

Intracellular Cl⁻ Dependence of Na-H Exchange in Barnacle Muscle Fibers under Normotonic and Hypertonic Conditions

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ABSTRACT We previously showed that shrinking a barnacle muscle fiber (BMF) in a hypertonic solution (1,600 mosM/kg) stimulates an amiloride-sensitive Na-H exchanger. This activation is mediated by a G protein and requires intracellular Cl⁻. The purpose of the present study was to determine (a) whether Cl⁻ plays a role in the activation of Na-H exchange under normotonic conditions (975 mosM/kg), (b) the dose dependence of [Cl⁻]_i for activation of the exchanger under both normo- and hypertonic conditions, and (c) the relative order of the Cl⁻- and G-protein-dependent steps. We acid loaded BMFs by internally dialyzing them with a pH-6.5 dialysis fluid containing no Na⁺ and 0–194 mM Cl⁻. The artificial seawater bathing the BMF initially contained no Na⁺. After dialysis was halted, adding 50 mM Na⁺ to the artificial seawater caused an amiloride-sensitive pH_i increase under both normo- and hypertonic conditions. The computed Na-H exchange flux ($J_{\text{Na-H}}$) increased with increasing [Cl⁻]_i under both normo- and hypertonic conditions, with similar apparent K_m values (~120 mM). However, the maximal $J_{\text{Na-H}}$ increased by nearly 90% under hypertonic conditions. Thus, activation of Na-H exchange at low pH_i requires Cl⁻ under both normo- and hypertonic conditions, but at any given [Cl⁻]_i, $J_{\text{Na-H}}$ is greater under hyper- than normotonic conditions. We conclude that an increase in [Cl⁻]_i is not the primary shrinkage signal, but may act as an auxiliary shrinkage signal. To determine whether the Cl⁻-dependent step is after the G-protein-dependent step, we predialyzed BMFs to a Cl⁻-free state, and then attempted to stimulate Na-H exchange by activating a G protein. We found that, even in the absence of Cl⁻, dialyzing with GTPγS or AlF₃, or injecting cholera toxin, stimulates Na-H exchange. Because Na-H exchange activity was absent in control Cl⁻-depleted fibers, the Cl⁻-dependent step is at or before the G protein in the shrinkage signal-transduction pathway. The stimulation by AlF₃ indicates that the G protein is a heterotrimeric G protein.

KEY WORDS: intracellular pH • cell volume regulation • shrinkage

INTRODUCTION

Cell swelling generally initiates a rapid sequence of events that results in the efflux of ions and water, a volume-regulatory decrease (VRD) that returns cell volume toward normal (for reviews see Hoffmann and Simonsen, 1989; Hallows and Knauf, 1994; Lang et al., 1995). The ion efflux may be mediated by K/Cl cotransport (Dunham and Ellory, 1981; Jennings and Schulz, 1990; Jennings and Schulz, 1991; Jennings and Al-Rohil, 1990), KCl efflux through parallel K⁺ and Cl⁻ channels (Knoblauch et al., 1989; Welling and O'Neil, 1990; Banderali and Roy, 1992), or K-H exchange (Cala, 1980, 1983, 1985). Conversely, cell shrinkage often initiates a volume-regulatory increase (VRI), a rapid sequence of events that results in the influx of ions and

water (Lang et al., 1995; Hoffman and Simonsen, 1989; Hallows and Knauf, 1994). The influx may be mediated by Na/K/Cl cotransport (Geck et al., 1980; Eveloff and Calamia, 1986) or Na-H exchange augmented by Cl-HCO₃ exchange (Kregenow et al., 1985; Grinstein et al., 1983; Jennings et al., 1986). It is believed that, in some cells, the VRD and VRI responses are reciprocal, with cell swelling stimulating VRD and inhibiting VRI, and cell shrinkage having the opposite effects (Lang et al., 1995).

Over a longer time frame, hypertonicity may stimulate osmotic-response elements in some cells, increasing the transcription of enzymes that catalyze the production of intracellular osmolytes. With a delay of ~48 h, mIMCD-3 (renal medullary collecting duct), PAP-HT25 (rabbit inner medulla), and MDCK cells respond to hypertonicity by inducing aldose reductase (Spring and Siebens, 1988; Garcia-Perez and Burg, 1991; Burg, 1995). This enzyme converts glucose to the relatively impermeant sorbitol, causing osmotic swelling. Other osmolytes, such as inositol, betaine, taurine, and glycerophosphocholine may also be concentrated, and thereby promote re-swelling (Burg, 1995; Garcia-Perez and Burg, 1991).

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A major unanswered question in cell physiology is how cells sense cell-volume changes and transduce them to the appropriate changes in ion transport. One important clue may be the observation, made by Parker (1986), that Cl^- is necessary for the shrinkage-induced activation of Na-H exchange in dog red blood cells. In earlier work on muscle fibers from the giant barnacle, we confirmed this observation and additionally showed that the shrinkage-induced activation of the Na-H exchanger specifically requires Cl^- in the intracellular fluid (Davis et al., 1994). The precise role of Cl^- in this process is unclear. However, there is precedent for involvement of Cl^- in other biological processes. For example, Cl^- increases the affinity of the α subunit of the heterotrimeric G protein G_o for GTP γ S (Higashijima et al., 1987).

A second important clue into the shrinkage signal-transduction system is that, in barnacle muscle fibers (BMFs),¹ the shrinkage-induced activation of the Na-H exchanger appears to be mediated by a G protein (Davis et al., 1992a). Thus, the effect of shrinkage on the exchanger is inhibited by dialyzing the fiber with GDP β S, and mimicked either by dialyzing with GTP γ S or by injecting the fibers with activated catalytic subunit of cholera toxin (CTX).

The purpose of the present study was to explore the role of Cl^- in the shrinkage-induced activation of Na-H exchange in internally dialyzed barnacle muscle fibers. We used microelectrodes to monitor intracellular pH (pH_i) and calculated the Na-H exchange rate ($J_{\text{Na-H}}$) from the rate of pH_i increase and the intracellular buffering power. Because internal Cl^- is required for the shrinkage-induced activation of Na-H exchange in BMFs, we hypothesized that the primary signal the cell senses during shrinkage may be an increase in $[\text{Cl}^-]_i$. To test this hypothesis, we determined the $[\text{Cl}^-]_i$ dependence of Na-H exchange, both under normo- and hypertonic conditions. We found that, even under normotonic conditions, the Na-H exchanger is inactive in the absence of Cl^- . Increasing $[\text{Cl}^-]_i$ causes a monotonic rise in $J_{\text{Na-H}}$ but, at a given $[\text{Cl}^-]_i$, $J_{\text{Na-H}}$ is always greater under hypertonic conditions. Thus, an increase in $[\text{Cl}^-]_i$ is not the primary shrinkage signal. In a second series of experiments, we asked whether the Cl^- -dependent step in the activation of the Na-H exchange is before or after the G-protein step. We found that, even in BMFs depleted of Cl^- , we could activate Na-H exchange with GTP γ S, AlF_3 , or CTX. Thus, the Na-H exchanger does not require Cl^- per se. Moreover, the Cl^- -dependent step precedes or is concurrent with the G-protein step in the signal-transduction cascade.

¹Abbreviations used in this paper: ASW, artificial seawater; BMF, barnacle muscle fiber; CTX, cholera toxin; DF, dialysis fluids; NMDG⁺, N-methyl-D-glucammonium.

METHODS

General

Barnacles were obtained from Bio-marine Enterprises (Seattle, WA) and kept in an aerated aquarium at 4°C. After dissection, barnacle muscle fibers were kept at 4°C for a period of up to 36 h in our standard artificial seawater (ASW) (see Solutions, below). Before experiments, fibers were incubated for at least 1 h in a Ca^{++} -free artificial seawater (see Solutions, below) to prevent contraction during the subsequent cannulation (see below). This Ca^{++} -free solution also contained 0.5 mM SITS (4-acetamido-4'-isothiocyanostilbene-2,2'-disulfate) (United States Biochemical Corp., Cleveland, OH) to permanently block the activity of the Na^+ -driven Cl-HCO_3 exchanger (Boron, 1977). The fibers were cannulated in a Ca^{++} -free (or a Ca^{++} - and Cl^- -free) ASW also containing SITS.

Solutions

Artificial seawaters. All ASWs were nominally HCO_3^- free. The standard ASW, in which fibers were incubated before the experiments, consisted of (mM): 440 Na^+ , 10 K^+ , 11 Ca^{++} , 45.5 Mg^{++} , 558 Cl^- , 5 EPPS⁻ (the anionic form of N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid; Sigma Chemical Co., St. Louis, MO), and 5 of the neutral form of EPPS ($\text{pK}_{\text{EPPS}} \cong 8.0$). The usual Ca^{++} -free ASW was made by replacing the Ca^{++} in the standard ASW with Mg^{++} . ASWs containing 0 Na^+ were made by replacing Na^+ , mole for mole, with N-methyl-D-glucammonium (NMDG⁺) that was produced by using HCl to titrate the free base N-methyl-D-glucamine (Sigma Chemical Co.). The ASW containing 50 mM Na^+ was made by diluting the standard ASW with the 0- Na^+ ASW.

Cl^- -free ASWs were made by replacing Cl^- , mole for mole, with gluconate (Sigma Chemical Co.). In Cl^- -free ASWs that also lacked Na^+ , we generated NMDG⁺ salts of gluconate by titrating NMDG free base with gluconic acid lactone. We made the 50- Na^+ /0- Cl^- ASW in the same way we made the 0- Na^+ /0- Cl^- ASW, except that we replaced 50 mM NMDG⁺/gluconate with Na^+ /gluconate.

We adjusted the pH values of all ASWs to pH 8.00 at 22°C. We decreased pH, in Cl^- -containing ASWs, with either EPPS or HCl, and in Cl^- -free ASWs, with EPPS. We increased pH, in Na^+ -containing ASWs, with NaOH, and in Na^+ -free ASWs, with NMDG-free base. The osmolalities were determined using a vapor pressure osmometer (Wescor Inc., Logan, UT), and adjusted to 975 ± 10 mosM/kg with mannitol or water. Solutions were made hypertonic ($1,600 \pm 10$ mosM/kg) by adding mannitol. Solutions were delivered to the chamber by peristaltic or syringe pumps, as previously described (Boron, 1985). Fibers were superfused at a rate of 1 ml min^{-1} .

Dialysis fluids. All the dialysis fluids (DFs) were Na^+ free. The standard pH-7.2 DF contained 34 mM Cl^- , and consisted of (mM): 243 K^+ , 7 Mg^{++} , 175 glutamate, 34 Cl^- , 2 EGTA, 44 of the anionic form of HEPES (United States Biochemical Corp.), 56 of the neutral form of HEPES, 0.5 phenol red, and 4.0 Tris/ATP. The standard pH-6.5 DF also contained 34 mM Cl^- , and consisted of (mM): 255.5 K^+ , 7 Mg^{++} , 160 glutamate, 34 Cl^- , 2 EGTA, 71.5 of the anionic form of MES (2-(N-morpholino)-ethanesulfonic acid; Sigma Chemical Co.), 28.5 of the neutral form of MES, 0.5 phenol red, and 4.0 Tris/ATP. Cl^- -free DFs were made by replacing all of the Cl^- with L-glutamate. DFs with $[\text{Cl}^-]$ values above 34 mM were made by replacing glutamate, mole for mole, with Cl^- . Dialysis fluid pH was adjusted upward with KOH and downward with either HCl (for Cl^- -containing DFs) or L-glutamic acid (for Cl^- -free DFs). In the DFs containing GTP γ S, 1 mM ATP was substituted with 1mM GTP γ S, keeping

the total nucleotide concentration at 4 mM. Aluminum fluoride was added to the DFs as 10 mM KF + 100 μ M AlCl₃.

Measurement of pH_i and Membrane Potential

The technique for measuring intracellular pH (pH_i) in internally dialyzed muscle fibers has been published elsewhere (Russell et al., 1983). Single, isolated BMFs were horizontally cannulated in a Ca⁺⁺-free solution. A length of cellulose-acetate dialysis tubing with a molecular weight cutoff of ~6 kD was threaded into one cannula, through the muscle fiber, and out the opposite cannula. The pH electrodes were fabricated according to the design of Hinke (1967). Glass microelectrodes used for measuring membrane voltage (V_m) were filled with 3 M KCl when the dialysis fluid contained Cl⁻, but with 1 M K-glutamate when the DF was Cl⁻ free.

Experimental Protocols

General. After cannulating the fiber and inserting the dialysis tubing, we began dialysis with a pH-7.2 DF at a rate of 5 μ l min⁻¹. All DFs were Na⁺ free. The pH and V_m electrodes were inserted through opposite cannulas so that their tips were within ~500 μ m of each other. The central region of the fiber was isolated from the cut ends with grease seals. We then began superfusing the fiber with a Na⁺-free ASW at a rate of 1 ml min⁻¹.

Studies on the [Cl⁻]_i dependence of Na-H exchange. In these experiments, after an initial ~30-min period of dialysis with a DF having a pH of 7.2 and a [Cl⁻] between 34 and 194 mM, dialysis continued for an additional ~50–60 min with a DF that was otherwise identical, except for having a pH of 6.5. Thus, the total time for dialysis was ~80–90 min. Previous work has shown that 60–90 min is sufficient for either ²²Na (Russell et al., 1983) or ³⁶Cl (Boron et al., 1978; Russell and Brodwick, 1988) in the DF to achieve isotopic equilibrium. After dialysis was halted, pH_i was allowed to stabilize for ~15 min in a Na⁺-free ASW.

Studies with GTP γ S or AlF₃ in Cl⁻-depleted cells. The protocol was similar to that above, except that the initial period of dialysis with the pH-7.2/Cl⁻-free DF continued for ~140 min to deplete the cell of Cl⁻. During this time, the fiber was superfused with a Na⁺- and Cl⁻-free ASW. This pH-7.2/Cl⁻-free DF was then switched to an identical solution that also contained either 1 mM GTP γ S or 10 mM AlF₃. After ~40 min, we switched to an identical solution

in which the pH of the DF was lowered to 6.5 to acid load the fiber. We continued dialyzing with this solution for ~60 min. Thus, the total dialysis time was ~240 min.

Studies on Cl⁻-depleted cells injected with CTX. This protocol was similar to the one in the GTP γ S and AlF₃ experiments. The major difference was that, ~160 min after initiating dialysis, we microinjected the BMFs with CTX, and only then inserted the microelectrodes. The injection fluid was the pH-7.2/0-Cl⁻ DF containing the dithiothreitol-activated CTX to a final intracellular concentration of ~3 \times 10⁻⁶ M. During the microinjection and electrode insertion, the fiber was briefly exposed to an ASW that lacked Ca⁺⁺ (to prevent contraction). Also, during the microinjection and electrode insertion, the fiber was dialyzed continuously with the pH-7.2/0-Cl⁻ DF. After an additional ~110 min dialysis with this DF, and an additional ~60 min with a pH-6.5/0-Cl⁻ DF, we halted dialysis and allowed the fiber to stabilize for 120 min before assaying as described above.

Statistics

Values are reported as means \pm SEM. Groups of data were compared using a two-sample *t* test assuming unequal variance.

RESULTS

Effect of Increasing Internal Cl⁻ under Normotonic Conditions

Fibers dialyzed with 34 mM Cl⁻ under normotonic conditions. Fig. 1 A illustrates an experiment in which a muscle fiber was acid loaded by internally dialyzing it with a Na⁺-free DF containing 34 mM Cl⁻ at pH 6.5. As noted in METHODS, all fibers were pretreated with SITS to eliminate Na⁺-driven Cl-HCO₃ exchange, and all were superfused with a Na⁺-free ASW for ~90 min. The terminal portion of this dialysis period is shown in Fig. 1 A. After we halted dialysis (Fig. 1 A, *a*), pH_i continued to drift downward (Fig. 1 A, *ab*), probably because the tip of the pH electrode in this experiment was rather dis-

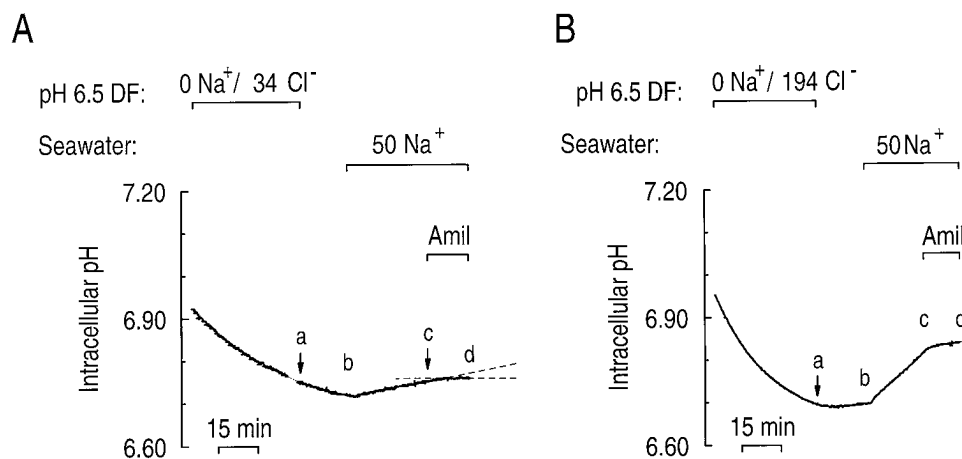


FIGURE 1. Effect on Na-H exchange of increasing [Cl⁻]_i under normotonic conditions. (A) 34 mM Cl⁻. Before the portion of the experiment shown here, we dialyzed a SITS-pretreated BMF for ~30 min with a pH-7.2/Na⁺-free DF containing 34 mM Cl⁻. The normotonic ASW was also Na⁺ free, but contained 558 mM Cl⁻. To acidify the cell, we then switched the DF to one with a pH of 6.5, the [Cl⁻] remaining at 34 mM. At *a*, we halted dialysis. We switched to an ASW containing 50 mM Na⁺ (975 mosM/kg) at *b*, initiating Na-H exchange, and added 1 mM amiloride at *c*

to block the exchanger. The dashed line before *a* represents the portion of the record from which we removed an artifactual spike. (B) 194 mM Cl⁻. The protocol was the same as in A, except that the dialysis fluids contained 194 mM Cl⁻. The ASWs contained 558 mM Cl⁻. We halted dialysis at *a*, added 50 mM Na⁺ to the ASW at *b*, and added 1 mM amiloride at *c*.

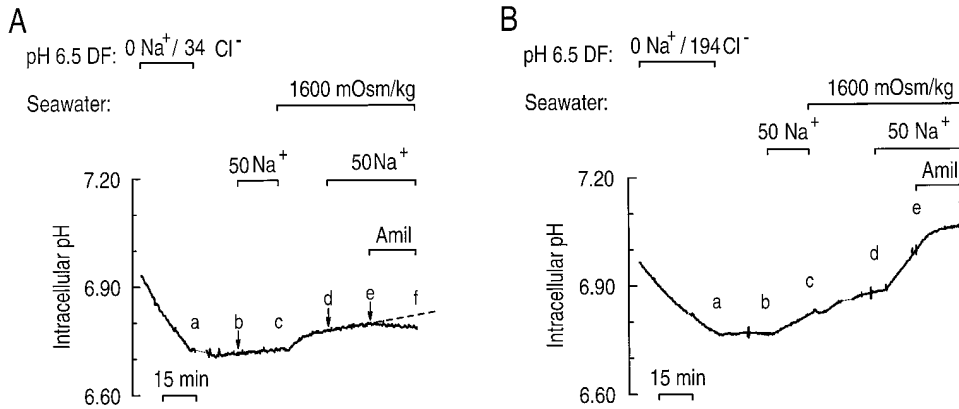


FIGURE 2. Effect on Na-H exchange of increasing $[Cl^-]_i$ under hypertonic conditions. (A) 34 mM Cl^- in the dialysis fluid. The protocol was the same as in Fig. 1 A, except that we added Na^+ to the ASW under both normo- and hypertonic conditions. The ASWs contained 558 mM Cl^- . We halted dialysis at *a*, and added 50 mM Na^+ to the ASW, which had an osmolality of 975 mosM/kg, at *b*. At *c*, we switched to a Na^+ -free ASW made hypertonic (1,600 mosM/kg) with mannitol. Cell shrinkage presumably increased $[Cl^-]_i$ from 34 to 56 mM. We added 50 mM Na^+ to the hypertonic ASW at *d*, and added 1 mM amiloride at *e*. (B) 194 mM Cl^- in the dialysis fluid. The protocol was the same as in A, except that the dialysis fluids contained 194 mM Cl^- . The ASWs contained 558 mM Cl^- . We halted dialysis at *a* and added 50 mM Na^+ to the ASW, which had an osmolality of 975 mosM/kg, at *b*. At *c*, we switched to a Na^+ -free ASW made hypertonic (1,600 mosM/kg) with mannitol. Cell shrinkage presumably increased $[Cl^-]_i$ from 194 to 318 mM. We added 50 mM Na^+ to the hypertonic ASW at *d*, and added 1 mM amiloride at *e*.

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tant from the dialysis tube. The mean pH_i at *b* was 6.72 ± 0.01 ($n = 20$). Inasmuch as the fiber had been dialyzed with a Na^+ -free DF, and superfused with a Na^+ -free ASW, $[Na^+]_i$ should have been extremely low. Exposing the fiber to an ASW containing 50 mM Na^+ produced a slow increase in pH_i (Fig. 1 A, *bc*), due mainly to the basal activity of the Na-H exchanger (Davis et al., 1994). We chose to use a modest level of Na^+ , 50 mM, because this concentration is high enough to support Na-H exchange, but not so high as to compete with amiloride for binding sites on the transporter. We computed the total acid-extrusion rate (J_{Total}) as the product of the pH_i recovery rate of segment *bc* and the previously measured intrinsic buffering power (Davis et al., 1994). The pH_i increase of Fig. 1 A, *bc* was inhibited by adding 1 mM amiloride to the 50- Na^+ ASW (Fig. 1 A, *cd*). The broken lines in the figure emphasize the slopes in the absence and presence of amiloride. The delay between the application and action of amiloride presumably reflects the time for the drug to reach the interstices of the BMF. For the purposes of this study, we will present values for Na-H exchange rate (J_{Na-H}) as the amiloride-sensitive component of J_{Total} ; that is, the difference in flux values between *bc* and *cd* in Fig. 1 A. For 20 fibers, the mean J_{Na-H} was $23 \pm 4 \mu M \min^{-1}$ (Table I).

Fibers dialyzed with 194 mM Cl^- under normotonic conditions. To address the question of whether increasing $[Cl^-]_i$ stimulates Na-H exchange under normotonic conditions, we performed the experiment shown in Fig. 1 B, in which we dialyzed the fiber with a Na^+ -free DF containing 194 mM Cl^- . After we halted dialysis, pH_i drifted upward very slowly (Fig. 1 B, *ab*). The mean pH_i at point *b* was 6.71 ± 0.02 ($n = 12$). Exposing the cell to 50 mM Na^+ produced a rapid intracellular alkalinization (Fig. 1 B, *bc*) that was largely blocked by amiloride

(Fig. 1 B, *cd*). The mean J_{Na-H} for these 12 experiments was $138 \pm 26 \mu M \min^{-1}$ (see Table I). Therefore, under normotonic conditions (975 mosM/kg), increasing $[Cl^-]$ in the DF from 34 to 194 mM dramatically stimulates the Na-H exchanger, increasing J_{Na-H} from 23 to $138 \mu M \min^{-1}$.

Effect of Increasing Internal Cl^- under Hypertonic Conditions

Effect of 34 mM Cl^- under hypertonic conditions. Fig. 2 A illustrates 1 of 13 experiments in which we examined the effect of hypertonicity on Na-H exchange in fibers dialyzed with 34 mM Cl^- . The first part of the protocol was identical to that of Fig. 1 A: the fiber was acid loaded by dialyzing with a Na^+ -free DF containing 34 mM Cl^- , dialysis was halted, and pH_i was allowed to stabilize (Fig. 2 A, *ab*). The mean pH_i at point *b* was 6.70 ± 0.02 ($n = 13$). As in Fig. 1 A, exposing the cell to an ASW containing 50 mM Na^+ elicited, at most, a very slow alkalinization (Fig. 2 A, *bc*).² At point *c*, we switched to a Na^+ -free

TABLE I
 J_{Na-H} at Different $[Cl^-]_i$ Values under Normotonic and Hypertonic Conditions

$[Cl^-]_i$	Activators of G proteins	Normotonic J_{Na-H}	Hypertonic J_{Na-H}
mM		$\mu M \min^{-1}$	$\mu M \min^{-1}$
0	—	-3 ± 7	—
34	—	23 ± 4	86 ± 17
194	—	138 ± 26	345 ± 43
0	1 mM GTP γ S	164 ± 23	—
0	10 mM KF + 100 μ M $AlCl_3$	296 ± 28	—
0	3×10^{-6} M CTX	100 ± 35	—
34	3×10^{-6} M CTX	135 ± 38	—

ASW made hypertonic (1,600 mosM/kg) by the addition of mannitol. The self-limited increase in pH_i (Fig. 2 A, *cd*) was presumably due to the concentration of intracellular buffers, as previously described. Indeed, in a previous study, we found that exposing a muscle fiber to the same hypertonic solution caused intracellular buffering power, measured at a pH_i of ~ 6.8 , to double (Davis et al., 1994). After pH_i stabilized, exposing the cell to a hypertonic ASW containing 50 mM Na^+ produced a slow pH_i increase (Fig. 2 A, *de*). Applying amiloride not only blocked this pH_i increase, it unmasked a slow acidification (Fig. 2 A, *ef*). The difference in the slopes of the pH_i recoveries in segments *de* and *ef* indicates that there was a modest rate of Na-H exchange when cells dialyzed with 34 mM Cl^- were shrunken. In a total of 13 similar experiments, the mean $J_{\text{Na-H}}$ was $86 \pm 17 \mu\text{M min}^{-1}$ (see Table I), a figure that takes into consideration the increased buffering power in hypertonic solutions. This mean $J_{\text{Na-H}}$, obtained under hypertonic conditions, is ~ 3.7 -fold higher than the mean $J_{\text{Na-H}}$ (see Fig. 1 A) obtained under normotonic conditions in cells dialyzed with 34 mM Cl^- .

Effect of 194 mM Cl^- under hypertonic conditions. To determine whether increasing $[\text{Cl}]_i$ also stimulates Na-H exchange under hypertonic conditions, we performed the experiment shown in Fig. 2 B. This experiment is identical to that shown in Fig. 2 A, except that the DF contained 194 rather than 34 mM Cl^- . The mean pH_i at point *b* was 6.73 ± 0.01 ($n = 8$). Exposing the cell to a normotonic ASW containing 50 mM Na^+ produced a rapid alkalinization (Fig. 2 B, *bc*), as observed above for fibers dialyzed with 194 mM Cl^- (Fig. 1 B). After we switched to a Na^+ -free hypertonic solution, and pH_i stabilized (Fig. 2 B, *cd*), increasing $[\text{Na}^+]_o$ to 50 mM produced an even more marked alkalinization (Fig. 2 B, *de*) that was largely blocked by amiloride (Fig. 2 B, *ef*). The mean $J_{\text{Na-H}}$ for these eight experiments was $345 \pm 43 \mu\text{M min}^{-1}$ (see Table I). Therefore, under hypertonic conditions, increasing the $[\text{Cl}^-]$ in the DF from 34 to 194 mM increased $J_{\text{Na-H}}$ fourfold, from 86 to $345 \mu\text{M min}^{-1}$.

$[\text{Cl}^-]_i$ Dependence of Na-H Exchange under Normo- and Hypertonic Conditions

We have already seen that, under normotonic conditions, increasing the $[\text{Cl}^-]$ of the DF from 34 to 194 mM caused an increase in $J_{\text{Na-H}}$ (Fig. 1, A vs. B). To de-

²The slope of the pH_i recovery in *bc* reflects not only the Na-H exchange rate, which tends to increase pH_i , but also the presence of background acid loading, which tends to decrease pH_i . In experiments in which the cell was later exposed to a hypertonic solution (*c-f*), it was not possible to inhibit the *bc* pH_i increase with amiloride because the amiloride is not fully reversible. Thus, we can draw no conclusions about the Na-H exchange rate in *bc* in this experiment.

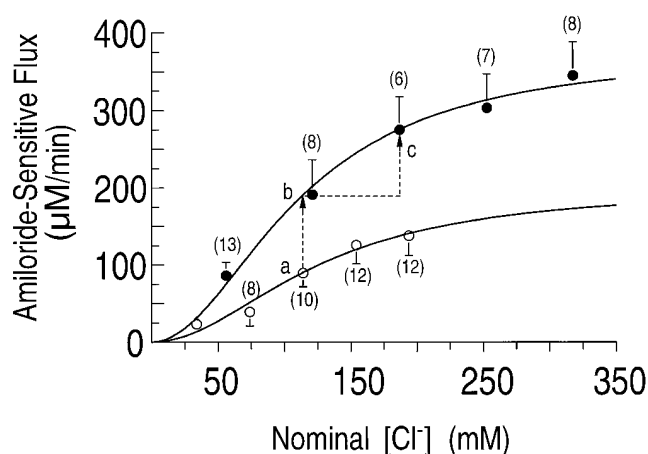


FIGURE 3. Cl^- -dependent activation of Na-H exchange under normo- and hypertonic conditions. The experiments summarized by the open circles and lower curve (975 mosM/kg) were performed with the same protocols as in Fig. 1, except that the dialysis fluids had $[\text{Cl}^-]$ values of 34, 74, 114, 154, or 194 mM. Similarly, the experiments summarized by the closed circles and upper curve (1,600 mosM/kg) were performed with the same protocols as in Fig. 2. We assume that cell shrinkage caused nominal $[\text{Cl}^-]_i$ to increase from 34 to 56 mM, from 74 to 121 mM, from 114 to 187 mM, from 154 to 253 mM, and from 194 to 318 mM. The curves through the data are the result of a nonlinear least-squares curve fit by a function of the form $V = V_{\text{max}} / (1 + [([\text{Cl}^-] / K_m)^2])$, using FigP software. For the normotonic data, K_m was 127 ± 44 mM, and V_{max} was $201 \pm 57 \mu\text{M min}^{-1}$. For the hypertonic data, K_m was 112 ± 24 mM, and V_{max} was $375 \pm 43 \mu\text{M min}^{-1}$. The number of experiments is given in parentheses. The standard error bars are omitted when smaller than the symbol.

termine the $[\text{Cl}^-]_i$ dependence of Na-H exchange under normotonic conditions, we performed additional experiments identical to those in Fig. 1 except that the $[\text{Cl}^-]$ of the DFs was 74, 114, or 154 mM. The data are summarized by the open circles in Fig. 3. In plotting the data, we have assumed that $[\text{Cl}^-]_i$ is the same as the $[\text{Cl}^-]$ of the DF.

We also performed additional experiments to determine the $[\text{Cl}^-]_i$ dependence of Na-H exchange under hypertonic conditions. These experiments were identical to those in Fig. 2, except that the $[\text{Cl}^-]$ of the DFs was 74, 114, or 154 mM. We have assumed that the cells behaved as perfect osmometers, so that increasing the osmolality from 975 to 1,600 mosM/kg increased the $[\text{Cl}^-]_i$ by a factor of 1,600/975, or 1.64. Thus, $[\text{Cl}^-]_i$ values for the hypertonic data, plotted as closed circles in Fig. 3, have values 1.64-fold higher than the corresponding $[\text{Cl}^-]_i$ values for the normotonic data, plotted as open circles. Fig. 3 shows that, under both normo- and hypertonic conditions, increasing nominal $[\text{Cl}^-]_i$ causes an increase in Na-H exchange activity. For normotonic conditions (Fig. 3, \circ), a nonlinear least-squares curve fit (Hill coefficient = 2) produced an apparent K_m for internal Cl^- of 127 mM, and an apparent

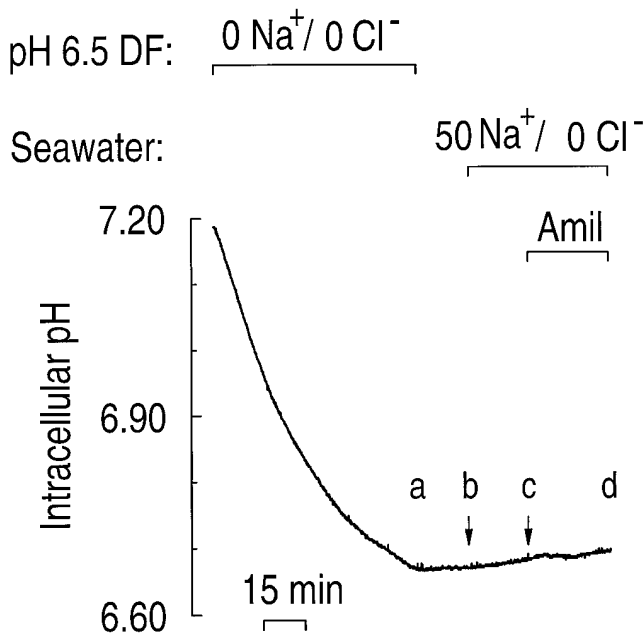


FIGURE 4. Effect of Cl^- depletion on Na-H exchange under normotonic conditions. Before the portion of the experiment shown, we dialyzed this SITS-pretreated BMF with a Na^+ - and Cl^- -free DF, first at pH 7.2, and then at pH 6.5. All ASWs were Cl^- free and normotonic (975 mosM/kg). We halted dialysis at *a*, added 50 mM Na^+ to the ASW at *b*, and added 1 mM amiloride at *c*.

V_{max} of 201 $\mu\text{M min}^{-1}$. For hypertonic conditions (Fig. 3, ●), the apparent K_m was ~ 112 mM, and the V_{max} was 375 $\mu\text{M min}^{-1}$. Therefore, we conclude that increasing the nominal $[\text{Cl}^-]_i$ stimulates Na-H exchange activity under both normo- and hypertonic conditions. Furthermore, although hypertonicity had little effect on the apparent K_m for internal Cl^- , it increased the V_{max} for Na-H exchange by nearly 90%.

Effect of Using Cl^- Substitutes other than Glutamate

From the above data, it would appear that increasing $[\text{Cl}^-]_i$ stimulates Na-H exchange, both under normo- and hypertonic conditions. However, to create DFs of increasing $[\text{Cl}^-]_i$, we simultaneously lowered [glutamate]. Thus, it is possible that the increased $J_{\text{Na-H}}$ we observed in high Cl^- DFs was due to decreasing [glutamate], rather than increasing $[\text{Cl}^-]_i$. Therefore, we examined the effect of using two other Cl^- substitutes, gluconate and sulfamate. Our standard DF contained 34 mM Cl^- and 160 mM glutamate. In the present series of experiments, done on matched fibers, we dialyzed BMFs with DFs containing 34 mM Cl^- plus 160 mM of either glutamate, gluconate, or sulfamate. We assayed the fibers for Na-H exchange as in Fig. 1 A, under normotonic conditions. If glutamate were inhibiting Na-H exchange at a $[\text{Cl}^-]_i$ of 34 mM, then replacing the glutamate with either gluconate or sulfamate should

substantially increase $J_{\text{Na-H}}$, that is, produce approximately the same $J_{\text{Na-H}}$ that we saw above at 194 mM Cl^- (i.e., $\sim 138 \mu\text{M min}^{-1}$), when we substituted 160 mM glutamate for 160 mM Cl^- . However, we found that although $J_{\text{Na-H}}$ was $16 \pm 5 \mu\text{M min}^{-1}$ ($n = 4$) in fibers dialyzed with 160 mM glutamate, it was no higher in fibers dialyzed with either 160 mM gluconate ($J_{\text{Na-H}} = 0 \pm 7$, $n = 4$) or 160 mM sulfamate ($J_{\text{Na-H}} = 14 \pm 4$, $n = 5$). Thus, because glutamate is not inhibitory, Cl^- must be stimulatory.

Where Does Cl^- Play a Role in Activation of Na-H Exchange?

Because we can use GTP γ S, AlF_3 , or CTX to activate the heterotrimeric G protein that ultimately activates the Na-H exchanger, we are in a position to ask whether the Cl^- -dependent step in the shrinkage signal-transduction cascade is after the G protein. Our approach was, first, to verify that complete Cl^- removal (i.e., removing Cl^- from both DF and ASW) does indeed block Na-H exchange, and then to determine whether the aforementioned G-protein activators are capable of stimulating Na-H exchange in the absence of Cl^- .

Effect of complete Cl^- removal on Na-H exchange under normotonic conditions. We Cl^- depleted fibers by exposing them to a Cl^- -free ASW and dialyzing them with a Cl^- -free DF for a minimum of 160 min, and an average of ~ 180 min. In the experiment shown in Fig. 4, we pretreated the fiber with SITS, and then dialyzed for 120 min with a pH-7.2 DF that was free of both Na^+ and Cl^- . During this time, the ASW was also free of Na^+ and Cl^- . We then switched the pH of the DF to 6.5 for an additional 60 min to acidify the cell. Fig. 4 picks up the experiment during this latter period of dialysis. After we halted dialysis, pH_i stabilized (Fig. 4, *ab*). Exposing the cell to an ASW containing 50 mM Na^+ did not significantly alter the trajectory of pH_i (Fig. 4, *bc*). Neither was the pH_i trajectory affected by applying 1 mM amiloride (Fig. 4, *cd*). In a total of six similar experiments, the mean $J_{\text{Na-H}}$ was $-3 \pm 7 \mu\text{M min}^{-1}$ (see Table I), which is not significantly different from zero. Thus, Cl^- depletion completely blocks Na-H exchange under normotonic conditions.

Effect of GTP γ S on Na-H exchange in Cl^- -depleted cells. We had previously shown that, in the presence of Cl^- and under normotonic conditions, GTP γ S activates Na-H exchange in barnacle muscle fibers (Davis et al., 1992a). To determine whether GTP γ S also activates the exchanger in Cl^- -depleted fibers, we performed a series of experiments similar to the one shown in Fig. 5 A. Our protocol was the same as for Fig. 4, except that the DF contained 1 mM GTP γ S for the final ~ 95 min of dialysis. In these experiments, the duration of dialysis with the Cl^- -free DF, before the introduction of GTP γ S, was as long as 145 min, and averaged 135 min. Fig. 5 A picks up the experiment during the latter part of dialy-

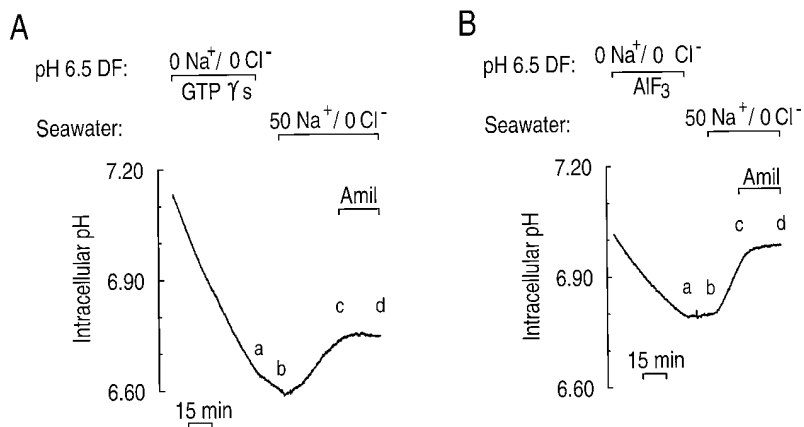


FIGURE 5. Effect of GTP γ S or AIF $_3$ on Na-H exchange in the absence of intracellular Cl $^-$. (A) GTP γ S. Before the portion of the experiment shown, we depleted the BMF of Cl $^-$ as described in Fig. 4. We then switched first to a pH-7.2 DF and then to a pH-6.5 DF, both containing 1 mM GTP γ S. The DF and ASWs were Cl $^-$ free and normotonic (975 mosM/kg). The figure shows the later part of the period of dialysis with the 1 mM GTP γ S at pH 6.5. We halted dialysis at *a*, introduced 50 mM Na $^+$ to the ASW at *b*, and added 1 mM amiloride at *c*. (B) AIF $_3$. The protocol was very similar to that in A, except that the DF contained AIF $_3$ (10 mM KF + 100 μ M AlCl $_3$), rather than GTP γ S. The DF and ASWs were Cl $^-$ free and normotonic (975 mosM/kg). We halted dialysis at *a*, introduced 50 mM Na $^+$ to the ASW at *b*, and added 1 mM amiloride at *c*.

sis with the pH-6.5 DF containing GTP γ S. When dialysis was halted, the pHi continued to drift downward (Fig. 5 A, *ab*) in this particular experiment. Exposing the cell to a Cl $^-$ -free ASW containing 50 mM Na $^+$ produced a substantial alkalization (Fig. 5 A, *bc*) that was blocked by amiloride (Fig. 5 A, *cd*). For the eight fibers in this study, the mean J_{Na-H} was $164 \pm 23 \mu\text{M min}^{-1}$ (see Table I), which is significantly greater than the above value for Cl $^-$ -depleted cells in the absence of GTP γ S, $-3 \pm 7 \mu\text{M min}^{-1}$ ($P < 0.0001$). Thus, even in Cl $^-$ -depleted fibers, GTP γ S markedly stimulates Na-H exchange.³

Effect of AIF $_3$ on Na-H exchange in Cl $^-$ -depleted cells. To obtain further evidence that G-protein activation will stimulate Na-H exchange even in the absence of Cl $^-$, we examined the effect of introducing AIF $_3$ into Cl $^-$ -depleted fibers. Our protocol was similar to that in Fig. 5 A, except that AIF $_3$ (10 mM KF plus 100 μ M AlCl $_3$) replaced GTP γ S for the final 100 min of dialysis. Before the introduction of AIF $_3$, muscle fibers in this group of experiments were Cl $^-$ depleted for as long as 175 min, and an average of 140 min. Fig. 5 B shows the terminal portion of dialysis with the pH-6.5, AIF $_3$ -containing DF. When we halted dialysis, pHi quickly stabilized (Fig. 5 B, *ab*). However, when we exposed the fiber to a Cl $^-$ -free ASW containing 50 mM Na $^+$, pHi rose very rapidly, as was the case for cells dialyzed with GTP γ S (see Fig. 5 A). The rapid pHi increase in the AIF $_3$ -dialyzed cells was greatly inhibited by amiloride (Fig. 5 B, *cd*). In a total of six experiments, the mean J_{Na-H} was $296 \pm 28 \mu\text{M min}^{-1}$

(see Table I), significantly greater than the control flux of $-3 \pm 7 \mu\text{M min}^{-1}$ in Cl $^-$ -depleted cells not dialyzed with AIF $_3$ ($P < 0.0001$). Thus, AIF $_3$ markedly stimulates Na-H exchange, even in Cl $^-$ -depleted fibers.

Effect of cholera toxin on Na-H exchange in Cl $^-$ -depleted cells. In previous experiments from this laboratory, we had shown that injecting CTX into BMFs the day before the experiment stimulates Na-H exchange. These previous studies were performed on cells dialyzed with 34 mM Cl $^-$ and superfused with an ASW containing Cl $^-$ (Davis et al., 1992a). To determine whether CTX can activate Na-H exchange in the absence of Cl $^-$, we needed to modify the protocol from the previous study so that the Cl $^-$ depletion, the CTX injection, and the Na-H exchange assay could all be done on the same day. Because the total time between CTX injection and Na-H exchange assay in the present study would be substantially less than in the previous one, we increased the final intracellular CTX concentration to 3×10^{-6} M. The protocol for the first portion of the experiment was similar to those shown in Fig. 5. The differences are detailed in METHODS; for example, the microelectrodes were not inserted until after CTX injection. In the experiment shown in Fig. 6 A, we exposed a fiber to a pH-7.2 DF that was free of both Na $^+$ and Cl $^-$. The Cl $^-$ depletion time before injection with CTX was as long as 170 min, and averaged 160 min. After we injected the CTX and inserted the microelectrodes, we continued to dialyze with the pH-7.2, 0-Cl $^-$ DF for an additional ~ 110 min. We then switched to a DF with a pH of 6.5 to acidify the cell. After halting dialysis, we allowed the fibers to incubate for an additional 120 min before assaying for Na-H exchange. Thus, we assayed the fibers ~ 290 min postinjection. Fig. 6 A picks up the experiment ~ 75 min after dialysis had been halted. Exposing the cell to a Cl $^-$ -free ASW containing 50 mM Na $^+$ caused an increase in pHi (Fig. 6 A, *bc*) that was re-

³Because we added the 1 mM GTP γ S as the tetra-lithium salt, the DF containing GTP γ S also contained 4 mM Li $^+$. Although previous work from this laboratory (Davis et al., 1992b) showed that 100 mM Li $^+$ stimulates Na-H exchange, here we found that dialyzing with 4 mM Li $^+$ (in the absence of GTP γ S) fails to stimulate Na-H exchange at pHi ~ 7.2 (not shown).

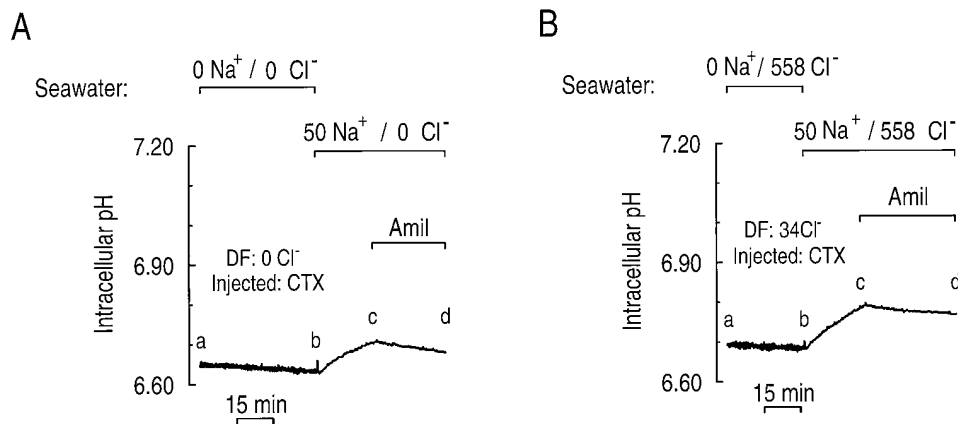


FIGURE 6. Effect of cholera toxin on Na-H exchange in the absence and presence of Cl⁻. (A) Absence of Cl⁻. The DFs and ASWs were Cl⁻-free throughout. Before the portion of the experiment shown, we depleted the BMF of Cl⁻ as described in Fig. 4, except that we had not yet inserted either the pH or voltage microelectrode. While continuing to dialyze with a Cl⁻-free DF, we microinjected CTX to a final concentration of 3×10^{-6} M, and then inserted the microelectrodes. Dialysis then continued, first with a pH-7.2 DF, and then

with a pH-6.5 DF. We halted dialysis and allowed the CTX to react with the cell for an additional 120 min, the final portion of which is shown as *ab*. At *b*, we added 50 mM Na⁺ to the ASW, and at *c*, we added 1 mM amiloride. (B) Presence of Cl⁻. Throughout the experiment, the ASWs contained 558 mM Cl⁻. The DFs contained 34 mM Cl⁻. Otherwise, the protocol was the same as in A.

versed by amiloride (Fig. 6 A, *cd*). The mean $J_{\text{Na-H}}$ for five similar experiments was $100 \pm 35 \mu\text{M min}^{-1}$ (see Table I), significantly greater than the control flux of $-3 \pm 7 \mu\text{M min}^{-1}$ in Cl⁻-depleted cells not injected with CTX ($P < 0.02$).

In the above CTX experiments, the $J_{\text{Na-H}}$ of $\sim 100 \mu\text{M min}^{-1}$ was substantially less than in comparable experiments with GTP γ S ($164 \mu\text{M min}^{-1}$) or AIF₃ ($296 \mu\text{M min}^{-1}$). One reason $J_{\text{Na-H}}$ may have been relatively low in the CTX experiments is that, even though we assayed ~ 290 min after injecting the CTX, we may not have allowed enough time for the CTX to have its maximal effect. Therefore, we asked whether, under our assay conditions (Fig. 6 A), CTX would activate Na-H exchange similarly in the presence and absence of Cl⁻. To answer this question, we performed a second series of experiments, identical to that shown in Fig. 6 A, except that the DF contained 34 mM Cl⁻, and the ASW contained 558 mM Cl⁻. Because increasing values of $[\text{Cl}^-]_i$ increase Na-H exchange, we chose a DF with a relatively low $[\text{Cl}^-]$ to minimize "background" Na-H exchange activity; that is, Na-H exchange independent of CTX. As with Cl⁻-depleted cells (see Fig. 6 A), cells dialyzed with 34 mM Cl⁻ and injected with CTX exhibited significant Na-H exchange (see Fig. 6 B). For five experiments, the mean $J_{\text{Na-H}}$ was $135 \pm 38 \mu\text{M min}^{-1}$ (see Table I). The background Na-H exchange rate for 34 mM Cl⁻ (see discussion of Fig. 1) was $\sim 23 \mu\text{M min}^{-1}$. Subtracting 23 from $135 \mu\text{M min}^{-1}$ produces a CTX-dependent $J_{\text{Na-H}}$ of $112 \mu\text{M min}^{-1}$ for Cl⁻-containing cells. This figure is indistinguishable from the $J_{\text{Na-H}}$ in CTX fibers that were Cl⁻ depleted (i.e., $100 \mu\text{M min}^{-1}$). Thus, under the conditions of our experiments, CTX produces a similar activation of Na-H exchange in the presence and absence of Cl⁻.

DISCUSSION

Role of Intracellular Cl⁻ in the Activation of Na-H Exchange

History. The first indication that Cl⁻ may play a role in the activation of the Na-H exchanger was Parker's observation, made in dog erythrocytes, that the Na-H exchanger fails to respond to cell shrinkage when extra- and intracellular Cl⁻ is replaced with either thiocyanate or nitrate (Parker, 1983). However, Cl⁻ is not required for Na-H exchange per se. When Parker activated the Na-H exchanger by shrinking the cells in the presence of Cl⁻, and then fixed the cells by briefly exposing them to glutaraldehyde, the Na-H exchanger remained activated even in the absence of Cl⁻ (Parker, 1984). Similarly, Motais et al. (1989) found that Cl⁻ is required for the cAMP-dependent activation of Na-H exchange by isoproterenol in trout erythrocytes. In these cells, once Na-H exchange was activated by cAMP in the presence of Cl⁻, the exchanger remained active even after NO₃⁻ replaced Cl⁻ (Motais et al., 1989). Thus, two signal-transduction processes leading to activation of the Na-H exchanger require Cl⁻, even though the exchanger itself does not require Cl⁻. More recently, a Cl⁻-dependent acid-base transporter, presumably an amiloride-resistant Na-H exchanger, has been found in the apical membrane of colonic crypt cells (Rajendran et al., 1995). There is one example in which Cl⁻ inhibits Na-H exchange. In salivary acinar cells, carbachol stimulates Cl⁻ channels, leading to Cl⁻ efflux and cell shrinkage. The simultaneous presence of carbachol, a decreased $[\text{Cl}^-]_i$ and cell shrinkage results in activation of Na-H exchange (Foskett, 1990; Robertson and Foskett, 1994).

Cl⁻ dependence of Na-H exchange under normotonic conditions. Perhaps our most unexpected result was that, even

under normotonic conditions, the Na-H exchanger is markedly stimulated by increasing $[Cl^-]_i$. As summarized by the open symbols in Fig. 3, increasing $[Cl^-]_i$ from its "normal" level of 34 mM to 194 mM caused J_{Na-H} to increase six-fold. This result suggests that the exchanger may be the target of a Cl^- -dependent signal-transduction system even under normotonic or "basal" conditions. If this were the case, then one might predict that reducing $[Cl^-]_i$ to zero would eliminate Na-H exchange under normotonic conditions, a prediction verified in Fig. 4. Thus, it appears that, at least in barnacle muscle, Cl^- is required to elevate the Na-H exchanger to even its basal level of activity, and to maintain this basal activity.

Cl⁻ dependence of Na-H exchange under hypertonic conditions. Three aspects of the data obtained under hypertonic conditions are noteworthy. First, as was the case under normotonic conditions, increasing the $[Cl^-]_i$ produces a graded increase in J_{Na-H} (Fig. 3, ●). Second, at any given $[Cl^-]_i$, the J_{Na-H} is always greater under hyper- than under normotonic conditions. For example, at a $[Cl^-]_i$ of 114 mM, J_{Na-H} was 89 $\mu M \text{ min}^{-1}$ under normotonic conditions (Fig. 3, a). Increasing the osmolality, while holding $[Cl^-]_i$ fixed at 114 mM, would be expected to increase J_{Na-H} approximately twofold to $\sim 191 \mu M \text{ min}^{-1}$ (interpolated point b). Thus, an increase in $[Cl^-]_i$ cannot be the primary signal for triggering the shrinkage-induced increase in J_{Na-H} . Third, shrinkage not only increases J_{Na-H} by shifting the exchanger from the normo- to the hypertonic curve in Fig. 3, it also increases J_{Na-H} because the loss of cell water increases $[Cl^-]_i$. Thus, in a cell dialyzed to a $[Cl^-]_i$ of 114 mM, increasing the osmolality from 975 to 1,600 mosM/kg, will increase $[Cl^-]_i$ to 187 mM. As shown in Fig. 3, this elevation in $[Cl^-]_i$ would increase J_{Na-H} by nearly 45%, from $\sim 191 \mu M \text{ min}^{-1}$ (Fig. 3, b) to 275 $\mu M \text{ min}^{-1}$ (Fig. 3, c). Thus, the increase in $[Cl^-]_i$ that accompanies shrinkage is an auxiliary shrinkage signal.

Model. As summarized in Fig. 3, the K_m values for intracellular Cl^- are similar under normo- and hypertonic conditions (127 vs. 112 mM). This observation is consistent with the notion that Cl^- plays similar roles in activating the Na-H exchanger at normal and low cell volume. We propose the following model for barnacle muscle fibers: the "shrinkage signal," which leads to activation of the Na-H exchanger, is amplified in such a way that the gain of the hypothetical amplifier increases with increasing $[Cl^-]_i$. In euvoletic cells, the basal shrinkage signal is small, but greater than zero. At a "normal" $[Cl^-]_i$ of 34 mM, the amplification of this small signal is weak. Thus, the combination of a euvoletic cell and a $[Cl^-]_i$ of 34 mM produces a very low J_{Na-H} (i.e., 23 $\mu M \text{ min}^{-1}$).⁴ However, raising $[Cl^-]_i$ to 194 mM in a euvoletic cell increases the amplification of even this weak shrinkage signal, producing a mod-

estly high J_{Na-H} (i.e., 138 $\mu M \text{ min}^{-1}$). In shrunken cells, the shrinkage signal is large. However, at a normal $[Cl^-]_i$ of 56 mM (produced by shrinking a cell with an initial $[Cl^-]_i$ of 34 mM), the amplification is weak, producing a rather modest J_{Na-H} (i.e., 86 $\mu M \text{ min}^{-1}$). However, the combination of a shrunken cell and a $[Cl^-]_i$ increased to 318 mM produces a robust J_{Na-H} (i.e., 345 $\mu M \text{ min}^{-1}$).

Activation of Na-H by GTP γ S, AIF₃, and CTX in the Absence of Cl⁻

In previous work, we showed that, at a pH_i of 6.8, Cl^- is required for the shrinkage-induced activation of Na-H exchange (Davis et al., 1994). Studying barnacle muscle fibers at a pH_i of ~ 7.2 , we also showed that a G protein is involved in this process (Davis et al., 1992a). In particular, we showed that dialyzing with GDP β S blocks the shrinkage-induced activation of the exchanger. The present study extends the previous G-protein work by demonstrating that GTP γ S and CTX both activate the exchanger at pH_i 6.8. In addition, we extend the earlier work by demonstrating that AIF₃ also activates the exchanger. Because AIF₃ stimulates heterotrimeric G proteins, but not low-molecular-weight G proteins (Kaziro et al., 1991; Bigay et al., 1985), our new observations with AIF₃ imply that activation of a heterotrimeric G protein can activate the exchanger.⁵ In earlier work, we showed that activating the PKA or PKC pathways fails to stimulate the exchanger (Davis et al., 1992a). Thus, the most straightforward explanation for our data is that the signal-transduction cascade triggered by cell shrinkage includes a heterotrimeric G protein.

One of the goals of the present study was to determine the order of the Cl^- -dependent and G-protein steps in the shrinkage signal-transduction cascade. One possibility is that Cl^- acts at a step somewhere in the G-protein cycle. Indeed, Cl^- appears to increase the affinity of α_o for GTP γ S (Higashijima et al., 1987). An increase in affinity of α for GTP would stabilize α in the GTP-bound or active state. A Cl^- requirement of the G protein in the signal-transduction cascade could explain the Cl^- -dependent activation of Na-H exchange in dog and trout RBCs, and in BMFs. To determine the order of the Cl^- -dependent and G-protein steps in our BMF experiments, we attempted to activate Na-H ex-

⁴We would also predict that this "basal" shrinkage signal is also G-protein dependent so that dialyzing with GDP β S should have the same effect as dialyzing with a Cl^- -free solution: inhibition of basal Na-H exchange.

⁵A recent report shows that AIF₃ can bind to a ras-GAP-GDP complex, inducing a shift in the absorbance spectrum of a fluorescent GDP analog (Mittal et al., 1996). The crystal structure of this ras-GAP-GDP-AIF₃ complex has been solved (Scheffzek et al., 1997). However, there is no evidence that AIF₃ activates a ras-related protein.

change with GTP γ S, AlF₃, and CTX in Cl⁻-depleted cells. As noted above, we found that Cl⁻ depletion blocks Na-H exchange under normotonic conditions, at pH_i \cong 6.8 (Fig. 4). Even in such Cl⁻-depleted cells, introducing GTP γ S (Fig. 5 A), AlF₃ (Fig. 5 B), or CTX (Fig. 6) activates the Na-H exchanger. Because each of

these three agents acts at the level of the G protein,⁶ and is nevertheless able to bypass the blockade introduced by Cl⁻ removal, we can conclude that the Cl⁻-dependent step must precede or be concurrent with the G-protein step.

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⁶In principle, introducing GTP γ S could also result in the de novo generation of ATP γ S within the muscle fiber. Such ATP γ S could lead to the generation of long lived phosphoproteins. However, our demonstration that cholera toxin and AlF₃ also activate the Na-H exchanger argues that, regardless of a possible phosphorylation, activation of a G protein can lead to activation of the exchanger.

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