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A Novel Ecto-Phosphatase Activity of *Herpetomonas muscarum muscarum* Inhibited by Platelet-Activating Factor

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In the present work ecto-phosphatase activity in *Herpetomonas muscarum muscarum* has been characterized using live parasites. This enzyme hydrolyzed *p*-nitrophenylphosphate at a rate of 4.27 nmol P_i/mg of protein · min. A pH curve was generated, in which these intact flagellates showed the highest phosphatase activity at pH 6.5. Classical inhibitors for acid phosphatase, such as sodium orthovanadate, sodium tartrate, and ammonium molybdate, were used in the experiments and showed different patterns of inhibition. Lithium fluoride, aluminum chloride, and fluoroaluminate complexes were also tested. Although lithium fluoride and fluoroaluminate complexes were capable of inhibiting the phosphatase activity, aluminum chloride stimulated this enzyme. Cytochemical analysis showed the localization of this enzyme on the parasite surface. This ecto-phosphatase activity was also significantly diminished when the parasites were treated with 10^{-6} M platelet-activating factor (PAF), a potent phospholipid mediator that promoted cellular differentiation in this parasite.

Herpetomonas muscarum muscarum is a nonpathogenic parasite of houseflies, which belongs to the family Trypanosomatidae. Some genera of this family undergo a process of cellular differentiation, which in the genus *Herpetomonas* produces three evolutive forms, characterized by the position of the kinetoplast relative to the nucleus (1). The developmental transformations that produce each life cycle stage of the parasite may be signaled in part by binding of environmental ligants to receptors, which mediate transduction of extracellular signals. Receptors for mammalian ligant proteins have been described for *Trypanosoma* and *Leishmania* (2).

Platelet-activating factor (PAF) is a potent phospholipid mediator that produces a wide range of biological responses including inflammation, allergy and cellular differentiation, through activation of a specific receptor on target cell surface (3). PAF is synthesized and released from a variety of stimulated cells and it seems to be involved with several pathologies such as anaphylaxis, thrombosis, asthma and endotoxemia (4, 5). Recently we have demonstrated that PAF triggers the process of cell differentiation of *H. m. muscarum* (6) and of *Trypanosoma cruzi* (7).

Parasite membranes are known to be involved in many cellular events, including transport of nutrients and ions and protection of the parasite against host immune responses (8). The process of phosphorylation/dephosphorylation is of major importance in regulating many cellular processes in eukaryotes (9). Ecto-protein kinases have been identified in *Leishmania major* (10), an ecto-ATPase has been shown in *L. tropica* (11) and ecto-phosphatase activities have been characterized in some members of the family Trypanosomatidae, such as *Leishmania* and *Trypanosoma* (12, 13). The gene for a *Leishmania mexicana* membrane-bound acid phosphatase has already been cloned and expressed (14). Although the physiological role for ecto-phosphatases has not been well established in parasites, they are supposed to be involved with nutrition, protection (15, 16) and with cell differentiation (17). Most *Leishmania* species are capable of secreting acid phosphatases, which can be inhibited by sodium tartrate, varying their degree of sensitivity (12). It has been suggested the involvement of these secreted enzymes with the interaction of the parasites with their host cells (18). In the present work we have characterized a phosphatase activity present on the external surface of *H. m. mus-*

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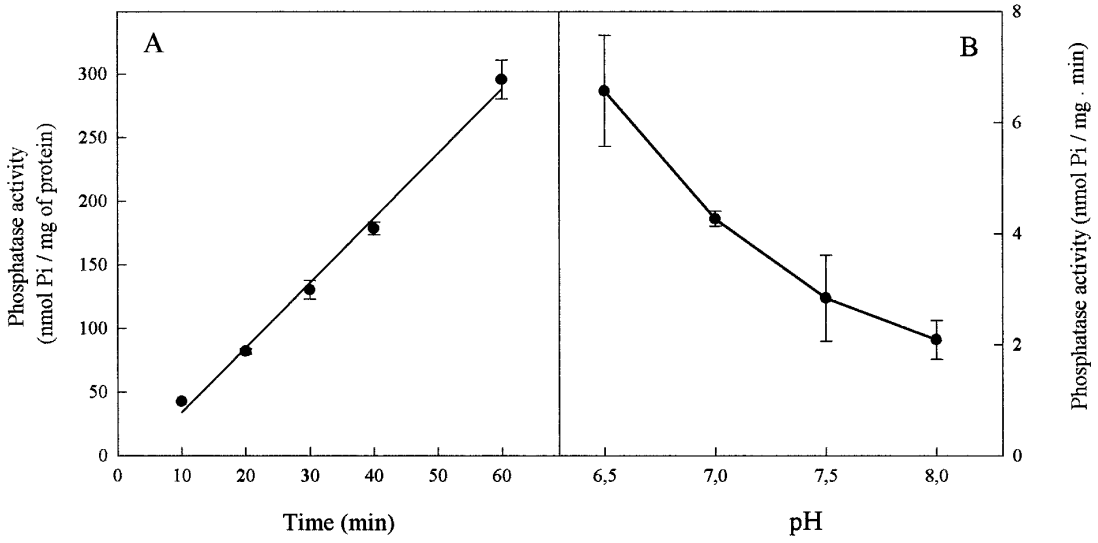

FIG. 1. (A) Temporal course of phosphatase activity of intact cells of *H. m. muscarum*. The reactions were performed at 30°C in a buffer containing 30 mM Tris–HCl/75 mM sucrose, pH 6.8, 10 mM *p*-NPP, and 1 mg of protein/ml (5.8×10^9 cells/ml). (B) Influence of pH on phosphatase activity of intact cells of *H. m. muscarum*. Reactions were performed under the same conditions described above varying only in the buffer pH (adjusted to range 6.5 to 8.0). The values represent the mean of at least three independent experiments which were performed in triplicate. The bars represent the standard errors.

carum and demonstrated that PAF inhibited this phosphatase activity.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA or from E. Merck, D-6100 Darmstadt, Germany (divalent cations and phosphatase inhibitors). In the present work we used *Herpetomonas muscarum muscarum* (ATCC 30260), cultured under chemically defined conditions (19) at 28°C. Three days after inoculation, cells 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 this buffer. Cellular viability was accessed, before and after incubations, by mobility and Trypan blue cell dye exclusion (20). The viability of the parasites was not affected by the conditions used in this work. Protein phosphatase activity was assayed in a reaction mixture (0.5 ml) containing 30 mM Tris-HCl/75 mM sucrose buffer pH 6.8, 10 mM *p*-nitrophenylphosphate (*p*-NPP) as substrate and 5.8×10^9 cells/ml (1 mg protein/ml). Incubations were carried out at room temperature for 60 min., unless otherwise specified. Reactions were initiated by the addition of cells [or supernatant obtained from the centrifugation of the parasites at 16,500 · g at 4° C, after incubation in the reaction mixture for one hour, or membrane enriched fraction, obtained as previously described (21)] and stopped by the addition of 2 ml 1N NaOH. Released *p*-nitrophenol (*p*-NP) was determined spectrophotometrically at 425 nm (13). A control, where cells were added after the reaction was stopped, was used as a blank (the extinction coefficient for *p*-nitrophenolate ions is $1.75 \cdot 10^4\ M^{-1} \cdot cm^{-1}$). Protein concentration was determined by the method of Lowry *et al*. (22), using bovine serum albumin (BSA) as standard. For cytochemical assays, parasites were cultivated in the absence and in the presence of 10^{-6} M PAF, collected by centrifugation, washed in 0.9% saline, briefly fixed for 20 minutes at 4°C with glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, washed in 0.1 M cacodylate buffer pH 7.2 and in 0.1 mM Tris-maleate buffer pH 5.0. Then, the pellet was incubated for 1 hour at 37 °C in 2 mM cerium chloride, 5 % sucrose, 0.1 mM Tris-acetate buffer pH 5.0 and 2 mM sodium β-glycerophosphate as substrate. The cells were then washed in Tris-maleate and cacodylate buffers, refixed with 2.5 % glutaraldehyde diluted in 0.1 M cacodylate buffer, post-fixed in 2 % 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. Ultra-thin sections were observed unstained in a Zeiss EM transmission electron microscope, operated at 80 kV. All experiments were performed in triplicate, with similar results obtained in three separate cell suspensions. V_{max} and apparent K_m for *p*-NPP were calculated using a computerized non-linear regression program (Sigma Plot 1.0 - Jandel Scientific, 1986-1993, USA) of the data to the Michaelis-Menten equation. The data were analyzed statistically by means of Fisher's test, as described (6).

RESULTS AND DISCUSSION

The time course of the phosphatase activity present on the external surface of *H. m. muscarum* was linear during the first hour (Fig. 1A). At pH 6.8 these parasites were able to hydrolyze *p*-NPP at a rate of 4.27 ± 0.01 nmol Pi/mg of protein · min. In the pH range from 6.5 to 8.0 the phosphatase activity reached a maximum at pH 6.5, decreasing concomitantly with the increase of pH (Fig. 1B), suggesting an acid phosphatase activity, as it has been previously described for other trypanosomatids (15, 17, 23). The dependence on *p*-NPP concentration for this ecto-phosphatase activity was observed. The values of V_{max} and apparent K_m for *p*-NPP were 4.90 ± 0.46 nmol P_i/mg/of protein · min and 0.76 ± 0.04 mM, respectively (Fig. 2).

Different phosphatase inhibitors were tested and the results are shown in Table 1. An alkaline phosphatase inhibitor (K_2HPO_4) and a serine-threonine phosphatase inhibitor (okadaic acid) (9) had no effect on this enzyme. The acid phosphatase inhibitors zinc chloride

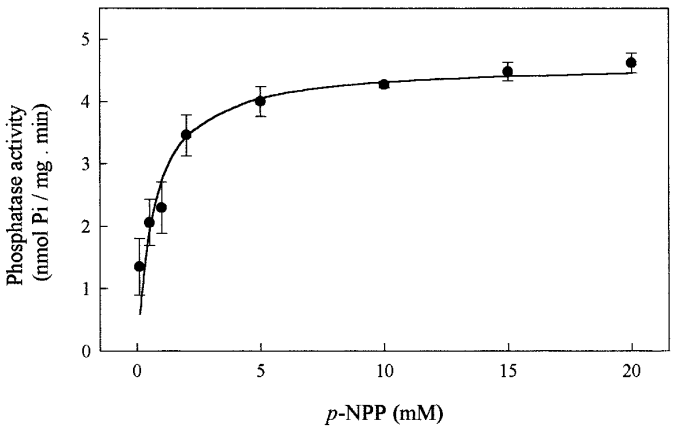

FIG. 2. Influence of *p*-NPP concentration on phosphatase activity of intact cells of *H. m. muscarum*. The reactions were performed at 30°C in a buffer containing 30 mM Tris–HCl/75 mM sucrose, pH 6.8, and 1 mg of protein/ml (5.8×10^9 cells/ml), and several *p*-NPP concentrations, as showed in abscissa. The curve represents the adjustment of kinetic parameters by nonlinear regression, using the Michaelis–Menten equation as described under Material and Methods. The values represent the mean of at least three independent experiments which were performed in triplicate. The bars represent the standard errors.

and ammonium molybdate (16) inhibited respectively 50% and 97% of this enzyme activity (Table 1). The classical phosphatase inhibitors sodium orthovanadate ($NaVO_3$) and lithium fluoride (LiF) (12), as well as fluoroaluminate complexes (24) were also tested (Fig. 3). Sodium orthovanadate inhibited about 95% of this activity in a dose dependent manner (Fig. 3A). Lithium fluoride was also an inhibitor to this phosphatase activity, reaching 85% of inhibition (Fig. 3B). The phosphotyrosine phosphatase activities of cellular extracts of *T. cruzi* and *T. brucei* were inhibited by sodium orthovanadate, while okadaic acid, had no effect on those parasites (17, 23). These data suggest that this ecto-phosphatase of *H. m. muscarum* could be a phosphotyrosin phosphatase. The fluoroaluminate complexes, which are originated by the addition of increasing concentrations of aluminum chloride to the reaction mixture (24), in the presence of 0.1 mM lithium fluoride, inhibited this enzyme activity in a dose dependent manner, although aluminum chloride by itself promoted an increase on this phosphatase activity (Fig. 3C). The fluoroaluminate complex seems to act as a $NaVO_3$ analog that strongly binds to the phosphatases (13) and ATPases (24) in a quasi-irreversible model that cannot be overcome by Pi high concentrations.

As PAF was shown to trigger the process of cell differentiation of *H. m. muscarum* (6) and of *Trypanosoma cruzi* (7), as well as phosphotyrosin phosphatases have been related to cell differentiation of *T. cruzi*, *T. brucei* and *L. mexicana* (17, 25), in this work we have tested if PAF was able of modulating the ecto-phosphatase activity of *H. m. muscarum*. The addition of PAF to the reaction mixture inhibited 50% of this activity (Fig. 4), at the same concentration (10^{-6} M) which triggered the process of cellular differentiation of these parasites (6). PAF added during the protozoa growth promoted 40 % inhibition of the phosphatase activity, but had no effect when added to membrane enriched fractions, during the course of reaction (data not shown). A specific PAF receptor antagonist, WEB 2086 (5), which abrogated PAF-induced cellular differentiation of *H. m. muscarum* and of *T. cruzi* (6, 7), was able to partially revert PAF effect, bringing this phosphatase activity to 70% of the control cells (Fig. 4). Our data suggest that in this system PAF must act through a complex signal transduction network, but not directly on the phosphatase enzyme, because PAF effect was partially reverted by the cell surface receptor antagonist WEB 2086 and it was only observed when PAF was used on intact viable cells. Little is known about the involvement of PAF with protein phosphatases, although it has already been reported that this lipid factor modulates phosphohydrolase activity in yeast vacuoles (26).

The supernatants of *H. m. muscarum* were able to hydrolyze *p*-NPP at a rate of 10.26 ± 1.88 nmol P_i/mg of protein · min, at the same conditions used for intact cells (Table 2). The presence of a secreted form of acid phosphatase, which is significantly more sensitive to sodium tartrate than the membrane-bound enzyme, was described in *L. donovani* (12, 16). Similarly, the phosphatase activity detected in the supernatant of *H. m. muscarum* was significantly inhibited (87.13% inhi-

TABLE 1

Influence of Divalent Cations and Phosphatase Inhibitors on Ecto-Phosphatase Activity of Intact Cells of *Herpetomonas muscarum muscarum*

Addition	% of activity
None	100.00
Cuprum sulfate (5 mM)	102.86
Magnesium chloride (5 mM)	100.66
Calcium chloride (5 mM)	99.60
Aluminum chloride (0.2 mM)	149.00
Okadaic acid 92.5 μM)	100.25
Monobasic potassium phosphate (10 mM)	99.87
Zinc chloride (1.0 mM)	42.04
Lithium fluoride (10 mM)	15.66
Sodium fluoride (10 mM)	5.01
Sodium orthovanadate (0.2 mM)	5.00
Ammonium molybdate (0.1 mM)	3.00

Note. The reactions were performed at 30°C in a buffer containing 30 mM Tris–HCl/75 mM sucrose, pH 6.8, 10 mM *p*-NPP, and 1 mg of protein/ml (5.8×10^9 cells/ml), in the absence or presence of divalent cations or phosphatase inhibitors. Phosphatase activity is expressed as a percentage of that measured under control conditions, i.e., without other additions. The phosphatase activity (4.27 ± 0.01 nmol P_i/mg of protein · min) was taken as 100%.

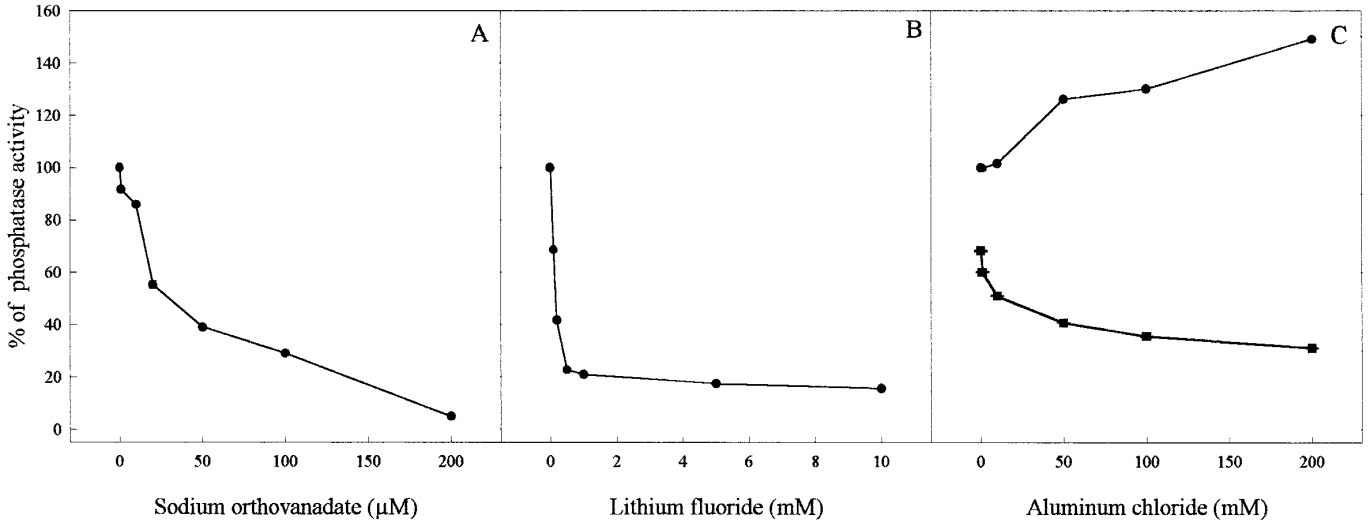

FIG. 3. Effect of phosphatase inhibitors on phosphatase activity of intact cells of *H. m. muscarum*. The reactions were performed at 30°C in a buffer containing 30 mM Tris–HCl/75 mM sucrose, pH 6.8, 10 mM *p*-NPP, and 1 mg of protein/ml (5.8×10^9 cells/ml), in the absence or the presence of some phosphatase inhibitors, as described under Material and Methods. The values represent the mean of at least three independent experiments which were performed in triplicate. The bars represent the standard errors. (A) Sodium orthovanadate ($NaVO_3$). (B) Lithium fluoride (LiF). (C) Aluminum chloride ($AlCl_3$) (●) and fluoroaluminate complexes [different amounts of $AlCl_3$ in the presence of 0.1 mM of LiF (■)]. The phosphatase activity (4.27 ± 0.01 nmol P_i/mg of protein · min) was taken as 100%.

bition) by 10 mM sodium tartrate, while the activity of intact cells was inhibited in 36.30% and that of membrane enriched fractions was inhibited in 25.42% (Table 2). The trypanosomatid flagellar pocket, a highly specialized area of the parasite membrane, as well as the Golgi apparatus, are involved in several processes including protein secretion to the external environment (27, 28). In *Leishmania*, cytochemical analysis of those phosphatases showed the presence of electrondense particles within the flagellar pocket and on the external surface of those parasites (18, 29). This pattern is very similar to that observed in *H. m. muscarum* control cells (Fig. 5A-5C), where electrondense cerium phosphate deposits were equally disposed on the parasite external surface and more concentrated within the flagellar pocket. Using cytochemical assay we observed electrondense cerium phosphate deposits on the Golgi apparatus (Fig. 5C), which is indicative of

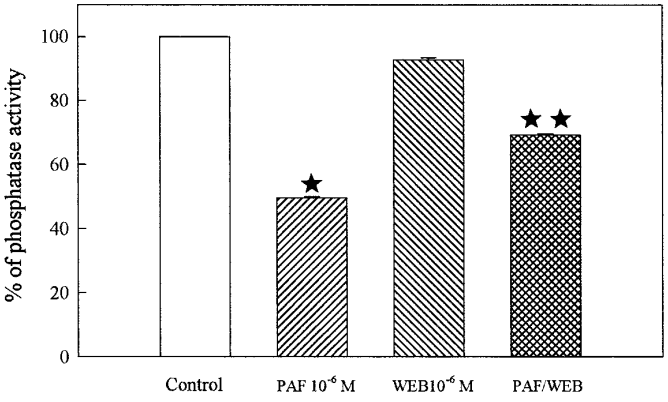

FIG. 4. Effect of PAF and PAF receptor antagonist (WEB 2086) on phosphatase activity of intact cells of *H. m. muscarum*. The reactions were performed at 30°C in a buffer containing 30 mM Tris–HCl/75 mM sucrose, pH 6.8, 10 mM *p*-NPP, and 1 mg of protein/ml (5.8×10^9 cells/ml), in the absence or the presence of 10^{-6} M PAF-acether and/or its specific receptor antagonist, WEB 2086, at the same concentration, as described under Material and Methods. The values represent the mean of at least three independent experiments which were performed in triplicate. The bars represent the standard errors; parasites treated with PAF had their rate of *p*-NPP hydrolysis significantly different from control cells (★) and from parasites treated with WEB 2086 previously to PAF treatment (★★); ($P < 0.05$, Fisher's test). The phosphatase activity (4.27 ± 0.01 nmol P_i/mg of protein · min) was taken as 100%.

TABLE 2

Influence of Sodium Tartrate on Phosphatase Activity of *Herpetomonas muscarum muscarum*

	Control	Sodium tartrate 10 mM	% of inhibition
Intact cells	4.27 ± 0.01	2.72 ± 0.08	36.30
Cells supernatant	10.26 ± 1.88	1.32 ± 0.61	87.13
Membrane enriched fraction	27.77 ± 0.22	20.71 ± 0.42	25.42

Note. The reactions were performed at 30°C in a buffer containing 30 mM Tris–HCl/75 mM sucrose, pH 6.8, 10 mM *p*-NPP, and 1 mg of protein/ml, in the case of intact cells, 0.22 mg of protein for assays with membrane enriched fractions, and 0.05 mg of protein for experiments with cell supernatants. The reactions were performed in the absence or the presence of 10 mM sodium tartrate. The values represent the mean ± standard errors of at least three independent experiments which were performed in triplicate and were expressed in nmol P_i/mg of protein · min.

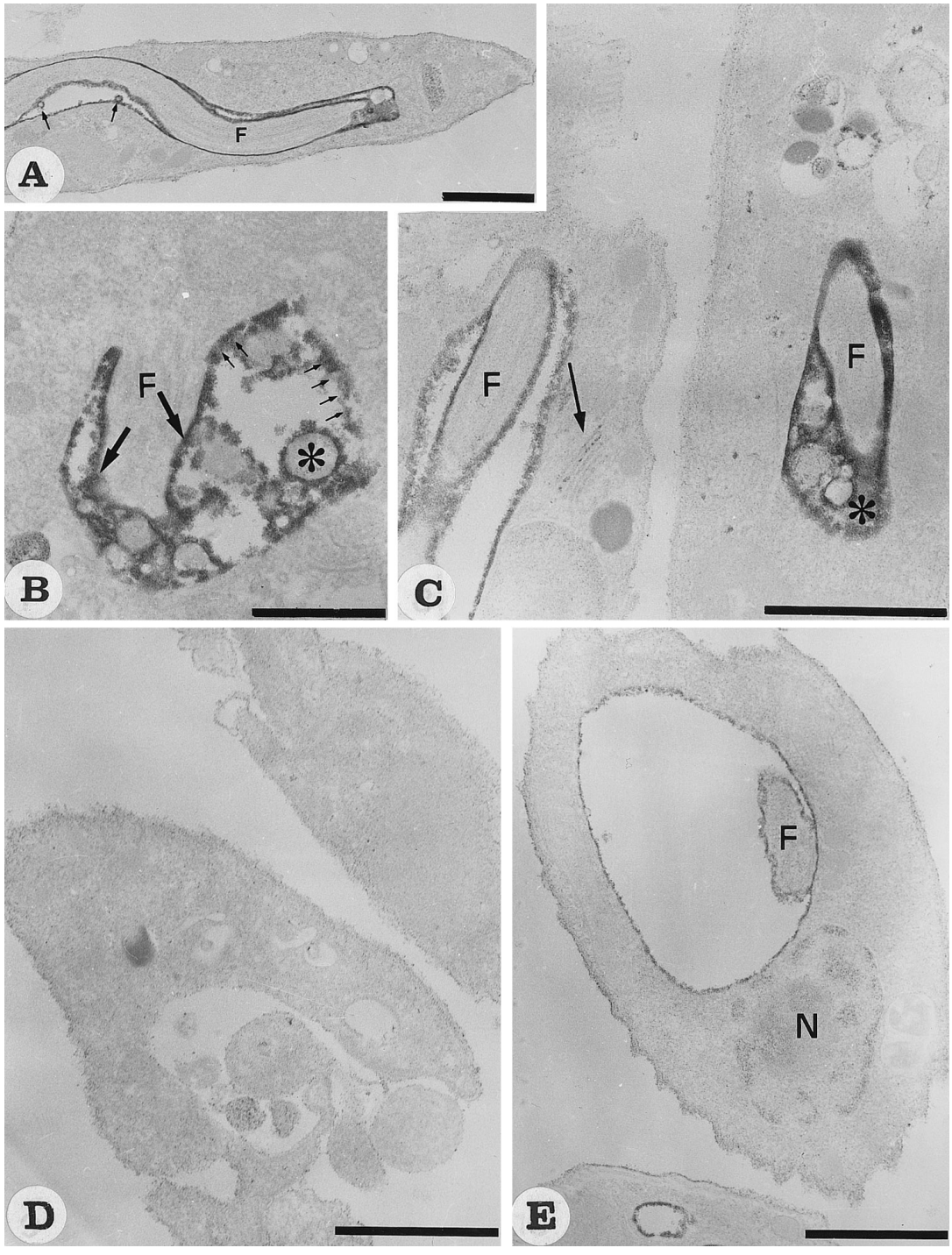

FIG. 5. Cytochemistry assay for localization of acid phosphatase activity of *H. m. muscarum*. The trypanosomatids were grown in a chemically defined medium (20), during 6 days, at 28°C, in the absence (A - Control cells) or the presence of 10^{-6} M PAF-acether (B, C, D, and E). The reactions were performed at 30°C in a buffer containing Tris-acetate, pH 5.0, using β-glicerophosphate as the substrate and cerium chloride as the capture agent. F, flagella; K, kinetoplast. The asterisk (*) indicates the flagellar pocket region. Bars, 1 μM.

a secreted phosphatase activity (28). The parasites grown in the presence of 10^{-6} M PAF showed two distinct patterns of cerium phosphate deposition (Fig. 5D and 5E). The most frequently observed pattern was a strong inhibition on phosphatase activity promoted by PAF, where no electrondense cerium phosphate deposits on *H. m. muscarum* external surface or within the flagellar pocket were observed (Fig. 5D). In the other pattern, PAF promoted some inhibition of this phosphatase activity, as the deposition of cerium phos-

phate was heterogeneous (Fig. 5E). These analysis confirmed our biochemical data and showed that PAF inhibited the phosphatase activity of *H. m. muscarum* intact cells. Reversible phosphorylation of proteins play a main role on the regulation of several cellular processes, including cell proliferation and differentiation in higher eukaryotic organisms (9), as well as in trypanosomatids (30). Our data suggest a relationship between cell differentiation and phosphatase activity, as it has been observed by other authors (17, 23). The precise role of ecto-phosphatases is not well established, but it has been related to cell growth, providing the cell with a source of inorganic phosphate, as well as protecting the parasite by preventing the protozoan digestion in the alimentary tract of the invertebrate host (15). Our data argue in favor of the occurrence of a specific receptor for PAF coupled to signal transduction pathway in *H. m. muscarum* similar to mammalian cells, somehow related to an ecto-phosphatase activity on the cell surface of these parasites. The same kind of PAF effect was also observed in *T. cruzi* (unpublished results). It would be extremely important to further investigate the modulation of ecto-phosphatase activities by PAF in parasites, the signal transduction pathways involved in this modulation, as well as the participation of these enzymes in the invertebrate-parasite complex.

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