
Received 31 October 1996

Protein kinase D: a novel target for diacylglycerol and phorbol esters
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Introduction
A variety of hormones, neurotransmitters and growth factors elicit their characteristic effects on cellular processes by binding to specific receptors on the surface of their target cells. A rapid increase in the synthesis of lipid-derived second messengers is an important mechanism for transducing these extracellular signals across the plasma membrane [1–4]. A key reaction in this process is the phospholipase C (PLC)-mediated hydrolysis of polyphosphoinositides to produce two second messengers: Ins(1,4,5)P3 and diacylglycerol (DAG). Ins(1,4,5)P3 induces mobilization of Ca2+ from intracellular stores [3]. DAG activates protein kinase C (PKC), originally described as a Ca2+-activated phospholipid-dependent protein kinase [5]. The early findings, that the potent tumour promoters of the phorbol ester family can substitute for DAG in PKC activation and that the phorbol ester receptor and PKC co-purify, supported the hypothesis that the cellular target of the phorbol esters is PKC [6]. A large number of cellular studies demonstrated that phorbol esters, presumably acting through PKC, induce a wide range of biological effects, including changes in ion flux, gene expression, cell-cell communication, cell differentiation and proliferation [7–10].

Subsequent studies revealed the diversity of the individual components of the DAG–PKC signal-transduction pathway. It has become apparent that PLC exists in several molecular forms which are regulated by G-proteins and tyrosine phosphorylation [11]. Furthermore DAG can be generated by several alternative routes, including the hydrolysis of phosphatidylincholine [12]. Molecular cloning has demonstrated the presence of multiple related PKC isoforms, which are differentially expressed in cells and tissues [2,13,14]. All members of the PKC family, i.e. conventional or classical PKCs (α, βI, βII, γ), novel PKCs (δ, ε, η, θ) and atypical PKCs (ζ, λ, i), possess a highly conserved catalytic domain. Most of the variation between the PKC subspecies occurs in the regulatory domain. The C1 region of this domain of both conventional and novel PKCs has a tandem repeat of zinc-finger-like cysteine-rich motifs that confers phospholipid-dependent phorbol ester and DAG binding on these PKC isoforms [15–20].

In contrast, atypical PKCs contain a single cysteine-rich motif and they neither bind phorbol esters nor are regulated by DAG [15,21,22]. However, other proteins, such as chimaerin [23], UNC-13 [24] and Vav [25], which possess a

Abbreviations used: PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; PKD, protein kinase D; CaM kinase, Ca2+/calmodulin-dependent protein kinase; PDB, phorbol 12,13-dibutyrate; PS, phosphatidylserine; MAPK, mitogen-activated protein kinase.

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single cysteine-rich domain, bind DAG and phorbol esters. These studies emphasized the complexity of the signalling pathways initiated by DAG but did not exclude the possibility that other protein kinases, unrelated to the PKC family in their catalytic domain, could also play a role in the mediation of the cellular effects of DAG and phorbol esters. Indeed, the identification of novel phorbol ester and DAG targets could have important implications for our understanding of signal transduction.

**Protein kinase D (PKD)**

Recently, we reported the molecular cloning, sequence and expression analysis of PKD, a novel mouse serine/threonine protein kinase with distinct structural features and enzymological properties [26]. PKD (accession no. Z34524 in the EMBL/GenBank database) is a protein of 918 amino acids with a predicted molecular mass of 102 kDa that consists of catalytic and regulatory domains.

The catalytic domain of PKD (residues 589–845) contains all 11 distinct subdomains characteristic of protein kinases [27,28], including an ATP-binding motif (GXGXXG) in subdomain I at residues 596–601 (GSGQFG), a lysine in subdomain II and the motifs DFG in subdomain VII and APE in subdomain VIII that are highly conserved in protein kinases. An unusual feature of the sequence is that the highly conserved arginine residue in the HRDL motif of subdomain VIIB of many protein kinases is replaced by a cysteine in PKD. This was observed previously only in two members of the protein kinase superfamily, the yeast Ras suppressor, YAK-1 [29], and a human putative protein serine kinase [27]. The products of these genes have not been shown directly to possess catalytic activity.

Comparison of the deduced amino acid sequence of the catalytic domain of PKD with that of other protein kinases indicates that PKD is a distinct protein kinase that is distantly related to Ca²⁺/calmodulin-dependent kinases [30]. The rank order of homologies within the catalytic domain is: myosin-light-chain kinase (*Dictyostelium*) > Ca²⁺/calmodulin-dependent protein kinase (CaM kinase) type II > CaM kinase type IV > cAMP-dependent protein kinase > phosphorylase *b* kinase. In particular, the catalytic domain of PKD exhibits only a low degree of similarity to the highly conserved regions characteristic of all members of the PKC family [30]. For example, the motif XDXLKSXXN/D in subdomain VI, which is important because it guides the peptide substrate into the correct orientation so that catalysis can occur [31], is YRDLKLDN in all PKCs, which differs from that of PKD (HCDLKPEN) in every variable residue (X).

The structural differences between the catalytic domains of PKD and the PKCs suggested that these proteins could have important functional differences. We examined the ability of a bacterially expressed fusion protein containing the catalytic domain of PKD to phosphorylate a variety of potential substrates [26]. These studies revealed that the catalytic domain of PKD phosphorylates the synthetic peptide syntide-2, a substrate also utilized by CaM kinases [32,33]. PKD isolated from COS cells transfected with a PKD cDNA construct (pCDNA3-PKD) also phosphorylated syntide-2 (Figures 1 and 2 and ref. [34]). In contrast, PKD did not catalyse significant phosphorylation of a number of substrates utilized by PKCs, such as histone and a peptide substrate based on the sequence of the pseudosubstrate region of PKC. We conclude that PKD is a protein kinase with distinct substrate specificity [26,34].

The N-terminal region of PKD contains a highly hydrophobic stretch of amino acids which is not found in any of the PKCs, two cysteine-rich zinc-finger-like motifs (cys-1 and cys-2) and a pleckstrin homology domain [36] inserted between the cysteine-rich motifs and the catalytic domain. Pleckstrin homology domains have recently been identified in a variety of intracellular signalling and cytoskeletal proteins, but are not present in PKCs. In contrast with all known PKCs, including mammalian, *Drosophila* and yeast isoforms [37], PKD does not contain sequences with similarity to a typical PKC pseudosubstrate motif upstream of cys-1. Interestingly, the length of the sequence separating the cysteine-rich motifs in PKD (95 residues) is substantially longer than that of classic PKCs (28 amino acids) or novel PKCs (35 amino acids). Furthermore the amino acids Ala-154 and Tyr-182 in cys-1 and Lys-298 and Cys-314 in cys-2 of PKD differ from the consensus of the cysteine-rich motif in PKCs. However, a fusion protein containing the zinc-finger-like domains of PKC bound [³H]phorbol 12,13-dibutyrate ([³H]PDB) with high affinity [26]. To substantiate that the binding measurements to the fusion protein *in vitro* reflect the physiological activity of PKD in intact cells, Van Lint et al.
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Figure I

Induction of PKD activation by phorbol 12,13-dibutyrate (PDB)

(a) PDB induction of PKD activation in Swiss 3T3 cells. Confluent and quiescent Swiss 3T3 cells were incubated in the absence (□) or presence (●) of 200 nM PDB for 10 min and lysed. The lysates were immunoprecipitated with PA-1 antiserum, and PKD was eluted at 4°C for 30 min by incubation of the immunoprecipitates with 0.5 mg/ml immunizing peptide as previously described [34]. Syntide-2 phosphorylation (bars) and autophosphorylation (autoradiogram) by eluted PKD was carried out as previously described [34,35]. The reactions were carried out in either the absence (—) or presence of 100 μg/ml phosphatidylserine (PS) and 200 nM PDB (PS/PDB), as indicated. The inset shows a representative autoradiogram of the 110 kDa autophosphorylated PKD. The results are means ± S.E.M. from three independent experiments each performed in duplicate.

(b) Kinetics of PKD activation induced by PDB in NIH 3T3 cells. Confluent and quiescent NIH 3T3 cells were treated for various times with 200 nM PDB and lysed. The lysates were immunoprecipitated with PA-1 antiserum, and PKD activity in the immunocomplexes was determined using an in vitro kinase assay followed by SDS/PAGE and autoradiography.

(c) Specificity of GF 1 and Ro 31-8220 inhibition of PDB-induced PKD activation in Swiss 3T3 cells. Confluent and quiescent Swiss 3T3 cells were incubated with GF 1 (GF; 3.5 μM). Ro 31-8220 (Ro; 3.5 μM). rapamycin (Rap; 20 nM). wortmannin (Wor; 50 nM) or PD098059 (PD; 10 μM) for 1 h, thapsigargin (TG 30 nM) for 30 min, cytochalasin D (Cyt; 2.5 μM) for 2 h or an equivalent amount of solvent (—). All cultures were subsequently challenged with 200 nM PDB for 10 min, except the control untreated cultures (——). The cultures were lysed and the lysates were immunoprecipitated with PA-1 antiserum and analysed by an in vitro kinase assay followed by SDS/PAGE and autoradiography.

[34] verified that transient expression of PKD confers increased [3H]PDB-binding activity on COS cells as compared with either COS cells transfected with the vector (pcDNA3) or untransfected cells. Furthermore addition of PDB in the presence of 1,2-α-phosphatidylserine (PS) markedly stimulated the activity of immunopurified PKD measured in a syntide-2 phosphorylation assay and in an autophosphorylation assay (Figures 1 and 2 and ref. [34]). These results provide evidence indicating that PKD is a novel target for the tumour promoters of the phorbol ester family as well as for the second messenger DAG.

Johannes et al. [38] recently cloned a human protein kinase termed PKCμ with 92% homology to PKD. It is highly likely that the two kinases are functional homologues. However, Johannes et al. [38] failed to demonstrate any phorbol ester binding and regulation and consequently concluded that this enzyme is an atypical PKC. These authors suggested that two amino acid substitutions in the cysteine-rich region could be responsible for these results. In addition, they suggested that the spacing of the cysteine-rich motifs could result in an inappropriate conformation for efficient PDB binding. Subsequent studies demonstrated that PKCμ, like PKD, is stimulated by phorbol esters and phospholipids [39]. These in vitro results indicate that PKD/ PKCμ is a novel phorbol ester/DAG-stimulated protein kinase that does not belong to any of the previously identified subfamilies of PKC.

PKD activation in living cells

Recently we examined the regulation of PKD activity in living cells. Zugaza et al. [35] demonstrated that exposure of intact cells to biologically active phorbol esters, membrane-permeant DAG and serum growth factors induces the rapid conversion of PKD into an activated state that persists during cell disruption and protein immunoprecipitation. PKD is activated by phorbol esters in immortalized cell lines such as Swiss 3T3, NIH 3T3 and Rat-1 cells as well as in secondary mouse embryo fibroblasts and in COS-7 cells transfected with a PKD expression construct (Figure 1 and ref. [35]). PKD recovered by immunoprecipitation from stimulated cells is fully active in the absence of lipid effectors (i.e. PS and PDB or DAG) as shown by autophosphorylation assays as well as phosphorylation of the exogenous substrate syntide-2 (Figure 1). Since PDB does not stimulate kinase...
activity in cells transfected with a PKD kinase-deficient mutant in which Lys-618 was mutated to Met, the inducible kinase activity in PKD immunoprecipitates is due to PKD activation rather than to the stimulation of a co-precipitating kinase. These findings identified an important functional difference between PKD and PKCs in their response to phorbol esters and DAG, since the stimulated state of PKCs is not maintained during cell lysis and protein isolation (e.g. see ref. [40]).

Phorbol esters induce PKD activation within seconds of stimulation of intact cells. Interestingly, PKD is activated more rapidly than other kinase cascades induced by phorbol esters, including p42MAPK, p70S6K or p125FAK [35,41]. Thus PKD activation is one of the earliest events induced by phorbol esters in intact quiescent cells.

To study the molecular mechanisms underlying PKD activation we examined whether this enzyme is regulated by phosphorylation. We found that PDB stimulation of intact cells caused marked retardation in the electrophoretic mobility of PKD and enhanced incorporation of 32P into this enzyme in metabolically labelled cells. Given that treatment with phosphatase reduces PKD activation, we conclude that PDB-induced PKD phosphorylation is essential to maintain the activated state of this enzyme. In accord with a dynamic regulation by phosphorylation-dephosphorylation in living cells, PKD is activated, deactivated and re-activated by addition, removal and re-addition of PDB [35].

At least two different mechanisms involving phosphorylation could be responsible for maintaining the activated state of PKD. Direct stimulation of PKD by phorbol esters or DAG in living cells could induce an activating autophosphorylation of this enzyme. Alternatively, PKD activation could be induced by trans-phosphorylation involving a different protein kinase. We found

**Figure 2**

**Effects of GF I on PKD and PKC**

(a) Effect of GF I on PKD activation in intact cells, as measured by syntide-2 phosphorylation. Confluent and quiescent Swiss 3T3 cells were incubated with 2.5 μM GF I for 1 h. Control cells received an equivalent amount of solvent (−). Cells were subsequently stimulated with 200 nM PDB for 10 min and lysed. The lysates were immunoprecipitated with PA-I antiserum. PKD was eluted from the immunoprecipitates with the immunizing peptide, and the eluted PKD activity was measured by a syntide-2 kinase assay. The results are means ± S.E.M. from three independent experiments, each performed in duplicate. (b) Effect of GF I on PKD activation by PS/PDB. Quiescent and confluent Swiss 3T3 cells were lysed and the lysates were immunoprecipitated with PA-I antiserum. Eluted PKD activity was measured by syntide-2 phosphorylation, in either the absence (−) or presence of 2.5 μM GF I and without (−) or with (+) 100 μg/ml PS and 200 nM PDB (PS/PDB), as indicated. The results are means ± S.E.M. from two independent experiments each performed in duplicate. (c) Effect of GF I on PKC activity. PKC (a mixture of α, β and γ isoforms) activity stimulated by 100 μg/ml PS and 200 nM PDB was determined by either syntide-2 phosphorylation (graph) in the absence or presence of different concentrations of GF I (0.25, 0.5, 1, 5 μM) or by autophosphorylation (inset) in the absence (−) or presence of 2.5 μM GF I.
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that treatment of Swiss 3T3 cells with the PKC inhibitors GF I and Ro 31-8220 before stimulation with PDB strikingly prevents PKD activation as shown by autophosphorylation (Figure 1c) or syntide-2 phosphorylation assays (Figure 2). In contrast, inhibition of a variety of kinases including p70<sub>60k</sub> with rapamycin, phosphatidylinositol 3-kinase with wortmannin, MEK-1 with PD098059, p125<sup>ITK</sup> tyrosine phosphorylation with cytochalasin D or the mobilization of Ca<sup>2+</sup> from intracellular stores with thapsigargin did not affect PKD activation in response to PDB (Figure 1c). It is noteworthy that GF I did not inhibit PKD activity when added directly in vitro, even at the concentrations used in intact cells to block PDB-induced PKD activation (Figures 2a and 2b). In parallel reactions, however, GF I potently inhibited the syntide-2-phosphorylating activity of a purified preparation of PKC (figure 2c, inset). These results strongly suggest that persistent PKD activation induced by phorbol esters, DAG and serum growth factors in living cells is mediated by PKC.

To substantiate the pharmacological evidence implicating PKC in PKD activation, we used transient co-transfection of COS-7 cells with cDNAs corresponding to PKD and constitutively activated PKC isoforms. Our results demonstrated that the PKCs ε and η potently activate PKD in living cells. In contrast, transfection with PKCζ did not induce PKD activation, indicating that PKD is activated by novel PKCs in a specific manner [35]. On the basis of these different experimental approaches, we conclude that PKD is activated in living cells through a PKC-dependent signal-transduction pathway.

Implications of dual pathways of PKD regulation

The preferential activation of PKD by PKCs ε and η suggests that PKD may transduce signals that distinguish these novel PKCs from other PKC isoforms. Interestingly, PKD is prominently expressed in lung, brain and heart [26], tissues in which PKCζ and PKCδ are also specifically expressed [13,42,43]. Furthermore both PKCμ and PKCε have been localized to the Golgi [44]. Overexpression of PKCε in rodent fibroblasts and in rat colon epithelial cells induces tumorigenesis, indicating a link between this PKC isoform and neoplastic transformation [45]. Future experiments should be directed to elucidating the role of PKD in PKCε-mediated malignant transformation. Further experimental work will also be required to elucidate whether PKCs directly phosphorylate and activate PKD or stimulate an intermediary kinase(s) that leads to activation of PKD. Regardless of the precise mechanism, the phosphorylation-dependent activation of PKD, which, like mitogen-activated protein kinases (MAPKs) and p70<sub>60k</sub>, is maintained during cell lysis and immunoprecipitation, will be invaluable in the identification of the extracellular signals that activate the novel PKC/PKD pathway. Currently, we are examining whether physiological activation of PKC via occupancy of specific membrane receptors for mitogenic neuropeptides and polypeptide growth factors can also promote activation of PKD.

Phosphorylation-dependent signal-transducing kinases, such as the MAPKs, the c-Jun N-terminal kinases and p70<sub>60k</sub>, sense environmental signals through upstream activating protein kinases [46,47]. The most proximal kinase in the cascade responds to a GTP-loaded small G-protein or to a second messenger [e.g. DAG, PtdIns(3,4,5)P<sub>3</sub>]. In contrast, PKD is regulated by a dual mechanism. Specifically, phorbol esters or DAG can stimulate PKD directly in the presence of phospholipids and/or induce PKD phosphorylation/activation via PKCs. This dual mechanism of PKD regulation has important functional implications. As shown in Figure 3, dual pathways of activation could serve to enlarge the repertoire of upstream signals that increase PKD activity. Recent reports demonstrate that PKCε and PKCη are stimulated by PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>, the putative second messengers generated by phosphatidylinositol 3-kinase [48,49]. At least, in theory, PKD could be activated through a PKC-dependent pathway, which is stimulated by PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> but does not require increased production of DAG. In addition, arachidonic acid, a product of phospholipase A<sub>2</sub> action or degradation of DAG, can potentiate DAG-mediated activation of certain isoforms of PKC including PKCε [2].

In view of the differences in substrate specificity between PKD and PKCs [26,34], the co-ordinated activation of PKC and PKD could also serve to diversify the downstream targets of the pathway (Figure 3). It is also conceivable that activated PKD, which is no longer dependent on lipid effectors, could function at sites in the cell...
Figure 3

Dual mechanism of regulation of PKD activity

Exogenously added phorbol esters (PDB, PMA (TPA)) or DAG endogenously generated by PLCβ or γ and/or PLD can stimulate PKD directly or induce phosphorylation-dependent PKD activation via PKC. PKD activated by phosphorylation is represented by PKD*. Arachidonic acid, a product of phospholipase A2 (PLA2) action or degradation of DAG, can potentiate DAG-mediated activation of certain PKC isoforms [2]. Other details are given in the text. PIP3, Ptdlns(3,4,5)P3; P13K, phosphatidylinositol 3-kinase.

that are different from the membranes where DAG is formed.

In conclusion, our results reveal an unsuspected connection between PKCs and PKD and imply that PKD can function downstream of specific PKCs in a novel signal-transduction pathway. Although elucidation of the precise role of the PKC-mediated PKD activation will require further work, the dual regulation of PKD reveals a novel theme in signal transduction that might be relevant for other second-messenger-regulated enzymes.

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Nuclear diacylglycerol, the cell cycle, the enzymes and a red herring (or how we came to love phosphatidylcholine)

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Introduction

Activation of phospholipase C (PLC) by a number of different types of receptors at the plasma membrane leads to the generation of at least three well-characterized second messengers [1,2]. Ins(1,4,5)P3 regulates the intracellular levels of Ca2+ via release from intracellular stores, whereas its 3-phosphotyrosyl 'brother' Ins(1,3,4,5)P4 may be involved in the regulation of Ca2+ influx via the regulation of a Ras GTPase-activating protein [3]. The other product of the phosphodiesterase cleavage of PtdIns(4,5)P2, namely diacylglycerol (DAG), is involved in the regulation of protein kinase C [4]. This is probably an oversimplification of the cycle, as it has been suggested that PtdIns(4,5)P2 may itself play a role as a second messenger and its hydrolysis may act to attenuate its signal.

Abbreviations used: PLC, phospholipase C; PLD, phospholipase D; DAG, diacylglycerol; PtdOH, phosphatidic acid; IGF-I, insulin-like growth factor I; MEL cells, murine erythroleukaemia cells; PtdCho, phosphatidylcholine.

Immunolocalization of PtdIns(4,5)P2 or subcellular fractionation suggests that it is not only present in the plasma membrane but also in the cytoskeleton, where it may play a role in regulating this structure [5], and in the nucleus, where its role is unknown [6,7].

The enzymes involved in the production of PtdIns(4,5)P2 have been shown to be present in isolated nuclei by incubation with [32P]ATP, which leads to the production of radiolabelled PtdIns4P, PtdIns(4,5)P2 and phosphatidic acid (PtdOH), suggesting the presence of not only the lipid substrates but also the enzymes that phosphorylate them [7–11]. Enzymes that hydrolyse PtdIns(4,5)P2 have also been shown to be present in the nucleus [12–15], suggesting that the cycle may be involved in the production of similar second messengers within this compartment.

Regulation of intranuclear DAG levels

Activation during proliferation

We demonstrated that treatment of Swiss 3T3 cells with insulin-like growth factor I (IGF-I), a

Received 20 December 1996