Osmosignalling in C6 glioma cells

Ralf Sinning, Freimut Schliess, Ralf Kubitz, Dieter Häussinger*

Medizinische Einrichtungen der Heinrich-Heine Universität, Klinik für Gastroenterologie und Infektiologie, Moorenstrasse 5, D-40225 Düsseldorf, Germany

Received 31 October 1996; revised version received 15 November 1996

Abstract The influence of aniso-osmolarity on the activity of the MAP kinases Erk-1 and Erk-2 was studied in C6 glioma cells. Hypo-osmotic treatment (205 mosmol/l) led to an increased activity of Erk-1 and Erk-2 within 3 min, which became maximal at 10 min and returned to basal level within 120 min. In contrast, Erk activity was reduced under hyper-osmotic conditions (405 mosmol/l), compared to the normo-osmotic control (305 mosmol/l). Erk activation was accompanied by a mobility shift of Raf-1. Hypo-osmotic exposure increased the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). Absence of extracellular Ca^{2+} largely abolished the $[Ca^{2+}]_i$ response to hypo-osmolarity, whereas Erk activation following hypo-osmotic stimulation remained unaffected, suggesting a Ca^{2+} independence of the osmosignalling pathway to the MAP kinases. Both the Ca^{2+} response as well as the Erk activation following hypo-osmotic exposure were maintained in the presence of the phospholipase C inhibitor U73122. Application of 8-CPT cAMP, forskolin/isobutylmethylxanthine or isoproterenol blocked Erk activation following hypo-osmotic treatment of the cells, suggesting a role of the Ras/Raf pathway upstream from Erk-1 and Erk-2. Protein kinase C (PKC) is unlikely to play a role in the hypo-osmolarity- induced signalling towards MAP kinases, as revealed by inhibition of PKC with Gö6850. Inhibition of pertussis- or cholera toxin-sensitive G-proteins as well as inhibition of tyrosine kinases with genistein and of PI3 kinase by wortmannin had no effect on the Erk response to hypo-osmolarity. It is concluded that osmosignalling in C6 glioma cells differs upstream of the MAP kinases from that observed in primary rat astrocytes, H4IIE rat hepatoma cells and isolated rat hepatocytes.

Key words: C6 glioma cell; Signal transduction; Calcium; MAP kinase; Protein kinase C; Phospholipase C; cAMP; Raf-1; Cell volume

1. Introduction

Changes of astrocyte volume are an early event in numerous pathological states such as brain ischemia, hyper-/hyponatremia and hepatic encephalopathy (for review, see [1]). Cell volume alterations induced by either aniso-osmotic environments or under the influence of hormones or cumulative substrate accumulation represent an independent signal which modulates cell function for example via changes in cyto skeleton, metabolic activities, protein phosphorylation and gene expression (for review see [2,3]). Astrocyte swelling was shown to stimulate glycogen synthesis [4] and was suggested to be a signal for proliferation and thus to play a role in gliosis [5]. In F98 glioma cells osmolarity affects the intracellular nucleoside triphosphate level, the rate of fatty acid biosynthesis and cytoplasmic pH [6]. Glutathione depletion due to diminished synthesis under hypo-natremic conditions was reported for C6 glioma cells [7]. In C6 glioma cells hypo-osmolarity-induced regulatory volume decrease (RVD) occurs by loss of KCl [8,6] and organic osmolytes due to an opening of volume-sensitive organic anion channels [9]. Hyper-osmolarity-induced regulatory volume increase (RVI) is initially performed by ionic mechanisms and a subsequent increase of *myo*-inositol transporter mRNA expression [10,11].

Hypo-osmotic astrocyte swelling leads to a biphasic response of the cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$ [12,13] and a calcium-mediated activation of the MAP kinases Erk-1 and Erk-2 [12]. Little is known about osmosignalling in glioma cells, which are often used as a model system for astrocytes. RVD of hypo-osmotically swollen C6 glioma cells was suggested to depend on the action of protein phosphatase type 1 and the presence of extracellular Ca^{2+}, although in suspensions of fura-2-loaded C6 glioma cells no increase in $[Ca^{2+}]_i$ following hypo-osmotic swelling was observed [6].

The extracellular signal-regulated kinases Erk-1 and Erk-2 belong to the family of mitogen-activated protein (MAP) kinases, which are central components of the growth factor-induced signal transduction via protein phosphorylation towards nuclear and cytoplasmic targets including transcription factors, cytoskeletal proteins and regulatory enzymes of protein and glycogen metabolism (reviewed in [14]). MAP kinases are activated by phosphorylation on Thr-183 and Tyr-185 by dual specificity MAP kinase kinases (MEK) [15]. The Ras/Raf pathway towards MEK shows sensitivity to activators of protein kinase A (PKA) [16–18]. Dual specificity MAP kinase phosphatases are able to inactivate the MAP kinases [19].

The present study shows aniso-osmolarity-induced changes in the phosphorylation and activity of endogenous Erk-1 and Erk-2 in C6 glioma cells and an increase in $[Ca^{2+}]_i$, resulting from Ca^{2+} influx across the plasma membrane. Osmosensitivity of Erk-1 and Erk-2 activity was independent of extracellular Ca^{2+} and PI3 kinase but sensitive to cAMP, pointing to a role of Ras/Raf in the pathway to Erk-1 and Erk-2. This contrasts the osmosignalling to Erk-1 and Erk-2 in primary astrocytes, which was shown to be dependent on influx of extracellular Ca^{2+} and PI3 kinase [12]. Thus, marked differences exist between osmosignalling in C6 glioma cells and primary astrocytes.

*Corresponding author. Fax: (49) (211) 81 18752.

Abbreviations: Erk, extracellular signal-regulated kinase; IP3, inositol-(1,4,5)-trisphosphate; MAP, mitogen-activated protein or microtubule-associated protein; MBP, myelin basic protein; PI3 kinase, phosphatidylinositol-3 kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; RVD, regulatory volume decrease; RVI, regulatory volume increase

PII S0014-5793(96)01376-2

2. Materials and methods

2.1. Materials

Anti-Erk-1 and anti-Erk-2 antibodies were obtained from UBI (Lake Placid, NY, USA); anti-Raf-1 from Santa Cruz Biotechnology. Genistein and daidzein were purchased from Calbiochem-Novabiochem GmbH (Bad Soden, Germany), 1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl-1*H*-pyrrole-2,5-dione (U73122), 1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl-1*H*-pyrrolidine-2,5-dione (U73343), 8-CPT-cAMP and isoproterenol were from Biomol Res. Lab. Inc. (Hamburg, Germany) and pertussis and cholera toxin from Research Biochemicals Incorporated (Natick, MA, USA). Gö6850 was a gift from Gödecke AG (Freiburg, Germany), Pluronic F-127 and the acetoxymethylester of fura-2 were purchased from Molecular Probes Inc (Eugene, OR, USA). Cell culture media and fetal calf serum were from Gibco Life Technologies (Gaithersburg, MD, USA). Sodium orthovanadate, forskolin and isobutylmethylxanthine were obtained from Sigma (Munich, Germany). ATP was from Boehringer (Mannheim, Germany) and γ-[^{32}P]ATP was from Amersham. (Braunschweig, Germany). All other chemicals were from Merck (Darmstadt, Germany).

2.2. Cell culture and aniso-osmotic treatment

C6 glioma cells (ATCC CCL107) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 95% air, 5% CO_2. When cells had reached 90% confluence, they were washed with Dulbecco's PBS and culture was continued in serum-free medium for an additional 24 h. Following 5 min preincubation with 100 μmol/l sodium vanadate aniso-osmolarity was achieved by dilution of the medium with the appropriate volume of NaCl-free medium (205 mosmol/l) or with medium of elevated NaCl content, thereby maintaining 100 μmol/l vanadate. In the normo-osmotic control (305 mosmol/l), the same volume of normo-osmotic medium, containing 100 μmol/l vanadate, was added. Normo-osmotic raffinose medium was prepared by substitution of 50 mmol/l NaCl against 100 mmol/l raffinose; hypo-osmolarity was adjusted by removing 100 mmol/l raffinose. Hyper-osmotic raffinose medium was prepared by addition of 100 mmol/l raffinose to normo-osmotic medium.

2.3. Detection of mobility-shifted Erk-1, Erk-2 and Raf-1

At the end of the incubation period, medium was removed and cells were immediately lysed at 4°C using 50 mmol/l Tris-HCl buffer (pH 7.2) containing 150 mmol/l NaCl, 40 mmol/l NaF, 5 mmol/l EDTA, 5 mmol/l EGTA, 1 mmol/l vanadate, 0.5 mmol/l phenylmethylsulfonyl fluoride (PMSF), 0.1% aprotinin, 1% Nonidet-P40, 0.1% sodium deoxycholate and 0.1% SDS. The homogenized lysates were centrifuged at 20 000 × *g* at 4°C and the supernatant was added to an identical volume of gel loading buffer containing 200 mmol/l dithiothreitol (DTT, pH 6.8). After heating to 95°C for 5 min, the proteins were subjected to gel electrophoresis (50 μg protein/lane, 9% gel). Following electrophoresis, gels were equilibrated with transfer buffer (39 mmol/l glycine, 48 mmol/l Tris-HCl, 0.03% SDS, 20% methanol). Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Pharmacia, Freiburg, Germany). Blots were blocked in 5% bovine serum albumin containing TBST (20 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 0.1% Tween 20) and then incubated overnight with 1:50 000-diluted antiserum against Erk-1, Erk-2 or Raf-1. Following washing with TBST and incubation with horseradish peroxidase-coupled anti-rabbit IgG antibody diluted 1:10 000 at room temperature for 1 h, the blots were washed three times and developed using enhanced chemiluminescent detection (Amersham, Braunschweig, Germany).

2.4. Immune complex kinase assay

The assay was performed as described previously [20]. Briefly, aliquots of cell lysate containing 20 μg protein (lysis buffer: 1% Triton X-100, 150 mmol/l NaCl, 10 mmol/l Tris-HCl pH 7.4, 1 mmol/l EDTA, 1 mmol/l EGTA, 20 mmol/l NaF, 0.2 mmol/l PMSF, 0.5% NP-40) were incubated with 1.5 μg of a monoclonal antibody against Erk-2 (class IgG 2a) for 2 h at 4°C. Immune complexes were collected using protein A-Sepharose 4B (Pharmacia), washed three times with lysis buffer and six times with kinase buffer (10 mmol/l Tris-HCl pH 7.4, 150 mmol/l NaCl, 10 mmol/l $MgCl_2$, 0.5 mmol/l DTT) and incubated with 1 mg/ml myelin basic protein (MBP), 25 μmol/l ATP and 5 μCi γ-[^{32}P]ATP for 30 min at 37°C. The activity of Erk-2 was monitored by autoradiography after SDS polyacrylamide electrophoresis (12.5% gel).

2.5. Ca^{2+} imaging at the single cell level

Astrocytes were grown on coverslips in DMEM+10% FCS. Following 24 h serum starvation sub-confluent cells were incubated with Krebs-Henseleit medium (KHB: 115 mmol/l NaCl/25 mmol/l $NaHCO_3$/5.9 mmol/l KCl/1.18 mmol/l $MgCl_2$/1.23 mmol/l NaH_2PO_4/1.2 mmol/l Na_2SO_4/1.25 mmol/l $CaCl_2$), containing 5 μmol/l of the fluorescent Ca^{2+} chelator fura-2 acetomethoxyester and 0.02% pluronic F-127, for 30 min at 37°C and 5% CO_2. For fluorescence recording, the coverslips were continuously superfused at a rate of 15 ml/min with KHB at 37°C, equilibrated with O_2/CO_2 (95/5, v/v), resulting in pH 7.4. Measurement of intracellular Ca^{2+} was performed as described [21].

3. Results

3.1. Activation of Erk-1 and Erk-2 and occurrence of mobility-shifted Raf-1 in response to aniso-osmotic challenge of C6 glioma cells

Alterations in the phosphorylation of endogenous Erk-1 and Erk-2 in C6 glioma cells in response to hypo-osmotic

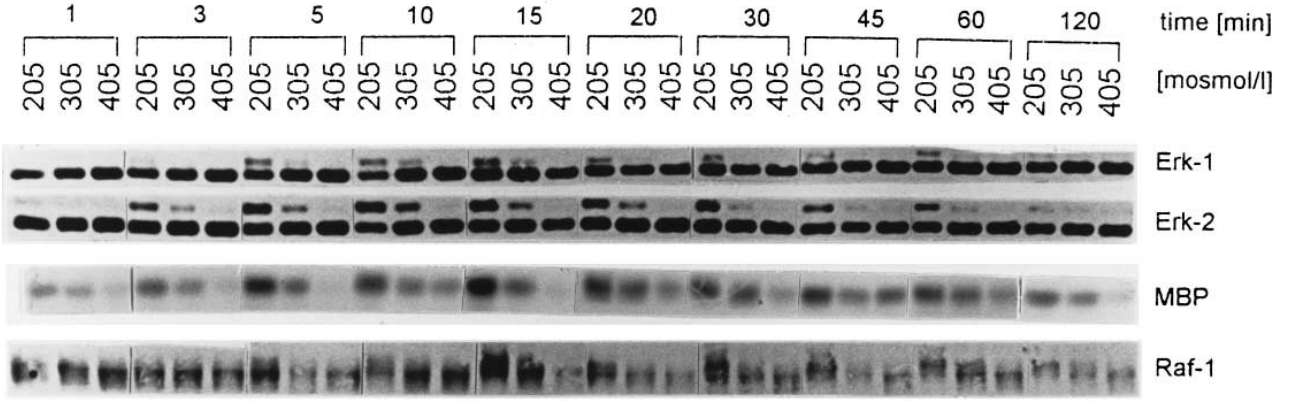

Fig. 1. Osmolarity dependence of Erk-1 and Erk-2 activity and Raf-1 phosphorylation in rat C6 glioma cells, determined by mobility shifts and immune-complex assay. Following 5 min pretreatment with 100 μmol/l vanadate, cells were treated with hypo-osmotic (205 mosmol/l), normo-osmotic (305 mosmol/l) or hyper-osmotic (405 mosmol/l) medium for the time indicated, thereby maintaining 100 μmol/l vanadate. Representative results from four independent experiments are shown. The extract of total protein was analyzed by Western blot, using antibodies specifically raised against Erk-1, Erk-2 or Raf-1. The immune complex assay was performed with MBP, using an antibody raised against Erk-2.

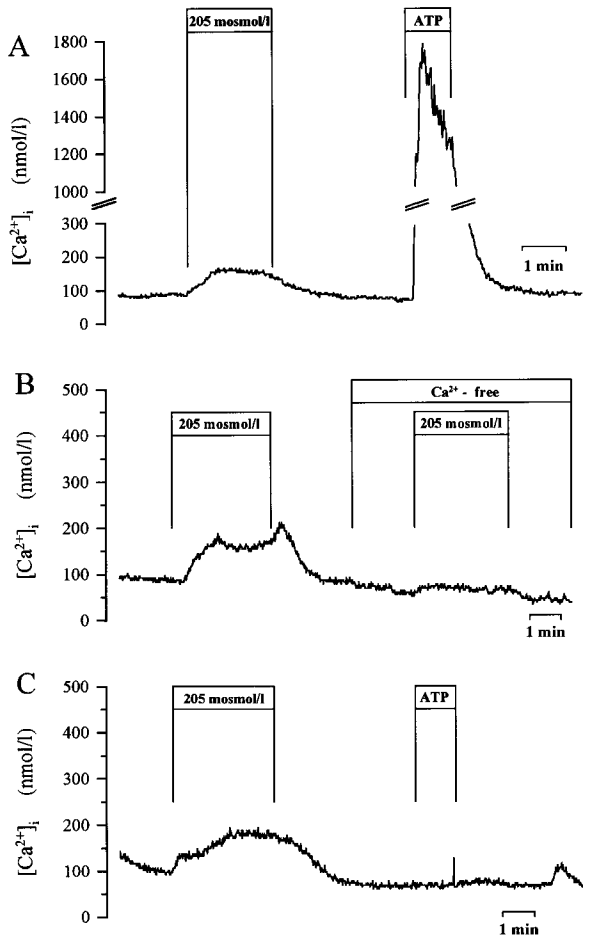

Fig. 2. Effect of hypo-osmolarity and extracellular ATP on intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ in C6 glioma cells, measured with fura-2 on single cell level. Normo-osmotic (305 mosmol/l) superfusion medium was suddenly changed to hypo-osmolarity (205 mosmol/l) as indicated. Representative traces from three experiments are shown in each case. A: Presence of 1.25 mmol/l extracellular Ca^{2+}. B: Ca^{2+}-free conditions: iso-osmotic replacement of 1.25 mmol/l $CaCl_2$ by NaCl and 5 mmol/l EGTA, 2 min before and during treatment of the cells. No desensitization of the signal was observed after twice application of hypo-osmolarity in presence of extracellular Ca^{2+} (data not shown). C: 20 min pretreatment of the cells with 1 μmol/l U73122. Following addition of this PLC inhibitor, the hypo-osmolarity-induced but not the ATP-induced $[Ca^{2+}]_i$ signal was preserved.

swelling or hyper-osmotic shrinkage of the cells were detected using the mobility shift assay. In order to amplify the phosphorylation signals, cells were preincubated with 100 μmol/l sodium vanadate, which was also present during aniso-osmotic stimulation and in the normo-osmotic control. Reduction of medium osmolarity from 305 to 205 mosmol/l induced a shift of Erk-1 and Erk-2 towards higher molecular masses within 3 min, which became maximal after 10 min and declined within 120 min to the basal level (Fig. 1; $n=4$). The mobility shifts observed under normo-osmotic conditions (305 mosmol/l) reflect the basal phosphorylation state of Erk-1 and Erk-2 in C6 glioma cells. On the other hand phosphorylation of Erk-1 and Erk-2 was diminished under hyper-osmotic conditions. The results obtained with the mobility shifts were confirmed by direct kinase activity measurements using MBP as substrate for Erk-2 (Fig. 1). In the absence of vanadate only weak phosphorylation signals were found, which were inconsistent and difficult to pick up. Erk activation was not due to a change of sodium or chloride concentration in the medium as no effect on Erk-1 and Erk-2 was obtained when 50 mmol/l NaCl was replaced by 100 mmol/l raffinose, thereby maintaining normo-osmolarity. Further, decreased activity of Erk-1 and Erk-2 was obtained when 100 mmol raffinose were added to normo-osmotic medium, thereby adjusting hyper-osmolarity (data not shown). With some delay Erk activation was accompanied by the occurrence of mobility-shifted Raf-1 (Fig. 1) which represents some phosphorylated forms of the Raf-1 kinase.

3.2. Elevation of $[Ca^{2+}]_i$ following hypo-osmotic exposure of C6 glioma cells

Hypo-osmotic (205 mosmol/l) treatment of the C6 glioma cells increased cytosolic free Ca^{2+} from basal levels of 84.2 ± 6.1 nmol/l to 203.9 ± 29.4 nmol/l ($n=3$). The elevation in $[Ca^{2+}]_i$ was maintained in a plateau-like manner as long as hypo-osmotic exposure was continued (Fig. 2A). In order to study the contribution of extracellular Ca^{2+} to the hypo-osmolarity-induced $[Ca^{2+}]_i$ response, extracellular Ca^{2+} was removed and 0.5 mmol/l EGTA was added 2 min before switching to hypo-osmotic medium. Under these conditions $[Ca^{2+}]_i$ remained unaffected (Fig. 2B; $n=3$). Following hypo-osmotic stress, the $[Ca^{2+}]_i$ increase was largely absent when extracellular Ca^{2+} was depleted (Fig. 2B; $n=3$). This suggests that the plateau may predominantly result from entry of extracellular Ca^{2+}. When C6 glioma cells were preincubated with U73122, which is a potent inhibitor of both PLCβ and PLCγ [22,12], the hypo-osmolarity-induced $[Ca^{2+}]_i$ response was preserved (Fig. 2C). As for control, the ATP-triggered Ca^{2+} signal completely disappeared in the presence of the PLC inhibitor (Fig. 2C) but remained unaffected in the presence of its inactive analog U73343 ($n=3$; not shown). Like in astrocytes [12] the $[Ca^{2+}]_i$ response to hypo-osmolarity was not affected by the presence of vanadate (100 μM; $n=3$, not shown). It is concluded that the hypo-osmolarity-induced changes in $[Ca^{2+}]_i$ are independent of PLC action.

3.3. Swelling-induced activation of Erk-1 and Erk-2 is independent of extracellular Ca^{2+} and requires cAMP-sensitive signalling steps

During Ca^{2+}-free conditions the osmosensitivity of Erk-1 and Erk-2 activity was completely maintained (Fig. 3, lanes 4–6; $n=4$), pointing to a signalling pathway to Erk-1 and Erk-2 independent of extracellular Ca^{2+}. Like the hypo-osmolarity-induced $[Ca^{2+}]_i$ response (Fig. 2B), the activity states of Erk-1 and Erk-2 were preserved in the presence of the PLC inhibitor U73122 (Fig. 3, lanes 7–9; $n=3$), indicating no requirement of PLC in triggering the osmosignalling to the MAP kinases. Application of Gö6850, a potent PKC inhibitor of broad specificity [23], did not abolish the activation of Erk-1 and Erk-2 following hypo-osmotic exposure of the C6 glioma cells (Fig. 3, lanes 10–12; $n=3$). Wortmannin at a concentration of 100 nmol/l, sufficient to specifically inhibit PI3 kinase in other cell types [24], was also without effect on the osmosensitivity of Erk-1 and Erk-2 (Fig. 3, lanes 13–15; $n=3$). The same was observed in the presence of LY294002 ($n=3$; not shown), another PI3 kinase inhibitor, which works in a mechanistically different manner from wortmannin [25].

When C6 glioma cells were pretreated with 8-CPT cAMP, the osmosensitivity of the MAP kinases was completely abol-

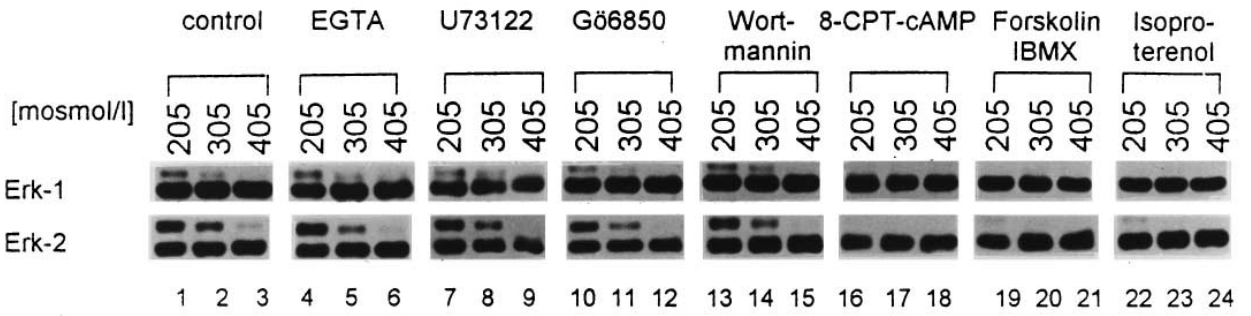

Fig. 3. Inhibitor sensitivity of the osmolarity dependence of Erk-1 and Erk-2 activity in C6 glioma cells. Representatives of at least three independent experiments are shown. Cells were pretreated with 100 μmol/l vanadate and aniso-osmolarity was adjusted without further pretreatment (controls) or after applying inhibitors as indicated. Conditions: control (lanes 1–3); EGTA: 5 mmol/l, 1 min (lanes 4–6); U73122: 10 μmol/l, 30 min (lanes 7–9); Gö6850: 1 μmol/l, 20 min (lanes 10–12); wortmannin: 100 nmol/l, 20 min (lanes 13–15); 8-CPT cAMP: 0.5 mmol/l, 20 min (lanes 16–18); forskolin (50 μmol/l) and isobuthylmethylxanthine (IBMX, 100 μmol/l) for 20 min (lanes 19–21); (−)isoproterenol: 5 μmol/l, 20 min (lanes 22–24).

ished (Fig. 3, lanes 16–18; $n=4$). The same was true for other maneuvers to increase cellular cAMP levels: forskolin and 3-isobutyl-1-methylxanthine, which act through activation of adenylylcyclase and inhibition of phosphodiesterase, respectively (Fig. 3, lanes 19–21; $n=4$). Also activation of adenylylcyclase-linked β-adrenoceptors by isoproterenol blocked the osmosensitive signal transduction pathway to the MAP kinases (Fig. 3, lanes 22–24; $n=4$).

4. Discussion

This study demonstrates that the phosphorylation and the activity of the endogenous MAP kinases Erk-1 and Erk-2 of C6 glioma cells increased with decreasing osmolarity and was associated with the occurrence of mobility-shifted Raf-1 (Fig. 1). The hypo-osmolarity-induced increase in $[Ca^{2+}]_i$ in C6 glioma cells (Fig. 2A) resulted from an influx from the extracellular space (Fig. 2B) and was independent of PLC action (Fig. 2C). The osmosignalling to Erk-1 and Erk-2 was independent of extracellular Ca^{2+} as well as from PLC (Fig. 3A), tyrosine kinases, PKC and PI3 kinase (Fig. 3B) but was abolished by elevated intracellular cAMP levels (Fig. 3C). Pertussis or cholera toxin-sensitive G proteins as well as genistein-sensitive tyrosine kinases are apparently not involved in the signalling steps upstream to Erk-1 and Erk-2 (not shown). The findings suggest a swelling-induced activation of MAP kinases via the Ras/Raf pathway. The occurrence of mobility-shifted Raf-1 following Erk activation may be due to inactivating phosphorylation of Raf-1 by the MAP kinases within a feedback loop [26].

Due to similarities in RVD between C6 glioma cells and primary astrocytes [11,27–29], C6 glioma cells were frequently used as a model system for astrocytes to study volume regulatory osmolyte fluxes [30,9,11] and osmotic stress-induced alterations in gene expression [10]. Hypo-osmolarity-induced activation of Erk-1 and Erk-2 was recently also observed in primary rat astrocytes [12]. Similar to C6 glioma cells this pathway was blocked by elevation of intracellular cAMP, suggesting a role of the Ras/Raf pathway in hypo-osmotic-induced signalling to Erk-1 and Erk-2 in both C6 glioma cells and astrocytes. However, upstream of Ras/Raf cell type-specific differences were found: in contrast to C6 glioma cells (this paper), in astrocytes the hypo-osmolarity-induced Erk activation was completely abolished in the absence of extracellular Ca^{2+} and by PI3 kinase inhibition [12]. Moreover, the plateau-like $[Ca^{2+}]_i$ increase following hypo-osmotic treatment of C6 cells (Fig. 2A) contrasts to the biphasic $[Ca^{2+}]_i$ response observed in astrocytes, which also involves Ca^{2+} release from intracellular stores [12,13], however, both responses occurred independent from inhibition of PLC by U73122 (this paper, [12]). The absence of a $[Ca^{2+}]_i$ response to hypo-osmolarity in C6 glioma reported earlier [28] may be due to the use of cell suspensions, which requires dissociation of the cells from their culture substrate and possible leads to altered cell function. The osmosignalling events in C6 glioma cells upstream of the Ras/Raf-MAP kinase pathway remain obscure. They apparently do not involve PI3 kinase and Ca^{2+} as observed in primary astrocytes [12], G-proteins and tyrosine kinases as observed in hepatocytes [31] and hepatoma cells [32] or the action of PKC and PLC.

The presence of vanadate, which among other things works as a protein tyrosine phosphatase inhibitor [33], was necessary to amplify the swelling-induced activation of Erk-1 and Erk-2 in C6 glioma cells (this paper) but not in astrocytes [12]. Likewise vanadate was necessary in hepatoma cells [32] but not in hepatocytes [31] in order to pick up the swelling-induced Erk activation. Whether the differences in sensitivity of Erk-1/Erk-2 are due to different levels of the constitutively expressed and Erk-specific MAP kinase phosphatase PYST2, which was cloned recently [34], remains to be established. Despite the presence of vanadate the swelling-induced Erk activation was transient in C6 glioma cells, which may reflect the induction of a MAP kinase phosphatase activity which overcomes the inhibitory potential of 100 μmol/l vanadate used in the present study.

Apart from hepatocytes [31], hepatoma cells [32], astrocytes [12] and C6 glioma cells (this paper), hypo-osmolarity-induced stimulation of Erk-type MAP kinases is also observed in the human intestine 407 cell line [35], in RAW 264.7 mouse macrophages, skin fibroblasts, vascular endothelial cells (F. Schliess and D. Häussinger, unpublished work) and yeast [36], and thus seems to be a general feature, which is in line with the growth factor-like action of cell swelling in many aspects [1,4,5].

Hyper-osmotic stress was shown to activate Erks in Madin-Darby canine kidney cells [37,38] while activation of both Erk-like MAP kinases and stress-activated protein kinases was found in rat 3Y1 fibroblasts, PC12 cells [39] and ven-

tricular rat myocytes [40]. In RAW 264.7 mouse macrophages p38, a MAP kinase which is related to the high osmolarity response gene (HOG1) of *Saccharomyces cerevisiae* [41] was activated in response to hyper-osmolar stress [42]. In contrast no activation of Erks was reported in RAW macrophages after hyper-osmotic treatment of the cells [42]. In primary astrocytes (R. Sinning and D. Häussinger, unpublished result) and C6 glioma cells (this paper) hyper-osmotic treatment led to a decrease of Erk-1 and Erk-2 activity below basal levels (Fig. 1), suggesting the induction within minutes of a phosphatase activity, whose identity remains to be clarified.

Taken together, astrocytes as well as C6 glioma cells responded with changes in $[Ca^{2+}]_i$ and Erk activity to anisoosmolarity but cell type-specific differences with respect to osmosensitivity and upstream signalling steps are obvious. Thus, extrapolation of findings obtained in C6 glioma cells to the situation in primary astrocytes may be problematic.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft, the Leibniz Prize and the Fonds der Chemischen Industrie, Frankfurt, Germany.

References

[1] Norenberg, M.D. (1994) J. Neuropathol. Exp. Neurol. 53, 213–220.
[2] Häussinger, D. (1996) Biochem. J. 321, 697–710.
[3] Häussinger, D. and Schliess, F. (1995) J. Hepatol. 22, 94–100.
[4] Dombro, R.S., Hutson, D.G. and Norenberg, M.D. (1993) Mol. Chem. Neuropathol. 19, 259- 268.
[5] Reichenbach, A. (1989) Glia 2, 71–77.
[6] Flogel, U., Niendorf, T., Serkowa, N., Brand, A., Henke, J. and Leibfritz, D. (1995) Neurochem. Res. 20, 793–802.
[7] Clark, E.C., Thomas, D., Baer, J. and Sterns, R.H. (1996) Kidney. Int. 49, 470–476.
[8] Mountian, I., Declercq, P.E. and Vandriessche, W. (1996) Am. J. Physiol. 39, C1319–C1325.
[9] Strange, K., Morrison, R., Shrode, L. and Putnam, R. (1996) Am. J. Physiol. 265, C244–C256.
[10] Paredes, A., McManus, M., Kwon, M. and Strange, K. (1992) Am. J. Physiol. 263, C1282–C1288.
[11] Strange, K., Morrison, R., Heilig, C.W., DiPietro, S. and Gullans, S.R. (1991) Am. J. Physiol. 260, C784–C790.
[12] Schliess, F., Sinning, R., Fischer, R., Schmalenbach, C. and Häussinger, D. (1996) Biochem. J. 319, 167–171.
[13] O'Connor, E.R. and Kimelberg, H.K. (1993) J. Neurosci. 13, 2638–2650.
[14] Waskiewicz, A.J. and Cooper, J.A. (1995) Curr. Opin. Cell. Biol. 7, 798–805.
[15] Crews, C.M., Alessandrini, A. and Erikson, R.L. (1992) Science 258, 478–480.
[16] Cook, S.J. and McCormick, F. (1993) Science 262, 1069–1072.
[17] Burgering, B.M., Pronk, G.J., van Weeren, P.C., Chardin, P. and Bos, J.L. (1993) EMBO J. 12, 4211–4220.
[18] Hafner, S., Adler, H.S., Mischak, H., Janosch, P., Heidecker, G., Wolfman, A., Pippig, S., Lohse, M., Ueffing, M. and Kolch, W. (1994) Mol. Cell. Biol. 14, 6696–6703.
[19] Alessi, D.R., Gomez, N., Moorhead, C., Lewis, T., Keyse, S.M. and Cohen, P. (1995) Curr. Biol. 5, 283–295.
[20] Samuels, M.L., Weber, M.J., Bishop, J.M. and McMahon, M. (1993) Mol. Cell. Biol. 13, 6241–6252.
[21] Schreiber, R. and Häussinger, D. (1995) Biochem. J. 309, 19–24.
[22] Salter, M.W. and Hicks, J.L. (1995) J. Neurosci. 15, 2961–2971.
[23] Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marmé, D. and Schächtele, G. (1993) J. Biol. Chem. 268, 9194–9197.
[24] Cross, D.A., Alessi, D.R., Vandenheede, J.R., McDowell, H.E., Hundal, H.S. and Cohen, P. (1994) Biochem. J. 303, 21–26.
[25] Cheatham, B., Vlahos, C.J., Cheatham, L., Wang, L., Blenis, J. and Kahn, C.R. (1994) Mol. Cell. Biol. 14, 4902–4911.
[26] Anderson, N.G., Li, P., Marsden, L.A., Williams, N., Roberts, T.M. and Sturgill, T.W. (1991) Biochem. J. 277, 573–576.
[27] Häussinger, D., Laubenberger, J., vom Dahl, S., Ernst, T., Bayer, S., Langer, M., Gerok, W. and Hennig, J. (1994) Gastroenterology 107, 1475–1480.
[28] Lohr, J.W. and Yohe, L.A. (1994) Brain Res. 667, 263–268.
[29] Isaacks, R.E., Bender, A.S., Kim, C.Y., Prieto, N.M. and Norenberg, M.D. (1994) Neurochem. Res. 19, 331–338.
[30] Jackson, P.S., Morrison, R. and Strange, K. (1994) Am. J. Physiol. 36, C1203–C1209.
[31] Noé, B., Schliess, F., Wettstein, M., Heinrich, S. and Häussinger, D. (1996) Gastroenterology 110, 858–865.
[32] Schliess, F., Schreiber, R. and Häussinger, D. (1995) Biochem. J. 309, 13–17.
[33] Gordon, J.A. (1991) Methods. Enzymol. 201, 477–482.
[34] Groom, L.A., Sneddon, A.A., Alessi, D.R., Dowd, S. and Keyse, S.M. (1996) EMBO J. 15, 3621–3632.
[35] Tilly, B.C., van den Berghe, N., Tertoolen, L.G., Edixhoven, M.J. and de Jonge, H.R. (1993) J. Biol. Chem. 268, 19919–19922.
[36] Thevelein, J.M. (1994) Yeast 10, 1753–1790.
[37] Terada, Y., Tomita, K., Homma, M.K., Nonoguchi, H., Yang, T.X., Yamada, T., Yuasa, Y., Krebs, E.G., Sasaki, S. and Marumo, F. (1994) J. Biol. Chem. 269, 31296–31301.
[38] Itho, T., Yamauchi, A., Miyai, A., Yokoyama, K., Kamada, T., Ueda, N. and Fujiwara, Y. (1994) J. Clin. Invest. 93, 2387–2392.
[39] Matsuda, S., Kawasaki, H., Moriguchi, T., Gotoh, Y. and Nishida, E. (1995) J. Biol. Chem. 270, 12781–12786.
[40] Bogoyevitch, M.A., Ketterman, A.J. and Sugden, P.H. (1995) J. Biol. Chem. 270, 29710- 29717.
[41] Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E. and Gustin, M.C. (1993) Science 259, 1760–1763.
[42] Han, J., Lee, J.D., Bibbs, L. and Ulevitch, R.J. (1994) Science 265, 808–811.