Cytosolic pH Regulation in Osteoblasts

Regulation of Anion Exchange by Intracellular pH and Ca^{2+} Ions

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ABSTRACT Measurements of cytosolic pH (pH_i), ^{36}Cl fluxes and free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were performed in the clonal osteosarcoma cell line UMR-106 to characterize the kinetic properties of Cl^-/HCO_3^- (OH^-) exchange and its regulation by pH_i and $[Ca^{2+}]_i$. Suspending cells in Cl^--free medium resulted in rapid cytosolic alkalinization from pH_i 7.05 to ~7.42. Subsequently, the cytosol acidified to pH_i 7.31. Extracellular HCO_3^- increased the rate and extent of cytosolic alkalinization and prevented the secondary acidification. Suspending alkalinized and Cl^--depleted cells in Cl^--containing solutions resulted in cytosolic acidification. All these pH_i changes were inhibited by 4',4',-diisothiocyano-2,2'-stilbene disulfonic acid (DIDS) and H_2DIDS, and were not affected by manipulation of the membrane potential. The pattern of extracellular Cl^- dependency of the exchange process suggests that Cl^- ions interact with a single saturable external site and HCO_3^- (OH^-) compete with Cl^- for binding to this site. The dependencies of both net anion exchange and Cl^- self-exchange fluxes on pH_i did not follow simple saturation kinetics. These findings suggest that the anion exchanger is regulated by intracellular HCO_3^- (OH^-).

A rise in $[Ca^{2+}]_i$, whether induced by stimulation of protein kinase C-activated Ca^{2+} channels, Ca^{2+} ionophore, or depolarization of the plasma membrane, resulted in cytosolic acidification with subsequent recovery from acidification. The Ca^{2+}-activated acidification required the presence of Cl^- in the medium, could be blocked by DIDS, and H_2DIDS and was independent of the membrane potential. The subsequent recovery from acidification was absolutely dependent on the initial acidification, required the presence of Na^+ in the medium, and was blocked by amiloride. Activation of protein kinase C without a change in $[Ca^{2+}]_i$ did not alter pH_i. Likewise, in H_2DIDS-treated cells and in the absence of Cl^-, an increase in

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/90/01/0121/25 $2.00 121
Volume 95 January 1990 121–145

$[Ca^{2+}]_i$ did not activate the Na^+/H^+ exchanger in UMR-106 cells. These findings indicate that an increase in $[Ca^{2+}]_i$ was sufficient to activate the Cl^-/HCO_3^- exchanger, which results in the acidification of the cytosol. The accumulated H^+ in the cytosol activated the Na^+/H^+ exchanger. Kinetic analysis of the anion exchange showed that at saturating intracellular OH^-, a $[Ca^{2+}]_i$ increase did not modify the properties of the extracellular site. A rise in $[Ca^{2+}]_i$ increased the apparent affinity for intracellular OH^- (or HCO_3^-) of both net anion and Cl^- self exchange. These results indicate that $[Ca^{2+}]_i$ modifies the interaction of intracellular OH^- (or HCO_3^-) with the proposed regulatory site of the anion exchanger in UMR-106 cells.

INTRODUCTION

Recent studies demonstrated the presence of a Cl^-/HCO_3^- exchange mechanism in various cells. The kinetic properties of this exchange mechanism have been extensively explored in red blood cells. Measurements of HCO_3^--coupled Cl^- fluxes and Cl^- self exchange in human red blood cells have established that these anions are translocated on 100-kDa transmembrane protein, band 3 (Cabantchik et al., 1978; Knauf, 1979). Several kinetic studies have shown that the exchange of anions by band 3 is an electroneutral process with a stoichiometry of 1:1 (Knauf et al., 1977). In both red blood cells and ghosts, the transport displays saturation kinetics (Dalmark, 1976), temperature dependence (Brahm, 1977), inhibition by stilbene derivatives (Cabantchik and Rothstein, 1974), and both competitive and noncompetitive inhibitions by various anions (Dalmark, 1976). The kinetic behavior of the exchanger suggests that anion translocation by the exchanger is best described by a ping-pong mechanism (Knauf et al., 1977; Gunn and Frohlich, 1979; Jennings, 1982). Thus, kinetically, the exchanger behaves as if it has a single anion translocating site which allows the transport of Cl^- or HCO_3^- in opposite directions, as determined by the chemical gradients of the anions across the plasma membrane.

Anion exchange in nucleated cells has only been studied to a limited extent. However, it appears that anion exchange similar to that found in erythrocytes, also operates in Ehrlich ascites cells (Aull, 1979), cardiac Purkinje cells (Vaughan-Jones, 1979), human neutrophils (Simchowitz and Roos, 1985; Simchowitz et al., 1986), renal collecting duct cells (Zeidel et al., 1986; Tago et al., 1986*a*), MDCK cells (Kurtz and Golchini, 1987), gastric parietal cells (Muallem et al., 1985), and Vero cells (Olsnes et al., 1986). For most of these cells, it is assumed that Cl^-/HCO_3^- exchange is mediated by a protein equivalent to the erythrocyte band 3, although variations in the properties of Cl^-/HCO_3^- exchange by various cells have been described (Schuster and Stokes, 1987).

In the present study we used the UMR-106 cells, a clonal rat osteosarcoma cell line with osteoblast phenotype (Martin et al., 1976; Partridge et al., 1981, 1983), to determine whether these cells possess a Cl^-/HCO_3^- exchanger and then to investigate the mechanisms that regulate its activity. We reasoned that in order for the osteoblast to carry out its function with regard to collagen synthesis and cross-linking as well as bone mineralization, it must have a base-secreting mechanism. During the process of hydroxyapatite formation, the main constituent of bone mineral, excess H^+ ions are generated and released into the bone extracellular fluid (BEF) (Parfitt and Kleerekoper, 1980). These protons must be either removed or neutral-

ized for the process of mineralization to continue and for collagen "maturation" to take place (Harris et al., 1974; Samachson, 1969). Obviously, a Cl^-/HCO_3^- exchanger that extrudes bicarbonate out of the cell into the BEF, could fulfil this task.

We report here the presence of an anion exchanger in UMR-106 cells and analyze the dependencies of the net Cl^- and HCO_3^- (OH^-) fluxes and Cl^- self exchange on intracellular pH. In view of recently available information regarding the effect of calciotropic hormones on free cytosolic Ca^+ in osteoblasts (Lieberherr, 1987; Yamaguchi et al., 1987*a*, 1988) we further investigated the effect of a rise in intracellular Ca^{2+} on the activity of the anion exchanger in the UMR-106 cells. Our results show that intracellular pH regulates the activity of the Cl^-/HCO_3^- exchanger and that a rise in free cytosolic Ca^{2+} stimulates the exchanger by reducing the concentration of base equivalent required for exchanger activation.

MATERIALS AND METHODS

Reagents

Nigericin, monensin, DIDS and 12-*O*-tetradecanoylphorbol-13 acetate (TPA) were purchased from Sigma Chemical Co., St. Louis, MO. 2',7'bis(carboxyethyl)-5(6)carboxyfluorescein (BCECF), the acetoxymethylester (AM) of fura 2 (fura 2/AM) and H_2DIDS were obtained from Molecular Probes Inc. (Eugene, OR). Amiloride was from Merck, Sharp and Dohme, West Point, PA. $Na^{36}Cl$ was purchased from Amersham Corp., Arlington Heights, IL.

The UMR-106 cell line was a generous gift of Dr. T. J. Martin, University of Melbourne, Melbourne, Australia to Dr. T. J. Hahn, The Veterans Administration Medical Center, West Los Angeles, Los Angeles, CA, who in turn generously supplied us with these cells.

Solutions

The following solutions were used during the experiments. Solution A (NaCl) contained the following (in millimolar): 140 NaCl, 1 $MgCl_2$, 4 KCl, 10 HEPES/Tris, 5 glucose. Solution B (KCl) and C (tetramethylammonium [TMA]-Cl) were prepared by isoosmotic replacement of NaCl by KCl or TMA-Cl, respectively, but were otherwise identical. Solution D (Na^+-gluconate) contained: 140 Na^+-gluconate, 1 $MgSO_4$, 4 K^+-gluconate, 10 HEPES/Tris, 5 glucose. In solution E (K^+-gluconate), K^+-gluconate replaced Na^+-gluconate with all other constituents remaining the same. The pH of each solution was adjusted with Tris base to that pH specified for each experiment.

Culture Conditions

UMR-106 cells were used between passages 10–12 and subpassages 3–14. Cells were seeded at a density of 2.5×10^4 cells/cm^2 in 75 cm^2 area flasks and grown at 37°C in a humidified 95% air 5% CO_2 atmosphere in Ham's F12: Dulbecco's modified Eagle's media (1:1) supplemented with 14.3 mM $NaHCO_3$, 1.2 mM L-glutamine, 7% fetal bovine serum, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. The cells reached confluence within 5–6 d in culture and were used on day 6–8 of growth. For the ^{36}Cl flux studies, cells were seeded at a density of 2.5×10^4 cells/cm^2 in 12-well disposable trays under the same conditions.

Measurement of Cytosolic pH

pH_i was measured fluorimetrically using the pH-sensitive fluorescent dye BCECF, essentially as described previously (Green et al., 1988*a*). Briefly, cells were released from tissue culture

flasks by trypsin/EDTA treatment and were incubated at 37°C for 10 min with 2 μM BCECF/AM. Cells were then collected by centrifugation, washed, and resuspended in solution A containing 1 mM $CaCl_2$ and 0.1% bovine serum albumin. Fluorescence was monitored with excitation at 500 nm and emission at 530 nm. Calibration of fluorescence signals to determine pH_i was performed as previously described (Green et al., 1988*a*).

Determination of $[Ca^{2+}]_i$

Measurements of free cytosolic calcium concentration ($[Ca^{2+}]_i$) were made by incorporating the calcium-sensitive fluorescent probe, fura 2, into UMR-106 cells. Cells were collected from tissue culture plates and washed as described for measurements of pH_i. The cells were incubated with 2 μM fura 2/AM in a shaking water bath at 37°C for 30 min. The cells were then washed and resuspended in the same medium. Fluorescence was measured in a Perkin-Elmer 650-40 spectrophotometer (Norwalk, CT) at excitation and emission wavelengths of 340 and 500 nm, respectively, with slits of 3 and 12 nm, respectively.

When desired, the cells were loaded with both dyes, fura-2 and BCECF, by an initial incubation with 5 μM fura 2/AM for 20 min at 37°C. Then 2 μM BCECF/AM was added and the incubation at 37°C continued for an additional 10 min. The cells were then washed and BCECF and fura 2 fluorescence was measured as described above. pH_i and $[Ca^{2+}]_i$ measurements of cells loaded with both dyes were performed with two separate cell samples. Calibration of the fura 2 signal was performed as previously described (Yamaguchi et al., 1987*a*). Briefly, medium Ca^{2+} was adjusted to 2 mM and the cells were lysed with digitonin (50 μg/ml) to obtain the maximal fluorescence. Next, 10 mM EGTA and sufficient NaOH to elevate the pH to 8.5 were added to obtain the minimum fluorescence. The dissociation constant for Ca^{2+}-fura 2 was assumed to be 220 nM and the calculation of $[Ca^{2+}]_i$ was similar to that previously described (Grynkiewicz et al., 1985).

Modification of Intracellular pH

Cells loaded with BCECF were washed twice and resuspended in 2 ml of solution B or E (KCl or K^+-gluconate). The pH of the medium was adjusted to the level desired in the cytosol and the cells were incubated with 0.5 μM nigericine and 1 μM monensin for 5 min at 37°C. The cells were then washed twice and resuspended in the same appropriate solution containing 10 mg/ml bovine serum albumin to scavenge the ionophores. The cells were kept at room temperature until used.

Loading the Cells with Base Equivalents

In some experiments, alkalinization of the cytosol and depletion of intracellular Cl^- were achieved by washing the BCECF-loaded cells twice and suspending them in solution D or E containing 4 mM HCO_3^- (Na^+-gluconate or K^+-gluconate). These cells were used to compare Cl^-/HCO_3^- exchange of ionophore-treated and -untreated cells and to study the dependency of Cl^-/HCO_3^- exchanger on extracellular Cl^- concentration.

Measurements of Intracellular K^+ Concentration

Cells were incubated for 10 min in either solution A or solution A containing 2 μM TPA. The solutions also contained 1.5 mM $CaCl_2$ and [^{14}C] inulin. At the end of the experiment wet weight was determined after collecting the cells by 10 s of centrifugation in an Eppendorf centrifuge. The pellets were dried by vacuum and the dry weight was determined. The pellets were dissolved in 1 ml of 1 M HNO_3. Samples were used to determine the [^{14}C]inulin content and K content by flame photometry. After correction for extracellularly trapped K^+, intracellular K^+ concentration was calculated as nmol/liter cell water.

Measurement of Intracellular Cl^- Concentration

Cells were suspended and incubated for 10 min in three different solutions: solution A, solution A containing 1.5 mM $CaCl_2$ to which 2 μM TPA was added, and solution B containing 1.5 mM $CaCl_2$. All solutions contained [^{14}C]inulin. Extracellular and cell water of the samples were determined as described above for determination of intracellular K^+. Cl^- content of the samples was measured by chloridometer and after subtracting external Cl^-, $[Cl^-]_i$ was calculated.

Measurement of $^{36}Cl^-$ Influx

To evaluate the effect of intracellular pH on Cl^-/Cl^- exchange, ^{36}Cl influx was measured after clamping of intracellular pH over a range of pH's from 6.5 to 7.8 under conditions where cells were not depleted of intracellular chloride. The experimental procedure entailed the following: UMR-106 cells in 12-well disposable trays were washed three times with solution A, pH 7.4. Then the cells were washed once and incubated in solution B (KCl) containing 0.5 μM nigericin and 1 μM monensin, for 5 min at 37°C. pH of the solution was adjusted to the final desired intracellular pH. During this incubation, some of the cells were incubated with 25 μM DIDS. The incubation solutions also contained 0.1 mM EGTA or 2 mM $CaCl_2$ as specified in the legend of each experiment. The cells were then transferred to isotonic K^+-gluconate buffer containing the same concentration of ionophores and 5 mM KCl, labeled with $^{36}Cl^-$. The pH of the uptake medium was adjusted to be the same as the intracellular pH, and the uptake media was with or without 25 μM DIDS. The time course of ^{36}Cl uptake in the presence and absence of DIDS was measured for each given value of intracellular pH. The uptake reaction was terminated by rapid wash with ice-cold K^+-gluconate solution containing 0.1 mM DIDS. The cells were then dissolved in 1 M NaOH, transferred to counting vials, and radioactivity was measured. The linear time course of ^{36}Cl influx, which was obtained from these studies for each individual pH_i, served as a basis for a complete study of the dependency of Cl^-/Cl^- exchange on intracellular pH.

RESULTS

Demonstration of Cl^-/HCO_3^- (OH^-) Exchange

To study whether an anion exchanger exists in UMR-106 cells, the effect of the Cl^- gradient across the plasma membrane on intracellular pH (pH_i) was measured. Dilution of cells suspended in solution A (NaCl) into Cl^--free medium (Na^+-gluconate) resulted in a rise of pH_i from a resting level of 7.05 ± 0.04 to 7.42 ± 0.07 within ~25 s (Fig. 1 *a*). Subsequently, pH_i was gradually reduced to a new steady-state level of 7.31 ± 0.07 within ~5 min. When the cells were preincubated for 5 min with 0.1 mM DIDS and then diluted into the Cl^--free buffer, cytosolic alkalinization was blunted (Fig. 1 *b*). 0.1 mM H_2DIDS was as effective as DIDS in inhibiting the pH_i changes recorded in Fig. 1 *a* (not shown). On the other hand, 0.25 mM amiloride had no effect of either the initial alkalinization or the subsequent acidification (Fig. 1 *c*). Preincubation of the cells with DIDS also inhibited the change in pH_i when amiloride was present in the medium (Fig. 1 *d*). The independence of cytosolic alkalinization on changes in membrane potential is demonstrated in Fig. 1, *e* and *f* where the cells were diluted into K^+-gluconate medium. Since the membrane potential in bone cells is close to the K^+ diffusion potential (Ferrier et al., 1987), suspending the cells in high K^+ medium leads to depolarization of the plasma membrane (see

below). It can be seen that the rate and extent of cytosolic alkalinization measured when the cells were added to K^+-gluconate medium, were not significantly different from those measured in Na^+-gluconate medium. In K^+-gluconate medium, cytosolic alkalinization is also DIDS and H_2DIDS inhibitable.

Fig. 2 depicts the effect of medium HCO_3^- concentration on the changes in pH_i. Dilution of the cells into HCO_3^- and Cl^--free medium resulted in a typical cytosolic alkalinization to pH_i of 7.3 and then acidification to pH_i of 7.22 (Fig. 2 *a*). Increasing HCO_3^- ion concentration in the ranges from 0 to 2 mM, gradually increased both the rate and extent of cytosolic alkalinization (Fig. 2, *a*, *c*, and *e*). Pretreatment of the cells with 0.1 mM DIDS largely inhibited cytosolic alkalinization also in the presence of HCO_3^- in the incubation medium (Fig. 2, *b*, *d*, and *f*). The effect of HCO_3^- on pH_i was identical in Na^+-gluconate or K^+-gluconate solutions, and it could not be blocked by amiloride (not shown). DIDS-sensitive cytosolic alkalinization in the virtual absence of HCO_3^- in the medium suggests that OH^- is also trans-

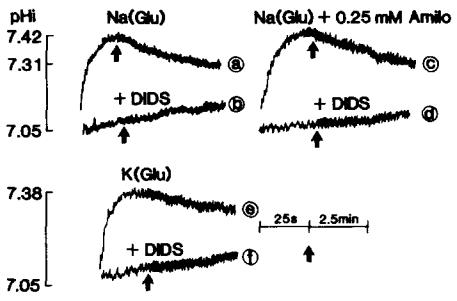

FIGURE 1. The presence of an anion exchanger in UMR-106 cells: effect of membrane potential. BCECF-loaded cells were added to 2 ml solution D, pH 7.4 (Na^+-gluconate) (*a*), 2 ml solution D containing 0.25 mM amiloride, pH 7.4 (*c*), or 2 ml solution E, pH 7.4 (K^+-gluconate) (*e*). Each set of experiments was repeated with cells preincubated with 0.1 mM DIDS before adding them to the indicated solutions (*b*, *d*, and *f*). Each experiment shown is representative of similar results obtained from three cell preparations. In each preparation two or three determinations were made.

ported by the exchanger. The increased rates of cytosolic alkalinization in the presence of HCO_3^- suggest that HCO_3^- is transported faster than OH^- in exchange for Cl^- by UMR-106 cells.

Fig. 3 shows that the changes in pH_i induced by the removal of Cl^- from the incubation medium are reversible. For these experiments, the cytosol was alkalinized and depleted of Cl^- and then the cells were exposed to Cl^--containing media. Direct measurement of Cl^- content (see Methods) shows that cells treated as described in Fig. 3 were completely depleted of Cl^-. In Fig. 3, *A* and *C*, loading with OH^- and depletion of Cl_i^- was achieved by suspending the cells in Cl^--free media to which 4 mM HCO_3^- was added. Then, the cells were collected by centrifugation and diluted into media of required composition. Fig. 3 *A* shows that when these cells were exposed to a medium containing 25 mM NaCl, the cytosol acidified. The rate and extent of the acidification was reduced by preincubation of the cells with DIDS and it was blunted when 4 mM HCO_3^- was present in the medium. After 4 min of

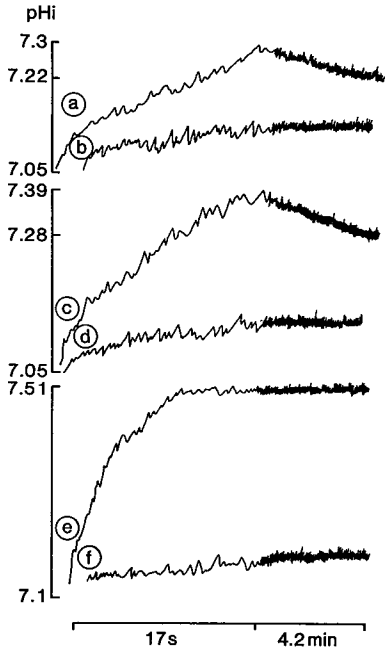

FIGURE 2. Effect of extracellular HCO_3^- on the rate and extent of cytosolic alkalinization. BCECF-loaded cells were added to 2 ml of solution D gassed with 100% O_2 (*a*). Cells were also added to 2 ml solution D (*c*), or solution D containing 2 mM HCO_3^- (*e*); both were equilibrated with air. HCO_3^- was added to the medium just before cell addition and medium pH was allowed to increase. Each experiment was repeated with a sample of cells that were preincubated with 0.1 mM DIDS (*b*, *d*, and *f*). Each experiment presented in the figure is one of three similar experiments.

incubation, the DIDS-sensitive acidification was 0.293 ± 0.03 (n = 3) pH units. The insensitivity of this mode of exchange to changes in membrane potential is shown in Fig. 3 *C*. Thus, the acidification pattern of cells added to a medium containing 25 mM KCl was not different from that observed in NaCl media. Cytosolic acidification as shown under the conditions of Fig. 3, *A* and *C* could result either from Cl^-/HCO_3^- exchange or Na^+/HCO_3^- cotransport, both of which are sensitive to DIDS

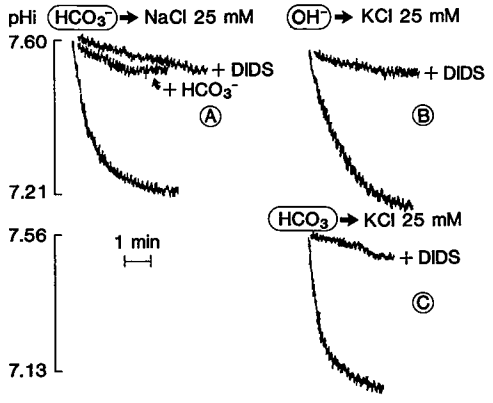

FIGURE 3. Cl_o^-/HCO_{3i}^- exchange in UMR-106 cells. BCECF-loaded cells were depleted of Cl^- and alkalinized as described under Methods. In *A* and *C*, cells were washed and suspended in Cl^--free media (solutions D and E, respectively) to which 4 mM HCO_3^- was added. In *B*, alkalinization was achieved by incubating the cells for 5 min at 37°C in solution E, pH 7.6, and ionophores. After this procedure, cells were added to 2 ml of a solution containing 25 mM NaCl (*A*) or 25 mM KCl (*B*, *C*) pH 7.2. Osmolality of the solutions was maintained by isosmotic replacement of Cl^- with gluconate. In *A*, experiments were performed in the presence or absence of 4 mM HCO_3^- in the medium. Each experiment was repeated with cells preincubated with 0.1 mM DIDS. Treatment with DIDS was initiated after cytosolic alkalinization was completed. Each experiment represents one out of three similar experiments.

and the HCO_3^- or OH^- gradients across the plasma membrane. To verify that most of the measured cytosolic acidification was due to Cl^-/HCO_3^- exchange, the cells were depleted of Na^+ and the initial pH_i was clamped at 7.6 by the ionophore technique (see Methods). Addition of these cells to a medium containing 25 mM KCl was followed by a DIDS-sensitive reduction of pH_i (Fig. 3 *B*) . Thus, the Cl^--dependent acidification shown in Fig. 3, was not due to a Na^+-dependent process. Further, this experiment shows that the ionophores were effectively extracted from the plasma membrane and ionophore-treated cells can be used to study the properties of HCO_3^- or OH^- transport.

The results presented in Figs. 1–3 demonstrate the presence of a reversible, Cl^--dependent, DIDS-sensitive, Na^+- and membrane potential–independent HCO_3^- (or OH^-) transport system in UMR-106 cells. These properties strongly suggest that we measured the activity of a Cl^-/HCO_3^- exchanger in these cells.

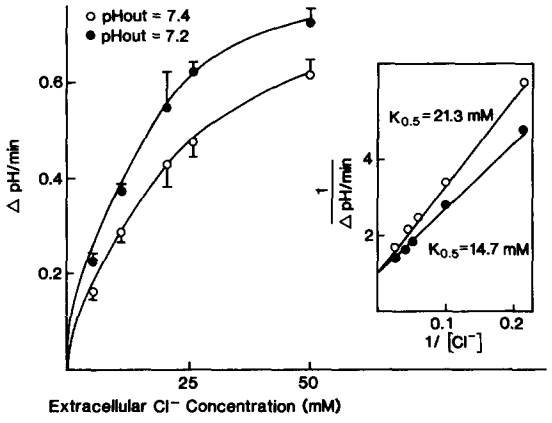

FIGURE 4. The dependence of the anion exchanger on extracellular Cl^- concentration: effect of pH_o. BCECF-loaded cells were depleted of Cl^- and alkalinized as described under Methods. Then the cells were added to 2 ml of solutions containing different concentrations of Cl^-; Cl^- had been replaced by equimolar concentrations of gluconate. In the absence of medium Cl^- a slow rate of acidification was observed. This rate was similar to that observed with DIDS-treated cells suspended in a solution containing 50 mM Cl^-. The rate of DIDS-sensitive pH_i change was recorded at two external pH's and plotted against external Cl^- concentration (*open symbols*, pH_o 7.4; *closed symbols*, pH_o 7.2). A double reciprocal plot of the results (*inset*) was used to obtain the $K_{0.5}Cl^-$ at each pH_o. The figure shows the mean ± SD of three experiments.

Extracellular Site

The protocol of HCO_{3i}^-/Cl_o^- exchange measurement in Fig. 3 *B* was used to study the dependence of base efflux on extracellular Cl^- (Fig. 4). Increasing the medium Cl^- concentration was followed by increased rates of DIDS-sensitive acidification. The dependency of the acidification rate on medium Cl^- concentration shows that the process was saturable and followed simple saturation kinetics. Further, raising the medium's pH from 7.2 to 7.4 decreased the apparent affinity of the exchanger for Cl_o^- without changing the overall V_{max}. A double reciprocal plot of the data (Fig. 4, *inset*) shows an apparent affinity for Cl^- ($K_{0.5}Cl^-$) of 14.7 mM at pH_o 7.2 and $K_{0.5}Cl^-$ of 21.3 mM at pH_o 7.4. Thus, there appears to be a competition between extracellular Cl^- and HCO_3^-, (or OH^-) for interaction with the extracellular face of the exchanger.

Dependency of the Exchange on pH_i

The effect of pH_i on net anion exchange was studied in cells depleted of Cl^- and Na^+ and whose pH_i was clamped at different levels by ionophore treatment. The rates of DIDS-sensitive acidification were measured by the addition of cells to Cl^--containing medium at pH 7.2. Since the concentration of internal substrate varies along with pH_i changes, acidification rates were determined from the slope of the tangent to the initial curves. Fig. 5 shows that increasing pH_i increased the rates of cytosolic acidification. However, the pattern of the pH_i dependence did not conform to a simple saturable behavior. Thus, a steep increase in acidification rates was observed when pH_i was varied between 7 and 7.4, with a fourfold increase in the rate of acidification over this range of pH_i. A Hill analysis of the data yielded a Hill coefficient of $n = 1.76$. The implication of these findings for regulation of the anion exchanger by pH_i will be discussed below.

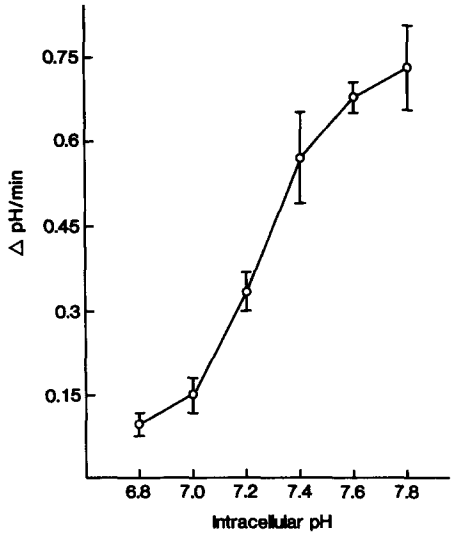

FIGURE 5. The dependency of net exchange on pH_i. BCECF-loaded cells were washed and incubated with K^+-gluconate solutions containing 0.1 mM EGTA, 0.5 μM nigericin, and 1 μM monensin for 5 min at 37°C. The pH of the solutions ranged from 6.8 to 7.8. The cells were then washed twice with similar solutions also containing 10 mg/ml albumin. Exchange measurements were initiated by dilution of cells into 2 ml solution B (KCl), pH 7.2, containing 0.1 mM EGTA with or without 0.1 mM DIDS. The rates of DIDS-sensitive pH_i changes were estimated from the change in fluorescence and plotted against pH_i. The figure shows the mean ± SD of three experiments.

Effect of Cytosolic Ca^{2+} on Anion Exchange Activity

To study further the mechanisms that regulate the activity of the exchanger we investigated the effect of a rise in $[Ca^{2+}]_i$ and stimulation of protein kinase C on the activity of the anion exchanger in UMR-106 cells. To study the temporal relationship between changes in $[Ca^{2+}]_i$ and pH_i, UMR-106 were loaded with both fura 2 and BCECF. In preliminary experiments we found that the fluorescence changes recorded from cells loaded with either dye alone were similar to those recorded from cells loaded with both dyes. It was necessary to load the cells with both dyes so that the relationship between changes in $[Ca^{2+}]_i$ and pH_i in the same cell preparation could be explored. Fig. 6 shows the effect of the phorbol ester, TPA, on $[Ca^{2+}]_i$ and pH_i. Exposure of the cells to 2 μM TPA in the presence of Ca^{2+} in the media leads to a rise in $[Ca^{2+}]_i$ from 112 to 436 nM within 45 s (Fig. 6 *a*). The cells were

able to partially reduce $[Ca^{2+}]_i$ to ~202 nM but not to the prestimulated level. This effect of TPA on $[Ca^{2+}]_i$ was due to stimulation of a protein kinase C-activated Ca^{2+} channel in UMR-106 cells (Yamaguchi et al., 1987*b*). Stimulation of the cells with TPA also induced a biphasic change in pH_i. After a lag period of ~30 (31 ± 6) s, the cytosol acidified from pH_i 7.15 to 6.86 over a period of 3 (3.41 ± 0.22) min (Fig. 6 *b*). The acidification was followed by a slow increase in pH_i so that within 15 min, near normal pH_i was restored. When calcium was removed from the incubation media and in the presence of 0.1 mM EGTA, neither a rise in $[Ca^{2+}]_i$ (Fig. 6 *c*) nor acidification of the cytosol (Fig. 6 *d*) was observed. Thus, the effect of TPA on pH_i appears to be secondary to the ability of TPA to raise $[Ca^{2+}]_i$.

To test whether a $[Ca^{2+}]_i$ increase is sufficient to trigger the changes in pH_i, we measured the effect of the Ca^{2+} ionophore, ionomycin, on $[Ca^{2+}]_i$ and pH_i. Fig. 7 shows that after the addition of 1 μM ionomycin to cells suspended in Ca^{2+}-contain-

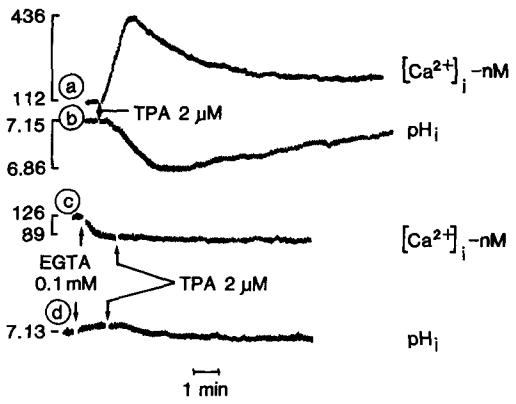

FIGURE 6. Effect of cell stimulation with TPA on $[Ca^{2+}]_i$ and pH_i. Cells were loaded with both fura-2 and BCECF as described under Methods. The cells were then added to 2 ml of solution A (NaCl) containing 1.5 mM Ca^{2+}. Then 2 μM TPA was added and the change of either $[Ca^{2+}]_i$ (*a*) or pH_i (*b*) was estimated from the change in fluorescence after appropriate calibrations. The same experiments were repeated in calcium-free solution A containing 0.1 mM EGTA and the changes in $[Ca^{2+}]_i$ (*c*) and pH_i (*d*) were measured. Separate samples of cells were used to measure $[Ca^{2+}]_i$ and pH_i. Each experiment represents one of eight experiments with similar results.

ing medium, there is an initial increase in $[Ca^{2+}]_i$ to 1,104 nM, after which the cells reduced $[Ca^{2+}]_i$ to ~ 356 nM (Fig. 7 *a*). $[Ca^{2+}]_i$ was maintained at this level for the duration of the experiment. The same concentration of ionomycin produced changes in pH_i similar to those induced by TPA stimulation (Fig. 7 *b*). Hence, after a lag period of ~45 s, pH_i was reduced from 7.2 to 6.92 over the first 3 min and then it gradually increased to 7.2 over the subsequent 10 min of the incubation with ionomycin. In Fig. 7, *c* and *d* the effect of a short but a large increase of $[Ca^{2+}]_i$ on pH_i was measured. Addition of 1 μM ionomycin to cells suspended in Ca^{2+}-free medium containing 0.1 mM EGTA increased $[Ca^{2+}]_i$ to 994 nM and then the cells reduced $[Ca^{2+}]_i$ to below resting level within 1.5 min (Fig. 7 *c*). Such a short-lived increase in $[Ca^{2+}]_i$ was not sufficient to trigger a change in pH_i (Fig. 7 *d*). This indicates that the elevated $[Ca^{2+}]_i$ must be maintained for the activation of cytosolic acidification. Conversely, when $[Ca^{2+}]_i$ was increased and maintained at ~2.5 μM,

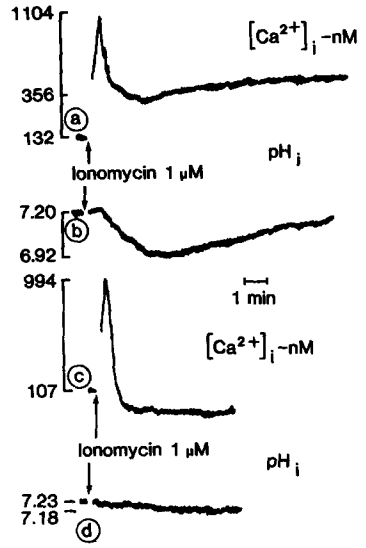

FIGURE 7. Effect of Ca^{2+} ionophore on $[Ca^{2+}]_i$ and pH_i. Cells loaded with fura 2 and BCECF were added to 2 ml of albumin-free solution A containing 1.5 mM Ca^{2+} (*a*, *b*) or 0.1 mM EGTA (*c*, *d*). Then, 1 μM ionomycin was added and the changes in $[Ca^{2+}]_i$ (a, c) and pH_i (b, d) were recorded. Separate samples of cells were used to measure $[Ca^{2+}]_i$ and pH_i. Each experiment shown is one of three similar experiments.

the changes in pH_i were blocked (not shown). It was necessary to adjust ionomycin and medium Ca^{2+} concentration to produce a Ca^{2+} signal similar to that induced by TPA in order to allow the changes in pH_i to occur.

To analyze the nature of the initial, Ca^{2+}-dependent acidification, the effect of medium Cl^- and H_2DIDS on the TPA-stimulated changes in $[Ca^{2+}]_i$ and pH_i was measured. Fig. 8 *a* shows that incubating the cells in Cl^--free medium (Na^+-gluconate) did not considerably change resting $[Ca^{2+}]_i$. Further, stimulation with TPA

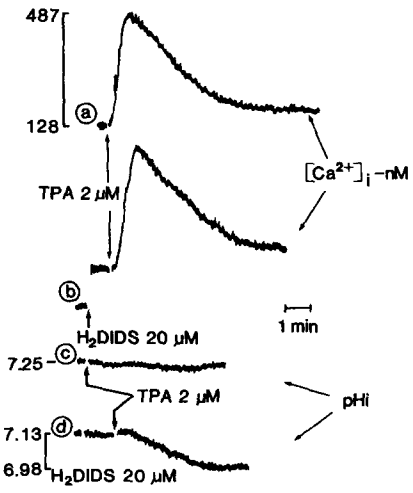

FIGURE 8. Effect of Cl^--free media and H_2DIDS on $[Ca^{2+}]_i$ and pH_i. Cells loaded with fura 2 and BCECF were washed and resuspended in solution D (Na^+-gluconate). After a 20-min incubation at room temperature in solution D, the cells were washed once and added to 2 ml of solution D containing 1.5 mM Ca^{2+} (*a*, *c*). Where indicated, the cells were stimulated with 2 μM TPA. The change in fluorescence was measured and calibrated to estimate $[Ca^{2+}]_i$ (*a*) and pH_i (*c*). Cells suspended in solution A (NaCl) were also added to 2 ml of solution A containing 1.5 mM Ca^{2+} and exposed to 20 μM H_2DIDS (*b*, *d*). Where indicated the cells were stimulated with 2 μM TPA. The experiments shown represent one of four others with Na^+-gluconate and one of three others with H_2DIDS.

resulted in a change in $[Ca^{2+}]_i$ similar to that observed with cells suspended in Cl^--containing medium. On the other hand, the changes in pH_i were largely blocked (Fig. 8 *c*). Likewise, when cells preincubated with 20 μM H_2DIDS were stimulated with TPA, $[Ca^{2+}]_i$ increased (Fig. 8 *b*) but the change in pH_i was blunted (Fig. 8 *d*). In these experiments 20 μM H_2DIDS was used to minimize the interference of H_2DIDS with the fura 2 signal. Table I summarizes the effect of H_2DIDS and Cl^- removal of TPA-induced cytosolic acidification. The effect of H_2DIDS was dose dependent where 25 and 250 μM H_2DIDS inhibited the TPA-induced acidification by ~48% and 70%, respectively. In four experiments performed in the absence of Cl^- there was little or no change in pH_i after stimulation with TPA. In addition, when TPA-mediated acidification was blocked we did not observe any cytosolic alkalinization despite stimulation of PKC and the $[Ca^{2+}]_i$ increase.

The experiments in Figs. 6 and 8 and Table I indicate that (*a*) the cytosolic acidification was due to a Cl^--dependent, H_2DIDS-sensitive mechanism, (*b*) the second-

TABLE I

Effect of H_2DIDS and Removal of Cl^- on TPA-induced pH_i Changes

Conditions	Additions	TPA-mediated acidification ΔpH/4 min
NaCl	—	0.274 ± 0.016 (11)*
NaCl	25 μM H_2DIDS	0.170 ± 0.021 (3)
NaCl	250 μM H_2DIDS	0.086 ± 0.022 (3)
Na^+-gluconate	—	0.007 ± 0.003 (4)
NaCl	Amiloride	0.502 ± 0.072 (6)
NaCl	Amiloride + 25 μM H_2DIDS	0.235 ± 0.028 (4)
NaCl	Amiloride + 250 μM H_2DIDS	0.093 ± 0.015 (3)

pH_i changes at the different conditions were measured as described in the legends of Figs. 8 (without amiloride) and 10 (with amiloride), respectively. When present, amiloride concentration was 0.2 mM. The table shows the mean ± SD for the indicated number of experiments. In all experiments inhibition by H_2DIDS and Cl^- removal was highly significant ($P < 0.01$ or better).

*The number in parentheses equals *n*.

ary recovery from acidification was dependent on the initial acidification and could not be triggered by either an increase in $[Ca^{2+}]_i$ or stimulation of protein kinase C.

Relationship between Cl^-/HCO_3^- and Na^+/H^+ Exchange

To evaluate the role of the Na^+/H^+ exchanger in the second-phase increase in pH_i seen upon stimulation with TPA, we studied the effect of Na^+-free medium and the Na^+/H^+ exchange inhibitor amiloride on the changes of pH_i. In Fig. 9, *a–c*, cells that had been washed twice with Na^+-free medium (TMA-Cl) were added to TMA-Cl medium containing 0.1 mM EGTA. Then 2.1 mM $CaCl_2$ was added to the medium, and the cells were stimulated with TPA. The protocol of washing the cells with Na^+- and Ca^{2+}-free medium with subsequent addition of $CaCl_2$ was selected to prevent $[Ca^{2+}]_i$ increase due to Na^+/Ca^{2+} exchange when cells are suspended in TMA-Cl medium. The $[Ca^{2+}]_i$ signal (Fig. 9 *a*) was comparable to that observed in NaCl

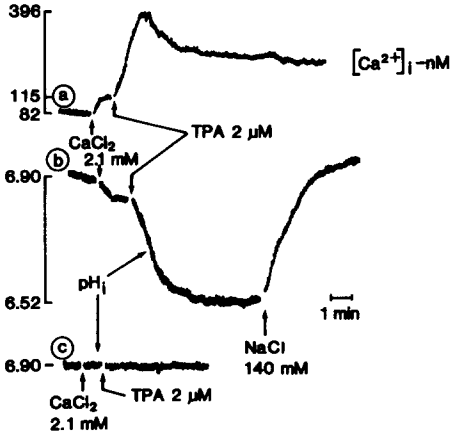

FIGURE 9. Effect of Na^+-free media on $[Ca^{2+}]_i$-induced pH_i changes. Cells loaded with fura 2 and BCECF were washed twice with solution C (TMA-Cl) containing 0.1 mM EGTA and then added to 2 ml of the same solution. Where indicated 2.1 mM $CaCl_2$ was added to the medium. Then the cells were stimulated with 2 μM TPA, and the changes in $[Ca^{2+}]_i$ (*a*) and pH_i (*b*) were measured. The effect of addition of 140 mM NaCl to the medium on pH_i was also measured (*b*). The experiment was repeated with cells washed as above except that the cells were preincubated with 0.1 mM DIDS for 5 min. Then the cells were added to 2 ml TMA-Cl medium containing 2.1 mM $CaCl_2$, TPA was added, and changes in pH_i were estimated (*c*). Each experiment in (*a, b*) represents one of six others while the experiment in (*c*) represents one of three similar experiments.

medium. Under these conditions, initial pH_i was lower than that of cells maintained in NaCl containing medium probably because during the washing with TMA-Cl the cytosol acidified because of Na^+/H^+ exchange (Fig. 9 *b*). However, after the administration of TPA, both the rate and extent of acidification were higher as compared with that measured in NaCl media (Fig. 9 *b*). In addition, in the absence of Na^+ in the medium, the recovery of pH_i was completely blocked and could be restored upon addition of 140 mM NaCl to the medium. Finally, cytosolic acidification was inhibited when cells were preincubated with 0.1 mM DIDS (Fig. 9 *c*).

The effect of amiloride on cytosolic acidification and subsequent recovery of pH_i is shown in Fig. 10 and is summarized in Table I. Cells were suspended in NaCl medium containing 2 mM $CaCl_2$ and then exposed to 0.2 mM amiloride. Stimulation by 2 μM TPA resulted in enhanced acidification and complete inhibition of the recovery phase (Fig. 10 *a*). In the presence of 0.2 mM amiloride in the incubation medium, stimulation with 2 μM TPA for 4 min resulted in acidification by 0.502 pH

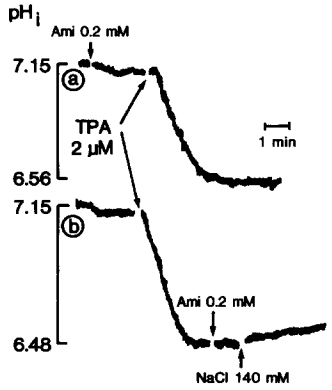

FIGURE 10. Effect of amiloride on $[Ca^{2+}]_i$-induced changes in pH_i. Cells loaded with BCECF were added to 2 ml of solution A (NaCl) (*a*) or solution C (TMA-Cl) (*b*). The solutions contained 1.5 mM Ca^{2+}. Where indicated, 0.2 mM amiloride, 2 μM TPA, or 140 mM NaCl were added to the medium. Each experiment is one of four or six similar experiments.

units. This acidification was inhibited by H_2DIDS in a dose-dependent manner similar to the inhibition of the acidification measured in the absence of amiloride (Table I). Dilution of the cells into Na^+-free medium (TMA-Cl) again resulted in augmented acidification after exposure to TPA. The recovery from acidification that was observed upon readdition of NaCl (as in Fig. 9 *b*) could be prevented by prior addition of 0.2 mM amiloride (Fig. 10 *b*). It appears, therefore, that the recovery from acidification that follows the initial acidification results from the activity of the Na^+/H^+ exchanger which is activated by the reduced pH_i.

Fig. 11 combines evidence for the importance of $[Ca^{2+}]_i$ in inducing cytosolic acidification, the effect of membrane potential on this process, and the role of Na^+/H^+ exchanger in the second phase increase in pH_i. In Fig. 11 *a*, cells were added to KCl medium in the presence of 2 mM Ca^{2+}. $[Ca^{2+}]_i$ rose from a resting level of 92 nM to 466 nM, which was due to Ca^{2+} influx through the depolarization-activated calcium channel present in these cells (Yamaguchi et al., 1989). The rise in $[Ca^{2+}]_i$ was followed by cytosolic acidification and inhibition of the secondary recovery from

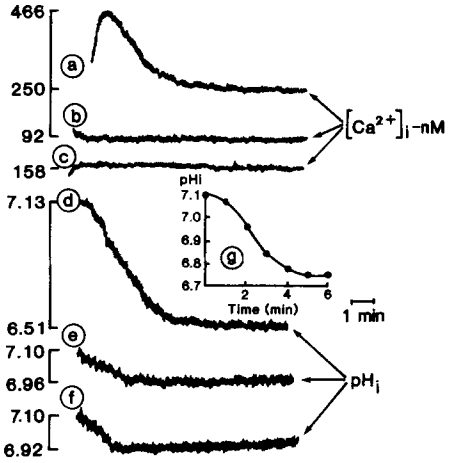

FIGURE 11. Effect of membrane depolarization on $[Ca^{2+}]_i$ and pH_i. Cells loaded with fura 2 and BCECF were added to 2 ml of solution B (KCl) containing 2 mM $CaCl_2$ (*a*, *d*), 0.1 mM EGTA (*b*, *e*) or 2 mM $CaCl_2$ and 25 μM verapamil (*c*, *f*). To obtain the acidification component due to Cl^-/HCO_3^- exchange (see text) the pH_i changes in *e* were subtracted from those shown in *d* (*g*). The experiments in *a*, *b*, *d*, and *e* represent one of seven and the experiments in *c* and *f* are one of two similar experiments.

acidification (Fig. 11 *d*). The pattern of pH_i change was similar to that observed when cells were added to TMA-Cl medium or during incubation with amiloride although the rate of acidification was slower. Hence, the Ca^{2+}-activated cytosolic acidification could be observed when the cells were depolarized. Also it is clearly shown in the figure that removal of Ca^{2+} from the medium (Fig. 11, *b* and *e*) or addition of the calcium channel blocker, verapamil (Fig. 11, *c* and *f*) inhibited both the $[Ca^{2+}]_i$ rise and cytosolic acidification. The protocol in Fig. 11 involves dilution of the cells into Na^+-free medium, which should result in some acidification due to Na_i^+/H_o^+ exchange. Indeed, the acidification shown in Fig. 11, *e* and *f* can be blocked by amiloride.

An attempt to directly evaluate the contribution of Na^+/H^+ exchange to cytosolic acidification shown in Fig. 11 *d* was unsuccessful since amiloride at 0.2 mM almost completely blocked the Ca^{2+} signal shown in Fig. 11 *a* and thus the acidification. However, acidification due to Cl^-/HCO_3^- exchange can be estimated from subtracting the acidification in the absence of $[Ca^{2+}]_i$ rise (Fig. 11 *e* and *f*) from the acidifi-

cation in the presence of $[Ca^{2+}]_i$ increase (Fig. 11 *d*). Fig. 11 *g* shows the results of such calculation. It can be seen that in the presence of high extracellular KCl concentration the rate and extent of Cl^-/HCO_3^- exchange is lower compared with that measured in TMA-Cl medium or medium containing amiloride (Fig. 10).

To determine the cause of the reduced rate of Cl^-/HCO_3^- exchange in KCl medium and the almost constant rate and prolonged cytosolic acidification in TPA-stimulated cells when added to TMA-Cl medium, the effect of TPA and cell depolarization on intracellular ionic content was determined. Table II shows that resting UMR-106 cells suspended in solution A contain ~32 mM Cl^- and 142 mM K^+. When stimulated with TPA for 10 min the cells did not gain any Cl^-. This was despite the activation of Cl^-/HCO_3^- exchange. From the acidification patterns shown in Figs. 9 and 10 (0.502 ± 0.072 pH units) and the buffer capacity of the cells (Green et al., 1988*a*) it was expected that the cells would gain ~43 mM Cl^-. The possible explanation for this discrepancy is suggested by the measurements of K^+ content of TPA-stimulated cells. While gaining no Cl^-, the cells lost ~49 mM K^+ (Table II). Therefore, it appears that Cl^- incorporated into the cells due to Cl^-/HCO_3^- exchange was extruded by a mechanism dependent on K_i^+. Such a mechanism

TABLE II

Effect of TPA and Cell Depolarization on Intracellular Ion Concentrations

Conditions	Intracellular ion concentration	
	Cl^-	K^+
	mM	*mM*
Control	31.83 ± 2.18	142 ± 7.9
2 μM TPA, 10 min	25.67 ± 1.67	93 ± 4.8
140 mM KCl, 10 min	52.34 ± 3.90	ND

Cl^- and K^+ content of cells incubated for 10 min at 37°C at the indicated experimental conditions were determined as detailed in Methods. The table shows the mean ± SD of three experiments. ND, not determined.

will prevent accumulation of Cl^- in the cytosol and allow continuous Cl^-/HCO_3^- exchange. In the presence of high extracellular KCl concentration, K^+ and Cl^- efflux are impaired and Cl^- slowly accumulates in the cytosol (Table II).

Effect of $[Ca^{2+}]_i$ on the Kinetic Properties of Cl^-/HCO_3^- Exchange

To explore some aspects of the mechanism by which Ca^{2+} ions activate the anion exchanger, we studied the effect of $[Ca^{2+}]_i$ on the apparent affinities of the extracellular and intracellular sites for the anions and on the overall maximal rate of exchange. Fig. 12 shows the measurement of $K_{0.5}$ for extracellular Cl^- at two $[Ca^{2+}]_i$. For these experiments the cytosol was alkalinized to pH 7.6 before the initiation of exchange. At this pH_i, the intracellular site is nearly saturated with base equivalent (see Fig. 5), so that an effect of $[Ca^{2+}]_i$ on $K_{0.5}Cl_o^-$ can be separated from an effect of $[Ca^{2+}]_i$ on pH_i dependency of the exchanger. Under the conditions of the experiments shown, $[Ca^{2+}]_i$ averaged 82 ± 11 nM (n = 5) in the presence of 0.1 mM EGTA and 257 ± 17 nM (n = 5) in the presence of 2 mM $CaCl_2$ in the medium. As shown in Fig. 12, at pH_o of 7.2, the same $K_{0.5}Cl_o^-$ was measured at these two

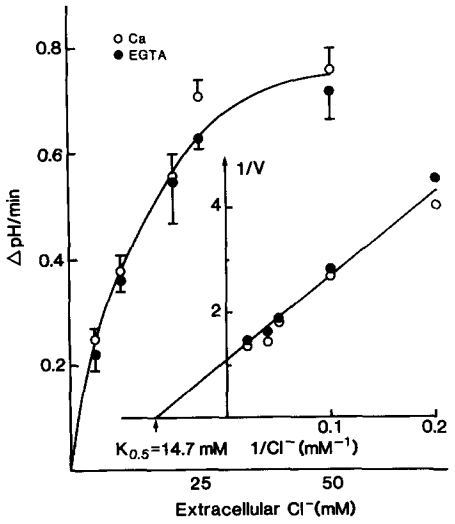

FIGURE 12. The dependency of anion exchange on extracellular Cl^- concentration at two $[Ca^{2+}]_i$. BCECF-loaded cells were depleted of intracellular Cl^- and alkalinized as described under Methods. The cells were then added to 2 ml of solutions containing different concentrations of Cl^- at pH 7.2. K^+-gluconate was replaced with equimolar concentrations of KCl. The solution contained either 1.5 mM Ca^{2+} (*open symbols*) or 0.1 mM EGTA (*closed symbols*). The rates of DIDS-sensitive pH_i changes were recorded and plotted against external Cl^- concentrations. A double reciprocal plot of the data is shown in the inset. The figure shows the mean ± SD of five experiments.

$[Ca^{2+}]_i$. The same results were obtained when similar experiments were repeated at pH_o of 7.4 (not shown). Since extracellular Cl^- and HCO_3^- (OH^-) appear to compete for interaction with the extracellular face of the exchanger (Fig. 4), it is likely that changes in $[Ca^{2+}]_i$ do not affect the external $K_{0.5}$ for base equivalents as well.

The effect of $[Ca^{2+}]_i$ on the pH_i dependency of net anion exchange is shown in Fig. 13. In one set of experiments, cells were loaded with BCECF and fura-2 and then pH_i was clamped in the range of 6.8–7.6 by treatment with ionophores in high K^+-gluconate medium. When the cells were suspended in Cl^--free or Cl^--containing medium also containing 0.1 mM EGTA, $[Ca^{2+}]_i$ averaged 67 ± 14 nM (n = 3) and

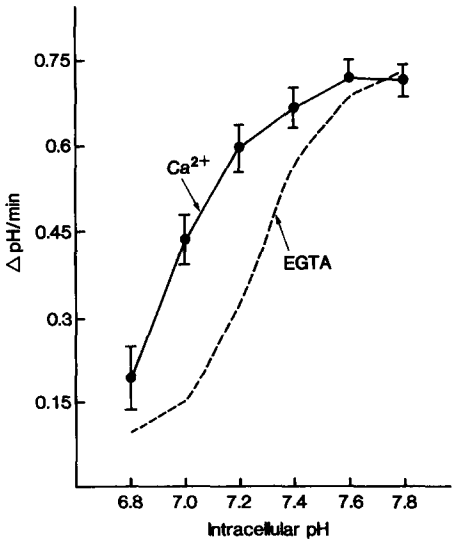

FIGURE 13. pH_i dependency of net anion exchange at two $[Ca^{2+}]_i$. pH_i of BCECF-loaded cells was clamped over the pH range of 6.8–7.8 as described under Methods and in the legend to Fig. 5. The cells were then added to 2 ml of KCl solution pH 7.2, which contained 1.5 mM Ca^{2+} (*closed symbols*). The rates of DIDS-sensitive pH_i changes were estimated from the change in fluorescence and plotted against pH_i. For comparison, the pH_i dependency measured in the absence of Ca^{2+} and in the presence of EGTA (see Fig. 5) is shown by the broken line. The figure shows the mean ± SD of three experiments.

was stable for the duration of net anion flux measurements. Suspending the cells in medium containing high K^+ and 2 mM $CaCl_2$ resulted in a rapid $[Ca^{2+}]_i$ rise to a peak of ~279 ± 21 nM (n = 3). $[Ca^{2+}]_i$ remained at this level for ~1.5 min. The indicated levels of $[Ca^{2+}]_i$ were not significantly affected by changes in pH_i over the range tested. This is in keeping with studies in excitable cells showing that the L-type Ca^{2+} channel is insensitive to pH_i in the range 6.0–9.0 (Iijima et al., 1986; Prod'hom et al., 1987). Thus, the rates of DIDS-sensitive pH_i changes were determined from the changes in pH_i during the first 1.5 min. Fig. 13 shows that an increase in $[Ca^{2+}]_i$ from 67 to 279 nM resulted in a clear shift in the pH_i dependency of HCO_3^-/Cl_o^- exchange. Half-maximal rate of exchange was measured at pH_i ~7.24 at $[Ca^{2+}]_i$ of

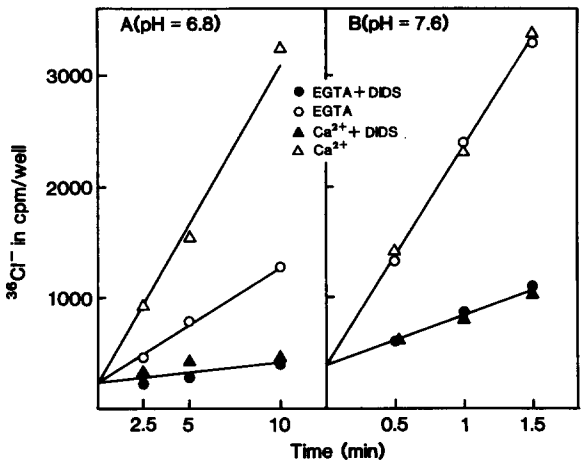

FIGURE 14. Measurements of Cl^- self exchange at two different pH_i. UMR-106 cells grown in 12-well disposable trays, were washed three times with solution A (NaCl), pH 7.4. Then the cells were incubated in solution B (KCl) containing 0.5 μM nigericin and 1 μM monensin for 5 min at 37°C. The pH of the solutions were adjusted to 6.8 or 7.6. During the incubation, samples of cells were also treated with 25 μM DIDS. At the end of this incubation, the medium was removed, and the cells were bathed in isotonic K^+-gluconate buffer containing the ionophores, 5 mM KCl labeled with $^{36}Cl^-$ and with or without DIDS. The pH of the uptake medium was adjusted to be the same as pH_i. At the times indicated in the figure, the reaction was terminated by rapid washing with ice-cold K^+-gluconate solution containing 0.1 mM DIDS. The cells were then dissolved in 1 M NaOH and transferred to counting vials for measurement of radioactivity. Washing and incubation of the cells were either in the presence of 1.5 mM Ca^{2+} in the media or in the absence of added calcium and in the presence of 0.1 mM EGTA. Similar time courses were performed for each pH in the range of 6.5–7.8.

67 nM and at pH_i 6.95 at $[Ca^{2+}]_i$ of 279 nM. The increase in $[Ca^{2+}]_i$ had no effect on the overall maximal rate of exchange. A Hill analysis of the results at high $[Ca^{2+}]_i$ yielded a Hill coefficient of 1.81 which was not significantly different from that measured at low $[Ca^{2+}]_i$ (n = 1.76).

Effect of $[Ca^{2+}]_i$ on Cl^-/Cl^- Exchange

The results presented thus far suggest that intracellular HCO_3^- and OH^- can activate the Cl^-/HCO_3^- exchange in UMR-106 cells. To obtain further evidence for such regulation, we assessed the effect of pH_i on a partial reaction of the exchanger, Cl^-/Cl^- exchange (Fig. 14). For Cl^-/Cl^- exchange measurements, the cells were loaded with Cl^- and clamped at different pH_i by pretreatment with ionophores in a

medium containing 140 mM KCl with either 0.1 mM EGTA or 2 mM $CaCl_2$. The high K^+ concentration, and the ionophores were kept in the incubation medium throughout the experiments to clamp pH_i during Cl^-/Cl^- exchange measurements. The Cl^--loaded cells were bathed in an isotonic K^+-gluconate medium containing 5 mM KCl labeled with $^{36}Cl^-$. Measurements of pH_i changes revealed that under these conditions, the rates of net Cl_o^-/HCO_3^- $(OH^-)_i$ exchange are negligible. Furthermore, in preliminary experiments, cells were depleted of Cl^- by treatment with K^+-gluconate medium and ionophores at different pH values and were used to measure net $^{36}Cl^-$ uptake from a medium containing 5 mM Cl^-. Under these conditions, ^{36}Cl flux measurements could detect net Cl^- uptake only at pH 7.2 and above in the presence of Ca^{2+} in the medium, and at pH 7.4 and above in the presence of EGTA in the medium (not shown). Therefore, most of the ^{36}Cl uptake under the conditions of Fig. 14 represents Cl^- uptake due to Cl^-/Cl^- exchange. Fig. 14 shows typical

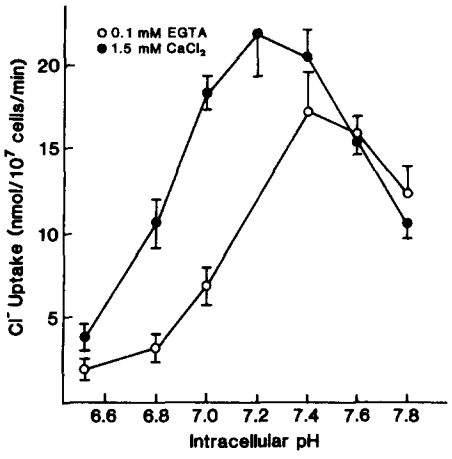

FIGURE 15. pH_i dependency of Cl^- self exchange at two $[Ca^{2+}]_i$. ^{36}Cl influx was measured at each indicated pH_i as described in the legend to Fig. 14. The linear period of uptake for each pH was determined from a series of experiments represented in Fig. 14. For the experiments shown, ^{36}Cl uptake was measured for 5 min at pH 6.8 and 7.0, 2.5 min at pH 7.2 and for 1.5 min at pH 7.4 and 7.6 in triplicate determinations. The component of DIDS-sensitive ^{36}Cl uptake was measured in the presence (*closed symbols*) or absence (*open symbols*) of Ca^{2+} in the media and plotted as a function of the indicated pH. The figure shows the mean ± SD of three experiments.

time courses of $^{36}Cl^-$ uptake at two different pH values and in the presence or absence of Ca^{2+}. Under all conditions extrapolation of $^{36}Cl^-$ uptake to zero time was different from zero. This probably represents ^{36}Cl absorbed to the plastic dishes and trapped in the extracellular spaces, since exposure of the cells to solutions containing ^{36}Cl for <2 s resulted in ^{36}Cl uptake similar to that obtained by extrapolating the ^{36}Cl uptake to zero time. Fig. 14 shows that at pH 6.8, ^{36}Cl uptake was linear for at least 10 min while at pH 7.6, ^{36}Cl uptake was linear for at least 1.5 min. The rate of DIDS-sensitive ^{36}Cl uptake at pH 7.6 was five to six times faster than the uptake at pH 6.8. Further, an increase in $[Ca^{2+}]_i$ increased the rate of ^{36}Cl uptake measured at pH 6.8 while it had no measurable effect on ^{36}Cl uptake at pH 7.6.

Having obtained the linear range of ^{36}Cl uptake for each pH, we studied the pH dependency of Cl^-/Cl^- exchange at two $[Ca^{2+}]_i$. Fig. 15 shows that, similar to the pH dependency of the net exchange (Fig. 5), the Cl^- self exchange does not obey

simple Michaelis-Menten kinetics. Again, a steep increase in the rate of DIDS-sensitive ^{36}Cl uptake was obtained over the pH range of 6.8–7.4. Beyond pH 7.4, an inhibition of ^{36}Cl uptake was observed. Thus, up to pH of 7.4, an increase in pH_i increased, rather than decreased, the rate of Cl^-/Cl^- exchange. An increase in $[Ca^{2+}]_i$ shifted the pH dependency of Cl^-/Cl^- exchange in a manner closely resembling that observed in the net exchange measurements (see Fig. 13).

DISCUSSION

The present study demonstrates the presence of an anion exchange mechanism in the osteosarcoma cell line UMR-106. The exchanger transports Cl^- in exchange for OH^- ions, Cl^- for HCO_3^- or acts as a Cl^- self exchanger. Similar to the band 3 protein in red blood cells (Cabantchik et al., 1978), the exchange process in UMR-106 cells is reversible, electroneutral, and can be inhibited by stilbene compounds. HCO_3^- or OH^- fluxes require Cl^- and were independent of intracellular or extracellular Na^+. These properties are consistent with a Cl^-/HCO_3^- or Cl^-/OH^- exchange process rather than Na^+/HCO_3^- cotransport. Inasmuch as UMR-106 cells share many properties with normal osteoblasts (Martin et al., 1976; Partridge et al., 1981, 1983), it is conceivable that a similar exchanger exists in normal osteoblasts. In the present studies, we characterized some of the kinetic properties of the exchanger and provided evidence for the regulation of the exchanger by intracellular $HCO_3^-(OH^-)$ and Ca^{2+} ions.

Extracellular Sites

The interaction of Cl^- ions with the external face of the exchanger obeys simple saturation kinetics. The apparent affinity ($K_{0.5}$) for Cl_o^- at pH 7.2 was 14.7 mM, which is similar to that value reported in parietal cells (Muallem et al., 1985). From the effect of pH_o on the $K_{0.5}$ for Cl_o^-, it appears that OH^- competes with Cl^- for interaction with an extracellular transport site. Similar behavior of the exchanger was described in MDCK cells, (Kurtz and Golchini, 1987) and Vero cells (Olsnes et al., 1986). Thus, it appears that the Cl^-/HCO_3^- exchanger has a single extracellular transport site that can accept Cl^-, HCO_3^-, and OH^-.

Intracellular Sites

The pH_i dependency of both net anion exchange and Cl^- self exchange did not follow simple saturation kinetics. A steep increase in the rate of the exchanger was observed over the pH range of 7.0–7.4. A similar pattern of behavior was observed for the pH_i dependency of Cl^-/Cl^- exchange in Vero cells (Olsnes et al., 1987) where almost a 10-fold increase in ^{36}Cl uptake occurred when pH_i was increased from 7.0 to 7.2. This finding indicates that OH^- or HCO_3^- ions interact more than once with the internal face of the exchanger for each turnover cycle of exchange. Such can occur if Cl^-/HCO_3^- exchange stoichiometry is higher than 1. This is considered highly unlikely since the rate of both Cl_o^-/HCO_3^- $(OH^-)_i$ and $Cl_i^-/HCO_3^-(OH^-)_o$ exchanges were independent of the membrane potential. Further, if the exchange was an electrogenic process, the depolarization of the plasma membrane should have alkalinized the cytosol in the absence of a $[Ca^{2+}]_i$ rise. This was not observed (Fig. 11). Alternatively, it is possible that Cl^-/HCO_3^- exchange oper-

ates as $2Cl^-/2HCO_3^-$ ($2OH^-$) exchanger. This is considered equally unlikely due to the properties of Cl^- and HCO_3^- (OH^-) interaction with the extracellular face of the exchanger. The Cl_o^- dependency of the exchange process obeys simple saturation kinetics with a single K_m for Cl^-; HCO_3^- (OH^-) appears to compete with Cl^- for binding to the same external site.

The results presented here of an electroneutral exchange process with a single external substrate site and more than one internal substrate sites are consistent with regulation of the Cl^-/HCO_3^- exchanger by internal $HCO_3^-(OH^-)$. Thus, on the internal face of the exchanger there is only a single transport site for $HCO_3^-(OH^-)$ but in addition, the exchanger possesses one or more modifier sites at which $HCO_3^-(OH^-)$ can bind and thereby activate the exchanger without being transported. Such regulation of the exchanger by internal HCO_3^- (OH^-) is strongly supported by the finding that increasing pH_i not only enhanced Cl^-/HCO_3^- exchange but also Cl^-/Cl^- exchange (Fig. 15). The fact that increasing pH_i stimulated Cl^- self exchange even though under these experimental conditions OH^- and HCO_3^- are not transported, is consistent with the concept that internal HCO_3^- (OH^-) could activate the anion exchanger. The effect of pH_i on Cl^-/Cl^- exchange in Vero, HeLa, OHIO, and MDCK cells (Olsnes et al., 1987), suggests that regulation of Cl^-/HCO_3^- exchanger by internal HCO_3^- (OH^-) is a general phenomenon, although more than one type of Cl^-/HCO_3^- exchanger may exist (Tago et al., 1986 *b*; Schuster and Stokes, 1987). Arguments similar to those raised here with regard to activation of the Cl^-/HCO_3^- exchanger by internal $HCO_3^-(OH^-)$ have been used to show the regulatory role of internal H^+ in activation of the Na^+/H^+ exchanger in microvillus membrane vesicles from rabbit kidney cortex (Aronson et al., 1982) and other cells (Grinstein et al., 1984; Vigne et al., 1984; Green et al., 1988 *a*).

The dependency of Cl^-/Cl^- and $Cl^-/HCO_3^-(OH^-)$ exchange on pH_i shows that beyond pH_i 7.4, the rate of Cl^-/Cl^- exchange is inhibited while that of Cl^-/HCO_3^- (OH^-) exchange continues to increase. This observation might be related to a competition between Cl_i^- and HCO_{3i}^- (OH_i^-) for binding to the intracellular transport site. A competition between HCO_{3i}^- and Cl_i^- has been clearly demonstrated in ^{36}Cl efflux studies in red blood cells (Dalmark, 1976). Such an interaction between the anions commits more of the exchangers to operate in the Cl^-/HCO_3^- (OH^-) mode and reduces the number of exchangers operating in Cl^-/Cl^- exchange mode. Since the *V*max of Cl^-/HCO_3^- (OH^-) exchange is severalfold lower than Cl^-/Cl^- exchange, an inhibition of ^{36}Cl uptake is observed. Alternatively, it is possible that the inhibition of Cl^-/Cl^- exchange at high pH_i could be due to a decrease in $[Cl^-]_i$ with increasing pH_i to below saturating concentrations.

Effect of $[Ca^{2+}]_i$ on the Anion Exchanger

We conducted a series of experiments that indicate that a rise in $[Ca^{2+}]_i$ activates the Cl^-/HCO_3^- exchanger in UMR-106 cells. Thus, when $[Ca^{2+}]_i$ was increased either by exposure of the cells to Ca^{2+} ionophore, depolarization of the plasma membrane, or activation of protein kinase C, the exchanger was activated. The properties of the $[Ca^{2+}]_i$-induced cytosolic acidification indicate that it is mediated by the Cl^-/HCO_3^- exchanger. Thus, cytosolic acidification required medium Cl^-, could be inhibited by DIDS and H_2DIDS, and was independent of the membrane potential.

The temporal relationship between the changes in $[Ca^{2+}]_i$ and pH_i shows that activation of the exchanger by $[Ca^{2+}]_i$ is a slow process. Thus, cytosolic acidification started after a lag period of 30–45 s after the $[Ca^{2+}]_i$ rise. Furthermore, even when $[Ca^{2+}]_i$ was increased to micromolar levels for short periods of time, the exchanger was not activated. Hence, a maintained elevation of $[Ca^{2+}]_i$ is necessary to keep the exchanger activated. Such a relatively slow process of activation suggests that Ca^{2+} ions do not interact directly with the exchanger. Rather, the increase in $[Ca^{2+}]_i$ might activate a biochemical pathway, like the Ca^{2+}- and calmodulin-dependent protein kinase, which modifies the properties of the exchanger.

Cl^-/HCO_3^- exchangers in different cells appear to be different in terms of their regulation by second messengers and protein kinases. The exchanger in UMR-106 cells can be activated by Ca^{2+} but not by activation of protein kinase C. This is concluded from the finding that stimulation of UMR-106 cells suspended in Ca^{2+}-free medium with TPA did not result in cytosolic acidification. On the other hand, in other cell types, activation of protein kinase C was followed by activation of the Cl^-/HCO_3^- exchanger (Olsnes et al., 1986). Thus, the site of the Cl^-/HCO_3^- exchanger that can be modified to activate the exchanger displays some differences among exchangers in different cells.

Kinetic analysis of the Ca^{2+}-activated Cl^-/HCO_3^- exchanger reveals that Ca^{2+} ions modify the interaction of the exchanger with intracellular HCO_3^- (OH^-). A $[Ca^{2+}]_i$ rise had no measurable effect on the interaction of the substrate with the extracellular face of the exchanger. The same $[Ca^{2+}]_i$ increase resulted in a shift of the pH_i dependency of both Cl^-/HCO_3^- (OH^-) and Cl^-/Cl^- exchange. Since the effect of pH_i on Cl^-/Cl^- exchange can be attributed to the regulatory role of HCO_3^- (OH^-), it is possible that an increase in $[Ca^{2+}]_i$ modifies the properties of the regulatory site for HCO_3^- (OH^-). It is likely that activation of Cl^-/HCO_3^- exchange by protein kinase C is also due to a similar modification of the kinetic properties of the exchanger, thus allowing a shift in pH_i dependency of Cl^-/Cl^- exchange (Olsnes et al., 1986).

Cl^-/HCO_3^- and Na^+/H^+ Exchangers and K^+ Efflux in Osteoblasts

Cytosolic acidification triggered by $[Ca^{2+}]_i$ elevation was followed by a cytosolic alkalinization. The secondary recovery from acidification required the presence of Na^+ in the incubation medium and was blocked by the diuretic amiloride. Hence, the secondary increase in pH_i is due to a Na^+/H^+ exchange process, the properties of which in UMR-106 cells have been reported previously (Green et al., 1988*a*, *b*). In the present study we demonstrate that unlike several other cells (Moolenaar et al., 1984; Grinstein et al., 1985*a*, *b*) the Na^+/H^+ exchanger in UMR-106 cells cannot be activated by protein kinase C. Thus, stimulation of cells suspended in Ca^{2+}-free medium with TPA did not alkalinize the cytosol as would be expected from activation of Na^+/H^+ exchanger by protein kinase C. In addition, inhibition of TPA-induced cytosolic acidification by either removal of Cl^- from the medium or pretreatment of the cells with H_2DIDS or DIDS was sufficient to prevent the initial acidification and the subsequent alkalinization. These observations indicate that the increased activity of the Na^+/H^+ exchanger of stimulated UMR-106 cells stemmed from acidification of the cytosol. In UMR-106 cells (Green et al., 1988*a*), as in other

cells (Aronson et al., 1982; Grinstein et al., 1984; Vigne et al., 1984), intracellular H^+ activates the Na^+/H^+ exchanger.

The sequential activation of both exchangers should result in net uptake of Cl^- and Na^+. This would lead to two undesirable effects: inhibition of Cl^-/HCO_3^- exchange due to the accumulation of Cl_i^- and cell swelling. To protect against such effects, the TPA-stimulated cells lost KCl. KCl efflux can occur either by a K^+/Cl^- cotransport system or by parallel K^+ and Cl^- conductive pathways. Osteoblasts express Ca^{2+}-activated K^+ conductive pathways in the plasma membrane (Ferrier et al., 1987). In high K^+ medium, which depolarized the cells, Cl^- accumulated in the cytosol. It is, therefore, possible that during activation of Cl^-/HCO_3^- and Na^+/H^+ exchange, UMR-106 cells lost KCl through K^+ and Cl^- conductive pathways. KCl efflux from TPA-stimulated cells and the slow rate of inactivation of an activated Cl^-/HCO_3^- exchanger (Olsnes et al., 1986) can account for the patterns of cytosolic acidification observed in Na^+-free medium or in the presence of amiloride (Figs. 9 and 10).

The increased activity of the Na^+/H^+ exchange was sufficient to restore near resting pH_i in the face of an activated Cl^-/HCO_3^- exchange. This, in turn, allows continuous secretion of base equivalents by the osteoblast. Without the Na^+/H^+ exchange–mediated increase in pH_i, the cytosol acidifies to a pH_i of ~6.5, at which HCO_3^- secretion stops even when $[Ca^{2+}]_i$ is elevated (Fig. 13). This relationship between Cl^-/HCO_3^- and Na^+/H^+ exchange might have an important physiological significance for the osteoblast as a bone-mineralizing cell. The process of bone formation includes the cross-linking of the collagen chains and the subsequent precipitation of hydroxyapatite (Parfitt and Kleerekoper, 1980). Both processes are pH dependent and require continuous and long-lasting secretion of base to the BEF by the osteoblasts (Samachson, 1969; Harris et al., 1974). HCO_3^- extrusion from the osteoblast into the extracellular environment can be mediated by the Cl^-/HCO_3^- exchanger. Thus, the activity of the Cl^-/HCO_3^- exchanger will increase during the bone formation phase of bone remodeling. Excessive and continuous HCO_3^- secretion by the osteoblast requires the removal of the excess H^+ generated in the cytosol during this process. Also, to maintain bone mineralization it is necessary that the H^+ ions be secreted to the systemic extracellular fluid (SEF) by a mechanism that responds to changes in pH_i. The Na^+/H^+ exchanger appears to fulfill this requirement. For such a system to play a role in bone remodeling, Cl^-/HCO_3^- and Na^+/H^+ exchangers should reside in opposite parts of the plasma membrane. Thus, the osteoblast should behave like a polarized cell in terms of acid- and base-secreting mechanisms. Although there is no experimental evidence as yet to support this hypothesis, recent data suggest that osteoblasts lining the bone are polarized cells. Plasma membrane Ca^{2+} pumps appear to exist only on the membrane facing the SEF and are absent from the membrane facing the BEF (Arisaka et al., 1988). A similar polarized arrangement of Cl^-/HCO_3^- and Na^+/H^+ exchangers can provide the osteoblast with the necessary mechanisms that will allow it to regulate the pH of the BEF and its own cytosolic pH during bone remodeling.

Mr. Timothy Beeker and Mr. Bradley Merritt provided skillful technical assistance. The authors are indebted to Ms. Jackie Williams and Ms. Rita Kaz for expert secretarial work.

This work was supported by National Institutes of Health grant AR-39245, the National Osteoporosis Foundation, The Max Factor Family Foundation, and The Veteran Administration Career Development Award. This work was done during the tenure of an established investigatorship from the American Heart Association to S. Muallem.

Original version received 9 May 1988 and accepted version received 9 June 1989.

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