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Immuno and Cytochemical Localization of *Trypanosoma cruzi* Nitric Oxide Synthase

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ABSTRACT-The localization and subcellular distribution of *Trypanosoma cruzi* nitric oxide synthase was investigated in epimastigote cells by immunocytochemistry at electron and light microscope level, using a polyclonal antibody to neuronal nitric oxide synthase, and also, at light microscope level, by the nicotinamide adenine dinucleotide phosphate-diaphorase histochemical reaction. The immunoreactivity was ultrastructurally localized by electron microscopy in the inner surface of cell membranes and in free cytosolic clusters in the body, flagellum and apical extreme. Light microscopy showed that immunoprecipitates, specific for the *Trypanosoma cruzi* nitric oxide synthase, co-localized with the formazan precipitates generated by the diaphorase reaction in the same areas identified by electron microscopy. These results, taken together with previous finding from our laboratory could help to explain the involvement of the nitric oxide transduction pathway in *T. cruzi* epimastigote motility.

Introduction

Nitric oxide (NO) is a free radical involved in signaling and cytotoxic responses in eukaryotic organisms (Moncada *et al.*, 1991). NO and citrulline are synthesized from L-arginine, oxygen and NADPH by different isoforms of the enzyme NO synthase (NOS). Two types of NOS have been described in mammalian tissues: that constitutively expressed in neural and endothelial cell, which is activated by Ca^{2+} and calmodulin, and the inducible NOS form, which is transcriptionally induced in several mammalian tissues in response to cytokines and inflammatory mediators (Marletta, 1994).

Although NOS activity was originally identified in mammalian tissues, it have been already found in metazoan such arthropods (Meyer, 1994), mollusks (Conte and Ottaviani, 1995), echinoderms (Martinez *et al.*, 1994) and plants (Ribeiro *et al.*, 1999). Recently, our laboratory demonstrated for the first time the presence of this enzyme activity in protozoa: *Trypanosoma cruzi*, the flagellate parasite responsible in humans of the Chagas' disease. In this parasite, it was found a nitric oxide transduction pathway similar to that in neural cells, integrated by putative NMDA/glutamate channel receptors, a Ca^{2+} stimulated NO synthase (tcNOS), and a nitroprusside-activable guanylyl cyclase (Paveto *et al.*, 1995). Agonists, precursor, intermediaries and effectors of this signaling pathway affect *T. cruzi* epimastigote motility (Pereira *et al.*, 1997).

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These techniques have been widely used to provide evidence of the presence of different isoforms of NOS in several eukaryotic cellular types (Lopez-Costa *et al.*, 1997).

In this study, the localization of NOS in *T. cruzi* epimastigote cells using cytochemical and immunocytochemical techniques is described.

Materials and Methods

Cell culture

T. cruzi epimastigote cells of the Tulahuen-2 strain were cultured 7 days at 28°C in a medium containing (w:v): 3.5% Bacto Liver, 1.0% tryptose, 3.0% yeast extract, 0.5% glucose, 0.8% Na_2HPO_4, 0.4% NaCl, and 0.04% KCl. The medium was supplemented with 20 mg/l hemine, 10% fetal calf serum (v:v), 0.1 mg/ml streptomycin and 100 U/ml penicillin (Wynne de Martini *et al.*, 1980). Before the addition of serum and antibiotics, the medium was adjusted to pH 7.8 and autoclaved 15 min at 118°C. Standing cultures were carried out 7 days at 28°C up to the late-exponential phase in 1-liter Erlenmeyer flasks containing 100 ml of medium. Parasites were collected by centrifugation at 1000 x*g*.

NADPH diaphorase cytochemistry

Cell pellets were washed with 0.1 M sodium phosphate buffer, pH 7.4, and then fixed in a solution containing 4% paraformaldehyde in the same buffer. After washing with 0.1 M phosphate buffer, the parasite pellet was resuspended in 1 ml of the same buffer and a drop of the cell suspension was smeared on gelatin coated glass slides which were air dried for one hour at room temperature. The slides were then incubated 1 h at 37°C in a solution containing 1 mg/ml β-NADPH and 0.02% nitro blue tetrazolium (w:v) in 0.1 M phosphate buffer containing 0.3% Triton X-100 (Vincent and Kimura, 1992). Controls were performed by omitting β-NADPH in the incubation mixture. Preparations were photomicrographed in a Zeiss Axiophot microscope using the Nomarski interference technique.

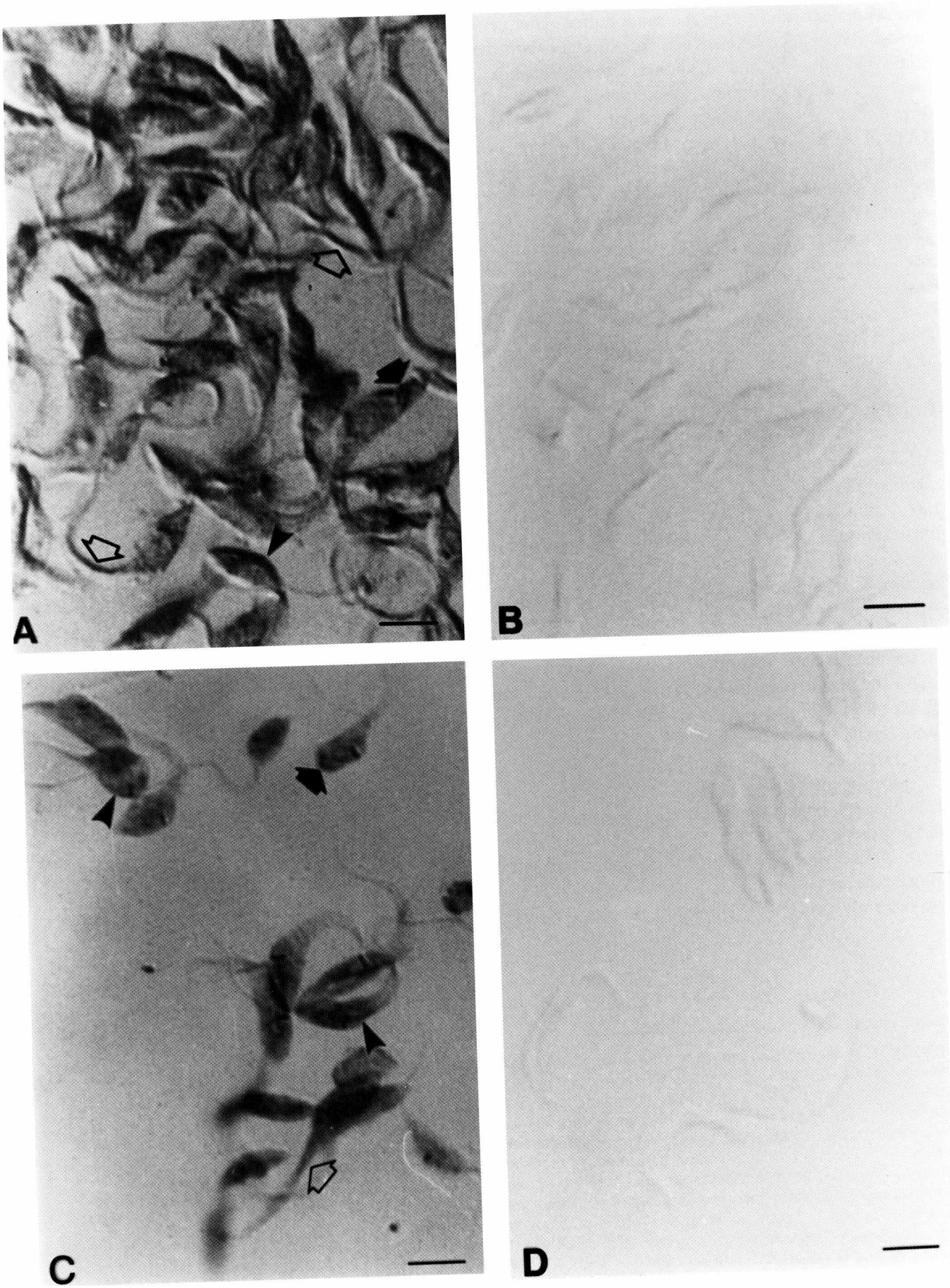
Immunocytochemistry

Parasites were processed to obtain glass smears as above mentioned. Then, for light microscope observations, smears were processed by the peroxidase/antiperoxidase (PAP) immunocytochemical procedure using an anti-human brain NOS polyclonal rabbit antibody that showed to be positive in human, rat and mouse tissue (Trasduction Laboratories, Lexington, USA.; Lloyd *et al.*, 1995). Glass smears were incubated at room temperature according to the following sequence: 10% normal sheep serum (Sigma Chemical Co., St. Louis, USA) 1 h, rabbit brain NOS antibody (dilution 1:250) at 4°C for 48 h, sheep anti rabbit IgG (Sigma; dilution 1:50) for 2 h and rabbit PAP (dilution 1:100) also at room temperature for 1 h. A washing step with phosphate buffer/saline (PBS) containing 0.2% Triton X-100 was included after each incubation. Sections were developed with a solution containing 0.05% diaminobenzidine (Sigma; w:v), 2.5% ammonium nickel sulfate (w:v), and 0.01% H_2O_2 (v:v) in PBS for 15 min at room temperature, and then dehydrated and mounted with Histomount (National Diagnostics, N. Jersey, USA). Control sections were processed following the same protocol but omitting the primary antibody. All sections were observed and photographed at light microscopy with a Zeiss Axiophot photomicroscope using the Nomarski interference technique.

Electron Microscopy

All immunocytochemical steps were performed as previously described except that immunoprecipitate pellets, instead of sections, were used. Parasites were previously washed with 0.1 M sodium phosphate buffer, pH 7.4, collected by centrifugation at 1000 xg and fixed in a solution containing 0.25% glutaraldehyde, 4%

FIGURE 1. A *T. cruzi* epimastigotes stained with the NADPHd HT; the reactivity seemed to be concentrated on inner surface of cell membranes (black arrowhead), flagellum (hollow arrow) and apical extreme (arrow). **B** Negative control. Observe absence of staining. **C** *T. cruzi* epimastigotes immunostained using a bNOS antibody. Parasites show immunoreactivity with a higher concentration attached to cell membranes (black arrowheads), flagellum (hollow arrowheads) and apical exterme (arrows). Compare with cytochemical results (Fig.1A). **D** Negative control. Observe absence of immunoreactivity. All parasites were photomicrographed using the Nomarski interference technique. **A, B, C** and **D** 2250X. Scale Bar: 5 μm.


A
B
C
D

paraformaldehyde (v:v) in the same buffer. After centrifugation pellets were incubated overnight with the anti-NOS antibody and two hours with the secondary antibody and PAP complex as above described but omitting Triton X-100 to preserve parasite ultrastructure. Control pellets were processed following the same protocol but omitting the primary antibody. Immunostained pellets were contrasted with 1% OsO_4 and 1% uranyl acetate (w:v), dehydrated and embedded in epoxy resine. Ultrathin sections were obtained from blocks containing parasite pellets. Ultrathin sections were then contrasted with lead citrate (Reynolds, 1963), and observed and photographed using a Zeiss 109 electron microscope (Priestley *et al.*, 1992).

Results

T. cruzi epimastigotes were subjected to light microscopy observation after reaction for NADPH diaphorase. Although positive diaphorase reactivity was dispersed through whole cells, a higher diaphorase reaction was found to be concentrated on inner surface of cell membranes, flagellum and the apical extreme of parasites (Fig. 1A). Negative controls showed the absence of staining (Fig. 1B). Immunocytochemistry confirmed these observations. A higher immunoreactivity was found attached to inner cell membranes, flagellum and apical extreme of parasites (Fig. 1C) whereas negative controls, showed a complete absence of immunostaining (Fig. 1D).

Electron microscopy showed dense immunoprecipitates clusters dispersed in epimastigote cytosol, but a higher immunoreaction was found concentrated in the inner surface of cell membranes, flagellar body and apical extreme (Fig. 2A and B). Also, dense NOS immunoreactive precipitate patches were clearly detected in the cell body, apical extreme and flagellum (Fig. 2A). In negative controls immunoprecipitates were absent, confirming the specificity of immunolabeling. In addition, the micrographs show some characteristic epimastigote structures: the distinctive network of microtubules subjacent to the surface membrane (Fig. 2A), the mitochondrial kinetoplast, and a flagellar pocket with the origin of flagellum (Fig. 2C).

Discussion

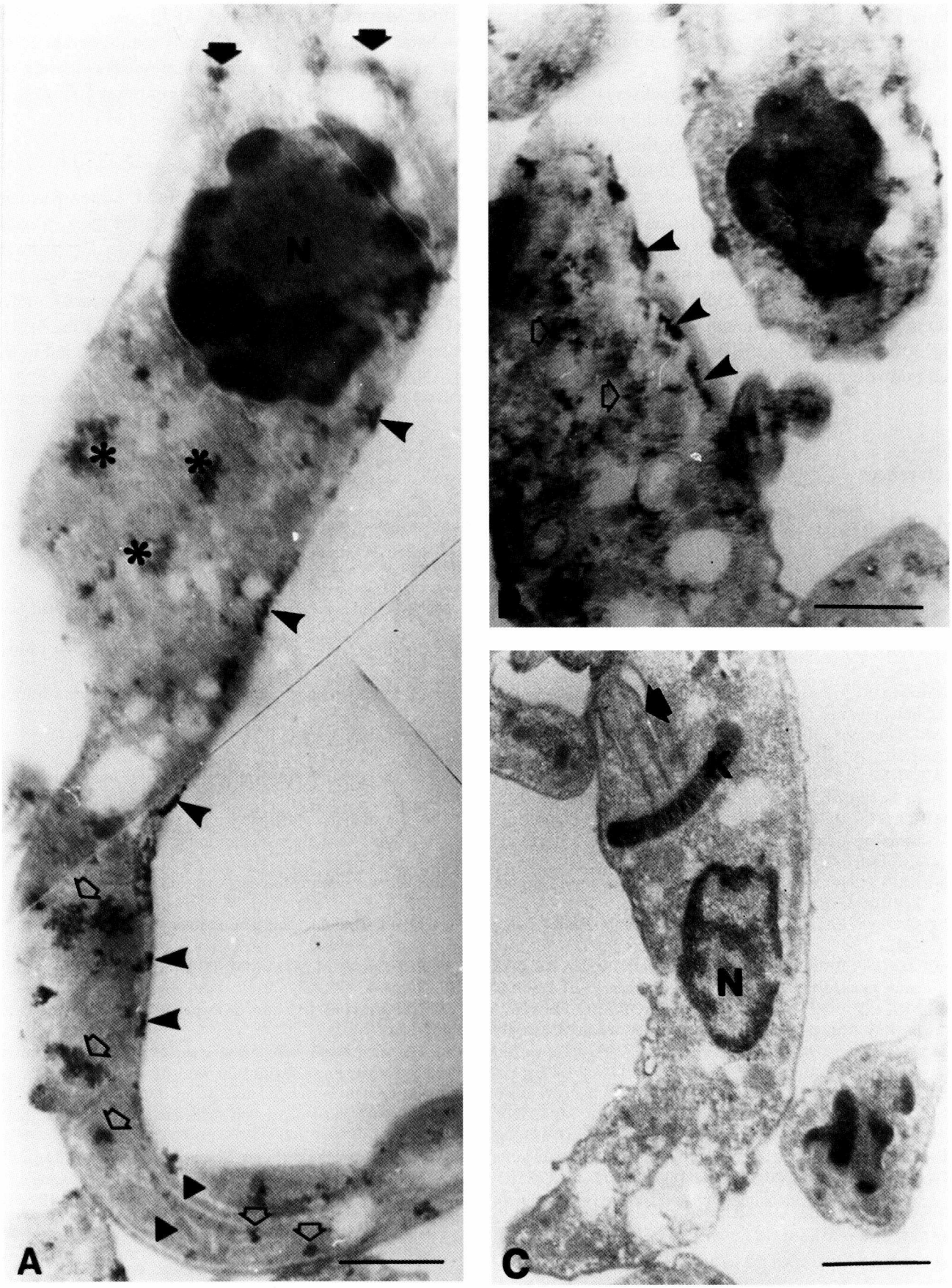
The cytochemical and immunocytochemical techniques added new information to previous biochemical evidences on the existence of nitric oxide synthase in *T. cruzi* epimastigotes (Paveto *et al.*, 1995; Pereira *et al.*, 1997).

The ultrastructural localization of *T. cruzi* NOS immunoprecipitates in inner cell membranes and flagellum support previous evidence that such pathway could play a regulatory role in epimastigote motility (Pereira *et al.*, 1997).

The NADPH-diaphorase histochemical technnique has shown to be a specific method to reveal the presence of NOS in neurons from vertebrate organisms (Hope *et al.*, 1991). Furthermore, this technique was recently employed in invertebrates to specifically detect this enzyme activity (Meyer, 1994; Martinez, 1995).

Some of the characteristics of the *T. cruzi* NOS indicated that this enzyme activity resembles the constitutive isoform of NOS (Paveto *et al.*, 1995). This suggestion is now reinforced by the fact that epimastigote NOS can be recognized by an antibody raised from mammalian brain NOS, one of the two constitutive enzyme isoforms. Further support on the existence of this enzyme in protozoan parasites was provided by the recent finding in *Leishnmania donovani* of a NOS activ-

FIGURE 2. A Electron micrograph from a longitudinal section of an entire *T. cruzi* epimastigote immunostained with a bNOS antibody. Observe a dense cluster of immunoprecipitates localized in free parasite cytosol, in the flagellum (hollow arrows), body (asterisks) and apical extreme (arrows). Also dense immunoprecipitates patches were clearly detected in inner surface of cell membranes all along the parasite (arrowheads). Black triangles show longitudinal section of flagellar microtubules. (N) nucleus. **B** Electron micrograph from the apical extreme of a *T. cruzi* epimastigote immunostained with a bNOS antibody. Observe here dense immunoprecipitate patches clearly detected in inner surface of cell membrane (arrowheads) and in cytosol clusters (hollow arrows). **C** Electron micrograph from a longitudinal section of a *T. cruzi* epimastigote. Primary antibody is omitted. Observe a complete absence of immunostaining. The epimastigote *T.cruzi* nucleus (N) kinetoplast (K) and pocket (arrow) may be observed. **A and B** 42000 X. Scale Bar: 0.4 mm. **C** 26,400. Scale Bar: 0.75 μm.


N
A
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ity with characteristics very similar to the *T. cruzi* enzyme. The *Leishmania* enzyme immunoreacted and was inhibited by an antibody raised from mammalian brain NOS (Basu *et al.*, 1997).

NOS is a soluble enzyme that possesses a PDZ code that links-attaches one extreme of the molecule to cell membrane. In the nervous system, this link ensures the localization of the enzyme close to the PSD-95 protein and to the NMDA receptor which is known as a Ca^{2+} channel responsible of the activation of the constitutive enzyme. Although the reason of this localization of NOS in the *Trypanosoma* it is not known, it may be speculated that this attachment could be related to the role of NO in parasite motility as microtubules were described highly concentrated in the flagellar axoneme and in the cell body organizing the subpellicular microtubule corset close to the cell membrane, (Gallo and Precigout, 1988; Martinez-Palomo *et al.*, 1976). Such role of NO in parasite motility was previously mentioned by some of us and now we found morphological evidence between both findings.

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