Published in final edited form as:

Cell Signal. 2009 November ; 21(11): 1680–1685. doi:10.1016/j.cellsig.2009.07.006.

Regulation of protein kinase C δ downregulation by protein kinase C ϵ and mammalian target of rapamycin complex 2

Alakananda Basu^{*}, Savitha Sridharan, and Shalini Persaud

From the Department of Molecular Biology & Immunology, University of North Texas Health Science Center, Fort Worth, Texas, 76107, USA

Abstract

Phosphorylation and dephosphorylation of PKCs can regulate their activity, stability and function. We have previously shown that downregulation of PKC δ by tumor promoting phorbol esters was compromised when HeLa cells acquired resistance to cisplatin (HeLa/CP). In the present study, we have used these cells to understand the mechanism of PKC δ downregulation. A brief treatment of HeLa cells with phorbol 12,13-dibutyrate (PDBu) induced phosphorylation of PKC δ at the activation loop (Thr505), turn motif (Ser643), hydrophobic motif (Ser662) and Tyr-311 sites to a greater extent in HeLa/CP cells compared to HeLa cells. Prolonged treatment with PDBu led to downregulation of PKC δ in HeLa but not in HeLa/CP cells. The PKC inhibitor Gö 6983 inhibited PDBu-induced downregulation of PKC δ , decreased Thr505 phosphorylation and increased PKC δ tyrosine phosphorylation at Tyr-311 site. However, knockdown of c-Abl, c-Src, Fyn and Lyn had little effect on PKC δ downregulation and Tyr311 phosphorylation. Pretreatment with the phosphatidylinositol 3-kinase inhibitor Ly294002 and mTOR inhibitor rapamycin restored the ability of PDBu to downregulate PKC δ in HeLa/CP cells. Knockdown of mTOR and rictor but not raptor facilitated PKC δ downregulation. Depletion of PKC ϵ also enhanced PKC δ downregulation by PDBu. These results suggest that downregulation of PKC δ is regulated by PKC ϵ and mammalian target of rapamycin complex 2 (mTORC2).

Keywords

PKC δ ; PKC ϵ ; rictor; mTOR; Src

1. Introduction

Protein kinase C (PKC), a family of phospholipid-dependent serine/threonine protein kinases, plays a critical role in regulating diverse cellular processes including cell proliferation, cell survival, apoptosis, cell migration and tumor promotion [1]. The PKC family can be categorized into three groups based on their structure, function and biochemical regulation [2–5]. Conventional PKCs (α , β I, β II and γ) require Ca²⁺ and diacylglycerol (DAG) for their activities. Novel PKCs (δ , ϵ , η and θ) are Ca²⁺-independent but DAG-dependent whereas atypical PKCs (ζ , λ /i) do not require Ca²⁺ or DAG for their activities. PKC serves as the receptor for tumor promoting phorbol esters, which are potent activators of conventional and novel

© 2009 Elsevier Inc. All rights reserved.

^{*}Corresponding author. Tel.: +1 817 735 2487; fax: +1 817 735 2118. abasu@hsc.unt.edu (A. Basu).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

PKCs and can substitute for DAG [6]. Prolonged treatment with tumor-promoting phorbol esters leads to degradation or downregulation of PKCs. Persistent activation or downregulation of PKCs by phorbol esters has been associated with tumor promotion [7]. Thus, understanding the mechanism of PKC downregulation is of critical importance.

PKCs are regulated not only by cofactors but also by phosphorylation. PKCs can be phosphorylated at three conserved Ser/Thr phosphorylation sites: (i) activation loop (A-loop), (ii) turn motif (TM), and (iii) hydrophobic motif (HM) [3,8]. The phosphorylation of PKCs primes them for activation by cofactors [3,8]. Phosphorylation of PKCs may involve both autophosphorylation [9] and transphosphorylation [10]. Most of the studies on PKC phosphorylation have been performed with conventional PKCs. It is generally believed that phosphorylation at the A-loop is mediated by phosphoinositide-dependent kinase-1 (PDK1) which has been shown to be the upstream kinase for several members of the AGC family of kinases, including PKC isozymes [11–], Akt/PKB [16] and p70S6K [17]. Once phosphorylated at the A-loop, PKCs are believed to undergo autophosphorylation at the TM and the HM [18]. However, recent studies suggest that Akt and conventional PKCs are phosphorylated at the TM and HM by the mammalian target of rapamycin complex 2 (mTORC2) [19–21].

There are controversies regarding the mechanism of phosphorylation of PKC δ at the A-loop (Thr505), TM (Ser643) and HM (Ser662). While it is generally believed PKC δ is phosphorylated at the activation loop by PDK1, it was also shown to be transphosphorylated by PKC ϵ [22]. Ser643 site is believed to be an autophosphorylation site whereas phosphorylation at the C-terminal hydrophobic domain of PKC δ and PKC ϵ is believed to be regulated by rapamycin-sensitive mTOR [23]. A recent study, however, suggests that PKC ϵ but not PKC δ is phosphorylated at both the TM and HM sites by mTORC2 [20]. PKC δ can also be phosphorylated at several tyrosine residues [24]. In contrast to serine/threonine phosphorylation, which is a common regulatory mechanism for PKC isozymes, tyrosine phosphorylation is a unique regulatory mechanism for PKC δ [24].

It is believed that priming phosphorylation of PKCs at serine/threonine sites maintains them in a closed, protease/phosphatase resistant form [3,8] and dephosphorylation predisposes them to downregulation [25–27]. In contrast to cPKCs, which are stabilized by phosphorylation, A-loop phosphorylation of PKC δ was shown to be necessary for phorbol ester-mediated downregulation of PKC δ [28]. On the other hand, phosphorylation of PKC δ at Y311 was reported to be important for downregulation of PKC δ by Src but not by phorbol esters [29]. It is now realized that the immunoreactivity of the PKC δ antibody used in these studies is altered by the PDBu treatment [30]. In addition, some of the studies on PKC δ phosphorylation and downregulation were performed in serum-deprived adherent cells grown in suspension [23] and the regulation of PKCs in suspension culture could be distinct from adherent cells [31]. Furthermore, the regulation of exogenously expressed PKC δ may be different from the endogenous PKC δ since the tight regulation of PKC δ is often lost during PKC δ overexpression [22]. Thus, the mechanism of PKC δ downregulation is yet to be unraveled.

PKC δ plays a critical role in apoptosis [32,33]. It is believed that proteolytic cleavage of PKC δ is essential for the induction of apoptosis. PKC δ is also a positive regulator of cell growth. It can function as an antiapoptotic protein and can contribute to cell transformation and metastasis [1,7,33,34]. We have shown that acquisition of resistance by HeLa cells to the chemotherapeutic drug cisplatin was associated with loss of regulation of PKC δ [35,36]. The ability of phorbol esters to downregulate PKC δ was compromised in cisplatin-resistant HeLa cells [35]. Thus, this system provides a unique tool to understand the mechanism of PKC δ downregulation by PKC activators. Our results show that downregulation of PKC δ is regulated by PKC ϵ and mTORC2.

2. Materials and methods

2.1. Antibodies and reagents

PDBu was purchased from LC Service Corporation (Woburn, MA). Polyclonal antibodies to tubulin, PKC δ and PKC ϵ , and monoclonal antibody to GAPDH were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody to PKC α was from Upstate Biotechnology, Inc. (Lake Placid, NY) and monoclonal antibody to PKC δ was from BD Transduction Laboratories (San Jose, CA). Polyclonal antibodies to phospho-PKC δ were obtained from Cell Signaling (Beverly, MA). Non-targeting siRNA and siRNA SMARTpool against PKC α , - ϵ , c-Src, Fyn, Lyn, c-Abl, PDK1, raptor, rictor and mTOR were obtained from Dharmacon (Lafayette, CO). Horseradish peroxidase conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from JacksonImmunoResearch Lab. Inc. (West Grove, PA). Poly(vinylidenedifluoride) membrane was from Millipore (Bedford, MA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL).

2.2. Cell Culture

Human cervical carcinoma HeLa cells and its cisplatin-resistant variants (HeLa/CP) were maintained in Dulbecco's modified minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine, and kept in a humidified incubator at 37°C with 95% air and 5% CO₂. HeLa/CP cells were maintained in drug-free media.

2.3. Immunoblot Analysis

Equivalent amounts of proteins were electrophoresed by sodium dodecyl sulfatepolyacrylamide gel electrophoresis and transferred electrophoretically to poly(vinylidene difluoride) membrane. Immunoblot analyses were performed as described before [37].

2.4. siRNA Transfection

Control non-targeting siRNA or SMARTpool siRNA against PKC isozymes, Src family tyrosine kinase, rictor, raptor or mTOR were introduced into HeLa or HeLa/CP cells using Lipofectamine 2000 (Invitrogen) and manufacturer's protocol. Briefly, cells were seeded one day before transfection. 48 h following siRNA transfection, cells were treated as indicated in the text and processed for Western blot analysis as described before [36,38].

3. Results

3.1. Comparison of the effects of PKC activators and inhibitors on PDBu-induced phosphorylation and downregulation of PKC δ

We have shown previously that downregulation of PKC δ by PDBu is compromised in HeLa cells that acquired resistance to the anticancer drug cisplatin (HeLa/CP) [35,36]. Since phosphorylation status of PKCs regulates their stability, we compared the effect of PDBu on the phosphorylation of PKC δ at the Ser/Thr and Tyr sites. Figure 1 shows that PDBu caused an increase in phosphorylation of PKC δ at the A-loop (T505), TM (S643), HM (S662) and Y-311 sites. The levels of phospho-PKC δ at all of these sites were greater in HeLa/CP cells compared to HeLa cells.

Since chronic treatment with PKC activators results in PKC δ downregulation, we examined the effects of several PKC inhibitors on PKC δ downregulation induced by PDBu. While Gö 6983 inhibits all PKCs, Gö 6976 inhibits conventional PKC α and - β I [39] and rottlerin inhibits PKC δ [40] although it has many other targets. Figure 2 shows that PDBu induced substantial downregulation of PKC δ in HeLa cells but not in HeLa/CP cells whereas PDBu led to

downregulation of PKC α in both HeLa and HeLa/CP cells. Both Gö 6976 and Gö 6983 prevented PKC δ downregulation by PDBu in HeLa cells although Gö 6976 was less effective than Gö 6983. In contrast, rottlerin had little effect on PDBu-induced downregulation of PKC δ . These results suggest that PKC δ downregulation is primarily regulated by novel PKCs.

It has been reported that phosphorylation of PKC δ at T505 is important for PKC δ downregulation [28]. We therefore examined the effects of PKC inhibitors on PDBu-induced phosphorylation of PKC δ at T505 site. Figure 3 shows a brief treatment with PDBu induced phosphorylation of PKC δ at T505 site. The general PKC inhibitor Gö 6983 inhibited PDBu-induced phosphorylation of PKC δ at T505 whereas Gö 6976 or rottlerin had no effect. Since atypical PKCs are phorbol ester-insensitive, this is consistent with an earlier report that PKC δ is transphosphorylated at the T505 site by PKC ϵ [22]. None of the inhibitors prevented phosphorylation of PKC δ at S643 site. PDBu caused a modest increase in PKC δ level when detected using the polyclonal antibody to PKC δ . However, PKC δ level in PDBu-treated cells was substantially less when monoclonal antibody from BD Transduction Laboratories was used to detect PKC δ . This decrease in PKC δ was blocked by Gö 6983 but not by Gö 6976 or rottlerin. In fact, PKC δ level detected using either monoclonal or polyclonal antibody to PKC δ was equivalent in cells treated with Gö 6983. These results suggest that the immunoreactivity of monoclonal antibody to PKC δ is decreased when PKC δ is phosphorylated at T505 site as has been reported [30].

Since PKC δ is not only regulated by Ser/Thr phosphorylation but also by tyrosine phosphorylation, we examined the effects of PKC inhibitors on PDBu-induced PKC δ tyrosine phosphorylation. As shown in Figure 3, Gö 6983 caused a substantial increase in PDBu-induced PKC δ Y311 phosphorylation; Gö 6976 also caused a modest increase whereas rottlerin had only a little effect on Y311 phosphorylation. Thus, an increase in PKC δ Y311 phosphorylation appears to correlate with inhibition of PKC δ downregulation by these PKC inhibitors (Fig. 2 vs. Fig. 3).

3.2. Effects of knockdown of Src family tyrosine kinases on PKC δ downregulation

It is believed that phosphorylation of PKC δ at Y311 is mediated by Src family tyrosine kinases (SFKs) [24,29,41]. Since PDBu failed to induce substantial downregulation of PKC δ in HeLa/CP cells, we depleted several SFKs, including c-Src, Fyn and Lyn using siRNA and examined the effect of SFK knockdown on PKC δ downregulation. A recent report suggested that c-Abl rather than c-Src phosphorylates PKC δ at Y311 site (42). Therefore, we also examined the effect of c-Abl knockdown on PKC δ downregulation by PDBu. As shown in Figure 4, specific knockdown of c-Abl and SFKs was achieved using siRNA although depletion of these kinases had little effect on PKC δ downregulation and Y-311 phosphorylation.

3.3. Effects of PKC ϵ knockdown on PKC δ downregulation

It has been reported that PKC ϵ regulates PKC δ phosphorylation at T505 site via Src-dependent PKC δ tyrosine phosphorylation [22,41]. We therefore examined if depletion of PKC ϵ affects PKC δ phosphorylation at Y311 and T505 sites. Figure 5 shows that siRNA silencing of PKC ϵ attenuated PDBu-induced Y311 phosphorylation in HeLa cells. Knockdown of PKC ϵ had little effect on the phosphorylation of PKC δ at T505 site when treated with PDBu for 30 min. However, when cells were treated with PDBu for 4 h and 18 h, the phosphorylation of PKC δ at T505 site was decreased in PKC ϵ -depleted cells. The levels of PKC δ following treatment with PDBu for 4 h and 18 h were also less in PKC ϵ -depleted cells compared to control siRNA-transfected cells. Thus, depletion of PKC ϵ enhanced PKC δ downregulation and this was associated with decrease in PKC δ Y311 phosphorylation.

3.4. Effects of PI-3 kinase and mTOR inhibitor on PKC δ downregulation

It is generally believed that phosphorylation of PKC δ at the activation loop is mediated by PDK1 [8] whereas phosphorylation at the hydrophobic site is mediated by rapamycin-sensitive mTOR [23]. To determine the importance of Ser/Thr phosphorylation on PKC δ downregulation, we examined the effect of Ly294002, a pharmacological inhibitor of PI3-K/PDK1 and rapamycin, an inhibitor of mTOR on PKC δ downregulation in HeLa/CP cells. Figure 6A shows that prolonged treatment with PDBu caused only a modest decrease in PKC δ in HeLa/CP cells although PDBu caused substantial downregulation of PKC α and PKC ϵ . Pretreatment with Ly294002 and rapamycin restored the ability of PDBu to downregulate PKC δ . These inhibitors also enhanced downregulation of PKC ϵ and PKC α by PDBu (Fig. 6A). PKC δ was phosphorylated at both Ser/Thr and Tyr residues and PDBu caused a substantial increase in Y311 phosphorylation, which was retained even when HeLa/CP cells were treated with PDBu for 16 h. Brief treatment with PDBu for 10 min or 30 min also caused a modest increase in PKC δ phosphorylation at Ser/Thr sites. Pretreatment with Ly294002 and rapamycin for a prolonged period had little effect on T505 phosphorylation but it attenuated phosphorylation of PKC δ at Y311, S643 and S662 sites by PDBu. Thus, dephosphorylation of PKC δ at both Ser and Tyr residues was associated with PKC δ downregulation.

To further rule out the involvement of PDK1 in mediating phosphorylation of PKC δ at T505, we depleted PDK1 using siRNA. Knockdown of PDK1 failed to inhibit T505 phosphorylation or enhance downregulation of PKC δ by PDBu (Fig. 6B). Thus, the effect of Ly294002 on PKC δ downregulation was not due to inhibition of PDK1.

3.5. Effects of mTOR and PKC ϵ on the phosphorylation and downregulation of PKC δ

mTOR can complex with either raptor or rictor to form mTORC1 and mTORC2, respectively [42]. Since rapamycin enhanced PKC δ downregulation, we compared the effects of knockdown of rictor, raptor or mTOR on PDBu-induced downregulation of PKC δ in HeLa/CP cells. As shown in Figure 7, knockdown of rictor and mTOR but not raptor facilitated PKC δ downregulation by PDBu. This downregulation was accompanied by a decrease in phosphorylation at Y311, S643 and S662 sites.

It has been reported that the phosphorylation of PKC ϵ at the TM and HM sites is regulated by mTORC2 [20]. We therefore compared the effect of depletion of PKC ϵ and rictor on PKC δ downregulation in HeLa/CP cells. Figure 8 shows that silencing of either PKC ϵ or rictor by siRNA enhanced PKC δ downregulation by PDBu and the effect was greater when both PKC ϵ and rictor were depleted. To determine if there was any association between phosphorylation of PKC δ at a particular site and PKC δ downregulation, we monitored PKC δ phosphorylation following a brief treatment (10 min) with PDBu. Depletion of either PKC ϵ or rictor caused a modest decrease in Y311 and S643 phosphorylation but knockdown of both PKC ϵ and rictor caused a substantial decrease in Y311, S643 and S662 phosphorylation. These results suggest that dephosphorylation at both Ser and Tyr sites may be important for PKC δ downregulation.

4. Discussion

Long-term cellular responses, such as cell proliferation, cell death and tumor promotion activities of PKC δ have been linked with PKC δ downregulation [7]. The dephosphorylation of priming sites of PKC α and PKC ϵ has been shown to be important for their downregulation [25–27]. Phosphorylation of cPKC at TM is important for protein maturation and stability [19,20]. mTORC2 has been shown to regulate TM and HM phosphorylation of PKC α and PKC ϵ but not of PKC δ [20]. The results of our present study demonstrate that downregulation of PKC δ by PDBu is also regulated by mTORC2. We have also made a novel observation that

PKC ϵ regulates activation-induced downregulation of PKC δ . Furthermore, dephosphorylation of PKC δ at both Ser/Thr and Tyr residues appears to be important for PKC δ downregulation.

In contrast to conventional PKC α or novel PKC ϵ , phosphorylation of PKC δ at the activation loop (T505) is believed to be required for its downregulation because the Ser/Thr phosphatase inhibitor calyculin A increased T505 phosphorylation and enhanced TPA-induced degradation of PKC δ [28]. We have also found that Gö 6983 inhibited T505 phosphorylation and blocked PKC δ downregulation (Fig. 2). However, Gö 6983 also caused a substantial increase in phosphorylation of PKC δ at Y311 site. In addition, it is now realized that the monoclonal antibody from BD Transduction laboratory does not recognize PKC δ phosphorylated at T505 site [30]. Even a brief treatment with PDBu that did not decrease PKC δ level detected using the polyclonal antibody to PKC δ in fact caused substantial decrease in PKC δ when detected using the monoclonal antibody to PKC δ (Fig. 3). This decrease in PKC δ level detected using monoclonal PKC δ antibody was reversed when T505 phosphorylation was inhibited by Gö 6983. Since calyculin A inhibits dephosphorylation of PKC δ at T505 site, it increases T505 phosphorylation in PDBu-treated cells. Because the monoclonal antibody does not detect T505 PKC δ , a decrease in PKC δ in calyculin A-treated cells in the earlier study could be explained by the loss of immunoreactivity of monoclonal PKC δ antibody towards T505 PKC δ rather than enhanced PKC δ downregulation.

PKC δ is phosphorylated not only at the Ser/Thr sites but also at Tyr residues [24]. It has been reported that phosphorylation of PKC δ at Y311 is important for downregulation of PKC δ by activated Src but not by phorbol esters [29]. Our results suggest that dephosphorylation of PKC δ at Y311 is associated with PDBu-induced PKC δ downregulation. First, PKC inhibitors that inhibited PKC δ downregulation by PDBu increased Y311 phosphorylation (Fig. 3). Gö 6983 caused a substantial increase in tyrosine phosphorylation of PKC δ at Y311 site. The cPKC inhibitor Gö 6976 also enhanced Y311 phosphorylation albeit less effectively than general PKC inhibitors. The ability of these inhibitors to induce PKC δ tyrosine phosphorylation correlated with their ability to prevent PKC δ downregulation. Second, the lack of PKC δ downregulation by PDBu in cisplatin-resistant HeLa cells was associated with increase in PKC δ tyrosine phosphorylation. Third, Ly294002 and rapamycin resorted PKC δ downregulation in HeLa/CP cells and attenuated PDBu-induced phosphorylation of PKC δ at Y311 site (Fig. 6A). Finally, knockdown of PKC ϵ and rictor by siRNA decreased PDBu-induced Y311 phosphorylation and enhanced PKC δ downregulation (Fig. 5, Fig. 7 and Fig. 8).

It is generally believed that PKC δ is phosphorylated at Y311 site by Src family tyrosine kinases [24,29,43]. However, knockdown of c-Src did not inhibit PDBu-induced PKC δ Y311 phosphorylation in HeLa/CP cells (Fig. 4), suggesting that phosphorylation at Y311 by PDBu may be mediated by a tyrosine kinase distinct from c-Src. Several Src family tyrosine kinases have been shown to complex with PKC δ [24]. It has been reported that Y311 phosphorylation in response to H₂O₂ is mediated by c-Abl and not by Src kinases [44]. However, knockdown of c-Abl, c-Src, Fyn or Lyn had little effect on Y311 phosphorylation and PKC δ downregulation in HeLa/CP cells (Fig. 4B). Most of the studies on PKC δ tyrosine phosphorylation have been performed in response to H₂O₂. It has been reported that PMA does not activate SFK activity directly and the identity of phorbol ester-activated kinase that phosphorylates PKC δ at Y311 remains to be determined [41].

It has been reported that there is a cross-talk between PKC δ and PKC ϵ . There is, however, controversy regarding how PKC ϵ regulates PKC δ phosphorylation. PKC δ protein expression and T505 phosphorylation were increased in PKC ϵ -/- mice compared to wild-type mice [45]. In contrast, adenoviral delivery of PKC ϵ in cardiomyocytes increased phosphorylation of PKC δ at T505 and Y311 sites [41]. However, the heterologously expressed PKC δ was not

influenced by PKC ϵ overexpression and the investigators questioned the suitability of studies using PKC overexpression. We have used siRNA silencing rather than PKC ϵ overexpression to investigate the involvement of PKC ϵ on PKC δ downregulation. We have found that depletion of PKC ϵ enhanced PDBu-induced downregulation of PKC δ in both HeLa and HeLa/CP cells (Fig. 5 and Fig. 8). We were unable to detect a consistent decrease in T505 phosphorylation by PKC ϵ depletion although knockdown of PKC ϵ did attenuate PDBu-induced phosphorylation of PKC δ at Y311 (Fig. 5 and Fig. 8). Neither Ly294002 nor PDK1 depletion affected phosphorylation of PKC δ at T505 site. The observations that Gö 6983 inhibited T505 phosphorylation (Fig. 3) and DN-PKC δ was dephosphorylated at T505 site (data not shown), suggest that PKC δ is autophosphorylated at T505 site.

Although Ly294002 did not inhibit phosphorylation of PKC δ at T505 site, it potentiated PKC δ downregulation by PDBu (Fig. 6). Ly294002 may influence PKC δ downregulation via PKC ϵ , which is also believed to be regulated by the PI3-K pathway [3,23]. In addition, PI3-K acts upstream of mTOR, which has been implicated in regulating phosphorylation of PKC δ at the hydrophobic site [23]. The earlier studies have focused on rapamycin-sensitive mTOR or mTORC1 [23]. We have found that brief treatment with Ly294002 or rapamycin had no effect on the phosphorylation of PKC δ at the A-loop, TM, HM or Y-311 sites (data not shown). However, prolonged treatment with Ly294002 or rapamycin decreased phosphorylation of PKC δ not only at the S662 site but also at S643 and Y311 sites. It has been reported that prolonged rapamycin treatment could inhibit the assembly of mTORC2 [46]. Thus, the effects of Ly294002 and rapamycin may be mediated via mTORC2 rather than mTORC1. Our results show that knockdown of rictor and mTOR but not raptor restored PDBu-induced downregulation of PKC δ in HeLa/CP cells (Fig. 7), suggesting the importance of mTORC2 in regulating PKC δ downregulation. Interestingly, knockdown of rictor not only caused a decrease in phosphorylation of PKC δ at the TM but also decreased Y311 phosphorylation.

We have found that an increase in PKC δ downregulation by knockdown of either PKC ϵ or rictor was associated with a decrease in both Y311 and S643 phosphorylation. Depletion of both PKC ϵ and rictor caused a substantial decrease in phosphorylation of PKC δ at S643, S662 and Y311 sites, and this was associated with a dramatic increase in PDBu-induced downregulation of PKC δ in HeLa/CP cells (Fig. 8). Since the tight regulation of PKC is lost when overexpressed, it is difficult to attribute a particular site responsible for PKC δ downregulation. Furthermore, phorbol ester has been shown to phosphorylate PKC δ at several newly identified Ser/Thr sites, including S299 and S304 at the V3 region (37) and the involvement of these sites on PKC δ downregulation remains to be established.

While mTORC2 is known to regulate phosphorylation of Akt, cPKCs and PKC ϵ at the TM and HM sites [19,20], we have found that Ly294002, rapamycin and rictor knockdown not only decreased phosphorylation at these sites but also attenuated phosphorylation at Y311 by PDBu (Fig. 6 and Fig. 8). It is conceivable that rictor regulates PKC δ Y311 phosphorylation via PKC ϵ , which is a binding partner of Src (47). Although knockdown of Src did not decrease Y311 phosphorylation, it is possible that PKC ϵ also interacts with a related tyrosine kinase, which phosphorylates Y311 in response to PDBu. While future studies should discern how rictor and PKC ϵ regulate phosphorylation of PKC δ at the Ser/Thr or Tyr sites, the present study provides evidence that like cPKC α and nPKC ϵ , the stability of PKC δ is also regulated by mTORC2. Furthermore, an interplay between PKC ϵ , mTORC2 and perhaps a member of the SFK regulates downregulation of PKC δ by PDBu.

The abbreviations used are

PARP, poly(ADP-ribose) polymerase
PDBu, phorbol 12,13-dibutyrate

PDK1, phosphoinositide-dependent protein kinase
 PI3-K, phosphoinositide 3-kinase
 PKC, protein kinase C
 aPKC, atypical PKC
 cPKC, conventional PKC
 nPKC, novel PKC
 SFK, Src family tyrosine kinases
 siRNA, short interfering RNA
 TPA, 12-*O*-tetradecanoylphorbol 13-acetate

Acknowledgements

This work was supported by the grant CA071727 from the National Cancer Institute.

The authors wish to thank Drs. Baohua Sun and Giridhar Akkaraju for cloning of PKC δ , Jie Huang, Haidi Tu and Chandreyi Basu for technical assistance, and Deepanwita Pal, Dr. Eswar Swamy and Dr. Qiang Zeng for help with the manuscript.

References

1. Griner EM, Kazanietz MG. *Nat Rev Cancer* 2007;7(4):281–294. [PubMed: 17384583]
2. Basu A. *Pharmacol Ther* 1993;59(3):257–280. [PubMed: 8309991]
3. Newton AC. *Biochem J* 2003;370(Pt 2):361–371. [PubMed: 12495431]
4. Nishizuka Y. *Science* 1992;258(5082):607–614. [PubMed: 1411571]
5. Parker PJ, Parkinson SJ. *Biochem Soc Trans* 2001;29(Pt 6):860–863. [PubMed: 11709088]
6. Sharkey NA, Leach KL, Blumberg PM. *Proc Natl Acad Sci U S A* 1984;81(2):607–610. [PubMed: 6320198]
7. Jackson DN, Foster DA. *Faseb J* 2004;18(6):627–636. [PubMed: 15054085]
8. Parekh DB, Ziegler W, Parker PJ. *Embo J* 2000;19(4):496–503. [PubMed: 10675318]
9. Flint AJ, Paladini RD, Koshland DE Jr. *Science* 1990;249(4967):408–411. [PubMed: 2377895]
10. Dutil EM, Keranen LM, DePaoli-Roach AA, Newton AC. *J Biol Chem* 1994;269(47):29359–29362. [PubMed: 7961910]
11. Cenni V, Doppler H, Sonnenburg ED, Maraldi N, Newton AC, Toker A. *Biochem J* 2002;363(Pt 3):537–545. [PubMed: 11964154]
12. Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, Parker PJ. *Science* 1998;281(5385):2042–2045. [PubMed: 9748166]
13. Balendran A, Hare GR, Kieloch A, Williams MR, Alessi DR. *FEBS Lett* 2000;484(3):217–223. [PubMed: 11078882]
14. Dutil EM, Toker A, Newton AC. *Curr Biol* 1998;8(25):1366–1375. [PubMed: 9889098]
15. Chou MM, Hou W, Johnson J, Graham LK, Lee MH, Chen CS, Newton AC, Schaffhausen BS, Toker A. *Curr Biol* 1998;8(19):1069–1077. [PubMed: 9768361]
16. Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P. *Curr Biol* 1997;7(4):261–269. [PubMed: 9094314]
17. Pullen N, Dennis PB, Andjelkovic M, Dufner A, Kozma SC, Hemmings BA, Thomas G. *Science* 1998;279(5351):707–710. [PubMed: 9445476]
18. Behn-Krappa A, Newton AC. *Curr Biol* 1999;9(14):728–737. [PubMed: 10421574]
19. Facchinetti V, Ouyang W, Wei H, Soto N, Lazorchak A, Gould C, Lowry C, Newton AC, Mao Y, Miao RQ, Sessa WC, Qin J, Zhang P, Su B, Jacinto E. *Embo J* 2008;27(14):1932–1943. [PubMed: 18566586]
20. Ikenoue T, Inoki K, Yang Q, Zhou X, Guan KL. *Embo J* 2008;27(14):1919–1931. [PubMed: 18566587]
21. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. *Science* 2005;307(5712):1098–1101. [PubMed: 15718470]

22. Rybin VO, Sabri A, Short J, Braz JC, Molkentin JD, Steinberg SF. *J Biol Chem* 2003;278(16):14555–14564. [PubMed: 12566450]
23. Parekh D, Ziegler W, Yonezawa K, Hara K, Parker PJ. *J Biol Chem* 1999;274(49):34758–34764. [PubMed: 10574945]
24. Steinberg SF. *Biochem J* 2004;384(Pt 3):449–459. [PubMed: 15491280]
25. Hansra G, Garcia-Paramio P, Prevostel C, Whelan RD, Bornancin F, Parker PJ. *Biochem J* 1999;342(Pt 2):337–344. [PubMed: 10455020]
26. Lee HW, Smith L, Pettit GR, Bingham Smith J. *Am J Physiol* 1996;271(1 Pt 1):C304–C311.
27. Lee HW, Smith L, Pettit GR, Smith JB. *Mol Pharmacol* 1997;51(3):439–447. [PubMed: 9058599]
28. Srivastava J, Procyk KJ, Iturrioz X, Parker PJ. *Biochem J* 2002;368(Pt 1):349–355. [PubMed: 12207561]
29. Blake RA, Garcia-Paramio P, Parker PJ, Courtneidge SA. *Cell Growth Differ* 1999;10(4):231–241. [PubMed: 10319993]
30. Rybin VO, Steinberg SF. *Am J Physiol Cell Physiol* 2006;290(3):C750–C756. [PubMed: 16236821]
31. England K, Watson J, Beale G, Warner M, Cross J, Rumsby M. *J Biol Chem* 2001;276(13):10437–10442. [PubMed: 11121415]
32. Basu A. *J Cell Mol Med* 2003;7(4):341–350. [PubMed: 14754503]
33. Brodie C, Blumberg PM. *Apoptosis* 2003;8(1):19–27. [PubMed: 12510148]
34. Perletti G, Terrian DM. *Curr Pharm Des* 2006;12(24):3117–3133. [PubMed: 16918438]
35. Huang J, Mohanty S, Basu A. *Biochem Biophys Res Commun* 2004;316(4):1002–1008. [PubMed: 15044084]
36. Mohanty S, Huang J, Basu A. *Clin Cancer Res* 2005;11(18):6730–6737. [PubMed: 16166454]
37. Basu A, Akkaraju GR. *Biochemistry* 1999;38(14):4245–4251. [PubMed: 10194341]
38. Lu D, Huang J, Basu A. *J Biol Chem* 2006;281(32):22799–22807. [PubMed: 16785234]
39. Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D, Schachtele C. *J Biol Chem* 1993;268(13):9194–9197. [PubMed: 8486620]
40. Gschwendt M, Muller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, Marks F. *Biochem Biophys Res Commun* 1994;199(1):93–98. [PubMed: 8123051]
41. Rybin VO, Guo J, Gertsberg Z, Elouardighi H, Steinberg SF. *J Biol Chem* 2007;282(32):23631–23638. [PubMed: 17569658]
42. Loewith R, Jacinto E, Wulschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN. *Mol Cell* 2002;10(3):457–468. [PubMed: 12408816]
43. Rybin VO, Guo J, Sabri A, Elouardighi H, Schaefer E, Steinberg SF. *J Biol Chem* 2004;279(18):19350–19361. [PubMed: 14970215]
44. Lu W, Finnis S, Xiang C, Lee HK, Markowitz Y, Okhrimenko H, Brodie C. *Biochem Biophys Res Commun* 2007;352(2):431–436. [PubMed: 17126298]
45. Klein G, Schaefer A, Hilfiker-Kleiner D, Oppermann D, Shukla P, Quint A, Podewski E, Hilfiker A, Schroder F, Leitges M, Drexler H. *Circ Res* 2005;96(7):748–755. [PubMed: 15761199]
46. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM. *Mol Cell* 2006;22(2):159–168. [PubMed: 16603397]

Fig. 1.

Comparison of the effects of PDBu on the phosphorylation of PKCδ in HeLa and HeLa/CP cells. Cells were serum-starved for 4 h and then treated without or with 1 μM PDBu for the indicated time. Western blot analyses were performed with total cell lysates. The blot was probed with GAPDH to control for loading differences. Results are representative of at least 3 independent experiments.

Fig. 2.

Comparison of the effects of PKC inhibitors on PDBu-induced downregulation of PKC δ in HeLa and HeLa/CP cells. Cells were pretreated without or with 1 μ M Gö 6983, 10 μ M rottlerin (Rot) or 1 μ M Gö 6976 for 1 h and then treated with 1 μ M PDBu for 17 h. Western blot analyses were performed with total cell lysates. The blot was probed with GAPDH to control for loading differences. Results are representative of at least 3 independent experiments.

Fig. 3.

Comparison of the effects of PKC inhibitors on PDBu-induced phosphorylation of PKC δ in HeLa and HeLa/CP cells. Cells were pretreated with or without 1 μ M Gö 6983, 10 μ M rottlerin or 1 μ M Gö 6976 for 1 h and then treated with 1 μ M PDBu for 15 min. Western blot analyses were performed with total cell lysates. Results are representative of at least 3 independent experiments.

Fig. 4.

Effect of SFK knockdown on PKC δ phosphorylation and downregulation. HeLa/CP cells were transfected with or without non-targeting siRNA (Con) or siRNA against c-Abl, c-Src, Fyn or Lyn. Cells were treated with or without 1 μ M PDBu for 8 h and Western blot analyses were performed with total cell lysates. Results are representative of 2 independent experiments.

Fig. 5.

Effect of PKC ϵ knockdown on PKC δ downregulation. HeLa cells were transfected with non-targeting siRNA (Con) or siRNA against PKC ϵ as described under Materials and methods. Cells were then treated with or without PDBu for indicated time periods. Western blot analyses were performed with total cell lysates. Results are representative of 3 independent experiments.

Fig. 6.
The effects of PDK1 and mTOR inhibition on PDBu-induced downregulation of PKC δ in HeLa/CP cells. **A**, Cells were pretreated with 25 μ M Ly294002 or 50 nM rapamycin for 45 min and then treated with 1 μ M PDBu for 16 h or PDBu was added 10 min or 30 min prior to harvesting cells. **B**, Cells were transfected with non-targeting siRNA (Con) or siRNA against PDK1 and treated with or without PDBu for 15 min or 18 h. Western blot analyses were performed with total cell lysates using indicated antibodies. Results are representative of 2 separate experiments.

Fig. 7. Effects of rictor, raptor and mTOR knockdown on PKC δ phosphorylation and downregulation. HeLa/CP cells were transfected with non-targeting siRNA (Con) or siRNA against rictor, raptor or mTOR and then treated with 1 μ M PDBu for 8 h. Western blot analyses were performed with total cell lysates. The blot was probed with GAPDH to control for loading differences. Results are representative of 2 independent experiments.

Fig. 8. Effects of PKC ϵ and rictor knockdown on PKC δ phosphorylation and downregulation. HeLa/CP cells were transfected with non-targeting siRNA (Con) or siRNA against PKC ϵ , rictor or both. Cells were treated with or without PDBu for indicated time periods and Western blot analyses were performed with total cell lysates. The blot was probed with GAPDH to control for loading differences. Results are representative of at least 2 independent experiments.