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Purine and pyrimidine nucleotides potentiate activation of NADPH oxidase and degranulation by chemotactic peptides and induce aggregation of human neutrophils via G proteins

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Whereas the chemotactic peptide, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe), induced NADPH-oxidase-catalyzed superoxide (O_2^-) formation in human neutrophils, purine and pyrimidine nucleotides *per se* did not stimulate NADPH oxidase but enhanced O_2^- formation induced by submaximally and maximally stimulatory concentrations of fMet-Leu-Phe up to fivefold. On the other hand, fMet-Leu-Phe primed neutrophils to generate O_2^- upon exposure to nucleotides. At a concentration of 100 μM, purine nucleotides enhanced O_2^- formation in the effectiveness order adenosine 5′-*O*-[3-thio]triphosphate (ATP[γS]) > ITP > guanosine 5′-*O*-[3-thio]triphosphate (GTP[γS]) > ATP = adenosine 5′-*O*-[2-thio]triphosphate (*Sp*-diastereomer) = GTP = guanosine 5′-*O*[2-thio]diphosphate (GDP[βS] = ADP > adenosine 5′-[β,γ-imido]triphosphate = adenosine 5′-*O*-[2-thio]triphosphate] (*Rp*-diastereomer). Pyrimidine nucleotides stimulated fMet-Leu-Phe-induced O_2^- formation in the effectiveness order uridine 5′-*O*-[3-thio]triphosphate (UTP[γS]) > uridine 5′-*O*-[2-thio]diphosphate (UDP[βS]) = uridine 5′-*O*[2-thio]triphosphate (*Rp*-diastereomer) (*Rp*)-UTP[βS]) = UTP > CTP. Uracil nucleotides were similarly effective potentiators of O_2^- formation as the corresponding adenine nucleotides. GDP[βS] and UDP[βS] synergistically enhanced the stimulatory effects of ATP[γS], GTP[γS] and UTP[γS]. Purine and pyrimidine nucleotides did not induce degranulation in neutrophils but potentiated fMet-Leu-Phe-induced release of β-glucuronidase with similar nucleotide specificities as for O_2^- formation. In contrast, nucleotides *per se* induced aggregation of neutrophils. Treatment with pertussis toxin prevented aggregation induced by both nucleotides and fMet-Leu-Phe. Our results suggest that purine and pyrimidine nucleotides act via nucleotide receptors, the nucleotide specificity of which is different from nucleotide receptors in other cell types. Neutrophil nucleotide receptors are coupled to guanine-nucleotide-binding proteins. As nucleotides are released from cells under physiological and pathological conditions, they may play roles as intercellular signal molecules in neutrophil activation.

Human neutrophils play a major role in host defense reactions against bacterial infections and in the pathogenesis of various diseases such as rheumatoid arthritis, glomerulonephritis, dermatoses, myocardial infarction and asthma [1]. The intercellular signal molecules, PAF, LTB_4 and the chemotactic peptide, fMet-Leu-Phe, stimulate NADPH-oxidase-catalyzed O_2^- formation [2–5]. Combinations of fMet-Leu-Phe plus PAF or LTB_4 synergistically induce O_2^- formation [2]. In addition to O_2^- formation, these agents induce degranulation and aggregation [2–5]. Receptors for fMet-Leu-Phe, PAF and LTB_4 interact with G proteins leading to the activation of phospholipase C. This lipase catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol trisphosphate. Diacylglycerol activates protein kinase C, and inositol trisphosphate mobilizes intracellular calcium [5]. ADP-ribosylation of G proteins by pertussis toxin prevents receptor-mediated cell activation [6, 7]. The mechanism by which fMet-Leu-Phe, PAF and LTB_4 induce cellular activation has been suggested to involve both protein kinase C and calcium mobilization [5, 8, 9].

We and others recently provided evidence that activation of NADPH oxidase involves more direct regulation by G

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Abbreviations. ADP[βS], adenosine 5′-*O*-[2-thio]diphosphate; (*Sp*)-ATP[αS], *Sp*-diastereomer of adenosine 5′-*O*-[1-thio]triphosphate; (*Rp*)-ATP[βS], *Rp*-diastereomer of adenosine 5′-*O*-[2-thio]triphosphate; (*Sp*)-ATP[βS], *Sp*-diastereomer of adenosine 5′-*O*-[2-thio]triphosphate; ATP[γS], adenosine 5′-*O*-[3-thio]triphosphate; [α,β-CH_2]ATP, adenosine 5′-[α,β-methylene]triphosphate; [β,γ-CH_2]ATP, adenosine 5′-[β,γ-methylene]triphosphate; [β,γ-NH]ATP, adenosine 5′-[β,γ-imido]triphosphate; fMet-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; G protein, guanine-nucleotide-binding protein; GDP[βS], guanosine 5′-*O*-[2-thio]diphosphate; GTP[γS], guanosine 5′-*O*-[3-thio]triphosphate; [β,γ-CH_2]GTP, guanosine 5′-[β,γ-methylene]triphosphate; [β,γ-NH]GTP, guanosine 5′-[β,γ-imido]triphosphate; LTB_4, leukotriene B_4; O_2^-, superoxide anion; PAF, platelet-activating factor; UDP[βS], uridine 5′-*O*-[2-thio]diphosphate; (*Rp*)-UTP[βS], *Rp*-diastereomer of uridine 5′-*O*-[2-thio]triphosphate; UTP[γS], uridine 5′-*O*-[3-thio]triphosphate].

Enzymes. Adenylate kinase (EC 2.7.4.3); β-glucuronidase (EC 3.2.1.31); lactate dehydrogenase (EC 1.1.1.27); NADPH oxidase (EC 1.6.99.6); nucleoside-diphosphate kinase (EC 2.7.4.6); protein kinase C (EC 2.7.1.37); superoxide dismutase (EC 1.15.1.1.).

proteins [10–14]. This was suggested by the finding that the phosphorothioate analogue of GTP, GTP[γS], enhanced O_2^- formation in cell-free systems severalfold and that the corresponding GDP analogue, GDP[βS], competitively antagonized the effects of GTP[γS] [10, 14]. Similar observations with GTP[γS] and GDP[βS] have been made for several G-protein-regulated effector systems including the regulation of phospholipase C in permeabilized cells [15–17].

Under physiological and even more so under pathological conditions such as inflammation, thrombosis, hemorrhage, hypoxia and trauma, nucleotides are released into the extracellular space from a variety of cellular systems including neurones, chromaffin cells, endothelium, platelets, and damaged tissues [18–22]. Released ATP binds to purinoceptors which regulate many cell functions [19, 23]. Purinoceptors can be divided into subtypes according to the effectiveness order of purinergic agonists [19, 23]. Phosphorothioate analogues of adenine nucleotides are useful tools to study the stereospecificity of purinoceptors [24, 25]. In addition to their direct effects on G proteins [10, 14–17], both GTP[γS] and GDP[βS] prevent activation of intact platelets via competitive antagonism with ADP at purinoceptors [26, 27].

In addition to ATP, UTP modulates cellular functions. UTP induces contraction or endothelium-dependent relaxation of blood vessels, prostacyclin formation, calcium mobilization and metabolic changes in various cell types [18, 21, 22, 28–30]. The existence of pyrimidinoceptors as a class of nucleotide receptors was suggested by the finding that UTP was similarly effective as ATP in inducing cellular activation [18, 28, 29].

Extracellular nucleotides play a role in the regulation of myeloid cells, as ATP and its phosphorothioate analogue, ATP[γS], induce calcium mobilization in HL-60 leukemic and U-937 myelo-monocytic cells and in mouse macrophages [31, 32]. In addition, nucleotides induce neutrophil aggregation [33, 34] and ATP stimulates the release of β-glucuronidase in guinea-pig neutrophils [35]. Furthermore, Kuhns et al. reported that ATP and UTP induced an increase in the intracellular calcium concentration and enhanced fMet-Leu-Phe-induced O_2^- formation in human neutrophils [36].

All these findings prompted us to study in detail regulation of neutrophil functions by extracellular nucleotides, particularly by pyrimidine nucleotides. We report that purine and pyrimidine nucleotides potentiate fMet-Leu-Phe-induced O_2^- formation and degranulation and *per se* induce aggregation in human neutrophils with similar effectiveness. Using various naturally occurring nucleotides and chemically modified derivatives, especially phosphorothioate analogues, we show that these activations are stereospecific and presumably involve nucleotide receptors and pertussis-toxin-sensitive G proteins.

MATERIALS AND METHODS

Synthesis of (Sp)-ATP[βS], (Rp)-ATP[βS], (Rp)-UTP[βS], UTP[γS] and UDP[βS]

UDP[βS] was synthesized in analogy to the method published for the synthesis of ADP[βS] [37]. UTP[γS] was prepared by incubation of GTP[γS] and UDP in the presence of nucleoside-diphosphate kinase from beef liver (Boehringer Mannheim, Mannheim, FRG) under the conditions described by Goody et al. [38]. UTP[γS] and UDP[βS] were purified by reverse-phase HPLC using a linear gradient of 0–15% (by vol.) acetonitrile in 100 mM triethylammonium bicarbonate, pH 7.0, within 20 min. The diastereomers of ATP[βS] were prepared as described [39]. The *R*p-diastereomer of UTP[βS] was synthesized accordingly. Purity of all compounds was checked by ^{31}P-NMR-spectroscopy, showing the expected chemical shift values.

Other materials

Inorganic pyrophosphate, A23187, *p*-nitrophenyl β-D-glucuronide, [α,β-CH_2]ATP, uridylyl (3′,5′)uridine and TTP were obtained from Sigma Chemie (Deisenhofen, FRG). All other nucleotides were of the highest degree of purity available and were obtained from Boehringer Mannheim (Mannheim, FRG). Stock solutions of nucleotides (10 mM) were prepared in 138 mM NaCl, aliquoted and stored at −20 °C. LTB_4 was a gift of Hoechst AG (Frankfurt/Main, FRG), and pertussis toxin was kindly provided by Dr. Motuyuki Yajima (Kyoto, Japan). PAF was a gift of Dr. Santosh Nigam (Abteilung für Gynäkologische Endokrinologie, Unversitätsklinikum Steglitz, Freie Universität Berlin). Sources of other materials have been described elsewhere [10–13, 40].

Preparation of human neutrophils

Heparinized blood was obtained by venous puncture from healthy volunteers of either sex who had taken no drugs for at least three weeks. Neutrophils were isolated by dextran sedimentation and centrifugation through Ficoll-Hypaque [10, 11, 40, 41]. Cell preparations consisted of more than 98% viable neutrophils as judged by trypan blue dye exclusion.

Assay for O_2^--generation

NADPH-oxidase-catalyzed O_2^- generation was monitored by continuous measurement of ferricytochrome *c* reduction inhibitable by superoxide dismutase, using an Uvikon 810 dual-beam spectrophotometer (Kontron, Eching, FRG). Reaction mixtures (500 µl) contained $1-2\times10^6$ neutrophils, 100 µM ferricytochrome *c* and a buffer consisting of 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose and 20 mM Hepes, pH 7.4. Reaction mixtures were preincubated for 3 min at 37 °C. O_2^- generation was initiated by the addition of stimuli [40]. All assays were performed in the absence of cytochalasin B. The formation of O_2^- was completed within 1–12 min, depending on the stimuli used. The total amounts of O_2^- generated were calculated according to Markert et al. [41], and these values are shown in the tables and figures.

Assay for release of β-glucuronidase

Neutrophils ($2.5-5\times10^6$ cells) were suspended in 500 µl of the buffer described above and were incubated for 5 min at 37 °C in the presence of cytochalasin B (5 µg/ml). Reactions were initiated by the addition of stimuli. After 10 min, reactions were terminated by placing the tubes onto melting ice. Reaction mixtures were centrifuged for 10 min at $250\times g$. The determinations of β-glucuronidase and lactate dehydrogenase activities of the supernatant fluids of reaction mixtures and of cell lysates were performed as described [42].

Aggregation of neutrophils

Aggregation was measured by turbidometry [4, 8]. Neutrophils (5×10^6) were suspended in 900 µl of the buffer described

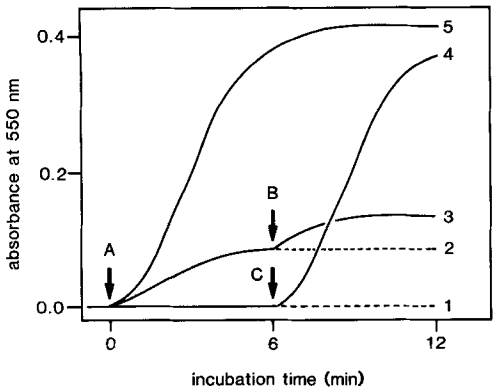

Fig. 1. *Kinetics of O_2^- formation in neutrophils.* (A) Addition of either 100 μM UTP[γS] (trace 1), 30 nM fMet-Leu-Phe (trace 2) or 100 μM UTP[γS] plus 30 nM fMet-Leu-Phe (trace 5); (B) addition of 100 μM UTP[γS] (trace 3) or solvent (trace 2, dotted line) to assays containing 30 nM fMet-Leu-Phe; (C) addition of 30 nM fMet-Leu-Phe (trace 4) or solvent (trace 1, dotted line) to assays containing 100 μM UTP[γS]. Superimposed original registrations from one single experiment are shown. Similar results were obtained in four experiments with neutrophils of different donors

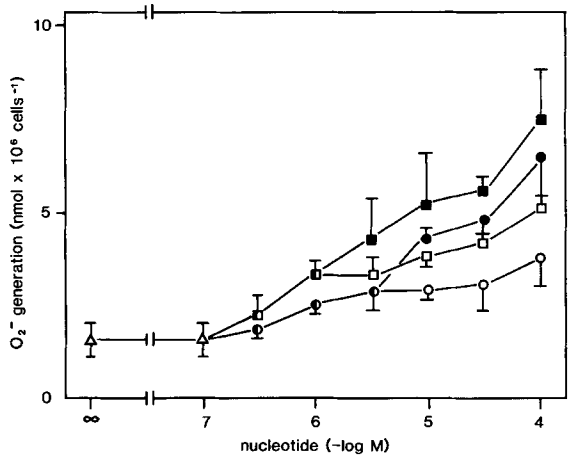

Fig. 2. *Concentration/response curves for ATP, ATP[γS], UTP and UTP[γS] on fMet-Leu-Phe-induced O_2^- formation.* Assays contained fMet-Leu-Phe (30 nM) and various concentrations of nucleotides. Nucleotides and fMet-Leu-Phe were simultaneously added to reaction mixtures. (○) ATP; (●) ATP[γS]; (□) UTP; (■) UTP[γS]. Data shown respresent the mean ± SEM of eight experiments performed with neutrophils of different donors

above. Cells were incubated for 3 min at 37 °C in the presence of cytochalasin B (1 μg/ml) prior to addition of stimuli. Aggregation experiments were carried out under constant stirring of cells at 10^3 rpm, using an Uvikon 810-dual beam spectrophotometer.

RESULTS

The effects of purine and pyrimidine nucleotides on O_2^- formation were studied. The kinetics of O_2^- formation are shown in Fig. 1. The phosphorothioate analogue of UTP, UTP[γS], *per se* did not activate O_2^- formation, even in the presence of cytochalasin B. However, at a concentration of 100 μM, UTP[γS] enhanced O_2^- formation induced by fMet-Leu-Phe (30 nM) fivefold with respect to V_{max} and the total amount of O_2^- generated. The effect of UTP[γS] was rapid in onset, as preincubation with UTP[γS] for 6 min prior to addition of fMet-Leu-Phe did not result in a greater enhancement of O_2^- generation than by simultaneous addition of stimuli. fMet-Leu-Phe made the cells responsive to UTP[γS]. After cessation of the fMet-Leu-Phe-induced O_2^- formation, UTP[γS] reactivated O_2^- formation to an extent amounting to 60% of that induced by fMet-Leu-Phe. The duration of the UTP[γS]-induced O_2^- formation was shorter than that induced by the chemotactic peptide. Similar results as with UTP[γS] were obtained with UTP, ATP and UTP[γS] (data not shown). After cessation of the O_2^- formation induced by UTP[γS] or ATP[γS] at a concentration of 10 μM, the re-exposure to a tenfold higher concentration of either UTP[γS] or ATP[γS] did not reactivate NADPH oxidase (data not shown). These results indicate that activations of NADPH oxidase by UTP[γS] and ATP[γS] were cross-desensitized as has been shown for several stimuli in neutrophils [43].

The concentration/response curves to stimulation of fMet-Leu-Phe-induced O_2^- formation by extracellular nucleotides are shown *in pars pro toto* for ATP, ATP[γS], UTP and UTP[γS] (Fig. 2). Nucleotides concentration-dependently enhanced fMet-Leu-Phe-induced O_2^- formation. The effects of nucleotides were evident at concentrations above 0.3 μM and did not reach a plateau at a concentration of 100 μM. This type of concentration/response curve has been observed for the activation of numerous cellular functions by purine and pyrimidine nucleotides [18, 24, 25, 44]. As nucleotide concentrations >100 μM are unlikely to occur in the extracellular space *in vivo* [18, 19, 22, 29], and as nucleotides at higher concentrations may permeabilize cells [45, 46], the effects of higher concentrations of nucleotides were not investigated. In neutrophils nucleotides at concentrations of up to 100 μM did not cause cell damage as revealed by trypan blue dye exclusion (data not shown) and the release of lactate dehydrogenase (see below).

The nucleotides specificities for NADPH oxidase activation by purine and pyrimidine nucleotides were investigated. At a concentration of 100 μM, the purine nucleotides, ATP[γS], ITP, GTP[γS], ATP, (*S*p)-ATP[βS], GTP, ADP, (*R*p)-ATP[βS] and [β,γ-NH]ATP significantly enhanced chemotactic-peptide-induced O_2^- formation in the effectiveness order given in Table 1. ADP[βS], (*S*p)-ATP[αS], [α,β-CH_2]ATP, [β,γ-CH_2]ATP, AMP, [β,γ-NH]GTP, [β,γ-CH_2]GTP and GDP were inactive. In contrast, adenosine inhibited O_2^- formation by more than 50%, presumably via adenosine A_2 receptors [47]. The pyrimidine nucleotides, UTP[γS], (*R*p)-UTP[βS], UDP[βS], UTP and CTP significantly enhanced fMet-Leu-Phe-induced O_2^- formation as shown in Table 1. In contrast, uridylyl (3′,5′)uridine, UDP, UDP-glucose, UMP, uridine, TTP, inorganic orthophosphate and pyrophosphate as well as the coenzymes, NAD^+ and $NADP^+$, were inactive. UTP[γS], (*R*p)-UTP[βS], UTP and UDP[βS] but not UDP were significantly more effective potentiators of O_2^- formation than the corresponding adenine nucleotides.

As far as regulation of G proteins by phosphorothioate analogues is concerned, a tenfold excess of GDP[βS] antagonizes the stimulatory effects of GTP[γS] [10, 14, 15]. Therefore, we investigated the possibility that phosphorothioate analogues of nucleoside diphosphates are partial antagonists at neutrophil nucleotide receptors, i.e. that they prevent potentiation of O_2^- formation by phosphorothioate analogues of nucleoside triphosphates. In platelets, both GDP[βS] and GTP[γS] are antagonists at

Table 1. *Nucleotide specificity for stimulation of fMet-Leu-Phe-induced O_2^- formation*
Nucleotides (100 μM each) or solvent (control) were added to reaction mixtures simultaneously with fMet-Leu-Phe (30 nM). Data shown represent the mean ± SEM of eight experiments performed with neutrophils of different donors. The numbers indicate the effectiveness order of nucleotides. Statistical significance of the effectiveness of nucleotides was assessed using the Wilcoxon test. Values of *P* are for comparison of nucleotides versus control (n.s. = not significant); values in brackets are comparison of UTP versus ATP, UTP[γS] versus ATP[γS], UDP versus ADP, UDP[βS] versus ADP[βS] and (*R*p)-UTP[βS] versus (*R*p)-ATP[βS]

Nucleotide		O_2^- generation	*P*
		nmol/10^6 cells	
Control		1.5 ± 0.5	
Purines	1. ATP[γS]	6.5 ± 1.0	<0.01
	2. ITP	6.0 ± 1.0	<0.01
	3. GTP[γS]	4.6 ± 1.5	<0.01
	4. ATP	3.9 ± 0.7	<0.01
	5. GTP	3.5 ± 0.7	<0.01
	6. (*S*p)-ATP[βS]	3.3 ± 0.4	<0.01
	7. GDP[βS]	3.2 ± 1.2	<0.01
	8. ADP	3.1 ± 0.6	<0.01
	9. [β,γ-NH]ATP	2.4 ± 0.8	<0.05
	10. (*R*p)-ATP[βS]	2.2 ± 0.3	<0.05
	ADP[βS]	1.9 ± 0.3	n.s.
	AMP	1.6 ± 0.4	n.s.
	[α,β-CH_2]ATP	1.4 ± 0.6	n.s.
	[β,γ-CH_2]ATP	1.3 ± 0.3	n.s.
	GDP	1.3 ± 0.4	n.s.
	[*S*p]-ATP[αS]	1.2 ± 0.6	n.s.
	[β,γ-CH_2]GTP	1.2 ± 0.6	n.s.
	[β,γ-NH]GTP	1.2 ± 0.5	n.s.
	Adenosine	0.5 ± 0.1	<0.01
Pyrimidine and other	1. UTP[γS]	7.5 ± 1.3	<0.01 (<0.05)
	2. UDP[βS]	5.2 ± 2.1	<0.01 (<0.01)
	3. UTP	5.1 ± 0.4	<0.01 (<0.05)
	4. (*R*p)-UTP[βS]	4.8 ± 0.5	<0.01 (<0.01)
	5. CTP	3.5 ± 0.5	<0.01
	UDP	2.2 ± 0.8	n.s. (<0.05)
	UDP-glucose	1.5 ± 0.6	n.s.
	$NADP^+$	1.6 ± 0.5	n.s.
	Pyrophosphate	1.5 ± 0.6	n.s.
	UMP	1.5 ± 0.5	n.s.
	NAD^+	1.4 ± 0.4	n.s.
	Orthophosphate	1.4 ± 0.2	n.s.
	TTP	1.3 ± 0.2	n.s.
	Uridine	1.2 ± 0.3	n.s.
	Uridylyl-uridine	1.2 ± 0.2	n.s.

purinoceptors [26, 27]. ADP[βS] at a concentration of 100 μM neither inhibited nor significantly enhanced activation of NADPH oxidase by fMet-Leu-Phe plus ATP[γS], GTP[γS] or UTP[γS] at a concentration of 10 μM (Table 2). In contrast to ADP[βS], GDP[βS] and UDP[βS] *per se* did not only potentiate fMet-Leu-Phe-induced O_2^- formation but also additively or synergistically enhanced NADPH oxidase activation by fMet-Leu-Phe plus ATP[γS], GTP[γS] or UTP[γS]. These results demonstrate that the effects of GDP[βS] and GTP[γS] on NADPH oxidase in intact cells differ from those in cell-free systems [10, 14].

The effects of UTP and UTP[γS] on NADPH oxidase activation by various concentrations of fMet-Leu-Phe were studied (Fig. 3). UTP and UTP[γS] did not prime the cells to respond to non-stimulatory concentrations of fMet-Leu-Phe (1 – 3 nM), indicating that a small but over-threshold activation of NADPH oxidase by fMet-Leu-Phe (10 – 30 nM) was required for synergistic interaction with UTP or UTP[γS]. The relative stimulatory effects of UTP and UTP[γS] were greater in the presence of submaximally stimulatory concentrations of fMet-Leu-Phe than in the presence of maximally stimulatory concentrations. Conversely, the absolute stimulatory effects of UTP and UTP[γS] were greater at high concentrations of fMet-Leu-Phe. In the experiment shown in Fig. 3, nucleotides lowered the EC_{50} for activation of NADPH oxidase by fMet-Leu-Phe from 130 nM to 60 – 75 nM. In two separate experiments with neutrophils of different donors UTP reduced the EC_{50} for NADPH oxidase activation by fMet-Leu-Phe from 60 nM to 40 nM and from 50 nM to 30 nM (data not shown). A similar range of interindividual variation of the EC_{50} for NADPH oxidase activation by fMet-Leu-Phe has been reported by Simpkins et al. [48]. In analogy to UTP and UTP[γS], ATP and ATP[γS] also lowered the EC_{50} for fMet-Leu-Phe (data not shown).

In addition to fMet-Leu-Phe, PAF and LTB_4 are well-known activators of neutrophils [1 – 3, 5]. Therefore, we studied the interactions of various stimuli on O_2^- formation (Table 3). In preliminary experiments, the concentration of PAF and LTB_4 used in the present study (300 nM for each) was found to be maximally effective to activate O_2^- formation. In agreement with previous results, we found that PAF and LTB_4 at maximally stimulatory concentrations activated O_2^- formation much less effectively than fMet-Leu-Phe, even when the latter agonist was present only at a submaximally stimulatory concentration (30 nM) [2, 3]. In addition, the activations of NADPH oxidase by PAF and LTB_4 were very brief and lasted less than 3 min. These differences in the capacities of the intercellular signal molecules to activate NADPH oxidase may be explained by the fact that in contrast to fMet-Leu-Phe, PAF and LTB_4 lead only to a transient generation of polyphosphoinositol-lipid-derived second messengers, the concentrations of which may be insufficient for a prolonged activation of NADPH oxidase [49 – 51]. fMet-Leu-Phe plus PAF synergistically activated NADPH oxidase to a similar extent as did fMet-Leu-Phe plus UTP[γS]. The combination of fMet-Leu-Phe plus LTB_4 resulted in a less pronounced synergistic activation of NADPH oxidase [2]. Interestingly, the combination of PAF plus LTB_4 was as effective in inducing O_2^- formation as was fMet-Leu-Phe plus LTB_4. In contrast, UTP[γS] caused only a small but significant enhancement of PAF- or LTB_4-induced O_2^- formation. Essentially the same results were obtained with ATP[γS] (data not shown).

The effects of nucleotides on degranulation in human neutrophils were studied. Similarly to activation of NADPH oxidase, UTP[γS] and ATP[γS] (data only shown for ATP[γS]) *per se* did not induce the release of β-glucuronidase (Fig. 4). At a maximally stimulatory concentration (1 μM), fMet-Leu-Phe induced the release of 40 ± 5% (mean ± SEM, $n = 11$) of cellular β-glucuronidase. The release of β-glucuronidase from neutrophils in the presence of cytochalasin B (5 μg/ml) and in the absence of agonists amounted to 6% of cellular content and paralleled the release of lactate dehydrogenase. In the presence of fMet-Leu-Phe at a submaximally stimulatory concentration (10 nM), the release of β-glucuronidase amounted to 12% of the cellular content. The effect of fMet-Leu-Phe was enhanced by ATP[γS] and UTP[γS] at concentrations above 1 μM without an increase in the release of lactate dehydrogenase. This specificity of nucleotides for the

Table 2. *Interaction of phosphorothioate analogues of nucleoside diphosphates (NDP[βS]) and nucleoside triphosphates (NTP[γS]) on fMet-Leu-Phe-induced O_2^- generation*
Nucleotides or solvent (control) were added to reaction mixtures simultaneously with fMet-Leu-Phe (30 nM). Data shown represent the mean ± SEM of eight experiments performed with neutrophils of different donors. The effect of phosphorothioate analogues of nucleoside diphosphates on O_2^- formation in the absence or presence of phosphorothioate analogues of nucleoside triphosphates was assessed statistically using the Wilcoxon test; n.s. = not significant

NTP[γS] (10 μM)	O_2^- generation with 100 μM NDP[βS] (*P*)			
	none	ADP[βS]	GDP[βS]	UDP[βS]
	nmol/10^6 cells			
none	1.5 ± 0.5	1.9 ± 0.3 (n.s.)	3.2 ± 1.2 (<0.05)	5.2 ± 2.1 (<0.01)
ATP[γS]	4.3 ± 1.5	4.4 ± 1.7 (n.s.)	7.0 ± 1.4 (<0.05)	9.4 ± 2.5 (<0.01)
GTP[γS]	3.0 ± 1.2	4.3 ± 1.4 (n.s.)	4.7 ± 0.6 (<0.05)	7.4 ± 0.2 (<0.01)
UTP[γS]	4.0 ± 1.0	5.3 ± 1.7 (n.s.)	8.5 ± 1.6 (<0.01)	10.7 ± 2.7 (<0.01)

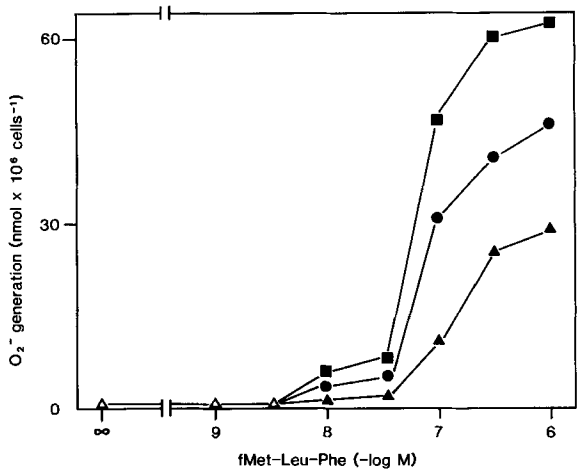

Fig. 3. *Concentration/response curve for fMet-Leu-Phe-induced O_2^- formation in the absence and presence of UTP or UTP[γS].* The effect of fMet-Leu-Phe at various concentrations was studied in the absence of nucleotides (▲) and in the presence of 100 μM UTP (●) or 100 μM UTP[γS] (■). Nucleotides and fMet-Leu-Phe were simultaneously added to reaction mixtures. Data shown represent the means of assay duplicates which varied less than 5%. Similar results were obtained in three experiments with neutrophils of different donors

Table 3. *Synergistic activation of O_2^- formation by combinations of fMet-Leu-Phe, PAF, LTB_4 and UTP[γS]*
The concentrations of stimuli in reaction mixtures were as follows. fMet-Leu-Phe, 30 nM; PAF and LTB_4, 300 nM; UTP[γS], 100 μM. In the combination experiments, stimuli were added simultaneously to reaction mixtures. Data shown represent the mean ± SEM of eight experiments performed with neutrophils of different donors. The statistical significance of the effects of combinations of stimuli versus the controls (one stimulus only) was assessed using the Wilcoxon test; n.s. = not significant

Stimulus	O_2^- generation
	nmol/10^6 cells
fMet-Leu-Phe	1.5 ± 0.5
PAF	0.2 ± 0.1
LTB_4	0.2 ± 0.1
UTP[γS]	0
fMet-Leu-Phe plus PAF	7.6 ± 2.5 (<0.01)
fMet-Leu-Phe plus LTB_4	2.3 ± 0.5 (<0.05)
fMet-Leu-Phe plus UTP[γS]	7.5 ± 1.5 (<0.01)
PAF plus LTB_4	2.1 ± 1.0 (<0.01)
PAF plus UTP[γS]	0.5 ± 0.1 (<0.01)
LTB_4 plus UTP[γS]	0.4 ± 0.2 (<0.05)

release of β-glucuronidase demonstrates that nucleotides did not increase the plasma membrane permeability. As was the case for potentiation of fMet-Leu-Phe-induced O_2^- formation, the concentration/response curves to nucleotides for degranulation did not reach saturation at 100 μM.

The nucleotide specificities for degranulation were similar to those for O_2^- formation, but the differences in the effectiveness of nucleotides were less marked (Table 4). At a concentration of 100 μM, ATP, ATP[γS], ITP, UTP, UTP[γS] and UDP[βS] were similarly effective potentiators of degranulation, whereas CTP, UDP, ADP, GTP, GTP[γS] and GDP[βS] were slightly less effective. In contrast, AMP, ADP[βS], (*Sp*)-ATP[αS], [β,γ-NH]ATP, [α,β-CH_2]ATP, [β,γ-CH_2]ATP, GDP, [β,γ-NH]GTP, [β,γ-CH_2]GTP, UMP and TTP did not significantly enhance the release of β-glucuronidase (data not shown). In analogy to activation of NADPH oxidase, the relative extent of synergistic degranulation by fMet-Leu-Phe and nucleotides was more pronounced at submaximally stimulatory concentrations of the chemotactic peptide. However, even in the presence of a maximally stimulatory concentration of fMet-Leu-Phe (1 μM), ATP[γS] (100 μM) stimulated the extent of β-glucuronidase release from 40% up to 50% of the cellular content (data not shown).

Finally, we investigated the effects of nucleotides on aggregation of human neutrophils. Typical superimposed aggregation traces are shown (Fig. 5). UTP *per se* induced aggregation, reaching a maximum at a concentration of 100 μM. Both V_{max} and the maximum extent of aggregation depended on the nucleotide concentration. The extent of aggregation induced by UTP (100 μM) amounted to 48% of that induced by a maximally stimulatory concentration of fMet-Leu-Phe (1 μM). Aggregation induced by UTP followed similar kinetics as that induced by the chemotactic peptide and was irreversible. In addition, UTP synergistically enhanced aggregation induced by a submaximally stimulatory concentration of fMet-Leu-Phe. At a concentration of 10 mM, fMet-Leu-Phe induced aggregation to an extent amounting to 34% of that of fMet-Leu-Phe at a concentration of 1 μM. UTP concentration-dependently enhanced aggregation induced by fMet-Leu-Phe (10 nM) up to the same extent as fMet-Leu-Phe at a maximally stimulatory concentration. The nucleotide

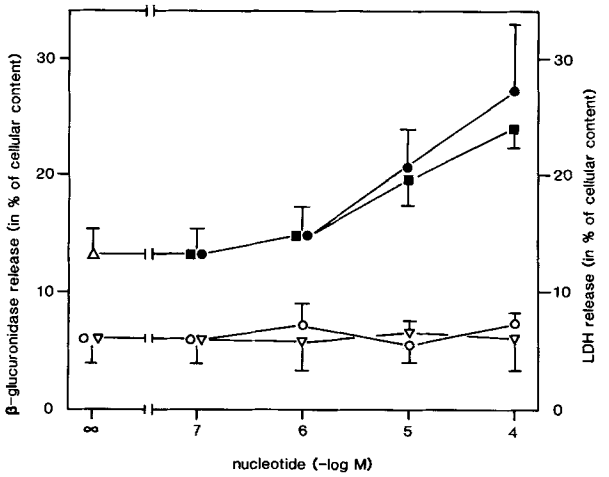

Fig. 4. *Concentration/response curves for ATP[γS] and UTP[γS] on fMet-Leu-Phe-induced release of β-glucuronidase.* The release of β-glucuronidase from neutrophils under various conditions during an incubation time of 10 min was determined. (●) Release of β-glucuronidase by fMet-Leu-Phe (10 nM) plus ATP[γS]; (▽) release of lactate dehydrogenase (LDH) by fMet-Leu-Phe (10 nM) plus ATP[γS]; (■) release of β-glucuronidase by fMet-Leu-Phe (10 nM) plus UTP[γS]; (○) release of β-glucuronidase by ATP[γS]. Data shown represent the mean ± SEM of seven experiments performed with neutrophils of different donors

specificities for the induction of aggregation are summarized in Table 5. ATP, ATP[γS], ITP, UTP and UTP[γS] at a concentration of 100 μM were similarly effective. ADP and CTP were less effective than ATP and UTP. GTP, GTP[γS], ADP[βS], TTP, UDP[βS] and UDP only marginally induced aggregation, and UMP as well as AMP were totally inactive.

The role of pertussis toxin-sensitive G proteins in the activation of neutrophils by extracellular nucleotides was studied. In agreement with previous results [6] we found that treatment of neutrophils with pertussis toxin at a concentration of 1 μg/ml for 3 h completely prevented fMet-Leu-Phe-induced O_2^- formation. In addition, pertussis-toxin-treated cells did not generate O_2^- upon exposure to fMet-Leu-Phe plus ATP[γS] or UTP[γS] (data not shown). Using these conditions of treatment pertussis toxin almost completely inhibited aggregation by fMet-Leu-Phe, ATP and UTP but not aggregation induced by the calcium ionophore, A23187, which circumvents receptor-stimulation by directly increasing the intracellular calcium concentration (Fig. 6). These results indicate that both the effects of fMet-Leu-Phe and of extracellular nucleotides are mediated via G proteins.

DISCUSSION

In addition to purine nucleotides, extracellular pyrimidine nucleotides are known to activate various cellular functions with similar effectiveness as purine nucleotides [18, 22, 28–30, 33]. Whereas much work has been performed to elucidate the mechanism by which purine nucleotides mediate their effects, much less attention has been paid to the effects of pyrimidine nucleotides. As ATP and UTP are known to induce aggregation and calcium mobilization in white blood cells [31–34], we investigated human neutrophils as a model system for purinergic and pyrimidinergic cell activation. Due to the low potency of nucleotides and the presence of ecto-nucleotidase activity in the plasma membrane of neutrophils

Table 4. *Nucleotide specificity for stimulation of fMet-Leu-Phe-induced β-glucuronidase release*
The release of β-glucuronidase induced by fMet-Leu-Phe at a concentration of 10 nM during an incubation time of 10 min was measured, as a percentage of cellular content, in the absence (control) and presence of various nucleotides at a concentration of 100 μM. The numbers indicate the effectiveness order of nucleotides. Data shown represent the mean ± SEM of seven experiments performed with neutrophils of different donors. The effectiveness of stimuli was assessed statistically using the Wilcoxon test. Values of *P* are for comparison of nucleotides versus control; n.s. = no significant; values in brackets are comparison of UTP versus ATP, UTP[γS] versus ATP[γS], UDP versus ADP and UDP[βS] versus ADP[βS]

Nucleotide	β-Glucuronidase release		*P*
		%	
Control		13 ± 2	
Purines	1. ATP	30 ± 7	<0.01
	2. ATP[γS]	27 ± 6	<0.01
	3. ITP	24 ± 3	<0.01
	4. GTP	21 ± 4	<0.01
	5. ADP	20 ± 6	<0.05
	6. GTP[γS]	19 ± 4	<0.05
	7. GDP[βS]	18 ± 3	<0.05
	ADP[βS]	16 ± 3	n.s.
Pyrimidines	1. UTP[γS]	24 ± 2	<0.01 (n.s.)
	2. UTP	23 ± 3	<0.01 (n.s.)
	3. UDP[βS]	23 ± 6	<0.05 (<0.05)
	4. UDP	19 ± 2	<0.05 (n.s.)
	5. CTP	19 ± 3	<0.05 (n.s.)

[52], binding studies with nucleotides are difficult to perform. Therefore, nucleotide receptors in neutrophils at present can only be assessed by measuring cell functions, as is the case for most other nucleotide-regulated cell systems [19, 23]. We measured O_2^- formation, release of β-glucuronidase and aggregation as parameters of neutrophil activation. We found that extracellular nucleotides activated neutrophils at concentrations between 0.1–100 μM. As the concentration of ATP has been shown to reach 20 μM in the systemic circulation after tissue injury [19], neutrophil activation by extracellular nucleotides obviously is of physiological relevance. The fact that nucleotides *per se* lead only to aggregation but not to activation of O_2^- formation and degranulation suggests that stimulations of these functions require different signal transduction pathways. In addition, activations of O_2^- formation and degranulation appear not to be prerequisites for induction of aggregation.

Several findings suggest that purine and pyrimidine nucleotides act via plasma membrane receptors. Most importantly, nucleotide-induced aggregation of neutrophils in pertussis-toxin-sensitive (see Fig. 6), suggesting that nucleotide receptors interact with G proteins. Interaction of purinoceptors with pertussis-toxin-sensitive G proteins has also been shown for inhibition of adenylyl cyclase in liver [53]. In addition, the desensitization of cell responses to nucleotides is a typical characteristic of receptor-mediated processes [43]. Furthermore, the stereospecificity of neutrophil activation by purine and pyrimidine nucleotides points to the recognition of nucleotides by specific receptors.

ATP, GTP and ITP are similarly effective agonists at neutrophil purinoceptors. In addition, neutrophil purinoceptors stereoselectively discriminate between phosphoro-

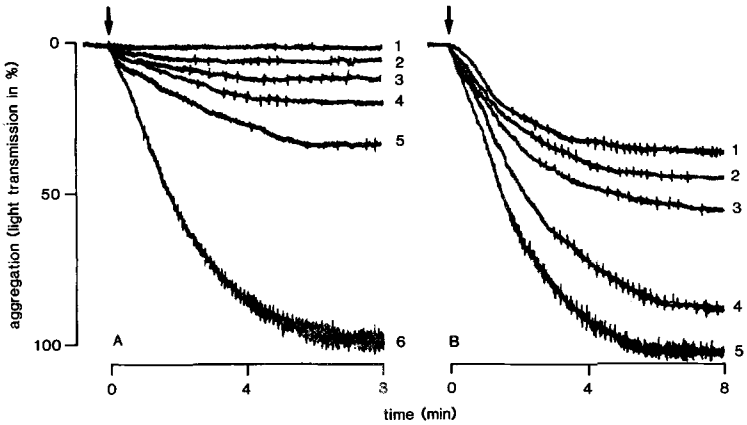

Fig. 5. *Induction of aggregation of neutrophils by UTP: synergism with fMet-Leu-Phe.* Arrows indicate the addition of stimuli to stirred suspensions of neutrophils. The extent of aggregation is referred to that induced by fMet-Leu-Phe (1 μM), which amounted to 100%. Superimposed original registrations from one representative experiment performed in duplicate are shown. The variation of responses within one experiment was less than 10%. Similar results were obtained in three experiments with neutrophils of different donors. (A) Trace 1, UTP 0 μM; trace 2, UTP 0.1 μM; trace 3, UTP, 1 μM; trace 4, UTP 10 μM; trace 5, UTP 100 μM or UTP 300 μM; trace 6, fMet-Leu-Phe 1 μM. (B) All assays contained fMet-Leu-Phe (10 nM) plus UTP at the indicated concentrations. Trace 1, UTP 0 μM; trace 2, UTP 0.1 μM; trace 3, UTP 1 μM; trace 4, UTP 10 μM; trace 5, UTP 100 μM

Table 5. *Nucleotide specificity for induction of neutrophil aggregation* To stirred suspensions of neutrophils, nucleotides were added at a concentration of 100 μM. The extent of aggregation (measured as percentage light transmission) is referred to that induced by fMet-Leu-Phe (1 μM), which effect amounted to 100%. The numbers indicate the effectiveness order of nucleotides. Data shown represent the mean ± SEM of 3–7 experiments performed with neutrophils of different donors. The effectiveness of UTP versus ATP, UTP[γS] versus ATP[γS], UDP versus ADP and UDP[βS] versus ADP[βS] was assessed statistically using the Wilcoxon test; n.s. = not significant

Nucleotides		Aggregation	*P*
		%	
Purines	1. ATP	41 ± 12	
	2. ATP[γS]	36 ± 3	
	3. ITP	29 ± 11	
	4. ADP	19 ± 5	
	5. ADP[βS]	9 ± 5	
	6. GTP	6 ± 2	
	7. GTP[γS]	5 ± 4	
	AMP	0	
Pyrimidines	1. UTP	48 ± 12	(n.s.)
	2. UTP[γS]	35 ± 11	(n.s.)
	3. CTP	19 ± 5	
	4. UDP[βS]	5 ± 4	(n.s.)
	5. UDP	3 ± 2	(<0.01)
	6. TTP	3 ± 1	
	UMP	0	

thioate analogues of ATP and ADP (see Table 1). In contrast, purinoceptors in smooth muscle cells do not show stereoselectivity for the diastereomers of ATP[βS], and both (*Sp*)-ATP[αS] and ADP[βS] are effective agonists at these receptors [24, 25]. In platelets, ADP is the most potent agonist, and in mast cells only ATP acts as an agonist at purinoceptors [19, 54]. In addition, GDP[βS], GTP[γS] and ATP are competitive antagonists at platelet purinoceptors but agonists at neutrophil purinoceptors [26, 27]. Furthermore, [α,β-CH_2]-ATP and [β,γ-CH_2]ATP are P_{2X}-purinoceptor agonists but not activators of human neutrophils [19, 23]. Finally, ATP[γS] is a less effective agonist at P_{2Y}-purinoceptors than ATP but not at neutrophil purinoceptors [44]. These data suggest that neutrophil purinoceptors are different from purinoceptors in other cell types. ATP, GTP, ITP and ADP may represent the endogenous ligands for neutrophil purinoceptors.

As pyrimidine nucleotides also stimulate neutrophils, the question arises whether pyrimidine nucleotides mediate their effects via purinoceptors or via distinct pyrimidinoceptors, the existence of which has been suggested in recent studies [18, 28, 29]. Uracil nucleotides are similarly effective stimulators of O_2^- formation, aggregation and degranulation as their corresponding adenine analogues (see Tables 1, 4 and 5). Whereas ATP, GTP and ITP are similarly effective activators of neutrophils, activation by pyrimidine nucleotides exhibits base specificity. UTP is more effective than CTP, and TTP is totally inactive. These results indicate that neutrophil nucleotide receptors do not only recognize the triphosphate but also the base of pyrimidine nucleotides. In addition, there is no stereochemical similarity between purine and pyrimidine nucleotides. The cross-desensitization between purine and pyrimidine nucleotides does not necessarily imply that purine and pyrimidine nucleotides act via the same receptor, as cross-desensitization between different receptors in neutrophils has been reported before [43]. We observed synergistic interaction of phosphorothioate analogues of purine and pyrimidine nucleotides on O_2^- formation (see Table 2). Synergism between signal transduction pathways activated by different receptors is a well-known phenomenon in neutrophils (see Table 3) [2, 5] and may also be true for purino- and pyrimidinoceptors. Considering the functional similarities between purine and pyrimidine nucleotides, it appears likely that pyrimidinoceptors represent a class of nucleotide receptors functionally related to purinoceptors. Finally, UTP appears to be the endogenous ligand for these putative pyrimidinoceptors.

The nucleotide effects are not specific for the triphosphates. Among the phosphorothioate analogues of nucleoside diphosphates, GDP[βS] and UDP[βS] are similarly active as the corresponding triphosphates with respect to O_2^- formation and degranulation (see Tables 1 and 4). These findings raise the question of whether the receptors can bind tri- as well as

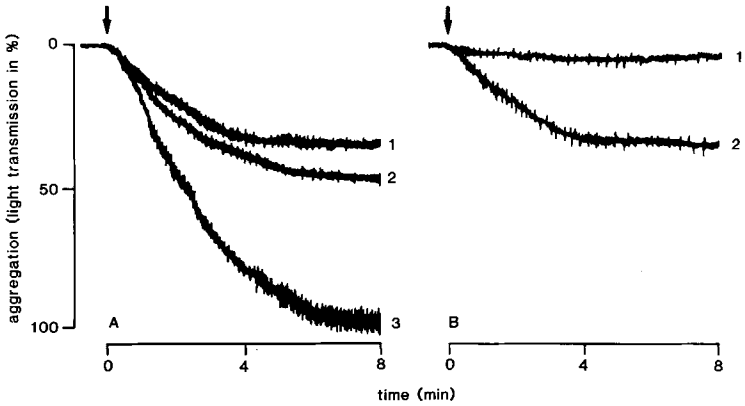

Fig. 6. *Inhibition of nucleotide- and fMet-Leu-Phe-induced neutrophil aggregation by pertussis toxin.* Neutrophils (5×10^6) were suspended in 900 µl assay buffer not supplemented with $CaCl_2$. Cells were incubated for 3 h at 37°C in the presence of pertussis toxin (1 µg/ml) or its carrier. Immediately following toxin treatment, aggregation assays were performed in the presence of $CaCl_2$ (1 mM). Arrows indicate the addition of stimuli to stirred neutrophil suspensions. The extent of aggregation is referred to that induced by fMet-Leu-Phe (1 µM), which effect amounted to 100%. Superimposed original registrations from one experiment performed in quadruplicate are shown. Assay variation was less than 10%. Similar results were obtained in six experiments. (A) Control cells. Trace 1, A23187 5 µM; trace 2, UTP or ATP 100 µM; trace 3, fMet-Leu-Phe 1 µM. (B) Pertussis toxin-treated cells. Trace 1, UTP or ATP 100 µM or fMet-Leu-Phe 1 µM; trace 2, A23187 5 µM

diphosphates. As phosphorothioate analogues of nucleoside diphosphates are extremely poor substrates for nucleoside-diphosphate kinases and adenylate kinase [15, 38], it is unlikely that these nucleotides act as their corresponding triphosphates. In addition, the transphosphorylation-resistant analogue of ATP, [β,γ-NH]ATP [13], caused a small but significant stimulation of O_2^- formation. Therefore, we conclude that purino- and pyrimidinoceptors recognize tri- as well as diphosphates.

As far as the effects of phosphorothioate analogues on O_2^- formation in intact neutrophils are concerned, our results suggest that these effects are mediated via purinoceptors and not directly via G proteins. First, nucleotides are hydrophilic molecules which do not sufficiently cross the plasma membrane [16, 17, 26, 27]. Second, nucleotides do not cause permeabilization of neutrophils as revealed by trypan blue dye exclusion and release of lactate dehydrogenase. Third, GTP[γS] is the most effective activator of G proteins [10–15, 55] but not of neutrophil purinoceptors. Fourth, [β,γ-NH]GTP is an effective activator of G proteins, whereas it is totally inactive at neutrophil purinoceptors. Fifth, GDP[βS] competitively antagonizes the effects of GTP[γS] at G proteins [7, 10, 14, 15] but additively enhances stimulation of intact neutrophils by GTP[γS] and *per se* is an agonist at neutrophil purinoceptors (see Table 2). It is also unlikely that nucleoside-diphosphate kinase reactions, leading to the formation of GTP[γS] [13], are involved in stimulation of NADPH oxidase by extracellular ATP[γS] and UTP[γS] in intact cells. ATP[γS] has recently been shown by us to serve as a thiophosphoryl group donor for the synthesis of GTP[γS] [13]. This reaction is time-dependent, but stimulation of O_2^- formation by extracellular nucleotides is rapid in onset without a significant lag time (see Fig. 1). In addition, GDP[βS] and UDP[βS] are activators of O_2^- formation in intact cells but no thiophosphoryl group donors in kinase-mediated reactions. Our results are in agreement with recent studies demonstrating that phosphorothioate analogues of nucleoside diphosphates and triphosphates act in the same direction at nucleotide receptors [24, 25].

There are certain functional similarities between nucleotides, PAF and LTB_4. In analogy to nucleotides, PAF and LTB_4 bind to specific recpetors and via G proteins activate neutrophils [5]. In agreement with previous results we found that PAF and LTB_4 are less effective activators of O_2^- formation than fMet-Leu-Phe [2, 3], while nucleotides *per se* are totally inactive (see Table 3). However, cells that have been stimulated with a low concentration of the chemotactic peptide become responsive to nucleotides. This activation of NADPH oxidase by nucleotides is small and short-lasting, similarly to activations of O_2^- formation by PAF and LTB_4 [2]. Nucleotides, PAF and LTB_4 synergistically enhance fMet-Leu-Phe-induced O_2^- formation, suggesting that their physiological function is to potentiate O_2^- formation induced by chemotactic peptides rather than to activate NADPH oxidase *per se*. This interpretation is also supported by the finding that combinations of PAF or LTB_4 plus nucleotides result only in small enhancements of O_2^- formation. In contrast, the combinations of fMet-Leu-Phe plus PAF or LTB_4 or nucleotides result in very marked synergism. However, the combination of PAF plus LTB_4 shows more pronounced synergistic activation of O_2^- formation than the combinations of lipid mediators with nucleotides. These data indicate that PAF and LTB_4 on one hand and nucleotides on the other hand are functionally related but not equivalent neutrophil activators.

Nucleotides potentiate chemotactic-peptide-induced O_2^- formation and β-glucuronidase release even with both classes of stimuli present at high concentrations, indicating that these agents induce neutrophil activation via different mechanisms. Agonists acting via distinct receptors may additively activate G proteins and thus may amplify activation of effector systems, i.e. NADPH oxidase and/or phospholipase C, finally leading to O_2^- formation, aggregation and degranulation. In addition, occupation of nucleotide receptors with agonists may increase the number and/or affinity state of formyl peptide receptors as is suggested by the nucleotide-induced reduction of the EC_{50} for NADPH oxidase activation by fMet-Leu-Phe. The relatively small synergistic activation of O_2^- formation by nucleotides plus lipid mediators in comparison to the combination of nucleotides plus fMet-Leu-Phe may be due to the fact that the former combinations lead only a small amplification of activation of signal transduction components. This interpretation is also supported by the fact

that PAF and LTB_4 lead only to transient activation of phospholipase C [49–51], as may also be true for nucleotides.

In summary, we present evidence that nucleotides which are released into the extracellular space under various physiological and pathological conditions may play roles as intercellular signal molecules in the activation of neutrophils. Taking into consideration the fact that extracellular nucleotides do not only activate neutrophils but also endothelium, smooth muscle cells, platelets, mast cells and lymphocytes, these agents may act simultaneously and in a coordinated way to regulate multiple functions of various cell types, which are located close together *in vivo*. Phosphorothioate analogues of purine and pyrimidine nucleotides are useful experimental tools to study nucleotide receptors in neutrophils. Finally, the development of competitive antagonists for neutrophil nucleotide receptors may be an interesting approach to interfere with inflammatory processes as has already been shown for PAF antagonists [55].

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