

Leishmania sp.: Growth and Survival Are Impaired by Ion Channel Blockers

Alicia Ponte-Sucre,* Yelitza Campos,† Marisol Fernandez,† Heidrun Moll,‡
and Alexis Mendoza-León†¹

*Laboratory of Molecular Physiology, I.M.E., Facultad de Medicina, and †Laboratory of Biochemical and Molecular Biology of Parasites, I.B.E., Facultad de Ciencias, Universidad Central de Venezuela, Caracas, Venezuela; and ‡Zentrum für Infektionsforschung, University of Würzburg, Germany

Ponte-Sucre, A., Campos, Y., Fernandez, M., Moll, H., and Mendoza-León, A. 1998. *Leishmania* sp.: Growth and survival are impaired by ion channel blockers. *Experimental Parasitology* **88**, 11–19. In the present work we examined the effect of ion transport blockers on the growth and viability of *Leishmania* sp. and on the infection of macrophages by the parasite. 4-aminopyridine and glibenclamide block voltage-dependent and K⁺ ATP channels, respectively; amiloride is used to detect Na⁺ channels and Na⁺/H⁺ antiporters; and anthracene-9-carboxylic acid affects chloride channels. The EC₅₀ for promastigote cultures of three strains of the *Leishmania* subgenus, namely, *Leishmania* (*Leishmania*) NR, *Leishmania* (*Leishmania*) *amazonensis* LTB0016, and *Leishmania* (*Leishmania*) *major*, at their stationary phase of growth, were, respectively, 39, 46, and 464 μM for 4-aminopyridine; 7, 0.8, and 10 μM for glibenclamide and 66, 170, and 10 μM for anthracene-9-carboxylic acid. The amiloride EC₅₀ for NR was 264 μM and 10 μM for *L. (L.) major*, but was never reached for LTB0016. Higher concentrations of the drugs impaired the exponential growth of *Leishmania* promastigotes. These results suggest the susceptibility of *Leishmania* sp. to blockers associated with K⁺ and Cl⁻ and to Na⁺ or Na⁺/H⁺ transport systems. Blockade of such systems might have impaired the survival of the parasites as promastigotes. In addition, it affected the persistence of parasites in host cells. Although the infection of the macrophage cell line J774 and peritoneal-exudate macrophages was not significantly decreased by concentrations of the drugs around the promastigotes' EC₅₀, the survival of intracellular parasites decreased significantly in the presence of these drugs without affecting the viability of the macrophages. Some blockers consistently gave small EC₅₀ and significantly decreased the infection process as well as the survival of intracellular parasites. Thus, elucidation of their mechanism of action in *Leishmania* is relevant, since they could represent a potential subject for the development of leishmanicidal drugs. © 1998 Academic Press

Index Descriptors and Abbreviations: BEL21, *Leishmania* (*Leishmania*) *mexicana*; PH8C5, *Leishmania* (*Leishmania*) *amazonensis*; LTB0016, *Leishmania* (*Leishmania*) *amazonensis*; JAP78, *Leishmania* (*Leishmania*) *garnhami*; *L. (L.) major*, *Leishmania* (*Leishmania*) *majo*.; NR, *Leishmania* (*Leishmania*) NR; M2903, *Leishmania* (*Viannia*) *braziliensis*; protozoa; parasitic; 4-AP, 4-aminopyridine; GLIB, glibenclamide; AMIL, amiloride; 9-CA, anthracene-9-carboxylic acid; ABC, Adenine Nucleotide Binding cassette; ion channel blockers; RPMI, culture medium RPMI; FCS, fetal calf serum; J774, macrophage cell line J774; PEC, peritoneal exudate cells; GABA, gamma amino butyric acid; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; IFN_γ, interferon gamma; DNA, desoxyribonucleic acid; RFLP, restriction fragment length polymorphism.

INTRODUCTION

The World Health Organization estimates that there are 12 million cases of leishmaniasis worldwide, with over 400,000 new cases every year (Olliaro and Bryceson, 1993). Although improvements have been made on the diagnosis of leishmaniasis and efforts to develop successful immunotherapy and chemotherapy are constant, the exponential increase of primary and secondary resistance of the parasitic strains to the classic chemotherapy stresses the necessity of precise methods for the accurate identification of the strains involved, the understanding of the host-parasite interactions, and the rational design of new and effective compounds with a better therapeutic index.

¹To whom correspondence and reprint requests should be addressed at Laboratory of Biochemical and Molecular Biology of Parasites, Instituto de Biología Experimental, Facultad de Ciencias, U.C.V., Apdo. 47577, Caracas 1041-A, Venezuela. Fax: (58)(2)(753.5897).

During its life cycle, *Leishmania* switches from invertebrate to vertebrate hosts in a series of obligatory developmental stages. The parasite's tolerance and probably various unknown mechanisms guarantee its survival in hostile environments characterized by various stress conditions such as differences in pH, temperature, osmolarity, nutrients, and serum components (Zilberstein and Shapira, 1994).

It has been documented that the intrinsic membrane proteins involved in the building of ion transporters are ubiquitous and found in the plasma membrane of excitable and nonexcitable cells (Chen and Hess, 1987; Hille, 1989). In multicellular as well as unicellular parasites such as *Ascaris suum* and *Plasmodium*, GABA-operated chloride channels, nutrient (lysine and gluconate) permeable channels, and Na^+/H^+ antiports have been defined electrophysiologically (Desai *et al.* 1993; Rheinallt *et al.* 1991; Bosia *et al.* 1993). In Trypanosomatidae, the best characterized studies on the role of the plasma membrane transport proteins in the physiology of the parasite are those related to the capacity of the cell to adapt to widely different extracellular pH values, through a surface membrane H^+ translocator ATPase (Bakker-Grunwald, 1992; Zilberstein and Dwyer, 1988); the maintenance of intracellular Ca^{2+} concentrations through Ca^{2+} buffering systems such as calmodulin $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPases or respiratory-dependent and independent intracellular Ca^{2+} transport systems (Benaim *et al.* 1991; Philosoph and Zilberstein, 1989), or the uptake of nutrients such as proline and glucose, by proton motive force-driven systems (Glaser and Mukkada, 1992; Zilberstein and Dwyer, 1985). Direct, as well as indirect data suggest the presence in *Leishmania sp.* of K^+ as well as anionic channels (Blum, 1992; DiFranco *et al.* 1995); their existence is further sustained because the surface membrane H^+ -ATPase is responsible only partially for the plasma membrane potential (Glaser *et al.* 1992). On the other hand, the identification of *P*-glycoproteins, members of the Adenine Nucleotide Binding Cassette (ABC) transporters have also been reported in *Leishmania* (Ouellette *et al.* 1990; Callahan and Beverly, 1991); their importance is evidenced by the fact that they can extrude drugs against concentration gradients, thus decreasing the intracellular drug concentration (Borst and Ouellette, 1995).

One approach for analyzing the presence of ion channels in diverse systems has been the use of specific toxins and inhibitors of the function of these proteins. Thus, voltage-dependent and voltage-independent K^+ -gated channels have been detected by the use of 4-aminopyridine (Castle *et al.* 1989); or glibenclamide (Schmid-Antomarchi *et al.* 1987); amiloride is used to detect both Na^+ channels and Na^+/H^+

antiporters (Dale, 1982; Bosia *et al.* 1993); and anthracene-9-carboxylic acid is used as a chloride channel inhibitor (DeLuca *et al.* 1990).

Using these classical ion transport blockers we investigated and demonstrated for the first time the susceptibility of *Leishmania* parasites, either cultured as promastigotes or hosted by macrophages of the cell line J774 or peritoneal exudate macrophages, to specific ion channel blockers. As the drug concentrations which affected intracellular *Leishmania* did not affect the viability of the host cell, the elucidation of the mechanism of action of these drugs could be relevant for the development of alternative chemotherapeutics with a better therapeutic index.

MATERIALS AND METHODS

Chemicals. All the drugs were obtained from Research Biochemicals International. 4-AP was dissolved in deionized water to 0.5 M and diluted in Schneider's *Drosophila* or RPMI medium to reach a concentration range between 10^{-9} and 10^{-2} M. 9-CA and AMIL were dissolved in DMSO to 0.1 M and diluted as above, to reach concentrations between 10^{-6} and 10^{-3} M. GLIB was dissolved in ethanol to 4×10^{-3} M as mentioned and diluted in Schneider's *Drosophila* or RPMI medium to reach a concentration range between 10^{-10} and 10^{-3} M. Control experiments done with the maximal DMSO and ethanol concentrations (0.001–0.025%) indicated that these solvent concentrations do not impair cell growth. As GLIB has an affinity for serum albumin of 96–98%, we have estimated the free drug concentration using the methodology described by Bennet *et al.* (1996).

Cell lines and cultures. All *Leishmania* strains used are listed in Table I. *Leishmania* NR is not a reference strain, it was originally isolated in 1980 from a Venezuelan patient with tegumentary diffuse leishmaniasis, and was kindly provided by Dr. Angel Hernández, Instituto de Biología Celular, Universidad Central de Venezuela. Therefore, it was important to identify this strain as a member of the *Leishmania* subgenus (Fig. 1). The *Leishmania* identification was carried out by restriction fragments length polymorphism (RFLP) of the β -tubulin gene region as previously described (Mendoza-León and Barker, 1996; Mendoza-León *et al.* 1995). Promastigotes of *Leishmania* strains were grown at 26°C in Schneider's *Drosophila* medium supplemented with 10% FCS, 20 $\mu\text{g ml}^{-1}$ of chloroamphenicol and 100 $\mu\text{g ml}^{-1}$ of ampicillin (Eresh *et al.* 1993; Mendoza-León *et al.* 1995). The origin and propagation of the *L. (L.) major* strain have been described elsewhere (Solbach *et al.* 1986). This strain was grown in blood agar.

The J774 cell line was maintained in RPMI medium supplemented with 10% FCS, 2 mM glutamine, 10 mM Hepes buffer, 100 $\mu\text{g ml}^{-1}$ penicillin, 160 $\mu\text{g ml}^{-1}$ gentamycin, 7.5% NaHCO_3 and 5×10^{-5} M 2-mercaptoethanol. For the preparation of the BALB/c PEC, thioglycolate-elicited peritoneal exudate cells were washed, resuspended in RPMI culture medium supplemented as above, and allowed to adhere

TABLE I
Designation of *Leishmania* Species

Subgenus	Species ^a	Strain designation ^b	Abbreviation	Origin
<i>Leishmania</i> (<i>L.</i>)	<i>mexicana</i>	MHOM/BZ/82/BEL21	BEL21	Belize
	<i>amazonensis</i>	IFLA/BR/67/PH8	PH8C5 ^c	Brazil
		MHOM/BR/77/LTB0016	LTB0016	Brazil
	<i>garnhami</i>	MHOM/VE/76/JAP78	JAP78	Venezuela
	<i>major</i> ^d	MHOM/IL/81		Israel
	<i>unknown</i> ^e	MHOM/VE/80/NR	NR	Venezuela
<i>Viannia</i> (<i>V.</i>)	<i>braziliensis</i>	MHOM/BR/75/M2903	M2903	Brazil

^a The nomenclature of *Leishmania* proposed by Lainson and Shaw (1987).

^b The majority of these strains have been designated as reference strains by WHO.

^c This is a cloned derivative of *L. (L.) amazonensis* PH8.

^d Solbach *et al.* (1986).

^e This Venezuelan strain has been identified as member of *Leishmania* subgenus.

for 4 hr at 37°C, 5% CO₂, and 95% air humidity. Nonadherent cells were removed by extensive washing with culture medium.

Drug effect during growth curve and stationary phase. The leishmanicidal activity of the compounds was studied with *Leishmania* promastigotes. To evaluate the capacity of the drugs to impair the exponential parasite growth, *Leishmania* were seeded at an initial density of 1.5×10^6 cells ml⁻¹ in the presence of a fixed concentration of each drug; their growth was then followed daily spectrophotometrically at 560 nm up to their stationary phase and compared to the growth of control vials seeded in parallel. To evaluate the capacity of the drugs to impair the survival of stationary phase *Leishmania*, the parasites were harvested at their stationary phase of growth and the cell suspension was adjusted to a density of 2×10^6 cells ml⁻¹ in a final volume of 0.1 ml of fresh Schneider's *Drosophila* medium. Aliquots of stationary phase *Leishmania* were then exposed to various concentrations of the drugs at room temperature for 2 or 4 hr. At the end of the incubation time, the number of alive cells was estimated in each of the experimental vials, compared to the number of alive cells of control vials incubated without drugs, and expressed as percentage of alive cells. To test the spontaneous death of the parasites, the viability was monitored at the beginning and at the end of the experiment in the control vials; this value never was higher than 1%. The viability of *Leishmania* was determined by a modification of a previously described procedure (Jackson *et al.* 1985). That is, at the end of the incubation time, the cells were washed twice in PBS, resuspended in the same buffer at the same density, stained for 2 min at 25°C with 125 µg ml⁻¹ fluorescein diacetate, and observed through a Nikon fluorescence microscope at 495 nm. To calculate the EC₅₀ the method of linear interpolation was used. This equation is commonly used in the field of parasitology and has been demonstrated to be valid for the calculation of the EC₅₀ (Huber and Koella, 1993).

Infection of macrophages. The leishmanicidal effect of the drugs was tested in cultures of the cell line J774 or BALB/c PEC. For these experiments, 2×10^5 macrophages ml⁻¹ were infected at a ratio parasites:macrophages of 5:1 with stationary phase promastigotes of *L. (L.) major*, in a final volume of 0.5 ml of RPMI culture medium.

Similar cultures were incubated with *Leishmania* only or with *Leishmania* plus 20 U ml⁻¹ of IFN_γ. At the ratio of 5:1 (*Leishmania*: macrophage), the macrophages were not extensively loaded with parasites. This was important because it prevented the massive dying of the infected macrophages due to the high parasite load. To ensure the survival of J774 and PEC, their sensitivity was explored at the same concentration range as for *Leishmania*. Thus, the viability of the macrophages at the drug concentrations tested was always greater than 96% (data not shown).

To evaluate the effect of the drugs on the process of infection of macrophages by *Leishmania*, the drugs were added to the macrophage-parasite culture at the beginning of an 18-hr incubation period. On the other hand, to explore the susceptibility of intracellular parasites to the drugs, the macrophage-parasite cultures were incubated without drugs for 18 hr; after removal of the excess of parasites by exhaustive washing with fresh RPMI culture medium, the drugs were added to the cultures which were then incubated for further 6 hr. Intracellular parasites were detected by a modification of a previously described procedure (Channon *et al.* 1984). That is, a mixture of acridine orange (0.005 mg ml⁻¹) and ethidium bromide (0.05 mg ml⁻¹) was added to the macrophage cultures, the plates were then placed for 10 min at room temperature, washed with PBS, fixed for 15 min with 1% paraformaldehyde prepared in PBS, and analyzed by fluorescence microscopy at 495 nm. In both types of experiments the frequency of infected macrophages and the ratio of alive vs dead parasites in infected macrophages were evaluated for each condition.

Statistical analysis of the data. In the promastigote culture assays the results are expressed as mean ± SEM of six replicas. The infection data are expressed as mean ± SEM of three to four replicas where at least 300 macrophages were analyzed. To express the significant decrease of macrophage infection, an index was calculated as described by Belosevic *et al.* (1988). Differences between the frequencies of infection of treated and control macrophages cultures were tested for statistical significance by the minimum χ² method (Scheffler, 1981). The alive vs dead data, obtained from the same experiments were analyzed by a one-way ANOVA analysis (Scheffler, 1981).

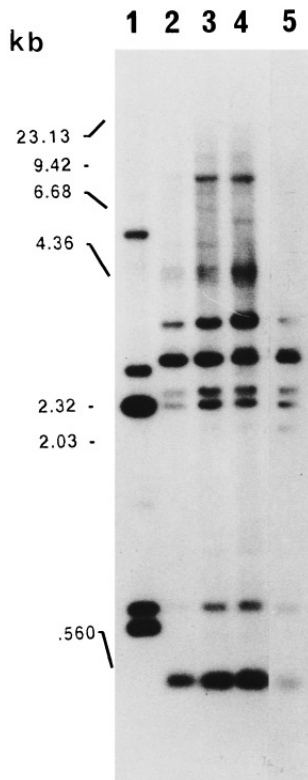


FIG. 1. *Leishmania* identification. Genomic DNA from New World *Leishmania* was digested with the endonuclease Sall, fractionated on 1% agarose gel, and transferred to a nylon membrane. The blot was hybridized at medium stringency conditions ($2\times$ SCC, 0.1% SDS $2\times$ Denhardt solution, $100\ \mu\text{g}/\text{ml}^{-1}$ calf thymus DNA, 67°C) to the *Leishmania* β -tubulin pLg β 4. *L. (V.) braziliensis* M2903 (Lane 1), *L. (L.) mexicana* BEL21 (lane 2), *L. (L.) garnhami* (Lane 4) and *L. (L.) amazonensis* PH8C5 (lane 5), were used to identify the Venezuelan *L. (L.)* strain NR (Lane 3). Molecular weight markers are from the HindIII digestions of DNA.

RESULTS

In order to evaluate the effect of ion transport blockers on the exponential growth of *Leishmania*, cultures of two *Leishmania* strains, NR and LTB0016, were exposed to concentrations of these drugs within the range known to affect these systems in other organisms (Fig. 2) (Castle *et al.* 1989; Dale, 1982). A concentration of 10 mM 4-AP impaired the growth of the cultured *Leishmania* from the beginning of the experiment. The effect of GLIB was evident at $16\ \mu\text{M}$ (as free drug) even at exponential phases of growth, a decrease of 50% occurred in both *Leishmania* strains. The drug 9-CA at 1 mM did not affect the growth of the NR strain, although

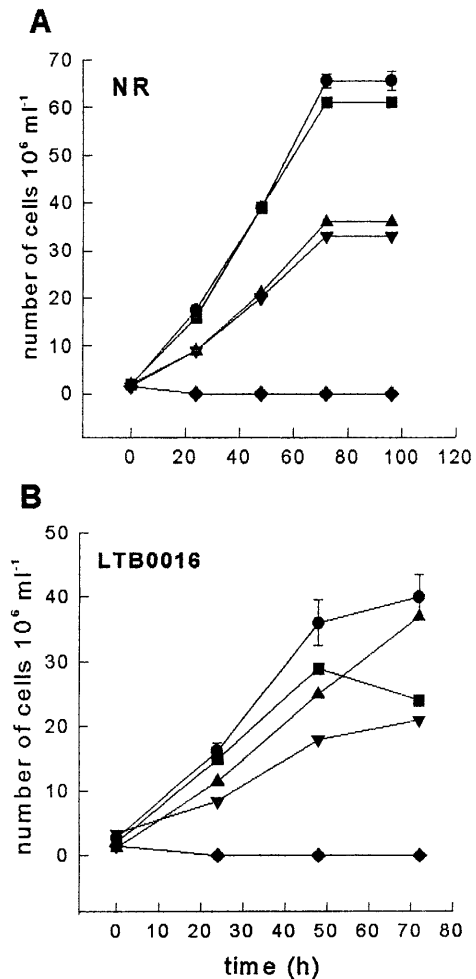


FIG. 2. Effect of ion channel blockers, 10 mM 4-AP (filled rhombus), 0.1 mM GLIB (filled upside down triangles), 1 mM 9-CA (filled squares), and 1 mM AMIL (filled triangles) on the growth curve of *Leishmania* sp. Data are presented as mean \pm SEM of 8 replicas. Filled circles, control cells.

a decrease of 40% was observed in the LTB0016. The drug AMIL at 1 mM impaired the growth of NR by 40%, but not of LTB0016. These results suggest that logarithmic *Leishmania* cells are sensitive to these ion channel blockers.

It has been demonstrated that the generation of infective or metacyclic promastigotes (metacylogenesis) in some species of *Leishmania* is associated with the stationary phase of growth (Sacks and Da Silva, 1987; Sacks, 1989). Since biochemical cell-surface changes are associated with this metacyclic form, we further evaluated the effect of ion channel blockers on stationary phase cultures. In preliminary experiments, stationary phase cultures of *Leishmania* exposed to high concentrations of 4-AP (10 mM), GLIB (16

μM), or 9-CA (1 mM) for 2 hr evidenced a decrease in viability of 70%; 4 hr were needed to evidence a decrease of 43–59% when AMIL (1 mM) was used. These incubation times were used to evaluate the effect of different concentrations of these drugs on the viability of stationary phase *Leishmania* cells. The dose-response curves for 4-AP showed an EC_{50} of 39 μM for NR, 46 μM for LTB0016, and 464 μM for *L. (L.) major*. The curves for GLIB for all the three strains are closely similar; the EC_{50} was 7 μM for NR, 0.8 μM for LTB0016, and 10 μM for *L. (L.) major*. The dose-response curves for 9-CA and AMIL show that the susceptibility among the three *Leishmania* strains to these drugs differed broadly. Different EC_{50} were found, and in the particular case of AMIL EC_{50} was never reached for the LTB0016 strain; at 1 mM, the maximal drug concentration assayed, the decrease in viability was 30%. A summary of EC_{50} obtained for each drug and for each strain is presented in Table II. The results presented herein suggest that stationary phase parasites are at least one order of magnitude more sensitive to the drugs than the parasites at exponential phases of growth and that stationary phase New World *Leishmania* promastigotes are more sensitive than *L. (L.) major* promastigotes to 4-AP and GLIB. Nevertheless, this last strain showed more susceptibility to 9-CA and AMIL than New World *Leishmania* promastigotes.

To further evaluate the potential effect of the ion transport blockers on the interaction of parasites with host cells, we studied their capacity to impair macrophage infection by *L. (L.) major*. The percentage of infection and the ratio of alive vs dead parasites were estimated in two series of experiments. To acknowledge if the drugs impair the entrance of

the parasites in the macrophages, the drug was added at the beginning of an 18-hr incubation of macrophage cells J774 or PEC with *Leishmania* parasites. There was a decrease in the percentage of infection which was significant for 3 μM GLIB in the cell line J774 and 10 μM AMIL in PEC (Table III) and which represents an impairment of infection of 64 and 39%, respectively. In the second series of experiments, and to evaluate if the drugs can interfere with the replication or survival of the intracellular parasites, the drugs were added when the macrophages were already infected by *Leishmania*. The decrease on the percentage of macrophages infected was significant in the cell line J774 for 4-AP (57%), GLIB (47%), and AMIL (41%) and for 4-AP (30%) and AMIL (41%) in the PEC (Table III). The effect of the drugs was also estimated by the ratio of alive vs dead parasites. This is an index of the survival of the parasites established inside the macrophage regardless of the percentage of infection found. This index, i.e., the survival of intracellular parasites, decreased significantly (82–97%) when the drugs were added at the beginning of the 18-hr incubation (Table IV). For established infections, the decrease in this ratio was also significant (72–96%) (Table IV). Thus, the drugs seem to decrease the percentage of infection more dramatically in an established infection and seem to impair the survival of intracellular parasites in both types of procedures without affecting the viability of the macrophages.

DISCUSSION

Ion transporters are essential for several physiological phenomena in all organisms including unicellular parasites (Bakker-Grunwald, 1992) and have been implicated in specific events such as drug resistance (Borst and Ouellette, 1995). In order to search for compounds that could affect the survival of *Leishmania* parasites, we have evaluated the efficacy of known inhibitors of ion channels on the viability of *Leishmania*.

During their life cycle parasites are subjected to different stresses; for instance, the intracellular environment within which amastigotes proliferate is acidic and imposes an homeostatic regulation which probably involves the participation of special ion channels or membrane elements related with them. Apparently, the susceptibility of *Leishmania* to ion channel blockers could be stage regulated since stationary phase *Leishmania* promastigotes, as well as intracellular amastigotes, have an increased susceptibility against these drugs compared to logarithmic phase promastigotes. We do

TABLE II
Effect of Ion Channel Blockers on the Viability of Stationary Phase Cultured *Leishmania*

Isolate	EC_{50} (μM)			
	4-AP	GLIB	9-CA	AMIL
NR	39 \pm 4.71	7 \pm 0.67	67 \pm 2.82	264 \pm 3.74
LTB0016	46 \pm 5.76	0.8 \pm 0.20	172 \pm 3.29	ND
<i>L. (L.) major</i>	464 \pm 13.29	10 \pm 0.52	10 \pm 0.32	10 \pm 1.41

Note. The data represents the mean \pm SEM of six replicas done in duplicate. ND, not determined. To calculate the EC_{50} the method of linear interpolation described by Huber and Hoella (1993) was used. This method is based on the use of two concentrations, x_1 and x_2 , such that the parasite density y_1 , at concentration x_1 , is more than half of the density found in the control vial (y_0) and the density found at x_2 (y_2) is less than half of y_0 . The EC_{50} is then found by the linear interpolation between x_1 and x_2 by the following equation: $\text{Log}(\text{EC}_{50}) = \log(x_1) + [(y_1 - (y_0/2))/(y_1 - y_2)][\log(x_2) - \log(x_1)]$

TABLE III

Effect of Ion Channel Blockers on the Percentage of Infection of Macrophages of the Cell Line J774 and Peritoneal Exudate Cells by *L. (L.) major*

	Fraction of infected macrophages [Impairment of infection](%)			
	J774		PEC	
	A	B	A	B
No drugs	17.75 ± 0.12	24.33 ± 0.10	26.33 ± 0.07	21.00 ± 0.22
4-AP ¹	12.00 ± 0.20	10.33 ± 0.19 ^a	18.80 ± 0.19	14.6 ± 0.38 ^b
	NS	[57]	NS	[30]
GLIB ²	6.40 ± 0.10 ^b	13.00 ± 0.24 ^a	21.00 ± 0.23	19.00 ± 0.14
	[64]	[47]	NS	NS
AMIL ³	14.00 ± 0.24	14.00 ± 0.35 ^a	16.00 ± 0.08 ^b	12.33 ± 0.43 ^b
	NS	[42]	[39]	[41]

Note. A: drugs present from 0–18 hr. B: drugs present from 18–24 hr. ¹100 μM 4-AP; ²3 μM GLIB (as free drug); ³10 μM AMIL. The data represent the mean ± SEM of 3–4 replicas, where at least 300 macrophages were analyzed. The frequency of infected macrophages of the experimental data was compared with the frequency of infected macrophages in nontreated macrophages (no drugs) by minimum χ^2 . A significant impairment of infection was calculated by the index developed by Belosevic *et al.* (1988). That is: [(% infected control macrophages—% infected treated macrophages)/(% infected control macrophages)] × 100. NS, not significant.

^a $P < 0.01$.

^b $P < 0.005$.

not know if this differential susceptibility is associated to the specific biochemical changes of the cell surface demonstrated in the metacyclic and amastigote forms of these parasites (Sacks, 1989). The expression of *Leishmania* surface glycoproteins (Sacks, 1989) and the preferential use of glucose as a source of energy (Cazzulo *et al.* 1985) are examples of biochemical changes related to stage regulation.

GLIB, a K⁺ ATP channel blocker, has a widespread use to identify K⁺ channels associated with insulin secretion (Panten *et al.* 1989; Schmid-Antomarchi *et al.* 1987). The EC₅₀ of 0.8 and 7 μM reported herein for New World *Leishmania* allows us to suggest the existence of low affinity sulfonylurea binding proteins in *Leishmania* (Nelson *et al.* 1992) and its associated Kir.6.2, which is a member of the

TABLE IV

Effect of Ion Channel Blockers on the Survival of Intracellular Established Parasites (Alive vs Dead *L. (L.) major*)^a

	J774		PEC	
	A ^b	B	A	B
No drugs	5.14 ± 0.14	1.46 ± 0.05	1.83 ± 0.04	1.57 ± 0.07
IFN γ ¹	0.75 ± 0.02	0.120 ± 0.01	0.22 ± 0.02	0.43 ± 0.01
4-AP ²	0.40 ± 0.04 ^b	0.23 ± 0.03 ^b	0.05 ± 0.006 ^b	0.38 ± 0.06 ^c
GLIB ³	0.32 ± 0.02 ^b	0.11 ± 0.012 ^b	0.09 ± 0.007 ^b	0.14 ± 0.01 ^c
9-CA ⁴	ND	0.09 ± 0.006 ^b	0.05 ± 0.004 ^b	0.28 ± 0.02 ^c
AMIL ⁵	0.93 ± 0.10 ^b	0.07 ± 0.006 ^b	0.08 ± 0.009 ^b	0.07 ± 0.01 ^c

Note. A: drugs present from 0–18 hr. B: drugs present from 18–24 hr. ¹20 U ml⁻¹ IFN γ ; ²100 μM 4-AP; ³3 μM GLIB (as free drug); ⁴10 μM 9-CA; ⁵10 μM AMIL. The data represent the mean ± SEM of 3–4 replicas, where at least 300 macrophages were analyzed. The alive vs dead results of infected macrophages of the experimental data (3, 4, and 5) were compared with the alive vs dead results of infected macrophages in nontreated macrophages (no drugs) by a one-way ANOVA. ND, not determined.

^a This ratio (alive vs dead) is an index of the survival regardless of the percentage of infection found.

^b $P < 0.005$.

^c $P < 0.010$.

inward rectifier K^+ channel family (Inagaki *et al.* 1995). The infection by *L. (L.) major* of J774 cells was significantly decreased when 3 μM GLIB was used. Also, the survival of intracellular parasites decreased significantly both in J774 and PEC. As the EC_{50} for promastigotes of this strain is 10 μM , these results suggest that there is a differential susceptibility of the intracellular parasites to this drug, compared to cultured promastigotes. Striking was the selective effect of glibenclamide on the viability of the intracellular parasite without affecting the survival of the macrophage. As this effect cannot be explained only by an increase of the antiparasitic activity, there is the possibility that the macrophage killing mechanisms could be synergistically potentiated by glibenclamide. This latter possibility is plausible as has been described for the action of stibogluconate, where an activation of the immune system has been reported as essential for the effect of the drug (Murray *et al.* 1988, Badaro *et al.* 1990).

4-AP has been used extensively for identifying a high variety of voltage-gated K^+ channels (Castle *et al.* 1989). In the present study, a concentration of 4-AP of 40 μM , 250 times smaller than the usual IC_{50} for mammalian tissues, impaired by 50% the survival of stationary phase New World *Leishmania* cultured as promastigotes. In conclusion, 4-AP and GLIB are effective in killing *Leishmania* either growing as promastigotes or while infecting macrophages. Although these results are suggestive of the presence of membrane systems which could resemble voltage-dependent K^+ channels and K^+ ATP channels in these parasites, additional experiments are needed to certify the existence of such molecular structures.

Anionic channels provide an electrical shunt and are important for the acidification of certain compartments and control of the cell homeostasis by regulating its volume, pH, transepithelial transport, and excitability (Jentsch, 1994). *In vivo*, 9-CA blocks Cl^- channels of developing and mature rat skeletal muscle cells and affects the differentiation of specific contractile properties at concentrations of 134 and 35 $\mu mol/kg$ (De Lucca *et al.* 1990). We were not able to detect consistent effects of 9-CA in our experiments. Nevertheless, we should not rule out the possibility of the presence of 9-CA-sensitive systems in these parasites. Their function would be very important during pH stress or in the development of multidrug resistance, a phenomena which has been associated with the *P*-glycoproteins and cell volume-regulated chloride channels (Hardy *et al.* 1995). Anionic channels have been assessed fluorometrically in *Leishmania* (Viera *et al.* 1994), but an association between chloride channels and drug resistance has not yet been found.

As *Leishmania* has to survive in environments characterized by nutritional stress and extreme pH conditions, the presence of transport systems involved in the intracellular pH homeostasis will be fundamental for its survival. A Cl^-/HCO_3^- antiporter has been described in *Leishmania* (Viera *et al.* 1994). This type of countertransport has been implicated in many physiological functions such as intracellular pH and cell volume regulation and must work in concert with a separate Na^+/H^+ antiport inhibited by AMIL at concentrations greater than 100 μM ; in unicellular parasites a Na^+/H^+ countertransport, totally blocked by 500 μM AMIL, has been described in *Plasmodium falciparum* (Bosia *et al.* 1993). Our results using AMIL suggest that intracellular *Leishmania* are more susceptible than the cultured promastigotes, a sensitivity indicative of the presence of molecular structures which could be related to Na^+ channels or to Na^+/H^+ antiports with a differential sensitivity for the drug. Additional experiments will help us to clarify this result.

In conclusion, in the present study we detected a susceptibility to K^+ , Cl^- , and Na^+/H^+ transport blockers in *Leishmania* promastigotes. Such susceptibility seems to be common to New World and Old World *Leishmania*. According to our experiments, the ion transport inhibitors used in this study are deleterious to cultured promastigotes. They also seem to affect the entry and/or the establishment of the parasite in their host cell since both the percentage of infection and the survival of intracellular parasites are decreased by some of the drugs. Indeed, as stated before, our data suggest either that intracellular parasites are more susceptible to some drugs than cultured promastigotes, or that the drugs promote an activation of the macrophages which increase the effectiveness of the cell in processing the intracellular parasites. Presently, experiments are being done to elucidate this issue. Preliminary data indicate that the nitric oxide production and the respiratory burst of infected PEC are not dramatically altered in the presence of the drugs but that an increase in the intracellular calcium could be important for the action of some of the drugs.

ACKNOWLEDGMENTS

This work was partially supported by Venezuelan Grants CDCH-UCV, 03.10.2831.94 and 09.33.0018.94, PC.09.010.94, CONICIT RPIV-110034, S1-96001411 and S1-2674, and the German Bundesministerium für Forschung und Technologie Grant KI8906/0. We thank Frau Carmen Bauer and Sandra Koster for technical assistance and Ana Herrera, Alejandro Pieters, Pedro Romero, and Horacio Vanegas for valuable discussion and critical comments on the manuscript. A.

I. Ponte-Sucre received a fellowship from the Universidad Central de Venezuela, CONICIT and DAAD to attend Dr. Heidrun Moll laboratory and perform the macrophage studies.

REFERENCES

- Bakker-Grunwald, T. 1992. Ion transport in parasitic protozoa. *Journal of Experimental Biology* **172**, 311–322.
- Badaro, R., Falcoff, E., Badaro, F. S., Carvalho, E. M., Pedral-Sampaio, D., Carvalho, J. S., Barral-Netto, M., Brandley, M., Silva, L., and Bina, J. C. 1990. Treatment of visceral leishmaniasis with pentavalent antimony and interferon-gamma. *New England Journal of Medicine* **322**, 16–21.
- Belosevic, M., Davis, C., Meltzer, M., and Nacy, C. 1988. Regulation of activated macrophage antimicrobial activities. *Journal of Immunology* **141**, 890–896.
- Benaim, G., Losada, S., Gadelha, F., and Docampo, R. 1991. A calmodulin-activated (Ca^{2+} - Mg^{2+})-ATPase is involved in Ca^{2+} transport by plasma membrane vesicles from *Trypanosoma cruzi*. *Biochemical Journal* **280**, 715–720.
- Bennet, L., Oie, S., and Schwartz, J. B. 1996. Design and optimization of dosage regimens; pharmacokinetic data. In “Goodman & Gilman’s The Pharmacological Basis of Therapeutics” (J. G. Hardman and L. E. Limbird, Eds.), 9th ed., pp. 1707–1711. McGraw-Hill, New York.
- Blum, J. L. 1992. Effect of osmolality on $^{86}\text{Rb}^{+}$ uptake and release by *Leishmania donovani*. *Journal of Cell Physiology* **152**, 111–117.
- Borst, P., and Ouellette, M. 1995. New mechanisms of drug resistance in parasitic protozoa. *Annual Review of Microbiology* **49**, 427–460.
- Bosia, A., Chigo, F., Turrini, F., Nissani, E., Pescarmona, G., and Ginsburg, H. 1993. Kinetic characterization of $\text{Na}^{+}/\text{H}^{+}$ antiport in *Plasmodium falciparum* membranes. *Journal of Cell Physiology* **154**, 527–534.
- Callahan, H., and Beverly, S. 1991. Heavy metal resistance: A new role for P-glycoproteins in *Leishmania*. *Journal of Biological Chemistry* **266**, 18427–18430.
- Castle, N., Haylett, D., and Jenkinson, D. 1989. Toxins in the characterization of potassium channels. *Trends in Neuroscience* **12**, 59–65.
- Cazzulo, J. J., Franke de Cazzulo, B. M., Engel, J. C., and Cannatta, J. J. 1985. End products and enzyme levels of aerobic glucose fermentation in trypanosomatids. *Molecular and Biochemical Parasitology* **16**, 329–343.
- Channon, J. Y., Roberts, M. B., and Blackwell, J. M. 1984. A study of the differential respiratory burst activity elicited by promastigotes and amastigotes of *Leishmania donovani* in murine resident peritoneal macrophages. *Immunology* **53**, 345–355.
- Chen, C., and Hess, P. 1987. Calcium channels in 3T3 and human fibroblasts. *Biophysical Journal* **51**, 226a.
- Dale, B. 1982. Amiloride: A molecular probe of sodium transport in tissues and cells. *American Journal of Physiology* **242**, C131–C145.
- De Lucca, A., Conte Camerino, D., Connold, A., and Vrbová, G. 1990. Pharmacological block of chloride channels of developing rat skeletal muscle affects the differentiation of specific contractile properties. *Pflügers Archives* **416**, 17–21.
- Desai, S., Korgstaad, D., and McCleskey, E. 1993. A nutrient-permeable channel on the intraerythrocytic malaria parasite. *Nature* **362**, 643–646.
- DiFranco, M., Villaroel, A., Ponte-Sucre, A., Quiñonez, M., Drujan, D., and Dagger, F. 1995. Incorporation of ion channels from the plasma membrane of *L. mexicana* into planar bilayers. *Acta Científica Venezolana* **46**, 206–207.
- Eresh, S., Mendoza-León, A., and Barker, D. C. 1993. A small chromosome of *Leishmania (Viannia) lainsoni* occupies a unique niche within the subgenus *Viannia*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **89**, 231–236.
- Glaser, T. A., Utz, G. L., and Mukkada, A. J. 1992. The plasma membranes electrical gradient (membrane potential) in *Leishmania donovani* promastigotes and amastigotes. *Molecular and Biochemical Parasitology* **51**, 9–16.
- Glaser, T., and Mukkada, A. 1992. Proline transport in *Leishmania donovani* amastigotes: Dependence on pH gradients and membrane potential. *Molecular and Biochemical Parasitology* **51**, 1–8.
- Hardy, S., Goodfellow, H., Valverde, M., Gill, D., Sepulveda, F., and Higgins, C. 1995. Protein kinase C-mediated phosphorylation of the human multidrug resistance P-glycoprotein regulates cell volume activated chloride channels. *The EMBO Journal* **14**, 68–75.
- Hille, B. 1989. Ionic Channels: Evolutionary Origins and modern roles. *Quarterly Journal of Experimental Physiology* **74**, 785–804.
- Huber, W., and Koella, J. 1993. A comparison of three methods of estimating EC_{50} in studies of drug resistance in malaria parasites. *Acta Tropica* **55**, 257–261.
- Inagaki, N., Gonoi, T., Clement, J., Namba, N., Inazawa, J., González, G., Aguilar-Bryan, L., Seino, S., and Bryan, J. 1995. Reconstitution of I_{KATP} : An inward rectifier subunit plus the sulfonylurea receptor. *Science* **270**, 1166–1170.
- Jackson, P., Pappas, M., and Hansen, B. 1985. Fluorogenic substrate detection of viable intracellular and extracellular pathogenic protozoa. *Science* **227**, 435–438.
- Jentsch, T. 1994. Molecular Physiology of anion transport. *Current Opinions in Cell Biology* **6**, 600–606.
- Lainson, R., and Shaw, J. J. 1987. Evolution, classification and geographical distribution. In “The Leishmaniasis in Biology and Medicine, Vol. I, Biology and Epidemiology” (W. Peters and R. Killick-Kendrick, Eds.), pp. 1–20. Academic Press, London/New York.
- Mendoza-León, A., Havercroft, J. C., and Barker, D. C. 1995. The RFLP analysis of the b-tubulin gene region in New World *Leishmania*. *Parasitology* **111**, 1–9.
- Mendoza-León, A., and Barker, D. C. 1996. Variation of a multigene family in New World *Leishmania*: The β -tubulin gene region. In “Molecular and Immune Mechanisms in the Pathogenesis of Cutaneous Leishmaniasis. Intelligence Unit Series” (F. J. Tapia, G. Cáceres Dittmar, and M. A. Sánchez, Eds.), pp. 107–127. Landes Bioscience Publishers, Texas.
- Murray, H. W., Berman, J. D., and Wright, S. D. 1988. Immunotherapy for intracellular *Leishmania donovani* infection. Interferon

- plus pentavalent antimony. *Journal of Infections Diseases* **157**, 973–978.
- Nelson, D. A., Aguilar-Bryan, L., and Bryan, J. 1992. Specificity of β -cell membrane proteins with an ^{125}I -labeled glyburide analog. *Journal of Biological Chemistry* **267**, 14928–14933.
- Olliaro, P. L., and Bryceson, A. 1993. Practical progress and new drugs for changing patterns of Leishmaniasis. *Parasitology Today* **9**, 323–328.
- Ouellette, M., Fase-Fowler, F., and Borst, P. 1990. The amplified H circle of methotrexate resistant *Leishmania tarentolae* contains a novel P-glycoprotein gene. *The EMBO Journal* **9**, 1027–1033.
- Panten, U., Burgfeld, J., Goerke, F., Rennicke, M., Schwanstecher, M., Walasch, A., Zücker, B., and Lenzen, S. 1989. Control of insulin secretion by sulfonylureas, meglitinide and diazoxide in relation to their binding to the sulfonylurea receptor in pancreatic islets. *Biochemical Pharmacology* **38**, 1217–1229.
- Philosoph, H., and Zilberstein, D. 1989. Regulation of intracellular calcium in promastigotes of the human protozoan parasite *Leishmania donovani*. *Journal of Biological Chemistry* **264**, 10420–10424.
- Rheinallt Parri, H., Holden-Dye, L., and Walker, R. J. 1991. Studies on the ionic selectivity of the GABA-operated chloride channel on the somatic muscle bag cells of the parasitic nematode *Ascaris suum*. *Experimental Physiology* **76**, 597–606.
- Sacks, D. L. 1989. Metacyclogenesis in *Leishmania* promastigotes. *Experimental Parasitology* **69**, 100–103.
- Sacks, D. L., and Da Silva, R. P. 1987. The generation of infective stage *Leishmania major* promastigotes is associated with the cell-surface expression and release of a developmentally regulated glycolipid. *J. Immunol.* **139**, 3–99–3106.
- Scheffler, W. 1981. Datos de enumeración: Distribuciones de ji cuadrado y de Poisson. In “Bioestadística” (W. Scheffler, Ed.), pp. 103–117. Análisis de varianza, pp. 122–157. Fondo Educativo Interamericano, México.
- Schmid-Antomarchi, H., Deweile, J., Fosset, M., and Lazdunski, M. 1987. The receptor for the antidiabetic sulfonylureas controls the activity of the ATP-modulated K^+ channel. *Journal of Biological Chemistry* **262**, 15840–15844.
- Solbach, W., Forberg, K., Kammerer, E., Bogdan, C., and Röllinghoff, M. 1986. Suppressive effect of cyclosporin A on the development of *Leishmania tropica* induced lesions in genetically susceptible BALB/c mice. *Journal of Immunology* **137**, 702–707.
- Viera, L., Lavan, A., Dagger, F., and Cabantchick, Z. 1994. The role of anions in pH regulation of *Leishmania major* promastigotes. *Journal of Biological Chemistry* **10**, 16254–16359.
- Zilberstein, D., and Dwyer, D. 1985. Protonmotive force-driven active transport of D-glucose and L-proline in the protozoan parasite *Leishmania donovani*. *Proceedings of the National. Academy of Sciences* **82**, 1716–1720.
- Zilberstein, D., and Dwyer, D. 1988. Identification of a surface membrane proton translocating ATPase in promastigotes of the parasitic protozoan *Leishmania donovani*. *Biochemical Journal* **256**, 13–21.
- Zilberstein, D. 1988. Calcium as a possible second messenger of external signals in *Leishmania donovani*. *Memorias do Instituto Oswaldo Cruz* **83**, 261.
- Zilberstein, D., and Shapira, M. 1994. The role of pH and temperature in the development of *Leishmania* parasites. *Annual Review of Microbiology* **48**, 449–470.

Received 24 February 1997; accepted with revision 28 July 1997