Evidence for the existence of an N_s -type regulatory protein in *Trypanosoma cruzi* membranes*

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The existence of a GTP-binding protein of the N_s type in *Trypanosoma cruzi* was explored. Epimastigote membranes were labelled by cholera toxin in the presence of [adenine- ^{14}C]NAD $^{+}$. After SDS/polyacrylamide-gel electrophoresis of extracted membrane proteins, a single labelled polypeptide band of apparent M_r approx. 45000 was detected. Epimastigote cells were treated with *N*-ethylmaleimide and electrofused to lymphoma S49 cells lacking the N_s protein. Evidence indicates that in such electrofusion-generated cell hybrids a heterologous adenylate cyclase system was reconstituted with the N_s protein provided by *T. cruzi* epimastigotes.

INTRODUCTION

Hormonal control of adenylate cyclase results from the interaction of four protein components: hormone receptors, two guanine-nucleotide-binding proteins, N_s and N_i (for enzyme stimulation and inhibition respectively), and adenylate cyclase catalytic subunits (Gillman, 1984). The N_s and N_i proteins seem to be heterotrimers of composition α , β and γ respectively. The M_r values ($\times 10^{-3}$) of vertebrate α_s , α_i , β and γ subunits are 42–45, 40–41, 35 and 5–8, respectively. Of these, the α_s and α_i polypeptides are substrates for the ADP-ribosylating activity of cholera and pertussis toxins respectively (Gillman, 1984).

Progress in the understanding of the structure and function of the adenylate cyclase system was achieved through the reconstitution of two different cell types, each one deficient in one of these components. Reconstitution may be carried out either by mixing membranes from one cell type with membrane detergent extracts from the other cell type (Ross *et al.*, 1978) or by cell membrane fusion (Orly & Schramm, 1976).

These approaches have been used in our laboratory in the study of the characteristics of the adenylate cyclase system of two lower eukaryotic organisms, *Neurospora crassa* and *Trypanosoma cruzi* (Flawiá *et al.*, 1983; Eisenschlos *et al.*, 1986), as representatives of Fungi and Protoctista Kingdoms (Whittaker & Margulis, 1978; Margulis, 1981). The catalytic subunits from both organisms interact with regulatory factors and receptors from avian erythrocytes or liver membranes, reconstituting heterologous systems showing responsiveness to isoprenaline (or glucagon) and guanosine 5'-[γ -imido]-triphosphate or fluoride (Flawiá *et al.*, 1983; Eisenschlos *et al.*, 1986). It is important to point out that *Neurospora* membranes have an adenylate cyclase that is almost inactive with the substrate MgATP, fully active with MnATP and insensitive to fluoride (Flawiá & Torres, 1972), which may indicate that *Neurospora* membranes are deficient in the guanine-nucleotide-binding protein

N_s . Properties of *T. cruzi* adenylate cyclase are similar to those of the *N. crassa* enzyme, except that the specific activity of the *T. cruzi* enzyme, measured with MgATP, is somewhat higher (Eisenschlos *et al.*, 1986). This, in turn, may indicate the presence in trypanosomatid membranes of a GTP-binding protein of the N_s type. If this is true, *T. cruzi* membranes might in theory provide enough N_s to reconstitute an isoprenaline-stimulated cyclase when fused to membranes from the lymphoma S49 cyc^{-} variant devoid of this guanine-nucleotide-binding protein. It is accepted that cell membranes from this variant provide the best assay system for N_s (Ross *et al.*, 1978).

Here we report that *T. cruzi* membranes have a 45 kDa polypeptide that can be labelled by cholera toxin in the presence of [^{14}C]NAD $^{+}$. In addition, after electrofusion of *T. cruzi* and lymphoma S49 cyc^{-} cells, an heterologous adenylate cyclase is reconstituted that is now sensitive to isoprenaline and fluoride.

MATERIALS AND METHODS

The origin of materials and the description of some general procedures used throughout this work have been given elsewhere (Flawiá *et al.*, 1983; Eisenschlos *et al.*, 1986).

Treatment of *T. cruzi* membranes with cholera toxin

T. cruzi membranes were prepared as previously described and resuspended in 1 mM-NaHCO $_3$. The membrane suspension (8 mg of protein) was incubated for 30 min at 37 °C in the presence of 20 mM-Tris/HCl buffer, pH 7.5, 2 mM-MgCl $_2$, 3 mM-dithiothreitol, 2 mM-adenine- ^{14}C]NAD $^{+}$ (5 μCi ; sp. radioactivity 500 $\mu\text{Ci}/\text{mmol}$), and 20 μg of activated cholera toxin (preincubated at a concentration of 100 $\mu\text{g}/\text{ml}$ for 20 min at 37 °C in the presence of 20 mM-dithiothreitol); the total volume was 2 ml. After incubation the mixture was diluted with 8 vol. of 1 mM-NaHCO $_3$ and centrifuged

* This paper is dedicated to Dr. L. F. Leloir on the occasion of his 80th birthday.

for 60 min at 105000 *g*. Membranes were then resuspended and centrifuged twice by the same procedure. Finally, the membrane pellet was extracted with 1% (w/v) sodium cholate for 2 h at 0 °C with magnetic stirring. After centrifugation for 60 min at 105000 *g*, a sample of the supernatant was treated for 2 min at 100 °C in the presence of 0.5 M-Tris/HCl buffer, pH 8.0, 0.6% dithiothreitol, 3% urea and 5% (w/v) SDS. Products were then subjected to SDS/polyacrylamide-gel electrophoresis (5–15% acrylamide gradient) (Hubbard & Cohn, 1975). The gel was then vacuum-dried and radioautographed.

Principles of electrofusion

Cell fusion is usually achieved *in vitro* by treating cell suspensions with inactivated Sendai virus (Okada, 1958; Harris & Watkins, 1965), lysophosphatidylcholine (Poste & Allison, 1973) or poly(ethylene glycol) (Kao & Michayluk, 1974). A main limitation of these 'fusogenic agents' is, however, the low yield and poor viability of the hybrids thus obtained.

Electrical-field-induced cell fusion has more recently been proposed as an alternative to the use of the above

fusogenic agents (Senda *et al.*, 1979; Zimmermann & Scheurich, 1981; Bisehoff *et al.*, 1982). The technique involves the orientation of cells under an asymmetric alternating-current field, a phenomenon called dielectrophoresis (Crane & Pohl, 1968; Mason & Townsley, 1971; Pohl, 1978), and the induction of the fusion process by electric breakdown pulses (Zimmermann & Scheurich, 1981; Bisehoff *et al.*, 1982). Evidence indicates that such fusion procedures yield amounts of cell hybrids that are several orders of magnitude higher than those obtained with methods using chemical fusogenic agents.

Cells

T. cruzi epimastigotes from the Tulahuen 0 strain and lymphoma S49 cyc⁻ cells were cultured and collected as indicated elsewhere (Torruella *et al.*, 1986; Eisenschlos *et al.*, 1986). In order to inactivate the catalytic component of *T. cruzi* adenylate cyclase, a cell suspension (10⁹ cells/ml) in 0.25 M-sucrose containing 5 mM-KCl was treated for 30 min at 37 °C with 1 mM-*N*-ethylmaleimide, followed by a further 10 min incubation under the same conditions in the presence of 5 mM-dithiothreitol.

T. cruzi and lymphoma cells were washed three times by suspension in 0.3 M-mannitol and centrifugation for 10 min at 6000 *g*. Finally, cells were mixed in the following two ratios of *T. cruzi* to lymphoma: 2 × 10⁹ and 5 × 10⁸ cells/ml (4:1 ratio) and 4 × 10⁹ and 5 × 10⁸ cells/ml (8:1 ratio).

Fig. 1. (a) Diagram of the electrofusion chamber with six parallel interconnected platinum electrodes of 0.3 mm diameter and (b) a block diagram of the electrofusion set-up

The ratio frequency generator was an LC oscillator tuned to produced a 600 kHz wave with variable amplitude from 0 to 60 V peak to peak. The pulse generator produced pulses up to 30 V peak with a duration of 1 μs to 10 ms. An inverted Olympus model CK microscope was used to monitor 'pearl chain' formation and cell-parasite fusion. A Tektonic 464 storage oscilloscope was used to determine the electrofusion conditions.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of proteins from *T. cruzi* membranes treated with [¹⁴C]NAD⁺ in the presence (a) or in the absence (b) of activated cholera toxin

M_r values (× 10⁻³) correspond to the following protein markers: phosphorylase *b*, 94; bovine serum albumin, 67; ovalbumin, 43; carbonic anhydrase, 30; soya-bean trypsin inhibitor, 20.1; α-lactalbumin, 14.4.

Table 1. Adenylate cyclase activities in membrane preparations from *T. cruzi*, lymphoma S49 cyc⁻ and electrofusion-generated hybrids

Additions to the assay mixtures were as follows: none (—); 10 mM-NaF (F); 10 μ M-guanosine 5'-[β -imido]triphosphate plus 0.1 μ M-isoprenaline (ISO); or 10 μ M-propranolol (PRO). Other conditions were given in the Materials and methods section. Results are means $\pm$ S.E.M.

Cells	Cell ratio	Cell treatment	Additions	Adenylate cyclase activity (pmol/30 min per mg of protein)
<i>T. cruzi</i>	—	—	—	14.5 $\pm$ 0.5
			F	21 $\pm$ 2
			ISO	14.6 $\pm$ 0.1
			ISO $\pm$ PRO	12.0 $\pm$ 0.4
<i>N</i> -Ethylmaleimide-treated <i>T. cruzi</i>	—	—	—	< 0.3
			F	3.1 $\pm$ 0.1
			ISO	< 0.3
			ISO + PRO	< 0.3
		Electrofusion	—	< 0.3
			F	2.7 $\pm$ 0.2
S49 cyc ⁻	—	—	ISO	< 0.3
			ISO + PRO	< 0.3
		Electrofusion	—	44 $\pm$ 5
			F	42 $\pm$ 2
			ISO	48 $\pm$ 1
			ISO + PRO	43 $\pm$ 1
<i>N</i> -Ethylmaleimide-treated <i>T. cruzi</i> plus S49 cyc ⁻	4:1	—	—	55 $\pm$ 4
			F	24 $\pm$ 9
			ISO	72 $\pm$ 9
			ISO + PRO	47 $\pm$ 3
		Electrofusion	—	60 $\pm$ 7
			F	45 $\pm$ 1
			ISO	60 $\pm$ 4
			ISO + PRO	54 $\pm$ 3
	8:1	—	—	29 $\pm$ 7
			F	377 $\pm$ 21
			ISO	194 $\pm$ 16
			ISO + PRO	31.6 $\pm$ 0.3
		Electrofusion	—	20 $\pm$ 3
			F	17 $\pm$ 3
			ISO	23 $\pm$ 3
			ISO + PRO	17 $\pm$ 1
			—	16 $\pm$ 1
			F	488 $\pm$ 11
			ISO	324 $\pm$ 3
			ISO + PRO	62 $\pm$ 3

Electrofusion procedure

Cell fusion was performed in a multi-electrode chamber having six parallel platinum electrodes with an average separation ($\pm 10\%$) of 100 μ m, mounted on a glass slide to allow observation with a microscope (Fig. 1a). The electrodes were connected to a function generator and a pulse generator (Fig. 1b). Both the electrode chamber and generators were designed and made in this laboratory.

The purpose of the function generator was to produce a high-frequency alternating field, resulting in cell dielectrophoresis and 'pearl chain' formation (Zimmermann & Scheurich, 1981; Bisehoff *et al.*, 1982; Senda *et al.*, 1979), and that of the pulse generator was the application of short constant-voltage pulses to induce the formation of pores within the membranes and subsequently their fusion.

A sample of the cell mixture (150 μ l) was pipetted into the chamber, between electrodes, followed by the application for 3 min of an alternating field (0.7 MHz; 650 V/cm, peak to peak. Ten successive electric pulses

(15 μ s each; 1100 V/cm field intensity) were then applied, with 2 s intervals between. During the pulse application, the alternating field was decreased to 200 V/cm. All these steps were carried out under microscopic observation at room temperature. Control suspensions containing *T. cruzi* or lymphoma cells alone were also electrofused.

Cell extracts

Material from several fusions was collected in a 15 ml conical plastic tube and chilled in ice. The suspensions were then subjected to four successive incubations for 5 min at 25 °C to allow lateral diffusion of membrane components. Each incubation was followed by chilling in ice. Thereafter, suspensions were centrifuged at 7000 *g* for 15 min. Cell pellets were then transferred with a spatula to a small ice-cooled mortar, mixed with about 2 vol. of glass Ballotini beads and ground with a pestle. The material was suspended in 1 mM-NaHCO₃ and centrifuged at 800 *g* for 15 min. The supernatants were then further centrifuged at 105000 *g* for 60 min, and the membrane pellets thus obtained were homogenized in

1 mM- NaHCO_3 to give a final protein concentration between 0.2 and 0.6 mg/ml. Control membrane pellets from cell mixtures not electrofused as well as membrane pellets from *T. cruzi* or lymphoma cells were also obtained.

Adenylate cyclase assay

The standard incubation mixtures contained 50 mM-Tris/HCl buffer, pH 7.5, 0.2 mM-3-isobutyl-1-methylxanthine, 1 mM-cyclic AMP, 2 mM- MgCl_2 , 0.5 mM- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 100–300 c.p.m./pmol), 2 mM-phosphocreatine, 0.2 mg of creatine kinase and the indicated additions (10 mM-fluoride, 10 μM -guanosine 5'- $[\beta\gamma\text{-imido}]\text{triphosphate}$, 0.1 μM -isoprenaline or 10 μM -propranolol). The final volume was 0.1 ml. Incubations were performed at 37 °C for 30 min. Procedures for stopping the reactions, purification of cyclic AMP from the mixtures and radioactivity counting are described in detail elsewhere (Flawiá *et al.*, 1983).

RESULTS AND DISCUSSION

Treatment of *T. cruzi* membranes with cholera toxin

Incubation of *T. cruzi* membranes with activated cholera toxin and $[\text{adenine-}^{14}\text{C}]\text{NAD}^+$ led to the appearance of a single labelled polypeptide product of apparent M_r approx. 45000 (Fig. 2). This value closely corresponds to that reported for the α_s subunit of the N_s guanine-nucleotide-binding protein of higher eukaryotic organisms (Gillman, 1984).

Adenylate cyclase activity in membranes from *T. cruzi*-lymphoma-S49-cell hybrids

Table 1 shows the values for adenylate cyclase specific activities in membrane preparations from *T. cruzi* cells not treated or treated with *N*-ethylmaleimide (not electrofused or electrofused) and from lymphoma S49 cyc^- cells (not electrofused or electrofused). In the presence of MgATP as substrate, adenylate cyclase from *T. cruzi* or lymphoma cells was stimulated by neither fluoride nor isoprenaline. Treatment of *T. cruzi* cells with *N*-ethylmaleimide completely abolished enzyme activity in the corresponding membrane preparation. Furthermore, adenylate cyclase in membrane preparations from lymphoma cells or from mixtures of lymphoma and *N*-ethylmaleimide-treated *T. cruzi* cells (not electrofused) also showed stimulatory responses to neither fluoride nor isoprenaline.

After electrofusion of *N*-ethylmaleimide-treated *T. cruzi* and lymphoma cells, mixed at two different ratios, the corresponding membrane preparations showed a clear stimulation of cyclase activity by fluoride or isoprenaline, indicating that an heterologous adenylate cyclase system was reconstituted. The β -adrenergic agonist effect was a specific receptor-mediated phenomenon, because it was blocked by a pharmacological concentration of the β -adrenergic antagonist propranolol. In addition, the maximal extent of stimulation by fluoride or isoprenaline was influenced by the cell ratio: the higher the proportion of *T. cruzi* cells, the larger the extent of enzyme activation. This supports the concept that *N*-ethylmaleimide-treated *T. cruzi* membranes provided limiting amounts of the factor required for the reconstitution of the heterologous system.

Fig. 3. Adenylate cyclase activity as a function of isoprenaline concentration

Membranes from electrofusion-generated hybrids were assayed in mixtures containing guanosine 5'- $[\beta\gamma\text{-imido}]\text{triphosphate}$ and the indicated isoprenaline concentrations in the absence (○) or in the presence (●) of propranolol. Other conditions were described in the Materials and methods section.

Fig. 3 shows cyclase activity in an electrofused cell mixture (4:1 ratio) as a function of isoprenaline concentration. The extent of the stimulatory response showed a maximum at 0.1 μM agonist, a value coincident with that giving maximal cyclase activation in wild-type lymphoma membranes (Ross *et al.*, 1978). Propranolol blocked the stimulation over all the range of isoprenaline concentrations explored.

The results reported here indicate that *T. cruzi* membranes have a polypeptide substrate for the ADP-ribosylating activity of cholera toxin. This polypeptide has an apparent M_r that is similar to that of the α_s subunit of the N_s adenylate cyclase regulatory protein of higher eukaryotes. In addition, evidence indicates that *T. cruzi* cells provide a regulatory component that is capable of interacting with the catalytic subunits of adenylate cyclase and with β -adrenergic receptors in the lymphoma S49 cyc^- cells. Membranes from these lymphoma cells provide an unambiguous assay system for the GTP-binding protein of the N_s type. The occurrence of this protein in *T. cruzi* might explain the fact that adenylate cyclase specific activity measured with MgATP in membranes from this parasite is higher than in *Neurospora* membranes.

These results may have some important implications in terms of the evolutionary aspects of hormone-regulated systems. Previous evidence from this laboratory indicates that *Neurospora* and *T. cruzi*, taken as representatives of the Fungi and Protoctista Kingdoms, have adenylate cyclase catalytic entities which are capable of interacting with N_s proteins from vertebrate tissues (Flawiá *et al.*, 1983; Eisenschlos *et al.*, 1986). Since these branches

diverged from the Metazoa Kingdom branch 750 million years ago (Whittaker & Margulis, 1978; Margulis, 1981), it appears that the occurrence of adenylate cyclase catalytic subunits, functionally similar to those found today in vertebrates, was almost coincident with the appearance of the eukaryotic cell structure. The occurrence of guanine-nucleotide-binding proteins of the *ras* type seems to be coincident with that of eukaryotic cells. These proteins have been described in Fungi and Protoctista (Powers *et al.*, 1984; Reymond *et al.*, 1984).

The next step is the appearance of proteins of the N_s type, the first evolutionary precedent of which is that found in the Protoctista *T. cruzi*. Fungi and Protoctista branches diverged 400 million years ago (Whittaker & Margulis, 1978; Margulis, 1981).

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