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Inhibition of K/HCO_3 Cotransport in Squid Axons by Quaternary Ammonium Ions

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Abstract. Previous squid-axon studies identified a novel K/HCO_3 cotransporter that is insensitive to disulfonic stilbene derivatives. This cotransporter presumably responds to intracellular alkali loads by moving K^+ and HCO_3^- out of the cell, tending to lower intracellular pH (pH_i). With an inwardly directed K/HCO_3 gradient, the cotransporter mediates a net uptake of alkali (i.e., K^+ and HCO_3^- influx). Here we test the hypothesis that intracellular quaternary ammonium ions (QA^+) inhibit the inwardly directed cotransporter by interacting at the intracellular K^+ site. We computed the equivalent HCO_3^- influx (J_{HCO3}) mediated by the cotransporter from the rate of pH_i increase, as measured with pH-sensitive microelectrodes. We dialyzed axons to pH_i 8.0, using a dialysis fluid (DF) free of K^+, Na^+ and Cl^-. Our standard artificial seawater (ASW) also lacked Na^+, K^+ and Cl^-. After halting dialysis, we introduced an ASW containing 437 mM K^+ and 0.5% CO_2/12 mM HCO_3^-, which (i) caused membrane potential to become transiently very positive, and (ii) caused a rapid pH_i decrease, due to CO_2 influx, followed by a slower plateau-phase pH_i increase, due to inward cotransport of K^+ and HCO_3^-. With no QA^+ in the DF, J_{HCO3} was ~58 pmole cm^{-2} sec^{-1}. With 400 mM tetraethylammonium (TEA^+) in the DF, J_{HCO3} was virtually zero. The apparent K_i for intracellular TEA^+ was ~78 mM, more than two orders of magnitude greater than that obtained by others for inhibition of K^+ channels. Introducing 100 mM inhibitor into the DF reduced J_{HCO3} to ~20 pmole cm^{-2} sec^{-1} for tetramethylammonium (TMA^+), ~24 for TEA^+, ~10 for tetrapropylammonium (TPA^+), and virtually zero for tetrabutylammonium (TBA^+). The apparent K_i value for TBA^+ is ~0.86 mM. The most potent inhibitor was phenylpropyltetraethylammonium ($PPTEA^+$), with an apparent K_i of ~91 μM. Thus, trans-side quaternary ammonium ions inhibit K/HCO_3 influx in the potency sequence $PPTEA^+ > TBA^+ > TPA^+ > TEA^+ \cong TMA^+$. The identification of inhibitors of the K/HCO_3 cotransporter, for which no inhibitors previously existed, will facilitate the study of this transporter.

Key words: Intracellular pH — Acid-base — Tetramethylammonium — Tetraethylammonium — Tetrapropylammonium — Tetrabutylammonium

Introduction

Intracellular pH (pH_i) regulation is the result of a delicate balance between processes that tend to alkalinize the cell ("acid extruders"), and others that tend to acidify it ("acid loaders"). Acid extruders include a number of transporters that move H^+ out of the cell and/or move HCO_3^- into the cell. These transporters include the Na-H exchanger (Wakabayashi, Shigekawa & Pouyssegur, 1997), the Na-driven Cl/HCO_3 exchanger (Roos & Boron, 1981) and Na/HCO_3 cotransporters (Deitmer & Schlue, 1989; Romero & Boron, 1999; Choi et al., 1999, 2000; Grichtchenko, Romero, & Boron, 2000) with Na^+:HCO_3^- stoichiometries of 1:2 and 1:1. The known acid-loading transporters generally move HCO_3^- out of the cell, and include the $Cl\text{-}HCO_3$ exchanger (Vaughan-Jones, 1979), the electrogenic Na/HCO_3 cotransporter with a Na^+:HCO_3^- stoichiometry of 1:3 (Boron & Boulpaep, 1983), and the K/HCO_3 cotransporter (Leviel et al., 1992; Hogan, Cohen & Boron, 1995a, 1995b; Zhao et al., 1995). Except for the K/HCO_3 cotransporter in squid axons, all of the aforementioned HCO_3^- transporters are inhibited by disulfonic stilbene derivatives such as DIDS and SITS. Note that the K/HCO_3 cotransporter activity reported in the rat kidney medullary thick ascending limbs is sensitive to DIDS (Leviel et al., 1992).

In the squid giant axon, the major acid extruder is

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the stilbene-sensitive Na^+-driven $Cl\text{-}HCO_3$ exchanger (Russell & Boron, 1976; Boron & Russell, 1983; Boron, Hogan & Russell, 1988). The major acid loader in squid axons appears to be a novel K/HCO_3 cotransporter that, under physiological conditions, would transport K^+ and HCO_3^- out of the cell. Internally dialyzing an axon to a pH_i of 8.0 with a dialysis fluid (DF) containing 400 mM K^+, and then halting dialysis, causes pH_i to decrease spontaneously. The "base efflux" that produces this pH_i decrease is blocked by the combination of K^+ and CO_2/HCO_3^- in the artificial seawater (ASW), but not by either K^+ or CO_2/HCO_3^- individually. These results are consistent with a coupled efflux of K^+ and HCO_3^-. On the other hand, dialyzing the axon with a K^+-free DF, and then introducing K^+ and CO_2/HCO_3^- into the ASW, causes a transient pH_i decrease, due to CO_2 influx, followed by a slower increase. The "base influx" that produces this pH_i increase requires both K^+ and CO_2/HCO_3^- in the ASW, but disappears when one introduces K^+ into the DF (presumably by eliminating the combined K^+/HCO_3^- gradient driving base influx). These data are consistent with a coupled influx of K^+ and HCO_3^-. Rb^+ substitutes for K^+ on the cotransporter, whereas Li^+, Na^+ or Cs^+ do not (Hogan et al., 1995b).

Because the K/HCO_3 cotransporter is not sensitive to stilbene derivatives, and because certain K^+ channels are sensitive to quaternary ammonium ions (QA^+), in the present study we investigated the possibility that QA^+ inhibits the cotransporter by interacting at the intracellular K^+ site. As an assay, we chose the alkalinization produced by K/HCO_3 influx in axons dialyzed with a K^+-free DF. This assay is technically simpler than the acidification produced by K/HCO_3 efflux. We found that, with the K/HCO_3 cotransporter operating in the inward direction, the most potent inhibitor we examined was phenyl-propyltetraethylammonium ($PPTEA^+$), with an apparent K_i of ~91 μM.

Materials and Methods

General Approach

The general approach in these experiments was similar to that used in previous studies on squid axons from this laboratory (Boron & Knakal, 1989, 1992; Hogan et al., 1995a, 1995b). Here we will only briefly summarize. Experiments were conducted at the Marine Biological Laboratory, Woods Hole, MA. We microdissected a 3- to 4-cm length of a giant axon, 400–700 μm in diameter, from squid (*Loligo pealei*), and stored the axon in natural seawater at ~4°C. We cannulated a single axon horizontally at both ends in an internal-dialysis chamber (Brinley, Jr. & Mullins, 1967), and inserted a length of cellulose acetate dialysis tubing (outer diameter = 140 μm; Fabric Research Laboratories, Dedham, MA) down the length of the axon. The dialysis capillary was perfused with dialysis fluid (DF) at a rate of ~2.1 μl/min. Voltage-sensitive microelectrodes (filled with 3M KCl) and pH-sensitive microelectrodes (Hinke, 1967) were inserted into the axon through opposite cannulas, and arranged with their tips within ~500 μm of one another. Electrode signals were amplified by high-impedance electrometers, and the data acquired every 2.5 sec by computer (Boron & Russell, 1983; Boron, 1985). The axon was superfused continuously with artificial seawater (ASW) maintained at 22°C.

We calibrated the pH microelectrodes with high-ionic strength pH buffers (Boron & De Weer, 1976). However, because quaternary ammonium ions at high concentration appeared to shift the offset of the pH-sensitive microelectrode, we regarded the absolute pH_i at the end of the period of dialysis as the same as the pH of the DF. Assuming that the electrode's slope did not change, we calculated all pH_i values for the experiment based on this single-point calibration.

Solutions

Artificial Seawaters

Our standard artificial seawater (0/0/0/0 ASW) was free of Na^+, K^+, Cl^- and HCO_3^-, and buffered to pH 8.00. Its composition was (in mM): 437.2 $NMDG^+$, 62.5 Mg^{++}, 3.0 Ca^{++}, 563 D-gluconate, 0.1 $EDTA^{2-}$, 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. The osmolality, measured with a vapor-pressure osmometer (model 5100C, Wescor, Logan, Utah), was adjusted to 968 ± 5 mOsm/kg with either mannitol or water. This and all other nominally CO_2/HCO_3^--free solutions were gassed with 100% O_2 to minimize dissolved [CO_2]. We made an ASW containing 437 mM K^+ and 12 mM HCO_3^-, but no Na^+ or Cl^-, by replacing $NMDG^+$ in the 0/0/0/0 ASW with K^+, and by replacing 12 mM gluconate with 12 mM HCO_3^- (ref. Hogan et al., 1995a). All ASWs contained 10^{-5} M ouabain. The ASWs were delivered to the chamber through Tygon tubing.

Dialysis Fluids

Our standard internal dialysis fluid (DF) contained no Na^+, K^+ or Cl^-, and was titrated to pH 8.00. Its composition was (in mM): 417.2 $NMDG^+$, 7 Mg^{++}, 16 $Tris^+$, 414 glutamate, 4 ATP^{4-}, 1 $EGTA^{2-}$, 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 L-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. DFs containing quaternary ammonium ions were made by using the quaternary ammonium hydroxide (Fluka, Ronkonkoma, NY), rather than NMDG free base, to titrate the glutamic acid. $PPTEA^+$ was a generous gift from Robert Rakowski (Chicago Medical School). Because the quaternary ammonium ions had apparent osmotic coefficients that were substantially greater than that of $NMDG^+$, we eliminated glycine from DFs containing high levels of quaternary ammonium ions.

Calculation of Acid-Base Transport Rates

We computed the equivalent "HCO_3^- influx" (J_{HCO3}) or H^+ efflux (J_H) from rates of pH_i increase and total intracellular buffering power (β_T),

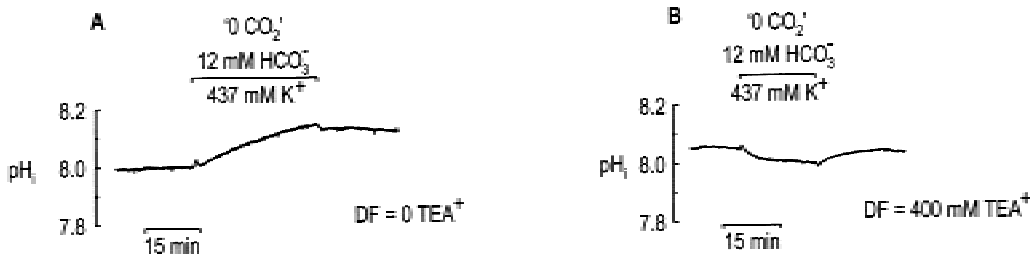

Fig. 1. Inhibition of K/HCO_3 cotransport by TEA^+ when exposing axons to out-of-equilibrium solutions. (*A*) Dialysis with $NMDG^+$ (TEA-free). Not shown is the portion of the experiment in which the axon was dialyzed to a pH_i of ~8.0 with a dialysis fluid containing no TEA^+, K^+, Na^+, Cl^- or HCO_3^-. The artificial seawater (ASW) similarly contained no K^+, Na^+, Cl^- or HCO_3^-. After halting dialysis and a brief stabilization period (~20 min), we switched the ASW to one containing 437 mM K^+, 12 mM HCO_3^-, (but no CO_2). The extracellular pH remained constant at 8.00. K/HCO_3 cotransport causes the pH_i to increase due to the coupled influx of K^+ and HCO_3^-. (*B*) Dialysis with 400 mM TEA^+. Using the same protocol as described in (*A*) the axon was dialyzed with DF containing 400 mM TEA^+. After halting dialysis and a brief stabilization period (~20 min), we switched the ASW to one containing 437 mM K^+, 12 mM HCO_3^-, and a constant extracellular pH of 8.00 but again no CO_2. In contrast to what is seen in (*A*), in the presence of 400 mM TEA^+, K/HCO_3 cotransport activity is blocked and pH_i does not increase.

as summarized previously (Boron & Knakal, 1989, 1992; Hogan et al., 1995b; Zhao et al., 1995).

Statistics

Results are expressed as the mean ± the standard error of the mean. 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. Calculation of apparent K_i values was accomplished with "Fig-P" software (FigP Biosoft Software, Durham, NC) and Sigmaplot (San Rafael, CA).

Results

In an earlier study on the K/HCO_3 cotransporter, we examined the effect of exposing a squid giant axon to an ASW containing 437 mM K^+ and 12 mM HCO_3^-, but no Na^+ or Cl^-. However, the ASW was unusual in that it was out-of-equilibrium with respect to CO_2/HCO_3^-: it contained virtually no CO_2 (Zhao et al., 1995). Figure 1*A* shows a similar experiment. The ASW initially contained no Na^+, K^+, Cl^- or HCO_3^-. We dialyzed the axon to a pH_i of 8.0 (*not shown*) with a DF that also contained no Na^+, K^+, Cl^- or HCO_3^-. The predominant cation in the DF was $NMDG^+$. After halting dialysis and allowing pH_i to stabilize, we switched the ASW to the aforementioned out-of-equilibrium solution. Because $[CO_2]_o$ was negligible, switching to this solution did not cause a fall in pH_i. However, the action of the K/HCO_3 cotransporter led to a slow rise in pH_i. Other experiments (*not shown*) indicate that this pH_i increase requires the extracellular K^+. To test whether the pH_i increase in Fig. 1*A* would occur regardless of the predominant intracellular cation, we performed one experiment in which we replaced $NMDG^+$ with TEA^+ in the DF. To our surprise, we found that the K^+-dependent alkalinization was completely absent (Fig. 1*B*)[1].We hypothesized that the TEA^+, presumably acting at the intracellular K^+ site, blocked the K/HCO_3 cotransporter.

K/HCO_3 Cotransport in the Absence of Quaternary Ammonium Ions

To test the above hypothesis, we undertook the experiments described in the remainder of this paper. For the sake of simplicity, our assay employed equilibrated CO_2/HCO_3^- solutions. Figure 2*A* shows one of 12 control experiments. The mean initial pH_i prior to internal dialysis for these axons was 7.35 ± 0.04. Not shown is the portion of the experiment in which we dialyzed for 80 min to achieve a pH_i of 8.0. The DF had a pH of 8.00 and was free of K^+, Na^+ and Cl^-. Until the time indicated in the figure, the axon was superfused with the 0/0/0/0 ASW, which had a pH of 8.00 and was also free of K^+, Na^+, Cl^- and HCO_3^-. After we halted dialysis, thereby returning control of pH_i to the axon, pH_i slowly drifted downward (segment *ab*), as described previously (Hogan et al., 1995a). The mean acid-extrusion rate (J_H) for this segment was -13.3 ± 2.6 pmole cm^{-2} sec^{-1} ($n = 12$), the negative sign indicating a net H^+ influx.

Replacing the ASW with a solution that was otherwise identical, except for containing 437 mM K^+ and 0.5% CO_2/12 mM HCO_3^-, caused a biphasic pH_i change. First, pH_i decreased rapidly by an average of 0.18 ± 0.01 (*bc*), reflecting the influx of CO_2, and the hydration of this CO_2 to form H_2CO_3, which then dissociates to form intracellular HCO_3^- and H^+. Second, pH_i increased slowly during the plateau phase (*cd*), presumably due to the coupled influx of K^+ and HCO_3^-. The calculated base

[1] The slight pH_i decrease during the exposure to K^+ and HCO_3^- probably reflects the presence of a very small amount of contaminating CO_2.

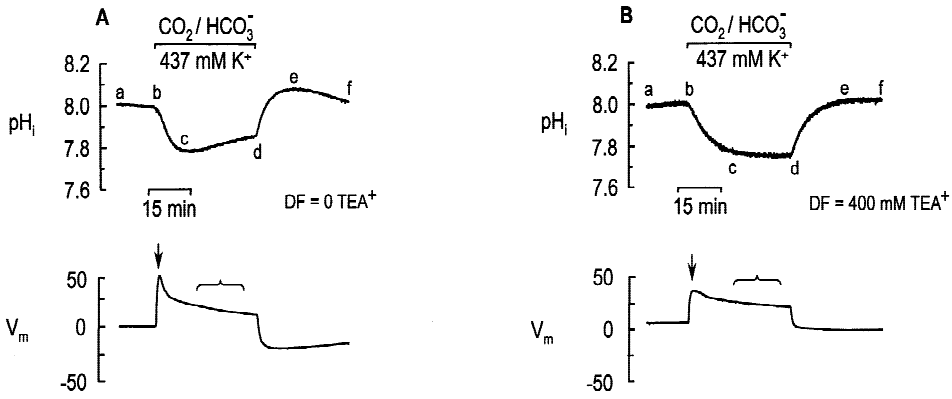

Fig. 2. Inhibition of K/HCO_3 cotransport by TEA^+ when exposing axons to equilibrated CO_2/HCO_3^- solutions. (*A*) Dialysis with $NMDG^+$ (TEA^+-free). The axon was dialyzed to a pH_i of ~8.0 with a dialysis fluid containing no TEA^+, K^+, Na^+, Cl^- or HCO_3^-. The artificial seawater (ASW) similarly contained no K^+, Na^+, Cl^- or HCO_3^-. Dialysis was halted shortly before point *a*, and an ASW containing 437 mM K^+ and 0.5% CO_2/12 mM HCO_3^- was introduced between points *b* and *d*. The pH_i initially decreases (*bc*) due to the influx of CO_2. The pH_i then increases (*cd*) due to the coupled influx of K^+ and HCO_3^- by the K/HCO_3 cotransporter. (*B*) Dialysis with TEA^+. The protocol for this experiment was identical to that in (*A*), except that the dialysis fluid contained 400 mM TEA^+. In contrast to the pH_i record in (*A*), where no TEA^+ was present, the pH_i increase during segment *cd* in the present figure was negligible. The average HCO_3^- influx in nine experiments similar to the one shown here (400 mM TEA^+) was reduced by 97% compared to the experiments in the absence of TEA^+. The braces above the V_m records indicate that the V_m, during the portion of the experiment used in the calculation of J_{HCO_3}, was approximately the same.

influx due to K/HCO_3 cotransport (J_{HCO3}) was 57.9 ± 5.3 pmole cm^{-2} sec^{-1}. The high-K^+ ASW also caused a rapid depolarization (arrow in lower panel of Fig. 2*A*), which averaged 41 ± 3 mV; membrane voltage (V_m) then relaxed (i.e., became more negative) by an average of 23 ± 3 mV over the course of segment *cd.*

When we switched back to the 0/0/0/0 ASW, pH_i increased rapidly, due to the efflux of CO_2 (*de*), and then decreased more slowly (*ef*). We have previously shown that this segment-*ef* pH_i decrease occurs only after the axon has been loaded with K^+ via the coupled uptake of K^+ and HCO_3^- (Hogan et al., 1995b), and thus probably reflects the coupled efflux of K^+ and endogenous HCO_3^- (footnote[2]). Removing K^+ from the ASW also caused V_m to shift rapidly in the negative direction (mean ΔV_m = −32 ± 2 mV).

Dose-dependent Inhibition of K/HCO_3 Cotransport by TEA^+

To determine the effect of intracellular TEA^+ on the K^+-dependent uptake of HCO_3^-, we performed experiments identical to that described above in Fig. 2*A*, except that the DF contained 400 mM TEA^+ (i.e., TEA^+ replacing $NMDG^+$). Figure 2*B* shows one of nine experiments with 400 mM TEA^+. After we dialyzed the axon to pH_i 8.0 and halted dialysis, pH_i drifted slowly upward (*ab*). However, for most axons in this group, pH_i drifted slowly downwards (mean J_H for segment *ab* = −10.2 ± 3.9 pmole cm^{-2} sec^{-1}). Switching from the 0/0/0/0 ASW to an ASW containing 437 mM K^+ plus 0.5% CO_2/12 mM HCO_3^- caused pH_i changes (*bcd*) and V_m changes similar in two respects to the ones described for Fig. 2*A.* First, the initial CO_2-induced decrease in pH_i (*bc* in Fig. 2*B*) averaged 0.22 ± 0.02 pH units. Second, the maximal K^+-induced ΔV_m (i.e., during the initial few seconds of exposure to the high-K^+ solution) was 26 ± 5 mV. However, a major difference was that the 400 mM TEA^+ totally blocked the pH_i recovery during the plateau-phase (*cd*). The mean J_{HCO3} in the presence of 400 mM TEA^+ was only 1.6 ± 4.3 pmole cm^{-2} sec^{-1}, a ~97% inhibition compared to the J_{HCO3} value in the absence of TEA^+ (57.9 ± 5.3 pmole cm^{-2} sec^{-1}). Switching back to the original 0/0/0/0 ASW produced a pH_i response that is noteworthy in that pH_i simply increased to its initial value (*de*), rather than overshooting it (compare *b* vs. *e*). Moreover, pH_i did not decline after its rapid increase (*ef*). Compared to the segment-*ef* acidification in the absence of TEA^+ (Fig. 2*A*), the 400 mM TEA^+ in Fig. 2*B* inhibited the mean J_H during segment-*ef* by 88%. Thus, 400 mM TEA^+ not only blocked the segment-*cd* plateau-phase alkalinization, it also blocked the segment-*ef* pH_i overshoot as well as the post-overshoot acidification, all of which are hallmarks of K/HCO_3 cotransport.

We performed experiments similar to those described for Fig. 2*B*, but in which we dialyzed axons with DFs containing 10–200 mM TEA^+. As summarized in Fig. 3*A*, the inhibition of K/HCO_3 cotransport by TEA^+ has an approximately hyperbolic dose dependence, with an apparent K_i of 78 ± 36 mM. It should be noted that,

[2] During the comparable portion of Fig. 1*A*, pH_i fell very slowly, possibly because the nonspecific entry of K^+ was less, leading to a lower $[K^+]_i$ after the removal of K^+ and HCO_3^- from the ASW.

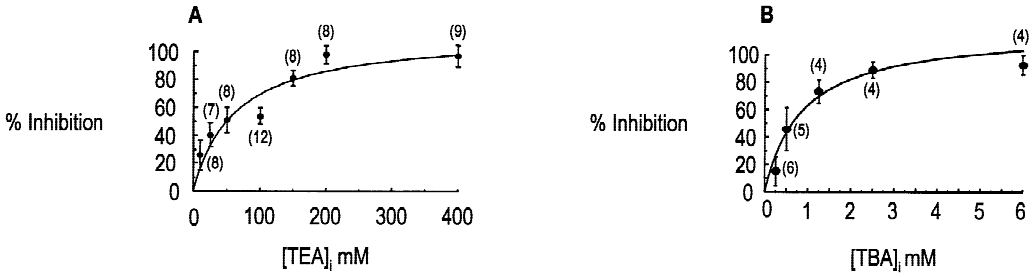

Fig. 3. Dose dependence of inhibition of K/HCO_3 cotransport by TEA^+ and TBA^+. (*A*) TEA^+. This panel summarizes data from 72 experiments similar to those shown in Fig. 2 for TEA^+ concentrations of 10, 25, 50, 100, 150, 200 and 400 mM. The vertical bars represent standard error. The number of experiments is given in parentheses. The curve through the data points is the function: % Inhibition $= [I/(I + K_i)] \times 100$, where I is the concentration of TEA^+ and K_i is the apparent inhibitory constant. We obtained the value for I by performing a nonlinear least-squares fit to the function: $J = J_{max} \times [1 - I/(I + K_i)] + J_{back}$, where J is the computed HCO_3^- influx, J_{max} is the HCO_3^- influx in the absence of TEA^+ and J_{back} is the background flux in the presence of saturating TEA^+. The best-fit values are: $J_{max} = 57.5 \pm 7.9$ pmole cm^{-2} sec^{-1}, $K_i = 77.8 \pm 35.9$ mM, and $V_{back} = -8.7 \pm 8.1$ pmole cm^{-2} sec^{-1}. (*B*) TBA^+. This panel is similar to (*A*), except that it summarizes data from 23 experiments for TBA^+ concentrations of 0.25, 0.5, 1.25, 2.5 and 6 mM. The best-fit values are: $J_{max} = 66.4 \pm 14.3$ pmole cm^{-2} sec^{-1}, $K_i = 0.86 \pm 0.58$ mM, and $V_{back} = -10.1 \pm 14.3$ pmole cm^{-2} sec^{-1}.

during the portion of the experiment used in the calculation of J_{HCO3} (i.e., segment *cd* in Fig. 2*A* and *B*, also denoted by the braces above the V_m record), V_m was approximately the same, regardless of the concentration of TEA^+ in the DF (Table 1). Thus, the changes in J_{HCO3} cannot be attributed to changes in V_m.

Inhibition of K/HCO_3 Cotransport by Other Quaternary Ammonium Ions

Effect of "Symmetrical" Quaternary Ammonium Ions at 100 mM

In experiments similar to that shown in Fig. 2*B*, we examined the effect of three other "symmetrical" quaternary ammonium ions: tetramethylammonium (TMA^+), tetrapropylammonium (TPA^+), and tetrabutylammonium (TBA^+), each at a concentration of 100 mM. TPA^+ and TBA^+ appeared to be toxic[3] at higher doses. As summarized in Table 2, 100 mM TMA^+ and TEA^+ were equally effective inhibitors of K/HCO_3 cotransport, decreasing J_{HCO3} by ~60%, compared to the control flux of 57.9 pmole cm^{-2} sec^{-1} in the absence of inhibitors. At a concentration of 100 mM, TPA^+ reduced J_{HCO3} ~80%, and 100 mM TBA^+ appeared to completely block K/HCO_3 cotransport. During the portion of the plateau phase of the CO_2/HCO_3^- exposure during which we computed J_{HCO3}, V_m was approximately the same, regardless of which, if any, of the four quaternary ammonium ions was present in the DF (Table 2). Thus, as was the case for various concentrations of TEA^+, the changes in J_{HCO3} with the other three symmetrical quaternary ammonium ions cannot be attributed to differences in V_m.

Table 1. Effect of TEA^+ (in the dialysis fluid) on mean V_m* in the presence of 437 mM extracellular K^+

$[TEA]_{DF}$ (mM)	V_m (mV)
0	+16 ± 1 (12)
10	+19 ± 2 (7)
25	+20 ± 1 (8)
50	+19 ± 2 (8)
100	+21 ± 2 (11)
150	+22 ± 1 (8)
200	+22 ± 2 (8)
400	+21 ± 2 (9)

* Mean ± SEM, number of observations in parentheses. V_m was taken as the mean V_m during the period indicated by the brace in Fig. 2*A* and *B*) during which we calculated J_{HCO3}.

Table 2. Effect of 100 mM quaternary ammonium ions on K^+-dependent HCO_3^- influx and V_m*

Condition	J_{HCO3} pmole cm^2 sec^{-1}	V_m mV
Control	57.9 ± 5.3	+16 ± 1(26)
TMA^+	20.4 ± 13	+21 ± 3 (3)
TEA^+	24.0 ± 3.4	+21 ± 1(12)
TPA^+	10.2 ± 8.7	+26 ± 2 (6)
TBA^+	−1.8 ± 7.8	+15 ± 2 (3)

* The V_m value is the mean, absolute membrane potential during the interval in which we computed J_{HCO_3}.

[3] The CO_2-induced acidification (e.g., *bc* in Fig. 2*A* and *B*), as well as the K^+-induced depolarization, were unusually small. In addition, the plateau-phase alkalinization (e.g., *cd* in Fig. 2*A* and *B*), as well as the V_m relaxation from the depolarization, were unusually rapid.

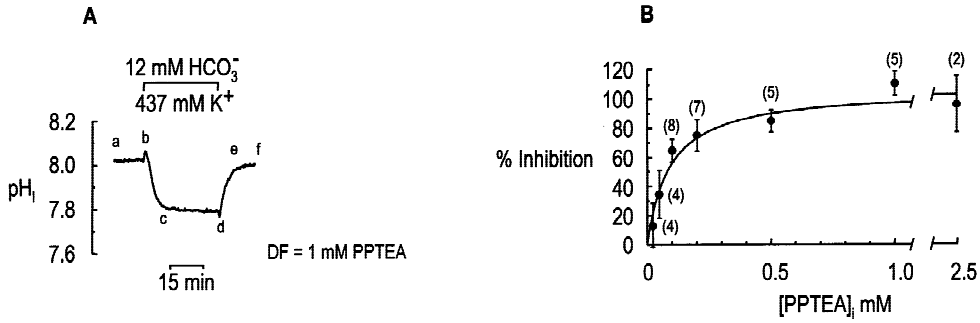

Fig. 4. Inhibition of K/HCO_3 cotransport by $PPTEA^+$. (*A*) Effect of 1 mM $PPTEA^+$. The protocol for this experiment is identical to the protocol used in Fig. 2*B*. The axon was dialyzed to a pH_i of ~8.0 with a dialysis fluid containing 1 mM $PPTEA^+$, but no K^+, Na^+, Cl^- or HCO_3^-. The artificial seawater (ASW) similarly contained no K^+, Na^+, Cl^- or HCO_3^-. Dialysis was halted shortly before point *a*, and an ASW containing 437 mM K^+ and 0.5% CO_2/12 mM HCO_3^- was introduced between points *b* and *d*. In contrast to the pH_i record in Fig. 1, where no $PPTEA^+$ was present, the pH_i increase during segment *cd* due to coupled K^+ and HCO_3^- influx was negligible. In a total of six such experiments, the initial CO_2-induced decrease in pH_i (*bc*) averaged 0.23 ± 0.02, and the K^+ induced ΔV_m was 32 ± 4 mV (*not shown*). The mean J_H during segment *ef* was −7.9 ± 8.2 pmole cm^{-2} sec^{-1}. (*B*) Dose dependence of inhibition. This figure summarizes data from 69 experiments similar to those shown in Fig. 4*A* (1 mM $PPTEA^+$), as well as others with intermediary $PPTEA^+$ levels (i.e., 0.025, 0.050, 0.100, 0.200, 0.500, 1.00 and 2.50 mM $PPTEA^+$). The vertical bars represent the standard error. The number of experiments is given in parentheses. The curve through the data points represents the best fit by the function: $V = V_{max} \times I/(I + K_i) + V_{back}$, where V is the computed HCO_3^- influx, V_{max} is the HCO_3^- influx in the absence of $PPTEA^+$, I is the concentration of $PPTEA^+$, K_i is the apparent inhibitory constant, and V_{back} is the background flux in the presence of saturating $PPTEA^+$. V_{max} was 60.2 ± 7.9 pmole cm^{-2} sec^{-1}, K_i was 0.091 ± 0.047 mM, and V_{back} was −3.6 ± 7.1 pmole cm^{-2} sec^{-1}.

Dose-dependent Inhibition by TBA^+

For TBA^+, we also examined the inhibition of J_{HCO3} over a broader range of concentrations. As summarized in Fig. 3*B*, the dose dependence of the inhibition of K/HCO_3 cotransport by TBA^+ is approximately hyperbolic, with an apparent K_i of 864 ± 581 μM.

Dose-dependent Inhibition by $PPTEA^+$

Because phenyl-propyltetraethylammonium ($PPTEA^+$) is a potent inhibitor of K^+ current in squid giant axons (Swenson, Jr., 1981), we examined its effect on K/HCO_3 cotransport. Figure 4*A* shows one of six experiments similar to that in Fig. 2*B*, except that the DF contained 1 mM $PPTEA^+$ and 399 mM $NMDG^+$ rather than 400 mM TEA^+. The average initial pH_i for these axons, before dialysis, was 7.32 ± 0.03. After we halted dialysis, pH_i slowly fell (*ab*); the average calculated J_H during this period was −8.7 ± 8.2 pmole cm^{-2} sec^{-1}. Switching from the 0/0/0/0 ASW to an ASW containing 437 mM K^+ plus 0.5% CO_2/12 mM HCO_3^- caused pH_i changes (*bcd*) and V_m changes (*not shown*) that were similar to those described for 400 mM TEA^+. Thus, $PPTEA^+$ at a concentration of only 1 mM completely blocked the plateau-phase pH_i recovery (*cd*). The mean J_{HCO3} was −7.3 ± 4.3 pmole cm^{-2} sec^{-1}, compared to 57.9 pmole cm^{-2} sec^{-1} in the absence of inhibitor. As seen with 400 mM TEA^+ (Fig. 2*B*) returning the axon to the original 0/0/0/0 ASW caused pH_i to simply increase to its initial value, reflecting the efflux of CO_2, with no pH_i overshoot (compare *b* vs. *e*). Moreover, pH_i did not decline after its rapid increase. The mean J_H during segment *ef* in the presence of $PPTEA^+$ was −7.9 ± 8.2 pmole cm^{-2} sec^{-1}, which is 86% lower than in the absence of inhibitor. Therefore, 1 mM $PPTEA^+$, like 400 mM TEA^+, eliminated the hallmarks of K/HCO_3 cotransport: the segment-*cd* alkalinization, the segment-*de* pH_i overshoot and the segment-*ef* post-overshoot acidification.

Figure 4*B* summarizes the results of a larger series of experiments in which we dialyzed axons with DFs containing 10 μM to 1 mM $PPTEA^+$. The apparent K_i for inhibition by $PPTEA^+$ was 90.5 ± 47.1 μM, ~850-fold lower than with TEA^+. As summarized in Table 3, during the portion of segment *cd* used to calculate J_{HCO3}, V_m was approximately the same as in the TEA^+ experiments (compare with Table 1). In particular, V_m tended to be more positive at the higher $PPTEA^+$ concentrations, where net HCO_3^- was the least. If $PPTEA^+$ had its pH_i effect by shifting V_m and thereby inhibiting a passive influx of HCO_3^-, the relationship between $PPTEA^+$ concentrations and J_{HCO3} should have been just the opposite.

Discussion

Rationale for Studying Quaternary Ammonium Ions

If the K^+:HCO_3^- stoichiometry of the K/HCO_3 cotransporter (Hogan et al., 1995a,b) is 1:1, then this transporter, under physiological conditions, would mediate the coupled efflux of K^+ and HCO_3^-. Functioning as an acid loader, the K/HCO_3 cotransporter would thus help the axon recover from intracellular alkali loads. In the normal steady state (e.g., $pH_i \cong 7.4$), the K/HCO_3 cotrans-

Table 3. Effect of $PPTEA^+$ on V_m changes* produced by adding 437 mM extracellular K^+

[PPTEA] μM	V_m mV
0	+16 ± 1(26)
0.025	+15 ± 3 (4)
0.050	+17 ± 2 (5)
0.100	+21 ± 2(10)
0.200	+25 ± 3 (9)
0.500	+25 ± 3(12)
1	+26 ± 2 (6)
2.5	+26 (2)

* Mean ± SEM, number of observations in parentheses. The V_m during segment *cd* during which we calculated J_{HCO3} (*cd* in Fig. 4*A*).

porter presumably counteracts the effects of the axon's major acid extruder, the Na-dependent Cl/HCO_3 exchanger. In the present experiments, for the sake of convenience, we studied the cotransporter while it was operating in reverse direction, mediating the influx of K^+ plus HCO_3^-.

Of the major functional classes of HCO_3^- transporters, the K/HCO_3 cotransporter is the only one not inhibited, to at least some extent, by the disulfonic stilbene derivatives DIDS or SITS. Because intracellular TEA^+ inhibits certain types of K^+ channels (Stanfield, 1983), including the delayed-rectifier K^+ conductance in squid axons (Armstrong & Hille, 1972) and Ca^{++}-activated K^+ channels (Villarroel et al., 1988), we decided to determine whether TEA^+ and other quaternary ammonium ions also inhibit the K/HCO_3 cotransporter, presumably by acting at the cotransporter's intracellular K^+ site.

Evidence that TEA^+ Acts Directly on the Cotransporter

The present work demonstrates that intracellular TEA^+ is indeed an inhibitor of the K/HCO_3 transporter. As shown in Fig. 3*A*, the inhibition appears to follow Michaelis-Menten kinetics, with an apparent K_i of ~78 mM. This K_i is ~200-fold greater than the K_i values (~0.4 mM) typical for inhibition of K^+ channels by TEA^+ (Stanfield, 1983; French & Shoukimas, 1981). Thus, it is very unlikely that TEA^+ exerts its effect on the cotransporter by simply inhibiting K^+ channels. If the pH_i increase we ascribe to the K/HCO_3 cotransporter (e.g., *cd* in Fig. 2*A*) were instead due to the passive influx of HCO_3^-, then one would expect that the pH_i increase would be increasingly inhibited by shifting V_m in the negative direction. However, over a wide range of concentrations, neither TEA^+ (Table 1) nor $PPTEA^+$ (Table 3) shifted V_m in this direction, compared to the situation in the absence of inhibitor. If anything, the higher levels of TEA^+ and $PPTEA^+$, which produced the greater inhibitions of K/HCO_3 cotransport, were associated with slightly more positive values of V_m. Thus, it is likely that TEA^+ directly inhibits the K/HCO_3 cotransporter, possibly by competing with intracellular K^+ at the cotransporter's K^+-binding site.

Relative Potency of Quaternary Ammonium Ions

The K^+ channels at the node of Ranvier, as well as the widely distributed Ca^{++}-activated K^+ channel, have both external and internal TEA^+ sites (Stanfield, 1983; Villarroel et al., 1988). Although the pharmacology of the internal site is similar to that of the delayed-rectifier K^+ conductance in the squid giant axon, the external site is highly selective for TEA^+. Even small increases in alkyl chain length decrease the inhibitory potency at the extracellular site of K^+ channels. However, when quaternary ammonium ions block K^+ channels from the intracellular side, the inhibitory potency increases with increasing alkyl chain length (Armstrong, 1971; French & Shoukimas, 1981; Swenson, Jr., 1981). The most potent inhibitor we have identified for the K/HCO_3 cotransporter is $PPTEA^+$ ($K_i \cong 91$ μM), which is 857-fold more potent than TEA^+ ($K_i \cong 78$ mM). Although the potency sequence for the inhibition of the K/HCO_3 cotransporter by quaternary ammonium ions is similar to that for inhibiting K^+ channels at their intracellular site, the inhibition of the transporter is different in two important respects. First, the apparent K_i values are much higher for the cotransporter. Second, the ratio of K_i values for TMA^+ vs. $PPTEA^+$ is much greater for the cotransporter (i.e., 857:1) than for K^+ channels (i.e., 57:1) (Hermann & Gorman, 1981; Swenson, Jr., 1981). These observations also argue that the quaternary ammonium ions do not inhibit the K/HCO_3 cotransporter by interacting with K^+ channels.

In summary, we have identified several inhibitors of the squid-axon K/HCO_3 cotransporter, for which no inhibitors previously existed. $PPTEA^+$ could prove to be a useful pharmacological tool for studying K/HCO_3 cotransport in other cell types.

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