Two pathways for ATP release from host cells in enteropathogenic Escherichia coli infection

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Crane, John K., Tonniele M. Naeher, Shilpa S. Choudhari, and Elisa M. Giroux. Two pathways for ATP release from host cells in enteropathogenic Escherichia coli infection. Am J Physiol Gastrointest Liver Physiol 289: G407–G417, 2005; doi:10.1152/ajpgi.00137.2005.—We previously reported that enteropathogenic Escherichia coli (EPEC) infection triggered a large release of ATP from the host cell that was correlated with and dependent on EPEC-induced killing of the host cell. We noted, however, that under some circumstances, EPEC-induced ATP release exceeded that which could be accounted for on the basis of host cell killing. For example, EPEC-induced ATP release was potentiated by noncytotoxic agents that elevate host cell cAMP, such as forskolin and cholera toxin, and by exposure to hypotonic medium. These findings and the performance of the EPEC espF mutant led us to hypothesize that the CFTR plays a role in EPEC-induced ATP release that is independent of cell death. We report the results of experiments using specific, cell-permeable CFTR activators and inhibitors, as well as transfection of the CFTR into non-CFTR-expressing cell lines, which discern the CFTR as a second pathway for ATP release from host cells. Increased ATP release via CFTR is not accompanied by an increase in EPEC adherence to transected cells. The CFTR-dependent ATP release pathway becomes activated endogenously later in EPEC infection, and this activation is mediated, at least in part, by generation of extracellular adenosine from the breakdown of released ATP.

Therefore, we proposed that EPEC-induced ATP release, followed by its conversion to the active secretagogue adenosine, could be a basis for EPEC-induced watery diarrhea.

In our previous study, we noted that EPEC-induced ATP release from host cells was tightly correlated with EPEC-induced cell death when nonintestinal cell lines (such as HeLa and HEK-293 cells) were studied. In intestinal cell lines, however, the tight correlation between cell death and ATP release seemed to break down. Specifically, ATP release from intestinal cells appeared to exceed what could be accounted for on the basis of EPEC-induced cell death. The EPEC espF mutant, for example, is quite attenuated in its ability to trigger cell death in cultured cell assays in all cell lines tested (18, 20). In HeLa cells, the espF mutant (strain UMD874) was also similarly attenuated in its ability to induce ATP release. However, in T84 cells, a colon cell line, the espF mutant was hardly attenuated at all in ATP release (see Fig. 1A and 19) compared with its parental wild-type strain, E2348/69. In addition, in intestinal cell lines, EPEC-induced ATP release was increased markedly by agents that elevated cAMP in the host cell, such as forskolin or cholera toxin (see Fig. 1, A and B). In nonintestinal cells, forskolin and cholera toxin had no enhancing effect on EPEC-induced ATP release (19). Therefore, we wondered whether in intestinal cells there was a mechanism or pathway for ATP release that was independent of EPEC-induced killing.

Consultation of the literature on ATP release from host cells revealed that there are several pathways for ATP liberation from host cells, which are completely independent of cell death or even cell damage. For example, ATP is present in high concentrations in secretory granules and synaptic vesicles, and this ATP is released along with other granule or vesicle contents during degranulation, exocrine and endocrine secretion, and synaptic transmission. However, the T84 cells we studied, similar to other enterocytes, do not contain secretory granules, so this mechanism did not seem an explanation for the discrepancy in ATP release we noted.

Another pathway for ATP release in epithelial cells involves the CFTR. In addition to its function as an apically localized chloride channel in respiratory and intestinal epithelia, CFTR also serves as a conduit for ATP efflux from the cell (29, 46, 59). CFTR-mediated ATP efflux is stimulated by intracellular cAMP, but maximal release of ATP via the CFTR usually requires the combination of two stimuli, such as exposure to hypotonic medium plus a cAMP-elevating agent (10, 29). Because T84 cells express CFTR, as do normal intestinal cells from the stomach to the rectum, it seemed that CFTR-depend-
dent ATP release might account for EPEC-induced ATP release over and above what could be explained by host cell killing, and we initiated this study to determine whether this was the case.

**MATERIALS AND METHODS**

**Materials.** The following reagents were obtained from Sigma (St. Louis, MO): α,β-methylene-ADP, creatine kinase, phosphocreatine, forskolin, adenosine, AMP, ADP, ATP, Tris-acetate, and type III collagen. CFTR<sup>wt</sup>-16 and CFTR<sup>mut</sup>-172 were kind gifts of Dr. Alan Verkman, Univ. of California at San Francisco, and were dissolved in DMSO.

**Cell culture.** T84 (human colon carcinoma) and HeLa (human cervical) cells were maintained as previously described (21), using a medium of DMEM/F-12, 18 mM NaHCO<sub>3</sub>, 7.5% fetal bovine serum, 20 μg/ml vancomycin, and 15 μg/ml gentamicin.

**Bacterial strains.** Bacterial strains included wild-type EPEC strains E2348/69 (O127:H6), B171–8 (O111:NM), all of which are described in previous publications (8, 18, 19, 39). JM194, a diffuse-adherent <i>E. coli</i> strain, was collected in Mexico and provided by John J. Mathewson (37, 38). Diffuse-adherent <i>E. coli</i> strain C1845 has been the subject of several publications (7, 57). The <i>Salmonella enterica</i> serovar Enteritidis was a clinical isolate from diarrheal feces from a patient at Erie County Medical Center, Buffalo, NY, and was previously described (19).

**ATP release assay.** ATP concentrations in conditioned medium from infected and uninfected cells were measured using ATP luminescence assay kits, either from Sigma or from Roche Molecular Biochemicals (Indianapolis, IN). Cells in 48- or 96-well plates were infected for 45 min, then the medium was changed to serum-free medium supplemented with an ATP-regenerating system and a nucleotidase inhibitor consisting of creatine kinase (20 mg/l), phosphocreatine (10 mM) and α,β-methylene-ADP (250 μM). It should be emphasized that, although the ATP regenerating system retards the breakdown of ATP, the phosphocreatine becomes depleted at later times, and so some breakdown of ATP can occur. At various times after the medium change, the plates were swirled to mix and 50- to 75-μl aliquots were removed from wells and sterile filtered. In transfected HeLa cells, the medium change step was omitted to avoid detachment of cells, and the infection was performed directly in medium containing the ATP-regenerating system. To avoid laborious filtering steps and increase the throughput, samples were filtered using a 96-well plate filter device (Unifilter, Whatman, Clifton, NJ) by centrifugation at 2,000 rpm for 5 min with collection of the filtrates in another 96-well plate placed below the Unifilter. These samples were then diluted six- or eightfold and assayed for ATP as previously described. Luminescence was measured in a Wallac 1450 Trilux Microbeta luminoimeter/scintillation counter that accommodates 96-well plates. Lactate dehydrogenase (LDH) release was measured as described previously (20) using a colorimetric method in kit form (Boehringer-Mannheim, now Roche Molecular Biochemicals).

**Transfection methods.** Preliminary transfection experiments were performed on HEK-293 and HeLa cells using different lipid transfection reagents, including Lipofectamine 2000, Fugene 6, Metafectene, and others. For brevity, only the HeLa cell transfections are reported here, but results with HEK-293 cells were in agreement with the HeLa results.

Eukaryotic expression plasmids encoding the wild-type and ΔPhe<sup>508</sup> mutant CFTR were kind gifts of Dr. William Guggino, Dept. of Physiology, Johns Hopkins University School of Medicine. Plasmid DNA was grown in <i>E. coli</i> strain DH5α under ampicillin selection, and plasmid DNA was purified using a Qiagen endo-free mega plasmid purification kit as previously described (18).

HeLa cells were plated on collagen-coated 24- or 48-well plates to reduce detachment during transfection and infection. One day after plating, when HeLa cells were at 80–90% confluency, they were transfected. For both 24- and 48-well transfections, the ratio of cationic lipid to plasmid DNA was 2.5 μg/μg DNA. For 24-well plates, each well was transfected with 2.5 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) that had been mixed with the 1 μg purified plasmid DNA. For 48-well plates, the ratio of lipid to DNA was maintained, but the amounts were reduced to 1 μl of Lipofectamine 2000 and 0.4 μg DNA. In some experiments, Fugene 6 (Roche) was substituted for Lipofectamine 2000. Transfection efficiency was monitored by transfecting other cell wells in parallel using the reporter plasmid pCMV-SPORT-βgal, followed by staining with X-gal (X-gal staining kit, Invitrogen) and examining the monolayer for blue cells by microscopy, as previously reported (18). Enhancing effects of forskolin and CFTR<sup>wt</sup>-16 on ATP release were generally only observed when the transfection efficiency exceeded 45%.

**Bacterial adherence assay.** HeLa cells were grown to confluency on collagen-coated 24-well plates to reduce detachment during transfection, infection, and washing steps. HeLa cells were transfected or not, then on the following day, the cells were infected with EPEC strains E2348/69 or B171–8 for 2 h, then washed twice with sterile PBS to remove unbond bacteria. HeLa monolayers were lysed in 1% Triton X-100 detergent in sterile water for 30 min, agitated to mix, then adhered bacteria were measured by dilution and plate counting. Adherence was done using duplicate wells per condition and two dilution plates per well, yielding four plates per condition.

**Ethidium homodimer uptake assay.** Uptake of ethidium homodimer, similar to that of propidium iodide, is a measure of damage to the plasma membrane. Ethidium homodimer uptake assays were performed as previously described (20), using 3 mg/l ethidium homodimer in living cells and reading on a fluorescence plate reader.

**Data analysis and statistics.** All of the experiments shown were performed at least three times. ATP assays were done in quadruplicate wells. Statistical analyses were performed using the Instat software program for the Macintosh computer (GraphPad software, San Diego, CA), and graphs were generated using the Prism 4.0 program by the same software company. All error bars shown in graphs are standard deviations. Statistical significance was by ANOVA using the Tukey-Kramer posttest for multiple comparisons. In figures, symbols such as an asterisk were used sparingly to emphasize that a particular condition was significantly different from the comparator. To avoid clutter and confusion, not all significant differences are flagged with a symbol; therefore, lack of an asterisk does not mean a condition was insignificant.

**RESULTS**

As shown in Fig. 1, A and B, forskolin increased EPEC-induced ATP release in the CFTR-expressing T84 cells but not in HeLa cells (19). One trivial explanation for this finding would be that forskolin is differentially toxic to EPEC-infected cells and therefore mediates its effects solely through increased host cell killing. Figure 1, C and D, shows the result of an experiment in which cell culture supernatants were analyzed both for ATP (Fig. 1C) and for LDH (Fig. 1D). LDH release from host cells is a well-described marker of bacterial-mediated cell killing (19, 41) and correlates well with other indexes of cell death such as propidium iodide uptake. As shown in Fig. 1C, forskolin potentiated ATP release triggered by infection with EPEC strain E2348/69 but had no effect on EPEC-induced LDH release (Fig. 1D). A large body of other literature supports the lack of toxicity of forskolin in T84 cells and in other cultured cell lines. Therefore, forskolin increased EPEC-induced ATP release without increasing EPEC-mediated cytotoxicity.

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CFTR-mediated chloride efflux. Unlike forskolin, CFTRact-16 activator CFTRact-16 directly interacts with and activates one of their most potent CFTR activators and one of their channel activity. Drs. Ma and Verkman generously supplied permeable activators and inhibitors of the CFTR chloride nously engineered reporter cell line, to find potent, cell-high-throughput screening system, together with an inge-

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One of the interesting and somewhat peculiar features of CFTR-dependent ATP release from cells is that it is stimulated by cell swelling, such as when cells are exposed to hypotonic medium (10, 59). Therefore, we tested whether hypotonic conditions affected EPEC-induced ATP release in T84 cells. Figure 2A shows that in uninfected T84 cells, neither hypotonicity alone nor forskolin alone had much effect on ATP release, but that the combination of hypotonicity plus forskolin did significantly increase ATP release, a result similar to that seen in other CFTR-expressing cells (10). ATP release trig-

gered by hypotonicity plus forskolin was not accompanied by an increase in uptake of ethidium homodimer, a measure of plasma membrane damage (Fig. 2B). Figure 2C shows that EPEC-induced ATP release was increased in hypotonic medium compared with control, isotonic medium. The results of Fig. 2 were therefore consistent with EPEC acting together with hypotonicity to activate a CFTR-dependent pathway for ATP release.

Recently, Ma et al. (33, 34) reported their results of using a high-throughput screening system, together with an ingen-

iously engineered reporter cell line, to find potent, cell-permeable activators and inhibitors of the CFTR chloride channel activity. Drs. Ma and Verkman generously supplied one of their most potent CFTR activators and one of their inhibitors for our use in this study. Ma et al. showed that the activator CFTRact-16 directly interacts with and activates CFTR-mediated chloride efflux. Unlike forskolin, CFTRact-16 does not stimulate adenylyl cyclase, does not elevate cAMP, does not affect PKA, and does not activate the CFTR via phosphorylation (34). Although Ma et al. did not test whether CFTRact-16 had any effect on CFTR-dependent ATP release, we reasoned that it might indeed also activate this function of the CFTR as well. Figure 3A shows the effects of CFTRact-16 on ATP release in T84 cells. Ten-micromolar CFTRact-16 alone had no effect on basal ATP release in uninfected cells, but CFTRact-16 did significantly increase ATP release trig-

gered by two EPEC strains, B171–8 and E2348/69 (Fig. 3A), in a manner similar to forskolin.

Aeromonas hydrophila and Aeromonas sobria produce pore-forming exotoxins that trigger ATP release from the host cells, including T84 cells (26). We tested whether ATP release by aerolysin, the toxin of A. hydrophila, also involved CFTR. Figure 3B shows that a crude sterile filtrate of A. hydrophila, containing aerolysin, did trigger a strong release of ATP from T84 cells. ATP release by the Aeromonas filtrate, however, was not stimulated by CFTRact-16 and was not inhibited by the thiazolidinone inhibitor of CFTR, CFTRinh-172. In fact, ATP release by the Aeromonas filtrate was paradoxically inhibited by CFTRact-16 (Fig. 3B) by unknown mechanisms. The results of Fig. 3B illustrate the specificity of the CFTR activator and inhibitor as probes of CFTR involvement by showing that some types of ATP release are completely unaffected by these reagents.

The experiments with hypotonicity (Fig. 2) and the CFTR activator (Fig. 3) were consistent with a role for the CFTR in EPEC-induced ATP release but did not rise to the level of proof. To definitively determine whether CFTR played a role in EPEC-induced ATP release, we tested whether transfection of cells lacking the CFTR with a plasmid encoding the CFTR would confer the sensitivity to activators such as forskolin and CFTRact-16 observed in T84 cells. We performed transfections of two human cell lines that do not express CFTR, HEK-293 cells and HeLa cells, with two variants of CFTR in a eukary-

otic expression vector: pRSV-wt-CFTR encoded wild-type CFTR, and pRSV-F508-CFTR encoded the nonfunctional ΔPhe508 mutant CFTR. HEK-293 cells were easier to transfect and yielded higher transfection efficiencies, but transfected HEK-293 cells were quick to detach on infection with EPEC bacteria, resulting in cell loss from the monolayer and relatively low ATP levels after experimental manipulations, such as medium changes. In contrast, HeLa cells were harder to transfect, but they adhered more tenaciously to tissue culture
In both cell lines, we noted greater cytotoxicity on transfection of either of the two CFTR plasmids (pRSV-wt-CFTR or pRSV-F508-CFTR) than with the reporter plasmid pCMV-SPORT-bgal or other plasmids we have used for transfections in the past (17). This greater toxicity is consistent with the biology and molecular biology of the CFTR. The eukaryotic expression vector encoding the CFTR is quite large (8.7 kb) as is the CFTR protein product (170 kDa for the mature, glycosylated form). The wild-type CFTR confers a new chloride channel activity on a cell that normally does not express it, and the ΔF508-CFTR is conformationally unstable, triggering retention in the endoplasmic reticulum and eventual degradation by proteasomes. Evidence of CFTR-mediated ATP release only seemed to be detectable when the transfection efficiency exceeded 45–50%, as monitored by parallel transfection with pCMV-SPORT-bgal followed by X-gal staining.

Figure 4 shows the results of HeLa cell transfections with the two CFTR plasmids mentioned. Figure 4, A–C, shows the results of transfection followed by infection with EPEC strain B171–8, whereas Fig. 4D shows an experiment in which EPEC strain E2348/69 was used. Untransfected HeLa cells (Fig. 4A) showed no enhancement of EPEC-induced ATP release by forskolin, as previously reported (19). Similarly, cells transfected with pRSV-ΔF508-CFTR also showed no enhancement of ATP release by forskolin (Fig. 4, B and D) or by CFTRact-16 (Fig. 4D, left). In fact, forskolin again showed a paradoxical inhibition of ATP release in the cells transfected with the two CFTR plasmids mentioned.
with pRSV-\textit{H}9004\textit{F}508-CFTR. In contrast, HeLa cells transfected with wild-type CFTR showed enhanced ATP release in the presence of forskolin (Fig. 4C) or CFTRact-16 (Fig. 4D). The fact that absolute ATP levels are not increased in Fig. 4C over those in Fig. 4A is due to the increased cell loss during transfection with the wild-type CFTR, as mentioned above.

\textit{S. enterica} serovar Typhi exploits the CFTR by using CFTR as a cellular receptor for adherence and invasion (32, 45, 60). We therefore tested whether transfection of the CFTR into a non-CFTR-expressing cell line affected EPEC adherence. Figure 5 shows the adherence of two EPEC strains to HeLa cells treated as shown in the figure labels. We also assessed the effect on adherence of mock transfection using the cationic lipid transfection reagents alone (without plasmid DNA). Cationic lipid transfection reagents contain phosphatidylethanolamine or phosphatidylethanolamine analogs. Phosphatidylethanolamine is recognized by Shiga-toxigenic \textit{E. coli} and EPEC as a receptor for adhesion (1, 4, 5). Figure 5 shows that adherence of EPEC E2348/69 was increased in HeLa cells mock transfected with Lipofectamine 2000 (Lipo) or Fugene 6 alone, although these increases in adherence fell short of reaching statistical significance. In contrast, there was no difference in EPEC adherence between cells transfected with the wild-type CFTR plasmid and the \textit{\textDelta F}508-CFTR for either

**Fig. 4. Effect of CFTR transfection on subsequent cellular responsiveness to EPEC infection, forskolin, and CFTRact-16.** HeLa cells were grown to 90% confluency in collagen-coated 48-well plates, then transfected with an expression plasmid encoding the wild-type CFTR (pRSV-wt-CFTR) or the \textit{\textDelta Phe}508 mutant CFTR (pRSV-\textit{\textDelta F}508-CFTR) as described in MATERIALS AND METHODS. Two days after transfection, the cells were changed into ATP assay medium and infected with wild-type EPEC strain E2348/69 or B171-8 at MOIs of 100:1; in these experiments, the medium change step was omitted after infection to reduce cell detachment; 20 \textmu M forskolin or 5 \textmu M CFTRact-16 were added as indicated in the axis labels. Supernatant aliquots were collected for ATP assay 2 h after infection. *Significantly increased compared with EPEC alone by ANOVA using the Tukey-Kramer posttest for multiple comparisons; †Not significantly increased compared with EPEC alone.

**Fig. 5. Effect of CFTR transfection on EPEC adherence to HeLa cells.** HeLa cells were transfected with wild-type CFTR or \textit{\textDelta Phe}508 mutant CFTR as described in MATERIALS AND METHODS and in the legend to Fig. 4. In some wells, the plasmid DNA was omitted and cells were treated with the cationic lipid transfection reagent alone [Lipofectamine 2000 (Lipo) or Fugene 6]. Twenty-four hours after the transfection, the HeLa cells were infected with the EPEC strain indicated for 2 h, then the wells were washed twice with sterile buffered saline, and the cell monolayer was solubilized with 1% Triton X-100 detergent in water. Bacterial counts were determined by dilution and plate counts.
EPEC strain tested, E2348/69 or B171–8. Therefore, in these transfection experiments, we could find no evidence that EPEC uses the CFTR as a point of attachment or adherence. This accords with a large body of published literature that EPEC adheres as well in vitro to non-CFTR-expressing cell lines such as HeLa and HEp-2 cells as to CFTR-expressing cell lines such as T84, Caco-2, HT-29, and so on (22, 48). Therefore, it appears that EPEC differs from *S. enterica* serovar Typhi in its interaction with the CFTR, namely that EPEC exploits the function of the CFTR channel as a conduit for augmented ATP release, whereas *S. typhi* exploits the structure of the CFTR as a point of adherence and invasion.

In experiments shown so far, we relied on potent activators of CFTR-dependent ATP release, such as cholera toxin, forskolin, or CFTRact-16 (Figs. 1–4) to maximally stimulate the function of the CFTR-dependent pathway for ATP release. In this more common situation, an important question is whether the CFTR-dependent pathway for ATP release above what is seen with EPEC infection alone. We are exploring whether other toxins that act via cyclic nucleotides, such as *E. coli* heat-labile toxin and heat-stable toxin, also potentiate EPEC-induced ATP release, because dual infections with enterotoxigenic *E. coli* and EPEC are fairly common (2, 3, 12, 35, 36). In the more typical case where EPEC is the sole pathogen, however, there would be no exogenous activator of the CFTR-dependent pathway for ATP release. In this more common situation, an important question is whether the CFTR-dependent pathway of ATP release can become activated during EPEC infection alone. Several inhibitors of CFTR-dependent chloride channel activity have been described, and these inhibitors also inhibit CFTR-dependent ATP release (10, 47, 59). We used these inhibitors and the highly selective, highly potent thiazolidinone CFTR inhibitor described by Ma et al. (33) to probe whether the CFTR-dependent pathway of ATP release is activated endogenously in EPEC infection.

Preliminary experiments were carried out with DIDS, and these did show inhibition of EPEC-induced ATP release. DIDS also inhibited growth of EPEC bacteria, however, so the results were difficult to interpret and are not shown. In contrast, GdCl3 and CFTRinh-172 did not inhibit EPEC growth, and they also did not interfere with the bioluminescent detection of ATP. In experiments conducted at early-to-middle time points (2–3 h after infection) and using the usual multiplicity of infection of 100–200:1, CFTR inhibitors such as GdCl3 (Fig. 6A) and CFTRinh-172 (Fig. 6B) did not inhibit ATP release triggered by EPEC alone, even in contrast, even at these earlier times and multiplicities of infection, it was apparent that these inhibitors reversed the effect of forskolin on ATP release (Fig. 6, A and B, right). At high multiplicities of infection, however, CFTRinh-172 did significantly inhibit EPEC-induced ATP release in T84 cells, even at early times (Fig. 6C). In addition, at later time points, CFTRinh-172 showed inhibition of EPEC-induced ATP release at usual multiplicities of infection (Fig. 6D). This led to the hypothesis that the CFTR-dependent pathway for ATP release becomes activated endogenously (i.e., without chemical activators such as forskolin or cholera toxin) later in the course of EPEC infection in this cultured cell model. On the basis of what has been learned about EPEC signaling to the host cell, at least two possible pathways could activate CFTR function in response to EPEC infection. First, EPEC infection activates PKC in the host cell (21). PKC, similar to cAMP-dependent protein kinase, directly phosphorylates the intracellular regulatory loop of CFTR and activates its channel activity (15, 27, 44). Second, ATP released from the host cell by the CFTR-independent pathway is broken down to adenosine. Adenosine receptors are linked via G protein Gs to adenylyl cyclase, triggering an elevation of intracellular cAMP (26, 53). This would lead to a positive feedback loop or “vicious circle” in which an initial CFTR-independent ATP release leads to generation of extracellular adenosine, which then acts via receptors to trigger an additional CFTR-dependent ATP release from the host cell. Of these two possibilities, the time at which CFTRinh-172 begins to have detectable inhibitory effects (2–4 h) favors the latter. PKC activation occurs rapidly after EPEC infection and then falls off to baseline 2–3 h after infection (21), whereas ATP release and subsequent breakdown to adenosine requires several hours of infection (19), a time course corresponding to the onset of detectable CFTRinh-172 effects on ATP release. If this is the case, then agents that potentiate the effects of adenosine, or addition of adenosine itself, should increase EPEC-induced ATP release and enhance the effects of the CFTRinh-172.

We reasoned that inhibitors of cyclic nucleotide phosphodiesterase (PDE) should potentiate adenosine’s effects on intracellular cAMP levels, and therefore on the CFTR. The potent xanthine PDE inhibitors, however, such as caffeine, theophylline, and so on, are also potent adenosine receptor antagonists. Therefore, we tested the effects of papaverine, a PDE inhibitor that lacks adenosine receptor blocking activity. Figure 6, E and F, shows that papaverine increased EPEC-induced ATP release and increased the amount of inhibition observed with CFTRinh-172. Addition of exogenous adenosine to T84 cells also increased EPEC-induced ATP release compared with EPEC alone (Fig. 6G), consistent with a role for adenosine in a CFTR-dependent pathway for ATP release. The enhancing effects of adenosine on EPEC-induced ATP release were not observed in HeLa cells (Fig. 6H), however, a difference that might be attributable to the absence of the CFTR in HeLa cells or to other differences such as adenosine receptor expression.

In our previous study of EPEC-induced ATP release, we compared the ATP-releasing ability of EPEC with that of *S. enterica* and enteroaggregative *E. coli* (EAEC) (19), and we have also tested diffuse adherent *E. coli* (DAEC; unpublished data). Whereas *S. enterica* serovar Enteritidis produced a strong ATP release from host cells, the EAEC and DAEC strains tested did not. We reexamined ATP release by these other enteric pathogens in the presence of the CFTR activators and inhibitors to determine whether a CFTR-dependent pathway for ATP release might be involved. Figure 7A shows that *S. enteritidis* triggered a strong release of ATP from the host cell at early time points that was only slightly and nonsignificantly increased by CFTRact-16. On the other hand, the Salmonella-induced ATP release was inhibited by CFTRinh-172, suggesting that a CFTR-dependent pathway for ATP release was already activated endogenously by Salmonella even at this early time point. At the other end of the spectrum are the diffuse-adherent *E. coli* strains JM194 and C1845, which by themselves triggered no release of ATP or such a tiny amount as to be biologically insignificant (Fig. 7, B and C). Despite the lack of ATP release by the diffuse-adherent *E. coli* alone, ATP release was still potentiated by CFTRact-16, suggesting that DAEC, similar to EPEC, exert some type of stress on the host cell plasma membrane in a manner similar to EPEC. This concept is plausible because DAEC strain C1845, similar to
Fig. 6. Use of CFTR inhibitors as evidence that the CFTR-dependent ATP release pathway is activated endogenously in EPEC infection. T84 or HeLa cells were infected with EPEC for 45 min, then changed into ATP assay medium, and samples were collected for ATP assay at 3 h unless indicated otherwise in the legend. Concentrations of inhibitors and activators used were 200 μM GdCl₃, 10 μM CFTRinh-172, and 20 μM forskolin. Experiments shown in A and H were done using cells in 48-well plates, whereas experiments in all of the other panels (B-G) were performed in 96-well plates using the same 0.25-ml volume of medium; therefore, absolute ATP levels in B-G are lower than in A and H. *Significantly decreased compared with the corresponding condition without inhibitor by ANOVA using the Tukey-Kramer posttest for multiple comparisons; #significantly decreased by 1-tailed t-test compared with E2348 alone. In G, the slope of the line shown for B171-infected cells, as determined by linear regression, was significantly different from 0 at a 95% confidence level.
EPEC, is known to possess the locus for enterocyte effacement, which encodes the type III secretion system (7, 23). The results of Fig. 7 suggest that EPEC is not unique in its ability to activate a CFTR-dependent pathway for ATP release from the host cell during infection and that additional pathogens may be found that exploit the ATP-exporting property of the CFTR in the future.

**DISCUSSION**

As mentioned in the introduction, certain discrepancies in ATP release by EPEC mutants, especially the espF mutant, and the effects of forskolin and cholera toxin on EPEC-induced ATP release led us to the hypothesis that EPEC-induced ATP release from host cells has two components, one which occurs in all host cells and is independent of the CFTR, and a second component that is dependent on the CFTR, activated by cell-shape changes such as swelling, and potentiated by cAMP. A comparison of the properties of the two pathways for ATP release is shown in Table 1. As summarized in Table 1 and based on current and previous work (19), both pathways for ATP release require a functional type III secretion system. Although the focus of this report has been on the CFTR-dependent pathway for ATP release, our work and that of others allow conclusions about the CFTR-independent pathway of ATP release as well.

Detailed studies of the mechanism of type III secretion by EPEC, Salmonella, Shigella, and other pathogens show that assembly of the type III “secretion” or “injectosome” in the host cell plasma membrane creates a pore of ~25 Å as estimated by osmotic protectants (9, 40, 52, 61) or even somewhat larger, ~30–50 Å, using atomic force microscopy (28). This pore is sufficiently large to allow permeability of the trisaccharide raffinose [molecular weight (MW) 504], which is very close in size to ATP (MW 507 for the free acid) (9). We have already shown that EPEC-infected cells become permeable to dyes such as trypan blue (a polyanion similar to ATP; MW 960) and propidium iodide and release LDH, a cytosolic enzyme (20). The concentration of ATP inside the mammalian cell cytosol is 3–10 mM (50), so ATP would exit by diffusion through this pore. Assuming the normal extracellular ATP concentration above a healthy cell monolayer is ~50 nM and that intracellular ATP concentration is 5 mM, ATP efflux by diffusion through the type III secretion pore would be driven by a 10,000,000-fold difference in concentration. Although the EPEC secretone differs from that of Salmonella, Shigella, and Yersinia by having a long filamentous portion composed of EspA, the common feature of all of the type III secretion pores, including those of plant pathogens, is the creation of a large, hydrophilic, nonselective channel across the host cell membrane (23, 24, 28, 51, 62).

The results shown above, including the transfection experiments and the effects of the CFTR activator and CFTR inhib-

### Table 1. Summary of features of CFTR-independent and -dependent ATP release

<table>
<thead>
<tr>
<th>Distinguishing Factors</th>
<th>CFTR-Independent ATP Release</th>
<th>CFTR-Dependent ATP Release</th>
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<tbody>
<tr>
<td>Occurrence</td>
<td>Occurs in all cell lines tested, including nonintestinal cells</td>
<td>Dependent on expression of CFTR</td>
</tr>
<tr>
<td>Relationship to EPEC-induced cell death</td>
<td>Dependent on or closely correlated with cell death; therefore, the espF mutant is markedly attenuated compared with wild-type</td>
<td>Not dependent on cell death; espF mutant is only slightly attenuated</td>
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<tr>
<td>Role of type III secretion</td>
<td>Dependent on type III secretion</td>
<td>Dependent on type III secretion</td>
</tr>
<tr>
<td>Sensitivity to inhibitors</td>
<td>Not inhibited by GdCl3 or CFTRinh-172</td>
<td>Inhibited by GdCl3 and CFTRinh-172</td>
</tr>
<tr>
<td>Sensitivity to agents that elevate cAMP or to CFTR activator</td>
<td>Unaffected, or paradoxically inhibited</td>
<td>Strongly potentiated</td>
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<tr>
<td>Effect of hypotonicity</td>
<td>Unaffected</td>
<td>Increased in hypotonic medium</td>
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<td>Efflux of other cytoplasmic contents and effects on plasma membrane</td>
<td>Accompanied by release of cytoplasmic proteins such as LDH; plasma membrane damage evident by ethidium homodimer uptake</td>
<td>Accompanied by chloride secretion, but not by proteins or LDH</td>
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EPEC, enteropathogenic *Eschericia coli*; LDH, lactate dehydrogenase.
itor, clearly demonstrate that there is a CFTR-dependent component of ATP release involved in EPEC infection. There is a controversy within the cystic fibrosis field as to whether the CFTR itself actually is the conduit for ATP efflux (46) or whether the CFTR activates another, widely expressed ATP efflux channel (10, 50, 55). Other members of the ATP-binding cassette family of membrane proteins are capable on their own, of mediating ATP release (49), so it is perhaps not farfetched to believe that the CFTR may also do so. Ma et al. (34) showed that the CFTRact-16 binds directly to and activates the CFTR itself. Because our data show that this same activator also potentiates EPEC-induced and hypotonicity-induced ATP release, the simplest interpretation of our results is that the CFTR itself functions as an ATP efflux transporter as well as a chloride channel.

Although our results clearly implicate the CFTR as a pathway for ATP release during EPEC infection, it is not yet clear exactly how EPEC activates the CFTR. CFTR-dependent ATP release usually appears to require a combination of two stimuli, such as hypotonicity plus forskolin, EPEC plus forskolin, EPEC plus cholera toxin, or EPEC plus CFTRact-16 (10). In this regard, the effects of EPEC early in infection closely mimic the effect of hypotonic medium (Fig. 2A). There appear to be two possible ways that EPEC could mimic hypotonic stress or exert true hypotonic stress on the host cell. First, EPEC intimate adherence triggers the formation by the host of protruding, cuplike pedestals, and it is possible that this outward distortion of the plasma membrane activates the CFTR just as cell swelling does. Second, the type III secretion system itself may trigger osmotic stress in the host cell by the infection into the host of bacterial products. Although the focus of research in type III secretion has been on the protein effectors that enter the host cell, small molecule solutes from the bacteria almost certainly are injected as well. The osmolarity of the E. coli cytosol, at 1,500 mosM (11, 42), is much higher than that of mammalian cells (300 mosM), so diffusion alone would provide a driving force for the flow of solutes from bacteria into the host cell. An increase in intracellular osmolarity in the host would trigger cell swelling in the same way as does hypotonicity, by stimulating an influx of water into the cell. Defining more exactly how EPEC exerts hypotonic stress on the host is limited by the lack of a clear theory of how cells perceive hypotonicity and osmotic stress in general, although progress is being made in this area (14). At late times after infection, EPEC would continue to exert osmotic stress on the host cell, but adenosine generated from breakdown of EPEC-released ATP would elevate cAMP via adenosine receptors (53). Therefore, at late times, EPEC alone could activate the CFTR-dependent ATP release pathway even without forskolin, cholera toxin, or the CFTR activator (Fig. 6, D–G).

In contrast to EPEC infection, the Aeromonas hydrophila toxin aerolysin (Fig. 3B) shows no sign of CFTR involvement in its ATP release. Although aerolysin releases ATP from the host cell and this ATP is converted into adenosine, adenosine is not sufficient to activate the CFTR-dependent pathway on its own (Fig. 6G, uninfected curve), demonstrating again that for effective CFTR-dependent ATP release, there must be a double stimulus (osmotic stress + a CFTR-activating agent).

Although resolution of many mechanistic questions is awaited, the finding of a CFTR-dependent ATP release pathway in response to infection with EPEC and other enteric pathogens (Fig. 7) would appear to have important implications even now. For example, the finding that the espF mutant is nearly equivalent to wild type in its ability to support CFTR-dependent ATP release (Fig. 1A) might explain why the espF mutant appears to be only moderately or slightly attenuated in virulence compared with wild type (25) instead of highly attenuated as suggested by our initial assays of cell death (18). Second, our findings might expand the range of diarrheal diseases in which thiazolidinone CFTR inhibitors are tested for efficacy; in addition to using these inhibitors in secretory diarrhea mediated by cAMP-elevating toxins, such as cholera toxin and the E. coli heat-labile toxin, there is now also a rationale for testing them in EPEC and Salmonella infection. Last, it is very likely that release of ATP occurs in mucosal infections other than those shown here. For example, exaggerated ATP release from bladder cells occurs in interstitial cystitis (56) and ATP acts via P2X purinergic receptors to trigger bladder contractions and urinary urgency in mice (13, 16). Therefore, it would seem possible that ATP release might occur in response to urinary tract infections. Extracellular ATP augments the killing of Candida albicans by antimicrobial histatins in saliva (30, 31), showing that extracellular adenine nucleotides may play a role in host defense and not just in the generation of injurious tissue responses.

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