

## Protein Tyrosine Phosphorylation Is Involved in Osmoregulation of Ionic Conductances\*

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Using the human Intestine 407 cell line as a model, we investigated a possible role for tyrosine kinase(s) in regulating the ion efflux pathways induced by hyposmotic stimulation (regulatory volume decrease, RVD). Pretreatment of <sup>125</sup>I- and <sup>86</sup>Rb<sup>+</sup>-loaded cells with the phosphotyrosine phosphatase inhibitor sodium orthovanadate (200 μM) potentiated isotope efflux triggered by mild hypotonicity (10–20%) but did not further increase the efflux in response to more vigorous osmotic stimulation (30% hypotonicity). The tyrosine kinase inhibitors herbimycin A and genistein largely reduced the osmotic shock-induced efflux in both control and vanadate-pretreated cells, while not affecting calcium-activated <sup>86</sup>Rb<sup>+</sup> efflux. Potentiation of the RVD response by vanadate was confirmed by direct measurements of hypotonicity-induced changes in cell volume. Hypotonic shock alone triggered a rapid and transient increase in tyrosine phosphorylation of several proteins as well as phosphorylation of mitogen-activated protein kinase. Furthermore, the potentiating effects of vanadate on hypotonicity-induced ion efflux and mitogen-activated protein (MAP) kinase phosphorylation were mimicked by epidermal growth factor. Neither vanadate nor epidermal growth factor provoked a RVD-like ionic response under isotonic conditions. These results indicate that tyrosine phosphorylation is an essential step in the RVD response and suggest a novel role of growth factors in the cellular defense against osmotic stress.

Most mammalian cell types, including intestinal epithelial cells, have to perform their physiological functions under a variable osmotic stress, either due to accumulation of osmotic substances (e.g., glucose, amino acids, and bile salts in the enterocytes) or to changes in the osmolality of the surrounding fluid. As a result, most cells have developed compensatory processes to maintain and restore their volume. Two

separate mechanisms can be distinguished, the regulatory volume increase (RVI)<sup>1</sup> and the regulatory volume decrease (RVD), activated by cell shrinking and cell swelling respectively (for review see Refs. 1–3). Whereas the RVI response leads to an accumulation of salt through the activation of ion pumps and carriers, the RVD response depends on the coordinate activation of K<sup>+</sup>- and Cl<sup>-</sup>-selective ionic channels, resulting in a net loss of salt and driving the efflux of water. Although swelling-induced activation of ionic channels is well documented for a number of cell types, including lymphocytes, hepatocytes, and epithelial cells (4–7), the underlying mechanism is still largely unclear.

The human fetal jejunum-derived Intestine 407 cell line (8) is particularly suitable for studying osmoregulation of chloride channels since no appreciable Ca<sup>2+</sup>- or cAMP-sensitive Cl<sup>-</sup> channel activity has been observed (7, 9).<sup>2</sup> Here we report that osmotic cell swelling triggers the phosphorylation of several proteins on tyrosine residues, as well as phosphorylation of MAP kinase. Furthermore, increased tyrosine phosphorylation, provoked by inhibition of phosphotyrosine phosphatases or by stimulation with EGF, potentiates the hypotonicity-induced ion efflux through K<sup>+</sup> and Cl<sup>-</sup> channels and limits osmotic swelling. Conversely, the swelling-induced ion efflux was blocked by tyrosine kinase inhibitors. Taken together, our results not only indicate that protein tyrosine phosphorylation is an essential step in the RVD response, but also suggest that (neuro-) hormones and growth factors acting through the MAP kinase signaling pathway may potentiate the RVD response and thereby prevent excessive cell swelling.

### EXPERIMENTAL PROCEDURES

**Materials**—Radio-isotopes and enhanced chemiluminescence (ECL) Western blotting detection kits were obtained from Amersham Netherlands B.V. (‘s Hertogenbosch, The Netherlands). Monoclonal IgG2bk anti-phosphotyrosine antibodies and polyclonal anti-ERK-1 antibodies were purchased from Upstate Biotechnologies, Inc. (Lake Placid, NY) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA) respectively. Other chemicals were from the following sources: DII-C14 from Molecular Probes (Eugene, OR), herbimycin A from Biomol (Plymouth Meeting, PA), and genistein from Sigma.

**Efflux Assays**—Confluent monolayers of Intestinal 407 cells were loaded with 5 μCi of <sup>125</sup>I- and 0.5 μCi of <sup>86</sup>Rb<sup>+</sup> for 2 h and washed three times with isotonic buffer (80 mM NaCl, 5 mM KCl, 1.3 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM glucose, 95 mM mannitol, and 20 mM Hepes, pH 7.4) prior to the assay. Isotope efflux was determined at 37 °C by replacing the medium at 1–2-min intervals. Hypotonic buffers were prepared by adjusting the concentration of mannitol. Radioactivity in the media was determined by  $\gamma$  radiation counting and expressed as fractional efflux per minute as previously described (10).

**Quantitation of Cell Volume**—Confluent cultures grown on glass coverslips were loaded with 1 μg/ml DII-C14 for 20 min at 37 °C prior to the experiment. Changes in cell volume were quantitated as increased cell height by constructing optical sections perpendicular to the substratum (step resolution 0.2 μm; 20 $\times$  water immersion objective, zoom 4 $\times$ ) at fixed time intervals, using a confocal scanner laser microscope (CSLM Bio-Rad Lasersharp mrc-600, Bio-Rad, Hempel Hempstad, United Kingdom). Cells were stimulated by rapid change of the incubation medium and scanned at exactly the same position as the recorded control.

**Immunoblotting**—Monolayers of cells were stimulated with hypotonic media and incubations were terminated by addition of boiling SDS

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<sup>1</sup> The abbreviations used are: RVI, regulatory volume increase; RVD, regulatory volume decrease; MAP, mitogen-activated protein; EGF, epidermal growth factor.

<sup>2</sup> B. C. Tilly, unpublished results.

sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. Phosphoproteins were identified by using monoclonal IgG2bk anti-phosphotyrosine antibodies or polyclonal anti-ERK-1 antibodies and an ECL Western blotting detection system according to the instructions provided by the manufacturers.

### RESULTS AND DISCUSSION

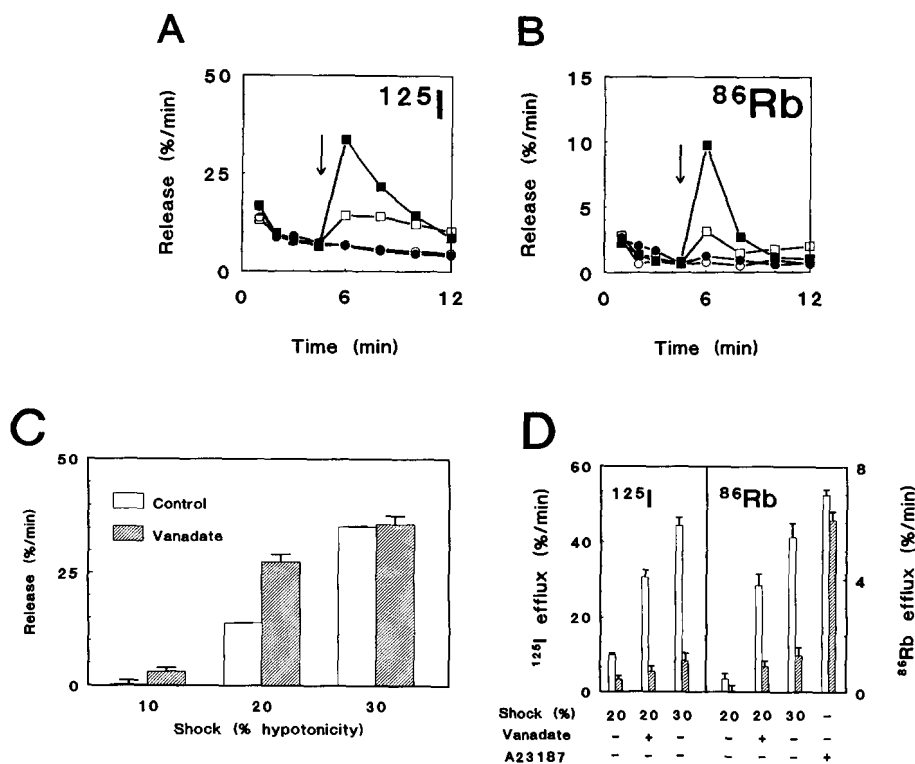
Passive swelling of Intestine 407 cells in hypotonic medium rapidly activates both  $\text{Cl}^-$  and  $\text{K}^+$  conductances, as evidenced by an increased efflux of radio-isotopes from  $^{125}\text{I}^-$  and  $^{86}\text{Rb}^+$ -loaded cells (Fig. 1, A and B). The efflux starts within 1–2 min after stimulation and is dose-dependent, a maximum being reached at 30–40% hypotonicity (data not shown). Pretreatment of the cells with vanadate (200  $\mu\text{M}$ ), a potent inhibitor of phosphotyrosine phosphatases (11), did not affect  $^{125}\text{I}^-$  and  $^{86}\text{Rb}^+$  efflux by itself but potentiated the efflux induced by a mild osmo-shock (10–20% hypotonicity) by approximately 2–3-fold (Fig. 1, A and B). No further enhancement of the isotope efflux, however, was observed at 30% hypotonicity (Fig. 1C). The facilitation of the shock-induced efflux by vanadate was dose-dependent, with maximal effects being observed at a concentration of 100  $\mu\text{M}$  ( $\text{EC}_{50}$  of 13 and 15  $\mu\text{M}$  for  $^{125}\text{I}^-$  and  $^{86}\text{Rb}^+$  efflux, respectively). Furthermore, the effects were specific for volume-regulated channels, since no potentiating effects of vanadate were noted on  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$  channels, as monitored by  $^{86}\text{Rb}^+$  efflux triggered by the  $\text{Ca}^{2+}$ -mobilizing hormones bradykinin and histamine or by the calcium ionophore A23187 (not shown). Comparable results were obtained when pervanadate was used instead of orthovanadate. To further substantiate a possible role for tyrosine phosphorylation in the RVD response, cells were treated with the specific tyrosine kinase inhibitor herbimycin A (12, 13). As shown in Fig. 1D, herbimycin A pretreatment (1  $\mu\text{M}$  for 48 h) did not affect the calcium ionophore-activated  $^{86}\text{Rb}^+$  efflux, but largely reduced the osmo-shock-induced efflux of  $^{125}\text{I}^-$  and  $^{86}\text{Rb}^+$ . The inhibition was observed in control as well as in vanadate-pretreated cells and within a broad range of hypotonicity (10–30%). Qualitatively similar but smaller effects (50–60% inhibition) were

observed using genistein (200  $\mu\text{M}$ ) as a tyrosine kinase inhibitor (not shown).

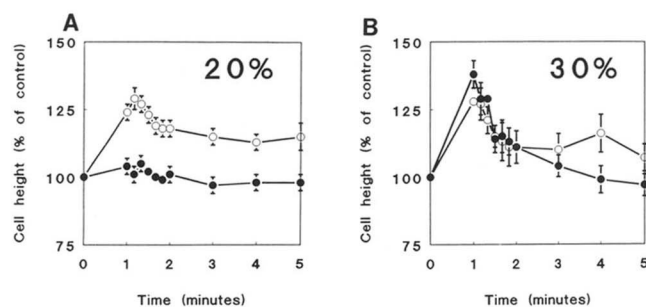
The effects of vanadate pretreatment on cell volume were studied more directly by quantitating cell height in confluent monolayers following hyposmotic stimulation. In control cultures, replacing an isotonic medium for a 20 or 30% hyposmotic one rapidly increased cell height by  $29 \pm 4\%$  (20% shock) and  $28 \pm 2\%$  (30% shock), respectively (Fig. 2, A and B). Pretreating the cultures with vanadate, however, prevented osmotic swelling almost completely when stimulated at 20% hypotonicity, but did not affect volume changes in response to a more vigorous osmotic shock (30% hypotonicity). Importantly, steady-state volume following hyposmotic stimulation was slightly lower in cells pretreated with vanadate as compared to control cultures ( $101 \pm 2\%$  versus  $116 \pm 2\%$  at  $t = 10$  min,  $p < 0.01$ ), most likely due to vanadate inhibition of a phosphotyrosine phosphatase that is part of the switch-off mechanism of volume-sensitive channels. No effects of vanadate were observed on cellular volume in the absence of hyposmotic stimulation.

The previous experiments raised the intriguing possibility that osmotic cell swelling by itself triggers tyrosine kinase activity. As shown on immunoblots using anti-phosphotyrosine antibodies (Fig. 3A), at least three distinct proteins (70, 74, and 125–130 kDa) became phosphorylated on tyrosine residues within 2 min following a hypotonic shock. Phosphorylation in response to a hypotonic shock alone was incomplete and showed an additional increase in the presence of vanadate. In contrast, vanadate caused only a slight enhancement of tyrosine phosphorylation under isotonic conditions. Tyrosine phosphorylation, even in the presence of vanadate, was transient rather than sustained; phosphatase action reversed the shock-induced protein phosphorylation to basal levels within 10–15 min. Among the proteins that were phosphorylated is a 125–130-kDa protein that co-migrates with immunoprecipitated p125<sup>FAK</sup> (results not shown), a tyrosine kinase associated with focal adhesions (14). p125<sup>FAK</sup> is proposed to be involved in signal transduction pathways triggered by integrins and mitogenic neuropeptides, finally leading to such diverse cellular

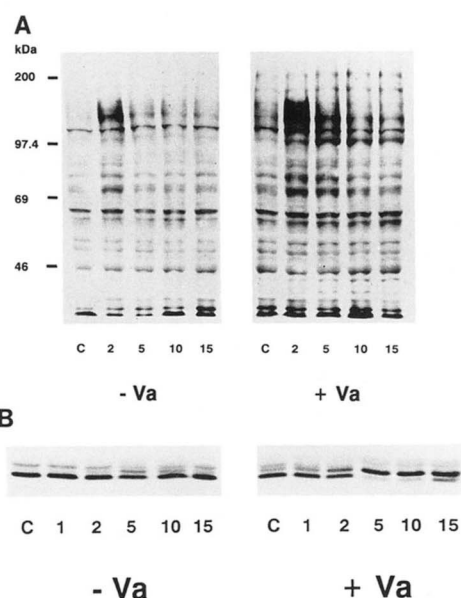
**FIG. 1. Potentiation of the volume-sensitive efflux of  $^{125}\text{I}^-$  and  $^{86}\text{Rb}^+$  by vanadate pretreatment (A–C) and its inhibition by herbimycin A (D).** A and B, time course of the fractional efflux of  $^{125}\text{I}^-$  (A) and  $^{86}\text{Rb}^+$  (B) in control (open symbols) and sodium vanadate-pretreated (200  $\mu\text{M}$ , 5 min) cultures (closed symbols). Circles represent unstimulated cells; boxes represent osmotically stimulated cells. Arrow indicates shift to a 20% hypotonic medium. C, dose dependence of osmo-shock-induced  $^{125}\text{I}^-$  efflux in control (open bars) and vanadate-pretreated (shaded bars) cultures. D, isotope efflux from control (open bars) and herbimycin A-pretreated cells (1  $\mu\text{M}$  for 48 h, shaded bars) after hyposmotic (20%, 30%) stimulation or addition of the  $\text{Ca}^{2+}$  ionophore A23187 (10  $\mu\text{M}$ ). Data (C and D) are expressed as mean  $\pm$  S.D. for  $n = 3$ .







**FIG. 2. Increase in cell volume following hypotonic stimulation.** Control (open symbols) and vanadate-pretreated (200  $\mu\text{M}$ , 5 min; closed symbols) cells were stimulated with 20% (A) or 30% (B) hypotonic media. Changes in cell volume were quantitated by measuring cell height and presented as percentage of the unstimulated control. Data are expressed as mean  $\pm$  S.E. for three independent experiments (total number of cells; 17 for 20% shock; 14 for 30% shock). Absolute cell height:  $4.0 \pm 0.2 \mu\text{m}$  ( $n = 23$ ) in control and  $4.2 \pm 0.2 \mu\text{m}$  ( $n = 21$ ) in vanadate-pretreated cultures.

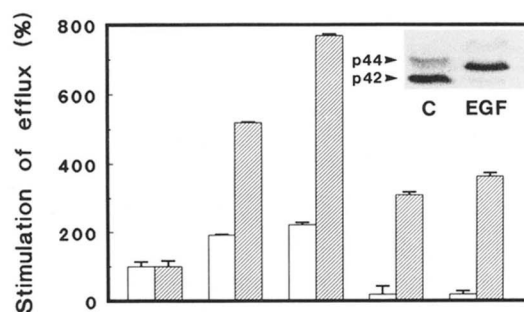


**FIG. 3. Time course (min) of phosphorylation of protein tyrosine (A) and MAP kinase (B) following hypotonic stimulation (30%) of control and vanadate-pretreated (200  $\mu\text{M}$ , 5 min) cells.** C, isosmotic control.

responses as cell adhesion, cell motility, and cell proliferation (15).

Another major family of protein kinases, known to be activated through a cascade of other kinases, including a tyrosine kinase, are the mitogen-activated protein kinases (for review see Ref. 16). Recently, these kinases have been suggested to be involved in the signal transduction pathway leading to activation of the  $\text{Na}^+/\text{H}^+$  exchanger (17). Like the 70-, 74-, and 125–130-kDa proteins, phosphorylation of MAP kinase, as evidenced by a shift in electrophoretic mobility, is observed within the first 2 min after applying a hypotonic shock in control as well as vanadate-pretreated cultures (Fig. 3B). Both the  $\text{p42}^{\text{mapk}}$  and (to a lesser extent)  $\text{p44}^{\text{mapk}}$  showed an altered mobility, indicating that both MAP kinase isozymes became phosphorylated and activated.

Among the cellular activators of MAP kinase are peptide growth factors such as EGF (18). To investigate whether EGF could mimic the effects of vanadate on osmoregulation, we stimulated the cells with EGF (50 ng/ml) 90 s prior to applying a submaximal hypotonic shock (20%). As shown in Fig. 4, EGF not only induced the mobility shift of MAP kinase, but also



Shock	+	+	+	-	-
EGF	-	-	+	+	+
Va	-	+	-	-	+

**FIG. 4. Potentiation of the volume-sensitive conductance by EGF.** Radio-isotope-loaded cells were pretreated with vanadate (200  $\mu\text{M}$ , 5 min) and/or EGF (50 ng/ml; 90 s) prior to hypotonic stimulation (20%). Data are expressed as percentage stimulation of the response to a 20% hypotonic shock in the absence of both vanadate and EGF (mean  $\pm$  S.D.,  $n = 3$ ; 100% values correspond with a fractional efflux of  $14.7 \pm 0.2\%/min$  for  $^{125}\text{I}^-$  and  $2.2 \pm 0.1\%/min$  for  $^{86}\text{Rb}^+$ ). Open bars represent  $^{125}\text{I}^-$  efflux; shaded bars represent  $^{86}\text{Rb}^+$  efflux. Inset shows EGF-induced shift in electrophoretic mobility of MAP kinase.

mimicked the potentiating effect of vanadate on  $^{125}\text{I}^-$  and  $^{86}\text{Rb}^+$  efflux. EGF alone, however, was not sufficient for inducing RVD-like ionic responses, since no increase in  $^{125}\text{I}^-$  efflux was observed in the absence of hypotonic stimulation. EGF did induce a moderate increase in  $^{86}\text{Rb}^+$  efflux by itself, additive to the volume-sensitive efflux and not potentiated by vanadate pretreatment, most likely through  $\text{Ca}^{2+}$  activation of a distinct class of  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$  channels (Fig. 4).

Potentiation of the ionic response by vanadate or EGF, as well as its blockade by tyrosine kinase inhibitors, affect both the  $^{125}\text{I}^-$  and  $^{86}\text{Rb}^+$  efflux. Two distinct conductances, however, are involved, since brief pretreatment of the cells with the  $\text{K}^+$  channel blocker quinidine (0.5 mM) completely inhibited the  $^{86}\text{Rb}^+$  efflux, while leaving the efflux of  $^{125}\text{I}^-$  unaffected (results not shown). The results fit into a model of ionic channel recruitment involving the fusion of sub-plasmalemma-localized vesicles containing  $\text{Cl}^-$  and  $\text{K}^+$  channels with the plasma membrane. This model is supported by the recent observation that in Intestine 407 cells whole cell capacitance, indicative for insertion of plasma membrane, increases upon osmotic swelling (19). Importantly, the synergistic action of growth factors and phosphotyrosine phosphatase inhibitors on the RVD response is not restricted to Intestine 407 cells. Qualitatively similar effects were observed in non-intestinal cell lines, including the SW-1573 S1 lung carcinoma cell lines used by others in studies of P-glycoprotein-associated volume-sensitive  $\text{Cl}^-$  channels (20, 21) (not shown).

Together, our results indicate that osmoregulation of ionic channels involves the activation of tyrosine kinases as well as phosphorylation of MAP kinase. An important role for MAP kinases as intermediates in volume regulatory responses is supported by the recent finding that, in yeast strains defective in responding to an increased tonicity of the medium, a member of the MAP kinase family (HOG1) is mutated (22). Although activation of the MAP kinase pathway is most likely an obligatory step in the cellular response to both hypo- and hyperosmotic stimulation, the experiments with EGF suggest that this pathway potentiates but does not initiate the RVD response. Since a protein co-migrating with  $\text{p125}^{\text{FAK}}$  is phosphorylated rapidly upon cell swelling, it is tempting to speculate that factors that couple to cytoskeletal elements (e.g. integrins; Refs. 15 and 23), by sensing the changes in membrane stretch that

occur during alterations of cell volume, may serve as primary triggers of the RVD response.

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