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Chloride accumulation in freshly isolated Ehrlich ascites tumor cells: the role of the Na/K/2Cl cotransporter

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Abstract

When Ehrlich ascites tumor cells are removed from the peritoneal cavity and incubated in a saline solution, cells lose water, sodium, lactate and hydrogen ions and gain chloride. The gain of intracellular chloride exceeds that predicted from passive distribution. As chloride has been purported to play a role in volume regulation, it was of interest to identify factors responsible for controlling or maintaining intracellular chloride out of electrochemical equilibrium in Ehrlich cells. The results demonstrate that chloride accumulation in freshly isolated Ehrlich cells is sensitive to bumetanide, low extracellular K^+ and low extracellular Na^+, and is insensitive to DIDS. We conclude that chloride accumulation occurs due to the activity of the Na/K/2Cl cotransporter.

Keywords: Ehrlich tumor cell; Cotransporter, $Na^+/K^+/Cl^-$; Chloride accumulation; Sodium ion transport; Potassium ion transport; Chloride ion transport

1. Introduction

Volume regulation, a fundamental property of animal cells, functions to maintain cell integrity by opposing potentially harmful alterations in cell volume. Numerous studies have been conducted in an effort to determine the mechanisms responsible for cell volume regulation. The focus of many has been the identification of those elements capable of serving as a volume sensor. Studies using Ehrlich ascites tumor cells [18,23–25], giant axon of the squid [4], vascular endothelium [30] and the rectal gland of the dogfish shark [13,27] have suggested a role for intracellular chloride ($[Cl^-]_i$). Therefore, $[Cl^-]_i$ may serve as a volume sensor and as such provide the signal that controls the activity of the membrane transport proteins closely associated with volume regulation, i.e., the Na/K/2Cl cotransporter.

Intracellular chloride concentration also appears to play a regulatory role in Na/K/2Cl activity in secretory cells. Studies in the rectal gland of the dogfish shark [27] and in mammalian airway epithelia [16] indicate that secretagogues increase basolateral cotransporter activity in response to the opening of chloride channels in the apical membrane. These two events are coordinated so that volume changes, if they occur, are not significant. In addition, a recent study by Xu and coworkers [36] in which a cDNA encoding for a Na/K/2Cl cotransport protein from shark rectal gland was stably transfected into human HEK-293 cells, indicates that the expressed cotransporter is quiescent in the host cells but is activated when intracellular chloride is depleted. These studies provide evidence that $[Cl^-]_i$ is involved in regulating Na/K/2Cl cotransporter activity.

The postulation that chloride serves as a volume sensor implies that the cellular concentration of the anion is a tightly regulated quantity and not passively distributed across the cell membrane. Therefore, the present study was undertaken to identify factors responsible for controlling or maintaining intracellular chloride.

The results demonstrate that Ehrlich cells, when removed from the peritoneal cavity, lose water, sodium, lactate and hydrogen ions and gain chloride. Chloride accumulation is sensitive to bumetanide, low extracelluar K^+ and low extracellular Na^+. We conclude that chloride accumulation occurs due to stimulation of the Na/K/2Cl cotransporter. In addition, the chloride accumulation that occurs results in a higher $[Cl^-]_i$ than that predicted from

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passive distribution. Some of the present data have been presented in abstract form [34].

2. Materials and methods

2.1. Reagents

Ouabain, gramicidin, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and the lactate diagnostic kit were products of Sigma Chemical Co., bumetanide was supplied by Hoffman-La Roche, tetra[^{3}H]phenylphosphonium bromide (TPP^+) by Amersham Life Science, L-[^{3}H]glucose and 5,5-[^{14}C]dimethyloxazolidine, 2,4-dione (DMO) were purchased from New England Nuclear. All other reagents were of the highest quality available.

2.2. Cell suspensions

Experiments were performed with Ehrlich Lettre' ascites tumor cells (hyperdiploid strain) maintained in male, Swiss white mice by weekly transplantation. Tumor bearing animals between 9 and 11 days were used. Two types of cell suspensions were prepared. In the first, ascites were removed from the peritoneal cavity by aspiration into a 10 ml syringe. The cell density was variable and ranged from 200–600 mg/ml ascitic fluid. At this point, cells were either assayed immediately for electrolytes, water content and pH, or added to the standard incubation medium (130 mM NaCl, 6 mM KCl, 10 mM Hepes-NaOH; pH 7.35, 290–300 mosM). In the latter case, 3 ml of cell suspension were placed in a pre-warmed flask (37°C) followed by the addition of 10 ml of medium. Subsequent changes in electrolyte, water content and pH were then measured during the next 30 min. Ehrlich cells used in this manner will be referred to as 'freshly isolated cells'.

The second preparation involved removal of ascites from the peritoneal cavity by aspiration and washing the cells in standard medium. The cells were then resuspended in standard medium and subsequently incubated at a density of 60 mg/ml under an air atmosphere at 22–23°C. After about 30 min a steady state with respect to cell volume, pH and electrolyte composition is established. Cells processed in this manner will be referred to as 'prepared cells'.

2.3. Sampling

Following the initial incubation of prepared cells or the addition of standard medium to freshly isolated cells, 0.7 ml aliquots of cell suspension were removed periodically and added to pre-weighed microcentrifuge tubes (1.5 ml capacity) containing 0.7 ml of cold choline dihydrogen citrate wash solution (CDHC; [35]) containing 0.23 μCi of L-[^{3}H]glucose. The samples were immediately centrifuged (12 000 × g; 15 s), the supernatants collected and the tubes subsequently weighed before the addition of 1 ml of 1% (v/v) perchloric acid (PCA).

2.4. Ion fluxes

Net fluxes of Na^+, K^+ and Cl^- were estimated as the slopes (mEq/kg dry wt. min^{-1}) of the initial, time-dependent change in cellular electrolyte content.

2.5. Intracellular pH

Intracellular pH was estimated from the distribution (cell/medium) of [^{14}C]DMO, as described previously [3].

2.6. Membrane potential

The membrane potential (E_m) was estimated from the initial uptake of the lipophilic cation [^{3}H]TPP^+ [20,31]. A calibration curve relating [^{3}H]TPP^+ uptake to the K-equilibrium potential (E_K) was established by suspending prepared cells in Na-free choline chloride media of varying [K^+]. After 15 min, gramicidin (0.5 μM) was added to insure high K^+ permeability and TPP^+ uptake was then measured between 1 and 5 min later. For each measurement, 1 ml cell suspension (40 mg wet wt.) was added to a glass tube maintained at 37°C containing 20 μl [^{3}H]TPP^+ (0.2 μCi; 5 μM TPP bromide). After incubation (30 s), 0.7 ml cell suspension was added to 0.7 ml cold CDHC and immediately centrifuged (12 000 × g; 15 s). The supernatant was collected and the cells lysed with 1 ml 1% perchloric acid. To correct for [^{3}H]TPP^+ trapped within the extracellular space, 0.7 ml cell suspension was added to 0.7 ml cold CDHC containing [^{3}H]TPP^+ and immediately centrifuged. These samples were then processed as described above. The calibration curve which is shown in Fig. 1 was obtained by plotting TPP^+ uptake (cpm/mg

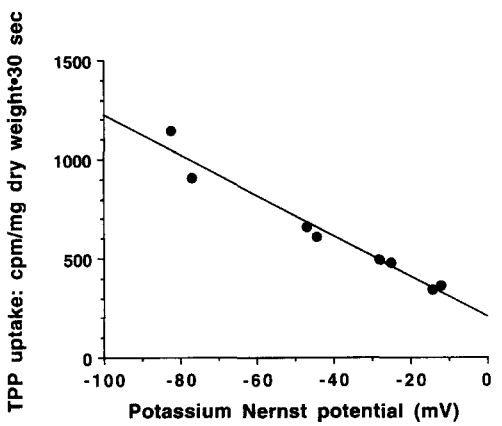

Fig. 1. Membrane potential calibration curve. Prepared cells were incubated in Na-free choline chloride media of varying K^+ and TPP^+ uptake was measured as described. Assuming that the total membrane conductance is dominated by K^+, the K^+ equilibrium potential (E_K) can then be considered to be equal to the membrane potential. Therefore, the relationship between initial, unidirectional TPP^+ influx to E_K was used to calculate the membrane potential of prepared cells suspended in standard medium. The figure shown is representative of other calibration curves generated in each experiment in which membrane potential was measured.

dry wt./30 s) versus the Nernst potential for K^+. Since gramicidin-induced K^+ conductance is assumed to dominate total membrane conductance, the Nernst potential for K^+ is presumed to be identical to the membrane potential. Although [^{3}H]TPP$^+$ uptake was measured in freshly isolated cells, calibration curves could not be generated using this cell population since the cellular electrolyte composition underwent continuous change. Time-dependent changes in uptake, however, were used to estimate the direction of change in the membrane potential.

2.7. Analytical methods

The cell pellets which were extracted with PCA for 60 min, were subsequently centrifuged 3 min at 12 000 × *g* to remove the PCA insoluble residue. Aliquots of the PCA extracts and medium were used to determine Na^+, K^+ and Cl^- as described previously [26]. Correction for Na^+, K^+ and Cl^- trapped within the extracellular space (ECS) was determined from the distribution of the non-penetrant, L-glucose. Cell water content corrected for the ECS was measured as described previously [2]. Data are reported as the means ± S.E.

3. Results

Table 1 compares the intracellular electrolytes, water and pH of freshly isolated and prepared cells. The freshly isolated cells were assayed immediately upon removal from the peritoneal cavity. These values are in agreement with previous reports [12,19,21,26] with the exception of K^+ which is higher than that reported by Ibsen and McKee [19] for tumor cells 9–11 days post-transplantation. Note that the water and intracellular Na^+ contents decrease by 18% and 54%, respectively, while the pH and Cl^- increase by 7.9% and 100%, respectively in prepared as compared with freshly isolated cells. Table 2 compares the extracellular electrolytes and pH of the ascitic fluid with standard medium. The values shown for ascitic fluid are consistent with previous reports [6,19]. The major differences between the ascitic fluid and standard medium is the extracellular pH (pH_o), chloride concentration ($[Cl^-]_o$), and that no lactate is added to the standard medium.

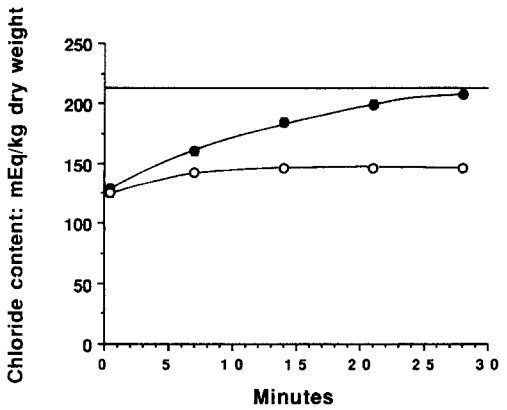

Fig. 2. Bumetanide sensitivity of Cl^- accumulation. Cells were removed from the peritoneal cavity and placed into standard medium with (open symbols; $n = 19$) or without (closed symbols; $n = 31$) 100 μM bumetanide, an inhibitor of the (Na/K/2Cl) cotransporter. Changes in Cl^- content were measured over the following 28 min. The horizontal line indicates Cl^- content in prepared cells (Table 1). Cl^- content was significantly lower ($P < 0.05$) in bumetanide-treated cells relative to control after 5 min.

Fig. 2 shows the chloride accumulation that occurs in the presence (open circles; $n = 19$) or absence (closed circles; $n = 31$) of 100 μM bumetanide, an inhibitor of the Na/K/2Cl cotransporter. At each point after 5 min, chloride was significantly lower in cells incubated with bumetanide ($P < 0.05$). Similar results were obtained in freshly isolated cells incubated in low extracellular K^+ ($[K^+]_o$; 1.2 mM) or Na^+ ($[Na^+]_o$; 9.4 mM) (data not shown). As Table 3 indicates, bumetanide, low $[K^+]_o$ and low $[Na^+]_o$ also significantly decreased the initial net chloride flux (J_{Cl^-}). In addition, chloride accumulation (data not shown) and J_{Cl^-} (Table 3) are moderately sensitive to DIDS (200 μM), an inhibitor of the anion exchanger. The results are consistent with the notion that it

Table 1
Intracellular electrolytes, water content and pH

	Freshly isolated	Prepared
H_2O/dry (kg water/kg dry weight)	4.20 ± 0.08 ($n = 25$)	3.46 ± 0.04 ($n = 78$)
(K) (mEq/kg dry wt.)	497.44 ± 12.53 ($n = 25$)	446.26 ± 9.85 ($n = 78$)
[K] (mmol/kg water)	118.57 ± 2.26 ($n = 25$)	129.08 ± 2.44 ($n = 78$)
(Na) (mEq/kg dry wt.)	161.00 ± 4.98 ($n = 25$)	74.59 ± 3.84 ($n = 78$)
[Na] (mmol/kg water)	38.70 ± 1.46 ($n = 25$)	21.35 ± 0.99 ($n = 78$)
(Cl) (mEq/kg dry wt.)	107.16 ± 2.99 ($n = 25$)	214.40 ± 3.07 ($n = 78$)
[Cl] (mmol/kg water)	25.76 ± 0.89 ($n = 25$)	62.18 ± 0.7 ($n = 78$)
(Lactate) (mEq/kg dry wt.)	74.68 ± 3.29 ($n = 20$)	ND
[Lactate] (mmol/kg water)	10.99 ± 0.55 ($n = 20$)	ND
pH	6.75 ± 0.03 ($n = 4$)	7.28 ± 0.01 ($n = 32$)

Ehrlich cells were removed from the peritoneal cavity and either assayed immediately for ion and water content and pH (freshly isolated), or washed and incubated for 60 min in standard medium (to establish an in vitro steady state) prior to assay (prepared). ND = not determined.

Table 2
Extracellular ion concentration and pH

	Ascitic fluid (mmol/kg water)	Standard medium (mmol/kg water)
[K]	6.85 ± 0.576 (n = 12)	5.59 ± 0.04 (n = 50)
[Na]	130.08 ± 2.49 (n = 12)	132.92 ± 0.513 (n = 50)
[Cl]	99.83 ± 1.62 (n = 12)	131.24 ± 0.652 (n = 50)
[Lactate]	14.04 ± 0.421 (n = 20)	ND
pH	6.83 ± 0.024 (n = 12)	7.36 ± 0.011 (n = 25)

Upon removal of ascites from the peritoneal cavity, the cells and fluid were separated by centrifugation. The pH and ionic composition of the ascitic fluid were assayed. The same analysis was carried out on aliquots of standard medium which had contained cells for at least 60 min. ND = not determined.

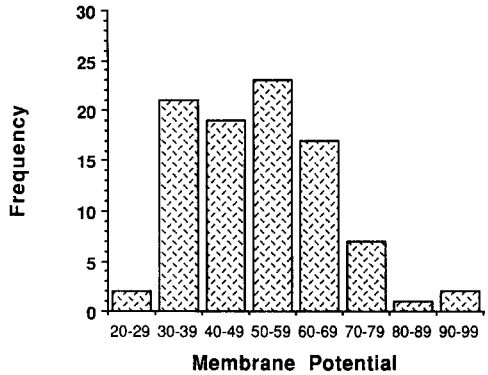

Fig. 3. Frequency distribution of E_m measurements. The E_m of prepared Ehrlich ascites tumor cells was measured as described, and the distribution is shown here. Each bar represents the number of E_m measurements that were calculated to be within the specified range. The mean E_m in prepared cells is 51.4 ± 1.53 mV, inside negative (n = 92).

is the Na/K/2Cl cotransporter that is responsible for chloride accumulation in freshly isolated Ehrlich cells.

To decide whether the final chloride distribution in prepared cells was due to passive processes, it was necessary to not only measure the chemical concentration gradient but also the membrane potential (E_m). Fig. 3 shows the frequency distribution of 92 Em measurements made in prepared Ehrlich ascites tumor cells. The mean E_m is 51.4 ± 1.53 mV, inside negative. This value is consistent with some studies reporting membrane potential in Ehrlich cells [15,21,22], but is inconsistent with others [8,33]. This is likely due to differences between Ehrlich cell strains and not to invalid techniques. A membrane potential of −51 mV predicts a chloride distribution ratio (r_{Cl}) of 6.75 ($[Cl^-]_o/[Cl^-]_i$). In fact, r_{Cl} in prepared cells is 2.11, which in turn predicts a E_m of 20 mV, inside negative. Fig. 4 shows the changes in r_{Cl} that occur when freshly isolated cells are incubated in standard medium. These data indicate that chloride is accumulated in freshly isolated cells to such an extent that it is out of electrochemical equilibrium.

The driving force acting on the cotransporter to mediate net movement of Na^+, K^+ and Cl^- is generally described as the sum of the transmembrane chemical potential gradients for these ions ($\Delta\mu_{Net}$). Net movement of Na^+, K^+ and Cl^- mediated by the cotransporter occurs when $\Delta\mu_{Net}$ is not equal to zero [17,24]. The $\Delta\mu_{Net}$ in freshly isolated cells is 0.66 kcal/mol (calculated from data in Tables 1 and 2), which indicates that there is a net inwardly directed driving force. When freshly isolated cells are placed into standard medium, the net transport of Na^+, K^+ and Cl^- is mediated by the cotransporter until $\Delta\mu_{Net}$ approximates

Table 3
Initial, net ion fluxes (J^{Ion}) in freshly isolated cells

	J^{Cl-} (mEq/kg dry weight/min)	J^{Na+} (mEq/kg dry weight/min)	J^{K+} (mEq/kg dry weight/min)
Control	4.09 ± 0.24 (n = 31)	−3.29 ± 0.17 (n = 29)	0.27 ± 0.48 (n = 29)
Bumetamide (100 μM)	1.5 ± 0.16 * (n = 19)	−4.07 ± 0.32 * (n = 19)	−0.85 ± 0.42 (n = 19)
DIDS (200 μM)	3.01 ± 0.5 (n = 8)	−3.35 ± 0.59 (n = 8)	−1.17 ± 0.53 (n = 8)
Bumetanide + DIDS	0.89 ± 0.29 * (n = 5)	−3.65 ± 0.68 (n = 5)	−4.08 ± 0.78 * (n = 5)
Ouabain (1 mM)	3.69 ± 0.5 (n = 4)	2.24 ± 0.3 * (n = 4)	−4.52 ± 0.74 * (n = 4)
Bumetanide + ouabain	1.59 ± 0.15 * (n = 4)	2.2 ± 0.31 * (n = 4)	−6.58 ± 0.78 * (n = 4)
Low K^+ (1.2 mM)	0.48 ± 0.26 * (n = 5)	ND	ND
Low Na^+ (9.4 mM)	1.75 ± 0.77 * (n = 5)	ND	ND

Ehrlich cells were removed from the peritoneal cavity and incubated in standard medium at 37°C under oxygen. Measurements of ion content and water were then made over 28 min. J^{Net} (net flux) for each ion was calculated from the time-dependent change in electrolyte content and reflects a net movement of that ion into our out of the cell. A (−) indicates a net loss of the ion, all others a gain. ND = not determined. (*) indicates a significant difference relative to control ($P < 0.05$).

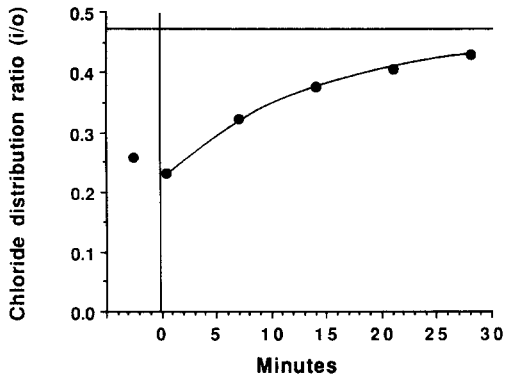

Fig. 4. Chloride distribution ratios. Chloride distribution ratios (r_{Cl}) were calculated from the average ($n = 31$) intracellular chloride concentration measured at each time point and the appropriate extracellular chloride concentration ($[Cl^-]_o/[Cl^-]_i$). The first point indicates r_{Cl} in freshly isolated cells before the addition of standard medium. The horizontal line represents r_{Cl} of prepared cells.

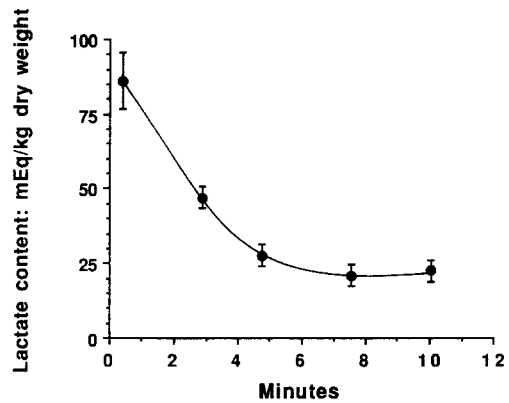

Fig. 6. Changes in lactate of freshly isolated cells. Cells were removed from the peritoneal cavity and placed into standard medium. Changes in intracellular lactate content were measured over the following 10 min. Results are the means ± S.E. of 6 experiments.

zero ($\Delta\mu_{Net} = -0.044$ kcal/mol at $t = 28$ min), as shown in Fig. 5. Once $\Delta\mu_{Net}$ reaches zero, the cotransporter becomes relatively quiescent (i.e., no further net chloride accumulation occurs), as has been reported previously in prepared Ehrlich cells [24].

One of the questions that arises from the present study is why intracellular chloride is relatively low in cells maintained within the peritoneal cavity, in spite of a gradient that favors movement of ions mediated by the Na/K/2Cl cotransporter. Since freshly isolated cells contain lactate and have a relatively low pH_i relative to prepared cells, it is possible that either the high lactate content or the low pH_i of freshly isolated cells may inhibit the cotransporter in vivo. Fig. 6 shows the loss of lactate from freshly isolated cells incubated in standard medium. Lactate has been shown to be transported out of the Ehrlich cell as lactic acid (either as H^+/lactate$^-$ symport or OH^-/lactate$^-$ antiport; [7]). The expectation is that once lactate begins to move out of the cell, pH_i increases reversing the inhibitory effect of H^+ or lactate on the Na/K/2Cl cotransporter and an accumulation of chloride.

As Fig. 7 indicates, freshly isolated cells immediately alkalinize upon the addition of standard medium (from 6.75 to 7.61), and then gradually regulate pH_i to the level observed in prepared cells. Based on the buffering capacity (27.7 mEq H^+/kg cell water/pH unit) of the Ehrlich cell reported by Bowen and Levinson [3], the initial change in pH_i is equivalent to a loss of 23.8 mmol H^+/kg cell water. This alkalinization occurs before a significant amount of lactate is lost and indicates that increasing extracellular pH (from 6.83 in ascitic fluid to 7.36 in standard medium) is sufficient to significantly alter pH_i and disinhibit the Na/K/2Cl cotransporter.

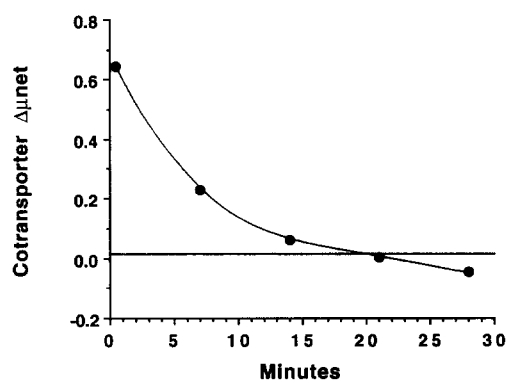

Fig. 5. Driving force for the cotransporter. The sum of the chemical potentials ($\Delta\mu_{Net}$) for Na^+, K^+ and Cl^- was calculated from the average concentrations ($n = 29$ for Na^+ and K^+; $n = 31$ for Cl^-) in freshly isolated cells incubated in standard medium for each time point according to: $\Delta\mu_{Net} = RT\ln([Na^+]_o \cdot [K^+]_o \cdot [Cl^-]_o^2)/([Na^+]_i \cdot [K^+]_i \cdot [Cl^-]_i^2)$. The horizontal line indicates the $\Delta\mu_{Net}$ for the cotransporter in prepared cells.

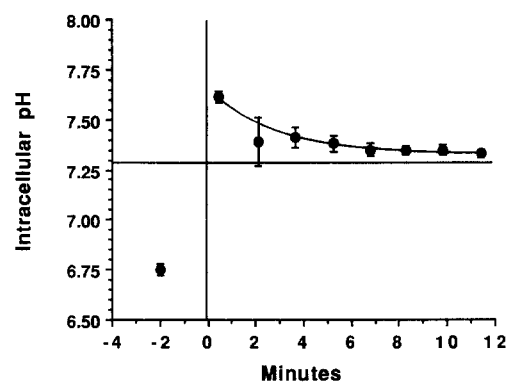

Fig. 7. Changes in pH_i of freshly isolated cells. Cells were removed from the peritoneal cavity and placed into standard medium. Changes in intracellular pH were measured over the following 12 min. The first data point represents the pH_i of freshly isolated cells before exposure to standard medium (Table 2). The horizontal line indicates the pH_i of prepared cells (Table 1). Results are the means ± S.E. of 7 experiments.

4. Discussion

The present study demonstrates that chloride is out of electrochemical equilibrium in Ehrlich mouse ascites tumor cells incubated in standard medium. Although for many years it was generally believed that chloride was in electrochemical equilibrium in most cells, there are a number of cell types including leukocytes [14], giant axon of the squid [4], vascular endothelial cells [30], vascular smooth muscle cells [10,11], cultured mesangial cells [28] and many types of secretory epithelia [1,5,9,13,27,29,32] in which chloride is reported to be out of electrochemical equilibrium. In Ehrlich cells, it is the Na/K/2Cl cotransporter that is responsible for the $[Cl^-]_i$ reaching a higher level than can be predicted from the membrane potential. This is based on the observations that chloride accumulation in freshly isolated cells is bumetanide-sensitive, requires the presence of both Na^+ and K^+ and is DIDS-insensitive.

Further evidence for the role of the Na/K/2Cl cotransporter in Ehrlich cell chloride accumulation is the significant decrease in J^{Cl^-} (indicating a decreased rate of influx) that occurs in the presence of bumetanide, low $[K^+]_o$ or low $[Na^+]_o$. In addition, J^{Na^+} becomes significantly more negative (indicating increased rate of efflux) in the presence of bumetanide. These data are consistent with a net movement of Na^+ into the cell through the cotransporter in the absence of bumetanide. While J^{K^+} in the presence of bumetanide was not significantly different relative to control, there is a 'leak' of K^+ out of the freshly isolated cells that was not present in control cells, indicating that, in the absence of bumetanide, the Na/K/2Cl cotransporter can move K^+ into the cell. In addition, when the Na^+/K^+-ATPase is inhibited by ouabain, there is a significant increase in the rate of K^+ efflux, which is increased further in the presence of both bumetanide and ouabain (Table 3). The effect of DIDS, in the presence of bumetanide, on J^{K^+} may reflect an activation of K^+ channels by DIDS which is masked by an active Na/K/2Cl cotransporter in the absence of bumetanide.

The significance of chloride being out of electrochemical equilibrium is not completely understood at this time. It has been suggested that $[Cl^-]_i$ plays a direct role in the regulation of Na/K/2Cl cotransport activity [36] and if this is the case, may be involved in the signaling process for volume regulation [4,13,18,23–25,27,30]. Studies in prepared Ehrlich ascites tumor cells have shown that the Na/K/2Cl cotransporter is involved in regulatory volume increase (RVI) only if $[Cl^-]_i$ is first depleted [18,23–25]. This is consistent with the notion that $[Cl^-]_i$ may interact with the regulation of the activity of the Na/K/2Cl cotransporter.

In summary, freshly isolated Ehrlich cells accumulate chloride to a greater extent than is predicted from the membrane potential. This chloride accumulation is sensitive to bumetanide, low $[K^+]_o$ and low $[Na^+]_o$, and is insensitive to DIDS which indicates that it is the Na/K/2Cl cotransporter that is responsible. The stoichiometry of the cotransporter approximates 1 Na/1 K/2 Cl. In addition, freshly isolated Ehrlich cells lose hydrogen ions and lactate.

Acknowledgements

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