A rapid stimulation of phosphatidylinositol metabolism in rabbit leukocytes by pseudomonal leukocidin

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The time course experiments of $^{32}P_i$-labelling and breakdown of phospholipids in rabbit leukocytes exposed to leukocidin from *Pseudomonas aeruginosa* suggested that the initial action of this toxin was to stimulate phosphatidic acid production, presumably by causing a rapid metabolic change of phosphatidylinositol (PI response) correlating with phosphatidylinositol-specific phospholipase C and 1,2-diacylglycerol kinase. It appears that a rapid formation of phosphatidic acid and degradation of polyphosphoinositides in leukocytes treated with the toxin might be related a Ca^{2+}-movement from extra- and intracellular spaces, resuling in the activation of Ca^{2+}-dependent enzymes involved in the leukocidic process.

Phosphoinositol metabolism *Phosphatidic acid* *Leukocidin* Pseudomonas aeruginosa
Rabbit leukocyte *Toxin action*

1. INTRODUCTION

Rapid phosphatidylinositol (PI) turnover in response to stimulation by hormones [1–3] or by neurotransmitters [4,5] is accompanied by an increase in the cytosolic [Ca^{2+}] for utilization of Ca^{2+} as their intracellular second messenger by way of Ca^{2+}-dependent protein phosphorylation in a similar manner to glycogen metabolism [6], smooth-muscle contraction [7], and ion transport [8]. In [9], the addition of *Pseudomonas aeruginosa* leukocidin to rabbit leukocyte homogenate, induced incorporation of radioactive phosphate moiety of [γ-^{32}P]ATP into a 28 kDa protein by Ca^{2+}-dependent, calmodulin-independent protein kinase. Ca^{2+} may play a significant role in leukocyte destruction by pseudomonal leukocidin.

Here we show that pseudomonal leukocidin induced a rapid metabolic change of PI (PI response), which gave rise to increased cytosolic free [Ca^{2+}] [10].

2. MATERIALS AND METHODS

2.1. *Materials*

[^{32}P]Orthophosphoric acid (carrier free in water) was obtained from New England Nuclear. Phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), PI, phosphatidylinositol 4-monophosphate (DPI), phosphatidylinositol 4,5-diphosphate (TPI) used as standard were purchased from Sigma. Silica gel 60 was from Merck. All other chemicals were analytical reagents.

2.2. *Preparation of leukocidin*

Purification and crystallization of pseudomonal leukocidin were done as in [9].

2.3. *Isolation of rabbit leukocytes*

Rabbit leukocyte suspension containing 98% polymorphonuclear leukocytes as assessed by Giemsa stain were prepared by means of Ficoll-Hypaque gradients [11] followed by dextran

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sedimentation and hypotonic lysis of erythrocytes with 0.85% ammonium chloride.

2.4. *Incorporation of $^{32}P_i$ into rabbit leukocytes*

Isolated leukocytes (1×10^8) were suspended in 3.5 ml 0.01 M Tris–HCl buffered saline (pH 7.2) containing 1 mM $MgCl_2$. A portion (150 μl) of this suspension was then added to plastic tubes each containing 50 μl of the same buffered saline containing 50 μCi $^{32}P_i$ and leukocidin (5 μg). After an appropriate incubation at 37°C, the reaction was terminated by addition of 720 μl chloroform/methanol/conc. HCl (100:200:2, by vol.) and the phases were separated by adding 245 μl chloroform and 245 μl 2 M KCl [12,13]. The lower organic phase was transferred, and the aqueous was washed once with 1 ml chloroform. The combined lower phases were dried under a flow of N_2. The lipids were separated in thin-layer plates (Silica gel 60 impregnated with 1% potassium oxalate containing 2 mM EDTA) using chloroform/methanol/4 N NH_4OH (45:35:10, by vol.) [12] or by two-dimensional chromatography (Silica gel 60) using chloroform/methanol/acetic acid/water (75:45:12:3, by vol.) in the first dimension and chloroform/methanol/conc. NH_4OH/water (70:30:0.5:4, by vol.) in the second dimension [14]. Lipids were localized by either autoradiography or iodine vapor and counted by scintillation with Aquasol II.

2.5. *Measurement of breakdown of [^{32}P]phospholipids*

Rabbit leukocytes (7.5×10^7) were suspended in 0.8 ml 0.01 M Tris–HCl buffered saline (pH 7.2) containing 1 mM $MgCl_2$ and then incubated with 300 μCi $^{32}P_i$ at 28°C for 60 min. Leukocytes were then washed with 50 ml buffered saline and gently resuspended in 3 ml of the same buffered saline. Triplicate samples (200 μl, 5×10^6 cells) were incubated with leukocidin (5 μg) at 37°C for different periods of time. The reaction was terminated by adding 720 μl chloroform/methanol/conc. HCl (100:200:2, by vol.). The extracted lipids were analyzed as above.

2.6. *Protein determination*

Estimation of protein was performed by the Lowry method [15], using bovine serum albumin as standard.

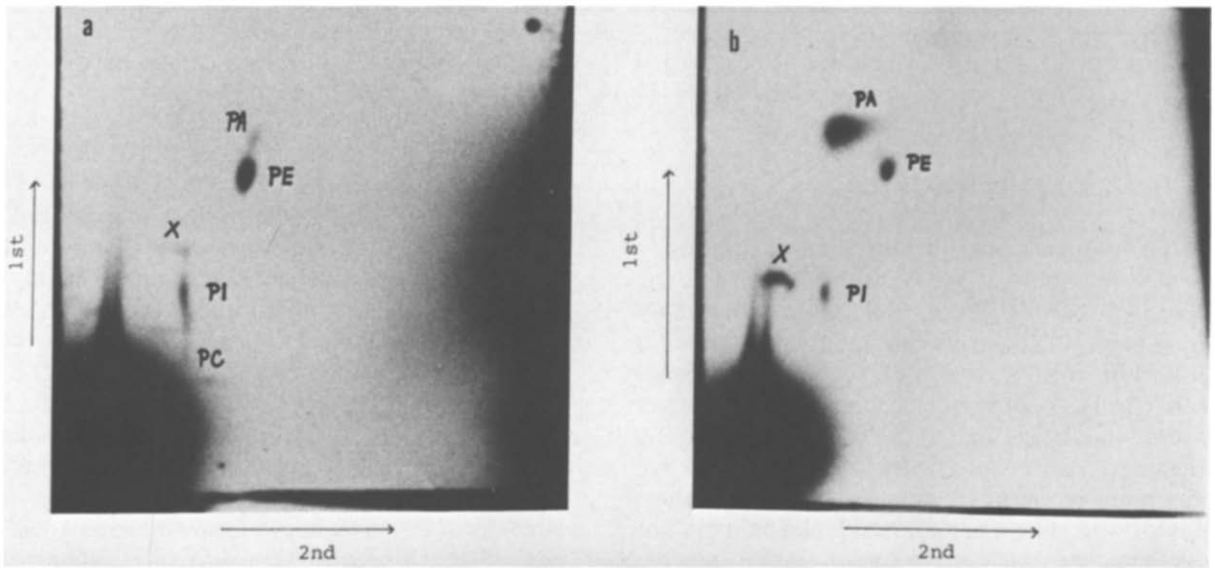

Fig.1. Two-dimensional thin-layer chromatography of [^{32}P]phospholipids extracted from control (a) and leukocidin-treated leukocytes (b). Rabbit leukocytes (4.3×10^6) were incubated with 50 μCi [^{32}P]phosphate in the absence or presence of leukocidin (5 μg). Labeled lipids extracted were separated by two-dimensional thin layer chromatography as described in text and detected by autoradiography. The origin contained DPI and TPI. Unidentified radioactive phospholipid is shown X.

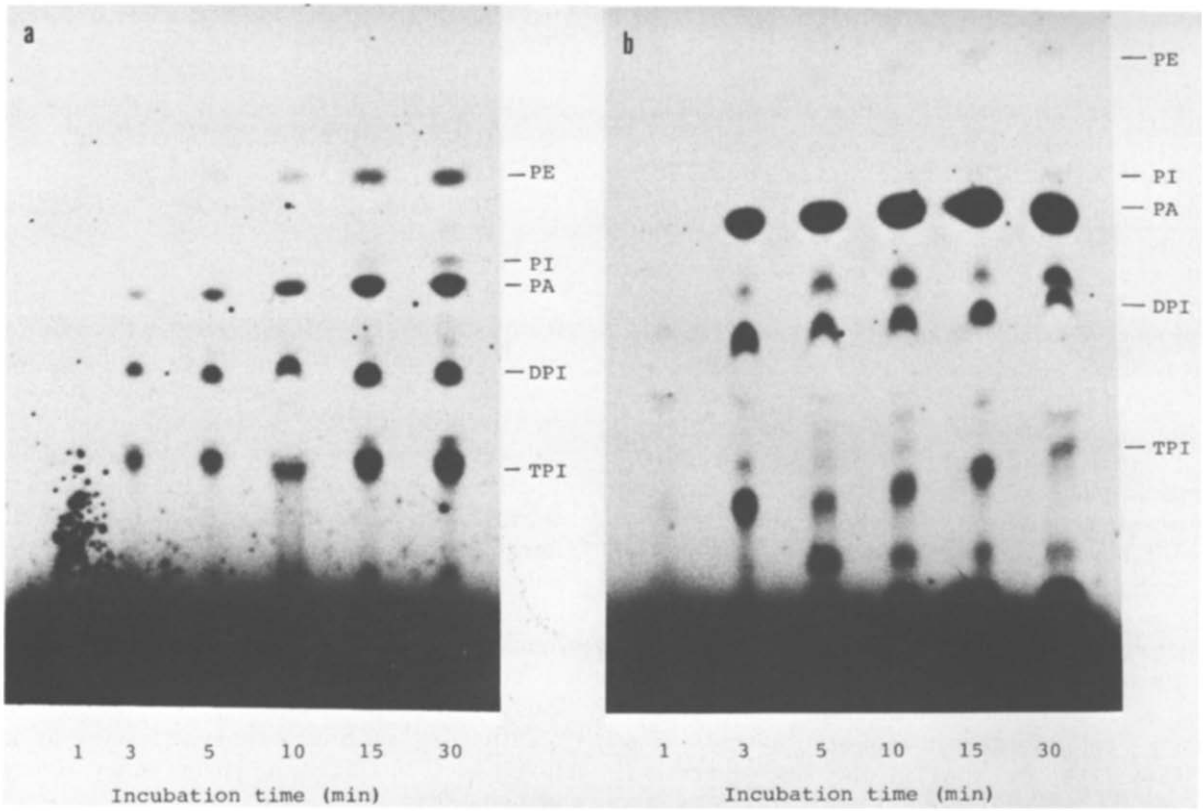

Fig.2. Formation of radioactive phospholipids in rabbit leukocytes. Aliquots (200 μl) of leukocytes (4.7×10^6) were incubated with [^{32}P]phosphate at 37°C for different periods of incubation time in the absence (a) or presence (b) of leukocidin (5 μg). At the time indicated, radioactive phospholipids were separated by thin layer chromatography with chloroform/methanol/4 N NH_4OH (45:35:10, by vol.) and identified by autoradiography.

3. RESULTS AND DISCUSSION

By two-dimensional thin layer chromatography for phospholipid analysis of rabbit leukocytes, the incorporation of $^{32}P_i$ into PA was remarkably stimulated in leukocytes exposed to pseudomonal leukocidin for 5 min, compared with non-treated cells (fig.1). Leukocidin treatment resulted in a 9-fold stimulation of $^{32}P_i$ incorporation into PA whereas the effects of this toxin on the radioactive incorporation into PI was <1.5-fold. Furthermore, it was shown in fig.1 that leukocidin did not significantly influence $^{32}P_i$ incorporation into PE and PC.

Fig.2 shows a typical example of autoradiography of leukocidin-treated leukocyte [^{32}P]phospholipids labeled for various times. The effect of pseudomonal leukocidin on the $^{32}P_i$-incorporation was apparently manifested in PA, DPI, and TPI. Incorporations of $^{32}P_i$ into these phospholipids were determined by counting the radioactivities of the bands and were then plotted against incubation time (fig.3). The rate of incorporation of $^{32}P_i$ into leukocyte phospholipids in the absence of leukocidin maximized at a plateau over 15 min. The treatment of leukocyte with leukocidin induced early formations of DPI and TPI which reached each maximum at 3 min accompanying rapid decreases of both phospholipids. Subsequently, labelling of PA in leukocytes exposed to leukocidin became maximal at 5 min and then diminished slowly accompanying a gradual increase in labelling of PI. This kinetic pattern is consistent with the idea that PA is a product from PI and polyphosphoinositides by combined action of PI-specific phospholipase C and 1,2-diacylglycerol

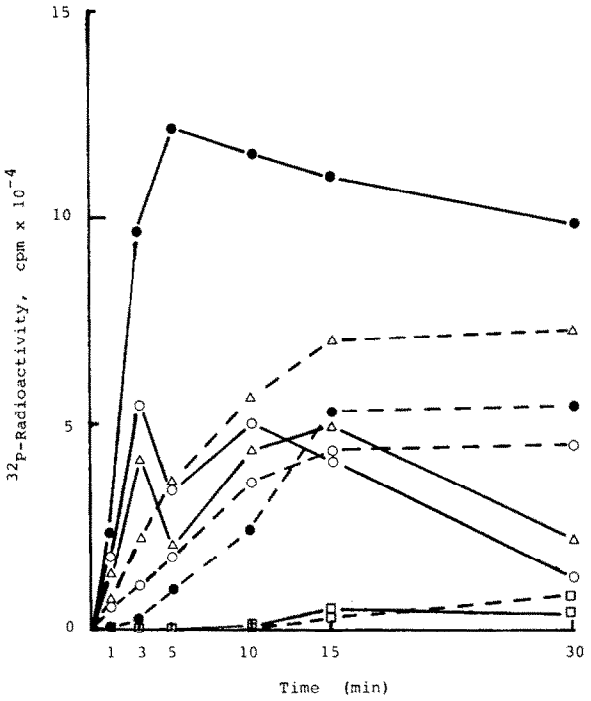

Fig.3. Time course of leukocidin-induced formation of phospholipids. The lipids analyzed by thin layer chromatography as shown in fig.2 were scraped into vials and the radioactivity measured by liquid scintillation counter. Results are representative of 3 similar experiments. Results obtained from (···) control leukocytes and (——) leukocidin-treated leukocytes: (○) DPI; (△) TPI; (●) PA; (□) PI.

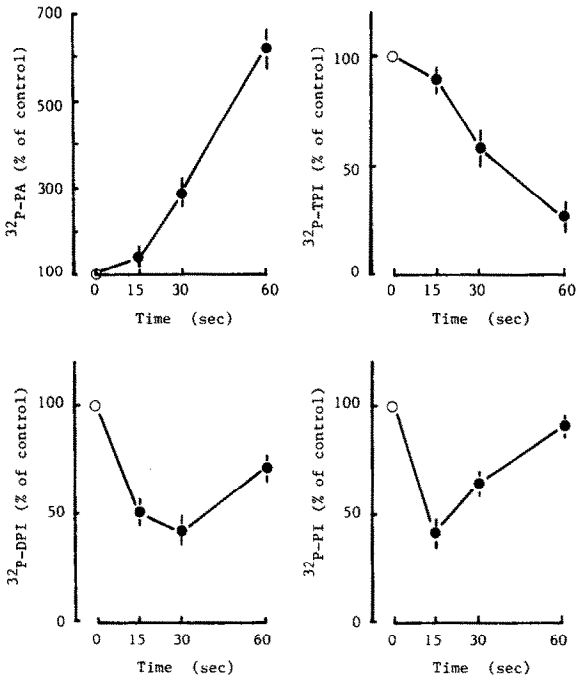

Fig.4. Time course of the changes in radioactivities of PI, DPI, TPI and PA after addition of leukocidin to leukocytes. Leukocytes were incubated with [^{32}P]phosphate for 60 min at 28°C and then reaction was started by addition of leukocidin (5 μg). The lipids were extracted and separated by thin layer chromatography as in fig.2. Separation of PA and PI from all other phospholipids was achieved by two-dimensional thin layer chromatography as in section 2. The spots containing PI, DPI, TPI and PA were scraped into vials to measure their radioactivities. Open symbols indicate zero time control, means ± SEM, n = 3; PA = 55100 ± 2500 cpm; TPI = 74600 ± 3500 cpm; DPI = 57300 ± 2000 cpm; PI = 38800 ± 1500 cpm. There were no detectable changes in the radioactivity of these phospholipids in control cells during the 60 s incubation period.

kinase [16]. In addition, phospholipid turnover induced by leukocidin was found to occur either in the absence or presence of added Ca^{2+} (1 mM), although this effect was abolished by addition of 5 mM EDTA (not shown). It was also noteworthy that leukocidin-treated platelets, in which no morphological change was induced by addition of higher amounts of leukocidin [17], exhibited similar rates of $^{32}P_i$ incorporation into phospholipids with those in control cells.

Incubation of leukocytes for 60 min, in the presence of $^{32}P_i$ resulted in a satisfactory incorporation of label into all of the major phospholipids to examine the breakdown of phospholipid in leukocytes exposed to leukocidin. Fig.4 shows the changes in the radioactivities of PI, DPI, TPI and PA over 60 s following addition of leukocidin to leukocytes which had been preincubated for 60 min in the presence of $^{32}P_i$ to label phospholipids and the ATP pool. Within 15–30 s of the addition of leukocidin, the radioactivities of PI and DPI had declined by ~60%. Thereafter, the radioactivity in both lipids gradually increased. A delayed loss of radioactivity from [^{32}P]TPI was followed by an increased incorporation of $^{32}P_i$ into PA. No significant effect upon other major phospholipids, principally PC and PE, was observed. [^{3}H]Glycerol purse-chase experiments (not shown) demonstrated that the radioactivities of

polyphosphoinositides as well as PI declined to a similar extent without the ensuring increase in radioactivity. These results indicate that the increase of radioactivity of PA in leukocidin-treated leukocytes is a reflection of rapid metabolic change of PI concerning the breakdown of polyphosphoinositides, but not an enhancement of de novo synthesis of PA.

In [17], we pointed out an enhancement of Ca^{2+}-influx and a significance of Ca^{2+}-mobilization from intracellular Ca^{2+} source on leukocyte destruction by pseudomonal leukocidin. It has been proposed that PA possesses Ca^{2+} ionophoretic property [18–20], and that DPI and TPI are very potent chelators of Ca^{2+} and Mg^{2+} [10,21,22]. Therefore, we concluded that the accumulation of PA and the breakdown of polyphosphoinositides in leukocidin-treated leukocytes might cause mobilization of Ca^{2+} from intracellular compartments and extracellular medium. Such enhanced level of cytosolic free Ca^{2+} seems to initiate activation of Ca^{2+}-dependent protein kinase [9]. However, further studies are still necessary to elucidate the direct proof of the toxic action of pseudomonal leukocidin on leukocyte enzymes responsible for PI metabolism.

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