An amino acid channel activated by hypotonically induced swelling of *Leishmania major* promastigotes

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Leishmania promastigotes accumulate amino acids (AAs) by an uphill transport mechanism that is dependent on membrane potential. The accumulated AAs provide the cell with an osmotic reservoir that can be utilized for osmoregulation. Exposure of *Leishmania* promastigotes to hypotonic media induced a rapid release of AAs that was proportional to the imposed osmotic gradients and independent of the ionic strength or the presence of Cl^- , K^+ , Na^+ or Ca^{2+} in the medium. The hypotonically activated AA release pathway was of relatively low chemical specificity. The solutes released included most of the zwitterionic and anionic AAs, predominantly alanine, hydroxyproline, glycine and glutamic acid, whereas cationic AAs were virtually excluded. AA release was markedly blocked by classical anion transport inhibitors such as the disulphonic stilbene 4,4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS) and its dihydro

derivative H_2DIDS and others, by restoration of isotonicity or by lowering the temperature (4°C). The temperature profile of AA release showed a low energy of activation (E_a 46 ± 1.3 (S.E.M.) kJ/mol) in the range $15\text{--}30^\circ\text{C}$ and a very high E_a (147 ± 8 kJ/mol) in the range $4\text{--}15^\circ\text{C}$. Parasites exposed to hypotonic media containing AAs also showed a hypotonically stimulated AA uptake under favourable solute concentration gradients. This uptake was analogous for L- and D-isomers of threonine. After hypotonic exposure, cells underwent a depolarization that was largely prevented by anion transport blockers. On the basis of all these results we propose that after hypotonic stress *Leishmania* promastigotes restore their internal volume by a regulated release of AAs, which involves activation of channels that allow the passage of both neutral and anionic AAs and possibly other anionic substances.

INTRODUCTION

Leishmania promastigotes develop, as part of their life cycle, in the digestive tract of the sandfly. In this environment the parasites face chemical fluctuations that depend on the nutritional habits of the sandfly [1] and the physiological properties of the particular section of the digestive tract in which they dwell. To cope with those fluctuations and maintain stable internal contents and cell volume, the parasites must operate appropriate corrective transport systems.

When challenged by hypotonicity, animal cells initially swell but generally recover their original volume [2–5]. This property has been referred to as regulatory volume decrease (RVD). It is associated, in most cases, with activation of parallel conductive pathways for K^+ and Cl^- [3,6,7], which result in net passive KCl efflux and an obligatory loss of water. In addition, some plants and animal cells respond to alterations in medium tonicity by releasing small organic solutes (osmolytes) [5,8–11]. The implicated osmolytes include: (1) monosaccharides (such as glucose and mannose), disaccharides (such as sucrose and trehalose) and amino acids (AAs) (such as proline and glutamate) in fungi, cyanobacteria and algae [8,12]; (2) taurine, alanine, glutamine and glycine in invertebrate and lower eukaryotes [9,13–15]; and (3) AAs (such as taurine, alanine), polyols (such as sorbitol, myo-inositol) and methylamines (such as betaine, glycerophosphocholine) in higher eukaryotes [5,10,16–18].

Although the mechanisms involved in the activation of RVD by specific transporters have remained elusive, a large body of evidence has recently implicated anion channels in the induced efflux of AAs and other small organic solutes [16,19–21]. The putative channels have been termed VSOAC, for volume-sensitive organic osmolyte anion channel [21].

Earlier studies indicated that hypotonic challenges of *Leishmania* promastigotes led to minor changes in K^+ fluxes [22]. On the basis of inhibiting the release of ^{86}Rb (used as a K^+ analogue) with the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone after hypotonic shock, it was suggested that K^+/H^+ exchange might have been activated on cell swelling; however, its contribution to RVD was not substantial [22]. Other studies indicated that *Leishmania* promastigotes release alanine and other ninhydrin-reactive substances on hypotonic challenge [23,24]. Intracellular pools of AAs were shown to be built up via a membrane potential (V_m)-driven mechanism [25] that is supported by a proton pump and a parallel anion-conductive pathway [26,27]. In this study we test the hypothesis that anion channel(s) might also be associated with RVD by providing a pathway for hypotonically activated AA release. By monitoring changes in AA levels in cells and medium after hypotonic stress, we show that the major component of RVD in *Leishmania* promastigotes is an anionic AA channel. We have termed this system HAAC, for hypotonically activated amino acid channel.

MATERIALS AND METHODS

Culture methods

Promastigotes of *Leishmania major* strain LCR-137 were grown as described previously [26]. The parasites used in this study were taken from cultures during their exponential phase of growth.

Chemicals

RPMI media and phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione (fluorescamine) were from Sigma Chemical Co., and fetal calf serum was purchased from Bio Technologies Inc. (Beth

Abbreviations used: AA, amino acid, DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulphonate; HAAC, hypotonically activated amino acid channel; RVD, regulatory volume decrease.

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Haemek, Israel). 4,4'-Di-isothiocyanostilbene-2,2'-disulphonate (DIDS) and its dihydro derivative H₂DIDS were procured from Molecular Probes. All other chemicals were obtained as products of the highest available grade.

Solutions

Isotonic Cl⁻ medium (iso-Cl⁻) was composed of (in mM): NaCl 137, KCl 4, KH₂PO₄ 1.5, Na₂HPO₄ 8.5, pH 7.4, and supplemented with Hepes 20, glucose 11, CaCl₂ 1 and MgSO₄ 0.8. Hypotonic Cl⁻ media (hypo-Cl⁻) were obtained by diluting iso-Cl⁻ with different volumes of water to the final desired osmolarity. Unless otherwise indicated, hypo-Cl⁻ was created by adding iso-Cl⁻ (300 ± 5 m-osM) to an equal volume of water to give a final osmolarity of 150 ± 2 m-osM. Osmolarity was checked with a Wescor osmometer (Wescor Inc., Logan, Utah, USA). All solutions were CO₂-free.

Determination of time-dependent release of AA following hypotonic stress

Cells were exposed to the indicated hypotonic media (30 °C, pH 7.4). At the indicated times, aliquots were withdrawn and quickly transferred to an isotonic buffer, pre-equilibrated at 4 °C, to stop the reaction. Subsequently, cells were centrifuged and the supernatants collected. To quantify the amount of AA released, an aliquot of 100 µl of cell supernatant was mixed with 900 µl of 0.2 M sodium borate, pH 9.0, at room temperature and quickly mixed with 500 µl of fluorescamine (0.2 mg/ml in acetone). Fluorescence intensity was determined in a SPEX Fluorolog II (DM1B) instrument at excitation and emission wavelengths of 390 and 475 nm respectively. To measure the total AAs remaining in cells, the cell pellet was extracted with 0.5 M HClO₄ for 1 h on ice and the supernatant was neutralized with 5 M KOH. The resulting pellet was used for protein measurement with the micro-BCA (bichinonic acid) method (Pierce Chemical Co.) adapted for a 96-well plate and read in an EL 309 autoreader (BioTek Instruments, Winooski, VT, U.S.A.) at 540 nm. The neutralized extract was again centrifuged and the supernatant was used for AA determination with fluorescamine as described above. Selected fractions of cell-remaining AAs were used for AA analysis (automatic precolumn fluoren-9-ylmethoxycarbonyl derivatization, HPLC column; Merck, Darmstadt, Germany). Unless otherwise specified, measurements were done in duplicate samples in at least five independent sets of equivalent experiments.

Cell treatments

Cells were preincubated for 20 min at 30 °C in the presence of various DIDS concentrations (100–250 µM), washed twice with the same incubation medium and assessed for time-dependent release of AAs as described above.

To examine the bidirectionality of AA fluxes in hypotonic media, cells were exposed to hypo-Cl⁻ medium containing 12.5 mM unlabelled threonine for 1 or 5 min. After being washed several times with iso-Cl⁻ medium to remove remaining external threonine, cells were processed for AA analysis as described above. To ensure that the measured threonine represented the AA taken up by cells, we used the non-transported ornithine (5 mM) as an external and contaminating marker.

Statistical analyses of unpaired data were done with the Sigmaplot program (Jandel Scientific, San Rafael, CA, U.S.A.). All data calculations, analyses and curve simulations were done with the Origin program (Microcal Software, Northampton, MA, U.S.A.) with IBM-compatible computers.

RESULTS

Microscopic observations showed that *Leishmania* promastigotes swell in hypotonic media but recover their resting shape and volume within 15–20 min. In virtually all the studies reported in this work we ascertained that in the course of the experiments, the cells remained motile and fully viable (results not shown).

Properties of hypotonically induced AA release

The induction of the release of AAs from *Leishmania* promastigotes exposed to hypotonic medium (150 m-osM) was determined in terms of the AA content of the cells and the medium (Figure 1). The appearance of amino-reactive material in hypotonic medium was detected within 5 s at 30 °C, and reached a maximum value within 5 min. The appearance of amino-reactive material in the medium was accompanied by a commensurate decrease of the material in cells. To ascertain that the amino-reactive material represented AAs we analysed the deproteinized cells by AA analysis. As shown in Table 1, the AAs identified and detected by the method described in the Materials and methods section account for almost all the amino groups detected by the fluorescent amino reagent fluorescamine.

Table 1 lists the predominant AAs present in cells after 1 min of exposure to either isotonic or hypotonic medium. Substantial and statistically significant decreases in the following AAs were found in hypotonic relative to isotonic medium: Glu 29 %, Gly 34 %, Ala 42 %, Asp 43 %, Hyp 45 %, Thr 60 %, Pro 60 %, Phe 67 %. Alanine, being the major osmolyte in cells, is probably the major contributor to RVD, as previously suggested by us and others [22,24,25]. Among the neutral AAs, serine was apparently released at a significantly lower rate than the others, when measured 1 min after hypotonic exposure. When measured over a 5–10 min period of exposure to hypotonicity, virtually all the neutral and acidic AAs showed a significant decrease in the cell (results not shown). The results imply that *Leishmania* parasites utilize part of their internal AA pool (negative AAs and most of the neutral AAs) for osmoregulation. We have found that the internal pool of AAs is subject to variations with the age of the culture; nevertheless the magnitude and specificity of release were largely maintained. In the experiments outlined below, we show primarily results related to the release of AAs into the

Figure 1 Effect of osmolarity on AA release

Leishmania promastigotes washed and then resuspended in iso-Cl⁻ medium were diluted with an equivalent volume of distilled water. The quantities of AAs released into the medium and remaining in cells were measured at different times, as indicated in the Materials and methods section. Traces are representative of results obtained in duplicate samples from at least seven independent experiments. Symbols: ◇, AAs in cells in iso-Cl⁻ medium; ●, AAs in cells in hypo-Cl⁻ medium; ○, AAs released into hypo-Cl⁻ medium.

Table 1 Amino acid profiles in cells in isotonic and hypotonic conditions

Cells exposed to isotonic (Iso) and hypotonic (Hypo) medium for 1 min at 30 °C were removed and processed for AA analysis as described in the Materials and methods section (see Figure 1). Results are given both as absolute values (nmol/mg of protein) and as a percentage of AAs released into hypotonic relative to isotonic medium. Values are means $\pm$ S.E.M. ($n = 3$, with samples taken in duplicate). A probability level $P < 0.05$ was considered statistically significant. Abbreviation: n.r.: AA release ≈ 0 .

Amino acid	Iso medium	Hypo medium	Release (%)	<i>P</i>
Asp	14 $\pm$ 2	8 $\pm$ 1	43	0.04
Hyp	33 $\pm$ 4	18 $\pm$ 3	45	0.02
Ser	30 $\pm$ 2	26 $\pm$ 2	13	0.35*
Glu	35 $\pm$ 5	25 $\pm$ 2	29	0.03
Thr	5 $\pm$ 2	2 $\pm$ 0.6	60	0.04
Gly	35 $\pm$ 5	23 $\pm$ 2	34	0.02
Arg	23 $\pm$ 4	25 $\pm$ 1	n.r.	0.50
Ala	134 $\pm$ 17	78 $\pm$ 20	42	0.01
Tyr	10 $\pm$ 2	5 $\pm$ 1	50	0.03
Pro	15 $\pm$ 5	6 $\pm$ 1	60	0.01
Phe	3 $\pm$ 1	1 $\pm$ 0.1	67	0.04
Leu	20 $\pm$ 3	12 $\pm$ 4	40	0.02
Orn	61 $\pm$ 10	50 $\pm$ 4	n.r.	0.30
Lys	23 $\pm$ 2	23 $\pm$ 1	n.r.	0.52

*Ser release was not significant at 1 min, but was highly significant at times > 5 min (30–40% release relative to control).

medium, although we have routinely measured the amino groups in both cell and medium.

Dependence on osmotic gradient

The hypotonically activated release of AAs depended on the tonicity of the medium (Figure 2). Whereas in isotonic conditions (300 m-osM) a negligible quantity of AAs was detected in the medium over a 10 min period at 30 °C (results not shown), AAs were easily detected in the medium within seconds at 250 m-osM (Figure 2, inset). The magnitude of the total AA release was found to be proportional to the osmotic gradient as visualized by the modified van't Hoff relationship shown in Figure 2. The intercept on the x-axis (approx. 264 m-osM) represents the threshold osmolarity required for the induction of AA release. Assuming that AA release is the corrective measure for hypotonically induced changes in cell volume, it can be inferred that parasites behave not only initially as perfect osmometers, but also as self-volume regulators, as observed in other cell types [28–30].

To ascertain that the hypotonically induced AA release was of a transient nature we also assessed the reversibility of the phenomenon. Cells challenged at 30 °C with hypotonic medium for 15 s were swiftly transferred either to 4 °C (same medium) or to isotonic medium at 30 °C. As shown in Figure 3, both restoration of isotonicity (Figure 3a) and decreasing the temperature to 4 °C (Figure 3b) led to an immediate arrest of AA release. These results indicate that hypotonically induced AA release was not caused by irreversible damage to cells. The complete arrest of AA release by restoration of isotonicity or decrease in temperature was used as a stopping device for assessing the time course of the phenomenon.

Dependence on temperature

The temperature dependence of hypotonically evoked AA release was studied by exposing cells to hypotonic medium pre-equilibrated at various temperatures (Figure 4). The initial rate of

Figure 2 Effect of external osmolarity on hypotonically stimulated AAs release

Promastigotes resuspended in iso-Cl⁻ medium were mixed with different solutions to give the final osmolarities shown in the inset. Aliquots were taken at the indicated times for estimation of AA release, as described in Figure 1. The maximum AA release attained at the indicated osmolarity after 10 min was plotted against the external osmolarity. A modified van't Hoff relationship was obtained. Abbreviation: mOsm, m-osM.

release relative to the rate at 30 °C (k/k_{30}) was plotted against the reciprocal of absolute temperature (Arrhenius plot; Figure 4, inset). The plot shows two major components with a sharp break at approx. 15 °C (transition temperature, T_c). Above T_c , the energy of activation (E_a) was 46 ± 1.3 kJ/mol, with a Q_{10} of approx. 2, whereas below T_c , E_a was 147 ± 8 kJ/mol, with a Q_{10} of approx. 8. The sharp change in slope observed at 15 °C might represent the existence of different limiting steps for the hypotonically induced AA release at different temperatures.

Membrane potential changes

To assess whether the hypotonically activated AA efflux was associated with changes in membrane conductivity, we monitored changes in V_m by following the fluorescence intensity of the potentiometric dye bis-oxonol, as previously described [27]. Under isotonic conditions (28 ± 0.1 °C, pH 7.4) V_m was -116 ± 2 mV. When medium osmolarity was lowered to 150 m-osM, cells underwent a pronounced membrane depolarization (-86 ± 3 mV). These changes in V_m were not observed when the ionic strength of the medium was varied by dilution of the iso-Cl⁻ medium with isosmotic mannitol (normoionic solution). When the inhibitor of anion transport systems, H₂DIDS, was present in the hypotonic medium, the V_m of cells remained hyperpolarized (-125 ± 3 mV). In general, values of V_m lower than -80 mV were approximate because the calibration curve was not linear in the region of -80 mV, as previously discussed [27]. These studies could not be performed with the more covalent reactive DIDS, owing to the fact that this probe is fluorescent and interfered with the signal of the potentiometric dye, whether or not it was

Figure 3 Effects of low temperature and restoration of isotonicity on AA release

Cells exposed to hypotonic medium at 30 °C, were rapidly transferred to either iso-Cl⁻ (Iso) medium at 30 °C (a) or hypotonic (Hypo) or isotonic medium at 4 °C (b) at the times indicated by the arrows (the values at 4 °C for both media were essentially superimposed). The control experiment represents a sample that was continuously exposed to hypotonic medium at 30 °C. Traces are representative of at least two separate experiments done under the same experimental conditions.

Figure 4 Effect of external temperature on AA release

Promastigotes resuspended in isotonic Cl⁻ medium, pH 7.4, were diluted in media pre-equilibrated at the indicated temperature to give a final osmolarity of 150 m-osM. The initial rate of release relative to the rate at 30 °C was plotted against the inverse of the absolute temperature (1/T, in K⁻¹) (inset). The activation energy, E_a, obtained from the respective slope was 46 ± 1.3 kJ/mol in the range 15–30 °C and 147 ± 8 kJ/mol in the range 4–15 °C.

Table 2 Effect of Cl⁻ transport blockers on hypotonically induced AA release from *Leishmania* promastigotes

Cells preincubated for 20 min at 30 °C in the absence (control) or presence of 250 μM DIDS were washed twice and then resuspended in iso-Cl⁻ and subsequently exposed for 1 min to hypotonic medium (150 mOsm). They were then analysed for AA content as described in Table 1. Values (means ± S.E.M.; n = 2) are given as the percentage release of each AA relative to the initial amount present in cells.

Amino acid	Control	DIDS
Asp	43 ± 5	7 ± 2
Hyp	45 ± 7	11 ± 2
Glu	29 ± 2	3 ± 0.1
Gly	34 ± 3	13 ± 3
Ala	42 ± 10	2 ± 0.2
Pro	60 ± 10	17 ± 2
Val	27 ± 13	14 ± 1
Leu	40 ± 13	6 ± 1

present in the medium or reacted with cells and removed from the medium.

Effect of anion transport blockers

The effect of disulphonic stilbenes [31] such as H₂DIDS on hypotonically evoked depolarization could be associated either with a direct inhibition of a putative HAAC pathway, or with putative Cl⁻ channels that support H⁺-pump activity under isotonic conditions [26]. We therefore studied the effect of both H₂DIDS and DIDS on the hypotonically stimulated AA release by measuring the quantity of AAs released into the medium and that remaining in the cellular pool. Results are shown for cells that were reacted with the more covalent reactive DIDS (100–250 μM), washed extensively and exposed to hypotonic medium. The hypotonic stress induced an AA release that was markedly inhibited by the disulphonic stilbene. Table 2 shows the percentage release of some of the major AAs in the presence and absence of 250 μM DIDS. No significant differences in AA content were observed in cells preincubated for 20 min in iso-Cl⁻ medium in the absence or presence of DIDS (results not shown). Other inhibitors were tested in terms of AA analysis of cells incubated in hypotonic conditions in the presence of the inhibitor. DNDS, the dinitro analogue of DIDS, gave at 100–250 μM similar results to those of DIDS (results not shown). Phlorizin and phloretin at 100 μM, although inhibitory, were considerably less potent than the disulphonic stilbenes (results not shown). Other anionic channel blockers such as nitrophenyl benzoate derivatives [31] were either highly toxic to cells and/or interfered with the assays.

Directionality properties and specificity

The AA uptake of cells exposed to hypotonic medium was also assessed under conditions of favourable concentration gradients. We used L-threonine, whose intracellular concentration is relatively low (4–5 mM), and D-threonine, as an AA that is not transported by constitutive transporters in isotonic conditions. When cells were placed in hypotonic medium a net decrease in cell L-threonine was detected after a 1 min incubation. However, when either D-threonine or L-threonine was included in the medium (12.5 mM), a markedly higher net uptake of the threonine (the AA analysis did not distinguish between the optical

Table 3 Uptake of threonine in *Leishmania* promastigotes exposed to different media

Cells exposed for 1 or 5 min to isotonic (Iso) or hypotonic (Hypo) medium containing 5 mM L-ornithine supplemented with (+) 12.5 mM threonine (thr) or unsupplemented (—) were quickly washed with cold isotonic medium and processed for AA analysis as described in Table 1. Values (means $\pm$ S.E.M.; $n = 2$) are expressed in nmol/mg of protein. Δ values represent the net change in D-Thr (obtained by subtraction of the corresponding iso(—) values at the indicated times).

Treatment	D-Thr	D-Thr				L-Orn	
		1 min	Δ (1 min)	5 min	Δ (5 min)	1 min	5 min
Iso	—	10 $\pm$ 1	—	10 $\pm$ 1	—	134 $\pm$ 10	134 $\pm$ 8
Hypo	—	8 $\pm$ 3	—2	6 $\pm$ 1	—4	135 $\pm$ 8	150 $\pm$ 4
Iso	+	16 $\pm$ 3	+6	66 $\pm$ 7	+56	137 $\pm$ 7	155 $\pm$ 7
Hypo	+	26 $\pm$ 5	+16	88 $\pm$ 3	+78	144 $\pm$ 10	148 $\pm$ 11

Figure 5 Kinetic analysis of AA release from cells

Data taken from Figure 1 were analysed kinetically by a two exponential decay function, but only those corresponding to 150 m-osM are portrayed graphically. The relationship between the fractional amount of AA present in the cells (F_A) as a function of time is given by $F_A = A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t})$, where A_1 and A_2 are coefficients ($A_1 + A_2 = 1$) and k_1 and k_2 are the corresponding rate constants for components 1 and 2 respectively. The best-fit parameters are given in Table 4 for all the four media used. Line 1 + 2 represents the regression line obtained by best-fit analysis, with lines 1 and 2 representing the respective individual components of AA release. In the range 100–200 m-osM, the A_1 and k_1 values for the various osmolarities did not differ significantly from each other ($P < 0.25$ and $P < 0.85$ respectively), nor did the A_2 and k_2 values differ significantly from each other ($P < 0.24$ and $P < 0.12$ respectively). However, in the range 100–200 m-osM, for a given osmolarity the A_1 value differed significantly from the corresponding A_2 value ($P < 0.02$) as the k_1 value differed from the corresponding k_2 value ($P < 0.03$).

isomers) was detected (Table 3): 16 nmol of threonine/mg of protein was found after incubation of cells in hypo-Cl[−] medium, compared with 6 nmol of threonine/mg of protein after incubation in iso-Cl[−] medium (after 5 min the values were 78 and 56 nmol/mg respectively). All these measurements represented significant increases in intracellular concentration of threonine ($P < 0.04$). Essentially similar results were obtained with L-threonine (results not shown). However, when L-ornithine was used instead of either D- or L-threonine, in either hypotonic or isotonic medium (Table 3), the intracellular concentration of ornithine remained practically constant under all experimental conditions (Table 3) ($P < 0.4$). This result is in agreement with the observation that cationic AAs are apparently excluded from the HAAC system (Table 1). Moreover these observations also

Table 4 Best-fit parameters for all the four media used

Osmolarity (m-osM)	Component 1		Component 2	
	A_1	k_1 (s ^{−1})	A_2	k_2 (s ^{−1})
250	0.40 $\pm$ 0.11	0.030 $\pm$ 0.011	0.65 $\pm$ 0.11	0.003 $\pm$ 0.005
200	0.74 $\pm$ 0.12	0.015 $\pm$ 0.007	0.17 $\pm$ 0.12	0.003 $\pm$ 0.004
150	0.74 $\pm$ 0.09	0.015 $\pm$ 0.007	0.17 $\pm$ 0.12	0.003 $\pm$ 0.004
100	0.84 $\pm$ 0.08	0.016 $\pm$ 0.002	0.13 $\pm$ 0.09	0.002 $\pm$ 0.002

support the observation that the intracellular measurements of AAs were not affected by possible contamination with extracellular material.

Kinetic analysis of AA release

The time profile of AA release obtained at different osmolarities shown in Figure 2 (inset) was analysed in terms of single- and double-exponential functions. Figure 5 shows the results obtained at 150 m-osM, which were best fitted by the sum of two exponential components that differed markedly in their rate constants (Table 4): $A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t})$, where A_1 and A_2 are coefficients that sum to unity. At 150 m-osM, $k_1 = 0.015$ s^{−1} ($t_{1/2}$ 4 s) and $k_2 = 0.003$ s^{−1} ($t_{1/2}$ 23 s). These values were very similar to those obtained for 100 and 200 m-osM ($P < 0.24$ and 0.3 respectively). However, the values describing the system at 250 m-osM were clearly different and carried with them large relative errors. This is because at 250 m-osM, which is very close to the threshold level required for AA release (approx. 264 m-osM), the AA release was relatively small. However, cell heterogeneity could also account for the observed differences found in cells exposed to a small osmotic gradient.

DISCUSSION

Organisms living in environments that undergo changes in tonicity and chemical composition have developed specific mechanisms allowing them to control their volume [12,29,32]. Some organisms use the dissipation of K⁺ and Cl[−] for correcting volume changes. Others build up osmotic reservoirs of organic solutes, operationally defined as osmolytes [2,15,19,29], which allow them to overcome tonicity changes both adaptively and in a regulated manner. *Leishmania* promastigotes, as well as other members of the trypanosomatid family, build up large reservoirs of AAs [22,25,33,34]. The major contributor to the intracellular accumulation of AAs is apparently the membrane potential generated by an electrogenic H⁺ pump [25]. In this work we have demonstrated that AA release is used as a means for correcting hypotonically induced changes in cell volume. Previous studies have monitored the release of ninhydrin-reactive material after hypotonic stress [23,24]. The mechanism ascribed to RVD was probably not associated with K⁺ efflux [22] because intracellular changes in this solute after hypotonic stress were relatively low (5 mM in 90 s). In our hands the hypotonically evoked AA release in *L. major* as well as in a variety of related species (E. Lafuente, L. L. Vieira and Z. I. Cabantchik, unpublished work) was found to be sufficient for the RVD response (150–200 mM). *Leishmania* promastigotes accumulate up to 900 nmol of AA/mg of protein. The concentration of alanine alone found in *L. major* (134 nmol/mg) is very similar to that obtained by Blum and co-

workers for *L. donovani* [24]. On the basis of estimates of total AA in nmol/mg of protein, the calculated intracellular concentration of all AAs would be as high as 300 mM (taking 1 mg protein as being equivalent to a cell-free volume of 4.74 μ l) [25,35] or lower, using other published values of cell volume and protein content [36]. The fact that AA analysis and fluorecamine gave similar results for total AAs indicates that AAs comprised the predominant fraction of the cells' amino-reactive material. In other protozoans, such as *Crithidia fasciculata*, the reported AA content of the cell was 150 mM [34], although it is not clear whether the determination included all the AA species and other possible osmolytes. Because most of the organisms also have a relatively high concentration of K⁺ (more than 100 mM) and a high concentration of inorganic and organic anions, the AA content might raise the cell osmotic potential to prohibitive levels unless the chemical activity of the AA is markedly reduced by ion-pairing or by other means. Our studies indicate that a substantial fraction of the AA content of *L. major* promastigotes can be mobilized for RVD swiftly (in seconds) and commensurately with the imposed osmotic gradient (Figure 2). Within the detection limits of the method used, the onset of release was extrapolated to a threshold osmolarity of about 264 m-osM. The major contributor to the RVD was alanine, the predominant AA, as suggested by us and others [22–25]. Similar studies were recently reported in the protozoan parasite *Giardia intestinalis* [15]. However, in *Leishmania*, not only neutral but also acidic AAs contributed to RVD. Although it is currently unclear whether one or more pathways accommodate the neutral and acidic AAs, the fact that all cationic AAs were excluded points to a relatively low substrate specificity. This is also apparent in the lack of discrimination between D-threonine and L-threonine and the admission of the non-metabolizable aminoisobutyric acid (J. J. Blum, personal communication). Although transport of aminoisobutyric acid was also detected in *Leishmania* in isotonic conditions (J. J. Blum, personal communication), in *Giardia* it was operative only in hypotonic conditions [15]. In the present studies, serine was unique among the neutral AAs in that its release kinetics was slower than for others. This and other results clearly indicate that substrate specificity varies for similar or analogous transport systems that operate in protozoans.

The relatively low substrate specificity of the hypotonically activated AA transport is characteristic of some membrane channels [37]. We have referred to this putative channel as HAAC, on the basis of various pieces of information that complement the chemical properties of the HAAC, as follows.

1. The idea of an HAAC is in line with the relatively low E_a (46 ± 1.3 kJ/mol) for AA egress in hypotonic conditions found in the 15–30 °C temperature range, although at lower temperatures (less than 15 °C) E_a increased enormously. Similar features were recently observed in *Giardia* [15]. We interpret the low temperature dependence of HAAC (46 ± 1.3 kJ/mol) as a property associated with solute diffusion through a relatively wide pore or open channel [37]. The high temperature dependence found at 4–15 °C (E_a 147 ± 8 kJ/mol) we attribute to the rate-determining signal transduction mechanisms, which apparently involve several protein kinases (L. L. Vieira, J. J. Blum and Z. I. Cabantchik, unpublished work).

2. The fast egress of AAs via HAAC was detected within 5 s of hypotonic exposure and could be completed within 5–10 min. Deactivation of HAAC was almost instantaneous, whether induced by restoration of isotonicity or by lowering the temperature (4 °C). Fast activation/deactivation mechanisms are compatible with the dynamics of channel operation and regulation [38].

3. The kinetics of AA release via HAAC could be best fitted to

a double-exponential decay function for osmolarities of 200 m-osM. Approx. 80 % of the response showed a similar fast rate constant at either osmolarity, whereas the remaining 20 % was slower and more variable (Figure 5). It is not clear at present whether both constants are associated with the same mechanism or pathway. It is conceivable that the slow component represents a late AA release from a cytosolic compartment that is in series with the major HAAC. At present we cannot exclude other possibilities, except that of non-specific leakage, because cationic AAs are apparently not released even at relatively high hypotonic challenges.

4. Given that AA release is blocked by anion transport inhibitors of the disulphonic stilbene family and to a smaller extent by other (less specific) anion transport blockers, and that hypotonic stress induces a disulphonic stilbene-sensitive anionic conductance, we suggest that HAAC might also accommodate anions. The hypotonically induced depolarization is in agreement with that view, although the relative contributions of inorganic and anionic AAs to the hypotonically induced depolarization have yet to be determined. Clearly, neither the hypotonically induced depolarization nor the AA release was affected by K⁺ or Na⁺ gradients, so that cationic conductances play a minor role, if any, in the RDV response of *Leishmania* promastigotes. As to anionic conductances, those have previously shown to be considerable in isotonic conditions [27], although they can be further enhanced after the hypotonic challenge. Moreover, it is conceivable that some of the observed hypotonically induced depolarization might be contributed by the anionic AAs, although this remains to be experimentally verified.

5. When exposed to hypotonic media, *Leishmania* promastigotes also showed the property of enhanced AA uptake under a favourable AA concentration gradient (Table 3). This property might also have some implications for nutrient uptake, particularly if, in the midgut of the sandfly, parasites become exposed to hypotonic AA-rich media.

In conclusion, the results obtained in the present work strongly suggest that hypotonically stimulated AA efflux in *Leishmania* promastigotes is mediated by a putative non-selective DIDS-sensitive anion channel. This view is in agreement with observations obtained in a variety of cell types [16,17,21,29,39,40]. Recent studies (L. L. Vieira, J. J. Blum and Z. I. Cabantchik, unpublished work) have indicated that specific signal transduction pathways are involved in the regulation of HAAC opening and closing in *L. major* promastigotes.

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