Endocytic Pathway in Mouse Cardiac Cells

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ABSTRACT. **Primary cultures of heart muscle cells provide powerful tools for cardiac cell biological research that permits both physiological and biochemical approaches. In the present study we analyzed the endocytosis of cardiac cells and presented morphological characterization of the endocytic machinery using markers, which enabled us to follow the fluid-phase, receptor-mediated endocytosis and the internalization of large particles. Our results demonstrated the route of the internalized cargo to early endosomes followed or not by its discharge in the late compartments. We also confirmed the ability of cardiac muscle cells to ingest large particles such as the mannosylated ligand zymosan A, and even internalize whole eukaryotic cells such as the protozoan parasite *Trypanosoma cruzi*. Since endocytosis is involved in many important cellular functions, the present work contributes to the knowledge of possible additional roles played by cardiac muscle cells besides their well known ability to act as physically energetic cells in the body, constantly contracting without tiring.**

Key words: cardiomyocytes/endocytosis/electron microscopy/*Trypanosoma cruzi*

The development of long-term culture systems useful in studying aspects of cellular biology and physiology of cardiac cells as well as their pathophysiologic states has been well explored (Eppenberger and Zuppinger, 1999; Piper *et al.*, 1986). Cell cultures can be prepared from fetal, neonatal or adult animals, the later being more difficult to establish and therefore less frequent (Eppenberger and Zuppinger, 1999). Some years ago, we established enzymatic protocols in order to dissociate and isolate embryonic mouse cardiac myoblasts, which follow *in vitro* differentiation into highly striated and functional myocytes (Meirelles *et al.*, 1986). Upon *in vitro* cardiomyogenesis, these primary cultured myocytes maintain their morphological integrity for several days and present major functional aspects of cardiac cells, including excitation-contracting coupling

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  Abbreviations: CM, primary cultures of embryonic cardiomyocytes; FITC-Dx, fluorescein isothiocyanate-Dextran; TRITC-Dx, rhodamin-dextran; CF, cationized ferritin; GA, glurataldehyde; HRP, horseradish peroxidase; Tf-Au, transferring-gold particles; zymosam A particles, Zy; TEM, transmission electron microscopy; ESI, electron microscopy imaging.

(Meirelles *et al.*, 1986), typical electrophysiologic and neurotransmitter responses (Aprigliano *et al.*, 1993). We have used these primary cultures of heart muscle cells to study some of the biological aspects of cardiac cells such as long-term host-parasite interaction (Meirelles *et al.*, 1999), surface recognition molecules (Soeiro *et al.*, 1999), cytoskeleton assembly (Pereira *et al.*, 1993; Pereira *et al.*, 2000) ; and surface charge (Soeiro *et al.*, 1995).

Endocytosis represents the internalization of small molecules, macromolecules, large particles and even whole cells (Clague, 1998). The material to be internalized is progressively enclosed by the plasma membrane with the formation of a new intracellular vesicle that pinches off from the cell surface. The distribution of the ligand after its internalization reveals the endosomal compartment as a complex set of heterogeneous tubulovesicular membranes extending from the cell surface to the perinuclear area, where it is often close to the Golgi apparatus (Mukherjee *et al.*, 1997). Upon internalization, the ligands are sorted and targeted to specific intracellular organelles following different fates such as final degradation, recycling back to the plasma membrane and other alternative routes like trafficking to organelles such as the Golgi (Clague, 1998; Desjardins *et al.*, 1994; Rabinovitch, 1995). Transport vesicles that bud off from one organelle and fuse with the designed target

organelles mediate the intracellular membrane trafficking, and several members of the Rab family of *Ras*-related small GTP-binding proteins participate in the transport events such as budding, docking and vesicle fusion (Seabra *et al.*, 2002).

Endocytosis plays a role in several important biological functions including the clearance of apoptotic bodies, lipid metabolism, antigen cell surface presentation, nutrient acquisition, and pathogen invasion (Clague, 1998). Up to now, little information is available concerning the endocytic pathway in cardiac muscle cells. Thus, in our present work we characterized, using the murine model, different aspects of the endocytosis in cardiomyocytes concerning i) fluid-phase, ii) receptor-mediated and last, iii) internalization of large ligands and whole eukaryotic cells such as the protozoan *Trypanosoma cruzi*.

Materials and Methods

Reagents

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA): Horseradish peroxidase (type II), zymosan A, human transferrin, and paraformaldehyde (PFA). Bovine serum albumin (BSA) was purchased from GIBCO (Long Island, New York, USA).

Cell cultures

Primary cultures of embryonic cardiomyocytes (CM) were purified following the method previously described (Meirelles *et al.*, 1986). Fibroblast-free CM cultures were obtained by differential plating of cells on gelatin-coated culture plates. All assays described here were run at least 4 times in triplicate. The animal procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals, resolution 242/99.

Endocytic assays

a) Fluid-phase endocytosis

For fluorescent approaches, the primary cultures of cardiomyocytes were allowed to take up 5 mg/ml^{-1} FITC- or rhodamin-dextran (FITC-Dx and TRITC-Dx, respectively) for 3 min at 37°C (5% CO_2 incubator), extensively washed at 4°C and then further chased for 5 and 60 min at 37°C in FITC-Dx free culture medium, as previously described (Rybak and Murphy, 1998). The samples were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton-X 100 in PBS for 2 to 3 minutes. They were incubated thereafter with DAPI and phalloidin-FITC for DNA and actin staining, respectively, and finally mounted with DABCO (anti-fading). For transmission electron microscopy (TEM) analysis, colloidal gold particles with a mean diameter of 15 nm were made according to the method of Frens (Frens, 1973) and 25 μg/ml^{-1} albumin was adsorbed to the gold particles (BSA-Au) at pH 5.5 as

previously described (Bendayan, 1984). During the internalization assays, the BSA-Au stock solution was diluted 10 times in culture medium (without addition of serum sources) and the cultures were incubated for different time periods varying from 15 min up to 24 h at 37°C (5% CO_2 incubator). After these incubations, the cultures were rinsed twice, fixed with 2.5% glutaraldehyde (GA) and processed for TEM.

b) Receptor-mediated endocytosis

In these studies, we followed three different protocols. i) We chased the internalization of anionic sites by labeling the cardiac cells with 100 μg/ml^{-1} cationized ferritin (CF) for 30 min at 4°C, followed by washing the cultures with cold PBS to remove the free tracer, and then maintaining in CF-free medium for 30 min to 24 h at 37°C. After these different periods of incubation, the cardiac cells were washed twice, fixed with GA and processed for TEM. ii) We followed the internalization of mannose receptors by using horseradish peroxidase (HRP) as mannosylated ligand. In these assays, 65 μg/ml^{-1} HRP was coupled to 15 nm colloidal particles (HRP-Au) at the pH 8.0 Frens, 1973 in the presence of 5 mM calcium chloride. Cardiac cells were first incubated for 30 min at 4°C with HRP-Au, washed to remove unbound ligand and further incubated for 30 min, 1 and 24 h at 37°C. Negative controls were prepared by co-incubating the cells with 500 mM D-mannose. The samples were then fixed and processed for TEM. iii) Transferrin receptors were analyzed by incubating cardiac cells for 30 min at 4°C with transferrin (dilution of 1:10 in medium without serum sources), which has been previously coupled to colloidal gold particles (at pH 5.5 with 65 μg/ml^{-1} of the purified protein), as previously described (Frens, 1973). The cultures were then rinsed to remove unbound transferrin-gold particles (Tf-Au), and then followed by additional incubation for 5 min and 60 min at 37°C. The cultures were then washed, fixed and processed for TEM.

c) Internalization of large particles

i) Zymosan A: To address the endocytic ability of ingesting large particles, cardiac cells were incubated with zymosan A particles, a well-known mannosylated ligand. The cultures were incubated at 37°C/180 min with 10^7 zymosan A particles (Zy), fixed with Bouin, stained with 10% Giemsa solution and analyzed at light microscopy level. ii) *Trypanosoma cruzi*: the endocytic ability of cardiomyocytes to internalize whole cells was next demonstrated by their interaction with trypomastigote forms of *T. cruzi* (Y stock). Bloodstream trypomastigote forms (Y strain) were harvested by heart puncture from *T. cruzi* infected Swiss mice at the parasitaemia peak day, as previously described (Meirelles *et al.*, 1982). After 24 h of plating, CM cultures were infected for 24 h at 37°C with trypomastigote forms employing a parasite:cell ratios of 10:1. Parasite internalization in CM was analyzed both by TEM and by fluorescent approaches, which employed DAPI stain as above described.

All fluorescent approaches were observed using a Zeiss Universal Photomicroscope equipped with epifluorescence.

Transmission electron microscopy (TEM)

The cardiac cultures were fixed for 1 h at 4°C with 2.5% GA in 0.01 M Na cacodylate buffer, pH 7.2, rinsed in the same buffer, and left overnight at 4°C. The cells were then carefully scraped off with a rubber policeman, collected by centrifugation, postfixed with 1% O_sO_4, dehydrated in acetone and embedded in Epon. For all TEM analysis, thin sections either unstained or stained with uranyl acetate and lead citrate were examined in an EM 10C Zeiss microscope.

Electron microscopy imaging (ESI)

ESI analysis was performed using Zeiss EM 902 transmission electron microscopy equipped with a prism-mirror-prism electron imaging spectrometer. This method enables detection of a particular chemical element in biological specimens, with high sensitivity and contrast. To obtain images of high contrast, an elastic image in a bright field was taken using E=0 eV and a spectroscopic image at the carbon edge with an energy loss of 245 eV.

Results

Electron microscopy view of cardiac muscle cells: The primary cultures of cardiomyocytes display morphological and physiological patterns of *in vivo* cardiac cells including striated myofibrils with rows of mitochondria profiles (Fig. 1A). The routine ultrastructural analysis of ventricular enriched cultures showed gap junctions, intercalated disks and the contractile apparatus (Fig. 1A, 1B).

We started our endocytic studies by analyzing the fluid-phase endocytosis through two different approaches and employing different ligands. In the first, we followed the uptake of fluorescent dextran conjugates as probe of fluid-phase endocytosis at 37°C. When CM were incubated with FITC-Dx for a short time point (5 min) typically required for materials to reach the early endosomal compartments (Rybak and Murphy, 1998), we noted a punctuate fluorescence pattern: small vesicles containing FITC-dextran scattered throughout the cell's cytoplasm, mostly accumulated in the periphery of the CM presumably corresponding to early endosomes (Fig. 2A). This dispersed localization was further confirmed by the association of actin filaments and TRITC-Dx labeled vesicles in the cardiac cells (Fig. 2B). When CM were further incubated at 60 min at 37°C to chase the phase-fluid ligand into late endocytic compartments, we found that large vesicles mostly accumulated in
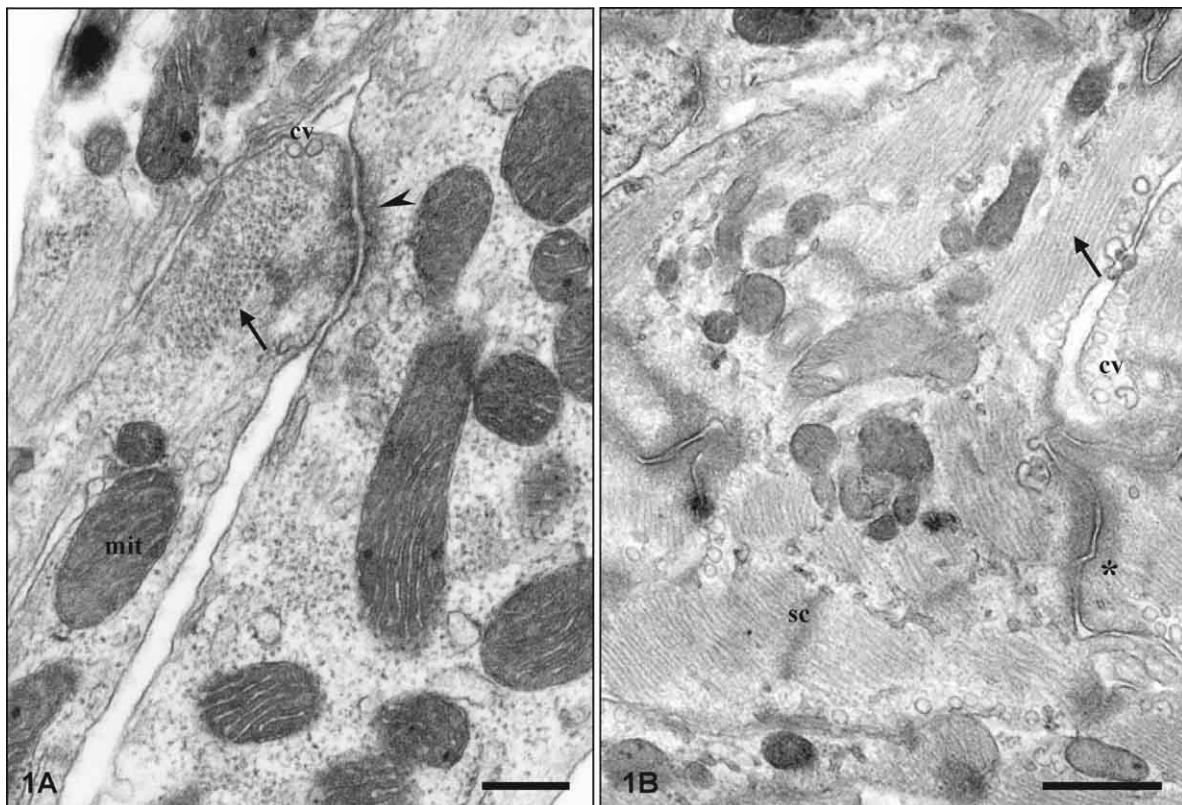

Fig. 1. Cell morphology of primary cultures of cardiac muscle cells. CM from mice embryos were plated in gelatin-coated coverslips and maintained at 37°C. Two days later, the culture displayed a high level of differentiated cardiac cells (>85%) displaying myofibrils (arrow), intercalated disks (asterisks) and gap junctions (arrowhead). Imaging of stained sections of cardiac cells showing myofibrils (→), caveolae (cv), gap junctions (➤) and mitochondria (mit) (A). TEM of typical cardiomyocytes showing sarcomers (sc) and intercalated disks (*) (B). Bar= 1 μm.
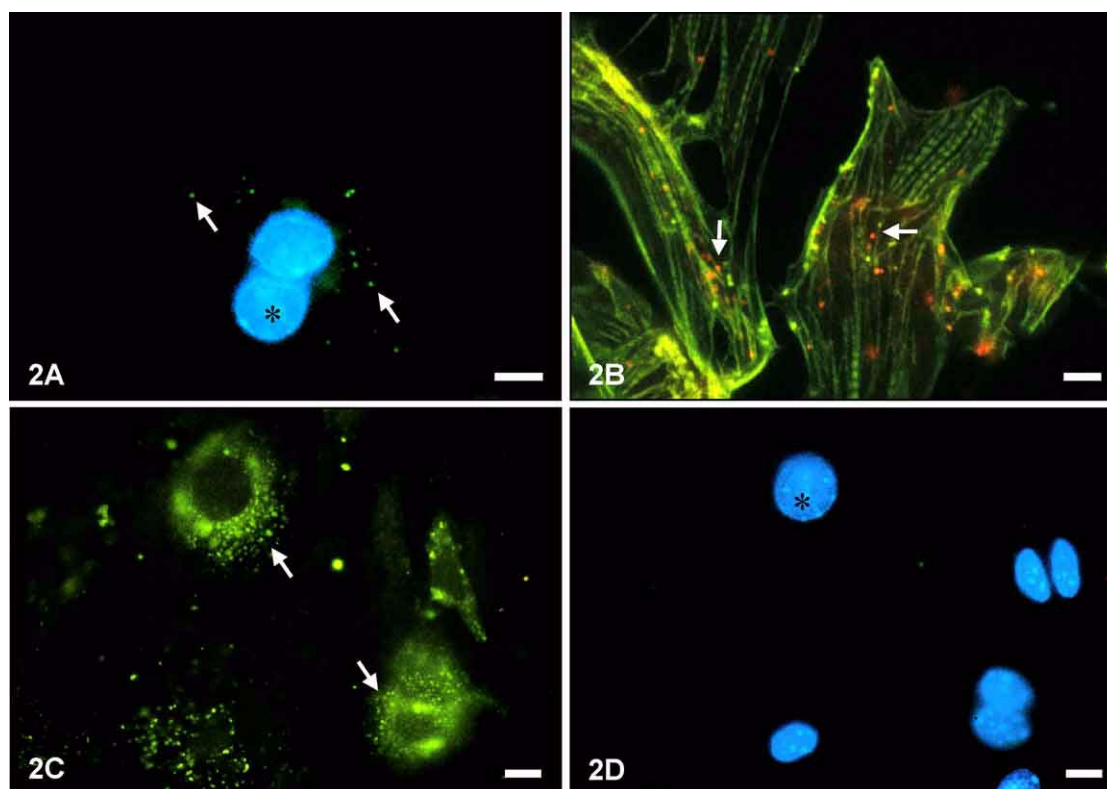
Fig. 2. Spatial distribution of FITC-Dx-labeled endosomes in cardiac muscle cells after 5 (A,B) and 60 min (C,D) of incubation at 37°C. Double labeling of dispersed endosomes with FITC-Dx (arrow) and cardiac cell nuclei with DAPI (asterisks) (A). Fluorescent image showing the association of early endosomal compartments (visualized by TRITC-Dx) with actin filaments of cardiac muscle cells, which were stained with phalloidin-FITC (arrow) (B). Cardiomyocytes that were chased for one hour with FITC-Dx (arrow) displayed labeled endosomal compartments, which were mostly localized at the cell perinuclear area (C). The correspondent cardiomyocyte nuclei are visualized by DAPI staining (asterisks) (D). Bar=0.25 μm.

the perinuclear area (Fig. 2C, D).

Concerning BSA-Au internalization in cardiac cells, we observed that after short time period of incubation (15 min up to 60 min), colloidal gold particles could be found at the cell surface and within small vesicles localized at the cell periphery, corresponding to early endosomes (Fig. 3A). After longer incubation times (from 1 h up to 24 h) at 37°C, gold labeled endosomes displaying different sizes and electrondensities could be found accumulated in the perinuclear region (Fig. 3B). These structures presumably correspond to late endosomes and lysosomes.

For ultrastructural analysis of receptor-mediated endocytosis in cardiac cells, we chose three ligands: HRP-Au, Tf-Au and CF particles. In order to characterize the early endosomal pathway (sorting and recycling compartments), we chased the Tf-Au internalization. After short periods of incubation (up to 15 min), we noticed a specific labeling in caveolae (Fig. 4A) as well as in coated pits (Fig. 4B, inset). From 15 min up to 60 min of incubation, the cargo could be found at the periphery of the CM within intracellular uncoated (Fig. 4C) and coated vesicles (Fig. 4D, inset). No labeling could be noticed close to nuclei (data not shown).

We next performed ultrastructural assays employing well-known receptor-mediated ligands, which have the final cargo destination in the late compartments. Then, the endocytic process through mannose receptors was followed using HRP coupled to colloidal gold particles (HRP-Au), as the mannosylated ligand. After 30–60 min of incubation at 37°C, the HRP-Au particles were found near and inside coated invaginations, within membrane specialized areas such as caveolae (Fig. 5A), as well as in coated and non-coated invaginations (Fig.5B). At this time, intracellular gold particles were concentrated inside small vesicles (Fig. 5C, inset) localized close to the sarcolemma suggestive of early endosome network. After chasing for 24 h at 37°C, HRP-Au particles were found in diverse intracellular vesicles, which displayed different sizes and were localized in the perinuclear area, possibly corresponding to late compartments (Fig. 5D). Control assays performed with the addition of D-mannose abolished the labeling (Fig. 5E).

For anionic sites, we noticed rapid tracer internalization through uncoated pits (Fig. 6A), reaching after 30 min to 3 h of incubation, smooth vesicles close to the plasma membrane closely related to early endosomes (Fig. 6B).
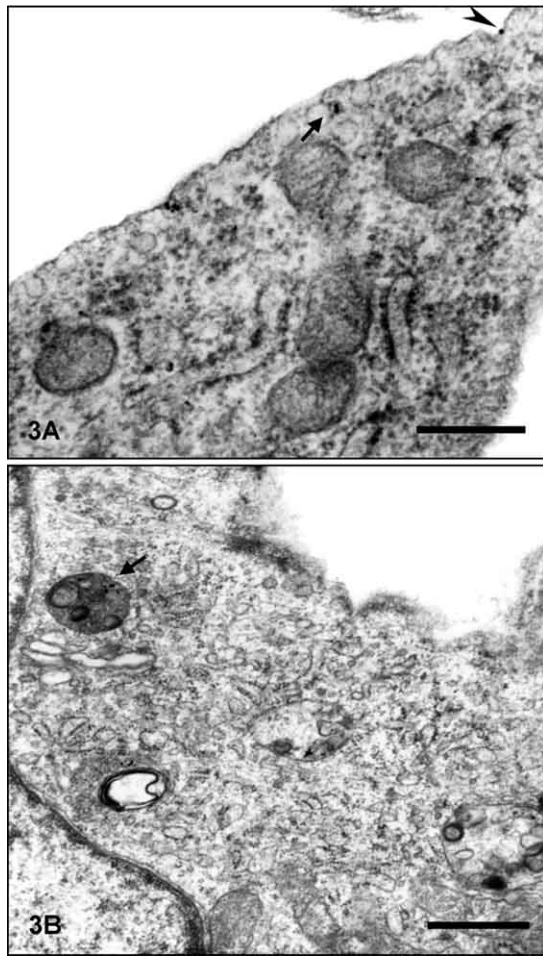
Fig. 3. Ultrastructural analysis of fluid-phase endocytosis in CM. After a short time period of incubation (15 min), a discrete labeling of BSA-Au particles is observed in the sarcolemma (arrowhead) and within intracellular vesicles localized in the cell periphery (arrow) (A); After longer periods of BSA-Au chase (1 h), labeled perinuclear endosomal compartments (arrow) can be seen in cardiac cells (B). Bar=2 μm.

Fig. 4. Transmission electron microscopy images of transferrin receptors chase in cardiac muscle cells. After 15 min of incubation at 37°C, the transferrin receptors (arrow) were localized at the sarcolemma within caveolae (A) and in coated pits (B). After 1 h of Tf-Au chase at 37°C, gold-labeled vesicles (arrow) localized at the cell periphery could be noted in uncoated (C) and coated vesicles (D), Bar= 4 μm.

After 24 h, the cationized ferritin could be noticed inside vesicles displaying different sizes and electron densities (late endosomes and lysosomes) localized mainly near the nucleus and sometimes containing myelin figures (Fig. 6C).

Next, we addressed the question of internalization of large ligands by CM, by assaying the ability of CM to internalize zymosan A particles. Our data showed that although at low rates and levels as compared to fibroblast cultures and macrophages (data not shown), the mannosylated particles could be found within large and tight vacuoles (Fig. 7A), which could almost occupy the whole cardiac cell cytoplasm. After 3 h of incubation at 37°C with 10^7 particles, around 18% of the CM exhibited internalized zymosan A particles (data not shown).

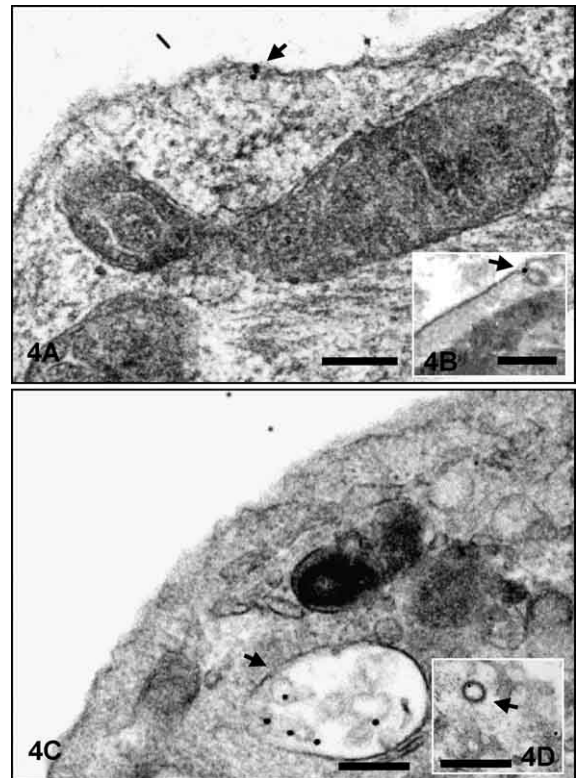When cardiac cells were interacted with trypomastigotes, we found and confrmied two well-known recognition sequences: the adhesion (data not shown) and the internalization steps (Fig. 7B). As described in the literature (Soeiro et al., 1999), the adhesion step was followed by the invagination of the sarcolemma, which will later on compose the parasitophorus vacuolar membrane (Fig. 7C).

Discussion

Isolated mouse myocytes maintain their functional integrity in *in vitro* cultures and are suitable for studying many cardiac cellular aspects such as intracellular Ca^{2+} dynamics, sarcoplasmic reticulum and its coupling to the plasma membrane, cytoskeleton assembly (Meirelles et al., 1999) and anionic sites distributions (Soeiro et al., 1994). Present ultrastructural and fluorescent analysis of the cultured cardiomyocytes showed typical images including sarcolemmal membrane with well organized T tubule-system, gap junctions, mitochondrial profiles and clear striation, which are in agreement with our previous published data (Meirelles et al., 1986; Pereira et al., 1993). This murine model has already been successfully used to investigate host physiological changes under pathogenic conditions

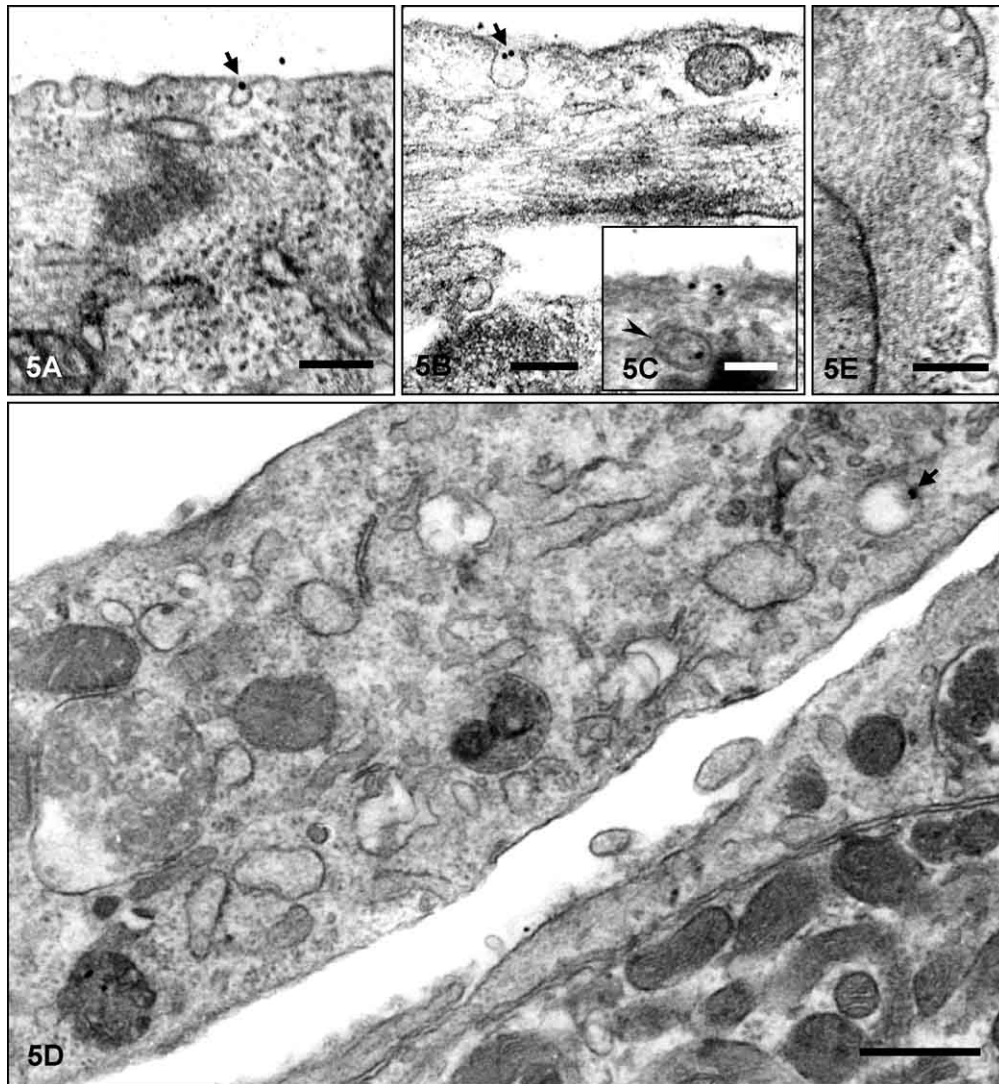**Fig. 5.** Cardiac myocytes were fed with HRP-Au for 30 min/4°C, washed and then chased in fresh medium for 1 h (A,B) and 24h (D) at 37°C. HRP-Au (arrow) can be seen associated to the sarcolemma caveolae (A), within uncoated invaginations (B) and inside intracellular endosomes localized at the cell periphery (arrowhead) (C, inset). After 24 h of HRP-Au chase, the ligand (arrow) was mostly found in vesicles close to the cell nuclei, putative of late endosomes and lysosomes (E). The addition of D-mannose blocked HRP-Au labeling in CM (D). Bar= 4 μm.

including surface charge alterations (Soeiro *et al.*, 1995), cytoskeleton destruction (Pereira *et al.*, 1993), mRNA expression modulation (Pereira *et al.*, 2000) and surface receptors down-regulation (Soeiro *et al.*, 1999).

Multiple endocytic mechanisms are present in mammalian cells including receptor-mediated endocytosis (regulated or not by clathrin), internalization by caveolae, fluid-phase endocytosis, macropinocytosis and phagocytosis (Henley *et al.*, 1999).

The current knowledge of cardiac endocytosis is still lacking, displaying only few reports. A recent study showed that an increased expression of Rab1 GTPase in myocardium distorts sub-cellular localization of proteins and is sufficient to cause cardiac hypertrophy and failure (Wu *et*

al., 2001). An enhanced acidification was observed in heart cell endosomes (pH 5.5) compared with kidney cell endosomes (pH 6.0) (Rybak and Murphy, 1998) suggesting that differences in endosomal pH may be important for the proper functioning of different cell types. Using rat cardiac myocytes it was demonstrated that the endocytic traffic is blocked in multivesicular carrier endosomes at and below 26°C, and that reduced temperature slows down transport in the recycling pathway, without a complete block (Punnonen *et al.*, 1998).

Our present results showed, by diverse tools, the endocytosis of ligands through different pathways, examining the distribution of early endosomes and late compartments by fluorescence and by electron microscopy. We began our
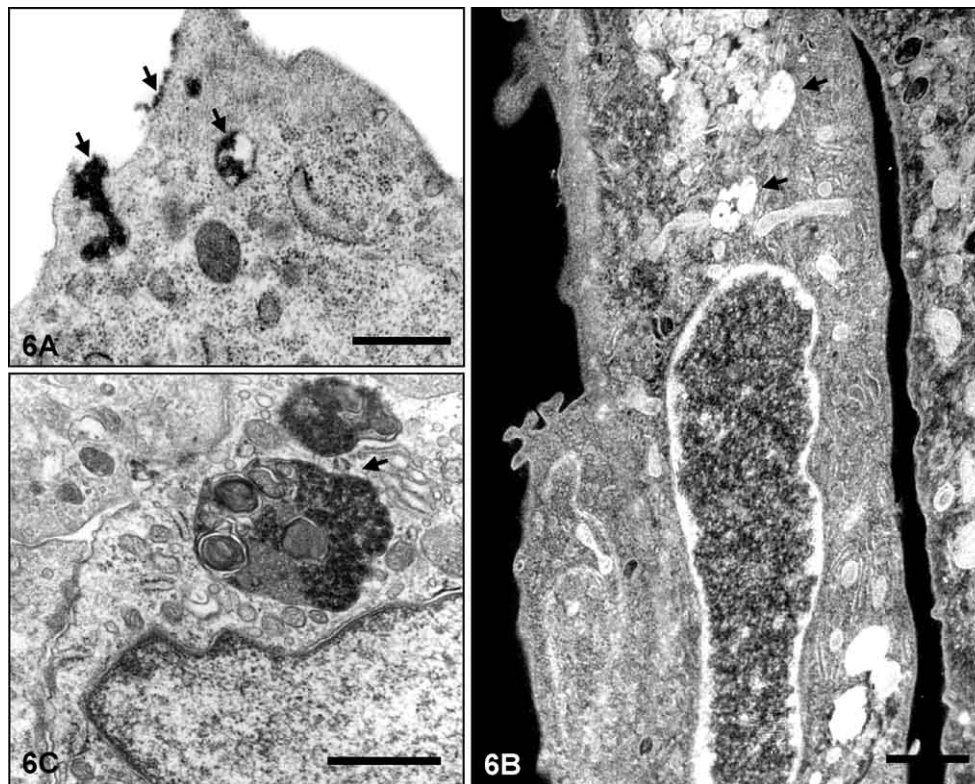
Fig. 6. Transmission electron micrograph of cardiac cells fixed after chasing with CF. After 30 min of ligand chase, CF particles were associated to the plasma membrane, within invaginations of the sarcolemma and intracellularly inside vesicles close to cell surface (arrow) (A). ESI image of thin unstained sections showing the labeled vesicles in cardiac cells after their chase for 3 h at 37°C (arrow) (B). After 24 h of incubation at 37°C, the CF particles could be noticed within late endosomes localized at the close proximity to the nucleus (arrow) (C). Bar= 2 μm.

studies by analyzing two well-characterized fluid phase markers: dextran (Kaisto *et al.*, 1999) and BSA (Soeiro *et al.*, 1992). FITC-Dx assays showed that the marker initially accumulated in peripheral compartments just beneath the sarcolemma and later on within compartments localized at the perinuclear region, suggesting their final cargo destination to late endosomes and lysosomes. We found the early endosomal compartment encircled by actin filaments, which travel longitudinally in the cardiac cells, composing the sarcomers. Our data concerning the kinetic and endosomal distribution of these fluid-phase cargo agree with previously published data done with skeletal muscle cells (Kaisto *et al.*, 1999), which characterized the endocytic compartments in fully differentiated cells by using single isolated fibers. Besides, Ellinger *et al.* (1998), using free-flow electrophoresis characterized the intracellular destination of FITC-Dx in the rat liver cells and found that after a short pulse (1–2 min) at 37°C, the ligand could be noticed in endosomes with the same electrophoretic mobility as early sorting endosomes, and that with increasing internalization time at 37°C, the FITC-Dx-labeled compartments co-localized with late containing endosomes. In order to better characterize early and late compartments in CM, double

immunofluorescent and proteomic approaches using GTPase markers are presently under way.

To further analyze the fluid-phase endocytosis, we presently performed electron microscopic analysis of the uptake of gold particles coupled to albumin. The kinetics of BSA-gold particles in cardiac cells was quite similar to the one observed with FITC-Dx, with the gold particles reaching later compartments as soon as 1 h after ligand incubation. In fact, the property of BSA-Au particles to be finally accumulated in late compartments allows its use in ultrastructural approaches in order to label lysosomes and to follow the process of lysosome-phagosome fusion (Soeiro *et al.*, 1992; De Carvalho and De Souza, 1989).

Later on, we followed the receptor-mediated endocytosis using the horseradish peroxidase coupled to colloidal gold particles as mannosylated tracer and the cationized ferritin as probe for the anionic sites labeling. HRP is a glycoprotein devoid of phosphorylated carbohydrates and rich in mannose and N-acetylglucosamine groups, which has been used as a current label for histochemical analysis of mannosyl binding sites (Straus, 1981). The fact that HRP-Au particles were also found in caveolae suggests the presence of mannose receptors in those invaginations. Actually, the
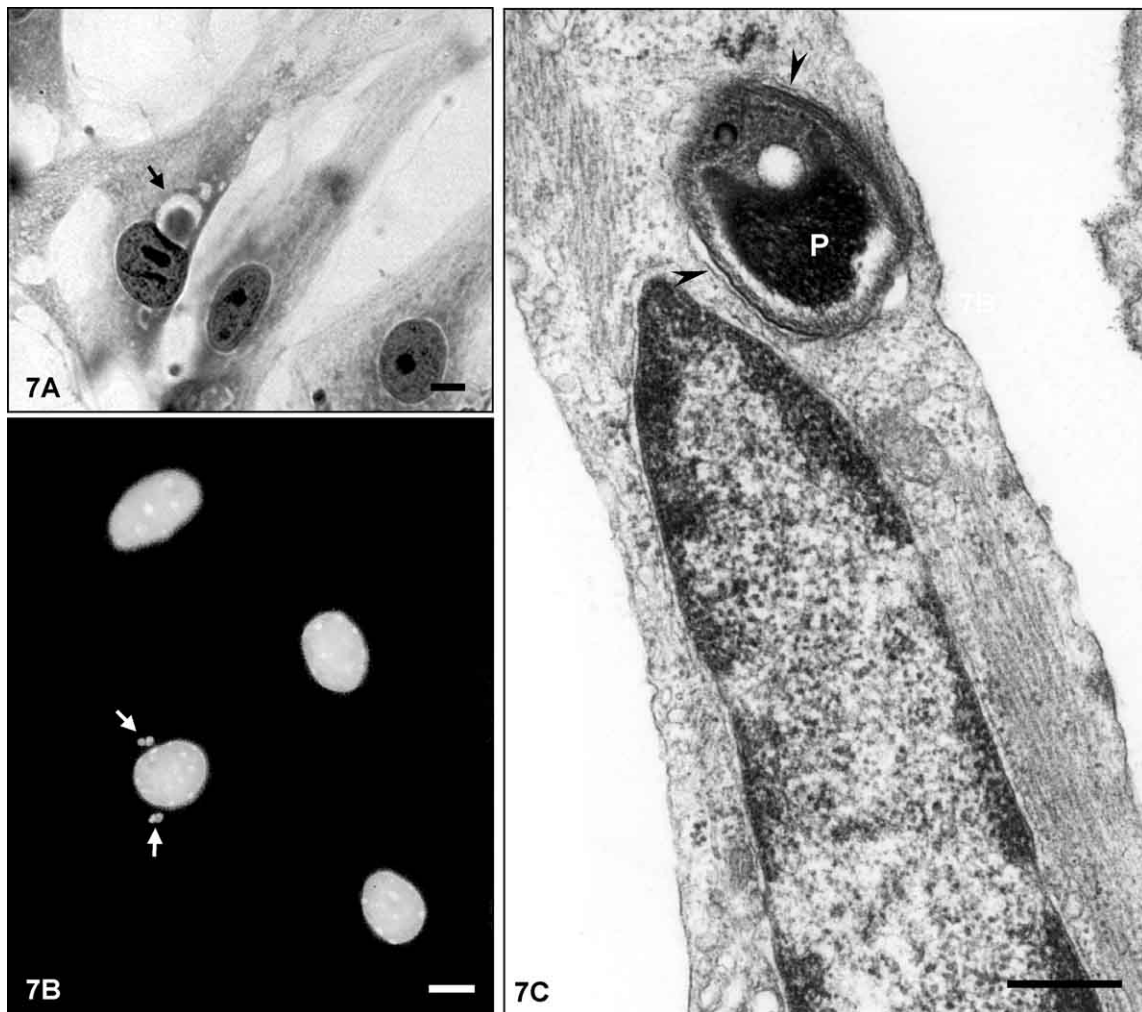
Fig. 7. Internalization of large particles and whole cells by cardiac muscle cells. Light microscopy illustration of Zymosam A particle uptake after 3 h at 37°C displaying internalized mannosylated particle (arrow) within large vacuoles (A). Fluorescent image of cardiac cells infected with *T. cruzi* tripomastigotes (arrow) for 24 h displaying intracellular parasites (B). Ultrastructural aspects of *T. cruzi* endocytosis by cardiac muscle cells. The intracellular parasite (P) is localized within the parasitophorous vacuole (arrowhead) until its escape to the host cell cytoplasm (C). Bar= 2 μm.

caveolae have been the subject of intensive study and to date many cellular roles have been proposed including signal transduction, potocytosis, cholesterol trafficking and endocytosis (Stan *et al.*, 2002). It has been demonstrated that elicited macrophages internalize larger amounts of HRP and some HRP-containing vesicles were also labeled with anti-caveolin, indicating that such vesicles should originate from caveolae (Kiss *et al.*, 2002). The authors also showed that the caveolae could pinch off from the plasma membrane and take part in the endocytic processes as alternative carriers in elicited phagocytes. HRP was also localized in caveolae of primary cultures of brain microvessel endothelial cells (Raub and Audus, 1990), and within caveolar and intercellular channels of vascular endothelial cells (Ogawa *et al.*, 2001). Therefore, since a thorough characterization of mannose receptors in caveolae of cardiac

cells becomes imperative raising interesting questions related to its possible intracellular destination, experimental approaches are now under way to clarify these points.

After longer incubation times, both the mannosylated ligand and the CF particles were associated with late endosomes and lysosomes characterized by their perinuclear localization and electron density pattern, which predicts their degradation by lysosomal hydrolases (Soeiro *et al.*, 1992, 1999). However, the delivery of receptor-mediated ligands to the late compartments in cardiac cells occurred later as compared to the fluid-phase tracers such as albumin and dextran, demonstrating that in cardiomyocytes different kinetics can be found according to the endocytic pathway and/or ligand sources.

On the other hand, the use of transferrin coupled to colloidal gold particles allowed the localization of the

early endosomal compartments (both sorting and recycling) in cardiac cells. During our assays, we localized a discrete transferrin receptor distribution localized mostly in clathrin-coated pits as well as in caveolae systems. Our data confirmed and extended previous published results within myofibers, which employed monoclonal antibodies to identify and characterize the transferrin receptors (Kaisto *et al.*, 1999). Further ultrastructural analyses employing specific probes for Rab proteins are under way to better characterize these pathways in cardiac cells.

Phagocytosis is the process by which leukocytes and other cells ingest particulate ligands whose sizes exceed about 0.5 to 1 μm, being critical for cellular immunity as well as tissue repair and morphogenetic remodeling (Greenbergö and Grinstein, 2002; Caron and Hall, 1998). Although professional phagocytes have an insatiable appetite being able to engulf multiple and larger particles, cumulative evidence shows that non-professional cells do have the ability to internalize such large particles under limited circumstances though much less efficiently (Brown, 1994). Our present results employing primary cultures of cardiac cells showed the mannose receptor-mediated phagocytosis of zymosan A, a carbohydrate-rich cell wall preparation derived from *S. cerevisiae*. The uptake of zymosan by macrophages has been investigated in detail (Lombard *et al.*, 1994), and it was demonstrated that this internalization is accompanied by secretion of the inflammatory mediator tumor necrosis-factor-α (Young *et al.*, 2001). The mannose receptors, which are lectin-like receptors, are implicated in receptor-mediated endocytosis of several microorganisms (Stahl, 1998). The invasion of *T. cruzi* in cardiac cells is mediated, at least in part, by mannose receptors, which are down-regulated after the parasite internalization (Soeiro *et al.*, 1999). Thus, since cardiac cells can produce pro-inflammatory cytokines (Machado *et al.*, 2000), the ability of zymosan A to promote inflammatory products by these cells deserves further investigation.

It is known that pathogenic microorganisms exploit key cellular functions in order to establish a successful infection. Several pathogens use the host endocytic pathway machinery to gain intracellular access (Clague, 1998; Mukherjee *et al.*, 1997). *Trypanosoma cruzi* is an obligate intracellular protozoan parasite that causes the incurable Chagas' disease, which mostly affects the heart (Brener and Gazzinell, 1997). Increasing data show that the infection of cardiac cells by *T. cruzi* occurs by an endocytic process with the formation of a parasitophorous vacuole (Soeiro *et al.*, 1999; Meirelles *et al.*, 1986) requiring the active participation of the host cytoskeleton machinery (Barbosa and Meirelles, 1995). The phagolysosomal fusion has been demonstrated in *T. cruzi* invasion in both phagocytic (De Carvalho and De Souza, 1989) and non-phagocytic cells (Meirelles *et al.*, 1986), without affecting the highly infective stages. In our present study, we demonstrated and confirmed the uptake of zymosan A and *T. cruzi* as clear

examples of the "phagocytic" competence of cardiac cells. Its relevance in cardiac function in combination with the ability of cardiomyocytes to secrete pro-inflammatory mediators (Machado *et al.*, 2000) is worthy of further investigation.

In summary, the novel concept that cardiac cells can ingest large particles and even whole cells raises important questions related to their ability to act as more than a physically energetic cell in the body, constantly contracting without tiring (Severs, 2000), but also contributing to other important cellular functions. In fact, recent studies speculate that during an infectious inflammation, the cardiomyocytes cannot be considered only as passive host cell since they can actively secrete many cytokines and chemokines (Machado *et al.*, 2000). Besides, they are also able to induct iNOS, produce high levels of nitrite, displaying trypanocidal activity (Machado *et al.*, 2000). It seems that the iNOS activation and the proinflammatory cytokines and chemokines produced by cardiomyocytes are likely to control parasite growth and cell influx, thus contributing to the pathogenesis of the chagasic cardiomyopathy seen in *T cruzi*-infected mice (Machado *et al.*, 2000). Further it is now accepted that during cardiomyopathy, cardiac cells can die both by necrosis and apoptosis (Zhang *et al.*, 1999), and it has been reported that the modulation of Bcl-2 and Bax complexes formation by insulin-like growth factor-1 (IGF-1) result in cardiac cell death protection (Wang *et al.*, 1998). It is possible that cardiac cells can produce mediators and express molecules involved in their own apoptosis, contributing to the progressive development of damage in the inflamed heart.

Our present data contributes to the understanding of how cardiac muscle cells work by presenting important morphological findings concerning endocytic pathways, which allows us to arrive at an overall understanding of physiological events and cell biology in cardiac cells. Since endocytosis is involved in diverse cellular functions, the knowledge of its several steps can contribute to the development of new future therapies for heart disease.

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