Biochemical Pharmacology, Vol. 51, pp. 957–965, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02403-4

Biphasic Effect of ATP on Neutrophil Functions Mediated by P_{2U} and Adenosine A_{2A} Receptors

Yu Zhang,[1]* *Jan Palmblad*[2] *and Bertil B. Fredholm*[1]

[1]Department of Physiology and Pharmacology, Section of Molecular Neuropharmacology, Karolinska Institute, S17177 Stockholm, Sweden and [2]Department of Medicine and Inflammation Research Center, Stockholm Söder Hospital, S11883 Stockholm, Sweden

ABSTRACT. In agreement with previous findings, the oxidative burst induced by fMet-Leu-Phe (fMLP) in polymorphonuclear neutrophils was enhanced by adenosine triphosphate (ATP) and uridine 5′-triphosphate (UTP), although these nucleotides were inactive as agonists *per se*. However, the enhancement by ATP was rapidly reversed to an inhibition by prolonged incubation. Adenosine diphosphate (ADP) was always inhibitory. Inhibition mediated by ATP coincided with its conversion into ADP, adenosine monophosphate (AMP), and adenosine. In addition, the inhibitory effects of ATP and ADP on the oxidative burst were virtually abolished by 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5] quinazolin-5-imine monomethanesulfonate (CGS 15943), a nonselective adenosine receptor antagonist, whereas the priming effect of ATP was antagonized by adenosine. Both ATP and UTP showed a similar potency in activating elastase release, intracellular inositol-(1,4,5)-triphosphate (IP_3) formation and an increase in cytosolic calcium. Neither ATP nor UTP affected the initial rise in cytosolic calcium induced by fMLP, but did enhance the secondary calcium increase. When added simultaneously with fMLP, ADP and adenosine abolished this second calcium peak without influencing the first. These results indicate that purine and pyrimidine nucleotides acting on P_{2U}-like receptors, which are coupled to the IP_3 pathway, can increase calcium, release elastase, and enhance the oxidative burst induced by chemokines. Adenosine formed by hydrolysis from ATP and ADP, by contrast, reduces the oxidative burst and the influx of extracellular calcium induced by fMLP. BIOCHEM PHARMACOL 51;7:957–965, 1996.

KEY WORDS. purine nucleotides; pyrimidine nucleotides; hydrogen peroxide; elastase; IP_3; intracellular calcium

PMNs† are major effector cells in inflammation and host defense, and there is evidence that nucleotides are important extracellular factors regulating neutrophil functions. ATP has been shown to enhance the surface expression of integrin adhesion molecules and augment adhesion of circulating leukocytes to the endothelium, as well as the migration of neutrophils into sites of inflammation [1]. ATP also acts as a secretagogue for enhanced exocytosis of primary granules from neutrophils [2, 3, 4, 5]. Although the purine nucleotide does not *per se* activate the formation of superoxide by human neutrophils, ATP does enhance the release of superoxide induced by fMLP, thereby acting as a priming agent for this response [6, 7, 8, 9]. These actions of ATP are shared by uridine 5′-triphosphate (UTP) [7, 8, 10], suggesting that the effect is mediated by the so-called P_{2U} receptor that is G protein coupled and linked to phospholipase C activation [11]. Extracellular nucleotides are rapidly broken down by ectonucleotidases [12]. These enzymes can convert ATP to adenosine, which exerts a marked effect on neutrophil function [13], whereas UTP is not converted to any known neutrophil receptor ligand. This might explain why ATP has sometimes been reported to produce a biphasic effect, involving both stimulation and inhibition of neutrophil function [14].

In the present work, we studied the effects of adenosine, ATP, ADP, and UTP on oxidative burst, elastase release, intracellular IP_3 formation, and calcium release to elucidate the contribution of P_2 and adenosine receptors in mediating neutrophil responses to extracellular ATP.

MATERIALS AND METHODS

Chemicals

Adenosine was obtained from Aldrich-Europe (Beerse, Belgium). CGS 15943 was a gift from Ciba Geigy (Summit,

* Corresponding author. FAX +46-8-34 1280.

† *Abbreviations:* CGS 15943, 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazoglo[1,5]quinazolin-5-imine monomethanesulfonate; IP_3, inositol-(1,4,5)-triphosphate; S-2484, L-pyroglutamyl-L-prolyl-L-valine-P-nitroanilide; fMLP, formyl-MetLeuPhe; PCA, perchloric acid; PMN, polymorphonuclear neutrophil granulocyte; HBSS, Hank's balanced salt solution; H_2O_2, hydrogen peroxide; and Fura-2AM, fura-2 acetoxymethylester.

Received 31 July 1995; accepted 17 November 1995.

NJ). D-myo-[2-^{3}H] inositol 1,4,5-triphosphate ([^{3}H] IP_3 51.4 Ci/mmol) was from Amersham (Amersham, U.K.). Percoll was from Pharmacia Fine Chemicals (Uppsala, Sweden). The elastase substrate (S-2484) was obtained from Pharmacia AB (Stockholm, Sweden). Fura-2 AM was from Calbiochem (La Jolla, CA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). fMLP, cytochalasine B, luminol, S-2484, and CGS 15943 were dissolved in DMSO. The other chemicals were dissolved in water.

Neutrophil Preparation

Human neutrophils were isolated from buffy coats prepared from citrated whole blood supplied by the Karolinska Hospital Blood Center or from heparinized blood from healthy donors by a one-step Percoll technique as described [15]. After centrifugation the neutrophil-rich band was collected and washed 3 times with PBS. Contaminating erythrocytes were lysed. The washed PMNs were resuspended to the desired cell concentration in HBSS. The final preparation contained more than 95% neutrophils as shown by Giemsa stain, 98% of which were assessed as viable by trypan blue exclusion.

Measurement of Hydrogen Peroxide Production (H_2O_2)

H_2O_2 production by neutrophils was determined using luminol-amplified chemiluminescence in the presence of azide and peroxidase as described by Wymann *et al.* [16]. This is a highly sensitive, although not fully specific, method to assess the respiratory burst in neutrophils. As a test solution, 250 μL each of 1 mM luminol, 10 mM sodium azide, and 1000 U/mL horseradish peroxidase were diluted in HBSS to 25 mL. Neutrophils were suspended in HBSS at a concentration of 10×10^6/mL. Neutrophil suspension (100 μL) was added to 850 μL of the test solution. The mixture was incubated with either buffer or agonists at a different concentration for 2 or 5 min in a plastic cuvette. Ten microlitres of 1 μM fMLP was added to the cuvette and luminol-amplified chemiluminescence was measured by a luminometer (Chronolog Corporation). Activity was expressed as the percentage of the uninhibited peak height. Because DMSO (at concentrations greater than 0.5%) *per se* inhibits H_2O_2 production, the concentration of DMSO in the suspension was kept below 0.1%. None of the agonists had any effect on the luminol system in the absence of cells.

Purine Metabolism in Neutrophil Leukocytes

Neutrophils (1×10^6/mL) were incubated with 10 μM ATP at 37°C for indicated times and then centrifuged for 1 min at 15,000 rpm. The supernatant was incubated for another 2 min at 85°C. After centrifugation for 10 min at 15,000 rpm at 4°C, all supernatants were frozen at −20°C until analyses. ATP, ADP, AMP, and adenosine were determined using HPLC as described [17]. Samples were injected on a reversed-phase HPLC system (Nucleosil 5 μM, 4 × 150 mm, Skandinaviska Gene-Tec AB, Kungsbacka, Sweden) with isocratic elution with 10 mM $(NH_4)H_2PO_4$, 13% methanol, pH 6.0 with NH_3 2 M (for adenosine assay) and with 0.06 M K_2HPO_4, 0.04 M KH_2PO_4, pH 6.0 with 85% phosphoric acid (for ATP assay). All assays were carried out in triplicate.

Measurement of Elastase

Neutrophils (2×10^6/mL) were suspended in 1 mL of HBSS and preincubated at 37°C for 5 min with 4 μg of cytochalasin B followed by varying concentrations of agonist. After 20 min of incubation, the suspension was centrifuged for 10 min at 1000 *g* at 4°C. The supernatant was carefully collected and used as the sample [18]. The sample (200 μL) was mixed with 200 μL of the buffer solution (Tris 100 mM, NaCl 960 mM, pH 8.3) and incubated at 37°C for 3 min. The substrate solution (200 μL) (S-2484, 2 mM) was added to the mixture. After exactly 180 sec of incubation at 37°C, the reaction was stopped by the addition of 200 μL of 20% acetic acid. The sample was transferred to a spectrophotometer (Beckman DU-64) and absorbance was read at 405 nM. Sample blanks were prepared by omitting the substrate or by adding acetic acid prior to substrate.

Measurement of IP_3

Neutrophil suspensions (8×10^6 cells in each tube) were preincubated for 20 min and then exposed to buffer or drugs. The reaction was terminated by the addition of 50 μL cold 2.8 M PCA at 30 sec. Samples were placed on ice for 60 min. After neutralization with 60 μL of buffer (2 mM KOH, 1 M Tris, 60 mM EDTA), samples were centrifuged for 20 min at 1000 *g* at 4°C.

The binding assay was performed essentially as described by Gerwins [19]. Twenty-five microliters of sample (or IP_3 standards), [^{3}H] IP_3 (3000–3500 cpm), binding protein (approximately 0.5 mg), and assay buffer (100 mM Tris-HCl, pH 9.4 mM EDTA, 4 mM EGTA, 4 mg/mL bovine serum albumin) were added to 96-well microtiter plates with U-shaped well bottoms and incubated at 4°C for 1 hr. Bound [^{3}H] IP_3 was separated from free by filtration using the Skatron semiautomatic cell harvester 7019 (Skatron AS, Tranby, Norway). Filters were then transferred to scintillation vials. Three millilitres of scintillation fluid (Optiphase "HiSafe3," Pharmacia-LKB, Sweden) was added and the vials left for 24 hr. Before counting, the vials were shaken. All assays were performed in triplicate.

Cytosolic Ca^{2+} Concentration

Neutrophils (5×10^6/mL) in HBSS supplemented with 20 mM HEPES, pH 7.4, were incubated at 37°C with 0.5 μM Fura-2AM for 30 min. Fura-2AM loaded cells were washed twice, reconstituted in HBSS (2.5×10^6/mL) and stored on ice until use. Then, 1.5 mL of cell suspension was added to

quartz cuvettes in a Hitatchi F-3000 fluorescence spectrophotometer (Tokyo, Japan). Fluorescence was excited at 340 and 360 nM and emitted light was measured at 510 nM. Measurements were made at 37°C with continuous stirring of the cell suspension. After a stable baseline had been established, stimulus was added and emitted light recorded. The system was controlled by addition of EGTA, Tris buffer, Triton X-100, and $CaCl_2$, after which calcium concentrations were calculated by the following equation $[Ca^{2+}]_i = (F_o - F_{min})/(F_{max} - F_o) \times 224$ (nM) as described [20, 21].

Statistics

All results are given as means ± standard errors. Results are compared by one-way analysis of variance or Student's *t*-test using the Primer program on a Macintosh computer.

RESULTS

In agreement with previous results [6, 7, 8, 14], ATP, ADP, and UTP were found to be ineffective *per se* as stimuli for H_2O_2 production (data not shown). Also in agreement with previous results, ATP and UTP caused a significant enhancement of fMLP-stimulated neutrophil chemiluminescence when PMNs were treated with these nucleotides for 2 min before the addition of 1 μM fMLP (Fig. 1a). Enhancement of H_2O_2 generation could be observed at 0.1 μM and maximum effects (160% of basal level for ATP, 150% of basal level for UTP) were detected at 10 μM. However, with prolonged treatment time, the enhancement of fMLP-induced chemiluminescence by ATP was rapidly reversed to an inhibition to 40% of that in untreated controls. Whereas UTP gave a similar enhancement of H_2O_2 production as did ATP during short preincubation, it was essentially inactive when incubated with PMNs for 5 min prior to addition of fMLP (Fig. 1b). ADP mediated only an inhibition of fMLP-induced H_2O_2 production at the 2 incubation times; the inhibition seen at 10^{-4} M amounted to 60% and 78% at 2 and 5 min, respectively. Enhancement of H_2O_2 production was noted when fMLP and ATP were added simultaneously, and a maximal enhancement was found when ATP was added 30 sec before fMLP (Fig. 2).

Neutrophil activation also results in exocytosis of azurophilic granule constituents, including the serine protease elastase. Therefore, we also investigated the effect of extracellular ATP, ADP, and UTP on elastase release in quiescent or fMLP-activated human neutrophils. ATP and UTP, but not ADP, stimulated the elastase release from the basal level of 6 ± 1 U/L to 28 ± 10 U/L for ATP, and to 24 ± 6 U/L for UTP (Fig. 3). The direct effect of ATP on elastase release was evident within 1 min and was not significantly altered by addition of 1 μM CGS 15943, a potent non-xanthine adenosine receptor antagonist (not shown). fMLP (at 1 μM) resulted in a marked increase in elastase release to 105 ± 20 U/L, but this response was unaffected by ATP,

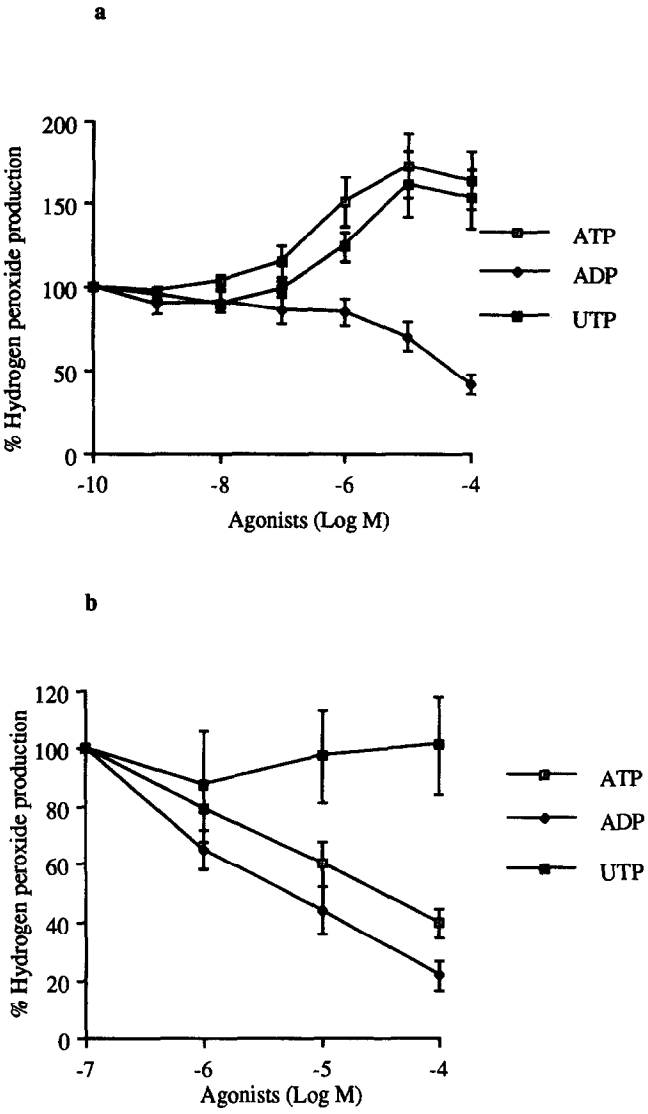

FIG. 1. The effects of ATP, ADP, and UTP on H_2O_2 production in fMLP-stimulated neutrophils. Drugs were added (a) 2 min or (b) 5 min before fMLP (1 μM). The data are expressed as means ± standard errors of 3 experiments using PMN from different donors. Results are normalized as percent of control values.

ADP, and UTP added together with fMLP (results not shown).

IP_3 levels, measured at 30 sec following ATP or UTP exposure, were increased in a dose-dependent fashion ($P < 0.05$) (Fig. 4). The magnitude of the IP_3 increase was half that induced by fMLP [22]. Because it is generally accepted that an increase in IP_3 leads to a rise in the free cytosolic concentration of calcium ($[Ca^{2+}]_i$), we tested the effect of ATP, ADP, and UTP (at 10 μM) on $[Ca^{2+}]_i$ fluxes. ATP and UTP, but not ADP, caused a rapid but transient rise in $[Ca^{2+}]_i$ from the basal level of 62 ± 8 nM to a peak of 160 ± 34 nM (ATP) or 151 ± 39 nM (UTP). $[Ca^{2+}]_i$ then returned rapidly to the basal level within 2 min (Fig. 5a). fMLP (at 0.1 μM) not only induced a marked increase in

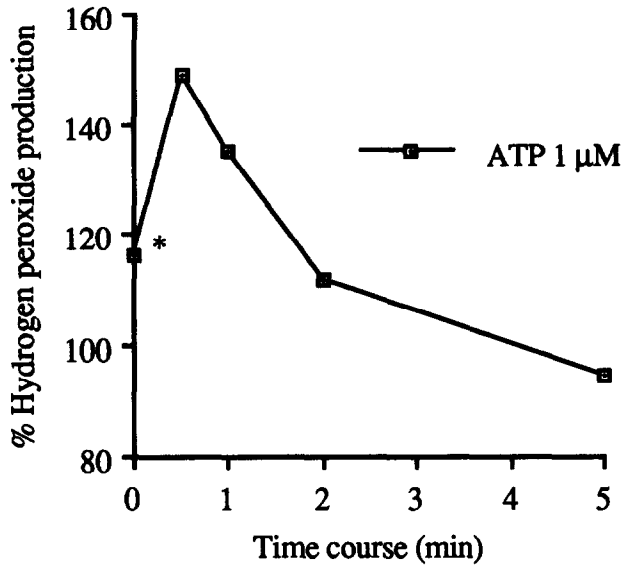

FIG. 2. Time course of the enhancement of H_2O_2 production by ATP. Neutrophils were preincubated with ATP (1 μM) for the indicated time. H_2O_2 was measured as described in the Methods section. *denotes the simultaneous addition of ATP and fMLP. The data are presented as percent of the control level of H_2O_2 production by neutrophils exposed only to fMLP. Each point represents the mean of 2 experiments.

$[Ca^{2+}]_i$ from a basal level to a peak of 338 ± 13 nM (approximately double that caused by ATP or UTP alone), but also induced a clear second peak, which often lasted up to 5 min [20]. The first peak was unaffected when ATP and

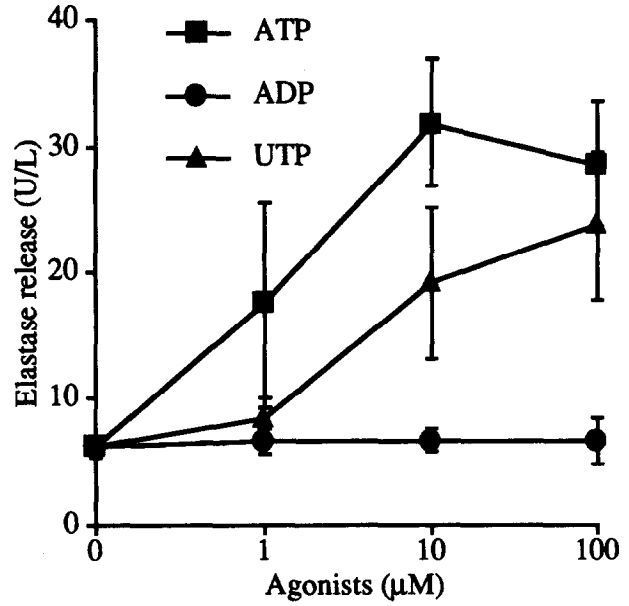

FIG. 3. The effect of ATP, ADP, and UTP on elastase release in nonactivated neutrophil leukocytes. PMNs were incubated with the indicated concentration of agonists and, after 20 min, the reaction was stopped by centrifugation. Elastase release was determined by spectrophotometry. The data are the means ± standard errors of 4 experiments using cells from different donors. Results presented as U/L.

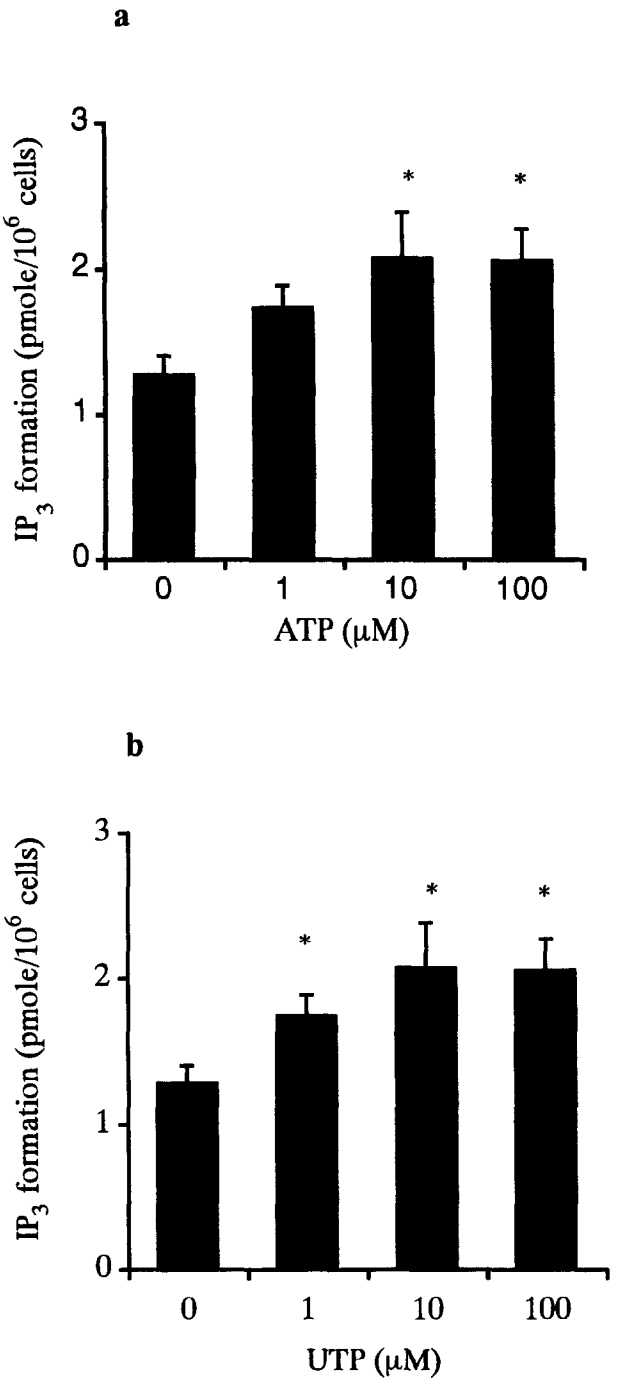

FIG. 4. The effect of ATP or UTP on IP_3 formation in neutrophils. Peak IP_3 mass was determined at 30 sec following addition of ATP and UTP. Data are means ± standard errors from (a) 2 or (b) 3 experiments, each performed in triplicate. *$P < 0.05$ (*t*-test).

UTP were added simultaneously with fMLP; however, the second peak tended to increase. Addition of ADP caused the disappearance of the second peak induced by fMLP (Fig. 5b).

Adenosine (at 1 μM) did not affect $[Ca^{2+}]_i$ over the 5-min observation period (data not shown). Treatment with adenosine for 5 min prior to fMLP stimulation did not influence the initial rise or the maximal level of $[Ca^{2+}]_i$, but

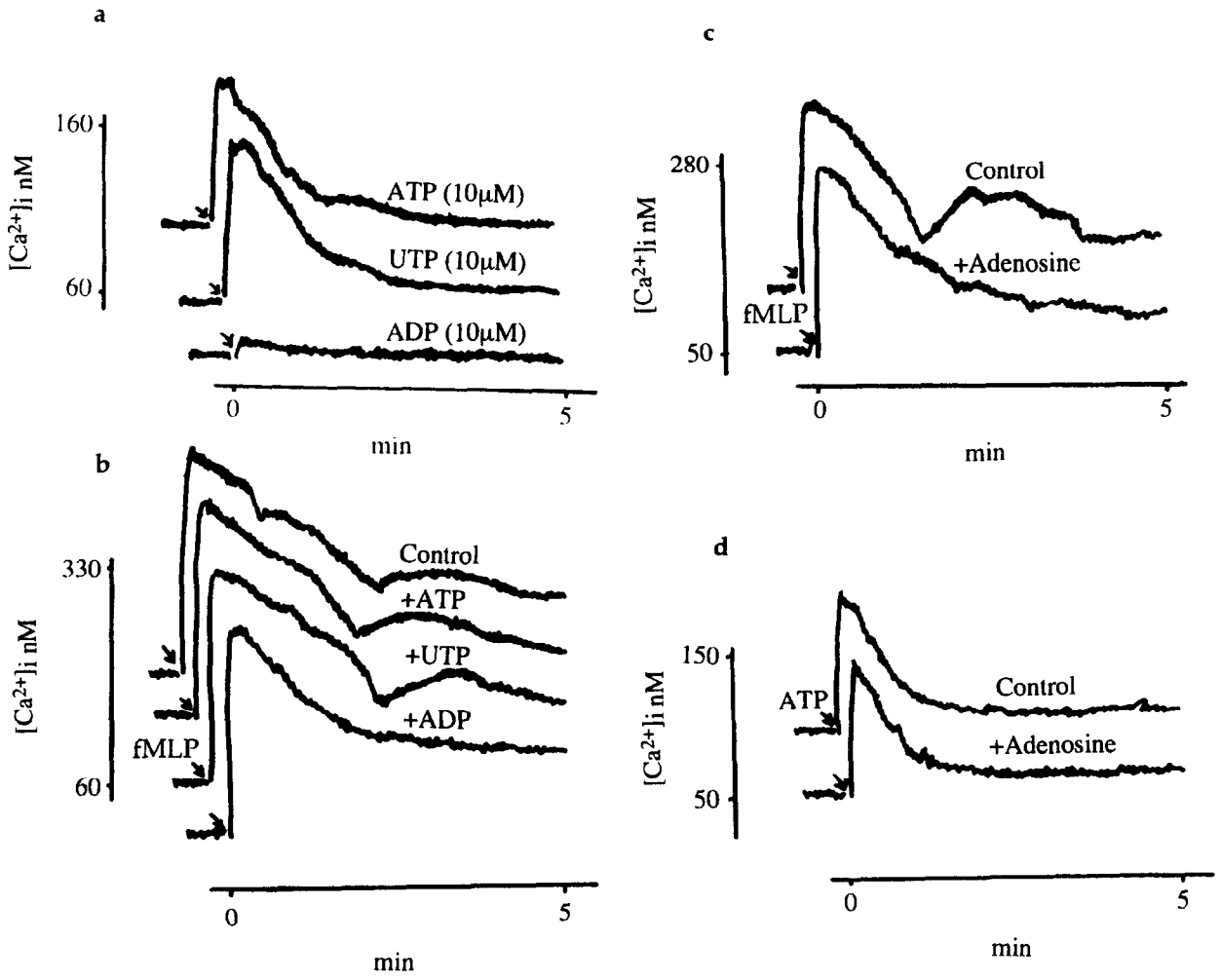

FIG. 5. (a) The effect of ATP or UTP on cytosolic calcium concentration; (b) the effect of fMLP on $[Ca^{2+}]_i$ in the presence of ATP, UTP, and ADP; (c) the effect of adenosine on calcium increase by fMLP; or (d) by ATP. Cytosolic calcium concentration was determined with fura-2 assay as described in the Methods section. These results are from a single experiment and are typical of 4 separate cell preparations.

caused a more rapid decline in the $[Ca^{2+}]_i$ transient compared to that in buffer-treated cells (Fig. 5c). This is in agreement with previous reports demonstrating that neither the generation of IP_3 nor the immediate increase in $[Ca^{2+}]_i$ by fMLP-stimulated neutrophils is affected by treatment with adenosine [22, 23]. However, the duration of the calcium response was shorter because adenosine pretreatment abolished the second $[Ca^{2+}]_i$ rise, suggesting that adenosine affected the fMLP-induced entry of extracellular calcium. Adenosine did not affect the change in $[Ca^{2+}]_i$ evoked by ATP (Fig. 5d).

The finding that ATP induced a transient enhancement of the oxidative burst, which was followed by a marked inhibition, might be compatible with the hypothesis that the inhibition is due to formation of adenosine. We, therefore, examined the metabolism of ATP in PMNs. Indeed, ATP was rapidly converted into ADP and AMP, which were further hydrolyzed into adenosine, starting from 2 min in nonactivated PMNs (Fig. 6).

We then studied whether the potent nonxanthine adenosine receptor antagonist CGS 15943 would modulate the chemiluminescence. It was found to virtually abolish the inhibitory effect of both ATP (Fig. 7a) and ADP (Fig. 7b) on the oxidative burst. Because the results suggest that ATP and adenosine might interact on H_2O_2 production we, therefore, examined the effect of ATP on adenosine-mediated inhibition, as well as that of adenosine on ATP-mediated enhancement of H_2O_2 formation. It is seen that adenosine caused a concentration-dependent inhibition of the H_2O_2-production by fMLP-activated neutrophils (Fig. 8a). In this set of experiments, ATP (at 10 μM) produced a 30% increase in fMLP-stimulated H_2O_2 production, and the combined effect of ATP and fMLP was inhibited by adenosine (Fig. 8a). ATP was able to enhance the H_2O_2-production induced by fMLP, even when the neutrophils had been preincubated for 3 min with a high concentration of adenosine (1 μM) (see Fig. 8b). The magnitude of the ATP-induced enhancement was larger when ATP was added only 30 sec before fMLP, but it was still reduced by adenosine (data not shown). Thus, adenosine does not selectively depress the actions of ATP but, rather, produces a general decrease in the magnitude of the oxidative burst.

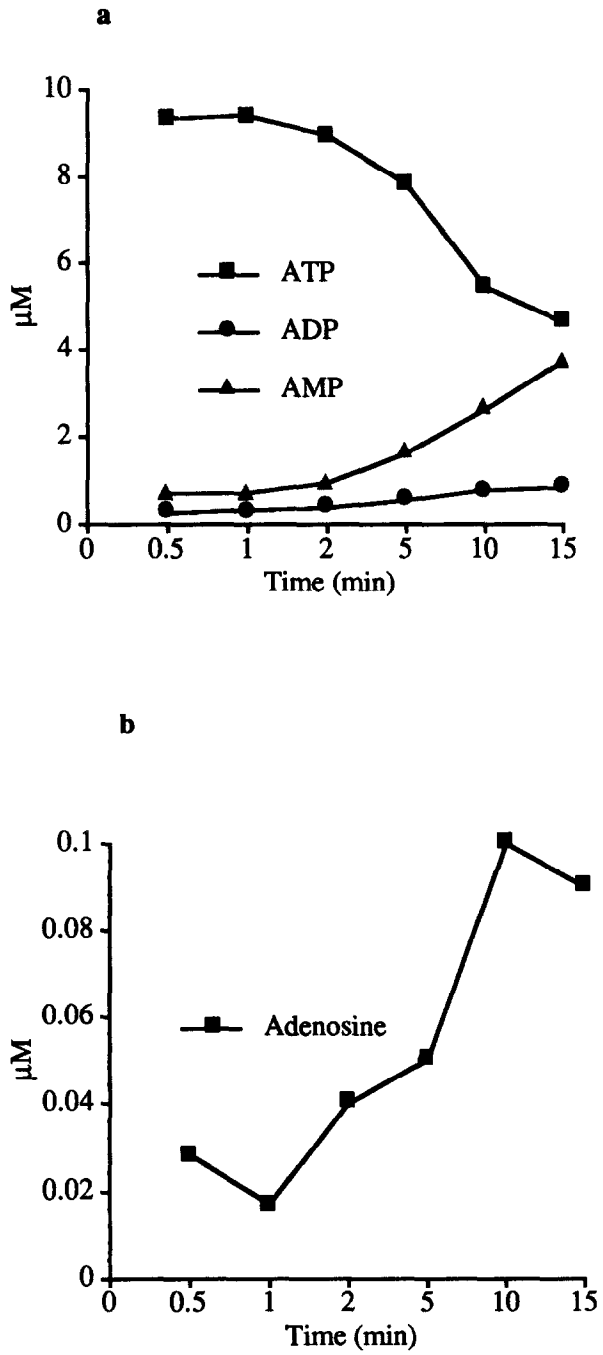

FIG. 6. Purine metabolism in neutrophil leukocytes. Neutrophils (1×10^6/mL) were incubated with ATP (10 μM) at 37°C for the indicated time. After centrifugation for 1 min, the supernatant was incubated at 85°C for 2 min and, then, centrifuged again for 10 min at 4°C. ATP, ADP, AMP, and adenosine were determined as described in the Methods section. The data are means ± standard errors from one experiment, performed in triplicate.

DISCUSSION

ATP and UTP caused a transient increase in cytosolic Ca^{2+}, in agreement with previous results [2, 6, 9, 24]. This was associated with, and probably caused by, a rise in IP_3. It has been clearly shown that the magnitude and rate of exocytotic release from neutrophil leukocytes is determined by the magnitude and rate of the rise in $[Ca^{2+}]_i$ [25], but it is also known that other receptor-mediated events contribute [26]. Accordingly, ATP and UTP induced elastase release, in agreement with previous results [2, 5, 27]. Compared to fMLP, ATP and UTP produced a correspondingly smaller increase in $[Ca^{2+}]_i$, IP_3, and elastase release. We have also confirmed that ATP, when added simultaneously

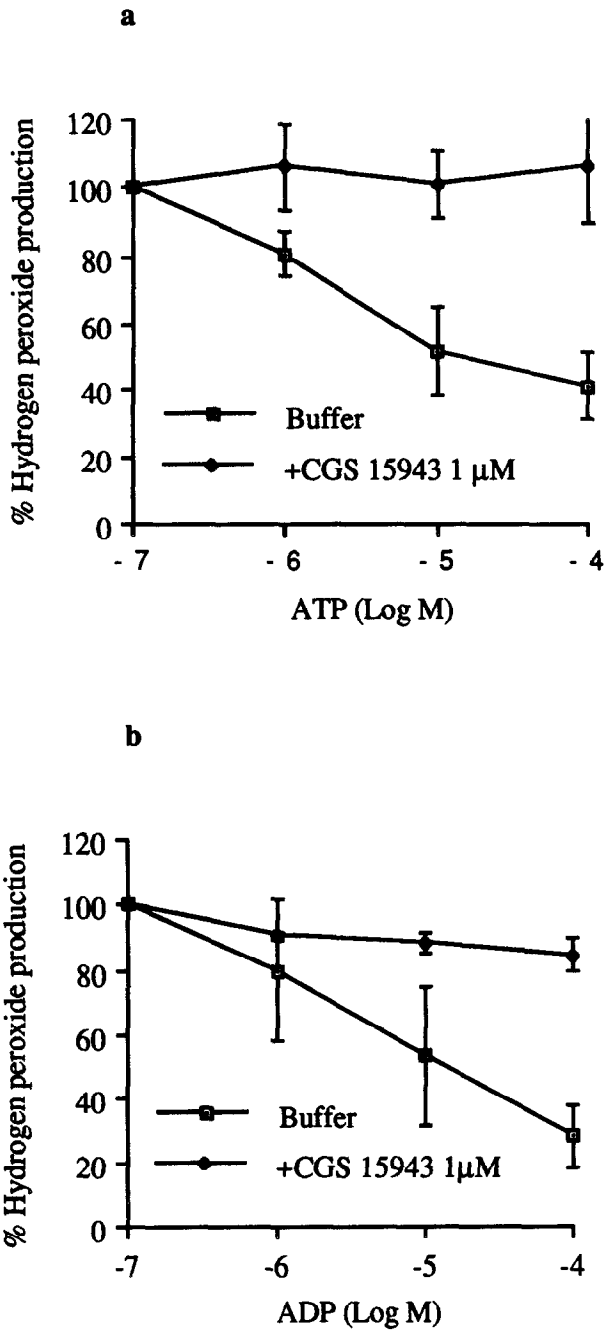

FIG. 7. The effects of ATP and ADP on H_2O_2 production in the absence or presence of CGS 15943. CGS 15943 (1 μM) was added together with ATP or ADP 5 min before fMLP (1 μM). The data are the means ± standard errors of 3 experiments using blood from different donors, normalized to percent of controls.

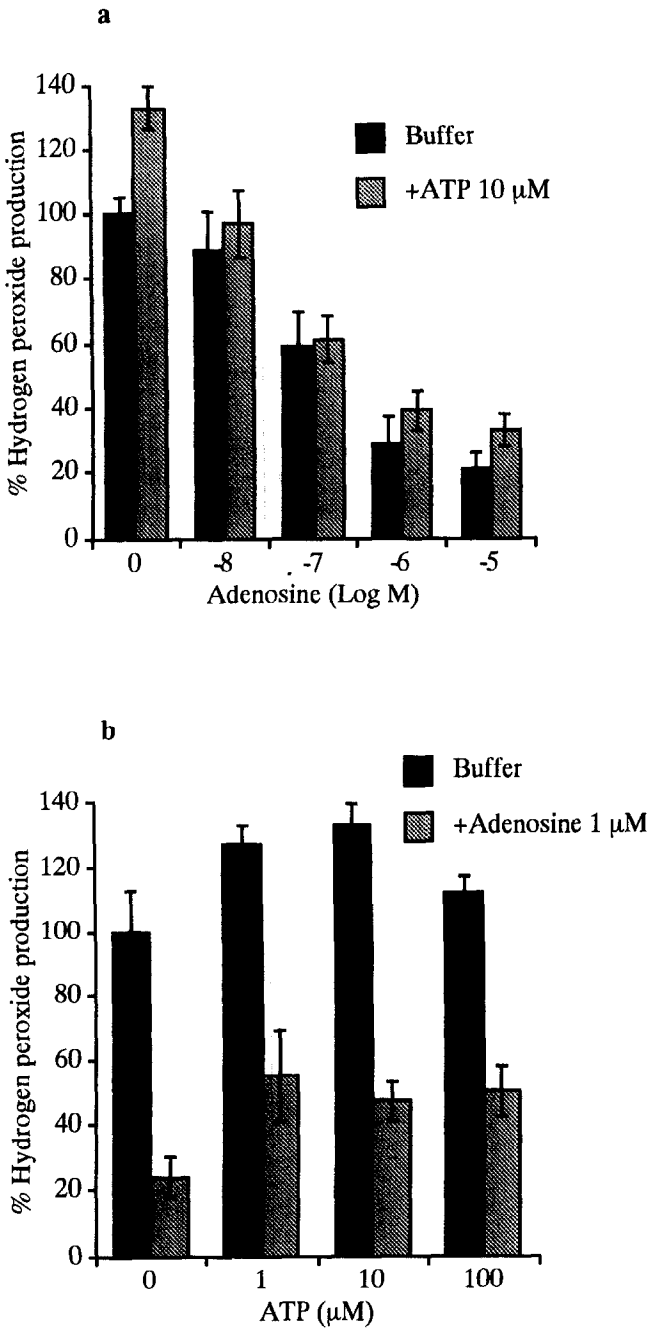

FIG. 8. The effect of ATP and adenosine on H_2O_2 production. (a) PMNs were preincubated with increasing concentrations of adenosine for 3 min, then 10 μM ATP was added 2 min before 1 μM fMLP. (b) PMNs were preincubated with adenosine (1 μM) for 3 min, then increasing concentrations of ATP were added 2 min before 1 μM fMLP. The data are the means ± standard errors of 3 (treatment group) to 6 (buffer group) experiments using blood from different donors, normalized as percent of control.

with or soon before fMLP, can increase the oxidative burst but has no effect when added alone [2, 6, 7, 8, 9, 14].

ATP is known to act on extracellular P_2 purinoceptors, which fall into at least 2 main families: G protein coupled receptors and intrinsic ion channels [11, 28]. The former, responsible for IP_3-mediated increase in $[Ca^{2+}]_i$, and which are tentatively named P_{2Y}, P_{2T}, and P_{2U} receptors [10, 11, 28] are the types that are responsible for the observed responses in neutrophils. Neither ADP, which is able to activate P_{2Y} and P_{2T} but not P_{2U} receptors [8, present data], nor 2MeSATP (2-methylthioadenosine 5′-triphosphate), which can stimulate P_{2X} and P_{2Y} receptors but not P_{2U} receptors [24, 29, 30] caused a strong stimulation [8]. By contrast, UTP, ITP (inosine 5′-triphosphate) and GTP (guanosine 5′-triphosphate), which potently stimulate P_{2U} purinoceptors but are weak agonists at all the other forms, were essentially as active as ATP [8]. Therefore, the receptor is best classified as a P_{2U}-type receptor.

The major finding in this study was that the effect of ATP on the oxidative burst was transient and rapidly converted into an inhibition. The time-course of this change corresponded to the conversion of ATP to AMP and adenosine. Adenosine is known to inhibit the effect of fMLP *via* an action on A_2 receptors [13]. Adenosine is quite potent in this respect, with clearcut inhibition being observed at 10 nM [22, 31]. Levels above this threshold were observed after a few min of incubation and it is likely that the adenosine concentration at the cell surface, which is both the site of action and the site of formation, is considerably higher than in the bulk of medium. The fact that the nonxanthine adenosine antagonist CGS 15943 blocked the late inhibition of H_2O_2 production by ATP provides further evidence that it is due to adenosine. ADP, which can be converted to adenosine as well, also caused an inhibition that was blocked by CGS 15943. By contrast, UTP, which cannot be converted to adenosine, did not produce a secondary inhibition. Even though UTP breakdown was not specifically determined, it is likely that there are ecto-nucleotidases that also rapidly degrade the pyrimidine nucleotides, and that such breakdown explains why the stimulatory effect of UTP was also transient.

Although the effect of ATP on the oxidative burst was biphasic, with an initial stimulation followed by a secondary adenosine-mediated inhibition, there was no direct evidence for an adenosine-mediated inhibition of ATP-stimulated elastase release. This is in agreement with previous results that adenosine is much more potent as an inhibitor of the oxidative burst than of exocytotic release of elastase [1, 9, 13, 22]. This could be related to the fact that adenosine has little or no effect on the initial $[Ca^{2+}]_i$ rise that is related to azurophilic granule release [22, 23].

It is well known that adenosine is able to cause inhibition of the oxidative burst by acting on an A_2 receptor [13]. Two A_2 receptors, a high-affinity A_{2A} and a low-affinity A_{2B} receptor, with different pharmacological characteristics have been identified [see 11]. The high potency of adenosine and the blockade by CGS 15943 suggest that the adenosine receptor is of the A_{2A} subtype. We have confirmed this by the use of selective agonists (e.g., 2-phenylaminoadenosine and CGS 21680) and antagonists (e.g., KF 17387) [11], as well as by the demonstration of A_{2A} receptor mRNA by PCR (Zhang, van der Ploeg and Fredholm, unpublished data).

Although ATP and UTP did not further enhance the $[Ca^{2+}]_i$ increase induced by fMLP, they tended to enhance the second peak of $[Ca^{2+}]_i$. Adenosine and ADP, by contrast, made this second peak disappear. These results indicate that generation of oxidative burst in response to fMLP in nucleotide-primed and adenosine-inhibited neutrophils could relate to the influx of extracellular calcium or alter a late step in the signal transduction pathway, such as the activity, translocation, or proteolysis of protein kinase C [31]. It has been found that protein kinase C activation is a potent stimulus and also a major pathway for agonist-induced phospholipase D activation [32, 33]. Phospholipase D acts primarily on phosphatidylcholine and generates phosphatidic acid, a potential second messenger that may be involved in the activation of NADPH oxidase [34, 35]. It is important in this regard that activation of P_{2U} receptors in smooth muscle cells has been shown to activate phospholipase D [36]. It is an interesting possibility that ATP, UTP, and adenosine may modulate the oxidative burst by interacting with phosoholipase D activation, but other mechanisms may also exist.

Potentiation of neutrophil responses to inflammatory mediators by ATP release from activated platelets and sites of tissue injury could be of major biological importance. It has already been shown *in vitro* that activated platelets can potentiate neutrophil responses [9, 14]. *In vivo*, free ATP concentration in blood can be as high as 20 μM following tissue injury [37]. Thus, it is likely that nucleotides influence neutrophil function at sites of inflammation by conferring a transient activation followed by a secondary inhibition.

In conclusion, purine and pyrimidine nucleotides mediate neutrophil function by a P_2 receptor that is distinct from P_{2X} and P_{2Y} and possibly represents a member of a P_{2U} family. The results also show that the effect of ATP is very transient and is rapidly converted from a stimulation to an inhibition in parallel with the hydrolysis of ATP to adenosine.

We wish to thank Agneta Wallman-Johansson for help with the purine determinations and Assoc. Professor Rigmor Stain-Malmgren for the use of the luminometer. These studies were supported by the Swedish MRC and by a grant from Hoechst AG.

References

1. Freyer DR, Boxer LA, Axtel RA and Todd III RF, Stimulation of human neutrophil adhesive properties by adenine nucleotides. *J Immunol* **141:** 580–586, 1988.
2. Cockcroft S and Stutchfield J, The receptors for ATP and fMetLeuPhe are independently coupled to phospholipases C and A_2 via G-protein(s). *Biochem J* **263:** 715–723, 1989.
3. Stutchfield J and Cockcroft S, Undifferentiated HL 60 cells respond to extracellular ATP and UTP by stimulating phospholipase C activation and exocytosis. *FEBS Lett* **262:** 256–258, 1990.
4. Xie MS, Jacobs LS and Dubyak GR, Regulation of phospholipase D and primary granule secretion by P_2-purinergic and chemotactic peptide-receptor agonists is induced during granulocytic differentiation of HL-60 cells. *J Clin Invest* **88:** 45–54, 1991.
5. Flezar M, Olivenstein R, Cantin A and Heisler S, Extracellular ATP stimulates elastase secretion from human neutrophils and increases lung resistance and secretion from normal rat airways after intratracheal instillation. *Can J Physiol Pharmacol* **70:** 1065–1068, 1992.
6. Axtel RA, Sandborg RR, Smolen JE, Ward PA and Boxer LA, Exposure of human neutrophils to exogenous nucleotides causes elevation in intracellular calcium, transmembrane calcium fluxes, and an alteration of a cytosolic factor resulting in enhanced superoxide production in response to fMLP and arachidonic acid. *Blood* **75:** 1324–1332, 1990.
7. Kuhns DB, Wright DG, Nath J, Kaplan SS and Basford RE, ATP induces transient elevations of $[Ca^{2+}]_i$ in human neutrophils and primes these cells for enhanced O_2^- generation. *Lab Invest* **58:** 448–453, 1988.
8. Seifert R, Wenzel K, Eckstein F and Schultz G, Purine and pyrimidine nucleotides potentiate activation of NADPH oxidase and degranulation by chemotactic peptides and induce aggregation of human neutrophils via G proteins. *Eur J Biochem* **181:** 277–285, 1989.
9. Ward PA, Cunningham TW, McCulloch KK, Phan SH, Powell J and Johnson KJ, Platelet enhancement of O_2^- responses in stimulated human neutrophils: Identification of platelet factor as adenine nucleotide. *Lab Invest* **58:** 37–47, 1988.
10. O'Connor SE, Dainty IA and Leff P, Further subclassification of ATP receptors based on agonist studies (Review). *Trends Pharmacol Sci* **12:** 137–141, 1991.
11. Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P and Williams M, Nomenclature and classification of purinoceptors. *Pharmacol Rev* **46:** 143–156, 1994.
12. Gordon JL, Extracellular ATP: effects, sources and fate. *Biochem J* **233:** 309–319, 1986.
13. Cronstein BN, Adenosine, an endogenous anti-inflammatory agent. *J Appl Physiol* **76:** 5–13, 1994.
14. Naum CC, Kaplan SS and Basford RE, Platelets and ATP prime neutrophils for enhanced O_2^- generation at low concentrations but inhibit O_2^- generation at high concentration. *J Leukocyte Biol* **49:** 83–89, 1991.
15. Ringertz B, Palmblad J, Rådmark O and Malmsten C, Leukotriene-induced neutrophil aggregation in vitro. *FEBS Lett* **147:** 180–182, 1982.
16. Wymann MP, von Tscharner V, Deranleau DA and Baggiolini M, Chemiluminescence detection of H_2O_2 produced by human neutrophils during the respiratory burst. *Anal Biochem* **165:** 371–378, 1987.
17. Fredholm BB, Dunwiddie TV, Bergman B and Lindström K, Levels of adenosine and adenine nucleotides in slices of rat hippocampus. *Brain Res* **295:** 127–136, 1984.
18. Adeyemi EO, Savage AP, Bloom SR and Hodgson HJ, Somatostatin inhibits neutrophil elastase release in vitro. *Peptides* **11:** 869–871, 1990.
19. Gerwins P, Modification of a competitive protein binding assay for determination of inositol 1,4,5-triphosphate. *Anal Biochem* **210:** 45–49, 1993.
20. Nilsson E, Andersson T, Fällman TM, Rosendahl K and Palmblad J, Effects of ethanol on the chemotactic peptide-induced second messenger generation and superoxide production in polymorphonuclear leukocytes. *J Infect Dis* **166:** 854–860, 1992.
21. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:** 3440–3450, 1985.
22. Zhang Y and Fredholm BB, Propentofylline enhancement of the actions of adenosine on neutrophil leukocytes. *Biochem Pharmacol* **48:** 2021–2032, 1994.
23. Walker BAM, Hagenlocker BE, Douglas VK and Ward PA, Effects of adenosine on inositol 1,4,5-triphosphate formation

and intracellular calcium changes in formyl-Met-Leu-Phe-stimulated human neutrophils. *J Leukocyte Biol* **48:** 281–283, 1990.
24. Walker BAM, Hagenlocker BE, Douglas VK, Tarapchak SJ and Ward PA, Nucleotide responses of human neutrophils. *Lab Invest* **64:** 105–112, 1991.
25. Lew PD, Wollheim CB, Waldvogel FA and Pozzan T, Modulation of cytosolic-free calcium transients by changes in intracellular calcium-buffering capacity: Correlation with exocytosis and O_2^- production in human neutrophils. *J Cell Biol* **99:** 1212–1220, 1984.
26. Lew PD, Monod A, Waldvogel FA, Dewald B, Baggiolini M and Pozzan T, Quantitative analysis of the cytosolic free calcium dependency of exocytosis from three subcellular compartments in intact human neutrophils. *J Cell Biol* **102:** 2197–2204, 1986.
27. O'Flaherty JT and Cordes JF, Human neutrophil degranulation responses to nucleotides. *Lab Invest* **70:** 816–821, 1994.
28. Dubyak GR and el-Moatassim C, Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. *Am J Physiol* **265:** C577–C606, 1993.
29. Merritt JE and Moores KE, Human neutrophils have a novel purinergic P_2 type receptor linked to calcium mobilization. *Cell Signal* **3:** 243–249, 1991.
30. Montero M, Garcia-Sancho J and Alvarez J, Biphasical and differential modulation of Ca^{2+} entry by ATP and UTP in promyelocytic leukaemia HL 60 cells. *Biochem J* **305:** 879–889, 1995.
31. Ward PA, Walker BAM and Hagenlocker BE, Functional consequences of interactions between human neutrophils and ATP, ATPγS, and adenosine. In *Biological Actions of Extracellular ATP*. (Ed. Dubyak GR and Fedan JS). *Ann NY Acad Sci* **603:** 108–119, 1990.
32. Natarajan V and Garcia JG, Agonist-induced activation of phospholipase D in bovine pulmonary artery endothelial cells: regulation by protein kinase C and calcium. *J Lab Clin Med* **121:** 337–347, 1993.
33. Pfeilschifter J and Merriweather C, Extracellular ATP and UTP activation of phospholipase D is mediated by protein kinase C-ε in rat renal mesangial cells. *Br J Pharmacol* **110:** 847–853, 1993.
34. Bellavite P, Corso F, Dusi S, Grzeskowiak M, Della-Bianca V and Rossi F, Activation of NADPH-dependent superoxide production in plasma membrane extracts of pig neutrophils by phosphatidic acid. *J Biol Chem* **263:** 8210–8214, 1988.
35. Rossi F, Grzeskowiak M, Della-Bianca V, Calzetti F and Gandini G, Phosphatidic acid and not diacylglycerol generated by phospholipase D is functionally linked to the activation of NADPH oxidase by fMLP in human neutrophils. *Biochem Biophys Res Commun* **168:** 320–327, 1990.
36. Gerwins P and Fredholm BB, Activation of phospholipase C and phospholipase D by stimulation of adenosine A_1, bradykinin or P_{2U} receptors does not correlate well with protein kianse C activation. *Naunyn-Schmiedeberg's Arch Pharmacol* **351:** 194–201, 1995.
37. Born GVR and Kratzer MAA, Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. *J Physiol* **354:** 419–429, 1984.