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Alteration of Biochemical Responses in Activated Human Neutrophils by ATP and Adenosine

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ABSTRACT

In both resting and opsonized zymosan activated neutrophils, ATP stimulated superoxide generation, whereas adenosine inhibited it slightly. The superoxide generation in activated neutrophils to ATP was greater than that of resting neutrophils. In Ca^{++} free medium, inhibitory effect of adenosine on superoxide generation was detectable, whereas ATP did not have any effect. The stimulatory effect of ATP on superoxide generation was inhibited by adenosine in a dose dependent manner. Neither ATP nor adenosine had any effect on NADPH oxidase acitivity. Effects of ATP or adenosine on superoxide generation were more prominent than that by other triphosphate nucleotides or nucleosides. ATP and ADP further stimulated Ca^{++} uptake and increased cytosolic free Ca^{++} level in neutrophils activated by opsonized zymosan, but adenosine inhibited a Ca^{++} mobilization. Verapamil effectively and tetrodotoxin slightly inhibited an increase of cytosolic free Ca^{++} level induced by ATP. Inhibitory effect of either verapamil or tetrodotoxin on superoxide generation in the ATP plus opsonized zymosan-activated neutrophils was greater than in the cells activated by opsonized zymosan alone. Tetraethylammonium chloride had no apparent effect on superoxide generation. CCCP, 2,4-dinitrophenol, diphenylhydantoin and procaine all inhibited superoxide generation in neutrophils activated by opsonized zymosan. Among these, CCCP only inhibited a stimulatory effect of ATP. ATP further stimulated a loss of sulfhydryl groups in activated neutrophils, whereas adenosine had no effect on it.

These results suggest that functional responses of neutrophils may be regulated at least partly by purines. ATP and adenosine may further alter functional responses of activated neutrophils through their effect on Ca^{++} uptake, membrane phosphorylation and oxidation of soluble sulfhydryl groups.

Key Words: ATP, Adenosine, Superoxide generation, Calcium mobilization, Sulfhydryl groups

INTRODUCTION

When neutrophils are exposed to a variety of soluble or particulate agents, molecular and functional changes take place in the plasma membrane or intracellular components. These changes included the sodium influx (Showell and Becker, 1976; Korchak and Weissmann, 1980), changes of the membrane potential (Mottola and Romeo, 1982), mobilization of calcium (Bareis *et al.*, 1982), phospholipid turnover (Gil *et al.*, 1982) and oxidation of surface and intracellular sulfhydryl groups (Voetman *et al.*, 1980). These changes are followed by the alteration of functional responses of neutrophils which consists of chemotaxis, phagocytosis, release of lysosomal enzymes and superoxide generation (Newburger *et al.*, 1980; Fantone *et al.*, 1982).

The functional responses of neutrophils to external stimuli can be altered by the change of cytosolic nucleotide level. It has been observed that agents which elevate the cAMP level within neutrophils inhibit the release of enzymes during feeding of opsonized zymosan, whereas agents which elevate the cGMP level within neutrophils enhance the release of enzymes (Zurier *et al.*, 1974; Weissmann *et al.*, 1975). It is suggested that atropine, phentolamine and propranolol may inhibit superoxide generation in activated neutrophils by decreasing calcium influx and

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this action is probably mediated through autonomic receptors (Lee *et al.*, 1988). Since the presence of β-adrenergic receptors on neutrophils is well known (Galant and Allred, 1981), these responses of neutrophils appear to be, at least partly mediated by adrenergic and muscarinic receptors.

On the other hand, it is recently reported that in neutrophils stimulated with fMLP, ATP enhances superoxide generation and this response is inhibited by adenosine (Cunningham *et al.*, 1988). Accordingly, the possibility is proposed that purinergic system may be involved in the expression of neutrophil responses.

The lines of evidence suggest that in various organs, ATP acts as a co-transmitter with the classical transmitters in autonomic fibers (Sneddon *et al.*, 1982). Also evidence is available for purinergic transmission in ganglia, and for purinergic modulation of transmitter release and postjunctional action (Su, 1983). The purine receptors have been classified into P_1 receptors and P_2 receptors that mediate effects of adenosine and ATP, respectively (Satchell, 1984). ATP and adenosine appear to cause opposite responses (Burnstock, 1985). In single sensory neurones, exogenously applied ATP produce a rapid and transient inwardly-rectifying Na^+ current (Krishtal *et al.*, 1983). It is demonstrated that ATP depolarize the membrane of the ganglion cell by decreasing resting K^+ conductance (Akasu *et al.*, 1983). Adenosine is suggested to inhibit release of transmitter from presynaptic terminals evoked by adrenergic nerve stimulation or elevated K^+ concentration through both stimulation of adenylate cyclase system and inhibition of the deploarization induced Ca^{++} uptake (Verhaeghe *et al.*, 1977; Vakade and Vakade, 1978; Ribeiro *et al.*, 1977. These findings also suggest that neutrophil responses can be modulated by ATP or adenosine. In addition, the report of Cunningham *et al.* (1988) shows that the presence of extracellular Ca^{++} may be prerequisite for stimulation of neutrophil responses by ATP, whereas adenosine stimulation does not require Ca^{++}. However, adenosine appears to inhibit the Ca^{++}-dependent transmitter release.

Thus, in this study, effects of ATP and adenosine on calcium uptake, intracellular free Ca^{++} level, change of intracellular sulfhydryl groups and superoxide generation were investigated to elucidate the role of purinenergic receptors in the expression of neutrophil function. Influences of ionic channel blockers, membrane phosphorylation inhibitors and suppressants of membrane depolarization on ATP stimulated neutrophil functional responses were also examined.

MATERIALS AND METHODS

Adenosine 5′-triphosphate, tris salt (ATP), adenosine 5′-diphosphate (ADP), cytidine 5′-triphosphate (CTP), guanosine 5′-triphosphate (GTP), uridine 5′-triphosphate (UTP), inosine 5′-triphosphate (ITP), adenosine, guanosine, inosine, verapamil, tetrodotoxin (TTX), tetraethylammonium chloride (TEA), carbonyl cyanide m-chlorophenylhydrazone (CCCP), potassium arsenate, diphenylhydantoin, procaine, ferricytochrome c, NADPH, quin 2/AM, carboxypyridine disulfide (CPDS), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), ethyleneglycol-bis (β-amino-ethylether), N,N,N′,N′,-tetraacetic acid (EGTA), zymosan (from Saccharomyces cerevisiae) and dextran (M.W. 465,000) were purchased from Sigma Chemical Co.. 2,4-Dinitrophenol (2,4-DNP) was obtained from Junsei Chemical Co., Ltd.; $CaCl_2$ from Kanto Chemical Co.. Other chemicals were of analytical reagent grade. $^{45}Ca^{++}$ was obtained from Dupont, NEM products, Boston Mass. U.S.A..

Preparation of neutrophils

Neutrophils were isolated from heparinized venous blood of healthy donors by dextran (average molecular weight 465,000) sedimentation of erythrocytes and treatment with 0.85% ammonium chloride (Trush *et al.*, 1978). The purity of neutrophil suspensions averaged 90% as judged by Wright-Giemsa stain.

Preparation of NADPH oxidase containing granule rich fraction neutrophils

Neutrophils activated by opsonized zymosan (Markert *et al.*, 1984) at 37°C for 15 min, or control neutrophils were centrifuged at 1,500g for 3 min and the pellets were resuspended in 0.25M sucrose to a concentration of 10^8 cells/ml. The cell suspension was disrupted by sonication for three 15 sec intervals at 25 watts power with a Branson sonifier cell disruptor (Mod. W 185D). Unbroken cells and nuclei were sedimented by centrifugation at 800g for 5 min. Sucrose was then added to the postnuclear supernatant with constant stirring and the final volume was adjusted to the sucrose concentration to 40% (W/V). The suspension was centrifuged at 48,000g for 1 h in a Beckman L5-50B ultracentrifuge. The supernatant completely removed and the pellets were

resuspended in 0.25M sucrose. The suspensions were centrifuged at 48,000g for 1 h and the pellets (granule rich fraction) were suspended in 25% ethylene glycol with a Teflon glass homogenizer (Hohn and Letrer, 1975; Gabig *et al.*, 1982). The protein concentration was determined by the method of Lowry *et al.* (1951).

Assay of superoxide radical generation

The superoxide dependent reduction of ferricytochrome c was measured by the method of Markert *et al.* (1984). Reaction mixtures in plastic microfuge tubes contained 10^6 neutrophils, 135mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 5mM dextrose, 75μM ferricytochrome c, 20mM HEPES-tris, pH 7.4 and 1mg opsonized zymosan in a total volume of 500μl. The reactions were performed in a 37°C·shaking water bath for 5 min. The reaction was then stopped by placing the tubes in melting ice and the cells were rapidly pelleted by centrifuging at 1,500g for 5 min at 4°C. The superntants were taken and the amount of reduced cytochrome c was measured at 550nm in a Gilford 260 U.V.-spectrophotometer. The amount of reduced cytochrome c was calculated by using an extinction coefficient of $1.85 \times 10^4 M^{-1}cm^{-1}$ at 550nm.

Assay of NADPH oxidase activity

The activity of NADPH oxidase was measured as reduction of ferricytochrome c by superoxide radicals produced from oxidation of NADPH by NADPH oxidase. The reaction mixture consisted of 0.2mg/ml granule rich fraction, 100μM NADPH, 75μM ferricytochrome c and 50mM Tris-HCl, pH 7.4 in a total volume of 500μl. The reaction mixture was preincubated for 5 min at 37°C and the reaction was initiated by adding NADPH. The reduction rate of ferricytochrome c was measured at 550nm (Lee *et al.*, 1987).

Measurement of Ca^{++} uptake by neutrophils

Ca^{++} uptake was measured by the millipore filter method using $^{45}Ca^{++}$ (Lee and Choi, 1966). The reaction mixtures contained 10^7 cells/ml of neutrophils, 135mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 5mM dextrose, 0.4 Ci/ml of $^{45}Ca^{++}$, 5mg/ml of opsonized zymosan and other compounds. After preincubation at 37°C for 5 min, the reaction was initiated by addition of opsonized zymosan concomitent with $CaCl_2$ and final volume was a 2.0ml. At the stated time, 0.3ml of aliquots were taken and under negative pressure, samples were filtered with a suction pump. Amount of $^{45}Ca^{++}$ in the filtered solution was detected with BECKMAN Liquid Scintillation Counter (Model LS 100C).

Measurement of cytosolic free calcium

Quin 2 loading and fluorescence measurement was performed by the modification of the method of Tsien *et al.* (1982). Neutrophils (approximately 10^8/ml) were loaded with 5μl of 20mM quin 2 /AM at 37°C for 20 min in 1.0ml of the reaction mixtures containing 135mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$ and 5mM dextrose. The suspension was then diluted to 10 fold with the above reaction mixture and further incubated at 37°C for 40 min. After loading, the suspension was centrifuged at 1,500g for 5 min and neutrophils were resuspended in the above reaction mixture as approximately 10^7/100μl.

Fluorescence measurement was done with a Turner Spectrofluorometer (Model 430). Preloaded neutrophils (10^7) were suspended in the same reaction mixture in a final volume of 2.0ml. After preincubation at 37°C for 5 min, reaction was initiated by addition of 4mg opsonized zymosan. The fluorescence change was read at an excitation wavelength of 339nm and emission wavelength of 492nm.

Assay of intracellular soluble sulfhydryl group content

Surface sulfhydryl groups of neutrophils were removed with carboxypyridinedisulfide (CPDS). Reaction mixtures (1.0ml) contained 10^7 neutrophils, 5mg opsonized zymosan, 10μM CPDS, HBSS buffer, pH 7.4 and ATP or adenosine. After 10 min of incubation, mixtures were centrifuged at 800g for 10 min.

After CPDS treatment, cell pellets obtained were washed with 1.0ml HBSS and centrifuged at 800g for 10 min. Pellets were resuspended in 1.0ml of deionized water for 20 min at room temperature and followed by adding 0.5ml of 0.2M phosphate buffer, pH 6.8. Suspension was centrifuged at 800g for 10 min and 1.0ml of supernatant was mixed with 75μl of 10mM DTNB. The absorbance was read at 412nm and content of sulfhydryl groups was estimated from the molar extinction coefficient of p-nitrothiophenol anion, 1.36×10^4/M/cm (Ellman, 1959; Mehrishi and Grassetti, 1969; Voetman *et al.*, 1980).

RESULTS

Alteration of superoxide generation in activated neutrophils by ATP or adenosine

Modulation of activated neutrophils response by ATP or adenosine was investigated. Amounts of superoxide generated in resting or activated neutrophils were 1.91 and 16.17 nmol/5 min/10^6 cells, respectively. Table 1 showed that ATP in a concentration of 50μM stimulated superoxide generation to 40.8%-59.1% in both resting and activated neutrophils. The response of activated neutrophils to extracellular ATP was greater than that of resting neutrophils. Adenosine slightly inhibited superoxide generation in both states of neutrophils and their responses to adenosine were relatively similar.

To explore the role of Ca^{++} in either ATP or adenosine induced alteration of superoxide generation, neutrophils were incubated in Ca^{++} free medium and effects of ATP or adenosine on superoxide generation were examined. In reaction mixtures containing 0.1mM Ca^{++} plus 0.5mM EGTA, ATP had no effect on superoxide generation in activated neutrophils, whereas adenosine inhibited it. On the other hand, in resting neutrophils the additional effect of ATP or adenosine on inhibitory action of EGTA was not detected. Table 2 indicates that adenosine may exert a inhibitory action on superoxide generation even at Ca^{++} free condition. The result shown in Fig. 1 indicated that ATP stimulated superoxide generation in activated neutrophils was inhibited by adenosine in a dose dependent manner.

Table 3 suggests that NADPH oxidase activity may be indirectly modulated by ATP or adenosine bound to sites other than NADPH oxidase, because ATP or adenosine did not affect NADPH oxidase activity.

Table 1. Alteration of superoxide generation by ATP or adenosine in activated neutrophils

Additions		Superoxide nmol/10^6 cells/5 min	
		Without zymosan	With zymosan
None		1.91 ± 0.25	16.17 ± 0.31
ATP	10μM	2.05 ± 0.21	22.35 ± 0.23
	50μM	2.69 ± 0.57	25.72 ± 0.45
	100μM	3.07 ± 0.24	27.01 ± 0.33
Adenosine	10μM	1.89 ± 0.46	13.93 ± 0.35
	50μM	1.71 ± 0.32	13.53 ± 0.19
	100μM	1.62 ± 0.31	13.01 ± 0.32

0.5ml of reaction mixtures contained 10^6 neutrophils, 1mg opsonized zymosan or not, 75μM ferricytochrome c and varying concentration of ATP or adenosine, and other experimental conditions were the same as described in Materials and Methods. Neutrophils were pretreated with ATP or adenosine for 5 min. The value represents mean ± S.E. of 6 experiments.

Table 2. Effect of ATP or adenosine on the suppressed superoxide generation in the absence of extracellular Ca^{++}

Additions		Superoxide nmol/10^6 cells/5 min	
		Without zymosan	With zymosan
None		1.84 ± 0.34	15.91 ± 0.51
EGTA	500μM	1.22 ± 0.31	6.19 ± 0.67
+ ATP	100μM	1.27 ± 0.21	6.85 ± 0.52
+ Adenosine	100μM	1.26 ± 0.30	5.35 ± 0.47
	500μM	—	5.03 ± 0.39
	1 mM	—	3.06 ± 0.43

Reaction mixtures did not contain exogenously added Ca^{++}, and remaining Ca^{++} was removed by EGTA. In this condition, neutrophils were pretreated with ATP or adenosine. The value represents mean ± S.E. of 4-6 experiments.

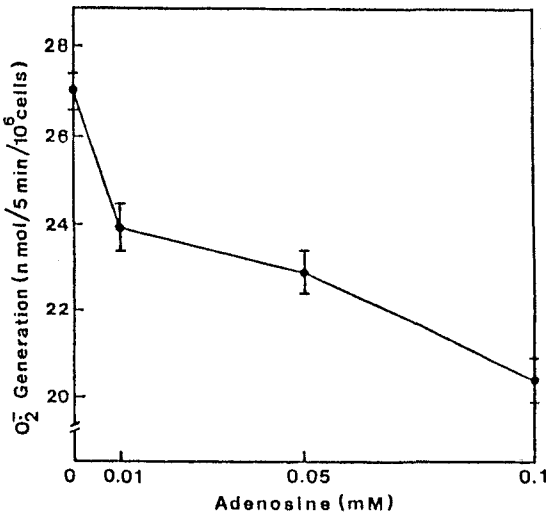

Fig. 1. Inhibition of ATP stimulated superoxide generation by adenosine. In the presence of opsonized zymosan and 100μM ATP, neutrophils were treated with varying concentration of adenosine. The point represents mean ± S.E. of 6 experiments.

Effects of triphosphate nucleotides and nucleosides on superoxide generation

Alteration of superoxide generation induced by ATP or adenosine was compared with effects of other compounds. As shown in Table 4, ADP, GTP, UTP and ITP except CTP stimulated superoxide generation in activated neutrophils, but in resting neutrophils, their stimulatory effects were not detectable. In addition, their stimulatory effects on superoxide generation in activated neutrophils were smaller than that of ATP.

Guanosine and inosine slightly inhibited superoxide generation in activated neutrophils but their inhibitory effect was smaller than that of adenosine (Table 5). In resting neutrophils, both guanosine and inosine had no effect on superoxide generation.

Effects of ATP and adenosine on Ca^{++} uptake and cytosolic free Ca^{++} level

Since Ca^{++} appears to play an important regulatory role in the functional responses of

Table 3. Effects of ATP and adenosine on NADPH oxidase activity

Additions		NADPH oxidase activity
		Superoxide nmol/mg protein/5 min
None		32.13 ± 3.09
ATP	100μM	32.14 ± 2.50
Adenosine	100μM	28.38 ± 2.98

Granule rich fraction which obtained from activated neutrophils was preincubated with ATP or adenosine for 5 min at 37°C and the reaction was initiated by addition of NADPH. Reduction of ferricytochrome c due to interaction of NADPH and NADPH oxidase was read at 550 nm. The value represents mean ± S.E. of 5 experiments.

Table 4. Effects of triphosphate nucleotides on superoxide generation in activated neutrophils

Additions	Superoxide nmol/10^6 cells/5 min	
	Without zymosan	With zymosan
None	1.90 ± 0.25	15.99 ± 0.48
ATP	3.06 ± 0.38	26.73 ± 0.45
ADP	2.21 ± 0.17	23.30 ± 0.48
CTP	2.15 ± 0.18	15.69 ± 0.33
GTP	2.03 ± 0.21	19.00 ± 0.45
UTP	2.10 ± 0.29	23.27 ± 0.59
ITP	2.36 ± 0.53	19.50 ± 0.47

Concentration of nucleotides is 100μM. Neutrophils were preincubated with nucleotides in the presence or absence of opsonized zymosna. The value represents mean ± S.E. of 3-6 experiments.

Table 5. Effects of nucleosides on superoxide generation in activated neutrophils

Additions	Superoxide nmol/10^6 cells/5 min	
	Without zymosan	With zymosan
None	1.90 ± 0.24	16.03 ± 0.35
Adenosine	1.60 ± 0.25	12.96 ± 0.36
Guanosine	1.78 ± 0.32	14.10 ± 0.42
Inosine	1.78 ± 0.22	14.12 ± 0.37

Concentration of nucleosides is 100μM. Neutrophils were preincubated with nucleosides in the presence or absence of opsonized zymosan. The value repersents mean ± S.E. of 3-6 experiments.

Table 6. Effect of ATP or adenosine on Ca^{++} uptake in activated neutrophils

Additions		Ca^{++} uptake (cpm of $^{45}Ca^{++}$)/10^7 cells/1 min
None		3159
ATP	100μM	5934
Adenosine	100μM	2819

Neutrophils were preincubated in Na^+ rich media and Ca^{++} uptake was initiated by addition of opsonized zymosan and ATP or adenosine. $^{45}Ca^{++}$ uptake by activated neutrophils was measured with liquid scientillation counter. The value represnets mean of 3 experiments.

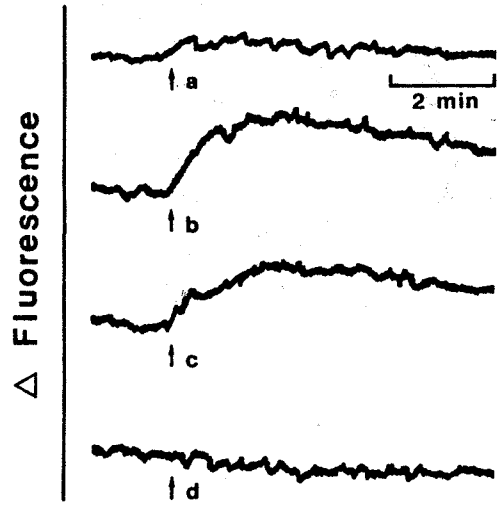

Fig. 2. Effects of ATP, ADP and adenosine on cytosolic free Ca^{++} level. Intracellular free Ca^{++} level in opsonized zymosan - activated neutrophils was measured as a fluorescence change of quin 2, a specific Ca^{++} chelator. Experimental conditions were the same as described in Materials and Methods. Fluorescence of quin 2-Ca^{++} complex was read at an excitation wavelength of 390nm and emission wavelength of 492nm. Concentration of ATP, ADP and adenosine is 100μM. Responses were initiated by adding the following agents at arrow point. a; no addition, b; ATP, c; ADP and d; adenosine in the presence of opsonized zymosan, respectively.

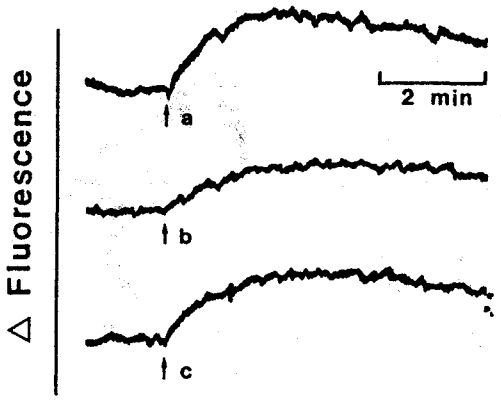

Fig. 3. Effects of verapamil and tetrodotoxin on increased cytosolic Ca^{++} level by ATP. Neutrophils were preincubated with 500μM verapamil or 10μM TTX. Response was initiated by opsonized zymosan plus verapamil or TTX at arrow point. a; ATP, b; ATP + verapamil and c; ATP + TTX, respectively.

Table 7. Effects of specific ion channel blockers on ATP stimulated superoxide generation in activated neutrophils

Additions		Superoxide nmol/10^6 cells/5 min	
		Without ATP	With ATP
None		16.03 ± 0.46	26.94 ± 0.35
Verapamil	100μM	13.47 ± 0.50	17.68 ± 0.43
TTX	10μM	15.37 ± 0.45	21.37 ± 0.59
TEA	100μM	16.17 ± 0.52	23.38 ± 0.31

Neutrophils were preincubated with ion channel blocker and then superoxide generation was initiated by either opsonized zymosan or opsonized zymosan plus ATP. The value represents mean ± S.E. of 6 experiments.

neutrophils (Goldstein *et al.*, 1975; Estensen *et al.*, 1976), effects of ATP and adenosine on Ca^{++} mobilization in activated neutrophils were examined. As can be seen in Table 6, ATP stimiulated a Ca^{++} uptake by activated neutrophils, whereas adenosine inhibited it.

The intracellular free Ca^{++} level was measured with quin 2/AM. When neutrophils were exposed to opsonized zymosan, fluorescence change due to complex formation of an increased Ca^{++} with quin 2 was small. However, after addition of ATP or ADP, fluorescence of quin 2 was remarkably increased (Fig. 2). In resting neutrophils, fluorescence changes due to addition of ATP or ADP were small (data not shown). On the other hand, adenosine interfered with the increase of fluorescence caused by opsonized zymosan.

Effects of verapamil, tetrodotoxin and tetraethylammonium chloride on cytosolic free Ca^{++} level and superoxide generation

To examine the alteration of membrane ion permeability by addition of ATP, effects of ionic channel blockers on activated neutrophil response were investigated. The results presented in Fig. 3 and Table 7 showed that verapamil, a Ca^{++} channel blocker effectively inhibited both the increase of quin 2 fluorescence and the enhanced superoxide generation in activated neutrophils by ATP plus opsonized zymosan. Tetrodotoxin, a Na^+ channel blockers slightly inhibited both the increase of quin 2 fluorescence and the enhanced superoxide generation by ATP plus opsonized zymosan. Inhibitory effects of verapamil or tetrodotoxin on the ATP plus opsonized zymosan-activated neutrophils were greater than that on the opsonized zymosan activated cells. However, 0.1mM of tetraethylammonium chloride, a K^+ channel blocker had no significant effect on responses of neutrophils activated by either ATP plus opsonized zymosan or opsonized zymosan alone.

Effects of inhibitors of membrane phosphorylation and depolarization on superoxide generation

Since the activation of receptors for neurotransmitters is thought to initiate cellular responses by opening the specific Ca^{++} channels in the cell membrane through membrane depolarization and protein phosphorylation (Lew *et al.*, 1984; Pontremoli *et al.*, 1986; Westwick and Poll, 1986), alteration of ATP stimiulated superoxide generation by inhibitors of membrane phosphorylation and depolarization was investigated. CCCP and 2,4-dinitrophenol, inhibitors of membrane protein phosphorylation inhibited superoxide generation in activated neutrophils by either ATP plus opsonized zymosan or opsonized zymosan alone (Table 8), while arsenate at the concentration of 100μM had no effect on superoxide generation. In opsonized zymosan activated neutrophils, the inhibitory effect of CCCP on ATP stimulated superoxide generation was greater than that in the absence of ATP. Diphenylhydantoin and procaine at the concentration of 1.0mM, inhibited superoxide generation by either ATP plus opsonized zymosan or opsonized zymosan alone and their inhibitory effects on both state of neutrophils were similar (Table 8).

Effects of ATP and adenosine on soluble sulfhydryl groups in activated neutrophils

Intracellular sulfhydryl groups of reduced form may be necessary for the expression of neutrophil responses. Sulfhydryl groups appear to involve in events that lead to neutrophil activation. As shown in Table 9, oxidation of soluble sulfhydryl groups in neutrophils was observed during phagocytosis and after 10 min of incubation with opsonized zymosan, soluble sulfhydryl groups were reduced to 30.7%.

Table 8. Effects of an inhibitors of membrane protein phosphorylation and membrane depolarization on superoxide generation

Additions		Superoxide nmol/10^6 cells/5 min	
		Without ATP	With ATP
None		16.03 ± 0.43	26.92 ± 0.47
CCCP	10μM	12.26 ± 0.23	13.69 ± 0.64
2,4-DNP	100μM	15.34 ± 0.63	21.80 ± 0.34
Arsenate	100μM	17.03 ± 0.28	24.57 ± 0.37
DPH	1 mM	5.08 ± 0.25	11.27 ± 0.71
Procaine	1 mM	8.10 ± 0.48	13.38 ± 0.84

Neutrophils were pretreated with above compounds and then response was initiated by either opsonized zymosan or opsonized zymosan plus ATP. The value represents mean ± S.E. of 6 experiments.

Table 9. Effects of ATP and adenosine on loss of soluble SH groups in activated neutrophils

Additions		Soluble SH groups (nmol)/10^7 cells
None		14.76 ± 0.37
Zymosan		10.23 ± 0.35
+ ATP	100μM	7.85 ± 0.59
+ Adenosine	100μM	10.99 ± 0.31

Neutrophils were incubated for 10 min in reaction mixtures which contained opsonized zymosan and ATP or adenosine at 37°C. Intracellular SH groups were spectrophotometrically measured with DTNB method. The value represents mean ± S.E. of 3-6 experiments.

ATP further stimulated this loss of sulfhydryl groups in activated neutrophils. However, adenosine had no effect on it.

DISCUSSION

ATP or adenosine is postulated to play a role of an endogenous feedback neuromodulator, regulating the adrenergic transmitter release as well as acting as a neurotransmitter, mediating central synaptic excitation of neurones both in the trigeminal nucleus and in the outer layer of the spinal cord (Su, 1983; Burnstock, 1985). It is reported that exogenously applied ATP produced a rapid and transient inwardly-rectifying Na^+ current in single sensory neurones that is enzymatically isolated from nodose, vestibular, trigeminal and spinal ganglia of rats (Krishtal *et al.*, 1983). Adenosine appears to inhibit release of transmitter from presynaptic terminals by stimulation of adenylate cyclase (Silinsky, 1984). Adenosine has also been shown to inhibit the voltage- dependent Ca^{++} current via activation of P_1-purinoceptors on postganglionic neurones of rat superior cervical ganglion (Henon and McAfee, 1983). Adenosine inhibits a norepinephrine efflux evoked by adrenergic nerve stimulation or elevated K^+ concentration, but does not affect that induced by tyramine in isolated blood vessels (Su, 1978; Burnstock, 1985) or heart (Khan and Malik, 1980). Thus, it is suggested that adenosine only interferes with the Ca^{++} dependent transmitter release.

The presence of β-adrenergic receptors on neutrophils is well known and many studies investigate functional changes of neutrophils following the binding of autonomic agents to their receptors or action sites (Zurier *et al.*, 1974; Westwick and Poll, 1986; Lee *et al.*, 1987). In addition, alteration of neutrophil responses by either ATP or adenosine was also reported (Cunningham *et al.*, 1988). Accordingly, investigation for role of purinergic system in the expression of neutrophil function seems to have a meaning. As can be seen in Table 1, superoxide generation in resting or activated neutrophils was stimulated by adding ATP in a dose dependent manner and response of activated neutrophils to ATP was greater than that of resting neutrophils. On the other hand, inhibition of either resting or activated neutrophil response by adenosine alone was not significant. Fig. 1 showed that in activated

neutrophils, the stimulatory effect of ATP was inhibited by adenosine in a dose dependent manner. This finding suggests that functional responses of neutrophils to external stimuli can be partially regulated by ATP and adenosine. Since Ca^{++} may be involved in adenosine induced change of cell function (Su, 1983; Cunningham *et al.*, 1988), the effect of adenosine on superoxide generation in Ca^{++} free medium was studied. In reaction mixtures containing 0.1mM Ca^{++} and 0.5mM EGTA buffer, adenosine further suppressed superoxide generation in neutrophils, whereas ATP did not show any significant effect on it (Table 2). Thus, it is postulated that the inhibitory action of adenosine on superoxide generation occurs independent of the extracellular Ca^{++}, but the stimulatory action of ATP requires the existence of extracellular Ca^{++}. In addition, purines probably act at certain sites other than NADPH oxidase, because this oxidase activity was not affected by ATP or adenosine (Table 3).

The different responses of neutrophils to several nucleotides and nucleosides show a substrate specificity for purine receptors in plasma membrane. As shown in Table 4 and 5, triphosphate nucleotides, such as ADP, GTP, UTP and ITP stimulated superoxide generation in activated neutrophils, but nucleotides including guanosine and inosine inhibited it. However, these effects of both triphosphate nucleosides and neucleosides were much less than that of ATP or adenosine. The result indicates that ATP or adenosine may act as a proper substrate for purine receptors than other compounds. A series of recent studies indicate that an increase of free cytoplasmic calcium is involved in the early triggering of the responses of neutrophils to surface stimulation by both particulate and soluble agents (Lew *et al.*, 1984; Young *et al.*, 1984). It is reported that fMet-Leu-Phe promotes influx of Ca^{++} across the plasma membrane of neutrophils by opening of receptor dependent Ca^{++} channels (Andersson *et al.*, 1986). In neuronal membrane, α-adrenergic agonists appear to stimulate Ca^{++} influx through increased phospholipid turnover (Michell, 1975; Exton, 1983). This suggestion is also made for muscarinic receptors (Doughney *et al.*, 1987). Previous experiment in our laboratory observes that atropine, phentolamine and propranolol interfere with Ca^{++} influx and inhibit superoxide generation in activated neutrophils. Carbachol stimulated superoxide generation is inhibited by atropine (Lee *et al.*, 1987). Thus, changes of Ca^{++} influx and intracellular free Ca^{++} level due to purine binding to the plasma membrane of neutrophils were investigated. Table 6 and Fig. 2 showed that ATP further stimulated Ca^{++} influx and increased the level of cytosolic free Ca^{++} in activated neutrophils, whereas adenosine inhibited this Ca^{++} mobilization. The intracellular Ca^{++} level appears to be increased by Na^{+} induced Ca^{++} release or probably Ca^{++} triggered Ca^{++} release from the intracellular Ca^{++} storage sites (Westwick and Poll, 1986). From above findings and suggestions, it is proposed that ATP and adenosine may affect the ion transport in the plasma membrane which is altered by external substance binding. In the present study, influences of ATP or adenosine on Ca^{++} release from intracellular Ca^{++} storge sites were not examined. However, their action on intracellular Ca^{++} mobilization may not be significant, because the stimulatory effect of ATP was not detectable in Ca^{++} free medium. In addition, it is postulated that either Ca^{++} binding or influx is needed for a signal transduction in neutrophils.

Ca^{++} influx and cytosolic Ca^{++} redistribution at neutrophils appear to be regulated by membrane depolarization and receptor binding to the plasma binding (Mottolà and Romeo, 1982; Lew *et al.*, 1984). In cardiac muscle, smooth muscle and neural tissues, elevation of the extracellular K^{+} concentration leads to membrane depolarization (Bolton, 1979; Cho *et al.*, 1988) then to the influx of Na^{+} and Ca^{++} into the cell which can induce release of transmitter and finally contraction (Crompton *et al.*, 1976; Powis, 1981). On the other hand, many of the neurotransmitters, hormones and drugs may modulate the efficiency of neurotransmitter release by modifying the permeability for Ca^{++} channels through their effects on cAMP-dependent protein kinases and subsequent phosphorylation in specific proteins at the plasma membrane (Reuter, 1983).

The enhanced Ca^{++} uptake and increased superoxide generation induced by ATP in opsonized zymosan activated neutrophils are inhibited by verapamil and tetrodotoxin but not by tetraethylammonium chloride (Fig. 3, Table 7). And their inhibitory effect on superoxide generation in the absence of ATP was greater than that in the absence of ATP. For example, the inhibitory effect of tetrodotoxin on superoxide generation in opsonized zymosan activated neutrophils in the absence of ATP was not detected. The result suggests that ATP binding to the plasma membrane further increases ion permeability for Ca^{++} and Na^{+} channels. Since membrane depolarization and phosphorylation of membrane protein due to activation of neutrophils is also reported, effects of phosphorylation inhibitors on the responses induced by adding ATP were investigated. As can be seen in Table 8, CCCP and 2,4-DNP in-

hibited superoxide generation in the presence or absence of ATP. Particularly, CCCP at the concentration of 10μM, effectively inhibited superoxide generation caused by adding ATP more than that in the absence of ATP. On the other hand, inhibitory effects of diphenylhydantoin and procaine were not significantly altered by the presence or absence of ATP. Thus, the possibility that phosphorylation of membrane protein by ATP binding may be implicated in the expression of neutrophil responses is proposed. However, it is unlikely that further depolarization of membrane due to ATP binding is attained.

Normal level of reduced form of sulfhydryl groups may be requisit for the expression of neutrophil functional responses. Oxidation of sulfhydryl groups, especially intracellular soluble sulfhydryl groups during phagocytosis is accompanied (Oliver *et al.*, 1976; Voetman *et al.*, 1980; Wedner *et al.*, 1981). It is reported that substances which can cause loss of soluble sulfhydryl groups probably affect neutrophil responses (Tsan *et al.*, 1976). Enhanced loss of soluble sulfhydryl groups by adding ATP in opsonized zymosan activatedneutrophils, as shown in Table 9, postulates that such change of sulfhydryl groups is partially involved in stimulated functional responses of neutrophils due to ATP binding.

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=국문초록=

활성화된 사람 중성 백혈구에서 ATP와 Adenosine 처리에 따른 생화학적 반응의 변경

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박 성 수 • 이 정 수

안정 상태 및 활성화된 중성 백혈구에서 ATP는 superoxide 라디칼 생성을 자극하였으나 adenosine은 약간 억제하였다. ATP에 대한 활성화된 중성 백혈구의 반응이 안정상태의 중성 백혈구에서보다 크게 나타났다. 칼슘이 제거된 반응액에서 superoxide 라디칼 생성에 대한 adenosine의 억제효과가 관찰되었으나 ATP는 영향을 주지않았다. Superoxide 라디칼 생성에 대한 ATP의 자극 효과는 adenosine에 의하여 용량에 따라 억제되었다. ATP와 adenosine은 NADPH oxidase 활성도에 영향을 주지 않았다. ATP 또는 adenosine에 의한 superoxide 라디칼 생성의 변경은 다른 triphosphate nucleotide나 nucleoside에 의한 것보다 현저하였다. 활성화된 중성 백혈구에서 ATP와 ADP는 칼슘이온의 흡수를 더 자극하였고 세포질 유리 칼슘농도를 증가시켰으나, adenosine은 칼슘이온의 이동을 억제하였다. APT에 의한 세포질 유리 칼슘이온 농도의 증가는 verapamil에 의하여 효과적으로, tetrodotoxin에 의하여 약간 억제되었다. ATP에 노출된 활성화된 중성 백혈구에서의 superoxide 라디칼 생성에 대한 verapamil과 tetrodotoxin의 억제 효과는 ATP의 영향이 없는 활성화된 중성 백혈구에서보다 크게 나타났다. Tetraethylammonium chloride는 superoxide 생성에 뚜렷한 영향을 미치지 못했다. CCCP, 2,4-dinitrophenol, diphenylhydantoin과 procaine은 활성화된 중성 백혈구에서 superoxide 라디칼의 생성을 억제하였다.

이들 가운데 CCCP만이 ATP의 자극 효과를 억제하였다. ATP는 활성화된 중성 백혈구에서의 sulfhydryl기의 손실을 더 자극하였으나 adenosine의 영향은 관찰되지 않았다.

이상의 결과로부터 중성 백혈구의 기능적 반응은 부분적으로 purine에 의하여 조절될 것으로 시사되었다. ATP와 adenosine은 칼슘 흡수와 그리고 아마도 세포막 인산화 반응 및 용해성 sulfhydryl기의 산화에 대한 영향을 통하여 활성화된 중성 백혈구의 반응을 더 변경 시킬 수 있을 것으로 추정된다.