# Relationships between phosphoinositide metabolism, $Ca^{2+}$ changes and respiratory burst in formyl-methionyl-leucyl-phenylalanine-stimulated human neutrophils

## The breakdown of phosphoinositides is not involved in the rise of cytosolic free $Ca^{2+}$

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The relationships between the changes of cellular $Ca^{2+}$, the activation of phosphoinositide turnover and the functional responses induced by the stimulus-receptor interactions in neutrophils are matter of controversy. By measuring the concentration dependency of different formyl-leucyl-methionyl-phenylalanine (FMLP)-induced changes, the following values of $ED_{50}$ were found: 1.6 and 0.8 nM for the rise in $[Ca^{2+}]_i$ monitored with Quin-2, in the presence and absence of exogenous $Ca^{2+}$, respectively; 20 nM for the activation of phosphoinositide metabolism, monitored as change in the $^{32}P_i$ of phosphatidate; 14 nM for membrane-bound $Ca^{2+}$ mobilization, monitored with chlorotetracycline (CTC); 34 nM for $^{45}Ca^{2+}$ influx and 32 nM for the respiratory burst. Furthermore, low dose of FMLP causes an increase in $[Ca^{2+}]_i$ in absence of activation of breakdown of phosphatidylinositol, phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-biphosphate monitored as changes in [$^3$H]glycerol radioactivity. The results clearly demonstrate that the increase in $[Ca^{2+}]_i$, due to the release from intracellular stores, is not caused by the breakdown of phosphatidylinositides. On the other hand, the data of the similarity of $ED_{50}$ are compatible with an involvement of phosphoinositide response in the release of membrane bound $Ca^{2+}$, monitored with CTC, and in the $^{45}Ca$ influx and in the respiratory burst.

*Phosphatidylinositide* *Phosphatidate* *$Ca^{2+}$ change* *Respiratory burst* *Human neutrophil*

## 1. INTRODUCTION

The functional responses of neutrophils to stimulus-receptor interactions are associated with an increase in the level of cytosolic $Ca^{2+}$ concentrations, $[Ca^{2+}]_i$, due to a change in the plasma membrane permeability and to a mobilization of intracellular stores, and with an increase in inositol phospholipids metabolism usually called 'phosphoinositide response' [1–12]. The coupling of these biochemical changes with neutrophil responses such as respiratory burst, secretion, aggregation, chemotaxis, etc., as well as the nature of the reactions involved in the phosphoinositide response, i.e., breakdown of phosphatidylinositol or phosphatidylinositoles by phospholipase C or phospholipase D type reaction [7,13–16] and their relationship with the changes of cellular $Ca^{2+}$ are not yet understood.

In analogy with other cell types [13,17–20], in neutrophils the activation of phosphoinositide response would initiate with the breakdown of phosphatidylinositol, or of phosphatidylinositol 4,5-biphosphate, to diacylglycerol and inositol-phosphate(s) [10–12,15,16,21–27]. Diacylglycerol would then stimulate the $Ca^{2+}$ and phospholipid

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dependent protein kinase C [17,19–21,23–25], while inositolphosphates would induce the release of $Ca^{2+}$ from intracellular stores and the opening of $Ca^{2+}$ channels in the plasma membrane [13, 14,18–20]. In this view the phosphoinositide response in neutrophils would cause the rise in cytosolic free $Ca^{2+}$, $[Ca^{2+}]_i$ [21,27]. However, according to others the increase in phosphoinositide turnover and the accompanying processes, like the phosphatidate formation and the diacylglycerol dependent activation of protein kinase C, are consequences and not causes of the rise in $[Ca^{2+}]_i$ [6,7,10].

In the course of investigations on the mechanism responsible for the desensitization, that occurs in neutrophils when formyl-methionyl-leucyl-phenylalanine (FMLP) is presented to the cells during some minutes instead of all at once, we have been able to show a dissociation between the changes in $[Ca^{2+}]_i$ and the phosphoinositide response. In fact, when the peptide is presented slowly the depressed magnitudes of the respiratory burst and of the activation of phosphoinositide response are not accompanied by a parallel depression of the magnitude of the rise in $[Ca^{2+}]_i$ [22].

In an attempt to clarify the relationships between the $Ca^{2+}$ changes and the activation of inositol phospholipids turnover in neutrophils we have investigated several responses ($Ca^{2+}$ influx, $Ca^{2+}$ mobilization, increase in $[Ca^{2+}]_i$, activation of polyphosphoinositides turnover and respiratory burst) following the interaction with different doses of FMLP. The results presented here demonstrate that the change in $[Ca^{2+}]_i$ is not the consequence of the activation of phosphoinositide response and show that the respiratory burst, the influx of $Ca^{2+}$, the mobilization of membrane-bound $Ca^{2+}$, monitored as chlorotetracycline fluorescence decrease, and the activation of phosphoinositide response are associated events, with similar values of $ED_{50}$ for FMLP.

## 2. MATERIALS AND METHODS

### 2.1. *Chemicals*

Quin-2 AM was purchased from Calbiochem-Behring (La Jolla, CA); $^{32}P_i$, [1,2,3-$^3$H]glycerol and $^{45}CaCl_2$ from New England Nuclear; FMLP, cytochalasin B, chlorotetracycline, and all phospholipids used as standard were purchased from Sigma (Taufkirchen). High performance TLC plates were from Merck (FRG).

### 2.2. *Preparation of neutrophils*

Human neutrophils were isolated from venous blood and suspended in Hank's balanced salt solution as previously described [28].

### 2.3. *Respiratory burst*

The activation of cell respiration by FMLP was measured at 37°C with a Clark oxygen electrode as previously described [28].

### 2.4. *Modification of cytosolic $Ca^{2+}$*

The changes in $[Ca^{2+}]_i$ were monitored with Quin-2 loaded cells according to [29] as previously described [22]. The amount of $Ca^{2+}$ released from intracellular pool was determined by comparing the $Ca^{2+}$ dependent fluorescence of Quin-2 loaded cells treated with 0.1% Triton to the fluorescence of a standard solution of Quin-2 free acid added to a batch of unloaded cells. The intracellular water content of neutrophils was assumed to be $0.35\ \mu l/10^6$ cells. The amount of $Ca^{2+}$ released from intracellular stores was measured knowing the concentration of the intracellular Quin-2 free acid and the percentage of saturation of intracellular Quin 2.

### 2.5. *Mobilization of bound $Ca^{2+}$*

The change in fluorescence of chlorotetracycline labelled cells was employed to monitor the mobilization of membrane-bound $Ca^{2+}$ as described in [30].

### 2.6. *Measurement of calcium influx*

The uptake of $^{45}Ca^{2+}$ was measured using the rapid Millipore filtration technique as described in [31].

### 2.7. *Phosphoinositide response*

The activation of phosphoinositide turnover was measured by following the changes in radioactivity of phospholipids labelled with $^{32}P_i$ or [$^3$H]glycerol. The labelling of phospholipids with [$^3$H]glycerol was performed by incubation of neutrophils ($70 \times 10^6$/ml) at 37°C in modified Hank's balanced salt solution containing 0.5 mM $CaCl_2$ with 20 $\mu$Ci/ml [$^3$H]glycerol (38.2 Ci/ mmol) for 90 min. The cells were washed twice and resus-

pended in the same buffer containing 0.5 mM $CaCl_2$ to a final concentration 30 × $10^6$/ml. The labelling of phospholipids with $^{32}P$ was performed by incubation of neutrophils in a buffered salt solution (pH 7.4) containing: 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes, 0.5 mM $CaCl_2$, 5.6 mM glucose and incubated (70 × $10^6$/ml) with 20 $\mu$Ci/ml $^{32}P_i$ for 60 min at 37°C. After labelling cells were washed once and resuspended at 30 × $10^6$/ml in the same buffer. Labelled neutrophils (1 ml) suspended in appropriate media were stimulated with different doses FMLP at 37°C for the indicated time. The lipids of duplicate or triplicate samples were extracted by adding 3.75 ml chloroform/methanol (2:1). In experiments where phosphatidylinositol 4,5-biphosphate and phosphatidylinositol 4-phosphate were to be examined the above solvent was replaced by chloroform/methanol/conc. HCl (20:40:1). The phases were split by adding 1.25 ml chloroform and 1.25 ml of 2 M KCl (or distilled water when HCl was present) and mixing. The

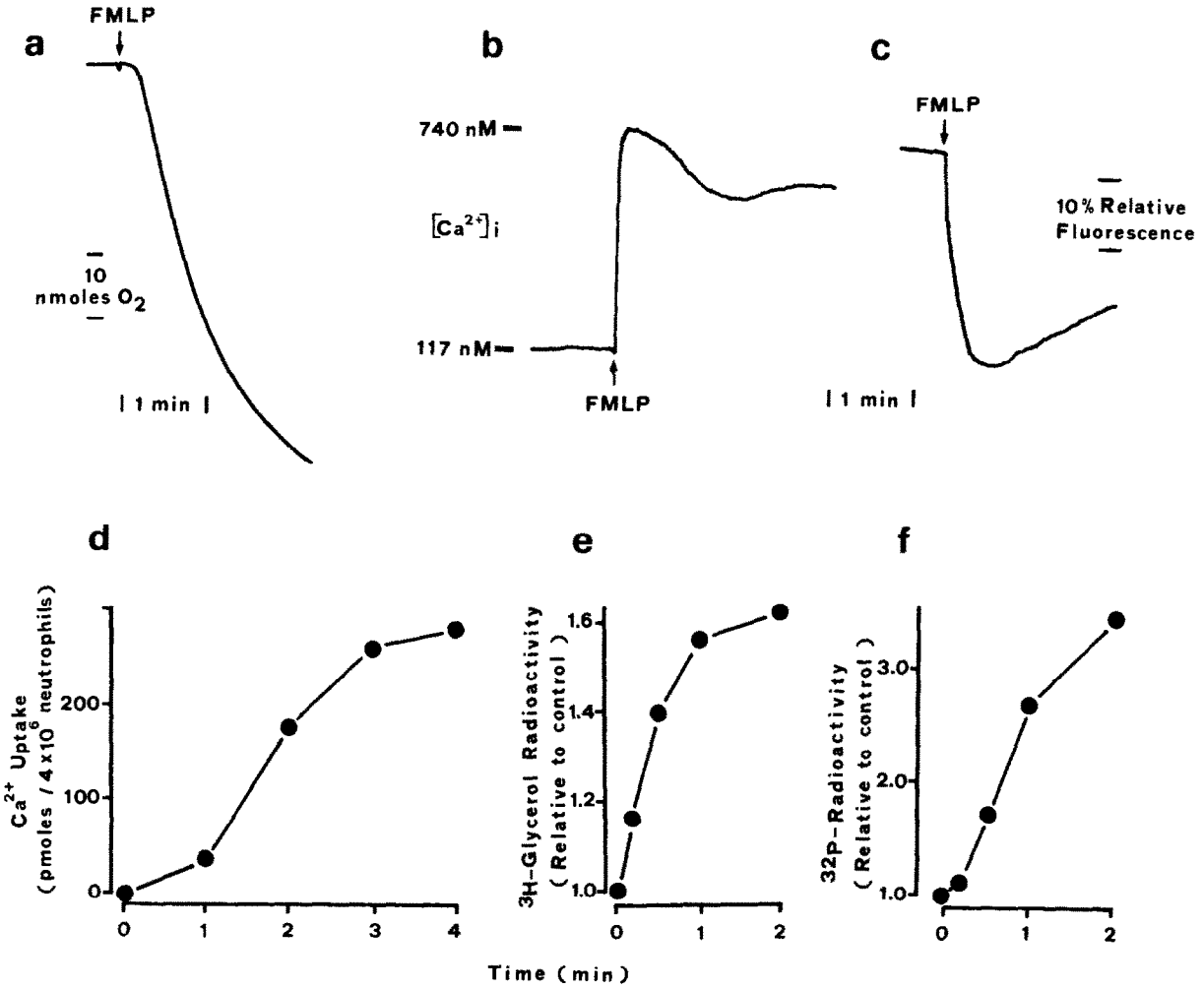


Fig.1. Time course and magnitude of different responses to FMLP ($10^{-7}$ M) in human neutrophils. (a) $O_2$ consumption (2 × $10^7$ cells/ml); (b) change in cytosolic $[Ca^{2+}]_i$ monitored by Quin-2 (5 × $10^6$ cells/ml); (c) release of membrane-bound $Ca^{2+}$ monitored by changes in CTC fluorescence (5 × $10^6$ cells/ml); (d) $^{45}Ca$ uptake; (e) change in [$^3$H]glycerol-labelled phosphatidic acid (3 × $10^7$ cells). The value of zero time was 9220 cpm; (f) change in $^{32}P$-labelled phosphatidic acid (2 × $10^7$ cells). The value of zero time was 3100 cpm. All the responses were measured in the same batch of cells and the data are of one experiment representative of five.

phases were separated by centrifugation and the lower phase was collected and dried under nitrogen. The phospholipids were separated on thin-layer chromatography (TLC) plates using a solvent system of methyl acetate/1-propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9) [32]. The $R_F$ values were 0.21 for phosphatidylcholine, 0.37 for phosphatidylserine, 0.43 phosphatidylinositol, 0.48 for phosphatidic acid, and 0.52 for phosphatidylethanolamine. Triglycerides move with the solvent front. The phosphatidylinositol 4,5-biphosphate (TPI) and phosphatidylinositol 4-phosphate (DPI) were separated on high performance TLC (HPTLC) plates impregnated with potassium oxalate (1%) in a solvent system of chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, v/v) [26]. The $R_F$ values for TPI and DPI were 0.33 and 0.39, respectively. $^{32}$P-phospholipids were detected by autoradiography and $^{3}$H-phospholipids stained with $I_2$ vapour in presence of carriers. The areas corresponding to the individual lipids were scraped into vials, eluted by addition of 0.3 ml of methanol followed by 4 ml of Instagel Scintillation liquid (Packard) and counted.

## 3. RESULTS AND DISCUSSION

Fig.1 shows the rapidity of onset and magnitude of the activation of the respiratory burst (a), of the rise in $[Ca^{2+}]_i$ monitored as increase in Quin-2 fluorescence (b), of the mobilization of the membrane-bound $Ca^{2+}$ monitored as decrease in chlorotetracycline fluorescence (c), of the influx of $Ca^{2+}$ monitored as increase in cell associated $^{45}Ca^{2+}$ (d) and of the increase in phosphoinositide turnover measured as [$^{3}$H]glycerol (e) and $^{32}P_i$ (f) incorporation into phosphatidate induced by 100 nM FMLP in human neutrophils. It can be seen that the various responses take place very early, the respiratory one being the more delayed.

Fig.2 reports the dose response curves of the FMLP-induced processes presented in fig.1. The data clearly show that the rise in $[Ca^{2+}]_i$ is elicited by very low dose of the peptide both in presence ($ED_{50}$ = 1.7 nM) and absence ($ED_{50}$ = 0.8 nM) of exogenous calcium. Similar values of $ED_{50}$ have been found by others [33,34]. Fig.2 also shows the dose response curve for phosphoinositide response

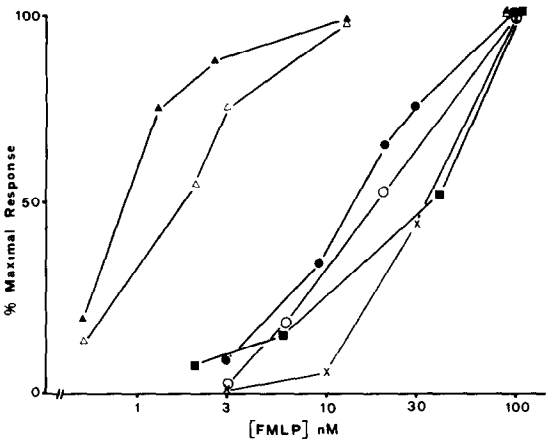


Fig.2. Concentration-dependency of FMLP-induced $O_2$ consumption (×); change in $[Ca^{2+}]_i$ in the presence (△), and absence of exogenous calcium (▲), of changes in CTC fluorescence (●), of $^{45}$Ca uptake (■) and of incorporation of [$^{32}$P]$P_i$ into phosphatidic acid (○) in human neutrophils. All the responses were measured in the same batch of cells and the data are of one experiment representative of five.

measured as change in the radioactivity of phosphatidate. It can be seen that the curve for this response is shifted to the right ($ED_{50}$ = 20 nM) when compared with that for the rise in $[Ca^{2+}]$ due to the release from intracellular stores. An $ED_{50}$ value of 40 nM for FMLP induced breakdown of phosphatidylinositol and phosphatidylinositol 4,5-biphosphate has been found recently by Dougherty et al. [12].

The dissociation between change of $[Ca^{2+}]_i$ and phosphoinositide response, shown also by Volpi et al. [34] as increase in radioactivity of phosphatidate, is more precisely demonstrated by the results of fig.3, which show the effects of two concentrations of FMLP (2 nM and 100 nM) on intracellular $Ca^{2+}$ mobilization and on turnover of [$^{3}$H]glycerol-labelled phosphatidylinositol, phosphatidylinositol 4-monophosphate, phosphatidylinositol 4,5-biphosphate and phosphatidate. At 100 nM concentration FMLP induces either an increase in $[Ca^{2+}]_i$ and an activation of phosphoinositide turnover, while at 2 nM the peptide causes only the changes in $[Ca^{2+}]_i$ and not the activation of phosphatidylinositide metabolism.

This finding is a direct demonstration that in neutrophils the activation of $Ca^{2+}$ mobilization from intracellular stores induced by FMLP is not

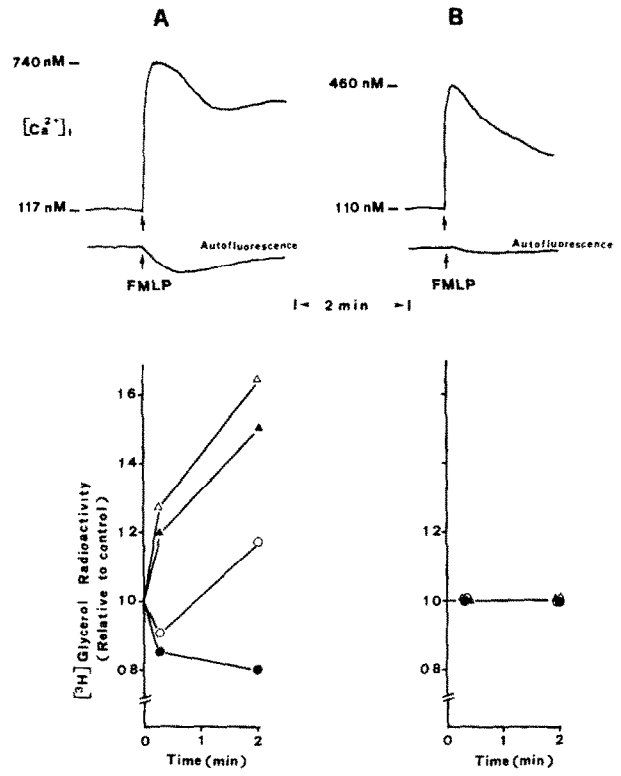


Fig.3. The effect of 100 nM (A) and 2 nM FMLP (B) on the cytosolic $[Ca^{2+}]_i$ monitored by Quin-2 and on the $[^3H]$glycerol-labelled phospholipids in human neutrophils. The values of zero time were: phosphatidylinositol, 83362 cpm (●); phosphatidylinositol 4-phosphate, 4732 cpm (▲); phosphatidylinositol 4,5-biphosphate, 2120 cpm (○); and phosphatidic acid, 9220 cpm (△). The data are of one experiment representative of three.

caused by the breakdown of phosphatidylinositides. It has been suggested that in these cells the changes in phosphoinositide metabolism are consequent on the mobilization of $Ca^{2+}$ [6,7,10,15,35]. Our finding that the increase in $[Ca^{2+}]_i$ induced by low dose of FMLP is not accompanied by the activation of phosphoinositide turnover might indicate that the two events are unrelated. However, if the hypothesis that the increase of the cytosolic $Ca^{2+}$ triggers the phosphoinositides turnover is correct, a level of $[Ca^{2+}]_i$, higher than that reached in the experiment reported in fig.3, is required.

Fig.2 also shows the dose response curves for other neutrophil responses to FMLP. All are shifted on the right when compared with that for the rise in $[Ca^{2+}]_i$ and are practically similar to that for phosphoinositide response. The $ED_{50}$ values are: 14 nM for $Ca^{2+}$ mobilization monitored as chlorotetracycline fluorescence changes, 34 nM for $^{45}Ca^{2+}$ influx and 32 nM for the respiratory burst.

The different $ED_{50}$ values for the changes in $[Ca^{2+}]_i$ assayed by Quin-2 and chlorotetracycline fluorescence clearly confirm that they monitor responses of different pools of $Ca^{2+}$. The release from intracellular stores is responsible for the rapid rise in cytosolic $Ca^{2+}$ concentration monitored by Quin-2 and the release from plasma membrane-bound $Ca^{2+}$ for the decrease in fluorescence of chlorotetracycline loaded neutrophils [34,36,38,39].

The similarity of $ED_{50}$ values for the change of chlorotetracycline fluorescence and phosphoinositide metabolism could indicate that phosphoinositide response is causally related with the mobilization of the plasma membrane pool of $Ca^{2+}$. Similar conclusions could be drawn for the relationship between phosphoinositide response and $Ca^{2+}$ gating, monitored by the increase of $^{45}Ca^{2+}$ influx, which also have similar $ED_{50}$ values for FMLP. If this is the case, the same molecular modifications of the plasma membrane would cause both $Ca^{2+}$ mobilization and opening of $Ca^{2+}$ gates. It has been postulated that phosphatidylinositol 4,5-biphosphate represents the site of plasma membrane-bound $Ca^{2+}$ and evidences have been provided that the breakdown of this phospholipid causes the release of $Ca^{2+}$ from platelet membrane [38]. However, the causal relationship between breakdown of phosphoinositides and $Ca^{2+}$ mobilization and gating should be further investigated in neutrophils since in some experimental conditions the hydrolysis of phosphoinositides is not accompanied by $Ca^{2+}$ changes. We have discussed this point in [39], where we reported that the breakdown of polyphosphatidylinositides caused in neutrophils by exogenous phospholipase C is not associated with the release of membrane-bound $Ca^{2+}$.

Also, the dose response curve for the activation of the membrane-bound NADPH oxidase is similar to those for the other plasma membrane events, phosphoinositide turnover and release of bound $Ca^{2+}$. As for their relationships, it is worth pointing out that the onset of the respiratory burst is clearly the more delayed. A dependency of the

activation of the NADPH oxidase by FMLP on the increase of phosphoinositide turnover and on mobilization of plasma membrane-bound $Ca^{2+}$, is compatible with the involvement of the $Ca^{2+}$ and phospholipid dependent protein kinase C as a key step in the transduction process for the respiratory response in neutrophils.

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