FEBS Letters 356 (1994) 275–278

FEBS 14896

Classical, novel and atypical isoforms of PKC stimulate ANF- and TRE/AP-1-regulated-promoter activity in ventricular cardiomyocytes

Juliette B.J. Decock[a], Judith Gillespie-Brown[a], Peter J. Parker[b], Peter H. Sugden[a], Stephen J. Fuller[a,*]

[a]*Department of Cardiac Medicine, National Heart and Lung Institute, University of London, Dovehouse Street, London SW3 6LY, UK*
[b]*Protein Phosphorylation Laboratory, Imperial Cancer Research Fund Laboratories, PO Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, UK*

Received 7 November 1994

Abstract Cultured neonatal rat ventricular myocytes were co-transfected with expression plasmids encoding protein kinase C (PKC) isoforms from each of the PKC subfamilies (classical PKC-α, novel PKC-ε or atypical PKC-ζ) together with an atrial natriuretic factor (ANF) reporter plasmid. Each PKC had been rendered constitutively active by a single Ala→Glu mutation or a small deletion in the inhibitory pseudosubstrate site. cPKC-α, nPKC-ε or aPKC-ζ expression plasmids each stimulated ANF-promoter activity and expression of a reporter gene under the control of a 12-*O*-tetradecanoylphorbol 13-acetate-response element (TRE). Upregulation of the ANF promoter is characteristic of the hypertrophic response in the heart ventricle and a TRE is present in the ANF promoter. Thus all subfamilies of PKC may have the potential to contribute to hypertrophic response in cardiomyocytes.

Key words: Atrial natriuretic factor; TRE/AP-1 sites; Regulation of promoter activity; Protein kinase C isoforms; Transfection; Neonatal rat ventricular cardiomyocytes in culture; Cardiac hypertrophy

1. Introduction

Postnatally, cardiac myocytes become amitotic and respond to a variety of trophic signals in vivo by increasing their size and myofibrillar content. Similarly, ventricular myocytes in culture display increased organisation of contractile proteins into sarcomeres and distinct changes in gene expression (reviewed in [1]). The latter includes rapid induction of immediate early genes such as c-*fos*, c-*jun* and *Egr*-1, re-expression of genes normally only expressed during foetal ventricular development (ANF, β-MHC, skeletal muscle α-actin) and the up-regulation of constitutively-expressed contractile protein genes such as ventricular MLC-2 and cardiac muscle α-actin.

The molecular mechanisms underlying these changes are still poorly understood, though participation of PKC is implicated. Many hypertrophic agonists activate PKC in cardiomyocytes [2–7]. TPA increases myocyte size, protein synthesis and promotes the changes in gene expression associated with the hypertrophic response [8–12]. Transfection of cardiomyocytes with plasmids in which PKC-α or PKC-β rendered constitutively-active by large N-terminal deletions induces up-regulation of co-transfected β-MHC [13], ANF- [3] or MLC-2-reporter [3] genes. However, these deletions remove structure (reviewed in [14]) involved in PKC regulation (substrate selection, subcellular localisation, etc.).

Although this evidence supports a role for PKC in the hypertrophic response, whether there is any specificity in terms of PKC isoform involvement has never been ascertained. PKC is a family of at least twelve phospholipid-dependent Ser-/Thr-protein kinases (reviewed in [14–16]) which can divided into the following subfamilies: (i) classical (cPKC-α, -β_1, -β_2 and -γ) which require both Ca^{2+} and diacylglycerol (or phorbol esters such as TPA) for activity; (ii) novel (nPKC-δ, -ε, -η, -θ and -μ) which have lost the requirement for Ca^{2+}; and (iii) atypical (aPKC-ζ, -ι and -λ) which are Ca^{2+}-independent and are not activated by diacylglycerol. Using isoform-specific antisera, we and others have shown that cPKC-α, nPKC-δ, nPKC-ε and aPKC-ζ are present in cultured neonatal rat cardiomyocytes [5,6,17].

The present work had two aims. First, to determine whether expression of PKC isoforms rendered constitutively-active by single amino acid mutations or small deletions in the ψSS (as opposed to large N-terminal deletions) induce the expression of a marker gene (ANF) for the hypertrophic response in cardiomyocytes. Secondly, whether a specific PKC subfamily stimulates its expression.

2. Experimental

2.1. Plasmid constructs

For each PKC isoform studied, expression vectors harboured: (i) wild type PKC; (ii) PKC with a ψSS (A→E) mutation producing a constitutively-active (A_{25}→E in cPKC-α [18], A_{159}→E in nPKC-ε [19], A_{119}→E in aPKC-ζ [20]; (iii) PKC with a $\Delta\psi$SS of residues 22–28 in cPKC-α [18], residues 154–163 in nPKC-ε [19] and residues 116–122 in aPKC-ζ (N.T. Goode and P.J. Parker, unpublished). cPKC-α and nPKC-ε were in vector pMT2 under the control of an SV40 promoter [21] and aPKC-ζ was in vector pCO2 (provided by Dr. Steve Goodbown, Imperial Cancer Research Fund Laboratories) under the control of a CMV promoter.

The ANF-LUX reporter vector, pANF(-638)LΔ5′ [22], the 2 × AP-1/LUX construct TRE2PRL(-36) [23], the corresponding control plasmids pSV0ALΔ5′ and PRL(-36), and an expression vector (pON249) in which the β-Gal gene is under the control of the human CMV promoter [24] were generously provided by Drs. Ken Chien, Geoff Rosenfeld and Joan Heller Brown (University of California, San Diego).

Plasmids were purified by polyethylene glycol precipitation following alkaline lysis of the bacterial host [25] and experiments used at least two different preparations of each plasmid.

*Corresponding author. Fax: (44) (71) 823-3392.

Abbreviations: ANF, atrial natriuretic factor; AP-1, activator protein-1; β-Gal, β-galactosidase; CMV, cytomegalovirus; LUX, luciferase; β-MHC, β-myosin heavy chain; MLC, myosin light chain; PBS, phosphate-buffered saline; PKC, protein kinase C; ψSS, pseudosubstrate site; $\Delta\psi$SS, ψSS deletion; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRE, TPA response element; wt, wild type.

SSDI 0014-5793(94)01283-0

2.2. Transfections and assays

Ventricular cardiomyocytes were isolated from the hearts of 1- to 2-day-old Sprague–Dawley rats by the method of Iwaki et al. [26] with minor modifications. After 24 h in culture, cardiomyocytes (10^6 cells/60 mm dish) were transfected. A few hours prior to transfection the medium on the cells was changed to 4% horse serum in 4 ml of maintenance medium (DMEM/medium 199 (4:1) containing 100 units/ml of both penicillin and streptomycin). Plasmids were diluted in 0.25 M $CaCl_2$ (500 μl) and an equal volume of 50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (pH 6.9), 280 mM NaCl, 1.5 mM Na_2HPO_4 was added. After a 20 min incubation to allow precipitate formation, 950 μl of suspension was added to the cells which were then incubated overnight. Each transfection used 15 μg of reporter plasmid, 4 μg of pON249 and 10 μg of PKC expression plasmid. The next day, the cells were washed once in 10% horse serum in maintenance medium and twice in maintenance medium. After incubation in maintenance medium for 48 h, cells were washed 3× in ice-cold PBS and extracted in 0.1 M potassium phosphate (pH7.9), 0.5% (v/v) Triton X-100, 1 mM dithiothreitol by incubation on ice for 15 min.

LUX was assayed by the addition of extract (20 μl) to 100 mM Tricine (pH 7.8), 10 mM $MgSO_4$, 2 mM EDTA, 75 mM luciferin, 5.5 mM ATP (0.5 ml). Light emitted was quantitated in an LKB 1219 RackBeta liquid scintillation counter with the photomultipliers set out of coincidence. β-Gal was assayed by addition of extract (100 μl) to 0.1 M sodium phosphate (pH7.3), 1.5 mM $MgCl_2$, 75 mM 2-mercaptoethanol, 2 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (200 μl) and incubated at 37°C for 1–3 h. The reaction was terminated by the addition of 0.5 ml of 0.5 M Na_2CO_3 and A_{410} measured.

Results are mean ± S.E.M. for 5–6 separate preparations (unless stated otherwise) of cardiomyocytes.

3. Results

cPKC-α(A_{25}→E) or cPKC-α(ΔψSS) stimulated expression of ANF-LUX in cardiomyocytes by 2- to 3-fold compared with cPKC-α(wt) or empty vector (Fig. 1A). nPKC-ε(A_{159}→E) and nPKC-ε(ΔψSS) stimulated ANF-LUX by 2- to 3-fold compared with empty vector (Fig. 1A). nPKC-ε(wt) also stimulated ANF-LUX expression significantly although stimulation was less than with the constitutively active constructs (Fig. 1A). Results were unchanged when normalised for β-Gal activity (Fig. 1A). aPKC-ζ(A_{119}→E) and aPKC-ζ(ΔψSS) caused the most marked stimulation of ANF-LUX (5- to 6-fold compared with empty vector) and again aPKC-ζ(wt) stimulated ANF-LUX though less than the constitutively active constructs (Fig. 1A). Stimulation by wild type constructs is presumably caused by over-expression of PKCs with low activity although more complex explanations are possible (e.g. their presence removes a repressor from the ANF promoter).

When aPKC-ζ-stimulated ANF-LUX activity was normalised for β-Gal activity, no stimulation by aPKC-ζ(wt), aPKC-ζ(A_{119}→E) or aPKC-ζ(ΔψSS) was detectable (Fig. 1B). This anomaly arose from inhibition of β-Gal expression from pON249 by the empty pCO2 vector which did not occur with the pCO2-aPKC-ζ constructs. The pCO2 vector and pON249 both use a CMV promoter and promoter competition is likely to have been a problem. There was no detectable LUX activity in cells co-transfected with PKC-expression plasmids and the promoterless backbone LUX vector pSV0ALΔ5′ (results not shown).

The sensitivity of TRE/AP-1 sites to activation by cPKC-α, nPKC-ε and aPKC-ζ was examined in cardiomyocytes using the TRE2PRL(-36) reporter plasmid [23]. LUX activity was stimulated 10- to 12-fold by cPKC-α(ΔψSS) or nPKC-ε(ΔψSS) compared with vector alone whereas aPKC-ζ(ΔψSS) stimulated activity about 4-fold (Fig. 2A). For nPKC-ε, constructs containing nPKC-ε(wt) or nPKC-ε(A_{159}→E) did not significantly stimulate LUX activity (Fig. 2A). Analogous constructs for aPKC-α and nPKC-ζ were not tested. Normalisation for β-Gal activity did not alter interpretation of these results, with the exception of aPKC-ζ where the stimulation was masked because of the inhibition of β-Gal expression by the empty pCO2 vector. There was no measurable LUX activity under any conditions when TRE2PRL(-36) was replaced by PRL(-36) (results not shown).

We compared the magnitude of the stimulation of ANF-LUX or TRE2PRL(-36) expression by PKC expression constructs with stimulation by 1 μM TPA. TPA stimulated ANF-LUX expression by 33.0 ± 14.6-fold and ANF-LUX/β-Gal by 14.3 ± 6.9-fold in cardiomyocytes transfected with ANF-LUX + pON249 ($n = 4$). Similarly, TPA stimulated TRE2PRL(-36) expression by 43.3 ± 4.3-fold and ANF-LUX/β-Gal by 13.6 ± 1.3-fold in cells transfected with TRE2PRL-(-36) and pON249 ($n = 3$).

4. Discussion

The 638 bp of the ANF gene regulatory sequence 5′ to the

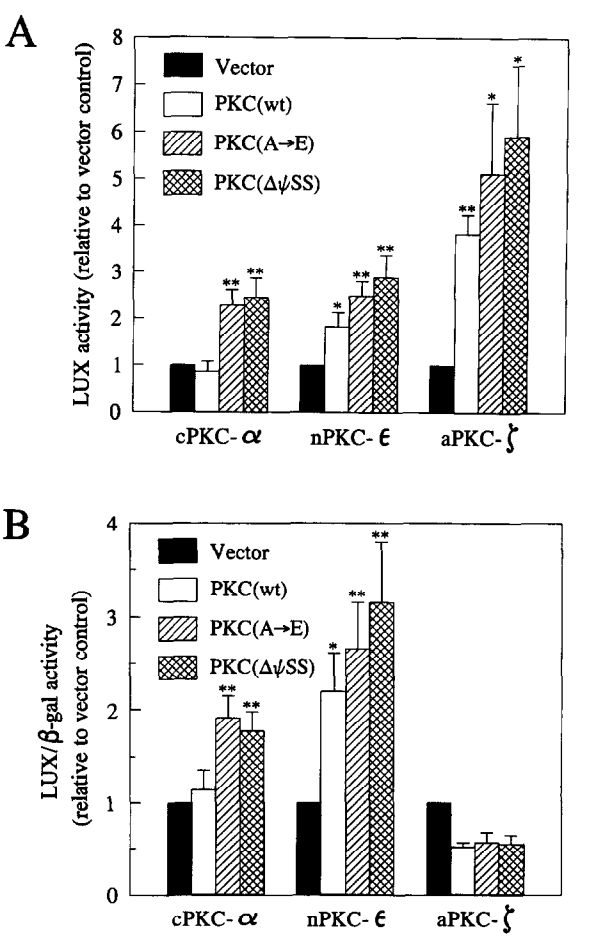

Fig. 1. Stimulation of ANF promoter activity in neonatal ventricular myocytes by expression of constitutively active isoforms of PKC. Transfection and extraction of cells was carried out as described in the Experimental section. Data are expressed relative to transfection with empty vector. (A) LUX activity. (B) LUX activity normalised to β-Gal. Statistical significance: $^*P<0.05$; $^{**}P<0.02$ by a 2-tailed Student's *t*-test for paired data.

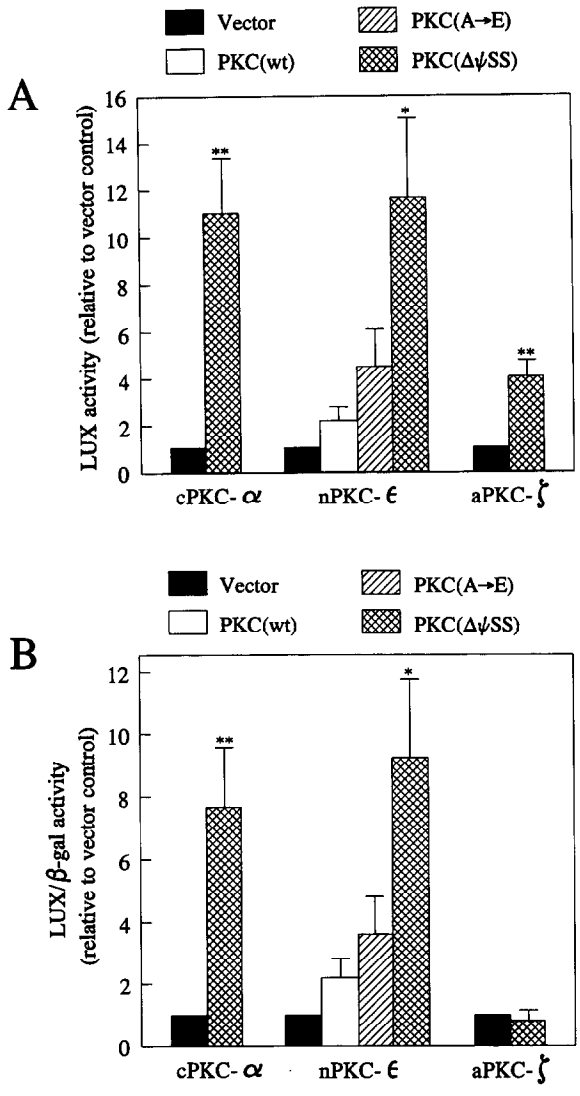

Fig. 2. Stimulation of TRE/AP-1-regulated promoter activity in neonatal ventricular myocytes by expression of constitutively active isoforms of PKC. Transfection and extraction of cells was carried out as described in section 2. Data are expressed relative to transfection with empty vector. (A) LUX activity. (B) LUX activity normalised to β-Gal. Statistical significance: $*P<0.05$; $**P<0.02$ by a 2-tailed Student's t-test for paired data.

transcriptional start site confer inducibility of ANF gene expression in response to the hypertrophic agonist phenylephrine in ventricular cardiomyocytes [22]. Ventricular re-expression of ANF is a marker for the hypertrophic response that is seen in vivo and in cultured cardiomyocytes (reviewed in [1]). Within the 638 bp sequence are CRE (cAMP response element), TRE/AP-1 (c-fos/c-jun heterodimer binding), AP-2, Egr-1 and CArG consensus sequences [22].

The most direct evidence of an involvement of PKC in the regulation of ANF gene transcription is the demonstration that transfection of cardiomyocytes with constitutively-active cPKC-α or cPKC-β stimulated ANF-LUX expression by 2- to 3-fold relative to β-Gal [3]. Other marker genes (MLC-2, β-MHC) are up-regulated by these PKC constructs [3,13]. This response is presumably mediated at least in part through TRE/AP-1 sites [3].

There are two difficulties with these studies. First, cPKC-α and cPKC-β were rendered constitutively active by deletion of more than half of N-terminal regulatory domain (residues 1–253 from cPKC-α and 6–159 from cPKC-β). The consequences of these deletions in terms of the properties of the PKCs expressed have not been fully assessed. However the regulatory domain contains regions that play a role in substrate selection [27], effector binding [20] and subcellular localisation [28] in addition to containing the φSS (reviewed in [14]). Secondly, of cPKC-α, cPKC-β_I/β_{II}, cPKC-γ, nPKC-δ, nPKC-ε and aPKC-ζ, the isoforms most readily detectable in cultured neonatal cardiomyocytes by immunoblotting are nPKC-δ, nPKC-ε and aPKC-ζ [5,6,17]. cPKC-α is more difficult to detect, and cPKC-β_I/β_{II} and cPKC-γ are not detectable [5,6,17]. Furthermore, when cultured neonatal cardiomyocytes are exposed to hypertrophic agonists (endothelin-1, phenylephrine), nPKC-ε is the isoform most readily activated (translocated) [7].

We used expression vectors encoding PKC isoforms rendered constitutively active by single amino acid mutations or small deletions within the φSS. The PKCs expressed are constitutively-active [18–20] but retain regulatory sequences associated with substrate selection [27] and determination of subcellular localisation [28]. In addition, the range of PKC subfamilies studied has extended to include an nPKC and an aPKC. ANF- and TRE-regulated-promoter activities were increased by cPKC-α, nPKC-ε and aPKC-ζ (Figs. 1 and 2). This implies that a TRE/AP-1 site may be at least partly responsible for the stimulation of ANF promoter activity. No significant difference between isoforms in terms of their ability to cause transactivation of ANF and TRE-regulated promoter activity was detected except that aPKC-ζ may be more efficient than cPKC-α or nPKC-ε at transactivating the ANF promoter (Fig. 1A) but less efficient than cPKC-α or nPKC-ε at transactivating at TRE sites (Fig. 2A). This pluripotency implies that any specificity in terms of mediation of the hypertrophic response by PKC isoforms would reside elsewhere in the signalling pathway. Thus, we have recently shown that there may be differences between isoforms in terms of their ability to be activated by hypertrophic agonists [7]. From the teleological standpoint, the demonstration of the sensitivity of the ANF promoter to nPKC-ε is important because this isoform is the most readily detectable in adult rat heart [4] which is known to re-express ANF on hypertrophy (reviewed in [1].

Acknowledgements: This work was supported by grants from the Biotechnology and Biological Sciences Research Council to S.J.F. and P.H.S., and the British Heart Foundation to S.J.F. S.J.F. is a British Heart Foundation Lecturer in Basic Science.

References

[1] Chien, K.R., Knowlton, K.U., Zhu, H. and Chien, S. (1991) FASEB J. 5, 3037–3046.
[2] Kaku, T., Lakatta, E. and Filburn, C. (1991) Am. J. Physiol. 260, C635–C642.
[3] Shubeita, H.E., Martinson, E.A., van Bilsen, M., Chien, K.R. and Brown, J.H. (1992) Proc. Natl. Acad. Sci. USA 89, 1305–1309.
[4] Bogoyevitch, M.A., Parker, P.J. and Sugden, P.H. (1993) Circ. Res. 72, 757–767.
[5]. Rybin, V.O. and Steinberg, S.F. (1994) Circ. Res. 74, 299–309.
[6] Pucéat, M., Hilal-Dandan, R., Strulovici, B., Brunton, L.L. and Brown, J.H. (1994) J. Biol. Chem. 269, 16938–16944.
[7] Clerk, A., Bogoyevitch, M.A., Andersson, M.B. and Sugden, P.H. (1995) J. Biol. Chem. (paper M4-5274, in press).

[8] Henrich, C.J. and Simpson, P.C. (1988) J. Mol. Cell. Cardiol. 20, 1081–1085.
[9] Fuller, S.J. and Sugden, P.H. (1989) FEBS Lett. 247, 209–212.
[10] Dunnmon, P.M., Iwaki, K., Henderson, S.A., Sen, A. and Chien, K.R. (1990) J. Mol. Cell. Cardiol. 22, 901–910.
[11] Allo, S.N., McDermott, P.J., Carl, L.L. and Morgan, H.E. (1991) J. Biol. Chem. 266, 22003–22009.
[12] Allo, S.N., Carl, L.L. and Morgan, H.E. (1992) Am. J. Physiol. 263, C319–C325.
[13] Kariya, K., Karns, L.R. and Simpson, P.C. (1991) J. Biol. Chem. 266, 10023–10026.
[14] Hug, H. and Sarre, T.F. (1993) Biochem. J. 291, 329–343.
[15] Nishizuka, Y. (1992) Science 258, 607–614.
[16] Dekker, L.V. and Parker, P.J. (1994) Trends Biochem. Sci. 19, 73–77.
[17] Bogoyevitch, M.A., Glennon, P.E., Andersson, M.B., Clerk, A., Lazou, A., Marshall, C.J., Parker, P.J. and Sugden, P.H. (1994) J. Biol. Chem. 269, 1110–1119.
[18] Pears, C.J., Kour, G., House, C., Kemp, B.E. and Parker, P.J. (1990) Eur. J. Biochem. 194, 89–94.
[19] Wotton, D., Ways, D.K., Parker, P.J. and Owen, M.J. (1993) J. Biol. Chem. 268, 17975–17982.
[20] Goode, N.T. and Parker, P.J. (1994) FEBS Lett. 340, 145–150.
[21] Schaap, D., Parker, P.J., Bristol, A., Kriz, R. and Knopf, J. (1989) FEBS Lett. 243, 351–357.
[22] Knowlton, K.U., Baracchini, E., Ross, R.S., Harris, A.N., Henderson, S.A., Evans, S.M., Glembotski, C.C. and Chien, K.R. (1991) J. Biol. Chem. 266, 7759–7768.
[23] Nelson, C., Albert, V.R., Elsholz, H.P., Lu, L.I.-W. and Rosenfeld, M.G. (1988) Science 239, 1400–1405.
[24] Cherrington, J.M. and Mocarski, E.S. (1989) J. Virol. 63, 1435–1440.
[25] Sambrook, J., Fritsch, E.F. and Maniatis, T. Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989.
[26] Iwaki, K., Sukhatme, V.P., Shubeita, H.E. and Chien, K.R. (1990) J. Biol. Chem. 265, 13809–13817.
[27] Pears, C., Schaap, D. and Parker, P.J. (1991) Biochem. J. 276, 257–260.
[28] Hyatt, S.L., Klauck, T. and Jaken, S. (1990) Mol. Carcinog. 3, 45–53.