Intracellular Ca$^{2+}$ and Zn$^{2+}$ signals during monochloramine-induced oxidative stress in isolated rat colon crypts

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Inflammatory bowel diseases are thought to result from inappropriate activation of the mucosal immune system in response to normal flora (58, 60, 68). During acute exacerbations of these illnesses, reactive species are generated through the interactions of bacteria in the lumen, activated granulocytes, and cells of the colon mucosa. In this study, we explored the ability of one such class of oxidants, represented by monochloramine (NH$_2$Cl), to serve as agonists of Ca$^{2+}$ and Zn$^{2+}$ accumulation within the colonocyte. Individual colon crypts prepared from Sprague-Dawley rats were mounted in perfusion chambers after loading with fluorescent reporters fura 2-AM and fluozin 3-AM. These reporters were characterized, in situ, for responsiveness to Ca$^{2+}$ and Zn$^{2+}$ in the cytoplasm. Responses to different concentrations of NH$_2$Cl (50, 100, and 200 µM) were monitored. Subsequent studies were designed to identify the sources and mechanisms of NH$_2$Cl-induced increases in Ca$^{2+}$ and Zn$^{2+}$ in the cytoplasm. Exposure to NH$_2$Cl led to dose-dependent increases in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_i$) in the range of 200–400 nM above baseline levels. Further studies indicated that NH$_2$Cl-induced accumulation of Ca$^{2+}$ in the cytoplasm is the result of release from intracellular stores and basolateral entry of extracellular Ca$^{2+}$ through store-operated channels. In addition, exposure to NH$_2$Cl resulted in dose-dependent and sustained increases in intracellular Zn$^{2+}$ concentration ([Zn$^{2+}]_i$) in the nanomolar range. These alterations were neutralized by dithiothreitol, which shields intracellular thiol groups from oxidation. We conclude that Ca$^{2+}$- and Zn$^{2+}$-handling proteins are susceptible to oxidation by chloramines, leading to sustained, but not necessarily toxic, increases in [Ca$^{2+}]_i$ and [Zn$^{2+}]_i$. Under certain conditions, NH$_2$Cl may act not as a toxin but as an agent that activates intracellular signaling pathways.

Key words

In addition, exposure to oxidants may activate intracellular signaling pathways that may exacerbate injury or, conversely, initiate protective responses in the inflamed mucosa. Affected pathways include protein kinase A and C activities, expression of heat-shock proteins, and modulation of NF-κB and effector pathways in apoptosis (24, 35, 52, 55, 58, 74). Of special interest is oxidant-induced release of the divalent cations, Ca$^{2+}$ and Zn$^{2+}$, to the cytoplasm. When uncontrolled and in excess, such divalent cation signals can exacerbate tissue injury (10, 56, 67). When coordinated and in moderation, however, such signals may elicit secretory or protective responses that would prevent or arrest inflammation-induced injury (36, 69, 72).

In this study, we explored the ability of one class of oxidants, chloramines, to serve as agonists of Ca$^{2+}$ and Zn$^{2+}$ release to the cytoplasm of the colonocyte. The prototype in this class of oxidants, monochloramine (NH$_2$Cl), is produced through the reaction of neutrophil-derived hypochlorous acid (HOCl) with bacteria-derived ammonia (NH$_3$; see Refs. 25 and 63). NH$_2$Cl is relatively stable in aqueous environments and cell permeant (24, 25). Potential targets of NH$_2$Cl oxidation include molecules and structures in intracellular compartments as well as those on the cell membrane. Other chloramine species are generated by transfer of the oxidizing Cl$^-$/radical to amine groups of small organic acids such as taurine, lysine, or histamine (63). These chloramine species retain oxidant capacity but may not be able to permeate cell membranes. Molecular species capable of consuming or neutralizing the oxidant Cl$^-$/of chloramines include glutathione and other peptides and proteins (59) in which thiol (S-H) groups or clusters are integral to structure or enzymatic functions. Recent studies have implicated such thiol groups in structural proteins and enzymes that regulate intracellular homeostasis of divalent cations such as Ca$^{2+}$ and Zn$^{2+}$ (16, 46, 48, 77). These considerations led us to hypothesize that exposure to NH$_2$Cl may elicit a distinct profile of disturbances in intracellular divalent cation homeostasis in epithelial cells of the colon crypt.

METHODS

Solutions and reagents. Ringer solutions contained (in mM) 145 NaCl, 2.5 KH$_2$PO$_4$, 1.0 MgSO$_4$ or MgCl$_2$, 1 CaCl$_2$, 10 HEPES, and 10 glucose, pH = 7.4. Ca$^{2+}$-free Ringer contained all of the compounds in standard Ringer except Ca$^{2+}$-, and, in addition, 500 µM EGTA was added. Intracellular buffer (ICB) contained (in mM) 125 KCl, 25 NaCl, 10 HEPES, 0.3 CaCl$_2$, 0.5 MgCl$_2$, 0.5 ATP, and 0.5 EGTA, pH = 7.25. Thapsigargin (THPS), ionomycin, and 1-hydroxy-pyridine-2-thione (pyrithione) were purchased from Sigma Chemical.

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and used from stock solutions containing DMSO. Carbachol (CCh; Sigma), dithiothreitol (DTT; Sigma), thimerosal (Sigma), N-nitroso-N-acetyl-D,L-penicillamine, (SNAP; Sigma), N,N,N',N'-tetrakis(2-pyridyl-methyl) (TPEN; Molecular Probes), and 2-aminooethoxydiphenylborate (2-APB; Sigma) were dissolved in stock water and then brought up in Ringer (1:1,000) or dissolved directly in Ringer.

NH₄Cl was prepared as described previously (24, 62). Briefly, a 200-μl solution containing 500 mM NaOCl in water was added dropwise to 10 ml of 20 mM NH₄Cl and 5 mM Na₂HPO₄ in water at 0°C. This procedure resulted in a 5 mM NH₂Cl solution. Use of NH₂Cl was completed within 6 h of preparation, since we observed that it remained stable in Ringer solution at concentrations ranging from 50 to 200 μM with <10% loss of absorbance at 242 nm. TaurNHCl was generated under similar conditions by including taurine instead of NH₂Cl in the reaction mixture. Concentrations were verified by measuring absorbance in an ultraviolet spectrophotometer at 242, 292, and 325 nm for NH₂Cl, HOCl, and TaurNHCl, respectively. NH₂Cl concentration ([NH₂Cl]), HOCl concentration, and TaurNHCl concentration were then quantified using molar extinction coefficients reported previously (76). Solutions containing chloramines did not include and were not mixed with solutions containing DMSO to avoid direct consumption of oxidants that has been reported previously (65). We also performed in vitro studies indicating that DMSO can consume chloramines and, at high concentration (>1:100 vol/vol), interfere with measurements of Zn²⁺ in solution.

Cryopreservation. Anesthetic and procedures for killing used in these experiments were approved according to policies of Harvard Medical School. Male Sprague-Dawley rats (Charles River Laboratories) weighing ~300 g were anesthetized and underwent laparotomy. The distal colon was identified, and a 1- to 2-cm segment was removed.

The viscus was opened and then rinsed in cold Ringer solution. As described previously (13), the colon was incubated in a Ca²⁺-free Ringer solution containing 1 mM EGTA, 5 mM Na₂HPO₄, and 0.1% BSA for 30 min at room temperature. After chelation, the tissue was manually shaken to liberate the crypts. The tissue was removed, and the solution was centrifuged at 200 rpm for 2 min. The supernatant was removed, and the pellet was resuspended in Ringer solution.

Dye loading: imaging and ratiometric measurements. Fura 2-AM and magfura 2-AM (Molecular Probes, Eugene, OR) were diluted in DMSO to a stock concentration of 1 mM. Suspended in 1.5 ml Eppendorf tubes, crypts were loaded in the dark at room temperature. After chelation, the tissue was manually shaken to liberate the crypts. The tissue was removed, and the solution was centrifuged at 200 rpm for 2 min. The supernatant was removed, and the pellet was resuspended in Ringer solution.

Studies were performed using monochromator-based excitation (Applied Scientific Instrumentation, Eugene, OR). Emitted light was collected at 520 ± 15 nm. During studies with fura 2, fluorescence was monitored by alternating excitation at 340 and 380 nm. Data are presented as a ratio of cation-sensitive intensity at 340 nm over cation-insensitive intensity at 380 nm. For studies utilizing fura 3, crypts were excited at 488 nm. Digital images of crypts were captured using a digital CCD camera (Hamamatsu ORCA-ER). Images were processed using compatible software (Universal Imaging, Downingtown, PA) to yield background-corrected pseudocolor images. Images were acquired every 10 s to minimize photobleaching. Contributions of autofluorescence were measured and were negligible.

Data collection, analysis, and statistics. Simultaneous fluorescence measurements from a whole crypt were obtained, since signals from individual cells could not be discerned at this level of magnification. It has been reported previously that the cells of the crypt function as a unit and thus that signals in individual cells are reflected in regional measurements. Regions of interest selected for analysis included the base of the crypt (the region of cells closest to the submucosa) and the apex (the region closest to the lumen). Although responses were more robust in basal regions, no clear differences emerged between regions in responses to ionophores or to NH₄Cl. Thus results are reported for signals collected from the entire crypt.

Concentrations of extracellular Ca²⁺ in calibration solutions containing TPEN and EGTA were calculated using the internet-based program maxchelator (http://www.stanford.edu/%7Epatton/webmaxes.htm). Dissociation constants (K₅) for fura 2 and fluozin 3 were calculated using the equation of Grynkiewicz et al. (26) formulated for both intensity-based and ratiometric dyes (http://www.molecularprobes/html). Data were summarized and reported as means ± SE. Comparisons were performed using ANOVA for multiple or sequential comparisons, as indicated.

RESULTS

Monitoring [Ca²⁺], in isolated colon crypts and controlling for interfering heavy metal divalent cations. Previous reports have demonstrated the feasibility of using dyes in the fura 2 and fluo 3 classes to monitor [Ca²⁺], levels in colon crypts in response to physiological stimuli (8, 40, 41). These approaches depend on assumptions (54) that have not been reported for primary cell preparations such as the colon crypt. Shown in Fig. 1, A and B, are digital images of a colon crypt loaded with fura 2-AM, recorded in visible light and during fluorescence...
excitation at 380 nm. The first assumption is that the dye is localized almost completely within the cytoplasm. Fluorescence intensity was monitored in crypts loaded with fura 2 at 340 and 380 nm, before and after exposure of crypts to 10 μM digitonin in ICB, an agent that permeabilizes the cell membrane without disturbing the integrity of organelles (31, 54). In five separate experiments, digitonin induced a marked decrease in fluorescence at both wavelengths, in all cases over 85%, indicating that fluorescence signals are the result of dye localized in the cytoplasm.

The second assumption of such fluorescence-based methods is that contributions of other interfering cations are negligible or can be controlled. All useful reporters respond to multiple divalent cations, although with different profiles of response (1, 26). In this regard, published reports suggest that, in cell-free systems, fura 2 increases fluorescence in response to Ca2+ (Kd ~145 nM), Zn2+ (Kd ~3 nM), and other metals not likely to interfere with fluorescence measurements, such as Cd2+ (Kd ~1 μM) and Co2+ (Kd ~9 nM; see Ref. 27). Responses to physiologically significant metals such as Cu2+ and Fe2+ are recognized but either cause quenching of signals or elicit responses opposite to those of Ca2+ and Zn2+ (1, 27, 38). However, release of such cations from intracellular pools or subcellular compartments could interfere with measurements of Ca2+ concentration ([Ca2+]i) when crypts are exposed to potentially toxic agents.

To evaluate such interference, we measured fura 2 signals during exposure of crypts to ionomycin, a cationophore able to translocate both Ca2+ and heavy metal divalent cations (15). In preliminary studies, crypts were exposed to 5 μM ionomycin in the presence of no added Ca2+ and 0.5 mM EGTA ([Ca2+]i ~1 nM). Exposure to ionomycin rapidly increased the excitation ratio, indicating release of divalent cations to the cytoplasm. In six experiments, the interval and magnitude of recovery to baseline was highly variable. Such a response is characteristic of binding of the dye to a ligand of higher affinity, for example, Zn2+.

To evaluate this possibility, we monitored responses to ionomycin and varying [Ca2+]i in the presence of TPEN, a known chelator for Zn2+ (Kd ~10^-15 M) and other metal divalent cations (1). TPEN also exhibits a very low affinity for Ca2+ (Kd ~100 μM; see Refs. 1 and 21), thereby screening out contributions of interfering metal cations while permitting fura 2 to respond to cytoplasm Ca2+ signals in the physiological range (100 nM-1 μM; see Ref. 29). As shown in Fig. 2A, the presence of 20 μM TPEN permitted direct correlation of fura 2 signals to [Ca2+]i. We found the greatest responsiveness in the range of 100–400 μM, with diminished responsiveness both above and below this range. As shown in Fig. 2B, the responses within this range provide a method for in situ calibration that controls for contributions from interfering metal polyvalent cations. Inserting the data in the relationship of Grynkiewicz et al. (26), we calculate that, in situ in the colonic crypt, the Kd of fura 2 for Ca2+ is 282 ± 32 nM (average ± SE, n = 9). This value is somewhat higher than those reported previously in cultured cells but quite close to those reported for primary epithelial cell preparations such as the gastric gland (26, 27, 54). Using similar methods in studies of five isolated colon crypts, we determined that the in situ Kd of fura 2 for Zn2+ is 4.7 ± 0.6 nM (average ± SE), quite close to reported values (27). In these studies (n = 5 crypts), fura 2-loaded crypts were exposed to 40 μM pyrithione, a heavy metal specific ionophore, in 0 Ca2+ Ringer with varying concentrations of Zn2+ (0, 2.5, 5, 7.5, 10, and 1,000 nM). Experiments were also undertaken to ensure that pyrithione does not transport Ca2+ in the cytoplasm from either extracellular solution or from the cellular organelles (data not shown).

We next performed studies to evaluate whether the presence of TPEN might interfere with the ability of fura 2 to monitor physiological increases in [Ca2+]i. Crypts were perfused with Ringer solutions under control conditions and then during exposure to a combination of 100 μM CCh and 1 μM THPS, agents that cause Ca2+ release from intracellular stores and prevent reuptake, thereby maximizing accumulation in the cytoplasm (29–31). In addition, irreversible release of intracellular stores activates capacitative entry of Ca2+ from the extracellular spaces to the cytoplasm, resulting in irreversible accumulation of Ca2+ beyond physiological tolerable limits. As shown in Fig. 3, the presence of TPEN did not significantly alter [Ca2+]i signals elicited by CCh/THPS or during capacitative entry. Together, these studies indicate that TPEN may be used to screen out contributions of other heavy metal cations while preserving the intracellular Ca2+ signals that are monitored by fura 2.

**Fig. 2.** Fura 2 signals in EGTA-isolated colon crypts during incremental increases in Ca2+ concentration ([Ca2+]i). Crypts were exposed to [Ca2+]i varying from 100 to 400 nM in the presence of ionomycin (10 μM) and N.N’,N’-tetrakis(2-pyridyl-methyl) (TPEN, 20 μM). A: recording of fura 2 fluorescence in an individual crypt. Individual data points collected every 10 s. B: summary of calibration curves in 9 crypts using the same protocol as in A. Results are expressed as mean ± SE fluorescence excitation ratios (340/380 nm). With the use of averaged values in B, Kd of fura 2 for Ca2+ is 282 ± 32 nM.
Effects of NH2Cl on [Ca2+], in isolated colon crypts. To evaluate the effects of NH2Cl, we initially exposed isolated colon crypts to solutions containing [NH2Cl] at 50, 100, and 200 μM. As summarized in Fig. 4, A and B, dose-dependent and largely irreversible increases were observed in the fluorescence excitation ratio. These basic observations led us to perform studies to determine the sources of these signals, including the contributions of 1) extracellular Ca2+, 2) intracellular pools of non-Ca2+ heavy metal divalent cations, and 3) physiologically regulated intracellular Ca2+ stores.

In the first set of studies, Ca2+ was removed from the perfusates, to which 0.5 mM EGTA was added to lower [Ca2+] to −1 nM. Crypts were exposed to different concentrations of NH2Cl [0 (control), 50, 100, and 200 μM]. After a 5-min exposure, NH2Cl was withdrawn. As shown in Fig. 5A, the excitation ratio decreased when Ca2+ was removed from the perfusate, confirming that extracellular Ca2+ plays an important role in preserving [Ca2+], under baseline conditions (17). During exposure to NH2Cl, the signal increased dose dependently to a plateau but then partially reversed when NH2Cl was withdrawn. The peak effects were greatly diminished compared with those observed in the presence of extracellular Ca2+ (Fig. 4), indicating that a significant component of the response to NH2Cl is because of influx of extracellular Ca2+.

In the second set of studies, we monitored fura 2 signals in crypts exposed to NH2Cl in the presence of TPEN. Crypts were first exposed to 0 Ca2+-Ringer and 20 μM TPEN to eliminate contributions from extracellular Ca2+ and to chelate labile metal cations present in intracellular pools, respectively. Crypts were then exposed to 200 μM NH2Cl for 5 min, allowed to recover in 0 Ca2+-Ringer, and then exposed to standard Ca2+-Ringer. As shown in Fig. 6, preexposure of crypts to TPEN significantly reduced and delayed the peak effects of NH2Cl, as summarized in Fig. 5B, C, and D, for each of the three NH2Cl concentrations (50, 100, and 200 μM). Each line represents averaged responses to NH2Cl at different doses [0 (control), 50, 100, and 200 μM].
NH₂Cl-induced signal is the result of heavy metal divalent are significantly different (P < 0.01 by linear regression). Note the exaggerated response to restoration of standard Ringer with physiological [Ca²⁺] and lack of inhibition of extracellular calcium entry by TPEN.

Effect of NH₂Cl, indicating that a significant component of the NH₂Cl-induced signal is the result of heavy metal divalent cations. To further characterize the sensitivity to TPEN, crypts were exposed to NH₂Cl and then TPEN at the peak of the response. As shown in Fig. 7, exposure to TPEN at the peak response to NH₂Cl caused a significantly more rapid dissipation in the signal. These findings indicate that TPEN chelates labile metal divalent cations released by NH₂Cl and confirm that the fura 2 response is the result of release of both Ca²⁺ and non-Ca²⁺ metal divalent cations from within the cell.

We then performed studies to determine whether intracellular pools of Ca²⁺ released by NH₂Cl might include physiologically regulated intracellular stores. To deplete these stores, crypts were perfused in 0 Ca²⁺ Ringer solution alone or containing, in addition, 100 μM CCh and 1 μM THPS. Crypts were then exposed to 200 μM NH₂Cl. As shown in Fig. 8, there was a significant decrease in the response to NH₂Cl in crypts pretreated with CCh/THPS. These findings suggest that, in the absence of extracellular Ca²⁺, release from intracellular stores is responsible for a component of the fura 2 signal achieved during exposure to NH₂Cl. In further studies, we first depleted the intracellular stores by exposing crypts to CCh/THPS and then monitored the response to NH₂Cl in the presence or absence of TPEN. As also shown in Fig. 8, the response to NH₂Cl was nearly abolished when TPEN was present. These findings indicate that, after elimination of extracellular Ca²⁺, the fura 2 signal in response to NH₂Cl can be attributed largely to release from THPS-sensitive intracellular Ca²⁺ stores and intracellular pools of heavy metal cations that are sensitive to TPEN.

Studies were performed to more fully characterize NH₂Cl-induced disturbances in Ca²⁺ homeostasis, using 2-APB, a recognized inhibitor of store-operated channels. It has been reported that, under some experimental conditions, 2-APB may inhibit inositol trisphosphate receptor (InsP₃) actions, including release of intracellular stores. When such stores have been depleted, however, 2-APB has also been shown to block capacitative entry, independent of its effects on InsP₃ receptors (23, 43, 61). In these studies, fura 2-loaded crypts were exposed to NH₂Cl (200 μM) in Ca²⁺-free Ringer containing TPEN (20 μM) to monitor an uncontaminated Ca²⁺ signal. After NH₂Cl-induced release of Ca²⁺ from intracellular stores, crypts were exposed to Ca²⁺-Ringer (containing ~20 μM free TPEN) alone or, in addition, 100 μM 2-APB. A representative recording is shown in Fig. 9A, comparing responses of glands from the same harvest. In both recordings, peak effects and time course in response to NH₂Cl (in Ca²⁺-free Ringer) and restoration of extracellular Ca²⁺ were similar to those observed previously in the presence of TPEN (Fig. 6). In the presence of 2-APB, added after store depletion by NH₂Cl, the increases in [Ca²⁺]; in response to restoration of extracellular Ca²⁺ were reduced to levels expected in the absence of store-operated entry. These findings are summarized for the comparison in Fig. 9B, indicating that 2-APB-induced reductions in [Ca²⁺], were significant (n = 7 crypts in control and 2-APB groups).
Measurements of intracellular Zn$^{2+}$ concentration in isolated colon crypts using fluozin 3. To more conclusively identify the TPEN-sensitive component of the divalent cation signal, we performed studies in isolated crypts loaded with a recently reported Zn$^{2+}$-sensitive fluorophore, fluozin 3 (27). This fluorophore has high affinity for Zn$^{2+}$ (reported $K_d \approx 10^{-20}$ nM) and little expected interference from physiological concentrations of Ca$^{2+}$ (20, 64). Upon excitation, as shown in Fig. 10A, loading appears similar to that observed with fura 2. However, little has been determined about the characteristics of fluozin 3 loading. To verify that the majority of the dye was loading in the cytoplasm and not organelles, experiments similar to those performed on fura 2-loaded crypts were undertaken to monitor cytoplasmic loss of dye during exposure to digitonin. In four separate experiments, crypts were loaded with 5 μM fluozin 3 and 5 μM magfura 2 simultaneously (data not shown). Fluorescence signals were monitored from both reporters by exciting fluozin 3 at 488 nm and magfura 2 at 340/380 nm. In these studies, the simultaneous presence of magfura 2 ensured permeabilization (36) and allowed us to compare the rates at which both reporters were lost during the permeabilization. Crypts were perfused with ICB containing 5 nM free Zn$^{2+}$ and 40 μM pyrithione to increase the baseline signal intensity of fluozin 3. Digitonin (10 μM) was then added to induce the leakage of cytoplasmic dye. In each case, the intensity of fluozin 3 decreased by at least...
85% after addition of digitonin, and the decline rates of signals were not visibly different (data not shown).

In preliminary studies (n = 4), we observed that when crypts were exposed to Ringer solutions containing ionomycin (10 μM), TPEN (20 μM), and [Ca2+]i (up to 1 μM), fluorescence signals were not altered, confirming that fluozin 3 signals are not influenced by increases in [Ca2+]i within the physiological range. Responses were then monitored during exposure of fluozin 3-loaded crypts to varying concentrations of Zn2+ (0 nM, 2.5 nM, 5 nM, 7.5 nM, 10 nM, and 1 μM) in the presence of pyrithione (40 μM), a cationophore that is highly selective of non-Ca2+ metals such as Zn2+ and Fe2+ (75, 78). As shown in the recording in Fig. 10B and the summary of eight crypts in Fig. 10C, incremental increases in fluorescence were observed during increases in intracellular Zn2+ concentration ([Zn2+]i). Based on these studies, we calculate that in situ the Kd of fluozin 3 for Zn2+ is 4 ± 0.3 nM.

Effects of NH2Cl on [Zn2+]i in isolated colon crypts. To evaluate the effects of NH2Cl, we exposed isolated colon crypts to solutions containing [NH4Cl] of 50, 100, and 200 μM. As shown in Fig. 11A, rapid increases were observed in response to NH2Cl, indicating that exposure to NH2Cl causes a marked release of intracellular pools of Zn2+ within the colonocyte. The addition of pyrithione in a Zn2+-free solution and in a 1-μM Zn2+ solution shows the ability of the ionophore to remove Zn2+ and the maximum signal capable under these experimental procedures, respectively. These signals were similarly quenched when TPEN was added to the perfusate (n = 4 experiments, data not shown), providing additional assurance that the signals were attributable to increases in [Zn2+]i. As shown in Fig. 11B, these effects were dose dependent. Based on calibration responses shown in Fig. 10C, it appears that labile [Zn2+]i may increase to as much as 4–6 nM during exposure to 200 μM NH2Cl.

Effects of NH2Cl reactants, membrane-impermeable chloramines, and other control studies of reagents. In a series of control studies, we monitored fura 2 and fluozin 3 signals in isolated crypts during exposure to NH3 (20 mM) and HOCl (200 μM), the reactants used to produce NH2Cl. At these concentrations, no changes in signal were observed for either NH3 (n = 4) or HOCl (n = 4), indicating that observed effects were the result of NH2Cl and not its reactants (data not shown). Similarly, crypts were exposed to 200 μM taurine-NH2Cl, a stable, but membrane-impermeant, chloramine species (24, 62). No alterations were observed in fura 2 or fluozin 3 signals (data not shown), indicating that the effects of NH2Cl on [Ca2+]i and [Zn2+]i homeostasis are the result of its permeability in the cell membrane.

Additional control studies were performed to evaluate the possibility that NH2Cl or its reactants might directly alter fluorescence properties of fura 2 or fluozin 3. In a cell-free chamber, each dye was placed in its free acid form, in the presence of EGTA-buffered solutions containing either 500 nM Ca2+ and 20 μM fura 2 or 2 nM Zn2+ and 20 μM fluozin 3. Addition of NH2Cl, HOCl, or NH3 all failed to alter baseline levels of fluorescence at the individual exciting wavelengths (340 and 380 nm for fura 2; 488 nm for fluozin 3). We also found no response to 100 μM peroxide, a precursor used by the neutrophil to generate HOCl. In addition, these reagents did not interfere with changes in fluorescence caused by doubling of divalent cation concentrations (data not shown).

We also performed studies to determine whether such effects might be observed in response to other recognized thiol oxidants, such as thimerosal (5, 45) and nitric oxide donors SNAP or 3-morpholinosydnonimine N-ethylcarbamide (SIN-1; see Refs. 18 and 19). In fura 2-loaded crypts (n = 4) exposed to thimerosal (100 μM), no responses were observed. In crypts exposed to the NO donor SNAP (1 mM), small but significant increases in fura 2 fluorescence were observed (n = 5 crypts, fura 2 excitation ratio 340/380 increasing from 1.00 ± 0.01 to 1.25 ± 0.05, P < 0.005; Fig. 12). Lower doses of SNAP (100 μM, n = 5) had no appreciable effect. These increases were not abolished in the presence of TPEN (n = 3 crypts). In addition, when fluozin 3-loaded crypts were exposed to SNAP (1 mM), little or no increase in fluorescence was observed. These findings indicate that SNAP-induced increases in the

![Fig. 12. Fura 2 fluorescence recording from an individual fura 2-loaded crypt exposed to the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP).](http://ajpgi.physiology.org/ by 10.220.33.1 on June 24, 2017)
concentration of NH₂Cl did not decrease in the presence of
of studies (22, 42, 46, 48, 49, 59, 77). In one set
clusters that have been associated with proteins that bind or
Ca²⁺ was added after a peak effect was seen during NH₂Cl exposure.
Ca²⁺ and Zn²⁺ are not observed uniformly in response to all
thiol oxidants.

**Intracellular thiols as targets of NH₃Cl oxidation.** We performed
studies to confirm that NH₃Cl-induced alterations in
Ca²⁺ and Zn²⁺ homeostasis were because of release from thiol
clusters that have been associated with proteins that bind or
transport divalent cations (22, 42, 46, 48, 49, 59, 77). In one set
of studies (n = 4), we pretreated crypts with DTT (1 mM), a
known sulfhydryl reducing agent (47, 62), before exposure to
200 μM NH₃Cl. As shown in Fig. 13, as long as DTT was
present in the perfusate, exposure to NH₃Cl did not elicit
alterations in fura 2 signals. When DTT was removed, how-
ever, responses to NH₃Cl were immediately apparent. In other
studies (n = 4), DTT reversed the effects of NH₃Cl when it
was added after a peak effect was seen during NH₃Cl exposure.
To determine whether the presence of DTT might alter the
concentration of NH₃Cl in solution, we mixed 200 μM NH₃Cl
and 1 mM DTT in a Ringer solution in a cell-free chamber. Measuring NH₃Cl absorbance at 242 nm, we found that the
concentration of NH₃Cl did not decrease in the presence of
DTT, indicating that the effects DTT were not attributable to
consumption of NH₃Cl.

In an additional set of control studies, we monitored the
ability of DTT to chelate Zn²⁺ or Ca²⁺. Using fluozin 3 (K₅ for
Zn²⁺ ~3 nM) and fluozin 2 (K₅ for Zn²⁺ ~1–2 μM; see Ref.
27), we monitored fluorescence (excitation 495 nm, emission
520 nm) of both reporters in cell-free KCl solutions (150 mM)
containing standard total concentrations of Zn²⁺ using a 96-
well plate fluorimeter. When fluozin 3 was present as the
reporter, concentrations were calculated (maxchelator) to pro-
vide free Zn²⁺ concentration ([Zn²⁺]) of 0.25 nM, 0.5 nM, 1.0
nM, 2.0 nM, 4.0 nM, 8.0 nM, and 16 μM if 500 μM EGTA
reported K₅ for Zn²⁺ ~1.24 nM) was present as a chelator/buffer. In other experiments, when fluozin 2 was used as the
reporter, concentrations were calculated (maxchelator) to pro-
vide free [Zn²⁺] of 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 16 μM if
1 mM citrate (reported K₅ for Zn²⁺ 17 μM) were present as a
chelator/buffer. Standard curves using each reporter/chelator
combination (fluozin 3/EGTA or fluozin 2/citrate) were gen-
erated, and fluorescence quenching of signals was compared
when 1 mM DTT was used to replace the chelator for each
reporter system. For two individual experiments, the relation-
ship of Grinkiewicz et al. permitted us to calculate that the
K₅ of DTT for Zn²⁺ was 9.6 and 13.5 μM, well above any levels
that might interfere with fluozin 3 measurements or that might
account for the effects of DTT on [Zn²⁺] in the nanomolar
range. In similar cell-free studies, the influence of DTT on
Ca²⁺ content of solutions was negligible, indicating that ob-
served effects of DTT on NH₃Cl-induced signals were not
attributable to chelation of divalent cations.

**DISCUSSION**

To our knowledge, measurements of [Zn²⁺], have not been
reported for epithelial cells of the colon mucosa. Moreover,
previous reports utilizing fluorescence-based measurements of
[Ca²⁺], in colonocytes have not taken into account contribu-
tions of other divalent cations, such as Zn²⁺. To monitor
isolated changes in [Ca²⁺], in the isolated colon crypt, we used
a well-recognized, ratiometric fluorescent reporter, fura 2. In
the presence of TPEN, which chelates non-Ca²⁺ metal divalent
cations without interfering with levels of [Ca²⁺], fura 2 would
monitor “pure” Ca²⁺ signaling responses to neurohumoral or
pathological stimuli. To monitor changes in [Zn²⁺], directly,
we used fluozin 3, a dye recently reported to be useful for
measurements in the subnanomolar to nanomolar range.

Our studies indicate that NH₃Cl causes dose-dependent
increases in [Ca²⁺], in native colon crypts of the rat. Further
studies indicate that NH₃Cl-induced accumulation of Ca²⁺ in
the cytoplasm is the result of release from intracellular stores,
as well as store-operated entry from extracellular fluid. The
duration of these increases is sustained beyond those associated
with physiological stimuli (8, 41), but are partly reversible,
even during exposure to NH₃Cl concentrations of up to 200
μM. We also noted that it was technically feasible to charac-
terize these responses only when contributions of other diva-
cient cations were screened out using the heavy metal chelator
TPEN. In the presence of 20 μM TPEN, the increases in
[Ca²⁺], are in the range of concentrations that are reported as
physiological signals (11, 17, 41, 54). A comparison between
responses to physiological agonists (Fig. 3) and to NH₃Cl (Fig.

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**Fig. 13.** Thiol reduction as a means of preventing or reversing NH₃Cl effects. A: recording in an individual crypt exposed to 200 μM NH₃Cl in 0 Ca²⁺ Ringer already containing 1 mM dithiothreitol (DTT). DTT was then removed with NH₃Cl remaining present. The crypt was allowed to recover in 0 Ca²⁺ Ringer, followed by addition of standard Ringer. B: recording in an individual crypt exposed to 1 mM DTT after initiation of responses to 200 μM NH₃Cl in 0 Ca²⁺ Ringer. NH₃Cl was later removed with DTT remaining present. Again, the crypt was allowed to recover in 0 Ca²⁺ Ringer, followed by addition of standard Ringer.
6) indicates that, when contributions of other divalent cations are taken into account, the responses (perhaps 200–300 nM above baseline) lie within the range expected in response to neurohumoral stimuli.

We also found that exposure of crypts to NH2Cl leads to dose-dependent increases in $[\text{Zn}^{2+}]$, in the range of 3–5 nM, which are sustained and not easily reversed. These effects on $[\text{Ca}^{2+}]$ and $[\text{Zn}^{2+}]$, are prevented by pretreatment with DTT, which shields intracellular thiol groups from oxidation by chlorinated oxidants. In addition, these effects are partially reversed by exposure to DTT, effects not attributable to chelation of $\text{Ca}^{2+}$ or $\text{Zn}^{2+}$. These findings indicate that thiol groups in $\text{Ca}^{2+}$- and $\text{Zn}^{2+}$-handling proteins are susceptible to oxidation by chloramines, leading to sustained increases in $[\text{Ca}^{2+}]$, and $[\text{Zn}^{2+}]$. These findings offer the possibility that there is a range of concentrations over which NH2Cl may not be toxic but instead stimulates release of physiological signals of oxidant stress in the colonocyte.

These studies raise three issues for discussion. The first issue is a technical one, namely, the strengths and limitations of fluorescence methods used to monitor intracellular divalent cation signals. $\text{Ca}^{2+}$-sensing fluorescent indicator dyes all are responsive to other polyvalent cations. Some potentially interfering ions (such as $\text{Cd}^{2+}$, $\text{Ba}^{2+}$, and $\text{La}^{3+}$) are excitatory (i.e., elicit responses similar to $\text{Ca}^{2+}$) but are not expected to interfere because they can only be added exogenously. In addition, some toxic cations (such as $\text{Mn}^{2+}$, $\text{Ca}^{2+}$, and $\text{Pb}^{2+}$) cause quenching or shifts in excitation spectra, rather than amplification of intensities; thus, they do not elicit responses similar to those of $\text{Ca}^{2+}$. Potentially interfering divalent cations include $\text{Fe}^{2+}$, $\text{Fe}^{3+}$, $\text{Cu}^{2+}$, and $\text{Zn}^{2+}$, which might be released from intracellular pools. The most widely used fluorescent reporter, fura 2, is quenched in response to $\text{Cu}^{2+}$, $\text{Fe}^{2+}$, and $\text{Fe}^{3+}$, but it responds to $\text{Zn}^{2+}$ in a similar manner to $\text{Ca}^{2+}$ (38). In fact, previous reports and manufacturer specifications indicate that, in vitro, fura 2 is more responsive to $\text{Zn}^{2+}$ ($K_d \approx 3–15$ nM) than to $\text{Ca}^{2+}$ ($K_d \approx 150–300$ nM; see Refs. 27, 38, and 54). Our calibration studies confirm these ranges of sensitivities to $\text{Ca}^{2+}$ and $\text{Zn}^{2+}$ in situ in cells of the rat colon crypt. These considerations suggest that, when fura 2 is used to monitor changes in $[\text{Ca}^{2+}]$, signals may include intracellular accumulation of $\text{Zn}^{2+}$ in the nanomolar range.

Our studies demonstrate that fura 2 monitors increases in $[\text{Zn}^{2+}]$, under two sets of conditions. First, it appears that $\text{Zn}^{2+}$ accumulates in the cytoplasm during exposure of the crypt to ionomycin, an ionophore frequently used to release $\text{Ca}^{2+}$ from intracellular stores or to equilibrate $\text{Ca}^{2+}$ in the cytoplasm with that in the extracellular fluid. Second, we observed release of $\text{Zn}^{2+}$ during exposure to NH2Cl, an oxidant that preferentially targets proteins containing thiolate clusters that bind or sequester heavy metal divalent cations. In both conditions, there are three considerations on which we base the conclusion that $\text{Zn}^{2+}$ is the interfering ion. First, of the likely contaminating heavy metals, only $\text{Zn}^{2+}$ elicits fluorescence responses similar to those of $\text{Ca}^{2+}$. Second, during exposure of crypts to ionomycin or NH2Cl, fura 2 signals are reduced in the presence of TPEN, a chelator with profound affinity for $\text{Zn}^{2+}$ ($K_d \approx 10^{-15}$ M) and other heavy metals but not $\text{Ca}^{2+}$ or $\text{Mg}^{2+}$. Third, exposure to ionomycin and NH2Cl led to increases in fluorescence in crypts loaded with fluozin 3, a reporter with a high degree of preference for $\text{Zn}^{2+}$ at nanomolar concentrations and virtual insensitivity to $\text{Ca}^{2+}$ in the submicromolar range. Our studies do not necessarily exclude release of other divalent cations (for example, $\text{Fe}^{2+}$, $\text{Fe}^{3+}$, or $\text{Cu}^{2+}$) during exposure to ionomycin or NH2Cl. However, they would argue that the release of $\text{Zn}^{2+}$, which excites fura 2 fluorescence with high affinity, outweighs the effects of other metal polyvalent cations that would tend to quench fura 2 signals. Our findings also send a fundamental message that, in using fluorescent reporters to explore changes in $[\text{Ca}^{2+}]$, during exposure to oxidants, toxins, and relatively uncharacterized neurohumoral agonists, it is important to control or take into account accumulation of interfering polyvalent cations.

The second issue raised by these studies involves the mechanisms by which NH2Cl causes accumulation of $\text{Ca}^{2+}$ and $\text{Zn}^{2+}$ within the cytoplasm of the colon crypt cell. With respect to $[\text{Ca}^{2+}]$, our studies indicate that NH2Cl-induced accumulations are the result of release of $\text{Ca}^{2+}$ from intracellular stores and influx from the extracellular microenvironment. When crypts were perfused with $0 \text{Ca}^{2+}$ Ringer and pretreated with CCh/THPS, exposure to NH2Cl diminished the increases in $[\text{Ca}^{2+}]$, implying that THPS-sensitive stores are the major intracellular sources for the $\text{Ca}^{2+}$ signal. These responses are attributable to thiol modification of $\text{Ca}^{2+}$ transport processes, since DTT prevented and reversed effects of NH2Cl on fura 2 signals.

The molecular basis for oxidant-induced release of $\text{Ca}^{2+}$ from intracellular stores is not clear. In addition to direct modification of $\text{Ca}^{2+}$ release channels in the membrane of the endoplasmic reticulum (16, 77), thiol oxidants might alter receptors regulating release of $\text{Ca}^{2+}$ from stores to the cytoplasm. In this regard, both InsP3 receptors (44) and ryanodine receptors (37) have been identified in colon crypts, the former having also been shown to support $\text{Cu}^{2+}$-dependent anion secretion (37). In other tissues, functional modification of either receptor has been demonstrated after exposure to thiol oxidants (14, 45), the latter in response to chloramines (14). These considerations suggest that NH2Cl may find a broad spectrum of targets in the apparatus that regulates store emptying. Future studies, beyond the scope of the current report, may thus provide insight into the role that thiol redox state plays in regulating both store emptying and refilling.

Subsequent responses to solutions containing physiological levels of $\text{Ca}^{2+}$, however, make it clear that entry of extracellular $\text{Ca}^{2+}$ is responsible for a major component of the NH2Cl-induced signal (Figs. 6 and 7). Entry of extracellular $\text{Ca}^{2+}$ might be because of influx through capacitative entry, activated when CCh- and THPS-sensitive stores are released (29–31). Alternatively, NH2Cl-induced entry of extracellular $\text{Ca}^{2+}$ might be independent of store emptying. In the present study, we found that 2-APB inhibits a significant component of NH2Cl-stimulated entry of extracellular $\text{Ca}^{2+}$, arguing that NH2Cl elicits its effects on extracellular influx through its effects on store depletion. It should be acknowledged, however, that, in the crypt configuration, it is difficult to directly evaluate the linkage between NH2Cl-induced store emptying and influx of extracellular $\text{Ca}^{2+}$. It is possible that exposure to NH2Cl may disengage store emptying and the “capacitative” entry that normally leads to store refilling. In cultured cell models, this separation of function (store release and store refilling) has been observed as a result of thiol modification through $\text{S}$-nitrosylation or alkylation (16, 42). Alternatively,
exposure to NH₂Cl might modify Ca²⁺ entry mechanisms that are independent of store emptying; in such cases, thiol modification has not been implicated (77). Further studies using reporters of intracellular stores may provide insight into these different possibilities (29–31). At the very least, our studies indicate that influx of extracellular Ca²⁺ supplies a major component of the sustained increase in [Ca²⁺], caused by NH₂Cl. It seems likely that the activation of such influx is at least partially independent of the effects of NH₂Cl on emptying of intracellular Ca²⁺ stores and is the result of thiol modification.

With respect to increases in [Zn²⁺], release of Zn²⁺ might be observed from specific Zn²⁺-enriched subcellular compartments or from cytoplasm pools of Zn²⁺-binding proteins that are susceptible to thiol modification. Subcellular compartments enriched in labile Zn²⁺ include acidic secretory compartments (20, 21) and under some conditions mitochondria (69). Cytoplasm pools of Zn²⁺-binding proteins include metallothioneins, glutathione, heat shock proteins, and other peptides enriched in cysteine or methionine (32, 46, 48, 49). A number of studies have indicated that Zn²⁺ may be released by accumulation of oxidants that tend to attack thiol clusters in metallothioneins (32, 49). Currently, very little is reported on oxidation of oxidants that preferentially target S-H groups, may be highly effective in eliciting these responses that are independent of store emptying; in such cases, thiol modification has not been implicated (77). Further studies using reporters of intracellular stores may provide insight into these different possibilities (29–31). At the very least, our studies indicate that influx of extracellular Ca²⁺ supplies a major component of the sustained increase in [Ca²⁺], caused by NH₂Cl. It seems likely that the activation of such influx is at least partially independent of the effects of NH₂Cl on emptying of intracellular Ca²⁺ stores and is the result of thiol modification.

In summary, we have adapted fluorometric approaches for monitoring changes in [Ca²⁺] and [Zn²⁺], in isolated crypts of the rat colon during exposure to oxidant stress. Our studies indicate that experimental approaches for monitoring [Ca²⁺], should control or take into account contributions from interfering polynuclear cations. When these factors are taken into account, it appears that NH₂Cl elicits increases in [Ca²⁺], that are sustained but certainly not much higher than those expected from normal signaling processes. These increases reflect contributions from emptying of physiologically regulated intracellular stores, as well as entry of extracellular Ca²⁺, most likely through store-operated channels. In addition, increases in [Zn²⁺], are observed in response to NH₂Cl and probably reflect release from cytoplasm pools of metal-binding proteins such as metallothionein. Increases in [Zn²⁺], may thus act as signals of oxidative stress, potentially activating downstream responses that are protective and anti-apoptotic. Further studies will determine the role played by these signaling responses to unique thiol-directed oxidants such as NH₂Cl in injury and protection in acute colitis.

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**REFERENCES**


G260 OXIDATIVE STRESS-INDUCED Ca2+ AND Zn2+ SIGNALS


