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INHIBITION OF CHLORIDE/BICARBONATE ANTIPORTS IN MONKEY KIDNEY CELLS (VERO) BY NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

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Abstract—Two chloride/bicarbonate antiport mechanisms are involved in the regulation of cytosolic pH (pH_i) in Vero cells, namely Na^+-dependent chloride/bicarbonate antiport to normalize pH_i after acidification of the cytosol, and Na^+-*in*dependent Cl^-/HCO_3^- exchange to regulate pH_i back to normal after alkalinization of the cytosol. We have tested the effects of the non-steroidal anti-inflammatory drugs acetylsalicylic acid (aspirin), salicylic acid, indomethacin and piroxicam on chloride/bicarbonate exchange and on chloride self exchange in Vero cells. All these drugs were found to inhibit both the Na^+-*in*dependent and the Na^+-linked chloride/bicarbonate antiport in a dose dependent manner. The Na^+-*in*dependent chloride/bicarbonate antiport was inhibited by lower doses of the drugs than the Na^+-linked antiport. The ability of the drugs to inhibit chloride self exchange did not vary much with varying external pH, indicating that the inhibitory effect is due to the anionic form of the drugs. Inhibition occurred immediately upon addition of the drugs, and it was rapidly reversible, indicating that the inhibitory effect is due to direct interaction of the drugs with chloride/bicarbonate antiport, and not to inhibition of prostaglandin synthesis. The relevance of our findings to the clinical effects of the drugs is discussed.

A number of investigations indicate that non-steroidal anti-inflammatory drugs (NSAIDs)† interfere with ion transport across cell membranes. Thus, all aspirin-like drugs may interfere with the absorption of salt by the kidneys [1–4], they may cause pH disturbances in tissues and blood [5, 6], partly due to inhibition of Cl^-/HCO_3^- exchange by capnophorin (band III) in erythrocytes [7–10], and they may give rise to ulcers in the gastric mucosa. The latter effect could be due to inhibition of transport of bicarbonate across plasma membrane of mucosal cells [11–13].

We have recently shown that two bicarbonate/chloride exchange mechanisms participate in the regulation of pH in the cytosol of Vero cells and several other nucleated mammalian cell lines [14–16]. One of these mechanisms is sodium dependent while the other is sodium *in*dependent. The Na^+-dependent exchange brings Na^+ and HCO_3^- into the cells in exchange with Cl^-, probably as $NaCO_3^-/Cl^-$ exchange. Due to the inwardly directed Na^+ gradient, HCO_3^- is transported into the cytosol where it reacts with protons, and the pH_i therefore increases. Thus, the Na^+-dependent chloride/bicarbonate antiport regulates pH back to normal after acidification of the cytosol. The Na^+-*in*dependent Cl^-/HCO_3^- exchange is involved in the normalization of the cytosolic pH after alkalinization. The gradients of Cl^- and HCO_3^- existing under normal conditions are such that extracellular Cl^- is exchanged with cytosolic HCO_3^-, and this antiport therefore tends to reduce the pH_i [14]. The activity of the sodium *in*dependent antiport is regulated by the cytosolic pH. In the majority of cell lines tested the rate of Cl^-/HCO_3^- exchange increases strongly when the intracellular pH is increased over a narrow range around neutrality [17–19]. In bicarbonate-containing buffers, the steady state pH_i in Vero cells appears to be the result of a balance between the alkalinizing activity of the Na^+-dependent $Cl^-HCO_3^-$ exchange the the acidifying activity of the Na^+-*in*dependent Cl^-/HCO_3^- antiport [14, 15].

To our knowledge there has been no studies on the effect of NSAIDs on the two Cl^-/HCO_3^- antiports in nucleated mammalian cells. As the monkey kidney cell, Vero, possess both Na^+-dependent and Na^+-*in*dependent chloride/bicarbonate antiport, this cell line is a useful model to study the influence of drugs on these mechanisms. It has earlier been shown that salicylate inhibits chloride transport by band III in erythrocytes [7]. The chloride/bicarbonate antiport in Vero cells differs from that in erythrocytes in many respects [14, 15, 17–19]. In the first place the rate of HCO_3^-/Cl^- antiport in Vero cells is increased approximately 10-fold when the internal pH is increased over a narrow range around neutrality, whereas this is not the case in erythrocytes. Furthermore, the capacity for antiport is much lower in Vero cells than in erythrocytes. Finally, whereas in erythrocytes the main anion antiport activity is due to HCO_3^-/Cl^- exchange, in Vero cells there is also

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† Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; BCECF, (2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein); MES, 2-(*N*-morpholino ethane)sulfonic acid; HEPES, *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid; Tris, Tris (hydroxy methyl)amino ethane; DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid; pH_i, cytosolic pH; pH_o, extracellular pH.

Table 1. Composition of buffers used

Buffer	Composition
Mannitol	260 mM mannitol, 2.5 mM $Ca(OH)_2$, 20 mM HEPES adjusted to the indicated pH with MES or Tris.
K-gluconate	140 mM K-gluconate, 2.5 mM Ca $(OH)_2$, 20 mM HEPES adjusted to the indicated pH with MES or Tris.
KCl	140 mM KCl, 2.5 mM $Ca(OH)_2$, 20 mM HEPES adjusted to the indicated pH with MES or Tris.
Choline chloride	140 mM choline chloride, 2.5 mM $Ca(OH)_2$, 20 mM HEPES adjusted to the indicated pH with MES or Tris.
PBS (phosphate buffered saline)	140 mM NaCl, 10 mM Na-phosphate, pH 7.4.

a Na^+-linked bicarbonate/chloride exchange mechanism [14, 15]. We therefore decided to test the effect of salicylate and other NSAIDs on chloride/bicarbonate antiport in the kidney derived Vero cells by measuring pH_i changes and relevant ion fluxes under different conditions.

MATERIALS AND METHODS

Materials. $H^{36}Cl$ (sp. act. 15.7 μCi/mg Cl) was obtained from the Radiochemical Centre (Amersham, U.K.). BCECF (2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein) was from Molecular Probes (Eugene, OR). Bumetanide was a generous gift from Leo Pharmaceuticals (Oslo, Norway). 2-(*N*-morpholino ethane)sulfonic acid (MES), *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid (HEPES), Tris (hydroxy methyl) amino ethane (Tris), 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid (DIDS), indomethacin, piroxicam, and nigericin were obtained from Sigma Chemical Co. (St Louis, MO). Acetylsalicylic acid was obtained from Bayer (Basel, Switzerland). Salicylic acid was from Merck (Darmstadt, F.R.G.). Amiloride was generously given to us by Merck, Sharp & Dohme (Drammen, Norway).

Buffers. HEPES medium: Minimal Essential Medium where the bicarbonate was replaced by 20 mM HEPES. The composition of the other buffers used is found in Table 1.

Cells. Vero cells (from African green monkey kidney) were kept as monolayer cultures in Minimal Essential Medium with 10% (v/v) fetal calf serum in an atmosphere containing 5% CO_2. The day before the experiment the cells were seeded into 24-well disposable trays or on coverslips placed in the bottom of Petri dishes at a density of 0.5–2 $\times$ 10^5 cells/cm^2.

Measurement of uptake of $^{36}Cl^-$. Cells in 24-well disposable trays were treated as indicated in legends to figures. Then the cells were washed twice with ice-cold Cl^--free buffer. Subsequently, the cells were incubated in the buffers indicated containing 0.17 μCi $H^{36}Cl$ and with or without additions as indicated. After the indicated periods of time the cells were washed twice with ice-cold PBS, and 0.3 ml of 5% (w/v) trichloroacetic acid was added per well. The cell-associated radioactivity extracted with trichloroacetic acid was measured.

Measurement of bicarbonate-linked Na^+ *uptake.* Cells in 24-well disposable trays were preincubated as indicated. The cells were washed three times with ice cold choline chloride buffer, and incubated with $^{22}NaCl$ in choline chloride buffer, pH 7.4, containing 10 mM choline bicarbonate. Then 0.3 ml liquid paraffin (Merck) was added to each well to prevent escape of CO_2, and the cells were incubated at 37°. After increasing periods of time, the paraffin and the buffer were removed by suction, each well was washed three times with ice-cold PBS, and then 0.3 ml of 5% (w/v) trichloroacetic acid was added. The radioactivity associated with the cells was measured.

Measurement of pH in the cytosol. Intracellular pH (pH_i) was measured with the pH dependent fluorescent probe BCECF as earlier described [14]. For this purpose cells were grown to confluence on 1 $\times$ 3 cm glass coverslips. Before the experiment, the coverslip was transferred to serum-free HEPES medium with 5 μM BCECF and incubated at 37° for 30 min. Then the coverslip with the cells was washed to remove the extracellular dye and 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 equipped with a thermostatic block to maintain the temperature at 37°. Band widths of 5 nm were used. The spectra were digitized and stored by a Perkin–Elmer 3600 data station. To calibrate the measurements the cytosolic pH was equilibrated with the extracellular pH by treating the cells with 10 μM nigericin in the presence of 140 mM KCl [20].

RESULTS

Effect of salicylate on the rate of $^{36}Cl^-$ *uptake*

We have earlier presented evidence that $^{36}Cl^-$ uptake by anion antiport measured as $^{36}Cl^-/Cl^-$ self-exchange reflects the activity of the Na^+-*in*dependent Cl^-/HCO_3^- exchanger [14, 15]. In these experiments the uptake of $^{36}Cl^-$ by antiport is measured in a buffer, pH 7.0, osmotically balanced with mannitol to avoid co-transport with Na^+ and K^+, and containing 0.5 mM of $^{36}Cl^-$. Under these conditions Vero cells accumulate $^{36}Cl^-$ 5–10 times

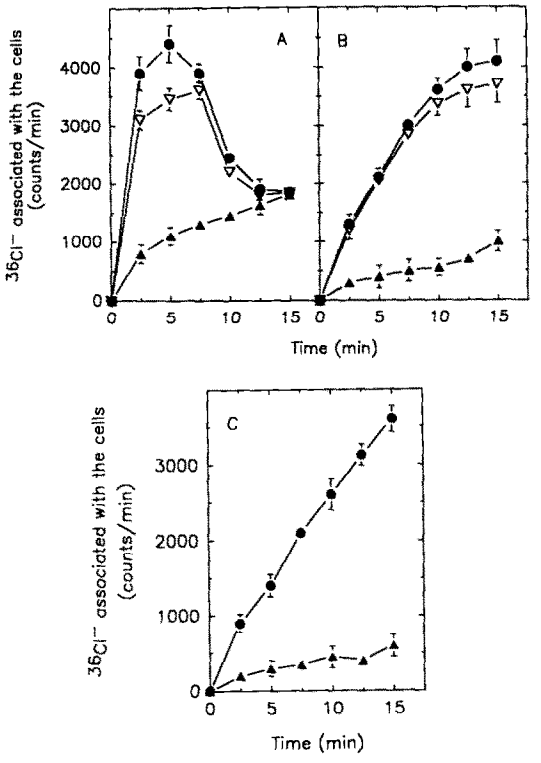

Fig. 1. Effect of salicylic acid and acetic acid on uptake of $^{36}Cl^-$. Vero cells in 24-well disposable trays were preincubated for 20 min in HEPES medium at 37°, pH 8.0 (A), or pH 6.0 (B, C). Then the medium was removed and the cells were washed twice with ice-cold mannitol buffer. Subsequently 0.3 ml/well of mannitol buffer (A, B) or K-gluconate buffer (C) at 37°, pH 7.0, containing 0.17 μCi/ml of $H^{36}Cl$ and the inhibitors indicated was added. After the indicated periods of time the buffer was removed and the cells were washed twice with ice cold PBS. Finally, 0.3 ml/well of 5% (w/v) trichloroacetic acid was added to extract $^{36}Cl^-$ from the cells, and the extracted radioactivity was measured. The additions were: (●), none; (▲), 2 mM salicylic acid; (▽), 2 mM acetic acid. The error bars represent SD in four experiments. Where no error bars are shown, the SD is less than the size of the symbol.

more rapidly at cytosolic pH 7.4 than at 6.7. The cells accumulate radioactivity to a concentration 5–10-fold higher than that in the surrounding buffer and the accumulation of $^{36}Cl^-$ is dependent upon the presence of intracellular Cl^-. The uptake is strongly inhibited by DIDS, which is a potent inhibitor of chloride/bicarbonate antiport. This strongly indicates that the uptake of $^{36}Cl^-$ is driven by the outward directed chloride gradient, as Cl^-/Cl^- self-exchange.

In the first set of experiments Vero cells were preincubated in HEPES medium, pH 8.0 or 6.0, for 20 min. The initial uptake of $^{36}Cl^-$ was much faster in cells preincubated at pH 8.0 (Fig. 1A) than in cells preincubated at pH 6.0 (Fig. 1B). Measurement of the internal pH by the fluorescent probe BCECF showed that in cells preincubated at pH 8.0 and pH 6.0 the internal pH was 7.4 and 6.7, respectively (data not shown).

It should be noted that at the high pH_i (Fig. 1A), the uptake rapidly levelled off and then the amount of $^{36}Cl^-$ associated with the cells started to decline. This is apparently due to the high Cl^- permeability induced at high pH_i [15, 19].

In the presence of 2 mM salicylate the chloride uptake was strongly inhibited both when the antiport was in the high activity state (Fig. 1A) and in the low activity state (Fig. 1B).

Salicylic acid is a membrane permeant weak acid (pK_a 3.9) that dissociate intracellularly and may acidify the cytosol [21]. Because the uptake of $^{36}Cl^-$ is critically dependent upon pH_i [14, 15, 18, 19], the possibility was considered that the reduced uptake of $^{36}Cl^-$ in the presence of salicylic acid could be due to reduction in pH_i caused by the weak acid, and not to a direct action on the chloride/bicarbonate antiport. pH_i in cells incubated at pH 7.0 in the presence of 2 mM salicylic acid was less than 0.05 pH unit lower than in the absence of the drug (data not shown). However, even this slight decrease of pH_i could reduce the $^{36}Cl^-$ uptake. We therefore tested if a similar acidification of the cytosol by acetic acid inhibited $^{36}Cl^-$ uptake to the same extent as salicylic acid. Experiments with BCECF-loaded cells showed that addition of 10 mM acetic acid caused essentially the same acidification as 10 mM salicylic acid (data not shown). The data in Fig. 1B show that addition of 2 mM acetic acid did not decrease the uptake of $^{36}Cl^-$ when the antiport was in the low activity state. In cells preincubated at pH 8.0, there was a slight inhibition of $^{36}Cl^-$ uptake in the presence of 2 mM acetic acid (Fig. 1A), but much less than in the presence of 2 mM salicylic acid. It may therefore be concluded that the strong inhibition of chloride/bicarbonate antiport by salicylic acid is not an effect due to acidification of the cytosol.

To test if the inhibition of $^{36}Cl^-$ uptake caused by the drugs could be related to a possible change in the membrane potential in the presence of drugs, the uptake of $^{36}Cl^-$ was measured in K-gluconate buffer which strongly reduces the membrane potential by abolishing the K^+ diffusion potential [22]. The uptake of $^{36}Cl^-$ was somewhat lower than in mannitol-balanced buffer, probably due to the inhibiting effect of gluconate on chloride uptake [23]. The extent of inhibition by the drugs was, however, essentially the same indicating that the inhibition is not due to a change in the membrane potential (Fig. 1C).

The possibility was considered that the decreased uptake of $^{36}Cl^-$ in the presence of drugs could be due to an increased Cl^- efflux and as a consequence of this a decreased driving force for $^{36}Cl^-$ uptake. Control experiments showed that the salicylic acid and other NSAIDs did not increase the rate of Cl^- efflux, but on the contrary strongly inhibited the efflux of $^{36}Cl^-$ into a chloride-free buffer (data not shown).

Effect of other anti-inflammatory analgesics on the uptake of $^{36}Cl^-$ under different ionic conditions

To study if also anti-inflammatory drugs other than salicylic acid inhibit chloride/bicarbonate antiport in nucleated cells, we tested indomethacin, piroxicam and acetylsalicylic acid for their ability to inhibit $^{36}Cl^-$ uptake. Indomethacin is, like salicylic acid, a carboxylic acid (pK_a 4.1), whereas piroxicam contains an enolic acid group (pK_a 6.3). For each drug

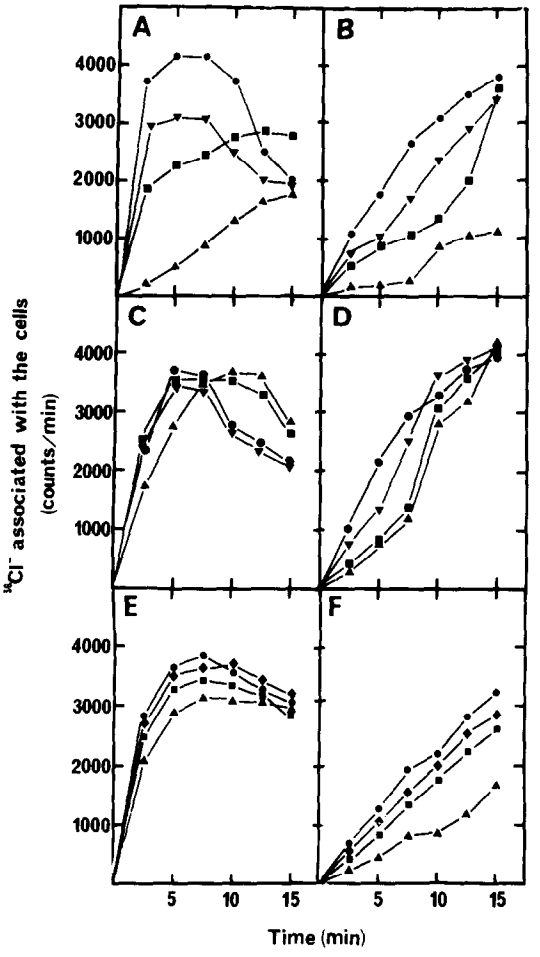

Fig. 2. Effect of indomethacin, piroxicam and acetylsalicylic acid on $^{36}Cl^-$ uptake by chloride/bicarbonate antiport. Experimental conditions as described in Fig. 1. The cells were preincubated for 20 min in HEPES medium at 37°, pH 8.0 (A, C, E), or pH 6.0 (B, D, F). In A and B the additions were: (●), none; (▼), 2 μM indomethacin; (■), 20 μM indomethacin; (▲), 200 μM indomethacin. In C and D the additions were: (●), none; (▼), 2 μM piroxicam; (■), 20 μM piroxicam; (▲), 200 μM piroxicam. In E and F the additions were: (●), none; (▼), 20 μM acetylsalicylic acid; (■), 200 μM acetylsalicylic acid; (▲), 2 mM acetylsalicylic acid.

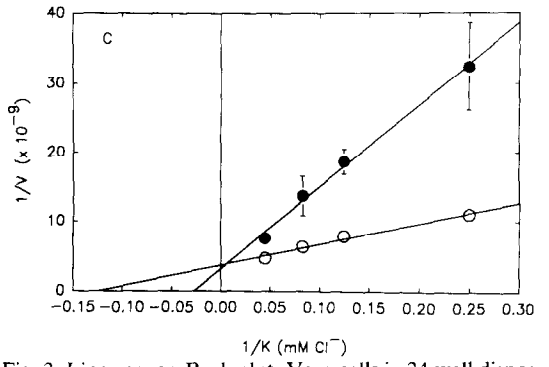

Fig. 3. Lineweaver–Burk plot. Vero cells in 24-well disposable trays were incubated for 20 min in HEPES medium, pH 6.0, in the absence of salicylic acid. The uptake of 0.64 μCi/ml of $^{36}Cl^-$ was measured after 3 min in mannitol buffer with increasing amount of unlabelled chloride in the absence and presence of 2 mM salicylic acid. The additions were: (○), none; (●), 2 mM salicylic acid. The error bars represent SD in three experiments. Where no error bars are shown, the SD is less than the size of the symbol. V = Cl^- ions/cell/sec.

two different sets of experiments were carried out. The cells were preincubated for 20 min either at pH 8.0 or pH 6.0, and the uptake of $^{36}Cl^-$ was measured in mannitol balanced buffer at pH 7.0 in the absence or presence of drugs.

We found that there was a dose dependent inhibition of $^{36}Cl^-$ uptake in the presence of indomethacin, piroxicam and acetylsalicylic acid, both when the antiporter was in the high activity (Fig. 2A, C and E) and in the low activity state (Fig. 2B, D and F). Indomethacin was found to be the most potent inhibitor. Thus at 20 μM it reduced the uptake rate of $^{36}Cl^-$ to approximately half the control value (Fig. 2A and B). In the case of piroxicam (Fig. 2C and D) and acetylsalicylic acid (Fig. 2E and F) 200 μM and 1–2 mM, respectively, was required to obtain the same extent of inhibition.

One interesting observation in Fig. 2B, D and F, is that the inhibition of the antiporter in the low activity state was most pronounced in the first 10 min of the experiment. Then, in spite of the drug being present, the rate of chloride uptake started to increase, suggesting that the antiporter was regulated to a state of higher activity [24].

To investigate if the inhibition of chloride uptake by the drugs was due to competition with chloride at the transport site, uptake of $^{36}Cl^-$ was measured in buffers containing increasing concentrations of unlabeled chloride, and the data were plotted according to Lineweaver and Burk. The results depicted in Fig. 3 showed that the drugs strongly increased the apparent K_m, whereas V_{max} was not substantially altered. Thus, in cells not treated with the drugs, K_m was 8 mM, and in cells treated with 2 mM salicylic acid the apparent K_m was 40–50 mM (Fig. 3). Similar results were found with 200 μM indomethacin (apparent $K_m > 60$ mM) and 200 μM piroxicam (apparent K_m 35–40 mM) (data not shown). This indicates that the inhibition was mainly competitive.

Effect of external pH on the inhibitory effect of anti-inflammatory analgesics on $^{36}Cl^-$ uptake

Since the anti-inflammatory drugs are weak acids, the concentration of protonated drug decreases strongly when the pH in the buffer is increased from 6 to 8, whereas the concentration of the anionic form does not change much. To investigate if the magnitude of inhibition changed with variations in the amount of protonated drug, we altered the external pH. For this purpose we measured the uptake of $^{36}Cl^-$ in mannitol buffer at different pH values (Fig. 4). The mannitol buffer does not contain permeant ions to exchange with H^+ or OH^-. Therefore the cells are not able to increase their pH_i to a large extent if the external pH is higher than the internal pH. However, if pH_o is lower than pH_i the internal pH may decrease due to efflux of Na^+ in exchange with H^+. To prevent this, the uptake of $^{36}Cl^-$ was

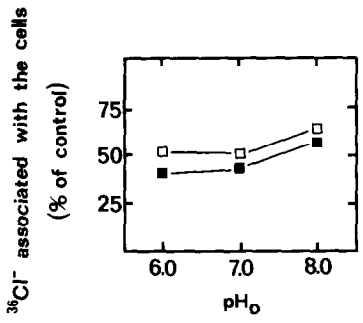

Fig. 4. Effect of external pH on the ability of indomethacin and piroxicam to reduce the rate of $^{36}Cl^-$ uptake. Vero cells in 24-well disposable trays were preincubated in HEPES medium, pH 7.4, at 37° for 25 min without drugs. The uptake of $^{36}Cl^-$ was then measured for 2 min in mannitol buffer containing 1 mM amiloride at 20° and the pH indicated on the abscissa in the absence or presence of indomethacin or piroxicam. The amount of cell-associated radioactivity in samples incubated in the presence of anti-inflammatory drugs is presented as per cent of that obtained in cells incubated without other drugs than amiloride. The uptake of $^{36}Cl^-$ in control samples were 800 counts/min at pH 6.0, 1650 counts/min at pH 7.0 and 750 counts/min at pH 8.0. (■), 20 μM indomethacin; (□), 200 μM piroxicam.

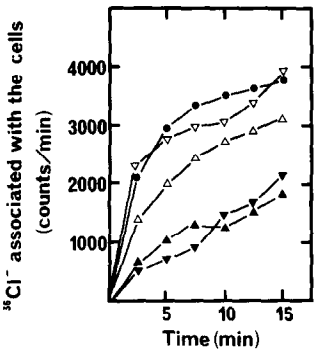

Fig. 5. Reversibility of the inhibitory effect of piroxicam and indomethacin on $^{36}Cl^-$ uptake. Vero cells in 24-well disposable trays were preincubated in HEPES medium, pH 6.0, for 20 min. Then the cells were washed twice with ice-cold mannitol buffer and incubated for 3 min in mannitol buffer, pH 7.0, at room temperature in the absence or presence of drugs as indicated. Subsequently, the cells were washed three times with ice-cold mannitol buffer, transferred to mannitol buffer, pH 7.0, containing 0.17 μCi $^{36}Cl^-$ and drugs as indicated. After incubation at 37° for the indicated periods of time, the amount of radioactivity associated with the cells was measured. The additions were: (●), none; (▽), 200 μM piroxicam only during preincubation; (△), 20 μM indomethacin only during preincubation; (▼), 200 μM piroxicam throughout experiment; (▲), 20 μM indomethacin throughout experiment.

measured in the presence of 1 mM amiloride which inhibit Na^+/H^+ exchange. Under these conditions the internal pH changed very slowly in response to alterations in the external pH. Therefore, the effect of variations in the external pH could be studied without much change in the internal pH, at least during the first 3 min of the experiments. In the absence of anti-inflammatory drugs the uptake of $^{36}Cl^-$ was higher at pH_o 7.0 than both at pH_o 8.0 and at pH_o 6.0. However, as shown in Fig. 3, the relative inhibition by indomethacin and piroxicam measured as percent of the uptake in cells not exposed to the drugs was essentially the same at the different pH_o values, indicating that the charged form of the drug is most important for the inhibitory effect.

Reversibility of the inhibitory effect

To test if the inhibitory effect of the anti-inflammatory drugs is reversible, cells were incubated in mannitol buffer in the presence of drugs at room temperature and then quickly washed with ice-cold mannitol buffer. Then the uptake of $^{36}Cl^-$ was measured in the absence or presence of drugs. Figure 5 shows that piroxicam did not inhibit chloride/bicarbonate antiport unless it was present during the uptake of $^{36}Cl^-$, indicating that the inhibitory effect of this drug is quickly reversible. In the absence of unlabelled chloride the inhibitory effect of indomethacin was not completely reversible. However, when 5 mM unlabelled chloride was present during the uptake of $^{36}Cl^-$, the inhibition by indomethacin was almost eliminated (data not shown). The finding that the inhibitory effect is reversible indicates that it is not due to a toxic effect of the drugs on the cells.

Effect of non-steroidal anti-inflammatory drugs on $^{36}Cl^-$ efflux

To test if the drugs also inhibited chloride/bicarbonate antiport at high chloride concentrations we measured the efflux rate of $^{36}Cl^-$ into buffers containing 140 mM Cl^-. Under these conditions more than 90% of $^{36}Cl^-$ efflux is DIDS-inhibitable and dependent on external chloride indicating that the efflux is through anion antiport in exchange with external Cl^-. The data in Fig. 6 show that the efflux was inhibited in a dose dependent manner by the drugs. It may therefore be concluded that the anti-inflammatory drugs tested inhibit both chloride uptake and chloride efflux by antiport.

Effect of anti-inflammatory analgesic drugs on bicarbonate dependent Na^+ uptake

We have earlier shown that in Vero cells Na^+ is transported by Na^+/H^+ exchange [1, 13, 6], Na^+-dependent chloride/bicarbonate antiport [15, 18] and Na^+,K^+,Cl^- cotransport (our unpublished data). Furthermore, sodium cotransport is involved in the uptake of sugars and amino acids.

To measure bicarbonate-dependent Na^+ uptake, we therefore used buffers without amino acids, sugars and potassium. Furthermore the buffers contained amiloride to inhibit the Na^+/H^+ exchanger and ouabain to inhibit the Na^+/K^+-ATPase. We have earlier shown that under these conditions the $^{22}Na^+$ uptake is strongly dependent upon the presence of bicarbonate, and the uptake is inhibited by DIDS. Furthermore, intracellular chloride is necessary for uptake [15].

As shown in Fig. 7, the anti-inflammatory analgesic drugs inhibited the bicarbonate dependent uptake of $^{22}Na^+$. Both 200 μM piroxicam, 200 μM indomethacin and 2 mM salicylic acid had an inhibi-

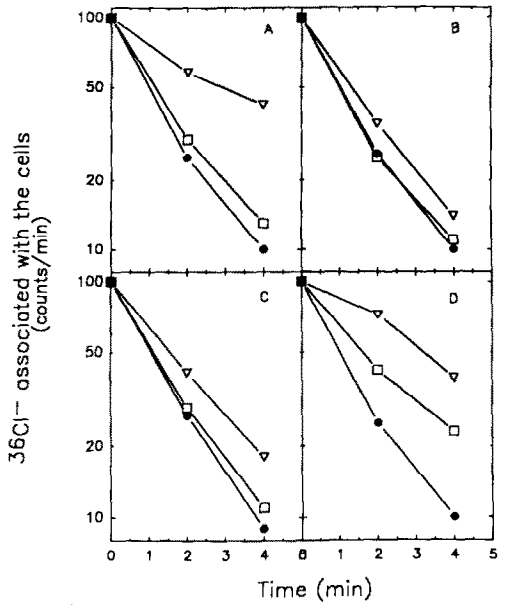

Fig. 6. Effect of NSAIDs on $^{36}Cl^-$ efflux. Vero cells were preincubated in HEPES medium, pH 6.0, for 20 min at 37°. Then the cells were washed twice with mannitol buffer, pH 7.0, and incubated with the same buffer containing 0.17 μCi/ml $^{36}Cl^-$ for 15 min at 37°. The cells were then transferred to KCl buffer, pH 7.0, at 24°. Drugs were present in the efflux buffer as indicated. The radioactivity associated with the cells was measured after increasing periods of time. The additions were: A: (●), none; (□), 0.2 mM salicylic acid; (▽), 2 mM salicylic acid. B: (●), none; (□), 0.2 mM acetylsalicylic acid; (▽), 2 mM acetylsalicylic acid. C: (●), none; (○), 20 μM piroxicam; (▽), 200 μM piroxicam. D: (●), none; (□), 20 μM indomethacin; (▽), 200 μM indomethacin.

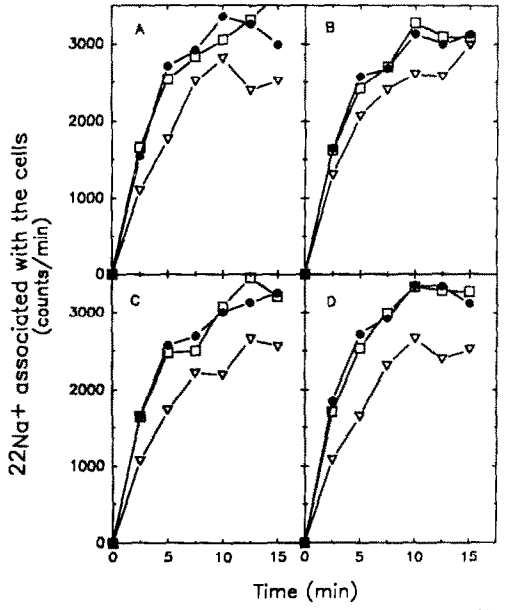

Fig. 7. Effect of NSAIDs on bicarbonate dependent $^{22}Na^+$ uptake. Vero cells in 24-well disposable trays were incubated in HEPES medium, pH 6.0, for 20 min at 37°. Then the medium was removed and 0.3 ml/well of choline chloride buffer with 10 mM choline bicarbonate, pH 7.3, and containing 1.0 μCi/ml $^{22}NaCl$ and drugs as indicated was added. Then the uptake of $^{22}Na^+$ was measured as described in Materials and Methods. The additions were: A: (●), none; (□), 0.2 mM salicylic acid; (▽), 2 mM salicylic acid. B: (●), none; (□), 0.2 mM acetylsalicylic acid; (▽), 2 mM acetylsalicylic acid. C: (●), none; (□), 20 μM piroxicam; (▽), 200 μM piroxicam. D: (●), none; (□), 20 μM indomethacin; (▽), 200 μM indomethacin.

tory effect, whereas one tenth of those doses were without effect.

To investigate if the Na^+-*in*dependent or the Na^+-linked antiport was more sensitive to the inhibition by drugs, the uptake of $^{22}Na^+$ and $^{36}Cl^-$ were measured under the same conditions in the absence and presence of drugs. As shown in Table 2, the uptake of $^{36}Cl^-$ was more strongly inhibited than the uptake of $^{22}Na^+$. Thus, the different anti-inflammatory analgesic drugs inhibit the two chloride/bicarbonate antiport systems to different extents.

Effect of anti-inflammatory analgesics on Na^+*-independent and* Na^+*-dependent bicarbonate linked changes of* pH_i

The two bicarbonate dependent pH regulating mechanisms can be studied independently by measuring pH_i under appropriate conditions. The activity of the Na^+-linked bicarbonate transport can be measured as bicarbonate dependent increase in pH_i in Na^+-containing buffers after acidification of the cytosol. When the pH_i is lowered, in Vero cells the activity of the Na^+-*in*dependent antiport is decreased to such a low level that it does not interfere with measurements of the activity of the Na^+-dependent process [14]. When the cytosol is acidified, H^+-extrusion also occurs by the Na^+/H^+ exchanger, but this can be inhibited by amiloride [14].

Table 2. Effect of indomethacin and piroxicam on the Na^+-independent ($^{36}Cl^-$) and the Na^+-linked ($^{22}Na^+$) chloride/bicarbonate antiport

	$^{36}Cl^-$	$^{22}Na^+$
	% of control	
Indomethacin 20 μM	55 ± 7	94 ± 8
Indomethacin 200 μM	13 ± 4	52 ± 9
Piroxicam 20 μM	69 ± 11	95 ± 8
Piroxicam 200 μM	39 ± 11	64 ± 12

Vero cells in 24-well disposable trays were preincubated in HEPES medium, pH 6.0, for 20 min. Then the cells were washed twice with ice-cold mannitol buffer, transferred to mannitol buffer, pH 7.0, containing either 0.17 μCi $^{36}Cl^-$ without HCO_3^- or 0.4 μCi/ml $^{22}Na^+$ with 10 mm HCO_3^-, and drugs as indicated. One mM amiloride was present to inhibit Na^+/H^+ exchange, and 50 μM ouabain was added to prevent extrusion of Na^+ by the Na^+, K^+, ATPase. Liquid paraffin was added to prevent escape of CO_2. After incubation at 37° for 3 min, the amount of radioactivity associated with the cells was measured. The results are expressed as per cent of radioactivity ± SD associated with cells not treated with indomethacin or piroxicam. The data are based on six measurements.

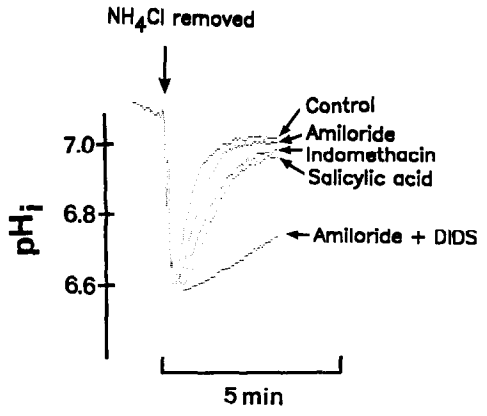

Fig. 8. Effect of indomethacin and salicylic acid on the regulation of pH_i in cells given an acid load. Vero cells loaded with BCECF as described in Materials and Methods were incubated in HEPES medium, pH 7.0, containing 15 mM NH_4Cl for 5 min. Then the cells were washed three times with HEPES medium, pH 7.0, and 10 mM choline bicarbonate was added in the same buffer with and without drugs as indicated. Amiloride was present together with indomethacin and salicylic acid. The fluorescence was recorded and calibrated as described in Materials and Methods.

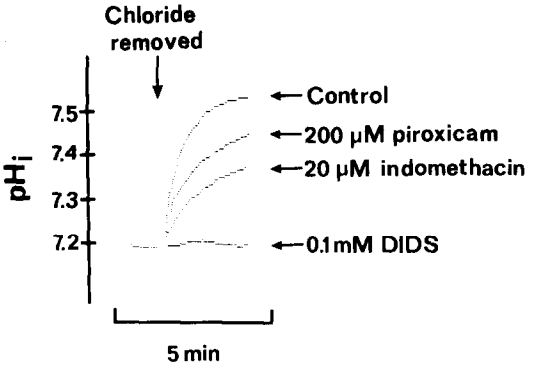

Fig. 9. Effect of piroxicam and indomethacin on the alkalinization induced by removal of extracellular chloride. Vero cells loaded with BCECF were incubated for 15 min at 37° in KCl buffer, pH 7.6, containing 10 mM choline bicarbonate. Subsequently, the cells were transferred to K-gluconate buffer, pH 7.6, containing 10 mM choline bicarbonate. When indicated, 200 μM piroxicam or 20 μM indomethacin were also present. The fluorescence was recorded and calibrated as described in Materials and Methods.

In the experiments in Fig. 8, an acid load was applied by using the ammonium prepulse technique as described earlier [14]. The data in Fig. 8 show that in the absence of inhibitors, normal pH_i was restored within 5 min. The regulation was partially inhibited by amiloride, whereas the combination of amiloride and DIDS strongly inhibited the regulation. The interesting observation in Fig. 8 is that also 200 μM indomethacin and 2 mM salicylic acid substantially inhibited the increase in pH_i after acidification in the presence of amiloride. 20 μM indomethacin and 0.2 mM salicylic acid was without effect. The data indicate that the pH_i-regulation by the Na^+-linked chloride/bicarbonate antiport is inhibited by the drugs.

We have earlier shown that at alkaline pH, removal of extracellular chloride induces a rapid increase in the pH_i due to Na^+-*in*dependent influx of bicarbonate driven by the outward directed chloride gradient [14]. To investigate if the non-steroidal anti-inflammatory drugs inhibit this increase in pH_i, BCECF-loaded cells were preincubated in a buffer containing 140 mM KCl, pH 7.6, and 10 mM choline-HCO_3^- and then transferred to a K-gluconate buffer with the same pH and the same concentration of bicarbonate. The K-gluconate buffer did not contain Na^+ to avoid interference from the Na^+-linked antiport.

The result in Fig. 9, show that the pH_i increased from 7.2 to 7.6 upon transfer to chloride-free buffer. As expected, the increase was blocked by 0.1 mM DIDS. The new observation is that also in the presence of 20 μM indomethacin or 200 μM piroxicam the increase in pH_i was considerably reduced. Altogether, the data indicate that the drugs here tested inhibit the pH_i regulation both by Na^+-dependent and Na^+-*in*dependent Cl^-/HCO_3^- exchange.

DISCUSSION

The mechanism of inhibition

The main observation presented here is that the four anti-inflammatory analgesic drugs tested all inhibit both Na^+-linked and Na^+-*in*dependent chloride/bicarbonate antiport in Vero cells. Other authors have found that the anti-inflammatory drugs acetylsalicylic acid and niflumic acid inhibit the activity of band III in erythrocytes [7, 8]. To our knowledge, there has been no studies on the effect of anti-inflammatory analgesic drugs on the two Cl^-/HCO_3^- antiports in nucleated mammalian cells. Structurally, the drugs here tested are anionic lipophilic compounds, and in this respect they resemble other inhibitors of chloride/bicarbonate antiport [25, 26].

One common mechanism of action for acetylsalicylic acid, indomethacin and piroxicam is the selective inhibition of prostaglandin synthesis from the precursor arachidonic acid [27, 28]. We do not think that this is the mechanism for the inhibition of chloride/bicarbonate antiport described in this paper. In the first place, inhibition of prostaglandin synthesis requires several minutes to affect cellular functions, because the prostaglandins already present must first be broken down. We found that preincubation with the drugs was not necessary to inhibit anion antiport. Secondly, the effect on chloride/bicarbonate antiport was rapidly reversible. If synthesis of prostaglandins were involved in restoration of the activity of the antiport, the inhibitory effect of the anti-inflammatory drugs should last for several minutes after the drugs were removed. Thirdly, we have earlier measured the inhibitory effect on prostaglandin synthesis by these drugs, and found that acetylsalicylic acid, piroxicam and indomethacin inhibited the synthesis, whereas salicylic acid exhibited no inhibitory effect [24]. The ability of the drugs to inhibit prostaglandin synthesis does not correlate with their capability to inhibit the two chloride/bicarbonate antiports as salicylic acid

is a more efficient inhibitor than acetylsalicylic acid. Finally, as discussed in another paper [24], prostaglandins inhibit rather than stimulate the Na^+-*in*dependent Cl^-/HCO_3^- exchange.

The ability of the drugs to inhibit Cl^-/HCO_3^- exchange did not vary strongly with the external pH. Thus, the relative inhibitory effect of the drugs was almost constant between pH_o 6.0 and 8.0. The concentration of the anionic form of the drugs is not very different at pH 6.0 and at pH 8.0. Only in the case of piroxicam is the anionic form approximately twice as high at pH 8.0 as at pH 6.0. In contrast to this, the concentration of the protonated form of the compound is approximately 100 times higher at pH 6.0 than at pH 8.0. The finding that almost the same inhibition was obtained at the two pH values therefore indicates that the inhibition of $^{36}Cl^-$ uptake is due to the anionic form of the drugs.

The anti-inflammatory analgesic drugs inhibit the Na^+-dependent and the Na^+-*in*dependent chloride/bicarbonate antiports to different extents. In general, higher concentrations were needed to inhibit the Na^+-linked antiport (measured as bicarbonate dependent uptake of $^{22}Na^+$) than to inhibit the Na^+-*in*dependent antiport (measured as $^{36}Cl^-$ uptake). This is in accordance with previous findings from this laboratory that various anion transport inhibitors inhibit the two kinds of antiport to different extent [29].

The action of NSAIDs on the activity of chloride/bicarbonate antiports are complicated. In addition to the inhibitory effect described in the present paper, we have found that preincubation with the drugs will under most circumstances activate the Na^+-*in*dependent antiport and inhibit the Na^+-dependent antiport [24]. The latter effects seem to require lower doses of the drugs under physiological ionic conditions than the direct inhibitory effects described in this paper.

Clinical relevance of the inhibition

To exert an anti-inflammatory effect in the tissues, the concentrations of the drugs are probably in the range 2 to 20 μM for indomethacin and piroxicam and 1 to 2 mM for salicylic acid [5]. In our experiments 20 μM of piroxicam had only a slight inhibitory effect on both chloride/bicarbonate antiports when the inhibition was measured at physiological concentrations of anions (see Figs 6 and 8). Twenty μM indomethacin considerably inhibited the Na^+-*in*dependent antiport, whereas the Na^+-linked antiport was not inhibited. On the other hand, 1–2 mM salicylic acid substantially inhibited both chloride/bicarbonate antiports. Thus, at anti-inflammatory concentrations of the drugs, piroxicam hardly affects chloride/bicarbonate antiport, whereas salicylic acid and probably also indomethacin is likely to inhibit Cl^-/HCO_3^- exchange.

Concentrations of all these drugs definitely high enough to inhibit anion transport are achieved in the body in a number of cases. Thus, after peroral intake of the drugs, 10–50 times higher concentrations than those mentioned above are regularly found in the upper gastrointestinal tract [5, 30, 31]. Interestingly, apical secretion of bicarbonate by gastric mucosa cells is partly mediated by Cl^-/HCO_3^- exchange [5, 11, 12]. Bicarbonate secretion is required to protect the cells against the luminal acid, and inhibition of chloride/bicarbonate antiport would therefore make the cells more susceptible to damage by the gastric acid. As such damage probably gives rise to peptic ulcers, it is interesting to note that a common side effect of the drugs here tested, is indeed upper gastrointestinal ulceration.

Overdose of the drugs here studied, and particularly of acetylsalicylic acid, is a common clinical problem, and in such situations tissue concentrations of the metabolite salicylic acid do reach concentrations of a few millimolar [6, 32], i.e. high enough to inhibit chloride/bicarbonate antiport. In fact, inhibition of Cl^-/HCO_3^- exchange mediated by the band III glycoprotein in erythrocytes is known to occur in salicylate-intoxicated patients [7–10]. HCO_3^-/Cl^- exchange is a rate limiting step in the transport of CO_2 from the periphery to the lungs [7–10]. Inhibition of this antiport will cause an increased CO_2 tension in the peripheral tissues as well as in the respiratory centre. This will result in a compensatory hyperventilation which is indeed found in patients moderately intoxicated with salicylate [5, 7–10, 32]. More severe inhibition of chloride/bicarbonate antiport in the erythrocytes is expected to cause acidosis because of accumulation of carbonic acid in the tissues. It is therefore likely that inhibition of Na^+-*in*dependent chloride/bicarbonate exchange in red blood cells is involved in the acid–base disturbances in patients with aspirin poisoning.

Chloride/bicarbonate antiport is probably also necessary for effective excretion of acid by the kidneys [33]. Cl^-/HCO_3^- antiport is believed to take place at the basolateral side of the tubules to compensate for H^+ excreted in the urine [33]. Inhibition of Cl^-/HCO_3^- antiport will therefore reduce the ability of the kidneys to excrete acid and to reabsorb HCO_3^- from the filtrate in the kidneys. Interestingly, increased urinary excretion of bicarbonate is regularly found in aspirin intoxicated patients [5, 6, 32].

Recently data have been presented indicating that a fraction of the active NaCl transport in the proximal convoluted tubule occurs via parallel Na^+/H^+ and Cl^-/HCO_3^- exchangers [34]. In accordance with this, intoxication with aspirin which inhibits chloride/bicarbonate antiport results in reduced Na^+ and Cl^- absorption by the kidneys. This may induce a loss of salt that may exaggerate the dehydration that regularly occurs in intoxicated patients [5, 6, 32]. Altogether it may be concluded that inhibition of chloride/bicarbonate antiport may be involved in several of the clinical effects of the non-steroidal anti-inflammatory drugs here studied.

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REFERENCES

1. Dunn MJ, Nonsteroidal antiinflammatory drugs and renal function. *Ann Rev Med* **35**: 411–428, 1984.
2. Scharschmidt L, Simonson M and Dunn MJ, Glom-

erular prostaglandins, angiotensin II and nonsteroidal antiinflammatory drugs. *Am J Med* **81**: 30–42, 1986.
3. Clive DM and Stoff JS, Renal syndromes associated with nonsteroidal antiinflammatory drugs. *N Engl J Med* **310**: 563–572, 1984.
4. Berg KJ, Acute effect of acetylsalicylic acid on renal function in patients with chronic renal insufficiency. *Eur J Clin Pharmacol* **11**: 111–116, 1977.
5. Flower RJ, Moncada S and Vane JR, Drug therapy of inflammation. Analgesic-antipyretics and anti-inflammatory agents; Drugs employed in the treatment of gout. In: *The Pharmacological Basis of Therapeutics* (Eds. Goodman Gilman A, Goodman LS, Rall TW and Murad F), pp. 674–715. Macmillan Publishing Company, New York, 1985.
6. Hill JB, Salicylate intoxication. *N Engl J Med* **288**: 1110–1113, 1973.
7. Wieth JO and Brahm J, The inhibitory effect of salicylate on chloride and bicarbonate transport in human red cells. A hypothesis for the stimulatory effect of salicylate on the respiration. *Ugeskr Laeg* **140**: 1859–1865, 1978.
8. Crandall ED, Winter HI, Schaeffer JD and Bidani A, Effects of salicylate on HCO_3^-/Cl^- exchange across the human erythrocyte membrane. *J Membr Biol* **65**: 139–145, 1982.
9. Crandall ED, Mathew SJ, Fleischer RS, Winter HI and Bidani A, Effects of inhibition of RBC HCO_3^-/Cl^- exchange on CO_2 excretion and downstream pH disequilibrium in isolated rat lungs. *J Clin Invest* **68**: 853–862, 1981.
10. Crandall ED and Bidani A, Effects of red blood cell HCO_3^-/Cl^- exchange kinetics on lung CO_2 transfer: theory. *J Appl Physiol* **50**: 265–271, 1981.
11. Flemström G, Gastroduodenal mucosal secretion of bicarbonate and mucus. Physiological control and stimulation by prostaglandins. *Am J Med* **81**: 18–22, 1986.
12. Flemström G and Turnberg LA, Gastroduodenal defence mechanisms. *Clin Gastroenterol* **13**: 327–354, 1984.
13. Fromm D, Ion selective effects of salicylate on antral mucosa. *Gastroenterol* **71**: 743–749, 1976.
14. Tønnessen TI, Ludt J, Sandvig K and Olsnes S, Bicarbonate/chloride antiport in Vero cells: I. Evidence for both sodium-linked and sodium-independent exchange. *J Cell Physiol* **132**: 183–191, 1987.
15. Olsnes S, Ludt J, Tønnessen TI and Sandvig K, Bicarbonate/chloride antiport in Vero cells: II. Mechanisms for bicarbonate-dependent regulation of intracellular pH. *J Cell Physiol* **132**: 192–202, 1987.
16. Reinertsen KV, Tønnessen TI, Jacobsen J, Sandvig K and Olsnes S, Role of chloride/bicarbonate antiport in the control of cytosolic pH. Cell-line differences in activity and regulation of antiport. *J Biol Chem* **263**: 11117–11125, 1988.
17. Olsnes S and Sandvig K, Interactions between diphtheria toxin entry and anion transport in Vero cells. I. Anion antiport in Vero cells. *J Biol Chem* **261**: 1542–1552, 1986.
18. Olsnes S, Tønnessen TI and Sandvig K, pH-regulated anion antiport in nucleated mammalian cells. *J Cell Biol* **102**: 967–971, 1986.
19. Olsnes S, Tønnessen TI, Ludt J and Sandvig K, Effect of intracellular pH on the rate of chloride uptake and efflux in different mammalian cell lines. *Biochemistry* **26**: 2778–2785, 1987.
20. Thomas JA, Buchsbaum RN, Zimniak A and Racker E, Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. *Biochemistry* **18**: 2210–2218, 1979.
21. Barker JL and Levitan H, Salicylate: effect of membrane permeability of Molluscan neurons. *Science* **172**: 1245–1247, 1971.
22. Sandvig K, Tønnessen TI, Sand O and Olsnes S, Requirement of a transmembrane pH gradient for the entry of diphtheria toxin into cells at low pH. *J Biol Chem* **261**: 11639–11644, 1986.
23. Knauf PA and Mann NA, Location of the chloride self-inhibitory site of the human erythrocyte anion exchange system. *Am J Physiol* **251**: C1–C9, 1986.
24. Tønnessen TI, Aas AT, Sandvig K and Olsnes S, Effect of anti-inflammatory analgesic drugs on the regulation of cytosolic pH by anion antiport. *J Pharmacol Exp Ther* **248**: 1197–1206, 1989.
25. Motais R and Cousin JL, The inhibitor effect of probenicid and structural analogues on organic anions and chloride permeabilities in ox erythrocytes. *Biochim Biophys Acta* **419**: 309–313, 1976.
26. Motais R, Baroin A, Motais A and Bady S, Inhibition of anion and glucose permeability by anaesthetics in erythrocytes. The mechanism of action of positively and negatively charged drugs. *Biochim Biophys Acta* **599**: 673–688, 1980.
27. Vane JR, Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs. *Nature* **231**: 232–235, 1971.
28. Ferreira SH, Prostaglandins, aspirin-like drugs and analgesia. *Nature New Biol* **240**: 200–203, 1972.
29. Madshus IH and Olsnes S, Selective inhibition of sodium-linked and sodium-independent bicarbonate/chloride antiport in Vero cells. *J Biol Chem* **262**: 7486–7491, 1987.
30. Brune K, Dietzel K, Nurnberg B and Schneider HT, Recent insight into the mechanism of gastrointestinal tract ulceration. *Eur J Rheumatol Inflamm* **9**: 8–14, 1987.
31. Semble EL and Wu WC, Antiinflammatory drugs and gastric mucosal damage. *Semin Arthr Rheum* **16**: 271–286, 1987.
32. Brenner BE and Simon RR, Management of salicylate intoxication. *Drugs* **24**: 335–340, 1982.
33. Breyer MD and Jacobson HR, Mechanisms and regulation of renal H^+ and HCO_3^- transport. *Am J Nephrol* **7**: 150–161, 1987.
34. Baum M, Evidence that parallel Na^+–H^+ and Cl^-–HCO_3^-(OH^-) antiporters transport NaCl in the proximal tubule. *Am J Physiol* **252**: F338–F345, 1987.