

## Amiodarone Inhibits *Trypanosoma cruzi* Infection and Promotes Cardiac Cell Recovery with Gap Junction and Cytoskeleton Reassembly *In Vitro*<sup>∇</sup>

Daniel Adesse,<sup>1</sup>† Eduardo Meirelles Azzam,<sup>1</sup>† Maria de Nazareth L. Meirelles,<sup>1</sup>  
Julio A. Urbina,<sup>2\*</sup> and Luciana R. Garzoni<sup>1\*</sup>

Laboratório de Ultraestrutura Celular, Instituto Oswaldo Cruz, Av. Brasil 4365, Manguinhos, 21045-900 Rio de Janeiro, Brazil,<sup>1</sup>  
and Laboratorio de Química Biológica, Centro de Biofísica y Bioquímica, Instituto Venezolano de  
Investigaciones Científicas, Apartado Postal 21.627, Caracas 1020A, Venezuela<sup>2</sup>

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**We present the results of the first detailed study of the antiproliferative and ultrastructural effects of amiodarone on *Trypanosoma cruzi*, the causative agent of Chagas' disease. Moreover, we report the effects of this compound on the recovery of F-actin fibrils, connexin43, and contractility in *T. cruzi*-infected cardiac myocytes. Amiodarone is the most prescribed class III antiarrhythmic agent and is frequently used for the symptomatic treatment of Chagas' disease patients with cardiac compromise. In addition, recent studies identified its antifungal and antiprotozoal activities, which take place through Ca<sup>2+</sup> homeostasis disruption and ergosterol biosynthesis blockade. We tested different concentrations of amiodarone (2.5 to 10 μM) on infected primary cultures of heart muscle cells and observed a dose- and time-dependent effect on growth of the clinically relevant intracellular amastigote form of *T. cruzi*. Ultrastructural analyses revealed that amiodarone had a profound effect on intracellular amastigotes, including mitochondrial swelling and disorganization of reservosomes and the kinetoplast and a blockade of amastigote-trypomastigote differentiation. Amiodarone showed no toxic effects on host cells, which recovered their F-actin fibrillar organization, connexin43 distribution, and spontaneous contractility concomitant with the drug-induced eradication of the intracellular parasites. Amiodarone is, therefore, a promising compound for the development of new drugs against *T. cruzi*.**

Chagas' disease is the largest parasitic disease burden and a major cause of heart disease and heart-related deaths in Latin America, where it affects approximately 16 to 18 million people (25, 38). The disease is caused by the protozoan parasite *Trypanosoma cruzi*, which possesses a life cycle involving a mammalian host and an insect vector (31).

Chemotherapy against *T. cruzi* is limited to two compounds, namely, benznidazole (a 2-nitroimidazole) and nifurtimox (a 5-nitrofurane), which are mostly active in acute- and early chronic-phase patients but are of limited efficacy in the prevalent chronic stage (8, 34). Moreover, *T. cruzi* exhibits considerable biological variability, indicating possible variations in virulence, pathogenicity, oxidative stress, and drug resistance (23, 27, 36), which may pose an important challenge in the search of safer and more effective chemotherapeutic agents for the specific treatment of Chagas' disease.

Studies in the last 2 decades have permitted the identification of several new drug targets for this parasite. Among the most promising are (i) the essential cathepsin L-like protease cruzipain, (ii) the unique Kinetoplastida enzymes trypano-

thione reductase and trypanothione synthase, and (iii) the inhibitors of *de novo* sterol biosynthesis pathways, such as imidazole and triazole derivatives (5, 33–35). There is also strong evidence that bisphosphonates can accumulate in the parasite's acidocalcisomes and interfere with the activity of enzymes involved in isoprenoid biosynthesis, such as farnesyl diphosphate synthase (13). Using *T. cruzi*-infected cardiomyocytes, we previously demonstrated that the bisphosphonate risedronate has a potent and selective *in vitro* effect against this parasite, resulting in recovery of the cardiac cells after the treatment (16). This compound also exhibited marked *in vivo* antiparasitic activity in a murine model of acute Chagas' disease (18). Another promising approach is the recent discovery of the anti-*T. cruzi* activity of the antiarrhythmic drug amiodarone, which is frequently prescribed for the symptomatic treatment of Chagas' disease patients (4). In the heart, the effects of this compound include inhibition of Na<sup>+</sup> channels, L-type Ca<sup>2+</sup> channels, K<sup>+</sup> channels, and the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, leading to its characteristic antiarrhythmic action. It was found that the *in vitro* and *in vivo* activity against *T. cruzi* was mediated by disruption of the parasite's Ca<sup>2+</sup> homeostasis and a blockade of ergosterol biosynthesis at the level of oxidosqualene cyclase (4).

Although the antiparasitic activity of amiodarone has been demonstrated previously, there is a lack of data regarding the effect of this compound on the ultrastructure of *T. cruzi* and its host cells and the recovery of these cells after the antiparasitic treatment. Primary cultures of murine cardiac myocytes have been the method of choice to demonstrate alterations in the host cell induced by this parasite. In these studies, many as-

\* Corresponding author. Mailing address for Luciana R. Garzoni: Laboratório de Ultraestrutura Celular, Instituto Oswaldo Cruz, FIOCRUZ, Avenida Brasil 4365, Pavilhão Carlos Chagas, 2º andar, 21040-900 Rio de Janeiro, Brazil. Phone: 55 (21) 2598-4535. Fax: 55 (21) 2260-4434. E-mail: largarz@ioc.fiocruz.br. Present address for Julio A. Urbina: 734 Springhill Lane, Cincinnati, OH 45226. Phone: (513) 321-2981. Fax: (513) 321-2981. E-mail: jurbina@mac.com.

† D.A. and E.M.A. equally contributed to this work and should be considered first coauthors.

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pects of this relationship were clarified, such as alterations in intracellular calcium dynamics (2, 17), changes in the cell cytoskeleton (24, 30), and cell-cell junction (1, 12). Gap junction channels are critical to maintaining cardiac homeostasis by allowing the free flow of ions and metabolites between cardiac myocytes, which contributes to the synchronized contraction of and signal exchange throughout the tissue. Gap junctions are composed of the connexin family of transmembrane proteins that assemble as end-to-end alignments of hexameric connexon subunits, thereby forming intercellular conduits for molecules of up to 1 kDa. Connexin43 (Cx43) is the most abundant gap junction protein in ventricular myocytes, being localized at intercalated disks in normal myocardium (15).

In the present study, we demonstrate the effects of amiodarone on the proliferation and ultrastructure of intracellular amastigote forms of *T. cruzi* growing in cardiomyocytes and the recovery of the host cells. We evaluated the recovery of spontaneous contractility of cardiac myocytes and the distribution of F-actin and Cx43 after treatment.

#### MATERIALS AND METHODS

**Parasites.** The Y (MHOM/BR/1950/Y) strain of *T. cruzi* was used in this work. Trypomastigote forms of *T. cruzi* were obtained from the supernatant of infected heart muscle cells grown in Dulbecco's modified Eagle medium (DMEM; Sigma Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS; Cultilab, São Paulo, Brazil), 1 mM CaCl<sub>2</sub>, 1 mM L-glutamine, 2% chicken embryo extract, 1,000 U/ml penicillin, and 50 µg/ml streptomycin. After 96 h of infection, the parasites were collected, centrifuged, and resuspended in DMEM.

**Cardiac cell cultures.** Hearts of 18-day-old Swiss Webster mouse embryos were submitted to mechanical and enzymatic dissociation as previously described (22). Briefly, cells were harvested using 0.05% trypsin and 0.01% collagenase in phosphate-buffered saline (PBS) at 37°C. Ventricular heart muscle cells (HMCs) were plated on 0.02% gelatin-coated plastic flasks, on glass coverslips, in 24-well plates, or in petri dishes. Cells were maintained at 37°C in a 5% CO<sub>2</sub> atmosphere in DMEM for 72 h before the experiments. Use and handling of the animals were approved by the Ethics Committee for the Use of Laboratory Animals, FIOCRUZ (CEUA), protocol P70/09.2.

**Infection of cultures and treatments.** Heart muscle cells were plated in 24-well plates at a density of  $1.5 \times 10^5$  cells/well in glass coverslips and infected with culture-derived trypomastigotes (20:1, parasites/host cells) in a final volume of 300 µl DMEM. After 2 h, the cultures were washed with PBS to remove non-adherent parasites and maintained in DMEM. Treatment with 2.5 to 10 µM amiodarone was performed by using the following two protocols: (i) addition immediately after the interaction step and (ii) addition at 48 h after infection. The total volume in each well was 500 µl. At specific times, coverslips (in triplicate) were collected, fixed with Bouin's fixative solution (Electron Microscopy Sciences, Hatfield, PA), and stained in Giemsa solution (Merck, Darmstadt, Germany). The percentage of infection was quantified by randomly counting at least 300 cells. In addition, supernatants were collected, and released parasites were counted in a hemocytometer.

**Ultrastructural studies.** Cells were plated in 35-mm plastic petri dishes and, at chosen times, were fixed (60 min/4°C) with 2.5% glutaraldehyde (Sigma-Aldrich), 2.5 mM CaCl<sub>2</sub>, and 0.1 M Na-cacodylate buffer (pH 7.2), followed by postfixation for 1 h in cacodylate buffer solution containing 1% OsO<sub>4</sub>, 0.8% potassium ferricyanide, and 2.5 mM CaCl<sub>2</sub>. Samples were dehydrated in acetone and then embedded in Poly/Bed 812 resin (Electron Microscopy Sciences). Thin sections (Ultracut UCT; Leica, Vienna, Austria) were stained with uranyl acetate and lead citrate and were examined by transmission electron microscopy using a Zeiss EM10C microscope.

**Immunofluorescence.** Cells were washed with PBS and fixed with 4% paraformaldehyde for 5 min at 20°C at the desired time points. After being washed in PBS, cells were permeabilized with 0.5% Triton X-100, and nonspecific staining was blocked with 4% bovine serum albumin (BSA). Primary anti-Cx43 antibody (Sigma-Aldrich, St. Louis, MO) was incubated overnight at 4°C, after which cells were washed and incubated with secondary polyclonal goat anti-rabbit Alexa Fluor 488 antibody (Invitrogen, Eugene, OR) for 1 h at 37°C. F-actin filaments were stained with Alexa Fluor 594 phalloidin (Invitrogen) for 30 min at 37°C, and

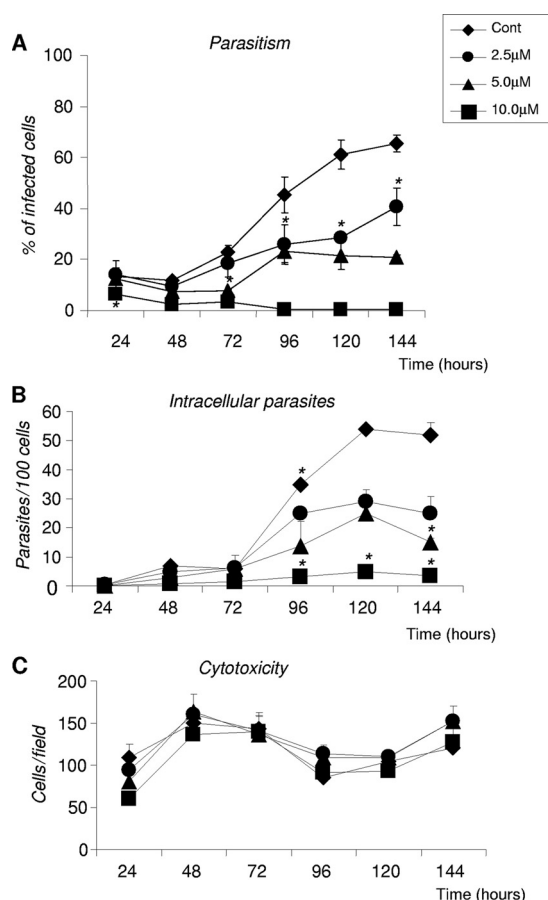


FIG. 1. Time and concentration dependence of the effects of amiodarone on infection of HMCs by *T. cruzi* (protocol i). Amiodarone (2.5 to 10 µM) was added to the cultures after 2 h of infection. (A, B) We observed significant effects of the drug on the percentage of infected cells (A) and the number of intracellular parasites (B), which were followed as a function of time. (C) The concentrations of the drug used in these studies were not toxic to the cardiac myocytes. Asterisks indicate statistical differences in relation to control cultures. The graphs show the means and standard deviations from triplicates of one representative experiment of four independent experiments. Cont, control.

DNA was stained with DAPI (4',6-diamidino-2-phenylindole). Images were acquired with an Olympus laser scanning confocal microscope.

**Statistical analysis.** Mean value comparisons were performed by using analysis of variance (ANOVA). *P* values below 0.05 were considered significant. IC<sub>50</sub>s (50% inhibitory concentrations) were calculated from dose-response curves by using nonlinear regression analysis with the GraFit software package.

**Materials.** Amiodarone was purchased from Sigma-Aldrich. Stock solutions were prepared in dimethyl sulfoxide and DMEM (pH 7.4) sterilized by using a 0.2-µm filter (Millipore, Billerica, MA). Trypsin was purchased from Sigma Chemical Co. (St. Louis, MO), and collagenase was purchased from Worthington Laboratories (Lakewood, NJ). All other reagents were analytical grade.

#### RESULTS

**Effect of amiodarone on intracellular *T. cruzi* amastigotes and host cells.** Using two different treatment protocols, amiodarone caused a strong inhibition of the infection of HMCs (Fig. 1 to 3). When the drug was added immediately after infection (protocol i) (Fig. 1), dose- and time-dependent reductions in the percentages of infected cells were observed.

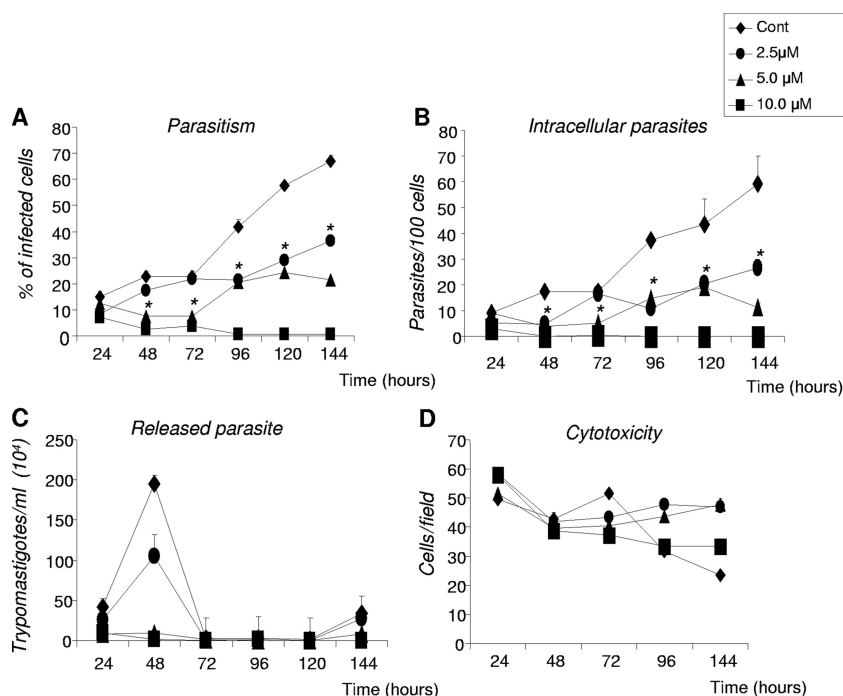


FIG. 2. Time and concentration dependence of the effects of amiodarone on infection of HMCs by *T. cruzi* (protocol ii). (A to C) Amiodarone (5 to 10  $\mu\text{M}$ ) was added to the cultures after 48 h of infection, and the percentage of infected cells (A), number of intracellular parasites per host cell (B), and number of released trypomastigotes in the supernatant (C) were followed as a function of time. (D) We did not observe significant cytotoxic effects of amiodarone with the concentrations used throughout the experiments. Asterisks indicate statistical differences in relation to control cultures. The graphs show the means and standard deviations from triplicates of one representative experiment of four independent experiments.

The inhibition was statistically significant after 24 h of treatment with 10  $\mu\text{M}$  amiodarone ( $P < 0.05$ ), 48 h with 5  $\mu\text{M}$  ( $P < 0.01$ ), and 96 h with 2.5  $\mu\text{M}$  ( $P < 0.01$ ) (Fig. 1A).  $\text{IC}_{50}$ s with protocol i were  $5.85 \pm 1.4 \mu\text{M}$  at 96 h and  $3.14 \pm 1.2 \mu\text{M}$  at 120 h. Amiodarone had also an inhibitory effect on the number of intracellular amastigotes (Fig. 1B), as revealed by light microscopy (Fig. 3), significant for 10  $\mu\text{M}$  amiodarone at 24 h of treatment ( $P < 0.05$ ). Addition of amiodarone to cultures at 48 h after infection (protocol ii) also led to a highly significant reduction in the percentage of infected cells (Fig. 2). After 48 h of treatment (96 h of infection), 5  $\mu\text{M}$  amiodarone already reduced significantly ( $P < 0.05$ ) the parasitism of cultures, which was also observed at 96 h treatment with 2.5  $\mu\text{M}$  amiodarone (Fig. 2A). In this protocol,  $\text{IC}_{50}$ s were  $4.47 \pm 0.3 \mu\text{M}$  and  $2.24 \pm 0.24 \mu\text{M}$  at 96 and 120 h, respectively. This inhibitory effect was also observed for the number of intracellular amastigotes in the HMCs (Fig. 2B). Moreover, amiodarone inhibited the release of trypomastigotes from infected cells in this model after completion of the intracellular cycle of the parasite. In control (untreated)-infected HMC cultures, the first two peaks of trypomastigote release to the supernatant occurred at 96 h and 192 h postinfection (Fig. 2C). Treatment with 5 and 10  $\mu\text{M}$  amiodarone, starting 48 h postinfection, drastically inhibited such release after 48 h of treatment (96 h postinfection), while at 96 and 144 h, no trypomastigotes were detected in the supernatant (Fig. 2C). Amiodarone at the concentrations used in the experiments (up to 10  $\mu\text{M}$ ) had no apparent cytotoxic effects on the myocytes, as shown in Fig. 1C and 2D.

Light microscopy observations showed that treatment with amiodarone induced drastic morphological alterations on intracellular amastigotes (Fig. 3B, C, and E, insets). We confirmed this observation through transmission electron microscopy of infected HMCs and released parasites after 144 h of treatment of infected HMCs with 5  $\mu\text{M}$  amiodarone. Infected untreated HMCs displayed abundant intracellular amastigotes in the cells' cytoplasm, with the expected kinetoplast morphology (bar shaped) (Fig. 4A). A drastic loss of cytoplasmatic content and the formation of membrane inclusions inside the amastigotes were visualized in treated cultures; these cells also exhibited mitochondrial swelling, disorganization of reservoirs and the kinetoplast, probably associated with the disruption of  $\text{Ca}^{2+}$  homeostasis (4), and a blockade of amastigote-trypomastigote differentiation (Fig. 4B). Untreated amastigotes spontaneously released from their host cells also displayed their characteristic morphology (Fig. 4C). There was a marked damage in amastigotes released to the medium from amiodarone-treated (5  $\mu\text{M}$ ) cultures, such as kinetoplast alteration, and in the Golgi apparatus (Fig. 4D).

**Cell physiology recovery after treatment with amiodarone.** In order to assess the recovery of host cell ultrastructure and physiology after the treatment of infected cultures with amiodarone, we evaluated gap junction protein Cx43 (detected by immunofluorescence) and actin filaments (stained with phalloidin). *T. cruzi* infection is known to disrupt gap junctional communication through Cx43 protein reduction (1). Figure 5 shows that infected untreated HMCs (Fig. 5D to F) totally lost Cx43 immunoreactivity at 192 h postinfection compared to that

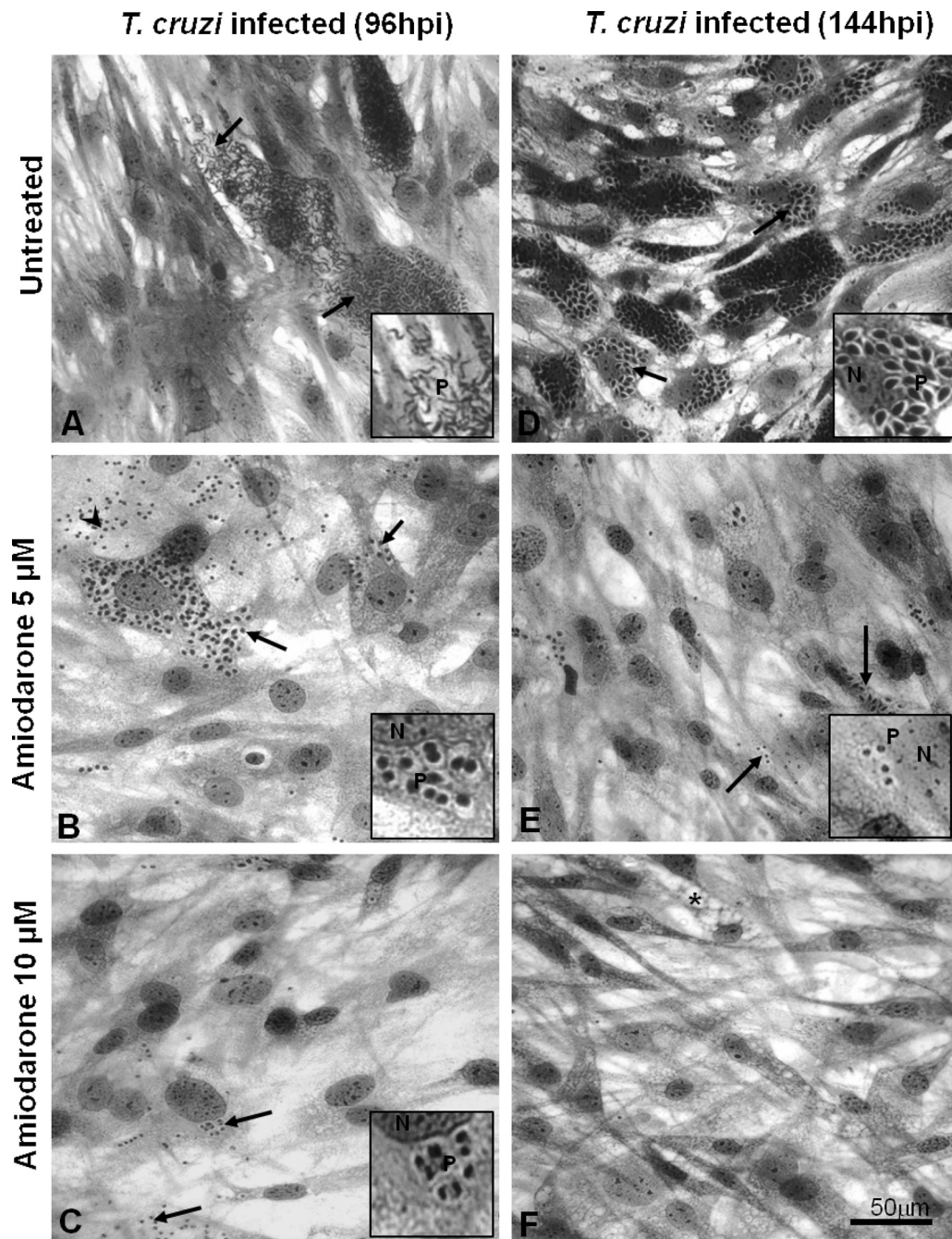


FIG. 3. Effects of amiodarone on the intracellular cycle of *T. cruzi*. Primary cardiac myocytes were obtained and infected with the Y strain of *T. cruzi*. After 2 h of infection, cultures were treated with 5 or 10  $\mu\text{M}$  amiodarone. At 96 h postinfection (hpi), untreated cultures (A) displayed cells with trypomastigote forms of the parasite already evading host cells. Cultures treated with 5  $\mu\text{M}$  (B) and 10  $\mu\text{M}$  (C) of amiodarone displayed intracellular parasites with severe morphological alterations (arrows). (C) Interestingly, an amiodarone concentration of 10  $\mu\text{M}$  induced a drastic reduction of parasitism. After 144 h of infection (corresponding to 142 h of treatment), we observed that in nontreated cultures (D), 70 to 80% of cardiomyocytes were infected. Treatment with 5  $\mu\text{M}$  amiodarone (E) drastically reduced the number of parasites, whereas the use of 10  $\mu\text{M}$  amiodarone (F) almost eliminated the parasitism of the cultures. Insets show details of intracellular parasites in higher magnification. P, parasites; N, nucleus.

of control uninfected cells (Fig. 5A to C), which displayed well-formed gap junction plaques (Fig. 5C) and abundant myofibrils, as revealed by phalloidin staining. After treatment with 5  $\mu\text{M}$  amiodarone for 144 h, infection was nearly abolished, as revealed by DAPI staining. In these cultures, Cx43 levels were

comparable to those of age-matched uninfected cultures (Fig. 5I), and phalloidin staining revealed the presence of both polygonal and filamentous structures, indicative of cytoskeleton reassembly (Fig. 5H).

We also assessed the impact of *T. cruzi* infection on cardiac

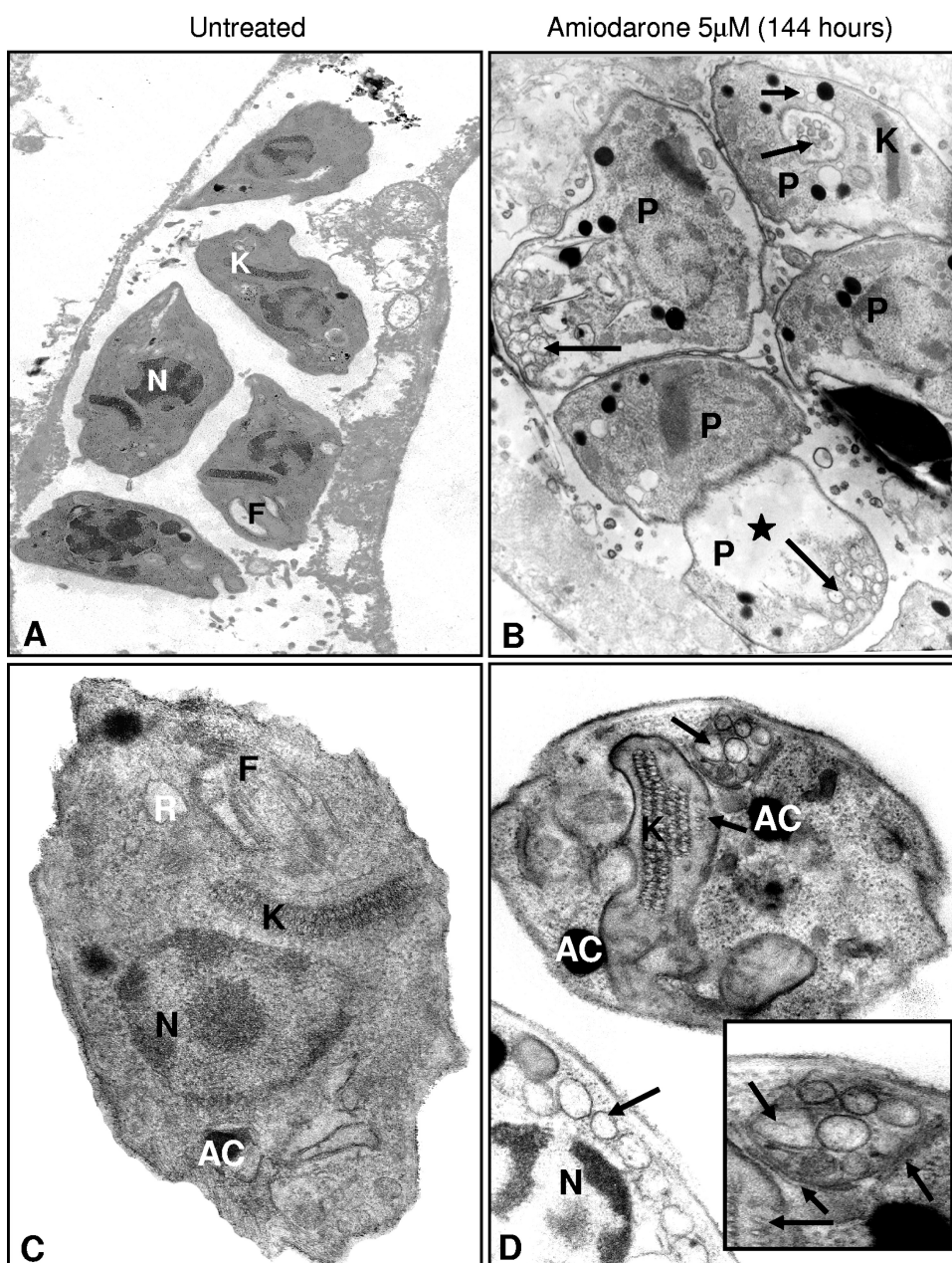
*T. cruzi* infected (192hpi)

FIG. 4. (A) Effects of amiodarone on the ultrastructure of intracellular *T. cruzi* (Y strain) amastigotes. Untreated HMCs after 192 h of infection with *T. cruzi*, displaying severe intracellular damage and intact amastigote forms (P) and presenting a bar-shaped kinetoplast (K), nucleus (N), and flagellum (F). (B) Infected cultures treated with 5  $\mu$ M amiodarone for 144 h showed parasites with membrane blebs (arrows), loss of intracellular material (stars), and kinetoplast (K) alterations. (C) Released parasites from untreated cultures displayed acidocalcisomes (AC) and a bar-shaped kinetoplast (K), reservosome (R), and flagellum (F), as expected. (D) Parasites obtained from cultures treated with 5  $\mu$ M amiodarone (144 h) showed important alterations in the kinetoplast and Golgi apparatus (arrow).

myocyte spontaneous electrocontractility, and the results are shown in Fig. 5J. The parasites' proliferation was associated with a progressive reduction in the cardiomyocytes' contraction, which followed the disorganization of the contractile apparatus as well as the disappearance of the gap junctions of these cells. After 48, 96, and 144 hours of infection (protocol i), untreated controls displayed a 28, 31, and 45% reduction in

spontaneous contractility ( $P < 0.05$ ), respectively, whereas treatment with amiodarone (5  $\mu$ M) restored the number of spontaneous beatings to levels indistinguishable from those of uninfected cultures at 96 ( $P < 0.05$ ) and 144 ( $P < 0.01$ ) hours of treatment. We observed no significant effect on contractility when the same dose of amiodarone was added to uninfected cultures (not shown).

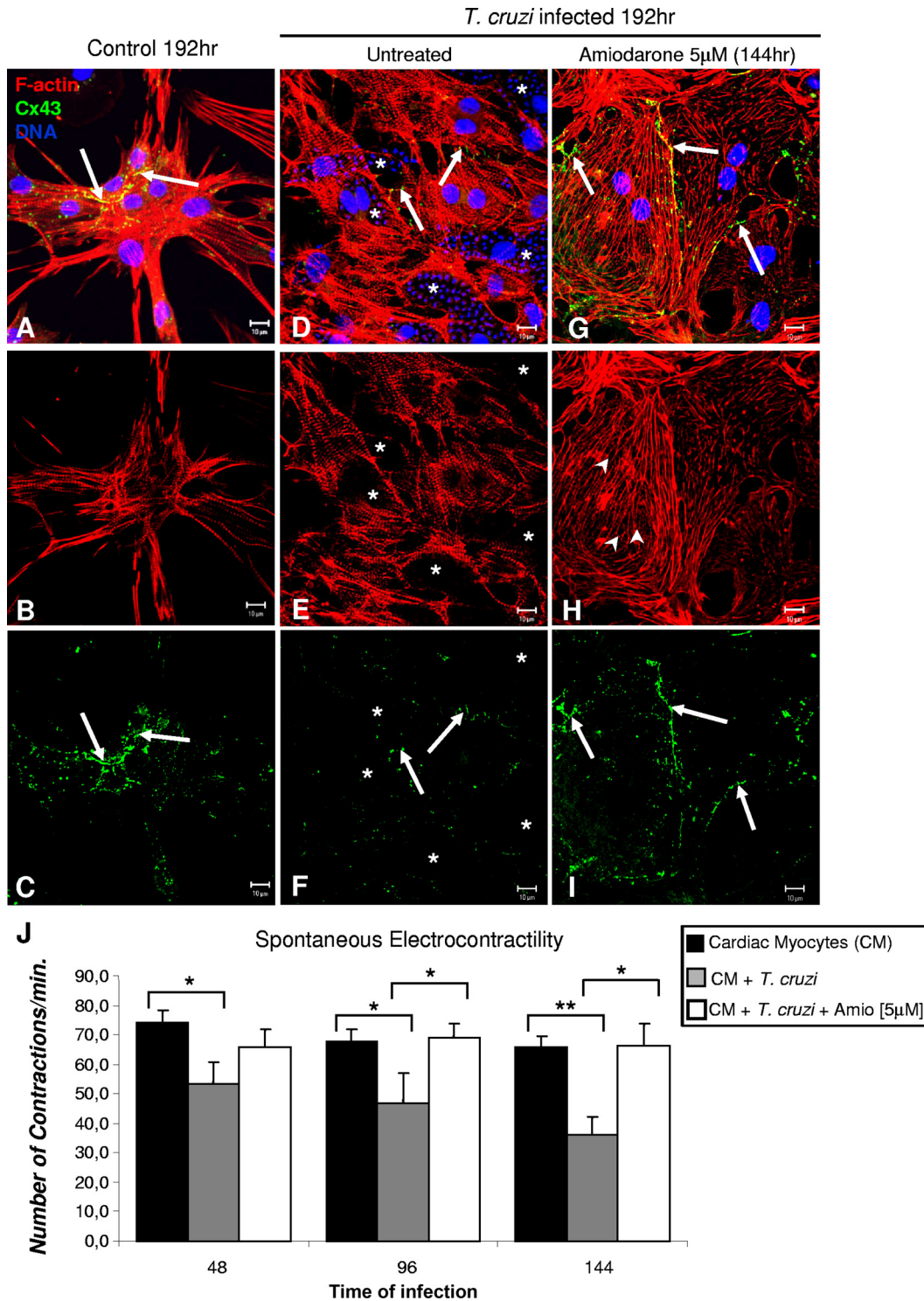


FIG. 5. Amiodarone induced the recovery of host cell homeostasis. After eradicating the infection, we observed the recovery of morphological and functional aspects of cardiac myocytes cultures, as assessed by immunofluorescence and measuring of the spontaneous electrocontractility of the cells. (A to C) Uninfected cultured myocytes displayed abundant connexin43 immunoreactivity (C) as well as striated patterns of F-actin staining (B). (D to F) Highly infected cultures, at 144 h of infection, presented destruction of the F-actin cytoskeleton (E) and loss of Cx43 plaques (F). (G to I) Treatment with 5 µM amiodarone for 142 h decreased parasitism and induced recovery of host cells, as evidenced by F-actin and Cx43 recovery, which included striations (H) and the presence of gap junction plaques (I). (J) The spontaneous electrocontractility of the myocytes culture was evaluated by counting the number of contractions per 10 s. We observed that *T. cruzi*-infected cultures had a progressive decrease in electrocontractility, whereas treatment with 5 and 10 µM amiodarone restored cultures to normal levels of electrocontractility. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (ANOVA).

## DISCUSSION

Human Chagas' disease results from infection by *T. cruzi*, and tissue damage arises from both direct parasite action and the inflammatory process that ensues (7). Cytolysis and fibrosis are key components associated with Chagas' disease's pathological manifestations, along with a sustained and diffuse inflammation of the affected organs (31). The growing perception that chronic-phase manifestations are associated with the persistence of the parasite in the mammalian tissues (32) and that the two available drugs (benznidazole and nifurtimox) have important limitations, particularly in the chronic phase (6), has stimulated the search for new trypanocidal compounds. Amiodarone is frequently used as an antiarrhythmic in chronic-phase Chagas' disease patients with cardiac compromise (21, 26), and it also has antifungal (9, 10) and antiprotozoal (4, 28) activities, which were recently described. It was shown that the drug disrupts the parasite's  $Ca^{2+}$  homeostasis and also blocks ergosterol biosynthesis, resulting in excellent parasitocidal activity with low cytotoxicity (4). The results obtained in this work clearly confirm that amiodarone has potent and selective activity against *T. cruzi*, with no significant effects on their preferred host cells, cardiomyocytes, and at doses that do not induce the well-known antiarrhythmic action of the drug on these cells. This selective action was confirmed by ultrastructural analysis of amiodarone-treated cultures (Fig. 4), which revealed massive alterations of the parasites, allowing at the same time the full structural and functional recovery of the cardiomyocytes (see below). The ultrastructural effects of amiodarone on intracellular amastigotes were similar to those previously described for other sterol biosynthesis inhibitors, such as risedronate (16), ketoconazole, and terbinafine (19, 37).

We also investigated whether amiodarone treatment would induce host-cell recovery, concomitant with the elimination of the intracellular parasites' burden. We have previously shown that infection by *T. cruzi* induces a cytoskeletal disruption in HMCs due to myofibrillar breakdown (24) and that treatment with risedronate and posaconazole allowed reassembly of cytoskeleton elements (16, 29). The present results show that amiodarone allowed the reorganization of the actin-containing myofibrils to their normal state, with the occurrence of characteristic polygonal arrangement. Lin and coworkers (20) have described this polygonal configuration in dissociated cardiac myocytes as an indicator of myofibrillar reassembly. Moreover, it is known that the infection of cultured cardiac myocytes with *T. cruzi* decreases gap junction communication and Cx43 expression (1, 11). These observations were extended to acutely infected mice, which displayed reduced cardiac Cx43 expression (1). Our present results demonstrate that Cx43 distribution among cardiac myocytes was fully restored after treatment of infected HMCs with amiodarone, and treated cultures displayed gap junction plaques comparable to those of uninfected controls. Cx43 has a turnover of approximately 1.5 h *in vitro* (3), and in *T. cruzi*-infected myocytes, Cx43 mRNA levels were largely unaffected (D. Adesse, unpublished data). This may provide evidence that the infection affects Cx43 translation and/or its subsequent trafficking to the plasma membrane, possibly due to microtubular damage (24). After the amiodarone-induced disappearance of the intracellular parasites, the host

cells were capable of synthesizing new Cx43 molecules and made their successful delivery to the plasma membrane. More importantly, HMCs restored their spontaneous contractility after treatment with 5  $\mu$ M amiodarone to levels comparable to those in control cultures. This observation is of interest since it permits us to speculate that during *in vivo* infection, *T. cruzi* disturbs synchronous contractility, which can be reverted with the use of amiodarone alone or in combination with another inhibitor of the parasite's sterol biosynthesis, such as posaconazole, which results in synergistic effects (4).

To summarize, our results showed that amiodarone has a selective antiproliferative effect on *T. cruzi* in an *in vitro* model of infection of cardiac cells. Treatment induced ultrastructural damage to intracellular amastigotes but promoted full structural and functional recovery of the host cells. This compound should be considered, beyond its known antiarrhythmic activity, as an important antiparasitic agent and a lead for the development of new specific treatments of this neglected disease.

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