

Mutual Enhancement of Virulence by Enterotoxigenic and Enteropathogenic *Escherichia coli*

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Enterotoxigenic *Escherichia coli* (ETEC) and enteropathogenic *E. coli* (EPEC) are common causes of diarrhea in children in developing countries. Dual infections with both pathogens have been noted fairly frequently in studies of diarrhea around the world. In previous laboratory work, we noted that cholera toxin and forskolin markedly potentiated EPEC-induced ATP release from the host cell, and this potentiated release was found to be mediated by the cystic fibrosis transmembrane conductance regulator. In this study, we examined whether the ETEC heat-labile toxin (LT) or the heat-stable toxin (STa, also known as ST) potentiated EPEC-induced ATP release. We found that crude ETEC culture filtrates, as well as purified ETEC toxins, did potentiate EPEC-induced ATP release in cultured T84 cells. Coinfection of T84 cells with live ETEC plus EPEC bacteria also resulted in enhanced ATP release compared to EPEC alone. In Ussing chamber studies of chloride secretion, adenine nucleotides released from the host by EPEC also significantly enhanced the chloride secretory responses that were triggered by crude ETEC filtrates, purified STa, and the peptide hormone guanylin. In addition, adenosine and LT had additive or synergistic effects in inducing vacuole formation in T84 cells. Therefore, ETEC toxins and EPEC-induced damage to the host cell both enhance the virulence of the other type of *E. coli*. Our in vitro data demonstrate a molecular basis for a microbial interaction, which could result in increased severity of disease in vivo in individuals who are coinfecting with ETEC and EPEC.

Along with rotavirus, enterotoxigenic *Escherichia coli* (ETEC) and enteropathogenic *E. coli* (EPEC) are among the most common causes of diarrhea in children in developing countries (29). Dual infections with both pathogens have been noted fairly frequently in studies of diarrhea around the world for many years (41, 42). More recent studies of diarrhea etiology have begun to describe multiple infections carefully, and evidence is emerging that patients with multiple pathogens are likely to experience more-severe diarrheal disease (2, 8, 24, 35, 40, 59).

One might expect dual infections with EPEC and ETEC to occur occasionally by chance alone, especially in poverty-stricken areas with poor hygiene. However, studies of diarrhea etiology in areas with good sanitation also noted dual infections with EPEC and ETEC. The tendency of EPEC and ETEC to occur in multiple infections has been noted in epidemiology surveys in developed countries such as northern Italy (8), Israel (24), and Yugoslavia prior to the Balkan war (13). EPEC and ETEC coinfection was also found in an investigation of a hospital outbreak in Durban, South Africa (1). Several of the children in the latter outbreak had a triple infection with EPEC, ETEC, and rotavirus.

Recent work from our laboratory suggested a molecular mechanism by which EPEC and ETEC might interact. Specifically, we found that cholera toxin (CT) and forskolin enhance EPEC-induced ATP release from the host cell (15) without any enhancement of EPEC-mediated cell killing. In a subsequent study, we determined that cholera toxin and forskolin enhance

cystic fibrosis transmembrane conductance regulator (CFTR)-dependent release of ATP from the host cell (14). CT and forskolin raise cyclic AMP (cAMP) levels in the host cell, activate cAMP-dependent protein kinase, and result in the phosphorylation and activation of CFTR (28, 45). Since the ETEC heat-labile toxin (LT) and ETEC heat-stable toxin STa (also called ST) also act by elevating cyclic nucleotides in the host cell, we suspected that ETEC toxins or ETEC infection might enhance EPEC-induced ATP release. In addition, we wished to determine if the chloride secretory responses triggered by adenine nucleotides in epithelial tissues were additive with those triggered by ETEC toxins.

MATERIALS AND METHODS

Bacterial strains used. Four well-studied classic human EPEC strains were used in this investigation: E2348/69 (serotype O127:H6), B171-8 (O111:NM), JCP88 (O119:B14) and E851/71 (O142:H6), as described in several publications (4, 23, 37, 46, 47). Enterotoxigenic *E. coli* strain H10407 (serotype O78: H11), which produces both LT-I and the heat-stable enterotoxin (STa), was used as a prototype ETEC strain and was a gift from James Fleckenstein, University of Tennessee (26). A genetically altered version of strain H10407 was also provided by James Fleckenstein, who had constructed a deletion of the gene encoding the A subunit of LT, *eltA*. James Fleckenstein designated the LT-deleted strain JF570, but in this report we have referred to it as H10407 Δ LT.

Materials. The following reagents were obtained from Sigma-Aldrich Chemicals (St. Louis, Mo.): α , β -methylene-ADP, creatine kinase, phosphocreatine, forskolin, adenosine, AMP, ADP, type III collagen, *E. coli* heat-stable toxin, carbachol, and phosphatidylinositol-specific phospholipase C (PI-PLC, from *Bacillus cereus*). Guanylin was purchased from Bachem Bioscience, Inc. (King of Prussia, Pa.), and cholera toxin was from List Biological Laboratories (Campbell, Calif.). U73122, an inhibitor of PI-PLC, was from Biomol (Plymouth Meeting, Pa.). Purified LT-II toxins were endotoxin free and were gifts from Terry Connell, Department of Microbiology, University at Buffalo.

Preparation of sterile filtrates of overnight cultures of ETEC strains. Strain H10407 or H10407 Δ LT were grown overnight in Casamino Acids-yeast extract medium (CAYE) supplemented with trace minerals. CAYE medium was 2%

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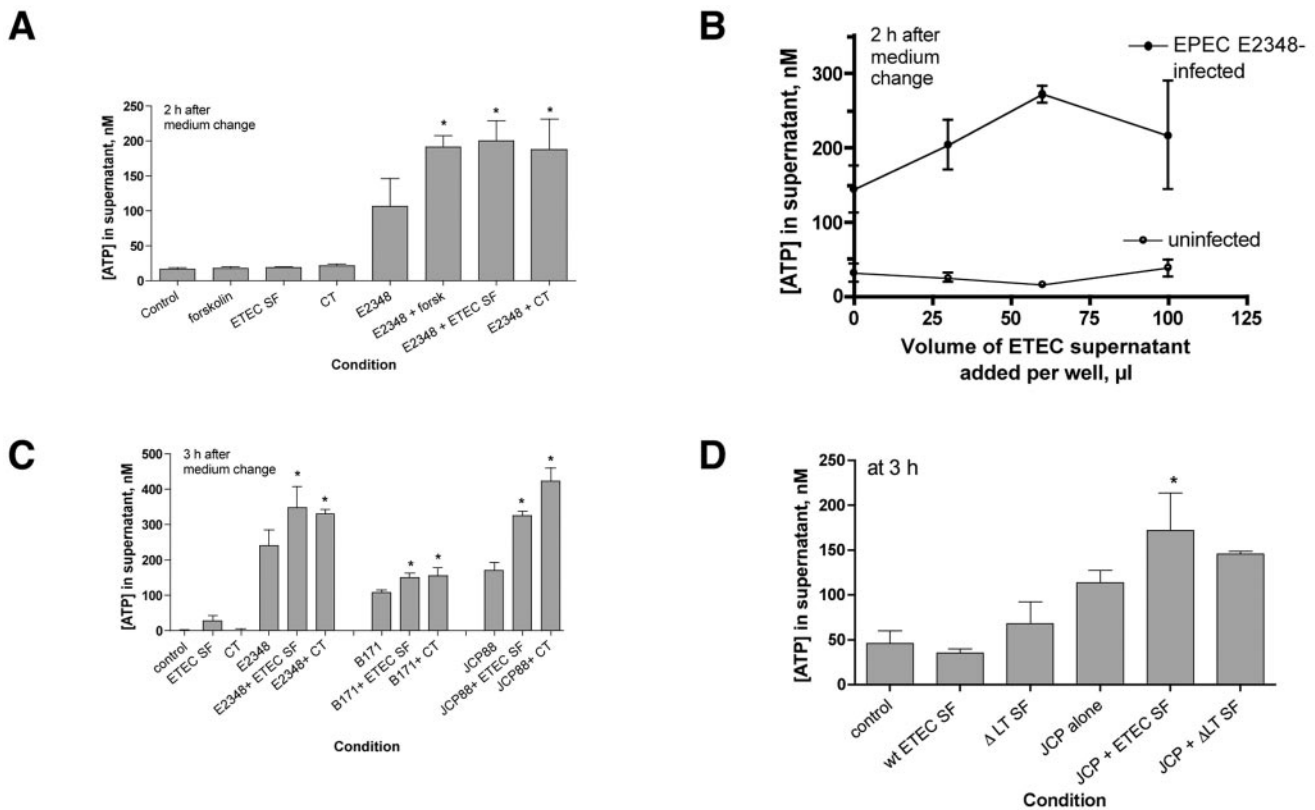


FIG. 1. Effect of ETEC culture filtrates on EPEC-induced ATP release from host cells. Culture filtrates were prepared from overnight cultures of ETEC strain H10407 or H10407 Δ LT (D) in CAYE medium as described in Materials and Methods. ETEC sterile filtrates were applied to T84 cells in 96-well plates for 18 to 20 h (60 μ l per well unless otherwise indicated). The medium was changed to serum-free medium without antibiotics, and cells were infected with EPEC at an MOI of 100:1. After 45 min to allow EPEC adherence, the medium was changed again to include an ATP-regenerating system (creatine kinase plus phosphocreatine) and the nucleotidase inhibitor α,β -methylene-ADP; incubation was continued for 2 to 3 h as shown on the figures. Then, the plates were swirled to allow mixing to occur; aliquots were collected, subjected to filtration via a 0.45- μ m filter to remove bacteria or detached host cells, and assayed for ATP. Abbreviations: forsk, 20 μ M forskolin added the day of the experiment; ETEC SF, ETEC sterile filtrate; CT, 200-ng/ml cholera toxin; E2348, EPEC strain E2348/69; B171, EPEC strain B171-8; JCP, EPEC strain JCP88; wt, wild type; ALT SF, sterile filtrate prepared from strain H10407 Δ LT.

Casamino Acids, 0.6% yeast extract, 43 mM NaCl, 38 mM K_2HPO_4 , 0.25% glucose, and 0.1% trace minerals as previously described (55). Cultures were at 37°C with shaking at 300 rpm. The overnight cultures were centrifuged at 3,000 \times g for 10 min to pellet the bulk of the bacteria, and then the supernatant was filtered through a 0.45- μ m syringe-tip filter. Aliquots of this sterile filtrate were made and frozen at -70°C and used for subsequent experiments up to 2 months after preparation.

Ussing chamber studies. Ussing chamber studies of secretion were performed on T84 cell monolayers grown in Snap-Well inserts (Corning Costar, Corning, NY). Caco-2 cells were also used for some Ussing chamber studies and gave similar results, but these results are not shown. The Snap-Well inserts, which had a 0.4- μ m pore size, were coated with 32 μ g collagen per well by the application of 0.16 ml of 0.2-mg/ml type III collagen (Sigma; dissolved in warm 0.2 M acetic acid) to the Snap-Well and allowing it to dry in the tissue culture hood under UV light.

T84 cells were seeded onto the Snap-Well inserts at $\sim 1.2 \times 10^6$ cells per well and allowed to grow to confluence for 7 to 9 days. At this time, the monolayers had transepithelial electrical resistances (TER) of 400 to 1,000 $\Omega \cdot \text{cm}^2$.

A Snap-Well insert containing a monolayer of T84 cells was placed in the plexiglass "slider" and inserted into the Ussing chamber (Physiologic Instruments, San Diego, CA) at 37°C and continuously short circuited by a four-electrode, automatic voltage clamp apparatus which measured short-circuit current (I_{sc}) and TER; chamber fluid resistance was automatically subtracted. The voltage clamp apparatus used was from Physiologic Instruments, model VCC MC6. Transepithelial resistance was determined by passing 10 s of 10-mV current pulses through the tissues. Short-circuit current was measured by passing sufficient current through the tissues via Ag-AgCl electrodes to reduce the

spontaneous transepithelial potential to 0. The composition of the tissue bathing solution was (in micromoles): 140 Na^+ , 124 Cl^- , 21 HCO_3^- , 5.4 K^+ , 2.4 HPO_4^{2-} , 0.6 $H_2PO_4^-$, 1.2 Mg^{2+} , 1.2 Ca^{2+} , and 10 glucose. The pH of this solution was 7.4 when gassed with a mixture of 95% O_2 to 5% CO_2 . To help maintain adhesion of the monolayer to the Snap-Well insert, the apical side of the chamber was filled with 5 ml of this bath solution, and the basolateral compartment was filled with 4 ml of bath solution. The chambers were bubbled slowly with a 95% O_2 to 5% CO_2 gas mixture.

Ussing chamber current and resistance data were collected in digital form on a Dell PC computer using Acquire & Analyze 2.0 software that accompanied the instrument. Raw I_{sc} values were converted to microamperes per square centimeters by dividing by the area of the monolayer (1.13 cm^2).

ATP release. ATP release was measured as previously described (14, 15) by preparing sterile filtrates of supernatant medium collected in the presence of an ATP-regenerating system and a nucleotidase inhibitor (α,β -methylene-ADP) and assaying for ATP with a luciferase luminescence assay kit (Roche Applied Science, Indianapolis, Ind.). The ATP-regenerating system is included to trap released ATP and prevent its degradation by nucleotidases. Some experiments (Fig. 1 and 2B) were done using cells grown in 96-well plates with 0.2 ml of medium per well; therefore, absolute ATP levels were lower than in other experiments performed in 48-well plates.

Vacuole formation studies. T84 cells were grown to near confluence on Permax plastic Lab-Tek chamber slides (Nunc-Intermed, Naperville, Ill.). Cells were treated with toxins or adenosine or inhibitors, fixed, stained with Giemsa stain as previously described (16), and then photographed at $\times 250$ magnification without oil. Latex beads (2 μ m in size; Sigma) were used for size determination.

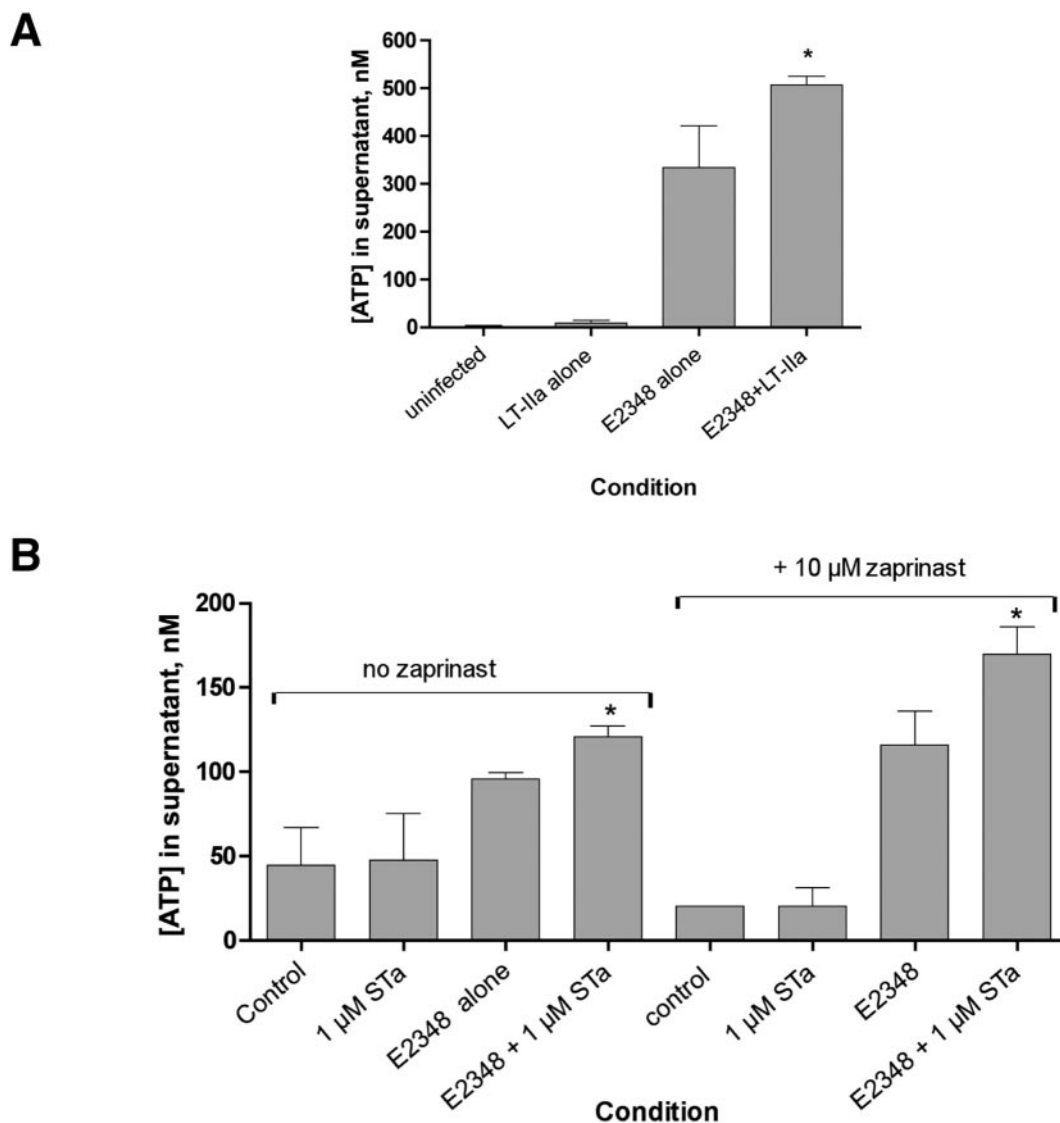


FIG. 2. Effect of purified *E. coli* enterotoxins on EPEC-induced ATP release. T84 cells were grown in 48-well (A) or 96-well (B) plates. (A) Purified, pyrogen-free LT-IIa toxin at 720 ng/ml was applied overnight; on the next day, the medium was changed as described in the legend to Fig. 1 and in Materials and Methods. After EPEC infection, supernatant medium was collected 2 h after the medium change. (B) Purified STa was applied to some wells just before infection with EPEC strain E2348/69, and then STa was added again to the same 1 μ M concentration after the medium change. The phosphodiesterase inhibitor Zaprinast (10 μ M) was added to some wells after the medium change (B, right). Supernatants were collected 3 h after the medium change shown in panel B.

Data analysis and presentation. All error bars shown in graphs and error values reported in the text are standard deviations (SDs). Significance was tested by one-way analysis of variance with the Tukey-Kramer posttest for multiple comparisons, using InStat software for Macintosh from GraphPad software (San Diego, Calif.). Graphs were prepared using Prism 4.0 software, also from GraphPad. Asterisks shown on graphs indicate a *P* value of <0.05.

RESULTS

In initial experiments, we tested whether a crude sterile filtrate of ETEC culture medium could potentiate EPEC-induced ATP release. As with cholera toxin and forskolin, ETEC sterile filtrate alone did not trigger any ATP release from T84 cells (Fig. 1A, left). However, the ETEC sterile filtrate did potentiate EPEC-induced ATP release, and the magnitude of

the potentiation was similar to that observed with full doses of cholera toxin and forskolin (Fig. 1A). When tested on T84 cell wells containing 200 μ l of medium, the enhancing effects of ETEC sterile filtrate peaked when about 60 μ l of filtrate was applied per well and then diminished if a larger volume was applied (Fig. 1B). The enhancing effect of ETEC sterile filtrate was observed with all wild-type EPEC strains tested (E2348/69, B171-8, and JCP88) (Fig. 1C), as well as EPEC strain E851/71 (Fig. 3). When a sterile filtrate was prepared from strain H10407 Δ LT, the enhancing effect on ATP release appeared to be less than that of the wild-type ETEC filtrate (Fig. 1D), suggesting that the heat-labile toxin is an important, biologically active component of the sterile filtrate in filtrates of strain H10407.

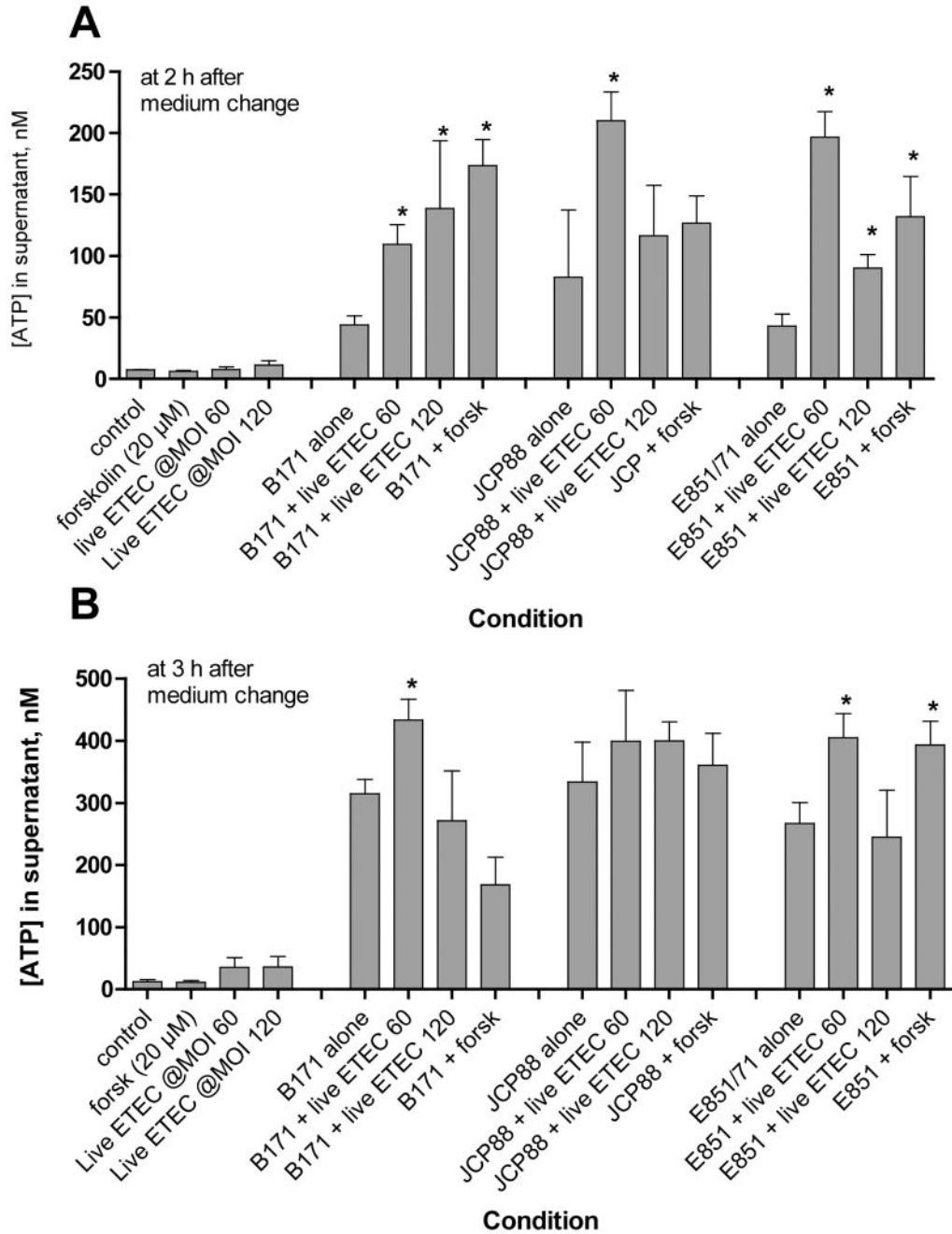


FIG. 3. Effect of infection with live ETEC on EPEC-induced ATP release from T84 cells. ETEC strain H10407 was grown overnight in CAYE medium, and then T84 cells were infected with 2.5 μl or 5 μl of overnight culture, resulting in an ETEC MOI of 60 or 120, respectively. Overnight cultures of the EPEC strains, in contrast, were subcultured for 2 h in serum-free Dulbecco's modified Eagle's medium and then used to infect T84 cells in 48-well plates. The MOIs were 140, 260, and 120 for EPEC strains B171-8, JCP88, and E851/71, respectively. In this experimental design, therefore, the ETEC had a 2-h "head start" relative to the EPEC strains. Supernatants were collected at 2 and 3 h for the ATP assay as shown in the graphs.

Figure 2A shows that purified heat-labile toxin, in this case LT-IIa, also potentiated EPEC-induced ATP release from host cells in a manner similar to that of the crude sterile filtrate. When purified STa toxin was tested by this assay, it was also able to enhance EPEC-induced ATP release (Fig. 2B); this enhancement was of even greater magnitude when an inhibitor of cyclic GMP phosphodiesterase, Zaprinast, was included in

the culture medium (Fig. 2B, right). Therefore, purified cholera toxin, a culture filtrate containing LT-I, purified LT-IIa, and purified STa were all able to potentiate EPEC-induced ATP release from T84 cells in this assay (Fig. 1 and 2).

The increased EPEC-induced ATP release by CT, LT, ETEC sterile filtrates, and STa toxin was not due to an increase in EPEC adherence to toxin-treated cells. EPEC adher-

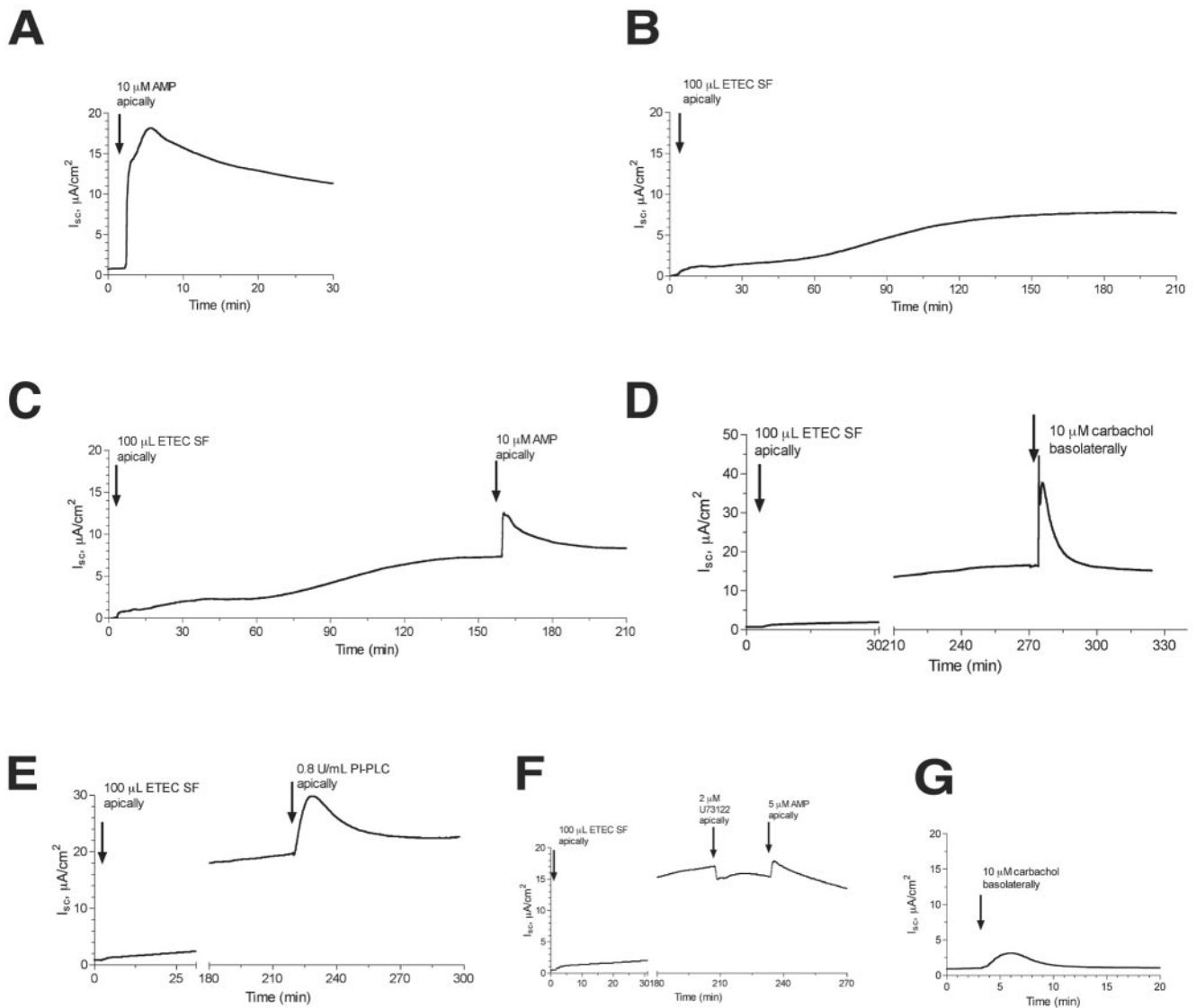


FIG. 4. Additive effects of AMP and ETEC toxins on short-circuit current in T84 cell monolayers in the Ussing chamber. T84 cells were grown on collagen-coated Snap-Well culture inserts as described in Materials and Methods for 7 to 9 days until they reached confluence and a high transepithelial resistance. AMP, ETEC toxins, and PI-PLC were applied on the apical (i.e., mucosal or luminal) side of the monolayer, while carbachol was applied on the basolateral side. U73122 is an inhibitor of PI-PLC. Raw data (current, voltage, and resistance) were collected in a digital file and then imported into GraphPad Prism for the creation and labeling of the graphs shown. All experiments shown were repeated at least three times, and representative tracings are shown. Using this cell line, preparation, and electrode configuration, a positive short-circuit current (shown as an upward deflection on the graph) represents chloride secretion toward the apical side of the tissue.

ence was the same in toxin-treated as in control T84 cells, as assessed by a visual adherence assay and by a quantitative adherence assay (measured as total cell-associated bacteria) (data not shown).

In the experiments shown in Fig. 1 and 2A, we applied ETEC sterile filtrates or purified LT or CT to the monolayers overnight to allow time for the full intoxication of cells before challenge with EPEC infection. We also wished to test whether infection with live ETEC bacteria would potentiate EPEC-induced ATP release. Figure 3 shows the result of one such experiment in which live ETEC bacteria were allowed to infect the T84 monolayer for 2 h before the cells were infected with

the indicated EPEC strain. As shown in Fig. 3, potentiation of ATP release in this dual infection was best observed at early times after EPEC infection and was most obvious when the ETEC infection was at a low multiplicity of infection (MOI) of 60:1. At later times or when a high ETEC MOI was used, ETEC potentiation of EPEC-induced ATP release was less prominent or was not observed at all (Fig. 3B and data not shown).

The results of Fig. 1 to 3 indicated that ETEC toxins could potentiate one type of cellular damage caused by EPEC, namely, ATP release. We also wondered if the interaction might act in the other direction, i.e., whether nucleotides re-

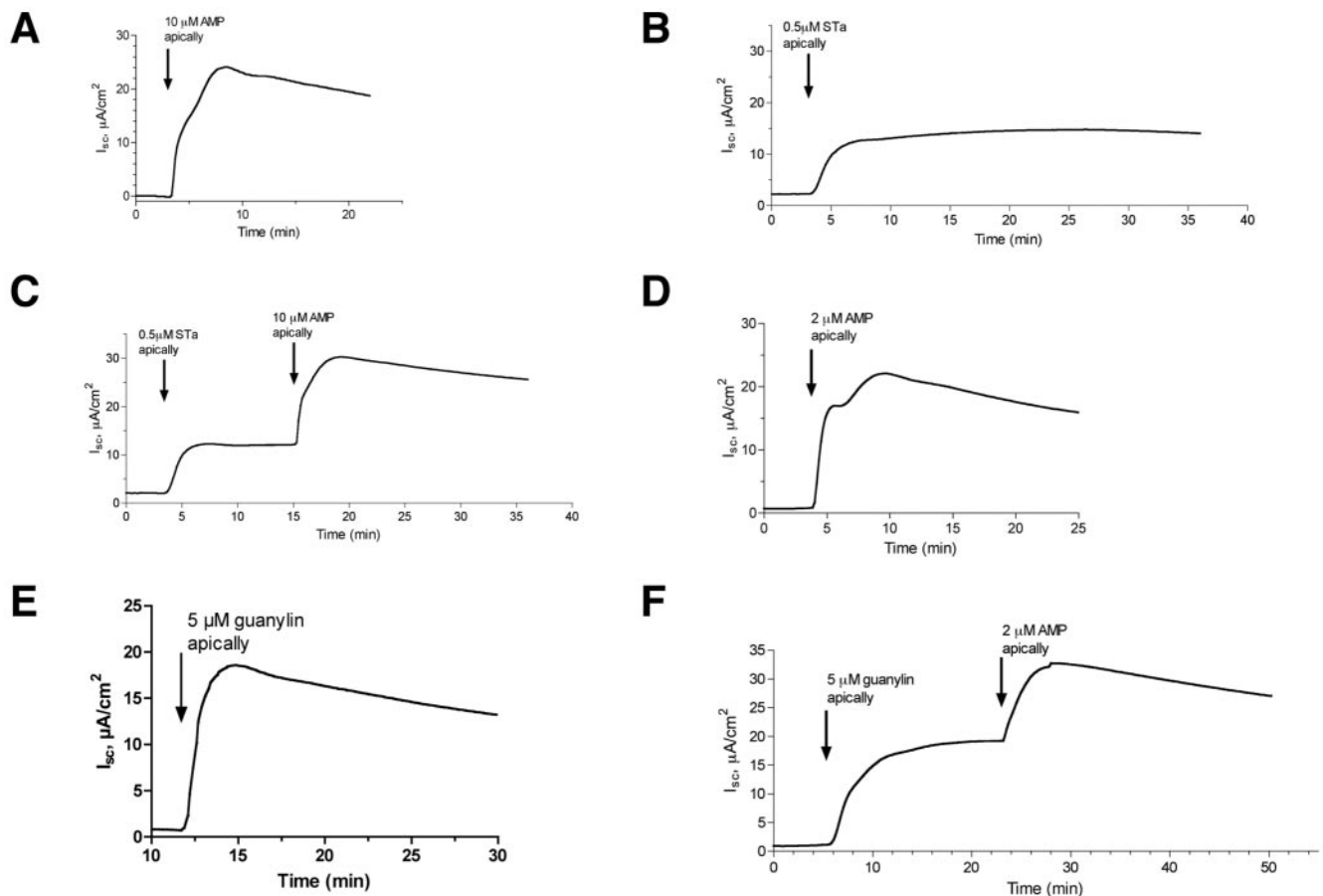


FIG. 5. Additive effects of AMP and STa toxin and guanylin on short-circuit current in T84 cell monolayers in the Ussing chamber. T84 cells were grown on collagen-coated Snap-Well culture inserts, as described in Materials and Methods and in the legend to Fig. 4. (A to C) Secretory effect of 10 μ M AMP alone, STa alone, and STa followed by AMP. (D to F) Parallel series of tracings, except that the AMP concentration was reduced to 2 μ M and guanylin was used instead of STa.

leased from the host by EPEC might enhance the effects of the ETEC toxins on secretory response in intestinal tissues studied in the Ussing chamber. Figures 4 and 5 show the results of those studies, in which short-circuit current reflects chloride secretion in T84 cell monolayers (20). Figure 4A shows that 10 μ M AMP produced an I_{sc} response that was brisk in onset, sustained in duration, and about as large as or even larger than that produced by the ETEC culture filtrate (Fig. 4B), purified STa toxin (Fig. 5B), or guanylin (Fig. 5E). To our knowledge, ours is the first study to directly compare the secretory effects of adenine nucleotides in a head-to-head manner to those of the better-studied ETEC toxins and guanylin. Although we used AMP as the test nucleotide in the tracings shown in Fig. 4 and 5, AMP is in fact converted to adenosine by ecto-5'-nucleotidase (CD73) on the apical surface of the monolayer, as previously described by others and by our own laboratory (15, 51).

Having shown that adenine nucleotides can trigger a strong short-circuit current on their own, we next tested if the effects of AMP were additive with those of the potent ETEC toxins (Fig. 4 and 5). Since the ETEC toxins, especially the LTs, take some time to intoxicate the host cell and develop a secretory response, they were applied first. When the secretion was fully

developed and had reached a plateau, AMP was added and the tracing was continued. In all cases, we noted that addition of AMP triggered an increase in I_{sc} , even when added to tissues already intoxicated with ETEC sterile filtrate (Fig. 4C). In experiments similar to those shown in Fig. 4C, the addition of 5 or 10 μ M AMP increased the peak I_{sc} by $43.6\% \pm 19.8\%$ above that observed with the ETEC sterile filtrate alone (data are means \pm SD of five separate experiments using matched tissues). To determine the mechanism of the interaction between AMP and ETEC sterile filtrate, we tested the additive effects of 10 μ M AMP in the presence of inhibitors and compared it to the additive effects of carbachol and other activators of secretory signaling. Figure 4D shows that the effect of carbachol, a muscarinic agonist, resembled that of AMP when carbachol was added with the ETEC sterile filtrate; in this case, carbachol was added to the basolateral side of the tissue because muscarinic receptors are localized there in polarized T84 monolayers. Likewise, the addition of purified PI-PLC to the apical side of the tissue mimicked the effect of AMP addition (Fig. 4E). In contrast, addition of 2 μ M U73122, a PI-PLC inhibitor, nearly abolished the additive effects of AMP in this tissue model (Fig. 4F). We conclude that the additive effects of AMP added after ETEC filtrate are most likely due to its

ability to raise intracellular calcium via activation of PI-PLC, rather than by further increasing intracellular cAMP levels (21, 38).

In Fig. 5, the effects of AMP added along with maximal concentrations of STa (Fig. 5C) or guanylin (Fig. 5F) were tested. Once again, the combination of AMP plus STa, or AMP plus guanylin, produced a short-circuit current larger than that observed with any of the agonists alone. In experiments similar to those shown in Fig. 5C and 5F, AMP plus STa increased the I_{sc} by $100\% \pm 44\%$ compared to STa alone, and AMP plus guanylin increased the I_{sc} to a value of $73\% \pm 68\%$ above that from guanylin alone (mean \pm SD for three experiments with STa and three experiments with guanylin). If AMP was added first, subsequent addition of guanylin or STa still resulted in an additive secretory response, showing that the order of addition was not critically important (data not shown). Also, the additive interaction was observed with concentrations of AMP as low as 2 μ M (Fig. 5D and 5F), concentrations shown to accumulate in response to EPEC infection in a tissue culture model (15).

In the experiments shown in Fig. 4 and 5, the combination of two secretory stimuli produced an I_{sc} that was generally less than the arithmetic sum of the two agonists applied separately but greater than either agonist alone. In other words, the effects of AMP plus ETEC toxins were not supra-additive or truly synergistic compared to each agonist studied separately, but they did show an enhancing interaction. The lack of a synergistic response between LT toxin and AMP, for example (Fig. 4C), is not surprising, since LT acts via cyclic AMP and adenosine receptors are coupled to secretion at least in part via cyclic AMP (52). The results of Fig. 4 and 5, however, do demonstrate that adenine nucleotides released from host cells by EPEC infection can increase intestinal secretion even in the presence of potent *E. coli* enterotoxins and the hormone guanylin.

While performing the experiments shown in Fig. 1 to 4, some of us (S.S.C. and T.M.N.) often noted the appearance of large vacuoles in T84 cells treated with cholera toxin or ETEC culture filtrate containing LT-I. We investigated this morphological change by fixing, staining, and photographing the cells (Fig. 6). Figure 6A shows that normal control T84 cells do not contain visible vacuoles, a finding confirmed at higher magnifications (not shown). Adenosine (20 μ M) triggers the formation of vacuoles after 3 h of incubation (Fig. 6B), as did 25 ng of CT/ml, a submaximal toxin dose (Fig. 6C). The combination of adenosine plus CT triggered formation of very large vacuoles throughout the monolayer (Fig. 6D), with vacuole size often exceeding 30 μ m in diameter. AMP, ADP, and ATP triggered vacuole formation in a manner similar to that of adenosine, and vacuole formation was also observed in Caco-2 cells treated with CT or adenosine. In contrast, COS-7 cells showed no vacuole formation with these treatments (photographs not shown). Semiquantitative image analysis revealed that T84 cells treated with adenosine plus CT showed both a greater number of vacuoles and an increase in vacuole size compared to T84 cells treated with adenosine alone or CT alone. Cells treated with adenosine, CT, ETEC sterile filtrate, or combinations of toxin plus adenosine still showed a high TER in the Ussing chamber (Fig. 4 and 5 and tracings not

shown), indicating that monolayer and tight junction integrity was maintained despite the dramatic morphological changes.

Similarly, the combination of ETEC culture filtrate plus adenosine resulted in greater vacuole formation than ETEC sterile filtrate alone (Fig. 6, compare panels E and F). T84 cells treated with a filtrate from H10407 Δ LT did not form visible vacuoles (Fig. 6G), indicating that it is the LT-I and not STa that is triggering vacuole formation.

Adenosine-induced vacuole formation was readily reversible within 24 h of removal of the adenosine, whereas CT-induced vacuolization persisted at least 24 h after a medium change to remove unbound toxin (data not shown). The giant vacuoles we observed in T84 cells (Fig. 6) closely resemble those reported by Barkla et al. in the LIM1863 colon carcinoma cells in terms of vacuole size, time of onset (1 to 3 h), and CT concentrations needed (10 to 100 ng/ml) (3). Using electron microscopy, Barkla et al. observed that CT triggered the formation of small vesicles at the cell apex that then ballooned to enormous size, which they felt was due to secretion of fluid into the vesicle lumen.

To determine if the theory proposed by Barkla et al. applied to the formation of large vacuoles in T84 cells, we investigated whether CT-induced vacuole production was blocked by inhibitors of the CFTR or other ion channels. Compared to cells treated with 50 ng of CT/ml alone (Fig. 7A), cells treated with CT plus 10 μ M CFTR_{inh}-172 showed a smaller average vesicle size (Fig. 7B). CFTR_{inh}-172 is a cell-permeant thiazolidinone that is a potent and specific inhibitor of the CFTR chloride channel, which is localized to the apical membrane (39). Panel 6C shows that 100 μ M glyburide (also called glibenclamide) inhibited CT-induced vacuole formation even more strongly than CFTR_{inh}-172. Glyburide is a sulfonylurea drug that blocks one type of K⁺ efflux channel (7), as well as chloride efflux via the CFTR (33, 49, 56). Potassium channels maintain a negative intracellular potential inside the cell and therefore provide a driving force for continued secretion of chloride (32). The results of the inhibitor experiments suggest that the giant vacuoles observed in response to CT are generated by secretion of ions and fluid into the vesicle lumen (Fig. 7D). Adenosine receptors are also linked to elevation of cyclic AMP in the host cell, so adenosine would also stimulate fluid secretion into vesicles (Fig. 6B, D, and F).

DISCUSSION

The work presented here shows that ETEC and EPEC bacteria mutually interact in a way that intensifies the pathogenic potential of both pathogens. In regard to ATP release from host cells, ETEC toxins and live ETEC bacteria enhance EPEC-induced ATP release in a supra-additive or synergistic fashion (Fig. 1 to 3). We have recently shown that the enhanced ATP release observed in response to cholera toxin and forskolin is via activation of an ATP efflux pathway mediated by the CFTR (14). CFTR-dependent ATP release in epithelial tissues had been previously described (5, 34, 54), but its activation in response to any microbial infection or toxin had not been previously noted. We believe that the enhancement of EPEC-induced ATP release by the LT and STa enterotoxins, as shown in Fig. 1 to 3, is via the same CFTR-dependent pathway.

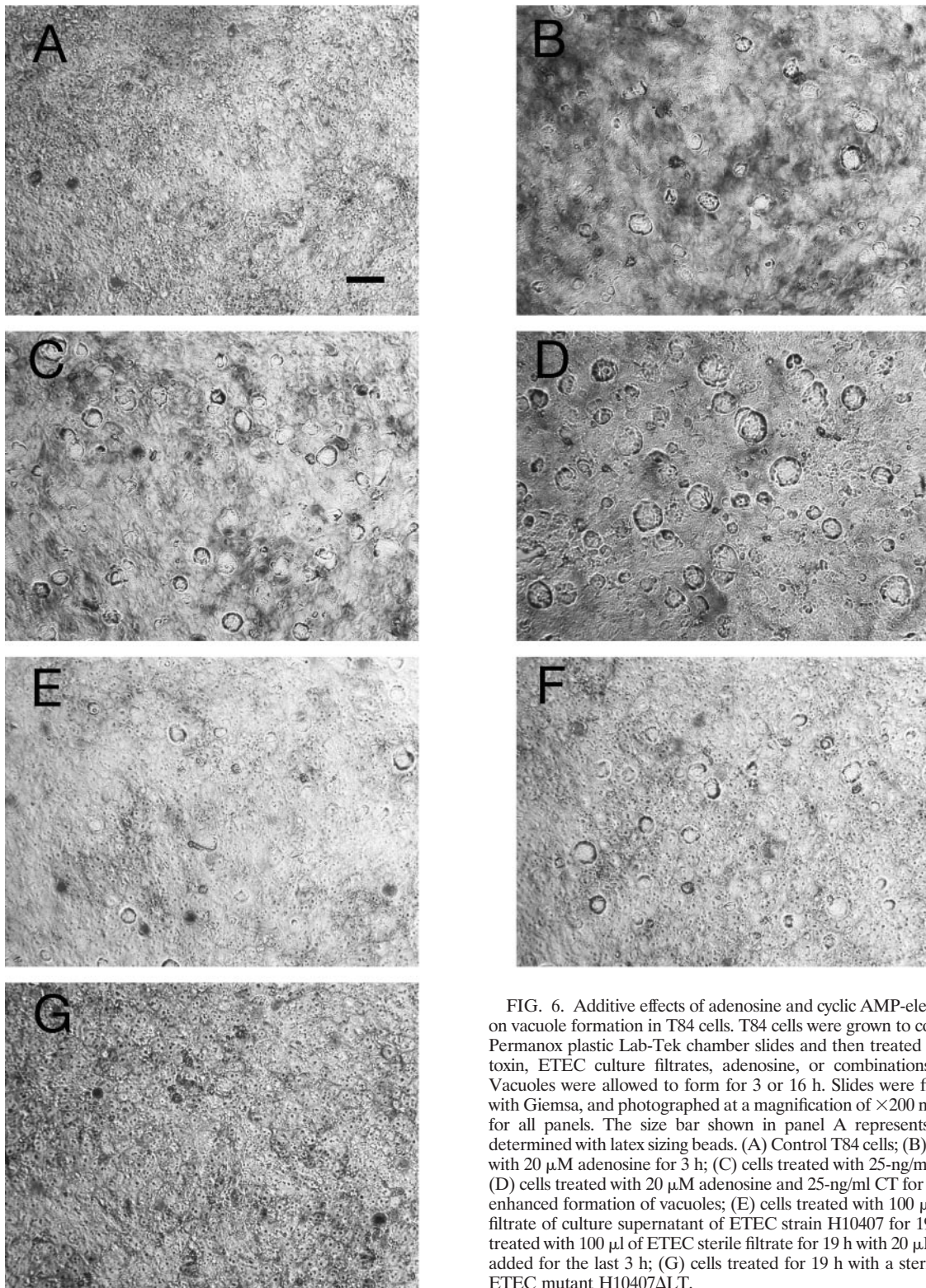


FIG. 6. Additive effects of adenosine and cyclic AMP-elevating toxins on vacuole formation in T84 cells. T84 cells were grown to confluence on Permanox plastic Lab-Tek chamber slides and then treated with cholera toxin, ETEC culture filtrates, adenosine, or combinations of stimuli. Vacuoles were allowed to form for 3 or 16 h. Slides were fixed, stained with Giemsa, and photographed at a magnification of $\times 200$ magnification for all panels. The size bar shown in panel A represents $30\ \mu\text{m}$, as determined with latex sizing beads. (A) Control T84 cells; (B) cells treated with $20\ \mu\text{M}$ adenosine for 3 h; (C) cells treated with 25-ng/ml CT for 3 h; (D) cells treated with $20\ \mu\text{M}$ adenosine and 25-ng/ml CT for 3 h, showing enhanced formation of vacuoles; (E) cells treated with $100\ \mu\text{l}$ of a sterile filtrate of culture supernatant of ETEC strain H10407 for 19 h; (F) cells treated with $100\ \mu\text{l}$ of ETEC sterile filtrate for 19 h with $20\ \mu\text{M}$ adenosine added for the last 3 h; (G) cells treated for 19 h with a sterile filtrate of ETEC mutant H10407 Δ LT.

In regard to chloride secretion, adenine nucleotides and adenosine released by EPEC infection act additively with ETEC toxins (Fig. 4 and 5); these additive effects are particularly strong with the heat-stable enterotoxin STa (Fig. 5A to C)

and its endogenous analog guanylin (Fig. 5D to F). This second type of EPEC-ETEC interaction also involves CFTR but instead affects CFTR's chloride channel function rather than its ability to export ATP. The CFTR is a major chloride channel

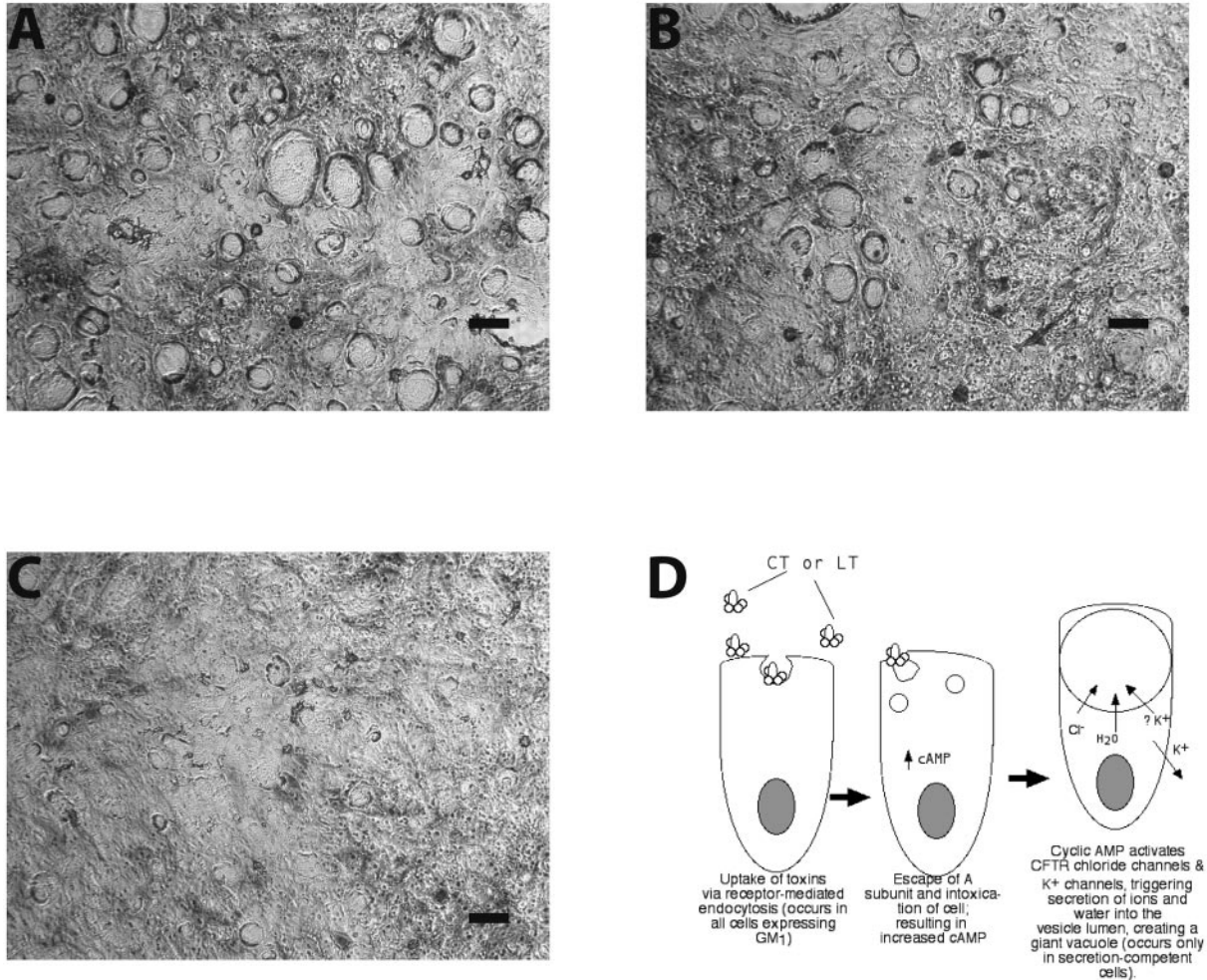


FIG. 7. Effect of ion channel inhibitors on cholera toxin-induced vacuole formation. T84 cells were grown as described in the legend to Fig. 6, treated with CT at 50 ng/ml for 3 h, fixed, stained, and photographed as before. Size bars, 30 μm. (A) Cells treated with CT alone; (B) cells treated with CT plus 10 μM CFTR_{inh}-172, a thiazolidinone CFTR inhibitor; (C) cells treated with CT plus 100 μM glyburide, a sulfonylurea which is a CFTR and potassium channel inhibitor; (D) sketch depicting the proposed mechanism for the formation of giant vacuoles in T84 cells in response to CT and LT (adenosine receptors are omitted from the diagram for the sake of simplicity).

in the small and large intestines, and its channel activity is regulated by cyclic nucleotides, as well as by intracellular calcium and protein kinase C (PKC). Cholera toxin, LT, and STa all produce diarrhea by triggering CFTR chloride channel activity (9, 25, 27). In addition, adenine nucleotides on epithelial surfaces are broken down to adenosine, act on adenosine receptors, and trigger chloride secretion via CFTR (12, 15, 52, 53). Therefore, the EPEC-EPEC interactions described here, with regard to ATP release (Fig. 1 to 3) and chloride secretion in the Ussing chamber (Fig. 4), all involve different aspects of the functions of CFTR. As shown in Fig. 5 and 6, the interaction between adenosine and toxins in triggering vacuole formation may also involve the CFTR, as well as potassium and other ion channels.

In previous studies we noted another, separate interaction between EPEC and the STa enterotoxin that did not directly involve CFTR. We showed that early in EPEC infection, host cells become hypersensitive to STa, as manifested by an increase in cyclic GMP accumulation to the toxin (17). This hypersensitiv-

ity is due to activation of host PKC by EPEC (17), followed by PKC-mediated phosphorylation of guanylyl cyclase C (GC-C), the receptor for STa (18, 19). PKC-mediated phosphorylation of GC-C occurs at residue Ser¹⁰²⁹ at the intracellular C-terminal domain of GC-C (57) and results in an increased number of GC-C molecules being expressed at the cell surface, as well as in increased guanylyl cyclase activity (19).

Therefore, including these earlier studies from our laboratory, we have now noted four different ways that EPEC and ETEC bacteria can interact. First, EPEC infection sensitizes cells to the effects of STa (17). Second, ETEC toxins synergistically increase EPEC-induced ATP release from host cells (Fig. 1 to 3). Third, adenine nucleotides act additively with ETEC toxins to trigger chloride secretion in intestinal tissues (Fig. 4 and 5). Fourth, adenosine and adenine nucleotides act additively or synergistically with CT and LT to induce vacuole formation in T84 cells (Fig. 6).

In the case of ETEC-EPEC interaction in vacuole formation, we cannot at present assert that vacuole formation di-

rectly causes disease or pathology induced by ETEC or EPEC infection. Vacuole formation was not noted in histological examination of the small intestine in naturally occurring cholera in humans (10) or in experimental cholera in dogs (22), although the colon was not studied in detail in those reports. In colonic cells, vacuole formation seems to be a distinctive morphological response to CT and LT along the lines of the CHO cell stretch response and Y1 adrenal cell rounding, which have been used as bioassays for the presence of these toxins. Since CT and LT are potent mucosal adjuvants, however, it is possible that enhanced vesicle and/or vacuole formation is a mechanism involved in the increased antigen presentation to immune cells in response to these toxins (11, 36, 43, 44, 50). Whether vacuole formation is viewed as pathological damage caused by the microbe or a host defense strategy, it is clear that there is an enhancing interaction between ETEC LT toxin and CT on the one hand and adenine nucleotides and adenosine on the other.

In all of these ways, *in vitro* studies of cultured cells indicate that EPEC and ETEC can mutually enhance the virulence of the other.

The importance of microbial interactions in disease in humans and animals has been a subject of increased interest recently; in many cases, the molecular bases for such interactions are coming to light (6). As mentioned in the introduction, dual infections involving EPEC and ETEC have frequently been noted in studies of diarrhea in children, but these were not recognized as examples of a microbial interaction because until now, no molecular mechanism had been identified for such an EPEC-ETEC interaction. Examples of possible EPEC-ETEC interactions have also been seen in animals. Wada and colleagues reported on a naturally occurring outbreak of unusually severe diarrhea among piglets, which was determined to be due to dual infection with EPEC ("attaching and effacing *E. coli*") and ETEC (58). Other examples of dual infections with EPEC and another pathogen have also been reported. For example, Schauer et al. noted an outbreak of enterocolitis in rabbits due to the combination of rabbit EPEC and an intracellular pathogen, *Lawsonia intracellularis* (48). In a study of turkeys, Guy et al. found that dual infection with an avian EPEC and turkey coronavirus produced a much higher mortality rate than with either microbe singly (30).

If EPEC and ETEC mutually enhance each other's virulence, one might wonder why *E. coli* strains have not emerged with the genetic characteristics of both, given the ability of *E. coli* for horizontal exchange of genetic material. One answer may be that it is difficult for bacteria to express both the type II secretion system (used by *Vibrio cholerae* and ETEC for the secretion of CT and LT) and the type III secretion system (used by EPEC, EHEC, *Salmonella*, and others for secretion of effectors). However, another answer may be that such EPEC-ETEC hybrid strains are already starting to emerge. Hedberg et al. described a foodborne diarrheal outbreak due to an *E. coli* strain not fitting into the usual categories for diarrheagenic *E. coli* (31). This strain, serotype O39:NM, had the characteristics of an atypical EPEC but also expressed the enteroaggregative heat-stable toxin EAST1, which has the same mechanism of action as STa. In many countries, microbiology laboratories (including many state, provincial, and national reference laboratories) no longer test for EPEC or ETEC in

fecal specimens. Therefore, EPEC-ETEC "hybrids" and strains similar to that described by Hedberg could already exist or could emerge without detection or recognition. Based on the results described here, etiologic and epidemiologic studies of diarrhea should be alert for dual infections with EPEC and ETEC, since our work and clinical observations would indicate that these dual infections could result in more-severe disease (1, 2).

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