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Characterization of Formylmethionyl-Leucyl-Phenylalanine Stimulation of Inositol Trisphosphate Accumulation in Rabbit Neutrophils

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SUMMARY

Inositol trisphosphate (IP_3) formed by phospholipase C-mediated breakdown of triphosphoinositide (PIP_2) may be a ubiquitous second messenger for a number of Ca^{2+}-mobilizing receptor agonists. Using [^{3}H]inositol-labeled rabbit peritoneal neutrophils, we report that radiolabeled inositol phosphates are generated in response to the chemotactic peptide, formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe). fMet-Leu-Phe-stimulated formation of [^{3}H]IP_3 occurs with a rapid time course and a concentration dependence which closely parallels that of stimulated lysosomal enzyme secretion. The synthetic peptide methionyl-leucyl-phenylalanine, which is unable to promote secretion, failed to elevate [^{3}H]IP_3 accumulation, and the competitive antagonist *t*-butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe depressed the stimulant action of fMet-Leu-Phe on [^{3}H]IP_3 levels and secretion. The Ca^{2+} ionophore ionomycin, which promotes secretion, was unable to enhance IP_3 levels, confirming that polyphosphoinositide hydrolysis is a specific receptor-mediated event that precedes calcium mobilization during neutrophil activation. The ability of leukotriene B_4 to also promote a rapid accumulation of [^{3}H]IP_3 suggests that there exists in the neutrophil an interaction between phospholipase A_2 and C-mediated events. These findings support the hypothesis that IP_3 may be a pivotal messenger for signal transfer by Ca^{2+}-mobilizing receptor agonists.

INTRODUCTION

Neutrophil activation is mediated in part by several bacterial peptides, the most potent being fMet-Leu-Phe[1] (1). fMet-Leu-Phe binds to a high affinity receptor(s) on the rabbit neutrophil membrane, resulting in the stimulation of several cellular functions including shape change, chemotaxis, phagocytosis, and degranulation (2).

Neutrophil degranulation studies have been facilitated by the use of cytochalasin B which enables lysosomal enzymes to be secreted into the extracellular medium rather than into phagocytic vacuoles (3). Although the fMet-Leu-Phe-induced secretion from cytochalasin B-treated cells is accompanied by and dependent upon an increase in intracellular Ca^{2+} (4, 5), the mechanism by which fMet-Leu-Phe and other chemotactic agents increase cytosolic Ca^{2+} levels is not understood. One expression of fMet-Leu-Phe-receptor interaction involves the phospholipase A_2-mediated formation of arachidonic acid metabolites, including the lipoxygenase product LTB_4, which can mobilize cellular Ca^{2+} and stimulate secretion (6–8). For other cell types in which stimulation results in elevated cytosolic Ca^{2+} levels, receptor-agonist interaction induces a breakdown of polyphosphoinositides via a phospholipase C-catalyzed reaction with the resultant increase in measurable IP_3 and IP_2 (9, 10). In permeabilized cells, exogenous 1,4,5-IP_3 can selectively mobilize nonmitochondrial pools of sequestered Ca^{2+} (11, 12).

Likewise, in the rabbit neutrophil, fMet-Leu-Phe stimulates a transient decrease of radiolabeled phosphoinositides (13) which is temporally correlated with radiocalcium flux (14). In this report, we used [^{3}H]inositol-labeled rabbit neutrophils to demonstrate that fMet-Leu-Phe and LTB_4 stimulate the production of water-soluble [^{3}H]inositol polyphosphates, with a time course and concentration dependence that closely parallel those for stimulated lysosomal enzyme secretion. The results are discussed in terms of the initial steps in neutrophil activation and of the possible interactions between phospholipase A_2 and C-mediated events in the neutrophil.

EXPERIMENTAL PROCEDURES

Peritoneal exudate from New Zealand White rabbits was collected by heparinized saline lavage 4 or 12 hr after intraperitoneal injection

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[1] The abbreviations used are: fMet-Leu-Phe, formylmethionyl-leucyl-phenylalanine; Met-Leu-Phe, methionyl-leucyl-phenylalanine; boc-Phe-Leu-Phe-Leu-Phe, *t*-butyloxycarbonylphenylalanyl-leucyl-phenylalanyl-leucyl-phenylalanine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP, inositol phosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; NAGA, *N*-acetyl-β-glucosaminidase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LTB_4, leukotriene B_4; ETYA, 5,8,11,14-eicosatetraynoic acid.

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of 150 ml of 0.1% shellfish glycogen in sterile saline as described previously (6). Cells (>90% neutrophils) were isolated by centrifugation (5 min, 200 × *g*), washed once, and resuspended at a cell density of 5 × 10^7/ml in modified Hanks' balanced salt solution (pH 7.4) of the following solute composition (in millimolar): NaCl (124), KCl (4), Na_2HPO_4 (0.64), KH_2PO_4 (0.66), $NaHCO_3$ (15.2), HEPES (10), $MgCl_2$ (0.2), $CaCl_2$ (0.5), and glucose (5.6). Neutrophils were maintained in this medium in an agitator water bath at 37° under an atmosphere of 5% CO_2 in oxygen and remained viable for several hours, as assessed by trypan blue exclusion and retention of lactate dehydrogenase.

For radiolabeled inositol incorporation, this medium also contained 0.025% bovine serum albumin. Neutrophils (1–2 × 10^8) were incubated with 50–100 μCi of [^{3}H]inositol (16 Ci/mmol; New England Nuclear) for 2 hr at 37° in a shaker bath under an O_2 atmosphere containing 5% CO_2. [^{3}H]Inositol-labeled cells were isolated by centrifugation, washed several times for a total of 15 min, and then resuspended in bovine serum albumin-free buffer. Aliquots (300 μl) of about 10^7 cells were added to tubes containing Hanks' buffer with reagents dissolved in 10% dimethyl sulfoxide (final concentration, 0.5%). Incubations at 37° were continued for the designated times and stopped by the addition of 2 volumes of ice-cold 4.5% perchloric acid. The samples were centrifuged at 200 × *g* for 5 min, and the supernatant was neutralized with a sufficient volume of 0.5 M KOH/9 mM $Na_2B_4O_7$/1.9 mM EDTA/3.8 mM NaOH to give pH 8–9. Elution of [^{3}H]inositol mono-, bis-, and trisphosphates from anion exchange columns of Dowex resin (AG 1-X8 formate form, Bio-Rad) was performed according to the procedure of Berridge *et al.* (10).

Measurements of lysosomal enzyme and lactate dehydrogenase release from nonradiolabeled neutrophils were performed using 5 × 10^6 cells/ml as previously described (6, 15). After 5 min, or other indicated times, incubations conducted at 37° were stopped by centrifugation. Enzyme activities were assayed in the supernatant and Triton X-100-lysed pellet. *N*-Acetylglucosaminidase and lysozyme secretion paralleled one another and only results of NAGA release are shown. In all cases, lactate dehydrogenase release was <5%.

fMet-Leu-Phe, Met-Leu-Phe, and boc-Phe-Leu-Phe-Leu-Phe were generously provided by Dr. Richard Freer of the Medical College of Virginia. Values are means ± standard errors of the means. Statistical comparisons were made by Student's *t* test. A value of $p < 0.05$ was taken as the criterion of statistical significance.

RESULTS

The data in Fig. 1 show that stimulation of [^{3}H] inositol-labeled neutrophils by 10 nM fMet-Leu-Phe resulted in a very rapid accumulation of [^{3}H]IP, [^{3}H]IP_2, and [^{3}H]IP_3. Within 20 sec, fMet-Leu-Phe enhanced the accumulation of [^{3}H]IP_3 by more than 3-fold. The stimulated level of [^{3}H]IP_3 declined slowly, reaching control values by 15 min. Likewise, the amount of [^{3}H]IP_2 increased 3- to 4-fold in response to fMet-Leu-Phe, although the peak level was not attained until 60 sec after stimulation. This level was maintained for 5 min (Fig. 1). In contrast, [^{3}H]IP accumulation increased more slowly and by only 50% during the first 60 sec of stimulation. In unstimulated controls, the levels of [^{3}H]inositol phosphates did not change by more than 15% throughout the time course of the experiment (data not shown).

In certain experiments, 10 mM lithium chloride was added to the medium 3 min prior to the addition of stimulus in order to inhibit the breakdown of IP by myoinositol 1-phosphomonoesterase (16, 17). After 5- and 15-min stimulation with fMet-Leu-Phe, the levels of [^{3}H]IP_2 and particularly [^{3}H]IP were increased in lithium-treated cells (Fig. 1). However, the stimulated levels of [^{3}H]IP_3 were unaffected by lithium and, moreover, no

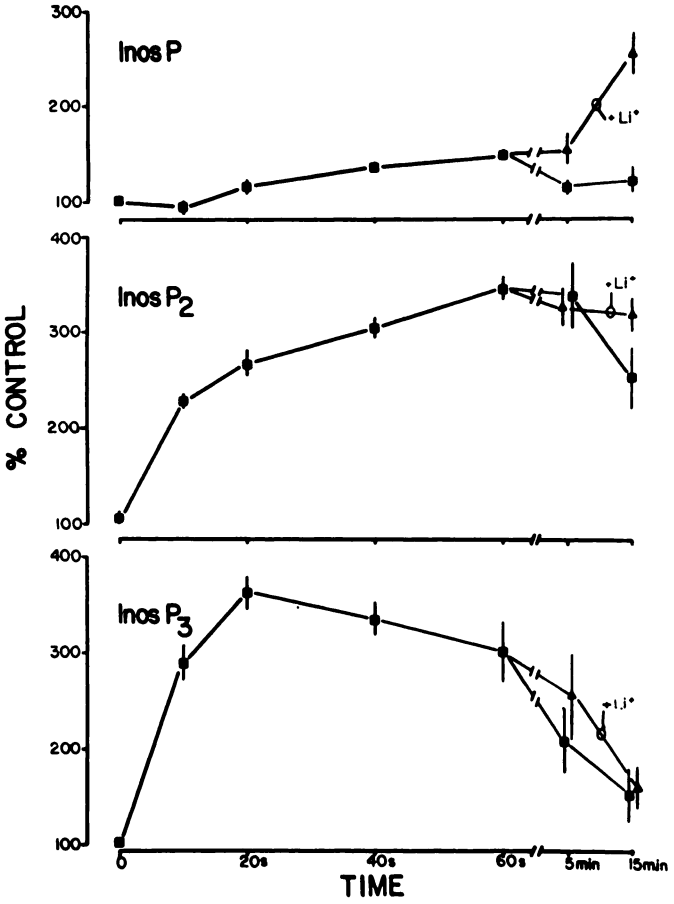

FIG. 1. *Time course of [^{3}H]inositol phosphate formation after stimulation with 10 nM fMet-Leu-Phe in presence and absence of 10 mM lithium chloride*

Mean control levels of [^{3}H]IP, [^{3}H]IP_2, and [^{3}H]IP_3 were 4800 ± 360, 1050 ± 140, and 650 ± 30 dpm or 1.76, 0.38, and 0.24% of total [^{3}H]inositol lipid, respectively, per 10^7 neutrophils. Values are means ± standard errors of the means of four independent determinations carried out in duplicate. The values obtained in the presence of lithium were identical to those obtained in lithium-free medium for the first 60 sec and thus for the sake of simplicity were excluded from the figure.

effect of lithium could be seen on the levels of any [^{3}H] inositol phosphate during the first min of stimulation. Lithium had no effect on basal levels of [^{3}H]inositol phosphates (data not shown). These data are consistent with the idea that measurement of [^{3}H]inositol polyphosphate levels represents the net effect of formation resulting from [^{3}H]inositol phospholipid hydrolysis plus degradation catalyzed by inositol phosphatases and that lithium augments inositol phosphate levels by inhibiting their degradation. However, the use of lithium as a research tool in these types of experiments appears limited in certain cell systems to studies of long term effects of receptor agonists (see also Ref. 18).

The levels of [^{3}H]inositol phosphates in fMet-Leu-Phe-stimulated neutrophils were dependent on the concentration of fMet-Leu-Phe (Fig. 2). Concentrations of fMet-Leu-Phe as low as 0.1 nM caused a significant accumulation of [^{3}H]IP, [^{3}H]IP_2, and [^{3}H]IP_3 after 60 sec. Maximal effects on inositol phosphate accumulation were observed with 100 nM fMet-Leu-Phe; the calculated

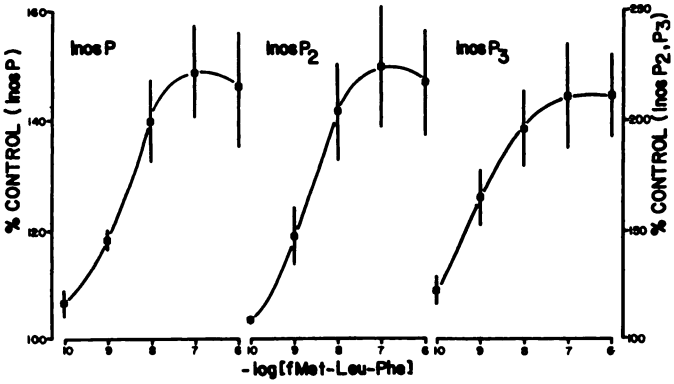

FIG. 2. *Dependence on fMet-Leu-Phe concentration of inositol phosphate accumulation*

[^{3}H]Inositol-labeled neutrophils were stimulated for 60 sec with fMet-Leu-Phe in the presence of 10 mM lithium and 5 μg/ml cytochalasin B. Mean control levels of [^{3}H]IP, [^{3}H]IP$_2$, and [^{3}H]IP$_3$ were 3200 ± 240, 450 ± 43, and 250 ± 45 dpm or 1.86, 0.33, and 0.15% of total [^{3}H]inositol lipid, respectively. Values are means ± standard errors of the means of three independent determinations conducted in duplicate.

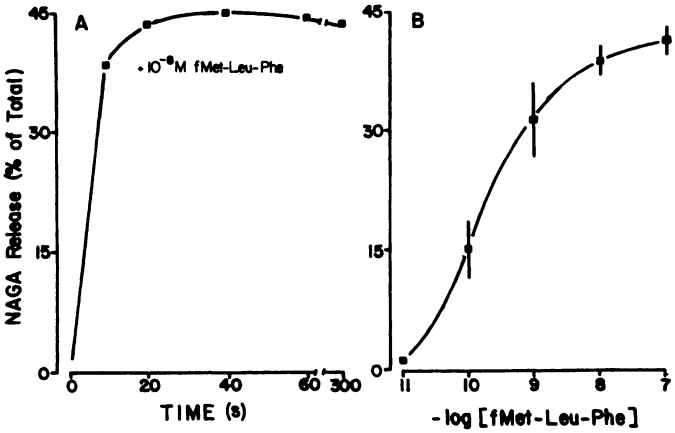

FIG. 3. *Time course and dose dependence of fMet-Leu-Phe-stimulated NAGA secretion*

Neutrophils were stimulated with the indicated concentration of fMet-Leu-Phe in the presence of cytochalasin B (5 μg/ml). In A, NAGA activity was measured in the supernatant and pellet after exposure to fMet-Leu-Phe for various time periods. Per cent release was determined after subtracting basal release. Values are means of duplicate determinations from a representative experiment. In B, values are means ± standard errors of the means from duplicate determinations obtained from three different preparations incubated for 5 min with various concentrations of fMet-Leu-Phe.

EC_{50} values for the stimulated formation of [^{3}H]IP, [^{3}H]IP$_2$, and [^{3}H]IP$_3$ were 1.8 (±0.6), 1.9 (± 0.7), and 0.7 (± 0.2) nM, respectively.

The rapidity and potency with which fMet-Leu-Phe stimulated the accumulation of [^{3}H]inositol phosphates, particularly [^{3}H]IP$_3$, reflect very closely the time course and concentration dependence of fMet-Leu-Phe-stimulated lysosomal enzyme secretion (Fig. 3). Evoked NAGA release was almost complete at 20 sec (Fig. 3A), a time when stimulated [^{3}H]IP$_3$ levels were maximal (Fig. 1). It is significant to note that, after 60 sec when enzyme release was complete, the levels of [^{3}H]IP$_2$ and [^{3}H]IP were maximal, suggesting that these inositol compounds are not directly involved in the secretory process. From

TABLE 1

Comparative effects of secretagogues on [^{3}H]IP$_3$ accumulation and lysosomal enzyme release

For [^{3}H]IP$_3$ analysis, incubations were conducted for 60 sec at 37° and are reported as percentages of DMSO-treated samples. Mean basal level of [^{3}H]IP$_3$ was 320 ± 48) dpm or 0.4% of total [^{3}H]inositol lipid. Values are means ± standard errors of the means of three to six independent determinations carried out in duplicate. For enzyme release, incubations were carried out as in Fig. 3. Values of NAGA secretion, all of which were obtained from cytochalasin B-treated neutrophils, are percentages of total cellular enzyme and represent means ± standard errors of the means of three determinations.

Treatment	[^{3}H]IP$_3$	NAGA release
	% *basal*	% *total*
fMet-Leu-Phe (10 nM)	321 ± 22*	
Met-Leu-Phe (100 nM)	97 ± 8	8 ± 1
boc-Phe-Leu-Phe-Leu-Phe (5 μM)	140 ± 16	14 ± 2
fMet-Leu-Phe + boc-Phe-Leu-Phe-Phe (5 μM)	171 ± 20†	28 ± 2
Cytochalasin B (5 μg/ml)	180 ± 24*	6 ± 1
fMet-Leu-Phe + cytochalasin B	425 ± 37†	43 ± 2
ETYA (5 μM)	113 ± 5	6 ± 1
fMet-Leu-Phe + cytochalasin B + ETYA (5 μM)	270 ± 65†	35 ± 3
LTB$_4$ (100 nM)	159 ± 29*	18 ± 5
Ionomycin (1 μM)	109 ± 18	48 ± 5

* $p < 0.05$ compared with dimethyl sulfoxide control samples.
† $p < 0.05$ compared with fMet-Leu-Phe alone.
‡ $p < 0.05$ compared with fMet-Leu-Phe plus cytochalasin B.

the data depicted in Fig. 3B, the EC_{50} for fMet-Leu-Phe-stimulated NAGA secretion was calculated to be 0.3 nM, a value consistent with that previously reported (6, 19), and comparable to the EC_{50} for stimulated [^{3}H]IP$_3$ accumulation (0.7 nM) (Fig. 2).

Although cytochalasin is not required for expressing the stimulatory effect of fMet-Leu-Phe on inositol phosphate accumulation (Fig. 1; Table 1), neutrophils fail to secrete in response to fMet-Leu-Phe in the absence of cytochalasin B (6). Therefore, this agent was included in the medium of experiments investigating the secretory response. Cytochalasin B alone had a slight stimulatory effect on NAGA release (Table 1) and also had a modest stimulatory effect on [^{3}H]IP$_3$ levels which was additive with the effect of fMet-Leu-Phe (Table 1).

The stimulation of [^{3}H]inositol phosphate formation by fMet-Leu-Phe was receptor-mediated as indicated by the inability of the inactive, nonformylated analogue Met-Leu-Phe to promote [^{3}H]IP$_3$ accumulation, and by the ability of the receptor antagonist boc-Phe-Leu-Phe-Leu-Phe (20), when added together with a near maximal stimulating concentration of fMet-Leu-Phe, to attenuate [^{3}H]IP$_3$ accumulation by 68% (Table 1). Similar effects on [^{3}H]IP and [^{3}H]IP$_2$ accumulation were also observed, although for the sake of simplicity these data are not shown. At this concentration of boc-Phe-Leu-Phe-Leu-Phe (5 μM), fMet-Leu-Phe-stimulated NAGA secretion was reduced by one-third (Table 1).

A near maximal concentration of the Ca^{2+} ionophore ionomycin, which is a potent stimulator of lysosomal enzyme release (Table 1), failed to significantly enhance the accumulation of [^{3}H]IP$_3$ (Table 1). This finding is

consistent with the contention that this agent stimulates pathways in the secretory process subsequent to agonist binding, receptor activation, and phospholipase C stimulation (21). In contrast to the lack of effect of ionomycin, LTB_4 significantly stimulated the accumulation of $[^3H]IP_3$, although to a lesser degree than did fMet-Leu-Phe. A 60-sec exposure to 100 nM LTB_4 produced about a 1.6-fold elevation in $[^3H]IP_3$ levels (Table 1). This lesser effect of LTB_4 relative to fMet-Leu-Phe in stimulating $[^3H]IP_3$ accumulation corresponds to their comparative abilities to evoke NAGA release (18 and 43%, respectively) from cytochalasin-treated cells (Table 1) and to elevate cytoplasmic ionic Ca^{2+} when employed at maximum secretory concentrations (22). Also consistent with a physiologically relevant role for LTB_4 in neutrophil function is the observation of significant stimulation of $[^3H]IP_3$ accumulation within 15 sec following the addition of LTB_4 (Table 2).

The effect of ETYA, an inhibitor of arachidonic acid metabolism, on the fMet-Leu-Phe-stimulated $[^3H]IP_3$ was also examined. ETYA alone did not significantly alter the amount of basal $[^3H]IP_3$; however, it depressed the fMet-Leu-Phe stimulation of $[^3H]IP_3$ accumulation by an average of 36% in paired preparations of cytochalasin-treated neutrophils (Table 1). This inhibitory action of ETYA on the fMet-Leu-Phe-stimulated $[^3H]IP_3$ accumulation was also observed in cytochalasin-untreated neutrophils but the magnitude of the inhibition was much reduced (data not shown). ETYA also inhibited fMet-Leu-Phe-induced lysosomal enzyme secretion from cytochalasin B-treated neutrophils by 19% (Table 1) ($p < 0.05$).

DISCUSSION

The rabbit neutrophil responds rapidly to fMet-Leu-Phe with a transient decrease in radiolabeled inositol lipids and a rise in labeled diacylglycerol and phosphatidic acid (13, 14, 23). These previous findings, which provide indirect evidence that a phospholipase C (phosphodiesterase) is responsible for triggering this reaction, are confirmed and extended in this report, by the direct demonstration that these cells also rapidly accumulate [^{3}H]inositol phosphates when stimulated by fMet-Leu-Phe. The potential physiologic significance of phosphoinositide hydrolysis and consequent inositol phosphate formation is indicated by the selective ability of 1,4,5-IP_3 to mobilize intracellular calcium stores in other cell systems (11, 12).

The fact that the accumulation of $[^3H]IP_3$ in response to fMet-Leu-Phe develops with the same rapid time course as does the secretory response and enhanced calcium uptake (15) is consistent with the idea that IP_3 is a key element for the coupling between receptor stimulation and the secretory response. On the other hand, the increases in $[^3H]IP_2$ and especially $[^3H]IP$ lagged behind the increases in $[^3H]IP_3$ and secretion, suggesting that the former two inositol phosphates are not intimately involved in the secretory process. Rather, they may be formed from the successive dephosphorylation of $[^3H]IP_3$, as previously suggested (9, 24). Recent studies have also shown that PI breakdown probably does not account for hydrolysis of inositol phospholipids during stimulation by calcium-mobilizing secretagogues (9, 25, 26). Nevertheless, while the slower accumulation of [^{3}H]IP is consistent with the hypothesis that the decrease in PI is secondary to the breakdown of the polyphosphoinositides, definitive proof of this postulate awaits direct chemical analysis of the phosphoinositides and inositol phosphates.

The ability of the specific receptor antagonist boc-Phe-Leu-Phe-Leu-Phe to inhibit the stimulation of [^{3}H]IP_3 accumulation by fMet-Leu-Phe, plus the inability of the inactive peptide Met-Leu-Phe (6) to enhance [^{3}H]IP_3 levels, provides another line of evidence demonstrating that the stimulation of $[^3H]IP_3$ accumulation by fMet-Leu-Phe is indeed a receptor-mediated event. It should be noted that the stimulation of $[^3H]IP_3$ levels by fMet-Leu-Phe appears closely coupled to the secretory response as indicated both by the fact that boc-Phe-Leu-Phe-Leu-Phe produced a parallel inhibition of $[^3H]IP_3$ accumulation and secretion, and that the EC_{50} for fMet-Leu-Phe-stimulated $[^3H]IP_3$ formation (0.7 mM) approximates the EC_{50} for secretion (0.3 nM).

The fact that fMet-Leu-Phe is capable of stimulating $[^3H]IP_3$ accumulation in cytochalasin-free cells, when there is a negligible secretory response (6), does not detract from the hypothesis that IP_3 serves as a second messenger to mobilize Ca^{2+}, since mobilization of cellular Ca^{2+} by fMet-Leu-Phe does not require the presence of cytochalasin (27, 28). Apparently then, activation of some critical step in the exocytotic process distal to increased Ca^{2+} availability is blunted in cells devoid of cytochalasin. The mechanism by which cytochalasin alone enhances $[^3H]IP_3$ accumulation is presently unknown. Nevertheless, the ability of cytochalasin to elevate $[^3H]IP_3$ levels, enhance Ca^{2+} mobilization (27), and promote secretion strengthens the link between these parameters.

Further confirmation that the stimulatory effect of fMet-Leu-Phe on $[^3H]IP_3$ accumulation is an expression of agonist-receptor interaction rather than a consequence of an increase in ionized cytosolic Ca^{2+} was obtained by the finding that 1 μM ionomycin was unable to stimulate the accumulation of $[^3H]IP_3$. Under the same conditions, this concentration of ionomycin increases the quin-2-sensitive cellular Ca^{2+} concentration from 0.2 to

TABLE 2

Time course of $[^3H]IP_3$ accumulation in leukotriene B_4-stimulated neutrophils

Values are means ± standard errors of the means of three determinations each done in duplicate and are reported as percentages of DMSO-treated control samples. [^{3}H]Inositol-labeled neutrophils were added to buffer containing leukotriene B_4 at a final concentration of 100 nM. Reactions were terminated after the indicated times by cold perchlorate extraction. Basal $[^3H]IP_3$ levels were 370 ± 34 dpm. All values represent $p < 0.05$ vs. control.

Time	$[^3H]IP_3$
	%
sec	*basal*
15	195 ± 20
30	158 ± 13
60	144 ± 8

0.8 μM[2] and also elicits near maximal NAGA secretion (Table 1). Although PI breakdown in these cells has been reported to be stimulated by a 5-fold higher ionomycin concentration (19), the results presented here indicate that PIP_2 hydrolysis and $[^3H]IP_3$ accumulation is not a Ca^{2+}-mediated event in the rabbit neutrophil. Ionomycin in all probability bypasses the polyphosphoinositide hydrolysis/IP_3 formation step of the neutrophil activation sequence, just as it does in most other secretory cells (29, 30).

The ability of LTB_4 to rapidly enhance $[^3H]IP_3$ accumulation, taken together with the ability of the eicosanoid synthesis inhibitor ETYA to attenuate fMet-Leu-Phe-stimulated $[^3H]IP_3$ formation, supports the involvement of arachidonate metabolites in neutrophil function. Arachidonate metabolites may amplify the fMet-Leu-Phe-induced breakdown of polyphosphoinositides by being exported from the neutrophil and subsequently acting as receptor agonists on neighboring cells. Indeed, recent studies have demonstrated that fMet-Leu-Phe stimulates human and rat neutrophils to secrete endogenously formed LTB_4 (7), which may act on surface receptors to regulate neutrophil function (5). Evidence is available favoring a similar positive feedback mechanism operative in the blood platelet (31).

In conclusion, this study provides additional support for the growing body of evidence implicating IP_3 as a causal mediator of signal transfer by Ca^{2+}-mobilizing receptor agonists. Still, in considering the messengers involved in signaling internal Ca^{2+} release in the neutrophil, one cannot ignore the phospholipase A_2-mediated activation of the arachidonic acid cascade, which is also a well documented sequela of peptidergic receptor activation (6, 29). A modulatory role of arachidonate metabolites in the generation of IP_3 as tentatively suggested by the present study, therefore, also merits close scrutiny. Further exploration of the interaction of phospholipases C and A_2 may provide important clues for elucidating the molecular basis of stimulus-secretion coupling.

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[2] P. B. Bradford and R. P. Rubin, unpublished findings.

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