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Mechanism of *Trypanosoma cruzi* death induced by *Cratylia mollis* seed lectin

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Abstract Incubation of *T. cruzi* epimastigotes with the lectin Cramoll 1,4 in Ca^{2+} containing medium led to agglutination and inhibition of cell proliferation. The lectin (50 μg/ml) induced plasma membrane permeabilization followed by Ca^{2+} influx and mitochondrial Ca^{2+} accumulation, a result that resembles the classical effect of digitonin. Cramoll 1,4 stimulated (five-fold) mitochondrial reactive oxygen species (ROS) production, significantly decreased the electrical mitochondrial membrane potential ($\Delta\Psi_m$) and impaired ADP phosphorylation. The rate of uncoupled respiration in epimastigotes was not affected by Cramoll 1,4 plus Ca^{2+} treatment, but oligomycin-induced resting respiration was 65% higher in treated cells than in controls. Experiments using *T. cruzi* mitochondrial fractions showed that, in contrast to digitonin, the lectin significantly decreased $\Delta\Psi_m$ by a mechanism sensitive to EGTA. In agreement with the results showing plasma membrane permeabilization and impairment of oxidative phosphorylation by the lectin, fluorescence microscopy experiments using propidium iodide revealed that Cramoll 1,4 induced epimastigotes death by necrosis.

Keywords *Trypanosoma cruzi* · Mitochondria · Calcium · ROS · Cell death · Cramoll 1,4 lectin

Abbreviations

AA	antimycin A
ADP	adenosine 5′-diphosphate
CCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
Cramoll 1,4	*Cratylia mollis* seed lectin (Isoform 1,4)
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
H_2DCF-DA	dichlorodihydrofluorescein diacetate
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

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PI	propidium iodide
ROS	reactive oxygen species
$\Delta\Psi_m$	electrical mitochondrial membrane potential

Introduction

Chagas' disease is caused by the protozoan *Trypanosoma cruzi*, and it is still an important health problem in Latin America affecting several million people in rural regions (http://www.who.int/tdr/diseases/chagas/swg_chagas.pdf). Current chemotherapeutics for Chagas' disease are based on the use of non-specific drugs, such as benznidazole, which appears to exert its trypanocidal effect through reactive oxygen species (ROS) generation and/or through the decrease in glutathione and trypanothione levels (Maya et al. 2007; Wilkinson et al. 2008). Unfortunately, treatment is associated with a high frequency of serious side effects in the host (Búa et al. 2004; Carraro et al. 2007; Pedrosa et al. 2001; Urbina and Docampo 2003).

The *T. cruzi* lifecycle was identified a hundred years ago (Chagas 1909), but until now, many aspects of its cell biology have remained a mystery. Therefore, a better understanding of the *T. cruzi* cell machinery could lead to the identification of targets for the development of more specific trypanocidal agents. The search for new compounds from natural sources is a widely used approach employed in the treatment of some parasitic diseases. Extracts and pure compounds obtained from plants have been reported to possess significant anti-protozoan activities without side effects (Güida et al. 2007). Artemisin and its derivatives are a potent new class of anti-malarials originating from *Artemisia annua*, L. (Asteraceae) (Kayser et al. 2003). Quinine is one of the best known alkaloids with anti-plasmodial activity, and licochalcone A from *Glycyrrhiza inflata* is a strong inhibitor of mitochondrial functions in *Leishmania* species (Kayser et al. 2003).

Compounds isolated from several plants from the Brazilian semi-arid region have revealed trypanocidal activity against *T. cruzi* epimastigotes (Vieira et al. 2008). In this regard, the seed lectin Cramoll 1,4 (Isoform 1,4) isolated from *Cratylia mollis*, a native forage from the semi-arid region in Northeast Brazil, is a protein that recognizes and binds to specific carbohydrates on the cell surface inducing mitogenic activity (Maciel et al. 2004). It also exhibits anti-tumour activity when encapsulated into liposomes (Andrade et al. 2004). Concanavalin A (ConA), a lectin with high homology to Cramoll 1,4, exerts a potent anti-hepatoma effect. After binding to the mannose moiety on hepatoma cell membrane glycoproteins, ConA is internalized and accumulates in the mitochondria causing cell death (Lei and Chang 2007). Some other lectins, including those from *Helix pomatia*, *Agaricus bisporus* and the *Arisaema helleborifolium* lectin (AHL), are being investigated for their possible use in cancer therapy (Kaur et al. 2006).

With regard to parasites, a lectin from *Synadenium carinatum* latex (ScLL) showed a protective effect against *Leishmania amazonensis* infection in BALB/c mice (Afonso-Cardoso et al. 2007). Binding of some lectins to specific oligosaccharides on the cell membrane seems to mediate cell death (Coelho et al. 2007; Macedo et al. 2007). Cells that do not possess these carbohydrates are resistant to the cytotoxic effect of these proteins (Gastman et al. 2004). Lectins can also be used to evaluate changes in the sugar composition and distribution on cell surfaces during the process of *T. cruzi* transformation from epimastigotes to the mammalian-infective metacyclic trypomastigotes (Bourguignon et al. 1998).

T. cruzi has only one mitochondrion, and the study on the mechanisms by which different compounds interfere with its bioenergetics could be useful for the search and development of new chemotherapies. Considering the property of ConA, a homologue of Cramoll 1,4, to cross the cell membrane and localize to mitochondria, we evaluated the effects of Cramoll 1,4 on *T. cruzi* epimastigote mitochondrial functions and cell viability.

Material and methods

Chemicals

Arsenazo III, MTT, Safranine O, Succinate, Digitonin, EGTA, CCCP, ADP, Antimycin A and Oligomycin were purchased from Sigma (St. Louis, USA). H_2DCF-DA was purchased from Molecular Probes Inc. (Eugene, Oregon, USA). CAT was purchased from Calbiochem of EMD Chemicals Inc. (Affiliate of Merck KGaA, Darmstadt, Germany). All other reagents were of the highest purity grade available.

Cell cultures

T. cruzi epimastigotes (Tulahuen 2 strain) were grown in LIT medium, containing 20 mg l^{-1} hemin and 10% fetal bovine serum, as described (Castellani et al. 1967). After 5 days, cells were harvested by centrifugation (1,000 *g* at 4 °C) and washed once in phosphate buffered saline (PBS), pH 7.2. The number of cells ml^{-1} was determined using a Neubauer chamber. *T. cruzi* trypomastigotes (Y strain) were grown and maintained as described (Andrews and Colli 1982). HaCaT keratinocyte, a spontaneously transformed human epithelial cell line, were grown in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum in a humidified atmosphere with 5%

CO_2 (v/v) at 37 °C. For the experiments, keratinocytes grown to ~80% confluence were used.

Preparation of *T. cruzi* mitochondrial fraction

Epimastigotes (log phase) were harvested by centrifugation (1,000 *g*, 10 min at 4 °C), washed in 0.12 M sodium phosphate, pH 8.0 buffer, containing 86.3 mM NaCl and 56 mM glucose. After centrifugation, the pellet was mixed with 3 ml of acid-washed glass beads (150–212 μm—SIGMA), 100 μl of protease inhibitor cocktail (Sigma) and 80 μl of 0.1 M phenylmethanesulphonyl fluoride (PMSF). Cells were disrupted by abrasion in a chilled mortar until 90% disruption was achieved (as determined under an optical microscope), resuspended in 4 ml of 50 mM HEPES-NaOH, pH 7.2 buffer, containing 0.27 M sucrose, 1 mM EDTA, and 1 mM $MgCl_2$, and centrifuged at 1,000 *g* for 5 min at 4 °C in order to remove the glass beads, unbroken cells and large debris. The supernatant was submitted to another centrifugation (1,000 *g* for 10 min at 4 °C) for total removal of the glass beads. The supernatant was then centrifuged at 16,000 *g* for 10 min at 4 °C to obtain the mitochondrial fraction, which was washed in 2 ml of 10 mM potassium phosphate, pH 7.5 buffer, containing 0.25 M sucrose and 1 mM EDTA. After another centrifugation at 16,000 *g* for 10 min at 4 °C, the pellet was resuspended in 200 μl of reaction medium (10 mM HEPES, pH 7.2, 125 mM sucrose, 65 mM KCl, 1 mM $MgCl_2$ and 2 mM K_2PO_4), and the total protein concentration was determined by the Biuret method (Gornall et al. 1949).

Lectin preparation

Seeds of *C. mollis* Mart. (camaratu bean) were collected in the State of Pernambuco (Brazil), and the lectin (Cramoll 1,4) was purified according to (Correia and Coelho 1995). The seed extract (10% w/v in 0.15 M NaCl) was ammonium sulphate-fractionated (40–60%) and then purified by affinity chromatography on a Sephadex G-75 column. Cramoll 1,4 elution was performed with 0.3 M glucose in 0.15 M NaCl, and protein concentration was determined according to (Lowry et al. 1951).

Microscopy

Epimastigotes or trypomastigotes (1.25×10^8 cells/ml) were incubated in PBS, pH 7.2, + 1 mM $MgCl_2$ with 2.5 or 50 μg/ml Cramoll 1,4 for 1 h. Phase contrast microscopic images were captured on a Leica DM IRB inverted microscope (Leica Microsystems, Wetzlar, Germany) coupled to an acquisition image system (CoolSnap-Pro/Colour). In order to evaluate cell death caused by necrosis, epimastigotes (1×10^7 cells/ml) were incubated in PBS, pH 7.2, + 1 mM $MgCl_2$ with 50 μg/ml Cramoll 1,4 for 2 h at 28 °C. Next, cells were stained with 10 μg/ml propidium iodide (590 excitation/535 emission) for 10 min, and images were captured using an epifluorescence microscope (Axioplan, Carl Zeiss, NY, USA).

DNA agarose gel electrophoresis

DNA fragmentation was evaluated based on the method described by (Piacenza et al. 2001). Briefly, epimastigotes (1.25×10^8 cells/ml) were incubated in PBS, pH 7.2, + 1 mM $MgCl_2$ with 50 μg/ml Cramoll 1,4 for 1, 2, 4, 8, 18 or 24 h. Parasites were then collected by centrifugation at 13,000 *g* for 5 min. Cell pellets were resuspended in 0.5 ml of TE buffer (10 mM Tris-HCl + 1 mM EDTA, pH 8.0) containing 5% Triton X-100 and kept on ice for 15 min. Next, samples were centrifuged at 13,000 *g* for 15 min at 4 °C to separate intact chromatin (pellet) from DNA fragments (supernatant). After the addition of 5 μl of 1 M $MgCl_2$ + 100 μl of 5 M NaCl + 1 ml of isopropyl alcohol, the supernatant was incubated overnight at −20 °C to precipitate DNA fragments. Intact chromatin and DNA fragment pellets were resuspended in 50 μl of TE buffer and treated with 100 μg/ml RNase at 37 °C for 1 h. Then, samples were treated with 500 μg/ml proteinase K at 55 °C for 1 h and separated by electrophoresis on a 2% agarose gel at 80 V for 3 h. DNA was stained with ethidium bromide (10 μg/ml for 15 min) and visualized in a Fuji Fla 3000G Laser Scanner (Fuji Film Co., Japan).

Determination of Cramoll 1,4 effect on cell viability and proliferation rates

T. cruzi epimastigotes (1.25×10^8 cells/ml) were incubated in PBS, pH 7.2, + 1 mM $MgCl_2$ with different lectin concentrations (1, 2.5, 5, 10, 20 or 50 μg/ml) for 1 h at 28 °C, and cell viability was verified by the MTT method (Muelas-Serrano et al. 2000). This method assesses viability by cellular conversion of the soluble tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into the insoluble formazan by mitochondrial enzymes. After cell treatment with the lectin as described above, cells were centrifuged (1,000 *g* for 5 min at 4 °C), and the pellet resuspended and incubated for 1.5 h in PBS containing MTT (2.5 mg/ml). Formazan extraction was performed using 10% SDS dissolved in 0.01 N HCl. Formazan formation was quantified in a spectrophotometer at 540 nm. In order to establish the effect of Cramoll 1,4 on cell proliferation rates after incubation with different lectin concentrations, as described above, cells were centrifuged (1,000 *g* for 5 min) and grown in LIT medium for 5 days. The number of cells was determined in a Neubauer chamber.

Treatment of the keratinocytes (1×10^6 cells/ml) with the lectin was performed after trypsinization (0.05% trypsin + 0.025% EDTA solution). Keratinocyte viability was analyzed by annexin V FITC and propidium iodide staining in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser and CellQuest software (version 4.1). Briefly, 10^6 cells were incubated with different lectin concentrations (1, 2.5, 5, 10, 20 or 50 μg/ml) for 1 h at 28 °C, washed in PBS and resuspended in binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) containing annexin V-FITC (1:500). After 20 min incubation in the dark at room temperature, cells were also stained with propidium iodide (PI, 1:50). Apoptosis was quantified by FACS analysis as the number of annexin V-FITC-positive and PI-negative cells divided by the total cell number, while necrosis was quantified as the number of PI-positive cells divided by the total cell number.

Measurement of Ca^{2+} movements

Variations in free Ca^{2+} concentrations in whole cells were followed by measuring the changes in the absorbance spectrum of arsenazo III (Scarpa 1979), using an SLM Aminco DW 2000 spectrophotometer at the wavelength pair 675–685 nm (Docampo et al. 1983). Calibration was performed by recording changes in absorbance after addition of known amounts of $CaCl_2$.

Estimation of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was estimated by following safranine O fluorescence (Vercesi et al. 1991a), recorded on a F-4500 Hitachi spectrofluorimeter operating at excitation and emission wavelengths of 495 and 586 nm, respectively, and a slit width of 2.5 nm. Relative changes in membrane potential were expressed as fluorescence arbitrary units (A.U.)

Cellular oxygen consumption

Oxygen consumption was measured in a high resolution respirometry OROBOROS Oxygraph-2 k (Oroborus Instruments, Innsbruck, Austria) in PBS, pH 7.2, + 1 mM $MgCl_2$ at 28 °C. DatLab 4 software was used for data acquisition (Gnaiger 2001).

Reactive oxygen species production

After treatment for 1 h under the conditions described in the Fig. 7 legend, cells were incubated for 40 min in the dark with 5 μM dichlorodihydrofluorescein diacetate (H_2DCF-DA) under constant stirring. Fluorescence was determined on an F-4010 Hitachi spectrofluorimeter at 488 and 525 nm for excitation and emission wavelengths, respectively, and a slit width of 5 nm.

Data analysis

All results are expressed as the mean ± the standard error of the mean (SEM) for at least four independent experiments. Statistical significance was determined by one way ANOVA followed by Tukey's test, with a significance level of $p<0.05$. The cell viability and proliferation experiments were analyzed by polynomial regression (SigmaStat®, version 2.0, 1992-1995, Jandel Corporation).

Results

Recognition of glycoconjugates on the *T. cruzi* cell surface by Cramoll 1,4

Using phase-contrast microscopy (Fig. 1), we observed that *T. cruzi* epimastigotes (*a*-*c*) or trypomastigotes (*a*'-*c'*) incubated for 1 h in the presence of lectin (either 2.5 or 50 μg/ml) underwent agglutination, likely due to the recognition of glycoconjugates present on parasite cell surface. In contrast, incubation of keratinocytes with Cramoll 1,4 did not generate the stable cell agglutination observed for *T. cruzi* (data not shown).

In order to evaluate whether the lectin-glycoconjugate interaction decreased parasite survival, we used the epimastigotes form in the following experiments.

Cramoll 1,4 inhibited *T. cruzi* epimastigote survival and proliferation but had no effect on keratinocyte viability

Figure 2a shows that treatment of epimastigotes with Cramoll 1,4 for 1 h caused a dose-dependent impairment of cell viability, reaching a maximum decrease of 60% upon treatment with 20 μg/ml. In order to ascertain whether the remaining cells were still capable of proliferating, parasites (treated with Cramoll 1,4 for determination of cell viability) were washed twice to remove unbound lectin, resuspended in culture medium, and after 5 days, the number of live parasites was determined. As can be seen in Fig. 2b, a significant decrease in the proliferation rate was observed with Cramoll 1,4 at concentrations higher than 20 μg/ml, reaching almost 100% inhibition at 50 μg/ml. These results indicate that the parasites able to reduce MTT to formazan after lectin treatment were not capable of proliferating in culture. This lectin had no significant effect on keratinocyte viability under the same incubation conditions (Fig. 3). In that case, cell viability was analyzed by flow cytometry because the agglutination promoted by

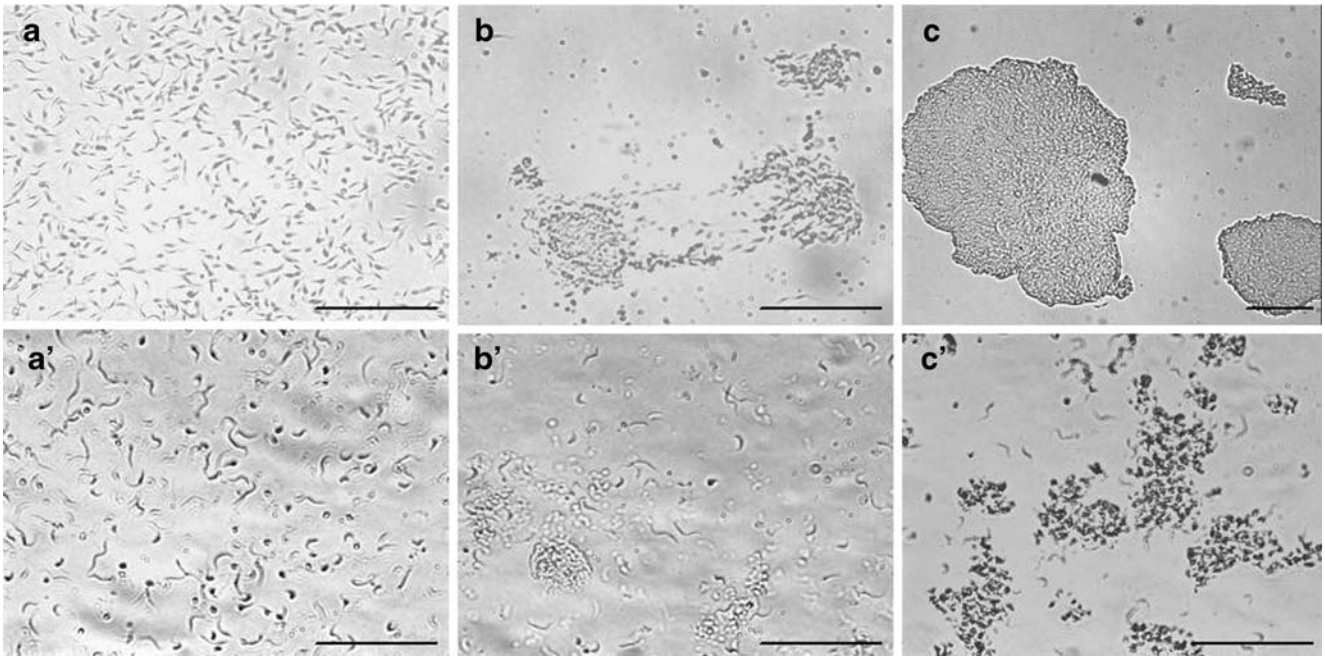

Fig. 1 Phase-contrast microscopy of untreated and Cramol 1,4-treated *T. cruzi* epimastigotes and trypomastigotes. Epimastigotes (*a*-*c*) or trypomastigotes (*a*'-*c*') were incubated in the absence (*a, a*'), or in the presence of 2.5 μg/ml Cramoll 1,4 (*b,b*') or 50 μg/ml Cramoll 1,4 (*c,c*') for 1 h. (*a, a*',*b, b*', and *c*'x 500); (*c* x 125). Bars represent 100 μM. The results shown are representative of four independent experiments

the lectin in these cells was not stable as in *T. cruzi* and could be reversed with gentle stirring.

Cramoll 1,4 promoted *T. cruzi* epimastigote plasma membrane permeabilization followed by medium Ca^{2+} internalization and accumulation by the mitochondrion

Digitonin has been used over the years to permeabilize the plasma membrane of trypanosomatids, allowing for the estimation of the mitochondrial membrane potential and the study of oxidative phosphorylation and Ca^{2+} transport by mitochondria *in situ* (Vercesi et al. 1991a, b; Vercesi et al. 1993). Indeed, when cells were incubated in the presence of digitonin (Fig. 4, *line a*) permeabilization of the plasma membrane was followed by mitochondrial Ca^{2+} uptake, as evidenced through the release of accumulated Ca^{2+} caused by the respiratory inhibitor antimycin A. Interestingly, incubation of *T. cruzi* epimastigotes in the same reaction medium containing Cramoll 1,4, instead of digitonin, was also followed by a slightly slower decrease in medium Ca^{2+} that was reversed by antimycin A. This indicates that, similar to digitonin, Cramoll 1,4 has the ability to permeabilize *T. cruzi* epimastigote plasma membranes to Ca^{2+} (Fig. 4, *line b*). In both cases, addition of the ionophore A23187 after antimycin A resulted in an additional release of Ca^{2+} from other intracellular stores (Moreno et al. 1992a, b). No Ca^{2+} uptake was observed when *T. cruzi* was incubated in the presence of heat-denatured lectin or in the absence of digitonin (Fig. 4, *lines c* and *d*).

Cramol 1,4 treatment decreased mitochondrial membrane potential ($\Delta\Psi_m$) and impaired oxidative phosphorylation

As illustrated in Fig. 5a, digitonin was used to permeabilize epimastigote plasma membrane allowing for the determination of $\Delta\Psi_m$ and ADP phosphorylation, as indicated by the fluorescence increase caused by ADP (100 μM) and reversed by carboxyatractylosyde (CAT, 5 μM), an inhibitor of the ADP/ATP carrier. At the end of the experiment, a total elimination of $\Delta\Psi_m$ was caused by the classical uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP, 1 μM) (*line a*). The *line b* of Fig. 5a shows that even after a preincubation period of 1 h with digitonin, epimastigotes could still phosphorylate added ADP. In contrast, when the parasites were submitted to lectin treatment for the same period (1 h), a very low increase in fluorescence was observed after ADP addition, indicating a significant impairment of oxidative phosphorylation (*line c*). On the other hand, when heat-inactivated Cramoll 1,4 was preincubated for 1 h with the epimastigotes, no safranine O uptake was observed, again indicating that denatured lectin had no ability to permeabilize the plasma membrane of the epimastigotes (*line d*). It should be noted

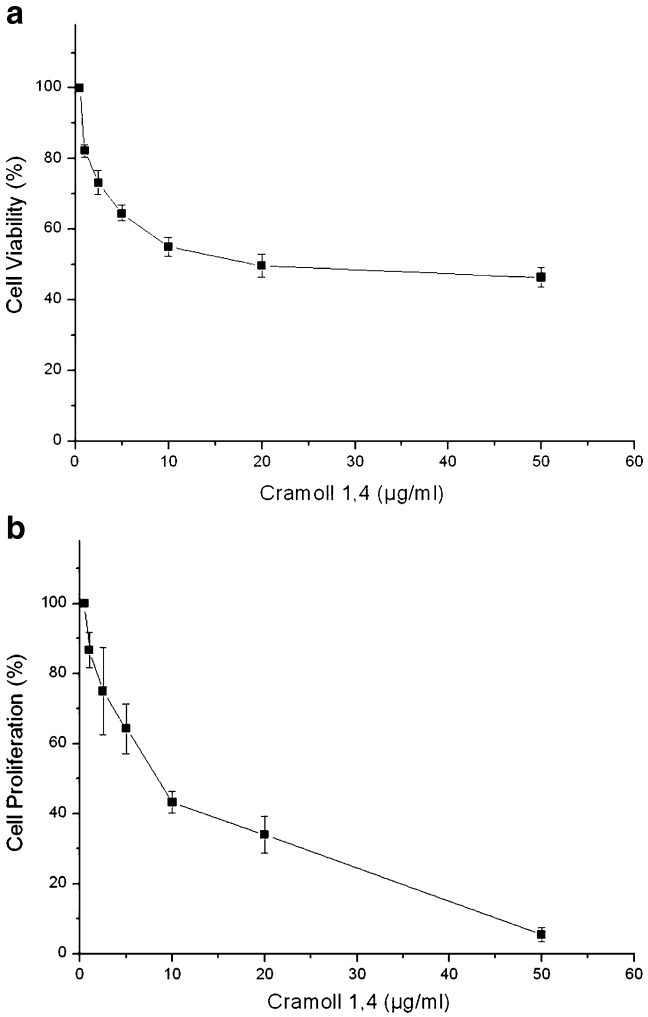

Fig. 2 Effect of Cramoll 1,4 on *T. cruzi* viability and proliferation rates. Epimastigotes (1.25×10^8 cells/ml) were incubated in PBS (pH 7.2), 1 mM $MgCl_2$ and 10 μM Ca^{2+} in the presence of different lectin concentrations for 1 h at 28 °C. **a** Cell viability was determined by the MTT assay and expressed as the percentage of viable cells related to control (r^2 = 0.90). **b** After treatment as in a, cells were washed in PBS, resuspended in culture medium, and after 5 days in culture, the number of cells was determined in a Neubauer chamber (r^2 = 0.97). Data are the average ± SEM of four independent experiments

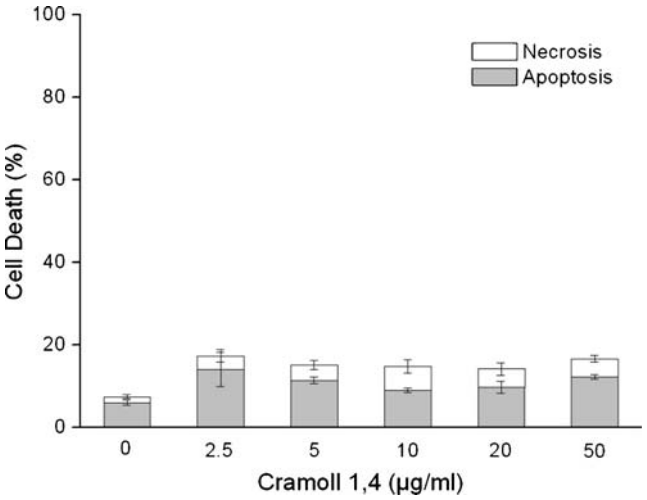

Fig. 3 HaCaT keratinocyte viability after Cramoll 1,4 treatment. After trypsinization, keratinocytes (1×10^6 cells/ml) were incubated in RPMI-1640 medium with 1% fetal bovine serum in the presence of different Cramoll 1,4 concentrations for 1 h. Data are the average ± SEM of four independent experiments. $p>0.05$

that all experiments were performed in the presence of 10 μM Ca^{2+}.

In the experiments of Fig. 5b, the parasites were incubated during a longer period of time (2 h), either in the presence of lectin (*line a*) or digitonin (*line b*), in reaction medium containing 10 μM Ca^{2+}. In both cases, $\Delta\Psi_m$ was significantly lower than in the experiments of Fig. 5a, and no ADP phosphorylation could be observed (*lines a* and *b*). The presence of EGTA significantly protected $\Delta\Psi_m$ and ADP phosphorylation in the presence of either digitonin (*line c*) or lectin (*line d*), indicating that Ca^{2+} had a major effect on mitochondrial dysfunction under both situations.

Cramoll 1,4 did not modify the uncoupled epimastigote respiration rate but released state 4 respiration

In the experiments of Fig. 6, we investigated the effect of Cramoll 1,4 (50 μg/ml for 1 h) on oligomycin-induced state 4 or CCCP-uncoupled epimastigote respiration. The lectin caused a 65% increase in state 4 respiration in the presence of Ca^{2+} and a 52% increase in free Ca^{2+} in the medium (EGTA present). In contrast, Cramoll 1,4 did not alter the

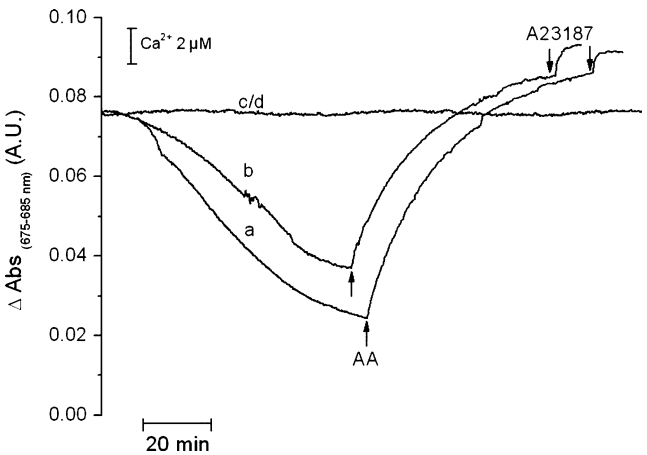

Fig. 4 Effect of Cramoll 1,4 or digitonin on Ca^{2+} uptake by *T. cruzi* epimastigotes. Parasites (1.25×10^8 cells/ml) were added to a reaction medium containing PBS (pH 7.2), 1 mM $MgCl_2$, 10 μM Ca^{2+}, 5 mM succinate and 40 μM arsenazo III in a total volume of 2 ml. Antimycin A (AA, 5 μM) and the ionophore A23187 (10 μM) were added at time points indicated by the arrows. Lines represent incubation with: 20 μM digitonin (*line a*), 50 μg/ml Cramoll 1,4 (*line b*), reaction medium (*line c*), or denatured lectin (*line d*). The results shown are representative of four independent experiments. A.U., arbitrary units

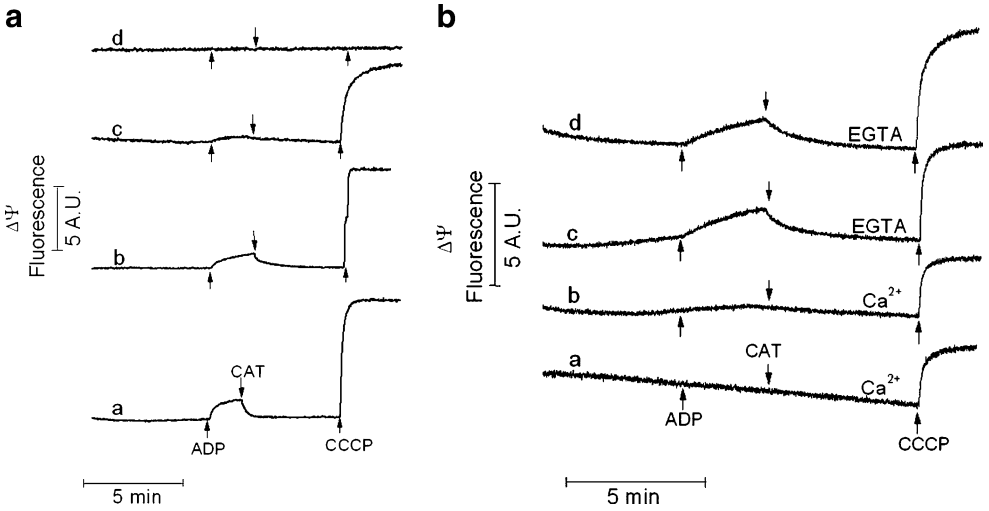

Fig. 5 *T. cruzi* epimastigote mitochondrial membrane potential ($\Delta\Psi_m$) after exposure to Cramoll 1,4. Epimastigotes (1.25×10^8 cells/ml) were added to a reaction medium containing PBS (pH 7.2), 1 mM $MgCl_2$, 5 μM safranine O and 5 mM succinate in a total volume of 2 ml. $\Delta\Psi_m$ was determined after 1 h (a) or 2 h (b) of treatment. **a** *Line a* represents the control with direct addition (no preincubation period) of digitonin (50 μM), *lines b-c* represent experiments performed after 1 h incubation with either 20 μM digitonin or 50 μg/ml Cramoll 1,4, respectively, and *line d* represents an experiment in the presence of denatured lectin. All of the experiments were performed in the presence of 10 μM Ca^{2+}. **b** *Line a* indicates cells incubated in the presence of 50 μg/ml Cramoll 1,4 + 10 μM Ca^{2+}, *line b* incubation with 20 μM digitonin + 10 μM Ca^{2+}, and *lines c-d* with 20 μM digitonin + 500 μM EGTA (Ca^{2+} free medium) or 50 μg/ml Cramoll 1,4 + 500 μM EGTA (Ca^{2+} free medium), respectively, for 2 h. Additions are indicated by the arrows: 100 μM ADP, 5 μM CAT and 1 μM CCCP. The results shown are representative of four independent experiments. A.U., arbitrary units

CCCP-uncoupled respiration rate either in the presence or absence of Ca^{2+}.

After 2 h incubation in the presence of Cramoll 1,4, respiration rates were similar in the presence of oligomycin and CCCP, indicating that respiration was completely uncoupled (data not shown). Accordingly, the results depicted in Fig. 5b (*line a*) showed that under these same experimental conditions, no changes in $\Delta\Psi_m$ was caused by ADP addition.

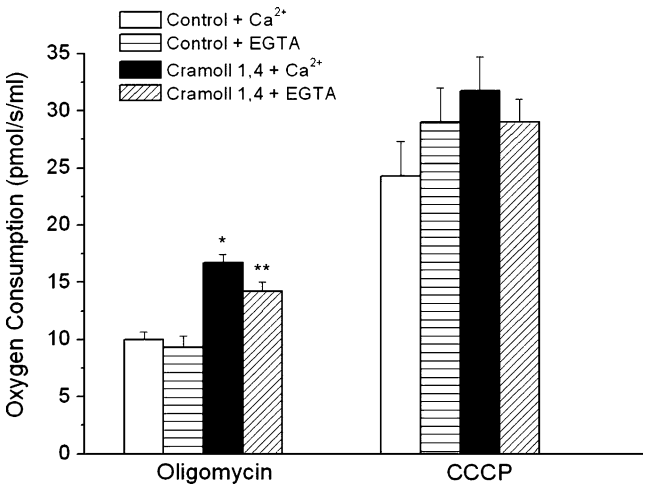

Fig. 6 O_2 consumption rates in epimastigotes exposed to Cramoll 1,4. Epimastigotes (1.25×10^8 cells/ml) were incubated with Cramoll 1,4 (50 μg/ml) in PBS (pH 7.2) + 1 mM $MgCl_2$ in the presence of 10 μM Ca^{2+} or 500 μM EGTA (Ca^{2+} free medium) for 1 h at 25 °C, in a total volume of 2 ml. Cells were then diluted to 1×10^7 cells/ml, and oxygen consumption was determined in the presence of oligomycin (2 μg/ml) or CCCP (0.5 μM). Data are the average ± SEM of four independent experiments performed in duplicate. *$p<0.05$ between the oligomycin control + Ca^{2+} vs. oligomycin + Cramoll 1,4 + Ca^{2+}. **$p<0.05$ between the oligomycin control + EGTA vs. oligomycin + Cramoll 1,4 + Ca^{2+}

Stimulation of ROS generation by epimastigotes treated with Cramoll 1,4 or digitonin: effect of Ca^{2+}

T. cruzi epimastigotes were treated with either lectin or digitonin for 1 h and subsequently loaded with H_2DCF-DA. Cramoll 1,4, but not denaturated Cramoll, increased ROS generation 5.4-fold, while digitonin increased it 4.2-fold (Fig. 7). ROS formation induced either by the lectin or digitonin was partially inhibited by EGTA. In agreement with the $\Delta\Psi_m$ experiments, Cramoll 1,4 had a higher effect on ROS production than digitonin (5.4- vs. 4.2-fold).

Cramoll 1,4, but not digitonin, decreased $\Delta\Psi_m$ in epimastigote mitochondrial fractions

All of the previous results suggested that, in addition to permeabilizing the epimastigote plasma membrane, Cramoll 1,4 also exerted a direct effect on mitochondria. This is possible because ConA, a plant lectin with high homology to Cramoll 1,4, is internalized via endocytosis and accumulates in mitochondria after it binds to the mannose moiety of the cell membrane glycoproteins (Lei and Chang 2007). Therefore, we investigated the direct effect of Cramoll 1,4 on isolated

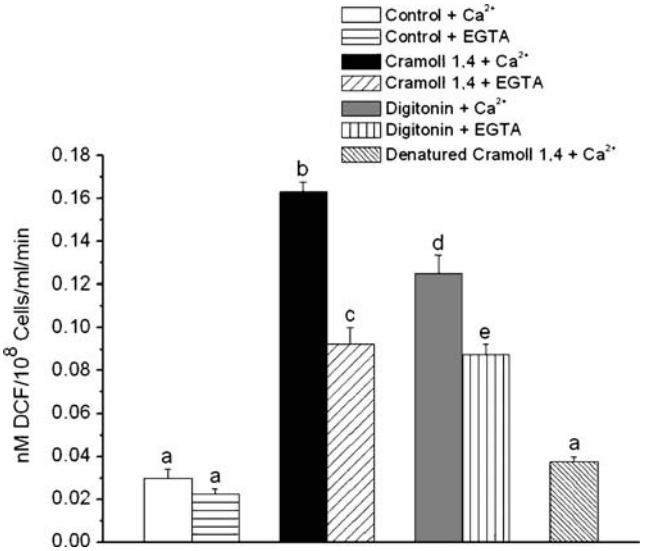

Fig. 7 ROS generation induced by Cramoll 1,4. Epimastigotes (1.25×10^8 cells/ml) were treated with 50 μg/ml Cramoll 1,4 or 20 μM digitonin for 1 h in the presence of 10 μM Ca^{2+} or 500 μM EGTA (Ca^{2+} free medium). After H_2DCF-DA loading, cells were added to the reaction medium containing PBS (pH 7.2), 1 mM $MgCl_2$ and 5 mM succinate in a total volume of 2 ml. Fluorescence was determined as described in the Materials and methods section. Data are the average ± SEM of four independent experiments. Statistical analysis: $p<0.05$ among distinct groups

epimastigote mitochondria (Fig. 8). Similar to the experiments performed with permeabilized epimastigotes, isolated mitochondria incubated in the presence of Cramoll 1,4 and EGTA (Ca^{2+}-free medium) generated a $\Delta\Psi_m$ similar to that exhibited by the control mitochondria, and both phosphorylated added ADP (Fig. 8a). In contrast, Fig. 8b shows that in the presence of 10 μM Ca^{2+}, Cramoll 1,4 significantly decreased $\Delta\Psi_m$, relative to the control experiment, confirming that it interacts directly with the organelle. This result is in agreement with the experiments showing that ConA penetrates in hepatoma cells and increases the permeability of the inner mitochondrial membrane (Lei and Chang 2007).

Cramoll 1,4-induces epimastigote death by necrosis

After epimastigote treatment with 50 μg/ml Cramoll 1,4 (2 h), the parasites were stained with propidium iodide (PI), and images were captured by fluorescence microscopy. The intense fluorescence signal obtained is compatible with cell death by necrosis (Fig. 9). This is in agreement with the results of the plasma membrane permeabilization (Fig. 4) and the impairment of epimastigote oxidative phosphorylation (Fig. 5a) after Cramoll 1,4 treatment. DNA fragmentation, which is characteristic of death by apoptosis, could not be detected by electrophoresis, even when the epimastigotes were treated with Cramoll 1,4 for up to 24 h (data not shown).

Discussion

Since the first description of the reactivity of epimastigotes and trypomastigotes with ConA (Alves and Colli 1974), lectins have been used as cell surface markers in order to evaluate changes in *T. cruzi* membrane glycoconjugate composition in different stages of the parasitic cell cycle (Bourguignon et al. 1998). Herein, we report that the interaction between Cramoll 1,4 and *T. cruzi* surface glycoconjugates results in plasma membrane permeabilization followed by medium Ca^{2+} influx and cell death. The action of Cramoll 1,4 on *T. cruzi* allowed for a better understanding of both the mechanisms of lectin toxicity and the molecular events that govern cell death in this parasite.

A large body of evidence demonstrates that mitochondrial calcium homeostasis plays an important role in the

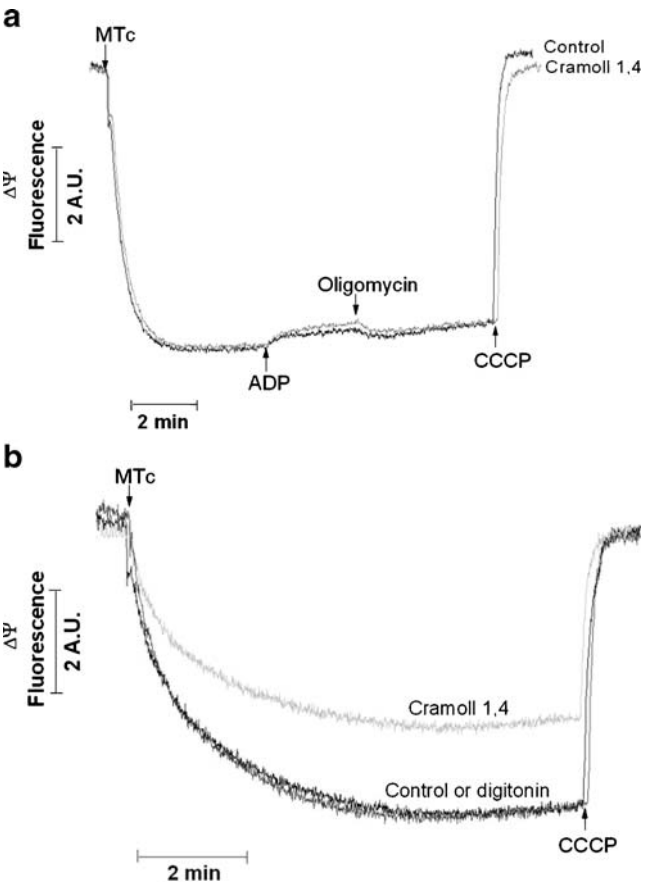

Fig. 8 Effect of Cramoll 1,4 on $\Delta\Psi_m$ of isolated epimastigote mitochondria. $\Delta\Psi_m$ was determined in the presence of 100 μM EGTA (**a**) or 10 μM Ca^{+2} (**b**). Cramoll 1,4 (50 μg/ml) or digitonin (20 μM) was added to the reaction medium. This reaction medium consisted of 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM K_2PO_4, 0.05% BSA, 5 μM safranine and 5 mM succinate in a total volume of 500 μl. *T. cruzi* mitochondrial fractions (MTc, 0.5 mg/ml), 200 μM ADP, 1 μg/ml oligomycin and 1 μM CCCP were added where indicated by the arrows. The results shown are representative of four independent experiments

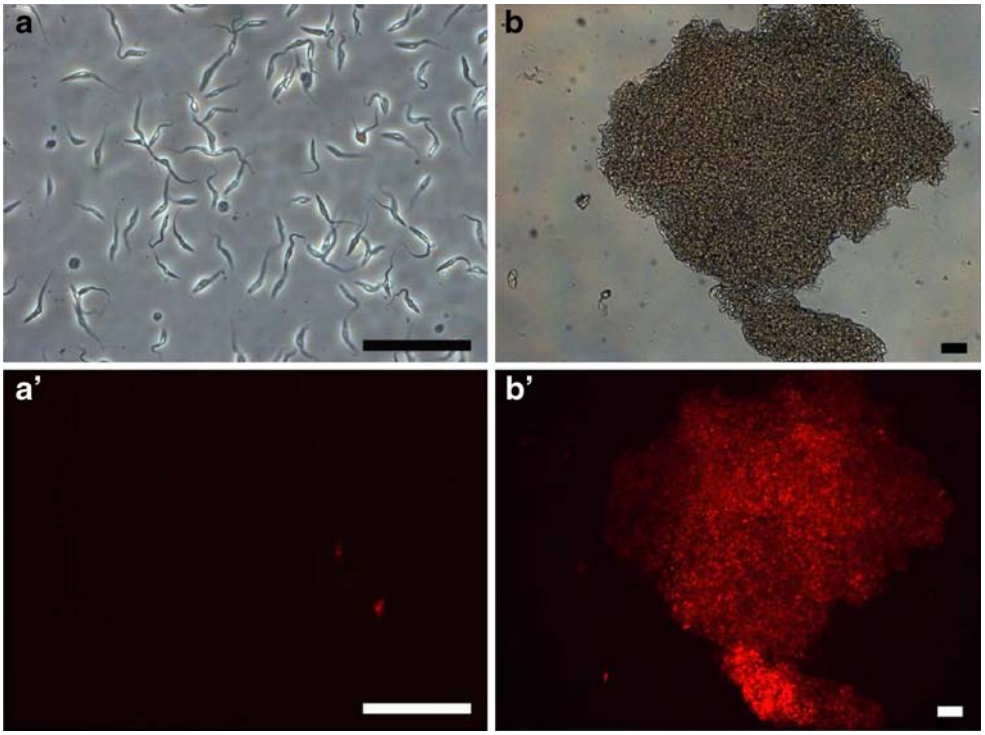

Fig. 9 Cramoll 1,4-induced epimastigote cell death occurs by necrosis. Epimastigotes (1×10^7 cells/ml) were incubated in the absence (a, a'), or in the presence of 50 μg/ml Cramoll 1,4 (b, b') for 2 h. The parasites were stained with 10 μg/ml propidium iodide. Bars represent 50 μM. The results shown are representative of two independent experiments performed in duplicate

modulation of numerous physiological cellular processes, including cell death (Giacomello et al. 2007). The *T. cruzi* mitochondrion is very active in calcium accumulation and has a high storage capacity (Docampo and Vercesi 1989; Moreno et al. 1992a, b), but under certain experimental conditions, *T. cruzi* mitochondrial calcium overload may lead to mitochondrial dysfunction. Indeed, it was observed that *T. cruzi* epimastigotes exposed to Ca^{2+} in the presence of fresh human serum underwent cell death mediated by permeabilization of the plasma membrane by membrane attack complex (MAC) deposition on the cell surface. This permeabilization was followed by cell Ca^{2+} influx and mitochondrial Ca^{2+} overload, a condition that stimulated ROS generation by the mitochondria (Irigoin et al. 2009). In this regard, Grijalba et al. (1999) demonstrated that mitochondrial Ca^{2+} overload alters the lipid organization of the inner mitochondrial membrane by interacting with the anionic head of cardiolipin, an abundant component of the inner mitochondrial membrane. These alterations in membrane organization may affect the respiratory chain function, including coenzyme Q mobility, favouring monoelectronic oxygen reduction (superoxide radical generation) at intermediate steps of the respiratory chain and leading to mitochondrial oxidative damage followed by cell death.

It has also been shown that *T. cruzi* epimastigote lysis by fresh human serum is dependent upon the alternative pathway of complement activation (Nogueira et al. 1975). In contrast, in trypomastigotes, the classical activation pathway of the human complement system is inhibited *in vitro* by parasitic calreticulin, a phosphoprotein phosphatase (Ferreira et al. 2004; Moreno et al. 2007) that renders this evolutionary form resistant to human serum.

In the current study, we showed that Cramoll 1,4, a protein of non-immune origin, is able to recognize glycoconjugates on the *T. cruzi* cell surface, leading to epimastigote agglutination (Fig. 1), thus decreasing cell viability and proliferation (Fig. 2). Cramoll 1,4 was also able to recognize *T. cruzi* trypomastigote surface membrane glycoconjugates causing agglutination (Fig. 1) and membrane permeabilization (data not shown). In contrast, this lectin was unable to agglutinate and kill keratinocytes, a human cell line. This indicates that the lectin-glycoconjugate interaction in this model was not strong enough to cause pore formation (Fig. 3).

As in the case of fresh human serum, Cramoll 1,4 induced plasma membrane permeabilization and mitochondrial calcium overload (Fig. 4). These processes were followed by increased production of ROS (Fig. 7), a $\Delta\Psi_m$ decrease and impairment in oxidative phosphorylation (Fig. 5) that culminated with cell death by necrosis (Fig. 9). As proposed by Grijalba et al. 1999, the attack of ROS to the inner mitochondrial membrane caused oxidative damage to lipids and proteins that led to increased membrane permeability with a consequent release of the resting respiration, as observed in Fig. 6.

In order to verify whether the lectin effect against *T. cruzi* epimastigotes was strictly the consequence of cell membrane permeabilization, we used digitonin as a positive control. The comparison indicated that although calcium uptake promoted by digitonin was slightly higher when

compared to that induced by the lectin (Fig. 4), the Cramoll 1,4 effects on mitochondria were stronger than that caused by digitonin, as illustrated by higher production of ROS (Fig. 7) and larger decreases of both $\Delta\Psi_m$ and oxidative phosphorylation (Fig. 5a). This prompted us to investigate a direct effect of the lectin on isolated epimastigote mitochondria. Indeed, the results indicated that Cramoll 1,4, but not digitonin, caused a significant decrease in $\Delta\Psi_m$ from isolated epimastigote mitochondria incubated in medium containing Ca^{2+} (Fig. 8b). In contrast, in Ca^{2+}-free medium, Cramoll 1,4 did not affect the ability of mitochondria to phosphorylate added ADP (Fig. 8a). The direct effect of Cramoll 1,4 on mitochondria may explain the different mechanism of epimastigote death caused by this lectin (necrosis) when compared to that caused by fresh human serum. The latter also permeabilizes the plasma membrane and causes Ca^{2+}-dependent *T. cruzi* epimastigote death by apoptosis (Irigoin et al. 2009).

To our knowledge, this is the first report investigating the mechanism of a seed lectin on *T. cruzi* mitochondrial function and cell death. We showed that Cramoll 1,4 toxicity to *T. cruzi* epimastigotes seems to result from a concerted action on the parasite's plasma and mitochondrial membranes, mitochondrial Ca^{2+} overload and ROS production. The nature of lectin interactions with the mitochondrial membranes is currently under investigation.

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