig. 2 *B*). We performed a total of 23 such experiments in which we determined base efflux in the nominal absence of CO_2/HCO_3^- at a mean pH_i of 7.74. As summarized by the left pair of bars in Fig. 9 *A*, the mean base efflux was 38.3 pmol cm^{-2} s^{-1}, which is significantly greater ($P < 0.0002$) than the flux observed for seven unpaired experiments in the presence of CO_2/HCO_3^- (right pair of bars). Although the mean pH_i was significantly greater in the absence of (pH_i 7.74) than in the presence of CO_2/HCO_3^- (pH_i 7.60), it is unlikely that the difference in fluxes was only a result of a difference in pH_i, inasmuch as the data summarized by the solid bars in Fig. 2 *D* suggest that base efflux does not fall steeply with decreases in pH_i.

Fig. 9 *B* summarizes the results from experiments similar to those shown in Fig. 9 *A*, except that the mean pH_i values at which the fluxes were determined were

higher than for Fig. 9 *A* and more closely matched. The data obtained in the absence of CO_2/HCO_3^- (left pair of bars) were obtained from axons dialyzed to a pH_i of 8.00, whereas the data obtained in the presence of CO_2/HCO_3^- (right pair of bars) were obtained after axons dialyzed to pH_i 8.30 were exposed to CO_2/HCO_3^-. At very similar mean values of pH_i (7.92–7.95) and V_m (−37 to −42 mV), the mean flux was somewhat higher in the presence of CO_2/HCO_3^-, although the difference did not reach statistical significance. Thus, applying CO_2/HCO_3^- did not significantly affect base efflux in the higher pH_i range, but probably inhibited base efflux in the lower pH_i range.

Analysis of hypotheses invoking the passive fluxes of H^+ or HCO_3^-. At point *c* in Fig. 8 *A*, pH_i was ~7.65, the computed nominal $[HCO_3^-]_i$ was ~5.4 mM, and V_m was ~−38 mV. Because the nominal $[HCO_3^-]_o$ was 12 mM, the equilibrium potential for HCO_3^- (and also for H^+) was −20.5 mV. Thus, the electrochemical gradients for both HCO_3^- and H^+ at point *c* would favor fluxes that would decrease pH_i, consistent with the observed pH_i decrease during segment *cd*. Similar conclusions can be reached for the mean CO_2/HCO_3^- data summarized in Fig. 8, *B* and *C*. Although a passive efflux of HCO_3^- could explain the pH_i decrease during *cd* in Fig. 8 *A*, it is not clear why adding CO_2/HCO_3^- to the ASW should have failed to increase net HCO_3^- efflux significantly. As will be seen in the Discussion, unless CO_2/HCO_3^- had an idiosyncratic effect on a passive HCO_3^- efflux, these data are inconsistent with the HCO_3^--efflux model.

Analysis of the K–H exchange hypothesis. Our observation that CO_2/HCO_3^- did not significantly affect base efflux at a pH_i of ~8.0 (Fig. 9 *B*) is consistent with K–H exchange, which should not be affected by CO_2/HCO_3^- per se. However, unless CO_2/HCO_3^- had an idiosyncratic effect on K–H exchange, the data suggesting inhibition of base efflux by CO_2/HCO_3^- at a pH_i of ~7.6–7.7 (Fig. 9 *A*) are inconsistent with the K–H exchange model.

Analysis of the K/HCO_3 cotransport model. If K/HCO_3 cotransport were governed by simple Michaelis–Menten kinetics, and if the actual concentrations of $[K^+]$ and $[HCO_3]$ in the unstirred layer around the axon were close to those assumed, then we would have expected K/HCO_3 cotransport to have been stimulated when we added CO_2/HCO_3^- to the ASW. However, as we note in the Discussion, if there was significant K^+ in the unstirred layer surrounding the axon, then it is easy to construct a model in which CO_2/HCO_3^- fails to stimulate, or even inhibits, K/HCO_3 cotransport. Thus, the data summarized in Figs. 8 and 9 are not inconsistent with the K/HCO_3 cotransport model.

Effect of Increasing Both Extracellular $[K^+]$ and $[HCO_3^-]$

If base efflux were mediated by a K/HCO_3 cotransporter, then the pH_i decrease observed in axons dialyzed with 400 mM K^+ ought to be blocked by the combination of high external $[K^+]$ and CO_2/HCO_3^-. In the experiment shown in Fig. 10 *A*, the axon had been dialyzed to a pH_i of 8.30 with a DF containing 400 mM K^+. The figure shows part of the experiment immediately after we halted dialysis, unmasking a relatively rapid intracellular acidification (*ab*). Although subsequently introducing an ASW containing 437 mM K^+ and 12 mM HCO_3^- caused an immediate decrease in pH_i (*bc*), because of the influx of CO_2, it prevented any further decrease in pH_i

after CO_2 equilibrated across the cell membrane (*cd*). In fact, pH_i drifted upward during *cd* in this experiment and a total of five of the nine similar experiments in which we dialyzed axons to pH_i 8.30, and 7 of the 13 in which we dialyzed axons to pH_i 8.0.

Fig. 10 *B* summarizes the paired results of 13 similar experiments in which axons were dialyzed to pH_i 8.00. Fig. 10 *C* does the same for axons dialyzed to pH_i 8.30. As noted in the presentation of Fig. 8, it is difficult to draw conclusions comparing the data from segments *ab*, *cd*, and *ef* in Fig. 10 *A* because the pH_i values are so different. Therefore, in Fig. 11 *A* we compare the data for segment *cd* from Fig. 10 *B* with

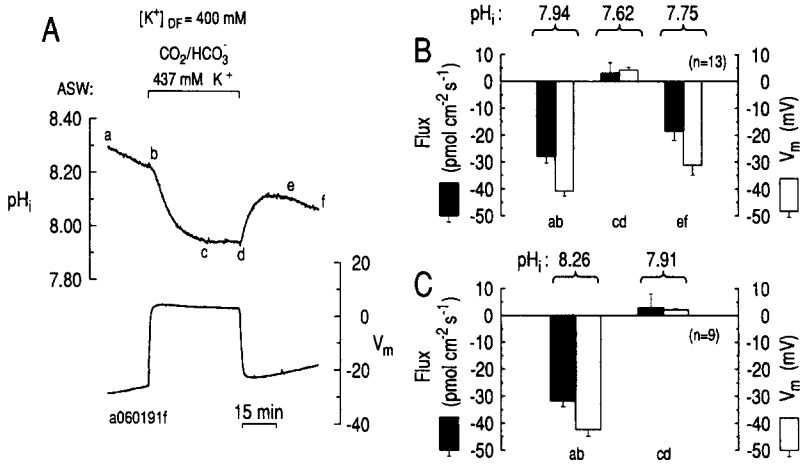

FIGURE 10. Effect of increasing both $[K^+]_o$ and $[CO_2/HCO_3^-]_o$ in experiments in which axons were dialyzed to a $[K^+]_i$ of 400 mM. (*A*) Experiment showing the time course of pH_i after dialysis with a pH-8.30 solution was halted. During the indicated time, K^+ and CO_2/HCO_3^- were added simultaneously without changing pH_o. (*B*) Summary of paired data, obtained in 13 experiments such as shown in *A*, in which the pH of the DF was 8.00. The left pair of bars indicates the mean flux and V_m before addition of CO_2/HCO_3^- (segment *ab* in *A*); the center pair, during the latter part of the CO_2/HCO_3^- exposure (*cd*); the right pair, after CO_2/HCO_3^- removal (*ef*). The mean pH_i values for the periods in which the flux and V_m data were computed are given above the braces. (*C*) Summary of similar paired data, obtained in nine experiments in which the pH of the DF was 8.30. In these experiments, the axons were not returned to a CO_2/HCO_3^-–free ASW.

pH_i-matched controls in which axons were dialyzed to pH_i 7.75 and fluxes were measured immediately after dialysis was halted. Fig. 11 *B* shows a similar comparison between the data for segment *cd* of Fig. 10 *C* and pH_i-matched controls in which axons were dialyzed to pH_i 8.00. In both cases, the net acid–base flux in the simultaneous presence of extracellular K^+ and HCO_3^- was not significantly different from zero. Thus, introduced together, K^+ and HCO_3^- eliminate base efflux.

Analysis of passive flux models. One explanation for why extracellular K^+ and HCO_3^- halted the pH_i decrease during segment *bc* in Fig. 10 *A* is that the segment-*ab* acidification was either a result of a passive H^+ influx or OH^-/HCO_3^- efflux, and

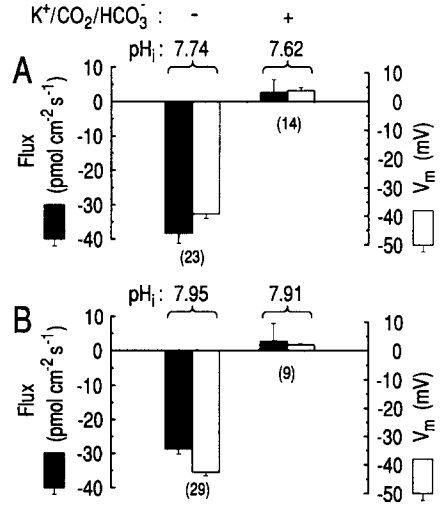

FIGURE 11. Effect of both K^+ and CO_2/HCO_3^-, comparing unpaired axons approximately matched to the same pH_i values. (*A*) The left pair of bars summarizes data for axons dialyzed to a pH_i of 7.75, in which flux and V_m values were obtained immediately after dialysis was halted. These are the same data summarized by the left pair of bars in Fig. 2 *D*. The right pair of bars summarizes the segment-*cd* data from 14 experiments similar to that shown in Fig. 10 *A*, except that the pH_{DF} was 8.00. (*B*) The left pair of bars summarizes data for axons dialyzed to a pH_i of 8.00, in which flux and V_m values were obtained immediately after dialysis was halted. These are the same data summarized by the middle pair of bars in Fig. 2 *D*. The right pair of bars summarizes the segment-*cd* data from nine experiments similar to that shown in Fig. 10 *A* in which the pH_{DF} was 8.30.

that the electrochemical gradients for these ions were erased or inverted by the K^+-induced depolarization. To test this hypothesis, we exposed an axon to CO_2/HCO_3^- while depolarizing with a combination of Na^+ and 250 μM veratridine; the latter blocks the inactivation of Na^+ channels and thereby increases Na^+ conductance. In

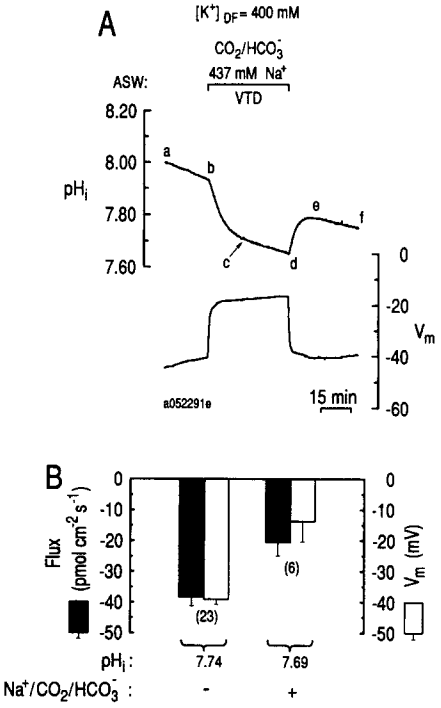

FIGURE 12. Effect of raising both $[Na^+]_o$ and $[CO_2/HCO_3^-]_o$, and simultaneously adding 250 μM veratridine, in experiments in which axons were dialyzed to a $[K^+]_i$ of 400 mM. (*A*) Experiment showing the time course of pH_i after dialysis with a pH 8.00 solution was halted. During the indicated time, Na^+, veratridine, and CO_2/HCO_3^- were added simultaneously without changing pH_o. (*B*) Summary of unpaired data, obtained from axons at approximately the same pH_i values. The left pair of bars summarizes data for axons dialyzed to a pH_i of 7.75, in which flux and V_m values were obtained immediately after dialysis was halted. These are the same data summarized by the left pair of bars in Fig. 2 *D*. The right pair of bars summarizes the segment-*cd* data from six experiments similar to that shown in *A*. The mean pH_i values for the periods in which the flux and V_m data were computed are given below the braces.

the experiment shown in Fig. 12 *A*, the axon had previously been dialyzed as in the previous experiments to a pH_i of 8.00 with a DF containing 400 mM K^+. Simultaneously introducing 437 mM Na^+, veratridine, and 0.5% CO_2/12 mM HCO_3^- caused a depolarization of ~25 mV as well as a rapid CO_2-induced acidification (*bc*), followed by a slower, continuing decline (*cd*). The data from six similar experiments are compared in Fig. 12 *B* to unpaired controls having approximately the same mean pH_i; the simultaneous presence of Na^+ and CO_2/HCO_3^- reduced base efflux by about half. This result is consistent with the hypothesis that a portion of the segment-*ab* pH_i decrease in Fig. 12 *A* was the result of a passive flux of H^+, HCO_3^-, or both, and that the depolarization caused by the introduction of Na^+ eliminated these passive fluxes and thereby slowed the acidification.

Fig. 12 *B*, however, also shows that, even in the presence of Na^+ and CO_2/HCO_3^-, substantial base efflux remained. In the six similar experiments summarized in Fig. 12 *B*, the average base efflux during segment *cd* was 20.5 pmol cm^{-2} s^{-1} at a time when the average V_m was −13.7 mV. Thus, the mean equilibrium potential for H^+ and HCO_3^- was −18.3 mV under these conditions, so that H^+ and HCO_3^- were very close to being in equilibrium across the plasma membrane. If anything, these ions were driven by slight gradients favoring fluxes that would have alkalinized the axon. Thus, our observation that axons acidified rather briskly in the 437-Na^+/12-HCO_3^- ASW implies that the segment-*cd* acidification in Fig. 12 *A* could not have been mediated by the passive flux of H^+ or HCO_3^-.

Analysis of the model of K–H exchange. If base efflux were the result of K–H exchange, then base efflux should have been blocked by introducing K^+ alone into the ASW. Instead, we found that base efflux was not blocked by K^+ alone (see Fig. 7), but only by the combination of K^+ and CO_2/HCO_3^-. These results are thus inconsistent with K–H exchange.

Analysis of the K/HCO_3 cotransport model. If base efflux were mediated by K/HCO_3 cotransport, then the pH_i decrease should have been abolished by simultaneously raising $[K^+]_o$ to approximately match $[K^+]_i$, and forcing $[HCO_3^-]_i$ to approximate $[HCO_3^-]_o$. This was indeed achieved at pH_i values of ~8.0 (Fig. 10 *C*). Thus, the data are consistent with the K/HCO_3 cotransport model.

Effect of Potential Inhibitors

Because SITS and DIDS interact with the $NMDG^+$ in the 0/0/0/0 ASW, we were unable to examine the possible effects of these compounds on base efflux. We found that neither 100 μM Sch28080 (n = 5), 1 mM Zn^{2+} (n = 2), nor 50 μM of the amiloride analogue HMA (n = 1) had an effect on the rate of acidification in axons either dialyzed to a $[K^+]$ of 400 mM or loaded with K^+ during a subsequent exposure to a high K^+ ASW.

DISCUSSION

Evidence Supporting Forward K/HCO_3 Cotransport in K^+-loaded Axons

The recovery of pH_i from an alkali load is supported by intracellular K^+ or Rb^+. The results described in this paper show that pH_i in squid giant axons recovers from an alkali

load via a mechanism that, at least in part, is stimulated by predialyzing the axon with a solution containing 400 mM K^+ or Rb^+. The lower rate of pH_i recovery (i.e., decrease) that occurs in axons dialyzed with a K^+-free DF may reflect the presence of intracellular K^+ not removed by dialysis, K^+ introduced by leakage from the V_m electrode, or both. However, we cannot rule out the possibility that a portion of this basal pH_i decrease reflects a K^+-independent process (e.g., H^+ production via metabolism). In axons dialyzed with K^+ or Rb^+, but not those dialyzed with Li^+, Na^+, or Cs^+, the pH_i recovery was accelerated by decreasing pH_o. This observation suggests that intracellular K^+ and Rb^+ support a mechanism that mediates the efflux of base equivalents from the axon.

Na^+-dependent Cl-HCO_3 exchange is unlikely to be involved. Because experiments were routinely conducted in the absence of intracellular and extracellular Na^+ and Cl^-, it is most unlikely that the Na^+-dependent Cl-HCO_3 exchanger previously identified in squid axons contributed to the pH_i recovery. Experiments described in the accompanying paper (Hogan et al., 1995) show that K^+-dependent base influx, which may be mediated by the same mechanism that mediates base efflux, is not blocked by either SITS or DIDS.

The combination of K^+ and HCO_3^- in the ASW block the pH_i recovery. Fig. 13 summarizes, for two different pH_i ranges, the effects of introducing into the ASW either K^+ alone, CO_2/HCO_3^- alone, K^+ plus CO_2/HCO_3^-, or Na^+ plus CO_2/HCO_3^-. Our observation that the pH_i recovery from the alkaline load in axons dialyzed with 400 mM K^+ was blocked by introducing K^+ and HCO_3^- simultaneously into the ASW, but not by other combinations, is consistent with the hypothesis that the pH_i decrease is a result of K/HCO_3 cotransport. An alternative hypothesis is that introducing K^+ and CO_2/HCO_3^- into the ASW blocks the pH_i decrease not by eliminating the gradient for net K/HCO_3 efflux, but by stimulating an entirely unrelated mechanism, the action of which opposes the acidifying effects of base efflux. However, consideration of data presented in the accompanying paper (Hogan et al., 1995)

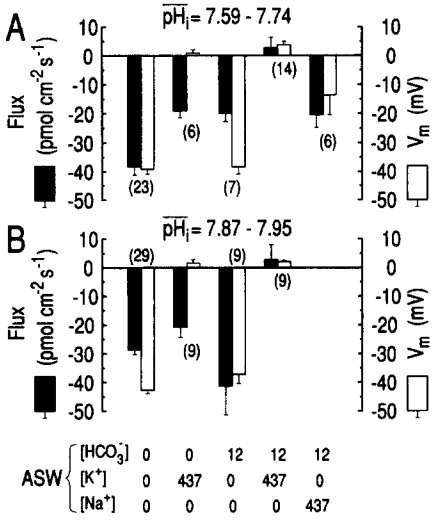

FIGURE 13. Summary of the effects on base efflux of adding to the ASW K^+ only, CO_2/HCO_3^- only, both K^+ and CO_2/HCO_3^-, or both Na^+ and CO_2/HCO_3^-. (*A*) Data obtained at a low range of mean pH_i values (i.e., 7.59–7.74). (*B*) Data obtained at a high range of mean pH_i values (i.e., 7.87–7.95). Solid bars are mean fluxes; open bars are mean V_m values obtained over comparable time periods; numbers of observations are given in parentheses; vertical hash marks indicate SEM values.

makes this explanation unlikely. In that paper, we demonstrate that, for axons previously dialyzed with a K^+-free fluid, simultaneously introducing K^+ and HCO_3^- to the ASW caused an initial CO_2-induced acidification that was followed by a robust pH_i increase. At a pH_i of 7.8, the rate of this pH_i increase corresponded to an equivalent net base influx of ~55 pmole cm^{-2} s^{-1}. Because base exited the axons at a mean rate of ~10 pmol cm^{-2} s^{-1} in the absence of K^+ and HCO_3^- (see Fig. 2 *C*), we conclude that the combination of 437 mM K^+ and 12 mM HCO_3^- in the ASW causes net base influx to increase by ~65 pmol cm^{-2} s^{-1}. In axons dialyzed with 400 mM K^+, introducing K^+ plus HCO_3^- into the ASW causes net base efflux to decrease by only ~41 pmol cm^{-2} s^{-1} at a pH_i of ~7.6–7.7 (see Fig. 10 *B*), from −38.3 to +2.6 pmole cm^{-2} s^{-1}. Thus, the net change in acid–base flux in the present experiments on K^+-dialyzed axons is only ~63% as large as in the experiments described in the accompanying paper on axons dialyzed with a K^+-free DF. Thus, it is most likely that the combination of K^+ and HCO_3^- blocks base efflux in the present experiments by eliminating the gradient favoring K/HCO_3 efflux.

K^+ in the ASW slowed the pH_i recovery. The slowing of base efflux caused by introducing extracellular K^+ (by itself) into the ASW is consistent with the K/HCO_3 efflux hypothesis. We envisage that, in axons dialyzed with 400 mM K^+, metabolically generated HCO_3^- supports a rather significant K/HCO_3 efflux. In the extracellular unstirred layer, accumulation of exiting K^+ and HCO_3^- would promote unidirectional K/HCO_3 influx and thus slow net base efflux. Adding 437 mM K^+ to the ASW would drastically increase $[K^+]$ in this extracellular unstirred layer and thus slow net base efflux, even if the bulk ASW was HCO_3^- free.

The Effect of CO_2/HCO_3^- on Base Efflux

An analysis of unpaired experiments suggests that adding CO_2/HCO_3^- to the ASW slowed the pH_i recovery at pH_i ~7.6–7.7 and had a statistically insignificant stimulatory effect at pH_i ~8.0. If our hypothesis that base efflux from K^+-loaded axons is mediated by K/HCO_3 cotransport is correct, then a major question is why CO_2/HCO_3^- appears to have inhibited base efflux at pH_i values in the range ~7.6–7.7 (see Fig. 8 *B*). That CO_2/HCO_3^- should have had any effect at all on base efflux argues strongly in favor of the involvement of HCO_3^- transport, inasmuch as we would not have expected CO_2/HCO_3^- to modulate K–H exchange or passive H^+/OH^- fluxes. But why should CO_2/HCO_3^- inhibit K/HCO_3 cotransport? We can offer two explanations that are not mutually exclusive:

1. The hypothetical K/HCO_3 cotransporter could saturate at relatively low levels of intracellular HCO_3^-. Under the conditions of our experiments, the hypothetical K/HCO_3 cotransporter may be fully active at the relatively low $[HCO_3^-]_i$ levels at or somewhat higher than those prevailing in the nominal absence of CO_2/HCO_3^-, but saturated by increasing $[HCO_3^-]_i$ to the levels achieved (e.g., 6–12 mM, depending on pH_i) when 0.5% CO_2/12 mM HCO_3^- is added to the ASW. One can also envision a scenario in which sufficient increases in $[HCO_3^-]_i$ could inhibit K/HCO_3 efflux.

2. Introducing CO_2/HCO_3^- into the ASW increases the reverse reaction (K/HCO_3 influx) more than it stimulates the forward reaction (K/HCO_3 efflux). Central to this argument is that $[K^+]$ in the extracellular unstirred layer ($[K^+]_{UL}$) is suffi-

ciently high to support a considerable K/HCO_3 influx when CO_2/HCO_3^- is added to the ASW. Imagine that an axon previously dialyzed with a 400 mM K^+ DF is exposed to CO_2/HCO_3^-–free ASW. As suggested above, the de novo generation of HCO_3^- via metabolism may be adequate to maintain $[HCO_3^-]_i$ at sufficiently high levels that K/HCO_3 cotransport functions in the net outward direction. We can only crudely approximate four key concentrations expected to affect the rate of a hypothetical K/HCO_3 cotransporter, $[K^+]_i$, $[K^+]_{UL}$, $[HCO_3^-]_i$, and $[HCO_3^-]_{UL}$.

$[K^+]_i$ at the time of our assays is probably somewhat less than the nominal value of 400 mM, because (*a*) $[K^+]_i$ may never have reached $[K^+]_{DF}$ during dialysis; and (*b*) some K^+ probably leaked out of the axon after we halted dialysis, but before we added CO_2/HCO_3^-.

$[K^+]_{UL}$ was probably in the range of several millimolar. Given a net K^+ efflux of 100 pmol cm^{-2} s^{-1}, Frankenhauser and Hodgkin (1956) estimated that the steady-state $[K^+]_{UL}$, which is proportional to the net K^+ efflux, is ~2 mM higher than in the bulk ASW. Because of the unusual makeup of our DF and ASWs, which caused the axons to be moderately depolarized (thereby favoring K^+ efflux via K^+ channels), which would have ensured a substantial K^+ loss via putative K/HCO_3 cotransport and which would have minimized K^+ uptake via either the Na-K pump or Na/K/Cl cotransporter, net efflux of K^+ could have been > 100 pmol cm^{-2} s^{-1}. Thus, $[K^+]_{UL}$ may have been even > 2 mM.

We will assume that $[HCO_3^-]_i$ was ~1 mM (the concentration in air-equilibrated ASW would be ~0.7 mM), and $[HCO_3^-]_{UL}$ was substantially less, 0.2 mM.

Introducing CO_2/HCO_3^- into the ASW would have increased both $[HCO_3^-]_i$ and $[HCO_3^-]_{UL}$, although it is likely that the fractional increase in $[HCO_3^-]_{UL}$ would have been substantially greater. For example, if pH_i were 7.7, equilibration with 0.5% CO_2 would have dictated a $[HCO_3^-]_i$ of 6 mM. If de novo formation of CO_2/HCO_3^- were enough to raise $[HCO_3^-]_i$ by an additional 1 mM (see previous paragraph), the total $[HCO_3^-]_i$ would have increased from 1 to 7 mM, or by sevenfold. Adding 0.5% CO_2/12 mM HCO_3^- to the ASW would have caused steady state $[HCO_3^-]_{UL}$ to increase from 0.2 to 12.2 mM, or by >60-fold. The effect of these increases in $[HCO_3^-]$ on unidirectional K/HCO_3 efflux and influx depends on the kinetics of these reactions, about which we have no information. However, if the apparent K_m values for HCO_3^- were the same as the axon's Na^+-dependent $Cl\text{-}HCO_3$ exchanger, 2.3 mM (Boron and Russell, 1983), then increasing $[HCO_3^-]_i$ from 1 to 7 mM would increase base efflux by a factor of ~2.5, whereas increasing $[HCO_3^-]_{UL}$ from 0.2 to 12.2 mM would increase base influx by a factor of >10. Thus, depending on the absolute values of base efflux and influx before the introduction of CO_2/HCO_3^-, introducing CO_2/HCO_3^- could easily have led to a greater increase in K/HCO_3 influx than in K/HCO_3 efflux, and thus caused net base efflux to decrease, as observed in the pH_i range 7.6–7.7 (Fig. 8 *B*).

It is interesting that the above analysis predicts that the stimulatory effect of added CO_2/HCO_3^- on K/HCO_3 efflux would be greater at higher pH_i values. This is because, at an elevated pH_i, more intracellular HCO_3^- would be formed from CO_2 entering from the ASW. Indeed, we found that added CO_2/HCO_3^- inhibited net base efflux at pH_i in the 7.6–7.7 range, but produced a modest although statistically insignificant stimulation when pH_i was ~8.0 (Fig. 8 *C*).

Preliminary work with an out-of-equilibrium CO_2/HCO_3^- ASW. If the above analysis is correct, then we should be able to stimulate net K/HCO_3 efflux from K^+-loaded axons by increasing $[HCO_3^-]_i$ but not $[HCO_3^-]_{UL}$. In a more recent study, Zhao et al. (1995) have extended the present work by using a rapid-mixing/stopped-flow technique to generate two out-of-equilibrium CO_2/HCO_3^- ASWs having a pH of 8.0, one with a $[CO_2]$ of 0.25% but an estimated $[HCO_3^-]$ of only ~61 μM, and the other with a $[CO_2]$ of 0.5% but an estimated $[HCO_3^-]$ of 122 μM. If these ASWs had been at equilibrium, the extracellular $[HCO_3^-]$ values instead would have been ~7 and 12 mM, respectively. Given a pH_i of 7.7, introducing the out-of-equilibrium ASW with a $[CO_2]$ of 0.25% should have caused $[HCO_3^-]_i$ to increase by ~3 mM, and the one with a $[CO_2]$ of 0.5% should have caused $[HCO_3^-]_i$ to increase by ~6 mM. However, in neither case should $[HCO_3^-]_{UL}$ have increased substantially. Indeed, both out-of-equilibrium ASWs increased base efflux by a factor of more than two. These data thus support the unstirred-layer hypothesis and demonstrate that intracellular HCO_3^- does indeed stimulate base efflux in K^+-loaded axons.

Evidence Against the Sole Involvement of Other "Base Efflux" Mechanisms

Evidence against the sole involvement of metabolism. Although we cannot rule out the possibility that the metabolic generation of acid is responsible for all or part of the baseline acidification in axons dialyzed with either $NMDG^+$ or TEA^+ (e.g., Fig. 2 *A*), it is unlikely for three reasons that metabolic events could mediate the enhanced acidification observed in axons dialyzed with K^+: (*a*) it is not clear why the metabolic generation of acid should be stimulated by intracellular K^+ or Rb^+ (Figs. 2 and 3). (*b*) It is not obvious why metabolism should be inhibited by CO_2/HCO_3^- in the 7.6–7.7 pH_i range, but not in the 8.0 pH_i range (Fig. 8). (*c*) One would not have expected metabolism to be blocked by adding to the ASW the combination of K^+ and CO_2/HCO_3^- (Fig. 10), especially when, by itself, intracellular K^+ stimulates base efflux (Fig. 2).

Evidence against the sole involvement of a H^+ conductance. Although it is possible that passive H^+ influx or passive OH^- efflux contributes to the background pH_i decrease observed in axons dialyzed with either $NMDG^+$ or TEA^+ (e.g., Fig. 2), it is unlikely for two reasons that passive fluxes could mediate all of the enhanced acidification observed in axons loaded with K^+: (*a*) the pH_i decrease in axons exposed to K^+ only in the ASW (Fig. 7) cannot be explained by a passive flux of H^+ or OH^-. (*b*) The pH_i decrease in axons exposed to Na^+, veratridine, and CO_2/HCO_3^- in the ASW (Fig. 12 *A*) also cannot be explained by a passive flux.

Evidence against the sole involvement of a HCO_3^- conductance. A passive efflux of HCO_3^- also could have contributed to the baseline pH_i decrease observed in axons dialyzed with cations other than K^+ or Rb^+. However, two lines of evidence suggest that HCO_3^- efflux is not the sole mechanism of the pH_i decrease observed in axons dialyzed with K^+: (*a*) the pH_i decrease observed in axons exposed to Na^+, veratridine and CO_2/HCO_3^- in the ASW (Fig. 12 *A*) goes against the HCO_3^- electrochemical gradient. (*b*) It is not clear why, if base efflux were produced by the passive egress of HCO_3^-, that the pH_i decrease in K^+-loaded axons was not substantially stimulated by 0.5% CO_2/12 mM HCO_3^- in all pH_i ranges. The Goldman-Hodgkin-Katz (GHK) equation (Goldman, 1943; Hodgkin and Katz, 1949) predicts that,

given reasonable estimates for $[HCO_3^-]_i$ and $[HCO_3^-]_{UL}$ in the nominal absence of HCO_3^-, adding 0.5% CO_2/12 mM HCO_3^- to the ASW should have markedly stimulated base efflux. For example, at a V_m of −50 mV and a temperature of 22°C, the GHK equation predicts that simultaneously increasing $[HCO_3^-]_i$ from 1 to 7 mM, and $[HCO_3^-]_{UL}$ from 0.2 to 12.2 mM, should have caused the passive HCO_3^- efflux to increase by a factor of ~5.4. If instead we assume that $[HCO_3^-]_{UL}$ increased from 1 to 13 mM, the fractional increase in computed HCO_3^- influx is ~6.0.

Evidence against sole involvement of a K–H exchanger. For four reasons, it is unlikely that an exchange of intracellular K^+ for extracellular H^+ made the major contribution to the accelerated pH_i decrease observed in K^+-loaded axons (e.g., Fig. 2): (*a*) the pH_i decrease in axons dialyzed with 400 mM K^+ was not eliminated when $[K^+]_o$ was increased from 0 to 437 mM, even though a K–H exchanger should have been equilibrated or reversed under such conditions. (*b*) It is not clear why base efflux should have been inhibited by CO_2/HCO_3^- at a pH_i of 7.6–7.7 if the pH_i decrease was mediated by a K–H exchanger. (*c*) It is not clear why base efflux should have been eliminated by the simultaneous introduction of K^+ and CO_2/HCO_3^- if the pH_i decrease were mediated by a K–H exchanger. Finally (*d*) one might have expected a K–H exchanger to have been inhibited by Sch28080 (Hofer and Machen, 1992).

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