Structural requirements for binding of adenosine-5'-*O*-(3-thiotriphosphate) (ATPγS) to human neutrophils

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Abstract: Human polymorphonuclear leukocytes (PMN) respond via pertussis toxin-sensitive pathways to extracellular nucleotides with an elevation in intracellular calcium ($[Ca^{2+}]_i$) and enhancement of the O_2^- generation induced by the chemotactic peptide N'-formyl-Met-Leu-Phe (fMLP). Binding studies with adenosine 5'-*O*-(3-thio[^{35}S]triphosphate) ([^{35}S]ATPγS) have recently identified at least two classes of receptors on intact human neutrophils. In this study, we further characterize nucleotide binding to this receptor with respect to its specific structural requirements. Utilizing [^{35}S]ATPγS as the primary ligand and various nucleotides and ATP analogues, competitive binding studies demonstrate that: (1) the triphosphate tail is essential for maximal receptor binding; (2) chemical modifications of the phosphate tail have profound effects on binding efficacy; (3) the base ring is recognized by the receptor, with purines being preferentially recognized; and (4) except for a spacing function, the ribose ring of nucleotides does not appear to be important for nucleotide binding. In addition, we demonstrate that the presence of divalent cations inhibits [^{35}S]ATPγS binding, suggesting that the tetraanionic form of ATP (ATP^{4-}) is the nucleotide species reactive with the receptor.

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Introduction

Extracellular nucleotides exert significant physiological effects on a number of biological processes, including platelet aggregation, vascular smooth muscle tone, cardiac function and neurotransmission (reviewed by Burnstock, 1981; Gordon, 1986). Several of these effects are assumed to be mediated via nucleotide-specific cell surface purinoceptors, which have been classified into two main groups on the basis of adenine

nucleotide agonist potency. P_1 purinoceptors are responsive to adenosine, whereas P_2 purinoceptors respond to ATP and ADP and are inhibited by AMP and adenosine (Burnstock et al., 1985). The P_2 class has been further subclassified into P_{2x} and P_{2y} subgroups according to their relative affinity for ATP and the ATP analogues, 2-methylthio ATP, β-γ-methylene ATP and α-β-methylene ATP (Burnstock et al., 1985). A purinoceptor of the P_{2y} subclass which has been proposed on rat hepatocyte plasma membranes is involved in the regulation of glycogenolysis via activation of glycogen phosphorylase (Keppens et al., 1986). In addition, a P_{2y} purinoceptor which mediates phospholipase C activation has recently been demonstrated on turkey erythrocyte membranes (Cooper et al., 1989). As a final subgroup of the P_2 class, P_{2z} receptors have been described in rat mast cells and murine macrophages (Cockcroft et al., 1979, 1980; Steinberg et al., 1987). In these cells, ATP^{4-} in near milli-molar concentrations has been identified as the reactive species causing non-selective cell permeabilization.

Extracellular ATP exerts a myriad of effects on a variety of inflammatory cells, including neutrophils, monocytes, macrophages and mast cells (reviewed by Daly, 1983; Landos et al., 1981; Paterson et al., 1981). In addition to ATP, other extracellular nucleotides (GTP, UTP, CTP, ITP) are capable of eliciting responses in neutrophils (Walker et al., submitted for publication). Induced responses include a transient elevation in intracellular calcium ($[Ca^{2+}]_i$) and 'priming' of the respiratory burst in neutrophils following stimulation by the chemotactic peptide N'-formyl-Met-Leu-Phe (fMLP) (Kuhns et al., 1988; Ward et al., 1988; Cowen et al., 1989). Additional phenomena observed in ATP-exposed PMN include azurophil and specific granule release (Balazovich et al., 1990; Cockcroft et al., 1989), enhanced expression of the neutrophil adhesion molecule CR3 (Walker et al., 1989), and translocation of protein kinase C (Balazovich et al., 1990).

The observation that a number of these responses are effectively blocked by pretreatment with pertussis toxin has led to the proposal that many of these effects are mediated via a guanine nucleotide-binding protein-associated cell surface receptor on PMN (Dubyak et al., 1988; Kuhns et al., 1988; Snyderman et al., 1988). Recently, we have presented evidence for such a nucleotide-specific receptor on the surface of human PMN which may be involved in the $[Ca^{2+}]_i$ elevation following cell contact with a variety of naturally occurring nucleotides. Binding studies in intact human PMN utilizing the radiolabelled ATP analogue adenosine 5'-O-(3-thio[^{35}S]triphosphate) ([^{35}S]ATPγS] have demonstrated a group of high-affinity receptors as well as a second group of low-affinity cell surface binding sites (Yu et al., submitted for publication). In the present study, the structural specificity of the nucleotide molecule is evaluated with respect to binding of ATPγS.

Materials and Methods

Reagents

Unless otherwise indicated, all compounds were purchased from Sigma Chemical Company (St. Louis, Missouri). Adenosine-5'-O-(3-thiotriphosphate) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). 2-Methylthio ATP was purchased from Research Biochemicals Inc. (Natick, MA). [^{35}S]ATPγS was purchased from New England Nuclear Research Products (Wilmington, Delaware).

Purification of human neutrophils

Whole blood was collected into acid citrated dextrose (Fenwal Laboratories) from healthy donors on the day of use and centrifuged at 400 × g for 15 min at room temperature. Platelet-rich plasma was discarded and the pellet was resuspended (75 ml total volume per 50 ml whole blood) in phosphate-buffered saline (PBS, 140 mM NaCl, 5 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4), layered onto density 1.077 g/ml Ficoll-Hypaque (Histopaque 1077, Sigma), and centrifuged at 400 × g

for 30 min at 4 °C. Supernatants were discarded and the pellet was resuspended twice in lysing solution (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA), for 10 min to lyse contaminating erythrocytes. Cells were then washed with PBS and counted in a hemacytometer. Isolates routinely contained > 98% neutrophils, and Giemsa stain revealed few contaminating mononuclear cells, erythrocytes or platelets. Viability by Trypan Blue exclusion was consistently greater than 97%.

[^{35}S]ATPγS binding to neutrophils

For binding assays, human neutrophils were resuspended in binding buffer (PBS + 2 mM Na_3SPO_3, pH 7.4) at a concentration of 4×10^7 PMN/ml. One hundred μl of this suspension were then added to polypropylene tubes containing 25 μl [^{35}S]ATPγS (1000–1500 Ci/mmol) and 25 μl of nucleotide solution or buffer. Final radioligand concentration for all competitive binding studies was 60 nM. Samples were incubated at 4 °C with constant agitation for 40 min, then rapidly filtered through polycarbonate filters (0.8 μm pore size; Nuclepore, Pleasanton, CA) following the addition of 4 ml cold PBS. Filters were washed three times with 4 ml PBS, then removed and counted in scintillation cocktail (Biosafe II). Sodium thiophosphate was included in order to eliminate functionally irrelevant, non-displaceable [^{35}S]ATPγS binding as recently described (Yu et al., submitted for publication). All experiments using the photoreactive compounds 3'-O-(4-benzoyl)benzoyl ATP and 8-azidoadenosine 5'-triphosphate were performed with shielding from ultraviolet light in order to prevent activation of these compounds. All conditions were performed in duplicate for each experiment. Non-specific binding to PMN was defined as radioactivity bound in the presence of excess ATPγS (1 mM), and ranged from 5 to 35% of total binding. The choice of a 40-min incubation period was based on the observation that at this interval binding was at equilibrium, and cell death by Trypan Blue exclusion minimal. The choice of polycarbonate filters was based on the negligible

amount of non-specific [^{35}S]ATPγS binding to these filters, as compared with that occurring with other filters (i.e. glass fiber). IC_{25} and IC_{50} values for each nucleotide were calculated by a von Krogh transformation of the data followed by linear regression analysis (von Krogh, 1916).

Results

Effect of phosphate tail structure on ability to compete with [^{35}S]ATPγS binding to PMN

Competitive binding assays were performed to examine the role of the nucleotide phosphate tail on competitive binding ability. Using [^{35}S]ATPγS as the primary ligand, various competing compounds of differing tail length and structure were tested. The structures of some of the competing compounds ATP, ADP, AMP, adenosine (Ado), 5'-adenylylimido-diphosphate (AMP-PNP) and β-γ-methylene ATP (AMP-PCP) are shown in Fig. 1. The resulting competitive binding curves and calculated IC_{50} values (concentration required to block 50% of specific [^{35}S]ATPγS binding) for each compound tested are shown in Fig. 2. The rank order of ATP > AMP-PNP ≥ ADP ≫ AMP-PCP ≫ AMP ≫ Ado suggests that the presence of the phosphate tail is essential for receptor binding. In addition, it appears that both phosphate tail length (ATP > ADP ≫ AMP ≫ Ado; Fig. 2a) and biochemical composition (AMP-PNP ≫ AMP-PCP; Fig. 2b) of the tail are critical to recognition by the receptor.

Effect of base ring structure on ability to block [^{35}S]ATPγS binding

Binding studies to assess the role of the base ring in receptor recognition were next performed. Studies examining the abilities of various purine and pyrimidine nucleotides to compete with the ability of [^{35}S]ATPγS to bind to neutrophils reveal the following rank order of potency (expressed as IC_{25} values rather than IC_{50} to allow
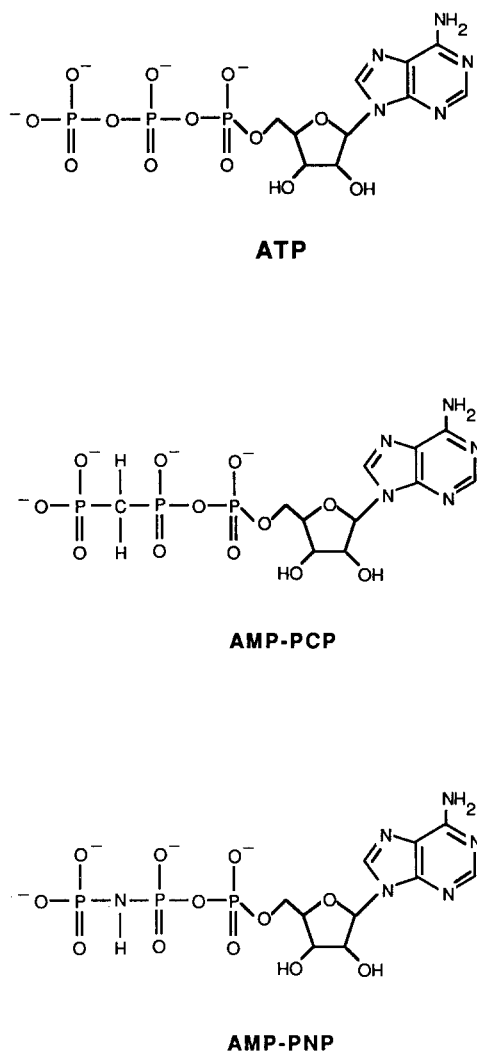
ATP

AMP-PCP

AMP-PNP

Fig. 1. Structure of ATP, AMP-PCP and AMP-PNP.

A.

	IC$_{50}$ ± SEM (µM)
Ado	>1mM
AMP	341.2 ± 19.1
ADP	13.3 ± 2.4
ATP	2.56 ± 0.88

Adenosine

AMP

ADP
ATP

Nucleotide [M]

B.

	IC$_{50}$ ± SEM (µM)
AMP-PCP	119.6 ± 10.2
AMP-PNP	9.87 ± 2.71
ATP	2.56 ± 0.88
ATPγS	0.58 ± 0.20

AMP-PCP

AMP-PNP
ATP
ATPγS

Nucleotide [M]

Fig. 2. Effect of phosphate tail length and composition on ability to block [^{35}S]ATPγS binding to PMN. Intact PMN (4 × 10^6 cells) were incubated with 60 nM [^{35}S]ATPγS and increasing concentrations of (A) adenosine, AMP, ADP and ATP. Similar competitive binding experiments were performed using ATPγS, AMP-PCP and AMP-PNP as competing nucleotides (B). The data represent the mean of duplicate determinations from one experiment and are expressed as a percent of maximal [^{35}S]ATPγS bound (specific binding in the absence of competing nucleotide, 1040 cpm). The data are representative of three separate experiments performed for each nucleotide, from which calculated IC$_{50}$ ± SEM values are shown.

greater discrimination between nucleotides; µM) ($\bar{x}$ ± SEM for µM concentration of nucleotide required to block 25% of [^{35}S]ATPγS binding): ATP (0.35 ± 0.10), ITP (13.2 ± 0.8), GTP (27.5 ± 2.5), TTP (82.1 ± 8.9), UTP (87.7 ± 8.1) and CTP (143 ± 7.1). Clearly, pyrimidine nucleotides are less effective in competing for binding of [^{35}S]ATPγS. These data confirm and extend our recent observations (Yu et al., submitted for publication). Various base ring substitutions on ATP were utilized in order to determine the effect of these structural changes on competition for binding of [^{35}S]ATPγS. The compounds 8-bro-

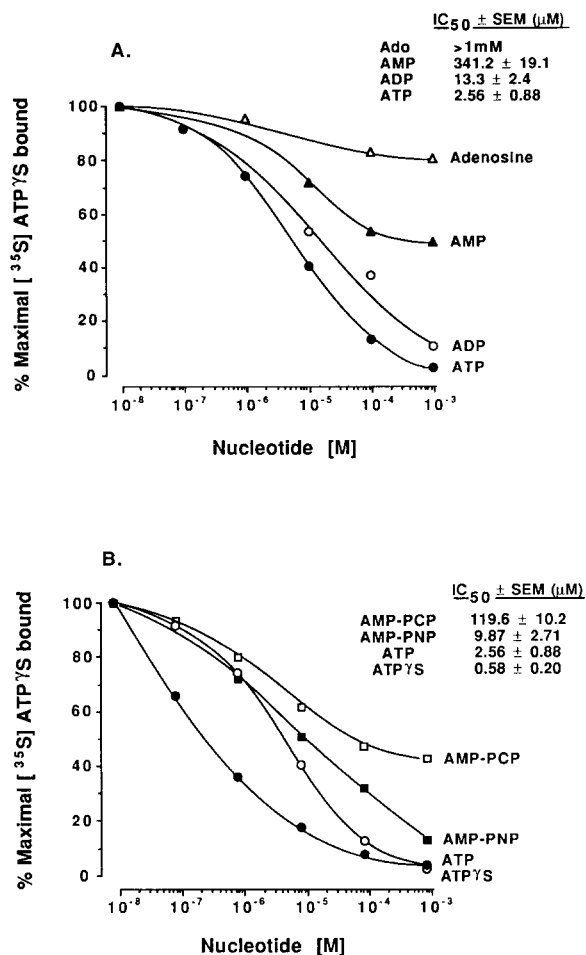mo ATP (8-Br ATP), 2-methylthio ATP (2-MeS ATP) and 8-azido ATP (8-N$_3$ATP) were employed. The position of these substitutions is shown in Fig. 3. The rank order potency obtained was: ATP > 2-MeS ATP > 8-Br ATP > 8-N$_3$-ATP (Fig. 4). These data demonstrate that the
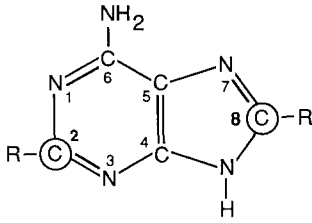
Fig. 3. Positions of substitution on adenine ring.

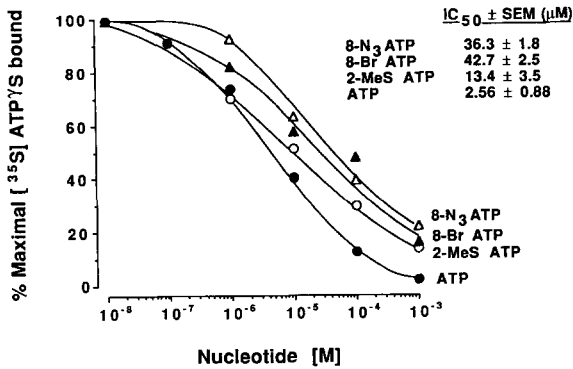|	$IC_{50} \pm$ SEM (μM)
8-N$_3$ ATP	36.3 ± 1.8
8-Br ATP	42.7 ± 2.5
2-MeS ATP	13.4 ± 3.5
ATP	2.56 ± 0.88

Fig. 4. Effect of base ring substitutions on competitive binding ability of nucleotides. PMN (4×10^6 cells) were incubated with 60 nM [^{35}S]ATPγS and increasing concentrations of 8-N$_3$ATP, 8-Br ATP, 2-MeS ATP and ATP. The data are expressed as a percent of maximal [^{35}S]ATPγS bound (1327 cpm in this experiment) and are representative of three independent experiments performed for each nucleotide, from which $IC_{50} \pm$ SEM values are calculated.

base ring is recognized by the ATPγS binding site, as base ring substitutions diminish the ability of the compound to compete with [^{35}S]ATPγS for binding. The data also suggest that the 8-position of the base ring may be more integral to interaction with the receptor than the 2-position.

Role of ribose moiety in receptor recognition

The role of the ribose group in the binding of nucleotide compounds to PMN was studied using a photoreactive analogue of ATP, 3'-O-(4-benzoyl)benzoyl ATP (BzATP) and ribose-5-phosphate (see Fig. 5). The competitive binding curves and calculated IC_{50} values are shown in Fig. 6. Ribose-5-phosphate was completely unable to compete with [^{35}S]ATPγS binding, even at con-
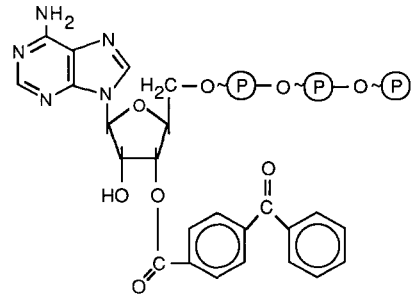
3'-O-(4-Benzoyl)benzoyl ATP
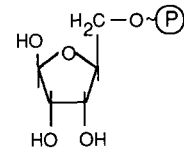
Ribose-5'-phosphate

Fig. 5. Structure of BzATP and ribose-5'-phosphate.

centrations of 1 mM. BzATP, however, was readily able to block [^{35}S]ATPγS binding to PMN, with a calculated IC_{50} within the range of that found for ATP (the data for which are reproduced from Fig. 2a), despite the large chemical

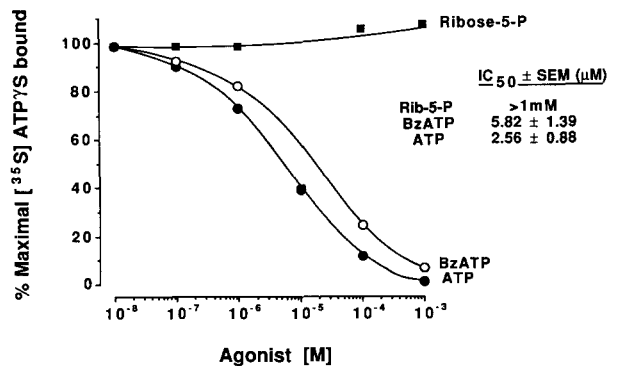|	$IC_{50} \pm$ SEM (μM)
Rib-5-P	>1mM
BzATP	5.82 ± 1.39
ATP	2.56 ± 0.88

Fig. 6. Role of ribose ring in ability to block [^{35}S]ATPγS binding. Intact PMN (4×10^6 cells) were incubated with 60 nM [^{35}S]ATPγS and increasing concentrations of ribose-5-phosphate, BzATP and ATP. The data, expressed as percent of maximal [^{35}S]ATPγS bound (1040 cpm), are representative of three separate experiments and allow calculation of $IC_{50} \pm$ SEM values.

group present on the ribose ring of this compound. Together, these data strongly suggest that the ribose moiety is not recognized by the active binding site of this receptor and may instead serve as a structural 'spacer' for the adenine ring and phosphate tail to allow optimal receptor binding.

Effect of divalent cations on [^{35}S]ATPγS binding to PMN

In order to determine which form of ATP is recognized by the PMN receptor, [^{35}S]ATPγS binding was assessed in both the absence and the presence of divalent cations over a wide range of radioligand concentrations (0.8–450 nM). As seen in Fig. 7, specific binding of [^{35}S]ATPγS to intact PMN (defined as total binding less binding in the presence of excess unlabelled ATPγS) was consistently reduced in the presence of Ca^{2+} and Mg^{2+} over all concentrations tested. Compared to the absence of cations, nonspecific binding values increased (from 17% to 35% of total counts) in the presence of cations (data not shown). These findings are consonant with functional studies by Walker et al. (submitted for publication), who have reported a relative decrease in the ability of ATP, as well as other
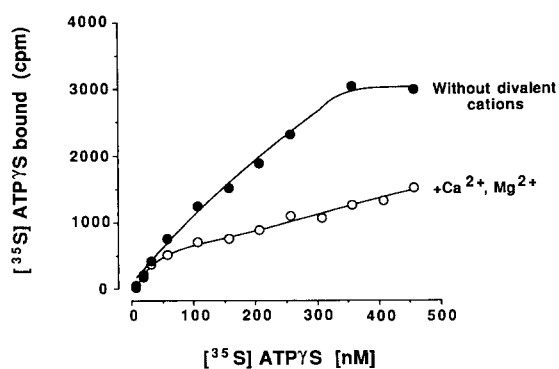
nucleotides, to induce [Ca^{2+}]$_i$ elevations in the presence of Ca^{2+} and Mg^{2+}. Together these data suggest that in human neutrophils, the active form of ATP is ATP^{4-}, an observation consistent with studies of putative purinoceptors in other systems. It has been suggested elsewhere that, under physiological concentrations of Ca^{2+} and Mg^{2+}, 2.63% of the total ATP is present as ATP^{4-} (Cockcroft et al., 1980).

Discussion

Extracellular nucleotides have a multitude of effects on human neutrophils, some of which are mediated by specific cell surface receptors. In this report, we have further characterized the structural specificity of the putative PMN receptor which mediates the [Ca^{2+}]$_i$ rise in human PMN in response to a number of nucleotides. We have shown that both the phosphate tail and the base ring are essential for recognition by the receptor. Phosphate tail length is critical for optimal binding, with the triphosphate tail preferentially recognized, as would be expected. In addition, the purine ring structure confers greater binding efficacy when compared to the pyrimidine ring. This specificity is demonstrated even when purine ring substitutions are present. In contrast, the active binding site of the receptor does not appear to recognize the ribose group of nucleotide compounds, based on the inability of an isolated ribose phosphate compound to block [^{35}S]ATPγS binding. This is confirmed by the observation that the presence of a relatively large chemical group on the ribose ring (BzATP) has no effect on competitive binding ability. We hypothesize that, rather than receptor recognition, the role of the ribose ring in nucleotide binding may be maintenance of proper 'spacing' between the base ring and the phosphate tail. Finally, we have provided evidence that this receptor requires the tetraanionic form of ATP (ATP^{4-}). This observation is consistent with functional studies of this receptor as well as studies of the purinergic receptor in mouse macrophages and rat mast cells, in

Fig. 7. Effect of divalent cations on [^{35}S]ATPγS binding to intact PMN. Intact PMN (4 × 10^6 cells) were incubated with increasing concentrations of [^{35}S]ATPγS (0.8–450 nM) in the absence and presence of Ca^{2+} and Mg^{2+} (1 mM CaCl$_2$, 2 mM MgCl$_2$). The data are expressed as absolute number of specific [^{35}S]ATPγS cpm bound.

which ATP-induced permeabilization of these cells requires ATP^{4-} and is inhibited by the presence of divalent cations (Steinberg et al., 1987; Cockcroft et al., 1979, 1980).

As referred to above, UTP is a potent agonist with respect to causing increases of intracellular calcium in human neutrophils. Our data (Yu et al., unpublished) demonstrate that UTP has very little ability to compete for binding of ATP γS to human neutrophils. The most straightforward explanation of these findings is that UTP interaction with the neutrophil involves engagement of a receptor that is structurally different from the receptor responsible for binding of ATP γS. Such a conclusion is quite different from the one to be derived from the data in Figs. 2 and 4 where derivatives of ATP effectively compete for binding of ATP γS, a finding that would be consistent with the speculation that these adenine nucleotides bind to a common receptor.

These findings may be applicable to other cells as well, as the binding site of these ATP-specific receptors may have similar structural characteristics. Indeed, others have reported that the functional potency of BzATP is not only comparable to but sometimes exceeds that of ATP in their systems (Gonzalez et al., 1989). Use of this class of photoaffinity labels therefore offers the best hope for receptor isolation in such cases.

In addition to providing a fundamental understanding of nucleotide binding and receptor–substrate interaction, these findings may be useful for future attempts to isolate the PMN receptor. The enormous number of surface-associated proteins on PMN capable of recognizing ATP includes ectonucleotidases, ectokinases and membrane-associated actin (Gordon, 1986; Dusenbery et al., 1988), making nonspecific surface radiolabelling followed by purification a difficult task. A more reasonable approach adopted by those in this field involves the use of specific radiolabelled ATP compounds complexed to photoreactive groups, or photoaffinity labels. Some photoaffinity labels currently used to identify ATP-binding proteins are 8-N_3ATP, BzATP and arylazido-β-alanyl adenosine 5'triphosphate

(Czarnecki et al., 1979; Guillory et al., 1977, 1983; Williams et al.; Jeng et al., 1975).

The findings reported above indicate that the optimal compounds used in the identification of this PMN receptor are those whose photoreactive group is located on the ribose ring (i.e. BzATP), rather than those with base ring substitutions (i.e. 8-N_3ATP). Use of compounds such as BzATP creates minimal interference with substrate recognition, thus allowing optimal binding and radiolabelling efficiency. This issue is particularly important for the PMN receptor, in which only ≈ 1500 receptors per cell have been calculated based on Scatchard analysis of dose-dependence binding experiments (Yu et al., submitted for publication). As would be expected, preliminary attempts at radiolabelling with the photoaffinity label 8-N_3ATP have resulted in a disappointingly low efficiency of PMN labelling (data not shown).

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