Increased Vesicle Recycling in Response to Osmotic Cell Swelling

Osmotic swelling of Intestine 407 cells leads to an immediate increase in cell surface membrane area as determined using the fluorescent membrane dye FM 1–43. In addition, as measured by tetramethylrhodamine isothiocyanate (TRITC)-dextran uptake, a robust (>100-fold) increase in the rate of endocytosis was observed, starting after a discrete lag time of 2–3 min and lasting for ~10–15 min. The hypotonicity-induced increase in membrane surface area, like the cell swelling-induced release of ATP (Van der Wijk, T., De Jonge, H. R., and Tilly, B. C. (1999) Biochem. J. 343, 579–586), was diminished after 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-acetoxyethyl ester loading or cytochalasin B treatment. Uptake of TRITC-dextran, however, was not affected. Treatment of the cells with the vesicle-soluble N-ethylmaleimide-sensitive factor attachment protein receptor-specific protease Clostridium botulinum toxin F not only nearly eliminated the hypotonicity-induced increase in membrane surface area but also strongly diminished the release of ATP, indicating the involvement of regulated exocytosis. Both the ATP hydrolase apyrase and the MEK inhibitor PD098059 diminished the osmotic swelling-induced increase in membrane surface area as well as the subsequent uptake of TRITC-dextran. Taken together, the results indicate that extracellular ATP is required for the hypotonicity-induced vesicle recycling and suggest that a positive feedback loop, involving purinergic activation of the Erk-1/2 pathway, may contribute to the release of ATP from hypo-osmotically stimulated cells.

In mammalian cells, hypotonic cell swelling leads to the activation of cell volume regulatory processes, which in general involves a transient increase in the K⁺ and Cl⁻ conductances (for reviews see Refs. 1–3). As a result, KCl leaves the cell, and cellular volume is rapidly restored (regulated volume decrease). In addition to ion channel activation, osmotic swelling, like many other forms of mechanical stress, is known to promote the release of ATP, a potentially autocrine or paracrine factor acting through plasma membrane purinoceptors (4–7). Extracellular ATP has been shown to regulate the regulatory volume decrease response in a number of different cell types (4, 5, 8, 9), either through the stimulation of a Ca²⁺-dependent K⁺ efflux (8) or by the activation of the volume-sensitive Cl⁻ channels (4, 9). In Intestine 407 cells, extracellular ATP is not required for the direct activation of volume-sensitive Cl⁻ channels (7). However, (sub)micromolar concentrations of extracellular ATP were able to potentiate the hypotonicity-provoked Cl⁻ efflux in a Ca²⁺-dependent manner (7). In addition, osmotically induced ATP release was found to be critically involved in the activation of extracellular signal-regulated protein kinase (Erk)1/2 in Intestine 407 cells (7). Although the role of Erk-1/2 activation in the regulatory volume decrease response remains to be elucidated, activation of these MAP kinases by cell swelling has been observed in all cell models studied so far (10–20).

To date, several potential mechanisms have been proposed to explain cellular release of ATP. These include the following: 1) leakage due to (local) membrane damage; 2) activation of specific channel(s) or transporter(s); and 3) exocytotic events. Previously, members of the ABC-superfamily of transporters were suggested to permeate ATP (for reviews see Refs. 21 and 22). Intestine 407 cells, however, lack CFTR expression, and in the subclone we use, P-glycoprotein expression was not detected (7, 10) arguing against a role for ABC transporters in the ATP release. Because the cell swelling-induced ATP release differs from the activation of osmo-sensitive Cl⁻ channels in both the time scale of activation/inactivation and in its sensitivity to inhibitors (6, 7), it was concluded that ATP does not permeate through volume-sensitive anion channels. In contrast, in Intestine 407 cells, the ATP release was found to depend largely on [Ca²⁺], as well as on an intact cytoskeleton (7).

In this study, we pursued the hypothesis that regulated exocytosis might be involved in the release of ATP. In a number of cell models it was found that disruption of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex assembly, by Clostridium botulinum toxins or N-ethylmaleimide, resulted in a specific inhibition of exocytotic events (for review see Ref. 25). Our results clearly show that in C. botulinum toxin F- or N-ethylmaleimide-treated cells, the increase in exposed membrane surface as well as the release of ATP is markedly reduced, suggesting the involvement of exocytosis. Furthermore, we found that extracellular ATP is required for both exocytosis and endocytosis to occur. Taken together, the results not only indicate that the hypotonicity-
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**EXPERIMENTAL PROCEDURES**

**Materials**—Luciferin/luciferase reagent was obtained from Promega Corporation (Madison, WI). FM 1–43 and A23187 were from Molecular Probes (Eugene, OR) and Roche Applied Science, respectively. PD098059 and C. botulinum toxin F were purchased from Calbiochem, respectively. Chariot™ protein delivery kit was obtained from Active Motif (Rixen, C. botulinum (Eugene, OR) and Roche Applied Science, respectively. PD098059 and C. botulinum toxin F were purchased from Calbiochem, respectively. Chariot™ protein delivery kit was obtained from Active Motif (Rixen, Belgium). Other chemicals were purchased from Sigma.

**Cell Culture**—Intestine 407 cells were routinely grown as monolayers in Dulbecco’s modified Eagle’s medium supplemented with 25 mM Hepes, 10% fetal calf serum, 1% non-essential amino acids, 40 mg/liter penicillin, and 90 mg/liter streptomycin under a humidified atmosphere of 95% O₂ and 5% CO₂ at 37 °C. Prior to the experiments, cells were serum-starved overnight. C. botulinum toxin F was introduced intracellularly by using the Chariot™ protein delivery kit according to the manufacturer’s instructions. Hypotonutopic buffers were prepared by adjusting the concentration of mannitol, and osmolality was measured using a cryoscopic osmometer (Osmomat 030, Salim & Kipp B.V., Breukelen, The Netherlands).

**Extracellular Release of ATP**—Cells were seeded at a concentration of 10,000/cm² and incubated under a humidified atmosphere of 95% O₂ and 5% CO₂ at 37 °C. Thereafter, cells were washed four times with isotonic buffer (80 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 95 mM mannitol, and 20 mM Hepes, pH 7.4). The cultures were stimulated with isotonic or hypotonic (60% tonicity) media for 15 min. Media were collected and centrifuged for 5 min at 300 × g, and the supernatants were transferred to fresh vials. ATP content was determined using a luciferin-luciferase luminescence kit Promega Corporation (Madison, WI) and a Topcount.NXT luminometer (Packard Instrument Co.).

**Membrane Staining with FM 1–43**—Loading experiments were performed with cells grown on coverslips, at ~50% confluency. During exposure to 1 mM FM 1–43, changes in fluorescence intensity were measured on-line (excitation wavelength = 479 nm; emission wavelength = 598 nm) in a fluorescence spectrophotometer (Hitachi F4500, Tiel, Holland) at 37 °C. Experiments were started after at least 5 min of exposure to the FM 1–43 to reach an equilibration of FM 1–43 dye partitioning into the plasma membrane.

**TRITC-Dextran Uptake**—Cells on coverslips at 80–90% confluency were incubated with 0.5 mg/ml TRITC-dextran (10,000 Da) in isotonic or hypotonic buffer (80 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 95 mM mannitol and 20 mM Hepes, pH 7.4; diluted in case of hypotonic stimulation). After incubation, cells were washed three times with ice-cold phosphate-buffered saline and fixed with 2% formaldehyde (20 min incubation). Confocal images of 512 × 512 pixels were constructed by summation of 15 optical sections parallel to the substratum and each 1 μm apart, using a 63× oil immersion objective (Axiolab 135M, Zeiss, Oberkochen, Germany). Endocytosis was quantified using KS400 software (Zeiss, Oberkochen, Germany) by counting the number of fluorescent spots with a size between 3 and 75 pixels, after subtraction of background fluorescence. Background fluorescence was determined by measuring the mean pixel intensity and adding five times its standard deviation of a circle drawn in the background.

**RESULTS**

Osmotic Cell Swelling Leads to an Increase in Cell Surface Labeling—The effects of hypo-osmotic stimulation on the cell surface membrane area was studied using the styryl dye FM 1–43.
1–43, a water-soluble probe that becomes intensely fluorescent at the lipid-liquid interface and does not pass the membrane (24). An increase in membrane surface area, due to exocytosis or to unfolding of FM 1–43-inaccessible plasma membrane, results in an increased binding of the dye and, consequently, in augmented fluorescence. As shown in Fig. 1, a shift from an isotonic to a hypotonic medium led to a rapid increase in fluorescence above control values. Like the release of ATP (7), the hypotonicity-provoked increase in FM 1–43 fluorescence was inhibited after loading the cells with BAPTA-AM or by treatment with cytochalasin B (Fig. 1A), implying that the increase in surface labeling is Ca\(^{2+}\)/H\(_{1001}\)-dependent and requires an intact actin cytoskeleton. Treating the cells with N-ethylmaleimide, an inhibitor of SNARE proteins involved in vesicle docking and fusion, almost completely abolished the cell swelling-induced increase in fluorescence, suggesting that exocytosis plays a crucial role. This notion was strengthened further by the finding that intracellularly delivered \(C.\) *botulinum* toxin F, resulting in proteolytic cleavage of the vesicle-associated SNARE protein VAMP2, almost completely prevented the increase in FM 1–43 fluorescence (Fig. 1B). Taken together, these results strongly indicate that osmotic swelling in this epithelial cell type is paralleled by rapid exocytosis.

### Table I

**Role of [Ca\(^{2+}\)] and F-actin in the regulation of osmotic cell swelling-induced endocytosis**

| Experimental conditions | TRITC-dextran uptake (% hypotonic control) |
|-------------------------|------------------------------------------|
|                         | Isotonic | Hypotonic |
| Control                 | 6 ± 2 (10) | 100 ± 6 (10) |
| BAPTA-AM                | 3 ± 1 (7)  | 86 ± 19 (7)  |
| A23187                  | 2 ± 1 (5)  | 91 ± 28 (5)  |
| Cytochalasin B          | 12 ± 3 (5) | 109 ± 20 (5) |
| Jasplakinolide          | 6 ± 2 (5)  | 32 ± 7* (5)  |

* Significant difference from the control (\(p < 0.05\); Student’s \(t\) test).

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**FIG. 3.** Time course of hypotonicity-induced TRITC-dextran uptake. Cells grown on coverslips were incubated for the times indicated with isotonic (closed symbols) or hypotonic medium (60% tonicity; open symbols) containing 0.5 mg/ml TRITC-dextran. After fixation, images were constructed as indicated under “Experimental Procedures” using confocal laser scanning microscope. The number of vesicles are determined as described under “Experimental Procedures.” Data are given as mean ± S.E. (\(n = 5\)).

**FIG. 4.** Hypotonicity-induced ATP release from control and \(C.\) *botulinum* toxin-treated cells. Control and \(C.\) *botulinum*-treated cultures were stimulated with isotonic (Iso) (open bars) or hypotonic (Hypo) (80% tonicity; hatched bars) medium for 15 min. Media were collected and ATP content was determined as described under “Experimental Procedures.” Data are expressed as mean ± S.E. (\(n = 6\)).
In many different cell models, mechanical stress, such as caused by osmotic cell swelling, leads to the release of ATP into the surrounding medium (4–7). Several potential mechanisms have been proposed to be involved, including anion channel activation (i.e. CFTR and/or volume-regulated anion channels), utilization of specific (ABC-type of) transporters, and exocytosis (26, 27). In Intestine 407 cells, results from previous studies argued against the involvement of anion channels because 1) these cells do not express CFTR and 2) there is a clear difference in the regulation of osmo-sensitive anion channels and the release of ATP (6, 7, 10, 28). In Intestine 407 cells, the hypotonicity-provoked release of ATP was found to depend critically on an increase in \([\text{Ca}^{2+}]_i\), and on an intact actin cytoskeleton (7), suggesting the involvement of exocytosis. This notion is supported by the observation that an increase in \([\text{Ca}^{2+}]_i\) alone is able to trigger the release of ATP (29).2

2 T. van der Wijk, unpublished results.

### TABLE II

| Experimental condition | TRITC-dextran uptake (% hypotonic control) |
|------------------------|-------------------------------------------|
|                        | Isotonic                                  | Hypotonic                               |
| Control                | 5 ± 1 (6)                                 | 100 ± 3 (13)                            |
| Apyrase                | 1 ± 1 (5)                                 | 11 ± 3* (7)                             |
| Suramin                | 5 ± 5 (3)                                 | 7 ± 5* (3)                              |
| ATP                    | 7 ± 2 (5)                                 | 135 ± 20 (5)                            |
| Genistein              | 3 ± 2 (5)                                 | 54 ± 11* (5)                            |
| PD098059               | 4 ± 1 (5)                                 | 45 ± 10* (10)                           |

* Significant difference from the control (*p* < 0.05; Student’s *t* test.)

Fig. 5. Apyrase and the MEK inhibitor PD09059 prevented a hypotonicity-induced increase in FM 1–43 fluorescence. Intestine 407 cells grown on coverslips were treated with Apyrase (3 IU/ml for 5 min), ATP (10 μM for 5 min), genistein (200 μM for 2 h), or PD098059 (50 μM for 2 h) prior to the experiments. Thereafter, the coverslips were placed in a cuvette containing 1 μM FM 1–43, and fluorescence was monitored continuously as described under “Experimental Procedures.” Arrow indicates shift in medium. Traces are representative of at least three experiments. Iso, isotonic; Hypo, hypotonic.

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DISCUSSION

In many different cell models, mechanical stress, such as caused by osmotic cell swelling, leads to the release of ATP into the surrounding medium (4–7). Several potential mechanisms have been proposed to be involved, including anion channel activation (i.e. CFTR and/or volume-regulated anion channels), utilization of specific (ABC-type of) transporters, and exocytosis (26, 27). In Intestine 407 cells, results from previous studies argued against the involvement of anion channels because 1) these cells do not express CFTR and 2) there is a clear difference in the regulation of osmo-sensitive anion channels and the release of ATP (6, 7, 10, 28). In Intestine 407 cells, the hypotonicity-provoked release of ATP was found to depend critically on an increase in \([\text{Ca}^{2+}]_i\), and on an intact actin cytoskeleton (7), suggesting the involvement of exocytosis. This notion is supported by the observation that an increase in \([\text{Ca}^{2+}]_i\) alone is able to trigger the release of ATP (29).2
Upon hypo-osmotic stimulation, a rapid increase in plasma membrane surface area was observed, as evidenced by an increased FM 1–43 fluorescence. This increase was abolished after treating the cultures with N-ethylmaleimide or C. botulinum toxin F, indicating that SNARE complex formation and subsequent exocytosis is involved. Like the hypotonicity-induced ATP release, the increase in total membrane area depends on an intact actin cytoskeleton and is regulated by intracellular free calcium. Activation of exocytosis by osmotic cell swelling is not unprecedented. An increase in membrane capacitance has been reported after hypo-osmotic stimulation of Intestine 407 cells (30). In addition, morphometric analysis of electron micrographs of rat hepatocytes revealed a marked enlargement of the membrane surface area within 5 min of hypotonic exposure (31). Furthermore, Bruck et al. (32) observed an increase in the release of horseradish peroxidase after hypotonic stimulation from a horseradish peroxidase-loaded perfused liver. Because the horseradish peroxidase release was found to be sensitive to colchicine, the involvement of exocytosis was suggested (32). In line with this concept, reducing the osmolarity of the surrounding medium promoted the release of fluorescein isothiocyanate-coupled dextran (72,000 Da) from preloaded inner medullary collecting duct kidney cells (33).

Subsequent to enhanced membrane surface labeling, an increased rate of endocytosis was observed after osmotic cell swelling, as evidenced by the robust uptake of TRITC-dextran. Uptake of dextran started after a discrete lag time of 2–3 min, reached maximal activity after ~5 min, and lasted for 10–15 min. The delayed onset of endocytosis in response to cell swelling may suggest that endocytosis is triggered by an increase in exocytosis. Smith and Betz (24) observed a similar lag time between the onset of Ca\(^{2+}\)-provoked exocytosis and the occurrence of endocytosis in adrenal chromaffin cells. They suggested that endocytosis starts only when the increase in cell size due to exocytosis reaches a certain threshold and limits a further increase (24). In addition, a negative correlation has been proposed between membrane tension and membrane expansion (34), implying that membrane expansion through exocytosis will result in an increased rate of endocytosis. In Intestine 407 cells, however, we found that exo- and endocytosis are regulated independently, because cytochalasin B treatment and BAPTA-AM loading, both strong inhibitors of exocytosis, did not affect the cell swelling-induced endocytosis. In contrast, our observation that the hypotonicity-provoked endocytosis is inhibited by inactivation of volume-regulated anion channels and promoted by K\(^+\) channel blockers and high extracellular K\(^+\) suggests that membrane depolarization, a known consequence of volume-regulated anion channels activation (35), may promote or trigger endocytosis.

The ATP hydrolyase apyrase completely inhibited the hyposhock-induced FM 1–43 fluorescence as well as the uptake of TRITC-dextran, suggesting that extracellular ATP is required for both the cell swelling-induced exocytosis and endocytosis. Extracellularly added ATP, however, does not induce an increase in either one of these parameters by itself, indicating that ATP acts as a permissive and propulsive factor and that an additional trigger is needed, perhaps an increase in membrane tension. Both an increase as well as a decrease in the rate of exocytosis has been reported after purinergic activation, the effects being attributed principally to its effect on [Ca\(^{2+}\)]\(_i\) (36–43). In addition, regulation of exocytosis by purinoreceptor activation through Ca\(^{2+}\)-independent and G-protein-dependent pathways have been demonstrated (38, 40, 41). Therefore, although extracellular ATP leads to an increase in [Ca\(^{2+}\)]\(_i\), in our cell system (7), the effect of ATP on exocytosis may be modulated by other Ca\(^{2+}\)-dependent pathways.

Extracellular ATP not only affects exocytosis but is also involved in the regulation of endocytosis. Nakasima et al. (44) reported that binding of epidermal growth factor and insulin to their receptors leads to the endocytosis of the receptor-ligand complex that is mediated by the activation of the small G protein p21\(^{Ras}\). Osmotic cell swelling rapidly activates the Erk-1/2 MAP kinases through a mechanism involving the Ras/Raf pathway, using extracellular ATP as an autocrine factor (7, 18). Therefore, it is tempting to speculate that ATP promotes endocytosis through the activation of p21\(^{Ras}\). This notion is supported by our observation that inhibition of the Erk-1/2 signaling cascade, using the MEK inhibitor PD098059, inhibited osmotic cell swelling-induced endocytosis.

Although the hypotonicity-induced endocytosis was completely inhibited in the presence of apyrase or suramin, inhibition of exocytosis by cytochalasin B or BAPTA-AM treatment did not significantly affect the uptake of TRITC-dextran (cf. Table I). However, as has been found for several other cell models (45), even under isotonic conditions, low levels of extracellular ATP are present. In addition, under hypotonic conditions, a small but significant increase in extracellular ATP levels was observed in cytochalasin B or BAPTA-AM-treated cells (7). Because complete removal of ATP by apyrase or the inhibition of purinoreceptors by suramin completely blocked both exo- and endocytosis, it is tempting to speculate that extracellular ATP, acting through purinoreceptor activation, serves two functions. First, basal concentrations of ATP are a prerequisite for vesicle recycling to occur, and second, elevated levels promote but do not trigger endocytosis through a pathway involving Erk-1/2.

To conclude, osmotic swelling of Intestine 407 cells rapidly leads to an increase in the rate of exo- and endocytosis. Subsequently to the exocytotic release of ATP, by using a signaling pathway that involves purinoreceptor activation, Erk-1/2 activation enhances the vesicle recycling and promotes the additional release of ATP. Extracellular ATP may then play an important role in autocrine potentiation of volume-sensitive Cl\(^-\) channels, as well as in cell-to-cell communication through activation of purinergic receptors in neighboring cells or tissues.

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