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Inositol phosphate formation in fMet-Leu-Phe-stimulated human neutrophils does not require an increase in the cytosolic free Ca^{2+} concentration

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The accumulation of inositol phosphates in *myo*-[^{3}H]inositol-labelled human neutrophils stimulated with the chemotactic peptide fMet-Leu-Phe was measured. The challenge with the chemotactic peptide caused the generation of inositol monophosphate (Ins*P*), inositol bisphosphate (InsP_2) and inositol trisphosphate (InsP_3). The formation of the three inositol phosphates followed a differential time course: InsP_3 accumulated very rapidly and transiently, whereas Ins*P* increased steadily for more than 2 min. Inositol phosphate formation was only partially decreased by procedures which prevented the fMet-Leu-Phe-dependent increase of cytosolic free Ca^{2+} concentration.

It has been proposed that the breakdown of the membrane inositol lipids plays a universal role in the activation of metabolic responses and in the mobilization of Ca^{2+} in the cytosol (Michell, 1975, 1983; Berridge, 1984). Evidence obtained in several cell types is consistent with this proposal.

Polymorphonuclear leukocytes seem to be a relevant exception. Data obtained in neutrophils by various authors support the idea of an agonist-stimulated metabolic cycle for phosphoinositides (Cockroft *et al.*, 1981; Dougherty *et al.*, 1984) and a role for InsP_3 in mobilizing intracellular Ca^{2+} (Prentki *et al.*, 1985). In this cell type, however, it has also been suggested that phosphoinositide breakdown can depend on the presence of intact intracellular Ca^{2+} stores (Cockroft *et al.*, 1981; Cockroft, 1984). This important observation would rule out a role for phosphoinositol metabolites in the control of Ca^{2+} mobilization. In addition, it is still doubtful whether inositol phosphates are generated at all in granulocytes stimulated with the chemotactic peptide fMet-Leu-Phe (Cockroft & Allan, 1984), even though neutrophils possess a plasma membrane polyphosphoinositide phosphodiesterase (Cockroft *et al.*, 1984).

In the present study we investigated phosphoinositide turnover and Ca^{2+} changes in *myo*-[2-^{3}H]inositol-labelled neutrophils. We found that inositol phosphates are formed in small but detectable amounts and that PtdIns breakdown does occur at basal levels of $[Ca^{2+}]_i$.

Our observations support the hypothesis that in neutrophils, as elsewhere, the PtdIns cycle plays a primary role in Ca^{2+} mobilization and cell activation.

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns(4)*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins*P*, inositol phosphate; InsP_2, inositol bisphosphate; InsP_3, inositol trisphosphate; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration.

Materials and methods

Neutrophil preparation

Human neutrophils were isolated (95% pure) from blood obtained from healthy donors according to standard procedures (Boyum, 1968). Cells were suspended in a medium containing 140 mM-NaCl, 5 mM-KCl, 1 mM-$MgSO_4$, 0.5 mM-$CaCl_2$, 1 mM-NaH_2PO_4, 5.5 mM-glucose, 20 mM-Hepes (pH 7.4 at 37°C). All experiments were performed in this medium, unless otherwise indicated. O_2 consumption was measured with a Clark oxygen electrode, as described previously (Di Virgilio *et al.*, 1984). Fluorescence measurements were performed in a Perkin–Elmer 650–40 fluorimeter

equipped with a thermostatted (37°C), magnetically stirred cuvette holder. Excitation and emission wavelengths were 339 ± 3 and 492 ± 10 nm, respectively. To minimize light scattering artefacts, two cut-off filters, UV D 25 and UV 35 for excitation and emission respectively, were used. Quin2 loading and calibration of fluorescence as a function of quin2 were performed as previously described (Tsien *et al.*, 1982; Pozzan *et al.*, 1983; De Togni *et al.*, 1984*a*).

Overnight incubation of neutrophils

Freshly prepared human neutrophils were resuspended in Eagle's basal medium, diploid-modified (inositol-free), at a concentration of 7.5×10^6 cells/ml, in silicon-treated glass flasks. The total volume was 10 ml. In some experiments an inositol-free RPMI medium was used in place of the Eagle's basal medium. Incubation media also contained 10 mM-Hepes, 5 mM-$NaHCO_3$, 2–3% fetal bovine or human serum and 1 μCi of *myo*-[^{3}H]inositol/ml). The incubation medium was changed once during the overnight incubation with fresh medium of the same composition. After 15–18 h of incubation at 37°C, the cells were washed, resuspended in RPMI containing 5% fetal bovine serum and further incubated at 37°C for 1 h before being used. Approx. 20–40% of the cells were lost during the overnight incubation. fMet-Leu-Phe-stimulated O_2 consumption in these cells varied between 50 and 80% of that of fresh control neutrophils.

Extraction and separation of water-soluble PtdIns metabolites

Total inositol phosphates were extracted as previously described (Berridge *et al.*, 1983; Vicentini & Meldolesi, 1984). Briefly, 300 μl of the cell suspension was quenched with an equivalent volume of ice-cold trichloroacetic acid (15%, w/v) and kept on ice for 15 min. The samples were then extracted four times with diethyl ether, neutralized with sodium tetraborate (final pH 8.0) and resuspended in 2 ml of distilled water. The inositol phosphates were then bound to AG resin (1-X8, 200–400 mesh, formate form) and eluted with 0.1 M-formic acid/1 M-ammonium formate. To separate individual inositol phosphates, the solution was applied to columns containing 1 ml of AG resin (1-X8, 200–400 mesh, formate form). The columns were washed six times with distilled water to remove *myo*-inositol, then the phosphate esters were eluted by the stepwise addition of solutions containing: (A) 5 mM-sodium tetraborate/60 mM-sodium formate; (B) 0.1 M-formic acid/0.2 M-ammonium formate; (C) 0.1 M-formic acid/0.5 M-ammonium formate; (D) 0.1 M-formic acid/1.0 M-ammonium formate (see Fig. 2). A 1 ml sample of each eluate was taken for liquid-scintillation counting.

Each value is representative of at least three similar experiments which gave similar results.

Materials

Quin2/AM was purchased from Calbiochem, *myo*-[^{3}H]inositol from Amersham International, fMet-Leu-Phe from Sigma, AG 1-X8 resin from Bio-Rad, diploid-modified basal Eagle's medium from Flow, inositol-free RPMI medium from Amimed, and ionomycin was kindly given by Dr. C. M. Liu of Hoffmann–La Roche.

Results

Investigations on phosphoinositide metabolism in neutrophils are hampered by the difficulty of labelling the phosphoinositide pool with *myo*-[^{3}H]inositol. In order to measure the accumulation of water-soluble products of PtdIns breakdown, cells are often either labelled for days in culture or prestimulated with agonists. Neither of these approaches is satisfactory in the case of neutrophils; these cells have a rather short life span even *in vivo* (half-life of approx. 24 h) and prestimulation often results in irreversible desensitization and/or dramatic changes in cell response (De Togni *et al.*, 1985). On the other hand, incubation with [^{3}H]inositol for a shorter time span (2–4 h) gives only a negligible labelling (see also Dougherty *et al.*, 1984).

We found that a 15–18 h incubation of neutrophils in culture medium containing *myo*-[^{3}H]inositol gives a sufficient degree of labelling of the phosphoinositide pool without a major impairment of cell responsiveness.

Fig. 1(*a*) shows that the NADPH oxidase activity of cells incubated overnight can still be stimulated (approx. 80% of the response of fresh control cells) by fMet-Leu-Phe. This chemotactic peptide can also trigger $[Ca^{2+}]_i$ changes in these cells (Fig. 1*b*), although the transients are smaller and of shorter duration as compared with those of freshly prepared neutrophils (see Pozzan *et al.*, 1983; De Togni *et al.*, 1984). Fig. 1(*c*) shows the time course of ^{3}H-labelled inositol phosphate accumulation in the same batch of quin2-loaded cells in which oxygen consumption and $[Ca^{2+}]_i$ changes were monitored in parallel. At the earliest time sampled (15 s), there is already a significant accumulation of water-soluble ^{3}H-labelled products, which increase steadily for more than 2 min. These experiments were performed in the presence of LiCl, which, by inhibiting the dephosphorylation of Ins*P*, leads to an accumulation of this phosphoinositol (Berridge *et al.*, 1982).

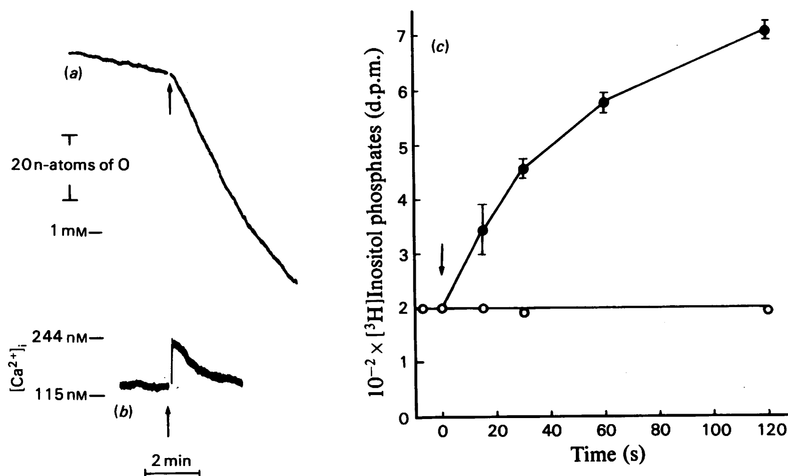

Fig. 1. *Oxygen consumption,* $[Ca^{2+}]_i$ *changes and [*^{3}*H]inositol phosphate accumulation in neutrophils incubated overnight*
Cell concentration was 2.5×10^6/ml in (*a*) and (*b*), 5.7×10^6/ml in (*c*); fMet-Leu-Phe (arrows) was 3×10^{-7} M. Intracellular quin2 concentration was 0.72 mM, and 10 mM-LiCl was added 2 min before the stimulus. Values in (*b*) are means ± S.D. for triplicate samples. ●, Stimulated cells; ○, control cells.

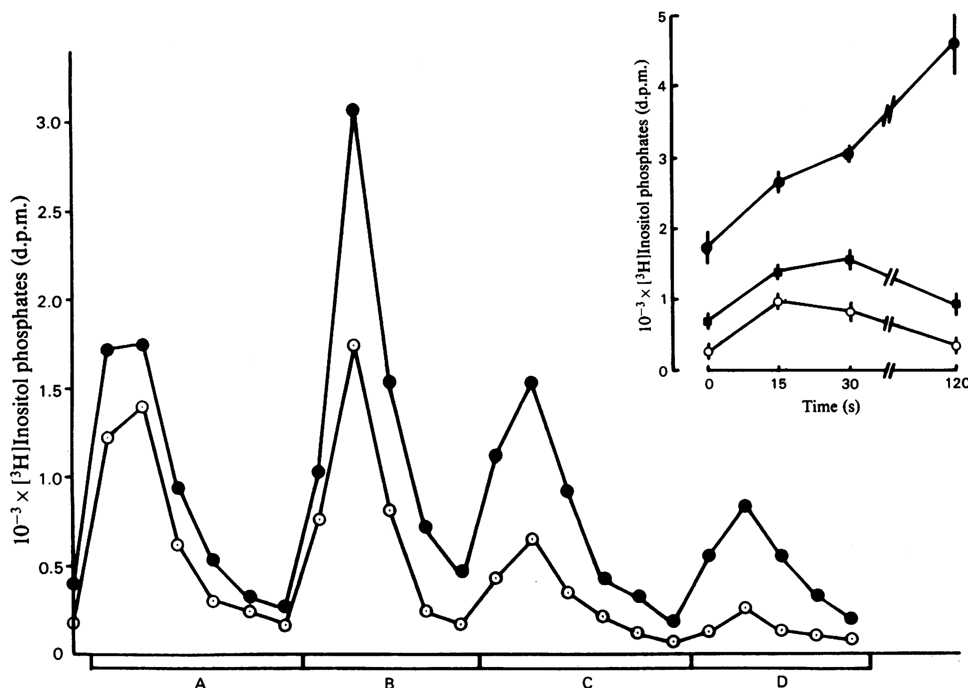

Fig. 2. *Elution profile of [*^{3}*H]inositol phosphates*
Extraction and separation were performed as described in the Materials and methods section. ○, Control; ●, 3×10^{-7} M-fMet-Leu-Phe. Cells were quenched after 30 s of incubation with or without the stimulus. Inset: time course of [^{3}H]inositol phosphate accumulation. Error bars indicate average deviation for duplicate samples. ●, Ins$P$; ■, InsP_2; ○, InsP_3. Cell concentration was 200×10^6/ml.

In Fig. 2 the elution profile of ^{3}H-labelled inositol phosphates in the absence of LiCl is shown. The data shown are duplicate determinations from a single experiment, representative of ten similar experiments. The inset of Fig. 2 shows the increase of Ins$P$, InsP_2 and InsP_3 as a function of time. Error bars indicate average deviation. In agreement with previous data obtained in other

cell types (Berridge *et al.*, 1983; Rebecchi & Gershengorn, 1983; Vicentini & Meldolesi, 1984; Vicentini *et al.*, 1985) and recently (Dougherty *et al.*, 1984) in a human myelomonocytic leukaemic cell line (HL-60), the various inositol phosphates seem to be generated with a differential time course. Ins*P* accumulates rather slowly, while InsP_3 is already maximal at 15s and decreases thereafter. Nevertheless, there is a significant overall accumulation of Ins*P* at 15s (from 1800 to 2600d.p.m.). It has been suggested that the primary substrate for the receptor-activated phospholipase C is PtdIns(4,5)P_2 (Michell *et al.*, 1981; Berridge & Irvine, 1984), rather than PtdIns and PtdIns(4)*P*. Our results are not inconsistent with this interpretation; however, we cannot discriminate as to whether Ins*P* and InsP_2 derive from the dephosphorylation of InsP_3 or rather from phospholipase C attack on PtdIns and PtdIns(4)*P*.

The hypothesis that PtdIns(4,5)P_2 breakdown generates the mediator (InsP_3) responsible for receptor-activated mobilization of intracellular Ca^{2+} rests on the assumption that InsP_3 generation should precede the $[Ca^{2+}]_i$ rise, i.e. InsP_3 has to be generated at basal $[Ca^{2+}]_i$. A clear demonstration of the independence of InsP_3 formation on the $[Ca^{2+}]_i$ rise is still lacking, and there are several hints to the contrary, particularly in neutrophils (Cockroft *et al.*, 1981; Cockroft, 1984; Volpi *et al.*, 1983).

In the experiments shown in Fig. 3, cells were incubated in LiCl/EGTA-supplemented medium, then ionomycin was added to deplete the intracellular Ca^{2+} stores. After 6min, when $[Ca^{2+}]_i$ was again at resting levels, the cells were challenged with fMet-Leu-Phe. Since extracellular Ca^{2+} was lower than $[Ca^{2+}]_i$ and the intracellular stores were depleted by the previous addition of ionomycin, the chemotactic peptide did not cause any further change in $[Ca^{2+}]_i$ (Lew *et al.*, 1984). In contrast there was a remarkable accumulation of inositol phosphates (sampled 60s after fMet-Leu-Phe addition). Radioactivity in inositol phosphates increased from 960 ± 24d.p.m. (before ionomycin) to 1238 ± 29d.p.m. (6min after ionomycin) to 1998 ± 27d.p.m. (1min after fMet-Leu-Phe). In a parallel sample challenged with fMet-Leu-Phe in the presence of 0.5mM extracellular Ca^{2+}, i.e. under conditions in which a significant increase in $[Ca^{2+}]_i$ occurs (Pozzan *et al.*, 1983), radioactivity increased from 1000 ± 25d.p.m. to 3026 ± 50d.p.m.

Fig. 4 shows the increase of Ins*P*, InsP_2 and InsP_3 in cells pretreated with ionomycin in an EGTA-containing medium and stimulated with fMet-Leu-Phe when $[Ca^{2+}]_i$ was again at resting level. At 15s after the challenge with the chemotactic peptide, Ins*P*, InsP_2 and InsP_3 increased by 23,

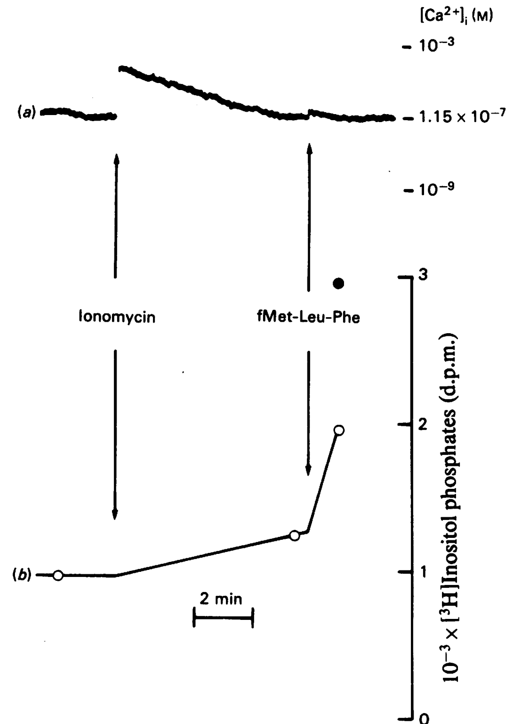

Fig. 3. *[^{3}H]Inositol phosphate formation at basal $[Ca^{2+}]_i$*
Cell concentration 2.5×10^6/ml (*a*) and 15×10^6/ml (*b*). The cells were taken from the same batch of quin2-loaded neutrophils. Ionomycin was 0.2μM and fMet-Leu-Phe was 3×10^{-7}M; 2mM-EGTA and 10mM-LiCl were added 2min before ionomycin. Open symbols, cells challenged in EGTA-containing medium and pretreated with ionomycin; closed symbols, cells challenged in the presence of 0.5mM extracellular Ca^{2+} without ionomycin pretreatment. Intracellular quin2 concentration was 0.54mM. Values are means of triplicate samples.

56 and 190% respectively. In agreement with the time course shown in Fig. 2, InsP_3 had already started to decline when measured at 30s, while Ins*P* increased steadily. Both Fig. 3 and Fig. 4 indicate that ionomycin induces some phosphoinositide breakdown, even in the absence of external Ca^{2+} and a larger phosphoinositol accumulation is observed with ionomycin when Ca^{2+} is present in the incubation medium (results not shown). A possible interpretation of this effect is that the $[Ca^{2+}]_i$ rise induced by the ionophore is sufficient to generate arachidonate metabolites, which are themselves agonists. In fact, it is known that the Ca^{2+} ionophore A23187 stimulates arachidonate metabolism in human polymorphonuclear leucocytes (Borgeat & Samuelsson, 1979). However, we cannot exclude that a $[Ca^{2+}]_i$ rise may indeed be sufficient, although not necessary, to activate the

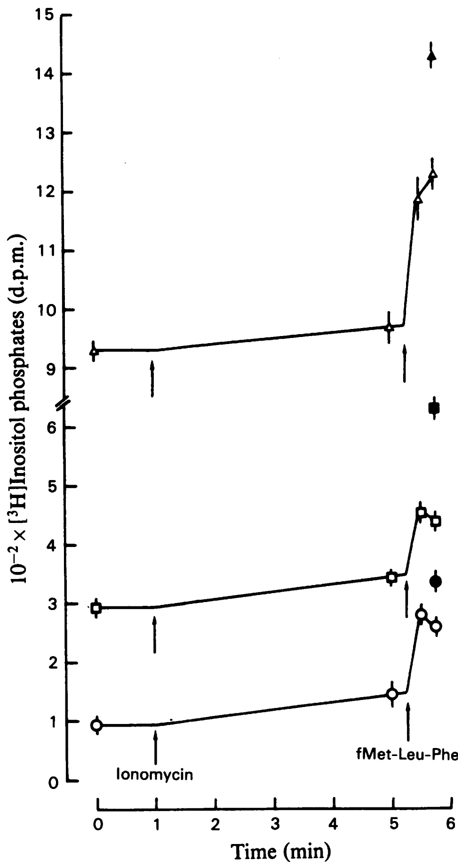

Fig. 4. *InsP, InsP$_2$ and InsP$_3$ accumulation at basal* [Ca^{2+}]$_i$
Cell concentration was 20×10^6 cells/ml. Quin2 content was 0.22 mM, fMet-Leu-Phe was 3×10^{-7} M and ionomycin was 0.2 μM; 1 mM-EGTA was added 1 min before ionomycin. △, Ins$P$; □, InsP_2; ○, InsP_3. Open symbols, cells challenged in EGTA-containing medium and pretreated with inomycin; closed symbols, cells challenged in the presence of 0.5 mM-extracellular Ca^{2+} without ionomycin pretreatment. Error bars represent average deviation for duplicate samples.

phosphoinositide cycle. That the rise of [Ca^{2+}]$_i$ can affect, directly or by means of arachidonate and its metabolites, the phosphatidylinositol cycle is indicated also be the larger hydrolysis induced by fMet-Leu-Phe in the presence of extracellular Ca^{2+}.

Discussion

During the last 2 years the 'phosphatidylinositol hypothesis' for stimulus–secretion coupling (Michell, 1975) has received widespread support (Nishizuka, 1984; Berridge & Irvine, 1984). Yet the crucial question of whether phosphoinositide turnover depends on a [Ca^{2+}]$_i$ rise has remained largely unanswered. In many cell types a requirement of extracellular Ca^{2+} for phosphoinositide breakdown has been demonstrated (Akhtar & Abdel Latif, 1980; Cockroft *et al.*, 1981; Cockroft, 1984; Volpi *et al.*, 1983; Laychock, 1983), and in other cells, in which extracellular Ca^{2+} was not needed, the mobilization of intracellular Ca^{2+} stores could not be ruled out (Jones & Michell, 1975; Fain & Berridge, 1979; Orchard *et al.*, 1984; Farese *et al.*, 1984). We know in fact, by direct measurement, that in a variety of cell types Ca^{2+} is rapidly discharged from intracellular stores into the cytoplasm following agonist–receptor interaction (Pozzan *et al.*, 1983; Tsien *et al.*, 1984). Cockroft (Cockroft *et al.*, 1981; Cockroft, 1984) attempted to circumvent this problem by depleting nuetrophils of endogenous Ca^{2+}. Under her experimental conditions, cells which had been most severely depleted of Ca^{2+} exhibited a small but detectable level of secretion when challenged with fMet-Leu-Phe, but were completely negative when examined for PtdIns breakdown. The situation is further complicated by the fact that, although it has been recently shown that InsP_3 can release Ca^{2+} from non-mitochondrial stores also in neutrophils (Prentki *et al.*, 1985), agonist-induced generation of InsP_3 could not be demonstrated in these cells (Cockroft & Allan, 1984). The only cells similar to neutrophils in which an increase in the accumulation of InsP_3 after chemotactic peptide stimulation was measured are HL-60 cells (Dougherty *et al.*, 1984). Polymorphonuclear leukocytes are therefore still considered an intriguing exception (Cockroft, 1981) that may negate the universality of the phosphatidylinositol hypothesis.

We think that these negative results can, at least in part, be explained by the difficulty of measuring the PtdIns cycle in this cell type. The standard technique involves either ^{32}P or [^{3}H]glycerol labelling of phospholipids. In both cases the activation of PtdIns turnover is measured as a change in the level of ^{32}P or ^{3}H-labelled phospholipids. Usually, only approx. 20% of the total inositol lipids undergo agonist-stimulated hydrolysis and the background against which these changes occur is always relatively high. It is, in particular, difficult to detect early and significant alterations in the level of PtdIns(4,5)P_2, since this phospholipid is not only a minor component of membrane phosphoinositides, but also the one which undergoes the most rapid breakdown and resynthesis. In particular, fast activation of the kinases which convert PtdIns and PtdIns(4)P to PtdIns(4,5)P_2 may mask the early and rapid hydrolysis of this latter phospholipid.

Moreover, as pointed out by Berridge (1983), polyphosphoinositides could decline, either by undergoing dephosphorylation back to PtdIns, or by being hydrolysed to polyphosphoinositols and diacylglycerol. In the first case a phosphomonoesterase and in the second case a phosphodiesterase would be operating, respectively. Only the second mechanism generates InsP_3 and is therefore compatible with the involvement of InsP_3 in intracellular Ca^{2+} mobilization.

In the present work we show that it is possible to label human granulocytes with *myo*-[^{3}H]inositol without drastically impairing cell responses; this can be achieved by incubating the cells overnight in culture medium enriched with 2–3% serum. Upon stimulation with fMet-Leu-Phe a fast accumulation of [^{3}H]phosphoinositols occurs and, remarkably, of InsP_3 as well. Although the incorporation of *myo*-[^{3}H]inositol into the phosphoinositide pool turned out to be rather variable from batch to batch of cells, the percentage increase of Ins$P$, InsP_2 and InsP_3 accumulation was remarkably constant upon fMet-Leu-Phe (3×10^{-7} M) stimulation. In ten separate experiments with neutrophils from ten different donors Ins$P$ increased at 15s by 56 ± 12%, InsP_2 by 98 ± 17% and InsP_3 by 268 ± 76%. About 10% of the radioactivity incorporated into the membrane inositol lipids was recovered in the inositol phosphates.

Using our experimental procedure, at any time we know real $[Ca^{2+}]_i$ levels in the cells in which inositol phosphate accumulation is measured. In addition, the controlled discharge of Ca^{2+} from intracellular stores with ionomycin allows to overcome the fMet-Leu-Phe-induced $[Ca^{2+}]_i$ transients. Our data are in contrast with previous results (Cockroft *et al.*, 1981; Volpi *et al.*, 1983). We do not see inhibition of phosphoinositol accumulation when the agonist-induced increase of $[Ca^{2+}]_i$ is prevented, but only a decrease (about 40%) with respect to parallel experiments run in the presence of extracellular Ca^{2+}. This discrepancy may be explained by two possibilities: (*a*) measurements of ^{3}H- or ^{32}P-labelled phospholipids might not be sensitive enough to allow the determination of minor, though significant changes; (*b*) in the experiments reported by Cockroft *et al.* (1981) a drastic and uncontrolled intracellular Ca^{2+} deprivation might have occurred. At the time these experiments were performed there was no reliable method for measuring $[Ca^{2+}]_i$; therefore it was impossible to determine the extent of $[Ca^{2+}]_i$ deprivation reached in those cells. Preliminary experiments (F. Di Virgilio & T. Pozzan, unpublished work) performed in neutrophils in which $[Ca^{2+}]_i$ was chelated at approximately 10^{-8} M, according to the procedure described previously (Di Virgilio *et al.*, 1984), suggest that drastic intracellular Ca^{2+} depletion can substantially diminish the generation of inositol phosphates.

On the other hand, our results are in agreement with the observation that the elevation of $[Ca^{2+}]_i$ may be sufficient to activate some phosphoinositide hydrolysis (Cockroft *et al.*, 1981; Cockroft, 1984). However we cannot discriminate between whether this effect is due to a direct activation of phospholipase C by Ca^{2+} or to the generation of arachidonic acid (and its metabolites) by phospholipase A_2.

In conclusion, our experiments show that fMet-Leu-Phe-dependent Ins$P$, InsP_2 and InsP_3 generation in human neutrophils does not require an increase in the level of $[Ca^{2+}]_i$ but can occur at resting $[Ca^{2+}]_i$ (i.e. around 120 nM). This is compatible with a role for InsP_3 as the messenger responsible for receptor-activated Ca^{2+} mobilization from intracellular stores.

'Black swans' (Cockcroft, 1981) may be turning grey, if not yet white.

Note added in proof (received 13 May 1985)

While this paper was undergoing revision a paper (Bradford & Rubin, 1985) appeared in which the formation of inositol phosphates was characterized in rabbit neutrophils.

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