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INVOLVEMENT OF Ca^{2+}-INDUCED Ca^{2+} RELEASE IN THE VOLUME REGULATION OF HUMAN EPITHELIAL CELLS EXPOSED TO A HYPOTONIC MEDIUM

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Exposure of cultured human epithelial cells (Intestine 407) to a hypotonic solution results in initial osmotic swelling and in a subsequent volume decrease near to the original level. The regulatory volume decrease was inhibited by reduction of the extracellular free Ca^{2+} concentration to 90 nM. Single epithelial cells responded to a hypotonic challenge with a biphasic increase in the cytosolic free Ca^{2+} level from about 90 to 200 nM. Both phases of the Ca^{2+} rise were abolished by reducing the extracellular Ca^{2+} to 90 nM. In the presence of caffeine (20 mM), the second-phase Ca^{2+} response to a hypotonic challenge occurred earlier immediately after the first-phase response. The second-phase Ca^{2+} response was selectively impaired by adenine (10 mM), procaine (1 mM) or ryanodine (5 to 10 μM). These blockers for Ca^{2+}-induced Ca^{2+} release channels inhibited volume regulation after osmotic swelling. It is concluded that Ca^{2+}-induced Ca^{2+} release from a ryanodine-sensitive store is a prerequisite for the volume regulation of human intestinal epithelial cells under hypotonic conditions.

In hypotonic media most animal cell species are known to readjust their volume after initial swelling due to osmotically-driven water influx, in spite of continued exposure to anisotonic media. The regulatory volume decrease (RVD) is generally accomplished by a dissipative loss in the cell content of KCl and water. Recent electrophysiological studies provided evidence for the involvement of activation of K^+ channels (1-3) as well as Cl^- channels (2,4,5). The former channel was shown to be activated by cytosolic Ca^{2+} in epithelial cells (1-3). Consistent with this fact, a significant increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was recently observed during the RVD process in epithelial cells (6,7). The volume-regulatory Ca^{2+} ions may be mobilized from the extracellular space and/or from some intracellular storage sites. The present results show that the RVD process involves ryanodine-sensitive Ca^{2+} release following Ca^{2+} influx upon osmotic swelling in human epithelial Intestine 407 cells.

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MATERIALS AND METHODS: Cultured epithelial Intestine 407 cells derived from human embryonic small intestine were purchased (from Flow Labs. Inc.) and cultured in Fischer medium supplemented with 10% newborn calf serum at 37°C. Neomycin-loaded cells were prepared by electroporation with three rectangular electric pulses (2 kV/cm, 150 μs) in the presence of 0.5 mM neomycin sulfate and 0.3 mg/ml fluorescein isothiocyanate (FITC), according to a previous report (8). Neomycin-injected cells were identified by cellular fluorescence of co-injected FITC and then subjected to study 1 - 2 h after electroporation.

The mean volume of cells in suspension was measured at room temperature (about 24°C) by an electronic sizing technique, as described previously (2).

Fluorescence Ca^{2+} measurements with fura-2 were made within single monolayer Intestine 407 cells cultured on coverslips in a microchamber (about 100 μl) made from two pieces of coverslip at room temperature, as described previously (9). A hypotonic challenge was made by replacing an isotonic bathing medium with a hypotonic medium (within 10 - 20 s) by perfusion. The ratio image was obtained by dividing the 340-nm image by the 360-nm (or 380-nm) image every 2 to 5 s by an image processor (Hamamatsu Photonics ARGUS-100/CA) using a silicon-intensified target camera (Hamamatsu Photonics C2400-08H). The mean cytosolic ratio values were calculated and subsequently plotted against time as a spline interpolated curve. This ratio method allowed us to estimate the $[Ca^{2+}]_i$ regardless of the variation in the cell thickness and the dye concentration during cell volume changes. Calibration of the free Ca^{2+} concentration was made by the *in vitro* method, as reported previously (9).

The control isotonic or hypotonic saline contained (mM) NaCl 137.5 or 54, KCl 4.2, $CaCl_2$ 0.9, $MgCl_2$ 0.5, mannitol 20 or 26.6, Na-HEPES 6, and HEPES 8 (pH 7.5; osmolarity, 293 or 161 mOsm). A low-Ca^{2+} solution was prepared by adding 10 μM EGTA (Nakarai Chem.) to above salines devoid of $CaCl_2$. The pCa value was calculated to be 7.05 by taking the purity of EGTA and the concentration of free Ca^{2+} ions contaminating in nominally Ca^{2+}-free solutions (4 μM: measured with Ca^{2+}-selective microelectrodes) into account. Adenine, caffeine, procaine, ryanodine and isobutylmethylxanthine (IBMX) were purchased from Nakarai Chem. Neomycin sulfate and FITC were from Sigma and Merck, respectively.

RESULTS AND DISCUSSION: When the extracellular osmolarity was reduced by 45%, Intestine 407 cells rapidly underwent swelling by 60 to 70% of the original cell volume within 20 s. After osmotic swelling, the cells gradually reduced their volume, despite the continued exposure to the hypotonic medium, to a steady level (of about 120%) within 3 min in the presence of 0.9 mM Ca^{2+} (Fig. 1A, open circles), as found previously (2). Deprivation of extracellular Ca^{2+} with 1 mM EGTA added to nominally Ca^{2+}-free media abolished the RVD (2). It could be argued that high concentrations of the Ca^{2+} chelator might reduce the cytosolic Ca^{2+} as well as external Ca^{2+} ions. With a low concentration of EGTA (10 μM), however, a reduction in the extracellular Ca^{2+} concentration down to pCa 7.05, which is nearly identical to the basal $[Ca^{2+}]_i$ value (see below), was also found to be effective in blocking RVD (Fig. 1A, open triangles). Thus, it appears that Ca^{2+} entry across the plasma membrane is essential for the volume regulation of hypotonically stressed cells.

Fura-2 fluorescence ratio measurements showed that a biphasic Ca^{2+} rise interposed by a transient fall was associated with the RVD process in

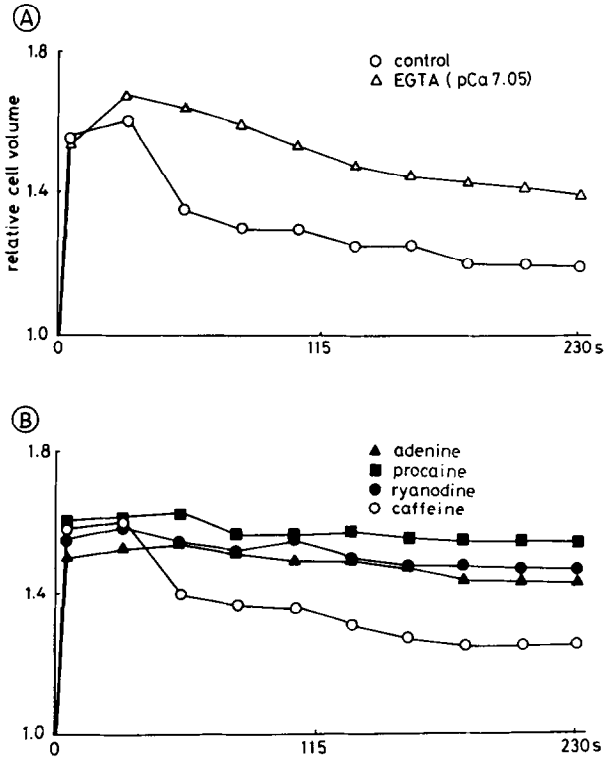

Fig. 1. Mean cell volume changes of Intestine 407 cells in suspension after a hypotonic challenge (55%, at zero time) in the absence (control) and presence of drugs. The effects of drugs were examined by pre-treatment with EGTA (10 μM, pCa 7.05), adenine (10 mM), procaine (1 mM), ryanodine (10 μM) and caffeine (20 mM) for 10 - 20 min. The mean cell volume before the hypotonic challenge was about 1100 μm^3. Representative data from 3 - 5 experiments. Effects of drugs, except for adenine, were fully reversible after washout.

the presence of extracellular Ca^{2+} ions at 0.9 mM (Fig. 2A, Table 1), as found previously (7). The $[Ca^{2+}]_i$ level gradually recovered within 2 - 3 min. Simultaneous microscopical observations showed that the volume regulation was accomplished within 100 - 150 s after transient swelling upon a hypotonic challenge. Both the first- and second-phase rises in $[Ca^{2+}]_i$ in the hypotonically stressed cells were markedly inhibited by reducing the extracellular free Ca^{2+} concentration to pCa 7.05 (Fig. 2B). Thus, the influx of Ca^{2+} ions may be required for both phases of the $[Ca^{2+}]_i$ rise upon osmotic perturbation. Ca^{2+} entry may induce a rise in $[Ca^{2+}]_i$ by itself and/or by triggering Ca^{2+} release from an intracellular Ca^{2+} store, such as the endoplasmic reticulum (ER).

To test the possibility that Ca^{2+} release from ER is involved in a biphasic $[Ca^{2+}]_i$ increase during the RVD process in Intestine 407 cells, fura-2 experiments were performed with adenine and procaine, which are known to inhibit Ca^{2+}-induced Ca^{2+} release in the presence of cytoplasmic ATP

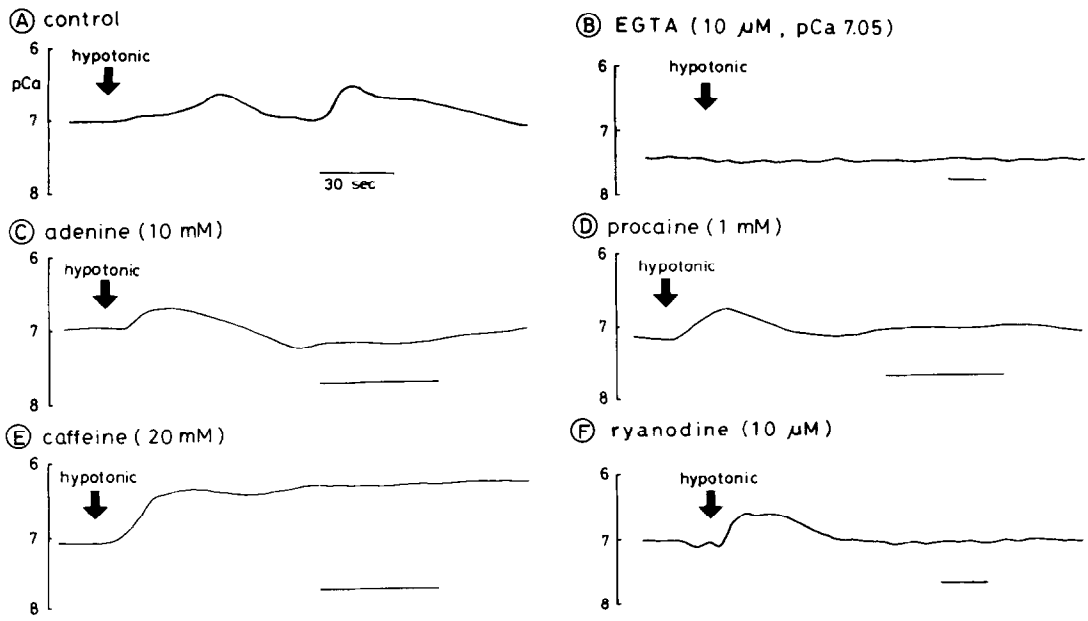

Fig. 2. Cytosolic Ca^{2+} measurements with fura-2 in single monolayer Intestine 407 cells before and after a hypotonic challenge (55%, at arrows). All drugs were applied 7 - 15 min before hypotonic challenges. Bar, 30 s.

(10,11) as well as Ca^{2+} release induced by inositol trisphosphate (IP_3) from sarcoplasmic reticulum (SR) (12). Adenine (10 mM) and procaine (1 mM) were found to suppress the second-phase $[Ca^{2+}]_i$ increase but not the first-phase

Table 1. Cytosolic free Ca^{2+} concentrations in Intestine 407 cells before and after hypotonic challenges

	number of observations	isotonic	hypotonic conditions		
		basal	first peak	trough	second peak
control	14	89 ± 19	201 ± 54*	102 ± 29	171 ± 48*
EGTA 10 μM (pCa 7.05)	10	81 ± 16	86 ± 17	---	---
adenine 10 mM	60	92 ± 8	246 ± 15*	---	---
procaine 1 mM	15	102 ± 7	173 ± 9*	---	---
ryanodine 10 μM	18	87 ± 4	206 ± 9*	---	---
caffeine 20 mM	10	76 ± 5	381 ± 22*	347 ± 18*	445 ± 14*

The data presented are the mean ± SEM. (in nM).
All drugs were applied 7 - 15 min before hypotonic challenges.
* Significantly different from the basal Ca^{2+} level ($P < 0.05$ evaluated by the F-test).

Ca^{2+} response in the hypotonically-swollen cells (Fig. 2C,D; Table 1). These results suggest that the first-phase and second-phase Ca^{2+} responses are due to Ca^{2+} influx and internal Ca^{2+} release, respectively. Since the second-phase rise in $[Ca^{2+}]_i$ was also abolished by reducing the extracellular free Ca^{2+} level to the intracellular level (Fig. 2B), the late Ca^{2+} release may be triggered by the preceding Ca^{2+} influx. The Ca^{2+} influx may induce Ca^{2+} release by triggering the Ca^{2+}-induced Ca^{2+} release (13) or by enhancing the IP_3-induced Ca^{2+} release (14), which is known to be $[Ca^{2+}]_i$-dependent (15). Neomycin-injected cells also responded with normal biphasic Ca^{2+} responses to a hypotonic challenge (15 observations, data not shown). Since neomycin is known to block the hydrolysis of inositol phospholipids by phospholipase C (16), IP_3-induced Ca^{2+} release may not be involved in the Ca^{2+} release upon hypotonic swelling. The pattern of $[Ca^{2+}]_i$ changes in response to hypotonic stress was affected by caffeine, which is believed to facilitate Ca^{2+}-induced Ca^{2+} release from SR by shifting the threshold to lower $[Ca^{2+}]_i$ (17). By pre-treatment of the cells with 20 mM caffeine, the second-phase Ca^{2+} response occurred much earlier, immediately after the first phase (Fig. 2E), and lasted for a longer time (200 - 300 s). Caffeine is also known to inhibit phosphodiesterase. However, prior treatment of the cells with IBMX (0.1 mM, 10-20 min), a potent phosphodiesterase inhibitor, failed to affect the pattern of biphasic Ca^{2+} responses to the osmotic perturbation (4 observations, data not shown). Therefore, it is likely that the second-phase $[Ca^{2+}]_i$ rise is due to Ca^{2+}-induced Ca^{2+} release triggered by the preceding Ca^{2+} influx.

No evidence, however, has been available for the existence of Ca^{2+}-induced Ca^{2+} release mechanism in the intestinal epithelial cell. Therefore, we next observed the effect of a plant alkaloid ryanodine on $[Ca^{2+}]_i$ in Intestine 407 cells. Ryanodine is known to block Ca^{2+}-induced Ca^{2+} release channels at doses over 1 μM (18,19) without affecting the IP_3-induced Ca^{2+} release in SR (20), and to be identical with the Ca^{2+}-induced Ca^{2+} release channel in SR (21-23). While ryanodine (5 - 10 μM) did not affect the basal $[Ca^{2+}]_i$ level, the second-phase rise in $[Ca^{2+}]_i$ was selectively impaired by pre-treatment (for 7 - 10 min) with 5 - 10 μM ryanodine (10 μM: Fig. 2F, Table 1). Apparently, the second-phase Ca^{2+} response to a hypotonic challenge is due to Ca^{2+}-induced Ca^{2+} release.

Ryanodine is known to lock the Ca^{2+}-induced Ca^{2+} release channel in the open state with doses below 1 μM (18,19). In fact, at 0.1 μM, the drug occasionally increased the basal $[Ca^{2+}]_i$ (to 174 $\pm$ 10 nM, n = 25). In most cases (41 of 66 cells), however, the basal $[Ca^{2+}]_i$ level was not affected by 0.1 μM ryanodine. Similarly, caffeine (10 - 20 mM) failed to induce significant increases in the basal $[Ca^{2+}]_i$. These facts may indicate that the Ca^{2+} content in the internal store may not be sufficiently high to

induce Ca^{2+} release in response to 0.1 μM ryanodine or 20 mM caffeine. After a hypotonic challenge, cytosolic Ca^{2+} from the initial Ca^{2+} influx may replenish the internal store, which in turn may be released again in response to elevated $[Ca^{2+}]_i$ in the vicinity of the store. The delay between the first phase due to Ca^{2+} influx and the second phase due to Ca^{2+} release may reflect the time to replenish to the threshold level for the release.

To examine whether the Ca^{2+}-induced Ca^{2+} release mechanism is involved in the RVD process, changes in the mean cell volume upon a hypotonic challenge were observed in the presence of drugs which were found to affect the late Ca^{2+} response. Prior treatment with adenine (10 mM), procaine (1 mM) and ryanodine (10 μM) inhibited volume regulation after osmotic swelling in Intestine 407 cells, as shown in Fig. 1B (filled symbols). In contrast, caffeine (20 mM) did not affect the volume regulation of hypotonically swollen cells (Fig. 1B, open circles). Therefore, it appears that the RVD process in these epithelial cells involves Ca^{2+}-induced Ca^{2+} release as an indispensable step.

Based on these observations, the following conclusions are deduced: 1. A human intestinal epithelial cell line (Intestine 407) responds to hypotonic stress with Ca^{2+} mobilization due to Ca^{2+} influx and Ca^{2+} release. 2. The Ca^{2+} influx triggers the Ca^{2+}-induced Ca^{2+} release from a ryanodine-sensitive intracellular store. 3. For the volume regulation of hypotonically swollen epithelial cells, the Ca^{2+} influx is necessary but not sufficient, unless it elicits Ca^{2+}-induced Ca^{2+} release.

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