Rapid activation of the novel serine/threonine protein kinase, protein kinase D by phorbol esters, angiotensin II and PDGF-BB in vascular smooth muscle cells

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Abstract Protein kinase D (PKD) is a novel serine/threonine kinase structurally distinct from all protein kinase C (PKC) isoforms but which like classic and novel PKCs is activated by phorbol esters and diacylglycerol. This study investigated the regulation of PKD in vascular smooth muscle cells (VSMC) by physiological regulators of VSMC function and growth factors. Treatment of rabbit aortic VSMC with phorbol ester, angiotensin II and PDGF-BB all stimulated PKD activity in a time- and concentration-dependent manner in VSMC. The effect of angiotensin II was particularly rapid and potent (maximum stimulation within 1 min and at 0.5 nM). In contrast, the maximum effect of PDGF-BB was obtained after 5 min. Other factors, including basic FGF, IGF-I, IGF-II, endothelin-1 and endothelin-2, had no effect on PKD activity in VSMC. These results show for the first time that PKD activity is regulated in VSMC, and is activated by the vasoconstrictor angiotensin II. PKD may be an important mediator for the biological function(s) of one or more PKC isoforms in VSMC and/or may represent a component of a novel PKC-independent signalling pathway in VSMC.

Key words: Signal transduction; Phospholipase C; Protein kinase Cμ; Vasoconstriction

1. Introduction

The activity of members of the protein kinase C (PKC) family is regulated by diverse extracellular growth factors, neurotransmitters, cytokines and hormones [1,2]. Classic and novel isoforms of PKC are activated by the second messenger diacylglycerol (DAG), generated through different pathways including phospholipase C (PLC)-catalysed hydrolysis of membrane inositol phospholipids. These PKCs are major cellular binding sites for and are activated by tumour-promoting phorbol esters [1–3]. PKCs are strongly implicated in a variety of biological processes in mammalian cells [1–3]. In particular, in arterial vascular smooth muscle cells (VSMC), PKC isoforms have been implicated in several biological functions including both the negative [4,5] and positive [6] regulation of cell proliferation, hypertrophy [7], the regulation of myosin light chain function [8,9] and the contraction of several types of arterial blood vessels [10–13]. Recently, a novel serine/threonine protein kinase, called protein kinase D (PKD), has been molecularly cloned which in common with conventional and unusual subtypes of PKCs contains two cysteine-rich, zinc-finger-like domains, binds phorbol ester and is activated by phorbol ester and DAG [14]. In other respects, however, PKD diverges from members of the PKC family and represents a novel kinase subtype. Thus, unlike all eukaryotic PKCs previously identified, PKD does not contain a typical pseudosubstrate domain, but possesses a plekstrin homology (PH) domain. The amino acid sequence of the catalytic domain of PKD also exhibits little homology to the catalytic domains of PKC isoforms and is most closely related to that of myosin light chain kinase (41% homology between murine PKD and myosin light chain kinase of *Dictyostelium*) [14]. A human kinase, termed PKCμ, has been cloned, exhibits striking homology to PKD (92% overall), and probably represents the human homologue of this enzyme [15].

Recent findings have implicated PKC isoforms in mediating phorbol ester- and DAG-induced activation of PKD [16,17]. Little is known, however, concerning the regulation of PKD in normal differentiated mammalian cells. In the present paper we investigated the occurrence and regulation of PKD in primary VSMC cultures. We report that PKD is rapidly activated in VSMC in response to biologically active phorbol esters, the vasoconstrictor angiotensin II and the growth factor PDGF-BB. These results suggest that PKD may play a role in intracellular signalling with implications for VSMC function.

2. Materials and methods

2.1. Cell culture

VSMC were cultured from explants of rabbit aortic tunica media obtained from 9-week-old New Zealand White rabbits as previously described [18]. For experimental purposes, primary cultures of aortic VSMC were rendered quiescent by incubation with DMEM containing 0.5% FCS for 40 h [18].

2.2. Immunoprecipitations

Cells were lysed at 4°C in 1 ml of a solution containing 1% Triton X-100, 50 mM Tris-HCl pH 7.5, 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride. Lysates were clarified by centrifugation at $15\,000 \times g$ for 10 min and then the supernatants were used for immunoprecipitation. Immunoprecipitations were routinely performed by incubating the lysates with 1 μg/ml anti-PKD polyclonal antibody (PA-1) overnight. Immunocomplexes were collected by incubating the lysates with protein A-agarose beads for a further 1 h.

2.3. Western blotting

After SDS-PAGE, proteins were transferred to Immobilon membranes [18]. Membranes were blocked using 5% non-fat dried milk in phosphate-buffered saline, pH 7.2, and incubated for 3–5 h in phosphate-buffered saline/0.05% Tween-20 containing antibody (1 μg/ml) as indicated. Immunoreactive bands were visualised either by chemiluminescence using HRP-conjugated anti-mouse or by anti-rabbit IgG and ECL reagent.

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2.4. PKD autokinase assay

Anti-PKD immunoprecipitates were washed three times with lysis buffer and twice with 30 mM Tris pH 7.4. The immunoprecipitates were assayed for kinase activity by incubating them in 20 μl of 30 mM Tris pH 7.4 containing 10 μCi/ml [γ-^{32}P]ATP, 100 μM ATP, 10 mM $MgCl_2$ for 10 min at 30°C. The reaction was terminated by adding an excess volume of lysis buffer at 4°C. The samples were washed three times in lysis buffer at 4°C and then proteins were extracted in 2×SDS-PAGE buffer at 95°C for 5 min. The samples were resolved by SDS-PAGE and then visualised by autoradiography. The autophosphorylation of PKD was semi-quantified by scanning densitometry of radiolabelled bands using an LKB Ultroscan XL densitometer.

2.5. Materials

PDGF-BB was obtained from R&D Systems. Angiotensin II and phorbol 12-myristate 13-acetate (PMA) were from Sigma Chemical Co., St. Louis, MO. Monoclonal PKCμ antibody was from Transduction Laboratories, Inc. Polyclonal antibody to PKD was raised against a synthetic peptide corresponding to the COOH-terminal region of the predicted amino acid sequence of PKD, as described previously [16]. Protein A-agarose, goat anti-rabbit IgG and goat anti-mouse IgG were from Oncogene Science, Inc. ECL reagents, HRP-conjugated anti-mouse IgG were from Amersham, UK. All other reagents used were of the purest grade available.

3. Results

Expression of PKD in primary cultures of rabbit aortic VSMC was assessed by immunoprecipitation of PKD using different amounts of a specific polyclonal antibody directed against murine PKD followed by Western blotting using a second specific monoclonal antibody raised against PKCμ, the human homologue of murine PKD. PKCμ antibody recognised a major 100 kDa band in PKD immunoprecipitates corresponding to the major 110 kDa PKD immunoreactive species previously reported (results not shown).

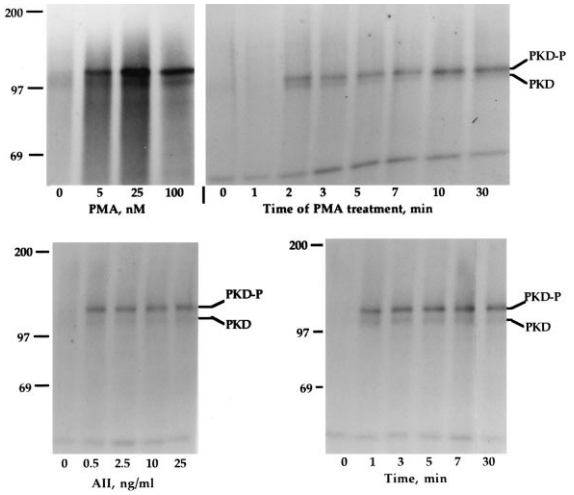

Fig. 1. Stimulation of PKD activity in VSMC by phorbol ester and angiotensin II. Top: VSMC were treated either with the indicated concentrations of PMA or with 100 nM PMA for the indicated times. PKD immunoprecipitates were prepared and assayed as described in Section 2. The positions of molecular weight standards ($\times 10^{-3}$) and phosphorylated and non-phosphorylated PKD forms are indicated. The results shown are representative of six independent experiments. Bottom: VSMC were treated either for 10 min with the indicated concentrations of angiotensin II (AII), or with 10 ng/ml angiotensin II for the times indicated. PKD immunoprecipitates were prepared and assayed as described in Section 2. The positions of molecular weight standards ($\times 10^{-3}$) and phosphorylated and non-phosphorylated PKD forms are indicated. The results shown are representative of three independent experiments.

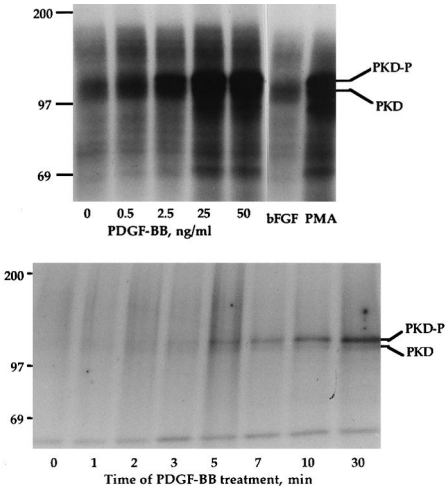

Fig. 2. Stimulation of PKD activity in VSMC by PDGF-BB. VSMC were treated for 10 min with the indicated concentrations of PDGF-BB or with 25 ng/ml PDGF-BB for the times indicated, PKD immunoprecipitates were prepared and kinase assays performed as described. The results shown are representative of five independent experiments.

Activation of PKD can be determined by autokinase assay of PKD in PKD immunoprecipitates [16]. Lysates and PKD immunoprecipitates were prepared from VSMC treated with or without the biologically active phorbol ester PMA and assayed for PKD autophosphorylation by incubation with [γ-^{32}P]ATP and Mg^{2+}. Kinase assay of control immunoprecipitates characteristically resulted in weak phosphorylation of two bands of approximately 110 kDa. In some experiments, phosphorylation of 110 kDa immunoreactive bands was not detectable in control immunoprecipitates. Stimulation of VSMC with PMA induced a striking increase in PKD autophosphorylation (10-fold, $n = 6$). In kinase assays of immunoprecipitates prepared from PMA-treated VSMC, a striking increase occurred in phosphorylation of a major band migrating more slowly than the PKD-immunoreactive bands seen in control immunoprecipitates. In some experiments, phosphorylation of additional bands in the M_r 110 000–120 000 range was observed. The effect of PMA was concentration-dependent with a detectable increase at 5 nM and a maximum effect at 25 nM (Fig. 1). At a concentration of 100 nM, it was consistently noted that the increase in PKD activity declined relative to the effect of 25 nM. As shown in Fig. 1, PMA-induced PKD activation reached a maximum within 2 min and was sustained at this level for up to 30 min.

Angiotensin II is a potent agonist for VSMC that induces rapid Gαq-mediated activation of β isoforms of PLC leading to the formation of the second messengers Ins(1,4,5)P_3 and DAG which cause mobilisation of intracellular Ca^{2+} and activate classic and novel isoforms of PKC, respectively. As the effects of angiotensin II on PKD were not previously investigated in any cell type, we examined whether angiotensin II regulates PKD in VSMC. Angiotensin II induced a striking increase in the autokinase activity of PKD (Fig. 1). The effect of angiotensin II was very rapid with a maximum stimulation achieved in 1 min which persisted for up to 30 min after its addition (Fig. 1). Angiotensin II was also extremely potent in inducing PKD autokinase activity with a maximum effect at a concentration as low as 0.5 nM (Fig. 1). Angiotensin II induced an increase in PKD autophosphorylation approxi-

mately equivalent to the effect of PMA. Mean maximum increases in autokinase PKD activity induced by angiotensin II were 11-fold ($n = 3$) above control unstimulated levels.

PDGF-BB, a potent mitogen and chemoattractant for VSMC, also increased PKD activity. Treatment of cells with different concentrations of PDGF-BB followed by PKD immunoprecipitation and autokinase assay showed that PDGF-BB caused a marked concentration-dependent increase in the autophosphorylation of PKD (Fig. 2). The major phosphorylated band detected in PKD immunoprecipitates prepared from PDGF-treated cells co-migrated exactly with the major phosphorylated band in immunoprecipitates prepared in parallel PMA-treated cells. PDGF-BB-induced PKD autokinase activity was detectable at 2.5 ng/ml and was maximum at 25 ng/ml. In contrast to the effect of PDGF-BB, basic FGF did not induce a significant increase in PKD activity in VSMC (Fig. 2). Treatment of rabbit aortic VSMC with bFGF induced a striking increase in activity of p42/p44 MAP kinases as judged by Western blotting with an antibody specific for the activated form of MAP kinases indicating that other early signalling events induced by bFGF were intact in these cells (results not shown). The effects of PMA and PDGF-BB were also time-dependent. PDGF-BB-induced PKD activation showed a detectable increase at 3 min, reached a near maximum level at 10 min and persisted above the control level for up to 30 min after addition of the factor (Fig. 2). The mean fold increases in PKD autokinase activity induced by PMA and PDGF-BB were approximately 10-fold ($n = 6$) and 5-fold ($n = 5$) above control unstimulated levels, respectively.

Several other polypeptide ligands for receptor protein tyrosine kinases and regulatory peptides known to act through phospholipase Cβ-mediated phosphoinositol lipid hydrolysis and generation of the second messenger DAG were also tested for their effect on PKD activity. In parallel cells and assays of PKD activity, IGF-I, IGF-II and the vasoconstrictor agonists endothelin-1 and endothelin-2 did not induce a significant increase in PKD activity, while angiotensin II and PMA both caused a striking activation of the enzyme (results not shown).

4. Discussion

PKD is a novel serine/threonine protein kinase which is regulated by phorbol esters and DAG, both activators of conventional and unusual PKCs, but possesses several unique features which distinguish it from all known members of the PKC family, including an unrelated catalytic domain, the absence of a pseudosubstrate motif in its regulatory region, and a PH domain between the second cysteine-rich domain and the catalytic domain. At present the function or functions of PKD in mammalian cells and tissues is unknown. As a first step to understanding the function of PKD in cells of the blood vessel wall, we have investigated the regulation of this enzyme in VSMC. The results presented here demonstrate that in primary cultures of aortic VSMC, phorbol esters, the vasoconstrictor angiotensin II and PDGF-BB strikingly and rapidly activate PKD. Activation of PKD was achieved at concentrations of these factors which correspond closely to those which produce biological responses in target cells, indicating that activation of this kinase through receptors is mediated through the same high-affinity receptors. PDGF and several peptides acting through G-protein-coupled receptors, including bombesin, vasopressin, endothelin and bradykinin, also activate PKD in the murine Swiss 3T3 cell line [19]. The results presented here are the first report that PKD activity can be regulated by the vasoconstrictor angiotensin II or is a response to regulatory factors for VSMC.

The mechanism by which PKD is activated in VSMC is unclear. It has previously been shown that PKD activation in intact cells is blocked by selective inhibitors of PKC. In in vitro assays, however, PKD is not susceptible to inhibition by these inhibitors, suggesting that the effects of PKC inhibitors in intact cells are indirect. Consistent with this interpretation, expression of constitutively activated forms of certain PKC isoforms induces activation of PKD expressed in COS-7 cells [16]. These results suggest that one mechanism by which PKD is activated in intact cells is via one or more isoforms of PKC. Since phorbol ester, PDGF-BB and angiotensin II all activate PKC in VSMC, it is possible that PKC(s) may also mediate PKD activation in these cells. Other polypeptide growth factors and peptides known to activate PLC isoforms and biologically active in rabbit aortic VSMC, including bFGF, IGF-I and endothelins, failed to activate PKD. It is plausible that selective activation of PKD may be determined by agonist-specific activation of particular PKC isotypes or may be due to cell-type differences in PKD regulation. The role of PKC in mediating PKD activation and the identification of the PKC isotypes which may be responsible for PKD activation in VSMC warrant further investigation.

The results revealed differences between the responses to PMA, PDGF-BB and angiotensin II. In particular, while PDGF-BB was a weaker activator of PKD than phorbol ester, angiotensin II induced an increase in PKD activity which was equivalent to the effect of PMA, at least as judged by autokinase assay. Interestingly, the kinetics of PKD activation also differed. PDGF-BB-stimulated PKD activation was noticeably slower than that induced by either PMA or angiotensin II. The basis for this slower activation by PDGF-BB is unclear, but it was previously reported that in Swiss 3T3 cells, PDGF-BB stimulation of mobilisation of intracellular Ca^{2+} stores also exhibited slower kinetics compared to the effect of bombesin and other regulatory peptides acting through G-protein-coupled receptors [20,21]. If PDGF-BB-induced PKD activity is mediated via PKC, then the slower kinetics of PKD activation by this factor might reflect a slower activation of the phospholipase-Cγ pathway leading to increased DAG generation and inositol phosphate formation.

Members of the PKC family have been implicated in the regulation of VSMC proliferation and in the responses of VSMC to vasoconstrictors such as angiotensin II as well as the contraction of several types of blood vessels and the control of myogenic tone [8–13,22,23]. Thus, the identification of PKD activation as a common response to PDGF-BB, a potent mitogen and chemoattractant for VSMC and the potent vasoconstrictor angiotensin II may have implications for intracellular signalling in both mitogenesis and contraction. It was recently reported that PKD may be preferentially activated via the Ca^{2+}-independent PKCε isoform [16]. In this context it is interesting that the ε isoform has been implicated in the control of contraction in neonatal rat cardiac myocytes [24] and in Ca^{2+}-independent phenylephrine-induced contraction in ferret aorta [25]. The PKC isoforms mediating angiotensin II-induced contraction are unknown. Given that PKD may be a downstream target for certain PKC isoforms, fur-

ther investigation of PKD regulation in VSMC may prove to be particularly valuable in elucidating PKC isoform-specific mechanisms of action and biological functions in these cells. Regardless of whether PKD is a downstream effector of PKCs and/or is regulated through PKC-independent pathways, the identification of PKD as a novel regulated kinase in VSMC is likely to provide new insights into the mechanisms mediating both PKC-dependent biological processes and the actions of PDGF-BB and angiotensin II in VSMC.

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