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ATP stimulates secretion in human neutrophils and HL60 cells via a pertussis toxin-sensitive guanine nucleotide-binding protein coupled to phospholipase C

Shamshad Cockcroft and Jane Stutchfield

Department of Experimental Pathology, School of Medicine, University College London, University Street, London WC1E 6JJ, England

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Human neutrophils and HL60 cells respond to extracellular ATP by causing exocytotic secretion. Secretion is accompanied by increases in inositol phosphates and a rise in cytosol Ca^{2+}. The responses to ATP are blocked by pertussis toxin pretreatment, indicating the involvement of a guanine nucleotide regulatory protein. Other nucleotides that are active in promoting secretion are ATPγS, UTP, ITP and AppNHp, whilst 8-bromo-ATP, AppCH_2p, ADP, AMP and adenosine are inactive.

ATP receptor; Ca^{2+}, cytosolic; Inositol phosphate; fMetLeuPhe; (HL60 cell, Human neutrophil)

1. INTRODUCTION

Human neutrophils and differentiated HL60 cells respond to a variety of soluble stimuli including fMetLeuPhe, platelet-activating factor, leukotriene B_4 and C5a. The best studied of these agonists is fMetLeuPhe which is known to stimulate a number of functional responses including secretion of azurophilic granules and specific granules. These physiological events are accompanied by hydrolysis of phosphatidylinositol bisphosphate and the consequent generation of inositol phosphates and a rise in cytosol Ca^{2+} [1].

It is now well-established that the inositol lipid-specific phospholipase C is coupled to the fMetLeuPhe receptor via a pertussis toxin-sensitive guanine nucleotide regulatory protein [2]. During our studies on the coupling of the fMetLeuPhe receptor to phospholipase C in HL60 membranes, we were surprised to observe that GTP alone between 1 and 100 μM was effective at stimulating phospholipase C activity. (In intact cells the level of GTP is approx. 500 μM and it is receptor occupation that normally catalyzes the exchange of bound GDP with GTP on the G-protein leading to the activation of the catalytic unit.) This result suggested to us that there may be a receptor-directed agonist present in our incubations that is capable of coupling to the phospholipase C. Mg-ATP was routinely present in our incubations to maintain the inositol lipids in the phosphorylated state and thus we considered the possibility that HL60 cells possess a receptor for ATP.

We report here that HL60 cells and also human neutrophils respond to externally applied ATP leading to increases in cytosol Ca^{2+} and inositol phosphates and exocytotic secretion.

Correspondence address: S. Cockcroft, Department of Experimental Pathology, School of Medicine, University College London, University Street, London WC1E 6JJ, England

Abbreviations: ATPγS, adenosine 5'-[γ-thio]triphosphate; AppNHp, adenosine 5'-[β,γ-imido]triphosphate; AppCH_2p, adenosine 5'-[β,γ-methylene]triphosphate; G-protein, guanine nucleotide-binding protein

2. MATERIALS AND METHODS

The sources of all nucleotides and pertussis toxin have been described [3,4].

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Human neutrophils and HL60 cells (differentiated with dibutyryl cyclic AMP) were obtained as in [3–5]. For measurement of inositol phosphates, HL60 cells were prelabelled with [^{3}H]inositol as in [3]. Cells were washed 3 times in a buffered salt solution (pH 7.2) comprising 20 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml bovine albumin and 5.6 mM glucose and then resuspended at 10^7 cells/ml. The buffer was supplemented with 10 mM LiCl when inositol phosphates were measured. After addition of cytochalasin B (5 μg/ml final), 100-μl aliquots of cells were transferred to tubes containing an equal volume of nucleotides or as indicated and the tubes incubated for 10 min at 37°C. At the end of incubation, the cells were sedimented by centrifugation at 600 × g and the supernatants sampled for β-glucuronidase and lysozyme as described [6]. For determination of inositol phosphates, the sedimented cells were quenched with chloroform/methanol and processed as in [4]. Pretreatment with pertussis toxin (500 ng/ml) was carried out for 2 h at 37°C where indicated.

For the measurement of levels of cytosol Ca^{2+}, cells (5×10^7 cells/ml) were loaded at room temperature with fura2-AM (10 μM). After 20 min the suspension was diluted 5-fold and left to recover for a further 40 min at room temperature. After centrifugation, the cells were resuspended at 10^7 cells/ml and kept at room temperature until use. Fluorescence was measured using a Perkin Elmer LS5 fluorimeter with excitation at 340 nm and emission at 510 nm. The cuvette was continuously stirred and maintained at 37°C during measurements. Calibration of the fluorescence signals was carried out as described previously [7].

3. RESULTS AND DISCUSSION

We initially examined the effect of extracellular ATP on secretion of β-glucuronidase (marker for azurophilic granules) and inositol phosphate production from dibutyryl cyclic AMP-differentiated HL60 cells (fig.1a,b). ATP stimulates secretion and inositol phosphate production in a concentration-dependent manner, with the half-maximal response being obtained at approx. 5 μM. Human neutrophils also respond to ATP and in this case, secretion of both β-glucuronidase and lysozyme (marker for specific granules) is evident (fig.1c,d). Although the concentration dependence for secretion is the same for both granule types (EC_{50} 5 μM), the extent of secretion of lysozyme is greater than that of β-glucuronidase. Compared to fMetLeuPhe, ATP is a less potent agonist for secretory function in both HL60 cells and neutrophils (table 1). However, ATPγS is comparable to fMetLeuPhe in promoting secretion from neutrophils (table 1).

It is well established that ADP ribosylation of the G-protein by pertussis toxin, in neutrophils and HL60 cells, uncouples the fMetLeuPhe receptor from phospholipase C leading to the inhibition of many of the neutrophil responses such as secretion, inositol phosphate formation and Ca^{2+} mobilization [8,9]. This is also the case for all the responses stimulated by ATP when these cells are pretreated with pertussis toxin for 2 h. Fig.1a–d illustrates that both secretion and formation of inositol phosphates stimulated by ATP are inhibited by pertussis toxin pretreatment.

The stimulation of inositol phosphate formation is normally associated with a rise in cytosol Ca^{2+} [10] and we therefore measured changes in intracellular Ca^{2+} caused by ATP using Fura-2 as the intracellular indicator (fig.2a–c). ATP causes a rise in cytosol Ca^{2+} in a concentration-dependent manner in HL60 cells (fig.2a,b) and neutrophils (fig.2c). The minimum concentration of ATP that gives a signal is 10^{-8} M for HL60 cells and 10^{-7} M for neutrophils. The initial rise in cytosol Ca^{2+} was also observed when extracellular Ca^{2+} was chelated with EGTA (not shown), indicating that the Ca^{2+} was mobilized from intracellular stores.

Since ATP is a poor agonist for stimulating secretion compared to fMetLeuPhe (see table 1), we also compared the rise in cytosol Ca^{2+} with both agonists. In ATP-stimulated HL60 cells, Ca^{2+} levels rose from a basal value of 100 nM to 3 μM within seconds, an increase that was comparable to that seen with fMetLeuPhe (fig.2b). As with fMetLeuPhe, the Ca^{2+} levels declined and plateaued at a new steady state. A similar pattern was observed with neutrophils but the maximal increase in Ca^{2+} achieved by ATP or fMetLeuPhe was in the region 600–700 nM (fig.2c). The low level of secretion caused by ATP, despite a rise in cytosol Ca^{2+} equivalent to that stimulated by fMetLeuPhe, may be due to lack of activation of additional signalling pathways such as phospholipase A_2 or D that are known to be stimulated by fMetLeuPhe [1,5,11]. That Ca^{2+} is not a sufficient signal for exocytosis in HL60 cells has been demonstrated previously [3] and is further reinforced here.

We next examined the specificity of the receptor to interact with other nucleotides. UTP, ITP and ATPγS and AppNHp were all effective in stimulating secretion (table 1). Others that were tested (highest concentration: 100 μM) and gave no stimulation of secretion were: $AppCH_2p$,

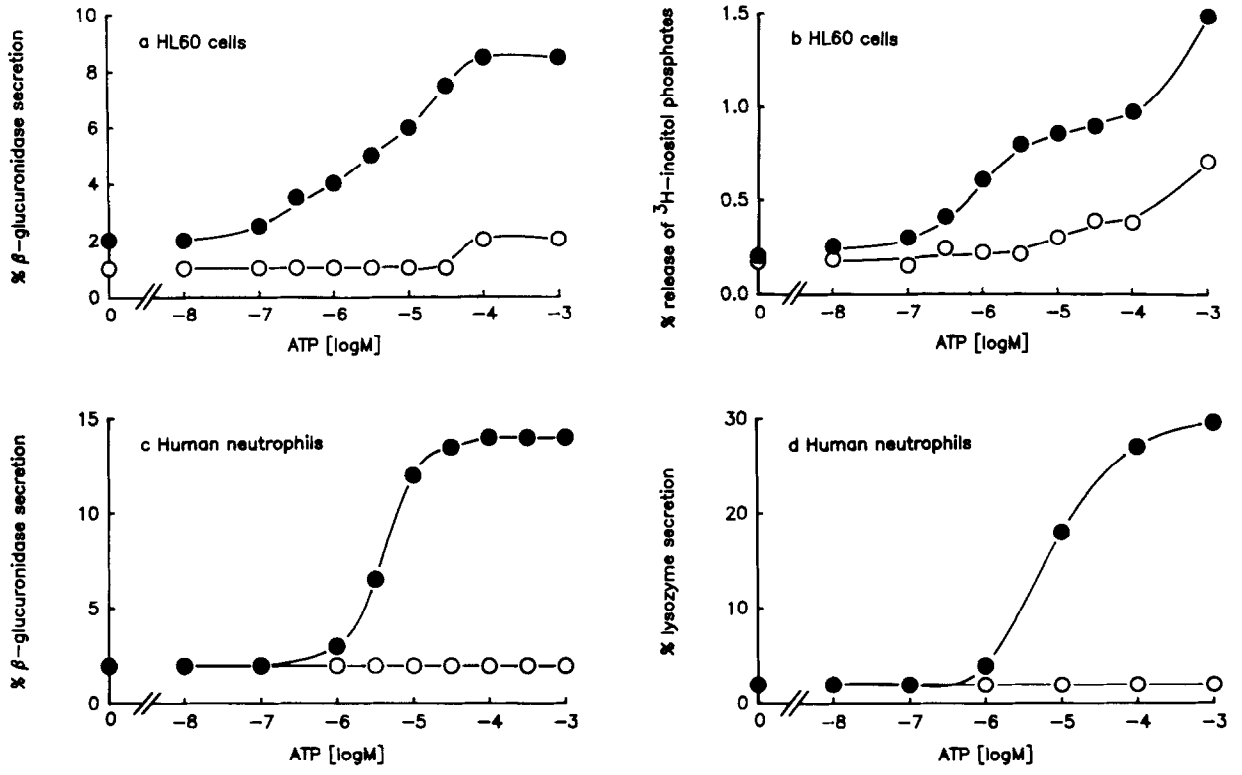

Fig.1. Effect of ATP on (a) β-glucuronidase secretion and (b) inositol phosphate production in differentiated HL60 cells and (c) β-glucuronidase secretion and (d) lysozyme secretion in human neutrophils: effect of pertussis toxin pretreatment. (•) Control cells, (○) cells pretreated with pertussis toxin. The increase in inositol phosphates is expressed as a percentage of dpm incorporated into total inositol lipids which was 1.6×10^5 in this experiment. Similar results were observed on three other occasions.

Table 1

% β-Glucuronidase secretion

Agonist	Concentration (μM)	HL60 cells	Human neutrophils
Control	0	1.5 ± 0.1 (20)	2.1 ± 0.3 (10)
ATP	100	11.6 ± 1.1 (20)	10.2 ± 1.6 (10)
UTP	100	16.1 ± 2.1 (8)	5.8 ± 1.1 (6)
ITP	100	8.0 ± 1.1 (7)	4.6 ± 1.0 (5)
ATPγS	100	6.1 ± 0.9 (7)	28.5 ± 1.8 (8)
AppNHp	100	3.7 ± 0.6 (9)	3.8 ± 0.8 (5)
$AppCH_2p$	100	1.7 ± 0.2 (5)	2.2 ± 0.6 (4)
8-Bromo-ATP	100	1.8 ± 0.4 (7)	2.4 ± 0.5 (5)
ADP	100	1.7 ± 0.2 (5)	2.2 ± 0.6 (4)
AMP	100	1.7 ± 0.2 (5)	2.2 ± 0.6 (4)
Adenosine	100	1.7 ± 0.2 (5)	2.2 ± 0.6 (4)
fMetLeuPhe	0.1	29.6 ± 1.3 (14)	32.4 ± 2.8 (9)

Nucleotide specificity of the ATP receptor in HL60 cells and human neutrophils. Results are presented as % secretion and are means ± SE (number of experiments in parentheses)

8-bromo-ATP, ADP, AMP and adenosine. Although the same agonist specificity was observed for HL60 cells and human neutrophils, the rank order of potency was different. For HL60 cells, the order is UTP > ATP > ITP > ATPγS > AppNHp whilst for neutrophils, it is ATPγS > ATP > UTP > ITP > AppNHp.

These differences in the rank order of potency suggest that the putative receptor in these two cell types may not be identical. This nucleotide specificity also suggests that this receptor may not fall into the purinergic classification of P_1 and P_2 [12,13] but may belong to a new class of ATP receptors. The observation that UTP, a pyrimidine, is also active, would suggest that this receptor cannot be simply classified as a purinergic receptor. Since UTP has not been used in many studies, it is not clear whether the effect of UTP is confined to this cell type. However, in Ehrlich

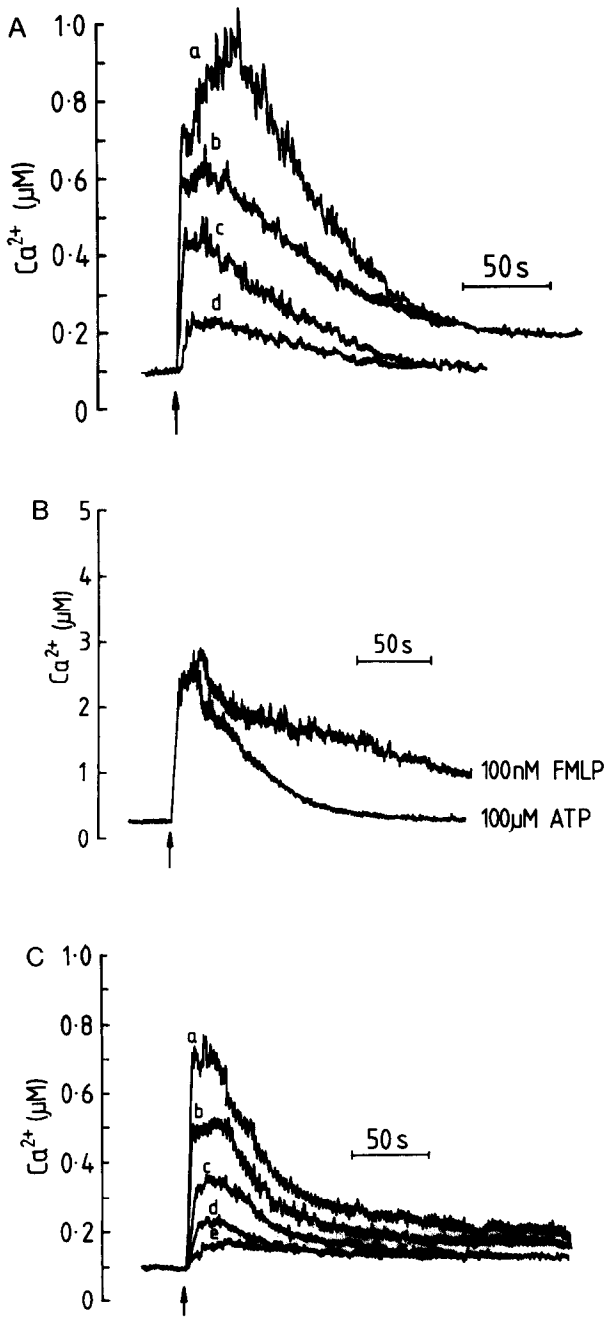

Fig.2. Changes in cytosol [Ca^{2+}] induced by ATP in (A) HL60 cells. a–d denote concentrations of ATP: a, 10^{-5}; b, 10^{-6}; c, 10^{-7}; d, 10^{-8} M. (B) HL60 cells. Comparison between ATP (10^{-4} M) and fMetLeuPhe (10^{-7} M). (C) Human neutrophils. a, fMetLeuPhe 10^{-7} M; b–e denote concentrations of ATP: b, 10^{-4}; c, 10^{-5}; d, 10^{-6}; e, 10^{-7} M. Similar results were observed on three other occasions.

ascites tumour cells, UTP was also found to be an agonist [14].

The presence of the ATP receptor on cells that are involved in host defense suggests that the physiological role for this receptor may be related to inflammation. ATP is stored in the granules of platelets, adrenal medulla and some neuronal cells and this can be discharged by secretion [15]. Since levels of ATP in plasma can easily rise to 20 μM following platelet activation [15], neutrophils are likely to be involved in haemostasis and also in pathological conditions such as thrombosis. In a recent study in human neutrophils, it has been shown that ATP, ITP or UTP although unable to stimulate O_2^- generation enhanced the response to fMetLeuPhe. ATP was also shown to cause a transient elevation of Ca^{2+} which was interpreted as being due to stimulated Ca^{2+} uptake rather than mobilization of intracellular stores [16].

Our results clearly demonstrate the presence of a receptor for ATP in human neutrophils and HL60 cells which is coupled to phospholipase C via a pertussis toxin-sensitive G-protein. Activation of the receptor results in increases in inositol trisphosphate which is most likely responsible for the rise in cytosol Ca^{2+}. Similar ATP receptors coupled to phospholipase C have been described recently in 4 other cell types, Ehrlich ascites tumour cells [14,17], turkey erythrocytes [18], hepatocytes [19] and FRTL-5 cells [20], although the pharmacological specificity is different in most cases as is the pertussis toxin sensitivity.

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