Bicarbonate ions and pH regulation of *Leishmania major* promastigotes

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Abstract ***Leishmania major*** **promastigotes are parasites endowed with a plasma membrane electrogenic H^+ pump and anionic channels. These systems have been thought to contribute to pH homeostasis of parasites and environmental adaptation by mediating extrusion of protons which are either generated metabolically or result from exogenous acid loads. In this work we show that HCO_3^- transport plays a physiological role in supporting pH regulation of parasites. Intracellular pH (pH_i) and the membrane potential (V_m) were assessed fluorometrically with pH sensitive and potentiometric dyes. We show that intracellular acidification, caused either by blocking the pump or the putative anion channel or by depleting Cl^- from cells, could be largely overcome by addition of HCO_3^-. Likewise, addition of HCO_3^- raises the steady state intracellular pH of untreated cells from 6.76 ± 0.01 to 6.98 ± 0.02 and induces membrane hyperpolarization in pump-inhibited cells. We provide evidence for the involvement of HCO_3^- transport systems that subserve pH homeostasis in *Leishmania* promastigotes. A major anionic pathway which is sensitive to anion transport blockers is apparently conductive in nature and accomodates ions such as HCO_3^- and Cl^-. In physiological conditions, the primary role of H^+ pumping is the generation of a relatively large membrane potential (V_m = −113 ± 4 mV) which subserves electrochemical-driven uptake of nutrients. The involvement of H^+ pumping in physiological pH regulation of promastigotes is apparently of a secondary nature.**

Key words: Leishmania; Promastigote; pH; Biocarbonate; Chloride; Channel; Fluorescence

1. Introduction

Leishmania parasites live and propagate in two distinct environments which differ primarily in their acid–base properties [1]: the pH in the midgut of the sandfly is 7.0–9.0 and in the phagolysosomes of mammalian macrophages is 4.5–5.5 . Adaptation and proliferation in such widely different environmental conditions demand appropriate regulatory mechanisms for maintaining a functional cell cytosol. These mechanisms also need to cope with acid loads generated by anaerobic metabolism of parasites [2]. A P-type H^+ pump was claimed to play a role in pH homeostasis and nutrient uptake in *Leishmania* parasites promastigotes [3,4]. Recently, we showed that a DCCD-sensitive electrogenic H^+ pump contributed to the maintenance of a highly hyperpolarized plasma membrane in *Leishmania* promastigotes [5,6]. In other systems, the operation of similar pumps demands a parallel conductive route for anions the function of which is to regulate H^+ pumping by dissipating the pump generated H^+ diffusion potential [7–9]. The operation of such Cl^- channels in *Leishmania* H^+ pumping was implicated on the basis of ion substitution studies, application of channel blockers and by fluorimetric measurements of membrane potential V_m [5,6].

All published work on pH homeostasis in *Leishmania* promastigotes described studies which were carried out in nominally CO_2-free media [3–5]. However, the environment in which promastigotes naturally develop, i.e. the gut of the sandfly, is characteristically alkaline and rich in $HCO_3^-/CO_3^=$ [1,10]. In such an environment, acid load is therefore expected to be generated only from within, by metabolic production of H^+ ions [2]. We therefore reassessed the contribution of transport mechanisms to pH homeostasis in *Leishmania major* promastigotes in physiologically relevant HCO_3^--containing media. Our studies provide evidence for the operation of HCO_3^--dependent mechanisms which play a primary role in pH homeostasis in these cells. These mechanisms include primarily a stilbene disulfonic-sensitive electrogenic pathway for HCO_3^- entry and possibly a Cl^-/HCO_3^- exchanger. The role of H^+ pumping in physiological pH regulation is therefore secondary and is apparently confined, physiologically, to the generation of a highly hyperpolarized membrane. Control of those transport mechanisms might be of therapeutic significance.

2. Materials and methods

Promastigotes of *Leishmania major* strain LCR-137 were grown as described elsewhere [5]. Measurements of cytosolic pH_i and membrane potential V_m were followed fluorimetrically at 30°C with the aid of the pH sensitive tetraacethoxymethyl 2′,7′-bis-(carboxyethyl)-5,6-carboxyfluorescein (BCECF) (ratio mode) and with the potentiometric dye bis-(1,3-diethylthiobarbituric acid) trimethine oxonol (DiSBAC2(3)) (bis-oxonol), as described previously [5,6]. Cl^--based media had the following basic composition (in mM): 137 NaCl, 4 KCl, 1.5 KH_2PO_4, 8.5 Na_2HPO_4, pH 7.4, supplemented with 20 HEPES, 11.1 glucose, 1 $CaCl_2$, and 0.8 $MgSO_4$. Cl^--free media had gluconate substituting for Cl. Cells were exposed either to either the anion transport blocker 4,4′-diisothiocyanodihydrostilbene-2-2′-disulfonic acid (H_2DIDS), the H^+-ATPase inhibitor dicyclohexylcarbodiimide (DCCD), or the uncoupler carbonylcyanide chlorophenylhydrazone (CCCP), as described previously [6]. All data shown were from individual experiments which were carried out three times independently (n = 3).

3. Results

In order to assess the role of HCO_3^- ions in supporting pH_i regulatory properties in *L. major* promastigotes, cells were initially exposed to ionic-balanced HEPES-buffered media. The cells maintained an intracellular pH_i of 6.76 ± 0.01, as previously shown by us and others [4,6]. However, upon addition of physiological concentrations of HCO_3^- (25 mM final) in a sealed cuvette, the steady-state pH_i rose to 6.98 ± 0.02 (Fig. 1A), while the extracellular pH remained in the range 7.4–7.5. The new pH_i attained by the cells in the presence of HCO_3^-, as well as the

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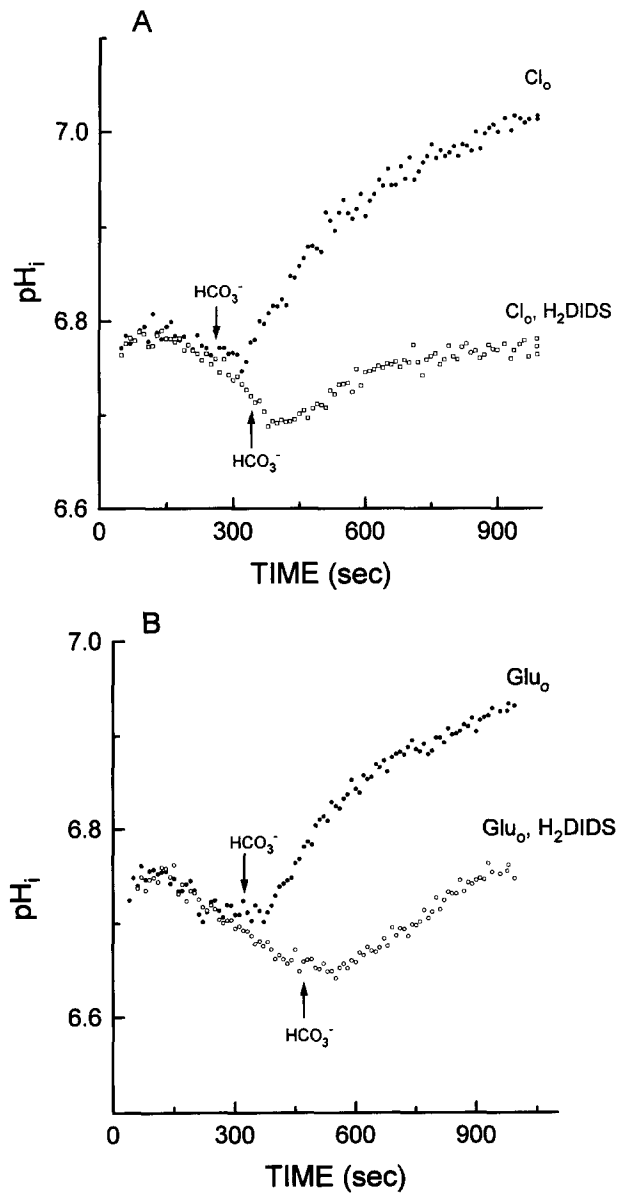

Fig. 1. The effect of HCO_3^- on intracellular pH_i. Cells preloaded with the pH indicator BCECF were suspended in either Cl^- medium (Cl_o^-) (A) or Cl^--free (gluconate) medium (Glu_o) (B) in the absence (●) or presence of the anion transport blocker H_2DIDS (○). pH_i was followed fluorimetrically (ratio mode) and computed from calibration curves as described previously [5]. The arrows indicate the time of addition of HCO_3^- (25 mM, final). Extracellular pH was maintained at 7.4–7.6 throughout the measurements.

extracellular pH in the cuvette (not shown), remained steady for a considerable period of time. In these conditions, pH_i was maintained close to neutrality (6.98 ± 0.02 pH units). Changes of extracellular pH from 6.5 to 8.0 by means other than HCO_3^- did not elicit comparable changes in pH_i. The intracellular alkalinization induced by addition of HCO_3^- was blocked by the membrane impermeant anion blocker, H_2DIDS (0.5 mM). In order to test for possible Cl^- dependency of the HCO_3^--induced alkalinization, cells were also pre-exposed to a Cl^--free (gluconate substituting for Cl^-) solution (Fig. 1B). In those conditions, pH_i fell steadily, as shown previously [5], but upon addition of HCO_3^- a new steady state, a more alkaline pH_i, was attained. In HCO_3^--supplemented Cl^--free medium, the cells attained a steady-state pH_i lower than in Cl^--containing medium (6.84 ± 0.01 vs 6.98 ± 0.02). However, the initial rate of HCO_3^--evoked alkalinization was similar (0.042 ± 0.01 and 0.039 ± 0.006 pH units/min, respectively) in either Cl^--containing or Cl^--free medium. That initial rate of alkalinization was markedly reduced in the presence of the anion blocker H_2DIDS (from 0.040 to 0.014 pH units/min). Although these results indicate the operation of HCO_3^- transport pathways, they do not provide sufficient information for discerning between electroneutral and conductive mechanisms.

The relative contribution of HCO_3^--dependent transport systems to cell pH homeostasis was studied under conditions known to arrest H^+ pump action. In the presence of the ATPase blocker DCCD (10 μM), cells underwent a gradual acidification (Fig. 2). However, pH_i partially increased upon addition of HCO_3^-, although at a rate relatively slower than in the absence of DCCD. The fact that cells substantially alkalinized after HCO_3^- addition indicated that there is a HCO_3^- transport pathway operative in the cells. That this pathway might be physiologically relevant is supported by the fact that HCO_3^- is seemingly present in substantial levels in the natural mediun were promastigotes dwell. Thus it can be deduced that in the presence of HCO_3^-, the contribution of H^+ pumping to pH homeostasis is secondary to that of buffering by HCO_3^-.

In order to study the nature of bicarbonate entry pathways into cells we studied the electrical properties of the anionic pathways present in *Leishmania* promastigotes with a potentiometric fluorescent dye [5]. In a recent study we demonstrated the operation of Cl^--conductive pathways which supported H^+ pumping. In Fig. 3A we show that in Cl^--containing, HCO_3^--free medium, the cells maintained a highly hyperpolarized membrane ($V_m = -113 \pm 4$ mV). In these conditions, i.e. marked hyperpolarization, assessment of HCO_3^- effects on V_m

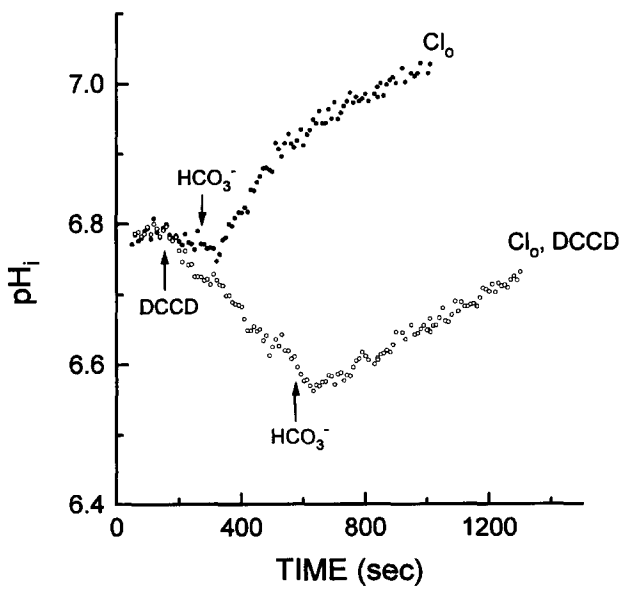

Fig. 2. The effect of HCO_3^- on intracellular pH_i in cells treated with DCCD. Cells loaded with BCECF were suspended in Cl^--based medium and pH was monitored as described in Fig. 1. The arrows indicate the times of additions of HCO_3^- (25 mM, final) and DCCD (20 μM, final).

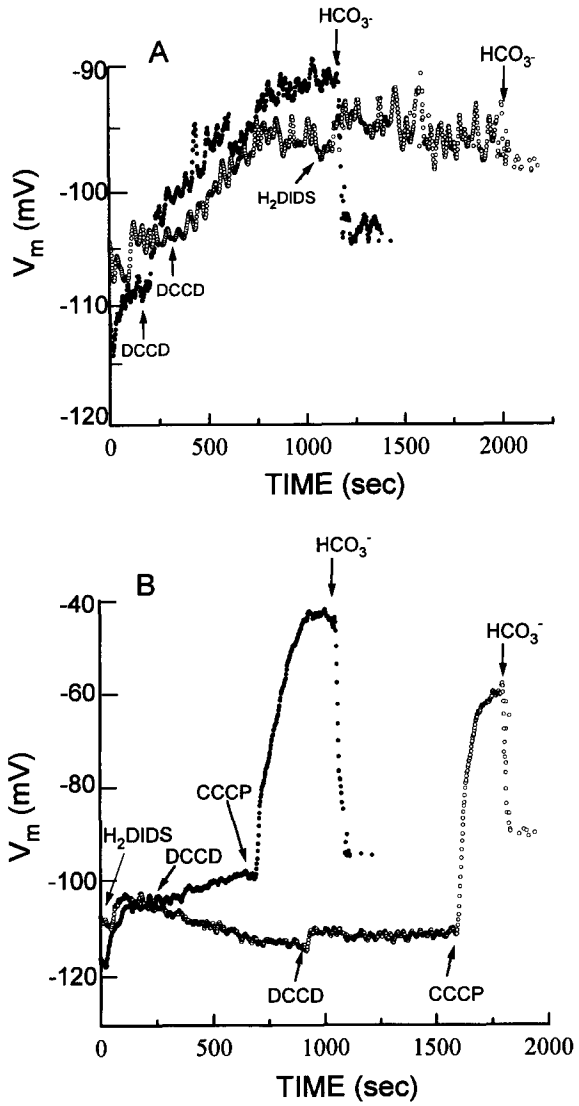

Fig. 3. Effect of HCO_3^- on cell membrane potential V_m. An aliquot of cells was added to a pre-equilibrated solution of 0.1 μM bis-oxonol in either Cl^- media (Cl_o^-), pH 7.4 (final suspension 5×10^6 cells/ml). The inhibitors DCCD (10 μM, final conc.) and CCCP (10 μM, final conc.) were added as indicated. HCO_3^- (25 mM, final) was added at the indicated points. H_2DIDS (500 μM) was added at the indicated times, just prior to HCO_3^- (A) or to DCCD (B) (○). Fluorescence determination of V_m values from calibration curves were as described earlier [6].

are limited by the sensitivity of the technique. We therefore opted for assessing HCO_3^- effects on V_m in cells predepolarized by treatment with DCCD and CCCP. As shown in Fig. 3A, DCCD depolarized the cell by about 20 mV in 10 min. HCO_3^- swiftly hyperpolarized the cells by about −5 mV in the presence of the anion blocker H_2DIDS and by −15 mV in the absence of blocker. Prolonged incubation with DCCD in the presence of H_2DIDS failed to hyperpolarize the cells (Fig. 3B), although it did not prevent CCCP from strongly (55 mV) depolarizing them. The DCCD + CCCP-treated cells also underwent hyperpolarization (−45 mV) when exposed to HCO_3^-, but H_2DIDS blocked that effect (−30 mV) only partially (30%). The HCO_3^--induced reduction in V_m in the presence of the electrogenic protonophore CCCP was not anticipated. Clearly, in the presence of DCCD and CCCP the cells markedly acidify and have therefore a low HCO_3^- content, thus creating a driving force for HCO_3^- entry. This is most likely to contribute to the anion diffusion potential which might counteract (partially) the effect of CCCP. However, the most compelling evidence resides in the effect of the anion blocker on V_m. Taken in toto, the results indicate the operation of a conductive pathway for HCO_3^- which might subserve pH homeostasis.

4. Discussion

Animal cells and microorganims experience intracellular acid–base loads which are generated either intrinsically by metabolism or extrinsically by environmental factors [2,11]. The heaviest loads are exerted on organisms which live and propagate in extreme acidic and alkaline conditions [11–13]. *Leishmania* parasites are organisms which obligatorily alternate between the neutral-alkaline HCO_3^--rich medium of the sandfly's midgut (the promastigote stage) and the acidic environment of the mammalian macrophage lysosome (the amastigote stage) [1,3–5,14]. The primary membrane mechanism invoked in pH regulation of *Leishmania* promastigotes has been associated with a P-type H^+ pump [3,4]. We have recently demonstrated in *Leishmania major* promastigotes the operation of a H^+ pumping mechanism which is electrogenic, DCCD sensitive, and regulated by the parallel action of a Cl^- channel [5,6]. Blockage of either component of the H^+ pumping mechanism, i.e. the H^+ pump itself or the Cl^-channel, led to a marked intracellular acidification, which resulted from accumulation of H^+ ions [2,6].

In near-to-neutral media (i.e. pH 7.2–7.4), the major acid load that free living promastigotes are likely to experience results from intracellular accumulation of metabolically generated H^+ ions [2]. In all previous studies of pH homeostasis in *Leishmania* (reviewed in [4]), the involvement of particular transport systems was assessed in non-physiological media, that is, media which were not supplemented with HCO_3^- (i.e. they were nominally CO_2-free media). In those conditions, H^+ pumping and an associated Cl^- channel activity were the major contributors to pH homeostasis [4–6]. However, since promastigotes naturally propagate in HCO_3^--rich media [1,4,10], it was of interest to explore the relative contributions of the above mentioned transporters to pH_i regulation in *L. major* promastigotes exposed to HCO_3^- ions. As shown in this work, addition of HCO_3^- to the media led to (i) cell alkalinization and to the maintenance of a near-to-neutral intracellular pH, which was about 0.25 pH units higher than in the absence of HCO_3^-, and (ii) substantial recovery of cells to baseline pH_i levels even in the presence of H^+ pump blockers.

The mechanism underlying the contribution of HCO_3^- to pH homeostasis is associated, in part, with an anion conductive pathway, as indicated by the hyperpolarizing effect of HCO_3^- on cells (Fig. 3). The putative anion channels apparently also conduct other anions [5], as supported by various pieces of evidence. First, we have recently shown a Cl^--induced hyperpolarization in Cl^--free media and in DCCD-predepolarized cells [6], and second, we showed here for HCO_3^- (Fig. 3) and elsewhere for Cl^- [6] that the anion-induced hyperpolarization is susceptible to blockage by classical anion transport blockers such as H_2DIDS [6]. The differences in V_m changes and pH elicited by HCO_3^- in Cl-containing vs. Cl-free (cell depleted)

media might result from the operation of other HCO_3^- transporters.

The mechanisms involved in regulating pH in vertebrate cells and in microorganims encompass a wide repertoire of exchangers, pumps and channels [7–9,11,15,16]. HCO_3^- transport mechanisms have been demonstrated to play an essential role in pH_i regulation in various cell systems [16]. These transport mechanisms involve primarily electroneutral co-transport systems such as a Na^+-dependent Cl^-/HCO_3^- exchanger [16,17], a Na^+-independent Cl^-/HCO_3^- exchanger [16,18–19], a Na^+/HCO_3^- co-transport system [16,20] and also a conductive pathway for HCO_3^- ions [21]. In this work we provided direct evidence for a primary physiological role of HCO_3^- ion transport in pH_i homeostasis of *Leishmania* promastigotes. The previously implied role of H^+ pumps in pH regulation of these cells was deduced on the basis of studies conducted in media lacking the important HCO_3^- ions. It is our view, that H^+ pumping might physiologically contribute to pH homeostasis depending on the CO_2/HCO_3^- concentration of the medium. However, in a HCO_3^--rich environment, such a contribution might only be secondary to that associated with HCO_3^- transporting systems, be they electrogenic, as shown in this work or electroneutral, as previously proposed [6]. Thus, in physiological media, the major role of electrogenic H^+ pumping would seem to be the generation of a marked hyperpolarized membrane, irrespective of the acid–base conditions of the environment. Such a mechanism could effectively subserve cation-dependent nutrient uptake [4] by providing a favorable electrochemical gradient for Na^+ and possibly H^+ ions, as amply demonstrated in various other microorganisms [22]. Pharmacological control of such mechanisms might be of therapeutic value.

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