Multisite dephosphorylation and desensitization of conventional protein kinase C isotypes

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The generation of antisera specific for the priming phosphorylation sites on protein kinase C α (PKC α) has permitted analysis of the dephosphorylation of these sites in relation to the down-regulation of the protein. It was demonstrated that these priming sites are subject to agonist-induced dephosphorylation, consistent with inactivation of the protein. Further, the process is shown to be blocked by a PKC inhibitor, indicating a requirement for PKC catalytic activity. This was corroborated by showing that a constitutively active fragment of PKC α is able to stimulate the dephosphorylation of wild-type PKC α in transfected cells. Consistent with a membrane-traffic event, the process controlled by PKC that leads to dephosphorylation is shown to be temperature-

sensitive and to correlate with transient accumulation of PKC α on cytoplasmic vesicular structures. It was established that the dephosphorylation of priming sites in PKC α is not unique and occurs with other conventional PKC isotypes, demonstrating that this is a general desensitization process for this subclass of kinases. The physiological importance of this desensitization is evidenced by the behaviour of PKC β 1 in U937 cells, where dephosphorylation of the activation loop site is shown to be a function of cell density.

Key words: protein phosphatase, vesicle traffic.

INTRODUCTION

The activation of protein kinase C (PKC) by diacylglycerol (DAG) has provided a cornerstone to a broadly employed signalling pathway [1–3]. DAG is produced acutely in response to agonists through activation of phosphoinositide-specific phospholipase C [4], as well as from phosphatidylcholine during more prolonged phases of DAG elevation [5]. That members of the PKC family are indeed activated by DAG *in vivo* has been established through the use of short-chain membrane-permeant DAG species (see [6]). Furthermore, the membrane-associated active PKC complex remains membrane-bound on hypotonic extraction (see [7]), permitting correlations to be made between agonist-induced DAG accumulation and PKC translocation. This allosteric control of PKC operates in concert with control through compartmentalization directed by interaction with targeting proteins, many of which recognize buried epitopes and interact exclusively with activated PKC (reviewed in [8,9]). That localization is important in PKC function has been established through use of translocation inhibitors [10] and analysis of behaviourally altered cell lines [11,12]. Such spatial control is a common feature of a number of key signal transducers (see [13]).

In addition to allosteric and compartmental control of PKC, it has become apparent that, at least for members of the conventional PKC (cPKC) class (α , β_1 and β_2), a series of conserved phosphorylation sites is occupied [14–17]. One of these is located in the activation loop in a region found to be phosphorylated in many different protein kinases and has been shown to be limiting for phosphotransfer in the cAMP-dependent protein kinase (see [18]); phosphorylation of cPKC in this domain is essential for catalytic activity [19,20]. Evidence indicates that, for cPKC, this activation loop phosphorylation does not occur through autophosphorylation [19], but involves a saturable 'PKC

kinase' probably polyphosphoinositide-dependent kinase 1 ('PDK1') [21,22]. Phosphorylation at the two well-defined C-terminal sites plays a more complex role in controlling the net phosphorylation and dephosphorylation rates [16,17] and, at least for PKC β , in controlling Ca²⁺ affinity [23]. While there is little evidence to date that the occupation of these sites in cPKCs is acutely increased, there is evidence that PKC α does become dephosphorylated in an agonist-dependent manner on prolonged stimulation [24,25], although the sites relevant to this process have yet to be defined. This process of dephosphorylation precedes, and appears to be necessary for, the subsequent degradation of PKC [24,25], which can occur via a ubiquitination and proteasome pathway [26,27]. However, the neutral-detergent insolubility of the dephosphorylated form of PKC α that appears has precluded analysis of its activity.

In order to understand the mechanisms involved in this primary desensitization response, we describe here the development and characterization of antisera selective for recognition of the three 'priming' phosphorylation sites in PKC α . These have been employed to monitor the induced dephosphorylation of PKC α , providing indirect evidence for inactivation of the protein prior to degradation. Further, the data establish that dephosphorylation is a feature common to cPKC isotypes, and evidence is provided that PKC-induced membrane traffic is required to facilitate PKC dephosphorylation.

EXPERIMENTAL

Peptide antigens and antibody production

The following peptides were synthesized by the Imperial Cancer Research Fund Peptide Synthesis Laboratory: VTTRT(P)FCG, PVLT(P)PPDQ, FGFS(P)YVNP and VELT(P)TDK [where (P) indicates phosphorylation]; dephosphorylated counterparts were

Abbreviations used: DAG, diacylglycerol; GFP, green fluorescent protein; (c)PKC, (conventional) protein kinase C; PKM α , PKC α catalytic domain fragment; PP2A, protein phosphatase 2A; T(P)497, antiserum selective for phosphorylated Thr⁴⁹⁷ [T(P)638 and S(P)657 are similarly defined].

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also synthesized. Peptides were coupled to keyhole-limpet haemocyanin and rabbits were immunized as previously described [28]. Other PKC antisera were as described elsewhere [28,29].

Cell culture and transfection

Expression vectors for wild-type PKC α , PKM α and PKC α phosphorylation-site mutants were as described previously [16,17]. PKC β_1 , PKC β_2 and PKC γ cDNAs were as described elsewhere [30]; these were subcloned into the expression vector pCO2 for the transfection experiments described. COS-7 cells were maintained at 37 °C in an air/CO $_2$ (9:1) atmosphere in Eagle's medium (E4) supplemented with 10% (v/v) foetal-calf serum. Transfections were carried out by calcium phosphate precipitation as described previously [31]. Treatments were initiated 48 h post-transfection. Where indicated, cells were exposed to 400 nM PMA ('tetradecanoylphorbol acetate', 'TPA'), with other additions as described in the text or Figure legends. For the experiments shown in Figure 6 (below), cells were incubated at 18 °C for 1 or 2 h as indicated. For some treatments, cells were switched back to 37 °C for 1 h.

U937 cells were maintained in RPMI medium with 10% foetal-calf serum under air/CO $_2$ (19:1). Cells were routinely seeded at 0.5×10^5 /ml and were passaged when subconfluent. For the experiments involving high-density cultures, cells were allowed to grow through to confluence ($\approx 1.2 \times 10^6$ /ml) and maintained without refeeding at this density [32]. Extraction of Triton X-100-soluble and -insoluble fractions from U937 cells was performed essentially as described previously [12], except that 1% (v/v) Triton X-100 was included in the initial extraction buffer and the insoluble fraction generated was extracted directly with Laemmli sample buffer.

Western analysis

Transfected cells were rinsed in PBS at 4 °C and lysed in Laemmli sample buffer [33]. For Western blotting, samples were run routinely on 10%-(w/v)-acrylamide/0.3%-(w/v)-methylenebisacrylamide resolving gels, followed by transfer as described previously [24]. Where indicated (Figures 1 and 5 below) samples were separated on low-bisacrylamide gels to optimize resolution of the phospho- and dephospho-PKC α species as described previously [16].

Antisera were used routinely at 1:2000 in 0.1% (v/v) Tween 20 in PBS, with incubation overnight at 4 °C. Immunoreaction was detected using anti-rabbit IgG-horseradish peroxidase or anti-mouse IgG-horseradish peroxidase conjugates and enhanced chemiluminescence (ECL[®], Amersham) according to manufacturer's protocol. Quantification of immunoreactive proteins was carried out by scanning and image analysis.

Localization of green fluorescent protein (GFP)-PKC α

PKC α was subcloned into the *Eco*R1 sites of a pZeoSV derived vector (Invitrogen), containing the coding sequence GFP, permitting in-frame fusion of GFP at the N-terminus of PKC α (details are available from P.J.P. on request). For transfections, COS-7 cells were seeded on coverslips at 2.5×10^5 cells per 35 mm-diameter plate and were grown for 18 h in 2 ml of Dulbecco's modified Eagle's medium containing 10% foetal-calf serum. The cells were transfected by addition of 250 μ l of a calcium phosphate solution (140 mM NaCl/5 mM KCl/0.75 mM Na $_2$ HPO $_4$ ·2H $_2$ O/6 mM glucose/25 mM Hepes/125 mM CaCl $_2$) containing 10 μ g of the PKC α construct. After 6 h, cells were transferred to fresh medium and incubated for a further 20 h at 37 °C. Incubations were then continued either at 37 °C or

18 °C as indicated in the Figures, in the presence or absence of PMA (10 nM) for 0, 10 or 120 min.

Following treatments, cells were washed twice in PBS and then fixed in 4% (w/v) paraformaldehyde in PBS. Coverslips were washed again twice in PBS and mounted in Mowiol (Calbiochem) containing 2.5% (w/v) 1,4-diazabicyclo[2,2,2]octane (Sigma). Confocal images were acquired on a confocal laser scanning microscope (model LSM410; Carl Zeiss Inc.) equipped with a $63 \times /1.4$ Plan-APOCHROMAT oil-immersion objective. The fluorescent tag Cy2 (Amersham) was excited with the 488 nm line of the argon laser. Each image represents a two-dimensional projection of sections in the Z-series, taken across the depth of the cell at 0.5 μ m intervals.

Other methods

Baculovirus production of recombinant PKC α , PKC β_1 , PKC β_2 and PKC γ were as described previously [34]. Protein determination was according to Bradford [35], with BSA as a standard. All experiments were been performed on at least three independent occasions, except for the data in Figure 7 (below; see the legend).

RESULTS

Previous evidence has indicated that phorbol ester (PMA) treatment of COS-7 cells induces the dephosphorylation of PKC α over a 1–2 h period [24]. In order to determine the phosphorylation sites involved, antisera specific to each of the three priming phosphorylation sites were generated. Initial screens for the efficacy of these sera employed PKC α -transfected COS-7 cells untreated or treated with PMA for 2 h. Extracts were prepared and run on highly resolving SDS/PAGE gels [16]. Western analysis of whole-cell extracts using MC5 (PKC α polypeptide) or site-specific antisera was performed. As exemplified in Figure 1 for an antiserum selective for phosphorylated Ser⁶⁵⁷ [S(P)657]-specific serum, immunoreaction was observed for the control sample only and not the dephosphorylated faster-migrating protein; by contrast, MC5 detected both phospho and dephospho forms. The specificity of these sera was further assessed by employing phosphorylation-site mutants previously described [16,17]. Lysates from transfected COS-7 cells were analysed with MC5, T(P)497, T(P)638 and S(P)657 site-specific sera. While wild-type PKC α was detected by all antisera,

Figure 1 Selective recognition of phosphorylated PKC α by S(P)657-specific antisera

Western blots for PKC α from transfected COS-7 cells are shown. PKC α protein was detected using the MC5 monoclonal antibody and phosphorylation at the Ser⁶⁵⁷ site using the S(P)657 antiserum (see the Experimental section). Prior to harvest, cells were treated for 0 or 2 h with 400 nM PMA ('tetradecanoylphorbol acetate', 'TPA') as indicated. The arrows indicate the slow-migrating phospho ($\leftrightarrow$) and dephospho ($\leftarrow$) species. This is one of 15 similar experiments.

Figure 2 Phosphorylation-site-specific antisera do not detect phosphorylation-site mutants

Western blots of COS-7 cells extracts expressing wild-type PKC α (wt), PKC α Thr⁴⁹⁷ → Glu (T497E), PKC α Thr⁶³⁸ → Ala (T638A) and PKC α Ser⁶⁵⁷ → Ala (S657A) mutants are shown. PKC α was detected with MC5. The site-specific antisera are as indicated to the right of each panel. The arrows signify the migration of the 80 kDa PKC α protein. Similar results have been obtained on at least four independent occasions.

Figure 3 PKC α dephosphorylation at the Thr⁴⁹⁷ and Ser⁶⁵⁷ sites precedes degradation

Western blots of PKC α -transfected COS-7 cells are shown. Cells were treated for 0, 1 or 2 h with PMA ('TPA') prior to harvest. Extracts were blotted with MC5 (to detect PKC α) or the two site-specific antisera shown. The arrows indicate the migration of the 80 kDa PKC α protein. This is one of three independent experiments.

the site-specific sera did not react with the phosphorylation-site mutants particular for each phosphopeptide (Figure 2). For the Ser⁶⁵⁷ → Ala PKC α mutant, no immunoreaction was observed with the T(P)638-specific serum, in addition to the lack of recognition of the Thr⁶³⁸ → Ala mutant. In these experiments the faster-migrating form of Ser⁶⁵⁷ → Ala PKC α was the dominant species (as indicated in the Figure), and we have found that this species is not phosphorylated at position 638 (F. Bornancin and P. J. Parker, unpublished work). These antisera thus react with the appropriately phosphorylated PKC α only.

These phosphorylation-site-specific antisera were employed to address the nature of, and mechanisms involved in, agonist-induced dephosphorylation of PKC α . For these studies the T(P)497 and S(P)657 sera were employed throughout, since the T(P)638 sera reacted weakly in whole-cell extracts. Following PMA (400 nM) stimulation of PKC α -transfected COS-7 cells, PKC α became dephosphorylated at both the 497 and the 657 phosphorylation site (Figure 3). Quantification of multiple

Figure 4 PMA-induced dephosphorylation of PKC α is blocked by PKC inhibition

The dephosphorylation of PKC α in transfected COS-7 cells was monitored by Western blotting with the T(P)497- and S(P)657-specific antisera. Cells were exposed to PMA ('TPA') (+) for 2 h in the presence of increasing concentrations (1 nM to 10 μ M) of Ro31-8220 (Ro) as indicated. For comparison, cells (—) not treated with PMA are shown in the first lane. The arrows indicate the migration of the 80 kDa PKC α protein. This inhibition has been reproduced in three independent experiments.

experiments ($n = 5$) has indicated that dephosphorylation is consistently more than 80 % complete after 1 h of treatment, while there is little change in total PKC α concentration. By 2 h of exposure to PMA there is a significant loss of PKC α protein (≈ 40 %). The loss of phosphate at Thr⁴⁹⁷ site provides evidence that PKC α is inactivated under these conditions, since phosphorylation at this site has been shown to be essential for activity [19].

To elucidate the mechanism involved in the PMA-induced dephosphorylation of PKC α , site-specific antisera were employed to monitor the effects of antagonists. We have established in a heterologous system that degradation of PKC is induced in an active fashion following PKC activation [36]. Since dephosphorylation precedes degradation [24,25], we determined the PKC activity requirement for dephosphorylation, using the PKC inhibitor Ro31-8220, which is a broad-specificity PKC inhibitor [37]. It is evident that Ro31-8220 inhibits PMA-induced dephosphorylation in wild-type PKC α -transfected COS-7 cells (Figure 4). The dose-dependency of inhibition by Ro31-8220 is consistent with its ability to inhibit PKC activity. A block to PKC α dephosphorylation was observed also for the PKC inhibitor bisindolylmaleimide I (results not shown).

The PKC inhibitor employed above is not entirely specific for PKC [38] and provides only consistent evidence on the involvement of catalytic activity. To establish this point directly, the constitutively active kinase domain of PKC α (PKM α) was employed. Co-expression of PKM α with wild-type PKC α induced the dephosphorylation of the latter (Figure 5). This effect is illustrated both by immunoreactivity with the T(P)497 antiserum (upper panel) and by monitoring the altered migration of the protein on SDS/PAGE (lower panel). This response was specific to active PKM α , since the kinase-inactive Lys³⁶⁸ → Met PKM α mutant [39] had no effect on wild-type PKC α phosphorylation (results not shown).

Previous studies have shown that down-regulation (i.e. degradation) of mammalian PKC expressed in *Schizosaccharomyces pombe* correlates with PKC activity-induced alterations in vesicle traffic [36]. The relevance of a budding/fusion step in PMA-induced dephosphorylation of wild-type PKC α in mammalian cells was assessed using transfected COS cells and temperature shift. Cells maintained at 18 °C (to inhibit trafficking processes)

Figure 5 Catalytically active PKM α induces wild-type PKC α dephosphorylation

Western blots of PKC α - and PKM α -transfected COS-7 cells are shown. PKC α protein was detected with MC5 and phosphorylation monitored with the T(P)497-specific antiserum. Extracts were fractionated into Triton X-100-soluble (S) and insoluble (P) fractions prior to SDS/PAGE, which was carried out in low bisacrylamide to effect resolution for the phospho and dephospho PKC α species (see the Experimental section). The left-hand lanes are from cells transfected with PKC α alone and the right-hand lanes for cells transfected with both PKC α and PKM α . The solid arrows represent the migration of phospho-PKC α and the broken arrow that of dephospho-PKC α . This is one of five similar experiments.

Figure 6 PMA-induced dephosphorylation of PKC α is temperature-dependent

(A) PKC α -transfected COS-7 cells were treated with 400 nM PMA ('TPA') for the times (in h) shown. Cells were maintained at 37 or at 18 °C, or 18 then 37 °C, as indicated. The phosphorylation state of PKC α was monitored with the T(P)497- or S(P)657-specific antisera. The arrows denote the migration of the 80kDa PKC α (phospho) species. (B) Cells treated as in (A) were subjected to SDS/PAGE in low-bisacrylamide gels (see the Experimental section) and analysed by Western for PKC α protein using the MC5 monoclonal antibody. The phospho- and dephospho-PKC α are indicated by solid and broken arrows respectively. This is one of three similar experiments.

display a decreased PMA-induced dephosphorylation of PKC α , as judged either by loss of T(P)497 and S(P)657 immunoreactivity or altered migration on SDS/PAGE (Figures 6A and 6B). However, on maintenance at 37 °C, efficient dephosphorylation was observed. This temperature-sensitive inhibition was reversible, since shiftback from 18 to 37 °C induced efficient dephosphorylation of PKC α . Direct evidence that PMA-induced dephosphorylation correlates with a traffic-dependent step in mammalian cells is provided by analysis of GFP-PKC α localization. In transfected COS-7 cells, GFP-PKC α shows a diffuse

Figure 7 Vesicular localization of GFP-PKC α in PMA-treated COS-7 cells

COS-7 cells were transfected with GFP-PKC α as described in the Experimental section. Cells were treated 48 h later with PMA (10 nM) for the times indicated and were maintained at 37 or 18 °C as shown. At 37 °C cells accumulated intensely fluorescent vesicles between 10 and 30 min after stimulation (see left-hand middle panel and inset); at 18 °C, no accumulation of the GFP-PKC α on vesicles was observed over the entire 2 h period. The vesiculation has been observed in more than 20 independent experiments; the effect of temperature shown is from one of two independent experiments.

cytoplasmic location with moderate concentration in a perinuclear compartment (Figure 7). Following PMA treatment at 37 °C, cells accumulate GFP-PKC α on vesicular structures. The precise timing of this response varies between cells, but is optimum between 10 and 30 min. This is evident in Figure 7, where two adjacent transfected cells display either intense vesicular fluorescence within 10 min of treatment (lower cell), or much less intense vesicular staining proximal to the plasma membrane (upper cell). By 2 h following treatment, when the protein has become largely dephosphorylated (see above), the GFP-PKC α is located predominantly in a perinuclear compartment. GFP itself displays a diffuse cytoplasmic and nuclear distribution that does not alter on PMA treatment (results not shown).

In untreated cells at 18 °C, GFP-PKC α shows a diffuse cytoplasmic location similar to cells maintained at 37 °C. However, following PMA treatment there is some loss of perinuclear signal with concomitant accumulation at the plasma membrane. This latter localization is more evident at 2 h, as is an accumulation of nuclear GFP-PKC α . At no stage following treatment with PMA do vesicles accumulate in the cells held at 18 °C, contrasting clearly with those at 37 °C. Thus, under conditions

Figure 8 PKC α phosphorylation-site-specific antisera detect the phosphorylation of baculovirus-expressed cPKCs

(A) An alignment of sequences for the four cPKC isotypes is shown from subdomain VII (DFG motif) to the C-termini. The three predicted phosphorylation sites are indicated by the arrows. Identical (★) and conserved (.) residues are shown under the alignment. (B) Baculovirus-expressed cPKCs α , β_1 , β_2 , and γ were subjected to Western analysis using the three PKC α phosphorylation-site-specific antisera. The arrow indicates the migration of the 80 kDa PKC α protein; PKC β_2 migrates slightly faster, and PKC γ slower, than PKC α . These Western blots are representative of three (PKC γ) or five (PKC $\beta_{1/2}$) independent experiments.

where the dephosphorylation is inhibited (at 18 °C), there is also an inhibition of PMA-induced vesicle accumulation. The more effective block in traffic (Figure 7) compared with dephosphorylation (Figure 6) implies that the plasma-membrane-associated PKC α can be dephosphorylated, but that this is inefficient compared with dephosphorylation in a distal compartment.

It has been established that cPKC α , cPKC β_1 and cPKC β_2 are phosphorylated at the equivalent three priming sites [14–17]. Since the primary sequences surrounding these sites and those predicted for PKC γ are largely conserved (Figure 8A), we determined the specificity of the phosphorylation-site-specific antiserum for these cPKC isotypes. It is evident that all the recombinant cPKC isotypes are recognized by these antisera, except for T(P)638 immunoreaction with PKC β_2 (Figure 8B). The sequence surrounding the site in PKC β_2 varies from that of the others with the introduction of a Glu residue at position –2

Figure 9 PMA-induced dephosphorylation and degradation of PKC β_2 and PKC β_1

(A) COS-7 cells were transfected with PKC β_2 were treated with PMA ('TPA'; 400 nM) for 0, 15 or 120 min as indicated. Cells were harvested and extracts subjected to SDS/PAGE. PKC β_2 protein was detected with a C-terminus-specific antiserum and the phosphorylation of the Thr⁵⁰⁰, Thr⁶⁴² and Ser⁶⁶² sites determined through immunoreaction with the T(P)497 (PKC α), T(P)642 (PKC β_2) and S(P)657 (PKC α) antisera. This is one of three independent experiments. (B) COS-7 cells were transfected with PKC β_1 and lysates subjected to SDS/PAGE. PKC β_1 protein was detected with a C-terminus-specific antiserum and the phosphorylation of the Thr⁵⁰⁰ site monitored with the T(P)497 (PKC α) antiserum. This is one of three independent experiments.

and replacement of a proline at +2 (see Figure 8A). Antibodies raised to an epitope specifically based upon the PKC β_2 T(P)642 site, nevertheless confirm this site as being phosphorylated in the recombinant protein (see below).

The behaviour of the non-PKC α cPKC isotypes was determined in order to assess whether or not PMA-induced dephosphorylation of PKC α reflected a general desensitization mechanism for this class of PKC isotypes. PKC β_2 was transiently expressed in COS-7 cells and cells treated with PMA for 0, 15 or 120 min. As shown in Figure 9(A), loss of immunoreaction with the T(P)497, T(P)642 and S(P)657 site-specific sera was faster than the loss of PKC β_2 protein, i.e. dephosphorylation was occurring prior to degradation. PKC γ was found also to undergo dephosphorylation at the T(P)514 and T(P)673 sites; immunoreaction with the T(P)638 serum (which would detect the PKC γ 656 site; see above) was inadequate to determine loss in these experiments (results not shown). In contrast with PKC β_2 , PKC β_1 was degraded too rapidly in response to PMA treatment to assess whether or not dephosphorylation had occurred prior to protein loss (Figure 9B).

We have established previously that the accumulation of a fast migrating, apparently dephosphorylated, form of PKC β_1 correlates with apoptosis in U937 cells [32]. To determine whether the activation loop site in PKC β_1 was affected in this process, we have blotted cell extracts from exponential-phase and high-density cultures of U937 cells with an antibody that recognizes the phosphorylated form of this site (see above). As shown in Figure 10, the antiserum to the protein itself recognizes two species in the insoluble fraction from high-density maintained cells that are slow- and fast-migrating. Of these two species, only the slow-migrating form is recognized by the phospho-specific antiserum. Previous studies demonstrated that the unphosphorylated primary translation product of the closely related PKC α is soluble [17], while the dephosphorylated form is

Figure 10 In high density U937 cells $\text{PKC}\beta_1$ becomes dephosphorylated at priming sites

U937 cells were grown to high density (H), harvested and separated into Triton X-100-soluble (S) and -insoluble (P) fractions; these were denatured in Laemmli sample buffer. For comparison, exponential ('log')-phase cells (L) were extracted directly into Laemmli sample buffer. $\text{PKC}\beta_1$ was detected with an antiserum to the C-terminus ($\text{PKC}\beta_1$) and the Thr^{500} phosphorylation with an antibody to the phosphorylated activation loop site [T(P)500]. This is one of three independent experiments.

insoluble [24]. The exclusive presence of the fast-migrating $\text{PKC}\beta_1$ in the insoluble fraction thus indicates that the protein becomes dephosphorylated in these dense cultures.

DISCUSSION

The results described here provide evidence that the PMA-induced dephosphorylation of $\text{PKC}\alpha$ occurs at defined priming sites. This is consistent with the notion that PMA-induced dephosphorylation is an inactivation process, since these sites have been shown to be important in cPKC function, in particular the activation loop site [19,20]. The trigger for dephosphorylation relies upon activation of PKC itself. This is not a passive, conformational requirement (although this may contribute to phosphatase sensitivity [24]), but an active catalytic one. The temperature-sensitivity of the process is consistent with the membrane trafficking of $\text{PKC}\alpha$ to a compartment where dephosphorylation is efficient. Direct evidence for a traffic step is provided by analysis of GFP- $\text{PKC}\alpha$ distribution following a PMA stimulus; the temperature-sensitivity of the vesicular localization observed is entirely consistent with the dephosphorylation data. It is also demonstrated that these three priming sites are conserved throughout the cPKC class and further that PMA-induced dephosphorylation of these sites is not peculiar to $\text{PKC}\alpha$ alone. Finally, we demonstrate that dephosphorylation is not the exclusive property of pharmacological agents, since dephosphorylation of $\text{PKC}\beta_1$ can occur in U937 cells without intervention. A summary of the 'processing' of the $\text{PKC}\alpha$ priming sites is provided in Scheme 1.

It is now well established that numerous kinases are phosphorylated in predicted loops linking conserved kinase domains VII and VIII [40]. This appears to be required for optimal catalytic activity and in many, but not all, cases is subject to acute regulation. In cPKC isotypes it has been established that phosphorylation in this activation loop is required for activity [19,20]. While not essential for activity, at least two other phosphorylation sites in the C-terminal (V5) domain of cPKC isotypes exist [14–17] and these play more subtle roles in control, at least for $\text{PKC}\alpha$ [16,17]. In effect the three phosphorylated residues in $\text{PKC}\alpha$ (Thr^{497} , Thr^{638} and Ser^{657}) maintain the protein in a closed, active conformer [16,17] that is functionally suppressed through occupation of the substrate-binding site by its pseudosubstrate site [41,42]. The absence of phosphate at any one of these sites promotes an open conformer that is phosphatase-sensitive, protease-sensitive and sensitive to oxidation [16,17]. These phosphorylations thus act in a co-operative fashion to maintain the active (latent) conformation. Additionally, in $\text{PKC}\beta_1$ the phosphorylation of the Ser^{660} site has been shown to

Scheme 1 $\text{PKC}\alpha$ phosphorylation and dephosphorylation

$\text{PKC}\alpha$ is synthesized as a soluble unphosphorylated protein [17] that accumulates phosphates at three priming sites: Thr^{497} , Thr^{638} and Ser^{657} . This phosphorylated species represents that usually found in cultured cells, either in the cytosol or, on activation, associated with membranes. Following sustained activation, $\text{PKC}\alpha$ promotes its own dephosphorylation through a temperature-dependent step that is a consequence of membrane traffic. The dephospho- $\text{PKC}\alpha$ is partly sequestered in a detergent-insoluble compartment, but the bulk is degraded.

influence Ca^{2+} affinity [23], consistent with the notion of a closed conformation involving catalytic-regulatory domain interactions.

In intact cells, dephosphorylation of $\text{PKC}\alpha$ has been shown to occur in an agonist-dependent manner [24,25] and to preclude degradation via a ubiquitin pathway [27]. Circumstantial evidence indicates that the dephosphorylation of $\text{PKC}\alpha$ is catalysed by a membrane-associated heterotrimeric complex of protein phosphatase 2A (PP2A) [24]. However, while complete inactivation of $\text{PKC}\alpha$ can be observed on dephosphorylation *in vitro*, the extent and nature of agonist-dependent dephosphorylation *in vivo* has not been determined. This is an important issue, since the dephosphorylated form of $\text{PKC}\alpha$ that accumulates is neutral-detergent-non-extractable and cannot be solubilized in a native state in order to assess activity. Thus it has not been clear previously whether the dephosphorylated protein is actually inactivated prior to its degradation. Through the development of the site-specific sera for $\text{PKC}\alpha$, we clearly establish that PMA-induced dephosphorylation is essentially complete at the key activation loop site, indicative of inactivation. Ser^{657} is also shown to be dephosphorylated in response to PMA. For $\text{PKC}\alpha$, the third priming site, Thr^{638} , has proven more difficult to assess

because antisera are less effective; nevertheless this site appears to be dephosphorylated in response to PMA (results not shown). The complete dephosphorylation in response to PMA is more clearly established for PKC β_2 , where all three priming sites are removed in parallel. Thus PMA-induced dephosphorylation of cPKCs is the primary desensitization response.

Previous studies on PKC down-regulation have provided evidence for the involvement of both passive and active events [36,39,43,44]. The active events correlate with altered vesicle traffic, albeit established in a heterologous cell context [36]. It is demonstrated here that the initiating dephosphorylation event is itself part of an active process, since dephosphorylation is blocked by PKC inhibitors and can be promoted by a catalytically active fragment of PKC α . The nature of the substrate(s) involved in promoting dephosphorylation is unknown, although it has been shown that there is no detectable change in the concentration or activity of the membrane-associated PP2A heterotrimeric complex [24]. On the basis of the effect of temperature shift on dephosphorylation and vesicular PKC α accumulation, in conjunction with previous studies in yeast [36], the regulated process that is required appears to be a membrane-traffic event that presumably causes effective co-localization of PKC α and PP2A. While an 'internalization' process is a likely possibility, blocking acidification of the endosomal compartment does not interfere with PMA-induced dephosphorylation of PKC α (G. Hansra and P. J. Parker, unpublished work). This contrasts with the dephosphorylation of the internalized β -adrenergic receptor, where endosomal acidification appears to be required for PP2A-catalysed dephosphorylation [45]. It has been argued that for the β -adrenergic receptor this acidification is required to alter receptor conformation, permitting dephosphorylation [45]. This is not a requirement for PKC α , since PMA interaction itself sensitizes the protein to phosphatases, and further the orientation of PKC α itself would not make it directly sensitive to endosomal acidification. The precise compartment involved in the PKC α dephosphorylation remains to be determined.

The generation of site-specific sera for PKC α has facilitated analysis of the behaviour of the other cPKC isotypes. The conservation of sequence at the phosphorylation sites in PKC β_1 , PKC β_2 and PKC γ is sufficient in all but one case to permit immunodetection with the reagents raised towards the PKC α sites. The sequence around T(P)642 site in PKC β_2 is divergent, requiring a further serum to be developed for immunodetection. These antisera have allowed us to establish that PMA induces the time-dependent dephosphorylation of PKC β_2 similar to its effect on PKC α . It is notable that PMA-induced degradation of PKC β_2 is faster than that of PKC α in these cells; however, dephosphorylation still precedes degradation (Figure 8A) as was also found for PKC γ (results not shown). PKC β_1 is degraded very rapidly in these cells in response to PMA. Protein loss effectively parallels loss of phosphorylation-site immunoreactivity (Figure 8B); for this isotype it is unclear whether dephosphorylation precedes degradation in this context.

It is noteworthy that PKC β_1 and PKC β_2 display distinct rates of degradation. These splice variants differ only in their C-terminal V5 domain, and even here they still retain $\approx 50\%$ identity. Since two of the three priming sites reside in this domain, dephosphorylation of PKC β_1 may have been more rapid than PKC β_2 , leading to a faster degradation. However we have shown elsewhere, using site-specific mutants of PKC α , that a mutant undergoing rapid dephosphorylation is degraded at the same rate as wild-type PKC α [24]. Thus while dephosphorylation seems to be required for degradation, it is not necessarily rate-limiting. It is concluded that the distinction between PKC β_1 and PKC β_2 is conferred by specific recognition of the different V5

domain sequences by a component(s) of the desensitization pathway.

PKC γ has not been analysed previously with respect to 'priming' phosphorylation, although studies on this isotype have demonstrated a requirement for priming in some manner [46]. It is shown here that baculovirus-produced PKC γ is reactive with sera recognizing T(P)514 [equivalent to α T(P)497], T(P)655 [equivalent to α T(P)638], and T(P)674 [equivalent to α S(P)657], indicating that these sites are at least partially occupied. As observed for PKC α , PMA treatment of PKC γ -transfected COS-7 cells induced dephosphorylation of PKC γ at these priming sites. Thus it appears that cPKC isotypes typically undergo agonist-induced dephosphorylation.

It has become apparent that the down-regulation of PKC is not a property restricted to pharmacological activation, but reflects the chronic operation of a cellular process, i.e. agonists causing sustained elevation of the endogenous activator, DAG, can also induce PKC degradation (see, for example, [47]). In line with this we demonstrate here that these dephosphorylation responses are relevant physiologically and can occur in U937 cells without drug treatments. As discussed above, the dephosphorylation of the cPKC isotypes precedes their degradation, and it will be important to assess this aspect of desensitization which would not be observed by simply following degradation. The site-specific antisera described here will no doubt facilitate such analyses in conjunction with isotype-specific reagents. The importance of these phosphorylations to PKC function and cellular properties is clearly evidenced by the use of a PKC α Thr⁴⁹⁷ mutant that shows dominant properties in blocking phosphorylation of another cPKC isotype [48,49]. Expression of this PKC α mutant induces apoptosis in COS-7 cells, demonstrating that the phosphorylation is important for cPKC function *in vivo*, and further that the consequence of function can have an essential role in survival [32].

In summary, it is concluded that all cPKC isotypes retain the same three priming phosphorylation sites and that these are subject to agonist-dependent dephosphorylation *in vivo*. The mechanism of dephosphorylation requires a PKC-dependent phosphorylation step that most likely involves membrane traffic of the PKC itself. The temporal restriction imposed on PKC function by its induced dephosphorylation and inactivation is anticipated to play a key role in determining cellular responses.

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