ELSEVIER

Biochemical Pharmacology

Biochemical Pharmacology 62 (2001) 1647–1651

Short Communication

Inhibition of protein kinase D by resveratrol

Robert S. Haworth*, Metin Avkiran

Centre for Cardiovascular Biology and Medicine, King's College London, London, United Kingdom

Received 5 June 2001; accepted 14 September 2001

Abstract

Protein kinase D (PKD) is a member of the protein kinase C (PKC) superfamily with distinctive structural, enzymological and regulatory properties. Identification of the cellular function(s) of PKD has been hampered by the absence of a selective inhibitor. Recently, Stewart et al. [9] showed that resveratrol inhibited PKD, but not various PKC isoforms, *in vitro*. Here we confirmed that the activity of PKD is indeed inhibited *in vitro* by resveratrol ($IC_{50} \sim 200\ \mu M$). Additionally, we assessed the inhibition by resveratrol of PKD activity in intact cells, by Western blotting with a phosphospecific PKD antibody which recognizes the autophosphorylated enzyme. In this setting, very high concentrations of resveratrol were required to achieve inhibition of PKD autophosphorylation ($IC_{50} \sim 800\ \mu M$). Since resveratrol produces other pharmacological effects (e.g., cyclooxygenase inhibition) at lower concentrations than those required to inhibit PKD in intact cells, its value as a selective tool to investigate the cellular function(s) of PKD is questionable.

Keywords: COS-7 cell; Protein kinase D; Resveratrol; Phorbol ester

1. Introduction

Protein kinase D (PKD[†]) is a recently identified serine/threonine kinase [1] which has distinct structural and enzymatic properties and is found in most tissues (see review by Rozengurt et al. [2]). PKD, which is also known as protein kinase C (PKC)μ [3], has a catalytic domain that is distantly related to Ca^{2+}-regulated kinases and shows little similarity to the highly conserved regions of the kinase subdomains of the PKC superfamily. Consistent with this, PKD does not phosphorylate a variety of substrates utilized by PKCs [1,4], indicating that PKD is a protein kinase with distinct substrate specificity. The N-terminal region of PKD contains a tandem repeat of cysteine-rich motifs which bind phorbol esters with high affinity [1,4] and immunopurified PKD is stimulated *in vitro* by either diacylglycerol or biologically active phorbol esters in the presence of phosphatidylserine [4]. More recently, a second mechanism of PKD activation has been identified, which involves phosphorylation of PKD via a PKC-dependent pathway [5]. Following the exposure of cells to agonists which activate PKC, PKD is rapidly phosphorylated, resulting in an activation which, unlike diacylglycerol-mediated activation, persists through an immunoprecipitation procedure. The finding that PKD can be activated in parallel with or downstream of PKC raises the possibility that some cellular responses that are thought to arise from PKC activation may be mediated by PKD.

Resveratrol is a phenolic compound with antitumour properties [6], which has been shown to exhibit potent antagonism of phorbol ester-mediated cellular responses [6–8] through unknown mechanisms. Recently, Stewart et al. have shown that resveratrol inhibits the autophosphorylation of PKD but not the autophosphorylation of the PKC isoforms PKCα, β1, γ, δ, ε or ζ, following activation with diacylglycerol and phosphatidylserine *in vitro* [9]. On this basis, the authors suggested that PKD may be the intracellular target through the inhibition of which resveratrol antagonises the effects of phorbol esters. The discovery of a selective inhibitor of PKD would represent a significant advance, since the absence of such a pharmacological tool has hampered the determination of the cellular function(s) of PKD. Although several functions of PKD have been proposed [10–12], these have been based on the consequences of overexpressing PKD or its kinase-inactive mutants. Such overexpression may lead to inappropriate localisation or regulation of PKD, leading to complications in interpreting the data. In the present study, we have investigated the ability of resveratrol to inhibit PKD both *in vitro*

* Corresponding author. Tel.: +44-20-7928-9292 ext. 2749; fax: +44-20-7928-0658.

E-mail address: robert.haworth@kcl.ac.uk (R. Haworth).

Abbreviations: PKD, protein kinase D; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PVDF, polyvinylidene difluoride

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00807-3

and in intact cells, to determine whether it might provide an alternative, pharmacological approach to the study of the cellular function(s) of PKD.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (370 MBq/mL), protein A-sepharose, polyvinylidene difluoride (PVDF) membrane, donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody and Enhanced Chemiluminescence reagents were from Amersham Pharmacia Biotech. Resveratrol was from Calbiochem-Novabiochem Ltd. PhosphoSer916 PKD antibody (catalogue no. 2051) was from New England Biolabs, USA. Dulbecco's modified Eagles medium and fetal bovine serum were from Life Technologies. Phorbol 12-myristate-13-acetate (PMA) was from Sigma.

2.2. Cell culture

Stock cultures of COS-7 cells were maintained at 37° in a humidified atmosphere containing 5% CO_2, in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum. For experimental purposes, cells were plated in 12 well tissue culture plates at a density of 5×10^4 cells/well.

2.3. In vitro kinase assay of PKD activity

Cells were exposed to 10 nM PMA for 5 min prior to harvesting. Ethanol, the vehicle for PMA, had no effect on PKD activity at its final concentration of 0.1% (data not shown). Following treatment, cells were washed with ice-cold phosphate buffered saline, and scraped into 1 mL of lysis buffer, which contained 50 mM Tris/HCl (pH 7.5), 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM 4-(2′-aminoethyl)-benzenesulfonyl fluoride hydrochloride and 1% Triton X-100. Following centrifugation (11,000 g for 30 min at 4°), PKD was immunoprecipitated from the supernatant (100 μL per tube) at 4° for 3 h with the PA-1 antiserum (1:100 dilution) [1,10,13]. Immune complexes were recovered by the addition of 50 μL protein A-sepharose (100 mg/mL), and pellets were washed three times with lysis buffer and three times with assay buffer (30 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$ and 1 mM dithiothreitol). The final pellet was resuspended to a total volume of 40 μL with assay buffer. Resveratrol or vehicle (dimethylsulphoxide, which had no effect on PKD autophosphorylation at its final concentration of 0.1%) was added to samples, followed by mixing and incubation for 10 min at room temperature. To initiate the phosphorylation reaction, 10 μL of phosphorylation mix (assay buffer containing 100 μM [γ-^{32}P]-ATP; 400–600 cpm/pmol) was added. The mixture was incubated at 30° for 5 min, and the reaction terminated by the addition of an equal volume of hot 2× Laemmli buffer. After boiling for 5 min, the samples were subjected to SDS-PAGE on 7.5% acrylamide gels; the gels were then dried and exposed to film for 6–18 hours at −80°.

2.4. Western blot analysis of PKD autophosphorylation

Cultured cells were pretreated with resveratrol for 10–60 min, followed by exposure to PMA (10 nM) for 5 min prior to harvesting. The cells were washed three times with ice-cold phosphate buffered saline and lysed in 100 μL of 1 × Laemmli buffer. Samples of cell lysate (15 μL) were then subjected to SDS-PAGE (7.5% acrylamide gel) and transferred to PVDF membrane, using a Pharmacia LKB Multiphor II transfer apparatus (0.8 mA/cm^2 for 90 min). Membranes were probed with a rabbit polyclonal antibody that recognizes PKD only when phosphorylated at Ser916 (1:500 dilution; procedure as recommended by the supplier), which has been previously shown to be a site of autophosphorylation upon activation [14,15]. Donkey anti-rabbit horseradish peroxidase-conjugated antibody (Amersham) was used at a dilution of 1:2000 to label bound antibody, and the antibody complex was detected using the Amersham Enhanced Chemiluminescence system, as recommended by the manufacturer.

3. Results and discussion

3.1. In vitro inhibition of PKD activity by resveratrol

To confirm that resveratrol inhibits PKD *in vitro*, cells were exposed to PMA, following which PKD was immunoprecipitated and assayed for activity by measuring its autophosphorylation in the presence of 0, 100 or 300 μM resveratrol. In this *in vitro* kinase assay, autophosphorylation of PKD was inhibited by the presence of resveratrol, as illustrated in Fig. 1 with 50% inhibition at approximately 200 μM. This confirms the findings of Stewart et al. [9], although the PKD inhibitory potency of resveratrol in the present study was somewhat less than that observed in that study ($IC_{50} \sim 50$ μM). In the present study, an intracellular method of PKD activation (by exposure of cells to PMA) was employed. It is possible that PKC-mediated activation of PKD in the present study (which potentially occurs through the phosphorylation of regulatory sites in the activation loop of PKD [16]) may interfere with the binding of resveratrol to PKD.

3.2. Ser916 phosphorylation status as an index of cellular PKD activity

Determination of PKD activity by the *in vitro* kinase assay would not be appropriate for determination of the PKD-inhibitory efficacy of resveratrol in intact cells, since

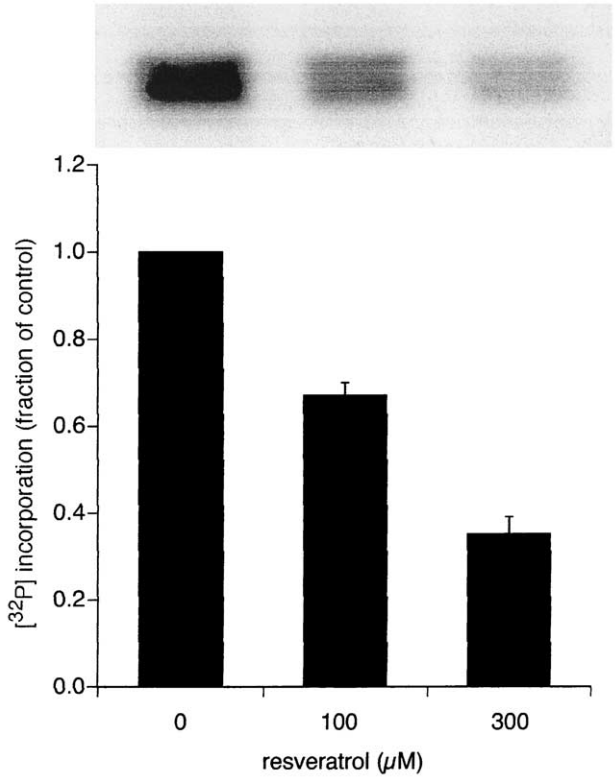

Fig. 1. *In vitro* inhibition of PKD by resveratrol. COS-7 cells were exposed to 10 nM PMA for 5 min, and lysed in lysis buffer. PKD was immunoprecipitated with PA-1 antiserum, and PKD activity was assessed following pretreatment with 0, 100 and 300 μM resveratrol, as indicated. The autoradiogram shown is representative of 4 independent experiments. The bar chart values are the mean ± S.E.M. (n = 4) of PKD activity obtained from scanning densitometry, expressed as a fraction of the DMSO-treated control.

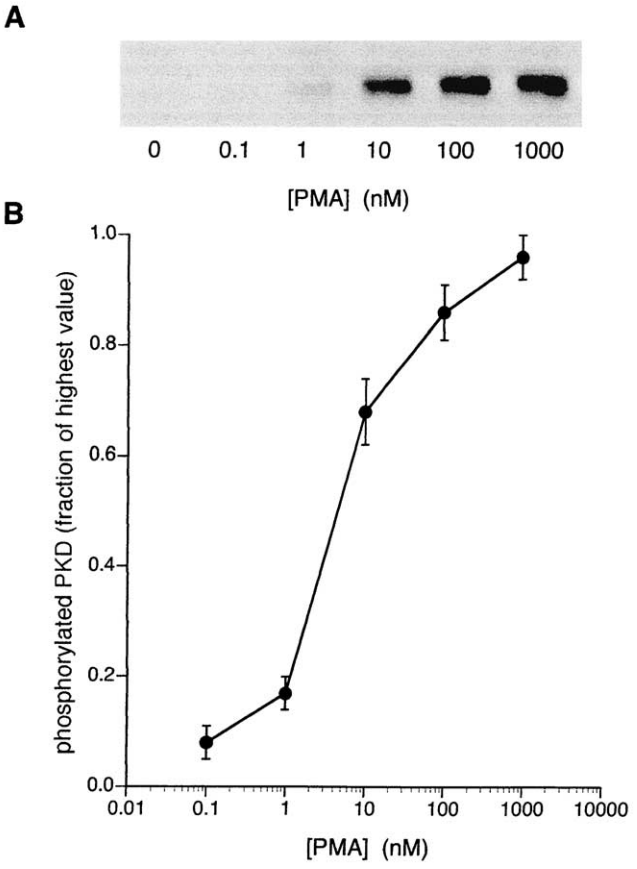

Fig. 2. PKD activation by PMA in intact cells. A: Western blot of protein from PMA-treated cells. COS-7 cells were exposed to various concentrations of PMA, as indicated. Equal amounts of protein were separated by SDS-PAGE, and subjected to Western blot analysis using the phosphoSer916 PKD antibody. The Western blot is representative of 4 independent experiments. B: Quantitative analysis of PKD phosphorylation. The results shown are the mean ± S.E.M. (n = 4) of PKD activity obtained from scanning densitometry, expressed as a fraction of the maximum increase in phosphorylation.

resveratrol would be lost during the immunoprecipitation procedure. Therefore, we validated an alternative method of indexing cellular PKD activity. Recently a phospho-specific antibody which recognises PKD only when phosphorylated at Ser916 has become commercially available, and Ser916 has previously been shown to be autophosphorylated upon the activation of PKD [14,15]. To test whether the antibody was suitable for assessing cellular PKD activity, COS-7 cells were exposed to different concentrations of PMA, and cell extracts were subjected to Western blot analysis. Fig. 2A shows a representative Western blot; as shown, there was little phosphorylated PKD detected in the absence of PMA treatment, but such treatment produced a dose-dependent increase in PKD phosphorylation. Quantitative data from these experiments (Fig. 2B) revealed an EC_{50} of 7 nM for PMA-induced PKD activation, which is comparable with EC_{50} values previously reported from alternative assays [1,13]. Thus, the present data show that PKD autophosphorylation in intact cells can be detected by Western blot analysis using the phospho-Ser916 antibody, and that the phosphorylation status is a good index for cellular PKD activity.

3.3. Inhibition of cellular PKD activity by resveratrol

To determine whether resveratrol was able to inhibit cellular PKD activity, COS-7 cells were pretreated with resveratrol (300 μM) for 10–60 min prior to exposure of cells to PMA (10 nM for 5 min). The results are shown in Fig. 3A. No significant inhibition of PKD autophosphorylation was observed with such pretreatment with resveratrol. To establish whether higher concentrations of resveratrol would be more effective, a dose-response analysis was also performed. Cells were pretreated with resveratrol (0–2000 μM) for 30 min prior to exposure of cells to PMA (10 nM for 5 min). The results are shown in Fig. 3B. A dose-dependent inhibition of PKD autophosphorylation was observed, with an IC_{50} of 760 μM. This is substantially higher than the IC_{50} estimated from our *in vitro* experiments (see Fig. 1B), possibly reflecting a limited access of resveratrol to PKD within the cell. An alternative explanation may be partial degradation of resveratrol during the 30 min incuba-

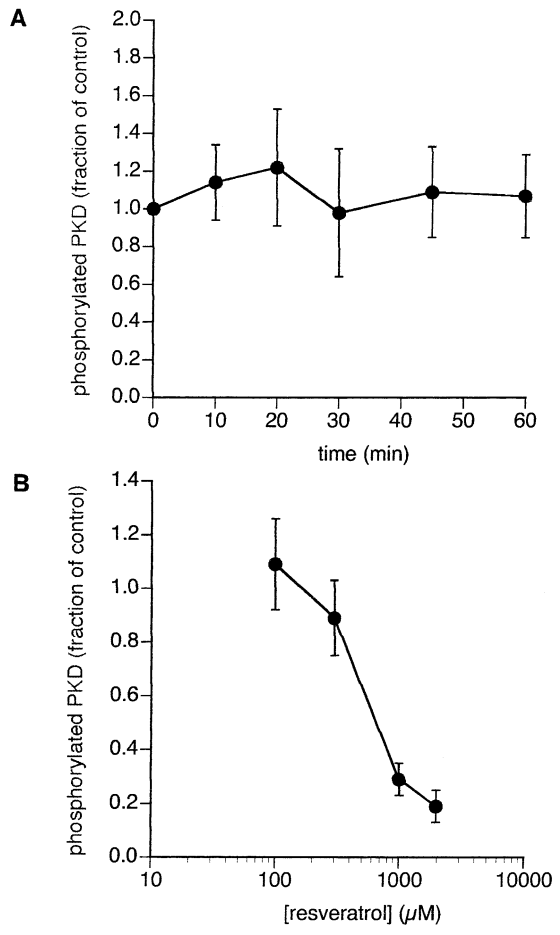

Fig. 3. Inhibition of cellular PKD activity by resveratrol. A: Quantitative analysis of PKD phosphorylation following exposure to PMA (10 nM, 5 min), after pretreatment with resveratrol (300 μM) for the times indicated. Equal amounts of protein were separated by SDS-PAGE, and subjected to Western blot analysis using the phosphoSer916 PKD antibody. The results shown are the mean ± S.E.M. (n = 4) of PKD activity obtained from scanning densitometry, expressed as a fraction of the maximum increase in phosphorylation. B: Quantitative analysis of PKD phosphorylation following exposure to PMA (10 nM, 5 min), after 30 min pretreatment with resveratrol at the concentrations indicated. Equal amounts of protein were separated by SDS-PAGE, and subjected to Western blot analysis using the phosphoSer916 PKD antibody. The results shown are the mean ± S.E.M. (n = 4) of PKD activity obtained from scanning densitometry, expressed as a fraction of the maximum increase in phosphorylation.

tion period; however, this seems unlikely since 60 min incubation with 50 μM resveratrol has been shown to inhibit protein tyrosine phosphorylation and mitogen-activated protein kinase activation by PMA in HeLa cells [17].

Although resveratrol has been shown to inhibit PMA-induced cellular responses in several cell types at concentrations of 15–50 μM [6–8,17], the mechanism by which this is achieved is not yet clear. Subbaramaiah et al. have shown that, in 184B5/HER cells, 15 μM resveratrol is sufficient to prevent PMA-induced redistribution of PKC activity from cytosolic to membrane fractions [7]. In addition, Yu et al. [17] have recently shown an inhibitory effect of resveratrol ($IC_{50} \sim 50$ μM) on the activity of PKC isolated from HeLa cells. These findings are at odds with the results reported by Stewart et al. [9], and suggest that PKC, rather than PKD, may be the key intracellular target through the inhibition of which resveratrol antagonises the effects of phorbol esters.

3.4. Conclusion

The concentrations of resveratrol required to inhibit PKD activity in intact cells are much higher than those previously shown to block PMA-induced biological effects [6–8,17], suggesting that PKD is not a mediator of these effects. The high concentrations of resveratrol required to obtain significant inhibition of PKD activity in intact cells also cast considerable doubt on the usefulness of resveratrol as a pharmacological tool to study the cellular function(s) of PKD, since resveratrol is already known to inhibit (e.g., PKC translocation and cyclooxygenase activity [7]) or promote (e.g., caspase activation [18]) other cellular processes at lower concentrations. Nevertheless, resveratrol may be of limited value as a pharmacological tool for the *in vitro* inhibition of PKD activity.

Acknowledgment

This work was supported by the British Heart Foundation (PG/98197). PA-1 antibody was kindly provided by Dr. Enrique Rozengurt (UCLA, California, USA).

References

[1] Valverde AM, Sinnett-Smith J, van Lint J, Rozengurt E. Molecular cloning and characterisation of protein kinase D: a target for diacylglycerol and phorbol esters with a distinctive catalytic domain. Proc Natl Acad Sci 1994;91:8572–6.

[2] Rozengurt E, Sinnett-Smith J, Zugaza JL. Protein kinase D: a novel target for diacylglycerol and phorbol esters. Biochem Soc Trans 1997;25:565–71.

[3] Johannes FJ, Prestle J, Eis S, Oberhagemann P, Pfizenmaier K. PKCμ is a novel, atypical member of the protein kinase C family. J Biol Chem 1994;269:6140–8.

[4] van Lint J, Sinnett-Smith J, Rozengurt E. Expression and characterisation of PKD, a phorbol ester and diacylglycerol-stimulated serine protein kinase. J Biol Chem 1995;270:1455–61.

[5] Zugaza JL, Sinnett-Smith J, Van Lint J, Rozengurt E. Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. EMBO J 1996;15:6220–30.

[6] Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CWW, Fong HHS, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 1997;275:218–20.

[7] Subbaramaiah K, Chung WJ, Michaluart P, Telang N, Tanabe T, Inoue H, Jang M, Pezzuto JM, Dannenberg J. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J Biol Chem 1998;273:21875–82.

[8] Hsieh TC, Wu JM. Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer. Exp Cell Res 1999;249:109–15.

[9] Stewart JR, Christman KL, O'Brian CA. Effects of resveratrol on the autophosphorylation of phorbol ester-responsive protein kinases. Biochem Pharmacol 2000;60:1355–9.
[10] Haworth RS, Sinnett-Smith J, Rozengurt E, Avkiran M. Protein kinase D inhibits plasma membrane Na^+/H^+ exchanger activity. Am J Physiol 1999;277:C1202–9.
[11] Sidorenko SP, Law CL, Klaus SJ, Chandran KA, Takata M, Kurosaki T, Clark EA. Protein kinase Cμ (PKCμ) associates with the B cell antigen receptor complex and regulates lymphocyte signaling. Immunity 1996;5:353–63.
[12] Tobias ES, Rozengurt E, Connell JMC, Houslay MD. Co-transfection with protein kinase D confers phorbol-ester-mediated inhibition on glucagon-stimulated cAMP accumulation in COS cells transfected to overexpress glucagon receptors. Biochem J 1997;326:545–51.
[13] Haworth RS, Goss MW, Rozengurt E, Avkiran M. Expression and activity of protein kinase D/protein kinase Cμ in myocardium: evidence for α_1-adrenergic receptor- and protein kinase C-mediated regulation. J Mol Cell Cardiol 2000;32:1013–23.
[14] Matthews SA, Rozengurt E, Cantrell D. Characterization of serine 916 as an *in vivo* autophosphorylation site for protein kinase D/protein kinase Cμ. J Biol Chem 1999;274:26543–9.
[15] Vertommen D, Rider M, Ni Y, Waelkens E, Merlevede W, Vandenheede JR, van Lint J. Regulation of PKD by multisite phosphorylation. J Biol Chem 2000;275:19567–76.
[16] Iglesias T, Waldron RT, Rozengurt E. Identification of *in vivo* phosphorylation sites required for protein kinase D activation. J Biol Chem 1998;273:27662–7.
[17] Yu R, Hebbar V, Kim DW, Mandlekar S, Pezzuto JM, Kong AT. Resveratrol inhibits phorbol ester and UV-induced activator protein 1 activation by interfering with mitogen-activated protein kinase pathways. Mol Pharmacol 2001;60:217–24.
[18] Tinhofer I, Bernhard D, Senfter M, Anether G, Loeffler M, Kroemer G, Kofler R, Csordas A, Greil R. Resveratrol, a tumor-suppressive compound from grapes, induces apoptosis via a novel mitochondrial pathway controlled by Bcl-2. Faseb J 2001;15:1613–5.