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Distributional Patterns of Phospholipase C Isozymes in Heart and Brain of Spontaneously Hypertensive and Normotensive Rats

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The phospholipase C (PLC)-mediated intracellular signal transduction pathway is considered to be involved in the regulation of blood pressure. However, little information is available concerning the distributional and functional significance of PLC in the genetic hypertensive rats. As the first step of knowing the role of PLC on hypertension, we investigated the distribution of 6 PLC isozymes (PLC-β1, -β3, -β4, -γ1, -γ2 and -δ1) in the heart and brain, which are concerned with hypertension, in the normotensive Wistar-Kyoto rat (WKY) and spontaneously hypertensive rat (SHR) using the western blotting and immunocytochemistry. The immunoreactivities of PLC isozymes in brain were detected, but there were no distributional and quantitative differences between the WKY and SHR. In the heart, but the immunoreactivities to PLC-β1 and -γ2 in the SHR were higher than those in WKY. In immunocytochemistry to confirm these western blotting data, PLC-β1 and -γ2 were localized in cardiac myocytes and the intensities of immunoreactivity in SHR were stronger than that in WKY. These results suggest that PLC-β1 and -γ2 would have possibility to concern with the establishment of spontaneous hypertension.

Key Words: PLC isozymes, Western blot, Immunocytochemistry, Heart, Brain, SHR

INTRODUCTION

The spontanously hypertensive rat (SHR) is widely used in cardiovascular research as a model of human essential hypertension. The mechanisms involved in the elevation of blood pressure in SHR are poorly understood. Recently several lines of evidence suggest a role for the PI second messenger system in the pathogenesis of hypertension in the SHR (Turla et al, 1990; Uehara et al, 1988; Yagisawa et al, 1991; Kawaguchi et al, 1992). Uehara et al. (1988) reported that the increase of vascular PLC activity precedes the development of hypertension and that the enhancement may be induced by both quantitative and qualitative changes in phospholipase C (PLC) in SHR. DNA sequencing of PLC-δ cDNA cloned from an aortic cDNA library of SHR revealed a total of three SHR-specific point mutations, two of which resulted in amino acid substitutions, situated in the putative catalytic X domain (Yagisawa et al, 1991). PLC isozymes can be divided into three structural types, β (β1, β2, β3 and β4), γ (γ1 and γ2) and δ (δ1, δ2, δ3 and δ4), on the basis of the relative location of the X and Y domains in the primary structure of the enzymes. The distinct structural features of the different PLC types appear to be related to specific mechanisms of receptor-mediated enzyme activation (John et al, 1993; Wahl et al, 1992; Yagisawa et al, 1994). PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messenger molecules, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG) (Teitelbaum I, 1992; Meisenhelder et al, 1989). These second messengers are the integral components of the intracellular Ca^{2+} messenger system. This signal transduction pathway has been considered to be involved in the regulation of

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vasoconstriction and blood pressure (Baraban et al, 1985; Takori et al, 1986; Griendling et al, 1986).

Although derangements of PLC-mediated PI metabolism have been suggested to play a role in the pathogenesis of essential hypertension, informations are still limitted, concerning the functional significance of PLC in other important tissues than aorta that control the blood pressure. Moreover, although Uehara et al. (1988) reported there was no difference in the cardiac PLC activity between adult WKY and SHR, Kawaguchi et al. (1992) found that the accumulations of IP_3, IP_4 and DG were significantly enhanced in isolated myocytes from SHR heart by the stimulation with norepinephrine, and PLC activity increased with age in SHRSP heart cells. Thus, it is not clear that PLC-mediated PI turnover pathways play a role in the pathogenesis of spontaneous hypertension.

Central autonomic dysfunction is also known to play an important role in the generation and maintenance of hypertension in the SHR (Birkenhager et al, 1984; McCarty et al, 1987; Judy et al, 1979). The neurons in the rostral ventrolateral medulla (RVLM) and rostral ventromedial medulla (RVMM) are critically involved in the generation of neurogenic vasomotor tone and hence the maintenance of the arterial pressure in various species (Reis et al, 1989; Guyenet et al, 1990). In microinjection of lidocaine and electrical stimulation, the RVLM and RVMM differentially control cardiovascular function (Brody et al, 1991). But, there was no study for the PLC distribution in the SHR brain.

As the first step, therefore, to know the role of PLC on tissues concerning with hypertension, we investigate the distributional difference of PLC isozymes which can be obtained antibodies of PLC isozymes (PLC-β1, -β3, -β4, -γ1, -γ2 and -δ1) in the brain and heart of the WKY and SHR using the western blotting and immnocytochemistry.

METHODS

Animals

Male WKY and SHR were purchased from Korea Food and Drug Administration (Seoul, Korea) and housed in commercial equipment in a conventional environment for at least 3 days prior to use. They were provided with pelleted standard diet and tap water *ad libitum.*

Chemicals

Trizma-base, ethylenediamine-tetraacetic acid (EDTA), ethylene glycol-bis (b-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), β-mercaptoethanol, phenylmethyl-sulfonyl fluoride (PMSF), leupeptin, Tween 20, bovine serum albumin (BSA, fraction V), sodium-periodate, lysine, paraformaldehyde and 3,3'-diaminobenzidine were purchased from Sigma (St. Louis, MO). NBT/BCIP was purchased from Boehringer mannheim (Germany). ^{125}[I]-protein A was purchased from Amersham (100 μCi/ml, Amersham, UK). All other chemicals were of analytical grade from commercial sources.

Antibodies

PLC isozymes were detected with immunoblotting and immunocytochemistry using rabbit polyclonal antibodies against PLC-β1, -β3, -β4 and -γ2 or mouse monoclonal antibodies against PLC-γ1 and -δ1. These antibodies were kindly gifted by Sue Goo Rhee, NIH, USA.

Preparation of tissue homogenates for western blotting

12-weeks-aged male WKY and SHR were decapitated and the heart and brain were immediately removed, weighed, transferred into ice-cold homogenizing buffer (10 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 0.02% leupeptin, 0.1% Triton X-100, pH 7.5), and homogenized. The homogenate were centrifuged at 20,000 x g for 20 min. A part of the supernatant was taken for protein determination (Smith et al, 1985), while the remainder was immediately mixed (1:1) with 2 x SDS sample buffer and incubated in a heat-block for 5 min. The boiled samples were either used immediately or frozen at -20°C for SDS-PAGE.

Western blot and phosphoimager analysis

PLC isozymes in 100 μg supernatant proteins were separated by 6% SDS-polyacrylamide gels and trans-

ferred to nitrocellulose sheets (Schleicher & Schuell, Germany). Nonspecific binding sites on nitrocellulose membrane were blocked by incubation with 3% BSA in TTBS (Tris; 100 mM, NaCl; 150 mM, Tween 20; 0.5%) for 1 hr at room temperature. Nitrocellulose sheets were then incubated overnight at 4°C in the primary antibody against PLC isozymes diluted in 0.1% BSA-PBS (1:1000). After being rinsed with TTBS, alkaline phosphatase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Kirkegaard & Perry Lab. Inc, Gaithersburg, Maryland, USA) at 1:1000 dilution for detecting polyclonal antibodies (anti-PLC-β1, -β3, -β4 and -γ2) and monoclonal antibodies (anti-PLC-γ1 and -δ1) respectively were added and incubated for 2 hr at room temperature. Immunoreactive bands were visualized with the NBT/BCIP (5-bromo-4-chloro-3 indolyl phosphate/Nitro-blue tetrazolium) method.

For phosphoimager analysis, ^{125}[I]-protein A, which was diluted 1:1000, was treated instead of secondary antibody for 2 hours at room temperature. After washing with TTBS, radioactivities were counted using phosphoimager (Fuji, BAS 2500, Japan). The unit of radioactivities was represented by PSL (photo stimulated luminescence)/mm^2.

Immunocytochemistry

The animals were anesthetized with an intraperitoneal injection of an urethane (1.2 g/kg b.w.). They were perfused transcardially with phosphate-buffered saline (PBS; 10 mM phosphate buffer, 150 mM NaCl,

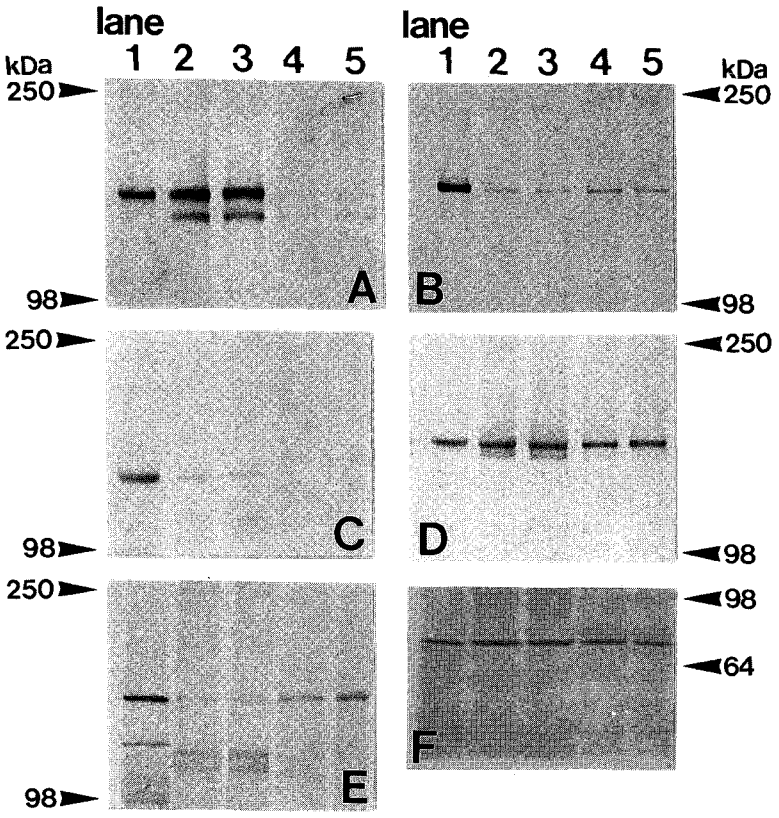

Fig. 1. Representative immunoblotting of PLC isozymes in brain and heart.
PLC-β1 (A), PLC-β3 (B), PLC-β4 (C), PLC-γ1 (D), PLC-γ2 (E) and PLC-δ1 (F) in the whole brain and heart (lane 1: standard, 2,3: whole brain of the WKY and SHR, 4,5: heart of the WKY and SHR). 50 μg protein content for PLC-β1 and -γ1, and 100 μg protein content for other enzymes.

pH7.4) followed preservation by perfusion with paraformaldehyde-lysine-periodate mixture (PLP; 10 mM periodate, 75 mM lysine, 2% paraformaldehyde, 37 mM phosphate buffer, pH 6.2) for 5 min. The heart was excised, and cut into slices that were fixed by immersion in the same PLP solution for 6 hr at room temperature and then overnight at 4°C. This tissue was embedded in wax for immunoperoxidase localization using the avidin-biotin-peroxidase method by using a VECTASTAIN *Elite* ABC kit. (Vector Laboratories, Burlingame, California, USA). Avidin-biotin-peroxidase method is following this ; The sections that were embedded in wax were cut at a thickness of 4 μm using a microtume (Technical Products International, St. Louis, MO). Sections were dewaxed, rehydrated, and blocked with normal blocking serum of the kit before staining. Sections were then incubated overnight at 4°C in the primary antibody against PLC-β1 (1:750), PLC-β3 (1:750), PLC-β4 (1:750), PLC-γ1 (1:100), PLC-γ2 (1:500) and PLC-δ1 (1:50) diluted in 0.1% BSA-PBS. After being rinsed in PBS, the sections were incubated with diluted biotinylated secondary antibody solution of the kit for 2 hr at room temperature. Sections were incubated with VECTASTAIN *Elite* ABC reagent for 30 min. For the detection of avidin-biotin-peroxidase, the sections were incubated in 0.05% 3,3'-diaminobenzidine containing 0.01% H_2O_2. After washing and dehydrating enough, the sections were mounted with canadabalsam and observed with a light microscope (Olympus, Japan)

RESULTS

Western blot and phosphoimager analysis

To know the distributional patterns of PLC isozymes in the heart and brain of the WKY and SHR, immunoreactivities to the PLC-β1, -β3, -β4, -γ1, -γ2 and -δ1 isoforms were determined in tissue-homogenates using the western blotting method. Whole rat brain and heart tissue homogenates from the WKY and SHR displayed protein bands corresponding to all of the 6 PLC isozymes, PLC-β1, -β3 and -β4, PLC-γ1 and -γ2, PLC-δ1. In the brain, immunoreactivities of 6 PLC isozymes were detected, but there were no differences between WKY and SHR (Fig 1). The 6 PLC isozymes were detected in heart. In contrast to the brain, the inten-

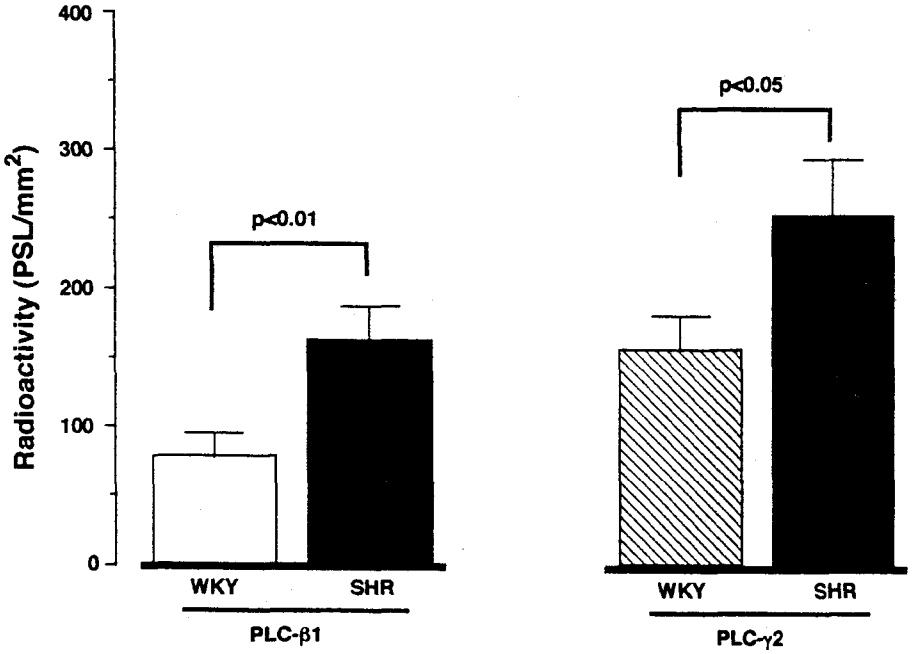

Fig. 2. Quantification of PLC-β1 and -γ2 in the heart homogenates from WKY and SHR. 50 μg protein content for PLC-β1 and 100 μg protein content for PLC-γ2. Values were represented by mean$\pm$SD from 3 rats.

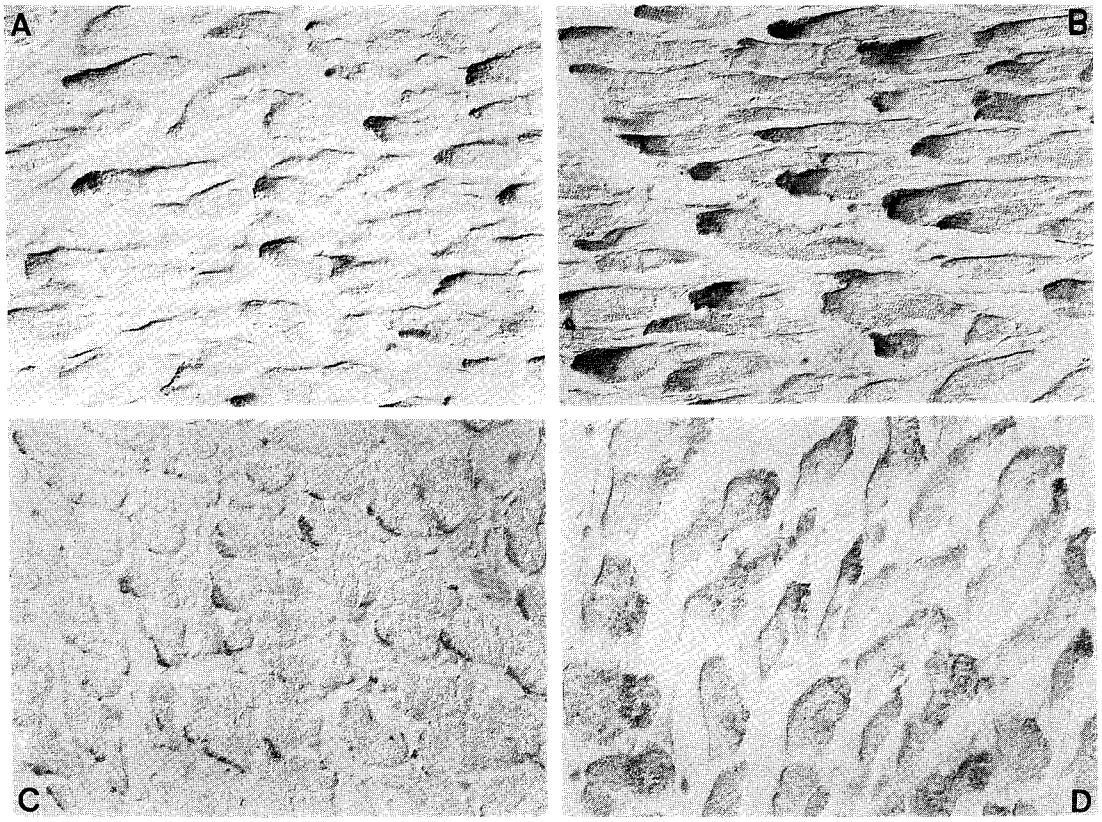

Fig. 3. Immunocytochemistry for PLC-β1 and -γ2 in heart of WKY and SHR. PLC-β1 (A: WKY and B: SHR) and -γ2 (C: WKY and D: SHR).

sities of PLC-β1 and -γ2 in SHR were stronger than those in WKY (Fig. 1-A and E). In order to quantify the PLC-β1 and -γ2, radioactivity was determined using ^{125}[I] labeled protein A by phosphoimager. The radioactivities of PLC-β1 and -γ2 were significantly enhanced by 2.1 folds and 1.6 folds in SHR against that of WKY, respectively. The radioactivities of PLC-β1 (loading content of protein: 50 μg protein) and -γ2 (loading content of protein: 100 μg) were 77.8 ± 17.1 and 156.0 ± 23.4 PSL/mm^2, respectively (Fig. 2).

Immunocytochemistry in heart

In order to clarify the distribution of PLC-β1 and -γ2 which had difference in western blotting experiments, immunohistochemical study using antibody to the PLC-β1 and -γ2 was perfomed in heart. Characteristrically PLC-β1 and -γ2 was only shown in cardiac myocytes of the both strains, and the staining intensities of PLC-β1 and -γ2 in SHR was stronger than those in WKY (Fig. 3-A~D).

DISCUSSION

To clarify the physiological and pharmacological role of PLC isozyme, it is needed to determine the distribution in its target organ. In the present study, we studied the distributional patterns of PLC isozymes in the brain and heart of the WKY and SHR using the western blotting and immunocytochemistry.

In whole brain, we did not observe any difference of isozyme-specific immunoreactivity between the WKY and SHR (Fig. 1). It has been reported several times that neurons in the rostral ventrolateral medulla are critically involved in the generation of neurogenic vasomotor tone and subsequently maintain the arterial pressure in various species (Guyenet et al, 1990; Brody et al, 1991). Mizuguchi et al. (1991) reported that the immunoreactivity of cultured neurons, astrocytes and oligodendrocytes was neurons > oligoden-

drocytes > astrocytes for PLC-β and -γ, and astrocytes > oligodendrocytes > neurons for PLC-δ. Interestingly in our results, there was no differences of immunoreactivity to PLC isozymes in the brain of the WKY and SHR (Fig. 1). These results suggest that the PLC of brain may play little role in genetic hypertension.

Hypertension and cardiac hypertrophy are commonly associated with heart failure and are two features that characterize SHR (Caird et al, 1971). The renin-angiotensin-aldosterone system is implicated in the transition to heart failure: SHR treated with the angiotensin converting enzyme inhibitor captopril starting at 12 months of age did not develop heart failure during the 18~24 month observation period (Weber et al, 1993; Brooks et al, 1992). Myocardial hypertrophy is mediated through angiotensin II AT1 type receptor (Sadoshima et al, 1993), which activate the G protein, PLC, DG and IP_3 pathway (Marrero et al, 1994), to increase the expression of *c-fos* protooncogenes (Sadoshima et al, 1993), and growth factors (Boluyt et al, 1995; Rosendorff et al, 1996). The p-angiotensinogen and steady-state mRNA levels for cardiac angiotensinogen increased in SHR during the development of hypertension (Tamura et al, 1996). Transforming growth factor-β1 (TGF-β1) mRNA levels increased in SHR with failing hearts, and transient increase in TGF-β1 mRNA abundance preceded the elevation in fibronectin and collagen mRNA levels after experimental aortic constriction in rats (Conrad et al, 1994; Villareal et al, 1992). The γ-isoforms of PLC are activated by tyrosine phosphorylation, and it is these forms of PLC that are regulated by growth factor receptors (Noh et al, 1995). In this study, immunoreactivity of PLC-γ2 was increased in cardiac myocyte of the SHR (Fig. 1-E and 3-C, D). This result indicates that the increase in PLC-γ2 in SHR may have a promotor activity in the development of hypertension as well as secondary changes in response to hypertension, such as cardiac hypertrophy.

PLC-β1 binds to the GTP-bound G_q, probably via the carboxyl-terminal regions of both proteins, resulting in the activation of PLC-β1 (Noh et al, 1995). Kanagy et al. (1994) reported that vascular reactivity to mastoparan, a G protein activator, in genetically hypertensive rats was significantly enhanced. In this point, there is the possibility that increased responsiveness of G proteins leads to elevated PLC-β1 activity and may contribute to the elevated vascular responsiveness of genetically hypertensive rat. cAMP independent pathway of sodium transport inhibition induced by activation of dopamine D1-like receptors is mediated by PLC, and intrarenal administration of norepinephrine or D1 agonist increases PLC-β1 expression and activity (Jose et al, 1995). Significant increases in dopamine and norepinephrine uptake V_{max} in the frontal cortex were shown in SHR (Hendley et al, 1992). The decrease of the serum calcium level in SHR induced the decrease of central dopamine level (Sutoo et al, 1993).

In this experiment, the quantitative increment of the PLC-β1 and -γ2 in the SHR heart would have possibility in the development of cardiovascular abnormalities, such as cardiac hypertrophy and myocardial fibrosis in genetic hypertensive rats.

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