Regulation of Cl/HCO_3 exchange in gastric parietal cells

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Microspectrofluorimetry of the fluorescent indicators 2',7'-bis-(2-carboxyethyl)-5(and -6)carboxyfluorescein and 6-methoxy-N-(3-sulfopropyl)-quinolinium was used to measure intracellular pH (pHi), intracellular Cl (Cli), and transmembrane fluxes of HCO_3 and Cl in single parietal cells (PC) in isolated rabbit gastric glands incubated in HCO_3/CO_2 -buffered solutions. Steady-state pHi was 7.2 in both resting (50 μM cimetidine) and stimulated (100 μM histamine) PCs. Transmembrane anion (HCO_3 or Cl) flux rates during Cl removal from or readdition to the perfusate were the same in resting and stimulated PCs. These rates increased at alkaline pHi, though this pHi dependence was small in the physiological range. Maximum velocity (V_{max}) for Cl influx or HCO_3 efflux was 80–110 mM/min at pHi 7.6–7.8, and the K_m for extracellular concentrations of Cl (Clo) was 25 mM; in the physiological range (pHi 7.1–7.3), V_{max} for anion fluxes was ≈ 50 mM/min. Steady-state Cli in the unstimulated PC was 62 ± 5 mM, but on histamine stimulation, Cli decreased rapidly to 25 mM and then increased back to a steady-state level of 44 mM. HCO_3 fluxes due to Cl removal or readdition were completely blocked by 0.5 mM 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid (H_2DIDS), but Cl fluxes were only inhibited by 80%. H_2DIDS did not inhibit the decrease in Cli that occurred with histamine treatment. Diphenylamine carboxylate (0.5 mM) inhibited Cl flux by only 50% and caused no additional inhibition of Cl flux when used in conjunction with H_2DIDS . Transmembrane anion fluxes during solution Cl removal or readdition occurred 80% through the anion exchanger at the basal membrane and 20% through other pathway(s), presumably the Cl channel in the apical membrane. We conclude that the increase in transport activity via the Cl/HCO_3 exchanger that occurs during histamine-induced increases in HCl secretion is due mostly to the decrease in Cli. In the resting cell with $\text{Cli} = 62$ mM, $\text{Clo} = 120$ mM, $\text{pHi} = 7.2$, and extra-

cellular $\text{pH} = 7.4$, the anion exchanger is poised near its thermodynamic equilibrium. During histamine stimulation Cli drops from 62 mM to 44 mM, the thermodynamic equilibrium of the anion exchanger at the basolateral membrane is disturbed, and the anion exchanger then exchanges cellular HCO_3 for extracellular Cl. Cli serves a crucial regulatory role in stimulus-secretion coupling in the PC.

Introduction

The parietal cell (PC) is a highly specialized cell that secretes nearly isotonic HCl into the gland lumen. The secretory activity of the PC is stimulated by a variety of secretagogues, with histamine being the most potent. Intracellular signaling events ultimately lead to the transition from the resting to the stimulated state. An H/K -ATPase is activated in parallel with permeability pathways for both Cl and K on the apical canalicular face of the PC, whereas an anion exchanger provides pathways for Cl entry and HCO_3 exit on the basolateral side of the cell (Ito and Schofield, 1974; Lee *et al.*, 1979; Forte *et al.*, 1981; Wolosin and Forte, 1984; Muallem *et al.*, 1985, 1988; Paradiso *et al.*, 1987; Ueda *et al.*, 1987).

Secretion of HCl into the gland lumen leaves the PC with an equivalent base load. The PC is able to accommodate this load by transporting HCO_3 into the blood, accounting for the "alkaline tide" that follows a meal. Experiments by Paradiso *et al.* (1989) indicated that in *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)-buffered (nominally HCO_3 -free) solutions histamine-stimulated/4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid (DIDS)-treated PCs alkalinized at a greater rate than unstimulated/DIDS-treated PCs. This suggested that the Cl/HCO_3 exchanger was activated by histamine, though the specific mechanism was not determined.

There are two ways in which the rate of HCO_3 and Cl movements through the exchanger could be increased. One, by altering the kinetic properties (i.e., maximum velocity [V_{max}] or K_m). This is the mode by which the Na/H exchanger is

activated by growth factors in fibroblasts and other cell types, and it likely involves a phosphorylation event (Sardet *et al.*, 1990) and an alteration (Moolenaar, 1986) in the affinity of the internal transport sites for H (Aronson *et al.*, 1982). We will refer to this as biochemical activation. The second mechanism involves a change in the transmembrane gradients for the transported ions, in this case Cl and HCO₃. Because extracellular concentrations of HCO₃ (HCO_{3o}) and Cl (Cl_o) remain roughly constant under physiological conditions, increasing the activity of the Cl/HCO₃ exchanger in PC could only be accomplished by either reducing intracellular Cl (Cl_i) and/or by increasing intracellular HCO₃ (HCO_{3i}). We refer to this as thermodynamic activation. Of course, it is possible that both activation mechanisms could be operative during the transition from the resting to the stimulated condition in the PC.

There have been three previous studies that are relevant here. Muallem *et al.* (1988) found that in HEPES-buffered solutions, histamine induced a cytosolic alkalization of 0.13 pH units in suspensions of PCs. They proposed that this elevation of intracellular pH (pHi) might directly activate the exchanger by raising [HCO₃]_i, and it could indirectly activate the exchanger if the exchanger exhibited a dependence on pHi (i.e., if the exchanger's activity was exquisitely sensitive to pHi such that this small increase in pHi would increase its activity several-fold). Paradiso *et al.* (1989), also working in HEPES-buffered solutions, showed in measurements on single PC that pHi increased during histamine stimulation in only ~60% of PC, and they concluded that increased pHi (and, consequently, HCO_{3i}) could not be the explanation for activation of anion exchange in all PC. Wenzl and Machen (1989), who also used HEPES-buffered solutions, found that even though the anion exchanger was indeed pHi sensitive, this pHi dependence was insufficient to account for the observed activation of the anion exchanger (i.e., with only an 0.13 pHi unit increase during stimulation).

With this as background, we initiated a further investigation of the regulation of Cl/HCO₃ exchange by measuring steady-state pHi and Cl_i as well as HCO₃ and Cl fluxes in both unstimulated and stimulated PCs. Microspectrofluorimetry with the fluorescent indicators 2',7'-bis-(2-carboxyethyl)-5(and 6)carboxyfluorescein (BCECF) and 6-methoxy-N-(3-sulfoethyl)quinolinium (SPQ) was used to measure pHi and Cl_i in single PC from isolated rabbit gastric glands. In contrast to previous studies on the Cl/HCO₃

exchanger (Muallem *et al.*, 1988; Paradiso *et al.*, 1989; Wenzl and Machen, 1989), in the present work we used HCO₃/CO₂ solutions to study the kinetics of anion transport and steady-state intracellular anion activities under more physiological conditions. Measurements of steady-state Cl_i, pHi, and HCO_{3i} (using the known [CO₂] and pHi in the Henderson-Hasselbalch equation, HCO_{3i} can be calculated) yield information about whether the anion exchanger might be thermodynamically activated. Biochemical activation was assessed by changing Cl_o and measuring the resulting rates of change of pHi and Cl_i. For example, when PCs were incubated with Cl-free solutions, adding Cl back caused pHi to decrease and Cl_i to increase at rates determined by the activity of the anion exchanger. If biochemical activation occurred during the resting-stimulated transition of PC, then it was expected that the rates of pHi decrease and Cl_i increase would be faster in stimulated versus resting PC. We found that pHi and kinetics of anion transport were not altered by histamine stimulation. However, a decrease in Cl_i, which is not inhibitable by H₂DIDS, does occur. We propose that this drop in Cl_i leads to a thermodynamic activation of the Cl/HCO₃ exchanger, and we discuss the overall role of Cl in stimulus-secretion coupling in the PC.

Results

Lack of effects of histamine on pHi and kinetics of the Cl/HCO₃ exchanger as measured with BCECF

A typical experiment in which PCs were perfused with Cl-containing and Cl-free solutions during resting and histamine-stimulated conditions is shown in Figure 1. Although there were occasionally small, transient changes in pHi during the initial stages of histamine treatment, there was no change in steady-state pHi between resting and 100 μM histamine-treated PCs (Figure 1 and Table 1, line 1). It is therefore unlikely that changes in pHi could be thermodynamically driving the increased activity of the anion exchanger (i.e., through a change in the OH/HCO₃ gradient across the basolateral membrane). Figure 1 also shows the protocol used to measure anion exchanger-mediated HCO₃ fluxes observed on Cl readdition to Cl-depleted cells, calculated as the initial rate of change of pHi × the buffering capacity.

When Cl was removed from or added to the perfusate, the pHi of the PC increased or decreased rapidly due to the activity of the Cl/

Figure 1. Effect of Cl removal and readdition on pHi in the resting (50 μ M cimetidine) and stimulated (100 μ M histamine) PC. BCECF measurements of pHi were made by perfusing the sample first with a cimetidine-containing Ringer's solution and then with a Cl-free solution, followed by a return to the Cl-containing solution. This same procedure was then repeated with histamine-containing solutions. pHi was 7.36 in the resting and 7.41 in the stimulated PC, and the rates of HCO₃ flux on Cl removal were 67 and 75 mM/min. When adding Cl back to Cl-depleted cells, pHi was 7.8 and 7.9 (resting and stimulated) and flux rates were 149 and 226 mM/min. Although HCO₃ flux was faster in the stimulated condition in this particular PC, part of this difference was due to the fact that pHi was higher in the stimulated PC in Cl-free solution and the anion exchanger is activated by increased pHi (see below). On average, steady-state pHi and HCO₃ fluxes were the same in the resting versus the stimulated condition (see Table 1, lines 1 and 3).

HCO₃ exchanger. For paired resting and stimulated PCs, the average HCO₃ flux rates on Cl removal at pHi $\approx$ 7.2 were 51 ± 20 mM/min ($n = 10$) and 57 ± 21 mM/min ($n = 9$), respectively. In all cases, for comparisons of resting and stimulated PCs, cells served as their own controls. It should be noted that although the pHi

was the same for resting and stimulated PCs, because Cli was different (as we determined with SPQ measurements, see below), the driving forces for anion exchange are slightly different when applying the Cl-removal maneuver to resting versus stimulated PC. However, when adding Cl back to resting and stimulated (Cl-depleted cells at the same pHi) the thermodynamic driving forces for the anion exchanger are equal. Under these conditions there were no differences in HCO₃ flux rates on Cl readdition to Cl-depleted cells: resting = 81 mM/min versus stimulated = 77 mM/min (pHi $\approx$ 7.6) (Table 1, line 3). Thus histamine treatment did not affect the ability of the exchanger to transport HCO₃ into or out of the cell, driven by an infinite gradient of Cl (i.e., Cl-containing to Cl-free or Cl-free to Cl-containing).

Over the course of these experiments, we measured HCO₃ flux rates when removing Cl or when adding Cl back to Cl-depleted cells under a variety of conditions, including resting versus histamine-stimulated and in Na-containing versus Na-free solutions. As shown in Figure 2, there was an increase in the HCO₃ flux rate at increasingly alkaline pHi. This is similar to what was observed previously except that the flux rates that occurred here in HCO₃-buffered solutions at 37°C were roughly 10 times greater than those that occurred in HEPES-buffered solutions at 25°C (Wenzl and Machen, 1989). In the present study we observed no consistent differences between this pHi dependence in resting and stimulated PCs. Also, over the physiological range of pHi, the pHi dependence of the HCO₃ flux rate was small. From these measurements we conclude that changes in

Table 1. pHi, Cli, and anion flux measurements in resting and stimulated parietal cells

	Resting	(n)	Stimulated	(n)
pHi	7.24 ± 0.1	(10, 3)	7.20 ± 0.18	(9, 3)
Cli (mM)	62 ± 5	(52, 13)	$44 \pm 9^*$	(11, 3)
HCO ₃ flux (mM/min):				
0–120 mM Cl	81 ± 17	(10, 3)	77 ± 21	(9, 3)
Cl flux (mM/min):				
0–120 mM Cl	71 ± 6	(10, 3)	70 ± 5	(6, 3)
0–10 mM Cl	25 ± 3	(6, 3)	27 ± 9	(11, 3)

Measurements of steady-state pHi and Cli and of HCO₃ and Cl fluxes during a change from Cl-free to Cl-containing (120 mM Cl) solutions were performed as described in Methods. All comparisons of resting vs. histamine (100 μ M)-stimulated conditions were performed on single PC. n, number of different PCs and number of animals.

* There were no differences between resting and histamine-stimulated PCs, except for steady-state Cli.

Figure 2. pH_i dependence of HCO_3 flux in resting and histamine-stimulated parietal cells under control conditions and in Na-free solutions. At increasingly alkaline pH_i , HCO_3 flux (calculated using BCECF measurements of pH_i and total buffering capacity, as described in Methods) increased, but there was no consistent difference between the pH_i dependence of HCO_3 fluxes in resting and stimulated PCs ($n = 22$).

pH_i , HCO_3 flux kinetics, and/or pH_i dependence are not occurring and are therefore not responsible for regulating the activity of the anion exchanger during histamine stimulation.

SPQ is used to measure steady-state Cl_i

The fluorescent indicator SPQ was used to measure Cl_i to investigate the possibility that Cl_i might play a role in the regulation of the activity of the anion exchanger. Figure 3 shows a typical trace measuring steady-state Cl_i and a subsequent calibration. Using the calibration technique described in Methods, Cl_i was estimated to be 60 mM in this PC. This can also be seen by noting that the relative fluorescence intensity in the steady-state unstimulated PC is close to that for the calibration solution containing 60 mM Cl. A calibration curve was made for each individual experiment, and these curves were then averaged to yield the curve in Figure 4. The relative fluorescence intensity of the signal (F_o/F) is plotted against Cl_i , and the ratio is linear over the range of Cl_i tested. The average Cl_i in unstimulated PCs was 62 ± 5 mM (see Table 1, line 2). As we will discuss further below, based on measurements of pH_i and Cl_i and known values of extracellular pH (pH_o) and Cl_o , the anion exchanger appears to be poised near equilibrium in the resting condition.

The kinetics of Cl fluxes as measured with SPQ do not change with histamine stimulation

We wanted to confirm the apparent lack of effect of histamine on HCO_3 flux as measured with

Figure 3. Steady-state Cl_i measurement and calibration with SPQ. Initially, the gland was perfused with Ringer's solution. Then the Ringer's solution was replaced with calibration solution containing 145 mM K, 10 μM TPT, 15 μM nigericin, and varying amounts of Cl (60, 25, 0 mM balanced by gluconate). Total quenchable fluorescence was determined by perfusing the sample with KSCN. This trace is representative of 21 such traces. See Table 1, line 2, for averages.

BCECF by measuring Cl flux with SPQ. We also hoped to gain some information regarding the kinetics of Cl/HCO_3 exchange. Cl flux was calculated by first depleting PCs of Cl and then adding back varying concentrations of Cl to the perfusate while measuring the initial rate of change of Cl_i . Figure 5 shows a trace in which PCs were incubated in a Cl-free medium and then 120 mM Cl was added back. The cells were returned to Cl-free and then 10 mM Cl was added back. Usually two such changes from Cl-free to Cl-containing solutions were made for each PC before calibration. Flux rates taken from 12 different PCs were compiled (Figure 6).

Figure 4. Calibration curve for SPQ fluorescence in PC at different Cl_i . The data were derived from a series of calibrations from 52 PCs and 13 animals and indicate that the ratio of F_o/F to Cl_i is linear over the range of Cl_i tested (0–60 mM).

Figure 5. Measurement of transmembrane Cl fluxes. Cl fluxes were measured with SPQ by perfusing glands with a Cl-free solution and then adding varying amounts of Cl (in this trace, 120 and 10 mM Cl) back to the perfusate. Cl flux was calculated as $\Delta\text{Cl}/\Delta t$, the initial rate of change of Cl. The second portion of the trace shows the calibration.

By transforming this data into a Lineweaver-Burke plot, the $V_{\max}$ for Cl influx was calculated to be 90 mM/min and the K_m for Cl_o was 25 mM (Figure 6, see inset). The Cl flux rate for adding 120 mM Cl back to Cl-depleted cells was 71 ± 6 mM/min (Figure 6 and Table 1, line 4), which was very close to the HCO₃ flux rate (81 ± 17 mM/min) calculated on the basis of BCECF measurements of pH_i (Table 1, line 3). The fact that the measured HCO₃ and Cl fluxes are well matched is an indication that the changes in pH_i and Cl_i that occur are likely to be carried out principally by the Cl/HCO₃ exchanger. We also compared Cl flux rates when adding 10 mM Cl back with resting versus histamine-stimulated Cl-depleted cells, and the rates were nearly identical (Table 1, line 5). Together these data confirm the tremendous capacity (large flux rate) of the transporter but indicate that histamine-induced changes in some kinetic parameter leading to an increase in exchange activity are unlikely.

Histamine treatment leads to a decrease in Cl_i

When 100 μM histamine was added to the perfusate, replacing cimetidine, a rapid drop in Cl_i occurred (Figure 7). Cl_i decreased from 60 to 25 mM and then increased back to 45 mM. Another pattern that was commonly observed was a slower drop from 60 to 45 mM Cl_i without the initial undershoot to 25 mM (not shown). On average, Cl_i dropped from 62 to 44 mM (see Table 1, line 2). This decrease in Cl_i was not inhibited by 0.5 mM H₂DIDS (5 min preincubation before addition of histamine; data not

Figure 6. Cl fluxes vs. Cl_o . Flux rates (switching from 0 to 10, 20, 60, and 120 mM Cl) as measured in Figure 5 (12 different PCs, 3 animals) were compiled. The inset shows the Lineweaver-Burke plot of this data, which yielded a $V_{\max}$ for Cl influx of 90 mM/min and a K_m for Cl_o of 25 mM.

shown) so it is unlikely to be mediated by the Cl/HCO₃ exchanger.¹

Cl fluxes are inhibited by H₂DIDS and diphenylamine carboxylate (DPC)

After a 5-min treatment with 0.5 mM H₂DIDS, Cl fluxes were inhibited by 80% (Figure 8). On removal of Cl from the perfusate, Cl efflux from the H₂DIDS-treated PC occurred at only 20% of the control rate, and, interestingly, Cl_i did not decrease to the same level as control. When Cl was added back to Cl-depleted PCs, Cl entry rate was only $\sim 20\%$ of control. DPC 0.5 mM (a known inhibitor of Cl channels) alone caused a 50% inhibition of Cl efflux, and the combination of 0.5 mM DPC and 0.5 mM H₂DIDS caused an 80% inhibition of Cl efflux. Because the effect of H₂DIDS plus DPC was no different from the effect of H₂DIDS alone, it seems likely that DPC at this concentration might also be affecting the Cl/HCO₃ exchanger. Because DPC was dissolved in a dimethyl sulfoxide (DMSO) stock solution, it was necessary to control for the effects of DMSO on SPQ fluorescence. This was achieved by running parallel experiments with DMSO alone. A small transient decrease in SPQ fluorescence occurred immediately after addi-

¹ In some cases, histamine seemed to initiate a rapid loss of dye from the PC, as indicated by a dramatic fall in the intensity of the signal. This could represent either stimulation of some mechanism that transports dye out of the cells or an increase in the passive leak due to an increase in the surface area to volume ratio that might occur during the resting to stimulated transition. In these cases it was not possible to calibrate the traces; however, there appeared to be an initial decrease in Cl_i preceding the loss of dye based on the observed increase in SPQ fluorescence after histamine stimulation.

Figure 7. Effect of histamine on Cl_i . In glands treated with 100 μM histamine, Cl_i decreased from 60 mM to roughly 25 mM and then increased back to 45 mM. The parallel lines drawn across the trace represent the rate of dye leakage that was linear throughout the experiment and the subsequent calibration. This trace is typical of 13 PC (5 animals).

tion of DMSO to the perfusate, but DMSO had no effect on the magnitude or rate of change of fluorescence in response to changes in Cl_o (data not shown). Our results with DPC are somewhat different from those of Malinowska (1990), who found that DPC inhibited aminopyrine uptake in digitonin-permeabilized PCs with a K_i of 240 μM . She proposed that this effect of DPC was due to blockage of apical Cl channels, which then indirectly inhibited H secretion (i.e., AP accumulation). The difference between the results of the two studies is likely due to the fact that Malinowska was studying events at the apical membrane, whereas we have studied anion exchange at the basolateral membrane.

Figure 8. Inhibition of Cl fluxes. Rate of Cl efflux on Cl removal was inhibited by 80% after a 5-min treatment with 0.5 mM H_2DIDS . Rates of Cl flux were calculated as described in Methods; the level of inhibition was measured as percent rate of Cl flux in the presence of inhibitor(s) vs. control.

Discussion

When the Cl concentration in the solution bathing the glands is altered, there are rapid changes in Cl_i and HCO_3^- of PC. In resting cells that have been incubated in Cl -free solutions and then exposed again to Cl , the rate at which Cl enters the cells (71 mM/min) was approximately equal to the rate of HCO_3^- loss (measured from the rate of pH_i decrease during the initial stages of Cl treatment, calculated to be 81 mM/min). In the histamine-stimulated cells, when it is expected that the Cl channels in the apical membrane have been activated (Demarest *et al.*, 1989), there was still good agreement between the Cl fluxes (70 mM/min) and the HCO_3^- fluxes (77 mM/min). In addition, it has been shown previously that 200–500 μM H_2DIDS largely blocks the Cl -induced changes of pH_i (Paradiso *et al.*, 1987), and the present experiments have shown that Cl fluxes were blocked by $\sim 80\%$ (Figure 9). The close agreement among these measurements indicates that during changes of Cl_o , influx and efflux of Cl from the cell are mediated primarily through the anion exchanger that is present in the basolateral membrane. This has allowed us to evaluate the effects of histamine stimulation on the kinetics of this transporter.

Using these measurements as an assay, we have found that Cl influx obeys Michaelis-Menten kinetics, with $K_m = 25$ mM. This value is similar to the value of 17.5 mM that Muallem *et al.* (1985) obtained from measurements of ^{36}Cl

Figure 9. Summary of the effects of H_2DIDS and DPC on Cl fluxes. Five-minute treatment with 0.5 mM DPC caused a 50% inhibition of Cl efflux on Cl removal, and the combination of 0.5 mM H_2DIDS and 0.5 mM DPC (together for 5 min) caused an 80% inhibition of Cl efflux. Both efflux and influx of Cl on Cl removal and readdition were 80% inhibited by 0.5 mM H_2DIDS .

fluxes in isolated basolateral membranes from the rabbit PC. Because in normal physiological conditions plasma [Cl] is >110 mM, it is apparent that the anion exchanger will be saturated at the outside aspect of the cell.

The $V_{\max}$ calculated from the Lineweaver-Burke plot using the Cl influx rate (with SPQ) was 90 mM/min, which was similar to the HCO₃ flux measured (with BCECF) under the same conditions (77 mM/min, Table 1). It should be noted that this rate is manifest during the transition of the cell from a Cl-free solution to a Cl-containing solution when pHi is in the range 7.5–7.8, which is higher than normally occurs under physiological conditions. Under more physiological conditions when pHi is 7.1–7.2, anion flux through the exchanger can occur at a rate of about 50 mM/min. The reason for this lower activity is that the anion exchanger exhibits a pHi dependence such that its activity increases as pHi increases (Figure 2).

As seen from both the Cl and HCO₃ fluxes, the ability of this transporter to move these anions across the basolateral membrane of the PC does not change during histamine stimulation. We will have more to say about this below. However, it should be noted that the anion exchanger of the osteosarcoma cell line UMR-106 is activated by raising Cai (Green *et al.*, 1990), so it appears that regulation of these transporters may differ depending on the cell type and specific anion exchanger. It is not clear whether this Ca-dependent activation in UMR-106 cells is mediated by direct action on the Cl/HCO₃ exchanger itself or via an intermediary protein(s) that in turn regulate anion exchange. It is also unknown whether the PC and UMR-106 Cl/HCO₃ exchangers are products from the same gene or are products of distinct but related genes. Although the precise molecular nature of the PC anion exchanger is not certain, we have recently identified a 185-kDa protein that bears some homology to the erythrocyte band 3 protein (Thomas *et al.*, 1989). One recent study (Kudrycki *et al.*, 1990) indicates that this protein may be the product of a gene related to but distinct from band 3. Band 3-like proteins are also found in high levels in kidney and choroid plexus as well as other nonepithelial tissues (Kay *et al.*, 1983; Drenckhahn and Zinke, 1984; Cox *et al.*, 1985; Schuster *et al.*, 1986; Drenckhahn *et al.*, 1987; Wagner *et al.*, 1987; Verlander *et al.*, 1988).

The rates determined here for the anion exchanger of the PC can be compared with those of the Na/H exchanger, which also exists in the basolateral membrane. In experiments in which

PCs were acidified to pHi 6.7 and pHi recovery through the Na/H exchanger was measured (Townsend and Machen, 1989), it can be calculated, using the intrinsic buffer capacity measurements published by Wenzl and Machen (1989), that the cation exchanger was operating at a rate of at least 3 mM/min. And in more recent experiments in which the activity of the Na/H exchanger was determined over a wider range of pHi values (M.C. Townsend, W. Reenstra, and T.E. Machen, unpublished data), it was found that the Na/H exchanger normally operates at a rate of 3 mM/min when the cell is at the resting pHi of 7.1–7.2. It has also been shown that there can be changes in the activity of the Na/H exchanger during histamine stimulation (Muallem *et al.*, 1988; Paradiso *et al.*, 1989), and these changes are likely due to biochemical changes in the Na/H exchanger because the transmembrane gradients for Na and H do not change very much during stimulation with histamine (present data and Negulescu *et al.*, 1990). Morell *et al.* (1990) have recently reported the existence of a putative cyclic AMP-dependent regulator of the Na/H exchanger in rabbit renal brush border membranes. This 42-kDa cofactor appears to confer protein kinase A-dependent inhibition to the renal exchanger. Whether this renal Na/H exchanger is the same protein as that in the PC and/or that recently cloned by Sardet *et al.* (1989) remains unknown. So it appears that tissue-specific differences in Na/H and Cl/HCO₃ exchange activity may be conferred by the existence of distinct transporters and/or regulatory factors.

In addition, there is an apparent Na/HCO₃ co-transport system in the basolateral membrane of PCs that has the ability to alkalinize the cells at a rate approximately equal to that of the Na/H exchanger (Townsend and Machen, 1989). Changes in the activity of the Na/HCO₃ co-transporter with stimulation have not been unequivocal (Machen *et al.*, 1989).

Thus there are at least three different pHi regulatory mechanisms in the basolateral membrane of the PC with the Cl/HCO₃ exchanger having a much larger (by a factor of ~10) capacity to transport H/OH. How can the cell accommodate the activities of these different transporters, which have such disparate transport capabilities, and then coordinate all of them plus the apical H/K ATPase in the stimulated cell? Our view is as follows. In the resting cell (when the H/K ATPase is inactivated) in 5% CO₂ solutions containing 123 mM Cl, 145 mM Na, 25 mM HCO₃, and pHo = 7.4, we have found that pHi = 7.1, HCO₃i = 12.5 mM (calculated

from pH_i using the Henderson-Hasselbalch equation), $Cl_i = 62$ mM (Table 1) and $Na_i = 8$ – 10 mM (Negulescu *et al.*, 1990). Under these conditions, the Na/H exchanger operates in the inward direction, driven by the inward-directed Na gradient at a rate of about 3 mM/min (M.C. Townsley, W. Reenstra, and T.E. Machen, unpublished data). The Cl/HCO₃ exchanger, which has a 10 times higher capacity to transport H/OH across the membrane, operates at a slow rate that is likely rather similar to that of the Na/H exchanger (Paradiso *et al.*, 1989) because the thermodynamic gradients for HCO₃ and Cl across the basolateral membranes are nearly matched, i.e., $Cl_o/Cl_i = HCO_{3o}/HCO_{3i} = \text{antilog}(pH_o - pH_i)$. Using the concentrations shown above, $Cl_o/Cl_i = 120/62 = 1.9$, $HCO_{3o}/HCO_{3i} = \text{antilog}(pH_o - pH_i) = 2.0$. We therefore conclude that the anion exchanger is operating at a slow rate in the resting cell not because the exchanger is incapable of transporting ions at a rapid rate but because the concentrations of the relevant ions on each side of the membrane keep it poised near equilibrium. Under these conditions, there is likely to be a very rapid unidirectional movement of Cl and HCO₃ across the membrane, but the net fluxes of the ions are small. The role of the Na/HCO₃ cotransporter may be similarly restricted by having it sit near its thermodynamic equilibrium. However, because we do not know the stoichiometry of this transporter or the membrane voltage, we do not have enough information to make any quantitative statements about how the activity of this transporter might be affected by the thermodynamic gradients involved.

When the PC is activated by histamine, the apical H/K-ATPase is stimulated. We have not been able yet to quantitate its activity, but previous work indicates that it does indeed turn on in these cells (Muallem *et al.*, 1988; Paradiso *et al.*, 1989), though its ability to affect pH_i appears to be small (Muallem *et al.*, 1988). To accommodate the accompanying increase in alkaline load to the cell (Muallem *et al.*, 1988; Paradiso *et al.*, 1989), the anion exchanger becomes activated but not by some biochemical change in the exchanger—at least the data in Figure 2 and Table 1 indicate that the ability of the anion exchanger to transport H/OH does not change during stimulation. Rather, the anion exchanger becomes activated because the Cl concentration gradient across the basolateral membrane changes. In the steady state, Cl_i decreases from 62 to 44 mM, so that $Cl_o/Cl_i = 120 \text{ mM}/44 \text{ mM} = 2.7 > HCO_{3o}/HCO_{3i} = \text{antilog}(pH_o - pH_i) = 2.0$. Under these conditions, there will be an

inward movement of Cl in exchange for intracellular HCO₃. The decrease in Cl_i compared with the resting cell is likely to be due to the opening of Cl channels in the apical membrane (Demarest *et al.*, 1989), because the decrease of Cl_i noted during histamine stimulation was not affected by the anion exchanger inhibitor H₂DIDS.

The interesting aspect of pH_i regulation in the PC is that it seems to be so precisely controlled, i.e., pH_i does not change during stimulation, yet there are large changes in the activities of at least three different pH_i regulatory mechanisms. In addition, the regulation of the different transporters is different in each case, and they are occurring at opposite sides of the cells. The H/K-ATPase is regulated by the insertion of membrane vesicles containing H/K pumps and Cl and K channels into the apical membrane. The basolateral Na/H exchanger is regulated through biochemical activation of already existing transporters that seem to alter their ability to transport H at a given pH_i , even when there are no changes in the transmembrane concentrations gradients for Na and H. And changes of the activity of the Cl/HCO₃ exchanger are mediated through thermodynamic activation of already existing transporters that have a very high capacity to move H/HCO₃ across the membrane. This thermodynamic activation occurs because the transmembrane concentration gradient for Cl is altered during stimulation, and this, in turn, seems to be regulated by the loss of Cl across the apical membrane (likely through a Cl channel). Thus, during stimulation of the PC, Cl_i serves a crucial regulatory role in coupling the apical with the basolateral pH_i regulatory mechanisms so that pH_i remains roughly constant in the face of large changes of transmembrane H/OH fluxes.

Methods

Chemicals and solutions

All chemicals were obtained from Sigma (St. Louis, MO) unless noted otherwise. BCECF/acetoxymethyl ester (AM), SPQ, and H₂DIDS were obtained from Molecular Probes (Eugene, OR) and DPC was a gift from Dr. William Reenstra. H₂DIDS 0.5 mM was prepared by heating in Ringer's solution. DPC 0.5 mM was prepared from a 200-mM stock that had been dissolved in DMSO. Ringer's solution contained 120 mM NaCl (or Na gluconate, for Cl-free solutions), 25 mM NaHCO₃/5% CO₂, 3 mM KCl (or K gluconate, for Cl-free solutions), 2 mM KH₂PO₄, 1 mM MgSO₄, 1 mM CaSO₄, 10 mM HEPES, and 10 mM glucose. This solution was bubbled with 95% O₂/5% CO₂. In the Na-free solution, Na was replaced with N-methyl-D-glucamine. Phosphate-buffered saline (PBS) contained 150 mM NaCl, 3 mM K₂HPO₄, 0.6 mM NaH₂PO₄, 1 mM CaCl₂, and 1 mM MgSO₄. Culture medium consisted of Eagle's Minimum Essential Medium

(MEM) (GIBCO, Grand Island, NY), pH 7.4, containing 50 μ M cimetidine and 20 mM HEPES. Solutions used in calibrating BCECF and SPQ fluorescence are described in the calibration section.

Preparation of gastric glands

Gastric glands were isolated from New Zealand White rabbits by high-pressure perfusion of the abdominal aorta and limited collagenase digestion as previously described (Paradiso *et al.*, 1986). Briefly, after perfusion with PBS, the fundic mucosa was scraped, minced, washed twice in PBS, digested for 25–30 min at 37°C in culture medium with 0.3 mg/ml collagenase type 1A, and 0.5 mg/ml bovine serum albumin (Calbiochem, San Diego, CA), and then washed four times in culture medium.

Dye loading

To measure intracellular pHi, glands were incubated in culture medium (MEM, pH 7.4, containing 50 μ M cimetidine and 20 mM HEPES) and 3 μ M BCECF/AM for 30 min at room temperature. The loading buffer was then replaced with dye-free culture medium, and the glands were allowed to recover from the inhibitory effects of the dye-loading process (Wolfbeis and Urbano, 1983; Illsley and Verkman, 1987; Krapf *et al.*, 1988; Negulescu *et al.*, 1989) for $\geq$ 30 min before experimentation.

For measurements of Cli, glands were incubated in culture medium containing 20 mM SPQ for $\geq$ 3 h at room temperature. This method was not toxic to PC based on exclusion of trypan blue, general morphology, and lack of effect on O₂ consumption rates of gastric glands in 50 μ M cimetidine- or 100 μ M histamine/10 μ M forskolin-containing solutions (data not shown). Experiments were performed in dye-free solutions. The rate of leakage was constant over the course of each experiment and was <5–10% in 30 min at 37°C.

Perfusion of cells

Loaded glands were allowed to settle on a glass coverslip that was then mounted into a stainless steel perfusion chamber. Perfusion solutions were equilibrated with 5% CO₂ and delivered from 60-ml syringes (suspended within a temperature-controlled lucite box) that were connected to the perfusion chamber by gas impermeable tubing (HCO₃/CO₂ experiments). All experiments were conducted at 37°C (unless noted); all perfusates were heated to 37°C and the perfusion chamber was heated to 37°C by a brass water jacket.

Microspectrofluorimetry

Measurements of pHi were made by exciting the BCECF-loaded glands with light provided by the microscope 75 W xenon bulb. A stepping motor (SLO-SYN; Superior Electric Company, Bristol, CT) alternated the appropriate filters (440 $\pm$ 5 and 490 $\pm$ 5 nm, Omega Optical, Brattleboro, VT) mounted in a paddle wheel at 2 Hz. The excitation light was passed through neutral density filters (to attenuate bleaching of the dye and to eliminate unnecessary exposure of the cells) and then through a 40 $\times$ oil immersion objective lens (Nikon Fluor/N.A. 1.30; Nikon, Garden City, NY) on a Zeiss IM35 microscope (Zeiss, Thornwood, NY). Emitted light was passed through a 520–550 nm bandpass filter and an image plane pinhole that restricted the emission to that from a single PC; this light was then collected with a photomultiplier tube (QL 30-F, APED-2 Thorn Emi Gencom Inc., Fairfield, NJ). The data were stored and processed by an IBM/AT computer and UMANS-software (User-friendly Microspec-

trofluorimetry Data Acquisition and Analysis System, Chester M. Regen, Urbana, IL).

SPQ fluorescence was excited at 350 nm with a fluorimeter (Fluoro-log2, Spex Industries, Edison, NJ). The light intensity was controlled by opening or closing the slit of the monochromator ($\approx$ 3–4 mm), and the emitted light from a single PC ($\geq$ 2–3 times greater than the level of autofluorescence) was transmitted through a 395-nm longpass filter and collected with a photomultiplier tube. The output from the photomultiplier was then stored in a data file on the Datamate computer.

Calibrations

Calibration of the BCECF fluorescence in terms of pHi was performed using the high K/nigericin method (Rink *et al.*, 1982; Thomas *et al.*, 1979). Briefly, the sample was perfused with solutions at various pH that contained 120 mM KCl, 30 mM Kgluconate, 10 mM HEPES, and 15 μ M nigericin to equilibrate pHi with pHo. The ratio of fluorescence at 490:440 at these known pHs was then used to establish a standard calibration curve to determine pHi under experimental conditions (Paradiso *et al.*, 1986).

SPQ fluorescence was calibrated in terms of Cli by perfusing the sample with a high K/nigericin/tri-phenyl-tin (TPT) solution containing varying amounts of Cl balanced by gluconate [145 mM K(Cl/gluconate), 2 mM KH₂PO₄, 1 mM MgSO₄, 1 mM CaSO₄, 10 mM HEPES, 10 mM glucose, 15 μ M nigericin, and 10 μ M TPT] to equilibrate both pHi with pHo and Cli with Clo. After each experiment, Cli was estimated by perfusing with calibration solutions containing known amounts of Cl (0–75 mM) and finally 150 mM potassium thiocyanate (KSCN) (to determine total quenchable fluorescence). Because there was variability from cell to cell in terms of the rate of dye leakage, in some cases it was necessary to draw lines extrapolating from the "experimental" portion of the trace to the "calibration" portion of the trace. The slope of the lines to be drawn was taken from the slope of the fluorescence intensity during control conditions and in the presence of calibration solutions and after transient changes of fluorescence due to changes in Cli caused by altering Clo had occurred. Only when the slope during the "experimental" portion of the trace was the same as that during the "calibration" portion was the experiment used for data collection. The level of remaining fluorescence in the presence of KSCN was usually 1.5–2 times the level of autofluorescence. A standard calibration curve for each experiment was made by plotting Fo/Fx versus Cli, where Fo = (fluorescence in the absence of Cl – fluorescence in the presence of KSCN) and Fx = (fluorescence in the presence of x mM Cl – fluorescence in the presence of KSCN).

Rates of anion flux

BCECF-loaded glands were mounted in the chamber and perfused with Cl-containing or Cl-free solutions (in the presence or absence of stimulants/inhibitors, as indicated for each experiment). HCO₃ flux at any given pHi was calculated by multiplying the total buffer capacity, Bt = BCO₂ + Bi = 2.3 [HCO₃] + Bi, by the initial rate of change of pH at the given pHi when switching to or from Cl-containing or Cl-free solutions. Bi at specific pHi was taken from Wenzl and Machen (1989), and [HCO₃]_i was estimated from the Henderson-Hasselbalch equation, assuming PCO₂ = 38 mmHg.

In SPQ-loaded glands, rates of transmembrane Cl flux were estimated from changes of SPQ fluorescence by measuring the slope of the tangential line immediately after the switch from Cl-free to Cl-containing solutions or vice-versa.

The method of statistical analysis used to compare steady-state pH_i, Cl_i, and rates of transmembrane anion fluxes was the Student's t-test.

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