Inhibition of growth-factor-induced phosphorylation and activation of protein kinase B/Akt by atypical protein kinase C in breast cancer cells

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The protein kinase B/Akt serine/threonine kinase, located downstream of phosphoinositide 3-kinase (PI-3K), is a major regulator of cellular survival and proliferation. Atypical protein kinase C (aPKC) family members are activated by PI-3K and also contribute to cell proliferation, suggesting that Akt and aPKC might interact to activate signalling through the PI-3K cascade. Here we demonstrate that blocking PKC activity in MDA-MB-468 breast cancer cells increased the phosphorylation and activity of Akt. Functional PI-3K was required for the PKC inhibitors to increase Akt phosphorylation and activation, potentially owing to the activation of specific PKC isoforms by PI-3K. The concentration dependence of the action of the PKC inhibitors implicates aPKC in the inhibition of Akt phosphorylation and activity. In support of a role for aPKC in the regulation of Akt, Akt and PKC ζ or PKC λ/ι were readily co-precipitated

from the BT-549 breast cancer cell line. Furthermore, the overexpression of PKC ζ inhibited growth-factor-induced increases in Akt phosphorylation and activity. Thus PKC ζ associates physically with Akt and decreases Akt phosphorylation and enzyme activity. The effects of PKC on Akt were transmitted through the PI-3K cascade as indicated by changes in p70 s6 kinase (p70^{s6k}) phosphorylation. Thus PKC ζ , and potentially other PKC isoenzymes, regulate growth-factor-mediated Akt phosphorylation and activation, which is consistent with a generalized role for PKC ζ in limiting growth factor signalling through the PI-3K/Akt pathway.

Key words: atypical PKC, epidermal growth factor, phosphoinositide 3-kinase, PKC inhibitor, p70^{s6k}.

INTRODUCTION

Three mammalian isoforms of protein kinase B (PKB)/Akt have been identified, termed PKB α , PKB β and PKB γ , or Akt1, Akt2 and Akt3. Akt1 and Akt2 are widely expressed in various tissues, whereas the expression of Akt3 is more restricted [1]. Akt regulates multiple biological functions including protein synthesis, apoptosis and glycogenesis [2,3]. The amplification of multiple components of the phosphoinositide 3-kinase (PI-3K) signalling cascade, including the catalytic subunit of PI-3K and Akt2 and mutational inactivation of the PTEN multifunctional phosphatase in human carcinomas, is in accordance with the importance of Akt and PI-3K in tumorigenesis [1].

The activation of Akt by growth factors is mediated, at least in part, by phosphorylation of Thr-308 in the catalytic domain and Ser-473 at the C-terminus. In many systems the phosphorylation of both Akt sites is blocked by pretreatment of the cells with the PI-3K inhibitors wortmannin or LY294002, indicating that they are phosphorylated as a consequence of PI-3K signalling [4,5]. PtdIns(3,4,5)P₃-dependent protein kinase 1 (PDK1) specifically phosphorylates Thr-308, whereas PDK-2 phosphorylates Ser-473. Ilk, PDK1 or autophosphorylation could account for the phosphorylation of Ser-473 [6–8]. PI-3K, by the generation of 3-phosphorylated PtdIns(3,4,5)P₃ and subsequent conversion into PtdIns(3,4)P₂ by SHIP, initiates a kinase cascade converging on Akt. In addition, heat shock and the activation of adenylate cyclase can modulate Akt activity by PI-3K-independent mechanisms [9]. A recent report showed that

protein kinase C ζ (PKC ζ) might negatively regulate Akt activity in a PI-3K-independent manner [10]. Thus both PI-3K-dependent and PI-3K-independent pathways might contribute to the regulation of Akt, depending on the ligand and the cell lineage analysed.

PKC is a family of structurally related serine/threonine protein kinases. The mammalian PKC isotypes have been grouped into three subfamilies, namely classical PKCs (cPKCs), novel PKCs (nPKCs) and atypical PKCs (aPKCs), on the basis of their structural and regulatory properties. PI-3K regulates aPKC, potentially through the activation of PDK1 by 3-phosphorylated phosphatidylinositols [11]. The multiple mechanisms of activation of PKC might account for the pleiomorphism and diversity of the cellular activities in which PKC has been implicated. It is also likely that specific PKC isoenzymes execute distinct cellular functions, including the regulation of anchorage-dependent and anchorage-independent growth, alterations in morphology and tumorigenicity [12–14]. The aPKC isotypes have been shown to be critically involved in cell proliferation and survival [15,16].

Here we demonstrate that PKC ζ and, to a smaller extent PKC λ/ι , physically and functionally interact with Akt. This interaction is associated with an inhibition of activation-dependent phosphorylation of Akt at both Ser-473 and Thr-308. This translates into a decrease in Akt activity and a subsequent decrease in phosphorylation at Thr-389 in p70 s6 kinase (p70^{s6k}) in intact cells. This process is PI-3K-dependent, probably as a consequence of PI-3K-dependent activation of PKC ζ . Thus the

Abbreviations used: aPKC, atypical PKC; cPKC, classical PKC; dn, dominant-negative; EGF, epidermal growth factor; HRP, horseradish peroxidase; LPA, lysophosphatidic acid; mAb, monoclonal antibody; nPKC, novel PKC; p70^{s6k}, p70 s6 kinase; PDK1, PtdIns(3,4,5)P₃-dependent protein kinase 1; PI-3K, phosphoinositide 3-kinase; PKC, protein kinase C.

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activation of PKC ζ through PI-3K serves to limit signalling through the PI-3K-Akt signalling cascade.

EXPERIMENTAL

Antibodies and reagents

Rabbit anti-PKC δ , anti-PKC ϵ , anti-PKC η , anti-PKC ι , anti-PKC ζ and mouse anti-PKC (MC5) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-(phospho-Akt), anti-(phospho-extracellular-signal-regulated protein kinase) and anti-(phospho-p70^{S6k}) antibodies were purchased from New England Biolabs (Beverly, MA, U.S.A.). Monoclonal anti-HA was a gift from Dr Bing Su (University of Texas M. D. Anderson Cancer Center, Houston, TX, U.S.A.). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were purchased from Bio-Rad (Hercules, CA, U.S.A.). HRP-conjugated Protein A was obtained from Amersham (Arlington Heights, IL, U.S.A.). Protein-A-conjugated Sepharose 4B was purchased from Pharmacia Biotech (Piscataway, NJ, U.S.A.). Ro-31-8220, GF109203X and LY294002 were purchased from Calbiochem (La Jolla, CA, U.S.A.). Wortmannin and monoclonal anti-(epidermal growth factor receptor) antibody were obtained from Sigma (St Louis, MO, U.S.A.). HA-epitope tagged wild-type Akt was a gift from Dr Julian Downward (London, U.K.). cDNA plasmids of PKC ζ and PKC λ/ι were purchased from Invitrogen (Carlsbad, CA, U.S.A.). For the construction of a kinase-deficient dominant-negative (dn) PKC ζ K275W mutant, site-directed mutagenesis of the full-length PKC ζ cDNA was performed as described previously [17]. 1-Oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (lysophosphatidic acid) 18:1 (LPA 18:1) was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.).

Cell lines

Human breast cancer cell lines MDA-MB-468 (University of Texas M. D. Anderson Cancer Center, Houston, TX, U.S.A.) and BT-549 from American Type Culture Collection (Manassas, VA, U.S.A.) were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) containing 1% (w/v) penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco) and 10% (v/v) fetal bovine serum (Sigma) at 37 °C in a humidified atmosphere.

Transient transfection

Cells were transiently transfected with various combinations of the expression vectors by FugeneTM 6 Transfection Reagent (Boehringer Mannheim Inc., Indianapolis, IN, U.S.A.) as recommended by the manufacturer.

Cell lysis, immunoprecipitation and immunoblotting

After transfection, cells were serum-starved overnight before stimulation with epidermal growth factor (EGF) (20 ng/ml) or LPA (5 μ M) for 10 min. Inhibition of PKC activity was performed by treatment of the cells with different doses of Ro-31-8220 or GF109203X for 1 h before cell harvest. PI-3K activity was inhibited by pretreatment of the cells with LY294002 or wortmannin for 1 h before stimulation. Cells were washed twice with cold PBS and lysed in ice-cold lysis buffer [50 mM Hepes (pH 7.4)/150 mM NaCl/1 mM EGTA/100 mM NaF/1.5 mM MgCl₂/10 mM sodium pyrophosphate/1% (v/v) Triton X-100/1 mM Na₃VO₄/10% (v/v) glycerol/1 mM PMSF/10 μ g/ml aprotinin]. Cellular protein concentration was determined by

bicinchoninic acid reaction (Pierce, Rockford, IL, U.S.A.). For immunoprecipitation, detergent lysates were incubated with 1 μ g of anti-HA mAb (monoclonal antibody) at 4 °C for 2 h. Immune complexes were captured by Protein A-Sepharose beads. Immunoprecipitates were washed with IP wash buffer [0.5% (v/v) Triton X-100/0.5% (v/v) Nonidet P40/10 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 mM EDTA/1 mM EGTA/1 mM Na₃VO₄/1 mM PMSF]. Proteins were separated by SDS/PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA, U.S.A.). Membranes were blocked with 5% (w/v) BSA for 1 h and then incubated for 2 h at room temperature or overnight at 4 °C with anti-(phospho-Akt) (1:1000 dilution), total Akt antibody (1:1000 dilution), anti-(phospho-p70^{S6k}) (1:1000 dilution) or PKC isoform antibodies (0.5 μ g/ml). Membranes were washed in TBS-T buffer [10 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.1% (v/v) Tween 20] and incubated with HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:2500 dilution) for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence (ECL[®]; Amersham).

Akt kinase activity

Cells were lysed in 1% (v/v) Nonidet P40 lysis buffer. Cell lysates, normalized for protein levels (bicinchoninic acid assay), were immunoprecipitated with anti-(total Akt) and Protein A-Sepharose. Akt kinase activity was determined as described [4].

RESULTS

Expression of PKC isoforms in breast cancer cell lines MDA-MB-468 and BT-549

To investigate a potential role of PKC in regulating the PI-3K signalling pathway in breast cancer cells, we first assessed the expression of different PKC isoforms in MDA-MB-468 and BT-549 breast cancer cell lines. Both cell lines expressed the δ , ϵ , η , ζ and λ/ι isoforms of PKC, with MDA-MB-468 cells having higher levels of each of these isoforms than BT-549 cells (Figure 1). Furthermore, the mAb MC-5, which recognizes all cPKC isoenzymes, failed to detect cPKC expression in MDA-MB-468 total cell lysates. Ready detection of cPKC in BT-549 cells suggests that MDA-MB-468 cells express low to absent levels of conventional PKC isoforms.

Inhibition of aPKC increases Akt phosphorylation and activity

To explore the regulatory effects of PKC on Akt, we assessed the effect of the PKC inhibitor Ro-31-8220 on Akt phosphorylation in MDA-MB-468 breast cancer cells, given that MDA-MB-468 cells expressed nPKCs and aPKCs but lacked substantive expression of cPKCs (Figure 1). Ro-31-8220 is a potent PKC inhibitor, binding to PKC isoforms competitively with ATP [18]. As shown in Figure 2, Ro-31-8220 at 1–6 μ M significantly increased both Ser-473 and Thr-308 phosphorylation of Akt, reaching maximum effects at 6 μ M. In contrast, the expression level of Akt was not altered by Ro-31-8220. As predicted by the increase in Ser-473 and Thr-308 phosphorylation of Akt, Ro-31-8220 enhanced Akt enzyme activity as assessed in kinase assays *in vitro* with histone 2B as substrate.

Because Ro-31-8220 demonstrates little selectivity for specific PKC isoforms [18] and might inhibit other kinases, this result raised the question of which subtype(s) of PKC could contribute to the regulation of Akt activity. As little or no cPKC is expressed in MDA-MB-468 cells, the effect of Ro-31-8220 on Akt phosphorylation and activation (Figure 2) is unlikely to be mediated by cPKCs.

Figure 1 Expression of PKC isoforms in BT-549 and MDA-MB-468 breast cancer cell lines

Cells were cultured and harvested as described in the Experimental section. Cells were lysed; proteins were then separated by SDS/PAGE and immunoblotted with mAb MC-5 (against cPKCs) or polyclonal rabbit anti-PKC δ , anti-PKC ϵ , anti-PKC η , anti-PKC ζ or anti-PKC λ/ι antibodies. The same membranes were stripped and reprobed with anti-(β -actin) mAb to confirm equal loading of proteins. The immunoblots were detected with enhanced chemiluminescence reagents. The results shown are representative of three independent experiments.

Figure 2 Increase in Akt phosphorylation and activation by Ro-31-8220 in a dose-dependent manner

MDA-MB-468 cells were starved overnight, then treated with the indicated amounts of Ro-31-8220 or vehicle [0.1% (v/v) DMSO] for 1 h. Cells were lysed and the samples were processed for Western blotting with anti-(phospho-Ser-473) or anti-(phospho-Thr-308) antibodies. The same membranes were stripped and re-probed with anti-Akt antibody to detect the expression levels of Akt. For assessment of Akt kinase activity, cells treated with Ro-31-8220 were lysed and Akt kinase activity was determined as described in the Experimental section. The assay mixture was separated by SDS/PAGE [12% (w/v) gel] and 32 P-labelled product was detected by autoradiography. Results were quantified by densitometry and the value from untreated cells was taken as unity. The results shown are representative of at least four independent experiments.

To gain further insight into the relative role of aPKCs and nPKCs in Akt regulation, we used GF109203X, which at a concentration of 0.2 μ M is sufficient to block approx. 90% of the kinase activity of cPKC and nPKC isoenzymes, and for aPKCs its IC_{50} is 6–10 μ M [19,20]. Incubation of MDA-MB-468 cells with GF109203X at 0.1, 1, 2, 5 and 10 μ M increased Akt phosphorylation at Thr-308 by 3.2-fold, 3.7-fold, 6.7-fold, 7.3-

Figure 3 GF109203X increases Akt phosphorylation

For dose-response studies, serum-starved MDA-MB-468 cells were incubated for 1 h with the indicated amounts of GF109203X. Cells were then lysed; proteins were then separated by SDS/PAGE and immunoblotted with anti-(phospho-Thr-308) (top panel) or anti-(phospho-Ser-473) (middle panel) antibodies. The membranes were stripped and reprobed with anti-Akt to confirm equal expression levels of Akt (bottom panel). Results were quantified by densitometry and the value from untreated cells was taken as unity. The results shown are representative of two independent experiments.

fold and 12.6-fold respectively; similar results were seen with Ser-473 (Figure 3). These results are most compatible with aPKCs' being major negative regulators of Akt. However, the concentration dependence of GF109203X also suggests that nPKC might contribute to the regulation of Akt phosphorylation and activity.

Activation of Akt by Ro-31-8220 is PI-3K-dependent

To determine whether activation of Akt by Ro-31-8220 was dependent on PI-3K activity, we pretreated MDA-MB-468 cells with PI-3K inhibitors LY294002 (1 μ M) or wortmannin (10 nM and 100 nM) for 1 h before incubation of the cells with Ro-31-8220 (4 μ M, 1 h). Western blot analysis with anti-(phospho-Ser-473) or anti-(phospho-Thr-308) antibodies demonstrated that inhibition of PI-3K activity inhibited Ro-31-8220-induced Akt phosphorylation and decreased basal Akt phosphorylation (Figure 4). Similar results were obtained when Akt enzyme activity was assessed (Figure 4). In contrast, pretreatment with rapamycin did not affect basal and Ro-31-8220-induced Akt phosphorylation and activation (Figure 4, lower panel), ruling out the involvement of the FRAP/mTOR (mammalian target of rapamycin) phosphatidylinositol kinase-related kinase, which is also sensitive to wortmannin and LY294002 [21]. When approximately 90% of basal Akt phosphorylation was inhibited by 1 μ M LY294002, as indicated in Figure 4 (upper panel), Ro-31-8220 induced a small but detectable increase in Akt phosphorylation, suggesting that a small PI-3K-independent effect of Ro-31-8220 might exist. However, the complete inhibition of Ro-31-8220-induced Akt phosphorylation and activation by 100 nM wortmannin indicates that most of the effect of Ro-31-8220 is dependent on PI-3K.

Stable association of PKC ζ and Akt in breast cancer cells

PKC ζ and Akt have been demonstrated to associate *in vitro* [22,23] and in intact cells [3,10] providing a potential mechanism for the effects of PKC inhibitors on Akt phosphorylation and activity. To investigate the possible functional link between aPKC isoforms and Akt in breast cancer cell lines, we examined the physical association of exogenous PKC ζ or PKC λ/ι with Akt in BT-549 breast cancer cells, because BT-549 cells expressed lower levels of aPKCs than MDA-MB-468 cells (Figure 1). We co-transfected BT-549 cells with expression constructs encoding

Figure 4 PI-3K dependence of Ro-31-8220-induced Akt phosphorylation and activation

Serum-starved MDA-MB-468 cells were pretreated for 1 h with LY294002 (upper panel) or rapamycin or wortmannin (lower panel), then incubated for 1 h with 4 μM Ro-31-8220. Cells were lysed; lysates were separated by SDS/PAGE [10% (w/v) gel], then immunoblotted with anti-phospho-Ser-473 or anti-phospho-Thr-308 antibodies. Equal loading of proteins was determined by assessing Akt levels with anti-Akt antibody. Equal amounts of cell lysates were subjected to Akt kinase assay as described in the Experimental section. ³²P-labelled products were separated by SDS/PAGE [12% (w/v) gel] and detected by autoradiography. Results were quantified by densitometry and the value from untreated cells was taken as unity. The results shown are representative of three independent experiments.

V5-tagged PKCζ or V5-tagged PKCλ/ι with a construct encoding HA-Akt and then assessed co-immunoprecipitation of the transfected PKC with Akt. As demonstrated in Figure 5 (top panel), the exogenous PKCζ was readily detected in Akt immunoprecipitates. PKCλ/ι was present in Akt immunoprecipitates but at lower levels than PKCζ despite similar levels of expression of PKCλ/ι, PKCζ and Akt in each transfection. Strikingly, EGF increased the ability to detect PKCζ in Akt immunoprecipitates approx. 5-fold, indicating that the interaction between these mediators was stabilized by activation of the EGF receptor (Figure 5, middle panel). Next we evaluated the ability of endogenous PKCζ and Akt to interact in breast cancer cells. As illustrated in Figure 5 (bottom panel), an EGF-dependent binding of endogenous PKCζ with Akt was readily observed in BT-549 cells, suggesting a direct association-mediated interaction of the two signalling proteins in intact cells. Thus the aPKCs PKCζ and, to a smaller extent, PKCλ/ι bind Akt, a process that is increased by the activation of cells by EGF. This is in contrast with a previous report that did not assess the interaction of PKCλ/ι with Akt and demonstrated a growth-factor-dependent dissociation of PKCζ and Akt in transfected cells [10].

Wild-type PKCζ, but not dn PKCζ, decreases EGF-induced Akt phosphorylation at Ser-473 and Thr-308

Phosphorylation of Akt at Ser-473 and Thr-308, which is indicative of Akt activation [4,24], was used to assess whether the

Figure 5 Co-immunoprecipitation of PKCζ with Akt

Top panel: BT-549 cells were cultured in a 60 mm dish until 80% confluent; they were then co-transfected with HA-Akt and V5-PKCζ or V5-PKCλ/ι at a ratio of 1:3 as described in the Experimental section, to ensure that all HA-Akt-containing cells also expressed PKCζ. Co-transfection of HA-Akt with empty vector (pCDNA3.1 GS) was performed as a control. At 24 h after transfection, cells were serum-starved overnight. Cells were lysed and cell lysates were subjected to immunoprecipitation (IP) with anti-HA mAb and subsequent immunoblotting (IB) with anti-V5 antibody. Equal loading of proteins was detected with anti-HA mAb. The transfection efficiency of PKCζ or PKCλ/ι was confirmed by immunoblotting whole cell lysates with anti-V5 antibody. Middle panel: BT-549 cells were co-transfected with HA-Akt and V5-PKCζ at a 1:3 ratio. After transfection, cells were serum-starved overnight followed by 10 min of stimulation with EGF (20 ng/ml) or no stimulation before lysis. HA-Akt was immunoprecipitated (IP) from cell lysates with anti-HA mAb. The presence of PKCζ in the immunoprecipitates was detected by immunoblotting (IB) with anti-V5 antibody, as indicated. The same membrane was stripped and reprobed with anti-HA mAb to confirm equal protein loading. Whole cell lysates were evaluated for expression of V5-PKCζ with anti-V5 mAb antibody. Bottom panel: endogenous Akt was immunoprecipitated (IP) with anti-Akt antibody from 500 μg of BT-549 cell lysate either untreated or treated with EGF (20 ng/ml) for 10 min. The presence of endogenous PKCζ was detected by immunoblotting (IB) with anti-PKCζ antibody. Rabbit anti-mouse (RAM) antibody was used as a negative control in the experiment. The results shown are representative of three independent experiments.

physical interaction of PKCζ with Akt altered Akt activity. As shown in Figure 6 (top panel), EGF treatment induced a modest increase in Akt phosphorylation at Ser-473 and Thr-308 in cells transfected with Akt. In contrast, a marked decrease in Akt

Figure 6 Akt phosphorylation induced by EGF or LPA is reversed by overexpression of wild-type PKC ζ , but not dn PKC ζ

The same co-transfection protocol was used as described in the legend to Figure 5 with either V5-PKC ζ (top panel) or dn V5-PKC ζ (middle panel). BT-549 cells were serum-starved overnight after transfection and were either left unstimulated or stimulated with EGF (20 ng/ml) for 10 min. Cells were lysed and HA-Akt was immunoprecipitated (IP) from the cell lysates by anti-

phosphorylation was observed in EGF-treated cells co-transfected with HA-Akt and wild-type PKC ζ . The expression of exogenous PKC ζ and Akt without altering the ability of EGF to activate the EGF receptor was determined by Western blotting of whole cell lysate with phosphotyrosine antibody as indicated in Figure 6 (top panel).

Expression of PKC ζ had little effect on basal Akt phosphorylation (Figure 6, top panel), potentially arguing that PKC ζ must be activated as a consequence of PI-3K for its activity to be manifest. To clarify this issue, we exploited dn PKC ζ , which has the critical lysine residue at the ATP-binding site replaced by tryptophan, resulting in a kinase-defective mutant [17], to inhibit the activity of endogenous PKC ζ . As illustrated in Figure 6 (middle panel), an approx. 3-fold increase in Akt phosphorylation at both Thr-308 and Ser-473 was observed in MDA-MB-468 cells co-expressing dn PKC ζ and HA-Akt, suggesting that inhibition of PKC ζ activity limited its effect on Akt phosphorylation. In addition, no decrease in Akt phosphorylation was observed in EGF-treated cells transfected with dn PKC ζ , compared with an empty vector control (Figure 6, middle panel). Equal transfection efficiency of dn PKC ζ was determined by Western blotting of cell lysate with anti-V5 antibody (results not shown). Thus wild-type PKC ζ , but not dn PKC ζ , limits the EGF-induced increase in Akt phosphorylation, which is compatible with the effect of Ro-31-8220 on Akt phosphorylation (Figures 2 and 3) being due to the inhibition of endogenous PKC ζ .

Wild-type PKC ζ decreases LPA-induced Akt phosphorylation

LPA is a bioactive lipid exhibiting potent growth factor activity; it induces multiple biological responses in breast cancer cells [25]. In addition, LPA can induce a PI-3K-dependent activation of PKC ζ and Akt through membrane-anchored G-protein-coupled receptors [26]. To determine whether the ability of PKC ζ to limit the activation of Akt can be generalized to multiple growth factors and G-protein-coupled receptors as well as tyrosine-kinase-linked receptors, we co-transfected MDA-MB-468 breast cancer cells with empty vector or wild-type PKC ζ with HA-Akt, and then treated cells for 10 min with 5 μ M LPA. Western blot analysis with the anti-(phospho-Ser-473) antibody indicated that LPA induced a 2.2-fold increase in Akt phosphorylation over control levels (Figure 6, bottom panel). Co-transfection of wild-type PKC ζ severely curtailed LPA-induced Akt phosphorylation without altering basal Akt phosphorylation (Figure 6, bottom panel). The ability of LPA to activate extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) in cells expressing exogenous PKC ζ and Akt was determined by Western blotting with anti-(phospho-ERK) antibody (Figure 6, bottom panel). Taken together, these results suggest that PKC ζ negatively

HA mAb. The immunoprecipitates were separated by SDS/PAGE [8% (w/v) gel] and immunoblotted (IB) with rabbit anti-(phospho-Ser-473) or anti-(phospho-Thr-308) antibodies. Equal loading of proteins and equal transfection efficiency were detected as indicated previously. Phosphorylation of the EGF receptor was examined by Western blotting of whole cell lysate with anti-phosphotyrosine mAb (top panel). Results were quantified by densitometry and the value from empty-vector-transfected and untreated cells was taken as unity. Bottom panel: equal amounts of HA-Akt were immunoprecipitated (IP) from lysates from MDA-MB-468 cells co-transfected with either empty vector or V5-PKC ζ with HA-Akt, then either unstimulated or stimulated with LPA (5 μ M) for 10 min as indicated. Phosphorylation of HA-Akt was detected by immunoblotting the immunoprecipitates with anti-(phospho-Ser-473) polyclonal antibody; the densitometry is presented as a histogram. The phosphorylation of ERK1 and ERK2 was examined by Western blotting of cell lysate with anti-(phospho-ERK) antibody. Values are means $\pm$ S.D. for three independent experiments.

Figure 7 Ro-31-8220-induced phosphorylation of p70^{S6k} at Thr-389

BT-549 cells were starved overnight and then treated with Ro-31-8220 at 4 μ M for 1 h. Cells were lysed; lysates were subjected to Western blotting with anti-(phospho-Ser-473) (Akt) or anti-(phospho-Thr-389) (p70^{S6k}) antibodies. The membrane was stripped and re-probed with anti-Akt or anti-p70^{S6k} antibodies to indicate that the levels of Akt and p70^{S6k} proteins were not changed by the treatment. Results were quantified by densitometry as described above. The results shown are representative of three independent experiments.

regulates Akt phosphorylation in a growth-factor-dependent manner.

Ro-31-8220 increases phosphorylation of Thr-389 on p70^{S6k}

Phosphorylation of Thr-389 in the linker domain between catalytic and autoinhibitory domains is obligatory for p70^{S6k} activity, whereas phosphorylation on sites located within the C-terminal autoinhibitory domain or the catalytic domain by FRAP/RAFT/mTOR, p38, PDK1 and possibly other kinases modulates the effect of phosphorylation of Thr-389 [27,28]. To determine whether the increased Akt activity associated with PKC inhibition was transmitted through the PI-3K/Akt signalling cascade, we assessed the effect of Ro-31-8220 on the phosphorylation of Thr-389 in p70^{S6k}. As indicated in Figure 7, Ro-31-8220 (4 μ M, 1 h) increased Akt phosphorylation on Ser-473 in BT-549 cells concomitantly with Thr-389 in p70^{S6k}, which is consistent with increased signalling through the PI-3K/Akt signalling cascade. Again, the PKC inhibitor Ro-31-8220 did not change expression levels of Akt and p70^{S6k}.

DISCUSSION

The production of three phosphorylated phosphatidylinositols by PI-3K results in the activation and recruitment to the membrane of a subpopulation of PH-domain-containing proteins [29]. In addition, three phosphorylated phosphatidylinositols bind to and activate a number of proteins containing C2 domains, such as the PTEN tumour suppressor gene product, some phosphatidylinositol kinases (not PI-3K) and nPKCs and cPKCs, but not aPKCs, which lack the C2 domain found in other PKCs [29–31]. The aPKCs are regulated by PI-3K through binding to PDK1 [32] and a PDK1-mediated phosphorylation of a negative regulatory site in the activation loop of aPKCs [32,33]. The role of each of the PI-3K targets in the positive or negative regulation of the flow of signals through the PI-3K pathway is only beginning to be elucidated. The difficulty in analysing the integration of signals through the PI-3K cascade is magnified when cross-talk between multiple signalling pathways is taken into account.

Most studies of the PI-3K pathway have focused on the positive flow of signals through the PI-3K signalling cascade contributing to cell survival, proliferation and differentiation through Akt, Ilk, PDK1, Tec kinases and their downstream targets Bad, p70^{S6k}, caspase 9, forkhead and GSK3 α/β [2,3,34,35]. However, the recent discovery that the PTEN tumour suppressor gene product, implicated in tumorigenesis in multiple cell lineages and through germline mutations in the Cowden's breast cancer predisposition syndrome, specifically dephosphorylates the same 3' site in the inositol ring that is phosphorylated by PI-3K has focused attention on the negative regulation of this pathway [36,37]. In addition to PTEN, SHIP, by dephosphorylating the 5' site on the inositol ring of PtdIns(3,4,5)P₃, also seems to act as a negative regulator of signalling through the PI-3K pathway [38–40].

Multiple PKC family members and in particular aPKCs, including PKC ζ and PKC λ/ι , are activated as a consequence of stimulation of PI-3K activity by many different growth factor receptors [32,41]. The present studies indicate that the aPKCs, particularly PKC ζ , participate in a negative feedback loop initiated by the activation of PI-3K by EGF and LPA and the subsequent activation of PKC ζ , which decreases the phosphorylation and activity of Akt. This activity of PKC ζ is transmitted through the PI-3K signalling cascade as a decrease in activation of p70^{S6k}. Because PKC ζ and p70^{S6k} can form a physical complex [42], PKC ζ might affect p70^{S6k} activity by several mechanisms. The results linking p70^{S6k} phosphorylation to Akt and PKC ζ extend a previous study that suggested that PKC ζ could decrease the ability of PDGF (platelet-derived growth factor) to activate Akt and transmit signals to GSK3 α [10]. The consequence of the feedback loop activated by PKC ζ is not clear. Although, as mentioned above, PKC ζ decreases signal transduction through PI-3K and Akt to p70^{S6k} and GSK3 α , which would be expected to decrease cellular proliferation and viability, PKC and probably PKC ζ also alter the phosphorylation of Bad [43], which would be predicted to increase cell survival. Thus the outcome of PI-3K-induced activation of PKC ζ probably depends on the intracellular machinery of the cell stimulated, the particular ligand activating the cell and the network of signalling cascades activated.

The mechanism(s) by which PKC ζ regulates the phosphorylation and activation of Akt also seem complex. PKC ζ interacts physically with Akt (Figure 5) [10,22,23], suggesting that Akt might be a direct target for phosphorylation by PKC ζ . Intriguingly, the effects of growth factors on this association might be quite different, depending on the receptor activated or the cell stimulated, because we have demonstrated that EGF markedly increases the association of PKC ζ with Akt in breast cancer cells (Figure 5), whereas PDGF has been reported to dissociate PKC ζ and Akt in Cos cells [10]. Our studies with inhibitors of PI-3K indicate that the effects of PKC ζ on Akt phosphorylation exhibit both PI-3K-dependent and PI-3K-independent components. The PI-3K-dependent component might indicate, at least in part, a requirement for activation of PKC ζ by PI-3K through PDK1, because the overexpression of PKC ζ did not alter basal Akt phosphorylation but rather blocked EGF-dependent or LPA-dependent Akt phosphorylation and activation. Doornbos et al. [10] suggest that the effects of PKC ζ are directly on Akt and are independent of upstream mediators such as PI-3K or SHIP, because they are exhibited in cells transfected with a membrane-targeted and growth-factor-independent PI-3K. However, because membrane-targeted PI-3K remains subject to regulation by the p85 subunit [44], this fails to rule out the possibility that PKC ζ might directly or indirectly have effects upstream of Akt. Although the physical interaction between PKC ζ and Akt suggests that Akt might be a direct target of PKC ζ , it is not

known whether the PI-3K-independent effect of PKC ζ is solely on Akt directly or whether other signalling molecules such as adenylate cyclase [9] are involved. Thus it remains possible and even likely that PKC ζ mediates multiple effects on the PI-3K signalling cascade upstream of Akt, at Akt and probably downstream of Akt.

An obligatory role of the PTEN tumour suppressor gene in the action of PKC ζ and PKC inhibitors on the phosphorylation of Akt is eliminated by the observation that both of the breast cancer cell lines used in these studies lack functional PTEN owing to homozygous mutations in the PTEN gene sequence that result in a complete lack of detectable PTEN protein [37]. Similarly, an obligatory role for conventional PKC in the effects of the PKC inhibitors on Akt phosphorylation and activation seems to be eliminated by the failure of MDA-MB-468 cells to express detectable levels of conventional PKCs (Figure 1). Although a lack of conventional PKCs might be unusual, it is not entirely without precedent, because we have previously identified an interleukin-2-dependent cell line apparently lacking conventional PKC protein and enzyme activity [45].

The concentration dependence of the effects of the PKC inhibitor GF109203X, which inhibits cPKC and nPKC at nanomolar concentrations and aPKC at micromolar concentrations [18,20], suggests that aPKC might have a major role in the effects of the inhibitor Ro-31-8220, although a small but significant (approx. 3.2-fold) increase in Akt phosphorylation was observed with 0.1 μ M GF109203X, indicating the possible involvement of nPKC in the regulation. Although the effects of the inhibitors implicate aPKC, as with all kinase inhibitors GF109203X and Ro-31-8220 might inhibit other kinases. However, the observations with the PKC inhibitors, when combined with the ability of PKC ζ to bind Akt in a growth-factor-dependent manner and the ability of PKC ζ to decrease growth-factor-induced Akt phosphorylation and activation, implicates PKC ζ as a major regulator of Akt phosphorylation and activation.

In summary, we have demonstrated that Akt and PKC ζ physically associate in a growth-factor-dependent manner. This association results in a decrease in the phosphorylation and activity of Akt, which is transduced through the signalling cascade as a decrease in p70^{S6} activation. Although PKC ζ interacts physically with Akt, potential additional interactions and cross-talk between aPKCs and the PI-3K signalling cascade at the level of PI-3K, SHIP or downstream substrates cannot be eliminated. Taken together, the results indicate that PI-3K-dependent activation of PKC ζ leads to a feedback inhibition of signalling through the PI-3K/Akt signalling cascade resulting in decreased Akt phosphorylation and activity, which in turn leads to decreased p70^{S6} and GSK3 α functionality. The role of this negative feedback loop in the functional outcomes of PI-3K signalling probably represents a complex interplay of positive and negative signals initiated by the PI-3K cascade as well as other signalling cascades.

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