Protein Kinase Cζ Is a Negative Regulator of Protein Kinase B Activity*

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Robert P. Doornbos‡§, Marga Theelen‡§, Paul C. J. van der Hoeven‡, Wim J. van Blitterswijk‡, Arie J. Verkleij‡, and Paul M. P. van Bergen en Henegouwen‡∥

From the ‡Institute of Biomembranes, Department of Molecular Cell Biology, Utrecht University, 3584 CH Utrecht, The Netherlands and ¶The Netherlands Cancer Institute, Department of Cellular Biochemistry, 1066 CX Amsterdam, The Netherlands

Protein kinase B (PKB), also known as Akt or RAC-PK, is a serine/threonine kinase that can be activated by growth factors via phosphatidylinositol 3-kinase. In this article we show that PKCζ but not PKCa and PKCd can co-immunoprecipitate PKB from CHO cell lysates. Association of PKB with PKCζ was also found in COS-1 cells transiently expressing PKB and PKCζ, and moreover we found that this association is mediated by the AH domain of PKB. Stimulation of COS-1 cells with platelet-derived growth factor (PDGF) resulted in a decrease in the PKB-PKCζ interaction. The use of kinase-inactive mutants of both kinases revealed that dissociation of the complex depends upon PKB activity. Analysis of the activities of the interacting kinases showed that PDGF-induced activation of PKCζ was not affected by co-expression of PKB. However, both PDGF- and p110-CAAX-induced activation of PKB were significantly abolished in cells co-expressing PKCζ. In contrast, co-expression of a kinase-dead PKCζ mutant showed an increased induction of PKB activity upon PDGF treatment. Downstream signaling of PKB, such as the inhibition of glycolysis, synthase kinase-3, was also reduced by co-expression of PKCζ. A clear inhibitory effect of PKCζ was found on the constitutively active double PKB mutant (T308D/S473D). In summary, our results demonstrate that PKB interacts with PKCζ in vivo and that PKCζ acts as a negative regulator of PKB.

Protein kinase B (PKB),1 also referred to as c-Akt or RAC-PK, is a 60-kDa serine/threonine kinase which is the cellular homologue of the viral onco gene v-Akt (1–3). So far, three isofoms of PKB have been isolated: PKBa, PKBβ, and PKBγ (1, 2, 4, 5). Overexpression of PKB family members has been correlated with different cancers such as breast cancer and some pancreatic and ovarian cancers (2, 6, 7). Recently, PKB has been found on the constitutively active double PKB mutant (T308D/S473D). In summary, our results demonstrate that PKB interacts with PKCζ in vivo, and that PKCζ acts as a negative regulator of PKB.

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§ Contributed equally to the results of this work.
∥ To whom correspondence should be addressed: Institute of Biomembranes, Dept. of Molecular Cell Biology, Utrecht University, 3584 CH Utrecht, The Netherlands. Tel.: 31-30-2533349; Fax: 31-30-2513655; E-mail: bergen@bio.uu.nl.

1 The abbreviations used are: PKB, protein kinase B; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PI, phosphatidylinositol; GSK-3, glycogen synthase kinase-3; GS, glycogen synthase; PH, pleckstrin homology; AH, Akt homology; PDK, phosphatidylinositol-3,4,5-triphosphate-dependent protein kinase; PIP(3,4,5)P3, phosphatidylinositol-3,4,5-triphosphate; PIP3, phosphatidylinositol-3,4-biphosphate; PIP2, phosphatidylinositol-4,5-biphosphate; PIP, phosphatidylinositol; PDGF, platelet-derived growth factor; PI-3 kinase, phosphatidylinositol 3-kinase; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; Btk, Bruton tyrosine kinase.

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The activation of PKB by both PI(3,4)P₂ and PDK-1 and -2 makes the activation of PKB a multistep process. Initial studies by Konishi and co-workers (25, 26) showed that the α, δ, and ζ isoforms of PKC are able to interact with PKB in vitro. In this paper we show that PKB can only be co-immunoprecipitated with PKCζ and in addition we found that this interaction is under control of PKB activity. To understand the possible function of the PKB-PKCζ association, we investigated whether the interacting kinases regulate the activity of the respective kinases. Although no effect was found of PKB on PKCζ activity, both PDGF- and p110-CAAX-induced activation of PKB is abolished by co-expression of PKCζ. The activity of GSK-3, a downstream target of PKB is also affected by PKCζ co-expression. Finally, we found that the constitutive active PKB mutant (T308D/S473D) is inhibited by PKCζ in a PDGF-independent fashion. The results obtained establish PKCζ as a negative regulator of PKB activity.

MATERIALS AND METHODS

Expression Constructs—The pSG5 (Stratagene, La Jolla, CA) constructs containing HA-tagged wild-type bovine PKBs, PKBδ, “kinese dead” and PKBζ (gift from Dr. Paul Cofer, Department of Pulmonary Diseases, University Hospital Utrecht, The Netherlands). The DNA fragments encoding the AH domain of PKBζ (PKBζAH) and PKBζ lacking the AH domain (PKBζBAH) were amplified by polymerase chain reaction and subcloned as a BamHI/KpnI fragment into the eukaryotic expression vector pBK-CMV (Stratagene, La Jolla, CA) containing a HA epitope tag (pBK-HA). p110-CAAX, p110-BR16-CAAX (PLAP-CAAX), and the pMT2SM constructs containing Myc-tagged wild-type mouse PKCζ and Myc-tagged kinase-dead PKCζ have been described earlier (27, 30).

Cell Culture, Transfections, and Immunoprecipitations—COS-1 and CHO cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 7.5% fetal calf serum (Life Technologies, Inc.) at 37 °C in a humidified atmosphere with 5% CO2. Transient transfections in COS-1 cells were performed at 40% confluency by a DEAE-dextran method. In short, DNA was diluted in 500 μl of DEAE-dextran (Sigma) in phosphate-buffered saline and added to the cells. Following a 30-min incubation at 37 °C, medium containing 80 μM chloroquine (Sigma) was added and the cells were incubated for 2.5–3 h at 37 °C and subsequently shocked with 10% dimethyl sulfoxide (Sigma) for 2.5 min. Lysates were centrifuged and supernatants were precleared with dextran-coated Salmonella beads (New England Biolabs, Beverly, MA) polyvinylidene fluoride membranes (NEN Life Science Products Inc., Boston, MA). For quantification of protein amounts a densitometer (Molecular Dynamics) and ImageQuant software were used.

In Vitro Kinase Assays for PKCζ, PKB, and GSK-3—PKCζ activity was measured with the e-peptide (ERMPPDKQGVSVRRRRV) as substrate as described previously (27, 28). Immunoprecipitations were incubated with 45 μl of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂) containing 50 μM e-peptide, 0.2 mM EDTA, 50 μM unlabeled ATP, and 5 μCi of [γ-32P]ATP (Amer sham International, United Kingdom). PKB activity was assayed with the Crodistide peptide (GRRPRTSSVVRP) as substrate as described previously (29). Cell lysates were incubated with 60 μg of GS peptide, 2 μg of MgCl₂, 100 μM ATP, and 2 μCi of [γ-32P]ATP. After incubation for 20 min at 30 °C under continuous shaking, reactions were stopped by addition of 200 μl of EDTA. Proteins were precipitated by the addition of 25% trichloroacetic acid and centrifuged for 1 min at 14,000 rpm. Supernatants containing the phosphorylated peptide were spotted onto p81 phosphocellulose filters (Whatman), washed three times with 1% (w/v) orthophosphoric acid, and analyzed by Cerenkov counting. Control experiments revealed that phosphorylation of the GS peptide is highly specific for GSK-3β and that neither PKB nor PKCζ is able to phosphorylate the peptide. Under the conditions used the kinase assays are linear for at least 60 min.

RESULTS

PKCζ Associates with PKB in CHO Cells—In vitro binding studies have recently shown that PKB associates with the α, δ, and ζ isoforms of PKC (5). In order to investigate the possible interaction of these PKC isoforms with PKB in vivo, we performed co-immunoprecipitation studies using CHO cells. Endogenous PKCa, PKCζ, and PKCζ were immunoprecipitated fromCHO cell lysates and Western blot analysis shows that similar amounts of the three PKC isoforms were precipitated (Fig. 1B).

The presence of PKB was analyzed by Western blot detection and, as shown in Fig. 1A, PKB is present in the PKCζ but not in the PKCa and PKCζ immunoprecipitates. As a control, normal rabbit serum was incubated with lysates of CHO cells and only a faint band is visible possibly reflecting aspecific binding to the non-immune control (Fig. 1A).

The binding of PKB with PKCζ was subsequently investigated in more detail by transient expression of HA-tagged PKB and Myc-tagged PKCζ (Myc-PKCaζ) in COS-1 cells. HA-PKB was immunoprecipitated using a monoclonal antibody against the HA-tag (12CA5) and co-immunoprecipitation of Myc-PKCaζ was observed on Western blot using a monoclonal antibody against the Myc-tag (9E10) (Fig. 1C). To identify the domain of PKB that is necessary for the association with PKCζ in vivo, we generated HA-tagged PKB constructs lacking the AH domain (HA-PKBAHΔ) or comprising the AH domain (HA-PKBAAH). Co-expression of these constructs with PKCζ revealed that the interaction of PKB with PKCζ depends entirely on the presence of the AH domain. This observation is in agreement with the

2 R. P. Doornbos, unpublished observations.
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PKCζ associates with PKB in CHO cells. A, CHO cells were lysed and incubated with normal rabbit serum (non-immune) or antibodies against PKCα, PKCδ, and PKCζ as described under “Materials and Methods.” Immunoprecipitates and a cell lysate (lysatc) from CHO cells were analyzed for the presence of PKB by Western blot using the monoclonal PKB/Akt 12CA5 and the monoclonal Myc antibody 9E10 to analyze separated by SDS-PAGE and immunoblotted using both the monoclonal m

PKC Activity Is Required for Complex Dissociation—To investigate the effect of PDGF on the PKB-PKCζ complex, COS-1 cells were transiently co-transfected with both HA-PKB and Myc-PKCζ. The cells were serum-starved overnight and either left untreated or stimulated with 25 ng/ml PDGF for 10 min. PKB was immunoprecipitated and co-immunoprecipitation of PKCζ was determined by Western blot analysis (Fig. 2A). Upon PDGF treatment the interaction decreased with approximately 75% indicating that PDGF induces the dissociation of the complex.

As previously reported, PDGF induces the activation of PKB and, albeit to a lesser extent, also of PKCζ (27). In order to establish whether the activity of these kinases is involved in PKB-PKCζ complex formation, we analyzed the effect of kinase-dead mutants of both PKB and PKCζ. In repeated experiments expression of kinase-dead PKCζ resulted in a reduction of complex formation which, however, can be explained by the reduction in PKCζ expression (Fig. 2B). In contrast, expression of kinase-dead PKB resulted in a dramatic increase in the PKB-PKCζ interaction (Fig. 2A). This demonstrates that PKB activity induces the dissociation of the complex, whereas PKCζ activity seems not to be required for the regulation of the complex. As a control experiment, we incubated the same blot with anti-HA antibodies showing that similar amounts of HA-PKB were precipitated (Fig. 2B).

PKCζ Activity Is Not Affected by PKB in Vivo—In order to establish the physiological role for the PKB-PKCζ interaction we investigated the effect on the activity of both kinases. To test a possible role for PKB on PKCζ activity, COS-1 cells were transiently transfected with PKCζ alone or co-transfected with PKB. After stimulation of the cells with PDGF, PKCζ was immunoprecipitated and its activity was measured by an in vitro kinase assay using the ε-peptide as a substrate (28). PDGF stimulation resulted in an increase of PKCζ activity (Fig. 3) which is in agreement with previous studies (27). Co-expression of PKB did not affect activation of PKCζ upon PDGF treatment, demonstrating that PKB has no effect on the PDGF-induced activity of PKCζ (Fig. 3). To demonstrate that PKCζ and not PKB activity accounts for the observed change in ε-peptide phosphorylation we co-transfected PKCζ with kinase-dead PKB. Similar results were obtained as with wild-type PKB showing that PKB activity does not influence the observed change in ε-peptide phosphorylation (Fig. 3).

PKCζ Is a Negative Regulator of PKB Activity—Using the same approach, we investigated whether PKCζ has an effect on PKB activity. For these experiments, COS-1 cells were transiently transfected with wild-type PKB or co-transfected with wild-type PKB and either wild-type PKCζ or kinase-dead PKCζ. After PDGF treatment, PKB was immunoprecipitated and its activity was measured by an in vitro kinase assay using Crotostide as substrate (10). Activity measurements showed that PKB activity was increased more than 3-fold upon stimulation of the cells with PDGF (Fig. 4A). However, PDGF-induced PKB activation was almost completely abolished when PKB was co-expressed with wild-type PKCζ (Fig. 4A). This indicates that PKCζ is able to inhibit PDGF-induced activity of PKB. In contrast, PKB activity was increased more than 7-fold by PDGF when the cells were co-transfected with the kinase-dead mutant of PKCζ (Fig. 4A). These data clearly show that PKB activity is negatively regulated by PKCζ.

An important step in the full activation of PKB is the phosphorylation of residues Thr308 and Ser473 by PDK1 and -2 (21). To establish whether the inhibition of PKB activation by PKCζ is due to a reduced increase in the phosphorylation of PKB we used a polyclonal antibody against PKB when phosphorylated on Ser473. As shown in Fig. 5, A and C, PDGF treatment induced an significant increase (p < 0.05) in Ser473 phosho-
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Fig. 2. Regulation of the PKB-PKCζ complex. COS-1 cells were transiently transfected with either wild-type PKB and wild-type PKCζ, serum starved for 16 h and left untreated or stimulated with 25 ng/ml PDGF-BB for 10 min as described under “Materials and Methods” (A). Alternatively, COS-1 cells were transiently transfected with wild-type PKB and wild-type PKCζ, wild-type PKB and kinase-dead PKCζ, or kinase-dead PKB and wild-type PKCζ (B). PKB was immunoprecipitated from the lysates using the monoclonal HA antibody 12CA5 and subsequently separated by SDS-PAGE and immunoblotted onto polyvinylidene difluoride. Co-immunoprecipitation (IP) of PKCζ was analyzed using the monoclonal Myc antibody 9E10. The amount of immunoprecipitated PKB was analyzed using the monoclonal HA antibody 3F10. Expression levels of PKB and PKCζ were analyzed by Western blot detection using the monoclonal 3F10 and 9E10 antibodies, respectively (as described under “Materials and Methods”).

Fig. 3. PKCζ activity is not affected by PKB. COS-1 cells co-expressing wild-type PKCζ (wt), wild-type PKCζ, and wild-type PKB or wild-type PKCζ and kinase-dead (kd) PKB were left untreated (gray bars) or stimulated with 25 ng/ml PDGF-BB (black bars) for 10 min as described under “Materials and Methods.” PKCζ was immunoprecipitated from the lysates using the monoclonal Myc antibody 9E10 and its activity was assayed with the e-peptide as substrate (see “Materials and Methods”). The results are presented as ± S.E. for six determinations (three independent experiments) related to the activity of PKCζ in unstimulated cells (100%). Asterisks indicate p < 0.05 (Student’s t test).

together, from these experiments it can be concluded that PKCζ is a negative regulator of PDGF-induced PKB activity.

PKCζ Inhibits p110-CAAX-induced PKB Activity—As already mentioned, both PKB and PKCζ are activated by PDGF most probably through the PI 3-kinase signaling pathway. In order to find out whether the negative regulation of PKB by PKCζ is mediated by the PI 3-kinase/PKB signal transduction pathway we expressed a catalytically active membrane-targeted PI 3-kinase (p110-CAAX) together with PKB in COS-1 cells. p110-CAAX caused a significant, ligand-independent increase in PKB activity (Fig. 4A). In contrast, a catalytically inactive membrane-targeted PI 3-kinase (PLAP-CAAX) was unable to do so (Fig. 4A), which is in agreement with the work of Didichenko and co-workers (30). Co-expression of PKCζ with p110-CAAX and PKB reduced the p110-CAAX-induced PKB activity with almost 80% (Fig. 4A). Interestingly co-expression of PKCζ with PLAP-CAAX and PKB also resulted in a decrease in basal PKB activity (Fig. 4A). These observations demonstrate that basal activity of PKCζ is already sufficient to inhibit PKB.

GSK-3, a Downstream Target of PKB, Is Also Affected by PKCζ—It has previously been shown that GSK-3 is phosphorylated and inactivated by PKB in vitro and in vivo (10, 11). In order to test whether this downstream effector of PKB is also affected by co-expression of PKCζ, we measured GSK-3 activities in cells expressing PKB alone, PKB and PKCζ, and PKB and kinase-dead PKCζ using a peptide phosphorylation assay (29). As expected, upon treatment of cells with PDGF the GSK-3 activity is decreased (Fig. 4B). However, when PKB is co-expressed with PKCζ the PDGF-induced reduction in GSK-3 activity is completely overcome (Fig. 4B). In contrast, cells co-expressing a kinase inactive PKCζ mutant exhibits a normal reduction in GSK-3 activity upon PDGF treatment (Fig. 4B). In addition, similar results were obtained when PKB was activated via the constitutively activated PI 3-kinase. While expression of p110-CAAX induces the activation of PKB, a significant decrease was found in the GSK-3 activity. Conversely, the expression of the catalytically inactive PLAP-CAAX did not
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Fig. 4. PDGF-induced PKB signaling is negatively regulated by PKCζ. A, COS-1 cells overexpressing wild-type (wt) PKB, wild-type PKB and wild-type PKCζ, or wild-type PKB and kinase-dead (kd) PKCζ were serum starved for 16 h and left untreated (gray bars) or stimulated with 25 ng/ml PDGF-BB (black bars) for 10 min as described under “Materials and Methods.” PKB was immunoprecipitated from the lysates using the monoclonal HA antibody 12CA5 and its activity was assayed with Crosstide as substrate (see “Materials and Methods”). The results are presented as average ± S.E. for six determinations (three separate experiments) related to the activity of PKB in unstimulated cells overexpressing PKB (100%). Asterisks indicate p < 0.05 (Student’s t test). B, corresponding GSK-3 activity measurements. Total cell lysates of untreated and PDGF-stimulated cells were analyzed for GSK-3 activity using a peptide phosphorylation assay (see “Materials and Methods”). The results are presented as average ± S.E. for four determinations (two independent experiments) and PDGF-stimulated values were related to their own control (100%).

induce a reduction of GSK-3 activity (Fig. 6B). Expression of PKCζ completely abolished the p110-CAAX-induced decrease in GSK-3 activity, whereas co-expression of PKCζ with PLAP-CAAX did not affect GSK-3 at all (Fig. 6B). These results clearly show that both PDGF- and p110-CAAX-induced PKB activity result in a decrease in GSK-3 activity. Furthermore, these experiments demonstrate that the inhibitory effect of PKCζ on PKB activity is also reflected in the GSK-3 activity.

PKCζ Acts Directly on PKB to Inhibit Its Activity—The question that remains is where PKCζ exactly affects the PKB signaling pathway. To find out whether PKCζ might act on PKB itself, we expressed an active PKB mutant (PKBDD) in which the two phosphorylation sites (Thr308 and Ser473) that are necessary for complete activation are replaced by aspartic acid resulting in a constitutively active form of PKB (21). This mutant exhibits high activity already in resting cells and as expected, PDGF treatment did not increase the activity any further (Fig. 7). Co-expression of PKCζ resulted in a reduction of the ligand-independent activity of the PKBDD mutant (Fig. 7). PDGF treatment did not contribute to this inhibitory effect since no difference could be observed between untreated and PDGF-stimulated conditions (Fig. 7). As a control, a PKB mutant (PKBΔA) in which the two phosphorylation sites are mutated into alanine was expressed and its activity was measured. Very little activity was detected for this mutant and neither PDGF treatment nor PKCζ co-expression changed its activity any further (Fig. 7). Together, these observations show that PKCζ does not act upstream of PKB in the PI 3-kinase signaling pathway to inhibit its activity but strongly suggest that PKCζ acts at the level of PKB kinase causing the reduction of its activity. In addition, these data show that PI 3-kinase is not necessarily required for the inhibitory effect of PKB by PKCζ.

DISCUSSION

In this report we show that PKCζ binds in vivo to the serine/threonine kinase PKB (c-Akt, RAC-PK). The PKB-PKCζ interaction was demonstrated by co-immunoprecipitation studies of both endogenous and transiently expressed PKCζ and PKB proteins. In contrast, we were not able to detect association of PKB with endogenous PKCζ and PKCζ in vitro in CHO cells. This latter observation is in disagreement with binding studies of Konishi and co-workers (5). These studies, however, were performed by in vitro binding studies using the PH domain of PKB fused to GST and lysates of COS-7 cells containing transiently expressed PKCζ isoforms. In addition, the PKCζ associ-
PKCζ has only been demonstrated in heat-treated cells (16). These results suggest that the affinity of PKB for PKCζ is higher than for PKCα or PKCδ. Although we were not able to detect the association of PKB with PKCα or PKBδ by co-immunoprecipitation studies we cannot exclude the association of these kinases in the in vivo situation. The association between PKB and PKCζ was observed in both serum-starved and PDGF-stimulated cells. Stimulation of the cells with PDGF resulted in a reduction in complex formation. An important question is how the association between PKB and PKCζ is regulated.

As shown in this paper, the PKB-PKCζ interaction is mediated by the AH domain of PKB. This was concluded from the observation that PKCζ co-precipitated with the AH domain while no co-precipitation was observed between PKCζ and the kinase-tail domain of PKB (PKBΔD). The AH domain of PKB largely consists of a PH domain, which has previously been identified as a lipid-binding domain (31). PH domains, however, including the PH domain of PKB, have also been described to bind to proteins such as PKC and the βγ subunit of the heterotrimeric G-protein (5, 32). The βγ subunit has been shown to bind to the carboxyl-terminal α-helix region of the βARK PH domain (33). In contrast, different isoforms of PKC including the Ca2+-dependent (α, βI, and βII) and Ca2+-independent (ε and η) isoforms have been shown to interact with the second and third β-sheet of the PH domain of the tyrosine kinase Bruton tyrosine kinase (Btk) (34, 35). PKCζ has been shown to bind to the first and second β-sheet of PKB (26). The β-sheets are part of the binding pocket of the PH domain for phosphoinositides. This raises the possibility that the PKB-PKCζ interaction is regulated by the PDGF-induced generation of D3-phosphoinositide lipids as they may compete with PKCζ for binding to the PKB-AH domain. This mechanism was implicated for the binding of PKCβII to the PH domain of Btk (35). Alternatively, PKB itself may regulate the dissociation of the

![Fig. 6. p110-CAAX induced PKB signaling is totally abolished by PKCζ. A, COS-1 cells overexpressing wild-type PKB, wild-type PKB together with either p110-CAAX or PLAP-CAAX in the presence or absence of PKCζ were serum starved for 16 h and left untreated. PKB was immunoprecipitated from the lysates using the monoclonal HA antibody 12CA5 and its activity was assayed with Crosstide as substrate (see “Materials and Methods”). Results are presented as average ± S.E. for four determinations (two independent experiments) related to the activity of PKB in unstimulated cells overexpressing PKB (100%). Asterisks indicate p < 0.05 (Student’s t test). The amount of immunoprecipitated PKB was analyzed using Western blot detection (as described under “Materials and Methods”). B, corresponding GSK-3 activity measurements. Total cell lysates were analyzed for GSK-3 activity using a peptide phosphorylation assay (see “Materials and Methods”). The results are presented as average ± S.E. for four determinations (two independent experiments) related to the GSK-3 value of mock-transfected cells (100%). The expression levels of PKB were analyzed by Western blotting of total cell lysates (as described under “Materials and Methods”).](image)

![Fig. 7. Constitutively active PKB is inhibited by PKCζ. A, COS-1 cells overexpressing wild-type (wt) PKB, constitutively active PKBΔD, PKBδ, and PKCζ, constitutively inactive PKB⁺⁺ or PKB⁺⁺ and PKCζ were serum starved for 16 h and either left untreated or stimulated with 25 ng/ml PDGF-BB for 10 min. PKB was immunoprecipitated from the lysates using the monoclonal HA antibody 12CA5 and its activity was assayed with Crosstide as substrate (see “Materials and Methods”). Results are presented as average ± S.E. for four determinations (two independent experiments) related to the activity of PKB in unstimulated cells overexpressing PKB (100%). Asterisks indicate p < 0.05 (Student’s t test).](image)
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complex. A huge increase was observed in the binding of PKCζ to a kinase-dead PKB mutant while no difference was found in the association with a PKCζ kinase-dead mutant. This indicates that PKB activity is very important for dissociation of the complex. The most straightforward explanation for this effect is that PKCζ is a substrate for PKB. In this situation the phosphorylation of PKCζ by PKB after stimulation of the cell with a growth factor would result in the dissociation of the complex. Alternatively, it is equally possible that another unknown protein in the complex, which is a substrate for PKB, is mediating this interaction. Current research is aimed at the determination of the role of inositol lipids and PKB substrates in complex formation between PKB and PKCζ.

An important observation of this study is the inhibitory effect of PKCζ on the PDGF- and p110-CAAX-induced activation of PKB. This effect is both measured on PKB activity and on the phosphorylation of serine 473 of PKB. Also the downstream signaling to GSK-3 is inhibited by PKCζ. These findings can be explained either by the inhibition of the upstream signaling of PKB or by a direct effect of PKCζ on PKB itself. Our results do not support the possibility that PKCζ inhibits PI 3-kinase, since PKCζ was shown to inhibit the constitutive active PKB mutant (PKBDD) even without stimulation of the cell with a growth factor. The PKBDD mutant is no longer a substrate for the PDK-1 and -2 kinases and is active without stimulation of the cell by growth factors. A possible PI 3-kinase independent mechanism for inhibition of PKB by PKCζ could be that PKCζ activates PP2A, the serine/threonine-specific phosphatase. PP2A activity has been described to inhibit PKB activity (15). However, the fact that PKCζ inhibits the activity of the PKBDD mutant that cannot be dephosphorylated does not favor this model. In conclusion, our data strongly suggest that the inhibiting activity of PKCζ is expressed directly on PKB.

The question, however, remains how PKCζ negatively regulates PKB activity. One of the first steps in the activation process of PKB is the translocation of PKB to the membrane. This process could be sensitive to PKCζ activity. Our data show that PKCζ inhibits the constitutively active PKB mutant already in quiescent cells, making this possibility unlikely. On the other hand, we have observed that a kinase-dead PKCζ normally binds to PKB but fails to inhibit PKB activity. This suggests that PKB is inhibited by phosphorylation rather than binding. An obvious model is that PKB is a direct substrate of PKCζ and that phosphorylation of PKB results in the inhibition of enzyme activity. A similar mechanism has been found for the regulation of Btk activity by PKC. Btk has been shown to bind to different PKC isoforms and they inhibit Btk autophosphorylation by direct phosphorylation (34). Preliminary experiments in our laboratory indeed show that there is a PKCζ-dependent phosphorylation of PKB in vitro when PKB was immunoprecipitated from cells expressing both PKB and PKCζ. More research is required to completely understand the mechanism by which PKCζ is inhibited by PKCζ.

Recently, PDK-1 has been described as the kinase that is responsible for the PI 3-kinase-dependent phosphorylation and activation of PKCζ (36, 37). PDK-1 was also found to associate with PKCζ in unstimulated cells (36, 37). This, together with our observation that PKB associates with PKCζ in quiescent cells, suggests that PDK-1 can form complexes with both PKB and PKCζ. This is an intriguing situation given the fact that PDK-1 stimulates the activation of PKB by direct phosphorylation and induces the inactivation of PKB via PKCζ. As shown in this paper, the dissociation of the PKB-PKCζ complex depends upon PKB activity, suggesting that the binding of the inositol lipids PI(3,4)P2 and PI(3,4,5)P3 to PKB results in the partial activation of PKB and subsequently in the dissociation of the complex. The same lipids stimulate PDK-1 resulting in the activation of PKCζ and the subsequent inactivation of PKB. On the other hand, the fraction of PKB that is not in complex with PKCζ can become completely activated also by PDK-1. This implies that both activity states of PKB, the inactive (PKB in complex with PKCζ) and the active state (PKB associated to the membrane), may be under the control of PKD-1 activity.

The link between PKCζ and the PKB pathway also has implications for the downstream effectors of PKB. As shown in this paper, the decrease in PKB activity by PKCζ leads to changes in GSK-3 activity. Furthermore, it has recently been shown that C2-ceramides inhibit PKB/Akt activity and induce apoptosis through an unknown mechanism (38, 39). In addition, C2-ceramides have also been shown to decrease glucose uptake through inhibition of PKB activity (40). Since ceramides have been described to activate atypical PKCζ (41), it is tempting to speculate that these effects are mediated by PKCζ. In addition, PKCζ has been described to regulate MAPK activity through Raf-1 (42). So, it seems that PKCζ may regulate the activity of different proteins from different signaling pathways.

In summary, we have shown that PKCζ associates in vivo with PKB through its AH domain. Both PDGF- and p110-CAAX-induced activation of PKB was inhibited by co-expression of PKCζ, which was also reflected in GSK-3 activity. Our data demonstrate that PKCζ negatively regulates PKB signaling, an effect that is regulated by direct action on PKB.

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