Biochemical Pharmacology, Vol. 51, pp. 1089–1093, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII 50006-2952(96)00046-9

SHORT COMMUNICATION

Tissue-Specific Developmental Regulation of Protein Kinase C Isoforms

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ABSTRACT. The limited amount of available information regarding the developmental control of protein kinase C (PKC) isoform expression restricts our understanding of the role of these enzymes in normal physiology. Accordingly, this study investigated PKC isoform expression in selected tissues from fetal, neonatal, and adult rats. PKCβ immunoreactivity was prominent in brain tissue, whereas the expression of PKCα, PKCδ, PKCε, and PKCζ was found to be widespread. Although no developmental change in any PKC isoform was evident in liver, striking tissue-specific age-dependent differences in PKC isoform abundance were noted in other tissues. For example, age-dependent increases in PKCα, PKCβ, and PKCδ in brain contrasted with age-dependent decreases in PKCα and PKCδ in lung, kidney, and heart. Immunoreactivity for PKCε was abundant in all fetal/neonatal tissues; PKCε was detected in the adult brain, heart, and liver, but not the adult kidney and lung. Finally, PKCζ was more abundant in fetal/neonatal than in adult brain, lung, kidney, and heart. These results indicate that the fetal/neonatal lung, kidney, and heart are enriched in PKCζ, PKCα, PKCδ, and PKCε, relative to the adult tissues. These age-dependent variations in the abundance of individual isoforms of PKC may critically influence tissue responsiveness to external stimuli. Moreover, the finding that PKCζ is particularly abundant in fetal tissues as well as the liver, the only tissue included in this study which retains regenerative capacity in the adult animal, is consistent with the notion that PKCζ may play a role in cell proliferation. BIOCHEM PHARMACOL 51;8:1089–1093, 1996.

KEY WORDS. protein kinase C; development

PKC† is a serine/threonine protein kinase implicated in a variety of vital cellular functions including the responses to hormones and drugs, proliferation, and cell differentiation [1]. Molecular cloning and biochemical studies have revealed a family of related PKC isoforms that differ in their cofactor requirements for enzymatic activity, tissue distribution, and subcellular localization. On the basis of structural criteria and enzymatic activity, individual PKC isoforms have been subdivided into three categories. PKCα, PKCβ, and PKCγ are referred to as conventional or calcium-dependent PKCs. Their activation by phosphatidylserine, DAG, or phorbol esters is enhanced in the presence of calcium, suggesting a role for increased cytosolic calcium in the *in vivo* regulation of conventional PKC. PKCδ, PKCε, PKCη/L, PKCθ are referred to as novel PKCs; these isoforms do not require calcium for maximal enzymatic activation. All conventional PKC and novel PKC isoforms are cellular receptors for DAG and tumor-promoting phorbol esters; agonist stimulation of cell-surface receptors and the release of DAG from membrane phospholipids (phosphatidylinositol 4,5-bisphosphate and/or phosphatidylcholine) have been linked to the activation of these PKC isoforms in several cell types. In contrast, PKCζ and PKCι (and the mouse homolog PKCλ) are referred to as atypical PKCs as they exhibit distinct structural properties and their kinase activity is not influenced by DAG/phorbol esters. Although the mechanism for atypical PKC isoform activation *in vivo* remains uncertain, there is evidence that phosphatidylinositol 3,4,5-trisphosphate (PIP_3) specifically stimulates PKCζ kinase activity *in vitro* [2]. This observation raises the possibility that lipid cofactors, which are downstream from activated growth factor receptors and are distinct from DAG, may act as physiologic activators of PKCζ. Finally, additional PKC isoforms with distinct structural and biochemical properties have been identified recently [3, 4], making it likely that the current classification of PKC will be expanded in the future to incorporate these newly identified species.

Studies on tissues from mature animals provide compelling evidence that PKC isoforms display distinct tissue, cellular, and subcellular distributions. For example, there is evidence that PKCα, PKCδ, PKCε, PKCζ, and PKCλ are expressed by many tissues in the adult animal [1, 5, 6]. Based upon their ubiquitous expression, these isoforms of PKC are presumed to subserve rather general cellular func-

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† *Abbreviations:* PKC, protein kinase C; and DAG, diacylglycerol.

Received 4 August 1995; accepted 22 November 1995.

tions. In contrast, PKCγ is abundant in brain [5], PKCη is localized primarily to lung and skin [7], and PKCθ is abundant in skeletal muscle [8]. The more tissue-selective expression of these PKC isoforms may suggest tissue-specific functions.

Our knowledge regarding PKC isoform expression in tissues from fetal/neonatal animals is considerably more limited. Studies to date have been confined to brain and cardiac tissue where striking isoform-specific developmental changes in PKC expression have been identified. For example, there have been numerous previous studies establishing that the abundance of calcium-dependent isoforms of PKC increases during post-natal development of the brain [9–13]. In only one of these studies was the analysis extended to the novel and atypical PKC isoforms. Here, the levels of PKCδ, PKCζ, and PKCε were reported to increase, decrease, and not change, respectively, between 6 days and 3 months of age [13]. These results suggest a very complex and isoform-specific regulatory control of PKC isoform expression during normal developmental maturation of the brain. The only other tissue in which the developmental regulation of PKC isoform expression has been considered is the heart, where a completely different age-dependent pattern of changes in PKC isoform expression occurs [14–17]. Here, the expression of conventional, calcium-sensitive isoforms of PKC (α, and perhaps β) and the atypical isoform PKCζ is confined to fetal and neonatal myocytes, whereas the novel, calcium-insensitive isoforms predominate in adult myocytes. Of note, the developmental decline in PKCζ expression occurs around the time of birth, precedes the decline in PKCα and PKCδ expression (which occurs during the first 2 weeks of post-natal life), and coincides with the loss of proliferative growth capacity of the myocytes. This has led to the speculation that PKCζ may play a specific role in the signal transduction pathway(s) leading to myocyte proliferation [14]. Indeed, the recent evidence that PKCζ may be an *in vivo* target for PIP_3, and thereby play a role in the phosphorylation cascade initiated by activated growth factor receptors [2], as well as recent evidence that PKCζ plays a critical role in mitogenic signaling in oocytes and fibroblasts [18, 19], is consistent with this hypothesis.

Given that previous analyses of the developmental control of PKC isoforms confined to brain and cardiac tissues provided strikingly divergent results (Refs. 9–13 vs Refs. 14–17), this study used immunoblot analysis to systematically analyze and compare the developmental changes in PKC isoform expression in several other rat tissues. Our goals were to identify developmental patterns of PKC isoform expression that would be anticipated to influence tissue growth and differentiation as well as tissue responsiveness to hormones and drugs.

MATERIALS AND METHODS

Materials

Polyclonal antibodies against PKCα, PKCβ, PKCδ, and PKCζ were purchased from Gibco-BRL (Grand Island, NY). Polyclonal anti-PKCε was the gift of Dr. Doriano Fabbro (Ciba Geigy, Basel, Switzerland). ^{125}I-Labeled goat anti-rabbit IgG F(ab′)$_2$ fragment was purchased from Du Pont NEN (Boston, MA). All chemicals were reagent grade.

Tissue Preparations

Fetal (day 20), neonatal (day 2), and adult (250 g) Wistar rats were used in this study. Organs were excised quickly, rinsed in cold physiologic saline solution, blotted with filter paper, weighed, and frozen rapidly in liquid nitrogen for subsequent preparation of total protein extracts. Four to twelve organs were pooled for each preparation. Total protein extracts were prepared from intact tissues by adding preheated (95°) homogenization buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 6 mM β-mercaptoethanol, 50 μg/mL aprotinin, 48 μg/mL leupeptin, 5 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium vanadate, and 50 mM NaF) containing 1% SDS to minced tissues (12 mL/g tissue) followed by homogenization with a polytron. Extracts were stored at −70° until used.

Immunoblot Analysis

Immunoblot analysis for individual PKC isoforms was performed according to methods published previously [14]. Five PKC isoform-specific antisera were used. These antisera were generated against synthetic peptides corresponding to amino acids 313–326 for PKCα, 313-329 for PKCβ (a sequence common to both splice variants of PKCβ), or unique sequences in the carboxy terminal variable region of PKCδ, PKCε, and PKCζ. However, it should be noted that the anti-PKCζ has been shown also to recognize PKCα ([20] and see Fig. 1). Moreover, additional atypical isoforms of PKC, which are structurally highly homologous to PKCζ in the carboxyl-terminal end of the molecule (PKCι [21] and PKCλ [6]), also are recognized by the anti-PKCζ antiserum [6, 21]. Thus, the identification of the protein detected with this antibody as PKCζ is tentative at this time. Primary PKC isoform specific antisera were used at a 1:250 (anti-PKCβ) or 1:500 (antisera to PKCα, PKCδ, PKCε, and PKCζ) dilution. The specificity of all immunoreactive proteins was established previously by immunoblot analysis in the presence and absence of competing immunizing peptide [14]. For quantification, the specific regions of the polyacrylamide gel corresponding to the labeled bands on the autoradiograms were excised and counted in an autogamma scintillation spectrometer.

RESULTS AND DISCUSSION

PKCα, PKCδ, PKCε, and PKCζ were detectable in each of the organs studied, although in some instances their expression was confined to a particular developmental stage (Figs. 1–3). In contrast, PKCβ immunoreactivity was detected in

brain (Fig. 1) and adult spleen (data not shown), but not in lung, kidney, heart, or liver (data not shown). Although it is possible that PKCβ may be present in these tissues at levels beneath the limit of detection in our assay system, the results are consistent with earlier reports that brain and spleen are particularly enriched in PKCβ [5, 9, 22, 23].

Results of western blotting with PKC isoform specific antisera and extracts from fetal, neonatal, and adult brain are shown in Fig. 1 and Table 1. PKCζ immunoreactivity was detected at the highest levels in the fetal brain and declined progressively in the neonatal and adult samples. The age-dependent decline in brain PKCζ expression was in marked contrast with the developmental changes in the abundance of the other PKC isoforms. Immunoreactivity for the conventional PKCs (PKCα and PKCβ) was greater in adult than in neonatal brains. Specific immunoreactivity for novel PKCδ also increased progressively from the fetal and neonatal to the adult samples, whereas brain PKCε immunoreactivity was abundant at all ages. The distinct patterns of conventional PKCα and atypical PKCζ expression are emphasized by the immunoblot with the anti-PKCζ antiserum. This antiserum, which also weakly recognizes PKCα, detected the rise in PKCα immunoreactivity concurrent with the decline in PKCζ immunoreactivity in the same immunoblot. These results provide independent confirmation of the findings of several other laboratories [9, 10, 13] and argue that distinct physiological mechanisms regulate the expression of individual PKC isoforms during normal development of the rat brain.

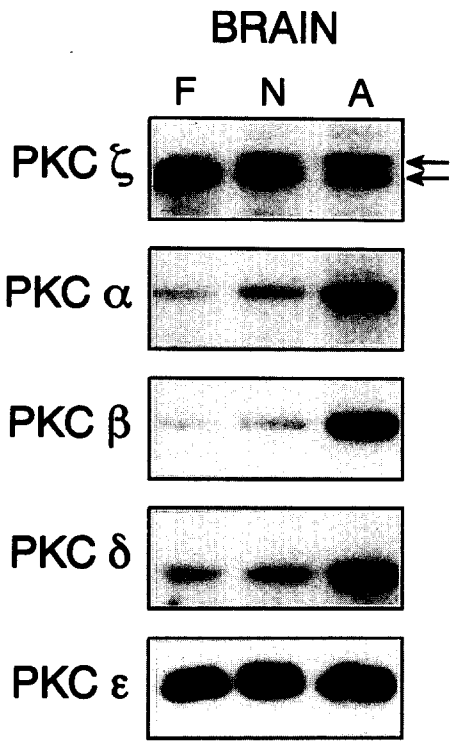

FIG. 1. PKC isoform expression in fetal, neonatal, and adult brain. Total protein extracts (150 μg) from fetal (F), neonatal (N), and adult (A) brains were subjected to SDS–PAGE and immunoblot analysis with PKC isoform-specific antibodies as described in Materials and Methods. The PKCζ antiserum recognized both PKCζ (lower arrow) and PKCα (upper arrow). Similar results were obtained in a second experiment performed on a separate set of samples.

TABLE 1. Developmental changes in PKC isoform expression

	PKC abundance (fetal level/adult level)				
	Brain	**Lung**	**Kidney**	**Heart**	**Liver**
PKCζ	1.9	2.2	3.9	5.0	1.1
PKCα	0.2	1.9	1.9	3.4	1.2
PKCδ	0.4	2.8	1.9	3.1	1.1
PKCε	0.7	2.3	4.5	1.4	1.2

Results are expressed as the ratio of PKC isoform immunoreactivity in fetal tissues relative to adult tissues and represent the average of data from experiments on two separate preparations. In each case, the variability was less than 15%.

Western blotting with extracts from the lungs, kidneys, and hearts of fetal, neonatal, and adult rats revealed a different developmental pattern of PKC isoform expression (Fig. 2, Table 1). For each organ, PKCζ immunoreactivity was highest in extracts from fetal tissues; PKCζ was barely detectable in the adult heart and was detectable at low levels in adult lung and kidney. However, the fetal/neonatal lung, kidney, and heart also were enriched in PKCα, PKCδ, and PKCε relative to the adult tissues, although the magnitude of developmental decline in the expression of individual PKC isoforms differed between tissues (for example, compare the trace levels of PKCε in adult lung and

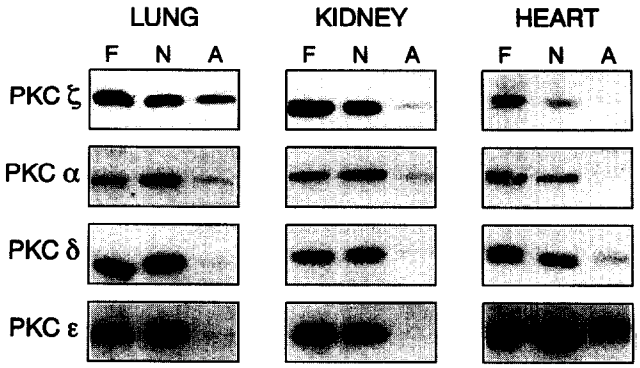

FIG 2. PKC isoform expression in fetal, neonatal, and adult lung, kidney, and heart. Total protein extracts (150 μg) from fetal (F), neonatal (N), and adult (A) tissues were subjected to SDS–PAGE and immunoblot analysis with PKC isoform-specific antibodies as described in Materials and Methods. Results are representative of two separate experiments performed on distinct sets of samples. A protein doublet for PKCε clearly was resolved in some experiments. This may represent PKCε species which differ in their phosphorylation state, since subtle differences in electrophoretic mobility due to differences in phosphorylation state have been described for this enzyme purified from brain [24]. In this regard, although the experiment depicted suggests that PKCε is more phosphorylated in the adult than in the neonatal heart, this was not a consistent observation.

kidney with the persistent PKCε expression in adult heart). Thus, in lung, kidney, and heart, fetal/neonatal tissues are enriched in atypical (ζ), conventional (α), and novel (δ and ε) isoforms of PKC.

A third pattern of PKC isoform expression was evident in extracts from fetal, neonatal, and adult livers. Here, no developmental changes in PKCα, PKCδ, PKCε, or PKCζ expression were detected (Fig. 3, Table 1). The absence of any age-dependent difference in PKC isoform expression in liver contrasts with the findings in other organs and suggests that the expression of these proteins is subject to multiple age- and tissue-specific regulatory mechanisms.

Although PKC isoform expression has been surveyed in many adult tissues [5], previous studies of the developmental regulation of PKC isoform expression were limited to brain and cardiac tissues and underestimated the complexity in the developmental regulation of these proteins. For example, the developmental increase in the abundance of conventional PKC isoforms (PKCα and PKCβ) and PKCδ in brain contrasted markedly with the developmental decrease of these isoforms in lung, kidney, and heart. Similarly, PKCε, which is abundant in all fetal/neonatal tissues included in this study, was readily detectable only in the brain, heart, and liver of the adult rat (not in kidney and lung). Thus, developmental changes in PKC expression do not conform to a single pattern, based upon either the particular isoform or tissue. Rather multiple factors (as yet largely unknown) must critically regulate the tissue- and age-specific expression of individual PKC isoforms. Moreover, these studies emphasize that age-dependent increases in PKC expression previously noted in the brain may not be representative of the developmental changes that occur in other tissues. In many other organs, neonatal/fetal tissues are enriched in PKC relative to adult tissues (similar to the situation previously described for the heart [14, 15, 17]). It is entirely possible that at least some component of the developmental changes in PKC isoform abundance results from age-dependent changes in cell composition in a particular tissue, rather than changes in the abundance of PKC isoforms within a defined cell type. However, our previous studies established that developmental changes in the abundance of PKC isoforms do occur in myocytes isolated from intact ventricles. These age-dependent differences in PKC isoform expression are likely to contribute importantly to the biological function of PKC. Thus, in addition to the complexity that arises as a result of the presence of multiple isoforms of PKC, which differ in their mode of activation, subcellular localization, and intrinsic substrate specificity, results reported herein lend support to the intriguing speculation that developmental changes in individual PKC isoforms also may underlie differences in hormonal modulation of cellular function.

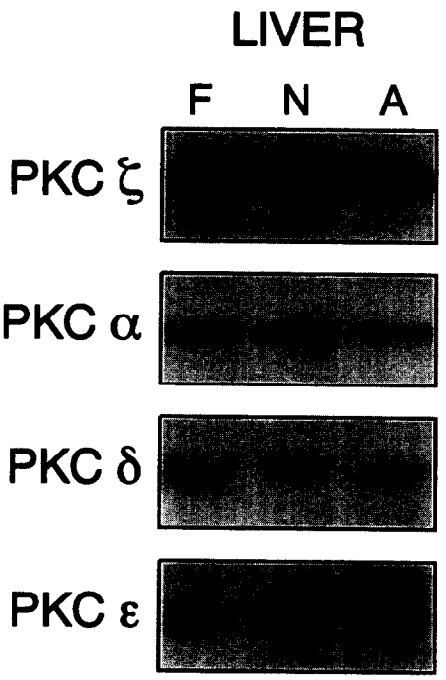

FIG. 3. PKC isoform expression in fetal, neonatal, and adult liver. Total protein extracts (150 μg) from fetal (F), neonatal (N), and adult (A) liver were subjected to SDS–PAGE and immunoblot analysis with PKC isoform-specific antibodies as described in Materials and Methods. Similar results were obtained in a second experiment performed on a separate set of samples. Any apparent minor age-dependent differences in PKC isoform expression were not consistent between the two preparations.

This study was predicated on the results of our previous experiments in rat cardiac myocytes [14], which indicated that fetal tissue is particularly enriched in PKCζ. Given the speculation that PKCζ may play a role in facilitating cell growth, we hypothesized that PKCζ would be abundant in all fetal tissues. Indeed, for brain, lung, kidney, and heart, the abundance of PKCζ was found to be greatest in fetal and/or neonatal tissues. Moreover, PKCζ was the only isoform of PKC that underwent a developmental decline in the brain. The finding that PKCζ was elevated in all fetal tissues examined in this study is consistent with the notion that PKCζ plays an important role in cell proliferation and maturation. While all fetal tissues examined appeared to be enriched in PKCζ, it is not exclusively a fetal isoform. In particular, the abundance of PKCζ was found to be similar in fetal, neonatal, and adult liver. This is not inconsistent with the hypothesis that PKCζ involved in a growth response, since the liver is the only tissue investigated in this study which retains regenerative capacity, even in the adult animal. The absence of any developmental decline in the abundance of PKCζ in the liver is intriguing and suggests that the role of PKCζ in cell proliferation requires further investigation.

This work was supported by U.S.P.H.S.-N.H.L.B.I. Grants HL-28958 (S.F.S.), HL-49537 (S.F.S.), and a National Institutes of Health post-doctoral training grant in pharmacological sciences (Grant 07271, M.G.).

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