Osmotic swelling-induced activation of the extracellular-signal-regulated protein kinases Erk-1 and Erk-2 in Intestine 407 cells involves the Ras/Raf-signalling pathway

Thea VAN DER WIJK*[1], Jannette DORRESTIJN†, Shuh NARUMIYA‡, J. Antonie MAASSEN†, Hugo R. DE JONGE* and Ben C. TILLY*

*Department of Biochemistry, Cardiovascular Research Institute COEUR, Faculty of Medicine and Health Sciences, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands, †Department of Molecular Cell Biology, Section Signal Transduction, Leiden University, Leiden, The Netherlands, and ‡Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto, Japan

Human Intestine 407 cells respond to hypo-osmotic stress with a rapid stimulation of compensatory ionic conductances accompanied by a transient increase in the activity of the extracellular-signal-regulated protein kinases Erk-1 and Erk-2. In this study, we examined the upstream regulators of hypotonicity-induced Erk-1/Erk-2 activation and their possible role in cell-volume regulation. The hypotonicity-provoked Erk-1/Erk-2 activation was greatly reduced in cells pretreated with the specific mitogen-activated/Erk-activating kinase inhibitor PD098059 and was preceded by a transient stimulation of Raf-1. Pretreatment of the cells with PMA, GF 109203X, wortmannin or *Clostridium botulinum* C3 exoenzyme did not appreciably affect the hypotonicity-provoked Erk-1/Erk-2 stimulation, suggesting the osmosensitive signalling pathway to be largely independent of protein kinase C and $p21^{rho}$. In contrast, expression of dominant negative RasN17 completely abolished the hypotonicity-induced Erk-1/Erk-2 activation. Stimulation of the swelling-induced ion efflux was independent of activation of these mitogen-activated protein kinases, as revealed by hypotonicity-provoked isotope efflux from $^{125}I^-$- and $^{86}Rb^+$-loaded cells after pretreatment with PD098059 and after expression of RasN17. In addition, the epidermal-growth-factor-induced potentiation of the hypotonicity-provoked anionic response did not depend on the increase in Erk-1/Erk-2 activity but, instead, was found to depend on Ca^{2+} influx. Taken together, these results indicate that hypotonic stress induces Erk-1/Erk-2 activation through the Ras/Raf-signalling pathway, and argue against a direct role for this pathway in cell-volume control.

INTRODUCTION

Most mammalian cells respond to changes in cellular volume with a net movement of water driven by a redistribution of salt and/or small organic molecules (for reviews see [1,2]). As a consequence of hyperosmotic stress, cells are triggered to accumulate osmolytes leading to a subsequent influx of water (regulatory volume increase). In reverse, hypo-osmotic stress induces a loss of cellular water due to osmolyte release [regulatory volume decrease (RVD)]. Despite the extensive research carried out on cell-volume regulation to date, the molecular mechanisms of osmosensing and osmosignalling are not completely resolved. Although differences may exist between cell types, a general feature of cell signalling in response to hypotonic stress is the rapid and transient activation of the extracellular-signal-regulated protein kinases Erk-1/Erk-2. Activation of these kinases has now been reported for a variety of mammalian cell types, including Intestine 407 cells [3], hepatoma cells [4], hepatocytes [5], cardiac myocytes [6], astrocytes [7] and C6 glioma cells [8], as well as for yeast [9].

Erk-1/Erk-2 are members of the superfamily of mitogen-activated protein kinases (MAP kinases), a group of serine/threonine kinases that are activated by dual-specificity protein kinases through phosphorylation on both threonine and tyrosine residues. Erk-1/Erk-2 are activated by diverse extracellular stimuli and affect many important cellular processes via protein phosphorylation of specific targets (for reviews see [10,11]). Several different pathways may lead to Erk-1/Erk-2 activation (reviewed in [12]). The pathway studied in most detail is the signalling cascade initiated by tyrosine kinase-containing receptors [e.g. epidermal growth factor (EGF) and platelet-derived growth factor receptors), which involves activation of the small G-protein $p21^{ras}$ (Ras). Conversion of Ras–GDP into the active GTP-bound state, via coupling of the Grb2–Sos complex to the receptor [13], recruits the Raf-1 kinase to the plasma membrane [14]. Subsequently, Raf-1 becomes activated and phosphorylates the mitogen-activated/Erk-activating kinase (MEK) [15], a dual-specificity protein kinase, which in turn activates Erk-1/Erk-2 [16].

In addition to receptor tyrosine kinases, several G-proteins coupled to serpentine receptors, as well as cell-surface adhesion receptors of the integrin family, have been identified as activators of Erk-1/Erk-2 [17,18]. These receptors have been suggested to activate the Ras/Erk pathway via activation of PtdIns 3-kinase (G-protein-coupled receptors) or by binding of the focal adhesion kinase $p125^{FAK}$ (integrin receptors) to the Grb2–Sos complex [19,20]. In addition, several Ras-independent pathways have been reported that lead to Erk-1/Erk-2 activation. For instance,

Abbreviations used: EGF, epidermal growth factor; Erk, extracellular-signal-regulated protein kinase; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, mitogen-activated/Erk-activating kinase; PKC, protein kinase C; RVD, regulatory volume decrease; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ECL, enhanced chemiluminescence.

[1] To whom correspondence should be addressed (e-mail vanderwijk@bc1.fgg.eur.nl).

Robbins et al. [21] and Chen et al. [22] found that expression of a dominant negative Ras mutant did not affect Erk-1/Erk-2 activation by G-protein-bound and integrin receptors. Furthermore, EGF has been reported to activate Erk-2 additionally via Ras-independent pathways, which involve protein kinase C (PKC) activation or intracellular Ca^{2+} $[Ca^{2+}]_i$ increase [23–25].

Using the human fetal jejunum-derived Intestine 407 cell line as a model, we have recently demonstrated that hypo-osmotic cell swelling is accompanied by a rapid and transient increase in tyrosine phosphorylation of several proteins, including $p125^{FAK}$ and Erk-1/Erk-2, as well as by activation of PtdIns 3-kinase [3,26]. Furthermore, increased tyrosine phosphorylation was found to be an essential step in eliciting an RVD response [3]. The finding that EGF not only activates the MAP kinases Erk-1/Erk-2 but also strongly potentiates the hypotonicity-induced ion efflux [3] suggested a role for Erk-1/Erk-2 in the activation and/or growth factor potentiation of the osmosensitive anion efflux. This notion was supported by the recent findings that both the yeast Hog1p and Mpk1p members of the MAP kinase family are critically involved in cell-volume regulation [9,27].

In this study we have investigated the regulatory pathway leading to hypotonicity-induced Erk-1/Erk-2 activation and evaluated the role of these MAP kinases in cell-volume regulation. The results presented indicate that the transient osmosensitive activation of Erk-1/Erk-2 in Intestine 407 cells is downstream of the Ras/Raf pathway, and independent of PKC, Ca^{2+} influx and PtdIns 3-kinase. Treatment of the cells with the specific MEK inhibitor PD098059 or expression of dominant negative RasN17 prevented the cell-swelling-induced Erk-1/Erk-2 activation, but neither affected the osmosensitive anion efflux or its potentiation by EGF. Taken together, the results argue against a direct role for Erk-1/Erk-2 in cell-volume regulation.

MATERIALS AND METHODS

Materials

Radioisotopes ($^{125}I^-$, $^{86}Rb^+$ and [γ-^{32}P]ATP) and enhanced chemiluminescence (ECL) detection kit were purchased from Amersham Netherlands B.V. ('s Hertogenbosch, The Netherlands). Antibodies and Syntide-2 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). The MEK inhibitor PD098059, myelin basic protein (MBP) and GF 109203X were purchased from Calbiochem (La Jolla, CA, U.S.A.), Sigma (St. Louis, MO, U.S.A.) and Biomol (Plymouth Meeting, PA, U.S.A.) respectively. Protein A–Sepharose was obtained from Pharmacia Biotech (Uppsala, Sweden). Recombinant *Cl. botulinum* C3 exoenzyme was isolated and prepared as described previously [28].

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/l penicillin and 90 mg/l streptomycin under a humidified atmosphere of 95% O_2 and 5% CO_2 at 37 °C. Before the experiments, cells were serum-starved overnight.

Assay of Erk-1/Erk-2 activity

After stimulation, cells were washed once with ice-cold PBS, and 1 ml of lysis buffer was added (1% Triton X-100, 150 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P40, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 1% aprotinin and 50 μg/ml leupeptin, pH 7.4). After 10 min (0 °C), lysates were collected, cleared by centrifugation and the supernatants were incubated with polyclonal anti-Erk-2 antibodies (recognizing both Erk-2 and, to a lesser extent, Erk-1) for 1 h. Thereafter, Protein A–Sepharose was added and the mixture was incubated for another hour. The immunoprecipitates were washed three times with lysis buffer and three times with kinase buffer (10 mM Tris/HCl, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, pH 7.4), and Erk-1/Erk-2 activity was determined by *in vitro* phosphorylation of MBP. Phosphorylation was carried out in 40 μl of kinase buffer supplemented with 0.5 mg/ml MBP and 25 μM ATP + 5 μCi of [γ-^{32}P]ATP (30 min; 37 °C). Reactions were terminated by the addition of SDS sample buffer. The samples were boiled for 5 min and subjected to SDS/PAGE. Radioactivity of MBP was quantified by a phospho-imaging system (Molecular Imaging System GS-363; Bio-Rad).

Assay of Raf-1 activity

Cells were lysed in 50 mM Hepes, pH 7.5, containing 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 1% aprotinin (10 min; 0 °C). Lysates were collected, cleared and incubated with 1 μg/ml polyclonal rabbit anti-Raf-1. Immune complexes were bound to Protein A–Sepharose and washed three times with lysis buffer, twice with 0.5 M LiCl/0.1 M Tris/HCl, pH 7.5 and once with kinase buffer (25 mM Tris/HCl, 10 mM $MnCl_2$, 1 mM dithiothreitol, 25 mM β-glycerolphosphate, pH 7.5). Raf-1 activity was determined using Syntide-2 as a substrate. Phosphorylation was carried out for 20 min at 30 °C in 40 μl of kinase buffer containing 125 μM Syntide-2 and 25 μM ATP + 5 μCi of [γ-^{32}P]ATP. Thereafter, samples were rapidly centrifuged and 15 μl portions of the supernatants were spotted on Whatman p81 phosphocellulose paper. After extensive washing of the paper with 0.85% phosphoric acid, ^{32}P incorporation was monitored by phospho-imaging.

Kinase mobility-shift assay

Monolayers of cells were stimulated as indicated in the legends and incubations were terminated by replacing the medium with boiling SDS sample buffer. Proteins were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose. Proteins were stained with polyclonal anti-Erk-1 antibodies (recognizing both Erk-1 and Erk-2) or polyclonal anti-Raf-1 antibodies and an ECL Western-blotting detection system according to the instructions provided by the manufacturers.

Vaccinia virus infection

Infections with recombinant vaccinia virus encoding the RasN17 mutant and wild-type vaccinia virus were performed as described previously [23]. After infection, the medium was replaced by Dulbecco's modified Eagle's medium containing 0.25% BSA. Experiments were performed 16–18 h after infection.

Efflux assays

Confluent monolayers of Intestine 407 cells were loaded with 5 μCi/ml $^{125}I^-$ or 0.5 μCi $^{86}Rb^+$ for 2 h in modified Meyler solution (108 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM $NaHCO_3$, 0.8 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 20 mM Hepes and 10 mM glucose, pH 7.4) under a 95% O_2/5% CO_2 humidified atmosphere. Subsequently, the cultures were washed three times with isotonic buffer (80 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 95 mM mannitol and 20 mM Hepes, pH 7.4), and radioisotope efflux

was determined by replacing the medium at 1–2 min intervals. Hypotonic buffers were prepared by adjusting the concentration of mannitol. Radioactivity in the medium was determined by γ-radiation counting and expressed as fractional efflux per minute as described previously [29].

RESULTS

Activation and phosphorylation of Erk-1/Erk-2 and Raf-1 in response to hypo-osmotic cell swelling

Previously, a transient increase in Erk-1 and Erk-2 phosphorylation, as demonstrated by a shift in electrophoretic mobility on SDS/PAGE, has been observed after hypotonic stimulation of Intestine 407 cells [3]. Recently, however, it has been reported that a mobility shift could already be observed after phosphorylation of either a tyrosine or a threonine residue of Erk-1/Erk-2, whereas phosphorylation of both residues is required to induce activation of Erk-1/Erk-2 [10]. Therefore we quantified hypo-osmotic swelling-induced Erk-1/Erk-2 kinase activity more directly, using MBP as a substrate. A time course of Erk-1/Erk-2 activity, as determined in confluent Intestine 407 cultures exposed to a 30% hypotonic shock (70% tonicity), is shown in Figure 1. Activation started without an apparent lag phase and peaked after 5 min. Thereafter, the Erk-1/Erk-2 activity slowly declined to prestimulatory levels. No further activation of Erk-1/Erk-2 was observed within 2–4 h of osmotic stimulation (results not shown). The osmosensitive stimulation of Erk-1/Erk-2 is a direct consequence of MEK activity, since pretreatment of the cells with the specific MEK inhibitor PD098059 [30], at concentrations that effectively block EGF-induced increase in MAP kinase activity, greatly reduced Erk-mediated MBP phosphorylation (Table 1).

In order to establish upstream signalling components of the hypotonicity-induced MEK/Erk activation, we studied the effects of osmotic cell swelling on both Raf-1 phosphorylation and activity. After a lag period of approx. 5 min, a marked increase in Raf-1 phosphorylation, as evidenced by a decreased electrophoretic mobility, was observed, which lasted for more than 30 min (Figure 2A). The hypotonicity-induced activation of Raf-1, however, as determined by *in vitro* phosphorylation using Syntide-2 as a substrate, started immediately and was very transient, peaking at 2 min after stimulation (Figure 2B). Subsequently, Raf-1 activity rapidly declined to levels even below the control values. Notably, the decrease in Raf-1 activity coincided with an increase in Raf-1 phosphorylation.

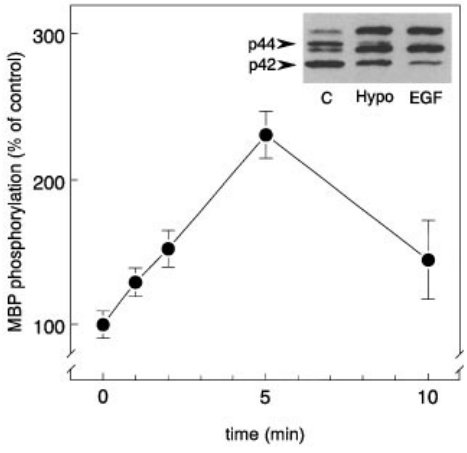

Figure 1 Time course of hypotonic shock-induced phosphorylation and activation of Erk-1/Erk-2 in Intestine 407 cells

Cells were exposed to 30% hypotonic medium for the times indicated in the presence of 200 μM Na_3VO_4 (total exposure time was 10 min at all time points). Erk-1/Erk-2 were immunoprecipitated and enzyme activity was measured using MBP as substrate. Data are expressed as percentage of activity in cells exposed to isotonic medium (mean $\pm$ S.E.M., $n = 3$). The inset shows the electrophoretic mobility shift of Erk-1/Erk-2 (p44/p42) induced by a 5 min exposure to 30% hypotonic medium or by EGF (50 ng/ml).

Table 1 Effect of the MEK inhibitor PD098059 on hypotonicity- and EGF-induced Erk-1/Erk-2 activation

Control or PD098059 (50 μM for 2 h)-pretreated cultures were exposed to isotonic medium, to 30% hypotonic medium or to 50 ng/ml EGF for 5 min. Thereafter, Erk-1/Erk-2 were immunoprecipitated and enzyme activity was determined using MBP as substrate. Data are expressed as percentage of activity in cells exposed to isotonic medium and not pretreated with PD098059 (mean $\pm$ S.E.M., $n = 3$).

	Erk-1/Erk-2 activity (% of control)		
Pretreatment	Isotonic	Hypotonic	EGF
None	100 $\pm$ 11	279 $\pm$ 19	303 $\pm$ 32
PD098059	46 $\pm$ 5	98 $\pm$ 26	108 $\pm$ 7

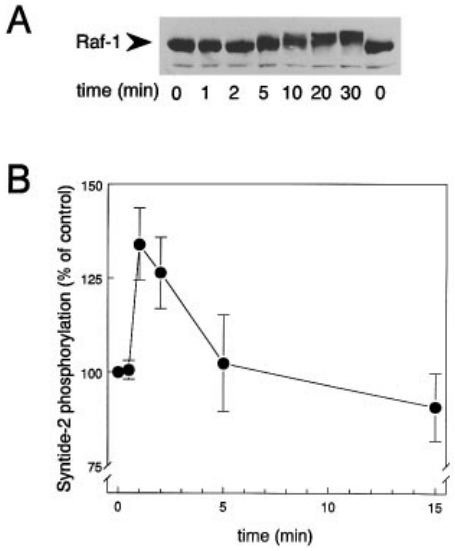

Figure 2 Time course of hypotonic shock-induced Raf-1 phosphorylation and activation

Cells were exposed to 30% hypotonic medium for the times indicated. (**A**) Electrophoretic mobility shift of Raf-1. Whole cell lysates were separated by SDS/PAGE followed by Western blotting. Immunoblots were incubated with anti-Raf-1 antibodies. (**B**) Raf-1 kinase activity. Raf-1 was immunoprecipitated and enzyme activity was measured using Syntide-2 as substrate. Data are expressed as percentage of activity in cells exposed to isotonic medium (mean $\pm$ S.E.M., $n = 4$).

Osmotic activation of Erk-1/Erk-2 is largely independent of PKC

PKC has previously been identified as an activator of MEK and Erk-1/Erk-2 through stimulation of Raf-1 kinase [31–33]. To investigate a possible regulatory role for PKC in the hypotonicity-induced activation of Erk-1/Erk-2, cells were pretreated with 100 nM PMA to either activate (5 min pretreatment) or down-regulate (24 h pretreatment) PKC. Whereas activation of PKC

Table 2 Effect of PMA on the hypotonicity-induced Erk-1/Erk-2 activation

Control and PMA-treated (100 nM for 5 min or 24 h) cells were exposed to 30% hypotonic medium or isotonic medium for 5 min. Thereafter the cells were lysed and Erk-1/Erk-2 enzyme activity was determined in immunoprecipitates using MBP as substrate. Data are expressed as percentage of activity in untreated cells (mean ± S.E.M., $n = 4$).

	Erk-1/Erk-2 activity (% of control)	
Pretreatment	Isotonic	Hypotonic
None	100 ± 15	373 ± 15
PMA (5 min)	325 ± 36	460 ± 16
PMA (24 h)	93 ± 31	267 ± 33

Table 3 Effect of GF 109203X on the hypotonicity-induced Erk-1/Erk-2 activation

Cells were exposed for 5 min to isotonic medium, 30% hypotonic medium or 100 nM PMA in control and GF 109203X-pretreated (1 μM for 20 min) cultures. Thereafter, Erk-1/Erk-2 were immunoprecipitated and enzyme activity was determined using MBP as substrate. Data are expressed as percentage of activity in untreated cells (mean ± S.E.M., $n = 6$).

	Erk-1/Erk-2 activity (% of control)		
Pretreatment	Isotonic	Hypotonic	PMA (5 min)
None	100 ± 5	331 ± 16	368 ± 27
GF 109203X	120 ± 16	262 ± 37	135 ± 16

by a brief treatment with PMA markedly stimulated Erk-1/Erk-2 under isotonic conditions, a further increase in activity of the MAP kinases was still observed after hypo-osmotic cell swelling (Table 2). Furthermore, down-regulation of PKC by prolonged PMA treatment only slightly affected hypotonicity-induced Erk-1/Erk-2 stimulation (Table 2). Pretreatment of cells with the specific PKC inhibitor GX 109203X (1 μM for 20 min) virtually abolished the PMA stimulation of Erk-1/Erk-2 activity, but caused only a minor inhibition of the hypotonicity-provoked increase in activity (Table 3). Taken together, these results suggest that hypotonic stress activates Erk-1/Erk-2 principally via a PKC-independent pathway.

Hypotonic stress-induced Erk-1/Erk-2 activation depends on active Ras

To investigate the putative involvement of the small GTPase Ras in the hypotonicity-induced Erk-1/Erk-2 activation, cells were infected with recombinant vaccinia virus encoding mutant dominant-negative RasN17 [23]. As compared with control cultures, infection of the cells with recombinant RasN17 virus completely abolished the hypotonic-stress-induced Erk-1/Erk-2 activation, whereas mock virus infection affected their stimulation only slightly (Figure 3). Unlike the hypotonicity-provoked Erk-1/Erk-2 stimulation, activation of these MAP kinases by EGF was only partially inhibited after expression of RasN17. This may be due to EGF-triggered activation of alternative Ras-independent pathways, as has been reported by others [23–25].

In wortmannin-treated (0.1–1 μM; 30 min) cells, neither basal nor the osmosensitive Erk-1/Erk-2 activities were affected (results not shown). In addition, pretreatment of the cells with *Cl. botulinum* C3 exoenzyme under conditions that inhibited the hypotonicity-induced anion efflux, p125^{FAK} phosphorylation as

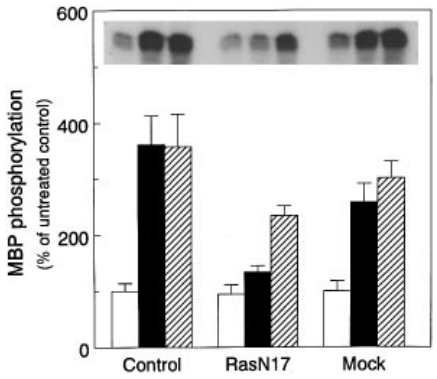

Figure 3 Effect of RasN17 expression on hypotonicity- and EGF-induced Erk-1/Erk-2 activation

Before exposure of the cells to isotonic (□), 30% hypotonic (■) or EGF (50 ng/ml)-containing (▨) medium, cells were transfected with recombinant vaccinia virus expressing RasN17 or with wild-type virus (Mock). Erk-1/Erk-2 were immunoprecipitated and activity was measured using MBP as substrate. Data are expressed as percentage of activity in uninfected (Control) cells exposed to isotonic medium (mean ± S.E.M., $n = 3$). The inset shows a representative autoradiograph of ^{32}P-labelled MBP separated by SDS/PAGE.

Table 4 Effect of *Cl. botulinum* C3 exoenzyme on hypotonicity-induced Erk-1/Erk-2 activation

Cells were exposed for 5 min to isotonic medium or 30% hypotonic medium in the absence or presence of 50 μg/ml C3 exoenzyme (48 h). After lysis of the cells, Erk-1/Erk-2 were immunoprecipitated and enzyme activity was measured using MBP as substrate. Data are expressed as percentage of activity in untreated cultures exposed to isotonic medium (mean ± S.E.M., $n = 3$).

	Erk-1/Erk-2 activity (% of control)	
Pretreatment	Isotonic	Hypotonic
None	100 ± 3	188 ± 4
C3 exoenzyme	50 ± 8	105 ± 17

well as the PtdIns 3-kinase activation (cf. [26]) markedly reduced basal Erk-1/Erk-2 activity but did not prevent Erk-1/Erk-2 stimulation by osmotic cell swelling (Table 4), arguing against a direct involvement of p125^{FAK} and PtdIns 3-kinase in hypotonicity-induced activation of the Ras/Erk cascade.

Erk-1/Erk-2 activation is not involved in triggering the compensatory ion efflux

Recent studies have demonstrated that in yeast strains defective in responding to alterations in the tonicity of the medium, the Hog1p kinase was mutated and inactive, suggesting an important role for members of the MAP kinase family in cell-volume regulation [27]. To investigate the role of Erk-1/Erk-2 in the activation of the osmosensitive anion conductance, hypotonicity-provoked $^{125}I^-$ efflux was determined in PD098059-pretreated cultures. As clearly shown in Figure 4(A), PD098059 (50 μM, 2 h) inhibition of MEK did not affect the swelling-induced $^{125}I^-$ efflux, whereas Erk-1/Erk-2 activation was almost completely abolished (cf. Table 1). Likewise, the osmosensitive $^{86}Rb^+$ efflux, indicative of K^+-channel activation, remained unaffected in the presence of PD098059. In addition, no significant decrease in

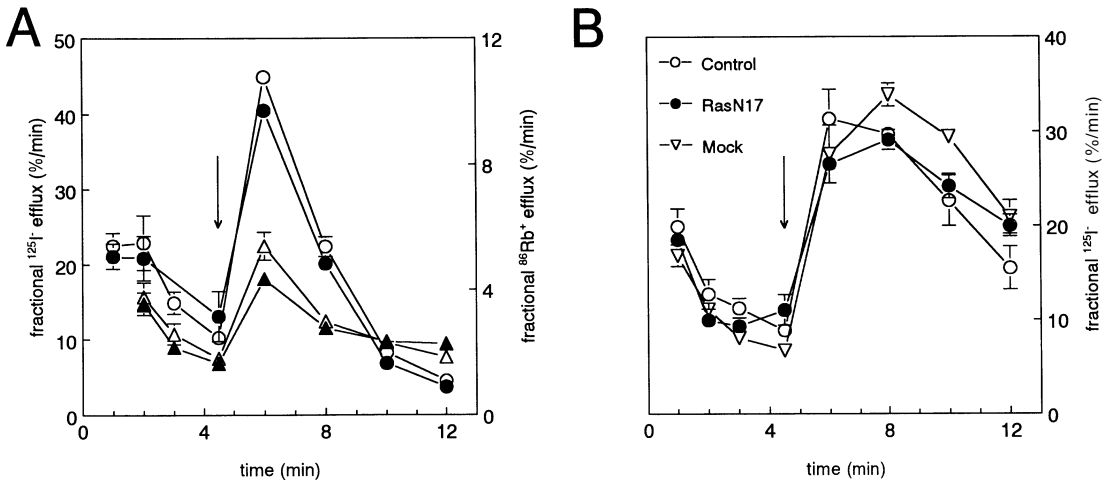

Figure 4 Effect of PD098059 and dominant-negative RasN17 on osmosensitive $^{125}I^-$ and $^{86}Rb^+$ efflux

(**A**) Radioisotope efflux was determined from control (○, △) and PD098059-treated (50 μM, 2 h; ●, ▲) cultures. Circles and triangles represent $^{125}I^-$ and $^{86}Rb^+$ efflux respectively. Data are expressed as fractional isotope efflux (mean ± S.E.M., $n = 3$). Arrow indicates a shift from isotonic to 30% hypotonic medium. (**B**) Control cultures and cells infected with either recombinant vaccinia virus expressing RasN17 or wild-type vaccinia virus (Mock) were osmotically stimulated (30% hypotonicity) and fractional $^{125}I^-$ efflux was determined. Data are expressed as mean ± S.E.M. ($n = 6$).

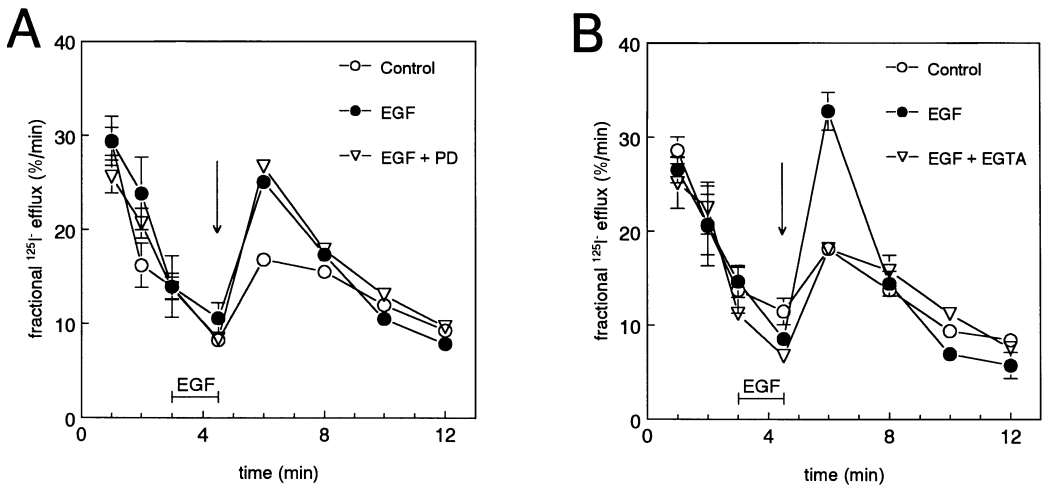

Figure 5 Effect of PD098059 and EGTA on EGF-induced potentiation of osmosensitive $^{125}I^-$ efflux

Control (○) and EGF-treated (50 ng/ml for 90 s before osmotic stimulation; ●, ▽) cells were exposed to a 20% hypotonic medium, and fractional $^{125}I^-$ efflux was determined. (**A**) Effects of pretreatment with 50 μM PD098059 (PD). (**B**) Effects of chelating extracellular Ca^{2+} by the addition of excess EGTA (1.3 mM) during the assay. Data are expressed as mean ± S.E.M. ($n = 3$). Arrow indicates a shift from isotonic to hypotonic medium.

osmosensitive iodide efflux was observed in cells expressing RasN17 (Figure 4B).

We previously reported an EGF-induced potentiation of the osmosensitive anion efflux in Intestine 407 cells [3]. Since, in addition to the activation of the Ras/Erk signalling cascade, EGF is also able to raise $[Ca^{2+}]_i$ by the release of Ca^{2+} from intracellular stores and by activation of plasma-membrane Ca^{2+} channels [34,35], we studied the effects of EGF in PD098059- and EGTA-treated cells. As shown in Figure 5, EGF potentiation of the hypotonicity-induced $^{125}I^-$ efflux was still present in PD098059-treated cells, but was completely absent from cultures pretreated with EGTA, indicating that the EGF-mediated increase in anion conductance is not due to activation of Erk-1/Erk-2 but instead involves a rise in $[Ca^{2+}]_i$. Importantly, Erk-1/Erk-2 activation by EGF or osmotic cell swelling was not reduced in EGTA-containing medium (results not shown). To summarize, these results indicate that Erk-1/Erk-2 activity is involved in neither eliciting nor potentiating the compensatory osmosensitive anion efflux.

DISCUSSION

Osmotic cell swelling rapidly leads to the phosphorylation and/or activation of a number of signalling enzymes concomitant with an increase in the conductance of compensatory ion channels. Among these enzymes, the Erk-1/Erk-2 members of the MAP kinase family are of particular interest since (1) they are rapidly and transiently activated, without an apparent lag phase; (2)

treatment of the cells with the Erk-1/Erk-2 activator EGF potentiates the hypotonicity-induced anion efflux [3]; (3) in yeast, the MAP kinases Hog1p and Mpk1p were found to be crucial for osmoresponse [9,27]. In this paper we analysed the signalling pathway(s) involved in hypotonicity-induced Erk-1/Erk-2 activation as well as their role in ion-channel activation.

Hypotonic activation of Erk-1/Erk-2 in Intestine 407 cells was accompanied by a rapid and transient increase in Raf-1 activity and was greatly reduced in cells pretreated with the MEK inhibitor PD098059. In addition, expression of dominant-negative RasN17 in the cells abolished hypotonicity-induced Erk-1/Erk-2 activation. These results clearly indicate that the osmotic cell-swelling-induced activation of Erk-1/Erk-2 is a direct consequence of Ras GDP/GTP exchange, analogous to the activation of Erk-1/Erk-2 by tyrosine kinase-containing receptors. This notion is supported by our observations that the osmosensitive Erk-1/Erk-2 stimulation was largely independent of PKC acting directly on Raf-1. A hypotonicity-induced phosphorylation of Raf-1, as reported in this study, was also found in H4IIE hepatoma and C6 glioma cells [4,8]. However, a clear discrepancy exists between Raf-1 activation and its phosphorylation. As is evident from our results, cell-swelling-induced Raf-1 activation was rapid and very transient, returning to a level even below prestimulatory activity within 2–5 min, whereas Raf-1 phosphorylation was only observed after 5 min of exposure to hypotonic medium. These observations are in agreement with the findings of Wartmann et al. [36], who recently reported that subsequent to its activation by Ras, Raf-1 is immediately inactivated by hyperphosphorylation.

It still remains to be established how osmotic cell swelling activates Ras. A possible mechanism involves the Ras-related GTPase $p21^{rho}$, which has been identified as an intermediate in the lysophosphatidic acid- and integrin-receptor-induced Erk-1/Erk-2 activation [28,37,38]. Using *Cl. botulinum* C3 exoenzyme-treated cells, we previously demonstrated the involvement of $p21^{rho}$ in the osmosensitive increase in anion conductance as well as in $p125^{FAK}$ phosphorylation and PtdIns 3-kinase activation [26]. Phosphorylated $p125^{FAK}$ as well as PtdIns 3-kinase are able to activate Ras by respectively direct binding to the Grb2-SH2/SH3 adaptor protein or by stimulating an Src-like kinase, which subsequently activates the Shc–Grb2–SOS complex [19,20]. Our results, however, argue against a role for $p21^{rho}$, $p125^{FAK}$ or PtdIns 3-kinase in the signalling cascade leading to Ras activation, since pretreatment of the cells with C3 exoenzyme did not prevent hypotonicity-induced Erk-1/Erk-2 activation. Furthermore, pretreatment of the cells with the PtdIns 3-kinase inhibitor wortmannin did not affect Erk-1/Erk-2 activation in Intestine 407 cells. PtdIns 3-kinase independence was also observed in C6 glioma cells [8]; however, Erk-1/Erk-2 activation in astrocytes was found to be completely abolished after PtdIns 3-kinase inhibition [7]. Alternatively, several heterotrimeric G-proteins have been identified as activators of Ras-type GTPase (reviewed in [39]). Both pertussis toxin-sensitive $G_i\beta\gamma$ subunits as well as pertussis toxin-insensitive $G\alpha_{12}$ and $G\alpha_{13}$ subunits have been implicated in the activation of MAP kinases. Whereas a G_i involvement in the hypotonicity-provoked Erk-1/Erk-2 phosphorylation was observed in hepatoma cells [4], G_i is not likely to be a candidate for Ras activation in Intestine 407 cells since pretreatment of the cells with pertussis toxin did not affect Erk-1/Erk-2 activation (T. van der Wijk and B. C. Tilly, unpublished work). These discrepancies in PtdIns 3-kinase and G_i involvement in the hypotonicity-induced Erk-1/Erk-2 activation clearly suggests that upstream regulators may differ between model systems.

In the yeast *Saccharomyces cerevisiae*, both the HOG1 and the PBS2 genes, which encode respectively a MAP kinase (Hog1p) and a MAP kinase kinase, are essential for adequately responding to an increase in extracellular tonicity [27]. Recently, the yeast osmosensor involved in activating the HOG1 cascade has been found [40,41]. The osmosensing mechanism belongs to the so-called 'two-component signal-transduction systems' and consists of the transmembrane histidine kinase Sln1p (the 'sensor') and the cytosolic Ssk1p response regulator. As, in most mammalian cell types studied, a rapid activation of Erk-1/Erk-2 is among the initial signalling events after osmotic cell swelling, it is tempting to propose a model in which the Ras/Erk cascade plays an important role in the RVD response. This notion is supported by several observations that cellular and oncogenic Ras can affect ion-transport systems [42–44]. Our results, however, using the specific MEK inhibitor PD098059 as well as the expression of dominant-negative RasN17, clearly demonstrate that the Ras/Raf/MEK/Erk cascade is not involved in the activation of compensatory ionic conductances in hypotonically stimulated Intestine 407 cells. Moreover, EGF potentiation of the hypotonicity-induced anion efflux occurs through elevation of $[Ca^{2+}]_i$ rather than Erk-1/Erk-2 activation. A potential role for Ca^{2+} signalling in the potentiation of the hypotonicity-induced ionic conductances in Intestine 407 cells was previously established by the observations that Ca^{2+}-mobilizing hormones as well as thapsigargin and the Ca^{2+} ionophore A23187 were able to enhance, but not to trigger, the osmosensitive ionic fluxes [45].

In addition to Erk-1/Erk-2, several other members of the MAP kinase family have recently been reported. Among these, the p38 MAP kinase is of particular interest because of its high homology to Hog1p and, in Intestine 407 cells, its rapid activation on osmotic cell swelling [46]. It is very unlikely, however, that p38 is involved in the activation of osmosensitive ionic channels, because inhibition of p38 MAP kinase, using the specific p38 inhibitor SB-203580, did not block the osmosensitive anion efflux and, unlike Erk-1/Erk-2, activation of p38 MAP kinase using cytokines or anisomycin did not potentiate the ionic response [46]. The role of c-Jun N-terminal kinase, a third member of the MAP kinase family which becomes activated on cell swelling in cardiac myocytes [6] as well as Intestine 407 cells (B. C. Tilly, unpublished work), in the regulation of osmosensitive ionic fluxes remains to be established.

To date, little is known about the physiological role of Erk-1/Erk-2 activation during the RVD response. An increased expression of the intermediate early genes c-*fos* and c-*jun* has been observed in osmotically stimulated hepatoma cells and cardiomyocytes [4,6], suggesting a role for Erk-1/Erk-2 in transcription regulation, maintaining cellular homoeostasis and/or long-term survival. Activation of the Ras/Erk-signalling cascade has been widely recognized as the key event leading to increased cell proliferation. In addition to a rapid and transient first phase, a second more sustained phase of Erk-1/Erk-2 activation as well as a subsequent translocation to the nucleus was found to be a prerequisite for G_0–S transition and cell division to occur [47,48]. Yet, in Intestine 407 cells, Erk-1/Erk-2 activation is transient and a second phase is lacking, rendering a role for cell-swelling-induced Erk-1/Erk-2 activation in cell proliferation unlikely. A more acute role for hypotonicity-activated Erk-1/Erk-2 has been proposed for hepatocytes [1,5]. In these cells, a relation has been found between Erk-1/Erk-2 activation and the rapid cell-swelling-induced excretion of taurocholate into bile. In addition, Erk-2 has been reported to activate phospholipase A_2, the enzyme regulating arachidonic acid release, suggesting a possible involvement of Erk-1/Erk-2 in intercellular signalling [49]. However, for epithelial cell lines such as Intestine 407, a functional role for hypotonicity-provoked Erk-1/Erk-2 activation remains to be established.

The results of this study also have implications for the interpretation of our original observation that protein tyrosine phosphorylation is an essential event in the activation mechanism of cell-swelling-induced compensatory ionic fluxes [3]. Since the Ras/Erk cascade is apparently not involved in the regulation of osmosensitive ionic channels, it follows that the tyrosine kinase(s) or phosphatase(s) involved in cell volume regulation are distinct from MEK.

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