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ACTION OF PHOSPHOLIPIDS AND LEUCOCIDIN ON THE *p*-NITROPHENYL PHOSPHATASE OF THE LEUCOCYTE MEMBRANE

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SUMMARY

1. The participation of phospholipids in the activity of the *p*-nitrophenyl phosphatase of the leucocyte membrane has been studied in relation to the mode of action of leucocidin and the unique character of the leucocyte enzyme.

2. Trypsin abolishes the sensitivity of the *p*-nitrophenyl phosphatase to K^+ but not the stimulation by leucocidin.

3. Phospholipase A does not prevent the stimulation by K^+ but prevents and reverses the stimulation by leucocidin.

4. Phospholipase A stimulates the *p*-nitrophenyl phosphatase activity at pH 5 but not at pH 7. It also induces an anomalous specific activity–concentration relationship in the presence of EDTA.

5. Acidic phospholipids inhibit the *p*-nitrophenyl phosphatase activity at pH 5 but only triphosphoinositide inhibits the activity at pH 7.2.

6. Acidic phospholipids reverse the stimulation of the activity at pH 5 induced by phospholipase A but do not reverse the stimulation induced by leucocidin.

7. The *p*-nitrophenyl phosphatase can be partially solubilised by lubrol or deoxycholate. The solubilised *p*-nitrophenyl phosphatase is not stimulated by leucocidin, it is inhibited by added acidic phospholipids but the phospholipids do not enhance the cation sensitivity. The solubilised ATPase does not become cation sensitive in the presence of added phosphatidyl serine.

8. It is concluded that the action of leucocidin on the *p*-nitrophenyl phosphatase is mediated through phospholipids but requires the phospholipids to be in a unique state of combination in the membrane.

9. The physiological function of the leucocyte *p*-nitrophenyl phosphatase is briefly discussed.

INTRODUCTION

The phosphatases of the polymorphonuclear leucocyte* surface membrane are of interest in relation to the transport of electrolytes and to the mode of action of leucocidin. The leucocyte, unlike most other mammalian cells, controls its electrolyte

* Called leucocytes in this paper.

balance by regulating the K^+ content and correlating with this is the absence of a Na^+-sensitive ATPase and the presence of a K^+-sensitive *p*-nitrophenyl phosphatase in the isolated surface membrane[1]. This led us to suggest that the *p*-nitrophenyl phosphatase is part of an electrogenic K^+ pump[2]. The two proteins that constitute the Staphylococcal toxin, leucocidin, synergistically after the permeability of the leucocyte to cations and the changes resemble those in excitable tissues during membrane depolarisation[3]. In the isolated membrane leucocidin stimulates the *p*-nitrophenyl phosphatase specifically and we have suggested that the permeability changes in the cell result from a structural change to the K^+ pump[2].

The *p*-nitrophenyl phosphatase of the leucocyte membrane has a maximal activity at pH 5 but smaller peak activities are also found at pH 6.2 and pH 7. The overall activity and the position of the pH optima are modified by treatment with dilute Tris buffer or with NaI solution and these reagents induce a K^+ sensitivity over the pH range 6–8. The stimulation by K^+ is far smaller than that found in membranes from electric organ[4] or brain[5] and is inhibited only by high concentrations ($1 \cdot 10^{-4}$ M) of ouabain.

In brain and electric organ it is probable that the K^+-sensitive phosphatase is part of an ATPase but the properties of the leucocyte *p*-nitrophenyl phosphatase suggest that it is distinct from the ATPase. TANAKA AND MITSUMATA[5] for example, have shown that phospholipids stimulate the *p*-nitrophenyl phosphatase of brain. In the present paper we show that the leucocyte *p*-nitrophenyl phosphatase is modified by phospholipids in a different fashion.

The action of leucocidin on the leucocyte or the isolated leucocyte membrane is accompanied by conversion of leucocidin to an inactive form but if the isolated membrane is treated with phospholipase A this does not occur[6]. Leucocidin can interact with the esterified fatty acids of phospholipids and triphosphoinositide can mimic, in part, the inactivation of leucocidin by the cell[7]. In the present paper we show that the stimulation of the *p*-nitrophenyl phosphatase by leucocidin is critically dependent on the presence of phospholipids.

MATERIALS AND METHODS

The F component and the S component of leucocidin were the crystalline products isolated from culture filtrates of the V 8 strain of *Staphylococcus aureus*[8]. In the experiments recorded here both components of leucocidin were present in equal amounts and the statement "*x* g of leucocidin" will mean "*x* g of each component of leucocidin". Russel's viper venom was obtained from Light and Co., Colnbrook, England. It was dissolved in water (6 mg/ml), boiled for 5 min, the insoluble material centrifuged off and the supernatant stored frozen. Samples were replaced weekly. Phospholipase A from pig pancreas was prepared by the method of DE HAAS *et al.*[9]. When assayed by the method of DE HAAS *et al.*[9] the pancreatic phospholipase was found to have a 4-fold greater specific activity than boiled Russel's viper venom. Before addition to the membranes the preparations were diluted to have equal activities. The purity of the pancreatic phospholipase A is described by DE HAAS *et al.*[9]. The purity of the phospholipase A in boiled Russel's viper venom was not investigated further but it acted on leucocyte membranes in a similar way as the pancreatic enzyme and was without effect in the absence of Ca^{2+}. Crystalline trypsin was obtained from

Armour Pharmaceuticals, Eastbourne, England. Lubrol W was obtained from I.C.I., London, England. Phosphatidyl ethanolamine from *Escherichia coli*, egg lecithin and lysolecithin (*ex* egg lecithin) were obtained from Light and Co., Colnbrook, England. Chemicals analysis and thin-layer chromatography indicated that these lipids were at least 90% pure. To obtain monophosphoinositide and triphosphoinositide, brain extracts were fractionated according to the method of HENDRICKSON AND BALLOU[10]. The triphosphoinositide was used without further treatment. The monophosphoinositide fraction was purified on alumina and then on DEAE-cellulose according to the method of ROUSER *et al.*[11]. Phosphatidyl serine from Light and Co., Colnbrook, England, was purified on DEAE-cellulose and silicagel by the method of WOODIN AND WIENEKE[7]. Triphosphoinositide, monophosphoinositide and phosphatidyl serine migrated as single spots on formaldehyde-treated paper under the conditions of HORHAMMER *et al.*[12]. The acidic phospholipids were stored as the ammonium salts. Before assessing their action on the *p*-nitrophenyl phosphatase they were converted to the free acids by treatment with chloroform–methanol–conc. HCl (800:400:3, by vol.) and shaking the solution with 0.2 vol. of 1 M HCl and then 3 times with chloroform–methanol–1 M HCl (3:48:47, by vol.) rejecting the upper phase each time. The final, lower phase, solution was dried *in vacuo*, the solids suspended in water and brought into solution by neutralisation with Tris base. Before addition to the membranes the solutions of phospholipids were ultrasonicated for 5 min at 0°.

Membrane preparations

Leucocyte surface membranes were prepared according to the methods of WOODIN AND WIENEKE[2,6,7]. To increase their K^+ sensitivity they were treated with NaI according to the method of ISRAEL AND TITUS[13]. The membranes were stored in 0.32 M sucrose solution at −12°. The concentration of the membranes is expressed in terms of their protein content. Trypsin treatment was carried out by digesting membranes (3.5 mg protein/ml) in 0.2 M Tris–chloride buffer (pH 7.2) with 0.70 mg/ml trypsin for 30 min at 37°. The digested membranes were washed 3 times with 50 vol. of 0.32 M sucrose, 1 mM sodium EDTA, solution by centrifuging at 80000 × *g* for 30 min at 0°. The washed membranes were suspended in 0.32 M sucrose solution and tested for their *p*-nitrophenyl phosphatase activity. Phospholipase A treatment was carried out by digesting membranes (3.5 mg protein/ml) in 0.145 M NaCl, 5 mM $CaCl_2$, 50 mM Tris–chloride buffer (pH 7.5) with Russel's viper venom (55 μg/ml) or phospholipase A from pancrease (14 μg/ml) at room temperature for 20 min. The digested membranes were then washed in the same way as the trypsin-treated membranes and assayed for *p*-nitrophenyl phosphatase activity. Treatment with leucocidin was carried out by incubating membranes (or membranes after trypsin or phospholipase A treatment) (3.0 mg protein/ml) in 40 mM NaCl, 10 mM Tris–chloride buffer (pH 7.2) with 10 μg/ml leucocidin for 20 min at room temperature and then diluting them 16-fold before determining the *p*-nitrophenyl phosphatase activity. High ionic strengths were used to treat the membranes with leucocidin and phospholipase A to simulate the conditions under which the membranes inactivate leucocidin and under which this inactivation is inhibited by phospholipase A.

Solubilisation of the p-nitrophenyl phosphatase

Lubrol solubilisation. Membranes (2.5 mg protein/ml) were treated with 1.2% lubrol in 0.32 M sucrose, 1 mM sodium EDTA, 50 mM Tris–chloride buffer (pH 7.2)

for 80 min at 0° and centrifuged at 102000 × *g* for 30 min. The clear supernatant (lubrol-soluble fraction) was removed and the pellet suspended in 0.32 M sucrose, 1 mM sodium EDTA, 50 mM Tris–chloride buffer (pH 7.2). After 1 h at 0° the suspension was centrifuged at 9000 × *g* for 15 min to give a slightly opalescent supernatant (lubrol-insoluble, sucrose-soluble fraction) and a pellet (lubrol- and sucrose-insoluble fraction). In some cases the preparations were made in the presence of 20 mM K^+. To determine the recovery of the *p*-nitrophenyl phosphatase activity and protein the fractions were diluted so that the solvent composition was 0.18 % lubrol, 0.32 M sucrose, 1 mM sodium EDTA, 5 mM Tris–chloride buffer (pH 7.2). (The concentration of the buffer was too low to affect the pH of the assay mixture at pH 5.) The effect of leucocidin and phospholipids was determined after the unbound lubrol had been removed. The lubrol-soluble fraction was diluted 2-fold with 1 mM sodium EDTA, 50 mM Tris–chloride buffer (pH 7.2) layered on top of a continuous gradient containing 0.32–0.83 M sucrose, 1 mM sodium EDTA solution and centrifuged at 120000 × *g* for 18 h in the S.W 39 rotor of the Spinco ultracentrifuge. Under these conditions the unbound lubrol floated and the *p*-nitrophenyl phosphatase sedimented about 3 cm. To remove unbound lubrol from the lubrol-insoluble, sucrose-soluble fraction and from the lubrol- and sucrose-insoluble fraction they were suspended in 0.16 M sucrose, 1 mM sodium EDTA, 50 mM Tris–chloride buffer (pH 7.2) and layered on top of 0.32 M sucrose, 1 mM sodium EDTA solution and centrifuged at 102000 × *g* for 4 h in the 40.2 rotor of the Spinco ultracentrifuge. The tubes were sliced and the pellets recovered. These procedures reduced the lubrol content of all three membrane fractions to less than 0.01 %.

Deoxycholate solubilisation. To remove sucrose, membranes (42 mg protein) were diluted with an equal volume of 1 mM sodium EDTA, 10 mM Tris–chloride buffer (pH 7.2) and centrifuged at 80000 × *g* for 30 min. The pellet was suspended in 0.5 % sodium deoxycholate, 10 mM Tris–chloride buffer (pH 7.2) at 0° for 1 h and centrifuged at 80000 × *g* for 30 min. The supernatant was removed and the pellet suspended in 0.5 % sodium deoxycholate, 10 mM Tris–chloride buffer (pH 7.2). The protein content and the *p*-nitrophenyl phosphatase activity of the supernatant and the pellet were determined. The supernatant was then fractionated by adding solid $(NH_4)_2SO_4$ and the material precipitated in the range 0–0.9 M $(NH_4)_2SO_4$ and 0.9–2.3 M $(NH_4)_2SO_4$ collected. Samples of these were adjusted to equal $(NH_4)_2SO_4$ concentrations and the protein, *p*-nitrophenyl phosphatase and the esterified fatty acid content determined. The remainder of the two fractions was dissolved in 10 mM Tris–chloride buffer (pH 7.2) and passed down a column of Sephadex G-25 suspended in this buffer. The effect of phospholipids and leucocidin on the *p*-nitrophenyl phosphatase and ATPase activity of the products was determined.

Chemical methods

p-Nitrophenyl phosphatase and ATPase activity was determined as described by WOODIN AND WIENEKE[2] except that Tris–acetate buffers were used. Protein was determined by the method of LOWRY *et al.*[14]. To determine the esterified fatty acids in the membranes, samples were extracted with chloroform–methanol–conc. HCl (800:400:3, by vol.) and the extract treated with 0.2 vol. of 1 M HCl. The lower layer was recovered, dried, extracted with ethanol–ether (2:1, v/v) and the esterified fatty acids in solution determined by the method of MORGAN AND KINGSBURY[15]. To deter-

mine lubrol a suspension of erythrocytes was prepared in 0.9 % NaCl solution, the suspension having $D^{1\,cm}_{430\,\mu} = 6.6$ after total haemolysis. Serially diluted lubrol, or lubrol-treated membrane supernatants were prepared in 0.9 % NaCl solution and 0.1 ml added to 0.1 ml of the erythrocyte suspensions. After 20 min at 37° the mixtures were diluted with 0.3 ml of 0.9 % NaCl solution, centrifuged and the adsorption at 430 mμ determined. Lubrol concentrations of 1–5 μg/ml could be determined in this way.

RESULTS

The effect of membrane concentration on the p-nitrophenyl phosphatase activity

The specific activity of normal, leucocidin-treated or phospholipase A-treated membranes, measured in 0.1 M Tris–acetate buffer (pH 7.2) was slightly dependent on concentration but the curves for the different preparations were parallel. If 1 mM sodium EDTA was present the specific activity–concentration relationship for phospholipase A-treated membranes was highly anomalous, the specific activity increasing 4-fold over a 3.5-fold increase in concentration (Fig. 1). The anomaly was completely suppressed by adding 5 mM Mg^{2+}. The anomaly was not due to the liberation of material into the membrane supernatants, thus membranes incubated in 1.0 mM sodium EDTA, 0.1 M Tris buffer (pH 7.0) centrifuged and suspended in their own supernatant or in fresh buffer, hydrolysed *p*-nitrophenyl phosphate at identical rates.

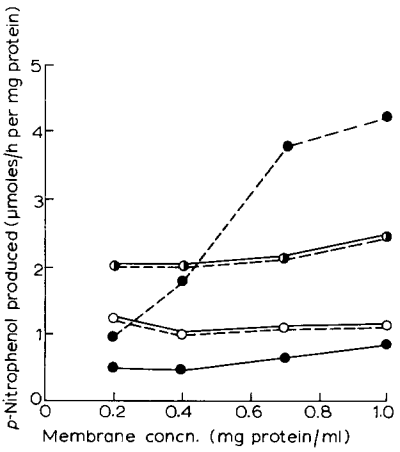

Fig. 1. Dependance of the *p*-nitrophenyl phosphatase specific activity on membrane concentration. The specific activity of the *p*-nitrophenyl phosphatase in normal membranes (○) or leucocidin-treated membranes (◑) or membranes treated with phospholipase A from Russel's viper venom (●) was determined in the presence of 1 mM sodium EDTA (– – – –) or in its absence (————).

The phenomenon was not suppressed by adding lysolecithin to a constant concentration (0.2 mM). As the preparation of the membranes involves washing by dilution to concentrations less than those employed in the assay mixtures the anomaly is not due to inactivation on dilution. It appears that the anomalous specific activity–concentration relationship reflects a co-operative effect between the membrane particles during hydrolysis of the substrate. In the present work comparison of the

properties of phospholipase A-treated membranes with other membrane preparations was usually carried out in the absence of EDTA.

The effect of trypsin and phospholipase A on the membrane p-nitrophenyl phosphatase

Digesting the membranes with trypsin solubilised about 30% of the protein and decreased the *p*-nitrophenyl phosphatase activity by a similar amount. The *p*-nitrophenyl phosphatase activity of the digested membranes was stimulated by leucocidin but the sensitivity to K^+ was lost (Table I). Treating membranes with excess phospholipase A from either Russel's viper venom or pancreas reduced the esterified fatty acid content to 60–70% of the normal value. The *p*-nitrophenyl phosphatase activity of the treated membranes was still sensitive to K^+ but was not stimulated by leucocidin (Table I). Phospholipase A from either source was without effect in the absence of Ca^{2+}.

The effect of phospholipase A treatment was manifested in different ways according to the pH at which the activity was subsequently measured. At pH values in the range 5–6.2 the activity of the treated membranes was greater than the controls while at pH 6.8–7.6 the activity was unchanged (six experiments) or reduced 10–20% (eight experiments). Leucocidin failed to stimulate the *p*-nitrophenyl phosphatase activity (at pH is 5, 6.2 or 7.2) of membranes treated with excess phospholipase A. Fig. 2 shows the effect of different concentrations of phospholipase A from Russel's viper venom on the *p*-nitrophenyl phosphatase activity at three different pH values.

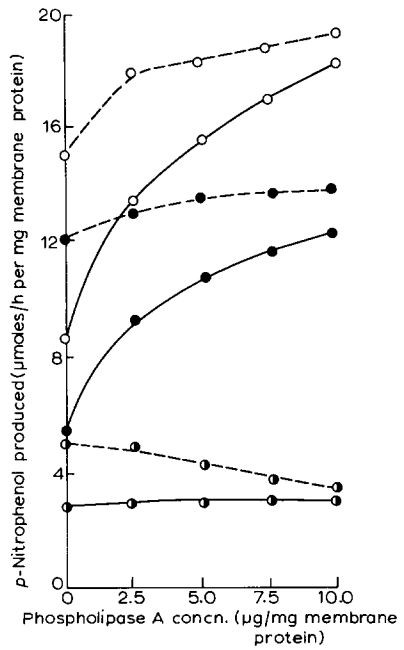

Fig. 2. Effect of phospholipase A on the *p*-nitrophenyl phosphatase activity measured at different pH values. Membranes were incubated alone or with different amounts of phospholipase A from Russel's viper venom. The membranes were washed and then incubated alone or with leucocidin and after dilution the *p*-nitrophenyl phosphatase activity determined in the presence of 5 mM Mg^{2+}. ———, normal membranes; ·····, leucocidin-treated membranes. The *p*-nitrophenyl phosphatase activity was measured at pH 7.2 (◑) or at pH 6.2 (●) or at pH 5.0 (○).

TABLE I

EFFECT OF TRYPSIN AND PHOSPHOLIPASE A ON THE MEMBRANE *p*-NITROPHENYL PHOSPHATASE AND ITS STIMULATION BY POTASSIUM AND LEUCOCIDIN

Membranes were treated alone, with trypsin (70 μg/mg membranes protein) or with phospholipase A from Russel's viper venom (10 μg/mg membranes protein) and washed. Samples were then treated alone or with leucocidin and after dilution the *p*-nitrophenyl phosphatase activity determined in the presence of 5 mM Mg^{2+}. The specific activity values are the means of duplicates for a representative experiment. The stimulation by leucocidin is the mean and range for six separate experiments.

pH	K^+ concn. (*mM*)	*Normal membranes*			*Trypsin-treated membranes*			*Phospholipase A-treated membranes*		
		p-Nitrophenol produced (μmoles/h per mg protein)		*Stimulation by leucocidin (%)*	*p-Nitrophenol produced (μmoles/h per mg protein)*		*Stimulation by leucocidin (%)*	*p-Nitrophenol produced (μmoles/h per mg protein)*		*Stimulation by leucocidin (%)*
		Control	*Leucocidin-treated*		*Control*	*Leucocidin-treated*		*Control*	*Leucocidin-treated*	
5.0	0	2.5	4.4	80 (70–110)	1.7	4.2	150 (120–160)	4.1	4.3	6 (0–14)
	10	2.5	4.5		1.7	4.5		4.3	4.4	
6.2	0	1.5	3.0	65 (35–80)	1.1	3.4	155 (90–200)	2.6	2.6	5 (0–9)
	10	2.0	3.8		1.1	3.4		2.9	3.0	
7.2	0	0.9	1.3	52 (35–65)	0.8	1.1	65 (40–75)	0.8	0.9	4.5 (0–9)
	10	1.4	1.9		0.8	1.1		1.5	1.1	

Similar results were obtained with phospholipase A from pancreas. The action of phospholipase A was mimicked by lubrol. Fig. 2 shows that this detergent stimulated the activity at pH 5 and 6.2 but not at pH 7.2.

Reversal of the effect of leucocidin

When normal and leucocidin-treated membranes were digested with trypsin the percentage stimulation of the *p*-nitrophenyl phosphatase by leucocidin was not changed. Fig. 4 shows that phospholipase A reduced the *p*-nitrophenyl phosphatase activity of leucocidin-treated membranes to values similar to those of normal membranes and that if EDTA was present in the assay medium the activity of normal and leucocidin-treated membranes was increased by phospholipase A to similar levels and in both cases the specific activity was strongly dependent on the membrane concentration.

The action of phospholipids on the p-nitrophenyl phosphatase activity

Acidic phospholipids inhibit the *p*-nitrophenyl phosphatase measured at pH 5. Fig. 5 shows the effect of two neutral phospholipids and two acidic phospholipids and in a further experiment it was found that phosphatidyl serine behaved similarly to monophosphoinositide. Acidic phospholipids reverse the stimulation induced by phospholipase A treatment (Fig. 5). At the concentrations used in the experiment recorded in Fig. 5, the phospholipids did not inhibit the activity measured at pH 7.2. With higher concentrations, only triphosphoinositide inhibits the activity at pH 7.2

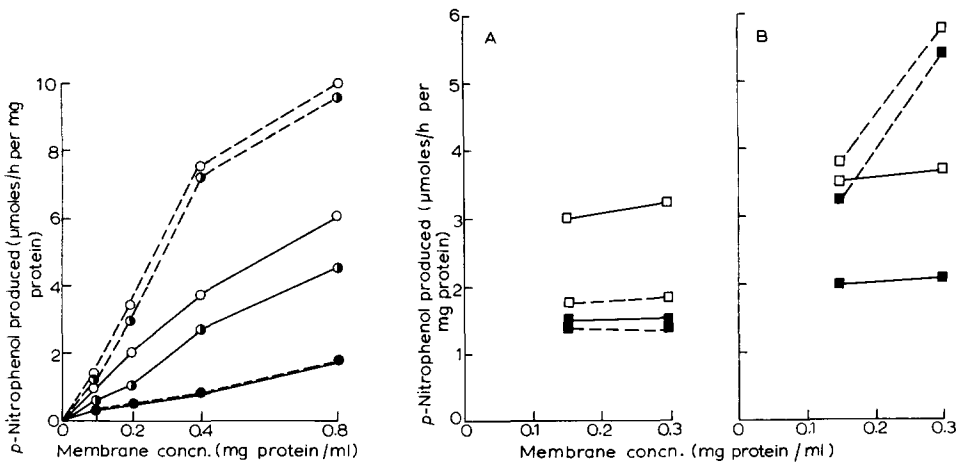

Fig. 3. The effect of lubrol on the *p*-nitrophenyl phosphatase activity measured at different pH values. Membranes (3.6 mg protein/ml) were treated with 1.2% lubrol in 0.05 M Tris–chloride buffer (pH 7.2) for 15 min at 0°. They were then diluted with 0.32 M sucrose solution to give the membrane concentrations indicated and the *p*-nitrophenyl phosphatase activity determined in the presence of 5 mM Mg^{2+} at pH 7.2 (●) or at pH 6.2 (◑) or at pH 5.0 (○). ———, normal membranes; – – – –, lubrol-treated membranes.

Fig. 4. The reversal by phospholipase A of the stimulation by leucocidin of the *p*-nitrophenyl phosphatase activity. Normal (■) or leucocidin-treated membranes (□) were treated alone or with excess phospholipase A from Russel's viper venom, washed and the *p*-nitrophenyl phosphatase activity determined at pH 7.2 in the presence of 5 mM Mg^{2+} (Graph A) or in the presence of 1 mM sodium EDTA (Graph B). ———, control membranes; — — —, phospholipase A-treated membranes.

(Table II). Acidic phospholipids reduced the *p*-nitrophenyl phosphatase activity of leucocidin-treated membranes measured at pH 5 and high concentrations of triphosphoinositide inhibited the activity measured at pH 7.2. However, in contrast to the effect on phospholipase A-treated membranes, acidic phospholipids did not reduce the activity of leucocidin-treated membranes to the levels found in normal membranes. The stimulation produced by leucocidin was not reversed (Table II).

The most effective inhibitor of the *p*-nitrophenyl phosphatase activity is triphosphoinositide. The inhibition is not due to the lipid acting as a substrate for the enzyme. When 0.2 mM triphosphoinositide was incubated with normal, leucocidin-treated or phospholipase A-treated membranes (4 mg protein/ml) at pH 5 or pH 7.0 less than 2 % was hydrolysed. The action of triphosphoinositide is due to irreversible adsorbtion. When normal and phospholipase A-treated membranes were treated with triphosphoinositide (0.15 μmole/mg membrane protein) and then washed, 30 % of the triphosphoinositide was adsorbed to the membrane in each case and the percentage inhibition of the *p*-nitrophenyl phosphatase activity at pH 5 was the same as with unwashed membranes.

Adsorbed triphosphoinositide did not restore the sensitivity of phospholipase A-treated membranes to leucocidin nor did it prevent the stimulation of normal mem-

TABLE II

THE ACTION OF PHOSPHOLIPIDS ON THE *p*-NITROPHENYL PHOSPHATASE ACTIVITY OF NORMAL AND LEUCOCIDIN TREATED-MEMBRANES

Normal and leucocidin-treated membranes were maintained alone or with the Tris salts of the phospholipids (0.6 mM; 1 μmole/mg membrane protein) in 0.01 M Tris–chloride buffer (pH 7.2) for 5 min at room temperature and then diluted 2-fold to determine the *p*-nitrophenyl phosphatase activity in the presence of 5 mM Mg^{2+} at pH 7.2 and 5.0.

Phospholipid present	*p-Nitrophenol produced at pH 5 (μmoles/h per mg protein)*		*p-Nitrophenol produced at pH 7 (μmoles/h per mg protein)*	
	Normal membranes	*Leucocidin-treated membranes*	*Normal membranes*	*Leucocidin-treated membranes*
None	7.0	11.0	0.85	1.25
Phosphatidyl choline	6.4	11.0	0.80	1.20
Phosphatidyl serine	3.7	9.1	0.75	1.20
Monophosphoinositide	2.3	7.0	0.75	1.18
Triphosphoinositide	1.4	4.0	0.2	0.3

TABLE III

SOLUBILISATION OF THE LEUCOCYTE MEMBRANE *p*-NITROPHENYL PHOSPHATASE WITH LUBROL

Leucocyte membranes were extracted with lubrol and fractionated as described in the text.

Fraction	*p-Nitrophenyl phosphatase activity at pH 5 (% amount recovered)*	*Specific activity at pH 5, p-nitrophenol produced (μmoles/h per mg protein)*
Lubrol-soluble	51	3.3
Lubrol-insoluble, sucrose-soluble	33	1.5
Lubrol- and sucrose-insoluble	16	0.6

branes by leucocidin. However, if leucocidin was treated with triphosphoinositide in solution it no longer stimulated the *p*-nitrophenyl phosphatase. Fig. 6 shows the concentration of triphosphoinositide required and it is similar to that needed to convert leucocidin to a biologically inactive form[7]. Thus the conversion of leucocidin to a biologically inactive form correlates with the loss of ability to stimulate the *p*-nitrophenyl phosphatase activity although the interaction between leucocidin and triphosphoinositide in the normal membrane only proceeds when the triphosphoinositide is in a unique state of combination.

Solubilisation of the p-nitrophenyl phosphatase

To confirm that the action of leucocidin on the *p*-nitrophenyl phosphatase is indirect and to provide a comparison with the *p*-nitrophenyl phosphatase of other tissues some preliminary experiments were made on the solubility of the leucocyte enzyme. Table III gives the recovery and specific activity of the *p*-nitrophenyl phosphatase in the three fractions with which the enzyme is associated after lubrol treatment. The specific activity was independent of concentration. None of the fractions were stimulated by K^+ but the lubrol-insoluble, sucrose-soluble fraction and the lubrol and sucrose-insoluble fractions prepared in the presence of K^+ had 80 % more *p*-nitrophenyl phosphatase activity than fractions prepared in the absence of K^+ and the stimulation was abolished $5 \cdot 10^{-4}$ M ouabain. The sensitivity to K^+ and ouabain of

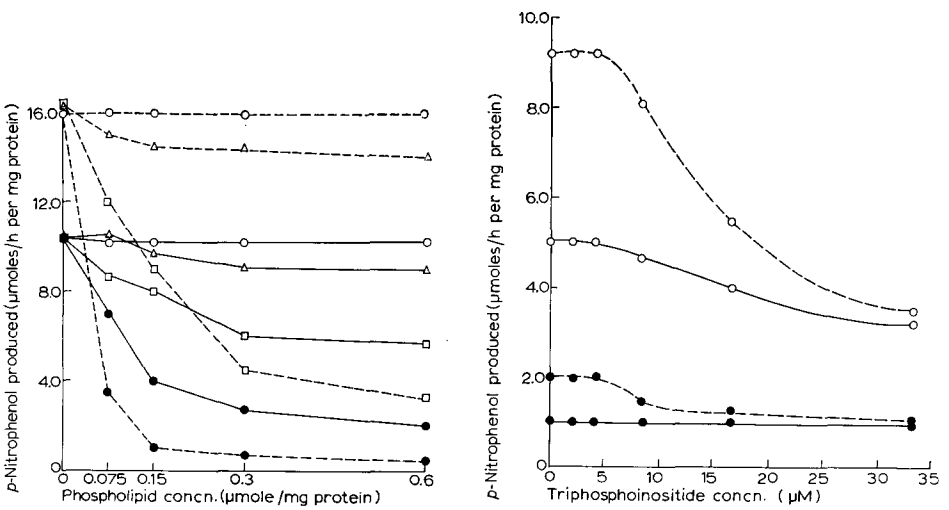

Fig. 5. Effect of phospholipids on the *p*-nitrophenyl phosphatase activity of normal and phospholipase A-treated membranes. Normal membranes or membranes treated with excess phospholipase A from Russel's viper venom were maintained alone or with various phospholipids for 5 min at room temperature and then the *p*-nitrophenyl phosphatase activity determined at pH 5. ———, normal membranes; — — —, phospholipase A-treated membranes. △, phosphatidyl choline; ○, phosphatidyl ethanolamine; □, monophosphoinositide; ●, triphosphoinositide.

Fig. 6. Inhibition by triphosphoinositide of the ability of leucocidin to stimulate membrane *p*-nitrophenyl phosphatase activity. Samples of triphosphoinositide alone or with leucocidin (3 μg/ml) were maintained in 0.01 M Tris–chloride buffer (pH 7.2) for 10 min at room temperature and then added to membranes (0.6 mg protein/ml). After a further 10 min at room temperature the *p*-nitrophenyl phosphatase activity was determined at pH 7.2 (●) and at pH 5 (○). ———, triphosphoinositide only; — — —, leucocidin and triphosphoinositide.

these two fractions was retained after unbound lubrol W was removed. The lubrol-soluble fraction was found to be insensitive to K^+ and ouabain under all the conditions studied. Triphosphoinositide (0.25–5 mM) added to all three fractions (freed from unbound lubrol) produced 50–80 % inhibition of the activity measured at pH 5. Leucocidin had no action on the activity of all three fractions alone or after treatment with triphosphoinositide.

Treatment with deoxycholate solubilised 95 % of the *p*-nitrophenyl phosphatase and 75 % of the protein. The bulk of the lipid and about 50 % of the *p*-nitrophenyl phosphatase was precipitated by 0.9 M $(NH_4)_2SO_4$ and increasing the $(NH_4)_2SO_4$ concentration to 2.3 M precipitated the remainder of the *p*-nitrophenyl phosphatase (Table IV). To test the effect of phospholipids and leucocidin the latter fraction was freed from unbound deoxycholate and $(NH_4)_2SO_4$ on a column of Sephadex G-25 with 58 % recovery of the protein. The *p*-nitrophenyl phosphatase activity of the product was insensitive to leucocidin. Table V shows that it was slightly stimulated

TABLE IV

COMPOSITION OF MATERIAL DERIVED FROM DEOXYCHOLATE-SOLUBILISED MEMBRANES

Samples of leucocyte membranes were solubilised in 0.5 % sodium deoxycholate and fractionated with $(NH_4)_2SO_4$. The protein and *p*-nitrophenyl phosphatase activity of the extract and the two $(NH_4)_2SO_4$ fractions was determined in the presence of a constant amount of deoxycholate and $(NH_4)_2SO_4$.

Fraction	*p-Nitrophenyl phosphatase at pH 5 recovered (% amount solubilised)*	*Protein recovered (% amount solubilised)*	*Esterified fatty acid content (mequiv/g protein)*
0–0.9 M $(NH_4)_2SO_4$	52	73	1.0
0.9–2.3 M $(NH_4)_2SO_4$	48	17	<0.05

TABLE V

ACTION OF PHOSPHATIDYL SERINE ON THE PHOSPHATASE ACTIVITY OF LEUCOCYTE MEMBRANES SOLUBILISED WITH DEOXYCHOLATE

Samples of leucocyte membranes were extracted with 0.5 % deoxycholate and fractionated with $(NH_4)_2SO_4$. A sample of the 0.9–2.3 M $(NH_4)_2SO_4$ fraction was freed from $(NH_4)_2SO_4$ and deoxycholate by treatment with Sephadex G-25 and the *p*-nitrophenyl phosphatase and ATPase determined in the presence and absence of 0.5 mM phosphatidyl serine.

Fraction	*p-Nitrophenol produced (μmoles/h per mg protein)*				*ATP hydrolysed (μmoles/h per mg protein)*
	pH 5	*pH 7*	*pH 7 5 mM Mg^{2+}*	*pH 7 5 mM Mg^{2+} 10 mM K^+*	*pH 7 5 mM Mg^{2+} 100 mM Na^+*
0.9–2.3 M $(NH_4)_2SO_4$ after Sephadex	15.0	1.6	1.8	2.4	5.2
0.9–2.3 M $(NH_4)_2SO_4$ after Sephadex assayed with 0.5 mM phosphatidyl serine	10.0	1.6	1.7	2.3	5.8

by potassium and that phosphatidyl serine inhibited the activity at pH 5. The ATPase activity was insensitive to 100 mM NaCl or 10 mM KCl alone or together and treatment with phosphatidyl serine did not induce sensitivity to these salts. These properties are different from those found with solubilised ATPase from brain[16].

DISCUSSION

The present results show that phospholipids affect the leucocyte *p*-nitrophenyl phosphatase activity, stress the difference of the phosphatases of the leucocyte membrane from those in other tissues and indicate that the action of leucocidin on the *p*-nitrophenyl phosphatase is mediated through an interaction with lipids.

We have previously shown[2] that, although some properties of the leucocyte *p*-nitrophenyl phosphatase resemble those of the *p*-nitrophenyl phosphatase in other tissues, the multiple pH maxima, the magnitude and pH dependence of the stimulation by K^+ and the high ouabain concentration required to prevent this stimulation indicate that the *p*-nitrophenyl phosphatases are not identical. In support of this we show here that in the leucocyte membrane the potassium stimulation is mediated through a protein component and that the *p*-nitrophenyl phosphatase activity is suppressed in the presence of phospholipids. The suppression is most marked when the activity is measured at pH 5. In contrast to this TANAKA AND MITSUMATA[5] have shown that brain *p*-nitrophenyl phosphatase is stimulated in the presence of phospholipids and that only the K^+-sensitive component, active at neutral pH, is affected.

Leucocyte membrane *p*-nitrophenyl phosphatase is stimulated by phospholipase A so esterified fatty acids are of importance. Stimulation of the enzyme also follows treatment with lubrol indicating that dissociation and not hydrolysis of esterified fatty acids from their neighbours in the membrane is all that is required. At pH 5 acidic phospholipids reduce the *p*-nitrophenyl phosphatase activity and at pH 7 only the highly charged triphosphoinositide is effective. It is possible that the entry of phospholipids into the membrane requires the dissociation of an electrostatic bond and that the p*K* of the negatively charged component is about pH 5.

In solution the F component of leucocidin interacts at low ionic strength with the esterified fatty acids of phospholipids and following its modification by phospholipids, the F component of leucocidin can interact with the S component to produce polymers[7]. This polymerisation is reversed by increasing the salt concentration to physiological levels. At physiological salt concentrations leucocidin is converted to an inactive form by leucocyte membranes but if the membranes are treated with phospholipase A this does not occur[6]. In the present paper it is shown that the stimulation of the *p*-nitrophenyl phosphatase by leucocidin is prevented by treating the membranes with phospholipase A and phospholipase A, but not trypsin, reverses the stimulation induced by leucocidin. It is probable that the stimulation of the *p*-nitrophenyl phosphatase by leucocidin leads to a concomitant conversion of leucocidin to an inactive form and that both phenomena result from an interaction with esterified fatty acids. The only phospholipids to mimic, in solution, the inactivation of leucocidin by membranes is triphosphoinositide[7] and it is of interest that triphosphoinositide is the only phospholipid to alter the *p*-nitrophenyl phosphatase activity at pH 7. Addition of triphosphoinositide to phospholipase A-treated membranes does not produce sensitivity to leucocidin and so, if the action of leucocidin with the membrane involves

interaction with triphosphoinositide, then in the normal membrane the triphosphoinositide must be in a unique state of combination or conformation. WOODIN AND WIENEKE[17], using an isotope dilution technique showed the presence of triphosphoinositide in the leucocyte membrane. There is also evidence that triphosphoinositide can adopt different conformational states or micellar forms in solution[7] and in the membranes[18].

The evidence that the *p*-nitrophenyl phosphatase of the leucocyte membrane may be involved in electrolyte control has been presented elsewhere[2]. The *p*-nitrophenyl phosphatase may have other physiological functions. The immediate response of the cell to leucocidin is an increased permeability to cations following activation of an ion pathway in the K^+ pump of the cell. The present results stress the importance of phospholipids in the interaction of leucocidin with the membrane and suggest that the ion pathway is activated by dissociating esterified fatty acids from the surface of the *p*-nitrophenyl phosphatase in the membrane. The dose response curve of leucocidin action is characterised by a plateau of no response at low leucocidin concentrations[19,20]. The conversion of leucocidin to an inactive form prevents multiple collisions from mimicking the effect of higher leucocidin concentrations. If the cell can reverse the separation of esterified fatty acids from the enzyme surface the phenomenon may be of physiological significance. Di-isopropylfluorophosphate and other organophosphorus compounds act as non-ionic detergents to enhance the cytotoxic action of leucocidin by preventing the cell from reversing the effect of suboptimal leucocidin concentrations[21]. As organophosphorus compounds inhibit chemotaxis and phagocytosis under the same conditions as they enhance leucocidin action the reversible separation of esterified fatty acids from the *p*-nitrophenyl phosphatase may be involved in these characteristic physiological phenomena.

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