We studied the kinetics of extracellular ATP (ATPe) in Escherichia coli and their outer membrane vesicles (OMVs) stimulated with amphipatic peptides melittin (MEL) and mastoparan 7 (MST7). Real-time luminometry was used to measure ATPe kinetics, ATP release, and ATPease activity. The latter was also determined by following \[^{32}\text{P}]\text{Pi}\) released from \[^{\gamma-32}\text{P}]\text{ATP}\). \textit{E. coli} was studied alone, co-incubated with Caco-2 cells, or in rat jejunal segments. In \textit{E. coli}, the addition of \[^{\gamma-32}\text{P}]\text{ATP}\) led to the uptake and subsequent hydrolysis of ATPe. Exposure to peptides caused an acute 3-fold (MST7) and 7-fold (MEL) increase in [ATPe]. In OMVs, ATPease activity increased linearly with [ATPe] (0.1–1 \(\mu\text{M}\)). Exposure to MST7 and MEL enhanced ATP release by 3–7 fold, with similar kinetics to that of bacteria. In Caco-2 cells, the addition of ATP to the apical domain led to a steep [ATP] increase to a maximum, with subsequent ATPease activity. The addition of bacterial suspensions led to a 6–7 fold increase in [ATP], followed by an acute decrease. In perfused jejunal segments, exposure to \textit{E. coli} increased luminal ATP 2 fold. ATPe regulation of \textit{E. coli} depends on the balance between ATPease activity and ATP release. This balance can be altered by OMVs, which display their own capacity to regulate ATPe. \textit{E. coli} can activate ATP release from Caco-2 cells and intestinal segments, a response which \textit{in vivo} might lead to intestinal release of ATP from the gut lumen.

Introduction

In bacteria, as in most cell types and organisms, cytosolic adenosine triphosphate (ATPcyt) acts as an ‘energy currency’; a regulated variable providing energy for metabolic reactions, serving as a substrate for nucleic acid synthesis, and regulating a variety of biological processes.

In \textit{Escherichia coli}, the ATPcyt concentration ranges from 1 to 5 mM, depending on the environmental and physiological conditions [1–3]. The nucleotide is synthesized \textit{de novo} or obtained from external purine compounds by salvage pathways [4].

ATPcyt of \textit{E. coli} can be transported through the inner membrane (IM) to the periplasm, where it can be metabolized by various enzymes [5]. The resulting adenosine and adenine can be transported back through exchangers to the cytoplasm [4]. Alternatively, periplasmic ATP, as well as ADP, AMP, and other small hydrophilic solutes, can diffuse across the outer membrane (OM) through water-filled...
porins which are permeable to solutes smaller than 600–700 Da [6,7]. At neutral pH, ATP is mainly an anionic species of 507 Da capable of crossing the OM via one or more porins whose selectivity, on the basis of chemical structure, is poor [7–9]. Thus, there may be a dynamic exchange allowing ATP to be released from the periplasm or be taken up from the extracellular space. In agreement with these findings, extracellular ATP (ATPe) accumulated in nanomolar concentrations in E. coli cultures under different experimental conditions [10–12].

E. coli possesses periplasmic nucleotidases and phosphatases capable of hydrolyzing ATP [5,13]. An enzyme encoded by the ushA gene displayed 5′-nucleotidase activity in E. coli [5,14], facilitating the hydrolysis of ATP, ADP, AMP, and other 5′ribo and 5′deoxyribonucleotides [15]. In addition, there are at least two periplasmic phosphatases capable of dephosphorylating ATP: AphA, an acid phosphatase exhibiting hydrolytic activity in an acid milieu [16] and PhoA, an alkaline phosphatase, exhibiting activity at pH 7–9. PhoA can be either transcriptionally up-regulated in the absence of Pi [17] or kinetically inhibited by high Pi [18,19].

Bacterial release of ATP may occur by lytic and non-lytic mechanisms. In E. coli, hypotonic treatment, carbon starvation, as well as exposure to glucose or carbon monoxide, promote the non-lytic release of ATP and other solutes [12,20,21]. Repression of 5′nucleotidases, on the other hand, leads to basal excretion of purine nucleotides, although ATP export was not tested [5].

Amphipatic peptides can enhance the permeability to small solutes in a variety of cells [9,22–30], either by creating disordered regions of the lipid bilayer and/or by forming pores [31–35]. Among these are small amphipatic peptides belonging to the mastoparan and melittin families, which display a broad spectrum of lytic activities in a variety of cells [36,37], inducing ATP release in erythrocytes [38,39] and adrenal chromaffin cells [40]. Mastoparans exhibit antimicrobial activity against both Gram-positive and -negative bacteria, though in E. coli the OM had to be previously disrupted in order for mastoparan to be effective [22,23]. Melittin, on the other hand, was rapidly taken up by E. coli, inducing morphological changes usually followed by leakage of the cytoplasm [9,37].

In principle, [ATPe] of E. coli may increase via cytoplasmic/periplasmic ATP release induced by physiological and pharmacological stimuli, and/or decrease via ATP hydrolysis due to periplasmic nucleotidases. Thus, in the present study we evaluated the relative contribution of ATPe hydrolysis and ATP release to the dynamic regulation of ATPe of E. coli. Melittin and mastoparan 7 (an active mastoparan analog; [41]) were used as ATP release inducers.

E. coli are able to form and release outer membrane vesicles (OMVs) both in vivo and in vitro [42–44]. Since the lumen of these vesicles may contain ATP as well as nucleotidases of periplasmic origin [45,46], our analysis of the dynamic ATPe regulation of E. coli took the contribution of OMVs into account.

Finally, we analyzed ATPe regulation in conditions closer to the in vivo situation, where E. coli lives as a commensal of the gut lumen, in close contact with intestinal epithelial cells. Accordingly, we studied the effect of E. coli on ATPe regulation of the human intestinal Caco-2 cell line and rat intestinal segments.

Materials and methods

Chemicals

All reagents were of analytical grade. Mastoparan 7 (MST7), mastoparan 17 (MST17), melittin (MEL), and firefly luciferase (EC1.13.12.7) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). D-luciferin was obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). [γ 32P]ATP (10 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Santa Clarra, California, U.S.A.). Phosphatidylglycerol, cardiolipin, and phosphatidylethanolamine were purchased from Avanti Polar Lipids (AL, U.S.A.).

Bacterial strains and culture conditions

E. coli DH5α was used in all experiments using bacteria and OMVs, the only exception being the experiments run to assess β-galactosidase activity (see M&M), where E. coli BL21 (DE3) was used. Both strains showed similar responses to MST7 and MEL (Supplementary Figure S1).

E. coli were cultured overnight in Luria–Bertani (LB) broth at 37°C, with shaking at 220 rpm. Overnight cultures were diluted 1:100 in fresh LB broth and cultured at 37°C with shaking. Growth was monitored by optical density at 600 nm (OD600). Bacteria were harvested when an OD600 of 0.6–0.9 was attained. Bacterial cultures were then centrifuged 2500xg for 5 min, the supernatant was removed, and the pellet resuspended at 2 × 10⁸ bacteria/ml in M9 medium containing: Na₂HPO₄ 42.3 mM, KH₂PO₄ 22.1 mM, NH₄Cl 18.7 mM, MgSO₄
7 μM, NaCl 108 mM, and CaCl₂ 0.1 mM; pH 7. Centrifugation and washing of the pellet was repeated three times.

For experiments run in the absence of phosphate, overnight cultured bacteria were harvested, washed, and resuspended in a similar way, except that (Hepes 10 mM, KCl 1 mM, NaCl 140 mM; pH = 7) was used.

In most cases, bacterial suspensions were preincubated in M9 or Hepes media for 60 min before use. In a few experiments (see the 'Rat intestinal perfusates' section below), intact jejunal segments were exposed to supernatants collected from _E. coli_ suspensions. In this case, _E. coli_ were cultured in the LB medium as above, centrifuged, and the resulting supernatant was collected.

**Isolation of OMVs**

Bacteria were cultured as above and OMVs were isolated from bacterial culture supernatants as described previously [47]. Bacteria were then pelleted (2500×g for 10 min at 4°C), and culture supernatants filtered using 0.22 μm pore size filters (Millipore Corporation, Billerica, MA, U.S.A.), followed by centrifugation at 166 000×g for 2 h at 4°C. The resulting pellet containing the OMVs was diluted in the M9 medium, and held at 4°C for up to 48 h before use. An aliquot was withdrawn to determine protein content using the Bradford assay. Total lipids of OMVs were extracted using a standard procedure [48] and subjected to analysis by high-performance TLC, using the following solvent system: chloroform/methanol/2-propanol/0.25% KCl/ethylacetate (30 + 9 + 25 + 6 + 18). Lipid spots were detected by spraying with sulfuric acid/ethanol (5:100, v/v) and charring at 120°C. Phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylethanolamine (PE), and lipopolysaccharide (LPS) were used as standards. Preliminary experiments showed that ATPase activity (assayed by the two methods described below, see 'Hydrolysis of ATP' section in M&M) of OMVs were similar for suspensions stored for 24 or 48 h at 4°C before use (data not shown).

Aliquots of OMVs suspensions (10 μl) were spotted on LB agar plates for overnight culture at 37°C. No bacterial colonies were observed.

**Electron microscopy**

OMVs were observed by electron microscopy. Vesicles were fixed with 1% (v/v) glutaraldehyde for 45 min at room temperature, centrifuged (166 000×g for 2.5 h at 4°C), and resuspended in 0.1 M ammonium acetate buffer (pH = 7). A drop of fixed vesicles was applied to a 400 mesh copper grid covered with a carbon film and the samples were stained with 2% (w/v) phosphotungstic acid (pH 5.2 with KOH). Electron micrographs were taken at 50 000× magnification.

**Culture of Caco-2 cells**

Caco-2 cells (passages 5–10, ATCC, Molsheim, France) were seeded on Snapwell tissue culture-treated polycarbonate membrane filters (6.5 mm diameter and 0.4 μm pore size; Corning, New York, U.S.A.). Cells were grown for 15–30 days on Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose supplemented with 10% v/v fetal bovine serum, 2 mM l-glutamine, and antibiotics in a humidified atmosphere of 5% CO₂ at 37°C. Experiments were performed on confluent cells that had developed a transepithelial electrical resistance greater than 250 w/cm². Under these conditions, cells formed a polarized epithelial cell monolayer with distinguished apical and basolateral domains. Membrane permeability was tested by adding dextran-FITC (MW = 59 000–77 000 Da, Sigma,) to the upper chamber (apical side) and measuring fluorescence (ex/em 490/520 nm) on the lower chamber (basolateral side).

**Rat intestinal perfusates**

Adult Wistar rats of either sex of 250–350 g body weight were anesthetized. Jejunum segments (approximately 10 cm long) were excised from animals and immediately placed in cold Ringer buffer containing (in mM): NaCl 130, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 24, glucose 6, oxygenated with a mixture of 95% O₂ and 5% CO₂, pH 7.4. The intestinal segment was flushed of luminal contents and placed in an organ bath containing 10 ml of Ringer solution kept at room temperature. The proximal and distal ends of the jejunum were cannulated to allow slow infusion of Ringer solution at 37°C with a flow rate of 1 ml/min using a peristaltic pump (Model E50, Elmeco Laboratory Instruments).

During the first 30 min the tissue was infused with Ringer solution and then replaced by a bacterial suspension, containing 5 × 10⁸ bacteria/ml in the same saline solution. Samples were collected at the distal end every 10 min during 60 min and the luminal ATP (ATPₜ) was measured by off-line luminometry immediately after
sample collection. In some experiments, before replacing the Ringer solution with the bacterial suspension, supernatants collected from *E. coli* suspensions were perfused.

**Treatments**

The ATPe kinetics of *E. coli* was assessed when bacteria were exposed to mastoparan 7 (MST7) or melittin (MEL). α,β-methyleneadenosine 5'-triphosphate lithium salt (AMP-CPP, a non-hydrolyzable ATP analog) was used as an inhibitor of ATPase activity via one or more enzymes.

ATPe kinetics of Caco-2 cells was assessed following exposure to bacteria (*E. coli*) or to an adrenergic cocktail denoted as 3V, which contained 10 mM isoproterenol (a β-adrenergic agonist), 30 mM forskolin (an adenylylate kinase activator), and 100 mM papaverine (a phosphodiesterase inhibitor). 3V was used to induce a fast increase in cytosolic cAMP. Experiments with Caco-2 cells exposed to either bacteria or 3V were carried out in the absence or presence of carbenoxolone, an inhibitor of ATP release.

Intact jejunum segments were continuously infused with either *E. coli* suspensions or supernatants collected from *E. coli* suspensions, and the perfusates were used for ATP determination by off-line luminometry.

**Extracellular ATP**

ATPe was measured by real-time luminometry with bacteria, OMVs, and Caco-2 cells using firefly luciferase, which catalyzes the oxidation of luciferin in the presence of ATP to produce light. In experiments using rat intestinal segments, the presence of ATP in the intestinal lumen was assessed by off-line luminometry. A custom-built luminometer of high sensitivity was used [49,50].

**On-line luminometry**

*E. coli* and OMVs

Real-time luminometry was carried out with bacteria or OMVs laid on coverslips that were mounted in the assay chamber of the luminometer. Measurements were performed in M9 medium containing a luciferin-luciferase mixture at 20°C. 2 × 10⁷ bacteria/40 or 10 µl OMVs (0.8–1.4 µg protein)/100 µl were used.

The time course of light emission was transformed into ATPe concentration versus time by means of a calibration curve. Increasing concentrations of ATP from 4 to 974 nM were sequentially added to the assay medium from a stock solution of ATP dissolved in the M9 medium. For the results shown in Figure 4C, the instantaneous rate of [ATPe] change (denoted as PATP) was calculated by taking the first derivative of the experimentally observed ATPe kinetics of Figure 4A,B.

**Caco-2 cells**

Caco-2 cells were grown and polarized on permeable supports as described above. Real-time luminometry was carried out by placing these supports, containing approximately 5 × 10⁵ cells, in the assay chamber of the luminometer. The configuration of the set-up is illustrated in Supplementary Figure S2. Measurements were run at 20°C. The basolateral side was loaded with 40 µl of DPBS medium (Gibco™), while the apical side was loaded with 40 µl of DPBS medium containing a luciferin-luciferase mixture. Using this configuration, the light-emitting luciferase reaction occurs in the apical domain.

The time course of light emission was transformed into ATPe concentration versus time by means of a calibration curve. Increasing concentrations of ATP (24–875 nM) were sequentially added to the assay medium from a stock solution of ATP dissolved in DPBS medium. Results were expressed as [ATPe] at every time point of a kinetic curve.

Increases in [ATPe] were evaluated as the difference between [ATPe] at 1 min post-stimulus and the basal [ATPe], and are indicated as ΔATP₁.

**Off-line luminometry of rat intestinal perfusates**

Aliquots (45 µl) of perfusates, were taken every 10 min, containing 0 (control) or 5 × 10⁸ bacteria/ml and used to measure ATP₁. A standard ATP curve was obtained for each sample by adding 1 µl of increasing concentrations of ATP from 1 to 20 µM. For each experiment, concentrations of ATP₁ were normalized to the control conditions in the absence of bacteria.
Intracellular ATP
The intracellular ATP content of *E. coli* was estimated using real-time measurements. Briefly, 10 μl of a bacterial suspension (2 × 10^8 bacteria) was placed on a coverslip. Samples were desiccated at room temperature to induce bacteriolysis [32]. The desiccated residue was dissolved in 40 μl of M9 medium containing a luciferin-luciferase mixture, and the total content of ATP was measured by luminometry as described in ‘On-line luminometry section in M&M’.

Hydrolysis of ATPe
The rate of ATPe hydrolysis was tested in bacteria, OMVs, and polarized Caco-2 cells. It was determined by following the accumulation of [γ-32P]Pi release from exogenous [γ-32P]ATP, and by real-time luminometry.

[γ-32P]ATP technique [38,51]
This technique was used for bacteria, OMVs, and Caco-2 cells. The reaction was started by adding [γ-32P]ATP (0.27 Ci/mmol; from 100 to 1000 nM) to bacterial or OMVs suspensions at 20°C. At different times, 100 μl aliquots of the suspension were withdrawn in duplicate and were poured into 750 μl of a stop solution containing 4.05 mM (NH₄)₆Mo₇O₂₄ and 0.83 mM HClO₄ (molybdate-perchloric solution). The ammonium molybdate solution formed a complex with the released phosphate, which was then extracted with 0.6 ml of isobutyl alcohol. Phases were separated by centrifugation at 1000×g for 5 min, aliquots of 200 μl of the organic phase containing [32P]Pi were transferred to vials with 2 ml of 0.5 M NaOH and radioactivity was measured using the Cerenkov effect. Any hydrolysis of [γ-32P]ATP into ADP + [32P]Pi in the bacterial or OMVs suspensions can be defined as ATPase activity; the time course of [32P]Pi accumulation yields a measure of the rate at which one or more nucleotidases or phosphatases of the samples hydrolyze ATPe.

To calculate ATPase activity, time-dependent levels of Pi were fitted to

\[ Y = Y_0 + A(1 - \exp^{-kt}), \]

where \( Y \) and \( Y_0 \) are the values of [32Pi] at each time \( t \) and at \( t = 0 \), respectively; \( A \) represents the maximal value for the increase in \( Y \) with time and \( k \) is a rate coefficient. The parameters of best fit resulting from the regression were used to calculate the initial rate of ATPase activity \( (vi) \) as \( k \times A \) (i.e. the first derivative of Eq. (1) evaluated at \( t = 0 \)). The [32P] Pi moles produced from [γ-32P]ATP were calculated using the ATP specific activity.

In a few experiments, instead of using submicromolar ATP concentrations, ATPase activity was detected at 500 μM ATP to provide an estimate of apparent maximal ATPase activity \( (Vi) \).

ATPase activity was expressed as: pmol/(10¹⁰ bacteria min) in *E. coli* suspensions, pmol/(μg prot) in OMVs, or nM/min in Caco-2 cells.

Uptake and hydrolysis of ATPe in *E. coli*
*E. coli* has cytosolic and periplasmic compartments. Unless otherwise stated, by uptake of ATPe we mean that ATPe should enter the periplasm, irrespective of its final location or metabolism inside the bacterium.

Aliquots (100 μl) of *E. coli* suspensions were incubated with 1 μM [γ-32P]ATP for 2 or 30 min, after which they were diluted in 1.4 ml of cold M9 medium, and centrifuged for 5 min at 2400×g. The resulting pellets were treated in two different ways: 1) they were transferred to vials containing 2 ml of 0.5 M NaOH and radioactivity was measured using the Cerenkov effect. Any hydrolysis of [γ-32P]ATP into ADP + [32P]Pi in the bacterial or OMVs suspensions can be defined as ATPase activity; the time course of [32P]Pi accumulation yields a measure of the rate at which one or more nucleotidases or phosphatases of the samples hydrolyze ATPe.

ATPe hydrolysis by real-time luminometry
This method was used for studies with OMVs and Caco-2 cells.
OMVs
Coverslips with 10 μl of OMV suspensions were mounted in the measuring chamber of the luminometer and exposed to the luciferase reaction mixture. Light emission was measured for 10 min, ATP was then added to the chamber at final concentrations of 200, 600, and 1000 nM (in separate experiments) and measurements were carried out for another 30 min.

Light output was transformed into ATPe concentration vs. time by means of a calibration curve and the slope of disappearance of ATPe (vi) was estimated from ATPe kinetics and multiplied by −1. ATPase activity was expressed as nM/(μg prot min).

Caco-2 cells
Measurements were performed on the apical side of polarized cells held on permeable supports as described in the ‘Culture of Caco-2 cells’ section.

Basal light emission was measured for 5 min, ATP (0.2–7 μM) or aliquots of E. coli suspensions were then added to the upper chamber at a given concentration and measurements were carried out for another 15–20 min. Light output was transformed into ATPe concentration vs. time by means of a calibration curve (‘On-line luminometry’ section) and the slope of disappearance of ATPe (vi) was estimated from the maximum value of ATPe kinetics until the end of the experiment, and multiplied by −1. ATPase activity was expressed as nM/min.

Hydrolysis of p-nitrophenyl phosphate
p-nitrophenyl phosphate (pNPP) was used as a substrate to estimate periplasmic phosphatase activity of E. coli suspensions (2 × 10⁹ bacteria/ml). pNPP phosphatase activity was measured using a spectrophotometric assay, based on the ability of phosphatases to catalyze the hydrolysis of pNPP to p-nitrophenol, a chromogenic product with absorbance at 420 nm [52]. Initial velocities were calculated as in the ‘Hydrolysis of ATPe’ section (Eq. 1).

Viability
Viability was tested by the capacity of E. coli to form colonies (culturability) and their IM integrity (β-galactosidase assay).

Culturability
Bacteria were grown overnight, harvested, and resuspended in M9 as explained in the ‘Bacterial strains and culture conditions’ section. Suspensions of bacteria (2 × 10⁷ bacteria/40 μl) were held in the absence of treatments (controls) or were exposed to MST7, MST17, or MEL for 1 or 20 min at 20°C. Afterwards, suspensions were diluted 1/25 000 and 10 μl aliquots were spotted on LB agar plates for overnight culture at 37°C. Colony formed units (CFU) were counted manually and expressed relative to control values.

β-galactosidase assay
This method was used to assess the IM integrity of E. coli. E. coli BL21 (DE3) were electroporated with a pGEM3ZF plasmid containing a gene encoding β-galactosidase. Transformed bacterial cultures were grown (37°C, 200 rpm) and β-galactosidase expression was induced by the addition of 1.0 mM IPTG to the culture medium. After induction for 3 h, bacterial suspensions were centrifuged at 2500×g for 5 min, the supernatant was removed, and the pellet was resuspended at 2 × 10⁹ bacteria/ml in the M9 medium. Centrifugation and washing of the pellet was repeated three times.

Transformed bacteria were exposed to the chromogenic lactose analog ONPG (o-nitrophenyl β-D-galactopyranoside) in the absence of any treatment (non-treated), or in the presence of MST7, MST17, or MEL.

Agents that compromise IM integrity would permit ONPG to diffuse into the bacterial cytoplasm, where it can be hydrolyzed to ONP (o-nitrophenol) by β-galactosidase activity. Accordingly, the concentration of ONP was followed by measuring the absorbance at 415 nm with 1 min intervals for 60 min at 20°C, using a Bio-Rad 550 plate reader. Data were normalized by subtracting absorbance at t = 0. The kinetics of ONP production was determined for each treatment.
Data analysis
Statistical significance was determined using one-way Analysis of Variance followed by a Tukey Multiple Comparison Test. A P value < 0.05 was considered significant. Numbers of determinations (n) from independent preparations (N) are indicated. In the experiments using Caco-2 cells, each filter containing polarized monolayers was considered an independent preparation. In experiments using rat intestinal segments, only one segment was excised from each rat. Each segment was considered an independent preparation.

Results
Experiments were performed using suspensions of E. coli, OMVs, Caco-2 cells, or intestinal rat segments. In these systems, regulation of [ATPε] may depend on the dynamic balance between the rates of ATP release and ATPε hydrolysis. The relative importance of both processes can be assessed by measuring the kinetics of ATPε accumulation (denoted as ATPε kinetics) and ATPase activity.

E. coli
ATPase activity
Bacterial suspensions were exposed to [γ-32P]ATP at various ATP concentrations (100–1000 nM), and the time course of [32P]Pi accumulation released from [γ-32P]ATP was determined (Figure 1A). The experimental

Figure 1. ATPase activity in E. coli.
(A) Time course of [32P]Pi accumulation released from [γ-32P]ATP (100–1000 nM) using E. coli suspended in the M9 medium. The dotted lines represent fittings of linear functions to experimental data for each [ATP] used. Results are expressed as pmol [32P]Pi/(10^{10} bacteria), and are means ± SEM (N = 6–10, n = 12–20). The inset shows the time course of [32P]Pi accumulation at 100 nM, in media with or without bacteria. (B) ATPase activity (vι) as a function of [ATP]. Each symbol represents ATPase activity calculated as the rate of Pi kinetics using data from A. The dotted line represents the fit of a linear function to experimental data. Results are expressed as pmol [32P]Pi/(10^{10} bacteria min), and are means ± SEM. The triangle symbol represents ATPase activity derived from ATP uptake experiments of C below. (C) Uptake of ATPε. E. coli suspensions were exposed to 1 μM [γ-32P]ATP for 2 and 30 min. Results show intracellular concentrations of [γ-32P]ATP or its hydrolysis product [32P]Pi; they are means ± SEM (N = 3, n = 12) expressed as pmol/(10^{10} bacteria). * and # denote P < 0.05 for [γ-32P]ATP and [32P]Pi, respectively. n, N denote number of replicates (n) and independent experiments (N).
design for assessing ATPase activity of the periplasm relies on the fact that ATPe is permeable to the OM, but not the IM [4,5]. The initial rate values of [32P]Pi production were used to calculate ATPase activity for each ATPe concentration. ATPase activity followed a linear function with ATPe concentrations (Figure 1B), with the slope of the curve ($K_{ATP}$ amounting to 0.04 min$^{-1}$ per $10^{10}$ bacteria. Spontaneous ATPe hydrolysis in the absence of bacteria was negligible (inset Figure 1A).

**Phosphatase activity**

At neutral pH, *E. coli* may exhibit periplasmic ATPase activity by 5'-nucleotidase and alkaline phosphatase. If the latter were active under experimental conditions, then it should be kinetically inhibited by Pi, and activated at alkaline pH [8,17,53].

Accordingly, we assessed and compared ATPase activity for bacteria suspended in the M9 medium, which contains phosphate, with that in Hepes medium, which lacked phosphate. Bacterial suspensions were grown in LB broth (see ‘Bacterial strains and culture conditions’ in the M&M section). They were then collected and resuspended in either M9 or Hepes media. Experiments were run at low [ATP] (600 nM, Figure 2A) or high [ATP] (500 μM, Figure 2B), the latter a measure of apparent maximal ATPase activity (Vi). ATPase activities in the Hepes medium were 3.3-fold (600 nM ATPe) and 16-fold (500 μM ATPe) higher than those in the M9 medium (Figure 2C,D). Vi was 3.27 ± 1.70 nmol/(10$^{10}$ bacteria min) in the Hepes medium and 0.24 ± 0.07 nmol/(10$^{10}$ bacteria min) in the M9 medium (Figure 2D).

As a further test for the inhibitory effect of Pi on ATPase activity of alkaline phosphatase, we incubated bacteria in Hepes medium containing the same Pi concentration as in the M9 medium. Results showed that, under these conditions, $^{32}$Pi production at 600 nM ATP was similar to that found in the M9 medium (Figure 2E). Moreover, while in the M9 medium the ATPase activity, due to 5'-nucleotidase activity, at pH = 9 was roughly similar to that found at pH = 7, in the Hepes medium ATPase activity was almost 2-fold higher at pH = 9 than at pH = 7.

Finally, we tested the capacity of bacterial suspensions to hydrolyze pNPP, a chromogenic product that acts as a substrate for phosphatases, but not for 5'-nucleotidase. As shown in Supplementary Figure S3, phosphatase activity was detected in both M9 and Hepes media. At alkaline pH, phosphatase activity was 2.14-fold higher in the absence of Pi (i.e. Hepes medium), than in its presence (M9 medium).

**Effect of AMP-CPP on ATPase activity**

AMP-CPP, a non-hydrolyzable ATP analog, was used as a potential inhibitor of ATPase activity. AMP-CPP is expected to permeate the OM since it is structurally similar to ATP but smaller than the 600–700 Da cut-off size for the permeability of main *E. coli* porins [54]. Bacterial suspensions were exposed to 600 nM ATPe both in Hepes and M9 media (Figure 3A). In media with or without AMP-CPP, ATPase activity in the Hepes medium was higher than that in the M9 medium. With 100 μM AMP-CPP, ATPase activity was reduced by approximately 60% (Hepes medium) and 82% (M9 medium) (Figure 3B).

**ATP uptake and ATPase activity**

The results above show that exogenous ATP can be hydrolyzed by *E. coli*. Since enzymes promoting ATPe hydrolysis are located in the periplasm, we checked whether ATPe can be incorporated into bacteria. Aliquots of *E. coli* suspensions were incubated with 1 μM [γ-32P]ATP for 2 or 30 min in the M9 medium, after which they were diluted and centrifuged to obtain a pellet. This pellet was then used to determine both the total amount of radioactivity incorporated and, in separate samples, the proportion of this radioactivity due to either [γ-32P]ATP or [γ-32P]Pi content.

Since M9 has millimolar concentrations of Pi, any nM [γ-32P]Pi accumulated in the extracellular medium will be highly diluted in unlabeled Pi, making direct [γ-32P]Pi uptake into the periplasm negligible. In the bacterial pellet, [γ-32P]ATP and [γ-32P]Pi were 1.5- and 2-fold higher at 30 min than at 2 min (Figure 1C), suggesting [γ-32P]-ATP uptake into the periplasm, with subsequent release of [γ-32P]Pi.

For each incubation time, the relative proportion of [γ-32P]Pi, with respect to [γ-32P]ATP, was similar. Total radioactivity of the pellets was not significantly different from the sum of the radioactivity obtained with [γ-32P]Pi + [γ-32P]ATP (Supplementary Figure S4).
Figure 2. Effect of phosphate (Pi) on ATPase activities of E. coli suspensions.

(A) Time course of Pi accumulation ([32P]Pi) released from [γ-32P]ATP (600 nM ATP), using E. coli suspended in media with Pi (M9, clear symbols) or without Pi (Hepes, gray symbols). The dotted lines represent the fit of linear functions to experimental data. Results are expressed as pmol [32P]Pi/(1×10^10 bacteria) (600 nM ATP), and are means ± SEM. (B) Time course of Pi accumulation ([32P]Pi) released from [γ-32P]ATP (500 μM ATP), using E. coli suspended in media with phosphate (M9) or without phosphate (Hepes). The dotted lines represent the fit of linear functions to experimental data. Results are expressed as nmol [32P]Pi/(1×10^10 bacteria) (500 μM ATP), and are means ± SEM. (C) Rates of Pi accumulation (data from A) were used to calculate ATPase activities (vi). Results are means ± SEM (#P < 0.001). Number of replicates (n) and independent experiments (N) are given in brackets on top of the bars (N, n). (D) Rates of Pi accumulation (data from B) were used to calculate apparent maximal activities (vi). Results are means ± SEM (#P < 0.001). Number of replicates (n) and independent experiments (N) are given in brackets on top of the bars (N, n). (E) Effect of Pi and pH on ATPase activity. Rates of Pi accumulation (Supplementary Figure S11) at 600 nM ATP were used to calculate vi. Experiments were run using E. coli suspended in media with phosphate (M9, clear symbols), without Pi (Hepes, gray symbols), and Hepes medium containing the same Pi concentration as in the M9 medium (Hepes + Pi, gray symbols with hatched lines), both at pH = 7 and pH = 9. Results are means ± SEM (^P < 0.01; #P < 0.001) of 3 independent experiments with 2 replicates each.
ATPe kinetics

In Figure 4A,B, the ATPe kinetics from E. coli is shown, which depends on both the rates of ATP release (promoting an increase in [ATPe]) and ATPe hydrolysis (promoting a decrease in [ATPe]).

In the absence of stimuli, [ATPe] remained steady at 24 ± 3 μM/(10^10 bacteria).

ATP release was then stimulated by adding MST7 and MEL. These amphipatic peptides can act as permeabilizing agents, compromising bacterial integrity to different extents. Thus, in principle, peptides may trigger ATP release by lytic and non-lytic mechanisms. With 10 μM MST7, an acute 3-fold increase in [ATPe] was observed, followed by a phase of slower increase. Ten μM MST17, an inactive analog of MST7, produced no effect (Figure 4A).

Both 3 and 30 μM MEL (denoted as MEL3 and MEL30, respectively) caused acute ATP release of different magnitudes. Under MEL3, a 1.4-fold increase in [ATPe] was observed within 1 min, which remained constant thereafter. On the other hand, MEL30 produced a fast 5-fold increase in [ATPe] within 1 min, followed by a phase of slower increase (Figure 4B). Values of ΔATP_{20}, i.e. values of [ATPe] attained at 20 min post-stimulus for each treatment, are shown as insets of Figure 4A,B.

To illustrate the transient nature of ATP release, Figure 4C shows the results obtained by taking the first derivative of ATPe kinetics from Figure 4A.B. In this way, the instantaneous changes of [ATPe], an indicator of bacterial ATP permeability (P_ATP), could be observed. Both for MST7 and MEL30, an acute steep increment of P_ATP, amounting to 53 (MST7) and 155 (MEL30) μM ATP/(10^10 bacteria min) was followed by a slower exponential decay.

Intracellular ATP content (ATPi) amounted to 296 ± 78 μM ATP/(10^10 bacteria). This means that ΔATP_{20} values (i.e. the increase in [ATPe] at 20 min post-stimulus) for MST7, MEL3, and MEL30 represented 22.3, 5.5, and 87.4% of ATPi, respectively.

Viability

The observed MEL- and MST7-dependent ATPe kinetics in Figure 4 could be induced by lytic and/or non-lytic release of ATPi. Therefore, we analyzed the effects of these peptides on the ability of bacteria to form colony units (CFU) and the IM integrity. The latter was measured in transformed E. coli BL21 expressing β-galactosidase, in the presence of ONPG.

Formation of colonies on a plate (denoted as culturability) does not necessarily reveal the viability of bacteria under assay conditions but rather shows that there were bacteria in the sample that, after the different treatments, were able to grow and divide.
MST7 and MEL3 decreased CFU formation by approximately 30% (Supplementary Figure S5). In both cases the effects were acute, since for each peptide, CFU values after 1 min exposure were not statistically different from those obtained after 20 min. MST17 did not change CFU values. Exposure to MEL30, on the other hand, decreased CFU values by 57 and 79% at 1 and 20 min, respectively (Supplementary Figure S5A).

The β-galactosidase assay showed similar activity values for control and MST17-treated samples. While exposure to MST7 or MEL3 caused no changes in β-galactosidase activity, under MEL30, a different pattern was observed. In this case, low increments in activity at relatively low incubation times (0–5 min) were followed by strong increases, of approximately 5.6-fold, at later times (5–20 min) (Supplementary Figure S5B,C). Thus, except for MEL30, the observed decrease in culturability using MST7 and 3 μM MEL occurs without viability (as assayed by β-galactosidase activity) being compromised.

OMVs
OMVs originate from *E. coli* under culture. Following purification, OMVs were detected by electron microscopy as spherical vesicles with diameters ranging from 50 to 200 nm, surrounding an electrondense center (Figure 5A), as previously described [55,56].

TLC analysis showed that OMVs were composed of LPS, PE, and CL (Figure 5B), the main phospholipid classes present in the OM of Gram-negative bacteria.

As, during formation, OMVs encapsulate portions of the bacterial periplasm with soluble proteins, they might exhibit ATPase activity due to originally periplasmic nucleotidases and phosphatases. Moreover, if significant ATP amounts were present in the vesicles’ lumen, then this pool might be released by MEL and MST7 exposure. Accordingly, as with *E. coli*, ATPase activity and ATPe kinetics of OMVs were studied.

ATPase activity
ATPase activity of OMVs was obtained by real-time luminometry after the addition of ATP. Figure 5C shows a steady pre-stimulus ATPe level, followed by an acute increase when ATP was added. The subsequent kinetics of [ATP] decrease could be used to estimate ATPase activity (see the ‘ATP hydrolysis by real-time luminometry’ section in M&M) at each [ATP], from which a substrate curve could be built (Figure 5D). Such a curve showed ATPase activity increased with [ATP]. Moreover, the determination of ATPase activity using [γ32P]-ATP at a single [ATP] provided similar results (see Figure 5D and Supplementary Figure S6).
ATP<sub>e</sub> kinetics

In the absence of stimuli, [ATP<sub>e</sub>] of OMVs amounted to 13 ± 5 nM/(µg protein). Under MST7 exposure, a 3 fold-increase in [ATP<sub>e</sub>] was observed within 1 min, followed by a phase of slower increase. Exposure to MEL30 caused an acute 7 fold-increase in [ATP<sub>e</sub>], followed by a continuous increasing phase (Figure 6). At more prolonged exposure times, [ATP<sub>e</sub>] decreased due to ATPase activity (Supplementary Figure S7). Values of ΔATP<sub>20</sub> showed a 4-fold higher [ATP<sub>e</sub>] for MEL30 than for MST7 (inset of Figure 6).
The OMV is a much simpler system than *E. coli*. Vesicles have a single internal compartment containing ATP, and mechanisms enabling ATP synthesis do not exist. On the other hand, the periplasmic ATP of *E. coli* can be influenced by cytosolic ATP (produced by an ATP synthase) as well as ATPe. Despite these differences, we tested whether ATPe kinetics of OMVs may mimic that of bacteria.

Accordingly, ATPe kinetics of OMVs and *E. coli* exposed to MST7 and MEL30 were compared by means of a correlation plot. Results showed a linear relationship for [ATPe] values of *E. coli* and OMVs for at least 5 min, although at later times this linearity was lost (Supplementary Figure S8A,B).

**Caco-2 cells**

Our results have shown that *E. coli* is capable of ATPe uptake and hydrolysis and, in the presence of amphipatic peptides, the release of intracellular ATP. Since *in vivo* *E. coli* may metabolically interact with intestinal epithelial cells of the human host, we evaluated the effect of *E. coli* on ATPe kinetics of Caco-2 cells, a model of epithelial enterocytes, and the potential effect of *E. coli* on these kinetics.

Caco-2 cells were grown as a polarized epithelial cell monolayer. ATPe kinetics was quantified on the apical side using real-time luminometry (Supplementary Figure S2). In the absence of bacteria, Caco-2 cells showed a basal rate of ATPe decrease amounting to 0.03 ± 0.003 nM/min. The addition of exogenous ATP (1.4–7 μM) led to an acute [ATPe] increase, followed by a decay phase (Figure 7A). The resulting decay kinetics were used to estimate ATPase activity. This was a linear function of [ATPe] over an ample nM–μM range (Figure 7B), with the slope of the curve (K_{ATP}) amounting to 0.023 min^{-1} for approximately 5 × 10^5 cells per well. Rates of ATPe hydrolysis, by assessing 32P-Pi production, provided similar results (Supplementary Figure S9).

Next, we determined the effect of *E. coli* and 3V on ATPe kinetics of Caco-2 cells. 3V is an adrenergic cocktail that can trigger ATP release via elevation of cAMP [38,50,57]. Experiments were run in the absence and presence of carbenoxolone (CBX), a generic blocker of ATP release of most eukaryotic cells [46,32]. Accordingly, stimuli were added to the apical medium and ATPe kinetics of Caco-2 cells was determined.

In the absence of stimuli, [ATPe] of Caco-2 cells amounted to 123 ± 5 nM (Figures 7C,D). Addition of 3V (Figure 7C) or *E. coli* (2 × 10^6 bacteria, Figure 7D) led to a 6 fold-increase in [ATPe] within 1 min to a maximum, followed by an acute decrease. These peaks were reduced 58–77% by CBX (inset Figure 7C,D), though the effect of CBX on 3V-induced ATP release was not significant (P = 0.12).
Intestinal rat segments

Bacterial suspensions were perfused through the lumen of jejunum rat segments. These conserved the intestinal wall structure and could therefore exhibit smooth muscle contractions that might influence ATP release. Luminal ATP$_L$ was monitored by off-line luminometry.

In the absence of bacteria [ATP$_L$] remained steady for 90 min at 13 ± 2 nM (N = 2, Supplementary Figure S10).

Then, ATP$_L$ was measured in perfusate samples in the absence of bacteria for 30 min at 10 min intervals. Subsequently, the Ringer solution was replaced by a bacterial suspension containing 5 × 10$^8$ bacteria/ml and

![Figure 7. ATPe kinetics and ectoATPase activity of Caco-2 cells.](image-url)
ATP$_L$ was measured every 10 min during the next 60 min (Figure 8A). Compared with basal pre-stimulated ATP$_L$ values, *E. coli* suspensions induced a 2-fold increase in the ATP$_L$ maximal value which was determined within the 30 min exposure to bacterial perfusion ($P < 0.05$, Figure 8B, $N = 4$). After 40 min, ATP$_L$ decreased to basal pre-stimulated levels (Figure 8A).

Next, we checked whether the observed increased in ATP$_L$ was caused by the ATP$_e$ content of the bacterial suspension. Thus, bacterial suspensions were centrifuged and the supernatant was collected. Intestinal segments were then perfused with Ringer solution, followed by the bacterial supernatant, and finally, the bacterial suspension (Figure 8C). Results show that maximal ATP$_L$ levels, compared with basal values, were 3-fold and 5-fold higher with the bacterial supernatant and the bacterial suspension, respectively (Figure 8D). This means that endogenous ATP$_e$ of bacteria, as well as bacteria themselves, cause increases of ATP$_L$ levels.

**Discussion**

The results of the present study show that ATP$_e$ regulation in *E. coli* results from a dynamic balance between the rates of ATP release and ATP$_e$ hydrolysis. Moreover, we show that OMVs, produced and released by *E. coli* under standard growth conditions, retain key features of the processes mediating ATP$_e$ regulation in bacteria.
In addition, we found that E. coli can strongly enhance ATPe release of Caco-2 cells and intestinal rat segments.

**Studies using E. coli and OMVs**

The addition of exogenous ATP to bacterial suspensions, which should promote ATP transport through OM porins into the periplasm [4,7], led to significant ATPe uptake and hydrolysis.

ATPase activity increased in proportion to [ATPe] over a wide nanomolar range (Figure 1B). A similar pattern was observed in OMVs, which is consistent with these vesicles encapsulating portions of the bacterial periplasm containing functional enzymes [44].

Based on previous reports [5,15] we propose periplasmic 5′-nucleotidase and alkaline phosphatase to be responsible for the observed ATPe hydrolysis at neutral pH. To further corroborate the functional presence of these enzymes, we evaluated the effect of medium Pi. 5′-nucleotidases from bacteria and various vertebrate species do not exhibit product inhibition [15,58]. Regarding alkaline phosphatase, although a transcriptional down-regulation of the enzyme in the presence of Pi was postulated, this mechanism was impeded under high Pi culture conditions. Nevertheless, several reports have showed that Pi can kinetically inhibit the alkaline phosphatase activity of E. coli [18,59].

Thus, in addition to experiments using the M9 medium (containing mM Pi), we also evaluated ATPase activity in media lacking phosphate (i.e. Hepes medium), where a strong increase in ATPe hydrolysis was found. Two components of ATPe hydrolysis were observed, one of which is relatively insensitive to Pi, consistent with 5′-nucleotidase activity, and a second component which was kinetically inhibited by Pi, and activated at alkaline pH, consistent with alkaline phosphatase.

Since in both, E. coli and OMVs, ATPe hydrolysis was a linear function of [ATPe], any increase in the nucleotide will at least be partially down-regulated by increases of periplasmic (bacteria) or intravesicular (OMV) ATPase activity.

Having shown that periplasmic ATPase activity is active under the experimental conditions, we turned our attention to bacterial ATP release. This was estimated by studying ATPe kinetics in the absence and presence of two amphipatic peptides, MST7 and MEL. In unstimulated conditions, a stable nanomolar level of ATPe was observed (Figures 4A,B), which is compatible with a potential low rate of ATP release being counterbalanced by periplasmic ATPase activity.

In line with this idea, other authors found no ATP secretion by E. coli in the absence of stimuli [58]. Moreover, when expression of periplasmic 5′-nucleotidase and alkaline phosphatase of E. coli was blocked, ATP release could be observed in the absence of stimulus [5], a process that would lead to [ATPe] accumulation.

Basal [ATPe] was challenged when E. coli were perturbed by exposure to MST7 and MEL. Addition of the peptides caused a 3-fold (MST7) and 7-fold (MEL30) increase in [ATPe] within 1 min, with the amplitude of ATPe response at 20 min post-stimulus ranging from 66 μM/(10^10 bacteria) (MST7) to 259 μM/(10^10 bacteria) (MEL30). Changes in [ATPe] were achieved by triggering a strong initial rate of ATP release (denoted as P_ATPe in Figure 4C) under MST7 (∼53 μM/min) and MEL30 (∼155 μM/min). These rates were approximately 30-fold higher than ATPase activities under the same conditions. Thus, the acute phase of ATPe kinetics mostly reflected the kinetics of ATP release. At later times, however, the rate of [ATPe] increase slowed down rapidly. While with MST7 a decrease in [ATPe] was not observed during 20 min exposure, with MEL30, [ATPe] started to decrease at approximately 12 min post-stimulus, an indication that ATP release is inactivated with time while ATPase activity progressively began to govern ATPe kinetics.

Overall, these results show that E. coli exhibit basal ATPe levels, which were greatly increased when bacteria were challenged by permeabilizers.

E. coli-derived ATPe may stimulate biofilm formation and adherence [60], promote bacterial growth, or even act as a quorum sensing signal [3].

Since bacterial ATP release can be caused by lytic and non-lytic mechanisms, two aspects of viability were analyzed, i.e. the capacity of bacteria to form colonies on solid agar plates (CFU) and the IM integrity, estimated by β-galactosidase activity.

Within 1 min, 10 μM MST7 and 3 μM MEL decreased CFU formation by approximately 30% (Supplementary Figure S5A), with no changes thereafter. Exposure to 30 μM MEL produced a stronger decrease in CFU. β-galactosidase activity showed no changes with 10 μM MST7 or 3 μM MEL, while 30 μM MEL produced a different pattern. In this case, low increments at short incubation times (0–5 min) were followed by strong increases at later times (5–20 min) (Supplementary Figure S5B). Thus MEL30, but not MST7,
compromised the IM. In agreement with these findings, Katsu et al. [23] showed that MST perturbed the IM of E. coli only when the OM was previously disrupted [23]. Instead, MEL was able to disrupt both membranes in a dynamic fashion; it can assemble to form transient pores in the OM and then translocate to the IM, inducing the leakage of cytoplasmic contents [9].

Therefore, ATP efflux induced by MST7 was compatible with changes only in the permeability of the OM, suggesting ATP release from the periplasm, even though subsequent culture led to a decreased culturability.

Under MEL30, on the other hand, the acute phase of [ATPe] increase would be mostly non-lytic, followed by a later phase of increase where the IM is compromised, a condition where the ATPcyt content can be released, thus leading to the observed high amplitude of ATPe kinetics. Accordingly, after 20 min of MEL30 exposure, 87% of intracellular ATP was released by a combination of lytic and non-lytic mechanisms. The late decrease in [ATPe] could be due to the ATPase activity of periplasmic and cytoplasmic nucleotidases and phosphatases.

Thus, ATP is released by a combination of lytic and non-lytic mechanisms. Under partial bacteriolysis, dying bacteria might release ATP and other nucleotides, which can be taken up and metabolized by viable bacteria.

The ATPe kinetics of OMVs was qualitatively similar to that obtained with E. coli; ATPe remained steady in the absence of stimuli, and acutely increased in the presence of the peptides.

Values of [ATPe] at each time point from OMVs were compared with similar values from E. coli. This correlation plot (Supplementary Figure S8A,B) showed a roughly linear relationship for [ATPe] values of E. coli and OMVs for at least 5 min (the acute phase), although at later times this linearity was lost. This could be due to several reasons. First, OMVs are smaller than E. coli and as such represent a simpler system where, unlike whole bacteria, ATPe regulation can be studied in the absence of cytoplasmic content, that is, with no synthesis or release of ATPcyt. In addition, several authors reported that some OM proteins can be disproportionately enriched in OMVs, whereas others can be excluded, so that an altered capacity for the proteins involved in ATPe regulation may occur.

OMVs may contain DNA, and proteins exhibiting various biological activities [44], such as proteases, peptidases, and β-lactamases [61].

In this context we show that OMVs contain intravesicular ATP, and that, similarly to bacteria, this nucleotide can be released when vesicles are exposed to permeabilizers. This is important inasmuch as OMVs were shown to lyse when exposed to external stimuli [62] or open transiently [63].

Bacteria, and therefore OMVs, lack purinergic receptors (for extracellular di- and trinucleotides), so that signaling via these receptors is assumed to be absent from E. coli and other microorganisms [3]. Although speculative, OMVs, acting as delivery vesicles containing ATP and ATPases, might modulate the purinergic signaling system of the host.

Figure 9 provides a qualitative model of ATPe regulation of E. coli and their OMVs. Under culture conditions, OMVs are formed from E. coli. ATP can be present in the cytosol, the periplasm, the extracellular space, or the lumen of OMVs. In both, OMVs or bacteria, regulation of ATPe depends on the dynamic balance between the rates of ATP hydrolysis, facilitated by nucleotidases/phosphatases of the periplasm (E. coli) or vesicular lumen (OMVs) and of ATP release. Permeabilizers can act on the OM alone (MST7) or both on the outer and inner membrane (MEL).

Effect of E. coli on the ATPe kinetics of Caco-2 cells

Caco-2 cells have been previously shown to release ATP when exposed to hypotonicity [64]. Our results also showed regulated ATP release, in this case when cells were exposed apically to an adrenergic cocktail containing isoproterenol. This was not unexpected, since in these cells isoproterenol was shown to increase cAMP and Ca\(^{2+}\) [65,66], two well-known second messengers triggering ATP release in different cell types [38,67,68], see Lazarowski [69]. ATPe concentration showed a fast and steep increase to a maximum, followed by a slower ATPe decay (Figure 7A). The latter is indicative of a high rate of extracellular ATPase activity, which could be corroborated by measuring ATPe consumption and \(^{32}\)P-Pi production under exposure to ATP (Supplementary Figure S9).

Thus, aliquots of E. coli were added to the apical side of Caco-2 cells, leading to a strong [ATPe] increase to a maximum, followed by an acute decrease (Figure 7D).

In Caco-2 cells, the initial rate of ATP release induced by E. coli (derived from data of Figure 7D), was approximately 30-fold higher than the rate of ATPe hydrolysis (Figure 7B) of these cells at similar ATPe levels. Therefore, during this brief phase of ATPe accumulation, quantified as ΔATPe (inset of Figure 7D), bacterial-induced ATPe kinetics was mainly governed by Caco-2 apical release of intracellular ATP. At later times, the kinetics of ATPe consumption and changes in cytoplasmic ATP concentration were dominated by ATPase activity of the cytosol and permeabilizers. The latter are not specifically targeted toward transmembrane transport of ATP, but are likely to exert an overall effect on this process.
times, however, [ATPe] decreased non-linearly, implying that ATPe release is down-regulated while ATPe hydrolysis began to dominate ATPe kinetics. Similarly, enteropathogenic E. coli-induced ATPe release of TC7 epithelial cells, although the experimental set-up did not allow distinguishing the apical and basolateral components of ATPe release [70].

What is the relative contribution of bacteria and Caco-2 cells to this late ATPe decay kinetics? Under the experimental conditions (Supplementary Figure S2), with approximately $5 \times 10^5$ Caco-2 cells exposed to $2 \times 10^7$ E. coli, the corresponding values of $K_{ATP}$ (which estimates the rate of ATPe hydrolysis, Figure 7B) would be $3 \times 10^{-2} \text{min}^{-1}$ (Caco-2) and $8 \times 10^{-5} \text{min}^{-1}$ (E. coli). Thus, the capacity to hydrolyze ATPe was approximately 300 times higher for Caco-2 cells than for bacteria. This is in agreement with Caco-2 cells exhibiting activities of ectonucleotidases and ectophosphatases, and their derived exosomes exhibiting strong ectoATPase activity [65,66]. Thus, the observed ATPe decay kinetics of Figure 7D was governed by the extracellular ATPase activity of Caco-2 cells, with no contribution from E. coli.

**Figure 9. Qualitative model of ATPe regulation of E. coli and their outer membrane vesicles (OMVs).**

Under normal growth conditions, OMVs are continuously formed from E. coli. These spherical vesicles form when small portions of the outer membrane bulge away from bacteria grown in broth, pinch off and release. ATP can be present in the cytosol, the periplasm, the extracellular space, or the lumen of OMVs. Permeabilizers can act on the OM alone (MST7) or both on the outer and inner membrane (MEL). E. coli was shown to hydrolyze ATPe and, in the presence of amphipatic peptides, release ATP. As, during formation, OMVs encapsulate portions of the bacterial periplasm with soluble proteins, they might exhibit ATPase activity due to originally periplasmic nucleotidases and phosphatases. In both, OMVs or bacteria, regulation of ATPe depends on the dynamic balance between the rates of ATP hydrolysis, facilitated by nucleotidases/phosphatases of the periplasm (E. coli) or vesicular lumen (OMVs), and of ATP release induced by MST7 and MEL.
As complementary proof for the effect of *E. coli* on intestinal cells, we used rat jejunum segments. Unlike Caco-2 cells, which represent an *in vitro* model of the enterocytes lining the intestinal cavity, rat intestinal segments conserve the complete intestinal wall structure. [ATP]e significantly increased shortly after *E. coli* injection into rat jejunum segments (Figure 8A). Similarly, injection of the pathogenic Gram-negative *Shigella flexneri* in rabbit ileum segments caused luminal ATP release [70]. Interestingly, the observed ATP_L increase was not only due to intestinal ATP release, but also to the endogenous ATPe of bacteria (Figure 8C).

The above considerations imply that *E. coli* are strong inducers of ATPe kinetics of intestinal cells. On the other hand, *E. coli* and their OMVs might release low micromolar ATPe under situations compromising their integrity (Figures 4,6). This could activate P2Y2 (EC50 for ATP ≈ 80 nM) [68], which are functional at the apical domain of Caco-2 cells [71,72]. Activation of P2Y2,4,6 receptors of intestinal cells and various intestinal cell lines has been shown to regulate transepithelial ion transport [73,74].

In short, the results of the present study show that *E. coli* can trigger apical ATP release from an intestinal epithelial cell model and rat intestinal segments, and simultaneously take up and hydrolyze submicromolar ATPe. ATPe hydrolysis can be further enhanced by the ectoATPase activity of Caco-2 cells, and potentially by ATPase activity of OMVs. This is metabolically important since *E. coli* is also permeable to ATPe hydrolysis products such as ADP and AMP [4].

Given that the intestinal mucosa is a purine-limited environment [75], ATPe metabolism by *E. coli* could be part of a nutritional program whereby successful colonization of the colon by at least five different, non-pathogenic, *E. coli* strains depend upon competition for nutrients with a dense and diverse microbiota, with up to 1000 different commensal species [76].

Alternatively, ATP release by bacteria may supply energy and hence facilitate the formation and survival of bacterial communities [3].

Future studies would allow elucidating the extent to which bacteria and OMVs can modulate nucleotide-dependent cellular and systemic responses of the host, such as inflammation, proliferation, vasodilation, apoptosis, and cell volume.

**Abbreviations**

AMP-CPP: α,β-methyleneadenosine 5'-triphosphate; AphA: acid phosphatase; ATPe: extracellular adenosine triphosphate; ATPL: luminal adenosine triphosphate; IM: inner membrane; OM: outer membrane; OMVs: outer membrane vesicles; MEL: melittin; MST7: mastoparan 7; PhoA: alkaline phosphatase

**Author Contribution**


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Competing Interests
The Authors declare that there are no competing interests associated with the manuscript.

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