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Receptor-mediated activation of a phospholipase A_2 in rabbit neutrophil plasma membrane

(chemotactic factor/calcium)

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ABSTRACT **Using the exogenous substrate [1-^{14}C]oleate-labeled autoclaved *Escherichia coli*, we have demonstrated that the chemotactic factors fMet-Leu-Phe, complement component C5a, and leukotriene B_4 [(5*S*,12*R*)-dihydroxy-6-*cis*,8-*trans*,11-*trans*,14-*cis*-icosatetraenoic acid] stimulate a phospholipase A_2 of isolated plasma membranes of rabbit peritoneal neutrophils. Each of the chemotactic factors shows a biphasic concentration dependence with the optimal concentrations occurring at 1, 10, and 0.1 nM, respectively. The specific antagonists of fMet-Leu-Phe binding, carbobenzoxy-Phe-Met and *t*-butoxycarbonyl-Phe-Leu-Phe, effectively block the stimulation by fMet-Leu-Phe, indicating that the activation is receptor mediated. Δ^6-*trans*-leukotriene [(5*S*,12*R*)-dihydroxy-all-*trans*-6,8,10,14-icosatetraenoic acid], a biologically inactive stereoisomer of leukotriene B_4, does not stimulate phospholipase activity, suggesting that the enhancement by leukotriene B_4 is also receptor mediated. The unstimulated and activated phospholipase exhibit a broad range of maximal activity between pH 7.0 and pH 8.5, both with an optimal pH of 8.5. The activation of the phospholipase by fMet-Leu-Phe is completely calcium dependent; no increase in activity is demonstrable if fMet-Leu-Phe is added in the absence of exogenous calcium or in the presence of EGTA. In contrast, the unstimulated plasma membrane activity of the phospholipase, as well as the activity arising after stimulation, is relatively insensitive to the concentration of calcium, being inhibited by <50% in the presence of 10 mM EGTA. The phospholipase hydrolyzes 1-[1-^{14}C]palmitoyl-2-acyl-*sn*-glycerophosphoethanolamine to form only radioactive lysophosphatidylethanolamine as the product, indicating that the enzyme has an A_2 specificity.**

Many factors, including peptides derived from complement, synthetic formylated peptides, and leukotrienes, are able to stimulate biologic functions in the rabbit polymorphonuclear leukocyte, such as chemotaxis, granule enzyme release, and aggregation (1–3). The various responses to these factors involve their interactions with specific receptors on the cell surface (4–6). Although the biochemical events translating receptor–ligand interaction into a given cell function remain poorly defined, changes in phospholipid metabolism, evidenced, in part, by the release and subsequent metabolism of arachidonic acid (7), are thought to be an important biochemical event in chemotactic factor activation of neutrophils. Most investigators have suggested that the release of arachidonic acid from the stimulated neutrophil is due to the activation of a phospholipase A_2. The evidence for this activation is both indirect and incomplete: phospholipase A_2 is present in neutrophils in both the granule and plasma membranes (8, 9); the phospholipase inhibitors, hydrocortisone and mepacrine, inhibit neutrophil chemotaxis and release arachidonic acid (10); stimulated neutrophils release labeled fatty acid from the 2-acyl, but not the 1-acyl, position of their phospholipids (11); and a breakdown of phosphatidylcholine and, to an even greater extent, phosphatidylethanolamine occurs shortly after chemotactic factor stimulation (12). However, there is no evidence for increased production of lysophosphatides in response to neutrophil stimulation (11), possibly due to the rapid reacylation of lysophospholipid, previously shown to occur in the neutrophil (13).

In the present study, we used [1-^{14}C]oleate-labeled autoclaved *Escherichia coli* as substrate to show that chemotactic factors activate a rabbit neutrophil plasma membrane phospholipase A_2. The use of exogenous substrate facilitates the characterization of the phospholipase with regard to activity and specificity, as the released fatty acid cannot be reincorporated into phospholipid in the absence of coenzyme A. The use of isolated plasma membranes not only provides direct evidence that receptor–ligand interaction activates a plasma membrane phospholipase A_2 but localizes an enzyme active in the metabolism of phospholipids and generation of arachidonic acid to the site of reception of chemotactic factors.

MATERIALS AND METHODS

Rabbit peritoneal neutrophils were obtained 12–14 hr after intraperitoneal injection of 0.1% glycogen in physiologic saline as described (14). Erythrocytes were routinely lysed with 150 mM NH_4Cl and suspended in Hanks' buffer. Subcellular fractions were prepared by a modification of the method of Woodin and Wieneke (15). Briefly, the cells were washed three times in 11.6% sucrose/1 mM EGTA/10 mM Hepes and homogenized in a glass hand-held homogenizer tube with a Teflon pestle at 1,200 rpm for 3 min. The homogenate was centrifuged at 700 × *g* for 10 min at 4°C and the resulting supernatant was layered on a discontinuous sucrose gradient made up in 1 mM EGTA/10 mM Hepes, pH 7.2, consisting of 10.8 ml of 30% sucrose, 8.4 ml of 40% sucrose, and 10.8 ml of 50% sucrose. This mixture was centrifuged at 120,000 × *g* for 2 hr at 4°C. The fraction used in these studies was isolated from the 30–40% sucrose interface and yields 0.65 mg of membrane protein per 10^9 cells. This fraction contains the following neutrophil plasma membrane markers: the Na,K-ATPase (16), the adenylate cyclase (17), the fMet-Leu-Phe receptor (18), and the site of leukocidin binding (19). By measuring protein markers specific for the plasma membrane, the latter was estimated to be enriched 25- to 45-fold over the homogenate (20). It is essentially free of granule contents, containing 0.1% of the cytoplasmic enzyme, lactic dehydrogenase.; <0.4% of the azurophil granule marker, β-glucuronidase; <1% of the specific granule membrane marker alkaline phosphatase; and <0.4% of lysozyme, an enzyme found in both specific and azurophil granules. By electron microscopy, the fraction was shown to consist of vesicles of heterogeneous size with some intravesicular filaments. The fraction was devoid of recognizable organelles except a few glycogen granules (20).

The [1-^{14}C]oleate-labeled autoclaved *E. coli* were prepared as described (9). The position of the label was deter-

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mined by treatment of the substrate with purified snake venom phospholipase A_2 (*Crotalus adamanteus*), the gift of D. Voelker (National Jewish Hospital, Denver, CO), and subsequent separation of the products by TLC, as described below. Greater than 95% of the label was in the 2-acyl position of the phospholipids. The preparation and use of 1-[1-^{14}C]palmitoyl-2-acyl-*sn*-glycerophosphoethanolamine in determining the positional specificity of the phospholipase was as described (21).

Because of its convenience, we measured the release of radioactive oleic acid by the rapid filtration assay of Vadas and Hay (22) except when high concentrations of fMet-Leu-Phe were used.* When pure phosphatidylethanolamine was used as substrate, 400 nmol of 1-[1-^{14}C]palmitoyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (10,000 cpm) was added as a sonicated suspension in water. The concentration of membrane protein was determined by the method of Lowry *et al.* (23). Reactions were stopped by the addition of 2 vol of CH_3OH and 1 vol of $CHCl_3$ and brought to $CHCl_3/CH_3OH$ (2:1) with additional $CHCl_3$ before extraction. $CHCl_3$ extracts were separated by TLC in $CHCl_3/EtOH/Et_3N/H_2O$, 30:35:34:8 (vol/vol) (24) on precoated silica gel plates (LHP-K, Whatman) that had been activated for 20 min at 120°C. The hydrolyzed [1-^{14}C]oleic acid was scraped and its radioactivity was determined in a Beckman LS-333 scintillation counting system.

In experiments testing the pH range, the enzyme was assayed as described above, with the substitution of 100 mM Tris·HCl buffer as follows: pH 4.4–6.0, 100 mM sodium acetate; pH 6.0–7.0, 100 mM Tris maleate; pH 7.0–9.0, 100 mM Tris·HCl; pH 9.0–10.0, 100 mM glycine.

[1-^{14}C]Oleic acid (50 mCi/mmol; 1 Ci = 37 GBq) was purchased from Amersham. Purified complement component C5a was a gift from P. Henson (National Jewish Hospital, Denver, CO). Leukotriene B_4 was a gift from P. Rokach (Merk Frost, Quebec City, PQ, Canada). The Δ^6-*trans*-leukotriene was a gift from P. Borgeat (Le Centre Hospitalier de l'Universite Laval, Sainte-Foy, PQ, Canada). fMet-Leu-Phe was purchased from Sigma. All other chemicals were of reagent grade.

RESULTS

Chemotactic Factor Stimulation of Rabbit Neutrophil Plasma Membrane Phospholipase. Unstimulated rabbit peritoneal neutrophil plasma membrane hydrolyzes [1-^{14}C]oleate-labeled autoclaved *E. coli* at the rate of 10%/20 min, indicating that the isolated plasma membrane from rabbit neutrophils, like that of human neutrophils (9), has phospholipase activity. Hydrolysis of the *E. coli* substrate is linear up to 100 μg of protein and 30 min of incubation or 40% hydrolysis, after which the curves plateau (data not shown). When the reaction mixture is incubated in the presence of increasing concentrations of the chemotactic peptide fMet-Leu-Phe, the phospholipase activity is enhanced up to 3-fold at the optimal concentration (1 nM) of fMet-Leu-Phe (Fig. 1). Higher concentrations of fMet-Leu-Phe induce progressively decreasing levels of phospholipase activity. The shape of the dose–response curve and the concentration of fMet-Leu-Phe (1 nM) that maximally enhances the phospholipase activity are similar to those described for fMet-Leu-Phe-stimulated chemotaxis of the rabbit polymorphonuclear leukocyte (22), suggesting a close correlation between phospholipase activation and biologic activity.

*fMet-Leu-Phe (0.1–1 μM) causes some of the radioactivity in the *E. coli* to nonspecifically pass through the filter, perhaps due to the hydrophobicity of the peptide. The activity of the phospholipase obtained with these higher concentrations of fMet-Leu-Phe was, therefore, assayed using TLC (19) to separate hydrolysis products rather than the rapid filtration method.

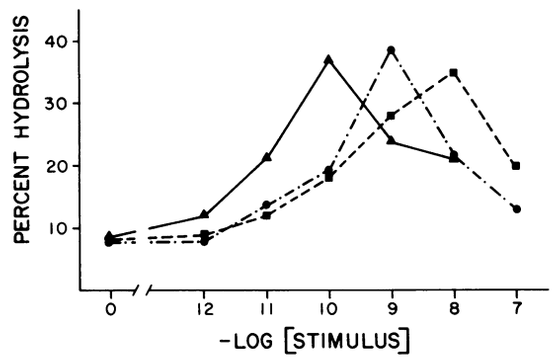

FIG. 1. Concentration dependence of the stimulation of rabbit neutrophil plasma membrane phospholipase. [1-^{14}C]Oleate-labeled *E. coli* (2.5×10^8) was incubated with 50 μg of plasma membrane protein in 100 mM Tris·HCl, pH 7.5/10 mM $CaCl_2$ at 37°C in a shaking waterbath for 20 min. Various concentrations of the chemotactic factors fMet-Leu-Phe (●), complement component C5a (■), and leukotriene B_4 (▲) were added prior to the addition of the membrane protein. Results are presented as percent hydrolysis per 50 μg of plasma membrane protein per 20 min. Each point represents the mean of at least three experiments. All values are corrected for nonenzymatic hydrolysis (<3%).

The complement-derived component C5a and the arachidonic acid metabolite leukotriene B_4 (5,12-dihydroxyicosatetraenoic acid) also increase the phospholipase activity approximately 3-fold, with optimal activity at 10 and 0.1 nM, respectively (Fig. 1). Like fMet-Leu-Phe, higher concentrations of both of these chemoattractants decrease phospholipase activation.

These data show directly that various chemotactic factors can stimulate a plasma membrane-associated phospholipase. All of the chemoattractants tested stimulate this activity to the same extent (3-fold) at optimal concentrations that closely correlate with the concentration at which they possess maximal chemotactic activity.

To determine whether the stimulation of phospholipase activity is mediated through chemotactic receptors, the effect of chemotactic antagonists and analogs was examined. Specific antagonists of fMet-Leu-Phe binding, carbobenzoxy-Phe-Met and *t*-butoxycarbonyl-Phe-Leu-Phe, when tested alone are without effect on the phospholipase activity. However, simultaneous addition of either of the antagonists with fMet-Leu-Phe abolishes the expected enhancement by the latter, indicating that the phospholipase was activated through interaction with the fMet-Leu-Phe receptor (Table 1). The chemoattractant leukotriene B_4 also enhances phospholipase activity 3-fold, whereas the biologically inactive all-*trans* stereoisomer, Δ^6-*trans*-leukotriene, has no effect over a wide range of concentrations (Table 1), suggesting that this chemoattractant is also stimulating phospholipase by receptor–ligand interaction.

Calcium Requirement for Activity and Activation. The calcium requirement for both the activity and the activation of the phospholipase is shown in Fig. 2. Unlike other phospholipase activities in both human and rabbit neutrophils (8, 9), the phospholipase activity in rabbit neutrophil plasma membranes is relatively insensitive to higher concentrations of both $CaCl_2$ and EGTA; 10 mM EGTA only decreases the basal or unstimulated phospholipase activity of the plasma membrane to 50% of that obtained in the presence of 20 mM $CaCl_2$. Furthermore, after the phospholipase activity is stimulated by 1 nM fMet-Leu-Phe in the presence of 5 mM $CaCl_2$, the addition of 10 mM EGTA has no effect (data not shown), indicating that the calcium requirements of the phospholipase activity before and after activation are similar.

In sharp contrast, the absence of exogenous calcium or the

Table 1. Effect of chemoattractant antagonists and analogs on phospholipase activity

Stimulant	% hydrolysis
None	9.2 ± 0.5
fMet-Leu-Phe	32.6 ± 5.1
Cbz-Phe-Met	9.0 ± 0.3
Boc-Phe-Leu-Phe	8.8 ± 0.6
fMet-Leu-Phe/Cbz-Phe-Met	12.0 ± 0.4
fMet-Leu-Phe/Boc-Phe-Leu-Phe	11.4 ± 0.6
Leukotriene B_4	37.4 ± 4.8
Δ^6-*trans*-leukotriene	9.8 ± 0.3

[1-^{14}C]Oleate-labeled autoclaved *E. coli* (2.5×10^8) was incubated with 50 μg of plasma membrane protein, 100 mM Tris·HCl, pH 7.5/10 mM $CaCl_2$ at 37°C in a shaking waterbath for 20 min. fMet-Leu-Phe (1 nM), benzyloxycarbonyl (Cbz)-Phe-Met (0.1 mM), *t*-butoxycarbonyl (Boc)-Phe-Leu-Phe (1 μM), leukotriene B_4 (0.1 nM), Δ^6-*trans*-leukotriene (1 μM), or combinations of fMet-Leu-Phe and antagonists were added prior to the addition of plasma membrane protein. Results (mean ± SEM for three experiments) are presented as percent hydrolysis per 50 μg of plasma membrane protein per 20 min.

presence of EGTA completely abolishes the activation of phospholipase activity by 1 nM fMet-Leu-Phe (Fig. 2). Maximal activation is observed at 10 mM $CaCl_2$. Increasing the Ca^{2+} concentration to 20 mM has no further effect. These data show directly that the presence of calcium is essential for phospholipase activation and that the activation process with respect to calcium is different from the mechanism that sustains unstimulated or enhanced activity.

Other Characteristics of Plasma Membrane Phospholipase Activity. The effect of pH on the phospholipase activity of the unstimulated and activated plasma membrane is shown in Fig. 3. Both unstimulated and fMet-Leu-Phe-stimulated phospholipase activities show similar curves, with maximal activity apparent over a broad pH range (7–8.5). The optimal pH for both is 8.5, with activity rapidly decreasing at pH values higher than 8.5 or lower than 7.0. No discernable phospholipase activity is detectable below pH 6.0.

To confirm the positional specificity of the phospholipase, plasma membrane with and without 1 nM fMet-Leu-Phe was incubated with 1-[1-^{14}C]palmitoyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (Table 2). In each case, >94% of the product formed comigrates with lysophosphatidylethanolamine on TLC.

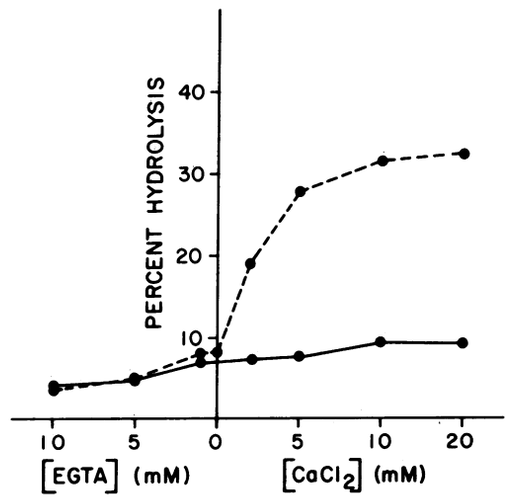

FIG. 2. Calcium requirement for unstimulated phospholipase activity and fMet-Leu-Phe stimulation of phospholipase. [1-^{14}C]Oleate-labeled *E. coli* (2.5×10^8) was incubated with 50 μg of plasma membrane protein in 100 mM Tris·HCl, pH 7.5, in the presence (●-----●) or absence (●——●) of 1 nM fMet-Leu-Phe at the indicated concentrations of EGTA or $CaCl_2$. Results are presented as percent hydrolysis per 50 μg of plasma membrane protein per 20 min. Each point represents the mean of four experiments.

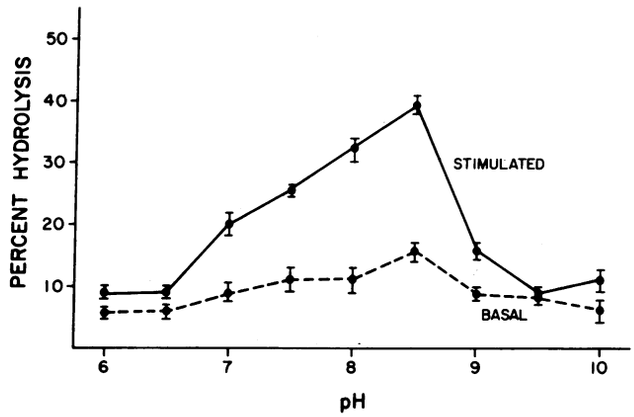

FIG. 3. Effect of pH on activity of basal and stimulated plasma membrane phospholipase activity. [1-^{14}C]Oleate-labeled *E. coli* (2.5×10^8) was incubated with 50 μg of plasma membrane protein in 10 mM $CaCl_2$ in the absence (●-----●) or presence (●——●) of 1 nM fMet-Leu-Phe at 37°C for 20 min. Buffering systems for each pH were as described in *Materials and Methods*. Results are presented as percent hydrolysis per 50 μg of membrane protein per 20 min. Each point is the mean of three experiments done in quadruplicate.

A small amount of radioactivity was measured at the solvent front with an R_f of 0.95. As the R_f of free fatty acid in our solvent system is 0.77, and it was felt that the measured label was nonspecific radioactivity that migrated with the solvent rather than a product of phospholipase A_1 hydrolysis. These data show that the phospholipase in both the unstimulated and activated rabbit neutrophil plasma membrane is a phospholipase A_2.

DISCUSSION

In the present study, we have directly demonstrated phospholipase A_2 activity in the plasma membrane of rabbit polymorphonuclear leukocytes by assaying the hydrolysis of [1-^{14}C]oleate-labeled autoclaved *E. coli*. Also, we have shown that the chemotactic factors fMet-Leu-Phe, C5a, and leukotriene B_4 activate the phospholipase A_2 in a biphasic concentration-dependent manner; optimal activation occurs at concentrations corresponding to their respective optimal chemotactic activity. These results, therefore, unequivocally show chemotactic factor-mediated phospholipase A_2 activation. At present, we have no explanation of why the activity should increase to a maximum with increasing concentrations of all three chemotactic factors and then decrease as the concentration is increased further. Of significance, in this regard, is the previous observation that this is also true

Table 2. Phospholipase specificity

	Unstimulated	fMet-Leu-Phe activated
Phosphatidylethanolamine hydrolyzed, nmol	2.1 ± 0.3	6.9 ± 0.8
Lysophosphatidylethanolamine, nmol	1.9 ± 0.3	6.5 ± 0.3

Reaction mixtures contained 400 nmol of 1-[1-^{14}C]palmitoyl-2-acyl-*sn*-glycero-3-phosphoethanolamine, 100 mM Tris·HCl, pH 7.5/10 mM $CaCl_2$, and 50 μg of plasma membrane protein in the presence or absence of 1 nM fMet-Leu-Phe. Mixtures were incubated at 37°C for 20 min; the reaction was terminated and the mixture was extracted with $CHCl_3/CH_3OH$ (2:1). Products were separated by TLC. Phospholipase activity is expressed as nmol of lysophosphatidylethanolamine formed per 20 min. Results represent mean ± SEM for two experiments.

of chemotactic factor activation of the Na,K-ATPase activity of these same membranes (16).

That the activity is found in our membrane fraction argues that the phospholipase studied here has a plasma membrane location. The use of isolated plasma membranes to demonstrate chemotactic factor activation eliminates the possibility that association of phospholipase activity with the plasma membrane is a result of redistribution of the phospholipase during activation. Moreover, the fact that the extent of phospholipase A_2 activation is tightly coupled to the concentration of chemoattractant, and thus the extent of receptor occupancy, allows us to investigate the mechanism of receptor-mediated enzyme activation directly.

Previous studies on the intact neutrophil have shown that extracellular calcium is required for the release of arachidonic acid (25). The present study shows directly that exogenous calcium is essential for activation of the plasma membrane phospholipase A_2. However, the phospholipase A_2 activities either before or after stimulation are much less sensitive to higher concentrations of calcium or the presence of EGTA than are other phospholipases (8, 9). This decreased sensitivity could be due to several factors including the presence of calcium in the membrane inaccessible to EGTA, the lipid environment of the enzyme in the lipid bilayer, or the conformation of the enzyme when embedded in the membrane.

The effect of calcium or EGTA on the activation of phospholipase A_2 in isolated plasma membranes is obviously different from their effects on the activity of the enzyme either before or after stimulation. This implies that the process of chemotactic factor-mediated activation in both the isolated plasma membrane and the intact neutrophil is more than merely supplying calcium to enhance the efficiency of an active phospholipase A_2. However, any concept of the process of activation requires a preliminary knowledge of the nature of the latent phospholipase. Both this and the nature of the activation process are currently unknown and require further investigation.

The 2-acyl specificity of the unstimulated and activated phospholipase is similar to the phospholipase activities previously described in human and rabbit neutrophils (8, 9). The positional specificity was confirmed by using phosphatidylethanolamine labeled in the 1-acyl position as substrate. Preliminary studies of substrate specificity have also shown that this phospholipase A_2 can cleave arachidonoyl-labeled *E. coli*. Arachidonoyl-labeled *E. coli* were not used in the present study, as the labeling of *E. coli* with arachidonic acid is only 10% as efficient as that with oleic acid. Alteration in pH appears to affect the unstimulated and activated phospholipase A_2 in a similar fashion. Both activities exhibit the same range of activity (pH 7.0–8.5) and an optimum activity at pH 8.5. This range of activity is also similar to that described for the phospholipase A_2 of the human neutrophil plasma membrane. However, the significance of the difference from the pH optimum of 7.5 observed for the human plasma membrane phospholipase is unknown. Furthermore, the fact that neither human nor rabbit plasma membranes exhibited any discernable phospholipase activity below pH 6.0 suggests that the acid-active (optimum pH, 5.5) phospholipase described in the neutrophil is specifically localized in the granule (26).

In summary, we have used an exogenous substrate, [1-^{14}C]oleate-labeled *E. coli*, to directly demonstrate that rabbit neutrophil plasma membrane phospholipase A_2 can be activated by chemotactic factors and that calcium is essential for this activation process. Although the data presented do not allow the conclusion that chemoattractant-stimulated liberation of arachidonic acid in the intact cell is mediated by a phospholipase A_2 pathway, the approach used in this study to define phospholipase A_2 activation in the neutrophil plasma membrane is necessary to dissect the series of events involving chemotactic factor-induced changes in the phospholipid of intact neutrophils. Furthermore, the same approach should be valuable in helping to resolve controversies concerning the stimulated release of free fatty acid in other cells, such as platelets and mast cells.

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