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ORIGINAL PAPER

Involvement of protein kinase C isoenzymes in *Trypanosoma cruzi* metacyclogenesis induced by oleic acid

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Abstract Previously, we showed that oleic acid (OA) induces *Trypanosoma cruzi* metacyclogenesis through a signaling pathway involving de novo diacylglycerol biosynthesis and simultaneous protein kinase C (PKC) activation. Herein, we demonstrated that OA also triggers a transient Ca^{2+} signal in epimastigotes, necessary for parasite differentiation, that could account for PKC activation. In addition, we found that this free fatty acid (FFA) directly stimulated in vitro the activity of *T. cruzi* PKC in a dose–response way. We determined the presence of classical and novel PKC isoenzymes that were differentially expressed in the infective amastigotes (α and δ) and tripomastigotes (α, β, and γ) and in the non-infective epimastigotes (α, β, γ, and δ). We also demonstrated that OA induced in epimastigotes the translocation of PKC α, β, γ, and δ to the membrane, indicating a selective effect of this FFA. To establish a correlation between *T. cruzi* metacyclogenesis induced by OA and the activation of a particular PKC isoenzyme, the specific PKC inhibitors Ro 32-0432 and Rottlerin (9–30 nM and 5–35 μM, respectively) were employed. These compounds, even at the lowest concentrations assayed, abrogated both epimastigote differentiation and membrane translocation of PKC β, γ, and δ. These findings strongly support a key role for classical and novel PKC isoenzymes in the signaling pathways involved in *T. cruzi* metacyclogenesis induced by OA.

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Introduction

Trypanosoma cruzi, the etiological agent of Chagas' disease, is an intracellular protozoan that undergoes a complex life cycle between a hematophagous insect vector, *Triatoma infestans*, and a mammalian host. In the intestinal tract of the vector, the replicative non-infectious epimastigotes differentiate to the infectious non-dividing metacyclic forms, a process denominated metacyclogenesis (Brener et al. 2000).

It is well known that free fatty acids (FFA) are important second messengers that differentially activate classical and novel protein kinase C (PKC) isoenzymes and mediate various cellular functions including differentiation (Liu and Heckman 1998; Nishizuka 1995). In earlier studies, we analyzed the lipid factors present in *T. infestans* intestinal extract demonstrating that among them, only the FFA displayed metacyclogenic activity on *T. cruzi* epimastigotes (Wainszelbaum et al. 2003). When oleic acid (OA), the most abundant of these FFA, was assayed at a similar concentration of that found in the intestinal extract (300 μM), a metacyclogenic effect was observed (Wainszelbaum et al. 2003). In addition, we previously reported that *T. infestans* intestinal extract generates an intracellular Ca^{2+} signal required for the triggering of this process (Lammel et al. 1996). Moreover, we also determined that OA triggered metacyclogenesis through a signaling pathway that involves diacylglycerol biosynthesis and PKC activation, providing the first evidence for a biological role of this kinase in a kinetoplastid protozoan (Wainszelbaum et al. 2003).

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PKCs are serine–threonine kinases that mediate a variety of physiological functions through signal transduction pathways and comprise a number of isoenzymes classified into three groups: classical (α, β, γ), novel (δ, ε, θ, η) and atypical (λ, ι, μ, ζ; Dempsey et al. 2000; Liu and Heckman 1998; Nishizuka 1995). Several reports illustrate the importance of PKC family in promotion of cell differentiation in eukaryotes (Dieter and Schwende 2000; Florin-Christensen et al. 1996; Ohba et al. 1998).

In the present work, we analyze the participation of OA in *T. cruzi* PKC activation and in the generation of the Ca^{2+} signal required for metacyclogenesis. We also study the expression of classical, novel, and atypical PKCs in the infective and non-infective *T. cruzi* stages and analyze which of these isoenzymes are involved in the signaling events of *T. cruzi* metacyclogenesis induced by OA.

Materials and methods

Materials

Fetal bovine serum (FBS), 199 medium and pre-stained protein ladders were obtained from Gibco BRL (Invitrogen, USA). Chemiluminescence (ECL) Western blot detection system was purchased from GE Healthcare (USA). OA, ethylene glycol tetraacetic acid (EGTA), dimethylsulfoxide (DMSO), phenylmethylsulfonylfluoride, leupeptin, aprotinin, benzamidine, soy bean trypsin inhibitor, Nα-Tosyl-Lys-chloromethylketone, Fura 2AM, and Grace medium were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Polyclonal antibodies against the different PKC isoenzymes were purchased from Abgent Biotechnology (CA, USA). Anti-rabbit peroxidase-conjugate, Rottlerin, and Ro 32-0432 were obtained from Santa Cruz Biotechnology (CA, USA).

Cells and cultures

T. cruzi RA lethal strain was used (González Cappa et al. 1981). Epimastigotes were axenically grown at 28°C in a biphasic medium and harvested by centrifugation at 1,000×*g* for 15 min after 48 h of culture and had less than 1% metacyclic forms (Isola et al. 1986). Amastigotes and trypomastigotes were purified from infected Vero cells (African green monkey kidney cells) grown in 199 medium + 10% FBS at 37°C with 5% CO_2 (Moreno et al. 1994).

Measurement of intracellular calcium concentration in OA-stimulated epimastigotes

The protocol described by Lammel et al. (1996) was employed. Briefly, intracellular calcium concentration ($[Ca^{2+}]_i$) was fluorometrically determined in epimastigotes previously loaded with 4 μM Fura-2AM (Grynkiewicz et al. 1985) and resuspended in phosphate buffer solution (PBS), both before (basal levels) and 30 s, 3, 5, and 10 min after receiving the additions of 10, 25, 50, and 100 μM OA. All the assays were performed with or without 5 mM EGTA. In each determination, 10^7 parasites were used and the final volume of the samples was 1.5 ml. $[Ca^{2+}]_i$ was measured using excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm in a JASCO PF-770 spectrofluorometer.

Preparation of homogenate supernatants from the different *T. cruzi* stages

Epimastigotes, amastigotes, or trypomastigotes (10^8/ml) were resuspended in 10 mM Tris/HCl, pH 7.4, in the presence of protease inhibitors (0.01% leupeptin; 2 mM phenylmethanesulfonyl fluoride; 25 U/ml aprotinin; 1 mM benzamidine; 0.5 mM Nα-Tosyl-Lys-chloromethylketone) and subjected to four freeze–thaw cycles. Disrupted parasites were then centrifuged at 27,000×*g* for 30 min and the resultant homogenate supernatants were aliquoted and stored at −80°C until used in immunoblot analysis of PKC isoenzymes (Belaunzarán et al. 2007).

Effect of OA on *T. cruzi* PKC activity

To evaluate the direct effect of OA on epimastigote PKC activity, we performed a partial purification of the enzyme following the method of Gómez et al. (1989). Briefly, parasites (10^9/ml) were washed thrice with 0.25 M sucrose + 5 mM KCl, resuspended in 20 mM Tris–HCl, 2 mM EDTA, 0.5 mM EGTA, 5 mM 2-mercaptoethanol, and 0.25 M sucrose, pH 7.4 (buffer A) in the presence of the protease inhibitors mentioned above and homogenized by three cycles of freezing and thawing. The parasite lysate was then centrifuged at 27,000×*g* for 60 min at 4°C and the resultant supernatant was immediately loaded onto a DEAE-cellulose column equilibrated with Buffer A. Elution was performed with buffer A + 0.15 M NaCl + 10% glycerol, fractions (3 ml) were collected, and PKC activity was assayed employing a modification of the protocol described by Gómez et al. (1989). Briefly, aliquots of each fraction (120 μl) were incubated with 25 μM Neurogranin (PKC panselect substrate) + 50 μM [γ^{32}P] ATP (specific activity 3,000 Ci/mmol) with a mixture of 0.5 mM $CaCl_2$, 60 μg/ml phosphatidylserine, and 3 μg/ml diacylglycerol or 0.5 mM EGTA at 30°C for 15 min. Reactions were stopped by spotting 30 μl of each mixture onto 2×2-cm square pieces of phosphocellulose paper and immediately soaked in 75 mM H_3PO_4, followed by three washes in the same solution. The papers were dried and the radioactivity was quantified by

RackBeta liquid scintillation counter (Pharmacia). The fractions containing the highest levels of PKC activity were pooled (fraction enriched in *T. cruzi* PKC) and immediately used to evaluate in vitro the direct effect of OA on the enzyme activity. With this aim, a modification of the PKC activity assay described above was performed using 50 to 500 μM OA instead of diacylglycerol in the reaction mixture.

Immunoblot analysis of PKC isoenzymes in *T. cruzi* stages

To study which PKC isoenzymes were expressed in the different *T. cruzi* stages, parasite homogenate supernatants (60–100 μg of protein per lane) were loaded on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (Laemmli 1970), transferred to nitrocellulose membranes, and incubated with specific polyclonal antibodies against PKC isoenzymes α, β, γ, δ, ε, and ζ. Commercial PKC antibodies were generated in rabbits immunized with a synthetic peptide selected from the C-terminal region, α (598–668), β (601–671), γ (600–671), and in the case of PKC ζ from the N-terminal (15–98) of the corresponding human PKC isoenzymes (Abgent Biotechnology). PKC δ and ε antibodies were generated in mice immunized with peptides corresponding to the sequences of human PKC δ (114–289) and human PKC ε (1–175), respectively (BD Biosciences). Immunoblots were visualized using a peroxidase-conjugate secondary antibody and ECL as substrate. Chemiluminescence was detected with the Storm® Gel and Blot Imaging System (Amersham Biosciences, USA). Homogenate from fresh bovine brain was used as PKC isoenzymes control.

Effect of OA on PKC isoenzyme translocation

Epimastigotes (10^7/ml) were stimulated with 300 μM OA for 15 min at 28°C (Wainszelbaum et al. 2003), centrifuged at 1,000×*g* for 15 min, resuspended in PBS (T0), and incubated at 28°C. Aliquots of this parasite suspension (1 ml) were collected every 15 min (T15, T30, and T45) and centrifuged at 1,000×*g* for 15 min. The pellets were then resuspended in PBS with the protease inhibitors described above, disrupted by four freeze–thaw cycles, and centrifuged at 14,000×*g* for 10 min at 4°C. The resultant supernatants were further centrifuged at 105,000×*g* for 1 h at 4°C and the pellets (microsomal fractions) and supernatants (soluble fractions) obtained were resuspended in PBS, aliquoted, and stored at −80°C (Wilkowsky et al. 2001).

To determine which PKC isoenzymes translocate from the cytosol to the membrane in response to OA stimulation, all these fractions were then analyzed by immunoblot employing the specific PKC isoenzyme antibodies described above. Non-stimulated parasites resuspended in PBS + 0.05% DMSO (vehicle) were used as controls. All the bands detected were quantified by densitometry using the Gel-Pro® Analyzer 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

Effect of specific PKC inhibitors on OA-induced metacyclogenesis

Epimastigotes (5×10^6 cells/ml) were pre-incubated with the PKC inhibitors Ro 32-0432 (9 and 30 nM; Wilkinson et al. 1993) and Rottlerin (5 and 35 μM; Gschwendt et al. 1994) for 1 h at 28°C. After this treatment, parasites were stimulated with 300 μM OA for 15 min at 28°C and transferred to modified Grace medium (Wainszelbaum et al. 2003). Growth curves were obtained by counting the parasites in a Neubauer chamber and metacyclogenesis was evaluated every 48 h, in quintuplicate samples by light microscopy, according to: (1) parasite motility and shape in live samples and (2) shape and relative kinetoplast-nucleus position in wet fixed-stained (May Grünwald-Giemsa) preparations. At least 200 cells were examined. Non-inhibited and non-stimulated parasites resuspended in Grace medium + 0.05% DMSO (vehicle) were used as controls.

Effect of PKC specific inhibitors on OA-induced isoenzyme translocation

Epimastigotes (5×10^6/ml) were pre-incubated with the PKC inhibitors Ro 32-0432 (9 and 30 nM; Wilkinson et al. 1993) and Rottlerin (5 and 35 μM; Gschwendt et al. 1994) for 1 h at 28°C. Parasites were then stimulated with 300 μM OA for 15 min at 28°C (Wainszelbaum et al. 2003), centrifuged at 1,000×*g* for 15 min, resuspended in PBS (T0), and incubated at 28°C. Aliquots of this parasite suspension (1 ml) were collected every 15 min (T15, T30, and T45) and processed as described above in order to obtain the corresponding membrane and cytosolic fractions. To determine the effect of the PKC inhibitors on isoenzyme translocation, all these fractions were then analyzed by immunoblot as previously described using the specific PKC isoenzyme antibodies. Non-inhibited and non-stimulated epimastigotes resuspended in PBS + 0.05% DMSO (vehicle) were used as controls.

Protein determinations

Protein contents were quantified by the method of Lowry et al. (1951).

Statistical analysis

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

Results

Oleic acid triggers a transient calcium signal in epimastigotes

Previously, we demonstrated that an intracellular Ca^{2+} signal is one of the requirements for the triggering of *T. cruzi* metacyclogenesis (Lammel et al. 1996) and that among the FFA present in *T. infestans* intestinal extract, OA induces this differentiation process (Wainszelbaum et al. 2003); therefore, we analyzed if OA could promote a Ca^{2+} signal.

Herein, we determined that the mean basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in epimastigotes was 94± 28 nm/l, and 30 s after the addition of OA, $[Ca^{2+}]_i$ increased to a maximum, which was dependent on the OA concentrations and thereafter declined without recovering its basal values. The rise in $[Ca^{2+}]_i$ observed at 30 s with the different OA concentrations assayed is shown in Fig. 1. An increase of approximately threefold was evident at concentrations of 25, 50, and 100 μM OA, with respect to the basal values. The addition of EGTA in the extracellular medium did not block the effect of OA on $[Ca^{2+}]_i$ (data not shown).

OA directly stimulates in vitro *T. cruzi* PKC activity

Having established that OA induces *T. cruzi* metacyclogenesis through a signaling pathway that involves de novo DG biosynthesis and concomitant PKC activation (Wainszelbaum et al. 2003), in the present work, we evaluated in vitro the direct effect of OA on parasite PKC activity. With this aim, we first partially purified this kinase from epimastigotes as described in "Materials and methods".

The parasite lysate was separated by ionic exchange chromatography on DEAE-cellulose and one main peak of PKC activity was resolved (Fig. 2a). The corresponding fractions were pooled (40–65 ml) and the fraction enriched in *T. cruzi* PKC obtained was immediately used to assay the enzyme activity. A significant increase on PKC activity was observed with concentrations over 100 μM OA, reaching a maximum at 400 μM OA (Fig. 2b). These results clearly indicate that OA directly activated in vitro *T. cruzi* PKC in a dose–response way.

PKC isoenzymes are differentially expressed in *T. cruzi* stages

Afterwards, we investigated which PKCs were expressed in the different parasite stages by immunoblot analysis of the corresponding homogenate supernatants. Results showed that PKC α, δ, and ε were expressed in the three *T. cruzi* stages, while β was expressed in epimastigotes and trypomastigotes and ζ in epimastigotes and amastigotes (Fig. 3a). Remarkably, PKC γ was only detected in the non-infective epimastigotes. The molecular weights of all PKC isoenzymes were between 70 and 120 kDa, as previously described for other cellular types (Nishizuka 1995); however, some differences were observed in PKC δ when comparing this isoenzyme for each parasite stage.

To corroborate the results mentioned above, we examined *T. cruzi* database (http://www.tcruzidb.org) in order to identify epitopes belonging to parasite PKC isoenzymes. With this aim, a BLASTP search was performed employing as query sequences the corresponding peptides used as immunogens by the manufacturer to obtain the commercial antibodies against human PKC isoenzymes here employed. As regards PKC α, β, γ, and δ, the alignments of the highest score sequences retrieved from *T. cruzi* database with their corresponding peptides used as immunogens, as mentioned above, provided molecular evidence for the presence of these isoenzymes in this protozoa (Fig. 3b). The *T. cruzi* sequences identified codify for putative protein kinases (Tc00.1047053510901.200) and rac serine–theronine kinases (Tc00.1047053508965.60, Tc00.1047053509047.110, and Tc00.1047053509007.60; Fig. 3b). These protein sequences shared 70–80% identity among them, with predicted molecular weights around 90 and 70 kDa. The comparison of the complete protein sequences of *T. cruzi* putative kinases with human PKC isoenzymes revealed a similarity of 64–74%. On the other hand, when the peptide sequences of human PKC ε and ζ were employed as query sequences in *T. cruzi* database for a BLASTP search, no significant

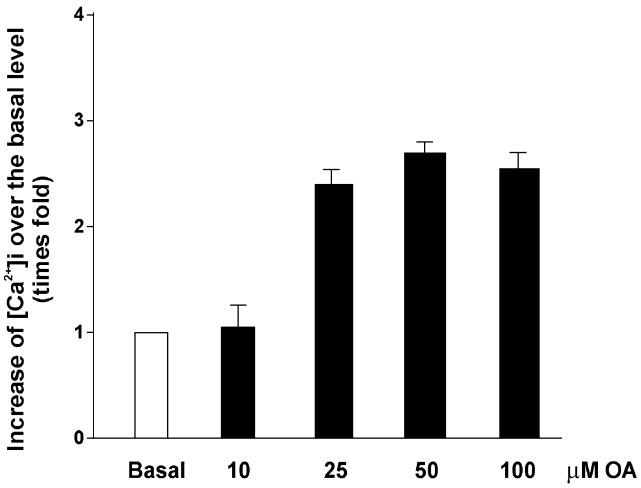

Fig. 1 Oleic acid triggers a transient Ca^{2+} signal in epimastigotes $[Ca^{2+}]_i$ was fluorometrically determined in epimastigotes previously loaded with 4 μM Fura-2AM and resuspended in PBS before (basal levels) and 30 s, 3, 5, and 10 min after receiving the additions of 10, 25, 50, and 100 μM OA. All the assays were performed with or without 5 mM EGTA. The *bars* represent the rise in $[Ca^{2+}]_i$ observed at 30 s. The data are representative of at least three measurements with different cell preparations

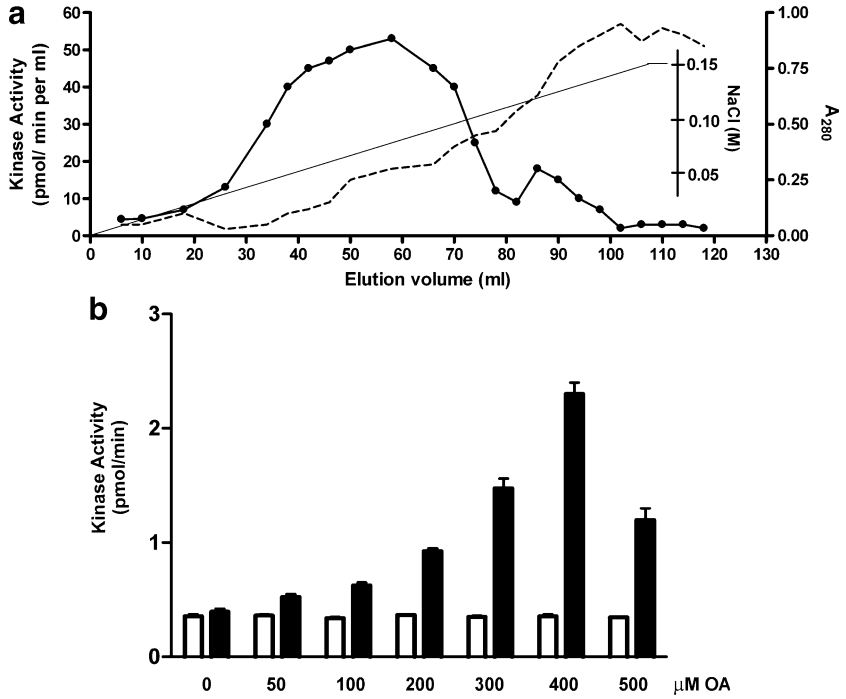

Fig. 2 Oleic acid stimulates in vitro *T. cruzi* PKC activity. **a** PKC was partially purified from an epimastigote lysate by DEAE-cellulose chromatography as described in "Materials and methods". The enzyme activity was determined every two fractions with Neurogranin as substrate and expressed as the difference between activities in the presence of 0.5 mM $CaCl_2$, 60 μg/ml phosphatidylserine, and 3 μg/ml diacylglycerol or in the presence of EGTA (*filled circle*). Protein determination by absorbance at 280 nm (*dashed line*); linear gradient of NaCl (*solid line*). **b** The fractions containing the highest levels of PKC activity were pooled, fraction enriched in *T. cruzi* PKC, and immediately used to evaluate in vitro the direct effect of OA on the enzyme activity. This activity was determined using Neurogranin as substrate with the addition of a mixture containing 50 to 500 μM of oleic acid (*shaded bars*), 0.5 mM $CaCl_2$, and 60 μg/ml phosphatidylserine or with 0.5 mM EGTA (*light bars*)

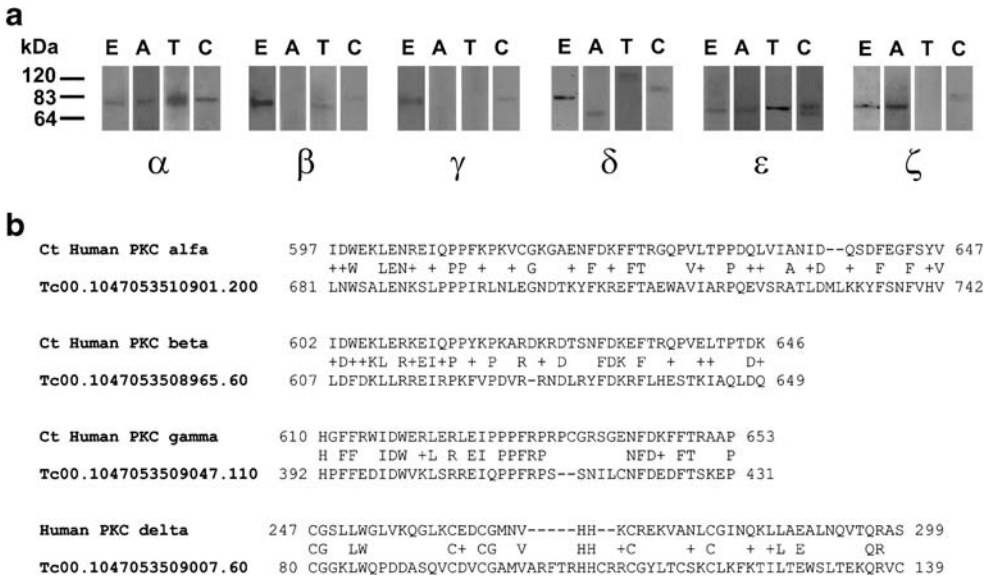

Fig. 3 PKC isoenzymes are differentially expressed in *T. cruzi* stages. **a** Parasite homogenate supernatants from epimastigotes (*E*), amastigotes (*A*), and trypomastigotes (*T*) were analyzed by immunoblot using specific polyclonal antibodies against PKC isoenzymes α, β, γ, δ, ε, and ζ and developed by chemiluminescence using the Storm® Gel and Blot Imaging System. Images are representative of three independent assays. Fresh bovine brain homogenate was used as control (*C*). **b** Alignments of the retrieved sequences with the highest score from *T. cruzi* database after BLASTP searches, employing as query sequences the corresponding peptides used as immunogens by the manufacturer to obtain the commercial antibodies against human PKC isoenzymes. *Letters* indicate conserved amino acids and *plus signs* similar amino acids

sequences were retrieved. Therefore, further studies will be necessary to corroborate the presence of these isoenzymes in *T. cruzi*.

OA stimulation promotes differential PKC isoenzymes translocation in epimastigotes

As we here demonstrated the presence of at least four PKC isoenzymes in epimastigotes and that OA stimulates in vitro PKC activity, we set up next to evaluate in this parasite stage which PKC isoenzymes translocated to the microsomal fraction by OA stimulation. PKC translocation from the cytosol to the membrane has served as the hallmark for PKC activation (Aihara et al. 1991; Liu and Heckman 1998; Quest 1996). As concerns *T. cruzi*, previous studies determined that in epimastigotes, this kinase is mainly located in the cytosolic and microsomal fractions, the latter being the localization of the physiologically active enzyme (Gomez et al. 1989).

Immunoblot analysis of microsomal and soluble fractions from OA-stimulated epimastigotes showed that α, β, γ, and δ isoenzymes differentially translocated to the microsomal fraction, suggesting that they were activated by OA (Fig. 4). PKC α showed a modest immunostaining at T30 in the microsomal fraction, with an increase at T45, while the soluble fraction significantly increased its mass at T15 (approximately tenfold higher with respect to control) and diminished after this time.

As regards PKC β, a significant increase was observed in the microsomal fraction at T0 and T15 (sevenfold higher with respect to control), with a decrease from T30 to T45, whereas in the soluble fraction, a weak immunostaining was detected with no significant modifications along the times assayed.

PKC γ was progressively visualized in the microsomal fraction with a maximum level at T45 (approximately 12 times higher with respect to control) with no immunoreactivity in the soluble fraction.

PKC δ behaved in the microsomal fraction in a similar way to PKC β, with a significant increase at T0 (fivefold higher with respect to control), decreasing from T15 to T45, while in the soluble fraction, no immunostaining was detected.

On the other hand, PKC ε and ζ were not detected in the microsomal fractions; meanwhile, in the soluble fractions, a modest immunoreactivity was observed with no variations at the times assayed (data not shown), indicating that these isoenzymes did not respond to OA under our experimental conditions.

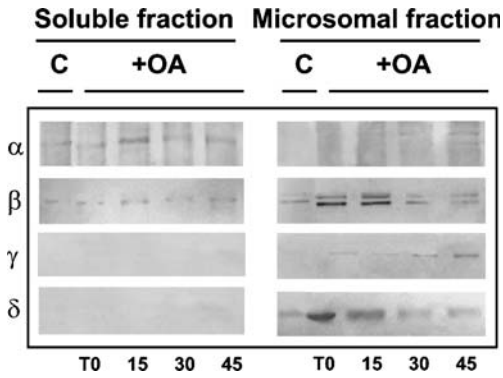

Fig. 4 Oleic acid promotes translocation of PKC α, β, γ, and δ in epimastigotes. Parasites (10^7/ml) were stimulated with 300 μM OA for 15 min at 28°C and then resuspended in PBS (T0) at 28°C. Aliquots were collected every 15 min (T15, T30, and T45) and processed in order to obtain the corresponding microsomal and soluble fractions. Each sample was analyzed by immunoblot using specific PKC antibodies. Parasites resuspended in PBS + 0.05% DMSO (vehicle) were used as controls (*C*). Images are representative of three independent experiments

Specific PKC inhibitors abrogate both OA-induced metacyclogenesis and PKC translocation

To determine which of the PKC isoenzymes that translocated to the microsomal fraction by OA stimulation were involved in *T. cruzi* metacyclogenesis induced by this FFA, we next examined the effect of the specific PKC inhibitors Ro 32-0432 (Wilkinson et al. 1993) and Rottlerin (Gschwendt et al. 1994) in this process as well as in PKC translocation.

Table 1 shows that both compounds, even at the lowest concentrations assayed (9 nM Ro 32-0432 and 5 μM Rottlerin), abrogated OA-induced epimastigote differentiation with respect to the control (non-inhibited OA-stimulated epimastigotes). The inhibitors lacked toxic effects in all the concentrations assayed, since parasite growth curves were similar to those obtained in control cultures without inhibition treatment (data not shown).

As regards the PKC isoenzymes translocation profiles, epimastigotes pretreated with Ro 32-0432 or Rottlerin and then stimulated with OA (Fig. 5) showed significant differences with respect to the OA stimulated parasites (Fig. 4). Immunoblot analysis of soluble and microsomal fractions of epimastigotes pre-incubated with each compound at concentrations for specific PKC α inhibition (9 nM Ro 32-0432, IC_{50}=9 nM and 35 μM Rottlerin, IC_{50}=30–42 μM) revealed that neither expression nor translocation to the microsomal fraction was affected (Fig. 5). The rapid and significant translocation to the microsomal fraction of PKC β observed at T0 in Fig. 4 was strongly inhibited when using 30 nM Ro 32-0432 (IC_{50}=28 nM; Fig. 5). Meanwhile, in the soluble fraction, this isoenzyme increased along the times assayed, suggesting that its expression was not affected by this compound. Similar results were obtained with 35 μM Rottlerin (IC_{50}=30–42 μM); however, traces of this isoen-

Table 1 Specific PKC inhibitors abrogate metacyclogenesis induced by oleic acid

Stimulus	Total parasites ($\times 10^6$/ml)	Metacyclic trypomastigotes ($\times 10^6$/ml)	% Metacyclogenesis
OA	81.0±2.0	24.9±2.6	30.8±3.2
5 μM Rottlerin + OA	81.5±1.9	2.2±0.6	2.7±0.8[a]
35 μM Rottlerin + OA	80.3±2.0	1.5±1.0	1.9±1.3[a]
9 nM Ro 32-0432 + OA	79.9±1.5	2.4±0.8	3.0±1.0[a]
30 nM Ro 32-0432 + OA	81.5±2.1	1.9±1.1	2.3±1.3[a]

Epimastigotes were pre-incubated with 5 and 35 μM Rottlerin or 9 and 30 nM Ro 32-0432 for 1 h at 28°C. Parasites were then stimulated to differentiate with 300 μM oleic acid (OA) for 15 min at 28°C and transferred to modified Grace medium. Non-inhibited but OA-stimulated parasites resuspended in Grace medium + 0.05% DMSO (vehicle) were used as control. All the inhibitors were assayed at non-toxic concentrations. Values recorded correspond to day 8 of the growth/differentiation curves in each case and represent the mean±SD of three different experiments

[a] Agents that induced statistically significant inhibition of metacyclogenesis with respect to the control ($p<0.01$)

zyme were detected in the microsomal fraction at T15. With respect to δ isoenzyme, 5 μM Rottlerin (IC_{50}=3–6 μM) abolished both expression and translocation to the microsomal fraction previously observed in Fig. 4 for OA-stimulated parasites. Similar results were found for PKC γ where 35 μM Rottlerin (IC_{50}=30–42 μM) also abrogated its translocation and protein expression. All these results strongly support the selective participation of β, γ, and δ isoenzymes in *T. cruzi* metacyclogenesis induced by OA.

Discussion

In the present work, we determined that OA, the main FFA present in *T. infestans* intestinal extract (Wainszelbaum et al. 2003), promotes in *T. cruzi* epimastigotes a transient Ca^{2+} signal. This fact correlates with previous findings where we established that this extract promotes a Ca^{2+} increase and that this is one of the requirements for *T. cruzi* metacyclogenesis (Lammel et al. 1996). The addition of EGTA in the external medium of epimastigotes stimulated with OA did not modify the Ca^{2+} signal; therefore, this increase could be produced by Ca^{2+} mobilization from the acidocalcisomes (Docampo et al. 1995). Accordingly, we already demonstrated that Ca^{2+} was mobilized from epimastigote intracellular storage sites when they were stimulated with *T. infestans* intestinal extract in the presence of different compounds such as calcium ionophores/chelators and agonist/antagonist of Ca^{2+} channels (Lammel et al. 1996).

Interestingly, it has been reported in *T. cruzi* amastigotes that another FFA, arachidonic acid, was able to promote Ca^{2+} mobilization from the acidocalcisomes (Catisti et al. 2000). In addition, it is well known that Ca^{2+} is involved in the regulation of classical PKCs (Liu and Heckman 1998; Nishizuka 1995); therefore, the Ca^{2+} signal induced by OA could account for the activation of parasite PKC. On the other hand, the participation of unsaturated fatty acids such as oleate, arachidonate, linolenate, and linoleate in PKC activation has already been described (McPhail et al. 1984). We found that the activity of the fraction enriched in *T. cruzi* PKC responds to OA in a dose-dependent way in the presence of Ca^{2+} and phosphatidylserine, reaching its maximum level at similar concentrations to those present in the *T. infestans* intestinal extract that promote metacyclogenesis (200–400 μM OA; Wainszelbaum et al. 2003). Accordingly, in human platelets where OA is able to activate classical, novel, and atypical PKCs, the activation of Ca^{2+}-dependent isoenzymes occurs at the highest

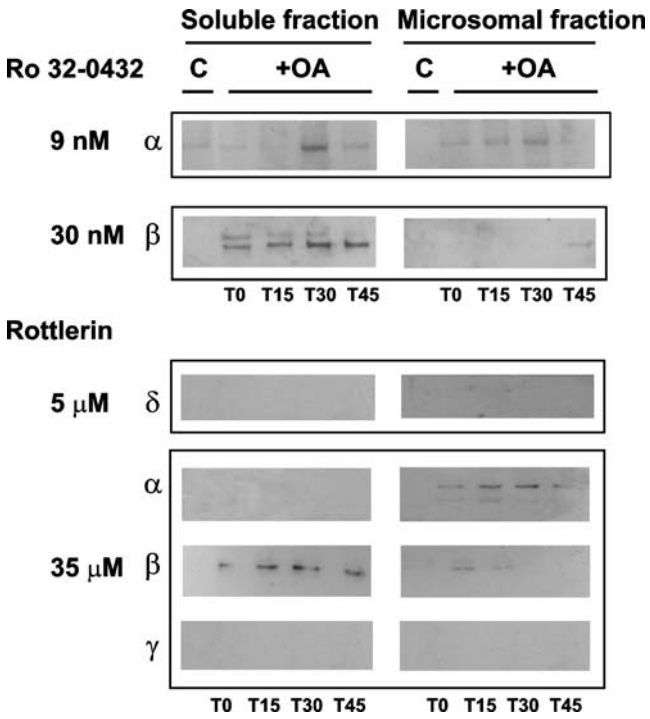

Fig. 5 Specific PKC inhibitors abrogate isoenzymes translocation in OA-stimulated epimastigotes. Parasites (5×10^6/ml) were pre-incubated with 9–30 nM Ro 32-0432 or 5–35 μM Rottlerin for 1 h at 28°C, stimulated then with 300 μM OA for 15 min, and resuspended in PBS (T0). Aliquots were collected every 15 min (T15, T30, and T45) and processed in order to obtain microsomal and soluble fractions. Samples were analyzed by immunoblot using specific PKC antibodies. Inhibited and non-stimulated epimastigotes were used as controls (*C*). Images are representative of three independent experiments

concentrations of this FFA (Khan et al. 1993). It is noteworthy that *T. cruzi* kinase activity was assayed with a partially purified PKC fraction using a PKC panselect substrate (Neurogranin) in the presence of Ca^{2+} and phosphatidylserine; therefore, the enzyme activity here determined could be attributed to the presence and simultaneous activation of different PKC isoenzymes.

We previously determined in vivo that OA promotes in epimastigotes de novo diacylglycerol biosynthesis and concomitant PKC activation (Wainszelbaum et al. 2003). Herein, we demonstrated that OA per se also activates in vitro *T. cruzi* PKC; this is in agreement with earlier studies where this kinase differentially responds in vitro to arachidonic acid and Ca^{2+} (Gomez et al. 1999). Therefore, the present result indicates that this FFA could mediate in vivo PKC activation in *T. cruzi* by two different ways, DG generation and/or direct effect of OA on this enzyme.

The present study documents by immunoblot analyses the existence in the three *T. cruzi* stages of PKC isoenzymes from the classical (α, β, γ), novel (δ, ε), and atypical (ζ) groups. Previous reports only described the presence of PKC α in epimastigotes and trypomastigotes (Gomez et al. 1999). The fact that we found additional isoenzymes could be due to the use of different polyclonal antibodies (raised against human PKC isoenzymes), other protein source (whole homogenates), as well as to the parasite strain here employed. Moreover, evidence for the existence of PKC α, β, γ, and δ isoenzymes was obtained from *T. cruzi* database. The protein sequences identified possess significant identity and homology with their human counterparts and correspond to putative rac serine–threonine kinases, the family to which PKC belongs, indicating that the polyclonal antibodies here employed recognized conserved PKC domains between mammalians and protozoa. Altogether, these results provide immunological and molecular evidence of the presence of PKC α, β, γ, and δ isoenzymes in *T. cruzi*.

With regard to PKC ε and ζ, even though these proteins were detected by immunoblot, no molecular evidences were obtained from the *T. cruzi* database. Therefore, the existence of these isoenzymes in *T. cruzi* remains to be elucidated. The molecular weights of the PKC isoenzymes here detected were similar to those reported for mammalian cells (Nishizuka 1995); however, some differences were observed when comparing PKC δ in the different *T.cruzi* stages. This finding could be attributed to stage-specific post-transductional modifications, like protein phosphorylation or different glycosylation patterns among others (Haynes and Cross 1996; Marques Porto et al. 2002).

Interestingly, PKC α, β, γ, and δ were differentially expressed in the infective and non-infective *T. cruzi* stages, suggesting that each isoenzyme possesses specific functions that could be related to the diverse environments that the parasite encounters through its complex life cycle. Up to now, little is known concerning the activation of PKC isoenzymes in response to physiologic stimuli that trigger *T. cruzi* metacyclogenesis. Activation of cellular PKC is associated with translocation of the enzyme from cytosol to membrane fractions in various cell systems (Aihara et al. 1991; Quest 1996; Liu and Heckman 1998). This is the first study in a protozoan hemoparasite that demonstrates the ability of OA, a metacyclogenic stimuli, to induce translocation of PKC α, β, γ, and δ, indicating a selective effect of this FFA on isoenzyme activation. This fact has already been described in macrophages, platelets, and pancreatic cells (Chen et al. 2002; Dey et al. 2006; Wrede et al. 2003).

With the purpose of establishing a correlation between metacyclogenesis and the activation of a particular PKC isoenzyme induced by OA, we employed the specific PKC inhibitors Ro 32-0432 and Rottlerin (Gschwendt et al. 1994; Wilkinson et al. 1993). These compounds, even at the lowest concentrations assayed, were held responsible for both inhibition of epimastigote differentiation and PKC β, γ, and δ membrane translocation, sustaining the involvement of these isoenzymes in *T. cruzi* metacyclogenesis. Accordingly, in *Giardia duodenalis*, an intestinal protozoan, the activation of PKC β during the induction of the encystment process was demonstrated (Bazan-Tejeda et al. 2007). In this sense, the participation of PKC β, γ, and δ in the signal transduction mechanisms that mediate morphogenesis has been intensively documented in other cell types, demonstrating that protein-kinase-mediated cell signaling pathways are crucial to activate stage-specific genes (Zhou et al. 2006; Boczan et al. 2000; Wachira et al. 2003).

As regards PKC α, Ro 32-0432 and Rottlerin, at concentrations for α specific inhibition (Gschwendt et al. 1994; Wilkinson et al. 1993), abrogated epimastigote differentiation, whereas isoenzyme translocation was not affected. Since up to now there is no other specific inhibitor commercially available for this isoenzyme, its contribution to *T. cruzi* metacyclogenesis deserves further analysis.

In conclusion, this study demonstrates that OA induces in *T. cruzi* a transient Ca^{2+} signal necessary for metacyclogenesis and regulation of PKC activity and the existence of at least four PKC isoenzymes differentially expressed in the parasite stages that selectively participate in the early events of OA-induced metacyclogenesis. All these findings will be relevant for future attempts to modulate parasite differentiation, providing new potential targets to block *T. cruzi* transmission.

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