Coupling of polyphosphoinositide breakdown with calcium efflux in formyl-methionyl–leucyl–phenylalanine-stimulated rabbit neutrophils

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Exposure of rabbit neutrophils to formyl-methionyl–leucyl–phenylalanine (FMLP) induced the efflux of $^{45}Ca^{2+}$ from pre-labeled cells which was almost complete within 30 s. On the other hand, FMLP-induced $^{45}Ca^{2+}$ influx did not become apparent until 60 s after stimulation. When [^{3}H]arachidonic acid-labeled neutrophils were stimulated with FMLP, the radioactivities in phosphatidylinositol 4,5-biphosphate (TPI) and phosphatidylinositol 4-phosphate (DPI) significantly decreased in parallel with the induction of $^{45}Ca^{2+}$ efflux. In contrast, degradation of polyphosphoinositides in [^{3}H]glycerol-labeled neutrophils was not significant until 60 s. Taken together, these results indicate that the early degradation of polyphosphoinositides, especially of those rich in arachidonic acid is closely associated with the initial efflux of calcium in FMLP-stimulated rabbit neutrophils. The study of resynthesis of polyphosphoinositides by measuring $^{32}P_i$ incorporation into these lipids is also presented.

Polyphosphoinositide *Ca^{2+} mobilization* *Formyl-methionyl–leucyl–phenylalanine* *Rabbit neutrophil*

1. INTRODUCTION

It has been proposed that the metabolic turnover of phosphatidylinositol (PI) known as 'PI cycle' may be essential to calcium mobilization in the receptor-media stimulus–secretion coupling [1]. In support of the proposal, recent studies demonstrated that phosphatidic acid (PA), one of the intermediates of the 'PI cycle' might act as a calcium-carrying ionophore [2,3]. However, in rabbit neutrophils stimulated with formyl-methionyl–leucyl–phenylalanine (FMLP) which is a synthetic chemotactic peptide, hydrolysis of PI and generation of PA were reported to be a consequence, not a cause, of calcium translocation [4].

In addition to PI, mammalian cells contain polyphosphoinositides such as phosphatidylinositol 4-phosphate (DPI) and phosphatidylinositol 4,5-biphosphate (TPI) which are phosphorylated derivatives of PI [5–13]. Although polyphosphoinositides exist in much smaller amounts than PI, much attention has been paid to the role of these lipids in regulating a variety of physiological functions [11–13]. Recently, studies with hepatocytes [12] and rat parotid acinar cells [13] have suggested that polyphosphoinositide breakdown may have a role in ligand-induced calcium mobilization similar to that proposed for PI.

Here, experiments were designed to pursue the possible link between the metabolism of polyphosphoinositides and calcium movement across plasma membranes of rabbit neutrophils utilizing FMLP as stimulus. The results provided evidence indicating a good correlation between the time

Abbreviations: TPI, phosphatidylinositol 4,5-biphosphate; DPI, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PA, phosphatidic acid; FMLP, formyl-methionyl–leucyl–phenylalanine; PBS, phosphate-buffered saline; HEPES, *N*-2-hydroxymethylpiperazine–*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol bis(*β*-aminoethylether)*N,N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; HPTLC, high-performance thin-layer chromatography

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course of polyphosphoinositide breakdown and that of calcium efflux from the cells.

2. MATERIALS AND METHODS

2.1. *Preparation of [^{3}H]arachidonic acid- and [^{3}H]glycerol-labeled rabbit neutrophils*

Rabbit peritoneal neutrophils (>90% pure) were collected as in [14]. Neutrophils (2–4 × 10^8) were obtained from one rabbit. Isolated rabbit neutrophils (2 × 10^8 cells) were washed once with phosphate-buffered saline (PBS, pH 7.4) containing 137 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl and 0.1% glucose supplemented with 1 mM EGTA and incubated in 30 ml the above buffer with 10 μCi [^{3}H]arachidonic acid (78.2 Ci/mmol, New England Nuclear) or 200 μCi of [^{3}H]glycerol (10 Ci/mmol, New England Nuclear) for 90 min at 37°C. After labeling, cells were washed 2 times with PBS containing 0.6 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (PBS-Ca/Mg) and then 0.6 ml neutrophil suspension was transferred to siliconized tubes (1 × 10^7 cells/tube).

2.2. *$^{32}P_i$ incorporation into phosphoinositides*

The isolated rabbit neutrophils were washed once with HEPES-buffered saline (pH 7.4) consisting of 20 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.1% glucose, 0.6 mM $CaCl_2$ and 1.0 mM $MgCl_2$. Before cell stimulation, the suspension of neutrophils (1 × 10^7 cells in 0.6 ml above buffer) was incubated with 40 μCi $^{32}P_i$ (Amersham) at 37°C for 60 min.

2.3. *Cell stimulation and lipid extraction*

Stimulation of neutrophils was initiated by the addition of 0.4 ml PBS-Ca/Mg (for cells prelabeled with [^{3}H]arachidonic acid or with [^{3}H]glycerol) or HEPES-buffered saline (for the $^{32}P_i$ incorporation study) with or without FMLP (Sigma). The final concentration of FMLP was 1 × 10^{-7} M. After incubation for various time periods at 37°C, the reaction was terminated by adding 4 ml chloroform/methanol/conc. HCl (20:40:1, by vol.) [15]. The organic phase was separated by the addition of 1 ml each of chloroform and distilled water, followed by centrifugation. The lower (organic) phase was collected and dried under nitrogen.

2.4. *Chromatography of lipids*

Separation and identification of phosphoinositides were achieved as in [16], using high-performance TLC (HPTLC) (Merck) impregnated with 1% potassium oxalate [17]. TPI, DPI, PI and PA were well separated from other lipids as shown in [16]. Counting of the radioactivities in the individual lipids was done as in [18].

2.5. *Measurements of calcium influx and efflux*

To examine the calcium influx, 0.6 ml of the neutrophil suspension (1 × 10^7 cells in PBS-Ca/Mg) was stimulated with FMLP (10^{-7} M) in the presence of 2 μCi $^{45}Ca^{2+}$ (Amersham). The reaction was stopped by the addition of 0.4 ml ice-cold 20 mM EDTA–PBS and 5 min centrifugation at 170 × *g* at 4°C. The resultant cell pellet was washed 2 times with ice-cold 5 mM EDTA–PBS and the radioactivity in the cells was counted as in

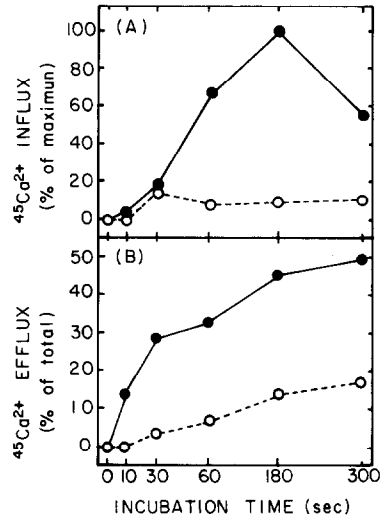

Fig.1. Time course of FMLP-induced $^{45}Ca^{2+}$ influx (A) and $^{45}Ca^{2+}$ efflux (B). Experimental conditions are in section 2: (A) data are expressed as percentage of the maximal influx observed at 3 min which amounted to 2210 cpm in radioactivity; (B) data are expressed as percentage of $^{45}Ca^{2+}$ released of the total radioactivity (38 300 cpm) incorporated into 1 × 10^7 neutrophils during the pre-labeling period. Open symbols indicate control incubations without FMLP and closed symbols incubations in the presence of FMLP (10^{-7} M). The figure illustrates a result of a typical experiment from two different experiments, both of which showing essentially similar results. Each value is the mean of duplicate determinations.

[19]. The effects of FMLP on the calcium efflux were examined on the neutrophils prelabeled with $^{45}Ca^{2+}$. The neutrophils (2×10^8 cells) were incubated at 37°C for 60 min in 20 ml PBS without any added calcium in the presence of 40 μCi $^{45}Ca^{2+}$. Subsequently, the cells were washed 2 times with PBS-Ca/Mg and the neutrophil suspension (1×10^7 cells/ml) was activated with FMLP (10^{-7} M) for various time periods at 37°C. Termination of the reaction was performed by a slight modification of the method in [20]. Briefly, after various periods of incubation, 1 ml neutrophil suspension was taken up and rapidly centrifuged at $1500 \times g$ for 1 min through 10 ml ice-cold 150 mM NaCl containing 1.5% (w/v) albumin (Sigma), 2.5 mM $LaCl_3$ and 5% (w/v) sucrose. The resultant cell pellet was washed 2 times with PBS and the radioactivity in the final pellet was determined as above.

3. RESULTS AND DISCUSSION

FMLP enhances both calcium influx and efflux in rabbit neutrophils [21]. Under our experimental conditions, FMLP-induced $^{45}Ca^{2+}$ influx (fig. 1A) became apparent at 60 s after stimulation whereas $^{45}Ca^{2+}$ efflux (fig. 1B) was markedly enhanced at 10 s and was almost complete by 30 s. This finding indicates that calcium efflux rather than calcium influx is an earlier event in FMLP-stimulated rabbit neutrophils. It has been suggested that polyphosphoinositide breakdown may be involved in agonist-induced calcium mobilization [13]. This suggestion appears to be interesting since polyphosphoinositides are known to reside at the plasma membrane where agonist–receptor interactions occur [22] and these phospholipids bind calcium with high affinity [23].

To examine as to whether polyphosphoinositide metabolism is related to calcium mobilization, rabbit neutrophils pre-labeled with [^{3}H]arachidonic acid or with [^{3}H]glycerol were stimulated with FMLP. The effects of FMLP on the redistribution of radiolabels in phosphoinositides are shown in fig. 2. In control incubations without FMLP, the radioactivities in TPI and DPI gradually increased and reached a plateau by 5 min. A similar result was observed in the study of human platelets from our laboratory [16]. Although we do not have any definite explanation for this, it is most likely that conversion of PI to polyphosphoinositides (PI→DPI→TPI) is moderately operating even in unstimulated cells. When [^{3}H]arachidonic acid-labeled neutrophils were stimulated with FMLP (fig. 2A), the significant loss of TPI and DPI occurred already at 10 s. The degradation of TPI was almost complete at 10 s while the level of DPI was further decreased by 30 s and was unchanged thereafter. In addition, FMLP caused a marked decrease in the radioactivity in PI from 20 700 dpm to 13 600 dpm at 30 s and the decreased level continued up to 5 min. FMLP-induced degradation of PI was also evident in [^{3}H]glycerol-labeled neutrophils (fig. 2B). PI lost the radioactivity by 27% at 60 s. However, the decrease in radioactivities in TPI and DPI was not apparent until 60 s after stimulation. Following the initial decline at 60 s, the levels of TPI and DPI tended to recover to those of unstimulated cells:

(i) These results indicate that polyphosphoinositides rich in arachidonic acid are more susceptible to hydrolysis;

(ii) The observation that [^{3}H]glycerol-TPI and -DPI were much more rapidly resynthesized

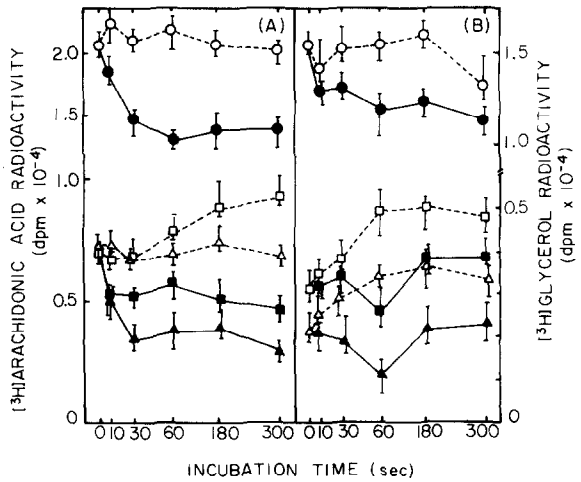

Fig.2. Time course of the effects of FMLP on [^{3}H]arachidonic acid (A)- or [^{3}H]glycerol (B)-labeled phosphoinositides. Experimental conditions are in section 2: ○,● phosphatidylinositol; (□,■) phosphatidylinositol 4,5-biphosphate; (△,▲) phosphatidylinositol 4-phosphate. Open symbols indicate control incubations and closed symbols incubations in the presence of FMLP (10^{-7} M). Each point represents the mean of 2 expt. performed in duplicate and each bar indicates the range of variations.

than [^{3}H]arachidonyl-TPI and -DPI is consistent with our finding [14] that PI synthesized after 5 min stimulation with FMLP following the initial degradation has a lower percentage of arachidonic acid. Thus, it could be deduced that at 5 min after stimulation, PI which is relatively poor in arachidonic acid was rapidly phosphorylated to DPI and to TPI by the sequential actions of PI- and DPI-kinases [24], respectively.

(iii) Results of fig. 1 and fig. 2 demonstrate correlation between time course of $^{45}Ca^{2+}$ efflux and that of degradation of polyphosphoinositides, particularly of those rich in arachidonic acid.

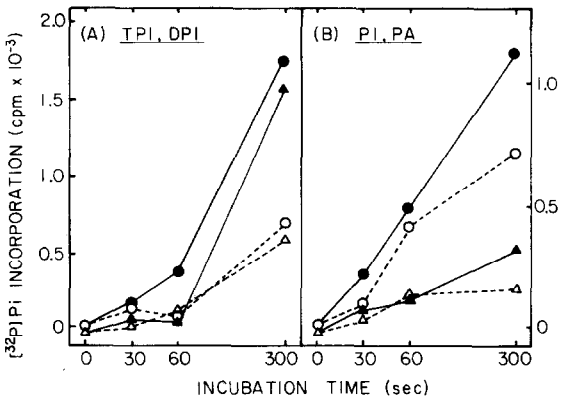

Fig.3. Time course of $^{32}P_i$ incorporation into phosphatidylinositol 4,5-bisphosphate (○,●) and phosphatidylinositol 4-phosphate (△,▲) in (A), and phosphatidylinositol (○,●) and phosphatidic acid (△, ▲) in (B). Experimental conditions are described in section 2. During the preincubation period (60 min) with $^{32}P_i$, TPI, DPI, PI and PA incorporated the radioactivity of 4470, 2150, 3310 and 520 cpm, respectively. Each point represents, therefore, the corrected value calculated as follows:

Corrected value =
- the radioactivity counted in the individual phospholipid at each time point
- the radioactivity incorporated into the corresponding phospholipid during the preincubation period

The figure illustrates a result of a typical experiment from two different experiments, both of which show essentially similar results. Each value is the mean of triplicate determinations. Open symbols indicate control incubations and closed symbols incubations in the presence of FMLP (10^{-7} M).

As mechanism(s) for degradation of polyphosphoinositides, two alternative pathways can be considered: polyphosphoinositides are dephosphorylated by phosphomonoesterases [25] or cleaved by phosphodiesterase(s) [26]. Phosphomonoesterase-catalyzed cleavage of polyphosphoinositides should be accompanied by the accumulation of PI, which was not the case here. Therefore, the action of phosphodiesterase(s) on polyphosphoinositides which give rise to diacylglycerol, is more likely. In [14], we demonstrated that [^{3}H]arachidonyl-diacylglycerol and -PA were formed upon addition of FMLP to [^{3}H]arachidonic acid-labeled rabbit neutrophils. This further supports the pathway to produce diacylglycerol.

Resynthesis of TPI and DPI was then ascertained by measuring $^{32}P_i$ incorporation into these lipids in response to FMLP. As in fig. 3A, 5 min stimulation with FMLP caused 2.5- and 2.8-fold increases in $^{32}P_i$ incorporation into TPI and DPI, respectively, compared to their respective controls. On the other hand, enhancement of incorporation of $^{32}P_i$ into PI (57% above control) and PA (110% above control) (fig. 3B) appeared to be smaller than that into polyphosphoinositides. This may be explained by the fact that the metabolic turnover of the monophosphate groups attached to the inositol rings of polyphosphoinositides is very rapid [22]. $^{32}P_i$ incorporation into other phospholipid fractions was not significantly enhanced by FMLP (not shown).

This study demonstrates that FMLP induces a rapid turnover of polyphosphoinositides and that degradation of these lipids, especially of those rich in arachidonic acid, is closely associated with calcium efflux from the rabbit neutrophils. The significant role of the rapid turnover of arachidonate-rich phosphoinositides in rabbit neutrophil functions was also suggested in [27]. An ultrastructural study [28] that showed FMLP causes calcium loss from the plasma membrane in human neutrophils. Calcium efflux observed here, therefore, may be a reflection of calcium release into the cytosol from the intracellular storage sites, most probably from the plasma membrane. Thus, these data offer a strong suggestion, although not a definite proof, that rabbit neutrophils may be categorized as one of such cells in which polyphosphoinositide metabolism is linked to the initial mobilization of calcium [12,13,29].

NOTE ADDED

After submitting this manuscript, we found the work of Volpi et al. [30] on the FMLP-induced rapid breakdown of polyphosphoinositides in rabbit neutrophils prelabeled with $^{32}P_i$. They raised a possibility that decreases in the amounts of these lipids may inhibit the activity of the calcium efflux pump. In contrast, the time course study reported here offers a suggestion that the breakdown of polyphosphoinositides would be coupled to the calcium efflux in the FMLP-stimulated rabbit neutrophils.

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