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Diadenosine polyphosphates induce intracellular Ca^{2+} mobilization in human neutrophils via a pertussis toxin sensitive G-protein

L. GASMI, A. G. MCLENNAN & S. W. EDWARDS *Cellular and Metabolic Regulation Group, Department of Biochemistry, University of Liverpool, Liverpool, UK*

SUMMARY

The diadenosine polyphosphates diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap_3A), diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A), diadenosine 5′,5‴-P^1,P^5-pentaphosphate (Ap_5A) and diadenosine 5′,5‴-P^1,P^6-hexaphosphate (Ap_6A) all stimulated increases in intracellular Ca^{2+} in human neutrophils. Maximal increases in intracellular Ca^{2+} of 650 nM were obtained at dinucleotide concentrations of 500–700 μM. These increases in intracellular Ca^{2+} were completely abolished by pre-treatment of the neutrophils with pertussis toxin and were hardly affected when the extracellular buffer was devoid of Ca^{2+}. On the other hand, adenosine triphosphate (ATP) could stimuate much greater increases in intracellular Ca^{2+} (up to 1·1 μM) at much lower concentrations (half maximal responses obtained at around 5 μM ATP). Receptor de-sensitization experiments indicate that human neutrophils may possess two types of P2-purinoceptors. The first of these may bind ATP (but not the dinucleotides) with high affinity whilst the second may bind the dinucleotides with lower affinity and also bind ATP.

INTRODUCTION

It is now recognized that the function of bloodstream neutrophils is carefully regulated by the local production of both pro-and anti-inflammatory mediators. Within the circulation, neutrophils are only poorly responsive to stimulation and need to become primed before their cytotoxic functions can be maximally expressed.[1,2] Priming thus up-regulates both the number and function of certain plasma membrane receptors, results in enhanced activity of the O_2^- generating reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, stimulates *de novo* biosynthesis and extends their functional lifespan via delayed apoptosis.[3–8] Many neutrophil priming agents exist but the effects of cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) have been most extensively studied.[1,4] However, whilst other immune cells and endothelial cells can generate such cytokines, bacterial products such as endotoxin are also effective priming agents. Many inflammatory conditions are associated with neutrophil–platelet interactions, and indeed it has been shown that activated platelets can also prime neutrophil functions.[9–11] There are a number of potential platelet products that may serve this function, but adenosine nucleotides such as adenosine triphosphate (ATP) can have priming effects *in vitro*.[12–14]

Apart from possessing high concentrations of ATP, platelet dense granules are also rich in dinucleoside polyphosphates, Ap_nA.[15,16] Whilst ATP has been shown to prime certain neutrophil functions and to mobilize intracellular Ca^{2+}, the dinucleoside polyphosphates have also been shown to exert stimulatory effects on neutrophils[17,18] and other cell types. These molecules may also be found in secretory granules of chromaffin cells and brain synaptosomes[19] and local concentrations in excess of 100 μM may be obtained under certain conditions.[15] There is now increasing evidence to show that these dinucleosides may function as novel extracellular mediators.

We have previously shown that the dinucleotides diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap_3A) and diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) can mobilize intracellular Ca^{2+} in neutrophils and prime respiratory burst activity.[17] Furthermore, these compounds can alone, or in combination with GM-CSF delay neutrophil apoptosis.[18] These effects on apoptosis are, however, observed at concentrations that are insufficient to generate intracellular Ca^{2+} transients. These observations thus indicate that diadenosine nucleotides may serve as novel regulators of acute inflammation under conditions in which platelet activation and neutrophil activation occur. The dinucleotides may be biologically more important than ATP because they are considerably more stable and hence longer lived.[20,21] The aims of this work were to investigate

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Abbreviations: Ap_3A, diadenosine 5′,5‴-P,$^1P^3$-triphosphate; Ap_4A, diadenosine 5′,5‴-P,$^1P^4$-tetraphosphate; Ap_5A, diadenosine 5′,5‴-P,$^1P^5$-pentaphosphate; Ap_6A, diadenosine 5′,5‴-P,$^1P^6$-hexaphosphate.

Correspondence: Dr S.W. Edwards, Department of Biochemistry, University of Liverpool, PO Box 147, Liverpool L69 3BX UK.

whether diadenosine 5',5'''-P^1,P^5-pentaphosphate (Ap_5A) and diadenosine 5',5'''-P^1,P^6-hexaphospahte (Ap_6A) were effective in activating intracellular Ca^{2+} transients in neutrophils and to explore the mechanisms that regulate the generation of this intracellular signal.

MATERIALS AND METHODS

Preparation of neutrophils

Neutrophils were isolated from heparinized human blood from healthy volunteers by a one-step centrifugation method[22] in neutrophil isolation medium (NIM, Cardinal Associates, Santa Fe, NM) as described previously.[23,24] After hypotonic lysis to remove contaminating erythrocytes, purified neutrophils were suspended in RPMI-1640 medium (Flow Laboratories, Rickmansworth, Herts, UK) and counted using a Fuchs–Rosenthal haemocytometer slide. Cell viability (>95%) and purity (>95%) were routinely assessed by trypan blue exclusion and Wright's staining, respectively.

Fluo-3 loading and fluorescence measurements

Neutrophils were suspended at 10^7 cells/ml in Ca^{2+}-free HEPES buffer and incubated with 2 μM Fluo-3AM (Calbiochem) for 30 min at 37°.[17] After this period, loaded cells were washed twice and re-suspended at the same cell density in Ca^{2+}-free HEPES buffer. As indicated in some experiments 1 mM Ca^{2+} was added (as $CaCl_2$) prior to stimulation and measurement of fluorescence, whilst in other experiments 1 mM EGTA was added. Loaded cells ($1{\cdot}5 \times 10^6$) were placed in a 3 ml fluorescence cuvette and fluorescence was measured (excitation 505 nm, emission at 530 nm) at 37° using a Perkin Elmer 3000 fluorimeter (Perkin Elmer, Beaconsfield, Bucks, UK). Quantification of intracellular Ca^{2+} increases from the changes in fluorescence was performed as previously described.[25] ATP (Sigma, Poole, Dorset) and diadenosine polyphosphate (Sigma) induced intracellular Ca^{2+} changes were measured at the indicated concentrations.

Incubation with pertussis toxin

Neutrophils were incubated at 1×10^7 cells/ml in Ca^{2+} free HEPES buffer with gentle agitation in the absence (control) or presence of pertussis toxin (Sigma) in the range 0·5–3 μg/ml. Experiments measuring the effects of pertussis toxin on fMet-Leu-Phe (Sigma) induced intracellular Ca^{2+} transients and activation of the respiratory burst[26] indicated that incubation periods of 2 hr were required for the toxin to exert its effects. Thus, after 1·5 hr incubation with pertussis toxin, the neutrophil suspensions were incubated for a further 30 min with 2 μM Fluo-3AM prior to washings and measurement of intracellular Ca^{2+} transients as described above. This treatment with pertussis toxin did not affect neutrophil viability nor phorbol 12-mysristate 13-acetate (PMA; Sigma)-induced activation of the respiratory burst.[26]

RESULTS

We have previously shown that the dinucleotides Ap_3A and Ap_4A mobilize intracellular Ca^{2+} levels in neutrophils at concentrations that also prime the ability of these cells to generate reactive oxygen metabolites in response to fMet-Leu-Phe.[17] We thus tested the ability of the dinucleotides Ap_5A and Ap_6A to mobilize intracellular Ca^{2+} in these cells. Figure 1 shows that the concentrations of Ap_5A and Ap_6A required to maximally mobilize intracellular Ca^{2+} were very similar to those of Ap_3A and Ap_4A. Maximal levels of intracellular Ca^{2+} were observed at around 500–700 μM and the maximal intracellular Ca^{2+} concentration achieved was around 650 nM. These results were different to those obtained using ATP. ATP mobilized much higher levels of intracellular Ca^{2+} (around 1·1 μM) and this was achieved at much lower concentrations. For example, 5 μM ATP resulted in an intracellular Ca^{2+} signal of around 600 nM but this concentration of dinucleotide did not stimulate any intracellular Ca^{2+} increase.

Effect of EGTA on Ap_nA induced intracellular Ca^{2+} increases

In order to establish if the increases in intracellular Ca^{2+} were due to mobilization of intracellular stores or instead to Ca^{2+} influx, experiments were performed in the presence and absence of extracellular Ca^{2+}/EGTA. When Fluo-3 loaded neutrophils were incubated in Ca^{2+}-free buffer, the addition of 1 mM EGTA caused a small decrease in the fluorescence signal, indicating that in resting cells, Ca^{2+} influx may play a small role in maintaining resting intracellular Ca^{2+} levels (Fig. 2a). Ap_4A addition then stimulated a rapid increase in fluorescence, the peak height of which (330 nM) was almost identical to that observed in neutrophils incubated in Ca^{2+} containing buffer (Fig. 2b). However, in Ca^{2+}-free medium, the fluorescence declined to basal levels by 2·5 min after addition of dinucleotide, whereas in Ca^{2+}-containing medium, the fluorescence was maintained above the basal levels for several minutes. Similar results were obtained in experiments using Ap_3A, Ap_5A and Ap_6A (data not shown). These data indicate that the initial rise in intracellular Ca^{2+} observed after addition of Ap_nA is almost entirely due to mobilization of intracellular stores; Ca^{2+} influx may be responsible for maintaining the stimulated intracellular Ca^{2+} level once the initial mobilization has subsided.

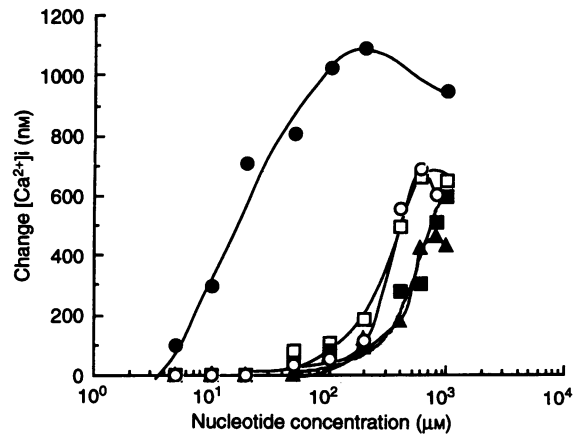

Figure 1. Concentration effects of ATP and diadenosine polyphosphates on intracellular Ca^{2+} levels in neutrophils. Neutrophils were loaded with Fluo-3 as indicated in Materials and Methods and intracellular Ca^{2+} levels were measured following the additions of: (●), ATP; (▲), Ap_3A; (□), Ap_4A; (■), Ap_5A and (○), Ap_6A. Values shown are the maximal increases in intracellular Ca^{2+} that were achieved (within 30 seconds of addition of nucleotide). Typical result of three independent experiments.

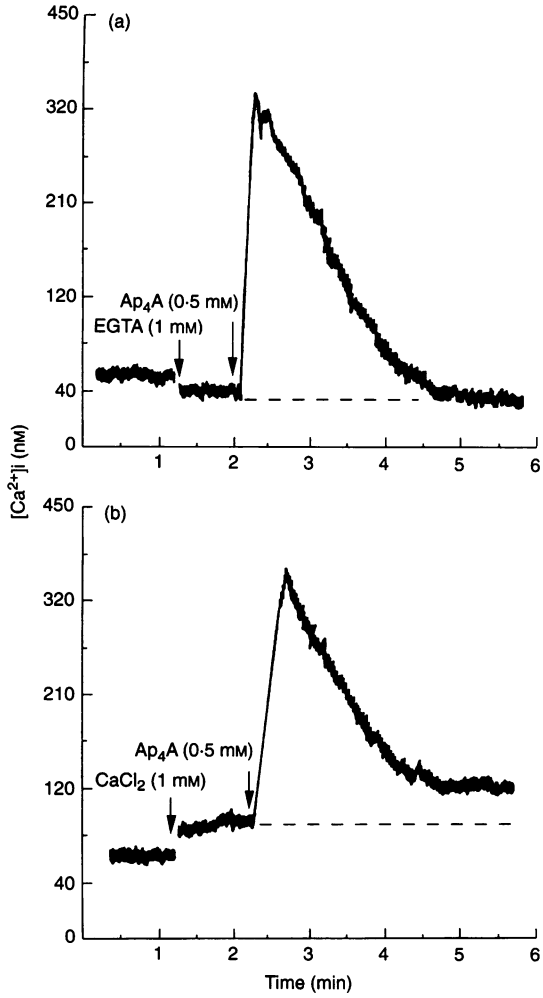

Figure 2. Effect of extracellular Ca^{2+} on intracellular Ca^{2+} transients. Neutrophils were loaded with Fluo-3 as described in Materials and Methods and suspended in Ca^{2+}-free HEPES buffer. In (a), 1 mM EGTA was added prior to the addition of 500 μM Ap_4 A and measurement of intracellular Ca^{2+}. In (b), 1 mM Ca^{2+} was added to the cell suspension prior to the addition of Ap_4A. This experiments is representative of five other experiments and similar results were obtained when cells were stimulated with Ap_3A, Ap_5A or Ap_6A.

Effect of pertussis toxin on intracellular Ca^{2+} increases

Previous work has shown that ATP-mediated increases in intracellular Ca^{2+} in neutrophils are inhibited by pertussis toxin and hence are mediated via a receptor that is coupled to a heterotrimeric G-protein of the G_i type.[12] It was therefore necessary to determine if the dinucleotides also acted via a similar G-protein-coupled process. Neutrophils were incubated with pertussis toxin at concentrations of 0·5–3·0 μg/ml for 2 hr and were loaded with 2 μM Fluo-3AM during the final 30 min of this incubation. When intracellular Ca^{2+} increases were stimulated by either ATP or Ap_nA (Fig. 3a and b) a pertussis toxin concentration of 3 μg/ml was required to completely abolish the intracellular Ca^{2+} transients. However, fMet-Leu-Phe stimulated intracellular Ca^{2+} transients were completely abolished by 2 μg/ml pertussis toxin (Fig. 3c). The sensitivity of the intracellular Ca^{2+} increases to pertussis toxin were

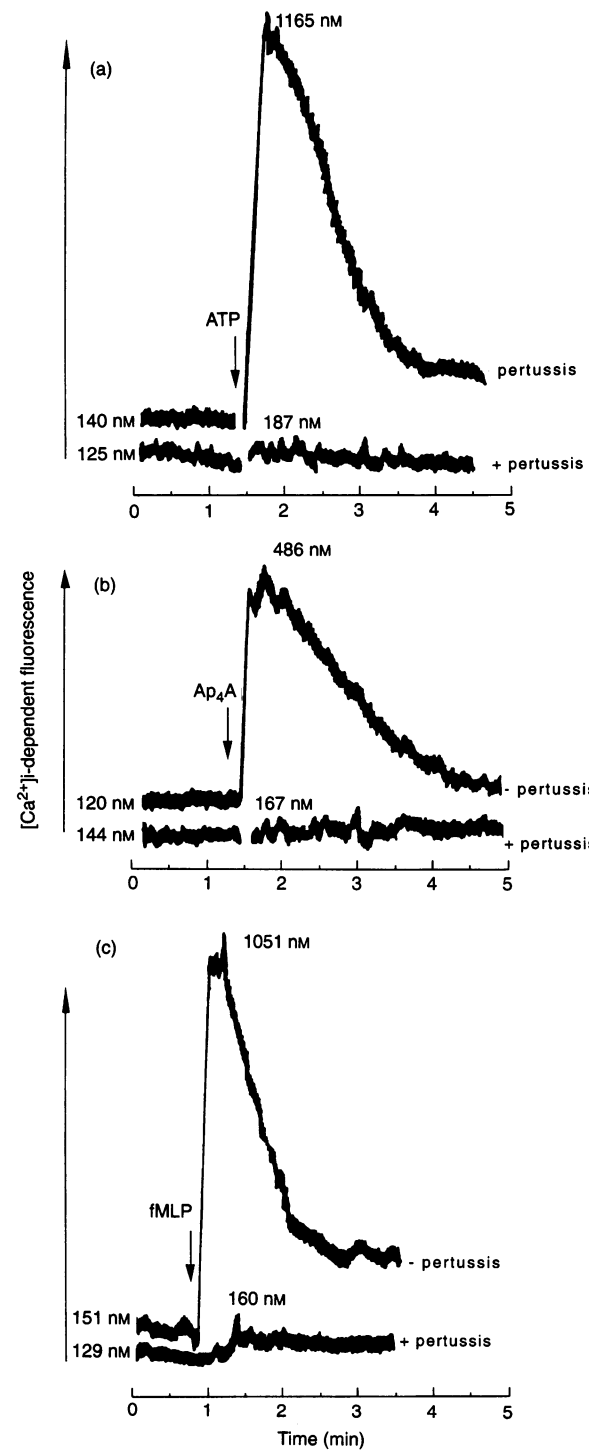

Figure 3. Effect of pertussis toxin on intracellular Ca^{2+} transients. Neutrophils were incubated in the absence (control) and presence of pertussis toxin within the range 0·5–3 μg/ml for 2 hr and in the final 30 min of this incubation, they were loaded with Fluo-3AM and suspended in Ca^{2+}-containing HEPES buffer prior to stimulation. Data shown are the results obtained after incubation with 3 μg/ml pertussis toxin (for ATP and Ap_4A) and 2 μg/ml for fMet-Leu-Phe. After incubation cells were stimulated by the addition of 10 μM ATP (a), 500 μM Ap_4A (b) and 0·1 μM fMet-Leu-Phe (c). Data are representative of three separate experiments.

identical when stimulated with either Ap_3A, Ap_4A, Ap_5A or Ap_6A (data not shown).

Receptor desensitization by ATP and Ap_nA

In order to gain some insight into whether ATP and Ap_nA bind to and activate the same receptor(s), the experiments shown in Fig. 4 were performed. Intracellular Ca^{2+} increases were stimulated by ATP and when the fluorescence had decreased to near basal levels, a second addition of ATP was made to the cells (Fig. 4a). However, this second addition of ATP failed to activate another intracellular Ca^{2+} rise. Because fMet-Leu-Phe could still activate an intracellular rise, the failure of ATP to induce a second Ca^{2+} signal is not due to depletion of intracellular stores, but more likely due to receptor desensitization. Similarly, a second addition of Ap_4A could not stimulate a second increase in intracellular Ca^{2+} (Fig. 4b), indicating receptor desensitization. However, when the Ca^{2+} signal stimulated by Ap_4A had declined to near basal levels, both ATP and fMet-Leu-Phe could stimulate further intracellular Ca^{2+} increases (Fig. 4c). When the order of additions was reversed, Ap_4A could not stimulate an intracellular signal following ATP addition, but fMet-Leu-Phe was still effective (Fig. 4d). The results obtained with Ap_4A were identical to those obtained in experiments using Ap_3A, Ap_5A and Ap_6A (data not shown). Furthermore, neither Ap_3A, Ap_5A nor Ap_6A could stimulate a second intracellular Ca^{2+} increase following initial Ap_4A addition (data not shown).

DISCUSSION

There is a growing awareness that diadenosine polyphosphates may serve as novel, extracellular regulators of cell function. Ap_3A and Ap_4A are present in high concentrations in the dense granules of platelets and in secretory granules of chromaffin cells and more recently, Ap_5A and Ap_6A have been reported in these cells.[15,16,27–29] These dinucleotides can elicit a variety of responses in several cells and tissues such as catecholamine release from chromaffin cells,[30] platelet aggregation,[31–33] vasoregulation,[34,35] increased intracellular Ca^{2+} levels in hepatocytes[36,37] and priming of the respiratory burst and elevated intracellular Ca^{2+} levels in neutrophils.[17] Furthermore, they can act either alone or in combination with GM-CSF to delay neutrophil apoptosis.[18] Indeed, they have been shown to interact with and increase the activity of a number of growth factors.[38] All dinucleotides are degraded much more slowly by cellular ectonucleotidases[15] and so whilst they may be secreted at lower levels than ATP they may be more important physiologically because of their enhanced stability and may act over greater spatial distances than ATP.

The cellular receptors responsible for nucleotide- and dinucleotide-mediated responses have not been fully defined. Based upon agonist-competition studies and pharmacological data, they appear to bind to P2-type purinoceptors that can also bind ATP and adenosine dinucleotide (ADP). It is thought that two major families of P2-purinoceptors exist in different cells and tissues: P2X (ligand gated) and P2Y (G-protein coupled).[39] Ap_4A and Ap_5A may bind to a P2Y-type receptor in chromaffin cells whilst Ap_6A may bind to a P2X-type receptor in bladder cells.[40,41] Many of these receptors have now been cloned and hence their molecular diversity and tissue distributions have been confirmed. There is, however, considerable debate as to whether the dinucleotides activate cells by the same receptor that binds ATP or ADP or whether unique dinucleotide receptors exist. There is some evidence to suggest that unique dinucleotide receptors may exist. For example, Ap_3A and Ap_4A exert effects on rabbit hearts that are not seen by addition of ATP, ADP, adenosine monophosphate (AMP) or adenosine,[34] while P_{2d} ($P2Y_7$) receptors have been described in rat brain synaptic terminals.[42]

In this report we show that Ap_3A, Ap_4A, Ap_5A and Ap_6A can all stimulate increases in intracellular Ca^{2+} in human neutrophils. There were no observed differences in either the

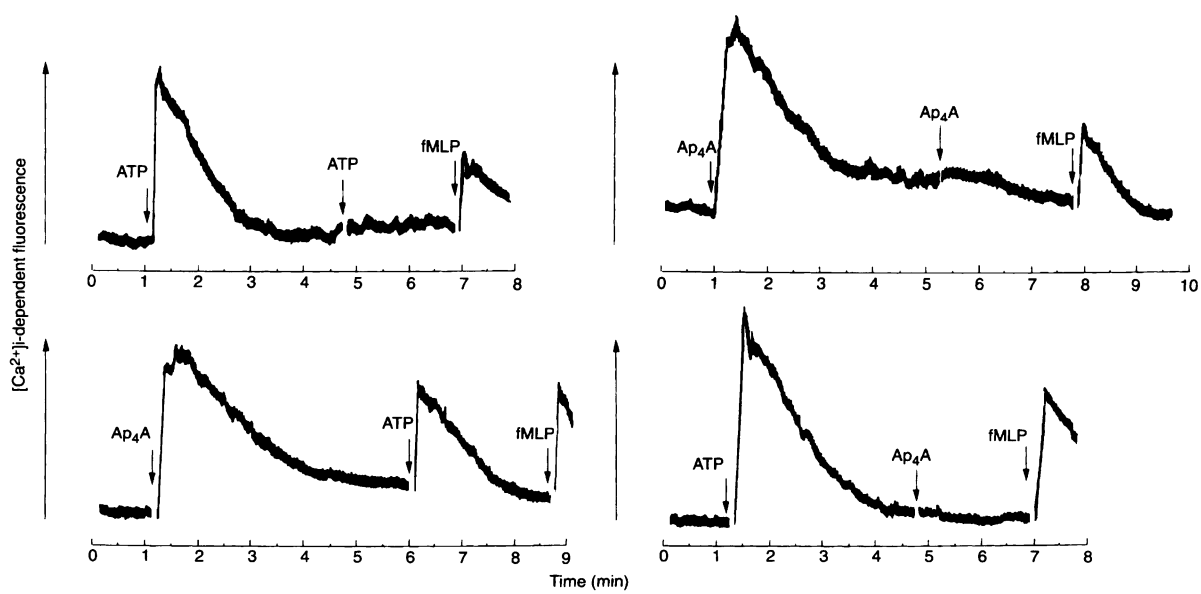

Figure 4. Receptor desensitization. Neutrophils were loaded with Fluo-3 and suspended in Ca^{2+}-containing hepes buffer. They were then stimulated by the additions of ATP (10 μM), Ap_4A (500 μM) and fMet-Leu-Phe (0·1 μM, as indicated). These results are typical of five separate experiments.

concentrations of nucleotides required to elicit these responses nor in the maximal levels of intracellular Ca^{2+} that could be mobilized by these different dinucleotides. Maximal increases in intracellular Ca^{2+} were observed at around 500 μM dinucleotide and these could induce increases of around 600 nM $Ca.^{2+}$ These concentrations are considerably higher than those required to elicit responses in other cell types (e.g.[38]). These intracellular Ca^{2+} transients were largely due to mobilization of intracellular stores because both the maximal levels of Ca^{2+} and the time to reach maximum were unaffected when cells were stimulated in Ca^{2+}-free buffer. In the absence of extracellular Ca^{2+}, however, the intracellular Ca^{2+} levels returned to basal levels more rapidly, indicating that increased Ca^{2+} influx may contribute to sustained increases in the intracellular levels of this cation. These intracellular Ca^{2+} transients were completely abolished by pretreatment of the cells with pertussis toxin. Thus, we conclude that all four dinucleotide polyphosphates tested probably interact with the same type of receptor on the neutrophil cell surface that is G-protein coupled.

However, the intracellular Ca^{2+} transients stimulated by ATP were quite different to those stimulated by the dinucleotides. Firstly, ATP could mobilize intracellular Ca^{2+} at much lower concentrations than the dinucleotides. For example, at concentrations of 5 μM, ATP could induce an intracellular Ca^{2+} signal of over 550 nM (>50% of the maximum response obtainable with this compound) whilst the dinucleotides were ineffective at this concentration. Secondly, the maximal levels of intracellular Ca^{2+} that could be stimulated by ATP were almost double those that could be maximally stimulated by the dinucleotides. Under our experimental conditions, the maximal increase in intracellular Ca^{2+} was observed within 30 seconds of addition of nucleotide or dinucleotide and so it is unlikely that any of these compounds were degraded during the timecourse of the measurement.

When ATP was used to stimulate intracellular Ca^{2+} increases, a subsequent addition of ATP could not re-stimulate a further Ca^{2+} transient. However, addition of fMet-Leu-Phe, which stimulates intracellular Ca^{2+} transients via a completely independent receptor-signalling pathway, could stimulate a transient when added after ATP. This indicates that the initial ATP addition did not fully deplete the intracellular Ca^{2+} stores and did not down regulate the functions of other receptors coupled to phospholipase C activation. Furthermore, addition of dinucleotides after the addition of ATP also failed to activate a second increase in intracellular Ca^{2+}. This may indicate therefore that the dinucleotides mobilize Ca^{2+} via ligation to a receptor(s) that also binds ATP. However, ATP could stimulate a second intracellular Ca^{2+} increase when added after the dinucleotide. Taken together with the differences in concentration dependencies of the ATP and dinucleotide induced Ca^{2+} transients, we propose that neutrophil possess two types of P2-purinoceptor. The first of these binds ATP (but not dinucleotide) with high affinity, whilst the second binds dinucleotides with low affinity but also binds ATP. Both types of putative receptor are G-protein coupled to phospholipase C to mobilize intracellular Ca^{2+}. Further work is necessary to test this hypothesis, to characterize the molecular properties of these putative receptors and to assign their biological functions.

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