Dephosphorylation of PKCδ by protein phosphatase 2Ac and its inhibition by nucleotides

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Abstract The protein phosphatases $PP1_c$, $PP2A_c$ and PP2Cα are shown to dephosphorylate protein kinase Cδ (PKCδ) in vitro; of these $PP2A_c$ displayed the highest specific activity towards PKCδ. The role of $PP2A_c$ in the dephosphorylation of PKCδ in cells was supported by the demonstration that these proteins could be co-immunoprecipitated from NIH3T3 cells. However the observation that binding of Mg-ATP to PKCδ could protect the enzyme from dephosphorylation by $PP2A_c$ in vitro indicates that an additional input/factor is required for dephosphorylation in vivo.

Key words: Protein kinase Cδ; Protein phosphatase; PP1; PP2C; PP2A; Dephosphorylation

1. Introduction

The protein kinase C (PKC) family of lipid-dependent serine/threonine kinases are known to play key roles in a whole range of cellular functions, including cell growth, differentiation and apoptosis. To unravel the molecular mechanisms underlying the diverse roles of the different members, it is important to define how each PKC isoform is regulated. In this regard much effort has been directed at defining the effectors involved in receptor-dependent activation. However it is evident that regulated loss of function, i.e. desensitisation, is critical in the response of cells. This is exemplified by the action of the PKC activator bryostatin I which can block responses to the PKC activator tetradecanoyl phorbol acetate (TPA), and induces an anomalous biphasic degradation of PKCδ [1]. This demonstrates clearly that the response of cells is dependent on the timing of PKC activation.

For PKCα, induced degradation appears to require the traffic of the membrane-associated protein to a perinuclear region, where dephosphorylation occurs, followed by ubiquitin conjugation and degradation through a proteasomal mechanism [2–5]. Evidence on the degradation of PKCδ indicates that this protein also undergoes dephosphorylation prior to a ubiquitin-dependent degradation [6]. The degradation of PKC (cPKC and nPKC isotypes) is not only a function of chronic pharmacological activation, but occurs in response to sustained activation by hormones and growth factors [7,8]. In particular for PKCδ this process is under cell cycle control. PKCδ overexpression induces a cell cycle arrest consistent with the notion that PKCδ degradation may contribute to cell cycle progression (recently reviewed [9]). However the mechanisms involved in this are unknown.

The mammalian cell contains four subfamilies of serine/threonine protein phosphatases, PP1-like, PP2A-like, PP2B and PP2C, which are responsible for the dephosphorylation of cellular proteins involved in a whole host of functions such as the regulation of cell metabolism, DNA replication, cell cycle control, development and transformation [10]. Both PP1 and PP2A exist in the cell as a variety of holoenzyme complexes, and are major phosphatases in the cell [11,12]. Dimeric PP2B, otherwise known as calcineurin, requires Ca^{2+}/calmodulin binding for activation and hence is an important player in Ca^{2+} signalling [13]. Unlike the above protein phosphatases, PP2C is a monomeric protein that is characterised by its dependence on Mg^{2+} or Mn^{2+} for activity. Although the functional roles of mammalian PP2C are not well defined, it is known to associate with p38 kinase and inhibit the p38 and JNK stress-activated MAP kinase pathway [14]. Studies to date analysing the mechanism of dephosphorylation of PKC have implicated the role of phospho-serine/threonine protein phosphatase PP2A. A heterotrimer form of PP2A was seen to dephosphorylate PKCα in vitro and inhibitors to PP2A have been shown to induce hyperphosphorylated forms of PKCα (and also PKCδ and ε) [2,3,15,16].

The studies here describe the specificity of protein phosphatases in vitro towards the dephosphorylation of PKCδ. This indicates that PP2A is likely to play the key role in vivo and this conclusion is supported by demonstration that PKCδ and PP2A can be co-immunoprecipitated from cells. However the protective effect of Mg-ATP in vitro implies that there must be a further regulatory step in initiating dephosphorylation in vivo.

2. Materials and methods

2.1. In vitro protein phosphatase treatment of PKCδ

As described previously [17], baculovirus-expressed PKCδ is phosphorylated at three regulatory phosphorylation sites (see Section 3). To assess the dephosphorylation of these sites, baculovirus-expressed recombinant PKCδ (Panvera) was treated with purified protein phosphatases. $PP1_c$ and $PP2A_c$ were purified from rabbit skeletal muscle [18]. The dimeric ($PP2A_D$) and trimeric ($PP2A_T55$ and $PP2A_T72$) holoenzyme forms with respectively PR55/B and PR72/B′ as the third variable subunit, were also purified from rabbit skeletal muscle [19]. PP2Cα was expressed and purified as a GST fusion protein from

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bacteria [20]. A total reaction volume of 50 μl was set up to contain the following: 20 mM Tris–HCl (pH 7.5), phosphatidylserine (PtdSer) and TPA (25 μg PtdSer, 2 μM TPA final), 8 ng–1 μg phosphatase (as indicated), 100–500 ng PKCδ recombinant protein; incubations were carried out at 30°C. The reactions were stopped by the addition of 4×SDS sample buffer, boiled and stored in liquid nitrogen. Samples were separated by SDS–PAGE for subsequent analysis by Western blotting.

2.2. Western blotting

Protein samples in SDS sample buffer were separated on 10% SDS–PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) using a wet transferring system (Bio-Rad). Membranes were blocked for 1 h at room temperature in PBS buffer containing 0.1% Tween 20 and 5% (w/v) skimmed milk powder [21]. When probing for phosphorylation sites, skimmed milk powder was substituted by 3% BSA. Membranes were incubated with primary antibody, either anti-PKCδ monoclonal (Transduction labs.) or polyclonal antiserum (1 in 5000) raised in rabbit against a synthetic peptide [22]. Phosphorylation-specific antisera (1 in 3500) were incubated in the presence of dephospho-peptide overnight at 4°C [23]. The membranes were washed, incubated with secondary antibody conjugated to HRP and cross-reactivity was visualised using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia). For a quantitative comparison of protein bands, an exposure of film within the linear range was scanned with the Adobe UMAX magic scan, and relative band intensities calculated using NIH image software.

2.3. Phosphatase activity assays

Protein phosphatase activity was determined either by using phosphorylase as a substrate as described in the protein phosphatase assay system (Life Technologies), or using *p*-nitrophenylphosphate (*p*NPP) as substrate. Using *p*NPP as substrate, a dilution of the phosphatase was added to a reaction mixture containing 25 mM Tris (pH 7.5), 2 mM $MgCl_2$ and 10 μM *p*NPP in a total volume of 1 ml. The reactions were carried out at room temperature and the optical density (OD) readings were taken at 420 nm at 30 s intervals.

2.4. Cell culture and transfection

NIH3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (60 μg/ml), streptomycin (100 μg/ml), and 10% (v/v) foetal calf serum in a 10% CO_2 humidified incubator at 37°C. The adherent cells were passaged three times a week by detaching cells with 0.25% (v/v) trypsin/versene and seeded at a density of 2×10^5 cells/ml. Cells (35 mm/100 mm plates) were transfected using Lipofectamine reagent (Gibco BRL) with 1–5 μg of plasmid DNA (myc-PKCδ in pEFlink or GFP-PKCδ in pEGFPC3 as indicated) for 6–18 h. Following transfection, the cells were placed in fresh low serum medium (0.05% FCS) and incubated for a further 24 h prior to experimentation. Whole cell extracts were harvested in 4×SDS sample buffer. Samples were boiled to 95°C and stored at −20°C or in liquid nitrogen (when analysing for phosphorylation status of proteins) prior to loading on SDS–PAGE.

2.5. Immunoprecipitation

Following a rinse with cold PBS, cells were scraped into 500 μl lysis buffer (20 mM Tris (pH 7.5), 2 mM EDTA, 0.2 M NaCl, 10 mM benzamidine, 0.2 mM PMSF, 10 μg/ml leupeptin, 100 μg/ml aprotinin, 10 μM microcystin LR and 1% Triton X-100 (Sigma and Alexis)) for a 100 mm dish. Samples were snap frozen in liquid nitrogen and homogenised using a glass Dounce homogeniser. After pre-clearing insoluble material, the lysate was incubated with 5–10 μg of antibody, followed by incubation with protein-G-Sepharose beads (Sigma). The beads–antibody complex was carefully washed three times in lysis buffer, boiled in SDS sample buffer and the immunoprecipitated proteins were subjected to SDS–PAGE. $PP2A_c$ was detected with a monoclonal antibody to $PP2A_c$ (S. Dilworth, manuscript in preparation).

3. Results

3.1. Dephosphorylation of PKCδ in vitro

In order to assess the intrinsic specificity of different classes of protein phosphatase towards PKCδ, purified proteins were tested in vitro. The dephosphorylation of the three critical Ser/Thr phosphorylation sites occupied in baculovirus-expressed PKCδ (T505, S643, S662) was monitored using site-specific antibodies. The phosphatase activities were titrated against PKCδ such that similar activities were compared (note the very different protein concentrations; see below). As illustrated in Fig. 1A–C, the purified catalytic subunits of PP1 (A), PP2A (B) and PP2C (C) all were able to dephosphorylate PKCδ in vitro. Furthermore none of these phosphatases displayed any marked site specificity, although in each case the S643 site, which resides within the sequence ...KPQLS(P)FSDK... was the most rapidly dephosphorylated.

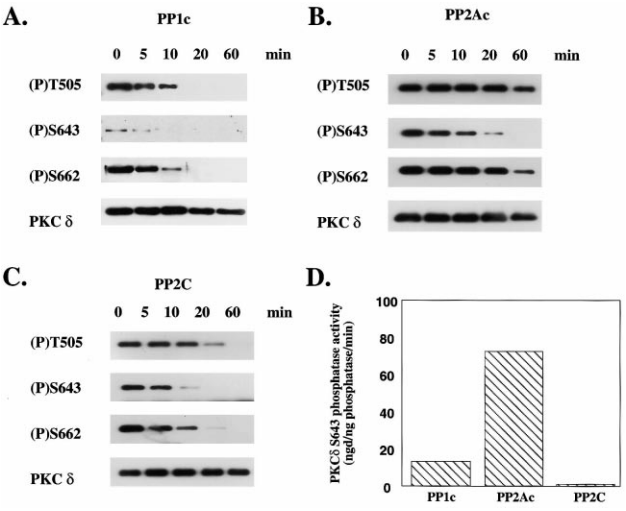

Fig. 1. Effect of protein phosphatases against PKCδ. Purified protein phosphatases were incubated with human recombinant PKCδ (500 ng) at 30°C for the times indicated in the presence of TPA (2 μM) and PtdSer (PS; 25 μg). Phosphatases and concentrations were as indicated in the panels. Reactions were stopped by addition of SDS sample buffer and analysed by Western blotting for phosphorylation at T505, S643 and S662 and also for PKCδ protein load. A: $PP1_c$ 125 ng was used; B: $PP2A_c$ 8 ng; C: PP2C 1000 ng; D: quantitation showing the specific activities of the three phosphatases indicated.

Despite the similar site specificity of these three phosphatases the specific activities varied greatly. Based upon the initial rates of dephosphorylation of PKCδ determined by scanning densitometry of linear exposures, the relative specific activities of PP2C:$PP1_c$:$PP2A_c$ are 1:13:72.

In view of the relative selectivity of $PP2A_c$ for PKCδ the activities of various PP2A holoenzyme forms were tested. The heterodimer and heterotrimeric complexes can have profound effects upon $PP2A_c$ specificity/activity [24–26]. As shown in Fig. 2, none of the oligomeric forms tested showed any significant difference in site selectivity for PKCδ dephosphorylation. Furthermore the relative rate of dephosphorylation of these complexes compared to $PP2A_c$ varied by no more than 1.4-fold.

3.2. Nucleotide inhibition of PKCδ dephosphorylation

The conditions providing optimum dephosphorylation of PKCδ in vitro is the TPA-induced active conformer, consistent with the TPA-induced effect in vivo. However, in vivo, substrates as well as allosteric activators are present. Surprisingly, inclusion of Mg-ATP in the in vitro incubation of PKCδ with PP2A completely blocked PKCδ dephosphorylation (Fig. 3A). This effect of Mg-ATP was not due to rephos-

phorylation of these sites by PKCδ itself or a contaminating kinase activity since it was also observed with Mg-ADP and the non-hydrolysable Mg-AMPPNP. Furthermore although unliganded ATP can inhibit PP2A [27], this was not a direct effect on PP2A itself since at these Mg^{2+} and ATP concentrations no significant inhibition of PP2A phosphorylase A phosphatase activity was observed (Fig. 3B). This potent effect of Mg-ATP in protecting PKCδ from dephosphorylation implies that in vivo either a different phosphatase activity is involved or that a further component/event is required to facilitate dephosphorylation.

3.3. Dephosphorylation of PKCδ in NIH3T3 cells

To distinguish whether or not PP2A is responsible for the activation-induced dephosphorylation of PKCδ in vivo, we investigated this response in intact cells. A role for PP2A action on PKCδ was consistent with the finding that TPA-induced PKCδ dephosphorylation was significantly inhibited at low concentrations of calyculin A (data not shown) or okadaic acid as judged by immunoreactivity and reduced migration of the protein (Fig. 4A). This effect was most evident at the activation loop (T505) and autophosphorylation (S643) sites. The hyperphosphorylated, slower migrating form of PKCδ induced by both okadaic acid (Fig. 4A) and calyculin A (data not shown) is likely to reflect phosphorylation at sites other than T505, S643 and S662, since these migrate more slowly than the initial phosphorylated protein. To assess the one class of protein phosphatase not tested in vitro the effects of the PP2B inhibitor cyclosporin A were investigated [28]. It

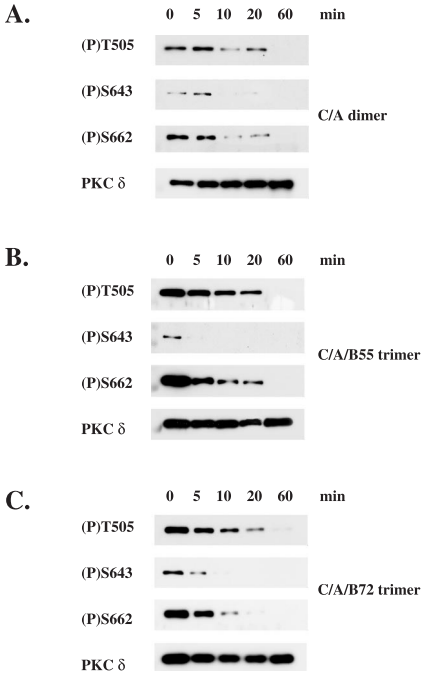

Fig. 2. Dephosphorylation of PKCδ by dimer and trimer forms of PP2A in vitro. Different $PP2A_c$ complexes (100 ng normalised to catalytic subunit content) were incubated with human recombinant PKCδ (50 ng) at 30°C for the times indicated in the presence of TPA (2 mM) and PtdSer (25 mg). Reactions were stopped by addition of SDS sample buffer and analysed by Western blotting for phosphorylation at T505, S643 and S662 and also for PKCδ protein load. A: PP2A (C/A) dimer. B: PP2A (C/A/B55) trimer. C: PP2A (C/A/B72) trimer.

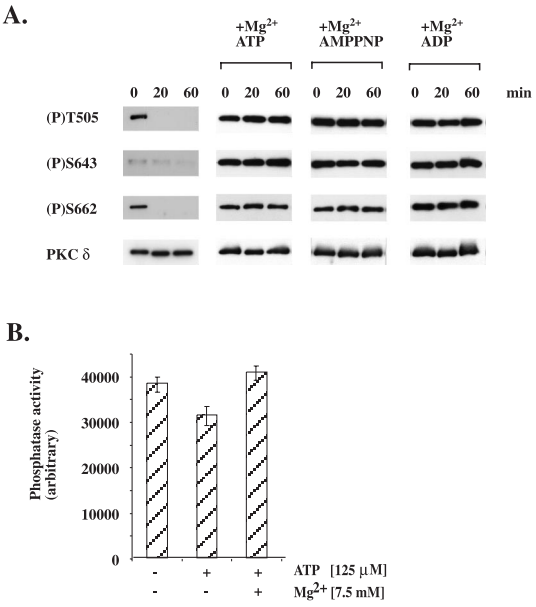

Fig. 3. Nucleotides prevent the dephosphorylation of PKCδ by PP2A. A: Different nucleotides (ATP, AMPPNP, ADP all at 125 μM final) and Mg^{2+} (7.5 mM), were present in the treatment of human recombinant PKCδ (500 ng) with PP2A (100 ng C/A/B55 trimer). Incubations were carried out at 30°C for the times indicated in the presence of TPA (2 μM) and PtdSer (25 μg). Reactions were stopped in SDS sample buffer and analysed by Western blotting for phosphorylation at T505, S643 and S662 and also for PKCδ protein load. This is representative of several similar experiments. B: The phosphatase activity of PP2A (2 ng C/A/B55 trimer, within the linear range) against phosphorylase A was monitored in the presence or absence of Mg^{2+}/ATP as indicated. Four independent experiments were performed.

was found that cyclosporin A did not effect the rate of degradation nor did affect the migration of PKCδ (Fig. 4B).

To determine more directly the potential for PP2A to account for TPA-induced PKCδ dephosphorylation, association between these two proteins was investigated. Subconfluent NIH3T3 cells were maintained in reduced serum overnight and then stimulated with TPA. Lysates were prepared and PKCδ immunoprecipitated and immunoblotted for $PP2A_c$. The 37 kDa catalytic subunit of PP2A was found associated with PKCδ and this was enhanced following TPA stimulation (Fig. 4C). This association supports the notion that PP2A plays the major role in TPA-induced PKCδ dephosphorylation.

4. Discussion

The evidence presented here demonstrates that of the protein phosphatases tested, $PP2A_c$ and complexes thereof are the most efficient in dephosphorylating PKCδ in vitro. Furthermore the effects of inhibitors and more directly the demonstration of a complex between PKCδ and $PP2A_c$ indicate that this catalytic activity is likely to be responsible for activation-induced dephosphorylation in vivo.

All the phosphatases tested displayed similar site specificity with the S643 site being the most sensitive to their action. Given the rather different primary sequence specificities exhibited by these proteins in other contexts it is surmised that this site may need to be dephosphorylated prior to the others, i.e. that dephosphorylation is ordered. This would be consistent

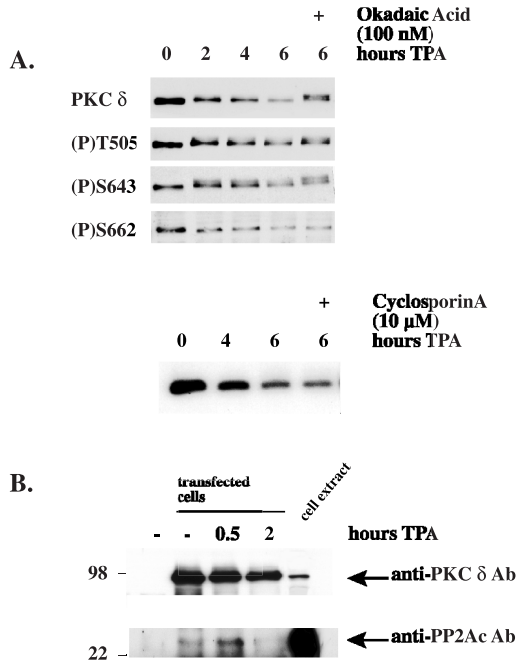

Fig. 4. Dephosphorylation of PKCδ in NIH3T3 cells. A: PKCδ over-expressing NIH3T3 cells were pre-treated with okadaic acid (100 nM) for 30 min prior to TPA stimulation (400 nM). Cell extracts were analysed by Western blotting for PKCδ protein expression and phosphorylation at T505, S643 and S662 at the times indicated. B: NIH3T3 cells were transiently transfected with GFP-tagged PKCδ. Cells (untransfected and transfected) were serum starved for 24 h followed by stimulation with TPA (400 nM) prior to immunoprecipitation of GFP-PKCδ using anti-GFP antibody. Immunoprecipitates were analysed by Western blotting for PKCδ protein (using anti-PKCδ antibody) and for the association of the $PP2A_c$ protein.

with the properties of PKCα where a mutual dependency on site occupation has been described [29,30]. The implication here is that for PKCδ the S643 is the most susceptible and that once removed the others become 'available'.

Activation of PKC requires the combinatorial effects of allosteric activators and phosphorylations in the catalytic domain. Kinase activity is also known to be required for their subsequent downregulation and degradation [6]. In addition to controlling activity, phosphorylations of PKCα co-operate to stabilise the kinase, keeping it in a phosphatase-resistant state [29]. The work described here suggests that binding of nucleotide can also keep PKCδ in a phosphatase-resistant state. Since the intracellular ATP concentration is relatively high (~1 mM), the finding that Mg-ATP (or related nucleotides) can induce a phosphatase-resistant state for PKCδ is of particular note. In further studies it was also observed that peptide/protein substrate binding to PKCδ (histone III S, protamine sulphate or a pseudo-substrate peptide), was sufficient to protect the kinase from dephosphorylation by PP2A (data not shown). It is surmised that PKCδ adopts a closed phosphatase-resistant conformation upon substrate (protein/Mg-ATP) binding. Thus, for PKCδ to become dephosphorylated in vivo a conformational change is required; this might be associated with a substrate release factor or through an independently induced change in conformation.

PP2A has previously been shown to be in multi-protein complexes with other protein kinases so that it has the ability to dephosphorylate. This includes p70S6K and CAM kinase IV [31,32]. These interactions, as that described here, provide direct evidence on the action of PP2A in the inactivation of these protein kinases. Whether these complexes are formed with the same PP2A subunits remains to be elucidated. Whilst it might be argued that PKCδ interaction with a particular complex of $PP2A_c$ might by-pass the resistance to dephosphorylation induced by substrate, the fact that PKCδ is in part complexed to PP2A prior to activation indicates that in fact an additional regulatory input is required for this purpose.

In conclusion, the evidence here indicates that PKCδ dephosphorylation and hence inactivation is effected by PP2A with which it forms a complex. Furthermore, this dephosphorylation requires an additional input in vivo to overcome the nucleotide/substrate bound state of the kinase.

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