A quantitative investigation into the dependence of Ca^{2+} mobilisation on changes in inositol 1,4,5-trisphosphate levels in the stimulated neutrophil

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1 The coupling of N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) receptor stimulation to Ca^{2+} mobilisation has been investigated in the human neutrophil by measuring the concentration-effect curves for inositol 1,4,5-trisphosphate (IP_3) formation and Ca^{2+} mobilisation.

2 fMet-Leu-Phe-dependent mobilisation of intracellular Ca^{2+} has been monitored in fluo-3-loaded human neutrophils by measuring increases in the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in the presence of extracellular EGTA. Fluo-3 was used in preference to fura-2 because it was found to be more sensitive to the high Ca^{2+} levels seen in stimulated neutrophils.

3 fMet-Leu-Phe induced a rapid mobilisation of intracellular Ca^{2+} ($\text{EC}_{50} = 2.9 \pm 0.1 \text{ nM}$) and increased $[\text{Ca}^{2+}]_i$ to a maximum of $1286 \pm 184 \text{ nM}$.

4 The amount of IP_3 in fMet-Leu-Phe-stimulated neutrophils was determined by competition with $[\text{^3H}]\text{-IP}_3$ for a specific IP_3 binding protein isolated from bovine adrenocortical microsomes. Basal IP_3 levels of $13.3 \pm 2.0 \text{ pmol per } 10^7 \text{ cells}$ were increased nearly 4 fold by maximally effective concentrations of fMet-Leu-Phe.

5 The EC_{50} for the IP_3 response ($95 \pm 18 \text{ nM}$) was much higher than that for mobilisation of intracellular Ca^{2+} , such that only a doubling in the concentration of IP_3 was required to fully mobilise intracellular Ca^{2+} .

6 As a result of this relationship IP_3 production was more sensitive than Ca^{2+} mobilisation to inhibition by demethoxyviridin, an inhibitor of phospholipase activation.

Keywords: Signal transduction; phospholipase C; phospholipase D; phospholipase inhibitor; demethoxyviridin

Introduction

Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by a specific phospholipase C (PLC) is a common step in the activation of many tissues by Ca^{2+} -mobilising ligands (Berridge, 1987; Berridge & Irvine, 1989). Both products of the reaction have second messenger roles. Inositol 1,4,5-trisphosphate (IP_3) mobilises intracellular Ca^{2+} from a non-mitochondrial pool (Berridge, 1987; Berridge & Irvine, 1989) whilst 1,2-diacyl-*sn*-glycerol represents the main physiological activator of the enzyme protein kinase C (Nishizuka, 1986; Berridge, 1987).

Ca^{2+} mobilisation by IP_3 has been studied extensively both in permeabilised cells and by micro-injection techniques and has been found to be mediated by a specific receptor associated with the endoplasmic reticulum (Streb *et al.*, 1984). Recent work has revealed that the IP_3 receptor and the IP_3 -sensitive Ca^{2+} channel are part of the same intrinsic membrane protein (Ferris *et al.*, 1989).

The actions of IP_3 have been reported to be highly co-operative, suggesting that just a small change in the cytoplasmic concentration could lead to a large effect on Ca^{2+} stores (Meyer *et al.*, 1988). Analysis of the concentration-dependence of IP_3 -mediated effects has identified a number of anomalies however. For example, in most permeabilised tissues exogenous IP_3 mobilises Ca^{2+} with concentrations for half-maximal effect (EC_{50}) being in the range $0.1\text{--}1.0 \mu\text{M}$ (Berridge, 1987). However, estimates of the affinity of IP_3 for its receptor are generally much higher than this e.g. 6 nM in bovine adrenocortical microsomes (Palmer & Wakelam, 1989). Furthermore, estimates of IP_3 concentrations in intact cells before

and after activation have been found to vary considerably between tissues (Shears, 1989). In anterior pituitary cells, IP_3 levels were increased 3 fold to 56 nM following stimulation with gonadotrophin-releasing hormone (Morgan *et al.*, 1987) whilst in rabbit neutrophils basal levels of $0.1 \mu\text{M}$ were increased 10 fold following stimulation by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) (Bradford & Rubin, 1986). Although it is clear that IP_3 mobilises intracellular Ca^{2+} it is not apparent what increase in IP_3 concentration is required to achieve complete mobilisation of the Ca^{2+} pool.

To address this question we have analysed the relationship between Ca^{2+} mobilisation and the amount of IP_3 produced in fMet-Leu-Phe-stimulated human neutrophils. In a similar study on vasopressin-stimulated rat hepatocytes, only 15% of the maximal inositol trisphosphate that could be generated was needed to mobilise intracellular Ca^{2+} fully (Thomas *et al.*, 1984). However, this particular study had the shortcoming that no distinction was made between the different inositol trisphosphate isomers. In the human neutrophil the concentration-effect curve for fMet-Leu-Phe-stimulated Ca^{2+} mobilisation has been reported to lie to the left of those for other fMet-Leu-Phe-dependent effects (Korchak *et al.*, 1984; Rossi *et al.*, 1985). However, in these studies the relationship between the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and IP_3 levels was not investigated. Furthermore, previous studies have used either quin2 or fura-2 to monitor the $[\text{Ca}^{2+}]_i$. The disadvantage of both these fluorescent probes is that they have a relatively high affinity for Ca^{2+} (120 nM and 224 nM respectively) and are therefore insensitive to high $[\text{Ca}^{2+}]_i$ such as those generated by maximally effective concentrations of agonist. To circumvent this problem we have made use of a new probe, fluo-3, which is reported to have a

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lower affinity for Ca²⁺ (864 nm) and therefore should measure such high [Ca²⁺]_i values more accurately (Minta *et al.*, 1989; Kao *et al.*, 1989; Merritt *et al.*, 1990).

Methods

Preparation of fura-2- and fluo-3-loaded human neutrophils

Human neutrophils were prepared as described previously (Tateson *et al.*, 1988) and suspended in N-[2-hydroxyethyl]-piperazine]N-[2-ethane sulphonic acid] (HEPES)-buffered (30 mM pH 7.2) Hank's balanced salt solution containing 1.6 mM Ca²⁺ (HBH). Neutrophils at 3×10^7 per ml were incubated with 0.5 μ M fura-2AM or 2 μ M fluo-3AM for 15 min at 37°C then diluted 5 fold with cold HBH before centrifugation at 300 *g* for 10 min. The cell pellet was then resuspended in HBH to 1×10^8 cells per ml and kept on ice until use.

Fluorescence measurements

Aliquots (0.1 ml) of loaded neutrophils were diluted 5 fold with HBH warmed to 37°C in fluorimetry cuvettes. Cytochalasin B (5 μ M) was then added together with 1 μ l of dimethylsulphoxide containing demethoxyviridin where indicated. After preincubation at 37°C for 4 min, 4 mM ethylene glycol-bis(β -aminoethylether) N,N,N',N'-tetraacetic acid (EGTA) was added and the mixture stirred continuously. This concentration of EGTA was calculated to reduce the free concentration of Ca²⁺ to <60 nM. fMet-Leu-Phe was then added and fluorescence followed until it reached a peak (after 10–20 s) at which point 100 μ M digitonin, 10 μ M diethylene-triaminepentacetic acid (DTPA) and 4 mM CaCl₂ were added to release the trapped probe into a saturating concentration of Ca²⁺. The fluorescence under these conditions was designated $F_{\max}$. EGTA (8.0 mM) was then added to reduce the free Ca²⁺ to <10 nM and obtain a minimum fluorescence value ($F_{\min}$). NaOH (26 mM) was added at this stage to maintain the pH > 7. Fluorescence was monitored with a Perkin-Elmer MPF2 spectrofluorimeter and protocols for experiments with fluo-3 and fura-2 were identical except that the monochromators were set at 340 nm excitation, 490 nm emission for fura-2 and 506 nm excitation 526 nm emission for fluo-3. The fluorescence from fura-2- and fluo-3-loaded cells was quantitated as described by Pollock *et al.* (1986) using K_d values of 224 nM for fura-2 and 864 nM for fluo-3 (Merritt *et al.*, 1990). To determine accurately the position of the concentration-effect curve, it was necessary to have a reliable estimate of the maximal increase in [Ca²⁺]_i. Stimulation of neutrophils with a maximally effective concentration of fMet-Leu-Phe (3 μ M), in the absence of extracellular Ca²⁺, increased [Ca²⁺]_i to $\geq 1.3 \mu$ M (Figure 1). At such high free Ca²⁺ concentrations fura-2 is >85% saturated with Ca²⁺ whilst fluo-3 is only 60% saturated. Thus fluo-3 fluorescence is a more sensitive measure of [Ca²⁺]_i in this range and was the preferred dye for measuring concentration-effect curves under these conditions. In order to quantitate the peak increase in fluorescence the cells were lysed approximately 10–20 s after addition of the stimulus (see above). Over longer periods of stimulation however, (>5 min), some loss of Ca²⁺-sensitive fluo-3 was observed, suggesting that caution should be used when experimental protocols require longer incubation periods than those used here.

Measurement of inositol 1,4,5-trisphosphate

Human neutrophils, prepared as described above were used at a concentration of 2.0×10^7 per ml in a total incubation

volume of 0.2 ml. Cells were treated with 5 μ M cytochalasin B for 5 min at 37°C before addition of demethoxyviridin where indicated. After a further 5 min cells were stimulated by addition of fMet-Leu-Phe and the reaction stopped 20 s later by addition of 50 μ l ice-cold HClO₄ (10% v/v). The samples were then kept on ice and subsequent operations were carried out at 0–4°C. Precipitated protein was removed by centrifugation and 0.2 ml aliquots of the supernatants neutralised, in the presence of a pH indicator (BDH '678') with approximately 300 μ l of KOH (22 mM) containing 50 mM-HEPES. The IP₃ content of the neutralised samples was measured with the adrenocortical binding protein assay as described by Palmer *et al.* (1988).

Curve fitting

The concentration-effect data for fMet-Leu-Phe was fitted to the equation $f(x) = (A \cdot x^n) / (B^n + x^n)$ using commercially available software (RS-1, BBN Software Products Corporation), where x = concentration of fMet-Leu-Phe, A = maximal effect, B = concentration giving half-maximal effect and n = slope. Each experiment was fitted separately and the curves displayed in Figure 3 were derived from the mean values of the above parameters.

Materials

Fura-2AM and fluo-3AM were obtained from Molecular Probes Inc., Oregon U.S.A. Cytochalasin B, fMet-Leu-Phe and DTPA were obtained from Sigma, Poole, Dorset. EGTA (puriss p.a. grade) was obtained from Fluka Chemicals Ltd, Derbyshire, U.K. 1 β -Hydroxy-18norandrosta-5,8,11,13-tetraeno[6,5,4-bc]furan-3,7,17-trione (demethoxyviridin, DMV) was the kind gift of Dr J. Hanson, Sussex University, Brighton, E. Sussex.

Results

Measurement of [Ca²⁺]_i with fluo-3

Stimulation of fluo-3-loaded cells by a maximally effective concentration of fMet-Leu-Phe (3 μ M) in the presence of excess extracellular EGTA initiated a rapid but transient increase in fluorescence at 526 nm, indicating release of intracellular Ca²⁺ (Figure 1b). The [Ca²⁺]_i in resting cells was estimated to be approximately 120 nM and was seen to increase, following fMet-Leu-Phe stimulation, to 1300 nM. These values are similar to estimates made from changes in fura-2 fluorescence (Figure 1a). The increase in [Ca²⁺]_i was identical in neutrophils which had not been pretreated with 5 μ M-cytochalasin B (result not shown).

Concentration-dependence of fMet-Leu-Phe-stimulated Ca²⁺ mobilisation and IP₃ production

We have shown previously that fMet-Leu-Phe induced a rapid and transient increase in the amount of IP₃ in human neutrophils (Thompson *et al.*, 1990). It can be seen from Figure 2 that this paralleled the mobilisation of intracellular Ca²⁺ measured in fluo-3 loaded cells. In the present studies fMet-Leu-Phe (3 μ M) caused a maximal increase in IP₃ (measured at 20 s) from basal levels of 13.3 ± 2.0 pmol per 10^7 cells to 50.3 ± 8.1 pmol per 10^7 cells ($n = 13$). The EC₅₀ for this response was estimated to be 95 ± 18 nM ($n = 3$) (Figure 3). The location of this concentration-effect curve was significantly to the right of that for mobilisation of Ca²⁺ by fMet-Leu-Phe (Figure 3). At concentrations of 30 nM and above

Figure 1 Fluorescence from fura-2-(a) or fluo-3-(b) loaded human neutrophils was measured in the presence of 4 mM EGTA as described in Methods. In both cases the neutrophils were pre-incubated for 5 min with 5 μ M cytochalasin B before the addition of 0.3 μ M fMet-Leu-Phe indicated by the arrow. F_{max} was determined in non-stimulated samples incubated alongside those shown. The results shown are representative of a large number of similar fluorescence traces from at least four different experiments.

Figure 2 Fluorescence from fluo-3-loaded neutrophils stimulated by 0.3 μ M fMet-Leu-Phe (solid line) was measured in the presence of 4 mM EGTA as described in Methods. Inositol 1,4,5-trisphosphate (IP_3) formed in fMet-Leu-Phe (0.3 μ M)-stimulated neutrophils (broken line) was measured at 5 s intervals by the procedure described in Methods. In all cases neutrophils were preincubated for 5 min with cytochalasin B before addition of fMet-Leu-Phe. The fluorescence trace is a single determination representative of a number of others whilst the IP_3 data represent the mean from three different experiments.

Figure 3 The peak increase in $[Ca^{2+}]_i$ in fluo-3-loaded neutrophils (●) was monitored in the presence of 4 mM EGTA as described in Methods. Inositol 1,4,5-trisphosphate (IP_3) formation (■) was measured 20 s after the addition of the indicated concentrations of fMet-Leu-Phe (FMLP). The neutrophils in both cases were pre-incubated with 5 μ M cytochalasin B for 5 min before addition of fMet-Leu-Phe. The results are mean from three experiments (s.e. mean shown by vertical bars) in which the data were expressed as a percentage of the maximal observed response. The lines were fitted to the data as described in Methods. The slope parameters as determined by this fit were 1.42 ± 0.11 for $[Ca^{2+}]_i$ and 0.82 ± 0.13 for IP_3 .

Figure 4 Neutrophils were pre-incubated with 5 μ M cytochalasin B and the indicated concentration of demethoxyviridin (DMV) for 5 min at 37°C before addition of 3 μ M fMet-Leu-Phe. The formation of inositol 1,4,5-trisphosphate (■) was determined 20 s after addition of fMet-Leu-Phe. The peak increase in $[Ca^{2+}]_i$ (●) was determined in fluo-3-loaded neutrophils in the presence of 4 μ M extracellular EGTA.

fMet-Leu-Phe induced a maximal increase in $[Ca^{2+}]_i$ to 1286 ± 184 nM ($n = 3$) in the presence of extracellular EGTA. The EC_{50} for this response was estimated to be 2.9 ± 0.1 nM, i.e. approximately 30 times lower than that for IP_3 formation (Figure 3).

Inhibition of fMet-Leu-Phe-stimulated responses by the phospholipase inhibitor, demethoxyviridin

The above result demonstrated that only a small part of the maximal fMet-Leu-Phe-stimulated IP_3 formation was required to release all of the intracellular Ca^{2+} . In order to investigate this further we have made use of demethoxyviridin (DMV), a newly discovered and potent inhibitor of fMet-Leu-Phe-dependent activation of both PLC and phospholipase D (PLD) in human neutrophils (Bonser *et al.*, 1991). DMV inhibited fMet-Leu-Phe (3 μ M)-stimulated IP_3 formation with half-maximal inhibition occurring at a concentration (IC_{50}) of 250 ± 130 nM (Figure 4). In contrast, DMV was much less effective as an inhibitor of the increase in $[Ca^{2+}]_i$ provoked by the same concentration of fMet-Leu-Phe (Figure 4). Significant inhibition of Ca^{2+} release was observed only at DMV concentrations ($> 1 \mu$ M) that inhibited the IP_3 response by $> 90\%$.

Discussion

Agonist-dependent formation of IP_3 and mobilisation of intracellular Ca^{2+} are part of the same signal transduction pathway linking receptor occupancy to increases in $[Ca^{2+}]_i$ (Berridge, 1987; Berridge & Irvine, 1989). In human neutrophils the EC_{50} for fMet-Leu-Phe-stimulated IP_3 formation is 30 times greater than that for Ca^{2+} mobilisation. From the location of the concentration-effect curves for these responses, it is apparent that only 30–40% of maximal IP_3 formed by fMet-Leu-Phe receptor stimulation is required to mobilise all the intracellular Ca^{2+} . This implies that $< 40\%$ of the fMet-Leu-Phe receptors need to be occupied to induce full Ca^{2+} mobilisation making this response very sensitive to low concentrations of fMet-Leu-Phe. A very low EC_{50} for fMet-Leu-Phe-stimulated Ca^{2+} mobilisation has been reported previously with quin2-loaded neutrophils (Korchak *et al.*, 1984; Rossi *et al.*, 1985).

A similar dissociation between $[^3H]$ -inositol trisphosphate formation and peak $[Ca^{2+}]_i$ was reported in vasopressin-

stimulated hepatocytes (Thomas *et al.*, 1984). In this case however, the inositol 1,4,5-trisphosphate isomer was not distinguished from other inositol trisphosphates. Neutrophils clearly have IP₃-sensitive Ca²⁺ stores (Prentki *et al.*, 1984), but we cannot rule out the possibility that Ca²⁺ is also mobilised from an IP₃-insensitive pool responding to an unknown second messenger. However, this would not alter the fact that release of Ca²⁺ from the IP₃-sensitive stores is dependent upon lower fMet-Leu-Phe concentrations than is IP₃ formation itself.

Assuming a neutrophil volume of 4.5 µl per 10⁷ cells (Tivey *et al.*, 1951), we have calculated that in fMet-Leu-Phe stimulated cells the IP₃ concentration increased from basal levels of approximately 3 µM to maximum levels of 10 µM, and that a doubling of the resting IP₃ concentration to only 6 µM was required to mobilise fully intracellular Ca²⁺. Such a large effect resulting from a small change in IP₃ concentration is consistent with the highly co-operative nature of IP₃-induced Ca²⁺ mobilisation (Meyer *et al.*, 1988). This property is reflected in the steep slope of the Ca²⁺ curve in Figure 3. The calculated concentrations of IP₃ in human neutrophils reported here are very much greater than the average K_D of IP₃ for its receptor (approximately 0.05 µM e.g. Ferris *et al.*, 1989; Palmer & Wakelam, 1989) and 10 times greater than the EC₅₀ for IP₃ dependent Ca²⁺ mobilisation in permeabilised human neutrophils (0.75 µM) (Prentki *et al.*, 1984). A similar discrepancy has been identified in a number of tissues and a satisfactory explanation is not available at present (for review, see Shears, 1989). The cellular IP₃ concentrations reported for other tissues using a similar assay for amounts of IP₃ are, in general, lower than those reported here for neutrophils (Palmer & Wakelam, 1989). The significance of this is also unclear.

The use of fluo-3 in human neutrophils has been characterized in detail recently (Merritt *et al.*, 1990) and we have found it to have a number of advantages over fura-2 as a measure of high levels of [Ca²⁺]_i (see Methods). The extent to which fluo-3 enters intracellular organelles has not been determined previously and was not assessed directly in the present study. However, release of fluo-3 from secretory granules does not contribute to the agonist-dependent fluorescence change observed in Figure 1 because this is unchanged in neutrophils which have not been treated with cytochalasin B which respond with a much reduced secretory granule release (result not shown).

We have found that DMV, an inhibitor of phospholipase activation, inhibited fMet-Leu-Phe-dependent IP₃ formation

at much lower concentrations than were required to inhibit Ca²⁺ mobilisation. This further supports the suggestion that only a fraction of the maximal amount of IP₃ produced by receptor stimulation was required to mobilise intracellular Ca²⁺. DMV inhibits fMet-Leu-Phe dependent activation of PLD in human neutrophils with an IC₅₀ of 31 ± 10 nM (Bonser *et al.*, 1991), indicating that at the level of phospholipase activation, this compound has a 10 fold selectivity for PLD over PLC (IC₅₀ = 250 ± 130 nM, Figure 4). Furthermore, it is apparent from the results in Figure 4 that functional responses which are dependent upon release of intracellular Ca²⁺ will be much less sensitive to inhibition by DMV than would be expected from its potency as an inhibitor of PLC activation. Therefore, at the functional level DMV would be expected to be very much more effective against PLD-dependent responses (e.g. superoxide anion generation), than against PLC-dependent responses mediated by intracellular Ca²⁺ mobilisation.

These observations predict that the functional selectivity of DMV towards PLD-dependent with respect to Ca²⁺-dependent responses results directly from the excess capacity in fMet-Leu-Phe-stimulated IP₃ production. The amount of IP₃ produced by agonist-dependent PLC activation will probably vary in different tissues and therefore the specificity of DMV towards PLD with respect to PLC-dependent responses is likely to be tissue-related. Thus the implication of this receptor reserve is that non-selective phospholipase inhibitors will have varying degrees of selectivity at the functional level depending on the tissue.

In summary, there are two main consequences of the ability of fMet-Leu-Phe to induce formation of IP₃ in excess of what is required to mobilise fully intracellular Ca²⁺. Firstly, Ca²⁺ mobilisation is more sensitive than IP₃ formation to fMet-Leu-Phe receptor occupancy. Secondly, fMet-Leu-Phe-dependent Ca²⁺ mobilisation, and we would predict Ca²⁺-dependent responses, are much less sensitive than PLC activation to the inhibitor of phospholipase activation, DMV. A further implication is that since the enzymic activity of PLC is not directly related to changes in [Ca²⁺]_i, inhibition of this enzyme is likely to be an inefficient way of inhibiting this signal transduction pathway.

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