Cl^- Secretion Induced by Bile Salts

A Study of the Mechanism of Action Based on a Cultured Colonic Epithelial Cell Line

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Abstract

When applied to the basolateral (serosal) side of the T_{84} colonic epithelial monolayer, taurodeoxycholate caused net Cl^- secretion in a dose-dependent manner with a threshold effect observed at 0.2 mM. In contrast, when applied to the apical (luminal) surface, concentrations of taurodeoxycholate below 1 mM had little or no effect. Only when the concentration of taurodeoxycholate present on the apical side was ≥ 1 mM did apical addition results in an electrolyte transport effect. This apical effect on electrolyte transport was associated with an abrupt increase in the permeability of the monolayer. Cyclic AMP and cyclic GMP in the T_{84} monolayers were not increased by the bile salt, but in the presence of extracellular Ca^{2+}, free cytosolic Ca^{2+} increased with a graded dose effect and time course that corresponded approximately to the changes in short circuit current (I_{sc}). The results suggest that luminal bile salts at a relatively high concentration (≥ 1 mM) increase tight junction permeability. Once tight junction permeability increases, luminal bile salts could reach the basolateral membrane of the epithelial cells where they act to increase free cytosolic Ca^{2+} from extracellular sources. The resulting increases in free cytosolic Ca^{2+}, rather than in cyclic nucleotides, appear to be involved in transcellular Cl^- secretion.

Introduction

Certain dihydroxy bile acids induce electrolyte and water secretion under experimental conditions in the large and small intestine of animals and man (1–7). This phenomenon is thought to be responsible for the diarrhea associated with bile acid malabsorption (8). However, the mechanism by which bile acids affect electrolyte and water transport is poorly understood. Based on in vivo and in vitro studies with intestinal mucosa, several hypotheses have been proposed. These hypotheses include mediation by cAMP (2, 9), enhancement of mucosal permeability (3, 4), mediation by local neuro-humoral responses (10), and enhancement of calcium influx through the apical membrane (11, 12). Each of these hypotheses is open to question. The intestinal mucosa is a rather complex and heterogeneous structure. It is, therefore, difficult to define the mechanisms of bile acid induced electrolyte and water secretion at the cellular level based on in vivo studies or in vitro studies using isolated intestine. Recently, our laboratory has utilized a well-differentiated colonic epithelial cell line as a model system to study the mechanisms of secretagogue-induced Cl^- secretion (13–25). The uniformity of this model epithelium allows a less ambiguous characterization of the secondary messengers involved in the Cl^- secretory process. Additional advantages are provided by the equal accessibility of the apical and basolateral surfaces, which allows for studies of the sidedness. Furthermore, the resistance of the monolayers, which reflects their integrity, can be readily monitored together with the Cl^- secretory response. This allows correlation of permeability changes with secretory responses. Because of these advantages, we used this model to study the effects of taurodeoxycholate, a bile acid capable of inducing secretion, in comparison with taurocholate, which at comparable concentrations has no effect on intestinal transport.

Methods

Growth and maintenance of T_{84} cells, transepithelial electrolyte transport studies, and measurement of cAMP were performed as described earlier (14–16). Measurement of cGMP utilized a similar procedure to that described for cAMP except that a cGMP standard and cGMP antiserum were used instead of the cAMP standard and cAMP antiserum (22).

Measurement of free cytosolic calcium. The method for free cytosolic calcium ($[Ca^{2+}]_i$) measurement follows that described by Tsien et al. (26–28) using the fluorescence probe Fura-2/AM, with some modifications. T_{84} monolayers were plated and grown on filters (Nuclepore Corp., Pleasanton, CA) attached to Lexan rings as previously described for Ussing chamber experiments (14, 15). These monolayers were rinsed free of culture medium and then incubated in regular Ringer's solution containing 1 μM Fura-2/AM for 90 min at 37°C. After the loading incubation, the entire ring assembly was incubated for 15 min at 37°C in the buffer used for fluorescence measurements. This buffer contained (in mM): NaCl, 136.9; KCl, 5.4; $CaCl_2$, 1.0; Na_2HPO_4, 0.4; $MgCl_2$, 0.5; $MgSO_4$, 0.4; $NaHCO_3$, 4.2; Hepes, 10.0; and glucose, 0.6, with the pH adjusted to 7.4 with NaOH.

In some experiments, the T_{84} monolayers were preincubated for 30 min in varying concentrations of an intracellular Ca^{2+} chelator, 1,2 bis-5-*methyl*-amino-phenoxylethane-*N,N,N'*-tetraacetoxymethyl acetate (MAPTAM).[1] In these experiments, the monolayers were first incubated in regular Ringer's solution containing 1 μM Fura-2/AM for 90 min at 37°C as described above. After this step, the monolayers were incubated in regular Ringer's solutions containing MAPTAM at the concentrations indicated for an additional 30 min at 37°C. The entire ring assembly was then incubated for 15 min at 37°C in the

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1. *Abbreviations used in this paper:* I_{sc}, short circuit current; MAPTAM, 1,2 bis-5-*methyl*-amino-phenoxylethane-*N,N,N'*-tetraacetoxymethyl acetate; STa, *Escherichia coli* heat stable enterotoxin; TDC, taurodeoxycholate; VIP, vasoactive intestinal polypeptide.

buffers used for fluorescence measurements with subsequent steps followed as described below.

Fluorescence measurements were carried out in a fluorescence spectrophotometer (model 650-105; Perkin-Elmer Corp., Norwalk, CT). Excitation monochromator settings were 340 and 380 nm with a slit width of 5 nm. Emission monochromator was set at 505 nm with a slit width of 10 nm. Emitted light first passed through a 495–515-nm interference filter before being recorded. Monolayers were mounted for fluorescence measurements as follows: the Nuclepore filter holding the monolayer was rapidly peeled off the Lexan ring and attached to a polystyrene support with silicon grease (high vacuum grease, Dow Corning Co., Midland, MI), which was then placed in a standard 10-mm plastic cuvette. The polystyrene support with the monolayer attached was exposed to the same buffer in the cuvette on both the apical and basolateral sides. Orientation of the monolayer within the cuvette itself was at 45° to the excitation beam and angled 10–20° from the vertical plane to minimize interference from reflected excitation light. Excitation monochromator settings were changed manually approximately every 10 s with the wavelength drive control.

Free cytosolic calcium levels were calculated from the equation: $[Ca^{2+}]_i = K_d(F_0/F_s)\,(R - R_0)/(R_s - R)$, where K_d is 224 nM, F_0 is the fluorescence at 380 nm in the absence of calcium and in the presence of 1 mM EGTA, F_s is the fluorescence at 380 nm in saturating calcium (i.e., 1 mM), R_0 is the ratio of the fluorescence at 340 nm and at 380 nm in the absence of calcium and in the presence of 1 mM EGTA, R_s is the ratio at saturating calcium (1 mM), and the experimental ratio, R, is obtained by dividing the fluorescence at 340 nm by that at 380 nm after subtracting the respective autofluorescence levels. Autofluorescence for individual monolayers was measured at the end of each experiment following the addition of 10 μg/ml ionomycin and 1 mM $MnCl_2$ to the buffer (Fig. 1).

To demonstrate that the apparent increase in $[Ca^{2+}]_i$ induced by taurodeoxycholate was not due to leakage of Fura-2 or interaction of taurodeoxycholate with Fura-2 or the monolayer itself, we performed the following experiments. Fig. 2 demonstrates that the lysis of the T_{84} monolayer (on polystyrene support in a cuvette) by 0.01% Triton X-100 led to a marked reduction of fluorescent signals at both 340 and 380 nm wavelengths after ~ 2 min. In contrast, an immediate and persistent increase of fluorescent intensity at 340 nm wavelength combined with a decrease of signal at 380 nm wavelength were observed with lysis of a cell suspension reflecting the higher extracellular $[Ca^{2+}]$. Such difference probably occurred because the volume of the monolayer that contained Fura-2 (in which Fura-2 fluorescence was measured) was much smaller than the cuvette volume. This is in contrast to a cell suspension when the volume that Fura-2 fluorescence was measured in was the volume of the cuvette. Furthermore, the number of cells on a monolayer is usually smaller. Thus, leakage of Fura-2 from lysis of cell monolayers resulted in a marked dilution of Fura-2 more than is usually encountered with a cell suspension (from ≃ 85 mM intracellularly to ≃ 0.1 mM in the cuvette media). Dilution of Fura-2 decreased fluorescent signals at both wavelengths to near the autofluorescent levels even though $[Ca^{2+}]$ was greater in the extracellular media than the cytosol. Such dramatic reduction in fluorescent intensities was not observed in our experiments with taurodeoxycholate, suggesting that there was no significant lysis of the cells or significant leakage of Fura-2 from the cells. Another experiment further demonstrates that leakage of Fura-2 from the monolayers during our experiments, if occurring, could not increase the fluorescent signal significantly. In this experiment, we monitored Fura-2 fluorescence after incubation of a monolayer with 0.5 mM taurodeoxycholate. Subsequently, we removed the monolayer and monitored the decrease in fluorescence in the cuvette's medium following the addition of 1 mM $MnCl_2$. The addition of $MnCl_2$ caused no significant reduction in fluorescence in the cuvette's medium (data not shown). Because Mn^{2+} quenches the Fura-2 signals, our findings that the $MnCl_2$ had no effect indicated that there was no measurable signals attributable to Fura-2 leakage in the media. A similar negative finding was observed in controls without the incubation with taurodeoxycholate.

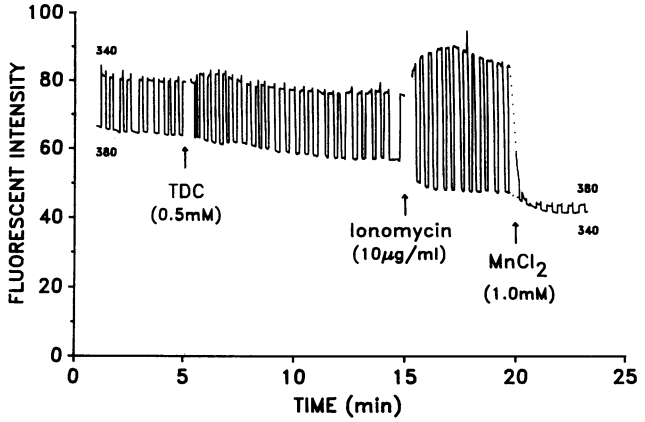

Figure 1. Measurement of free cytosolic Ca^{2+} in T_{84} monolayers. The figure shows a representative tracing from a Fura-2-loaded monolayer prepared as described in Methods on a polystyrene support placed in a standard 10-mm plastic cuvette. Horizontal tracings show the intensity of the emission at 505 nm from excitation at 340 nm or 380 nm as indicated. Vertical tracings resulted from the movement of the pen while excitation monochromator settings were changed manually approximately every 10 s with the wavelength drive control. The results are shown as relative fluorescent intensity over the time course of the study. Dotted lines represent the time that tracings were interrupted to add 0.5 mM taurodeoxycholate, 10 μg/ml ionomycin or 1 mM $MgCl_2$ at 5, 15, or 20 min, respectively. Autofluorescent levels at both wavelengths were determined at the end of the experiment after the addition of 1 mM $MnCl_2$ to quench the signals. The experimental ratio, R, is obtained by dividing the fluorescent intensity at 340 nm by that at 380 nm after subtracting the respective autofluorescent levels. Taurodeoxycholate caused a small increase in the ratio of fluorescence at 340 nm and 380 nm reflecting an increase in $[Ca^{2+}]_i$ as summarized in Fig. 11, whereas ionomycin caused a much larger increase. 10 μg/ml ionomycin increased $[Ca^{2+}]_i$ by 1,835±380 nM (mean±SE, n = 6) 3 min after the addition.

We have also demonstrated that Fura-2 did not interact with taurodeoxycholate or the T_{84} monolayer itself. After taurodeoxycholate was added to cuvettes containing Fura-2 without a monolayer, there was no change in the fluorescent signals compared to before the addition (data not shown). Taurodeoxycholate was also added to cuvettes containing monolayers, but not loaded with Fura-2 to show that there was no change in fluorescent signals measured at the Fura-2 wavelengths after the addition (data not shown). Therefore, neither leakage of Fura-2 nor interaction of Fura-2 with taurodeoxycholate or the monolayer itself could account for the apparent sustained increase in $[Ca^{2+}]_i$ after the addition of taurodeoxycholate in this study.

Materials. Taurodeoxycholate, taurocholate, and ionomycin were obtained from Calbiochem Biochemicals, La Jolla, CA. Bumetanide was a gift from Dr. P. W. Feit, of Leo Pharmaceutical Products, Ballerup, Denmark. Barium chloride dihydrate was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. Ouabain was from Fluka Chemical Corp., Hauppauge, NY. cAMP and cGMP radioimmunoassay kits and radiolabeled compounds were purchased from New England Nuclear, Boston, MA. Fura-2/AM and Fura-2 pentapotassium salt were purchased from Molecular Probes, Inc., Junction City, OR. PGE_1 was purchased from Sigma Chemical Co., St. Louis, MO. *E. coli* heat stable enterotoxin was kindly provided by Dr. Ralph Giannella, Cincinnati, OH. Carbachol was from ICN Biochemicals, Cleveland, OH. MAPTAM was from Calbiochem-Behring Corp., La Jolla, CA.

Statistical analysis. Student's t tests and analysis of variance were used as indicated (29).

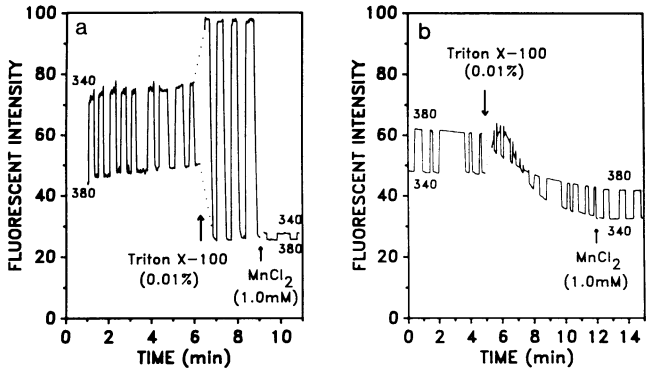

Figure 2. The effect of cell lysis with Triton X-100 on fluorescent signals from Fura-2 loaded cell suspensions and monolayers. Tracings are as described in Fig. 1. (*a*) A representative tracing from a T_{84} cell suspension in a standard 10-mm plastic cuvette. The cuvette contained a 2-ml medium with $\sim 6 \times 10^6$ Fura-2-loaded cells per ml. Intracellular Fura-2 was estimated to be ~ 85 μM. Cells were lysed with 0.01% Triton X-100 to allow the intracellular Fura-2 to leak into the cuvette buffer. Dotted lines represent the time that tracings were interrupted to add Triton X-100. As expected, lysis of cells in suspension led to an increased fluorescent ratio at 340 nm and 380 nm wavelengths reflecting the higher $[Ca^{2+}]_i$ in the medium. 1 mM $MnCl_2$ was added at the end of the experiment to determine the level of autofluorescence. (*b*) A representative tracing from a T_{84} cell monolayer in a cuvette, the monolayer contained $\sim 4 \times 10^5$ cells. In contrast to a cell suspension, the intensities of both fluorescent signals rapidly dropped to approximate the background levels within a few minutes after lysis of the cells by exposure to 0.01% Triton X-100. Addition of 1 mM $MnCl_2$ did not reduce the signals further. These experiments indicate that leakage of Fura-2 from a T_{84} monolayer, if occurring, would reduce the fluorescent intensities and could not account for the persistent increase in $[Ca^{2+}]_i$ observed in this study with taurodeoxycholate.

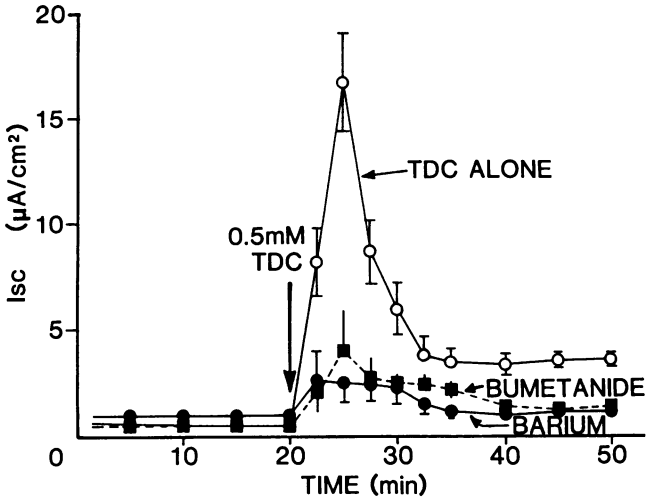

Figure 3. Time course of the I_{sc} response of T_{84} cell monolayer to taurodeoxycholate (TDC). 0.5 mM TDC was added at 20 min after mounting in the absence of an inhibitor (○ -), in the presence of 3 mM $BaCl_2$ (● -), or in the presence of 0.1 mM bumetanide (■ -). The inhibitors if present were added at time zero. Values are means±SE in $\mu A/cm^2$ of at least four experiments each. Both $BaCl_2$ and bumetanide inhibited the action of TDC. 0.1 mM ouabain, added 40 min before the addition of TDC, also inhibited the effect of TDC (see text).

Results

The effect of taurodeoxycholate and taurocholate on electrolyte transport across the T_{84} cell monolayer. The time course of short circuit current (I_{sc}) response, the graded dose effect, and unidirectional isotope flux results are summarized in Figs. 3 and 4 and in Table I, respectively. As noted in Table I, the addition of taurodeoxycholate at 0.5 mM caused an immediate increase in net Cl^- secretion. Conductance across the monolayers also increased. Consistent with an increase in tight junction permeability, there was a trend for unidirectional Na^+ fluxes to increase equally in both directions with time, with net Na^+ flux remaining near zero throughout the study. Based on isotopic flux experiments, the changes in I_{sc} could be totally attributed to net Cl^- secretion. Hence, the changes in I_{sc} were used to monitor the Cl^- secretory response in subsequent experiments. While 0.5 mM taurodeoxycholate caused Cl^- secretion with a corresponding increase in the I_{sc}, an equivalent concentration of taurocholate had no effect on I_{sc} as expected (data not shown). At 0.5 mM, the effects of taurodeoxycholate on I_{sc} and $[Ca^{2+}]_i$ were reversible as shown in Fig. 5.

The increase in monolayer's conductance by taurodeoxycholate, which to a large extent reflected tight junction permeability, was also reversible. Conductance of the monolayers gradually declined to near baseline levels within 10 min after removal of the bile acid from the bathing media. In the experiment shown in Fig. 5, the conductance of the monolayers were 1.1±0.1, 1.4±0.1, and 1.1±0.1 mS/cm² before the addition of taurodeoxycholate, at 10 min after the addition of 0.5 mM taurodeoxycholate and at 10 min after replacement with medium free of the bile salt, respectively. In comparison, con-

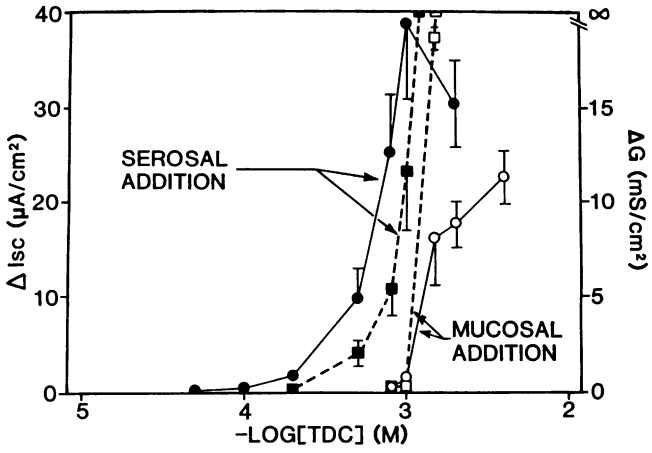

Figure 4. Graded dose effect of taurodeoxycholate (TDC) on the changes in ΔI_{sc} and conductance (ΔG) on T_{84} cell monolayers. Closed figures represent results obtained with serosal addition and open figures with mucosal addition. Values are means±SE in microamperes per square centimeter for ΔI_{sc} (●, ○) or millisiemans per square centimeter for ΔG (■, □) of at least three experiments at each concentration. Each monolayer was exposed to only one concentration of TDC. The peak ΔI_{sc} and corresponding ΔG are shown (corresponding with flux period 2 in Table I). Serosal addition of TDC was more effective than mucosal addition. While the dose-response curve for ΔI_{sc} was to the left of that for ΔG for serosal addition, the ΔG appeared to precede ΔI_{sc} for mucosal addition.

Table I. $^{22}Na^+$ and $^{36}Cl^-$ Flux in Response to 5 × 10^{-4} M TDC in the T_{84} Cell Line

Group	Experimental condition	Flux period	$J^{Na}_{m\to s}$	$J^{Na}_{s\to m}$	J^{Na}_{net}	$J^{Cl}_{m\to s}$	$J^{Cl}_{s\to m}$	J^{Cl}_{net}	I_{sc}	G
I	No addition	1	0.38±0.04	0.35±0.02	0.03±0.04	0.36±0.03	0.41±0.04	−0.05±0.4	0.02±0.01	0.71±0.06
(n = 4)	No addition	2	0.39±0.05	0.33±0.03	0.06±0.05	0.38±0.04	0.35±0.02	0.03±0.05	0.02±0.01	0.68±0.05
	No addition	3	0.36±0.05	0.30±0.05	0.06±0.07	0.39±0.05	0.35±0.02	0.04±0.06	0.02±0.01	0.66±0.05
II	No addition	1	0.27±0.01	0.33±0.05	−0.06±0.05	0.29±0.03	0.34±0.02	−0.05±0.05	0.02±0.01	0.58±0.02
(n = 5)	5 × 10^{-4} M TDC	2	0.31±0.04	0.34±0.06	−0.03±0.04	0.34±0.02*	0.64±0.07*‡	−0.30±0.06*‡	0.29±0.03*‡	0.81±0.04
	5 × 10^{-4} M TDC	3	0.44±0.06*	0.42±0.04*‡	0.02±0.09	0.41±0.03*	0.53±0.05*‡	−0.12±0.07	0.13±0.02*‡	0.83±0.05

Results are expressed as mean±SE in $\mu eq \cdot h^{-1} \cdot cm^{-2}$ except for G which is in $mS \cdot cm^{-2}$; the number of paired monolayers for each experimental group (n) is indicated in parentheses. Period 1 is the average of two consecutive 5-min flux periods starting 10 min after mounting and addition of isotope to the Ussing chambers, and ending just before addition of TDC (10–20 min). Period 2 is the average of three consecutive 5-min intervals starting right after the addition of TDC (20–35 min). Period 3 is the average of two consecutive periods, one 5-min and one 10-min period, starting 15 min after the addition of TDC (35–50 min). * $P < 0.05$ with Student's unpaired t test as compared to the same period of group I. ‡ $P < 0.05$ with Student's paired t test as compared to period 1 in the same group.

trols showed a persistently elevated conductance after the addition of 0.5 mM taurodeoxycholate with corresponding conductances of 1.0±0.1, 1.3±0.2, 1.3±0.2 mS/cm², respectively.

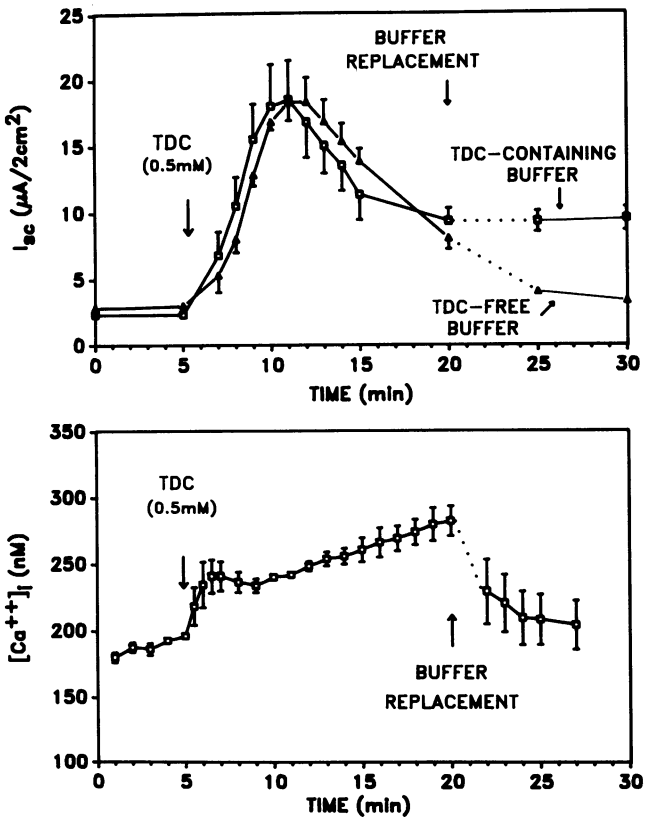

Figure 5. Reversibility of the effect of taurodeoxycholate. The effects of 0.5 mM taurodeoxycholate added at 5 min were shown followed by removal of taurodeoxycholate from the media at 20 minutes when both I_{sc} and $[Ca^{2+}]_i$ reached relatively stable levels. Removal of taurodeoxycholate was achieved by replacing the bathing media with similar media free of taurodeoxycholate. Removal of taurodeoxycholate returned the sustained increase in I_{sc} and $[Ca^{2+}]_i$ to the basal level in ~ 10 and 5 min, respectively. After the removal of taurodeoxycholate, the conductance also gradually reversed back to a basal level within 10 min (see text). Values are means±SE of four experiments for each condition.

The effect of taurodeoxycholate on I_{sc} could be significantly inhibited by 0.1 mM bumetanide or 3 mM $BaCl_2$ when these inhibitors were applied serosally but not mucosally (Fig. 3). Apamin had no effect; the responses to 0.5 mM taurodeoxycholate alone and the responses to taurodeoxycholate in the presence of 1 μM apamin were 21.1±5.3 and 23.8±5.0 μA/cm², respectively (n = 5 each). The graded dose effect of $BaCl_2$ for the inhibition of taurodeoxycholate-induced I_{sc} resembled the graded dose effect for the inhibition of A23187-induced I_{sc} (15, 18). I_{sc} induced by either taurodeoxycholate or A23187 was less sensitive to Ba^{2+} than I_{sc} induced by a cyclic-nucleotide mediated process (Fig. 6). Neither taurodeoxycholate or A23187-induced I_{sc} were sensitive to apamin. Serosal ouabain also significantly reduced the action of the bile salt. The peak I_{sc} response to 0.5 mM taurodeoxycholate was 7.5±0.7 μA/cm² in the presence of 0.1 mM ouabain added 40

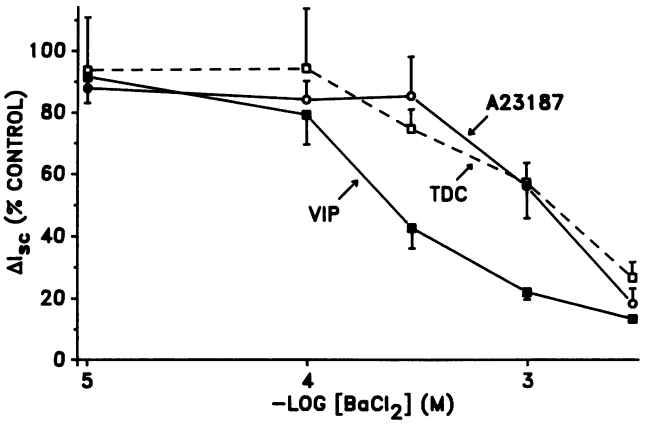

Figure 6. Graded dose effect of $BaCl_2$ for the inhibition of taurodeoxycholate-induced I_{sc}. $BaCl_2$, at the concentration indicated, was added to the basolateral reservoir 15 min before the addition of 0.5 mM taurodeoxycholate. Values are means±SE of four to six experiments and expressed as percentage of the ΔI_{sc} response in controls, which was 17.2±5.6 μA/cm². The graded dose effect of $BaCl_2$ for the inhibition of I_{sc} induced by A23187 and VIP reported elsewhere (15) were included for comparison. The dose response for taurodeoxycholate-induced I_{sc} was not different from that for A23187-induced I_{sc} ($P > 0.5$), but significantly different from that for VIP-induced I_{sc} ($P < 0.01$) by analysis of variance.

min earlier, which was significantly less than the peak response to 0.5 mM taurodeoxycholate alone ($P < 0.05$).

The graded dose effect of taurodeoxycholate on I_{sc} is shown in Fig. 4. Of note is the preferential action of taurodeoxycholate on the basolateral side. Addition of taurodeoxycholate to the basolateral reservoir elicited a response at concentrations as low as 0.2 mM. However, addition of taurodeoxycholate to the apical reservoir had no effect at concentrations below 1 mM. Resistance of the monolayers was minimally affected by concentrations of bile salt below 0.5 mM. However, if the bile salt concentrations were increased above 1 mM, the conductance of the monolayers increased dramatically and rapidly (Fig. 4). This change in conductance coincided with detachment of the cells from the filter support into the bathing medium and the observation of free floating debris. Apical application of taurodeoxycholate, which had no effect at lower concentrations, increased the I_{sc} only at a relatively high concentration of 1 mM or higher. The increase in I_{sc} elicited by apical application, once observed, was near maximal. These findings suggest that apical application of taurodeoxycholate causes Cl^- secretion only when the taurodeoxycholate is in contact with the basolateral membrane.

Effect of taurodeoxycholate on cyclic nucleotide-mediated Cl^- secretion and Ca^{2+}-mediated Cl^- secretion. Since cAMP- or cGMP-mediated secretion and Ca^{2+}-dependent effector mechanisms are synergistic in this cell line, we determined which of these mechanisms potentiated the secretory response of T_{84} cells to taurodeoxycholate. PGE_1, *Escherichia coli* heat stable enterotoxin and carbachol, whose mechanisms of action have been characterized in this cell line (16, 21, 22), were selected as the cAMP-, cAMP-, and Ca^{2+}-mediated secretagogues, respectively. The results of secretory responses shown in Fig. 7 demonstrate a synergistic effect of taurodeoxycholate and *E. coli* heat-stable enterotoxin, and an additive effect of taurodeoxycholate and PGE_1. In contrast, the effects of taurodeoxycholate and carbachol were not additive. The combined effect of taurodeoxycholate and carbachol did not differ from the effect of taurodeoxycholate alone. These findings suggest that carbachol and taurodeoxycholate share similar mechanisms of action, while PGE_1 and *E. coli* heat-stable enterotoxin have different mechanisms of action.

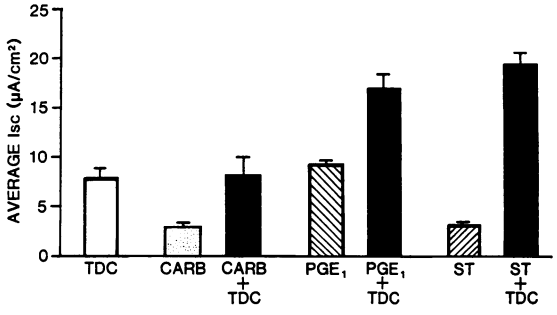

Figure 7. Effect of TDC in the presence of other secretagogues. 0.5 mM TDC was added alone or in the presence of 0.1 mM carbachol (CARB), 10 μM PGE_1 or 0.3 μM *E. coli* heat-stable enterotoxin (ST). The average I_{sc} during the first 15 min after the addition is shown. Values are mean±SE in microamperes per square centimeter of experiments for each condition. The concentrations of agents used, except for taurodeoxycholate, were those that caused maximal I_{sc} responses. The effect of carbachol and taurodeoxycholate combined did not differ significantly from that of taurodeoxycholate alone ($P > 0.2$). In contrast, the effects of PGE, combined with taurodeoxycholate were additive (no significant difference as compared to the predicted additive effect, $P > 0.9$; and larger than the effect of either one alone, $P < 0.05$ each). The effects of *E. coli* heat stable enterotoxin and taurodeoxycholate were synergistic being larger than the predicted additive effect ($P < 0.01$).

We then examined whether the effect of taurodeoxycholate required extracellular Ca^{2+}. Exclusion of Ca^{2+} from the basolateral bathing media (together with the introduction of 1 mM EGTA to the basolateral side) for 5 min decreased the I_{sc} response to 0.5 mM taurodeoxycholate. Without basolateral Ca^{2+}, the I_{sc} response was 5.8±1.4 μA/cm² vs. 22.9±4.4 μA/cm² when Ca^{2+} was present on both sides of the monolayer ($P < 0.05$). The change in conductance was 47.0±7.4 mS/cm² without basolateral Ca^{2+} vs. 1.1±0.6 mS/cm² when Ca^{2+} was present on both sides ($P < 0.05$). Experiments in which basolateral Ca^{2+} was lowered simultaneously with the addition of taurodeoxycholate, a marked attenuation of ΔI_{sc} was still observed. We were unable to measure electrical parameters when Ca^{2+} was totally removed from both sides of the monolayer as the conductance of the monolayer increased rapidly under these conditions. Blockers of the various Ca^{2+} handling systems were then used to further evaluate the source of $[Ca^{2+}]_i$. Dantrolene and TMB-8, compounds which block the release of Ca^{2+} from intracellular stores, were without an effect on taurodeoxycholate-induced I_{sc} as expected. Peak I_{sc} response to 0.5 mM taurodeoxycholate alone, taurodeoxycholate in the presence of dantrolene (saturated solution contained ~ 25 μM dantrolene according to reference 30) and taurodeoxycholate in the presence of 50 μM TMB-8 were 22.3±5.6 ($n = 4$), 21.0±7.8 ($n = 6$), and 17.2±2.3 ($n = 5$) μA/cm², respectively. Verapamil and nifedipine were also used to determine if extracellular Ca^{2+} enter the cell via a Ca^{2+} channel sensitive to these agents. Neither verapamil or nifedipine affect the response of 0.5 mM taurodeoxycholate. Taurodeoxycholate alone, taurodeoxycholate in the presence of 25 μM verapamil, and in the presence of 1 μM nifedipine were 16.0±3.1 ($n = 3$), 18.6±4.7 ($n = 5$), 19.4±3.0 ($n = 5$) μA/cm², respectively.

Measurement of secondary messengers. cAMP, cGMP, and free cytosolic Ca^{2+} were measured. The results of cAMP and cGMP measurement are summarized in Fig. 8. Cyclic nucleotides were not measurably increased by taurodeoxycholate. Taurodeoxycholate itself did not interfere with the radioimmunoassays, and control experiments demonstrated that cAMP and cGMP could be readily increased by PGE_1 and *E. coli* heat stable enterotoxin (ST), respectively.

Free cytosolic Ca^{2+} measurements were carried out with taurodeoxycholate and taurocholate. The time course and graded dose effect of taurodeoxycholate on free cytosolic Ca^{2+} are shown in Figs. 9 and 10. Although the increase in $[Ca^{2+}]_i$ was relatively small, a graded dose effect could be readily observed. Further, free cytosolic Ca^{2+} was increased by taurodeoxycholate with a graded dose effect that correlated approximately with the changes in I_{sc}. In contrast to the increase in free cytosolic Ca^{2+} observed with carbachol and histamine (21, 23, 25), the increase observed with taurodeoxycholate required extracellular calcium and was more persistent similar to those induced by Ca^{2+} ionophores (25). In experiments where monolayers were incubated with 0.5 mM taurodeoxycholate for 15 min, the removal of taurodeoxycholate from the media resulted in the return of $[Ca^{2+}]_i$ to the resting levels within 4 to

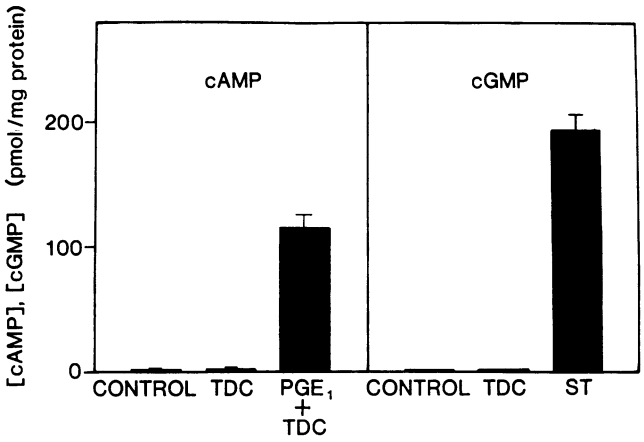

Figure 8. Measurement of cyclic nucleotides. cAMP or cGMP were measured 5 min after the addition of 0.5 mM TDC. Values represent the means±SE of three experiments in each group in picomoles per milligram protein. 0.5 mM TDC added to the radioimmunoassay did not decrease the ability of the assay to measure standards (data not shown). 0.5 mM TDC did not increase cellular cAMP or cGMP. In contrast, 10 μM PGE_1 in the presence of 0.5 mM TDC and 0.25 μM *E. coli* heat stable enterotoxin (ST) increased cAMP and cGMP, respectively, as expected. 10 μM PGE_1, in the absence of TDC, caused a higher increase in cellular cAMP, 341±58 pmol/mg protein ($P < 0.05$) suggesting that taurodeoxycholate partially inhibits adenylate cyclase activities. In addition, no increase in cAMP or cGMP were observed with serosal application of 1 mM TDC or mucosal addition of 2 mM TDC (data not shown).

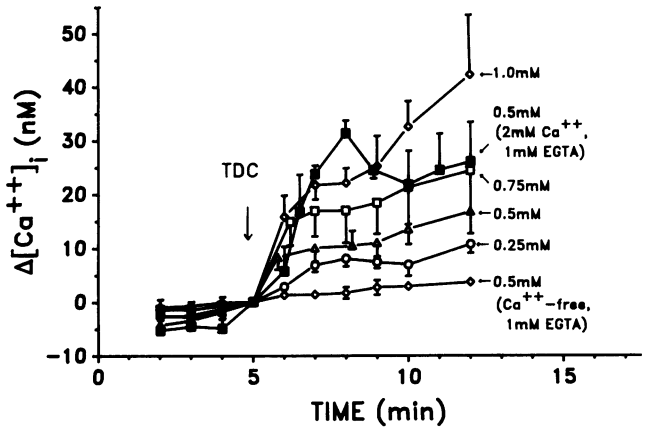

Figure 9. Time course of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) responses in the presence of increasing concentrations of taurodeoxycholate. Free cytosolic Ca^{2+} was measured as described in the Methods using intact monolayers preincubated with Fura-2/AM. The results shown are means±SE of four experiments each and are expressed as the changes of free cytosolic Ca^{2+} from the baseline value before the addition of TDC at the concentrations indicated. The measurements were carried out in the presence of 1 mM $CaCl_2$ in the extracellular bathing medium unless otherwise indicated. 0.5 mM TDC in the Ca^{2+}-free medium containing 1 mM EGTA was without an effect. EGTA did not interfere with the measurement of $[Ca^{2+}]_i$ because in media containing 2 mM $CaCl_2$ and 1 mM EGTA, an effect of 0.5 mM TDC was still observed. In other experiments not shown here, the addition of 1 mM EGTA to the medium containing 1 mM $CaCl_2$ rapidly reduced the apparent $[Ca^{2+}]_i$ by 18±2 nM at 1 min after the addition and remained relatively constant thereafter with $\Delta[Ca^{2+}]_i$ being −21±4 nM 15 min after the addition. Baseline free cytosolic Ca^{2+} values before the addition of TDC was 67±7 nM for the experiments carried out in the presence of 1 mM $CaCl_2$ and 18±2 nM for the experiments in the absence of $CaCl_2$. The results suggest that TDC caused a graded increase in free cytosolic Ca^{2+} only in the presence of extracellular calcium, while taurocholate had no effect even at 1 mM (data not shown).

5 min after the removal (Fig. 5). Taurocholate had no effect on free cytosolic Ca^{2+} as expected (data not shown).

Effect of an intracellular Ca^{2+} chelator on taurodeoxycholate-induced responses. Preincubation of T_{84} monolayers with varying concentrations of MAPTAM for 30 min reduced the $[Ca^{2+}]_i$ and I_{sc} responses to 0.5 mM taurodeoxycholate in parallel. The $[Ca^{2+}]_i$ and I_{sc} responses to taurodeoxycholate were reduced to near zero after T_{84} monolayers were preincubated with 10^{-3} M MAPTAM for 30 min (Fig. 11). In contrast, the monolayers preincubated with 10^{-3} M MAPTAM for 30 min still responded rapidly to 10^{-7} vasoactive intestinal polypeptide (VIP) plus 5 μg/ml ionomycin with an increase in I_{sc} of 24±5 $\mu A/cm^2$ (mean±SE, $n = 4$) 15 min after the addition.

Discussion

We have demonstrated that the cultured T_{84} colonic epithelial monolayer responded to taurodeoxycholate by secreting Cl^- ions and increasing paracellular permeability, while taurocholate had no effect. Besides description of the effects of taurodeoxycholate on Na^+ and Cl^- fluxes and identification of the transport pathways involved in the secretory process, the present study was designed to answer a number of questions pertinent to bile salt-induced secretion: (*a*) Do bile salts exert their effect on the luminal (apical) membrane or the basolateral membrane? (*b*) What is the relationship between Cl^- secretion and epithelial permeability? (*c*) Whether cAMP, cGMP or free cytosolic Ca^{2+} is the secondary messenger involved in bile salt-induced Cl^- secretion? (*d*) How does taurodeoxycholate interact with other endogenous or exogenous secretagogues whose action is mediated by cAMP, cGMP, or Ca^{2+}?

Taurodeoxycholate caused Cl^- secretion across the T_{84} cell monolayers. The changes in I_{sc} fully reflected net Cl^- secretion as demonstrated by unidirectional isotopic flux experiments. The transient nature of the secretory effect of taurodeoxycholate resembles those of other "Ca^{2+} agents" e.g., carbachol, histamine, and A23187 (18, 21, 23), all of which can be potentiated by a cAMP- or cGMP-mediated response as discussed below. Taurodeoxycholate also decreased the resistance of the monolayer but with a slightly more delayed time course compared to its effects on free cytosolic Ca^{2+} and Cl^- secretion. Such delay was obscured at higher concentrations of taurodeoxycholate (≥ 1 mM) when the decrease in resistance occurred more rapidly. Due to an increase in monolayer permeability, there were also gradual increases in unidirectional Na^+ fluxes equally in both directions after the addition of taurodeoxycholate.

Regarding the transport pathways involved in the secretory process, our studies suggest that Cl^- secretion induced by taurodeoxycholate occurs via transport pathways similar to those previously characterized in T_{84} cells for A23187 (15). Using specific blockers, we have demonstrated that Cl^- secretion induced by the bile salt involved the bumetanide-sensitive Na^+,K^+,Cl^- cotransport pathway, the ouabain-sensitive

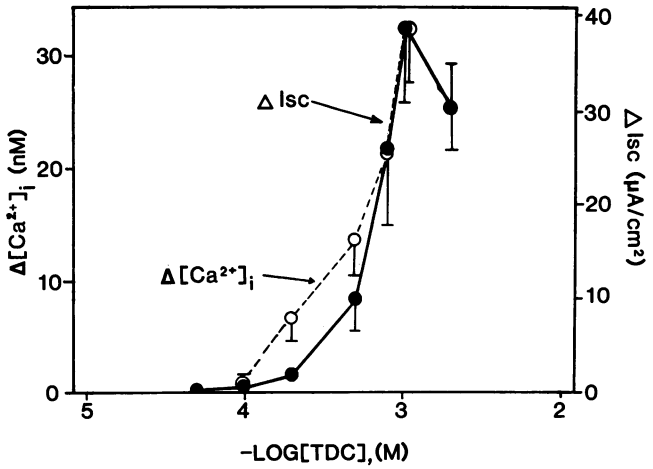

Figure 10. Graded dose effect of TDC on $\Delta[Ca^{2+}]_i$ (o -). The increases in $[Ca^{2+}]_i$ at 5 min after the addition, which in many cases were near the peak values, were used for calculation. The spontaneous increases in $[Ca^{2+}]_i$ at 5 min before the addition, if present, were subtracted. Values are means±SE of four experiments at each concentration of TDC. The peak increases of I_{sc} within 10 min after the addition of TDC (same as in Fig. 4) were included for comparison. Concentrations of TDC > 1 mM disrupted the monolayers causing interference with fluorescent measurements and, therefore, are not shown. The graded dose effect for $\Delta[Ca^{2+}]_i$ approximates those of ΔI_{sc}.

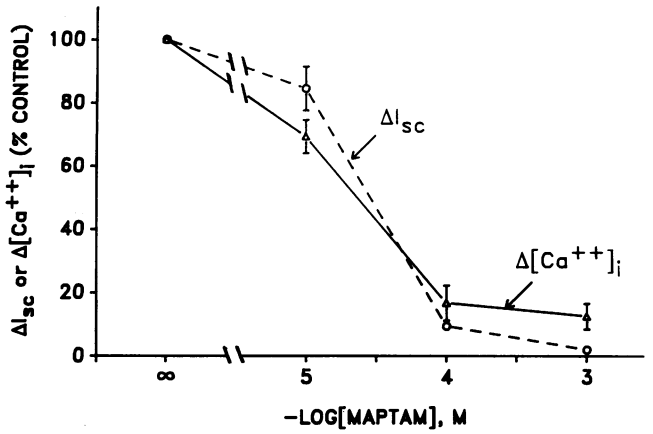

Figure 11. Graded dose effect of MAPTAM for the inhibition of $[Ca^{2+}]_i$ and I_{sc} responses induced by 0.5 mM taurodeoxycholate. Preincubation of T_{84} monolayers with MAPTAM were at the concentrations indicated for a duration of 30 min as described in Methods. Following the preincubation steps, the measurements of $[Ca^{2+}]_i$ and I_{sc} in response to taurodeoxycholate were carried out in the same manner as for other $[Ca^{2+}]_i$ measurements. The peak increase in I_{sc} and $[Ca^{2+}]_i$ at 10 and 5 min after the addition of 0.5 mM taurodeoxycholate were used for calculation. For $[Ca^{2+}]_i$, the spontaneous increase in $[Ca^{2+}]_i$ 5 min before the addition, if present, were subtracted. Values are means±SE of four to eight experiments at each concentration of MAPTAM and are expressed as a percentage of the $\Delta[Ca^{2+}]_i$ and ΔI_{sc} response in controls which were 51±8 nM and 19±5 $\mu A/cm^2$, respectively. MAPTAM reduced the $[Ca^{2+}]_i$ and I_{sc} response to 0.5 mM taurodeoxycholate in parallel with changes in I_{sc} approximated those of $[Ca^{2+}]_i$. Monolayers preincubated with 10^{-3} MAPTAM for 30 min, which did not respond to 0.5 mM taurodeoxycholate, still responded markedly to 10^{-7} M VIP plus 5 μg/ml ionomycin (see text).

Na^+,K^+-ATPase pump, and the Ca^{2+}-dependent K^+ channel. Involvement of the apical Cl^- channel (19) can be postulated since an electrogenic Cl^- secretion was observed. Our finding that taurodeoxycholate activates a K^+ channel which is relatively resistant to barium, similar to that activated by A23187 (15, 18) supports a role for $[Ca^{2+}]_i$ as discussed below.

Whether bile salts exert their effect on the apical or basolateral membrane was investigated by comparing the graded dose effect of taurodeoxycholate in the apical and basolateral reservoirs and the relationship between Cl^- secretion and epithelial permeability. The lower threshold for the I_{sc} response to taurodeoxycholate after application to the basolateral side (Fig. 4) suggests that taurodeoxycholate exerts its effect on Cl^- secretion by activating a membrane-related mechanism localized at the basolateral side. Application of taurodeoxycholate to the basolateral reservoir caused a detectable change in I_{sc} at a concentration as low as 0.2 mM. In contrast, taurodeoxycholate applied apically had no effect at this relatively low concentration. It was only at a critical concentration of 1 mM or higher that apical taurodeoxycholate caused a secretory response. At these higher concentrations, taurodeoxycholate rapidly and dramatically increased the conductance of the monolayer. The increase in monolayer permeability appeared to precede or parallel the changes in I_{sc}, with apical application, whereas with basolateral application, the monolayer permeability increased slightly later in the time course, especially with lower concentrations (≤ 0.5 mM). The increased permeability may result from disruption of the tight junctions between cells since, as time passed, free floating debris was observed. The results suggest that luminal application of bile salts causes Cl^- secretion by first disrupting the tight junction of the monolayer, thereby allowing the bile salts to reach the basolateral membrane where they may then activate a Cl^- secretory mechanism. The results are similar to observations in isolated intestinal tissues where the threshold concentration of bile acids that causes secretion was lower during serosal application compared to mucosal application (31). The data would also explain in vivo observations that water and electrolyte secretion in response to bile acids is closely related to enhancement of mucosal permeability (3, 4).

Regarding the secondary messengers involved in bile acid-induced secretion, we measured cAMP, cGMP, and $[Ca^{2+}]_i$. In contrast to previous studies in the intestine, we did not observe an increase in cellular cAMP in response to taurodeoxycholate (2, 9). Our studies are in agreement with some in vitro studies where no rise in mucosal cAMP was observed (32), and with the observations that bile acids actually inhibit adenylate cyclase (33, 34). The lack of a cAMP response in our studies is not due to a possible interference of the bile acid with the assay because taurodeoxycholate added to the radioimmunoassay did not decrease the ability of the assay to measure standards. Thus, it is interesting that the cAMP content in the presence of both taurodeoxycholate and PGE_1 was lower than that observed after exposure to PGE_1 alone. A possible explanation for this phenomenon is the inhibition of adenylate cyclase activity by taurodeoxycholate (33, 34) which may be secondary to an increase in protein kinase C activities. The activities of protein kinase C, which increased with taurodeoxycholate (35), have been shown to blunt PGE_2-induced increase in cAMP in T_{84} cells (36).

Because our studies indicated that cyclic nucleotides are not intracellular mediators of the taurodeoxycholate-stimu-

lated Cl^- secretory response, we next investigated whether intracellular Ca^{2+} mediates this response. Several findings in the present study suggest that $[Ca^{2+}]_i$, at least in part, mediates the taurodeoxycholate-stimulated Cl^- secretory response. First, taurodeoxycholate increased $[Ca^{2+}]_i$ as well as the I_{sc} responses. Removal of taurodeoxycholate reversed changes in both $[Ca^{2+}]_i$ and I_{sc} to basal levels. Further, the dose-response curves for taurodeoxycholate-induced increases in $[Ca^{2+}]_i$ and I_{sc} were superimposed. Thus, there was a good correlation between $[Ca^{2+}]_i$ and I_{sc} responses. Second, both responses were dependent on extracellular Ca^{2+}. Removal of extracellular Ca^{2+} abolished the $[Ca^{2+}]_i$ response and at least dramatically diminished (or possibly also abolished) the I_{sc} response. These findings suggest that taurodeoxycholate increases the flux of Ca^{2+} across the plasma membrane into the cytosol and this increase in Ca^{2+} flux contribute to the I_{sc} response. Third, like the other "Ca^{2+}-agents" (i.e., carbachol, histamine, A23187), taurodeoxycholate augmented the I_{sc} response to PGE_2 and *E. coli* heat stable enterotoxin but did not add to the I_{sc} response caused by carbachol. Fourth, taurodeoxycholate activated a "Ca^{2+}-dependent" K^+ channel, which was less sensitive to barium, similar to A23187. Fifth, although taurodeoxycholate increased $[Ca^{2+}]_i$ by only $\simeq$ 30 nM, our studies with carbachol and histamine demonstrates that these agents increased I_{sc} when $[Ca^{2+}]_i$ was increased within this range of magnitude (25). Finally, an intracellular Ca^{2+} chelator, MAPTAM, inhibited and abolished the $[Ca^{2+}]_i$ as well as the I_{sc} response induced by taurodeoxycholate in parallel in a dose-dependent manner.

The above findings suggest a role for Ca^{2+} in the taurodeoxycholate-stimulated Cl^- secretory response, however, our data suggests that Ca^{2+} is not the only intracellular messenger responsible for the effect. There was not a direct relationship in the time course of the responses between an increase in $[Ca^{2+}]_i$ and I_{sc} caused by taurodeoxycholate. This is also true for carbachol and histamine. This suggests that for these agents, Ca^{2+} probably accounts for only part of the response with other "messengers" serve as a coactivator. In fact, other data indicates that a much larger increase in $[Ca^{2+}]_i$ alone is required to elicit a given I_{sc} response by 4-Br-A23187 (25). The nature of other intracellular messengers mediating the remainder of the response with agents such as taurodeoxycholate, histamine, and carbachol remains to be investigated. Potential candidates include arachidonic acid (37) and protein kinase C (38, 39).

In summary, the findings in the present study demonstrate that taurodeoxycholate but not taurocholate causes Cl^- secretion from the T_{84} cell line by specifically interacting with the basolateral membrane of the T_{84} monolayer. The intracellular mechanism is not mediated by cyclic nucleotides but is in part mediated by flux of extracellular Ca^{2+} into the cytosol of the T_{84} cell resulting in an increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ then promotes the opening of a Ca^{2+}-dependent K^+ channel on the basolateral membrane to increase Cl^- secretion.

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