**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 (pHi), ^36^Cl fluxes and free cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) 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 pHi and [Ca\(^{2+}\)]. Suspending cells in Cl\(^{-}\)-free medium resulted in rapid cytosolic alkalinization from pHi 7.05 to ~7.42. Subsequently, the cytosol acidified to pHi 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 O\(^{-}\)-containing solutions resulted in cytosolic acidification. All these pHi changes were inhibited by 4',4'-diisothiocyano-2,2'-stilbene disulfonic acid (DIDS) and H\(^{+}\)DIDS, 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 pHi did not follow simple saturation kinetics. These findings suggest that the anion exchanger is regulated by intracellular HCO\(^{3-}\) (OH\(^{-}\)).

A rise in [Ca\(^{2+}\)], 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\(_2\)DIDS 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\(^{2+}\)], did not alter pHi. Likewise, in H\(_2\)DIDS-treated cells and in the absence of Cl\(^{-}\), an increase in...
[Ca$^{2+}$], did not activate the Na$^+$/H$^+$ exchanger in UMR-106 cells. These findings indicate that an increase in [Ca$^{2+}$], 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$^{2+}$] increase did not modify the properties of the extracellular site. A rise in [Ca$^{2+}$] increased the apparent affinity for intracellular OH$^-$ (or HCO$_3^-$) of both net anion and Cl$^-$ self exchange. These results indicate that [Ca$^{2+}$], 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., 1986a), MDCK cells (Kurtz and Golchini, 1987), gastric parietal cells (Mallem et al., 1985), and Vero cells (Ohnes 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⁻/HCO₃⁻ 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 HCO₃⁻ (OH⁻) fluxes and Cl⁻ self exchange on intracellular pH. In view of recently available information regarding the effect of calcitropic hormones on free cytosolic Ca⁺ in osteoblasts (Lieberherr, 1987; Yamaguchi et al., 1987a, 1988) we further investigated the effect of a rise in intracellular Ca²⁺ on the activity of the anion exchanger in the UMR-106 cells. Our results show that intracellular pH regulates the activity of the Cl⁻/HCO₃⁻ exchanger and that a rise in free cytosolic Ca²⁺ 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 acetoxymethylenelester (AM) of fura 2 (fura 2/AM) and H₂DIDS were obtained from Molecular Probes Inc. (Eugene, OR). Amiloride was from Merck, Sharp and Dohme, West Point, PA. Na⁶⁴Cl 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 MgCl₂, 4 KCl, 10 HEPES/Tris, 5 glucose. Solution B (KCl) and C (tetramethylammonium [TMA]-Cl) were prepared by isosmotic replacement of NaCl by KCl or TMA-Cl, respectively, but were otherwise identical. Solution D (Na⁺-gluconate) contained: 140 Na⁺-gluconate, 1 MgSO₄, 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 × 10⁴ cells/cm² in 75 cm² area flasks and grown at 37°C in a humidified 95% air 5% CO₂ atmosphere in Ham's F12: Dulbecco's modified Eagle's media (1:1) supplemented with 14.3 mM NaHCO₃, 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 NaCl flux studies, cells were seeded at a density of 2.5 × 10⁴ cells/cm² in 12-well disposable trays under the same conditions.

Measurement of Cytosolic pH

pHₗ was measured fluorimetrically using the pH-sensitive fluorescent dye BCECF, essentially as described previously (Green et al., 1988a). 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 CaCl₂ 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 pHᵢ was performed as previously described (Green et al., 1988a).

**Determination of [Ca²⁺]ᵢ**

Measurements of free cytosolic calcium concentration ([Ca²⁺]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 pHᵢ. 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. pHᵢ and [Ca²⁺]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., 1987a). Briefly, medium CaCl₂ 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 Ca²⁺-fura 2 was assumed to be 220 nM and the calculation of [Ca²⁺]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, alkalization 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 HCO₃⁻ (Na⁺-gluconate or K⁺-gluconate). These cells were used to compare Cl⁻/HCO₃⁻ exchange of ionophore-treated and -untreated cells and to study the dependency of Cl⁻/HCO₃⁻ 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 CaCl₂ and [¹⁴C] 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 HNO₃. Samples were used to determine the [¹⁴C] 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 CaCl₂ to which 2 μM TPA was added, and solution B containing 1.5 mM CaCl₂. All solutions contained [¹⁴C]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⁻], was calculated.

Measurement of ⁴⁰Cl⁻ Influx

To evaluate the effect of intracellular pH on Cl⁻/Cl⁻ exchange, ⁴⁰Cl⁻ 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 CaCl₂ 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 ⁴⁰Cl⁻. 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 ⁴⁰Cl⁻ 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 ⁴⁰Cl⁻ influx, which was obtained from these studies for each individual pH, served as a basis for a complete study of the dependency of Cl⁻/Cl⁻ exchange on intracellular pH.

RESULTS

Demonstration of Cl⁻/HCO₃⁻ (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 (pHi) was measured. Dilution of cells suspended in solution A (NaCl) into Cl⁻-free medium (Na⁺-gluconate) resulted in a rise of pHi from a resting level of 7.05 ± 0.04 to 7.42 ± 0.07 within ~25 s (Fig. 1 a). Subsequently, pHi 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 H₂DIDS was as effective as DIDS in inhibiting the pHi 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 pHi 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 H2DIDS inhibitable.

Fig. 2 depicts the effect of medium HCO3 concentration on the changes in pH. Dilution of the cells into HCO3 and Cl--free medium resulted in a typical cytosolic alkalinization to pH of 7.3 and then acidification to pH of 7.22 (Fig. 2 a). Increasing HCO3 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 HCO3 in the incubation medium (Fig. 2, b, d, and f). The effect of HCO3 on pH 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 HCO3 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.](https://jgp.rupress.org/)
incubation, the DIDS-sensitive acidification was $0.293 \pm 0.03$ (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⁻/HCO₃⁻ exchange or Na⁺/HCO₃⁻ cotransport, both of which are sensitive to DIDS.

**FIGURE 2.** Effect of extracellular HCO₃⁻ on the rate and extent of cytosolic alkalinization. BCECF-loaded cells were added to 2 ml of solution D gassed with 100% O₂ (a). Cells were also added to 2 ml solution D (c), or solution D containing 2 mM HCO₃⁻ (e); both were equilibrated with air. HCO₃⁻ 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.

**FIGURE 3.** Cl⁻/HCO₃⁻ 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 HCO₃⁻ 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 HCO₃⁻ 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 HCO₃⁻ or OH⁻ gradients across the plasma membrane. To verify that most of
the measured cytosolic acidification was due to Cl⁻/HCO₃⁻ exchange, the cells
were depleted of Na⁺ and the initial pH was clamped at 7.6 by the ionophore tech-
nique (see Methods). Addition of these cells to a medium containing 25 mM KCl was
followed by a DIDS-sensitive reduction of pHᵢ (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 HCO₃⁻
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 HCO₃⁻ (or
OH⁻) transport system in UMR-106 cells. These properties strongly suggest that we
measured the activity of a Cl⁻/HCO₃⁻ exchanger in these cells.

**FIGURE 4.** The dependence
of the anion exchanger on
extracellular Cl⁻ concentra-
tion: effect of pHₒ. BCECF-
loaded cells were depleted of
Cl⁻ and alkalinized as
described under Methods.
Then the cells were added to 2
ml of solutions containing dif-
ferent concentrations of Cl⁻;
Cl⁻ had been replaced by equi-
molar concentrations of glu-
conate. 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 con-
taining 50 mM Cl⁻. The rate of DIDS-sensitive pHᵢ change was recorded at two external pH's
and plotted against external Cl⁻ concentration (open symbols, pHₒ 7.4; closed symbols, pHₒ 7.2).
A double reciprocal plot of the results (inset) was used to obtain the K₀ₛ₃Cl⁻ at each pHₒ. The
figure shows the mean ± SD of three experiments.

**Extracellular Site**

The protocol of HCO₃⁻/Cl⁻ 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
Clₒ without changing the overall Vₘₐₓ. A double reciprocal plot of the data (Fig. 4,
inset) shows an apparent affinity for Cl⁻ (K₀ₛ₃Cl⁻) of 14.7 mM at pHₒ 7.2 and K₀ₛ₃Cl⁻
of 21.3 mM at pHₒ 7.4. Thus, there appears to be a competition between extracel-
ular Cl⁻ and HCO₃⁻ (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.

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+}], 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+}], 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+}], and pH_i, in the same cell preparation could be explored. Fig. 6 shows the effect of the phorbol ester, TPA, on [Ca^{2+}], 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+}], from 112 to 436 nM within 45 s (Fig. 6a). The cells were
able to partially reduce $[Ca^{2+}]_i$ to $\sim 202$ 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., 1987b). Stimulation of the cells with TPA also induced a biphasic change in $pH_i$. After a lag period of $\sim 30 (31 \pm 6)$ s, the cytosol acidified from $pH_i$ 7.15 to 6.86 over a period of 3 (3.41 $\pm$ 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 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 $\mu$M ionomycin to cells suspended in $Ca^{2+}$-containing medium, there is an initial increase in $[Ca^{2+}]_i$ to 1,104 nM, after which the cells reduced $[Ca^{2+}]_i$ to $\sim 356$ 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 $\sim 45$ 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 $\mu$M 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 $\sim 2.5$ $\mu$M,
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$_2$DIDS on the TPA-stimulated changes in [Ca$^{2+}$]$_i$ and pH$_i$ was measured. Fig. 8a shows that incubating the cells in Cl$^-$-free medium (Na$^+$-gluconate) did not considerably change resting [Ca$^{2+}$]$_i$. Further, stimulation with TPA...
resulted in a change in \([\text{Ca}^{2+}]_i\), 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 \(\mu\text{M}\) H\(_2\)DIDS were stimulated with TPA, \([\text{Ca}^{2+}]_i\) increased (Fig. 8 b) but the change in pH\(_i\) was blunted (Fig. 8 d). In these experiments 20 \(\mu\text{M}\) H\(_2\)DIDS was used to minimize the interference of H\(_2\)DIDS with the fura 2 signal. Table I summarizes the effect of H\(_2\)DIDS and Cl\(^{-}\)-removal of TPA-induced cytosolic acidification. The effect of H\(_2\)DIDS was dose dependent where 25 and 250 \(\mu\text{M}\) H\(_2\)DIDS 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 \([\text{Ca}^{2+}]_i\) increase.

The experiments in Figs. 6 and 8 and Table I indicate that (a) the cytosolic acidification was due to a Cl\(^{-}\)-dependent, H\(_2\)DIDS-sensitive mechanism, (b) the second-

| Conditions | Additions | TPA-mediated acidification \(\Delta\text{pH}/4\text{ min}\) |
|------------|-----------|---------------------------------------------------------|
| NaCl       | —         | 0.274 ± 0.016 (11)*                                     |
| NaCl       | 25 \(\mu\text{M}\) H\(_2\)DIDS | 0.170 ± 0.021 (3)                                     |
| NaCl       | 250 \(\mu\text{M}\) H\(_2\)DIDS | 0.086 ± 0.022 (3)                                     |
| Na\(^{-}\)-gluconate | —         | 0.007 ± 0.003 (4)                                     |
| NaCl       | Amiloride | 0.502 ± 0.072 (6)                                     |
| NaCl       | Amiloride + 25 \(\mu\text{M}\) H\(_2\)DIDS | 0.235 ± 0.028 (4)                                     |
| NaCl       | Amiloride + 250 \(\mu\text{M}\) H\(_2\)DIDS | 0.093 ± 0.015 (3)                                     |

\(pH\) 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\(_2\)DIDS and Cl\(^{-}\)-removal was highly significant \((P < 0.01 \text{ or better})\).

*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 \([\text{Ca}^{2+}]_i\) or stimulation of protein kinase C.

**Relationship between Cl\(^{-}\)/H\(^{+}\) 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 \([\text{Ca}^{2+}]_i\) increase due to Na\(^{+}\)/Ca\(^{2+}\) exchange when cells are suspended in TMA-Cl medium. The \([\text{Ca}^{2+}]_i\) signal (Fig. 9 a) was comparable to that observed in NaCl.
FIGURE 9. Effect of Na\(^{+}\)-free media on [Ca\(^{2+}\)]\_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 \(\mu\)M TPA, and the changes in [Ca\(^{2+}\)]\_ and pH\(_i\) 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 \(\mu\)M 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 \(\mu\)M TPA for 4 min resulted in acidification by 0.502 pH

FIGURE 10. Effect of amiloride on [Ca\(^{2+}\)]\_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 \(\mu\)M 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 H2DIDS 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

Fig. 11 combines evidence for the importance of [Ca2+]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. In Fig. 11 a, cells were added to KCl medium in the presence of 2 mM Ca2+. [Ca2+]i rose from a resting level of 92 nM to 466 nM, which was due to Ca2+ influx through the depolarization-activated calcium channel present in these cells (Yamaguchi et al., 1989). The rise in [Ca2+]i was followed by cytosolic acidification and inhibition of the secondary recovery from acidification (Fig. 11 d). The pattern of pH 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 Ca2+-activated cytosolic acidification could be observed when the cells were depolarized. Also it is clearly shown in the figure that removal of Ca2+ from the medium (Fig. 11, b and e) or addition of the calcium channel blocker, verapamil (Fig. 11, c and f) inhibited both the [Ca2+]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+/H+ 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 Ca2+ signal shown in Fig. 11 a and thus the acidification. However, acidification due to Cl−/HCO3− exchange can be estimated from subtracting the acidification in the absence of [Ca2+]i rise (Fig. 11 e and f) from the acidifi-
cation in the presence of \([\text{Ca}^{2+}]_i\) increase (Fig. 11d). Fig. 11g shows the results of such calculation. It can be seen that in the presence of high extracellular KCl concentration the rate and extent of \(\text{Cl}^-/\text{HCO}_3^-\) 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 \(\text{Cl}^-/\text{HCO}_3^-\) 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 \(\sim 92 \text{ mM Cl}^-\) and \(142 \text{ mM K}^+\). When stimulated with TPA for 10 min the cells did not gain any Cl\(^-\). This was despite the activation of \(\text{Cl}^-/\text{HCO}_3^-\) exchange. From the acidification patterns shown in Figs. 9 and 10 \((0.502 \pm 0.072 \text{ pH units})\) and the buffer capacity of the cells (Green et al., 1988a) it was expected that the cells would gain \(\sim 43 \text{ mM Cl}^-\). 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 \(\sim 49 \text{ mM K}^+\) (Table II). Therefore, it appears that Cl\(^-\) incorporated into the cells due to \(\text{Cl}^-/\text{HCO}_3^-\) exchange was extruded by a mechanism dependent on \(K^+\). Such a mechanism will prevent accumulation of Cl\(^-\) in the cytosol and allow continuous \(\text{Cl}^-/\text{HCO}_3^-\) exchange. In the presence of high extracellular KCl concentration, \(K^+\) and Cl\(^-\) efflux are impaired and Cl\(^-\) slowly accumulates in the cytosol (Table II).

**Table II**

| Conditions                  | Intracellular ion concentration |     |     |
|-----------------------------|---------------------------------|-----|-----|
|                             | Cl\(^-\) (mM)                  | K\(^+\) (mM) |
| Control                     | 31.83 ± 2.18                   | 142 ± 7.9   |
| 2 \(\mu\)M TPA, 10 min     | 25.67 ± 1.67                   | 93 ± 4.8    |
| 140 mM KCl, 10 min          | 52.94 ± 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.

**Effect of \([\text{Ca}^{2+}]_i\), on the Kinetic Properties of \(\text{Cl}^-/\text{HCO}_3^-\) Exchange**

To explore some aspects of the mechanism by which \(\text{Ca}^{2+}\) ions activate the anion exchanger, we studied the effect of \([\text{Ca}^{2+}]_i\), 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 \([\text{Ca}^{2+}]_i\). For these experiments the cytosol was alkalinized to pH 7.6 before the initiation of exchange. At this pH, the intracellular site is nearly saturated with base equivalent (see Fig. 5), so that an effect of \([\text{Ca}^{2+}]_i\) on \(K_{0.5}\) can be separated from an effect of \([\text{Ca}^{2+}]_i\) on pH dependency of the exchanger. Under the conditions of the experiments shown, \([\text{Ca}^{2+}]_i\) averaged \(82 ± 11 \text{ nM (n = 5)}\) in the presence of 0.1 mM EGTA and \(257 ± 17 \text{ nM (n = 5)}\) in the presence of 2 mM CaCl\(_2\) in the medium. As shown in Fig. 12, at pH 7.2, the same \(K_{0.5}\) 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 CaCl₂ resulted in a rapid [Ca²⁺]i rise to a peak of ~279 ± 21 nM (n = 3). [Ca²⁺]i remained at this level for ~1.5 min. The indicated levels of [Ca²⁺]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 Ca²⁺ channel is insensitive to pH 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 [Ca²⁺]i from 67 to 279 nM resulted in a clear shift in the pH_i dependency of HCO₃⁻/Cl⁻ exchange. Half-maximal rate of exchange was measured at pH_i ~7.24 at [Ca²⁺]i of 67 nM and at pH_i 6.95 at [Ca²⁺]i of 279 nM. The increase in [Ca²⁺]i had no effect on the overall maximal rate of exchange. A Hill analysis of the results at high [Ca²⁺]i yielded a Hill coefficient of 1.81 which was not significantly different from that measured at low [Ca²⁺]i, (n = 1.76).

**Effect of [Ca²⁺] on Cl⁻/Cl⁻ Exchange**

The results presented thus far suggest that intracellular HCO₃⁻ and OH⁻ can activate the Cl⁻/HCO₃⁻ 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 CaCl₂. The high K⁺ concentration, and the ionophores were kept in the incubation medium throughout the experiments to clamp pHᵢ during CI⁻/CI⁻ exchange measurements.

The Cl⁻-loaded cells were bathed in an isotonic K⁺-gluconate medium containing 5 mM KCl labeled with ³⁶Cl⁻. Measurements of pHᵢ changes revealed that under these conditions, the rates of net CI⁻/HCO₃⁻ (OH⁻) 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 ³⁶Cl⁻ uptake from a medium containing 5 mM Cl⁻. Under these conditions, ³⁶Cl⁻ flux measurements could detect net Cl⁻ uptake only at pH 7.2 and above in the presence of Ca²⁺ in the medium, and at pH 7.4 and above in the presence of EGTA in the medium (not shown). Therefore, most of the ³⁶Cl uptake under the conditions of Fig. 14 represents Cl⁻ uptake due to Cl⁻/Cl⁻ exchange. Fig. 14 shows typical time courses of ³⁶Cl⁻ uptake at two different pH values and in the presence or absence of Ca²⁺. Under all conditions extrapolation of ³⁶Cl⁻ uptake to zero time was different from zero. This probably represents ³⁶Cl absorbed to the plastic dishes and trapped in the extracellular spaces, since exposure of the cells to solutions containing ³⁶Cl for <2 s resulted in ³⁶Cl uptake similar to that obtained by extrapolating the ³⁶Cl uptake to zero time. Fig. 14 shows that at pH 6.8, ³⁶Cl uptake was linear for at least 10 min while at pH 7.6, ³⁶Cl uptake was linear for at least 1.5 min. The rate of DIDS-sensitive ³⁶Cl uptake at pH 7.6 was five to six times faster than the uptake at pH 6.8. Further, an increase in [Ca²⁺], increased the rate of ³⁶Cl uptake measured at pH 6.8 while it had no measurable effect on ³⁶Cl uptake at pH 7.6.

Having obtained the linear range of ³⁶Cl uptake for each pH, we studied the pH dependence of Cl⁻/Cl⁻ exchange at two [Ca²⁺]. Fig. 15 shows that, similar to the pH dependence 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 increased, rather than decreased, the rate of Cl$^{-}$/Cl$^{-}$ exchange. An increase in [Ca$^{2+}$], 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$^{-}$ 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$_{i}$/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 alkalized 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 $2\text{Cl}^-/2\text{HCO}_3^-$ (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$_i^-$ 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 exchange 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$_3^-$ (OH$_i^-$) for binding to the intracellular transport site. A competition between HCO$_3^-$ 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 Vmax 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$^{2+}$], on the Anion Exchanger**

We conducted a series of experiments that indicate that a rise in [Ca$^{2+}$], activates the Cl$^-$/HCO$_3^-$ exchanger in UMR-106 cells. Thus, when [Ca$^{2+}$], 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$^{2+}$],-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$_2$DIDS, and was independent of the membrane potential.
The temporal relationship between the changes in [Ca²⁺]i and pH³ shows that activation of the exchanger by [Ca²⁺]i is a slow process. Thus, cytosolic acidification started after a lag period of 30-45 s after the [Ca²⁺]i rise. Furthermore, even when [Ca²⁺]i was increased to micromolar levels for short periods of time, the exchanger was not activated. Hence, a maintained elevation of [Ca²⁺]i is necessary to keep the exchanger activated. Such a relatively slow process of activation suggests that Ca²⁺ ions do not interact directly with the exchanger. Rather, the increase in [Ca²⁺]i might activate a biochemical pathway, like the Ca²⁺- and calmodulin-dependent protein kinase, which modifies the properties of the exchanger.

Cl⁻/HCO₃⁻ 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 Ca²⁺ but not by activation of protein kinase C. This is concluded from the finding that stimulation of UMR-106 cells suspended in Ca²⁺-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⁻/HCO₃⁻ exchanger (Olsnes et al., 1986). Thus, the site of the Cl⁻/HCO₃⁻ exchanger that can be modified to activate the exchanger displays some differences among exchangers in different cells.

Kinetic analysis of the Ca²⁺-activated Cl⁻/HCO₃⁻ exchanger reveals that Ca²⁺ ions modify the interaction of the exchanger with intracellular HCO₃⁻ (OH⁻). A [Ca²⁺]i rise had no measurable effect on the interaction of the substrate with the extracellular face of the exchanger. The same [Ca²⁺]i increase resulted in a shift of the pH³ dependency of both Cl⁻/HCO₃⁻ (OH⁻) and Cl⁻/Cl⁻ exchange. Since the effect of pH³ on Cl⁻/Cl⁻ exchange can be attributed to the regulatory role of HCO₃⁻ (OH⁻), it is possible that an increase in [Ca²⁺]i modifies the properties of the regulatory site for HCO₃⁻ (OH⁻). It is likely that activation of Cl⁻/HCO₃⁻ exchange by protein kinase C is also due to a similar modification of the kinetic properties of the exchanger, thus allowing a shift in pH³ dependency of Cl⁻/Cl⁻ exchange (Olsnes et al., 1986).

Cl⁻/HCO₃⁻ and Na⁺/H⁺ Exchangers and K⁺ Efflux in Osteoblasts

Cytosolic acidification triggered by [Ca²⁺]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 pH³ is due to a Na⁺/H⁺ exchange process, the properties of which in UMR-106 cells have been reported previously (Green et al., 1988a, b). In the present study we demonstrate that unlike several other cells (Moolenaar et al., 1984; Grinstein et al., 1985a, b) the Na⁺/H⁺ exchanger in UMR-106 cells cannot be activated by protein kinase C. Thus, stimulation of cells suspended in Ca²⁺-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 H₂DIDS 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., 1988a), 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−/HCO3− 
exchange due to the accumulation of Cl− 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 Ca2+-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−/HCO3− 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−/HCO3− 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 rest-
ing pH in the face of an activated Cl−/HCO3− exchange. This, in turn, allows con-
tinuous secretion of base equivalents by the osteoblast. Without the Na+/H+ 
exchange-mediated increase in pH, the cytosol acidifies to a pH of ~6.5, at which 
HCO3− secretion stops even when [Ca2+]i is elevated (Fig. 13). This relationship 
between Cl−/HCO3− and Na+/H+ exchange might have an important physiological 
significance for the osteoblast as a bone-mineralizing cell. The process of bone for-
mation includes the cross-linking of the collagen chains and the subsequent precipi-
tation 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). HCO3− extrusion from the 
osteoblast into the extracellular environment can be mediated by the Cl−/ 
HCO3− exchanger. Thus, the activity of the Cl−/HCO3− exchanger will increase dur-
ing the bone formation phase of bone remodeling. Excessive and continuous HCO3− 
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. The Na+/H+ exchanger appears to fulfill this 
requirement. For such a system to play a role in bone remodeling, Cl−/HCO3− 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 Ca2+ 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−/HCO3− 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.

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