Calcium-dependent activation of Erk-1 and Erk-2 after hypo-osmotic astrocyte swelling

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The influence of hypo-osmotic cell swelling on the activity of the mitogen-activated protein (MAP) kinases Erk-1 and Erk-2 (where Erk stands for extracellular signal-regulated protein kinase) was studied in cultured rat astrocytes. Hypo-osmotic treatment led within 10 min to an increased activity of Erk-1 and Erk-2, which became maximal at 20 min and returned to the basal level within 60 min. Moreover, exposure to hypo-osmotic conditions induced a biphasic increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$): a rapid peak-like increase was followed by a sustained plateau. The absence of extracellular Ca^{2+} completely abolished Erk activation as well as the plateau of the $[Ca^{2+}]_i$ response after hypo-osmotic stimulation. Application of wortmannin and agents to elevate intracellular cAMP levels also completely blocked Erk activation but were without effect on the biphasic $[Ca^{2+}]_i$ response to hypo-osmotic treatment of the cells, suggesting a role of PtdIns 3-kinase and the Ras/Raf pathway downstream of the calcium signal. Protein kinase C (PKC) and Ca^{2+}/calmodulin (CaM)-dependent kinases are unlikely to play a role in the hypo-osmolarity-induced signalling towards MAP kinases, as revealed by the blockage of PKC and CaM kinases. Inhibition of tyrosine kinases, pertussis-toxin- or cholera-toxin-sensitive G-proteins and phospholipase C had no effect on the $[Ca^{2+}]_i$ response; the Erk response to hypo-osmolarity was also largely unaltered. This is different from the swelling-induced MAP kinase activation in hepatocytes, which was shown to occur via a calcium-independent but G-protein- and tyrosine kinase-dependent mechanism. Thus osmo-signalling towards MAP kinases might exhibit cell-type-specific features.

INTRODUCTION

Cell swelling induced either by hypo-osmotic environments or under the influence of hormones or cumulative substrate accumulation has been recognized as a signal that modulates cell function [1,2]. Astrocyte swelling is an early event in numerous pathological states such as brain ischaemia, hyponatraemia and hepatic encephalopathy (reviewed in [3]). Astrocyte swelling triggers volume-regulatory ion fluxes as well as the release of osmolytes such as aspartate, glutamate, taurine and *myo*-inositol [4–7], leading to 'regulatory volume decrease' (RVD). Moreover, astrocyte swelling was shown to stimulate glycogen synthesis [8] and was suggested to be a signal for proliferation and thus to play a role in gliosis [9]. In astroglioma cells hypo-osmotic exposure resulted in the down-regulation of the expression of osmolyte transporter mRNA [10] as well as elevated nucleoside triphosphate levels, stimulation of fatty acid biosynthesis and a decrease in cytoplasmic pH [11].

Signal transduction processes that are triggered by astrocyte swelling are largely unknown. Hypo-osmotic astrocyte swelling has been reported to elicit a biphasic response of the cytosolic calcium concentration ($[Ca^{2+}]_i$): a rapid transient increase is followed by a sustained plateau ([5], and this paper). It was suggested that the plateau phase is essential in mediating the loss of K^+ and Cl^- during RVD, whereas the accompanying osmolyte release occurs independently of extracellular calcium. Phospholipase C (PLC)-catalysed Ins(1,4,5)P_3 generation and Ca^{2+}/calmodulin (CaM) effectors have been reported to be involved in calcium signalling towards RVD [12,13].

The kinases Erk-1 and Erk-2 (where Erk stands for extracellular signal-regulated protein kinase) 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 (MEKs) [15]. The Ras/Raf pathway towards MEK shows sensitivity to activators of protein kinase A (PKA) [16–18]; however, alternative pathways might exist [19,20]. Dual-specificity MAP kinase phosphatases (MKP-1, also known as 3CH134 or CL100) are able to inactivate the MAP kinases [14]. Erk activity correlates with a shift to apparently higher molecular masses of the proteins during SDS gel electrophoresis, which can be detected by Western blotting with specific antibodies. Furthermore, the ability of the immunoprecipitated Erk proteins to phosphorylate the myelin basic protein (MBP) can be monitored by autoradiography.

The aim of this study was to characterize swelling-induced signalling events in astrocytes. The activity of the endogenous MAP kinases Erk-1 and Erk-2 was studied by using the model of cultured rat astrocytes that were exposed to hypo-osmotic media. Here a calcium-dependent activation of Erk-1 and Erk-2 in response to hypo-osmotic astrocyte swelling is reported, which is distinct from the osmo-signalling to Erk-1 and Erk-2 observed in hepatoma cells [21] and hepatocytes [22].

MATERIALS AND METHODS

Materials

Antibodies were obtained from UBI (Lake Placid, NY, U.S.A.), Genistein and erbstatin analogue were purchased from Calbiochem-Novabiochem (Bad Soden, Germany), 1-{6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl-1*H*-pyrrole-2,5-

Abbreviations used: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CaM, calmodulin; Erk, extracellular signal-regulated protein kinase; FCS, fetal calf serum; MAP, mitogen-activated protein; MBP, myelin basic protein; MEK, MAP kinase kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; RVD, regulatory volume decrease.

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dione (U73122), 1-{6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl-1*H*-pyrrolidine-2,5-dione (U73343), KN-62, 8-Br-cAMP, wortmannin and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) were from Biomol Research Laboratory (Hamburg, Germany) and pertussis and cholera toxins from Research Biochemicals Incorporated (Natick, MA, U.S.A.). Gö6850 was a gift from Gödecke (Freiburg, Germany). Pluronic F-127 and the acetoxymethyl ester of fura 2 were purchased from Molecular Probes (Eugene, OR, U.S.A.). Cell culture media and fetal calf serum (FCS) were from Gibco Life Technologies (Gaithersburg, MD, U.S.A.). Sodium orthovanadate, forskolin, isobutylmethylxanthine, ionomycin and phorbol 12-myristate 13-acetate 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).

Astrocyte preparation and culture

Primary astrocytes were prepared from cerebral hemispheres of newborn Wistar rats as described and were cultured for 2–3 weeks in Dulbecco's minimal essential medium, supplemented with 10% (v/v) FCS in a humidified atmosphere of air/CO_2 (19:1). The purity of the cell culture, as determined by immunohistochemical staining with glial fibrillary acidic protein, was more than 95%. After starvation for 24 h in serum-free medium, hypo-osmolarity (205 m-osmol/l) was achieved by dilution of the medium with the appropriate volume of NaCl-free medium. In the normo-osmotic control (305 m-osmol/l), the same volume of normo-osmotic medium was added. Normo-osmotic raffinose medium was prepared by the replacement of 50 mM NaCl with 100 mM raffinose.

Mobility shift kinase assay

At the end of the incubation period, medium was removed and cells were immediately lysed at 4 °C with 50 mM Tris/HCl buffer (pH 7.2) containing 150 mM NaCl, 40 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM vanadate, 0.5 mM PMSF, 0.1% aprotinin, 1% (v/v) Nonidet P40, 0.1% sodium deoxycholate and 0.1% SDS. The homogenized lysates were centrifuged at 20000 ***g*** at 4 °C for 20 min and the supernatant was added to an identical volume of gel-loading buffer containing 200 mM dithiothreitol (pH 6.8). After heating to 95 °C for 5 min, the proteins were subjected to gel electrophoresis [50 μg of protein per lane; 9% (w/v) gel]. After electrophoresis, gels were equilibrated with transfer buffer [39 mM glycine/48 mM Tris/HCl (pH 8.3)/0.03% SDS/20% (v/v) methanol]. Proteins were transferred to nitrocellulose membranes with a semi-dry transfer apparatus (Pharmacia, Freiburg, Germany). Blots were blocked in 5% (w/v) BSA containing TBST [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.1% Tween-20] and then incubated overnight with antiserum against Erk-1 or Erk-2 diluted 1:50000. After being washed with TBST and incubation for 1 h at room temperature with horseradish peroxidase-coupled anti-(rabbit IgG) antibody diluted 1:10000, the blots were washed three times and developed by enhanced chemiluminescent detection (Amersham, Braunschweig, Germany).

Immune-complex kinase assay

The assay was performed as described previously [23]. Briefly, aliquots of cell lysate containing 20 μg of protein [lysis buffer: 1% (w/v) Triton X-100/150 mM NaCl/10 mM Tris/HCl (pH 7.4)/1 mM EDTA/1 mM EGTA/20 mM NaF/0.2 mM PMSF/0.5% Nonidet P40] 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 by using Protein A–Sepharose 4B (Pharmacia), washed three times with lysis buffer and six times with kinase buffer [10 mM Tris/HCl (pH 7.4)/150 mM NaCl/10 mM $MgCl_2$/0.5 mM dithiothreitol] and incubated with 1 mg/ml MBP, 25 μM ATP and 5 μCi of [γ-^{32}P]ATP for 30 min at 37 °C. The activity of Erk-2 was monitored by autoradiography after SDS/PAGE [12.5% (w/v) gel].

Calcium imaging at the single-cell level

Astrocytes were grown on coverslips in Dulbecco's minimal essential medium containing 10% (v/v) FCS. After 24 h of serum starvation, sub-confluent cells were incubated with Krebs–Henseleit medium (KHB; 115 mM NaCl/25 mM $NaHCO_3$/5.9 mM KCl/1.18 mM $MgCl_2$/1.23 mM NaH_2PO_4/1.2 mM Na_2SO_4/1.25 mM $CaCl_2$), containing the fluorescent Ca^{2+} chelator fura 2 acetomethoxy ester (5 μM) and 0.02% Pluronic F-127, for 30 min at 37 °C in 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 (19:1), resulting in pH 7.4. Measurement of intracellular calcium was performed as described [24].

Analysis of results

Results are expressed as means ± S.E.M. (*n*, number of coverslips studied). For each series, astrocytes were prepared from at least three different brains. Results were compared with Student's *t* test: $P < 0.05$ was considered statistically significant.

RESULTS

Activation of Erk-1 and Erk-2 in response to hypo-osmotic astrocyte swelling

Alterations in phosphorylation of endogenous Erk-1 and Erk-2 in primary rat astrocytes in response to hypo-osmotic cell swelling were detected with the mobility shift assay. A decrease in medium osmolarity from 305 to 205 m-osmol/l induced a shift of Erk-1 and Erk-2 towards higher molecular masses within 10 min, which became maximal after 20 min and declined within 60 min to the basal level (Figure 1). The mobility fluctuations observed under normo-osmotic control conditions reflect the cellular stress induced by adding normo-osmotic medium. The time-dependent hypo-osmolarity-induced MAP kinase activation was confirmed for Erk-1 by direct measurement of the enzymic activity towards

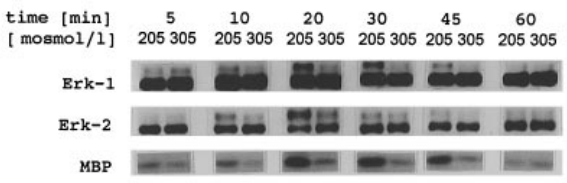

Figure 1 Hypo-osmolarity-induced activation of Erk-1 and Erk-2 in rat astrocytes, determined by mobility shifts and immune-complex assay

Cells were treated with hypo-osmotic medium (205 m-osmol/l) or normo-osmotic control medium (305 m-osmol/l) for the times indicated. Representative results of four independent experiments are shown. The extract of total protein was analysed by Western blot, with antibodies specifically raised against Erk-1 and Erk-2. The immune-complex assay was performed with MBP, using an antibody raised against Erk-2.

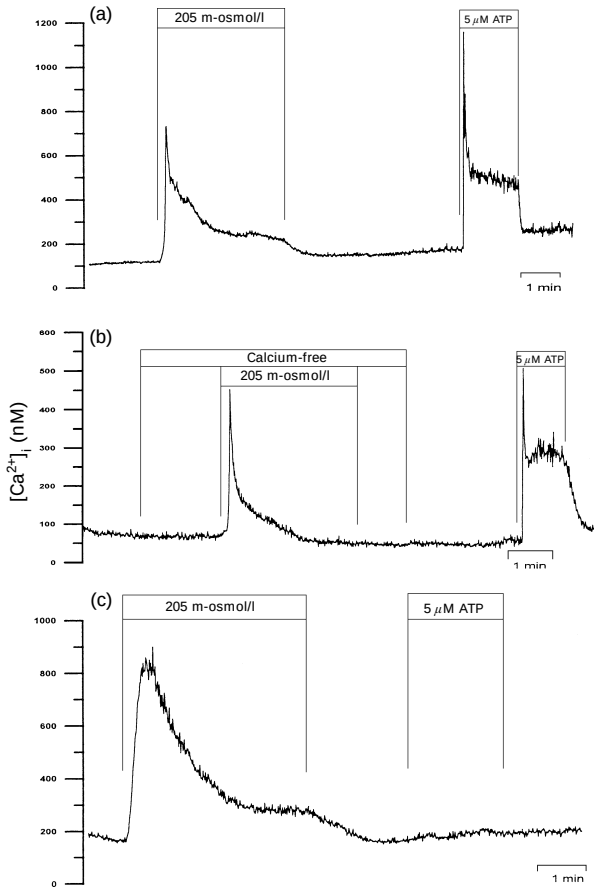

Figure 2 Effect of hypo-osmolarity and extracellular ATP on $[Ca^{2+}]_i$ in astrocytes, measured with fura 2 on single cells

Normo-osmotic (305 m-osmol/l) superfusion medium was suddenly changed to hypo-osmolarity (205 m-osmol/l) as indicated. Restoration of normo-osmolarity is followed by application of 5 μM ATP for control. Representative traces from at least seven experiments (compare with Table 1) are shown. (**a**) Presence of 1.25 mM extracellular Ca^{2+}; (**b**) calcium-free conditions (5 mM EGTA); (**c**) 20 min pretreatment of the cells with 10 μM U73122.

Table 1 Inhibitor sensitivity of the biphasic $[Ca^{2+}]_i$ response in astrocytes exposed to hypo-osmolarity or extracellular ATP

Fluorescence ratio-imaging was performed as described in the Materials and methods section. Hypo-osmotic exposure of the astrocytes was achieved by lowering the extracellular osmolarity from 305 to 205 m-osmol/l. Conditions used: calcium-free, medium without calcium, containing 5 mM EGTA for 1 min before and during hypo-osmotic stimulation; Gö6850 (1 μM for 20 min); KN-62 (20 μM for 30 min); genistein (100 μM for 20 min); erbstatin (1 μM for 1 h); vanadate (20 μM for 5 min before and during hypo-osmotic stimulation); pertussis toxin (250 ng/ml for 24 h); cholera toxin (5 μg/ml for 8 h); U73122 and U73343 (5 μM for 30 min); wortmannin (100 nM for 20 min), dibutyryl-cAMP (0.5 mM for 20 min). As an alternative to hypo-osmotic treatment, cells were exposed to 5 μM ATP. $\Delta[Ca^{2+}]_i$ (peak) = $[Ca^{2+}]_i$ (peak) − $[Ca^{2+}]_i$ (basal); $\Delta[Ca^{2+}]_i$ (plateau) = $[Ca^{2+}]_i$ (plateau) − $[Ca^{2+}]_i$ (basal); results represent the means ± S.E.M. for the number of experiments indicated in parentheses. *Statistically significant deviation from the control value at the $P < 0.05$ level (Student's t test). †The corresponding $[Ca^{2+}]_i$ value is statistically indistinguishable from $[Ca^{2+}]_i$ (basal) at the $P < 0.05$ level.

	m-osmol/l			5 μM ATP
Pretreatment	$[Ca^{2+}]_i$ (basal) (nM)	$\Delta[Ca^{2+}]_i$ (peak) (nM)	$\Delta[Ca^{2+}]_i$ (plateau) (nM)	$\Delta[Ca^{2+}]_i$ (ATP) (nM)
None	113 ± 5.8 (30)	591 ± 37 (30)	89 ± 9 (25)	634 ± 106 (30)
Calcium-free	105 ± 7 (14)	385 ± 22 (14)*	6 ± 2.3 (14)*†	244 ± 36 (14)*
Gö6850	105 ± 6.4 (8)	469 ± 60 (7)*	61 ± 23 (7)	640 ± 100 (8)
KN-62	109 ± 5 (7)	523 ± 38 (7)	47 ± 16 (7)*	774 ± 124 (6)
Genistein	99 ± 5.4 (15)	556 ± 37 (11)	59 ± 9 (11)	659 ± 61 (14)
Daidzein	114 ± 7 (9)	451 ± 27 (8)	55 ± 7(8)	516 ± 36 (8)
Erbstatin	104 ± 8.5 (15)	502 ± 42 (12)	70 ± 8 (14)	701 ± 150 (15)
Vanadate	127 ± 13.8 (11)	494 ± 49 (10)	96 ± 22 (9)	584 ± 153 (10)
Pertussis toxin	96 ± 12 (20)	356 ± 62 (15)*	90 ± 19 (15)	557 ± 77 (19)
Cholera toxin	101 ± 8.3 (21)	361 ± 33 (15)*	75 ± 15 (15)	505 ± 60 (18)
U73122	167 ± 64 (7)	401 ± 80 (7)*	92 ± 44 (7)	4 ± 65 (7)*†
U73343	125 ± 18 (8)	331 ± 89 (7)*	59 ± 23 (7)	473 ± 86 (7)
Wortmannin	80 ± 9 (10)*	432 ± 31 (9)*	73 ± 13 (9)*	504 ± 44 (10)
Dibutyryl-cAMP	90 ± 4 (9)*	394 ± 52 (9)*	66 ± 16 (9)*	508 ± 96 (9)

the MBP by using an immune-complex assay (Figure 1). Erk activation was not due to a decreased sodium or chloride concentration in the medium, as there was no activation when 50 mM NaCl was replaced by 100 mM raffinose, thereby maintaining normo-osmolarity (results not shown).

Biphasic elevation of $[Ca^{2+}]_i$ after hypo-osmotic exposure of astrocytes

Hypo-osmotic treatment of the astrocytes led to a rapid increase in cytosolic calcium from approx. 110 to 700 nM, followed by a decrease within 1 min to a sustained plateau phase, which was maintained as long as hypo-osmotic exposure was continued and which was approx. 90 nM above the baseline level (Figure 2a). To study the contribution of extracellular calcium to the biphasic phenotype of the $[Ca^{2+}]_i$ response, extracellular calcium was chelated with 5 mM EGTA 1 min before switching to hypo-osmotic medium. Under these conditions intracellular calcium stores [25] as well as $[Ca^{2+}]_i$ (Table 1) remained unaffected. After hypo-osmotic stress, the initial peak was significantly decreased by approx. 30 % and the plateau phase was completely absent when extracellular calcium was depleted (Figure 2b and Table 1). This suggests that the initial transient rise in $[Ca^{2+}]_i$ is only partly due to calcium influx, whereas calcium release from intracellular stores contributes mostly to the peak. In contrast, the plateau phase might result predominantly from calcium entry.

Formation of Ins(1,4,5)P_3 catalysed by either receptor-tyrosine kinase-coupled PLCγ or G-protein-coupled PLCβ from PtdIns(4,5)P_2 is a well-described path leading to elevated $[Ca^{2+}]_i$ by stimulating the release of Ca^{2+} from Ins(1,4,5)P_3-sensitive stores [26]. When astrocytes were preincubated with U73122, which is a potent inhibitor of both PLCβ and PLCγ [27,28], the hypo-osmolarity-induced sustained $[Ca^{2+}]_i$ response was preserved (Table 1 and Figure 2c), although the initial peak was slightly smaller. However, the diminution of this peak is probably not due to the inhibition of PLC because it was also observed in presence of the inactive analogue U73343 (Table 1). As for control, the ATP-triggered calcium signal disappeared completely in the presence of the PLC inhibitor but remained unaffected in presence of its inactive analogue U73343 (Table 1 and Figure 2c; compare with Figure 2a) [27]. It is concluded that the hypo-osmolarity-induced changes in cytosolic calcium occurred independently of PLC action.

Hypo-osmolarity-induced activation of Erk-1 and Erk-2 requires extracellular calcium upstream of wortmannin- and cAMP-sensitive signalling steps

During Ca^{2+}-free conditions the hypo-osmolarity-induced activation of Erk-1 and Erk-2 was completely abolished (Figure 3A, lanes 3 and 4), pointing to an important role of extracellular calcium in the osmo-signalling pathway upstream of the MAP

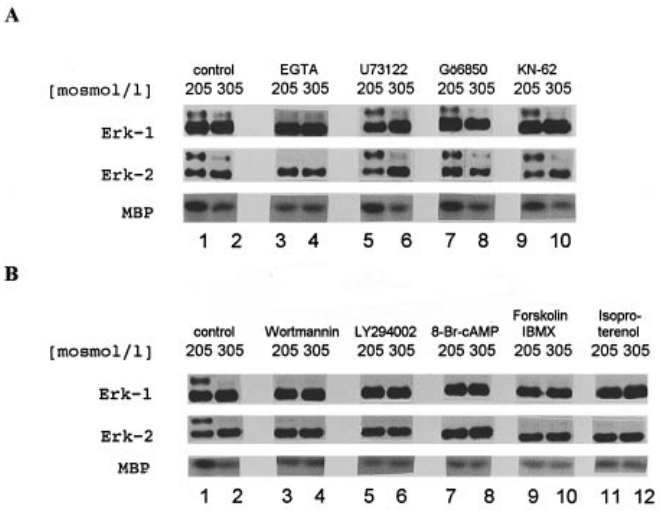

Figure 3 Pharmacological characterization of the hypo-osmolarity-induced activation of Erk-1 and Erk-2

Representative results from at least three independent experiments are shown. (**A**) Dependence on extracellular calcium but independence of PLC, PKC and CaM kinase. Cells were exposed to hypo-osmolarity (205 m-osmol/l) or normo-osmolarity (305 m-osmol/l) for 20 min without pretreatment (lanes 1 and 2) or after treatment with EGTA (5 mM for 1 min; lanes 3 and 4), U73122 (10 μM for 30 min; lanes 5 and 6), Gö6850 (1 μM for 20 min; lanes 7 and 8) or KN62 (20 μM for 30 min; lanes 9 and 10). (**B**) Dependence on wortmannin- and cAMP-sensitive signalling steps. Before hypo-osmotic (205 m-osmol/l) or normo-osmotic (305 m-osmol/l) treatment, astrocytes remained untreated (lanes 1 and 2) or were pretreated with wortmannin (100 nM for 20 min; lanes 3 and 4), LY294002 (10 μM for 20 min; lanes 5 and 6), 8-Br-cAMP (0.5 mM for 20 min; lanes 7 and 8), forskolin (50 μM)/isobuthylmethylxanthine (IBMX; 100 μM) for 20 min (lanes 9 and 10) or (−)isoprenaline (isoproterenol) (5 μM for 20 min; lanes 11 and 12).

kinases. The role of PLC and potential calcium effectors within this pathway was examined. Like the hypo-osmolarity-induced $[Ca^{2+}]_i$ response (Table 1 and Figure 2c), the activation of Erk-1 and Erk-2 was preserved in presence of the PLC inhibitor U73122 (Figure 3A, lanes 5 and 6), confirming that there is no requirement of PLC for triggering the hypo-osmolarity-induced calcium signalling to the MAP kinases. Application of Gö6850, a potent protein kinase C (PKC) inhibitor of broad specificity [21,29], as well as inhibition of CaM kinases by KN-62, did not abolish the activation of Erk-1 and Erk-2 after hypo-osmotic exposure of the astrocytes (Figure 3A, lanes 7–10). In addition, the calcium response accompanying astrocyte swelling remained largely unaffected by these treatments (Table 1), indicating that there is no requirement of PKC or CaM kinases for both the hypo-osmolarity-induced alterations in cytosolic calcium and the activation of MAP kinase.

Application of wortmannin at a concentration of 100 nM, sufficient to inhibit PtdIns 3-kinase specifically in other cell types [30], completely abolished the swelling-induced activation of Erk-1 and Erk-2 (Figure 3B, lanes 3 and 4). The same was observed in the presence of LY294002, another PtdIns 3-kinase inhibitor, which works mechanistically differently from wortmannin [31]. Wortmannin lowered the resting $[Ca^{2+}]_i$ to approx. 80 nM but had little effect on the swelling-induced changes in $[Ca^{2+}]_i$ (Table 1). This suggests that PtdIns 3-kinase is involved in the hypo-osmolarity-triggered signalling cascade downstream of the calcium signal but upstream of the MAP kinases.

When astrocytes were pretreated with 8-Br-cAMP, the activation of MAP kinases after hypo-osmotic treatment was completely abolished (Figure 3B, lanes 7 and 8). The same was true of other manoeuvres to increase cellular cAMP levels: forskolin and 3-isobutyl-1-methylxanthine, which act through the activation of adenylate cyclase and the inhibition of phosphodiesterase respectively. Furthermore, the activation of adenylate cyclase-linked β-adrenoceptors by isoprenaline blocked the osmosensitive signal-transduction pathway to the MAP kinases (Figure 3B, lanes 9–12). The biphasic $[Ca^{2+}]_i$ response remained largely unaffected after treatment with cAMP (Table 1), indicating that the inhibitory effect of PKA activation on hypo-osmolarity-induced Erk-1 and Erk-2 activation occurred downstream of the calcium signal.

Genistein and erbstatin analogue, which are potent inhibitors of tyrosine kinases [32,33], were without effect on hypo-osmolarity-triggered signalling at the levels of MAP kinases (results not shown) and cytosolic calcium (Table 1) respectively. Moreover, sodium vanadate, a tyrosine-phosphatase inhibitor [34], was without effect on the $[Ca^{2+}]_i$ response to hypo-osmolarity (Table 1), confirming the absence of protein-tyrosine phosphorylation/dephosphorylation steps in the generation of the swelling-induced calcium signal. In the presence of pertussis toxin or cholera toxin, a decrease in the hypo-osmotically induced calcium peak by approx. 35% was observed but the plateau phase (Table 1) as well as the activation of Erk-1 and Erk-2 remained unaltered (results not shown).

DISCUSSION

This study demonstrates the activation of the endogenous MAP kinases Erk-1 and Erk-2 of primary rat astrocytes after hypo-osmotic exposure (Figure 1). This activation requires the presence of extracellular calcium but is independent of the action of PLC, PKC and CaM kinase (Figure 3A). Neither tyrosine kinases nor pertussis- or cholera-toxin-sensitive G-proteins are apparently involved in the signalling steps upstream to Erk-1 and Erk-2. However, the PtdIns 3-kinase inhibitors wortmannin and LY294002, as well as the elevation of intracellular cAMP levels (Figure 3B), led to an inhibition of the hypo-osmolarity-induced activation of Erk-1 and Erk-2 in rat astrocytes without affecting the $[Ca^{2+}]_i$ response.

The most important protein kinase cascade leading to Erk activation is the Ras/Raf pathway, which involves first the activation of the GTP-binding protein Ras and then the protein serine/threonine kinases Raf-1 and MEK [14]. Recent reports have demonstrated that PKA can inhibit the Ras/Raf pathway towards Erk-1 and Erk-2 in astrocytomas [35] and other cell types [16–18] and the block was mapped to occur at the level of Raf-1 activation. Thus Raf-1 might be involved in the swelling-induced MAP kinase activation in astrocytes. The role of PtdIns 3-kinase within the Ras/Raf-mediated phosphorylation cascade leading to Erk-1 and Erk-2 is not well defined. In L6 myoblasts wortmannin blocked the insulin-like growth factor-1-activated MAP kinase cascade at the level of Raf-1 activation but was without effect on the formation of GTP-Ras [30]. In 3T3-L1 adipocytes, however, the insulin-induced MAP-kinase cascade was blocked by inhibition of GTP-Ras formation in the presence of wortmannin [36]. This suggests that PtdIns 3-kinase inhibition might interfere with the GTP-Ras-dependent recruitment of Raf-1 kinase to the plasma membrane. How the increase in calcium in response to astrocyte swelling is translated into a protein phosphorylation cascade leading to the MAP kinases remains unclear. Neither protein tyrosine phosphorylation steps sensitive to genistein or erbstatin analogue, nor cholera- or pertussis-sensitive G-proteins, are involved in the hypo-osmotically stimu-

lated signalling to Erk-1 and Erk-2 in astrocytes (results not shown). In 3T3 cells Ca^{2+} was shown to activate Raf in a Ras- and PKC-independent manner [19]. Another possibility is the direct interference of calcium with the N-terminal SH2 domains of the p85 subunit of the PtdIns 3-kinase, which has been demonstrated *in vitro* to alter the enzyme's affinity for phosphotyrosine residues [37]. Thus increased $[Ca^{2+}]_i$ could induce the activation of PtdIns 3-kinase after binding of p85 to pre-existing phosphotyrosine residues of as yet unidentified proteins. However, whether this applies to the hypo-osmolarity-triggered signalling in astrocytes is unclear.

Although the swelling-induced Erk activation in astrocytes was completely resistant to the inhibition of PKC and CaM kinases by Gö6850 and KN-62 respectively (Figure 3A, lanes 7–10), the Erk activation by ionomycin (1 μM) (results not shown), which channels calcium through membranes from non-specific sources, was diminished by these treatments. Moreover, elevation of intracellular cAMP levels as well as wortmannin treatment of the cells only partly abolished the ionomycin-induced Erk activation (results not shown), which contrasts with the total inhibition of the swelling-induced activation (Figure 3B, lanes 3, 4 and 7–12). This suggests that, in general, multiple pathways towards Erk-1 and Erk-2 might be triggered by elevated $[Ca^{2+}]_i$ and underlines the specificity of the signalling pathway induced by astrocyte swelling.

In contrast with astrocytes, a calcium-independent mechanism of swelling-induced stimulation of Erk-1 and Erk-2 was found in rat hepatoma cells and hepatocytes [21,22,24]. In these cells the hypo-osmolarity-induced Erk activation is independent from PKC but tyrosine kinases and G-proteins are involved in upstream signalling to Erk-1 and Erk-2 [21,22]. Moreover the swelling-induced Erk activation in H4IIE cells is found to be resistant to wortmannin and elevation of intracellular cAMP (F. Schliess and D. Häussinger, unpublished work). Hypo-osmolarity-induced stimulation of Erk-type MAP kinases is also observed in the human intestine 407 cell line [38] and yeast [39]. According to their pleiotropic regulatory potential, a role of Erk-1 and Erk-2 in mediating the major effects of cell-swelling is suggested. In H4IIE cells the activation of Erk-1 and Erk-2 precedes the phosphorylation of c-Jun and the subsequent auto-induction of c-*jun* transcription [21,40]. In liver the swelling-induced increase in taurocholate excretion into bile and the alkalinization of endocytotic vesicles are linked to Erk-1 and Erk-2 activation [22,24]. In intestine 407 cells and yeast, activation of the MAP kinases is related to volume-regulatory responses [38,39]. The involvement of Erk-1 and Erk-2 in signalling towards astrocyte RVD seems unlikely because inhibition of PLC and antagonizing of CaM, which are known to block RVD [12,13], are without effect on the hypo-osmolarity-induced Erk activation (Figure 3A, lanes 7–10). A role of Erk-1 and Erk-2 in linking astrocyte swelling to a stimulation of proliferation, alterations in gene expression and energy metabolism, including a pathogenetic role in hepatic encephalopathy [4], is conceivable but remains to be established.

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