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The Regulation of Intracellular pH in Monkey Kidney Epithelial Cells (BSC-1)

ROLES OF Na^+/H^+ ANTIPORT, Na^+-HCO_3^--($NaCO_3^-$) SYMPORT, AND Cl^-/HCO_3^- EXCHANGE*

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Using the pH-sensitive absorbance of 5 (and 6)-carboxy-4',5'-dimethylfluorescein, we investigated the regulation of cytoplasmic pH (pH_i) in monkey kidney epithelial cells (BSC-1). In the absence of HCO_3^-, pH_i is 7.15 ± 0.1, which is not significantly different from pH_i in 28 mM HCO_3^-, 5% CO_2 (7.21 ± 0.07). After an acid load, the cells regulate pH_i in the absence of HCO_3^- by a Na^+ (or Li^+)-dependent, amiloride-inhibitable mechanism (indicative of Na^+/H^+ antiport). In 28 mM HCO_3^-, while still dependent on Na^+, this regulation is only blocked in part by 1 mM amiloride. A partial block is also observed with 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) (1 mM). With cells pretreated with DIDS, 1 mM amiloride nearly totally inhibits this regulation. Cl^- had no effect on pH_i regulation in the acidic range.

In HCO_3^--free saline, Na^+ removal leads to an amiloride-insensitive acidification, which is dependent on Ca^{2+}. In 28 mM HCO_3^-, Na^+ (and Ca^{2+}) removal led to a pronounced reversible and DIDS-sensitive acidification. When HCO_3^- was lowered from 46 to 10 mM at constant pCO_2 (5%), pH_i dropped by a DIDS-sensitive mechanism. Identical changes in pH_o (7.6 to 6.9) in the nominal absence of HCO_3^- led to smaller changes of pH_i.

In the presence but not in the absence of HCO_3^-, removal of Cl^- led to a DIDS-sensitive alkalinization. This was also observed in the nominal absence of Na^+, which leads to a sustained acidification.

It is concluded that in nominally bicarbonate-free saline, the amiloride-sensitive Na^+/H^+ antiport is the predominant mechanism of pH_i regulation at acidic pH_i, while being relatively inactive at physiological values of pH_i. In bicarbonate saline, two other mechanisms affect pH_i regulation: a DIDS-sensitive Na^+-HCO_3^- symport, which contributes to cytoplasmic alkalinization, and a DIDS-sensitive Cl^-/HCO_3^- exchange, which is apparently independent of Na^+.

An important mechanism of pH_i[1] regulation in most mammalian cells is an amiloride-sensitive Na^+/H^+ antiport (Murer *et al.*, 1976; Roos and Boron, 1981; Boron, 1983) stimulated by acidic pH_i (Aronson *et al.*, 1982). Growth factors activate this antiport, leading to a rapid cytoplasmic alkalinization (Schuldiner and Rozengurt, 1982; Moolenaar *et al.*, 1983; Rothenberg *et al.*, 1983). PS120 fibroblasts, a mutant lacking Na^+/H^+ antiport activity (Pouysségur *et al.*, 1984), did not raise pH_i in response to mitogens (L'Allemain *et al.*, 1984) and were unable to grow at physiological pH_o in the absence of HCO_3^-. Growth was restored in HCO_3^--containing medium. This indicated an important role for pH_i in growth control (Pouysségur *et al.*, 1985) but suggested that Na^+/H^+ activity is not strictly necessary. Regulation of pH_i could be performed by a Na^+-dependent Cl^-/HCO_3^- exchange (L'Allemain *et al.*, 1985), which might be identical to Na^+-HCO_3^-/Cl^--H^+ exchangers previously described for several invertebrates (Thomas, 1977; Boron *et al.*, 1981; Boron and Russel, 1983) and postulated to be present in A431 cells, a human epidermoid cancer cell line (Rothenberg *et al.*, 1983).

Here we investigate intracellular pH regulation of kidney epithelial cells (BSC-1) (Hopps *et al.*, 1963) both in the absence and presence of HCO_3^-. We have previously shown by $^{22}Na^+$ flux measurements (Jentsch *et al.*, 1985b) that this cell line expresses an electrogenic, DIDS-sensitive Na^+-HCO_3^- symport (or $NaCO_3^-$ symport).[2] This symport had been previously described only in the proximal tubule of the salamander kidney (Boron and Boulpaep, 1983) and in the bovine corneal endothelium (Jentsch *et al.*, 1984a, 1985c), but recent evidence suggests that it is present in the mammalian kidney too (in addition to BSC-1, in the proximal tubules of the rat (Yoshitomi *et al.*, 1985; Yoshitomi and Frömter, 1985; Alpern, 1985) and the rabbit (Sasaki *et al.*, 1985; Biagi and Sohtell, 1986). Since these epithelia are involved in transcellular transport of acid equivalents (which, however, is not known from BSC-1), regulation of pH_i in these cells is likely to be an important special case of pH_i regulation.

In this work, we explore the roles of Na^+/H^+ antiport and Na^+-HCO_3^- symport in the regulation of pH_i in BSC-1 cells. Moreover, our data indicate a DIDS-sensitive Cl^-/HCO_3^- exchange activity in these cells.

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[1] The abbreviations used are: pH_i, intracellular pH; pH_o, extracellular pH; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CDMF, 5 (and 6)-carboxy-4',5'-dimethylfluorescein; NMDG, *N*-methyl-D-glucamine; V_m, plasma membrane voltage; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

[2] T. J. Jentsch, P. Schwartz, B. S. Schill, B. Langner, A. P. Lepple, S. K. Keller, and M. Wiederholt, (1986) *J. Biol. Chem.* **261**, 10673-10679.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and supplements were purchased from Biochrom KG, Berlin, Federal Republic of Germany. DIDS was from Fluka, Neu-Ulm, Federal Republic of Germany, and amiloride was a generous gift from MSD Sharp and Dohme, Munich, Federal Republic of Germany. 5 (and 6)-Carboxy-4′,5′-dimethylfluorescein diacetate was obtained from Molecular Probes, Junction City, OR, and nigericin, SITS, and digitonin were purchased from Sigma.

Cells—The monkey kidney epithelial cells used in this study (BSC-1) were of the same strain as described previously (Jentsch *et al.*, 1985b) (obtained from Flow Laboratories, Irvine, United Kingdom, at passage 52). They were maintained in Eagle's minimal essential medium with Earle's salts and 2.2 g/liter $NaHCO_3$, supplemented with nonessential amino acids, 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells used in this study were from passages 58–72. For the experiments, they were seeded on plastic cover slips in Leighton tubes (Costar, Cambridge, MA) and were used at least 3 days after having reached confluency.

Determination of Intracellular pH—Intracellular pH was determined using the pH-sensitive absorbance of 5 (and 6)-carboxydimethylfluorescein (CDMF) generated *in situ* (Simons *et al.*, 1982), which allows better resolution of pH changes in the physiological range than 5 (and 6)-carboxyfluorescein (Thomas *et al.*, 1979). Cells grown on plastic cover slips were loaded with dye by incubation at room temperature for approximately 40 min in saline (150 mM Na^+, 5 mM K^+, 1.7 mM Ca^{2+}, 1 mM Mg^{2+}, 160 mM Cl^-, 1 mM SO_4^{2-}, 1 mM $H_2PO_4^-$, buffered with 10 mM HEPES to pH 6.7) containing 100 μmol/liter CDMF-diacetate, which was added from a stock solution in dimethyl sulfoxide (final concentration: 0.15% dimethyl sulfoxide). Control cells from the same cover slip, which was cut into two parts, were incubated in saline free of CDMF-diacetate under otherwise identical conditions.

pH_i was determined continuously using a dual beam dual wavelength photometer built for this purpose. Two pieces of plastic slips covered with CDMF-loaded cells and control cells, respectively, were inserted (on top of each other) in a cuvette and were continuously superfused with test solutions at 37 °C. This allows a fast exchange of the medium bathing both control and dye-loaded cell monolayers (90% fluid exchange in about 2 s). Two chopped light beams, passing through dye-loaded and control cells, respectively, are split by a beam-splitter and are focused onto two photodiodes. Interference filters (10 nm half-width, type PIL-1, Schott Glaswerke, Mainz, Federal Republic of Germany) of 509 and 470 nm, respectively, are placed in front of each photodiode, and white light intensity was measured for both beams as reference. The absorbance of CDMF has maximum pH sensitivity at about 509 nm, while being pH-insensitive at 470 nm (isosbestic point) (Rosoff and Cantley, 1985). Changes in absorbance caused by loss of intracellular dye can be corrected by calculating the ratio of absorbance at 509 nm *versus* 470 nm. This was done by a personal computer (Apple IIe, Cupertino, CA) connected to the photometer. Measurement of both dye-loaded and control cells allows corrections for absorbance changes of the cells (*e.g.* in Ca^{2+}-free medium) or for absorbance of the solutions bathing the cells to be made. Some experiments of this study were made before implementation of computerized data acquisition. In these cases, light intensities of both channels were subtracted by analog circuitry and recorded by a pen writer, and data are presented as transmittance as a function of time. Calibration curves (performed at the end of the experiments as described below) allow a semiquantitative evaluation to be made, which, however, cannot be easily extrapolated to early time points of the experiment (due to dye leakage). These calibration curves are either directly shown (Figs. 2 and 5), or an approximate pH scale is given at the right end of the figure. Usually, the calibration was performed 5–15 min after the end of experiments.

Alternatively, the availability of two independent channels (two beams) allows a direct comparison of pH_i regulation in two different cell populations (*e.g.* DIDS-treated and untreated cells). Absorbance was calibrated in terms of pH using the nigericin method (Thomas *et al.*, 1979) at the end of each experiment and by determining the extent to which CDMF contributes to the total absorbance by removing the trapped dye with digitonin (80 μM). The nigericin method was also used to establish a calibration curve for absorbance of CDMF intracellularly trapped in BSC-1 as a function of pH (Fig. 1), demonstrating the suitability of this dye for studies of pH_i.

Solutions—The standard bicarbonate saline, SBS, contained 151 mM Na^+, 5 mM K^+, 1.7 mM Ca^{2+}, 1 mM Mg^{2+}, 123 mM Cl^-, 1 mM SO_4^{2-}, 1 mM H_2PO_4, 28 mM HCO_3^-, and 5 mM glucose, and was gassed with 5% CO_2 in air to pH 7.4. The sodium, bicarbonate, and chloride concentrations of this solution were varied by replacing lithium, choline, or NMDG for Na^+, Cl^- for HCO_3^-, and cyclamate, gluconate, or sulfate for Cl^-. In the latter saline, osmolality was adjusted by mannitol. Nominally bicarbonate-free solutions were buffered with 10 mM HEPES to pH 7.4. In solutions designed to change pH_i by nonionic diffusion, 20 mM NaCl was replaced by 20 mM NH_4Cl, or 50 mM NaCl was replaced by 50 mM sodium acetate.

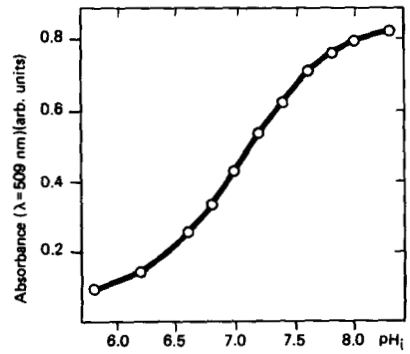

FIG. 1. **Calibration curve for CDMF trapped intracellularly in BSC-1 cells.** Cells were dye-loaded as described under "Experimental Procedures," and absorbance at 509 nm was recorded as a function of pH_i, which was equilibrated with pH_o using the nigericin method as described in Thomas *et al.* (1979).

DIDS Pretreatment of Cells—Cell monolayers were pretreated with DIDS by incubation at 37 °C in SBS with 1 mM DIDS for at least 30 min. This irreversibly inhibits the Na^+–HCO_3^- symport (Jentsch *et al.*, 1985b, 1985c). Afterwards, the cells were loaded with CDMF as described above and used for the experiments.

RESULTS

Regulation of pH_i in Nominally Bicarbonate-free Saline

Recovery from Acid Load—The average steady state intracellular pH of BSC-1 cells in nominally bicarbonate-free saline, buffered with 10 mM HEPES to pH 7.4, was 7.15 ± 0.1. The regulation of pH_i was investigated by imposing an acid load on the cell using the NH_4Cl prepulse technique (Roos and Boron, 1981). Sudden addition of extracellular NH_4Cl leads (by nonionic diffusion of NH_3, Fig. 2, *inset*) to an intracellular alkalinization. When sufficient NH_4^+ has accumulated in the cell (*e.g.* by direct transport of NH_4^+, see Fig. 2, *inset*), subsequent removal of NH_4Cl leads to an overshoot intracellular acidification (Fig. 2). In the acidic pH range, the cells start to extrude acid equivalents, shifting pH_i toward more alkaline values. As shown by the effect of extracellular Na^+ removal, which stopped or reversed the regulation of pH_i, the acid-extruding mechanism depends on extracellular sodium (Fig. 2). In other experiments, we observed that lithium could partially substitute for sodium in this respect (data not shown). Amiloride at 1 mM, a concentration high enough to cause significant inhibition of Na^+/H^+ antiport in several systems (Benos, 1982), largely blocked this regulation of pH_i in Na^+-saline (Fig. 2) as well as in Li^+-saline (data not shown). Thus, these results strongly suggest that under nominally HCO_3^--free conditions Na^+/H^+ antiport is the main mechanism for H^+ extrusion following an acid load.

Effect of Na^+ Removal and Amiloride at Steady State pH_i—To assess the role of Na^+/H^+ antiport in the maintenance of pH_i under steady state conditions in HCO_3^--free media, we replaced extracellular Na^+ by NMDG. This resulted in a reversible cytoplasmic acidification (Fig. 3), which might be explainable by a reversal of Na^+/H^+ antiport. However, amiloride (1 mM) did not reduce the acidification caused by

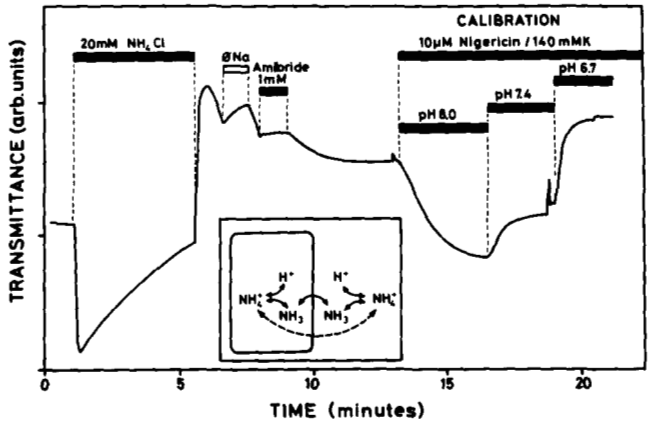

FIG. 2. **Regulation of pH_i in the nominal absence of HCO_3^- following an acid load.** Cells were acid-loaded by preincubation for 5 min in 20 mM NH_4Cl (which replaced equal amounts of NaCl) in nominally bicarbonate-free saline (obtained from SBS by substituting NaCl for $NaHCO_3$). Removal of Na^+ (replaced by NMDG) reversed this regulation, which could also be blocked by 1 mM amiloride in Na^+ saline. At the end of the experiment, transmittance was calibrated in terms of pH_i using the nigericin method (Thomas *et al.*, 1979). Since the data were not corrected for dye leakage, the pH scale thus obtained may not be easily extrapolated to the beginning of the experiment, as described under "Experimental Procedures." *Inset,* schematic diagram of the mechanism by which addition of NH_4Cl causes intracellular alkalinization.

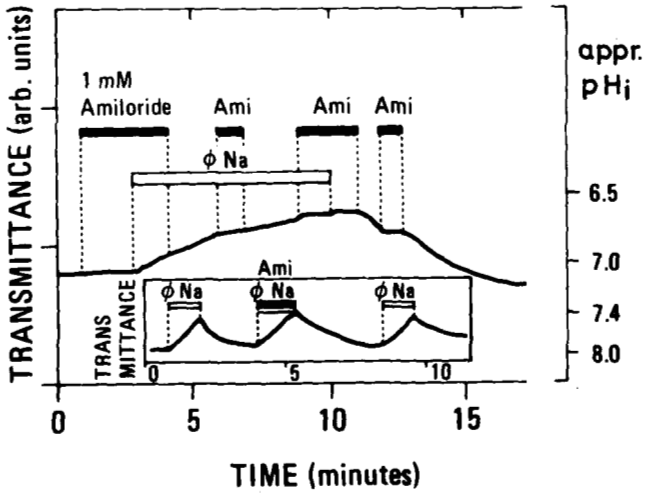

FIG. 3. **Effect of Na^+ removal and amiloride on pH_i in the absence of HCO_3^- starting from resting conditions.** *Inset,* removal of Na^+ (replaced by NMDG) resulted in an acidification which was largely unaffected by simultaneously applied amiloride (1 mM). Amiloride had no visible effect on pH_i for at least 2 min (*main panel*) under resting conditions, but inhibited recovery from acidification caused by Na^+ removal and slightly inhibited acidification induced by Na^+ removal when pH_i had dropped below physiological levels.

removal of Na^+ near physiological values of pH_i (Fig. 3 and *inset*) nor did amiloride (1 mM) applied under steady state conditions have an effect on pH_i. On the other hand, when Na^+ was removed during some minutes, which caused the cell to acidify below about pH 6.9, an effect of amiloride on the rate of acidification became apparent (Fig. 3, $t \approx 7$ min). When Na^+ was readded, pH_i recovered toward more alkaline values by an amiloride-sensitive mechanism (Fig. 3). Thus, these results suggest that Na^+/H^+ antiport, while being responsible for acid extrusion following an acid load, is relatively inactive at physiological values of pH_i (above 7.1).

These experiments virtually excluded a significant role of Na^+/H^+ antiport in acidification upon Na^+ removal in HCO_3^--free saline. We therefore hypothesized that Na^+ removal might raise intracellular Ca^{2+} by a Na^+/Ca^{2+} antiport, which might affect pH_i. Therefore, we removed extracellular Na^+ together with Ca^{2+} (Fig. 4). This indeed greatly reduced the intracellular acidification associated with Na^+ removal in HCO_3^--free medium. Removal of extracellular Ca^{2+} alone in the presence of Na^+, however, had no significant effect on pH_i within some minutes. Since this suggested that, in bicarbonate-free medium, Na^+ removal might acidify the cytoplasm by a Ca^{2+}-dependent mechanism, we performed experiments similar to the one shown in Fig. 2, but removed both Na^+ and Ca^{2+} during the recovery from the acid load. Again, this maneuver totally blocked recovery of pH_i, lending support to our contention that Na^+/H^+ exchange is responsible for this regulation.

Regulation of pH_i in Bicarbonate Saline

Recovery from Acid Load—In standard bicarbonate saline containing 28 mM HCO_3 (gassed with 5% CO_2 to pH 7.4) pH_i averaged at 7.21 ± 0.07. We investigated recovery from acid loads in bicarbonate saline in an experiment identical to that of Fig. 2 except for the presence of 28 mM HCO_3^- and 5% CO_2 (Fig. 5). After the cytoplasm is acidified with the NH_4Cl prepulse technique, pH_i returns to physiological values. As in the absence of HCO_3^-, this regulation is Na^+-dependent, since removal of Na^+ led to a blockade or reversal of this return (Fig. 5*A*). To exclude an effect of the Ca^{2+}-dependent mechanism described in the previous section, we repeated this experiment, removing Na^+ and Ca^{2+} simultaneously, with identical results (data not shown). In contrast to the bicarbonate-free system, however, amiloride (1 mM) inhibited this regulation only in part (Fig. 5*A*). When DIDS (1 mM) was tested on the recovery from acid load in the presence of HCO_3^-, a similar (although larger) partial inhibition was observed (Fig. 5*B*). DIDS, an aminoreactive reagent, is an irreversible inhibitor of several anion transport processes (*e.g.* Cl^-/HCO_3^- exchange of red blood cells (Cabantchik and Rothstein, 1972)). However, in several systems, short term inhibition is due to electrostatic interaction, leading to reversible inhibition, while more time is needed for irreversible inhibition due to formation of covalent bonds (Lepke *et al.*, 1976; Edelman *et al.*, 1978). With BSC-1, the inhibition caused by 1 mM DIDS was still partially reversible after exposure for 5 min. When cells were pretreated with DIDS (1 mM) for a prolonged time (30 min at 37 °C), a maneuver known to block the Na^+–HCO_3^- symport of BSC-1 irreversibly (Jentsch *et al.*,

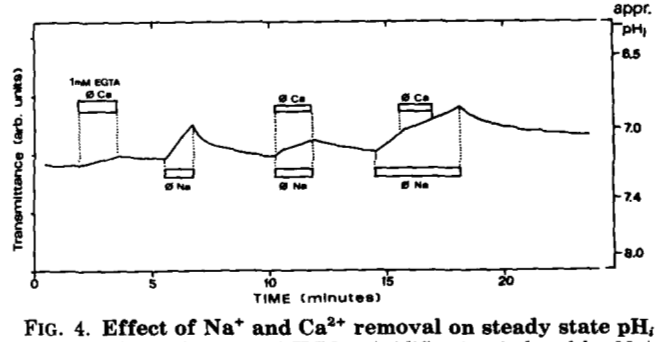

FIG. 4. **Effect of Na^+ and Ca^{2+} removal on steady state pH_i in the nominal absence of HCO_3^-.** Acidification induced by Na^+ removal (replaced by NMDG) was largely reduced by concomitant removal of Ca^{2+} (which was omitted from the saline). Although the first Na^+ removal caused a larger change in transmittance than the third one, this probably does not reflect a larger change in pH_i, but may rather be due to dye leakage which was not corrected for in this experiment.

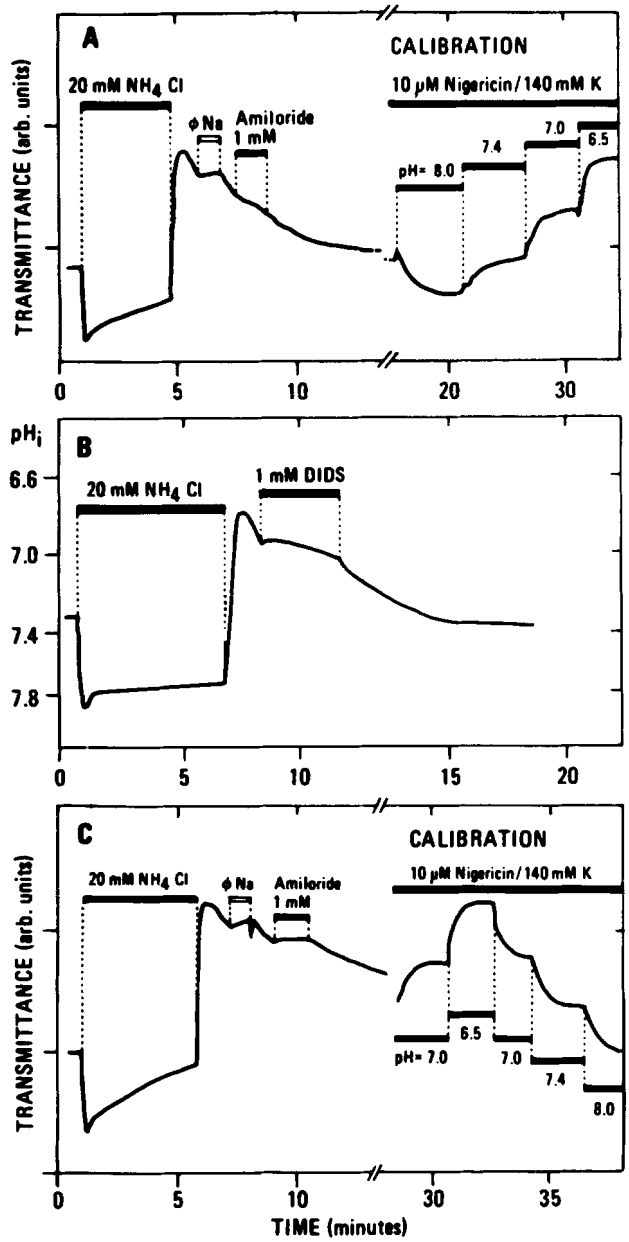

FIG. 5. **Recovery from acid load in bicarbonate saline.** Cells were acid-loaded in SBS by preincubation with 20 mM NH_4Cl, and the subsequent recovery of pH_i was studied in SBS. *A*, effects of Na^+-free bicarbonate saline (sodium replaced by NMDG and 28 mM choline) and of 1 mM amiloride on pH_i regulation; *B*, effect of 1 mM DIDS on recovery from acid load; *C*, effect of amiloride on pH_i recovery with cells pretreated with DIDS (as specified under "Experimental Procedures").

1985b), amiloride (1 mM) caused a nearly complete block of acid extrusion (Fig. 5*C*). This suggests that at least two mechanisms contribute to H^+ extrusion in HCO_3^- saline, both being dependent on sodium. The amiloride-sensitive mechanism is independent of bicarbonate and is probably identical to a Na^+/H^+ antiport, while the DIDS-sensitive process depends on bicarbonate and may be identical to the Na^+–HCO_3^- symport postulated earlier for BSC-1 (Jentsch *et al.*, 1985b).

An alternative explanation for a DIDS-sensitive, bicarbonate- and sodium-dependent recovery from acid load could be a Na^+–HCO_3^-/Cl^-–H^+ exchange. We therefore removed Cl^- during recovery from acid load in HCO_3^--saline, which had no significant effect (data not shown). However, with the postulated mechanism recovery from acid load should depend on intracellular Cl^-, which leaves the cell in exchange for HCO_3^-. Therefore, the cells were chloride-depleted by preincubation (for 60 min) in Cl^--free saline and were acid-loaded by exposure to 20 mM $(NH_4)_2SO_4$ in the absence of chloride. If the two processes for acid extrusion were Na^+/H^+ antiport and Na^+–HCO_3^-/Cl^-–H^+ exchange, the regulation of pH_i should be (nearly) totally blocked by 1 mM amiloride in the absence of Cl^-. This has indeed been observed with A431 cells postulated to express these two processes (Rothenberg *et al.*, 1983). With BSC-1, however, amiloride only partially blocked the regulation (Fig. 6*A*); with DIDS-pretreated cells, this inhibition is much more complete (Fig. 6*B*). This supports the notion that the DIDS-sensitive mechanism is a Na^+–HCO_3^- symport.

Effect of Na^+ Removal—In the experiment of Fig. 7, we compared the effect of Na^+ removal on pH_i both in the absence and presence of HCO_3^-. To avoid the Ca^{2+}-dependent effects described above, Na^+ and Ca^{2+} were simultaneously removed. This had only minimal effects on pH_i in HCO_3^--free solution, but led to a conspicuous acidification in the presence of HCO_3^-. Upon readdition of Na^+ (and Ca^{2+}), pH_i returned to control values. In the experiment of Fig. 8 we directly com-

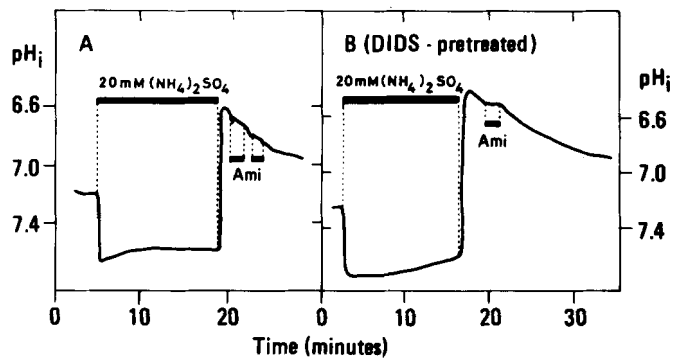

FIG. 6. **Effect of amiloride on recovery from acid load in Cl^--free bicarbonate saline with Cl-depleted cells.** *A*, untreated cells; *B*, DIDS-pretreated cells. Cells were pretreated with DIDS as described under "Experimental Procedures," and depleted of Cl^- prior to the experiment by loading the cells with CDMF for about 40 min in Cl^--free saline (obtained from the solution specified under "Experimental Procedures" by substituting SO_4^{2-} for Cl^-, with osmolality adjusted by mannitol). During the experiments, chloride was replaced by gluconate (Ca^{2+} concentration was raised to 6 mM to compensate for formation of complexes with gluconate).

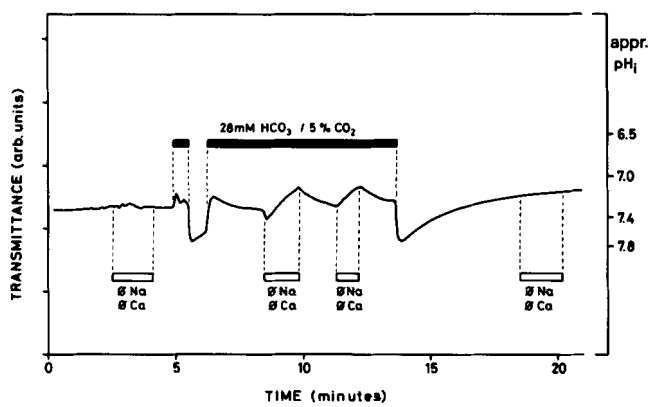

FIG. 7. **Effect of Na^+ (and Ca^{2+}) removal on pH_i in the absence and presence of HCO_3^-.** Na^+ (replaced by 28 mM choline and 123 mM NMDG) was removed together with Ca^{2+} (omitted from medium) in nominally bicarbonate-free saline buffered with HEPES to pH 7.4 and in SBS (containing 28 mM HCO_3^- and gassed with 5% CO_2 to pH 7.4).

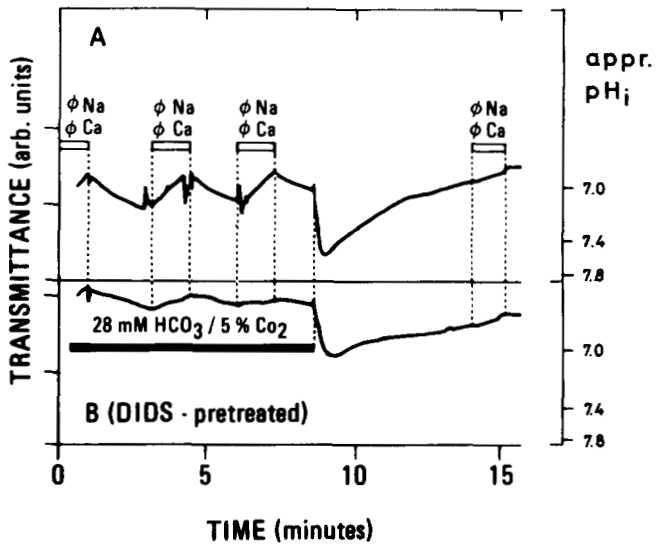

FIG. 8. **Effect of DIDS pretreatment on acidification induced by Na^+ and Ca^{2+} removal in bicarbonate saline.** pH_i regulation of DIDS-pretreated cells (as specified under "Experimental Procedures") and untreated cells was measured simultaneously exploiting the dual beam capability of the setup. Solutions are identical to those given in the caption to Fig. 7.

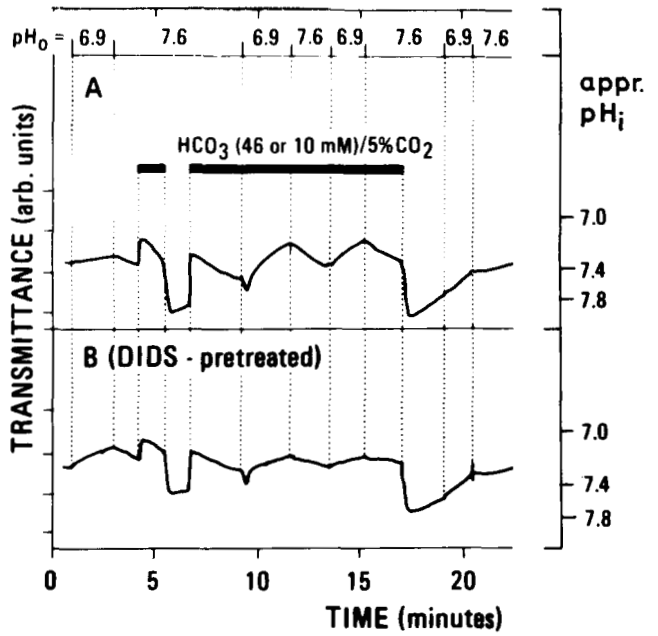

FIG. 9. **Effect of changes of pH_o on pH_i in the presence and the absence of HCO_3^-.** Identical changes of pH_o were performed (using the dual beam capability of the setup) with DIDS-pretreated cells (*B*) and untreated cells (*A*) in nominally bicarbonate-free saline (buffered with 10 mM HEPES to the indicated values of pH) and in bicarbonate saline (46 or 10 mM HCO_3^-) gassed with 5% CO_2. HCO_3^- was replaced by Cl^-. The high initial bicarbonate concentration was chosen to keep pH_o relatively close to physiological values during the experimental procedure and to enable a direct comparison to electrophysiological studies.[3] The transient alkalinization observed shortly after addition of 10 mM HCO_3^-, 5% CO_2 (pH 6.9) was probably due to insufficient equilibration with CO_2. When freshly equilibrated saline had filled the tubings during the following experimental cycles, this effect disappeared.

pared the effect of Na^+ (and Ca^{2+}) removal between untreated and DIDS-pretreated cells, exploiting the dual beam capability of the photometer. The acidification caused by Na^+ (and Ca^{2+}) removal in bicarbonate saline is inhibited in DIDS-pretreated cells, suggesting a role for a Na^+- and HCO_3^--dependent process inhibitable by DIDS. This is compatible with a Na^+–HCO_3^- symport.

Effect of Lowering Bicarbonate Concentration—We lowered HCO_3^- concentration at constant pCO_2, since changes in CO_2, by intracellular conversion into carbonic acid, affect pH_i independently of membrane processes (see, *e.g.* Fig. 7 at ~6 and ~12 min). Under these conditions, reduction of HCO_3^- leads to a decreased value of pH_o which might *per se* influence pH_i. Thus, to elucidate whether the effect is directly due to HCO_3^- concentration changes, we performed identical changes of pH_o (from 7.64 to 6.94) in nominally HCO_3^--free saline in the same experiment (Fig. 9*A*). While pH_i dropped slightly with extracellular acidification in the absence of HCO_3^-, the fall in pH_i was significantly larger when identical changes in pH_o were performed in HCO_3^- saline, which is equivalent to a reduction of HCO_3^- from 46 to 10 mM at constant 5% CO_2. The intracellular buffering power is presumably higher in the presence of HCO_3^-/CO_2, and hence the pH_i changes would tend—on these grounds—to be smaller, contrary to the observed results. This points to an additional acid transfer process emerging in the presence of HCO_3^-. In cells pretreated with DIDS, the presence of HCO_3^- had no effect on changes in pH_i elicited by pH_o (Fig. 9*B*), suggesting a DIDS-sensitive HCO_3^- transport process.

Evidence for Chloride-Bicarbonate Exchange—In bicarbonate saline, removal of extracellular Cl^- led to an intracellular alkalinization (Fig. 10). In nominally bicarbonate-free saline, no significant alkalinization was observed. Such effect of chloride is also observed after intracellular alkalinization induced by a prepulse of 50 mM acetate (Fig. 11*A*). (By nonionic diffusion as acetic acid addition of acetate leads to a drop of pH_i. After sufficient intracellular accumulation of acetate (either by direct transport of the anion, or by a combined effect of nonionic diffusion and pH_i regulation, which increases total intracellular acetate concentration), subsequent extracellular acetate removal leads to an overshoot intracellular alkalinization.) With cells pretreated with DIDS, however, we were unable to detect an effect of Cl^- on pH_i both after alkalinization (Fig. 11*B*) and under resting conditions (data not shown). While the data of Fig. 11 might suggest that Cl^-/HCO_3^- exchange activity may contribute to pH_i regulation after base loading, we were unable to detect an effect of either 1 mM DIDS or SITS on the recovery from an acetate prepulse (data not shown). As expected, also 1 mM

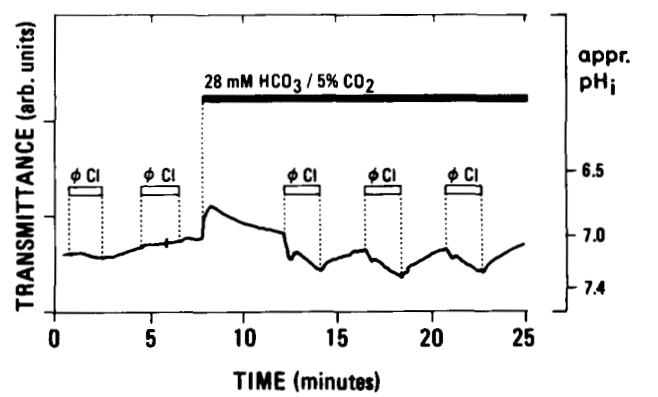

FIG. 10. **Effect of Cl^- removal on pH_i in the absence and presence of HCO_3^-.** Cl^- was replaced by gluconate in the nominal absence and the presence of HCO_3^- (SBS). Similar effects were also observed when SO_4^{2-} was used as a substitute. The fast intracellular alkalinization observed during the first removal of Cl^- is probably due to insufficient saturation of the saline with CO_2. This effect disappeared when freshly equilibrated saline reached the cuvette during the subsequent cycles of Cl^- removal.

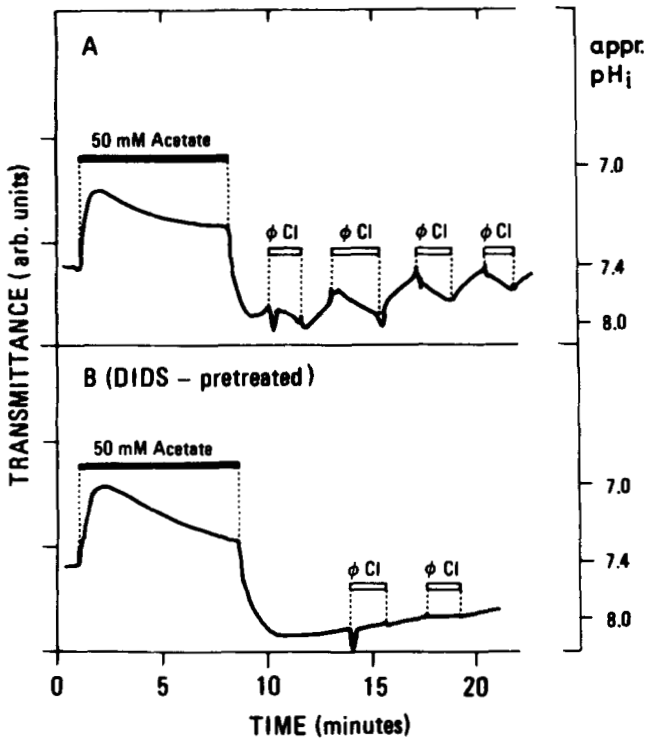

FIG. 11. **Effect of Cl^- removal in bicarbonate saline following a base load.** *A*, untreated cells; *B*, DIDS-pretreated cells. pH_i was shifted to alkaline values by preincubation with 50 mM sodium acetate, which replaced equal amounts of NaCl in SBS. Gluconate was used as a substitute for Cl^-. Although in this experiment, the alkalinization upon acetate removal appears to be larger in DIDS-pretreated cells, this effect was not reproducibly observed.

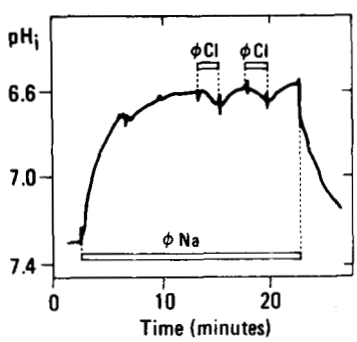

FIG. 12. **Effect of Na^+ removal on Cl^-/HCO_3^- exchange.** Na^+ was replaced by 123 mM NMDG and 28 mM choline in saline containing 28 mM HCO_3^-, gassed with 5% CO_2. In Na^+-free saline, chloride was totally replaced by gluconate, which led to an intracellular alkalinization.

amiloride proved ineffective in impairing recovery from base loading (data not shown).

To investigate whether Cl^-/HCO_3^- exchange activity is dependent on sodium, we removed sodium for more than 10 min in bicarbonate saline and removed Cl^- in nominally Na^+-free saline (Fig. 12). Na^+ removal led to a sustained acidification, from which the cells failed to recover within 20 min. This acidification might be due to a combined effect of Na^+–HCO_3^- symport, Na^+/H^+ antiport (activated at lower values of pH_i), and the Ca^{2+}-dependent effect described above. Cl^- removal led to an alkalinization even under those conditions, suggesting that Cl^-/HCO_3^- exchange activity in BSC-1 is not (totally) dependent on Na^+ (or, alternatively, that the transporter has a high affinity for Na^+).

DISCUSSION

In this study, we have demonstrated three major processes involved in cytoplasmic pH regulation in monkey kidney epithelial cells (BSC-1): a Na^+/H^+ antiport, a Na^+–HCO_3^- symport, and a Cl^-/HCO_3^- exchange. Moreover, the data suggest an effect of Ca^{2+} on pH_i independent of these mechanisms.

Amiloride-sensitive Na^+/H^+ exchangers have been identified in virtually every mammalian cell examined to date, where they play an important role in pH_i regulation (Boron, 1983), volume regulation (Grinstein *et al.*, 1985), in the response to growth factors (Schuldiner and Rozengurt, 1982; Rothenberg *et al.*, 1983; Moolenaar *et al.*, 1983), and, in specialized epithelial cells, in transcellular transport of acid equivalents (Boron, 1983). Since the available Na^+ gradient across the plasma membrane by far exceeds the H^+ gradient established under physiological conditions, this process should be relatively inactive under steady state conditions. Indeed, a modifier site on the cytoplasmic domain has been postulated (Aronson *et al.*, 1982), which activates the antiport when pH_i falls below a given threshold. BSC-1 cells also express such antiport, as already suggested from $^{22}Na^+$ uptake studies (Rothenberg *et al.*, 1982; Jentsch *et al.*, 1985b). Compatible with activation of Na^+/H^+ antiport by low pH_i, we could demonstrate its activity during recovery from acid loading. In nominally bicarbonate-free saline, this recovery could be totally blocked or reversed by removal of Na^+ (which could partially be replaced by Li^+ as in other systems (Kinsella and Aronson, 1980; Paris and Pouysségur, 1983)) and largely blocked by 1 mM amiloride, a known inhibitor of this process (Benos, 1982). This strongly suggests that Na^+/H^+ antiport is the predominant mechanism for acid extrusion in the absence of HCO_3^-.

We were unable to demonstrate significant Na^+/H^+ exchange activity at normal pH_i. Amiloride (1 mM) had no significant effect on steady state pH_i when applied for some minutes, and removal of Na^+ (together with Ca^{2+}, as discussed later) had only a small effect. This is compatible with the assumption that in BSC-1 Na^+/H^+ antiport does not significantly contribute to resting pH_i in bicarbonate-free saline. This is not a general characteristic of mammalian cells, since *e.g.* in human fibroblasts amiloride elicited an acidification under steady state conditions (Moolenaar *et al.*, 1984). However, with other cells (proximal tubule of the salamander kidney), even a slight alkalinization has been observed with 2 mM amiloride (Boron and Boulpaep, 1983), which was interpreted as permeation of the base form of amiloride. Thus, the missing effect of amiloride on steady state pH_i *per se* is not conclusive. With BSC-1, the plasma membrane voltage is very low in nominally bicarbonate-free saline (about −15 mV),[3] predicting $pH_i = 7.15$ by pure passive distribution of H^+ ($pH_o = 7.4$). This may obviate regulation of pH_i by Na^+/H^+ antiport in HCO_3^--free saline under resting conditions.

Investigations of the role of Na^+/H^+ antiport at normal values of pH_i were complicated by a Ca^{2+}-dependent effect. Extracellular Na^+ removal led to an amiloride-insensitive acidification. This virtually excludes a reversal of Na^+/H^+ antiport as cause of the acidification, especially since 1 mM amiloride (which acts by competing with Na^+ (Mahnensmith and Aronson, 1985)) should be very potent in Na^+-free saline. When Na^+ was removed together with Ca^{2+}, however, pH_i remained nearly constant, suggesting that Na^+ removal reverses a Na^+/Ca^{2+} antiport, raising intracellular Ca^{2+}, which in turn causes pH_i to drop. However, since Ca^{2+} ionophores

[3] T. J. Jentsch, H. Matthes, S. K. Keller, and M. Wiederholt, *Am. J. Physiol.*, in press.

act by catalyzing $Ca^{2+}/2H^+$ exchange (Kauffman *et al.*, 1980), we were unable to demonstrate a fall in pH_i using Ca^{2+} ionophores. Intracellular acidification caused by raising Ca_i^{2+} has been observed in other cells (Meech and Thomas, 1980; Vaughan-Jones *et al.*, 1983), although in fibroblasts an alkalinization was observed with the Ca^{2+} ionophore A23187 (Muldoon *et al.*, 1985). The mechanism by which Ca^{2+} lowers pH_i is not clear. A possibility is a release of H^+ from intracellular stores, *e.g.* mitochondria (Vercesi *et al.*, 1978; Meech and Thomas, 1980).

In the presence of bicarbonate, additional mechanisms of pH_i regulation were observed. Following an acid load, acid extrusion was due both to an amiloride-sensitive mechanism (Na^+/H^+ antiport) and to a DIDS-sensitive process, the latter depending both on Na^+ and HCO_3^-, suggesting a Na^+–HCO_3^- symport (Jentsch *et al.*, 1985b). Our inability to demonstrate an effect of Cl^- during recovery from acid load suggests a predominant role for Na^+–HCO_3^- symport (independent of Cl^-) and Na^+/H^+ antiport in acid extrusion, although the presence of a Na^+–HCO_3^-/Cl^-–H^+ exchange cannot be definitely ruled out (*e.g.* when the intracellular affinity for chloride is very high, the time of chloride depletion (60 min) might not be sufficient to block this process). Furthermore, since the DIDS-sensitive Na^+-dependent process made a large contribution to pH_i regulation after an acid load in the presence of HCO_3^-, this suggests that the stoichiometry of Na^+–HCO_3^- symport is such to allow net inward transport.

Na^+ (and Ca^{2+}) removal in the presence of HCO_3^-, as well as lowering HCO_3^- at constant pCO_2, led to a DIDS-sensitive acidification. Thus, Na^+–HCO_3^- symport is not inactivated at resting pH_i. This is also suggested by the large electrical effects when concentrations of these ions are changed starting from control conditions (Jentsch *et al.*, 1985b).[3]

In the presence of HCO_3^- we observed also a DIDS-sensitive alkalinization upon Cl^- removal. This suggests a Cl^-/HCO_3^- exchange. In these cells, it should be more efficient than Cl^-/OH^- exchange, since virtually no such effect was observed in nominally HCO_3^--free saline. Cl^-/HCO_3^- exchange activity was observed both in the alkaline pH range (after an acetate prepulse) and in the acidic pH range (Na^+-free). The latter result also suggests that Cl^-/HCO_3^- exchange is not (totally) dependent on Na^+ (or, alternatively, that the process should have a large affinity for sodium). A coupling to Na^+ would have important consequences for the net transport direction of chloride-bicarbonate exchange. With Na^+–HCO_3^-/Cl^-–H^+ exchange, as found in invertebrates (Thomas, 1977; Boron *et al.*, 1981; Boron and Russel, 1983) and probably also present in A431 cells (Rothenberg *et al.*, 1983) and in mammalian fibroblasts (L'Allemain *et al.*, 1985), the Na^+ gradient provides the driving force for net inward transport of HCO_3^- (resulting in a pH_i-regulating mechanism), whereas a pure Cl^-/HCO_3^- exchange should mediate a net efflux of HCO_3^- under most physiological conditions (Boron, 1983). In some cells, this efflux of HCO_3^- is important for transcellular transport of acid equivalents (Fischer *et al.*, 1983), while the associated influx of Cl^- has been postulated to be important for other cells (Vaughan-Jones, 1979; Aickin and Brading, 1984). A parallel operation of Na^+/H^+ and Cl^-/HCO_3^- exchange has been postulated to mediate net electroneutral NaCl uptake by *some epithelial cells* (Reuss and Costantin, 1984). It is interesting to note that indications for Cl^-/HCO_3^- exchange have also been observed for other cells expressing a Na^+–HCO_3^- symport, such as the bovine corneal endothelium (Jentsch *et al.*, 1985a) or the proximal tubule of the salamander (Boron and Boulpaep, 1983), but no significant Cl^-/HCO_3^- exchange activity has been found in the rat proximal tubule (Cassola *et al.*, 1983; Alpern, 1985).

The mechanisms of pH_i regulation after alkaline load remain obscure. Although Cl^- removal stopped or reversed the return of pH_i to resting levels after an acetate prepulse, we could not detect an effect of 1 mM DIDS or SITS on this regulation. Furthermore, pH_i regulation after base loading occurred also in nominally bicarbonate-free saline. Thus, remaining possible explanations may be that both passive ionic permeabilities (such as conductive pathways for H^+ or OH^-, for which electrophysiological data provide some evidence[3]) and accumulation of metabolic acid equivalents (as reviewed in Roos and Boron (1981)) contribute to recovery from alkaline load.

The net transport direction of electrogenic Na^+–HCO_3^- symport is not totally clear, especially since this transporter has been discovered only recently (Boron and Boulpaep, 1983; Jentsch *et al.*, 1984a, 1984b, 1985b). In order to account for the known direction of transepithelial transport, a net efflux of HCO_3^- and Na^+ has been postulated for the proximal tubule (Boron and Boulpaep, 1983; Yoshitomi *et al.*, 1985) and the corneal endothelium (Jentsch *et al.*, 1984a). However, there is no convincing evidence for this claim except possibly for the salamander kidney (Boron and Boulpaep, 1983). Since the symport is thought to proceed passively, transport direction is linked to the stoichiometry of HCO_3^- to Na^+ binding and to ionic gradients and values of V_m in the respective cells. A 2:1 stoichiometry is compatible with a net outward transport in the salamander system (Boron and Boulpaep, 1983), while a stoichiometry of 3:1 would be necessary to allow this transport direction in the rat (Yoshitomi *et al.*, 1985) (however, see Alpern, 1985). Since V_m is about −55 mV in these cells,[3] a stoichiometry of 2:1 or 3:1 would probably lead to an influx or efflux, respectively, of Na^+ and HCO_3^-. The present data indicate that net inward transport occurs during recovery from acid load, while no outward transport could be detected after alkalinization (missing effect of DIDS and SITS on recovery from alkaline load). A continuous influx should contribute to intracellular alkalinization, whereas an efflux would acidify the cell. Then, a block of the process by DIDS (or by the absence of HCO_3^-) should elicit an acidification, or alkalinization, respectively, if pH_i were not dominated by other regulatory processes. An alkalinization upon these maneuvers has indeed been described for the salamander kidney (Boron and Boulpaep, 1983), supporting the postulated efflux. For BSC-1, these maneuvers yield less clear results. With DIDS or in the absence of HCO_3^-, steady state pH_i was not significantly different from control conditions. However, V_m permanently decreases upon DIDS or HCO_3^- removal[3] (V_m on the order of −15 mV), which would lead to the observed value of pH_i assuming even a passive distribution of H^+. Moreover, both DIDS and HCO_3^- removal affect Cl^-/HCO_3^- exchange, giving rise to further difficulties in interpretation.

In summary, pH_i regulation in monkey kidney epithelial cells (BSC-1) in the absence of HCO_3^- is primarily due to Na^+/H^+ antiport, which is activated at acidic pH. In HCO_3^- saline, two additional mechanisms emerge: a Cl^-/HCO_3^- exchange, which apparently is not dependent on Na^+, and a Na^+–HCO_3^- symport. The latter process may mediate net influx of both ions, contributing to pH_i regulation after acid loading. At the values of pH_i and V_m determined for BSC-1, this is compatible with an apparent 2:1 coupling of bicarbonate to sodium. This in turn could be easily interpreted in terms of the ion pair model in which $NaCO_3^-$ is the transported species, which is compatible with kinetic data obtained from $^{22}Na^+$ uptake studies in BSC-1.[2]

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