pH-regulated Anion Antiport in Nucleated Mammalian Cells

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Abstract. The uptake of $^{36}Cl^-$ into cells was measured after preincubation in medium containing nigericin and KCl to allow control of the intracellular pH. When the pH was increased from pH 7.0 to pH 7.3 there was a 10-fold increase in the rate of $^{36}Cl^-$ uptake. The increase was half maximal at pH 7.15 in Vero and L-cells, whereas in phorbol 12-myristate 13-acetate–treated Vero cells the increase was half maximal at pH 6.9. Kinetic studies showed that in cells preincubated with nigericin and isotonic KCl, both at pH 7.0 and at pH 8.0, the K_m for Cl^- was 7 mM. In the two cases the J_{max} was 1.7×10^8 Cl^- ions × $cell^{-1}$ × s^{-1} and 1.6×10^9 Cl^- ions × $cell^{-1}$ × s^{-1}, respectively. Bicarbonate inhibited $^{36}Cl^-$ uptake with a K_i of 5–6 mM. Probably, the anion antiporter plays a role in the regulation of the intracellular pH.

THE pH in the cytosol is partly regulated by the Na^+/H^+ antiporter which is sensitive to the internal pH (16, 17, 22, 23, 26, 31). In chick muscle cells this antiporter is activated when the intracellular pH drops below pH 7.7 (half maximal activation at pH 7.4). The large electrochemical Na^+ gradient over the plasma membrane is then allowed to drive H^+ out of the cells until the pH is normalized (8, 31). It occurred to us that Cl^-/HCO_3^- antiport, which is also believed to play a role in the pH regulation of the cytosol of nucleated cells (1, 6, 10, 11, 30), may respond to alterations in intracellular pH in a similar fashion as the Na^+/H^+ antiport.

In our recent studies on the uptake mechanism of diphtheria toxin, we found that anion transport is required for toxin entry into Vero cells (28) and that the toxin partly inhibits anion antiport in these cells (19, 20). In the course of our studies we found that when the cytosol had been alkalinized by preincubating Vero cells at pH 8.0 in the presence of bicarbonate, the uptake of $^{36}Cl^-$ occurred much more rapidly than in cells preincubated at pH 6.5 (18). We proposed that this difference in uptake rate could be due to regulation of the anion antiporter by the intracellular pH.

To test this in more detail we have now taken advantage of the fact that the carboxylic ionophore, nigericin, can exchange K^+ for H^+ across the membrane (9, 31). Therefore, when the K^+ concentration is equal at both sides of the plasma membrane, and when Na^+ is absent from the medium, the pH in the cytosol should be equal to the pH in the medium. On the other hand, if the K^+ concentration on the two sides of the membrane is different, the H^+ concentration should show a corresponding asymmetrical distribution (9, 31).

In the present paper, we have tested the rate of $^{36}Cl^-$ uptake in cells preincubated with nigericin under conditions in which the pH in the cytosol was varied. We have also measured the effect of the preincubation on the kinetics of chloride uptake and on the ability of bicarbonate to inhibit the uptake.

Materials and Methods

Materials

$H^{36}Cl$ (specific activity 9.2 μCi/mg Cl) was obtained from The Radiochemical Centre, Amersham, UK; 2-(*N*-morpholino)ethane sulfonic acid (MES)[1], Hepes, Tris, choline chloride, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), DIDS (4,4′-diisothiocyanato-2,2′-stilbene disulfonic acid), and nigericin were obtained from Sigma Chemical Co., St. Louis, MO. BCECF (2′,7′-bis[carboxyethyl]-5,6-carboxyfluorescein) was from HSC Research Development Corp., Toronto, Canada.

Cells

Vero cells (from African green monkey kidney), L-cells, and fetal hamster kidney cells are strains that have been growing in this laboratory for years. HeLa OHIO-cells were obtained from Flow Laboratories LTD, Irvine, Scotland, and Hep_2 cells from Dr. Patrice Boquet, The Pasteur Institute, Paris. The cells were kept in minimum essential medium with 10% (vol/vol) fetal calf serum in an atmosphere containing 5% CO_2. The day before use the cells were seeded out into 24-well disposable trays or on coverslips placed in the bottom of petri dishes in the same medium (pH 7.3–7.6) at a density of $0.5–2 \times 10^5$ cells/cm^2. The next day the medium was changed to minimum essential medium that contained 20 mM Hepes, pH 7.4, instead of bicarbonate, and the cells were incubated for 1 h without CO_2. Then the medium was removed, and buffers as described below were added.

Chloride Uptake Measurements

Cells in 24-well disposable trays were preincubated as indicated below and then washed twice with ice-cold mannitol buffer (260 mM mannitol, 1 mM $Ca[OH]_2$ and 20 mM MES adjusted with Tris to pH 7 when not otherwise indicated). Then 0.3 ml was added per well of the same buffer containing 0.17 μCi $H^{36}Cl$. The cells were incubated at 24°C for the indicated period of time, then rapidly washed twice with ice-cold phosphate-buffered saline (PBS) (140 mM NaCl, 20 mM Na-phosphate, pH 7.4), and finally 0.3 ml per well of 5% (wt/vol) trichloroacetic acid was added. After 10 min at room temperature the trichloroacetic acid was transferred to counting vials, and the radioactivity was measured.

[1] *Abbreviations used in this paper:* BCECF, 2′,7′-bis (carboxyethyl)-5,6-carboxyfluorescein; DIDS, 4,4′-diisothiocyanato-2,2′-stilbene disulfonic acid; MES, 2-(*N*-morpholino)ethane sulfonic acid; TPA, phorbol 12-myristate 13-acetate.

Measurements of pH in the Cytosol

Intracellular pH was measured with the fluorescent probe BCECF, which is a membrane-permeable ester that is hydrolyzed and trapped in the cytosol. The fluorescence of the dye is pH dependent (21). Cells were grown to confluence on 1 × 3-cm glass coverslips. Before the experiments the coverslips were transferred to Hepes medium without serum, and then BCECF was added to a final concentration of 10 μM. After 1 h at 37°C, the cells were washed to remove extracellular dye, and the coverslip was placed in a cuvette at an angle of 30° to the excitation source. Buffers were changed by perfusion. Fluorescence was measured with a Perkin-Elmer LS-5 fluorescence spectrometer (Perkin-Elmer Corp., Instrument Div., Norwalk, CT) equipped with a thermostatic block to maintain the temperature at 37°C. Band widths of 2.5 nm were used. Spectra were digitized and stored by a Perkin-Elmer 3600 data station (Perkin-Elmer Corp., Data Systems Group, Oceanport, NJ). After each experiment the cytosolic pH was equilibrated with the extracellular pH by treating the cells with 10 μM nigericin in the presence of 140 mM KCl to calibrate the measurements.

Results

Effect of External and Internal pH on the Rate of $^{36}Cl^-$ Uptake

When cells are incubated with isotonic KCl in the presence of nigericin, the pH of the cytosol is close to that of the buffer surrounding the cells (31). It is shown in Fig. 1 that when Vero cells were preincubated at pH 7.0 under such conditions, and then transferred to a buffer osmotically balanced with mannitol pH 7, and containing a trace amount of $^{36}Cl^-$, the cells accumulated $^{36}Cl^-$ at a rate that remained constant for 30–40 min and then, after the intracellular $^{36}Cl^-$ concentration had reached a level 5–6 times higher than that in the surrounding buffer, the amount of radioactivity associated with the cells started to decline.

The driving force for this uphill transport of $^{36}Cl^-$ against the concentration gradient is apparently efflux of anions from the cells by antiport, as previously described (18). In addition to efflux of anions by antiport a certain leakage occurs. Therefore, with time the driving force for $^{36}Cl^-$ entry is reduced and the rate of $^{36}Cl^-$ accumulation levels off and then the curves start to decline (18). Although we believe that the antiporter normally operates as a HCO_3^-/Cl^- antiporter, it is likely that it acts mainly by Cl^-/Cl^- exchange under the conditions used here (18).

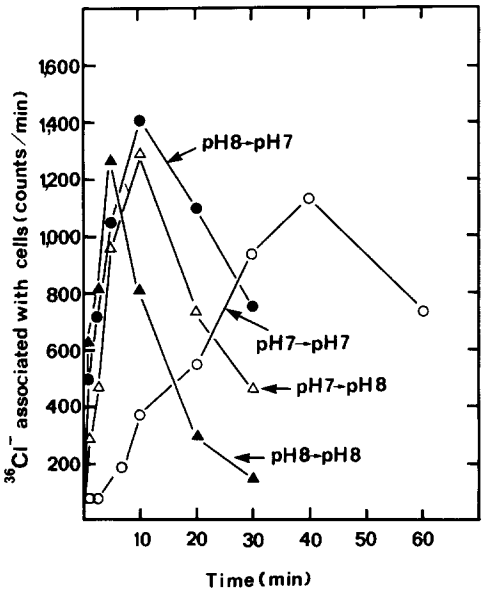

Figure 1. Effect of preincubation at pH 7 and pH 8 on the rate of chloride uptake. Vero cells in 24-well disposable trays were incubated for 10 min at 37°C in isotonic KCl buffer (0.14 M KCl, 1 mM $CaCl_2$, 20 mM MES) that contained 1 μM nigericin and adjusted with Tris to pH 7 (*open symbols*) or pH 8 (*closed symbols*). Then the cells were washed and the uptake of $^{36}Cl^-$ was measured, as described in Materials and Methods, in mannitol buffer adjusted to pH 7 (○ and ●) or pH 8 (△ and ▲).

When the preincubation with KCl and nigericin was done at pH 8.0, the uptake of $^{36}Cl^-$, measured at pH 7.0, occurred at an initial rate that was approximately 10 times higher than when the preincubation was at pH 7.0. Also, the amount of radioactivity associated with the cells started to decline more rapidly after preincubation at pH 8.0 than when the preincubation was at pH 7.0. This is in accordance with our previous findings that when the cytosol was alkalinized, the rate of anion efflux from the cells was strongly increased (18).

When the uptake was measured at pH 8.0 rather than at pH 7.0, there was much less difference in the initial uptake rate in cells preincubated under the two conditions, and the amount of radioactivity associated with the cells started to decline more rapidly (Fig. 1). In contrast, when the uptake was measured at pH 6 it occurred at the slow rate irrespective of the pH during the preincubation (data not shown). This is in accordance with our previous findings (18). Clearly the rate of $^{36}Cl^-$ uptake is influenced both by the pH during the preincubation and by the pH of the mannitol buffer during the uptake measurement. In the following experiments we have measured the uptake of $^{36}Cl^-$ in mannitol-balanced buffer at pH 7.0.

It is evident from the data in Fig. 1 that when the uptake was measured at pH 7.0 the difference in radioactivity associated with cells preincubated at pH 7.0 and pH 8.0 was maximal after 2–5 min. To determine more exactly at which pH during the preincubation the transition from the state of slow to the state of rapid uptake rate occurred, we preincubated cells at different pH in the presence of nigericin, valinomycin, and isotonic KCl and then measured the ability of the cells to accumulate $^{36}Cl^-$ during 3 min. Valinomycin was present in this experiment to ensure equal concentration of K^+ on the two sides of the membrane. It is shown in Fig. 2*A* that there was a strong increase in the uptake of $^{36}Cl^-$ in Vero cells between pH 7.0 and 7.3 and that the increase was half maximal at approximately pH 7.15. In the absence of ionophores the external pH influenced the rate of uptake to a lesser extent. The uptake of $^{36}Cl^-$ was in all cases strongly inhibited in the presence of 10 μM DIDS. Similar results to those with Vero cells were also obtained with L-cells (Fig. 2*B*). Also in HeLa OHIO cells, Hep_2 cells, and in fetal hamster kidney cells there was a pH-dependent increase in the chloride uptake rate, but the extent of increase differed among the different cell lines (data not shown).

It has been shown that the Na^+/H^+ antiporter is activated in cells treated with a number of growth factors, as well as with the tumor promoter TPA (2, 6, 8, 15, 22, 25, 29). After treatment with these agents the intracellular pH was therefore increased by 0.1–0.2 pH units (15, 25). The data in Fig. 2*A* show that TPA treatment of Vero cells also affected the anion antiporter. Thus, in TPA-treated cells the pH-induced increase in $^{36}Cl^-$ uptake occurred at ~0.2 pH units lower than in untreated cells. Although this difference is small, it was

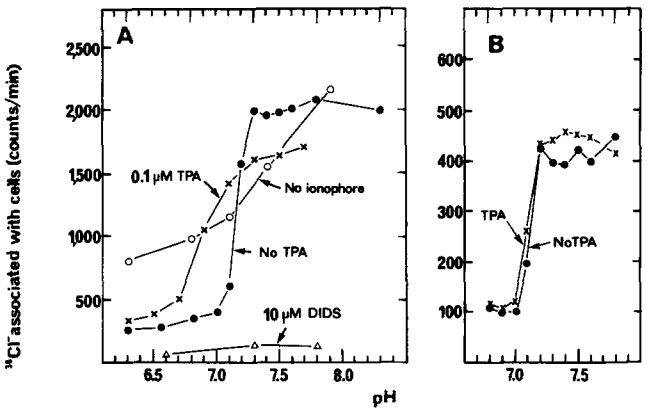

Figure 2. Effect of pH during the preincubation on the initial rate of $^{36}Cl^-$ uptake in Vero (*A*) and L-cells (*B*). Cells were preincubated for 15 min in isotonic KCl buffer that contained 1 μM nigericin and 10 μM valinomycin, when not otherwise indicated, and adjusted to the pH given on the abscissa. Then the cells were washed and their ability to accumulate $^{36}Cl^-$ during 3 min was measured in mannitol buffer adjusted to pH 7. In one case (△), 10 μM DIDS was added to the mannitol buffer that contained the isotope, and in one case (×) the cells had been incubated for 2 h with 0.1 μM TPA before the onset of the experiment. ○, no inophore; ●, ionophores present, no TPA.

consistently found in 12 independent experiments. In L-cells, TPA essentially had no effect on chloride uptake (Fig. 2*B*).

To test if it is the external or the internal pH during the preincubation, which is decisive for the rate of $^{36}Cl^-$ uptake, we took advantage of the observation that in the presence of nigericin it is possible to vary the intracellular pH by varying the extracellular K^+ concentration at constant extracellular pH (31). For this purpose we replaced part of the KCl with choline chloride to ensure isotonicity. Intracellular pH was measured with the membrane-permeable ester BCECF (21). The data in Fig. 3*A* show that also in Vero cells the internal pH varied with the K^+ concentration. The correlation between the internal pH and the logarithm of the K^+ concentration deviated from a straight line both at pH 7.8 and pH 7.4 in the surrounding buffer. This is probably due to loss of intracellular K^+ at low extracellular K^+ concentration in the presence of nigericin, as recently described (12, 31).

We then measured the ability of Vero cells to accumulate $^{36}Cl^-$ after preincubation in buffer with different KCl concentrations. As shown in Fig. 3*B*, the increase in $^{36}Cl^-$ uptake was half-maximal when 10 mM KCl was present in buffer adjusted to pH 7.8, whereas 70 mM was required when the external buffer was adjusted to pH 7.4. It can be estimated from Fig. 3*A* that in both cases this corresponds approximately to internal pH 7.1, which is equal to the value measured in Fig. 2. Clearly, therefore, the internal pH is decisive for the transition in chloride uptake rate under these conditions.

Rate of Transition between the States of High and Low Antiport Rate

If the change in the rate of anion antiport plays a role in the pH regulation in the cells, the cells should be able to respond rapidly with altered antiport rate upon changes in the internal pH. To test this, cells were preincubated with isotonic KCl in the presence of nigericin and valinomycin for 10 min at pH 7 and then they were transferred to the same buffer adjusted to different pH values. After increasing periods of time, the ability of the cells to accumulate $^{36}Cl^-$ during 3 min was then measured. It is shown in Fig. 4*A* that upon transfer to buffer with pH above 7.1 the ability of the cells to increase the uptake rate was rapidly established and that the rate of the transition increased with increasing pH. When cells preincubated at pH 7.8 were transferred to buffer adjusted to pH below 7.3, the opposite transition took place at a rate that increased with decreasing pH (Fig. 4*B*). In L-cells (Fig. 4*C*)

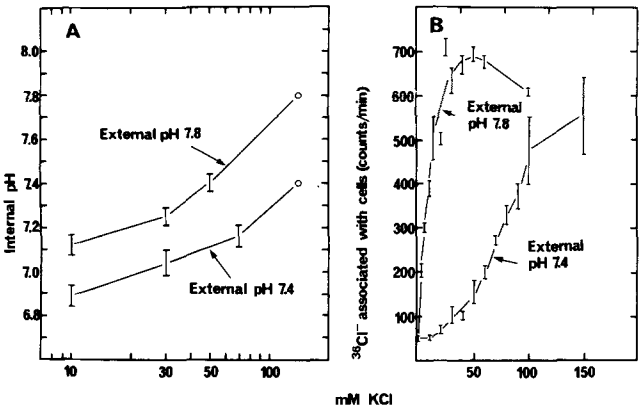

Figure 3. Effect of the concentration of KCl on the internal pH and on $^{36}Cl^-$ uptake. (*A*) Vero cells grown on coverslips were loaded with BCECF, and then the internal pH was measured as described in Materials and Methods after incubation for 10 min in a buffer that contained 5 μM nigericin, 1 mM $CaCl_2$, 20 mM MES adjusted to pH 7.4 or pH 7.8 with Tris, and either 0.14 M choline chloride, 0.14 M KCl, or a mixture of the two to give the concentration of KCl indicated on the abscissa. (*B*) Vero cells growing in 24-well disposable trays were incubated for 10 min with the same buffers as in *A*, and then their ability to take up $^{36}Cl^-$ during 3 min was measured. The data in *A* represent the average ± SD in five experiments, whereas the data in *B* represent the range in duplicate experiments.

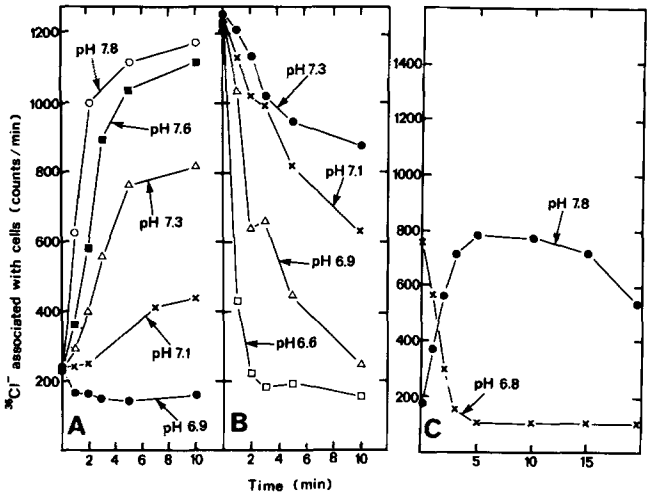

Figure 4. Rate of transition between high and low Cl^- uptake rate in Vero cells (*A* and *B*) and L-cells (*C*). Cells in 24-well disposable trays were incubated with isotonic KCl buffer that contained 1 μM nigericin and 10 μM valinomycin and adjusted to pH 7 or pH 7.8 as in Fig. 1. After 10 min at 37°C the cells were transferred to the same buffer adjusted to the pH indicated and, after increasing periods of time, the ability of the cells to take up $^{36}Cl^-$ during a 3-min interval was measured. In *A* the cells had been preincubated for 15 min at pH 7, in *B* at pH 7.8, whereas in *C* one sample (●) had been preincubated at pH 7.8 and one (×) at pH 6.8.

the transition occurred as rapidly as in Vero cells. Clearly, therefore, changes in the internal pH allow the cells to quickly increase and decrease the rate of anion antiport.

Kinetics of Chloride and Bicarbonate Binding and Uptake in Vero Cells Preincubated at pH 7 and pH 8

We have found earlier that after preincubation of Vero cells at high pH the efficiency (J_{max}) of the antiporter for chloride increased (18). To test this under conditions in which the internal pH was better controlled, we preincubated Vero cells for 10 min at pH 7 and pH 8 in buffer that contained nigericin and isotonic KCl, and then we measured the initial rate of chloride uptake in the presence of increasing concentrations of unlabeled NaCl. The data were transformed to Lineweaver-Burk plots as shown in Fig. 5. The results showed that both in cells preincubated at pH 7 and at pH 8 the K_m was ~7 mM. On the other hand the maximal transport rate was approximately 10-fold lower in cells preincubated at pH 7 (J_{max} = 1.7 × 10^8 Cl^- ions × $cell^{-1}$ × s^{-1}) than in cells preincubated at pH 8 (J_{max} = 1.59 × 10^9 Cl^- ions × $cell^{-1}$ × s^{-1}).

If the antiporter plays a role in the pH regulation of the cytosol, it is likely to involve transfer of HCO_3^- ions. Unfortunately, it is difficult to carry out uptake studies with labeled bicarbonate due to transmembrane transport of CO_2 as well as exchange with CO_2 in the air. We therefore attempted to estimate the affinity of the antiporter for bicarbonate by measuring the ability of bicarbonate to inhibit $^{36}Cl^-$ uptake. For this purpose we measured the initial rate of $^{36}Cl^-$ uptake in the presence of increasing concentrations of $NaHCO_3$ and at two different concentrations of NaCl. Although the experiments were not carried out in a CO_2-incubator, the concentration of HCO_3^- and the pH remained essentially constant during the experiments, which lasted for only 0.5–10 min at 24°C.

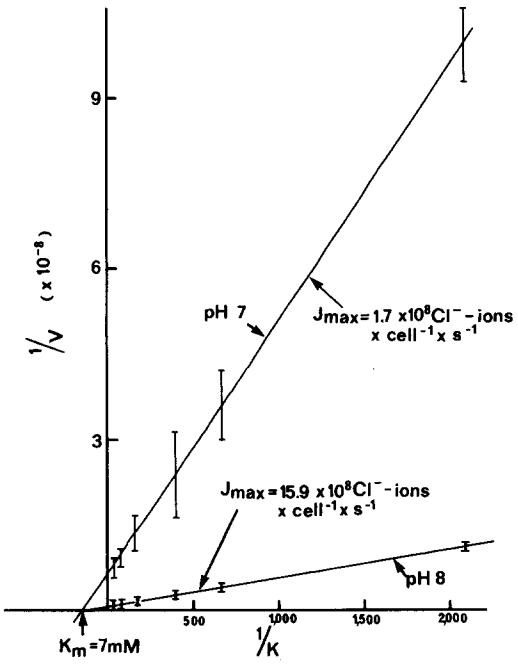

Figure 5. Lineweaver-Burk plot of Cl^- uptake in cells preincubated at pH 7 and pH 8. Vero cells in 24-well disposable trays were preincubated for 10 min at 37°C in isotonic KCl buffer that contained 1 μM nigericin adjusted to pH 7 or pH 8 as in Fig. 1, and then the initial rate of $^{36}Cl^-$ uptake was measured at 24°C in mannitol buffer, pH 7, that contained increasing concentration of unlabeled NaCl. In the case of cells pretreated at pH 8 the first time points were taken within the first minute. The symbols represent average ±SD in four independent experiments. V = Cl^- ions × $cell^{-1}$ × s^{-1}; K = M Cl^-.

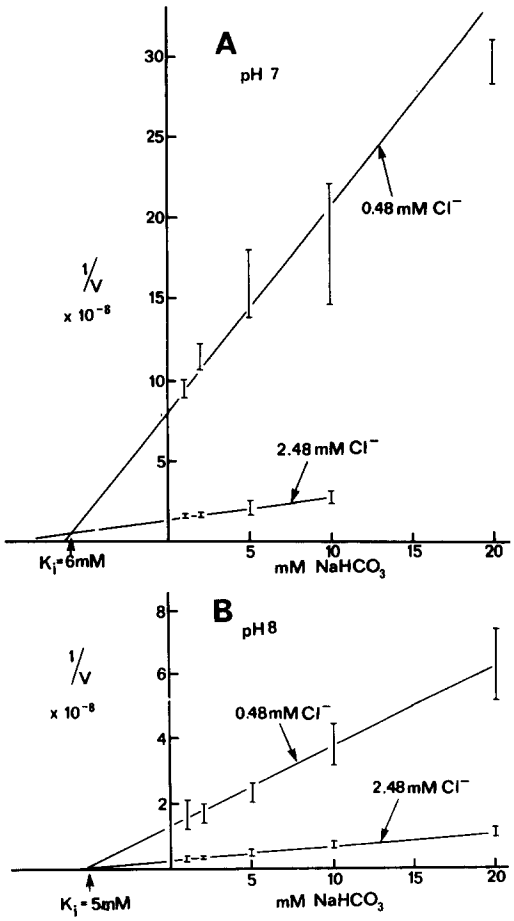

Figure 6. Dixon plots of the inhibition of Cl^- uptake by bicarbonate in cells preincubated at pH 7 (*A*) and pH 8.0 (*B*). Vero cells in 24-well disposable trays were preincubated for 10 min at 37°C in isotonic KCl buffer that contained 1 μM nigericin as in Fig. 1, and then the initial rate of $^{36}Cl^-$ uptake was measured at 24°C in mannitol buffer, pH 7, that contained increasing concentrations of $NaHCO_3$ as given on the abscissa and either 0.48 mM or 2.48 mM NaCl. The data represent average ±SD in 2–6 independent experiments. V = Cl^- ions × $cell^{-1}$ × s^{-1}; K = mM $NaHCO_3$.

Thus, control experiments with ^{14}C-bicarbonate showed that incubation for 60 min was required to reduce the bicarbonate concentration by one-half.

The uptake data obtained were transformed to Dixon plots, and the K_i for bicarbonate was measured in cells preincubated at pH 7 and pH 8. The data in Fig. 6, *A* and *B* show that the K_i was 5–6 mM in the two cases. Altogether, the data indicate that the affinity of the antiporter for bicarbonate is approximately the same as that for chloride, and that the increased rate of anion exchange at internal pH above pH 7.1 is due to increased transport capacity and not to increased affinity for the anions.

Discussion

We have presented here evidence that in Vero cells and in L-

cells there is an anion antiporter that is sensitive to changes in the intracellular pH. The activity of the antiporter increased approximately 10-fold over a very narrow pH interval of only 0.3 pH units. Also in HeLa cells, Hep_2 cells and, to a lesser extent in fetal hamster kidney cells, the rate of chloride uptake was increased after treatment with alkaline pH. Possibly, therefore, a pH-regulated anion antiporter is ubiquitous in nucleated cells.

The best characterized anion antiporter is band 3 in erythrocytes which rapidly exchanges Cl^- for HCO_3^- in peripheral tissues and in the lung (for reviews see references 5 and 27). At 25°C the rate of exchange by the erythrocyte exchanger was found to be 1.26×10^{10} Cl^- ions $\times$ $cell^{-1}$ s^{-1} (4). Since the red cells contain $\sim 10^6$ band 3 molecules per cell, this correspond to ~12,000 Cl^- ions exchanged per band 3 molecule per second. We found in Vero cells, pretreated at pH 8, that 1.59×10^9 Cl^- ions $\times$ $cell^{-1} \times s^{-1}$ were taken up at 24°C. If the anion antiporter in Vero cells is identical with the diphtheria toxin binding site as our data suggest (19, 20), the number of antiporter molecules could be identical with the number of toxin binding sites, which is $\sim 10^5$ per Vero cell (13, 14). In this case the uptake rate would be ~16,000 Cl^- ions $\times$ s^{-1} per antiporter molecule, a value very close to that found with the erythrocyte exchanger.

The Na^+/H^+ antiporter is believed to play a role in increasing the internal pH after acidification of the cytosol (16, 26, 29). In several systems a Na^+-linked Cl^-/HCO_3^- antiporter has been shown to participate in increasing the intracellular pH after artificial acidification (3, 10, 11, 24, 30). Possibly the anion antiporter here described has the opposite effect in decreasing the pH when the cytosol has become too alkaline. The rapidity with which it is turned on and off is in accordance with this possibility. As will be discussed in a forthcoming paper, DIDS in fact inhibits the normalization of the pH in alkali-loaded cells kept in a bicarbonate-containing medium.

We thank Mrs. Jorunn Jacobsen for her excellent technical assistance with the cell cultures.

Received for publication 23 September 1985, and in revised form 12 November 1985.

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