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Platelet-Activating Factor Induction of Secreted Phosphatase Activity in *Trypanosoma cruzi*

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The effects of platelet-activating factor (PAF) on the ecto-phosphatase activity of *Trypanosoma cruzi* were investigated. Living parasites hydrolyzed *p*-nitrophenyl phosphate (*p*-NPP) at a rate of 5.71 ± 0.37 nmol P_i mg^{-1} min^{-1}. This ecto-phosphatase activity increased to 8.70 ± 1.12 nmol P_i mg^{-1} min^{-1} when the cells were grown in the presence of 10^{-9} M PAF. This effect was probably due to stimulation of the release of the ecto-phosphatase and/or the secretion of an intracellular phosphatase to the extracellular medium, as suggested by cytochemical analysis. Modulation of the ecto-phosphatase activity was also observed when PAF was added during the time course of the reaction. WEB 2086, a competitive PAF antagonist, was able to revert PAF effects when both were used at the same concentration. When PAF was added to a membrane enriched fraction preparation of *T. cruzi,* no alteration on the phosphatase activity was observed. This result suggests an involvement of intracellular signaling, as PAF was only effective on intact cells. Sphingosine and phorbol-12-myristate-13-acetate (PMA) were then used to investigate a possible involvement of protein kinase C (PKC) with PAF-induced phosphatase secretion. Sphingosine by itself stimulated the secretion of a phosphatase but did not significantly interfere with PAF effects on this enzyme. On the other hand, PMA was able to abrogate PAF-induced release of this phosphatase. These data are highly suggestive of a putative involvement of signal transduction mediated by a ligand of mammalian origin (PAF), through PKC and a specific receptor located on the cell surface of the human parasite *Trypanosoma cruzi.*

Trypanosoma cruzi, the etiological agent of Chagas' disease, is a parasitic protozoan with a complex life cycle involving morphological and functionally different stages that enable these parasites to adapt to a variety of conditions imposed by the insect vectors and mammalian hosts (1).

Cell surface components play a key role in the survival of protozoan parasites in hostile environments and in confrontation with host immune responses. Despite the disease processes caused by these parasites being extremely important, the biochemistry, physiology and molecular biology of these surface constituents remains largely unexplored (2). Protein phosphorylation is a major mechanism for controlling the activities of enzymes and other proteins, and it is involved in the regulation of many cellular processes. The steady-state level of phosphorylation of any cellular protein is dependent on the balance of the activities of the protein kinases and protein phosphatases (3). Protein kinases and phosphatases have been detected at the cell surface of trypanosomatids (4–10) and in other intracellular pathogens such as *Coxiella burnetti* (11) and *Yersinia* (12, 13). Several biological roles for these ecto-phosphatase activities have been proposed. These enzymes may provide the parasite with a source of inorganic phosphate by hydrolyzing phosphomonoester metabolites (5–7) and protect the parasite by suppressing the respiratory burst, normally induced upon entry of microorganisms into phagocytic cells, by dephosphorylation of critical substrates that enable the macrophage to become activated (11, 12, 14). More recently, a role for these ecto-enzymes in cell differentiation (15, 16) and infection of host cells (8, 9, 17, 18) has been suggested.

Platelet-activating factor (PAF) is a potent mediator of cellular functions with a wide range of physiological and pathological activities (19–21). At the cellular level, PAF exerts its effects through specific receptors located at the cell surface. Binding of PAF to these

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receptors generates a cascade of signal transduction events leading to the activation of protein kinases and culminating in the modulation of several biological processes (20). PAF is not restricted to mammalian cells, but has also been found to be produced by a variety of living organisms, including lower eukaryotes (21). Also, the dihydroxyacetone pathway for biosynthesis of ether lipids has been identified in *Leishmania* (22) and a plasmenylethanolamine, that is a PAF analog (23), was characterized in *T. cruzi* (24). The physiological role of PAF in lower eukaryotes is still unknown. PAF may play a morphoregulatory role in the slime mold *Dictyostellium discoideum* (25, 26) and may be involved in the regulation of cell cycle in the yeast *Saccharomyces cerevisae* (27). In the protozoan *Tetrahymena pyriformis,* some biochemical effects, such as increase in Ca^{2+} influx and enhancement of glycogenolysis, were observed upon treatment with PAF (28).

Previously, we have shown that PAF is able to promote some effects on trypanosomatids such as triggering cell differentiation in *T. cruzi* and in *Herpetomonas muscarum muscarum* (29, 30) and modulating ecto-phosphatase activity in *H. m. muscarum* (7). In the present work we demonstrate that PAF promotes the modulation of an ecto-phosphatase activity and the secretion of a phosphatase in epimastigote forms of *T. cruzi,* a stage of the parasite that lives within the reduviid bug midgut in nature. These effects seem to occur through a cascade of signal transduction events, including PKC activity and PAF receptors on the cell surface of this parasite.

MATERIALS AND METHODS

Platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), sphingosine, phorbol-12-myristate-13-acetate (PMA) and *p*-nitrophenylphosphate (*p*-NPP), were purchased from Sigma Chemical Company. WEB 2086 was kindly provided Dr. H. Heurer (Boehringer Ingelhein, Germany). All other reagents were analytical grade. Epimastigotes of *T. cruzi* (Dm 28 C clone) were maintained by weekly transfers in Brain Heart Infusion (BHI) medium supplemented with 10% fetal bovine serum, at 28°C. The parasites grown in the presence or in the absence of 10^{-9} M PAF for 6 days were harvested by centrifugation, washed twice with 0.9% saline and once with 30 mM Tris-HCl/75 mM sucrose buffer pH 6.8 and kept in the same buffer before the assays. Cellular viability was accessed, before and after incubations, by motility and cell dye exclusion (Trypan blue method) (31). The viability of the parasites was not affected by the conditions used in this work. The phosphatase activity was measured by the rate of inorganic phosphate production. Intact cells and membrane enriched fractions (32) were incubated for 20 min at room temperature in 0.5 ml of a reaction mixture containing 30 mM Tris-HCl/75 mM sucrose buffer pH 6.8, 10 mM *p*-NPP as substrate and 1 mg of protein. Reactions were started by the addition of the substrate and stopped by the addition of 2 ml 1 N NaOH. A control, where the substrate was added just after the reactions were stopped, was used as blank. For determining the concentration of released *p*-nitrophenol (*p*-NP), product of *p*-NPP hydrolysis, the tubes with cells or membrane enriched fractions were centrifuged at 1,500*g* for 20 min and the supernatant was measured spectrophotometrically at 425 nm, using a *p*-NP curve as standard. For detection of secreted phosphatase activity, intact cells were incubated in the presence or in the absence of 10^{-9} M PAF for 1 h. Then the supernatant was collected by two centrifugation steps at 16, 500*g* for 20 min at 4°C and assayed for phosphatase activity as described above. Protein concentration was determined by the method of Lowry *et al.* (33) using bovine serum albumin (BSA) as standard. In the experiments with PAF antagonist and PKC modulators, the parasites were treated for one hour with the PAF antagonist WEB 2086 (10^{-9} M) or with the PKC inhibitor sphingosine (50 ng/ml) prior to the addition of PAF, while the PKC stimulator PMA (20 ng/ml) was added to the reaction mixture 20 min prior to the addition of PAF. For cytochemical analysis, parasites grown for 6 days were harvested and then briefly fixed for 20 min at 4°C with glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, and then washed 1× in 0.1 M cacodylate buffer and in 0.1 mM Tris-maleate buffer pH 5.0. After that, the pellet was incubated for 20 min at 37°C in Tris-acetate buffer pH 5.0 containing 2 mM cerium chloride, 5% sucrose, 2 mM β-glycerophosphate, as substrate, in the presence or in the absence of 10^{-9} M PAF. The cells were then washed in Tris-maleate and cacodylate buffers, refixed with 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in a graded acetone series and embedded in Epon. As a control, the same number of cells was incubated in the absence of substrate. Ultrathin sections were observed unstained in a Zeiss 900 EM transmission electron microscope, operated at 80 kV.

TABLE I

Effect of PAF on Ecto-Phosphatase Activity of *Trypanosoma cruzi*

	Control	10^{-9} M PAF
Intact cells	5.71 ± 0.37	8.70 ± 1.12
Supernatant	0.86 ± 0.09	1.70 ± 0.17
Membrane enriched fraction	21.30 ± 3.05	20.74 ± 3.22

Note. The reactions were performed at 25°C in a buffer containing 30 mM Tris–HCl/75 mM sucrose, pH 6.8, 10 mM *p*-NPP, and 1 mg of protein. The phosphatase assays were carried out at room temperature for 20 min, when intact cells and membrane enriched fractions were used, and for 60 min when supernatants were used. For detection of phosphatase activity of living parasites, those were grown for 6 days in the presence or in the absence of 10^{-9} M PAF. For detection of secreted phosphatase activity, living parasites (6 days culture) were incubated for 1 h in the presence or in the absence of 10^{-9} M PAF. Then the supernatants were collected by two centrifugation steps at 16,500*g* for 20 min at 4°C before the phosphatase assays. The values are expressed in nmol P_i mg^{-1} min^{-1} and represent the mean of at least three independent experiments, which were performed in triplicate.

RESULTS

Recently, the involvement of secreted and ecto-phosphatases of trypanosomatid parasites in cell differentiation (15, 16) and infection of host cells (8, 9, 17, 18) has been suggested. Following the procedures used for demonstrating PAF-induced cell differentiation in *T. cruzi* (29), these parasites were grown in the presence of PAF for 6 days, which resulted in significant alteration on the phosphatase activity present on the external surface (Table I). To measure the PAF-induced increase in total extracellular phosphatase activity, the parasites that had been incubated for 6 days in the presence and in the absence of PAF were pel-

leted, washed, and ressuspended in phosphatase reaction mixture. After a 20 min incubation in reaction mixture the reaction was stopped and the cells were removed by centrifugation. The absorbance at 425 nm in the supernatant fraction was measured. Phosphatase activity was increased 52% in PAF treated cells compared to control cells (Table I).

In the preceding assays, the phosphatase activity may have resulted from both the enzyme present on the surface of the parasites and the enzyme secreted into the medium during the course of the assay. Taking this into consideration, we also measured the phosphatase activity in the cell-free supernatants. These latter assays were performed after an incubation of the washed parasites with 10^{-9} M PAF for one hour. The supernatants were then collected employing two sequential centrifugation steps at 16,500*g* for 20 min. The supernatants were subsequently incubated for 60 min in the same reaction mixture described above. The phosphatase activity detected in these supernatants was 97% higher than that measured in supernatants obtained from control parasites that were not incubated with PAF (Table I). These results clearly indicate that the secreted phophatase activity increased by a 1-h incubation with PAF.

Cytochemical analysis confirmed these effects induced by PAF on ecto-phosphatase activity of *T. cruzi*. The cytochemical reaction was detected by electron-dense cerium phosphate deposits, the product of the reaction between cerium chloride and the inorganic phosphate cleaved from β-glycerophosphate by phosphatase activity. Cells treated with PAF showed different patterns of cytochemical reaction when compared to control cells (Fig. 1). Untreated cells showed electron-dense deposits of cerium phosphate homogeneously disposed on the parasite external surface (Fig. 1a). After treatment with 10^{-9} M PAF the parasites presented electron-dense deposits of cerium phosphate inside a large vacuole (Fig. 1b), always close to the flagellar pocket (Fig. 1c) and also as concentrated patches on the cell surface (Fig. 1d). These results are highly suggestive of the release of the ecto-phosphatase from the membrane (Fig. 1d) as well as the presence of a secreted enzyme (Fig. 1b and 1c), since the exocytic process in trypanosomatids takes place within the flagellar pocket (17).

Addition of PAF during the time course of the reaction induced the same effects observed when the parasites were pre-incubated with PAF for 6 days (Fig. 2). This result suggests that PAF achieves its effects rapidly, i.e., within the 20 min time course of the assay. WEB 2086, a specific antagonist of PAF receptors, reverted the PAF-induced effect on the ecto-phosphatase activity (Fig. 2) and partially reverted PAF-induced secreted phosphatase (Fig. 3).

When plasma membrane enriched fractions were incubated in the presence of PAF, no alteration on the phosphatase activity was detected (Table I). These results show that the effects induced by PAF were only observed when living cells and supernatants obtained from those cells were used, which is highly suggestive that they occur through binding of PAF to the parasite surface, followed by transduction of this signal to the interior of the cell, and not via a direct effect on the enzyme.

A secreted acid phosphatase of *L. mexicana* mediates the infection of mononuclear phagocytes, which was modulated by sphingosine and PMA (17). In order to investigate if PAF effects occur through signal transduction pathway involving protein kinase C, the effects of two modulators of PKC on the PAF-induced secretion of phosphatase were investigated (Fig. 4). The phosphatase activity detected in the supernatants showed that 50 ng/ml sphingosine, known to inhibit PKC activity, induced a secretion of a phosphatase into the extracellular medium, similarly to the results obtained when 10^{-9} M PAF was used, although sphingosine was not able to promote any additive effect on this PAF-induced secretion. The phosphatase activity detected in the supernatants from parasites pretreated with the PKC stimulator PMA (20 ng/ml), was 25% lower than that measured in the supernatants of control cells. Our results also show that 20 ng/ml PMA was able to suppress PAF-induced phosphatase secretion.

DISCUSSION

In previous results we have demonstrated that PAF triggers the process of cell differentiation in *T. cruzi* (Dm 28c) and in *H. m. muscarum*, without interfering with their cell growth (29, 30). In the present study we have demonstrated that PAF promoted an enhancement of the ecto-phosphatase activity of *T. cruzi* (Dm 28c) when the parasites were grown in the presence of 10^{-9} M PAF (Table I). PAF effects were only observed in living cells and in supernatants obtained from those cells, but not when membrane enriched fractions were used, which is highly suggestive that they occur through transduction of signals from PAF receptors to the interior of the cells (Table I). These results further support the possibility that PAF produced in either the insect vector or the vertebrate host modulates biochemical activities in *T. cruzi* via signal transduction pathway.

The results obtained by cytochemical analysis, when *T. cruzi* epimastigotes were treated with 10^{-9} M PAF, are indicative of secretion of a phosphatase (Fig. 1). Similar to these results, PAF stimulated the secretion of a lysosomal acid phosphatase in neutrophils (34). Also, PAF is known to stimulate secretion of intracellular components in monocytic leukemia cells, including some soluble factors considered to be related to cell differentiation and proliferation (35).

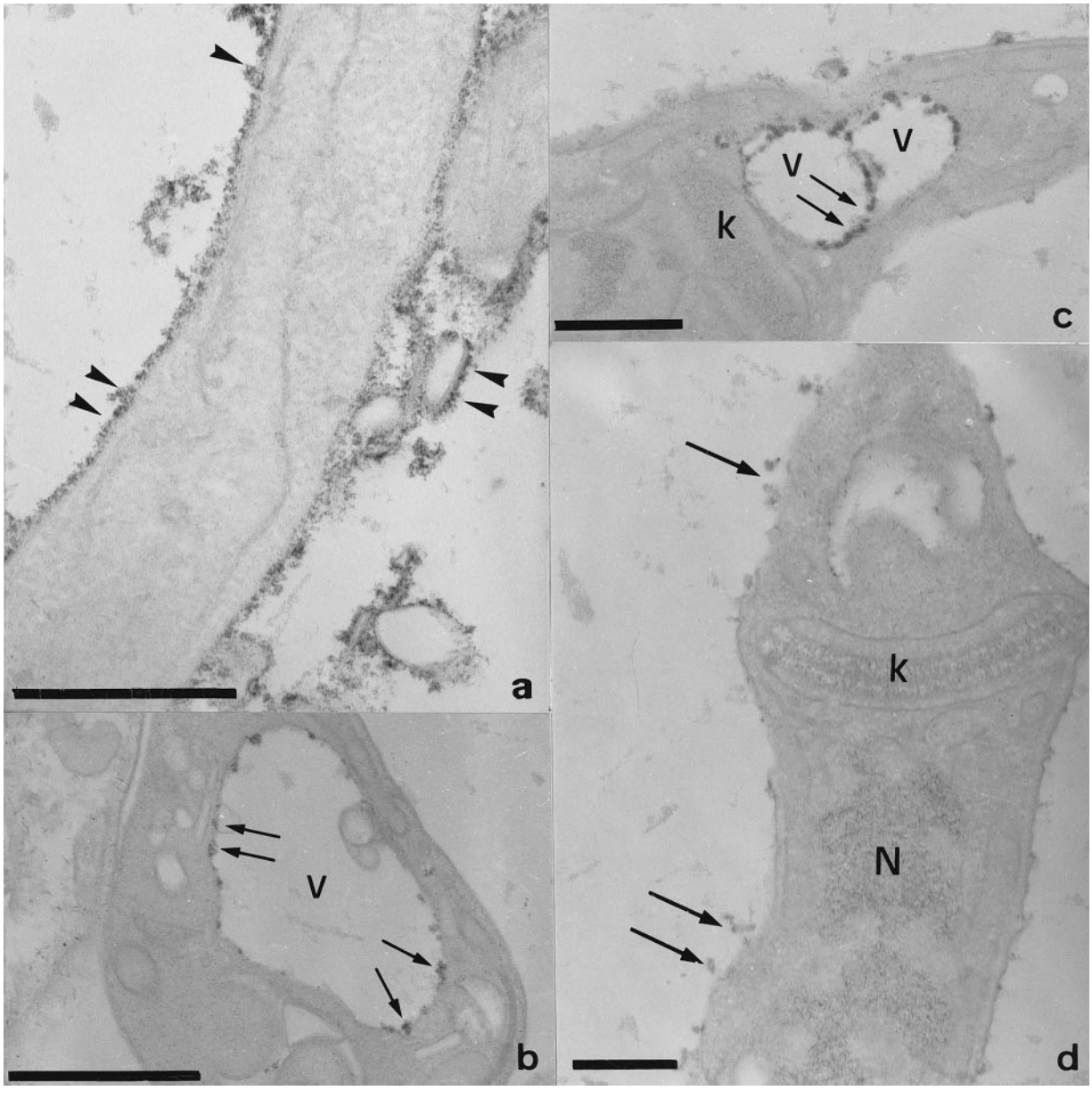

FIG. 1. Cytochemical assay for localization of acid phosphatase activity of *T. cruzi.* Parasites grown for 6 days were incubated for 20 min in the absence (a) or in the presence of 10^{-9} M PAF (b–d). The reactions were performed at 25°C in a buffer containing Tris–acetate, pH 5.0, using β-glycerophosphate as substrate and cerium chloride as capture agent. K, kinetoplast; N, nucleus; V, vacuole. The arrows indicates the cerium chloride deposition. Bars, 1 μm.

Although PAF receptors have not been described in lower eukaryotes, our previous results suggest the presence of such receptors in *T. cruzi* (29) and in *H. m. muscarum* (7, 30). WEB 2086 is a competitive PAF antagonist that binds specifically to PAF receptors (36). WEB 2086 abrogated PAF effects on cell differentiation of *T. cruzi* (29) and of *H. m. muscarum* (30) and on ecto-phosphatase activity of *H. m. muscarum* (7). Similarly, in this work we show that WEB 2086 clearly reverted (Fig. 2) or partially reverted (Fig. 3) PAF effects on phosphatase activities of *T. cruzi.* The partial reversion observed in Fig. 3 may be compared to previous results, including the failure of WEB 2086 to revert the paw edema caused by PAF in mice and to abrogate the synthesis of PAF by the ionophore A23187 (37, 38).

Following activation of PAF receptors in mammalian cells, diverse intracellular responses are elicited, including activation of protein kinase C (PKC) and gene expression (20). Although these mechanisms of transduction of signals from PAF can be considered, to a certain extent, universal in mammalian cells (39), it is

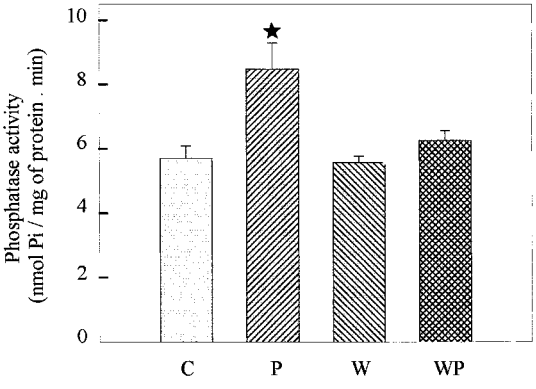

FIG. 2. Effect of PAF and PAF receptor antagonist (WEB 2086) on phosphatase activity of intact cells of *Trypanosoma cruzi*. The reactions were performed at 25°C in a buffer containing 30 mM Tris–HCl/75 mM sucrose, pH 6.8, 10 mM *p*-NPP, and 1 mg of protein. Living parasites were incubated for 1 h in the presence or in the absence of 10^{-9} M WEB 2086, prior to the phosphatase assays, and then with 10^{-9} M PAF during the time course of the reaction. Living parasites without any drug addition were used as control. The values represent the mean of at least three independent experiments, which were performed in triplicate and were expressed in nmol P_i mg^{-1} min^{-1}. The bars represent the standard errors; parasites treated with PAF had rates of *p*-NPP hydrolysis significantly different from control cells (★); ($P < 0.05$, Fisher's test).

possible that PAF may function in different ways in lower eukaryotes. In contrast to most vertebrate cells, PAF produced by *Dictyostellium discoideum* is not re-

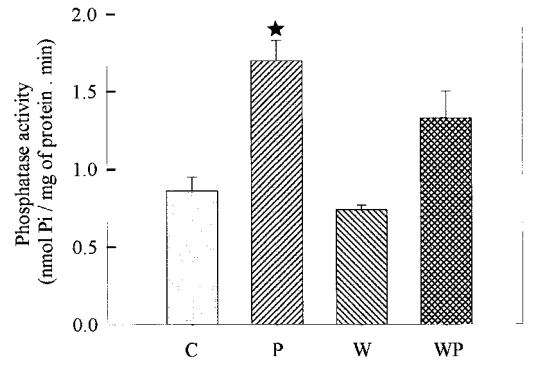

FIG. 3. Effect of PAF and PAF receptor antagonist (WEB 2086) on the secreted and/or released phosphatase activity of *Trypanosoma cruzi*. The reactions were performed at 25°C in a buffer containing 30 mM Tris–HCl/75 mM sucrose, pH 6.8, 10 mM *p*-NPP, and 1 mg of protein. Living parasites were incubated in the presence of 10^{-9} M WEB 2086 for 1 h, centrifuged and incubated for 1 h in a fresh reaction mixture containing 10^{-9} M PAF. Then the supernatants were collected by two centrifugation steps at 16,500*g* for 20 min at 4°C, prior to the phosphatase assays. Supernatants obtained from cells incubated in the reaction mixture without any drug addition were used as control. The values represent the mean of at least three independent experiments, which were performed in triplicate and were expressed in nmol P_i mg^{-1} min^{-1}. The bars represent the standard errors; parasites treated with PAF had rates of *p*-NPP hydrolysis significantly different from control cells (★); ($P < 0.05$, Fisher's test). C, control; P, PAF; W, WEB; and WP, WEB plus PAF.

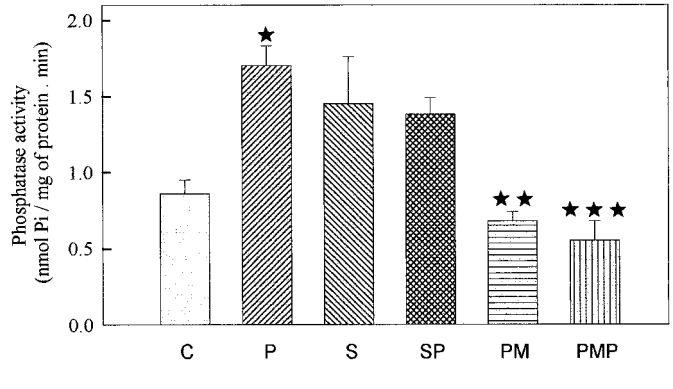

FIG. 4. Effect of PAF and the protein kinase C modulators sphingosine and PMA on the secreted and/or released phosphatase activity of *Trypanosoma cruzi*. The reactions were performed at 25°C in a buffer containing 30 mM Tris–HCl/75 mM sucrose, pH 6.8, 10 mM *p*-NPP, and 1 mg of protein. Living parasites were incubated in the presence of 50 ng/ml sphingosine for 60 min or 20 ng/ml PMA for 20 min, centrifuged and incubated for 1 h in a fresh reaction mixture containing 10^{-9} M PAF. Then the supernatants were collected by two centrifugation steps at 16,500*g* for 20 min at 4°C, prior to the phosphatase assays. Supernatants obtained from cells incubated in the reaction mixture without any drug addition were used as control. The values represent the mean of at least three independent experiments, which were performed in triplicate and were expressed in nmol P_i mg^{-1} min^{-1}. The bars represent the standard errors; parasites treated with PAF had rates of *p*-NPP hydrolysis significantly different from control cells (★), from parasites treated with PMA (★★) and from parasites pretreated with PMA and then with PAF (★★★); ($P < 0.05$, Fisher's test). C, control; P, PAF; S, sphingosine; SP, sphingosine plus PAF; PM, PMA; and PMP, PMA plus PAF.

leased to the extracellular medium (25) and an intracellular role for PAF involving signal transduction was postulated, as there are evidences for the presence of PAF receptors inside those cells (26).

Our results show that the PKC stimulator PMA abrogated the PAF-induced phosphatase secretion in *T. cruzi* epimastigotes (Fig. 4). Similarly, the reversion of PAF effects by PMA has already been described in other biological systems, such as in human neutrophils and in bone marrow-derived macrophages (40, 41). Also, it was shown that PMA interfered with the binding of PAF to its receptor, although it was not elucidated weather this effect was mediated by activating PKC or by a direct conformational change in PAF receptors (40). PMA stimulated a PKC activity (42) and induced filopodium-like projections (43) in *T. cruzi* epimastigotes.

It is intriguing that PAF modulates the activity of extracellular phosphatases in *T. cruzi*. A secreted acid phosphatase in the related parasite *Leishmania*, also modulated by sphingosine and PMA, mediates the infection of mononuclear phagocytes, (17). Furthermore, PAF and sphingosine presented similar effects on stimulating a vacuolar alkaline phosphatase in the yeast Saccharomyces *cerevisae* (44), which is comparable to the results presented here (Fig. 4). Some metabolic pathways shared by PAF and sphingosine could ex-

plain these results (45), as the biosynthesis of sphingosine by PAF has been described in HL-60 cells (46).

Receptors for mammalian ligand proteins have been postulated to exist in *Trypanosoma* and *Leishmania* (47, 48). Furthermore, the gene for a putative receptor-adenylate cyclase has been cloned and expressed in *L. donovani* (49) and several signal transduction mechanisms have been identified in trypanosomatids, in general (9, 17, 50), and in *T. cruzi* (8, 18, 51) in particular. Our results suggest that 10^{-9} M PAF induces the secretion of an intracellular phosphatase and stimulates the release of the ecto-phosphatase from the membrane of these parasites and that these responses occur via signal transduction pathways involving PKC and PAF receptors on the membrane of *T. cruzi*. Secreted enzymes or ecto-enzymes with kinase and phosphatase activities in *Leishmania* and *Trypanosoma* have been shown to be involved in cell differentiation and cell invasion processes through signal transduction networks (8, 9, 17, 18, 52). PAF-induced secreted phosphatase in *T. cruzi* could possibly play a role in host–parasite interactions, as these flagellates might be in contact with PAF during their life cycle either the insect vector or the vertebrate host.

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