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Fluorescence measurements of cytosolic free Na concentration, influx and efflux in gastric cells

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Regulation of cytosolic free Na (Na_i) was measured in isolated rabbit gastric glands with the use of a recently developed fluorescent indicator for sodium, SBFI. Intracellular loading of the indicator was achieved by incubation with an acetoxymethyl ester of the dye. Digital imaging of fluorescence was used to monitor Na_i in both acid-secreting parietal cells and enzyme-secreting chief cells within intact glands. In situ calibration of Na_i with ionophores indicated that SBFI fluorescence (345/385 nm excitation ratio) could resolve 2 mM changes in Na_i and was relatively insensitive to changes in K or pH. Measurements on intact glands showed that basal Na_i was 8.5 ± 2.2 mM in parietal cells and 9.2 ± 3 mM in chief cells. Estimates of Na influx and efflux were made by measuring rates of Na_i change after inactivation or reactivation of the Na/K ATPase in a rapid perfusion system. Na/K ATPase inhibition resulting from the removal of extracellular K (K_o) caused Na_i to increase at 3.2 ± 1.5 mM/min and 3.5 ± 2.7 mM/min in parietal and chief cells, respectively. Na buffering was found to be negligible. Addition of 5 mM K_o and removal of extracellular Na (Na_o) caused Na_i to decrease rapidly toward 0 mM Na. By subtracting passive Na efflux under these conditions (the rate at which Na_i decreased in Na-free solution containing ouabain), an activation curve (dNa_i/Na_i) for the Na/K ATPase was calculated. The pump demonstrated the greatest sensitivity between 5 and 20 mM Na_i. At 37°C the pump rate was <3 mM/min at 5 mM Na_i and 26 mM/min at 25 mM Na_i, indicating that the pump has a great ability to respond to changes in Na_i in this range. Carbachol, which stimulates secretion from both cell types, was found to stimulate Na influx in both cell types, but did not have detectable effects on Na efflux. dbcAMP+IBMX, potent stimulants of acid secretion, had no effect on Na metabolism.

Introduction

The concentration of free Na is a carefully regulated component of the cytosol. The plasma membrane Na/K ATPase establishes and maintains an inwardly directed Na gradient that can be utilized by the cell in a variety of ways, including the control of membrane potential and cell volume and intracellular pH and Ca. Na movement into the cell through channels and secondary active transport mechanisms represents a load on the pump such that in the steady state the level of cytosolic free Na (Na_i) reflects a balance between Na entry and extrusion. After blockade of the Na/K pump, the rate at which Na_i increases indicates the permeability of the cells to Na. Similarly, the rate of Na_i decrease on reactivation of the pump can be used to estimate Na/K pump activity. Thus, a probe sensitive to Na_i could be used to study the contributions of both Na influx and efflux pathways to the regulation of steady-state Na_i in intact cells.

Until recently, direct, continuous measurement of Na_i in living cells has been technically difficult, the options being limited to Na-selective microelectrodes (Thomas, 1978; Sejersted *et al.*, 1988) and nuclear magnetic resonance (NMR) (Springer, 1987). NMR requires dense preparations of cells so it cannot monitor Na_i at the single cell level. Electrode measurements of Na_i require impalement by double-barrel electrodes and are generally not suited for experiments on small cells. The use of ion-selective fluorescent probes has proven to be a useful method for measuring dynamic changes in cytosolic concentrations of H and Ca in individual cells (Tsien and Poenie, 1986; Tsien, 1988, 1989). The purpose of the present study, then, was to characterize and evaluate the ability of a recently described fluorescent indicator for sodium, SBFI (sodium-binding benzofuran isophthalate; see Harootunian *et al.*, 1989 and Minta and Tsien, 1989) to monitor Na_i regulation at the single-cell level.

Gastric glands contain at least two secretory cell types. Parietal cells secrete HCl, whereas chief cells secrete pepsinogen. Cholinergic

stimulation induces both cell types to secrete (Berglindh *et al.*, 1980; Koelz *et al.*, 1982) through a Ca-regulated pathway (Hersey *et al.*, 1984; Chew and Brown, 1986, 1989; Negulescu *et al.*, 1989). Agents that elevate adenosine 3',5'-cyclic monophosphate (cAMP) have also been shown to be extremely potent stimulants of acid secretion. Although neither cell type is involved in transcellular Na transport, stimulation of these cells might be expected to affect Na turnover. For example, cell stimulation may activate a basolateral K conductance that should hyperpolarize the cell, thus aiding Cl secretion (Ueda *et al.*, 1987) and resulting in a loss of cellular K. For the cell to maintain K under these conditions, entry of K via the Na/K pump needs to be stimulated, and this requires corresponding increases in Na influx, either directly or indirectly. Furthermore, there is evidence that stimulation of basolateral K channels in other Cl-secreting tissues causes concomitant stimulation of Na/K ATPase activity (Welsh *et al.*, 1982; Smith and Frizzell, 1984). Using SBFI, we monitored steady-state Na_i, as well as influx and efflux, to study the regulation of Na_i in gastric cells.

Results

Sensitivity and selectivity of SBFI fluorescence in glands

The characteristics of SBFI in vitro have been described elsewhere by Minta and Tsien (1989). However, these and other initial studies (Harootunian *et al.*, 1989; Negulescu and Machen, 1990a) indicated that the dye has different spectral properties inside the cells, including decreased sensitivity to [Na], and a excitation spectrum shift toward longer wavelengths. Therefore, control experiments were designed to test the sensitivity and selectivity of SBFI for Na in gastric cells. After AM ester loading of glands (see Methods), SBFI fluorescence in the cytoplasm of both chief and parietal cells was uniform. There were, however, differences in the loading between the two cell types. Parietal cells accumulated approximately twice as much SBFI as chief cells. In addition, parietal cells appeared to exclude dye from a small circular region near the base of the cell that corresponded with the location of the nucleus, as determined by propidium iodide staining.

The behavior of SBFI in the two cell types showed small, but significant, quantitative differences. Figure 1 shows the average basal and calibration ratio values for SBFI fluorescence in parietal and chief cells as determined by analysis of digitally processed video images. Each trace represents the average ratio for five cells within a single gland. The average ratio of chief cells was significantly higher than that of parietal cells, but calibration of the fluorescence signal using the pore-forming ionophore gramicidin (see Methods) indicated that the resting Na_i values of the two cell types were actually quite similar. On average, Na_i in parietal cells was 8.5 ± 2.2 mM (n = 230 cells, 20 animals) and 9.2 ± 3 mM in chief cells (n = 121 cells, 20 animals). The sensitivity of SBFI in the two cell types was similar, with a 10-mM change in Na_i yielding a change of 0.13 ± 0.02 ratio units. However, because chief cells contained less dye than parietal cells, the resolution of Na changes was somewhat less in chief cells than in parietal cells. The ratio imaging system could detect in-

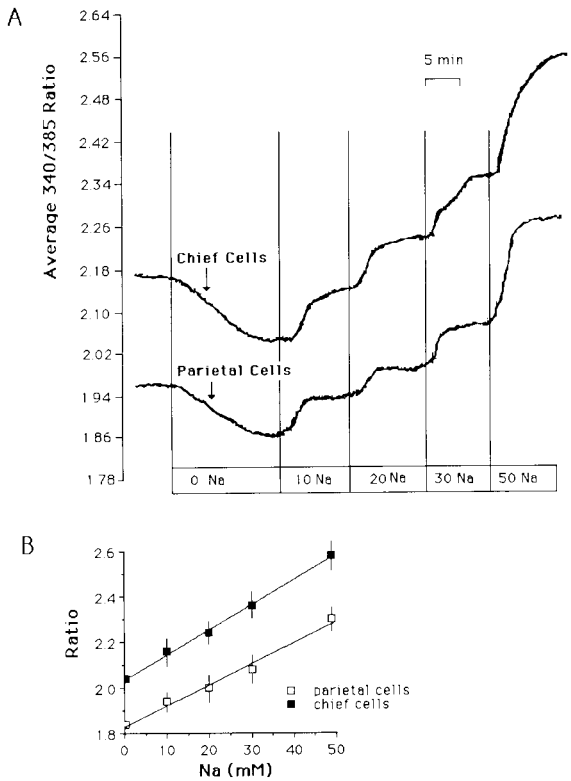

***Figure 1.* Calibration of SBFI fluorescence in parietal and chief cells of intact gastric glands.** Fluorescence emission was collected from a single gland by a low-light-level camera and digital image processing was used to distinguish fluorescence from each cell type (see Methods). (A) The ratio of the emission intensity at excitation wavelengths of 345 and 385 is displayed graphically. Each trace represents the average of five cells within the gland. At the times indicated, Na was removed from the perfusate and gramicidin was added. Then step changes in Na were made. (B) The ratio changes approximately linearly (r = 0.991) between 0 and 50 mM Na.

crements as small as 0.02 ratio units, which permitted resolution of Na_i of $\approx$2.0 mM in parietal cells and $\approx$3.0 mM in chief cells. Because of the superior Na_i resolution in parietal cells, most figures show data from parietal cells. In gramicidin-treated cells, the ratio responded approximately linearly between 0 and 50 mM Na_i (Figure 1B).

Acidic environments decrease the SBFI ratio (see Harootunian *et al.,* 1989; see below). Although compartmentation was not visible in either cell type of gastric glands, it was possible that the lower ratio observed in parietal cells was due to dye accumulation in diffuse acidic compartments within the parietal cell. To test this possibility, glands treated with gramicidin D—which primarily affects the plasma membrane (Rink *et al.,* 1980)—were subsequently exposed to the H/K ionophore nigericin, and the Na/H ionophore monensin. Together, 5 μM nigericin and 5 μM monensin should equilibrate pH, Na, and K across all intracellular membranes (Harootunian *et al.,* 1989). The addition of these ionophores had no additional effect on the SBFI ratio in either cell type, suggesting that the lower ratio in parietal cells was not due to significant accumulation of dye in acidic spaces. It is unclear what factors account for the differences observed between parietal and chief cells, but variability in fluorescence responses among different cell types is a generally observed phenomenon and emphasizes the importance of performing in situ calibrations. This issue is discussed in more detail by Negulescu and Machen (1990a).

Figure 2 demonstrates the selectivity of SBFI for Na over K and H in parietal cells. In these experiments, Na_i was clamped using ionophores and then step changes in either K or pH were made to determine their independent effects on SBFI. Figure 2A examines the sensitivity of SBFI to K in situ. Glands were treated with gramicidin in a solution containing 10 mM NaCl and 150 mM KCl. After removal and readdition of Na for reference, KCl was removed in steps and replaced with CsCl, which has negligible effects on SBFI (Minta and Tsien, 1989). Because Cs and K both permeate the gramicidin pore, Cs exchanges with K and maintains the ionic balance of the cell. Under these conditions, removal of 50 mM K had no detectable effects on SBFI. In fact, removal of all the K from the perfusate resulted in an "apparent" decrease of only 4 mM Na. In these experiments, when intracellular K was changing dramatically, it was crucial to use Cs as the cationic replacement for K. As shown in Figure 2A, if *N*-methyl-D-

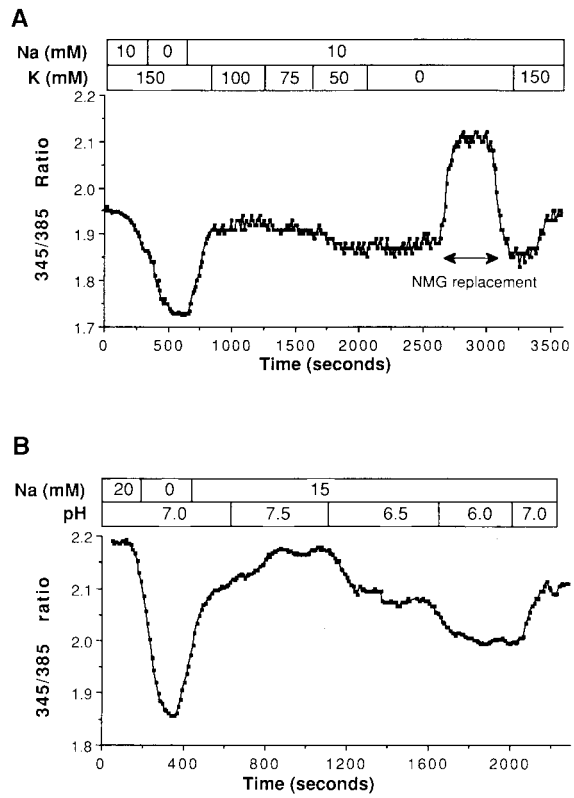

Figure 2. **Selectivity of SBFI fluorescence for Na over K and pH.** (A) Effect of removing K (isotonic Cs replacement) in the presence of constant Na in gramicidin-treated glands. Note that replacement of permeant Cs with the impermeant NMG cation results in an increase in the SBFI ratio. (B) Effect of pH on SBFI ratio in the presence of constant Na. Nigericin (20 μM) was used to equilibrate pH_i and pH_o. Trace represents the average of eight parietal cells within a single gland. Similar results were obtained in three other experiments.

glucamine (NMG) was substituted for Cs, the ratio increased significantly. This effect may be due to a drastic change in ionic strength because Cs (as well as Cl) rapidly leaves the cell and cannot be replaced by the impermeant NMG cation.

Figure 2B shows the effect of altering pH_i in the presence of constant Na_i. In addition to gramicidin, 10 μM nigericin (a K/H ionophore) was added to the high-K calibration medium to assure that $pH_i = pH_o$. In parietal cells, SBFI showed a small but significant sensitivity to pH_i. Increasing pH from 7.0 to 7.5 caused a ratio increase of 0.05 units, corresponding to an apparent increase of 4 mM Na_i. A total change of 1.0 pH unit (7.5–6.5) caused the SBFI fluorescence to decrease by 0.1 ratio units, which would appear as a decrease of $\approx$8 mM Na_i. pH changes as small as 0.20 pH units caused apparent, though small, changes in Na_i. Changes

in Ca_i between 100 nM and 10 μM caused no detectable changes in SBFI fluorescence (not shown).

Na_i responds to changes in Na influx and efflux

To study regulation of Na_i metabolism in intact cells, we had to show that SBFI responds predictably to changes in Na influx and efflux. A simple experiment to demonstrate this behavior in cells within an intact gland is shown in Figure 3. On removal of Na_o (NMG replacement), Na influx is nil, and Na exits the cell through both active and passive pathways. Under these conditions, Na_i decreased from a resting value of 10 mM to 0 mM within 5 min. The influx pathways appeared to be saturated at 80 mM Na_o, because decreasing Na_o in steps from 150 to 80 mM caused no perceptible decreases in Na_i (not shown). Addition of Na_o caused Na_i to increase quickly to its previous basal value.

Figure 3 also shows that Na efflux through the Na/K ATPase could be quickly and reversibly blocked by the removal of K_o. The K-free medium caused Na_i to increase within 30 s at an average rate of 3.2 ± 1.5 mM/min in parietal cells (n = 210 cells, 13 animals) and 3.5 ± 2.7 mM/min in chief cells (n = 130 cells, 13 animals). Similar rates of influx were observed in cells treated with ouabain (150 μM) or its more reversible analogue dihydroouabain (not shown). After inactivation of the pump, Na_i would increase to ≈50 mM Na, at which point the rate of influx decreased despite the presence of 150 mM Na_o (e.g., Figure 5A). Readdition of K to the perfusate then caused very rapid extrusion of the accumulated Na as Na_i decreased to resting levels within 120 s. The Na decrease was acutely dependent on extracellular K and was blocked by ouabain (not shown), indicating that it was entirely due to the action of the Na/K ATPase.

Na buffering by the cytoplasm is negligible

The net unidirectional Na flux (JNa) at any Na_i is a function of both the rate of change of Na_i and the buffering capacity of the cytoplasm (β) such that $JNa = (\beta)dNa_i/dt$. Thus, an estimate of β is required to calculate JNa from changes in Na_i. β was determined by measuring the change in Na_i during a protocol that placed a defined Na load on the cell. The Na load was calculated by measuring H fluxes through the Na/H exchanger and assuming that for every H that moved out of the cell, one Na entered. To prevent the accumulated Na from leaving the cell the experiments were performed in ouabain-containing solutions. Also, basal Na influx was reduced by performing these experiment at 30°C. The experimental protocol was to acid-load the ouabain-treated cells with the use of an ammonium pulse. Then the amiloride-sensitive pH_i recovery was used as a basis for estimating the H flux. The H flux was calculated by multiplying the total pH_i recovery (measured with BCECF) with the average H buffering capacity over that pH range as determined previously (Wenzl and Machen, 1989).

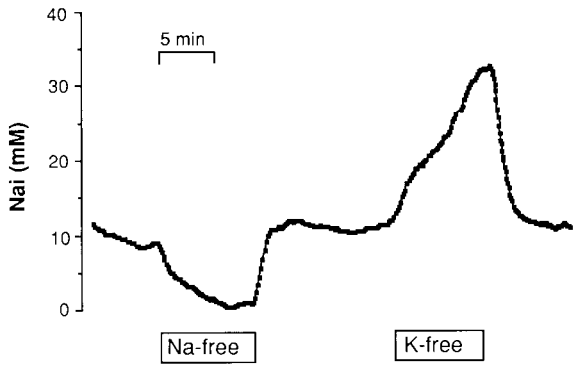

Figure 3. **Effect of Na or K removal on Na_i.** Na was removed from the bathing solution (replaced with NMG) to eliminate Na influx and cause Na_i to decrease. K was removed to inactivate the Na/K ATPase and cause Na_i to increase. Trace represents average of eight parietal cells recorded simultaneously from a single gland.

A typical experiment is shown in Figure 4A. A 4-min pulse of 30 mM NH_4Cl caused pH_i to drop to 6.40 in the presence of 200 μM amiloride. Note that amiloride blocks the recovery of pH_i. After removal of amiloride, pH_i recovered to 7.20. The average β over this pH range was 36 mM/pH (Wenzl and Machen, 1989), so H flux resulting from the recovery = 0.80 × 36 = 29 mM H. Because Na/H exchange has a stoichiometry of 1:1, Na influx during this maneuver is estimated to be 29 mM. When Na_i was measured in a parallel experiment using SBFI (Figure 4B), Na_i increased from 17 to 45 mM, a change of 28 mM. Amiloride was not used in the Na experiment both because of its fluorescence and because its absence minimizes pH_i changes after NH_4 removal. However, because the acid load on the cell is dependent only on the length of the NH_4 pulse, there should not be any difference in the H flux whether or not the cell is pretreated with amiloride. In four similar experiments, the average H flux was 32 ± 4 mM and the average Na flux was 30 ± 6 mM. Because the change in Na_i was nearly identical to the

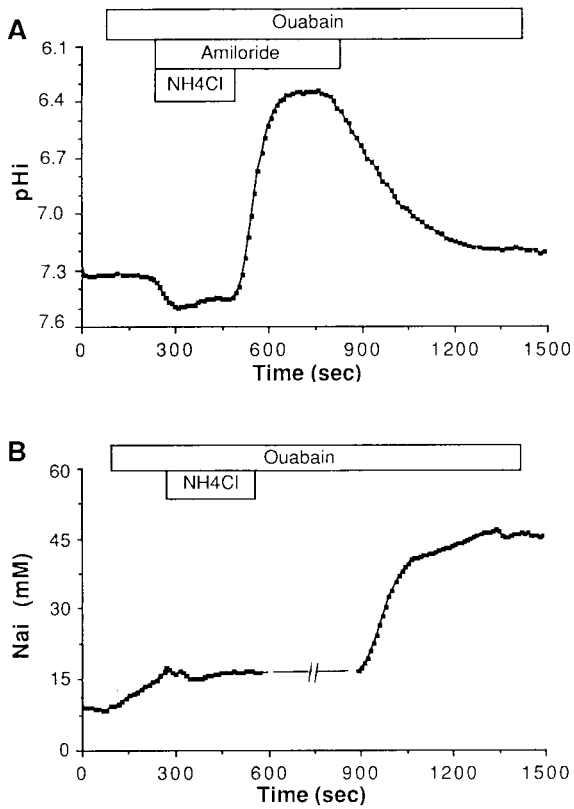

Figure 4. **Delivery of a defined Na load through the Na/H exchanger for measuring Na buffering capacity.** (A) BCECF-measured changes in pH_i. Following a 4-min pulse of 30 mM NH_4Cl, parietal cells acidified to 6.4 in the presence of amiloride. Removal of amiloride allowed pH to recover through Na/H exchange to pH 7.2. The experiment was conducted in 300 μM ouabain for comparison with Na measurements. (B) Effect of a similar acid load on Na_i. Amiloride could not be used with SBFI, so the trace is broken to align the Na increase with the pH recovery. The Na buffering capacity was determined by comparing H (A) and Na fluxes (B) in parallel experiments (see Results). Trace represents data from 10 parietal cells and is typical of six similar experiments.

calculated Na load through the Na/H exchanger, we conclude that β for Na is equal to 1.

Na_i dependence of Na pump

The easily detected active Na extrusion (Figure 3) and the lack of Na buffering (Figure 4) were favorable conditions for analysis of Na/K ATPase activity. Previous studies of Na/K ATPase in cells have used radioactive tracers or Na-sensitive microelectrodes to demonstrate that the pump binds three Na ions and is very sensitive to Na_i (Garay and Garrahan, 1973; Sejersted *et al.*, 1988; Skou *et al.*, 1988). Figure 5 shows an experiment designed to determine the sensitivity of the Na pump to Na_i in intact, individual parietal cells by the use of the fluorescence technique. The protocol, shown in Figure 5A, involved measurement of total and ouabain-insensitive Na efflux from Na-loaded cells. Cells were Na-loaded by Na/K pump inactivation in K-free Ringer's. The pump was then reactivated by switching to K-containing solution. The pump was reactivated in a Na-free solution to determine total Na efflux (pump activity + passive efflux). Under these conditions Na rapidly decreases to 0 mM Na. To account for passive Na exit under these conditions, the experiment was repeated on the same gland in the presence of ouabain. As seen in Figure 5A (and in expanded time scale in Figure 5B), the rate of Na efflux in a K-containing, Na-free medium was approximately sixfold faster in the absence of ouabain. These rates of efflux are plotted as a function of Na_i in Figure 5C. By subtracting the rate of passive (ouabain-insensitive) Na efflux from the total Na efflux at various Na_i, the activity of the pump as a function of Na_i can be determined. As shown in Figure 5C, the Na pump was found to have a steep sensitivity to Na_i. The pump increased its activity sharply above 5 mM Na_i and appeared to be saturated near 35 mM Na_i. On average (45 cells, 5 animals), the pump increased its activity eightfold between 5 and 20 mM Na, was half maximally activated at 12 mM $\pm$ 2 Na_i and had a maximal rate of Na transport of 26 $\pm$ 5 mM/min. Thus, the pump exhibited maximum sensitivity to Na_i at levels near resting Na_i and thus appeared well-suited to resist changes in Na_i. The average Hill coefficient was 2.71 $\pm$ 0.21.

Effects of stimulants of acid-secretion on Na_i and Na fluxes

Addition of the cholinergic agonist, carbachol (100 μM), caused Na_i to increase in both chief and parietal cells within 0.5–1.5 min and took between 1.0 and 10.0 min to reach a new steady-state level. On average, Na increased from 8.0 to 13.1 mM (n = 190 cells, 16 animals). After removal of carbachol, Na_i usually decreased back to control levels within 20 min. In addition to the increase in Na_i, Na influx was also seen to increase. In paired experiments, Na influx increased from 3.2 $\pm$ 1.2 mM/min to 7.9 $\pm$ 2.8 mM/min. These effects are illustrated in Figure 6A. Carbachol had no detectable effect on Na efflux (i.e., Na/K ATPase activity).

Carbachol is known to stimulate acid secretion via a Ca_i-dependent pathway (Chew and Brown, 1989; Negulescu *et al.*, 1989). However, agents that elevate cAMP are even more potent

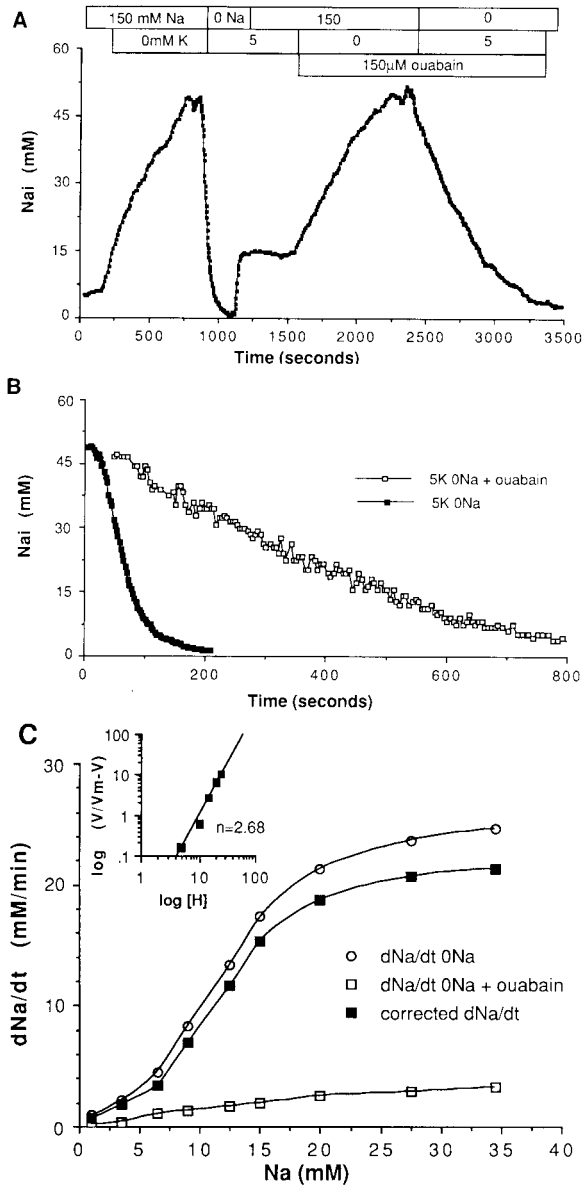

Figure 5. **Measurement of ouabain-sensitive Na efflux and Na dependence of the Na pump.** (A) After Na loading of cells in K-free medium, the rate of Na efflux in Na-free medium was measured in the presence and absence of 150 μM ouabain. (B) Na efflux on expanded time scale. (C) Rate of Na efflux plotted as a function of Na_i. Rates of Na efflux were calculated from B. Subtraction of ouabain-insensitive efflux (open squares) from total efflux (open circles) yields the activation curve of the Na/K pump (filled squares). Inset in (C): Hill plot of Na pumping versus Na_i. Data averaged from seven parietal cells within a single gland and are typical of five similar experiments.

stimulants of secretion. To determine whether Na_i metabolism changed during stimulation via the cAMP-dependent pathway, glands were perfused with a combination of the membrane-permeant dibutyrylcAMP (dbcAMP) and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Figure 6B shows that addition of these secretagogues had no effect on either steady-state Na_i or the rates of Na influx and efflux.

Discussion

Measurement of Na_i using SBFI

The results from this study indicate that SBFI is a useful probe for the study of Na_i regulation at the single-cell level. In gastric cells the probe was capable of responding to changes in Na_i as small as 2 mM. Sensitivity appears to vary among cell types because changes of <1 mM Na were detectable in fibroblasts (Harootunian *et al.*, 1989). The selectivity for Na over K was excellent: when Na_i was clamped at 10 mM Na, complete removal of K (replacement with Cs) produced only a 4-mM apparent change in Na_i. Assuming that intracellular K decreased by ~100 mM, the selectivity of Na:K in the cell is 25:1. A slight pH sensitivity was detected. The SBFI ratio decreased or increased as pH decreased or increased, respectively. Changes as small as 0.20 pH units would affect the SBFI ratio.

Unidirectional Na fluxes measured with SBFI in gastric glands

In addition to measurement of steady-state Na_i, SBFI was particularly useful in determining the rates of Na influx and efflux from cells. Unidirectional influx was estimated by measuring the rate of increase of Na_i after inactivation of the Na pump on either removal of K from or addition of ouabain to the solution. Ouabain-sensitive Na efflux was used to determine Na pump fluxes.

The conversion of SBFI-measured fluorescence changes to Na fluxes depends on two parameters: the Na sensitivity of the SBFI fluorescence ratio and Na buffering by the cytoplasm. Na buffering was not detected, and, because the dye calibration was nearly linear between 0 and 50 mM Na, no correction was needed to convert ratio changes to Na changes at various Na_i in gastric cells. Taken together, these findings indicate that the rates of SBFI ratio changes can be directly converted to Na fluxes.

The conclusion that Na buffering is negligible needs to be resolved with Na measurements using flame photometry which have indicated that total cellular Na in gastric glands is 40 mM (Koelz *et al.*, 1981). Similar observations—that

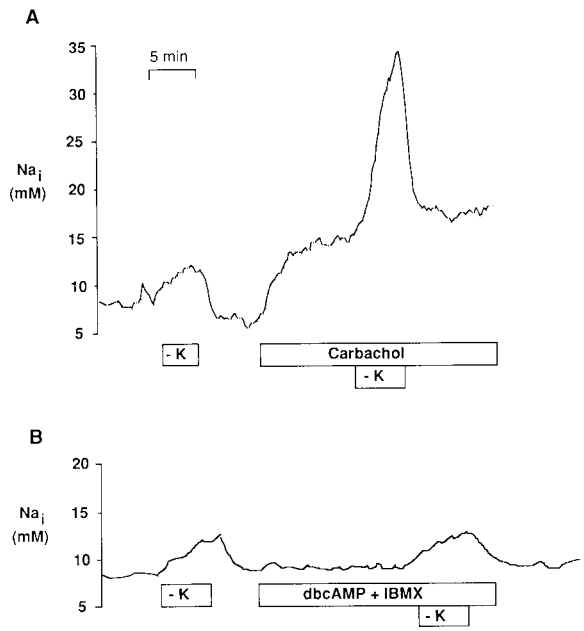

***Figure 6.* Effect of secretagogues on Na metabolism in parietal cells.** (A) 100 μM carbachol caused steady-state Na_i to increase and increased the rate of Na influx in K-free solutions. (B) dbcAMP+IBMX (100 μM each) had no apparent effect on Na metabolism. Similar results were obtained from chief cells. Trace is the average of eight parietal cells in one gland. Similar results were obtained in 14 other experiments.

total cellular Na exceeds free Na_i, whereas increments of total Na are fully visible as free Na—have also been made in oocytes (Horowicz and Paine, 1979) and fibroblasts (Harootunian *et al.,* 1989). This seemingly general phenomenon may be explained by a fixed quantity of several tens of millimolar tightly sequestered or bound Na in coexistence with the pool of unbuffered free Na_i.

Nature of Na influx pathways

Both parietal and chief cells demonstrated large permeabilities to Na. With an influx of 3.2 mM/min and resting Na_i of 8.7 mM, Na turnover in the parietal cell occurs every 2.7 min. In chief cells Na turnover would occur every 2.6 min. The nature and significance of the Na entry pathway(s) are unclear, but a number of possibilities can be ruled out. K-coupled Na transport (e.g., $NaKCl_2$) contransport was not detected, because Na_i increased at the same rate in 0K and in ouabain. Na/HCO_3 cotransport (Townsley and Machen, 1989) is not a factor, because these experiments were conducted in nominally HCO_3-free solutions. Finally, Na/Ca exchange can be ruled out, because it has not been detected in either cell type (Muallem *et al.,* 1988; Negulescu and Machen, 1990b). The amiloride-sensitive Na/H exchanger, which has been characterized in both cell types by Paradiso *et al.,* (1987a,b), undoubtedly accounts for a portion of the Na influx. In principle, the degree to which Na/H exchange contributes to overall Na influx could be determined directly by measuring Na influx in amiloride-containing solutions. In practice, such experiments must await the availability of nonfluorescent amiloride analogues. However, two pieces of evidence suggest that Na/H exchange is the major contributor to Na influx in gastric cells. In ouabain-treated cells, NH_4Cl caused Na influx to cease entirely (Figure 4B). Aronson *et al.* (1982) have shown that the Na/H exchanger is allosterically inhibited by alkaline pH_i and that NH4 can compete for Na on the Na/H exchanger (Aronson, 1985), so it is possible that NH4-induced inhibition of Na influx is due to inhibition of the Na/H exchanger. Another estimate can be based on measurements of H flux through the Na/H exchanger using BCECF. According to measurements of intrinsic H buffering in parietal cells (β = 16 mM/pH at 7.1) (Wenzl and Machen, 1989) and the rate of amiloride-induced acidification (0.13 pH/min) (P.A. Negulescu and T.E. Machen, unpublished) H flux = 0.13 pH/min $\times$ 16 mM/pH = 2.1 mM H/min. Because Na/H exchange is 1:1, this value is also an estimate of Na flux through the exchanger. By comparing this value with the total Na influx, Na/H exchange accounts for 2.1/3.2 or 64% of the total influx. Thus, Na/H exchange accounts for two-thirds or more of Na entry in the resting cell.

Na pump kinetics measured with SBFI

The entry of Na must be balanced by Na extrusion in the steady state. Active Na efflux was K-dependent and ouabain-sensitive, indicating that it was accomplished by the Na/K ATPase. Na/K pump kinetics were determined by measuring Na efflux rates in Na-free solutions in the presence and absence of ouabain. This method provided a correction for passive Na fluxes and allowed for determination of pump rates below baseline Na_i.

There are several possible sources of error with this method, all related to measuring Na fluxes in Na-free solutions. First, Na-loaded cells will shrink as they lose Na and Cl. This would occur primarily in ouabain-containing solutions, where none of the lost Na is exchanged for K. Cell shrinkage would tend to concentrate the remaining Na_i and result in an elevation of Na_i.

Thus, the actual Na loss in ouabain-treated cells may be greater than the measured loss, resulting in a possible overestimation of ouabain-insensitive efflux and a subsequent underestimation of pump rates. In addition to volume considerations, Na-free solutions will cause pH_i to decrease because of reverse operation of the Na/H exchanger. Acidification of the cytoplasm has been shown to inhibit the Na pump (Eaton *et al.,* 1984; Breitweiser *et al.,* 1987), which would also result in an underestimate of Na pump fluxes. Both the effects of volume and the effect of low pH on the pump would be most significant at low Na_i, because the difference between pump activity and passive efflux is small and pH_i would be the most acidic. Finally, acidification would cause the SBFI ratio to drop in both ouabain-treated and control cells. If Na_i were underestimated as a result of this, the pump rate at a given Na_i would be overestimated.

One way to assess the magnitude of these effects is to compare the observed rate of Na_i influx when K was removed from the cell with the calculated pump values in the same gland. For example, in Figure 5A, resting Na_i was ~7 mM and the rate of Na increase on K removal was 5 mM/min. (This gland had somewhat larger resting influx than the average of 3.2 mM/min but was useful in this case because this resulted in good Na loading.) Because Na efflux is equal to Na influx at steady-state Na_i, the pump rate at 7 mM Na_i should also be 5 mM/min. The calculated pump rate (Figure 5C) at 7 mM Na_i, obtained independently by subtracting ouabain-sensitive Na efflux from total Na efflux in Na-free solutions, was 4 mM/min. Thus, at 7 mM Na_i, the calculated pump rate was only underestimated by 20%. This indicates good accuracy with this method.

The resolution of pump fluxes with this technique was sufficient to determine that the rate of Na/K pumping was saturable and dependent on Na_i, with a K_m of 12 mM, a V_{max} of 26 mM/min and a Hill coefficient for Na of 2.71. All these parameters are consistent with an enzyme well suited to resist changes in Na_i and possessing multiple Na-binding sites. These values are similar to those obtained in cardiac Purkinje fibers by Sejersted *et al.* (1988) with the use of Na-sensitive microelectrodes. These investigators calculated a K_m of 14 mM Na, V_{max} as high as 27 mM/min ($x = 13.3$), and a Hill coefficient of 1.94. In experiments on rabbit urinary bladder, Lewis and Wills (1983) inferred the pump rate from measurements of pump current and also found Na pumping to be saturable with a K_m of 14 mM and a Hill coefficient for Na of 2.8. The similarity of these data suggests these tissues all possess the same enzyme.

Na metabolism and secretagogues

Na metabolism increased in both chief and parietal cells on stimulation with carbachol. In parietal cells, steady-state Na_i increased from 8.0 to 13.1 mM, whereas Na influx increased by 2.4-fold. The steady-state measurement may be overestimated by ≈1 mM because carbachol increases pH_i in gastric cells by ≈0.15 pH units (P.A. Negulescu and T.E. Machen, unpublished). The rather small change in Na_i can be explained by the steep activation of the Na/K pump at elevated Na_i. According to the activation curve in Figure 5C, an increase in Na_i from 8.0 to 13.1 would result in a 2.2-fold increase in efflux, which accounts for nearly the entire observed influx change on carbachol stimulation. Thus, our data are consistent with a model in which carbachol stimulated Na influx, and the resulting increase in Na_i caused the transport rate of the pump to increase. Because it was seen in both HCl-secreting parietal cells and protein-secreting chief cells, this may be a general feature of cholinergic stimulation. Carbachol does not appear to affect the transport properties of the pump directly. The nature of the stimulated influx pathway was not examined, although a likely candidate is the Na/H exchanger.

In contrast to the effect of carbachol, dbcAMP+IBMX, a more potent stimulus of HCl secretion than carbachol, had no detectable effect on either Na influx or efflux. This indicates that a change in Na metabolism is not necessary for stimulation of HCl secretion from parietal cells. In this regard, parietal cells differ from other Cl-secreting epithelia, which show increased Na influx and Na/K pump activity on stimulation. In tracheal cells, for example, increased Na influx is due to activation of Na-coupled Cl uptake. In this case, increased Na/K pump activity serves two roles: it prevents accumulation of Na while increasing the uptake of K. The latter effect helps the cell maintain K in the presence of increased K permeability across the basolateral membrane. The parietal cell uncouples Na and Cl transport in two ways. First, Cl uptake is not Na coupled but rather is accomplished by a Na-independent Cl/HCO_3 exchanger. This eliminates any Na load resulting from increased Cl transport. Second, the uptake of K by the apical H/K ATPase, rather than the basolateral Na/K ATPase, may be sufficient to compensate for K loss across both basal and apical membranes.

Materials and methods

Materials

All chemicals were reagent grade and, unless otherwise specified, obtained from Sigma. The fluorescent indicator SBFI and its acetoxymethyl ester were obtained from Molecular Probes.

Isolation of gastric glands

Gastric glands from New Zealand White rabbits were prepared as described by Berglindh and Obrink (1976). After sacrifice, the stomach was perfused with phosphate-buffered saline via retrograde perfusion of the descending aorta. The stomach was then excised and the mucosa stripped from the underlying muscularis. After mincing, the mucosa was placed in a digestion medium that contained 0.3 mg/ml type 1A collagenase in an Eagle's minimum essential medium (GIBCO), supplemented with 1 mg/ml bovine serum albumin (Calbiochem), 10^{-4} M cimetidine, a histaminergic (H2) blocker to ensure that glands remain unstimulated, and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). The solution was stirred and gassed (100% O_2) at 37°C. Glands were formed within 45 min. These glands were settled (1 g) and were then rinsed several times at room temperature in Eagle's medium containing 40 μM cimetidine but without enzymes or albumin.

Solutions

Experiments were performed in a Ringer's solution containing 150 mM NaCl, 2.5 mM K_2HPO_4, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 10.0 mM glucose, and 20 mM HEPES, pH 7.45. In Na-free or K-free solutions the replacement cation was NMG.

SBFI loading and calibration

The membrane-permeant acetoxymethyl ester of SBFI/AM was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM. Before introduction to cells, the dye ester was mixed 1:1 with a 25% w:v solution of Pluronic F-127 (Molecular Probes) in DMSO. The final concentration of dye in the loading solution was 7 μM. Loading took place in a 5% cytocrit for 30 min at room temperature and resulted in ~75 μM intracellular dye. SBFI exhibits a peak excitation shift to shorter wavelengths on binding Na. This shift can be detected by alternating the excitation wavelength sent to the sample and measuring the emission intensity from each wavelength. By dividing these intensities one obtains, in principle, a ratio that is independent of dye concentration and therefore is unaffected by absolute fluorescence intensity changes resulting from dye leakage or bleaching. This is supported by the observation in control experiments that the 345/385 ratio was seen to be stable for over an hour, during which time fluorescence decreased by 25%. Excitation light was provided by a xenon arc lamp and filtered by two narrow bandpass filters (345 ± 10 and 385 ± 10 nm) purchased from Omega Optical (Brattleboro, VT). The filters were alternated by a computer-controlled filter wheel. Excitation light was passed to the cells via a 395 dichroic reflector. Emitted light above 450 was collected. To correct for day-to-day variations in illumination of the field, the 345/385 ratio was normalized each day to a ratio of 1.0 using a droplet of fura-2 in a Ca-free/EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid) calibration standard. (A sample of fura-2 was used instead of SBFI because the relative 345/385 intensities of SBFI in cells at 0 [Na] were more similar to those of fura-2 in 0 [Ca] than SBFI at 0 [Na] in vitro.) Calibration of the SBFI fluorescence ratio was performed after each run by exposing glands to various concentrations of Na in a calibration solution (Na + K = 150 mM, 120 mM gluconate, 30 mM Cl, 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.0) containing 5 μM of the pore-forming antibiotic gramicidin D (in DMSO). (This concentration of gramicidin seemed sufficient to equalize Na_i and Na_o because 5 μM and 15 μM gramicidin gave equivalent calibration curves.) Gramicidin D is selective for monovalent cations and should equilibrate Na_o and Na_i, as well as K_i and K_o, without disturbing gradients across internal organelles (Harootunian *et al.*, 1989). In experiments where Na_i was kept constant and K_i was varied, CsCl was used to replace K_o. Unless otherwise noted, Na_i values in figures represent the mean of multiple cells recorded simultaneously within a given gland. Where given in the text, averages are mean ± SD for all cells in all experiments.

Fluorescence measurements

Dye-loaded cells were mounted in a perfusion chamber and placed over the objective (×40) of a Zeiss IM35 inverted microscope at 37°C. SBFI fluorescence from individual glands was measured by the use of digital image processing of video images of the fluorescence at each excitation wavelength, as described previously (Tsien and Poenie, 1986). Black-and-white fluorescence images were acquired by a Silicon Intensified Target (SIT) camera (Dage 66) and relayed to an image processor (Gould FD5000) that was controlled by a microcomputer. The intensity of each pixel at a single wavelength was typically averaged over eight video frames (264 ms). After applying a correction for background and dark current, the fluorescence intensity ratio was calculated for each pixel and displayed as one of 32 pseudocolors. These ratios were then calibrated as described above. Analysis and plotting of ratio versus time for individual cells was accomplished with the use of a graphics emulation terminal. The imaging system allows identification and recording of responses from individual cells. The perfusion system has the advantage of allowing rapid solution changes (<2 s turnover) and eliminates any fluorescence of extracellular dye that has leaked out of the cells. Because the isolated gland is a closed tube, only the basolateral membranes of the cells are exposed to the perfusion solution. Thus, changing the composition of the perfusate permits selective investigation of basolateral membrane transport mechanisms. At 37°C, the loss of SBFI because of leakage and/or photobleaching was ≈25% of the total signal per hour, and experiments up to 3 h in length were possible. Control experiments showed that similar calibrations were obtained after 0.5- and 2-h experiments. Experiments were generally not longer than 2 h. Because the SBFI ratio was sensitive to changes in temperature, all experiments and calibrations were performed at 37°C unless otherwise noted.

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