P2U Agonists Induce Chemotaxis and Actin Polymerization in Human Neutrophils and Differentiated HL60 Cells*

Margith W. Vergheesef§, Tracy B. Kneislert, and J oyce A. Boucheront

From the fDepartment of Cell Biology, Glaxo Wellcome Inc., Research Triangle Park, North Carolina 27709 and the fDepartment of Radiation, Pathophysiology and Toxicology, Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20889-5603

Human neutrophils or HL60 cells express P2U receptors and respond to micromolar concentrations of ATP, adenosine 5′-O-(thiotriphosphate) (ATPγS), or UTP with immediate increases in intracellular Ca2+ through activation of phosphoinositide phospholipase C (Cowen, D. S., Lazarus, H. M., Shurin, S. B., Stoll, S. E., and Dubyk, G. R. (1989) J. Clin. Invest. 83, 1651–1660). P2U agonists reportedly induce limited enzyme secretion and enhance the respiratory burst in response to chemotactic factors. We demonstrate here that P2U agonists are chemotactic for neutrophils or differentiated HL60 cells. Rhodamine phalloidin staining indicates that ATPγS treatment induces actin polymerization and shape changes similar to those seen when these cells are treated with chemotactic peptide fMet-Leu-Phe. Although undifferentiated HL60 cells fail to mount a rise in Ca2+ when challenged with fMet-Leu-Phe, they increase Ca2+ in response to P2U agonists. However, functional expression of phospholipase C-coupled receptors is not sufficient for chemotaxis since HL60 cell migration in response to these agonists or to fMet-Leu-Phe occurs only after exposure to differentiating agents such as BT2cAMP. In addition to the well known G protein-linked receptors for lipid or peptide chemotactic factors, neutrophils apparently also can utilize G protein-linked purino/pyrimidino receptors to recognize nucleotides as chemoattractants. High concentrations of ATP and ATPγS generated at sites of platelet aggregation and tissue injury could thus be important mediators of inflammation.

Extracellular ATP and other purine nucleotides bind to P2 receptors, which are classified pharmacologically into five distinct subgroups of P2X, P2U, P2Y, and P2Z (2–4). P2U and P2Z receptors have a restricted cellular distribution, whereas receptors for the other three subgroups are widely distributed. The physiological role of P2Z and P2Y receptors has been studied extensively in nervous and cardiovascular systems. The typical order of potency for P2X receptors is AMP-PCP > ATP = 2-methylthio-ATP, whereas that for P2Y receptors is 2-methylthio-ATP > ADPβS > ATP > AMP-PCP. Interestingly, P2U receptors recognize not only purine nucleotides but also UTP as demonstrated recently by expression of cloned murine and human P2U, cDNA in human leukemia or astrocytoma cell lines (4, 5). Receptors of this subclass are present on many cells including those of the immune system and the vasculature, but not much is known about their physiological functions (6). Recent studies with isolated cell systems demonstrate that P2U receptors of the U, X, and Y subclasses are members of the G protein-linked superfamily and are coupled to various effectors including adenylyl cyclase or phospholipases (1, 7–11).

Human neutrophils or promyelocytic HL60 cells in various stages of differentiation respond to ATP, ATPγS, or UTP with an immediate increase in intracellular Ca2+. This response is mediated through the activation of a pertussis toxin-sensitive G protein, which in turn activates the phosphoinositide phospholipase C (PI-PLC) cascade (1, 8, 10, 11). The magnitude and the time course of these two early biochemical responses are very similar to those observed with the chemotactic factor fMet-Leu-Phe (12). In contrast to fMet-Leu-Phe, P2U agonists induce only a limited secretary response and are poor activators of O2 formation in neutrophils or differentiated HL60 cells and their effects on chemotaxis are unknown (8, 13).

Since high concentrations of ATP and UTP are present at sites of platelet aggregation (2) and neutrophils are the initial cell type found at sites of tissue injury, we wanted to determine whether these nucleotides could be important for the recruitment of neutrophils in the absence of other known peptide and lipid chemoattractants. Our results demonstrate that activation of P2U receptors on human peripheral blood neutrophils induces a typical chemotactic response, accompanied by actin polymerization and changes in cell shape characteristic of those seen during chemotaxis in response to fMet-Leu-Phe. By comparing the effects of fMet-Leu-Phe and P2U receptor agonists on differentiating HL60 cells, we show that Ca2+ mobilization is an insufficient signal for chemotaxis, since undifferentiated HL60 cells fail to migrate in response to ATPγS in spite of a robust Ca2+ response.

EXPERIMENTAL PROCEDURES

Cells—Human peripheral neutrophils were isolated from fresh venous blood from healthy volunteers as described previously (12). HL60 cells were cultured in supplemented RPMI 1640 medium (Life Technologies, Inc.) under normal culture conditions and differentiated with 0.5 mM db-cAMP for 1–4 days as reported (12).

Ca2+ Mobilization—Neutrophils or HL60 cells were labeled with the fluorescent Ca2+ indicator Fura-2/AM (Molecular Probes, Eugene, OR), and changes in fluorescence were monitored in a Perkin-Elmer LS-5B spectrofluorimeter as described (12). For desensitization studies, the second agonist was added 5 min after addition of the first stimulus and fluorescence was monitored for an additional 3–5 min.

 Chemotaxis—Neutrophils or HL60 cells were suspended at 2 × 106 cells/ml of Hepes-buffered Hanks’ balanced salt solution, and chemotaxis was assayed in modified bipartite Boyden chemotaxis chambers as described previously (12). Briefly, P2U agonists or the synthetic peptide

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Present address: Dept. of Medicine, University of North Carolina, Chapel Hill, NC 27599.

1 The abbreviations used are: AMP-CPP, α,β-methyleneadenosine 5′-triphosphate; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; AMP-PNP, S-adenosyl-β-γ-imidodiphosphate; PI-PLC, phosphoinositide phospholipase C; ATPγS, adenosine 5′-O-(thiotriphosphate); db-cAMP, BT2cAMP; ADPβS, adenosine 5′-(O-thiodiphosphate).
P$_{2U}$ Agonist-induced Chemotaxis

**Fig. 1. Effect of purine nucleotides and UTP on Ca$^{2+}$ mobilization in human neutrophils.** Fura-2 fluorescence traces A-D and E-H are from two separate donors and are representative of at least three experiments. An arrow indicates the addition of stimulus, and individual tracings are superimposed for ease of comparison. Panels A and B, dose response to ATP$_γ$S (A) or UTP (B) at 100, 10, or 1 µM (trace 1, 2, or 3, respectively). Panels C and D, desensitization to ATP$_γ$S (second arrow) following the initial addition (first arrow) of ADP$_γ$S or AMP-PNP (C, trace 1 or 2, respectively); AMP-CP or AMP-CPD (D, trace 1 or 2, respectively). Panels E and F, desensitization to ATP$_γ$S or UTP (second arrow for trace 1 or 2, respectively) following the initial addition (first arrow) of ATP$_γ$S (E) or UTP (F). Panels G and H, desensitization to fMet-Leu-Phe (second arrow) following the initial addition of UTP or ATP$_γ$S (G, trace 1 or 2, respectively) or fMet-Leu-Phe (H). Nucleotides were used at 100 µM and fMet-Leu-Phe at 100 nM concentration.

fMet-Leu-Phe was added to the bottom compartment, and cells were placed in the upper compartment and allowed to migrate through a polycarbonate filter at 37 °C for 90 min. In some experiments, agonists also were present in the upper compartment to distinguish directed from random migration (1A). Filters were fixed and stained, and cells that had migrated through the filter were counted under ≥100 magnification in 10 different fields of each filter.

Fluorescence Staining of F-actin—Neutrophils or differentiated HL-60 cells were prepared as for chemotaxis but exposed to agonists in polystyrene tubes for 5 min in a shaking water bath kept at 37 °C. The cells were then fixed in the presence of 3.7% formaldehyde, permeabilized with 0.1 mg/ml of lysophosphatidylcholine, and stained with 0.16 µM rhodamine-labeled phalloidin for 15 min at 37 °C (15). Cytosin preparations from these cells were viewed under oil in a Leitz Diaplan microscope (x 63) equipped with a mercury lamp and photographed with a Wild camera.

**RESULTS**

Pharmacological Characterization of P2 Receptors on Human Neutrophils—Fluorescent probes such as Fura-2 provide a convenient assay system to monitor changes in intracellular Ca$^{2+}$ and to characterize surface receptors that are coupled to the P1-PLC pathway (12). As reported previously (1), the tracings in Fig. 1 demonstrate the presence of P$_{2U}$ receptors on human neutrophils, since ATP$_γ$S, a nonhydrolyzable analog of ATP (Fig. 1A) and UTP (Fig. 1B) caused an immediate, concentration-dependent rise in intracellular Ca$^{2+}$. In contrast, the most potent agonists for P$_{2Y}$ receptors (ADP$_γ$S, Figs. 1C) or P$_{2X}$ receptors (AMP-CP; Fig. 1D) failed to elicit any changes in intracellular Ca$^{2+}$ in human neutrophils. Similarly, weaker agonists for these receptors like AMP-PNP (Fig. 1C) or AMP-CP (Fig. 1D), also were inactive in this assay, further supporting the absence of P$_{2Y}$ or P$_{2X}$ receptor-mediated activation of neutrophils. Desensitization studies showed that neither of these inactive nucleotides interfered with subsequent effects of ATP$_γ$S. In contrast, ATP$_γ$S (Fig. 1E) desensitized neutrophils against a second challenge with the same agonist (trace 1) or UTP (trace 2). The same results were obtained when UTP was used as the first stimulus (Fig. 1F), indicating that ATP$_γ$S and UTP shared the same receptors. Ca$^{2+}$ mobilization in response to the chemotactic peptide fMet-Leu-Phe (Fig. 1G, second ar-

**Fig. 2. Migration of human neutrophils in response to ATP$_γ$S.** ATP$_γ$S at the concentrations indicated was present in only the bottom (filled bars) or top and bottom (hatched bars) compartments of Boyden chemotaxis chambers to measure chemotaxis or chemokinesis, respectively. The number of neutrophils migrating through the filter was standardized relative to the number of cells migrating in response to 100 µM ATP$_γ$S (58 ± 16 cells/field, n = 5).

The data in Fig. 2 (solid bars) demonstrate that ATP$_γ$S induced chemotaxis at concentrations ranging from 10 to 100 µM. The chemotactic response to 100 µM ATP$_γ$S (58 ± 16 cells/field, n = 5), the highest concentration tested, was approximately 95% of that observed with 1 nM fMet-Leu-Phe (87 ± 12 cells/field, n = 9). That the observed migration in response to ATP$_γ$S was indeed directional chemotaxis and not simply increased random motility was demonstrated by the diminished migration seen when the concentration gradient was reduced by adding ATP$_γ$S to the upper compartment of the chemotaxis chambers (hatched bars). For comparison, chemotaxis in response to 1 or 10 nM fMet-Leu-Phe was reduced by 65 and 37% respectively, when equal concentrations of the peptide were present in the top compartment of the chambers (data not shown).

Relationship between Ca$^{2+}$ Mobilization and Chemotaxis of P$_{2U}$ Agonists in Human Neutrophils—To determine whether increases in intracellular Ca$^{2+}$ were predictive of chemotactic activity, we compared the effects of a series of purinergic agonists and UTP on these two assays in aliquots from the same neutrophil preparations. The Fura-2 data were averaged from the peak excursions (distance in millimeters) of the fluorescence signal from the base line observed immediately after the addition of stimulus. Chemotaxis data were standardized relative to the responses observed using 1 nM fMet-Leu-Phe. As shown in Fig. 3, UTP was as effective as ATP$_γ$S in eliciting chemotaxis (bottom panel) in human neutrophils, indicating that P$_{2U}$ receptors were functionally linked not only to Ca$^{2+}$ mobilization (top panel) but also to an important physiological response of neutrophils. Although ATP and UTP raised intracellular Ca$^{2+}$ levels more than ATP$_γ$S, migration in response to
ATP was only about 50% of that seen with these two P₂U agonists. Chemotaxis in response to AMP-PNP reached only 10% of that stimulated by 1 nM fMet-Leu-Phe, and small agonists. Chemotaxis in response to AMP-PNP reached only 10% of that seen with these two P₂U agonists. Preparations.

Concentrations below 100 μM for the latter P₂U agonists were completely ineffective in this assay (data not shown). Adenosine did not elevate intracellular Ca²⁺ or induce chemotaxis at the concentrations tested (10–100 μM), and the same was true for AMP (data not shown). Although P₂U agonists were not tested in the chemotaxis assay, they did not induce any changes in cell shape normally seen with chemotactic factors (see below).

P₂U receptors on HL60 cells induce Ca²⁺ mobilization, but differentiation is required for stimulation of Chemotaxis—Undifferentiated HL60 myeloid precursor cells are known to express P₃U receptors that stimulate PI-PLC and Ca²⁺ mobilization (1). In contrast, fMet-Leu-Phe receptor-mediated changes in intracellular Ca²⁺ and functional responses such as chemotaxis, enzyme secretion, and O₂⁻ production were detectable only after HL60 cells were forced to differentiate toward a neutrophil phenotype with db-cAMP (8, 16). To determine whether coupling of P₂U receptors to PI-PLC was sufficient to transmit a chemotactic signal, we followed chemotaxis and Ca²⁺ mobilization response of human neutrophils and differentiated HL60 cells—Neutrophils readily change their shape from a round, resting form to a triangular shape containing a broad leading edge and a narrower trailing edge when exposed to fMet-Leu-Phe in suspension (14). These changes are accompanied by increased actin polymerization near the cell periphery as visualized with rhodamine-labeled phalloidin (15). Such changes from round to irregular shapes as well as increases in phalloidin staining also were apparent when neutrophils or db-cAMP-differentiated HL60 cells were exposed to ATPγS. These morphological changes were similar to those observed when the cells were treated with fMet-Leu-Phe, indicating that ATPγS could function as a typical chemotactic factor.

**DISCUSSION**

Our observations that ATPγS and UTP caused a complete cross-desensitization of the Ca²⁺ mobilization response of human neutrophils support recent reports that P₂₃U receptors recognize both of these agonists (4, 5). Only the P₂₃U subclass of P₂ receptors appeared to be coupled to Ca²⁺ mobilization, since potent agonists for P₂₃U receptors (ADPβS) or P₂₄ receptors (AMP-CPP) were ineffective by themselves and did not affect
subsequent changes in intracellular Ca\(^{2+}\) by ATP\(\gamma S\), UTP, or ATP (data shown only for ATP\(\gamma S\)). Similar P2U receptors also were identified on myeloid progenitor cells but not on mature lymphocytes or lymphocytic leukemia cells (1).

In spite of the robust Ca\(^{2+}\) mobilization elicited by P2U agonists, the functional significance of P2U receptors for neutrophil activation was not obvious. Occupancy of P2U receptors caused only a partial secretory response and did not enhance the respiratory burst unless fMet-Leu-Phe was also added (Ref. 13; our data not shown). Our data demonstrate that high micromolar concentrations of P2U agonists elicited a chemotactic response in neutrophils or differentiated HL60 cells that approached that observed with the classical chemoattractant fMet-Leu-Phe. Similarly high concentrations of agonists were typically required for activation of P2 receptors in other systems, including the vasculature, neurotransmission, and cardiac function (2). Although most currently known peptide or lipid chemoattractants are active at nanomolar concentrations, ATP and UTP are present in cells at millimolar concentrations. Leakage of these nucleotides from damaged cells or degranulating platelets could lead to sufficiently high accumulations to be physiologically relevant.

Classical chemotactic factors, including lipids (leukotriene B\(_4\), platelet-activating factor), synthetic peptides (fMet-Leu-Phe), the complement fragmentation products (C5a), and cytokines (interleukin-8) are coupled to the PI-PLC pathway to mobilize intracellular Ca\(^{2+}\) (12, 17, 18). In the present studies, the correlation between Ca\(^{2+}\) mobilization and chemotaxis breaks down for ADP\(\beta S\), a potent P2\(\gamma\) agonist, where chemotaxis of neutrophils is observed in the complete absence of Ca\(^{2+}\) mobilization. Whether this is an example where chemotaxis is dissociated from a G protein/PI-PLC/Ca\(^{2+}\) pathway, as recently described for transforming growth factor \(\beta\), cannot be ascertained from our experiments (15). Since P2U receptors typically stimulate the formation of Ca\(^{2+}\)-mobilizing phosphoinositides in other cells, we speculate that migration in response to ADP\(\beta S\) more likely results from conversion of ADP\(\beta S\) to another agonist such as ATP\(\gamma S\) during the prolonged incubation necessary for chemotaxis assays. Similarly, we attribute the decreased neutrophil chemotaxis in response to ATP as compared with ATP\(\gamma S\) to the instability of ATP during the chemotaxis assay. These two purine triphosphates were equipotent in the Ca\(^{2+}\) mobilization assay, while UTP was more potent than either of these. However, the absence of PI-PLC coupling does not completely rule out the possibility that human neutrophils express other P2 receptors.

Interestingly, myeloid progenitor cells such as undifferentiated HL60 cells express P2U receptors that appeared to be fully coupled to the PI-PLC pathway and subsequent Ca\(^{2+}\) mobilization (1). In spite of this, undifferentiated HL60 cells failed to migrate in response to ATP\(\gamma S\), indicating that Ca\(^{2+}\) mobilization was not sufficient for chemotaxis. When HL60 cells were
induced to differentiate toward a neutrophil-like phenotype, they began to express receptors for fMet-Leu-Phe and other chemoattractants (platelet-activating factor), and chemotaxis in response to fMet-Leu-Phe, ATPγS, or platelet-activating factor was detectable 24 h after adding db-cAMP as a differentiating agent. As these cells acquired the proficiency to migrate in response to fMet-Leu-Phe, they apparently also gained the capability of coupling their P2U receptors to a migratory response. Interestingly, the functional coupling of phospholipase D to P2U or fMet-Leu-Phe receptors during HL60 differentiation followed a time course that is very similar to the one we observed for the appearance of chemotaxis mediated by these receptors (11). Phospholipase D may thus provide the amplification signal necessary for the expression of fully functional receptors, since Ca2+ mobilization was obviously insufficient to generate a chemotactic response.

Directed or random migration involves the rearrangement of the cytoskeleton, and changes in actin polymerization are typically observed for all chemoattractants described so far (14, 15). In our studies, ATPγS induced changes in cell shape in neutrophils or differentiated HL60 cells that were very similar to those observed with the classical chemotactic factor fMet-Leu-Phe. Moreover, patterns of actin polymerization as visualized with rhodamine-labeled phalloidin staining confirmed the similarities between fMet-Leu-Phe and ATPγS elicted changes. Interestingly, G protein-linked receptors for cAMP are coupled to the PI-PLC pathway in Dictyostelium, where extracellular cAMP induces changes in cell shape and chemotaxis (14). In contrast to Dictyostelium, cAMP does not activate the PI-PLC pathway in neutrophils and can actually inhibit chemotaxis of these cells (19).

To our knowledge, our results provide the first evidence that neutrophils can migrate in response to increasing concentration gradients of nucleotides such as P2U agonists. Recent reports indicate that human P2U receptors share more sequence homology with G protein-coupled receptors for peptides than for cAMP or adenosine (5). G protein-linked receptors that recognize such diverse signals as P2U agonists, lipid chemoattractants, or peptide chemoattractants thus appear to utilize similar biochemical pathways leading to neutrophil chemotaxis.

Acknowledgment—We thank Dr. David Bruckenstein of Glaxo Research Institute for valuable microscopy and photography.

REFERENCES
1. Cowen, D. S., Lazarus, H. M., Shurin, S. B., Stoll, S. E., and Dubyak, G. R. (1989) J. Clin. Invest. 83, 1651–1660
2. Gordon, J. L. (1986) Biochem. J. 233, 309–319
3. Pirotton, S., Motte, S., Cote, S., and Boeynaems, J.-M. (1993) Cell. Signalling 5, 1–8
4. Erb, L., Lustig, K. D., Sullivan, D. M., Turner, J. T., and Weisman, G. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10449–10453
5. Parr, C. E., Sullivan, D. M., Paradiso, A. M., Lazarowski, E. R., Burch, L. H., Olsen, J. C., Erb, L., Weisman, G. A., Boucher, R. C., and Turner, J. T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3275–3279
6. Seifert, R., and Schultz, G. (1989) Trends Pharmacol. Sci. 10, 365–369
7. Boyer, J. L., Downs, P., and Harden, T. K. (1989) J. Biol. Chem. 264, 884–890
8. Cockcroft, S., and Stutchfield, J. (1989) Biochem. J. 263, 715–723
9. Cooper, C. L., Morris, A. J., and Harden, T. K. (1989) J. Biol. Chem. 264, 6203–6206
10. Okajima, F., Tokumitsu, Y., Kondo, Y., and Ui, M. (1987) J. Biol. Chem. 262, 13483–13490
11. Xie, M., Jacob, L. S., and Dubyak, G. R. (1991) J. Clin. Invest. 88, 45–54
12. Verghese, M. W., Charges, L. J., akol, L., Dillon, S. B., and Snyderman, R. (1987) J. Immunol. 138, 4374–4380
13. Axtell, R. A., Sandborg, R. R., Smolen, J. E., Ward, P. A., and Boxer, L. A. (1990) Blood 75, 1324–1332
14. Devreotes, P. N., and Zigmond, S. H. (1988) Annu. Rev. Cell Biol. 4, 649–686
15. Reibman, J., Meixler, S., Lee, T. C., Gold, L. I., Cronstein, B. N., Haines, K. A., Kolahinski, S. L., and Weissman, G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6805–6809
16. Chaplinski, T. J., and Niedar, J. (1982) J. Clin. Invest. 70, 953–964
17. Cowen, D. S., Baker, B., and Dubyak, G. R. (1990) J. Biol. Chem. 265, 16181–16189
18. Wu, D., LaRosa, G. J., and Simon, M. I. (1993) Science 261, 101–103
19. Harvath, L., Robbins, J. D., Russell, A. A., and Seamon, K. B. (1991) J. Immunol. 146, 224–232
P2U Agonists Induce Chemotaxis and Actin Polymerization in Human Neutrophils and Differentiated HL60 Cells
Margrith W. Verghese, Tracy B. Kneisler and Joyce A. Boucheron

J. Biol. Chem. 1996, 271:15597-15601.
doi: 10.1074/jbc.271.26.15597

Access the most updated version of this article at http://www.jbc.org/content/271/26/15597

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 19 references, 12 of which can be accessed free at http://www.jbc.org/content/271/26/15597.full.html#ref-list-1