

Ca-mediated Stimulation of Cl Secretion by Reactive Oxygen Metabolites in Human Colonic T84 Cells

Hiroshi Tamai, Timothy S. Gaginella, James F. Kachur, Mark W. Musch,* and Eugene B. Chang*

Gastrointestinal Disease Research, Searle Research and Development, Skokie, Illinois 60077; and *Department of Medicine, Gastroenterology Section, The University of Chicago, Chicago, Illinois 60637

Abstract

Monochloramine (NH_2Cl), a granulocyte-derived reactive oxygen metabolite (ROM), increases short-circuit current (Isc) in cultured T84 monolayers in a concentration-dependent manner up to nonlethal concentrations of 75 μM . Isc increases slowly after NH_2Cl , reaching a peak value of $18 \pm 2 \mu\text{A}/\text{cm}^2$ 20 min after addition. The Isc changes are persistent (lasting over 20–30 min), depend on medium Cl, and are inhibitable with bumetanide. ^{36}Cl flux studies demonstrated that NH_2Cl increases serosa-to-mucosa flux of Cl without changing mucosa-to-serosa flux, consistent with stimulation of electrogenic Cl secretion. Isc responses to NH_2Cl , but not PGE_2 , are dependent on medium calcium. As demonstrated in fura-2-loaded T84 cells, NH_2Cl increases free cytosolic calcium by influx of extracellular Ca^{2+} and by release of Ca^{2+} from endogenous stores. However, NH_2Cl had no effect on phosphatidylinositol metabolism or cyclic nucleotide levels. We conclude that ROM directly stimulate electrolyte secretion, an effect in part mediated by increases in cytosolic Ca^{2+} , possibly through increasing Ca^{2+} permeability of cellular membranes. (*J. Clin. Invest.* 1992. 89:301–307.) Key words: Cl transport • oxygen radicals • intracellular calcium

Introduction

Reactive oxygen metabolites (ROM)¹ are major mediators of the inflammatory process and are formed by activated granulocytic as well as phagocytic cells (1). These cells generate superoxide anion and also secrete myeloperoxidase, the former being converted to hydrogen peroxide either enzymatically (by superoxide dismutase) or nonenzymatically. Hydrogen peroxide can then be metabolized by catalase, resulting in the formation of water or to hypochlorous acid (HOCl) in the presence of

myeloperoxidase and Cl. In the presence of ammonia, HOCl is rapidly converted to monochloramine (NH_2Cl). The half lives as well as the oxidative potential of the various ROM differ. Superoxide anion is very short lived whereas hydrogen peroxide, hypochlorous acid, and NH_2Cl are stable. Of these four ROM, hypochlorous acid and monochloramine are more potent oxidants than superoxide and hydrogen peroxide (2).

The effects of ROM on intestinal ion transport have recently received great attention. We, and others, have shown that numerous ROM evoke short-circuit current (Isc) responses in rat colon (2–5), reflecting stimulated Cl secretion. In rat distal colon, H_2O_2 or NH_2Cl stimulate a biphasic secretory response (2–5), i.e., there is a rapid increase in Isc followed by a second more prolonged Isc increase. The early peak can be partially inhibited by atropine (and also by tetrodotoxin [TTX]), suggesting acetylcholine release as the mechanism of action. However, the latter peak is unaffected by either atropine or TTX, suggesting a direct action of NH_2Cl . Thus, ROM appear to stimulate secretion by direct actions on epithelial cells and also by release of neurotransmitters which then stimulate mucosal epithelial cells.

To better define the cellular mechanisms mediating the direct actions of ROM on epithelial ion transport, the Cl-secreting cultured intestinal cell line T84 was studied. T84 cells grow as electrically resistive monolayers on collagen-coated permeable supports and are capable of secreting Cl when stimulated by a variety of secretagogues (6–11). These agents either elevate cGMP such as heat-stable *Escherichia coli* toxin (7), elevate cAMP such as PGE_1 and vasoactive intestinal peptide (VIP) (8, 10), or elevate cytosolic calcium such as carbachol and the calcium ionophore ionomycin (11–13).

In this study, we chose to study the relatively more stable and cell-permeant ROM NH_2Cl which has been shown to be synthesized in inflamed colonic mucosa (14). NH_2Cl , although not extensively studied in this setting, may have a primary role in the pathogenesis of diarrhea and tissue destruction in patients with inflammatory bowel diseases. We demonstrate that NH_2Cl , at noncytotoxic concentrations below 75 μM , is a potent stimulant of electrogenic Cl secretion in T84 cells, an effect mediated by stimulated increases in cytosolic Ca^{2+} .

Methods

Synthesis of NH_2Cl . NH_2Cl was synthesized as previously described (15) by adding 5 ml 40 mM NH_4Cl in 10 mM Na_2HPO_4 (pH 8.0) to 5 ml water, 50 μl NaOCl was added four times waiting 30 s between additions. The concentration of NH_2Cl was determined assuming an extinction coefficient of 429 M/cm at 242 nm. As a vehicle control, 20 mM NH_4Cl in 5 mM Na_2HPO_4 (pH 8.0) was used.

Isc and ^{36}Cl flux measurements. T84 cell stocks and monolayers were grown and maintained as described previously (12) and were between passages 49 and 58. Monolayers were always used 7–8 d after plating. Electrical measurements (transepithelial resistance and Isc)

This work was presented in abstract form at the American Gastroenterological Association Meeting, San Antonio, TX, May 1990.

Address correspondence to Eugene B. Chang, M.D., Department of Medicine, Gastroenterology Section, The University of Chicago, 5841 South Maryland Avenue, Box 400, Chicago, IL 60637.

Received for publication 5 February 1991 and in revised form 6 September 1991.

1. Abbreviations used in this paper: AM, acetoxymethyl ester; IP, inositol phosphates; IP3, inositol-1,4,5-trisphosphate; Isc, short-circuit current; LDH, lactate dehydrogenase; ROM, reactive oxygen metabolites; STa, heat stable *E. coli* enterotoxin; TTX, tetrodotoxin.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/92/01/0301/07 \$2.00

Volume 89, January 1992, 301–307

were made using automatic voltage clamps (World Precision Instruments, Sarasota, FL). The bathing medium used for these studies was a HCO₃⁻-Ringer solution (composition in mmol/liter: 114 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 1.65 NaH₂PO₄, 0.3 NaH₂PO₄, 25 NaHCO₃, and gassed with 5% CO₂ in O₂ to maintain pH at 7.4 at 37°C). Monolayers were short-circuited except when resistance measurements were taken. For ³⁶Cl flux studies, monolayers were mounted in Ussing chambers and ³⁶Cl added 5 min after mounting. Baseline fluxes were measured 15 min later over a period of 30 min, NH₂Cl was then added and fluxes were measured over the next 30 min.

Lactate dehydrogenase (LDH) release. T84 cells were grown in six-well plates and plated at the same density as for monolayer studies (600,000 cells/cm²). Cells generally reached confluence 5 d after plating at this density and for LDH studies were only used at day 8, that is 3 d postconfluence. Cells were left attached to the culture surface and washed twice with HBSS. The monolayers were then stimulated with NH₂Cl at varying concentrations and 30 min later the medium removed. One set of wells was scraped into HBSS and the LDH content measured by the colorimetric assay of Cabaud-Wroblewski (16) in both the homogenate and medium. Cells in another set of wells treated precisely as above were removed with 0.1% (wt/vol) trypsin and 2 mM EDTA in HBSS for 15 min at 37°C and diluted into 0.4% trypan blue to count.

Cyclic nucleotide measurements. Monolayers were mounted in Ussing chambers and stimulated with NH₂Cl. After 15 min the monolayers were removed from the chamber, rapidly (< 15 s) punched from the support ring, placed into ice cold 10% trichloroacetic acid, and processed for cAMP and cGMP as described previously (17). Cyclic nucleotides in the samples were measured by acetylated cyclic nucleotide radioimmunoassays (New England Nuclear, Boston, MA). Proteins in the trichloroacetic acid precipitate were measured by the method of Bradford (18).

Intracellular calcium measurements. For experiments using suspensions, T84 cells were loaded with 10 μM fura-2 acetoxymethylester (AM) for 30 min in media without serum and then rinsed with HBSS (composition in mmol/liter: 137 NaCl, 5 KCl, 0.2 NaH₂PO₄, 0.45 MgSO₄, 10 glucose, 25 Hepes, pH 7.4 with Tris base). The cells were released from the plastic by brief (3 min) treatment with trypsin (0.1% (wt/vol) in HBSS) and resuspended at a density of 2 × 10⁶ cells/ml in HBSS with 1 mM CaCl₂. 1 ml of the cell suspension was used for each measurement. Fluorescence measurements were made in a spectrofluorometer (F-2000; Hitachi America Ltd., Brisbane, CA) in a gassed, water jacketed cuvette. Intracellular [Ca_i] was calculated from fura-2 epifluorescence using excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm. In all cases, measurements were made in the same passages of cells used for Isc measurements.

For single cell recordings, T84 cells were grown on 25-mm glass cover slips. Cells were loaded with fura-2 (60 min at 10 μM fura-2 AM) in medium and then rinsed with HBSS. The monolayers were mounted in a specially designed stage-mounted cell perfusion chamber and perfused with HBSS containing 1 mM CaCl₂ for 5 min before measurements. Intracellular calcium was calculated as previously described (19). Briefly, fura-2 fluorescence was measured from single cells with a microspectrofluorometer in the epi-illumination mode. Cells were excited with 340 and 380 nm light at 60 Hz. Emissions were stored and averaged every second. Ca_i was calculated from calibrated solutions as previously described in detail (19).

Inositol phosphate formation. Monolayers were prelabelled in medium 199 with [³H]inositol (1 μCi/ml) which has significantly less inositol per liter than DMEM/F12 mixture (0.05 versus 12.6 mg/liter). After 2 d of labelling, monolayers were rinsed in HBSS and stimulated for varying times with 50 μM NH₂Cl. The monolayers were extracted by punching the monolayer from the support and immediately placing it in 500 μl 5% trichloroacetic acid. Trichloroacetic acid was removed by eight extractions with an equal volume of water saturated diethylether. The samples were neutralized with Tris base and applied to Dowex AG1×8 anion exchange columns. Fractions were eluted as previously

described (20). Protein in the trichloroacetic acid precipitates was measured by the method of Bradford (18).

Materials. T84 cells were obtained both from American Type Culture Collection, Rockville, MD, and James McRoberts, UCLA, Torrance, CA. Media and supplements were from JRH Biosciences, Lenexa, KS, and newborn calf serum from Gibco Laboratories, Grand Island, NY. NaOCl and NH₄Cl were from Aldrich Chemical Co., Milwaukee, WI. Fura-2 acetoxymethylester was from Molecular Probes, Inc., Eugene, OR. Cyclic nucleotide radioimmunoassay kits, ³⁶Cl, and [³H]inositol were from New England Nuclear, Boston, MA. LDH measurements were performed with a kit from Sigma Chemical Co., St. Louis, MO. Heat stable *E. coli* enterotoxin was a gift of Dr. Ralph Gianella, University of Cincinnati, Cincinnati, OH.

Results

NH₂Cl effect on Isc. When added to the serosal solution, NH₂Cl stimulated a gradual increase in Isc (Fig. 1) reaching a peak by 20 min. Monolayers that were used nine or more days after plating had smaller and more variable responses than monolayers only 7–8 d after plating. Additionally, monolayers from earlier passages of cells tended to have a shorter lag period between addition of NH₂Cl and Isc increase. For these reasons, monolayers from earlier passages (less than passage 60) and of 7–8 d postplating were routinely used for these studies.

The concentration dependence of the Isc response to NH₂Cl is shown in Fig. 2. Data presented are maximal Isc changes. Although peak changes did not always occur at the same time after addition of NH₂Cl, the variability between “early” and “late” responders was significantly reduced by confining studies to selected cell passages and to cell 7–8 d after plating. NH₂Cl at concentrations as low as 10 μM stimulated Isc. Stimulation was maximal at 50–75 μM and decreased at 100 μM. Although mucosal addition of NH₂Cl (50 μM) also stimulated secretion of Cl, the peak Isc change was only 7 ± 3 μAmp/cm² compared to an increase of 21 ± 5 μAmp/cm² in cells stimulated by serosal addition of NH₂Cl (*n* = 5). There

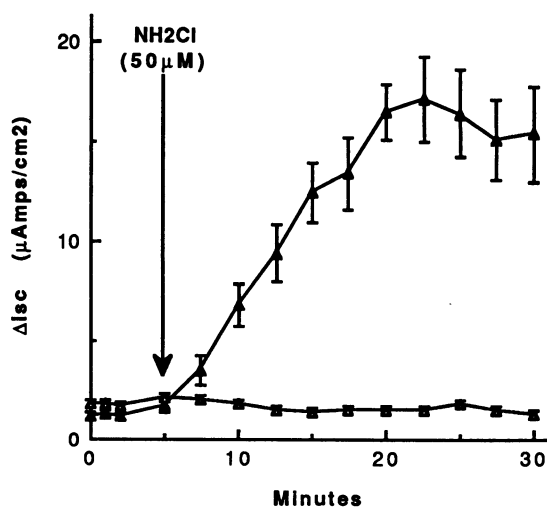


Figure 1. Effect of NH₂Cl (50 μM, serosal) on Isc (▲). T84 monolayers of passage 52–56 were mounted in a modified Ussing chamber 7 d after plating and electrical parameters measured as described in Methods. Values shown are means ± SE of five monolayers. Also shown is the mean ± SE Isc of five control T84 monolayers treated only with vehicle on the serosal side (Δ).

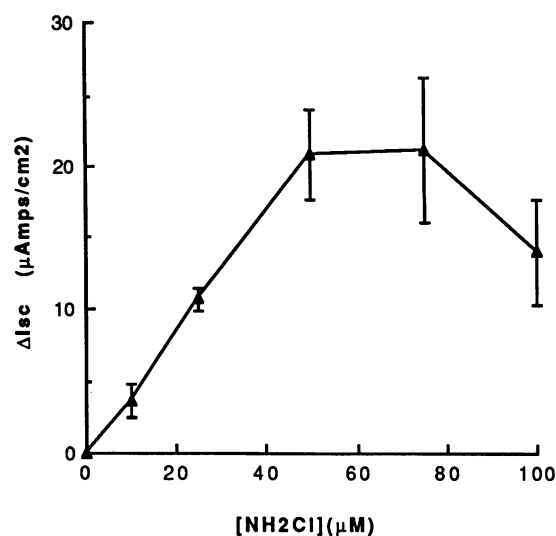


Figure 2. Concentration-response curve for serosal NH_2Cl -stimulated Isc. Basal Isc in monolayers was generally $< 5 \mu\text{Amp}/\text{cm}^2$. Values shown are maximal changes (Δ was the change in Isc between pre-treatment and stimulated values) and are means \pm SE for six experiments.

were no significant differences in the time needed to achieve a maximal response between tissues stimulated mucosally or serosally.

To determine whether changes in Isc were dependent on Cl^- , media Cl^- was replaced by SO_4^{2-} . Isc responses of cells in SO_4^{2-} buffer were inhibited by $92 \pm 4\%$ ($n = 5$), suggesting NH_2Cl stimulated electrogenic Cl^- secretion. To confirm this, the Na-K-2Cl cotransport inhibitor bumetanide ($100 \mu\text{M}$), which inhibits the Cl^- secretory response in T84 monolayers (7), was added to the serosal side in Cl^- -containing buffer. The Isc response to NH_2Cl in the presence of bumetanide was blocked $90 \pm 6\%$ ($n = 5$), again consistent with Isc changes being due to stimulated Cl^- secretion. Finally, non-steady-state trans-epithelial unidirectional flux studies with ^{36}Cl also demonstrated that NH_2Cl stimulated the serosa-to-mucosa flux, but had no effect on the mucosa-to-serosa flux (Table I). Since the T84 cell has only been reported to secrete Cl^- , the latter findings would be additional evidence that NH_2Cl stimulates net Cl^- secretion in these cells.

NH_2Cl stimulation of Isc was not mediated by stimulation of prostaglandin production. Pretreatment of the monolayer

with 10^{-5} M piroxicam, an arachidonic acid cyclooxygenase inhibitor, did not alter the maximal Isc response to $50 \mu\text{M}$ NH_2Cl (19 ± 4 in the absence and $18 \pm 6 \mu\text{Amp}/\text{cm}^2$ in the presence of piroxicam).

Cytotoxicity studies. In the present studies, NH_2Cl has been used at concentrations below those believed to be cytotoxic (15). However, to readdress this issue, the cellular content of the cytosolic enzyme LDH was measured after exposure to concentrations of NH_2Cl from 1 to $100 \mu\text{M}$ for 30 min. In this instance, the cells were left adherent to the plastic culture wells and NH_2Cl was added to the medium, thus the application in the case may be considered mucosal. Although the media was saved in each case, LDH levels were below the level of detection after all stimulations. As shown in Table II, NH_2Cl at concentrations at or below $75 \mu\text{M}$ had no effect on cellular LDH levels. At $100 \mu\text{M}$ NH_2Cl , LDH levels decreased by $\sim 20\%$, suggesting some compromise in cellular viability. In all cases, cell counts were performed to ascertain the number of cells remaining to the plastic following stimulation. Cells were removed from the plastic by addition of trypsin and EDTA after the 30-min exposure to NH_2Cl . Even at $100 \mu\text{M}$ NH_2Cl , there was no detectable decrease in the number of cells, the number of cells averaging 2.5 million in 10 cm^2 wells. In addition, the number of cells which took up the vital dye trypan blue did not increase up to $75 \mu\text{M}$ monochloramine (averaging $\sim 5\%$). At $100 \mu\text{M}$ monochloramine a greater number of cells did take up trypan ($14.3 \pm 3.9\%$, $n = 6$), suggesting that $100 \mu\text{M}$ may be the concentration at which cellular viability is compromised.

Mechanism of action. Increases in cAMP, cGMP, or cytosolic Ca^{2+} in T84 are reported to be associated with stimulated Cl^- secretion (7, 11, 13). To examine whether these second messengers mediated the effects of NH_2Cl on Cl^- secretion and Isc, cyclic nucleotides were measured in monolayers stimulated with 5 or $50 \mu\text{M}$ NH_2Cl , $0.1 \mu\text{M}$ VIP to elevate cAMP levels, or $0.1 \mu\text{M}$ heat stable *E. coli* enterotoxin (StA) to elevate cGMP levels. As shown in Table III, neither concentration of NH_2Cl stimulated increases in cAMP levels, although VIP elevated cAMP 100-fold. Cyclic GMP was below the limit of detection in control, VIP, or NH_2Cl -stimulated cells, increased levels could easily be detected following stimulation with StA.

Since stimulated increases in intracellular calcium cause Cl^- secretion in T84 cells, the effects of NH_2Cl on cytosolic calcium were investigated. Initially these experiments were performed on suspensions of T84 cells from the same passages used to measure Isc (Fig. 1). These cells were loaded with fura-2 ($10 \mu\text{M}$

Table I. Effect of NH_2Cl on Transepithelial Cl Fluxes

Condition	Jms	Jsm	Jnet	Isc	R
Pretreatment	0.45 ± 0.06	0.36 ± 0.03	0.09 ± 0.06	0.16 ± 0.03	362 ± 48
+ NH_2Cl	0.49 ± 0.05	$0.73 \pm 0.06^*$	$-0.24 \pm 0.04^*$	$0.38 \pm 0.03^*$	326 ± 46
Δ	0.04 ± 0.08	$0.37 \pm 0.07^*$	$-0.33 \pm 0.06^*$	$0.22 \pm 0.05^*$	-36 ± 66

T84 monolayers were mounted in Ussing chambers, ^{36}Cl added 5 min after mounting, and pretreatment fluxes period (30 min flux period, samples taken only at beginning and end of period) begun 15 min later. NH_2Cl ($50 \mu\text{M}$, serosal) was then added and fluxes measured over next 30 min with samples removed only at beginning and end of period. Isc and R measurements, however, are averages of values recorded every 10 min during each period. Flux values and Isc are expressed in $\mu\text{eq}/\text{h} \cdot \text{cm}^2$ and R in $\Omega \cdot \text{cm}^2$. Δ represents the difference in measurements between stimulated and pretreatment values. Values are means \pm SE of five experiments. $^* P < 0.05$ and $^* P < 0.01$ compared with pretreatment value by paired Student's *t* test.

Table II. Concentration-Response Relation of NH_2Cl Effect on LDH Release

$[\text{NH}_2\text{Cl}]$, μM	0	10	25	50	75	100
LDH	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.2	1.1 ± 0.1
Number cells	2.5 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	2.7 ± 0.1	2.5 ± 0.1	2.6 ± 0.1

LDH activity is expressed as units LDH $\times 10^6/10 \text{ cm}^2$, cell counts as number of cells $\times 10^6$ per 10 cm^2 well. LDH was measured colorimetrically by the method of Cabaud-Wroblewski (16). Monolayers were treated for 30 min with varying concentrations of NH_2Cl , washed, and remaining LDH extracted as described in Methods. Values are means \pm SE for six experiments.

for 30 min) as described in Methods, washed in HBSS, and then resuspended in warmed, oxygenated HBSS. The cells were used immediately in a warmed, oxygenated cuvette in a spectrofluorometer. In fura-2-loaded T84 cells in suspension, basal cytosolic calcium concentrations averaged $96 \pm 14 \text{ nM}$ ($n = 5$). Following addition of NH_2Cl ($50 \mu\text{M}$), gradual increases in cytosolic Ca^{2+} were generally observed, reaching a maximum of 153 ± 17 ($n = 5$) by 15–20 min. This time course was similar to the observed changes in I_{sc} of monolayers and is shown in Fig. 3.

The concentration-response relationship in cell suspension of Ca responses to NH_2Cl is shown in Fig. 4. Each point represents the means of peak responses to NH_2Cl , $n = 6$. The ED_{50} was $33 \mu\text{M}$ as determined by least squares analysis, similar to that observed for I_{sc} responses ($32 \mu\text{M}$ as calculated by least squares analysis, see Fig. 2).

The effects of NH_2Cl on intracellular Ca^{2+} were also determined in T84 cells grown on cover slips mounted in a special microfluorometer perfusion chamber. This setup allowed rapid changes in media concentrations of Ca^{2+} . Shown in Fig. 5 are representative tracings of intracellular calcium in single T84 cells stimulated with NH_2Cl in the presence and absence of extracellular calcium. NH_2Cl -stimulated changes in free cytosolic calcium were dependent both on extra- as well as intracellular calcium. There was no change in $[\text{Ca}_i]$ when changing from a Ca-containing to Ca-free solution (Fig. 5 A). When NH_2Cl was included in the Ca-free perfusion solution, in approximately half of the cells measured (11 of 20 cells), an in-

crease of $[\text{Ca}_i]$, albeit reduced, was observed (Fig. 5 A). In other cells, the Ca response to NH_2Cl was completely dependent on extracellular calcium (9 of 20 cells). In these cells, the addition of Ca^{2+} (1 mM) back to the perfusion medium stimulated an increase of $[\text{Ca}_i]$ when NH_2Cl was still present (Fig. 5 B). In contrast, no changes in $[\text{Ca}_i]$ were observed when Ca^{2+} was added back to the perfusion medium in untreated control cells (data not shown).

The calcium dependence of the I_{sc} response is shown in Table IV. When calcium concentration in the medium was reduced to 0.1 from 1 mM, peak I_{sc} responses were only 15% of those in 1 mM calcium medium. However, reduction of calcium did not affect the I_{sc} response to PGE_2 , a cAMP-mediated secretagogue, indicating a selective inhibition of the NH_2Cl response. These results suggest that stimulated increases in Ca_i are pivotal to NH_2Cl stimulation of Cl secretion.

Many agents which stimulate release of calcium from intracellular stores do so via stimulation of phosphatidylinositol breakdown and the formation of inositol phospholipids (20). In particular, the formation of 1,4,5-IP₃ from phosphatidylin-

Table III. Effect of NH_2Cl on Cyclic Nucleotide Content

Condition	cAMP	cGMP
Vehicle	2.48 ± 0.11	nd
$5 \mu\text{M}$ NH_2Cl	2.63 ± 0.15	nd
$50 \mu\text{M}$ NH_2Cl	2.47 ± 0.21	nd
$0.1 \mu\text{M}$ VIP	$226.2 \pm 21.3^*$	nd
$0.1 \mu\text{M}$ STa	2.72 ± 0.18	$62.3 \pm 6.4^*$

Both cAMP and cGMP are expressed in picomoles cyclic nucleotide/mg protein. T84 monolayers were grown on permeable supports, washed, and stimulated on the serosal side with above agents (at designated concentrations) or vehicle ($30 \mu\text{l}$ 20 mM NH_4Cl in pH 8.0, 5 mM Na_2HPO_4) until maximal I_{sc} changes occurred (generally 10–15 min). At this time monolayer was removed from Ussing chamber and placed into ice cold trichloroacetic acid for RIA as described in Methods. Values are means \pm SE for six experiments. nd, not detected (limit of sensitivity 0.25 pmol cGMP/mg protein and 0.5 pmol cAMP/mg protein). * $P < 0.001$ by analysis of variance.

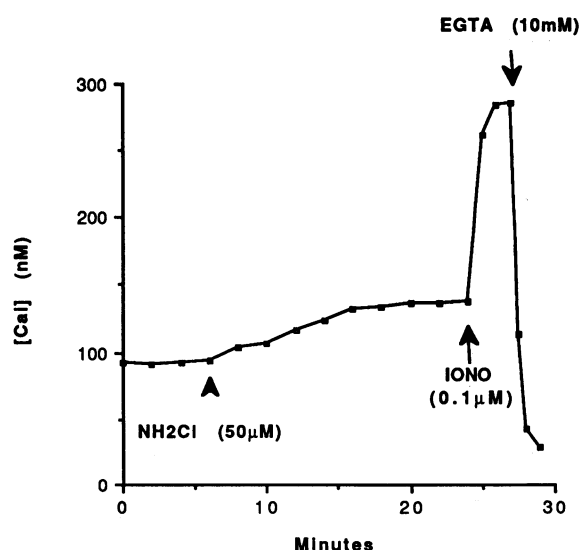


Figure 3. Representative tracing of the effect of NH_2Cl on intracellular Ca^{2+} in T84 cells in suspension (buffer $[\text{Ca}]$, 1 mM). T84 cells from the same passages used for I_{sc} measurements in Fig. 1 were loaded with fura-2 AM as described in Methods and harvested by trypsinization. Cells were resuspended at $\sim 2 \times 10^6$ cells/ml and used immediately. Ionomycin ($0.1 \mu\text{M}$) was added to maximally increase cytosolic Ca and EGTA to obtain the minimum fluorescence as described by Grynkiewicz (27).

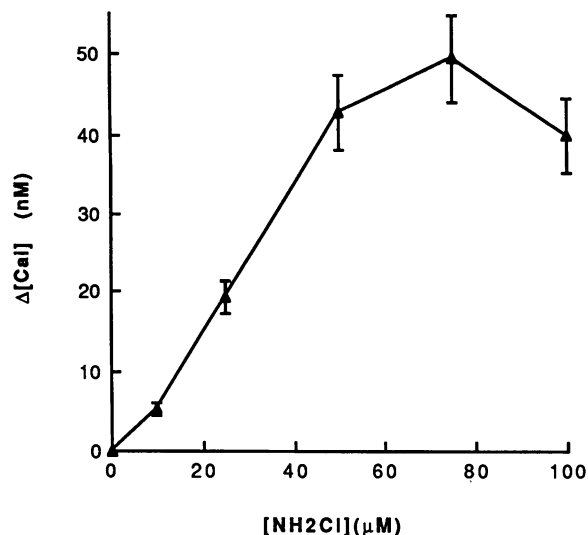


Figure 4. Concentration-response relationship for NH_2Cl stimulated $[\text{Ca}]_i$ increases of cells in suspension. Values represent maximal increases in ΔCa_i after each concentration which generally occurred 10–15 min after addition and are means \pm SE for six experiments.

inositol,4,5-bisphosphate stimulates release of calcium from a nonmitochondrial intracellular store, the calciosome (21). This has been demonstrated for the muscarinic agonist carbachol in T84 cells (12). Cells were stimulated for varying time with 50 μM NH_2Cl and formation of inositol phosphates (IP) including inositol-1,4,5-trisphosphate (IP₃) measured. A spontaneous, but reproducible increase in IP₃ was noted in control cells (Fig. 6). NH_2Cl , even after 30 min, was no different from control even at time points where the increase in cytosolic calcium had plateaued. In contrast, bethanechol (10 μM) stimulated a significant, but transient increase in IP₃ compared to controls, with levels declining after 10 min (Fig. 6). Although not shown in Fig. 6, NH_2Cl did not stimulate increases in either inositol monophosphate or inositol bisphosphates. These findings suggest that other mechanisms might be responsible for the stimulated increases in cytosolic calcium by NH_2Cl .

Discussion

In the present study we show that the granulocyte/phagocyte-derived ROM, NH_2Cl , directly stimulates Cl secretion in the human colonic cell line T84 at concentrations which do not appear to compromise cell viability. The mechanism of action appears to be via elevation of cytosolic Ca^{2+} which may cause Cl-secretion by activating K^+ efflux of the basolateral membrane as suggested previously (11). Although the results in single cell recordings of intracellular Ca_i varied, it should be emphasized that in the intact monolayer, Isc changes stimulated by NH_2Cl were always dependent on medium calcium. In addition, some dependence on extracellular Ca^{2+} could always be demonstrated in single cell measurements. This suggests that although in certain T84 cells where NH_2Cl stimulated the release of intracellular calcium as well that this is not sufficient to stimulate Isc of the monolayer.

The mechanism(s) by which NH_2Cl increases $[\text{Ca}]_i$ may be through its actions on membrane permeability to Ca^{2+} . In T84 cells, the NH_2Cl -stimulated increases in cytosolic Ca^{2+} appear to arise from release of Ca^{2+} from endogenous stores as well as

influx of extracellular Ca^{2+} . The precise mechanisms of NH_2Cl -induced increases in cytosolic Ca^{2+} remain unknown. Oxidants have been shown to alter Ca^{2+} sequestration of plasma membrane vesicles of hepatocytes (22). Oxidants have also been reported to stimulate Ca^{2+} release from sarcoplasmic reticulum (23) and inhibit sarcolemmal Na-K-ATPase via activation of Na/Ca exchange activity (24) and depressing Ca

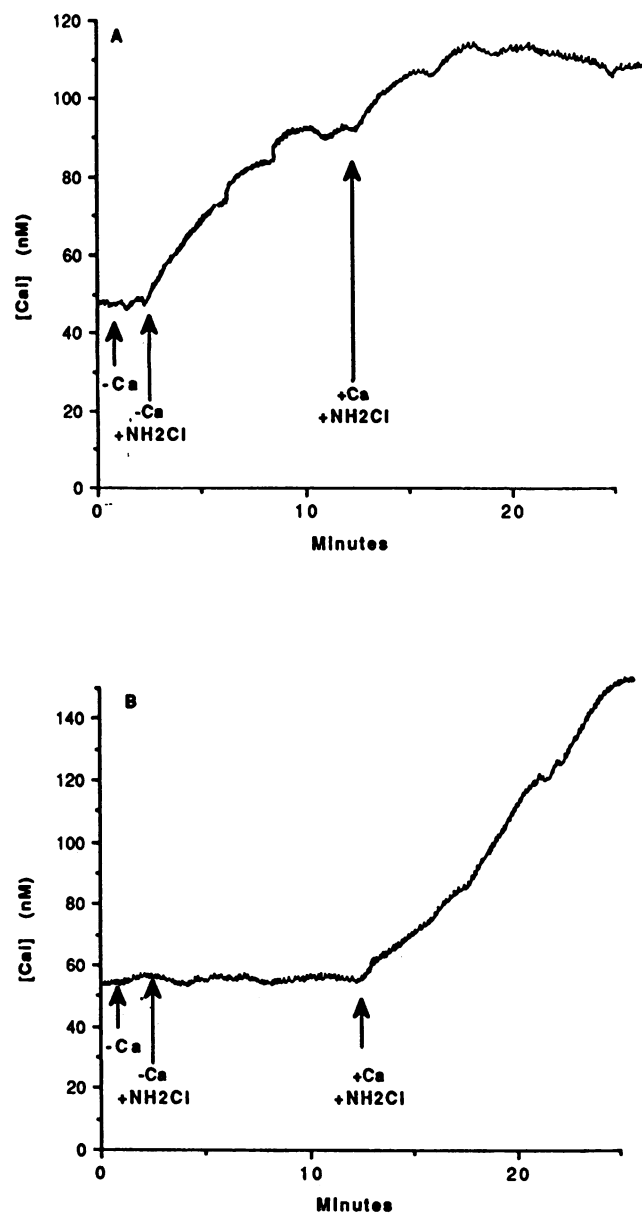


Figure 5. Representative tracings of Ca_i response to NH_2Cl (50 μM) from single T84 cells grown on glass cover slips and mounted in a microfluorimeter perfusion chamber. (A) Example of a cell where the NH_2Cl -stimulated Ca_i increase was only partially dependent on extracellular Ca^{2+} . Cells were initially perfused with Ca-containing buffer, then with Ca^{2+} -free buffer and 2 min later stimulated with NH_2Cl (50 μM). Addition of Ca^{2+} (1 mM) to the perfusion buffer at 12 min produced a small increase in the response. (B) Example of a Ca_i response to NH_2Cl entirely dependent on extracellular Ca^{2+} . Addition of Ca^{2+} at 13 min produced an increase in Ca_i . In the absence of NH_2Cl , addition of Ca^{2+} to the perfusate of untreated control cells had no effect on Ca_i (data not shown).

pump activity (25). However, the possibility cannot be excluded that oxidants cause selective changes in Ca permeability of the plasma or organelle membranes (26), possibly due to lipid peroxidation.

The rather heterogeneous Ca response of T84 cells to NH_2Cl was very striking. Cells attached to the same cover slip, for example, displayed variable dependency on extracellular Ca^{2+} and different time courses of response. We speculate that those cells whose responses appeared to depend exclusively on extracellular Ca^{2+} may have been depleted of their intracellular Ca^{2+} pools by the fura-2/AM incubation which is performed under Ca-free conditions. Alternatively, it is possible that the plasma membrane is more susceptible to NH_2Cl than organelles, perhaps because it is exposed to higher NH_2Cl concentrations present in ambient extracellular milieu. Finally, similar heterogeneity in Ca_i response of individual cells have been reported previously (13) and presumably reflect, in part, the polyclonal nature of T84 cells.

The sidedness of NH_2Cl stimulated Isc changes is also of interest and bears comment. We speculate that NH_2Cl permeability across the basolateral and luminal membranes might be different. Alternatively, the luminal membrane may be less susceptible to NH_2Cl induced increases in Ca permeability.

Increases in cytosolic Ca^{2+} are believed to stimulate Cl secretion in T84 cells by opening K^+ channels in the basolateral membrane (11). Our studies indicate that removal of extracellular Ca^{2+} significantly inhibits NH_2Cl -stimulated Isc and increases in Ca_i . Our attempts to increase T84 Ca-buffering activity with quin-2 to blunt or negate increases in Ca_i due to release from endogenous stores or influx across the plasma membrane were unfortunately nonspecific. Quin-2 loading of cells indiscriminately inhibited both NH_2Cl and PGE_2 stimulated Isc, the latter mediated through the stimulated increases in cellular cAMP content (11).

It has been difficult to define the importance of ROM in electrolyte secretion observed in inflammatory states of the intestine. ROMs have diverse effects in the inflammatory process. They increase capillary permeability at tissue concentrations $< 5 \mu\text{M}$ (14). In the acetic acid induced experimental colitis in rat, the tissue concentration of NH_2Cl has been estimated to be 0.2–0.3 mM by the production of HOCl and NH_4 (14). The concentrations used in this study were well below this range and at concentrations that did not appear to be cytotoxic to T84 cells judged by measurements of cellular LDH content, minimal changes in monolayer conductance, and absence of

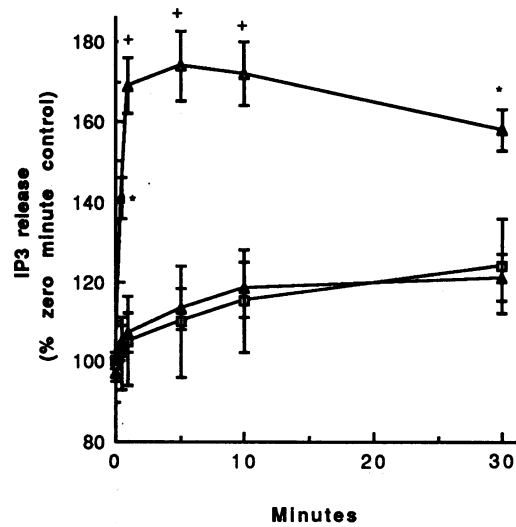


Figure 6. Formation of tritiated inositol trisphosphates (IP3) in T84 cells. Monolayers were labelled for 2 d in [^3H]inositol-containing medium, washed with HBSS, and stimulated for varying times with vehicle (40 mM NH_4Cl in pH 8.0 10 mM Na_2HPO_4 , serosal) (□), NH_2Cl (50 μM , serosal) (Δ), or bethanechol (10 μM , serosal) (Δ). IP3 responses to bethanechol were measured as a positive control. Reactions were stopped by punching out the monolayers and extracting the inositol phosphates in trichloroacetic acid as described in Methods. Values are means \pm SE of six determinations. * $P < 0.05$, * $P < 0.01$ compared with vehicle treated monolayer at same time by paired Student's *t* test.

changes in Jms of Cl^- . H_2O_2 has also been shown to stimulate Cl secretion in rat colon as well as T84 monolayers; however, larger concentrations are required (4). This may be related to the less potent oxidizing potential of H_2O_2 compared with NH_2Cl (15).

The effect of H_2O_2 in intact rat colon appears to be complex. Approximately 70% of the early peak of the H_2O_2 -induced response can be blocked with cyclooxygenase inhibitors (4) and also by TTX, hexamethonium, or atropine (4). However, the late peak of the response in colon is only poorly inhibited by cyclooxygenase inhibitors or by hexamethonium or atropine ($< 50\%$ inhibition) (4). NH_2Cl also stimulates a similar biphasic Isc response in rat colon (2, 5). The early peak can be inhibited $\sim 25\%$ by TTX, atropine, or cyclooxygenase inhibitors whereas the late peak is unaffected by TTX or atropine and inhibited 30% by cyclooxygenase inhibition (5). The differences in these results may be due to the use of different ROM or concentrations of ROM used. In T84 cells, however, piroxicam (10^{-5} M), a selective cyclooxygenase inhibitor, had no effect on basal or NH_2Cl -stimulated Isc.

ROMs, therefore, may have an important role in the pathogenesis of diarrhea in inflammatory bowel diseases. In vivo and in intact mucosa, they activate neurotransmitter release which stimulates active secretion. However, our studies support the hypothesis that a second or delayed phase of ROM-stimulated secretion results from a direct effect of ROMs on epithelial ion transport.

Acknowledgments

This study was supported by National Institutes of Arthritis, Diabetes, and Digestive and Kidney Diseases grants DK-35810 and DK-40922

Table IV. Effect of Low Ca^{2+} Buffer on NH_2Cl -stimulated Isc

Condition	ΔIsc (NH_2Cl)	ΔIsc (PGE_2)	R
Control	10.0 \pm 1.6	28.9 \pm 2.1	543 \pm 136
0.1 mM Ca^{2+}	1.4 \pm 0.2*	33.3 \pm 3.2	482 \pm 99

T84 monolayers were mounted in Ussing chambers and electrical parameters measured as described in Methods. R values presented are before stimulation of Isc by either agent. NH_2Cl (50 μM , serosal) was added after basal Isc equilibrated (~ 10 min). After NH_2Cl response, PGE_2 (10 μM , serosal) was added and changes in Isc recorded. Values are maximal increase in Isc after stimulation and are means \pm SE for five monolayers. * $P < 0.01$ compared with 1 mM Ca^{2+} containing buffer.

(E. B. Chang), DK-42086 to the Digestive Disease Center of the University of Chicago, and a grant from the Crohn's and Colitis Foundation of America (M. W. Musch).

References

1. Agner, K. 1972. Biological effects of hypochlorous acid formed by "MPO"- and peroxidation in the chloride ion. In *Structures and Function of Oxidation-reduction Enzymes*. A. Akeson and A. Ehrenberg, editors. Pergamon Press, London, 329-335.
2. Tamai, H., J. F. Kachur, M. B. Grisham, and T. S. Gaginella. 1990. Scavenging effect of 5-aminosalicylic acid on neutrophil-derived oxidants: possible contribution to mechanism of action in inflammatory bowel disease. *Biochem. Pharmacol.* 41:1001-1006.
3. Bern, M. J., C. W. Sturbaum, S. S. Karayalcin, H. M. Berschneider, J. T. Wachsman, and D. W. Powell. 1989. Immune system control of rat and rabbit colonic electrolyte transport: role of prostaglandins and enteric nervous system. *J. Clin. Invest.* 83:1810-1820.
4. Karayalcin, S. S., C. W. Sturbaum, J. T. Wachsman, J. H. Cha, and D. W. Powell. 1990. Hydrogen peroxide stimulates rat colonic prostaglandin production and alters electrolyte transport. *J. Clin. Invest.* 86:60-68.
5. Tamai, H., J. F. Kachur, D. A. Baron, M. B. Grisham, and T. S. Gaginella. Monochloramine, a neutrophil-derived oxidant stimulates rat colonic secretion. 1990. *J. Pharmacol. Exp. Ther.* 257:884-894.
6. Barrett, K., J. A. Cohn, P. A. Huott, S. I. Wasserman, and K. Dharmasathaphorn. 1990. Immune-related intestinal chloride secretion II. Effect of adenosine on T84 cell line. *Am. J. Physiol.* 258:C902-C912.
7. Huott, P. A., W. Liu, J. A. McRoberts, R. A. Giannella, and K. Dharmasathaphorn. 1988. Mechanism of action of *Escherichia coli* heat stable enterotoxin in a human colonic cell line. *J. Clin. Invest.* 82:514-523.
8. Warhurst, G., N. B. Higgs, M. Lees, A. Tonge, and L. A. Turnberg. 1988. Activation of protein kinase C attenuates prostaglandin E2 responses in a colonic cell line. *Am. J. Physiol.* 255:G27-G32.
9. Wasserman, S., P. Huott, K. Barrett, G. Beuerlein, M. Kagnoff, and K. Dharmasathaphorn. 1988. Immune-related intestinal Cl secretion. I. Effect of histamine on the T84 cell line. *Am. J. Physiol.* 254:C53-C62.
10. Mandel, K. G., J. A. McRoberts, G. Beuerlein, E. S. Foster, and K. Dharmasathaphorn. 1986. Ba²⁺ inhibition of VIP- and A23187 stimulated Cl-secretion by T84 cell monolayers. *Am. J. Physiol.* 250:C486-C494.
11. Cartwright, C. A., J. A. McRoberts, K. G. Mandel, and K. Dharmasathaphorn. 1985. Synergistic action of cyclic adenosine monophosphate and calcium-mediated chloride secretion in a colonic epithelial cell line. *J. Clin. Invest.* 76:1837-1842.
12. Dharmasathaphorn, K., K. G. Mandel, J. A. McRoberts, L. D. Tisdale, and H. Masui. 1984. A human colonic tumor cell line that maintains vectorial electrolyte transport. *Am. J. Physiol.* 246:C204-C208.
13. Reinlib, L., R. Mikkelsen, D. Zahniser, K. Dharmasathaphorn, and M. Donowitz. 1989. Carbachol-induced cytosolic free Ca²⁺ increases in T84 colonic cells seen by microfluorimetry. *Am. J. Physiol.* 257:G950-G960.
14. Grisham, M. B., T. S. Gaginella, C. V. Ritter, H. Tamai, R. M. Be, and D. N. Granger. 1990. Effects of neutrophil-derived oxidants on intestinal permeability electrolyte transport and epithelial cell viability. *Inflammation.* 14:531-542.
15. Thomas, E. L., M. B. Grisham, and M. M. Jefferson. 1986. Preparation and characterization of chloramines. In *Methods in Enzymology*. 132: Part J. D. S. Giovanni and E. Johannes, editors. Academic Press, Inc., Orlando, FL. 569-584.
16. Cabaud, P. G., and F. Wroblewski. 1958. Colorimetric measurement of lactic dehydrogenase activity of body fluids. *Am. J. Clin. Pathol.* 30:234-238.
17. Musch, M. W., A. M. Nahkla, and E. B. Chang. 1990. Phorbol ester stimulated secretion in chicken ileum: role of arachidonic acid metabolites. *Gastroenterology.* 99:393-400.
18. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
19. Murthy, S. N., and R. J. Miller. 1989. Regulation of Ca⁺⁺ influx into striatal neurons by kainic acid. *J. Pharmacol. Exp. Ther.* 249:184-193.
20. Chang, E. B., and M. W. Musch. 1990. Calcium mediated neurohumoral inhibition of chicken enterocyte Na influx: role of phosphatidylinositol metabolites. *Life Sci.* 46:1913-1921.
21. Volpe, P., K. H. Krause, S. Hashimoto, F. Zorzato, T. Pozzan, J. Meldolesi, and P. D. Lew. 1988. "Calciosome", a cytoplasmic organelle: the inositol 1,4,5-trisphosphate sensitive Ca²⁺ store of nonmuscle cells? *Proc. Nat. Acad. Sci. USA.* 85:1091-1095.
22. Bellomo, G., R. Mirabelli, P. Richelmi, and S. Orrenius. 1983. Critical role of sulfhydryl group(s) in ATP-dependent Ca²⁺ sequestration by the plasma membrane fraction from rat liver. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 163:136-139.
23. Kako, K., M. Kato, T. Matsuoka, and A. Mustapha. 1988. Depression of membrane-bound Na-K-ATPase activity induced by free radicals and by ischemia of kidney. *Am. J. Physiol.* 254:C330-C337.
24. Kaneko, M., R. E. Beamish, and N. S. Dhalla. 1989. Depression of heart sarcolemmal Ca²⁺ pump activity by oxygen free radicals. *Am. J. Physiol.* 256:H368-H374.
25. Okabe, E., M. L. Hess, M. Oyama, and H. Ito. 1983. Characterization of free radical-mediated damage of canine cardiac sarcoplasmic reticulum. *Arch. Biochem. Biophys.* 255:164-177.
26. Watanabe, H., A. Kobayashi, T. Yamamoto, S. Suzuki, H. Hayashi, and N. Yamazaki. 1990. Alterations of human erythrocyte membrane fluidity by oxygen-derived free radicals and calcium. *Free Radical Biol. & Med.* 9:507-514.
27. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1984. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 3440-3449.