Role of calcium in adaptive cytoprotection and cell injury induced by deoxycholate in human gastric cells

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Kokoska, Evan R., Gregory S. Smith, Andrew B. Wolff, Yashwant Deshpande, Christopher L. Rieckenberg, Ali Banan, and Thomas A. Miller. Role of calcium in adaptive cytoprotection and cell injury induced by deoxycholate in human gastric cells. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G322–G330, 1998.—We have developed an in vitro model of adaptive cytoprotection induced by deoxycholate (DC) in human gastric cells and have shown that pretreatment with a low concentration of DC (mild irritant, 50 µM) significantly attenuates injury induced by a damaging concentration of DC (250 µM). This study was undertaken to assess the effect of the mild irritant on changes in intracellular Ca²⁺ and to determine if these perturbations account for its protective action. Protection conferred by the mild irritant was lost when any of its effects on intracellular Ca²⁺ were prevented: internal Ca²⁺ store release via phospholipase C and inositol 1,4,5-trisphosphate sustained Ca²⁺ influx through store-operated Ca²⁺ channels or eventual Ca²⁺ efflux. We also investigated the relationship between Ca²⁺ accumulation and cellular injury induced by damaging concentrations of DC. In cells exposed to high concentrations of DC, sustained Ca²⁺ accumulation as a result of extracellular Ca²⁺ influx, but not transient changes in intracellular Ca²⁺ content, appeared to precede and induce cellular injury. We propose that the mild irritant disrupts normal Ca²⁺ homeostasis and that this perturbation elicits a cellular response (involving active Ca²⁺ influx) that subsequently provides a protective action by limiting the magnitude of intracellular Ca²⁺ accumulation.

Understanding the role of calcium in adaptive cytoprotection is critical in maintaining mucosal integrity. Direct cytoprotection was defined as the ability of exogenous PG cytokines, independent of effects on acid secretion, to protect mucosae of the gastrointestinal (GI) tract against damage induced by various injurious agents and noxious insults. Adaptive cytoprotection, in contrast, is that process whereby administration of a low concentration of a damaging agent (not damaging by itself), termed a “mild irritant,” is able to attenuate injury to GI mucosa on subsequent exposure to higher concentrations of the same or even differing damaging agents.

The majority of early work investigating adaptive cytoprotection utilized in vivo models. Chandhury and Robert (11) and others (2, 25, 38) demonstrated that orally administered pretreatment with mild irritants (ethanol, bile salts, or hydrochloric acid) protected gastroduodenal mucosa against injury induced by a variety of dissimilar necrotizing agents and that a certain dose (or concentration) of the mild irritant was required to elicit this protective response. In addition, because indomethacin blocked this protective response and some mild irritants were purported to stimulate PG synthesis, it was argued that adaptive cytoprotection was in large part mediated by endogenous PGs (2, 11, 45). This view, however, has been challenged by a number of investigators (15, 31, 49), and recent studies have suggested that other mediators may play contributory roles in adaptive cytoprotection, independent of PGs, including nitric oxide, glutathione, dopamine, the internal enteric reflex, mucus and/or bicarbonate secretion, salivary secretions, and the formation of a physical protective covering of surface debris (10, 24, 31–33, 37).

We have recently investigated adaptive cytoprotection under in vitro conditions in gastric cells derived from a human carcinoma cell line. We determined that pretreatment of these cells with a low concentration of deoxycholate (DC) significantly attenuated both cell injury and permeability changes induced by subsequent exposure to damaging concentrations of DC (34). This protection was dependent on both the concentration and duration of mild irritant exposure. In addition, whereas DC exposure increased PG synthesis, the concentrations required were much higher than those required to initiate protection. We did not observe enhanced PG synthesis in response to a mild irritant concentration. This work suggested that adaptive cytoprotection exists in AGS cells under in vitro conditions independent of intact blood flow, neural innervation, or circulating humoral mediators. Furthermore, our findings indicated that stimulation of endogenous PG synthesis was not a prerequisite in mediating this protective response.

It has been proposed that Ca²⁺ homeostasis is critical in maintaining mucosal integrity (52) and that this cation plays a major role in promoting mucosal injury induced by various noxious agents, such as indomethacin, ethanol, and excessive nitric oxide (22, 51, 53). The relationship between Ca²⁺ and cellular injury is by no means specific to GI mucosa and has been described in many other cell types in which injury was initiated by diverse causes including ischemia and/or reperfusion, chemical exposure, radiation, and infection (20). Schanne and associates (47), in the late 1970s, suggested that the influx of Ca²⁺ across damaged cell membranes may be the final common pathway to cell death.

In view of these considerations and the results of our aforementioned studies (34) validating the existence of adaptive cytoprotection under in vitro conditions, we initiated the present study to explore the potential role that maintenance of intracellular Ca²⁺ homeostasis might play in this protective response. The major
objective of this study was to assess the effect of a mild irritant concentration of DC on changes in intracellular Ca\textsuperscript{2+} and to determine if these effects account for its protective action. We also investigated the relationship between Ca\textsuperscript{2+} accumulation and cellular injury induced by damaging concentrations of DC. Our prior work enabled us to use a well-defined, in vitro model of adaptive cytoprotection in human gastric cells.

MATERIALS AND METHODS

Cells. A human gastric cell line, known as AGS and derived from carcinoma cells, was obtained from American Type Culture Collection (Rockville, MD) at passage 49. We have previously characterized this cell line and have determined AGS cells to be morphologically similar to gastric mucous cells (Pas\textsuperscript{+}, alcin blue\textsuperscript{+}), with an ability to differentiate when postconfluent (34). Cells were maintained at 37°C in an atmosphere of 5% CO\textsubscript{2} and 100% relative humidity. Cells were split on a weekly basis at a ratio of 1:6 on reaching confluence. Cells were detached using 0.5 g porcine trypsin and 0.2 g EDTA tetrasodium combined with Hanks' balanced salt solution (HBSS) and then plated into either 24- or 48-well plates (Costar, Cambridge, MA) for experiments, four-well cover-glass chambers (Nunc, Naperville, IL) for confocal microscopic imaging, or into 150-cm\textsuperscript{2} flasks for propagation. All experiments were performed at 80–90% confluence. Cell passage was maintained between 50 and 65, and medium was changed every 2–3 days. AGS media consisted of Ham's F-12 supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Solutions. Before all experiments, medium was aspirated and replaced with HBSS plus 10 mM HEPES (Sigma Chemical, St. Louis, MO), consisting of (in mM) 137 NaCl, 5.7 NaHCO\textsubscript{3}, 5.3 KCl, 1.26 CaCl\textsubscript{2}, and 0.8 MgSO\textsubscript{4}. Experiments involving Ca\textsuperscript{2+}-free buffer utilized HBSS plus 10 mM HEPES and 2 mM 1,2-bis(2-aminophenoxy)ethane-N,N',N'\textsuperscript{-}tetraacetic acid (Sigma), consisting of (in mM) 137 NaCl, 5.7 NaHCO\textsubscript{3}, and 5.3 KCl. All test compounds were dissolved in NaHCO\textsubscript{3}, and 5.3 KCl. All test compounds were dissolved in HBSS and subsequently added to HBSS plus 1% fetal bovine serum (NaHCO\textsubscript{3}) consisting of (in mM) 137 to obtain F\textsubscript{MnCl\textsubscript{2}}. The fluo 3 signal was quenched to obtain F\textsubscript{MnCl\textsubscript{2}} and 50 µM digoxigenin, N,N',N',N'\textsuperscript{-}tetraakis(2-pyridylmethyl) ethylenediamine (50 µM, Molecular Probes) was used in all solutions as a heavy metal scavenger (30).

It is well accepted that manganese (Mn\textsuperscript{2+}) can be used as a Ca\textsuperscript{2+} surrogate to estimate extracellular Ca\textsuperscript{2+} influx through the plasma membrane (3). In separate experiments, Mn\textsuperscript{2+} uptake was monitored by quenching fluo 3 fluorescence with the addition of 2 mM MnCl\textsubscript{2} to all solutions (control and experimental). Data are presented as mean fluorescence.

Continuous fluorescent signals during both protocols were quantitated using a CYTOFLUOR II Fluorescent Multi-well Plate Reader (PerSeptive Biosystems) employing 485 nm and 530 nm as the excitation and emission spectra, respectively. Cells were maintained throughout the experiments at a temperature of 37°C with a heated stage.

Measurement of Ca\textsuperscript{2+} efflux. AGS cells were incubated overnight with 45CaCl\textsubscript{2} (10 µCi/ml), washed three times with HBSS, and subsequently treated with either control or test solutions. The supernatant was then removed at the designated time points, and cells were lysed with 1% Triton X-100. Radioactivity in the supernatant and within the lysed cells was determined by scintillation counting (Beckman LS 5000CE; Irvine, CA). Results are expressed as percent Ca\textsuperscript{2+} efflux based on the equation

\[
\text{Calcium efflux} (\%) = \frac{\text{cpm}_{\text{supernatant}}}{\text{cpm}_{\text{supernatant}} + \text{cpm}_{\text{cells}}}
\]

where cpm is counts per minute.

Measurement of cell injury. Cellular injury was quantitated using two different assays, one measuring plasma membrane integrity and the other measuring cytoplasmic enzyme leakage. We employed the fluorescent agent ethidium homodimer-1 (Et, 4 µM; Molecular Probes) to monitor plasma membrane integrity. Et enters cells through damaged membranes and exhibits enhanced fluorescence on binding to nucleic acids. This fluorescent probe produces a bright red fluorescence in dead cells, which was measured with a Fluorescent Multi-well Plate Reader at 485 nm excitation and 620 nm emission wavelengths (26, 40). Injury is reported as relative fluorescence.

Cell injury was also assessed by release of lactate dehydrogenase (LDH) into the buffer. Measurement of LDH release in cultured cells has been deemed a reliable and reproducible indicator of cellular injury (5, 41). LDH content was determined using the equation

\[
[K_d] = \frac{F - F_{\text{min}}}{F_{\text{max}} - F}
\]

where the minimum fluo 3 signal (F\textsubscript{min}) equals 1.25 F\textsubscript{MnCl\textsubscript{2}} minus 0.25 F\textsubscript{max} and the dissociation constant (K\textsubscript{d}) equals 400 nM (29).

The maximum fluo 3 signal, or F\textsubscript{max}, was determined by permeabilizing AGS cells with 50 µM digitonin (Sigma). The fluo 3 signal was quenched to obtain F\textsubscript{MnCl\textsubscript{2}} and 50 µM digoxigenin, N,N',N',N'\textsuperscript{-}tetraakis(2-pyridylmethyl)-ethylenediamine (50 µM, Molecular Probes) was used in all solutions as a heavy metal scavenger (30).
metrically by measuring its absorbance at 490 nm (Bio-Rad model 3550 microplate reader, Hercules, CA) in 96-well plates (Costar). Experimental values are reported as the percentage of LDH released from control cells.

Confocal imaging. Confocal images were acquired with a Zeiss LSM 410 laser-scanning confocal microscope system utilizing a Zeiss Airviolet 1.35 optical microscope. The samples were viewed with a Zeiss Plan-Neofluar 103×, 1.25 NA oil objective. The samples were then illuminated with an argon-krypton multiline laser, and confocal images were recorded with dual, simultaneous fluorescence detectors. The data system was operated with Zeiss software LSM (Rev. 3.92). Time-lapse images were acquired using the time series macro, which are part of this software package.

Experimental design. Previous work suggested that protection conferred by the mild irritant was dependent on both concentration and duration of exposure. We determined that pretreating cells with 50 µM DC for 20 min before subsequent exposure to a damaging concentration (250 µM) of DC provided optimal protection compared with other mild irritant concentrations or pretreatment durations (34). Therefore, we initially investigated the mechanism(s) whereby 50 µM DC elicits changes in intracellular Ca\(^{2+}\). The roles of phosphoinositide-specific phospholipase C (PLC) and subsequent inositol 1,4,5-trisphosphate (IP\(_3\)) generation were determined using the aminosteroid U-73122, an inhibitor of IP\(_3\) generation (9, 18). The mechanism of sustained influx of extracellular Ca\(^{2+}\) was investigated with Ca\(^{2+}\)-free buffer, the store-operated Ca\(^{2+}\)-channel (SOC) blocker lanthanum (3), and the voltage-operated Ca\(^{2+}\)-channel (VOCC) antagonists verapamil and nifedipine. Ca\(^{2+}\) efflux after 50 µM DC treatment was then measured, and the effect of the plasma membrane Ca\(^{2+}\)-ATPase inhibitors quercetin and vanadate (36) was quantitated. The above studies were then repeated, and cells were subsequently exposed to 250 µM DC to determine what effect mild irritant Ca\(^{2+}\) mobilization had on protection against injury induced by a damaging concentration of DC.

The second series of experiments investigated the role of Ca\(^{2+}\) on cellular injury induced by 250 µM DC. We initially assessed the temporal relationship between intracellular Ca\(^{2+}\) elevation and cell injury. This was achieved, in separate studies, with the simultaneous measurement of changes in intracellular Ca\(^{2+}\) content and Et uptake or LDH release. We then examined the effect of Ca\(^{2+}\)-removal on injury induced by DC. Ca\(^{2+}\)-free buffer was utilized to determine the role of extracellular Ca\(^{2+}\). To investigate the role of intracellular Ca\(^{2+}\), separate cells were pretreated with TG (10 min) and subsequently exposed to DC in Ca\(^{2+}\)-free buffer. TG, a microsomal Ca\(^{2+}\)-ATPase inhibitor, rapidly depletes intracellular Ca\(^{2+}\) stores (8).

Statistics. Statistical evaluation was performed by ANOVA with a Scheffé post hoc test. Data (n = 6–12 per group) are reported as means ± SE. P < 0.05 was taken to represent statistical significance.

RESULTS

Intracellular Ca\(^{2+}\) changes induced by mild irritant: role of PLC-1P\(_3\). AGS cells exposed to 50 µM DC demonstrated an initial increase in intracellular Ca\(^{2+}\) present within 2 min, followed by a sustained elevation lasting up to 10 min, and then a gradual decrease to baseline. Cells pretreated with neomycin (100 µM, 10 min) or U-73122 (1 µM, 15 min) and subsequently treated with 50 µM DC displayed no such elevation in intracellular Ca\(^{2+}\), and their levels were noted to be quite similar to control cells throughout the period of study. In contrast, cells pretreated with the inactive analog U-73343 (1 µM, 15 min) and subsequently exposed to 50 µM DC demonstrated no difference with regard to changes in intracellular Ca\(^{2+}\) compared with cells treated with only 50 µM DC. Data from these studies are depicted in Fig. 1. These data suggest that the initial increase in intracellular Ca\(^{2+}\) content induced by a low concentration of DC involves the release of intracellular Ca\(^{2+}\) stores via a PLC- and IP\(_3\)-related mechanism.

Intracellular Ca\(^{2+}\) changes induced by mild irritant: role of SOCl. Sustained intracellular Ca\(^{2+}\) elevations (2–10 min) were not observed in cells treated with 50 µM DC in the absence of extracellular Ca\(^{2+}\). Quenching of fluo fluorescence by Mn\(^{2+}\) was first evident 6 min after 50 µM DC exposure, which suggests the influx of extracellular Ca\(^{2+}\) over this time period. Pretreatment of AGS cells with either La\(^{3+}\) (25 µM, 15 min), verapamil (20 µM, 15 min), or nifedipine (10 µM, 15 min) alone did not significantly affect intracellular Ca\(^{2+}\) levels (data not shown). The SOCC blocker La\(^{3+}\), but not the VOCC antagonists verapamil or nifedipine, inhibited both the sustained intracellular Ca\(^{2+}\) plateau and Mn\(^{2+}\) uptake after 50 µM exposure. Data from these studies are shown in Fig. 2, A and B. These data suggest that the sustained Ca\(^{2+}\) elevation, as induced by 50 µM DC, is mediated by the influx of extracellular Ca\(^{2+}\) through SOCCs, observations that are consistent with store-operated Ca\(^{2+}\) influx (SOCl).

Ca\(^{2+}\) efflux induced by mild irritant. Compared with controls, AGS cells exposed to 50 µM DC demonstrated initial signs of net Ca\(^{2+}\) efflux at 10 min postexposure followed by significant efflux at 20 min (Fig. 3A). In separate studies, Ca\(^{2+}\) efflux at 20 min after 50 µM DC exposure was not observed in cells pretreated with the plasma membrane Ca\(^{2+}\)-ATPase inhibitors quercetin (10 µM, 10 min) or vanadate (10 µM, 10 min) (Fig. 3B).
Furthermore, in cells pretreated with quercetin or vanadate and subsequently exposed to 50 µM DC, intracellular Ca²⁺ content did not return to baseline but remained elevated during the entire 20-min time period (Fig. 4). These data suggest that under normal conditions 50 µM DC elicits active Ca²⁺ efflux at later time points (10–20 min), causing a return toward resting Ca²⁺ levels. Injury, as quantitated by Et uptake and LDH release, was subsequently measured in cells exposed to graded concentrations of DC for 20 min. The higher concentrations of DC that elicited sustained

Intracellular Ca²⁺ accumulation and cellular injury induced by DC. DC exposure elicited a dose-dependent rise in Ca²⁺ accumulation in AGS cells. A large, initial surge of intracellular Ca²⁺ was observed within the first 2 min after DC exposure (100–500 µM) followed by a lower, sustained elevation, which persisted for at least 20 min. However, cells exposed to the lowest DC concentration, 50 µM, demonstrated an initial intracellular Ca²⁺ elevation followed by a return toward resting Ca²⁺ levels. Injury, as quantitated by Et uptake and LDH release, was subsequently measured in cells exposed to graded concentrations of DC for 20 min. The higher concentrations of DC that elicited sustained

Mild irritant Ca²⁺ mobilization and adaptive cytoprotection. AGS cells, with or without pretreatment by the aforementioned inhibitors, were then exposed to 50 µM DC for 20 min, washed three times to remove any remaining inhibitor, and subsequently exposed to 250 µM DC for 20 min. Compared with controls, cells pretreated with buffer and subsequently exposed to 250 µM DC demonstrated sustained intracellular Ca²⁺ accumulation and significant injury as measured by both Et uptake and LDH release. In contrast, the elevation in intracellular Ca²⁺ content and cellular injury induced by 250 µM DC were significantly attenuated when cells were pretreated with 50 µM DC, with or without the inactive PLC inhibitor U-73343. Furthermore, protection conferred by the mild irritant was lost when any of its effects on changes in intracellular Ca²⁺ were prevented: internal store release via PLC and IP₃ (neomycin or U-73122 pretreatment), sustained extracellular Ca²⁺ influx through SOCCs (Ca²⁺-free buffer or La³⁺ pretreatment), or eventual Ca²⁺ efflux (quercetin or vanadate pretreatment). These data are depicted in Table 1.

Fig. 2. Effect of lanthanum, verapamil, or nifedipine pretreatment on changes in intracellular Ca²⁺ concentration (A) or manganese influx (B) induced subsequently by 50 µM DC. *P < 0.001 vs. control, †P < 0.01 vs. control, n = 6–12 per group.

Fig. 3. Ca²⁺ efflux in AGS cells under either control conditions or after treatment with 50 µM DC (A). *P < 0.001 vs. control, n = 6–12 per group. In a separate experiment, the effect of the plasma membrane Ca²⁺-ATPase inhibitors quercetin or vanadate on Ca²⁺ efflux induced by 50 µM DC was measured 20 min after treatment (B). *P < 0.001 vs. control, †P < 0.001 vs. cells exposed to 50 µM DC, n = 6–12 per group.
elevations in intracellular Ca\textsuperscript{2+} (100–500 µM) were associated with significant cellular damage, whereas 50 µM DC did not appear to induce cell injury compared with control cells. These data are depicted in Table 2. There was a strong correlation between [Ca\textsuperscript{2+}]\textsubscript{i} and both Et uptake (r\textsuperscript{2} = 0.949, P < 0.001) and LDH release (r\textsuperscript{2} = 0.746, P < 0.001). Whereas these data suggest that there was a significant association between intracellular Ca\textsuperscript{2+} accumulation and cell injury in response to damaging concentrations of DC, cause and effect remain unclear.

Fig. 5A demonstrates the simultaneous measurement of intracellular Ca\textsuperscript{2+} accumulation and cell injury (Et uptake). Although not shown, intracellular Ca\textsuperscript{2+} levels and Et uptake remained constant over the 20-min time period in control cells. Treatment with 250 µM DC elicited a large, initial intracellular Ca\textsuperscript{2+} surge followed by a decline toward basal levels. However, at 20 min the intracellular Ca\textsuperscript{2+} content remained elevated. AGS cells showed initial signs of significant Et uptake at 10 min postexposure followed by significant Et uptake at 20 min. In a separate experiment, similar patterns were observed with the simultaneous measurement of intracellular Ca\textsuperscript{2+} accumulation and LDH release (Fig. 5B). However, a significant increase in LDH release was first evident at an earlier time point (6 min postexposure).

Table 1. Mild irritant Ca\textsuperscript{2+} mobilization and adaptive cytoprotection

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Intracellular Ca\textsuperscript{2+}, nM</th>
<th>Ethidium Uptake, relative fluorescence</th>
<th>LDH Release, %Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>125 ± 14</td>
<td>136 ± 7</td>
<td>100</td>
</tr>
<tr>
<td>HBSS</td>
<td>245 ± 16*</td>
<td>365 ± 5*</td>
<td>165 ± 10*</td>
</tr>
<tr>
<td>DC (50 µM)</td>
<td>172 ± 11*†</td>
<td>237 ± 7*†</td>
<td>113 ± 3*†</td>
</tr>
<tr>
<td>Neomycin (100 µM); DC (50 µM)</td>
<td>256 ± 17*†</td>
<td>360 ± 8*†</td>
<td>137 ± 9*†</td>
</tr>
<tr>
<td>U-73122 (1 µM); DC (50 µM)</td>
<td>247 ± 19*†</td>
<td>351 ± 11*†</td>
<td>140 ± 7*†</td>
</tr>
<tr>
<td>U-73343 (1 µM); DC (50 µM)</td>
<td>164 ± 13*†</td>
<td>245 ± 4*†</td>
<td>112 ± 5*†</td>
</tr>
<tr>
<td>DC (Ca\textsuperscript{2+} free) (50 µM)</td>
<td>242 ± 9*†</td>
<td>357 ± 17*†</td>
<td>153 ± 13*†</td>
</tr>
<tr>
<td>Lanthanum (25 µM); DC (50 µM)</td>
<td>260 ± 17*†</td>
<td>363 ± 6*†</td>
<td>150 ± 8*†</td>
</tr>
<tr>
<td>Quercetin (10 µM); DC (50 µM)</td>
<td>287 ± 19*†</td>
<td>420 ± 15*†</td>
<td>138 ± 12*†</td>
</tr>
<tr>
<td>Vanadate (10 µM); DC (50 µM)</td>
<td>291 ± 16*†</td>
<td>426 ± 12*†</td>
<td>145 ± 8*†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–12 per group. Pretreatment was followed by exposure to 250 µM sodium deoxycholate (DC) for 20 min. LDH, lactate dehydrogenase; HBSS, Hanks’ balanced salt solution. *P < 0.01 vs. control. †P < 0.01 vs. HBSS pretreatment.

Table 2. Intracellular Ca\textsuperscript{2+} accumulation and cellular injury induced by DC

<table>
<thead>
<tr>
<th>DC, µM</th>
<th>Intracellular Ca\textsuperscript{2+}, nM</th>
<th>Ethidium Uptake, relative fluorescence</th>
<th>LDH Release, %Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>124 ± 13</td>
<td>131 ± 9</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>131 ± 9</td>
<td>133 ± 10</td>
<td>94 ± 9</td>
</tr>
<tr>
<td>100</td>
<td>179 ± 12*†</td>
<td>263 ± 7*†</td>
<td>151 ± 10*†</td>
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<tr>
<td>250</td>
<td>256 ± 17*†</td>
<td>381 ± 12*†</td>
<td>178 ± 8*†</td>
</tr>
<tr>
<td>500</td>
<td>342 ± 18*†</td>
<td>477 ± 14*†</td>
<td>287 ± 11*†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–12 per group. Cells were exposed to DC for 20 min. *P < 0.01 vs. control.
Confocal imaging of Ca\(^{2+}\) changes and Et uptake. Living AGS cells previously loaded with fluo 3 were subsequently exposed to 250 µM DC plus Et for time-lapse confocal imaging. Before DC exposure, images demonstrated a low background intracellular Ca\(^{2+}\) signal (green) with minimal fluo 3 compartmentalization. A minority of injured cells demonstrating Et uptake (red) could be detected at this early time point. Two minutes after DC exposure, the majority of AGS cells demonstrated a large increase in green fluorescence (or [Ca\(^{2+}\)]) at 5 min postexposure. At this time point, no increase in the number of injured cells was apparent. Ten to twenty minutes after exposure to 250 µM DC, AGS cells demonstrated slight increases in green fluorescence compared with the same cells at baseline, and an increasing number of cells demonstrated Et uptake (red cytosolic staining).

Removal of extracellular and intracellular Ca\(^{2+}\). Preliminary data indicated that the effect of TG on changes in intracellular Ca\(^{2+}\) was not concentration dependent within the range of 500 nM to 5 µM. We therefore employed the lowest concentration (500 nM). Pilot studies in AGS cells indicated that TG, in Ca\(^{2+}\)-containing buffer, elicited SOCl (or capacitive Ca\(^{2+}\) entry). Cells treated with TG in Ca\(^{2+}\)-free buffer elicited an initial increase in [Ca\(^{2+}\)], followed by a steady decline (at 5 min postexposure) to baseline values. These experiments suggested that intracellular Ca\(^{2+}\) stores were effectively depleted (data not shown).

AGS cells were exposed to 250 µM DC in 1) Ca\(^{2+}\)-containing buffer, 2) Ca\(^{2+}\)-free buffer, and 3) Ca\(^{2+}\)-free buffer after pretreatment with TG. In the presence of extracellular Ca\(^{2+}\), 250 µM DC exposure caused a large, initial increase in intracellular Ca\(^{2+}\) content followed by a lower, sustained elevation. Interestingly, in the absence of extracellular Ca\(^{2+}\), 250 µM DC elicited an initial intracellular Ca\(^{2+}\) surge followed by a rapid return to baseline values. In AGS cells pretreated with TG, subsequent exposure to 250 µM DC in Ca\(^{2+}\)-free buffer did not appear to evoke any changes in intracellular Ca\(^{2+}\) content (data not shown).

Cellular injury was quantitated, with both Et uptake and LDH release, 20 min after the aforementioned experiment. In control cells, the absence of extracellular Ca\(^{2+}\), with or without TG pretreatment, did not appear to induce significant cellular injury. Removal of extracellular Ca\(^{2+}\) reversed injury induced by 100 µM DC and significantly attenuated injury elicited by 250 µM DC. In AGS cells exposed to 500 µM DC, elimination of extracellular Ca\(^{2+}\) only marginally reduced cellular injury. Further depletion of intracellular Ca\(^{2+}\) stores with TG, compared with only extracellular Ca\(^{2+}\) removal, did not appear to further decrease cellular injury with any of the concentrations of DC employed. These data are shown in Fig. 6, A and B.

**DISCUSSION**

A considerable body of data suggests that Ca\(^{2+}\) is critical in maintaining GI integrity and may be a major mediator involved in mucosal injury induced by ethanol, nitric oxide, indomethacin, or stress (22, 43, 51–53). We therefore investigated the role of Ca\(^{2+}\) in both adaptive cytoprotection and cell injury induced by DC. Our data indicate that pretreatment with a mild irritant (50 µM DC) significantly attenuated both Ca\(^{2+}\) accumulation and cell injury induced by damaging concentrations of DC (250 µM). This protection was not observed when the effects of the mild irritant on changes in intracellular Ca\(^{2+}\) were prevented. Our findings suggest that the mild irritant initially disrupts normal Ca\(^{2+}\) homeostasis and that this perturbation elicits a cellular response (involving active Ca\(^{2+}\) efflux) that subsequently protects cells from injury induced by damaging concentrations of DC by limiting Ca\(^{2+}\) accumulation. In contrast, sustained Ca\(^{2+}\) accumulation as a result of extracellular Ca\(^{2+}\) influx, but not transient changes in intracellular Ca\(^{2+}\) content, appeared to precede and induce cellular injury by high concentrations of DC. A schematic diagram depicting these proposed events is shown in Fig. 7.

The mechanism whereby a low, nondamaging concentration of DC elicits changes in intracellular Ca\(^{2+}\) appears to initially involve the release of internal Ca\(^{2+}\)
stores through PLC activation and/or IP₃ generation. This is followed by a period of sustained intracellular Ca²⁺ accumulation resulting from the influx of extracellular Ca²⁺ through SOCCs. Finally, the intracellular Ca²⁺ content returns to resting values as a result of active Ca²⁺ efflux from the cytosol into the extracellular space (see Fig. 7).

The aforementioned proposed mechanism of a mild irritant concentration of DC-induced Ca²⁺ mobilization is consistent with previously published observations. Although similar work has not been reported specific to gastric cells, extensive investigation has been performed in hepatocytes and colonocytes. Combettes et al. (12, 13) reported in hepatocytes that bile salts initially mobilize IP₃-sensitive intracellular Ca²⁺ pools. This is followed by the passive entry of extracellular Ca²⁺ through a process that appears to be independent of bile salts themselves. DC, at concentrations ranging from 10 to 600 µM, has been shown to significantly increase PLC activity in HT-29 colon tumor cells and rat colonic mucosa (4, 6). Devor et al. (16), employing both whole cell patch-clamp techniques and fluorescent measurements in T84 colonic cells, demonstrated that taurodeoxycholate initially induces the release of Ca²⁺ from intracellular stores via an IP₃-dependent mechanism. Craven et al. (14) reported that DC increases diacylglycerol and IP₃ generation in rat colonic cells. It is currently unknown whether the effect of DC on PLC is direct or related to its detergent properties, which may alter the interaction of PLC with membrane substrates.

Several groups have also reported that low concentrations of bile salts stimulate Ca²⁺ efflux in hepatocytes (7, 13). Because Na⁺/Ca²⁺ exchangers are present in very low levels or absent in nonexcitable cells, Ca²⁺ extrusion in human gastric cells is likely Ca²⁺-ATPase mediated. Currently the mechanism whereby bile salts stimulate Ca²⁺ efflux is unclear. This effect may be indirect and regulated by the mere increase in intracellular Ca²⁺ content (1). Ca²⁺-ATPase is regulated by calmodulin and fatty acids-acidic phospholipids, and perhaps these are mobilized by low concentrations of DC. Whereas the current work suggests that DC stimulates Ca²⁺ efflux, other mechanisms may also partially account for a decrease in the intracellular Ca²⁺ elevation; these include Ca²⁺ buffering and sequestration.

Our observations are consistent with other reports that suggest that bile salt toxicity involving concentrations in excess of a mild irritant may evoke substantial perturbations in intracellular Ca²⁺ content (19, 39, 50). Dziki et al. (19) demonstrated cellular hypercalcemia in DC-induced injury in rabbit gastric cells. They reported that 200 µM DC was the minimal concentration required to elicit intracellular Ca²⁺ changes and observed an initial rise in intracellular Ca²⁺ content 2 min after exposure, followed by a continuous rise to maximal concentrations at 20 min (19, 39). We also observed that damaging concentrations of DC elicit a large increase in intracellular Ca²⁺ within 2 min. However, in contrast to the Dziki study, this initial increase was noted to be only transient and was followed by a lower but sustained elevation over the remaining 20 min. Our findings suggested that whereas DC elicited early, transient intracellular Ca²⁺ changes, sustained elevation is required to induce cell injury. In support of this contention is the observation that when cells were exposed to a high concentration of DC (250 µM) in the absence of extracellular Ca²⁺, injury was significantly attenuated despite a significant but transient Ca²⁺ elevation. Perhaps one reason our observed Ca²⁺ trends differed from that of Dziki and associates is that they used a primary rabbit gastric cell preparation, in which sev-
eral different cell types were represented, each of which may have differing Ca\textsuperscript{2+} responses on exposure to DC than would occur with a single cell type (19, 39). The AGS cell line, on the other hand, is morphologically homogeneous. Furthermore, because AGS cells are of human origin (and therefore have human relevance) and involve cells that are commonly exposed to noxious luminal substances under in vivo conditions, this cell line is an excellent model for the study of GI damage.

Dziki et al. (19) also reported minimal cellular injury in response to 200 µM DC exposure, and this group and others have utilized higher concentrations of DC (500 µM-1 mM) while studying injury to rabbit gastric cells and hepatocytes (19, 23, 39). In contrast, we observed a mild degree of injury with 100 µM DC, a moderate degree of cell injury induced by 250 µM DC, and extensive cell injury induced by DC concentrations exceeding 500 µM. Moreover, DC concentrations in human stomach remnants after distal gastric resections are of the order of 370 µM or lower (17). Hence, we employed concentrations that are likely to occur under physiological conditions. The differences in DC concentrations required to induce injury in our human cell line and that of the rabbit stomach cell line are most likely species specific.

The causal connection between intracellular Ca\textsuperscript{2+} accumulation and cell death remains moot. The cellular Ca\textsuperscript{2+} gradient between the extracellular and intracellular compartments is among the largest in mammalian cells. Cells normally maintain (through plasma membrane impermeability and active extrusion) a steep gradient such that an [Ca\textsuperscript{2+}]i of 10\textsuperscript{-7} M is preserved despite being surrounded by an extracellular Ca\textsuperscript{2+} concentration that approaches 10\textsuperscript{-3} M (21). Some investigators believe that irreversible cellular injury is followed by the influx of Ca\textsuperscript{2+}, which then leads to the classic morphological appearance of Ca\textsuperscript{2+}-toxicity (coagulation necrosis), whereas others contend that a reversible cellular injury causes a loss of the maintenance of the Ca\textsuperscript{2+} gradient, leading to intracellular Ca\textsuperscript{2+} accumulation and subsequent irreversible cellular injury (20, 21). Ca\textsuperscript{2+} has been implicated in converting reversible to irreversible cellular injury in models investigating hepatic injury induced by ischemia-reperfusion, phalloidin, galactosamine, and silica (20, 27, 28). Our data indicate that intracellular Ca\textsuperscript{2+} accumulation precedes cell injury induced by DC. Our studies also suggest that sustained rises in [Ca\textsuperscript{2+}]i are required for cellular injury and that rapid, transient changes are well tolerated by AGS cells. These observations are consistent with the findings of others (20, 42).

Several mechanisms have been proposed whereby sustained increases in intracellular Ca\textsuperscript{2+} may cause cellular toxicity. Ca\textsuperscript{2+} disrupts the normal cellular cytoskeleton by dissociating of actin microfilaments from various protein structures (35). Phospholipases are also activated by Ca\textsuperscript{2+} with the subsequent hydrolysis of phospholipids and the disruption of membrane stability (35). Furthermore, Ca\textsuperscript{2+} has been implicated to activate proteases and endonucleases with resultant degradation of cytoskeletal elements (e.g., microtubules and actin), membrane proteins, and nucleic acids (42). Finally, it has been reported that excessive Ca\textsuperscript{2+} impairs mitochondrial function (42).

In summary, our data indicate that Ca\textsuperscript{2+} plays a significant role in both adaptive cytoprotection and cell injury induced by DC. Mild irritant pretreatment attenuated subsequent injury induced by damaging concentrations of DC through mechanisms that resisted intracellular Ca\textsuperscript{2+} accumulation. The mild irritant appeared to provide this protective effect through Ca\textsuperscript{2+} mobilization involving PLC and IP\textsubscript{3} activation, sustained Ca\textsuperscript{2+} influx through SOCCs, and eventual Ca\textsuperscript{2+} extrusion through the plasma membrane. Finally, sustained Ca\textsuperscript{2+} influx preceded cell injury induced by damaging concentrations of DC and may be a significant etiologic factor in bile salt toxicity.

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REFERENCES


