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A commonly-used ecto-ATPase inhibitor, ARL-67156, blocks degradation of ADP more than the degradation of ATP in murine colon

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Abstract

Background—Adenosine 5'-triphosphate (ATP) is released extracellularly as a neurotransmitter and an autocrine or paracrine mediator in numerous systems, including the gastrointestinal tract. It is rapidly degraded to active and inactive metabolites by membrane-bound enzymes. Investigators frequently use inhibitors of ATP hydrolysis such as ARL-67156 and POM-1 to suppress the catabolism of ATP and prolong its effects in pharmacological studies. Our aim was to investigate directly the effects of ARL-67156 and POM-1 on the degradation of ATP and ADP in mouse colonic muscles.

Methods—The degradation of ATP and ADP was evaluated by superfusing tissues with 1,*N*⁶-etheno-ATP (eATP) and 1,*N*⁶-etheno-ADP (eADP) as substrates and monitoring the decrease of substrate and increase of products (i.e., eADP, eAMP, and e-adenosine) by high-performance liquid chromatography techniques with fluorescence detection. Relaxation responses to etheno-derivatized and non-derivatized ATP and ADP were examined in isometric tension experiments.

Key results—ARL-67156 inhibits the degradation of ADP but not of ATP whereas POM-1 inhibits the degradation of ATP but not of ADP in murine colonic muscles. Consequently, ARL-67156 enhances relaxation responses to both ATP and ADP whereas POM-1 reduces relaxation to ATP and does not affect relaxation to ADP.

Conclusions & Inferences—Studies that use ARL-67156 to inhibit ATP degradation in smooth muscle likely evaluate responses to accumulated ADP rather than ATP. POM-1 appears to be a more selective inhibitor of ATP degradation in the mouse colon. The choice of pharmacological tools in studies on extracellular ATP signaling may affect the interpretation of experimental data in functional studies.

Keywords

ARL-67156; POM-1; nucleotidases; colon; ATP; ADP; ATP degradation

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Introduction

ATP is traditionally assumed to be released in the extracellular space as a neurotransmitter and a paracrine and autocrine mediator in a large variety of systems, including the gastrointestinal (GI) tract (1, 2, 3, 4). Once released in the extracellular space ATP is rapidly hydrolyzed to adenosine 5'-diphosphate (ADP) and adenosine 5'-monophosphate (AMP) by ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), which is then metabolized to adenosine by 5'-nucleotidase (5, 6). Rapid ATP degradation results in very transient responses of target tissues to ATP and in likely monitoring responses to biologically-active ATP metabolites (e.g., ADP and adenosine) that may differ from the direct effects of released ATP. To avoid such complications and to prolong the effects of released ATP, investigators frequently use inhibitors of ATP hydrolysis (7, 8, 9, 10, 11, 12). 6-N,N-diethyl-beta, gamma-dibromomethylene-D-ATP (ARL-67156, formerly known as FPL 67156) (13) is the first inhibitor of ectoATPase introduced for research (14) and is frequently utilized to inhibit ATP hydrolysis in tissue preparations. The majority of studies however do not validate the inhibitory action of ARL-67156 on the degradation of ATP and other purines although it is acknowledged that same enzymes could be involved in the degradation not only of ATP but also of ADP (15). Moreover, recent studies indicate that ARL-67156 may be a weak inhibitor of different forms of the membrane-bound E-NTPDases (16), suggesting that assumptions for enzyme inhibitory activity of ARL-67156 in the absence of direct validation of such activity might be erroneous.

E-NTPDase 1, 2, 3 and 8 are cell surface members of the NTPDase/CD39 family (17, 18, 19). In the GI tract in particular, NTPDase 1, 2 and 3 are responsible for ecto-ATPase activity (20). Thus, mRNA for NTPDase 1, 2 and 3 is present throughout all regions of the mouse intestine, including the colon (20). Immunohistochemistry techniques have shown localization of NTPDase 2 and NTPDase 3 in enteric nerves (20). NTPDase 2 also appears to be localized in enteric glia (20, 21) whereas NTPDase 3 is found in epithelial cells and connective tissue in the GI mucosa and submucosa (20). Finally, NTPDase 1 is expressed in blood vessels (22) and in immune cells of the lamina propria (23) and does not appear to be co-localized with NTPDase 3 in the mouse colon (20). Despite diverse cellular localization of NTPDases 1, 2 and 3, it is recognized that the three enzymes are together responsible for the major part of membrane-located nucleotidase activities in many tissues including the colon (20). NTPDase 1 hydrolyzes both ATP and ADP, NTPDase 2 mainly hydrolyses ATP, and NTPDase 3 has intermediate properties (24). ARL-67156 shows weak competitive inhibition of recombinant human NTPDase 1 and 3 and mouse NTPDase 8 and even weaker inhibition of NTPDase 2 (25). Interestingly, in mouse brain, higher levels of ADP than of ATP were observed when brain tissue was incubated with ATP in the presence of ARL-67156 (26), suggesting that ARL-67156 might be a more effective inhibitor of the degradation of ADP than of ATP. Therefore, it is possible that effects that are obtained in the presence of ARL-67156 are mediated by ADP and not by ATP. The effectiveness of ARL-67156 as an inhibitor of the enteric purinergic neurotransmitter degradation (27) or of ATP catabolism in the colon (28) has been questioned, but direct validation of ARL-67156 effects on ATP or ADP degradation in the GI tract has not been done previously.

In the present study, we compared the inhibitory effects of ARL-67156 on the degradation of ATP and ADP in whole *tunica muscularis* isolated from mouse colon. The effects of ARL-67156 were also compared with the effects of another frequently used ecto-ATPase inhibitor, sodium polyoxotungstate (POM-1) (29, 30). Our observations demonstrate differential effects of ARL-67156 and POM-1 on the degradation of extracellular ATP and ADP in the colon and suggest that results from studies using ARL-67156 to inhibit the extracellular metabolism of ATP should be interpreted with great caution.

Methods and Materials

Drugs

ATP, adenosine, ADP and AMP were purchased from Sigma-Aldrich, St. Louis, MO, USA, whereas ARL-67156 and POM-1 were purchased from Tocris Bioscience (Ellisville, MO, USA). Stock solutions were prepared in distilled water and drugs were further diluted in Krebs solution to the final concentrations.

Tissue preparation

C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were euthanized by sedation with isoflurane followed by cervical dislocation and exsanguination. The entire GI tract was removed and placed in oxygenated cold Krebs solution for further dissection. Krebs solution (at 37°C) had the following composition (mM): 118.5 NaCl, 4.2 KCl, 1.2 MgCl₂, 23.8 NaHCO₃, 1.2 KH₂PO₄, 11.0 dextrose, 1.8 CaCl₂ (pH 7.4). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nevada. For experiments monitoring purine degradation, colon preparations were dissected free from the remaining GI tract and opened along the mesenteric border. After removing mucosa and submucosa, the *tunica muscularis* preparations were placed in vertical 200- μ l chambers of a Brandel superfusion system and were continuously maintained at 37 $\pm$ 0.5 °C by continuous perfusion of oxygenated Krebs solution (2, 25). For mechanical experiments, circular muscle strips of colon muscularis were placed in 10-ml vertical organ baths containing oxygenated Krebs solution at 37 °C.

Degradation of ATP and ADP

To determine enzymatic degradation of ATP and ADP, colonic preparations were superfused with 1,*N*⁶-etheno-ATP (eATP) and 1,*N*⁶-etheno-ADP (eADP) substrates as previously described (31). The process of etheno-derivatization involves insertion of acyl group between nitrogen 1 and 6 of the adenine moiety and renders the molecule fluorescent but leaves unaltered the phosphate chain. Therefore, 1,*N*⁶-etheno-nucleotides and non-derivatized nucleotides share identical sites for hydrolysis (Fig. 1A). This was also verified in preliminary experiments in which tissues were superfused with non-derivatized ATP and the superfusate samples were etheno-derivatized after the enzymatic reactions. As shown in Fig. 1B, 1,*N*⁶-etheno-ATP (eATP, 50 nM) and non-derivatized ATP (50 nM) are metabolized to the same products during the contact with colonic tissue. However, use of 1,*N*⁶-etheno-nucleotides as exogenous substrates for nucleotidases offers greater sensitivity in detecting substrate reduction and product formation and avoids possible complications from endogenously released purines as these remain under detection threshold (31, 32, 33, 34,

35). To determine $V_{\max}$ and K_m of ATPase and ADPase activities in the murine colon, tissues were superfused with eATP or eADP at 10 nM, 25 nM, 50 nM, 100 nM and 200 nM for 30 s and the decrease in substrate concentration was evaluated (Fig. 1C,D). Tissues were then superfused with selected concentrations of each substrate, dissolved in Krebs solution, for 30 s in the absence of E-NTPDase inhibitors or after 30-min perfusion with either ARL-67156 (10-100 μ M) or POM-1 (100 μ M). In the majority of experiments, the substrates eATP and eADP were used at 50 nM, which is in the range of maximum saturation concentrations (Fig. 1C, D). In some experiments eATP and eADP substrates were used at either 25 nM, which is close to the half-maximal saturation concentrations (K_m) in the colon (Fig. 1C,D) or 30 μ M to match concentrations used in the mechanical experiments. Three hundred μ l of the superfusion solution were collected from the beaker containing the substrate (S0, (-) *tissue*). After treating tissues with substrate, contents of the chambers were collected in ice-cold Eppendorf tubes (S1, (+) *tissue*) and the enzymatic reactions were stopped by immersing the tubes in liquid nitrogen. The degradation of ATP was evaluated by assaying the decrease in eATP amounts after contact with tissue and the production of eADP, eAMP and e-adenosine (eADO), whereas the degradation of ADP was determined by evaluating the decrease in eADP and increase in eAMP and eADO in tissue superfusates.

HPLC measurements of etheno-nucleotides and adenosine

A reverse-phase gradient Agilent Technologies 1200 liquid chromatography system (Agilent Technologies, Wilmington, DE, USA) was used to detect the 1, N^6 -etheno-products as described previously (2, 31). The mobile phase consisted of 0.1 M KH_2PO_4 (pH 6.0) as eluent A. Eluent B consisted of 65% eluent A and 35% methanol. Gradient elution was employed according to the following linear program: time 0, 0% eluent B; 18 min, 100% eluent B. The flow rate was 1 ml/min and run time was 20 min. Column and autosampler temperatures were maintained at 25°C and 4°C, respectively. The fluorescence detector was set to record 1, N^6 -etheno-derivatized nucleotide and nucleoside signals at an excitation wavelength of 230 nm and emission wavelength of 420 nm. The amount of nucleotide/nucleoside in each sample was calculated from calibration curves of nucleotide standards run simultaneously with each set of unknown samples. Results were normalized for sample volume and tissue weight and product formation was expressed in fmol/mg tissue (femtomoles/mg tissue).

Preparation of eATP and eADP

ATP and ADP were etheno-derivatized as follows: 0.2 mM ATP or ADP (dissolved in double distilled water) was acidified to pH 4.0 with citrate phosphate buffer. Chloroacetaldehyde (1 M) was added and substrates were heated to 80°C for 40 min to form 1, N^6 -ethenoderivatives eATP and eADP (35, 36). Substrates were further diluted in the superfusion solution to final concentration needed.

Isometric force measurements

Strips of colon muscle (~ 5 mm $\times$ 2 mm) were cut in the long axis of the circular muscle fibers and attached to a Fort 10 (World Precision Instrument, Sarasota, FL, USA) isometric strain gauge in organ baths filled with Krebs solution. A resting force of 3 mN was applied as described previously (2). This was followed by an equilibration period of 1 h, before

experiments were initiated. Drugs were added to tissues via the bathing solution at the concentrations stated. Signals were recorded onto a PC via Acknowledge software (Biopac Systems Inc, Goleta, CA, USA) and areas under the trace (AUT) were calculated. Etheno-derivatized and non-derivatized ATP and ADP were used at a concentration (i.e., 30 μM) that produces incomplete relaxation so that possible enhancement in relaxation responses in the presence of E-NTPDase inhibitors could be detected.

Statistics

Data are presented as means $\pm$ SEM. Means are compared by a two-tailed, unpaired t test or by one-way ANOVA for comparison of more than two groups followed by a post hoc Bonferroni multiple comparison test (GraphPadPrism, v. 3, GraphPad Software, Inc, San Diego, CA). A probability value of less than 0.05 was considered significant. The Michaelis-Menten equation was used to determine maximum velocity (V_{max}) and Michaelis constant (K_m) for eATP and eADP metabolism in the colon (GraphPad Prism).

Results

Concentration-dependent catabolism of eATP and eADP in murine colon

Maximum velocity (V_{max}) and the half-maximal saturation concentration (K_m) for ATPase and ADPase in colon *muscularis* were determined by the Michaelis-Menten equation (Fig. 1C, D). The V_{max} for eATP degradation was $0.045 \text{ pmol.mg}^{-1}.\text{min}^{-1}$ and the K_m was 21.24 nM (Fig. 1C). The V_{max} for eADP degradation was $0.041 \text{ pmol.mg}^{-1}.\text{min}^{-1}$ and the K_m was 24.15 nM (Fig. 1D). At a concentration approximately two times the K_m of eATP and eADP (i.e., 50 nM) both substrates were at saturation concentrations. Therefore 50 nM concentration of substrate was used in the majority of subsequent studies.

Effects of ARL-67156 and POM-1 on the degradation of ATP

Under control conditions, superfusion of mouse colon preparations with eATP (50 nM for 30 s) resulted in decreased amounts of eATP ($P < 0.001$, $n = 15$) and increased amounts of products eADP+eAMP+eADO ($P < 0.0001$, $n = 15$) (Fig. 2A,B). The individual products of eATP were as follows: eADP was $17.75 \pm 0.82 \text{ fmol/mg}$ and $28.1 \pm 3.2 \text{ fmol/mg}$ before and after contact of eATP with tissue, respectively ($P < 0.001$); eAMP was 0.84 ± 0.08 and $10.7 \pm 1.6 \text{ fmol/mg}$ before and after eATP contact with tissue, respectively ($P < 0.001$), and eADO was 0 ± 0 and $11.94 \pm 2.12 \text{ fmol/mg}$ tissue before and after eATP contact with tissue, respectively ($P < 0.001$).

When colon tissues were incubated with ARL-67156, 100 μM for 30 min prior to eATP, the amount of eATP was reduced to about the same amount as in the absence of ARL-67156 (Fig. 2), suggesting that ARL-67156 failed to inhibit the degradation of eATP. In the same samples, the amount of total e-product (i.e., eADP+eAMP+eADO) was similarly increased despite the presence of ARL-67156 (Fig. 2). The amounts of eADP produced from eATP in contact with colonic muscles in the presence of 100 μM ARL-67156 was $37.34 \pm 8.3 \text{ fmol/mg}$ and was not significantly different from the amount of eADP produced from eATP in the absence of ARL-67156 ($P > 0.05$, $n = 10$). Thus, the formation of eADP from eATP was not inhibited by ARL-67156. However, in the presence of ARL-67156 (100 μM) eAMP was

3.54±0.36 fmol/mg which was significantly lower than eAMP formed from eATP in the absence of ARL-67156 ($P<0.001$, $n=10$), suggesting that ARL-67156 likely inhibited the formation of eAMP from eADP.

Likewise, eADO was 3.63±0.5 fmol/mg in the presence of ARL-67156 100 µM, which was significantly lower than eADO formed from eATP in the absence of ARL-67156 ($P<0.001$). Comparable results were obtained with lower concentrations of ARL-67156 (10 and 50 µM, data not shown), suggesting that ARL-67156 (10-100 µM) does not appear to inhibit the degradation of eATP in the mouse colon, but may inhibit the degradation of eADP.

In the presence of POM-1 (100 µM), another inhibitor of ecto-nucleotidases, the amount of eATP was not significantly decreased from the amount of eATP in the absence of tissue ($P>0.05$, $n=5$), but was significantly higher than eATP in the presence of ARL-67156 100 µM ($P<0.05$) (Fig. 2), suggesting that POM-1 likely inhibited the eATP degradation in this tissue. Moreover, eADP and eAMP were not formed from eATP in the presence of POM-1 ($P>0.05$, $n=5$) and the formation of eADO and total products (eADP+eAMP+eADO) were significantly reduced than when tissue was in contact with eATP in the absence of inhibitors ($P<0.05$, respectively, $n=5$, Fig. 2).

Similar effects of ARL-67156 and POM-1 on the eATP degradation were observed when a lower substrate concentration that was close to K_m (Fig. 2C) was used. Thus, ARL-67156 failed to inhibit the degradation of 25 nM eATP as demonstrated by the similar formation of the products in the absence or presence of ARL-67156 ($P>0.05$, Fig. 2C). POM-1 significantly reduced the formation of eADP+eAMP+eADO from 25 nM eATP ($P<0.05$ vs. absence of inhibitors, Fig. 2C) which was consistent with the results obtained using 50 nM substrate. Therefore, the lack of inhibitory effects of ARL-67156 was not due to the use of high substrate concentration.

Effects of ARL-67156 and POM-1 on the degradation of ADP

Next we examined the degradation of eADP in colonic muscles. Under control conditions superfusion with eADP (50 nM for 30 s) resulted in decreased amounts of eADP ($P<0.001$, $n=14$) and increased amounts of the products eAMP and eADO ($P<0.001$, $n=14$) (Fig. 3).

Superfusion of colonic muscles with eADP in the presence of ARL-67156 (100 µM) resulted in reduced degradation of eADP than in the absence of ARL-67156. Thus the amount of total product (eAMP+eADO) was significantly decreased compared to control conditions ($P<0.01$, $n=6$) (Fig. 3). In particular, eAMP was 11.75±0.47 fmol/mg ($n=31$), 14.32±0.88 fmol/mg ($n=14$), and 12.4±0.97 ($n=6$) in absence of tissue, in presence of tissue, and in presence of tissue with ARL-67156 (100 µM), respectively ($P<0.05$ presence of tissue vs. no tissue, $P>0.05$ presence of tissue plus ARL-67156 vs. no tissue). eADO was 0±0 fmol/mg ($n=31$), 10.43±1.3 fmol/mg ($n=14$) and 3.5±0.68 fmol/mg ($n=6$) in the absence of tissue, in presence of tissue, and in presence of tissue with ARL-67156 (100 µM), respectively ($P<0.001$ presence of tissue vs. no tissue, $P<0.001$ presence of tissue vs. presence of tissue plus ARL-67156). Similar effects were observed with ARL-67156 10 µM and 50 µM (data not shown).

POM-1 did not affect the degradation of eADP in the mouse colon. Thus superfusion of tissues with eADP in the presence of POM-1 resulted in reduced eADP ($P<0.001$, $n=8$) and increased formation of eAMP and eADO which was not different from formation of products in the absence of POM-1 (Fig. 3).

To determine whether the lack of effect of POM-1 on eADP degradation was due to an inability of the inhibitor to compete with maximum saturation concentrations of eADP, we performed experiments using half-maximal saturation concentration of eADP, i.e., 25 nM. The effects of ARL-67156 and POM-1 on the degradation of 25 nM eADP were similar to the effects of these inhibitors on the degradation of 50 nM eADP. Thus, ARL-67156 inhibited the degradation of 25 nM eADP whereas POM-1 did not inhibit degradation of 25 nM ADP (Fig. 3C).

Relaxation responses of murine colon to non-derivatized and etheno-derivatized ATP and ADP in the presence of ARL-67156 and POM-1

ATP and ADP (each at 30 μ M) caused partial relaxation of murine colon (Fig. 4). Relaxation responses to both ATP and ADP were enhanced in the presence of 100 μ M ARL-67156 ($n=3$) (Fig. 4). POM-1 inhibited the relaxation responses to ATP and did not affect the relaxation responses to ADP ($n=4$) (Fig. 4).

Similar responses were observed with 1, N^6 -etheno-derivatized ATP and ADP that were used as substrates in the purine degradation studies. At concentrations used in the degradation experiments (i.e., 25 and 50 nM) eATP and eADP caused no effect on the tone of isolated colonic muscles (not shown). eATP and eADP (each at 30 μ M) caused partial relaxation of murine colon (Fig. 5). Relaxation responses to both eATP and eADP were enhanced in the presence of 100 μ M ARL-67156 (Fig. 5). POM-1 inhibited the relaxation responses to eATP and did not affect the relaxation responses to eADP (Fig. 5). Therefore, the process of 1, N^6 -derivatization of ATP and ADP does not appear to affect the sites of hydrolysis or the sites of action of eATP and eADP when compared with non-derivatized ATP and ADP (Figs. 4 and 5).

Effects of ARL-67156 and POM-1 on the degradation of 30 μ M eATP and eADP

We next performed experiments examining the effects of ARL-67156 and POM-1 on the degradation of 30 μ M eATP and eADP to match the concentrations of ATP and ADP (or eATP and eADP) that were required to produce colon relaxations. Similar to the results obtained with 50 nM eATP, ARL-67156 did not inhibit the degradation of 30 μ M eATP whereas POM-1 reduced the degradation of 30 μ M eATP. Thus, amounts of eADP+eAMP +eADO from 30 μ M eATP were 11.15 ± 0.48 fmol/mg ($n=12$), 19.29 ± 1.35 fmol/mg ($n=6$), 13.95 ± 0.83 fmol/mg ($n=3$) and 10.61 ± 2.06 fmol/mg ($n=3$) in the absence of tissue, in the presence of tissue ($P<0.001$ vs. absence of tissue), in the presence tissue with ARL-67156 ($P<0.05$ vs. absence of tissue), and in the presence of tissue with POM-1 ($P>0.05$ vs. absence of tissue), respectively.

Likewise, ARL-67156 reduced the formation of eAMP+eADO from 30 μ M eADP whereas POM-1 did not inhibit eAMP+eADO formation from 30 μ M eADP. Thus, amounts of eAMP +eADO from 30 μ M eADP were 11.21 ± 0.65 fmol/mg ($n=12$), 18.59 ± 1.66 fmol/mg ($n=6$),

12.67±1.14 fmol/mg (n=3) and 14.64±0.94 fmol/mg (n=3) in the absence of tissue, in the presence of tissue (P<0.001 vs. absence of tissue), in the presence of tissue with ARL-67156 (P>0.05 vs. absence of tissue), and in the presence of tissue with POM-1 (P<0.001 vs. absence of tissue), respectively.

Discussion

In this report, we provide evidence for differential effects of two commonly used E-NTPDase inhibitors on the degradation of ATP and ADP in colonic muscles. Using direct chromatography analysis, we demonstrate that ARL-67156, a pharmacological tool commonly used to suppress the extracellular catabolism of ATP, suppresses the degradation of ADP but not of ATP in the colon. In contrast, POM-1 demonstrates a more selective inhibitory activity for the degradation of ATP over ADP than ARL-67156. Furthermore our data suggests that relaxation to ATP in the colon is likely mediated by metabolism to ADP as responses to exogenous ATP are reduced in the presence of POM-1. These findings may assist investigators in interpreting experimental results obtained in functional studies that use ARL-67156 and POM-1 to inhibit purine degradation.

ATP is a well-established extracellular signaling molecule in a large number of systems, including the GI tract (4). Levels of extracellular ATP are controlled by the action of E-NTPDases that degrade ATP to ADP and AMP, which is then metabolized to adenosine by 5'-nucleotidase (15, 37). ATP and ADP are biologically active and despite some overlap, these two substances have quite distinct pharmacology profiles in terms of receptor mechanisms that mediate tissue and cell responses to the two adenine nucleotides (38). For example, ATP activates the ligand-gated P2X receptors and some G-protein-coupled P2Y receptors (e.g., P2Y1, P2Y2, P2Y11, and P2Y12), whereas ADP stimulates primarily P2Y1, P2Y12, and P2Y13 receptors (38). Ecto-nucleotidases have the ability to either terminate P2 receptor-mediated responses to ATP and/or to favor the activation of ADP and adenosine-sensitive receptors (19). To prolong the action of ATP that is released in the extracellular space either as a neurotransmitter or an autocrine and paracrine mediator, many investigators use E-NTPDase inhibitors such as ARL-67156 or POM-1 (see Introduction). The interpretation of such results, however, could be complicated by the facts that multiple purines are substrates for the same enzyme or that enzyme inhibitors may have different potencies for each E-NTPDase. The majority of functional studies does not control for site of action of E-NTPDase inhibitors or for accumulation of specific substrates or products although, at times, the effectiveness of such inhibitors has been questioned due to unanticipated observations (27, 28).

In the present study, we utilized etheno-derivatized ATP and ADP to monitor the effects of ARL-67156 and POM-1 on the degradation of ATP and ADP in murine colon since 1) etheno-purine substrates are degraded in a manner identical to that of non-derivatized purines (demonstrated in the present study, Fig. 1), 2) etheno-purines mimic the biological activities of non-derivatized purines when applied exogenously (demonstrated in the present study, Figs. 4 and 5), and 3) etheno-purine substrates have been used routinely by others and us to evaluate the degradation of adenine nucleotides in living systems as they provide high sensitivity in detecting substrate reduction and product formation (31, 32, 33, 34, 39). Using

highly-sensitive chromatography methodologies we demonstrate here that ARL-67156 (10, 50 and 100 μM) inhibited more the ADP degradation than ATP degradation, causing accumulation of ADP rather than ATP in the mouse colon muscularis. In contrast, POM-1 inhibited more the degradation of ATP than ADP and caused accumulation of ATP rather than ADP in the same system. As one would anticipate from the degradation studies, ARL-67156 enhanced the relaxation responses caused by ADP as presumably more ADP was accumulated due to inhibited ADP degradation by ARL-67156. Interestingly, the relaxation responses to ATP were also enhanced in the presence of ARL-67156 although ARL-67156 was shown to inhibit the degradation of ADP but not of ATP. This apparent discrepancy in responses to ATP is likely due to the accumulation of ADP that was formed from ATP but could not be further degraded in the presence of ARL-67156. In accordance with the degradation studies, POM-1 reduced the relaxation responses to ATP and had no effect on the relaxation responses to ADP. These results strongly suggest that relaxation responses to ATP in the colon are likely mediated by metabolism to ADP. The specific receptor subtype(s) mediating relaxation to exogenous ATP or ADP were not examined in the current study as the responses are likely mediated by several purine receptor subtypes on various cell types that are activated upon bath application of purines. For example, P2Y1 receptors are the predominant receptors mediating the inhibitory responses to purine neurotransmitters in the colon (40, 41); however, the relaxation responses to either ATP or ADP in the colon were not inhibited by P2Y1 receptor antagonists or in colons isolated from *P2ry1*^{-/-} mice (28, 40, 41), suggesting that receptors in addition to the P2Y1 receptors were responsible for the relaxation responses to ATP or ADP. Further studies would be required to identify the underlying mechanisms of the relaxation responses to extracellular purines in the murine colon.

For years ARL-67156 was considered to be the most effective drug to inhibit ecto-nucleotidases and particularly the degradation of ATP (13). However, ARL-67156 appears to be a weak competitive inhibitor of human recombinant NTPDase1, NTPDase3 and nucleotide pyrophosphates/phosphodiesterase 1 (NPP1) with K_i of 11 ± 3 , 18 ± 4 and 12 ± 3 μM , respectively (25). At concentrations used in the literature (50-100 μM), ARL-67156 “partially but significantly inhibited the mouse and human forms of these enzymes, but NTPDase2, NTPDase8, NPP3 and ecto-5'-nucleotidase activities were less affected” (21). Also, Muller et al. 2006 (29) demonstrated that ARL-67156 has no action on NTPDase 2 and had K_i 27 and 112 μM on NTPDases 1 and 3, respectively. POM-1 on the other hand appears to be a more potent ecto-nucleotidase inhibitor than ARL-67156 as it has a reported K_i of 2.58 μM at NTPDase 1, 28.8 μM at NTPDase 2 and 3.26 μM at NTPDase 3 (29). In the present study we used eATP and eADP substrates at 50 nM which was determined to be in the range of maximum saturation concentrations for both nucleotides (Fig. 1). Care was taken not to use too high substrate concentrations because previous studies examining the effects of ARL-67156 on recombinant mouse and human E-NTPDases have demonstrated that ARL-67156 was not efficient in blocking any nucleotidases in experiments where high concentrations of the substrate ATP were involved (25). However, concentrations higher than 50 nM (i.e., 30 μM) were required in organ bath studies to elicit relaxation responses in smooth muscle strips. Consistent with the data obtained using 50 nM etheno-substrates, ARL-67156 inhibited the degradation of 30 μM eADP more than the degradation of 30 μM

eATP whereas POM-1 inhibited the degradation of eATP more than the degradation of eADP at the 30 μ M substrate concentration. Moreover, data obtained with half-maximal saturation concentrations of eATP and eADP, i.e., 25 nM, were also consistent with the results obtained using 50 nM and 30 μ M substrates, suggesting that the inability of ARL-67156 to inhibit eATP degradation (or of POM-1 to inhibit eADP degradation) was not due to competition of high substrate concentrations.

While the present study was not designed to distinguish between the E-NTPDase isoforms affected by ARL-67156 and POM-1 in the complex tissue (i.e., mouse colon) that is comprised of multiple cell types expressing multiple nucleotidases, it demonstrates that effects observed in the presence of ARL-67156 in the colon may in fact be due to accumulation of ADP and not of ATP. Since ATP and ADP may activate different receptor sites or target cells the interpretation of the results may be different. This phenomenon may not be restricted to the mouse colon as studies showed that effects in the presence of ARL-67156 may not always be explained with accumulation of ATP (27, 28) and ARL-67156 caused accumulation of ADP (not ATP) in the mouse brain (26).

In conclusion, ARL-67156 inhibited the degradation of ADP but not ATP in the mouse colon. Investigators who use ARL-67156 to inhibit degradation of ATP in the colon likely observe responses to ADP and not to ATP as probably assumed. POM-1 appears to be a more selective inhibitor of ATP degradation than ARL-67156 in the colon. Results obtained using E-NTPDase inhibitors in functional studies should be interpreted with caution in the absence of direct validation of the identity of accumulated extracellular nucleotides in whole tissues.

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Key Points

- Adenosine 5'-triphosphate (ATP) is rapidly degraded to active and inactive metabolites in the extracellular space by enzymes on cell membranes.
- ARL-67156 and POM-1 are presumed to inhibit the catabolism of extracellular ATP and are therefore commonly used in functional studies to suppress the degradation of ATP and prolong its effects. Their effects on ATP and ADP degradation were evaluated in mouse colonic muscles using high-performance liquid chromatography techniques.
- ARL-67156 suppresses the degradation of ADP but not of ATP in the mouse colon whereas POM-1 appears to be a more selective inhibitor of ATP catabolism in this tissue.

Figure 1. Metabolism of non-derivatized and 1,N⁶-etheno-derivatized ATP and enzyme kinetics in murine colon

(A) Chemical structures of non-derivatized ATP (top) and 1,N⁶-etheno-derivatized ATP (eATP, bottom). Cleavage sites for NTPDases and 5' nucleotidase, indicated by the scissors signs, remain unchanged by etheno-derivatization. (B) Superfusion of murine colon tissues with 50 nM eATP substrate (upper chromatogram) results in formation of metabolites eADP, eAMP and e-adenosine, eADO. Likewise, superfusion of tissues with 50 nM non-derivatized ATP substrate (bottom chromatogram), followed by etheno-derivatization after the enzymatic reaction, results in production of eADP, eAMP and eADO. LU, luminescence units. (C) Diagram of reaction rate and Michaelis-Menten kinetics of ATPase activities in the murine colon assessed by the decrease in eATP substrate (10-200 nM) after contact with tissue for 30 s; n=6-12. (D) Diagram of reaction rate and Michaelis-Menten kinetics of ADPase activities in the murine colon assessed by the decrease in eADP substrate (10-200 nM) after contact with tissue for 30 s; eADP n=2-9. In both (C) and (D) the velocity of substrate reduction (pmol.mg tissue⁻¹.min⁻¹) was plotted against substrate concentration [nM]. The K_m values (in nM) are indicated on the graphs.

Figure 2. Degradation of eATP in the murine colon

(A) Original chromatograms of eATP (50 nM) in the absence of tissue [(-) tissue] (blue trace), in presence of tissue with no drug [(+) tissue] (red trace), and in the presence of tissue pre-treated with either ARL-67156 (100 μ M) (green trace) or POM-1 (100 μ M) (purple trace). Note that the eATP substrate contained small amount of eADP, negligible amount of eAMP and no eADO. eATP was decreased and eADP, eAMP and eADO were increased in the (+) tissue samples. In the presence of ARL-67156, the decrease in eATP was unchanged, eADP was increased more than in (+) tissue control, and the formation of eAMP and eADO

were decreased. In the presence of POM-1, the decrease of eATP and the formation of eADP, eAMP and eADO were inhibited. LU, luminescence units *(B)* Graphic representation of substrate (eATP 50 nM) reduction and product (eADP+eAMP+eADO) formation in (–) tissue control, (+) tissue control, (+) tissue plus ARL-67156 (100 µM) and (+) tissue plus POM-1 (100 µM). Asterisks denote significant differences from the amounts of substrate or products in (–) tissue samples (***P<0.001); number of experiments in parenthesis. *(C)* Graphic representation of product (eADP+eAMP+eADO) formation from 25 nM eATP substrate in (–) tissue control, (+) tissue control, (+) tissue plus ARL-67156 (100 µM) and (+) tissue plus POM-1 (100 µM). Asterisks denote significant differences from the amounts of products in (–) tissue samples (***P<0.001); open circle denotes significant difference from the amounts of e-products in (+) tissue control samples (^oP<0.05); number of experiments in parentheses.

Figure 3. Degradation of eADP in murine colon

(A) Original chromatogram of eADP (50 nM) in the absence [(-) tissue] (blue trace), in presence of tissue [(+) tissue, 30 s contact of substrate with tissue] (red trace) and in tissue being in contact with either ARL-67156 (100 μ M) (green trace) or POM-1 (100 μ M) (purple trace). Note that the eADP substrate contained negligible amounts of eAMP and no eADO. eADP was decreased and eAMP and eADO were increased in the (+) tissue samples (red trace). In the presence of ARL-67156, the decrease in eADP and formation of eAMP and eADO were inhibited. In the presence of POM-1, the decrease of eADP and formation of

eAMP and eADO were unchanged compared with (+) tissue control. LU, luminescence units *(B)* Graphic representation of substrate (eADP 50 nM) reduction and product (eAMP +eADO) formation in (–) tissue control, (+) tissue control, (+) tissue plus ARL-67156 and (+) tissue plus POM-1. Asterisks denote significant differences from the amounts of substrate or e-products in (–) tissue controls (***P<0.001, **P<0.01); number of experiments in parenthesis. *(C)* Graphic representation of product (eAMP+eADO) formation from 25 nM eADP substrate in (–) tissue control, (+) tissue control, (+) tissue plus ARL-67156 (100 µM) and (+) tissue plus POM-1 (100 µM). Asterisks denote significant differences from the amounts of e-products in (–) tissue samples (***P<0.001, **P<0.01); number of experiments in parentheses.

Figure 4. ARL-67156 potentiates the relaxation responses to ATP and ADP whereas POM-1 reduces the response to ATP and has no effect on response to ADP

(A) Original traces of spontaneous contractions in mouse isolated colonic muscle. Black lines under the traces indicate when ATP or ADP was added to the bath. Inhibitors were added to the bath for 25 mins before ATP or ADP were added (presence of inhibitors indicated by black lines above traces); w, washout. (B) Summaries of contractile responses to ATP or ADP in the absence and presence of ARL-67156 (100 μM) and POM-1 (100 μM) as area under the trace (AUT). Asterisks denote significant differences from the responses in the absence of inhibitors (**P<0.01, ***P<0.001); number of experiments in parentheses.

Figure 5. ARL-67156 potentiates the relaxation responses to eATP and eADP whereas POM-1 reduces the response to eATP and has no effect on the response to eADP

(A) Original traces of spontaneous contractions in mouse isolated colonic muscle. Black lines under the traces indicate when eATP or eADP were added to the bath. Inhibitors were added to the bath for 25 mins before eATP or eADP were added (presence of inhibitors indicated by black lines above traces); w, washout. (B) Summaries of contractile responses to eATP or eADP in the absence and presence of ARL-67156 (100 μ M) and POM-1 (100 μ M) as area under the trace (AUT). Asterisks denote significant differences from the responses in the absence of inhibitors (*** P <0.001); number of experiments in parentheses.