Release of ATP during host cell killing by enteropathogenic *E. coli* and its role as a secretory mediator

JOHN K. CRANE,1 RUTH A. OLSON,1 HEATHER M. JONES,2 AND MICHAEL E. DUFFEY2

Departments of 1Medicine and 2Physiology and Biophysics, University at Buffalo, State University of New York, Buffalo, New York 14214

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Crane, John K., Ruth A. Olson, Heather M. Jones, and Michael E. Duffey. Release of ATP during host cell killing by enteropathogenic *E. coli* and its role as a secretory mediator. *Am J Physiol Gastrointest Liver Physiol* 283: G74–G86, 2002. First published March 13, 2002; 10.1152/ajpgi.00484.2001.—Enteropathogenic *Escherichia coli* (EPEC) causes severe, watery diarrhea in children. We investigated ATP release during EPEC-mediated killing of human cell lines and whether released adenine nucleotides function as secretory mediators. EPEC triggered a release of ATP from all human cell lines tested: HeLa, COS-7, and T84 (colon cells) as measured using a luciferase kit. Accumulation of ATP in the supernatant medium was enhanced if an inhibitor of 5′-ectonucleotidase was included and was further enhanced if an ATP-regenerating system was added. In the presence of the inhibitor/regenerator, ATP concentrations in the supernatant medium reached 1.5–2 μM 4 h after infection with wild-type EPEC strains. In the absence of the inhibitor/regenerator system, extracellular ATP was rapidly broken down to ADP, AMP, and adenosine. Conditioned medium from EPEC-infected cells triggered a brisk chloride secretory response in intestinal tissues studied in the Ussing chamber (rabbit distal colon and T84 cell monolayers), whereas conditioned medium from uninfected cells and sterile filtrates of EPEC bacteria did not. The short-circuit current response to EPEC-conditioned medium was completely reversed by adenosine receptor blockers, such as 8-(p-sulfophenyl)-theophylline and MRS1754. EPEC killing of host cells releases ATP, which is broken down to adenosine, which in turn stimulates secretion via apical adenosine A2b receptors. These findings provide new insight into how EPEC causes watery diarrhea.

extracellular nucleotides; adenosine receptors; ATP efflux; purinergic receptors; apoptosis

ENTEROPTHAGENIC *Escherichia coli* (EPEC) is a common cause of diarrhea in children in developing countries. Unlike other types of diarrheagenic *E. coli*, EPEC produces no toxins, and the way it produces watery diarrhea is unknown. EPEC kills host intestinal cells in vitro, and this cell death has mixed features of apoptosis and necrosis (2, 12, 14). Intestinal cell death also occurs in vivo in rabbits infected with rabbit EPEC (REPEC) and rabbit diarrheagenic *E. coli* (RDEC-1; E. Boedeker and J. Crane, unpublished data).

Many EPEC virulence factors are encoded on a pathogenicity island known as the locus of enterocyte effacement (LEE), including the type III secretion machinery and the EPEC secreted proteins. Type III secretion is a property of certain gram-negative bacteria, including *Salmonella*, *Shigella*, *yersinia*, and EPEC, that allows secretion of bacterial proteins into the host cell cytosol or plasma membrane (26). One LEE-encoded protein, EPEC-secreted protein F (EspF), is necessary for host cell killing (11). EspF is one of a growing list of effector proteins secreted into the host cell during the process of infection, in EspF's case, into the cytosol (33). EspF is also required for the EPEC-mediated drop in transepithelial electrical resistance (TER) seen in response to EPEC infection (34).

While investigating the potential role of ectoprotein kinases in the pathophysiology of EPEC infection, we examined whether EPEC would trigger a release of ATP from the host cell. In this study, we report the rate and extent of ATP release from the host cell in response to EPEC infection, demonstrate the rapid breakdown of released ATP to less phosphorylated adenine nucleotides and adenosine, and show that these extracellular nucleotides and nucleosides trigger a brisk chloride secretory response in intestinal tissues studied in the Ussing chamber. EPEC-infected host cells release nucleotide mediators capable of triggering a secretory response from neighboring, healthy cells. These findings confirm and extend the importance of host cell killing by EPEC and suggest a mechanism for the watery diarrhea produced by this pathogen.

MATERIALS AND METHODS

**Materials.** The following reagents were obtained from Sigma-Aldrich: α,β-methylene-ADP, creatine kinase, phosphocreatine, forskolin, adenosine, AMP, ADP, Tris-acetate, tetrabutylammonium dihydrogen phosphate, and type III collagen. 8-(p-sulfophenyl)-theophylline was from Research Biochemicals International (Natick, MA).

**Bacterial culture, tissue culture, and animal tissue.** Bacterial strains used in this study are described in Table 1. Bacteria were grown overnight in LB broth supplemented with 1% mannose at 37°C with 300 rpm shaking. The next morning, the strains were subcultured 1:8 into EPEC adhe-
Table 1. *Bacterial strains used in this research*

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>General Description</th>
<th>Mutations</th>
<th>Other, Comments</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2348/69</td>
<td>Wild-type EPEC</td>
<td></td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>JPN15</td>
<td>Plasmid cured derivative of E2348/69</td>
<td>EAF−</td>
<td>Nonadherent due to loss of BFP (pilus)</td>
<td>28</td>
</tr>
<tr>
<td>UMD874</td>
<td>Derived from E2348/69</td>
<td>espF</td>
<td>At far right end of the LEE; defective in host cell killing</td>
<td>18, 33</td>
</tr>
<tr>
<td>CVD206</td>
<td>Derived from E2348/69</td>
<td>eae</td>
<td>Intimin mutant</td>
<td>17</td>
</tr>
<tr>
<td>SE796</td>
<td>Derived from E2348/69</td>
<td>ler</td>
<td>LEE encoded regulator</td>
<td>19</td>
</tr>
<tr>
<td>SE1010</td>
<td>Derived from E2348/69</td>
<td>sepZ</td>
<td>In LEE; component of type III secretion</td>
<td>20, 44</td>
</tr>
<tr>
<td>B171-8</td>
<td>Wild-type EPEC</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>JCP88</td>
<td>Wild-type EPEC</td>
<td></td>
<td></td>
<td>47</td>
</tr>
<tr>
<td>Enterohaemorrhagic E. coli</td>
<td>Enterohaemorrhagic E. coli (STEC)</td>
<td>None known</td>
<td>Clinical isolate; Argentina</td>
<td></td>
</tr>
<tr>
<td>H.S</td>
<td>Normal stool flora E. coli</td>
<td></td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>HB101</td>
<td>Laboratory E. coli</td>
<td>O9:H4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strains other than E. coli</td>
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<td></td>
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<tr>
<td><em>Salmonella enterica</em>, serotype enteritidis</td>
<td>None known</td>
<td>Clinical isolate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shigella sonnei</td>
<td>Clinical isolate</td>
<td>None known</td>
<td>From traveller returning from Cuba</td>
<td></td>
</tr>
</tbody>
</table>

EPEC, enteropathogenic *Escherichia coli*; LEE, locus of enterocyte effacement; ler, LEE-encoded regulator; EAF, EPEC adherence factor; BFP, bundle-forming pilus; STEC, Shiga-toxigenic *E. coli*.

Bacterial culture medium consisting of DMEM/F-12 (GIBCO-BRL, Grand Island, NY) supplemented with 40 mM HEPES, pH 7.4, 18 mM NaHCO₃, 2% heat-inactivated newborn calf serum, and 1% mannose for 2 h with the same temperature and shaking conditions. EPEC adherence medium was warmed overnight at 37°C. T84 cells were infected at a multiplicity of infection of 100:1 and HeLa cells at 200:1 unless otherwise stated.

T84 and HeLa cells were maintained as previously described (13), using a medium of DMEM/F-12, 18 mM NaHCO₃, 5% newborn calf serum, 5% fetal bovine serum, 20 μg/ml vancomycin, 15 μg/ml gentamicin, and 0.5 μg/ml amphotericin B-deoxycholate. HeLa cells are a human cervical cancer line, and T84 is a human colon cancer cell line. For experiments, cells grown in multiwell plates were rinsed with sterile PBS, then refed with EPEC adherence medium; for 24-well plates, this was 0.5 ml/well. T84 cells used for the ATP-release experiments were from ATCC and were at passage 53 on receipt. In general, conventionally grown T84 cells and HeLa cells were used for ATP release experiments and ATP breakdown experiments, whereas polarized, low-passage T84 cells were used for Ussing chamber experiments. Low-passage (30–40) T84 cells were a kind gift of Dr. Cynthia Sears (Johns Hopkins University, Baltimore, MD).

For Ussing chamber measurements, T84 cells were seeded into collagen-coated, 12-mm, 0.4-μm pore size Snap-Well tissue culture inserts (model 3407; Corning Costar, Corning, NY). Snap-Well inserts were coated with 200 μl of sterile, 0.2 g/l type III calf-skin collagen dissolved in 0.05 M warm acetic acid. After the collagen was allowed to dry overnight, low-passage T84 cells were plated into the Snap-Well at a density of ~650,000 cells per Snap-Well insert. Snap-Well inserts were fed with medium containing 10% fetal calf serum every 3 days for 9 to 12 days, when TER reached values of >1,000 Ω·cm².

**Preparation of colon tissue for Ussing chamber experiments.** Male New Zealand White rabbits were killed by exposure to 100% CO₂. The descending colon was removed, opened into a flat sheet, and washed in standard tissue bathing solution (see below). The epithelium was stripped from its underlying musculature with a glass microscope slide.

**ATP assay.** ATP concentrations in conditioned medium from infected and uninfected cells were measured using an ATP bioluminescence assay kit (model HS II; Roche Molecular Biochemicals, Indianapolis, IN). Cells in 24-well plates were infected for 1 h, then the medium was changed to serum-free adherence medium to remove most of the unbound bacteria. At various times after the medium change, the plates were swirled to mix, and 100-μl aliquots were removed from wells and placed into the upper chamber of Spin-X tubes (Corning Costar, Corning, NY). Spin-X tubes are microcentrifuge tubes containing a 0.45-μm filter insert and were used to remove bacteria and any detached human cells. Before adding the experimental sample, the lower portion of the Spin-X tube was loaded with 100-μl of preservation/lysis buffer supplied with the ATP assay kit. As soon as possible after the collection of the experimental sample, the Spin-X tubes were centrifuged for 1 min at 14,000 g in an Eppendorf centrifuge, then the filter insert was removed and discarded. ATP samples so collected were then stored frozen at −70°C until the day of the ATP assay, when they were diluted an additional fivefold in 100 mM Tris-acetate-2 mM EDTA. One hundred microliters of sample was mixed together with 100 μl of luciferase reagent and immediately read on a Bio-Orbit 1250 lumimeter (LKB-Wallac, Turku, Finland). ATP standards used to prepare the standard curve were 0, 0.01, 0.03, 0.05, and 0.1 μM. To exclude the possibility of an artifactual inhibition of ATP measurement by antibiotics, as in Fig. 4, the luminescence reading observed from 100 nM ATP was compared in the presence and absence of antibiotics. The addition of 75 μg/ml ciprofloxacin and 80 μg/ml polymixin B (final concentrations 50 times higher than achieved in Fig. 4 after dilution) gave readings 94 and 95% of the no-antibiotic standard, respectively.

For many of the experiments shown, an inhibitor/regenerator system was added to the adherence medium during the
accumulation phase of the experiment to retard ATP breakdown. The 5’-ectonucleotidase inhibitor α,β-methylene-ADP was added from a concentrated stock to yield 250 μM in the culture medium. Similarly, creatine kinase and phosphocreatine were premixed and added together to yield final concentrations of 20 mg/l and 10 mM, respectively.

Induction of apoptosis by UV irradiation, and LDH release assay. Ultraviolet (UV) light was used to induce apoptosis in HeLa cells using 2 min of irradiation on a UV transilluminator box as previously described (11). Lactate dehydrogenase (LDH) release was measured as described previously (12).

Transepithelial electrophysiological measurements. Monolayers of T84 cells were inserted into a modified Ussing chamber (model DCH; NaviCyte, San Diego, CA) at 37°C and continuously short circuited by a four-electrode, automatic voltage clamp apparatus (model 616C; Department of Bioengineering, University of Iowa, Iowa City, IA), which measured short-circuit current (Isc) and TER; chamber fluid resistance was automatically subtracted. Isc was measured by passing a constant current through the tissues via Ag/AgCl electrodes to reduce the spontaneous transepithelial potential to zero. Transepithelial resistance was determined by

RESULTS

Initial experiments to detect ATP release during EPEC infection were performed in ordinary, serum-free medium without any special additives, in HeLa and T84 cells (Fig. 1A and 1C). ATP release in these conditions was readily detected in HeLa cells (Fig. 1A), but not in T84 cells (Fig. 1C), which was surprising, because the extent of EPEC-induced cell death is about the same in these two cell lines (12). Because the literature indicated that T84 cells, like other intestinal cells, have abundant amounts of several ectonucleotidases (46, 51), we felt that the failure to detect ATP in EPEC-infected T84 cell supernatants might be due to its rapid destruction, rather than due to actual lack of release. The addition of an inhibitor of 5’-ectonucleotidase, α,β-methylene-ADP, by itself did not increase the amount of detectable ATP a great deal, nor did addition by itself of an ATP-regenerating system consisting of creatine kinase and phosphocreatine (Table 2). The combination of α,β-methylene-ADP and the ATP regenerating system, however, resulted in significantly higher ATP concentrations in the supernatant medium of EPEC-infected cells (Table 2 and Fig. 1, B and D; note the difference in scales on the ordinate). Thus when ATP was “trapped” by the inhibitor/regenerator system the ATP accumulated to concentrations exceeding 1.5 μM in both cell lines. ATP release was markedly less in response to infection with the cell-death deficient espF mutant (strain UMD874), the plasmid-cured strain JPN15, or nonpathogenic E. coli strain H.S. ATP concentrations in the medium above uninfected cells remained negligible even in the presence of the inhibitor/regenerator system. Other wild-type EPEC strains tested, including E851/71, B171–8, and JCP88 triggered a release of ATP similar in magnitude to that of strain E2348/69 (Table 2).

Performance of the espF mutant UMD874 relative to wild-type strains depended on the host cell line used. In HeLa cells, ATP release by strain UMD874 was 32.8 ± 13% of that released by the wild-type strains (mean of 3 separate experiments each done in triplicate; see also Fig. 1B), whereas in T84 cells, ATP release by UMD874 was 68.9 ± 10% of the wild-type strain (mean of 5 separate experiments; P = 0.005 compared with HeLa; see also Fig. 1D). In COS-7 cells, the performance of UMD874 relative to wild-type strains was similar to that in HeLa cells (data not shown).

To determine whether ATP breakdown was indeed rapid enough in T84 cells to account for the lack of detectable ATP accumulation seen in Fig. 1C, ATP levels were measured after treatment with exogenous ATP (Fig. 2). Because EPEC and other diarrheagenic E. coli infections can alter intestinal cell surface enzymes, the fate of ATP was compared in uninfected cells, cells infected for 1 h with EPEC, and for 1 h with enterohaemorrhagic E. coli strain 042. Figure 2A shows that ATP was rapidly destroyed by T84 cells and the rate of disappearance was the same in infected as in uninfected monolayers.

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Figure 2B shows that the addition of the inhibitor/regenerator system prevented the destruction of exogenous ATP over the short term. Other experiments (not shown) demonstrated that the regenerator system was eventually exhausted 4–6 h after infection but could be replenished by readdition of the creatine kinase and phosphocreatine.

Although the luminescence method used in Fig. 2, A and B documented the rapid breakdown of ATP in T84 cells, that method could not determine the products of ATP hydrolysis. To accomplish this, HPLC analysis was used (Fig. 2C). In these experiments, a higher concentration of exogenous ATP was added (0.2 mM) to unequivocally identify products generated. The HPLC tracings confirmed the rapid breakdown of ATP and showed, in agreement with the work of others (16), that first ADP, and then AMP and adenosine appear in the cell supernatants as a result of ATP breakdown. The loss of ATP from the culture medium (Fig. 2, B and C) was well fit by a single-component exponential decay

Table 2. Comparison of ATP accumulation in the presence and absence of various inhibitor-regenerator combinations and comparison with other wild-type EPEC strains in T84 cells

<table>
<thead>
<tr>
<th>Infection Condition</th>
<th>Inhibitor or Regenerator Condition</th>
<th>[ATP] in Supernatant, 4 h after Medium Change, Mean (nM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>None</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>Uninfected</td>
<td>+α,β-methylene-ADP and CK + PC</td>
<td>9.5 ± 1.2</td>
</tr>
<tr>
<td>E2348/69-infected</td>
<td>CK + PC alone, no αβ-methylene-ADP</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>E2348/69-infected</td>
<td>+α,β-methylene-ADP + CK + PC</td>
<td>188.7 ± 15.4</td>
</tr>
<tr>
<td>E2348/69-infected</td>
<td>+α,β-methylene-ADP + CK + PC + 10 U/ml myokinase</td>
<td>754.9 ± 63</td>
</tr>
<tr>
<td>B171-8-infected</td>
<td>+α,β-methylene-ADP + CK + PC</td>
<td>1461.6 ± 98.8</td>
</tr>
<tr>
<td>JCP88-infected</td>
<td>+α,β-methylene-ADP + CK + PC</td>
<td>880.8 ± 24.9</td>
</tr>
</tbody>
</table>

CK, 20 μg/ml creatine kinase; PC, 10 mM phosphocreatine.
curve with an ATP half-life of 22–27 min. To extend the analysis further, exogenous AMP was also added to T84 monolayers, and its fate was determined by HPLC. Figure 2D shows that AMP is rapidly broken down to adenosine; at later times, another peak presumptively identified as inosine monophosphate is also detected.

Figure 3A shows that, in contrast with EPEC infection, ATP was not released from HeLa cells by a purely apoptotic stimulus, i.e., irradiation with UV light. Similarly the cytosolic enzyme LDH was not released in response to UV irradiation (Fig. 3B), although this UV treatment did trigger widespread apoptosis by morphologic criteria (Fig. 3C). These results are in accord with a large body of data showing that cytoplasmic contents are not released in apoptosis (7). Therefore, the release of ATP by EPEC illustrates a nonapoptotic feature of EPEC-mediated killing.

We considered the question of the origin of the ATP released during EPEC infection and whether the bacteria themselves could release ATP. The first and most persuasive evidence on this question is quantitative. EPEC, like all bacteria, contains ATP, and this ATP content is readily measurable. The amount of ATP detectable in T84 or HeLa supernatants, as shown in Fig. 1, is far greater than can be accounted for by the bacterial ATP content. For example, in our typical assay well of 0.5 ml, if 100% of the bacterial inoculum adhered (which does not occur) and if 100% of the bacteria spontaneously lysed (which does not occur (12)), the amount of ATP released from the bacteria would only yield a concentration of \( \frac{1}{100} \) nM. Thus the amount of ATP actually released is \( \frac{1}{20} \) times that which could possibly be contributed by the bacteria, even in the most unrealistic, worst-case scenario. The addition of antibiotics after infection also indicates that the source of the released ATP is of host cell origin (Fig. 4). Antibiotics with diverse mechanisms of action all inhibit the ATP release observed in EPEC infection. This inhibition is observed even with polymixin B, which triggers bacterial lysis by binding to the bacterial outer membrane. Other antibiotics not shown in Fig. 4, including ampicillin and tetracycline, also inhibited ATP release by 50–75%. This is consistent with our previous report that EPEC-induced cell death required prolonged contact of live bacteria with the host cell (12). If, in contrast, the ATP were coming from the...
bacteria, the antibiotic treatment should increase the amount liberated via bacterial cell lysis. Also, there are the results with the espF mutant, deficient in host cell killing. The espF mutant UMD874 adheres like the wild-type strain, but ATP release is markedly less (Fig. 1, A, B, and D). Again, these results are only consistent with ATP release from the host cell. Finally, we have measured the ATP content of the monolayers after infection with wild-type EPEC. In T84 cells, the ATP content of the monolayer decreased from 3.7 ± 0.1 nmol/well in control cells to 3.0 ± 0.3 nmol/well 4 h after infection. The decline in the monolayer ATP accounted for the concentration of ATP measured in the supernatant medium for wells containing 0.5 ml of medium.

In experiments with cells grown in 6.5 mm Transwell inserts, EPEC infection triggered a release of ATP only into the upper, apical compartment. No ATP was detected in the basolateral chamber in polarized cells, even when the inhibitor/regenerator was added on both sides (ATP in the lower chamber 1.2 ± 0.8 vs. 310 ± 54 nM in the upper chamber after EPEC infection). This indicated that ATP release was directed to the same (apical) side of the cell exposed to the bacteria.

The experiment shown in Fig. 5A compared the ATP-releasing ability of EPEC with that of other known enteric pathogens and the nonadherent laboratory strain HB101. Of the strains tested, only three triggered a release of ATP greater than that of HB101: EPEC E2348/69, E. coli O157:H7, and Salmonella enterica. The failure of Shigella sonnei to trigger any detectable ATP release is well explained by the inability of Shigella species to kill epithelial cells, as opposed to macrophages and lymphocytes, which Shigellae kill quickly (35). More surprising was the lack of detectable ATP release by enterohaemorrhagic E. coli strain 042. Enterohaemorrhagic E. coli are known to damage host cell enterocytes in vitro and in vivo (38, 40, 41), but the kind of damage inflicted by strain 042 must differ qualitatively in such a way as to not release ATP. The low amount of ATP released by E. coli O157:H7 is consistent with the much lower adherence observed with this pathogen, which lacks the bundle-forming pilus. The ATP release seen with E. coli O157:H7 reflects a poorly studied, contact-mediated damage, not the effects of the Shiga-like toxins (SLTs) in the T84 cells (2). T84 cells lack the Gb3 glycolipid receptor for SLT-I and SLT-II, and furthermore we showed above that a purely apoptotic stimulus does not trigger ATP release (Fig. 3). Finally, it is worth noting that ATP release by EPEC was 66% of that released by S. enterica. This illustrates a recurring theme in EPEC-mediated host cell killing: that although EPEC is rel-

**Fig. 3.** Lack of ATP or lactate dehydrogenase (LDH) release in response to a purely apoptotic stimulus in HeLa cells. HeLa cells in 24-well plates were exposed to UV light for 2 min on a UV transilluminator box, then ATP (A) or LDH activity (B) was then measured in aliquots of supernatant medium at the times indicated. C: cells grown on glass Lab-Tek slides were fixed 4 h after UV irradiation, stained with Giemsa, then photographed at magnification ×600. Cells show shrinkage, nuclear condensation, and marked membrane blebbing, demonstrating that apoptosis is induced by this method. PMA, 1 μM phorbol myristate acetate.

**Fig. 4.** Effect of antibiotics on EPEC-induced ATP release in T84 cells. T84 cells were infected with EPEC E2348 or normal stool flora E. coli strain H.S. for 1 h and then changed to fresh medium with the inhibitor/regenerator (I/R). Immediately after the medium change, polymixin B (16 mg/l final concentration) or ciprofloxacin (cipro; 15 mg/l final) was added from concentrated stocks and the incubation was continued. ATP release in the two antibiotic-treated conditions was significantly less than E2348 alone at 2.5 and 4 h (P < 0.05). uninf, Uninfected.
activating slow and inefficient in assays of cell death that measures the final completion of cell death (such as nuclear fragmentation and DNA cleavage), EPEC performs quite well in assays that reflect earlier, membrane changes in the host, such as externalization of phosphatidylserine and phosphatidylethanolamine, membrane permeability to dyes, and ATP release.

Figure 5B illustrates the ATP-releasing abilities of several mutants derived from EPEC strain E2348/69. The reduced ATP release of the plasmid-cured derivative, JPN15, and the eae (intimin-deletion) mutant, CVD206, are consistent with their previously described defects in host cell killing and adherence (11, 12). Strains with mutations in the LEE-encoded regulator (ler) and sepZ (a part of the type III secretion machinery) were also markedly reduced in ability to trigger ATP release from the host cell. Results with these mutants demonstrate the importance of genes encoded in the EPEC LEE in the EPEC-mediated ATP release just as they are in EPEC-induced cell death and other types of EPEC-mediated damage (34). The espF mutant is particularly instructive, because this mutant is equivalent to wild-type EPEC in total adherence, intimate adherence, actin condensation, tyrosine phosphorylation, and secretion of the other EPEC-secreted proteins yet is deficient in its ability to induce host cell death (11).

ATP release from EPEC-infected cells could occur by the formation of large pores in the host plasma membrane, as occurs with other pathogens possessing the type III secretion system (5, 24), or by some other mechanism. Several laboratories have reported that the cystic fibrosis transmembrane regulator (CFTR) may allow efflux of ATP when it is activated by cell swelling or by cAMP (25, 45, 54). Other members of the ATP-binding cassette family, such as the P-glycoprotein, which mediates the multidrug resistance phenotype, also may allow ATP efflux, albeit in a cAMP-insensitive manner (1). To determine whether cAMP elevation affected EPEC-induced ATP release, we compared the effects of forskolin, a direct stimulator of adenylyl cyclase, on ATP release in cells with and without CFTR. Figure 6 shows that although forskolin alone did not trigger detectable ATP release in either cell line, in T84 cells, which express CFTR, forskolin dramatically increased the amount of ATP effluxed in response to EPEC infection (Fig. 6A). In HeLa cells,
which do not express CFTR, forskolin had no effect on EPEC-induced ATP release (Fig. 6B). As with forskolin, cholera toxin treatment of T84 cells increased ATP efflux in response to EPEC infection (Fig. 6C). These findings show that, in some cell lines, cAMP elevations significantly potentiate EPEC-induced ATP release. The enhanced ATP release is not due to increased cytotoxicity, i.e., forskolin did not increase EPEC-induced cell death as measured by the propidium iodide uptake method (data not shown). The CFTR is one plausible target of cAMP that could be involved in the increased ATP release. If EPEC is shown to interact with the CFTR, it would join *Salmonella* as a bacterial pathogen that exploits the activity of the CFTR during intestinal infection (43).

Results showed that EPEC infection triggered ATP release from the host cell (Figs. 1 and 4–6). Over the past decade, investigators from many laboratories have shown that extracellular nucleotides may trigger anion secretion in respiratory and gastrointestinal tissues and cell lines (3, 31, 46, 53). Therefore, we sought to determine whether the ATP released in response to EPEC infection was sufficient to cause chloride secretion in intestinal tissues, because such a response could have relevance to the secretory diarrhea seen in EPEC infection. We studied intestinal tissues in the Ussing chamber using the voltage-clamp technique to measure $I_{sc}$. Initial Ussing chamber experiments were carried out using stripped distal colon from the normal rabbit. Rabbit colon was chosen because agonist-stimulated $I_{sc}$ in this tissue is predominantly due to chloride secretion and because it has been studied in the Ussing chamber with regard to adenosine-induced secretion (21). In these experiments, 5 ml of conditioned medium from EPEC-infected T84 cells collected in the absence and presence of the inhibitor/regenerator triggered $I_{sc}$ responses of 13.8 ± 5.4 and 29.5 ± 3.5 μA/cm², respectively, when added to the mucosal side of the tissue (data not shown, results from 7–8 colon tissues from 4 separate rabbits). Conditioned medium from uninfected HeLa and T84 cells did not trigger $I_{sc}$ (<1.5 μA/cm²), nor did sterile filtrates of EPEC bacteria ($n = 2$ separate experiments).

On the basis of the results of the Ussing chamber experiments with rabbit colon, we extended those observations with similar experiments using T84 cell monolayers grown on Snap-Well inserts. This model was chosen because it has been well characterized and offers the potential of less tissue-to-tissue variability than with native colon, and the $I_{sc}$ in T84 cells reflects chloride secretion and not cation absorption. (15, 30).

Using T84 cell monolayers as the test tissue in the Ussing chamber, we again tested conditioned media from EPEC-infected HeLa and EPEC-infected T84 cells for their ability to stimulate chloride secretion.

Figure 7 shows the results obtained with conditioned medium from HeLa cells. Figure 7A shows that conditioned medium from uninfected HeLa cells failed to trigger an $I_{sc}$ response, whether collected in the absence (Fig. 7A, 1) or presence of the inhibitor/regenerator (Fig. 7A, 2). Similarly, a filtrate of EPEC bacteria did not trigger chloride secretion (Fig. 7B, 1). In contrast, conditioned medium from EPEC-infected HeLa cells, without any inhibitor/regenerator, triggered a brisk and sustained $I_{sc}$ (Fig. 7C). Figure 7C demonstrates the response observed when the test sample

![Fig. 7. Effect of EPEC-HeLa-conditioned medium on secretory responses in the Ussing chamber. T84 cells grown in Snap-Well inserts were used as the test tissue in the Ussing chamber for measurements of resistance and short-circuit current ($I_{sc}$) as described in MATERIALS AND METHODS. Transepithelial electrical resistance was $>1,000$ Ω/cm² for these monolayers. The $y$-axis is $I_{sc}$. Because the Snap-Well monolayers have an area of 1.1 cm², the current flux in μA/cm² is the raw $I_{sc}$ divided by 1.1. Vertical arrows with numbers indicate the time of addition of a test substance. A, 1: conditioned medium from uninfected HeLa cells collected in the absence of the inhibitor/regenerator (0.6 ml). A, 2: conditioned medium from uninfected HeLa cells collected in the presence of the inhibitor/regenerator (0.6 ml). A, 3: AMP (1 μM) as a positive control to demonstrate responsiveness of the tissue. B, 1: sterile filtrate (1 ml) of EPEC E2348 subcultured for 2 h in EPEC adherence medium. B, 2: AMP (10 μM) as a positive control to demonstrate responsiveness of the monolayer. C, 1: EPEC-HeLa-conditioned medium (0.6 ml) collected in the absence of the inhibitor/regenerator. C, 2: 8-(p-sulfophenyl)-theophylline (8-SPT; 50 μM), an adenosine receptor antagonist, to the mucosal side only.

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was diluted 10-fold (0.6 ml of sample into a hemichamber of 6 ml volume). In four separate experiments, the $I_{sc}$ generated by the application of 1 ml of EPEC-HeLa-conditioned medium was $5.3 \pm 1.8 \mu A/cm^2$ in the absence of the inhibitor/regenerator and $9.3 \pm 2.3 \mu A/cm^2$ in its presence ($P = 0.02$). For EPEC-T84-conditioned media, the $I_{sc}$ values observed were $3.0 \pm 1.9$ and $8.4 \pm 3.4 \mu A/cm^2$ for media collected in the presence and absence of the inhibitor/regenerator, respectively. In general, the amplitude of the $I_{sc}$ response was proportional to the amount of test medium applied over the range of 0.5 to 1.8 ml. As a basis for comparison, maximal doses of adenosine (10–20 μM) in this system produce $I_{sc}$ responses of 23–33 μA/cm². $I_{sc}$ triggered by EPEC-HeLa- and EPEC-T84-conditioned media were quickly and completely reversed by 8-((p-sulfophenyl)-theophylline, a non-cell-permeable adenosine receptor antagonist (Figs. 7C and 8F). Complete reversal of the secretory response by 8-((p-sulfophenyl)-theophylline indicates that it is adenosine, not AMP, ADP, or ATP, that is the final agonist acting on the T84 cells to trigger chloride secretion. This finding is consistent with the reports of several other laboratories indicating that apically applied adenine nucleotides in T84 cells trigger secretion via adenosine receptors, specifically the adenosine A₂b, adenosine receptor subtype (3, 31, 52, 53).

Fig. 8 shows the results obtained with conditioned medium from EPEC-infected T84 cells. Once again, conditioned medium from uninfected T84 cells triggered no secretion whether the inhibitor/regenerator was present or not (Fig. 8A, I and 2). Conditioned medium from T84 cells infected with wild-type EPEC E2348/69 did trigger a secretory response, and it was larger with medium collected in the presence of the inhibitor/regenerator (Fig. 8C) than in its absence (Fig. 8B). Conditioned medium from cells infected with the espF mutant UMD874 triggered an $I_{sc}$ less than that from E2348-infected cells (Fig. 8, D and E, without and with inhibitor/regenerator, respectively). In four separate experiments similar to Fig. 8, C and E, the ratio of the $I_{sc}$ triggered by UMD874-conditioned medium was 66.4 ± 22% of that triggered by wild-type EPEC-conditioned medium (UMD874 significantly less than wild EPEC by paired t-test, $P = 0.02$). The decreased $I_{sc}$ observed after infection with the espF mutant UMD874 is quantitatively similar to the reduced ATP release induced by this mutant (Figs. 1 and 5B). Results shown in Figs. 7 and 8 also demonstrated that the amount of ATP released during EPEC infection was sufficient to trigger chloride secretion, not only when added at full strength, but even after dilution 6- to 10-fold.

Fig. 8. Effect of EPEC-T84-conditioned media on chloride secretion in the Ussing chamber. Four-hour-conditioned media were collected from T84 cell monolayers infected with wild-type EPEC strain E2348, espF mutant UMD874, or left uninfected. Some of the T84-conditioned medium were collected from T84 cell monolayers infected with wild-type EPEC E2348, espF mutant UMD874, or left uninfected. Some of the T84-conditioned medium were collected in the absence of the inhibitor/regenerator (A, 1; B, and D) and some with the inhibitor/regenerator (A, 2; C, E; and F, I). To test the response of the tissue to conditioned medium, 1 ml of Ringer buffer was removed from the 6-ml mucosal hemichamber and replaced with 1 ml of the test substance, and the response was recorded. Each panel represents the tracing from one monolayer. The size bar for the y-axis indicates 5 μA, and the x-axis size bar represents 2 min in A–G. Current flux in μA/cm² is the raw $I_{sc}$ shown divided by 1.1. The conditioned media shown in the figure are as follows: A, 1: conditioned medium from uninfected T84 cells, collected in the absence of the inhibitor/regenerator. A, 2: conditioned medium from uninfected T84 cells, collected in the presence of the inhibitor/regenerator. A, 3: AMP (10 μM) added a positive control to demonstrate the viability and responsivity of the test tissue. B: EPEC E2348-T84-conditioned medium, collected in the absence of the inhibitor/regenerator. C: EPEC E2348-T84-conditioned medium, collected in the presence of the inhibitor/regenerator. D: EspF mutant UMD874-T84-conditioned medium, collected in the absence of the inhibitor/regenerator. E: EspF mutant UMD874-T84-conditioned medium, collected in the presence of the inhibitor/regenerator. F, 1: EPEC-T84-conditioned medium with inhibitor/regenerator. F, 2: 8-SPT (25 μM). G, 1: EPEC-T84-conditioned medium. G, 2: MRS1754 (200 nM), an adenosine A₂b, receptor antagonist.

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Additional experiments were performed to determine whether the secretagogue activity in conditioned medium from EPEC-infected cells was accounted for by its content of adenine nucleotides or adenosine, as opposed to some other bioactive molecule. The secretagogue in the conditioned medium retained its activity after filtration through a 10,000 mol weight cut-off membrane, consistent with the low molecular size of a nucleotide or nucleoside. After boiling, conditioned media from EPEC-infected cells retained 75 ± 3% of their original activity (4 separate experiments), which is consistent with the properties of AMP or adenosine (37).

The presence of the inhibitor/regenerator had more prominent effects in EPEC-T84-conditioned medium than in EPEC-HeLa-conditioned medium (compare Fig. 7C with Fig. 8, B and C). Initially, the enhancing effect of the inhibitor/regenerator led us to believe that phosphorylated adenine nucleotides, such as ATP or ADP, might be triggering a secretory response in the Ussing chamber. Instead, however, the discrepancy is most likely explained by more avid reuptake of adenosine in T84 cells. Mun et al. (36) found that T84 cells took up adenosine via both the apical and basolateral surfaces of the cell, although the capacity of the latter was much larger. Because EPEC infection markedly increases transmembrane permeability in the 5-h duration of infection we used (6, 34, 42), adenosine could reach the basolateral space and be susceptible to reuptake via the basolateral transport system. This system is of sufficient affinity and capacity to reduce extracellular adenosine levels below the threshold for activation of adenosine receptors (36). Thus the avid reuptake ability of T84 cells limits the accumulation of extracellular adenosine in media collected in the absence of the inhibitor/regenerator. In contrast, ATP, ADP, and AMP are taken up poorly or not at all by either mammalian or bacterial cells. The inhibitor/regenerator temporarily traps nucleotides in their phosphorylated forms, in which they are not susceptible to reuptake, but the phosphocreatine in the regenerating system becomes exhausted toward the end of the 4-h collection period. In addition, injection of the sample into the Ussing chamber results in a 6- to 10-fold dilution, such that the concentration of the α, β-methylene ADP inhibitor drops well below its IC50 for 5’-nucleotidase. Evidently, nucleotidases in the test tissue are able to convert some of the nucleotides to adenosine and generate a secretory response. In further support of the hypothesis that adenosine is the mediator of secretion, apyrase treatment of EPEC-T84-conditioned medium (5 U/ml apyrase for 5 min, 37°C) did not abolish the I_{sc} response in the Ussing chamber, but instead actually increased the I_{sc} response. In four experiments, the ratio of the I_{sc} observed after apyrase treatment relative to sham-treated control was 154 ± 42% (significantly increased; \( P = 0.018 \) by paired t-test). Furthermore, the I_{sc} triggered by EPEC-T84-conditioned medium in the presence of the inhibitor/regenerator was completely reversed by 8-(p-sulfophenyl)-theophylline (Fig. 8F, 2) an adenosine receptor antagonist. In addition, the secretory response of EPEC-T84-conditioned medium was reversed by 200 nM MRS1754, a highly selective adenosine A2b receptor blocker (29). Figure 9 shows the dose-response relationship for reversal of the I_{sc} by MRS1754. The apparent IC50 of MRS1754 in Fig. 9 (~50 nM) is somewhat higher than values reported in the literature (2–20 nM) (29). MRS1754, a hydrophobic compound, binds strongly to plastic, so the modestly decreased potency observed in Fig. 9 may be due to absorption to the Ussing chamber itself, which has a large internal surface area and is composed of acrylic plastic. In contrast, the I_{sc} triggered by EPEC-conditioned medium was not blocked by 10 μM MRS1719, a potent antagonist of P2Y1 receptors and a weaker antagonist of P2X receptors (data not shown). These findings indicate again that it is adenosine, and not a phosphorylated adenine nucleotide, that triggers chloride secretion when applied to the apical side of T84 cells (3, 31, 53), and this adenosine effect is mediated via adenosine A2b receptors (52).

**DISCUSSION**

The results presented here show that EPEC infection of cells triggers a release of ATP from host cells that is large in magnitude and, in polarized cells, is solely into the apical culture medium. Extracellular ATP is quickly broken down to less phosphorylated nucleotides and adenosine. These products accumulate in sufficient quantity that they are capable of triggering a chloride secretory response in intestinal tissues in the Ussing chamber (Figs. 7C and 8B). An inhibitor/regenerator system was used as a nonphysiological way of trapping ATP to allow its convenient and accurate measurement. On the basis of the data shown, concentrations of ATP measured in the presence of the inhibitor/regenerator appear to provide a reasonable estimate of the total adenine nucleotide pool (ATP, ADP, and AMP) that is large in magnitude and, in polarized cells, is solely into the apical culture medium. Extracellular ATP is quickly broken down to less phosphorylated nucleotides and adenosine. These products accumulate in sufficient quantity that they are capable of triggering a chloride secretory response in intestinal tissues in the Ussing chamber (Figs. 7C and 8B). An inhibitor/regenerator system was used as a nonphysiological way of trapping ATP to allow its convenient and accurate measurement. On the basis of the data shown, concentrations of ATP measured in the presence of the inhibitor/regenerator appear to provide a reasonable estimate of the total adenine nucleotide pool (ATP, ADP, and AMP).
ADP, AMP, and adenosine), which accumulates in the absence of the inhibitor/regenerator.

Because the regenerating system can rephosphorylate ADP to ATP, it is formally possible that release of ADP contributes to the extracellular adenine nucleotide accumulation. This seems unlikely, however, for two reasons. First, ATP release is observed in HeLa cells in the absence of the inhibitor/regenerator (Fig. 1A). Second, the ratio of ATP to ADP in healthy cells is >5:1, a situation that applies early in the course of infection. Even after 4 h of EPEC infection, the ATP content of the monolayer is still ~80% of the uninfected control, so a severe alteration in the “energy charge,” or ATP:ADP ratio, of the cell is unlikely. However, our data do not rule out the possibility of ADP efflux from the infected host cell, especially late in the course of EPEC infection.

The mechanism of ATP release in response to EPEC infection clearly involves the type III secretion system of this pathogen but may be regulated in more subtle ways as well. The reduced ATP release observed with the EPEC sepZ, ler, and espF mutants clearly implicates the type III secretion system in general and the EspF in particular in the ATP release process.

Detailed studies (5) of the mechanism of type III secretion by *Shigella* show that assembly of the *Shigella* “secretion” or “injectosome” in the red blood cell membrane creates a pore of ~25 Å. This pore is sufficiently large to allow permeability of the trisaccharide raffinose (504 mol weight), which is very close in size to ATP (507 mol weight for the free acid). The concentration of ATP inside a mammalian cell is as high as 3–5 mM, so ATP could exit by passive diffusion through this pore. We (12) have already shown that EPEC-infected cells become permeable to dyes such as trypan blue and propidium iodide. Ide et al. (24) recently showed that the pore created in the host cell membrane by EPEC resembles that of *Shigella*, with an estimated pore size of 30–50 Å.

The finding that forskolin and cholera toxin markedly increase the ATP efflux from EPEC-infected T84 cells (Fig. 6A) suggests that, in addition to the bacteria-produced pore, other cellular mechanisms may influence or regulate the rate of ATP release. Research from other laboratories shows that the CFTR itself, or a molecule closely regulated by the CFTR, permits ATP efflux in response to nonlethal stimuli such as cell swelling, stretching, or cAMP (25, 45, 54). Even in the absence of exogenous stimulators of cAMP, this CFTR-dependent pathway could be activated in response to EPEC infection. For example, EPEC infection could activate this pathway by cell swelling triggered by type III secretion, because both Shigella and EPEC have been shown to induce a contact-dependent osmotic lysis of red blood cells (5, 48, 58). Interestingly, two laboratories have shown that the espF mutant was fully competent in this osmotic hemolysis (48, 58). If EPEC-induced osmotic swelling allows a second, CFTR-dependent pathway of ATP release, this could explain why the performance of the espF mutant was so different in cell lines with and without CFTR. According to this hypothesis, in T84 cells, the CFTR-dependent pathway of ATP release is still activated by the espF mutant, so the mutant appears less attenuated than in cells lacking CFTR.

Although EPEC strains do not produce cAMP-elevating toxins, enterotoxigenic (ETEC) strains do produce the labile toxin. Dual infections with EPEC and ETEC are common in developing countries (32); our results suggest a possible interaction between two distinct types of diarrheagenic *E. coli*, which could result in more severe disease with dual infection.

The findings presented here may have relevance to the pathogenesis of diarrhea by EPEC, which has remained a puzzle despite decades of research. Malabsorption was one of the earliest explanations proposed for EPEC diarrhea. Indeed, malabsorption has been documented in human cases of EPEC infection (56, 57) as well as in rabbits infected with RDEC-1 (55). However, a purely malabsorptive diarrhea would not explain the rapid onset of diarrhea observed in fasting humans in volunteer challenge studies nor the persistence of diarrhea for weeks in children maintained on total parenteral nutrition without oral intake (47). Therefore, there must be a secretory component to EPEC diarrhea, but how this is triggered is not known.

The idea that adenine nucleotides released from EPEC-infected cells could trigger a fluid secretory response has several attractive features. Biopsy and autopsy specimens from cases of human EPEC infection and from rabbits infected with REPEC or RDEC show that EPEC infection is patchy, with many spared, apparently normal areas (56, 57). Furthermore, EPEC seem to preferentially adhere to villi, whereas it is the crypt cells that possess the secretory capacity to generate a watery diarrhea. Furthermore, several laboratories have shown that after a few hours of EPEC infection, the transmembrane electrical potential of intestinal cells collapses (50) and cells show decreased secretory responses to agonists such as carbachol and forskolin (22, 42). This makes it unlikely that the intestinal cells actually adhered to by EPEC are capable of generating the sustained fluid secretion necessary to explain an ongoing diarrhea. In contrast, a new paradigm for EPEC-induced diarrhea is suggested by the current report. According to this hypothesis, adenine nucleotides released from EPEC-infected cells act on nearby, uninfected cells, including crypt cells, to generate a secretory response in the intestine.

Invasive pathogens, such as *Salmonella* and *Shigella*, trigger an influx of polymorphonuclear leukocytes (PMNs) into the gut epithelium, and these translocating PMNs release 5’-AMP, the neutrophil-derived secretagogue (30, 31). EPEC, in contrast, behaves clinically as a noninvasive organism and does not trigger an influx of fecal leukocytes (27). Our finding that large concentrations of adenine nucleotides can be released directly from intestinal cells, without the need for PMNs, shows that these nucleotides may play a pathophysiological role in diarrhea even for noninflammatory pathogens such as EPEC.
Theoretically, adenine nucleotides could trigger chloride secretion from epithelial cells by interacting with several types of purinergic receptors on both the apical and basolateral surfaces of the cell. Adenosine A2 receptors coupled to chloride secretion are associated with a stimulation of adenyl cyclase. Purinergic P2Y receptors, which respond to ADP, ATP, and UTP, are also found in the intestine and are coupled to chloride secretion via various intracellular signaling pathways (46). P2Y receptors capable of responding to adenine nucleotides are apparently not found on the apical surface of T84 cells (3, 16, 53), although one recent report did describe an $I_{sc}$ response to UTP applied apically to T84 cells (49). P2X receptors are found on sensory neurons in intestine and other hollow organs and may mediate the sensation of pain as well as alterations in peristalsis (9, 10) but are apparently not linked to ion secretion. Our results indicate that adenosine A2 receptors are responsible for the chloride secretory response to EPEC-conditioned media applied apically to T84 cells in agreement with others (53) who investigated the effects of ATP on this cell line.

Although the focus in this study has been on the effects of released adenine nucleotides on the host, it has not escaped our attention that the release of nucleotides may provide benefits to the pathogen. E. coli, including EPEC, is capable of scavenging adenosine from the environment with an even higher avidity than mammalian cells (to concentrations <0.1 $\mu$M) and utilizing the adenosine in lieu of de novo purine biosynthesis (59), which is energetically expensive. This is particularly relevant in light of increasing appreciation that the intestinal mucosa is a highly purine-limited environment, not just for invasive bacteria (23) but also for noninvasive pathogens such as Vibrio cholerae (8).

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REFERENCES


