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Rapid Report

Exocytosis upon osmotic swelling in human epithelial cells

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Upon osmotic swelling human epithelial cells exhibited significant increases in the membrane capacitance. Evidence for exocytosis includes its dependency on temperature, cytosolic Ca^{2+} and ATP as well as its sensitivity to guanosine 5'-*O*-(3-thio)triphosphate (GTPγS) and *N*-ethylmaleimide (NEM). A role of the osmotic exocytosis in the subsequent cell volume regulation is suggested.

It is known that a stretch of the urinary bladder epithelium may promote fusion of intracellular vesicles to the luminal membrane [1]. Recently, a mechanical stretch has been shown to induce exocytosis in lung epithelial cells [2]. New membrane incorporation at the extending edge of the plasma membrane was observed in migrating epithelial and fibroblastic cells [3]. Therefore, there is a possibility that membrane expansion due to osmotic swelling may also cause such 'membrane-reformative' exocytosis in epithelial cells exposed to a hypotonic solution. Here, we examined this possibility by modifying the technique of time-resolved capacitance measurements [4] to allow real-time monitoring of the membrane capacitance and currents even during changes in the zero-current potential (E_{rev}) due to activation of volume-sensitive ion channels [5,6].

A human small intestinal epithelial cell line, Intestinc 407 (Flow Labs.), was cultured in Fischer medium supplemented with 10% newborn calf serum. Spherical Intestine 407 cells in suspension were prepared by detaching from the plastic substrate and culturing with agitation for 1–3 h. The cells were placed in a chamber (0.5 ml) and perfused at a flow rate of 3–5 ml/min. The isotonic or hypotonic bathing solution was composed of (in mM): 137.5 or 54 NaCl, 4.2 KCl, 0.9 $CaCl_2$, 0.5 $MgCl_2$, 20 or 26.6 mannitol, 6 Ha-Hepes and 8 Hepes (pH 7.4; osmolality 293 or 161 mosM).

Phase-sensitive capacitance measurements were performed using the whole-cell patch-clamp technique with an amplifier (LIST, EPC-7) and Sylgard-coated Pyrex pipettes after compensating for the stray capacitance. The experimental system is illustrated in Fig. 1. A 20 mV peak-to-peak sinusoidal voltage (800 or 300 Hz) was added to the holding potential using an oscillator (Kikusui Electronics, Model 454). The resultant sinusoidal currents at 0° and 90° were measured by a two-phase lock-in amplifier (NF Electronic Inst., 5610A) and fed to a personal computer (NEC, 9801VM) via an AD converter (Canopus ADX-98E). The holding potential was intermittently changed by 14 mV at 500-ms intervals (V'_{dc}, V''_{dc}) using a pulse generator (Nihon Kohden, SEN7203). The voltage steps as well as the resultant current steps (I'_{dc}, I''_{dc}) were also fed to the computer after filtering out the sinusoidal component via low-pass filters (NF: P-83, P-84). These signals were sampled every 120 μs and time-averaged over 120 ms. The values of the membrane capacitance (C_m), conductance (G_m), current (I_m) and the access conductance via the patch pipette (G_a) were calculated on line, according to Lindau and Neher [4]. In the present system, however, the assumption of constant E_{rev} is not necessary for the calculations, because $b = G_a \cdot G_m/(G_a + G_m)$ could be calculated by canceling out E_{rev} using the following equations: $I'_{dc} = b(V'_{dc} - E_{rev})$ and $I''_{dc} = b(V''_{dc} - E_{rev})$. The data were displayed in real-time and stored on a magnetic disk. Error analyses with dummy circuits indicated that measured C_m values were little affected by the change in G_m of less than 20 nS or in G_a of less than 30 nS when G_m and G_a were more than 1 and 40 nS, respectively. Data were ac-

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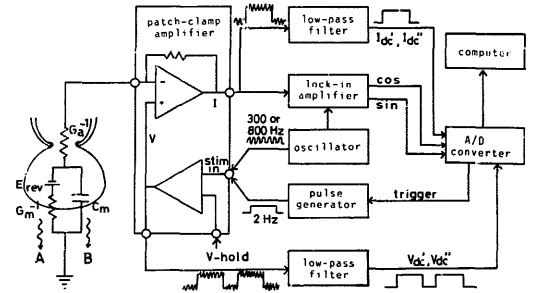

Fig. 1. Block diagram of the measuring instruments and the minimal equivalent circuit for the cell-pipette assembly. This system allows real-time monitoring of C_m, I_m and G_m even during changing the E_{rev} value.

cepted when the G_a value was always over 50 nS and its change was less than 30 nS during the experiment. The control pipette solution contained (in mM): 127 K-gluconate, 20 KCl, 10 Hepes, 10 Na-Hepes, 5.4 $MgSO_4$, 0.2 EGTA, 3 Na_2-ATP, 0.05 Na_2-GTP and 0.1093 $CaSO_4$ (pCa 7, pH 7.3). In some experiments, 10 mM EGTA was added to the pipette solution devoid of $CaSO_4$ (pH 7.4 with NaOH). When necessary, guanosine 5′-*O*-(3-thio)triphosphate (GTPγS, 100 μM, Sigma) or *N*-ethylmaleimide (NEM, 0.2 mM, Nacalai Tesque) was added to the control pipette solution, and 5′-adenylyl imidodiphosphate (AMP-PNP, 3 mM,

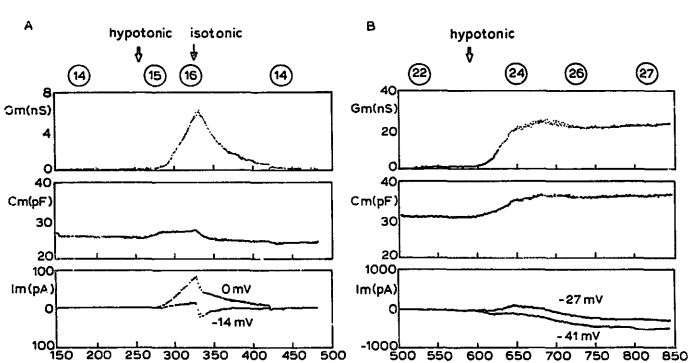

Fig. 2. Representative real-time displays of changes in the membrane capacitance (C_m), current (I_m) and conductance (G_m) in response to osmotic perturbation in single Intestine 407 cells. The extracellular osmolality was changed by superfusing with a hypotonic or isotonic solution (at arrows). The cell diameter is given in the circle (in μm). Alternating holding potentials are given on I_m traces. Inward and outward currents activated upon osmotic cell swelling at voltages between the equilibrium potential to K^+ (E_K −80 mV) and that to Cl^- (E_{Cl} −10 mV) chiefly represent volume-regulatory Cl^- and K^+ currents, respectively [7]. Transient inward currents activated upon restoration of extracellular osmolality may represent Ca^{2+}-permeable cation channels [8]. Upon restoring the extracellular osmolality, the capacitance rapidly decreased from 28.8 ± 1.8 to 24.1 ± 1.6 pF ($n = 10$, $P < 0.01$), while the cell size decrease from 19.7 ± 0.9 to 15.9 ± 1.0 μm in diameter ($P < 0.01$). In contrast, a hypertonic challenge (133% osmolality) to the cell equilibrated with an isotonic solution consistently induced cell shrinkage (from 16.8 ± 0.6 to 14.5 ± 0.5 μm, $n = 6$, $P < 0.01$) with no change in the membrane capacitance (22.3 ± 2.7 to 22.3 ± 2.9 pF, $n = 6$, $P > 0.1$).

Sigma) was added to the ATP-free pipette solution.

In single-cell size measurements, AMP-PNP, GTPγS or NEM was applied to the cytosol via a patch pipette filled with (in mM): 147 KCl, 3.5 $MgCl_2$, 3 (zero in the case of AMP-PNP) ATP, 0.05 GTP, 15 Na-Hepes, 10 Hepes (pH 7.4), 1 mM EGTA and 0.4658 mM $CaCl_2$ (pCa 7) under the whole-cell configuration maintained for 1.5–2 min. For the control experiment, the cytosol was equilibrated with the above pipette solution (devoid of AMP-PNP, GTPγS and NEM) under the whole-cell mode. After detaching the pipette by mechanical vibration, the cells were provided for single-cell size measurements. Measurements of the cell membrane resistance under the whole-cell mode had proved that complete membrane reseals could be accomplished in these cells. Single cell diameters before (d_0) and after osmotic perturbation (d) were measured under a phase-contrast microscope and a video monitor system. The relative cell volume was estimated from $[d/d_0]^3$.

Data are given as means ± S.E. Statistical significance was evaluated by Student's t-test. The experiments were performed at room temperature (24 ± 1°C).

Upon brief application of hypotonic stress, small increases in C_m, I_m and G_m were rapidly observed in association with visible osmotic swelling under the whole-cell configuration (Fig. 2A). The onset of a capacitance increase preceded that of an increase in G_m or I_m. Upon long-term exposure to a hypotonic solution, prominent increases in the cell diameter, C_m, I_m and G_m were observed (Fig. 2B). Osmotic swelling attained to a steady state within several min. Under the whole-cell configuration, cells exposed to a hypotonic solution cannot exhibit volume regulation, because the

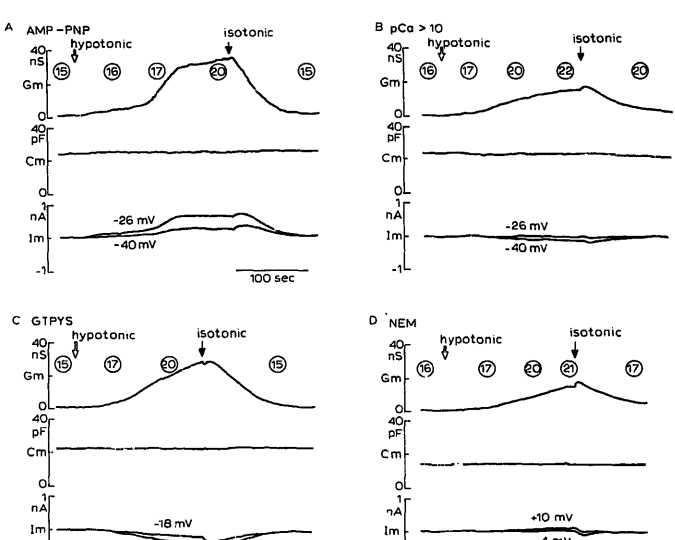

Fig. 3. Representative traces of changes in G_m, C_m and I_m upon osmotic swelling of single spherical intestine 407 cells loaded with AMP-PNP (3 mM, A), a high concentration (10 mM) of EGTA and no Ca^{2+} (pCa > 10, B), GTPγS (100 μM, C) or NEM (0.2 mM, D). Hypotonic challenges were made 10–15 min after treatment of cells with these chemicals. Arrows and numbers in circles are the same as in Fig. 2. Holding potentials are given on current traces. The data represent three to six similar experiments. The capacitance change was not statistically significant ($P > 0.5$) in each kind of experiments. In a total of 18 cells subjected to one of the above maneuvers there was little change in mean membrane capacitance (from 23.5 ± 2.2 to 23.9 ± 2.3 pF, $P > 0.05$), while the mean cell conductance and diameter significantly increased (from 0.9 ± 0.2 to 13.6 ± 2.8 nS and 15.8 ± 0.4 to 20.4 ± 0.7 μm, $P < 0.01$) at peak osmotic swelling.

intracellular osmolality is clamped at a certain high level by an isotonic pipette solution as well as non-diffusible cytoplasmic osmolytes. At the steady swelling state, the mean membrane conductance and capacitance increased from 1.5 ± 0.3 to 27.4 ± 3.9 nS ($n = 16$, $P < 0.01$) and 23.2 ± 1.3 to 28.8 ± 1.6 pF ($P < 0.01$), respectively, while the cell diameter increased from 15.6 ± 0.8 to 21.2 ± 1.2 μm ($P < 0.01$). The capacitance increase by 5.6 pF would correspond to the incorporation of membrane from more than 4000 vesicles of 0.2 μm diameter, provided that it was solely due to exocytotic insertion of intracellular membrane vesicles into the plasma membrane.

The hypothesis that the capacitance increase is due to exocytosis was supported by its dependency on ATP, Ca^{2+} and temperature. When ATP in the pipette solution, which dialyzed the cytosol, was replaced with a non-hydrolyzable ATP analog, AMP-PNP (3 mM), a hypotonic challenge induced cell swelling and activation of ionic conductances (Fig. 3A, top trace) and marked activation of outward currents at -26 to -40 mV (bottom trace) but little increased the membrane capacitance (middle trace). When most cytosolic Ca^{2+} ions were chelated with 10 mM EGTA, the cells failed to respond to a hypotonic challenge with sizable increases in the capacitance (Fig. 3B, middle trace). However, increases in G_m (top trace) and inward currents at -26 to -40 mV (bottom trace) were still observed in association with osmotic swelling. The capacitance response was also inhibited by lowering ambient temperature. Upon osmotic swelling no change in the capacitance was detected (30.3 ± 2.3 to 30.2 ± 2.7 pF, $P > 0.5$), while the conductance largely increased (from 0.9 ± 0.4 to 6.5 ± 1.3 nS, $P < 0.1$, $n = 3$) at 9.5°C.

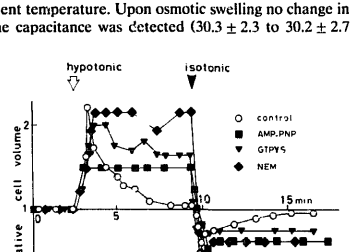

Fig. 4. Representative traces of inhibition of RVD and RVI in single spherical Intestine 407 cells by maneuvers which prevented osmotic exocytosis. Control (open circles), intracellular AMP-PNP 3 mM (filled squares), intracellular GTPγS 100 μM (filled, reversed triangles) and intracellular NEM 0.2 mM (filled diamonds). Before starting records cells were pretreated with these chemicals for about 10 min. Hypotonic challenges are made at arrows and isotonic challenges were subsequently at arrowheads. The data represent five to twelve similar experiments. The mean cell diameter was 17.6 ± 0.3 (S.E.) μm before osmotic perturbation, 21.9 ± 0.4 μm at peak osmotic swelling and 15.3 ± 0.3 μm at peak osmotic shrinkage ($n = 42$).

Small Ras-like GTP-binding proteins are known to mediate the transfer of secretory vesicles from the endoplasmic reticulum (ER) to the Golgi complex [10] and the fusion of secretory vesicles with the plasma membrane [11]. In fact, a non-hydrolyzable thiophosphate analog of GTP, GTPγS, inhibits the transfer and fusion of secretory vesicles in mammalian cell-free reconstituted systems [12,13] and Ca^{2+}-driven secretion from some secretory cells [14]. However, GTPγS has been reported to stimulate exocytosis in many secretory cells [15,16]. In Intestine 407 cells, GTPγS (100 μM) applied in the pipette solution, which had contained 50 μM GTP, did not stimulate but inhibited the capacitance response to a hypotonic challenge (Fig. 3C, middle trace). However, the GTP analog failed to block increases in G_m and I_m during osmotic swelling.

Recently, it has been demonstrated that NEM-sensitive fusion protein (NSF) plays an essential role in inter-organelle fusion events [17,18]. However, it is not as yet known whether NSF is also involved in the fusion of exocytotic vesicles to the plasma membrane. The intracellular application of NEM (0.2 mM) via patch pipettes was found to abolish the capacitance increase without abolishing increases in G_m and I_m upon osmotic cell swelling (Fig. 3D, middle trace).

It is known that parallel activation of K^+ and Cl^- currents takes place during osmotic cell swelling in Intestine 407 cells and that the K^+ channel is pre-existing and activated by cytosolic Ca^{2+} [7]. In the present study, prominent activation of outward currents, which represent mainly K^+ currents (see in the legend for Fig. 2), was actually observed even when osmotic exocytosis was inhibited by replacement of intracellular ATP with AMP-PNP (Fig. 3A). Since Cl^- channels are known to be present in secretory granules in some cells [19-21], one might speculate that exocytosis during osmotic swelling may insert new Cl^- channels which may in turn participate in the volume-regulatory Cl^- flux. However, this inference is at variance with the fact that volume-sensitive activation of Cl^- currents is independent of cytosolic Ca^{2+} [22], whereas the osmotic exocytosis is dependent of Ca^{2+} (Fig. 3B). In fact, activation of Cl^- currents was observed even when the exocytotic process had been blocked by GTPγS (Fig. 3C). These results indicate that activation of not only volume-sensitive K^+ but also Cl^- channels is independent of the membrane insertion upon osmotic swelling.

Single-cell size measurements showed that Intestine 407 cells can exhibit a regulatory volume decrease (RVD) after transient osmotic swelling upon reducing extracellular osmolality and a regulatory volume increase (RVI) after transient osmotic shrinkage upon restoring osmolality (Fig. 4, open circles). Both the

RVD and the RVI were impaired by intracellular applications of AMP-PNP, GTPγS and NEM (Fig. 4, filled symbols). Since these maneuvers inhibited osmotic exocytosis (Fig. 3), it is possible that the insertion of new membranes during cell swelling is involved in the subsequent volume regulation in the epithelial cells (without inserting volume-regulatory K^+ and Cl^- channels). Before the precise role of exocytosis associated with osmotic swelling in cell volume regulation is determined, simultaneous observations of the exocytosis and volume regulation processes will be needed.

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