Secretory state regulates Zn\(^{2+}\) transport in gastric parietal cell of the rabbit

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IN RESPONSE TO SECRETORY STIMULATION, the gastric parietal cell undergoes a striking morphological transformation. Its apical membrane becomes highly infolded when secretory canaliculi are formed from the fusion of the tubulovesicles (TVs), membrane-bound structures that harbor H\(^{+}\)-K\(^{+}\)-ATPase that mediates luminal acid secretion. Uptake was suppressed by removal of external ATP or blockade of H\(^{+}\)-K\(^{+}\)-ATPase from the cytoplasm into the TV compartment and secretory stimulation with forskolin enhanced vectorial movement of cytoplasmic Zn\(^{2+}\) into the tubulovesicle/lumen (TV/L) compartment. Our findings suggest that Zn\(^{2+}\) accumulation in the TV/L compartment is physiologically coupled to secretion of acid. These findings offer novel insight into mechanisms regulating Zn\(^{2+}\) homeostasis in the gastric parietal cell and potentially other cells in which acidic subcellular compartments serve signature functional roles.

gastric gland; tubulovesicles; zinc; acidity regulation

MATERIALS AND METHODS

Gland isolation. Anesthesia and euthanasia for New Zealand White rabbits were approved by an independent review board according to the policies of Harvard Medical School. Gastric mucosa was harvested after aortic perfusion with phosphate-buffered saline, as described previously (7, 19, 42). Glands were prepared as described and suspended in fresh Dulbecco’s modified Eagle’s medium (DMEM) until being used (7, 19, 42). Preparation of tubulovesicles from rabbit gastric mucosa. At 4°C, gastric mucosa was minced and then subjected to mechanized trituration as described previously (34). Supernatants were serially subjected to low-speed centrifugation (4,500 g\(_{\text{max}}\)) for 10 min, high-speed centrifugation (14,600 g\(_{\text{max}}\)) for 10 min, and then ultra-high centrifugation (48,000 g\(_{\text{max}}\)). TVs were separated from the pellet by centrifugation through a 4-h centrifugation (100,000 g\(_{\text{max}}\)) that yielded the TVs on top of a 20% sucrose gradient. TVs were kept overnight at 4°C for use the next day or frozen at −80°C for up to 3 wk in isotonic suspension medium (in mM: 300 sucrose, 0.2 EDTA, 5 Tris-HCl, pH 7.3).

Western blot. Purity of the TV preparation was evaluated by Western blot (7.5% SDS-PAGE, samples 3–10 μg each; confirmed by Bradford assay) using antibodies to detect enrichment of H\(^{+}\)-K\(^{+}\)-ATPase (mouse anti-human α-subunit antibody from Chemicon, Temecula, CA) and depletion of Na\(^{+}\)-K\(^{+}\)-ATPase (mouse anti-human α1-subunit antibody from Upstate Technology, Lake Placid, NY) compared with content in whole mucosa or isolated gastric glands.

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Monitoring Zn$^{2+}$ uptake by isolated tubulovesicles. Similar to observations in other subcellular secretory compartments (17, 24), we observed that isolated TVs load with a Zn$^{2+}$-sensitive fluorescent reporter, N-(6-methoxy-8-quinolylo)-para-toluene sulfonamide (TSQ), that does not depend on cleavage of an acetoxymethyl ester (AM) group for uptake and fluorescence. In preliminary studies in a simulated intracellular buffer (ICB) solution, we found that TSQ has a high affinity for Zn$^{2+}$ ($K_d \sim 10^{-10}$ M), which can be quenched by addition of the chelator TPEN ($K_d \sim 10^{-15}$ M). Furthermore, we found that TSQ fluorescence is markedly attenuated in free solution when the ambient pH falls below 4.5 but is stable at higher pH levels. In preliminary studies, the following characteristics were also observed: first, when aliquots of TVs (15 μl; 10–15 μg) were suspended minimal background fluorescence was observed in ICB solutions containing TVs alone or TSQ alone when free Zn$^{2+}$ was depleted by the presence of 0.5 mM EGTA; second, relative fluorescence signals were 1:3:10 for TVs in the absence of TSQ, TVs plus TSQ in ICB depleted of free Zn$^{2+}$ (0.5 mM EGTA), and TVs plus TSQ in the presence of an excess of free Zn$^{2+}$ (10 μM), respectively; third, in ICB solution not containing TVs, independent effects on fluorescence of TSQ were not observed in the presence of Ca$^{2+}$, Mg$^{2+}$, and other soft metal divalent cations (Cu$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, Ni$^{2+}$) up to concentrations of 1 μM. We also observed that, at concentrations above 1 μM, Cu$^{2+}$ and Fe$^{2+}$ interfere with binding of TSQ to Zn$^{2+}$ (2.5 and 25 mM) in free solution. These observations confirm the low potential for nonspecific enhancement of TSQ fluorescence when TV membranes are present and the specificity of TSQ for Zn$^{2+}$.

To assay content of Zn$^{2+}$ in TV preparations, aliquots of TVs (15-μl aliquots containing 10–15 μg of concentrated TVs) were placed in individual well of a 96-well plate coated with polylysine and then partially filled with 300 μl of ICB (containing in mM) 125 KCl, 25 NaCl, 0.3 CaCl$_2$, 0.5 MgCl$_2$, 10 HEPES, 0.5 Na$_2$ATP, and 0.5 EGTA, pH 7.25; calculated free [Ca$^{2+}$] of 190 nM. After exposure to experimental conditions, TVs were exposed to ICB containing 40 μM TSQ (dissolved first in ethanol and then diluted 1:1,000) for 20 min, at which time fluorescence (excitation 360 nm; readout 520 nm) was measured. Corrections for background fluorescence were performed.

By analogy to the accumulation of weak bases (such as aminopyrine or acridine orange that have been used to monitor rates of acid secretion in TVs and intact gastric glands), we found that the accumulated TSQ dissipates from the TVs within 20 min once it has been removed from the ICB; thus it is not feasible to utilize TSQ fluorescence in order to monitor real-time changes in Zn$^{2+}$ fluxes into or out of TVs. A modified protocol was thus developed, analogous to those used for $[^{14}C]$aminopyrine measurements of acid secretion (7), in which accumulation of TSQ and immediate readout of fluorescence were used to monitor uptake of Zn$^{2+}$ in TV preparations. In these protocols, aliquots of concentrated TVs were suspended in wells containing ICB (total volume 300 μl) with variable levels of Zn$^{2+}$ (0, 0.25, 2.5, 25, 250 nM), under baseline conditions and during exposure to reagents or changes in conditions on the cytoplasmic side of the TVs. When concentrations of reagents with potential chelating activity were being altered (e.g., removal of ATP, which has affinity for several diveral cations), WEBMAX algorithms (see below) were utilized to adjust content of Zn$^{2+}$, Ca$^{2+}$, and Mg$^{2+}$ required in order to preserve free concentrations for each divalent cation.

Acidity of isolated tubulovesicles. To monitor steady-state acidity levels within TV preparations, aliquots of concentrated TVs were placed in individual wells of a 96-well plate coated with polylysine and then partially filled with 300 μl of ICB. Aliquots of TVs were exposed to ICB solution (300 μl) containing valinomycin, to enhance activity of the proton pump, and LysoSensor Yellow-Blue DND-160 (8 μM) for 30 min and then washed twice with fresh ICB. Excitation was performed at 360 nm, and emission was read at 460 nm (acidity quenches fluorescence) or at 528 nm (acidity enhances fluorescence), to provide a report of acidity at steady state. Corrections for background fluorescence were included.

Monitoring p-ATPase activity in tubulovesicle preparations. Activity of enzymes cleaving p-nitrophenylphosphate (pNPase activity, a surrogate for ATPase activity) was measured as published previously (41). Aliquots of TVs were suspended in solutions containing (in mM) 7.5 Tris-Cl (pH 8.3), 3.5 MgSO$_4$, 0.1 ouabain, 30 sucrose, and 50 KCl with 20 μM EGTA. Activity was measured under control conditions or in the presence of orthovanadate, a general inhibitor of P-type ATPase activity (39), at a concentration of 1 or 2 mM (19).

Loading of gastric glands with fluorescent reporters. Fluozin-3 AM and mag-fura-2 AM (Invitrogen-Molecular Probes, Eugene, OR) were dissolved separately in dimethyl sulfoxide (DMSO, 1:1,000 concentration) and then loaded (30 min at room temperature) into glands suspended in DMEM containing 100 μM cimetidine (pH 7.4), with dye concentrations between 4 and 8 μM. Subsequently, glands were rinsed in dye-free DMEM, mounted on glass coverslips, and transferred to the microscope stage (Nikon TE-2000), where they were superfused with Ringer solution (in mM: 145 NaCl, 2.5 K$_2$HPO$_4$, 1.0 MgSO$_4$ or MgCl$_2$, 1 CaCl$_2$, 10 HEPES, 10 glucose, pH 7.4) at room temperature.

Gland permeabilization. Glands were permeabilized with digitonin (10, 21) after they had been mounted on coverslips and were under microscopic observation. After an initial rinse and stabilization in Ringer solution, glands were exposed to experimental manipulations and then superfused with ICB solution containing 10 μM digitonin (19, 22).

Monitoring [Zn$^{2+}$] in cytoplasm and [Ca$^{2+}$] in intracellular stores. Fluorescence was monitored concurrently in 6–10 individual parietal cells in each isolated gland. To monitor [Zn$^{2+}$], glands were loaded with 4–8 μM fluozin-3 AM for 30 min, mounted on coverslips, and secured in the perfusion chamber. Glands loaded with fluozin-3 AM were excited at 488 nm, with emission measurement at 520 ± 15 nm. To monitor the integrity of intracellular Ca$^{2+}$ stores ([Ca$^{2+}$]$_{i}$), glands were loaded with mag-fura-2 AM (22) and excited alternately at 340 and 380 nm, with collection of emitted light at 520 ± 15 nm. Digital images of glands were captured with a digital charge-coupled device (CCD) camera (Hamamatsu). Images were processed by using compatible software (Universal Imaging, Downingtown, PA) to yield background-corrected pseudocolor images reflecting the intensity of fluorescence (fluozin-3) or the ratio of fluorescence intensities (fura-2). Images were acquired every 10 s to minimize photobleaching. Contributions of autofluorescence were measured and taken into account.

Additional reagents and methods. Unless otherwise specified, all reagents were purchased from Sigma-Aldrich. Reagents were dissolved in vehicle (ethanol or DMSO) and then added to Ringer solutions in dilutions no more concentrated than 1:1,000. TSQ was obtained from Molecular Probes and was dissolved in Ringer solutions directly. All solutions were checked for changes in pH and adjusted if necessary to baseline pH. For Ringer and ICB solutions, calculations of free and bound concentrations of Ca$^{2+}$, Zn$^{2+}$, TPEN, and EGTA were performed with Internet-based WEBMAX programs and algorithms (http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm).

Data summary and statistical analysis. Fluorescence intensities were monitored continuously throughout each experiment. At discrete time intervals, measurements were summarized as means ± SE. For comparison between treatments, unless stated otherwise, measurements in different regions of interest (6–10 cells for each gland) were combined to provide a single integrated value at each time point for each gland. Unless stated otherwise, comparisons were performed with analysis of variance for sequential or multiple measurements and purchased software (Sigma Stat, version 2.0, Jandel).
RESULTS

Characterization of tubulovesicle preparation and its ability to load with Zn\(^{2+}\)-sensing reporter TSQ. Shown in Fig. 1A is a digital image of TVs seen in bright field. As reported previously (14), there was >10-fold enrichment of H\(^+-K^+\)-ATPase and near-absence of Na\(^+-K^+\)-ATPase compared with whole mucosa or isolated gastric glands (Fig. 1B). As shown in Fig. 1C, TSQ accumulates readily in association with the TVs and fluoresces brightly after exposure to ICB solutions containing Zn\(^{2+}\) (5 \(\mu\)M). We noted the increased affinity of TSQ for Zn\(^{2+}\) in the presence of lipid, as reported previously (3a, 43) and suggesting that TSQ may partition between aqueous and lipid phases. Thus, after a period of timed uptake, the magnitude of TSQ fluorescence is likely to reflect Zn\(^{2+}\) content within the entire vesicle—including the limiting lipid membrane and lumen.

Endogenous ATPase activity was also confirmed in these preparations, by monitoring hydrolysis of para-nitrophenylphosphate (pNPP). When normalized against baseline levels, pNPPase activity was decreased from 100 \(\pm\) 4% to 52 \(\pm\) 3% in the presence of 100 \(\mu\)M omeprazole (\(P < 0.001\)), a classic proton pump inhibitor. In addition, activity was decreased to 23% \(\pm\) 7% in the presence of 2 mM vanadate, a general inhibitor of \(\alpha\)-ATPases (39, 40) (\(P < 0.001\), \(n = 16\) observations for each condition in 3 separate experiments), including the gastric proton pump (19). These findings confirm that, under these conditions, it is feasible to measure activity of the proton pump (H\(^+-K^+\)-ATPase) (16, 26).

Uptake of Zn\(^{2+}\) in tubulovesicle preparations depends on ATP and is coupled to secretory activity. Studies were then performed to determine whether Zn\(^{2+}\) accumulation in the TVs is influenced by activity of H\(^+-K^+\)-ATPase. As shown in Fig. 2A, stepwise increases in TSQ accumulation/fluorescence were observed after 30-min incubation with standard ICB solutions containing varying concentrations of Zn\(^{2+}\) (0, 0.25, 2.5, and 25 nM). This standard protocol was used to determine whether steady-state loading of Zn\(^{2+}\) was altered in the absence of ATP in the ICB solution. As also shown in Fig. 2A, in

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**Fig. 1.** Characterization of tubulovesicles (TVs) isolated from rabbit gastric mucosa. A: brightfield visualization of isolated TVs prepared as reported in text (\(\times 40\), oil immersion). B: Western blot using anti-Na\(^+-K^+\)-ATPase antibodies (\(\alpha\)-subunit) and anti-H\(^+-K^+\)-ATPase antibodies (\(\alpha\)-subunit) of 3 preparations: whole mucosa (M), isolated gastric glands (G), and isolated TVs (T). Depletion of Na\(^+-K^+\)-ATPase and enrichment of H\(^+-K^+\)-ATPase in the TV preparation are observed. C: visualization of fluorescence of isolated TVs loaded with N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ, 40 \(\mu\)M) for 20 min, with excitation at 360 nm and emission at 520 nm in intracellular buffer (ICB) solution adjusted to contain 5 \(\mu\)M Zn\(^{2+}\) (image \(\times 40\), oil immersion; same scale as A).

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p-ATPase activity and did not reveal any underlying non-p-ATPase activity that is responsive to Zn\(^{2+}\). These observations indicate that accumulation of Zn\(^{2+}\) is strongly influenced by conditions (ATP depletion) or reagents that can inhibit activity of the endogenous H\(^+-K^+\)-ATPase but does not itself directly alter ATPase activity in isolated TVs.

Relationship between Zn\(^{2+}\) movements and acidity in tubulovesicle compartment. The observation that Zn\(^{2+}\) uptake is suppressed during exposure to specific and nonspecific inhibitors of H\(^+-K^+\)-ATPase, but not totally eliminated, led us to hypothesize that the proton gradient across the TV membrane might influence its accumulation of Zn\(^{2+}\). To address this hypothesis, we evaluated the relationship between acidity and Zn\(^{2+}\) content in the TVs with two sets of studies. In the first set of studies, we used the protonophore FCCP (20 µM) to equalize the pH across the TV membrane with that of the cytoplasm, theoretically diminishing the gradient for uptake of

the absence of ATP significant reductions in Zn\(^{2+}\) accumulation were observed at all concentrations tested. As shown in Fig. 2B, Zn\(^{2+}\) accumulation in isolated TVs was also reduced during exposure to increasing [Zn\(^{2+}\)] in the presence of different inhibitors of acid secretion. Significant suppression of Zn\(^{2+}\) uptake was observed in response to omeprazole (100 µM), which inhibits the catalytic site by irreversibly binding to cysteine residues in the α-subunit (8), and in response to Sch-28080, which inhibits K\(^+\) binding and translocation in H\(^+-K^+\)-ATPase (5).

To confirm that Zn\(^{2+}\) does not directly stimulate ATPase activity, we measured pNPPase activity during exposure of TVs to modified ICB solutions containing varying [Zn\(^{2+}\)]. In preliminary studies, we observed that exposure to the general p-ATPase inhibitor orthovanadate (1 mM) suppresses Zn\(^{2+}\) uptake by isolated TVs (Fig. 3A), similar to the effects of ATP depletion or exposure to proton pump inhibitors (Fig. 2, A and B). As shown in Fig. 3B, pNPPase activity was not influenced by changes in extravesicular [Zn\(^{2+}\)]. In addition, exposure to orthovanadate (1 mM) led to almost complete suppression of
Preparations of TVs were exposed to ICB containing FCCP and different concentrations of Zn\(^{2+}\) for 30 min. In five separate studies (Fig. 4) dissipation of the pH gradient significantly reduced uptake of Zn\(^{2+}\), consistent with a process of cation exchange (Zn\(^{2+}\) for H\(^{+}\)).

A second set of studies was performed to determine whether changes in Zn\(^{2+}\) might acutely alter pH within the isolated TVs. To perform these studies, the fluorescent reporter LysoSensor DND-160 was used to monitor acidity within isolated TVs. Initially, we evaluated sequestration of this reporter and its ability to monitor pH ratiometrically in isolated TVs (excitation 360 nm; emission at 460 nm and 528 nm) (3, 6, 19, 31). In these protocols, the ratio of emission intensities (460 nm/528 nm) was measured in TVs (8- to 10-mg aliquots) loaded with the reporter in 96-well plates and then exposed to ICB containing the protonophores FCCP (20 \(\mu\)M) and nigericin (7 \(\mu\)M), adjusted to clamp pH levels over the range 3.4–7.4. As shown in Fig. 5, fluorescence of the reporter LysoSensor DND-160 at different emission wavelengths (460 nm or 528 nm) behaved in accordance with the recognized responses (3, 31) and suggested the feasibility of emission ratiometric reporting. In control studies, no fluorescence was observed when TVs were not present, indicating that the measurements were not attributable to residual dye.

Preliminary studies also revealed, however, that dye accumulation was increased in the presence of agents that lead TVs to accumulate H\(^{+}\) (valinomycin 10 \(\mu\)M) and decreased in the presence of agents that decrease such accumulation (omeprazole 100 \(\mu\)M) (data not shown). The ratio of emission intensities (460 nm/528 nm) is thus required in order to compensate for changes in dye accumulation. In a set of 12 experiments \((n = 77\) wells), the baseline emission ratio (460 nm/528 nm) in the TV preparation was a mean 2.02 (SD 0.11), corresponding to a vesicular pH of 6.2 (SD, 0.6 pH units). Under these conditions, a statistically significant change in the emission ratio (460 nm/528 nm) of 0.05 would correspond to a pH change of 0.3 units (a doubling or halving of [H\(^{+}\)]).

To address the influence of extravesicular Zn\(^{2+}\) on intravesicular acidity, TVs were loaded with LysoSensor DND-160 for 20 min. As a preliminary verification of active acid secretion, we monitored the emission ratio (460 nm/528 nm) in TV preparations \((n = 4\) experiments, \(n = 6\) wells for each condition) over time (5–30 min) under control conditions or in the presence of 100 \(\mu\)M omeprazole. As previously reported in intact gastric glands (19) the emission ratio in the isolated TV preparation began to rise slowly (Fig. 6A), the increase becoming a trend as early as after 10 min after incubation with omeprazole and becoming statistically significant by 25 min after exposure. Under baseline conditions, in the absence of the proton pump inhibitor, no significant alterations were observed in the emission ratio.

Monitoring of changes in pH within the TVs was then performed after brief exposures (10 min) to concentrations of Zn\(^{2+}\) (~0 to 25 nM) under baseline conditions or in the presence of omeprazole. As shown in Fig. 6B, under these standard conditions, significant decreases were observed in response to [Zn\(^{2+}\)] at 2.5 and 25 nM. As noted above, a small deviation from baseline, as little as 5% in the emission ratio, correlates to substantial changes in acidity within the TV; these

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**Fig. 4.** Relationship between Zn\(^{2+}\) and H\(^{+}\) gradient across the membranes of isolated TVs. Isolated TVs were exposed to ICB solutions alone or those containing the protonophore FCCP (20 \(\mu\)M), which dissipates the H\(^{+}\) gradient across the vesicular membrane. Uptake of Zn\(^{2+}\) was monitored in solutions containing different levels of free Zn\(^{2+}\). Results are expressed as means ± SE; \(n = 25\) wells in 5 separate experiments. \(*P < 0.05\) compared with baseline (ICB containing EGTA and no added Zn\(^{2+}\)); \(P < 0.05\) compared with ICB containing no FCCP.

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**Fig. 5.** Responses of TVs loaded with LysoSensor DND-160 to incremental changes in pH. TVs were loaded with the reporter after equilibration with ICB solution and the protonophores FCCP and nigericin. A: fluorescence at different emission wavelengths (460 nm or 528 nm) after excitation at 360 nm, showing inverse responses to incremental increases in pH \((n = 30\) wells in 6 individual experiments). Results are expressed as means ± SE. B: ratio of responses at each pH shown in A; arrow indicates location of the average level in wells before any experimental manipulation and corresponding to a “baseline” pH of 6.2 ± 0.6.
findings are consistent with a hypothesis that, under baseline conditions when acid secretion is not inhibited, availability of Zn\(^{2+}\) insulates the TV against substantial proton leak (19) and preserves intracellular acidity. In contrast, in the presence of omeprazole (100 μM), significant increases in pH were observed in response to increases in [Zn\(^{2+}\)] in the ICB solution, indicating that when the proton pump is inhibited entry of Zn\(^{2+}\) is associated with a small but detectable alkalization of the compartment. These findings suggest that inhibition of H\(^{+}\) secretion in the TV unmasks an exchange of Zn\(^{2+}\) (uptake) for H\(^{+}\) (extrusion).

Role of tubulovesicle compartment and secretory state in regulating [Zn\(^{2+}\)] in parietal cell of isolated gastric gland. The foregoing observations suggested that there are conditions under which activity of the gastric proton pump might regulate cytoplasm concentrations of [Zn\(^{2+}\)] ([Zn\(^{2+}\)]) in the gastric parietal cell. Specifically, the results suggested that Zn\(^{2+}\) movements into the TV/L compartment are suppressed when the proton pump is inhibited. Thus we predicted that exposure to a proton pump inhibitor would impair disposal (i.e., extrusion) after artificial loading of the cytoplasm with Zn\(^{2+}\).

Measurements of [Zn\(^{2+}\)] in parietal cells of isolated gastric gland. We utilized the fluorescent reporter fluo-3 to monitor [Zn\(^{2+}\)] in individual parietal cells of the isolated gastric gland. This reporter has been used successfully in a variety of cell types, including pancreatic islet cells (18), neurons (11), and epithelial cells of colon crypts (10). Figure 7A demonstrates fluo-3 loading in parietal cells of an isolated gastric gland, with intracellular distribution similar to that of fura-2.

An important component of the characterization of any dye is an evaluation of potential contributions to its signal from noncytoplasmic compartments. To confirm that fluo-3 localizes largely in the cytoplasm, glands were loaded with fluo-3 and then permeabilized with 10 μM digitonin in ICB. However, baseline levels of fluorescence were so low (because of such low levels of free Zn\(^{2+}\)) that loss of dye was not detectable with permeabilization.

We then performed an experiment to determine the potential contributions of fluo-3 localized in noncytoplasmic compartments, under conditions in which significant levels of free Zn\(^{2+}\) might be present throughout the cell. Glands were loaded with fluo-3 (excitation 488 nm). In these experiments, the fluorescent reporter mag-fura-2 (excitation 340 nm/380 nm) was coloaded with fluo-3. Mag-fura-2 has affinity for Ca\(^{2+}\) at K\(_d\) \(\sim\) 25 μM (20). In the parietal cell it accumulates both in cytoplasm and in intracellular stores with high Ca\(^{2+}\) content; after permeabilization of the cell membrane the component of dye in the cytoplasm leaks out and the time course of washout can be monitored. In addition, its selective responses to excitation in the UV range permit coloading with reporters that selectively respond to excitation in the visible range, such as fluo-3. Its presence in these studies served as a control, that is, as a coloaded reporter to demonstrate completeness of the permeabilization.

As shown previously, exposure to digitonin results in washout of fluorescent reporters from the cytoplasm (22), with the residual fluorescence being attributable to dye sequestered in intracellular compartments (19, 22). Thus glands loaded with both dyes were exposed to the Zn\(^{2+}\)-selective ionophore pyrithione in the presence of ICB solutions containing Zn\(^{2+}\) at a concentration of 5 nM. A subsequent exposure to ICB solutions containing digitonin (10 μM) was utilized to deplete the cytoplasmic component of fluorescence (Fig. 7B). In three experiments performed under these conditions, permeabilization reduced total fluo-3 fluorescence by 50%, 56%, and 59% (Δ% from before digitonin to after digitonin). Taking into account only the increases (Δ) in fluorescence above baseline, 76%, 89%, and 90% of the increase in signal was reduced by permeabilization. Thus noncytoplasmic contributions appear to constitute 10–20% of the total Zn\(^{2+}\)-specific response.

Studies were then performed to determine the K\(_d\) of fluo-3 for Zn\(^{2+}\). In these experiments (n = 3 glands, 22 cells), pyrithione (50 μM) was used to clamp [Zn\(^{2+}\)], at different concentrations (0, 0.25, 2.5, 5.0, 7.5, 10, 25 nM) with EGTA-buffered Ringer solutions. Results are summarized as F/F\(_0\), the ratio of fluorescence at time \(t\) over that at baseline \(t_0\). As shown in Fig. 7C, the dye is responsive over the range 0.25–10 nM, with a K\(_d\) of 4.7 ± 0.4 nM.
In control studies, fluozin-3-loaded glands were exposed to Ringer solutions (Ca\(^{2+}\) 1 mM) containing ionomycin (10 \(\mu\)M) and TPEN (free concentration \(\sim 10 \mu M\)). In other studies, glands were exposed to carbachol (100 \(\mu\)M) and thapsigargin (2 \(\mu\)M) in the presence of TPEN (free concentration \(\sim 10 \mu M\)). In both sets of studies, no disturbances in baseline levels of fluozin-3 fluorescence were detected, despite high levels of Ca\(^{2+}\) accumulation in the cytoplasm during these maneuvers, as detected by fura-2 (42). These findings confirm prior studies (11) showing that fluozin-3 signals are not altered by isolated changes in [Ca\(^{2+}\)].

**Measurements of \([Zn^{2+}]_i\) during secretory inhibition.** To evaluate the role of acid secretion, we first defined a set of conditions in which it would be possible to monitor loading of the cytoplasm with free Zn\(^{2+}\). Gastric glands were mounted on coverslips and initially equilibrated with perfusates containing Ca\(^{2+}\)-Ringer solutions. Glands were then perfused with Ringer solutions containing EGTA and no Ca\(^{2+}\), in order to simplify preparation of solutions containing enough Zn\(^{2+}\) to provide free Zn\(^{2+}\) concentrations between 1 and 100 \(\mu M\). Under these conditions, we found that responses were observed during exposure to solutions containing free [Zn\(^{2+}\)] as low as 10 \(\mu M\), but most consistently when the concentration exceeded 50 \(\mu M\).

In all subsequent experiments monitoring uptake of Zn\(^{2+}\), glands were exposed to Ca\(^{2+}\)-depleted Ringer solutions containing free [Zn\(^{2+}\)] of 60 \(\mu M\). Shown in Fig. 8A is a recording from individual parietal cells from a single gastric gland loaded with fluozin-3, monitored during exposure to the Zn\(^{2+}\)-containing perfusate. After an initial upsurge, relatively linear increases were observed in [Zn\(^{2+}\)], corresponding to increases from the subnanomolar baseline levels to 3–5 nM over a 20-min time course. At the end of the experiment, the gland is exposed to TPEN (10 \(\mu M\)), which quenches the signal completely and demonstrates that it is due to the accumulation of Zn\(^{2+}\).

To evaluate the impact of secretory inhibition on [Zn\(^{2+}\)], glands were exposed to Ca\(^{2+}\)-depleted Ringer solutions containing free [Zn\(^{2+}\)] of 60 \(\mu M\) for a period of 10 min and then exposed, also for 10 min, to solutions containing proton pump inhibitors omeprazole (100 \(\mu M\)) or Sch-28080 (10 \(\mu M\)). A similar recording of fluozin-3 fluorescence in individual parietal cells in an isolated gastric gland is shown in Fig. 8B. In this recording, a striking acceleration is noted in the rate at which Zn\(^{2+}\) accumulates within the cells. Similar results were observed when glands were exposed to Sch-28080, which inhibits the proton pump by an entirely different mechanism than that utilized by omeprazole. At the end of each recording, signals were ablated by exposure to TPEN, demonstrating their specificity for Zn\(^{2+}\). We also performed studies to exclude the possibility that either inhibitor would independently elicit increases in [Zn\(^{2+}\)]. To do this, glands loaded with fluozin-3 were exposed to perfusates containing omeprazole (\(n = 4\)) or Sch-28080 (\(n = 4\)), but in the absence of added Zn\(^{2+}\). In none of these experiments was an increase observed in [Zn\(^{2+}\)]; (data not shown).

These overall results are summarized in Fig. 8C for a statistical comparison between the different groups: first, glands exposed only to Zn\(^{2+}\) for 10 min and then vehicle (DMSO 1:1,000) for 10 min (\(n = 14\)); second, glands exposed to Zn\(^{2+}\) for 10 min and then omeprazole (\(n = 8\)) for 10 min; third, glands exposed to Zn\(^{2+}\) for 10 min and then Sch-28080.
Impairment of Zn\(^{2+}\) disposal from the cytoplasm during inhibition of acid secretion. 

**A**: recording of fluozin-3 fluorescence in a single gastric gland during exposure to Ringer solutions containing 0 Ca\(^{2+}\) and 60 \(\mu\)M Zn\(^{2+}\). After exposure to Zn\(^{2+}\), exposure to N,N,N',N'-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN, 10 \(\mu\)M) quenches all fluozin-3 activity, demonstrating that signals are due to Zn\(^{2+}\). Each line represents a recording of a single parietal cell in the gland.

**B**: recording of fluozin-3 fluorescence in a single gastric gland under conditions similar to those in A, but with introduction of omeprazole (100 \(\mu\)M) midway during the exposure to Zn\(^{2+}\). Again, exposure to TPEN quenches all fluozin-3 activity at the end of the experiment.

**C**: summary of studies. Control studies summarize results of exposure of gastric glands to Zn\(^{2+}\) alone for 20 min, showing results (means ± SE) for measurements at 4 time points: 1) baseline; 2) at the end of the first 10-min exposure to Zn\(^{2+}\); 3) at the end of the second 10-min exposure to Zn\(^{2+}\) (at baseline or in the presence of a proton pump inhibitor); 4) after quenching by TPEN. Each column represents \(n = 6–8\) individual experiments. *Comparison with baseline; †comparison with first 10 min peak effect; ‡comparison with responses under control conditions.

**DISCUSSION**

With the use of approaches in isolated TVs of rabbit gastric glands, the studies reported here indicate that Zn\(^{2+}\) movements into the TV/L compartment are regulated by secretion of H\(^+-\)K\(^+-\)ATPase that mediates luminal acid secretion. These studies suggest that the proton gradient across the TV membrane may regulate uptake of Zn\(^{2+}\); furthermore, when H\(^+-\)K\(^+-\)ATPase activity is blocked, exposure to Zn\(^{2+}\) leads to...
The studies reported here argue that uptake of Zn\(^{2+}\) is not directly mediated by ATPase activity but may involve exchange with H\(^+\) extruded to the cytoplasm side of the TV. With a novel application of the acid-sensitive reporter Lyso-Sensor DND-160, such extrusion from isolated TVs was only detectable when the proton pump was inhibited (Fig. 6). At baseline, when H\(^+\)-K\(^+\)-ATPase was not inhibited, the presence of Zn\(^{2+}\) tended to preserve acidity within the isolated TV, consistent with the hypothesis that Zn\(^{2+}\) content insulates the TV/L compartment from back-leak of H\(^+\) (19). These considerations suggest that the rate of proton extrusion accompanying uptake of Zn\(^{2+}\) is quite low and does not influence the overall downstream benefit of Zn\(^{2+}\) uptake to insulate the TV/L compartment against leakage of protons.

A second novel message from these studies is that acidic subcellular compartments might, under some circumstances, be important in buffering accumulations of labile Zn\(^{2+}\) in the cytoplasm. Prior reports have provided evidence that Zn\(^{2+}\) plays a role in regulating acidity in subcellular organelles that harbor proton pumps (19, 23, 35). It has not been suspected, however, that such compartments would prove to be major sites of sequestration for labile Zn\(^{2+}\). Our studies provide evidence that inhibition of secretory activity leads to interruption of the flow of labile Zn\(^{2+}\) from the cytoplasm to the lumen of the gastric gland (Fig. 8). Moreover, the capacity of the TV compartment for uptake of Zn\(^{2+}\) seems to amplify when acid secretion is accelerated (Fig. 9). These considerations suggest that when the proton pump is disabled accumulation of Zn\(^{2+}\) during different forms of cellular stress (38, 43) could lead to undue or untimely accumulation of Zn\(^{2+}\) in the cytoplasm, and thence to a variety of disturbances in cell viability and function (36, 37).

These studies raise three issues for discussion. The first issue is a technical one, namely, the strengths and limitation of fluorescence methods used to monitor Zn\(^{2+}\) signals in isolated TVs and the cytoplasm of the parietal cell. Measurement of Zn\(^{2+}\) uptake into isolated TVs poses a particularly challenging problem, since these vesicles are not likely to contain esterase suitable for conversion of fluorescent reporters tagged with the AM group to facilitate membrane permeability. In addition, most reporters for divalent cations are pH sensitive (20) and the pH of the TV/L compartment is not readily controllable. Finally, the method used to isolate TVs requires the presence of chelating agents (EDTA) leading to leaching of all divalent cations in order to obtain good separation.

Preliminary studies indicated that the reporter TSQ can overcome the most crucial of these obstacles, since its activity does not require chemical conversion because it is taken up by the TVs and its brightness is stable at pH levels as low as 4.5. Studies using LysoSensor DND-160 indicate that the resting pH within the TVs is above 5, suggesting that TSQ responses are reliable for the experimental conditions evaluated in this study. However, once TSQ was removed from the medium, pH within the isolated TV, is quite low and does not influence the overall downstream benefit of Zn\(^{2+}\) uptake to insulate the TV/L compartment against leakage of protons.

In individual parietal cells, the capacity of the TV/L compartment to buffer against increases in intracellular Zn\(^{2+}\) has also been demonstrated: inhibition of H\(^+\)-K\(^+\)-ATPase leads to significant immobility and accumulation of Zn\(^{2+}\) loads within the cytoplasm (Fig. 8); acceleration of secretion with forskolin enhances baseline levels of accumulation of extracellular Zn\(^{2+}\) in the cytoplasm and vectorial movements toward the TV/L compartment (Fig. 9). These findings are novel in two respects. First, to our knowledge these are the first reported assessments of Zn\(^{2+}\) movements into subcellular compartments that are as acidic as the gastric TVs and the first reported measurements of [Zn\(^{2+}\)] in the cytoplasm of the gastric parietal cell. Our observations are consistent with a novel concept, namely, that acid secretion and Zn\(^{2+}\) uptake into the TV/L compartment of the gastric gland are closely coupled.
Two potential confounders in this assay are the enhancement of TSQ fluorescence in the presence of lipid and pH levels within the TV. Prior reports (17, 20) and our preliminary studies indicate that association with the lipid membranes of TVs enhances baseline fluorescence of TSQ, even when Zn\(^{2+}\) has been depleted in the ICB solution. It seems likely that TSQ uptake monitors accumulation of Zn\(^{2+}\) in both the lipid phase and the lumen of the TV. That this assay reflects specific transport activity, rather than nonspecific accumulation of Zn\(^{2+}\), is suggested by its responsiveness to removal of ATP and exposure to proton pump inhibitors.

With respect to acidity within the TV, we have noted that TSQ fluorescence is not influenced at pH levels higher than 4.5. This is a limitation on the ability of TSQ to monitor Zn\(^{2+}\) content in a highly acidic environment. Our studies (Fig. 5) suggest that baseline pH within the isolated TV (as measured with LysoSensor DND-160) are above this threshold pH level. Prior studies in our laboratory (19) indicated that baseline pH is ∼3.5 in the intact TV/L compartment of isolated rabbit glands. It is not surprising that the pH gradient generated in the absence of cytosol components would be different from that generated in parietal cells organized within an intact gastric gland. Such differences might be attributable to differences in osmolality of the ICB, an admittedly artificial solution, compared with that of the cytosol or to the absence of cytosolic regulatory factors in optimizing acid secretion in permeabilized glands (2). For the purposes of this study, the generation of a weaker H\(^+\) gradient in isolated TVs was advantageous in evaluating characteristics of Zn\(^{2+}\) uptake. However, these considerations emphasize the importance of controlling or taking into account effects of acidity on activity of any fluororescent reporter for divergent cations. Despite its limitations, measurement of TSQ uptake appears to be reproducible and capable of detecting responses to changes in conditions on the cytoplasm side of the TV.

The use of fluozin-3 to monitor [Zn\(^{2+}\)], has been reported in several cell types (10, 11) since its description by Gee et al. (18) to monitor release of Zn\(^{2+}\) extracellularly from islet cells. In the parietal cell, the dominant component of the fluozin-3 signal appears to be confined to the cytoplasm. Its in situ \(K_d\) (3–4 mM) makes it suitable for measuring changes in intracellular Zn\(^{2+}\) in the range <1 to 10 nM. In addition, the reporter is not sensitive to levels of Ca\(^{2+}\) as high as 10 mM (10, 45). In these studies, a protocol was developed for calibrating fluozin-3 measurements in the presence of the zinc ionophore pyrithione (Fig. 6) that permitted cytoplasm loading of Zn\(^{2+}\) (Fig. 7). Utilizing solutions free of added Ca\(^{2+}\) and containing EGTA also minimized the influence of Ca\(^{2+}\) and simplified the calculations of Zn\(^{2+}\) requirements for solution preparation and use of chelators such as TPEN, which have a modest but definite affinity for Ca\(^{2+}\) at neutral and acidic pH (4, 19). The main limitation of the reporter is that it might be sensitive to changes in other divalent cations (Fe\(^{2+}/Fe^{3+}\), Cu\(^{2+}\)) that are known to quench its responses (20). Moreover, it is not feasible for ratiometric measurements that might control for dye loss or blanching. Used here for the purpose of measuring rates of Zn\(^{2+}\) accumulation in the cytoplasm of the parietal cell in the isolated gastric gland, the reporter provided specific and reproducible results with respect to calibration and loading of Zn\(^{2+}\) from the extracellular solution.

The second issue for consideration is the likely mechanism of Zn\(^{2+}\) export from cytoplasm to the TV/L compartment of the gastric gland. The studies reported here provide novel evidence that H\(^+\) is a candidate counterion for exchange, permitting uptake of Zn\(^{2+}\) into the highly acidic TV compartment. The observation that this exchange is obscured when acid secretion is present and unmasked when acid secretion is inhibited highlights the role of the proton gradient in motivating movements of Zn\(^{2+}\) into the lumen of the TV. Our studies suggest that the rate of acid secretion and the acidity of the TV/L compartment both drive uptake of Zn\(^{2+}\). Such a mechanism of regulation of Zn\(^{2+}\) transport by exchange with H\(^+\) might also account for transport of Zn\(^{2+}\) into secretory compartments of other cells characterized by an interior in which acidity is maintained by proton pumps, a principle demonstrated in presynaptic vesicles of neurons (16) and recently in a model system utilizing the vacuoles of yeast cells (25).

In recent years, considerable progress has been made in identifying families of Zn\(^{2+}\) transporters, including those that import Zn\(^{2+}\) to the cytoplasm (ZIP or SLC39A family) and those that export Zn\(^{2+}\) from the cytoplasm to other compartments or the outside (ZnT or SLC30A family) (13, 29, 36). Of note, transporters in bacteria and yeast that are closely related to the ZnT family appear to function as antiporters, exchanging Zn\(^{2+}\) for H\(^+\) (29); however, similar reports of the energetics and cofactors of transport have not emerged in mammals. Recent work (44) in a mouse model has revealed intracellular localization of two isoforms of the SLC30A family, ZnT4 and ZnT5, in the gastric parietal cell. Because the studies reported here have been conducted in tissues and isolated glands of the rabbit, currently available cDNA libraries and antibodies do not permit definitive identification and localization of the ZnT isoform(s) involved. However, in light of the reported differences in localization of ZnT4 to endosomes and ZnT5 to secretory compartments in other cell types (13, 29, 36), ZnT5 is a reasonable candidate for localization to the TV compartment.

The final issue relates to the consequences of regulation of Zn\(^{2+}\) movement into the TV/L compartment by the secretory activity of H\(^+\)-K\(^+\)-ATPase. One implication of the present observations, and those from prior studies (19), is that Zn\(^{2+}\) flows from the cytoplasm to the lumen in conjunction with secretion of H\(^+\). The content of labile Zn\(^{2+}\) in secretory vesicles of the gastric parietal cell has not been determined; however, measurements of total Zn\(^{2+}\) in gastric juice have been reported, suggesting levels as high as 10–15 μM (32). Moreover, secretory compartments in other exocrine and endocrine cells have also been implicated as potential pools for similar levels of Zn\(^{2+}\) sequestration and secretion (28, 33). The availability of Zn\(^{2+}\) in the lumen of the gastrointestinal tract has been implicated in infectious diarrheas of childhood (27, 30) and in the regulation of activity of bacterial enterotoxins (9). Moreover, in adults, therapy with proton pump inhibitors has been implicated in infectious diarrheas, such as those caused by \textit{Clostridium difficile} (12), the causes remaining unclear. The findings in this report offer the possibility that endogenous release of Zn\(^{2+}\), from natural secretions in the stomach and organs of the upper gastrointestinal tract, may serve important homeostatic functions within the lumen of downstream regions.
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GRANTS

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REFERENCES


