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Activation of extracellular signal-regulated kinase ERK after hypo-osmotic stress in renal epithelial A6 cells

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Abstract

Activation of mitogen-activated protein (MAP) kinases has been reported to occur after a hypo-osmotic cell swelling in various types of cells. In renal epithelial A6 cells, the hypo-osmotic shock induced a rapid increase in the phosphorylation of an extracellular signal-regulated kinase (ERK)-like protein that was maximal 10 min after osmotic stress. Activation of ERK was significantly increased when hypo-osmotic stress was performed in the absence of extracellular Ca^{2+}, a condition that inhibits regulatory volume decrease (RVD). Exposure of cells to PD98059, an inhibitor of the MAP kinase kinase MEK, at a concentration that fully cancelled ERK activation, did not inhibit RVD. On the contrary, RVD was abolished when osmotic shock was induced in the presence of SB203580, an inhibitor of stress-activated protein kinases (SAPKs). These results suggest that different MAP kinases are activated after hypo-osmotic stress in A6 cells. SAPKs would be involved in the control of RVD, while ERK would lead to later events, such as gene expression or energy metabolism.

Keywords: Osmotic shock; A6 cell; ERK; MAP kinase; Calcium; RVD; Cell volume

1. Introduction

Most cell types respond to changes in cellular volume after osmotic stress by evoking specific compensatory mechanisms. Generally, a hypo-osmotic shock induces a rapid uptake of water that leads to cell swelling. As a consequence, cells regulate their volume by activating K^+ and Cl^- channels [1–4]. During regulatory volume decrease (RVD), most cells also activate transporting pathways for various organic osmolytes such as polyols, methylamines and amino acids [1,5,6]. The question of a common pathway or of two separate pathways for Cl^- and organic osmolytes through a volume activated anion channel is still under discussion. In addition, the cellular mechanisms controlling the volume sensitive Cl^- channel that are activated during RVD are still elusive. [7–11].

Various data obtained in various types of cells argue that activation of a mitogen-activated protein (MAP) kinase (MAPK) pathway occurs after hypo-osmotic stimulation. MAPKs are implicated in the control of a broad spectrum of cellular events. Stimulation by various extracellular signals of signal transduction pathways that regulate morphology, proliferation, differentiation or survival often triggers the activation of at least one member of the MAPK family, for instance the extracellular signal-regulated kinase (ERK)-1/2 or the p38 MAP kinase [12,13]. ERK1 and ERK2 have been shown to be activated after a hypo-osmotic shock in cardiac myocytes [14], intestine 407 cells [15–17], glioma cells [18], astrocytes [19,20], hepatoma cells [21], murine renal cells [22] but not in fibroblats [23]. p38 MAP kinase seems to be activated concomitantly with ERK1/2 in intestine 407 cells [24], but does not seem to be involved in cardiac myocytes [14], renal cells [22] and fibroblasts [23]. Finally, the ERK1/2 cascade involving the MAP kinase kinase MEK has been reported to be either crucial [19] or not necessary [16] in the osmo-sensitive anion efflux, or just partly involved in RVD [22]. Activation and role of ERK1/2 and/or p38 MAP kinase after a hypo-osmotic shock probably differ between cells and are not necessarily involved in RVD.

Amphibian renal A6 cells form highly polarized monolayers and have been used for many years for studying Na^+ and Cl^- transports in epithelia. When submitted to a hypo-

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osmotic shock, they swell and subsequently regulate their volume. The Cl^- and taurine transport pathways have been shown to play a role during RVD [24,25]. However, less is known concerning the cellular mechanisms that lead to activation of these transporters and are stimulated during RVD in these cells. The only report so far shows that stress-activated protein kinases (SAPKs), p38 MAP kinase and c-jun N-terminal kinase (JNK) are activated after a hypo-osmotic shock [26]. We have then undertaken this study to investigate whether ERKs were also stimulated after hypo-osmotic stress in these cells. Our results suggest that although hypotonic stress induces ERK activation, this pathway is not involved in cell-volume control.

2. Materials and methods

2.1. Cell culture

A6 cells, a line derived from the distal tubule of *X. laevis* kidney, were a gift from Dr. Rossier (Lausanne, Switzerland). They had originally been obtained from the American Type Culture Collection and subsequently cloned (clone A6-2F3) by limiting dilution [27]. Cells were grown on plastic culture flasks at 28 °C in a humidified atmosphere of 5% CO_2 in air. The amphibian cell medium (AM, pH 7.4) was supplemented three times weekly with antibiotics and 10% fetal calf serum (IBF, Paris, France) for cell nourishment. When cells reached confluence, they were rinsed with a saline solution containing (mM): NaCl 85, $NaHCO_3$ 17.5, KCl 4, KH_2PO_4 0.8, glucose 11, treated with trypsin (0.25%), and diluted four times with the saline solution. Cells were then centrifuged 5 min at 1000 rpm, placed in AM without serum, and let 1 h at 28 °C in a humidified atmosphere of 5% CO_2 in air.

All experiments were performed using cells in suspension. Cells were placed (time zero of the experiment) either in isotonic solution (normal Ringer solution, in mM: NaCl 83, $NaHCO_3$ 24, KCl 2.5, $CaCl_2$ 2, $MgSO_4$ 2, Na_2HPO_4 3.4, KH_2PO4 1.2, glucose 11, HEPES 5, pH 7.4 after bubbling with 5% CO_2, osmolality: 247 mosM/kg H_2O) or in normal Ringer solution diluted to 2/3 with water (osmolality: 165 mosM/kg H_2O) to induce the hypo-osmotic shock.

Experiments in Ca-free conditions were performed in Ringer solution deprived of Ca^{2+} (same composition, except that $CaCl_2$ was omitted and 0.1 mM EGTA were added).

Finally, cells were treated or not for 15 min with PD 98059 (Calbiochem, 15 mM stock in DMSO) or with 25 μM SB203580 (Biomol. Research Lab., Plymouth PA, USA, 50 mM stock in DMSO) before osmotic stress.

2.2. Cell volume measurements

A Coulter counter multisizer II connected to a PC-type computer was used to measure the volume of isolated cells prepared as described above. Volume changes were measured as previously described [28].

2.3. Western blotting

One-milliliter cell suspension was taken at different times and rapidly centrifuged with a bench centrifuge. The cell pellet was dissolved in 50-μl denaturation buffer (5% SDS, 15% glycerol, 15 mM Na_2HPO_4, 0.5M β-mercaptoethanol, 0.25% bromophenol blue, pH = 6.8) and immediately boiled for 10 min at 95 °C. Fifteen microliters containing 10–20-μg proteins was separated by SDS-PAGE and then transferred onto nitrocellulose as described by Chiri et al. [29]. Western blotting was performed as previously described [29], using an anti-ERK1 antibody (C-16, Santa Cruz Biotechnology, Inc.) diluted 1:1000 in blocking buffer

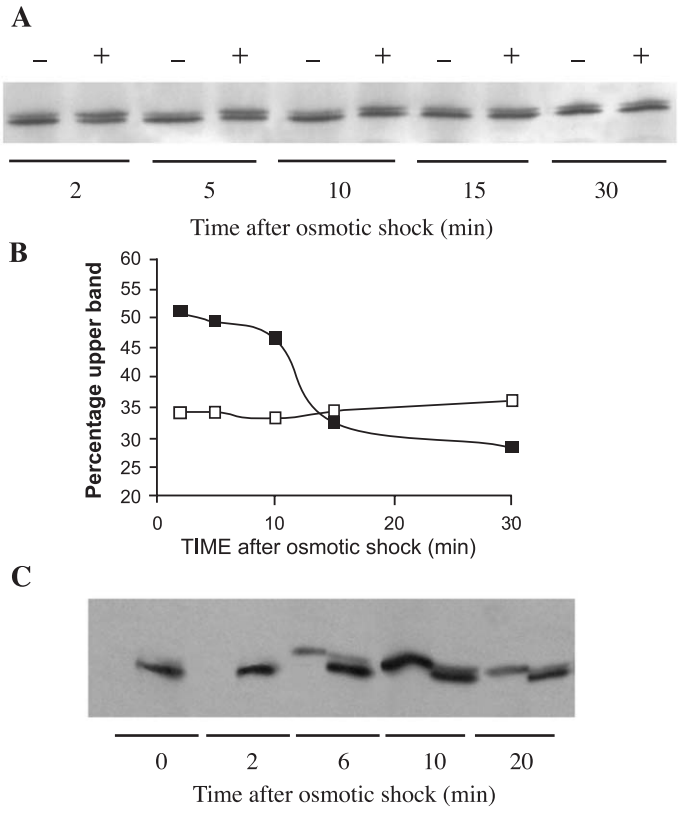

Fig. 1. Changes after a hypo-osmotic shock in ERK phosphorylation. (A) Cells were submitted (+) or not (−) to hypo-osmotic stress for different times, and Western blot performed with an anti-ERK1 antibody after separation of proteins on SDS-PAGE. (B) Time course after osmotic stress of the upper band signals obtained in A that were quantified after scanning and densitometry. Cells were submitted (full symbols) or not (empty symbols) to hypo-osmotic. The experiment shown here is representative of three experiments that gave similar results. (C) Cells were arrested at different times after stress, and proteins separated on the same SDS-PAGE. After transfer, bands of nitrocellulose corresponding to each time were cut in two halves that were each blotted using an anti-active p42/44 MAPK antibody (A) and an anti-ERK1 antibody (B). Bands were then properly aligned before ECL revelation. Four experiments were performed that gave similar results.

[29] or a mouse anti-phospho-42/44MAPK (Thr202/Tyr204, Cell Signaling Technology, Inc., 9106) E-10 monoclonal antibody diluted 1:2000 in blocking buffer. The membrane was incubated for 1 h with secondary antibody conjugated to horseradish peroxidase (ICN, 67428) diluted 1:5000, rinsed three times for 5 min in washing buffer [29], and proteins were revealed by enhanced chemoluminescence (ECL, Amersham).

3. Results and discussion

3.1. Hypo-osmotic stress activates an ERK-like protein

ERK have been shown to be activated after a hypo-osmotic shock in various types of cells. In order to detect ERK proteins in A6 cells, we used two different commercial antibodies. An anti-ERK1 antibody revealed a protein doublet migrating around 44 kDa (Fig. 1). These proteins probably correspond to the active (higher band) and the inactive (lower band) forms of an ERK-like protein, as has already been described by others [30]. An anti-active p42/44 MAPK monoclonal antibody raised against the dually phosphorylated forms of human ERK1 and ERK2 revealed a single protein that migrated at a MW (molecular weight) similar to that of the upper band of protein doublet detected with the anti-ERK1 antibody (Fig. 1C). This result suggests that one ERK only can be detected in A6 cells, as has already been reported by others [31]. Fig. 1C shows that the intensity of the protein detected with the anti-active p42/44 MAPK antibody increased with time after osmotic shock to reach a maximum 10 min after stimulation. This fits with results obtained with the anti-ERK1 antibody which detected a protein doublet showing an upper band that increased after stimulation and then faint 10 min after osmotic shock (Fig. 1A and C). This time course of ERK activation also corresponds to the change in A6 cell volume induced after the hypo-osmotic shock (Fig. 3). A more precise kinetics showed that the increase in ERK phosphorylation revealed with the anti-active p42/44 MAPK antibody could be observed as soon as 2 min after osmotic shock (Fig. 2). We performed six experiments that showed a similar MAPK activation, with a maximum phosphorylation always attained between 6 and 10 min after osmotic shock. However, a clear decrease in MAPK phosphorylation was observed 10 min after osmotic shock and basal level was

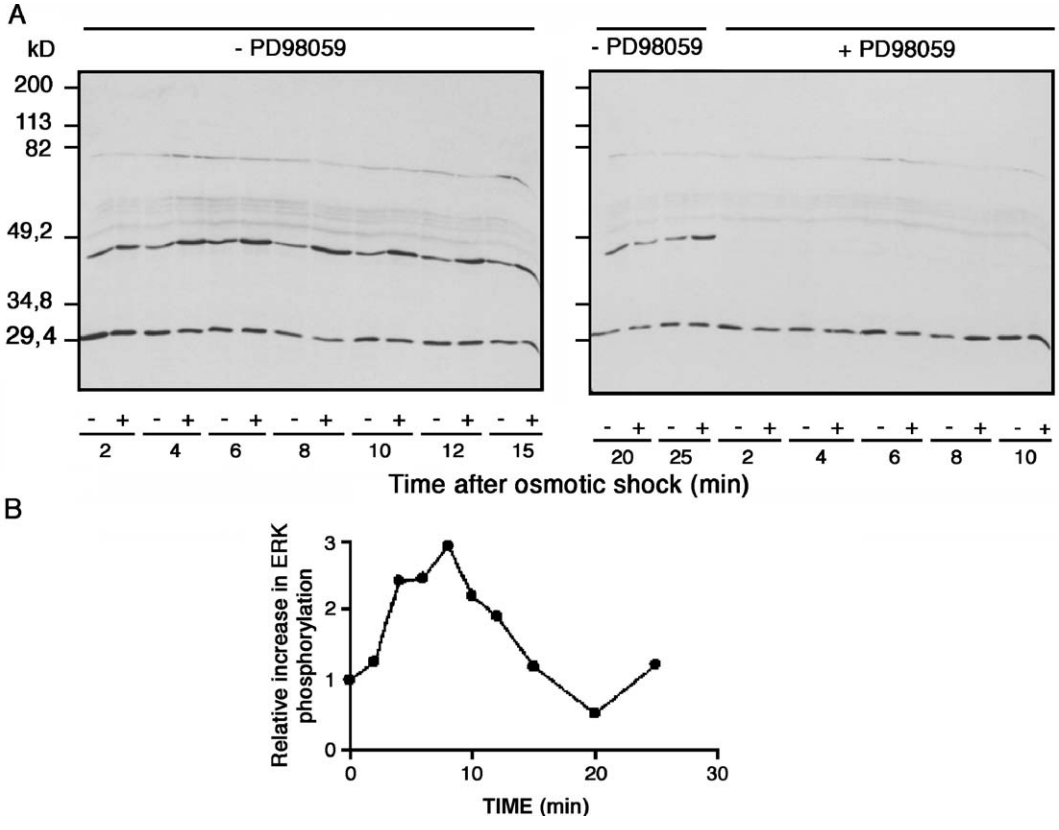

Fig. 2. Sensitivity to PD98059 of ERK phosphorylation. Cells that were pretreated (+PD98059) or not (−PD98059) for 15 min with 75 μM PD98059 were submitted (+) or not (−) to hypo-osmotic stress and arrested at different times after shock. Proteins were separated by SDS-PAGE analysis and Western blot performed using the anti-active p42/44 MAPK antibody (A). We verified that the same amount of proteins was loaded in each band by revealing with Ponceau red the nitrocellulose after transfer of proteins (not shown). The anti-active MAPK antibody also revealed nonspecifically a protein around 30 kDa. Three experiments were performed that gave similar results. (B) Time course after osmotic stress of signals obtained in A that were quantified after scanning and densitometry.

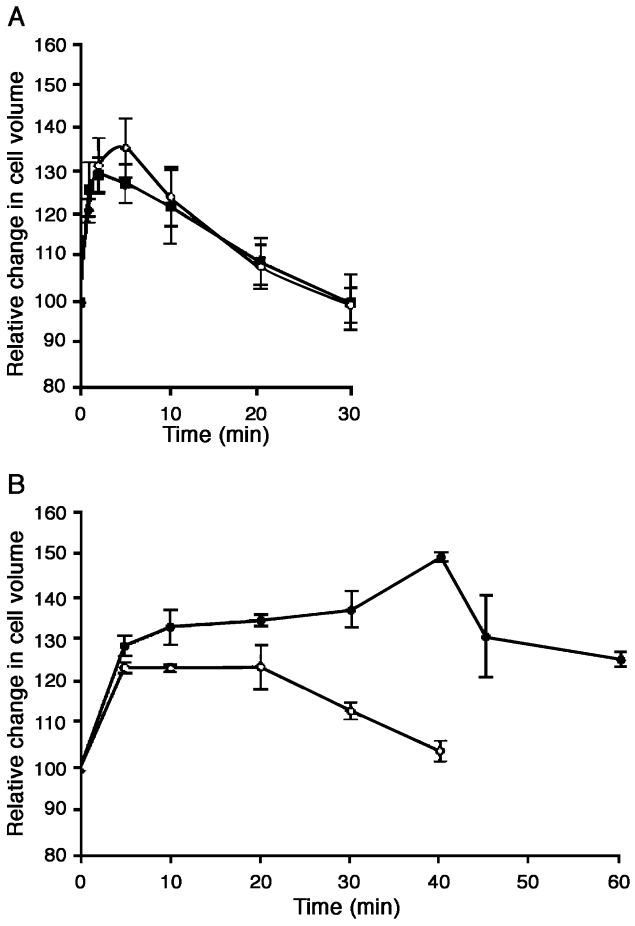

Fig. 3. Effect of 75 μM PD 98059 (A) or 25 μM SB 203580 (B) on RVD. A6 cells were submitted to a hypo-osmotic shock at zero time. Cells were pretreated (full symbols) for 15 min with either 75 μM PD98059 (A) or 25 μM SB203580 (B) before the osmotic shock and drugs remained present during the osmotic stress. Controls (empty symbols) were treated with an amount of DMSO similar to that of medium containing the inhibitors. Results are means ± S.E. of N experiments ($N=6$ for control experiments and $N=4$ for PD98059 and SB203580 experiments).

recovered after 30 min in four experiments, while in two other experiments MAPK phosphorylation only slightly decreased after 10 min and remained elevated for at least 30 min after osmotic shock.

3.2. Role of ERK in the control of RVD

In order to test whether ERK activation was a consequence or a trigger of RVD, we measured time-dependent changes in ERK phosphorylation and cell volume when A6 cells were pretreated for 15 min with 75 μM PD98059, an inhibitor of MEK PD98059, and then exposed to hypo-osmotic solution containing the same amount of inhibitor.

We observed that 75 μM PD98059 abolished both the basal level of ERK phosphorylation in non-stimulated cells and the increase in ERK phosphorylation after hypo-osmotic stress (Fig. 2). This suggests that the pathway stimulated after a hypo-osmotic shock that leads to ERK activation involves MEK. However, RVD was not significantly altered in these conditions (Fig. 3A). Therefore, it is unlikely that ERK activation is involved in the control of A6 cell volume decrease after hypo-osmotic stress.

Finally, we tested whether other MAPK cascades, such as JNK or p38 MAPK cascades, could be involved in RVD. Indeed, these MAPK have been found to be transiently activated in A6 cells submitted to hypo-osmotic stress [26]. We found that 25 μM SB203580, which is known to inhibit specifically p38 MAPK [32], cancelled RVD response, resulting in continued cell swelling after 1-h osmotic stress (Fig. 3B). This suggests a role of p38 MAPK in RVD induced after hypo-osmotic stress in A6 cells.

3.3. Role of calcium in the activation of ERK after hypo-osmotic stress

A cell calcium increase has been reported in A6 cells following an osmotic stress and RVD was found to be impaired by Ca-free solutions [28,33]. The transient peak of Ca^{2+} that occurs during RVD following the hypo-osmotic shock in A6 cells could stimulate a MAP kinase pathway. We then tested whether stimulation of ERK after hypo-osmotic stress was dependent on the presence of external Ca^{2+}. We observed that the absence or presence of external Ca^{2+} did not change the basal level of ERK phosphorylation detected with the anti-MAPK antibody

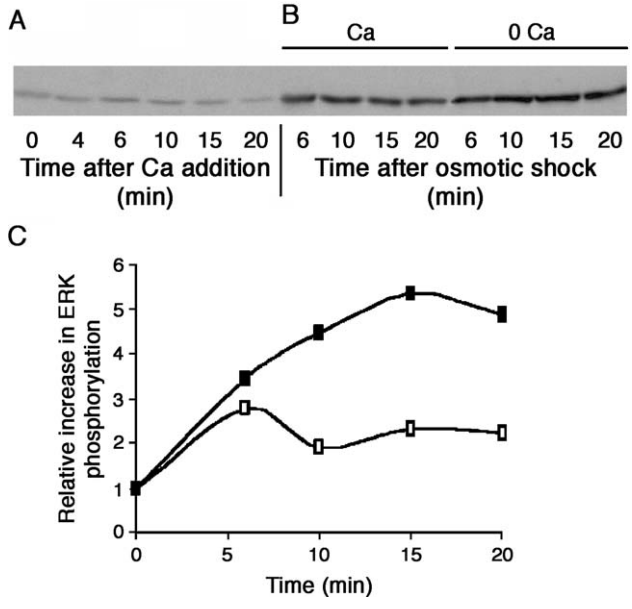

Fig. 4. Effect of calcium on ERK phosphorylation. Proteins were separated by SDS-PAGE analysis and Western blot performed using the anti-active p42/44 MAPK antibody. (A) Cells were placed in a calcium-free (0 Ca) medium and arrested at different times after Ca addition. (B) Cells were submitted to hypo-osmotic stress in a medium containing calcium (Ca) or not (0 Ca) and arrested at different times after shock. (C) Time course after osmotic stress of signals obtained in B that were quantified after scanning and densitometry. Cells were submitted to hypo-osmotic stress in a medium containing calcium (empty symbols) or not (full symbols). The experiment shown here is representative of three experiments that gave similar results.

(Fig. 4A). A large increase in the phosphorylation of ERK could be stimulated when osmotic stress was induced in absence of external Ca^{2+}, a condition that partly inhibits RVD and keeps cells swelling [28]. A similar Ca-independence of the osmo-signaling pathway to ERK has been reported in C6 glioma cells [18]. On the contrary, activation of ERK1/2 after hypo-osmotic stress depends on the presence of external Ca^{2+} in astrocytes [20], suggesting that mechanisms that lead to MAP kinase activation exhibit cell-type-specific features. It has recently been reported that stimulation of ERK1/2 occurs in A6 cells after long-term stimulation of protein kinase C (PKC) [34]. Stimulation of a similar pathway after osmotic shock would necessarily imply a Ca-independent PKC isoform [35]. However, we observed that the absence of external Ca^{2+} led to a maximum level in ERK phosphorylation after osmotic shock that was significantly higher than that observed in the presence of Ca^{2+} (Fig. 4B and C). These results are similar to those reported by Niisato et al. [26] who showed that SAPK was maintained activated in A6 cells when the hypo-osmotic shock was induced in the presence of 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), a Cl^- channel blocker which inhibited the RVD and kept the cells continuously swelling. ERK and SAPK pathways might be activated after hypo-osmotic stress by a common mechanism. A direct activation of SAPK and ERK pathways by the mitogen-activated protein Kinase/ERK kinase kinase 3 (MEKK3) has, for example, been reported in NIH3T3 and Cos7 cells [36]. The overstimulation of ERK that occurs in absence of external Ca^{2+} could be due to an intense activation of the stress sensor upstream the ERK pathway in these conditions where cells keep swelling, which is a Ca^{2+} indirect effect.

Altogether, these data suggest that hypo-osmotic stress leading to cell swelling induces activation of different MAP kinases that might play different roles. The present data and those reported by Niisato et al. [26] demonstrate a role of SAP kinase in RVD. It is unlikely that this short-term effect after hypo-osmotic stress is induced by the ERK pathway since RVD was not affected by the MEK inhibitor PD 98059. Furthermore, the RVD associated K^+ permeability increase was also not modified by this inhibitor following hypo-osmotic stress (unpublished data). In the renal collecting duct, Na^+ absorption was found to be down-regulated by EGF through the ERK pathway which reduced the Na^{2+} channel subunit mRNA levels [37]. In addition, a similar long-term inhibitory effect of the ERK cascade, starting after 1 h, was also described in the renal A6 cells in response to PKC activation [34]. In A6 cells, the sodium transport is rapidly stimulated by a hypo-osmotic stress, with a maximum stimulation reached after 20 min [28,38]. Since inhibition of the ERK pathway does not affect the K^+ permeability necessarily associated to the Na^{2+} transport, a possibility remains that this cascade is involved in long-term effects, such as alterations in gene expression, including that of ENaC subunits, or energy metabolism. Further studies will be necessary to assess this hypothesis.

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