J. Membrane Biol. 116, 239–248 (1990)

The Journal of
Membrane Biology

Lowering Extracellular Sodium or pH Raises Intracellular Calcium in Gastric Cells

Paul A. Negulescu and Terry E. Machen
Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, California 94720

Summary. The dependence of cytoplasmic free [Ca] (Ca_i) on [Na] and pH was assessed in individual parietal cells of intact rabbit gastric glands by microfluorimetry of fura-2. Lowering extracellular [Na] (Na_o) to 20 mM or below caused a biphasic Ca_i increase which consisted of both release of intracellular Ca stores and Ca entry across the plasma membrane. The Ca increase was not blocked by antagonists of Ca-mobilizing receptors (atropine or cimetidine) and was independent of the replacement cation. Experiments in Ca-free media and in Na-depleted cells indicated that neither phase was due to reversal of Na/Ca exchange. The steep dependence of the Ca_i increase on Na_o suggested that the response was not due to lowering intracellular [Na] (Na_i). The effects of low Na_o on Ca_i were also completely independent of changes in intracellular pH (pH_i). Ca_i was remarkably stable during changes of pH_i of up to 2 pH units, indicating that H and Ca do not share a cytoplasmic buffer system. Such large pH excursions required determination of the pH dependence of fura-2. Because fura-2 was found to decrease its affinity for Ca as pH decreased below 6.7, corrections were applied to experiments in which large pH_i changes were observed. In contrast to the relative insensitivity of Ca_i to changes in pH_i, decreasing extracellular pH (pH_o) to 6.0 or below was found to stimulate release of intracellular Ca stores. Increased Ca entry was not observed in this case. The ability of decreases in Na_o and pH_o to stimulate release of intracellular Ca stores suggest interactions between Na and H with extracellular receptors.

Key Words gastric glands · fura-2 · calcium · sodium · pH

Introduction

Cytoplasmic free [Ca] (Ca_i) is a critically regulated component of the cytosol. The concentration of free-Ca ions in the cytoplasm represents a pool of Ca which, in the steady state, reaches equilibrium with intracellular Ca-binding sites, some of which serve regulatory functions. A variety of hormones, peptides and neurotransmitters are believed to exert their effects by altering Ca_i. In the case of the gastric parietal cell, for example, histamine, gastrin and the cholinergic agonist, carbachol, each cause elevation of Ca_i (Chew & Brown, 1986). The agonist-stimulated Ca increases are biphasic, consisting of an initial release of Ca from intracellular stores and a sustained Ca influx from the extracellular space (Negulescu & Machen, 1988*a*). The elevation of Ca_i has an important role in regulating HCl secretion from the parietal cell (Negulescu, Reenstra & Machen, 1989).

In addition to the ability of agonists to raise Ca_i, it is known that the intracellular concentrations of Na (Na_i) and H (pH_i) can affect Ca_i in some cell types. For example, in excitable cells increasing Na_i (or removing Na_o) causes Ca_i to rise due to the decreased driving force for Ca extrusion via Na/Ca exchange (e.g., Dipolo & Beauge, 1983). The effects of pH on Ca_i are more complex: decreasing pH_i has been observed to increase, decrease, or have no effect on Ca_i, depending on the cell type. In squid axons and *Xenopus* oocytes there is an inverse relationship between pH_i and Ca_i such that decreasing pH_i increases Ca_i (Baker & Honerjager, 1978; Rink, Tsien & Warner, 1980), while increasing pH_i decreases Ca_i (Mullins & Requena, 1979). These results suggest reciprocity between intracellular buffer systems for Ca and H. In contrast, decreasing pH_i in *Helix* neurones resulted in a small Ca_i decrease (Alvarez-Leefmans, Rink & Tsien, 1981) while increases in pH_i result in increases of Ca_i in lymphocytes (Grinstein & Goetz, 1985) and smooth muscle cells, (Siskind et al., 1989). Finally, alterations of pH_i had no apparent effect on Ca_i in fibroblasts (Ives & Daniel, 1987). One complication of studying the relationships between Na and Ca or H and Ca in intact cells is that it is difficult to change Na and pH selectively because Na_i and pH_i are linked through Na/H exchange. Since decreases in pH_i could result in increases in Na_i and vice versa, it is important to control for the effects of such interactions.

The original intention of this study was to evaluate the role of Na in regulation of Ca_i in parietal cells from intact rabbit gastric glands. This included testing for the presence of Na/Ca exchange. Ca_i was measured using the fluorescent, Ca-sensitive probe fura-2. Fluorescence measurements from individual cells within glands were made using microfluorimetry. Removal of extracellular Na (Na_o) caused Ca_i to increase. Surprisingly, this increase occurred in both Ca-containing and Ca-free bathing solutions. Furthermore, the Ca increase in Ca-containing solutions was found to be biphasic, consisting of both Ca release from hormone-sensitive internal stores and entry of extracellular Ca. Thus, having set out to investigate a Na-dependent mechanism to lower Ca_i (Na/Ca exchange), a Na-sensitive mechanism for raising Ca was identified. The release appeared to be independent of Na_i. Because removal of Na_o acidifies cells through reverse operation of Na/H (Paradiso, Tsien & Machen, 1987) exchange, we were concerned with identifying possible relationships between pH_i and Ca_i. pH_i was monitored with BCEF or 5-(and 6-)biscarboxyfluorescein diacetate. Decreasing pH_i by up to 2 pH units at normal pH_o had very little effect on Ca_i, even when the pH dependence of fura-2 Ca binding was taken into account. However, lowering extracellular pH (pH_o) to 6.0 or below caused release of intracellular Ca stores. Thus, lowering either extracellular Na or pH caused changes in Ca_i which resembled the action of agonists. All evidence indicates that the effects of low Na or pH are mediated by action at some external site.

During the course of this study, two papers appeared which pertained to extracellular Na removal and Ca mobilization. Muallem, Beeker and Pandol (1988) showed that replacement of Na_o with tetramethylammonium (TMA) or choline caused elevation of Ca_i in gastric glands and pancreatic acini by releasing intracellular Ca stores. Because this effect could be blocked with atropine, it was concluded that the organic cations were binding to cholinergic receptors. More recently Smith, Dywer and Smith (1989*a*) reported that removal of Na_o mobilizes Ca_i and triggers inositol phosphate turnover in a variety of cultured cells which do not possess cholinergic receptors. This latter group concluded that Ca_i elevation could be caused by Na_o removal itself. In the present study we show that, although parietal cells possess cholinergic receptors which can be stimulated by choline, removal of Na_o itself can raise Ca_i through a mechanism distinct from cholinergic receptor activation. (Portions of this work have appeared in abstract form (Negulescu & Machen, 1988*b*).)

Materials and Methods

Materials

All chemicals were reagent grade and, unless otherwise specified, obtained from Sigma.

Isolation of Gastric Glands

Gastric glands from New Zealand White rabbits were prepared as described previously (Berglindh & Obrink, 1976). After sacrifice, the stomach was perfused with phosphate-buffered saline (100% O_2, pH 7.4) via retrograde perfusion of the descending aorta. This facilitated separation of the gastric mucosa from the underlying muscularis. After mincing, the mucosa was placed and stirred in a digestion medium that contained 0.3 mg/ml Type 1A Collagenase in an Eagle's minimum essential medium (Gibco, Grand Island, NY), supplemented with bovine serum albumin (BSA) (Calbiochem, San Diego, CA), 10^{-4} M cimetidine (Sigma, St. Louis, MO) and 20 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.45, 100% O_2, 37°C). Glands were formed within 45 min. These isolated glands were allowed to settle and were then washed several times at room temperature in the same medium without enzymes or albumin.

Dye Loading and Calibration

For Ca_i measurements, a suspension of isolated gastric glands (5% cytocrit) was loaded with fura-2/AM (Molecular Probes, Eugene, OR) in Eagle's medium containing 10^{-4} M cimetidine and 10 μM dye for 30 min at 24°C. Following loading, the glands were washed in Eagle's medium and left at room temperature until use. Excitation light was provided by two monochomators set at 340 and 385 nm which were rapidly alternated by a rotating chopper mirror and passed to the sample via a 395 dichroic. The emitted light passed through a UV filter (450 nm long-pass) before counting.

Ca_i was calibrated in each cell using the formula derived by Grynkiewicz, Poenie and Tsien (1985) for dual-wavelength measurements.

$$Ca_i = K(R - R_{min})/(R_{max} - R) \quad (1)$$

where R_{min} is the ratio of fluorescence intensities at 340 and 385 nm obtained at 0 Ca, R_{max} is the ratio at saturating Ca, and R is the measured ratio. At the end of each experiment R_{min} and R_{max} were determined by treating cells with 10–15 μM digitonin in the presence and absence of $CaCl_2$. Digitonin was used because the R_{max} achieved in this manner was higher than that achieved by the Ca ionophore, ionomycin. In Eq. (1) K represents $K_d(F_{min}/F_{max})$, where K_d is the dissociation constant for fura-2 under the appropriate conditions and F_{min} and F_{max} are the fluorescence intensities at 385 nm minus and plus Ca, respectively. The effective dissociation constant for fura-2 in parietal cells has been calculated to be 300 nM at 37°C, pH 7.0 (Negulescu et al., 1989).

Because fura-2 is a tetracarboxylic acid it is possible that its affinity for Ca is sensitive to H. In the original description of the dye only a small change in fluorescence was noted when pH was changed from 7.05 to 6.75 in the presence of 200 nM free Ca. Because pH_i approached 6.0 in some of our experiments, we

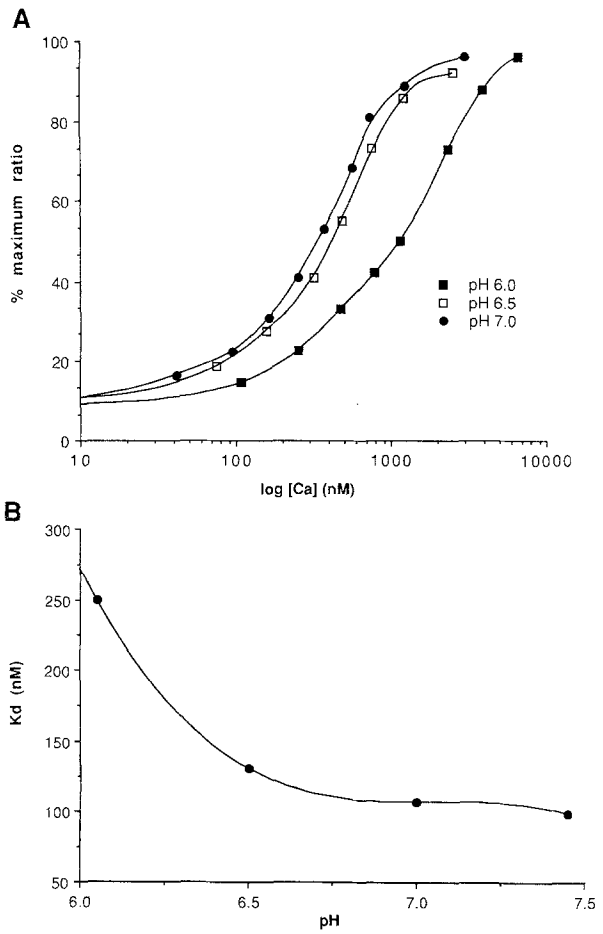

Fig. 1. Fura-2 sensitivity to pH in vitro. (*A*) Ca titrations in the presence of 5 μM fura-2 are shown at pH 7.0, 6.5 and 6.0 as described in Materials and Methods. Since the 340/385 nm ratio at saturating Ca decreased as pH decreased, the ratios obtained at different Ca values are expressed as a percentage of the maximum ratio so that the curves are comparable. (*B*) K_d for fura-2 (calculated as in Materials and Methods) as a function of pH

extended the characterization of the pH sensitivity of fura-2. Ideally, this characterization would be performed on dye in cells. However, we felt the Ca_i titration, which required precise control of both Ca_i and pH_i could not be performed on parietal cells. Therefore, the pH dependence of fura-2 between pH 7.5 and 6.0 was evaluated by in vitro titration of fura-2 free acid in cuvettes. The experimental conditions were similar to those described by Grynkiewicz et al. (1985). Briefly, a solution containing 100 mM KCl, 5 mM MOPS, 5 mM MES, 10 mM K_2H_2EGTA and 2 μM fura-2 was titrated to a given pH and then serially diluted with an identical solution in which K_2H_2EGTA was replaced with 10 mM CaH_2EGTA. Excitation spectra were obtained at each dilution using a SPEX fluorolog fluorimeter (emission 505 nm). Because the solutions contained no Mg, calculation of free Ca at each dilution was given by

$$[Ca] = K_d([CaEGTA]/[EGTA]) \quad (2)$$

in which K_d is the dissociation constant of EGTA at the pH of the titration. The K_ds for EGTA at various pH's were given by Tsien and Pozzan (1989). An indication of the effect of pH on fura-2 can be seen in Fig. 1*A*, where the ratios of fluorescence intensities at 340/385 are plotted as a function of Ca. It is apparent that decreasing pH_i shifts the sensitivity of the probe to the right, which indicates decreased affinity for Ca at low pH.

In order to calculate the K_d for fura-2 at a single pH at least three ratios are necessary—at 0 Ca, saturating Ca, and some intermediate Ca. With these values, the K_d for fura-2 can be calculated by rearrangement of Eq. (1).

$$K_d = [Ca]/(R - R_{min}/R_{max} - R)(F_{min}/F_{max}) \quad (3)$$

where [Ca] is the free [Ca] given by Eq. (2). Calculated in this manner the K_ds for fura-2 at pH 7.5, 7.0, 6.5 and 6.0 were 105, 117, 135 and 248 nM, respectively. These values are plotted in Fig. 1*B*.

Although these values were obtained at room temperature in free solution, and therefore are different from absolute values which would be obtained in cells, the basic pH sensitivity of the dye is unlikely to be altered by the intracellular environment. Therefore, an approximation of the effect on the dye in cells can be made. In order to apply a correction for pH to the calibration of Ca_i the % change of K_d from pH 7.0 was calculated from Fig. 1b and then multiplied by the apparent K_d of the dye in cells at pH 7.0 (300 nM). For example, if pH_i changed from 7.0 to 6.0, one would predict an 83% increase in the K_d (i.e., from 300 to 549 nM). Ca_i can now be calculated based on the new K_d. In experiments where pH_i changed by more than 0.3 pH units the corrected Ca_i is shown as a dashed line.

For measurements of pH_i, glands were incubated with either 2 μM BCECF/AM or 15 μM 5-(and 6-)biscarboxyfluorescein diacetate (Molecular Probes) for 30 min at room temperature. BCECF (pK_a 7.0) was used when pH_i was expected to remain above 6.4 while fluorescein diacetate (pK_a 6.3) was used to monitor pH_i less than 6.4. Excitation of the fluorescein-based indicators was alternated between 440 and 490 nm (510 dichroic). Emitted light above 520 nm was collected. Calibration of pH_i was performed in a nigericin-containing (20 μM) high K solution at various pH_o's.

Microfluorimetry

Ca_i or pH_i was measured at 37°C in single parietal cells within intact rabbit gastric glands using microspectrofluorimetry dye-loaded glands (*see above*) were settled onto a cover glass, which was mounted into a temperature-controlled rapid perfusion chamber. The chamber was placed on the stage of an inverted microscope (Zeiss IM35), and a 63× oil objective (Zeiss Neofluar 1.25NA) permitted identification of parietal cells in intact gastric glands. Excitation of the dye was achieved using a modular fluorimeter system (Spex fluorolog 2, model F2C) as described previously. Fluorescence emission from a single cell was obtained using an image plane pinhole which corresponded to a spot diameter of 20 μM. Emission intensity from a parietal cell was measured using a photomultiplier attached to a Spex Datamate microcomputer which stored the two alternating fluorescence signals in separate memories. Data points were recorded every second.

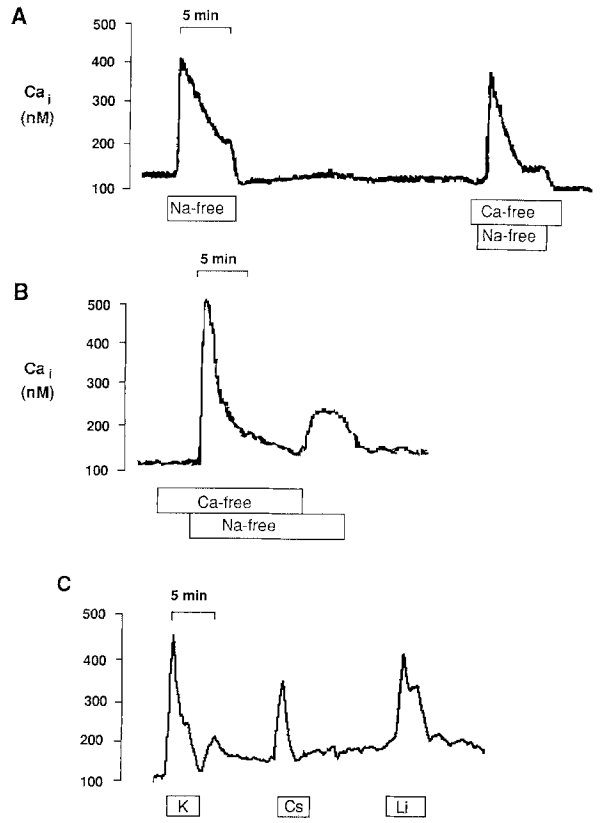

Fig. 2. Removal of extracellular Na (NMG replacement) causes an increase of Ca_i in parietal cells. (*A*) Na removal elevates Ca_i in both Ca-containing and Ca-free (0.1 mM EGTA) solutions. (*B*) Relative contributions of intracellular and extracellular Ca to Na-free-induced increase in Ca_i. Na-free solutions appear to release internal stores and increase Ca entry. (*C*) Increases in Ca_i are independent of the replacement cation for Na. Increases were seen in Na-free solutions containing either 150 mM K, 150 mM Cs, or 150 mM Li

Solutions

Experiments were performed in a Ringer's solution containing (in mM): 150 NaCl, 2.5 K_2HPO_4, 1.0 $CaCl_2$, 1.0 $MgSO_4$, 10.0 glucose, 10.0 HEPES/NaOH, pH 7.45. In Na-free solutions, Na was replaced with either 150 mM N-methyl-D-glucamine (NMG), 150 mM K or 150 mM Li or 150 mM Cs. In low Na solutions NaCl was replaced isosmotically with NMG-Cl. For example, a solution containing 20 mM NaCl consisted of (in mM): 130 NMG-Cl, 20 NaCl, 2.5 K_2HPO_4, 1.0 $CaCl_2$, 1.0 $MgSO_4$, 10.0 glucose, 10 HEPES/NMG, pH 7.45. NMG-Cl stock solutions were made by weighing out enough NMG free base to make 100 ml of 1 M NMG. Then a 1 N HCl solution was used to dissolve the NMG base powder and bring the volume to 95 ml. pH was then titrated to 7.0 using a concentrated HCl solution and brought to 100 ml with H_2O. For NH_4 pulse experiments, NH_4Cl replaced NaCl. The high K calibration solution for BCECF calibration consisted of (in mM): 100 K-gluconate, 30 KCl, 10 Na-gluconate, 10 HEPES/NaOH, 1.0 $CaCl_2$ and 1.0 $MgSO_4$.

Results

Effect of Low Na_o on Ca_i in Parietal Cells

Figure 2 shows the effect of removing extracellular Na (Na_o) on a single parietal cell within an intact gastric gland. Figure 2*A* compares the responses obtained in Ca-containing (1 mM) and Ca-free (0.1 mM EGTA). Within 30 sec of Na removal Ca_i began to increase rapidly and reached similar levels in both Ca-containing and Ca-free solutions, indicating release of Ca from intracellular stores. On average, total removal of Na_o caused Ca_i to increase from a resting level of 93 ± 12 nM to 427 ± 24 nM. Following the peak, Ca_i decreased back toward baseline. In Na-free, Ca-containing solutions Ca_i usually did not return all the way to control levels, suggesting that Ca may be entering the cell from extracellular space.

Figure 2*B* shows the relative contributions of intracellular and extracellular Ca to the Ca_i increase stimulated by Na_o removal. First, a cell was exposed to a Ca-free, Na-free solution. This caused a transient increase in Ca_i. Then, after Ca_i had decreased to close to resting levels, Ca_o was introduced in the continued absence of Na_o. This caused a second, sustained elevation of Ca_i. Under these conditions elevation of Ca_i in the presence of Ca_o cannot be due to the reverse operation of Na/Ca exchange because Na_i had been depleted due to prolonged exposure (at least 10 min) to Na-free solutions. Previous measurements of Na_i in parietal cells using the fluorescent dye, SBFI, have shown that this length of time is sufficient to deplete cells of Na_i (Negulescu et al., 1990). Ca_i returned back to baseline upon readdition of Na_o. These results indicate that removal of Na_o causes both release of Ca from internal stores and entry of extracellular Ca.

In order to control for possible effects of Na removal on agonist receptors as described by Muallem et al. (1988), control experiments were conducted in the presence of 10 μM atropine and 100 μM cimetidine. These antagonists block the effects of cholinergic and histaminergic stimuli on Ca_i (Chew & Brown, 1986; Negulescu & Machen, 1988*a*). We found that there was no difference in the Na-free responses in the presence of the antagonists (*not shown*). Although most experiments were conducted in NMG-containing solutions, similar results were obtained by replacing Na_o with K, Cs, or Li (Fig. 2*C*).

The nature of the released Ca was investigated by sequentially exposing parietal cells to Na-free and carbachol-containing solutions and vice versa. As shown in Fig. 3, if the cell was exposed to Na-

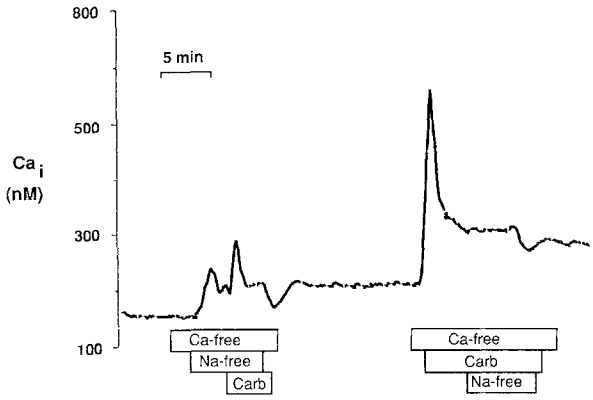

Fig. 3. Na-free solutions release Ca from hormone-sensitive intracellular Ca stores. Pretreatment of parietal cells with Na-free solutions attenuated Ca_i increases due to carbachol (100 μM), while treatment with carbachol prevents increases due to Na-free solutions. These experiments were conducted in Ca-free solutions to selectively observe effects on intracellular Ca stores

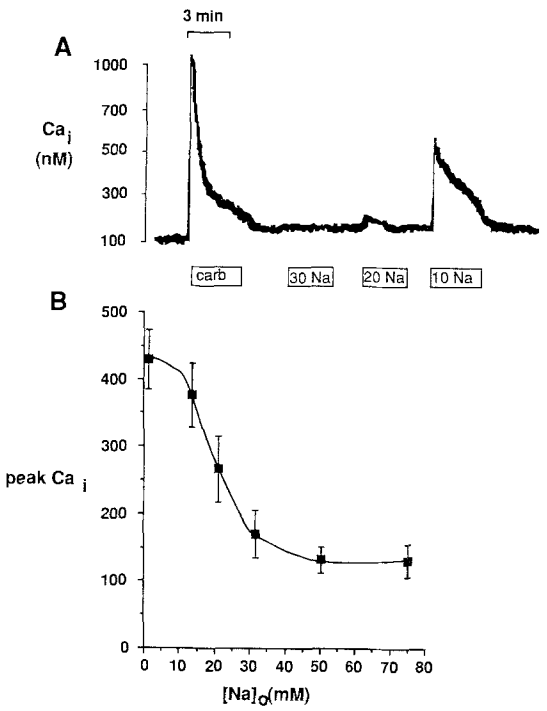

Fig. 4. Na-dependence of the Ca_i response to low Na solutions. (*A*) Ca_i increases where only detected when Na_o at 20 mM or below. Response to 100 μM carbachol shown for comparison. (*B*) Na concentration dependence of Ca_i response to low Na. Values represent averages (±SEM) for cells exposed only once, at a single concentration of Na_o. Averages based on responses from at least five cells in two separate preparations. Half-maximal responses were obtained at 16 mM Na_o

free solutions first, the Ca_i response to carbachol was small. Conversely, if the cell was treated with carbachol first, Na-free had no effect. These experiments were conducted in Ca-free solutions to prevent refilling of the internal store between treatments (Negulescu & Machen, 1988*c*) and to rule out the contributions of Ca entry mechanisms to the response. These findings suggest that Na-free and carbachol are releasing Ca from the same intracellular store.

The response to Na-free solutions in Figs. 2 and 3 involved the complete removal of Na_o. To test the sensitivity of the response to various levels of Na_o, the concentration dependence of the response was studied. Figure 4*A* shows a typical experiment in which varying amounts of Na_o were removed from the perfusate. Generally, no effects were seen when Na_o was greater than 20 mM. However, at 15 mM Na_o or lower a rather sharp concentration dependence was noted. For the cell shown in Fig. 4, 20 mM Na_o caused only a very slight increase in Ca_i, whereas 10 mM Na_o produced a maximal effect. The Ca_i response to a maximal dose of 100 μM carbachol is shown for comparison. Figure 4*B* shows the average peak Ca_i achieved by various Na_o. Similar results were obtained in cells that had been treated with carbachol (e.g., Fig. 4*A*) and in cells that had never been exposed to carbachol (e.g., Fig. 2*A*).

Initially, removal of Na_o might be expected to hyperpolarize the cell due to the loss of Na through conductive pathways. A secondary depolarization would occur due to eventual inactivation of the Na/K ATPase. The effects of both depolarization and hyperpolarization of the membrane potential were investigated by altering external K. Neither high K (50 mM) nor low K (0.5 mM) had any significant effect on Ca_i (*not shown*).

Ca_i Increases upon Na_o Removal Are Independent of Changes in pH_i

Gastric parietal cells are known to possess an Na/H exchanger which normally functions to extrude H from the cytoplasm and is responsible for pH_i recovery following acid loads in HEPES-buffered (HCO_3-free) solutions (Paradiso et al., 1987). Removal of Na_o results in the reverse operation of the exchanger and causes pH_i to acidify. Acidification of the cytoplasm elevates Ca_i in squid axon (Baker & Honerjager, 1978). Therefore, it was important to determine to what extent changes in Ca_i correlated with changes in pH_i when Na_o was removed. This was done by performing parallel experiments in fura-2 and BCECF-loaded cells. In order to distinguish between the effects of pH_i and Na_o on Ca_i, cells were acidified using maneuvers which did not

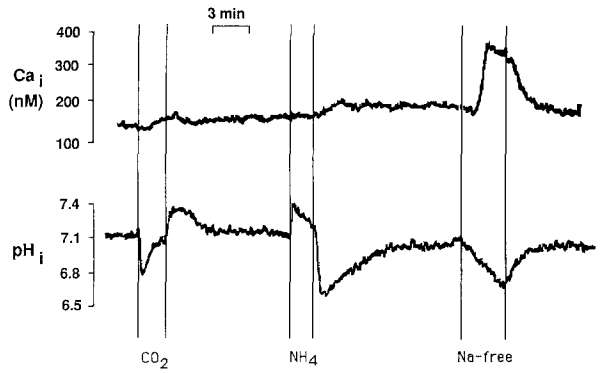

Fig. 5. Ca_i increases caused by Na removal are independent of changes in pH_i. pH_i was acidified by pulsing the cells with either 5% CO_2, 20 mM NH_4Cl, or Na removal. Na removal caused the largest increase, which occurred before pH_i had dropped by more than 0.10 pH units. pH_i and Ca_i were measured in parallel experiments

involve removal of Na_o. Figure 5 shows that acidification of cells, produced by either a pulse of 5% CO_2 or 30 mM NH_4, had small effects on Ca_i when compared to the effect of Na_o removal. Taking the pH-dependent affinity shift of fura-2 into account, Ca_i increased by 15% at pH 6.5 (i.e., increased by 20 nM). In contrast to these relatively small changes, removal of Na_o increased Ca_i to 380 nM. In addition, note that increases in Ca_i due to Na_o removal occurred before pH_i had decreased by more than 0.05 pH units. Thus, while large decreases in pH_i elicit small increases in Ca_i, it does not appear that pH_i contributes to the rapid increases in Ca_i due to Na-free solutions.

Effect of Low pH_o on Ca_i

Another way to acidify cells is to lower the pH of the external solution. In contrast to the maneuvers shown in Fig. 5, in which pH_i was changed at constant pH_o, decreasing pH_o caused large increases of Ca_i. Figure 6*A* shows that decreasing pH_o from 7.4 to 5.7 or lower caused progressive increases in Ca_i. No effect was detected at pH_o above 6.2. The Ca_i increase was due entirely to release of intracellular Ca. Note that there was a lag of about 45 sec between the time pH_o was decreased and the time of the response (Fig. 6*B*). Complete release of the internal store, assessed by the ability of carbachol to cause additional Ca release, was observed at a pH_o of about 5.0. Figure 6*C* shows the dose response for external protons. The Ca_i increases were due to release from hormone-sensitive internal stores, since the response was observed in Ca-free solutions (Figs. 6*A* and *B* and 7) and was blocked by pretreatment of carbachol (Fig. 7). Pretreatment of cells with atropine had no effect on the ability of low pH to increase Ca_i (*not shown*).

Data presented in Fig. 5 show that lowering pH_i to 6.7 had only small effects on Ca_i. However, when pH_o was lowered to 6.0 or below, pH_i was expected to drop rapidly below 6.7. To determine pH_i at the time of the Ca elevation, pH_i was measured with fluorescein diacetate, which has a pK_a of 6.3 and is appropriate for measuring more acidic pH_i's. Figure 8 shows parallel experiments in which pH_i and Ca_i were measured when cells were subjected to a large acidification using either 50 mM NH_4 or by lowering pH_o to 5.5. A pulse of 50 mM NH_4Cl followed by incubation in 250 μM amiloride and 500 μM H_2DIDS (which blocked the pH regulatory response) acidified cells to pH_i 6.0, which had minimal effects on the fura-2 ratio. Once corrected for decreased affinity of fura-2 at low pH (Fig. 1), Ca_i still only increased to 200 nM. Thus, the effect of low pH_o appears to be largely due to an effect of H at an external site.

Interactions Between Low Na_o and pH_o

Figure 9*A* shows that Ca_i increases resulting from 20 mM Na_o and pH_o 5.7 are additive. An interesting finding was that while ouabain by itself had no effect on Ca_i, it augmented Ca_i increases induced by low pH_o (Fig. 9*B*). Since the transmembrane Na gradient appeared to affect the response to pH_o, one possibility was that these effects were mediated through the Na/H exchanger. However, neither amiloride nor alterations in pH_i had any effect on the effect of low Na_o (Fig. 10), which indicates that Na/H exchange is not directly responsible for Ca_i increases.

Discussion

Lowering Na_o to less than 10–20 mM rapidly causes a biphasic increase in Ca_i by releasing internal Ca stores and stimulating Ca influx in parietal cells. Release of internal stores was shown by Na-free-induced increases in Ca_i in the absence of Ca_o (Fig. 2). Ca entry was observed in Na-depleted cells and therefore was not due to Na/Ca exchange operating in reverse. Because removal of Na_o increased Ca influx, it was difficult to determine directly whether Na/Ca exchange exists in parietal cells. However, the fact that reducing the transmembrane gradient for Na entry (by lowering Na_o to 30 mM or by exposing cells to ouabain) had no effect on Ca_i suggests that Na/Ca exchange does not contribute sig-

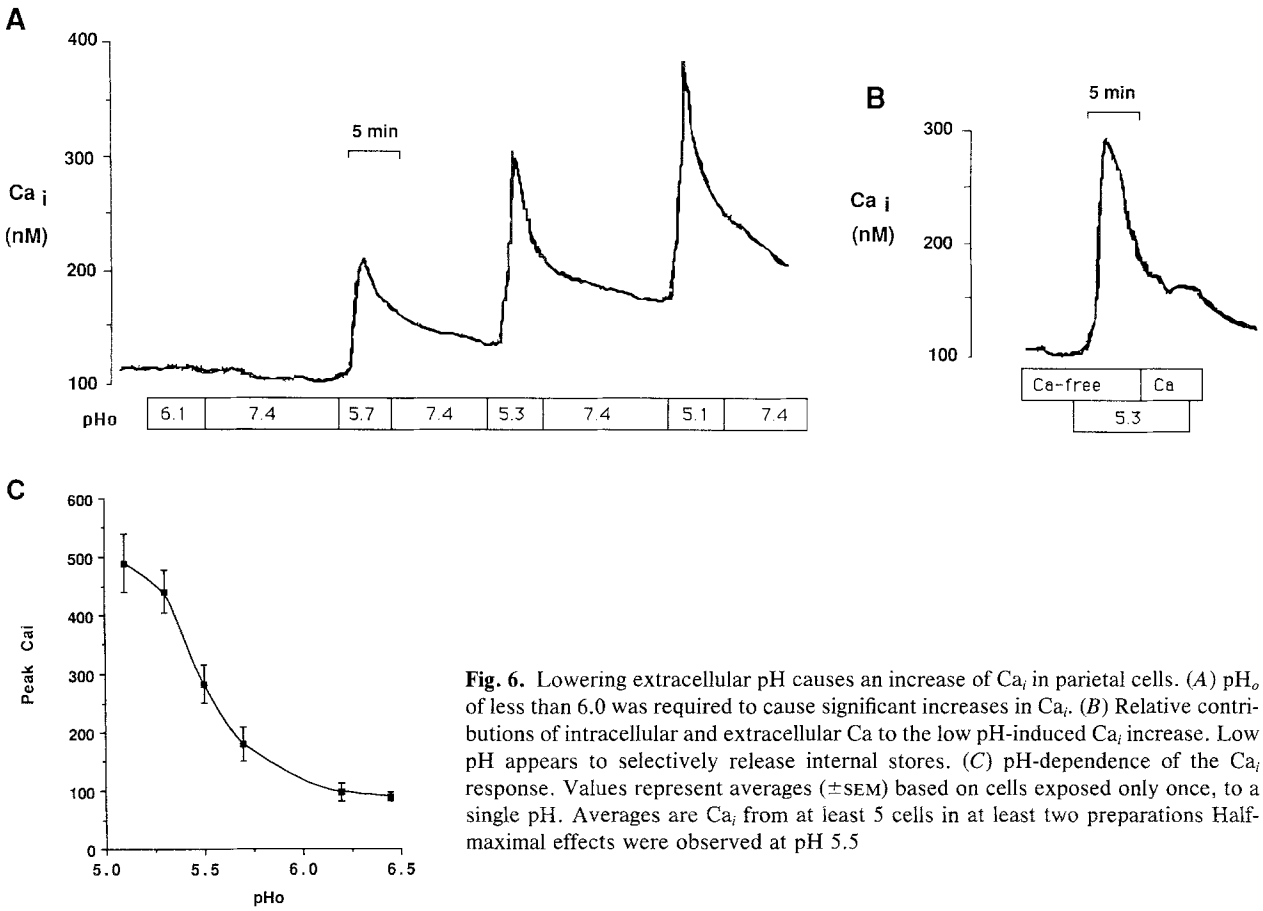

Fig. 6. Lowering extracellular pH causes an increase of Ca_i in parietal cells. (*A*) pH_o of less than 6.0 was required to cause significant increases in Ca_i. (*B*) Relative contributions of intracellular and extracellular Ca to the low pH-induced Ca_i increase. Low pH appears to selectively release internal stores. (*C*) pH-dependence of the Ca_i response. Values represent averages (±SEM) based on cells exposed only once, to a single pH. Averages are Ca_i from at least 5 cells in at least two preparations Half-maximal effects were observed at pH 5.5

nificantly to Ca regulation under these conditions. This agrees with the conclusions of Muallem and Sachs (1985). With regard to Ca_i metabolism, these effects of low Na_o are similar to the effects of carbachol or histamine in that Na-free (NMG-containing) solutions were found to release Ca_i from a carbachol-sensitive pool (Fig. 4) and cause Ca entry (Fig. 2*B*).

The effects of Na-free solutions on Ca_i were not due to interactions of the replacement cation with cholinergic or histaminergic receptors since the response persisted in the presence of either atropine or cimetidine (an H2-histaminergic receptor blocker). In addition, Ca_i increased in solutions where Na was replaced with either K, Cs, or Li, none of which would be expected to stimulate hormone receptors (although Li is known to affect phosphoinositide metabolism). It should be noted, however, that cholinergic receptors can be stimulated if the replacement cation is choline or TMA as shown by Muallem et al. (1988) and confirmed in our own control experiments (*not shown*).

While the data indicate that the increase in Ca_i upon Na removal is not due to interactions with cholinergic or histaminergic receptors, our observations are nevertheless most consistent with action at some external site. The Ca_i increase after Na_o removal was rapid, exhibited a steep dose dependence, and was independent from effects on mem-

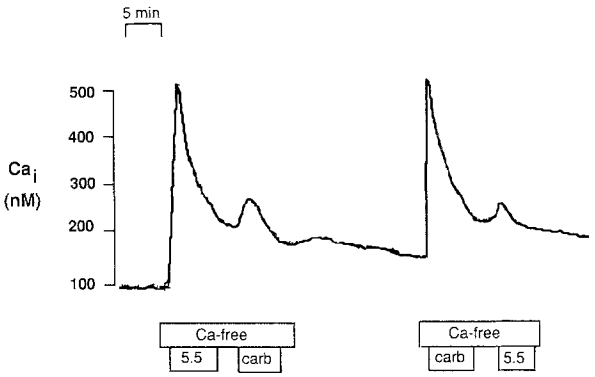

Fig. 7. Low pH_o releases Ca from carbachol-sensitive intracellular stores. Experiments were conducted in Ca-free solutions to selectively observe effects on intracellular stores. Ca_i increases due to carbachol were markedly attenuated if Ca stores had previously been released by perfusing cells with solution at pH 5.5. Conversely, if parietal cells were first treated with carbachol, Ca_i responses to low pH were blocked

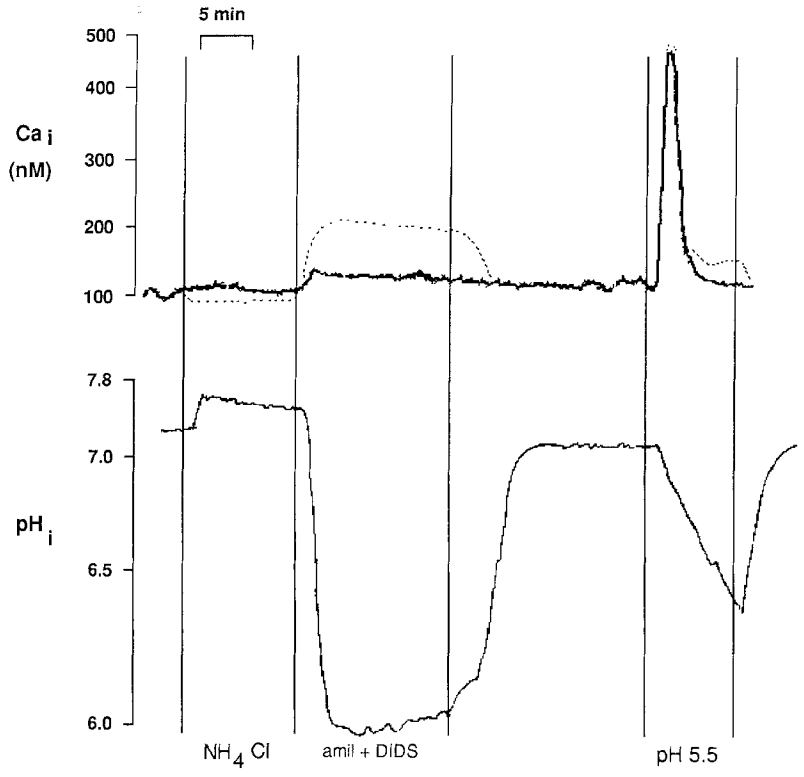

Fig. 8. Ca increases caused by lowering pH_o are largely dependent of changes in pH_i. The dashed lines represents the calculated Ca_i when the pH dependence of fura-2 is accounted for. Extreme changes in pH_i, caused by pulsing cells with 50 mM NH_4Cl, had very little effect on Ca_i. By the time Ca_i had peaked due to exposure to pH_o 5.5, pH_i had only acidified to 6.8

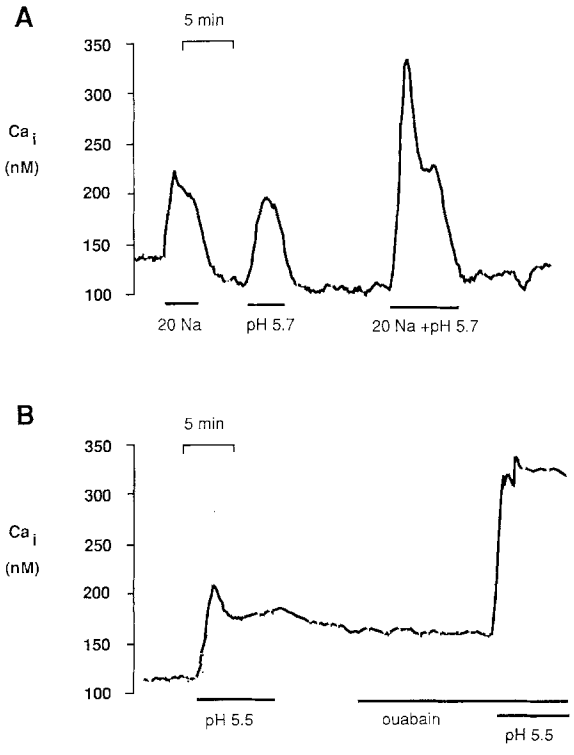

Fig. 9. Interactions between low pH and low Na. (*A*) Ca_i increases caused by low Na and pH are additive. (*B*) Raising Na_i increased the effectiveness of low pH

brane potential (as elicited by 10-fold increases or decreases of K_o), and pH_i (elicited by pulsing cells with CO_2 or NH_4Cl). Since volume or Na_i changes are probably similar at 30 mM (which had no effect on Ca_i) and 20 mM Na_o (which did), it is unlikely that either Na_i or volume are triggers for Ca_i increases following Na_o removal. Although not shown in the results, similar responses to low Na and pH were obtained in chief cells, which also possess hormone-sensitive Ca stores (Chew & Brown, 1986). The similarity between the responses of these two cell types and the similarity of the low Na results with those of Smith et al. (1989*a*) suggest that this may be a feature common to many cell types.

Precedents exist for several possible mechanisms to explain the effects of low Na_o on Ca metabolism. Smith et al. (1989*a*) have suggested that a Na-sensitive "receptor" is responsible for triggering release of internal Ca stores, based on the observation that Na removal triggers phosphoinositide breakdown. If such receptors exist, they would need to be sensitive to the loss of Na and their physiological relevance with regard to Ca mobilization is unclear since cells would never be exposed the large decreases in Na_o needed to elicit a measurable Ca_i rise. With regard to Ca entry across the plasma membrane, Na-free solutions have been ob-

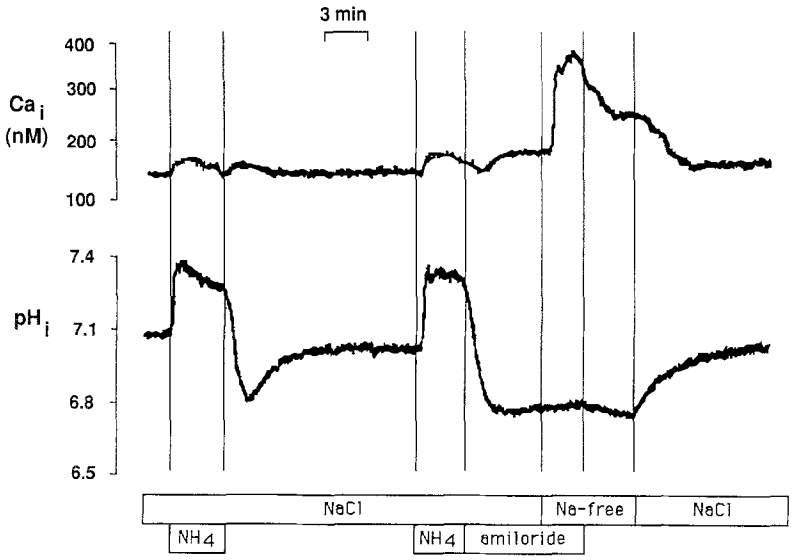

Fig. 10. The Na-free effect is unaffected by amiloride and independent of pH_i. To determine whether low Na_o effects were dependent on Na/H exchange or pH_i, Na-free solutions were exposed to cells which were held acidic following a 30 mM NH_4Cl pulse with 100 μM amiloride. Under these conditions Na-free solutions elicited a typical response, despite different pH_i conditions

served to increase Ca influx in phorbol ester-stimulated neutrophils (Nasmith & Grinstein, 1987). In this case removal of Na_o may have opened Na-sensitive Ca channels. It is possible that either or both of these mechanisms are responsible for the effects on Ca_i in gastric cells. As pointed out by Smith et al. (1989*a*), the apparently direct effects of Na removal on Ca_i in the present study are distinct from findings that Na can affect the affinity of agonists and antagonists for their receptors (Motulsky & Insel, 1983; Nunnari et al., 1987), since agonists were not required to observe Ca_i increases.

The effect of low pH_o on Ca_i was similar in several ways to the effect of low Na_o. For example, decreases in pH_o produced a rapid (although delayed) Ca_i increase which was due to release from internal stores. Second, the response exhibited a steep "dose response." Also, the effect of H was due mostly to action at some external site since drastic excursions of pH_i in either alkaline or acidic directions had little apparent effect on Ca_i, even when the decreased affinity of fura-2 for Ca_i at pH values below 6.5 were considered. This independence implies the lack of a shared cytoplasmic buffer system between H and Ca. There were two differences between the effects of low pH_o and low Na_o. First, the sensitivity to pH occurred as [H] increased, whereas the Na response was observed as Na concentrations decreased. A second difference was that low pH_o did not seem to enhance Ca entry from outside the cell.

The physiological significance of the response to low pH_o is unclear, since it occurred at pH values lower than the basolateral membranes of these cells are likely to encounter under normal physiological conditions. However, it may reflect a general phenomenon since it has also been observed in fibroblasts (Smith, Dwyer & Smith, 1989*b*).

The finding that both low pH and low Na stimulated Ca_i increases prompted the suggestion that the effects could be mediated through the same mechanism. One such candidate would be the Na/H exchanger, especially since it would be expected to reverse directions at about 20 mM Na_o. This is due to equilibrium conditions of the exchanger described by

$$Na_i/Na_o = [H_i]/H_o] = \text{antilog}(pH_i - pH_o). \quad (4)$$

Since Na_o = 150 mM, pH_o = 7.4 and pH_i = 7.1 under control conditions, the exchanger will extrude protons at normal Na_i, which has been determined to be 10 mM (Negulescu et al., 1988*c*, 1990). However, at 20 mM Na_o or below, the exchanger should reverse. The finding that ouabain treatment of cells augmented Ca_i release due to low pH_o (Fig. 9*B*) is consistent with an effect on the Na/H exchanger since the transmembrane gradient of Na would be smaller, resulting in more reverse flux through the exchanger at low pH_o. However, the lack of blockade with amiloride (Fig. 10) argues against such a mechanism and in favor of a "receptor."

That cells are sensitive to low Na_o or pH_o has implications for experiments in which the Na gradient is altered to assess the activity of Na-dependent transporters or where pH_o is altered to study pH-dependent processes. For example, one must consider the possible effects of stimulating Ca_i metabolism, including emptying of intracellular Ca stores,

when investigating Na-dependent transporters, particularly Na/Ca exchange. Ideally, such experiments would be conducted at sufficiently high Na or pH as to avoid complications due to the phenomena described here.

References

Alvarez-Leefmans, F.J., Rink, T.J., Tsien, R.Y. 1981. Free calcium ions in neurones of *helix aspera* measured with ion-selective micro-electrodes. *J. Physiol. (London)* **315:**531–548

Baker, P.F., Honerjarger, P. 1978. Influence of carbon dioxide on levels of ionized calcium in squid axons. *Nature (London)* **273:**458–461

Berglindh, T.H., Obrink, K.J. 1976. A method for preparing isolated glands from the rabbit gastric mucosa. *Acta Physiol Scand.* **96:**150–159

Chew, C.S., Brown, M.R. 1986. Cholesystokinin, carbachol, gastrin, histamine, and forskolin increase [Ca^{2+}] in gastric glands. *Am. J. Physiol.* **250:**G814–G823

Dipolo, R., Beauge, L. 1983. The calcium pump and sodium-calcium exchange in squid axons. *Annu. Rev. Physiol.* **45:**313–324

Grinstein, S., Goetz, J.D. 1985. Control of free cytoplasmic calcium by intracellular pH in rat lymphocytes. *Biochim. Biophys. Acta* **819:**267–270

Grykiewicz, G., Poenie, M., Tsien, R.Y. 1985. A new generation of Ca indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:**3440–3450

Ives, H.E., Daniel, T.O. 1987. Interrelationship between growth factor-induced pH changes and intracellular Ca. *Proc. Natl. Acad. Sci. USA* **84:**1950–1954

Motulsky, H.J., Insel, P.A. 1983. Influence of sodium on the a_2-adrenergic receptor system of human platelets. *J. Biol. Chem.* **258:**3913–3919

Muallem, S., Beeker, T., Pandol, S.J. 1988. Role of Na/Ca exchange and plasma membrane Ca pump in hormone-related Ca efflux from pancreatic acini. *J. Membrane Biol.* **102:**155–162

Muallem, S., Sachs, G. 1985. Ca metabolism during cholinergic stimulation of acid secretion. *Am. J. Physiol.* **248:**G216–G228

Mullins, L.J., Requena, J. 1979. Calcium measurement in the periphery of an axon. *J. Gen. Physiol.* **74:**393–413

Nasmith, P.E., Grinstein, S. 1987. Phorbol ester-induced changes in cytoplasmic Ca in human neutrophils. *J. Biol. Chem.* **262:**13558–13566

Negulescu, P.A., Harootunian, A.T., Tsien, R.Y., Machen, T.E. 1990. Fluorescence measurements of cytosolic free Na concentration, influx and efflux in gastric cells. *Cell. Reg.* **1:**259–268

Negulescu, P.A., Machen, T.E. 1988*a*. Intracellular Ca regulation during secretagogue stimulation of the parietal cell. *Am. J. Physiol.* **254:**C130–C140

Negulescu, P.A., Machen, T.E. 1988*b*. Lowering extracellular sodium releases calcium from hormone-sensitive intracellular stores in gastric parietal cells. *Biophys. J.* **53:**524*a*

Negulescu, P.A., Machen, T.E. 1988*c*. Release and reloading of intracellular Ca stores following cholinergic stimulation of the parietal cell. *Am. J. Physiol.* **254:**C498–C504

Negulescu, P.A., Reenstra, W.W., Machen, T.E. 1989. Intracellular Ca requirements for stimulus-secretion coupling in the parietal cell. *Am. J. Physiol.* **256:**C241–C251

Nunnari, J.M., Rapaske, M.G., Brandon, S., Cragoe, E.J., Limbird, L.E. 1987. Regulation of porcine brain alpha 2-adrenergic receptors by Na, H, and inhibitors of Na/H exchange. *J. Biol. Chem.* **262:**12387–12392

Paradiso, A.M., Tsien, R.Y., Machen, T.E. 1987. Digital image processing of intracellular pH in gastric oxyntic and chief cells. *Nature (London)* **325:**447–450

Rink, T.J., Tsien, R.Y., Warner, A.E. 1980. Free calcium in *Xenopus* embryos measured with ion-selective microelectrodes. *Nature (London)* **283:**658–660

Siskind, M.S., McCoy, C.E., Chobanian, A., Schwartz, J.H. 1989. Regulation of intracellular calcium by cell pH in vascular smooth muscle cells. *Am. J. Physiol.* **256:**C234–C240

Smith, J.B., Dwyer, S.D., Smith, L. 1989*a*. Decreasing extracellular Na concentrations triggers inositol polyphosphate production and Ca mobilization. *J. Biol. Chem.* **264:**831–837

Smith, J.B., Dwyer, S.D., Smith, L. 1989*b*. Lowering extracellular pH evokes inositol polyphosphate formation and calcium mobilization. *J. Biol. Chem.* **264:**8723–8728.

Tsien, R.Y., Pozzan, T. 1989. Measurements of cytosolic Ca using quin-2. *Methods Enzymol.* **172:**230–262

Received 29 September 1989; revised 6 February 1990