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Disruption of Ca^{2+} Homeostasis in *Trypanosoma cruzi* by Crystal Violet

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ABSTRACT. We have demonstrated previously that crystal violet induces a rapid, dose-related collapse of the inner mitochondrial membrane potential of *Trypanosoma cruzi* epimastigotes. In this work, we show that crystal violet-induced dissipation of the membrane potential was accompanied by an efflux of Ca^{2+} from the mitochondria. In addition, crystal violet inhibited the ATP-dependent, oligomycin-, and antimycin A-insensitive Ca^{2+} uptake by digitonin-permeabilized epimastigotes. Crystal violet also induced Ca^{2+} release from the mitochondria and endoplasmic reticulum of digitonin-permeabilized trypomastigotes. Furthermore, crystal violet inhibited Ca^{2+} uptake and the (Ca^{2+}-Mg^{2+})-ATPase of a highly enriched plasma membrane fraction of epimastigotes, thus indicating an inhibition of other calcium transport mechanisms of the cells. Disruption of Ca^{2+} homeostasis by crystal violet may be a key process leading to trypanosome cell injury by this drug.

Supplementary key words. Mitochondria, plasma membrane Ca^{2+}-ATPase, fura-2.

BLOOD transfusion is the second most important mechanism of transmission of Chagas' disease [9, 26, 30]. Recent studies have shown that the prevalence of blood with positive serology for *Trypanosoma cruzi* ranges from 0.5% to 60% [30]. This latest figure indicates that it would be practically impossible, in some areas of Latin America, to discard blood with positive serology for use in transfusion [30].

Crystal violet is a triphenylmethane dye described as effective against *T. cruzi* trypomastigotes in the blood by Nussenzweig and colleagues a number of years ago [26]. This dye is currently used in blood banks in some endemic areas in attempts to eliminate blood transmission of Chagas' disease [9]. Previous studies on the mode of action of this dye have demonstrated an uncoupling action of crystal violet on *T. cruzi* epimastigotes [11, 32] and mammalian mitochondria [22]. Crystal violet induces a rapid dose-related collapse of the mitochondrial membrane potential [32] and uncoupling of oxidative phosphorylation in digitonin-permeabilized epimastigotes [11]. This mitochondrial damage would lead, at a later stage, to ATP depletion [11], thus suggesting that the mitochondria are a main target of crystal violet toxicity.

Cell viability requires a perfect functioning of the processes controlling ATP and Ca^{2+} homeostasis. It is known that Ca^{2+} plays a determinant role in a variety of pathological and toxicological processes and that cell death caused by a variety of toxins or pathological conditions is associated with disruption of ATP and Ca^{2+} homeostasis [25]. Ca^{2+} accumulates in necrotic tissue [31], and a disruption of intracellular Ca^{2+} homeostasis is related to loss of cell viability [18]. This irreversible cell injury may be due to interactive effects of increased cytosolic Ca^{2+} and decreased ATP levels [17]. While increased cytosolic Ca^{2+} plays a role in many irreversible Ca^{2+}-dependent catabolic processes, adequate ATP levels might allow repair or recovery mechanisms to proceed. In fact, increase in cytosolic Ca^{2+} and decline in ATP have been correlated with cell injury caused by ischemia [17] and damage to tissue culture cells subjected to hypoxia [5] and oxidative stress [14]. While the mechanisms underlying cell injury under these conditions are controversial, several interventions limiting the depletion of ATP and the increase in cytosolic Ca^{2+} have been shown to have a protective effect on the cells [25].

In this paper we report that, in addition to the mitochondrial damage, and by independent mechanisms, crystal violet causes disruption of Ca^{2+} homeostasis in *T. cruzi* epimastigotes and trypomastigotes.

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MATERIALS AND METHODS

Culture methods. *Trypanosoma cruzi* epimastigotes (Y strain) were grown at 28° C in a liquid medium described previously [32]. Five days after inoculation, cells were collected by centrifugation and washed twice with Eagle's minimum essential medium (MEM) supplemented with 30 mM Na-Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer pH 7.2 (MEM-Hepes buffer). *Trypanosoma cruzi* trypomastigotes (Y strain) were obtained from the culture medium of L_6E_9 myoblasts as described previously [29]. The trypomastigotes were washed twice in Dulbecco's PBS. The contamination with amastigotes and intermediate forms of the preparations used was always less than 5%. The final concentration of cells was determined using a Neubauer chamber. The protein concentration was determined by the biuret assay [12] in the presence of 0.2% deoxycholate.

Isolation of plasma membrane vesicles. *T. cruzi* epimastigotes were harvested and washed once with 11 mM KCl, 140 mM NaCl, 75 mM Tris-HCl pH 7.6. Plasma membrane vesicles were then prepared as described before [2].

Chemicals. ATP, oligomycin, antimycin A, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), Dulbecco's phosphate buffer saline (D-5573), Eagle's minimum essential medium (MEM) (M-3024), ethylene-bis(oxyethylenitrilo)-tetraacetic acid (EGTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), digitonin, crystal violet, arsenazo III, and calcium ionophore A23187 were from Sigma Chemical Co. All other reagents were analytical grade.

Determination of Ca^{2+} movements. Variations in free Ca^{2+} concentration were followed by measuring the changes in the absorbance spectrum of the metallochromic indicator arsenazo III, using the SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm [28] at 28° C. Metallochromic indicators are substances that undergo color changes when the concentration of free metal ion in the solution changes. When the absorbance of arsenazo III changes are measured as a function of time at 675–685 nm, only Ca^{2+} produces absorbance changes [28]. No free radical formation from arsenazo III (i.e. one electron reduction of the indicator) [6, 21] occurred under the conditions used in the experiments described below.

Determination of ATPase activity. This was carried out as described previously [1, 2]. Briefly, aliquots of plasma membrane vesicles (about 0.5 mg protein/ml) were incubated in a medium containing 150 mM KCl, 75 mM Hepes-KOH (pH 6.8), 1 mM ATP, 1 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 mM EGTA, 1 μg/ml oligomycin, 1 μg/ml calcium ionophore A23187, and the appropriate concentrations of $CaCl_2$ to obtain the desired free Ca^{2+} concentration. Concentrations of the ionic spe-

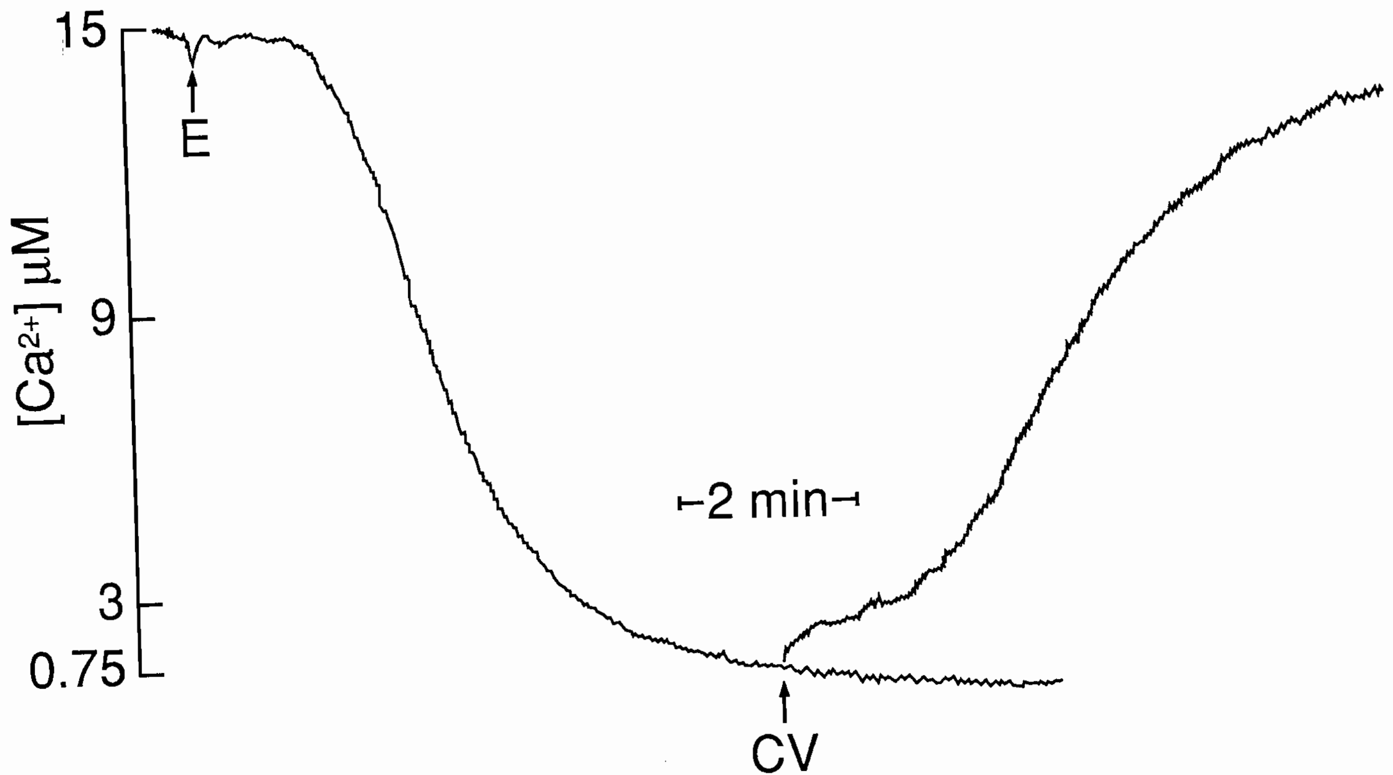

Fig. 1. Effect of crystal violet on Ca^{2+} transport by epimastigotes mitochondria in situ. The reaction medium (3 ml) contained: 125 mM sucrose, 65 mM KCl, 10 mM Hepes buffer, pH 7.2, 2.5 mM potassium phosphate, 1 mM $MgCl_2$, 2.0 mM succinate, 2 μg/ml oligomycin, 26 μM digitonin, epimastigotes (E, 0.70 mg protein/ml) and 40 μM arsenazo III. Crystal violet (CV, 50 μM) was added where indicated.

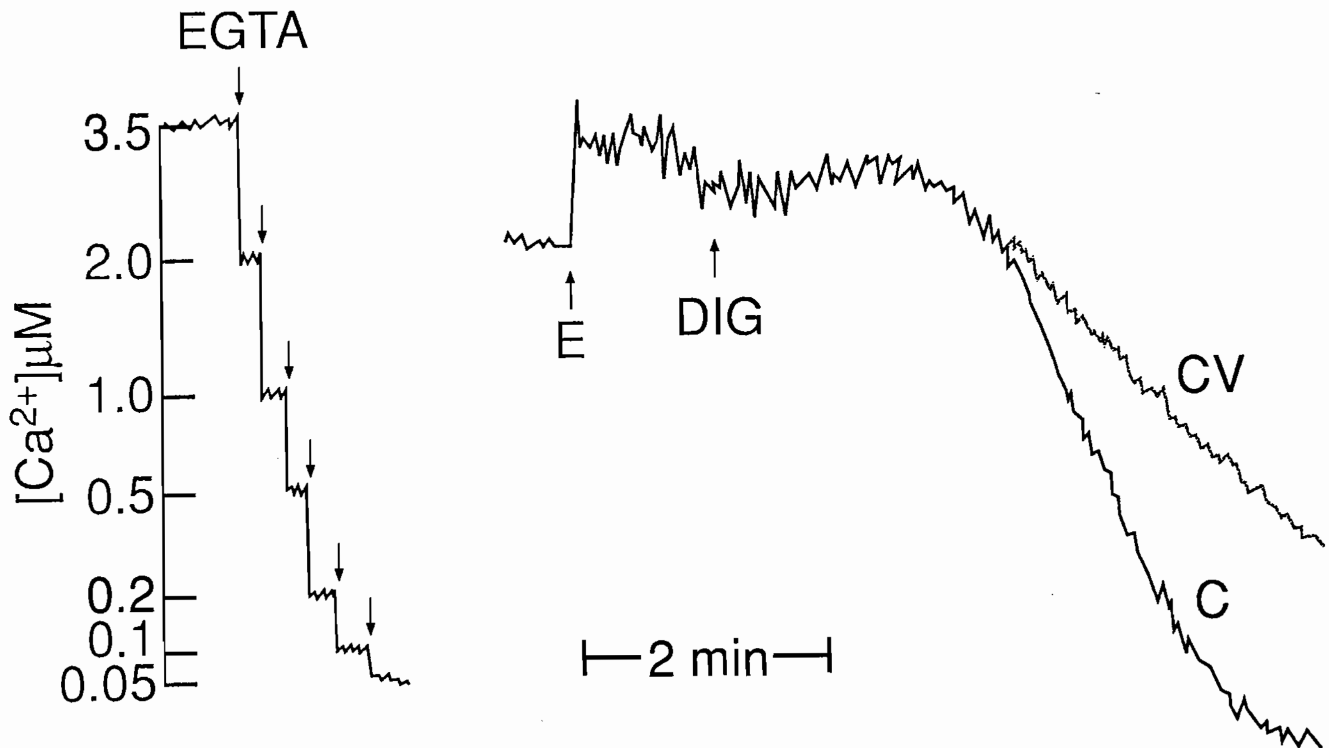

Fig. 2. Effect of crystal violet on ATP-dependent Ca^{2+} uptake by digitonin-permeabilized epimastigotes. The reaction medium (3 ml) contained: 125 mM sucrose, 65 mM KCl, 10 mM Hepes buffer, pH 7.2, 2.5 mM potassium phosphate, 1 mM $MgCl_2$, 1 mM ATP, 1 μM FCCP, and 40 μM arsenazo III. The calibration was performed by the sequential addition of known concentrations of EGTA. Epimastigotes (E, 0.70 mg/ml) and digitonin (DIG, 26 μM) were added where indicated. C, control epimastigotes. CV, epimastigotes in the presence of 50 μM crystal violet (CV).

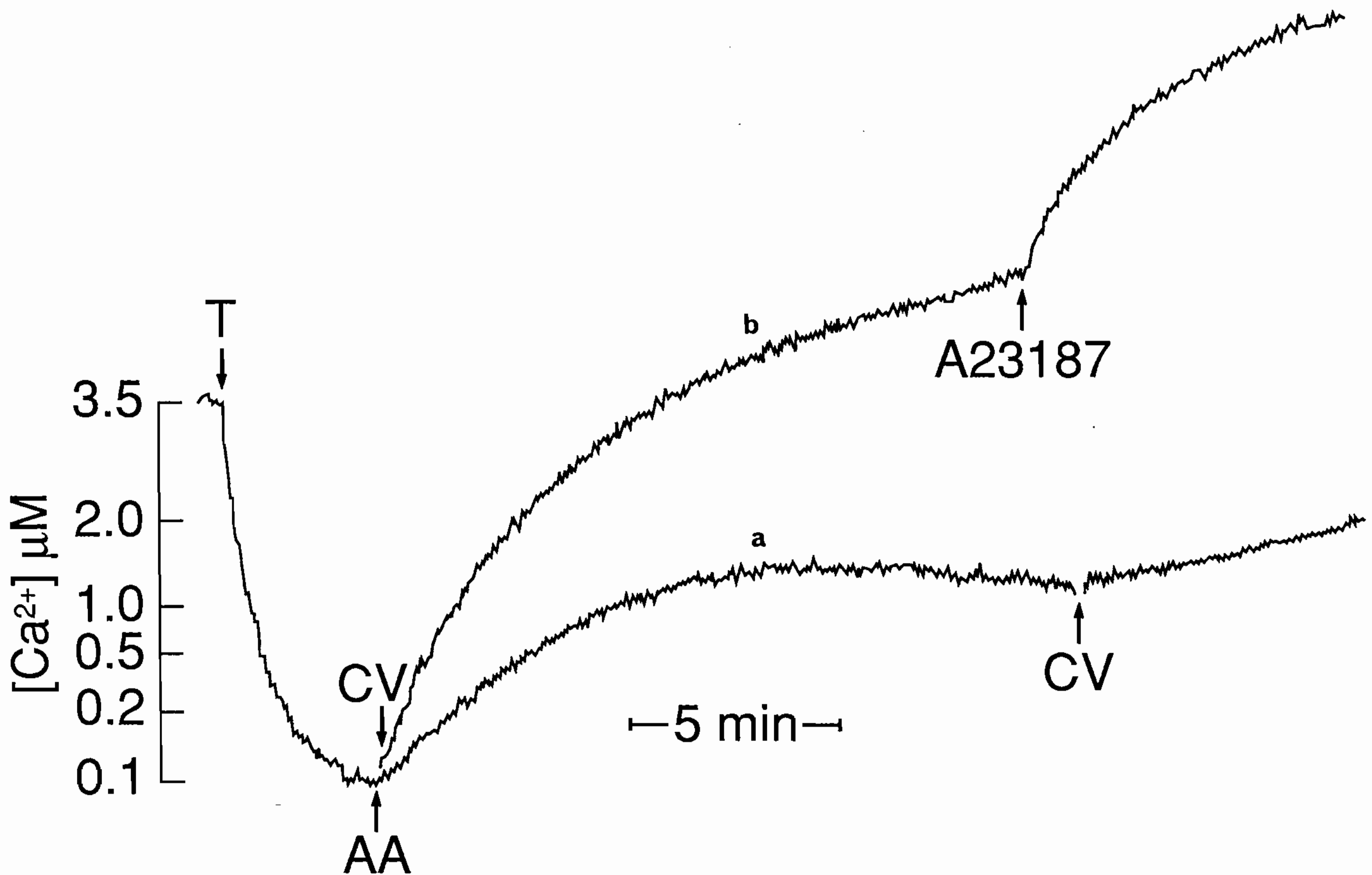

Fig. 3. Effect of crystal violet on Ca^{2+} uptake by digitonin-permeabilized trypomastigotes. The reaction medium (3 ml) contained 125 mM sucrose, 65 mM KCl, 10 mM Hepes buffer, pH 7.2, 2.0 mM potassium phosphate, 1 mM $MgCl_2$, 2.0 mM succinate, 1.0 mM ATP, 40 μM arsenzao III, and 20 μM digitonin. Trypomastigotes (T, 0.3 mg protein/ml), antimycin A (AA, 1 μg/ml), crystal violet (CV, 40 μM), and calcium ionophore A23187 (1 μM) were added where indicated.

cies and complexes at equilibrium were calculated employing an iterative computer program as described before [2]. After 45 min incubation at 28° C, the reaction was arrested by the addition of 8% (final concentration) cold trichloroacetic acid. The mixture was centrifuged and the supernatant was kept for inorganic phosphate determination. The latter was carried out according to the method of Fiske & Subbarrow [10], modified by the use of ferrous sulfate as reducing agent.

RESULTS

We have previously reported [11] that after incubation of *T. cruzi* epimastigotes in the presence of low concentrations of crystal violet (50 μM for 30 min), the most significant change was mitochondrial damage as observed by electron microscopy [11]. Since uncouplers of oxidative phosphorylation are known to release calcium from mitochondria [27], we investigated the effect of crystal violet on Ca^{2+} uptake and retention by *T. cruzi* mitochondria in situ using the digitonin-permeabilization technique [7, 8, 23, 33]. As illustrated in Fig. 1, digitonin-permeabilized epimastigotes, in the presence of succinate as respiratory substrate, were capable of taking up the Ca^{2+} present in the incubation mixture within 6 min, and to buffer external free Ca^{2+} at concentrations in the range of 0.75 μM, a concentration compatible with the buffering characteristics of mitochondria [4]. This level of Ca^{2+} could be maintained within the cells for at least 10 min. The addition of crystal violet after a steady state was attained initiated release of the accumulated Ca^{2+}. The delay between crystal violet addition and Ca^{2+} release was dose-dependent, higher concentrations of crystal violet causing a significantly shorter period of retention (not shown).

Figures 2, 3 show that crystal violet has significant effects on other Ca^{2+} transporting systems of *T. cruzi* epimastigotes and trypomastigotes. In this regard, we have found that both life cycle forms behave similarly concerning the presence of two intracellular Ca^{2+} pools, namely the mitochondrion and the endoplasmic reticulum (RD, SNJM & AEU, unpubl. data). Figure 2 shows Ca^{2+} uptake by digitonin-permeabilized epimastigotes in the presence of FCCP to prevent Ca^{2+} uptake by mitochondria, and supplemented with ATP to stimulate Ca^{2+} uptake by the endoplasmic reticulum [33]. Addition of crystal violet reduced the ability of the endoplasmic reticulum to sequester Ca^{2+} without causing efflux of the Ca^{2+} previously taken up, thus indicating that in addition to the depletion of Ca^{2+} from the mitochondria, crystal violet inhibited Ca^{2+} uptake by the endoplasmic reticulum.

The experiment depicted in Fig. 3 illustrates that when *T. cruzi* trypomastigotes were added to a medium containing ATP, succinate, and digitonin, a decrease in medium Ca^{2+} was observed until the ambient free Ca^{2+} concentration was lowered to less than 0.1 μM. The subsequent addition of antimycin A (line a) was followed by an increase in medium Ca^{2+}, indicating the existence of a mitochondrial Ca^{2+} pool. Once Ca^{2+} release

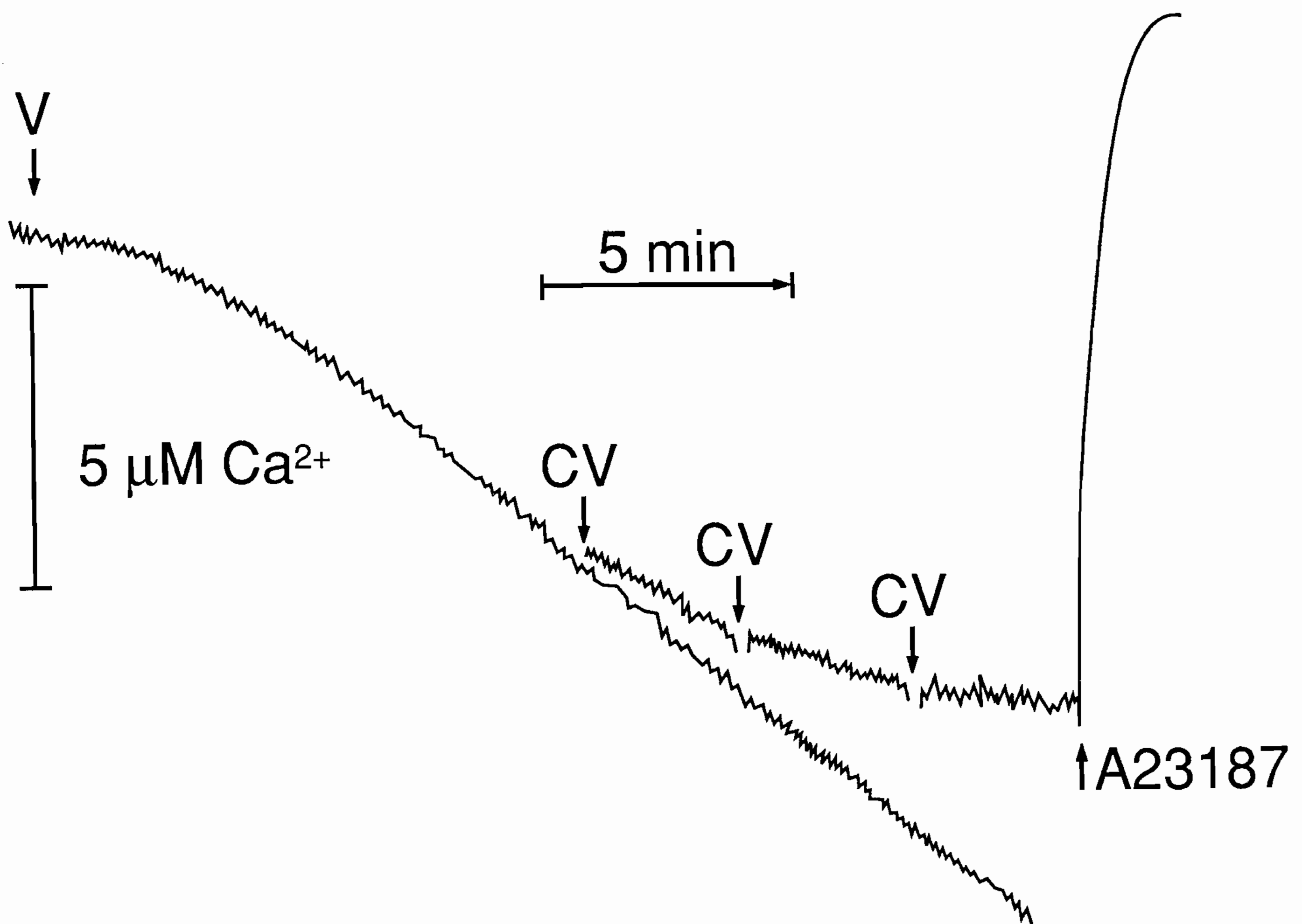

Fig. 4. Effect of crystal violet on Ca^{2+} uptake by plasma membrane vesicles from epimastigotes. The reaction medium (1 ml) contained 150 mM KCl, 75 mM Hepes, (pH 6.8), 2 mM β-mercaptoethanol, 1 mM ATP, and 1 mM $MgCl_2$. The arrows indicate the addition of vesicles (V, 0.8 mg protein/ml), different concentrations of crystal violet (CV, 25, 50 and 100 μM final concentration) or 1 μM calcium ionophore A23187.

was completed, a slow nonmitochondrial Ca^{2+} uptake was detected, probably due to the endoplasmic reticulum [33]. When crystal violet (CV, 40 μM) was subsequently added a slow increase in the ambient Ca^{2+} concentration was observed, thus indicating not only uptake inhibition but also Ca^{2+} release from this compartment. In agreement with these results, when crystal violet was added in the absence of antimycin A (line b), the increase in the ambient Ca^{2+} concentration was higher than with antimycin A alone, thus indicating that crystal violet not only prevented Ca^{2+} uptake by the nonmitochondrial compartment but also released Ca^{2+} from it. The subsequent addition of the calcium ionophore A23187 resulted in an additional Ca^{2+} release from a crystal violet-insensitive endogenous compartment.

In order to investigate the effect of crystal violet on the plasma membrane calcium transport activity, a subcellular fraction highly enriched in plasma membrane vesicles was prepared from *T. cruzi* epimastigotes [2]. This form was chosen because a great amount of cells was required to prepare this membrane fraction. The plasma membrane fraction obtained showed a (Ca^{2+}-Mg^{2+})-ATPase activity with general characteristics common to plasma membrane ATPases involved in Ca^{2+} transport such as stimulation by calmodulin, and inhibition by trifluoperazine [2]. Figure 4 shows that crystal violet caused a concentration-dependent inhibition of Ca^{2+} uptake by the plasma membrane vesicles. In contrast, addition of 1 μM FCCP did not cause any significant inhibition of Ca^{2+} uptake by these vesicles [2] (not shown). In agreement with these results, crystal violet inhibited the (Ca^{2+}-Mg^{2+})-ATPase activity of these plasma membrane preparations at similar concentrations to those that affected calcium transport (Fig. 5). In contrast, the Mg^{2+}-ATPase activity of the plasma membrane fraction was not affected by crystal violet. This result is in agreement with the results previously reported on the lack of effect of crystal violet on the activity of the plasma membrane Mg^{2+}-ATPase of epimastigotes as detected by cytochemical methods [11].

It has been reported [18, 25] that impairment of Ca^{2+} sequestration in the mitochondria and endoplasmic reticulum and of Ca^{2+} extrusion by the plasma membrane (Ca^{2+}-Mg^{2+})-ATPase could lead to an increase in cytosolic Ca^{2+}. However, attempts to detect an increase in cytosolic Ca^{2+} in either *T. cruzi* epimastigotes or trypomastigotes using fluorescent indicators, such as fura-2/AM [23, 33], were unsuccessful because of the extinction of the fluorescence of the indicator dyes by crystal violet [16].

DISCUSSION

The effects of crystal violet on digitonin-permeabilized epimastigotes and trypomastigotes are in agreement with the observations made in these cells [11, 32] and in isolated rat liver mitochondria [22] indicating uncoupling of oxidative phosphorylation. Crystal violet caused an efflux of Ca^{2+} from *T. cruzi* mitochondria in situ, which was apparently secondary to the decrease in their membrane potential [32]. Mitochondrial Ca^{2+} uptake occurs via an electrogenic process through the Ca^{2+} uniporter, which is driven by the membrane potential [4, 13, 24]. Under conditions of decreased membrane potential, Ca^{2+} efflux can occur by reversal of the uniport system [4, 13, 24].

In addition to these effects on mitochondria, crystal violet inhibited Ca^{2+} uptake by the endoplasmic reticulum as indicated by the inhibition of the ATP-dependent, oligomycin- and antimycin A-insensitive Ca^{2+} uptake by digitonin-permeabilized epimastigotes and the Ca^{2+} release from the antimycin A-insensitive pool of digitonin-permeabilized trypomastigotes. This effect on Ca^{2+} sequestration by the endoplasmic reticulum could be related to the potent inhibitory effect of crystal violet on protein synthesis by *T. cruzi* epimastigotes and trypomastigotes (MEH, SNJM & RD, unpubl. data). In this regard, it has been reported [3, 19] that the rate of protein synthesis is regulated by Ca^{2+} in a wide variety of cell types and that inhibition of microsomal calcium sequestration causes an impairment of initiation of protein synthesis [19].

Crystal violet also inhibited Ca^{2+} transport and the (Ca^{2+}-Mg^{2+})-ATPase activity of the *T. cruzi* epimastigotes plasma membrane. In this regard, it is known that Ca^{2+}-ATPases involved in Ca^{2+} transport by the plasma membrane are membrane-bound enzymes that require phospholipids for maximal activity [4]. Lipid bilayers are solvents for apolar and amphipatic compounds such as crystal violet. These agents, in turn, perturb biological membranes [15, 20, 34]. The interaction of crystal violet, as it has been described for other cationic amphiphilic drugs [15, 20, 34] with the plasma membrane, could explain in part this inhibitory effect. However, taking into account that the plasma membrane Mg^{2+}-ATPase is not inhibited by crystal violet, a direct inhibitory effect on the (Ca^{2+}-Mg^{2+}) ATPase cannot be ruled out. In addition, depletion of the ATP content of the cells caused by crystal violet [11] should cause an additional decrease in the capacity of plasma membrane and endoplasmic reticulum Ca^{2+}-ATPases to maintain calcium homeostasis.

In conclusion, disruption of Ca^{2+} homeostasis caused by the collapse of the mitochondrial membrane potential, and the inhibition of Ca^{2+} transport by the endoplasmic reticulum and the plasma membrane together with ATP depletion [11] caused by crystal violet, may be the key processes leading to trypanosome cell injury by this drug.

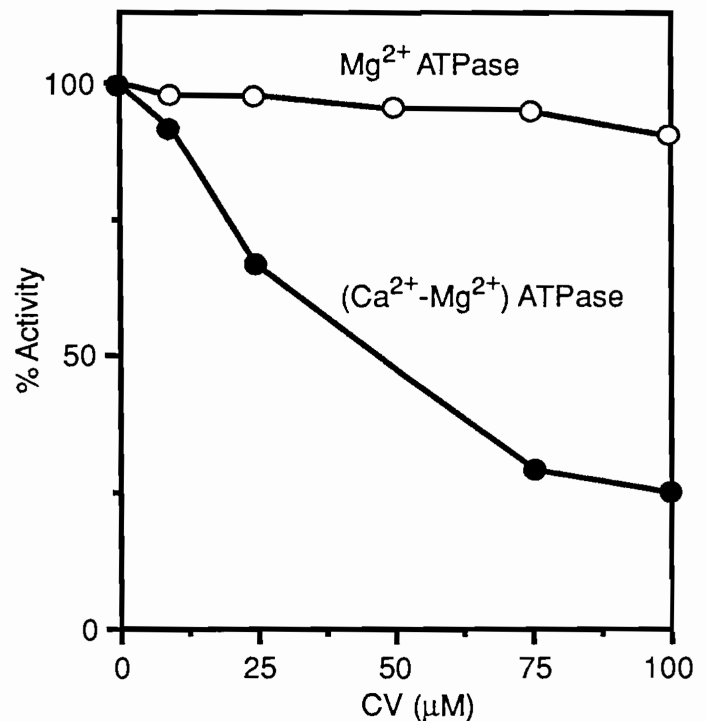

Fig. 5. Effect of crystal violet on the Mg^{2+} ATPase and (Ca^{2+}-Mg^{2+})-ATPase activities of plasma membrane vesicles from epimastigotes. Plasma membrane vesicles from epimastigotes (0.5 mg protein/ml) were incubated in a medium containing 150 mMKCl, 75 mM Hepes-KOH (pH 6.8), 1 mM ATP, 1 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 mM EGTA, 1μg/ml oligomycin, 1 μg/ml calcium ionophore A23187, the appropriate concentration of $CaCl_2$ to obtain a free Ca^{2+} concentration of 1μM, and different concentrations of crystal violet. After 45 min incubation at 28° C, the reaction was stopped by the addition of 8% trichloroacetic acid. The mixture was centrifuged and the supernatant was kept for phosphate determinations as described under Materials and Methods. The control activities of the Mg^{2+} ATPase and the (Ca^{2+}-Mg^{2+}) ATPase were 36.8 ± 5.2 and 5.1 ± 1.5 nmol/min mg protein, respectively.

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