Cardiac protein kinase C expression in two models of cardiac hypertrophy associated with an activated cardiac renin–angiotensin system: effects of experimental hyperthyroidism and genetic hypertension (the mRen-2 rat)

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Abstract

There is evidence for a role of protein kinase C (PKC) in the development of cardiac hypertrophy. We examined the expression of individual PKC isoforms in the adult rat heart in two distinct, well-characterised *in vivo* models of cardiac hypertrophy associated with an activated cardiac renin–angiotensin system, namely experimental hyperthyroidism and the TGR(mRen2)²⁷ rat. The cardiac expression of a range of PKC isoforms (PKC- α , PKC- δ , PKC- ϵ , PKC- γ , and PKC- ι) was examined by immunoblotting. Our work demonstrates that the expression of total cardiac nPKC- δ and nPKC- ϵ relative to protein is

selectively and differentially modified in these models. A consistent up-regulation of nPKC- δ in conjunction with overall down-regulation of nPKC- ϵ was observed in both models. The expression of other PKC isoforms was unaffected. The divergent responses of the expression of the two nPKC isoforms to an activated cardiac renin–angiotensin system *in vivo* in adulthood suggest that these individual nPKC isoforms subserve specific roles in the response.

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Introduction

Members of the protein kinase C (PKC) family are important for regulating cell growth and are intracellular signalling molecules for a variety of Gq protein-coupled receptor agonists acting through hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (reviewed by Nishizuka 1992, 1995). The PKC family contains three subfamilies differing in structure, regulatory properties and tissue distribution (Ono *et al.* 1988, Dekker & Parker 1994, Sugden & Bogoyevitch 1995). The conventional PKCs (cPKCs) (α , β I/II and γ) are activated by calcium, phosphatidylserine and diacylglycerol (DAG)/phorbol esters. The novel PKCs (nPKCs) (δ , ϵ , η /L and θ) are activated by phosphatidylserine and DAG/phorbol esters, but are insensitive to calcium. The atypical PKCs (aPKCs) (ζ , ι , and the mouse homologue λ) are insensitive to DAG/phorbol esters and calcium (Nakanishi *et al.* 1993). PKC isoform switching occurs during development and, even in adulthood, the PKC isoform expression profile responds to environmental and hormonal stimuli. The nPKC isoforms nPKC- δ and nPKC- ϵ predominate in normal adult rat heart, and the Ca²⁺-sensitive cPKC isoforms are not consistently detected (Bogoyevitch *et al.*

1993, Rybin & Steinberg 1994, but see Rybin & Steinberg 1997).

PKC has been implicated as a signalling molecule mediating cardiac hypertrophy (Housey *et al.* 1988, Dunnmon *et al.* 1990, Sei *et al.* 1991) and overexpression of certain PKC isoforms has been associated with cardiac hypertrophy and altered cardiac performance. For example, targeted overexpression of the PKC- β isoform in hearts of transgenic mice results in left ventricular hypertrophy (LVH), cardiac myocyte necrosis and decreased left ventricular performance (Wakasaki *et al.* 1997). Pressure overload (aortic banding), another hypertrophic stimulus, also leads to re-expression of cPKC isoforms (Gu & Bishop 1994), normally only abundant in the immature heart (Rybin & Steinberg 1994, Clerk *et al.* 1995). In addition, there is also a selective quantitative increase in nPKC- ϵ (Gu & Bishop 1994). However, the expression of nPKC- δ is unchanged (Gu & Bishop 1994), raising the possibility that overexpression of one or more of the nPKC isoforms normally found in the adult heart may influence myocyte growth and/or heart performance.

Factors evoking LVH and changes in cardiac performance in adulthood include increased sympathoadrenal tone

and increased workload (Chien *et al.* 1991, Komuro & Yazaki 1993). The effect of increased workload may be signalled by the autocrine, paracrine or juxtacrine release of vasoactive peptides, notably angiotensin II (Baker & Aceto 1990, Schelling *et al.* 1991, Lee *et al.* 1993, Sadoshima & Izumo 1993, Lip *et al.* 1994). The renin-angiotensin system contributes to hyperthyroidism-induced cardiac hypertrophy (Kobori *et al.* 1997) and RIAs and RT-PCR revealed increased cardiac levels of renin and angiotensin II and enhanced cardiac expression of renin mRNA in hyperthyroid rats, with regression of LVH in response to losartan treatment (Kobori *et al.* 1997). Similarly, significant expression of the Ren-2 gene leads to overproduction of angiotensin II in the hearts of hypertensive transgenic TGR(mRen2)27 rats (Bohm *et al.* 1995, Lee *et al.* 1995) and it appears that pathomorphological changes in the hypertrophied hearts of these transgenic animals occur secondary to an activated intracardiac renin-angiotensin system (Bohm *et al.* 1995, Lee *et al.* 1995).

The present study used antibodies generated against synthetic peptides derived from unique sequences of individual PKC isoforms to identify and compare the effects of an activated cardiac renin-angiotensin system induced by experimental hyperthyroidism (triiodothyronine (T_3) injection) or introduction of the mouse Ren-2 gene into the rat genome (the TGR(mRen2)27 transgenic rat) on the cardiac PKC isoform expression profile in adulthood. The study was restricted to measurement of PKC protein expression as our primary interest was in protein abundance, rather than the regulation of PKC mRNA expression or of PKC activity.

Materials and Methods

Materials

Radiochemicals, prestained molecular mass standards, enhanced chemiluminescence (ECL) Western blotting detection reagents, autoradiography film (Hyperfilm ECL), horseradish peroxidase-linked anti-mouse immunoglobulin and intensifying screens were from Amersham International, Amersham, Bucks, UK. SDS-PAGE reagents and reagents for protein assay (Bradford method) were from Bio-Rad (Hemel Hempstead, Herts, UK). Nitrocellulose (0.45 mm) was from Schleicher and Schuel (Dassal, Germany). Other laboratory chemicals were from Merck (Poole, Dorset, UK). Mouse monoclonal anti-peptide antibodies against PKC- α , - δ , - ϵ , - γ , and - ι were from Transduction Laboratories (Lexington, KY, USA). Rabbit anti-PKC- δ and - ϵ polyclonal antibodies were from Gibco BRL (Paisley, Scotland). Horseradish peroxidase-linked anti-rabbit immunoglobulin was from Sigma (Poole, Dorset, UK).

Animals and hormone treatment

Female Wistar rats (200–250 g) from Charles River, Margate, Kent, UK were given free access to food and water. Rats were made hyperthyroid by daily subcutaneous injection of T_3 (100 mg/100 g body weight per day in 200 to 250 μ l) for 3 days. This resulted in an approximately 40% increase in left ventricular mass over the treatment period (results not shown; see also Sugden *et al.* 1992). Control rats were injected with equivalent amounts of hormone solvent (10 mM NaOH/0.03% BSA). Heterozygous female TGR(mRen2)27 rats (281 ± 8 g) and TGR(mRen2)27 transgene negative controls (265 ± 6 g) were from the Hatter Institute, University College London and were transferred to Queen Mary and Westfield College 2–3 weeks before use. Hypertension in the TGR(mRen2)27 rats was verified by systolic blood pressure measurements (tail-cuff plethysmography) from 5 to 12 weeks, over which period mean blood pressure increased from 127 ± 4 mmHg to 236 ± 6 mmHg ($P < 0.001$). Mean blood pressure in transgene negative rats did not exceed 140 mmHg (results not shown). Mean heart weights (g/100 g body weight) were 4.40 ± 0.17 ($n=6$) and 3.07 ± 0.04 ($n=6$) respectively in the TGR(mRen2)27 and transgene negative rats ($P < 0.001$).

Sample preparation

Whole hearts were excised under anaesthesia and freeze-clamped. For each animal, the entire frozen heart was ground under liquid nitrogen, and homogenised with five volumes of 20 mM Tris pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 10% glycerol, 1% Igepal, 0.5 mM Na_3VO_4 , 0.2 mM PMSF, 10 mg/ml leupeptin, 1.5 mg/ml benzamidine, 50 mg/ml aprotinin, 50 mg/ml pepstatin A. The homogenates were placed on ice for 20 min and then microfuged at 10 000 g for 20 min at 4 °C to pellet cell debris and connective tissue. The supernatant, representing the total cell extract, was used for immunoblotting. Protein concentrations were determined by the Bradford method (Bradford 1976).

SDS-PAGE and immunoblotting

The supernatants were prepared for SDS-PAGE by boiling for 5 min in Laemmli sample buffer containing 5% 2-mercaptoethanol. Protein aliquots (40 μ g) were loaded onto 7.5% (w/v) acrylamide gels and subjected to SDS-PAGE in a Bio-Rad Mini Protean gel apparatus. Protein was transferred electrophoretically to nitrocellulose membrane (0.45 mm) for 1 h at 100 V using a Bio-Rad Mini Protean transfer apparatus with 0.01% (w/v) SDS added to the transfer buffer. The nitrocellulose was blocked by incubation in 5% (w/v) non-fat domestic milk powder ('Marvel') (or 3% BSA for PKC- δ and - ϵ polyclonal

antibodies) dissolved in 20 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5 (TTBS) overnight at 4 °C.

Blocking buffer was decanted and primary antibody, dissolved in blocking buffer at the recommended dilution, added and incubated at room temperature for 2 h. Blots were washed in TTBS for 45 min with at least five changes of buffer. Horseradish peroxidase-linked second antibody diluted in blocking buffer was added (anti-mouse for monoclonal antibodies; anti-rabbit for polyclonal antibodies). Nitrocellulose was incubated for 2 h at room temperature with the secondary antibody. Following washing of the blots, protein bands were visualised using Amersham ECL reagents and exposure to Hyperfilm for between 30 s and 10 min. Immunoblots were quantified by laser scanning densitometry.

Statistical methods

Results are presented as mean $\pm$ S.E.M. Statistical significance was assessed using a two-tailed Student's *t*-test. $P < 0.05$ was taken to indicate statistical significance of differences between groups.

Results

PKC isoform expression in hearts from adult euthyroid and hyperthyroid rats

Using immunoblot analysis, we first examined PKC isoform expression in total heart extracts from control, euthyroid adult Wistar rats. An initial characterisation using competing antigenic peptides was performed to determine the specificity of the antibodies. Preliminary experiments established the specificity of immunoreactivity by the use of the appropriate competing peptide antigen (2 μ g/ml). Several antibodies recognised more than one protein, but in each case PKC protein bands were identified by their specific reduction in immunoreactivity when competing peptide was included (results not shown). We detected bands corresponding in apparent molecular mass to the cPKC isoforms cPKC- α (apparent molecular mass, 82 kDa) and cPKC- γ (apparent molecular mass, 80 kDa), the nPKC isoforms nPKC- δ (apparent molecular mass, 78 kDa) and nPKC- ϵ (apparent molecular mass, 90 kDa), and the aPKC isoform aPKC- ι (apparent molecular mass, 74 kDa) in extracts of hearts from adult euthyroid rats. Representative immunoblots are shown to the right of Fig. 1. The presence of cPKC- α (Ca^{2+} -dependent) and the two Ca^{2+} -independent PKC isoforms (nPKC- δ and nPKC- ϵ) in adult rat hearts is consistent with previous reports (Bogoyevitch *et al.* 1993, Rybin & Steinberg 1994). Other PKC isoforms, including PKC- β , were not detected.

Although T_3 suppresses immunodetectable cPKC- α in cultures of neonatal rat ventricular myocytes (Rybin &

Steinberg 1996), the abundance (relative to protein) of cPKC- α in extracts of whole hearts was unchanged by experimental hyperthyroidism *in vivo* (Fig. 1, top panel). cPKC- γ expression was also unchanged (Fig. 1, top panel). In contrast, nPKC- δ expression (relative to protein) was almost doubled ($P < 0.01$) in response to experimental hyperthyroidism *in vivo*, whereas the expression of PKC- ϵ in hearts of hyperthyroid rats was almost halved ($P < 0.001$) (Fig. 1, middle panel). Although there was a trend towards increased aPKC- ι expression in the heart of hyperthyroid rats, this did not achieve statistical significance (Fig. 1, bottom panel).

PKC isoform expression in hearts from TGR(mRen2)27 rats

PKC isoform expression was determined in adult TGR(mRen2)27 rats and in transgene negative (Hanover Sprague-Dawley $\times$ Wistar-Kyoto) controls. Immunoblot analysis revealed the presence of PKC- α , - γ , - δ , - ϵ , and - ι in hearts from the transgene negative controls, as in hearts from Wistar rats. Representative immunoblots are shown to the right of Fig. 2. There were no statistically significant changes in expression of PKC isoforms PKC- α , - γ or - ι (relative to protein) in the hearts of TGR(mRen2)27 positive rats (Fig. 2, top and bottom panels). The abundance of nPKC- δ (relative to protein) in hearts of TGR(mRen2)27 rats was increased 2-fold ($P < 0.01$) relative to the transgene negative controls (Fig. 2, middle panel). In contrast, the relative expression of nPKC- ϵ in hearts of TGR(mRen2)27 rats was only 70% ($P < 0.05$) of that of the controls (Fig. 2, middle panel).

Discussion

The major specific PKCs identified in the neonatal rat heart to date (Bogoyevitch *et al.* 1993, Puceat *et al.* 1994, Rybin & Steinberg 1994) are the cPKC isoforms cPKC- α and cPKC- β , the nPKC isoforms nPKC- δ and nPKC- ϵ , and aPKC- λ (previously identified as the structurally highly homologous PKC- ζ ; Rybin *et al.* 1997). The expression of the (Ca^{2+} -sensitive) cPKC isoforms, cPKC- α and cPKC- β , and of the nPKC- δ isoform is progressively down-regulated during the first 2 postnatal weeks (Rybin & Steinberg 1994, Clerk *et al.* 1995), and recent studies have provided evidence for regulation of both PKC- α and - δ at the level of transcription during this developmental period (Rybin *et al.* 1997). In the present study, we were able to reproduce the developmental decline in cardiac cPKC- α expression previously reported to occur during late foetal and early neonatal development (Rybin & Steinberg 1994, Clerk *et al.* 1995). In our hands, abundances (relative to protein) were 100 ± 14 ($n=3$), 64 ± 7 ($n=3$) and 22 ± 7 ($n=3$) in hearts of 20-day foetal, 4-day neonatal and adult hearts respectively. Although there is also a relatively modest (30%) developmental

Figure 1 Quantification of PKC isoform expression in hearts from euthyroid and hyperthyroid rats. Total protein extracts from hearts were denatured and subjected to SDS-PAGE (40 µg protein/lane) and immunoblotting with the isoform-specific antibodies indicated, as described in Materials and Methods. Results were analysed by laser densitometry. Results for hyperthyroid rats (three to eight per group) are expressed relative to PKC isoform expression in hearts from euthyroid rats when samples from euthyroid and hyperthyroid rats were run in parallel. Results for euthyroid rats are normalised to 100% and the S.E.M. for this group represents the variation observed between individual hearts (means for at least four rats). Data from representative single experiments (left hand blots, euthyroid; right hand blots, hyperthyroid) are shown to the right.

Figure 2 Quantification of PKC isoforms in hearts from TGR(mRen2)27 transgene negative or positive rats. Total protein extracts of hearts were denatured and subjected to SDS-PAGE (40 µg/lane) and immunoblotting with the indicated isoform-specific antibodies, as described in Materials and Methods. Results for transgene positive rats are expressed relative to those from transgene negative rats, which have been normalised to 100%. The S.E.M. for the transgene positive group thus gives the relative change observed when samples from transgene negative and positive rats were run in parallel, with five to ten rats in the transgene positive group. The S.E.M. for the transgene negative group represents the variation observed between hearts from five to ten rats. Data from representative single experiments are shown to the right (left hand blots, transgene negative; right hand blots, transgene positive).

decline in nPKC- ϵ expression, nPKC- ϵ continues to be expressed at high levels in adulthood (Rybin & Steinberg 1994, 1996, Clerk *et al.* 1995). The developmental decline in expression of cPKC- α and nPKC- δ (Rybin & Steinberg 1994, 1996) coincides with the maturation of the sympathetic innervation of the heart and a postnatal surge in thyroid hormone levels (see Rybin & Steinberg 1996) and it has recently been established using cultured cardiac myocytes that the change in thyroid status could account for the down-regulation of nPKC- ϵ expression that occurs during normal cardiac development (Rybin & Steinberg 1996).

The present study analysed the changes in expression of individual PKC isoforms in the adult rat heart induced by 3 days of experimental hyperthyroidism *in vivo*. The study is therefore of interest with respect to both the developmental influence of changes in thyroid status and the well-established actions of hyperthyroidism to activate the cardiac renin-angiotensin system (Kobori *et al.* 1997) and elicit LVH in adulthood (Litten *et al.* 1982, Chilian *et al.* 1985, Klein 1990). Our finding that nPKC- ϵ protein expression is down-regulated in hearts of adult hyperthyroid rats complements previous results from Steinberg's group demonstrating increased nPKC- ϵ expression in hearts from adult hypothyroid rats (Rybin & Steinberg 1996). The results provide clear evidence that the cardiac expression of nPKC- ϵ in the adult rat heart is responsive to changes in thyroid hormone status *in vivo*. In addition, we demonstrated up-regulation of cardiac nPKC- δ protein expression in hearts of adult hyperthyroid rats. This response to elevated T_3 levels in adulthood contrasts with down-regulation of nPKC- δ during the neonatal period (Rybin & Steinberg 1996). It therefore appears that down-regulation of cardiac nPKC- ϵ (as previously suggested by Rybin & Steinberg 1996) is a consistent response to a rise in circulating thyroid hormone concentrations, but it is unlikely that neonatal down-regulation of nPKC- δ is necessarily a consequence of increased thyroid hormone concentrations. It is not known to what extent the state of activation of the cardiac renin-angiotensin system is modulated by changes in thyroid status during developmental growth.

The TGR(mRen2)27 transgenic rat represents a non-invasive model for cardiac hypertrophy and hypertension with a clearly defined genetic alteration (Mullins *et al.* 1990, Bohm *et al.* 1995, Lee *et al.* 1995, Veniant *et al.* 1995). TGR(mRen2)27 rats develop severe fulminant hypertension (200–260 mmHg) as a consequence of introduction of the mouse Ren-2 renin gene into the rat genome (Mullins *et al.* 1990). Pathomorphological alterations of cardiovascular organs, including an increased left ventricular mass index, are observed in young rats aged <12 weeks (Veniant *et al.* 1995) and have been attributed, at least in part, to an activated cardiac renin-angiotensin system (Bohm *et al.* 1995, Lee *et al.* 1995). The current studies demonstrate an approximately 2-fold increase in

cardiac nPKC- δ expression relative to protein in adult TGR(mRen2)27 hypertensive rats compared with age-matched transgene negative controls. There is a concomitant moderate decline in cardiac PKC- ϵ expression, whereas the cardiac expression of the other PKC isoforms, including those mainly expressed during early development, is unchanged. Thus, as in hyperthyroidism, the cardiac isoform profile of the adult TGR(mRen2)27 rat still contains mainly nPKC isoforms, but there is a switch towards the nPKC- δ isoform at the expense of nPKC- ϵ in the absence of re-expression of cPKC isoforms.

In conclusion, we examined the protein expression of individual PKC isoforms *in vivo* in hearts of adult rats using two quite distinct models of LVH (experimental hyperthyroidism and genetic hypertension) associated with an activated cardiac renin-angiotensin system. Our work demonstrates that cardiac nPKC- δ and nPKC- ϵ expression is selectively and differentially modified, and the responses are similar in the two models. The similarity between the effects of genetic hypertension and hyperthyroidism in altering the PKC isoform balance within a specific PKC isoform class (the nPKCs) implies a common regulatory influence and strongly suggests that modulation of the cardiac PKC isoform profile is a specific response to an activated renin-angiotensin system. However, it should be emphasised that the findings of altered protein levels of these two important signalling molecules does not provide insight into the activation state of the enzymes, nor can conclusions be drawn as to whether enzyme levels change in response to increased activation. A consistent up-regulation of cardiac nPKC- δ was observed in conjunction with overall down-regulation of cardiac nPKC- ϵ . The divergent responses of the two PKC isoforms argue in favour of the hypothesis that these individual nPKC isoforms subserve different roles in mediating the response to an activated renin-angiotensin system. It is notable that these two isoforms both belong to the novel sub-family of PKC isoforms, and that the reason for differences in their response can not be simply attributed to the sub-family to which they belong.

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