Inactivation and dephosphorylation of protein kinase Bα (PKBα) promoted by hyperosmotic stress

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To study the role of protein kinase B (PKB) in response to cellular stress, we examined PKBα activity following different stress treatments. Hyperosmotic but not chemical stress resulted in inactivation of PKBα and prevented activation by pervanadate and mitogens. Hyperosmotic shock did not affect the MAP kinase pathway, suggesting that this inhibitory effect was specific for PKB. Our data further indicate that down-regulation occurs via dephosphorylation of Thr308 and Ser473, the major regulatory phosphorylation sites of PKBα. Indeed, calyculin A, which inhibits protein phosphatases 1 and 2A, effectively blocked hyperosmotic stress-mediated inactivation (dephosphorylation) of PKBα. High osmolarity did not affect phosphatidylinositol 3-kinase activity but led to a marked increase in PI(3,4,5)P3 and a decrease in PI(3,4)P2, formation after pervanadate stimulation, suggesting that hyperosmotic stress has an inhibitory effect on a phosphatidylinositol 5-phosphatase which converts PI(3,4,5)P3 into PI(3,4)P2. Immunofluorescence studies revealed that membrane translocation, a prerequisite for PKB activation, was not affected by hyperosmotic stress. Our results indicate that hyperosmotic stress can act at two levels: (i) inhibition of phosphorylation of Thr308 and Ser473 by upstream kinases and (ii) by promoting rapid dephosphorylation of these regulatory sites.

Keywords: osmotic shock/phosphorylation/protein kinase B/protein phosphatase 2A/signal transduction

Introduction

Protein kinase B (PKB), also termed RAC kinase (Jones et al., 1991) or Akt (Bellacosa et al., 1991), represents a family of 3-phosphoinositide-regulated serine/threonine kinases (Hemmings, 1997a). PKBα, β and γ become phosphorylated and activated in response to mitogens and survival factors in a phosphatidylinositol 3-kinase (PI 3-kinase)-dependent manner (Burgering and Coffer, 1995; Franke et al., 1995; Alessi et al., 1996; Didichenko et al., 1996; Meier et al., 1997; Andjelković et al., 1997; Walker et al., 1998). Phosphorylation of PKBα and β occurs on the two residues Thr308 (Thr309 in PKBβ) in the activation loop and Ser473 (Ser474 in PKBβ) within the C-terminal activation domain. Whereas the upstream kinase phosphorylating Ser473 (Ser474 in PKBβ) is still unknown, phosphorylation of the A-loop site by 3-phosphoinositide-dependent protein kinase-1 (PDK1) is dependent on the product of PI 3-kinase, phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3] and, to a lesser extent, phosphatidylinositol-3,4-bisphosphate [PI(3,4)P2] (Alessi et al., 1997; Stokoe et al., 1997), which is produced from PI(3,4,5)P3 by polyphosphate-5-phosphatase activities (Kavanaugh et al., 1996; Woischils and Parker, 1997). Additionally, PI(3,4,5)P3 and PI(3,4)P2 are thought to provide docking sites for PKB and PDK1, which both become targeted to the plasma membrane mediated by their pleckstrin homology (PH) domains (Hemmings, 1997b).

A dynamic balance between positive and negative regulatory mechanisms is crucial to setting the threshold at which extracellular stimuli trigger signal transduction pathways. In insulin receptor signalling, changes in PKBα activity control glycolysis synthesis by phosphorylation and inactivation of glycogen synthase kinase-3 (GSK3) (Cross et al., 1995), increase in protein synthesis by indirect inactivation of 4E-BP1 (Gingras et al., 1998) or the regulation of glucose uptake by GLUT4 translocation (Kohn et al., 1996). Furthermore, PKBα is necessary for cell survival and the prevention of apoptosis (Ahmed et al., 1997; Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Kulik et al., 1997), which may occur by phosphorylation of the Bel-x inhibitor BAD (Datta et al., 1997; Del Peso et al., 1997).

In contrast to the activation of PKB, downregulation of this kinase is less well understood. Inactivation of PKBα has been described in cells which detach from the extracellular matrix (anoikis), implying that PI 3-kinase is a mediator of the aberrant survival of Ras-transformed epithelial cells which are no longer attached to the extracellular matrix (Khwaja et al., 1997). In the current study, we examined the effect of high osmolarity on the regulation of PKBα. We demonstrate that hyperosmotic stress not only activates the stress-induced Jun N-terminal kinase (JNK) cascade (Galcheva-Gargova et al., 1994) but also strongly inhibits PKBα activation and activity. Inactivation of PKBα was due to dephosphorylation of the regulatory phosphorylation sites by protein phosphatase 2A (PP2A).

Results

Cellular stresses do not activate PKBα

To gain further insights into PKB regulation, we transiently transfected HEK-293 and COS-1 cells with haemagglutinin (HA) epitope-tagged wild-type PKBα (HA-PKBα) and tested the effect of different stress conditions on kinase activation (Figure 1). In agreement with our previous results (Andjelković et al., 1996; Meier et al., 1997), pervanadate potently stimulated PKBα. However, we did not detect any PKBα activation by 0.5 M sorbitol.
Osmotic stress-induced PKB inactivation

(Figure 1), 0.5 M sodium chloride (data not shown) or 10 μg/ml anisomycin (Figure 1). Furthermore, in Swiss 3T3 cells, we did not detect PKBα activation when cells were treated by heat shock at 42°C for 30 min (M. Andjelković and B.A. Hemmings, unpublished data). In contrast, treatment with 0.5 M sorbitol (Figure 1) or 0.5 M sodium chloride (data not shown) reduced basal PKBα activity to 20–25% of the control in both HEK-293 and COS-1 cells. Basal PKBα activities were not reduced when cells were treated with either heat shock or anisomycin, indicating that osmotic shock selectively inhibits PKBα activity. We therefore decided to characterize the regulation of PKB in response to stress in more detail.

**High osmolarity both prevents activation and inactivates PKBα**

To test whether high osmolarity mediates inactivation of activated PKBα, we transiently transfected HA-PKBα into HEK-293 or COS-1 cells. Pervanadate-induced activation of PKBα was almost completely reversed when 0.5 M sorbitol or 0.5 M sodium chloride were added 15 min after stimulation (Figure 2A and B). Moreover, pre-treatment of the cells with 0.5 M sorbitol or 0.5 M sodium chloride prevented pervanadate-induced activation of PKBα. Similar results were obtained when platelet-derived growth factor (PDGF) was used to activate HA-PKBα (Figure 2C). Activation of HA-PKBα by PDGF was not detectable when cells were pre-treated with 0.5 M sorbitol. Furthermore, HA-PKBα activity was decreased dramatically when 0.5 M sorbitol was added to the cells after PDGF stimulation. To confirm the data obtained with overexpressed PKBα, we assayed endogenous PKBα from Swiss 3T3 cells immunoprecipitated with a polyclonal antibody raised against the C-terminus of PKBα (Figure 3). A 5-fold activation of endogenous PKBα was obtained after PDGF stimulation, which was similar to the activation of overexpressed PKBα. Sorbitol treatment moderately decreased the basal activity of endogenous PKBα but almost completely abolished PDGF-mediated stimulation (Figure 3).

Hyperosmotic and chemical stress are potent inducers of the stress kinase (JNK/p38) pathways (Han et al., 1994; Kyriakis et al., 1994; Meier et al., 1996). We therefore looked for a link between activation of stress kinases and inactivation of PKBα in response to cellular stress by comparing the effects of sorbitol and anisomycin on both JNK and PKBα activation (Figure 2D). Pervanadate treatment caused JNK activation (2- to 3-fold over the basal level), which was enhanced further upon pre-treatment of HEK-293 cells with 10 μg/ml anisomycin or 0.5 M sorbitol (Figure 2D). Interestingly, whilst pre-treatment with sorbitol abolished PKBα activation by pervanadate, pre-treatment with anisomycin had no effect on PKBα activity after pervanadate stimulation (Figure 2A).

**Inactivation of PKBα occurs by dephosphorylation of Thr308 and Ser473**

We reasoned that the inhibition of activation and inactivation of PKB α activity could be due to effects on the two key regulatory phosphorylation sites Thr308 and Ser473 (Alessi et al., 1996). We therefore transiently transfected wild-type HA-PKBα and the constitutively active mutant HA-PKBα-T308D/S473D into HEK-293 cells and tested the effect of sorbitol upon PKBα activation (Figure 4A). Compared with HA-PKBα, the HA-PKBα-T308D/S473D mutant showed an 18-fold higher basal activity in unstimulated cells. Pervanadate treatment promoted a 25-fold stimulation of the wild-type activity but did not increase the activity of the HA-PKBα-T308D/S473D mutant. Moreover, pre-treatment with 0.5 M sorbitol for 15 min abolished the effect of pervanadate stimulation in HA-PKBα but had no effect on the HA-PKBα-T308D/S473D mutant. Immunoblotting of wild-type PKBα with an antibody specific for either phosphothreonine 308 or phosphoserine 473 revealed that both sites were phosphorylated in response to pervanadate but remained in a dephosphorylated state following pre-treatment of the cells with 0.5 M sorbitol for 15 min (Figure 4B). Taken together, these data suggest that dephosphorylation of both Thr308 and Ser473 was involved in the sorbitol-induced inactivation of PKBα.
Fig. 2. Hyperosmotic but not chemical stress prevents activation and mediates inactivation of PKBα. (A and B) HEK-293 (A) and COS-1 (B) cells were transiently transfected with HA-PKBα, serum starved (24 h) and treated with 0.1 mM pervanadate for 15 min, 0.1 mM pervanadate for 15 min followed by 0.5 M sorbitol (0.5 M NaCl) for a further 15 min, pre-treated with 0.5 M sorbitol (0.5 M NaCl) for 15 min followed by 0.1 mM pervanadate for a further 15 min, or 10 μg/ml anisomycin for 15 min followed by 0.1 mM pervanadate for a further 15 min. (C) COS-1 cells transiently transfected with HA-PKBα and stimulated with 50 ng/ml PDGF for 15 min, pre-treated with 0.5 M sorbitol for 15 min followed by 50 ng/ml PDGF for 15 min, or treated with 50 ng/ml PDGF for 15 min before adding 0.5 M sorbitol (15 min). After stimulation, cells were lysed and assayed for in vitro PKBα kinase activity as described (Alessi et al., 1996). Activities are the mean (± SD) of two independent experiments assayed in duplicate. Relative activities are based on the activities found in unstimulated cells (control). (D) In parallel experiments, samples from HEK-293 cells (A) were used to assay endogenous JNK. JNK activity was measured in pull-down assays using GST-cJun(5–89) as substrate (Meier et al., 1996).

**PP2A mediates osmotic shock-dependent dephosphorylation of PKBα**

To understand the mechanism of Thr308/Ser473 dephosphorylation in the sorbitol-induced inactivation of PKBα, we predicted that PP2A dephosphorylates and inactivates PKBα in vivo, since PP2A inactivates PKBα in vitro whilst phosphatase 1 (PP1) does not (Andjelković et al., 1996). To test this, we used calyculin A, a cell-permeable inhibitor of PP1 and PP2A. HEK-293 cells transiently transfected with wild-type HA-PKBα were stimulated with pervanadate and sorbitol in the presence or absence of calyculin A (Figure 5A). As described previously for okadaic acid (Andjelković et al., 1996), treatment with calyculin A alone led to a 30-fold stimulation of PKBα. Pervanadate plus calyculin A resulted in a slightly higher activation of PKBα. In both cases, activation of PKBα was no longer inhibited by sorbitol. In addition, we performed a time-course of PKBα reactivation with calyculin A (Figure 5B). In the presence of sorbitol and pervanadate, inactive PKBα became reactivated 5 min after calyculin A addition, peaking at 20 min. Thus, calyculin A caused a robust activation of PKBα which was completely insensitive to sorbitol, indicating that PP2A is involved in mediating downregulation of PKBα activity through the dephosphorylation of the regulatory phosphorylation sites. However, activation of PKBα by calyculin A was sensitive to wortmannin (data not shown), showing that calyculin A not only activates PKBα by inactivating PP2A but also by the activation of upstream elements of PI 3-kinase. To gain further insight into this mechanism, we performed a time-course of PKBα activation with calyculin A in the presence or absence of
sorbitol (Figure 5C). We found that during the first 10 min of calyculin A stimulation, sorbitol pre-treatment caused a lower PKBα activity compared with cells which were not treated with sorbitol. After 10 min of calyculin A stimulation, the PKBα activity of sorbitol-treated cells increased relative to untreated cells and slightly exceeded it after 20 min of calyculin stimulation. Thus, we conclude that sorbitol either activates PP2A directly or that an upstream element is inhibited by high osmolarity, thereby affecting PP2A activity or targeting. To investigate this idea, we first tested whether PP2A is upregulated by osmotic shock. HEK-293 cells, transiently transfected with HA-PP2Ac were stimulated and assayed for PP2A activity (Figure 5D). Sorbitol (0.5 M) did not affect PP2A activity in either unstimulated or pervanadate-treated cells, although pervanadate caused a 40% reduction of PP2A activity. Second, we tested whether sorbitol promotes targeting to PKBα. We were unable to detect any PP2A activity or protein associated with endogenous PKBα in immunoprecipitates from unstimulated, stimulated or sorbitol-pre-treated HEK-293 cell extracts (data not shown).

**Hyperosmotic stress does not inhibit PI 3-kinase, PDK1 and MAP kinase**

To study the specificity of osmotic stress-induced inactivation of PKBα, we examined the effect of sorbitol on PI 3-kinase and PDK1 (Burginger and Coffer, 1995; Franke et al., 1995; Alessi et al., 1997; Stokoe et al., 1997). Endogenous PI 3-kinase was assayed from HEK-293 (Figure 6D) and Swiss-3T3 (data not shown) cells stimulated either with pervanadate or PDGF. HEK-293 cells were pre-treated with or without 0.5 M sorbitol for 15 min followed by incubation with 0.1 mM pervanadate for 15 min, or were left untreated. PI 3-kinase activity was determined in anti-p85 immunoprecipitates as described by Whitman et al. (1985). Immunoprecipitates obtained from pervanadate-stimulated cell lysates showed a 7.5-fold increase of PI 3-kinase activity [as determined by phosphatidylinositol 3-phosphate (PI3P) production] compared with extracts from unstimulated cells (Figure 6A). The same extracts were also assayed in parallel for PKBα activation (Figure 6B, first three bars). As expected, stimulation with pervanadate resulted in a 3-fold activation of endogenous PKBα. Furthermore, sorbitol treatment before addition of pervanadate, which prevented activation of PKBα (Figure 6B), led to an 8-fold higher PI3P formation similar to pervanadate-stimulated cells, indicating that PI 3-kinase activity was unaffected by sorbitol treatment. The same experiments were repeated with Swiss 3T3 cells stimulated with 50 ng/ml PDGF for 15 min, with or without pre-treatment with 0.5 M sorbitol, or left untreated. Both, PKBα and PI 3-kinase activities were stimulated with PDGF. However, following pre-treatment with sorbitol, PDGF failed to activate PKBα but still
Inhibition of PP2A/PP1 by calyculin A blocks sorbitol effect on PKBα. (A) Calyculin A blocks sorbitol-mediated inactivation of PKBα. HEK-293 cells were transiently transfected with HA-PKBα, serum starved and treated with 0.5 M sorbitol (15 min), followed by 0.1 mM pervanadate stimulation for a further 15 min (first three bars) as an internal control. Last three bars: in the presence of calyculin A (100 nM), cells were either not treated, stimulated with 0.5 M sorbitol for 15 min, or stimulated with 0.1 M pervanadate (15 min) or with pervanadate (15 min) and 0.5 M sorbitol (30 min). (B) Calyculin A reactivates sorbitol-induced inactivated PKBα. First three bars: internal control as described in (A). Next three bars: cells pre-treated with 0.5 M sorbitol for 15 min then stimulated with 100 nM calyculin A in the presence of 0.1 mM pervanadate for the times indicated. (C) Time-course of PKBα activation in the presence or absence of 0.5 M sorbitol. HEK-293 cells were transiently transfected with HA-PKBα, pre-treated with 0.5 M sorbitol for 15 min (●) or left untreated (□) and then stimulated with calyculin A (100 nM) for the times indicated. The inset shows a better resolution of the first three time points. (D) HEK-293 cells were transiently transfected with HA-PP2Acα, stimulated with 0.5 M sorbitol for 30 min, 0.1 mM pervanadate (15 min) and 0.5 M sorbitol (15 min) prior to treatment with pervanadate for a further 15 min, or left untreated (control). After stimulation, HA-PP2Acα was immunoprecipitated and assayed as described in Materials and methods. The data shown are the average (± SD) of two independent experiments in duplicate.

Next, we investigated whether osmotic stress results in a general inactivation of growth factor-induced signalling pathways. We focused our attention on the MAP kinase pathway involved in transmitting the mitogenic signals which result in cell growth but which is poorly activated in HEK-293 cells in response to cellular stress (Brunet and Pouyssegur, 1996). HEK-293 cell extracts were assayed for MAP kinase activities (Figure 6B and C). Using the polyclonal antibody C-16, activation of p42 and p44 MAPK was detected either by mobility shifts on Western blots after SDS–PAGE or by in vitro kinase assays using myelin basic protein (MBP) as a substrate. As expected, both p42 and p44 MAP kinases were activated following pervanadate treatment. Moreover, treatment of HEK-293 cells with 0.5 M sorbitol had no effect on basal or pervanadate-stimulated p42 and p44 MAP kinase activities. The finding that sorbitol selectively inhibited PKBα but did not affect the MAP kinase pathway, PDK1 or PI 3-kinase activity suggests that the target of hyperosmotic stress is specific for the PKB signalling pathway acting downstream or independent of PI 3-kinase and PDK1.

**High osmolarity elevates PI[3,4,5]P3 and lowers PI[3,4]P2 levels in pervanadate-stimulated HEK-293 cells**

Because we found no correlation between PKBα and PI 3-kinase activity after hyperosmotic stress, we studied changes in 3'-phosphoinositide levels after hyperosmotic...
stress-induced inactivation of PKBα. PI(3,4)P$_2$ and PI(3,4,5)P$_3$ have been shown to participate in the regulation of PKBα activity at the level of targeting PKB to the membrane and by direct binding to the PKBα-PH domain (Frech et al., 1997; Stokoe et al., 1997). As expected, 15 min pervanadate treatment caused an increase in both PI(3,4)P$_2$ and PI(3,4,5)P$_3$, which was accompanied by a decrease in the level of the PI 3-kinase substrate PI(4,5)P$_2$ (Figure 7). Pre-treatment of the cells with the PI 3-kinase inhibitor wortmannin (which blocks PKBα activation) for 15 min markedly inhibited the production of 3′-phosphoinositides whilst attenuating PI(4,5)P$_2$ consumption. Moreover, we found that sorbitol alone caused only modest changes in phosphoinositide levels. Pre-treatment with 0.5 M sorbitol for 15 min, which causes a marked ablation of pervanadate-stimulated PKBα activity, substantially inhibited pervanadate-stimulated PI(3,4)P$_2$ formation by 41% and surprisingly caused a 50% higher accumulation of PI(3,4,5)P$_3$.

**Influence of PI(3,4,5)P$_3$ and PI(3,4)P$_2$ on PP2A activity in vitro**

To test whether the sorbitol-induced decrease of PI(3,4)P$_2$ or increase of PI(3,4,5)P$_3$ affects PP2A activity, we assayed various PP2A heterodimers and trimers (PP2A$_{2}$, PP2A$_{1}$, PP2A$_{3}$ and PP2A$_{0}$) in the presence of different ratios of PI(3,4)P$_2$ and PI(3,4,5)P$_3$ using $^{32}$P-labelled HA-ΔPH-PKBα as a substrate (Figure 8). To detect inhibition of phosphatase activity by phospholipids, we standardized the assays to achieve dephosphorylation of HA-ΔPH-PKBα in the absence of phospholipids which was fully reversed in the presence of 100 nM okadaic acid. With every form of PP2A tested, we were unable to detect activity changes in response to different ratios of PI(3,4)P$_2$ and PI(3,4,5)P$_3$, indicating that PKBα inactivation was not due to interactions and activity changes of PP2A with PI(3,4)P$_2$ or PI(3,4,5)P$_3$. 

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**Fig. 6.** Effect of hyperosmotic stress in PKB specific. HEK-293 cells were treated with 0.1 mM pervanadate for 15 min, pre-treated with 0.5 M sorbitol for 15 min before stimulation with 0.1 mM pervanadate for 15 min, or left untreated. Total cell lysates were assayed for (A) PI 3-kinase activities (B) PKBα, MAP kinase activities and (C) for p42 and p44 MAP kinase activation-associated band shifts.

**Fig. 7.** Changes of phosphoinositide levels upon pervanadate/sorbitol stimulation in HEK-293 cells. HEK-293 cells were labelled with $[^{32}$P]orthophosphate for 120 min. After 15 min incubation in fresh medium, cells were stimulated with sorbitol (15 and 30 min), pervanadate (15 min) or were pre-treated with either sorbitol or wortmannin for 15 min before pervanadate was added for a further 15 min. After stimulation, $[^{32}$P]PI(4,5)P$_2$ was added to the combined material, and lipids were extracted as previously described (Didichenko et al., 1996). Lipids were deacylated and the resulting glyceroinositides separated by HPLC.

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**Osmotic stress-induced PKB inactivation**
Membrane translocation is an important initial step in PKBα activation (Andjelković et al., 1997) but it is still unclear whether PI(3,4)P₂ and/or PI(3,4,5)P₃ mediate membrane translocation. Our finding that PI(3,4)P₂ levels are reduced by hyperosmotic stress prompted us to study PKBα membrane translocation in response to 0.5 M sorbitol (Figure 9). HEK-293 cells transiently transfected with HA-PKBα were pre-treated with or without 0.5 M sorbitol for 15 min followed by stimulation with 0.1 mM pervanadate for 4 min, or were left untreated. Unstimulated cells showed a clear cytoplasmic distribution of HA-PKBα (Figure 9A). Incubation of the cells with 0.5 M sorbitol, which reduces basal PKBα activity, did not alter its subcellular localization (Figure 9D). As expected, pervanadate treatment resulted in PKB membrane translocation within 4 min of stimulation (Figure 9B) and, although pre-treatment of the cells with 0.5 M sorbitol prior to pervanadate stimulation prevents activation of PKB, translocation to the plasma membrane was unaffected (Figure 9C).

**Discussion**

The general mechanism for PKB activation is well established (Hemmings, 1997b). Stimulation of many receptor tyrosine kinases results in an increase in PI(3,4,5)P₃ and PI(3,4)P₂ levels, which in turn mediate targeting of PKBα, PDK1 and probably the not yet fully characterized Ser473
kinase to the plasma membrane by their PH domains (Andjelković et al., 1997; Anderson et al., 1998). PKBα then becomes phosphorylated and activated by PDK1 (and the Ser473 kinase) on Thr308 and Ser473. After activation, PKBα detaches from the plasma membrane and translocates to the nucleus (Andjelković et al., 1997; Meier et al., 1997). The new results presented in this report support the concept that negative regulatory signal transduction pathways also operate to modulate PKB activity.

We demonstrate here that hyperosmotic stress (0.5 M sorbitol or 0.5 M NaCl), but not chemical stress (anisomycin), inactivates endogenous and overexpressed PKBα in three different cell lines. This is in contrast to previous studies by Konishi et al. (1996), who reported activation of PKB by hyperosmotic shock. The probable explanation for this difference is that they used a general substrate which could, under certain circumstances, be phosphorylated by co-precipitating protein kinases. To avoid this, we and others (Cross et al., 1995) took advantage of a specific peptide as a substrate for PKB. High osmolarity rapidly inactivates PKBα to a level of 20%. Similarly, it was reported that p70 S6K, a downstream effector of PDK1 and probably PKBα (Alessi et al., 1998; Pullen et al., 1998), becomes completely inactivated after the cells are treated with sorbitol (Chou and Blenis, 1996). The inhibitory effect of hyperosmotic stress on PKBα is selective, since JNK, a stress-activated protein kinase, and the classical MAP kinases (p42 and p44) remain unregulated in the presence of pervanadate and 0.5 M sorbitol. All tested stress conditions activated JNK, whereas only hyperosmotic stress inhibited PKBα, suggesting that the stress-activated protein kinases are not involved in the regulation of PKBα.

The measurement of PI 3-kinase activity in vitro and the in vivo levels of PI(3,4)P2 and PI(3,4,5)P3 revealed no inhibition of PI 3-kinase activity in response to 0.5 M sorbitol. Instead, sorbitol treatment resulted in increased formation of PI(3,4,5)P3 accompanied by a reduction in PI(3,4)P2 production, indicating that hyperosmotic stress may inhibit the conversion of PI(3,4,5)P3 to PI(3,4)P2 by a PH domain of PKBα, which is downregulated by sorbitol. Moreover, we showed that PKBα is dephosphorylated at Thr308 and Ser473 after treatment with sorbitol. Since PP2A dephosphorylates PKBα in vitro and calyculin A prevented inactivation of pervanadate-activated PKBα by hyperosmotic shock, we reasoned that hyperosmotic stress may also regulate PP2A targeting or activity. However, upon sorbitol treatment, we detected no direct binding of PP2A to PKBα or activation of PP2A, although pervanadate led to a significant decrease of PP2A activity. This result is similar to the observation of Srinivasan and Begum (1994) who showed a decrease in PP2A activity in rat skeletal muscle cells upon insulin stimulation. Further evidence that PP2A activity is not upregulated directly by high osmolarity comes from the fact that the MAP kinase pathway, which is sensitive to PP2A (Alessi et al., 1995), is not downregulated by osmotic shock. Furthermore, we were unable to find an effect of phospholipids on various forms of PP2A, suggesting that PI(3,4)P2, which is downregulated by sorbitol, does not act to inhibit PP2A.

The precise role of different 3-phosphoinositides in the regulation of PKBα is somewhat controversial. PI(3,4)P2, which is downregulated by sorbitol, has been shown to inhibit PKBα in vitro and in vivo (Franke et al., 1997; Frech et al., 1997; Klippel et al., 1997). Furthermore, PI(3,4,5)P3, which accumulates in response to hyperosmotic stress, does not activate but even inhibits PKBα activity in vitro (Franke et al., 1997; Frech et al., 1997; Klippel et al., 1997), and allows direct activation by PDK1 in vitro (Alessi et al., 1997; Stokoe et al., 1997). Consequently, PI(3,4,5)P3 could mediate translocation of PKBα but is not sufficient for its activation in the presence of hyperosmotic stress, because the latter process requires PI(3,4)P2 levels above a certain threshold. The requirement for PI(3,4)P2 production may be at the level of PKB or upstream kinases. However, the PH domain of PKB does not appear to mediate this process, as hyperosmotic stress resulted in a complete loss of APH-PKBα activity (data not shown). Although PDK1 activity appears not to be affected by hyperosmotic stress, we cannot exclude the possibility that its localization, as well as that of the Ser473 kinase, is sensitive to PI(3,4)P2.

It has been shown that PKBα activation is important for the suppression of apoptosis (Datta et al., 1997; Del Peso et al., 1997). Furthermore, inactivation of PKBα following hyperosmotic stress appears to be similar to inactivation of PKBα upon activation of PKBα by hyperosmotic stress, because the latter process requires PI(3,4)P2 levels above a certain threshold. The requirement for PI(3,4)P2 production may be at the level of PKB or upstream kinases. However, the PH domain of PKB does not appear to mediate this process, as hyperosmotic stress resulted in a complete loss of APH-PKBα activity (data not shown). Although PDK1 activity appears not to be affected by hyperosmotic stress, we cannot exclude the possibility that its localization, as well as that of the Ser473 kinase, is sensitive to PI(3,4)P2.

Following hyperosmotic stress, PKBα is inactivated rapidly accompanied by inhibition of PI(3,4)P2 production, whereas the stress kinase pathways (JNK, p38) are activated. Thus, hyperosmotic stress may promote apoptosis at two independent levels: first, through activation of the JNK pathway (Verheij et al., 1996) and, second, by inhibition of PKBα, a major promoter of cell survival (Kennedy et al., 1997) acting through phosphorylation of BAD on Ser136. Phosphorylation of BAD promotes its dissociation from Bcl-xL and its association with 14-3-3. Released Bcl-xL then suppresses cell death pathways such as the cytochrome c-activated caspase protease cascade (Datta et al., 1997; Del Peso et al., 1997; Hengartner, 1998). The elucidation of the inhibitory pathways leading to PKB inactivation will provide further insights into the control of apoptosis.

Materials and methods

Cell culture, transient transfection and stimulation conditions

Human embryonic kidney HEK-293, monkey kidney COS-1 and Swiss 3T3 mouse fibroblasts were maintained as described previously (Meier et al., 1997). Plasmid DNA (10 μg) was transiently transfected into HEK-293 cells using the calcium phosphate method (Chen and Okayama, 1988). For transfection of COS-1 cells, 3 μg of plasmid DNA was introduced using a DEAE–dextran method (Seed and Aruffo, 1987). After transfection, cells were serum starved for 24 h and then stimulated with 0.1 mM pervanadate (Andjelkovic et al., 1996), 10 μg/ml anisomycin (Sigma), 50 ng/ml PDGF-BB (Gibco-BRL) or combinations as described in the figure legends.
**Construction of expression vectors**
The cytomegalovirus (CMV)-based expression constructs encoding wild-type HA-PKBα, HA-3T3-D-PKBα, HA-S473D-PKBα and HA-T308D/S473D-PKBα have been described (Alessi et al., 1996). Human HA-P2Acot was cloned into the pCMV5 vector (D.Evans and B.A. Hemmings, unpublished).

**Immunoprecipitation and Western blotting**
Cells were lysed in lysis buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 40 μM NaF, 20 μM β-glycerophosphate, 20 μM β-glycerophosphate pH 7.4, 1 mM microcystin-LR (Biomol), 0.1 mM sodium orthovanadate, 1 mM benzamidine and 0.5 mM phenylmethylsulfon fluoride (PMSF). Extracts were centrifuged at 12 000 g for 15 min at 4°C prior to Western blotting or immunoprecipitation.

**Immunoblotting of p42 and p44 MAP kinases was carried out with the polyclonal antibody C16 (Santa Cruz Biotechnology).**

**Preparation of 32P-labelled α-PKBα**
HA-APK-PKBα and Myc-PDK1 (both bound to beads) were incubated at 30°C in kinase buffer (50 mM Tris–HCl pH 7.4, 20 mM β-glycerophosphate pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 0.03% Brij 35) with the presence of 10 mM MgCl2 and 50 μM ATP (sp. acc. 5000 c.p.m./nmol).

**References**


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Received June 25, 1998; revised September 29, 1998; accepted October 16, 1998.

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