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Electrical Membrane Properties in the Model *Leishmania*-Macrophage

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1. Introduction

Leishmaniasis, a disease caused by parasites of the genus *Leishmania*, constitutes a worldwide health problem. Since 1993 the disease has spread over wider areas of the world, and the situation has further deteriorated due to the AIDS pandemic. No vaccine is available to control the disease, and current therapies have problems of resistance, therapeutic failure, and cost (González et al., 2009). In Colombia, the recent incidence of most parasitic diseases has tended to stabilize, but in contrast the last decade has seen a doubling of the incidence of cutaneous Leishmaniasis (INS, 2009). Several reasons explain this, among which climate changes, deforestation, migration, and vector changes are in common with other areas of the world, but local circumstances such as illegal cultivars, the internal armed conflict, and recent health reforms are also important.

The parasite *Leishmania* transits between different environments in its life cycle, from the mosquito gut to the salivary glands, and for a short period in the vertebrate skin, before entering a compartment known as the parasitophorous vacuole (PV) inside macrophages and dendritic cells. The transformation between the two major parasite stages, from promastigote to amastigote, is the result of some of the changes that occur between the mosquito gut and the PV, among which temperature and pH have been implicated (Zilberstein & Shapira, 2004). On the other hand, osmolarity and ionic concentration are known to affect the ability of any cell, presumably including *Leishmania*, to control membrane potential, ion and nutrient transport, osmolarity, volume, and pH, though the precise effects are yet poorly understood. Our major interest is based on the assumption that *Leishmania* survival in the macrophage is also the result of the integrated function of the three concentric membranes found by the intracellular (amastigote) form of this parasite: the host cell plasma membrane (i.e., the macrophage plasma membrane), the parasitophorous vacuole membrane (PVM), and the *Leishmania* plasma membrane.

2. Macrophage plasma membrane

In the vertebrate host, *Leishmania* is an obligatory intracellular parasite that infects cells of macrophagic and dendritic lineage. In other intracellular parasite-host cell relationships, in particular *Plasmodium*, the demands of the replicating parasite are met by incorporating parasite membrane channels and transporters, or by modulating those of the host cell

(Ginsburg & Stein, 2005; Staines et al., 2007; Martin et al., 2009). Altered calcium homeostasis in erythrocytes and muscle cells has been reported in *Plasmodium* and *Trypanosoma* infection (Tanabe, 1990; Olivier, 1996; Tardieux et al., 1994) as well as in macrophages infected with *Leishmania* (Eilam, 1985; Olivier, 1996). Though there is no evidence of *Leishmania* induced alterations of macrophage nutrient transport, changes in macrophage membrane permeability, particularly in the electrical membrane properties, may alter its ability to activate and present antigen, therefore affecting the immune response.

Several functional activation stages have been proposed for the macrophage (Gordon, 2003; Mosser & Edwards, 2008). For example, macrophages stimulated *in vitro* with lipopolysaccharide (LPS) and interferon gamma (INF-γ) activate in a way that has been designated as classical. This functional stage is characterized by morphological changes, increased macrophage surface area, nitric oxide (NO) production, upregulation of tumour necrosis alpha (TNF-α) secretion, and induction of interleukin 12, among others (reviewed by Gordon, 2003). There is so far no coherent view of macrophage electrical membrane properties and their functional significance and association with the different activation stages, but some associations are now becoming apparent.

2.1 Macrophage passive membrane properties

Macrophage passive electrical membrane properties have been recorded in primary macrophages of mouse (Buisman et al., 1988; Vicente et al., 2003), man (Gallin & Gallin, 1977; Holevinsky & Nelson, 1998), various mouse (Randriamampita & Trautmann, 1987; Buisman et al., 1988; Holevinsky & Nelson, 1998; Gallin, 1991; Forero et al., 1999; Camacho et al., 2008; Quintana et al., 2010; Villalonga et al., 2010) and human (McCann et al., 1987) cell lines. Differences between some measurements of electrophysiological parameters may be related to cell culture conditions, though some authors argue that they represent genuine innate characteristics of currents expressed by macrophages (Randriamampita & Trautmann, 1987). Several studies support this hypothesis (Randriamampita & Trautmann, 1987; McKinney & Gallin, 1990; Forero et al., 1999; Camacho et al., 2008). In this view, variations in characterization are explained by differences in the ionic solutions used and the corresponding change to the Nernst equilibrium and the electrochemical force imposed on each ion, and of course by differences in the ion channel populations expressed (Vicente et al., 2006; Villalonga et al., 2007; Vicente et al., 2008).

In our experience, adherence onto glass, a step used to differentiate monocytes to macrophages, critically affects the electrical properties of these cells. The passive electrical membrane properties of J774.A1, a mouse macrophage-like cell line, vary with time after adherence onto glass, where increased membrane capacitance (Cm) and hyperpolarization have been observed (Gallin & Sheehy, 1985; McKinney & Gallin, 1990; Camacho, unpublished data). Active electrical properties also vary, with large outward currents (I_{OUT}) recorded during the first 8 hours post-adherence with minimal inward rectifying current (I_{KIR}) (Gallin & Sheehy, 1985; Randriamampita & Trautmann, 1987). I_{OUT} and I_{KIR} have similar amplitudes after 24 hours post-adherence (Gallin & Sheehy, 1985), but after 48 hours the situation reverses, with predominant I_{KIR} and negligible I_{OUT} (McKinney & Gallin, 1990). We have therefore controlled for time of adherence onto glass in some of our experiments to guarantee similar amplitudes for I_{OUT} and I_{KIR} (Forero et al., 1999; Camacho et al., 2008; Quintana et al., 2010).

Membrane capacitance is an electrical parameter that is proportional to surface area because the ability to store charge of a capacitor depends on this geometry. The lipid bilayer of the cell membrane can be modelled as a capacitor of two parallel conducting plates separated by a dielectric, so changes in Cm reflect direct changes in membrane area. Cm varies with post-adherence time (McKinney & Gallin, 1990) and *Leishmania amazonensis* post-infection time (Forero et al., 1999; Camacho et al., 2008; Quintana et al., 2010). J774.A1 cells exposed to cytochrome C to induce apoptosis are smaller than control cells, but have a similar Cm (Clavijo et al., 2009) suggesting loss of volume without decrease in surface area.

Macrophages are professional phagocytes, and phagocytosis implies the remodelling of the plasma membrane by the cytoskeleton and the incorporation of the phagocytic load into an intracellular compartment, the phagosome (PG). Entry of any load results in plasma membrane donation and should impact macrophage Cm. Phagocytosis of immune complexes or inert particles is associated with a reduction of Cm proportional to the load. After phagocytosis of 3 μm particles, Cm dropped around 7-10% (Holevinsky & Nelson, 1998; Quintana et al., 2010). *L. amazonensis* infected macrophage-like cells lose nearly a third of their Cm by 3 hours post-entry (Quintana et al., 2010), corresponding well to an average parasite load of two to three promastigotes (Hoyos et al., 2009). Cm recovers in this model, and by 24 hours post-infection the values are above control levels (Forero et al., 1999). Membrane donation by the macrophage plasma membrane upon phagocytosis requires recruitment of intracellular membranes from endosomes and lysosomes (Pitt et al., 1992; Desjardins et al., 1994a, Desjardins et al., 1994b, 1995; Beron et al., 1995; Idone et al., 2008). The fusion of these membranes fulfils two purposes: extra membrane for the nascent PG, and early release of immune effectors as shown for TNF-α (Murray et al., 2005, 2005b). Recruitment of endoplasmic reticulum (ER) membrane (Gagnon et al., 2002; Becker et al., 2005) may be important for PG formation when phagocytosing large loads, though this proposed role is controversial. After *L. amazonensis* entry we observe positive labelling with anti-LAMP antibodies, a lysosomal marker, as well as labelling of PV membranes with anti-IP_3 receptor (Perez, 2008), a marker of ER, suggesting membrane donation from this organelle.

Other processes in which the macrophage recruits more membrane to the macrophage plasma membrane should polarize more charge and increase Cm. During classical activation, J774.A1 becomes larger and adds membrane (Camacho et al., 2008), but LPS alone surprisingly reduces this parameter in RAW 264.7 (Villalonga et al., 2010), suggesting reduction of surface area. In the *Leishmania*-macrophage model, evidence of reduction of endosomal and lysosomal compartments (Barbiéri et al., 1990) has been interpreted as a way to concentrate lysosomal activity into the PV to control the parasite. This phenomenon may also indicate alterations in the ratio of membrane fusion/fission in the exocytic/endocytic rate. Assuming that the dielectric properties of J774.A1 plasma membrane do not change during *Leishmania* infection, the augmentation of macrophage Cm (Forero et al., 1999) may constitute a defect in macrophage plasma membrane recycling.

In most animal cells, the resting Vm is set by the activity of leak channels selective for K^+, along with the contribution of the electrogenic activity of ion pumps. Thus the resting potential of many animal cells is close to E_{K+}. Most data suggest a Vm for the macrophage close to E_{K+} that varies with changes in $[K^+]_o$ (Gallin & Sheehy, 1985; Judge et al., 1994). The slope was -49 mV/ 10 fold $[K^+]_o$, indicating higher permeability to K^+ under resting

conditions (Gallin & Sheehy, 1985), but suggesting the contribution of other ions. A contribution of the electrogenic activity of a Na^+/K^+ ATPase pump (Gallin & Livengood, 1983) of about 6 mV to the macrophage Vm could explain the deviation found. The depolarization obtained after inhibition of I_{KIR} by Ba^{2+} has implicated this current in the macrophage Vm value (Randriamampita & Trautmann, 1987), particularly after long periods of adherence (Gallin & Sheehy, 1985). More recently it has been proposed that Kv1.3 voltage gated channels establish macrophage Vm (Mackenzie et al., 2003) as shown in T lymphocytes (Panyi et al., 2004).

Early measurements of Vm were misinterpreted as action potentials (McCann et al., 1983). Values of around -75 mV have been recorded (McCann et al., 1987; Gallin & Sheehy, 1985; Buisman et al., 1988), and it has been suggested that time of adherence is not important because rapid hyperpolarization was observed after only 30 minutes post-adhesion (Gallin & Sheehy, 1985). Values of -60 to -70 mV were recorded in J774 and in peritoneal macrophages (Randriamampita & Trautmann, 1987). In whole cell configuration we have measured Vm when the membrane current is zero during the first minute after attaining this configuration, and have recorded potentials between -40 and -50 mV (Forero et al., 1999; Camacho et al., 2008; Quintana et al., 2010) at 24 or less hours post-adherence onto glass. Similar values (-42 to -58 mV) were reported in the same cell line by McKinney & Gallin, (1990). In T lymphocytes, Vm is close to the gating potential of Kv1.3 channels and this argument has been used to suggest that one of the functions of this channel is to establish Vm (Panyi et al., 2004). After 24 hours post-phagocytosis the average Vm found was depolarized compared to control macrophages (Camacho et al., 2008). Similar Vm values and depolarizations were found in macrophages stimulated with LPS and INF-γ (Camacho et al., 2008) at 24 hours post-treatment or exposed to cytochrome C to induce apoptosis 2 hours post-treatment (Clavijo et al., 2009).

Macrophage membrane resistance (Rm) has been measured. We have found membrane resistance of 2 GΩ in macrophage-like cells not altered by *L. amazonensis* infection (Forero et al., 1999). After reaching the whole cell configuration, a 10 mV step pulse was applied to the macrophage from a holding potential of -60 mV and the recorded current averaged to calculate Rm from Ohm's law. ATP^{4-} exposure reduced Rm by a factor of 10 in a related macrophage cell line (J774.2), where membrane depolarization was also observed (Buisman et al., 1988). Functional evidence of a poorly selective conductance was recorded, based on the reversal potential measurement (0 mV; Buisman et al., 1988). This is compatible with the permeation effect achieved after the ATP exposure used to load macrophages with anionic fluorescent probes (Steinberg et al., 1987a), explaining the reduction in Rm.

2.2 Macrophage ion currents

With respect to active electrical properties of the macrophage plasma membrane, Cl^- currents (I_{Cl}), K^+ outward (K^+ I_{OUT}) and inward currents (I_{KIR}) have been described. Large conductance chloride channels were recorded in patches of J774 and peritoneal macrophages (Randriamampita & Trautmann, 1987). Thioglycolate-recruited mouse peritoneal cells dialyzed in K^+-glutamate containing $InsP_3$ showed a rapidly activating outward current that depolarized the cells, suggesting that part of the current was carried out by Cl^- (Judge et al., 1994). In human monocyte-derived macrophages, rises of $[Ca^{2+}]_i$ induced an I_{Cl} current of properties similar to those described previously, and sensitive to

DIDS (Holevinsky et al., 1994). A third of the total current recorded in our model was inhibited by DIDS involving an anion, most probably Cl^- (Camacho et al., 2008).

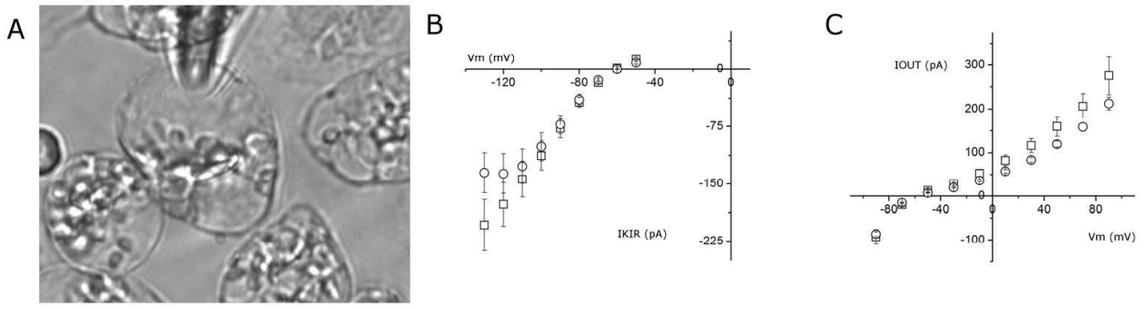

Fig. 1. Currents in J774.A1 macrophage-like cells. A. Light microscopy image of J774.A1 macrophage-like cells infected with *Leishmania amazonensis*. Note the tip of the recording pipette and the intracellular parasites in the parasitophorous vacuoles. B. I/V curve of inward rectifying potassium currents. C. I/V curve of outward currents. (□) Mean I peak at the beginning of the trace and after the capacitive transient. (O) Mean I of the steady state current in the last 20 s of the recording. Typical recordings of currents elicited by applying 9-10 pulses of 1 s (B) or 100 ms (C) at 10-20 mV intervals, from -50 to -130 mV (B) or -90 to 90 mV (C) from a holding potential of -60 mV. Data represent the mean values ± SE with n = 10.

I_{KIR} is induced by hyperpolarizing voltages and is inactivating (Gallin & Sheehy, 1985; Randriamampita & Trautmann, 1987; McKinney & Gallin, 1988; Judge et al., 1994; Forero et al., 1999). The voltage range of activation changes to less negative potentials with rises in $[K^+]_o$, following a square root relation between the peak G_{KIR} and $[K^+]_o$ (Gallin and Sheehy, 1985; Randriamampita & Trautmann, 1987). I_{KIR} inactivation is accelerated by voltage (Gallin & Sheehy, 1985; Randriamampita & Trautmann, 1987; Judge et al., 1994; Forero et al., 1999; Figure 1B), and its amplitude inhibited by extracellular Ba^{2+} (Gallin & Sheehy, 1985; Randriamampita & Trautmann, 1987; Forero et al., 1999) and Cs^+ (Gallin & Sheehy, 1985; Randriamampita & Trautmann, 1987; Judge et al., 1994). I_{KIR} goes to zero over time during recording (Gallin and Sheehy, 1985; Randriamampita and Trautmann, 1987), but the current is preserved in time with the addition of ATP and GTP to the pipette solution (McKinney & Gallin, 1990; Judge et al., 1994), suggesting dependence on metabolism (Judge et al., 1994). I_{KIR} was reduced by H_2O_2 production (Judge et al., 1994) although the authors did not indicate this. Colony stimulating factor was reported to have no effect on this current (Judge et al., 1994) but more recent data shows induction of this current by macrophage colony stimulating factor (M-CSF; Vicente et al., 2003). Kir2.1 channels are held responsible for this current (Vicente et al., 2003).

K^+ I_{OUT} appears to be the result of at least three different types of ion channel populations: inactivating, non-inactivating and calcium dependent K^+ current(Ca^{2+} K^+ I_{OUT}; Gallin, 1991). Total K^+ I_{OUT} is rapidly activated with depolarization (Figure 1C) and inactivates over time. The selectivity was studied in tail currents with different $[K^+]_o$; the reversal potential was also shifted to lower potentials with rises in $[K^+]_o$, implicating this ion in these currents (Gallin & Sheehy, 1985). K^+ I_{OUT} is inhibited by 4-aminopyridine (4-AP) and tetraethyl ammonium (TEA; Gallin & Sheehy, 1985; Camacho et al., 2008;), two classical potassium channel blockers, by margatoxin, a specific inhibitor of Kv1.3, and by charybdotoxin, a specific inhibitor of calcium dependent potassium channels (Camacho et al., 2008). Ca^{2+} K^+ I_{OUT} attains a large amplitude with $[Ca^{2+}]_i$ of 1 μM and is inhibited by quinine (Randriamampita & Trautmann, 1987). The

relative contributions of these currents may however vary with cell type, culture conditions, and cell status (Randriamampita & Trautmann, 1987; Gallin, 1991; DeCoursey et al., 1996; Eder et al., 1997; Camacho et al., 2008). More recently it has been shown that transcription of specific genes and expression of Kv1.3, Kv1.5 are associated with K^+ I_{OUT} (Mackenzie et al., 2003; Vicente et al., 2006; Vicente et al., 2006; Park et al., 2006).

Despite several reports that outward currents are altered when macrophages are activated (Nelson et al., 1992; Ichinose et al., 1992; McKinney & Gallin, 1992), a coherent view of macrophage functional status and electrical membrane properties is just emerging. Using primary cultured cells and a macrophage cell line, Felipe's group initially showed that the majority of the K^+ I_{OUT} in mouse bone marrow macrophages is generated almost exclusively by Kv1.3 voltage gated potassium ion channels (Vicente et al., 2003). In an elegant set of experiments they characterised the electrophysiological response of these cells and showed K^+ I_{OUT} and I_{KIR}. The currents were associated with transcription of mRNA, protein expression, and localization to the macrophage plasma membrane of Kv1.3 and Kir2.1. They showed the expression pattern of these ion channels after stimulation with M-CSF, LPS, and TNF-α. Over-expression of Kv1.3 and Kir2.1 translated functionally to approximately three times more K^+ I_{OUT} and around four times more I_{KIR} during macrophage proliferation (M-CSF), contrasting with nearly 15 times more K^+ I_{OUT} and a reduction to half in I_{KIR} after macrophage activation with LPS (classical activation; Vicente et al., 2003). The differences in the timing of mRNA transcription resulted in electrophysiological differences. Furthermore, they recorded evidence of the impact of differential expression of Kvβ subunits in macrophages during proliferation and differential activation between LPS and TNF-α (Vicente et al., 2005). The expression of Kv1.5 resulted in associations with Kv1.3 to conform hetero-dimers to distinct biophysical properties (Vicente et al., 2006; Vicente et al., 2007). They also observed modulation by Kv1.5 and LPS of intracellular traffic of Kv1.3 and Kv1.3/Kv1.5 channels (Vicente et al., 2008). Their suggestion is that expression of Kv1.5 does not generate homodimers, but rather the heterodimers Kv1.3/Kv1.5 with different stoichiometries that alter macrophage electrical properties and trafficking to the membrane. In humans, hKv1.5 is transcribed and expressed and a drop is observed in its typical outward current after exposure of antisense oligonucleotides against this channel; functional consequences followed, with a 50% reduction in migration of human alveolar macrophages (Park et al., 2006). Using the same conditions reported by Vicente et al. (2003), we have amplified products the expected weight for Kir2.1 and Kv1.3 in J774.A1 cells from mRNA (Figure 2).

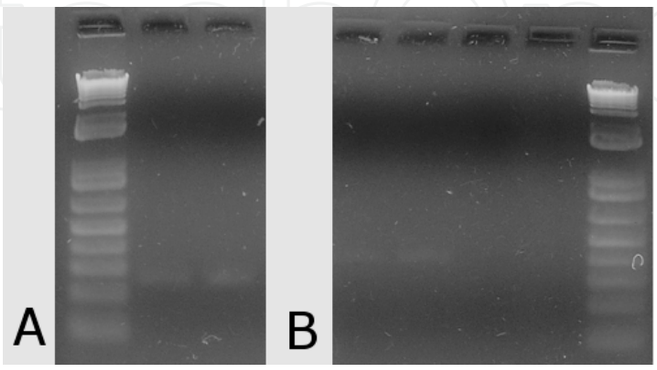

Fig. 2. Transcripts of ion channels in J774.A1. A. Transcripts of the inward rectifier potassium channel Kir2.1. B. Transcripts of the voltage gated potassium ion channel Kv1.3. Primers and PCR conditions are as reported by Vicente et al., (2003).

TNF-α stimulation alters expression of different Kvβ, inducing more inactivation of K^+ I_{OUT} but less expression to the plasma membrane of Kv1.3 after 24 hours compared to LPS stimulation (Vicente et al., 2005). In the mouse macrophage cell line RAW 264.7, there is evidence of transcription, protein expression, protein localization, and functional expression of Kv1.3 and Kv1.5 (Vicente et al., 2006; Villalonga et al., 2007). RAW 264.7 has K^+ I_{OUT} of smaller amplitude, expresses more Kv1.5 and therefore has less sensitivity to margatoxin (Vicente et al., 2006). Stimulation with LPS of this line resulted in higher transcription and protein expression and increases in Kv1.3 K^+ I_{OUT}, in contrast to the downregulation in transcription and protein expression and lower K^+ I_{OUT} inactivation after exposure to the immunosuppressor agent dexamethasone (Villalonga et al., 2010). However, it has been suggested that, by inducing Kv1.3 and repressing Kir2.1 after activation, macrophages reduce Ca^{2+} driving force and intracellular K^+ concentration (Vicente et al., 2003). In the proposed model of the T lymphocyte, the influx of Ca^{2+} after activation depolarizes the membrane and reduces the electrical driving force in the absence of a counterbalancing cation flux, suggesting that Kv1.3 is involved in maintenance of Ca^{2+} entry (Panyi et al., 2004). A biphasic intracellular increase of calcium has been observed in mouse macrophages. The initial transient is attributed to release of Ca^{2+} from endoplasmic reticulum, and the second transient, with less amplitude but longer time course, to Ca^{2+} influx through the plasma membrane (Randriamampita et al., 1991). In TRPV2 (Transient Receptor Potential Vanilloid 2) knockout mice, this cation channel generates a biphasic entry of Ca^{2+}, with a rapid initial transient followed by a sustained increase. The lack of this channel alters macrophage phagocytosis (Link et al., 2010). We have seen evidence of higher Ca^{2+} concentration in activated macrophage-like cells (Camacho, unpublished data) in which the Vm measured was less than in control cells, suggesting depolarization (Holevinsky & Nelson, 1995; Camacho et al., 2008). The currents show increased K^+ I_{OUT} and lowered I_{KIR} (Camacho et al., 2008), similar to the description by Vicente et al., 2003. Thus far unavailable simultaneous measurements of calcium and other potential currents could bolster support for this hypothesis, but a proposal in macrophages similar to that of T lymphocytes is more coherent. Thus, some of the functional stages of the macrophage (phagocytosis, classical activation) generate an increase in intracellular Ca^{2+}. The entry of this divalent cation depolarizes the membrane, inducing gating of Kv1.3 channels and counterbalancing Ca^{2+} entry, thereby maintaining the driving force for a sustained response.

We have studied the impact of *Leishmania* infection on the electrical properties of infected macrophage-like cells using the classical whole cell configuration of the patch clamp technique (Hamill et al., 1981). The model initially chosen was infection of J774.A1 by *L. amazonensis* because this parasite induces a large PV that is easily recognised in a light microscope (Figure 1A). The pipette solution included ATP and GTP to maintain stable currents, particularly I_{KIR}, and glutamate was the predominant anion (Forero et al., 1999; Camacho et al., 2008; Quintana et al., 2010). The solution flow during pharmacological testing was carefully controlled to avoid rises of the total I_{OUT} (Randriamampita & Trautmann, 1987). The recordings were easier at earlier times but seal stability was compromised with time post-infection.

Leishmania infection alters macrophage plasma membrane electrical properties and K^+ ion currents. However, there are differences between the state induced during the first hours post-infection and the state of an established infection of more than 24 hours. Macrophages were seen to depolarize during the first 12 hours post-infection. A decrease in Vm was

associated with a change in conductance and lower amplitude of I_{KIR} density, and a rise of I_{OUT} density (Quintana et al., 2009). Reductions of Cm and depolarization, with similar ion current density, were found in macrophages after phagocytosis of latex beads (Quintana et al., 2009), suggesting that the changes observed during early *Leishmania* infection are associated with phagocytosis. In contrast, we have shown that an established infection with *L. amazonensis* of non-activated J774A.1 is associated with increased I_{KIR} density (Forero et al., 1999) and a rise in K^+ I_{OUT} (Camacho et al., 2008), which would tend to counter activation (Vicente et al., 2003) and would be consistent with observed suppression of activation by *Leishmania* infection (Liew et al., 1998). There is evidence that depolarization is associated with less I_{KIR} and a rise in K^+ I_{OUT}, leading to hyperpolarization (Randriamampita & Trautmann, 1987). The depolarization during early infection (Quintana et al., 2010) can be explained by lower I_{KIR}, and the hyperpolarization observed in established infection is explained by higher K^+ I_{OUT} (Camacho et al., 2008).

Scott et al., 2003, working with macrophages stimulated with LPS and INF-γ, have shown that *Leishmania major* infection, or treatment with a wide range of K^+ channel blockers, suppresses NO production, consistent with a role for K^+ currents in the deactivation effect of *Leishmania*. We found no differences between non-activated control and infected macrophages in either the K^+ I_{OUT} density, its Iss/Ip ratio over the period studied, or susceptibility to 4-AP inhibition. However, the K^+ I_{OUT} time to peak and sensitivity to TEA were altered by infection, suggesting greater contribution of some potassium channels to the I_{OUT} (Camacho et al., 2008). This is significant because alteration of K^+ currents may compromise the ability of the macrophage to phagocytose, to be activated (Randriamampita & Trautmann, 1989; Buchmüller-Rouiller & Mauël, 1991; Fischer & Eder, 1995; Holevinsky & Nelson, 1995; Vicente et al., 2003; Villalonga et al., 2010), to present antigen (McKinney & Gallin, 1992), and to do transmigration (Gendelman et al., 2009). In particular, inhibition of K^+ I_{OUT} has been associated with reduced phagocytic ability (Hara et al., 1990) and less NO production (Scott et al., 2003; Vicente et al., 2003), suggesting that these currents may be critical in control of infections in macrophages.

Plasma membrane hyperpolarization has been associated with calcium replenishment, production of oxygen radicals (Gamaley et al., 1998; Hattori et al., 2003; Hanley et al., 2004), myoblast membrane fusion (Liu et al., 1998), and protection against apoptosis (Dallaporta et al., 1999; Liu et al., 2005) as well as *Leishmania* infection (Forero et al., 1999; Fajardo et al., 2007). *Leishmania* infection raises macrophage intracellular calcium (Eilam et al., 1985; Olivier, 1996), and promotes production of oxygen radicals (Sousa-Franco et al., 2005). Fusion to form giant cells is a physiological response in macrophages (Vignery, 2005; McNally & Anderson, 2005; Cui et al., 2006) and proceeds via mechanisms similar to those in phagocytosis and with the participation of ER membranes (McNally & Anderson 2005). There is evidence of protection against apoptosis in macrophages infected with *Leishmania* (Aga et al., 2002; Lisi et al., 2005). We found that 1 μM staurosporin induces apoptosis of J774A.1, but that infection with *Leishmania braziliensis* protects these cells against this agent. Macrophage apoptosis was accompanied by depolarization of the mitochondrial and macrophage plasma membrane (Clavijo et al., 2009). *L. amazonensis* and *L. braziliensis* infection induce macrophage plasma membrane hyperpolarization (Forero et al., 1999; Fajardo et al., 2005). The significance of this hyperpolarization is not clear, though it may contribute to macrophage deactivation. Interestingly, we have observed that the changes in volume of the PV are associated with this membrane potential change (Fajardo et al., 2005).

We intend to continue with the characterisation of macrophage membrane properties, to understand them in relation to the cell's repertoire of functional stages.

3. Parasitophorous vacuole membrane

Leishmania is an intracellular parasitic protozoon of macrophages and dendritic cells, confined in an endolysosomal compartment, the parasitophorous vacuole (PV). Assuming that the parasitophorous vacuole membrane (PVM) permits ion and nutrient exchange between the lumen of the PV and the infected cell cytoplasm, the PVM will be involved in parasite survival and replication. The majority of studies on the biogenesis and membrane composition of phagolysosomes have been made on the model of phagocytosis of latex beads by macrophages (Desjardins & Griffiths, 2003). These studies have shown highly regulated sequential acquisition into the phagosome of proteins from the plasma, endosomal and lysosomal membranes of the macrophage (Pitt et al., 1992; Desjardins et al., 1994a, 1994b; Desjardins, 1995; Andrews, 1995; Beron et al., 1995; Idone et al., 2008). The compartment is thereby acidified, and with concentrated hydrolytic activity becomes a microbicidal environment. Moreover, once the phagosome (PG) matures to a phagolysosome, enzymatic activity allows protein degradation of intracellular pathogens and coupling of small peptides for antigen presentation in the MCH-II context (Harding et al., 1995; Germain, 1995). The correct presentation is vital to orchestrate the immune response. *Leishmania* delays PG maturation and phagolysosomal formation (Burchmore & Barrett, 2001). This delay is associated with lipophosphoglycan (LPG), a component of the complex parasite glycocalyx. It has been proposed that LPG disorganises PVM lipid domains and interferes with recruitment of synaptotagmin V, a regulator of the exocytic pathway, delaying the arrival of cathepsin F and V-Type H^+ pumps to the PVM (Vinet et al., 2009). The consequence is altered PV enzymatic activity and acidification. Furthermore, *Leishmania* sequesters in the interface between the PVM and the parasite MCH-II molecules, altering antigen presentation (Antoine et al., 1998).

The permeability of the PVM depends on whether it interacts with the endocytic pathway of the host cell, which varies with different intracellular pathogens (Meirelles & De Souza, 1983). *Toxoplasma gondii* PV appears to be excluded from the phagosome maturation process within the endocytic pathway (Lingelbach & Joiner, 1998). Its PVM comes from host-cell membranes, plasma membrane, ER and mitochondria, in addition to membranes from specialized parasite organelles that appear to interfere with fusion of the PVM with endocytic components (Lingelbach & Joiner, 1998; Marti et al., 2007). Schwab et al., (1994) have documented bidirectional movement of charged and non-charged molecules of less than 1900 Da between the lumen of the *Toxoplasma* PV and the host-cell cytoplasm. It has been suggested that this permeability could be involved in nutrient transport, as proposed in *Plasmodium* where ion channel activity of small cationic and anionic molecules was recorded (Desai et al., 1993; Desai & Rosenberg, 1997). However, *Chlamydia trachomatis* PVM, located to the same level in the endocytic pathway as that of *Toxoplasma*, is able to exclude anionic molecules of low molecular weight (Heinzen & Hackstadt, 1997), suggesting differences in the transporter repertoire. The molecules described in the PVM of *T. gondii* and *Plasmodium* are poorly selective, similar to porin molecules. In the case of *Trypanosoma cruzi* such molecules are implicated in the lysis of the PVM that sets the parasite free into the host cell cytoplasm (Andrews, 1990). Porin-like molecules that induce ion currents (Noronha

et al., 2000) have been implicated in *Leishmania* exit from the macrophage (Horta, 1997; Aleida-Campos & Horta, 2000).

The *Leishmania* PV matures in the endosomal pathway to a late endo-lysosomal compartment and has features such as acidic pH, acid phosphatase and hydrolytic activity, and late endosomal and lysosomal membrane markers (Shepherd et al., 1983; Rabinovitch et al., 1985; Prina et al., 1990; Russell et al., 1992; Russell, 1995; Antoine et al., 1998). Despite this apparent hostility, the amastigote is adapted to the pH, and the PV could also be seen as a land of milk and honey since this compartment is rich in products from the degradation of sugars, proteins and nucleic acids (Prina et al., 1990; reviewed by Burchmore & Barrett, 2001).

The acidification of the PV is assumed to be the result of the V-Type H^+ pumps in charge of endosomal acidification (Sturgill-Koszycki et al., 1994; Lamb et al., 2009), with a contribution from the parasite metabolism. The pump function may be associated with cationic as well as anionic shunt currents (Harvey & Wieczorek, 1997; Grabe & Oster, 2001; Haggie et al., 2007; Carraro-Lacroix et al., 2009; Wienert et al., 2010; Steinberg et al., 2010; Dong et al., 2010) for proper function. In macrophages the presence of the V-Type H^+ pump is also vital for efficient phagocytosis (Gagnon et al., 2002) and macrophage fusion into giant cells (McNally & Anderson, 2005).

Leishmania PVM permeability depends on transporters present in the donating membranes: the macrophage plasma membrane (Antoine et al., 1998, Quintana et al., 2009), the endosomal membranes (Veras et al., 1992, 1994, 1995, 1996; Russell et al., 1992; Collins et al., 1997; Schaible et al., 1999; Cortázar et al., 2006), the parasite membrane (Henriques et al., 2003), membrane transporters from the endoplasmic reticulum (Gagnon et al., 2002; Becker et al., 2005; McNally & Anderson, 2005), and transporters from compartments that exchange membrane with the PVM, endosomes and autophagic vacuoles (Schaible et al., 1999). Thus, the possible membrane transporters present on the PVM include macrophage plasma membrane ion pumps and channels where K^+ channels (Randriamampita & Trautmann, 1987; Gallin, 1991; Nelson et al., 1992; Ichinose et al., 1992; McKinney & Gallin, 1992; Holevinsky & Nelson, 1995; DeCoursey et al., 1996; Eder et al., 1997; Forero et al., 1999; Scott et al., 2003; Vicente et al., 2003; Mackenzie et al., 2005; Vicente et al., 2006; Park et al., 2006; Hanley et al., 2004; Scheel et al., 2005; Camacho et al., 2008; Villalonga et al., 2010) and transporters of the ABC family (Di et al., 2006) are important. Transporters present in the macrophage intracellular membranes that could contribute to the PV are V-Type H^+ pumps (Lamb et al., 2009), ionic channels (Dong et al., 2010), H^+ channels (Grabe & Hoster, 2001), iron Nramp transporters (Hackam, 1998; Jabado, 2000; Gomez et al., 2007), voltage gated chloride ClC channel/H^+ transporters (Jentsch, 2007; Steinberg et al., 2010; Wienert et al., 2010), and ABC transporters (Russell et al., 1992; Cortázar et al., 2006; Di et al., 2006), not to mention the repertoire of ion channels and pumps present on the ER membrane. The conditions on the PVM in terms of membrane potential, pH and electrochemical driving force are different for transporters coming from the macrophage membrane, but relatively similar for those coming from intracellular membranes, suggesting that many of the latter are functional.

Several transporters have been found in the *Leishmania* PVM. The acidification of the compartment is associated with the activity of a V-Type H^+ pump (Sturgill-Koszycki et al., 1994). The distribution of anionic fluorescent probes of different sizes into *in situ* and isolated PVs suggests the expression of transporters of the ABC superfamily (Steinberg et

al., 1987; Russell et al., 1992; Cortázar et al., 2006), which could be involved in nutrient transport or could function as anionic shunts. There are also Narmp1 iron transporters that in some mouse models compromise *Leishmania* survival (Huynh & Andrews, 2008).

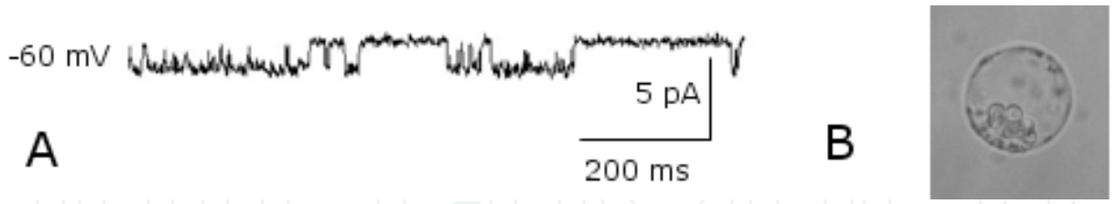

Fig. 3. Patch clamp recording on the parasitophorous vacuole membrane of *Leishmania amazonensis*. A. Inside-out single channel recording in symmetric solutions consisting of 150 mM KCl, 100 μM $CaCl_2$ and 20 mM HEPES, pH 7.2 in the presence of 50 μM DIDS. B. Light microscopy of an isolated PV. Note the parasites polarized to the membrane (Perez et al., 2009).

We have recorded ion currents through the PVM (Cortázar et al., 2006; Perez et al., 2009) in the giant PV induced by *L. amazonensis* in J774.A1 using the PV-attached configuration of the patch clamp technique (Hamill et al., 1981). Isolation of the PV can be achieved by rupturing macrophages with hypotonic solutions and mechanical force (Chakraborty et al., 1994). We substituted a hypotonic solution for an isotonic one in the presence of protein inhibitors and mechanical force (Cortázar et al., 2006), because acidic pH and probenecid-sensitive Lucifer yellow load were better maintained. Though PV purification can be achieved with either sucrose (Chakraborty et al., 1994) or Percoll gradients (Cortázar et al., 2006), we chose the latter method as in our experience the PV deteriorates in presence of sucrose gradients, most probably due to high osmotic pressure. The purified PV maintains low pH and ABC transport activity (Cortázar et al., 2006), though washout of excess Percoll from the PVM has proved difficult, particularly in patch clamp experiments in which seal formation is compromised. We explored the use of differential centrifugation and more recently Split Flow Lateral Transport Thin Cell Fractionation (SPLITT) techniques to enrich the PV fraction, avoiding the problem of further centrifugation and wash (Perez et al., 2007) and preserving the PVM for electrophysiological recording. We used bath solutions with high Na^+ as well as high K^+, with relatively high calcium, and with high Na^+ in the pipette, and recorded a current with 46 pS conductance in the PV-attached configuration (Cortázar et al., 2006). In high symmetrical K^+ in the PV-attached configuration there are bursts of anion currents induced by hyperpolarized potentials that are sensitive to broad range anion channel inhibitors but not to probenecid (Perez et al., 2009), ruling out the presence of some ABC transporters. In inside-out patches bathed in high K^+ and DIDS 50 μM, cationic single channel activity was recorded that was sensitive to broad range potassium channel blockers (Perez et al., 2009; Figure 3). We are now refining the electrophysiological characterization of these currents before determining the molecular nature of the transporters responsible for them.

4. *Leishmania* plasma membrane

Leishmania adaptation and proliferation during its life cycle demands appropriate regulatory mechanisms for parasite survival. During its life cycle the parasite encounters environmental differences in ion concentrations, osmolarity, pH, temperature and nutrient

availability, among others. Adaptation to some of these changes requires the expression of ion channels and transporters, and it has been suggested that the parasite will therefore have higher demands for energy (ter Kulle, 1993).

Leishmania relies on purine transport by specific transporters (Ogbunude & Dzimidi, 1993), has a biopterin transporter capable of also moving folate (Ouellette et al., 2002, Dridi et al., 2010), imports Fe by specific transporters (Huynh et al., 2006; Jacques et al., 2010) to warranty its replication within the PV, and is able to extrude drugs (Ouellette et al., 2001). Of particular importance in *Leishmania* is the presence of a homologue of the mammalian AQP9 aquaglyceroprotein (Figarella et al., 2007) associated with parasite resistance to one of the first line therapies (Gourbal, 2004; Figarella et al., 2007; Maharjan et al., 2008). LmAQP1 has been shown to contribute to parasite water and glycerol transport (Figarella et al., 2007). More recently a family of aquaporins was described in *Leishmania donovani*. They localise intracellularly, have differential expression, and can potentially contribute to parasite osmolarity regulation by transporting water because once transfected into yeast they alter the osmolarity properties of these cells (Biyani et al., 2011).

Molecules involved in nutrient uptake are found in *Trypanosomatidae* (Glaser & Mukkada, 1992; ter Kulle, 1993; Vieira et al., 1996; Tetaud et al., 1994). Transport of D-glucose and D-proline is mediated by H^+ symporters in *Leishmania* (Zilberstein & Dwyer, 1985; Zilberstein, 1993). The transport of 2-deoxy-D-glucose was used as a measure of D-glucose and it was shown that *Leishmania donovani* secondarily transports glucose. Glucose can be concentrated to a level of about 80 times the extracellular concentration by an electrochemical gradient of protons (Zilberstein & Dwyer, 1985). Sucrose is similarly concentrated by a sucrose/H^+ symporter in promastigotes of *L. donovani* (Singh & Mandal, 2011) and forms a potential source of nutrients relevant for this form living in the mosquito gut. Arginine is an essential amino acid for *Leishmania. Leishmania donovani* LdAAP3 permease has highly specific arginine transport activity in *Saccharomyces cerevisiae,* optimal at low pH, and when coupled to GFP localises to the *Leishmania* plasma membrane (Shaked-Mishan et al., 2006). Lysine is an essential amino acid of many eukaryotes including *Leishmania*. Unlike mammalian cells, *Leishmania* has independent permeases for arginine and lysine. LdAAP7 expressed in *S. cerevisiae* transports lysine, but when over-expressed in promastigotes has no impact on the uptake of this amino acid (Inbar et al., 2010). LdAA7 is essential for parasite survival and localises to the promastigote plasma membrane and flagella (Inbar et al., 2010).

Leishmania as other eukaryotes is capable of maintaining lower levels of cytoplasmic Ca^{2+}. The parasite stores this ion in the usual compartments, the ER and mitochondria (Philosoph & Zilberstein, 1989), but also in specialized vesicles, the acidocalcisomes (Lu et al., 1997), which are important during parasite invasion, differentiation and replication (Moreno & Docampo, 2003). Evidence suggests that calmodulin (Moreno & Docampo, 2003) and calcium ATPases are present in the ER membrane (Philosoph & Zilberstein, 1989) and in the *Leishmania* plasma membrane (Mandal et al., 1997; Corte-Real et al., 1995), where they will pump calcium out of the parasite cytoplasm. In addition to controlling calcium, *Leishmania* controls volume and osmolarity. The parasite transits from the insect gut, where fluctuations in osmolarity are expected, to the PV, which is believed to be hypo-osmotic compared to the macrophage cytoplasm (LeFurgey et al., 2005). In hypotonic solutions, water enters *Leishmania,* increasing its volume, which is later restored to normal levels. The recovery is associated with extrusion of ions, saccharides and amino acids (Darling et al., 1990;

LeFurgey et al., 2005), as well as with minor changes of K^+ and efflux of alanine through a DIDS sensitive transporter that alters Vm (Vieira et al., 1996). This latter transporter possesses ion channel kinetics (Vieria et al., 1996) and is regulated by protein kinases A and C and arachidonic acid (Vieira et al., 1997). In addition to acidocalcisomes, other intracellular organelles may be involved, by moving Na^+ and Cl^- (LeFurgey et al., 2001). *Leishmania* also maintains a large pool of free intracellular amino acids that appears to help the parasite to respond to osmolarity changes (Shaked-Mishan et al., 2006).

Ion and nutrient transport are fundamental in all cells for function and survival. Cells build up ion gradients by investing energy, generating an electrochemical force that when dissipated allows secondary transport of ions, sugars and amino acids and indirect regulation of volume, pH and osmolarity. In eukaryotic cells the pumps that acidify in the endocytic pathway are V-Type (Lamb et al., 2009). H^+ concentration induces a proton motive force that opposes further acidification. Cationic and anionic shunt currents present in the endocytic membranes counteract the effect of this force on pump function (Harvey & Wieczorek, 1997; Graben & Oster, 2001; Scheel et al., 2005; Carraro-Lacroix et al., 2009; Wienert et al., 2010; Steinberg et al., 2010; Dong et al., 2010). Recent evidence suggests that anion entry may not be required for maintenance of the electromotive force for pump functioning and, surprisingly, that the H^+ gradient may serve to concentrate Cl^- that might be important in the progression in the endocytic pathway (Novarino et al., 2010). The maintenance of intracellular pH within the physiological range is important in this parasite, particularly when adapting to the low pH of the PV (Antoine et al., 1990). The acidification of the PV results from V-Type H^+ pump activity (Sturgill-Koszycki et al., 1994) coupled to anionic or cationic currents. In macrophages, the currents generated by chloride channels CFRT (Di et al., 2006) and ClC/H^+ transporters (Steinberg et al., 2010) are the candidate anion current shunts.

Leishmania maintains an intracellular pH that ranges between 6.4 and 6.8 (Vieira et al., 1994; Marchesini & Docampo, 2002) independent of the environmental pH. Amastigotes show tighter pH regulation compared to promastigotes (Marchesini & Docampo, 2002). In promastigotes, the insect form, a proton electrochemical gradient drives secondary transport of D-glucose and D-proline (Zilberstein & Dwyer, 1985); this gradient is built up by the activity of P-type K^+/H^+ pumps that are Mg^{2+} dependent and orthovanadate sensitive (Jiang et al., 1994; Mukherjee et al., 2001; Burchmore & Barrett, 2001). This pump indirectly effects pH regulation by keeping the chemiosmotic energy constant (Zilberstein, 1993). Two P-Type H^+ pumps have been cloned with differential expression between amastigotes and promastigotes (Meade et al., 1987), one of which is expressed on the parasite plasma membrane (Anderson & Mukkada, 1994; Marchesini & Docampo, 2002). Functional complementation of *S. cerevisiae* with the putative P-Type H^+ pumps LDH1A and LDH1B induces an electrochemical gradient and allows survival of this yeast at low pH (Grigore & Meade, 2006).

Other pumps and transporters are also found in *Leishmania* membranes. A different H^+ pump has been suggested in *Leishmania* pH regulation. The use of DCCD has implicated H^+ pump activity in parasite pH control. It has been suggested that, in *Leishmania major* promastigotes, a H^+ pump and a Cl^- channel contribute to acid secretion and to the maintenance of a hyperpolarized membrane potential (Vieira et al., 1994; Vieira et al., 1995). These authors refer to the inhibition of a H^+ pump by the use of DCCD, which is more

compatible with the V-PP-H^+ pump (vacuolar proton-pumping pyrophosphatase) described in plants and protozoa. The V-PP-H^+ pump activity of digitonin-permeabilized *L. donovani* promastigotes is K^+ dependent, and inhibited by Na^+, DCCD and N-ethylmaleimide, but not by vanadate (a P-Type H^+ pump inhibitor) or bafilomycin-A (a V-Type H^+ pump inhibitor). The pump localises to subfractions of electron-dense organelles with contents similar to acidocalcisomes, and it is suggested that it acts to degrade cytosolic pyrophosphatase (Rodrigues et al., 1999). Docampos´ group later described, in amastigotes of *L. amazonensis*, a H^+ pump sensitive to DCCD and N-ethylmaleimide but not to bafilomycin-A; based on its apparent K^+ independence, they concluded it has P-Type H^+ pump activity. The positive labelling of the parasite plasma membrane with antibodies against the TcHA2 of *Trypanosoma*, that recognises LDH1 in promastigotes and amastigotes, suggests that the H^+ pump seen by them is the P-Type H^+ pump described by Zilberstein´s group and cloned by Meade's group, but the activity reported corresponds to the V-PP-H^+ pump described by Rodrigues et al., 1999. Besides the two type pumps, a Na^+/K^+ pump has been suggested on the *Leishmania mexicana* plasma membrane based on ouabain sensitivity (Felibertt et al., 1995). Also P-type K^+ and a Na^+ pumps (Stiles et al., 2003), Na^+/H^+ exchanger (Vercesi et al., 2000) and Mg^{2+}/H^+ pump activities were found in *L. donovani*. The antiporter accumulates H^+ in everted vesicles in the presence of ATP and Mg^{2+}, releases it when exposed to FCCF, and is unable to transport Rb^+, therefore implicating Mg^{2+} as the co-transported ion (Mukherjee et al., 2001).

Leishmania Vm has been measured in promastigotes and amastigotes. Very hyperpolarized Vm values are found (-113 mV; Vieira et al., 1995; Marchesini & Docampo, 2002) after bisoxonol distribution in promastigotes, and less hyperpolarized Vm are found in amastigotes (-75 mV; Marchesini & Docampo, 2002). The activity of DCCD on the electrochemical gradient created by H^+ pumps coupled to DIDS sensitive anion transporters have been implicated in the promastigote Vm (Vieira et al., 1995; Marchesini & Docampo 2002) and Vieira et al., (1995), concluded that there is no contribution from K^+ or Na^+ to the *Leishmania* Vm, despite previous data where distribution of tetraphenylphosphonium bromide points to K^+ (Glaser et al., 1992) as in other eukaryotes. Again the presumed DCCD-sensitive H^+ pump activity was shown in intracellular compartments of *Leishmania* promastigotes, but the proposed model couples its function to DIDS sensitive transporters on the parasite plasma membrane.

Direct measure of an anion conductance was recorded in plasma membranes of *Leishmania mexicana* reconstituted in lipid planar bilayers (DiFranco et al., 1995). These authors described two anionic currents but suggested the presence of other channels. Their finding is consistent with *Leishmania* anion fluxes involved in parasite survival (Ponte-Sucre et al., 1998) and electron transport by redox enzymes (Bera et al., 2005). We have been interested in the study of ion channels of *Leishmania*, with a particular emphasis on chloride channels, assuming that these molecules are relevant in parasite survival and are an adaptation to the acidic pH of the PV. Direct recordings on the parasite have not been possible due to its small size, shape, movement, and the presence of a complex glycocalyx on its surface that interferes with seal formation. To overcome this difficulty we have used amphibian oocytes to express total mRNA from *Leishmania*. In combination with the model of *Xenopus* oocytes (Stühmer, 1992) we have shown that other species are capable of expressing ion channels efficiently (*Bufo marinus*). However, we have encountered some difficulties in both models in particular low oocyte production and viability compared to other reports (Chaves et al.,

2003). We attribute these to reduced partial oxygen pressure due to the altitude of Bogotá (2600 m above sea level) where the laboratory is located, because control of temperature and hormone replacement did not improve production or oocyte viability (Arroyo & Camacho, 2006). Despite these difficulties it is possible to record the expected potassium currents after injection of cRNA of the ion channel Kv1.1 (Vargas et al., 2004).

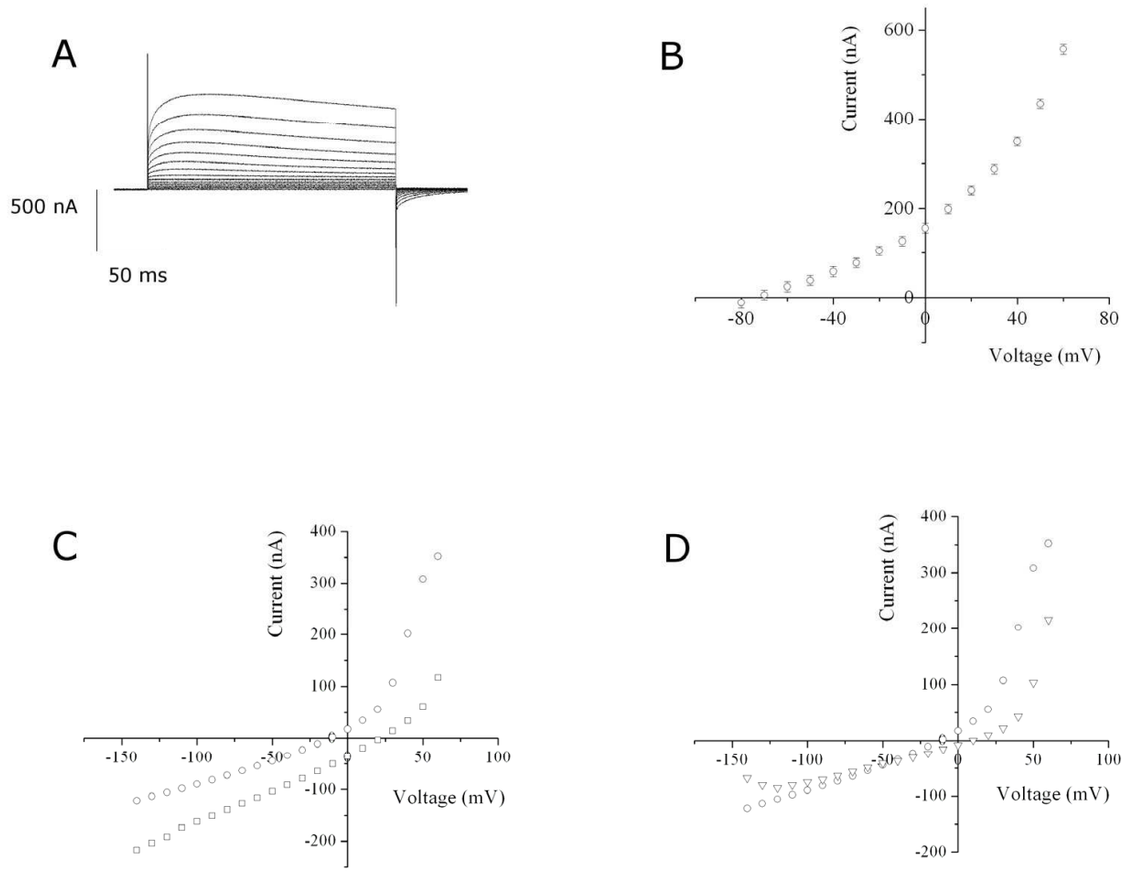

Fig. 4. Two electrode voltage clamp currents of *Bufo marinus* oocytes. A. A representative current recording. Whole cell membrane currents were recorded from a holding potential of -80 mV. Pulses were applied with 10 mV steps during 0.5 seconds. B. Mean I/V curves of control oocytes. C. Inhibition of control currents in the presence of DIDS. D. Inhibition of control currents in the presence of EGTA. Mean current of control oocytes, (□) DIDS and (∇) EGTA (Arroyo, 2005).

B. marinus can be kept in captivity at intermediate altitudes (e.g. in Bogotá) in controlled temperature environments and 12 hour light cycles. They can be fed with insects or earthworms, and oocyte production can be induced with gonadotropic hormone (Arroyo & Camacho, 2006). The basal oocyte ion currents of *B. marinus* recorded with two-electrode voltage clamp are mainly due to chloride. The reversal potential of the currents is close to the chloride equilibrium potential, which becomes more negative in chloride-free medium, suggesting that potassium permeability is also important (Figure 4). The currents are sensitive to broad anion inhibitors of these channels such as DIDS, which caused the

inhibition (Figure 4C). These currents are also calcium dependent because recording in the presence of the chelator EGTA reduces their current amplitude (Figure 4D). The electrophysiological properties found resemble those of the currents described for *X. laevis* oocytes (Miledi et al., 1989).

Using *X. laevis* and *B. marinus* oocytes we have studied the ion currents induced after injection of polyA mRNA of *L. amazonensis* (Arroyo, 2005; Lagos et al., 2007) and *Leishmania braziliensis* (Garzon et al., 2009). We recorded three types of chloride currents, one of which (Type 3) appears to be specific to parasite mRNA injection. Type 3 currents were characterized by a slow activation to a stationary level, without any inactivation. Reduction of extracellular Cl^- resulted in an altered current, indicating that the current was mediated by chloride ions. Substitution of extracellular Cl^- by other halogens indicated a relative anion permeability sequence $I^->Br^->Cl^-$. Negative results with the broad spectrum K^+ channel inhibitors TEA and 4-AP eliminated the possibility of contribution of K^+ currents. Conversely, these currents showed sensitivity to the broad spectrum Cl^- channel blockers DIDS and niflumic acid; one-third of the current was blocked by these two inhibitors. Reduction of extracellular divalent cations using a nominally Ca^{2+}-free solution with 1 mM EGTA resulted in only a 10% decrease of the amplitude of this current (Lagos et al., 2007). There are several classes of chloride channels and three molecularly distinct chloride channel families (ClC, CFTR and ligand-gated GABA and glycine receptors; Jentsch et al., 2005). In animal cells, the activity of V-Type H^+ pumps in endosomal compartments is associated with ClC chloride channel activity.

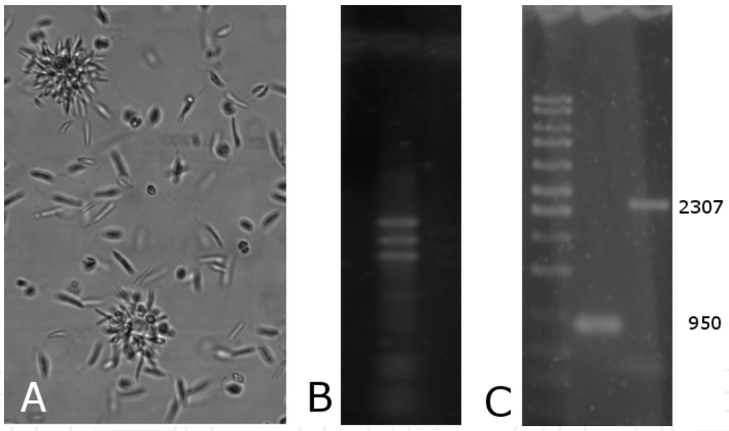

Fig. 5. Transcription of the putative chloride channel LbrM01 V2.0210. A. Promastigotes of *Leishmania braziliensis*. Note typical rosettes. B. Total RNA of *Leishmania*. A triple band of ribosomal RNA is found and is typical of this parasite. Agarose gel of a partial and total product of the putative ClC channel (Lozano et al., 2009).

The *Leishmania* amastigote inside the PV is constantly flooded by protons, and ClC channel/H^+ transporter expression will contribute to efflux of H^+, alleviating pH changes, and may concentrate Cl^- or other relevant anions. With the information available on the *L. braziliensis* genome we have identified four potential putative genes for chloride channels, one of which is classified as ClC. We have recently cloned the putative ClC channel of *L. braziliensis* (Lozano et al., 2009; Figure 5) which is homologous to intracellular mammalian ClC channels, some of which are co-transporters of Cl^-/H^+. We have inserted this putative channel into an expression

vector and recorded HEK293 cells with patch clamp in the whole-cell configuration after transfection of LbrM01 V2.0210. A Cl^- current 10 times the amplitude of control currents was recorded (Lozano et al., 2011). We are further characterising this channel and hope to understand its importance for *Leishmania* physiology.

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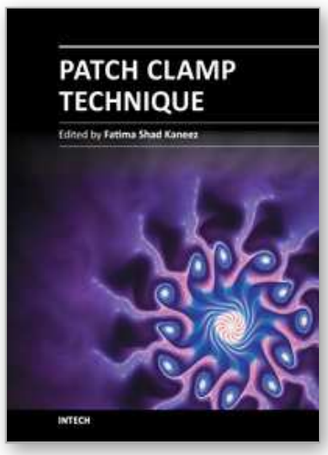

Patch Clamp Technique
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This book is a stimulating and interesting addition to the collected works on Patch clamp technique. Patch Clamping is an electrophysiological technique, which measures the electric current generated by a living cell, due to the movement of ions through the protein channels present in the cell membrane. The technique was developed by two German scientists, Erwin Neher and Bert Sakmann, who received the Nobel Prize in 1991 in Physiology for this innovative work. Patch clamp technique is used for measuring drug effect against a series of diseases and to find out the mechanism of diseases in animals and plants. It is also most useful in finding out the structure function activities of compounds and drugs, and most leading pharmaceutical companies used this technique for their drugs before bringing them for clinical trial. This book deals with the understanding of endogenous mechanisms of cells and their receptors as well as advantages of using this technique. It covers the basic principles and preparation types and also deals with the latest developments in the traditional patch clamp technique. Some chapters in this book take the technique to a next level of modulation and novel approach. This book will be of good value for students of physiology, neuroscience, cell biology and biophysics.

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