

# Free fatty acids induce cell differentiation to infective forms in *Trypanosoma cruzi*

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Intestinal extracts of *Triatoma infestans* induce cell differentiation of *Trypanosoma cruzi* epimastigotes into the infective metacyclic form. Part of this effect can be explained by the presence of haemoglobin fragments, which stimulate trypanosomal adenylate cyclase. In this work we examined the metacyclogenetic activity of lipids present in this intestinal extract. We found that lipid extracts of the intestinal extract have significant stimulatory effects that reside with the free-fatty-acid fraction, especially oleic acid. These compounds stimulate *de novo* diacylglycerol formation and protein kinase C activity in the parasite. Moreover, meta-

cyclogenesis is stimulated by phorbol esters and cell-permeant diacylglycerol, while protein kinase C down-regulation or incubation with inhibitors of this kinase abrogates this effect. These results indicate that free fatty acids are a novel signal, inducing metacyclogenesis, acting through a pathway involving diacylglycerol biosynthesis and protein kinase C activation.

Key words: epimastigote, fatty acid, kinetoplastid, metacyclic trypomastigote, protein kinase C.

## INTRODUCTION

*Trypanosoma cruzi*, the protozoan parasite that causes Chagas' disease, undergoes a complex life cycle. It replicates as intracellular amastigotes in the mammalian host and as extracellular epimastigotes in the digestive tract of insect haematophagous vectors. Epimastigotes in the insect midgut can either replicate as such, to generate additional epimastigotes, or differentiate into infective metacyclic trypomastigotes [1]. This is a non-replicative stage. Trypomastigotes are released with the faeces during the feeding of the insect on the mammalian host, thus enabling the infection of the latter and the completion of the parasite's life cycle. The decision on which course the cell takes depends on the specific set of signals it encounters in the surrounding medium. It should be noted that, as trypomastigotes are non-replicative, it is of adaptive advantage that only a fraction of the epimastigotes differentiate into the metacyclic forms at any one time. In this way perpetuation of the infection in the insect vector is ensured.

The concept of differentiation to terminal forms has similarities among kinetoplastid protozoans. Thus both in African and South American trypanosomes, the trypomastigote forms that are able to infect the next host are non-replicative, and in this respect they resemble terminal differentiation. However, upon reaching an appropriate environment, trypomastigotes can again differentiate into replicative forms [1]. The signals involved in triggering these morphological and physiological changes are likely to implicate the activation of protein kinase cascades, and a variety of complex physiological signals [2,3].

The process of metacyclogenesis has been studied by several authors. A number of *in vitro* systems have been proposed to induce metacyclogenesis, including the use of artificial triatomine urine media [4,5]. Such systems, however, are effective on sele-

cted *T. cruzi* strains only. In our laboratory, an *in vitro* system that employs *Triatoma infestans* (kissing bug) intestinal extract has been established, which proved effective in all tested strains [6]. Therefore it allows the study of the general processes modulating differentiation in *T. cruzi* [7,8]. A partial explanation for the metacyclogenetic effects of *T. infestans* intestinal extracts is that they contain peptides derived from haemoglobin breakdown, which stimulate adenylate cyclase and induce metacyclogenesis. However, the percentages of differentiation obtained with these peptides are lower than those observed with whole *T. infestans* intestinal extract [9], suggesting the occurrence of other metacyclogenetic factors.

In this work, we investigate the generation of lipid-soluble molecules from *T. infestans* intestinal extract with metacyclogenetic activity, and how they exert their action. We demonstrate that FFA (free fatty acids), present in this intestinal extract, play a key role in this process, which can be explained through the *de novo* formation of DG (diacylglycerol) and the activation of PKC (protein kinase C).

## EXPERIMENTAL

### Materials

[1-<sup>14</sup>C]Oleate (56 mCi/mmol), [<sup>32</sup>P]sodium orthophosphate (10  $\mu$ Ci/ $\mu$ l), [d-glucose-1-<sup>14</sup>C]lactose (51 mCi/mmol) and [methyl-<sup>3</sup>H]thymidine (79 Ci/mmol) were obtained from Amersham Biosciences (Piscataway, NJ, U.S.A.). OA (oleic acid), palmitic acid, stearic acid, linoleic acid, linolenic acid, soya bean PC (phosphatidylcholine) type II-S, BSA (fraction V), lipid standards, EGTA, Grace medium, carboxyfluorescein diacetate and DMSO were from Sigma (St. Louis, MO, U.S.A.). Phosphate-free

Abbreviations used: DG, diacylglycerol; dibutyryl-cAMP, N<sup>6</sup>,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate sodium salt; FFA, free fatty acids; HA1004, N-(2-guanidioethyl)-5-isoquinolinesulphonamide; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; OA, oleic acid; OAG, 1-oleoyl 2-acetyl-sn-glycerol; PC, phosphatidylcholine; PKA, protein kinase A; PKC, protein kinase C.

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RPMI 1640 medium, fetal bovine serum, bisindolylmaleimide, HA1004 [*N*-(2-guanidioethyl)-5-isoquinolinesulphonamide] and H7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine] were from Gibco-BRL (Rockville, MD, U.S.A.). Modified Grace medium is composed of Grace medium, 10 % whole egg ultrafiltrate, 7 % fetal bovine serum and 1 % albumin [10]. Chicken blood was drawn under sterile conditions with heparin as an anticoagulant. TLC plates were from Merck (Darmstadt, Germany).

### Parasites

Epimastigotes of *T. cruzi*, RA lethal strain [11], maintained by weekly passages in biphasic medium, were harvested after 48 h of culture [12] and comprised less than 1 % of the metacyclic form.

### Preparation of *T. infestans* intestinal extract

A method described previously [6] was used. Briefly, hindguts from adult triatomines were removed 48 h after feeding and homogenized in Grace medium when used as a culture supplement, or in PBS, pH 7.2. After centrifugation for 30 min at 12 000 *g* and sterile filtration of the supernatants through 0.2  $\mu$ , extracts were stored at  $-70^{\circ}\text{C}$  until used. Protein concentration was determined by the method of Lowry et al. [13] and, based on these determinations, the final intestinal extract protein concentration added to the Grace medium was adjusted to 2 mg/ml in the differentiation assays [8].

### *T. infestans* intestinal contents

These were obtained by gently squeezing the abdomen of adult triatomines 48 h after feeding. The intestinal content was then resuspended in PBS, pH 7.2. Protein concentration was determined by the method of Lowry et al. [13] and the final concentration was adjusted to 2 mg/ml.

### Lipids of chicken blood, *T. infestans* intestinal extract and *T. infestans* intestinal contents: extraction, analysis and quantitation

The method of Bligh and Dyer [14] was used for lipid extraction in all cases. The solvents were evaporated under nitrogen to a constant weight. Lipids from *T. infestans* intestinal extracts were then resuspended in DMSO for the metacyclogenesis assay.

To analyse the lipid contents of the samples, they were separated by TLC on silica gel plates using two sequential solvent systems: first, chloroform/methanol/water (65:35:2.5, by vol.) to separate polar lipids, allowed to run up to the middle of the plate, and then hexane/diethylether/acetic acid (70:30:1, by vol.), allowed to run up to the top of the plate to separate neutral lipids in the upper half [15,16]. Lipids were identified by comparison with authentic standards. Plates were then dried, sprayed with 10 %  $\text{CuSO}_4$  in 8 %  $\text{H}_3\text{PO}_4$  and charred by exposure to  $150^{\circ}\text{C}$  for 13 min [17]. The plates were scanned in a Umax-Astra 610S apparatus and densitometry was measured using SigmaGel software. Each lane was scanned three times and the average values were determined. The density of known amounts of different marker lipids was used as reference to determine the quantity of FFA present in the sample.

### Analysis of FFA present in *T. infestans* intestinal extract

Lipid extraction and separation was performed as described above. The portion of silica gel corresponding to FFA was scraped off the plate and the lipids were eluted with two portions of methanol (2 ml each). Methylation was conducted after adding 5 % (v/v)

$\text{H}_2\text{SO}_4$  for 2 h under nitrogen, in the dark at  $64^{\circ}\text{C}$ . After adding 2 vol. of water and 2 vol. of hexane, the fatty acid methyl esters extracted in the upper phase were analysed by GC using Hewlett Packard 5840A equipment, as described previously [18]. The identity of each peak was confirmed by GC/MS analysis.

### Lipid fraction from *T. infestans* intestinal extract without FFA

The remaining lipids from the silica gel, after removing the FFA mentioned above, were scraped off together from the plates and eluted with two portions of methanol (2 ml each). The solvent was evaporated under nitrogen and the lipids were finally resuspended in DMSO to be used in metacyclogenesis assays at the indicated concentrations.

### Metacyclogenesis assays

The differentiation protocol already described by Isola et al. [6] was employed. Epimastigotes ( $5 \times 10^6/\text{ml}$ ) were incubated in Grace medium or in the presence of the following compounds for 15 min at  $28^{\circ}\text{C}$ : (a) 0.5 % DMSO (vehicle), (b) *T. infestans* intestinal extract, protein concentration 2 mg/ml, (c) haemolysed chicken blood, protein concentration 2 mg/ml, (d) lipid fraction from *T. infestans* intestinal extract, 300  $\mu\text{g}/\text{ml}$ , (e) FFA from *T. infestans* intestinal extract, 300  $\mu\text{M}$ , (f) lipid fraction from *T. infestans* intestinal extract without FFA, 300  $\mu\text{g}/\text{ml}$ , (g) OA, 300  $\mu\text{M}$ , (h) OAG (1-oleoyl 2-acetyl-*sn*-glycerol), 10  $\mu\text{g}/\text{ml}$ , and (i) PMA, 32 nM. In each case, parasites were then transferred to modified Grace medium and incubated at  $28^{\circ}\text{C}$ . Growth curves were obtained by counting the parasites in a Neubauer chamber and metacyclogenesis was evaluated by parasite motility and shape in live samples and by shape and relative kinetoplast-nucleus position in wet-fixed/stained (May-Grünwald-Giemsa) preparations, every 48 h. More than 200 cells were examined in each case. In addition, determination of transialidase activity (a marker of the trypomastigote stage) in culture supernatants was performed, using sialyl-lactose as a donor and [ $^{14}\text{C}$ ]lactose as an acceptor [19]. Each result is representative of five similar experiments.

### Cell viability of parasites in the presence of FFA

To test this aspect two different approaches were used. The first was thymidine uptake and incorporation during the metacyclogenesis assay of epimastigotes stimulated with OA [20]. Parasites ( $5 \times 10^6/\text{ml}$ ) were stimulated with 300  $\mu\text{M}$  OA or 0.5 % DMSO (vehicle) for 15 min at  $28^{\circ}\text{C}$ , and then transferred to modified Grace medium and incubated at  $28^{\circ}\text{C}$ . Aliquots of the parasite suspensions ( $1 \times 10^6/\text{ml}$ ) were labelled with [*methyl*- $^3\text{H}$ ]thymidine 2  $\mu\text{Ci}/\text{ml}$  and incubated at  $28^{\circ}\text{C}$  for 2 h. After this period samples were filtered on 0.45- $\mu\text{m}$  filters. The filters were then washed twice with 0.9 % (v/v) NaCl and once with 10 % and once with 5 % (v/v) trichloroacetic acid. Radioactivity was measured in a Beckman IS liquid scintillation counter. This protocol was performed daily during the metacyclogenesis assay.

The second approach was carboxyfluorescein diacetate dyeing [21]. Epimastigotes were stimulated with 300  $\mu\text{M}$  OA or 0.5 % DMSO (vehicle), for 15 min at  $28^{\circ}\text{C}$ . After this period, 5 % (v/v) of carboxyfluorescein diacetate was added to the parasites. A UV-light microscope (Eclipse E600; Nikon) was used to visualize the cells and images recorded using Spot RT software, version 3.4 (Spot Diagnostic Instruments).

### Effect of PKC inhibitors on metacyclogenesis

Epimastigotes ( $5 \times 10^6/\text{ml}$ ) were incubated for 15 min at  $28^{\circ}\text{C}$  in Grace medium in the presence of *T. infestans* intestinal extract

(protein concentration, 2 mg/ml). Simultaneously, the following PKC inhibitors were added: (a) 15 nM bisindolylmaleimide, (b) 40  $\mu$ M HA1004, (c) 6  $\mu$ M H7 and (d) 6  $\mu$ M H7 + 40  $\mu$ M HA1004 [22–26]. In each case, parasites were then transferred to modified Grace medium and incubated at 28 °C. Growth curves and metacyclogenesis were evaluated as described above.

#### Effect of PKC down-regulation on metacyclogenesis

Epimastigotes ( $5 \times 10^6$ /ml) were incubated in Grace medium with 100 nM PMA for 24 h [27–29] at 28 °C. After this period, the compound was removed by centrifugation and metacyclogenesis was assayed after stimulation with Grace medium in the presence of *T. infestans* intestinal extract (protein concentration, 2 mg/ml) or 300  $\mu$ M OA, for 15 min at 28 °C. Parasites were then transferred to modified Grace medium and incubated at 28 °C. Growth curves and metacyclogenesis were evaluated as described above.

#### Protein phosphorylation patterns in stimulated parasites

*In vivo* activation of protein kinases was determined according to Brattsand et al. [30]. Briefly, epimastigotes ( $1 \times 10^7$ /ml, 41  $\mu$ g of protein) were incubated with 100  $\mu$ Ci/ml [ $^{32}$ P]sodium orthophosphate for 15 h, at 28 °C in RPMI 1640 phosphate-free medium. After this period, the parasites were washed three times by centrifugation and resuspended in the same volume of Grace medium. Aliquots of 500  $\mu$ l of the radiolabelled cells were incubated for 15 min at 28 °C with (a) 0.5 % DMSO (vehicle), (b) 32 nM PMA, (c) *T. infestans* intestinal extract (protein concentration, 2 mg/ml), (d) 300  $\mu$ M OA and (e) 1 mM dibutyryl-cAMP (*N*<sup>6</sup>,2'-*O*-dibutyryl-adenosine 3',5'-cyclic monophosphate sodium salt). The pellets obtained after centrifugation were resuspended in 100  $\mu$ l of Laemmli's sample buffer [31], analysed by SDS/PAGE and transferred to nitrocellulose membranes. Radioactivity was detected using the Storm Gel and Blot Imaging System (Amersham Biosciences).

#### Examination of PKC down-regulation by change in protein phosphorylation patterns

Epimastigotes ( $1 \times 10^7$ /ml, 41  $\mu$ g of protein) were incubated in Grace medium with 100 nM PMA or 0.5 % DMSO (vehicle) for 24 h [27–29] at 28 °C. After this period, the compounds were removed by centrifugation and parasites were incubated with 100  $\mu$ Ci/ml [ $^{32}$ P]sodium orthophosphate for 15 h in RPMI 1640 phosphate-free medium. The radiolabelled cells were then washed three times by centrifugation and resuspended in Grace medium alone or supplemented with *T. infestans* intestinal extract (protein concentration, 2 mg/ml) for 15 min at 28 °C. To evaluate protein phosphorylation patterns in these parasites, a similar protocol to that described above was used.

#### Radiolabelled lipid patterns of epimastigotes incubated with [ $1$ - $^{14}$ C]oleate

Epimastigotes ( $1 \times 10^7$ /ml) were labelled with 1  $\mu$ Ci/ml [ $1$ - $^{14}$ C]oleate in Grace medium for 5 or 30 min at 28 °C. The lipids were then extracted [14] and neutral and polar lipids were separated by TLC on silica gel plates, as described above. Radioactive spots were detected using the Storm Gel and Blot Imaging System.

#### Effect of *T. infestans* intestinal extract on DG biosynthesis in epimastigotes

Parasites ( $1 \times 10^7$ /ml) were incubated with 1  $\mu$ Ci/ml [ $1$ - $^{14}$ C]oleate, in Grace medium supplemented with *T. infestans* in-

testinal extract (protein concentration, 2 mg/ml) or 0.5 % DMSO (vehicle) as a control, for 30 min at 28 °C. The lipids were then extracted [14] and the neutral lipids separated by TLC on silica gel plates, using hexane/diethylether/acetic acid (70:30:1, by vol.). Radioactive spots were detected using the Storm Gel and Blot Imaging System. The plates were also charred as describe above and total DG mass quantified by densitometry, using Sigma Gel software.

#### Effect of OA on DG metabolic labelling in epimastigotes

Parasites ( $1 \times 10^7$ /ml) were stimulated with 10, 30, 100 or 300  $\mu$ M OA or 0.5 % DMSO (vehicle) for 15 min at 28 °C and then exposed to a 30-min pulse of 1  $\mu$ Ci/ml [ $1$ - $^{14}$ C]oleate. Lipids were extracted [14] and two solvent systems were used to investigate labelling patterns. In the first, separation of polar and neutral lipids system described previously was used [15]. Briefly polar lipids were first separated on the lower half of the TLC plates with chloroform/methanol/water (65:35:2.5, by vol.), and then neutral lipids were separated by developing the chromatograms to the top of the plates with the solvent system used for the neutral lipid separation described above. The second solvent mixture was the one described by Takuwa et al. [32], which allows complete separation of DG from cholesterol, and employs benzene/chloroform/methanol (80:15:5, by vol.). Quantification of lipid spots was conducted with the Storm Gel and Blot Imaging System.

#### Fatty acid uptake assay

Epimastigotes ( $1 \times 10^7$  or  $1 \times 10^8$ /ml) were incubated in a mixture of 1  $\mu$ Ci/ml [ $1$ - $^{14}$ C]oleate plus 300  $\mu$ M unlabelled OA, in Grace medium, for 30 min at 28 °C. For a control, vehicle without parasites was used. Incubation was terminated by centrifugation and the radioactivity remaining in the supernatants was measured. The loss of radioactivity in the parasite containing samples, was used to evaluate the amounts of OA taken up by the cells [33].

#### Statistical analysis

The statistical significance of the results was analysed using the Student's *t* test.

## RESULTS

In this study the metacyclogenetic lipid factors present in *T. infestans* intestinal extract were examined. Most of the intestinal contents arise from the blood meal ingested by the insect. Therefore, we first investigated the effects of whole chicken blood on *T. cruzi* cell differentiation and found that it is devoid of metacyclogenetic activity. By contrast, *T. infestans* intestinal extracts obtained after 48 h of feeding acquired significant activity (Table 1). Whole-lipid fractions from this extract were tested in the *in vitro* assay showing significant metacyclogenetic effect, amounting to about half of that observed with the complete intestinal extract (Table 1).

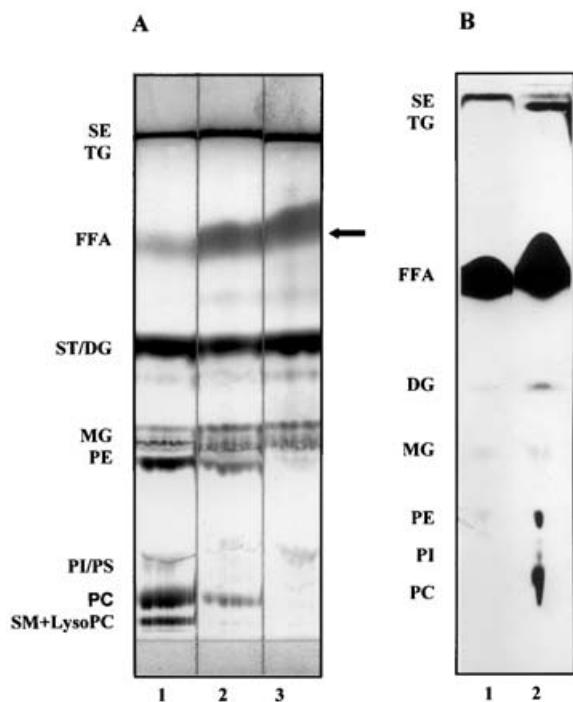
We studied the lipid composition of intact chicken blood and that of *T. infestans* intestinal extract using TLC. Striking differences were observed: in particular, the amount of FFA was 4-fold higher in the intestinal extract than in chicken blood (300 versus 75  $\mu$ M, respectively; Figure 1A). In addition, phospholipids were strikingly reduced. By contrast, no differences were detected in the amounts of other lipids like sterols, triacylglycerol and steryl esters. If, instead of whole intestinal extract, only the insect gut contents were analysed, almost no phospholipids were found (Figure 1A, lane 3).

**Table 1** FFA have significant effects on *T. cruzi* metacyclogenesis

Total lipid extracts from metacyclogenetic intestinal preparations induced significant differentiation on epimastigotes into infective metacyclic trypomastigotes. When the lipid extracts were analysed by TLC and tested separately in the *in vitro* metacyclogenesis assay described in the Experimental section, only FFA displayed metacyclogenetic activity. Epimastigote suspensions ( $5 \times 10^6$ /ml) having less than 1% metacyclic forms, were incubated in Grace medium in the presence of the following compounds for 15 min at 28 °C, after which they were transferred to modified Grace medium and incubated at 28 °C. Control, 0.5% DMSO; IE, *T. infestans* intestinal extract (protein concentration, 2 mg/ml); CB, haemolysed chicken blood (protein concentration, 2 mg/ml); LF, lipidic fraction from *T. infestans* intestinal extract (300 µg/ml); FFA, FFA present in *T. infestans* intestinal extract (300 µg/ml); LF<sub>r</sub>, lipidic fraction from *T. infestans* intestinal extract without FFA (300 µg/ml); LF<sub>r</sub> + FFA, 300 µg/ml; OA, 300 µM OA. Values recorded correspond to day 8 of the growth/differentiation curves in each case and are the means  $\pm$  S.D. from five samples.

Stimulus	Total parasites ( $\times 10^6$ /ml)	Metacyclics ( $\times 10^6$ /ml)	Metacyclogenesis (%)
Control	81.0 $\pm$ 3.2	2.8 $\pm$ 0.6	3.5 $\pm$ 0.7
IE	80.5 $\pm$ 3.0	53.1 $\pm$ 4.4	66.0 $\pm$ 5.5*
CB	80.7 $\pm$ 2.9	4.8 $\pm$ 1.2	5.9 $\pm$ 1.5
LF	80.5 $\pm$ 2.7	24.2 $\pm$ 3.7	30.1 $\pm$ 4.7*
FFA	80.6 $\pm$ 2.1	24.6 $\pm$ 1.9	30.5 $\pm$ 2.3*
LF <sub>r</sub>	80.4 $\pm$ 2.2	5.6 $\pm$ 1.8	6.9 $\pm$ 2.2
LF <sub>r</sub> + FFA	80.9 $\pm$ 2.5	25.2 $\pm$ 2.9	31.1 $\pm$ 3.6*
OA	79.9 $\pm$ 2.1	22.0 $\pm$ 3.3	27.5 $\pm$ 4.1*

\*  $P < 0.01$ ; agents that induced statistically significant increases in metacyclic trypomastigotes with respect to their corresponding control values.

**Figure 1** Phospholipids are rapidly deacylated in the gut of the insect vector and the FFA rapidly incorporated into *T. cruzi* lipids

(A) FFA accumulate in the gut of the insect vector upon digestion. TLC of total lipids from (lane 1) intact chicken blood, (lane 2) *T. infestans* intestinal extract, 48 h after a chicken-blood meal, and (lane 3) *T. infestans* intestinal contents at the same time point. Plates were charred for visualization. Note the breakdown of polar lipids in the intestinal tract, with accumulation of FFA (arrow). (B) *T. cruzi* epimastigotes rapidly incorporate FFA into cellular lipids. Autoradiogram of a TLC separation of total epimastigote lipids after labelling the parasites with [<sup>14</sup>C]oleate (1 µCi/ml) for 5 min (lane 1) and 30 min (lane 2). LysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphoserine; MG, monoacylglycerol; ST/DG, free sterols + DG; TG, triacylglycerol; SE, steryl esters; SM, sphingomyelin.

As shown in Table 1, the lipid fraction had metacyclogenetic effects. This was not accompanied by any toxic action, since total cell numbers showed a similar increase to control cultures. The remarkable accumulation of FFA in this preparation suggested that these lipids could account for the metacyclogenetic effect induced by the lipid fraction. This hypothesis was tested by comparing the metacyclogenetic effects of FFA isolated by TLC with those of the remainder lipids eluted together (Table 1). Only the FFA fraction displayed metacyclogenetic activity. Re-addition of FFA to the rest of the lipids of the intestinal extract fully restored the biological activity. GC analysis of the fraction corresponding to FFA obtained after methylation indicated that OA was the most abundant component (35.9%), followed by stearic acid (34.3%). Other components of that fraction were palmitate (19.3%), linoleate (9.6%) and linolenate (0.9%). If authentic OA, the most abundant of the FFA observed, was added to the cultures at comparable concentrations to the FFA found in *T. infestans* intestinal extract, a similar level of differentiation was observed (Table 1). A metacyclogenetic role for FFA is clearly demonstrated by these experiments.

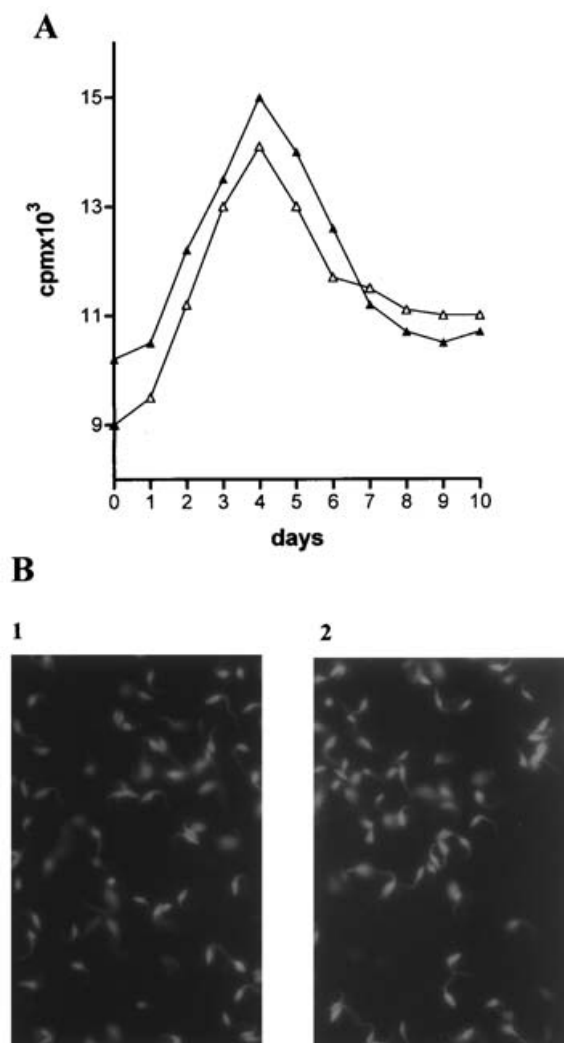
Parasites, in the presence of 300 µM OA, remain functionally and morphologically viable. As shown in Figure 2(A), thymidine incorporation in epimastigotes, in the presence of OA, is similar to that of the control samples. Moreover, carboxyfluorescein diacetate staining clearly demonstrates the integrity of the cell membrane (Figure 2B). Viability is also indicated by parasite motility. Thus OA lacks toxic effect at the levels used in our experiments.

We examined *T. cruzi* fatty acid uptake from the medium at the biologically relevant concentration observed here, i.e. 300 µM OA. We found that parasite suspensions of  $1 \times 10^7$  cells/ml were able to capture  $18.0 \pm 1.9\%$  of OA in 30 min from the medium. Increasing the cell density 10-fold, to  $1 \times 10^8$  cells/ml, only resulted in a doubling of the fatty acid removal from the medium ( $38.6 \pm 2.4\%$ ).

We examined the possible mechanism through which FFA could induce metacyclogenesis. To address this matter we investigated the metabolic fate of OA in epimastigotes. Radiolabelled [<sup>14</sup>C]oleate rapidly incorporates into cellular lipids (Figure 1B). When the cells were stimulated with *T. infestans* intestinal extract, DG labelling was particularly enhanced (Figure 3A). Thus an effect of this extract is to stimulate *de novo* DG biosynthesis. If, instead of *T. infestans* intestinal extract, epimastigotes were stimulated with an equivalent amount of OA, a similar increase in DG biosynthesis, of about 3-fold, was observed (Figure 3B, lane 5). Accompanying DG biosynthesis a significant rise in the level of PC was found. Therefore, the increase of DG formation by the intestinal extract (Figure 3A) can be explained on the basis of its FFA contents.

In addition to the radiotracer experiments we investigated whether the DG mass was also enhanced by stimulation with OA. We used the solvent system of Takuwa et al. [32] to separate DG from sterols using TLC. The results are shown in Figure 4. It is evident that DG mass was increased concomitantly with labelling upon stimulation with OA (about 3-fold).

The possibility that the observed rise in cellular DG, due to *de novo* biosynthesis, could result in the activation of PKC, was tested using a number of different approaches. First, we evaluated the effect of different PKC activators on metacyclogenesis. We used PMA and the cell-permeant DG, OAG, which stimulate this kinase *in vivo* and *in vitro* [34–36]. Table 2 shows that both compounds induce significant metacyclogenesis. Second, we tested whether PKC inhibitors could block the metacyclogenetic effects of *T. infestans* intestinal extract. Table 2 shows that strong inhibition was obtained with bisindolylmaleimide, as well as the

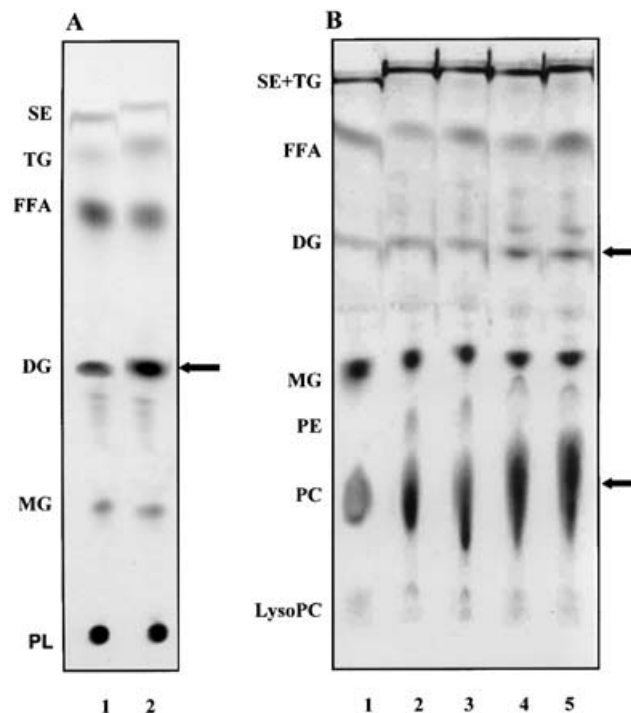


**Figure 2** OA does not affect cell viability of parasites at the concentrations used

(A) [*methyl*-<sup>3</sup>H]Thymidine uptake and incorporation during the metacyclogenesis assay. Parasites were stimulated with 300 μM OA or 0.5% DMSO (vehicle) for 15 min at 28 °C and transferred to modified Grace medium at 28 °C. Aliquots of parasites were labelled with 2 μCi/ml [*methyl*-<sup>3</sup>H]thymidine and incubated at 28 °C for 2 h. After this period, radioactivity was measured. This protocol was performed daily for 10 days. The points represent the means from three parallel experiments, with differences between samples being less than 15%. ▲, Parasites stimulated with 300 μM OA; △, 0.5% DMSO. (B) Carboxyfluorescein diacetate dyeing. Epimastigotes were stimulated with 300 μM OA or 0.5% DMSO (vehicle) for 15 min at 28 °C. After this period, 5% (v/v) carboxyfluorescein diacetate was added to the parasites. UV light was used to visualize the cells and images were recorded. Panel 1, epimastigotes stimulated with 300 μM OA; panel 2, 0.5% DMSO.

combination of H7 and HA1004. It should be noted that separately the inhibitory activity of the two latter compounds was relatively low. Higher concentrations had toxic effects, precluding their use in the assay on live cells. However, H7 and HA1004 act on different sites of PKC [25,26]. Thus their combination could block the enzyme through additive mechanisms, explaining the considerably greater effect of their joint addition.

Direct evidence of stimulation of PKC by the intestinal extract and FFA on live cells was obtained by comparing protein phosphorylation patterns. We used parasites stimulated with PMA, *T. infestans* intestinal extracts or OA. Figure 5(A) shows that OA and



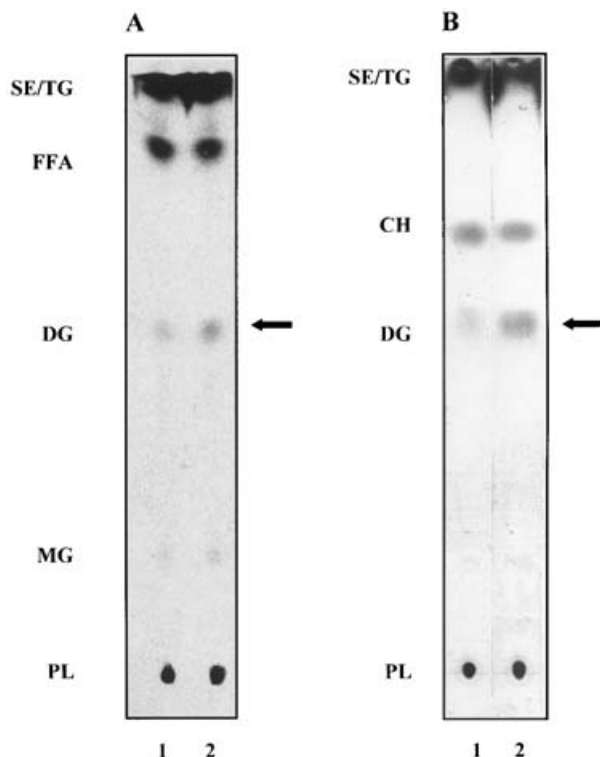
**Figure 3** *T. infestans* intestinal extract and OA have marked effects on lipid biosynthesis, particularly PC and DG, in *T. cruzi* epimastigotes

Autoradiograms of total lipids from [<sup>14</sup>C]oleate-labelled epimastigotes after incubation with (A) *T. infestans* intestinal extract (lane 2) or (B) 10 μM (lane 2), 30 μM (lane 3), 100 μM (lane 4) and 300 μM (lane 5) OA. Lanes 1 correspond to control (epimastigotes without stimulation, 0.5% DMSO). PL, polar lipids; other abbreviations are as in Figure 1.

PMA induced similar phosphorylation patterns, which were also found in the cells stimulated by *T. infestans* intestinal extracts. In particular, bands of approximate molecular masses of 180, 120 and 26 kDa were conspicuously phosphorylated in all these cases. In the 50-kDa region, different profiles between PMA, *T. infestans* intestinal extracts and OA were observed. We also analysed the phosphorylation profile obtained after the incubation of the cells with dibutyl-*c*-AMP, a permeant analogue of *c*-AMP, which activates PKA (protein kinase A; Figure 5A, lane 5). In this case, we observed a different protein-phosphorylation pattern, with a major band of approx. 38 kDa. Thus *T. infestans* intestinal extracts and OA act mainly through the *in vivo* stimulation of PKC. Finally, the importance of the role of PKC was shown by experiments in which this kinase was down-regulated by prolonged exposure to PMA (24 h) [27–29]. In these cells, metacyclogenesis induced by either *T. infestans* intestinal extracts or OA was abrogated (Table 2). PKC down-regulation was documented by the lack of the characteristic enhanced protein-phosphorylation pattern found in control cells (Figure 5B). Collectively, these results strongly support a key role for PKC activation by the physiological metacyclogenetic stimuli found in *T. infestans* intestinal extracts.

## DISCUSSION

In the present study, we found that lipids from the digested blood meal have a metacyclogenetic effect. Our experiments show that this action is due to the FFA present among these lipids. Such effects are not the result of a non-specific membrane-perturbing action or specific toxicity on epimastigotes. This is supported by the fact



**Figure 4** Parasites stimulated with OA increase their DG levels

Parasites ( $1 \times 10^7$ /ml) were stimulated with  $300 \mu\text{M}$  OA or 0.5% DMSO (vehicle) for 15 min at  $28^\circ\text{C}$  and then exposed to a 30-min pulse of  $1 \mu\text{Ci/ml}$  [ $1\text{-}^{14}\text{C}$ ]oleate. Lipids were extracted and separated by TLC using the solvent system benzene/chloroform/methanol (80:15:5, by vol.). This TLC plate was revealed in two different ways: (A) autoradiography and (B) charring. Lanes 1, 0.5% DMSO; lanes 2, parasites stimulated with  $300 \mu\text{M}$  OA.

that the total cell numbers and viability are similar in control and stimulated parasites. The results documenting this point include unaltered thymidine incorporation, carboxyfluorescein diacetate staining and parasite motilities. Thus differentiation, and not selective destruction of epimastigotes, underlies the relative decrease of this cellular stage, as well as the corresponding increase in trypomastigote forms. It is likely that the fatty acids in our experimental conditions are bound to either serum proteins or BSA present in the differentiation medium, preventing membrane disruption. We conclude that the effects of the FFA in the intestinal medium is accurately reproduced in the *in vitro* experimental system used here, i.e. FFA effects appear as a physiological stimulus for cell differentiation in *T. cruzi*.

It is noteworthy that intact chicken blood lacks metacyclogenetic activity. This appears only after a period of digestion in the insect gut. We found that the accumulation of FFA is accompanied by a virtually complete disappearance of phospholipids suggesting high phospholipase levels in the intestinal medium. In this way, this digestive process appears to link the physiology of the insect vector to the completion of the cell cycle in *T. cruzi*.

What is the mechanism by which FFA induce metacyclogenesis? These lipids have been shown previously to be efficiently incorporated into *T. cruzi* epimastigotes [18,37]. Here we have found evidence that only a fraction of the OA present in the medium is taken up by the cells, and a 10-fold increase in cell density only doubles OA uptake. These results suggest that most of the FFA are tightly bound to the proteins from the medium and not readily available to the cells. This could explain the relatively high

**Table 2** PKC agonists and antagonists have marked effects on *Trypanosoma cruzi* metacyclogenesis

Epimastigote suspensions ( $5 \times 10^6$ /ml) were incubated in Grace medium in the presence of the following compounds. Control, 0.5% DMSO; IE, *T. infestans* intestinal extract (protein concentration, 2 mg/ml); OAG,  $10 \mu\text{g/ml}$  OAG; PMA, 32 nM PMA for 15 min at  $28^\circ\text{C}$ , after which they were transferred to modified Grace medium and incubated at  $28^\circ\text{C}$ . To evaluate the effect of PKC inhibitors on metacyclogenesis, epimastigotes ( $5 \times 10^6$ /ml) were incubated for 15 min at  $28^\circ\text{C}$  in Grace medium in the presence of *T. infestans* intestinal extract (protein concentration, 2 mg/ml) with the following compounds. IE + Bm, 15 nM bisindolylmaleimide; IE + H7,  $6 \mu\text{M}$  H7; IE + HA1004,  $40 \mu\text{M}$  HA1004; IE + H7 + HA1004,  $6 \mu\text{M}$  H7 +  $40 \mu\text{M}$  HA1004. All the inhibitors were assayed at non-toxic concentrations. To evaluate the effect of down-regulation, epimastigotes ( $5 \times 10^6$ /ml) were incubated with 100 nM PMA for 24 h at  $28^\circ\text{C}$ . PMA was then removed by centrifugation and parasites were stimulated with Grace medium supplemented with *T. infestans* intestinal extract [protein concentration, 2 mg/ml; PMA (24 h) + IE] or  $300 \mu\text{M}$  oleic acid [PMA (24 h) + OA], for 15 min at  $28^\circ\text{C}$ , transferred to modified Grace medium and incubated at  $28^\circ\text{C}$ . Values recorded correspond to day 8 of the growth/differentiation curves in each case and are the means  $\pm$  S.D. from five samples.

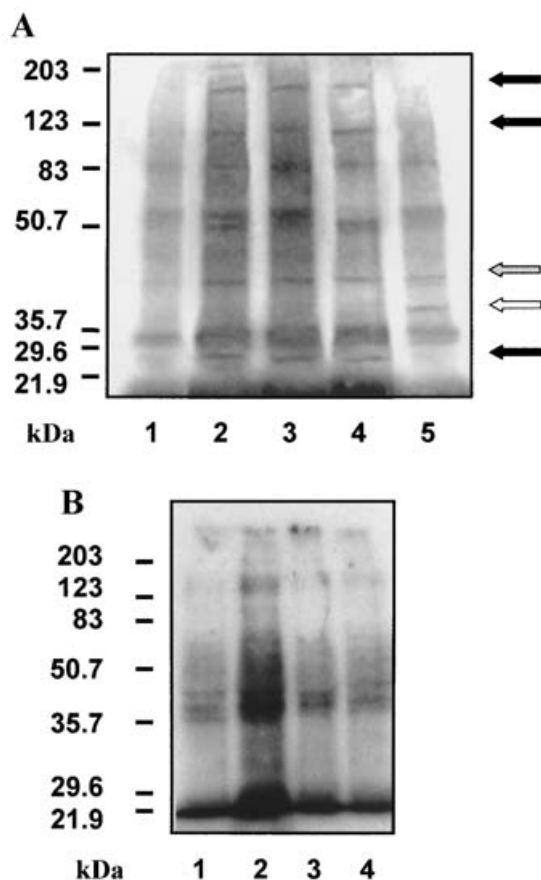
Stimulus	Total parasites ( $\times 10^6$ /ml)	Metacyclics ( $\times 10^6$ /ml)	Metacyclogenesis (%)
Control	$80.2 \pm 0.8$	$6.3 \pm 0.6$	$7.8 \pm 0.7$
IE	$80.7 \pm 2.1$	$51.2 \pm 3.9$	$63.5 \pm 4.8^*$
OAG	$81.0 \pm 1.9$	$43.2 \pm 4.2$	$53.3 \pm 5.2^*$
PMA	$80.5 \pm 2.0$	$36.2 \pm 4.7$	$45.0 \pm 5.8^*$
IE + Bm	$80.9 \pm 3.1$	$31.9 \pm 2.8$	$39.4 \pm 3.5^\dagger$
IE + H7	$78.6 \pm 2.2$	$43.8 \pm 3.1$	$55.7 \pm 3.9$
IE + HA1004	$79.9 \pm 3.0$	$39.0 \pm 3.8$	$48.8 \pm 4.7^\dagger$
IE + H7 + HA1004	$80.7 \pm 2.8$	$14.8 \pm 5.9$	$18.4 \pm 7.3^\dagger$
PMA (24 h) + IE	$81.0 \pm 0.9$	$14.7 \pm 2.8$	$18.1 \pm 3.4^\dagger$
PMA (24 h) + OA	$78.9 \pm 1.5$	$14.1 \pm 1.5$	$17.3 \pm 1.9$

\* Agents that induced statistically significant increases in metacyclic trypomastigotes with respect to their corresponding control values ( $P < 0.01$ ).

† Agents that induced statistically significant inhibition of metacyclogenesis ( $P < 0.01$ ).

concentrations of OA required for the metacyclogenetic effects. It is however noteworthy that comparison of OA uptake in *T. cruzi* with that of several mammalian cultured cell lines indicates that this parasite has a remarkably higher ability to take up OA. For instance, it has been reported that macrophages, a cell type with a high rate of fatty acid uptake, exposed to unbound  $50 \mu\text{M}$  OA, take up around  $86.0 \pm 7.2$  nmol/mg of protein in 30 min [33]. By contrast, *T. cruzi* epimastigotes take up around 15 times more OA in the same period ( $1350.0 \pm 37.0$  nmol/mg of protein in 30 min). Precise conclusions from this comparison are difficult because of the fact that the exact amount of unbound fatty acid in our differentiation medium (*T. infestans* intestinal extract) is not known. However, our uptake experiments suggest that the order of magnitude of unbound extracellular FFA appear comparable with those present in the experiments of Augé et al. [33].

Cell shape is an important consideration when analysing plasma-membrane surface amounts per cell. Indeed, an important difference between cultured mammalian cells and epimastigotes is that the latter have a thread-like slender shape with a long flagellum. Therefore, epimastigotes offer a higher surface of plasma membrane/mg of protein exposed to the medium than mammalian cells do. This difference probably enhances the chances of FFA diffusion by a spontaneous flip-flop process in *T. cruzi*. However, the striking rates of OA acquisition by this parasite strongly suggests the presence of specific fatty acid transporters, like those described in other cell types, such as fatty acid translocase/CD36 and others [38,39]. Fatty acid transporters in *T. cruzi* emerge clearly as an important area now open to research.



**Figure 5** Protein-phosphorylation analysis reveals that PKC is involved in *T. cruzi* metacyclogenesis

(A) Effect of metacyclogenesis agonists. [ $^{32}$ P]Sodium orthophosphate-labelled epimastigotes were incubated with (lane 1) 0.5% DMSO (vehicle), (lane 2) 32 nM PMA, (lane 3) *T. infestans* intestinal extract (protein concentration, 2 mg/ml), (lane 4) 300  $\mu$ M OA or (lane 5) 1 mM dibutyryl-cAMP, for 15 min, after which protein-phosphorylation patterns were analysed by SDS/PAGE and autoradiography. Black arrows indicate PKC-phosphorylated proteins, the grey arrow shows differences in the 50-kDa region and the white arrow indicates PKA-phosphorylated proteins. (B) Effect of PKC down-regulation in *T. cruzi* epimastigotes. [ $^{32}$ P]Sodium orthophosphate-labelled epimastigotes were subjected to the following treatments (all at 28  $^{\circ}$ C): (lane 1) 0.5% DMSO (vehicle) for 15 min, (lane 2) *T. infestans* intestinal extract (protein concentration, 2 mg/ml) for 15 min, (lane 3) PKC down-regulation by exposure to 100 nM PMA for 24 h, (lane 4) PKC down-regulation (100 nM PMA, 24 h) followed by exposure to *T. infestans* intestinal extract for 15 min. Protein-phosphorylation patterns were analysed as above. Note the lack of phosphorylation enhancement upon incubation with *T. infestans* intestinal extract in PKC down-regulated parasites. Molecular masses of protein standards are indicated on the left.

We have found that, associated with the addition of FFA or *T. infestans* intestinal extract, strong stimulation of *de novo* DG biosynthesis is observed. This is also reflected in an increase in DG mass (Figures 3 and 4), demonstrating that cellular DG levels are significantly elevated. Concomitantly PC formation is strongly enhanced. Previous work from other authors has shown that *de novo* DG biosynthesis can stimulate PKC [40–42]. This seems to be the mechanism of action of FFA in *T. cruzi*. Therefore, *de novo* DG formation appears to have arisen early in the evolution of eukaryotic cells as a mechanism for DG second-messenger generation.

The activity of a compound as a cell signal requires the existence of an efficient termination mechanism. In our experiments we found that OA has strong stimulatory effects on PC formation.

This is consistent with a pathway that generates DG ending in the biosynthesis of PC. The latter thus appears to be a key DG-signal-elimination mechanism. DG signal termination by conversion to PC has been demonstrated in mammalian cells [15,16]. This also seems to be the case in *T. cruzi*, therefore suggesting an ancient appearance of this mechanism as well.

Cell differentiation is known to be modulated by PKC in different cell types. For instance, stimulation by phorbol esters induces terminal differentiation in HL-60 leukaemia cells [43] and in keratinocytes [44].

The rise in cellular DG observed in this work suggested a possible role of PKC in the regulation of metacyclogenesis in *T. cruzi*. This hypothesis was investigated in different ways. These include the direct demonstration of metacyclogenetic activity of known PKC agonists, the blockade of the process by inhibitors of the kinase as well as down-regulation of PKC on prolonged exposure to phorbol esters. Moreover, comparison of the phosphorylation patterns induced by OAG, OA and *T. infestans* intestinal extract, show striking similarities, especially at the level of the bands corresponding to 180, 120 and 26 kDa. Some differences are observed in the bands of the region of about 50 kDa. These differences may reflect activation of different isoforms of the kinase, or the occurrence of a variety of stimulating factors present in the *T. infestans* intestinal extract, which could account for the multiplicity of bands observed in this case. Our results, however, allow us to conclude that stimulation of PKC is indeed a key step in the metacyclogenetic process.

We also analysed the phosphorylation profile obtained after the incubation of the cells with dibutyryl-cAMP, a permeant analogue of cAMP, which activates PKA (Figure 5A, lane 5). In this case, no PKA activation is observed on exposure to the *T. infestans* intestinal extract. After addition of dibutyryl-cAMP, we observed a different protein-phosphorylation pattern, with a major band of approx. 38 kDa. These results indicate that *T. infestans* intestinal extract and OA mainly act through the *in vivo* stimulation of PKC and that the PKA pathway does not appear to be activated in these cells. It is possible that activation of the PKA pathway, observed on exposure to isolated haemoglobin fragments [9], is blocked in the presence of the PKC-activating stimuli. Thus activation of one signalling pathway may result in the blocking of an alternative one, leading to the same differentiating effects.

In conclusion, our results strongly point to FFA, and particularly OA, as a novel metacyclogenetic signal. These lipids appear to arise as a result of the digestion of blood meal lipids, and exert their action by the generation of DG, to activate trypanosomal PKC. Thus differentiation of *T. cruzi* is here shown to critically involve PKC, providing the first evidence for a biological role of this kinase in a kinetoplastid protozoan.

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## REFERENCES

- 1 Brener, Z., Andrade, Z. A. and Barral-Netto, M. (2000) O parasito e sua interaçao com os hospedeiros. In *Trypanosoma cruzi* e Doença de Chagas, 2nd edn (Guanabara Koogan, S. A., ed.), pp. 41–47. Editora Guanabara Koogan S. A., Rio de Janeiro
- 2 Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature (London)* **334**, 661–665
- 3 Black, J. D. (2000) Protein kinase C-mediated regulation of cell cycle. *Frontiers Biosci.* **5**, 406–423
- 4 Isola, E. L. D., Lammel, E. M. and González Cappa, S. M. (1983) Influencia de la hemolinfa de *Triatoma infestans* en la morfogénesis del *Trypanosoma cruzi*. *Rev. Arg. Microbiol.* **15**, 181–185

- 5 Contreras, V. T., Morel, C. M. and Goldemberg, S. (1985) Stage specific gene expression precedes morphological changes during *Trypanosoma cruzi* metacyclogenesis. *Mol. Biochem. Parasitol.* **14**, 83–96
- 6 Isola, E. L. D., Lammel, E. M. and González Cappa, S. M. (1986a) *Trypanosoma cruzi*: differentiation after interaction of epimastigotes and *Triatoma infestans* intestinal homogenate. *Exp. Parasitol.* **62**, 329–335
- 7 Isola, E. L. D., Lammel, E. M. and Redruello, M. (1989) *Trypanosoma cruzi*: conditions required to improve metacyclic differentiation in axenic culture. *Rev. Arg. Microbiol.* **21**, 9–14
- 8 Isola, E. L. D., Lammel, E. M., Giovaniello, O., Katzin, A. M. and González Cappa, S. M. (1986) *Trypanosoma cruzi* morphogenesis: preliminary purification of an active fraction from hemolymph and intestinal homogenate of *Triatoma infestans*. *J. Parasitol.* **72**, 467–469
- 9 Fraidenaich, D., Peña, C., Isola, E. L. D., Lammel, E., Coso, O., Díaz Añel, A., Pongor, S., Baralles, F., Torres, H. and Flawiá, M. (1993) Stimulation of *Trypanosoma cruzi* adenylyl cyclase by an  $\alpha^{\text{P}}$ -globin fragment from *Triatoma* hindgut. Effect on differentiation of epimastigote to trypomastigote forms. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10140–10144
- 10 Junker, C. E., Vaughn, J. and Cory, J. (1967) Adaptation of an insect cell line (Grace's Antheraea cells) to medium free of insect hemolymph. *Science* **155**, 1565–1566
- 11 González Cappa, S. M., Bijovsky, A. T., Freilij, H., Muller, L. A. and Katzin, M. A. (1981) Aislamiento de una cepa de *Trypanosoma cruzi* a predominio de formas delgadas en la Argentina. *Medicina (Buenos Aires)* **41**, 119–120
- 12 Isola, E. L. D., Lammel, E. M., Katzin, V. J. and González Cappa, S. M. (1981) Influence of organ extracts of *Triatoma infestans* on differentiation of *Trypanosoma cruzi*. *J. Parasitol.* **67**, 53–58
- 13 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
- 14 Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917
- 15 Florin-Christensen, J., Florin-Christensen, M., Delfino, J. M., Stegmann, T. and Rasmussen, H. (1992) Metabolic fate of plasma membrane diacylglycerol and phosphatidic acid in NIH 3T fibroblasts. *J. Biol. Chem.* **267**, 14783–14789
- 16 Florin-Christensen, J., Florin-Christensen, M., Delfino, J. M. and Rasmussen, H. (1993) New patterns of diacylglycerol metabolism in intact cells. *Biochem. J.* **289**, 783–788
- 17 Baron, C. B., Cunningham, M., Strauss, 3rd, J. F. and Coburn, R. F. (1984) Pharmacomechanical coupling in smooth muscle may involve phosphatidylinositol metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6899–6903
- 18 Florin-Christensen, M., Florin-Christensen, J., Isola, E. L. D., Lammel, E. M., Meinardi, E., Brenner, R. R. and Rasmussen, L. (1997) Temperature acclimation of *Trypanosoma cruzi* epimastigote and metacyclic trypomastigote lipids. *Mol. Biochem. Parasitol.* **88**, 25–33
- 19 Leguizamón, M. S., Campetella, O., González Cappa, S. M. and Frasch, A. C. C. (1994) Mice infected with *Trypanosoma cruzi* produce antibodies against the enzymatic domain of trans-sialidase that inhibit its activity. *Infect. Immun.* **62**, 3441–3446
- 20 Lammel, E. M., Turyn, D. and Isola, E. L. D. (1992) *Trypanosoma cruzi*: incorporation of macromolecule precursors during the morphogenic process. *Rev. Arg. Microbiol.* **24**, 81–85
- 21 Goodall, H. and Johnson, M. H. (1982) Use of carboxyfluorescein diacetate to study formation of permeable channels between mouse blastomeres. *Nature (London)* **295**, 524–526
- 22 Way, K. J. (2000) Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol. Sci.* **21**, 181–187
- 23 Hidaka, H. and Kobayashi, R. (1992) Pharmacology of protein kinase inhibitors. *Annu. Rev. Pharmacol. Toxicol.* **32**, 377–397
- 24 Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F. et al. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* **266**, 15771–15781
- 25 Kawamoto, S. and Hidaka, H. (1984) 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H7) is a selective inhibitor of protein kinase C in rabbit platelets. *Biochem. Biophys. Res. Commun.* **125**, 258–264
- 26 Crowley, M. R., Fineman, J. R. and Soifer, S. J. (1994) HA1004, an intracellular calcium antagonist, selectively attenuates pulmonary hypertension in newborn lambs. *J. Cardiovasc. Pharmacol.* **23**, 806–813
- 27 Florin-Christensen, J., Florin-Christensen, M., Meinardi, E. and Calle, R. (1996) Diversity of roles of protein kinase C $\alpha$  in the proliferation of Swiss 3T3 cells. *Biochem. J.* **315**, 513–516
- 28 Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. *Science* **233**, 305–312
- 29 Rodríguez-Pena, A. and Rozengurt, E. (1984) Disappearance of Ca<sup>2+</sup>-sensitive, phospholipid dependent protein kinase activity in phorbol-ester-treated 3T3 cells. *Biochem. Biophys. Res. Commun.* **120**, 1053–1059
- 30 Brattsand, G., Friedrich, B. and Gullberg, M. (1989) A rapid separation procedure to analyze phosphorylation of two putative protein kinase C substrates in intact lymphocytes. *Scand. J. Immunol.* **30**, 233–240
- 31 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–684
- 32 Takuwa, N., Takuwa, Y., Bollag, W. E. and Rasmussen, H. (1987) The effects of bombesin on polyphosphoinositide and calcium metabolism in Swiss 3T3 cells. *J. Biol. Chem.* **262**, 182–187
- 33 Augé, N., Santanam, N., Mori, N., Keshava, C. and Parthasarathy, S. (1999) Uptake of 13-hydroperoxylinoleic acid by cultured cells. *Arterioscler. Thromb. Vasc. Biol.* **19**, 925–931
- 34 Nishizuka, Y. (1995) Protein kinase C and lipid signalling for sustained cellular responses. *FASEB J.* **9**, 484–496
- 35 Newton, A. C. (1997) Regulation of protein kinase C. *Curr. Opin. Cell. Biol.* **9**, 161–167
- 36 Gómez, M. L., Ochatt, C. L., Kazanietz, M. G., Torres, H. N. and Téllez-Iñón, M. T. (1999) Biochemical and immunological studies of protein kinase C from *Trypanosoma cruzi*. *Int. J. Parasitol.* **29**, 981–989
- 37 de Lema, M. G. and Aeberhard, E. E. (1986) Desaturation of fatty acids in *Trypanosoma cruzi*. *Lipids* **21**, 718–720
- 38 Glatz, J. F. and Storch, J. (2001) Unravelling the significance of cellular fatty acid-binding proteins. *Curr. Opin. Lipidol.* **12**, 267–274
- 39 Brinkmann, J. F., Abumrad, N. A., Ibrahim, A., van der Vusse, G. J. and Glatz, J. F. (2002) New insights into long-chain fatty acid uptake by heart muscle: a crucial role for fatty acid translocase/CD36. *Biochem. J.* **367**, 561–570
- 40 Farese, R. V., Konda, T. S., Davis, J. S., Standaert, M. L., Pollet, R. J. and Cooper, D. R. (1987) Insulin rapidly increases diacylglycerol by activating *de novo* phosphatidic acid synthesis. *Science* **236**, 586–589
- 41 Farese, R. V., Davis, J. S., Barnes, D. E., Standaert, M. L., Babichkin, J. S., Hock, R., Rosic, N. K. and Pollet, R. J. (1985) The *de novo* phospholipid effect of insulin is associated with increases in diacylglycerol, but not inositol phosphates or cytosolic Ca<sup>2+</sup>. *Biochem. J.* **15**, 269–278
- 42 Farese, R. V. (2001) Insulin-sensitive phospholipid signaling systems and glucose transport. *Update II. Exp Biol Med.* **226**, 283–295
- 43 Aihara, H., Asaoka, Y., Yoshida, K. and Nishizuka, Y. (1991) Sustained activation of protein kinase C is essential to HL-60 cell differentiation to macrophage. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11062–11066
- 44 Denning, M. F., Dlugosz, A. A., Howett, M. K. and Yuspa, S. H. (1993) Expression of an oncogenic *ras<sup>H</sup>* gene in murine keratinocytes induces tyrosine phosphorylation and reduced activity of protein kinase C  $\delta$ . *J. Biol. Chem.* **268**, 26079–26081

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