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Extracellular ATP itself elicits superoxide generation in guinea pig peritoneal macrophages

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Extracellular ATP itself elicited the generation of superoxide (O_2^-) in guinea pig peritoneal macrophages associated with an increase in cytosolic calcium ($[Ca^{2+}]_i$). The ATP-induced O_2^- generation was completely inhibited by pretreatment with pertussis toxin (PT) accompanied by the suppression of $[Ca^{2+}]_i$ mobilization. Pre-exposure to a small amount of phorbol myristate acetate (PMA) primed the ATP-induced generation of O_2^- without a change of $[Ca^{2+}]_i$. The results suggest that ATP-induced O_2^- generation is mediated by $[Ca^{2+}]_i$ mobilization and by PT-sensitive G protein.

Macrophage; ATP receptor; Superoxide generation; $[Ca^{2+}]_i$ mobilization

1. INTRODUCTION

A variety of actions of extracellular adenosine triphosphate (ATP) have been described on lymphocytes [1,2], neutrophils [3,4], macrophages [5,6], vascular endothelial cells [7], type II pneumocytes [8], mast cells [9] and HL60 human promyelocytic leukemia cells [10]. It is thought that these effects of ATP are elicited via a P_2 purinergic receptor distinct from the P_1 receptor [11].

Kuroki and Minakami [12] recently reported that, in the presence of cytochalasin B, ATP can trigger the generation of superoxide anion (O_2^-) by human neutrophils. Theirs was the first report to describe a direct effect of extracellular ATP on O_2^- generation. However, no information was reported on the effect of ATP on macrophages.

This paper describes the effects of extracellular ATP on the generation of O_2^- by macrophages from the peritoneal exudate of guinea pigs. We showed that extracellular ATP itself, could elicit O_2^- production which was mediated by the mobilization of cytosolic calcium ($[Ca^{2+}]_i$) and by pertussis toxin (PT)-sensitive G protein.

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Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; AMPPCP, adenosine, β, γ-methyleneadenosine 5′-triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); GTP, guanine triphosphate; CTP, cytosine triphosphate; fura-2/AM, fura-2 acetoxymethyl ester; Cyt *c*, ferricytochrome *c*; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase; FCS, fetal calf serum; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; $[Ca^{2+}]_i$, intracellular calcium; O_2^-, superoxide anion

2. MATERIALS AND METHODS

2.1. *Reagents*

Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, β, γ-methyleneadenosine 5′-triphosphate (AMPPCP), adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), guanine triphosphate (GTP), cytosine triphosphate (CTP), fura-2 acetoxymethyl ester (fura-2/AM), ferricytochrome *c* (Cyt *c*) from horse heart (type IV), phorbol 12-myristate 13-acetate (PMA) and superoxide dismutase (SOD) were obtained from Sigma Chemical Company (St. Louis, MO). RPMI1640 medium and Hank's balanced salt solution (HBSS) were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Heat-inactivated fetal calf serum (FCS) was obtained from Gibco Laboratories (Grand Island, NY). Pertussis toxin (PT) was from Seikagaku Kogyou Ltd. (Tokyo, Japan). HEPES buffer was prepared from 20 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM dextrose (pH 7.3).

2.2. *Animals*

Hartley guinea pigs weighing 300–500 g were used in these experiments.

2.3. *Preparation of cells*

Macrophages were collected from the peritoneal exudate of guinea pigs 4–7 days after the intraperitoneal injection of 20 ml liquid paraffin as previously described [13]. The purity of the macrophages exceeded 95%.

2.4. *Determination of superoxide generation*

O_2^- was detected as described previously [13]. Briefly, 1 ml of the reaction mixture containing 100 μM Cyt *c* and 1×10^6 cells in 1 ml of HEPES buffer was preincubated in a plastic cuvette for spectrophotometric analysis at 37°C for 5 min. ATP or another nucleotide was then added to the reaction mixture and the rate of SOD-inhibitable reduction of Cyt *c* was measured continuously by recor-

ding the increase in absorption at 550–540 nm using Hitachi 556 double beam spectrophotometer.

2.5. *Determination of cytosolic calcium ($[Ca^{2+}]_i$) mobilization*

Cells were suspended in RPMI1640 medium with 10% FCS and incubated with 5 μM fura-2/AM for 30 min at 37°C as previously described [14]. The cells were washed and resuspended in HEPES buffer (1×10^6 cells/ml). Fluorescence was measured with the Shimazu RF-5000 Intracellular Calcium Measurement System (Tokyo, Japan), in a stirred plastic cuvette maintained at 37°C. The excitation wavelengths were 335 nm and 362 nm and the emission wavelength was 500 nm. $[Ca^{2+}]_i$ was calculated from the following equation: $[Ca^{2+}]_i = K_d(F - F_{min})/F_{max} - F)$, where F was the fluorescence intensity of the dye in the cells and F_{max} and F_{min} the intensities at saturating and zero concentrations of calcium, respectively. The dissociation constant (K_d) of fura-2 was assumed to be 224 nM. F_{max} and F_{min} were determined by the addition of 0.1% triton X-100 and 6.6 mM EGTA, respectively.

2.6. *Treatment of cells with PMA or PT*

In the assay for O_2^- or $[Ca^{2+}]_i$, 100 pg/ml of PMA, which itself did not generate O_2^- or change the $[Ca^{2+}]_i$, was added to the cell suspension 1 min before stimulation with ATP. One μM of PT was pre-exposed to the cell suspension in RPMI1640 containing 10% FCS for 90 min at 37°C. This was followed by two washes and resuspension in HEPES buffer for O_2^- assay, or followed by resuspension in RPMI1640 and loading with fura-2/AM for $[Ca^{2+}]_i$ assay.

3. RESULTS

3.1. O_2^- generation with ATP

We studied the effect of ATP on the generation of O_2^- by macrophages from the peritoneal exudate of guinea pigs. One mM of ATP itself induced O_2^- generation in the absence of other stimulants. Fig. 1 shows the representative time course of ATP-induced O_2^- generation. The response to ATP was characterized by a prompt increase in O_2^- generation with no lag time following the addition of ATP, and by the rapid termination of O_2^- generation within 2–3 min, as reported with human neutrophils [12]. The complete inhibition of O_2^- generation was obtained by adding 180 U/ml superoxide dismutase (Fig. 1). The responses obtained with various concentrations of ATP are demonstrated in Fig. 2. ATP induced the generation of

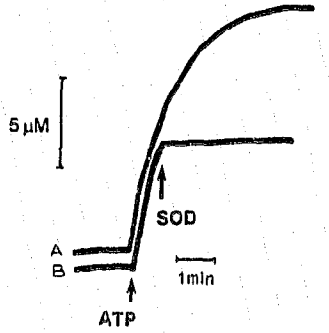

Fig. 1. ATP-induced O_2^- generation by guinea pig peritoneal macrophages. (A) Guinea pig macrophages (1×10^6 cells/ml) were incubated with 100 μM Cyt *c* for 5 min and were then stimulated by 1 mM ATP. (B) SOD (180 U/ml) was added to the reacting cells following challenge with ATP.

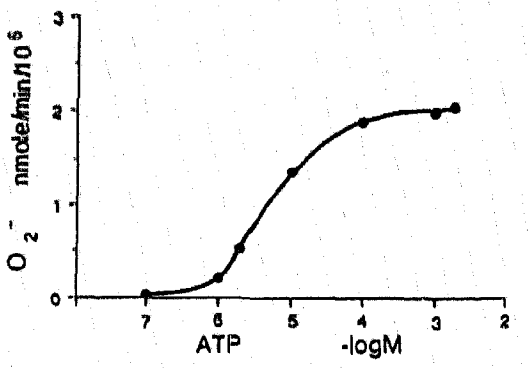

Fig. 2. Dose-dependent effect of extracellular ATP on O_2^- generation in guinea pig macrophages. Guinea pig macrophages were stimulated by various concentrations of ATP under the conditions described in Section 2. The points give a mean value from triplicated assays performed on a single preparation of cells.

O_2^- by the peritoneal macrophages in a dose-dependent manner. A maximal response was observed with 1 mM ATP. The response obtained with various nucleotides, including non-hydrolysable analogues of ATP, was examined on peritoneal macrophages (Table I). ATP and ADP induced the generation of O_2^- by the macrophages, whereas AMP and adenosine did not. These potency characteristics indicated P_2 purinergic receptor on the guinea pig peritoneal macrophages, although ADP was more potent than ATP. ATPγS, a non-hydrolysable analogue of ATP, induced O_2^- generation much like ATP or ADP. GTP had a small effect but CTP showed almost none.

3.2. *Detection of $[Ca^{2+}]_i$ increase by ATP*

We examined the effects of ATP on the mobilization of $[Ca^{2+}]_i$ in peritoneal macrophages. The addition of 1 mM ATP to macrophages loaded with fura-2 produced a rapid but transient increase from a basal level of 101 mM to a peak of 300 nM within 10 s. $[Ca^{2+}]_i$ returned to the basal level after 2–3 min. The maximal response was observed with 1 mM ATP, a concentration comparable to the optimal concentration for O_2^- generation (Fig. 3).

Table I

Production of O_2^- by different nucleotides

Nucleotide	Concentration	*n*	O_2^- generation
PMA	10 ng/ml	3	2.52 ± 0.19
ATP	1 mM	6	0.80 ± 0.06
ATPγS	1 mM	6	1.85 ± 0.16
AMPPCP	1 mM	2	0.03 ± 0.02
ADP	1 mM	6	1.62 ± 0.13
AMP	1 mM	4	0.04 ± 0.01
Adenosine	100 μM	4	0.02 ± 0.01
GTP	1 mM	6	0.24 ± 0.05
CTP	1 mM	5	0.08 ± 0.03

O_2^- generation (nmol/min/1×10^6 cells) induced by various nucleotides was measured as in section 2. Data represent mean ± SD.

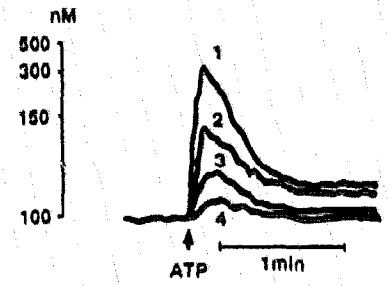

Fig. 3. Effect of ATP concentration on the increase in $[Ca^{2+}]_i$ by guinea pig peritoneal macrophages. Fura-2 loaded guinea pig macrophages (1×10^6 cells/ml) were challenged by 1 mM (1), 100 μM (2), 1 μM (3) and 100 nM (4) ATP.

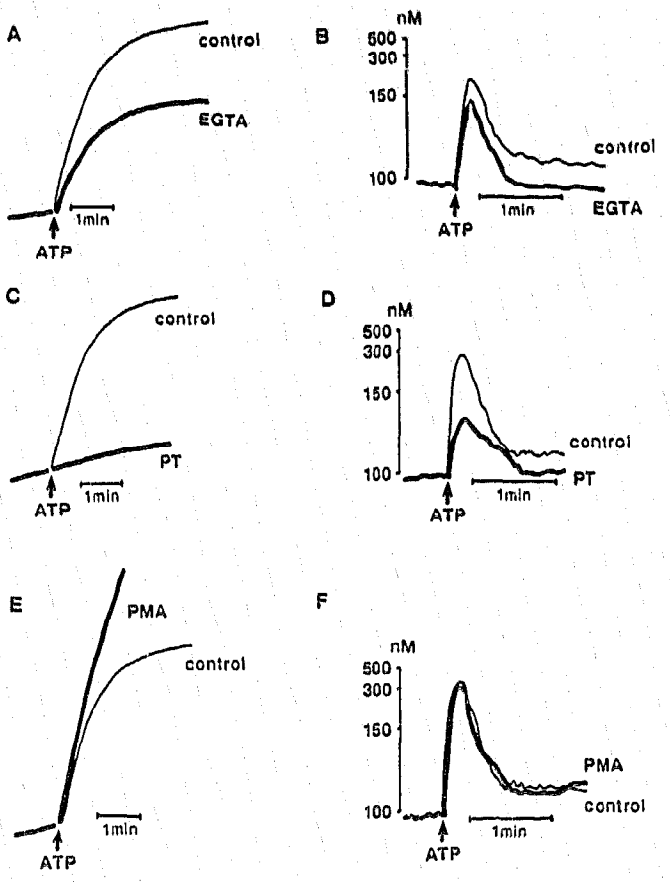

Fig. 4. Effects of EGTA, pertussis toxin and a low dose of PMA on O_2^- generation and $[Ca^{2+}]_i$ increase in guinea pig macrophages. Guinea pig macrophages were prepared as described in Section 2 for O_2^- (A,C,E) and $[Ca^{2+}]_i$ (B,D and F) assays. (A,B) EGTA (6.6 mM) was added to the cell aliquots 1 min before ATP (1 mM) challenge. (C,D) Guinea pig macrophages were pretreated with or without PT (1 μg/ml) in RPMI1640 containing 10% FCS for 90 min. Cells were then incubated in HEPES buffer with Cyt *c* (100 μM) for O_2^- assay (C), or loaded with fura 2 (5 μM) for 30 min and resuspended in HEPES for $[Ca^{2+}]_i$ assay (D). One mM of ATP was then added as a challenge to cell aliquots. (E,F) A low dose (100 pg/ml) of PMA, which itself did not elicit O_2^- production or $[Ca^{2+}]_i$ increase, was added to cell aliquots 1 min prior to the ATP (1 mM) challenge.

3.3. *Effect of EGTA on O_2^- generation and $[Ca^{2+}]_i$ increase*

If extracellular divalent ions were chelated by 6.6 mM EGTA, the generation of O_2^- was remarkably inhibited (Fig. 4A), but the initial increase of $[Ca^{2+}]_i$ was not influenced except for a faster return to the basal level (Fig. 4B). These results indicated that O_2^- generation was partially dependent on the influx of calcium from the extracellular matrix in agreement with a previous report [12].

3.4. *Effect of PT on O_2^- generation and $[Ca^{2+}]_i$ increase*

The ATP-induced generation of O_2^- was completely inhibited by preincubation with 1 μg/ml PT for 90 min (Fig. 4C). This ATP-induced $[Ca^{2+}]_i$ increase was incompletely suppressed, both in the initial phase and in a second phase, by pretreatment with PT (Fig. 4D).

3.5. *Effect of PMA on O_2^- generation and $[Ca^{2+}]_i$ increase*

When macrophages were pretreated with a small amount of PMA (100 pg/ml) for only 1 min and then stimulated with ATP, the rapid termination of the ATP-induced O_2^- generation was abolished. However, the rate of the initial increase of O_2^- generation did not differ significantly from that of the control cells (Fig. 4E). The increase in $[Ca^{2+}]_i$ elicited by ATP in the PMA-pretreated macrophages had the same pattern and magnitude as the control cells (Fig. 4F).

4. DISCUSSION

Previous studies have shown that extracellular ATP primed human neutrophils for the O_2^- generation induced by *N*-formyl-Met-Leu-Phe (fMLP) [4], and that ATP itself stimulated O_2^- generation on cytochalasin B-treated human neutrophils [12]. However, there had been no report on ATP-induced O_2^- generation by macrophages. In this study, we showed that extracellular ATP could elicit the generation of O_2^- and the mobilization of $[Ca^{2+}]_i$ by macrophages obtained from guinea pig peritoneal exudate.

The effective concentration of ATP on human neutrophils was 2 μM in the study by Kuhns et al. [4] and 10 μM in that by Kuroki et al. [12]. In our study on macrophages obtained from the guinea pig peritoneal cavity, the maximal effect on O_2^- generation was obtained with 1 mM ATP (Fig. 2). We determined in the other experiments that 10 μM ATP was optimal for generation of O_2^- on 10% casein-induced peritoneal neutrophils of the guinea pig, and that cytochalasin B had no effect on the ATP (1 mM)-induced O_2^- generation on macrophages (data not shown). These data indicate that the affinity of the P_2 purinergic receptor on macrophages to ATP may differ from that of neutrophils.

Treatment with EGTA, which inhibited the influx of Ca^{2+} due to the deletion of extracellular calcium (Fig. 4B), did not completely suppress O_2^- generation (Fig. 4A). On the other hand, pretreatment with PT suppressed both components of $[Ca^{2+}]_i$ increase and completely inhibited O_2^- generation (Fig. 4E,F). These results suggest that O_2^- generation depends more on the release of calcium from the intracellular stores than on the influx of calcium from the extracellular matrix, although O_2^- generation might not simply depend on the $[Ca^{2+}]_i$ increase [15]. We also confirmed the previous findings that the receptors for ATP were coupled to phospholipase C via pertussis toxin-sensitive G-protein on human neutrophils and HL60 human promyelocytic leukemia cell line [4,16].

It has previously been thought that a low concentration of PMA (under 10^{-9} M) did not induce O_2^- generation and the stable translocation of cytosolic protein kinase C (PKC) to the membrane [17,18,19], although a high concentration of PMA could elicit these reactions [18,20]. On the other hand, such low concentrations of PMA have a priming effect on the O_2^- generation induced by WGA, immune complex or fMLP in guinea pig phagocytes [13] and human neutrophils [20]. Our results also indicate that a low concentration of PMA primed the generation of O_2^- induced by ATP on guinea pig peritoneal macrophages with no effect on $[Ca^{2+}]_i$ increase, although further studies concerning the priming effect of PMA are required. A few publications have reported the stimulation of O_2^- by ATP in human neutrophils [4,12] but not in macrophages. However, the neutrophils required a priming stimulus such as cytochalasin B [12]. In contrast, guinea pig macrophages required no stimulation or priming of the ATP-induced generation of O_2^-. The role of extracellular ATP on phagocytic cells is presently unclear. Platelets and vascular endothelial cells, which are in contact with the phagocytes in the blood vessel, can secrete ATP into the plasma. ATP secreted by these cells which have been aggregated or damaged by an injurious event may play an important role in producing further damage to the adjacent tissue. Another possible role of extracellular ATP is on the activation of the alveolar macrophages that float in the alveolar surfactant. Rice et al. [21] suggested that the ATP content of the rat alveolar surfactant would be in the order of 1 mM, sufficient to activate the alveolar macrophages.

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