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Short Communication

Effect of the ecto-ATPase inhibitor, ARL 67156, on the bovine chromaffin cell response to ATP

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Abstract

Bovine chromaffin cells contain an ecto-ATPase ($K_m = 1.57 \pm 0.27 \times 10^{-4}$ M) which can hydrolyze ATP present in the culture media. ARL 67156 is a competitive inhibitor of this ATPase ($K_i = 2.55 \pm 1.36 \times 10^{-7}$ M). A small increase in potency (threefold) is seen when ARL 67156 is included during measurement of ATP-stimulated inositol phosphate formation. ARL 67156 also acts on chromaffin cell P2Y receptors to increase inositol phosphate formation ($EC_{50} = 4.9 \times 10^{-5}$ M). It is useful as an ecto-ATPase inhibitor in studies with bovine chromaffin cells since it exhibits a 300-fold selectivity for the ecto-ATPase versus the P2Y receptor.

Keywords: Ecto-ATPase; Chromaffin cell; ARL 67156; Inositol phosphate; P2Y receptor

1. Introduction

ATP is an important signaling molecule that mediates diverse biological effects including changes in vascular reactivity and central nervous system stimulation (Brake and Julius, 1996). It can be released from cat adrenal medulla by splanchnic nerve or acetylcholine stimulation (Douglas and Poisner, 1966) and bovine chromaffin cells in response to K^+-induced membrane depolarization or nicotinic acetylcholine receptor stimulation (Castillo et al., 1992; Rojas et al., 1985; White et al., 1987). The addition of this nucleotide to media bathing bovine chromaffin cells can elevate intracellular Ca^{2+} concentrations by increasing Ca^{2+} influx across the cell membrane (Diverse-Pierluissi et al., 1991) or by stimulating inositol phosphate (InsP) formation (Kim and Westhead, 1989; Nakahata et al., 1986; Sasakawa et al., 1989; Zheng et al., 1997). ATP can also stimulate cyclic AMP (cAMP) accumulation in bovine chromaffin cells (Zhang et al., 2001). The ATP-stimulated increases in InsP and cAMP can be enhanced by the concurrent presence of neuropeptide Y (Zhang et al., 2001; Zheng et al., 1997). Bovine chromaffin cells have been shown to possess both P2X and P2Y receptor subtypes (Reichsman et al., 1995).

The effects of secreted neurotransmitters are terminated by either enzymatic degradation or reuptake into the nerve terminal. The effects of ATP have been shown to be decreased by the presence of ectonucleotidases (Cheung et al., 1992; Schwarzbaum et al., 1998; Todorov et al., 1997). Ligand-potency studies can be useful in the characterization of the P2Y receptor, a Gq-coupled receptor, mediating the effects of ATP including the InsP formation from phosphatidyl inositol in bovine chromaffin cells. However, since ectonucleotidase activity has previously been demonstrated in these cells (Torres et al., 1990), the rank order of potency of the various nucleotides could be altered by this activity. The nucleotidase activity in bovine chromaffin cells has been shown to consist of ecto-ATPase, ecto-ADPase and ecto-5′ -nucleotidase activities (Torres et al., 1990). ARL 67156 (FPL 67156, Fisons), an ATP analogue, is a selective inhibitor of ecto-ATPase (Crack et al., 1997). We examined the effectiveness of this ecto-ATPase inhibitor as a potential tool in our characterization of chromaffin cell P2Y receptor(s).

2. Materials and methods

2.1. Chromaffin cell culture

Isolation of bovine adrenal chromaffin cells was performed as previously described (Li et al., 2002). Cells were

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plated in 24-well plastic plates (5×10^5 cells/well) or in 35-mm dishes (3×10^6 cells/dish) in an atmosphere of 5% CO_2 at 37 °C. Viability and purity as determined by Trypan blue exclusion and neutral red staining, respectively, were greater than 95%. The medium was replaced every other day. Cells were used between 3 and 8 days after plating.

2.2. Ecto-ATPase assay

Culture media was removed from chromaffin cells plated on 24-well plastic plates and the cells washed with 0.5 ml of Krebs–Ringer–HEPES buffer (KRH) (pH 7.4) (37 °C) containing 25 mM HEPES, 125 mM NaCl, 1.2 mM $CaCl_2$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 5.6 mM glucose. To begin the assay, the KRH was removed and 0.5 ml KRH containing 3 mM ATP and 4 μCi γ-[^{33}P]ATP (3000 Ci/mmol) was added and incubated for 10 min except as noted. An aliquot (0.2 ml) of conditioned media was removed and added to an equal volume of 10% trichloroacetic acid. Inorganic phosphate (P_i) was determined using a modified Fiske–SubbaRow method (Schwarzbaum et al., 1998) where 0.67 ml of 350 mM H_2SO_4 containing 2 mM $Mo_7O_{24}(NH_4)_6$ was added to form [^{33}P]phosphomolybdic acid. The complex was extracted into isobutyl alcohol and phases separated by centrifugation for 5 min at $1000 \times g$ prior to the determination of ^{33}P by scintillation spectroscopy.

2.3. Inositol phosphate determination

Assays were essentially as described previously (Nakahata et al., 1986) with minor modifications. Cells plated on 35-mm dishes were labeled for 18 h (37°) with 2 μCi [^{3}H]inositol in 1 ml inositol-free high-glucose DMEM. After labeling, cells were rinsed twice with DMEM, 20 mM HEPES, pH 7.4, (DMEM–HEPES) prior to being subjected to further treatments, e.g., ATP stimulation for 20 min (37°). DMEM–HEPES was used to wash cells; LiCl (10 mM) was added to the DMEM–HEPES when it was used as the preincubation medium or the dissolving medium for the stimulating agents. Labeled compounds were then extracted into methanol using chloroform/methanol/H_2O (1:1:0.9). The resulting aqueous methanol phase was added to a Dowex 1-X8 (formate form) column and the inositol

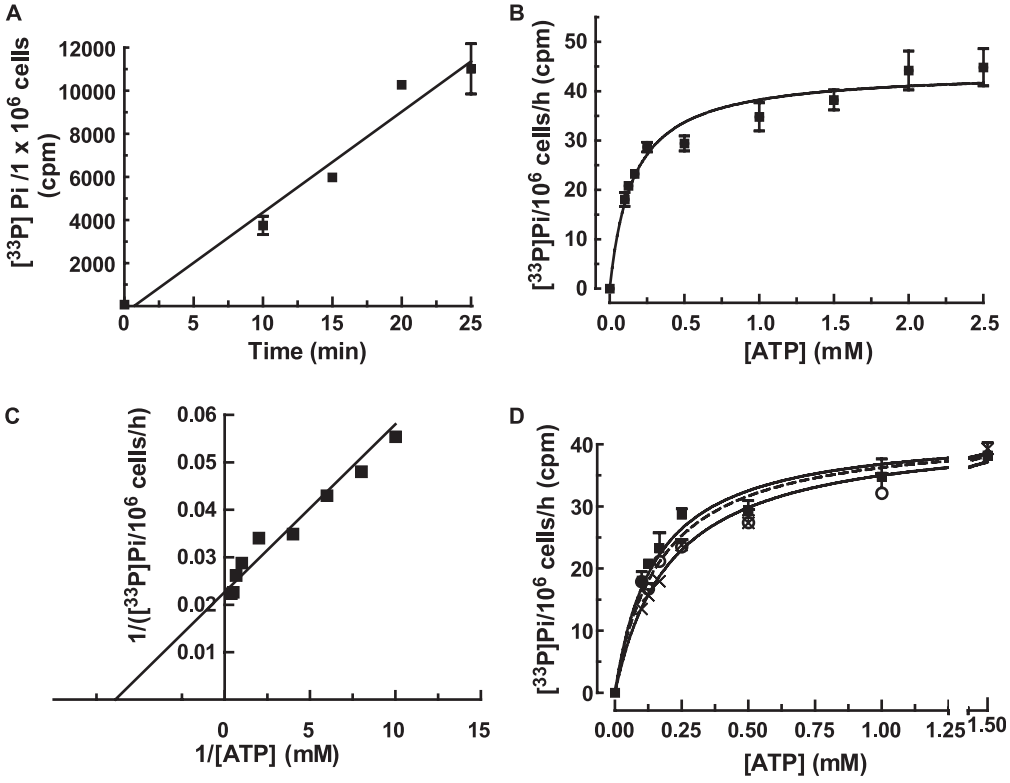

Fig. 1. Bovine chromaffin cells possess ecto-ATPase activity. In each case (A–D), the reaction was started by the addition of Na_2ATP and stopped by adding an aliquot of the conditioned media to 10% trichloroacetic acid and [^{33}P]P_i determined. (A) Time dependence of γ-[^{33}P]ATP hydrolysis ($R^2 = 0.9675$). Bovine chromaffin cells were incubated for various times with Na_2ATP (3 mM) containing 4 μCi γ-[^{33}P]ATP for 10 min. (B) Saturation curve for ATP ($R^2 = 0.9647$). Increasing concentrations of Na_2ATP containing γ-[^{33}P]ATP were added and the reaction continued at 37° for 10 min ($K_m = 1.57 \pm 0.27 \times 10^{-4}$ M). (C) Lineweaver–Burk plot of data in (B). (D) Effect of ARL 67156 on bovine chromaffin cell ecto-ATPase. Increasing concentrations of Na_2ATP containing γ-[^{33}P]ATP were added at various ARL 67156 concentrations, and the reaction continued at 37° for 10 min ($K_i = 2.55 \pm 1.36 \times 10^{-7}$ M). No ARL 67156, $R^2 = 0.9755$ (■-■); 30 nM ARL 67156, $R^2 = 0.9389$ (○-○); 100 nM ARL 67156, $R^2 = 0.9701$ (×-×). Each experiment was done in triplicate. Data were plotted using GraphPad Prism, version 4.

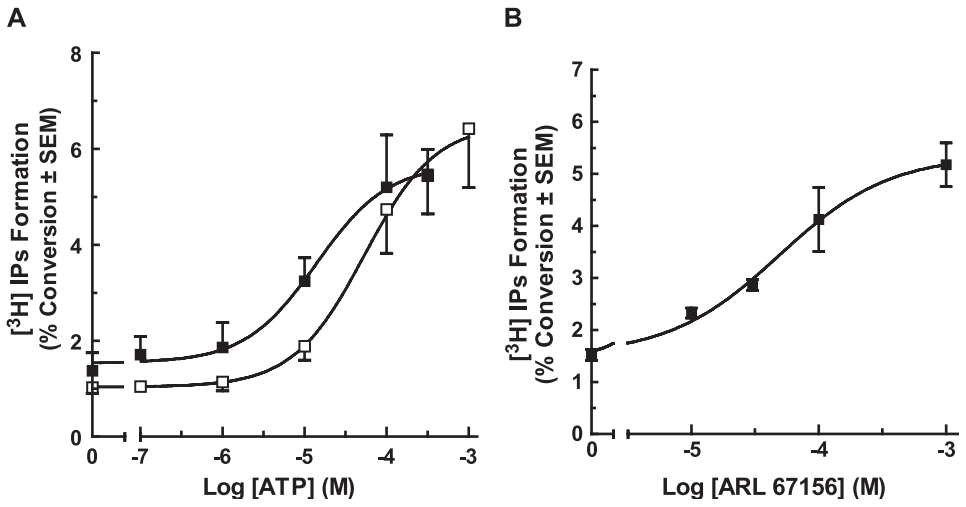

Fig. 2. ARL 67156 alters InsP formation in bovine chromaffin cells. Cells were loaded with [^{3}H]inositol (18 h, 37 °C) and incubated with increasing concentrations of agonist for 20 min at 37 °C. Labeled compounds were extracted, separated and the radioactivity determined. The percent conversion of inositol phospholipids (b) to inositol phosphates (a) was calculated using the formula $a/(a+b)\times 100$. Each point is the average of three experiments with triplicate determinations. Data were plotted using GraphPad Prism, version 3. (A) Concentration–response curve for ATP stimulation of InsP formation in the absence (□-□) and presence (■-■) of ARL 67156 (1×10^{-7} M). (B) Concentration–response curve for ARL 67156 stimulation of InsP formation.

phosphates eluted with 8 ml of 1 M ammonium formate containing 0.1 M formic acid. Radioactivity in a 3-ml aliquot (8 ml total volume) of the eluate (a) and a 0.375-ml aliquot (1 ml total volume) of the organic phase containing the inositol phospholipids (b) were determined by liquid scintillation counting. The percent conversion of inositol phospholipids to inositol phosphates was calculated by the formula $a/(a+b)\times 100$.

2.4. Materials

γ-[^{33}P]ATP was purchased from ICN. [^{3}H] *myo*-inositol and inositol-free DMEM (Dulbecco's Modified Eagle's Medium) were purchased from ICN Biomedicals, Irvine, CA. All other chemicals including ARL 67156 (6-*N*,*N*-diethyl-beta-gamma-dibromomethylene-D-adenosine-5-triphosphate) were purchased from Sigma, St. Louis, MO.

2.5. Statistical analysis

Data were analyzed using GraphPad Prism, versions 3.02 and 4.00.

3. Results

The hydrolysis of γ-[^{33}P]ATP by bovine chromaffin cells is linear with time up to 25 min (Fig. 1A). Initial velocity studies with increasing substrate concentrations showed ATP to be saturating at 2 mM (Fig. 1B). A Lineweaver–Burk plot of the data yielded a K_m for ATP of $1.57\pm0.27\times10^{-4}$ M (Fig. 1C). The inclusion of the ecto-ATPase inhibitor, ARL 67156, in the initial velocity studies yielded a competitive inhibition pattern (constant V_{max}) with a K_i for ARL 67156 of $2.55\pm1.36\times10^{-7}$ M (Fig. 1D).

The usefulness of ARL 67156 as an ecto-ATPase inhibitor in ligand-potency studies designed to characterize the P2Y receptor depends on whether this compound (an ATP analog) is a potent agonist or antagonist. ARL 67156 (1×10^{-7} M) produced a left shift in the concentration–response curve for ATP stimulation of InsP formation ($EC_{50}=1.39\times10^{-5}$ M with ARL 67156 compared to 3.94×10^{-5} M without ARL 67156) (Fig. 2A). A 100-fold higher concentration of ARL 67156 (1×10^{-5} M) produced the same shift in the concentration curve for ATP as did the lower concentration of ARL 67156 ($EC_{50}=1.10\times10^{-5}$ M, data not shown). However, increasing concentrations of ARL 67156 alone produced a concentration-dependent increase in InsP formation ($EC_{50}=4.9\times10^{-5}$ M) (Fig. 2B). The phospholipase C inhibitor, U73122, completely antagonized ARL 67156 stimulated InsP formation (data not shown).

4. Discussion

Bovine chromaffin cells contain ecto-ATPase, ecto-ADPase and ecto-5′ -nucleotidase activities (Torres et al., 1990). Our data reveal a K_m for ATP of $1.57\pm0.27\times10^{-4}$ M which is in good agreement with that reported previously ($K_m=2.50\times10^{-4}$ M) for the bovine chromaffin cell ecto-ATPase (Torres et al., 1990). This activity can potentially confound data from ligand affinity studies with purines due to their degradation. However, ARL 67156, an ATP analog, has been reported to be a novel and selective inhibitor of ecto-ATPase (Crack et al., 1997). Varying ATP concentrations in the presence of two ARL

67156 concentrations showed that ARL 67156 is a competitive inhibitor of ecto-ATPase ($K_i = 2.55 \pm 1.36 \times 10^{-7}$ M). Studies with a rabbit ear artery preparation yielded a dissociation constant (pK_I) of 5.2 for ARL 67156 as an ecto-ATPase inhibitor (Crack et al., 1997). Therefore, ARL 67156 can be a useful inhibitor with which to examine the potency of various ligands on P2Y receptor function if nucleotide degradation is occurring.

The inclusion of ARL 67156 (1×10^{-7} M) in a study of InsP formation with increasing ATP concentrations produced a left shift in the concentration–response curve (an apparent threefold increase in ATP potency). Increasing the ARL 67156 concentration to saturation levels did not increase the apparent potency of ATP further. This suggests that a small amount of ATP in the culture medium is being degraded by the ecto-ATPase. The low rate of degradation is consistent with the low affinity of the ecto-ATPase for ATP ($K_m = 1.57 \pm 0.27 \times 10^{-4}$ M), i.e., relatively large concentrations of ATP are required to reach the half-maximal velocity of the enzyme. Thus, ecto-ATPase activity does not produce any significant nucleotide degradation during ligand-potency studies with bovine chromaffin cells. Interestingly, when ARL 67156 alone was examined for its ability to stimulate InsP formation, it was as effective as ATP ($EC_{50} = 1.2 \times 10^{-5}$ M, Zheng et al., 1997) with an EC_{50} of 4.9×10^{-5} M. However, since ARL 67156 has greater than a 300-fold selectivity as an enzyme inhibitor versus affinity for the receptor, it provides a useful tool in studies where nucleotide degradation by ecto-ATPase activity can confound data interpretation.

In conclusion, bovine chromaffin cells contain a low affinity ecto-ATPase activity that hydrolyzes ATP present in culture medium. ARL 67156 is an effective inhibitor of this enzyme.

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