cAMP stimulates protein kinase B in a Wortmannin-insensitive manner

Carol L. Sable[a], Nathalie Filippa[a], Brian Hemmings[b], Emmanuel Van Obberghen[a,*]

[a]*Institut National de la Santé et de la Recherche Médicale (INSERM) U 145, Faculté de Médecine, Avenue de Valombrose, 06107 Nice Cédex 2, France*

[b]*Friedrich Miescher Institute, CH 4002 Basel, Switzerland*

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Abstract Activation of protein kinase B (PKB) by growth factors has been demonstrated to proceed via phosphatidylinositol 3-kinase (PI3-kinase). Here, we show that agents which raise intracellular cAMP can also stimulate PKB. However, this effect is not sensitive to wortmannin, indicating that it is PI3-kinase independent. This activation does not appear to result from direct phosphorylation by protein kinase A (PKA) since GST–PKB is not an effective PKA substrate. In addition, the activation pathway of PKB by cAMP seems to be linked to that of growth factors, albeit downstream of PI3-kinase. Evidence for this is that a constitutive active PKB, T308D, S473D, containing activating mutations in the serine and threonine residues which are phosphorylated subsequent to PI3-kinase activation, cannot be further stimulated by cAMP elevations. Hence, these data suggest that, in addition to growth factors, cAMP can also lead to activation of PKB. This cAMP stimulatory action appears to require phosphorylation of T308 and S473, and hence would indicate that cAMP modulates the phosphorylation event of these PKB regulatory sites.

Key words: PKB; cAMP; PI3-kinase; Phosphorylation

1. Introduction

Protein kinase B (PKB), also referred to as akt or RAC kinase, is a serine/threonine protein kinase which was cloned by virtue of its homology to the A and C protein kinases, and is the cellular homolog of the product of the *v-akt* oncogene [1–3]. Structurally, the N-terminus contains a PH domain which is thought to be involved in the interaction of PKB with phospholipids [4–6], dimerization [7], and possibly interaction with other proteins [8]. The kinase domain is localized to the center of the protein. Regulatory sites for phosphorylation are found in the C-terminus [9]. The kinase is activated in response to treatment of cells with polypeptides acting through tyrosine kinase receptors such as PDGF, insulin, bFGF and EGF [4,10,11]. Stimulation of PKB in response to these agents is strictly dependent on the activity of PI3-kinase for the following reasons: (i) it is inhibitable by wortmannin [4,10–12], (ii) PDGF receptor mutants which cannot interact with PI3-kinase fail to activate PKB [4,11], and (iii) constitutively active forms of PI3-kinase are able to stimulate PKB [13,14]. Currently, a two-step model has been proposed to explain activation of PKB in response to growth factors [15,16]. Firstly, stimulation of PI3-kinase subsequent to engagement of growth factor receptors leads to the production of PtdIns-3,4,5-P_3, which can then be dephosphorylated by a specific phospholipid phosphatase to PtdIns-3,4-P_2. The latter product has been shown to bind to the PH domain of PKB and is thought both to localize it to membranes and to directly activate the kinase. Next, PKB is phosphorylated on threonine 308 and serine 473 by unidentified kinase(s) [9].

Several lines of evidence implicate PKB in cell growth; the product of the *v-akt* oncogene is capable of transforming cells [17], overexpression of PKB in ovarian carcinomas has been demonstrated [18], and recently, data linking PKB with protection from apoptosis has been accumulating. PKB can mediate the anti-apoptotic effects of IGF-1 in neuronal cells [19], Rat-1 and COS-7 cells [20,21]. In addition, constitutively active PKB results in the activation of $p70^{S6k}$, another protein implicated in cellular growth processes [11]. However, at present the only physiological substrate known for PKB is glycogen synthase kinase 3 (GSK3) [22]. Because, to date, GSK3 has not been implicated in any processes regulating cell growth, this implies that other substrates are likely to exist for PKB which are important in controlling these pathways.

Elevation in the intracellular cAMP level can have variable consequences on cellular proliferation depending on cell type and the nature of the activation [23]. In many cells, the effect of cAMP is to diminish cell growth and promote differentiation, and in certain conditions cAMP is antagonistic to the effect of growth factors. In many cases the inhibition of cell growth by cAMP is thought to result from inhibition of MAP kinase cascade, which is crucial to cell proliferation [24–29]. However, this is clearly not the only mechanism through which cAMP is exerting its antiproliferative effects, since several reports have demonstrated that it is possible to disassociate MAP kinase activity from cell proliferation after treatment of cells with cAMP [30–32]. In addition to the MAP kinase cascade, cAMP has also been reported to negatively regulate the activity of $p70^{S6k}$ [33] and $p27^{Kip1}$ [34], proteins which are implicated in the progression of the cell cycle [35]. Increased levels of cAMP have also been implicated in the promotion of differentiation, including the conversion of 3T3-L1 fibroblasts to adipocytes [36]. The overall picture from these studies is that in many cell types cAMP is inhibitory to cell growth. However, this does not hold true in all systems; in several cell types cAMP can mediate the action of growth promoting factors [37]. Moreover, the effects of cAMP on activation of $p70^{S6k}$ are not always inhibitory. In Swiss 3T3 cells cAMP is mitogenic and can activate $p70^{S6k}$ [31].

Because cAMP and PKB are both implicated in processes controlling growth and differentiation, we were interested in

*Corresponding author. Fax: (33) 4-93-81-54-32

Abbreviations: bFGF, basic fibroblast growth factor; CPT–cAMP, 8-(4-cholorophenylthio)-cyclic AMP; EGF, epidermal growth factor; GSK-3, glycogen synthase kinase-3; IGF-1, insulin-like growth factor-1; MAP kinase, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PGE_1, prostaglandin E_1; PH domain, pleckstrin homology domain; PI3-kinase, phosphatidylinositol 3′-kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKB-CA, protein kinase B constitutively active; $p70^{S6k}$, p70 ribosomal S6 protein kinase

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determining if cAMP exerted some of its effects through the modulation of PKB activation. To do this, we examined the effect of agents which can increase intracellular cAMP concentrations on the activity of PKB overexpressed in 293-EBNA cells. We found that cAMP-elevating agents can increase the activity of PKB through a mechanism which is independent of PI3-kinase. This activation does not appear to result from a direct phosphorylation of PKB by PKA, but it appears to converge on the same pathway which is utilized by growth factors.

2. Materials and methods

2.1. Materials

Culture media and Geneticin were from Life Technologies, Inc. (Gaithersburg, MD). All chemicals not otherwise noted were from Sigma (St. Louis, MO). Insulin was a kind gift from Novo-Nordisk (Copenhagen, Denmark). [γ-^{32}P]ATP was purchased from ICN (France). The QuickChange™ Site Directed Mutagenesis Kit was from Stratagene (LaJolla, CA). All oligonucleotides were from Eurogentec (Seraing, Belgium). The ^{T7}Sequencing™ Kit was from Pharmacia (Uppsala, Sweden) and Plasmid Maxi Plasmid Purification Kits were from Quiagen® (Chatsworth, CA). GST–PKB was a gift from Jean-Francois Tanti (INSERM U145).

2.2. Culture and transfection of 293-EBNA cells

293-EBNA cells are human embryo kidney cells that constitutively express the EBNA-1 protein from the Epstein Barr virus (Invitrogen, San Diego, CA). These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum and 500 μg/ml geneticin. Exponentially growing cells were trypsinized, seeded at 1.25×10^5 cells/well in 6-well tissue culture dishes (3.5-mm diameter), and incubated for 3 days in 2 ml of growth medium. One microgram of supercoiled DNA (PKB constructs in pECE) was mixed with 100 μl of 0.25 M $CaCl_2$ and 100 μl of 2×BES (buffered saline containing 50 mM *N,N*-bis-2-hydroxyethyl-2-aminoethane sulfonic acid, pH 6.95, 280 mM NaCl, and 1.5 mM Na_2HPO_4). The mixture was incubated for 30 min at room temperature before being added dropwise to the cells. After incubation for 15–18 h at 35°C under 3% CO_2, the cells were then removed to an incubator at 37°C, 5% CO_2 for 8 h before starvation in DMEM containing 0.2% (w/v) BSA for 14 h.

2.3. Immunoprecipitation and in vitro kinase assay

293-EBNA cell extracts were prepared by lysing cells in a buffer containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM vanadate, 100 mM NaF, 0.5 mM PMSF, 100 UI/ml aprotinin, 20 μM leupeptin, 1% (v/v) Triton X-100 for 15 min at 4°C. The lysates were clarified by centrifugation at $15\,000\times g$ for 15 min at 4°C, and immunoprecipitated using an anti-HA (12CA5) antibody coupled to protein G-sepharose. After washing of the immunocomplexes, kinase activity was assayed using Crosstide [22] as a substrate in a reaction mixture containing 50 mM Tris, 10 mM $MgCl_2$, 1 mM DTT, 5 μM ATP, 30μM Crosstide, and 3.3 μCi [γ-^{32}P]ATP. The phosphorylation reaction was allowed to proceed for 30 min at 30°C, then stopped by spotting 40 μl onto Whatman P81 filter papers, and immersing in 1% (v/v) orthophosphoric acid. The papers were washed several times, rinsed in ethanol, air-dried, and the radioactivity was determined by Cerenkov-counting. Background values obtained from a mixture lacking cell lysate were subtracted from all values.

2.4. Construction of constitutive active PKB

Constructs encoding HA-tagged PKB and ΔPH–PKB in the mammalian expression vector pECE were provided by Brian Hemmings, and have been described previously [12]. Mutations which changed both T308 and S473 to aspartate residues were made using the QuickChange Mutagenesis Kit. Mutations were confirmed by sequencing.

2.5. Phosphorylation of GST–PKB by PKA

GST–PKB was phosphorylated in vitro using 10 U of PKA catalytic subunit in a reaction mix containing 40 mM HEPES, pH 7.2, 20 mM $MgCl_2$, 100 mM ATP, and 10 μCi [γ-^{32}P]ATP. The reaction continued 10 min at room temperature before stopping in Laemmli buffer and subjecting to SDS-PAGE. Gels were stained, destained and autoradiography was performed. Radioactive bands were excised and quantified by Cerenkov counting.

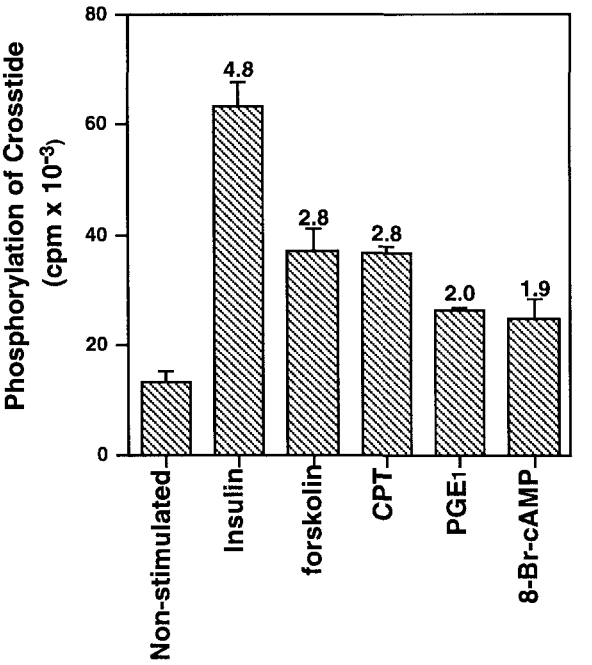

Fig. 1. Stimulating effect of agents raising intracellular cAMP on the activity of ΔPH-PKB in transfected 293-EBNA cells. Serum-starved 293-EBNA cells overexpressing ΔPH-PKB were incubated for 15 min with the various agents at the following concentrations: insulin, 1 μM; forskolin, 10 μM; CPT-cAMP, 1 mM; PGE_1, 2.5 μM; and 8-Br-cAMP, 1 mM. At the end of the incubation period, cells were lysed, and ΔPH-PKB was immunoprecipitated and kinase activity was determined using Crosstide as a substrate as described in Section 2. ΔPH-PKB activity is expressed as cpm of ^{32}P incorporated into Crosstide, and fold-stimulation of basal level of nonstimulated cells is indicated above each column. Data are means ± SD of a representative of two separate experiments, each performed in triplicate.

3. Results and discussion

3.1. Compounds that increase intracellular cAMP activate PKB

In an attempt to look for a role of the cAMP-dependent signaling pathway in the activation of PKB, we tested, in 293-EBNA cells transfected with ΔPH–PKB, the effect of compounds which are known to elevate intracellular cAMP levels. As can be seen in Fig. 1, all of the cAMP elevating agents led to a significant increase in the activity of ΔPH–PKB. Forskolin (10^{-5} M) and CPT–cAMP (1 mM) both increase the activity of PKB by 2.8-fold. Prostaglandin E_1 (2.5×10^{-6} M) and 8-bromo-cAMP (1 mM) were the least efficacious, leading to 2.0- and 1.9-fold increases in the activity with respect to nonstimulated cells. Although all the cAMP elevating compounds led to significant increases in PKB activity, none were as efficient as insulin, which increased the kinase activity of ΔPH–PKB by a factor of 4.8. These different agents increase cAMP by various mechanisms: forskolin directly activates adenylyl cyclase, PGE_1 binds to a G_s coupled receptor which leads to activation of adenylyl cyclase, and 8-bromo-cAMP and CPT–cAMP are cell permeable analogs of cAMP.

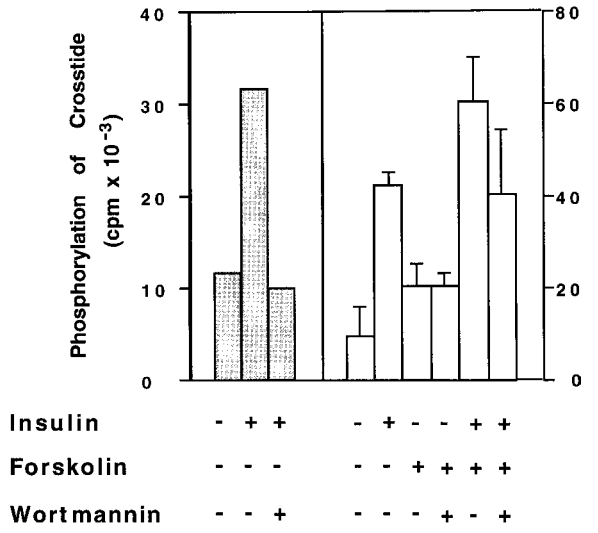

Fig. 2. Effect of pre-incubation with wortmannin on the kinase activity of PKB. 293-EBNA cells transfected with PKB were serum-starved prior to pre-incubation or not with 100 nM wortmannin. Subsequently, the cells were stimulated with either forskolin (10 μM, 15 min), insulin (1 μM, 5 min), or a combination of both. The cells were then lysed, PKB was immunoprecipitated and kinase assays were performed as described, and results are expressed as cpm of ^{32}P incorporated into Crosstide. Data are expressed as means ± SD of a representative of five comparable experiments, each performed in triplicate.

Since the only common element to all these different drugs is the ability to elevate cAMP, we conclude that cAMP can activate ΔPH–PKB in transfected 293-EBNA cells. Similar results were obtained with the wild-type PKB construct using forskolin as an agonist (data not shown).

3.2. *cAMP activates PKB in a PI3-kinase-independent mechanism*

Stimulation of PKB in response to growth factor receptors has been shown to be dependent on PI3-kinase activation, since this stimulation is completely inhibited by wortmannin. To attempt to determine if the mechanism of activation of PKB by cAMP-elevating agents is similar to that of growth-factor receptors, that is, dependent on PI3-kinase activity, we examined the effect of wortmannin on the cAMP-induced activation of PKB. As can be seen in Fig. 2, the activity of PKB induced by insulin in 293-EBNA cells transfected with a full-length construct of PKB is abolished by pretreatment of the cells with wortmannin. In contrast, the activity induced by forskolin was not significantly altered in the presence of wortmannin, indicating that the mechanism of activation of PKB by forskolin occurs by a PI3-kinase-independent mechanism. In addition, the PKB activity induced by the co-stimulation with insulin and forskolin was additive, and the component of the activity which was contributed by forskolin was not sensitive to wortmannin. Comparable results were obtained with cells transfected with ΔPH–PKB (data not shown). These data demonstrate that PKB can be activated by two separate pathways: one which is dependent on activity of PI3-kinase, and one which is not.

3.3. *Activation of PKB by cAMP producing agents does not appear to result from a direct phosphorylation of PKB by PKA*

The most direct mechanism through which cAMP-elevating agents could lead to activation of PKB would be by a direct phosphorylation of PKB by PKA. Indeed, PKB contains a putative site for phosphorylation by PKA at serine-422. Since this site lies between the two phosphorylation sites, which have been shown to be involved in regulating the activity of PKB, phosphorylation of this site by PKA could lead to a conformational change in PKB, resulting in an increased activity of PKB.

To determine whether direct phosphorylation of PKB by PKA could account for the effect of cAMP elevating agents on PKB activity, we looked at phosphorylation of a GST–PKB fusion protein by PKA catalytic subunit in vitro. Fig. 3 shows the results from these experiments where differing quantities of GST–PKB were phosphorylated by PKA. These data show that apparently PKB can serve as a substrate for PKA in vitro. In order to be considered a physiological substrate, the stoichiometry of phosphorylation must approach 1 mole of phosphate transferred per mole of protein. However, we calculated that only 0.0036 moles of phosphate are transferred per mole of GST–PKB. In addition, we were unable to demonstrate that PKB immunoprecipitated from over-expressing 293-EBNA cells can be phosphorylated by PKA (data not shown). Therefore, PKB is unlikely to serve as a direct physiological substrate for PKA. This implies that a protein(s) other than PKA are activated in response to elevation in intracellular cAMP which are involved in the PI3-kinase-independent activation of PKB.

3.4. *Effect of cAMP producing agents on the activation of various PKB mutants*

Activation of PKB has been proposed to proceed by a two-step mechanism. Initially, phospholipid products of PI3-kinase are thought to bind to the PH domain of PKB serving

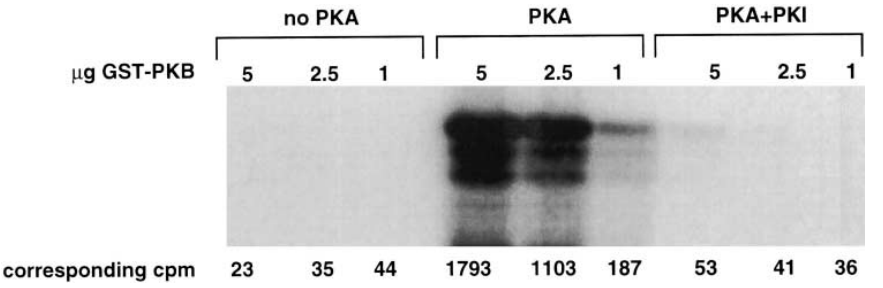

Fig. 3. Phosphorylation of GST-PKB by PKA. Indicated quantities of GST-PKB were incubated with 10 U of PKA catalytic subunit as noted, for 10 min as described in Section 2. Bands were excised and quantified by Cerenkov counting and cpm are noted below the corresponding bands.

to localize the kinase to the membrane. Once in the membrane, PKB is activated by phosphorylation at two residues in the C-terminus. To further ascertain the mechanism by which cAMP elevation leads to activation of PKB, we examined the effect of forskolin on the activation of different mutants of PKB. As was shown in Figs. 1 and 2, activation of a PKB mutant which lacks the N-terminal 106 amino acids, the region comprising the PH domain, is identical to that of the full-length PKB. This indicates that the PH domain is not necessary for activation of PKB by forskolin, and therefore indicates that binding of phospholipids to the PH domain of PKB is not essential for its activation.

Secondly, we examined the activation by forskolin of a mutant of PKB which contains an aspartate residue at threonine-308, and at serine-473. This mimics the effect of phosphorylation of these residues, and this mutant has been shown to be constitutively active [9]. If activation of PKB by cAMP converged on the same signaling pathway through which PKB is activated by insulin, except at a site which is downstream of PI3-kinase, it would be expected that stimulation of the cells with forskolin would have no effect on the activation of this mutant, which we have named PKB-CA However, in contrast, if cAMP activates PKB by a mechanism which is distinct from that of the phosphorylation of S473 and T308, then forskolin should be able to further activate the constitutively active PKB mutant. Fig. 4 shows the activation of PKB in cells overexpressing the constitutively active PKB. As can be seen, forskolin fails to lead to a further increase in the kinase activity of PKB-CA, indicating that the mechanism of activation of PKB by cAMP-elevating agents is similar to that which leads to phosphorylation at S473 and T308. This is also consistent with our data showing that PKB is not a direct substrate for PKA.

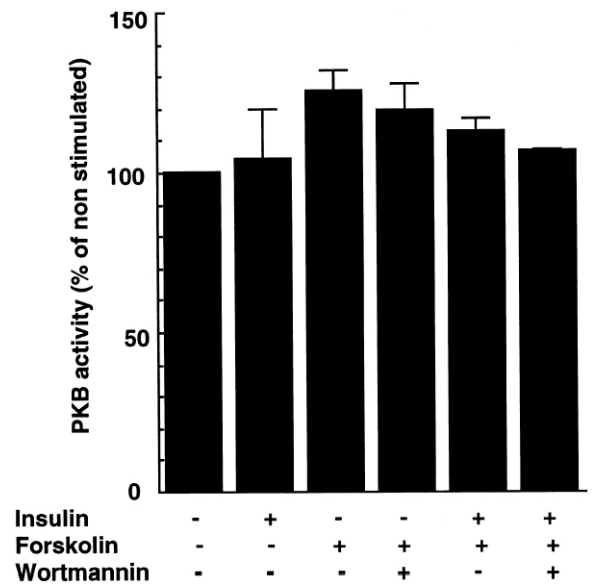

Fig. 4. Effect of forskolin on the kinase activity of a constitutively active mutant of PKB. 293-EBNA cells transfected with PKB-CA were serum-starved prior to stimulation with either forskolin (10 μM, 15 min), insulin (1 μM, 5 min), or a combination of both. The cells were then lysed, PKB was immunoprecipitated and kinase assays were performed as described, and results are expressed as % activity as compared to nonstimulated cells. Data are expressed as means ± SEM of two independent experiments each performed in triplicate.

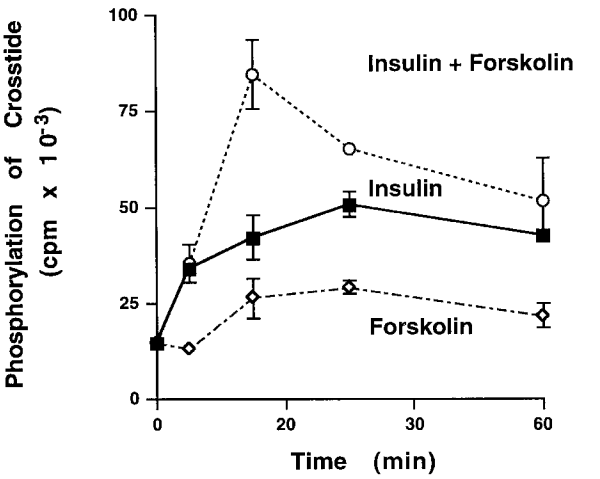

Fig. 5. Kinase activity of ΔPH–PKB with respect to time. 293-EBNA cells transfected with ΔPH–PKB were serum-starved prior to stimulation with either forskolin (10 μM, ◇) insulin (1 μM, ■), or the simultaneous addition of both (○). The cells were then lysed, ΔPH–PKB was immunoprecipitated and kinase assays were performed as described. Results are expressed as cpm of ^{32}P incorporated into Crosstide. Data represent means ± SD of two independent experiments each performed in triplicate.

3.5. Time course of activation ΔPH–PKB by forskolin

Recent data have demonstrated that, in some cell systems, the effects of cAMP-elevating agents can alter the time course but not the magnitude of the activation of MAP kinase by growth factors [30]. In an attempt to define the conditions which lead to a stimulation of PKB by cAMP elevation, we wished to examine the effect of forskolin on the activation of PKB over time. Shown in Fig. 5 is the activity of ΔPH–PKB as a function of time in response to insulin, forskolin, and insulin plus forskolin. As compared with reports in the literature of the time course of activation of the wild-type PKB which peaks 5 min after insulin stimulation, ΔPH–PKB activity seems to reach a maximum between 15 and 30 min. In addition, activation of ΔPH–PKB by cAMP elevating agents parallels that of the activation by insulin, and at all time points except 5 min, the activity induced by both insulin and cAMP elevating agents is close to additive. Therefore, the effect of cAMP on PKB activity does not vary as a function of time.

Data in this report show that, in addition to the described activation of PKB which is dependent on PI3-kinase, PKB can also be activated by increases in intracellular cAMP concentrations in a manner which is independent of PI3-kinase. However, this activation does not appear to be due to a direct phosphorylation of PKB by PKA, and appears to ultimately be related to the same mechanism as the one used by PI3-kinase, that is, phosphorylation of T308 and S473. Support of this is given by the fact that the constitutively active PKB, which contains mutations mimicking phosphorylation of T308 and S473 is unable to be further activated in response to cAMP. Previous reports have shown that PKB can be activated independently of PI3-kinase by cellular stress [8]. In contrast with the effect of cAMP elevation, this process required a functional PH domain, implying that the mechanisms are different or cell type specific.

Further experiments are required to determine the mechanism through which cAMP is activating PKB. We favor a

pathway which converges on that which is used by insulin at a point which is downstream of PI3-kinase. Since the activation of PKB by insulin in 293-EBNA is not maximal, if both cAMP and insulin resulted in activation of the same kinase(s) which phosphorylate T308 and S473, the effects would be additive. Of course, other potential mechanisms exist, for example, cAMP could be inhibiting a phosphatase which is responsible for dephosphorylating, and thereby inactivating, PKB. It has long been known that cAMP can inactivate protein phosphatase 1, due to the phosphorylation and activation of inhibitor-1 by PKA [38]. In addition, okadaic acid, an inhibitor of phosphatases 1 and 2A [39], is also a strong activator of PKB [12]. However, if cAMP was stabilizing PKB in an active form due to the inhibition of a phosphatase, it would be expected that the stimulatory effects of cAMP would be greater at later time points. Since this is not the case, we do not favor this mechanism.

In summary, we have shown that activation of PKB does not only proceed through PI3-kinase dependent signals, and multiple parallel, and/or convergent mechanisms appear to exist to lead to its activation. It will thus be of interest to determine physiologically the functional consequences of the interplay between these various different signal transduction pathways.

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