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Review Paper

Targeting calcium homeostasis as the therapy of Chagas' disease and leishmaniasis – a review

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Abstract. Ca^{2+} has been largely recognized as an essential messenger in all eukaryotes, from mammals to parasites. The disruption of Ca^{2+} homeostasis in any cell usually drives to lethal effects resulting in cell death by apoptosis or necrosis. This appears also to be the case in human trypanosomatids, such as *Trypanosoma cruzi*, the causative agent of Chagas' disease, *Trypanosoma brucei*, which produces "sleeping sickness" and *Leishmania sp*, responsible for leishmaniasis. The aim of this review is to describe the intracellular Ca^{2+} regulation and the cytotoxic effect of new drugs regarding the disruption of Ca^{2+} homeostasis in these parasites. With regard to intracellular Ca^{2+} regulation, all these trypanosomatids possess a single mitochondrion that occupies 12% of the total volume of the parasite which is able to accumulate large amounts of Ca^{2+}. The endoplasmic reticulum is also involved in Ca^{2+} regulation. These parasites also possess acidocalcisomes, an unusual organelle involved in the bioenergetics of these cells in accumulating large amounts of polyphosphates together with Ca^{2+} ions. Trypanosomatids possess relatively large amounts of calmodulin. While this well conserved protein is identical among all vertebrates, there is 89% amino acid sequence identity between *T. cruzi* and vertebrate calmodulin. Recently, this protein has been cloned and expressed from *T. cruzi*, allowing a further characterization corroborating significant differences between calmodulin from *T. cruzi* and mammals. It has also been reported that a commonly used antiarrhytmic, amiodarone, which is used in chronic Chagas' patients with heart problems, is able to produce a large trypanocidal effect. The intracellular compartments responsible for the increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) upon the addition of amiodarone are the single large mitochondrion and the acidocalcisomes. Amiodarone is also capable to inhibit the oxidosqualene cyclase, a key enzyme in the synthesis of ergosterol. The effect of amiodarone was highly synergistic with posaconazole, a known potent inhibitor of the synthesis of ergosterol. Interestingly, posaconazole by itself is able to produce an increase in the $[Ca^{2+}]_i$. Concerning putative treatments in humans, amiodarone was reported to induce the cure of a patient with Chagas' disease, when used in combination with itraconazole. Seemingly, a recent case indicated the cure of a patient with Chagas' disease by the administration of posaconazole. Miltefosine, an orally active alkyl-lysophospholipid with potent anti-*Leishmania* activity, represents a major advance in the treatment of leishmaniasis. Recently it was reported that miltefosine also disrupts the parasite's intracellular Ca^{2+} homeostasis, by inducing a large increase in $[Ca^{2+}]_i$, through the activation of a plasma membrane Ca^{2+} channel. It has been found that the combination of miltefosine and amiodarone have synergistic effects on the proliferation of amastigotes growing inside macrophages and this led to 90% of parasitological cure in a murine model of leishmaniasis, as revealed by a PCR assay. More recently, posaconazole has been used successfully in a case of a human Old World cutaneous leishmaniasis. All these findings strongly suggest that the alteration of the intracellular Ca^{2+} homeostasis of these parasites is a promising strategy as a target of new as well as repurposed old-known drugs.

INTRODUCTION

Ca^{2+} has been largely recognized as a paramount messenger in all eukaryotes so far studied, from non-mammals to parasites (Benaim, 1996; Garcia *et al.*, 2008). The disruption of Ca^{2+} homeostasis in any cell usually drives to lethal effects conducting to cell death including apoptosis and necrosis (Zhivotovsky & Orrenius, 2011). This appears to be also the case of human trypanosomatids. The aim of this review is to describe the intracellular Ca^{2+} regulation and the cytotoxic effects regarding the disruption of Ca^{2+} homeostasis and other related intracellular signals in these parasites.

Intracellular Calcium Homeostasis in Trypanosomatids

In trypanosomatids as well as in all eukaryotic cells, intracellular Ca^{2+} is finely regulated. This is warranted by the presence of different transport mechanisms, some of them located in intracellular organelles, limited by their capacity as compartments, and others in the plasma membrane, which are responsible for the long-term Ca^{2+} regulation with virtual infinite capacity for extruding Ca^{2+}.

Regarding intracellular Ca^{2+} regulation in trypanosomatids, for example, *Trypanosoma cruzi* possesses an extracellular stage in the human bloodstream where the Ca^{2+} concentration is around 2 mM, while after invading host cells they find an intracellular Ca^{2+} concentration of about 30-100 nM. These parasites themselves are able to maintain a cytoplasmic Ca^{2+} concentration between 20-50 nM (Benaim, 1996). *Leishmania* parasites also have two different stages; an extracellular form (promastigotes, in the insect midgut) and an intracellular form (amastigotes) in the interior of infected macrophages. This fact implies that these parasites should possess very efficient mechanisms for the regulation of this cation during each of these stages, as well as during the differentiation process.

Mitochondrial Ca^{2+}-transport in different species of trypanosomatids including, *T. cruzi* (Docampo & Vercesi, 1989) and several *Leishmania* species (Benaim *et al.*, 1990a; Vercesi & Docampo 1992; Bermudez *et al.*, 1997) have been extensively studied. These parasites possess a single large mitochondrion which occupies 12% of the total volume of the parasite. In fact, the DNA content of this mitochondrion is so large that it is visible under light microscope, looking like a second small nucleus, named kinetoplast, which characterize the whole family. Albeit these apparent difference with mitochondria from mammals, they also possess in its internal membrane a Ca^{2+} electrophoretic uniporter, very similar in nature to that present in higher eukaryotes (Benaim, 1996). In fact, very recently the Ca^{2+} electrophoretic uniporter has been identified as a forty-kilodalton protein in humans, being very conserved through evolution, including in kinetoplastids (*Trypanosoma* and *Leishmania*) (Di Stefani *et al.*, 2011). Thus, identical to the mitochondria of mammals, the driving force for Ca^{2+} entry is the electrochemical gradient formed by the transference of H^+ by the cytochromes chain at the inner mitochondrial membrane. This system is able to accumulate large amounts of Ca^{2+} in the mitochondrial lumen, but with rather very low affinity. However, this uniporter can easily work when large amount of the cation, as a consequence of a sudden increase in the cytoplasm, approaches its low affinity Ca^{2+}- binding site (Benaim, 1996).

As another intracellular compartment, but with high affinity for Ca^{2+}, the endoplasmic reticulum has also been shown to be involved in the intracellular Ca^{2+} regulation in these parasites. In this regard, an over-expression and characterization of a gene for a Ca^{2+}-ATPase from the endoplasmic reticulum in *T. brucei* has been characterized (Noland *et al.*, 1994), being analogous to the well characterized sarco(endo)plasmic Ca^{2+}-ATPase (SERCA) present in other eukaryotic cells. Interestingly, the over-expression of this SERCA enzyme in *Leishmania amazonensis* has been associated with enhanced virulence of this parasite against macrophages (Rodriguez *et al.*, 2002).

Plasmodium also displays molecular mechanisms to deal with Ca^{2+} homeostasis (Passos & Garcia, 1997; Hotta *et al.*, 2003; Gazarini & Garcia, 2004; Beraldo & Garcia,

2005; Beraldo *et al.*, 2007). Moreover, sensing the environment and using the ion to transduce external signals might be one of the strategies for parasite controlling its cellular cycle.

Concerning this Ca^{2+}-transporting compartment and different to the system from mammals, the second messenger IP_3, which has been identified in these parasites (Docampo & Pignataro, 1991), have been reported to lack any effect on intracellular calcium release in *T. cruzi*. Thus, even though there are many similarities between these parasites and mammals concerning Ca^{2+} regulation, some interesting differences are remarkable. In this regard, another non-mitochondrial intracellular calcium pool has been identified in *T. brucei*, which is sensitive to changes in intracellular pH (Ruben *et al.*, 1991; Vercesi *et al.*, 1994). This acidic compartment has been also identified in *T. cruzi* and has been called acidocalcisome (Docampo *et al.*, 1995). These authors reported that *T. cruzi* possesses most of its intracellular Ca^{2+} in the acidocalcisomes. The biochemical characterization of this organelle has provided evidence that is acidified by a vacuolar-type proton-translocating (V- H^+-ATPase) pump and that it also possesses a Ca^{2+}/H^+ for counter-transporting these two ions and also a Ca^{2+}-ATPase for Ca^{2+}. The function of these acidocalcisomes has been associated to osmoregulation and pH regulation (Ulrich *et al.*, 2010). Interestingly, these organelles constitute also a deposit of polyphosphates, and amongst them pyrophosphates (see Docampo & Moreno, 2008 for a comprehensive review). Membrane pyrophosphatase activity is associated to vectorial H^+-transport. Since pyrophosphate is an alternative source of energy in these parasites and even is more abundant than ATP, this organelle undoubtedly deserves more investigation. In fact, a proton pumping pyrophosphatase is present in the acidocalcisomes, but its presence has been also demonstrated in the plasma membrane and in Golgy apparatus in *T. cruzi* (Martinez *et al.*, 2002).

All the mentioned transport mechanisms are present in intracellular organelles, and thus, are limited by its capacity as compartments. So, they are able to regulate intracellular Ca^{2+} only at short terms. For long-term regulation these parasites possess a plasma membrane Ca^{2+}-ATPase (PMCA) which is capable to pump the cation out to the cell without any physical constraint. By the use of a highly enriched fraction of plasma membrane vesicles, it has been identified a plasma membrane Ca^{2+}-ATPase (PMCA) in *Leishmania braziliensis* (Benaim & Romero, 1990), *Leishmania mexicana* (Benaim *et al.*, 1993a), *T. cruzi* (Benaim *et al.*, 1991; Benaim *et al.*, 1995), and *T. brucei* (Benaim *et al.*, 1993b). Calmodulin is the natural regulator of the PMCA in all species so far studied, and it is also the case for this enzyme from the different trypanosomatids mentioned above (Benaim & Romero, 1990b; Benaim *et al.*, 1991).

Vanadate is the classical inhibitor of the so-called "P-type ATPases", which forms an aspartyl-phosphate intermediate in its catalytic cycle (Carafoli, 1987). Both the Ca^{2+} transport and the Ca^{2+}-ATPase activity were inhibited by low concentration of vanadate (Benaim & Romero, 1990b), indicating that the parasite Ca^{2+}- pump also belongs to this family of ATPases.

A Ca^{2+}-ATPase (Tca1) has been cloned and characterized in the plasma membranes and acidocalcisomes of *T. cruzi* (Lu *et al.*, 1998). However, Tca1 appears to lack a typical CaM-binding domain (Lu *et al.*, 1998), suggesting that this enzyme possesses a non-canonical CaM-binding site or there is still another PMCA in these parasites not yet identified, taking into consideration that this enzyme can be isolated by a CaM-agarose column (Benaim *et al.*, 1995) and it is indeed, as mentioned, stimulated by CaM.

The possibility of the existence of a Ca^{2+}/Na^+ exchanger mechanism in the plasma membrane has been evaluated in many of these parasites (Benaim & Romero, 1990b; Benaim *et al.*, 1991, 1993b) The results indicate that there is not a Ca^{2+} release driven by Na^+ entrance in any of the different parasites so far studied. This finding, however, was not surprising since this mechanism for Ca^{2+} extrusion is much less ubiquitous than the PMCA, being present

mainly in excitable cells (Carafoli, 1987). This fact also points that the long-term intracellular Ca^{2+}-regulation in these parasites is so far a responsibility of the plasma membrane Ca^{2+}-ATPase.

Calmodulin as the mediator of Ca^{2+} functions in different trypanosomatids

A crucial role of Ca^{2+} as a second messenger in trypanosomatids has become apparent. Thus, many important functions such as variant surface glycoprotein release in *T. brucei* (Bowles & Voorheis, 1982), regulation of cAMP levels through the regulation of the adenylate cyclase in *T. brucei* (Voorheis & Martin, 1981) and the cAMP phosphodiesterase in *T. cruzi* (Tellez-Iñon *et al.*, 1985), and cellular differentiation in *T. brucei* (Stodlg & Clarke, 1996) and in *L. major* (Besteiro *et al.*, 2008). Importantly, calcium is also related to cell invasion since the cytoplasmic Ca^{2+} concentration of trypomasigotes from *T. cruzi* increases during interaction with host cells (Moreno *et al.*, 1994).

In addition, calmodulin (CaM), the ubiquitous intracellular calcium binding regulator has been identified and characterized in American and African trypanosomes, as well as in *L. braziliensis*, *L. mexicana* and *L. donovani*. It has been associated to different functions in trypanosomatids, as cAMP-dependent phosphodiestherase stimulation in *T. cruzi* (Tellez-Iñon *et al.*, 1985), CaM-dependent protein kinase in *T. cruzi* (Ogueta *et al.*, 1998), signal transduction in the cGMP-nitric oxide pathway in *T. cruzi* (Paveto *et al.*, 1995), besides the regulation of the PMCA mentioned above.

CaM is a very well characterized Ca^{2+}-binding protein which possesses 148 amino acids with a very peculiar composition. Thus, 50 amino acids are acidic (aspartate and glutamate), which confers to the protein a characteristic low Pka (about 4). The absence of cysteine disulfide bonds and tryptophan is another well known hallmark of this curious protein. The tertiary structure of CaM resembles a dumb bell with two

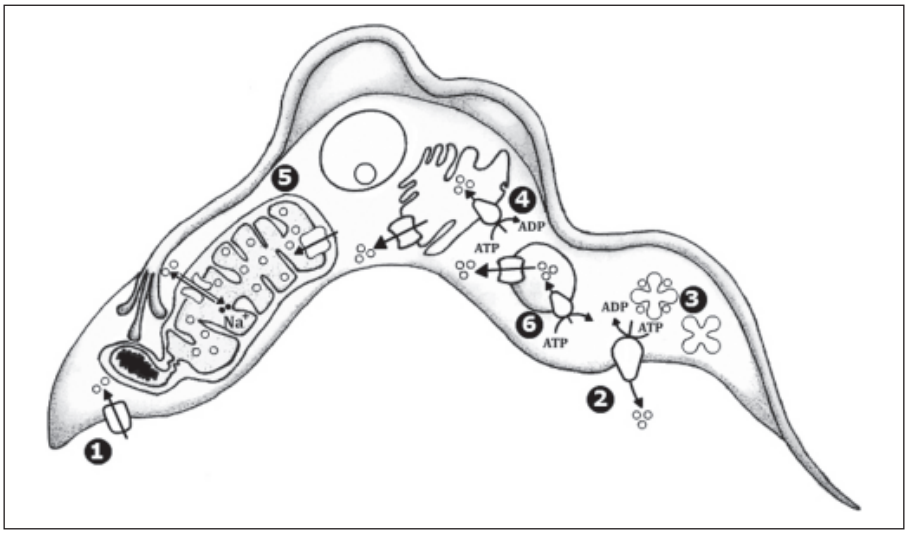

Figure 1. Schematic model of the Ca^{2+} regulation in trypanosomatids

Ca^{2+} enters the cell through an unidentified Ca^{2+} channel (1), and is extruded out of the cell by a plasma membrane Ca^{2+} pump (PMCA) (2) which is stimulated by calmodulin (CaM) upon binding Ca^{2+} (3). Inside the cell, Ca^{2+} can be sequestered with high affinity by the endoplasmic reticulum through a SERCA pump (4), leaving this organelle by a not yet identified Ca^{2+} channel. The large single mitochondrion (5) can transport Ca^{2+} with low affinity, by a mitochondrial Ca^{2+} uniporter (CMU), which is very similar to the recently characterized from humans, and also energized by the H^+ electrochemical potential. The intramitochondrial Ca^{2+} could be delivered to the citosol by a non-electrogenic Ca^{2+}/Na^+ (or Ca^{2+}/H^+) exchanger not well defined yet. Finally, the acidocalcisomes (6) can accumulate large amounts of Ca^{2+} by a PMCA, facilitated by the large amount of polyphosphates inside this organelle.

lobules, each containing two high affinity (EF hand) Ca^{2+} binding domains (Carafoli, 1987). The binding of Ca^{2+} to these 4 sites is highly cooperative and induces an increase in the, already high, content of α-helix structure, followed by an significant increase in the degree of hydrophobicity of the protein, essential for enzyme recognition. This conformational behavior of CaM in the presence of different Ca^{2+} concentrations allows this protein to effectively behave as a very sensitive Ca^{2+} sensor. Even more, CaM is able to be phosphorylated by several protein kinases, which change the affinity of this protein toward several enzymes, and also its stimulatory properties (Benaim & Villalobo, 2002). This property could explain its plasticity in the recognition of so many different target enzymes.

Besides being the most ubiquitous, CaM is the best conserved protein, concerning Ca^{2+} regulation. Interestingly however, when compared with other groups, CaM from trypanosomatids results to be remarkably different. Thus, albeit all CaMs so far studied possess the same number of amino acids, *T. brucei* CaM contains 17 amino acids substitutions with respect to vertebrates CaM while *T. cruzi* CaM contains 16 substitutions (Chung & Swindle, 1990).

When CaM is compared to distinct trypanosomatids with the protein from vertebrates, a significant difference in electrophoretic mobility in SDS-PAGE was observed (Ruben *et al.*, 1983; Benaim *et al.*, 1991; Benaim *et al.*, 1998), despite that CaM from both sources possess the same molecular weight. This difference was also observed on the typical Ca^{2+}-shift observed when the protein was run in SDS-PAGE in the presence or absence of Ca^{2+}. These results indicate that, in fact, there are significant differences between these two proteins. Another related result found was that a monoclonal antibody designed to the C-terminal region from mammalian CaM was unable to recognize purified CaM from *L. mexicana* and *T. cruzi* (Benaim *et al.*, 1998). All these results taken together suggest potentially important differences of CaMs from parasites with respect to its function. Conceivably, these unusual structural features of CaM from trypanosomatids may contribute to distinct interaction with proteins or pathways unique to these parasites.

CaM shares 100% amino acid sequence identity between different vertebrates and 99% between trypanosomatids. However, there is 89% amino acid sequence identity between *T. cruzi* and vertebrate CaMs. Recently, it has been cloned and expressed from *T. cruzi*, thus allowing a further characterization of the protein (Garcia-Marchan *et al.*, 2009). The results corroborated significant differences between calmodulin from *T. cruzi* and mammals. Thus, a polyclonal antibody developed in an egg-yolk system to the *T. cruzi* CaM recognizes the autologous CaM but not the CaM from rat. CaM from *T. cruzi* undergoes a larger increase in the α-helix content upon binding with Ca^{2+}, when compared to CaM from vertebrates, as evidenced by circular dichroism. Finally, two classic CaM antagonists, calmidazolium and trifluoperazine, capable of inhibiting the action of CaM in several target enzymes in mammals, when assayed on the plasma membrane Ca^{2+} pump from human erythrocytes, showed significant less activity when compared with experiments assayed upon stimulation with the *T. cruzi* CaM (Garcia-Marchan *et al.*, 2009). The results confirm unique biochemical characteristics of this protein from *T. cruzi*. These key differences between CaM from trypanosomatids and vertebrates make this protein not only an excellent object of study from the therapeutic point of view, but also could contribute to the understanding of the Ca^{2+} signaling in these parasites.

Calcium as a mediator of the citotoxic action of different drugs in trypanosomatids

As mentioned, large evidence has been accumulated demonstrating that Ca^{2+} exert a crucial role in the mechanisms undergoing cell death, including apoptosis, by the action of different deleterious effectors (Zhivotovsky & Orrenius, 2011). This appears to be also the case in trypanosomatids. Thus, a direct role of Ca^{2+} has been demonstrated in the trypanocidal effect of high density

lipoproteins (HDL) from normal human serum. Also, melarsoprol, a commonly used drug against *T. brucei* infections, and the combination of salicylhydroxamic acid with glycerol as well in *T. brucei*, has been showed, since larger concentrations of the drugs are necessary for a trypanocidal action, when calcium is omitted from the assay medium (Clarkson & Amole, 1982). This first report has led to the sight by many authors through a rational approach to the study of the effect of different drugs capable to affect different Ca^{2+} transport mechanisms in these parasites. Accordingly, it has been demonstrated that pentamidine, a drug of common use in different trypanosomiasis, inhibits the plasma membrane Ca^{2+}-ATPase and the associated Ca^{2+} transport in *T. brucei*, and interestingly, without affecting its human counterpart (Benaim *et al.*, 1993). Crystal violet, another well known trypanocidal drug, inhibits the plasma membrane Ca^{2+} pump, and also the mitochondrial Ca^{2+} transport, demonstrating that disruption of Ca^{2+} homeostasis is also lethal for *T. cruzi* (Docampo *et al.*, 1993a, 1993b).

More recently it has been reported that a commonly used antiarrhytmic amiodarone (a halogenated bis-aryl-ketone), which is used in chronic Chagas' patients with heart problems, is able to produce a large trypanocidal effect through the disruption of the intracellular Ca^{2+} homeostasis in epimastigotes (insect form) from *T. cruzi* (Benaim *et al.*, 2006). This effect was also revealed in amastigotes-infected VERO cells, with larger effectiveness. This fact is interesting since intracellular amastigotes are the clinically relevant form of this parasite. Amiodarone acts directly on intracellular organelles of the parasite, since if the experiments are performed in the absence of extracellular Ca^{2+} (i.e., in the presence of EGTA), the drug is unable to induce an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). One of the main intracellular compartments responsible for the increase in the $[Ca^{2+}]_i$ upon addition of amiodarone is the single large mitochondrion of these parasites, as could be inferred by confocal microscopy experiments designed to evaluate compartmentalized Ca^{2+} in isolated living trypanosomes (amastigotes) in the host cell interior (Benaim *et al.*, 2006).

Amiodarone was also capable to inhibit the oxidosqualene cyclase, a key enzyme in the synthesis of ergosterol (Benaim *et al.*, 2006). At this point it is important to mention that these parasites posses in their membranes this sterol, instead of cholesterol, showing an absolute requirement of ergosterol to warrant their survival. This has been indeed one of the most recent strategies in the search for new drugs against these parasites. In this context it was interesting to notice that the effect of amiodarone was highly synergistic with posaconazole, a known inhibitor of the sterol 14α-demethylase, another crucial enzyme in the synthesis of ergosterol (Urbina, 2009). Thus, isobolograms constructed on amastigotes-infected macrophages with combinations of amiodarone and posaconazole showed a large potentiating effect of these drugs when administered together. Remarkably, posaconazole by itself was able to produce an increase in the $[Ca^{2+}]_i$ of promastigotes from *T. cruzi*, after a period of incubation large enough (4 days) to allow the depletion of the endogenous ergosterol of the parasites (Benaim *et al.*, 2006).

Recently, the effects of this compound on recovering myocardial contractility in *T. cruzi*-infected cardiac myocytes through its direct action over F-actin fibrils and gap junction proteins such as connexin43 has been reported (Adesse *et al.*, 2011).

Concerning putative treatments in humans using these drugs, amiodarone was reported to induce the cure of a patient with Chagas' disease, when used in combination with itraconazole, another azole derivative, which inhibits the sterol 14α-demethylase (Paniz-Mondolfi, 2009). Seemingly, a recent case was reported that indicates the cure of a patient with Chagas' disease by administration of posaconazole (Pinazo *et al.*, 2010). In this case, the patient presented systemic lupus erythematosus that required immunosuppression to control the auto-immune process, and showed the sudden manifestation of Chagas' disease. A 400 mg per 12 hour for 90 days posaconazole treatment was given, resulting in the cure of

the patient. Consequently, these drugs are very promising as new strategies for the treatment of Chagas' disease.

It has been recently demonstrated that amiodarone is also effective against *L. mexicana*, even at lower doses to that required to inhibit the proliferation of *T. cruzi* (Serrano *et al.*, 2009a). The drug is effective against promastigotes (insect form) and amastigotes-infected macrophages, even with larger effectiveness. The mechanism of action is also through the disruption of Ca^{2+} homeostasis, releasing Ca^{2+} from the mitochondrion, as visualized by confocal microscopy. In that work it was also demonstrated that the acidocalcisomes are involved in the large increase in the $[Ca^{2+}]_i$ observed upon addition of amiodarone. This drug was able to alkalinize the interior of acidocalcisomes, releasing acridine orange, a weak base fluorescent dye, which accumulates in acidic compartments, and therefore is an acidocalcisome marker. Miltefosine, an orally active alkyl-lysophospholipid with potent anti-*Leishmania* activity, represents a major advance in the treatment of leishmaniasis (Croft & Engels, 2006). Recently it was reported that miltefosine also disrupts the parasite's intracellular Ca^{2+} homeostasis, by inducing a large increase in intracellular Ca^{2+} levels (Serrano *et al.*, 2009b). But different to amiodarone, the elevation of the $[Ca^{2+}]_i$ upon addition of miltefosine was only observed in the presence of extracellular Ca^{2+}, indicating that this drug does not induce the release of Ca^{2+} from intracellular compartments, but should exert its effect through the activation of a plasma membrane Ca^{2+} channel, not yet identified. In this sense, it has been demonstrated that anti-*Trypanosoma evansi* VSG antibodies also produced an increase on the parasite $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} when added to a suspension of *T. evansi*, a parasite related to *T. brucei*, which infects cattle (Mendoza *et al.*, 2008). It is very likely that these anti-*T. evansi* VSG antibodies exert its effect by inducing the opening of a plasma membrane Ca^{2+} channel.

The *in vitro* and *in vivo* activities of amiodarone and miltefosine on *L. mexicana* have been also investigated (Serrano *et al.*, 2009b). It was found that the drug combination had synergistic effects on the proliferation of intracellular amastigotes growing inside macrophages and led to 90% of parasitological cure in a murine model of leishmaniasis, as revealed by a PCR assay using a novel DNA sequence specific for *L. mexicana* (Serrano *et al.*, 2009b). Concerning its possible use in humans, amiodarone was also successfully administrated in a clinical case of cutaneous leishmaniasis (Paniz-Mondolfi *et al.*, 2008).

Very recently, posaconazole has been used orally in a case of a human Old World cutaneous leishmaniasis. *Leishmania* parasites were identified as *Leishmania infantum* by PCR. In this treatment, 400 mg of posaconazole was administered orally twice daily for 14 days. Despite the short course of treatment, there have been no signs of primary recurrence or visceral involvement for more than 15 months following completion of treatment. (Paniz-Mondolfi *et al.*, 2011).

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