Different regulation by pH_i and osmolarity of the rabbit ileum brush-border and parietal cell basolateral anion exchanger

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1. The purpose of this study was to look for evidence of a pH-sensitive modifier site on the parietal cell basolateral anion exchanger, determine the pH range in which allosteric regulation takes place, investigate the effect of the osmolarity on internal pH (pH_i) dependence and compare it with that of the ileum brush-border anion exchanger.

2. When the pH_i in parietal cell basolateral membrane (BLM) vesicles was increased, the rate of Cl^--gradient-driven $^{36}Cl^-$ uptake increased from $6{\cdot}03 \pm 2{\cdot}24$ to $38{\cdot}09 \pm 3{\cdot}33$ nmol (mg protein)$^{-1}$ with the steep increase in anion exchange rates occurring within a narrow pH range between pH_i 7·0 and 7·5. This was due to allosteric activation by internal OH^- and not due to a change in driving force, since the driving force for maximal exchange rates was provided by the outwardly directed Cl^- gradient.

3. The pH_i dependency curve of parietal cell BLM anion exchange rates was shifted to the left by 0·25 pH units by increasing the osmolarity of the intra- and extravesicular solutions from 300 to 380 mosmol l^{-1}. Thus cell shrinking may activate the parietal cell anion exchanger without a change in pH_i and without phosphorylation of the anion exchanger protein.

4. In ileum brush-border membranes, the pH_i-dependent increase in the rate of Cl^--gradient-driven $^{36}Cl^-$ uptake was more gradual and the half-maximal anion exchange rate was attained at lower pH_i (pH 6·5). Increasing the osmolarity from 300 to 500 mosmol l^{-1} had no effect on pH dependence.

5. We conclude that the parietal cell basolateral and ileum brush-border anion exchangers possess an internal modifier site for allosteric activation by OH^-, but the pH range in which allosteric regulation occurs differs between the two exchangers, as does the effect of an increase in osmolarity. Since current evidence suggests that both the parietal cell basolateral and the ileum brush-border anion exchanger are encoded by the AE2 gene, the differences in pH_i dependence between the two may be due to alternative splicing, post-transcriptional modification, or the different membrane environment.

6. The pH_i range for allosteric activation found in this study would suggest that for both the ileum and the parietal cell anion exchanger, but especially for the latter, a potentiating effect of the allosteric activation and the HCO_3^- availability occurs within the physiological pH_i range and can cause dramatic increases in maximal anion exchange rates with increasing pH_i.

Gastric parietal cells secrete HCl by proton secretion through the K^+,H^+-ATPase and the concomitant secretion of Cl^- through a Cl^- channel (Forte & Machen, 1986). The basolateral parietal cell anion exchanger is necessary for base extrusion and Cl^- uptake during acid secretion (Rehm & Sanders, 1975). It has been shown that anion exchange activity increases during stimulation of acid secretion (Muallem, Blissard, Cragoe & Sachs, 1988; Paradiso, Townsley, Wenzl & Machen, 1989; Yanaka, Carter, Lee & Silen, 1990; Seidler, Roithmaier, Classen & Silen, 1992), and different theories have been developed regarding the mode of activation: (1) direct stimulus-mediated change of activity (Muallem *et al.* 1988; Paradiso *et al.* 1989; Yanaka *et al.* 1990); (2) a decrease in intracellular Cl^- (Thomas & Machen, 1991); and (3) a rise in internal pH (pH_i), mediated by the operation of the basolateral Na^+–H^+ exchanger (Muallem *et al.* 1988) or the apical K^+–H^+ pump which in turn increases the anion exchange rate by either raising the intracellular HCO_3^- concentration and thus the driving force (Michelangeli,

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1978; Hersey, 1979; Ekblad, 1980; Debellis, Curci & Frömter, 1992) or by shifting the pH_i value from one in which the exchanger is inactive to one in which it is highly active (Muallem *et al.* 1988). The latter theory is very attractive because a pH_i dependence of the anion exchanger has been shown in all cell types investigated so far (Olsnes, Tonessen & Sandvig, 1986; Olsnes, Tonessen, Ludt & Sandvig, 1987; Mason, Smith, de Jesus Garcia-Soto & Grinstein, 1989; Wenzl & Machen 1989; Lin, Ahluwalia & Gruenstein, 1990; Green, Yamaguchi, Cleeman & Muallem, 1990; Mugharbil, Knickelbein, Aronson & Dobbins, 1990; Lambert, Bradley & Mircheff, 1991). Both the pH_i range and the magnitude of the pH_i-dependent activation vary widely, however, when different studies in different cell types are compared. We wondered whether this was due to different experimental approaches or due to differences in the regulation of the anion exchangers. Therefore we designed and evaluated a method which allowed us to assess the pH_i dependence of the parietal cell anion exchanger without a change in the driving force for anion exchange. This was achieved by preparing parietal cell basolateral membranes from rabbit gastric mucosa and measuring the initial rates of 4,4′-diisothiocyanostilbene-2′,2-disulphonic acid (DIDS)-inhibitable $^{36}Cl^-$ uptake into Cl^--loaded vesicles with intravesicular pH ranging from 5·5 to 8·0.

We then determined the pH_i dependence of the ileum brush-border anion exchanger. This anion exchanger was chosen because there is evidence that the parietal cell basolateral and the ileum brush-border anion exchanger are both encoded by the gene for the AE2 isoform of the anion exchanger gene family (Kudrycki, Newman & Shull, 1990; Chow, Dobbins, Aronson & Igarashi, 1992; Rossmann, Seidler & Classen, 1993), although the reported molecular weight of both is different (Thomas, Machen, Smolka, Boron & Kopito, 1989; Chow *et al.* 1992), as is the described pH_i dependency (Wenzl & Machen, 1989; Mugharbil *et al.* 1990; Thomas & Machen, 1991; Seidler *et al.* 1992). Since different methodological approaches may play a role in these reported discrepancies, we used identical techniques to compare the properties of both anion exchangers.

A part of this work was presented at a meeting of the American Gastroenterology Association in May 1992 (Nader, Seidler & Classen, 1992).

METHODS

Preparation of parietal cell basolateral membranes

Male New Zealand White rabbits (2–3 kg) were anaesthetized and pretreated as previously described (Seidler *et al.* 1992; Lamprecht, Seidler & Classen, 1993). In brief, the rabbit was anaesthetized using a mixture of 100 mg ketamine, 0·5 mg atropine and 8 mg 5,6-dihydro-2-(2,6-xylidino)4H-1,3-thiazin (Rompun; Bayer), given I.M. An ear vein was then cannulated and 50 mg ranitidine was given I.V. 45 min before killing to ensure that parietal cell ultrastructure was in the 'resting state'. After opening the abdomen, the rabbit was killed with an overdose of 50–100 mg phenobarbitone I.V., the aorta was cannulated and the stomach was perfused under high pressure as previously described (Lamprecht *et al.* 1993). Preparation of rabbit parietal cell basolateral membranes was done exactly as described in detail recently (Lamprecht *et al.* 1993), by a combination of differential, Ficoll-step and linear sucrose gradient centrifugation. The final membrane pellet was suspended in 300 mM sucrose, 10 mM *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulphonic acid (Hepes), pH 7·5, and used on the same day or stored in liquid nitrogen until further use.

Preparation of ileum brush-border membranes

For the preparation of ileum brush-border membranes animals were pretreated and killed as above, and 1·5 m of the terminal ileum were removed, opened and washed thoroughly with ice-cold 0·9% saline solution. The mucosa was scraped off and transferred to ice-cold preparation buffer C (300 mM mannitol, 10 mM tris(hydroxymethyl)aminomethane (Tris), 16 mM Hepes, at pH 7·4). All the following steps were performed at 4 °C in buffer C. Homogenization was performed using a Polytron homogenizer at low speed for 3 min followed by homogenization in a Teflon–glass homogenizer, twenty strokes at 1000 r.p.m. Further preparation was done according to the method of Knickelbein, Aronson, Atherton & Dobbins (1983). After homogenization $CaCl_2$ was added to a final concentration of 10 mM and the suspension was incubated for 15 min. Then the homogenate was centrifuged at 3000 *g* for 10 min, the pellet was discarded, the supernatant centrifuged at 27000 *g* for 60 min and the resulting pellet was resuspended. Magnesium gluconate was added to the suspension at a final concentration of 10 mM and the procedure was repeated twice. The final pellet, P, was further treated and used for uptake studies the same day as described later. The entire membrane preparation was done at 4 °C.

Enzyme assays

Protein determination was performed by the method of Lowry with modifications as described by Peterson (1983). Na^+,K^+-ATPase activity, used as a marker enzyme for basolateral membranes, was measured as described by Forbush (1983). The method of Hirst & Forte (1985) was used for measuring K^+,H^+-ATPase activity, a marker enzyme for tubulovesicles. The activity of alkaline phosphatase was used as a marker for brush-border (BB) membranes since, although there is a small quantity of alkaline phosphatase activity on basolateral membranes, most alkaline phosphatase activity is located on the BB membrane. Enzyme activity can be assayed conveniently, and was determined by measuring the hydrolysis of *p*-nitrophenyl phosphate at pH 10·3 (Hanna, Mircheff & Wright, 1979). In addition, the activity of sucrase, as a pure brush-border enzyme, was determined as described by Dahlquist (1964).

Transport studies

The membranes were suspended in the appropriate 'intravesicular' buffer solution and pelleted at 30000 *g*. The pellet was revesiculated by twenty-five passes through a 25 gauge needle in the intravesicular buffer solution plus 0·2 mM valinomycin and the suspension was incubated for 1·5 h at room temperature. A 5 μl aliquot of vesicles was added to 45 μl extravesicular buffer containing $^{36}Cl^-$. At varying time intervals isotope uptake was stopped by the addition of 3 ml ice-cold stop solution. The solution was filtered immediately through 0·45 μm Millipore HAWP filters and washed twice

with 3 ml stop solution. Filters were transferred to 5 ml scintillation cocktail and radioactivity was measured using a β-scintillation counter. All media were gassed with 100% N_2 except those containing HCO_3^-, which were gassed with the appropriate mixture of N_2–CO_2. The compositions of the intra- and extravesicular solutions and the stop solution are listed in detail in the figure legends. Unless specifically mentioned, the concentration of extravesicular Cl^- ($[Cl^-]_o$) is calculated as the Cl^- concentration after mixing the extravesicular solution (containing $^{36}Cl^-$) with the vesicle suspension (containing only unlabelled Cl^-). The osmolarity of each solution was checked with an osmometer and it was ensured that the respective intra- and extravesicular solutions had exactly the same osmolarity. To clamp the transvesicular membrane potential to zero the K^+ ionophore valinomycin was added as mentioned above and the K^+ concentration was always the same in the intra- and extravesicular solution. For the determination of pH_i dependence, different pH values of the intravesicular buffer were obtained by appropriate mixtures of Tris, 2-(*N*-morpholino)ethanesulphonic acid (Mes) and Hepes while the $[Cl^-]_i$ was kept constant over the tested pH range. The pH of the extravesicular solution (pH_o) was titrated to a predetermined value, so that the indicated pH_o was achieved after mixing with the vesicle suspension. All transport studies were performed at room temperature.

Each experiment was performed at least in triplicate on each of at least three separate membrane preparations; n is the number of separate rabbit membrane preparations. Values are given as means ± S.E.M. Error bars are not shown when included within the symbol. Statistical analysis was performed using Student's t test and $P < 0.05$ was considered significant, if the appropriate number of experiments required for statistical evaluation had been performed. Many experiments, however, were repeated only three times, since they served the purpose of validation of our methodology and were not done to generate new data.

Materials

$H^{36}Cl$ was obtained from Amersham–Buchler (Braunschweig, Germany), HAWP filters from Millipore (Eschborn, Germany), scintillation cocktail from Packard (Frankfurt/Main, Germany) and Ficoll from Pharmacia (Uppsala, Sweden). Valinomycin and 4,4′-diisothiocyanostilbene-2′,2-disulphonic acid (DIDS) were obtained from Sigma (Deisenhofen, Germany) and all other chemicals were purchased from Merck (Darmstadt, Germany) at the highest purity available.

RESULTS

Basolateral membrane (BLM) preparation

Our aim was to obtain a highly enriched basolateral membrane fraction with little contamination by other cell organelles. The final 'basolateral membrane' pellet ($n = 21$) consisted of 0·94 ± 0·07% of the initial protein in the postnuclear supernatant and its Na^+,K^+-ATPase activity was enriched 16·5 ± 0·9-fold. K^+,H^+-ATPase activity was enriched 3·74 ± 0·7-fold in that fraction and all other intracellular membranes were present only in minor quantities, as has been reported in detail previously (Lamprecht *et al.* 1993).

Brush-border membrane (BBM) preparation

Pilot experiments of ileum brush-border membrane preparations ($n = 6$) yielded a pellet, P, which consisted of 2·59 ± 0·49% initial mucosal homogenate protein and its alkaline phosphatase activity was enriched 10·80 ± 0·87-fold and sucrase activity 10·00 ± 0·57-fold, both brush-border marker enzymes. Little contamination by basolateral membranes occurred as indicated by a 2·08 ± 0·48-fold enrichment of Na^+,K^+-ATPase activity. This BBM preparation could be purified further on a sucrose gradient, with almost complete removal of basolateral membrane contaminants. We found that the kinetic parameters obtained using these highly enriched BBM vesicles were not better but worse than when the original procedure described by Knickelbein *et al.* (1983) was used, probably because of the longer time required for preparation. Therefore, we did not pursue this higher purification method further. All other BBM preparations (excluding the pilot experiments) were tested only for alkaline phosphatase activity (since the sucrase activity had always paralleled the alkaline phosphatase activity in the pilot experiments) and discarded when the enrichment was lower than 9-fold. The mean enrichment of alkaline phosphatase activity for the experiments reported in this paper was 11·9 ± 1·7-fold ($n = 18$) and is in good agreement with the enrichment of brush-border membranes used for transport studies in other laboratories (Knickelbein *et al.* 1983; Mugharbil *et al.* 1990).

Transport studies

Time course of $^{36}Cl^-$–Cl^- and $^{36}Cl^-$–HCO_3^- exchange in parietal cell BLM and ileum BBM vesicles

Figure 1*A* shows the time course of $^{36}Cl^-$ uptake into parietal cell BLM vesicles with a $[Cl^-]_i$ of 50 and 100 mM. The plateau was the same, but the initial rise in Cl^- uptake was somewhat faster with 100 mM $[Cl^-]_i$. The possibility of $^{36}Cl^-$ uptake through a Cl^- conductance was eliminated by measuring Cl^- uptake against its concentration gradient and using valinomycin and equal transmembrane K^+ gradients to clamp the transmembrane voltage to zero. The initial rapid uptake of $^{36}Cl^-$ was blocked by 2 mM of the known anion exchange inhibitor DIDS.

Figure 1*B* shows the time course of $^{36}Cl^-$ uptake into Cl^--loaded ileum BBM vesicles with a $[Cl^-]_i$ of 100 mM. The Cl^- uptake into ileum BBM vesicles was somewhat slower than into parietal cell BLM vesicles. Therefore, we selected two different uptake times (5 and 9 s) to evaluate the pH_i dependency of anion exchange into BBM vesicles. This set of experiments was performed under the conditions used by Mugharbil *et al.* (1989), that is with hypertonic solutions, which are thought to be superior for isotope flux studies into membrane vesicles. However, there was no significant difference in the time course or

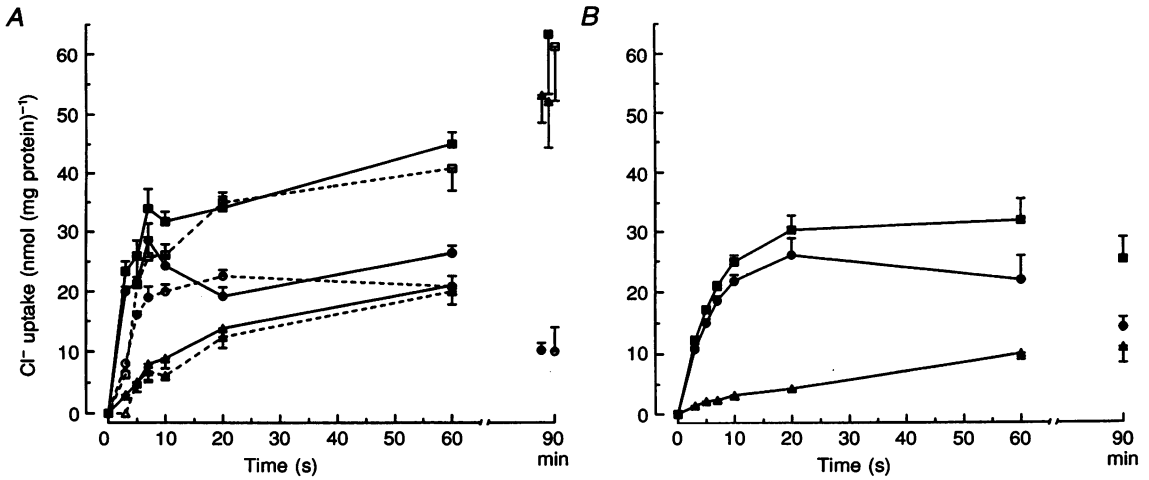

Figure 1. Time course of $^{36}Cl^-$ uptake into parietal cell BLM and ileum BBM vesicles preloaded with Cl^-

A, parietal BLM vesicles preloaded with 50 (open symbols, dashed lines) or 100 mM (filled symbols, continuous lines) Cl^-. $[Cl^-]_o$, 19 mM after diluting 5 μl vesicles in 45 μl extravesicular buffer solution. Vesicles were loaded with 60 mM mannitol, 50 or 100 mM KCl, 50 mM or no potassium gluconate, and the appropriate mixture of 40 mM Tris–Hepes–Mes at pH 7·5. Extravesicular buffer consisted of 40 mM mannitol, 100 mM potassium gluconate, 15 or 10 mM $^{36}Cl^-$ (the final $[Cl^-]_o$ was reached after mixing the vesicle suspension with the extravesicular medium) and 40 or 45 mM Tris–Hepes at pH 5·5 with (triangles) or without (squares) 2 mM DIDS. In the absence of DIDS, sodium was kept constant at 4 mM with sodium gluconate. DIDS-inhibitable uptake is indicated by circles. Stop solution contained 60 mM mannitol, 100 mM potassium gluconate, 40 mM Mes–Tris at pH 5·5. $n = 3$. *B*, ileum BBM vesicles preloaded with Cl^- under the same conditions as used by Mugharbil *et al.* (1989). $[Cl^-]_o$, 14 mM. Vesicles were loaded with 150 mM mannitol, 50 mM potassium gluconate, 100 mM KCl and 40 mM Tris–Hepes–Mes, at pH 7·5. Extravesicular buffer consisted of 35 mM mannitol, 150 mM potassium gluconate, 5 mM $^{36}Cl^-$, 16 mM Tris, 20 mM Hepes, 104 mM Mes at pH 5·5 with or without 7 mM DIDS (symbols as for *A*). In the absence of DIDS, sodium concentration was kept constant at 14 mM with sodium gluconate. Stop solution consisted of 170 mM mannitol, 150 mM potassium gluconate, 16 mM Hepes and 10 mM Tris, at pH 7·5. $n = 3$.

maximal values for Cl^- uptake into BBM vesicles in Fig. 4*B*, in which we have used isotonic conditions.

We next compared $^{36}Cl^-$–HCO_3^- and $^{36}Cl^-$–OH^- exchange with $^{36}Cl^-$–Cl^- exchange in parietal cell BLM vesicles. Figure 2 shows the DIDS (2 mM)-inhibitable Cl^- uptake into BLM membrane vesicles loaded with either 100 mM Cl^-, HCO_3^- or gluconate. We found a rapid DIDS-inhibitable $^{36}Cl^-$ uptake into the vesicles loaded with Cl^- or HCO_3^-, but little DIDS-inhibitable $^{36}Cl^-$ uptake into gluconate-loaded vesicles. The small portion of DIDS-sensitive $^{36}Cl^-$ uptake into gluconate-loaded vesicles probably represents $^{36}Cl^-$–OH^- exchange, but may also be

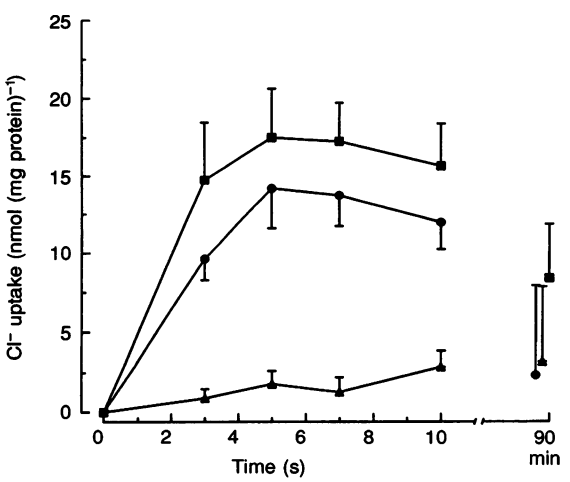

Figure 2. DIDS (2 mM)-inhibitable Cl^- uptake into parietal cell BLM vesicles loaded with 100 mM Cl^- (■), HCO_3^- (●) or gluconate (▲)

Intravesicular buffer contained 60 mM mannitol, 100 mM KCl or $KHCO_3$ or potassium gluconate and 40 mM Tris–Hepes at pH 8·0. $[Cl^-]_o$, 19 mM. Extravesicular buffer consisted of 50 mM mannitol, 100 mM potassium gluconate, 10 mM $^{36}Cl^-$ and 40 mM Tris–Mes at pH 5·5. Stop solution contained 100 mM potassium gluconate, 60 mM mannitol, 40 mM Mes–Tris at pH 5·5. $n = 3$.

due to another membrane-altering effect of DIDS, since we consistently observed a smaller apparent intravesicular volume in DIDS-treated vesicles (Lamprecht *et al.* 1993). The absolute $^{36}Cl^-$ uptake values in these experiments are lower than those in Fig. 1, because we cannot take into account the extravesicular anions (other than Cl^-) which are added to the uptake medium with the vesicle suspension. Since the added HCO_3^-, Cl^- or gluconate will compete with $^{36}Cl^-$ for uptake, the overall $^{36}Cl^-$ uptake values are lower and anion exchange rates are underestimated.

Dependence of $^{36}Cl^-$–Cl^- exchange rates on pH_i

Time course experiments. In a CO_2–HCO_3^--buffered system it is impossible to change pH_i without changing $[HCO_3^-]_i$ and thus the driving force for Cl^-–HCO_3^- exchange. As we have demonstrated that Cl^- and HCO_3^- are transported equally well by both anion exchangers tested, we determined the pH_i dependence of $^{36}Cl^-$–Cl^- instead of $^{36}Cl^-$–HCO_3^- exchange. This allowed us to establish identical transmembrane Cl^- concentration gradients over the whole tested pH range and therefore selectively measure the influence of pH_i on exchange rates, but only if our assumptions were correct that the outwardly directed Cl^- gradient provided the driving force for $^{36}Cl^-$–Cl^- exchange and that a transmembrane pH gradient *per se* is not necessary to activate anion exchange. We therefore repeated the time course experiments for both types of membrane vesicles with (pH_o 5·5–pH_i 7·5) and without (pH_o 7·5–pH_i 7·5 and pH_o 5·5–pH_i 5·5) a transmembrane pH gradient. The results for parietal cell BLM vesicles are shown in Fig. 3*A*. There is no significant difference in $^{36}Cl^-$ uptake rates with or without a pH gradient when the intravesicular pH is 7·5. The inhibition of $^{36}Cl^-$ uptake by DIDS is less when pH_o is 7·5. We do not know the reason for this finding, but there may be several explanations. (1) DIDS inhibits anion exchange less at higher pH values. This is supported by our finding that increasing the DIDS concentration at this high pH value from 2 to 4 and 7 mM increased the DIDS inhibition of anion exchange at 20 s uptake time from 40 to 60%, which is approximately the same as found at pH_o 5·5. (2) Our BLM vesicles may have a mixed orientation (Kinne, Haase, Gmaj & Murer, 1978), and DIDS binds to an extracellular site (Jesse, Sjöholm & Hoffmann, 1986). In the inside-out vesicles, the DIDS binding site is hidden, and therefore $^{36}Cl^-$–Cl^- exchange is not inhibited at high pH_o but is inhibited at low pH_o because of inactivation of the modifier site. In this case, however, one would expect the Cl^- uptake to be higher in the pH_i 7·5–pH_o 7·5 (all vesicles take part in anion exchange) than in the pH_i 7·5–pH_o 5·5 vesicles (only the right side-out vesicles take part in anion exchange), which was not the case. At a pH_i of 5·5 $^{36}Cl^-$ uptake is distinctively less and lacks the rapid initial uptake, indicating inhibition of the anion exchanger at low pH_i. For ileum BBM vesicles (illustrated in Fig. 3*B*) the lack of a pH gradient also does not influence the $^{36}Cl^-$ uptake rates at pH_i 7·5. DIDS inhibition at pH_o 7·5 is also not complete at 4 mM, but at 7 mM shows no significant difference to the values at pH_o

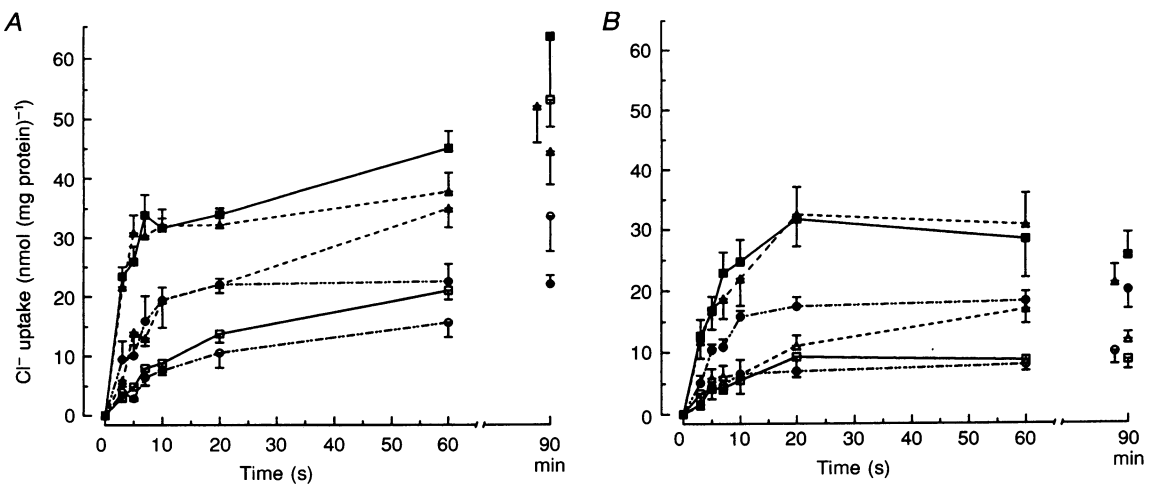

Figure 3. Time course of Cl^- uptake into Cl^--loaded parietal cell BLM and ileum BBM vesicles with (pH_i 7·5–pH_o 5·5; squares) and without ($pH_i = pH_o = 7·5$ (triangles) or 5·5 (circles)) transmembrane pH gradient

A, parietal cell BLM vesicles. *B*, ileum BBM vesicles. For both *A* and *B*, intravesicular buffer contained 60 mM mannitol, 100 mM KCl and 40 mM Tris–Hepes–Mes at pH 7·5 or 5·5. $[Cl^-]_o$, 19 mM. Extravesicular buffer consisted of 40 mM mannitol, 100 mM potassium gluconate, 10 mM $^{36}Cl^-$ and 40 mM Tris–Hepes–Mes at pH 7·5 or 5·5. Stop solution contained 100 mM potassium gluconate, 60 mM mannitol, 40 mM Mes–Tris at pH 5·5 with (open symbols) or without (filled symbols) 2 (*A*) or 4 mM (*B*) DIDS. $n = 4$.

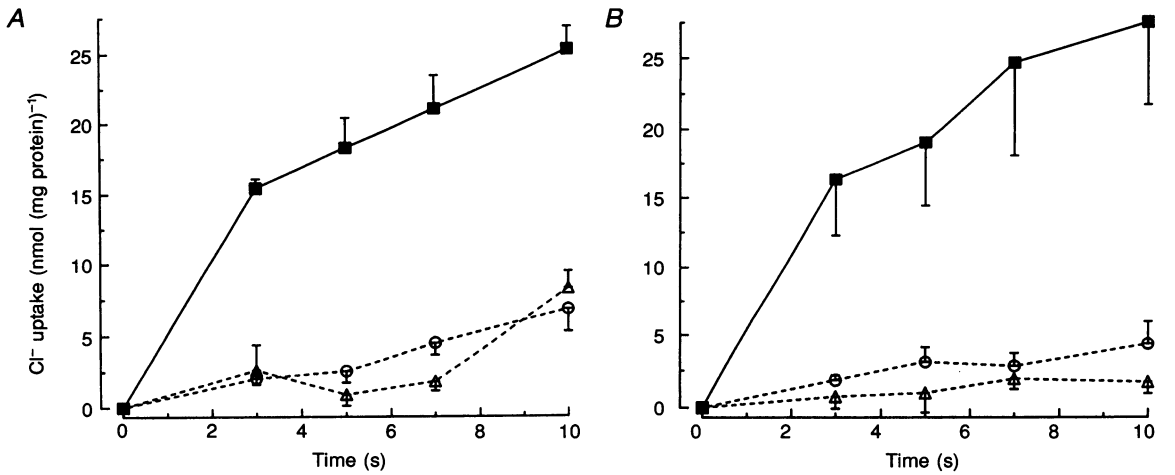

Figure 4
Time course of Cl^- uptake into parietal cell BLM (*A*) and ileum BBM (*B*) vesicles loaded with Cl^- at pH_i 7·5 (■) or gluconate at pH_i 5·5 (○) or 7·5 (△). $[Cl^-]_o$, 19 mM. For exact composition of media see the legend to Fig. 3. $n = 3$.

5·5 (data not shown), supporting the first explanation discussed above, since in BBM vesicles the vesicle orientation is presumably right-side out (Haase, Schäfer, Murer & Kinne, 1978). As in BLM vesicles there is very little $^{36}Cl^-$ uptake at pH_i 5·5. These experiments demonstrate that a transmembrane pH gradient *per se* is not a necessary requirement for maximal obtainable anion exchange rates in our model.

The maximal proportion of $^{36}Cl^-$ uptake in exchange for OH^-_i that may take place in addition to $^{36}Cl^-$ uptake in exchange for Cl^-_i was determined for our experimental setting. Figure 4*A* and *B* shows $^{36}Cl^-$ uptake into parietal

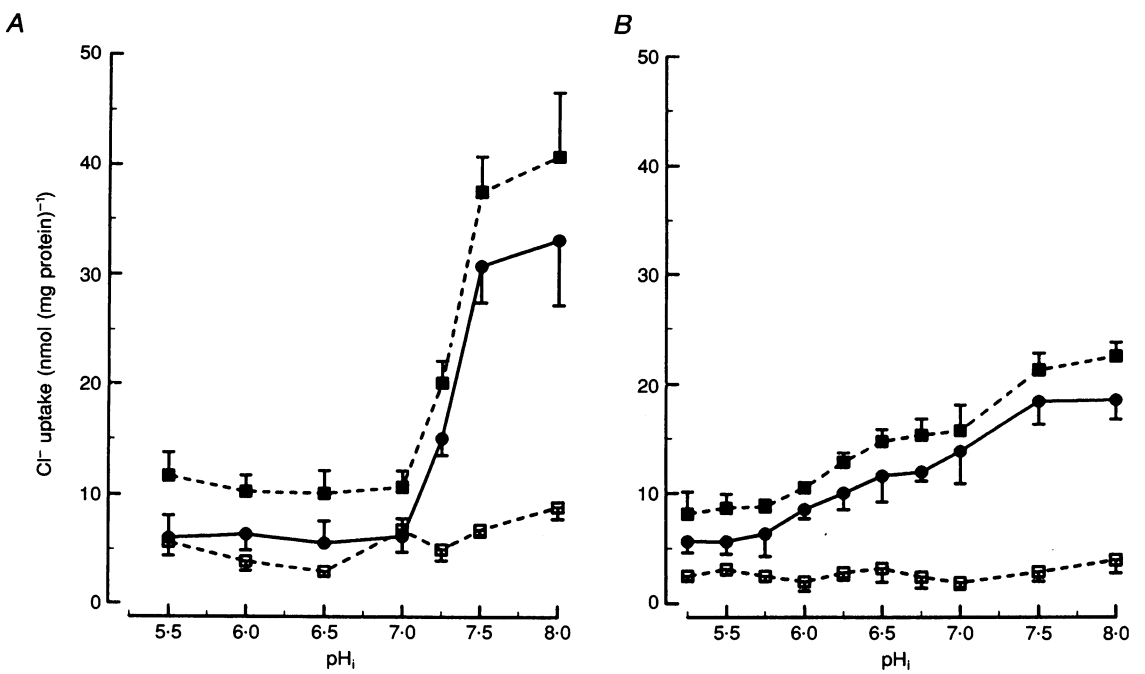

Figure 5. Effect of pH_i on Cl^- uptake into Cl^--loaded parietal cell BLM (*A*) and ileum BBM (*B*) vesicles at pH_o 5·5
Various pH_i values were obtained by using appropriate mixtures of Tris, Hepes and Mes. $[Cl^-]_o$, 19 mM. Uptake time was 5 s. □, with 2 (*A*) or 4 mM (*B*) DIDS; ■, without DIDS; ●, DIDS-inhibitable uptake. For exact composition of media see the legend to Fig. 3. $n = 5$

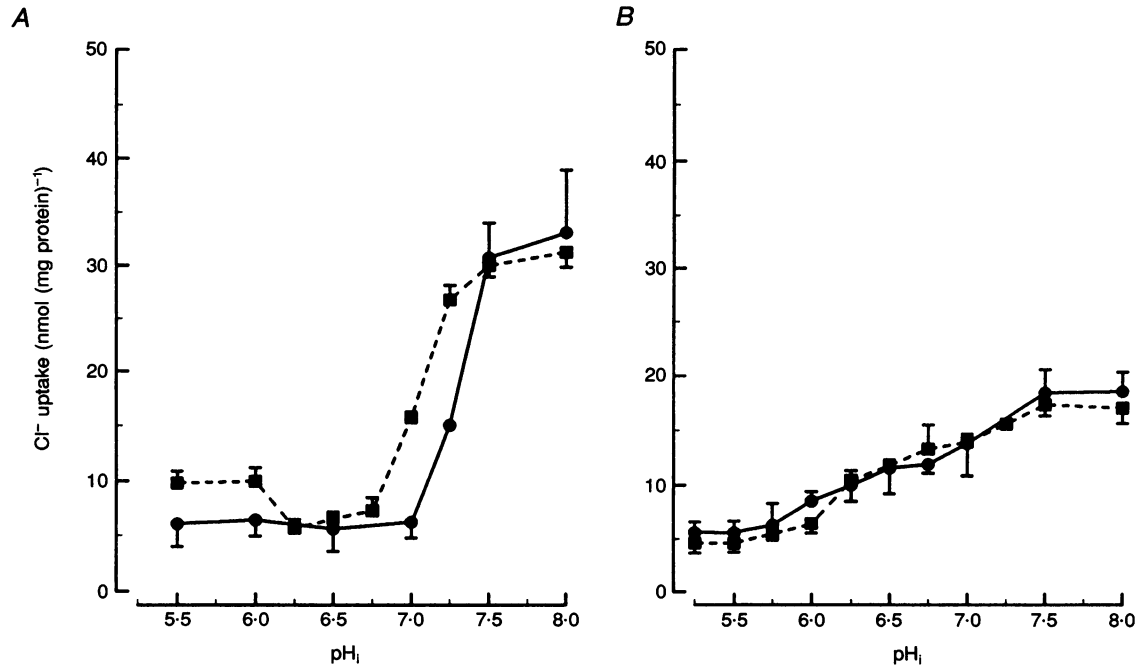

Figure 6. Influence of the osmolarity on the pH_i dependence of Cl^- uptake into parietal cell BLM (*A*) and ileum BBM vesicles (*B*)

$[Cl^-]_o$, 19 mM. Osmolarity in the intra- and extravesicular buffer was adjusted to the desired value with mannitol (in *A*: 300 (●) and 380 mosmol l^{-1} (■); in *B*: 300 (●) and 500 mosmol l^{-1} (■)). Uptake time was 5 s. For composition of media see legend to Fig. 3. $n = 5$.

cell BLM and ileum BBM vesicles loaded with gluconate at pH_i 5·5 and 7·5 in comparison with $^{36}Cl^-$ uptake into Cl^--loaded vesicles at pH_i 7·5. It is evident that the maximal proportion of $^{36}Cl^-$–OH^- exchange at 5 and 9 s is one-tenth of the $^{36}Cl^-$–Cl^- exchange. We can therefore presume that if we do observe changes in anion uptake rates at different pH_i values, it is not due to activation of anion exchange by the transmembrane pH gradient *per se*, and not due to a change in the driving force provided by the additional presence of OH^- anions for $^{36}Cl^-$–OH^- exchange at high pH_i.

Initial $^{36}Cl^-$–Cl^- exchange rate at intravesicular pH 5·5–8·0. The effect of intravesicular pH on $^{36}Cl^-$ uptake into Cl^--loaded parietal cell BLM vesicles is illustrated in Fig. 5*A*. When the intravesicular pH was increased from 5·5 to 8·0 the DIDS-inhibitable uptake rates of $^{36}Cl^-$ were increased approximately 5-fold in an S-shaped manner. The steep increase of $^{36}Cl^-$-uptake rates occurred in a narrow pH_i range between 7·0 and 7·5. Increasing the pH_i above 7·5 had little influence on uptake rates, indicating full activation of the anion exchanger at this pH value.

Figure 5*B* shows the influence of pH_i on $^{36}Cl^-$ uptake into ileum brush-border membrane vesicles. $^{36}Cl^-$ uptake increased 3·5-fold from pH_i 5·25 to 8·0. However the pH_i-dependent rise in activity occurred over a much wider pH_i range between 5·75 and 7·5. Half-maximal inhibition of anion exchange rates occurred at distinctively lower pH_i values than in parietal cell basolateral membrane vesicles, pH 6·5 *versus* pH 7·25. An identical pH_i dependency was obtained when the uptake times were 9 instead of 5 s (data not shown).

Influence of the osmolarity on the pH_i dependence of $^{36}Cl^-$–Cl^- exchange. We next studied the effect of increasing the osmolarity of both the intra- and extravesicular solution from 300 to 380 and 500 mosmol l^{-1}. The resulting DIDS-inhibitable uptake rates are compared in Fig. 6*A* for parietal cell BLM vesicles and Fig. 6*B* for ileum BBM vesicles. In parietal cell BLM vesicles, the pH_i dependency was significantly ($P < 0·02$) shifted to the left, that is to more acidic values. Half-maximal $^{36}Cl^-$ uptake was shifted from 7·25 to 7·0 with an increase in osmolarity from 300 to 380 mosmol l^{-1}. The time course of $^{36}Cl^-$ uptake at pH 7·5 was not different at 300 and 380 mosmol l^{-1}. Surprisingly, there was no shift in the pH_i dependency for ileum BBM vesicles with an increase in the osmolarity up to 500 mosmol l^{-1} (Fig. 6*B*).

DISCUSSION

In this study, we investigated the pH_i dependence of two gastrointestinal anion exchangers, the basolateral parietal cell and the ileum brush-border anion exchanger under identical experimental conditions, and measured the effect

of an increase in osmolarity on this pH_i dependence. What we found was a marked pH_i sensitivity of $^{36}Cl^-$–Cl^- exchange rates both in parietal cell basolateral and ileum brush-border membrane vesicles. The parietal cell anion exchanger displayed a strong increase in $^{36}Cl^-$–Cl^- exchange rates over the narrow pH range between very low activity below pH 7·0 and almost maximal activity at pH 7·5 for intra- and extravesicular solutions of 300 mosmol l^{-1}. The ileum brush-border anion exchanger also showed a strong increase in exchange rates with increasing pH_i, but surprisingly, the increase in activity was more gradual, and the half-maximal activation was reached at a lower pH value (pH 6·5). For both anion exchangers the pH_i at which half-maximal allosteric activation was seen was lower than previously described (Mugharbil *et al.* 1989; Thomas *et al.* 1991). This is probably due to the fact that we measured only $^{36}Cl^-$–Cl^- exchange and largely ruled out interference with HCO_3^- or base exchange, the availability of which is strongly pH dependent. In the physiological pH range, $^{36}Cl^-$–HCO_3^- (but not $^{36}Cl^-$–Cl^-) exchange depends on pH_i in a 2-fold fashion, because of the allosteric activation of anion exchange by pH_i and the increase of HCO_3^- concentration with increasing pH_i. The pH_i range for allosteric activation found in this study would suggest that for both the ileum and the parietal cell anion exchanger, but especially for the latter, a potentiating effect of the allosteric activation and the HCO_3^- availability occurs within the physiological pH_i range and can cause dramatic increases in maximal anion exchange rates with increasing pH_i. A shortcoming of our study is that we have not ruled out the possibility that changes in the Michaelis–Menten constant (K_m) values for Cl^- and HCO_3^- in the opposite direction occur with decreasing pH_i.

The fact that an alkaline pH_i increases anion exchange rates has been demonstrated in a number of cell types and experimental systems (Olsnes *et al.* 1986, 1987; Mason *et al.* 1989; Wenzl *et al.* 1989; Lin *et al.* 1990; Mugharbil *et al.* 1990; Green *et al.* 1990; Lambert *et al.* 1991; Seidler *et al.* 1992). The pH_i range in which activation occurred and the degree of activation varied considerably, however. If these differences were cell-type or anion exchanger isoform specific, it could mean that different cell types have different modes of pH_i-dependent anion exchange regulation serving their specific functions. On the other hand, the differences could be due to methodological differences. In most studies in which anion exchange rates were studied in intact cells, a constant and maximal driving force (transmembrane concentrations gradients for Cl^- and base together) over the tested pH_i range was not present (Olsnes *et al.* 1986, 1987; Mason *et al.* 1989; Green *et al.* 1990; Lambert *et al.* 1991), the concentration of exchangeable base might have been rate limiting for anion exchange (Wenzl *et al.* 1989; Seidler *et al.* 1992), or it was impossible to keep the driving force constant due to the strong pH_i dependency of HCO_3^- availability (Thomas *et al.* 1991; Seidler, Lamprecht, Roithmeier & Classen, 1991). Thus, all studies performed in intact cells have the problem that due to a limited manipulability of the intracellular ionic environment, the transmembrane anion concentrations cannot be strictly controlled. Furthermore, the manipulations of the extracellular anion concentrations needed to stimulate maximal anion exchange rates (e.g. external Cl^- removal and readdition) are known to induce cell volume changes, which in turn may activate anion exchange (Hoffmann & Simonsen, 1989).

Mugharbil *et al.* (1990) used an ileum brush-border membrane vesicle preparation to measure the pH_i dependence of Cl^- uptake into HCO_3^--loaded vesicles. Although their study definitely showed that allosteric activation of the ileum anion exchanger by the pH_i takes place, they did not show in which pH range allosteric regulation occurs, since HCO_3^- availability below pH 7·0 is too low to create a driving force for $^{36}Cl^-$ uptake in the experimental settings used. This explains why they found the steep rise in Cl^- uptake at a pH_i of approximately 7·3, where HCO_3^- concentration is in the range for half-maximal saturation of the transport site.

Therefore, we validated and applied a method that would allow us to measure the dependence of maximal anion exchange rates on pH_i in the absence of significant changes in driving force or the concentration of exchangeable anions. This was done by measuring the uptake of $^{36}Cl^-$ into Cl^--loaded membrane vesicles at different intravesicular pH. The driving force for $^{36}Cl^-$ uptake was created by the outwardly directed Cl^- gradient. To test the feasibility of this approach, we had to clarify several points: (1) since we wanted to measure $^{36}Cl^-$–Cl^- instead of $^{36}Cl^-$–HCO_3^- exchange, we had to ascertain that Cl^- was exchanged equally as well as or better than HCO_3^-; (2) we had to ascertain that the Cl^- gradient and not the pH gradient, or the Cl^- gradient in conjunction with a pH gradient, was sufficient to establish the maximal anion exchange rate; and (3) we had to find out what percentage of $^{36}Cl^-$–OH^- exchange may increase overall Cl^- uptake rates in the high pH_i range. The results of the experiments are found in the Results section and indicated to us that: (1) internal Cl^- is exchanged equally as well as HCO_3^-; (2) a transmembrane pH gradient *per se* does not significantly increase anion exchange rates; (3) the potential percentage of $^{36}Cl^-$–OH^- exchange is at best one-tenth of the $^{36}Cl^-$–Cl^- exchange rate at a pH_i of 7·5. Thus, we should be able to determine selectively the effect of pH_i on maximal anion exchange rates.

In a number of cell types, anion exchange is activated during volume regulation (Hoffmann *et al.* 1988). Hypertonicity of the external solution results in a shift of the pH_i dependence of anion exchange rates in *Xenopus* oocytes that express AE2 (S. Alper, personal communication). Therefore, we tested whether a high osmolarity *per se* influences the pH_i dependence of anion exchange rates. In our experimental setting, the

osmolarity was increased in both the intra- and extravesicular solutions and no transmembrane gradient existed. Interestingly, the pH_i dependence of the parietal cell BLM anion exchanger was shifted to more acidic values by an increase in osmolarity from 300 to 380 mosmol l^{-1}, whereas we found no change in the pH_i dependence of the ileum BBM anion exchanger. This shows that hypertonicity *per se* influences the pH_i dependence of the parietal cell anion exchanger. Thus cell shrinking could activate the anion exchanger without a change in pH_i and without phosphorylation of the anion exchanger protein.

Chow *et al.* (1992) have cloned a cDNA from rabbit ileum cells with high homology to the AE2 isoform of the anion exchanger gene family, and we have strong evidence from our laboratory that parietal cells express the AE2 mRNA (U. Seidler, unpublished data). Moreover, both our functional data suggest that the parietal cell anion exchanger is an AE2 isoform, and an antibody directed against a fusion protein constructed with the help of the ileum AE2 sequence stains the basolateral membrane of the parietal cell (but not the ileum BB membrane). Unless Chow *et al.* (1992) happened to clone an ileum basolateral anion exchanger, one must conclude that both the parietal cell BLM and the ileum BBM anion exchangers are encoded by the AE2 gene. The different pH dependencies of allosteric activation and the different effect of an increase in osmolarity between the parietal cell and the ileum brush-border anion exchanger therefore seemed surprising to us. A similar controversy has recently been resolved: type A intercalated cells in the collecting duct secrete protons via vacuolar-type H^+ pumps, and extrude base via a basolateral anion exchanger protein which is an alternative splice variant of the AE1 gene. Type B intercalated cells secrete base via an apical anion exchanger. This apical anion exchanger protein was not recognized by a variety of antibodies against band 3 and other AE isoform epitopes, had a different stilbene sensitivity and a different dissociation constant (K_d) for Cl^-_o than the basolateral anion exchanger of type A cells, and was therefore believed to be encoded by a different gene (Kopito, 1990). A recent publication by van Adelsberg, Edwards & Al-Awqati (1993) has convincingly demonstrated that the apical anion exchanger of type B intercalated cells is also an AE1 isoform. This is, to our knowledge, the first demonstration of apically and basolaterally localized ion exchanger proteins being the identical gene product. In the Na^+–H^+ exchanger gene family, different isoforms have been localized to the apical and basolateral domains of epithelial cells. The different kinetic parameters of anion exchange in type A and B intercalated cells are as difficult to explain as the different characteristics of the parietal cell and ileum anion exchanger. Van Adelsberg *et al.* (1993) speculated that the binding of inhibitors and substrates may be altered by the characteristics of the plasma membrane domain in which the protein is located, and a similar situation may exist for the ileum brush-border and the parietal cell basolateral anion exchanger. Another explanation may be differences in post-translational modification. Northern blot hybridization of parietal cell mRNA with AE2 cDNA yields two bands (Rossmann *et al.* 1993), and these two bands are exclusively found in the stomach (Kudrycki *et al.* 1990). Thus, the parietal cell anion exchanger may be an alternative splice variant with some unique properties.

What might be the physiological significance of the observed pH_i dependence of the ileum brush-border and parietal cell anion exchanger? The ileum villus cell anion exchanger is involved in NaCl reabsorption, and the coupling of brush-border Na^+–H^+ exchange and $^{36}Cl^-$–HCO_3^- exchange activities is believed to occur via slight changes in the pH_i (Knickelbein, Aronson, Atherton & Dobbins, 1985). The crypt cell anion exchanger is involved in Cl^- secretion. The physiological relevance of pH_i dependency in such a low pH_i range is difficult to understand, since the base availability in that pH_i range is rather low. On the other hand, potentiating activation of anion exchange rates by allosteric activation and HCO_3^- avaliability would also occur in a pH_i range below 7 and, since the pH_i in the ileum cells may be variable, may allow greater flexibility of NaCl absorption and Cl^- secretion.

The parietal cell anion exchanger is involved in contralateral base extrusion during apical HCl secretion. Could the allosteric regulation contribute to the known activation of $^{36}Cl^-$–HCO_3^- exchange upon stimulation of the parietal cell? The first prerequisite for an answer to this question is to know parietal cell pH_i and the change in pH_i during stimulation. The available data vary widely. For example, in frog oxyntic cells, the findings range from a resting pH_i in 5% CO_2/HCO_3^- of 7·02 (Yanaka, Carter, Goddard & Silen, 1991) to pH_i 7·4 (Debellis, Curci & Frömter, 1992) and a change in pH_i during stimulation from no change at all (Saario, Carter & Silen, 1987) to rather dramatic increases in pH_i of 0·5 to 1·0 units (Hersey, 1979; Ekblad, 1980). Our own pH_i measurements have demonstrated that in a population of freshly isolated parietal cells, changes in parietal cell acid formation and an increase in the basolateral Cl^-–base exchange rate can occur in the complete absence of changes in overall pH_i in that population (Seidler *et al.* 1992). However, recent measurements in our laboratory have shown that pH_i in single parietal cells in culture varies considerably within cells from the same rabbit under identical culture conditions, as does the response to secretagogues, both in changes in acid formation as well as in pH_i (U. Seidler, unpublished observations). The pH_i of the majority of cells was clearly in the pH_i range between 7·0 and 7·5, in which allosteric regulation is maximal. Thus, it is likely that under physiological conditions, a secretagogue-induced shift in pH_i and a subsequent allosteric activation of anion exchange will be one of several modes of anion exchange regulation during stimulation of acid formation. We are

presently working on the establishment of experimental models in which we can study pH_i and acid formation simultaneously in the same cell in culture, and in the same intact epithelium. If these models become available, one will be better able to delineate the mechanisms of ion homeostasis in the parietal cell.

REFERENCES

Chow, A., Dobbins, J. W., Aronson, P. S. & Igarashi, P. (1992). c-DNA cloning and localization of a band 3-related protein from ileum. *American Journal of Physiology* **263**, G345–352.

Dahlquist, A. (1964). A method for assay of intestinal disaccharidases. *Analytical Biochemistry* **7**, 18–25.

Debellis, L., Curci, S. & Frömter, E. (1992). Microelectrode determination of oxyntic cell pH in intact frog gastric mucosa. Effect of histamine. *Pflügers Archiv* **422**, 253–259.

Ekblad, E. B. M. (1980). Increase of intracellular pH in secreting frog gastric mucosa. *Biochimica et Biophysica Acta* **632**, 375–385.

Forbush, B. III (1983). Assay of Na/K-ATPase in plasma membrane preparations: increasing the permeability of membrane vesicles using sodium dodecyl sulphate buffered with bovine serum albumine. *Analytical Biochemistry* **128**, 159–163.

Forte, J. G. & Machen, T. E. (1986). Ion transport by gastric mucosa. In *Physiology of Membrane Disorders*, 2nd edn, ed. Andreoli, T. E., Hoffman, J. F., Fanestil, D. D. & Schulz, S. G., pp. 535–558. Plenum Press, New York.

Green, J. U., Yamaguchi, D. T., Cleeman, C. R. & Muallem, S. (1990). Cytosolic pH regulation in osteoblasts. *Journal of General Physiology* **95**, 1121–145.

Haase, W., Schäfer, A., Murer, H. & Kinne, R. (1978). Studies on the orientation of brush border membrane vesicles. *Biochemical Journal* **172**, 57.

Hanna, S., Mircheff, A. K. & Wright, E. M. (1979). Alkaline phosphatase of basal lateral and brush border plasma membranes from intestinal epithelium. *Journal of Supramolecular Structure* **11**, 451–466.

Hersey, S. J. (1979). Intracellular pH measurements in gastric mucosa. *American Journal of Physiology* **237**, E82–89.

Hirst, B. H. & Forte, J. G. (1985). Redistribution and characterization of K/H-ATPase in membranes from resting and stimulated gastric parietal cells. *Biochemical Journal* **231**, 641–649.

Hoffmann, E. K. & Simonsen, L. O. (1989). Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiological Reviews* **69**, 315–382.

Jesse, F., Sjöholm, C. & Hoffmann, E. K. (1986). Identification of the anion exchange protein of Ehrlich cells: a kinetic analysis of the inhibitory effects of 4,4-diisothiocyano-2,2-stilbene disulfonic acid (DIDS) and labeling of membrane proteins with ^{3}H-DIDS. *Journal of Membrane Biology* **92**, 195–205.

Kinne, R., Haase, W., Gmaj, P. & Murer, H. (1978). ATP hydrolysis as driving force for transport processes in isolated renal plasma membrane vesicles. In *Biochemical Nephrology*, ed. Guder, W. G. & Schmidt, U., pp. 178–185. Hans Huber, Bern.

Knickelbein, R., Aronson, P. S., Atherton, W. & Dobbins, J. W. (1983). Sodium and chloride transport across ileal brush border. I. Evidence for Na–H exchange. *American Journal of Physiology* **245**, G666–670.

Knickelbein, R., Aronson, P. S., Atherton, W. & Dobbins, J. W. (1985). Sodium and chloride transport across ileal brush border. II. Evidence for Cl–HCO_3 exchange. *American Journal of Physiology* **249**, G236–245.

Kopito, R. R. (1990). Molecular biology of the anion exchanger gene family. *International Review of Cytology* **123**, 177–199.

Kudrycki, K. E., Newman, P. R. & Shull, G. E. (1990). cDNA cloning and tissue distribution of mRNAs for two proteins that are related to the band 3 Cl^-/HCO_3^- exchanger. *Journal of Biological Chemistry* **265**, 462–471.

Lambert, R. W., Bradley, M. E. & Mircheff, A. K. (1991). pH-sensitive anion exchanger in rat lacrimal acinar cells. *American Journal of Physiology* **260**, G517–523.

Lamprecht, G., Seidler, U. & Classen, M. (1993). pH_i-regulating ion transport mechanisms in parietal cell basolateral membrane vesicles. *Americal Journal of Physiology* **265**, G903–910.

Lin, P., Ahluwalia, M. & Gruenstein, E. (1990). An alkaline pH-activated Cl^--anion exchanger regulates pH homeostasis in fibroblasts. *American Journal of Physiology* **258**, C132–139.

Mason, M. J., Smith, J. D., de Jesus Garcia-Soto, J. & Grinstein, S. (1989). Internal pH-sensitive site couples Cl/HCO_3 exchange to Na/H antiport in lymphocytes. *American Journal of Physiology* **256**, C428–433.

Michelangeli, F. (1978). Acid secretion and intracellular pH in isolated oxyntic cells. *Journal of Membrane Biology* **38**, 31–50.

Muallem, S., Blissard, D., Cragoe, E. J. & Sachs, G. (1988). Activation of the Na/H and Cl/HCO_3 exchange by stimulation of acid secretion in the parietal cell. *Journal of Biological Chemistry* **263**, 14703–14711.

Mugharbil, A., Knickelbein, R. G., Aronson, P. S. & Dobbins, J. W. (1990). Rabbit ileal brush-border membrane Cl/HCO_3 exchanger is activated by an internal pH-sensitive modifier site. *American Journal of Physiology* **259**, G666–670.

Nader, M., Seidler, U. & Classen, M. (1992). Differences in the internal pH-sensitive "modifier site" of the ileal brush border and the parietal cell basolateral anion exchanger in the rabbit. *Gastroenterology* **94**, A1077.

Olsnes, S., Tonessen, T. I., Ludt, J. & Sandvig, K. (1987). Effect of intracellular pH on the rate of chloride uptake and efflux in different mammalian cell lines. *Biochemistry* **26**, 2778–2785.

Olsnes, S., Tonessen, T. I. & Sandvig, K. (1986). pH-regulated anion antiport in nucleated mammalian cells. *Journal of Cell Biology* **102**, 967–971.

Paradiso, A. M., Townsley, M. C., Wenzl, E. & Machen, T. E. (1989). Regulation of intracellular pH in resting and stimulated parietal cells. *American Journal of Physiology* **257**, C554–561.

Peterson, G. L. (1983). Determination of total protein. *Methods in Enzymology* **91**, 95–119.

Rehm, W. S. & Sanders, S. S. (1975). Implications of the neutral carrier Cl/HCO_3 exchange mechanism in gastric mucosa. *Annals of the New York Academy of Sciences* **264**, 442–445.

Rossmann, H., Seidler, U., Nader, M., Classen, M., Obermaier, B. & Kopito, R. (1993). Expression of the AE2 isoform of the anion exchanger gene family in rabbit parietal cells. *Pflügers Archiv* **420**, R71.

Saario, I., Carter, K. & Silen, W. (1987). Intracellular pH (pH_i) in stimulated *vs.* inhibited parietal cells in frog gastric mucosa. *Gastroenterology* **92**, A1607.

Seidler, U., Lamprecht, G., Roithmeier, S. & Classen, M. (1991). Cl^-/base exchange in isolated rabbit parietal cells and parietal cell basolateral membranes. *Pflügers Archiv* **418**, suppl. A288.

Seidler, U., Roithmaier, S., Classen, M. & Silen, W. (1992). Influence of acid secretory state on Cl/base and Na/H exchange and pH_i in isolated rabbit parietal cells. *American Journal of Physiology* **262**, G81–91.

Stieger, B. & Murer, H. (1983). Heterogenity of brush-border membrane vesicles from rat small intestine prepared by a precipitation method using Mg/EGTA. *American Journal of Biochemistry* **135**, 95–101.

Thomas, H. A. & Machen, T. E. (1991). Regulation of Cl/HCO_3 exchange in gastric parietal cells. *Cell Regulation* **2**, 727–737.

THOMAS, H. A., MACHEN, T. E., SMOLKA, A., BARON, R. & KOPITO, R. R. (1989). Identification of a 185 kDa band 3-related polypeptide in oxyntic cells. *American Journal of Physiology* **257**, C537–544.

VAN ADELSBERG, J. S., EDWARDS, J. C. & AL-AWQATI, Q. (1993). The apical Cl/HCO_3 exchanger in β-intercalated cells. *Journal of Biological Chemistry* **268**, 11283–11289.

WENZL, E. & MACHEN, T. E. (1989). Intracellular pH dependence of buffer capacity and anion exchange in the parietal cell. *American Journal of Physiology* **257**, G741–747.

YANAKA, A., CARTER, K. J., LEE, H.-H. & SILEN, W. (1990). Influence of Cl^- on pH_i in oxyntopeptic cells of *in vitro* frog gastric mucosa. *American Journal of Physiology* **258**, G815–824.

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