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Regulatory volume decrease in Ehrlich ascites tumor cells is not mediated by a rise in intracellular calcium

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Ehrlich ascites tumor cells suspended in hyposmotic solution initially swell and then shrink back towards normal volume, a process known as regulatory volume decrease (RVD). RVD is characterized by a specific loss of KCl, although the mechanism for this is currently unknown. The hypothesis that a rise in intracellular calcium ($[Ca^{2+}]_i$) activates calcium-sensitive ion conductances to initiate RVD was investigated. The results indicate that in the Ehrlich cell no rise in $[Ca^{2+}]_i$ occurs when the extracellular osmolality is reduced from 300 mosM to 180 mosM. These findings were substantiated by the lack of sensitivity of RVD to the Ca^{2+}-sensitive K^+ channel blockers charybdotoxin (CTX) and nifedipine. In contrast, the ionophore ionomycin induced a cell shrinkage that was sensitive to CTX and nifedipine indicating that a rise in $[Ca^{2+}]_i$ could play a role in cell volume reduction but that this occurred by a mechanism different from that observed in RVD. The conclusion from these experiments is that Ca^{2+} does not act as a second messenger for RVD in the Ehrlich cell.

Introduction

Animal cells when suspended in hyposmotic medium (less than 300 m osM) initially swell but then exhibit regulatory volume decrease (RVD) whereby they return towards normal volume within a few minutes [1–3]. The decrease in cell volume is due to the loss of solute, primarily K^+ and Cl^-, and water that is osmotically obligated to it. A rise in $[Ca^{2+}]_i$ has often been hypothesized to be responsible for increasing the K^+ permeability, presumably by activating Ca^{2+}-sensitive K^+ channels [2–6] as well as increasing Cl^- permeability [2,5]. However, $[Ca^{2+}]_i$ has been measured during RVD in only a few cell types. For example, in MDCK cells a rise in $[Ca^{2+}]_i$ during RVD has recently been demonstrated [4]. Beck et al. [7], have also reported a transient rise in $[Ca^{2+}]_i$ following hyposomotic challenge in proximal convoluted tubule cells, but were unable to relate this directly to the RVD response observed. On the other hand, Grinstein and Smith [8] measured $[Ca^{2+}]_i$ in human lymphocytes directly and found that intracellular calcium did not rise during hyposmotic challenge.

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The present study was undertaken to investigate the role of Ca^{2+} in regulatory volume decrease in Ehrlich cells. The results show that during a hyposmotic challenge $[Ca^{2+}]_i$ does not increase, but rather decreases in proportion to the volume increase. Furthermore, agents known to block Ca^{2+}-sensitive K^+ channels are without effect on RVD. Thus, we conclude that intracellular Ca^{2+} apparently does not mediate regulatory volume decrease in Ehrlich cells. Some of the present data have been presented in abstract form [9].

Materials and Methods

Reagents

Fura-2 acetoxymethyl ester (fura-2/AM) was supplied by Molecular Probes (Junction City, OR) poly(L-lysine) and nifedipine by Sigma (St. Louis, MO), charybdotoxin (CTX) by Research Biochemicals (Natick, MA), ionomycin by Calbiochem (San Diego, CA), and [^{3}H]sucrose (12.50 Ci/mmol) by New England Nuclear (Boston, MA). All other reagents were of the highest quality possible.

Cell suspension

Experiments were performed with Ehrlich Lettré ascites tumor cells (hyperdiploid strain) maintained in white male mice (Hauschka/Institute for Cancer Research; Ha/IRC) by weekly transplantation. Cells grown for 9 to 11 days were removed from the peri-

toneal cavity by aspiration and washed (twice) free of ascitic fluid. Cells were subsequently incubated at a density of 60 or 100 mg wet weight/ml under air for 30 min at 21–23°C in standard, nominally Ca^{2+}-free solution containing (mM): 150 NaCl, 5 KCl, 10 Hepes-NaOH (pH 7.3, 290–300 m osM). Additional incubations and all experiments were conducted under air at 21–23°C.

Regulatory volume decrease (RVD)

Following the initial incubation period, the osmolarity of the extracellular medium was reduced to 180 mosM by the addition of 10 mM NaHepes. At periodic intervals during the next 25 min, 0.7-ml aliquots of cell suspension were removed and placed in pre-weighed 1.5 ml microcentrifuge tubes containing 0.7 ml ice-cold choline dihydrogen citrate solution (CDHC, 180 mosM; [10]) plus 0.35 μCi [^{3}H]sucrose. The samples were immediately centrifuged (15 s at 15 000 $\times g$), the supernatant collected, and the tubes subsequently weighed. The cell pellets were then extracted with 1 ml of 1% (v/v) perchloric acid (PCA).

RVD experiments were also conducted in the presence or absence of 25 nM CTX or 100 μM nifedipine [11] which are known to inhibit Ca^{2+}-activated K^+ channels. These substances were added to the cell suspension several minutes prior to the hyposmotic challenge and subsequent procedures.

Ionomycin-induced volume changes

Ehrlich cells suspended in standard medium in the presence or absence of 25 nM CTX or 100 μM nifedipine were incubated with either 2 or 5 μM ionomycin. Aliquots were then removed and processed to measure cell water and ion content as described above.

Analytical methods

The cell pellets were extracted with PCA at room temperature for 60 min and then centrifuged (2 min 15 000 $\times g$) to remove the PCA-insoluble residue. Aliquots of the PCA extracts and medium were used to determine Na^+ and K^+ by emission flame photometry using Li^+ as an internal standard. Cl^- was assayed with an autotitrator [12]. Additional aliquots of the PCA extract and the CDHC supernatant were assayed for [^{3}H]sucrose with a liquid scintillation spectrometer. Correction for Na^+, K^+ and Cl^- trapped in the extracellular space (ECS) was based on the distribution of the [^{3}H]sucrose, a nonpenetrating solute. Cell volume was measured as described previously [13] and is reported as kg cell water (after correction for ECS) per kg dry cell weight. Steady-state cell volume was determined from cells incubated in standard solution for at least 30 min and was equivalent to 3.57 ± 0.16 ($n = 30$) kg water/kg dry weight. Data are reported as the means $\pm$ S.E.

Measurement of cytosolic free Ca^{2+}

Cytosolic calcium concentration ($[Ca^{2+}]_i$) was determined by digitized fluorescent video image microscopy using the Ca^{2+}-sensitive fluorophore fura-2/AM as described previously [14]. Stock solutions of fura-2/AM were prepared in dimethyl sulfoxide (DMSO; 1 mg/ml). Cells were loaded with the fura-2/AM (2 μM) solution for 30 min at 23°C following which a 5 μl aliquot was plated on specially designed poly(L-lysine)-coated wells containing 0.7 ml standard incubation medium (300 m osM). Fluorescent images of fields containing more than 20 cells, excited at 340 and 380 nm and emitting at greater than 510 nm were taken before and at intervals after the osmolarity was reduced to 180 m osM by the addition of a small volume of 10 mM NaHepes or after ionomycin (2 μM) was added to the isotonic medium in the presence or absence of 25 nM CTX or 100 μM nifedipine. Corrected images were obtained by subtracting unfocused, cell-free images while masks were prepared to eliminate intercellular areas. The average 340/380 ratio was determined for each cell and converted to $[Ca^{2+}]$ according to Grynkiewicz et al. [15].

Changes in the cell volume, in response to variations in environmental osmolarity results in variations in cytoplasmic ionic strength. Consequently, the effect of altered ionic strength on the fura-2/Ca dissociation constant (K_d) was determined. Standard curves for calibration of free $[Ca^{2+}]$ were prepared as described by Grynkiewicz et al. [15] using Ca^{2+}-EGTA buffers. Separate curves were determined for different conditions of cytoplasmic ionic strength. Normal ionic strength medium was prepared to mimic the intracellular activities previously reported for these cells (156 mM KCl, 11 mM NaCl, 10 mM Hepes, pH 7.1; [16]). Media of reduced (93 mM KCl, 7 mM NaCl, 10 mM Hepes, pH 7.1) and elevated (225 mM KCl, 25 mM NaCl, 10 mM Hepes, pH 7.1) ionic strength were prepared to mimic the expected intracellular contents after cell swelling and shrinkage, respectively. Apparent association constants (K_{CaEGTA}) for the Ca^{2+}-EGTA complex were calculated from the corrections given by Harrision and Bers [17] for reduced and normal ionic strengths ($K_{CaEGTA} = 4.43 \times 10^6$ M^{-1} and 3.81×10^6 M^{-1}, respectively) and from the data of Grynkiewicz et al. [15] for high ionic strength ($K_{CaEGTA} = 3.19 \times 10^6$ M^{-1}). The relationship between K_d and ionic strength ($T = 21$–23°C) is described by:

$$-\log(K_d) = 7.217 - 0.0045 \cdot I$$

where I is the ionic strength of the cytoplasm. If it is assumed that changes in cytoplasmic ionic strength parallel those in the environment, then for cells in normal saline environment, $K_d = 192$ nM, while for cells in 60% normal osmolarity, $K_d = 118$ nM.

Results

The upper panel of Fig. 1 shows a typical change in cell water content during RVD and is similar to that previously reported [18]. 30 s after the osmolarity is reduced to 180 mosM the cells swell, increasing their volume from 3.51 kg H_2O/kg dry weight to 5.35 kg H_2O/kg dry weight. This is less than that predicted from the theoretical swell (5.85 kg H_2O/kg dry weight), and is due, at least in part, to an incomplete mixing [19,20] and in large part to the presence of osmotically inactive water [21]. The swelling phase is followed by a return of the volume towards the isotonic level. The reduction in cell volume correlates with the loss of KCl (Table I) and is consistent with the idea that volume increase activates conductive pathways for K^+ and Cl^-, resulting in the loss of KCl driven by the net outward gradient [5]. This would allow water to leave the cell down the osmotic gradient.

Steady-state intracellular calcium levels in Ehrlich cells incubated in nominally Ca^{2+}-free medium are 85.5 ± 1.5 nM (n = 329), which is somewhat depressed compared to cells incubated with 2 mM Ca^{2+} in the extracellular phase ($[Ca^{2+}]_i$ = 105 ± 2.9 nM (n = 51)). The lower portion of Fig. 1 shows that following the reduction of osmolarity from 300 to 180 mosM, $[Ca^{2+}]_i$ decreased from 85 ± 3.3 nM to 60 ± 1.9 nM. This decrease in $[Ca^{2+}]_i$ parallels the increase in cell volume. Measurements of $[Ca^{2+}]_i$ during the interval immediately following the osmotic perturbation or during volume recovery failed to demonstrate any increase in $[Ca^{2+}]_i$. The presence of 2 mM Ca^{2+} in the extracellular phase had no effect on the volume response or the Ca^{2+} change, with $[Ca^{2+}]_i$ levels decreasing from 101 ± 3.9 nM to 71 ± 4.1 nM (data not shown).

Exposure of Ehrlich cells to the Ca^{2+} ionophore ionomycin, causes the selective loss of KCl and water, a response that mimics RVD, at least superficially. The underlying mechanism for the ionomycin response presumably involves the activation of Ca^{2+}-sensitive K^+ channels secondary to the increase in $[Ca^{2+}]_i$. This permits a net loss of K^+, a conductive loss of Cl^-

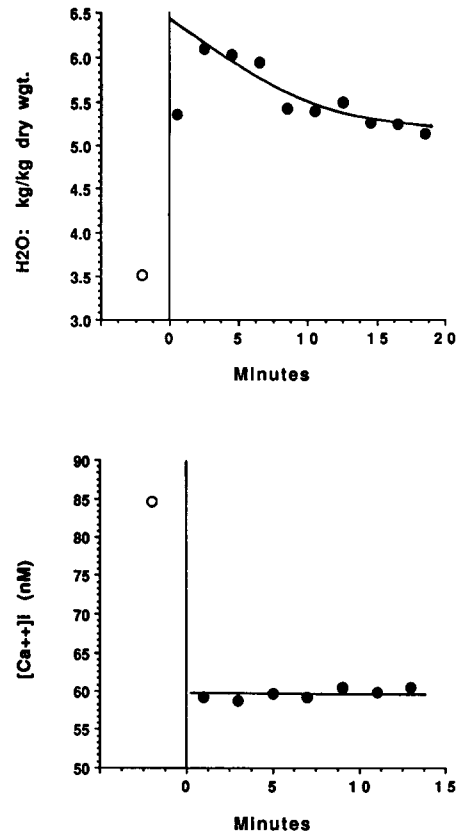

Fig. 1. Regulatory volume decrease (RVD). The osmolarity of the standard suspending medium (6 mM K^+, 135 mM Na^+, 140 mM Cl^-; pH 7.3; 300 mosM) was reduced from 300 mosM to 180 mosM by the addition of 10 mM NaHepes at time zero. Upper panel: Change in cell volume (water content) was measured during the next 20 min. Representative experiment displayed; seven others gave similar results. Lower panel: Change in $[Ca^{1+}]_i$ during RVD was measured by fluorescent video image microscopy during the next 15 min. Representative experiment displayed; nine others gave similar results. Cell $[Ca^{2+}]$ and water content prior to the reduction in osmolarity are shown for reference (open symbol).

TABLE I

Intracellular ionic composition following cell volume changes

Ehrlich cell suspension was incubated at 23°C in standard medium (see Materials and Methods) in the presence or absence of 5 μM ionomycin or in hyposmotic medium (180 mosM). Aliquots of cell suspension were removed from each incubation protocol for the measurement of ion and water content (see Methods). 'After RVD' indicates the electrolyte and water content of cells after 20 min of hyposmotic challenge. 'After ionomycin' indicates ion and water content after 30 s of exposure to ionomycin.

	K^+ (mequiv./kg dry wt.)	Na^+ (mequiv./kg dry wt.)	Cl^- (mequiv./kg dry wt.)	H_2O (kg/kg dry wt.)
Steady-state control	521 ± 6 (145.3 ± 2) [a]	107 ± 3 (30.1 ± 10)	194 ± 3 (54.2 ± 0.9)	3.59 ± 0.2
After RVD	428 ± 17 (85.2 ± 3)	113 ± 7 (22.8 ± 2)	149 ± 6 (29.7 ± 1)	5.02 ± 0.1
After ionomycin	452 ± 14 (144.9 ± 5)	100 ± 4 (31.7 ± 1)	137 ± 6 (43.8 ± 2)	3.14 ± 0.1

[a] Ion concentrations are expressed as mequiv./kg water. Values reported represent a minimum of seven separate determinations.

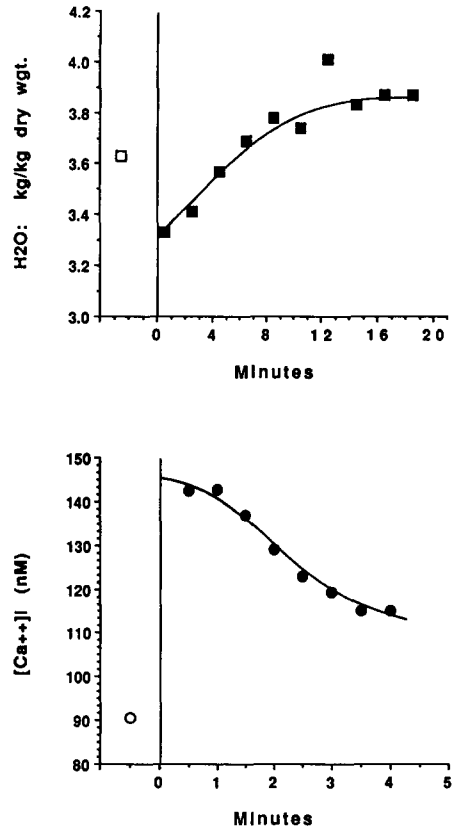

Fig. 2. Ionomycin-induced volume response. Cells in standard suspending medium were exposed to ionomycin at time zero. Upper panel: Change in cell water content was measured during the next 20 min following exposure to 5 μM ionomycin. Representative experiment displayed; nine others gave similar results. Lower panel: Change in $[Ca^{2+}]_i$ after exposure to 2 μM ionomycin. Representative experiment displayed; one other gave similar results. Cell $[Ca^{2+}]_i$ and water content before the addition of ionomycin are shown for reference (open symbol).

driven by the net outward gradient and obligatory osmotic shrinkage [22,23].

Fig. 2 demonstrates that the addition of ionomycin to cells at isotonic volume results in a transient shrinkage and an elevation of intracellular Ca^{2+}. To demonstrate that $[Ca^{2+}]_i$ could be mobilized during a hyposmotic challenge in the absence of added extracellular calcium, ionomycin (2 μM) was added to cells incubated in hyposmotic medium. In this case the hyposmotic challenge again results in a reduction in $[Ca^{2+}]_i$ (Fig. 3) but the subsequent addition of ionomycin is effective in mobilizing Ca^{2+} ($[Ca^{2+}]_i = 186 \pm 8.0$ nM).

The sensivity of Ehrlich cells to CTX and nifedipine was investigated in order to elucidate the role of Ca^{2+}-sensitive K^+ channels in RVD. The upper panel of Fig. 4 indicates that there is no difference in the RVD response in the presence or absence of CTX or nifedipine. If intracellular calcium were involved in activating Ca^{2+}-sensitive K^+ channels, leading to the selective loss of KCl during hyposmotic challenge, these drugs would be expected to inhibit RVD. The results

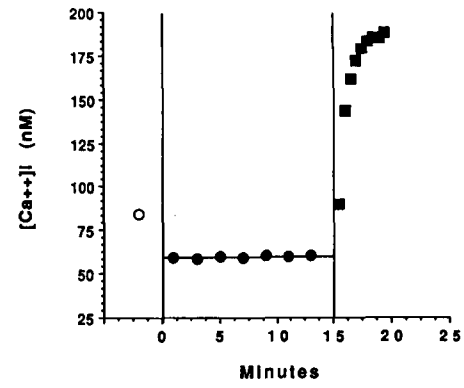

Fig. 3. Ca^{2+} availability during hyposmotic challenge. The extracellular osmolarity of cells loaded with fura-2/AM and plated in standard, nominally Ca^{2+}-free medium (300 m osM) was reduced to 180 mosM by the addition of an appropriate amount of 10 mM NaHepes (closed circles). After 15 minutes of hyposmotic challenge, cells were then exposed to 2 μM ionomycin (closed squares). Change in $[Ca^{2+}]_i$ was measured by fluorescent video image microscopy. $[Ca^{2+}]_i$ before the reduction in osmolarity and the addition of ionomycin is shown for reference (open symbol).

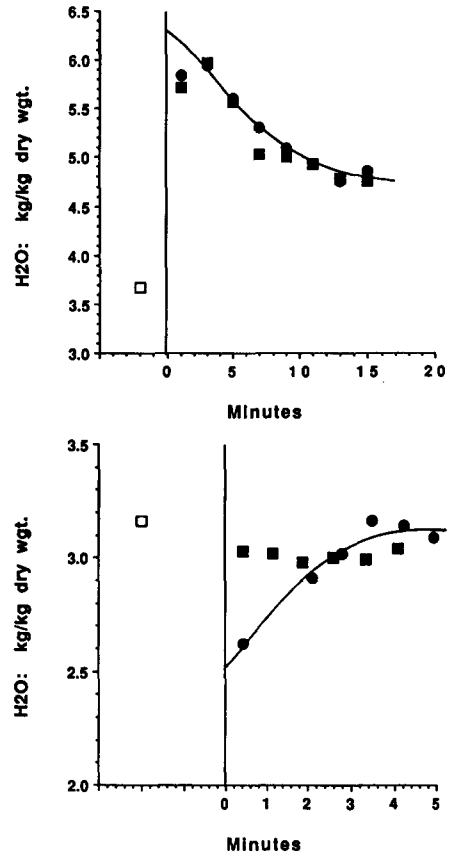

Fig. 4. Effect of CTX and nifedipine on volume changes. Upper panel: The osmolarity of the standard suspending medium was reduced from 300 to 180 mosM in the presence (squares) or absence (circles) of 25 nM CTX or 100 μM nifedipine. Changes in cell water content were measured during the next 15 min. Lower panel: Cells in standard medium were exposed to 5 μM ionomycin in the presence (squares) or absence (circles) of CTX or nifedipine. Changes in cell water content were measured during the next 4.5 min. Cell water content before the reduction in osmolarity and the addition of ionomycin is shown for reference (open symbols). Representative experiment displayed; five others gave similar results.

indicate that this is not the case. However, the ionomycin-induced volume response is inhibited by either CTX or nifedipine suggesting that the Ca^{2+}-sensitive K^+ channels are not activated in the presence of these compounds (Fig. 4, lower panel).

Discussion

The role of intracellular calcium as a second messenger mediating RVD has been examined in other cell types including MDCK cells [4], toad bladder cells [24], rabbit proximal convoluted tubule cells [7] and human lymphocytes [8]. In MDCK cells and toad bladder cells, RVD is dependent on extracellular Ca^{2+} and is associated with a transient increase in $[Ca^{2+}]_i$ [4,24]. In rabbit proximal convoluted tubule cells subjected to a hyposmotic challenge, Ca^{2+} rises transiently although this response is not necessary for RVD [7]. In contrast, there is no change in $[Ca^{2+}]_i$ during RVD in lymphocytes [8]. The experiments reported here were directed to identifying changes in $[Ca^{2+}]_i$ that occur during regulatory volume decrease in Ehrlich cells subjected to a hyposmotic challenge.

Reduction of the extracellular osmolarity from 300 mosM to 180 mosM results only in the decrease in $[Ca^{2+}]_i$ expected from the increase in cell volume (Fig. 1). No rise in $[Ca^{2+}]_i$ could be detected before or during RVD. It is possible that small, localized areas of Ca^{2+} mobilization occur in the Ehrlich cell and are undetected by our methodology. However, the failure of the two Ca^{2+}-sensitive K^+ channel blockers, CTX and nifedipine, to affect RVD argue against this possibility (Fig. 4, upper panel). In addition, RVD in Ehrlich cells is similar in the presence of nominally Ca^{2+}-free and physiological extracellular Ca^{2+} levels although RVD in MDCK and toad bladder cells was shown to be dependent on extracellular Ca^{2+} [4,24]. The fact that the $[Ca^{2+}]_i$ level fails to increase in proportion to the volume recovery during RVD has also been reported by Grinstein and Smith in human lymphocytes [8] and may indicate a change in the cytoplasmic Ca^{2+} set-point due to the decrease in ionic strength. Deansfield et al. [25], have reported that reduced $[Na^+]_i$ enhances Ca^{2+} sequestration by muscle mitochondria. It has also been reported that the calcium affinity of erythrocyte plasma membrane Ca^{2+}-ATPase is increased by decreased $[Mg^{2+}]_i$ [26] and that alkalinization increases the Ca^{2+}-ATPase activity in rat liver microsomes [27]. Similar responses may occur during the dilution of intracellular contents during hyposmotic challenge in Ehrlich cells resulting in a lower $[Ca^{2+}]_i$ level than expected.

The response to ionomycin suggests that a rise in $[Ca^{2+}]_i$ can lead to a cell shrinkage with the selective loss of KCl (Table I). However, ionomycin-induced shrinkage is sensitive to both CTX and nifedipine which contrasts the lack of sensitivity demonstrated by Ehrlich cells to these agents during RVD (Fig. 4). This is consistent with the existence of two different mechanisms or channel populations which may be involved in the two different volume perturbations. It is interesting to note that while Grinstein and Smith [8] report a Ca^{2+}-independent, CTX-sensitive population of K^+ channels active in RVD in the human lymphocyte, no indication of such a channel was found in the Ehrlich cell.

During RVD, the loss of K^+ exceeds that of Cl^- by approximately two times. Hoffmann and Simonsen [2] have suggested that this is due to an increased H^+ uptake via the Jacobs-Stewart cycle in order to offset the loss of K^+ concomitant with the activation of the Cl^--HCO_3^- exchanger. Therefore, the loss of K^+ in excess of the loss of Cl^- will depend on the buffering capacity of Ehrlich cells. However, the data presented here are not consistent with this as the Cl^--HCO_3^- exchanger would reach electrochemical equilibrium at a higher $[Cl^-]_i$ than is found during RVD. It is more likely that the excess loss of potassium is due to the loss of organic osmolytes [2]. In the case of the Ca^{2+}-induced loss of KCl the stoichiometry is 1:1.

Hoffman et al. [5] have previously reported that RVD is due to a transient increase in $[Ca^{2+}]_i$ which then activates separate K^+ and Cl^- conductances resulting in the net loss of KCl. This hypothesis was based on the findings that the calcium ionophore A23187-induced loss of KCl and the KCl loss seen during RVD were similar in that they were both unaffected by nitrate substitution and inhibited by quinine or pimozide. The present study uses both direct measurement of $[Ca^{2+}]_i$ and specific inhibitors of Ca^{2+}-sensitive K^+ channels to show that the responses to hyposmotic challenge and Ca^{2+} ionophores are similar in terms of the end result but occur by different mechanisms.

In summary, although a direct role for Ca^{2+} as a second messenger in regulatory volume decrease has been established in other cells, it is apparent that this is not the case in the Ehrlich cell. This conclusion is supported by: (1) the lack of a measurable rise in $[Ca^{2+}]_i$ following a hyposmotic challenge; and (2) the insensitivity of RVD to CTX and nifedipine which are known to inhibit Ca^{2+}-sensitive K^+ channels.

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