Regulation of AE2-mediated Cl^- Transport by Intracellular or by Extracellular pH Requires Highly Conserved Amino Acid Residues of the AE2 NH_2-terminal Cytoplasmic Domain

A.K. Stewart, M.N. Chernova, B.E. Shmukler, S. Wilhelm, and S.L. Alper

Department of Medicine, Harvard Medical School, Molecular Medicine and Renal Units, Beth Israel Deaconess Medical Center, Boston, MA 02215

ABSTRACT We reported recently that regulation by intracellular pH (pH_i) of the murine Cl^-/HCO_3^- exchanger AE2 requires amino acid residues 310–347 of the polypeptide's NH_2-terminal cytoplasmic domain. We have now identified individual amino acid residues within this region whose integrity is required for regulation of AE2 by pH. $^{36}Cl^-$ efflux from AE2-expressing *Xenopus* oocytes was monitored during variation of extracellular pH (pH_o) with unclamped or clamped pH_i, or during variation of pH_i at constant pH_o. Wild-type AE2–mediated $^{36}Cl^-$ efflux was profoundly inhibited by acid pH_o, with a value of $pH_{o(50)} = 6.87 \pm 0.05$, and was stimulated up to 10-fold by the intracellular alkalinization produced by bath removal of the preequilibrated weak acid, butyrate. Systematic hexa-alanine [$(A)_6$]bloc substitutions between aa 312–347 identified the greatest acid shift in $pH_{o(50)}$ value, ~0.8 pH units in the mutant $(A)_6$342–347, but only a modest acid-shift in the mutant $(A)_6$336–341. Two of the six $(A)_6$ mutants retained normal pH_i sensitivity of $^{36}Cl^-$ efflux, whereas the $(A)_6$ mutants 318–323, 336–341, and 342–347 were not stimulated by intracellular alkalinization. We further evaluated the highly conserved region between aa 336–347 by alanine scan and other mutagenesis of single residues. Significant changes in AE2 sensitivity to pH_o and to pH_i were found independently and in concert. The E346A mutation acid-shifted the $pH_{o(50)}$ value to the same extent whether pH_i was unclamped or held constant during variation of pH_o. Alanine substitution of the corresponding glutamate residues in the cytoplasmic domains of related AE anion exchanger polypeptides confirmed the general importance of these residues in regulation of anion exchange by pH. Conserved, individual amino acid residues of the AE2 cytoplasmic domain contribute to independent regulation of anion exchange activity by pH_o as well as pH_i.

KEY WORDS: Cl^-/HCO_3^- exchange • weak acids • *Xenopus* oocytes • isotopic flux • pH-sensitive microelectrodes

INTRODUCTION

The SLC4 bicarbonate transporter gene superfamily includes the AE gene family of Na^+-independent Cl^-/HCO_3^- exchangers AE1, AE2, and AE3. These anion exchangers contribute to the regulation of cell pH_i, cell volume, tonicity, and intracellular Cl^- homeostasis in vertebrate eukaryotic cells (Alper, 1994; Bevensee et al., 2000; Alper et al., 2002). All AE polypeptides share a highly conserved hydrophobic, polytopic, COOH-terminal transmembrane domain of >500 amino acids (aa),* with a short COOH-terminal cytoplasmic tail capable of binding carbonic anhydrase II (Vince and Reithmeier, 2000; Sterling et al., 2002b). This transmembrane domain is preceded by a less extensively conserved hydrophilic NH_2-terminal cytoplasmic domain of 400–700 aa (Alper, 1994). The COOH-terminal transmembrane domain suffices to mediate anion exchange in the absence of nearly the entire NH_2-terminal cytoplasmic domain (Grinstein et al., 1978; Kopito et al., 1989). Although the cytoplasmic NH_2-terminal domain of AE1 is important for its binding to multiple cytoskeletal proteins, glycolytic enzymes, and hemoglobin (Zhang et al., 2000), the functions of the cytoplasmic NH_2-terminal domains of AE2 and AE3 remain less extensively investigated.

The polypeptide products of the various AE genes differ in their acute regulation by pH. Native AE1-mediated Cl^-/HCO_3^- exchange in erythrocytes (Funder and Wieth, 1976) and heterologous AE1–mediated Cl^-/Cl^-exchange in *Xenopus* oocytes (Humphreys et al., 1994; Zhang et al., 1996) displays a broad pH versus activity profile, consistent with its primary role in facilitating CO_2/HCO_3^- exchange between the respiring tissues and lungs (Jennings, 1992). In contrast, nonerythroid Na^+-independent Cl^-/HCO_3^- exchange in many tissue culture cells is sensitively regulated by changes in pH_i (Reinertsen et al., 1988), consistent with its proposed

A.K. Stewart's present address is University Laboratory of Physiology, University of Oxford, Parks Road, Oxford OX1 3PT, UK.

Address correspondence to Seth L. Alper, RW763 East Campus, Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, MA 02215. Fax: (617) 667-8040; E-mail: salper@caregroup.harvard.edu

Abbreviation used in this paper: aa, amino acids.

J. Gen. Physiol. © The Rockefeller University Press • 0022-1295/2002/11/707/16 $5.00
Volume 120 November 2002 707–722
http://www.jgp.org/cgi/doi/10.1085/jgp.20028641

role in recovery from alkaline loads. Similarly, the recombinant nonerythroid anion exchanger AE2 is highly sensitive to changes in pH_i when expressed in tissue culture cells (Lee et al., 1991; Jiang et al., 1994) or in *Xenopus* oocytes (Humphreys et al., 1994; Zhang et al., 1996; Stewart et al., 2001). In contrast, recombinant AE3 expressed in 293 cells has been reported to be insensitive to changes in pH_i (Sterling and Casey, 1999).

Zhang et al. (1996) compared regulation by pH of anion influx mediated by AE1 and AE2 in *Xenopus* oocytes, and localized to the AE2 transmembrane domain a "pH sensor site" which conferred increased pH-sensitivity of anion transport. These structure-function studies demonstrated that the extracellular proton sensitivity ($pH_{o(50)}$ value) of AE2-mediated $^{36}Cl^-$ influx (~7.0 under conditions of unclamped pH_i) was acid-shifted 0.7 pH units by truncation of the cytoplasmic NH_2-terminal 510 aa. This result suggested a role for the cytoplasmic NH_2-terminal domain in regulation of AE2 transport by changing pH, and localized between aa 99 and 510 of the 705 aa AE2 NH_2-terminal cytoplasmic domain, a "pH modifier site" that modulated the $pH_{o(50)}$ value of the transmembrane domain.

More recently, we demonstrated that variation of pH_o regulates AE2 activity in efflux assays and requires the integrity of two noncontiguous regions in the NH_2-terminal cytoplasmic domain, encompassing aa 328–347 and aa 391–510 (Stewart et al., 2001). We further showed by varying pH_i at constant pH_o that removal of the NH_2-terminal 310 aa greatly reduced AE2 regulation by pH_i, but was without effect on AE2 regulation by varying pH_o. In the present work we have defined individual amino acid residues within the NH_2-terminal cytoplasmic domain whose mutation alters the regulation of AE2-mediated Cl^- transport activity by changing pH_o with constant or minimally changing pH_i, and by varying pH_i at constant pH_o. We demonstrate that a delimited, highly conserved region of the AE2 NH_2-terminal cytoplasmic domain is involved in AE2 regulation by both pH_i and pH_o. We further demonstrate that some of these conserved residues in corresponding regions of related anion exchanger polypeptides contribute similarly to their functional regulation by pH_i.

MATERIALS AND METHODS

Reagents

$Na^{36}Cl$ was purchased from ICN. Other chemical reagents were of analytical grade and obtained from Sigma-Aldrich, Calbiochem, or Fluka. Restriction enzymes and T4 DNA ligase were from New England BioLabs, Inc. Taq DNA polymerase and dNTPs were from Promega.

Solutions

ND-96 medium consisted of (in mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, and 2.5 sodium pyruvate, pH 7.40. Flux media lacked sodium pyruvate. pH values of 7.0, 8.0, and 8.5 in room air flux media were achieved with 5 mM HEPES. 5 mM Mes was used was used for room air flux media of pH values 5.0 and 6.0. In Cl^--free solutions, NaCl was replaced isosmotically with 96 mM sodium isethionate, and equimolar K, Ca, and Mg gluconate were substituted for the corresponding Cl^- salts. CO_2/HCO_3^--buffered solutions of pH 7.4 were saturated with 5% CO_2–95% air at room temperature for ~1 h and differed from Cl^- free ND-96 in replacement of 24 mM sodium isethionate with 24 mM $NaHCO_3^-$. pH of CO_2/HCO_3^--buffered solutions was verified before each experiment. Addition to flux media of the weak acid salt sodium butyrate was in equimolar substitution for NaCl.

Mutant AE2 cDNAs

Murine AE2 encoded in plasmid pΔX (Zhang et al., 1996) was used as template for PCR. The AE2 hexa-Ala bloc substitution mutants $(A)_6$312–317, $(A)_6$318–323, $(A)_6$324–329, $(A)_6$330–335, $(A)_6$336–341, and $(A)_6$342–347 were constructed by a four primer PCR method as described (Zhang et al., 1996; Chernova et al., 1997a,b; Stewart et al., 2001). Single point mutations were constructed by the same method to generate single residue missense mutants. Oligonucleotide primers were obtained from Biosynthesis; primer sequences are available upon request. Integrity of PCR products and ligation junctions was confirmed by DNA sequencing of both strands.

cRNA Expression in Xenopus Oocytes

Mature female *Xenopus* (NASCO) were maintained and subjected to partial ovariectomy as described (Humphreys et al., 1994). Stage V-VI oocytes were manually defolliculated following incubation of ovarian fragments with 2 mg/ml collagenase A (Boehringer) for 60 min in ND-96 solution containing 50 ng/ml gentamycin and 2.5 mM sodium pyruvate. Oocytes were injected on the same day with cRNA or with 50 nl H_2O. Capped cRNA was transcribed from linearized cDNA templates with the T7 MEGAscript kit (Ambion), and resuspended in diethylpyrocarbonate-treated water. RNA integrity was confirmed by agarose gel electrophoresis in formaldehyde, and RNA concentration was estimated by A_{260}. Injected oocytes were then maintained for 2–6 d at 19°C.

$^{36}Cl^-$ Efflux Measurements

Individual oocytes in Cl^--free ND-96 were injected with 50 nl of 130 mM $Na^{36}Cl$ (10,000–12,000 cpm). After a 5–10 min recovery period, the efflux assay was initiated by transfer of individual oocytes to 6 ml borosilicate glass tubes, each containing 1 ml efflux solution. At intervals of 3 min, 0.95 ml of this efflux solution was removed for scintillation counting and replaced with an equal volume of fresh efflux solution. After completion of the assay with a final efflux period in the presence of the anion transport inhibitor 4,4'-di-isothiocyanatostilbene-2,2' disulfonic acid (DIDS; 200 μM), each oocyte was lysed in 100 μl of 2% SDS. Samples were counted for 3–5 min such that the magnitude of 2SD was $<5\%$ of the sample mean.

Experimental data were plotted as ln (percentage cpm remaining in the oocyte) versus time. $^{36}Cl^-$ efflux rate constants were measured from linear fits to data from the last three time points sampled for each experimental condition. All single time point values for $^{36}Cl^-$ efflux from AE2 cRNA–injected oocytes into chloride medium exceeded 150 cpm. Efflux cpm values for water-injected oocytes (40–90 cpm) were indistinguishable from those for AE2 cRNA–injected oocytes in the presence of DIDS, and both less than threefold higher than machine background values (typically 20 cpm, peak 30 cpm). Within each experiment,

water-injected and AE2 cRNA–injected oocytes from the same frog were subjected to parallel measurements. On each experimental day, activity of tested mutant AE2 polypeptides was compared with wt AE2 activity at pH 7.4. Each AE2 mutant was tested in oocytes from at least three frogs.

Individual oocytes maintained in Cl^--free solution at pH_o 7.4 were exposed sequentially to (Cl^--containing) ND-96 at pH 5.0, 6.0, 7.0, 8.0, and 8.5, then to solution of pH 8.5 in the presence of DIDS. During this variation of bath pH between pH_o 5 and 8.5, oocyte pH_i varied between extreme values of ~7.1 and ~7.6, but usually to a lower degree (Zhang et al., 1996; Stewart et al., 2001). Rate constants measured at each pH_o value for wt AE2 and for the tested AE2 mutants in each individual experiment were fit (Sigma Plot) to the following first-order logistic sigmoid equation:

$$v = (V_{max} \times 10^{-K})/(10^{-K} + 10^{-x}) + d, \quad (1)$$

where v = AE2-mediated Cl^- efflux rate constant, V_{max} = the maximum AE2-mediated Cl^- efflux rate constant, x = pH_o at which rate constant was measured, K = $pH_{o(50)}$, the pH_o at which v is half-maximal, and d = 0 in all cases except that of the AE2 mutant $(A)_6$342–347, for which d = 24.3 ± 6.9% (see Fig. 2 D). Rate constants for each mutant were normalized to the fit parameter V_{max} calculated for each individual oocyte (100%), and the normalized data were fit to the same equation (Eq. 1). Differences in mean $pH_{o(50)}$ values for individual mutants were subjected to analysis of variance (Zhang et al., 1996) and by comparison for all pairs using Tukey-Kramer analysis (JMP for Macintosh). pH_o dependence of wild-type AE2-mediated $^{36}Cl^-$ efflux did not differ when the experiment was performed with the order of pH_o change reversed (Stewart et al., 2001).

To vary pH_i at constant pH_o, $^{36}Cl^-$-injected oocytes were preincubated for 30 min before the start of the experiment in pH 7.4 Cl^--free solution containing 40 mM sodium butyrate. $^{36}Cl^-$ efflux was then initiated by transfer of oocytes into Cl^--containing solution in the continued presence of butyrate. The oocytes were then transferred into efflux medium containing Cl^- but lacking weak acid. In these conditions, pH_i increases 0.50 ± 0.03 pH units after butyrate removal, with a time constant of ~6 min (Stewart et al., 2001). Neither the addition of butyrate nor its subsequent removal led to changes in the intracellular-free Ca^{2+} concentration (unpublished data) as measured by fluorimetric ratio imaging in FURA2-AM–loaded oocytes (Vandorpe et al., 2001). pH_i sensitivity of AE2-mediated $^{36}Cl^-$ efflux was expressed for individual oocytes as fold stimulation of AE2 activity after weak acid removal (rate constant after removal of weak acid/rate constant in presence of weak acid). We have shown previously that butyrate is neither an inhibitor of nor a substrate for AE2 (Stewart et al., 2001).

To maintain constant pH_i during variation of pH_o concentrations of extracellular butyrate were chosen for each pH_o value according to the Henderson-Hasselbalch equation to yield a uniform total intracellular butyrate concentration of 0.5 mM at steady-state pH_i. pH_i was assumed to be 7.0 when pH_o was 7.4, and the pK_a of butyrate was taken as 4.82. Sodium butyrate was substituted isosmotically with NaCl. Bath butyrate concentrations were 0.005 mM at bath pH 5.0, 0.05 mM at bath pH 6.0, 0.5 mM at pH 7.0, 5 mM at pH 8.0, and 20 mM at pH 8.5.

Measurement of Oocyte pH_i

Oocyte pH_i was measured during bath superfusion using pH microelectrodes as described previously (Romero et al., 1997; Stewart et al., 2001). Oocyte pH_i was measured during bath superfusion using BCECF ratio fluorimetry, also as described previously (Zhang et al., 1996).

Statistics

Analysis of variance followed by Tukey-Kramer analysis was performed among groups of mutants compared with wild-type AE2. Positive values indicated means that were significantly different. Statistical significance was also assessed by Student's paired and unpaired *t* tests, for which level of significance was $P < 0.05$.

RESULTS

AE2-mediated Cl^- Transport Is Sensitive to Changing pH_o when pH_i Is Clamped

We have shown previously that AE2 function in *Xenopus* oocytes is highly sensitive to changes in pH_i within the physiological range when pH_o is held constant, using introduction and removal of the permeant weak acid butyrate that is neither inhibitor nor substrate of AE2 (Stewart et al., 2001). Our earlier studies had demonstrated the high sensitivity of AE2 function to changes in pH_o (Zhang et al., 1996; Stewart et al., 2001), but under conditions in which near-steady-state pH_i was unclamped and changed in parallel with pH_o. Although the range of pH_o change in these experiments far exceeded the range of unclamped pH_i change, the ability of changing pH_o alone to regulate AE2 function at constant pH_i remained in question. We therefore devised conditions in which variation of pH_o in tandem with variation of bath butyrate concentration might effectively clamp pH_i during changes in pH_o.

Fig. 1 A depicts microelectrode measurements of changing pH_i in a representative AE2-expressing oocyte subjected to sequential 15 min exposures to varying pH_o, matching the $^{36}Cl^-$ efflux protocol. Exposure of a different AE2-expressing oocyte (Fig. 1 B) to the same pattern of pH_o change while simultaneously varying bath butyrate concentration almost completely damped the corresponding changes in pH_i.[1] Fig. 1 C summarizes the pH_i versus pH_o relationship in the absence (top slope = 0.02) and presence of adjusted butyrate concentrations (bottom slope = 0.002). Only in the absence of changing [butyrate] did oocyte pH_i change during an increase in pH_o from 5.0 to 8.5 ($P = 0.03$, Student's unpaired *t* test). Parallel measurement of oocyte pH_i by BCECF ratio fluorimetry in the absence (n = 4–8) or presence of step changes in graded extracellular [butyrate] (n = 6–12) similarly showed that butyrate damped pH_i change in response to changing pH_o (unpublished data). Thus, simultaneous variation of bath butyrate concentration and pH_o allowed evaluation of AE2 regulation by changing pH_o while pH_i remained nearly constant (nominal "pH_i clamp").

Fig. 1, D–F, show traces of $^{36}Cl^-$ efflux from oocytes expressing wild-type (wt) AE2 and maintained under pH_i clamp conditions during three protocols of pH_o change: sequentially from 5.0 to 8.5 (1D), sequentially

from 8.5 to 5.0 (1E), and nonsequentially (1F). As shown in Fig. 1 G, the dependence of AE2 activity on pH_o was essentially indistinguishable for these three pH_i clamp protocols, and none of the three differed significantly from the pH_o dependence of AE2 activity measured in the absence of pH_i clamp (Fig. 1 H). These experiments confirm that changing pH_o can regulate AE2-mediated Cl^- transport even when pH_i remains essentially unchanged. Moreover, imposition of pH_i clamp conditions does not alter the apparent pH_o dependence of AE2 activity.

Regulation of AE2 NH_2-terminal Hexa-alanine Substitution Mutants by Varying Bath pH

We have shown previously that the region encompassing amino acids 310–347 within the NH_2-terminal cytoplasmic domain of the murine AE2 anion exchanger is required for normal regulation of AE2-mediated Cl^- transport by pH_o and by pH_i (Stewart et al., 2001). We studied this region in greater detail through the systematic substitution of hexa-alanine blocs into consecutive six-amino acid stretches of AE2, as illustrated in the schematic of Fig. 2 A. Fig. 2 B presents the $^{36}Cl^-$ efflux rate constants measured at pH_o 7.4 exhibited by oocytes expressing AE2 hexa-alanine bloc substitutions. With the sole exception of $(A)_6$318–323, all mutants exhibited $^{36}Cl^-$ efflux activity statistically indistinguishable from that of wild-type AE2. The low $^{36}Cl^-$ efflux activity of the $(A)_6$318–323 mutant at pH 7.4 precluded subsequent evaluation of its regulation by pH_o (Fig. 2 E), but sufficed to evaluate its regulation by pH_i (Fig. 2 G).

To define those portions of the region encompassing residues 312–347 with importance for AE2 regulation by pH_o, we measured $^{36}Cl^-$ efflux activity of the hexa-Ala bloc mutants under conditions of varying pH_o at unclamped pH_i. Fig. 2 C shows a representative $^{36}Cl^-$ efflux trace from three AE2-expressing oocytes and from one water-injected oocyte as a function of sequentially increasing pH_o. Wild-type AE2–mediated $^{36}Cl^-$ efflux was minimal at low pH_o, increased at higher pH_o values, and was inhibited by 200 μM DIDS added at the final pH_o of 8.5. Whereas AE2 $(A)_6$330–335 exhibited a pattern of $^{36}Cl^-$ efflux similar to that of wild-type AE2, $(A)_6$342–347 showed reduced inhibition of $^{36}Cl^-$ efflux activity at lower pH_o compared with wild-type AE2. Fig. 2 D profiles $^{36}Cl^-$ efflux activity (normalized as described in MATERIALS AND METHODS) as a function of pH_o for wild-type AE2 and two representative hexa-Ala bloc mutants, $(A)_6$330–335 and $(A)_6$342–347. The pH_o value at which the rate constant for wild-type AE2–mediated $^{36}Cl^-$ efflux was half-maximal [$pH_{o(50)}$] was 6.87 ± 0.05 (n = 36; Fig. 2, D and E). The hexa-Ala bloc mutant $(A)_6$330–335 exhibited pH_o dependence indistinguishable from that of wild-type AE2, whereas the $pH_{o(50)}$ value of the AE2 mutant $(A)_6$342–347 was shifted to a more acidic pH value of 6.11 ± 0.11 (n = 11, $P < 0.05$).[2] Fig. 2 E summarizes the $pH_{o(50)}$ values measured as shown in Fig. 2 D for all hexa-Ala bloc mutants. The data suggest that amino acid residues 342–347 are particularly important for regulation of AE2 by pH_o. The hexa-Ala bloc mutant $(A)_6$336–341 displayed a $pH_{o(50)}$ value only marginally acid shifted from that of wild-type AE2, as assessed by all pairs Tukey-Kramer analysis. Nonetheless, the high degree of conservation among these amino acid residues (see below) warranted their inclusion among those investigated in greater detail.

Regulation of AE2 NH_2-terminal Hexa-alanine Substitution Mutants by Varying pH_i at Constant pH_o

We next investigated regulation of the same set of AE2 mutants by changing pH_i at constant pH_o, using the weak acid butyrate as described previously (Stewart et

[1]Lowering bath pH from 7.4 to 5.0 in the presence of 5 μM butyrate lowered pH_i by 0.13 ± 0.017 pH units (n = 6) within 15 min (a single case is shown in Fig. 1 B), but only 0.07 ± 0.01 pH units (n = 3) within the same period in the absence of extracellular butyrate (Fig. 1 A presents a representative single trace). Thus, an acidification of ~0.06 pH units can be attributed to butyric acid entry over this time period in this condition. With mean resting pH_i = 7.34 ± 0.02 (n = 11) at pH_o 7.40 (7.21 in example of Fig. 1 C), addition of 5 μM butyrate at pH_o 5.0 is predicted to lead to an equilibrium intracellular butyrate concentration of 0.66 mM. (This is a minimum estimate that assumes free diffusion of butyric acid and no permeability of butyrate anion.) Proton flux represented by this acidification requires knowledge of oocyte buffer capacity. The intrinsic buffer capacity of the oocyte has been estimated by the fall in pH_i after response to a CO_2 pulse of a single concentration (5% CO_2/95% air). In eight oocytes with mean resting pH_i of 7.24 ± 0.06 under HEPES-buffered (i.e., nominally CO_2/HCO_3^- free) conditions, intrinsic buffer capacity was 18.9 ± 1.8 mM/pH unit (n = 8) measured at the midpoint of the 5% CO_2-induced pH_i change, 7.00 ± 0.04. This value for oocyte intrinsic buffer capacity agrees with that of 19.8 mM/pH unit (Cooper and Boron, 1998), and with the value for *Xenopus* early embryo of 18 mM/pH unit (Turin and Warner, 1980). The magnitude of butyric acid entry required to acidify oocytes with intrinsic buffer capacity of 18.9 mM/pH unit is predicted to be 1.13 mM. This value is not much greater than the minimum estimate of 0.66 mM butyrate calculated to have entered the average oocyte in our experiments, especially considering the variation in recorded pH_i with depth of microelectrode penetration, and the strong dependence of intrinsic buffer capacity upon oocyte pH_i prior to CO_2 pulse (buffer capacity values estimated from multiple published pH_i traces of individual oocytes range between 9 and 21 mM/pH unit).

[2]AE2 $(A)_6$342-347 is the only hexa-Ala substitution mutant among those tested that failed to approach zero activity at pH_o 5.0. Therefore, the curve fit to the data of the AE2 mutant $(A)_6$342-347 (inverted triangles) differed from all others computed for this work, such that an extra parameter was used in the sigmoid fit, in order not to force the fit through a zero point (see MATERIALS AND METHODS). The $pH_{o(50)}$ value of 6.11 ± 0.11 arising from this fit for AE2 $(A)_6$342-347 and shown in Fig. 2 D can be considered a maximum estimate. The curve fit using the standard sigmoid equation yields a predicted $pH_{o(50)}$ value of 5.62 ± 0.20, with lower confidence limits.

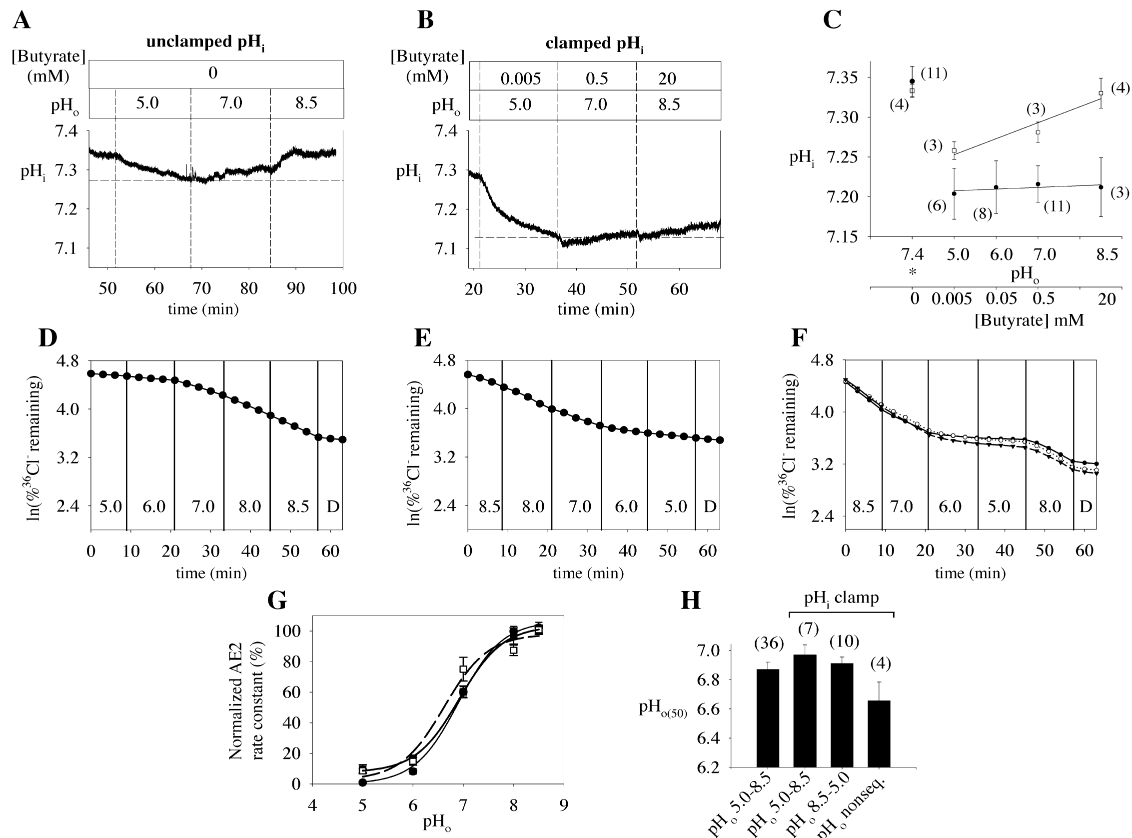

FIGURE 1. AE2-mediated Cl^- transport is regulated by varying pH_o independent of pH_i. (A) pH_i measured by pH-sensitive microelectrode in an AE2-expressing oocyte during the indicated changes in pH_o. (B) pH_i measured by pH-sensitive microelectrode in an AE2-expressing oocyte during the same pH_o changes in the presence of the indicated butyrate concentrations, resulting in nominal "pH_i clamp." Initial condition for both A and B was pH_o 7.4 in absence of butyrate. (C) Summary of pH_o versus pH_i relationship in the presence (filled circles) and absence (open squares) of nominal "pH_i clamp." Initial resting pH_i is indicated by the asterisk. (D) Representative $^{36}Cl^-$ efflux timecourse for wild-type AE2 oocyte measured during sequential increases in pH_o under pH_i clamp conditions. D = 200 μM DIDS. (E) Representative $^{36}Cl^-$ efflux timecourse for wild-type AE2 oocyte measured during sequential decreases in pH_o under pH_i clamp conditions. (F) Representative $^{36}Cl^-$ efflux timecourse for wild-type AE2-expressing oocytes measured during nonsequential order of pH_o changes under pH_i clamp conditions. (G) pH_o versus activity profile for oocytes expressing wild-type AE2 under pH_i clamp conditions when pH_o is changed from 5.0 to 8.5 (filled circles), from 8.5 to 5.0 (filled squares), and in nonsequential order (open squares). (H) $pH_{o(50)}$ values exhibited by wild-type AE2 where pH_o has been changed under conditions of unclamped pH_i and under the pH_i clamp conditions indicated, calculated from fits of pH_o versus $^{36}Cl^-$ efflux activity plots as in G for *n* individual oocytes (means ± SEM). pH nonseq. = nonsequential order of bath pH changes. The $pH_{o(50)}$ values did not differ as assessed by ANOVA.

al., 2001). Fig. 2 F shows a representative efflux trace in which $^{36}Cl^-$ efflux activities of wild-type AE2 and of AE2 $(A)_6$330–335 are reduced at low pH_i and subsequently stimulated when pH_i is elevated (by butyrate removal). In contrast, AE2 $(A)_6$342–347-mediated $^{36}Cl^-$ efflux activity was insensitive to changes in pH_i imposed by butyrate addition and removal. Fig. 2 G summarizes similar experiments for all hexa-Ala bloc mutants, expressed as fold stimulation of $^{36}Cl^-$ efflux rate constant after butyrate removal. The data reveal the importance of two discrete regions encompassing aa 318–323 and 336–347 for wild-type regulation of AE2-mediated Cl^- transport by varying pH_i. Hexa-Ala substitutions in these regions reduced AE2 stimulation by butyrate removal from the wild-type 10-fold to below 1.5-fold ($P < 0.002$, Student's unpaired *t* test). In contrast, the mutants $(A)_6$324–329 and $(A)_6$330–335 retained wild-type stimulation. The $(A)_6$312–317 mutant retained significant, though reduced, sensitivity to pH_i elevation, showing 3.5-fold stimulation by butyrate removal ($P < 0.002$).

Determination of Individual Amino Acid Residues Important for AE2 Regulation by pH_o and pH_i

The hexa-Ala bloc substitution mutants implicated AE2 aa 336–347 as important for regulation of AE2 activity by pH. To define the individual residues required for this regulation, we tested the functional properties of point mutants created as part of a modified alanine scan of this region (Fig. 3 A). Threonine 339, a weak candidate

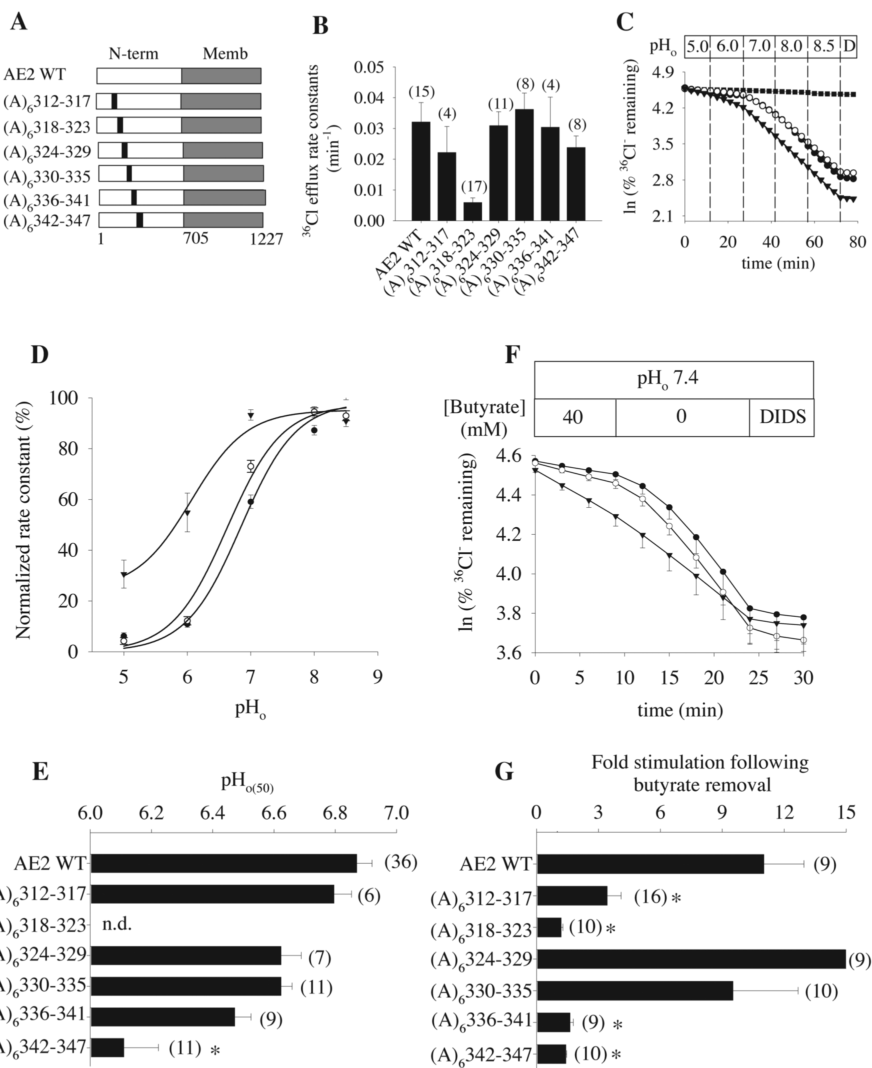

FIGURE 2. Hexa-alanine bloc substitution mutants within residues 312–347 of AE2 define segments important for regulation of Cl^- transport by pH_o and by pH_i. (A) Schematic of wild-type AE2 (top) and (below) hexa-alanine $[(A)_6]$ substitution mutations (black boxes) spanning AE2 residues 312–347 of the NH_2-terminal cytoplasmic domain (white box), preceding the AE2 transmembrane domain (gray box). (B) $^{36}Cl^-$ efflux rate constants for n oocytes measured at pH_o 7.4 in oocytes expressing wild-type AE2 or the indicated AE2 $(A)_6$ mutants (values are means ± SEM). (C) Representative $^{36}Cl^-$ efflux timecourse for three wild-type AE2 oocytes (bottom traces) and one H_2O-injected oocyte (top trace) measured during stepwise increases in pH_o (top bar). (D) Regulation by pH_o of normalized $^{36}Cl^-$ efflux from oocytes expressing wild-type AE2 (filled circles), mutant AE2 $(A)_6$330–335 (open circles), and mutant AE2 $(A)_6$342–347 (filled inverted triangles). Values are means ± SEM; curves were fit to data as described in MATERIALS AND METHODS. (E) $pH_{o(50)}$ values exhibited by wild-type AE2 and the indicated AE2 $(A)_6$ mutants, calculated from fits of pH_o versus $^{36}Cl^-$ efflux activity plots such as that in D for n individual oocytes (means ± SEM). Asterisk indicates $P < 0.05$. AE2 $(A)_6$318–323 Cl^- efflux rate constant (B) was too low for measurement of an inhibitory $pH_{o(50)}$ value (n.d., not done). (F) Representative time course of $^{36}Cl^-$ efflux from oocytes expressing wild-type AE2 (closed circles), AE2 mutant $(A)_6$330–335 (open circles), and AE2 mutant $(A)_6$342–347 (filled inverted triangles) during elevation of pH_i by removal of bath butyrate (40 mM) and subsequent inhibition by DIDS (200 μM). (G) Mean fold stimulation of Cl^- efflux (±SEM) after bath butyrate removal from n oocytes expressing wild-type AE2 or the indicated AE2 $(A)_6$ mutants. Asterisk indicates $P < 0.002$.

PKC site, was mutated to glutamate and (not depicted) to valine. Alanine 340 was mutated to glycine. Fig. 3 B shows that none of the mutants exhibited significant reduction of $^{36}Cl^-$ efflux activity at pH_o 7.4. Fig. 3 C compares representative pH_o versus Cl^- efflux activity profiles of three AE2 point mutants with that of wild-type AE2. AE2 R337A exhibited a pH_o dependence indistinguishable from that of wild-type AE2, whereas the mutants E346A and E347A displayed more acidic $pH_{o(50)}$ values of 6.10 ± 0.07 (n = 14, $P < 0.05$) and 6.20 ± 0.05 (n = 11, $P < 0.05$), respectively. Fig. 3 D summarizes the $pH_{o(50)}$ values for all point mutants measured as in Fig. 2 C. The gray bars indicate the four residues (W336, R341, E346, and E347) for which alanine substitution led to significant acid-shifts in $pH_{o(50)}$ value relative to that of wild-type AE2. Alanine substitution of other residues did not significantly shift $pH_{o(50)}$ values.

We tested the importance of the same individual amino acid residues of AE2 in its regulation by pH_i. Fig. 3 E shows a $^{36}Cl^-$ efflux trace in the initial presence and subsequent absence of the weak acid butyrate, thus alkalinizing pH_i at constant pH_o. The ΔpH_i following butyrate removal was 0.56 ± 0.04 pH units with a time constant ($t_{0.5}$) of 322 ± 92s (n = 4). Thus, there was a lag time between t = 9 min and t = 12–15 min, during which $^{36}Cl^-$ efflux accelerated to its new steady-state. Rate constants for the new steady-state were calculated between t = 18 min and 24 min (Fig. 3 E). $^{36}Cl^-$ efflux activity of the AE2 mutants R337A and E346A exhibited pH_i sensitivity identical to that of wild-type AE2, whereas $^{36}Cl^-$ efflux mediated by the AE2 mutant E347A was insensitive to this change in pH_i. Fig. 3 F summarizes the pH_i sensitivity of the AE2 point mutants compared with wild-type AE2. Stimulation of

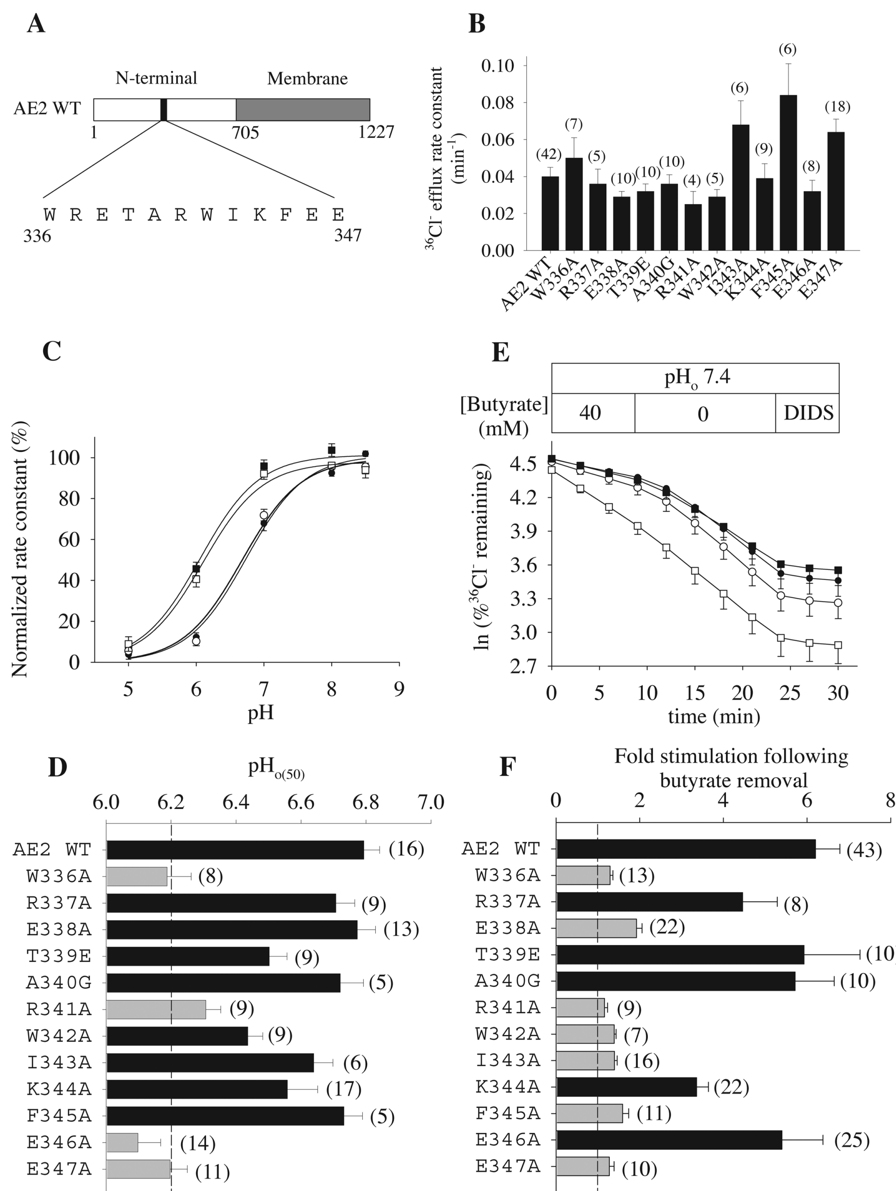

FIGURE 3. Systematic point mutagenesis of AE2 residues 336–347 identifies amino acids important for regulation of Cl^- transport by pH_o and by pH_i. (A) Schematic of point mutations in the NH_2-terminal cytoplasmic domain of AE2. (B) $^{36}Cl^-$ efflux rate constants measured at pH_o 7.4 in *n* oocytes expressing wild-type AE2 or the indicated AE2 substitution point mutants (mean ± SEM). (C) Regulation by pH_o of normalized $^{36}Cl^-$ efflux from oocytes expressing wild-type AE2 (filled circles) or the AE2 mutants R337A (open circles), E346A (filled squares), and E347A (open squares). Values are means ± SEM. (D) $pH_{o(50)}$ values for the indicated single codon mutants (means ± SEM). Gray bars indicate $pH_{o(50)}$ values significantly different from wild-type AE2 ($P < 0.05$, Student's unpaired *t* test). (E) Representative time course of $^{36}Cl^-$ efflux from oocytes expressing wild-type AE2 (closed circles) or the AE2 mutants R337A (open circles), E346A (filled squares), or E347A (open squares) during elevation of pH_i by removal of bath butyrate (40 mM) and subsequent inhibition by DIDS (200 μM). (F) Mean fold stimulation (±SEM) of Cl^- efflux following bath butyrate removal from *n* oocytes expressing wild-type AE2 or the indicated AE2 point mutants. Gray bars indicate mutants for which stimulation values differed significantly from wild-type AE2 ($P < 0.05$, Student's unpaired *t* test).

$^{36}Cl^-$ efflux by butyrate removal was indistinguishable from wild-type level (black bars) in 4 of the 12 mutants, and was only slightly reduced for K344A. In contrast, 7 of the 12 alanine substitution mutants exhibited severely attenuated stimulation by butyrate removal (gray bars for W336, E338, R341, W342, I343, F345, and E347) ($P < 0.05$, Student's unpaired *t* test).

The AE2 mutants W336A, R341A, and E347A were concordant in their mutant phenotypes of regulation by pH_i and by pH_o, whereas mutants R337A, T339E, T339V (not depicted), A340G, and K344A were concordant in their wild-type or near wild-type phenotypes. Four AE2 mutants, E338A, I343A, F345A, and W342A, were insensitive to changing pH_i while maintaining wild-type or near wild-type pH_o sensitivity (Fig. 3, D and F). AE2 E346A was the only mutant that displayed acid-shifted pH_o sensitivity (Figs. 3 C and 2 D) together with wild-type pH_i sensitivity (Fig. 3 E). Alanine substitution of the neighboring residue E347 yielded both acid-shifted pH_o sensitivity and loss of pH_i sensitivity. These data define several amino acid residues whose mutation can alter the wild-type regulation of AE2 activity by pH. The differential consequences for regulation by pH_o and by pH_i based on the point mutations studied further supports the hypothesis that AE2 activity is regulated independently by extracellular and by intracellular protons.

The Effects of Missense Mutations on AE2 Sensitivity to Changing pH_o Are Not Altered Under Conditions of pH_i Clamp

We compared the effect of the mutation E346A on pH_o dependence of AE2 function in conditions of "unclamped" and "clamped" pH_i. The pH_o versus Cl^-

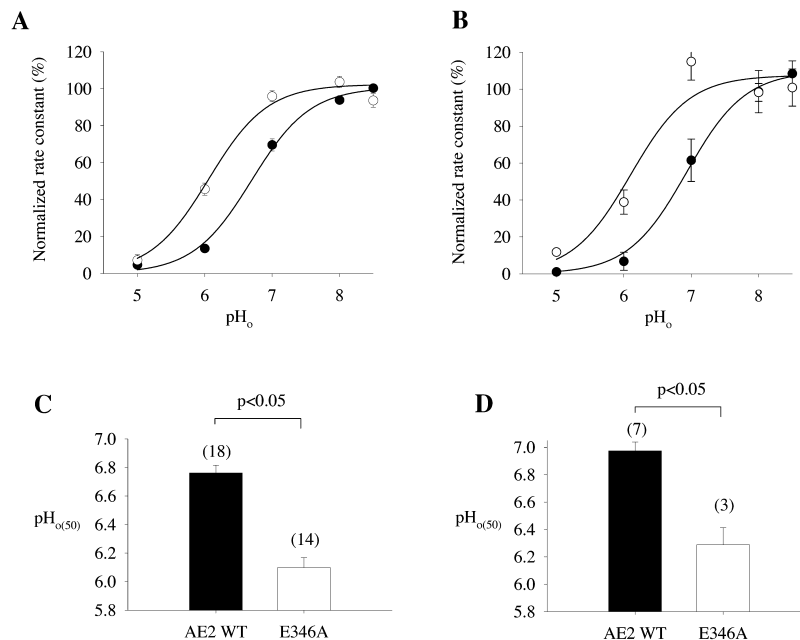

FIGURE 4. AE2 residue E346 is important for AE2 regulation by changing pH_o at constant pH_i. (A) pH_o versus activity profile for oocytes expressing wild-type AE2 (filled circles) or AE2 E346A (open circles). (B) pH_o versus activity profile for oocytes expressing wild-type AE2 (filled circles) or AE2 E346A (open circles), measured under the panel B conditions of nominal "pH_i clamp." (C) Comparison of $pH_{o(50)}$ values for wild-type AE2 and AE2 E346A. (D) Comparison of $pH_{o(50)}$ values for wild-type AE2 and AE2 E346A in conditions of nominal "pH_i clamp." Values in C and D are means ± SEM for n oocytes.

efflux activity profile in the absence of butyrate (Fig. 4 A) revealed respective $pH_{o(50)}$ values for wild-type and mutant AE2 of 6.76 ± 0.05 (n = 18) and 6.10 ± 0.07, n = 14, P <0.05), similar to values presented in Fig. 3 E. Fig. 4 B show that this difference between the pH_o sensitivity of mutant and wild-type AE2 did not change when pH_o was varied during conditions of "pH_i clamp." In these conditions, the difference between the $pH_{o(50)}$ values of wild-type AE2 (6.97 ± 0.06, n = 7), and of AE2 E346A (6.29 ± 0.13, n = 3) was maintained (P < 0.05). Fig. 4, C and D, summarize the pH_o sensitivity data for wild-type AE2 and E346A and further confirm that AE2-mediated Cl^- transport can be regulated by changing pH_o when pH_i remains nearly constant. Moreover, at least one residue of the AE2 NH_2-terminal intracellular domain among the several important for AE2 regulation by changing pH_o is also important for regulation by changing pH_o at near constant pH_i.

Comparison of Charge and Steric Effects at AE2 Residues 346 and 347

Mutation of any one of the three glutamate residues among aa 336–347 of AE2 altered at least one aspect of AE2 regulation by pH. We therefore compared the consequences of substituting Ala in place of E346 and E347 with those of the charge-conserving Asp substitution (Fig. 5 A). Mutant transport activity at pH 7.4 was not significantly reduced from wild-type level (not depicted). The $^{36}Cl^-$ efflux traces of Fig. 5 B reveal that, in contrast to wild-type AE2, neither mutant E346D nor E347D was sensitive to the intracellular alkalinization produced by butyrate removal. Fig. 5 C shows a similar pH_i phenotype for the double mutant E346D/E347D and emphasizes its contrast with the wild-type pH_i sensitivity of the mutant E346A. These data suggest that both structure and charge of the amino acid side chain at position 346, if not equally so at position 347, can be important in conferring wild-type regulation of AE2 by pH_i.

Regulation of AE2-mediated Cl^-/HCO_3^- Exchange by pH_i

The above mutagenesis experiments investigating structure-function relationships in the AE2 anion exchanger examined effects on nominal Cl^-/Cl^- exchange activity in room air. However, the experimental advantages offered by measurement of Cl^-/Cl^- exchange in *Xenopus* oocytes may not reflect the physiological function of Cl^-/HCO_3^- exchange by AE2 in intact vertebrates or chordates. We therefore investigated whether the findings of Fig. 3 concerning the influence of single amino acid residues on the regulation of AE2-mediated Cl^-/Cl^- exchange by pH_i might also apply to AE2-mediated Cl^-/HCO_3^- exchange. Fig. 6 A shows representative $^{36}Cl^-$ efflux traces of oocytes subjected to the butyrate removal assay in Cl^--free solutions equilibrated with 5% CO_2/24 mM HCO_3^-, pH 7.40. Whereas addition and removal of 40 mM butyrate first reduced and then stimulated wild-type AE2–mediated $^{36}Cl^-$ efflux, AE2 E347A–mediated Cl^-/HCO_3^- exchange was insensitive to butyrate-associated changes in pH_i. These data, summarized in Fig. 6 B, suggest that at least some of the AE2 structure-function relationships derived from study of Cl^-/Cl^- exchange regulation by pH_i in *Xenopus* oocytes apply equally to regulation of AE2-mediated Cl^-/HCO_3^- exchange. The result also suggests that pH_i rather than $[HCO_3^-]$ regulates AE2 activity in the butyrate removal assay.

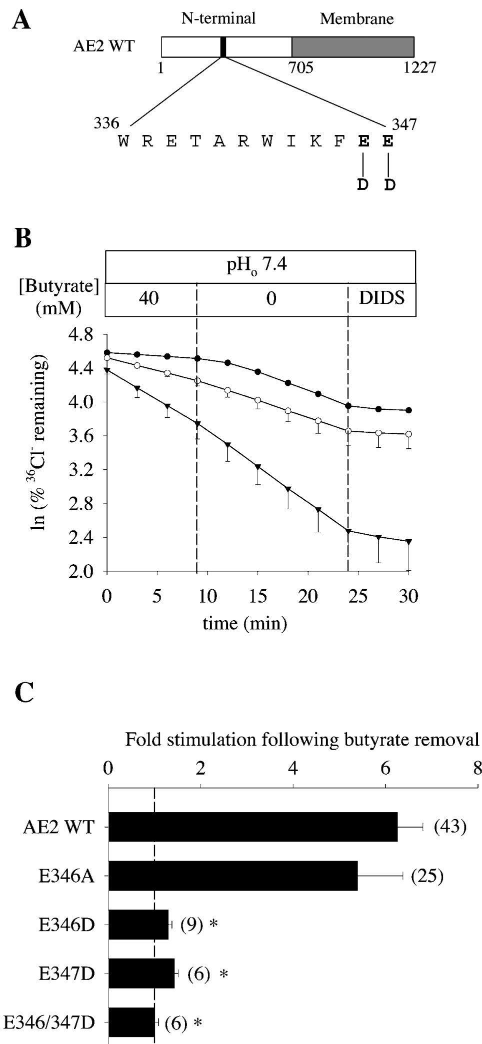

FIGURE 5. The importance of AE2 residues E346 and E347 to regulation of Cl^- transport by pH_i is not restricted to side chain charge. (A) Schematic of AE2 E346 and E347 substitution mutants. (B) Representative time course of $^{36}Cl^-$ efflux from oocytes expressing wild-type AE2 (filled circles) or the AE2 mutants E346D (open circles) or E347D (closed inverted triangles) during elevation of pH_i by removal of bath butyrate (40 mM) and subsequent inhibition by DIDS (200 μM). (C) Mean fold stimulation (± SEM) after butyrate removal measured in n oocytes expressing wild-type AE2 or the indicated mutants. Asterisk indicates $P < 0.05$ (Student's unpaired t test).

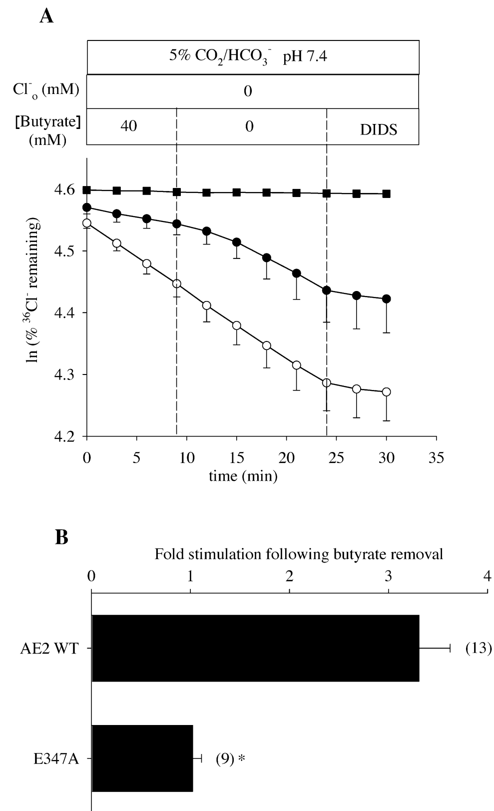

FIGURE 6. AE2 residue E347 is important also for the regulation of Cl^-/HCO_3^- exchange by pH_i. (A) Representative time course of Cl^-/HCO_3^- exchange in oocytes previously injected with water (closed squares) or with cRNA encoding wild-type AE2 (filled circles) or the AE2 mutant E347A (open circles). $^{36}Cl^-$ efflux into bath solution containing HCO_3^- as the only nominal permeant anion is measured first in the presence of butyrate, then after its removal and during subsequent inhibition by DIDS (200 μM). (B) Cl^-/HCO_3^- exchange mediated by wild-type AE2 is stimulated by intracellular alkalinization, whereas Cl^-/HCO_3^- exchange mediated by the mutant AE2 E347A is not stimulated. This phenotype resembles that for Cl^-/Cl^- exchange mediated by these polypeptides. Values are means ± SEM for n oocytes. Asterisk indicates $P < 0.05$ (Student's t test).

The Amino Acid Residues Important for Regulation of AE2 by pH_o and by pH_i Are Conserved Among AE2-related Anion Transporters

Alignment of the AE2 region shown above to be important to AE2 regulation by pH_o and pH_i with corresponding regions of other SLC4 bicarbonate transporter polypeptides reveals absolute sequence conservation with the Na^+-independent chloride bicarbonate exchanger, AE3. This AE2 sequence also shows a high degree of sequence identity with Na^+-dependent bicarbonate cotransporters, including several not known to transport chloride (Fig. 7). In contrast, no homologous region is present in the very divergent NH_2-terminal putative cytoplasmic domain of BTR1 (Parker et al., 2001). The acute regulation by pH of most of these anion transporters has yet to be studied. However, the evident sequence conservation suggests that these residues may make similar contributions to regulation of these AE2-related anion transporters. We tested this hypothesis for regulation by pH_i of the Na^+-independent anion exchangers, AE3 and AE1.

As shown in Fig. 8, B and C, cardiac AE3 (cAE3) can be stimulated by intracellular alkalinization, whereas AE1 is insensitive to this change in pH_i. The cAE3 residues E150 and E151 (corresponding to AE2 E346 and

	336											347
	*		*			*	*	*		*	*	*
mAE2	**W**	R	**E**	T	A	**R**	**W**	**I**	K	**F**	**E**	**E**
mAE3	**W**	R	**E**	T	A	**R**	**W**	**I**	K	**F**	**E**	**E**
mNCBE	**W**	R	**E**	T	A	**R**	**W**	L	K	**F**	**E**	**E**
hNDCBE1	**W**	K	**E**	T	A	**R**	**W**	L	K	**F**	**E**	**E**
DmNDAE1	**W**	K	**E**	T	A	**R**	**W**	**I**	K	**F**	**E**	**E**
hNBCe2	**W**	K	**E**	S	A	**R**	**W**	**I**	K	**F**	**E**	**E**
hNBCe1	**W**	K	**E**	T	A	**R**	**W**	**I**	K	**F**	**E**	**E**
rNBCn1	**W**	K	**E**	T	A	**R**	**W**	L	K	**F**	**E**	**E**
hAE4	**W**	R	**E**	T	G	**R**	**W**	V	L	**F**	**E**	**E**
hAE1	**W**	M	**E**	A	A	**R**	**W**	V	Q	L	**E**	**E**
mAE1	**W**	V	**E**	A	A	H	**W**	**I**	G	L	**E**	**E**
trAE1	**W**	Q	**E**	T	G	**R**	**W**	V	G	Y	**E**	**E**
hBTR1	T	N	T	E	N	E	A	T	S	G	G	C

Figure 7. Amino acid sequence alignment of AE2 aa 336–347 with corresponding regions of other members of the SLC4 bicarbonate transporter superfamily. Asterisks mark conserved residues in which mutation to alanine alters regulation of AE2-mediated Cl^- transport by pH_i (butyrate removal method). Boldface marks conservation of those pH_i-related residues. E346 is marked by an asterisk to indicate change to a mutant pH_i phenotype when mutated to aspartate. BTR1 lacks this conserved region (alignment as presented in Parker et al., 2001). EMBL/GenBank/DDBJ accession nos. for these sequences are: mAE2 (murine anion exchanger 2; J04036), mAE3 (murine anion exchanger 3; AAA40692), mNCBE (murine sodium-dependent chloride/bicarbonate exchanger; BAB17922), hNDCBE1 (human sodium-dependent chloride/bicarbonate exchanger 1; AF069512), DmNDAE1 (*D. melanogaster* sodium-dependent anion exchanger 1; AF047468), hNBCe2 (human electrogenic sodium bicarbonate cotransporter 2; AF293337), hNBCe1 (human electrogenic sodium bicarbonate cotransporter 1; AF007216), rNBCn1 (rat electroneutral sodium bicarbonate cotransporter 1; AF070475), hAE4 (human anion exchanger 4; AF332961), hAE1 (human anion exchanger 1; CAA31128), mAE1 (murine anion exchanger 1; J02756), trAE1 (trout anion exchanger 1; Z50848), hBTR1 (human bicarbonate transporter-related protein 1; AF336127).

E347) were then mutated to alanine, individually and in tandem. Fig. 8, D and E, show that stimulation of cAE3-mediated $^{36}Cl^-$ efflux by intracellular alkalinization is severely attenuated by each of these mutations. This suggests that pH_i regulates cAE3 in oocytes as suggested previously for brain AE3 (bAE3) in 293 cells (Lee et al., 1991), and that such regulation requires integrity of residues corresponding to those required by AE2.

The AE1 NH_2-terminal cytoplasmic domain shows partial conservation of the AE2 residues demonstrated above to be critical for normal regulation of AE2 by pH_i (Fig. 7), yet wild-type AE1-mediated Cl^- transport is pH_i insensitive. However, the $AE1_{cyto}/AE2_{memb}$ chimera exhibits regulation not only by changing pH_o (Zhang et al., 1996) but also by changing pH_i (Fig. 9, B and C). In contrast, the converse chimera $AE2_{cyto}/AE1_{memb}$ exhibited an AE1-like phenotype, whether in response to changing pH_o (Zhang et al., 1996) or to intracellular alkalinization by butyrate removal (Fig. 9 C). The NH_2-terminal AE2 truncation mutant $\Delta_N 659$ displayed similar lack of stimulation by intracellular alkalinization. Thus, stimulation by intracellular alkalinization requires defined residues from the NH_2-terminal cytoplasmic domain in combination with yet-to-be defined portions of the AE2 transmembrane domain. The pH_i sensitivity of the $AE1_{cyto}/AE2_{memb}$ chimera thus allowed experimental test of the potential importance of the AE1 region depicted in Fig. 7 when present in the "permissive environment" of proximity to the AE2 transmembrane domain (Fig. 9 A). Fig. 9, B and C, show that introduction into the $AE1_{cyto}/AE2_{memb}$ chimera of the double Ala substitution mutation E99A/E100A severely attenuated stimulation of Cl^- efflux by intracellular alkalinization ($n = 14$, $P < 0.05$).

DISCUSSION

Ion exchanger and cotransporter polypeptides are important contributors to cellular and compartmental pH homeostasis. This homeostasis requires that transporters of H^+ and HCO_3^-, as well as pH-sensitive transporters of other solutes, be subject to regulation by pH_i, and in some cases also by pH_o. Regulation by pH_i and by pH_o of several K^+, Na^+, and Ca^{2+} channels is partially understood at the molecular level in terms of titration by protons of critical amino acid residues, similar to classical models invoked for pH-regulatory transporters (Gunn et al., 1973; Milanick and Gunn, 1984). However, the mechanisms by which pH acutely regulates the activities of pH-regulatory ion exchangers and cotransporters remain for the most part obscure. Even regulation by pH_i of the Na^+/H^+ exchanger, NHE1, perhaps the most extensively studied among these transporters, is not yet understood at the level of individual amino acid residues. Similarly, the molecular mechanisms by which protons regulate activity of Cl^-/HCO_3^- exchangers of the SLC4 gene family have until recently remained uninvestigated.

The current work has shown that AE2-mediated Cl^- transport is sensitive to changing pH_o under conditions of constant pH_i (Fig. 1), complementing our earlier demonstration that AE2 activity is also sensitive to changing pH_i at constant pH_o (Stewart et al., 2001). We have found two stretches of amino acid sequence in the middle of the AE2 NH_2-terminal cytoplasmic domain (aa 318–323 and aa 336–347) that are required for wild-type pH sensitivity of AE2-mediated anion exchange in *Xenopus* oocytes (Fig. 2). These two regions are present in all five NH_2-terminal variant AE2 polypeptide products arising from alternate promoter usage or splicing of the AE2 gene (Stuart-Tilley et al., 1998; Medina et al., 2000). Alanine scan mutagenesis of aa 336–347 has identified individual amino acid residues whose mutation modulates AE2 regulation by pH_o or abolishes AE2 regulation by pH_i under the conditions studied (Fig. 3). The AE2

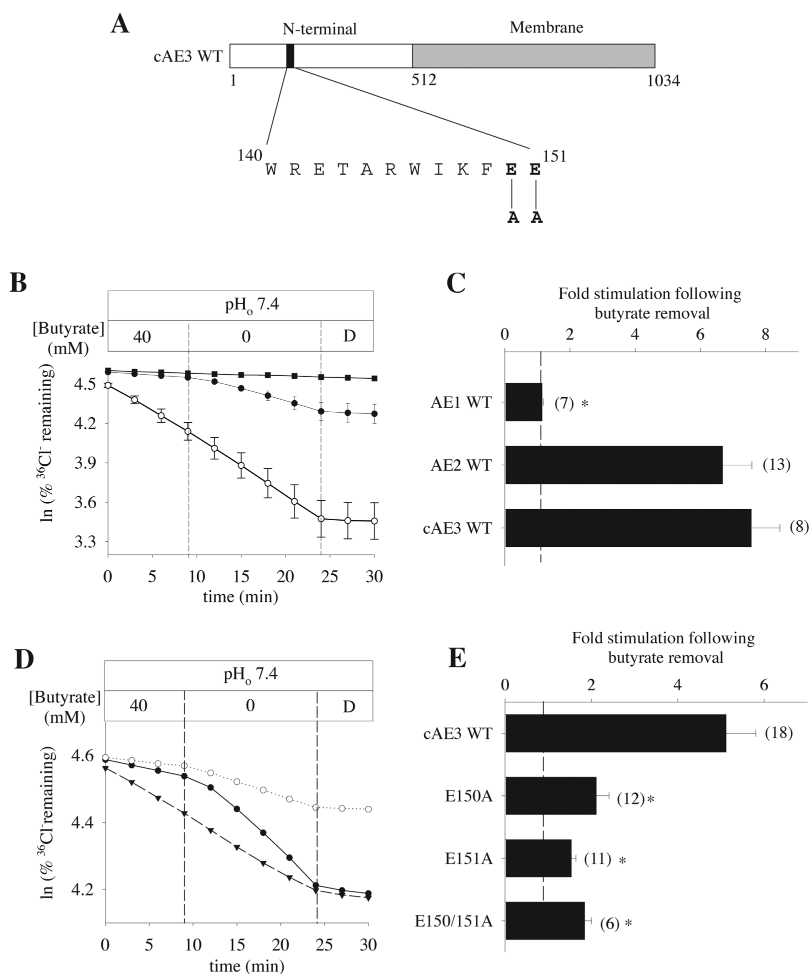

Figure 8. Mutation of corresponding glutamate residues in the cAE3 NH_2-terminal cytoplasmic domain similarly alters regulation by pH_i. (A) Schematic showing site of alanine substitutions for conserved glutamate residues in the NH_2-terminal cytoplasmic domain of rat cAE3. (Rat cAE3 residues E150 and E151 correspond to rat bAE3 residues E347 and E348.) (B) Representative $^{36}Cl^-$ efflux time course from oocytes previously injected with water (filled squares), with cRNA encoding mouse kidney AE1 (open circles), or rat cAE3 (filled circles) during removal of butyrate and subsequent inhibition with DIDS (D, 200 μM). (C) Mean fold stimulation of $^{36}Cl^-$ efflux by intracellular alkalinization (butyrate removal) in oocytes expressing either AE1, AE2, or cAE3. (D) Representative $^{36}Cl^-$ efflux time course from oocytes expressing wild-type cAE3 (filled circles) or the cAE3 mutants E150A (open circles) or E151A (closed triangles) during butyrate removal and subsequent inhibition by DIDS (D, 200 μM). (E) Mean fold stimulation of $^{36}Cl^-$ efflux by intracellular alkalinization (butyrate removal) in oocytes expressing either wild-type cAE3, the cAE3 mutants E150A or E151A, or the double mutant E150A/E151A. Values in C and E are means ± SEM for *n* oocytes; dashed lines at onefold indicate no stimulation by butyrate removal. Asterisk indicates $P < 0.05$.

amino acid residues required for wild-type regulation by pH_o and pH_i are not identical (Fig. 3). Although mutations in some residues altered both modes of regulation, mutations in other residues altered selectively either the response to changing pH_i or the response to changing pH_o (Figs. 3–5). At least one structural requirement for regulation of AE2-mediated Cl^-/Cl^- exchange by pH_i applies equally to pH_i regulation of Cl^-/HCO_3^- exchange (Fig. 6). Regulation by pH_i of Cl^-/Cl^- exchange mediated by the related polypeptide cAE3 (Fig. 8) and by the $AE1_{cyto}/AE2_{memb}$ chimera (Fig. 9) was severely attenuated by mutations corresponding to those which alter AE2 regulation.

Defined individual amino acid residues contribute to the independent regulation of AE2-mediated Cl^- transport by pH_o and by pH_i. Earlier studies with large deletions and chimeras (Zhang et al., 1996) and with smaller NH_2-terminal and internal deletions (Stewart et al., 2001) focused attention upon AE2 residues 312–347 as a region required for wild-type AE2 regulation by independent variation of pH_o and of pH_i. Subsequent hexa-Ala bloc substitution mutagenesis (Fig. 1) within this NH_2-terminal region focused attention upon residues 336–347. Systematic alanine scan mutagenesis of this region (Fig. 3) then identified several individual amino acid residues important for AE2 regulation by pH_o only, by pH_i only, or for both. Individual mutation to alanine of the four residues W336, R341, E346, and E347 significantly shifted the AE2 $pH_{o(50)}$ to more acidic values. Uniquely among these four mutants, AE2 E346A retained wild-type pH_i sensitivity. However, individual mutation to alanine of the seven residues W336, E338, R341, W342, I343, F345, and E347 eliminated AE2 regulation by pH_i in the butyrate removal assay. Among these, E338A, W342A, I343A, and F345A retained wild-type or near wild-type pH_o sensitivity. These distinct effects of individual mutations upon regulation of AE2 by pH_i and by pH_o reinforce the hypothesis that AE2 independently senses and responds to protons on both sides of the plasma membrane.

Contribution of Cytoplasmic Amino Acid Residues to AE2 Regulation by pH_o

Regulation by pH_i of AE2-mediated anion transport, itself a pH-regulatory process in the physiological pres-

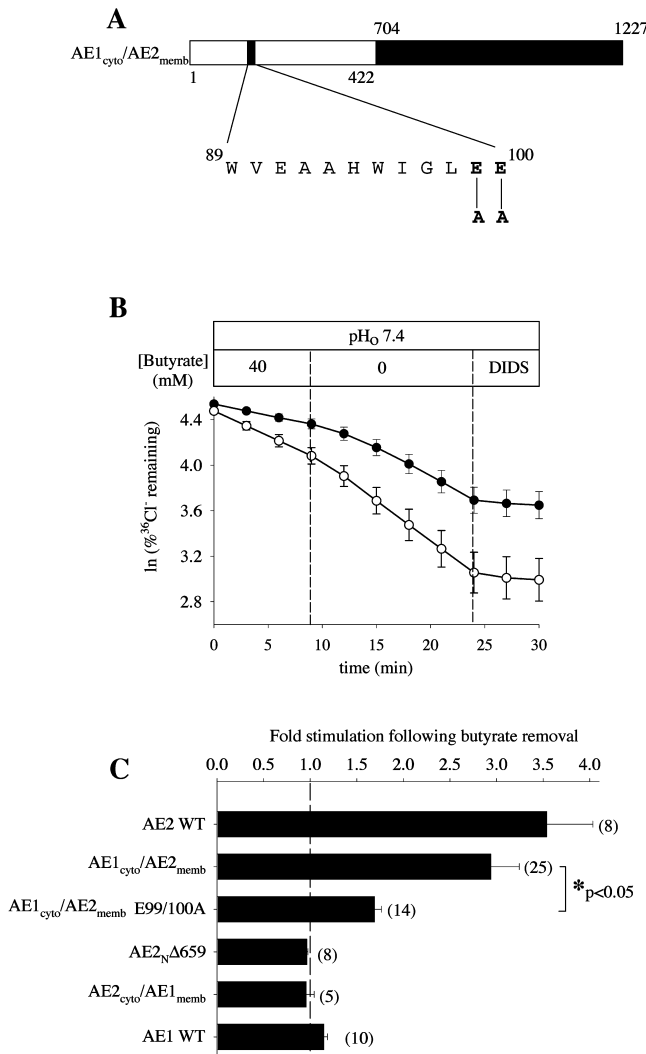

Figure 9. Mutation of corresponding glutamate residues in the NH_2-terminal cytoplasmic domain of the chimeric anion exchanger $AE1_{cyto}/AE2_{memb}$ similarly alters regulation by pH_i. (A) Schematic showing site of alanine substitutions for conserved glutamate residues in the NH_2-terminal cytoplasmic domain of the chimera $AE1_{cyto}/AE2_{memb}$. (B) Representative $^{36}Cl^-$ efflux time course from oocytes expressing "wild-type" $AE1_{cyto}/AE2_{memb}$ (filled circles) or the corresponding E99A/E100A double mutant (open circles) during intracellular alkalinization (butyrate removal) and subsequent inhibition by DIDS (200 μM). (C) Mean fold stimulation (± SEM) of $^{36}Cl^-$ efflux by intracellular alkalinization (butyrate removal) in *n* oocytes expressing either wild-type AE2, wild-type AE1, or the indicated chimeras or mutant polypeptides.

ence of CO_2/bicarbonate buffers, is intuitively reasonable across the normal range of pH_i. The NH_2-terminal cytoplasmic domain of AE2 is appropriately situated to contribute to such regulation. AE2's function as an acid loader in physiological conditions suggests a rationale for inhibition of AE2-mediated anion transport not only by reduced pH_i, but also by reduced pH_o. AE2 itself mediates or contributes to the intracellular acidification produced by extracellular acidification. Regulation of AE2 by pH_o would allow the cell to down-regulate acid loading when first faced with extracellular acidosis, preferably before the cell has begun to experience adverse effects of an intracellular acid load. Nonetheless, an important role for NH_2-terminal cytoplasmic domain residues is less easily envisioned for AE2's response to changing pH_o than to changing pH_i.

pH_o regulates AE2-mediated Cl^- transport across a physiological range with a $pH_{o(50)}$ value of ~6.9, in contrast to the AE1 $pH_{o(50)}$ values of <5.0 (Zhang et al., 1996) or 5.8 (as measured by a different method by Muller-Berger et al., 1995). As we show here for AE2, Muller-Berger et al. (1995) also found that AE1-mediated Cl^- transport activity is inhibited by lowering pH_i at constant pH_o, or by lowering pH_o at near-constant pH_i (although at much lower pH values than for AE2).[3] Defined residues of the NH_2-terminal cytoplasmic domain are absolutely required for inhibition of AE2-mediated Cl^- transport by acidic pH_i as assessed by the butyrate removal assay. These residues must either interact with or be required to maintain integrity of the pH sensor of the AE2 transmembrane domain (Zhang et al., 1996). The postulated interaction with or conformational change in the transmembrane sensor (resulting from either mutation or deletion of these critical residues of the NH_2-terminal cytoplasmic domain) must sufficiently reduce the effective pK of sensor residues to render it insensitive to the 0.5 pH unit intracellular acidification produced by addition of 40 mM butyrate to a pH_o 7.4 bath (Stewart et al., 2001). Among the candidate residues contributing to this transmembrane pH sensor might be E1007 (Sekler et al., 1995), the residue corresponding in AE1 to human E681 and mouse E699. In AE1, mutation of this glutamate to

[3]Muller-Berger et al. (1995) found that reduction of bath pH to 6.1 either from 7.2 or from 8.3 did not change oocyte pH_i when measured after 90 min preincubation (in 110 mM KCl Barth's solution). Thus, 90 min preincubation at the desired pH_o achieved conditions of nominal pH_i-clamp which were suitable for their experiments in which each oocyte was exposed first to control pH and then to a single altered bath pH. Our experimental protocol exposed each oocyte to the complete range of bath pH values in the course of a single efflux experiment (Stewart et al., 2001 and the current work). In this setting, pH_i measured either by pH-sensitive microelectrode (Fig. 1) or by BCECF ratio fluorimetry (Zhang et al., 1996; unpublished data) did change acutely in parallel with changing pH_o. Thus, our experimental conditions required coincident changes in pH_o and butyrate concentration to achieve oocyte pH_i clamp. These and other experimental differences might contribute to the difference of ≥1 pH unit between the AE1 $pH_{o(50)}$ values reported by Muller-Berger et al. (1995) and Zhang et al. (1996). Muller-Berger et al. (1995) lowered pH_i at constant pH_o through use of 40 or 60 mM NH_4Cl. In our experiments, 20 mM NH_4Cl did not inhibit AE1. The use of NH_4Cl for intracellular acidification at constant pH_o could not be applied to experiments with AE2, since NH_4Cl activates AE2 despite its accompanying acidification of the oocyte (Humphreys et al., 1997).

glutamine (Chernova et al., 1997b) or its chemical modification to hydroxynorvaline with Woodward's reagent K (Jennings, 1995) not only alters anion selectivity, but also converts H^+-sulfate cotransport into proton-independent sulfate transport. This observation has led to the hypothesis that this glutamate at or near the cytoplasmic face of putative transmembrane span 8 is or contributes to the proton binding site. Such direct, pH-dependent interactions (with their presumed conformational changes) have been demonstrated in K^+ channels, where it is suggested that at acidic pH, specific residues are protonated in the COOH-terminal cytoplasmic tail, leading to interaction with the NH_2-terminal tail and in turn causing conformational changes in the protein (Qu et al., 2000; for review see Jiang et al., 2002).

Regulation of AE2 by pH_o involves protonation of exofacial residues, likely distinct from external Cl^- binding site(s) (Milanick and Gunn, 1984). These protonation(s) could alter Cl^- binding affinity or Cl^- translocation rate, a function of the conformational change between inward-facing and outward-facing forms of the transporter. Substitution of the entire AE2 NH_2-terminal cytoplasmic domain with that of AE1 (Zhang et al., 1996; Stewart et al., 2001), or deletion or mutation of those NH_2-terminal cytoplasmic domain residues essential for wild-type AE2 response to changing pH_o (including many residues not subject to titration by protons near the physiological range) shift the wild-type AE2 $pH_{o(50)}$ to more acidic values by 0.5–0.8 pH units. This shift, representing altered proton affinity of the external pH sensor (Gunn et al., 1973), might be accomplished through loss of its direct interaction with the cytoplasmic face of the AE2 transmembrane domain, resulting in altered conformation (and pKa) of protonatable residues at AE2's extracellular face or within the aqueous vestibule thought to have E1007 at its base. Alternately, a distinct polypeptide may mediate this interaction. The effects of AE2 mutations in the mutations in the NH_2-terminal cytoplasmic domain upon AE2 pH_o sensitivity resemble the alterations in ligand binding affinity of transmembrane hormone receptors secondary to mutations in receptor cytoplasmic loops or tail, or to receptor interaction with cytoplasmic regulatory polypeptide(s).

Putative Structure of the pH-regulatory Region of the AE2 NH_2-terminal Cytoplasmic Domain

AE2 polypeptide has been purified from porcine stomach (Zolotarev et al., 1996, 1999), and is present in gastric membranes as a dimer or higher order oligomer (Zolotarev et al., 1999), but its structure remains unknown. We have mapped the AE2 sequences implicated in regulation of AE2 by pH upon the X-ray structure of crystallized erythroid AE1 NH_2-terminal cytoplasmic domain reported by Zhang et al. (2000). This alignment predicts that AE2 aa 336–347 occupies a region corresponding to most of the AE1 structure's β strands β2 and β3 along with their short connecting loop. This region is adjacent to AE1 residue 356, the COOH-terminal extent of structured sequence. Although structure remains uncertain for the disordered aa 357–379 in the AE1 NH_2-terminal cytoplasmic domain and the ~20 subsequent amino acids that link the cytoplasmic domain to the first transmembrane span of AE1, the loop connecting β strands β2 and β3 is plausibly oriented close to the cytoplasmic face of the AE1 transmembrane domain (Zhang et al., 2000). We postulate that AE2 aa 336–347, although hundreds of residues away (in linear sequence) from the first transmembrane domain of AE2, may adopt a similar conformation with respect to elements of the internal pH sensor proposed to reside in the AE2 transmembrane domain. The pattern of altered pH_i-regulatory phenotype produced by the alanine scan of AE2 aa 336–347 is consistent with a structure of two short β sheets connected by a loop, showing one short patch of alternating phenotype within each predicted β-sheet. This pattern is distinct from that of altered pH_o sensitivity across the same region.

The decreased pH_i sensitivity exhibited by the (β3 strand) double mutant E99A/E100A of the chimeric $AE1_{cyto}/AE2_{memb}$ transporter (Fig. 8) further supports this hypothesis. The absence of pH_i dependence for AE1-mediated Cl^- efflux in the butyrate removal assay highlights the functional interaction between pH-regulatory residues in NH_2-terminal cytoplasmic domain and the yet undefined pH sensor structure present in the AE2 transmembrane domain, but lacking in that of AE1.

The different effects on AE2 regulation by pH_i of E346 substitution by alanine or by aspartate suggest that in this position side chain packing may be more important than charge to the structure of a functional regulatory domain. A similar result has been presented for the electrogenic SGLT1 Na^+-glucose cotransporter (Quick et al., 2001). The importance of packing in the folding of this regulatory domain is further suggested by the modified pH regulation exhibited by alanine substitution of conserved tryptophan residues. In contrast, substitution of the adjacent residue E347 with Ala or with Asp each led to altered regulation by pH_i (Figs. 3 F and 5 C). These two adjacent glutamates likely comprise part of a β-sheet structure, projecting their side chains in opposite directions (Zhang et al., 2000). Thus, the different consequences of mutagenesis of these adjacent residues likely reflects their different nearest neighbors.

Important roles for glutamate residues in conferring sensitivity to changing pH_o and pH_i have been demon-

strated previously in potassium channels (Xu et al., 2000), chloride channels (Stroffekova et al., 1998), and the capsaicin receptor (Jordt et al., 2000). Although histidine residues play major roles in regulation of several K^+ channels by pH_i (Coulter et al., 1995; Doi et al., 1996; Chanhevalap et al., 2000; Qu et al., 2000; Xu et al., 2000), alanine substitution of AE2 His residues 314 and 317, either individually or in tandem, was without effect on AE2 regulation by pH_i (not depicted). The lack of effect of T339 mutation to Val or to Glu similarly failed to support a role for T339 phosphorylation in AE2 regulation by pH.

Conserved Amino Acids of the NH_2-terminal Cytoplasmic Domain Contribute to the pH_i Regulation of other Anion Exchangers

The region of aa 336–347 is among the most highly conserved of the NH_2-terminal cytoplasmic domains of SLC4 gene family members. Its functional importance was confirmed in the AE2 homologue cAE3 by mutating the two glutamate residues in cAE3 corresponding to E346 and E347 of AE2. Both mutations greatly attenuated the pH_i sensitivity of cAE3-mediated Cl^- efflux from *Xenopus* oocytes. However, whereas the AE2 mutant E346A exhibited a wild-type response to butyrate removal, the corresponding mutation in cAE3 (E150) showed considerably reduced pH_i sensitivity (only twofold increase upon butyrate removal) compared with the fivefold increase exhibited by wild-type cAE3. This difference between the functional consequences of mutation within this highly conserved region in AE2 and AE3 may reflect sequence differences in immediately adjacent flanking regions, but very likely reflects different effects on interacting structures. These binding sites may be at the cytoplasmic face of the AE2 and AE3 transmembrane domains, or may represent other polypeptides.

These results are consistent with the initial report of pH_i-sensitive Cl^-/HCO_3^- exchange in 293 cells transiently transfected with AE3 (Lee et al., 1991), but contrast with the pH-insensitive AE3-mediated Cl^-/nitrate exchange in 293 cells reported by Sterling and Casey (1999). The latter measurements were made in high K^+/nigericin pH-clamp conditions in which $pH_i = pH_o$. The activation of AE3 by intracellular alkalinization in *Xenopus* oocytes is consistent with its hypothesized role in cardiac myocytes in recovery from alkaline load (Leem et al., 1999). As anion exchange in guinea pig cardiomyocytes is oppositely regulated by pH_i and by pH_o (Sun et al., 1996; Vaughan-Jones, 1986; Leem and Vaughan-Jones, 1998), the individual effects of pH_i and pH_o on heterologous AE3 in transfected 293 cells (Sterling and Casey, 1999) may have been masked by the chosen experimental conditions. Interestingly, whereas the pH_i dependence of anion exchange in guinea pig myocytes is similar to that exhibited by mouse AE2 expressed in *Xenopus* oocytes their responses to varying pH_o differ. In guinea pig myocytes, low pH_o stimulates and high pH_o inhibits Cl^-/HCO_3^- exchange, with a $pH_{o(50)}$ value of 7.03 (Wilson and Vaughan-Jones, 2000), a value similar to that of AE2-mediated Cl^-/Cl^- exchange oppositely regulated in oocytes by pH_o. The functionally dominant anion exchanger protein of guinea pig myocytes has not yet been identified, although AE3 (Kopito et al., 1989; Kudrycki et al., 1990; Yannoukakos et al., 1994) and AE1 (Richards et al., 1999) have both been proposed.

The pH-regulatory subdomains within the AE2 NH_2-terminal cytoplasmic domain whose function requires the integrity of aa 318–323 and aa 336–347 likely interact with or control the conformation of effector moieties. These may include binding site(s) at the cytoplasmic face of the AE2 transmembrane domain not present in AE1, binding sites elsewhere within the AE2 cytoplasmic NH_2-terminal domain, and/or binding surfaces on separate, regulatory polypeptides. These possibilities are currently under investigation. Although the structure of the transmembrane domain of AE1 is known only to a resolution of 18 Å, several mutations of human AE1 associated with distal renal tubular acidosis (dRTA) (Alper et al., 2002) suggest sites at the cytoplasmic face of the transmembrane domain that are sensitive to the structure of the NH_2-terminal cytoplasmic domain, and so either influence or themselves might represent sites of intramolecular interaction. Thus, the AE1 mutants R589H (Bruce et al., 1997; Jarolim et al., 1998) and R901X (Karet et al., 1998) are both functional in the context of the erythroid AE1 NH_2-terminal cytoplasmic domain. In contrast, within the context of the renal type A intercalated cell, in which the NH_2-terminal cytoplasmic domain of kidney AE1 lacks the first 65 residues present in erythroid AE1, these mutants can exhibit impaired surface accumulation (Toye et al., 2002) and/or a dominant-negative trafficking phenotype (Quilty et al., 2002).

As judged by the butyrate removal assay, pH_i regulates both Cl^-/Cl^- and Cl^-/HCO_3^- exchange mediated by AE2. The recent demonstrations that Cl^-/HCO_3^- exchange mediated by AE2 and by AE3 can be regulated by bound carbonic anhydrase II (Sterling et al., 2002a) and by carbonic anhydrase IV (Sterling et al., 2002b) adds to the potential complexity of AE regulation by pH. These AE binding proteins might in some contexts exert allosteric effects on AE activity, in addition to their presumed functions in channeling transport substrate to and from the anion translocation pathway through the polypeptide.

Conclusion

The present study has defined individual, conserved amino acid residues in the NH_2-terminal cytoplasmic

domain of the AE2 anion exchanger whose mutation alters the independent regulation of AE2 activity by pH_o and by pH_i. The corresponding conserved glutamate residues were shown also to be critical for regulation by pH_i of anion exchange mediated by cAE3 and of the $AE1_{cyto}/AE2_{memb}$ chimera. The importance of one of these glutamates was further demonstrated for the regulation of AE2-mediated Cl^-/HCO_3^- exchange by pH_i. The conservation of these residues in yet other bicarbonate transporters involved in intracellular and extracellular compartmental pH regulation suggests similar mechanisms of functional control. These results confirm that integrity of residues in both the NH_2-terminal cytoplasmic domain and in the COOH-terminal transmembrane domain of AE2 is necessary for wild-type regulation of AE2 by pH_i and by pH_o. Integrity of individual residues of the NH_2-terminal cytoplasmic domain which contribute to the "pH-modifier site" previously proposed by Zhang et al. (1996) is necessary but not sufficient for the wild-type pattern of independent sensing of pH_i and pH_o. Also required is the presence of the appropriate "pH_i/pH_o sensor(s)" in the transmembrane domain which mediates anion translocation. Future experiments will seek to identify the transmembrane domain pH sensor(s) of AE2 and their modes of communication directly with modulatory residues of the AE2 NH_2-cytoplasmic domain, or indirectly via distinct regulatory polypeptides.

We thank Dr. Lianwei Jiang for BCECF fluorescence ratio imaging measurements, and Professor Richard Vaughan-Jones and Alan Stuart-Tilley for helpful discussions.

A.K. Stewart was supported by an International Prize Travelling Fellowship of the Wellcome Trust. S.L. Alper was supported by National Institutes of Health grants DK43495 and DK34854 (The Harvard Digestive Diseases Center).

Submitted: 4 June 2002
Revised: 26 September 2002
Accepted: 30 September 2002

REFERENCES

Alper, S.L. 1994. The band 3-related AE anion exchanger gene family. *Cell. Physiol. Biochem.* 4:265–281.

Alper, S.L., R.B. Darman, M.N. Chernova, and N.K. Dahl. 2002. The AE gene family of Cl^-/HCO_3^- exchangers. *J. Nephrol.* 15:S41–S53.

Bevensee, M.O., S.L. Alper, P.S. Aronson, and W.F. Boron. 2000. Control of intracellular pH. *In* The Kidney: Physiology and Pathophysiology. Third edition. D.W. Seldin, and G. Giebisch, editors. Philadelphia, Lippincott, Williams, and Wilkins. 391–442.

Bruce L.J., D.L. Cope, G.K. Jones, A.E. Schofield, M. Burley, S. Povey, R.J. Unwin, O. Wrong, and M.J. Tanner. 1997. Familial distal renal tubular acidosis is associated with mutations in the red cell anion exchanger (Band 3, AE1) gene. *J. Clin. Invest.* 100:1693–1707.

Chanhevalap, S., Z. Yang, N. Cui, Z. Qu, G. Zhu, C. Liu, L.R. Giwa, L. Abdulkadir, and C. Jiang. 2000. Involvement of histidine residues in proton sensing of ROMK1 channel. *J. Biol. Chem.* 275:7811–7817.

Chernova, M.N., B.D. Humphreys, D.H. Robinson, A.K. Stuart-Tilley, A.M. Garcia, F.C. Brosius, and S.L. Alper. 1997a. Functional consequences of mutations in the transmembrane domain and the carboxy-terminus of the murine AE1 anion exchanger. *Biochim. Biophys. Acta.* 1329:111–123.

Chernova, M.N., L. Jiang, M. Crest, M. Hand, D.H. Vandorpe, K. Strange, and S.L. Alper. 1997b. Electrogenic sulfate/chloride exchange in *Xenopus* oocytes mediated by murine AE1 E699Q. *J. Gen. Physiol.* 109:345–360.

Cooper, G.J., and W.F. Boron. 1998. Effect of PCMBS on CO_2 permeability of *Xenopus* oocytes expressing aquaporin 1 or its C189S mutant. *Am. J. Physiol.* 275:C1481–C1486.

Coulter, K.L., F. Perier, C.M. Radeke, and C.A. Vandenberg. 1995. Identification and molecular localization of a pH-sensing domain for the inward rectifier potassium channel HIR. *Neuron.* 15:1157–1168.

Doi, T., B. Fakler, J.H. Schultz, U. Schulte, U. Brandle, S. Weidemann, H.P. Zenner, F. Lang, and J.P. Ruppersberg. 1996. Extracellular K^+ and intracellular pH allosterically regulate renal Kir1.1 channels. *J. Biol. Chem.* 271:17261–17266.

Funder, J., and J.O. Wieth. 1976. Chloride transport in human erythrocytes and ghosts: a quantitative comparison. *J. Physiol.* 262:679–698.

Grinstein, S., S. Ship, and A. Rothstein. 1978. Anion transport in relation to proteolytic dissection of band 3 protein. *Biochim. Biophys. Acta.* 507:294–304.

Gunn, R.B., M. Dalmark, D.C. Tosteson, and J.O. Wieth. 1973. Characteristics of chloride transport in human red blood cells. *J. Gen. Physiol.* 61:185–206.

Humphreys, B.D., L. Jiang, M.N. Chernova, and S.L. Alper. 1994. Functional characterization and regulation by pH of murine AE2 anion exchanger expressed in *Xenopus* oocytes. *Am. J. Physiol.* 267:C1295–C1307.

Humphreys, B.D., M.N. Chernova, L. Jiang, Y. Zhang, and S.L. Alper. 1997. NH_4Cl activates AE2 anion exchanger in *Xenopus* oocytes at acidic pH_i. *Am. J. Physiol.* 272:C1232–C1240.

Jarolim, P., C. Shayakul, D. Prabakaran, L. Jiang, A. Stuart-Tilley, H.L. Rubin, S. Simova, J. Zavadil, J.T. Herrin, J. Brouillette, et al. 1998. Autosomal dominant distal renal tubular acidosis is associated in three families with heterozygosity for the R589H mutation in the AE1 (band 3) Cl^-/HCO_3^- exchanger. *J. Biol. Chem.* 273:6380–6388.

Jennings, M.L. 1992. Cellular anion transport. *In* The Kidney: Physiology and Pathophysiology. Second edition. D.W. Seldin and G. Giebisch, editors. Raven Press, New York. 113–145.

Jennings, M.L. 1995. Rapid electrogenic sulfate-chloride exchange mediated by chemically modified band 3 in human erythrocytes. *J. Gen. Physiol.* 105:21–47.

Jiang, L., A. Stuart-Tilley, J. Parkash, and S.L. Alper. 1994. pH_i and serum regulate AE2-mediated Cl^-/HCO_3^- exchange in CHOP cells of defined transient transfection status. *Am. J. Physiol.* 267:C845–C856.

Jiang, C., Z. Qu, and H. Xu. 2002. Gating of inward rectifier K^+ channels by proton-mediated interactions of intracellular protein domains. *Trends Cardiovasc. Med.* 12:5–13.

Jordt, S.E., M. Tominaga, and D. Julius. 2000. Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc. Natl. Acad. Sci. USA.* 97:8134–8139.

Karet, F.E., F.J. Gainza, A.Z. Gyory, R.J. Unwin, O. Wrong, M.J. Tanner, A. Nayir, H. Alpay, F. Santos, S.A. Hulton, et al. 1998. Mutations in the chloride-bicarbonate exchanger gene AE1 cause autosomal dominant but not autosomal recessive distal renal tubular acidosis. *Proc. Natl. Acad. Sci. USA.* 95:6337–6342.

Kopito, R.R., B.S. Lee, D.M. Simmons, A.E. Lindsey, C.W. Morgans, and K. Schneider. 1989. Regulation of intracellular pH by a neu-

ronal homolog of the erythrocyte anion exchanger. *Cell.* 59:927–937.

Kudrycki, K.E., P.R. Newman, and G.E. Shull. 1990. cDNA cloning and tissue distribution of mRNAs for two proteins that are related to the band 3 Cl^-/HCO_3^- exchanger. *J. Biol. Chem.* 265:462–471.

Lee B.S., R.B. Gunn, and R.R. Kopito. 1991. Functional differences among nonerythroid anion exchangers expressed in a transfected human cell line. *J. Biol. Chem.* 266:11448–11454.

Leem, C.H., D. Lagadic-Gossmann, and R.D. Vaughan-Jones. 1999. Characterization of intracellular pH regulation in the guinea-pig ventricular myocyte. *J. Physiol.* 517:159–180.

Leem, C.H., and R.D. Vaughan-Jones. 1998. Sarcolemmal mechanisms for pH_i recovery from alkalosis in the guinea-pig ventricular myocyte. *J. Physiol.* 509:487–496.

Medina, J.F., J. Lecanda, A. Acin, P. Ciesielczyk, and J. Prieto. 2000. Tissue-specific N-terminal isoforms from overlapping alternate promoters of the human AE2 anion exchanger gene. *Biochem. Biophys. Res. Commun.* 267:228–235.

Milanick, M.A., and R.B. Gunn. 1984. Proton-sulfate cotransport: external proton activation of sulfate influx into human red blood cells. *Am. J. Phys.* 247:C247–C259.

Muller-Berger, S., D. Karbach, D. Kang, N. Aranibar, P.G. Wood, H. Ruterjans, and H. Passow. 1995. Roles of histidine 752 and glutamate 699 in the pH dependence of mouse band 3 protein-mediated anion transport. *Biochemistry.* 34:9325–9332.

Parker, M.D., E.P. Ourmozdi, and M.J. Tanner. 2001. Human BTR1, a new bicarbonate transporter superfamily member and human AE4 from kidney. *Biochem. Biophys. Res. Commun.* 282:1103–1109.

Quick, M., D.D. Loo, and E.M. Wright. 2001. Neutralization of a conserved amino acid residue in the human Na^+/glucose transporter (hSGLT1) generates a glucose-gated H^+ channel. *J. Biol. Chem.* 276:1728–1734.

Qu, Z., Z. Yang, N. Cui, G. Zhu, C. Liu, H. Xu, S. Chanchevalap, W. Shen, J. Wu, Y. Li, and C. Jiang. 2000. Gating of inward rectifier K^+ channels by proton-mediated interactions of N- and C-terminal domains. *J. Biol. Chem.* 275:31573–31580.

Quilty, J.A., J. Li, and R.A. Reithmeier. 2002. Impaired trafficking of distal renal tubular acidosis mutants of the human kidney anion exchanger kAE1. *Am. J. Physiol.* 282:F810–F820.

Reinertsen, K.V., T.I. Tonnessen, J. Jacobsen, K. Sandvig, and S. Olsnes. 1988. Role of chloride/bicarbonate antiport in the control of cytosolic pH. Cell-line differences in activity and regulation of antiport. *J. Biol. Chem.* 263:11117–11125.

Richards, S.M., M.E. Jaconi, G. Vassort, and M. Puceat. 1999. A spliced variant of AE1 gene encodes a truncated form of Band 3 in heart: the predominant anion exchanger in ventricular myocytes. *J. Cell Sci.* 112:1519–1528.

Romero, M.F., M.A. Hediger, E.L. Boulpaep, and W.F. Boron. 1997. Expression cloning and characterization of a renal electrogenic Na^+/HCO_3^- cotransporter. *Nature.* 387:409–413.

Sekler, I., R.S. Lo, and R.R. Kopito. 1995. A conserved glutamate is responsible for ion selectivity and pH dependence of the mammalian anion exchangers AE1 and AE2. *J. Biol. Chem.* 270:28751–28758.

Sterling, D., and J.R. Casey. 1999. Transport activity of AE3 chloride/bicarbonate anion-exchange proteins and their regulation by intracellular pH. *Biochem. J.* 344:221–229.

Sterling, D., D.V. Alvarez, and J.R. Casey. 2002a. The extracellular component of a transport metabolon: extracellular loop 4 of the human AE1 Cl^-/HCO_3^- exchanger binds carbonic anhydrase IV. *J. Biol. Chem.* In press.

Sterling, D., R.A. Reithmeier, and J.R. Casey. 2002b. A transport metabolon. Functional interaction of carbonic anhydrase II and chloride/bicarbonate exchangers. *J. Biol. Chem.* 276:47886–47894.

Stewart, A.K., M.N. Chernova, Y.Z. Kunes, and S.L. Alper. 2001. Regulation of AE2 anion exchanger by intracellular pH: critical regions of the NH_2-terminal cytoplasmic domain. *Am. J. Physiol.* 281:C1344–C1354.

Stroffekova, K., E.Y. Kupert, D.H. Malinowska, and J. Cuppoletti. 1998. Identification of the pH sensor and activation by chemical modification of the ClC-2G Cl^- channel. *Am. J. Physiol.* 275:C1113–C1123.

Stuart-Tilley, A.K., B.E. Shmukler, D. Brown, and S.L. Alper. 1998. Immunolocalization and tissue-specific splicing of AE2 anion exchanger in mouse kidney. *J. Am. Soc. Nephrol.* 9:946–959.

Sun, B., C.H. Leem, and R.D. Vaughan-Jones. 1996. Novel chloride-dependent acid loader in the guinea-pig ventricular myocyte: part of a dual acid-loading mechanism. *J. Physiol.* 495:65–82.

Toye, A.M., L.J. Bruce, R.J. Unwin, O. Wrong, and M.J. Tanner. 2002. Band 3 Walton, a C-terminal deletion associated with distal renal tubular acidosis, is expressed in the red cell membrane but retained internally in kidney cells. *Blood.* 99:342–347.

Turin, L., and A.E. Warner. 1980. Intracellular pH in early *Xenopus* embryos: its effect on current flow between blastomeres. *J. Physiol.* 300:489–504.

Vandorpe, D.H., M.N. Chernova, L. Jiang, L.K. Sellin, S. Wilhelm, A.K. Stuart-Tilley, G. Walz, and S.L. Alper. 2001. The cytoplasmic C-terminal fragment of polycystin-1 regulates a Ca^{2+}-permeable cation channel. *J. Biol. Chem.* 276:4093–4101.

Vaughan-Jones, R.D. 1986. An investigation of chloride-bicarbonate exchange in the sheep cardiac Purkinje fibre. *J. Physiol.* 379:377–406.

Vince, J.W., and R.A. Reithmeier. 2000. Identification of the carbonic anhydrase II binding site in the Cl^-/HCO_3^- anion exchanger AE1. *Biochemistry.* 39:5527–5533.

Wilson, D.A., and R.D. Vaughan-Jones. 2000. Kinetic distinction between chloride/hydroxyl and chloride/bicarbonate exchangers in the guinea pig ventricular myocyte. *Biophys. J.* 78:1320A.

Xu, H., Z. Yang, N. Cui, S. Chanchevalap, W.W. Valesky, and C. Jiang. 2000. A single residue contributes to the difference between Kir4.1 and Kir1.1 channels in pH sensitivity, rectification and single channel conductance. *J. Physiol.* 528:267–277.

Yannoukakos, D., A. Stuart-Tilley, H.A. Fernandez, P. Fey, G. Duyk, and S.L. Alper. 1994. Molecular cloning, expression, and chromosomal localization of two isoforms of the AE3 anion exchanger from human heart. *Circ. Res.* 75:603–614.

Zhang, D., A. Kiyatkin, J.T. Bolin, and P.S. Low. 2000. Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3. *Blood.* 96:2925–2933.

Zhang, Y., M.N. Chernova, A.K. Stuart Tilley, L. Jiang, and S.L. Alper. 1996. The cytoplasmic and transmembrane domains of AE2 both contribute to regulation of anion exchange by pH. *J. Biol. Chem.* 271:5741–5749.

Zolotarev, A.S., B.E. Shmukler, and S.L. Alper. 1999. Chemical cross-linking demonstrates homo-oligomeric interaction of AE2 anion exchanger polypeptide in pig gastric membranes. *Biochemistry.* 38:8521–8531.

Zolotarev, A.S., R.R. Townsend, A. Stuart-Tilley, and S.L. Alper. 1996. HCO_3^--dependent conformational change in gastric parietal cell AE2, a glycoprotein naturally lacking sialic acid. *Am. J. Physiol.* 271:G311–G321.