K⁺- and HCO₃⁻-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 (pHi) after using internal dialysis to load squid giant axons with alkali to pHi 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 pHi. Under these conditions, halting dialysis unmasked a slow pHi 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 (pHo) 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 pHo. 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 HCO₃⁻ in the ASW, which eliminates the gradient favoring a hypothetical K⁺/HCO₃⁻ efflux, blocked pHi recovery in K⁺-dialyzed axons. However, the pHi recovery was not blocked by the combination of 437 mM Na⁺, veratridine, and CO₂/HCO₃⁻ in the ASW, a treatment that inverts electrochemical gradients for H⁺ and HCO₃⁻ and would favor passive H⁺ and HCO₃⁻ fluxes that would have alkalinized the axon. Similarly, the recovery was not blocked by K⁺ alone or HCO₃⁻ alone in the ASW, nor was it inhibited by the K⁺ pump blocker Sch28080 nor by the Na–H exchange inhibitors amiloride and hexamethylenaminolride. Our data suggest that a major component of base efflux in alkali-loaded axons cannot be explained by metabolism, a H⁺ or HCO₃⁻ conductance, or by a K–H exchanger. However, this component could be mediated by a novel K/HCO₃⁻ cotransporter.

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INTRODUCTION

It has been known for some time that intracellular pH (pHi) in squid giant axons is regulated, at least in part, by an ion transporter that appears to exchange extracellular Na⁺ and HCO₃⁻ 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-HCO₃ exchangers are blocked by stilbene derivatives such as DIDS, and respond to decreases in pHi by extruding acid equivalents from the cell, thereby returning pH₃ toward its initial value. Although the squid axon uses a Na⁺-dependent Cl-HCO₃ 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 HCO₃⁻, and is sensitive to disulfonic stilbene derivatives (Vaughan-Jones, 1982; Chaillet, Amsler, and Boron, 1986). In contrast to the Na⁺-dependent Cl-HCO₃ exchanger, this Na⁺-independent Cl-HCO₃ exchanger can function in the total absence of Na⁺ and is stimulated by increases in pH₃. Certain epithelial cells, including renal proximal tubule cells (Boron and Boulpaep, 1983), possess an electrogenic Na/HCO₃ cotransporter that normally moves Na⁺ and HCO₃⁻ out of cells and thereby decreases pH₃. Similar to the Na⁺-dependent Cl-HCO₃ exchanger, the electrogenic Na/HCO₃ cotransporter is blocked by DIDS. However, it does not transport Cl⁻. Finally, Leveil, Borensztein, Houillier, Paillard, and Bichara (1992) suggested that a DIDS-sensitive K/HCO₃ cotransporter can contribute to the recovery of pH₃ from alkali loads in medullary thick ascending limbs from rat kidney.

The present study was initiated in an attempt to identify a Cl-HCO₃ exchanger in squid axons internally dialyzed to a pH₃ of ~8.0 (initial pH₃ ~ 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 pH₃ recovery (i.e., decrease) that requires intracellular K⁺ (or Rb⁺). This pH₃ decrease can be blocked by the simultaneous presence of K⁺ and HCO₃⁻ in the seawater, but not by a combination of Na⁺, veratridine, and HCO₃⁻ that produces similar pH₃ and Vₘ changes, nor by either K⁺ or HCO₃⁻ alone. Thus, a major component of this “base efflux” is most easily accounted for by a novel K/HCO₃ 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 CO₂/HCO₃⁻ to the seawater caused a rapid pH₃ decrease (because of the influx of CO₂), followed by a sustained pH₃ increase, at least part of which appears to be the result of “base influx.” This pH₃ increase is not inhibited by disulfonic stilbene derivatives, even at high doses. Because base influx cannot be produced by introducing either K⁺ alone or CO₂/HCO₃⁻ alone, it is most likely mediated by the same novel K/HCO₃ cotransporter that is responsible for the pH₃ 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 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 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 HCO₃⁻-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 Mg²⁺, 3.0 Ca²⁺, 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 CO₂/HCO₃⁻-free solutions were gassed with 100% oxygen to minimize the concentration of dissolved CO₂. All artificial seawaters contained 10⁻⁵ M ouabain.
We made an ASW free of K⁺, Na⁺, and Cl⁻ but containing 12 mM HCO₃⁻ by replacing 12 mM gluconate in the 0/0/0/0 ASW with 12 mM HCO₃⁻. This solution (0/0/0/12-HCO₃⁻ ASW) was made by (a) adding all the components except CO₂/HCO₃⁻ 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% CO₂ until the pH stabilized at 8.00. As discussed previously, the actual [HCO₃⁻]₀ is less than the nominal [HCO₃⁻]₀, because of the formation of CO₂ ion pairs with Na⁺, Mg²⁺, and Ca²⁺ (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-HCO₃⁻ ASW by replacing 12 mM gluconate in the 437-K⁺ ASW with 12 mM HCO₃⁻.

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 NH₄NO₃.

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 CO₂-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 Mg²⁺, 16 Tris⁺, 414 glutamate, 4 ATP⁻, 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), pHi data were acquired by computer. Rates of pHi change (dpHi/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 HCO₃⁻ (or other base). J is thus positive for fluxes that produce a pHi increase (base influx), and negative for fluxes that produce a pHi decrease (base efflux). We computed J as the product of dpHi/dt, total intracellular buffering power (β₀), and volume-to-surface ratio. β₀ was taken as the sum of the intrinsic buffering power (βᵢ, measured as described below) and the open-system CO₂/HCO₃⁻ buffering power (βₜHCO₃⁻, which we assumed to be the theoretical value of ln10 × [HCO₃⁻]). More recent work, in which we actually measured βᵢ in experiments in which axons were exposed to CO₂/HCO₃⁻, confirms the assumption that βᵢ is the sum of β₀ and the computed βₜHCO₃⁻ (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 NH3/NH4+. Before the exposure to, NH3/NH4+ the axons were internally dialyzed with either a 400 mM K+ or a 400 mM NMDG+ dialysis fluid. So that we could determine the pHi dependence of βh, the DFs had pH values ranging from 7.70 to 8.30. So that the NH3/NH4+ induced pHi increase was consistently ~0.2, we matched the total NH3/NH4+ concentration to the pH of the DF: 2.5-5 mM for a pHDF of 7.70 or 7.75, 5-10 mM for a pHDF of 8.00, and 10-40 mM for a pHDF of 8.30. Fig. 1A shows a buffering-power experiment in which an axon was dialyzed to a pHi of 8.30 with a K+-free fluid. After dialysis was halted and pHi drifted slowly downward (see Results), exposing the axon to an ASW containing 40 mM total NH3/NH4+ caused a pHi increase owing to the influx of the weak base NH3, whereas removing the NH3/NH4+ had the opposite effect. We computed βh from the equation βh = Δ[NH4+]i/ΔpHi. To compensate for drift in the pHi baseline after application of the NH3/NH4+ ASW, we fitted the pHi vs.-time record after NH3/NH4+ removal (after a time when all NH3 was judged to have left the axon) with a line and back-extrapolated this line to a time late during the NH3/NH4+ exposure (see inset of Fig. 1A). The [NH4+]i at the end of the NH3/NH4+ exposure was calculated from the final pHi, the pHo, and [NH4+]o, assuming that NH3 was equilibrated across the axon membrane: [NH4+]i = [NH4+]o × 10([pH]i - [pH]o). ΔpHi was taken as the difference in pHi values between the extrapolated line and the final pHi record during the NH3/NH4+ exposure, as shown in the figure. In each experiment, the pHi associated with the βh value was the average of these two pHi values.

Fig. 1B summarizes the pHi dependence of βh for the two dialysis fluids noted above. βh 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, βh increased with pHi in the presence of...
NMDG. In our calculations of acid-base fluxes (see above), we assumed that β₁ 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 [3t for axons dialyzed with 400 mM TEA⁺ is approximately the same as for axons dialyzed with 400 mM K⁺. Although the β₁-vs.-pHi relationship for 400 mM K⁺ extended to a pHi of only ~8.1, we used the line of best fit for flux calculations at pHi 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 pHᵢ 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 pHᵢ 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 pHᵢ 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 pHᵢ 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 HCO₃⁻. Under these conditions of ion replacement, all known acid-loading HCO₃ transporters (i.e., Cl⁻-HCO₃ exchangers and electrogenic Na/HCO₃ cotransporters) should have been blocked. After dialysis with a K⁺-free DF was halted in the experiment shown in Fig. 2 A, pHᵢ decreased at a rather low rate (bc). Moreover, the rate of intracellular acidification was not affected appreciably by decreasing the pH of the ASW (pHₒ) 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 pHᵢ decrease after the cessation of dialysis (bc) was far higher than in the first case. In addition, the rate of intracellular acidification twice¹ was increased by reducing pHₒ from 8.00 to

¹. We found in other experiments that, even at a fixed pHₒ of 8.00, the rate of intracellular acidification gradually decreased as pHᵢ 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 pHₒ 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 pHᵢ was lowered to 7.0 for a second time (ef vs. de), and slowed again when pHₒ 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_i 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 (pHDF 7.75 bars), 7.991 ± 0.006 (pHDF 8.0 bars), and 8.287 ± 0.004 (pHDF 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 (pHDF 7.75 bars), 7.948 ± 0.007 (pHDF 8.0 bars), and 8.244 ± 0.007 (pHDF 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 \( \text{pH} \) of 8.00 (\( n = 59 + 29 = 88 \), middle pair of bars in Fig. 2, C and D) and in axons dialyzed to \( \text{pH} \) 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 tetroethylammonium (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.

**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 \( \text{pH} \) 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 \( \text{pH} \) 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 \( \sim \)19 pmol cm\(^{-2}\) s\(^{-1}\) greater than the baseline flux, and for Na\(^+\), \( \sim \)24 pmol cm\(^{-2}\) s\(^{-1}\).

**Effect of decreasing \( \text{pH}_o \) to 7.00 in axons dialyzed with Li\(^+\), Na\(^+\), Rb\(^+\), or Cs\(^+\).** Although each of the Group-IA cations tested supported a postdialysis \( \text{pH} \) decrease, the data of Fig. 5 do not address the issue of whether the mechanism of the \( \text{pH} \) decrease was the same in all cases. For example, loading an axon with a particular cation could lead to metabolic changes that produce a \( \text{pH} \) 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 \( \text{pH} \).
because of the displacement of H+ from Ca2+/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 pHo 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 bcddefg in Fig. 2 B. As can be seen by comparing the rates of pH decrease at a pHo of 7.00 with those immediately before and after (i.e., at a pHo of 8.00), extracellular acidification increased the rate of pH decrease only when the cation was Rb+. It is of interest that in the experiment shown with Na+, we removed Ca2+ from the ASW; in this experiment, apparent base efflux was indistinguishable from that observed in axons dialyzed with NMDG+ or TEA+, and decreasing pHo seems to have slowed the pH decrease.

The paired analyses (pHo 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 pHo, 8.00 in the immediate postdialysis period, when pH was relatively high; (b) during the exposure to the pHo 7.00 ASW, when pHo was somewhat lower; and (c) after returning pHo to 8.00, when pHo was lowest. We averaged the fluxes as well as pH and Vm values in periods a and c, and compared these averages with the comparable values for period b. For each cation, average pH and Vm 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 pHo. Decreasing pHo 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 pHo actually caused a decrease in base efflux. We conclude that the pH decreases observed in axons dialyzed with Li+, Na+, or Cs+ are unrelated mech-
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 ($P<0.05$).

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. 5), 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$^+$-dia
dyzed 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$^+$], or [Rb$^+$].

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-
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+]Dv to 400 mM would stimulate K-H exchange and speed the pHi decrease. This model makes two important predictions: (a) base efflux should be blocked or even reversed (depending on the pHi and pHo values) by sufficiently increasing [K+]o. For example, if pHi were the same as pHo (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 CO2/HCO3.

A fourth possibility is that the pHi decrease in Fig. 2 B was mediated by a novel K/HCO3 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 CO2/HCO3 into the ASW. (b) Base efflux should be blocked by simultaneously increasing both [K+]o and [HCO3]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 pHi of 8.00. The figure shows the portion of the experiment after dialysis had been halted and pHi began to fall (ab). Because the ASW had a pH of 8.00, pHi was

![Figure 7](image-url)
pH throughout this experiment. If the pH 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 $\gg$ [K$^+$]i. However, Fig. 7A shows that, by itself, raising [K$^+$]o to 437 mM modestly slowed but did not abolish the pH decrease (bc vs. ab and cd). Similar observations were made in axons dialyzed to a pH of 7.75. On average, the inhibition of base efflux was $\sim$37% in paired experiments among axons dialyzed to a pH of 7.75 (Fig. 7B), and $\sim$30% for axons dialyzed to a pH of 8.00 (Fig. 7C).

**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. 7A) cannot be the result of a passive flux. For example, in axons dialyzed to a pH of $\sim$8.0, the mean pH at which base efflux was computed (i.e., segment bc) was 7.87. Thus, the mean Eh was $-7.4$ mV, slightly more negative than the mean Vm of $+1.6$ mV. This difference is $\sim0$ mV, which is probably larger than the cumulative errors in measuring pH and Vm, and suggests that a passive flux should have produced, if anything, a pH increase during bc, rather than the decrease that we consistently observed. The case against passive fluxes is stronger for axons dialyzed to a pH of 7.75. Because these cells had a mean pH of 7.59 during bc, their mean Eh was $-24.1$ mV. This is substantially more negative than the mean Vm of $+1.0$ mV. Thus, we can conclude that the pH 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 decrease observed in K$^+$-loaded axons exposed to a K$^+$-free ASW (ab in Fig. 7A), it did not contribute to the pH decrease observed when K$^+$ was added to the ASW (bc in Fig. 7A). For axons dialyzed to a pH 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 at time of analysis (i.e., 7.87) with the pHo of 8.00; thus, the outward H$^+$ chemical gradient is 2.6:1 to go along with the inward K$^+$ chemical gradient of 1.09:1. For axons dialyzed to a pH of 7.75, the thermodynamic argument against K–H exchange is even stronger. Because the mean pH at the time of analysis was 7.59 (pHo = 8.00), there would be an outward H$^+$ chemical gradient of $\sim2.6:1$ to go along with the inward K$^+$ chemical gradient of 1.09:1. Thus, at both pH values, the pH 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$_3$]o, 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/HCO₃ cotransport. Thus, the data from Fig. 7 are consistent with both the HCO₃-efflux and the K/HCO₃-cotransport models.

**Effect of Increasing Extracellular and Intracellular [HCO₃⁻]**

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% CO₂/12 mM HCO₃⁻. The figure shows the part of the experiment after dialysis had been halted, and pHᵢ decreased at the fairly rapid pace (ab) characteristic of a 400-mM internal K⁺. The subsequent exposure to 0.5% CO₂/12 mM HCO₃ elicited a rapid pHᵢ decrease (bc), because of the influx of CO₂, the subsequent hydration to form H₂CO₃, and the dissociation of H₂CO₃ to form intracellular HCO₃⁻ and H⁺. However, pHᵢ continued to fall fairly rapidly in the presence of CO₂/HCO₃⁻ (cd). Removing the CO₂/ HCO₃ caused a rapid pHᵢ increase (de), because of CO₂ 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 pHᵢ 8.00 in which we measured the acid-base
flux before (ab), during (cd), and after (ef) an exposure to CO₂/HCO₃⁻. Fig. 8 C summarizes results for experiments on nine axons dialyzed to pH 8.30 in which we measured the flux before (ab) and during (cd) an exposure to CO₂/HCO₃⁻. Comparing the fluxes computed for segments ab and cd is difficult because the pH data were obtained at very different pH values, and acid-base transport can be very sensitive to differences in pH. One approach is to compare the cd and ef values for the axons dialyzed to pH 8.00 (Fig. 8 B), inasmuch as the mean pH values are rather similar. Although the net base efflux was somewhat greater in the presence of than in the absence of CO₂/HCO₃⁻, the difference was not statistically significant (paired t test, two tail).

Another approach is to compare data obtained in the presence of CO₂/HCO₃⁻ (e.g., segment cd in Fig. 8 A) with those from other experiments in which we dialyzed axons to pH 7.75–7.81, halted dialysis, and then determined base efflux in the absence of CO₂/HCO₃⁻ (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 CO₂/HCO₃⁻ at a mean pH 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 CO₂/HCO₃⁻ (right pair of bars). Although the mean pH was significantly greater in the absence of (pH 7.74) than in the presence of CO₂/HCO₃⁻ (pH 7.60), it is unlikely that the difference in fluxes was only a result of a difference in pH, 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.

Fig. 9 B summarizes the results from experiments similar to those shown in Fig. 9 A, except that the mean pH 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 CO₂/HCO₃ (left pair of bars) were obtained from axons dialyzed to a pHᵢ of 8.00, whereas the data obtained in the presence of CO₂/HCO₃ (right pair of bars) were obtained after axons dialyzed to pHᵢ 8.30 were exposed to CO₂/HCO₃. At very similar mean values of pHᵢ (7.92–7.95) and Vᵥm (−37 to −42 mV), the mean flux was somewhat higher in the presence of CO₂/HCO₃, although the difference did not reach statistical significance. Thus, applying CO₂/HCO₃ did not significantly affect base efflux in the higher pHᵢ range, but probably inhibited base efflux in the lower pHᵢ range.

Analysis of hypotheses invoking the passive fluxes of H⁺ or HCO₃. At point c in Fig. 8 A, pHᵢ was ~7.65, the computed nominal [HCO₃]ᵢ was ~5.4 mM, and Vᵥm was ~−38 mV. Because the nominal [HCO₃]ₒ was 12 mM, the equilibrium potential for HCO₃ (and also for H⁺) was ~−20.5 mV. Thus, the electrochemical gradients for both HCO₃ and H⁺ at point c would favor fluxes that would decrease pHᵢ, consistent with the observed pHᵢ decrease during segment cd. Similar conclusions can be reached for the mean CO₂/HCO₃ data summarized in Fig. 8, B and C. Although a passive efflux of HCO₃ could explain the pHᵢ decrease during cd in Fig. 8 A, it is not clear why adding CO₂/HCO₃ to the ASW should have failed to increase net HCO₃ efflux significantly. As will be seen in the Discussion, unless CO₂/HCO₃ had an idiosyncratic effect on a passive HCO₃ efflux, these data are inconsistent with the HCO₃-efflux model.

Analysis of the K–H exchange hypothesis. Our observation that CO₂/HCO₃ did not significantly affect base efflux at a pHᵢ of ~8.0 (Fig. 9 B) is consistent with K–H exchange, which should not be affected by CO₂/HCO₃ per se. However, unless CO₂/HCO₃ had an idiosyncratic effect on K–H exchange, the data suggesting inhibition of base efflux by CO₂/HCO₃ at a pHᵢ of ~7.6–7.7 (Fig. 9 A) are inconsistent with the K–H exchange model.

Analysis of the K/HCO₃ cotransport model. If K/HCO₃ cotransport were governed by simple Michaelis–Menten kinetics, and if the actual concentrations of [K⁺] and [HCO₃] in the unstirred layer around the axon were close to those assumed, then we would have expected K/HCO₃ cotransport to have been stimulated when we added CO₂/HCO₃ 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 CO₂/HCO₃ fails to stimulate, or even inhibits, K/HCO₃ cotransport. Thus, the data summarized in Figs. 8 and 9 are not inconsistent with the K/HCO₃ cotransport model.

Effect of Increasing Both Extracellular [K⁺] and [HCO₃]

If base efflux were mediated by a K/HCO₃ cotransporter, then the pHᵢ decrease observed in axons dialyzed with 400 mM K⁺ ought to be blocked by the combination of high external [K⁺] and CO₂/HCO₃. In the experiment shown in Fig. 10 A, the axon had been dialyzed to a pHᵢ 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 HCO₃ caused an immediate decrease in pHᵢ (bc), because of the influx of CO₂, it prevented any further decrease in pHᵢ.
after CO₂ equilibrated across the cell membrane (cd). In fact, pHᵢ drifted upward during cd in this experiment and a total of five of the nine similar experiments in which we dialyzed axons to pHᵢ 8.30, and 7 of the 13 in which we dialyzed axons to pHᵢ 8.0.

Fig. 10 B summarizes the paired results of 13 similar experiments in which axons were dialyzed to pHᵢ 8.00. Fig. 10 C does the same for axons dialyzed to pHᵢ 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 pHᵢ values are so different. Therefore, in Fig. 11 A we compare the data for segment cd from Fig. 10 B with pHᵢ-matched controls in which axons were dialyzed to pHᵢ 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 pHᵢ-matched controls in which axons were dialyzed to pHᵢ 8.00. In both cases, the net acid–base flux in the simultaneous presence of extracellular K⁺ and HCO₃⁻ was not significantly different from zero. Thus, introduced together, K⁺ and HCO₃⁻ eliminate base efflux.

Analysis of passive flux models. One explanation for why extracellular K⁺ and HCO₃⁻ halted the pHᵢ 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⁻/HCO₃⁻ efflux, and
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 CO₂/HCO₃⁻ 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
the experiment shown in Fig. 12 A, the axon had previously been dialyzed as in the
previous experiments to a pH of 8.00 with a DF containing 400 mM K+. Simulta-
neously introducing 437 mM Na+, veratridine, and 0.5% CO2/12 mM HCO3 caused a depolarization of ~25 mV as well as a rapid CO2-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; the simultaneous presence of Na+ and CO2/HCO3 reduced base efflux by about half. This result is consistent with the hypothesis that a portion of the segment-ab pH decrease in Fig. 12 A was the result of a passive flux of H+, HCO3, 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 CO2/HCO3, 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 Vm was ~13.7 mV. Thus, the mean equilibrium potential for H+ and HCO3 was ~18.5 mV under these conditions, so that H+ and HCO3 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-
HCO3 ASW implies that the segment-cd acidification in Fig. 12 A could not have been mediated by the passive flux of H+ or HCO3.

Analysis of the model of K-H exchange. If base efflux were the result of K-H ex-
change, 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 CO2/HCO3. These results are thus inconsistent with K-H exchange.

Analysis of the K/HCO3 cotransport model. If base efflux were mediated by K/HCO3
cotransport, then the pH decrease should have been abolished by simultaneously raising [K+]o to approximately match [K+]i, and forcing [HCO3]i to approximate [HCO3]o. This was indeed achieved at pH values of ~8.0 (Fig. 10 C). Thus, the data are consistent with the K/HCO3 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 Zn2+ (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/HCO3 Cotransport in K+-loaded Axons

The recovery of pH from an alkali load is supported by intracellular K+ or Rb+. The results described in this paper show that pHr 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 pHᵢ 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 Vₘ electrode, or both. However, we cannot rule out the possibility that a portion of this basal pHᵢ 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 pHᵢ recovery was accelerated by decreasing pHₒ. This observation suggests that intracellular K⁺ and Rb⁺ support a mechanism that mediates the efflux of base equivalents from the axon.

Na⁺-dependent Cl⁻-HCO₃ 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⁻-HCO₃ exchanger previously identified in squid axons contributed to the pHᵢ 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 HCO₃⁻ in the ASW block the pHᵢ recovery. Fig. 13 summarizes, for two different pHᵢ ranges, the effects of introducing into the ASW either K⁺ alone, CO₂/HCO₃⁻ alone, K⁺ plus CO₂/HCO₃⁻, or Na⁺ plus CO₂/HCO₃⁻. Our observation that the pHᵢ recovery from the alkaline load in axons dialyzed with 400 mM K⁺ was blocked by introducing K⁺ and HCO₃⁻ simultaneously into the ASW, but not by other combinations, is consistent with the hypothesis that the pHᵢ decrease is a result of K⁺/HCO₃⁻ cotransport. An alternative hypothesis is that introducing K⁺ and CO₂/HCO₃⁻ into the ASW blocks the pHᵢ decrease not by eliminating the gradient for net K⁺/HCO₃⁻ 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](image-url)

**Figure 13.** Summary of the effects on base efflux of adding to the ASW K⁺ only, CO₂/HCO₃⁻ only, both K⁺ and CO₂/HCO₃⁻, or both Na⁺ and CO₂/HCO₃⁻. (A) Data obtained at a low range of mean pHᵢ values (i.e., 7.59–7.74). (B) Data obtained at a high range of mean pHᵢ values (i.e., 7.87–7.95). Solid bars are mean fluxes; open bars are mean Vₘ 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 HCO₃⁻ to the ASW caused an initial CO₂-induced acidification that was followed by a robust pHᵢ increase. At a pHᵢ of 7.8, the rate of this pHᵢ 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 HCO₃⁻ (see Fig. 2 C), we conclude that the combination of 437 mM K⁺ and 12 mM HCO₃⁻ in the ASW causes net base influx to increase by ~65 pmole cm⁻² s⁻¹. In axons dialyzed with 400 mM K⁺, introducing K⁺ plus HCO₃⁻ into the ASW causes net base efflux to decrease by only ~41 pmol cm⁻² s⁻¹ at a pHᵢ 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 HCO₃⁻ blocks base efflux in the present experiments by eliminating the gradient favoring K/HCO₃⁺ efflux.

**K⁺ in the ASW slowed the pHᵢ recovery.** The slowing of base efflux caused by introducing extracellular K⁺ (by itself) into the ASW is consistent with the K/HCO₃ efflux hypothesis. We envisage that, in axons dialyzed with 400 mM K⁺, metabolically generated HCO₃⁻ supports a rather significant K/HCO₃ efflux. In the extracellular unstirred layer, accumulation of exiting K⁺ and HCO₃⁻ would promote unidirectional K/HCO₃ 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 HCO₃⁻ free.

**The Effect of CO₂/HCO₃⁻ on Base Efflux**

An analysis of unpaired experiments suggests that adding CO₂/HCO₃⁻ to the ASW slowed the pHᵢ recovery at pHᵢ ~7.6–7.7 and had a statistically insignificant stimulatory effect at pHᵢ ~8.0. If our hypothesis that base efflux from K⁺-loaded axons is mediated by K/HCO₃ cotransport is correct, then a major question is why CO₂/HCO₃⁻ appears to have inhibited base efflux at pHᵢ values in the range ~7.6–7.7 (see Fig. 8 B). That CO₂/HCO₃⁻ should have had any effect at all on base efflux argues strongly in favor of the involvement of HCO₃⁻ transport, inasmuch as we would not have expected CO₂/HCO₃⁻ to modulate K-H exchange or passive H⁺/OH⁻ fluxes. But why should CO₂/HCO₃⁻ inhibit K/HCO₃ cotransport? We can offer two explanations that are not mutually exclusive:

1. The hypothetical K/HCO₃ cotransporter could saturate at relatively low levels of intracellular HCO₃⁻. Under the conditions of our experiments, the hypothetical K/HCO₃ cotransporter may be fully active at the relatively low [HCO₃⁻] levels at or somewhat higher than those prevailing in the nominal absence of CO₂/HCO₃⁻, but saturated by increasing [HCO₃⁻]; to the levels achieved (e.g., 6–12 mM, depending on pHᵢ) when 0.5% CO₂/12 mM HCO₃⁻ is added to the ASW. One can also envision a scenario in which sufficient increases in [HCO₃⁻]i could inhibit K/HCO₃ efflux.

2. Introducing CO₂/HCO₃⁻ into the ASW increases the reverse reaction (K/HCO₃ efflux) more than it stimulates the forward reaction (K/HCO₃ influx). Central to this argument is that [K⁺] in the extracellular unstirred layer ([K⁺]UL) is suffi-
ciently high to support a considerable K/HCO₃ influx when CO₂/HCO₃ is added to the ASW. Imagine that an axon previously dialyzed with a 400 mM K⁺ DF is exposed to CO₂/HCO₃-free ASW. As suggested above, the de novo generation of HCO₃ via metabolism may be adequate to maintain [HCO₃]ᵢ at sufficiently high levels that K/HCO₃ cotransport functions in the net outward direction. We can only crudely approximate four key concentrations expected to affect the rate of a hypothetical K/HCO₃ cotransporter, [K⁺]ᵢ, [K⁺]ₓL, [HCO₃]ᵢ, and [HCO₃]ₓL.

[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⁺]ₓ during dialysis; and (b) some K⁺ probably leaked out of the axon after we halted dialysis, but before we added CO₂/HCO₃.

[K⁺]ₓL 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⁺]ₓL, 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/HCO₃ 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⁺]ₓL may have been even > 2 mM.

We will assume that [HCO₃]ᵢ was ~1 mM (the concentration in air-equilibrated ASW would be ~0.7 mM), and [HCO₃]ₓL was substantially less, 0.2 mM.

Introducing CO₂/HCO₃ into the ASW would have increased both [HCO₃]ᵢ and [HCO₃]ₓL, although it is likely that the fractional increase in [HCO₃]ₓL would have been substantially greater. For example, if pHᵢ were 7.7, equilibration with 0.5% CO₂ would have dictated a [HCO₃]ᵢ of 6 mM. If de novo formation of CO₂/HCO₃ were enough to raise [HCO₃]ᵢ by an additional 1 mM (see previous paragraph), the total [HCO₃]ᵢ would have increased from 1 to 7 mM, or by sevenfold. Adding 0.5% CO₂/12 mM HCO₃ to the ASW would have caused steady state [HCO₃]ₓL to increase from 0.2 to 12.2 mM, or by >60-fold. The effect of these increases in [HCO₃] on unidirectional K/HCO₃ efflux and influx depends on the kinetics of these reactions, about which we have no information. However, if the apparent Km values for HCO₃ were the same as the axon's Na⁺-dependent Cl-HCO₃ exchanger, 2.3 mM (Boron and Russell, 1983), then increasing [HCO₃]ᵢ from 1 to 7 mM would increase base efflux by a factor of ~2.5, whereas increasing [HCO₃]ₓL 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 CO₂/HCO₃, introducing CO₂/HCO₃ could easily have led to a greater increase in K/HCO₃ influx than in K/HCO₃ efflux, and thus caused net base efflux to decrease, as observed in the pHᵢ range 7.6-7.7 (Fig. 8 B).

It is interesting that the above analysis predicts that the stimulatory effect of added CO₂/HCO₃ on K/HCO₃ efflux would be greater at higher pHᵢ values. This is because, at an elevated pHᵢ, more intracellular HCO₃ would be formed from CO₂ entering from the ASW. Indeed, we found that added CO₂/HCO₃ inhibited net base efflux at pHᵢ in the 7.6-7.7 range, but produced a modest although statistically insignificant stimulation when pHᵢ was ~8.0 (Fig. 8 C).
Preliminary work with an out-of-equilibrium $\text{CO}_2/\text{HCO}_3^-$ 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 [$\text{HCO}_3^-$]$_{\text{i}}$ but not [$\text{HCO}_3^-$]$_{\text{e}}$. 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 [$\text{HCO}_3^-$] of only $\sim$61 $\mu$M, and the other with a [CO$_2$] of 0.5% but an estimated [$\text{HCO}_3^-$] of 122 $\mu$M. If these ASWs had been at equilibrium, the extracellular [$\text{HCO}_3^-$] 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 [$\text{HCO}_3^-$]$_{\text{i}}$ to increase by $\sim$3 mM, and the one with a [CO$_2$] of 0.5% should have caused [$\text{HCO}_3^-$]$_{\text{i}}$, to increase by $\sim$6 mM. However, in neither case should [$\text{HCO}_3^-$]$_{\text{e}}$ 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 $[\text{HCO}_3^-]$ and $[\text{HCO}_3^-]_{UL}$ 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$^\circ$C, the GHK equation predicts that simultaneously increasing $[\text{HCO}_3^-]$, from 1 to 7 mM, and $[\text{HCO}_3^-]_{UL}$ from 0.2 to 12.2 mM, should have caused the passive HCO$_3^-$ efflux to increase by a factor of $\sim 5.4$. If instead we assume that $[\text{HCO}_3^-]_{UL}$ increased from 1 to 13 mM, the fractional increase in computed HCO$_3^-$ influx is $\sim 6.0$.

**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 pHi decrease observed in K$^+$-loaded axons (e.g., Fig. 2): (a) the pHi 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 pHi of 7.6--7.7 if the pHi 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 pHi 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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