Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 218-224

PRIMING OF THE RESPIRATORY BURST OF HUMAN NEUTROPHILS BY THE DIADENOSINE POLYPHOSPHATES, AP_4A AND AP_3A: ROLE OF INTRACELLULAR CALCIUM

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Received May 30, 1994

The diadenosine polyphosphates, Ap_3A and Ap_4A,prime the respiratory burst of human neutrophils after stimulation with fMet-Leu-Phe. Maximal priming of oxidase activity occurred at 600-800 μM Ap_3A and Ap_4A, compared with maximal priming observed at 200 μM ATP. The time course of priming of the oxidase by all 3 nucleotides was very rapid, being detectable if added within 10 s of fMet-Leu-Phe. All 3 nucleotides also elicited increases in intracellular Ca^{2+} levels and there was a close concentration-dependency between the extent of priming and the increase in intracellular Ca^{2+}. However, at low concentrations of nucleotides (<50 μM Ap_3A and Ap_4A and < 0.1 μM ATP) priming of the oxidase was observed without detectable increases in intracellular Ca^{2+}. These observations indicate that diadenosine polyphosphates may be novel regulators of neutrophil function and that priming of oxidase activity may occur via mechanisms that are either dependent or independent of increases in intracellular Ca^{2+}.

It is now recognised that neutrophil function during inflammation is regulated by a variety of mediators which "prime" processes such as the respiratory burst (1). Thus, primed neutrophils generate higher levels of reactive oxidants when they are subsequently stimulated with receptor-mediated agonists such as the chemotactic peptide, fMet-Leu-Phe (2). Many priming agents have now been identified and these include cytokines (such as γ-interferon, tumour necrosis factor and granulocyte-macrophage colony-stimulating factor), bacterial lipopolysaccharide and substance P (1). The mechanisms by which priming agents up-regulate neutrophils are not fully understood, but changes in receptor number and function, phosphorylation on tyrosine residues and activation of phospholipase D have all been implicated (3-6).

Recently, it has been shown that platelets can also prime neutrophils and that released ATP (which is normally located within dense platelet granules) is likely to be the priming agent involved (7).

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Abbreviations used are: Ap_3A, diadenosine 5',5'''-P^1,P^3-triphosphate; Ap_4A, diadenosine 5',5'''-P^1,P^4-tetraphosphate; Ap_5A, diadenosine 5',5'''-P^1,P^5-pentaphosphate; Ap_6A, diadenosine 5',5'''-P^1,P^6 hexaphosphate; fMet-Leu-Phe, *N*-formylmethionyl-leucyl-phenylalanine.

0006-291X/94 $5.00

Indeed, incubation of neutrophils with ATP leads to increases in intracellular Ca^{2+} and priming of O_2^- generation (8). These observations are of pathological importance because platelets are often found associated with neutrophils at sites of inflammation. Indeed, there is considerable interest in the role of extracellular adenine nucleotides in the regulation of cell function, because P_2-type receptors are present on the surfaces of many cell types (9). Activation of P_2 purinergic receptors can result in phospholipase C-mediated inositol phosphate formation in many cell types such as hepatocytes, erythrocytes and neutrophils (10-13).

In addition to ATP, platelets are also known to store and release the diadenosine polyphosphates Ap_3A, Ap_4A, Ap_5A and Ap_6A. These compounds have vasomotor activity and may be important regulators of blood pressure (14,15). Ap_4A, Ap_5A and Ap_6A are also stored in the secretory granules of chromaffin cells, *Torpedo* synaptic vesicles and rat brain synaptosomes and may have additional activity as neurotransmitters (16). The longer extracellular half-lives of these compounds compared to ATP (17,18) and the existence of specific dinucleotide P_2-type receptors (19-21) indicate that, in addition to having various possible intracellular functions, the diadenosine polyphosphates are also physiologically important, extracellular mediators (22). Because neutrophil:platelet interactions appear to be important in certain inflammatory reactions, the aims of this work were to determine the effects of the major platelet dinucleotides, Ap_3A and Ap_4A, on the respiratory burst of human neutrophils.

MATERIALS AND METHODS

Materials. Mono-Poly Resolving Medium and RPMI 1640 medium were from Flow Laboratories, whilst Fluo-3 AM was from Calbiochem. fMet-Leu-Phe, ATP, Ap_3A, Ap_4A luminol and cytochrome were from Sigma.

Purification of neutrophils. Neutrophils were isolated from heparinised human blood from healthy volunteers by centrifugation through Mono-Poly Resolving Medium, exactly as described previously (23). After hypotonic lysis to remove contaminating erythrocytes, neutrophils were suspended in RPMI 1640 medium and counted using a Fuchs-Rosenthal haemocytometer slide. Cell viability (>95 %) and cell purity (>97 % neutrophils) were routinely measured by Trypan blue exclusion and Wright's staining, respectively.

Neutrophil priming and activation. Neutrophils (0.5 x 10^6 cells/ml) were incubated in the presence and absence of priming agents (ATP, Ap_3A and Ap_4A) with gentle agitation at 37 °C, in RPMI 1640 medium. Times of incubation were as indicated in the figure legends. After incubation, suspensions were stimulated by the additions of fMet-Leu-Phe (1 μM).

Chemiluminescence. This was measured in primed and unprimed neutrophil suspensions (0.5 x 10^6 cells/ml) containing 10 μM luminol (24). After stimulation, photon emission was measured either using an LKB Wallac 1251 luminometer (using 1 ml suspensions) or a Dynatech ML-1000 plate reader (in 200 μl suspensions).

Superoxide secretion. This was monitored as the rate of superoxide dismutase (SOD)-inhibitable cytochrome c reduction (25,26). Suspensions contained 0.5 x 10^6 neutrophils and 75 μM cytochrome c. After stimulation, increases in absorption were monitored at 550 nm using a Bio-Rad 3550 plate reader. Control wells additionally contained 30 μg/ml SOD.

Intracellular Ca^{2+} measurements. These were performed using Fluo-3 as described previously (27). Briefly, neutrophil suspensions (10^7 cells/ml) in Ca^{2+} -free Hepes buffer (pH 7.4) were incubated with gentle agitation at 37 °C with 2 μM Fluo-3 acetomethoxy ester for 30 min. After loading, cells were washed twice and re-suspended in Ca^{2+}-containing Hepes buffer (1 mM Ca^{2+})

and kept at room temperature until use. Fluo-3 loaded cells were placed in fluorimeter tubes (0.5 x 10^6 cells/ml, total volume of 3.0 ml) and fluorescence measurements were performed at 37 °C using a Perkin-Elmer 3000 Fluorescence spectrometer (excitation 505 nm, emission 530 nm).

RESULTS

Effect of Ap_3A, Ap_4A and ATP on the respiratory burst. The addition of Ap_3A, Ap_4A and ATP at concentrations up to 2 mM did not stimulate reactive oxidant production in human neutrophils, as assessed by luminol chemiluminescence or cytochrome c reduction. However, all compounds were found to prime reactive oxidant production in a dose-dependent manner, in response to stimulation by fMet-Leu-Phe. Figure 1 shows that concentrations of ATP at < 1 μM primed the fMet-Leu-Phe stimulated respiratory burst, with maximal priming (approx 2.3 fold increase) occurring at 200 μM ATP. The diadenosine nucleotides Ap_3A and Ap_4A also primed the respiratory burst, but these were not as potent as ATP. For example, maximal priming occurred at 600-800 μM concentrations

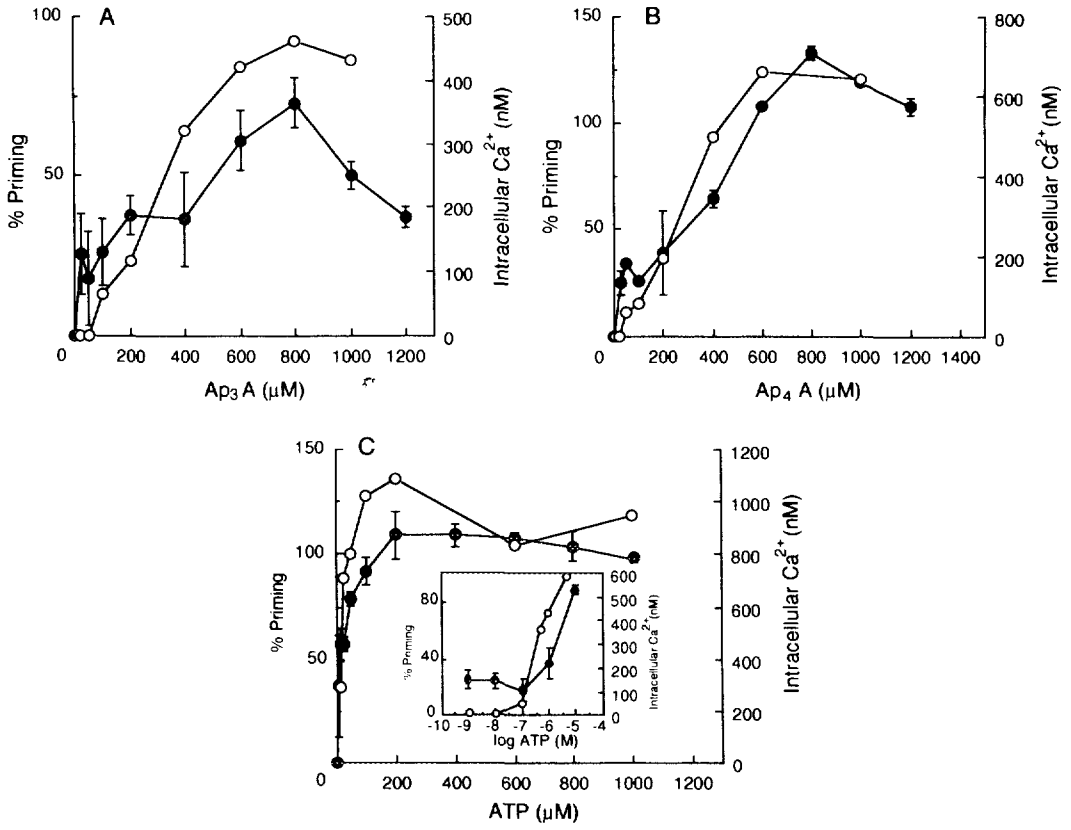

Figure 1. Correlation between priming of oxidase activity and intracellular Ca^{2+} levels. Neutrophils were loaded with Fluo-3 as described in Methods. The effects of different concentrations of Ap_3A (A), Ap_4A (B) or ATP(C) on intracellular Ca^{2+} levels were then monitored (o) whilst in (●) the ability of these nucleotides to prime fMet-Leu-Phe stimulated oxidase activity was measured by chemiluminescence. Inset in (C) shows the effects of low concentrations of ATP on intracellular Ca^{2+} levels and oxidase priming. Values for Ca^{2+} measurements are expressed as increases above basal (unstimulated) levels. Similar results have been obtained in 3 other separate experiments.

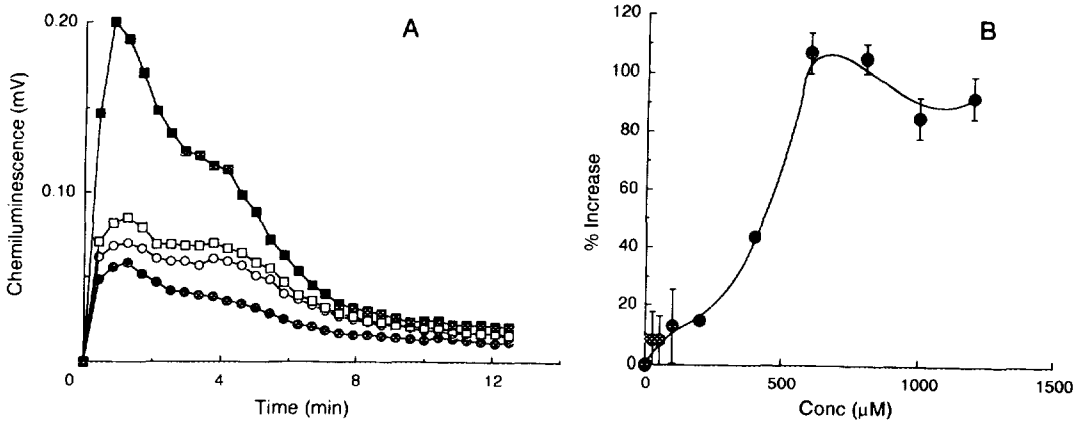

Figure 2. Priming of fMet-Leu-Phe stimulated oxidase activity by adenine nucleotides. In A, neutrophils were incubated for 1 min in the absence (●) or presence of 50 μM Ap_3A (○), Ap_4A (□) or ATP (■) before stimulation with 1 μM fMet-Leu-Phe and measurement of luminol chemiluminescence. Similar results were obtained in 3 other separate experiments. In B, neutrophils were incubated with Ap_4A (at the indicated concentration) and fMet-Leu-Phe stimulated O_2^- secretion was measured by following the rate of SOD-inhibitable cytochrome c reduction. Values (mean ± SD, n= 6) are presented as % increase over control rates incubated under identical conditions in the absence of Ap_4A.

of both dinucleotides, with maximal increases of 1.7 fold and 2.5 fold for Ap_3A and Ap_4A, respectively.

All 3 nucleotides could prime the oxidase after only very short pre-incubation times. For example, if Ap_3A or Ap_4A were added just 10 s before the addition of fMet-Leu-Phe, then priming of the oxidase was observed (data not shown): maximal priming by these dinucleotides occurred after 1 min incubation and the priming response was still observed if the lag between addition of nucleotide and addition of fMet-Leu-Phe was extended to 25 min (data not shown). ATP also primed the oxidase very rapidly, but maximal priming occurred 3 min before the addition of fMet-Leu-Phe. Typical chemiluminescence traces showing the unprimed fMet-Leu-Phe response and the responses obtained after priming with 50 μM ATP, Ap_3A and Ap_4A are shown in Figure 2A.

Priming of O_2^- secretion (as assayed by the cytochrome c reduction assay) was also observed after pre-incubation with these 3 nucleotides. The dose-dependencies and pre-incubation times were identical to those observed for priming of chemiluminescence. Fig. 2B shows results for Ap_4A.

Changes in Intracellular Ca^{2+}. Previous work has shown that ATP can activate changes in intracellular Ca^{2+} in human neutrophils and that diadenosine polyphosphates can stimulate Ca^{2+} changes in other cell types (28,29). We therefore measured increases in intracellular Ca^{2+} induced by these nucleotides and corellated these changes with priming of the oxidase.

Figure 1 shows that the nucleotides Ap_3A (Fig.1A), Ap_4A (Fig. 1B) and ATP (Fig.1C) all activated increases in intracellular free Ca^{2+} and these increases were dose-dependent. Maximal increases in intracellular Ca^{2+} were 450 nM, 700 nM and 1 μM for Ap_3A, Ap_4A and ATP,

respectively. There was a remarkable correlation between the increase in intracellular Ca^{2+} and the extent of priming of fMet-Leu-Phe stimulated reactive oxidant production for all 3 nucleotides. Maximal priming occurred at concentrations of nucleotide which also caused the maximal increases in intracellular Ca^{2+}. However, low concentrations of nucleotides (up to 50 μM for Ap_3A and Ap_4A, and up to 0.1 μM for ATP) could prime fMet-Leu-Phe stimulated reactive oxidant production, without detectable increases in intracellular Ca^{2+}. In our experimental system we can detect changes in intracellular Ca^{2+} as low as 20 nM. This disparity between priming and elevations in Ca^{2+} is highlighted in Figure 3 (A and B), where 20 μM Ap_4A primed fMet-Leu-Phe stimulated oxidant production by approx 50 % but this concentration of nucleotide did not elicit detectable changes in intracellular Ca^{2+}. In contrast, 600 μM Ap_4A primed the oxidase by over 2.5 fold (Fig. 3C) and this concentration of nucleotide caused a substantial increase in intracellular Ca^{2+} (Fig.3D).

DISCUSSION

In this report, we have shown that, in common with ATP, the adenine dinucleotides Ap_3A and Ap_4A prime the respiratory burst of neutrophils. Priming by all of the nucleotides appears to occur via mechanisms that are quite distinct from those by which cytokines prime these cells. For example, GM-CSF fails to activate phospholipase C to alter intracellular Ca^{2+} levels and incubation times in excess of 20-30 min are required for the oxidase to be primed. In contrast, the priming effects of ATP and the dinucleotides were detectable within 10 s of addition and maximal by 3 min (for ATP) or 1 min (for Ap_3A and Ap_4A) prior to the addition of fMet-Leu-Phe. Furthermore, these nucleotides all activated dose-dependent increases in intracellular Ca^{2+}. Hence, it is extremely unlikely that the nucleotides and cytokines prime the respiratory burst via analogous processes.

For all 3 nucleotides tested, there was a marked correlation between the extent of priming and the increase in intracellular Ca^{2+} at high concentrations: this relationship existed for Ap_3A and Ap_4A at >50 μM whilst for ATP this was observed at >0.1 μM. Such elevations in intracellular Ca^{2+} were rapid and thus it is tempting to propose that ATP priming is at least causally related to intracellular levels of this second messenger. However, when added at lower concentrations, these nucelotides could prime reactive oxidant production without detectable increases in intracellular Ca^{2+}. For example, at concentrations of <50 μM Ap_3A and Ap_4A could prime the oxidase, but increases in intracellular Ca^{2+} were not observed. In our assay, increases in intracellular Ca^{2+} of <20 nM would have been detected. Thus, 2 possibilities exist to explain these observations. First, the nucleotides may prime the respiratory burst of neutrophils by two separate mechanisms namely a Ca^{2+}-dependent process (at high nucleotide concentrations) and a Ca^{2+}-independent mechanism (at low nucleotide concentrations). Alternatively, the observed changes in intracellular Ca^{2+} may be consequential and not directly related to the priming process. Further work will be required to evaluate these proposals by manipulating intracellular Ca^{2+} levels and measuring the effects of the dinucleotides on priming.

It has been estimated that the concentrations of platelet-derived Ap_3A and Ap_4A exceed 100 μM in the cleft between a platelet thrombus and a wounded blood vessel wall (30). If similar local

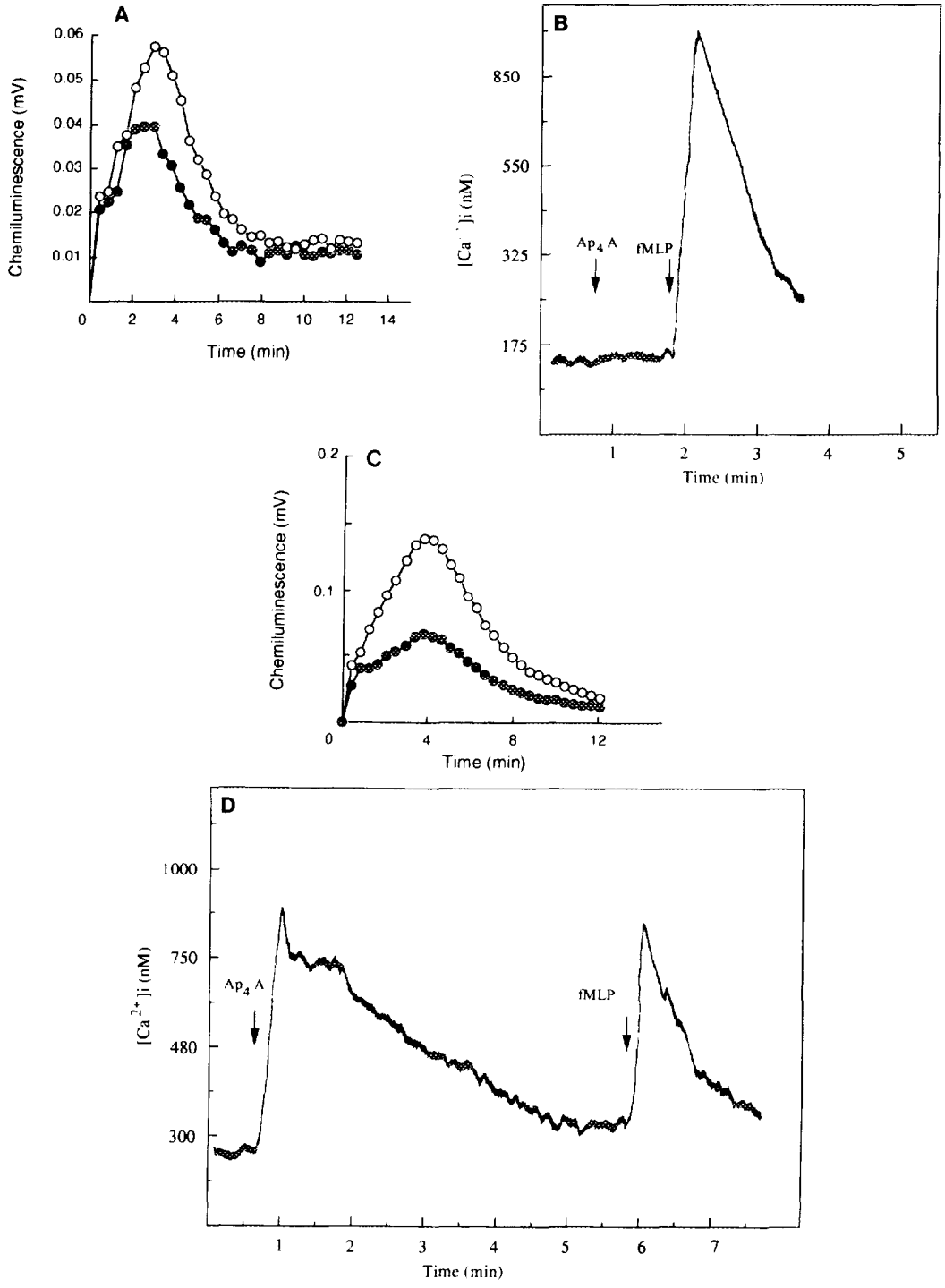

Figure 3. Effect of Ap_4A on intracellular Ca^{2+} and priming of oxidase activity.
In B and D, neutrophils were loaded with Fluo-3 and as indicated by the arrows, intracellular Ca^{2+} levels were monitored following the additions of Ap_4A at 20 μM (B) or 0.6 mM (D), and 1 μM fMet-Leu-Phe. In A and C, the effects of Ap_4A at 20 μM (A) and 0.6 mM (C) on fMet-Leu-Phe stimulated oxidase activity were measured by luminol chemiluminescence. In A and C (●) shows the unprimed response whilst (○) shows the response primed by Ap_4A. Similar results have been obtained in 3 other separate experiments.

concentrations were achieved at sites of inflammation, this would suggest that the Ca^{2+}-independent mechanism may be of particular physiological significance. Since Ap_4A appears to bind to at least two types of P_2-purinoceptor, one with properties similar to the Ca^{2+}-mobilizing P_{2y} receptor (27,31) and one with a high specificify and affinity for adenine dinucleotides, termed P_{2d} (20,21), it is tempting to suggest that the Ca^{2+}-dependent and Ca^{2+}-independent responses operate in neutrophils via these two different subclasses of P_2 receptor.

Acknowledgment: We thank the Medical Research Council for financial support.

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