Reactive oxygen species activate a Ca^{2+} -dependent cell death pathway in the unicellular organism *Trypanosoma brucei brucei*

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Here we examine a cell death process induced by reactive oxygen species (ROS) in the haemoflagellate *Trypanosoma brucei brucei*. Ca^{2+} distribution in cellular compartments was measured with stable transformants expressing aequorin targeted to the cytosol, nucleus or mitochondrion. Within 1.5 h of ROS production, mitochondrial Ca^{2+} transport was impaired and the Ca^{2+} barrier between the nuclear envelope and cytosol was disrupted. Consequently the mitochondrion did not accumulate Ca^{2+} efficiently in response to an extracellular stimulus, and excess Ca^{2+} accumulated in the nucleus. The terminal transferase deoxytidyl uridine end labelling assay revealed that, 5 h after treatment with ROS, extensive fragmentation of nuclear DNA occurred in over 90% of the cells. Permeability changes in the plasma membrane did not occur until an additional 2 h had elapsed. The intracellular

Ca^{2+} buffer, EGTA acetoxymethyl ester, prevented DNA fragmentation and prolonged the onset of changes in cell permeability. Despite some similarities to apoptosis, nuclease activation was not a consequence of caspase 3, caspase 1, calpain, serine protease, cysteine protease or proteasome activity. Moreover, trypanosomes expressing mouse Bcl-2 were not protected from ROS even though protection from mitochondrial dysfunction and ROS have been reported for mammalian cells. Overall, these results demonstrate that Ca^{2+} pathways can induce pathology in trypanosomes, although the specific proteins involved might be distinct from those in metazoans.

Key words: apoptosis, aequorin, Bcl-2, Ca^{2+} flux, mitochondria.

INTRODUCTION

Trypanosomes of the brucei group infect humans and livestock throughout sub-Saharan Africa. These flagellated protozoa evade the host immune system by a combination of antigenic variation [1] and immune suppression [2,3]. Consequently trypanosome infections are invariably lethal unless some form of chemotherapeutic intervention is administered. Innate resistance to trypanosome infections occurs in some species owing to non-immune-mediated mechanisms of parasite killing. Human resistance to *Trypanosoma brucei brucei* could result from the production of reactive oxygen species (ROS) within the trypanosome after transport of the human haptoglobin-related protein to the lysosome [4–7]. *T. brucei rhodesiense* resists lysis and is infectious to humans [8,9]. Cape buffalo and N'dama cattle are also tolerant of *T. brucei brucei* infections. Trypanotolerance correlates with large quantities of ROS-producing proteins in the serum, especially polyamine oxidase in N'dama cattle [10] and xanthine oxidase (XO) in Cape buffalo [11,12]. The use of ROS to protect against trypanosome infection is an intriguing adaptation to a parasite that lacks catalase and peroxidase. Maintenance of redox potential depends in large part on trypanothione and trypanothione reductase [13]. The mechanisms by which ROS cause pathology to *T. b. brucei* are unknown.

The present study evaluates the process of ROS-mediated death in African trypanosomes. In mammalian cells, ROS-dependent injury and changes in Ca^{2+} homeostasis together initiate programmed cell death or necrosis in a variety of cell types [14–18]. However, trypanosomes are well protected against changes in Ca^{2+} homeostasis. No fewer than four organelles have been identified that transport Ca^{2+} in an energy-dependent manner, including the plasma membrane [19], endoplasmic

reticulum [20–22], mitochondrion [21] and novel acidocalcisome [23–25]. Targeted aequorins have been used to study the dynamic interactions of Ca^{2+} between these compartments [26–28]. The trypanosome mitochondrion maintains a low resting level of Ca^{2+} , but transiently accumulates large quantities of Ca^{2+} from the cytosol after influx across the plasma membrane [27], or after release from the acidocalcisome [27,28]. Collectively, the Ca^{2+} -transporting organelles were shown to form a fail-safe system in which the disruption of one compartment is compensated for by the others [27,28]. Here we evaluate for the first time whether the Ca^{2+} pathways might nevertheless be exploited to induce pathology in trypanosomes. The targeted aequorins are used to monitor changes in organelle Ca^{2+} homeostasis *in vivo* during the cell death process. We report that the death of trypanosomes is associated with changes in the ability of the mitochondrion to modulate $[\text{Ca}^{2+}]$, along with ROS-mediated disruption of the slight barrier properties of the trypanosome nuclear envelope. At later times, Ca^{2+} -dependent loss of motility and Ca^{2+} -dependent fragmentation of nuclear DNA was observed. Further studies are required to determine whether activation of this cell death pathway by agents other than ROS might serve as a therapy against trypanosomes.

MATERIALS AND METHODS

Treatment with XO

T. b. brucei procyclic forms were cultured in SDM-79 as described previously [26]. At mid-exponential phase, cells were washed once in Dulbecco's phosphate-buffered saline [D-PBSG: 2.7 mM KCl/1.5 mM KH_2PO_4 /137 mM NaCl/18.4 mM Na_2HPO_4 /1% (w/v) glucose (pH 7.3)] (Gibco) and resuspended at a density of

Abbreviations used: AM, acetoxymethyl ester; $[\text{Ca}^{2+}]_{\text{cyt}}$, $[\text{Ca}^{2+}]_{\text{mit}}$, $[\text{Ca}^{2+}]_{\text{nuc}}$, intracellular free Ca^{2+} concentration in the cytoplasm, mitochondrion and nucleus respectively; BAPTA, bis-(o-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; D-PBSG, Dulbecco's phosphate-buffered saline; kDNA, kinetoplast DNA; ROS, reactive oxygen species; TUNEL, terminal transferase deoxytidyl uridine end labelling; XO, xanthine oxidase.

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5×10^6 cells/ml in the same buffer containing 10 % (v/v) fetal bovine serum. XO from buttermilk (Sigma) and catalase (Sigma) were dissolved in D-PBSG before being added to the trypanosome cultures. Where indicated, cells in D-PBSG containing 10 % (v/v) fetal bovine serum were loaded with 25 μ M EGTA acetoxymethyl ester (AM) (Molecular Probes) for 1 h at room temperature. Cells were washed once to remove DMSO and unincorporated EGTA/AM, then suspended in the same buffer. Cell motility was evaluated with a Neubauer haemocytometer and Olympus BH-2 microscope. For each experimental condition, 50–100 cells were counted and the percentage of motile cells was determined. Each experiment was repeated four times.

Terminal transferase deoxynucleotidyl uridine end labelling (TUNEL) assay

Trypanosomes were treated with xanthine (1 μ M) and XO (25 m-units/ml) for variable periods. Aliquots of cells (200 μ l) were removed and allowed to settle on poly-(L-lysine)-coated coverslips for 1 h at 4 °C. Excess liquid was aspirated and cells were fixed with 3 % (w/v) paraformaldehyde in D-PBSG for 10 min and permeabilized with 0.1 % (v/v) Triton X-100 for 2 min as described previously [27]. The exposed 3'-OH ends of nicked or fragmented DNA were labelled with the Apoptosis Detection System, fluorescein (Promega) in accordance with the manufacturer's instructions. After the cells had been washed once with $2 \times$ SSC and twice in D-PBSG, they were mounted on slides with Mowiol [27]. The percentage of labelled cells was determined by counting the number of nuclei with maximum fluorescence divided by the total number of cells in a field. At least 100 cells were counted for each measurement and each experiment was repeated three times.

Stable transformants expressing Bcl-2

The mouse Bcl-2 cDNA in plasmid no. 3027 [29] was kindly provided by S. J. Korsmeyer (Washington University School of Medicine, St. Louis, MO, U.S.A.). The plasmid was digested with *Xho*I/*Sma*I and the 865 bp fragment containing the Bcl-2 cDNA was ligated into the *Xho*I/*Eco*RV sites of pTSA-Hyg2 [30]. The resulting plasmid, pTSA-Bcl-2, was linearized with *Mlu*I and used to transform procyclic cells as described previously [26]. Hygromycin-B-resistant cells were cloned once by limiting dilution. Bcl-2 expression was verified by immunoblots as described below. Transformed cell line Bcl-2/clone 8 expressed the highest level of Bcl-2 and was used in all further experiments. Cellular fractionation of Bcl-2/clone 8 was performed after lysis in a N_2 bomb as described previously [27]. The 17 500 g pellet, 100 000 g pellet and 100 000 g supernatant were separated by SDS/PAGE [12 % (w/v) gel] and analysed for Bcl-2 by immunoblot analysis.

Immunoblots

Nitrocellulose blots were blocked with 5 % (w/v) non-fat dried milk and 0.1 % (v/v) Tween 20 for 1 h in buffer A [50 mM Tris/HCl/150 mM NaCl (pH 7.5)], rinsed briefly in buffer A and incubated with antibodies in buffer A containing 0.1 % gelatin, 0.01 % (v/v) Tween 20 and 0.1 % non-fat dried milk. Recombinant Bcl-2 was detected with the rabbit anti-Bcl-2 polyclonal antibody bcl-2(Ab-2) (Calbiochem) diluted 1:350. Duplicate blots were also incubated with rabbit anti-(cytochrome c_1) [31] [kindly provided by Dr. S. Hajduk (School of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, AL, U.S.A.)] and diluted 1:1000. Incubations were overnight at 4 °C. Blots were washed in buffer A containing 0.1 % (v/v)

Tween 20, twice for 15 min each and three times for 5 min each. Horseradish peroxidase-conjugated secondary antibodies were diluted 1:50 000 in buffer A/0.1 % gelatin/0.01 % (v/v) Tween 20/0.1 % non-fat dried milk and incubated with blots for 90 min at room temperature. Blots were washed as before and proteins were detected with the enhanced chemiluminescence (ECL[®]) kit (Amersham).

Aequorin transformants and the measurement of compartmentalized Ca^{2+}

Procyclic cells were stably transformed with different aequorin constructs, as described previously [26–28]. The night before each experiment, aequorin transformants were diluted to $(2-5) \times 10^6$ cells/ml. The next day, cells at a density of $(1-2) \times 10^7$ cells/ml in SDM-79 containing 10 % (v/v) fetal bovine serum were incubated with 2.5 μ M coelenterazine (Molecular Probes) for 3.5 h. Treated cultures received 1 mM xanthine and 50 m-units/ml XO for the last 1.5 h. Cells were washed once in reaction buffer [116 mM NaCl/5.4 mM KCl/0.8 mM $MgSO_4$ /5.5 mM D-glucose/50 mM Hepes (pH 7.0)] and resuspended at a density of $(5-8) \times 10^6$ cells/ml in the same buffer plus 1 mM $CaCl_2$. Luminescence was recorded in the macrochamber of a home-built luminometer and converted off-line into $[Ca^{2+}]$ as described previously [27].

Measurements with fura 2

Cells were loaded with fura 2 for 1 h at 27 °C in SDM-79 as described previously for bloodstream forms [23]. In experiments in which EGTA/AM was present, cells were co-incubated with fura 2/25 μ M EGTA/AM for 1 h. Cells were washed once in fresh SDM-79 and left to incubate for an additional 1.5 h, without (control), or with xanthine (1 mM) and XO (50 m-units/ml). Cells were washed and resuspended in luminometer buffer as described above. Fura 2 fluorescence and quantitation of the signal was as described previously [23].

Cell viability assays

At different times after exposure to ROS, 3 ml of cells in D-PBSG supplemented with 10 % (v/v) fetal bovine serum were incubated with 3 μ M propidium iodide for 5 min. Fluorescence was recorded with a Perkin-Elmer LS-5B luminescence spectrometer at an excitation wavelength of 535 nm and an emission wavelength of 617 nm. Cells were lysed by the addition of 0.1 % (v/v) Triton X-100. The background fluorescence of propidium iodide in the absence of cells was subtracted from each value. The percentage of viable cells was determined by dividing the initial fluorescence by the maximum fluorescence obtained after total cell lysis and subtracting from 100 %.

Statistical analyses

Data sets were compared by Student's two-tailed *t* test with two samples of unequal variance. Data sets were said to be significantly different for $P < 0.01$.

RESULTS

ROS and cell motility

To evaluate the mechanism of ROS-mediated cell death in *T. b. brucei*, the actively moving flagellated trypanosomes were scored for motility by visual inspection in a Neubauer haemocytometer after treatment for 5 h with 1 μ M xanthine and variable quantities of XO (Figure 1A). Motility was chosen as an easily recognizable

Figure 1 Inhibition of cell motility in response to ROS

Procyclic trypanosomes were suspended in D-PBSG/10% (v/v) fetal bovine serum. **(A)** To the medium was added 1 μ M xanthine and various amounts of XO as indicated (U, units). After 5 h of treatment, the percentage of motile cells was determined with a Neubauer haemocytometer. Each point is the mean $\pm$ S.D. for four separate experiments with 50 cells counted in each experiment. **(B)** To the medium of each culture was added 1 μ M xanthine and 25 m-units/ml XO; motility was evaluated at the times indicated. Control cells received no further addition; catalase cells received 100 units/ml catalase; EGTA/AM cells were loaded with 25 μ M EGTA/AM for 1 h before treatment with XO/xanthine. Each point is the mean $\pm$ S.D. for three separate experiments with 50 cells counted in each experiment.

energy-dependent process and indicator of cell change. A dose-dependent inhibition of cell motility was observed (Figure 1A). Unless indicated otherwise, future experiments in this study used 25 m-units/ml XO. This amount is similar to the concentration of XO occurring naturally in Cape buffalo serum [12] and ensured motility loss *in vitro* within 5 h. Loss of motility was due to the catabolism of xanthine and was inhibited by 1 mM allopurinol, a selective inhibitor of XO (results not shown).

The time course of motility inhibition and the effects of several modulators on cell motility are shown in Figure 1(B). XO/xanthine was without effect on cell motility during the initial 1 h of exposure. However, by 3 h nearly 80% of the cells were non-motile and by 5 h 100% of the cells were non-motile. Loss of motility resulted from the ROS generated by the catabolism of xanthine because the effects were blocked by the addition of 100 units/ml catalase (Figure 1B). The motility of catalase-treated cells was not significantly different from that of untreated cells at

the end of 5 h (see the Materials and methods section for a description of the statistical analyses). When trypanosomes were washed free of the XO medium after a 1 h exposure, motility loss still occurred, albeit at a later time (results not shown). Therefore once the process of ROS-dependent motility loss was initiated, the outcome was irreversible. A role for Ca²⁺ in mediating the cell response to ROS was suggested by experiments in which cytosolic free Ca²⁺ concentrations ($[Ca^{2+}]_{cyt}$) were stabilized with 25 μ M EGTA/AM. Intracellular EGTA was used in these studies to eliminate the possibility that chelation of cations from the medium affected the XO/xanthine system. In separate studies it was determined with fura 2-loaded cells that 25 μ M EGTA/AM effectively buffered $[Ca^{2+}]_{cyt}$ below the basal level, even after exposure to XO/xanthine (results not shown). The cells loaded with EGTA/AM exhibited only a 17% loss of motility by 3 h and an approx. 40% loss of motility by 5 h. These cells were significantly improved over the XO/xanthine-treated cells and were not significantly different from the catalase-treated cells ($P < 0.01$). Similar results were obtained when $[Ca^{2+}]_{cyt}$ was stabilized with 10 μ M bis-(*o*-aminophenoxy)ethane-*N,N',N',N'*-tetra-acetic acid (BAPTA)/AM. In this instance, loss of motility occurred in only 25% of BAPTA-loaded cells after 5 h of treatment with XO, whereas 100% of the cells without BAPTA were non-motile (results not shown). These results implicate Ca²⁺ in the pathway leading to loss of motility. Previously, 2 μ M of the Ca²⁺ ionophore 4Br-A23187 was shown to elevate $[Ca^{2+}]_{cyt}$ approx. 3-fold above the resting level [23]. However, in the present experiments, 4Br-A23187 had no effect on cell motility during the same 5 h period (results not shown). Therefore Ca²⁺ is required but might not be sufficient to produce the observed changes in cell motility.

ROS and the fragmentation of nuclear DNA

Endonuclease activity was evaluated with a TUNEL assay [32] (Figure 2). Figure 2(A) shows FITC labelling of control cells after 5 h of growth in D-PBSG containing 10% (v/v) fetal bovine serum. The nucleus is relatively free of label, whereas the kinetoplast DNA (kDNA) is intensely labelled (bright spots in Figure 2). Replication of kDNA involves the release of catenated minicircles from the network with the subsequent production of reactive 3'-OH ends [33]. In contrast, cells exposed to ROS for 5 h exhibited intense FITC labelling of the nuclear DNA and kDNA (Figure 2B). A ladder was not seen in agarose gels, suggesting that the DNA was nicked but might not be completely fragmented (results not shown). When EGTA/AM-loaded cells were exposed to ROS for 5 h, the nuclear labelling was greatly inhibited, whereas the kDNA labelling was not affected (Figure 2C). The time course of nuclear DNA nicking was determined by evaluating the percentage of cells that exhibited the maximum fluorescence seen in ROS-treated cells (Figure 2D). The nuclei of control cells never became extensively labelled (Figure 2D, bottom curve). In contrast, approx. 35% of the ROS-treated cells were maximally labelled by 3 h and 95% were maximally labelled by 5 h. Nuclease activity in the EGTA/AM-loaded cells was decreased; by 5 h only 10% of the cells exhibited maximum labelling. The fluorescence of EGTA/AM-treated cells was not significantly different from that of untreated cells, although both data sets were significantly different from XO/xanthine-treated cells. The time course of DNA nicking (Figure 2D) and of motility loss (Figure 1B) were similar.

Ca²⁺ homeostasis in ROS-treated cells

To quantify Ca²⁺ changes *in vivo* that occurred in ROS-treated trypanosomes, recombinant aequorins were used. The targeted

Figure 2 ROS and the nicking/fragmentation of nuclear DNA

(A–C) Paraformaldehyde-fixed and permeabilized cells were labelled with the TUNEL procedure and examined by fluorescence microscopy after 5 h incubation in medium alone (A) or medium plus XO/xanthine (B), or after preloading with 25 μ M EGTA/AM for 1 h before incubation in medium plus XO/xanthine (C). (D) The time course of DNA nicking/fragmentation was determined. Each point represents the percentage of cells exhibiting the maximum fluorescence. At least 100 cells were randomly evaluated at each time and the experiment was repeated three times (plots show the means $\pm$ S.D.).

aequorins have been described previously and allow us to monitor $[Ca^{2+}]$ in the cytosol, nucleus ($[Ca^{2+}]_{nuc}$) and mitochondrion ($[Ca^{2+}]_{mit}$) [26–28]. Aequorin was reconstituted with coelenterazine *in vivo* for 3.5 h in SDM-79 containing 10% (v/v) fetal bovine serum, as described previously [26]. This medium permitted the uninterrupted expression of aequorin and its subsequent reconstitution with coelenterazine. XO/xanthine was added to this medium for the final 1.5 h of coelenterazine loading. Because pyruvate in the SDM-79 medium served as an antioxidant, it was necessary to double the concentration of XO to 50 m-units/ml and increase the concentration of xanthine to 1 mM. It was determined in separate experiments that cell exposure to ROS for 1.5 h under these conditions did not inhibit motility, although the cells moved more slowly. Therefore this dose of XO/xanthine was functionally equivalent to an early time point under the original conditions. Before making luminescence measurements, cells were washed from the medium and suspended in reaction buffer containing 1 mM Ca^{2+} .

We used the aequorin transformants to evaluate early changes in Ca^{2+} homeostasis. Within 1.5 h of exposure to ROS, the basal level of Ca^{2+} within the mitochondrion became significantly elevated from 517 ± 81 nM ($n = 12$) to 917 ± 195 nM ($n = 13$) (Figure 3B). Transport properties of the mitochondrion were measured *in vivo* after Ca^{2+} homeostasis had been perturbed with 200 nM melittin. In *T. b. brucei*, 200 nM melittin causes a selective (La^{3+} -inhibitable) influx of Ca^{2+} across the plasma membrane [34]. The mechanism involves the activation of phospholipase A_2 . Arachidonic acid (eicosatetraenoic acid), but not eicosatrienoic acid or palmitic acid, can mimic the effects of melittin [35]. On addition of melittin, the Ca^{2+} transport properties of the mitochondrion were shown to be significantly impaired (Figure 3B). The mitochondrion from control cells transiently accumulated Ca^{2+} to a peak of 8.03 ± 2.09 μ M ($n = 12$), whereas the mitochondrion from ROS-treated cells could only accumulate Ca^{2+} to a peak level of 5.56 ± 0.59 μ M ($n = 13$).

We determined whether excess Ca^{2+} might accumulate in the

Figure 3 Effects of ROS on Ca²⁺ homeostasis in *T. b. brucei*

Stable transformants were created that expressed aequorin targeted to the cytoplasm (**A**), mitochondrion (**B**) or nucleus (**C**). Control cells (solid lines) and cells exposed to ROS for 1.5 h (dotted lines) were compared. Melittin (200 nM) was added to disrupt homeostasis at the times indicated with an arrow. Each experiment was repeated from 6 to 13 times with means $\pm$ S.D. for basal and peak Ca²⁺ levels indicated in the text. Student's *t* test was used to compare data sets for control cells and those treated with XO/xanthine. XO/xanthine significantly altered basal Ca²⁺ and peak Ca²⁺ between the data sets ($P < 0.01$).

nucleus (Figure 3C). The ROS-treated cells exhibited a significantly higher basal level of [Ca²⁺]_{nuc} than control cells [609 $\pm$ 112 nM ($n = 9$) and 406 $\pm$ 48 nM ($n = 10$) respectively]. However, the most marked change occurred in response to melittin. We have previously demonstrated that the nuclear envelope of *T. b. brucei* is a slight barrier to the free exchange of

Ca²⁺ with the cytosol [27,28]. This situation contrasts with some mammalian cells, in which the rapid equilibration of cytosolic and nuclear Ca²⁺ occurs [36,37]. In trypanosomes the addition of 200 nM melittin to control cells elevated [Ca²⁺]_{nuc} to a lower level than [Ca²⁺]_{cyt} [compare Figure 3(C), in which the peak [Ca²⁺]_{nuc} in control cells is 0.99 $\pm$ 0.22 μ M ($n = 10$), with Figure 3(A), in which the peak [Ca²⁺]_{cyt} in control cells is 2.65 $\pm$ 0.72 μ M ($n = 9$)]. However, ROS disrupted the barrier properties of the nuclear envelope; the subsequent addition of melittin elevated [Ca²⁺]_{nuc} to 2.36 $\pm$ 0.78 μ M (Figure 3C).

Although Ca²⁺ levels in the mitochondrion and nucleus were affected by ROS, the aequorin procedure did not measure any significant change in the basal level of [Ca²⁺]_{cyt} [control cells, 416 $\pm$ 95 nM ($n = 9$); ROS-treated cells, 498 $\pm$ 31 nM ($n = 6$)] (Figure 3A). However, differences in the basal level of [Ca²⁺]_{cyt} might have occurred without being detected, because the Ca²⁺-independent oxidation of coelenterazine makes the calculation of [Ca²⁺] difficult in the range 200–300 nM [27]. The Ca²⁺ measurements were therefore repeated with fura 2 (results not shown). Fura 2-loaded cells were incubated for 1.5 h with XO/xanthine as described for the aequorin clones; fluorescence was measured in the luminometer buffer. The basal level of [Ca²⁺]_{cyt} after treatment with XO/xanthine was 583 $\pm$ 169 nM ($n = 16$ for six separate cultures), in general agreement with the value that we obtained with aequorin. However, the basal level for control cells incubated for 1.5 h with no additions was significantly lower than in measurements made with aequorin [190 $\pm$ 85 nM ($n = 15$ for four separate cultures)]. Therefore inaccuracies in the aequorin measurements at the lower range of [Ca²⁺] obscured changes in basal levels of [Ca²⁺]_{cyt} that occurred after treatment with XO/xanthine. We conclude that at early times after treatment with XO/xanthine, an elevation in the basal level of [Ca²⁺]_{cyt} occurred. Fura 2-loaded cells were also used to verify the ability of EGTA/AM to stabilize [Ca²⁺]_{cyt} near the basal level. Cells were loaded simultaneously with 25 μ M EGTA/AM and 25 μ M fura 2/AM. After 1.5 h of exposure to XO/xanthine, the basal level of [Ca²⁺]_{cyt} was maintained at 101 $\pm$ 57 nM ($n = 16$ for 3 separate cultures).

Changes in cell permeability

The temporal relationship between changes in cell [Ca²⁺], loss of motility, DNA nicking and an increase in cell permeability was evaluated. Disruption of membrane integrity was detected by the increase in fluorescence of membrane-impermeant propidium iodide (Figure 4). During the initial 5 h of exposure to ROS, when motility was completely inhibited, cells were still intact as determined by the propidium iodide assay. After 7.5 h of exposure to ROS, approx. 35% of the cells were intact; by 24 h only 9% were intact. Loading of cells with EGTA/AM prolonged the onset of permeability changes; after 7.5 h of exposure to ROS, approx. 97% of the cells were still intact. This value is significantly different from XO/xanthine-treated cells but not from untreated cells. However, EGTA/AM could not prevent permeability changes and was itself toxic to the cells during long-term incubations. Nevertheless, after 24 h of exposure to ROS, approx. 40% of the EGTA/AM-loaded cells were intact; this value was significantly higher than cells that did not receive the cell-permeant Ca²⁺ chelator, although it was significantly lower than untreated control cells.

Effects of apoptosis inhibitors

The results presented so far demonstrate that cell death in *T. b. brucei* could be associated with endonuclease activation brought about by elevated ROS and accompanied by changes in mito-

Figure 4 Cell permeability changes after treatment with ROS

At the indicated times, cells were incubated for 5 min with 3 μ M propidium iodide and the percentage of maximum possible fluorescence was recorded. Cells were untreated (control), exposed to 1 μ M xanthine and 20 m-units/ml XO (XO) or loaded with EGTA/AM for 1 h before treatment with XO/xanthine (EGTA/AM). Each experiment was repeated three times; means $\pm$ S.D. are plotted.

chondrial Ca^{2+} stores. In mammalian cells, protease activation can occur downstream of mitochondrial dysfunction and is responsible for endonuclease activation (reviewed in [38]). In these instances, cysteine proteases of the caspase family, Ca^{2+} -sensitive calpains or proteasomes can initiate cell death. However, we report that inhibitors of caspase 3 (inhibitory peptide Z-DEVD-SMK, 5 μ M), caspase 1 (inhibitory peptide Z-VAD-FMK, 5 μ M), calpain (calpain I inhibitor, 20 μ M), serine protease (tosyl-lysylchloromethane, 100 μ M; PMSF, 1 mM), cysteine protease [E-64d, (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester, 20 μ M] or proteasomes (lactacystin, 10 μ M) were without effect on cell death brought about by ROS (results not shown).

Proteins of the Bcl-2 family can protect cells from caspase-dependent and caspase-independent cell death (reviewed in [39,40]). Several mechanisms of Bcl-2 activity have been described, including the ability to prevent mitochondrial dysfunction by blocking the opening of the mitochondrial permeability transition pore complex [39,40]. Cells that overexpress Bcl-2 are protected from the lethal effects of ROS [41]. Importantly, the effects of Bcl-2 are not species-specific; mouse Bcl-2 and Bcl-xL can protect yeast from Bax-induced cell death [42]. Moreover, association with Bax was not required in all cases to suppress cell death [42]. Consequently we prepared stable trypanosome transformants expressing mouse Bcl-2. Western blots demonstrated the expression and distribution of mouse Bcl-2 in cell fractions from stable trypanosome transformants (Figure 5A). The protein was not present in control procyclic cells (results not shown) and occurred in the 17000 g pellet but not the 100000 g pellet or the 100000 g supernatant. Its distribution was identical with that of cytochrome c_1 (Figure 5A). The low-speed pellet (1000 g at 15 min) was not evaluated for Bcl-2 because this fraction was contaminated with unbroken cells. Bcl-2 transformants exhibited normal motility (Figure 5B, Bcl-2 control). However, the transformants were not protected from the effects of ROS (Figure 5B, Bcl-2 XO), and the loss of motility was not significantly different from that of control procyclic cells (Figure 5B, Proc XO). Similarly, the TUNEL assay revealed no signifi-

Figure 5 Response of Bcl-2 transformants to ROS

(A) Cellular distribution of Bcl-2 in procyclic cells was determined by Western blot analysis of cell fractions. Samples (approx. 15 μ g of each) were separated by SDS/PAGE and transferred to nitrocellulose. The blots were incubated with anti-Bcl-2 (α -Bcl-2) or anti-cytochrome c_1 (α -Cyt c_1), as indicated. Lane a, whole-cell homogenate; lane b, 17000 g pellet; lane c, 100000 g pellet; lane d, 100000 g supernatant. (B) The effects of ROS on trypanosome motility. Bcl-2 transformants (Bcl-2 XO) and normal procyclic cells (Proc XO) were treated with 1 μ M xanthine and 25 m-units/ml XO; motility was evaluated at the times indicated. Bcl-2 transformants without XO/xanthine were also evaluated (Bcl-2 control). Each point is determined from an analysis of 50 cells. Values plotted are means $\pm$ S.D. for three separate experiments. (C) The effects of ROS on DNA nicking/fragmentation was tested with the TUNEL assay. The percentage of cells exhibiting maximal fluorescence was determined at different times after treatment with ROS. The treatment and labelling of the individual curves were as described for (B). Each point is determined from an analysis of 50–100 cells. Values plotted are means $\pm$ S.D. for three separate experiments.

cant change in the susceptibility of the nuclear DNA to nicking/fragmentation in response to ROS (Figure 5C).

DISCUSSION

Ca^{2+} flux is a hazardous method of propagating signals in eukaryotic cells. Although small quantities of Ca^{2+} can trigger rapid changes in cell activity, disruption of homeostasis can also be toxic. In a wide variety of multicellular organisms, ROS production, in conjunction with changes in Ca^{2+} homeostasis, is a common feature of programmed cell death and necrosis [14–18]. If applicable to pathogenic protozoa, these same Ca^{2+} pathways

might be targeted in the design of novel therapies. The present study has examined the process by which ROS kills the unicellular pathogen *T. b. brucei*. ROS production might be an important negative regulator of the trypanosome infection cycle, as evidenced by the role of ROS in innate resistance to trypanosome infections of humans [4–7] and livestock [10–12]. Ca²⁺ might also have a role in infection because the virulence of *T. b. brucei*, as defined by the ability to infect the mammalian host, depends on the ability to elevate [Ca²⁺]_{cyt}. Reagents that stabilize [Ca²⁺]_{cyt}, or disrupt downstream targets of Ca²⁺, prevent infection (L. Ruben, unpublished work). Similar results have been seen with BAPTA-loaded *T. cruzi* [43,44]. In the present study we show that Ca²⁺ stabilization protects *T. b. brucei* from the deleterious effects of ROS. Moreover, elevated [Ca²⁺] in conjunction with ROS can trigger a sequence of events culminating in cell death.

We used procyclic-form trypanosomes in the present study because we have previously constructed procyclic cell lines that express targeted aequorins in the cytosol, mitochondrion and nucleus [26–28]. Although procyclic cells are found within the insect host, initial observations suggest that the process of ROS-dependent death also applies to bloodstream-form trypanosomes [12]. In particular, exposure of bloodstream-form trypanosomes to XO/xanthine results in a time-dependent loss of motility. The motility loss is irreversible and precedes by several hours the permeability changes in the plasma membrane [12]. We show in procyclic forms that fragmentation/nicking of the nuclear DNA also precedes cell death. Intracellular Ca²⁺ buffers protect the cells from early loss of motility, DNA fragmentation/nicking and late-onset changes in plasma membrane permeability. Aequorin transformants permit the direct measurement of Ca²⁺ redistribution at an early time in the cell death process. The large single mitochondrion ordinarily retains low levels of Ca²⁺ but in response to a Ca²⁺ signal accumulates Ca²⁺ to levels up to 11-fold those in the cytosol [27,28]. The accumulated Ca²⁺ is retained transiently and, within 60 s of the stimulus, [Ca²⁺]_{mit} returns to basal levels. In the present study, a stable elevation in the basal level of [Ca²⁺]_{mit} was reported. The stable change might result from Ca²⁺ efflux followed by uptake in a futile cycling of Ca²⁺ across the mitochondrial membrane; this is consistent with altered permeability properties. Similar results have been reported for both isolated mitochondria and intact tissues during the ROS-mediated programmed cell death of mammalian cells (reviewed in [17]). In *T. b. brucei*, a consequence of mitochondrial damage is the inability to accumulate Ca²⁺ effectively in response to an extracellular signal (Figure 3B). Peak [Ca²⁺]_{mit} is significantly lower in the ROS-treated cells.

The decreased signal with aequorin targeted to the mitochondrion is not a consequence of ROS-mediated destruction of the aequorin or coelenterazine. This conclusion is supported by the greater luminescence signal of aequorin targeted to the nucleus in the presence of ROS than in its absence (Figure 3C). The increase in [Ca²⁺]_{nuc} results from the loss of the slight barrier properties of the nuclear envelope [26,28]. In the presence of ROS, the peak [Ca²⁺]_{nuc} is equivalent to [Ca²⁺]_{cyt} (compare Figures 3A and 3C). The basal levels of [Ca²⁺]_{cyt} and [Ca²⁺]_{nuc} are also elevated after treatment with ROS. These changes in [Ca²⁺]_{nuc} are presumed to contribute to the activation of the endonuclease responsible for nicking/fragmentation of the nuclear DNA. In general, these results are the first to demonstrate the ability of Ca²⁺ to produce pathology in *T. b. brucei*.

Interestingly, several aspects of the cell death pathway in response to ROS are similar to programmed cell death in mammalian cells, including commitment to cell death hours before permeability changes occur, disruption of mitochondrial

function, redistribution of cell Ca²⁺, changes in permeability properties of the nuclear envelope, and Ca²⁺-dependent nicking/fragmentation of the nuclear DNA. Concanavalin A has also been reported to induce an apoptosis-like process in *T. b. brucei* over the course of 48–72 h [45]. Changes in cytoskeletal organization, vacuolization of the cytosol and fragmentation of nuclear DNA were reported. In addition, the steady-state levels of some RNAs were shown to increase [46] and the cDNA species corresponding to two transcripts were cloned. These cell-death-related genes encode the mitochondrial protein, prohibitin, and the trypanosome receptor for activated C kinase, TRACK [47]. Apoptosis-like changes have also been reported for *T. cruzi* in response to conditioned medium or antibiotic G418 [48]. A shift in the distribution of EF-1 α to a nuclear localization was reported as one of the changes accompanying cell death [49]. In the related kinetoplastid *Leishmania donovani*, apoptosis-like changes were reported in response to heat shock [50]. Despite the precedence for apoptosis-like changes in kinetoplastids, the events that we report differ in several respects. Protease activation is an important component of the death process in multicellular organisms [38]. However, cell-permeant protease inhibitors were without effect on *T. b. brucei*. Although inhibitors of some caspases were not tried, the inhibitors used are known, at least, to block the caspases directly and indirectly responsible for the activation of endonuclease activity (caspase 3 and caspase 1 respectively). The lack of involvement of calpains, proteasomes, serine proteases and cysteine proteases was surprising [38]. Bcl-2 is known to suppress programmed cell death for a wide range of initiators, including ROS [39–41]. However, the expression of Bcl-2 in trypanosomes was not protective. Because Bcl-2 seemed to localize to the correct compartment, it seems that protein targets of mammalian Bcl-2 were not involved in the trypanosome response to ROS. Overall, these results demonstrate that in *T. b. brucei* ROS initiates a Ca²⁺-dependent sequence of events that culminates in cell death. An understanding of the orderly process leading up to cell death might ultimately reveal biochemical pathways that can be used to induce parasite destruction.

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