K⁺- and HCO₃⁻-dependent Acid–Base Transport in Squid Giant Axons

II. Base Influx

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ABSTRACT We used microelectrodes to determine whether the K/HCO₃ co-transporter tentatively identified in the accompanying paper (Hogan, E. M., M. A. Cohen, and W. F. Boron. 1995. Journal of General Physiology. 106:821–844) can mediate an increase in the intracellular pH (pHi) of squid giant axons. An 80-min period of internal dialysis increased pHi to 7.7, 8.0, or 8.3; the dialysis fluid was free of K⁺, Na⁺, and Cl⁻. Our standard artificial seawater (ASW), which also lacked Na⁺, K⁺, and Cl⁻, had a pH of 8.0. Halting dialysis unmasked a slow pHi decrease. Subsequently introducing an ASW containing 437 mM K⁺ and 0.5% CO₂/12 mM HCO₃ had two effects: (a) it caused membrane potential (V_m) to become very positive, and (b) it caused a rapid pHi decrease, because of CO₂ influx, followed by a slower plateau-phase pHi increase, presumably because of inward cotransport of K⁺ and HCO₃⁻ ("base influx"). Only extracellular Rb⁺ substituted for K⁺ in producing the plateau-phase pHi increase in the presence of CO₂/HCO₃⁻. Mean fluxes with Na⁺, Li⁺, and Cs⁺ were not significantly different from zero, even though V_m shifts were comparable for all monovalent cations tested. Thus, unless K⁺ or Rb⁺ (but not Na⁺, Li⁺, or Cs⁺) somehow activates a conductive pathway for H⁺, HCO₃⁻, or both, it is unlikely that passive transport of H⁺, HCO₃⁻, or both makes the major contribution to the pHi increase in the presence of K⁺ (or Rb⁺) and CO₂/HCO₃⁻. Because exposing axons to an ASW containing 437 mM K⁺, but no CO₂/HCO₃⁻, produced at most a slow pHi increase, K–H exchange could not make a major contribution to base influx. Introducing an ASW containing CO₂/HCO₃⁻, but no K⁺ also failed to elicit base influx. Because we observed base influx when the ASW and DF were free of Na⁺ and Cl⁻, and because the disulfonic stilbene derivatives SITS and DIDS failed to block base influx, Na⁺-dependent Cl–HCO₃⁻ exchange also cannot account for the results. Rather, we suggest that the most straightforward explanation for the pHi increase we observed in the simultaneous presence of K⁺ and CO₂/HCO₃⁻ is the coupled uptake of K⁺ and HCO₃⁻.

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INTRODUCTION

As described in the accompanying paper (Hogan, Cohen, and Boron, 1995), we found that when axons were dialyzed with an alkaline fluid containing 400 mM K⁺ or Rb⁺, pH decreased rather rapidly after dialysis was halted. The “base efflux” that produced this pH recovery could be blocked by the combination of extracellular K⁺ and CO₂/HCO₃⁻, but not by either K⁺ or CO₂/HCO₃⁻ alone. This led us to suggest that base efflux might be mediated by a novel K/HCO₃⁻ cotransporter, inasmuch as alternative hypotheses (i.e., metabolic production of acid, passive fluxes of H⁺/OH⁻, passive efflux of HCO₃⁻, and K-H exchange) failed to account for the data. If the K/HCO₃-cotransporter hypothesis is correct, then it should be possible to elicit an intracellular alkalinization by dialyzing axons with a K⁺-free fluid and then exposing them to an ASW containing both K⁺ and HCO₃⁻. In the experiments described in this paper, we explore the conditions under which such a predicted “base influx” could be produced. We found that a substantial base influx occurs when we simultaneously introduce CO₂/HCO₃⁻ and K⁺ or Rb⁺ (but not Li⁺, Na⁺, or Cs⁺) into the ASW, but not when we introduce CO₂/HCO₃⁻ alone or K⁺ alone. Our results support the hypothesis that squid axons possess a novel K/HCO₃⁻ cotransporter.

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

METHODS

General

We used microelectrodes to measure pH and Vm in internally dialyzed squid giant axons, as described in the accompanying paper (Hogan et al., 1995).

Solutions

Artificial seawaters. Four of the extracellular fluids in this study were the same as those described in the accompanying paper. All ASWs had a pH of 8.00 at 22°C. Our standard ASW (0/0/0/0) contained no Na⁺, K⁺, Cl⁻, or HCO₃⁻. We also employed a variant containing 437 mM K⁺, another containing 0.5% CO₂/12 mM HCO₃⁻, and a third containing both 437 mM K⁺ and CO₂/HCO₃⁻. In addition, we used a series of ASWs containing 0.5% CO₂/12 mM HCO₃⁻ in which the major cation was Li⁺, Na⁺, Rb⁺, or Cs⁺. For the Na⁺-containing ASW, we simply replaced K⁺ with Na⁺ in the 437-K⁺/12-HCO₃⁻ ASW. For the ASWs containing Li⁺, Rb⁺, or Cs⁺, we replaced 425 mM NMDG⁺ in the 12-HCO₃⁻ ASW with the appropriate alkali-metal cation. We made these solutions by titrating 425 mM gluconic acid lactone with sufficient LiOH, RbOH, or CsOH to bring the pH to 8.00. The LiOH was added as a powder (Sigma Chemical Co., St. Louis, MO); the Rb and Cs hydroxides were added as 5% aqueous solutions (obtained from Sigma Chemical Co.). After titrating the solution to pH 8.00, we added an additional 12 mM NMDG free base and titrated the solution back to 8.00 by gassing with 0.5% CO₂ as described above. We added 12 mM NMDG, rather than alkali-metal hydroxides, for this titration by CO₂ because we judged that it would be more accurate to weigh NMDG powder than estimate the OH⁻ content of an aqueous alkali-metal hydroxide solution. However, with this approach, the Li⁺, Rb⁺, and Cs⁺ solutions had only 425 mM of the alkali metal cation, with the other 12 mM being represented by NMDG⁺. We expected the error involved in comparing the effects of the 437-K⁺ (or Na⁺)/12-HCO₃⁻ ASW with the 425-Li⁺ (or Rb⁺).
Cs+/12-HCO₃ ASW to be negligible. We made upward adjustments to pH with the hydroxides of Li⁺, Na⁺, Rb⁺, or Cs⁺. All artificial seawaters contained 10⁻³ M ouabain.

SITS was obtained from United States Biochemical Corp. (Cleveland, OH). Sch28080 was obtained from Schering Corporation (Kenilworth, NJ). Hexamethylenemiloride (HMA) was purchased from E. Cragoe (Nacogdoches, TX). Tenidap was provided by Pfizer, Inc. (Groton, CT). Other chemicals were obtained from Sigma Chemical Co.

**Dialysis fluids.** Our standard internal dialysis fluid (DF) lacked Na⁺, K⁺, and Cl⁻ and was titrated to pH 8.00, as described in the accompanying paper (Hogan et al., 1995).

**Calculation of Acid–Base Transport Rates**

Rates of pH change (dpH/dt) were determined from linear curve fits, performed by computer, to the data, which were acquired by computer. We define acid–base flux (J) as 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 pH increase (base influx), and negative for fluxes that produce a pH decrease (base efflux). We computed J as the product of dpH/dt, total intracellular buffering power (β₁), and volume-to-surface ratio, as described in the accompanying paper (Hogan et al., 1995).

**Statistics**

Results are expressed as the mean ± SEM. For statistical comparisons, we used the paired or unpaired Student's t tests, as indicated in the text, considering P values <0.05 as statistically significant.

**RESULTS**

**Effect of Adding Both K⁺ and CO₂/HCO₃⁻ to the ASW**

In the experiment shown in Fig. 1 A, we dialyzed an axon to a pH of 8.00 using a K⁺-free dialysis fluid (DF), and then halted dialysis, thereby returning control of pH to the axon. As described in the accompanying paper (Hogan et al., 1995), halting dialysis unmasked a pH recovery (i.e., the pH decrease in segment ab) that was rather slow, inasmuch as the intracellular fluid was nominally K⁺ free. We then switched the ASW to one containing 437 mM K⁺ and 0.5% CO₂/12 mM HCO₃⁻. If axons possess a K/HCO₃ cotransporter, then the combination of both K⁺ and HCO₃⁻ in the ASW ought to lead to an influx of K⁺ and HCO₃⁻, and thus an increase in pH. As expected of any cell with a membrane permeable to CO₂, introducing CO₂ caused a rapid pH decrease (bc), because of the influx of CO₂, the hydration of this CO₂ to form H₂CO₃, and the subsequent dissociation of the H₂CO₃ to form HCO₃⁻ and H⁺. As predicted by the K/HCO₃-cotransporter model, this initial acidification was followed by a pH increase during the plateau phase (cd). After the removal of the K⁺ and CO₂/HCO₃⁻, pH increased rapidly, because of the efflux of CO₂ (de), and then declined more slowly (ef). The segment-ef acidification occurred over a pH range in which pH had been relatively stable before the addition of K⁺ and CO₂/HCO₃⁻ (compare ef and ab). Because the accompanying paper showed that dialyzing axons with K⁺ leads to a rather rapid pH decrease (Hogan et al., 1995), we surmise that the pH decrease in segment ef in Fig. 1 A reflects the build-up of K⁺ during segment bed.

Fig. 1 B summarizes the paired flux and Vₘ data for 21 experiments in which the axon was dialyzed to a pH of 8.00; Fig. 1 C summarizes the paired results of six ad-
ditional experiments in which the axon was dialyzed to a pH$_i$ of 8.30. For the axons dialyzed to a pH$_i$ of 8.00, the net influx of base in the presence of K$^+$ and HCO$_3^-$ during segment cd averaged ~58 pmol cm$^{-2}$ s$^{-1}$. In the absence of K$^+$ and HCO$_3^-$ during segment ab, the net acid–base flux was in the opposite direction (i.e., a base efflux), and averaged ~17 pmol cm$^{-2}$ s$^{-1}$. Thus, the addition of K$^+$ and HCO$_3^-$ to the ASW caused the net acid–base flux to shift by 75 pmol cm$^{-2}$ s$^{-1}$ in the direction of base influx. Similarly, for axons dialyzed to pH$_i$ 8.30, introducing K$^+$ and CO$_2$/HCO$_3^-$ caused net base influx to increase by ~42 pmol cm$^{-2}$ s$^{-1}$. Note, however, that comparing the data corresponding to segments ab and cd is complicated by rather different pH$_i$ values during the two segments (see below).

Figure 1. Effect of increasing [K$^+$]$_o$ and [CO$_2$/HCO$_3^-$] in experiments in which axons were dialyzed with a K$^+$-free DF. (A) Experiment showing the time course of pH$_i$ after dialysis with a pH 8.00 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 21 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 (solid bar) and V$_m$ (open bar) 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 six 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.

In the axons dialyzed to a pH$_i$ of 8.00, the net base efflux during segment ef was ~49 pmol cm$^{-2}$ s$^{-1}$, ~32 pmole cm$^{-2}$ s$^{-1}$ higher than the baseline base efflux during segment ab ($P < 0.005$). These data for segments ef and ab were obtained at similar pH$_i$ values.

Because, for the experiment shown in Fig. 1 A, the pH$_i$ values prevailing in the absence of CO$_2$/HCO$_3^-$ (segments ab and ef) were so different from those in the presence of CO$_2$/HCO$_3^-$ (cd), and because acid–base transporters can be very sensitive to changes in pH$_i$, it may not be appropriate to compare the paired flux data
from segments ab and cd as we did above in the discussion of Fig. 1, B and C. Therefore, in Fig. 2 A, we compare data for segment cd from 25 experiments on axons dialyzed to pH 8.00 with pH7-matched controls that consist of segment-ab-type data from 28 experiments on other axons dialyzed to a pH of ~7.8. Comparing experiments matched to a pH of ~7.8 shows that the simultaneous introduction of K+ and CO2/HCO3− shifts a net base efflux of ~10 pmol cm−2 s−1 to a net influx of ~56 pmol cm−2 s−1 (P < 0.0001). For experiments matched to a pH of ~8.0 (Fig. 2 B), K+ and HCO3− caused the flux to shift from an efflux of ~15 to an influx of ~29 pmol cm−2 s−1 (P < 0.002). Thus, comparing data obtained at comparable pH7 values, we see that the simultaneous introduction of K+ and CO2/HCO3− elicits a sizable shift in acid–base transport in the direction of net base influx.

**Effect of Adding both Na+ and CO2/HCO3− to the ASW**

The above data are consistent with the hypothesis that the influxes of K+ and HCO3− are coupled. However, as summarized in Fig. 3, the plateau-phase alkalinization in Fig. 1 A (segment cd) could have at least three other origins. First, the segment-cd pH7 increase could have been the result of the passive efflux of H+. Indeed, an analysis of the data summarized in Fig. 1 B shows that the electrochemical gradient for H+ favored the passive efflux of this ion. The mean pH7 during segment cd was 7.81, so that the mean $E_H$ was $\sim-11$ mV. The mean $V_m$ during segment cd was +16.4 mV. Thus, there existed a ~27-mV gradient favoring the passive efflux of H+ during segment cd, consistent with the observed alkalinization. Second, the segment-cd pH7 increase could have been a result of the passive influx of HCO3−.
cause $E_{HCO_3^-}$ is the same as $E_H$ when CO$_2$ is equilibrated across the cell membrane (Roos and Boron, 1981), there also existed a ~27-mV gradient favoring the passive influx of HCO$_3$. Third, the segment-cd pH$_i$ increase could have been the result of K–H exchange.

To examine more closely the possibility that the passive flux of H$^+$, HCO$_3^-$, or both could account for the data for segment cd in Fig. 1 A, we compare in Fig. 4 A the effects of applying Na$^+$ and CO$_2$/HCO$_3$ with those of applying K$^+$ and CO$_2$/HCO$_3$. In the first CO$_2$/HCO$_3$ pulse of the experiment, we achieved the V$_m$ shift by introducing Na$^+$ and veratridine (which blocks inactivation of Na$^+$ channels) into the ASW. Even though the V$_m$ shift during segment cd of Fig. 4 A (Na$^+$/HCO$_3$)
was as large as that during segment gh (K+/HCO₃⁻), there was no plateau-phase alkalinization during the Na⁺ exposure, but a sizable alkalinization during the K⁺ exposure. Moreover, the acidification after CO₂/HCO₃⁻ washout was much slower after the Na⁺ exposure (ef) than after the K⁺ exposure (ij). Fig. 4 B summarizes the paired Na⁺ data for 10 experiments, and Fig. 4 C summarizes the paired K⁺ data from the same 10 axons. Even though the mean Vₘ values during the CO₂/HCO₃⁻ exposures (segments cd vs. gh) were indistinguishable statistically, the mean acid–base flux was virtually zero during the Na⁺ exposure (segment cd), but ~55 pmole cm⁻² s⁻¹ during the K⁺ exposure (segment gh; P < 0.002). Thus, we can conclude that the segment-cd alkalinization in Fig. 1 A was not a result of the K⁺-induced shift in Vₘ, but rather the presence of extracellular K⁺ per se.

If either a passive efflux of H⁺ or a passive influx of HCO₃⁻ could occur to an appreciable extent in the absence of K⁺, these fluxes should have produced an alkalinization during segment cd in Fig. 4 A. The mean pHᵢ prevailing during the Na⁺ exposure (segment cd) was 7.78, so that E_H and E_HCO₃⁻ were ~−13 mV. Because the mean Vₘ was +14 mV during the Na⁺ exposure, there existed a 27-mV gradient favoring passive fluxes of H⁺ and HCO₃⁻ that would have alkalinized the axon during the Na⁺ exposure.

Because the pHᵢ values prevailing in the presence and absence of Na⁺/CO₂/HCO₃⁻ in the above experiments were rather different, we repeated the analysis of the Na⁺ data to match the fluxes obtained in the presence of Na⁺/CO₂/HCO₃⁻ with controls at a similar pHᵢ. This repeated analysis is similar to that presented in Fig. 2 for K⁺/CO₂/HCO₃⁻ data. In Fig. 5, we compare unpaired data for segment cd obtained on 11 axons dialyzed to a pHᵢ of 8.00 and then exposed to Na⁺/CO₂/HCO₃⁻ with data for segment ab from 28 other axons dialyzed to a pHᵢ of ~7.8. The difference between the acid–base fluxes under the two conditions is not statistically significant.

Unless K⁺ but not Na⁺ can somehow activate a conductive pathway for H⁺, HCO₃⁻, or both, the results presented in Figs. 4 and 5 rule out the passive-H⁺-efflux and passive-HCO₃⁻-influx models as explanations for the plateau-phase pHᵢ increases observed in the simultaneous presence of K⁺ and CO₂/HCO₃⁻.

Effect of Adding Li⁺, Rb⁺, or Cs⁺ Together with CO₂/HCO₃⁻ to the ASW

Rb⁺, but not Li⁺ or Cs⁺, can substitute for K⁺ in supporting base influx in the presence of HCO₃⁻. To determine the selectivity of the putative K/HCO₃ cotransporter
for extracellular cations, we replaced the K⁺ in the K⁺/HCO₃ ASW with either Li⁺, Rb⁺, or Cs⁺. The protocol was similar to that in Fig. 1A. The axon was dialyzed with a DF in which NMDG⁺ was the major cation, dialysis was halted, and then the axon was exposed to an ASW containing 0.5% CO₂/12 mM HCO₃⁻ and one of the aforementioned cations. Typical results for Li⁺, Rb⁺, and Cs⁺ are shown in Fig. 6, A–D.

![Figure 6](image)

**Figure 6.** Ability of various extracellular monovalent cations to support putative base influx. (A) Li⁺. (B) Rb⁺. (C) Cs⁺ in an experiment in which pHᵢ was stable during the plateau phase of the CO₂/HCO₃⁻ exposure. (D) Cs⁺ in an experiment in which pHᵢ increased during the plateau phase of the CO₂/HCO₃⁻ exposure.

In the Li⁺/HCO₃⁻ experiments, pHᵢ was stable during the plateau phase, whereas in the Rb⁺/HCO₃⁻ experiments, pHᵢ invariably increased. Although pHᵢ was often stable during the plateau phase of Cs⁺/HCO₃⁻ experiments, in three of seven cases pHᵢ drifted upward. Interestingly, the upward drift of pHᵢ was correlated with a Vₓ that slowly became more negative during the Cs⁺ exposure and which undershot
the initial $V_m$ after the removal of Cs$^+$. These $pHi$ and $V_m$ data are consistent with the hypothesis that, in some axons, the K/HCO$_3$ cotransporter is capable of accumulating Cs$^+$ and HCO$_3^-$. It should be noted that a correlation between a negative drift in $V_m$ and an alkaline drift in $pHi$ does not hold for cations in general. For example, $V_m$ drifted in the negative direction during an exposure to Na$^+$, even though the acid–base flux was negligible (see Fig. 4).

Average data for all the alkali-metal cations are summarized in Fig. 7. The results show that only extracellular Rb$^+$ can consistently substitute for extracellular K$^+$. The mean fluxes supported by Li$^+$, Na$^+$, and Cs$^+$ were not significantly different from zero, even though the $V_m$ values prevailing in these experiments were not very different from those in the K$^+$ and Rb$^+$ experiments.

**Effect of Increasing Extracellular [K$^+$] in the Absence of CO$_2$/HCO$_3^-$**

Fig. 8 A shows an experiment in which an axon had been dialyzed with a pH-8.00, K$^+$-free DF in which NMDG$^+$ was the major cation. After dialysis was halted, pH$_i$ was rather stable (ab) as long as NMDG$^+$ was the major extracellular cation (see discussion of Fig. 1 A). If the segment-cd $pHi$ increase in Fig. 1 A were mediated by a K–H exchanger, then increasing only [K$^+$]$_o$ should lead to a sizable alkalinization. However, when we replaced 437 mM of the NMDG$^+$ with K$^+$ in the experiment shown in Fig. 8 A, we observed, after a delay of ~15 min, only a very slow pH$_i$ increase. In other experiments, pH$_i$ was stable or decreased slowly during the latter portion of segment bc. In a few experiments (not shown), the K$^+$ exposure evoked a somewhat different series of pH$_i$ changes. After a delay of several minutes (during which time the decay in $V_m$, $b' b''$ in Fig. 8 A was nearly completed), pH$_i$ increased by a small amount and then slowly decreased.

Fig. 8 B summarizes the paired data for 10 experiments in which we dialyzed axons to a pH$_i$ of ~7.8; Fig. 8 C similarly summarizes data from seven other axons dialyzed to a pH$_i$ of 8.00. In the lower pH$_i$ range, introducing K$^+$ did not significantly affect the acid–base flux (see left and center bars in Fig. 8 B); in paired comparisons, K$^+$ caused net base influx to increase by $0.5 \pm 4.2$ pmol cm$^{-2}$ s$^{-1}$. This is substantially less than the increase of 75 pmol cm$^{-2}$ s$^{-1}$ observed when K$^+$ and HCO$_3^-$ were increased simultaneously (see Fig. 2 A). In the higher pH$_i$ range, K$^+$ increased base influx by $20.1 \pm 3.6$ pmol cm$^{-2}$ s$^{-1}$, although this is significantly less than the
increase in base influx observed in a comparable pHi range when K⁺ and CO₂/HCO₃ were simultaneously increased (see Fig. 2 B), 42.1 ± 10.6 pmol cm⁻² s⁻¹ (P < 0.05).

After the extracellular K⁺ was removed in the experiment shown in Fig. 8 A, pHi declined more rapidly than it had before [K⁺], had been increased to 437 mM (compare cd and ab), perhaps reflecting modest K⁺ loading of the axon during the exposure to extracellular K⁺. Consistent with the K⁺-loading hypothesis, Vᵢ was more negative after K⁺ removal (point d) than before the application of K (point a). The rightmost flux bars in Fig. 8, B and C, summarize the data on apparent base efflux during the control period after the exposure to 437 mM K⁺, confirming that

![Figure 8](image-url)

**Figure 8.** Effect of increasing only [K⁺], in axons dialyzed with a K⁺-free DF. (A) Experiment showing the time course of pHi after dialysis was halted. During the indicated time, 437 mM extracellular NMDG⁺ was replaced with K⁺. (B) Summary of paired data, obtained in 10 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 Vᵢ 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). The mean pHi values for the time interval over which the flux and Vᵢ data were obtained are shown above the braces. (C) Summary of similar paired data, obtained in seven experiments in which the pH of the DF was 8.00.

The data summarized in Fig. 8 are not consistent with either the H⁺-conductance or the K-H exchange models for base influx. If the base influx observed in axons dialyzed with a K⁺-free DF and exposed to K⁺/HCO₃ ASW were a result of H⁺ efflux (or OH⁻ influx), then the depolarization caused by the increase in [K⁺], in the experiment shown in Fig. 8 A should have favored a sizable passive efflux of H⁺, and thus a substantial pHi increase. If the base influx observed in axons dialyzed with a K⁺-free DF and subsequently exposed to K⁺/HCO₃, were a result of K-H ex-
change, then the increase in $[K^+]_o$ in the experiment shown in Fig. 8 A should have favored the efflux of $H^+$ in exchange for extracellular $K^+$, and thus caused a substantial pHi increase.

**Effect of Introducing CO$_2$/HCO$_3^-$ in the Absence of Extracellular [K$^+$]**

Fig. 9 A shows an experiment similar to that in Fig. 8 A, except that the axon was exposed to an ASW containing 0.5% CO$_2$/12 mM HCO$_3^-$ but no K$^+$. Applying CO$_2$/HCO$_3^-$ caused a rapid pHi decrease ($bc$), because of the influx of CO$_2$, followed by a slow pHi decline during the plateau phase ($cd$). Removing the CO$_2$/HCO$_3^-$ caused the opposite pHi changes ($def$). Fig. 9, B and C, summarizes paired data from experiments in which we dialyzed axons to pH$_i$ 8.00 and 8.50, respectively. In both pH$_i$ ranges, the mean acid-base flux in the presence of CO$_2$/HCO$_3^-$ ($cd$), was indistinguishable from that in paired control measurements made in the absence of HCO$_3^-$ ($ab$). However, because the mean pH$_i$ values were so different in the presence versus the absence of CO$_2$/HCO$_3^-$, we compared the CO$_2$/HCO$_3^-$ data with segment-$ab$ data from other axons that had been dialyzed to a comparable pH$_i$. The results of this analysis, summarized in Fig. 10, show that net base efflux is not significantly different in the presence versus the absence of CO$_2$/HCO$_3^-$. 

![Diagram](image_url)
Effect of Potential Inhibitors

In three experiments (not shown), we examined how the pH increase elicited by the simultaneous presence of K⁺ and CO₂/HCO₃⁻ was affected by adding either 500 μM SITS or 100 μM DIDS to the ASW. In a fourth instance, we compared, in axons from the same animal, the base influx produced by K⁺ and CO₂/HCO₃⁻ under control conditions with that in the presence of the unusually large dose of 500 μM DIDS. There was no substantial difference in plateau-phase alkalinization rates in the presence (Fig. 11 A) vs the absence of DIDS (Fig. 11 B). The average inhibition for the four cases was −1% ± 11%, indicating that the base influx supported by K⁺ and HCO₃⁻ is not stilbene sensitive. We made similar observations (n = 1 for each case) with 1 mM amiloride, which inhibits Na−H exchange; 50 μM HMA, which also inhibits Na−H exchange; 100 μM Sch28080, which inhibits the gastric...
K-H pump; and 50 μM Tenidap, which inhibits several types of anion transporters (McNiff, Svensson, Pazoles, and Gabel, 1994).

**DISCUSSION**

**Evidence Supporting “Reverse” K/HCO₃ Cotransport in K⁺-depleted Axons**

In the presence of CO₂/HCO₃, base influx is supported by K⁺ or Rb⁺. In the companion paper (Hogan et al., 1995), we found that pHᵢ recovered from alkali loads substantially faster when axons were predialyzed with a fluid containing 400 mM K⁺ or Rb⁺, compared with when they were dialyzed with NMDG⁺ or TEA. Moreover, we found that the pHᵢ decrease supported by intracellular K⁺ was blocked when K⁺ and CO₂/HCO₃ were present together in the ASW, but not when either K⁺ or CO₂/HCO₃ was present alone. These results led us to suggest that a K/HCO₃ cotransporter, functioning in the “forward” or base-efflux direction, can mediate the recovery of pHᵢ from an alkali load. In the present study on axons dialyzed with a K⁺-free fluid, we found that simultaneously introducing CO₂/HCO₃ and either K⁺ or Rb⁺ into the ASW had the opposite effect: After the initial CO₂-induced pHᵢ decrease, both K⁺/CO₂/HCO₃ and Rb⁺/CO₂/HCO₃ elicited a substantial intracellular alkalinization. These results are suggestive of K/HCO₃ cotransport in the “reverse” or base-influx direction.

Fig. 12 summarizes unpaired acid-base flux and Vₘ data for five extracellular conditions: (a) in the absence of K⁺ (or Rb⁺) and CO₂/HCO₃; (b) in the presence of only extracellular K⁺; (c) in the presence of only extracellular CO₂/HCO₃; (d) in the presence of both K⁺ and CO₂/HCO₃; and (e) in the presence of Na⁺, veratridine, and CO₂/HCO₃. Data obtained over a moderately alkaline pHᵢ range

1. These control data were presented in the accompanying paper (Hogan et al., 1995); the remainder of the data summarized in the figure are from the present paper.
(mean pH\textsubscript{i} values between 7.73 and 7.81) are summarized in Fig. 12 A, and data for a more alkaline pH\textsubscript{i} range (mean pH\textsubscript{i} values between 7.99 and 8.04), in Fig. 12 B. It is apparent that only the combination of K\textsuperscript{+} and CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} can elicit base influx, even though comparable depolarizations were produced by K\textsuperscript{+} alone and by the combination of Na\textsuperscript{+}, veratridine, and CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}.

The combination of intracellular K\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-} blocks base influx. As noted above, in the companion paper (Hogan et al., 1995) we found that the recovery of pH\textsubscript{i} from alkali loads in K\textsuperscript{-}-dialyzed axons was blocked by simultaneously introducing K\textsuperscript{+} and CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} into the ASW (see Fig. 11 in the companion paper). Because HCO\textsubscript{3}\textsuperscript{-} is present in both the intracellular and extracellular fluid under such conditions, we took these observations as evidence that coupled K/HCO\textsubscript{3}\textsuperscript{-} efflux is blocked by nullifying the transmembrane gradients for both K\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-}. In the context of the present study, the data from the companion paper can be viewed from the opposite perspective: coupled K/HCO\textsubscript{3}\textsuperscript{-} influx is blocked by nullifying the transmembrane gradients for both K\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-}.

Na\textsuperscript{+}-dependent Cl-HCO\textsubscript{3}\textsuperscript{-} exchange is unlikely to be involved. Although the plateau-phase pH\textsubscript{i} increase that we observed with K\textsuperscript{+} (or Rb\textsuperscript{+}) and CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} present simultaneously in the ASW (see Figs. 1 A and 6 B) is reminiscent of the pH\textsubscript{i} recovery mediated by the Na\textsuperscript{+}-dependent Cl-HCO\textsubscript{3}\textsuperscript{-} exchanger in acid-loaded axons (Boron and De Weer, 1976) three lines of evidence suggest that this similarity is only apparent. In the first place, the present experiments were routinely conducted in the absence of intracellular and extracellular Cl\textsuperscript{-}. The Na\textsuperscript{+}-dependent Cl-HCO\textsubscript{3}\textsuperscript{-} exchanger has an absolute requirement for Cl\textsuperscript{-} (Boron and Russell, 1983), although we note that leakage of Cl\textsuperscript{-} from the V\textsubscript{m} electrode could support a modest degree of Na\textsuperscript{+}-dependent Cl-HCO\textsubscript{3}\textsuperscript{-} exchange. Second, in experiments on acid-loaded axons, the apparent maximal velocity of the Na\textsuperscript{+}-dependent Cl-HCO\textsubscript{3}\textsuperscript{-} exchanger is \(\sim20\) pmol cm\textsuperscript{-2} s\textsuperscript{-1} (Boron and Russell, 1983; Boron and Knakal, 1989, 1992), about one third of the base influx we measured in alkali-loaded axons exposed to K\textsuperscript{+} (or Rb\textsuperscript{+}) and CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}. Third, the pH\textsubscript{i} increase supported by K\textsuperscript{+} and CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} in the present experiments was not blocked by SITS or DIDS, even at very high doses. The Na\textsuperscript{+}-dependent Cl-HCO\textsubscript{3}\textsuperscript{-} exchanger is very stilbene sensitive (Thomas, 1976; Boron and Russell, 1983; Boron and Knakal, 1989).

Evidence Against the Sole Involvement of Other Base-influx Mechanisms

Evidence against a sole contribution from a H\textsuperscript{+} conductance. As noted in the companion paper (Hogan et al., 1995), the axon may indeed have a moderate passive permeability to H\textsuperscript{+}/OH\textsuperscript{-} under the conditions of our experiments. However, if such a pathway for the passive flux of H\textsuperscript{+}/OH\textsuperscript{-} does exist, it can account for only a part of the base influx observed in the present study, and even then, only in one pH\textsubscript{i} range. In the present work, the strongest evidence for a passive H\textsuperscript{+} efflux is the observation that introducing K\textsuperscript{+} (in the absence of CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}) produces a statistically significant decrease in base efflux (increase in base influx) at a pH\textsubscript{i} of \(\sim8.0\) (see Fig. 8 C for paired data, Fig. 12 B for unpaired data). However, even at this pH\textsubscript{i}, K\textsuperscript{+} by itself elicited a far smaller base influx (\(\sim20\) pmol cm\textsuperscript{-2} s\textsuperscript{-1} in a paired comparison) than did the combination of K\textsuperscript{+} and CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} (\(\sim42\) pmol cm\textsuperscript{-2} s\textsuperscript{-1}). At a pH\textsubscript{i} of \(\sim7.75\), the base influx evoked by K\textsuperscript{+} (0.5 pmol cm\textsuperscript{-2} s\textsuperscript{-1}) was statis-
tically insignificant, and far less than the base influx evoked by the combination of $K^+$ and $\text{CO}_2/\text{HCO}_3^-$ ($\sim 75 \text{ pmol cm}^{-2} \text{s}^{-1}$). Thus, in the more alkaline pH$_i$ range (i.e., pH$_i = \sim 8.0$), a passive $H^+$ efflux could account for less than half of total base influx. In the less alkaline pH$_i$ range, a passive $H^+$ efflux cannot account for any of the total base influx.

**Evidence against a major contribution from a HCO$_3^-$ conductance.** The Na$^+$/CO$_2$/HCO$_3^-$ data summarized in Fig. 4 B (paired data) and Fig. 12 (unpaired data) are consistent with a small HCO$_3^-$ or $H^+$ conductance that, at most, could make a minor contribution to total base influx. In paired experiments at a pH$_i$ of $\sim 7.75$, we found that introducing Na$^+$, veratridine, and CO$_2$/HCO$_3^-$ produced a sizable positive shift in $V_m$ ($\sim 27 \text{ mV}$), but a net base influx of only $\sim 17 \text{ pmol cm}^{-2} \text{s}^{-1}$. On the other hand, introducing K$^+$ and CO$_2$/HCO$_3^-$ produced a similar positive shift in $V_m$ ($\sim 23 \text{ mV}$), but a net base influx of $\sim 78 \text{ pmol cm}^{-2} \text{s}^{-1}$, $\sim 4.6$-fold greater than in the case of Na$^+$.

The above data show that when $V_m$ is made more positive by exposing the axon to K$^+$, net base influx in the presence of CO$_2$/HCO$_3^-$ is far larger than when $V_m$ is made more positive by exposing the axon to Na$^+$. However, in both the K$^+$ and Na$^+$ experiments, the electrochemical gradient for HCO$_3^-$ favored a passive HCO$_3^-$ influx. Thus, these data do not rule out the following model: In the presence of K$^+$, the presumed influx of HCO$_3^-$ is mediated via a HCO$_3^-$ conductance that is activated only in the presence of K$^+$ (or Rb$^+$), but not Li$^+$, Na$^+$, or Cs$^+$. However, Fig. 11 in our companion paper, which summarizes experiments in which we exposed K$^+$-loaded axons simultaneously to K$^+$ and CO$_2$/HCO$_3^-$ in the ASW, makes this scenario unlikely. The data summarized in that figure show that the net acid–base flux was approximately zero at a time when $V_m$ averaged $\sim 5 \text{ mV}$ and pH$_i$ averaged 7.62. Because the computed equilibrium potential for HCO$_3^-$ under these conditions is $\sim 22 \text{ mV}$, the gradient driving HCO$_3^-$ into the axon was $\sim 25 \text{ mV}$. Thus, there was no net influx of HCO$_3^-$, even though HCO$_3^-$ was driven by a fairly large gradient, and even though K$^+$ was present both inside and outside the axon in these experiments. Fig. 1 C in the present paper shows that, with K$^+$ present in the ASW but not the DF, net base efflux was rather sizable ($\sim 28 \text{ pmol cm}^{-2} \text{s}^{-1}$) at a time when the HCO$_3^-$ gradient was only $\sim 17 \text{ mV}$. Thus, it is very unlikely that, in experiments such as that shown in Fig. 1 A (segment cd) in the present paper, extracellular K$^+$ promotes a pH$_i$ increase in the presence of CO$_2$/HCO$_3^-$ by activating a HCO$_3^-$ conductance.

**Evidence against a major contribution from K–H exchange.** Two lines of evidence indicate that a K–H exchanger could have made, at most, a small contribution to base influx. First, as noted above in the discussion on a potential H$^+$ conductance, introducing only K$^+$ into the ASW either had no effect on base influx (pH$_i = \sim 7.75$) or produced a modest increase (pH$_i = \sim 8.0$). Second, introducing K$^+$ and CO$_2$/HCO$_3^-$ together produced a major increase in base influx. There is no reason to expect K$^+$ to influence a K–H exchanger only in the presence of CO$_2$/HCO$_3^-$.

**Evidence for K/HCO$_3^-$ Cotransport in Renal Tubules**

Based on experiments in which they studied suspensions of alkali-loaded rat medullary thick ascending limbs, Levisel and colleagues concluded that these cells pos-
sess a DIDS-sensitive K/HCO₃ cotransporter (Leviel, Borenztein, Houillier, Paillard, and Bichara, 1992), presumably at the basolateral membrane. These authors alkali-loaded the renal tubule cells by diluting a 0.2-ml aliquot of a CO₂-equilibrated tubule suspension into a 3-ml vol of CO₂/HCO₃⁻-free saline. The alkali load was produced as intracellular HCO₃⁻ combined with H⁺ to form CO₂, which then rapidly exited the cell and accumulated to a low level (~6% of the physiological [CO₂]) in the stagnant extracellular fluid. This CO₂ washout produced a near-instantaneous pHᵢ increase from ~7.2 to ~7.8 (the alkali load), followed by a slower decrease over the next 60 s to ~7.4 (the pHᵢ recovery). These pHᵢ changes are qualitatively the same as those in segment def of our Fig. 1 A. In the Leviel experiments, [HCO₃⁻]ᵢ would have decreased during the pHᵢ recovery from ~3.7 (pHᵢ ≈ 7.8) to ~1.8 mM (pHᵢ ≈ 7.4). Among the lines of evidence that led Leviel et al. to conclude that the pHᵢ recovery (corresponding to ef in our experiment) was mediated by a DIDS-sensitive K/HCO₃ cotransporter were as follows. First, the rate of pHᵢ recovery was slowed by DIDS but insensitive to removal of extracellular Cl⁻, ruling out Cl⁻-HCO₃⁻ exchange. Second, the pHᵢ recovery in the Leviel experiments was slowed by raising [K⁺]₀ or previously depleting cells of K⁺. These results are consistent with K/HCO₃ cotransport, but do not rule out the possibility that changes in [K⁺] inhibited transport at least in part by producing positive shifts in Vₘ.

Third, extracellular K⁺ activity (a₀ K), monitored with an ion-sensitive electrode, increased as pHᵢ recovered after the CO₂/HCO₃⁻ washout. Although these a₀ K data do not address the issue of whether the time course and magnitude of the a₀ K changes were precisely those expected, they lend qualitative support to the existence of a renal K/HCO₃ cotransporter.

An observation that was inconsistent with K/HCO₃ exchange was that 10⁻⁴ M acetazolamide did not inhibit the pHᵢ recovery, which proceeded at a rate of 0.03 s⁻¹. This rate of pHᵢ decrease was far too fast² to be mediated solely by the uncatalyzed CO₂ hydration reaction. If acetazolamide did indeed block carbonic anhydrase, the observed pHᵢ decrease could have been produced by H⁺ influx or metabolic generation of H⁺.

Thus, a K/HCO₃ cotransporter may be present in the kidney. The DIDS-sensitivity observed in the renal experiments suggests that the transport processes studied by Leviel et al. (1992) and by us are different.

Possible Physiological Role for K/HCO₃ Cotransport

The putative K/HCO₃ cotransporter identified in the present study could help mediate the recovery of pHᵢ from alkali loads, such as those that would occur as the result of a decrease in extracellular Pco₂ (respiratory acidosis). We would expect the Na⁺-dependent Cl⁻-HCO₃⁻ exchanger (the only significant acid extruder in squid ax-

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². If buffering power were 10 mM, base efflux would be 0.3 mM s⁻¹, the rate at which H⁺ appeared in the intracellular fluid because of the reaction sequence CO₂ + H₂O → H₂CO₃ → H⁺ + HCO₃⁻. These reactions would be driven by HCO₃⁻ efflux. The rate-limiting step for H⁺ formation in the absence of carbonic anhydrase would be CO₂ + H₂O → H₂CO₃, which has a rate constant of ~0.08 s⁻¹. Given an intracellular [CO₂] of 0.075 mM, the uncatalyzed hydration reaction could have formed H₂CO₃, and thus H⁺, only at a rate of 0.006 mM s⁻¹, which is 2% of the observed acidification rate.
ons) would be inhibited by such an alkali load. It is intriguing to speculate that the hypothetical K/HCO₃ cotransporter has just the opposite pH dependence, so that the cotransporter might be stimulated by an alkali load. In the steady state, we would expect the K/HCO₃ cotransporter and acid extruder to counteract one another. The hypothetical K/HCO₃ cotransporter could contribute to the recovery of pH from alkali loads in other cell types, beside the squid axon. For example, many cell types exposed to nominally CO₂/HCO₃-free solutions exhibit a background acid-loading mechanism that is unmasked by blocking Na-H exchange (Boyarsky, Ganz, Cragoe, and Boron, 1990). Investigators have long noted that, in a variety of cell types, removing a CO₂/HCO₃ buffer causes a rapid pH increase (because of CO₂ efflux), followed by a slower pH recovery, as described above for the Leviel experiments. Although in principle this pH decrease could be mediated by a Cl-HCO₃ exchanger functioning with ambient HCO₃, in pyramidal neurons isolated from the rat hippocampus these pH recoveries are not blocked by disulfonic stilbene derivatives (Bevensec, M. O., and W. F. Boron, unpublished observations).

Why should cells have evolved a K/HCO₃ cotransporter if intracellular acid loading could be accomplished just as effectively via Cl-HCO₃ exchange? One consideration is that the availability of two acid loaders gives the cell the option of lowering pH either with an accompanying accumulation of Cl⁻ or a depletion of K⁺. A second consideration revolves around effects on cell volume. Because transported HCO₃ is not fully active osmotically, Cl-HCO₃ exchange (in the physiological direction of Cl⁻ uptake) is expected to lead to cell swelling. K/HCO₃ cotransporter, on the other hand, is expected to lead to cell shrinkage. The availability of acid loaders with opposite effects on cell volume would allow the cell to regulate cell pH independently of volume. A third consideration is that the pH and/or pHo dependence of K/HCO₃ cotransport could be substantially different from that of Cl-HCO₃ exchange.

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