Journal of Cell Science 107, 3477-3483 (1994)
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The protein phosphatase inhibitor okadaic acid induces defects in cytokinesis and organellar genome segregation in *Trypanosoma brucei*

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SUMMARY

Mitosis and cytokinesis are events that are highly coordinated in most eukaryotic cell cycles. African trypanosomes possess a single mitochondrion and must additionally coordinate the organellar division cycle. Here we report that okadaic acid, a potent and specific inhibitor of protein phosphatases PP1and PP2A, uncouples these cycles in living trypanosomes. Cell cycle analysis of treated cells revealed elevated DNA content. Microscopic examination indicated that okadaic acid treatment yielded multinucleate cells with a single mitochondrial network indicating these cells have undergone mitosis but failed to complete cytokinesis. Immunofluorescence analysis of 5-bromo-2-deoxyuridine incorporation demonstrated that the mitochondrial DNA was replicated but did not segregate. The dose response curve for inhibition of the normal cell cycle paralleled that for the in vitro inhibition of protein phosphatase activities with IC_{50}s of approximately 20 nM okadaic acid. These results suggest the involvement of a PP1/PP2A-like activity in coordinating mitosis, mitochondrial DNA division and cytokinesis in trypanosomes.

Key words: cell cycle, mitochondrion, okadaic acid, protein phosphatase, *Trypanosoma brucei*

INTRODUCTION

Protein phosphorylation and dephosphorylation play key regulatory roles in the eukaryotic cell cycle. The essential roles of phosphatases in eukaryotic cell division have been established through studies of cell division cycle defective mutants. The lesions in several mutants defective in mitosis have been traced to phosphatase genes in *Aspergillus nidulans* (Doonan and Morris, 1989), *Schizosaccharomyces pombe* (Ohkura et al., 1989) and *Drosophila melanogaster* (Baksa et al., 1993; Mayer-Jaekel et al., 1993). The phosphatase genes thus identified encode products highly related to the catalytic subunit of type 1 serine threonine phosphatases (PP1s) of mammalian cells. Evidence indicates that PP1 may negatively regulate the $p34^{cdc2}$/cyclin mitotic kinase complex and the entry into mitosis. Phosphatase genes that encode proteins related to the catalytic subunit of type 2A protein phosphatase (PP2A) have also been identified in fission and budding yeasts (Blacketer et al., 1993). Recent studies on the role of protein phosphatases on cellular processes have been greatly facilitated by the use of okadaic acid (OKA), a cell permeant polyether derivative of fatty acid. OKA is known to be a potent, highly specific, reversible inhibitor of protein phosphatases PP1 and PP2A. It is considerably less effective towards Ca-dependent protein phosphatase PP2B and has no effect upon the structurally unrelated Mg-dependent protein phosphatase, PP2C (Cohen, 1989). No molecules other than PP1 and PP2A have been shown to be directly inhibited by OKA, despite extensive testing (Haystead et al., 1989; Cohen et al, 1990). In mammalian cells, OKA uncouples replication from mitosis and induces premature onset of mitosis during S phase of the cell cycle (Yamashita et al., 1990).

While numerous studies have demonstrated the involvement of protein phosphatases in the cell cycle of higher eukaryotes, much less is known about the phosphatases of more divergent eukaryotes such as the kinetoplastid *Trypanosoma brucei*. Trypanosomes, which diverged from the anthrocentric arm of the eukaryotic lineage about twice as long ago as yeast (Sogin et al., 1989), possess a single nucleus and also a single mitochondrion; the latter contains a catenated network of DNA, known as kinetoplast DNA (kDNA). In higher eukaryotes that possess multiple mitochondria, organellar replication is asynchronous. In contrast, organisms that have a single mitochondrion, such as *T. brucei*, must couple nuclear and mitochondrial division during the cell cycle. In *T. brucei*, nuclear DNA and kDNA are duplicated exactly once in each cell cycle, making the organism a useful model for studying the interrelationship of nuclear division and organellar division. At the structural level, the division of *T. brucei* progresses temporally through the appearance of a new flagellum, the division and migration of the kinetoplast, mitotic division of the nucleus and finally cytokinesis. The period of DNA synthesis comprises replication of both nuclear DNA (S_n) and kDNA (S_k). A kinetic study on the cell cycle of *T. brucei* (Woodward and Gull, 1990) established that although S_n and S_k begin at approximately the same time, S_k is completed first. In addition, there is an

extended period of post-mitotic cytokinesis that is likely due to the elongated structure of the cells and is controlled in part by the subpellicular array of microtubules.

Several trypanosome genes encoding phosphatases homologous to PP1 and PP2A have been cloned (Evers and Cornelissen, 1990; Erondu and Donelson, 1991). These putative PP1 and PP2A homologues show about 66% and 55% amino acid identity with mammalian PP1 and PP2A, respectively. Similarly, a PP2C gene from the related parasite *Leishmania chagasi* has been characterized (Burns et al., 1993). While this PP2C is clearly insensitive to OKA, *L. chagasi* has a prominent PP1-like activity (Burns et al., 1993). The interaction of the trypanosome PP1 and PP2A isoforms with OKA has not been studied, nor have previous studies reported on the in vivo functions of these molecules. In this report we show that the treatment of *T. brucei* procyclic form organisms with OKA yielded cells with multiple nuclei and a single kinetoplast. Biochemical studies demonstrated that a protein phosphatase PP1-like activity present in trypanosome lysates was inhibited by OKA. These data suggests that trypanosome phosphatases may be involved in coordinating mitosis, mitochondrial DNA segregation and cytokinesis.

MATERIALS AND METHODS

Materials

OKA and 1-nor-okadaone were purchased from LC Laboratories, Woburn, MA. 5-Bromo-2-deoxyuridine (BrdU), 5-deoxycytidine, 4,6-diamino-2-phenylindole (DAPI), 1,4-diazobicyclo-(2,2,2)octane were purchased from Sigma Chemical Co., St Louis. Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody was obtained from Beckton Dickinson. [^{32}P]ATP was synthesized using a Gamma Prep kit (Promega) and $^{32}P_i$ (NEN Corp). All other chemicals were of reagent grade.

Parasites, cell culture and metabolic labeling

Procyclic forms of TREU667 (Michelotti and Hajduk, 1987) strain of *T. brucei* were grown in SDM79 (Brun and Schononberger, 1979) plus 10% fetal bovine serum. Cells were metabolically labeled overnight with $^{32}P_i$ as previously described (Parsons et al., 1991). Cell lysates were analyzed by 10% SDS-PAGE and autoradiography. For in vivo treatment, OKA was added in the solvent DMSO; the final concentrations of DMSO were 0.1% or less. At these concentrations DMSO had no effect on any of the parameters studied.

In vitro phosphatase assay

Cells were harvested by centrifugation, washed once in phosphate buffered saline, pH 7.2 (PBS) and lysed at a concentration of 10^8 cells/ml in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EGTA, 1 mM orthovanadate, 0.05 mg/ml leupeptin, 0.5% aprotinin and 1 mM phenylmethylsufonyl fluoride (PMSF) containing 1% nonidet P-40 (NP-40) and 0.25% deoxycholate on ice for 10 minutes. The solubilized proteins were separated from the pellet by centrifugation at 10,000 rpm for 10 minutes at 4°C. The phosphatase assay was carried out with 20 μg/ml of soluble extract using a protein phosphatase assay system (Gibco BRL Life Technologies, Inc). The substrate, ^{32}P-glycogen phosphorylase a, was radiolabeled using [^{32}P]ATP and phosphorylase kinase according to the manufacturer's protocol. Cell extracts were incubated for 15 minutes with various concentrations of OKA before the addition of ^{32}P-phosphorylase. No divalent cations were added to the assay buffer.

Kinase renaturation assay

Lysates of control and OKA-treated cells were electrophoresed, the proteins were transferred to polyvinylidene difluoride membranes and subjected to kinase renaturation assay as described (Parsons et al., 1993).

Flow cytometry

DNA content of fixed, RNase-treated cells was measured by flow cytometry as described (Gale et al., 1994) except that the cells were stained with propidium iodide overnight.

Fluorescence microscopy

For DAPI staining, the cells were washed once in PBS and allowed to settle on glass slides. Following fixation in 70% ethanol, the slides were stained with 1 μg/ml of DAPI for 15 minutes, and mounted in 50% glycerol in PBS. For BrdU labeling, the cells were incubated in serum-free medium containing 50 μM BrdU and an equimolar amount of 2-deoxycytidine for either overnight or 3 hours. Cells were fixed on slides as above and then incubated in 1.5 M HCl at room temperature for 30 minutes to denature the DNA, allowing the antibody access to BrdU-substituted groups. Slides were washed extensively in PBS to remove the acid and then incubated for 2 hours with FITC-anti-BrdU. After further washing the cells were stained with DAPI as described above and were mounted in 50% glycerol with PBS containing 0.1 M Tris-HCl (pH 9.0) with 1 mg/ml of 1,4-diazobicyclo-(2,2,2)octane as an antifade agent.

RESULTS

OKA inhibits *T. brucei* PP1/PP2A activities in vitro

To determine if OKA inhibits PP1/PP2A activities of trypanosomes, phosphatase activity was assayed in soluble trypanosome extracts. Since PP1 and PP2A are the only phosphatases with significant activity toward glycogen phosphorylase (Cohen, 1989), the assay was based on measuring the dephosphorylation of this substrate. The observed activity was not affected by the presence of either EDTA or Mg^{2+} (not shown), confirming that the phosphatase activity measured in this assay was not due to PP2B or PP2C. The results show that 90% of the phosphatase activity measured in the soluble extract was inhibited by 500 nM OKA (Fig. 1) whereas the negative control compound 1-nor-okadaone (Nishiwaki et al., 1990) had no inhibitory effect. A 50% inhibition of the PP1/PP2A activity was achieved at a concentration of 20 nM OKA; similar results were obtained at higher and lower concentrations of cell extract. Data obtained from other organisms has shown that PP2A is completely inhibited at 1 nM OKA while PP1 requires approximately 10 nM OKA for 50% inhibition (Cohen, 1989). Thus our data suggest that primary activity measured in trypanosome extracts using this assay is PP1-like.

OKA does not change the total phosphorylation profile of the treated cells

Treatment of mammalian cells with OKA globally increases the phosphorylation of cellular proteins (Haystead et al., 1989). In order to test the effects of OKA on phosphorylation of trypanosome proteins the cells were labeled overnight with $^{32}P_i$ in various concentrations of OKA. Surprisingly, OKA-treated cells exhibited the same overall phosphoprotein profile as that of control cells (Fig. 2). Similarly, OKA did not affect total phosphorylation of pp44/46, a serine/tyrosine-phosphorylated protein (Parsons et al., 1994) revealed by immunoprecipitation analysis of ^{32}P-labeled cell extracts (data not shown). Thus,

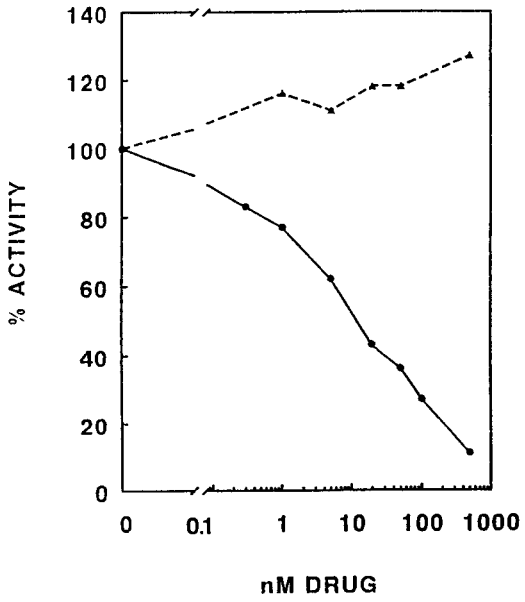

Fig. 1. PP1/PP2A activities in trypanosome extracts are inhibited by OKA. Cell extracts were prepared as described in Materials and Methods and then incubated in the presence of various concentrations of either OKA or 1-nor okadaone as indicated in the figure. The phosphatase assay was carried out using a protein phosphatase assay kit from BRL according to the manufacturer's protocol. Results are presented as average of triplicate measurements. —●—, OKA; - -▲- -. NOR-OKA.

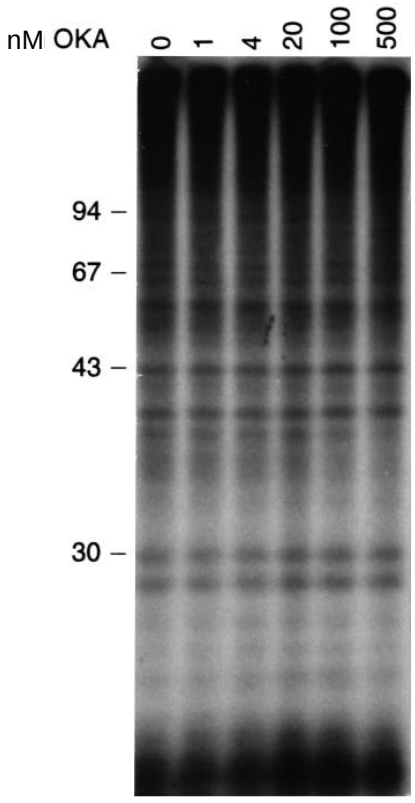

Fig. 2. OKA does not augment phosphorylation of cellular proteins. Cells were grown overnight in the presence of OKA and $^{32}P_i$, lysed in sample buffer and then analyzed by 10% SDS-PAGE. Phosphorylated proteins were visualized by autoradiography. The migration of molecular mass standards (kDa) is indicated at left.

while OKA inhibits a PP1-like activity in vitro, it does not have global effects on total protein phosphorylation in vivo. At these concentrations, OKA did not inhibit protein synthesis (data not shown). OKA did not appear to modulate the level of the PP1-like molecules, since extracts from cells treated overnight with 100 nM OKA had normal phosphatase activity in the in vitro assay described above (the packed cells were diluted over 1000-fold for the assay, reducing OKA to non-inhibitory levels).

Effects of OKA on protein kinase activity

It has been reported that in mammalian cells, OKA treatment activates $p34^{cdc2}$/H1 protein kinase activity (Yamashita et al., 1990). No reagents are currently available to study $p34^{cdc2}$ of trypanosomes. To investigate the effects of OKA on individual protein kinases in *T. brucei*, we examined the activity of eight protein kinases that were previously identified by a kinase renaturation assay (Parsons et al., 1993). Kinase renaturation analysis of total extracts prepared from cells grown overnight in OKA revealed that the activity of a 73 kDa protein kinase, as well as 42 kDa protein kinase, was gradually induced with increasing concentrations of OKA, while the activity of a 71 kDa protein kinase was concomitantly decreased (Fig. 3). The 73 kDa and 71 kDa protein kinases partitioned into NP-40 soluble and insoluble fractions, respectively. It appears that the

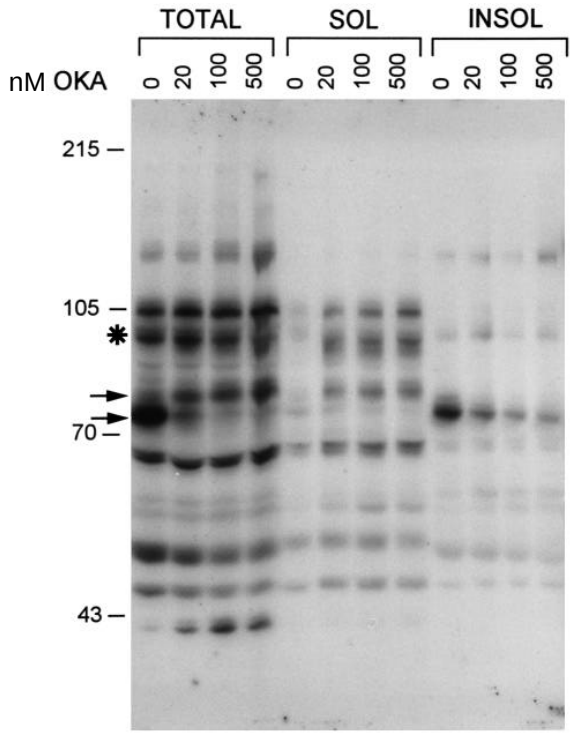

Fig. 3. Effects of OKA on cellular protein kinases. Cells were grown overnight in various concentrations of OKA. The cells were either directly lysed in SDS sample buffer (total) or separated into NP-40 soluble and insoluble fractions. Samples separated by SDS-PAGE were subjected to kinase renaturation analysis. Arrows indicate the positions of renaturable protein kinases of 71 kDa and 73 kDa. The asterisk marks protein kinase SPK89. The first lane containing the soluble extract from control cells (sol, 0) is underloaded. The migration of molecular mass standards (kDa) is indicated at left.

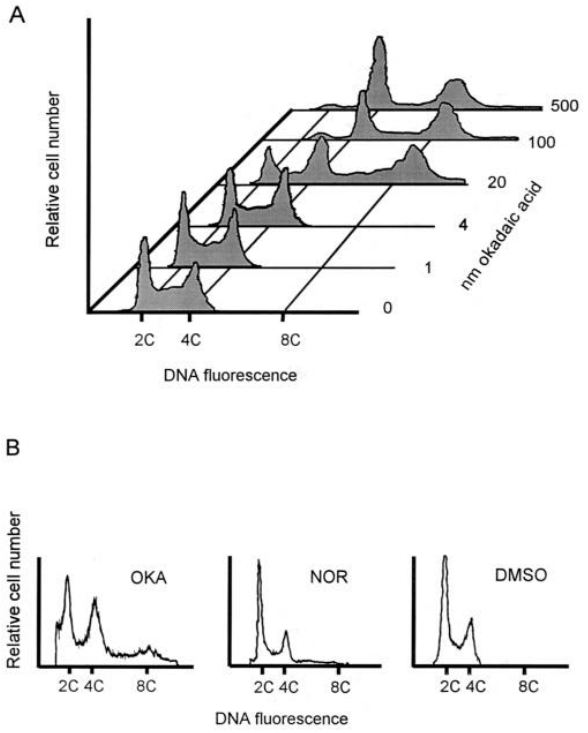

Fig. 4. Flow cytometry reveals that OKA blocks cytokinesis. Cells were stained with propidium iodide and analyzed by flow cytometry. The data are plotted on a linear scale. (A) Cells were treated with various concentrations of OKA as indicated for 18 hours. (B) Cells were incubated with 50 nM OKA, 250 nM 1-norokadaone or 0.1% DMSO (the solvent for the drugs), respectively.

42 kDa protein kinase was unstable during separation of soluble and insoluble fractions (compare total versus soluble and insoluble lanes). Interestingly, the cell cycle-regulated protein serine/threonine kinase SPK89 is active in the OKA-treated cells. SPK89 activity is virtually undetectable in G_0/G_1, is highly induced in S phase, and drops to moderate levels in G_2/M (Gale et al., 1994).

Effects of OKA on the trypanosome cell cycle

Overnight treatment of cultures with OKA revealed reduced proliferation with increasing concentration of OKA (not shown). To determine whether OKA affected progression through a specific phase of the cell cycle, we analyzed cellular DNA content using flow cytometry. The results obtained are shown in Fig. 4A. Untreated cells show a classical cell cycle curve, having a predominant G_0/G_1 population (2C chromosome number) with relatively fewer cells in S phase and G_2/M (DNA synthesis and 4C chromosome number, respectively). This profile remains unchanged following ovenight incubation with 4 nM OKA. When the concentration of the drug was increased to 20 nM and higher, the fluorescence profile was drastically changed, showing an accumulation of cells with 4C and 8C DNA content. The IC_{50} (20 nM) obtained via in vitro phophatase assay correlated well with the concentration of the drug at which the normal cell cycle progression was inhibited. In contrast, following treatment with 250 nM of nor-okadaone, an analogue of OKA (shown in Fig. 1 not to affect PP1-like activity), the DNA content of the cells appeared normal (Fig. 4B). These results indicate that OKA blocks cytokinesis but does not inhibit nuclear DNA synthesis. This is compatible with our finding of continued SPK89 protein kinase activity in OKA-treated cells.

OKA treatment yields multinucleate cells

Since the flow cytometry results demonstrated that OKA interfered with trypanosome cell division, we investigated whether OKA-treated cells were multinucleate or rather contained a single polyploid nucleus, as would be predicted from studies in higher eukaryotes (Yamashita et al., 1990). Furthermore, we determined the fate of mitochondrial DNA during cell division. Control and OKA-treated cells were stained with the DNA-specific dye, DAPI, and analyzed by fluorescence microscopy. Fig. 5A shows a control cell that possesses a single nucleus and a single kinetoplast, representing an early point in the cell

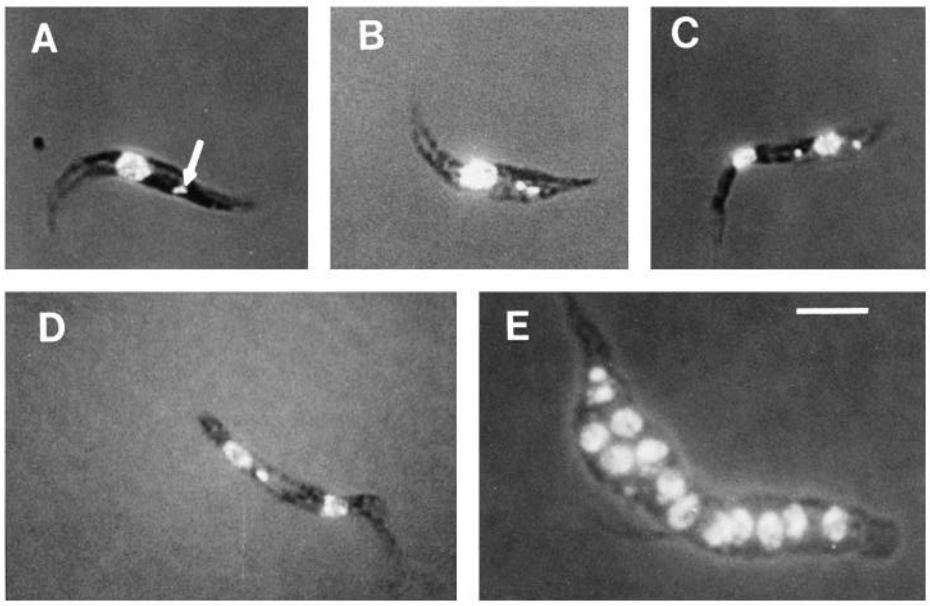

Fig. 5. In vivo treatment with OKA results in multinucleate cells. Cells incubated in the presence of OKA, fixed in methanol, stained with Giemsa and then with DNA-specific dye DAPI. The cells were visualized under the microscope using visible light and epifluorescence. (A,B,C) Control cells at progressive stages in the *T. brucei* cell cycle. Arrow marks the kinetoplast. (D) Typical cell after 18 hour incubation in OKA (20 nM). (E) Typical cell after long-term OKA treatment (20 nM for 10 days, then 50 nM for 5 days). Bar, 10 μm.

Table 1. Nuclei and kinetoplasts of okadaic acid-treated cells*

	Normal forms			Abnormal forms		
nM OKA	1N, 1K	1N, 2K	2N, 2K	2N, 1K	>2N	0N, 1K
0	84	10	3	2	0	1
20	40	1	5	30	19	7
100	8	0	9	60	20	3
500	9	0	11	66	10	1

*Nuclei (N) and kinetoplasts (K) of at least 100 DAPI-stained cells were counted following a 16 hour incubation in the drug. The percentage of cells with each staining pattern is indicated.

cycle. Fig. 5B and C show control cells in subsequent stages: one cell having two kinetoplasts and a single nucleus and finally one having two kinetoplasts and two nuclei.

Analysis of cells that had been grown overnight in the presence of OKA revealed a majority of binucleate cells with only one kinetoplast (Fig. 5D). The kinetoplast was consistently positioned between the two nuclei and appeared somewhat larger than that of control cells. In addition, these binucleate cells were larger than control cells and very often contained a second smaller flagellum. These results show that the nuclear division clearly occurred while kinetoplast segregation and cytokinesis were inhibited in the presence of the drug. Analysis of DAPI fluorescence of control and OKA-treated cells allowed us to quantitate the number of nuclei and kinetoplasts in cytologically distinct cells (Table 1). These results show that the number of abnormal cells gradually increased with increasing concentration of OKA. In experiments in which flow cytometry and microscopy were performed in parallel, the data were congruent with the interpretation of the FACs profile as an overlay of two cell cycles, one going from 2C (G_1) to 4C (G_2/M) and the second going from 4C (G_1) to 8C (G_2/M). When the treatment with OKA continued for several days, multinucleate cells containing most typically a single kinetoplast were generated (Fig. 5E). In these cultures, the number of nuclei per cell varied from a minimum of two to a maximum of twelve. After about one week of OKA treatment, the cells died, even if the drug was removed. The effects of OKA are not restricted to the insect stage of parasite development, since OKA-treated *T. brucei* strain 427 bloodforms grown in culture showed the same abnormalities upon DAPI staining (data not shown).

DNA synthesis in OKA-treated cells

To determine if the mitochondrial division cycle was blocked at the stages of DNA synthesis or segregation, cultures were grown overnight in the presence of OKA and BrdU (which is incorporated into DNA in place of thymidine). BrdU incorporation was detected via immunofluorescence with a monoclonal antibody specific for BrdU-substituted DNA. The doubling time of the trypanosomes under our conditions is approximately 12 hours, thus all cells should have sufficient time to proceed through S phase in the overnight incubation. The cells were counterstained with DAPI. As expected, all nuclei and kinetoplasts of control cells incorporated BrdU (not

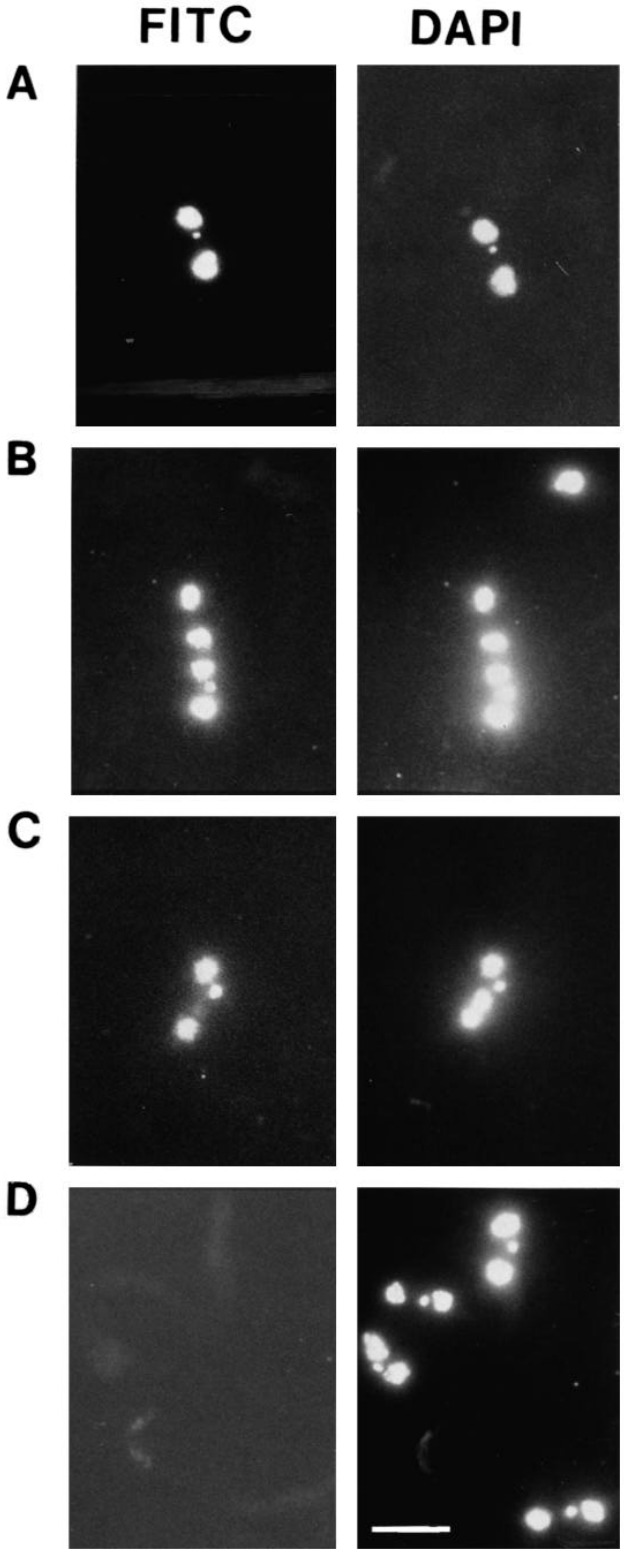

Fig. 6. Nuclear and kDNA synthesis in the presence of OKA. Cultures were grown in 100 nM OKA and in the presence or absence of BrdU (as indicated). BrdU incorporation was detected via immunofluorescence with a monoclonal antibody specific for BrdU-substituted DNA. The cells were counterstained with the DNA-specific dye DAPI. (A) Cells grown overnight in the presence of OKA and BrdU. (B,C) Cells grown in the presence of OKA for 30 hours and then labeled with BrdU for 3 hours. Note the nucleus in C, which is not replicating synchrously with the other two nuclei. (D) Negative control. Cells grown in the presence of OKA but in the absence of BrdU shows no staining with anti-BrdU. Bar, 5 μm.

shown). The nuclei and kinetoplasts of OKA-treated cells also reacted with anti-BrdU, indicating the presence of newly replicated DNA (Fig. 6A). The intensity of anti-BrdU staining of kDNA was not decreased in OKA-treated cells compared to control cells suggesting that at least the bulk of kDNA synthesis proceeded normally. Thus, OKA blocks a subsequent step in the kDNA cycle.

The cytoplasm exerts a powerful influence on the entry of resident nuclei into S phase (Blow and Laskey, 1988). In order to determine if the multiple nuclei of OKA-treated cells were cycling synchronously, cells were grown in the presence of OKA for approximately 30 hours to generate a high proportion of cells with greater than two nuclei and then pulse labeled with BrdU for 3 hours. Approximately 30% of the cells incorporated BrdU during the pulse indicating that only these cells were in S phase for a significant part of the labeling period. We examined those cells that had three or more nuclei and that showed evidence of DNA synthesis. Interestingly, kDNA continued to be labeled in these multinucleate cells. In 75% of labeled cells, all nuclei were concordant for staining with anti-BrdU (Fig. 6B). However, in 25% of labeled cells at least one nucleus failed to synthesize DNA synchronously with the other nuclei (Fig. 6C).

DISCUSSION

Our studies have relied on the use of a well known cell-permeant phosphatase inhibitor, OKA, which inhibits PP1 and PP2A of mammalian cells with IC_{50} values of about 10 nM and 0.1 nM, respectively. In our experiments, both in vitro phosphatase activity and normal cell cycle progression showed 50% inhibition at 20 nM OKA. Nor-okadaone, which is structurally identical to OKA except for a single R group, was not an inhibitor of the PP1-like activity nor did it induce alterations in the trypanosome cell cycle.

Trypanosomes treated with OKA were defective in segregation of the organellar genome and cytokinesis, but not in mitosis. OKA treatment of mammalian cells induces a distinctly different phenotype. Treatment of BHK21 cells in early S phase with 0.5 mM OKA resulted in a transient activation of $p34^{cdc2}$ kinase activity, rapid cytoskeletal reorganization reminiscent of mitosis (including aster formation), and later extensive chromosome condensation (Yamashita et al., 1990). In starfish oocytes however, OKA induced MPF activity and prevented its inactivation (Picard et al., 1989). Since at least one mitotic cycle proceeded normally in trypanosomes treated with OKA, this may point to differences in the phosphorylation network regulating a putative trypanosome p34/cdc2-cyclin kinase activity and mitosis. Analysis of mutant cells lacking specific PP1-like catalytic subunits also indicates an involvement of PP1 in transition through mitosis in higher eukaryotes. PP1 mutants in *D. melanogaster* (Baksa et al., 1993), *S. pombe* (Booher and Beach, 1989; Ohkura et al., 1989; Kinoshita et al., 1990) and *A. nidulans* (Doonan and Morris, 1989) all show excessive chromosome condensation and defective chromosome disjunction. Effects of PP1 mutations or OKA treatment on organelles in these organisms have not been reported. Several *S. pombe* mutants that are defective in cytokinesis, but are mitotically competent, have been described. The genes identified thus far are not protein phosphatases but rather cytoskeletal components (Balasubramanian et al., 1992).

If OKA acts by inhibiting trypanosome phosphatases in vivo why does it not augment total protein phosphorylation? Even under conditions where virtually all protein phosphorylation is inhibited (Gale et al., 1994), in pulse-chase experiments we have been unable to see dephosphorylation of $^{32}P_i$-labeled trypanosome phosphoproteins over the course of 24 hours, (A. Das, unpublished results). Apparently the in vivo targets of phosphatase action in procyclic form trypanosomes are relatively minor species. Since we are unable to detect dephosphorylation occuring in vivo, it is unlikely that we could detect any effect of OKA on this process. Thus we cannot determine at this time whether OKA exerts its effects on trypanosomes through its only known mechanism of action - inhibition of protein phosphatases - or if it acts upon another target.

Nevertheless, OKA induced specific biochemical changes in the cells, modulating the activities of at least two protein kinases. Activity of an NP-40-soluble 73 kDa protein kinase increased, while activity of a 71 kDa insoluble protein kinase decreased. Since there is no evidence in the literature that okadaic acid affects protein kinase activity directly, we believe these effects are mediated via inhibition of a protein phosphatase, which either directly or indirectly modulates the activity of these protein kinases. It is intriguing to speculate that the decrease in activity of the 71 kDa protein kinase, which is apparently associated with a macromolecular structure such as the cytoskeleton, may be related to the defects in the kDNA segregation and cytokinesis that we observed. The kinetoplast has been demonstrated to be physically complexed to the flagellum and the basal body and treatment with antimicrotubular drug ansamitocin blocks both basal body and kDNA segregration (Robinson and Gull, 1991). Okadaic acid does not disrupt the association of kDNA with the flagellum (A. Das, unpublished results). We postulate that protein phosphorylation affects cytoskeletal processes involved in segregation of the organellar genome and possibly the basal bodies. Although there is no evidence from other systems that OKA affects topoisomerase II, an alternative hypothesis might be that OKA indirectly modulates topoisomerase II-mediated decatenation of the kDNA network, thus blocking kDNA segregation.

Our data suggest that OKA treatment not only blocks kDNA segregration and cytokinesis, but also allows re-replication of the organellar genome in the absence of organellar division. A potential alternative explanation is that the kDNA is not being replicated but rather repaired following this toxic insult. However, DNA repair is very limited in mitochondria (Wallace, 1992). Furthermore, unlike DNA repair that is expected to occur throughout the cell cycle, the kDNA labeling was cell cycle-specific since it occurred concurrently with nuclear labelling. Therefore we believe that re-replication of kDNA is the most likely interpretation of our results. Two models have been proposed for preventing rereplication of kDNA within the trypanosome cell cycle. One of these invokes the existence of a licensing factor (Woodward and Gull, 1990), such as that proposed by Blow and Laskey (1988) to prevent re-replication of nuclear DNA of mammalian cells. According to this hypothesis, a licensing factor enters the nucleus during mitosis; only DNA bound to this factor would be available for replication during the next cycle. If this model holds true for kDNA, our data suggest that segregation of kDNA is not

required for functional activation of licensing factor. The second model for kDNA re-replication control postulates that the nicks that punctuate newly synthesized minicircles may serve to mark DNA that has been replicated within an S phase (Ryan et al., 1988). These nicks are sealed at the end of S_k. If there is not an additional checkpoint, then the blocking of kDNA segregation would later result in kDNA re-replication, such as we observed. Future analysis of the effects of OKA on kDNA structure should resolve which point(s) in the kDNA cycle are blocked.

Our data suggest that the role that protein phosphorylation plays in cell division may also include a role in the organellar cycle. Cytokinesis is also blocked by OKA, suggesting that kDNA segregation and cytokinesis may be interrelated. In trypanosomes, as in most eukaryotic cells, cytokinesis follows every mitosis. The available data indicate that these events are coordinated within the cell cycle (Woodward and Gull, 1990), although little is known about the specific molecules involved. Since treatment with OKA overrides this coordination, we suggest that a protein phosphatase may function in the coupling of the mitotic and cytoplasmic cell cycle of trypanosomes.

The authors thank Dr Theo DeVos for critical reading of the manuscript and Dr C. Michael Turner for advice on BrdU labeling of trypanosomes. This work was supported in part by NIH grant no. AI31077.

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(Received 17 March 1994 - Accepted, in revised form, 11 August 1994)