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Protein Kinase C Receptor Binding Assay for the Detection of Chemopreventive Agents from Natural Products

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Abstract – Protein Kinase C (PKC) is generally believed to play a central role in signal transduction, cellular growth control, gene expression, and tumor promotion. And it has been suggested that inhibitors of PKC might play important roles for the prevention and treatment of cancer. In order to investigate the possible inhibitors of PKC from natural products, PKC receptor binding assay was performed using bovine brain particulate as a source of PKC and the amount of [^{3}H]Phorbol 12,13-dibutyrate (PDBu) bound to PKC was measured in the presence of test materials. Total methanol extracts from 100 kinds of natural products were partitioned into 3 fractions (n-hexane, ethyl acetate and aqueous layer) and their binding ability to the regulatory domain of PKC was evaluated. The ethyl acetate fractions of *Morus alba* (roots, IC_{50}: 156.6 μg/ml), *Rehmannia glutinosa* (roots, IC_{50}: 134.3 μg/ml), *Lysimachia foenum-graecum* (roots, IC_{50}: 167.8 μg/ml), *Polygonum cuspidata* (roots, IC_{50}: 157.3 μg/ml), *Cnidium officinale* (aerial parts, IC_{50}: 145.2 μg/ml), and the hexane (IC_{50}: 179.3 μg/ml) and the EtOAc fraction of *Symplocarpus nipponicus* (roots, IC_{50}: 155.9 μg/ml) showed inhibitory activity of [^{3}H]PDBu binding to PKC.

Keywords – Protein kinase C, Receptor binding assay, [^{3}H]phorbol 12,13-dibutyrate (PDBu), Cancer chemoprevention.

Introduction

PKC is a Ca^{2+} and phospholipid-dependent enzyme (Liyange *et al.*, 1992) that is activated by diacylglycerol (Takai *et al.*, 1979; Kishimoto *et al.*, 1980). Once extracellular agonists bind to receptors, phospholipase C is activated with the aid of a G-protein and phosphatidylinositol is hydrolyzed to form two second messengers which are diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). The second messengers have independent signal pathway for cell activation. IP_3 binds to a receptor that triggers calcium mobilization, as a result, it modulates calcium-dependent cellular processes. DAG increases the affinity of PKC for calcium and phospholipid and directly activates PKC (Castogna, 1987; Weinstein, 1988).

Two-stage chemical carcinogenesis in mouse skin model has proved that phorbol ester, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is a potent tumor promoting agent (Diamond *et al.*, 1980) and tumor-promoting phorbol esters activate PKC directly both *in vitro* and *in vivo* (Castagna, 1982;

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Yamanishi, 1983). The distribution of PKC activity was similar to phorbol ester binding activities (Minakuchi *et al.*, 1981) and PKC activity was also co-purified with phorbol ester binding activity during chromatographic procedures (Ashendel *et al.*, 1983), which suggest that PKC is the functional receptor of phorbol ester (Kikkawa *et al.*, 1983; Niedel *et al.*, 1983). Therefore, the specific inhibitor of PKC was very useful for studying the role of PKC in the regulation of various cellular functions and understanding the mechanism of tumor promotion and it may be used as a tool for developing agents useful for cancer chemoprevention. Several compounds such as 18-*O*-β-glycyrrhetinic acid (Kitagawa *et al.*, 1986), calphostin C isolated from *Cladosporium cladosporioides* (Kobayashi *et al.*, 1989) and quercetin (Nishino *et al.*, 1984a) have been reported to inhibit PKC by binding to PKC, but the specificity of most of them displays a poor selectivity both *in vitro* and *in vivo* (Hidaka *et al.*, 1984; Hannun and Bell, 1989; Johnson and Clark, 1990; Love *et al.*, 1989). The purpose of this study is to find novel agents capable of binding to the regulatory domain of PKC from natural products, which might regulate the function of PKC, for the development of cancer chemopreventive agents.

Experimental

Chemicals and reagents – $[^3H]$PDBu (20 Ci/mmol) was purchased from DuPont Company, USA. All other chemicals were obtained from Sigma Chem. Co., USA. Polysafe as a scintillation cockfail was obtained from Wallac Oy, Finland.

Preparation of bovine brain particulate – The bovine brain particulates, as a source of PKC, were prepared based on the method described with some modification (De Vries *et al.*, 1988). Fresh bovine brains were obtained from a local abattoir in Seoul and all the procedures were carried out at 0-4℃. Fresh bovine brains were homogenized for 30 sec at 4℃ in 5 vol of homogenizing buffer [20 mM Tris-HCl (pH 7.5), 2 mM ethylenediamine-tetraacetic acid (EDTA), 10 mM ethylene glyco-*bis*-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol]. The homogenate was centrifuged for 20 min at 15,000 g. The pellet was resuspended in homogenizing buffer, rehomogenized, and centrifuged. The pellet was suspended in 10 mM Tris-HCl buffer (pH 7.4) to yield a concentration of 200 mg wet tissue/ml buffer and stored as aliquots at -80℃.

Plant materials – Plant materials were purchased from a herb market in Seoul, and voucher specimens have been deposited at Herbarium of Natural Products Research Institute, Seoul National University, Seoul, Korea. Each of the dried herbs were sliced, and then extracted 3 times with 100% MeOH at room temperature. The MeOH extracts were concentrated under reduced pressure below 40℃, and then the concentrated MeOH extracts were partitioned into *n*-hexane, ethyl acetate, and water layers.

$[^3H]$PDBu binding assay – The binding assay performed with bovine brain particulates as a source of PKC was based on the method described (Beutler *et al.*, 1989). Each reaction mixture (200 μl) contained bovine brain particulates (473.2 μg protein/well) as a source of PKC, 50 mM Tris-HCl buffer (pH 7.4), 0.2 mM $CaCl_2$ and 2 μl of diluted $[^3H]$PDBu (20 *n*Ci, 5 nM final concentration, diluted with EtOH).

Plant extracts were added to the reaction mixture to yield four different concentrations (10 μl, dissolved in DMSO, final 5% DMSO concentration). Incubation was performed for 1hr at 37℃ while shaking.

The amount of $[^3H]$PDBu bound in the presence of nonradioactive PDBu (4 μM) was used to measure the nonspecific binding. The IC_{50} values were calculated by using semi-

log plots. Unbound [^{3}H]PDBu was removed by filtration with 50 mM Tris-HCl (pH 7.4) through glass fiber filtermats (Wallac Oy, Finland) using cell harvester (Tomtec, Inc., USA). The filtermat was dried for 1hr at 50℃ in dry oven. The amount of [^{3}H]PDBu bound to glass fiber filtermat was measured by a liquid scintillation counter (Microbeta 1450, Wallac Oy, Finland).

Protein determination – The protein concentration was measured based on the method of Lowry (Lowry *et al.*, 1951). Bovine serum albumin was used as a standard.

Results and Discussion

The PKC contains two domains, one is regulatory domain which interacts with calcium, phosphatidylserin and DAG, and the other is catalytic domain that interacts with ATP and substrate. These regulatory and catalytic domains of PKC are considered as pharmacological targets for the design of inhibitors for the development of cancer prevention and treatment. Many inhibitors for these two different domains have already been found (Kobayashi *et al.*, 1989; Loomis and Bell, 1988; Tamaoki *et al.*, 1986) with less selectivity both *in vitro* and *in vivo* (Hidaka *et al.*, 1984; Hannun and Bell, 1989; Johnson and Clark, 1990; Love *et al.*, 1989).

Several compounds which have shown to interact with PKC were evaluated with the PKC binding assay system (Table 1). D-Sphingosine, a long-chain sphingoid base of sphingolipid breakdown products, has been reported to be a potent and reversible inhibitor of PKC by competitive inhibition of PDBu binding site of PKC (80 μM, 120 μg/ml) when tested with a mixed-micelle assay system (Hannun *et al.*, 1986; Hannun and Bell. 1989). As expected, D-Sphingosine and its isomers D-threo-sphingosine and L-threo-sphingosine inhibited the [^{3}H]PDBu binding to PKC at a concentration of 17, 21, 18 μg/ml, respectively. 18-*O*-β-Glycyrrhetinic acid

Table 1. Evaluation of antitumor-promoting agents and structurally-related compound to inhibit [^{3}H]PDBu binding to PKC

Compound	Inhibition of [^{3}H]PDBu binding (IC_{50}, μg/ml)
Glycyrrhizic acid	>200
18-*O*-α-Glycyrrhetinic acid	143
18-*O*-β-Glycyrrhetinic acid	>200
D-Sphingosine	17
D-threo-Sphingosine	21
Quercetin	18
Querctrin	>200
Nordihydroguaiaretic acid	>200

(glycyrrhetic acid), an aglycone of glycyrrhizic acid (glycyrrhizin), is an anti-inflammatory agent isolated from licorice root (*Glycyrrhiza urelensis*, Leguminosae), and it is known to inhibit TPA-induced tumor promotion (Nishino *et al.*, 1984a). 18-*O*-β-Glycyrrhetinic acid also inhibited the binding of [^{3}H]TPA to mouse epidermal membrane fraction with an IC_{50} value of around 500 μM (235 μg/ml) (Kitagawa *et al.*, 1986), suggesting that its anti-promoting activity is due to the interaction with PKC. Glycyrrhizic acid (glycyrrhizin) was also reported to demonstrate weaker antagonistic effects in several TPA-induced biological systems (Nishino *et al.*, 1984c; Kitagawa *et al.*, 1984; Kitagawa *et al.*, 1976). In our assay system, 18-*O*-β-glycyrrhetinic acid (glycyrrhetic acid) showed a similar IC_{50} values (143 μg/ml) on the inhibition of [^{3}H]PDBu-binding to PKC, however, glycyrrhizic acid (glycyrrhizin) and 18-*O*-α-glycyrrhetinic acid lacked binding affinity to PKC, suggesting the aglycone and the stereo-configuartion of the glycosidic linkage (β form) may play important roles in binding to the regulatory domain of PKC.

Quercetin, a flavonoid, has an inhibitory effect on DMBA-initiated teleocidin-promoted mouse skin papiloma formation (Nishino *et al.*, 1984d), and it has been shown that quercetin binds to the catalytic domain of PKC (Gshwendt *et al.*, 1984). In our assay system which uses [^{3}H]PDBu as a ligand, quercetin

did not reveal binding effect to the regulatory domain of PKC.

Nordihydroguaiaretic acid, a known lipoxygenase inhibitor, inhibited TPA-induced tumor formation (Nakadate *et al.*, 1982) This compound was also effective when treatment was prior to the 7,12-dimethylbenz[α]anthracen (DMBA) exposure of mammary glands ien the DMBA-initiated-TPA promotion system, i.e., it worked as an anti-initiator (Metha and Moon, 1991). When nordihydroguaiaretic acid was tested, it showed a strong inhibitory effect on the binding of PDBu to PKC (IC_{50} : 14 μg/ml).

In order to find a potential chemopreventive agents from natural sources, we evaluated plant extracts with PKC binding assay system using [^{3}H]PDBu as a ligand. In screening a large number of samples, this binding assay system has advantages over other systems using PKC enzyme activities, due to its being a relatively simple and rapid assay procedure. With this [^{3}H]PDBu binding assay system, 100 kinds of natural products were evaluated, and the results were summarized in Table 2. Among the 300 kinds of solvent fractionated plant extracts, the EtOAc fraction of *Morus alba* (roots, IC_{50}: 156.6 μg/ml), *Rehmania glutinosa* (roots, IC_{50}: 134.3 μg/ml), *Lysimachia foenum-graecum* (roots, IC_{50}: 167.8 μg/ml), *Polygonum cuspidata* (roots, IC_{50}: 157.3 μg/ml), *Cnidium officinale* (aerial parts,

Table 2. Evaluation of the potential of plant extracts to inhibit [^{3}H]PDBu binding to PKC

Scientific name/Family name	Plant part[a]	Inhibition of [^{3}H]PDBu binding to PKC (IC_{50}, μg/ml)		
		Ethyl acetate fraction	Aqueous fraction	Hexane fraction
Acanthopanax senticosus/Araliaceae	Rb	>200	>200	>200
Achyranthes japonica/Amaranthaceae	Rt	>200	>200	>200
Aconitum koreanum/Ranunculaceae	Rt	>200	>200	>200
Aconitum kusnezoffii/Ranunculaceae	Rt	>200	>200	>200
Akebia quinata/Lardizabalaceae	Tu	>200	>200	>200
Albizzia julibrissin/Leguminosae	Bk	>200	>200	>200
Alisma orientale/Alismataceae	Rh	>200	>200	>200
Anemarrhena asphodeloides/Liliaceae	Rh	>200	>200	>200
Angelica dahurica/Umbelliferae	Rt	>200	>200	>200
Angelica gigas/Umbelliferae	Rt	>200	>200	>200
Angelica koreana/Umbelliferae	Rt	>200	>200	>200
Angelica tenuissima/Umbelliferae	Rt	>200	>200	>200
Anthriscus sylvestris/Umbelliferae	Wp	>200	>200	>200
Aralia continentalis/Araliaceae	Rt	>200	>200	>200
Artemisia argyri/Compositae	Lf	>200	>200	>200
Asparagus cochinchinensis/Liliaceae	Bk	>200	>200	>200
Atractylodes japonica/Compositae	Rh	>200	>200	>200
Benicasa cerifera/Cucurbitaceae	Fr	>200	>200	>200
Boswellia carteriz/Bruseraceae	Wp	>200	>200	>200
Bupleurum falcatum/Umblliferae	Rt	145.2	>200	>200
Campanula takesimana/Campanulaceae	Wp	>200	>200	>200
Cassia tora/Leguminosae	Sd	>200	>200	>200
Cimicifuga heracleifolia/Ranunculaceae	Rh	>200	>200	>200
Citrus Unshiu/Rutaceae	Fb	>200	>200	>200
Cnidium officinale/Umbelliferae	Ap	>200	>200	>200
Cocculus *trilobus*/Menispermaceae	Rh	>200	>200	>200
Codonopsis pilosula/Campanulaceae	Fr	>200	>200	>200
Coptis chinensis/Ranunculaceae	Rt	>200	>200	>200

Table 2. Continued.

Scientific name/Family name	Plant part[a]	Inhibition of [^{3}H]PDBu binding to PKC (IC_{50}, μg/ml)		
		Ethyl acetate fraction	Aqueous fraction	Hexane fraction
Corydalis ternata/Papaveraceae	Rh	>200	>200	>200
Crataegus pinnatifida/Rosaceae	Fr	>200	>200	>200
Broussonetia kazinoki/Moraceae	Sd	>200	>200	>200
Cuscuta australis/Convolvulaceae	Sd	>200	>200	>200
Cyperus rotundus/Cyperaceae	Rh	>200	>200	>200
Dianthus chinensis/Caryophyllaceae	Wp	>200	>200	>200
Eucommia ulmoides/Eucommiaceae	Bk	>200	>200	>200
Fritillaria verticillata/Liliaceae	Rh	>200	>200	>200
Gardenia jasminoides/Rubiaceae	Rt	>200	>200	>200
Gastrodia elata/Orchidaceae	Rh	>200	>200	>200
Gentiana scabra/Gentianaceae	Rt	>200	>200	>200
Panax ginseng/Araliaceae	Rt	>200	>200	>200
Gleditschia japonica/Leguminosae	Fr	>200	>200	>200
Hydnocarpus anthelmintica/Flacourtiaceae	Sd	>200	>200	>200
Juglans sinensis/Juglandaceae	Sd	>200	>200	>200
Kalopanax pictum/Araliaceae	Bk	>200	>200	>200
Kochia scoparia/Chenopodiaceae	Fr	>200	>200	>200
Leonurus sibiricus/Labiatae	Lf,St	>200	>200	>200
Ligusticum delavayi/Umbelliferae	Rt	>200	>200	>200
Ligustrum japonicum/Oleaceae	Fr	>200	>200	>200
Loncicera japonica/Caprifolaceae	Fl	>200	>200	>200
Lorydalis speciosa/Fumariaceae	Fl	>200	>200	>200
Lycium chinense/Solanaceae	Fr	>200	>200	>200
Lysimachia foenum-graecum/Primulaceae	Rt	167.8	>200	>200
Machilus thunbergii/Lauraceae	Bk	>200	>200	>200
Mentha arvensis/Labiatae	Ap	>200	>200	>200
Morus alba/Moraceae	Rt	156.6	>200	>200
Nelumbo nucifera/Nymphaeaceae	Fr	>200	>200	>200
Nepeta japonica/Labiatae	Ap	>200	>200	>200
Paeonia japonica/Ranunculaceae	Rt	>200	>200	>200
Paeonia obovata/Ranunculaceae	Rt	>200	>200	>200
Perilla sikokiana/Labiatae	Lf	>200	>200	>200
Phlomis umbrosa/Labiatae	Rt	>200	>200	>200
Phytolacca esculenta/Phytolaccaceae	Rt	>200	>200	>200
Pinellia ternata/Araceae	Tu	>200	>200	>200
Plantago asiatica/Plantaginaceae	Sd	>200	>200	>200
Platycodon grandiflorum/Campanulaceae	Rt	>200	>200	>200
Polygonatum falcatum/Liliaceae	Rh	>200	>200	>200
Polygonum cuspidata/Polygonaceae	Rt	157.3	>200	>200
Poncirus trifoliata/Rutuceae	Fr	>200	>200	>200
Poria cocos/Polyporaceae	Rb	>200	>200	>200
Prunella vulgaris/Labiatae	Fl	>200	>200	>200
Prunus armeniaca/Rosaceae	Sd	>200	>200	>200
Prunus persica/Rosaceae	Sd	>200	>200	>200
Raphanus sativus/Cruciferae	Sd	>200	>200	>200
Rehmannia glutinosa/Scrophulariaceae	Rt	134.3	>200	>200
Rubus coreanus/Rosaceae	Fr	>200	>200	>200
Salvia miltiorrhiza/Labiatae	Rt	>200	>200	>200
Sanguisorba officinalis/Rosaceae	Rt	>200	>200	>200
Sanicula rubriflora/Umbelliferae	Wp	>200	>200	>200

Table 2. Continued.

Scientific name/Family name	Plant part[a]	Inhibition of [^{3}H]PDBu binding to PKC (IC_{50}, μg/ml)		
		Ethyl acetate fraction	Aqueous fraction	Hexane fraction
Saussurea lappa/Compositae	Rt	>200	>200	>200
Scrophularia ninpoensis/Scrophulariaceae	Rt	>200	>200	>200
Scutellaria baicalensis/Labiatae	Rt	>200	>200	>200
Smilax china/Liliaceae	Rh	>200	>200	>200
Sophora flavescens/Leguminosae	Rt	>200	>200	>200
Spiraea salicifolia/Rosaceae	Lf,St	>200	>200	>200
Strychnos ignatii/Loganiaceae	Sd	>200	>200	>200
Symplocarpus npponicus/Araceae	Rt	155.9	>200	179.3
Taraxacum ohwianum/Compositae	Wp	>200	>200	>200
Taraxacum platycarpum/Compositae	Rt	>200	>200	>200
Torillis Japonica/Umbelliferae	Fr	>200	>200	>200
Torreya nucifera/Taxaceae	Fr	>200	>200	>200
Trichosanthes kirilowii/Cucurbitaceae	Rt	>200	>200	>200
Trichosanthes kirilowii/Cucurbitaceae	Sd	>200	>200	>200
Tussilago farfara/Compositae	Sd	>200	>200	>200
Valeriana fauriei/Valerianaceae	Wp	>200	>200	>200
Veratum patulum/Liliaceae	Ap	>200	>200	>200
Vitex rotundifolia/Verbenaceae	Fr	>200	>200	>200
Xanthium strumarium/Umbelliferae	Fr	>200	>200	>200
Zingiber officinale/Zingiberaceae	Rh	>200	>200	>200

[a]Ap: Aerial parts; Bk: Bark; Fb: Fruit bark; Fl: Flower; Fr: Fruits; Lf: Leaves; Rb: Root bark; Rh: Rhizome; Rt: Root; Sd: Seeds; St: Stem; Wp: Whole plants.

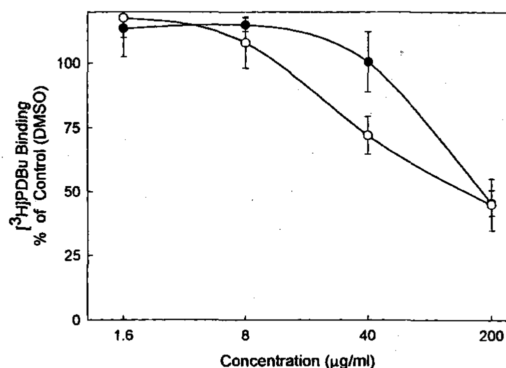

Fig. 1. Inhibitory effects of [^{3}H]PDBu binding to protein kinase C by the EtOAc fractions of *Lysimachia foenum-graecum* (roots, ●) and *Morus alba* (roots, ○). The results represent mean±S.D. performed in duplicate.

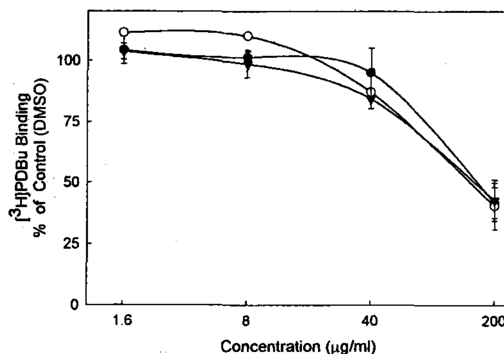

Fig. 2. Inhibitory effects of [^{3}H]PDBu binding to protein kinase C by the hexane fraction of *Symplocarpus nipponicus* (roots, ●) and the EtOAc fractions of *Symplocarpus nipponicus* (roots, ▼) and *Cnidium officnale* (aerial parts, ○). The results represent mean±S.D. performed in duplicate.

IC_{50}: 145.2 μg/ml), and the hexane (IC_{50}: 179.3 μg/ml) and the EtOAc (IC_{50}: 155.9 μg/ml) fraction of *Symplocarpus nipponicus* (roots) showed inhibitory activity of [^{3}H]PDBu binding to PKC. The dose dependent inhibition of [^{3}H]PDBu binding to PKC by these active fractions are shown in Fig. 1, 2 and 3. These active fractions are under investigation by using activity-guided fractionation method with

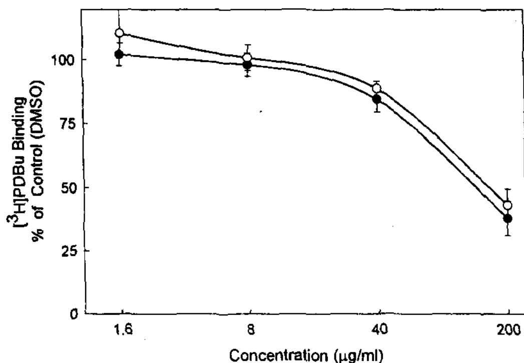

Fig. 3. Inhibitory effects of [^{3}H]PDBu binding to protein kinase C by the EtOAc fractions of *Rehmannia glutinosa* (roots, ●) and *Polygonum cuspidata* (roots, ○). The results represent mean±S.D. performed in duplicate.

column chromatography to find active principles, however, in order to prove the PKC-related mode of action (activation or inhibition) or tumorigenic action (promotion or inhibition) of active principles detected with this PKC-binding assay system, additional experiments need to be performed such as catalytic study with PKC and animal studies with two-stage carcinogenesis model systems. Nontheless, the discovery of active compounds in this system will be useful for the development of cancer chemopreventive agents.

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