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Hypo-osmotic cell swelling activates the p38 MAP kinase signalling cascade

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Abstract Hypo-osmotic swelling of human Intestine 407 cells leads to a significant increase of intracellular MAPKAP-kinase 2 activity and Hsp27 phosphorylation. Pre-treatment of the cells with the p38 MAP kinase inhibitor SB-203580 blocks this activation, indicating that the hypotonicity-induced activation of MAPKAP kinase 2 is, similarly to that described for hyperosmotic treatment, the result of an activated p38 MAP kinase cascade. The activation of MAPKAP kinase 2 proceeds with kinetics similar to that of one of the first physiological responses of hypo-osmotic treatment, the opening of compensatory Cl^- channels. However, inhibition of the p38 MAP kinase cascade does not block the osmo-sensitive anion efflux and, vice versa, activation of p38 MAP kinase by cytokines and anisomycin does not increase the efflux. These results indicate that the p38 MAP kinase cascade is not directly involved in Cl^- channel activation but instead may play a role in subsequent cellular repair processes.

Key words: Regulatory volume decrease; Chloride channel; Reactivating kinase; MAP kinase; Heat shock protein; Chloride channel; Cytokine

1. Introduction

Human Intestine 407 cells respond to hypo-osmotic stimulation with a rapid increase in Cl^- and K^+ permeability, resulting in a net efflux of salt and, subsequently, cellular water. Although the conductive efflux pathways triggered in response to osmotic cell swelling have been described in many different cell systems, the molecular mechanism(s) involved in the activation of osmo-sensitive ion channels and transporters have not been clarified in detail and may differ between cell types (for reviews see [1–3]). Previously, we demonstrated that stimulation of the volume-sensitive Cl^- efflux in Intestine 407 cells was independent of a rise in $[Ca^{2+}]_i$, but, in contrast, was found to rely completely upon protein tyrosine phosphorylation and on the activity of the small GTP-binding protein $p21^{rho}$ [4–6].

Among the proteins phosphorylated in response to hypotonic stimulation are the ERK1 and ERK2 members of the MAP kinase family [4,7]. In Intestine 407 cells, both isoenzymes are rapidly and transiently phosphorylated following osmotic cell swelling [4]. Furthermore, activation of ERK1 and ERK2, by pre-treating the cells with peptide growth factors such as EGF, was associated with potentiation of the osmo-sensitive anion efflux [4]. EGF activation of these MAP kinases in the absence of a hypotonic shock, however, was unable to elicit an ionic response, suggesting that the osmo-sensitive chloride channel is not directly activated by ERK1 and/or ERK2 [4]. Recently, several other members of the MAP kinase family have been described, which are part of distinct pathways and activated by specific external signals (for reviews see [8,9]). Among these the p38 MAP kinase/reactivating kinase (RK) is of particular interest, since activation of this kinase has been observed following exposure of cells to such diverse forms of cellular stress as heat shock, hyperosmotic stimulation and treatment with cytokines and xenobiotics [10–12]. Furthermore, p38 MAP kinase was found to be highly homologous to the HOG1 protein, a yeast member of the MAP kinase family critically involved in the adaptation to hyperosmotic stress [13,14]. In intact cells, p38 MAP kinase stimulation leads to an activation of MAPKAP kinase 2 and subsequent phosphorylation of the low molecular weight heat shock proteins Hsp25 and Hsp27 [11,12,15,16].

Here, we report that osmotic cell swelling activates MAPKAP kinase 2 and subsequently induces Hsp phosphorylation. We show that this activation by hypo-osmotic treatment is rapid, starting within 30 s of stimulation, and sustained for more than 10 min. Using the specific p38 MAP kinase inhibitor SB 203580, we demonstrate that the increased MAPKAP kinase 2 activity is the result of an activated p38 MAP kinase cascade. With the help of this inhibitor, we further analyse the role of activated p38 MAP kinase for one of the first physiological responses to hypotonicity, the opening of osmo-sensitive anion channels.

2. Materials and methods

2.1. Cell culture

Intestine 407 cells were grown as monolayers on plastic in Dulbecco's modified Eagle's Medium (DMEM) 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 95% O_2/5% CO_2 atmosphere.

2.2. $^{125}I^-$ efflux assays

Intestinal 407 cells were loaded with 5 μCi $^{125}I^-$ (Amersham) in modified Meyler (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) for 2 h under a 95% O_2/5% CO_2 atmosphere. Prior to the assay, the cells were washed 3 times with isotonic buffer (80 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 95 mM mannitol and 20 mM HEPES, pH 7.4). 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 min as previously described [17].

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2.3. MAPKAP kinase 2 activity assay

Cultures were stimulated as indicated in the legends and washed three times with ice-cold phosphate-buffered saline (PBS). Thereafter, cells were scraped off, sedimented and stored at −80°C prior to the assay. The pellet was re-dissolved in lysis buffer (0.27 M sucrose, 50 mM NaF, 20 mM Tris-acetate, 10 mM β-glycerophosphate, 5 mM pyrophosphate, 1 mM EGTA, 1 mM Na_3VO_4, 0.1 mM EDTA, 1 mM benzamidine, 0.2 mM phenylmethane sulfonylfluoride, 2 μg/ml leupeptin, 1% Triton X-100 and 0.1% β-mercaptoethanol, pH 7.0) and incubated for 15 min at 0°C. Lysates were cleared by centrifugation and the supernatants were incubated with an anti-MAPKAP kinase 2 antiserum overnight prior to addition of protein A-Sepharose. 1 h later the immunoprecipitates were washed three times with TBS (20 mM Tris-HCl, 0.154 M NaCl, pH 7.5) containing 50 mM NaF, 1 mM Na_3VO_4 and 1% Triton X-100 and finally resuspended in a buffer consisting of 50 mM Na-β-glycerophosphate, 4 mM Mg-acetate, 1 mM EDTA, 0.4 mg/ml Hsp25, 20 μM H7 (Calbiochem), 20 μM HA1077 (Calbiochem) and 2.5 μM protein kinase A inhibitor (Gibco). The reaction was started by the addition of ATP (0.1 mM ATP containing 2 μCi [γ-^{32}P]ATP) and terminated 10 min later by the addition of SDS-stopmix. Samples were subjected to SDS-PAGE and radioactivity in the Hsp25 band was quantitated by phospho-imaging (BAS 2000, Fuji or Molecular Imaging System GS-363, Bio-rad).

2.4. Hsp27 phosphorylation

Nearly confluent cultures of Intestine 407 cells were washed twice with phosphate-free modified-Meyler and incubated in the same medium containing 0.4 mCi/ml $^{32}PO_4^{3-}$ (Amersham) for 3 h. Thereafter, the cells were stimulated, washed 3 times (150 mM NaCl, 20 mM Na_3PO_4, pH 7.4, 0°C) and Hsp27 phosphorylation was determined as described by Cuenda et al. [18]. Briefly, after solubilization of the cells (lysis buffer, 10 min), the lysates were collected, cleared by centrifugation and protein G-Sepharose-conjugated anti-Hsp27 antibodies (StressGen, Victoria, Canada) were added to the supernatants. After 2 h of incubation at 0°C, the beads were centrifuged, washed 3 times with 1 ml lysis buffer containing 0.5 M NaCl and twice with lysis buffer, resuspended in sample mix and subjected to SDS-PAGE. Radioactivity in the Hsp27 band was quantitated by phospho-imaging as described above.

3. Results

3.1. Activation of MAPKAP kinase 2 and the p38 MAP kinase cascade by osmotic stimulation

Confluent cultures of Intestine 407 cells were treated with anisotonic media and activation of the p38 MAP kinase signalling cascade was assessed by quantitating the enzymatic activity of MAPKAP kinase 2 in the immunoprecipitates (in vitro Hsp25 phosphorylation assay). After stimulating the cells with a mild hypo-osmotic shock (30% hypotonicity, 10 min) a large increase in in vitro Hsp25 phosphorylation was observed, indicative of increased MAPKAP kinase 2 activity (Fig. 1). In contrast, a comparable hyperosmotic shock (30% hypertonicity, 10 min) did not enhance kinase activity. However, in line with a previous report [18], clear activation of MAPKAP kinase 2 was observed in response to a more severe hyperosmotic shock (Fig. 2).

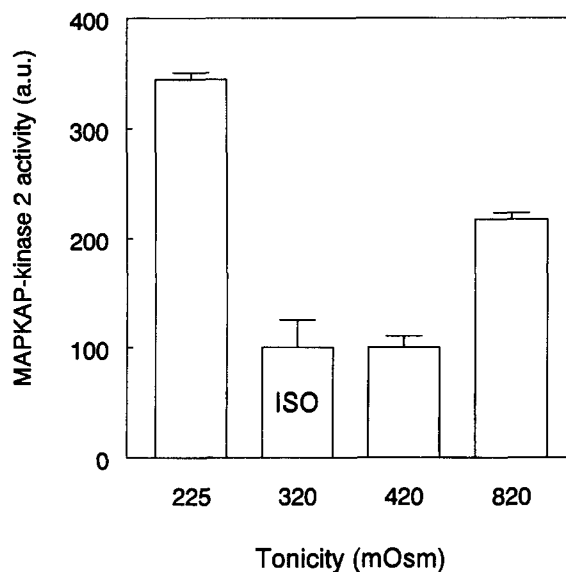

Fig. 1. Activation of the p38 MAP kinase signalling cascade by anisotonic media. Enzymatic activity of MAPKAP kinase 2 in immunoprecipitates obtained from cultures of Intestine 407 cells stimulated for 10 min with hypo- and hypertonic media. Data are expressed as percentage stimulation relative to the iso-osmotic (ISO, 320 mOsm) control (mean ± S.D.). a.u., arbitrary units.

The time course of the hypotonicity-induced p38 MAP kinase stimulation was studied by determining the in vitro activity of MAPKAP kinase 2 as well as by measuring Hsp27 phosphorylation in intact cells. As indicated in Fig. 2, both the enzymatic activity of MAPKAP kinase 2 as well as the amount of Hsp27 phosphorylated showed a rapid increase following hypo-osmotic stimulation. Phosphorylation was observed as early as 30 s after osmotic stimulation and reached a plateau value after 10 min (Fig. 3 and results not shown).

3.2. Activation of osmo-sensitive anion efflux

Osmo-sensitive anion efflux activation following stimulation of Intestine 407 cells with anisotonic media was investigated by quantitating changes in $^{125}I^-$ efflux from radio-isotope loaded cultures. As clearly seen in Fig. 3, a prompt and large increase in $^{125}I^-$ release was observed after stimulating the cells with a hypotonic medium (30% hypotonicity, 225 mOsm) but not after replacing isotonic medium (320 mOsm) by mildly 30% (420 mOsm) or severely 250% (820 mOsm) hyperosmotic media. The small sustained increase in $^{125}I^-$ efflux following hypertonic stimulation is most likely caused by an increase in intracellular tracer concentration, due to

Table 1
Effects of cytokines, LPS and anisomycin pre-treatment on MAPKAP kinase 2 activity and hypotonicity-provoked $^{125}I^-$ efflux

	MAPKAP kinase 2 activity (% of control)	Hypotonicity-induced $^{125}I^-$ efflux (% of untreated control)
IL-1β	409 ± 128[a]	90 ± 11
TNF-α	626 ± 10[a]	115 ± 16
LPS	427 ± 57[a]	90 ± 10
Anisomycin	1306 ± 65[a]	133 ± 33

Intestine 407 cells were pretreated for 20 min with the various compounds (IL-1β, 20 ng/ml; TNF-α, 50 ng/ml; LPS, 1 μg/ml and anisomycin, 20 μg/ml), followed by measurements of MAPKAP kinase 2 activity and hypotonicity (20%)-induced $^{125}I^-$ efflux (cf. Fig. 4, legend). Data are expressed as percentage relative to the untreated control (mean ± S.D.).
[a]Significant difference from the control ($p < 0.01$).

decreased cell volume. These results indicate that osmo-sensitive anion channels are triggered by hypo-osmotic stress only and imply that the signal transduction pathway(s) involved in channel recruitment/activation contains at least one component specifically stimulated by hypotonic but not by than hypertonic challenge.

3.3. Role of p38 MAP kinase in the activation of hypotonicity-induced anion efflux

To investigate a possible role of the p38 MAP kinase signalling cascade in the regulation of osmo-sensitive Cl^- channels, cells were pre-treated with the specific p38 MAP kinase inhibitor SB 203580 [18,19] or with cytokines/xenobiotics reported to activate p38 MAP kinase [10–12,18]. As shown in Fig. 4, pre-treating the cells with SB 203580 completely inhibited the hypotonicity-induced Hsp27 phosphorylation in intact cells and largely reduced the enzymatic activity of MAPKAP kinase 2 in immunoprecipitates. In contrast, the cell swelling-provoked $^{125}I^-$ efflux was not affected by SB 203580. Importantly, the inactive analogue SK&F 105809 [18] appeared unable to inhibit hypotonicity-induced MAPKAP kinase 2 activation or $^{125}I^-$ efflux at similar concentrations (results not shown).

Although not operating directly as an activator of osmo-sensitive anion channels, p38 MAP kinase, similar to ERK1/ERK2 [4], could be involved in potentiating the ionic response to hypo-osmotic stimulation and in this way promote the ability of the cells to correct adequately changes in cell volume. Pre-treatment of the cells with cytokines (IL-1β or TNF-α), LPS or the xenobiotic anisomycin resulted in a rapid activation of MAPKAP kinase 2 (Table 1). However, unlike growth factors that activate ERK1/ERK2 MAP kinases [4], these MAPKAP kinase 2 agonists did not potentiate $^{125}I^-$ efflux after mild (20%) hypotonic stimulation (Table 1), suggesting that p38 MAP kinase does not play a role in the mechanism of activation of osmo-sensitive Cl^- channels.

4. Discussion

Osmotic swelling of Intestine 407 cells has profound effects on multiple signal transduction pathways which coincide with

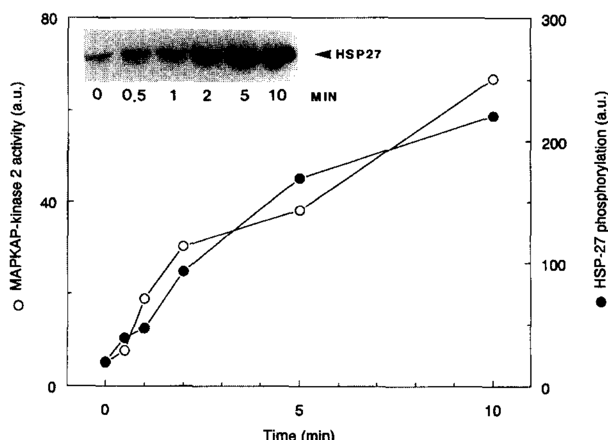

Fig. 2. Time course of the hypotonicity-provoked activation of MAPKAP kinase 2 activity (open symbols, arbitrary units) and intact cell Hsp27 phosphorylation (closed symbols, arbitrary units). Inset shows ^{32}P-labelled Hsp27 immuno-precipitated from osmotically stimulated cells pre-loaded with $^{32}PO_4^{3-}$.

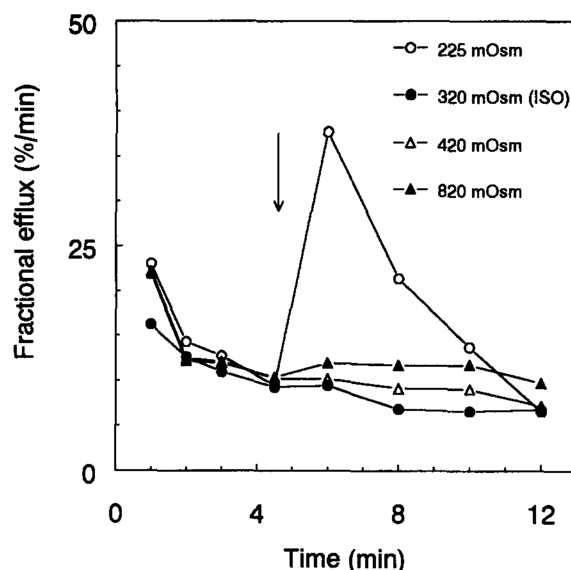

Fig. 3. Time course of fractional efflux of $^{125}I^-$ loaded cultures of Intestine 407 cells. Arrow indicates shift to an anisotonic medium (either hypotonic, 225 mOsm or hypertonic, 420 and 820 mOsm). The isotonic control (320 mOsm, ISO) is indicated by open circles. Data are representative of 3 independent experiments.

a rapid increase in ionic conductances (regulatory volume decrease or RVD). We have previously demonstrated an important role of the Ras-related GTP-binding protein $p21^{rho}$ as well as phosphatidylinositol-3-kinase in the activation of osmo-sensitive anion channels [6]. Other signalling molecules were also affected, including $p125^{FAK}$ and ERK1/ERK2, but their precise role in ion channel regulation has not yet been elucidated [4,7]. In this report we present evidence for osmotic activation of another MAP kinase, p38 MAP kinase, as well as its target enzyme MAPKAP kinase 2.

The family of MAP kinases consists of multiple, highly homologous isoforms of enzymes, activated by specific extracellular stimuli and participating in parallel signalling cascades with little cross activation [7,8]. In Intestine 407 cells both ERK1/ERK2 and p38 MAP kinase were activated following osmotic stimulation, however, clear differences were observed. First, in the absence of the phosphotyrosine phosphatase inhibitor vanadate, ERK1/ERK2 phosphorylation was slow, starting after 2–5 min, and transient, lasting only 10–15 min [4], whereas activation/phosphorylation of downstream components of p38 MAP kinase (MAPKAP kinase 2, Hsp27) started more rapidly (≤30 s) and reached a plateau value after 10 min. In addition, cell swelling-induced ERK1/ERK2 phosphorylation, but not p38 MAP kinase activation, was found to be sensitive to *C. botulinum* C3 exoenzyme pretreatment (B.C. Tilly, unpublished results), suggesting a role for $p21^{rho}$ upstream of ERK1/ERK2 activation, but not as a component of the p38 MAP kinase's signalling cascade. Finally, growth factors that activate ERK1/ERK2 were found to potentiate osmo-sensitive anion efflux [4], whereas activators of p38 MAP kinase, i.e. cytokines, LPS and anisomycin, were ineffective.

In comparison with TNF-α and IL-1β, as well as with a moderate hyperosmotic stimulation (~30% hypertonicity), a 30% hypo-osmotic shock is a relatively strong activator of the p38 MAP kinase signalling cascade. However, the results presented argue against a direct or indirect role of p38 MAP

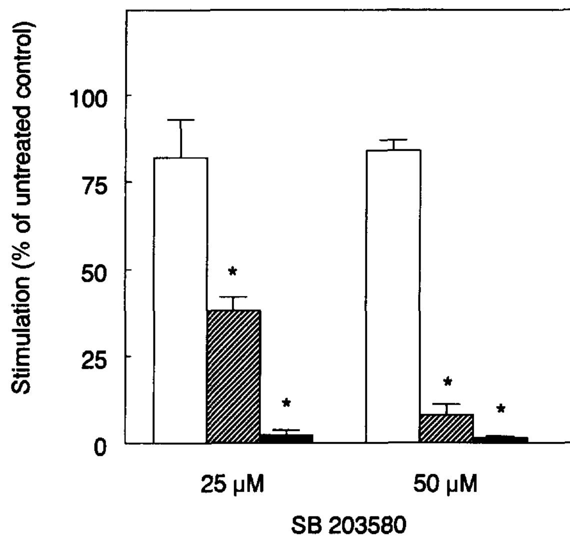

Fig. 4. Effects of SB 203580 on the hypotonicity-induced anion efflux, MAPKAP kinase 2 activity and Hsp27 phosphorylation. Cultures of Intestine 407 cells were pretreated with either 25 or 50 µM SB 203580 for 30 min and 2 h, respectively. Thereafter, the cells were stimulated with a 30% (225 mOsm) hypotonic medium. Open bars represent the increment in fractional $^{125}I^-$ efflux measured at $t = 1.5$ min after hypotonic stimulation (cf. Fig. 1); hatched bars, MAPKAP kinase 2 activity; solid bars, Hsp27 phosphorylation. Data are expressed as percentage stimulation relative to untreated controls (mean ± S.D.). Asterisk indicates a significant difference from the untreated control ($p < 0.05$).

kinase in the activation of osmo-sensitive anion channels. This suggests that p38 MAP kinase and/or its effectors serve other important cellular functions, for instance in preserving or restoring cellular integrity after osmotic swelling. The small heat shock proteins Hsp25 and Hsp27, both substrates for MAPKAP kinase 2, have been implicated as molecular chaperones [20] and inhibitors of F-actin polymerization [21]. In contrast to their chaperone function, which has been shown to be independent of protein phosphorylation [22], inhibition of actin polymerization occurs only in the presence of non-phosphorylated Hsp25 and was abolished after its phosphorylation [23,24]. Since the F-actin cytoskeleton is rapidly and transiently re-modelled during osmotic cell swelling, leading to the formation of numerous small and poorly organized F-actin stress fibers [6], activation of p38 MAP kinase and subsequent phosphorylation of heat shock proteins might facilitate the formation of full size stress fibers and thereby the restoration of the cytoskeleton.

Although speculative, cell swelling induced activation of p38 MAP kinase might serve an additional function. Evidence is now rapidly accumulating that F-actin or proteins associated with the actin cytoskeleton might be involved in ion channel activation (e.g. see [3]). Indeed, rapid activation of apical Na^+ channels has been demonstrated upon addition of exogenous actin in excised membrane patches of toad kidney A6 cells [25]. Furthermore, disruption of the actin cytoskeleton, by pre-treating the cells with cytochalasins, was found to potentiate the activity of volume-sensitive Cl^- channels [6,26]. It is therefore tempting to speculate that the small heat shock proteins might not only be involved in restoring the actin cytoskeleton but, by reducing the amount of short F-actin fibers, might also play a role in closure of the swelling-activated ion channels.

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