INTERACTION OF TIGLIANE AND DAPHNANE DITERPENOID ESTERS WITH PROTEIN KINASE C ISOZYMES *IN VITRO*

Submitted by
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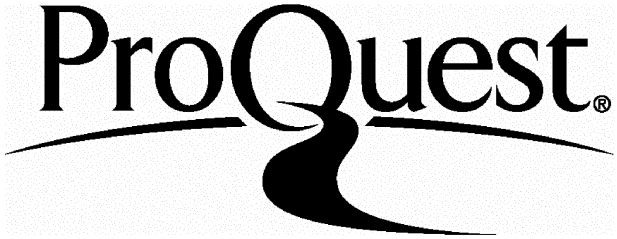

To my parents, Nevena and Milorad, with love, respect and gratitude

(Мојим Родитељим, Невени и Милораду са љубављу, поштовањем и захвалношћу)

To my Dejana with love and gratitude

(Мојој Дејани са љубављу и захвалношћу)

ABSTRACT

Different biological effects elicited by different phorbol esters in mammalian cells are thought to be due mainly to their differential interaction with, and modification of, the cellular pool of PKC isozymes. However, in most investigations only TPA, as the most potent tumor-promoting phorbol ester, has been used. Inspite of observations that other phorbol esters exert more specific biological effects *in vivo*, relatively few studies have been reported so far on their interaction with individual PKC isozymes *in vitro* or *in vivo*.

In vivo interaction of different phorbol esters with PKC involves penetration of the phorbol ester molecule into the inner layer of the cellular membrane and binding to the regulatory domain of PKC followed by redistribution of the PKC pool from the cytosolic to the membrane bound fraction. It is thought that PKC redistribution ("translocation") is followed by an activation step and phosphorylation of substrate(s).

Tigliane (TPA, PdBu, DOPP, DOPPA and Sap A) and daphnane (Thy A and Rx) diterpenoids of the phorbol ester group, were investigated for their ability to interact with purified recombinant protein kinase C (PKC) isozymes. Representative compounds of distinct biological activity were chosen in an attempt to establish a correlation between their *in vivo* effects and their ability to interact with individual PKC isozymes.

Utilising PKC isozymes α, β_1, β_2, γ, δ, ε and ζ purified from a baculovirus / Sf9 insect cell expression system and a phosphatidylserine / Triton X-100 mixed micellar system as an *in vitro* cell membrane model, binding and activation of PKC isozymes by seven different phorbol esters was studied. Binding affinity and activation potency of individual compounds were found to correlate well with high tumor promoting activity of TPA and PdBu on one side and with the non-promoting action of DOPPA and Rx. However, the non-promoters DOPP and Sap A and a second stage tumor promoter Thy A were effective agonists of PKC isozymes.

To study the ability of some PEs to induce association of PKC isozymes with

cellular membranes (i.e. "translocation"), a membrane fraction obtained from HL-60 cells was used, in order to approach *in vivo* conditions. Although the ability of the investigated PEs to induce "translocation" of PKC isozymes corresponded to their ability to induce PKC activation, the ability of micromolar Ca^{2+} concentrations to induce membrane association of n-PKCs, ε and δ, was not in agreement with our activation results and current theory of Ca^{2+} independency of the n-PKC isozymes.

These results suggested that specific biological effects of different phorbol esters could not solely be explained through differences in their interaction with PKC isozymes *in vitro*. It is possible that an intracellular component, absent in an artificial system, is responsible for modulation of phorbol ester effects *in vivo*.

Additionally a daphnane diterpene and a second stage tumor promoter mezerein, was isolated from previously uninvestigated *Daphne blagayana*, a plant indegenous to the Balkan. For the first time, detailed one and two dimensional NMR (^1H, ^{13}C, COSY and NOESY) experiments were conducted to confirm the previously determined structure of mezerein. Computer assisted molecular modelling and structure analysis enabled determination of molecular minimum free energy and interatomic distances of the pharmacophore's functional groups. These values were similar to those obtained for a highly potent tumor promoter TPA. As an activator of individual PKC isozymes *in vitro*, mezerein appeared to be different from TPA. Mezerein was relatively less potent (when compared with TPA) as an activator of the novel PKC isozymes δ and ε. This suggested that differences in biological activity of mezerein and TPA could be, in part, due to differences in their ability to activate the PKC isozymes δ and ε.

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GLOSSARY

AA = Arachidonic acid
ADP = Adenosine diphosphate
ATP = Adenosine triphosphate
cAMP = Cyclic Adenosine monophosphate
BSA = Bovine serum albumin
Ca^{2+} = Calcium ion
CNS = Central nervous system
DAG = Diacylglycerol
DEAE = Diethylaminoethyl cellulose
DNA = Deoxyribonucleic acid
DTT = Dithiothreitol
DOG = 1,2-sn-Dioleoylglycerol
EDTA = Ethylene diamine tetraacetic acid
EGF = Epidermal growth factor
EGTA = Ethylene glycol bis (b-aminoethyl ether)-N,N,N',N',-tetraacetic acid
FCS = Foetal calf serum
FPLC = Fast protein liquid chromatography
G-protein = GTP-binding regulatory protein
H^+ = Hydrogen ion
HCl = Hydrochloric acid
HEPES = 4(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
HL-60 = Human leukaemic cell line
HPLC = High pressure liquid chromatography
Ig = Immunoglobulin
IP_3 = Inositol-1,4,5,-triphosphate
K^+ = Potassium ion
kD = Kilodalton
M = Molar
MAPK = Mitogen activated protein kinase
Mr = Relative molecular mass
MS = Mass spectrometry
MWCO = Molecular weight cut off
Na^+ = Sodium ion
NaCl = Sodium chloride
NMR = Nuclear magnetic resonance
OAG = sn-1-Oleoyl-2-acetylglycerol
^{32}P = Radiolabeled phosphate group
PA = Phosphatidic acid
PBS = Phosphate buffered saline
PC = Phosphatidyl choline
PDGF = Platelet derived growth factor
PE(s) = Phorbol ester(s)
PGE_2 = Prostaglandin E_2
Pi = Inorganic phosphate
PI = Phosphatidylinositol

PIP_2 = Phosphatidylinositol-4,5-bisphosphate
PKA = cAMP-dependent protein kinase
PKC = Protein kinase C
PKM = Proteolytic fragment of PKC (catalytic domain)
PLA_2 = Phospholipase A_2
PLD = Phospholipase D
PLC = Phospholipase C
PMSF = Phenyl methyl sulphonyl fluoride
PS = Phosphatidylserine
S.E.M. = Standard error of mean
SDS/PAGE = Sodium dodecyl sulphate/Polyacrylamide gel electrophoresis
TCA = Trichloracetic acid
Tris = Tris(hydroxymethyl)aminoethane
UV = Ultra-violet

Phorbol ester abbreviations used:

TPA = 12-O-tetradecanoylphorbol-13-acetate = 13-Acetyl-12-tetradecanoyl-4β,9α,12β,13α,20-pentahydroxy-1,6-tigliadien-3-one
PdBu = phorbol-12,13-dibutyrate = 12,13-dibutanoyl-4β,9α,12β,13α,20-pentahydroxy-1,6-tigliadien-3-one
DOPP = 12-deoxyphorbol-13-phenylacetate = 13-O-Phenylmethyl-4β,9α,13α,20-tetrahydroxy-1,6-tigliadien-3-one
DOPPA = 12-deoxyphorbol-13-phenylacetate-20-acetate = 20-Acetyl-13-O-phenylmethyl-4β,9α,13α,20-tetrahydroxy-1,6-tigliadien-3-one
Sap A = Sapintoxin A = 13-Acetyl-12-(2-methylaminobenzoyl)-9α,12β,13α,20-tetrahydroxy-1,6-tigliadien-3-one
Thy A = Thymeleatoxin A = 12-(3-Phenyl-2E-propanoyl)-12β-hydroxydaphnetoxin
Mez = Mezerein = 12-(5-Phenyl-2E,4E-pentadienoyl)-12β-hydroxydaphnetoxin
Rx = Resiniferatoxin = 9,13,14,-orthophenylacetylresiniferonol-20-homovanilate

CHAPTER 1. INTRODUCTION

1.1. PHORBOL ESTERS

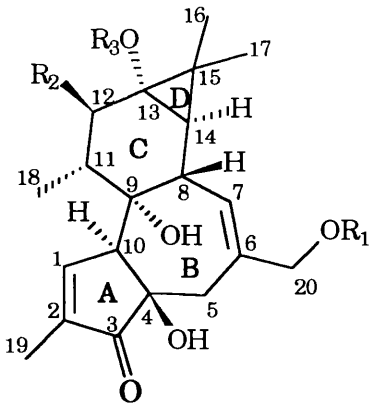
1.1.1. Chemistry

Phorbol esters (PEs) are naturally occuring *tri-* and *tetra*-cyclic diterpenoids isolated from only two plant families; the *Euphorbiaceae* and *Thymelaeaceae* (reviewed in 1-3). According to their hydrocarbon skeleton PEs can be divided into three groups, Tiglianes, Daphnanes and Ingenanes (Figure 1.1.1. A., B. and C., p. 20).

Tigliane diterpenes

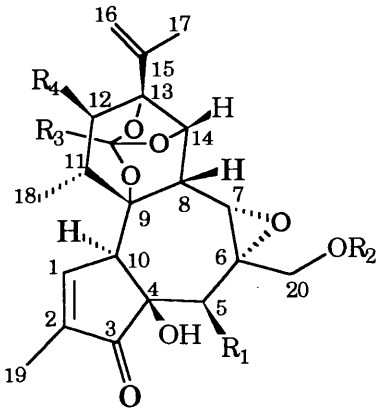
Detailed structure elucidation studies (ref. 1 and references cited therein) revealed that the structure of the parent tigliane derivative, phorbol, was composed of a semi-rigid carbon backbone arranged into 4 rings (Figure 1.1.1. A.). The five-membered ring A is trans-linked to ring B and is non-planar. The seven-membered ring B is stabilised into an "envelope" conformation and is *trans*-linked to ring C. The cyclohexane ring C is in the "chair" conformation imposed by the *cis* linkage to the small cyclopropane ring D. There are six oxygen atoms in the phorbol structure, one as C-3 α,β unsaturated ketone and five forming hydroxyl groups at C-4 (tertiary, α or β), C-9 (tertiary, α), C-12 (secondary, β), C-13 (tertiary, α) and C-20 (primary). Tigliane diterpenoids were isolated first from the seed oil of *Croton tiglium* L., *Euphorbiaceae* (4). This oil contained phorbol diesters esterified at positions C-12, C-13 and triesters esterified at C-12, C-13 and C-20. C-12, C-13 and C-20 triesters are also termed "cryptic esters", since removal of C-20 acyl group is required before these esters can induce an inflammatory response. Subsequently tigliane esters of deoxyphorbol have been isolated, including 4-deoxy, 4-deoxy-5-hydroxy, 4,20-dideoxy, 4,20-dideoxy-5-hydroxy, 12-deoxy, 12-deoxy-5-hydroxy, 12-deoxy-16-hydroxy, 12,20-dideoxy and 16-hydroxy derivatives (reviewed in ref. 1). Acid residues found in ester groups are highly variable, with saturated and unsaturated aliphatic (of a different carbon chain length), and aromatic residues being identified so far.

A.

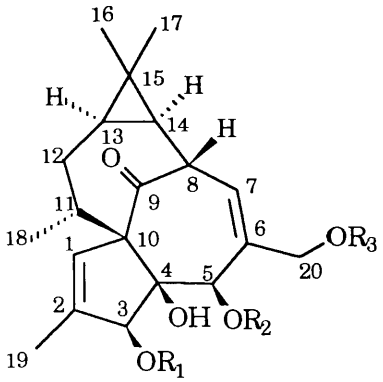


TIGLIANE

B.



DAPHNANE



C.

INGENANE

Figure 1.1.1. Structures of Tigliane, Daphnane and Ingenane polyol diterpenoids.

Daphnane diterpenes

The daphnane diterpenes are found in both plant families *Euphorbiaceae* and *Thymelaeaceae* but their distribution is limited. Esters based upon 5-deoxydaphnetoxin and 1α-alkyldaphnane nuclei are present only in the *Thymelaeaceae,* resiniferonol esters are only present in the *Euphorbiaceae,* whilst daphnetoxin and 12-hydroxydaphnetoxin esters are present in both plant families. This may provide some chemosystematic evidence for a relationship between these families (5).

The daphnane diterpenes have a slightly different carbon skeleton to the tiglianes, with a cyclopropane D ring being opened to give the (β) 2-propenyl residue in position C-13 (Figure 1.1.1. B.). In these compounds the hydroxyl groups of the C ring (α hydroxyls of C-9, C-13 and C-14) in the molecule of a metabolic precursor are linked in an orthoester bond to aromatic or aliphatic groups. Some of the daphnane group members have lost the C-6/C-7 double bond as a result of epoxidation and have an extra C-5 (secondary, β) hydroxyl group (e.g. Thymeleatoxins and Mezerein) (2,5).

Ingenane diterpenes

The ingenane diterpenes are found only in genus *Euphorbia* (*Euphorbiaceae*) and their isolation has been found to be the most technically difficult. The parent alcohol ingenol, exhibits a five membered A ring and cycloheptane ring B in the *cis* configuration, the C ring is also *cis* fused to cyclopropane D ring. The structure of the ingenane carbon skeleton also has many basic differences to the tigliane nucleus, with ring C attached to ring B at position C-10 (instead of C-9) and formation of a C-9 ketone bridge between C-8 and C-10 (Figure 1.1.1. C.). The C_{1-2} and C_{6-7} double bonds, the C-4-β-tertiary hydroxyl group and C-20 primary hydroxyl group are still present, but the arrangement of hydroxyl groups is different from the tigliane nucleus, in that the C-12 and C-13 hydroxyls are absent and two secondary (β) hydroxyl groups at C-3 and C-5 are present. Hence different esterification profiles are seen when compared with the tigliane and daphnane phorbol esters (ref. 1 and references therein). The ingenol nucleus occurs naturally in various states of oxygenation, including: 5-

deoxyingenol, 20-deoxyingenol, 16-hydroxy-20-deoxyingenol, 13-hydroxyingenol, 16-hydroxyingenol and 13,19-dihydroxyingenol with the possibility of esterification of all additional hydroxy groups.

Apart from tumor promoting activity of some ingenols (6), and antileukemic activity of ingenol-3,20-dibenzoate (7), the biological effects of the ingenanes have not been extensively studied and are in general considered to be of similar activity to the tiglianes.

The structures of the different tigliane and daphnane esters used in the present studies are presented in Figure 1.1.2, p. 24.

1.1.2. Biological actions of phorbol esters: structure-activity relationship

Diterpenoids of the phorbol ester group are known to exert a range of different biological effects, including skin irritation and inflammation, tumor promotion, induction of cell differentiation and proliferation, platelet aggregation and muscle contraction. The biological-activity profiles, of different phorbol esters, are known to be controlled by their structure.

The biological actions of the phorbol esters at nano-molar concentrations suggested the existence of specific binding sites to mediate such actions. Specific receptors were shown to have a wide distribution in intact cells, mouse epidermis and membrane fractions (9-11) as well as soluble fractions (12). TPA and other phorbol esters were then shown to directly activate a phospholipid dependent, serine / threonine protein kinase, termed Protein Kinase C (PKC) *in vitro* (13) by substituting for diacylglycerol in increasing the calcium affinity of the enzyme (see later, Section 1.2.). Subsequently the phorbol ester binding activity and PKC activity were found to co-purify to apparent homogeneity in mouse, rat and bovine brain (14-18). *In vitro* work showed that the phorbol ester binding was dependent on phosphatidyl serine (PS) and calcium and could be competitively inhibited by diacylglycerol (19-22, and Section 1.2.).

The identification and isolation of this enzyme as the receptor site for tumor promoting PEs (144,13-17) and the involvement of this enzyme in intracellular signal transduction has intensified research into the biochemical mechanisms of

cancer. The use of phorbol esters as selective and potent PKC activators has contributed to making the PKCs among the most thoroughly studied families of protein kinases in cellular signalling.

However, the recent discovery of other PE binding molecules like chimaerins (n(α1), α2, β1 and β2)(145-147), Vav (457) and unc-13 (148) suggests that not all pharmacological effects of PEs may be channelled through activation of PKC kinases.

The biological actions of the phorbol esters have largely been investigated with commercially available preparations and has been limited by the availability of source plant material. Consequently investigators have largely concentrated on TPA and PdBu as active derivatives and 4-α-PDD as an inactive control.

Comparative studies conducted on a range of phorbol derivatives have elucidated many structural requirements for biological activity (e.g. for tumour promotion requirements see Table 1.1.1., p. 26). Substitution of the primary hydroxyl group at C-20 (e.g. esterification), decreases bioactivity in proportion to the type of substitution (23,24). The conformation of the C-20, CH_2OH group seems to be important since changes (e.g. double bond shift from C-6/C-7 to C-7/C-8) result in a loss of activity. Similarly a configurational change (β OH to α OH) or derivatisation on the C-4, results in lessening of activity. The importance of such hydrophylic groups appears to involve orientating the phorbol ester to facilitate interaction with PKC binding site groups and/or with phospholipid head groups in the membrane. The phorbol esters also require lipophilic moieties in the molecule (typically as part of the ester groups at C-12 and C-13) whose function, at least partly, is to allow anchorage and spatial organisation in the lipid bilayer and is a function of saturation (including the presence of aromatic groups) and acyl chain length.

Similar studies on diacylglycerols have revealed an optimum length for acyl chains (6-10 carbon atoms) and the presence of the free hydroxyl group at C-3 of the glycerol backbone as being essential for activity (25).

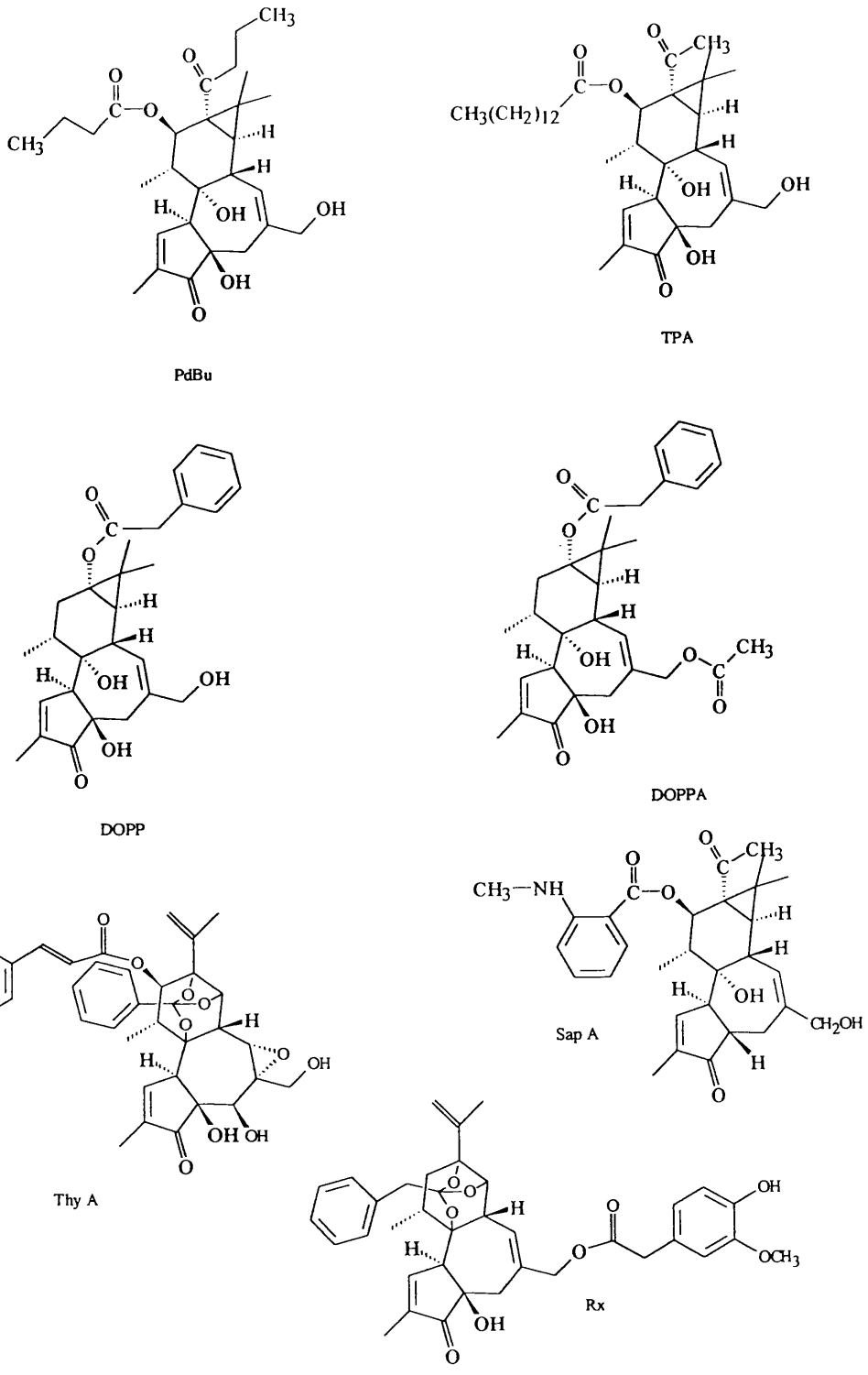


Figure 1.1.2. Structures of phorbol esters used in our studies. TPA, 12-O-tetradecanoylphorbol-13-acetate; **PdBu**, phorbol-12,13-dibutyrate; **DOPP**, 12-deoxyphorbol-13-phenylacetate; **DOPPA**, 12-deoxyphorbol-13-phenylacetate-20-acetate; **Sap A**, Sapintoxin A; **Thy A**, Thymeleatoxin A; **Rx**, Resiniferatoxin.

The synthetic diacylglycerol analogue *sn* 1-oleoyl-2-acetylglycerol (OAG) has also been found to be a good activator of PKC and similarly the monoesters of ingenol at the C_3 suggest that a single ester acyl group is sufficient for activity and is not restricted to the C-12 or C-13 position on the phorbol esters (26).

The *trans*-configuration of the A and B ring linkage is also considered necessary for activity. Esters of 4α-phorbol, because of *cis* A/B ring fusion, have a different shape to the *trans* phorbol derivatives and this alteration in stereochemistry has been proposed as the reason for lack of biological activity of these compounds. However, α-Sapintoxin (cis) has been found to be biologically active at high concentrations (27).

Computer assisted modelling techniques have recently been employed to compare energetically favourable structures of different phorbol esters and diacylglycerols (28). Interestingly according to this study the C-1 ester and C-3 hydroxyl group in diacylglycerols correspond to the C-13 ester and C-9 hydroxy group (rather than C-20)(29), of the phorbol derivatives in terms of spatial positioning. Furthermore it was suggested that the C-4 group (and possibly other parts of the molecule such as C-20) which did not have a correlating group in the diacylglycerol molecule, may play functional roles on separate receptors or impose selectivity for PKC isoforms.

A more recent study of the structural basis of PKC activation by diacylglycerols and a range of different tumour promoters (DAGs, debromoaplysiatoxins, tiglianes, ingenanes, bryostatins and teleocidins), based on experimental rather than on computer driven hypotheses proposed a different scenario for PKC activation using the DAG structure as a template (30). Here the structural components found to be responsible for tumor-promoting activity of tiglianes were C-3 ketone (as hydrogen bond acceptor), C-9 (as hydrogen bond acceptor) and C-20 (as hydrogen bond donor), the hydroxylic group in the tigliane structure (e.g. TPA) and C-9 ketone (as hydrogen bond acceptor), C-20 hydroxylic (as hydrogen bond donor) and C-3 ester carbonyl group (as hydrogen bond acceptor) in ingenanes (e.g. Ingenol-3-O-tetradecanoate). On minimalist grounds and on the basis of structure-activity studies, it was assumed that the interaction of only three hydrophylic atoms is required. However, it was postulated that

secondary interactions with other hydrophylic atoms may occur and that, in addition, spatially corresponding hydrophobic moieties were obviously required, if only to ensure that the activator is membrane soluble. This model (and deduced pharmacophores) was found to be consistent with all the structure-activity observations on the DAGs and tumour promoters.

Table 1.1.1. Structure / activity requirements for tumor-promoting and hyperplastic activity of the phorbol esters.

(1) Required for activity:

- *trans*-configuration of the A/B ring linkage (cis-isomers are inactive).
- Free primary hydroxyl group at C-20.
- At least one ester group at C-12 or C-13.
- Oxygen (ether or hydroxyl) bearing group at C-4 (4-deoxyphorbol esters are inactive as tumor promoters and hyperplastic agents).

(2) Affecting the activity

- Increasing unsaturation in an ester moiety (C-12 and/or C-13) results in decreased complete tumor-promoting activity but increased stage 2 tumor-promoting activity.
- A tertiary hydroxyl group at the C-4 is necessary for complete tumor-promoting activity. O-methylation of the C-4 hydroxyl group results in the loss of complete promoting activity, although stage 1 tumor-promoting activity is retained.

(3) Not necessary for activity:

- Cyclopropane (D) ring.
- Secondary hydroxyl group at C-16.
- Ester group at C-12 is not required for activity (although a secondary hydroxyl group at same position reduces activity dramatically and 12-deoxy PEs are anti tumour-promoters).

Tumor promotion

From a medical point of view the most interesting biological action of phorbol esters is tumour promotion. As early as 1941 Berenblum reported that oil from *Croton tiglium* applied with small doses of carcinogen was observed to potently promote the formation of skin tumors (papillomas) on mouse (31), which become progressively dysplastic, aneuploid and finally malignant (32-34). This led to the establishment of a two-stage model for carcinogenesis (35) where the carcinogen is applied in a single subthreshold dose (initiation) followed by repetitive application of the croton oil (promotion) leading to formation of premalignant lesions called papillomas. The effect was found to occur in mouse but not rat, rabbit or guinea pig skin (36). Subsequently the phorbol-12, 13-diesters were found to be the agents in croton oil responsible for its tumor-promoting activity (121), with TPA being the most potent. Tumor-promoting phorbol esters have been found among tigliane, daphnane as well as ingenane derivatives. Structure / activity relationships for phorbol ester induced tumour promotion beyond those noted in Table 1.1.1., are ill defined. C-12 deoxygenation leads to production of weak (non) promoters. Unsaturation of the acyl ester side chain is thought to impart second stage promoting ability (73). Methylation of C-4 OH imparts first stage promoting ability and C-4 deoxy PEs (e.g. Sap A) are not active as tumour-promoters.

A schematic representation of multistage carcinogenesis in mouse skin is depicted in Figure 1.1.3., p. 28. The initiation stage of mouse skin tumorigenesis is effected by single application or exposure to a subcarcinogenic dose of a skin carcinogen such as 7,12-dimethylbenz[a]anthracene (DMBA). The initiation occurs as a result of interaction of a reactive form of a skin carcinogen with the DNA of an epidermal target cell and is a rapid process that produces no apparent morphological alterations in epidermis (37 and references therein). As a result of interaction of a reactive carcinogenic intermediate with DNA one can measure a transient inhibition of epidermal DNA synthesis. This interaction ultimately leads to mutations in critical target genes of epidermal stem cells. The c-Ha-ras, and to a limited extent, N-ras genes have been identified as target genes for certain tumor

initiators. One of the hallmarks of tumor initiation in mouse skin is that it persists for the life time of the animal (irreversible effect), despite the fact that the epidermis renews itself approximately once every 6-8 days.

In contrast to the initiation stage, treatment of mouse skin with a tumor promoting agent produces dramatic morphological and biochemical effects which are reversible in the absence of continuated treatment, although some effects may persist for longer periods of time after discontinuation of promoter treatment. Thus tumour promoters must be given at an optimal frequency and duration in order to effect tumour development. The process of tumour promotion in mouse skin is believed to involve the selective clonal expansion of initiated cells into visible clonal outgrowths (papillomas) by one or a combination of several mechanisms.

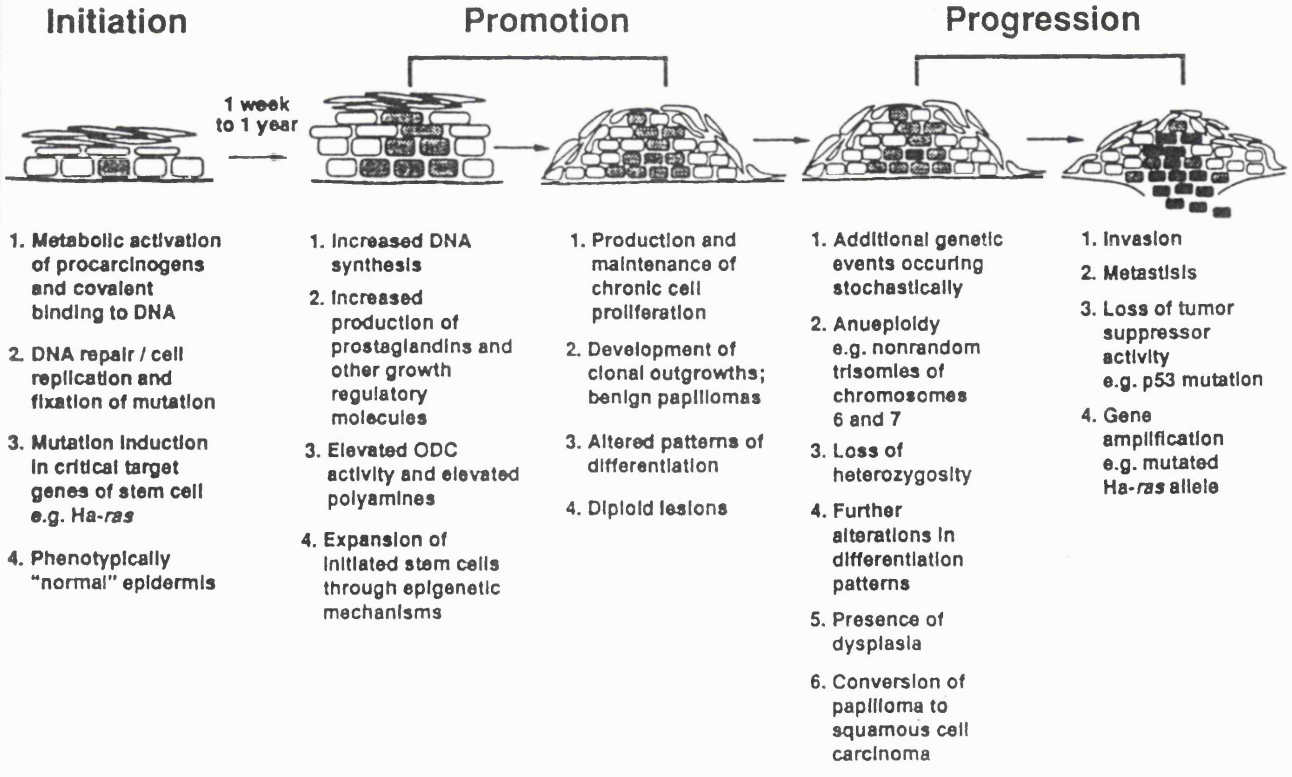


Figure 1.1.3. Overview of multistage carcinogenesis in mouse skin.

While genetic mechanisms have been postulated to play a role in the tumour promotion stage (38,39) the eventual reversibility of promoter-induced effects argues that this stage is accomplished primarily through epigenetic mechanisms.

The promoting stage has been further subdivided operationally into stage I (conversion) and stage II promotion (progression) (37), thus if the doses of complete promoter (e.g. TPA) were limited to give a low tumor incidence, doses of a weak promoter (e.g. mezerein) following would give an equal response to that of high TPA doses (40). Currently, the standard two-stage promotion protocol involves initiation followed by one to four applications of TPA (stage I) and then by multiple applications of a weak papilloma-promoting agent, such as mezerein or 12-retinoylphorbol-13-acetate (RPA) (stage II) (37). At a cellular level the complete tumor promoter TPA induces several distinct changes in both the mouse dermis and epidermis (41).

Cellular changes

Within a few hours after application of a single effective dose of the TPA to mouse skin, localized edema and erythema, characteristic of inflammation and irritation are evident, and by 24 hr there is leukocytic infiltration of the dermis. At that time there is also a 5- to 10-fold increase in the percentage of dark basal cells (DCs) in the interfollicular epidermis. These DCs are characterised by their strong basophilia, dense chromatin and large numbers of free ribosomes. They increase in number in TPA-induced hyperplasia to a greater extent than in hyperplasia induced by mezerein or more weakly promoting hyperplastic agents. Within 1-2 days after a single promoter treatment, an increased number of nucleated cell layers is observed. This is followed by a phase of increased keratinisation of the upper layers of the epidermis. Without additional promoter treatments, all these responses gradually subside and the epidermis regains its normal appearance within approximately 2-3 weeks of treatment. Repeated promoter treatment, however, prevents this decrease in response, and the skin appears to be in a chronic state of irritation and regenerative hyperplasia. In fact repeated treatment with TPA leads to potentiation of the hyperplasia response.

This ability to produce a potentiated hyperplasia after multiple treatments and the magnitude of this response appear to correlate most closely with the tumor promoting ability of various compounds. It has been argued that induction of cell proliferation and hyperplasia was a necessary but not a sufficient condition for tumour promotion in the mouse skin model. This argument was based on the observation that certain chemicals could produced dramatic epidermal hyperplasia after a single application (e.g. mezerein, acetic acid, ethyl phenyl propyiolate) and yet these compounds exhibited only poor papilloma promoting ability. However, careful examination of these compounds revealed that they are unable to maintain a potentiated epidermal hyperplasia and cell proliferation when given repeatedly due, in part, to severe epidermal toxicity (41,42). These observations as well as the fact that regenerative hyperplasia and cell proliferation alone can promote skin tumors, supports the hypothesis that epidermal hyperplasia and cell proliferation of a specific type, magnitude, and duration is sufficient for skin tumour promotion in susceptible mouse strains and stocks.

With repeated TPA treatment of initiated skin, benign tumors begin to appear in about 6 weeks and, with some mouse stocks such as selectively bred SENCAR, there can be as many as 20-30 papillomas per mouse after 15 weeks of promoter treatment. This is accompanied by with persistant hyperplasia, decrease in Thy-1+ epidermal dendritic cells, an increase in dark keratinocytes and infiltration of the area with small mononuclear lymphocytes, neutrophils and macrophages.

The stage 2 tumour promoter mezerein, induced only slow, transient hyperplasia, did not affect Thy-1+ cell levels but did increase 1aP+ epidermal dendritic cell levels and induced infiltration of eosinophils. The initial application of a stage 1 promoter (e.g. 4-O-methyl TPA) has some permanent effects since the commencement of stage 2 promoter application could be delayed for up to 2 months without impairing its response. However, if the time between repetition of the stage 2 application was too long (allowing hyperplasia to reverse) the tumor response greatly diminished (43). The possible involvement of various cell types (e.g. phagocytic cells stimulated by TPA to produce reactive oxygen species) supplementing the direct epidermal cell effects of the promoting phorbol esters has been suggested (37). The first tumours to appear during a two-stage mouse skin

tumorigenesis are papillomas, only a small proportion of which, will "progress" to an invasive squamous cell carcinoma (SCC). However, other tumour types have been noted to develop in mouse skin to various extents, such as keratoacanthomas, melanomas and adnexal tumours (67 and references therein). The appearance of these tumour types may be highly dependent on the type of initiator and/or promoter used and on the genetic background of the mouse stock or strain utilized.

Biochemical changes and the role of protein kinase C

PEs with promoting activity produce an initial inhibition, that is soon followed by greatly increased rates of nucleic acid and protein synthesis. Promoter-treated skin shows an increase in phospholipid turnover, a decreased responsiveness to epidermal chalones (reversible tissue-specific mitotic inhibitors) and β-adrenergic agonists, a decrease in basal activities of epidermal superoxide dismutase (SOD) and catalase, a decrease in glucocorticoid receptors and an increase in the activity of xanthine oxidase. Tumour promoter application also gives rise to a decrease in epidermal histidase and histidine decarboxylase, modification of epidermal keratins and keratin expression, increased synthesis and phosphorylation of histones and a large induction of ornithine decarboxylase (ODC), the rate limiting enzyme in polyamine biosynthesis. As a result of the induction of epidermal ODC, the levels of putrescine and especially spermidine in the epidermis become elevated. The elevated ratio of spermidine / spermine that occurs after TPA treatment is tightly linked to the DNA synthesis response induced by this promoter (37).

Evidence is also emerging that generation of free radicals may be involved in the skin tumor promoting actions of several classes of tumor-promoting agents, including phorbol esters. For example TPA stimulates production of $O^{\cdot}_2$ and possibly other free radicals by polymorphonuclear leukocytes (PMNs) probably by activating the ubiquitous membrane bound NADPH oxidase system. Fisher *et al* demonstrated (44) the production of $O^{\cdot}_2$ in isolated epidermal cells by active, but not inactive, phorbol esters and its supression by antipromoters. More direct evidence of the involvement of free radicals in tumor promotion comes from the

studies with free radical generating compounds (such as the organic peroxides and anthrones (45) and the finding that antioxidants are effective inhibitors of chemical carcinogenesis and skin tumor promotion. However, the exact biochemical mechanism of free radical mediated skin tumor promotion remains unknown, and both genetic and epigenetic mechanisms have been postulated (reviewed in 46).

The observation that PKC is a major cellular receptor (for other receptors see below) for the phorbol esters has led to investigation of its involvement in tumour promotion.

Using normal and TPA-resistant mice strains no differences were apparent in epidermal PKC levels or activities (47,48). On the other hand not all PKC activators are able to promote tumours. Sapintoxin A was an efficient and potent activator of heterogenous preparations of PKC isozymes *in vitro* but was inactive as both a Stage 1 and Stage 2 tumor-promoter, unless coapplied with a calcium ionophore (A23187) (49). Conversely the sesquiterpene lactone Thapsigargin, is a tumor promoter which does not activate PKC but has been found to raise cell calcium by inhibition of the endoplasmic reticulum calcium ATP-ase (50). While PKC activation alone therefore, seems insufficient for tumor promotion, there is however much evidence implicating PKC as playing a supportive role in the transformation of cells. Non-phorbol activators of PKC such as Teleocidins and Aplysiatoxins have been found to be potent tumor promoters (51,52) and diacylglycerol (DOG) was found to be a potent stage 2 promoter in mouse skin (53). Cancer chemotherapeutics (e.g. nonsteroidal antiestrogens such as tamoxifen and vitamin A analogues such as retinal) have been found to inhibit PKC-activity *in vitro* (34,54). Similarly a metabolite of AMGP (1-O-alkyl-2-O-methylglycero-3-phosphocholine) an anti-tumour agent, has been shown to inhibit PKC activity (55). Ultraviolet radiation was found to induce point mutations of the PKC-α gene in a murine fibrosarcoma cell line (56). However, these mutations were not found to be responsible for induction of mouse Balb/c 3T3 fibroblasts transformation (459) and induction of tumours in immunologically "nude" mice by those cells (as initially reported, (56)). The effect correlated with increased levels and activity of

PKC in the transformed cells as has been observed after ultraviolet irradiation of normal cells (57). Increases of diacylglycerol levels have been observed in kidney or fibroblast cells transformed by Kirsten and Simian sarcoma retroviruses (ras-genes) (58,59) and modification of the PI cycle is observed in rous and avian sarcoma virus transformed cells (60,61) suggesting that alterations in the PI/PKC pathway may be one mechanism for tumorogenesis in some situations.

As noted above, tumour progression in mouse skin is the process whereby papillomas and possibly other premalignant skin lesions progress to SCC. It is generally accepted that additional genetic changes within cells that comprise papillomas are necessary for tumor progression (62). Recent studies have implicated the possible role of mutated Ha-ras gene dosage in the process of malignant tumor progression (63,64). Yuspa and coworkers (37,65) have also reported that changes in the fos gene may be involved in skin tumor progression and that Ha-ras and fos may cooperate in converting papilloma-derived keratinocytes to malignant tumors.

The expression of recessive mutations (i.e. tumour suppressor genes) has been shown to be involved in carcinogenesis in both humans and animals (66). These data suggest that tumour supressor gene located on chromosome 7 could be important in malignant progression in the mouse model system. Finally, Ruggeri *et al* (67) have reported that alterations in the putative tumour supressor gene p53, occur in 25-50 % of murine SCCs induced by the two stage initiation-promotion protocol.

Some of the above mentioned morphological and biochemical changes have been associated with individual stages in a two-stage promotion concept. The induction of dark keratinocytes (DCs) and the synthesis of prostaglandins and possibly other metabolites of arachidonic acid as well as the growth factors TGFα and TGFβ are reported to be associated with stage I ("conversion"). Stage I promotion also has some persistent effects that last 5-8 weeks, while stage II promotion is apparently immediately reversible (37). The induction of epidermal ODC, elevated levels of polyamines, and the maintenance of chronic cellular proliferation have been reported to be associated more specifically with stage II promotion (37 and

references therein). These observations, provide strong support for the hypothesis that those operational stages of tumour promotion have a distinct mechanistic basis. On the other hand differences of effects observed in different mouse stocks have raised questions about the generality and the mechanistic basis of this phenomenon (68). Additionally, Shoyab *et al* (72) proposed that a phorbol-12,13-diester-12-ester-hydrolase may be responsible for prevention of phorbol ester induced tumor promotion in skin. This enzyme converts 12,13-diesters to 13-monoesters and was shown to be present in rat, guinea pig, hamster and rabbit but not in mouse, cat, dog, monkey or human. They proposed that species possessing this enzyme were resistant to tumor promotion.

However, understanding the tumour response in genetically different mouse strains to specific initiating and promoting agents may also facilitate the study of other tumour types. Many concepts now currently applied to other tissues and model systems, including cell culture models for multistage carcinogenesis and transformation, were originally derived from the mouse skin model. That these concepts also apply to human cancer has been confirmed by epidemiological studies indicating that human carcinogenesis also occurs via a multistep process involving initiation and promotion mechanisms (37 and references therein). In terms of more direct relevance to human skin cancer, comparison of skin tumors in man and mouse has shown both similarities and differences in the incidence and biological behavior of certain tumour types.

Additionally, it was postulated that the multistep nature of human tumorigenesis may involve the activation of proto-oncogenes as well as the inactivation of one or more tumor suppressor genes. Recent studies have shown that ras gene activation can be detected in a significant proportion of both melanomas (20 %, primarily N-ras)(69) and nonmelanomas (46 % SCCs, 31 % basal cell carcinomas, primarily Ha-ras) (70).

All this data suggest that the mouse skin model under appropriate conditions is relevant for studying molecular mechanisms of skin carcinogenesis in humans.

Inflammation

The irritancy of phorbol esters manifests itself by classical symptoms in severe reddening, swelling, pain and blistering thickening of the skin when applied externally, and a burning sensation on mucous membranes, intestinal pain and severe purging/vomiting if ingested (74). The inflammatory response is the acute effect of phorbol esters which has been utilised to screen plant material for their presence and for bio-assay guided PE purification. Observation of mouse ear erythema caused by single application of irritant phorbol esters detected two types of response. A transient response (e.g. as elicited by resiniferatoxin with 100 times potency of TPA) peaking within 1-2 hours and then declining to normal and a persistent response (e.g. elicited by DOPP) evident in one hour and able to remain for 24 hours. TPA was able to achieve either non-persistent (at low doses) or persistent (at high doses) responses (75). Examination of phorbol ester effects at a cellular level in rabbit skin detected vasoconstriction (24) whereas in mouse skin vasodilatation, oedema and cellular infiltration were evident (76). The response as monitored by ear thickness indicated that oedema peaked within 6 hours for TPA and declined over 24 hours whereas that of Rx peaked within 1 hour and disappeared within 4 hours (77). The possibility of a neurogenic effect for the phorbol esters, by causing polymodal nociceptors to release multiple peptides (such as substance P, a PI cycle agonist) which then potently mediate neurogenic inflammation (78) has been suggested (79). Resiniferatoxin (Rx) has been suggested to act as an ultrapotent analogue of capsaicin (a pungent compound from *Capsicum anuum*) which elicits inflammation by neuropeptide release (80) in experiments on an isolated population of dorsal root ganglion neurones. However while pretreatment of mouse ear with Rx causes desensitisation to Rx and capsaicin-induced oedema (80), capsaicin pretreatment only inhibited early (transient) erythema induced by Rx and had an enhancing effect on persistent erythema (81). Higher doses of Rx could reduce the latency time to erythema and caused the erythema to be prolonged (over 12 hours) whereas higher doses of other irritant phorbol esters (e.g. Sapintoxin D) only prolonged the duration and had little effect on latency time. Inflammation appears to be a complex interaction

recruiting endogenous mediators from neurogenic and inflammatory cell sources which synergise in the final observable response.

There is however general agreement on the structural requirements for pro-inflammatory activity (24,82). The structure-activity relationships for induction of inflammation like the requirements for tumour promotion are generalised and not absolute; A/B rings in the trans configuration and an acyl residue at C-12/C-13 or C-13 are the only requirements (24). Features increasing potency include: a primary hydroxyl at C-20, unsaturation in the C-12/C-13 residue and an aromatic residue at C-12/C-13. Whilst all tumour-promoting phorbol esters are pro-inflammatory, not all pro-inflammatory phorbol esters are tumour-promoting. The sapintoxins and resiniferatoxin are representative examples. Sap A is a tumour promoter only in the presence of calcium ionophore (49) yet it is of similar inflamatory potency to TPA (83); resiniferatoxin is not a tumour promoter yet it is 100 times more potent at inducing erythema than TPA (84). The mechanisms of resiniferatoxin induced erythema have recently been extensively studied. Whilst it has been proposed that Rx acts as an ultrapotent analogue of capsaicin, (79,80), recent results from our laboratory implicate the existence of a new protein kinase that can serve as a specific receptor for Rx (449).

Mitogenic effects

Phorbol esters have been found to induce proliferation in skin (85), cultured B and T-lymphocytes (86,87) and fibroblasts (88). Phorbol esters have also been found to influence growth and activation of lymphocyte specialized subpopulations; e.g. LAK (lymphokine activated killer) T-lymphocytes (89), lymphoid supressor T-lymphocytes (90) and cytotoxic T-lymphocytes (91). Additionally phorbol esters induce macrophages to produce a variety of cytokines (monokines and lymphokines) which themselves act on lymphocyte populations as mitogens (92,93). Without accessory cells (macrophages and monocytes) TPA-induced mitogenic activity in lymphocyte cultures is poor, but addition of calcium ionophores (94), lectins (89,95), or cytokines (92) can serve to augment the response. In heterogeneous lymphocyte cultures from peripheral blood, the mitogenic effects of phorbol esters have been found to vary between species (96). Thus TPA on its

own is mitogenic in chimpanzee, marmoset, baboon, rhesus monkey and human cultures but not those from guinea pig, rat, mouse and dog. However, mouse thymocytes are mitogenically responsive to TPA alone (97).

The effectiveness of phorbol esters on mitogenesis does not correlate with the ability and degree of tumor promoting activity as has been suggested (89) since both promoting and non-promoting phorbol esters are mitogenic in human lymphocytes cultures (98). The non-promoting DOPPA and Sap A could induce 95 % and 70% of the mitogenic effect of TPA. Similarly these effects of DOPPA do not fit with its poor activation of PKC *in vitro*. In co-mitogenic experiments on allogenic mixed lymphocyte cultures, DOPPA was more potent than Sap A or TPA in achieving the maximal response. Some investigations (89,99), however imply, that non-promoting phorbol esters may produce mitogenic effects only through accessory cell-mediated mechanisms.

Differentiation

In certain transformed or leukemic cell lines derived from proerythroid cells the effects of phorbol esters integrate with the stage of differentiation or maturation which the cell has reached and exerts effects reflecting the different developmental histories of these cell lines.

Growth inhibition is a comon response in many cell lines treated with TPA (e.g. human promyelocytic leukemia cells HL-60, (100), human colon cancer cell lines (101), U937 monocytic leukemia cell line (102) and Daudi human B-cell line (103). Not all of these cells undergo differentiation as a result (e.g. Daudi cells appear to be arrested in G1/G0 phase of the cell cycle). In HL-60 cells differentiation has been linked with the translocation and down-regulation of PKC induced by TPA, since resistant strains did not exhibit such a decline (104). Furthermore, bryostatin 1 actively translocates PKC and induces some down-regulation, but does not induce HL-60 cells to differentiate and blocks the effect of TPA (105). Immunocytochemical studies indicated that the differentiating action of TPA coincided with the ability to induce PKC translocation toward the plasma membrane whereas in other fibroblastic and leukemic cell lines resistant to differentiation, TPA induced translocation to the nucleus (88,106). However, some

studies (107) indicated that plasma membrane translocation was a proliferative response whereas nuclear translocation of PKC induced by PEs plays an important role in differentiation. The generation of PKM seems to be vital, since in TPA-resistant cell lines PKM persists whereas in sensitive cells TPA removes PKC rapidly from the cell membrane. *sn* 1-oleoyl-2-acetylglycerol (OAG), even when applied to compensate for metabolic loss, was unable to induce differentiation in HL-60 cells (29,108). The effects of phorbol esters on this system are not confined to tumor promoting PEs (109). Weak promoters (e.g. Thy A) and non-promoters (e.g. Sap A and DOPP) could equally induce differentiation while a non promoter DOPPA had some effect at high doses . However, the non-promoting Rx was unable to induce differentiation but shared the effect of immediate inhibition of cell growth/division with other compounds mentioned above.

Nuclear events

PEs have been found to alter gene expression in many systems, e.g. in mouse skin c-fos, c-myc and ODC genes are induced by TPA as detected by increased mRNA levels (87), Epstein-Barr and oncogenic herpes viral antigens in virally infected cells of lymphoblastoid lines (e.g. Raji) (47,87), c-fos and c-myc in fibroblast (Swiss 3T3) cells (110), p40 in HTLV infected T-lymphocytes (111), CD2 and CD5 antigenes in T-lymphocytes (112), c-fos in HL-60 and U937 (113) cell lines. The effect of TPA in several cell types is also to cause gene suppression, e.g. p34 protein kinase in Daudi lymphoblastoid cell line (103) and c-myc in medullary thyroid carcinoma (114). These effects are not believed to be due to direct interaction of PEs with DNA (34,115). The events which link activation of PKC and the nuclear and genetic events which follow are uncertain, and the elements (whether it is PKC itself or some downstream signalling molecule) which transduce the message are unknown.

It has been found that genes inducible by TPA have one or more TPA responsive elements (TREs) consisting of short stretches of bases which act as recognition sites on the DNA for at least 3 transacting factors (activating proteins in the cell)(450). TPA treatment appears to induce or suppress gene transcription by a combination of modification of transcriptional stimulatory activity of DNA-

bound activating proteins and modification of the DNA affinity for soluble activating proteins. Induction of cellular proto-oncogenes (e.g. c-fos, c-myc, c-sis) could be responsible for tumor promoting effect of phorbol esters. The cysteine-rich region in C1 region of the regulatory PKC domain has been suggested to bear homology to the DNA binding regions ("zinc fingers") of other DNA binding proteins (see below). However some investigations suggested that DNA binding motif was not necessary to promote gene expression and that active PKC was required (116).

Several substrates of PKC which could transduce the message from PKC to the nuclear machinery have been postulated. These include translation factors (117,464,465) and topoisomerase (118)(see below, p. 73).

Gene expression changes depending on the state of the cell cycle (e.g. whether genetically programmed towards differentiation or growth), hence PEs will affect gene transcription and processing according to the cellular factors then present in the cell. Induction of gene expression has been found with non-promoters and weak activators of PKC in addition to TPA in Raji cells (measured by Epstein-Barr virus early antigen induction) (119), indicating that the correlation with tumor promoting ability was poor.

Other effects

Platelet aggregation

TPA was the first PE to be shown capable of inducing dose-dependent aggregation of human platelets (120). It was shown to cause vacuole formation within storage granules and dilation of the open canicular system of platelets (121,122). Studies on the aggregating ability of a range of PEs indicated that daphnane ortho-esters were unable to induce aggregation of platelets (123) yet 12-deoxyphorbol esters could induce platelet aggregation 124). In order to induce platelet aggregation PEs were required to posses: A/B rings in the trans configuration, a free C-20 primary hydroxyl group and a C-13 ester function (125). Biochemical studies using phorbol esters possessing different abilities to induce

platelet aggregation have revealed that this activity may correlate with phosphorylation of 47 kDa substrate (pleckstrin) for protein kinase C (126).

Secretion

Phorbol esters have a variety of effects on cellular secretion. Some of these effects are slow, taking many hours to manifest themselves (e.g. enhancement of collagenase secretion in U937 monoblast cell line (102), Interleukin-1 in macrophages (92)) and reflect the induction of genes, or indirectly reflect the influence of cells communicating amongst each other (e.g. suppression of immunoglobulin secretion in lymphocyte preparations (127)). Rapid effects are seen in cells with specific secretory functions whose actions are primed and occur through exocytosis and cells which secrete excess metabolites as they accumulate. Exocytosis has been most extensively studied in platelets (thromboxanes, serotonin, ATP, 5-HT, transferable aggregating substance-TAS, 128) and neutrophils ($O^{\cdot}_2$ and H_2O_2, 129,130) and invoke in each, different mechanisms.

Arachidonic acid metabolism

TPA has been found to influence arachidonic acid metabolism and prostaglandin production in many cell types. In dog kidney (MDCK) cells TPA stimulates deacylation of cellular phospholipids through enhanced PLA_2 activity resulting in arachidonic acid production and release, and subsequent prostaglandin production and release (131). Such evidence points to the central involvement of PKC in eicosanoid synthesis, implicating these biologically active derivatives of arachidonic acid in inflammation, and allergic reaction, smooth muscle contraction, platelet aggregation and possibly tumour promotion (131).

Ionic and electrophysiological effects

The phorbol esters have been found to influence ionic parameters in cells specialised for synaptic transmission as well as non excitablecells through PKC dependent and independent pathways.

PKC mediated modulation of ion channels has been suggested as a possible mechanism in cell types where calcium and potassium conductances are central

to their physiology and the putative L-type calcium channel has been found to be a PKC substrate *in vitro* (132). The enhancement by PEs of electrically stimulated neurotransmitter release in a variety of neuronal tissues and excitable cells has also been reported (132,133). However, ionic effects of some PEs which are not found to activate PKC (e.g. 4-α-PDD) were similar to effects of TPA. Some of the ionic effects of the PEs may therefore be independent of PKC activation (134).

PEs have also been found to change calcium flux in some non-neurological excitable cells. While TPA had no effect on resting calcium levels in an insulin secreting cell line, it inhibited depolarisation-induced calcium influx through a voltage-gated calcium channel (133). In smooth muscle cells calcium "transients" induced by depolarisation were increased by TPA, involving a Na^+/Ca^{2+} antiport mechanism (135).

Many nonexcitable cells such as thymocytes, fibroblasts, HL-60 cells, neuroblastoma cells and T-lymphocytes showed no calcium change but showed a slight alkalinisation of the cytosol when treated with TPA, OAG or DOG (86,136-138) thought to be generated through a Na^+/H^+ antiport mechanism.

Receptor transmodulation

Experiments using combinations of physiological ligands (especially growth factors) and phorbol esters have revealed an important role for PKC in alteration of the cellular response to many physiological ligands (termed receptor transmodulation).

Many of the effects of the phorbol esters appear to be of a negative feedback nature on receptors which themselves can induce PI turnover but variation in the mechanism between different receptor types and even similar receptor types in different tissues is evident (for the review see 139). PKC mediated transmodulations can be: direct desensitization of the receptor (e.g. EGF receptor, 140; and α-adrenergic receptor, 141), an indirect uncoupling of the receptor from signalling pathways distal to receptor occupancy (e.g. uncoupling of β-adrenergic receptor from adenylate cyclase in T-cells, (142) or a reduction in the number of the receptors on the cell surface (e.g. internalization of the occupied and unoccupied transferrin receptor, 143).

The consequences of transmodulation between PE-stimulated PKC activity and physiological agonist-receptor systems are largely unknown but the deregulation of cellular systems may contribute to wider biological effects of the phorbol esters.

Although most of the above mentioned biological effects of phorbol esters were interpreted through their interaction with their main intracellular receptor, PKC, the recent discovery of other PE binding molecules, like chimaerins (n(α1), α2, β1 and β2)(145-147), Vav (457) and Unc-13 protein from nematode *Caenorhabditis elegans* (148,456) implies that not all pharmacological effects of PEs are channelled through activation of PKC kinases.

It has been shown recently that TPA binds and activates a Ras guanine nucleotide exchange factor (Ras GEF) - Vav (457), via its cysteine-rich, PKC-homologous domain. PE-DAG-mediated Vav activation pathway is an alternative to the proteintyrosinekinase (PTK)-dependent Vav stimulation, allowing receptors that are linked to different signal transduction pathways to become coupled to Ras.

The observation that α-and β-chimaerins are themselves involved in intracellular signalling (it functions as a GAP protein for the small GTP binding protein $p21^{rac}$) (145,149). This interaction appears to alter actin polymerisation and cytoskeletal organisation in mammalian cells, and these chimaerins are more tissue or cell type-restricted than the other GAPs. n(α1)- and α2-chimaerin are largely present in hippocampus, thalamus and cerebral cortex neurons, while only α1 chimaerin was expressed in Purkinje cells and only α2-chimaerin was expressed in rat testes, early pachytene spermatocytes (146). β-1 chimaerin was expressed only in the late stage of germ cell development in rat testes (145) while β2 was found in granule cerebellar cells (147). This implies that spatial and temporal expression of chimaerins may be essential for the development and function of these cells. In addition α2 and β2 chimaerins are found to contain an N-terminal SH2 (Src homology 2) domain which could give them other signalling functions. The exact *in vivo* function of chimaerins is not yet known, although there are some indications of their involvement in actin assembly/cytoskeletal

organisation (145), and the possibility of PEs interacting and producing biological effects through non-PKC signalling pathways is under investigation.

Unc-13 is a potential target for the pathways regulated by DAG/PE. However, its funcion in *C. elegans* is unknown and its mammalian homolog has not been identified so far (148,456).

1.2. PKC

1.2.1. History

Originally Protein Kinase C (PKC) (EC 2.7.1.37) was discovered by Nishizuka and coworkers as a histone protein kinase from rat brain that could be activated by limited proteolysis (150), Ca^{2+} and (phospho)lipids (151) or phorbol esters and phospholipids (13). It has been proposed that PKC activation by diacylglycerol was due to release of this substance from phosphatidylinositol metabolism (152). Furthermore, activation of the enzyme by Ca^{2+} resulted in its conversion from a soluble to a membrane bound form.

Intensive research efforts in the following 15 years resulted in the discovery that PKC takes part in cellular responses to various agonists including hormones, neurotransmitters and growth factors, and is a key regulatory molecule involved in many cellular processes, e.g. cell proliferation and differentiation, exocytosis (e.g. hormone and neurotransmitter secretion) and cell motility.

Subsequently PKC was found not to be a single molecular entity but a family of closely related isozymes, perhaps providing an explanation for the wide range of processes in which PKC is involved.

1.2.2. PKC family

At the present the PKC family is the largest serine/threonine-specific kinase family known. So far, the cDNAs coding for ten different PKC isozymes have been cloned from different species and tissues or cell lines. At present, the mammalian PKC family consists of 11 different polypeptides, α, β_1, β_2, γ, δ, ε, ζ, η, θ, ι(l) and μ, coded by ten genes (153). PKC β_1 and β_2 are derived from a single gene by alternative splicing of the 3' exons (154). The isozymes differ in their enzymatic properties (cofactor and activator dependency and substrate affinity), and in their tissue and intracellular distribution. According to their cofactor dependency and structure, PKC isozymes are now grouped into three classes. Both the classical (conventional) PKC isozymes (c-PKCs; α, β_1, β_2 and γ, showing calcium dependency) and novel PKC isozymes (n-PKCs; δ, ε, θ, η and μ, showing no calcium dependency)

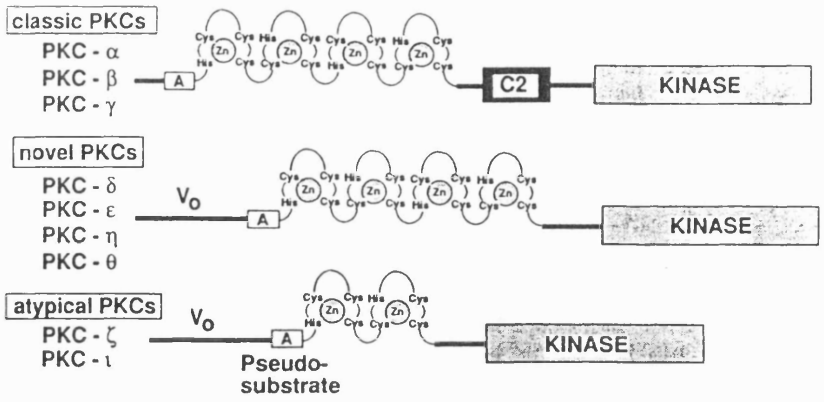
are phorbol ester (TPA) responsive, whereas the atypical PKC isozymes (a-PKCs; ζ and ι(λ)) are phorbol ester unresponsive (153,155,156,334). Mouse PKC λ is 98 % homologous to human PKC ι and hence appears to be the mouse homologue of PKC ι (334).

Structure

The primary amino acid structure, deduced from cDNA sequences available, can be divided into conserved (C1-C4) regions which are separated by variable (V1-V5) regions (Figure 1.2.1., p. 46). All PKC isozymes contain these constant and variable regions in a single polypeptide chain (molecular mass of around 80 kDa), although the n-PKC isozymes lack the conserved C2 region and a-PKC isozymes lack one of two cysteine-rich Zn^{2+} finger motifs in the C1 region. These regions are organised into two functional domains within the PKC molecule. The C-terminal regions C3-V5 have been defined in all PKC isozymes as the catalytic domain (of approx. 50 kDa) which contains the catalytic site, which binds MgATP and the substrate. The catalytic domain is separated by the V3 region from the N-terminal regulatory domain (V1-C3) of approx. 30 kDa, to which cofactors and activators bind.

Recently, first visualisation of crystals of PKC β_1 by electron microscopy, suggested a kidney bean-shaped catalytic domain of about 7.0 nm x 4.5 nm in diameter (157). The same study suggested that the regulatory domain lies above the catalytic domain, and that it could be represented by a sphere of approx. 2.5 nm diameter (Figure 1.2.2., p. 47). Calculations based on circular dichroism (CD) data additionally suggest that PKC maintains a highly ordered structure, and that the protein has a significant α-helical content (Table 1.2.1., p. 47) (158-160).

The V_0 region is an amino-terminal extension present in PKC-δ, –ε, –ζ and –η but not in PKC–α, –β, and –γ. Little conservation is seen between -δ, –ε, and –ζ in this region although significant homology exists for –ε and η (161,162). There is no direct evidence available concerning the role of these extended amino-terminal domains. However, evidence has been presented, suggesting that the V_0 region plays a role in restricting the effector-dependent substrate specificity (163-165).



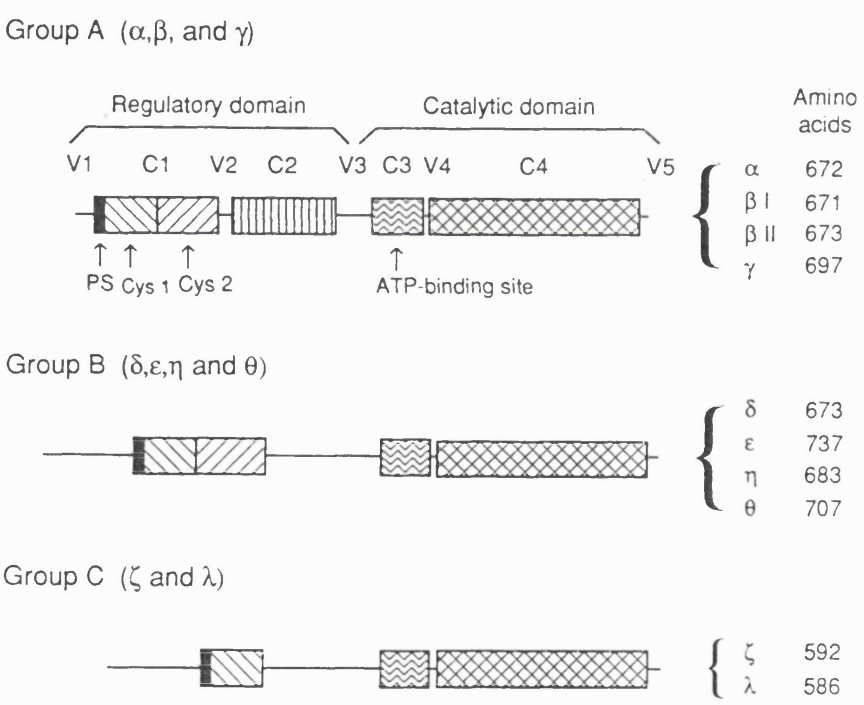


Figure 1.2.1. Schematic structure of PKC members. All PKC isozymes consist of constant (C) and variable (V) regions. Three structurally distinct groups of PKC isozymes (classic, novel and atypical-PKCs) are shown. The cysteine rich repeats in the C1 region are depicted in **A** together with chelated Zn^{2+} ions.

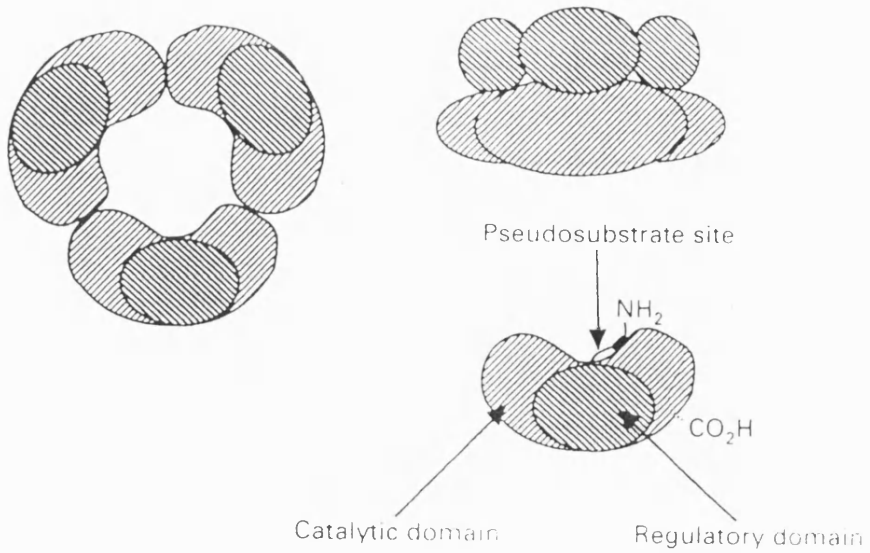


Figure 1.2.2. Schematic representation of the proposed domain structure of PKC-β_1 (from ref. 157.).

Table 1.2.1. Predicted contribution of α-helix, β-sheet, β-turn and random coil conformations to the secondary structure of PKC-α, β and γ (based on circular dichroism experiments, acc. to ref 160.).

PKC-isozyme	α-helix, %	β-sheet, %	β-turn, %	random coil,%
α	39.8	26.3	26.2	7.7
β	39.4	27.1	25.3	8.2
γ	37.6	31.5	26.0	4.9

The N-terminal V1 region of c-PKCs is relatively short (~ 20 amino acids) and no function has been attributed to this region. On the other hand, the V1 region of n- and a-PKC isozymes is extended (see above) and may influence or modulate the function of the conserved domain or may confer substrate specificity of these enzymes.

Recently discovered PKC μ was found to contain two unique hydrophobic amino terminal domains that could potentially function as a putative NH_2-terminal transmembrane anchor (332,333).

At the begining of the **C1 region**, a sequence motif is located that is similar to the consensus sequence XRXXS/TXRX found in the phosphorylation sites of PKC substrates (166). However, the serine or threonine residue found in the substrate motif is changed to alanine in all PKC isozymes. Thus, this motif cannot be phosphorylated and is very likely to represent a pseudosubstrate site that exhibits autoregulatory features (167) by blocking the catalytic (substrate binding) site. In fact, pseudosubstrate peptides are rather efficient inhibitors of PKC both *in vitro* and *in vivo* (168,169). Similarly, synthetic peptides containing this sequence with alanine replaced by a serine residue can be used as *in vitro* substrates (163,170,171). Deletion of the entire PKC-α pseudosubstrate site (PKC-α Δ19-31) produces a constitutively active enzyme (172). In addition, anti - pseudosubstrate antibodies could be used to activate PKC *in vitro* in the absence of effectors (173), presumably by binding to and sequestering this inhibitory region.

On the basis of the pseudosubstrate sequence, substrate specificity of PKC isozymes could be speculated. However, a comparison of these sites does not reveal any significant differences and nor do the c-PKC isozymes α, β_2 and γ show any distinct differences when probed with the respective substrate peptides (171). It is possible, however, that within natural (holoprotein rather than the peptide) substrates of PKC, structures of higher order may determine substrate specificity (174). There are now a number of other protein kinases including the cAMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase II where

specific sequences within the respective regulatory domains/subunit interact with the catalytic site as a means of controlling activity (167).

PKC μ is the only PKC isozyme that lacks a typical pseudosubstrate site (332,333). This, together with other structural and functional characteristics (see below), indicate that regulation of PKC μ kinase activity differs from that of the other known PKC isozymes, suggesting a novel, yet to be defined, role of PKC μ in intracellular signal transduction pathways.

The Cys-rich subregion within the C1 region consists of two Zn-finger-like motifs (except a-PKCs which contain only one motif), which mediate DNA binding of other proteins (175), each with six cysteine and two histidine residues, which confers the PE or DAG binding (176-178). Purified PKC-β_2 has been shown to contain four tightly bound Zn^{2+} ions that may stabilise a particular conformation by interaction with one histidine nitrogen and three cysteine sulfur atoms (179,180) (see Figure 1.2.1.A.). Recombinant fusion proteins of glutathione S-transferase and the cysteine-rich domains of PKC bind Zn^{2+} and PE, and removal of Zn^{2+} inhibits PE-binding (181). Mutation of one of the conserved cysteines (that is likely to coordinate Zn^{2+}) in each of the repeats destroys PE binding activity (177). In the presence of PE, binding of additional Zn^{2+} to PKC also enhances the attachment of PKC to the membrane cytoskeleton (182). The cysteine-rich Zn^{2+} finger motifs, similar to those found in PKC [$HX_{12}CX_2CX_{10\text{-}14}CX_2CX_4HX_2CX_7C(C_6H_2)$], have been found in over 200 proteins, e.g. transcriptional factor IIIA (183) and GAL4 (184), and human glucocorticoid (185) and estrogen receptors (186). However, for PKC isozymes no DNA-binding activity has been demonstrated, but the regulatory subunit alone, generated by proteolytic cleavage in the V3 region, may bind DNA. On the other hand there is evidence of PKC-activity that is associated with the cell nucleus (187), hence, nuclear proteins involved in transcription, replication and repair may be subject to phosphorylation (see below). A similar Zn^{2+} finger sequence motif is also found in the protooncogenes c-raf and A-raf (188), n-chimaerin (189), 80-kDa diacylglycerol kinase (190), two members of CDC24 family of guanine nucleotide exchange factors (GEFs) - Vav and Lfc (457,458), phospholipase A_2 (191) and *Caenorhabditis elegans* protein Unc-13 (148,456). Diacylglycerol kinase (192), c-Raf kinase isozymes (193) and n-chimaerin

(194) also, show no DNA-binding activity. The use of deletion mutants of different PKC isozymes revealed that the Cys-rich region is necessary for DAG and phorbol ester binding (195,196), and the C1 region, expressed on its own in *E. coli*, exhibited phospholipid-dependent phorbol ester binding (177). PKC-ζ contains only one zinc finger and does not bind DAG or PEs (197), is not activated by PEs and does not translocate or down-regulate in response to PEs or membrane-permeant DAG (198). Second a-PKC isozyme ι (λ) was found to have similar properties (331,334). This is in agreement with the finding that, at least *in vitro*, PKC-ζ exhibits a constitutive protein kinase activity (335,155) which may be influenced *in vivo* by other unknown factors. It seems important that all PKC isozymes, even PKC-ζ exhibit a distance of 15 amino acids from the end of the pseudosubstrate box to the beginning of the zinc finger motif.

In recently discovered PKC isozyme - μ, an atypically long spacing of 74 amino acids (in contrast to a 15- and 22-amino acid spacer in c-PKCs and n-PKCs, respectively) was associated with limited phorbol ester activation and considerable constitutive *in vitro* kinase activity (332,333). The presence of C1-like regions in other proteins (194,201) infers the presence of a DAG/PE binding site. In the case of n-chimaerin, PDbu has been shown to bind the bacterially expressed protein and this is dependent upon refolding in the presence of Zn^{2+} (see above, 181). However, DAG kinase is not inhibited at submicromolar concentrations of TPA (while presumably binding DAG) implying that the divergence of these sequences has led to changes that can distinguish DAG and PDBu (or possibly other PE).

It is possible that differences between c-and n-PKCs, and between chimaerin and the non-phorbol ester-binding a-PKCs, may be due in part to differences in hydrophobicity. The first Zn^{2+} finger of c-PKCs and chimaerin have a strongly hydrophobic region around the third and fourth conserved Cys residues. In contrast, the first and single Zn^{2+} fingers of the non-phorbol ester-binding DAG kinase and PKC-ζ, respectively, are weakly hydrophobic in the third and fourth conserved Cys region and are strongly hydrophobic in the N-terminal region of the fingers. PKC-δ, –ε, –η and –θ have reduced hydrophobicity at the third to fourth conserved Cys region of their first Zn^{2+} fingers relative to those of the c-PKCs and chimaerin. The second Zn^{2+} fingers of PKC–α, –β and –γ, and –δ, –ε and –η are

weakly hydrophobic to non-hydrophobic at their third to fourth conserved Cys region, yet have moderate hydrophobicity at the mid-portion of their sequences. Because PKC deletion constructs containing one or the other Zn^{2+} finger, expressed in insect cells, bind [^{3}H]PDBu tightly (Kd = 21-41 nM), but 10- to 20-fold less than the native enzyme (196), and because individual Zn^{2+} fingers from PKC can bind phorbol ester, its binding may be controlled by the overall hydrophobicity of each finger. Although there are two PE-binding motifs, binding of more than one PE per PKC has not been demonstrated. The binding of a single PE molecule to native PKCs may sterically block a second PE binding site on the second Zn^{2+} finger. However, the possibility of having two nonequivalent second-messenger sites in c- and n-PKCs has been hypothesised to explain the differential effects of several PKC-activating tumor promoters (202).

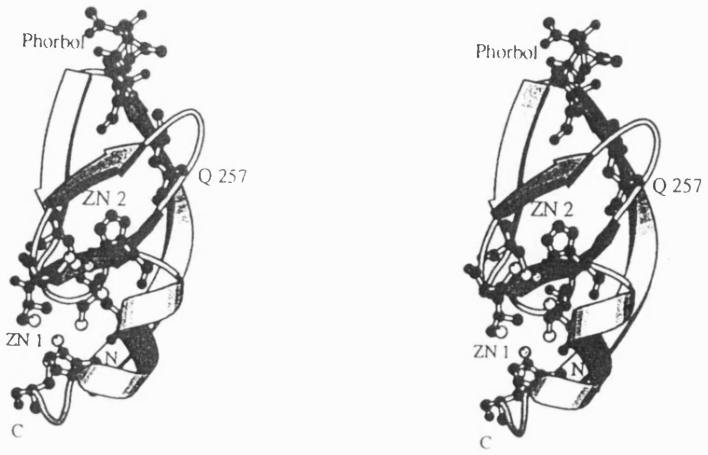
A recent crystallographic study of PKC-δ second cysteine-rich region (Cys2, residues 231-280) and of the Cys2 complexed with phorbol 13-acetate provided a closer description of the PE binding to the cysteine rich region of a PKC molecule (203). According to this model the Cys2 domain of the PKC-δ consists of two small β sheets and a short C-terminal α helix. The same model proposes that PE binds between residues 239-242 and 251-254 in a groove between two pulled-apart β strands (formed by the main chain from 239-242 and 250-254 and the side chains of Met-239, Pro-241, Thr-242, Leu-250 and Leu-254) at the tip of the domain (Figure 1.2.3. A. and B., p. 53). The four rings of phorbol are inserted length-wise into a narrow groove between the two β strands with the C20 OH group buried in the bottom (formed by the side chains of Tyr-238, Leu-251 and Gln-257). In the absence of phorbol binding, the gap between the strands contains bound water molecules that form bridging hydrogen bonds. Bound PE displaces these water molecules, and the C3, C4 and C20 oxygens replace the lost bridging hydrogen bonds. Five hydrogen bonds are formed between three of the phorbol oxygens and the protein (Figure 1.2.3.). The C3 oxygen accepts a hydrogen bond from the main-chain amide of Gly-253. The C4 hydroxyl donates a hydrogen bond to the main-chain carbonyl of Gly-253. Lack of ability to donate a hydrogen bond to the Gly-253 could explain the diminished activity of 4-deoxy PEs and changed activity of 4-O-methyl PEs (first stage promoters, see above). The C20 hydroxyl accepts a

hydrogen bond from the main-chain amide of Thr-242 and donates bifurcated hydrogen bond to the main chain carbonyls of Thr-242 and Leu-251, acting as an interstrand bridge (Figure 1.2.3. B.). It has been also suggested that C20 OH is analogous to the 3-hydroxyl of DAG. The C12 hydroxyl faces outward from the binding site, and lack of interaction involving the C12 hydroxyl could explain the high affinity of 12-deoxyphorbol esters (see above). Neither C9 OH nor the C13 acetyl hydrogen-bond with the protein, but form an intramolecular hydrogen bond instead, and are important because removal of either one of these polar moieties separately would leave the other with an unsatisfied hydrogen bond in the lipid phase. Since both of the normal positions of esterification point away from the protein, the tails of long-chain phorbol esters probably do not interact directly with the protein.

The determined Cys2 structure shows how membrane insertion (204) by PKC might occur (see below). The "top" third of the proposed cys2 surface is composed almost entirely of hydrophobic residues and is completely devoid of charged groups. The "middle" third is overwhelmingly composed of positively charged side chains. The chain termini are close together at the "bottom" of the domain, suggesting that connections to the soluble domains are well above the membrane.

The layered surface suggests that the Cys2 domain could bury itself top down 6-8 Å into the bilayer, with some of the basic side chains from the middle of the domain positioned to interact with acidic phospholipid headgroups. The PKC activator domain partitioning into the membrane is strongly favoured by the presence of PE or DAG (196,204,205). This behavour can be explained by the structure of the activator-binding site and by the nature of the protein surface. Membrane insertion of the unliganded activator-binding site would have lead to either a loss of hydration between the unzipped strands or to the insertion of bound water molecules into the membrane along with the protein. Either of these situations involve several unsatisfied hydrogen bonds at the protein-membrane interface, which is highly unfavorable. By contrast, PE binding satisfies the hydrogen bonding potential of the activator-binding groove. The activator forms a hydrophobic cap over the exposed polar main-chain groups.

A



B

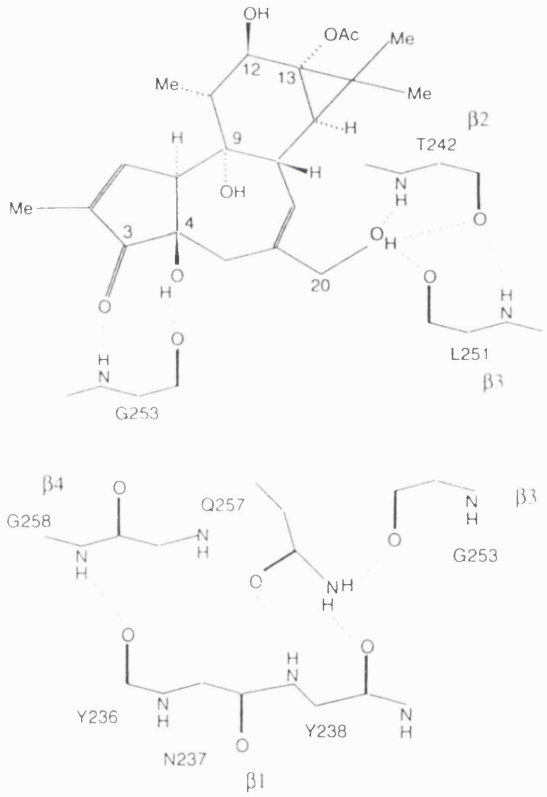


Figure 1.2.3. (A) Stereoview of phorbol ester binding to the Cys2 domain of PKC C1 region. **(B)** Hydrogen-bonding interactions in the phorbol-binding site (from ref. 203).

When an activator is bound, the domain can fully bury its hydrophobic exterior surface in the membrane without suffering an unfavorable loss of hydrogen bonds in the groove.

Only the water-insoluble long-chain PEs are potent PKC activators. Because these compounds are completely partitioned into the membrane, PKC-PE binding is directly linked to membrane association by the protein (see below).

The C2 region is believed to confer the Ca^{2+}-dependency, because activation of the n-, a-PKC isozymes and PKC μ, which lack this region, is insensitive to Ca^{2+} (206,207,332,333). A recombinant polypeptide fragment containing the C1 region can bind PE in the presence of PS, whereas that containing both C1 and C2 regions requires both Ca^{2+} and PS. This region does not contain a typical Ca^{2+} binding site (e.g. E-F hand binding motif, like in calmodulin), and the high-affinity Ca^{2+} binding site might form through appropriately spaced amino acid side chains or through coordination with bound PS (208). There is clear evidence that PKC-δ and -ε do not interact with membranes in a Ca^{2+}-dependent fashion while PKC-α and -β do (163,209). However, using fusion proteins with the C1/C2 domains of c-PKCs and n-PKCs, it has been reported that the C1 region (rather than the C2 region) of PKC-ε can bind Ca^{2+} in place of Mg^{2+} as a divalent cation, whereas the C2 region imparts calcium selectivity (210).

Sequence segments homologous to the PKC C2 region have also been identified in a synaptic vesicle-specific Ca^{2+}/PS-binding protein, synaptotagmin (p65) (211), cytosolic phospholipase A_2 (212), phospholipase C γ (213), and GAP (214).

The V3 or hinge region functions as a link between the regulatory and catalytic domains. As a long stretch of variable (i.e. isoform specific) sequence, this region has proved useful for the generation of selective antisera. Proteolytic activation of PKC by calpain I and II (Ca^{2+} dependent neutral proteases) or trypsin, proceeds through cleavage within the V3 region (201,215). Limited cleavage with these proteases releases two functional domains, with constitutive kinase activity (catalytic domain) and a phospholipid-dependent phorbol ester

binding activity (regulatory domain) (216,217). This region appears to be fairly flexible to permit dislodging of the pseudosubstrate region from the catalytic domain on binding of the activators. It is of interest that this region appears to be more susceptible to proteolysis in the effector activated state (215) implying that the region becomes more exposed/accessible to the protease. PKC-α is more resistant to proteolytic cleavage than PKC-β and γ (218,219). Further experimental evidence will be necessary to determine whether or not the V3 region of the different PKC isozymes exhibit certain features that modulate the susceptibility to proteolytic cleavage. Recent findings indicate that the hinge region (and the catalytic domain) may be involved in the nuclear targeting of PKC-α (220). Selective translocation or localisation of PKCs may provide a means of regulating substrate specificities and differential susceptibilities to proteolysis.

The C3 region is the putative ATP-binding domain that includes Gly X Gly XX Gly X_{16} Lys consensus sequence for ATP binding. The invariant lysine residue that contributes to the predicted ATPbindingsite was found to be essential for kinase activity (221-223).

The V4 region is a short insert separating C3 and C4 regions and is found in PKC-γ but not in other isozymes.

C4 region is a part of the catalytic domain that contains the substrate binding site and the phosphate transfer region and is highly conserved amongst protein kinases (174,224).

The V5 region at the C-terminal is relatively poorly conserved between PKC isozymes. This region also covers the different sequences in the PKC-β gene splice variants. These two exons encode the C-terminus of the PKC-β_1 and β_2 proteins (154,225).

PKC activation model

Initial studies with partially purified PKC from rat brain indicated that several components were required for the activation of the enzyme (151). In addition to Ca^{2+}(later studies identified PKC family members that could be activated in the absence of Ca^{2+}, n-PKCs), two other physiological cofactors were identified as lipid-soluble effectors. Purified phosphatidyl-L-serine (PS) substituted most effectively for the naturally occurring membrane phospholipids in supporting the activity of PKC. In general, most lipid effectors of PKC are negatively charged, while many lipid inhibitors are positively charged (226). PKC interacts with neutral phospholipids such as phosphatidylcholine (PC), but apparently only anionic phospholipids (i.e. phosphatidyl-L-serine, phosphatidylglycerol, cardiolipin, phosphatidic acid and phosphatidylinositol 4,5-bisphosphate) are capable of providing a suitable environment for PKC activation (208)(although, some activation was observed with short chain PC, 470). Only PS, however, can fully support PKC activity. Studies using PS analogs revealed a high degree of specificity for the functional groups within the phospholipid's polar head group (227). Both the carboxyl and amino functional groups in the head group are absolutely critical for function, additionally activation was stereospecific for phosphatidyl-L-serine. Therefore, a minimum of three points of contact between PS and PKC were suggested and these points are inferred to be carboxyl, amino and phosphate groups of PS (228).

Neutral lipid, diacylglycerol (DAG) was also required in conjunction with Ca^{2+} and PS for maximal activation of the kinase. DAG is considered to be the major physiological activator of PKC (while tumour-promoting PEs can readily substitute as activators of PKC), and it also displays an extremely high degree of specificity in its activation. PKC is stereospecifically activated only by sn-(S)-1,2-DAGs and not sn-1,3- or sn-2,3- isomers or (R)-DAGs (229). DAG is also a transient activator, and its activity is terminated when DAG is either phosphorylated by a specific kinase, or hydrolyzed at the sn-2 position (230). Structure-activity relationship studies have suggested a three point attachment of DAGs to PKC, and it has been demonstrated that the primary hydroxyl group

and the two ester groups with fatty acyl chains of sn-1,2-DAGs are essential for its function.

A comparison with other lipid-dependent proteins (e.g. n-chimaerin, c-raf, vav, diacylglycerol kinase, PLA_2, PLC, GAP, unc-13) has identified at least four regions of significant lipid-protein interactions within the structure of c-PKCs and only three such regions are present in the n-PKC family members. The first potential lipid-protein region encompasses both cysteine-rich regions of the regulatory domain C1 region, the second region is linked with the C2 PKC region and is present only in the c-PKC isozymes, the third lipid-protein region may be located in the catalytic domain of the PKC molecule, based on the evidence that the activity of the catalytic domain of PKC can be influenced by phospholipids in a substrate- and pH-dependent manner (231,232) and the fourth, lipid-protein interaction site, may reside within the pseudosubstrate segment of the C1 PKC region (since synthetic peptides which mimic the PKC pseudosubstrate site, are able to bind membranes composed of acidic lipids) (233).

Circular dichroism studies (see above, Table 1.2.1., p. 47), showed that PKC can interact with Ca^{2+} in the absence of PS and this binding introduces a slight disorder in its conformation. A large and specific conformational change (i.e. large increase in β-structure at the expense of α-helix) is observed upon binding of PKC to PS vesicles (159), however, in the presence of mixed phospholipid (PS/PC or PS/PC/DAG) vesicles, the helical content increases (158). Analysis of the mean hydrophobicity (160) and hydrophobic moment profiles does not predict any particular location of a structural domain which could definitely associate with the plasma membrane. However, the cysteine-rich regions of the regulatory domain have some short hydrophobic stretches. Lester *et al* suggested (234) that the region of PKC sequence which penetrates the monolayer contains cysteines leading to the conclusion that the cysteine-rich region is responsible for the lipid binding.

Proposed mechanism of PKC activation

By means of several membrane-model systems, activation properties of partially or highly purified fractions of PKC isozymes were studied *in vitro*. As a major advance, the use of DAG/PS vesicles (generated by sonication) as the

classical activator of PKC (151,13) has been substituted by so-called mixed micelles as the membrane-model system. Mixed micelles, composed of (phospho)lipid(s) embedded in Triton X-100 micelles (Figure 1.2.4.), have proven to functionally mimic the situation of the cellular membrane environment of PKC and has minimized artefacts such as damage of vesicle structure by the presence of Ca^{2+} (208).

A model for the *in vivo* activation of the c-PKCs is presented in Figure 1.2.5., p. 59. In the inactive (resting) state, the pseudosubstrate site of PKC is bound to the substrate binding site. In response to intracellular Ca^{2+}, PKC interacts with membranes (mediated by the C2 region of the regulatory PKC domain) where it remains in an inactive, but conformationally distinct membrane-associated state (208,236).

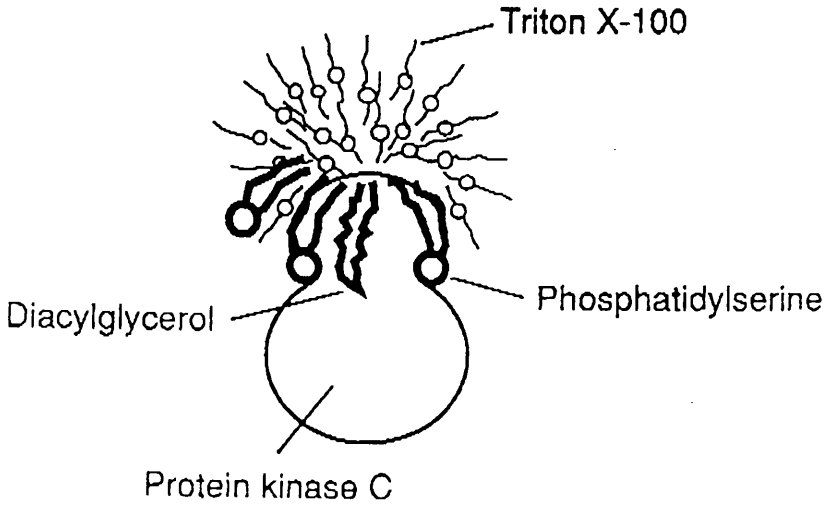


Figure 1.2.4. Schematic representation of Triton X-100 / lipid mixed micelles, showing that one molecule of protein kinase binds to one micelle (from ref. 205).

Association of PKC with membrane phospholipids is not sufficient to elicit activation of the enzyme (237). Activation of PKC occurs only when activators such as DAG or PEs become accessible to the membrane-bound enzyme at the

intracellular membrane surface. These activator molecules appear to exert their effect by promoting the insertion of the inactive membrane associated PKC into the membrane (234,238,239). During this insertion, PKC undergoes an additional conformational change such that the pseudosubstrate site is dislodged (unmasked) from the substrate site, thus rendering the enzyme active and capable of phosphorylating cellular substrates (237). A disturbance of the membrane structure is obviously not the only factor required for PKC activation (if this were the case, any membrane destabilizer would potentially activate PKC). Presumably, the specificity of activation is derived from the ability of PKC to directly interact with DAG or PEs during or following the insertion process.

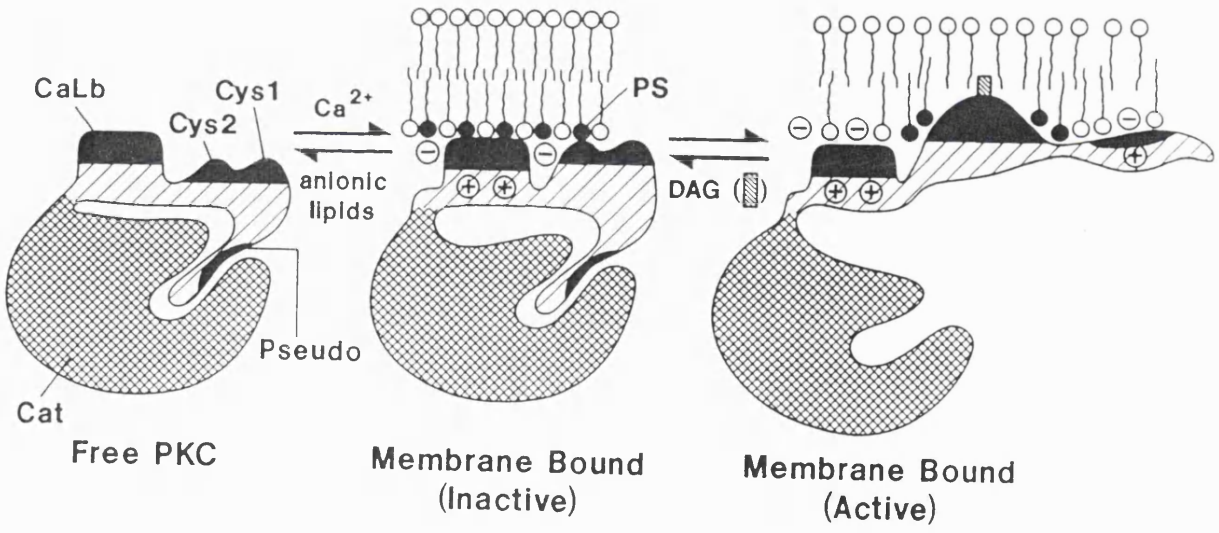


Figure 1.2.5. Activation mechanism of PKC. Pseudo, pseudosubstrate motif of C1 region; Cys1, Cys2, cysteine-rich motifs of C1 region; CaLB, calcium-dependent lipid-binding site in C2 region, see text for details (from ref. 235).

This model for PKC activation can be modified to account for the behavior of the n-PKC group (Ca^{2+}-independent). Since these PKCs lack the C2 region, their association with membranes is not dependent upon intracellular Ca^{2+} concentrations. After membrane attachment, activation of these family members occurs in a manner identical to c-PKCs. Relative to c-PKCs, n-PKC isozymes also have a much larger V1 region. Apparently, this extended regulatory domain can still modulate the substrate selection of the catalytic domain, even after activation of the PKC (164). Alternatively, n-PKC isozymes may already be associated with membranes or cytoskeletal elements in an inactive state awaiting activation. There is also additional evidence that n-PKCs, specifically PKC-δ and ε, can be primarily in the particulate fraction of cells (240,241). The proposed overall shape of the PKC molecule, in which there could be close proximity of the regulatory N-terminal pseudosubstrate and the catalytic C-terminal domain substrate binding site, lends support to the above model for PKC function (157). However, this model does not adequately address the activation of the PKC family members by effectors such as arachidonic acid or other fatty acids which appear to exert their effects in the absence of membranes.

The structure of the activator-binding domain-PE complex helps clarify the role of PEs in PKC activation (see above and ref. 203). PE binding does not produce a significant conformational change within the activator-binding domain. Rather, it acts to cover the polar interior of the groove and completes a continuous hydrophobic surface over a large portion of the domain. The long-chain lipid tails of PKC activators are probably not directly involved in binding to PKC, but instead retain the activator within the membrane. The retention of the long-chain PEs in the membrane means that the favorable free energy of protein-PE binding can be used to drive the insertion of the protein into the membrane. The present structural results suggest that PEs activate PKC by altering the nature of the protein surface and by stabilizing the membrane-inserted state, rather than by inducing a conformational change at the activator-binding site.

PKC autophosphorylation and phosphorylation

Most of the protein kinases described so far exhibit pronounced autophosphorylation which is often but not necessarily linked to a modulation of kinase activity. Autophosphorylation has also been reported for all PKC isozymes known (156 and references therein).

A detailed study on PKC-β_1 revealed certain features of autophosphorylation: 1) all phosphorylated residues were found in variable regions of both regulatory and catalyticdomains of PKC isozymes, 2) the surrounding amino acid sequence did not exhibit any consensus sequence nor any similarity to the pseudosubstrate/ substrate consensus motif (see above), 3) the distribution of autophosphorylation sites over the N-terminus, the hinge region and the C-terminus indicates a close proximity of these regions to the catalytic centre of PKC. A comparative phospho-amino acid analysis of highly purified PKC-α, β_2 and ζ revealed almost exclusive autophosphorylation of PKC-α and ζ on serine and, in contrast, PKC-β_2 phosphorylates primarily on threonine residues (156). The localisation of autophosphorylation target residues might, therefore, indicate a role that is quite diverse between PKC isozymes.

Reports on a potential role of autophosphorylation for the proteolytic activation and degradation of PKC have been controversial (164,222).

Several reports suggest a post- or co-translational phosphorylation of PKC prior to activation (242,243) and that without this initial phosphorylation, PKC is inactive and cannot be activated. This observation is supported by the finding that PKC expressed in *E. coli* is inactive (244) due to lack of an as yet unidentified PKC kinase present only in eukaryotic cells. In this respect, protein phosphatases become relevant (245), and it is notable that, at least with PKC-α, potato acid phosphatase is capable of dephosphorylating PKC without loss of PKC activity, whilst protein phosphatases 1 and 2A can completely abolish PKC activity (242). A recent report (451) indicated that in PKC α isozyme, Thr-497 plays the dominant role in the regulation of PKC α activity and that Thr-495 may also be important.

Expression of PKC isozymes

The tissue distribution of the PKC isozymes has been determined mostly by Northern blot analyses and, more recently, by Western blotting using isozyme-specific antibodies. PKC-α, β_1, β_2, δ, ε, and ζ seem to be ubiquitously distributed (although α and ε seem not to be present in liver and β could not be detected in kidney, i.e. renal mesangial cells), e.g. in brain, lung, spleen, thymus and skin (246-249), whilst PKC-γ is exclusively found in the central nervous system, e.g. brain (246,247,250) and PKC-η is strongly expressed in epithelia of skin, digestive tract and respiratory tract and only slightly in brain and spleen (161,162) (see Table 1.2.2., p. 63). PKC-θ is predominantly expressed in skeletal muscle, thymus, lung and kidney and, to a clearly lower extent, in testes, skin and brain (251,461). It is also important to mention that all PKC isozymes known so far are expressed in brain tissue.

In a variety of different cell lines in culture, PKC-α, β_1, β_2, δ, ε, and ζ seem to be ubiquitous isozymes whilst PKC-γ is restricted to neuronal cell lines. Though both PKC-α and β are present in most cell lines investigated, in none of them are both missing. Amongst cells of the haematopoetic system, however, PKC-α is absent from myeloid cells of different origin (where PKC-δ is the predominant isozyme) (252), megakaryocytes (HEL) and macrophages (253,254), whilst PKC-β isozymes are absent from mouse and rat fibroblasts, renal mesangial cells, murine erythroleukaemia and neuroblastoma cells.

Highest levels of PKC-θ were found in all T-cell lines and many hematopoetic cell lines, however no transcripts were detected in several carcinoma, melanoma, schwanoma or astrocytoma cell lines (461,462). On the other hand, PKC-η was reported to be expressed in several carcinoma or keratinocyte cell lines (161,162).

The absence of PKC-ε appears to be a feature of cells from the myeloid (i.e. megakaryocytes, macrophages, platelets and the promyelocytic leukaemia cell line HL-60) but not erythroid lineage of the haematopoetic system.

Soon after the detection of the various PKC isozymes it became clear not

only that there are tissue-specific patterns of expression but that the amount and number of PKC isozymes varied within a given tissue depending on its developmental stage (reviewed in 246,249 and 255). This also indicates that a certain set of PKC isozymes is necessary to guarantee the ordered sequence of proliferation and differentiation events which leads to and maintains the characteristics of a given tissue. Due to the limited information on the gene and promoter structure, little is known about how the expression of PKC isozymes is regulated.

Since a true picture of PKC isozyme expression in different tissues and cell lines is rapidly emerging, it can be anticipated that the role of a given PKC isozyme in a distinct signalling pathway may become evident once it is possible to define a given cell type unequivocally by its PKC isozyme expression pattern and by its signal response potential (see Table 1.2.3., p. 65).

Table 1.2.2. PKC subspecies in mammalian tissues (modified from ref. 155).

PKC-isozyme	Amino acid residues	Molecular weight (calculated, daltons)	Tissue expression
α	672	76,799	Most tissues
β_1	671	76,790	Most tissues
β_2	673	76,933	Most tissues
γ	697	78,366	Brain only
δ	673	77,517	Most tissues
ε	737	83,474	Most tissues
η(L)	683	77,972	Lung, skin, Sk. muscle, heart
θ	707	81,571	Sk. muscle; T cells, thymus, kidney
ζ	592	67,740	Universal
λ	586	67,200	Ovary, testis

Intracellular distribution and translocation of PKC isozymes

Resting (unstimulated) cells of various origins have characteristic profiles of intracellular PKC isozyme distribution. Upon treatment of these cells with specific (e.g. growth factors, hormones or cytokines) or unspecific (e.g. serum, phorbol esters) stimuli, a redistribution of PKC isozymes can be observed. After prolonged treatment, the proteolytic degradation and downregulation (see below) could also be demonstrated. These processes could be demonstrated by analysis of the particulate (i.e. membrane) and soluble (i.e. cytosolic) fractions of the cells, by means of PKC activity measurement or immunoblotting. Immuno-cytofluorescence studies have provided very detailed information on the responsiveness of PKC isozymes to extracellular signals, showing redistribution of PKC isozymes to the membranes as well as to the cytoskeleton- known to contain a variety of good *in vitro* PKC substrates (256).

According to current opinion, translocation to cellular membranes has been regarded as an equivalent to the activation of the respective PKC isozyme. However in various cell types, significant portions of certain PKC isozymes are constitutively present in the particulate fraction, and it would be hard to believe that this indicates a permanent and persistent activation as has been proposed (257,258).

Recently, intracellular PKC receptors (RACKs) have been described that presumably contribute to the subcellular localisation of PKC (259). It is theoretically possible that these proteins could have specificity for PKC isozymes and thus participate in the dynamics of the subcellular distribution of PKC isozymes and response upon activation.

In most cell lines investigated, PKC-α seems to be located in the cytosol and is translocated to the cellular membrane and down-regulated upon TPA treatment (260-264). With more specific stimuli, differences in translocation can be observed. In GH_4C_1 pituitary gland cells, PKC-α is translocated to the membrane fraction, but not downregulated upon treatment with thyrotropin-releasing hormone (TRH) or TPA (265,209).

Table 1.2.3. Responses of PKC isozymes to physiological stimuli in various cell types (from ref. 153).

Cell type	Stimulus[a]	Second messenger[a]	PKC-α	PKC-β	PKC-δ	PKC-ε	PKC-ζ
IIC9 fibroblasts	α-Thrombin	DAG from PtdIns(4,5)P_2, Ca^{2+}; DAG from PtdCho	Rapid transient membrane association	NE[c]	NE	Rapid sustained membrane association	No response
	α-Thrombin	Nuclear DAG from PtdCho	Rapid nuclear translocation	NE	NE	No nuclear translocation	No nuclear translocation
	PDGF	DAG from PtdCho	No response	NE	NE	Slow membrane association	No response
GH4C1 pituitary cells	TRH (acute)	DAG from PtdIns(4,5)P_2, Ca^{2+}	Rapid membrane association	Rapid membrane association	Rapid membrane association	Rapid membrane association	ND
	TRH (6 h)	DAG (from PtdCho?)	No response	No response	No response	Downregulation	ND
Swiss 3T3 fibroblasts	Bombesin	DAG from PtdIns(4,5)P_2, Ca^{2+}; DAG from PtdOH	Induction after 30 h treatment	NE	Rapid membrane association followed by downregulation	Rapid membrane association followed by downregulation	No response
	PDGF	DAG from PtdIns(4,5)P_2, Ca^{2+}; DAG from PtdCho	No response	NE	Rapid membrane association followed by downregulation	Rapid membrane association followed by downregulation	No response
RBL-2H3 basophilic cells	Antigen – Ca^{2+}	ND[b]	No response	No response	Rapid membrane association	Rapid membrane association	No response
	Antigen + Ca^{2+}	ND	Rapid membrane association	Rapid membrane association	Rapid membrane association	Rapid membrane association	No response
Blood platelets	α-Thrombin + Ca^{2+}	DAG from PtdIns(4,5)P_2, Ca^{2+}; DAG (from PtdCho?)	Rapid sustained membrane association	Rapid sustained membrane association	No response	NE	Rapid sustained membrane association
	α-Thrombin – Ca^{2+}	DAG from PtdIns(4,5)P_2, Ca^{2+}	Rapid transient membrane association	Rapid transient membrane association	No response	NE	Rapid transient membrane association

[a]DAG, diacylglycerol; PtdOH, phosphatidic acid; PtdCho, phosphatidylcholine; PtdIns(4,5)P_2, phosphatidylinositol (4,5)-bisphosphate; PDGF, platelet-derived growth factor; TRH thyrotropin-releasing hormone.
[b]ND, not determined.
[c]NE, not expressed in this cell type.

In NIH 3T3 fibroblasts, PKC-α is reported to translocate to the nucleus upon TPA treatment (266). Similarly in Swiss 3T3 fibroblasts PKC-α seems to associate with the nuclear fraction upon treatment with insulin-like growth factor I, but with the cellular membrane when treated with bombesin (267). As reported recently, a PKC-α mutant lacking the regulatory domain was associated primarily with the nuclear envelope (220).

PKC-β also appears to be a cytosolic isozyme in unstimulated cells which is sensitive to downregulation by TPA (218,265,209,263,261,268,254). However, this was not found to be a general role, and it was reported recently (269) that PKC-β_2 was resistant to TPA treatment in a human colonic cell line. In GH_4C_1 cells, PKC-β_1 is downregulated by TPA, but not by TRH treatment (265,209). In rat liver and in HL-60 cells, a distinct portion of PKC-β is reported to be found in the nuclear fraction (270,271,264).

Depending on the cell type, PKC-δ seems to be differentially distributed within the cell. Whilst located in the cytosol in human platelets (253), in rat6 fibroblasts (260) and renal mesangial cells (272), the majority of PKC-δ was found associated with the particulate fraction. In the latter cell lines, the δ isozyme can be completely downregulated by TPA treatment. Also in rat brain and 3Y1 cells PKC-δ is found to be predominantly membrane associated (273,274) while when expressed in COS-1 cells, PKC-δ is distributed relatively equally between cytosolic and membrane fraction.

PKC-ε which is mostly cytosolic in GH_4C_1 pituitary (209), rat brain (276), rabbit brain(276) and human neuroblastoma cells (156) was found to be membrane-associated to a certain extent in U937 cells (277). Downregulation of ε isozyme upon cell stimulation was found to depend on the cell type and nature of stimulator (263,209,278).

PKC-η, has been reported to be present in the cell nucleus of several human tumour cell lines. TPA treatment of these cell lines did not lead to downregulation (279). In contrast to this observation, a recent report (460) indicates that in transiently expressing COS1 cells and constitutively expressing keratinocytes PKC-η is localized on the rough endoplasmic reticulum and is absent in the

nucleus. When overexpressed in NIH 3T3 fibroblasts, PKC-η was found to colocalize with Golgi apparatus and to relocalize to cell membrane and nuclear pores upon TPA treatment (454).

When PKC-θ was expressed in COS cells, the majority of the protein was found in the particulate fraction (251). However, in unstimulated Jurkat T cells PKC-θ was detected almost exclusively in the cytosolic compartment and stimulation with TPA caused rapid and complete cytosol-to-membrane translocation of PKC-θ (462).

In several cell lines investigated so far, PKC-ζ seems to be present as a cytosolic isoenzyme (260,261,272), only in HL-60 cells has it been reported to be mostly in the particulate fraction (198). Again, translocation and downregulation by TPA appears to be dependent on the cell type. Sensitivity towards TPA treatment that was observed in several cell lines (e.g. rat6 fibroblasts, 260 and human platelets, 261) seems quite contradictory to the fact that PKC-ζ neither binds nor can be activated by PEs (280,197,199). However, especially for the nPKC isozymes, mechanisms of activation and degradation must be postulated which might involve other cellular (maybe cell type-specific) components. Recently, membrane- and cytoskeleton-associated PKC-binding proteins have been reported which may serve to anchor or compartmentalize PKC isozymes to different intracellular membranes (281,282,264).

For the PKC λ(ι) a second member of the a-PKC group no data on intracellular localisation is available at the present.

PKC μ is the recently discovered member of the PKC family (332,333), and was found to be associated with the particulate fraction when expressed in HeLa cells. This was in agreement with the postulated role of the hydrophobic amino terminus as an integral membrane anchor (333). However, observed Mg^{2+} dependent subcellular localisation (333), suggests that additional factors may contribute to or determine intracellular localisation.

In addition, recent immunochemical investigation of intracellular localization of eight PKC isozymes overexpressed in NIH 3T3 fibroblasts (454) revealed that before activation the majority of the PKCs are not cell-membrane-

)ound and are diffusely distributed throughout the cytoplasm. In resting state PKCs α, βI, γ and ζ were found to have pancytoplasmic distribution, PKC βII was found perinuclearly, PKC ε had punctate cytoplasmic distribution (see however 455) and PKC δ and η were found associated with the Golgi network. Stimulation of NIH 3T3 cells with 100 nM TPA resulted in rapid and selective redistribution of the different PKC isozymes (with the exception of PKC ζ) to distinct subcellular structures. Within minutes after TPA stimulation PKC α associates with ER, cell membrane and focal adhesions; βI translocates to cell membrane; PKC βII associates with microfilaments and cell membranes; γ accumulates in Golgi organelles and cell membranes; PKC δ and ε associate with perinuclear membranes, cell membranes and cell junctures; and PKC η translocates to Golgi, cell membrane and nuclear pores.

From above discussion, it is obvious that a clear picture of the distribution, translocation and degradation does not exist at the present. However, a connection of the above findings with data on PKC-mediated cellular events may bring us closer towards connecting PKC localisation and migration with function.

PKC - role in signal transduction mechanisms

Signal transduction mechanisms represent a highly organised and complex set of mechanisms that regulates or modulates cell functions including gene expression in response to different extracellular signals (e.g. hormones, growth factors or neurotransmitters), resulting ultimately in cell proliferation, differentiation or secretion (Figure 1.2.6., p. 70) (283,284). It is therefore not surprising, that most if not all known proto-oncogenes have turned out to code for proteins involved in signal transduction mechanisms (285).

Most of the components of signal transduction pathways are proteins whose activity is altered by either ligand or second messenger binding, by covalent modifications and by subsequent changes in conformation or subunit number. The majority of covalent modifications observed are phosphorylations on tyrosine or serine/threonine residues, and both Tyr- or Ser/Thr-specific kinases and their protein substrates (being sometimes protein kinases themselves) are found to be components of a signal transduction pathways.

PKC as a phospholipid dependent, Ser/Thr-protein kinase family is known to play one of the central roles in signal transduction mechanisms.

As explained above PKC is thought to be activated *in vivo* under physiological conditions, by increased amounts of DAG in cellular membranes that result from agonist-induced hydrolysis of inositol phospholipids (Figure 1.2.7., p. 71) (155). This was once thought to be the sole mechanism to produce the DAG that links extracellular signals to intracellular events through activation of protein kinase C. However, it is becoming clear that agonist-induced hydrolysis of other membrane phospholipids, particularly choline phospholipids, *via* phospholipase D and phospholipase A_2 may also take part in cell signalling. The products of hydrolysis of these phospholipids may enhance and prolong the activation of PKC. Such prolonged activation of PKC is essential for long-term cellular responses such as cell proliferation and differentiation.

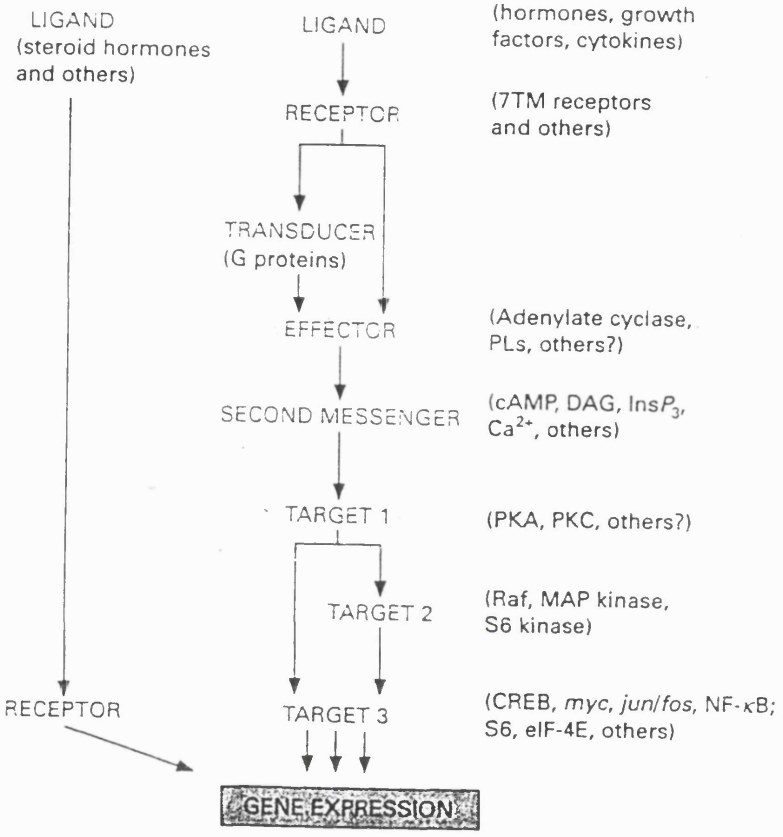


Figure 1.2.6. Schematic overview of the signal transduction pathways (from ref. 156).

Although arachidonic acid regulates many physiological processes through its conversion to various eicosanoids, several *cis* unsaturated fatty acids and lysophosphatidylcholine, which are the primary products of PC hydrolysis catalyzed by PLA_2, potentiate PKC activation and thereby may contribute to signal transduction through the PKC pathway (155 and references therein).

Recently it has been shown that other components of glycerolipid metabolism can be activators of PKC, at least *in vitro* (219,286). Cardiolipin is able to activate PKC-α, β_2 and –ε (156 and references therein). cis-unsaturated fatty acids were found to activate PKC–ε and –ζ (274,200) but not the PKC–δ isozyme (275). Arachidonic acid (274) and even better lipoxin A (146), another lipoxygenase metabolite, are capable of activating PKC–β_1, –γ and –ε, even in a Ca^{2+} independent fashion. PKC–α can be activated by arachidonic acid only in the presence of Ca^{2+}, whilst PKC–β_2 and δ do not respond at all (287,288).

Surprisingly, PtdIns (4,5)P_2 can substitute for DAG as an activator of PKC–α, –β_2 and –γ (289,290,219), but not of β_1 (289), and PtdIns can replace PS as a cofactor of activation, at least for PKC–α and β_2 (219).

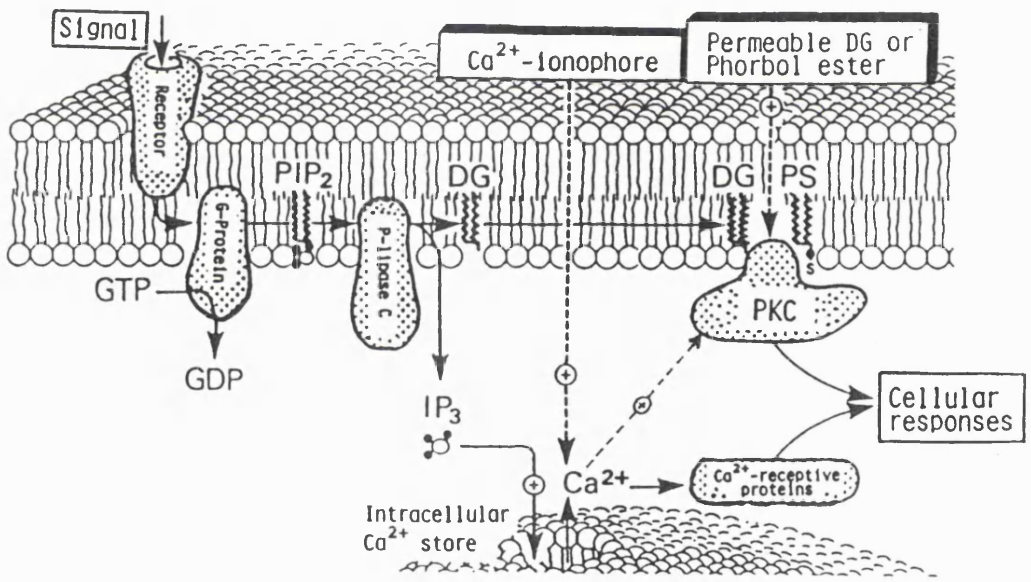


Figure 1.2.7. Schematic representation of postulated physiological PKC activation mechanism. Mode of action of non-physiological agents (Ca^{2+} ionophore, PEs and permeable DG) is also suggested (from ref. 71).

There is evidence that several PKCs (among them PKC-ζ) can be stimulated by phosphatidylinositol (3,4,5)-trisphosphate (291,292), indicating that cell

signalling pathways that involve activation of phosphatidylinositol 3-kinase (PI 3-kinase) might be involved in activation of some PKC isozymes. These findings, together with observations that DAG can be generated in an inositol lipid-independent way, e.g. from PC (293,294), may indicate that some if not all PKC isozymes may be activated by different second messengers in a distinct way.

Different cellular mechanisms that control intracellular Ca^{2+} levels could also influence activation status of at least c-PKC isozymes. In most stimulated cells, the concentration of Ca^{2+} increases transiently, whereas physiological responses persist long after the Ca^{2+} concentration returns to basal levels. On the other hand, activation of cell surface receptors often results in the generation of regular oscillations in the intracellular Ca^{2+} concentration (295). It has been suggested that Ca^{2+} mobilisation and PKC activation act synergistically to cause a variety of cellular responses, but the biochemical mechanism of this synergism is not fully understood (296). In the presence of high Ca^{2+} concentrations, activation of PKC requires less phospholipid degradation, whereas in the presence of intense phospholipid degradation, less Ca^{2+} concentration is needed to activate the enzyme. On the other hand PKC may participate in the generation of Ca^{2+} oscillations by exerting negative feedback control over agonist-induced inositol phospholipid hydrolysis and setting a new cycle of production of IP_3 (297). This hypothesis is primarily based on the observation that Ca^{2+} oscillations are inhibited by PEs, and this inhibition is prevented by PKC inhibitors (156). A consequence of this proposed mechanism is that the activities of phospholipases and PKC and the amounts of DAG and IP_3 must also be oscillating with a rate or pattern similar to that of Ca^{2+} (indeed, in thrombin-stimulated human platelets, the amount of DAG does oscillate, 298).

Members of the PKC family probably respond differently to various combinations of Ca^{2+}, PS, DAG and other phospholipid degradation products *in vivo*. Thus, the patterns of activation of the enzymes may vary in extent, duration and intracellular localisation. Detailed spatio-temporal aspects of phospholipid degradation and activation of PKC isozymes within the cell are still poorly understood. However, it is clear that the different isozymes are, to some extent, present in different places and potentially activated by different factors. On the

other hand, there is as yet no definitive proof of particular PKC isozymes being activated in a specific location leading to the phosphorylation of a defined substrate. This may largely be due to our limited knowledge of well defined substrates, in particular of those that are specific for individual PKC isozymes.

Physiological substrates of PKC isozymes

In vivo studies by [^{32}P]orthophosphate labelling of cells treated with TPA or other stimuli involving PKC signalling pathways have revealed such a large number of putative physiological substrates (reviewed in 299) that it is rather difficult to assess their significance in PKC-mediated cellular events. They can be arbitrarily divided into three major classes: 1) proteins involved in signal transduction and PKC activation (e.g. the EGF, T cell insulin receptors, Ras and GAP), 2) proteins involved in metabolic pathways (channels, pumps) and 3) proteins involved in regulatory function concerning gene expression (transcription factors, translation factors, reviewed in 300). It should be noted that amongst PKC substrates there might be several other protein kinases like S6 kinase or the Raf kinase.

Three prominent substrates, most likely to be involved in the control of cell functions mediated by PKC are: myristoylated alanine rich C kinase substrate (MARCKS), DNA topoisomerase I (301) and lamin B (302,303), although little is known about specificity of PKC isozymes towards them. MARCKS is phosphorylated by PKC under several conditions such as macrophage activation or growth-factor-dependent mitogenesis. Its phosphorylation leads to a redistribution of MARCKS from the actin filaments of the membrane to the cytoplasm (304 and references therein) and a reduction in its binding affinity to Calmodulin (CaM)(305). MARCKS belongs to a group of CaM binding proteins that binds CaM in the presence of Ca^{2+}, and has been used widely as a marker or index of PKC activation in intact cells (306,307). MARCKS protein also appears to have a role in reversibly linking the actin cytoskeleton to the plasma membrane (308).

DNA topoisomerase I and lamin B are nuclear proteins and thought to be involved in the control of DNA synthesis and are thought to be substrates after

translocation of activated PKC isozymes to the cell nucleus.

For some substrates, differential phosphorylation *in vitro* by different PKC isozymes has been reported. Perhaps the clearest example so far is provided by glycogen synthase kinase-3β (GSK-3β), a kinase that phosphorylates c-Jun (among other substrates) (309). There is a striking rank order of ease by which different PKC isozymes phosphorylate GSK-3β, with PKC-α, β_2 and γ being roughly equipotent and significantly more potent than PKC-β_1. The difference between PKC-β_1 and β_2 is particularly intriguing in view of the fact that these proteins differ only in their carboxy-terminal 50 amino acids, inferring that substrate selectivity of these PKC isozymes is influenced by this region of the catalytic domain, in addition to the pseudosubstrate site and catalytic site recognition determinants. Additional examples of polypeptides phosphorylated to varying extents by different PKC isozymes include the neuronal PKC substrate GAP-43 (also termed neuromodulin, B-50, F-1, CaM binding protein) where phosphorylation follows the order: PKC-β_1>β_2>α>γ, (310), the epidermal growth factor receptor (PKC-α>β>γ, 311) and the vitamin D receptor (PKC-β>α≈γ, 312). Neuromodulin is a CNS-specific phosphoprotein that appears to play a role in neuronal growth, synaptic plasticity, neurotransmitter release and phosphoinositide metabolism. (313). Unfortunately detailed kinetics of the various PKC isozymes for these substrates are lacking, as are data on the relative potency of active catalytic-domain fragments towards them. Thus it is not possible to evaluate the present data in the light of the model for PKC activation presented above.

Neurogranin (RC3) is a recently discovered CNS-specific PKC substrate (314), and it is shown not to be phosphorylated *in vitro* by any other protein kinases (315). The expression of neurogranin in developing rat brain parallels that of PKC-γ and both of these proteins are concentrated in the cell bodies and dendrites of cortical and hippocampal neurons (314).

Many cytoskeletal proteins are PKC substrates, like vinculin (316), filamin (317), profilin (340), desmin (318), troponin T, troponin I, C-protein associated with cardiac myofibrils (319), dystrophin (452) and the previously mentioned MARCKS (304). PKC appears to be intimately involved in regulation of the cytoskeletal

function. c-PKCs, have been found to associate with the cytoskeleton (316,320) and the myofilament (321). PKC-α in rat embryo fibroblasts is associated with focal contacts of the cytoskeleton and co-localizes with vinculin and talin (316) and activation of PKC by TPA treatment of REF cells results in actin filament depolymerisation and reorganisation of vinculin (316). Thyrotropin-releasing hormone (TRH) treatment of GH_4C_1 cells results in translocation of PKC-α, β, δ, and ε to the cytoskeletal fraction (256). Stimulation of heart cells with α-adrenergic agonist results in the association of PKC with myofibrils (322).

Protein kinases phosphorylate a number of transcription factors including c-jun, c-fos, CREB, SRF, SP1, Oct2, Myb, Max, myogenein, HSF, Gal-4, ADR1, C/EBP α and β, and vitamin D receptor (for reviews see 323-325). Since various PKC isozymes have been found in and can translocate to the nucleus depending on cell type, and because PKC activation is associated with altered gene expression it can be expected that PKC phosphorylates some transcription factors and in that way regulates gene expression. It has been found that PKC phosphorylates CREB and C/EBP α (324,326) and it was predicted that GCN4, CPC-1, HBP-1, TGA-1, Opague 2 and v-jun (but not c-jun) should be PKC substrates (324). Transcription factor, NF-κB, in the resting cell, is predominantly cytosolic as a complex with inhibitory protein, I-κBα. On activation of PKC by TPA, I-kBα becomes phosphorylated (probably by an indirect mechanism), the complex dissociates, and NF-κB translocates into the nucleus as an active transcription factor (327). NF-κB is a transcriptional factor that regulates a variety of genes, including immune-related and viral genes. A recent report (453) suggests that PKC ε (but not α, δ or θ) isozyme is responsible for NF-κB activation in rat 3Y1 fibroblasts. Additionally, PKC-ζ, has been reported to bind and stimulate a putative 50 kD I–κB kinase which phosphorylates and inactivates I-κB α *in vitro* (463,464). c-jun, a TPA-responsive transcription factor, is also regulated by PKC (328). PKC-β was found to phosphorylate the vitamin D_3 receptor (VDR), a member of the steroid/thyroid hormone receptor superfamily (329) and TPA treatment of Swiss-3T3 cells resulted in a dramatic decrease in both VDR mRNA and protein levels, while substantially stimulating proliferation (330).

The ribosomal protein S6 (of the 40 S subunit) as well as the initiation factors eIF-3, eIF-4E, eIF-4B and eIF-4F (the mRNA cap binding complex) have been reported to be phosphorylated in cells treated with insulin or PEs (467). Whilst S6 phosphorylation is exerted by a S6 kinase which seems to be downstream of PKC in a protein kinase cascade (468), at least eIF-4F and -4E appear to be direct substrates of PKC (469).

Biological action of PKC

Since the discovery of phospholipid dependent Ser/Thr protein kinase, PKC, more than fifteen years ago by Nishizuka and co-workers (150), and its identification as a major receptor for phorbol esters (18,22), a multitude of physiological and pathological signaling mechanisms have been ascribed to PKC. Since the accumulated evidence and literature of involvement of PKC in most of cellular functions is vast, and is out of the scope of this review, biological roles of PKC will be described only in general. The reader is refered to a recent book and references cited there (338) for comprehensive reviews on biological functions of PKC.

One of the crucial roles of PKC is its role in transcriptional activation, cell transformation, differentiation and control of cell death (including the control of cell selection by apoptosis) (339). Although involvement of PKC isozymes in these processes was and is extensively studied, detailed mechanisms are still not known.

One of the most widely investigated biological roles of PKC is undoubtedly its role in nervous system. Increasing evidence suggests that PKC plays a key role in neuronal growth and differentiation, in regulation of neurotransmitter release, various forms of synaptic plasticity (including long-term potentiation of synaptic efficacy, long-term depression of synaptic transmission, facilitation of

neurotransmitter release, altered synaptic coupling and kindling-induced epileptogenesis), ligand-regulated ion channels (e.g. nicotinic acetylcholine, $GABA_A$ NMDA receptors), voltage-dependent Ca^{2+}, K^+, Na^+ and Cl^- channels (reviewed in 336).

The role of PKC in mediating the response to extracellular stimuli was first demonstrated in studies of serotonin secretion from platelets and was then found in many other secretory systems (reviewed in 155,335). This effect was one of the initial indications of the importance of the enzyme in signaling pathways. PKC activation can alter secretory responses by affecting the intracellular secretory pathway or by modulating extracellular signaling through changes in function of plasma membrane receptors and ion channels (335 and references therein). There is compelling evidence that the activation of PKC results in enhanced secretory response because of alterations in the intracellular pathway of regulated exocytosis. Despite a wealth of information concerning the effects of PKC activation on secretion, the substrates responsible for these effects are uncertain. The major limitation is the lack of understanding of the biochemical machinery involved in exocytosis. Effects of PKC on secretion may vary with PKC isozyme (335).

Additionally, PKC is thought to play important roles in the control of smooth-muscle functions (reviewed in 337), and although activation of PKC is postulated to be involved in smooth-muscle contraction, the role of this kinase is controversial and the precise mechanism is still obscure.

Existence of a number of PKC isozymes, cell-type specific isozyme profile and a complexity of mechanisms (cofactor-activator dependancy, substrate specificity, intracellular localisation and phosphorylation) involved in regulation of PKC activity, could explain the multitude of roles that PKC plays in the regulation of a number of cellular functions in different cell types.

PEs were found to interfere with same biological processes, with structure-dependent differences in their effects and potency (see Section 1.1.). Differences

in PE interaction with characteristic intracellular pool of PKC isozymes or interference with a complex PKC regulatory mechanisms could provide a possible explanation for differences in their *in vivo* effects.

Therefore, in attempt to establish a correlation between different biological effects of PEs and their interaction with individual PKC isozymes, we decided to investigate different aspects (binding, activation, translocation) of PKC-PE interaction. This could, in addition, provide more information about the role of individual PKC isozymes in specific biological processes.

Additionally, metabolic transformation of some deoxyPEs was studied, as a process that may influence the effects of PEs *in vivo*.

CHAPTER 2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1.Chemicals

From Sigma Chemical Co., Poole, UK:

Hydrochloric acid (HCl)
Potassium hydroxide (KOH)
Sodium hydroxide (NaOH)
Trichloracetic acid (TCA)
Dithiothreitol (DTT)
β-Mercaptoethanol
Hydrogen peroxide
Phenylmethylsulphonylfluoride (PMSF)
Histone IIIS ("Type 1")
Leupeptin
sn-1,2-Dioleoylglycerol
Ethylene diamine tetraacetic acid (EDTA)
Ethyleneglycol bis (b-aminoethyl ether)-N,N,N',N'-tetra acetic acid
N-2-Hydroxyethylpiperazine-N-ethane sulphonic acid (HEPES)
Trypan blue stain
Brilliant blue G (coomasie blue)
Trisma (TRIS)
Glycerol
Phosphate buffered saline (PBS)
Adenosine triphosphate (ATP)
Bovine serum albumine (BSA)
Bromophenol blue

From Merck-BDH, UK:

Sucrose
Acetic acid
Ethanol
Methanol
Chloroform
Diethyl ether
Acetone
Sulphuric acid
Magnesium chloride ($MgCl_2$)
Calcium chloride ($CaCl_2$)
Triton X-100
Tween-20
Butanol
Glycine
Sodium chloride (NaCl)
Hydrochloric acid
Potassium dihydrogen orthophosphate (KH_2PO_4)
Sodium dihydrogen orthophosphate (NaH_2PO_4)
Dipotassium hydrogen orthophosphate (K_2HPO_4)
Disodium orthophosphate (Na_2HPO_4)

From Romil Chemicals Ltd., Shepshed, U.K.: HPLC grade solvents (methanol, chloroform, acetone, hexane and cyclohexane)

From Merck, Darmstat, Germany: 0.25 mm, aluminium-backed 60 F_{254} silica gel plates

From Whatman: Glass microfibre filters (2.5 cm GF/C) Cation exchange chromatography paper (p81) Diethylaminoethyl cellulose (DEAE), DE52, (anion exchange)

From Millipore, Herts, U.K.: Prefilters AP25

From Spectrum, Houston, Texas, USA: Spectra/Por^{r} 4, regenerated cellulose membrane with MWCO of 12,000-14,000

From Gibco BRL, Paisley, UK: Gentamycin
L-Glutamine
Foetal bovine serum (FBS)

From ICN Biomedicals, Oxfordshire, U.K.:

RPMI 1640 culture medium
Adenosine-5-[γP^{32}]-triethylammonium salt (^{32}P-γ-ATP at 4500 Ci/mmol).

From New England Nuclear:

[^{3}H]-Thymidine (1mCi/ml)

From Du Pont UK Ltd.,:

Phorbol-12,13-dibutyrate, [20-^{3}H(N)], (20-^{3}H(N)-PdBu at 20.7 Ci/mmol).

From Lipid Products, South Nutfield, Surrey, U.K.:

L-α-Phosphatidylserine (PS), chloroform solution (20 mg/ml) stabilised with 3% methanol.

All reagents were of "Analar" grade, except solvents for HPLC which were of HPLC purity grade, and were handled according to the suppliers recommendations.

Other reagents:

PKC pseudosubstrate site based (PSS) peptides were generously donated by Prof. Peter Parker, Protein phosphorylation Laboratory, Imperial Cancer Research Fund, London. The sequence and molecular weights of PSS peptides are given in the table below.

PKC PSS	molecular weight	sequence
α	1959.36	DVANRFARKGSLRQKNV
δ	1401.80	MNRRGSIKQAKI
ε	2067.60	ERMRPRKRQGSVRRRV
η	1666.11	RKRQRSMRRRVH
ζ	1542.10	IYRRGSRRLRKL

Phorbol Esters:

The phorbol esters used (exept PdBu and TPA) were isolated and purified from plant sources in the Department of Pharmacognosy, The School of Pharmacy.

DOPP = 12-deoxyphorbol-13-phenylacetate, 13-O-Phenylmethyl-4β,9α,13α,20-tetrahydroxy-1,6-tigliadien-3-one ($C_{28}H_{34}O_6$, Mw = 466.58)
DOPPA = 12-deoxyphorbol-13-phenylacetate-20-acetate, 20-Acetyl-13-O-phenylmethyl-4β,9α,13α,20-tetrahydroxy-1,6-tigliadien-3-one ($C_{30}H_{36}O_7$, Mw = 508.62)
Sap A = Sapintoxin A, 13-Acetyl-12-(2-methylaminobenzoyl)-9α,12β,13α,20-tetrahydroxy-1,6-tigliadien-3-one ($C_{30}H_{39}NO_7$, Mw = 511.634)
Thy A = Thymeleatoxin A, 12-(3-Phenyl-2E-propanoyl)-12β-hydroxydaphnetoxin ($C_{36}H_{36}O_{10}$, Mw = 628.68)
Mezerein = 12-(5-Phenyl-2E,4E-pentadienoyl)-12β-hydroxydaphnetoxin ($C_{38}H_{38}O_{10}$, Mw = 654.712)
Rx = Resiniferatoxin, (9,13,14,-orthophenylacetylresiniferonol-20-homovanilate ($C_{37}H_{40}O_9$, Mw = 628.718)
PdBu = phorbol-12,13-dibutyrate, 12,13-dibutanoyl-4β,9α,12β,13α,20-pentahydroxy-1,6-tigliadien-3-one ($C_{28}H_{40}O_8$, Mw = 504.63) was purchased from Sigma.
TPA = 12-O-tetradecanoylphorbol-13-acetate, 13-Acetyl-12-tetradecanoyl-4β,9α,12β,13α,20-pentahydroxy-1,6-tigliadien-3-one ($C_{36}H_{56}O_8$, Mw = 616.834) was purchased from Sigma.

All phorbol esters were stored as acetone solutions, and kept in the dark at - 20^0 C.

2.1.2. Plasticware

For measurement and cell culture work plasticware was supplieed by: Sterilin, Eppendorf, Beckman, Gilson, BCL, Bacton Dickinson, Flow, Packard, Nalgene and Nunclon.

2.1.3. Apparatus

General

pH meter - Pye Unicam PW9418

Vortex - Rotamixer

Bath sonicator - Ultrasonics Ltd. type 6442AE

Filtering - A Millipore 12-well-manifold, using glass fibre filters was used for redioactive ligand-PKC complex recovery and cell harvesting.
For ultracleaning of HPLC samples, a Millipore OM110 filtering unit (13 mm) was used.

Centrifuge - Techno Spin R and Microspin245 from Sorval Instruments, Du Pont, U. K.; TL-100 ultracentrifuge, Beckman, USA

Radioactivity measuring - Beckman LS 6000IC counter

Cell Work

Sterile cabinet - Microflow Class 2, biological safety cabinet, M.D.H., Inter Med, U K.

CO_2 cabinet - Water Jacketed Incubator, Forma Scientific, U K

Cell counting - A hemocytometer (Weber Scientific International Ltd.) was used to count cells and to monitor trypan blue exclusion using microscope inspection.

Chromatography:

FPLC - Protein chromatography was conducted on a Fast Protein Liquid Chromatography (FPLC) system (LCC 500 plus, Frac-100 fraction collector, Pharmacia, Uppsala, Sweden) using different prepacked (1 ml, HiTrap Heparin and 1 ml, HiTrap Q, Pharmacia, Uppsala, Sweden) or hand-packed (DE52) columns. FPLC was conducted at 4^0 C.

HPLC - High performance liquid chromatography (HPLC) of phorbol esters was conducted on a system composed of: two Altex 110 A pumps controlled by an Altex 421 Controller (Altex, Berkeley, CA, U.S.A.) and Waters 991 Photodiode-array detector (Millipore, U.K.). For analytical HPLC an Apex Apex Silica column (4.6 mm x 25 cm, 5 μm particle size, Jones Chromatography, U.K.) was used. Semi-Preparative HPLC was performed on an Apex Prepsil column (10 mm x 25 cm, 8 μm particle size, Jones Chromatography, U.K.).

TLC - TLC plates (0.25 mm, aluminium-backed 60 F_{254} silica gel plates, Merck, Darmstat, Germany)

2.2. METHODS

2.2.1. HL 60 cell culturing

Human promyelocytic leukemia HL-60 cells (ICRF - London) were grown in RPMI 1640 medium supplemented with 2 mM glutamine and 10 % heat inactivated fetal calf serum (L-glutamine was added befor use and foetal calf serum was heat inactivated at 56 ^{0}C for 30 minutes before addition) in a 5 % CO_2 humidified atmosphere at 37 ^{0}C.

Cells were maintained at a concentration of between 5 and 10 x 10^5 cells/ml under which conditions viability was > 90 % (as assessed by trypan blue exclusion) and doubling time was between 36 and 48 hours.

2.2.2. Analytical and Preparative HPLC of phorbol esters

The HPLC system used consisted of: two Altex 110 A pumps controlled by an Altex 421 Controller (Altex, Berkeley, CA, U.S.A.) and Waters 991 Photodiode-array detector (PDA)(Millipore, U.K.). For analytical HPLC an Apex Apex Silica column (4.6 mm x 25 cm, 5 μm particle size, Jones Chromatography, U.K.) was used. Semi-Preparative HPLC was performed on an Apex Prepsil column (10 mm x 25 cm, 8 μm particle size, Jones Chromatography, U.K.). All PEs were injected as solutions in $CHCl_3$. Isocratic elution was carried out with the mobile phase $CHCl_3$/MeOH (99:1) at the flowrate 1 ml/min. These conditions were found to be optimal using a range of solvent systems and an analytical HPLC column. All solvents were of HPLC grade (Romil Chemicals Ltd. U.K.) and were degassed by sonication prior to use. Absorbances between 230 and 400 nm were detected and data were processed using PDA software (version 6.22.). The use of PDA allowed us to record UV spectra of analysed compounds during chromatography and to evaluate the sample purity. Photodiode-array shares many elements with conventional UV/VIS detectors but the essential difference is that it can record the entire spectral range (190-800 nm) during analysis enabling spectral and chromatographic profiles to be recorded simultaneously. Post-run analyses, using new algorithms, provide extremely sophisticated methods of visualizing data (e.g. three-dimensional topographical plots Figure 3.5.4. A.-B.; and contour plot Figure 3.5.4. C. in Section 3.5.) enabling quick analysis and judgement of eluant purity. Spectral library features enables comparison of experimental spectra with standards, whilst integration and hence quantitation can be achieved once peak areas have been calibrated with standards.

2.2.3.Purification of PKC isotypes

PKC isotypes α, β_1, β_2 , γ, δ, ε, and ζ were expressed in Sf9 insect cells as previously described (26). For purification of individual isotypes a cell pellet (~10^8 cells) was resuspended in ice-cold homogenisation buffer (20 mM Tris-HCl, pH 8 , 5 mM EGTA, 10 mM benzamidine, 0.3% (v/v) β-mercaptoethanol, 1% (v/v) Triton X-100, 50 mM phenyl-methyl sulphonyl fluoride and 100 μg/ml leupeptin), and

disrupted by 3 cycles of freeze/ thaw in liquid nitrogen and all further procedures conducted at 4^0C. The homogenate was centrifuged at 40000 x g for 15 mins and the supernatant was diluted 3-fold with homogenisation buffer before being loaded onto a DE 52 column, previously equilibrated in elution buffer and linked to an FPLC system (Pharmacia, Uppsala, Sweden). Active fractions (see assay) were eluted from this column with a linear gradient of 0 - 1M NaCl in elution buffer (20 mM Tris-HCL, pH 7.5, 2 mM EDTA, 10 mM benzamidine, 0.3% (v/v) β-mercaptoethanol, 0.02% (v/v) Triton X-100). Pooled activity was applied successively to a Hi-Trap Heparin column and then a Hi-Trap Q column using the same gradient of NaCl in elution buffer. Active fractions were pooled and stored at - 20 ^{0}C after being dialysed for 4 hours against: 50 % (v/v) glycerol, 20 mM Tris-HCL, pH 7.5, 1.25 mM EDTA, 10 mM benzamidine and 1 mM dithiothreitol at 4 ^{0}C.

2.2.4. PKC assay

PKC activity was assayed by measuring the incorporation of ^{32}P from ^{32}P-γ-ATP into substrate as previously described (14). The substrates used were salmon protamine sulphate, calf thymus Histone IIIs (Sigma), myelin basic protein (MBP) and PKC δ, ε, η and ζ pseudosubstrate site-based peptides as in Olivier *et al* (163) (synthesised by the peptide synthesis laboratory at ICRF, London). PKC isotypes were diluted prior to use in 20 mM Tris-HCL, pH 7.5, 1.25 mM EDTA, 10 mM benzamidine and 1 mM dithiothreitol before use. Once diluted, enzyme was kept on ice and added just prior to initiation, being utilised invariably within 1 hour. Before use, each PE was investigated for its purity and weighted accurately. Purity of PEs used was investigated by analytical normal phase HPLC coupled with photodiode array detector (see section 2.4.2.). Samples containing impurities were cleaned using the same HPLC methodology on a preparative scale.

The assay consisted of:

1. Substrate/cofactor mix (mixture of substrate, Mg^{2+}/Ca^{2+} and micelle mix in the ratio 1:1:1):

- Substrate: 5mg/ml (Histone, MBP or Protamine sulphate) or 0.5 mM (pseudosubstrate site based peptides) in 200 mM Hepes, pH=7.5, 2.5 mM EGTA.
- Mg^{2+}/Ca^{2+}: 50 mM $MgCl_2$ ± 3.75 mM $CaCl_2$ in H_2O

2. Micelle mix:

- Phosphatidylserine 20mg/ml in $CHCl_3$/MeOH (95:5), phorbol esters, dioleoyl glycerol (concentration varies), dried under stream of nitrogen and resuspended in micelle buffer (2 mM Hepes, pH=7.5, 1 % v/v Triton X-100) by vortexing.

3. ATP mix:

- 500 μM ATP in H_2O
- 100,000 cpm/μl ^{32}P-γ-ATP

The assay was prepared by mixing:
- 15 μl of substrate/cofactor mix
- 5 μl of enzyme

The assay was started by mixing in 5 μl of ATP mix (for final concentrations of mixture components see Table 1.). Alternatively, when enzyme activity was low, the assay was scaled up (using the same ratio of substrate/cofactor mix : enzyme : ATP mix of 3:1:1).

Phosphate incorporation was measured over a 6 minute period on 35 ^0C, which was within the linear region of the time course (data not shown). Unit of PKC activity represented the amount of enzyme that incorporated 1 nmol of ^{32}P per minute.

For termination of the assay 15 μl aliquots were spotted onto a cation exchanger paper (Whatman P81) and washed with 30 % acetic acid (3x10 minute washes). Wet papers were then counted for radioactivity (see below).

Table 2.1. Final concentrations of reagents used in PKC activition assays

Reagent	Final Concentration
Buffer (Hepes)	40 mM
ATP	100 μM
^{32}P-γ-ATP	~ 200 cpm/pmol ATP
$MgCl_2$	10 mM
$CaCl_2$ (free Ca^{2+})*	0.75 mM (100 μM)*
EGTA	0.5 mM
EDTA	0.25 mM
Substrate	1mg/ml**, 100 μM***
DTT	0.2 mM
Triton X-100	2 mg/ml (70 mol %)
PS	1 mg/ml (30 mol %)
Phorbol esters	2-2000 nM (0.00006-0.06 mol%)
Final Volume	25 μl

* Where present in the assay , free Ca^{2+} concentration was calculated using "Chelate" software

** Histone IIIs and Protamine sulphate

*** **Pseudosubstrate site peptides**

2.2.5. Radioactivity measuring

For [^{32}P] counting, wet filters or ion exchange strips were placed in plastic vials and counted with the open window method for Cerenkov radiation in a Beckman LS 6000IC counter.

For [^{3}H]PdBu counting, wet filters with radioactive material on them were placed in plastic vials and 4ml of liquid scintillant was added and vials vigorously vortexed. Vials were then counted for liquid scintillation for 2 minutes in a Beckman LS 6000IC counter.

2.2.6. HL-60 cell membrane preparation

Cells were typically harvested at a concentration of 10^6 cells/ml by centrifugation (2000 rpm for 5 minutes), washed in buffer A (20 mM Hepes pH 6.9, 3 mM EGTA, 2mM $MgCl_2$, 20 mM NaCl, 100 mM KCl, 100 μg/ml leupeptin, 50 μg/ml PMSF and 1 mM DTT) at room temperature. Cells were finally resuspended in buffer A (2-5 x 10^8 cells/ml) and lysed by 3 repetitive freeze/thaw cycles in liquid nitrogen. Unbroken cells and nuclei were pelleted by centrifugation (2000 rpm for 10 minutes). The resulting supernatant (cell free, as judged by light microscopy) was subjected to further centrifugation (10,000 x g for 5 minutes) on a benchtop eppendorf - test tube centrifuge (Microspin245, Sorval Instruments, Du Pont) and the pellet resuspended in buffer A (2 mg/ml of membrane proteins). Aliquots (1 ml) of membrane suspension were "snap frozen" in liquid nitrogen and kept at - 20 ^{0}C.

Further techniques are given in each chapter.

CHAPTER 3. EXPERIMENTAL

3.1.Characterisation of [^{3}H]PdBu binding to PKC isotypes α, β_1, β_2, γ, δ, ε and ζ

3.1.1. Introduction

It is thought that phorbol esters exert their different biological effects through interaction with members of the protein kinase C family (13,14,17).

Recent results from our laboratories indicate substantial differences in interaction between different phorbol esters and PKC isotypes *in vitro* (347,348). In addition it is known that the ability of phorbol esters to induce different biological effects is strongly related to their structure (349,350). Selective use of different phorbol esters as probes could therefore be useful for identifying the pathways in which separate PKC isotypes are involved (351,352).

In continuation of studies on the ability of a range of phorbol esters to activate different PKC isotypes *in vitro* (347), it was important to examine the binding affinity of different phorbol esters to pure recombinant PKC isotypes α, β_1, β_2, γ, δ, ε and ζ *in vitro*.

3.1.2. Experimental procedures

Purification of PKC isotypes

PKC isotypes α, β_1, β_2 , γ, δ, ε, and ζ were expressed in Sf9 insect cells as previously described (355), and purified as described in Section 2.2.3. A typical chromatographic profile for a MonoQ purification step is shown on Figure 3.1.1.,]p. 92. Extracts of "vector only" transfected Sf9 cells showed no contaminating PKC activity (data not shown).

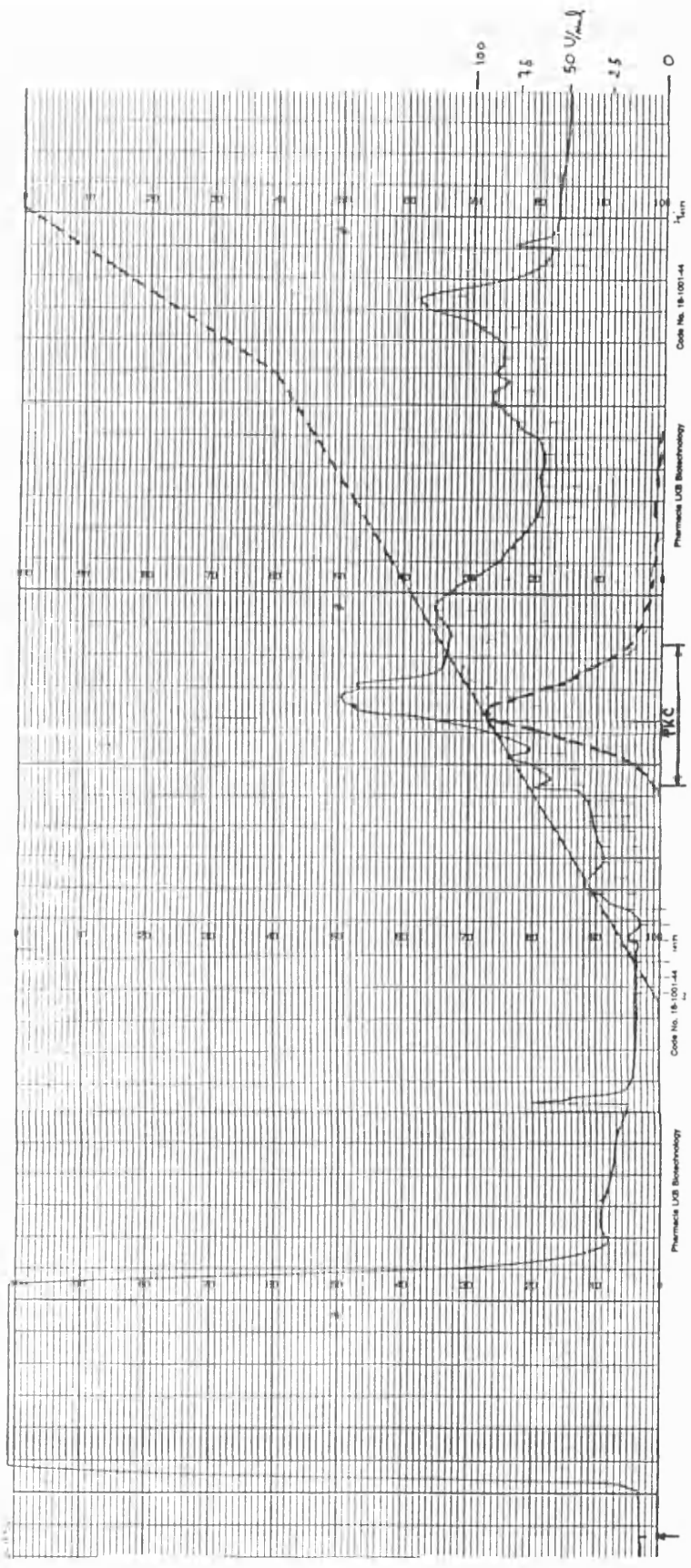


Figure 3.1.1. Representative example of FPLC - MonoQ chromatographic profile. Example of 280 nm absorbance (——) chart recording for MonoQ FPLC of PKC-γ isozyme. Active fractions from Hi-Trap Heparin column (see Chapter 2.4.3.) were pooled and applied to a MonoQ column using a linear gradient of 0 - 1M NaCl (----) in elution buffer (Chapter 2.4.3.). (↑) marks the application of the sample.

PKC assay

PKC activity was assayed by measuring the incorporation of ^{32}P from ^{32}P-γ-ATP into substrate as previously described in Section 2.2.4. The substrates used were salmon protamine sulphate, calf thymus Histone IIIs (Sigma), and PKC δ, ε, and ζ pseudosubstrate site-based peptides as in Olivier *et al* (163). PKC isotypes were diluted in buffer (20 mM Tris HCl, pH 7.5, 1mM EDTA, and 5 mM dithiothreitol) before use. For binding / competition studies 10^{-3}-10^{-2} units of enzyme activity were employed per assay (1 unit incorporated 1 nmol ^{32}P into protamine sulphate per minute under kinase assay conditions in the absence of calcium, lipids and phorbol esters).

Binding of [^{3}H]PdBu to recombinant PKC isotypes

[^{3}H]PdBu binding to PKC isotypes was measured using a combination of the mixed micellar protocol (163) and DE 52 binding protocol (14). The binding assay mixture (50 μL) contained 20 mM Tris HCL, pH 7.5, 1 mg/mL BSA, 0.5 mM $CaCl_2$ (or 1 mM EGTA), mixed Triton X-100/PS micelles (2.3 mM and 1 mM final concentration respectively for a 70/30 mol % mixed micelles), [^{3}H]PdBu (with or without varying concentrations of non-radioactive PdBu) and enzyme. PS and PdBu (radioactive and non-radioactive) were incorporated into Triton X-100 micelles by first drying stock solutions under a stream of nitrogen gas and then adding Triton micelles (5.75 mM in 20 mM Tris HCL, pH 7.5) and vortexing (2 mins) and bath sonicating (2 mins). Micelle solutions were clear. Incubation of PKC isotypes was carried out at room temperature for 20 mins and terminated by addition of an ice cold suspension of DE-52 anion exchange cellulose (20% w/v in 20 mM Tris HCL, pH 7.5). The mix was allowed to stand for 20 mins at 4^{0}C to allow the PKC/[^{3}H]PdBu to bind to the DE-52. After this time the suspension was rapidly filtered through Whatman GF/C glass fibre filters and washed (4 x 5 mL of 20 mM Tris HCL, pH 7.5 at 4^{0}C). Filters were transferred into liquid scintillant and counted for radioactive scintillation in a Beckman LS 6000IC counter.

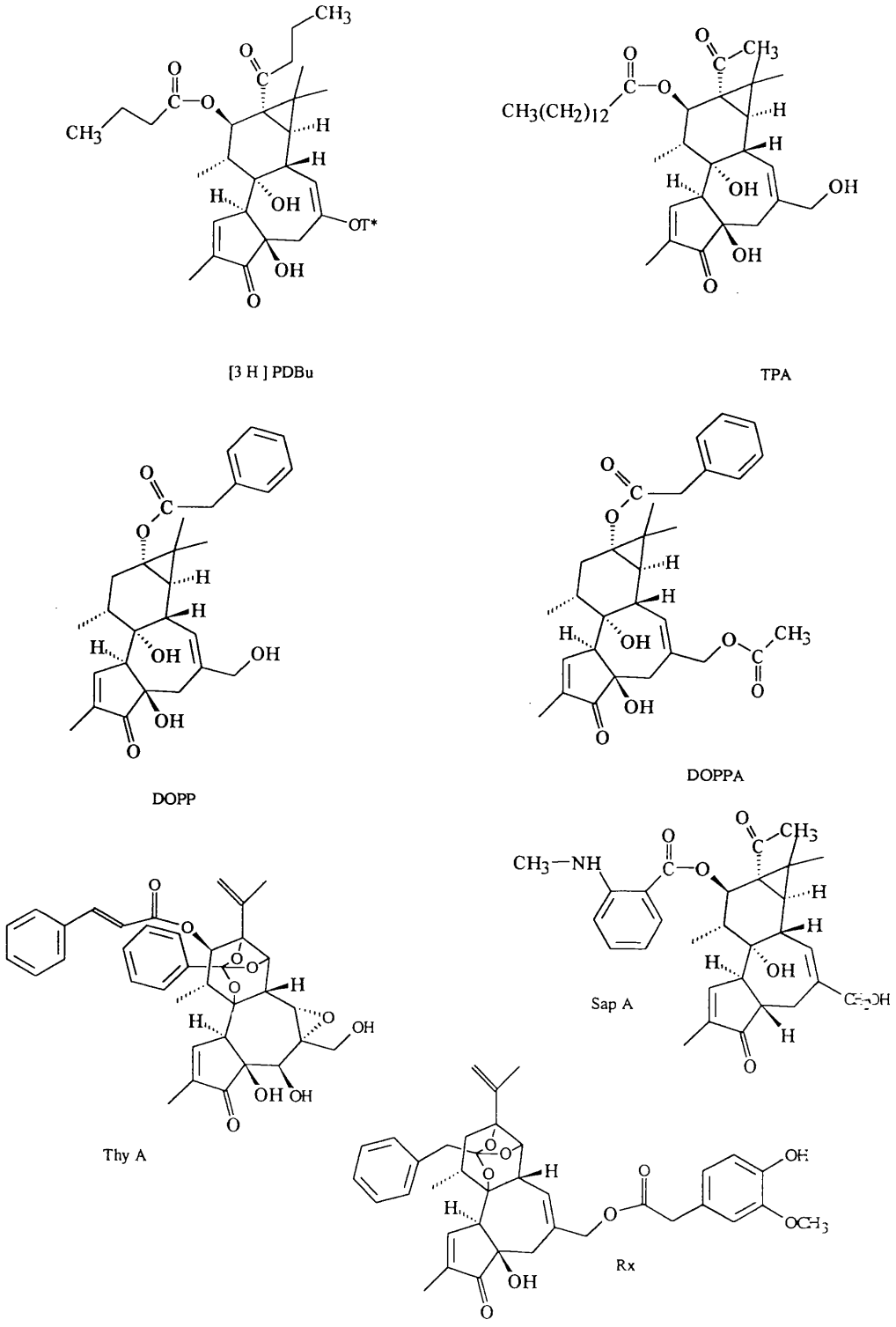


Figure 3.1.2. Structures of the phorbol esters used in this study. TPA, 12-O-tetradecanoylphorbol-13-acetate; **PdBu**, phorbol-12,13-dibutyrate (T*, Tritium); **DOPP**, 12-deoxyphorbol-13-phenylacetate; **DOPPA**, 12-deoxyphorbol-13-phenylacetate-20-acetate; **Sap A**, Sapintoxin A; **Thy A**, Thymeleatoxin A; **Rx**, Resiniferatoxin.

Each binding point was performed in triplicate assays in two groups to determine total binding and non-specific binding (determined using a 1000-fold excess of non-radioactive PdBu). Specific binding at each concentration of [^{3}H]PdBu was calculated as the difference between the total and the non-specific binding. To determine the dissociation constants (Kd) and the maximum number of binding sites (B_{MAX}) for different PKC isotypes Scatchard analysis was conducted on the specific binding curves at concentrations of [^{3}H]PdBu varying between 4 and 200 nM. Lines of best fit for Scatchard plots were calculated using least squares linear regression analysis.

Competition of binding of [^{3}H]PdBu to recombinant PKC isotypes

Competition of binding of [^{3}H]PdBu to recombinant PKC isotypes was performed under the same conditions as above using a fixed concentration of [^{3}H]PdBu (30 nM), and varying the non-radioactive phorbol ester competitor. The phorbol esters used in competition were PdBu, TPA, Sap A, DOPP and DOPPA from the Tigliane series, and Thy A and Rx from the Daphnane series (for structures see Figure 3.1.2., p. 94)(343). Varying concentrations of phorbol ester competitors were incorporated into the micelles with [^{3}H]PdBu (30 nM). The amount of specific [^{3}H]PdBu bound in the absence of competitor was taken as 100% binding, and the inhibition of this binding was calculated from this value for each isotype. Competition experiments were performed in triplicate for each point, and each experiment was conducted 3 times.

3.1.3. Results

Binding of [^{3}H]PdBu to recombinant PKC isotypes.

The binding of [^{3}H]PdBu to purified recombinant PKC isotypes α, β_1, β_2 , γ, δ, ε, and ζ was performed throughout with a modified mixed micellar system originally described by Hannun and Bell (357). We decided to use this system because it appears to be a physically defined system consisting of approximately 140 molecules of Triton X-100 per micelle, whereas sonic dispersion of phospholipids alone results in an heterogeneous mix of unilamellar and multilamellar vesicles. This system has also been used in previously reported work on PKC isotype activation by phorbol esters (347). The retrieval of PKC from the binding assay was adapted from the method of Parker *et al* (14) using DE-52, and we found all PKC activity was completely removed from solution under the conditions used. The binding of [^{3}H]PdBu to PKC isotypes was completed within 10 minutes at room temperature and stable for at least 30 minutes (see Figure 3.1.3., p. 97, for a representative example). In the absence of PS, we found no binding of [^{3}H]PdBu to PKC in the presence of Triton X-100 micelles alone, irrespective of the calcium/EGTA concentration used. The total and non-specific binding of [^{3}H]PdBu to PKC α, in the presence or absence of calcium are shown in Figure 3.1.4.A., p. 98, and the specific binding of [^{3}H]PdBu to PKC α is shown in Figure 3.1.4.B., p. 99, [^{3}H]PdBu binding to PKC α and ε did not appear to be saturable at the doses of [^{3}H]PdBu used here reflecting what may be a second binding site. However the limits of determination of the non-specific binding at higher [^{3}H]PdBu concentrations precluded a quantitative analysis of any such low-affinity binding site.

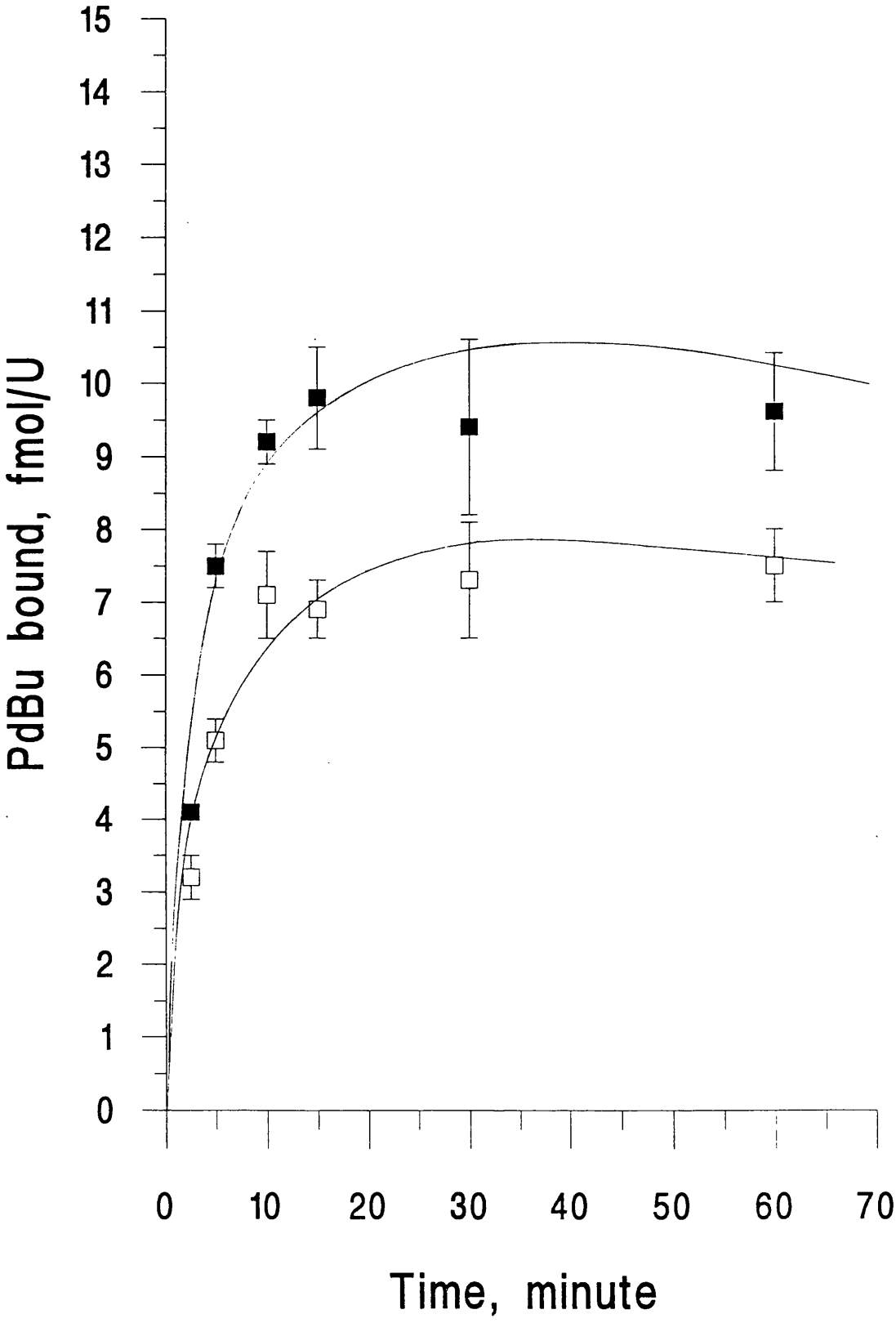


Figure 3.1.3. Time course for [^{3}H]PdBu binding to PKC α. Specific binding of [^{3}H]PdBu (25 nM) in the presence (closed symbols) and absence (open symbols) of calcium.Similar profiles were obtained for other concentrations of [^{3}H]PdBu and with other PKC isotypes.

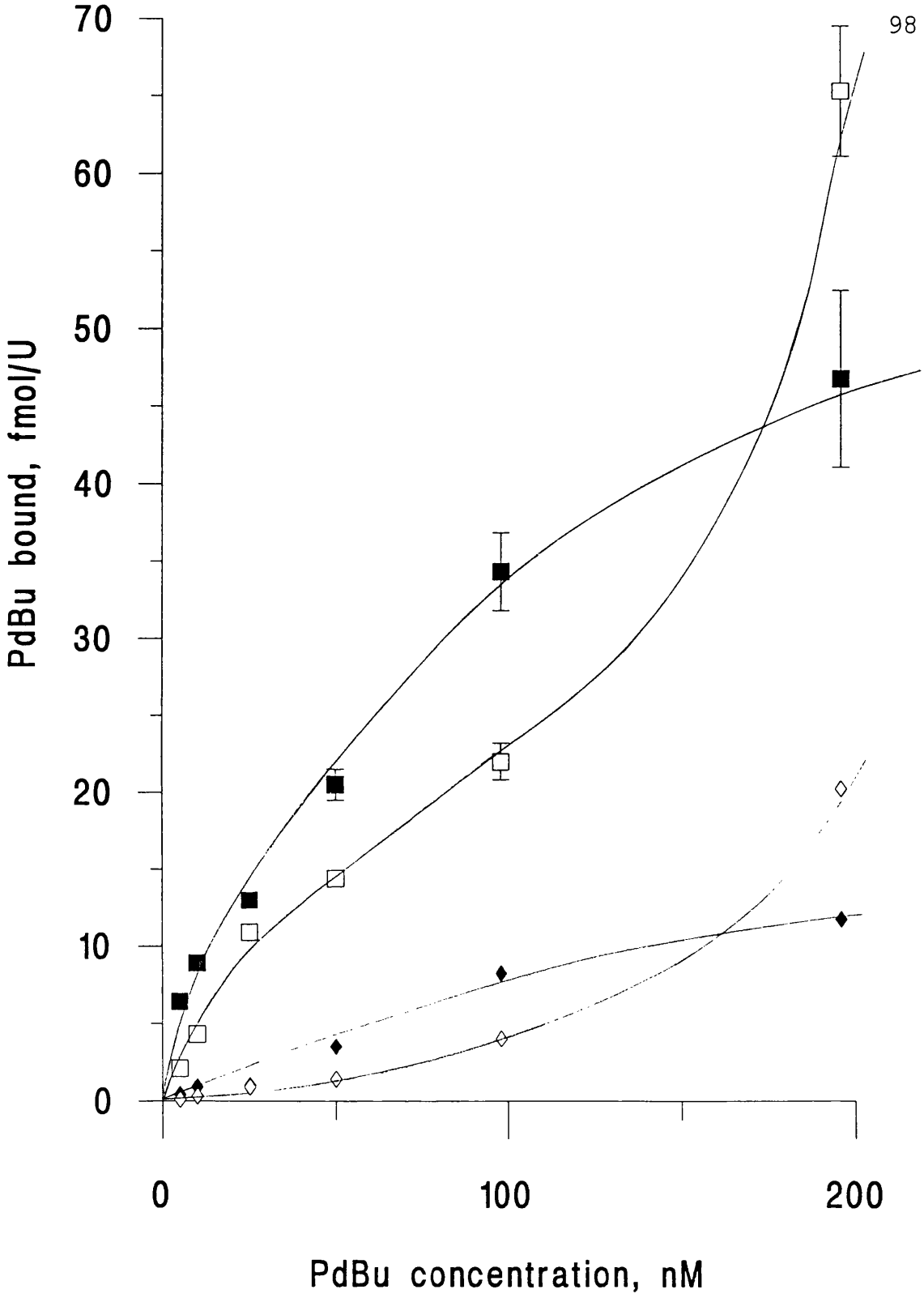


Figure 3.1.4. Binding of [H^3]PdBu to Protein kinase C α. A. The binding of increasing doses of [H^3]PdBu in the presence (closed symbols) and absence (open symbols) of calcium. Total binding (■,□) and non-specific binding with 1000-fold unlabelled PdBu (◆,◊) are shown for [H^3]PdBu doses up to 200 nM. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

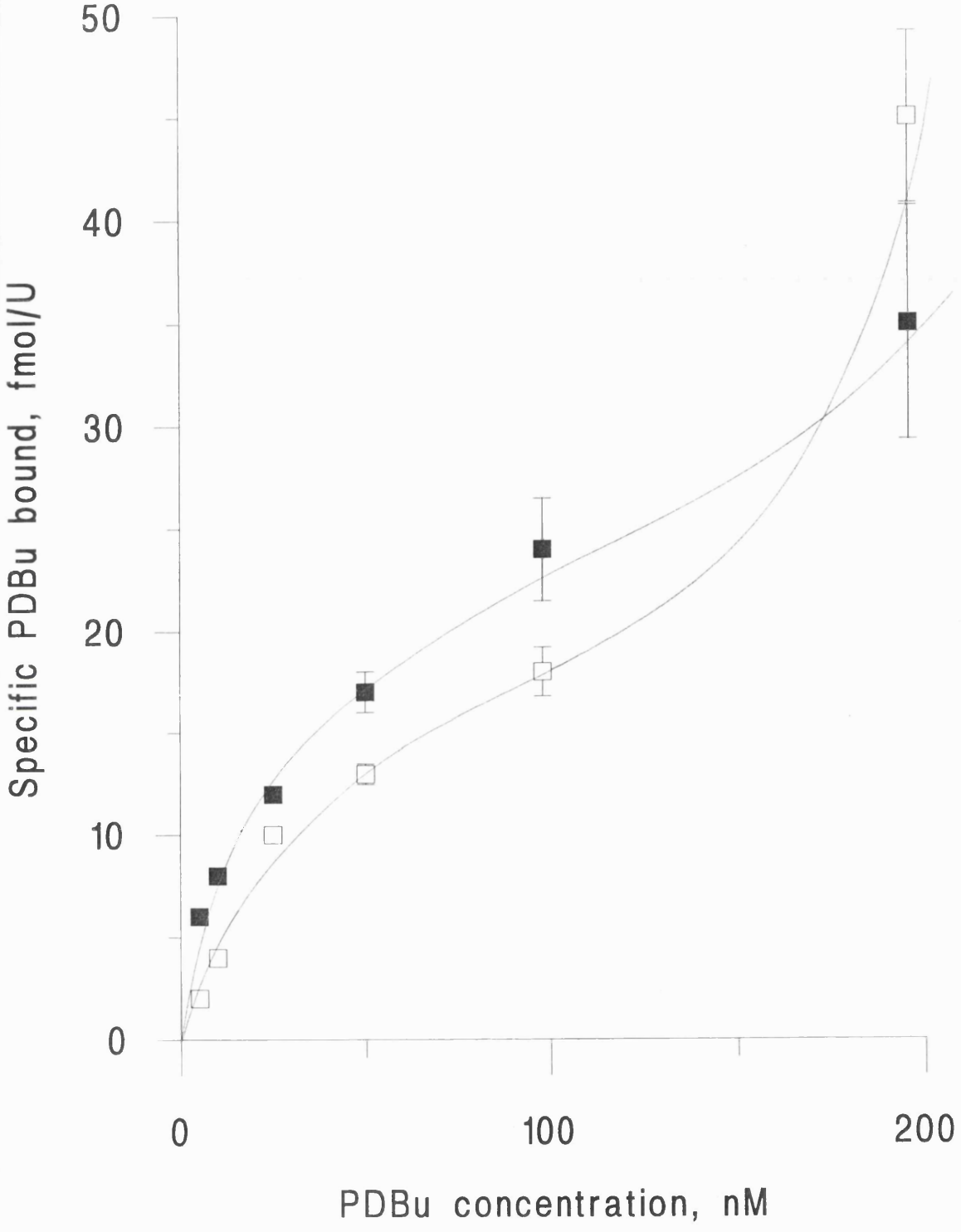


Figure 3.1.4. B. Specific binding of [H^3]PdBu to Protein kinase C α. From the values shown above (Figure 3.1.4.A., p. 98), the specific binding curves of [H^3]PdBu to Protein kinase C α, in the presence (■) and absence (□) of calcium is shown. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

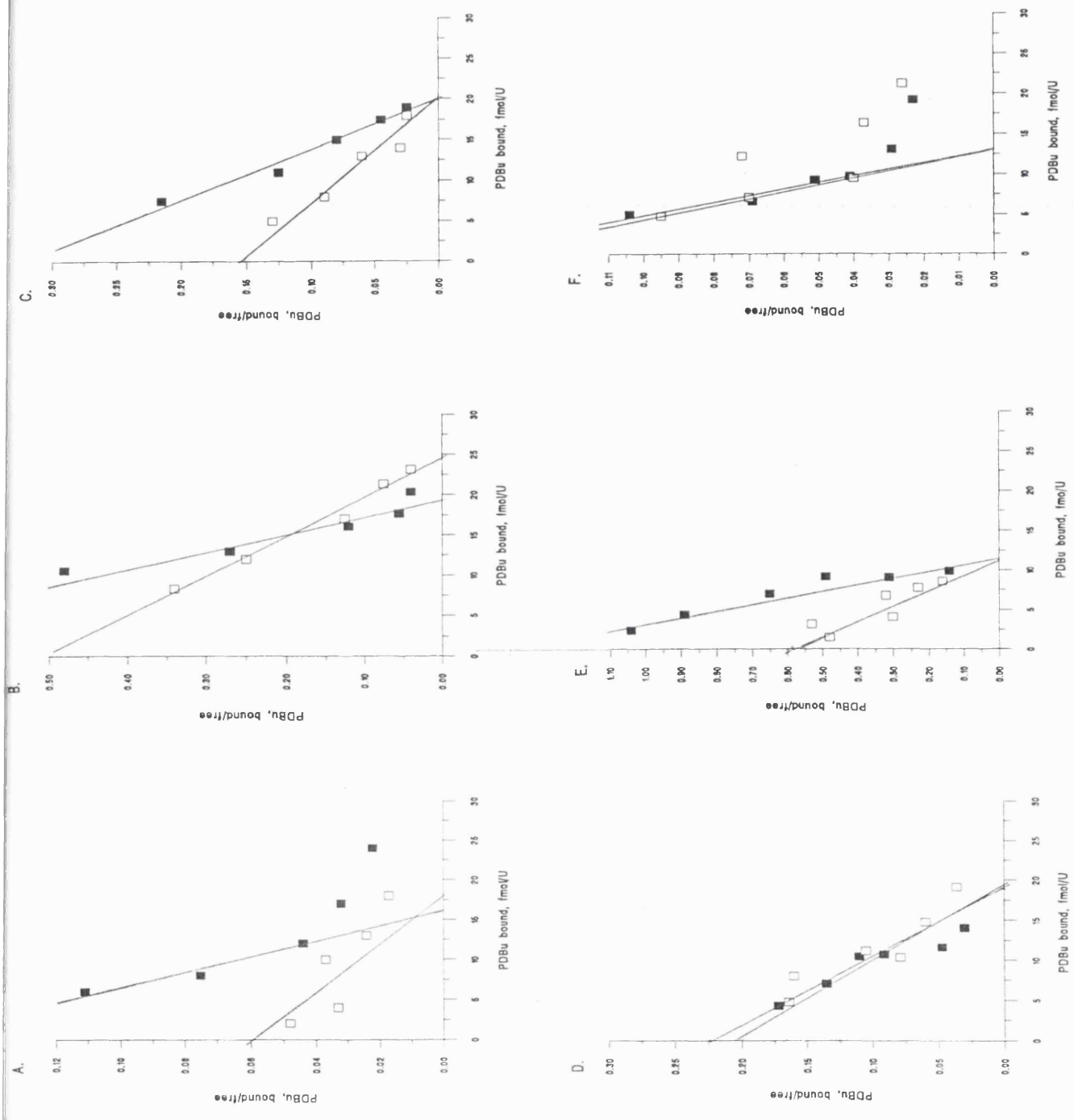


Figure 3.1.5. Scatchard analysis of the specific binding of [H^3]PdBu to Protein kinase C isotypes. Scatchard analysis (bound/free vs PdBu bound per PKC enzyme unit present in the assay) from mean values from 3 separate experiments, calculated from the specific binding curves in the presence (■) and absence (□) of calcium. **A. PKC** α. **B.** PKC β_1. **C.** PKC β_2. D. PKC γ. **E. PKC** δ. **F. PKC** ε. The Kd and B_{MAX} from these graphs are shown in Table 3.1.1.

PKC ζ failed to show any binding activity above non-specific even at 1000 nM [^{3}H]PdBu (data not shown). Scatchard analysis was conducted on the specific binding of [^{3}H]PdBu to PKC α, β_1, β_2, γ, δ and ε (Figure 3.1.5. A.-F., p. 100). The Scatchard plot is based on the the equation **(3)** derived (Figure 3.1.6.) from equation **(2)** which, under equilibrium conditions, describes a simple bimolecular association reaction **(1)**.

$$R + L^* \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} RL^* \qquad (1)$$

$$RL^* = \frac{R_t \cdot L^*}{(K_d + L^*)} \qquad (2)$$

$$K_d = 1/K = k_{12}/k_{21}$$

$$\frac{RL^*}{L^*} = \frac{R_t}{K_d} - \frac{1}{K_d} RL^* \qquad (3)$$

Figure 3.1.6. Equations used in analysis of binding data. (K, association constant, K_d, dissociation constant, L*, free ligand concentration, R_t, total receptor concentration = B_{max}, RL*, bound ligand concentration)

These showed that the dissociation constants (Kd) of PdBu at equilibrium ranged from 1.6 to 18.5 nM in the presence of calcium, and 3.9 to 60 nM in the absence of calcium (see Table 3.1.1., p. 102), although PKC α and ε appeared to possess a possible second binding site. The specific PdBu binding to all PKC isotypes, as a percentage of total binding, was between 86 and 95% when

determined for [H^3]PdBu concentrations at the Kd values (see Table 3.1.1.). The (B_{MAX}) values, based on kinase activity with protamine sulphate (requiring no lipid or calcium cofactors) was similar for all PKC isotypes (12-24 fmole PdBu bound per unit of enzyme activity). When units of activity were calculated in moles of enzyme present, the stoichiometry of the binding at the B_{MAX} values ranged from 1.4 to 2.8 molecules of PdBu per molecule of enzyme present.

Table 3.1.1. Values of Kd and B_{MAX}, from Scatchard analyses of the specific binding of [H^3]PdBu to Protein kinase C isotypes, and percentage of specifically bound [H^3]PdBu at Kd. The values for PKC α and ε suggest there may be 2 binding sites, only the strongest of which is shown. Each value represents the mean ± S.E.M of 3 separate experiments.

Table 1

PKC Isotype	+Calcium			- Calcium		
	Kd (nM)	B_{MAX} (f.moles/U)	% Bound[A]	Kd (nM)	B_{MAX} (f.moles/U)	% Bound[A]
α	18 ± 2.1	16 ± 0.9	88 ± 6.4	60 ± 8.1	18 ± 0.7	90 ± 7.1
β_1	1.6 ± 0.4	19 ± 1.3	95 ± 7.3	3.9 ± 0.8	24 ± 4.2	89 ± 4.3
β_2	4.5 ± 0.6	20 ± 1.4	86 ± 4.2	9.5 ± 1.2	20 ± 3.1	85 ± 5.1
γ	16 ± 0.9	18 ± 0.7	89 ± 5.3	18 ± 3.1	18 ± 2.0	86 ± 2.1
δ	1.7 ± 0.2	12 ± 0.6	94 ± 7.8	4 ± 0.9	12 ± 0.9	92 ± 6.6
ε	17 ± 2.2	13 ± 2.1	89 ± 6.6	18 ± 1.1	13 ± 1.8	91 ± 8.1
ζ	No specific [H^3]PdBu binding.					

[A]. Specific binding as % of total binding at Kd.

Competition of binding of [^{3}H]PdBu to recombinant PKC isotypes.

To compare the enzyme-ligand interactions for different PKC isotypes we quantified the competition for specific [^{3}H]PdBu binding for a range of naturally occurring tigliane and daphnane esters, known to possess differing biological activities (71,341-343). The phorbol esters (see Figure 3.1.2.,, p. 94) TPA, PdBu, Sapintoxin A (Sap A), DOPP, DOPPA, Thymealeatoxin (Thy) and Resiniferatoxin (Rx) were used in final concentrations of 1 nM-50 μM (0.00003 - 1.5 mol % micelle).

Table 3.1.2. IC_{50} values (nM) for phorbol esters competing for specific [H^3]PdBu binding sites (30 nM) to Protein kinase C isotypes. Each value represents the mean ± S.E.M of 3 separate experiments.

Table 2

PKC Isotype	Ca^{2+}	IC_{50} values (nM)						
		PdBu	TPA	Dopp	Doppa	Sap A	Thy	Rx
α	+	25 ± 3.1	4 ± 0.6	25 ± 3.1	>10μM	2.5 ± 0.4	5μM ± 0.4	>10μM
	-	25 ± 2.0	4 ± 0.7	25 ± 2.4	>10μM	2.5 ± 0.7	5μM ± 0.4	>10μM
β_1	+	75 ± 4.2	70 ± 7.1	10 ± 1.1	9μM ± 1.1	3 ± 0.1	20 ± 4.1	9μM ± 2.1
	-	75 ± 8.4	70 ± 4.2	10 ± 1.0	6μM ± 0.8	3 ± 0.6	20 ± 3.8	11μM ± 3.4
β_2	+	7 ± 1.0	8 ± 1.1	20 ± 1.0	5μM ± 1.0	2.5 ± 0.5	50 ± 7.2	45μM ± 3.2
	-	16 ± 2.4	17 ± 2.3	20 ± 0.8	15μM ± 2.3	2.5 ± 0.1	50 ± 8.1	45μM ± 4.1
γ	+	100 ± 15	50 ± 1.2	50 ± 4.2	>50μM	2 ± 0.4	300 ± 45	>50μM
	-	400 ± 24	250 ± 43	50 ± 7.1	>50μM	2 ± 0.4	500 ± 61	>50μM
δ	+	25 ± 1.1	30 ± 5.1	25 ± 0.9	>50μM	35 ± 5.1	95 ± 11	>50μM
	-	25 ± 2.4	30 ± 2.3	25 ± 3.2	>50μM	35 ± 4.3	95 ± 15	>50μM
ε	+	60 ± 8.1	25 ± 3.1	15 ± 2.1	>50μM	2 ± 0.9	3μM ± 0.7	>50μM
	-	60 ± 4.2	40 ± 5.1	15 ± 3.4	>50μM	2 ± 0.3	3μM ± 0.6	>50μM
ζ	+/-	No specific [H^3]PdBu binding.						

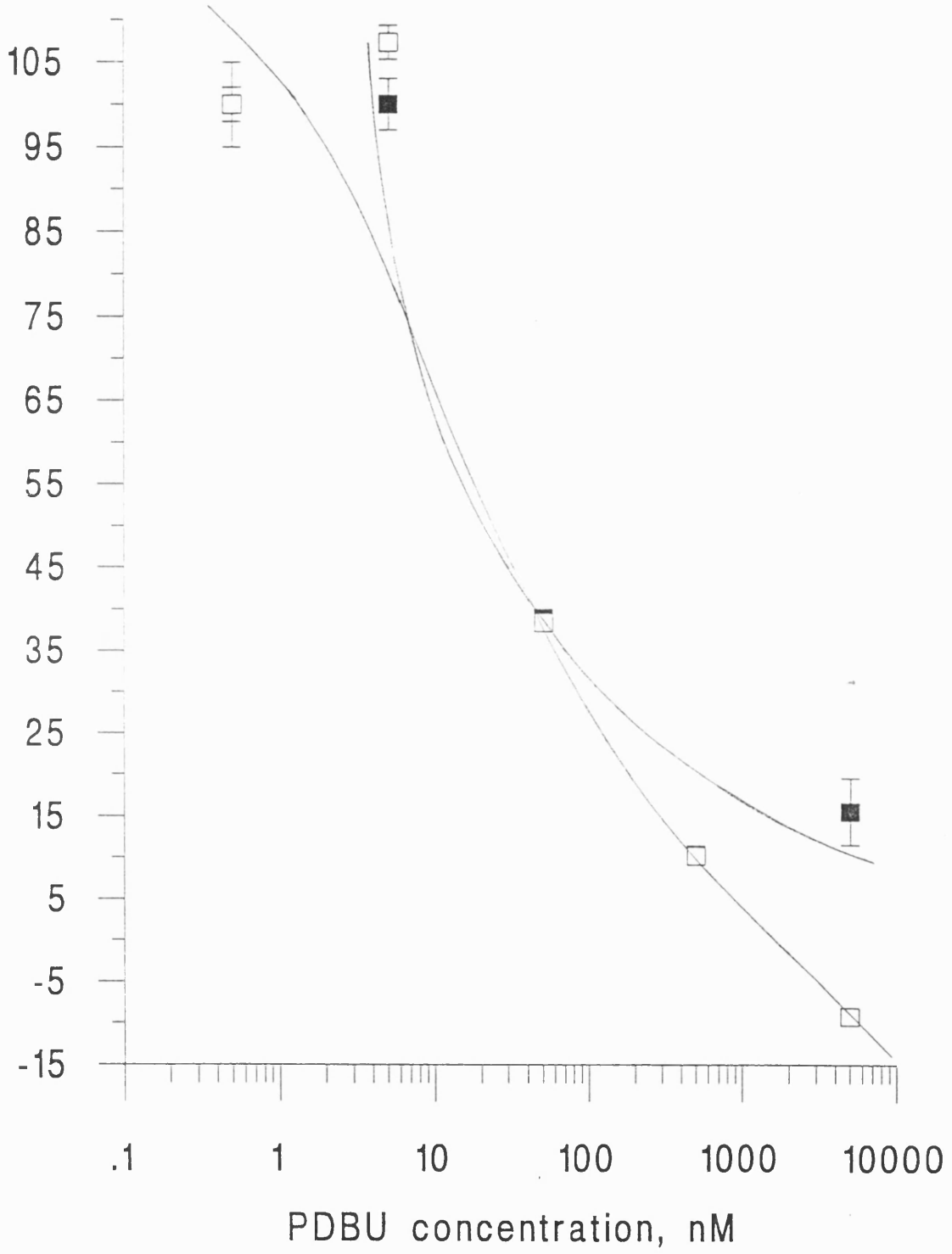


Figure 3.1.7. A. Competition of specifically bound [H^3]PdBu to Protein kinase C α by PdBu in the presence (■) and absence (□) of calcium. [H^3]PdBu, present at 30 nM throughout, was challenged with increasing doses of phorbol esters. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

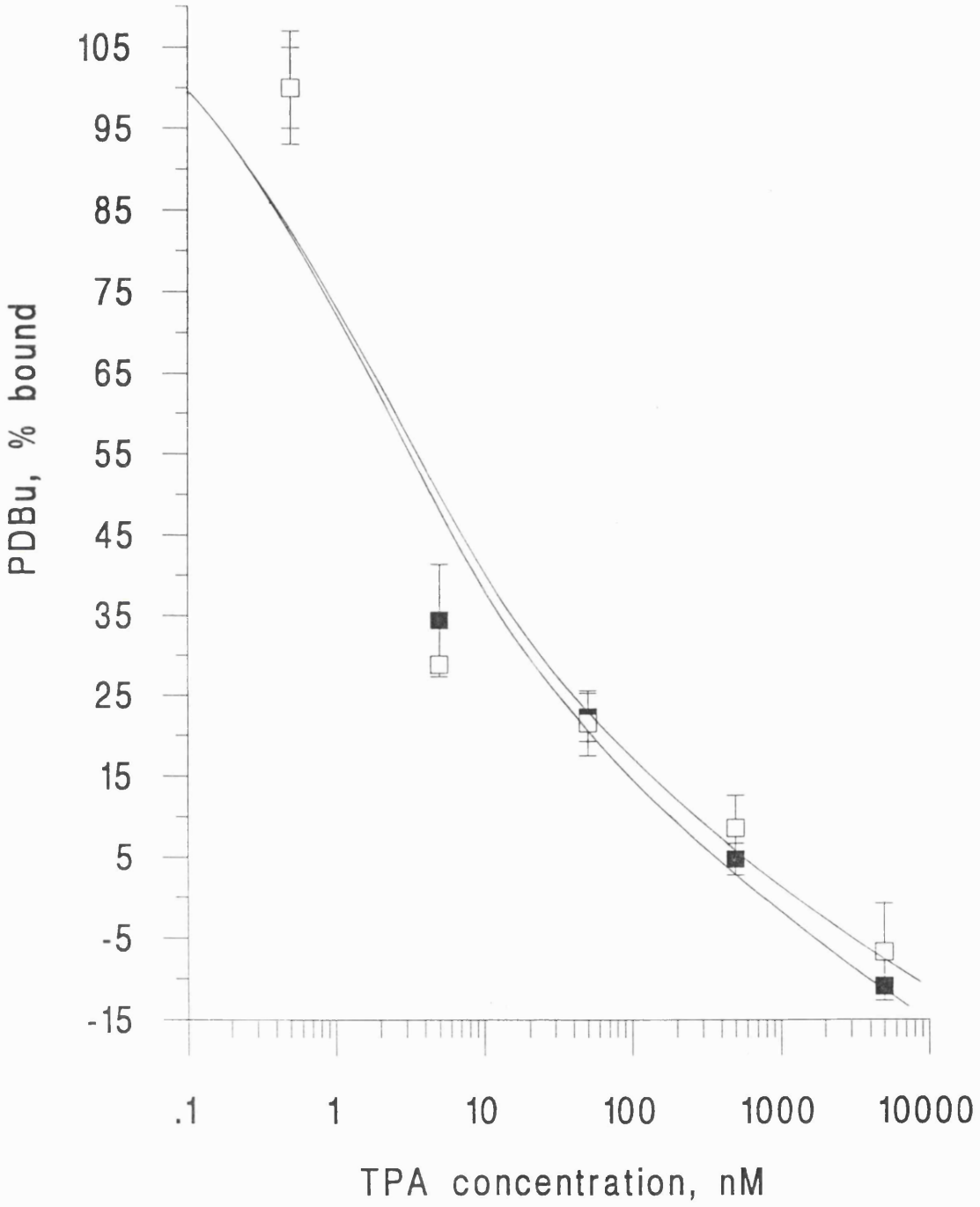


Figure 3.1.7. B. Competition of specifically bound [H^3]PdBu to Protein kinase C α by TPA in the presence (■) and absence (□) of calcium. [H^3]PdBu, present at 30 nM throughout, was challenged with increasing doses of phorbol esters. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

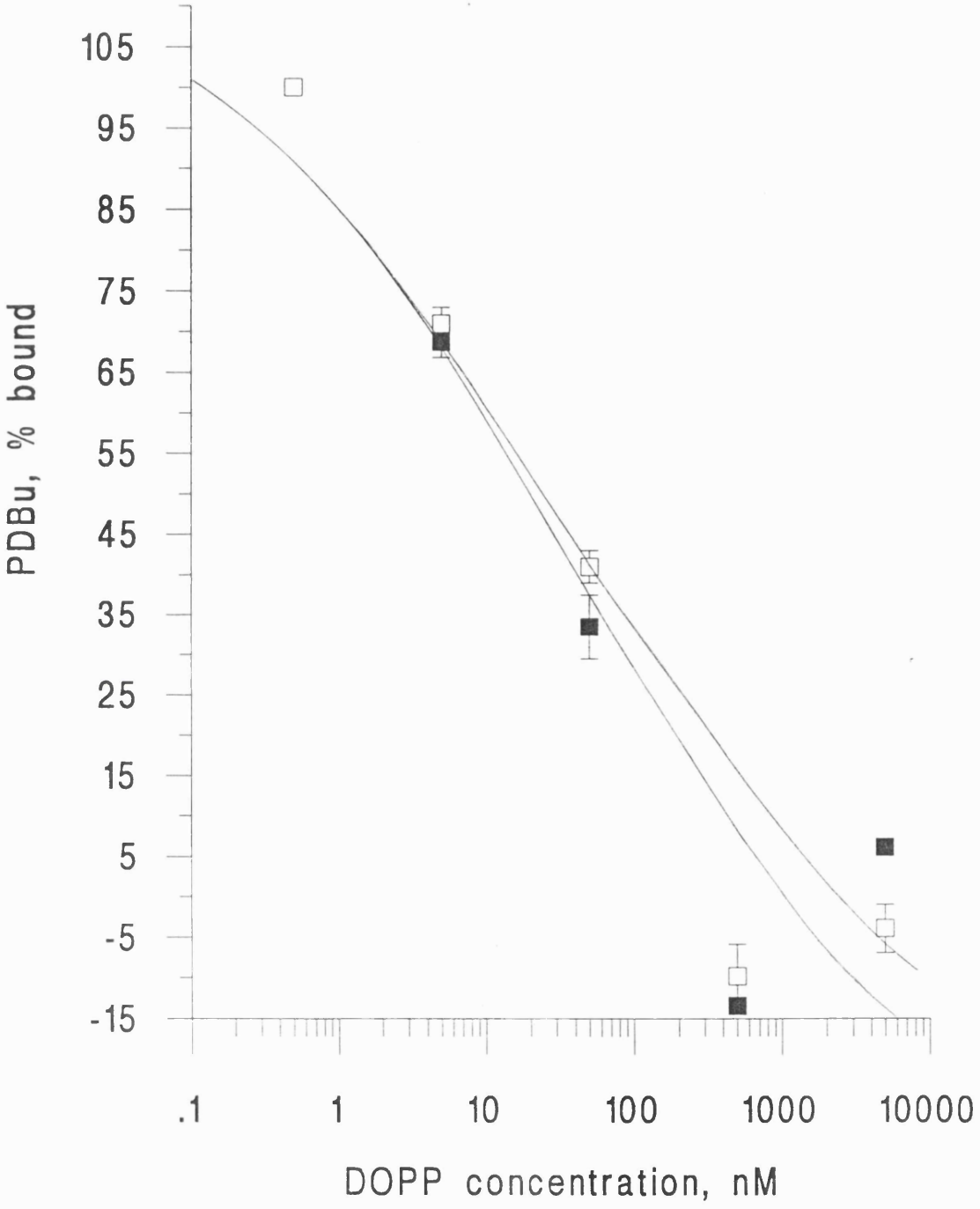


Figure 3.1.7. C. Competition of specifically bound [H^3]PdBu to Protein kinase C α by DOPP in the presence (■) and absence (□) of calcium. [H^3]PdBu, present at 30 nM throughout, was challenged with increasing doses of phorbol esters. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

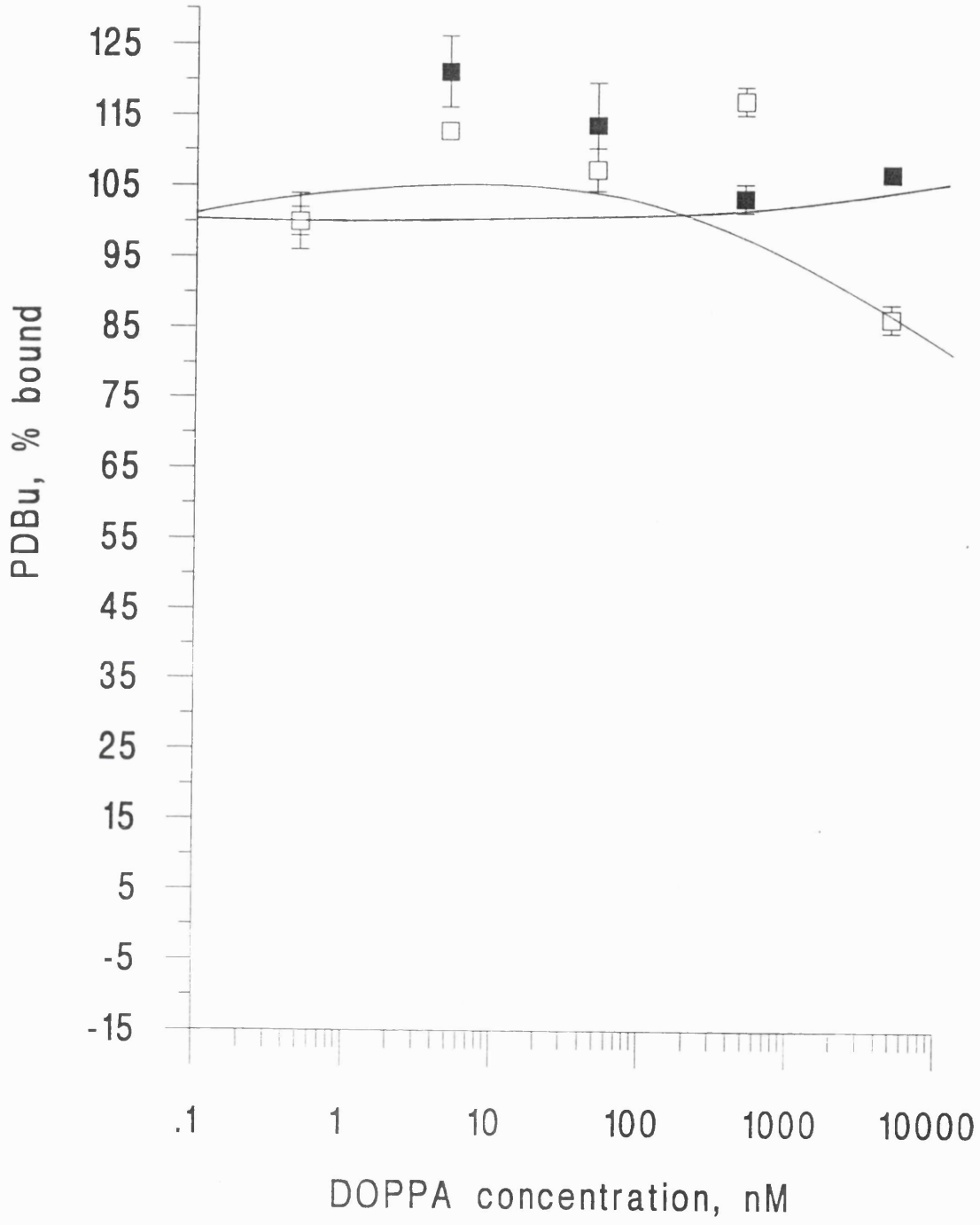


Figure 3.1.7. D. Competition of specifically bound [H^3]PdBu to Protein kinase C α by DOPPA in the presence (■) and absence (□) of calcium. [H^3]PdBu, present at 30 nM throughout, was challenged with increasing doses of phorbol esters. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

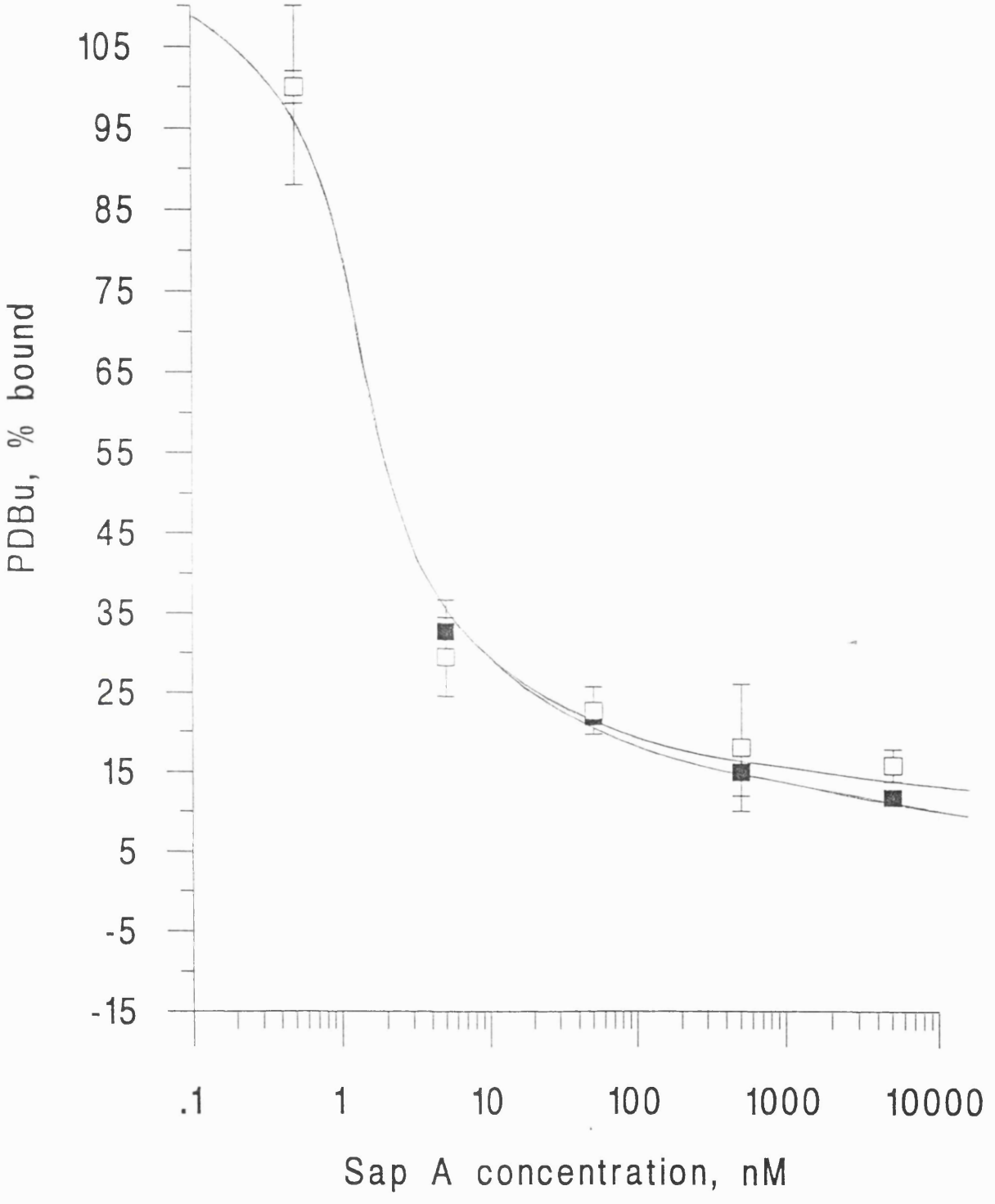


Figure 3.1.7. E. Competition of specifically bound [H^3]PdBu to Protein kinase C α by Sap A in the presence (■) and absence (□) of calcium. [H^3]PdBu, present at 30 nM throughout, was challenged with increasing doses of phorbol esters. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

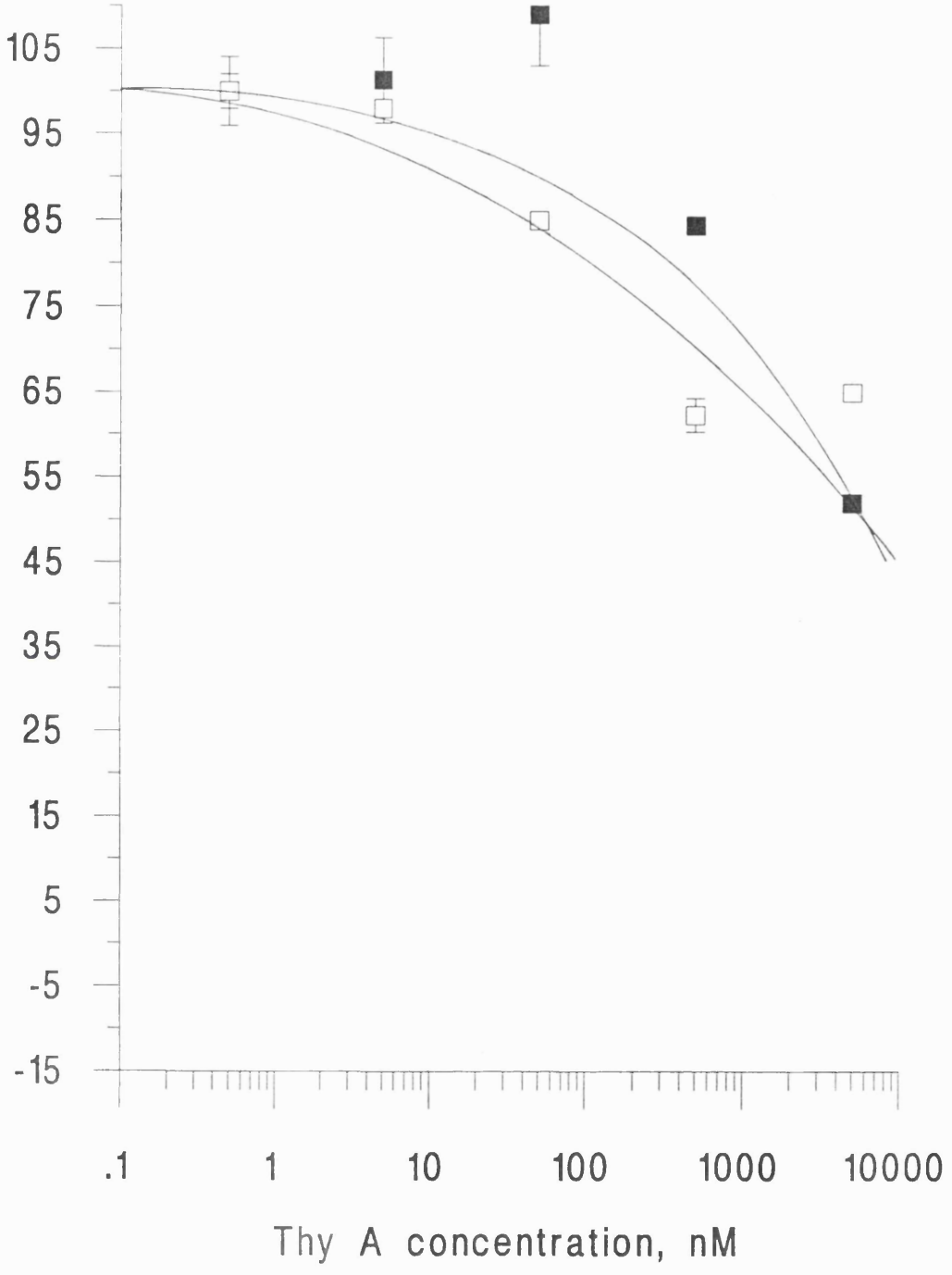


Figure 3.1.7. F. Competition of specifically bound [H³]PdBu to Protein kinase C α by Thy A in the presence (■) and absence (□) of calcium. [H³]PdBu, present at 30 nM throughout, was challenged with increasing doses of phorbol esters. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

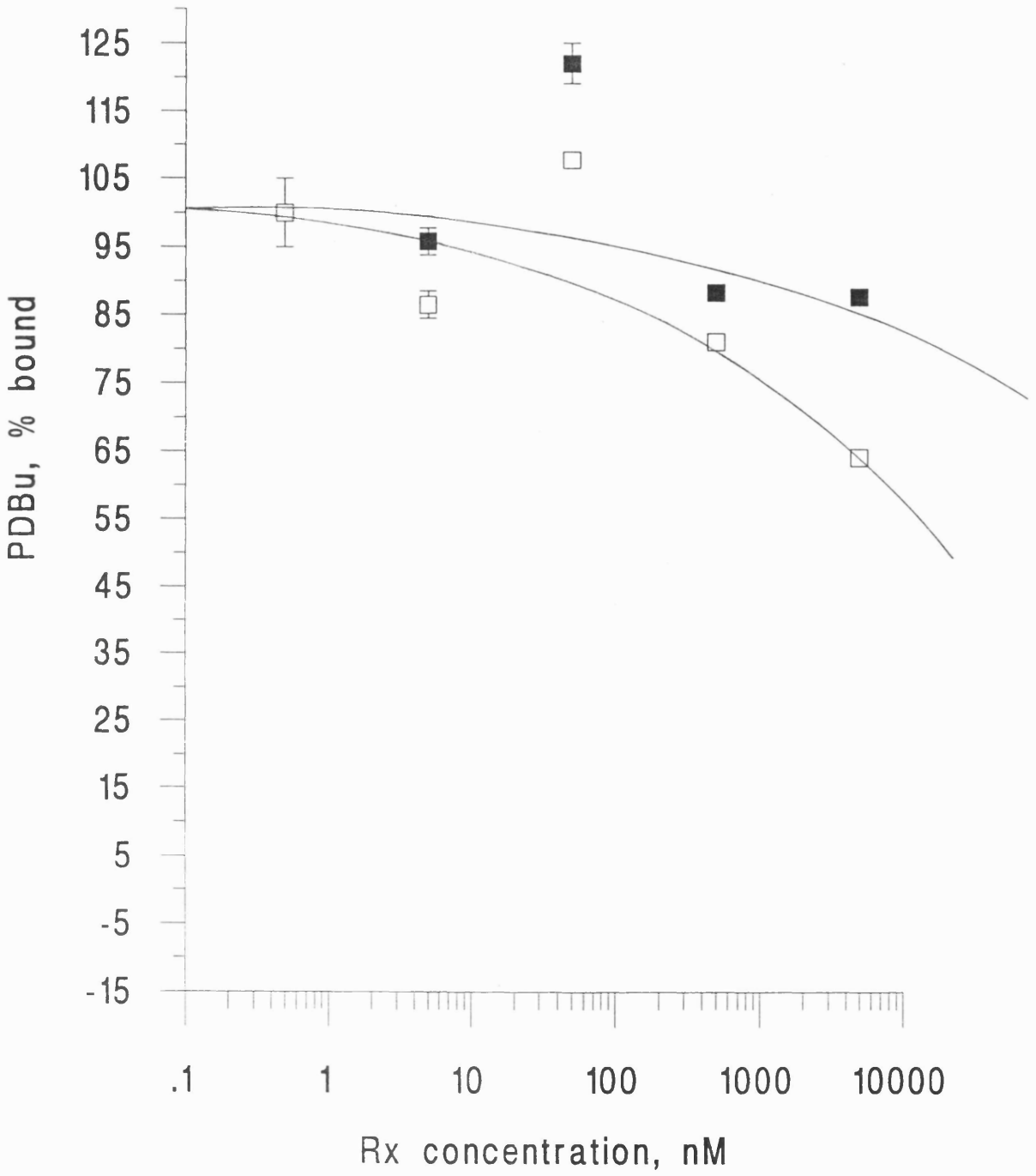


Figure 3.1.7. G. Competition of specifically bound [H^3]PdBu to Protein kinase C α by Rx in the presence (■) and absence (□) of calcium. [H^3]PdBu, present at 30 nM throughout, was challenged with increasing doses of phorbol esters. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

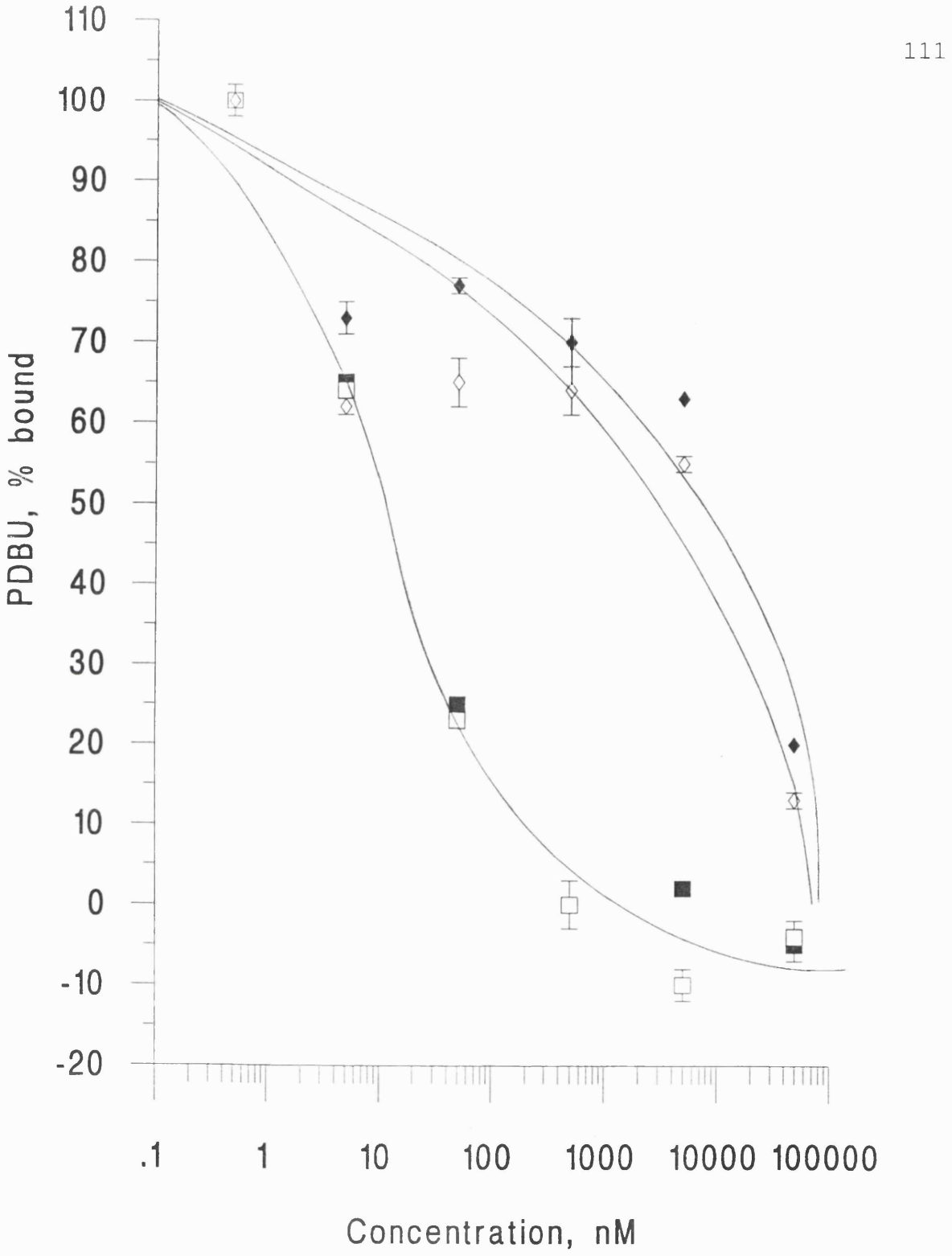


Figure 3.1.8. A. Competition of specifically bound [H^3]PdBu to PKC-β_1 by the 12-deoxyphorbols, DOPP and DOPPA. Increasing doses of DOPP (■,□) or DOPPA (◆,◊) were added to [H^3]PdBu (30 nM) bound to PKC-β_1 in the presence (closed symbols) and absence (open symbols) of calcium. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

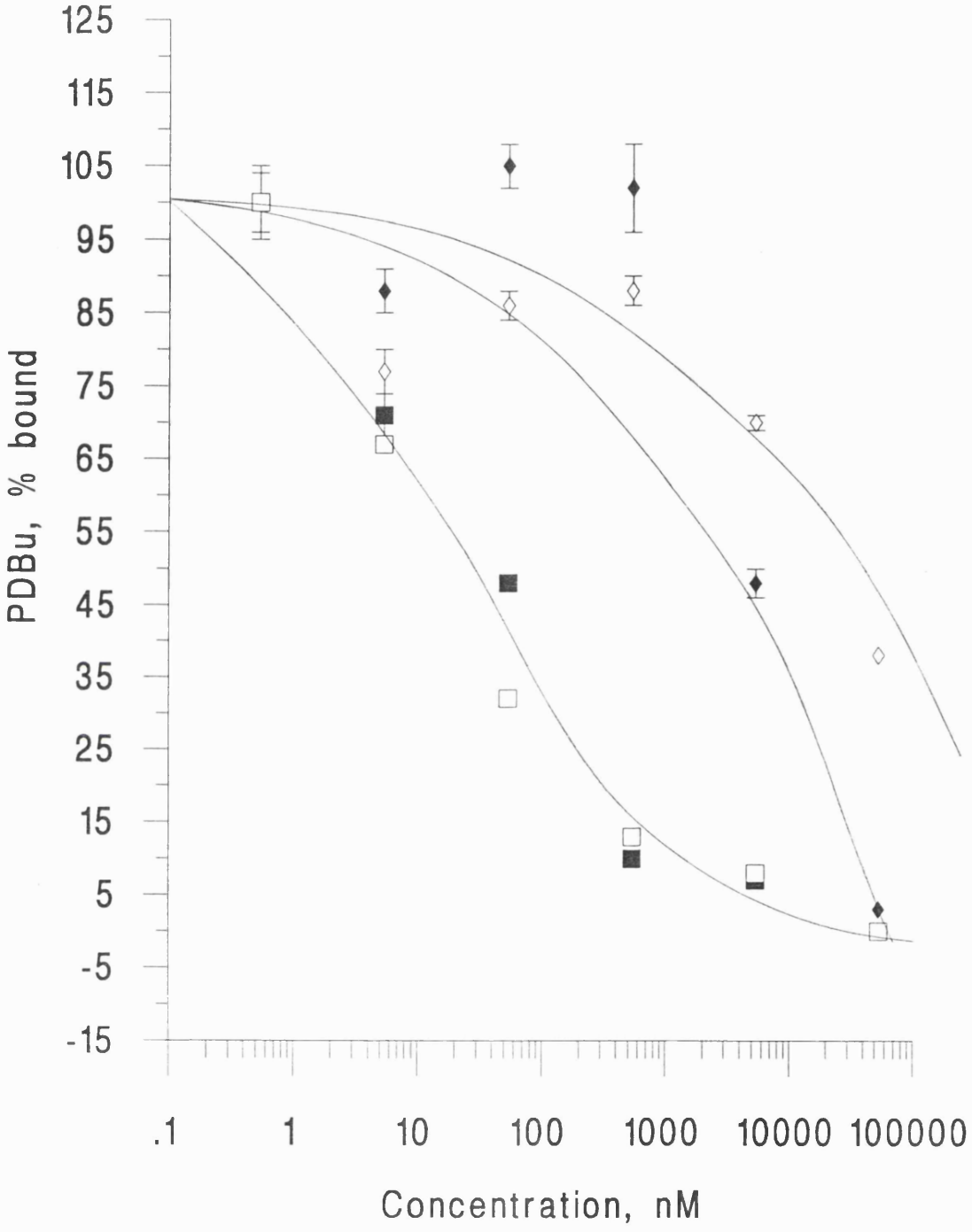


Figure 3.1.8. B. Competition of specifically bound [H^3]PdBu to PKC-β_2 by the 12-deoxyphorbols, DOPP and DOPPA. Increasing doses of DOPP (■,□) or DOPPA (◆,◊) were added to [H^3]PdBu (30 nM) bound to PKC-β_2 in the presence (closed symbols) and absence (open symbols) of calcium. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

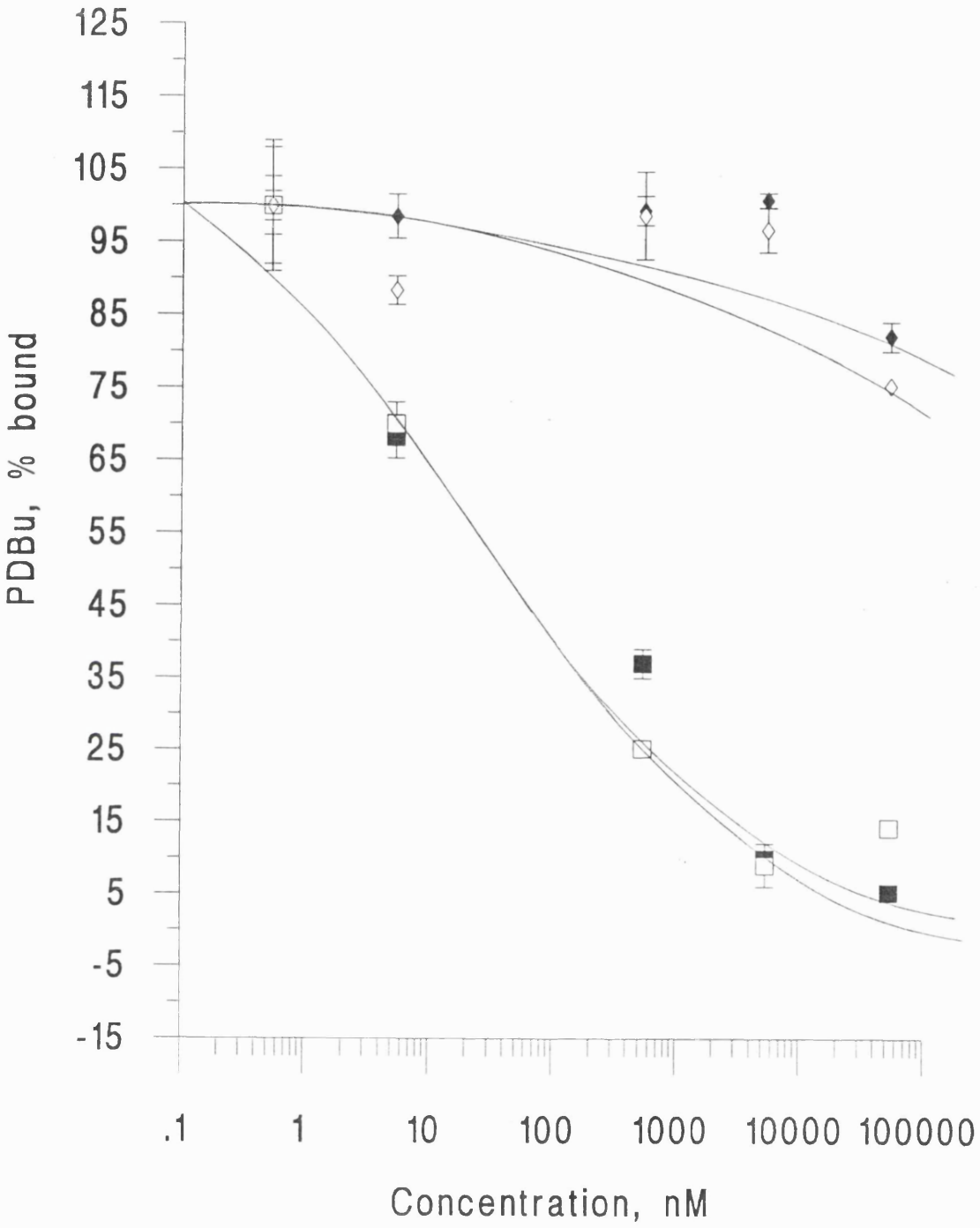


Figure 3.1.8. C. Competition of specifically bound [H^3]PdBu to PKC-γ by the 12-deoxyphorbols, DOPP and DOPPA. Increasing doses of DOPP (■,□) or DOPPA (◆,◊) were added to [H^3]PdBu (30 nM) bound to PKC-γ in the presence (closed symbols) and absence (open symbols) of calcium. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

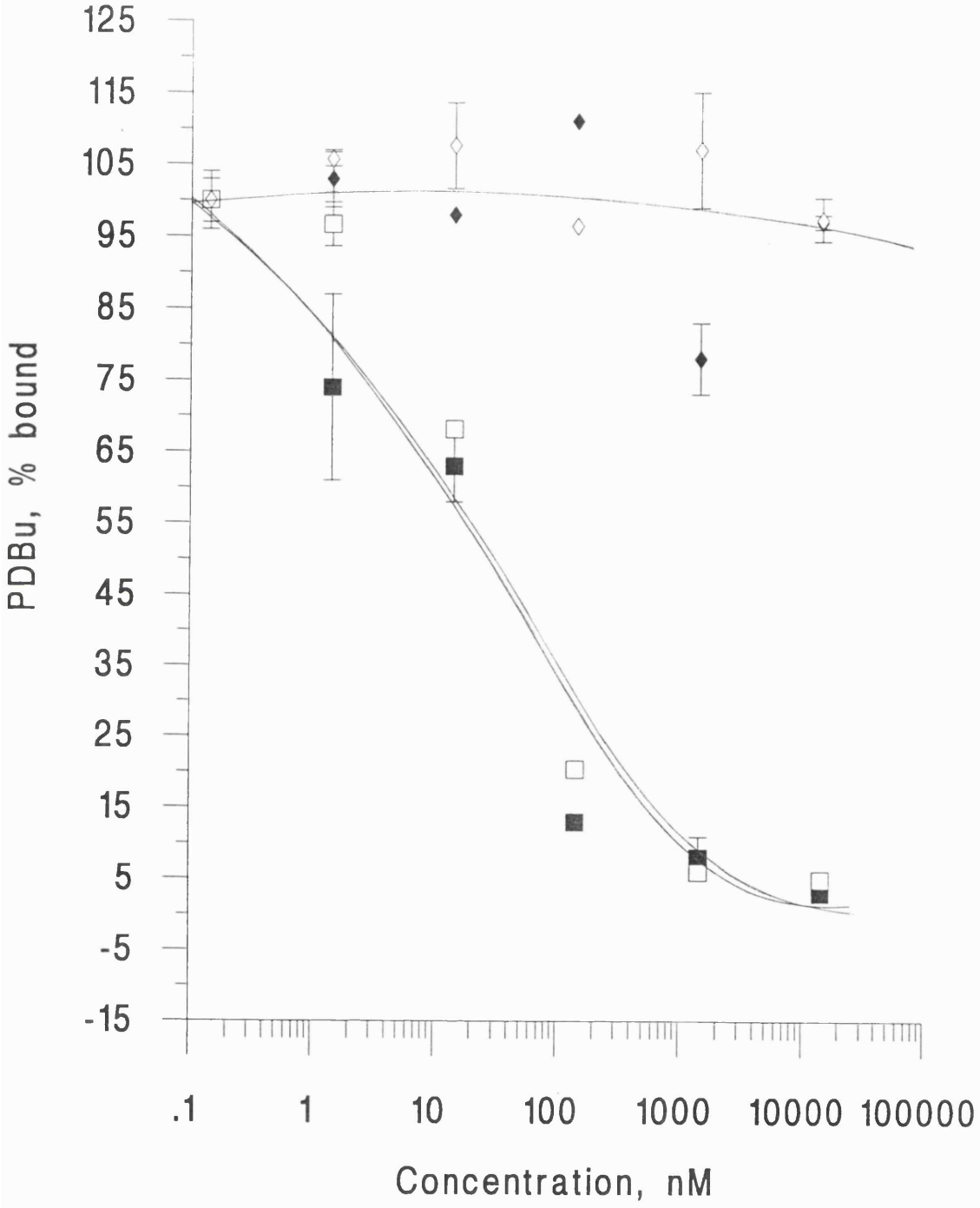


Figure 3.1.8. D. Competition of specifically bound [H^3]PdBu to PKC-δ by the 12-deoxyphorbols, DOPP and DOPPA. Increasing doses of DOPP (■,□) or DOPPA (◆,◊) were added to [H^3]PdBu (30 nM) bound to PKC-δ in the presence (closed symbols) and absence (open symbols) of calcium. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

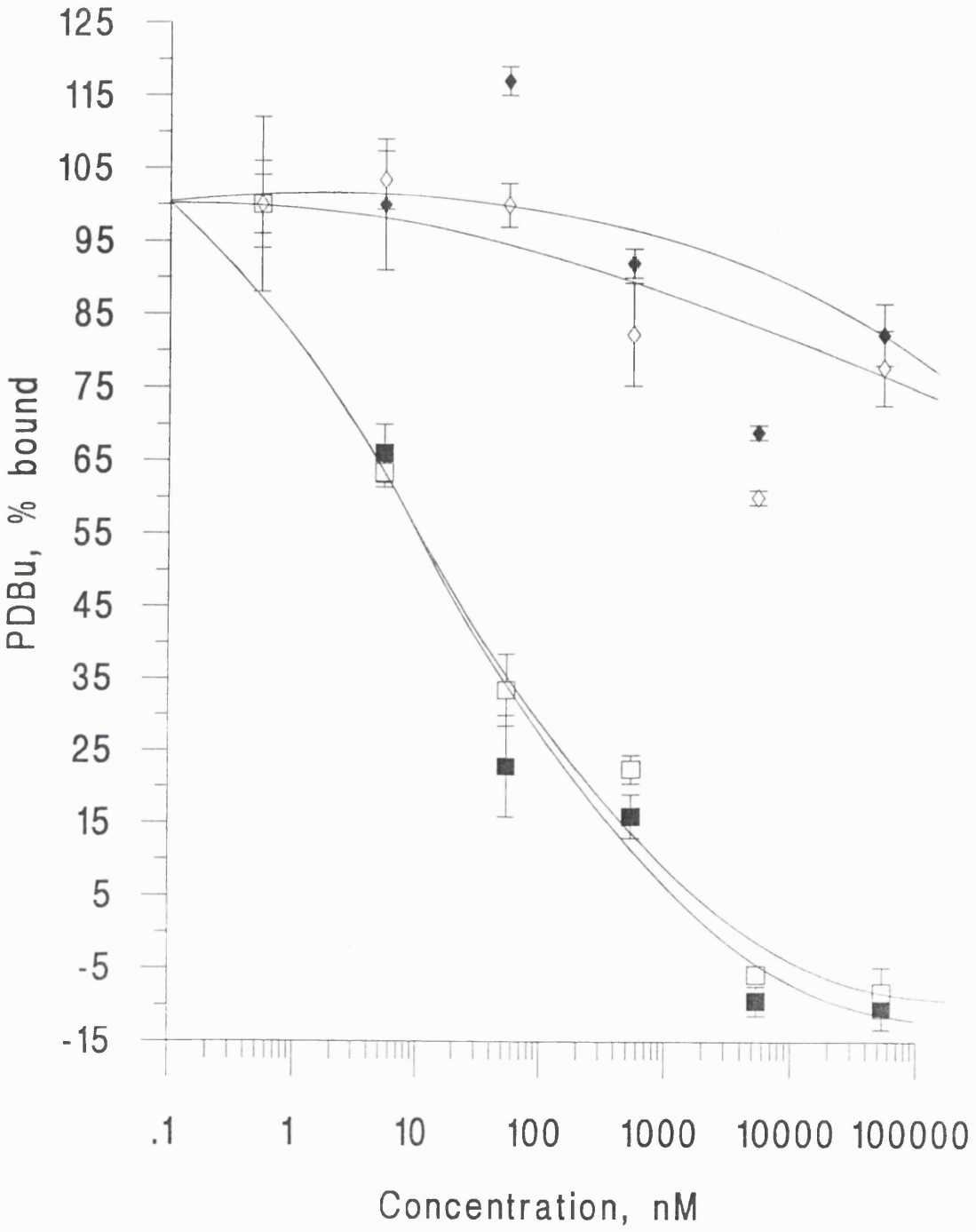


Figure 3.1.8. E. Competition of specifically bound [H^3]PdBu to PKC-ε by the 12-deoxyphorbols, DOPP and DOPPA. Increasing doses of DOPP (■,□) or DOPPA (◆,◊) were added to [H^3]PdBu (30 nM) bound to PKC-ε in the presence (closed symbols) and absence (open symbols) of calcium. The specifically bound PdBu was termed 100% and the competition depicted as reduction in this value. The mean values and S.E.M range is shown for triplicate determinations of a representative experiment.

An example of a typical competition experiment of specific [^{3}H]PdBu binding to PKC α in the presence and absence of calcium by these phorbol esters (1 nM to 10 μM) is shown in Figure 3.1.7. A.-G., pgs. 104-110. Here TPA, PdBu, DOPP and Sap A were potent competitors for [^{3}H]PdBu binding (~3-30 nM), largely unaffected by the presence of calcium, whereas Thy, Rx and DOPPA were only weakly able to compete at the doses tested. The concentrations at which 50% of specific [^{3}H]PdBu binding was abolished (IC_{50}) by these phorbol esters for PKC α and the other PKC isotypes are shown in Table 3.1.2. A, p. 103, comparison of the two 12-deoxyphorbols used in this study (DOPP and DOPPA) in competition for [^{3}H]PdBu binding to PKC β_1, β_2, γ, δ and ε are shown in Figure 3.1.8. A.-E., pgs. 111-115 DOPP is able to fully compete for binding to all these isotypes (IC_{50} range from 10-50 nM), whereas DOPPA is only fully effective for the PKC β_1 and β_2 isotypes and is much less potent than DOPP (IC_{50} ~ between 5-15 μM).

3.1.4. Discussion

The spectrum of biological effects elicited by different phorbol esters in mammalian cells is thought to be due mainly to their differential interaction with, and modification of, the cellular complement of PKC isotypes engaged in signal transduction. Initial studies using a range of phorbol esters to stimulate *in vitro* kinase activity of PKC isotypes purified from bovine brain, suggested that the ability to stimulate activity was related to the structure of substituent groups at the C-20 and C13/14 areas of the phorbol ester nucleus (347). In this present study we have measured the binding of [^{3}H]PdBu to recombinant PKC α, β_1, β_2, γ, δ, ε, and ζ isotypes, purified after baculoviral expression in the insect (*Spodoptera frugiperda*) Sf9 cell system, and the inhibition of specific [^{3}H]PdBu binding to these isotypes by seven phorbol esters. We decided to use the Sf 9 cell baculovirus-expression system because it has certain advantages over other systems. First, each expressed isozyme was a single genetically defined species (288). Second, high levels of protein expression were achieved (288) with a significantly simplified

purification by the high concentration of PKC in the initial cell extract. Third, these insect cells do not appear to express a detectable endogenous PKC activity, adding an advantage of being able to generate isotype pure PKCs (288,397,199). Some reports also showed that crude extracts of infected insect cells and purified recombinant protein show identical properties with decreased possibility of generating proteolytic fragments (288,199) during purification. Furthermore, using the same crude preparations of infected insect cells for all isozymes under identical conditions may even minimize variables introduced through different purification schemes.

It is well known that binding of a ligand with its receptor does not necessarily lead to stimulation of receptor function (activation in the case of enzyme receptors) but can also lead to its blocking or inhibition. In order to further investigate differences in the ability of different PEs to activate PKC isotypes we studied here their affinity to bind to these isotypes.

The conditions for [^{3}H]PdBu binding employed an artificial Triton/PS micelle suspension to emulate the requirements for phorbol ester binding, previously used in the isotype kinase stimulation assays (347), developed by Hannun *et al* (356,357). Using this protocol, binding of [^{3}H]PdBu rapidly reached equilibrium and remained stable for at least 30 minutes, enabling Scatchard analysis to be performed (see Figure 3.1.5. A.-F., p. 100). The DE-52 recovery step efficiently bound all PKC activity and limited non-specific [^{3}H]PdBu binding (at [PdBu] = Kd) to less than 15% of total bound for PKC α, β_1, β_2 , γ, δ and ε isotypes. PKC ζ isotype did not show any [^{3}H]PdBu binding capacity above background, even when PdBu was used at 1 μM, suggesting that even low PdBu affinity sites are not available in this isotype, in agreement with previous studies (358). The Scatchard analysis of receptor-ligand interaction allows Kd and B_{MAX} values to be calculated at equilibrium for conditions where ligand binding is not saturated (linear part of a binding curve). The concentration of each PKC isotype was not determined here but expressed instead in terms of kinase activity when protamine sulphate was used as a substrate (requiring no lipid, calcium or phorbol esters for maximum activity - (359)). Using this approach we found that the B_{MAX} values, determined from the Scatchard analyses for the PKC isotypes, ranged from between 12 and

24 fmoles of PdBu per unit of activity, and were unaffected by the presence of 0.5 mM calcium or 1mM EGTA. PdBu binding to PKC α and ε was found to have incomplete saturation and the resulting Scatchard plots suggested the presence of a second binding site of low affinity for PdBu. However the technical limitations of determining non-specific binding at higher [^{3}H]PdBu concentrations, under the conditions used here (1mM PdBu used for determination of nonspecific binding at 1 μM [^{3}H]PdBu was found to disturb the micellar system significantly), precluded a quantitative analysis of any such low-affinity binding site. When units of activity were calculated in moles of enzyme present the stoichiometry of the binding at the B_{MAX} values ranged from 1.4 to 2.8 molecules of PdBu per molecule of enzyme present.

The Kd values for specific PdBu binding, determined for each PKC isotype, ranged from 1.6 to 60 nM. In the absence of calcium the Kd values of the cPKCs α, β_1 and β_2 were approximately 2 to 3-fold higher than in its presence, indicating that calcium can assist one or more parameters of the binding kinetics. PKC γ showed no decrease in Kd with added calcium, although other researchers have found evidence for a calcium effect (360). The Kd observed for the novel PKC ε was also unaffected by the presence of calcium, in agreement with earlier [^{3}H]PdBu binding observations (240), however calcium did appear to lower the Kd of PKC δ in a similar manner to the cPKC's α, β_1 and β_2. This was unexpected in that the nPKC isotypes lack the calcium binding ability associated with the C2 region, and do not show calcium sensitivity in their kinase activity (153,155,246,345,347; see however, Section 3.2).

The composition of the artificial membrane has been found to exert a great influence in the kinetics of [^{3}H]PdBu binding to PKC, and several laboratories using 100% PS micelles have found evidence of a tighter binding in such an environment (361,362). With this membrane system the Kd values were less than 1 nM for classical PKC isotypes, and appeared to be unaffected by the presence of calcium. Work on artificial membranes composed of PS and phosphatidylcholine (PC), in 20/80 mol % ratio, found two types of association of PKC to the membrane (361,363). In another report, an isolated membrane-associated PKC was found to have kinase activity independent of calcium and DAG or phorbol ester cofactors,

yet was still able to tightly bind PdBu with a Kd of < 0.5 nM, and was termed membrane-inserted. The weaker and reversible binding of PKC with such membranes exhibited cofactor dependence for both phorbol ester binding and stimulation of kinase activity, and could be converted to the membrane inserted form by high concentrations of phorbol ester (239). However some recent *in vivo* studies showed no apparent "irreversible " association of PKC α and δ with cellular membranes (368). With Triton/PS micelles the cofactor-sensitive, reversible association of PKC similar to that seen in PS/PC vesicles and inverted erythrocyte vesicles (286,364) is readily observed, whereas the membrane-inserted form has not been observed, even in the presence of PdBu (239,362,363). By using a Triton/PS mixture we observe Kd values that are well above those seen in systems where membrane-insertion occurs, and our PdBu binding appears to be cofactor-sensitive for several of the PKC isotypes, suggesting that we are measuring the weaker, reversible binding in our system. The *in vivo* PE/PKC interaction has been found to bear similarities with the Triton/PS micellar system, in that a removal of PE causes redistribution of membrane PKC to the cytosol (368).

We investigated the ability of seven different phorbol esters to compete with a fixed amount of [^{3}H]PdBu for specific binding to each PKC isotype. Since these phorbol esters were not available as radiolabeled molecules, we used competition experiments in order to determine their relative affinities for PKC isotypes. Competition studies with either cPKC or nPKC isotypes in the presence of calcium showed similar [^{3}H]PdBu titration curves to those seen in the absence of calcium. The functional group at the C20 carbon of these phorbol esters was found to be the most critical factor in the ability to compete with PdBu binding for all isotypes tested, with a free alcohol group conferring potent competition (seen with TPA, DOPP, Sap A), whereas an acetyl group (e.g. DOPPA) or a bulky aromatic group (e.g. Rx) significantly decreased or abolished competing ability. The PKC β isotypes (β_1 and β_2) were still able to show Doppa and Rx competition, however, suggesting that these isotypes have a less definite requirement for a free C20 than the other isotypes for competition, and hence their kinase activity may be more easy to stimulate by these less biologically potent phorbol esters. At the C12/C13

region, one aliphatic or aromatic ester group was adequate for potent competition with PdBu, possessing butyrate groups at both these positions. The presence of a hydroxyl group at the C5 (Thy A) position did not appear to affect the ability to compete with PdBu binding to the PKC isotypes.

Recently Kazanietz *et al* showed that DOPP had potent competing ability for PdBu bound to all isotypes, in agreement with this report, but Rx had no competing ability (362,365). They found that PdBu binding to the PKC isotypes using 100% PS vesicles was greater than the case with 20% PS vesicles, however, and we suggest that the difference between these reports and the present results may reflect the tighter membrane-inserted binding of PdBu in the former system.

Whilst competition for specific PdBu binding to the PKC isotypes is not directly comparable with earlier activation studies in the same system, the potency of TPA, DOPP, DOPPA and Rx to compete for binding was similar to activation potency in the presence of calcium (i.e. TPA and DOPP have similar, potent activation of all isotypes, but both DOPPA and Rx were only able to activate β_1, with β_2 untested (347)). The tigliane Sap A and the daphnane Thy did not conform to their kinase activation profiles, however, in that Sap A was able to potently compete for PdBu bound to PKC δ, and Thy was a poor competitor for PdBu bound to PKC α. However, further investigation of the kinase activities of these isotypes conducted on the purified recombinant protein from Sf9 cells, showed that the PKC δ preparation was found to be stimulatable by Sap A, that both PKC-δ and ε were activated with Thy A and that neither of the PKC-β isozymes was stimulatable by DOPPA (Section 3.2.). These results indicate that source of enzymes could be responsible for differences observed (bovine brain purified and COS cell-expressed preparation were used in previous work; see also ref. 367). Additionally we found that with the preparations of PKC α from recombinant Sf9 cells, a rapid loss of phorbol ester-stimulated kinase activity occurred, although no evidence of proteolysis to PKM or loss of PdBu binding ability was experienced. The discrepancy in PKC δ and α behaviour may lie in post translational processing differences of these isotypes between the two systems from which these isotypes were purified; this may also explain the shift in Kd in PdBu binding apparent with calcium for PKC δ. It has recently been discovered using fusion proteins of the

C1/C2 domains of c- and n-PKCs that the C1 domain of PKC ε can bind calcium in the place of magnesium as a divalent cation (rather than the C2 region), while the C2 region imparts calcium selectivity (210). The divalent metal ion dependence of the C1 region may offer an explanation of the change in the Kd of PKC δ in the presence of calcium when interacting with an artificial membrane system.

The significance of different states of PKC association with membranes in the physiological situation is not known. Whilst transient translocation of cytosolic PKC can be observed with a variety of agonists, this does not exclude a membrane-inserted PKC playing a role in the PKC signalling system.

3.2. In vitro calcium and phorbol ester induced translocation of PKC isotypes

3.2.1. Introduction

In vivo interaction of different PKC activators (including phorbol esters and physiological activator-diacylglycerol) with PKC involves redistribution of the PKC pool from the cytosolic (soluble) to the membrane bound (particulate) fraction. This phenomenon is known as PKC translocation (345,372-374). It is thought that PKC translocation is followed by an activation step and phosphorylation of substrate(s). Using artificial detergent/phospholipid or mixed phospholipid membrane systems, recent studies revealed differences in the ability of structurally distinct phorbol esters to bind and activate purified PKC isotypes (347,371). However similar *in vivo* studies revealed some discrepancies with *in vitro* experiments (380-382). Additionally a greater complexity of the *in vivo* PKC translocation-activation relationship has been reported (375-379).

These observations prompted us to investigate the reason(s) for *in vivo/in vitro* differences in phorbol ester-PKC interaction.

Using human promyelocytic (HL-60) cell membranes in an *in vitro* model, we studied calcium and phorbol ester induced PKC translocation. Additionally, ionic conditions (found to influence PKC-membrane interaction) were chosen to match those inside the cell.

3.2.2. Experimental procedures

Preparation of membranes

Human promyelocytic leukaemia HL-60 cells (ICRF - London) were grown as described in Section 2.4.1. Cells were typically harvested at a concentration of 10^6 cells/ml by centrifugation (2000 rpm for 5 minutes), washed twice in buffer **A** (20 mM Hepes pH 6.9, 3 mM EGTA, 2mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 100 µg/ml leupeptine, 50 µg/ml PMSF and 1 mM DTT) at room temperature. Cells

were finally resuspended in buffer **A** (2-5 x 10^8 cells/ml) and lysed by 3 repetitive freeze/thaw cycles in liquid nitrogen. Unbroken cells and nuclei were pelleted by centrifugation (2000 rpm for 10 minutes). Supernatant (cell-free, as judged by light microscopy) was subjected to further centrifugation (10,000 x g for 5 minutes) on a benchtop eppendorf - test tube centrifuge (Microspin245, Sorval Instruments, Du Pont) and the pellet resuspended in buffer **A** (at a concentration of 2 mg/ml of membrane proteins). Aliquots (1 ml) of membrane suspension were snap frozen in liquid nitrogen and kept at - 20 ^{0}C. Before the assay, an aliquot of HL-60 membrane suspension was thawed and incubated at 70 ^{0}C for 10 mins in order to eliminate any indigenous kinase activity (similar results were obtained without heat treatment, however, indigenous kinase present in membranes contributed to a high "background" activity). "Heat inactivated" membranes were pelleted (10 000 x g, 5 min) separated from the supernatant and finally resuspended in the same volume of fresh buffer **A**.

Purification of PKC isotypes

PKC isotypes α, β_1, β_2, γ, δ, ε and ζ were expressed in Sf 9 cells using baculovirus expression system (25). For purification of individual isotypes the same procedure as described earlier (Section 2.2.3.) was used, exept that, because of the instability of PKC α, only one-step purification on the Hi-Trap Mono Q column has been used. Active fractions were pooled and stored at - 20 ^{0}C after being dialysed for 4 hours against: 50 % (v/v) glycerol, 20 mM Tris-HCl, pH 7.5, 1mM EDTA, and 5 mM dithiothreitol. In order to investigate the effects of cation chelators on enzyme stability upon storage, a portion of the active pool was also dialysed with the same buffer lacking EDTA and supplemented with 1 mM $ZnCl_2$.

PKC assay

PKC activity was assayed by measuring the incorporation of ^{32}P from ^{32}P-γ-ATP into substrate as previously described in Section 2.2.4. (26). The substrate used was protamine sulphate. PKC isotypes were diluted in 20 mM Tris-HCl, pH 7.5, 1mM EDTA, and 5 mM dithiothreitol before use. In translocation experiments 0.1 unit (1 unit incorporated 1 nmol ^{32}P into protamine sulphate per

minute under kinase assay conditions in the absence of calcium, lipids and phorbol esters), of enzyme activity was used per assay.

PKC Translocation assay

The assay mixture was prepared by mixing 50 µl of membrane suspension (equivalent of 100 µg membrane proteins, see Section 2.2.6.) and 1 µl of $CaCl_2$ (in H_2O) or phorbol ester solution (in EtOH). Solutions of different $CaCl_2$ and phorbol ester concentrations were used in order to achieve the desired final concentration. The mixture was thorougly mixed by repetitive vortexing and left to equilibrate for 15 min at 25 ^{0}C. The assay was started by adding 25 µl (0.1 U) of purified enzyme and the assay mixture was incubated at 25 ^{0}C for 15 minutes. The assay was terminated by pelleting the membranes (10 000 x g, 5 min) at 4 ^{0}C. For determination of total enzyme activity, 5 µl of reaction mixture was taken before centrifugation and treated in the same way as the pellet. Supernatant containing unbound PKC was removed and an aliquot used to determine PKC activity. The pellet was washed twice with ice cold buffer **A** (20 mM Hepes pH 6.9, 3 mM EGTA, 2mM $MgCl_2$, 20 mM NaCl, 100 mM KCl, 100 µg/ml leupeptin, 50 µg/ml PMSF and 1 mM DTT) containing corresponding concentrations of calcium or phorbol ester. To determine the amount of PKC asociated with membranes, the membrane pellet was solubilised in 70 µl of 20 mM Hepes pH 7.5, 1 % (v/v) Triton X-100 and 1 mg/ml protamine sulphate. PKC activity was assayed by measuring the incorporation of ^{32}P from ^{32}P-γ-ATP into protamine sulphate (see above). The percentage of induced translocation was calculated using values of induced translocation at highest Ca^{2+} (2 mM) or phorbol ester (20 µM) concentration as 100 %.

3.2.3. Results

Utilising isolated HL-60 cell membranes and purified recombinant PKC isotypes α, β_1, β_2, γ, δ, ε, ν and ζ we studied the ability of calcium and 7 different phorbol esters to induce association of these isotypes with cellular membranes.

We decided to use HL-60 cellular membrane fractions because this human leukemic cell line has been extensively studied by our research group. Additionally the membrane fraction (10 000 x g, 5 min), obtained using a benchtop eppendorf - test tube centrifuge (Microspin245, Sorval Instruments, Du Pont) allowed us to develop a relatively fast and reproducible assay. The amount of membrane used per assay (equivalent to 100 μg membrane proteins and ~ 7 x 10^6 cells) was determined to be a minimum required for reliable manipulation and pellet visualisation.

The amount of PKC (free and membrane associated) was determined through an activation assay using protamine sulphate as substrate. PKC phosphorylation of protamine sulphate has been shown to be independent of the presence of calcium and/or phorbol esters (239). This allowed us to determine the amount of PKC activity, regardless of differences in cofactor/activator dependency of different PKC isotypes, and use this as a measurement of translocation.

All PKC isotypes used showed spontaneous (in the absence of Ca^{2+} and phorbol esters) association with HL-60 membranes, ranging from 8-68 % of total enzyme in the assay (see Table 3.2.1., p. 126). PKC-η appeared to behave in a significantly different manner from other isotypes,with up to 68 % spontaneous membrane association, reflecting its higher affinity for the membranes used.

The percentage of membrane associated enzyme was calculated using total activity of PKC added in the assay as 100 %. Residual enzyme activity determined in the supernatant containing unbound PKC was found to add up, with the corresponding membrane associated fraction, to 100 % with an error of less than 15 %.

Table 3.2.1. Association of PKC isozymes α, β_1, β_2, γ, δ, ε, η and ζ with HL-60 cell membranes. PKC membrane association in the absence of added phorbol ester or Ca^{2+} (min) and 20 μM phorbol ester or 2mM free Ca^{2+} (max).

PKC	membrane association (min/max), % total enzyme*							
	TPA	PdBu	DOPP	DOPP A	Sap A	Thy A	Rx	Ca^{2+}
α	18/82	18/65	18/94	18/39	18/75	18/53	18/18	15/83
β_1	16/98	15/89	13/78	20/27	21/99	23/99	22/28	18/98
β_2	10/74	10/54	10/49	10/14	10/68	10/60	10/13	10/70
γ	9/40	9/43	9/44	9/10	9/30	9/31	9/9	7/55
δ	10/70	10/56	10/62	10/18	10/66	10/52	10/11	17/71
ε	20/57	20/42	20/44	20/33	20/54	20/53	20/20	20/44
η	68/92	68/88	68/98	68/77	68/86	68/77	68/74	67/68
ζ	8/10	8/8	8/11	8/10	8/10	8/7	8/10	8/11

*Mean values of three separate experiments are shown. S.E.M. values did not exceed 15% of mean.

Under the experimental conditions used, induced translocation of all isotypes (except ζ) was a rapid process reaching equilibrium within 10 minutes (see Figure 3.2.1. A. and B., pages 129-130) for all PKC izotypes investigated. We also found that incubation of up to 60 min at room temperature did not affect the stability of PKC translocation.

Ca^{2+} induced translocation

While Mg^{2+} at concentrations of up to 2 mM did not induce any translocation of PKC isotypes (data not shown), micromolar concentrations of Ca^{2+} were able to induce translocation of all investigated PKC isotypes exept η and ζ. Free Ca^{2+} concentration (buffered with EGTA) ranged between 0.02 μM and 2 mM (as calculated with Chelate software). EC_{50} values (Ca^{2+} concentration inducing 50 % maximum association) for Ca^{2+}-induced translocation were determined to be between 2.8 and 6.8 μM (see Table 3.2.2. p. 128). A representative example of Ca^{2+} induced membrane association of PKC-α is presented in Figure 3.2.2. H., p. 139. In all cases (PKC α, β_1, β_2, γ, δ and ε) Ca^{2+} at a concentration of 1 μM began to induce translocation, reaching saturation (maximum of translocation) at approximately 100 μM. This is in agreement with the *in vivo* situation where, upon stimulation, intracellular Ca^{2+} levels are reaching micromolar levels. It is thought that this rise in Ca^{2+} concentration is able to induce translocation of cPKCs. Calcium induced translocation also proved to be a rapid process reaching equilibrium within 10 minutes (see Figure 3.2.1. A., p. 129) for all PKC isotypes investigated (exept η and ζ).

Calcium induced translocation of PKC isotypes ε and to some extent δ was unexpected. These isotypes are thought not to be dependent on Ca^{2+} for activation. In addition several recent reports confirmed that association, of only c PKCs α, β_1 and β_2 but not δ, with cellular membranes *in vivo,* was Ca^{2+} dependent (240,384,385). However one report on calcium dependent translocation of PKC ε *in vivo* was in agreement with the findings described here (209).

Table 3.2.2. Phorbol ester and Ca^{2+} induced association of PKC isotypes with HL-60 cell membranes.

PKC	Tot_{50}*, μM (EC_{50})**, μM							
	TPA	PdBu	DOPP	DOPPA	Sap A	Thy A	Rx	Ca^{2+}
α	0.61 (0.61)	1.5 (0.9)	0.2 (0.24)	>20 (0.06)	4.2 (3.5)	19 (2.3)	>20 (>20)	5 (4.5)
β_1	0.24 (0.64)	0.84 (2.1)	2.2 (1.9)	>20 (0.01)	0.3 (0.6)	0.52 (1.9)	>20 (0.1)	2 (2.8)
β_2	2.2 (1.9)	9 (2.0)	20 (1.8)	>20 (0.23)	10.2 (5.4)	10.1 (9.0)	>20 (>20)	7.9 (3.1)
γ	> 20 (2.1)	> 20 (6.0)	>20 (3.5)	>20 (>20)	>20 (6.9)	>20 (5.5)	>20 (>20)	69 (6.8)
δ	0.31 (0.03)	>20 (0.18)	>20 (0.16)	>20 (0.39)	6 (0.25)	16 (1.2)	>20 (>20)	>2mM (2.0)
ε	0.98 (0.47)	4.5 (0.74)	2.8 (0.89)	>20 (0.25)	7.1 (2.8)	13.1 (2.0)	>20 (>20)	2.2 (1.8)
η	(1.00)	(0.32)	(0.25)	(2.2)	(1.5)	(0.4)	(0.14)	>2mM
ζ	>20 (>20)	>20 (>20)	>20 (>20)	>20 (>20)	>20 (>20)	>20 (>20)	>20 (>20)	>2mM >2mM

* Concentration required to induce translocation of 50 % of total enzyme in the assay.

** Concentration required to induce 50 % of maximum translocation.

Mean values of three separate experiments are shown. S.E.M. values did not exceed 15 % of mean.

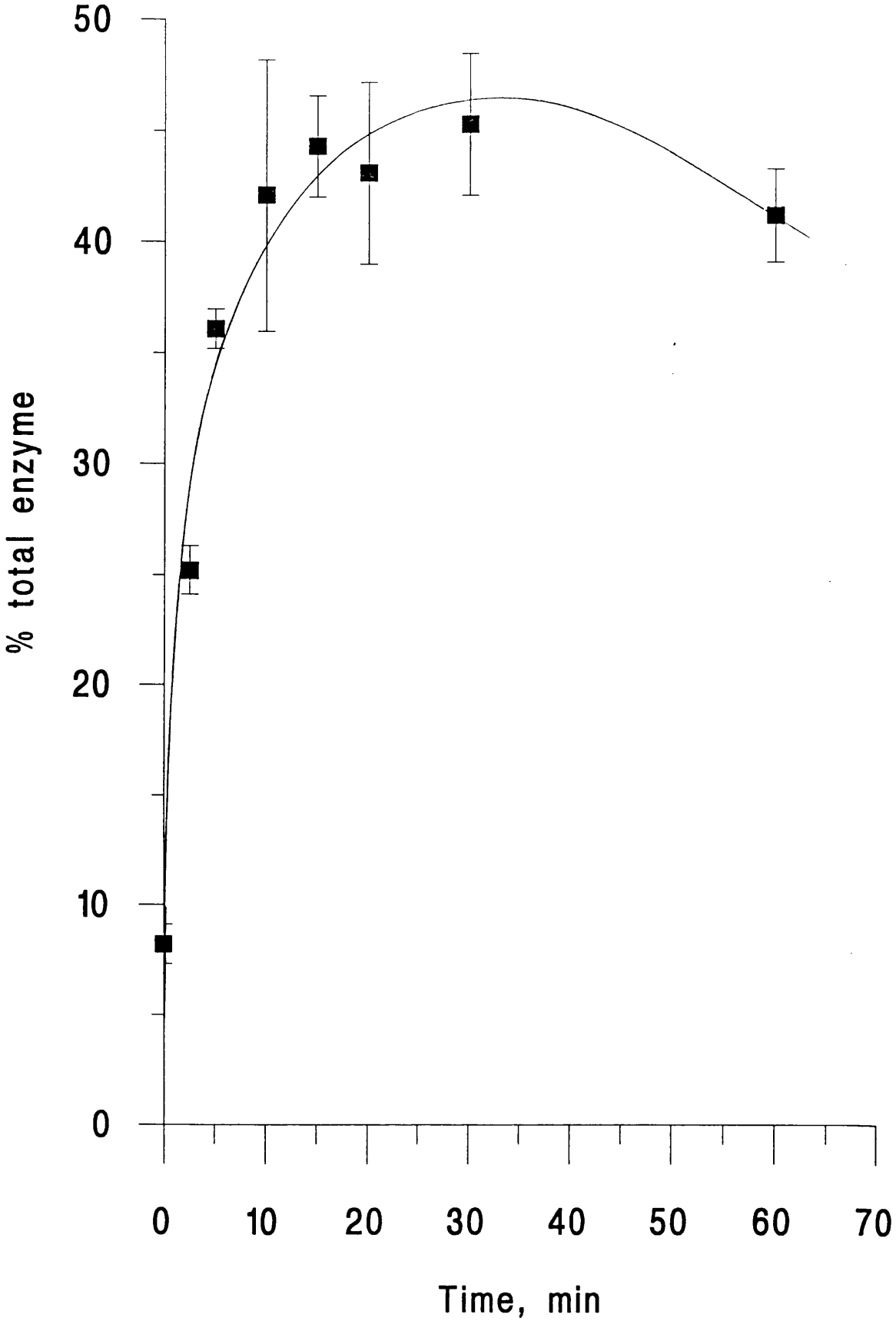


Figure 3.2.1. A. Ca^{2+} induced association of PKC-α with HL-60 cell membranes. Ca^{2+} (3 μM) induced PKC-α association with HL-60 membranes was measured over 60 minutes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment

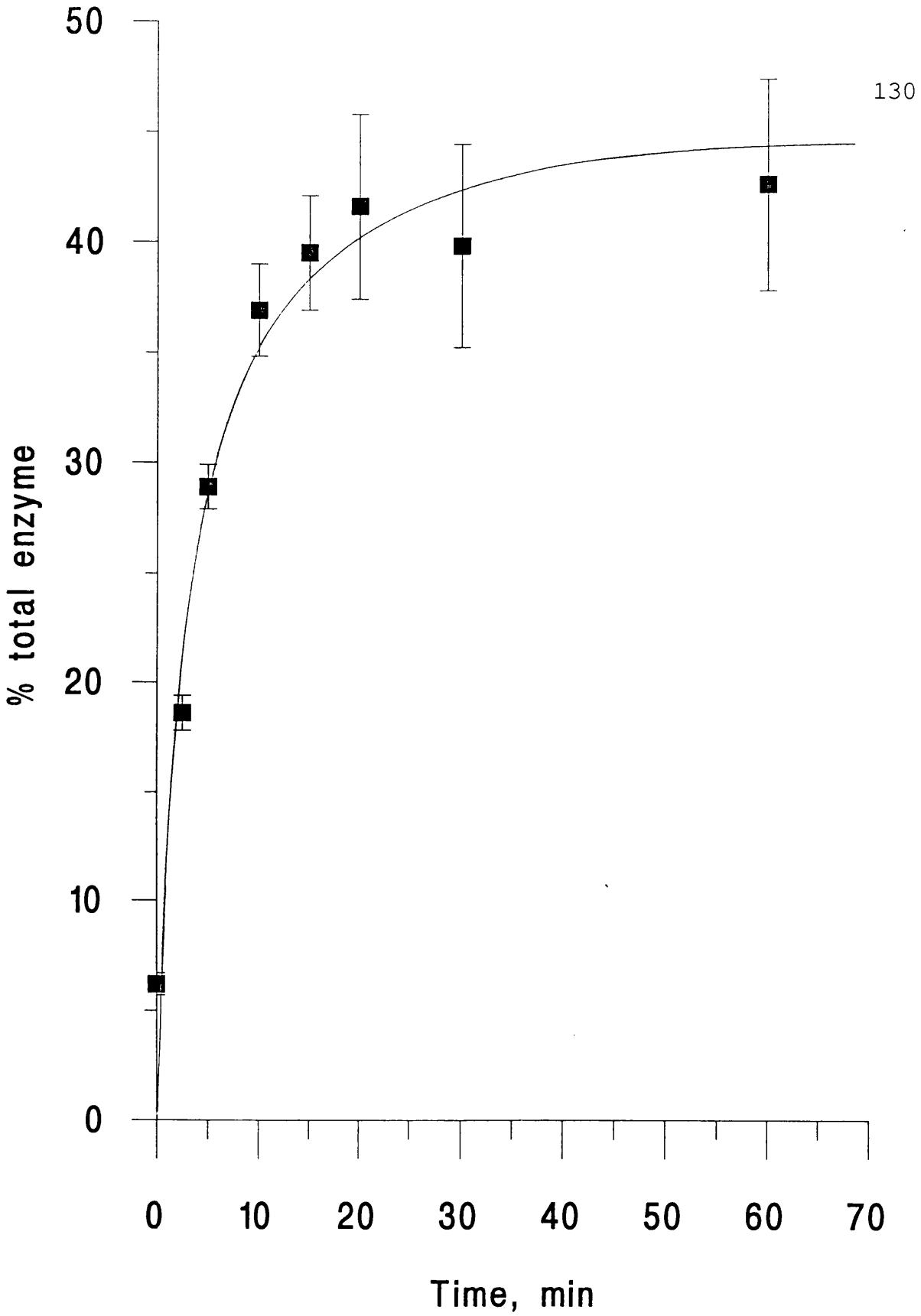


Figure 3.2.1. B. TPA induced association of PKC-α with HL-60 cell membranes. TPA (200 nM) induced PKC-α association with HL-60 membranes was measured over 60 minutes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment

Phorbol ester induced translocation

We have studied the ability of seven phorbol esters (TPA, PdBu, DOPP, DOPPA, Sap A, Thy A and Rx, for structures see Section 3.1., p. 94) to induce association of different PKC isotypes with HL-60 membranes in suspension.

Phorbol esters were used at final concentration of 0.002 - 20 μM, with no free Ca^{2+} present. As in the case of calcium, phorbol estrer induced translocation also proved to be a rapid process reaching equilibrium within 10 minutes (see Figure 3.2.1. B., p. 130) for all PKC izotypes investigated (except for PKC-ζ).

An example of a typical experiment of phorbol ester induced translocation of PKC-α is shown on Figure 3.2.2. A.-G., pgs. 132-138. Here TPA, PdBu, DOPP, Sap A, Thy A and to some extent DOPPA were able to induce association of PKC α with HL-60 membranes, whereas Rx showed no effect. Values for spontaneous and maximum induced translocation (as percent of total enzyme) are given in Table 3.2.1., p. 126. Concentrations at which 50 % of total enzyme amount was translocated (TOT_{50}) or 50 % maximal translocation (EC_{50}) of different PKC isozymes was induced by a range of phorbol esters are presented in Table 3.2.2., 128.

A comparison of two 12-deoxy phorbols used in this study (DOPP and DOPPA) in induction of PKC α, β_1, β_2, γ, δ, ε and η translocation is presented in Figure 3.2.3. A.-H., pgs. 140-147. In all cases exept for PKC α and δ, in the concentration range used, DOPPA showed to be unable to induce enzyme association with the membranes. On the other hand with all PKC isotypes investigated, DOPP was very efficient at inducing enzyme translocation, EC_{50} range from 0.16-3.5 μM.

Under conditions used none of the phorbol esters tested was able to induce any translocation of PKC ζ.

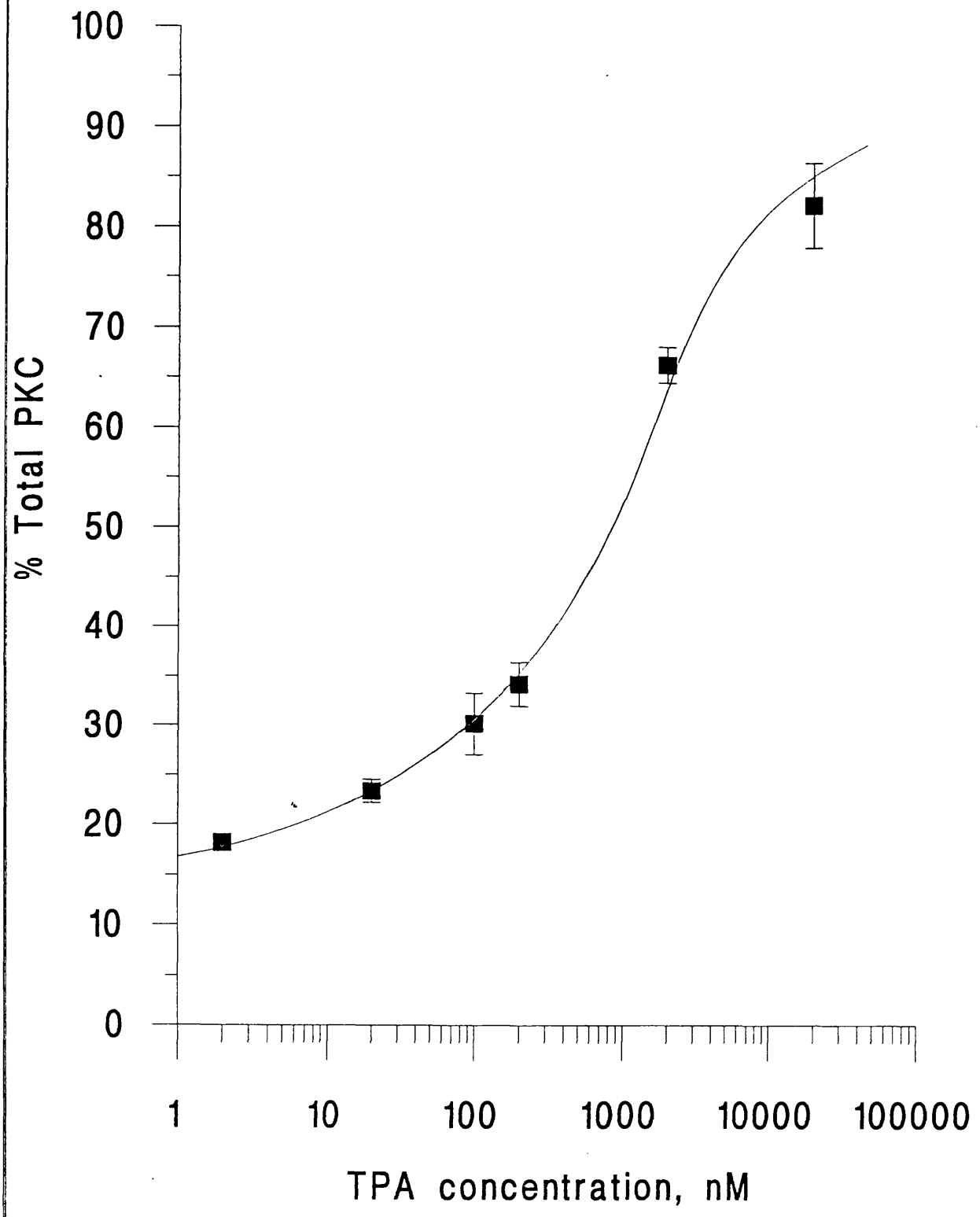


Figure 3.2.2. A. TPA induced association of PKC-α with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

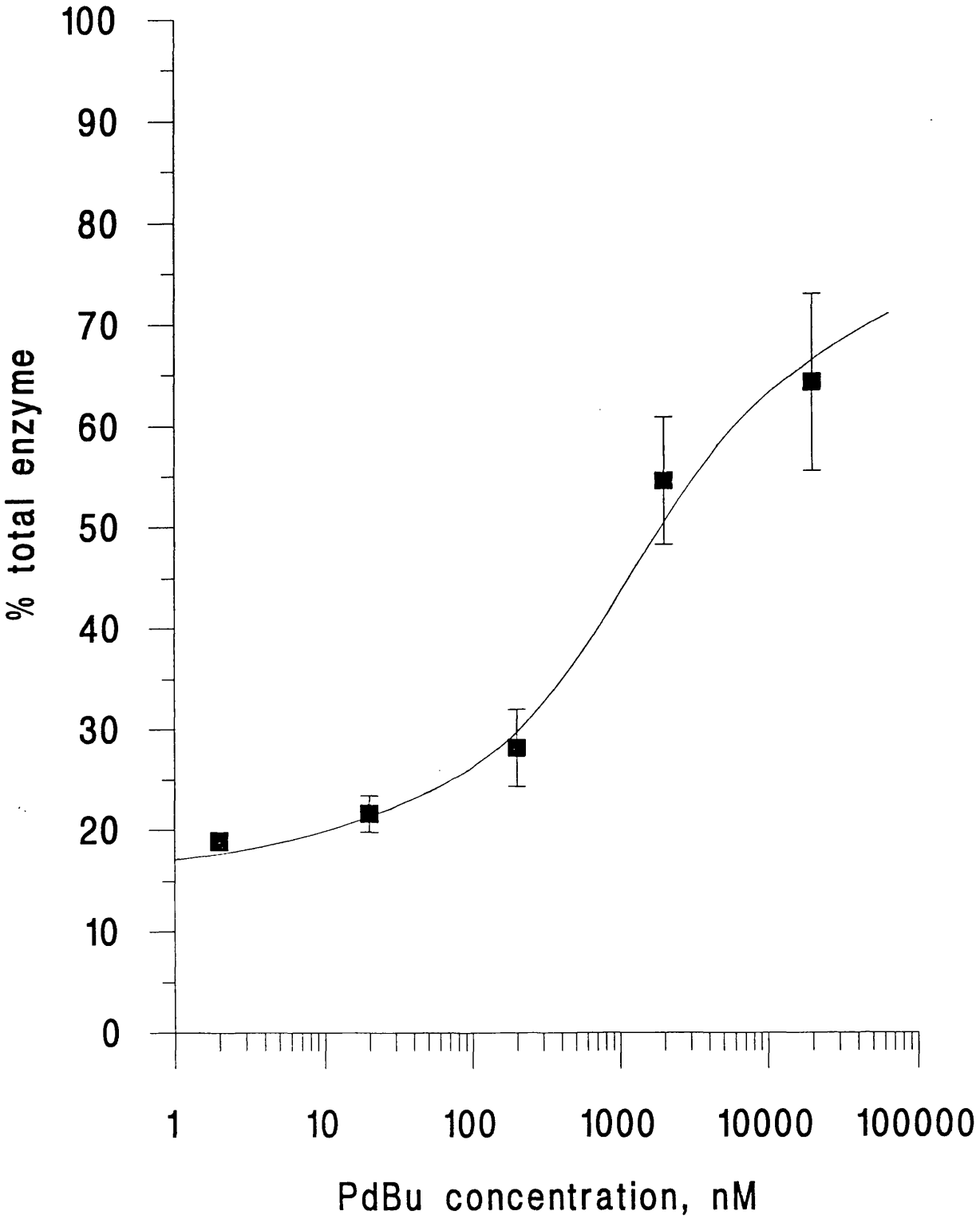


Figure 3.2.2. B. PdBu induced association of PKC-α with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

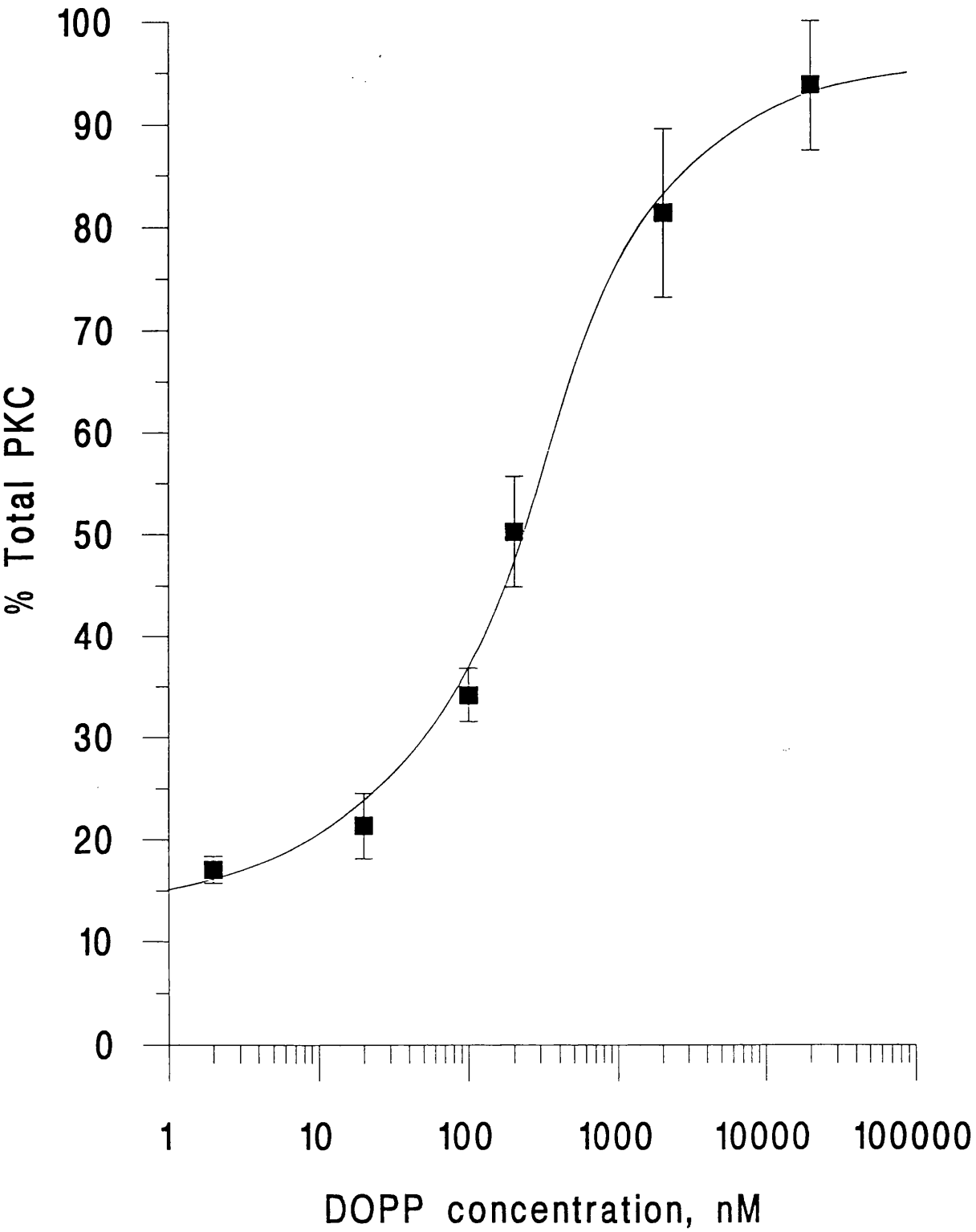


Figure 3.2.2. C. DOPP induced association of PKC-α with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

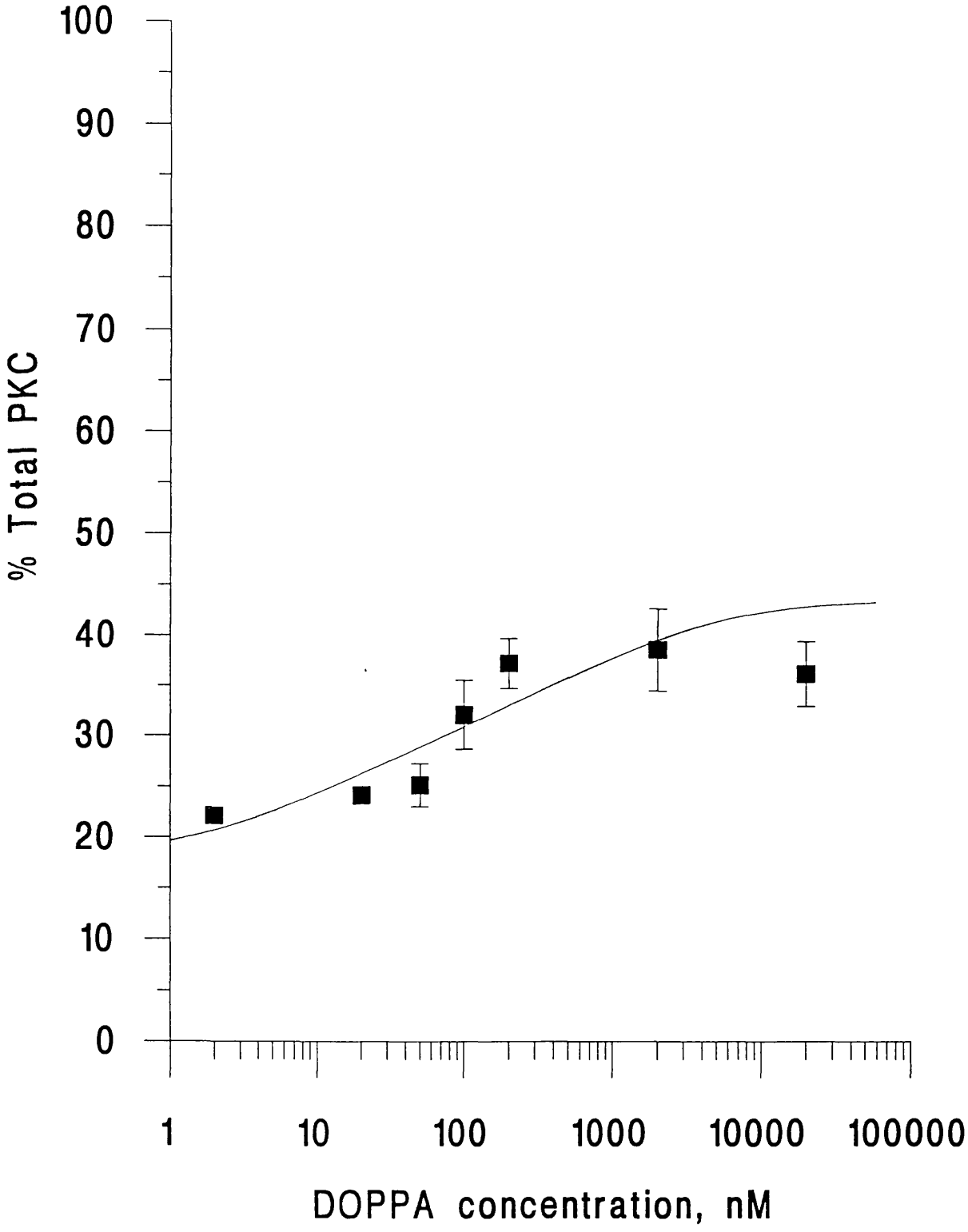


Figure 3.2.2. D. DOPPA induced association of PKC-α with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

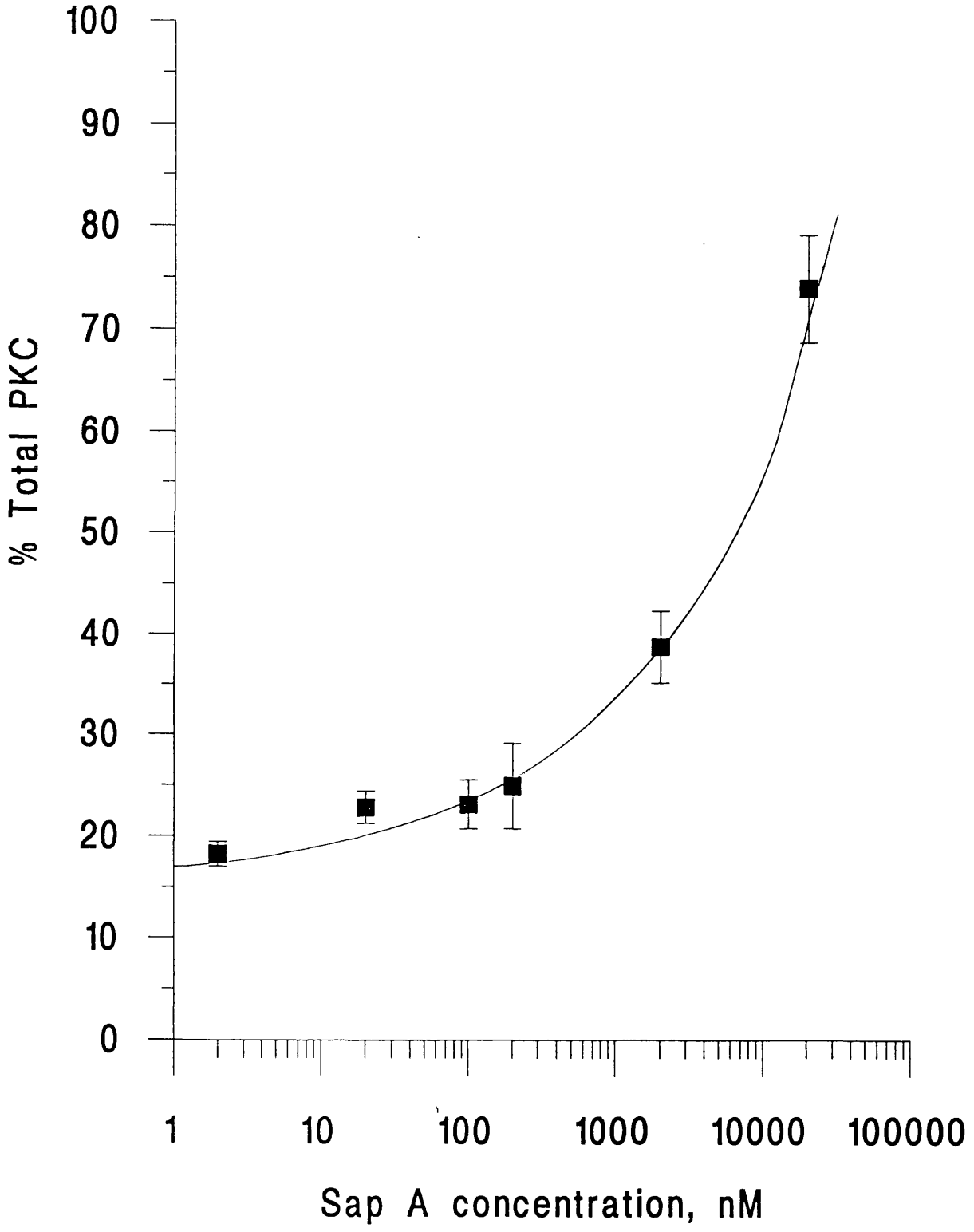


Figure 3.2.2. E. Sap A induced association of PKC-α with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

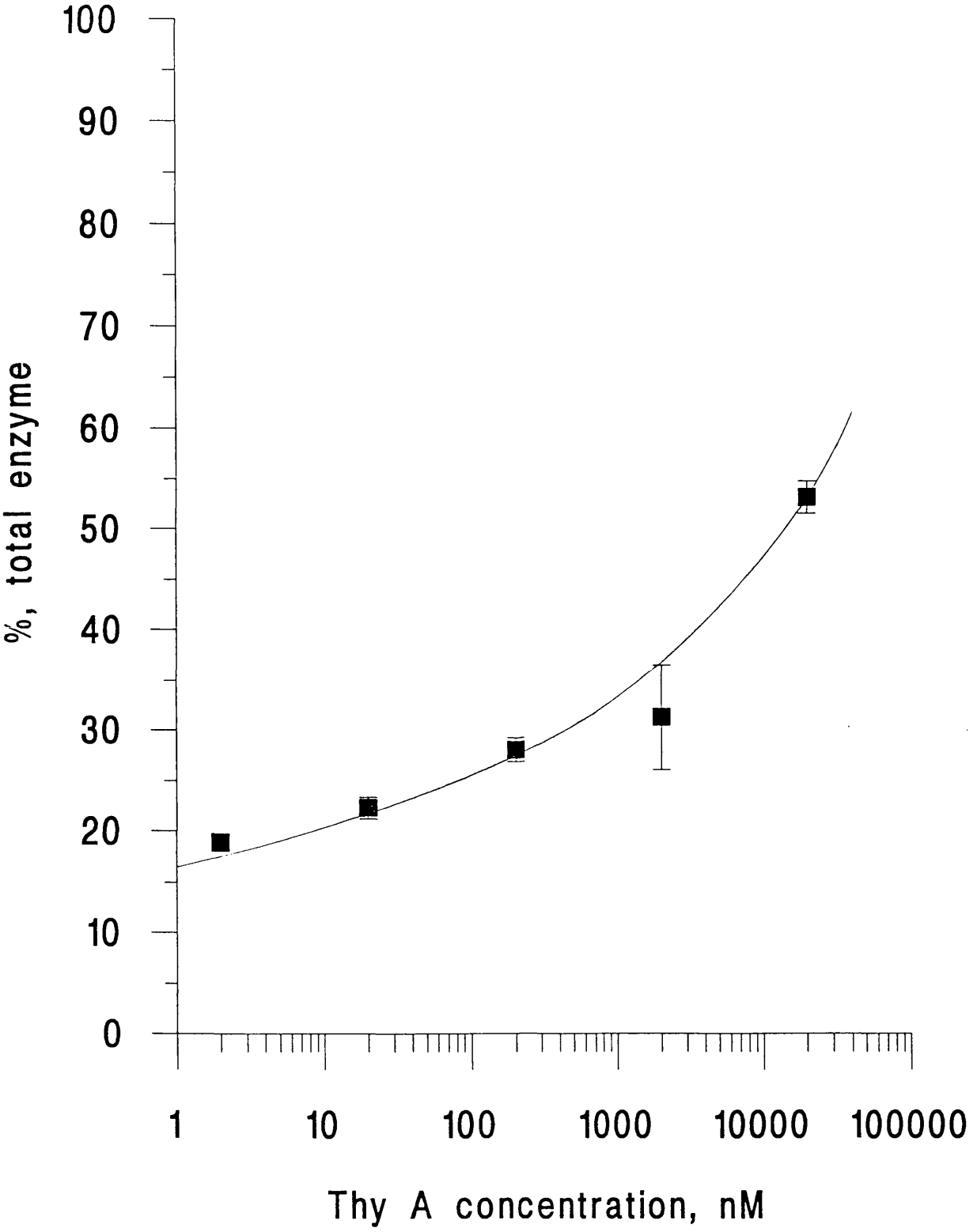


Figure 3.2.2. F. Thy A induced association of PKC-α with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

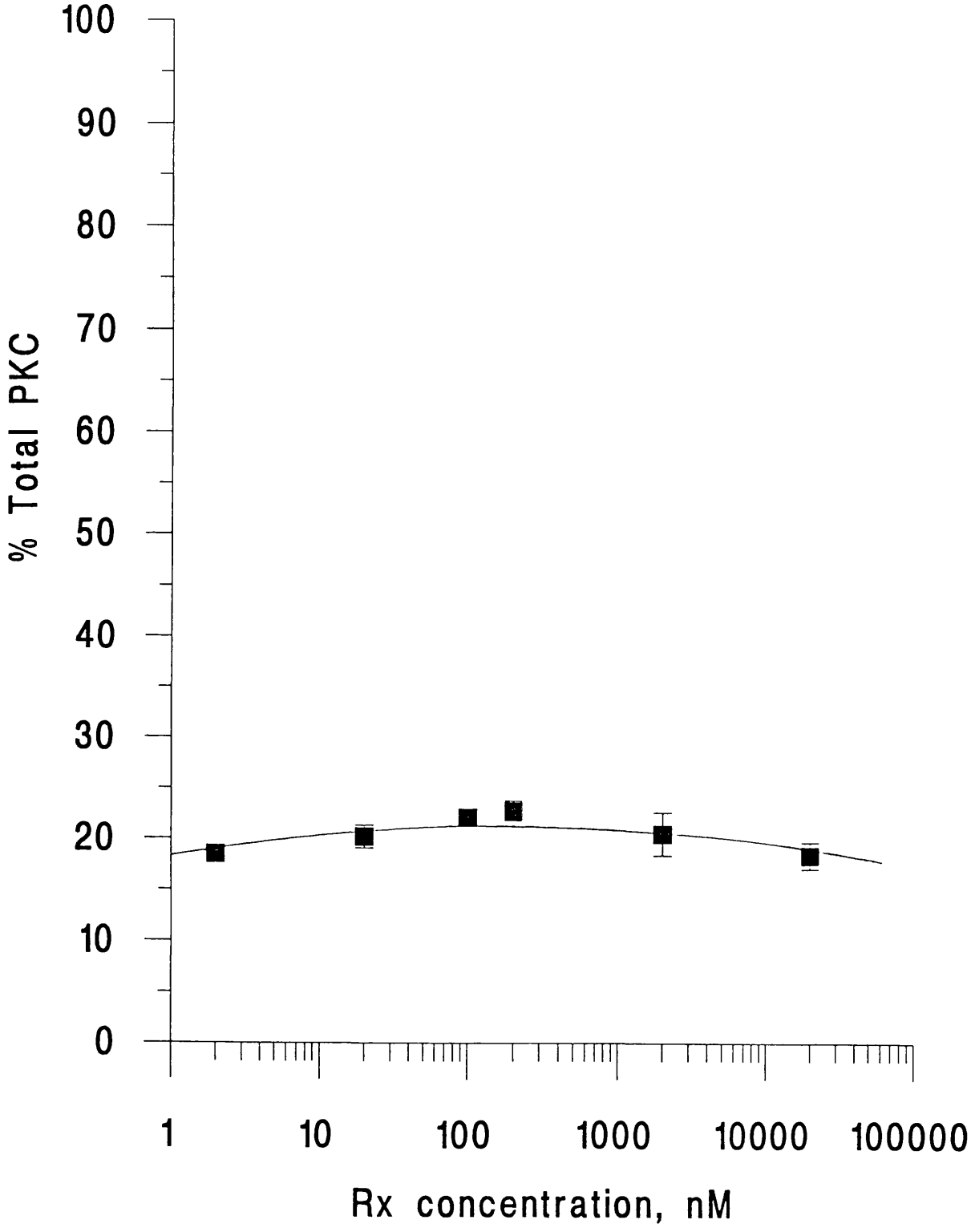


Figure 3.2.2. G. Rx induced association of PKC-α with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

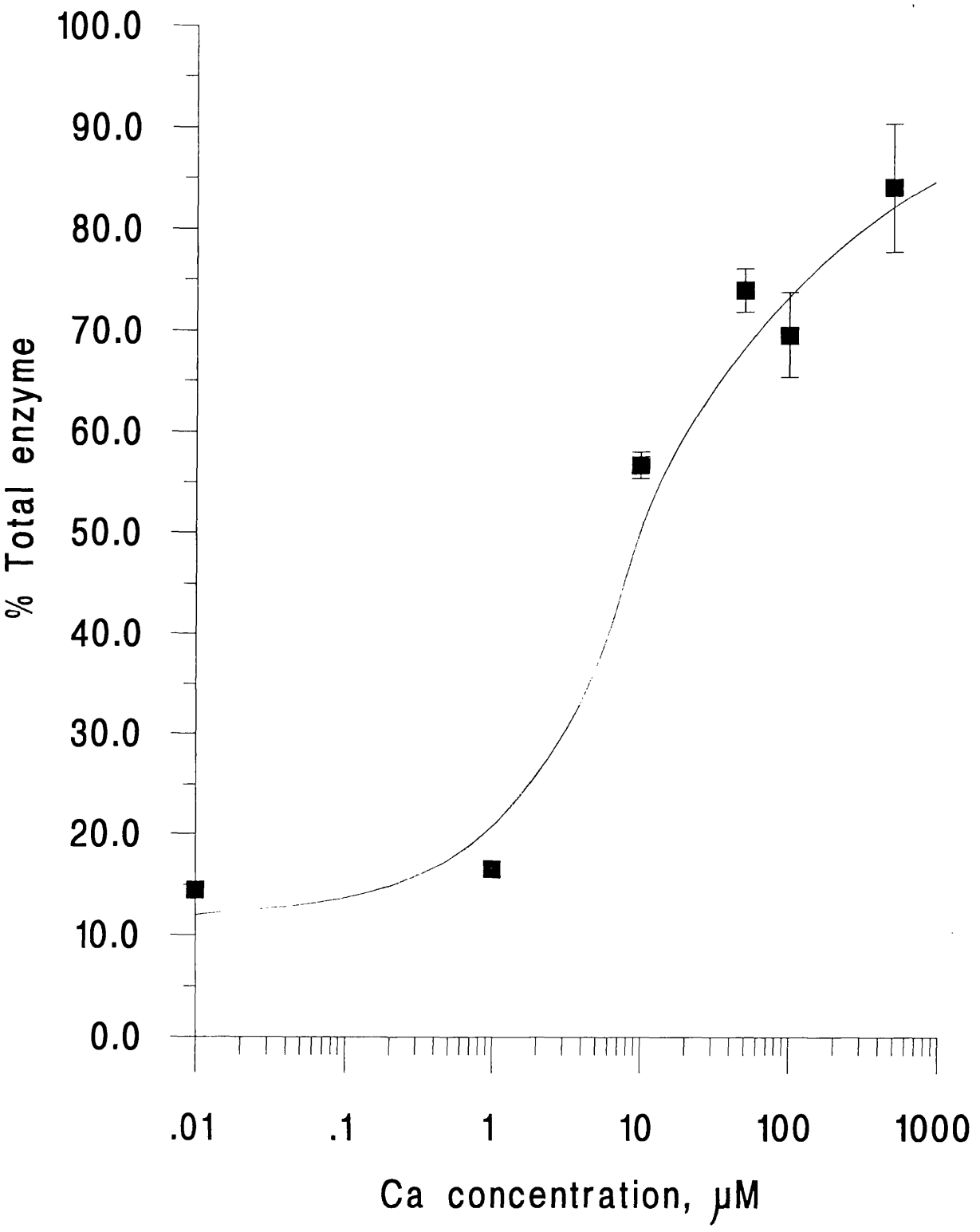


Figure 3.2.2. H. Ca^{2+} induced association of PKC-α with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

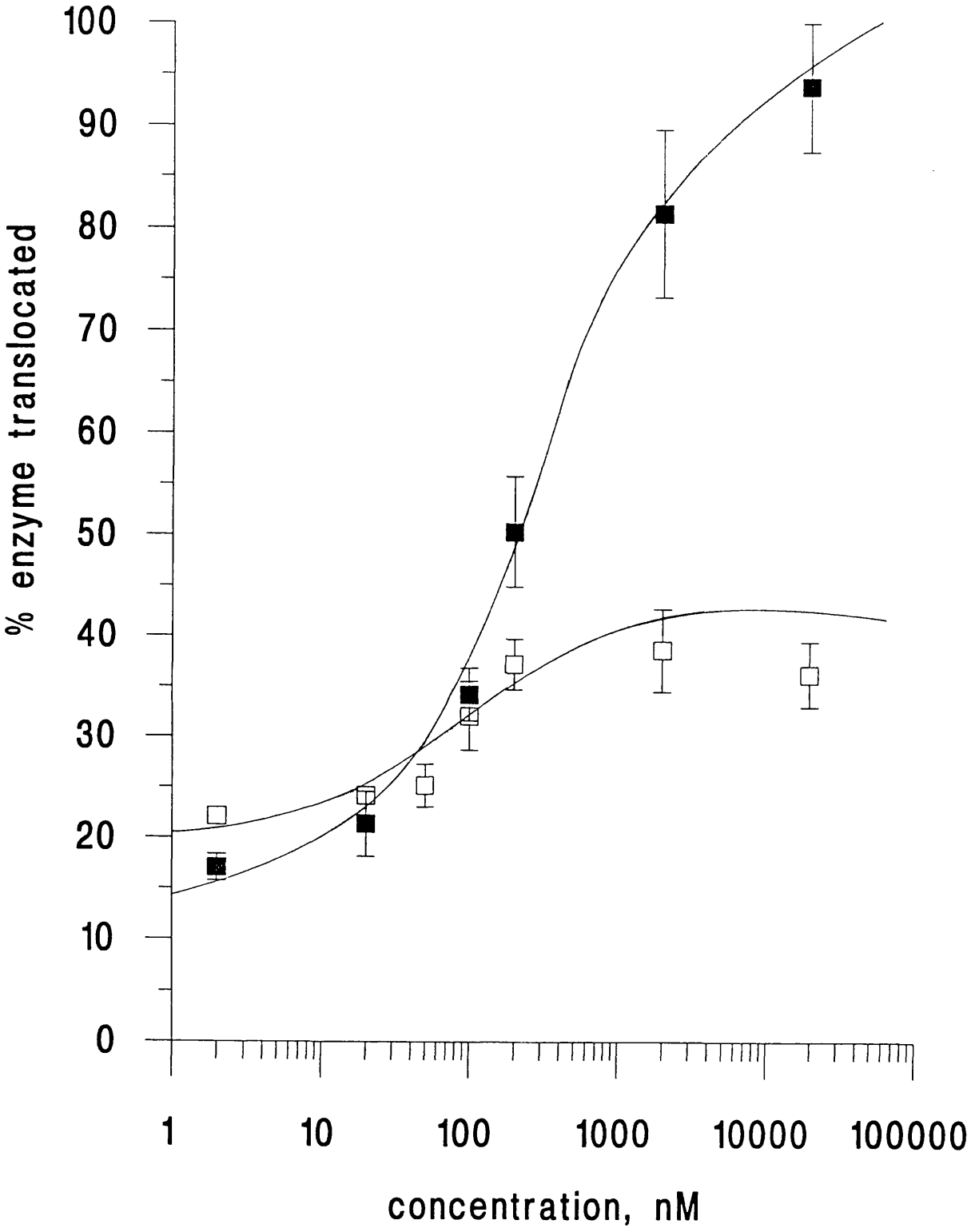


Figure 3.2.3. A. DOPP (■) and DOPPA (□) induced association of PKC-α with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

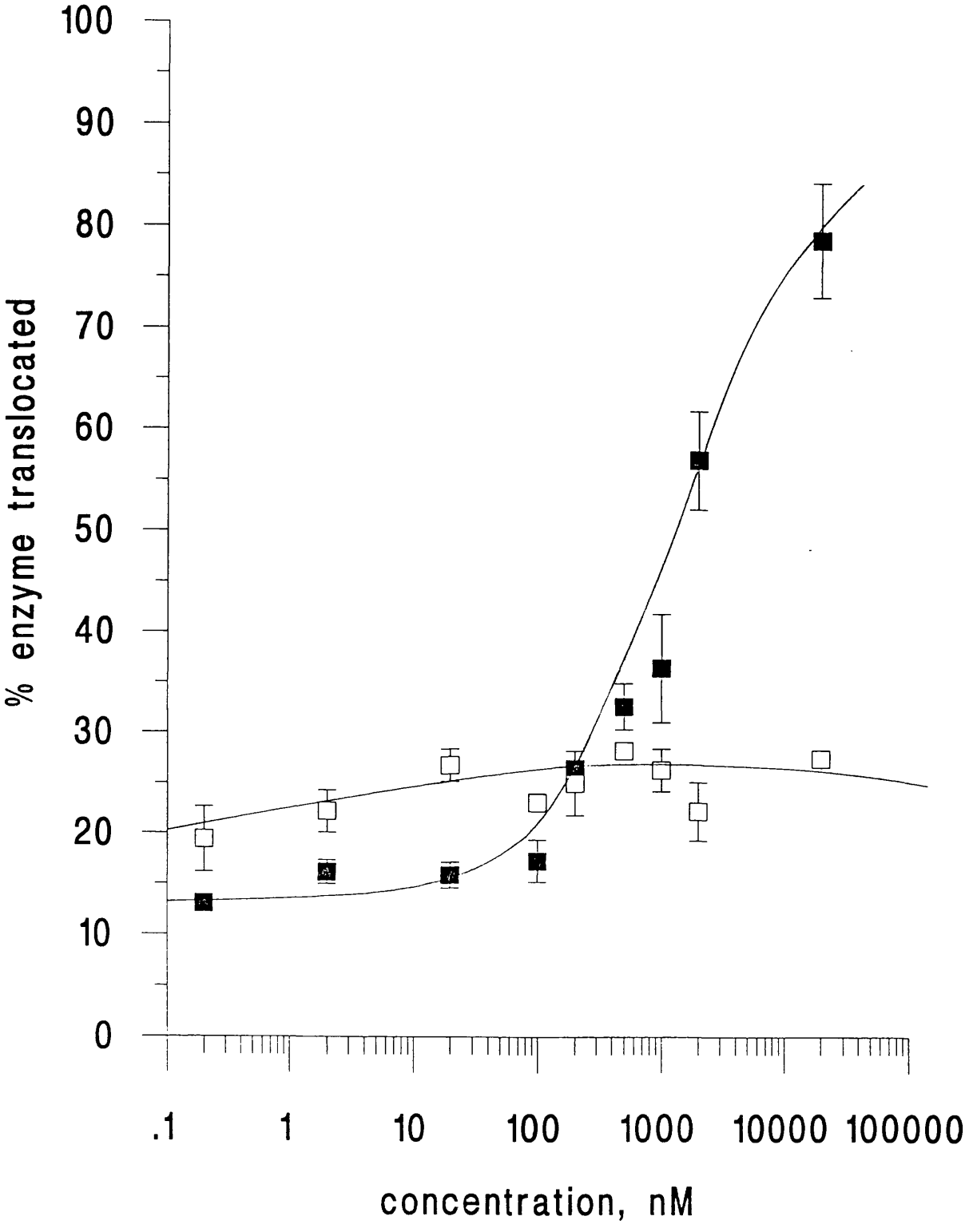


Figure 3.2.3. B. DOPP (■) and DOPPA (□) induced association of PKC-β_1 with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

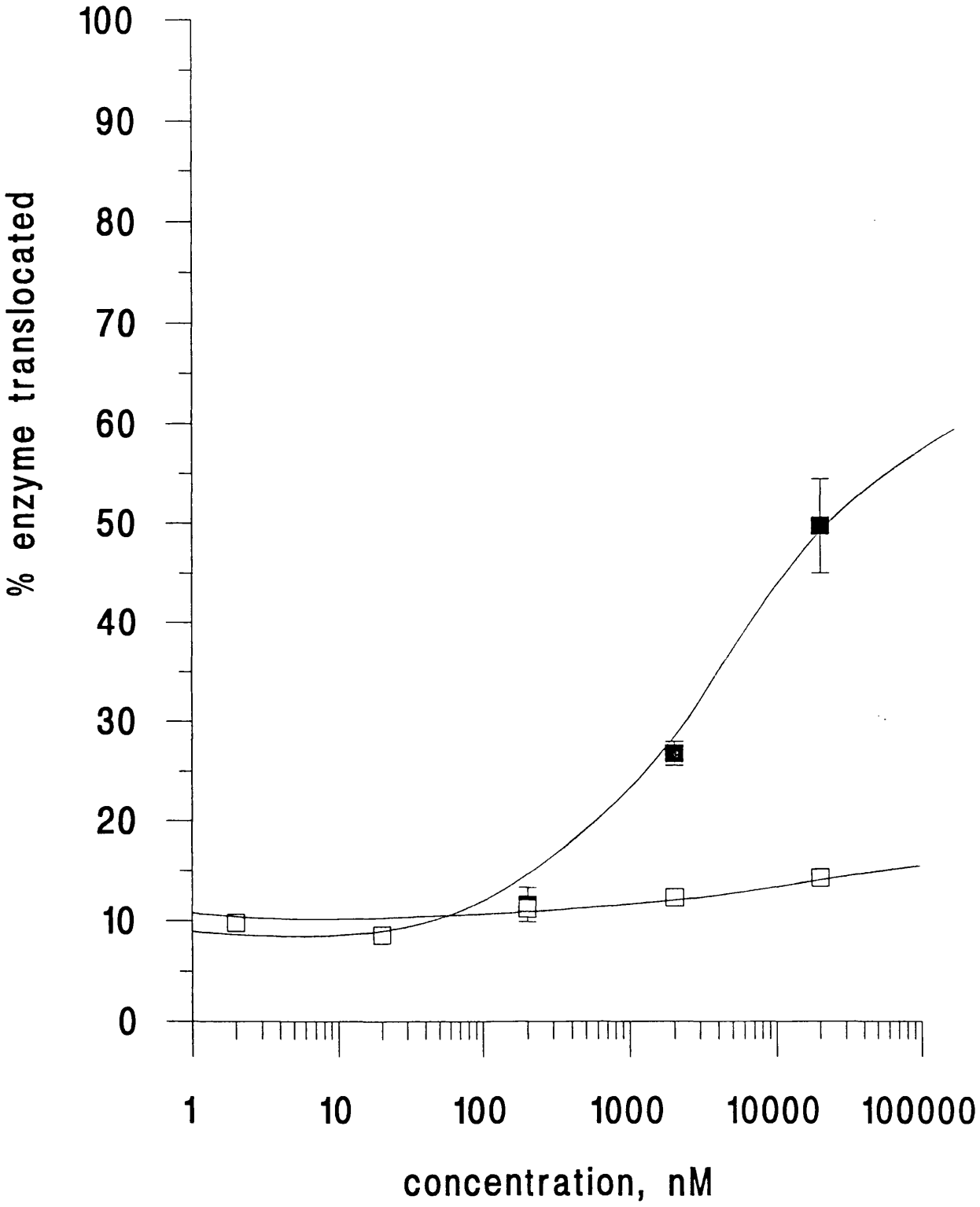


Figure 3.2.3. C. DOPP (■) and DOPPA (□) induced association of PKC-β_2 with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

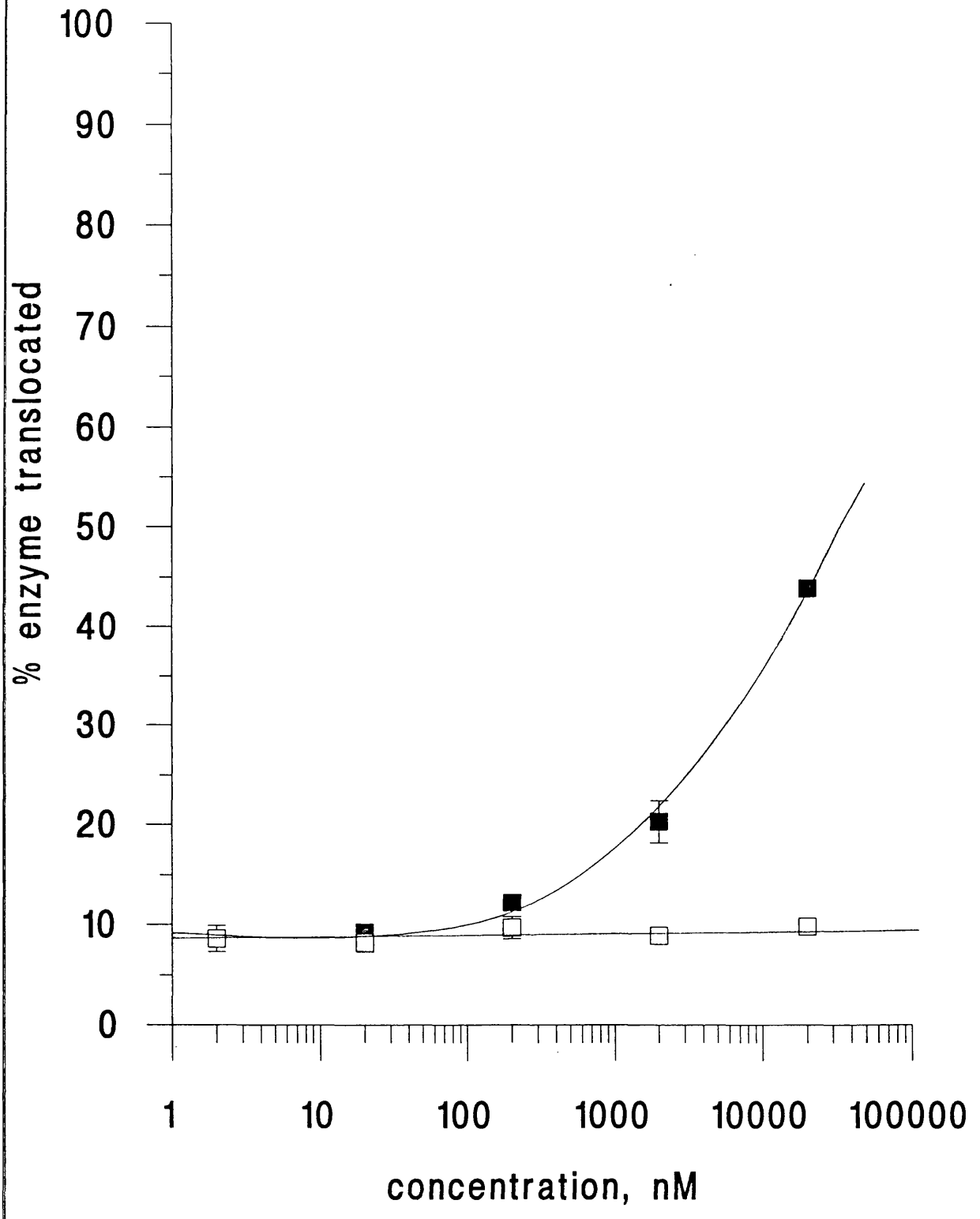


Figure 3.2.3. D. DOPP (■) and DOPPA (□) induced association of PKC-γ with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

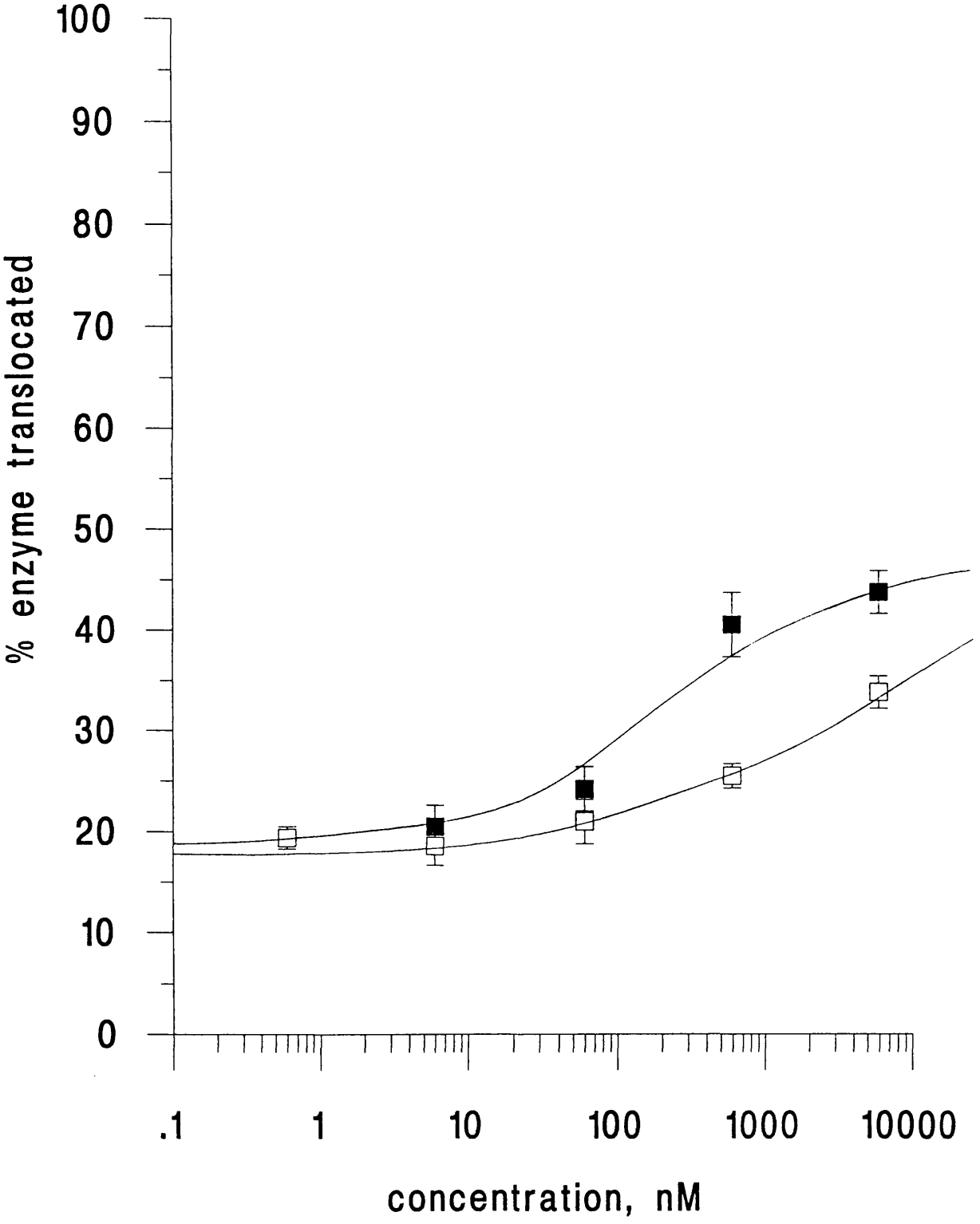


Figure 3.2.3. E. DOPP (■) and DOPPA (□) induced association of PKC-δ with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

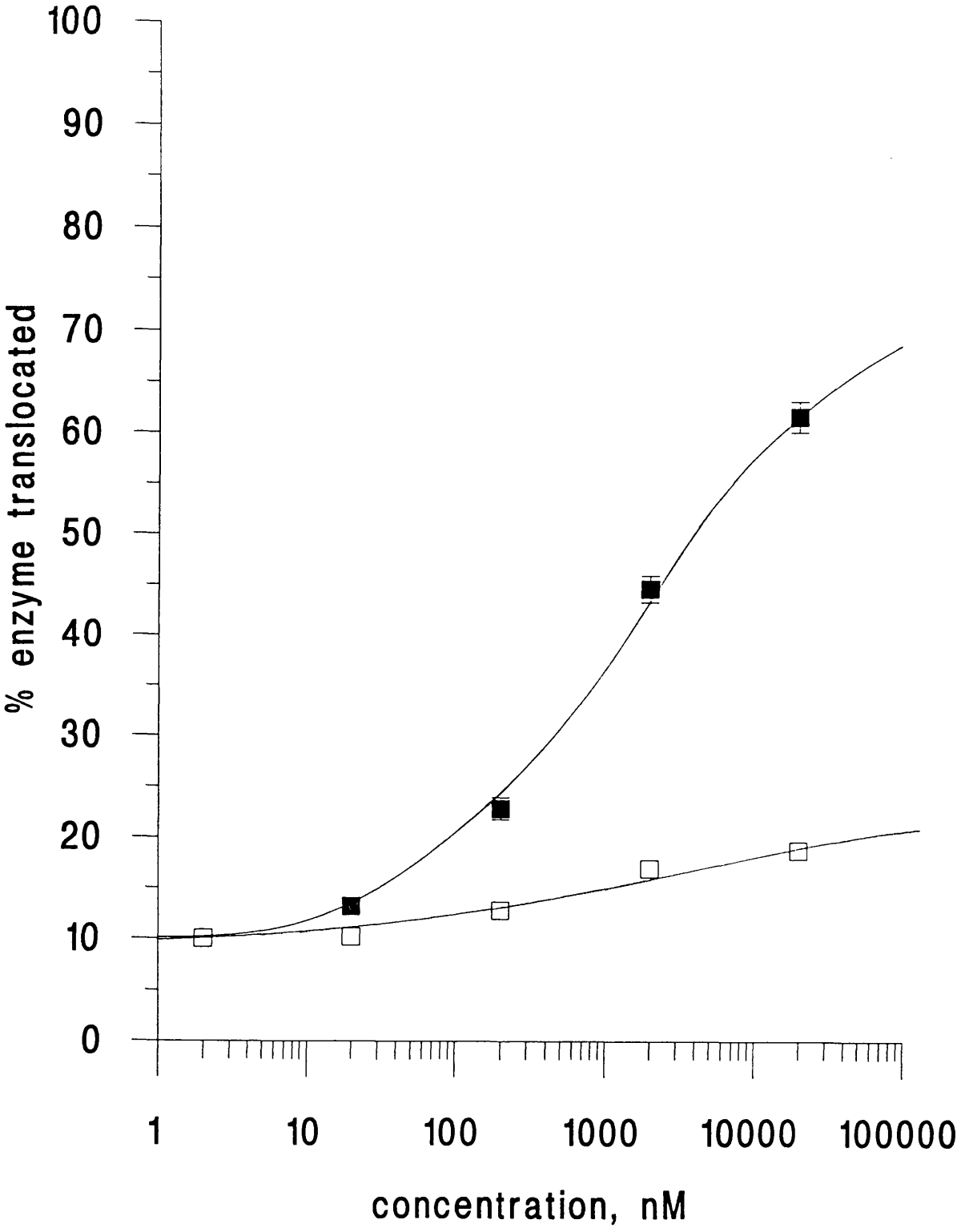


Figure 3.2.3. F. DOPP (■) and DOPPA (□) induced association of PKC-ε with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

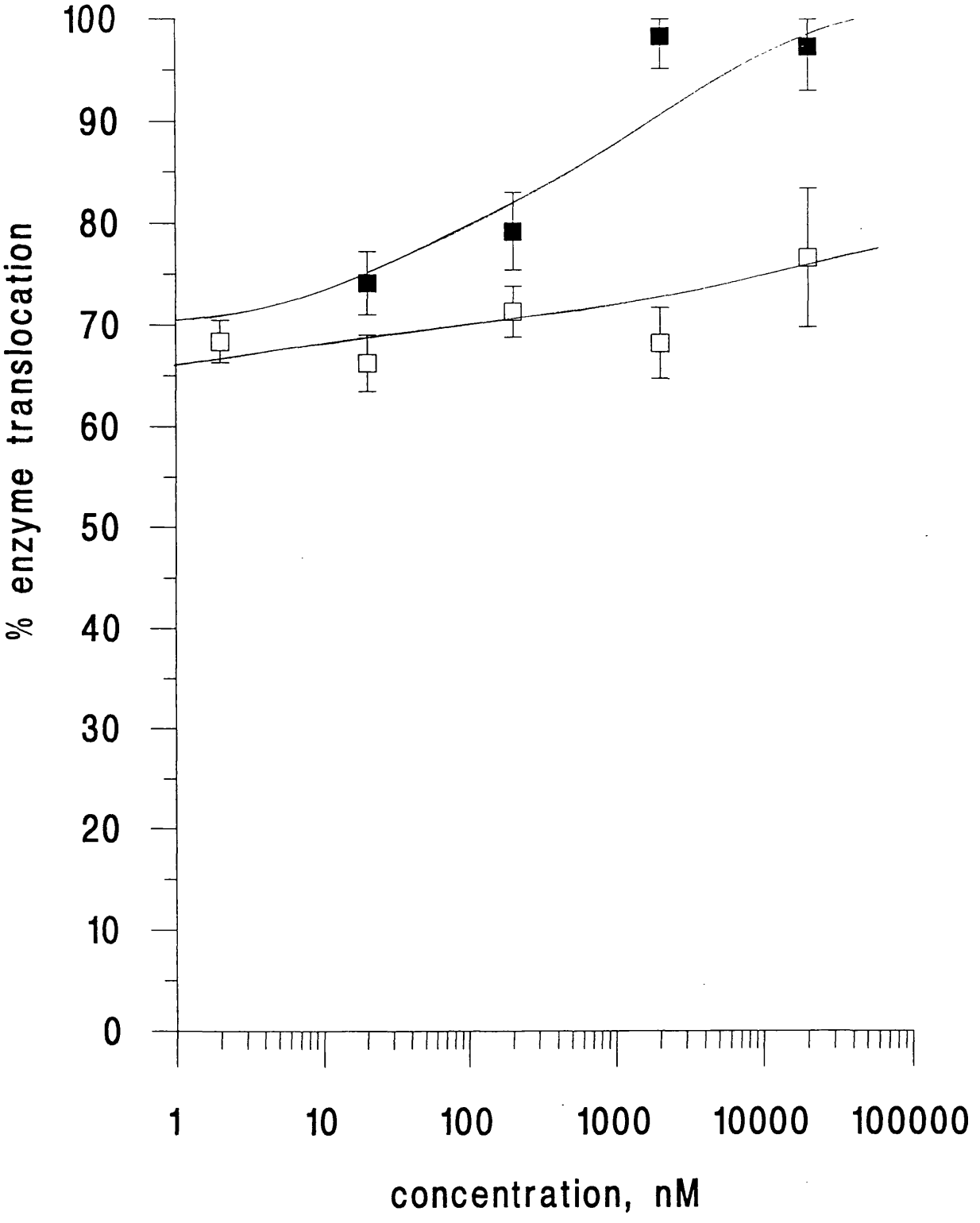


Figure 3.2.3. G. DOPP (■) and DOPPA (□) induced association of PKC-η with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

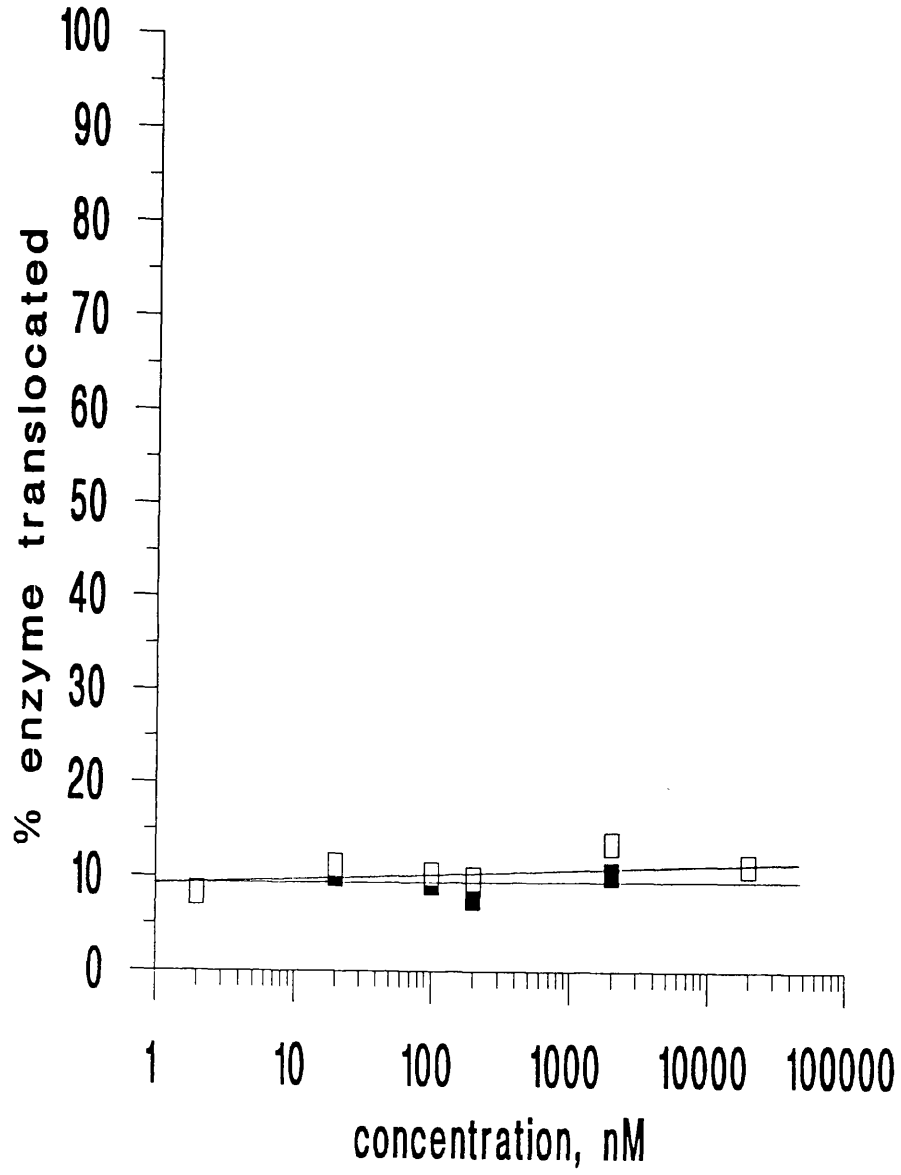


Figure 3.2.3. H. DOPP (■) and DOPPA (□) induced association of PKC-ζ with HL-60 cell membranes. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

3.2.4. Discussion

In mammalian tissues each cell was found to express a defined set of PKC isozymes that are specifically located within the cell (155). Physiological activators (diacylglycerols) and tumour promoting phorbol esters, can significantly alter the intracellular distribution of PKC. This effect known as "translocation" is thought to be followed by PKC activation and phosphorylation of substrate(s).

Initial studies using a range of PE, showed differences in their selectivity and ability to bind and activate different PKC isotypes *in vitro* (347,371,362). On the other hand it is known that different PKC activators are able to induce distinct responses *in vivo* , involving different PKC isotypes (209,351,374,380-382,385,386-388).

In this study we measured the ability of calcium and seven phorbol esters to induce association of recombinant PKC isotypes α, β_1, β_2, γ, δ, ε, η and ζ with HL-60 membranes *in vitro*. We chose HL-60 cells as a source of membranes in this study, because this human leukaemic cell line has been extensively studied by our research group (351).

In various resting cells, different profiles of membrane associated PKC isotypes have been found (187,209,264,368,374,378-382,385,387-389,390,391). Therefore it was not unexpected to find that different PKC enzymes used in this study exert different "spontaneous membrane association" in the absence of free Ca^{2+} and PE. This association was found to comprise 8 to 70 % of total enzyme in the assay (Table 3.2.1., p. 126). These findings are in agreement with predominantly cytosolic localisation of PKC isozymes α, β_1 and ζ found in resting HL-60 cells(264). Additionally high level (70 %) of PKC η basal association is in agreement with most (387,391) but not all (392) reports, on *in vivo* distribution of the η isotype. It could be therefore that this isotype has a generally higher affinity for cellular membranes.

Supportive effects of calcium ions on activation of c PKCs α, β and γ *in vitro* has been well documented. In addition several reports have confirmed calcium effects on induced translocation of some c PKCs but not n PKCs during cell stimulation (240,384,385). However one recent report does suggest some calcium

induced translocation of PKC ε *in vivo* (209). Most *in vitro* studies (using artificial membranes) agreed that in the absence of activators and under ionic strength conditions (~100 mM K^+) similar to that found in intact cells, PKC-membrane association was unstable even in the presence of micromolar Ca^{2+} concentrations (236,369,370,393).

Results shown here demonstrate that calcium at micromolar levels is able to induce association of PKC isotypes α, β_1, β_2, γ, ε and to some extent δ with HL-60 membranes in suspension under ionic strength conditions close to that *in vivo*. Calcium EC_{50} values determined here were in the range of 1.8-4.5 μM and induced translocation between 24 and 80 % (Table 3.2.1., p. 126 and 3.2.2., p. 128). PKC η and ζ did not show any significant Ca^{2+} induced membrane assocation under experimental conditions used.

The observed calcium induced translocation of PKC isotypes ε and to some extent δ was not in agreement with current theory that these two n PKC isotypes do not require calcium as a cofactor. Moreover we were not able to observe any Ca^{2+} effect on activation of ε and δ PKCs, using standard mixed micelle assay (see Section 3.3.).

Previous *in vitro* studies of the Ca^{2+} role in PKC association with lipid membranes were done using an artificial lipid systems (micelles and vesicles) or were done utilising semipurified mixtures of PKC isozymes (236,364,369,370,393-395). Previous *in vivo* studies on Ca^{2+} induced translocation of PKC, were conducted after fractionation of disrupted cells and immunoblot analyses of proteins after SDS-PAGE (13,19-21,29,31). Fractionation artefacts and difficulties linked with precise quantitative analyses of immunoblot results could, at least partially, be responsible for some of the *in vitro/in vivo* discrepancies observed. However one possible explanation for non-selective Ca^{2+} induced translocation, under experimental conditions used here might lay in the loss of some "factor(s)" from HL-60 membrane during preparation. Such factor(s) may either stabilise, in theory, a cPKC-membrane -Ca^{2+} complex, or the Ca^{2+} may be able to allow such a factor to bind PKC to the membrane.

Seven phorbol esters of the tigliane (TPA, PdBu, DOPP, DOPPA and Sap A) and daphnane (Thy A and Rx) series were investigated for their ability to induce PKC-membrane association.

Rx and DOPPA were generally incapable of inducing translocation of the PKC isotypes studied, suggesting the importance of a free C_{20}-OH group for *in vitro* effects of phorbol esters. However DOPPA showed some effect on PKC α and δ but with only 10-15 % induced translocation and at higher concentrations (> 300 nM). This finding was partly in agreement with our recent *in vitro* results (347,371) but not in agreement with recent reports on DOPPA induced translocation of different PKC isotypes *in vivo* (380-382), or the *in vitro* activation studies. Since translocation by PEs performed here was in the absence of free calcium, it is possible that these PEs cannot induce PKC isotypes to associate with the membranes. The effect of DOPPA/Rx on displacing PdBu from β PKC's in vitro (Section 3.1.), would then indicate that PdBu inserts the enzyme to the membrane where Rx/DOPPA are able to displace PdBu.

The Phorbol esters, TPA, PdBu, DOPP, DOPPA and Sap A were similar in their ability to induce PKC translocation, showing however some differences in potency (see Table 3.2.2.). Thy A when compared with them, exerted a similar effect on PKC β_1, γ, δ and ε but was less potent in the case of α, β_2 and η isotypes. PKC γ, δ and η appeared to be generally less responsive to the phorbol esters used, translocating for only 20-35 % above basal level at highest PE concentration (Table 3.2.1.). While PKC-η had high basal association and could not be translocated for more than an additional 30 %, δ and γ might not be properly "supported" to associate with membranes originating from the cells that do not express these isotypes.

Some recent *in vivo* studies, utilising TPA, PdBu, DOPPA, Sap A and Thy A, revealed that all of them were able to translocate unselectively all PKC isotypes found in cells studied (380-382). Kiley *et al* (382) reported that in addition to induced translocation, DOPPA was able to activate PKC and cause phosphorylation of specific substrates *in vivo*. Moreover, studies in this laboratory

(W.J. Ryves unpublished) showed that, DOPPA but not Rx was able to induce macrophage-like differentiation of HL-60 cells. This effect might be due to some extent to metabolic transformation of DOPPA to DOPP over time (351), but dose dependent translocation of endogenous PKCs with DOPPA were observed within 5 minutes.

Again as in the case of Ca^{2+} induced translocation one possible explanation, for differences between *in vitro* and *in vivo* results, could be that a "cofactor" might exist *in vivo,* that is lost in artificial systems or disabled under *in vitro* conditions. Indeed, a "lipid factor" that can selectively activate PKC β_1 isotype, was reported recently to be present in nuclear membranes of HL-60 cells (388). On the other hand interpretation of *in vivo* results, obtained through different methods, must be done cautiously.

In conclusion, our aim was to establish a convenient and reproducible *in vitro* method, that could be used in simulation of PE-PKC interaction *in vivo.* To some extent results obtained with our system were in agreement with effects found in living cells. However, because of some discrepances, we conclude that translation of results from one system to the other could not be done completely.

3.3. Activation of recombinant PKC isotypes α ,β_1, β_2, γ, δ, ε, η and ζ by TPA, PdBu, DOPP, DOPPA, Sap A, Thy A and Rx. Investigation of substrate affinity of different PKC isozymes.

3.3.1. Introduction

Differences in biological effects of different PEs (see introduction) are thought to be, at least partly, due to their interaction with different isotypes of the protein kinase C family of enzymes.

PE-PKC interaction, *in vivo*, involves several steps. These are likely to be: PE penetration to the inner leaflet of the cellular membrane, binding to the PKC molecule, activation of PKC kinase activity and consequent phosphorylation of specific substrates.

Recent investigation in our laboratory showed that PEs vary in their ability to activate some PKC isozymes (347). Aditionally we have studied the binding affinity of a range of PEs for different PKC isozymes (Section 3.1.) (371). Results of these two studies did not correlate completely. Sources of PKC isotypes used in these two studies were different, with bovine brain PKC (α, β_1 and γ) and COS-1 cells expressed recombinant PKC (δ and ε) in activation studies (347) and Sf 9 cells expressed recombinant PKC (α, β_1, β_2, γ, δ, ε and ζ) in binding experiments (371). We decided to repeat the activation screening, with PKC isotypes purified from Sf 9 cells. In this section, an activation study of PKC isotypes α ,β_1, β_2, γ, δ, ε, η and ζ by TPA, PdBu, DOPP, DOPPA, Sap A, Thy A, Rx and DOG is presented. In addition, substrate affinity of different PKC isozymes has also been studied.

3.3.2. Experimental procedures

PKC isotypes purification

PKC isotypes, exept PKC-η, were purified as described earlier (Section 2.2.3.) and were kept at - 20 ^{0}C. PKC-η, was expresseed in mammalian COS-1 cells and was donated by Dr L. Dekker.

PKC activation assay

Essentially the same assay as described in Section 2.2.4. was used. All PEs were tested at 2, 20, 200 and 2000 nM final concentration range (0.00006-0.06 mol % in micelle). Dioleoyl glycerol (DOG) was used at a final concentration of 4, 8, 16 and 32 μM (0.13- 1.04 mol % in micelles). Substrates used in this study were: calf thymus Histone IIIs, myelin basic protein (MBP) and salmon protamine sulphate (all at 1mg/ml final), and PKC δ, ε, η and ζ pseudosubstrate site-based peptides (100 μM, final). When the ability of different PKC isotypes to phosphorylate a range of different substrates was investigated, a fixed concentration of TPA (200 nM) was used in the presence and absence of calcium, was employed as a positive control.

Triplicates of each test point were assayed in the presence or absence of added calcium (100 μM free) for each isozyme. Controls (without PEs) were conducted in parallel. Each experiment was repeated 3 times independently.

3.3.3. Results

Results of stimulation of PKC α, β_1, β_2, γ, δ, ε, η and ζ activity by TPA, PdBu, DOPP, DOPPA, Sap A, Thy A, Rx and DOG are presented in Table 3.3.1., p. 154. For comparative purposes, at each point, PKC activity was determined and compared with the maximum activation with TPA as activator. Background phosphorylation (PS only) was substracted from all values and the results are expressed as a percentage of the maximum TPA stimulation in the presence of calcium (100 %). The concentration of PE or DOG required to give 50 % of the maximal TPA stimulation, for each isozyme, is also presented in Table 3.3.1. as Max 50 values. The stimulation of all purified isotypes was found to be wholly dependent on the presence of PS, no stimulation being observed at any concentration (2-2000 nM) without this phospholipid (data not shown). PKC isotype ζ did not show any increase in activity (above background) in the presence of any activator (PE or DOG) and at any concentration (data not shown). PKC α was found to lose its PE/DOG-stimulated kinase activity rapidly after purification

from Sf 9 cells and was not investigated further. PKC η activation profiles with phorbol esters and DOG in the presence and absence of calcium are presented for the first time and are shown in Figures 3.3.1. A.-H. pgs. 157-164. DOPP and DOPPA stimulation of PKC β_1, β_2, γ, δ, ε and η in the presence and absence of calcium are shown in Figures 3.3.2. A.-F. pgs. 165-170, respectively.

Table 3.3.1. A. Activation of purified PKC isotypes β_1 and β_1 by phorbol esters and DOG *in vitro*, using Histone III-S as a substrate.

PKC	Activator	Activation* % TPA max.		Max 50* nM	
		+ Ca^{2+}	- Ca^{2+}	+ Ca^{2+}	- Ca^{2+}
β_1	TPA	100	80	3	68
	PdBu	92	79	2.8	93
	DOPP	86	67	2.4	140
	DOPPA	0	0	> 2 μM	> 2 μM
	Sap A	103	61	3.5	490
	Thy A	81	67	13	71
	Rx	0	0	> 2 μM	> 2 μM
	DOG	53	39	24 μM	> 24 μM
β_2	TPA	100	84	25	42
	PdBu	95	98	38	120
	DOPP	77	97	18	41
	DOPPA	4	2	> 2 μM	> 2 μM
	Sap A	97	74	130	225
	Thy A	96	90	58	138
	Rx	0	0	> 2 μM	> 2 μM
	DOG	2	1	> 24 μM	> 24 μM

Mean values for triplicate determinations of a representative experiment are shown. S.E.M. values did not exceede 10 % of the mean. Each experiment was repeated three times with results of separate experiments not differing more than 15 %.
*See text (results).

Table 3.3.1. B. Activation of purified PKC isotypes γ and δ by phorbol esters and DOG *in vitro*, using Histone III-S (PKC-γ) and δ pseudo substrate site based peptide (PKC-δ) as substrate.

PKC	Activator	Activation* % TPA max.		Max 50* nM	
		+ Ca^{2+}	- Ca^{2+}	+ Ca^{2+}	- Ca^{2+}
γ	TPA	100	68	3	130
	PdBu	109	80	3	79
	DOPP	107	73	6	148
	DOPPA	23	3	> 2 μM	> 2 μM
	Sap A	98	61	15	1250
	Thy A	99	59	7	890
	Rx	10.1	0	> 2 μM	> 2 μM
	DOG	84	3	19	> 24 μM
δ	TPA	100	98	41	65
	PdBu	90	92	205	290
	DOPP	60	65	190	210
	DOPPA	5	8	> 2 μM	> 2 μM
	Sap A	79	81	240	255
	Thy A	72	65	385	390
	Rx	3	3	> 2 μM	> 2 μM
	DOG	31	31	> 24 μM	> 24 μM

Mean values for triplicate determinations of a representative experiment are shown. S.E.M. values did not exceede 10 % of the mean. Each experiment was repeated three times with results of separate experiments not differing more than 15 %.

*See text (results).

Table 3.3.1. C. Activation of purified PKC isotypes ε and η by phorbol esters and DOG *in vitro*, using ε and η pseudo substrate site based peptides (respectively) as substrates.

PKC	Activator	Activation* % TPA max.		Max 50* nM	
		+ Ca^{2+}	- Ca^{2+}	+ Ca^{2+}	- Ca^{2+}
ε	TPA	100	94	30	31
	PdBu	98	97	110	150
	DOPP	97	95	14	18
	DOPPA	6	7	> 2 μM	> 2 μM
	Sap A	89	87	85	93
	Thy A	105	104	72	73
	Rx	9	6	> 2 μM	> 2 μM
	DOG	45	45	> 24 μM	> 24 μM
η	TPA	100	104	3	6
	PdBu	99	122	4.2	4.5
	DOPP	105	103	6	5
	DOPPA	6	7	> 2 μM	> 2 μM
	Sap A	68	77	18	11
	Thy A	73	77	220	290
	Rx	6	7	> 2 μM	> 2 μM
	DOG	95	75	11 μM	15 μM

Mean values for triplicate determinations of a representative experiment are shown. S.E.M. values did not exceede 10 % of mean. Each experiment was repeated three times with results of separate experiments not differing more than 15 %.

*See text (results).

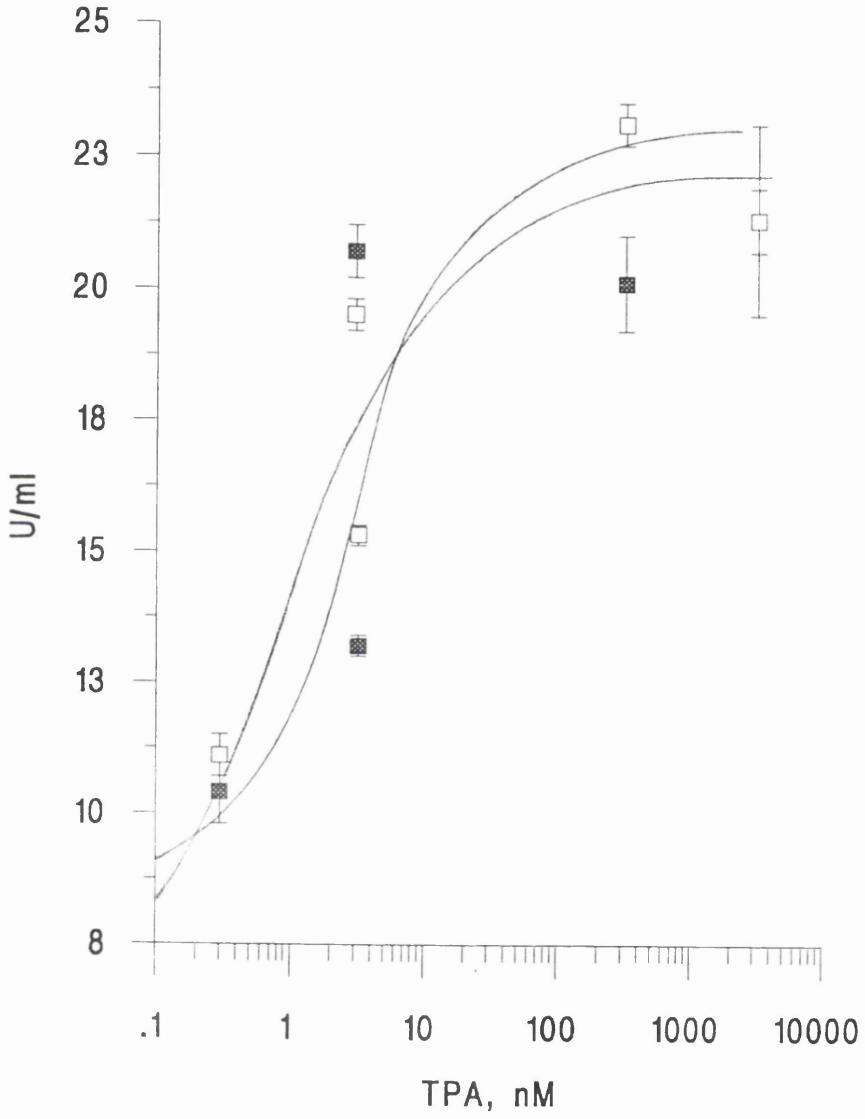


Figure 3.3.1. A. TPA activation of PKC η in the presence (■) and absence (□) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted three times and results did not differ more than 15 % between individual experiments.

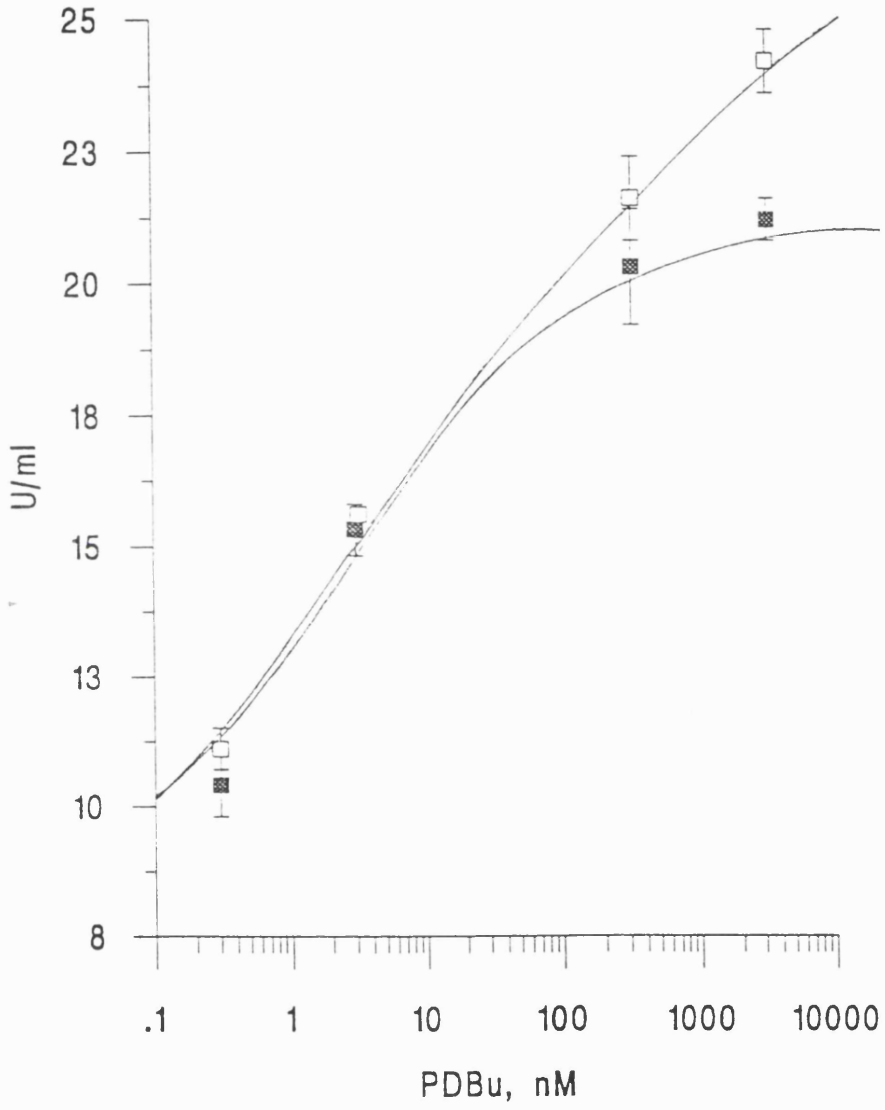


Figure 3.3.1. B. PdBu activation of PKC η in the presence (■) and absence (□) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted three times and results did not differ more than 15 % between individual experiments.

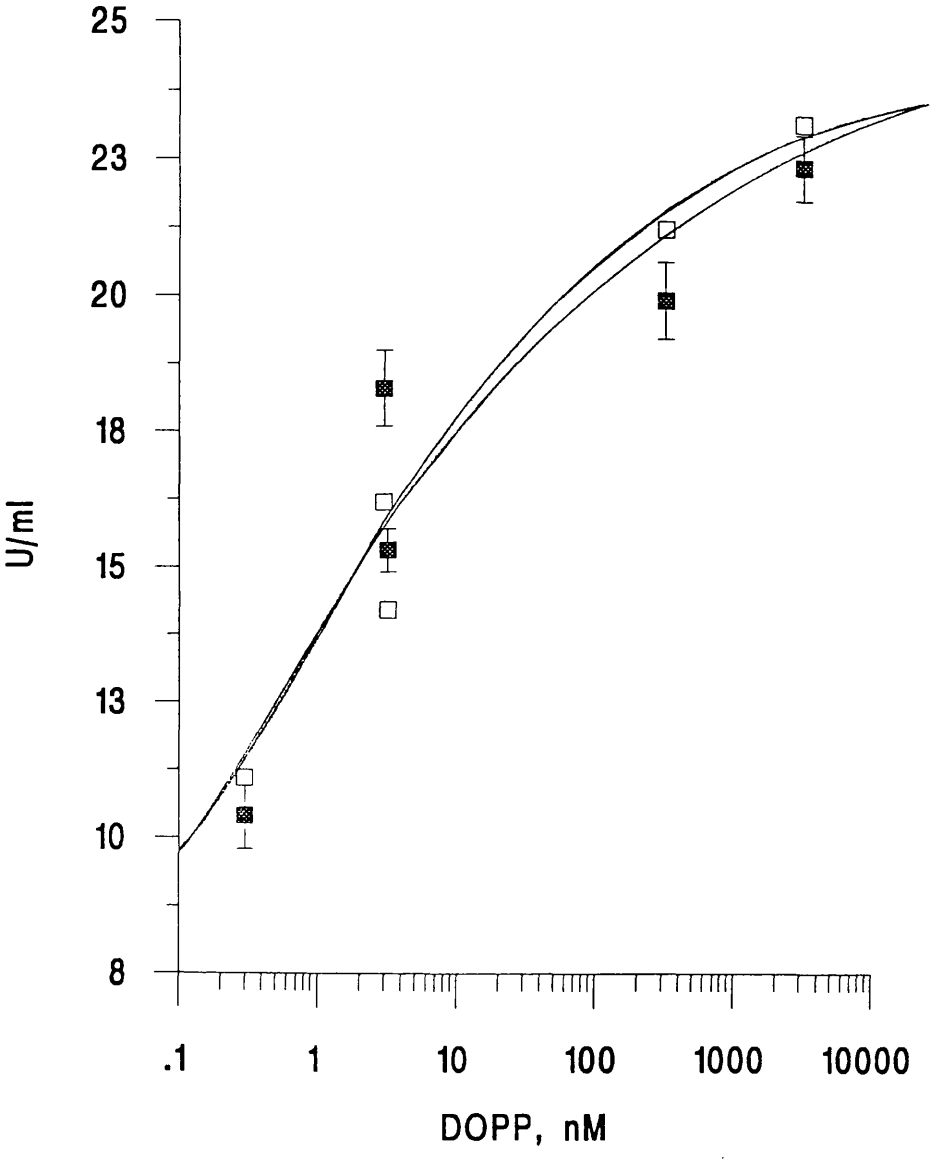


Figure 3.3.1. C. DOPP activation of PKC η in the presence (■) and absence (□) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted three times and results did not differ more than 15 % between individual experiments.

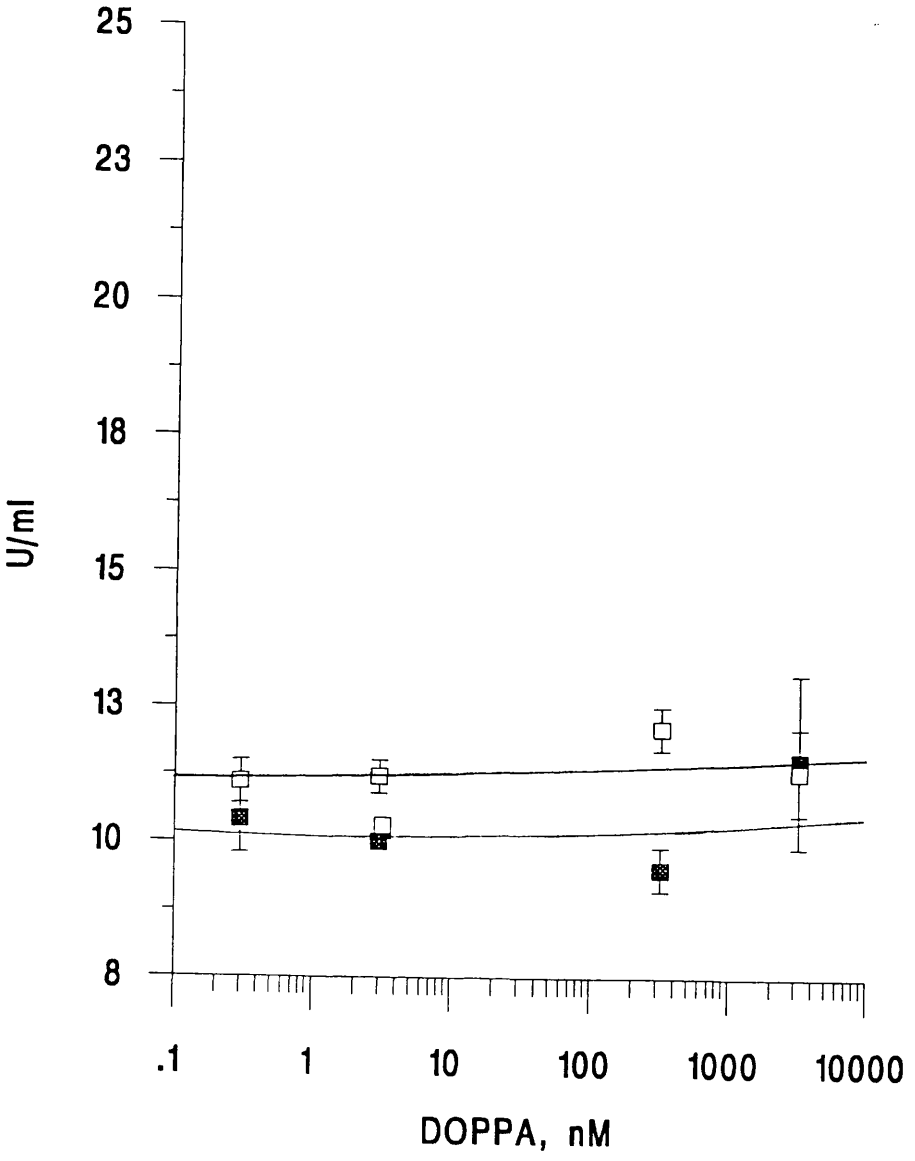


Figure 3.3.1. D. DOPPA activation of PKC η in the presence (■) and absence (□) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted three times and results did not differ more than 15 % between individual experiments.

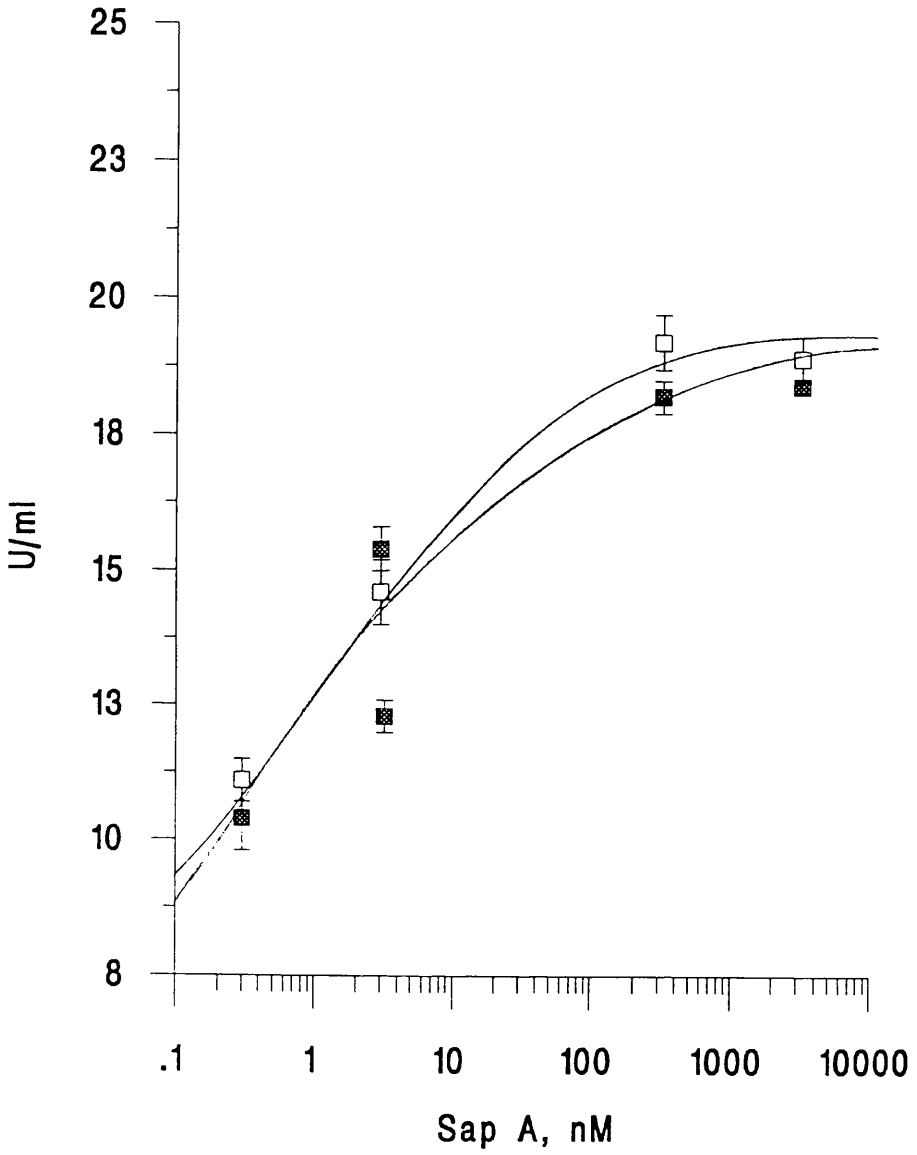


Figure 3.3.1. E. Sap A activation of PKC η in the presence (■) and absence (□) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted three times and results did not differ more than 15 % between individual experiments.

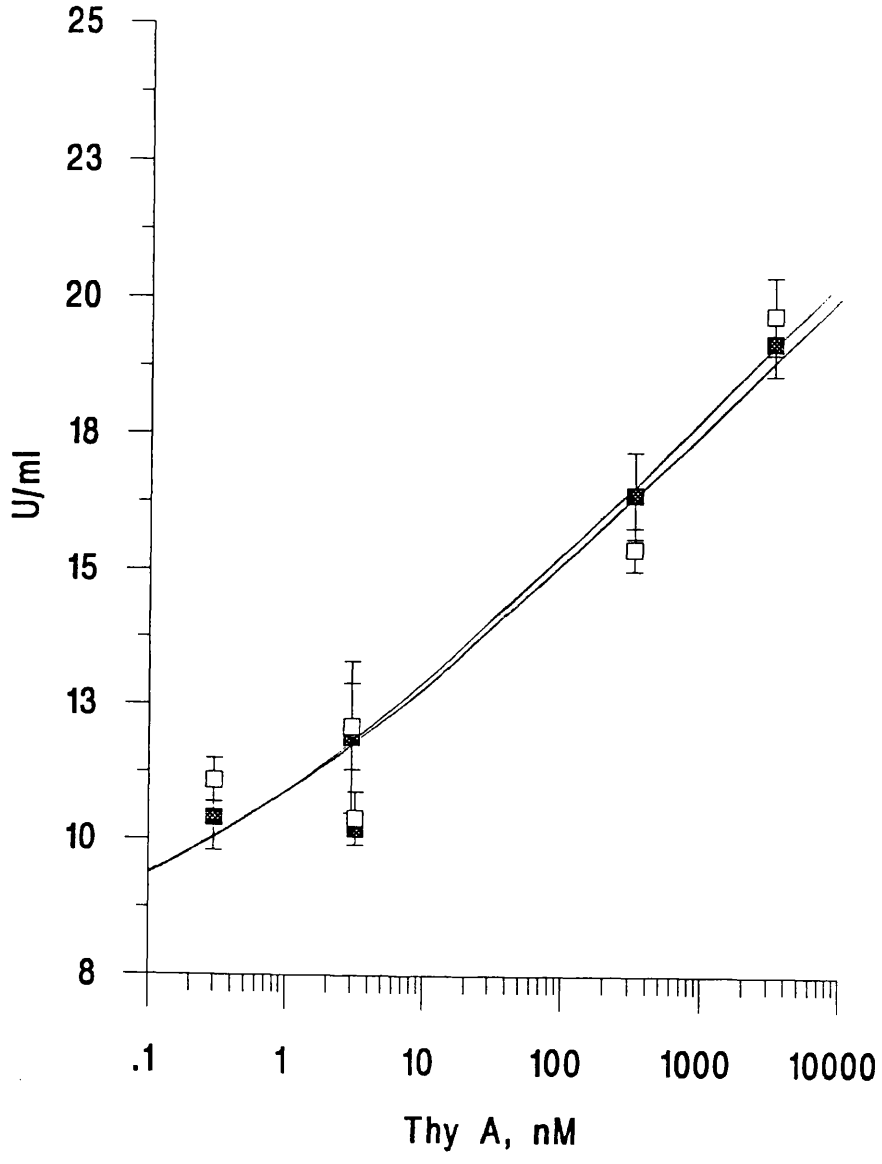


Figure 3.3.1. F. Thy A activation of PKC η in the presence (■) and absence (□) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted three times and results did not differ more than 15 % between individual experiments.

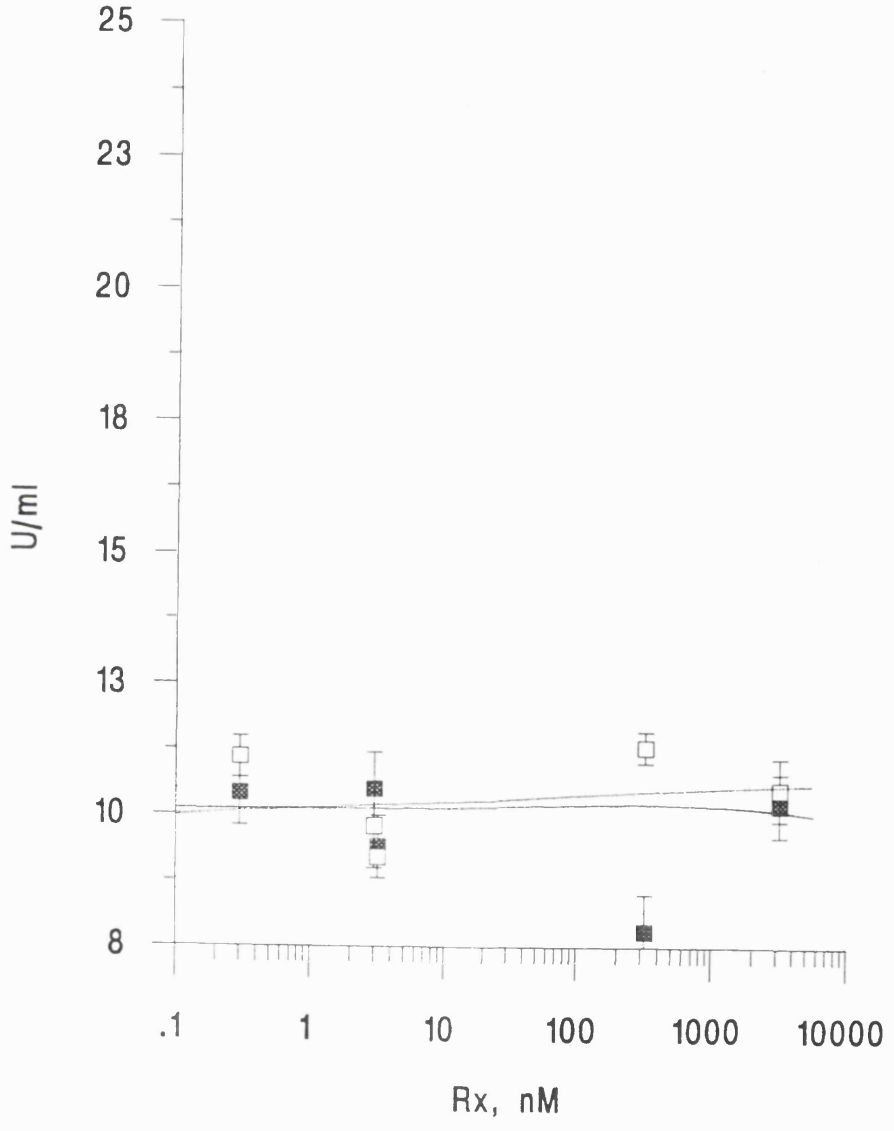


Figure 3.3.1. G. Rx activation of PKC η in the presence (■) and absence (□) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted three times and results did not differ more than 15 % between individual experiments.

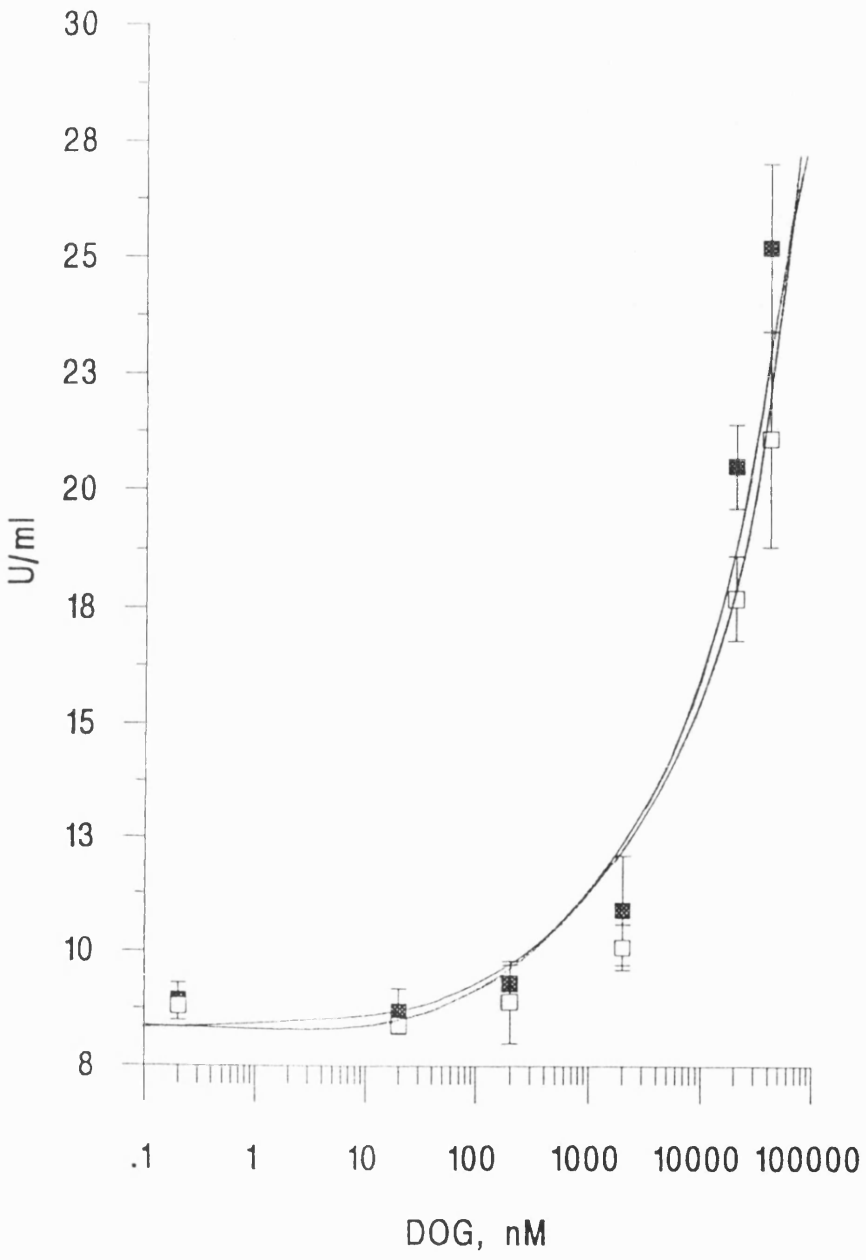


Figure 3.3.1. H. DOG activation of PKC η in the presence (■) and absence (□) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted three times and results did not differ more than 15 % between individual experiments.

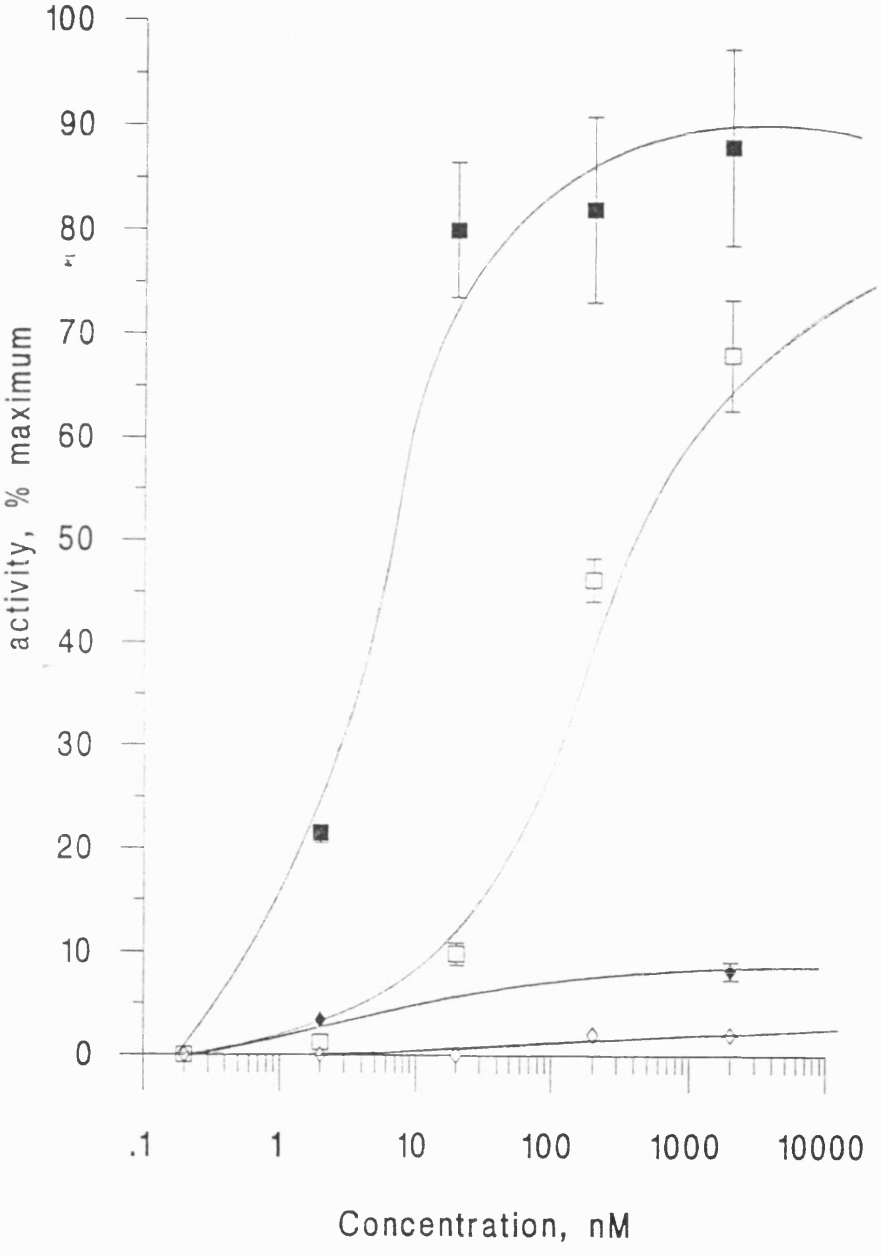


Figure 3.3.2. A. DOPP (■, □) and DOPPA (◆, ◊) activation of PKC β_1 in the presence (closed symbols) and absence (open symbols) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted two times and results did not differ more than 10 % between individual experiments.

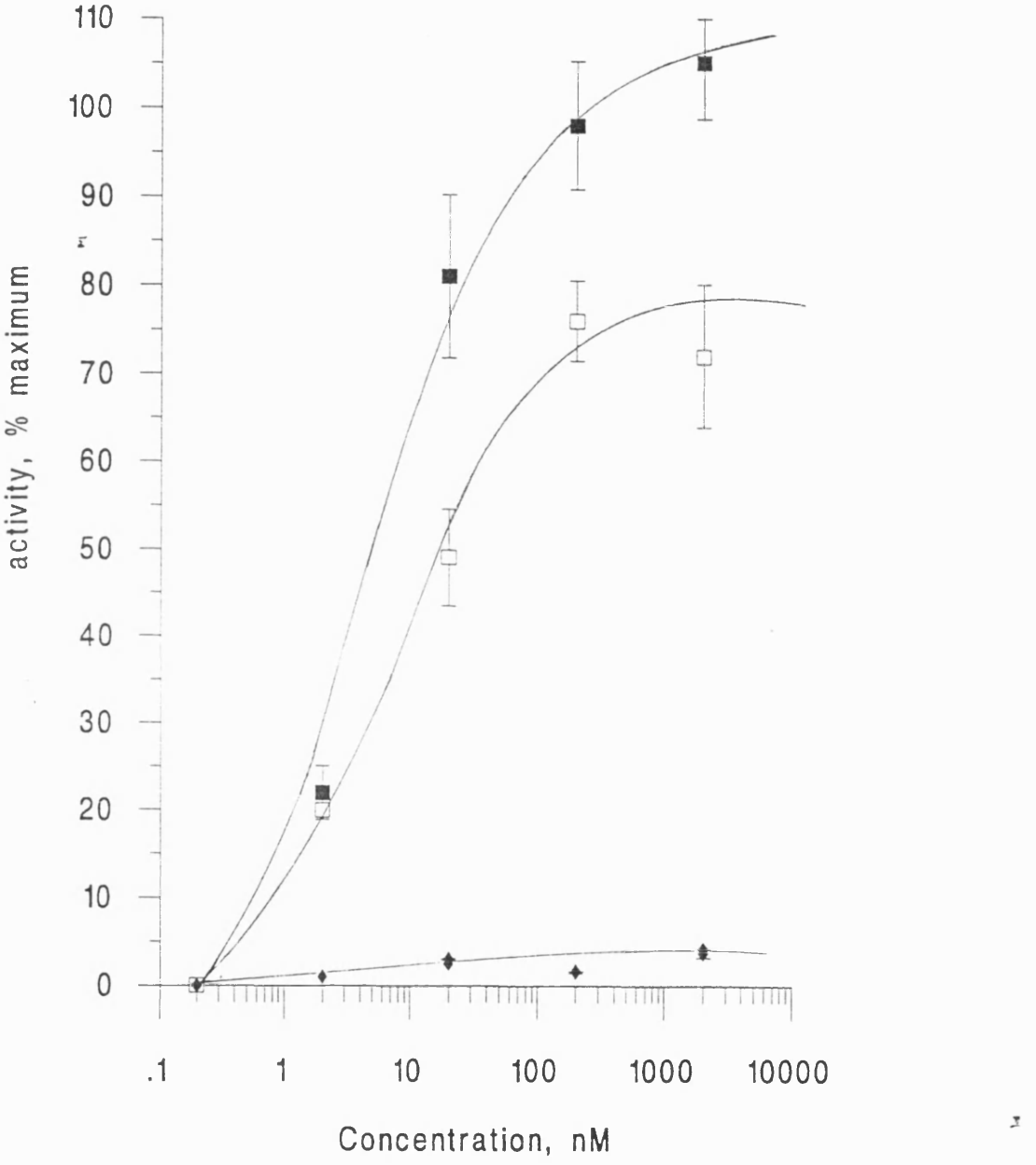


Figure 3.3.2. B. DOPP (■, □) and DOPPA (◆, ◊) activation of PKC β_2 in the presence (closed symbols) and absence (open symbols) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted two times and results did not differ more than 10 % between individual experiments.

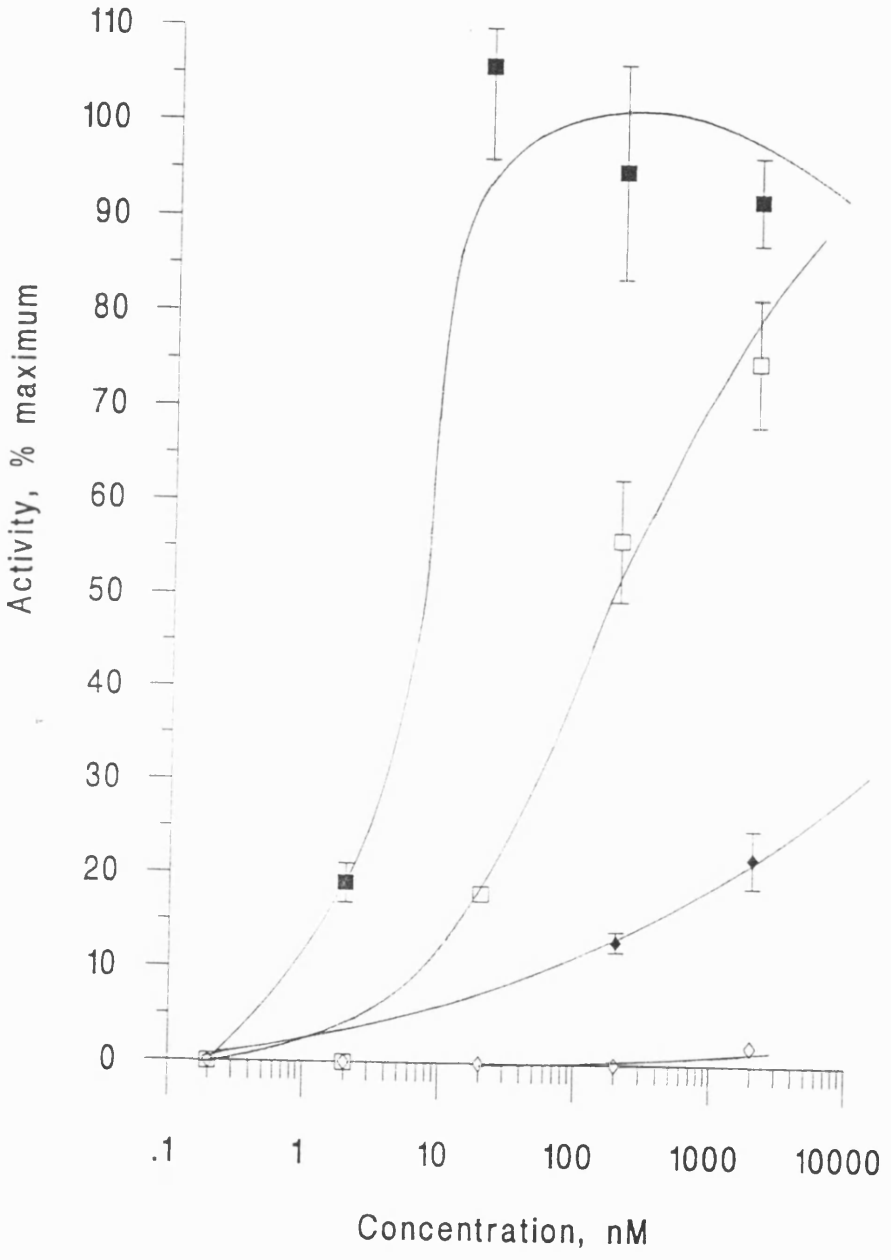


Figure 3.3.2. C. DOPP (■, □) and DOPPA (◆, ◊) activation of PKC γ in the presence (closed symbols) and absence (open symbols) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted two times and results did not differ more than 10 % between individual experiments.

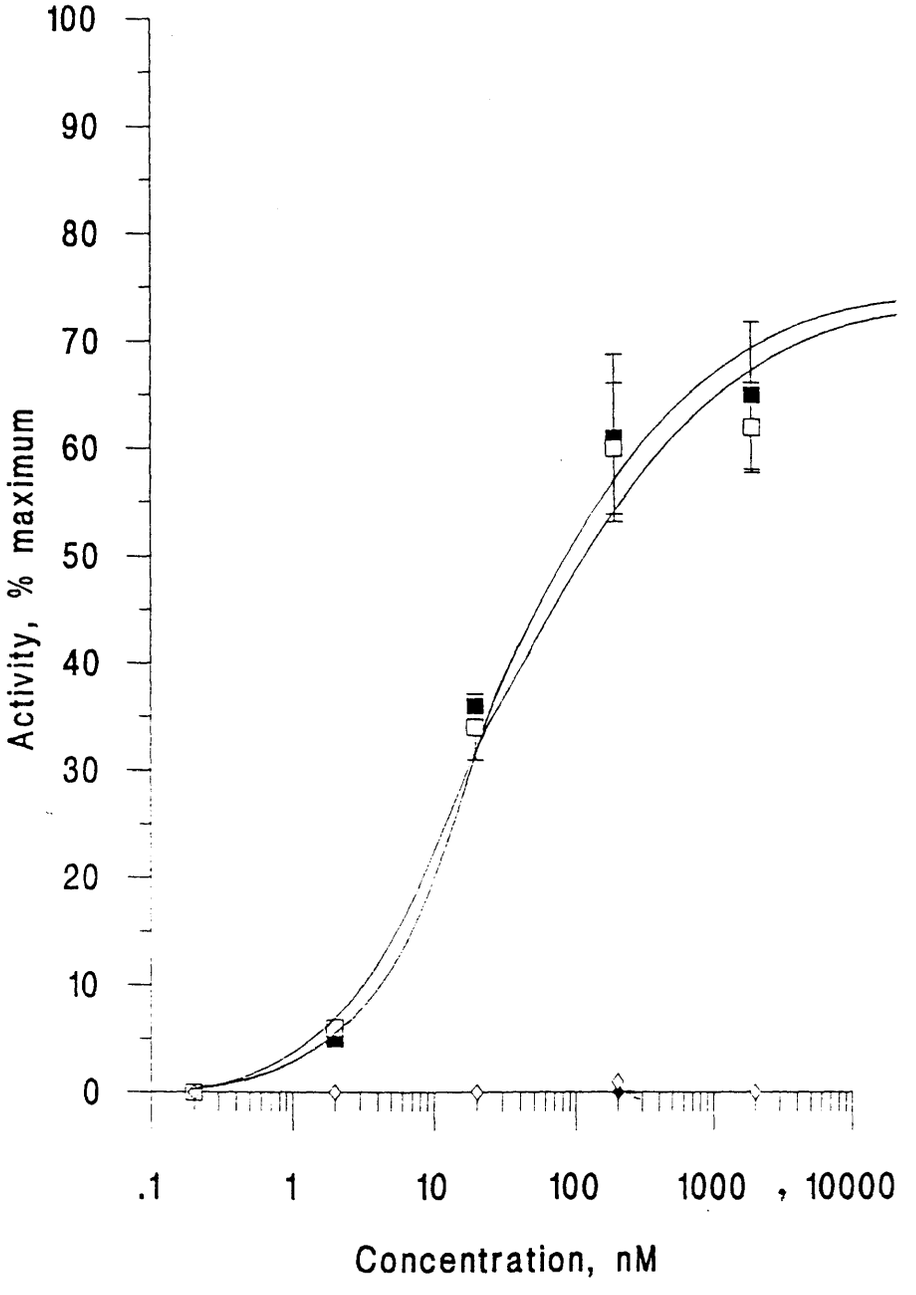


Figure 3.3.2. D. DOPP (■, □) and DOPPA (◆, ◊) activation of PKC δ in the presence (closed symbols) and absence (open symbols) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted two times and results did not differ more than 10 % between individual experiments.

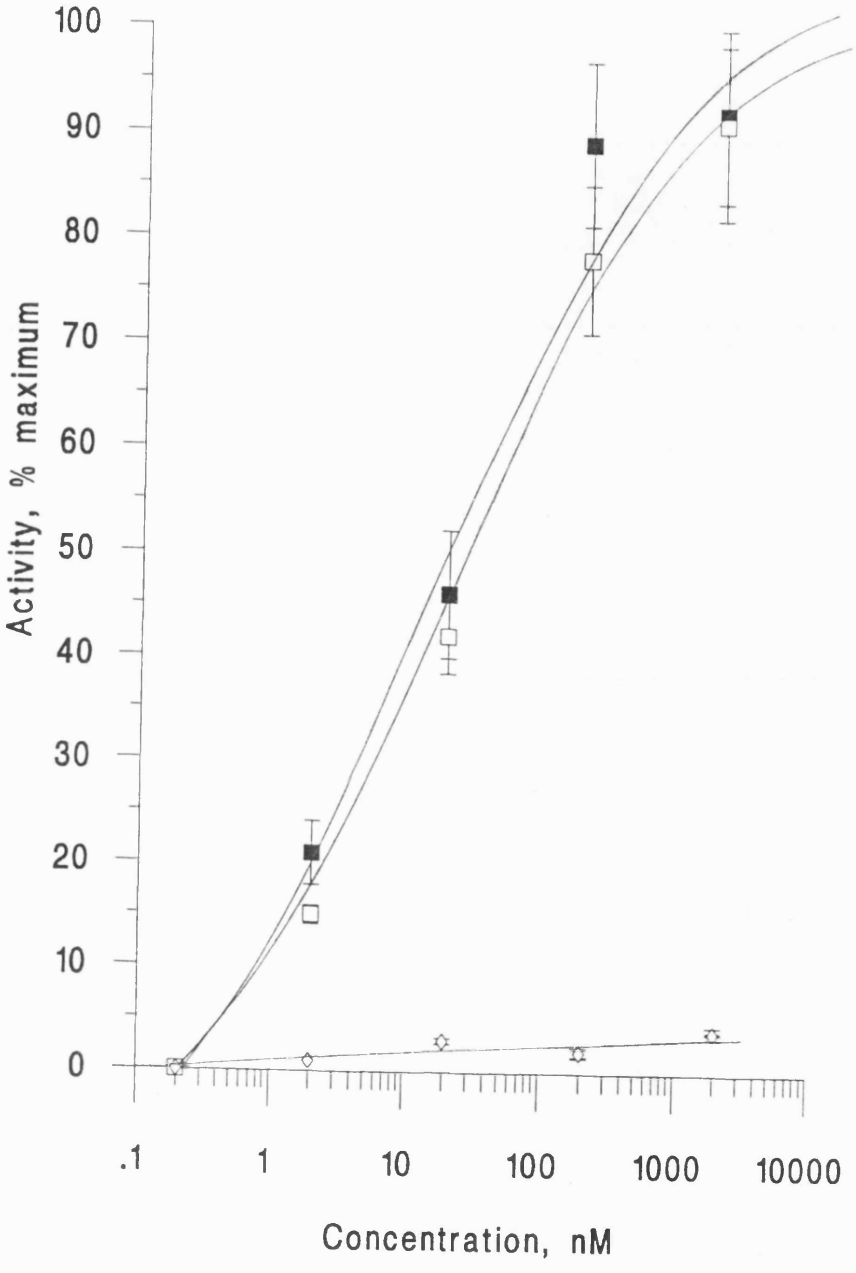


Figure 3.3.2. E. DOPP (■, □) and DOPPA (◆, ◊) activation of PKC ε in the presence (closed symbols) and absence (open symbols) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted two times and results did not differ more than 10 % between individual experiments.

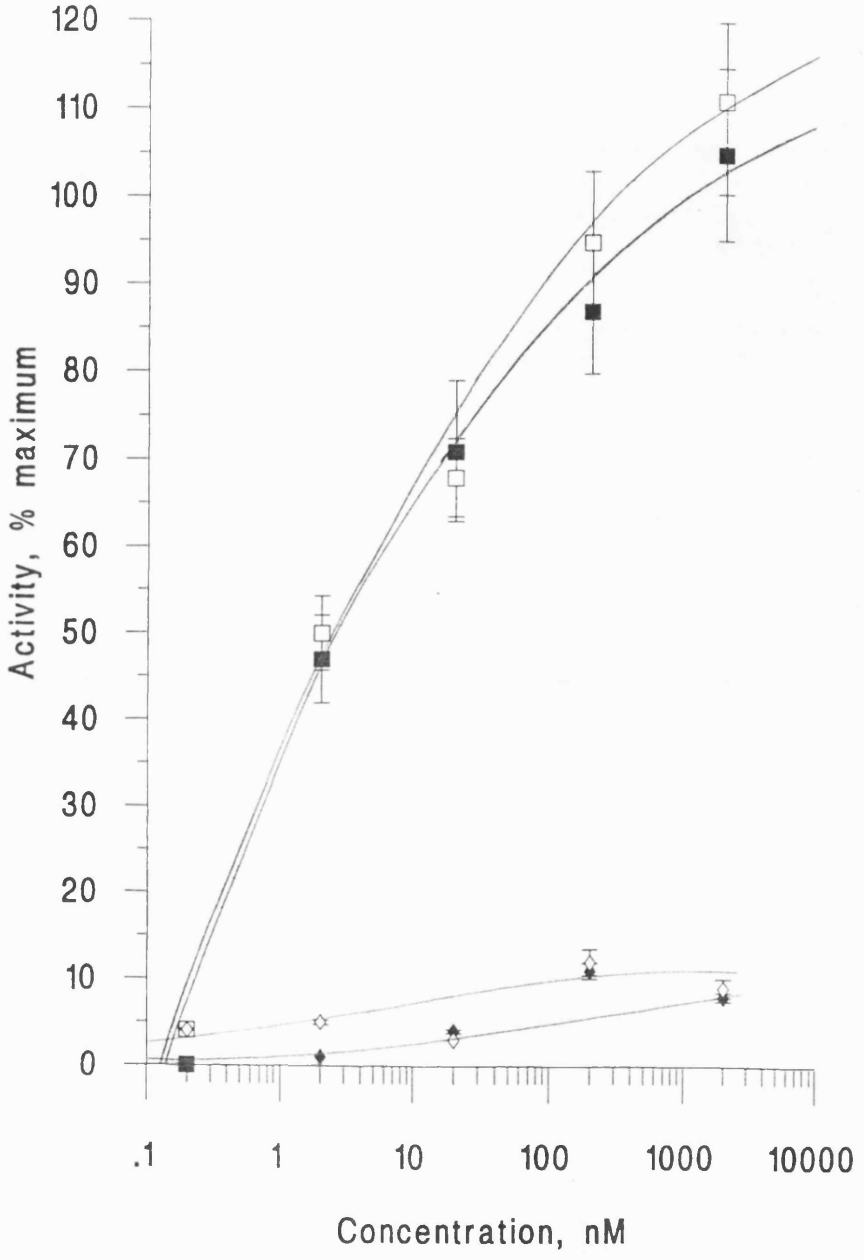


Figure 3.3.2. F. DOPP (■, □) and DOPPA (◆, ◊) activation of PKC η in the presence (closed symbols) and absence (open symbols) of calcium. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment. Each experiment was conducted two times and results did not differ more than 10 % between individual experiments.

The ability of PKC isotypes β_1, β_2, γ, δ, ε and η to phosphorylate a range of substrates was investigated and results are presented in Table 3.3.2. Phosphorylation of all substrates, ecxept protamine sulphate, was found to be dependent on the presence of PS (data not shown). Phosphate incorporation by each PKC isotype into different substrates was expressed as a percentage of Histone IIIs phosphorylation, using phosphorylation of Histone IIIs stimulated with 200 nM TPA and in the presence of 100 μM free Ca^{2+} as 100 % phosphorylation.

Table 3.3.2. A. Phosphorylation of Histone III-S, Protamine sulphate and Myelin basic protein (MBP) by PKC isozymes β_1, β_2, γ, δ, ε and η.

PKC	Substrate Cofactor	Histone IIIs* + Ca^{2+}	Histone IIIs* - Ca^{2+}	Protam. Sulphate* + Ca^{2+}	Protam. Sulphate* - Ca^{2+}	MBP* + Ca^{2+}	MBP* - Ca^{2+}
β_1	PS	9.6	7.7	132.2	138.1	22.3	15.1
	PS/TPA	100	61.3	129.1	133.4	161.3	152.1
β_2	PS	7.2	6.1	262.3	268.2	23.1	25.2
	PS/TPA	100	51	258.1	259.3	230.8	130.8
γ	PS	18.9	8.2	289.2	295.1	38.2	17.4
	PS/TPA	100	63.1	302.1	299.2	163.2	152.9
δ	PS	23.1	25.2	310.1	315.2	53.1	47.1
	PS/TPA	100	105.1	306.4	303.2	228.1	235.1
ε	PS	41	40.2	510.2	505.3	134.2	45.1
	PS/TPA	100	102.1	505.3	514.3	470.2	271.2
η	PS	66.2	65.4	335.1	328.2	98.2	75.6
	PS/TPA	100	84.2	325.6	322.1	310.1	308.2

* % phosphorylation, using phosphorylation of Histone IIIs stimulated with 200 nM TPA (PS/TPA) and in the presence of 100 μM free Ca^{2+} as 100 %. All substrates were used at final concentration of 1 mg/ml.

Table 3.3.2. B. Phosphorylation of δ, ε, η and ζ pseudosubstrate site based peptides by PKC isozymes β_1, β_2, γ, δ, ε and η.

PKC	Substrate Cofactor	δ pss*		ε pss*		η pss*		ζ pss*	
		+ Ca^{2+}	- Ca^{2+}	+ Ca^{2+}	- Ca^{2+}	+ Ca^{2+}	- Ca^{2+}	+ Ca^{2+}	- Ca^{2+}
β_1	PS	29.0	29.1	28.1	25.3	58.1	52.3	41.2	44.3
	PS/TPA	77.4	75.3	84.3	80.2	245.2	251.2	215.3	223.
β_2	PS	62.1	60.2	69.2	71.3	167.1	89.2	69.1	72.3
	PS/TPA	165.4	166.1	202.3	212.1	315.4	310.2	292.3	271.
γ	PS	26.0	29.1	54.4	41.2	163.0	72.4	34.5	36.1
	PS/TPA	152.4	145.1	152.2	147.2	281.4	245.4	220.1	218.
δ	PS	142.9	141.6	208.6	215.1	128.4	135.1	257.1	265.
	PS/TPA	514.3	495.1	828.6	811.3	371.4	366.2	1057	956
ε	PS	385.7	214.3	214.3	105.1	585.4	143.1	114.3	111
	PS/TPA	585.7	442.9	485.7	343.2	685.7	705.3	605.3	486
η	PS	98.3	82.4	122.3	115.3	205.3	199.1	84.3	89.2
	PS/TPA	360.2	355.3	390.2	378.3	427.3	420.3	367.3	371.

* % phosphorylation, using phosphorylation of Histone IIIs stimulated with 200 nM TPA (PS/TPA) and in the presence of 100 μM free Ca^{2+} as 100 %. All substrates were used at final concentration of 100 μM.

3.3.4. Discussion

Different aspects (cofactor dependency and substrate specificity) of purified PKC isotypes β_1, β_2, γ, δ, ε, η and ζ were investigated. All PKC isotypes investigated were purified from baculovirus infected insect Sf 9 cells, except PKC-η which was expressed in mammalian COS-1 cells and was donated by Dr L. Dekker.

All isotypes investigated were found to be dependant on phosphatidylserine for activation and were effectively inactive in its absence. For each isozyme, kinase activity stimulated with different activators (PEs or DOG) was compared to the TPA elicited kinase activity (after PS control values had been substracted from them). Phosphorylation properties of different substrates used in this investigation was compared with Histone IIIs as standard substrate used in PKC studies.

In agreement with current opinion (200), both, phorbol esters and DOG were found to be unable to stimulate PKC ζ activity above the control (PS) level (data not shown).

cPKC isotypes β_1, β_2, and γ

Activation of PKC isozymes α, β_1, β_2, and γ is considered to be stimulated with PEs/DAG and to be dependent on the presence of free calcium ions (171,219,288). Unfortunately PKC α which was purified from Sf 9 cells showed rapid loss (12-24 hours) of activator (PE or DOG) stimulated activity and did not give reproducible results, therefore it was excluded from further investigation.

The results demonstrated (Table 3.3.1. A and B.) that full tumor promoters (TPA and PdBu), weak tumor promoters (Thy A) and non-promoting phorbol esters - Sap A and DOPP, share the ability to potently activate PKC isozymes β_1, β_2, and γ in a calcium dependent manner. The cofactor action of calcium was found to exert the greatest effect on the PKC - γ, when TPA was the activator, as indicated by the potency shift between the presence and absence of 100 μM free calcium

(max 50 values in Table 3.3.1. A. and B., pgs. 154-155). The calcium effect (with TPA as activator) followed the order PKC-γ > PKC-β_1 > PKC-β_2 (Table 3.3.1. A. and B.). The same order was found for Thy A while other phorbol esters (PdBu, DOPP and Sap) showed different calcium-stimulation profiles following the order of PKC-β_1 > PKC-γ > PKC-β_2.

Phorbol esters of limited biological activity (DOPPA and Rx) were almost ineffective at the stimulation of cPKC isozymes investigated here. Although showing some stimulation of PKC γ at higher concentrations (>200 nM), this behavour of DOPPA (see below) and Rx is not in agreement with previous reports on isozymes isolated from bovine brain (347), where PKC isozyme β_1 was found to be selectively stimulated with DOPPA and higher concentrations of Rx (although much weaker activation was also observed with PKC-γ).

1,2-sn-Dioleoylglycerol (DOG) was a much weaker activator of these isotypes, with (as in the case of DOPPA and Rx) calcium sensitive higher stimulation of PKC-γ.

In agreement with the literature (359), phosphorylation of protamine sulphate (Table 3.3.2. A., p. 171), under the conditions used, was found to be independent of the presence of calcium and/or TPA. Histone IIIs phosphorylation (Table 3.3.2. A.) appeared to be most sensitive to the presence of calcium ions (both in the case of PS and PS/TPA) and activator (200 nM TPA) stimulation (as a difference between PS and PS/TPA effect). MBP and pseudosubstrate site based peptides generally showed higher phosphate incorporation than histone (Table 3.3.2. A. and B., pgs. 171-172). However, this phosphorylation (MBP, δ, ε, η and ζ pseudosubstrate site peptides) was not dependent on the presence of calcium and additionally showed to be less sensitive to activator (200 nM TPA) stimulation (as a difference between PS and PS/TPA phosphorylation). These results are mostly in agreement with results reported recently (171,219,288). However, because of differences in experimental conditions in some cases, direct comparison of results was not possible.

It is important to point out cofactor and substrate differences between two PKC-β isozymes (Table 3.3.1. A., B. and C. pgs. 154-156 and Table 3.3.2. A. and B. pgs. 171-172), which are alternatively spliced products of the same gene. To our

knowledge this is the first report on comparative investigation of activation profiles of PKC-β_1 and β_2 isozymes. Observed differences in activation properties of these two closely related isozymes support the opinion that small structural differences between similar PKC isozymes could result in different activation requirements, suggesting possible differences in their activation *in vivo* and consequently differences in their intracellular signalling role. Unfortunately none of the phorbol esters investigated here exerted distinct selectivity towards one of these β isozymes, preventing the use of these compounds as selective probes in investigation of individual isozymes.

n-PKCs δ and ε

In agreement with earlier reports (347,199,248,274,275,367,397), both n-PKC isotypes showed calcium independent activation with phorbol esters and DOG. Activation profiles of PKC-δ and ε with a range of activators is presented in Table 3.3.1. B. and C., pgs. 155-156. Although Sap A (~ 3 and 5 x for ε and δ respectively) and Thy A (~ 2.5 and 7.5 x for ε and δ respectively) were found to be less potent activators than TPA the effects observed here were different from results observed in an earlier study (347), where Thy A was found to be unable to activate both isotypes, while Sap A was found to be a selective PKC-δ non-activator. In agreement with our results is a recent report on the Thy A effect *in vivo* (380), where this second stage tumor promotor was shown to cause translocation and downregulation of multiple PKC isozymes, including δ and ε. However, it can be speculated that, as in the case of mezerein (see Section 3.5.), weaker activity of Thy A towards nPKC isozymes (see also below for PKC-η) could, at least partly, be responsable for its activity as a second-stage tumor-promoter. Interestingly PKC-ε was unique in that DOPP appeared to be a more potent (~ 2 x) activator than TPA (Max 50 values in Table 3.3.1. A.-C., pgs. 154-156). With different substrates similar phosphorylation profiles were observed for both isotypes with ζ psudosubstrate based peptide being the best and histone IIIs the poorest substrate (Table 3.3.2. A. and B., pgs. 171-172). These results are essentially in agreement with recently reported investigations (199,248,274,275,367,397).

PKC η

Activation of novel PKC-η with a range of phorbol esters (reported here for the first time) is of particular importance regarding the predominant localisation of this isozyme in skin (162,398), a model-organ used for the investigation of tumour-promoting properties of different phorbol esters. Results presented in Figure 3.3.1. A.-H., pgs. 157-164, and Table 3.3.1. C., p. 156, suggest that activation of this isozyme by DOG and a range of phorbol esters is not dependent on calcium, as expected for a member of n-PKC subfamily. Activation with TPA, PdBu, DOPP, DOPPA, Sap A and Rx showed comparably similar profiles as with other n-PKCs. However, Thy A, a weak second stage tumor promoter, appeared to be a comparably much weaker activator(~ 70 and 50 x as compared with TPA, for + an - Ca^{2+} respectively) of PKC-η (Table 3.3.1. C.). This suggests the possible use of Thy A as a selective non-activator of this PKC isozyme *in vitro* and a possible explanation (at least partly) for its distinct effects *in vivo*. Unfortunately no data on Thy A effects on the PKC-η isotype *in vivo* is available at the present. DOG was a relatively (as compared with TPA) better activator of PKC-η than of other isozymes investigated, implying possible selectivity in activation of different PKC isozymes with physiological activators (DAGs) *in vivo*. The ability of non-promoting phorbol esters , Sap A and DOPP to potently activate PKC-η could not support an explanation for their inactivity as tumor-promoters *in vivo*, leaving still an open possibility that their selective interaction with other, yet uninvestigated or undiscovered, PKC isozymes (or some other unknown receptors) could explain their specific biological effects. Substrate phosphorylation profiles of PKC-η appeared to be similar to profiles observed for PKC-δ and ε (Table 3.3.2. A. and B., pgs. 171-172) and results reported recently (162,398,399).

Demonstration of strict structural requirements for a PKC activator is demonstrated by the two non-promoting 12-deoxyphorbol esters, DOPP and DOPPA. Esterification of the free primary hydroxyl group at the C_{20} position in DOPPA, as the only structural difference with DOPP, resulted in almost complete loss (exept with PKC-γ at higher concentrations) of ability to activate PKC

isozymes (Figure 3.3.2. A.- F., pgs. 165-170). This observation was not in agreement with an earlier report (347), in that neither of the PKC-β isozymes appeared to be selectively stimulated with DOPPA under the experimental conditions used. Additionally, this observation could not explain the recently reported ability of DOPPA to nonselectively interact with some PKC isotypes *in vivo* (380-382). However, our preliminary observations, using cellular membranes isolated from HL-60cells, as a membrane model, indicate that DOPPA could selectively (at least when compared with PKC-γ) activate PKC-β isozymes in the presence of Ca^{2+}. Differences between these *in vitro* and *in vivo* experiments could suggest the possible existence of some, as yet unidentified, "membrane factor" that is present in some or all cell types and that may be responsible for DOPPA activity *in vivo* (see also Murray *et al,* 388). The PKC-β isotype used in a previous study was isolated from tissue (bovine brain) in which this isozyme is normally present, and one might speculate that possibly some fraction of such "factor" could remain copurified with PKC-β. Such a "factor" could not necessarily be present in cells which do not normally express PKC or a particular PKC isozyme.

The mode of activation and the substrate specificites of different PKC family members have been studied with isozymes purified from different tissues, or different cell systems after expression of recombinant enzymes (162,171,199,219,248,274,275,288,367,397-399). These studies revealed that different PKC isozymes show different requirements for phospholipid, phospholipid metabolites and Ca^{2+} *in vitro,* that is likely to reflect differential responsivenes for second messangers after receptor activation *in vivo.* In addition to differences in cofactor dependence, PKC isozymes also show differences in substrate phosphorylation, suggesting possible divergence in their signalling pathways.

Differences in activation profiles of phorbol esters reported here and observed earlier (347) are not completely unexpected, since recent investigation of PKC-δ have shown species-related differences in biochemical properties of this PKC isozyme (275). Discrepances between studies on one particular isozyme may be accounted for by differences in assay methods (e.g. presence of detergent,

substrate used etc.), purity of enzyme, purity of cofactors and activators, post translational modifications of the enzyme (e.g. different phosphorylation states) and alternatively the primary structure differences between PKC isozymes obtained from different sources. Since experimental procedures applied here and used earlier (347) were essentially the same, it is likely that differences in PKC isozymes (different source) used is the most probable reason for the differences observed between these two studies. Behavour of PKC α, losing rapidly its activator (PE or DOG) stimulated activity is important in this context.

Discovery that tumor-promoting phorbol esters (such as TPA) directly activate protein kinase C *in vitro,* literally "promoted" experiments to study the involvement of PKC in a variety of biological responses in various cell types that are mimicked by phorbol esters. However, in most of these investigations only TPA, as a most potent tumor-promoting phorbol ester, was used. Inspite of observations that other phorbol esters exert more specific biological effects *in vivo,* relatively few studies have been reported so far (347,380-383) on their interaction with individual PKC isozymes *in vitro* or *in vivo.*

Observed distinct biochemical properties of the PKC isozymes *in vitro* are consistent with the hypothesis that each isoform may have distinct roles in signal transduction processes. However, the specific roles of distinct PKC isozymes in different cellular responses and signal transduction pathways *in vivo* await further elucidation and experimental evidence is limited and frequently conflicting. On the other hand, because of discrepances between some *in vivo* and *in vitro* results, an important question has arisen, of whether or not these *in vitro* characteristics can be extended to *in vivo* situations. In this respect, the use of phorbol esters of different biological activity as probes in both *in vivo* and *in vitro* investigations of any particular biological system, is of crucial importance.

3.4. DOPPA metabolism in HL 60 cell cultures

3.4.1. Introduction

To understand the mechanism of action of different phorbol esters and to correlate results obtained from *in vitro* and *in vivo* experiments, knowledge of their possible transformation (isomerisation and metabolism) *in vivo*, is extremely important.

Earlier reports (72,400-413) showed, animal species and cell type dependent, metabolism of different PEs, mainly through hydrolysis of ester bonds in a PE molecule.

Our recent investigation of DOPPA activity *in vivo*, suggested a non-selective interaction with some PKC isotypes (380,382). This finding was not in agreement with our recent report (347) of DOPPA showing selective activation of the PKC β isotype *in vitro*, or with our *in vitro* binding and translocation studies (see Section 3.1., 3.2. and 3.3.) .

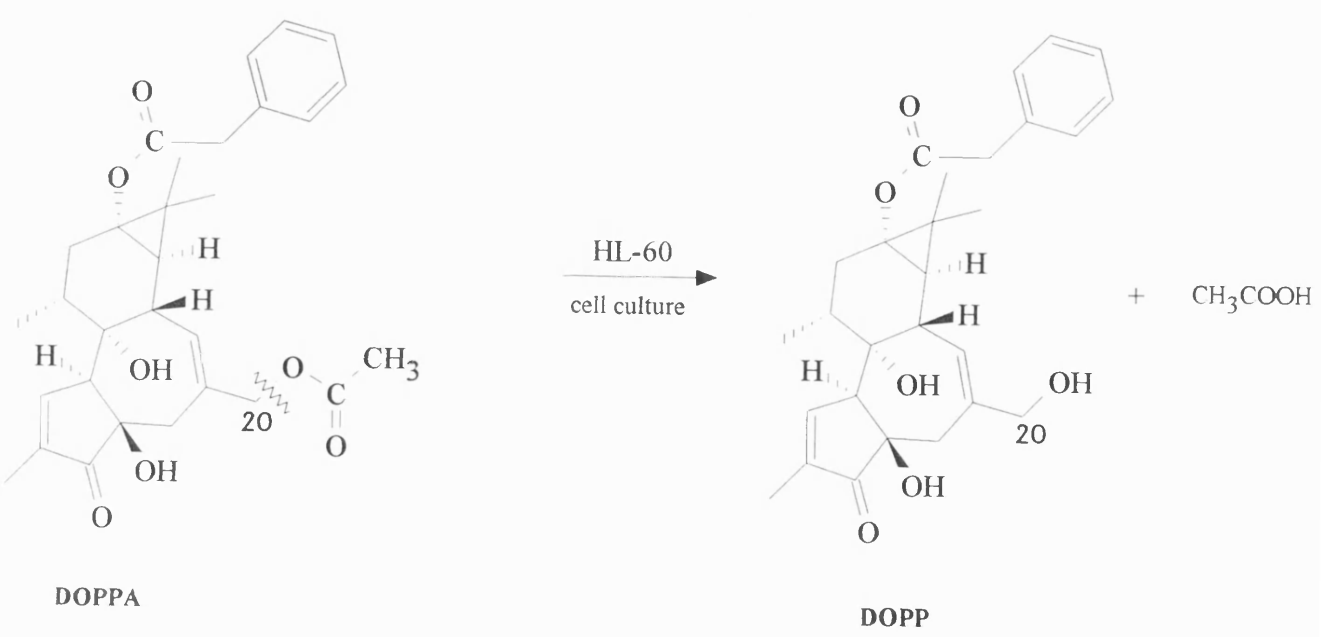


Figure 3.4.1. Metabolic deacetylation of DOPPA to DOPP. Equation shows hydrolysis of C20 ester bond of DOPPA generating DOPP and acetic acid.

This prompted us to investigate possible metabolic transformation of DOPPA to the more potent and non selective 12-deoxyphorbol DOPP (Figure 3.4.1., p. 179). Here we investigated DOPPA to DOPP metabolism using HL-60 cell culture as a model system. This study was a part of our wider study of PE effects on leukemic HL-60 cell line (351).

3.4.2. Experimental procedures

Cell culturing and incubation with DOPPA

HL-60 cells were cultured as described earlier in Section 2.2.1., p. 84. Experiments were conducted on HL-60 cell cultures at a density of 3 x 10^5 cells/ml. DOPPA (5 µl of acetone stock giving a final concentration of 1.96 µM in cell culture) was added to 5 ml of HL-60 cells or culture media (fresh or preconditioned for 3 days with HL-60 cells). After incubation for 0-96 h the culture was extracted three times with 10 ml of diethylether and the combined extracts were dried with anhydrous sodium sulphate and filtered through a Millipore AP25 prefilter. The extract was then evaporated under a stream of nitrogen and redissolved in 50 µl of chloroform for HPLC analysis.

HPLC analysis

HPLC methodology used is described earlier in detail in Section 2.2.2., p. 85, and was initially developed for phorbol ester purification and analysis. Typically, 20 µl of chloroform extracts were injected onto an Apex Silica column (Jones Chromatography; 25 cm x 4.6 mm i.d., 5 µm particle size). The mobile phase used was chloroform : methanol (99:1) which was found to give best separation. Absorbance between 240 and 400 nm were detected with a Waters 991 Photodiode-Array detector and data were processed using PDA software (as described in Section 2.2.2.). Standard solutions of DOPP and DOPPA were analysed using the same HPLC method and results were processed and analysed using PDA software in order to obtain calibration curves.

3.4.3. Results

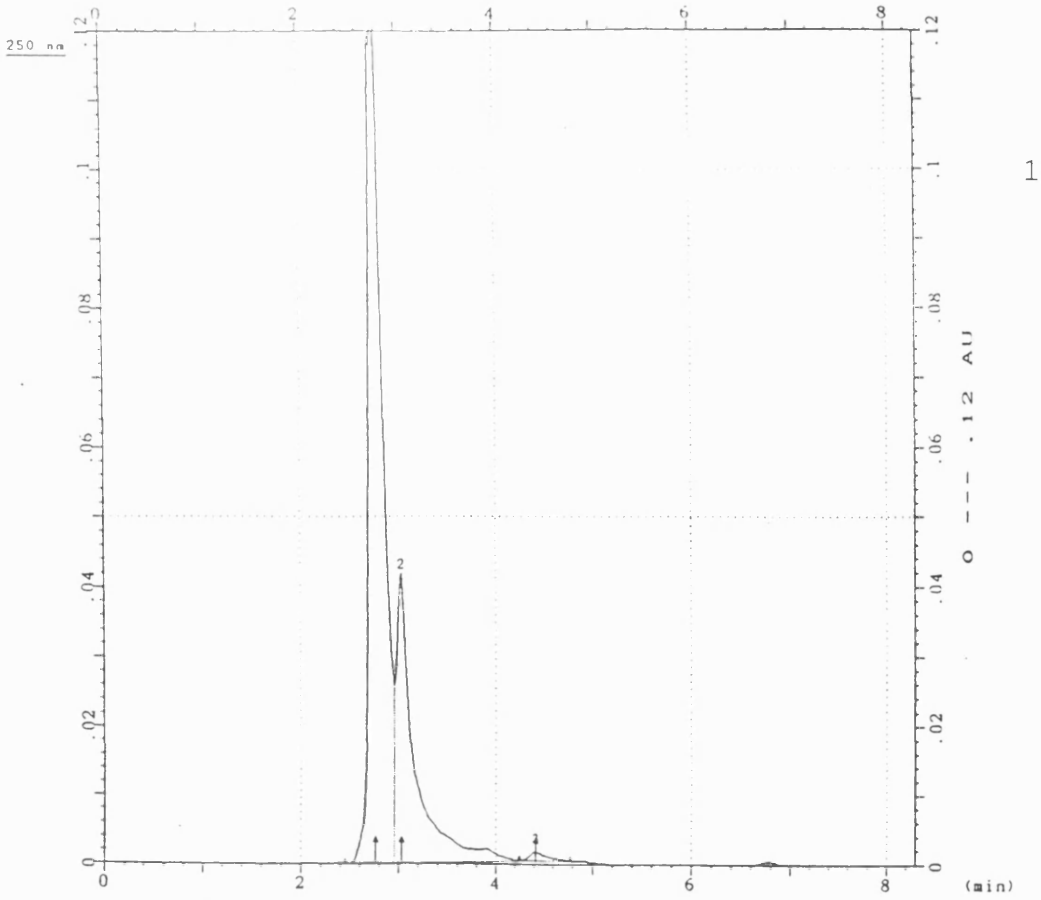
At the set time intervals, after DOPPA had been added to the HL-60 culture or media alone, cell cultures were partitioned against diethylether to extract DOPPA and any other phorbol ester formed by metabolism. Diethyl ether proved to be the best solvent for extraction, giving better recovery of phorbol esters, with less impurities extracted.

Using HPLC analysis of cell extracts supplemented with DOPPA or DOPP we found that whereas DOPPA co-eluted within a region of contaminant peaks (Figure 3.4.2. A. and B., page 182) after 3.05 min, the DOPP peak was separated away from other peaks after 7.25 min. It was therefore possible to detect and secure identification of a DOPP peak in the chromatogram of extracts seen at 250 nm, by comparison with a characteristic DOPP absorbance profile between 230 - 400 nm (Figure 3.4.3. A., p. 183) obtained for pure, standard compounds. The area under the peak for DOPP was used to quantify its presence by comparison with a calibration curve prepared with DOPP standards (Figure 3.4.3. B., p. 183). Under these conditions, the yield of extractable phorbol ester was 80-90 % and the detection limit was 100 and 400 pmol of DOPPA and DOPP respectively.

Metabolism of DOPPA to DOPP detectable in HL-60 cell cultures or culture medium alone is shown in Figure 3.4.4., p. 184, as the percentage of DOPPA converted over the 4 day study. In cell culture, appearance of DOPP was observed within 2 h, had reached 30 % of added DOPPA by 24 h and reached 80 % by 96 h. In the absence of cells, media alone caused a slow conversion of DOPPA to DOPP with low levels (5 %) detectable by 48 h and 30 % by the end of the study.

No other phorbol ester metabolite than DOPP was observed in the chromatogram throughout the investigation. It is unlikely that other de-esterification products (e.g. of C13 ester group) were formed and had passed undetected, because their higher polarity would confer a longer elution time, separating them from contaminant peaks. Additionaly, no changes in absorption profile of contaminant peaks was observed throughout the study, ruling out the presence of some new coeluting component(s).

A



B

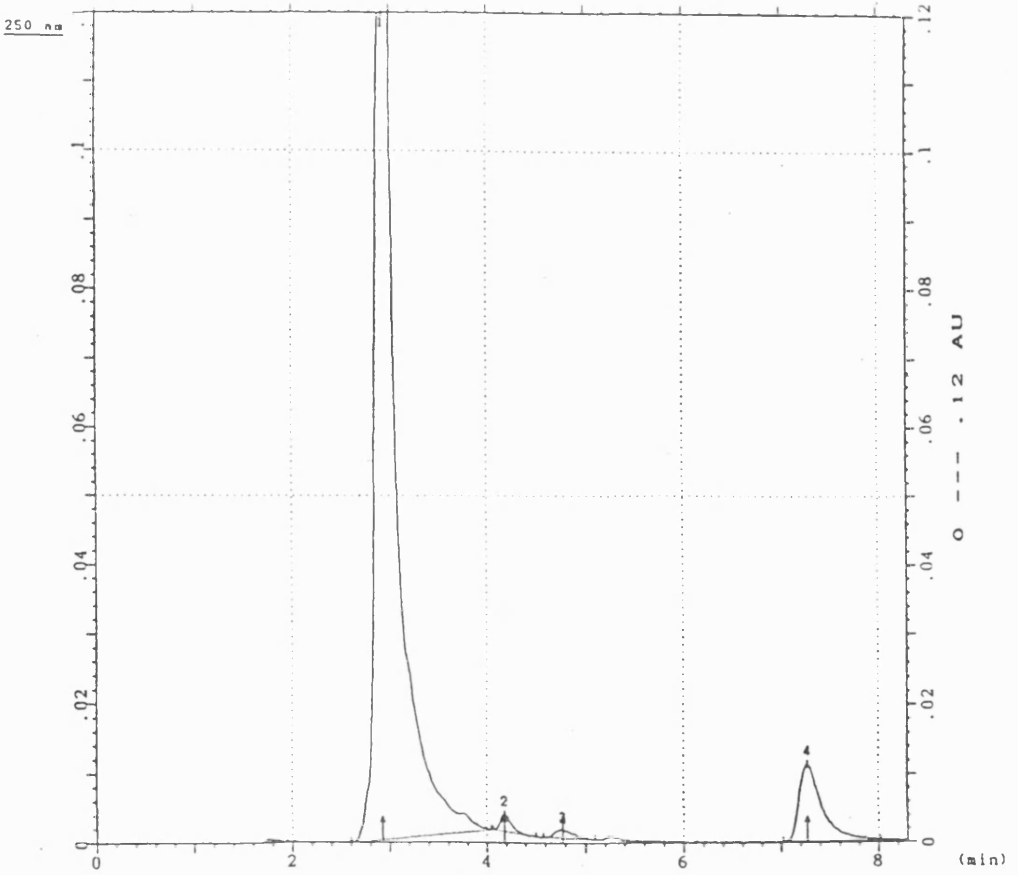
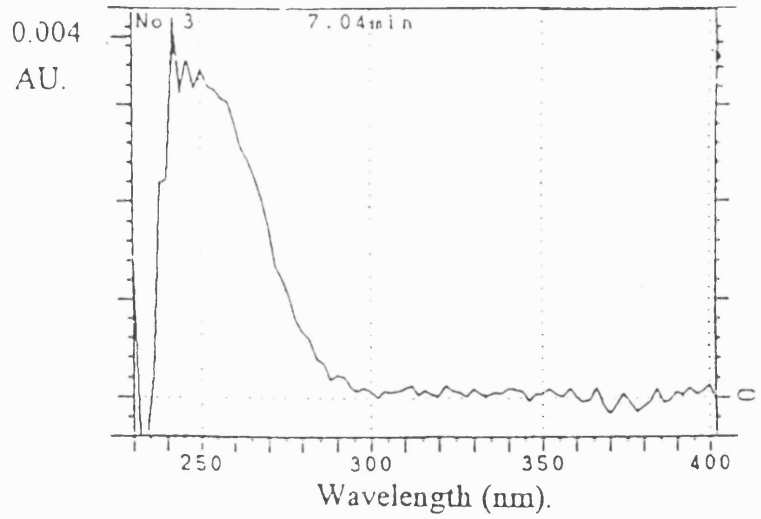


Figure 3.4.2. HPLC chromatograms of HL-60 cell culture extracts. Representative HPLC chromatograms at 250 nm obtained at time 0 (**A**) and after 48 h incubation (**B**) of DOPPA in HL-60 cultures. Peak labelled as 2 in **A** represents DOPPA and peak labelled as 4 in **B** is one of DOPP.

B

A



B

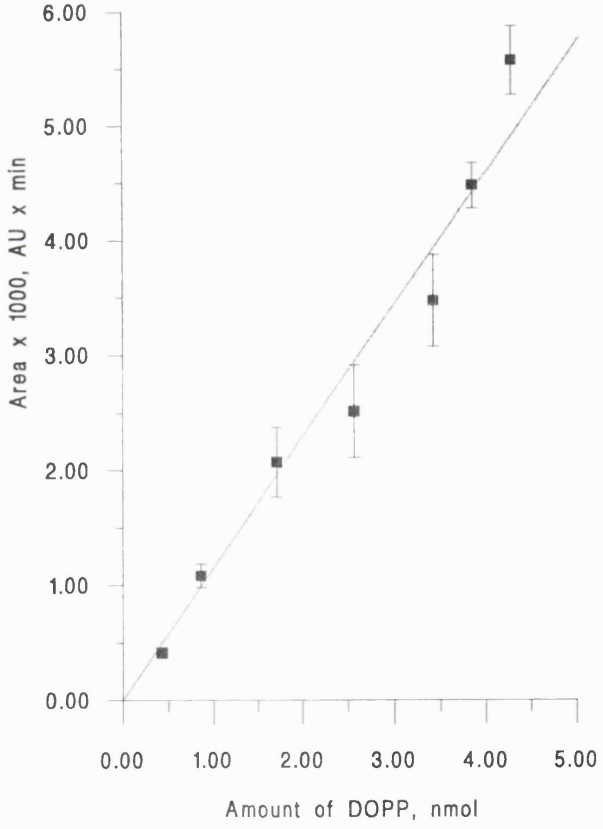


Figure 3.4.3. Absorbance spectrum and calibration curve for DOPP. (**A**) Absorbance spectrum, between 230 and 400 nm, of DOPP peak shown on Figure 3.4.2. and (**B**) Calibration curve for DOPP standards, used to quantify DOPP in culture and media extracts. The mean values and S.E.M. is shown for triplicate determinations.

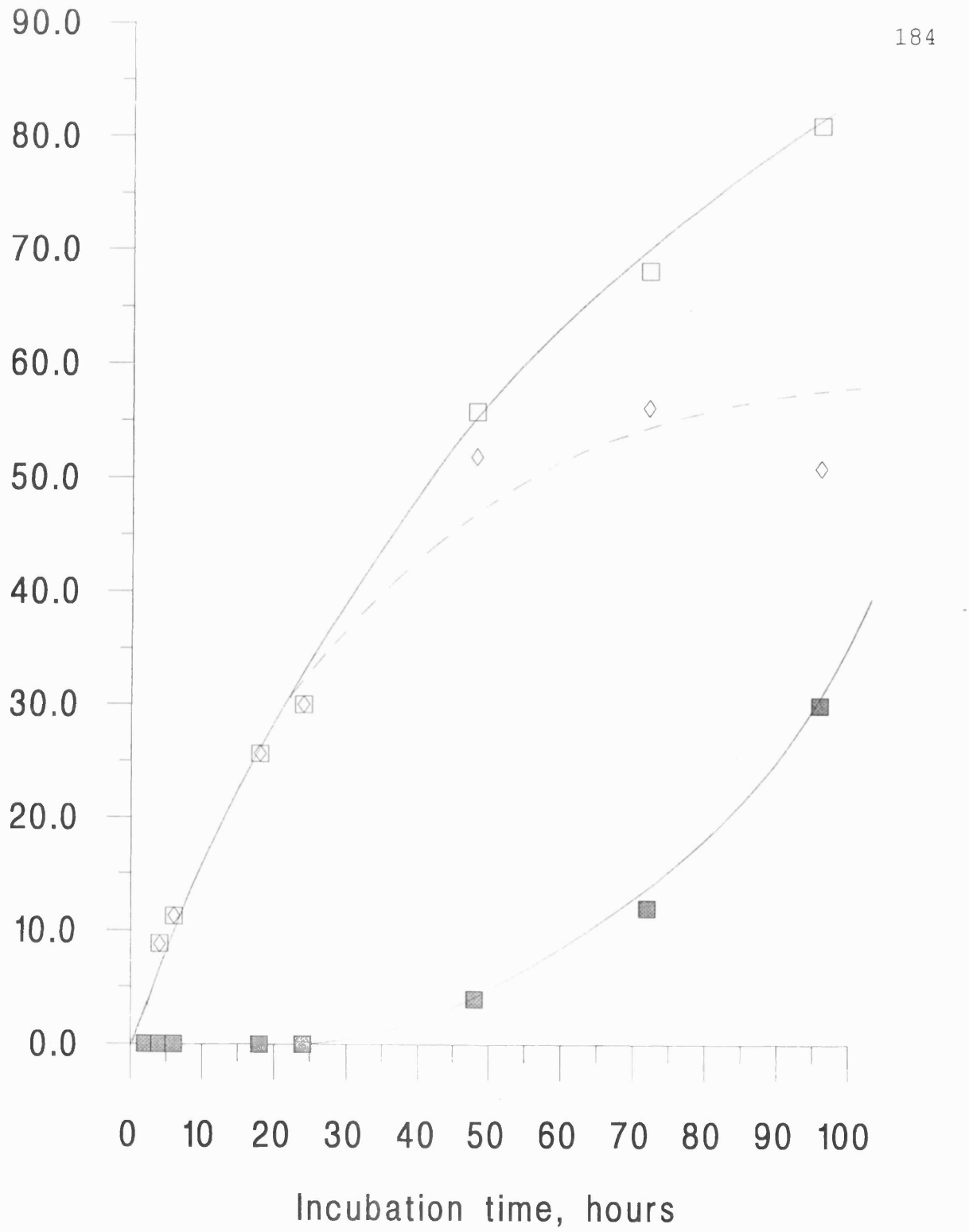


Figure 3.4.4. Time course of DOPPA metabolism in HL-60 cells. DOPPA (2 μM) was added to HL-60 cell cultures (□) or with media alone (■) for varying times prior to extraction. Results are shown as a percentage of DOPPA converted to DOPP, with specific metabolism (◊) obtained from the differences between these values. Data from a representative experiment are given (typically values obtained from separate experiments did not vary more than 10 %).

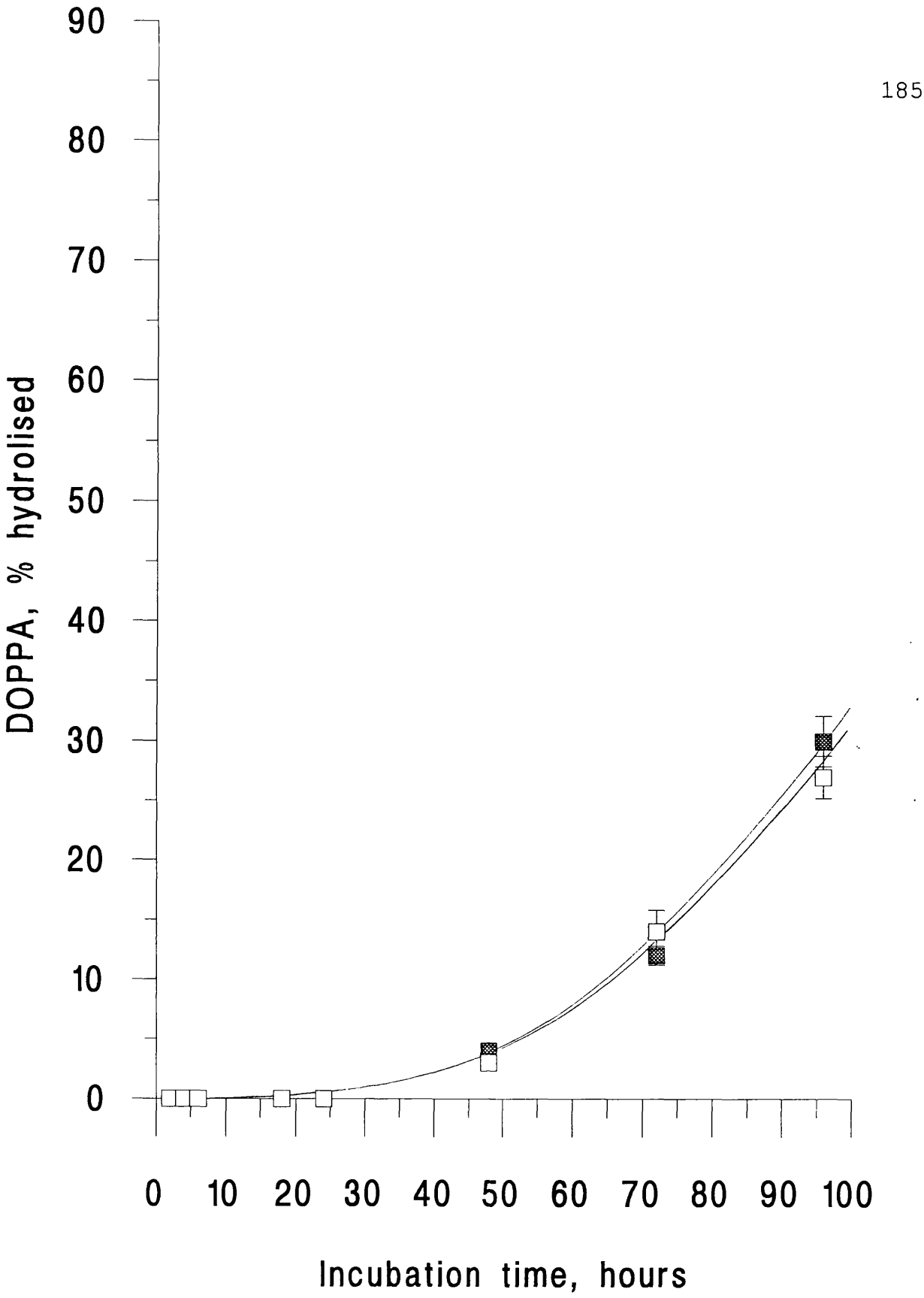


Figure 3.4.5. Time course of DOPPA metabolism in culture medium. DOPPA (2 μM) was added to HL-60 cell conditioned RPMI 1640 medium (□) or normal media (■) and incubated for varying times prior to extraction. Results are shown as a percentage of DOPPA converted to DOPP. Each value represents the mean ±S.E.M. of two separate experiments.

Cell conditioned media obtained after 3 days incubation and pelleting of cells from a suspension, did not show any metabolising effect on DOPPA different from normal media (Figure 3.4.5., p. 185). Additionally when 24h DOPPA treated HL-60 cultures were separated into a cell pellet and media prior to ether extraction of each, both DOPPA and DOPP were only detectable in the media and not the cells.

3.4.4.Discussion

According to their chemical nature phorbol esters may be metabolised by several enzymatic pathways (400):
Oxidation, e.g. dehydratation of free hydroxyl groups, epoxydation of double bonds, C-hydroxylation
Reduction, e.g. hydrogenation of the carbonyl group and of double bonds
Hydrolysis of existing or *formation* of additional *ester functions*
Formation of ether functions

Studies of PE metabolism so far have demonstrated hydrolysis of ester functions on position C12 and C13 (72,403-408,410-413), C3 carbonyl group reduction (402) and glucuronide formation (409). These metabolic transformations were found to be species, tissue and cell type dependent. However, in most of the studies, product of C12 ester bond hydrolysis was found to be a major or the only metabolite. Additionaly, lipases responsible for ester bond hydrolysis were characterised from rat liver (407,413), skin of several species (72) and sera of several mammalian species (410-412). This led to a theory that resistance to tumor-promotion by phorbol esters by some species could, at least partly, be explained with their metabolic transformation to less active or inactive compounds.

In contrast to extensive studies of phorbol-12,13 diesters, only two studies have reported on metabolism of DOPPA (380,382), a deoxyphorbol ester with acetylated C20 primary hydroxyl group. However in both studies, using cultures of U 937 (382) and PC 12 cells (380), no accurate identification or quantification

of metabolic product was reported.

Here using HPLC coupled with photodiode array detector it was possible to assess quantitatively DOPPA loss from HL-60 cell cultures and the appearance of its C20 deacylation product - DOPP. The breakdown of DOPPA in cell free medium, incubated under normal cell culture conditions, was very slow, with significant quantities of DOPP (30 %) appearing only after 4 days (Figure 3.4.5., p. 185). Similar slow breakdown was also observed when DOPPA was incubated with media obtained from 3 days old HL-60 cultures, indicating that conversion to DOPP is catalysed by an intracellular mechanism rather than in the medium (Figure 3.4.5.).

Interestingly, when cells incubated with DOPPA for 24 h were separated from the media, HPLC analysis revealed that both the product DOPP and the remaining DOPPA were only detectable in the media fraction, indicating that the amount of these phorbol esters within cells was too low to detect (less than 100 or 500 pmol for DOPPA and DOPP respectively). This suggested that whilst DOPPA metabolism required cells, both DOPP and DOPPA can freely exchange between the media and the cell.

The use of a photodiode array detector enabled UV-VIS spectral analysis of eluted fractions and their comparison (together with chromatographic profiles) with spectral profiles of standard compounds.

Through analysis of topographic and three-dimensional chromatogram plots (usingPDA software) DOPP has been identified as the only DOPPA metabolite in HL-60 cell culture. This was in agreement with previous reports, where the major or only metabolic product of 12,13-phorbol diesters was found to be 13-monoester (probably for steric reasons), suggesting that 12-deoxy-13-phorbol esters will be less susceptible to hydrolysis of C13 ester bonds.

As a part of the same study we investigated DOPPA and DOPP for their effects on HL-60 differentiation, and compared them to the tumor-promoting phorbol ester TPA.

All phorbol esters were able to exert a maximal effect on cell cultures (with qualitative and quantitative differences) as monitored by parameters of culture density, inhibition of radiolabeled thymidine incorporation, induction of cell-cell

and cell-substratum adherence and morphological changes consistent with acquisition of macrophage-like characteristics (351). DOPPA, in these experiments, followed a different time course, with a delayed time to achieve 50 % inhibition of thymidine incorporation. Taken with the metabolism time course, the characteristics of the inhibition of thymidine incorporation (Figure 3.4.6.) by DOPPA can be interpreted as being due to the appearance of DOPP in HL-60 cultures.

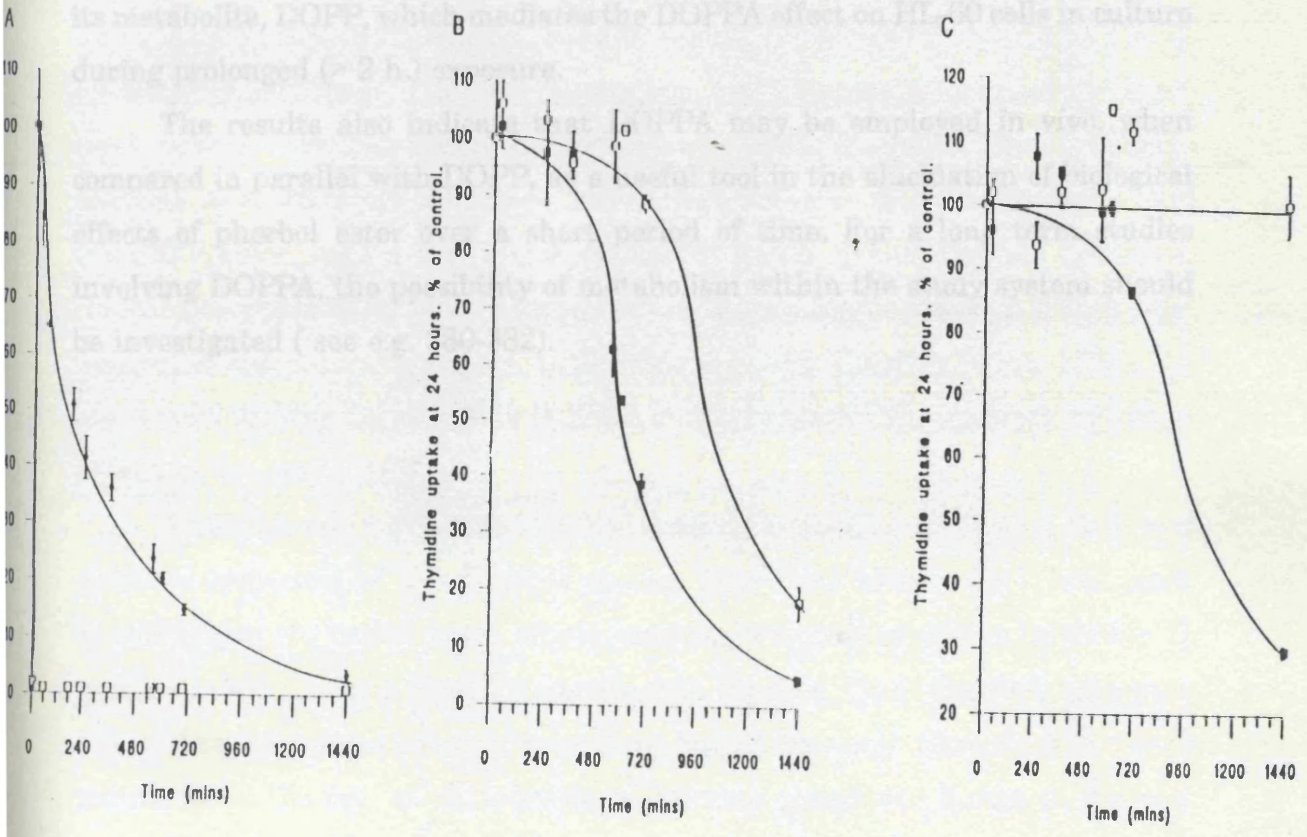


Figure 3.4.6. Effect of timed exposure of HL-60 cells to TPA, DOPP and DOPPA. The phorbol esters TPA (A), DOPP (B) or DOPPA (C) were added to HL-60 cultures and pulsed with [^{3}H]thymidine 2 h prior to harvesting. The final concentrations of phorbol ester used were 10 nM (◆), 100 nM (□) or 1000 nM (■). Incorporated radioactivity is expressed as a percentage of control HL-60 cultures with the range shown (from ref. 351).

Effects of phorbol esters are thought to be mediated by members of the PKC family of kinases of which PKC - α and PKC β_1 are known to be present in HL-60 cells (264), although a third PKC - ζ type kinase has also been reported (414,415). A previous study from our laboratory has assesed the ability of TPA, DOPP and DOPPA to stimulate PKC α, β_1, γ, δ and ε isotypes *in vitro* (347). In that study DOPPA was only able to activate PKC β_1 which would suggest that this isotype may mediate the differentiating effects of DOPPA in HL-60 (see however, our recent study, Section 3.1., 3.2. and 3.3., and ref. 371). Our DOPPA metabolism study, however suggest that it is the metabolism of DOPPA which initiates, and its metabolite, DOPP, which mediates the DOPPA effect on HL-60 cells in culture during prolonged (> 2 h.) exposure.

The results also indicate that DOPPA may be employed *in vivo,* when compared in parallel with DOPP, as a useful tool in the elucidation of biological effects of phorbol ester over a short period of time. For a long term studies involving DOPPA, the possibility of metabolism within the study system should be investigated (see e.g. 380-382).

3.5. Isolation of Mezerein from *Daphne blagayana*, Frayer *Thymeleaceae*, its chemical and biochemical characterisation

3.5.1.Introduction

Botany and ethnopharmacology

Daphne blagayana Frayer, belongs to *Spermato*phytes **phylum**, **division** - *Angiospermae*, **class** - *Dicotyledoneae*, **subclass** - *Archichlamydeae*, **order** - *Thymelaeales*, **family** *Thymelaeaceae* and **genus** - *Dap*hne (416). Synonims are: *D. lerchenfeldiana* Schur.; *D. alpina* Baumg. non L.; *D. "manzellii"* or *"mazellii"* Hort. not *D. mazeli* Carr. The family *Thymeleaceae* to which the genus *Daphne* belongs contains approximately 90 genera with about 500 species from temperate and tropical regions, with a high concentration in Africa. Genus *Daphne* Linnaeus, contains approximately 70 species which are mainly concentrated in Europe and temperate and subtropical Asia, with a few representatives in North Africa. All are shrubs varying considerably in habit and may reach three or more meters in height in the wild (416).

Although only a limited number of species have been investigated, toxic and curative properties of the *Daphne* species have been known for a long time. Theophrastus (he called these plants kneoron, κνεορον) mentions (probably *D. gnidium*) their use as a relief for constipation (berries, *Cocca Gnidiae*, have been kneaded up in a peace of bread to prevent mouth burning). Gerard (XVII cent.) comments on the use, of *D. laureola* in "purging phlegmatic humours, drawing water from the head and provoking vomit" and of *D. gnidium* in abortion (423). Linnaeus certainly knew of the poisonous properties of *D. mezereum*. Poisonous properties of these plants must have made their use in medicine somewhat hazardous. The symptoms of poisoning include intense irritation and burning pains in the mouth and stomach, gastro-enteritis and convulsions which in severe cases may be followed by death. Application to the skin causes irritation and inflammation accompained with blistering. Luckily, the unpleasant taste of the

fruit (and other parts) of *Daphnes* is a sufficient deterrent in the majority of instances to prevent serious poisoning. However, until recently mezereon (dried bark of various *Daphne* species) was still recognised in the *British Pharmaceutical Codex 1949* (422). At one time decoctions (of mainly *D. mezereum* and *D. laureola*) were thought to be a cure for cancer, worms, toothache, rheumatism and various skin deseases (417).

In Serbian popular tradition, *D. mezereum* (vučja lika, vučja oputa, maslinica, ajdučka oputa, likovac), is known to posses vesicant (causes blisters) properties and has been used for treating rheumatism, skin diseases and for inducing abortions, although because of its toxicity internal use has been abandoned. It used to be a constituent of *Emplastrum Mezerei cantharidatum* (418). Its toxic and irritant properties are well known in traditional medicine as well as of *D. gnidium* L.(maslinica), *D. laureola* (vučje uho, divlja lovorika, zelenika, veliki, žuti jeremičak, zimzeleni likovac, ličac, lovorčica, ljubčac vazdazelen, vazdazelena maslinica, ušitak). it is also known that animals do not eat these plants. Sometimes berries of *Daphnes* were found in adulterated pepper (418).

Type locality (Locus classicus) *Daphne blagayana* Freyer (in Flora Genensb. 21: 176, 1838), Thymeleaceae (Figure 3.5.1.A. and B., p. 192), was originally discovered in 1837 by Count Blagay in woods of St. Laurent near Biligrac west from Ljubljana (Slovenia, Former Yugoslavia) (419).

Distribution This beautiful evergreen shrub is a subendemic species of the central Balkans, with small enclaves found in the southern Alps, southern Karpats, on Stara Mt. in Bulgaria and the Greek mountains (northern parts of Pindus and Olympus). It is widespread in Slovenia (Lower Stajerska and Koruska), Croatia (Hrvatska), Bosnia and Hercegovina (Bosna i Hercegovina), Macedonia (Makedonija), Yugoslavia (Jugoslavia), Romania, Bulgaria, Greece and Albania (Figure 3.5.2., p. 193). Central location is in the region of the serpentine massives of west Serbia (Srbija)(serpentine massif of Ibar) and central Bosnia. It is also present, although in less frequent populations, in central and south Dinarides, whilst in other parts of distribution areal this species is relatively rare (419).

A

B

Figure 3.5.1. A. and B. *Daphne blagayana* Freyer, *Thymeleaceae*

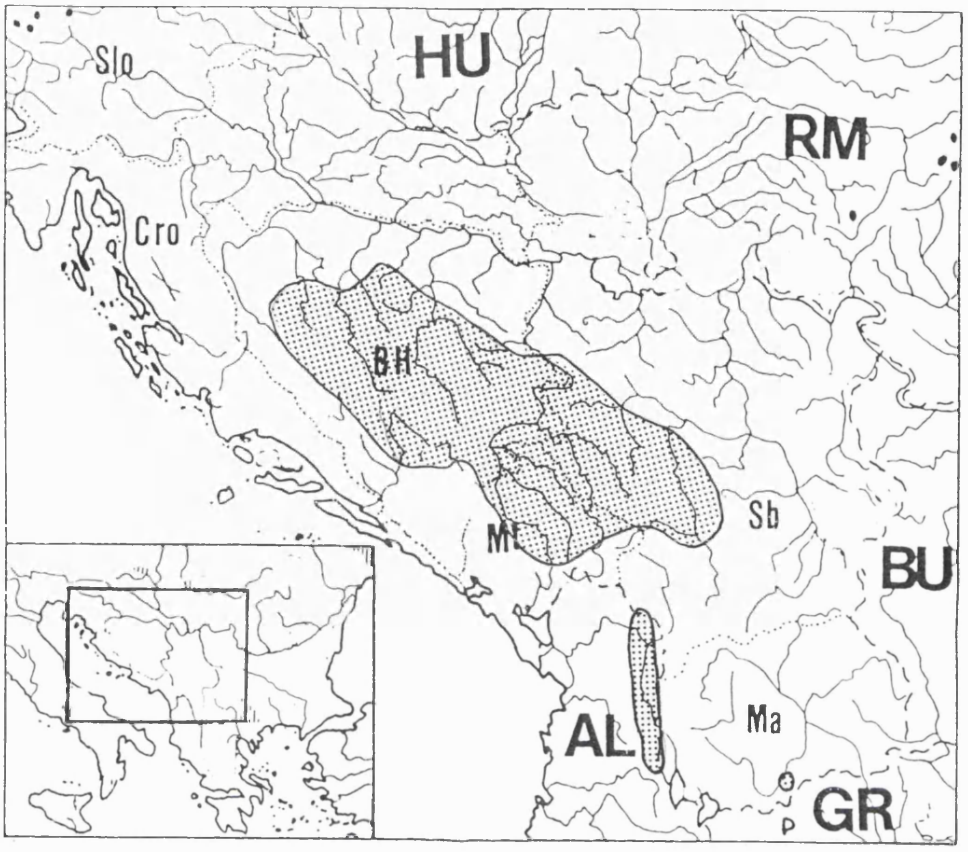


Figure 3.5.2. Distribution areal of *Daphne blagayana* Freyer, *Thymeleaceae*. AL - Albania, BU - Bulgaria, BH - Bosnia and Hercegovina, Cro - Croatia, GR - Greece, Hu - Hungary, Ma - Macedonia, MI - Monte Negro, RM - Romania, Sb - Serbia, and Slo - Slovenia (acc. to Meussel et al. 1978: mapp 295d) (423).

Description Trailing evergreen shrub less than 30 cm in height with long bare stems; bark dark purplish-brown, becoming purplish-gray with age; leaves alternate, shortly stalked, dark green, glabrous, leathery, broadly ovate or obovate, rounded at the apex, often minutely apiculate, clustered towards the tips of the more or less upright young shoots, 2-5 cm long, 1-1.5 cm wide. Flowers creamy-white (rarely pinkish), in dense terminal heads of 20-30, very fragrant; floral bracts very conspicuous, pale greenish and silky-pubescent, about 1 cm long, 0.6 cm wide; perianth lobes ovate, obtuse or subacute to acute, 4-6 mm long, 2-3 mm wide. Fruit is berry, whitish or pinkish, rarely produced in cultivation (419).

Chromosome No. 2n=18. (in ref 417, Nevling 1962); **Flowering period** April-June (in the wild).

Habitat Natural habitat is in light deciduous and pine woods, Buxus thickets, forest clearings, pastures and rocky grounds on limestone, dolomitic and serpentine formations. It is found, optimum, in pine wood vegetation on serpentinits. It is distributed from mountain (600-700 m) to subalpine regions (2000 m) (420,421).

The plant is known in Serbian popular tradition (Jeremičak, Borika, Blagojev likovac) as toxic for humans and live-stock (418).

Apparently this species reached Britain about 1875, being introduced by Messrs. Veitch as an horticultural species and can still be rarely found (417).

Chemistry , pharmacology and biochemistry

Plants of *Daphne* genus have been investigated, and data of phytochemical investigations of *D. mezereum, genkwa, odora, gnidium, kiusiana, transcaucasica, cneorum, cannabina* (syn. for *papyracea*), *oleoides, papyracea, juliae, pontica, altaica, giraldii, glomerata, gnidioides, pseudogenkwa, pseudomezereum, sericea, tangutica, feddei, bholua, mucronata* and *arisanensis* (source, Chemical Abstract from 1907-1995), have been reported so far (23 species all together). Various diterpenoids, triterpenoids, steroids, polyphenols and their glycosides, flavonoids and their glycosides, cumarins and their glycosides and polysaccharides have been isolated and characterised from different *Daphne* species (5 and refences therein).

However, to our knowledge no data on chemistry and phytochemical investigations, of *D. balagayana,* have been published to date. Here we report on isolation of mezerein from *D. blagayana* and its biochemical characterisation.

Mezerein is an ester of 12-Hydroxydaphnetoxin and belongs to the daphnane diterpene group (Figure 3.5.3., p. 195). Its occurance has been so far reported only in *Daphne mezereum* (425,426). Due to its activity as a second stage tumor promoter (in two-stage mouse skin model) and as a PKC activator, mezerein has been widely used in biochemistry and pharmacology. However, inspite of its wide use in research and its established structure (425-427), no extensive NMR investigation (400 MHz ^{1}H NMR, ^{13}C NMR, 2D-NMR: COSY, NOESY), has been

published todate. Also, inspite of its known ability to activate (51,73,209,362,365,441) a mixed PKC population, few studies on its interaction with individual PKC isotypes have been reported.

In this study we report on detailed structural investigation of mezerein using a range of NMR methods and on its activation of PKC isotypes α, β_1, β_2, γ, δ, ε and ζ.

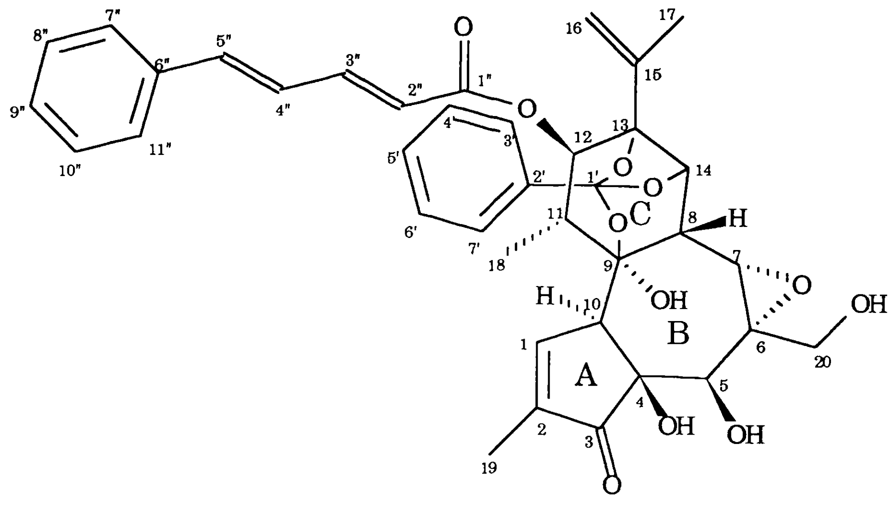


Figure 3.5.3. Structure of mezerein

3.5.2. Experimental procedures

Plant material

Aerial parts (branches and leaves) of *Daphne blagayna* Freyer were collected on Mount Golija (Western Serbia, Yugoslavia) in October 1992. Identification was done by Dr Dmitar Lakušić (Institute for Botany, Faculty of Biology, University of Belgrade, Belgrade, Serbia, Yugoslavia) and a voucher sample was deposited at the Herbarium of Botanical Garden and Institute for Botany, Belgrade, Serbia, Yugoslavia.

Analytical and Preparative HPLC

HPLC system used was composed of: two Altex 110 A pumps controlled by an Altex 421 Controller (Altex, Berkeley,CA,U.S.A.) and Waters 991 Photodiode-array detector (Millipore,U.K.). For analytical HPLC an Apex Apex Silica column (4.6 mm x 25 cm, 5 μm particle size, Jones Chromatography, U.K.) was used. Semi-Preparative HPLC was performed on Apex Prepsil column (10 mm x 25 cm, 8 μm particle size, Jones Chromatography, U.K.). All samples were injected as solutions in $CHCl_3$. Isocratic elution was carried out with the mobile phase $CHCl_3$/MeOH (99:1) at the flowrate 1 ml/min. All solvents were of HPLC grade (Romil Chemicals Ltd. U.K.) and were degassed by sonication prior to use. Absorbances between 230 and 400 nm were detected and data were processed using PDA software (version 6.22.).

Irritancy testing

Irritant activity measurement of substances was based on acute pro-inflammatory effect of diterpene esters and the "mouse ear erythema assay" (75,446).

Assay was performed on male CD-1 mice (20-25 g body weight) and 5 μl of acetone solution of a sample (1mg/ml, 5 μg dose) was applied to the inner surface of the right ear of the animal; 5 μl of acetone was applied to the inner surface of the left ear as a control.

Erythema was assessed subjectively at appropriate time intervals by

comparison of the treated ear with the control ear. Erythema was assigned a score at each reading as follows: - no reddening, ± reddening just visible, + distinct reddening, ++ reddening with edema and +++ reddening and edema accompanied with haemorrhagia. Animals were monitored every hour within the first 12 hours, every six hours in the next 36 hours and then daily for seven days.

PKC activation assay

The same phospholipid/detergent mixed micellar assay as previously described (Section 2.2.4.) has been used to investigate the ability of mezerein to activate purified recombinant PKC isotypes α, β_1, β_2, γ, δ, ε and ζ. Activation was also conducted with TPA, used as a comparison, for each particular PKC isotype using a concentration of 2 μM in the presence of Ca^{2+} (100 μM) (100% stimulation). Triplicates of each test point were assayed in the presence or absence of added calcium (100 μM free) for both, mezerein and TPA. Each experiment has been repeated 3 times independently.

Mass spectroscopy

Several mass spectroscopic methods were used during this study:

Fast atom bombardment MS (FAB MS). High resolution FAB MS was performed on VG ZAB SE double focusing mass spectrometer (Fisons, Manchester, U.K.), using Xenon atoms for ionisation (8 keV, ~ 1 mA) and (MNOBA (3-nitrobenzyl alcohol) as matrix to aid ionosation. (resolution set up at 10 000)

Electron ionisation MS (EI MS). EI MS was performed on VG ZAB SE double focusing mass spectrometer (Fisons, Manchester, U.K.), using accelerating voltage of 40 eV and source at 200 ^{0}C.

Matrix-assisted laser desorption/ionisation (MALDI) MS using nitrogen laser at 337 nm on VG TOFSpec (V1.2C) time-offlight (TOF) mass spectrometer. 2,5-dihydrohybenzoic acid was used as matrix (0.7 μl in AcCN/H_2O, 7:3), mixed with an equal volume of sample and allowed to dry, EtOH was added in order to recrystallise the sample and to produce a more uniform crystal surface (increasing signal to noise ratio). Multiple laser shots were fired and signals were averaged. The experiment was done in reflection mode (accelerating voltage - 20 KeV and

reflection voltage 23 KeV) and positive mode (acquisition score - 10 mV and acquisition sampling 250 MHz)

Nuclear magnetic resonance spectroscopy

400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR, 2D-NMR: COSY, NOESY were done on a Bruker AMX 400 NMR spectrometer.

UV spectroscopy

UV absorption spectra were recorded on an Wallac Compuspec UV/VIS spectrophotometer with a resolution of 0.1 nm.

Computer assisted molecular modelling

For computer assisted molecular modeling SYBYL 6.1 (R 3000) mollecular modeling software (Tripos Associates Ltd., Bracknell, U.K.) was used on an Iris Indigo XZ 4000 workstation (Silicon Graphics U.S.A.).

3.5.3. Results

Purification of mezerein from Daphne blagayana Frayer.

Dried plant material (800 g) was crushed and milled in a grinder and extensively extracted with acetone (2 x 2.5 L for 2 days). Combined acetone extracts were filtered and the acetone evaporated under vacuum below 40 ^{0}C to give a tar-like dark green substance (25.3g). This residue was dissolved (with brief repetitive sonication in an ultrasound bath) in 500 ml 70 % aqueous methanol and filtered. Methanol solution was extracted with n- Hexane (4 x 150 ml) and the methanol portion further investigated (data not shown). However, the n-hexane extract, which is usually discarded (usually contain pigments, lipids, triterpenoids, steroids, jatrophane and casbane diterpenoids) as inactive, showed irritant activity in a "mouse ear erythema test" (m.e.e.t.) and was taken as a subject of our investigation. After removal of n-hexane under reduced pressure (< 40 ^{0}C), the dark green, tar-like residue (11.1 g) was subjected to a series of purification steps.

These included a series of gravitational and positive pressure column chromatographies on silica gel. In the first step "m.e.e.t." guided gravitational column chromatography on silicagel (7 x 65 cm). Using a successive gradient of solvents of increasing polarity (petroleum ether, hexane, diethyl ether, chlorophorm, acetone and methanol), a large proportion of green pigments (10.5 g, inactive in "m.e.e.t") was removed. Fractions (10 ml) were monitored by TLC (silica gel, $CHCl_3$/Acetone, 8:2) and similar fractions were combined and tested for irritancy.

Table 3.5.1. Proinflammatory activity of *D. blagayana fraction* 1-VII

time (hours)	mouse ear reaction
1	±
2	+
3	++
4 → 23	++
23 → 80	+++
96	++
5 → 7 days*	++

Acetone solution (5 µl) of a sample (100 µg dose) was applied to the inner surface of the right ear of the animal; 5 µl of acetone was applied to the inner surface of the left ear as a control. Erythema was assigned a score at each reading as follows: - no reddening, ± reddening just visible, + distinct reddening, ++ reddening with edema and +++ reddening and edema accompanied with haemorrhagia.

* ear remained inflammed for 12 days, and did not recover from necrosis.

We decided to further purify the $CHCl_3$ / Acetone (1:1) fraction (46 mg, bright yellow resinous substance, fraction 1-VII) because it showed the highest irritant activity, leading eventually (28 h) to complete necrosis of the mouse ear (Table 3.5.1., p. 199).

On TLC the $CHCl_3$/ Acetone, 8:2 fraction contained at least 15 components as judged from the number of bands visualised under 366 nm UV light, after spraying with 60 % H_2SO_4 and warming at 90 ^{0}C. After a succesive gravitational and positive pressure column chromatography on silica gel using $CHCl_3$/ Acetone gradient elution, we were able to isolate a major active component -"X" (2.1 mg, white solid substance) that appeared as a single TLC-homogenous fraction (using a range of different mobile phases).

X showed yellow fluorescence under 366 nm UV light, after spraying with 60 % H_2SO_4 and warming at 90 ^{0}C and an Rf value of 0.25 (silica gel TLC, $CHCl_3$ / Acetone, 8:2). However when X was applied on an analytical HPLC using $CHCl_3$ / MeOH, 99:1 as a mobile phase and a Photodiode array detector, it appeared to contain impurities. These were well separated from the main component enabling its purification on a semipreparative column. From computer analyses of contour and 3D chromatogram plots (see Section 2.4.2. and Figure 3.5.4. A - D, pgs. 202-203) the purity of X was confirmed. After removal of the solvent under a stream of nitrogen, compound X appeared to be a white solid substance (1.8 mg, 0.0002 % yeld) with an Rf value of 0.25 (silica gel TLC, $CHCl_3$ / Acetone, 8:2) and appeared as a single spot of yellow fluorescence under 366 nm UV light, after spraying with 60 % H_2SO_4 and warming at 90 ^{0}C.

Purified compound X was subjected to spectroscopic (^{1}H NMR, COSY, NOESY; ^{13}C NMR, MS and UV) analysis in order to elucidate its structure.

UV spectroscopy of X

Ultraviolet absorbtion maxima in absolute ethanol λ^{EtOH}_{max} and corresponding molar absorbances were (log ε): 226.4(4.19), 234.4(4.23), 240.2(4.20) and 314.7(4.55) (Figure 3.5.5., p. 204).

Mass spectroscopy of X

HR FAB mass spectometry exhibited (Figure 3.5.6., p. 205) an ion $(M + Na)^+$ at m/e (mass/charge) 654.2371 suggesting M^+ of 654.2473 (after substraction of 22.9898 for Na) and $C_{38}H_{38}O_{10}$ (calculated 654.2465). Laser desorption time of flight mass spectrometry, LDTF MS exhibited M^+ + Na at m/e 677.462, EI mass spectrometry gave peaks as shown in Figure 3.5.7., p. 206.

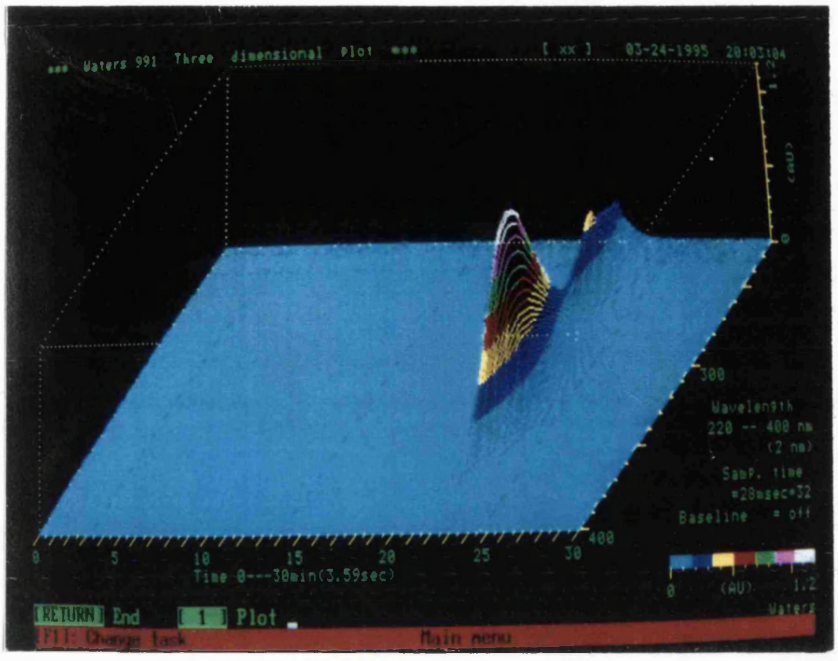
^1H NMR of X

The 400 MHz ^1H NMR of X is given in Figure 3.5.8., p. 207, and analysis of the spectra in Table 3.5.2., p. 210. Additionally through-bond and through-space ^1H-^1H connectivities were obtained by two-dimensional NMR experiments (COSY and NOESY), and presented in Figure 3.5.9., p. 208, and 3.5.10., p. 209.

^{13}C NMR of X

The 100 MHz ^{13}C NMR of X is presentad in Figure 3.5.11., p. 211 and Table 3.5.3., p. 212.

A



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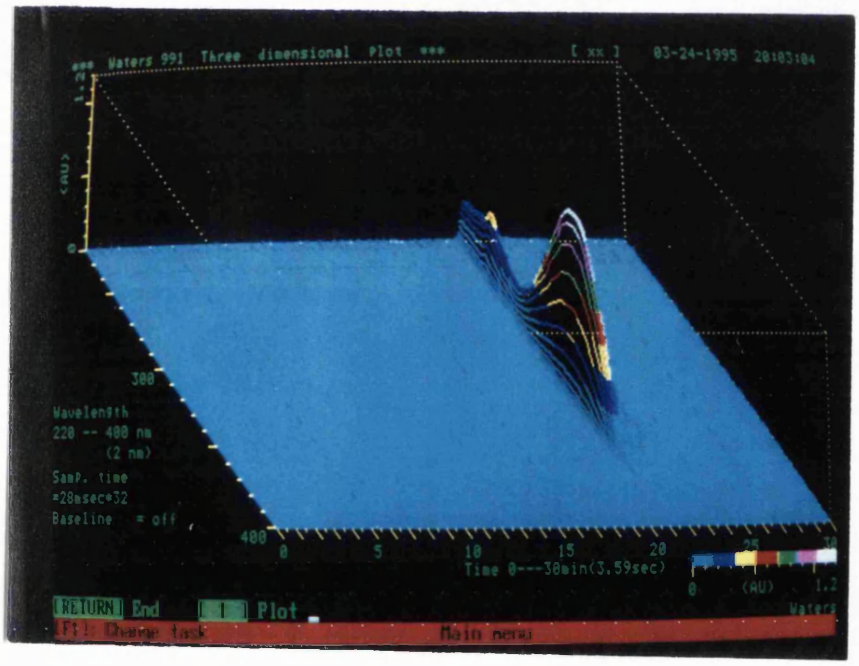
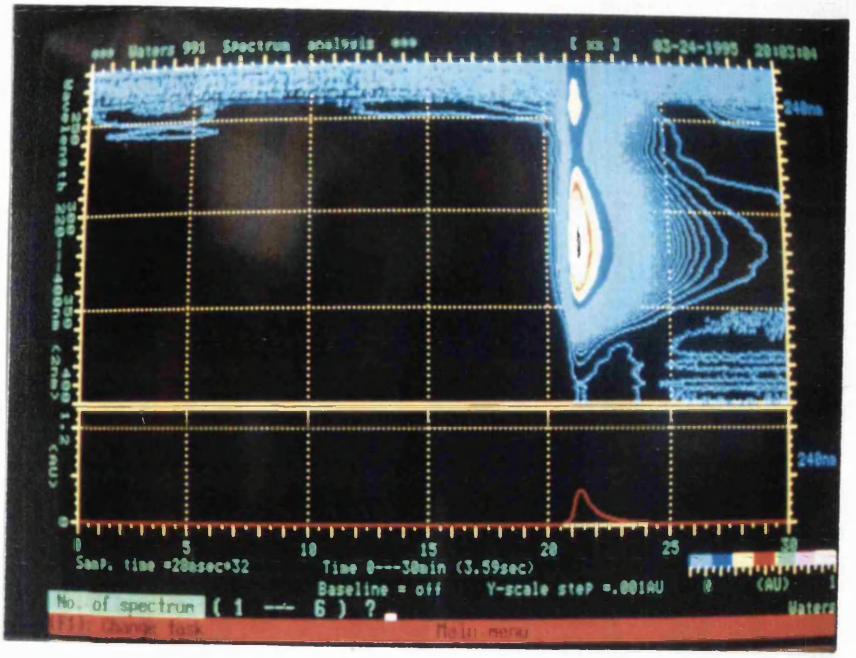


Figure 3.5.4. (A) and (B). Photodiode array three dimensional chromatogram plots of mezerein (Column: Apex Silica, Jones Chromatography; $CHCl_3$/MeOH, 99:1 as a mobile phase; flow rate 1 ml/min; Waters 991 Photodiode array detector).

C



D

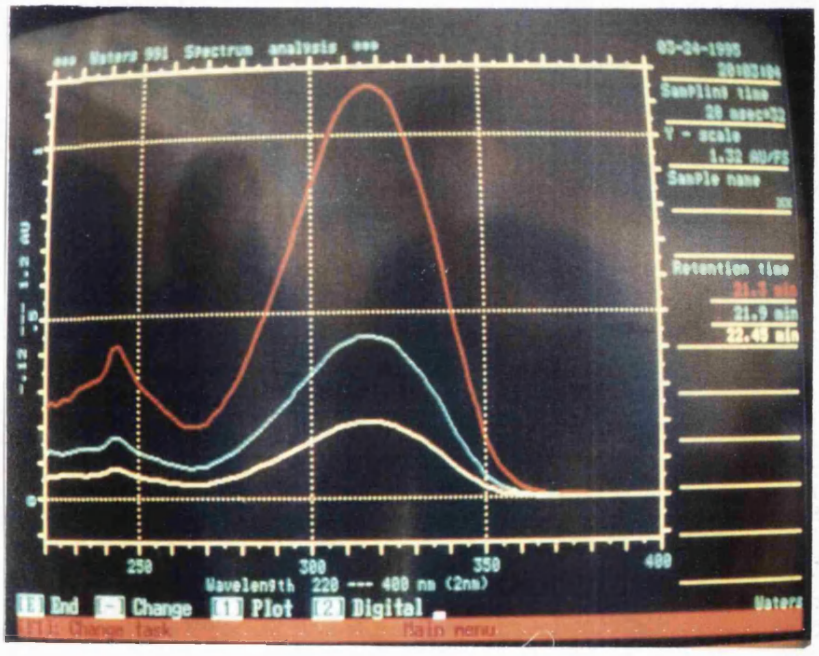


Figure 3.5.4. Photodiode array contour chromatogram plot (C) and chromatogram spectrum analysis (D) of mezerein (Column: Apex Silica, Jones Chromatography; $CHCl_3$/MeOH, 99:1 as a mobile phase; flow rate 1 ml/min; Waters 991 Photodiode array detector).

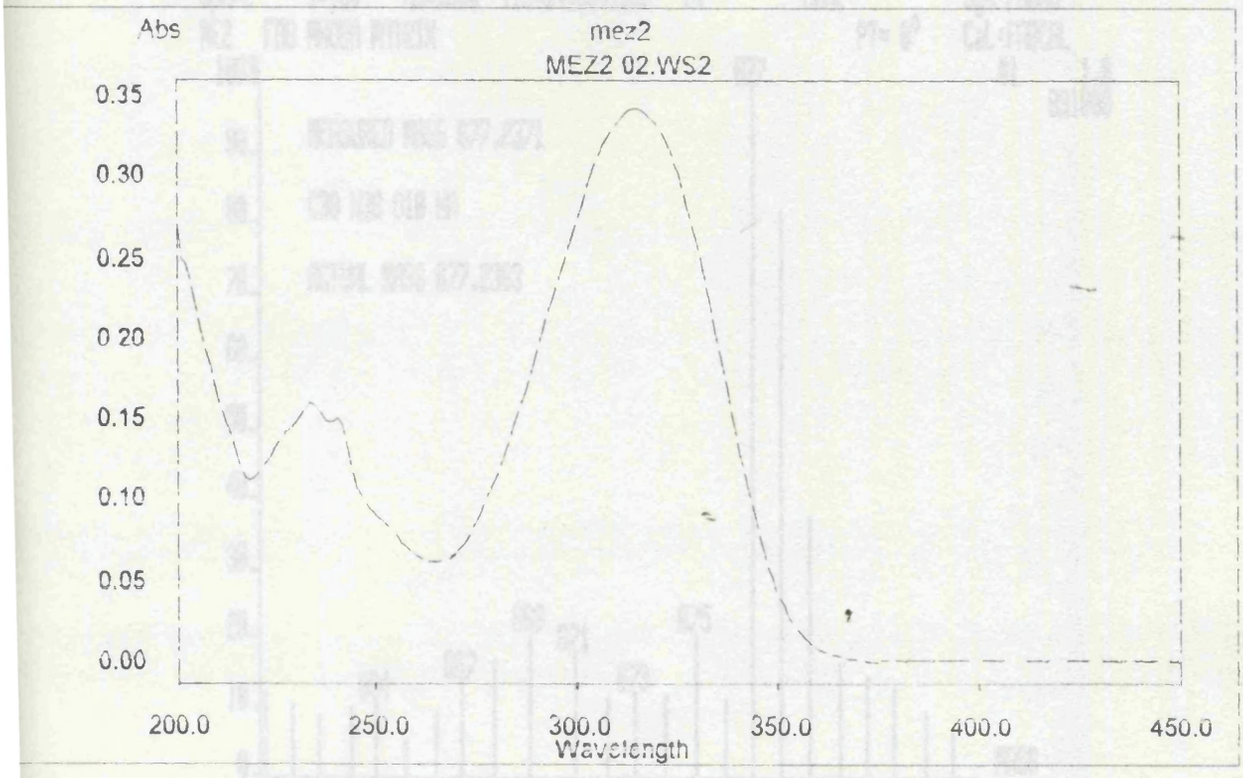


Figure 3.5.5. UV absorbtion spectra of mezerein (Wallac Compuspec UV/VIS spectrophotometer, resolution: 0.1 nm, solvent EtOH).

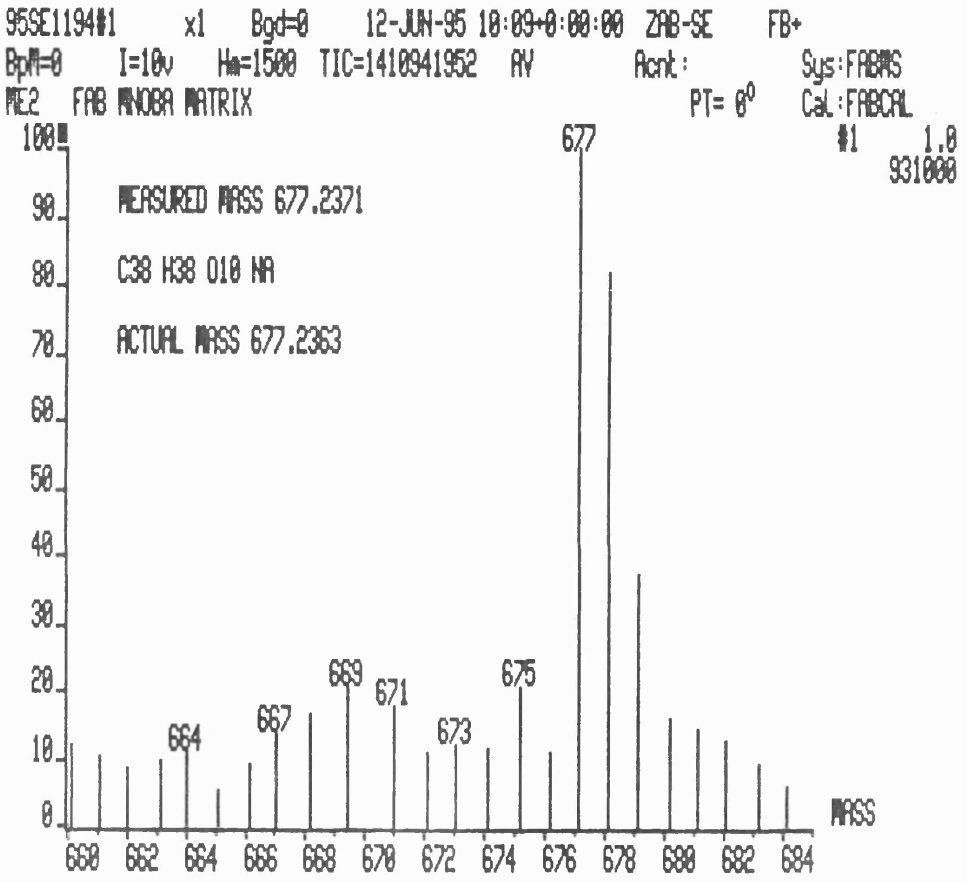


Figure 3.5.6. HR FAB mass spectra of mezerein (Value of 22.9898 for Na should be substracted from measured mass of 677.2371 to gave the real accurate molecular mass of 654.2473).

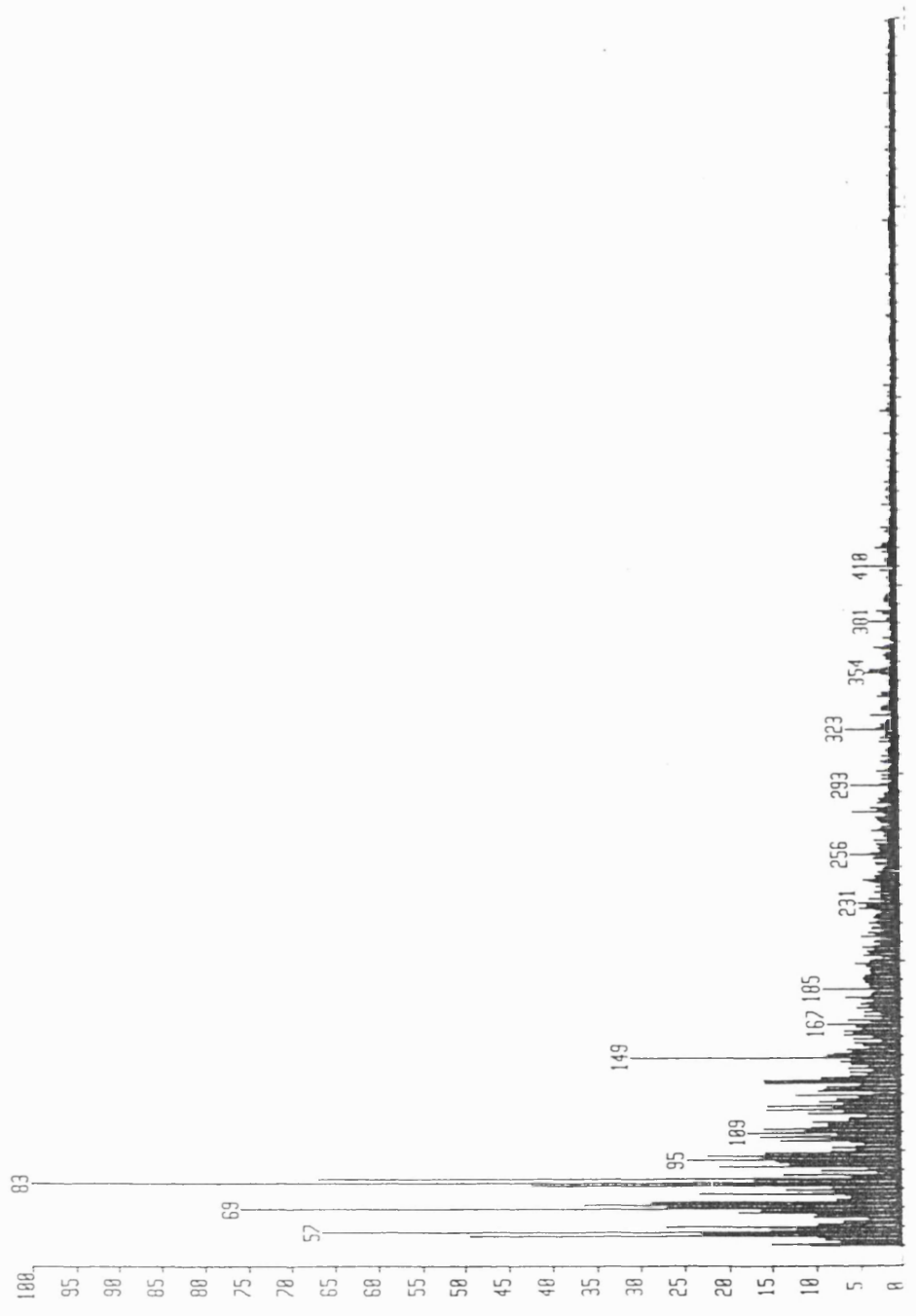
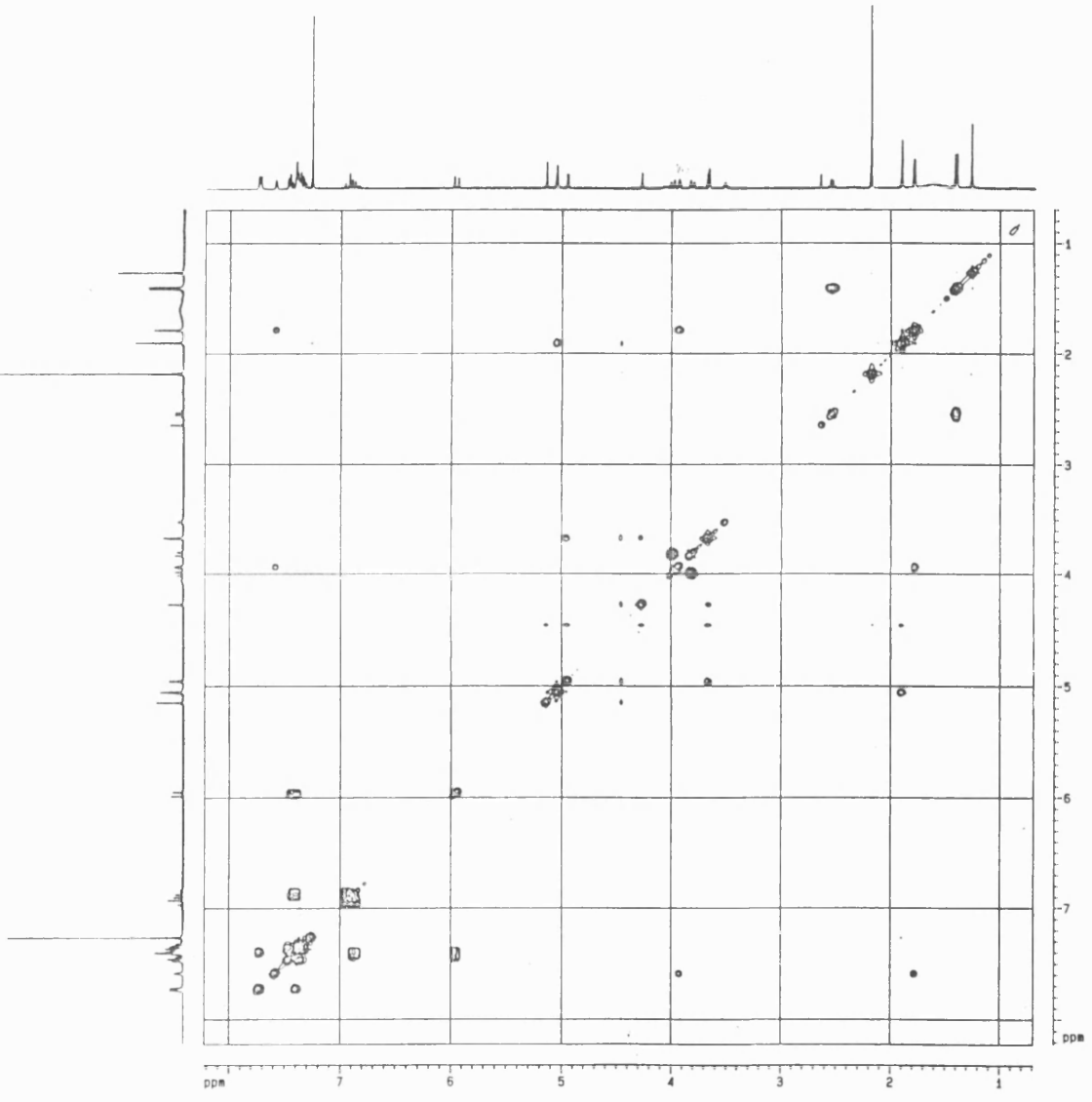


Figure 3.5.7. EI Mass spectra of mezerein.

Figure 3.5.8. 400 MHz ^{1}H NMR spectra of mezerein.



XX COSY 45

Current Data Parameters

NAME	apr2795.001
EXPNO	20
PROCNO	1

F2 - Acquisition Parameters

Date	950427
Time	11.46
PULPROG	cosy45
SOLVENT	CDCl3
AQ	0.1700040 sec
FIDRES	2.941453 Hz
DW	166.0 usec
RG	4096
NUCLEUS	1H
HL1	1 dB
D1	0.9539203 sec
P1	6.5 usec
D0	0.0000030 sec
DE	172.9 usec
SFO1	400.1361762 MHz
SWH	3012.05 Hz
TD	1024
NS	8
DS	4
IN0	0.0003320 sec

F1 - Acquisition parameters

ND0	1
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SFO1	400.1362 MHz
FIDRES	11.765813 Hz
SW	7.528 ppm

F2 - Processing parameters

SI	512
SF	400.1343931 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.40

F1 - Processing parameters

SI	512
MC2	QF
SF	400.1343931 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

2D NMR plot parameters

CX2	20.00 cm
CX1	20.00 cm
F2PLO	8.220 ppm
F2LO	3289.20 Hz
F2PHI	0.693 ppm
F2HI	277.17 Hz
F1PLO	8.220 ppm
F1LO	3289.20 Hz
F1PHI	0.693 ppm
F1HI	277.17 Hz
F2PPMCM	0.37638 ppm/cm
F2HZCM	150.60175 Hz/cm
F1PPMCM	0.37638 ppm/cm
F1HZCM	150.60175 Hz/cm

Figure 3.5.9. ^{1}H-^{1}H COSY of mezerein.

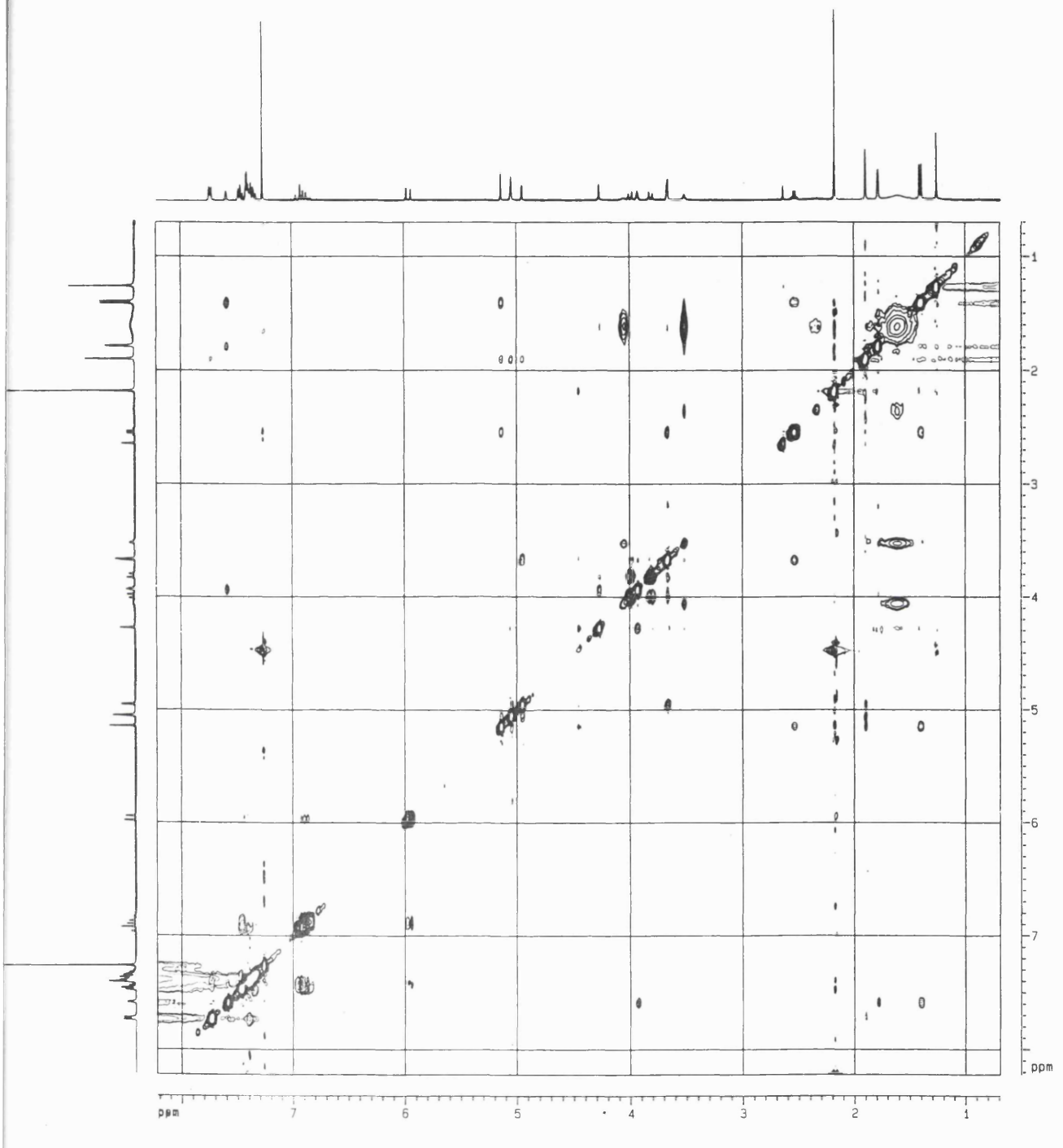


Figure 3.5.10. ^{1}H-^{1}H NOESY of mezerein.

Table 3.5.2. 400 MHz ^{1}H NMR of mezerein.

δ, ppm	Multiplicity	Integration	Inference
7.70-7.75	m	3H	H-8",H-9",H-10"
7.59	bs(q)	1H	H-1
7.42-7.50	m	3H	H-4',H-5',H-6'
7.30-7.42	m	7H	aromatic olefinic*
6.8-7.0	m	2H	H-4", H-5"
5.96	d, J=15 Hz	1H	H-2"
5.14	s	1H	H-12
5.05	s	2H	H-16
4.945	d, J=2.5 Hz	1H	H-14
4.27	s	1H	H-5
4.99	d, J=12.5	2H	H-20 A
3.93	t→p, J=2.58	1H	H-10
3.80	d, J=12.5	2H	H-20 B
3.665	d, J=2.68	1H	H-8
3.66	s	1H	H-7
2.54	q, J=7.22 Hz	1H	H-11
1.90	s	3H	3H-17
1.78	m (q)	3H	19-H
1.405	d, J=7.24 Hz	3H	3H-18

* Multiplet includes signals from H-2', H-3' and H-7' (δ = 7.30-7.35 ppm), H-3" (d, δ = 7.37, 7.41, J=15 Hz) and H-6", H-7" and H-11" (δ = 7.3-7.42 ppm).

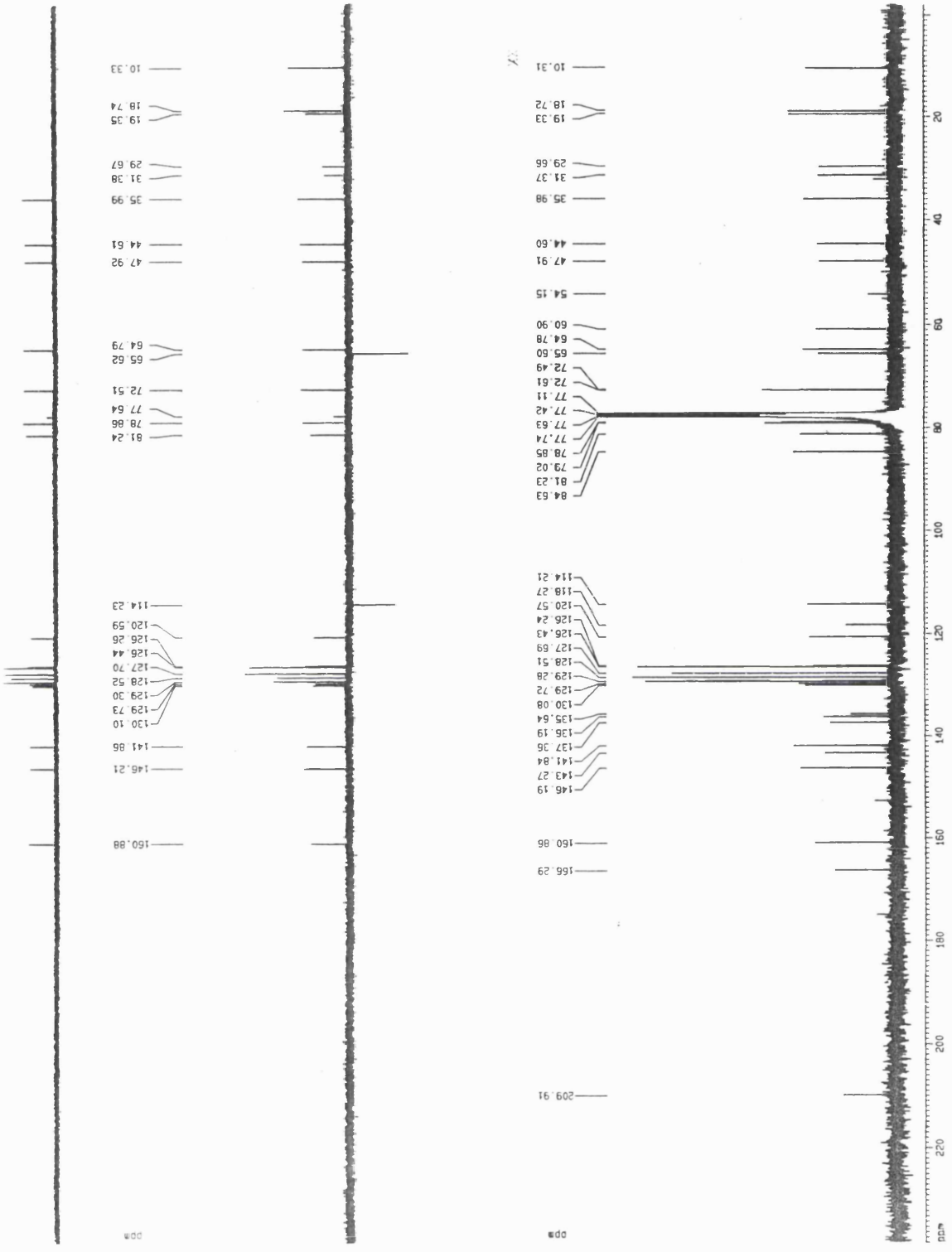
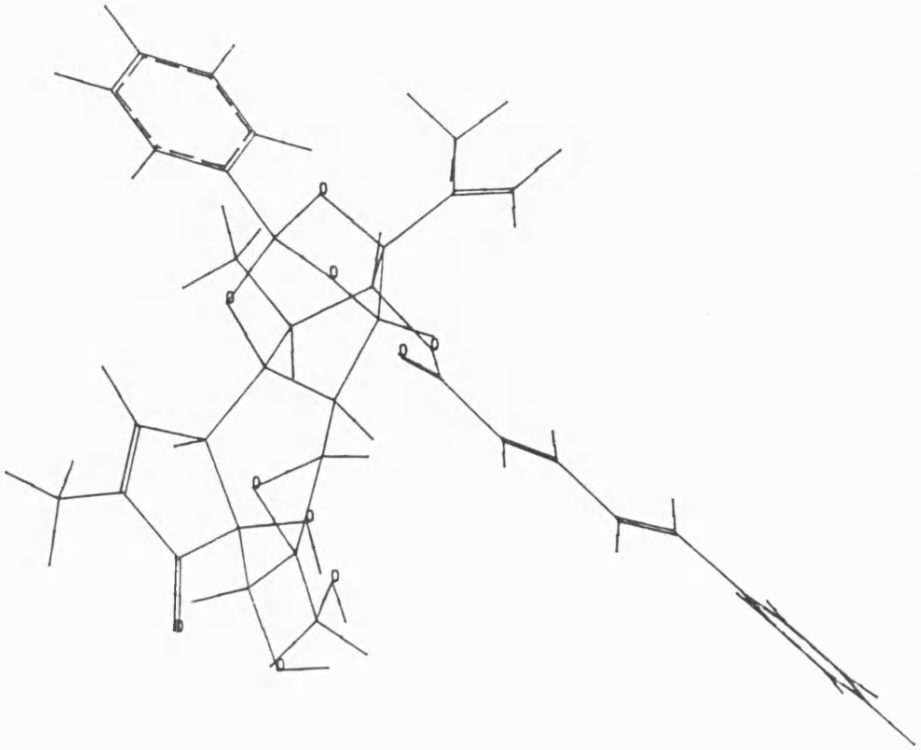


Figure 3.5.11. 100 MHz ^{13}C NMR spectra of **mezerein.**

Table 3.5.3. 100 MHz ^{13}C NMR of mezerein.

δ, ppm	inference	δ, ppm	inference
209.91	C-3	118.27	C-1'
166.29	C-1"	114.21	C-16
160.86	C-1	84.63	C-13
146.19	C-2"	81.23	C-14
143.27	C-15	79.02	C-4
141.84	C-3"	78.55	C-12
137.36	C-2	72.61	C-9
136.19	C-6"	72.49	C-5
135.64	C-2'	65.60	C-20
130.08	C-9"	64.78	C-7
129.73	C-5'	60.90	C-6
129.28	C-11", C-7"	47.90	C-10
128.52	C-4', C-6'	44.60	C-11
127.7	C-8", C-10"	35.98	C-8
126.44	C-3'	19.33	C-19
126.26	C-7'	18.72	C-17
120.59	C-4", C-5"	10.31	C-18

A



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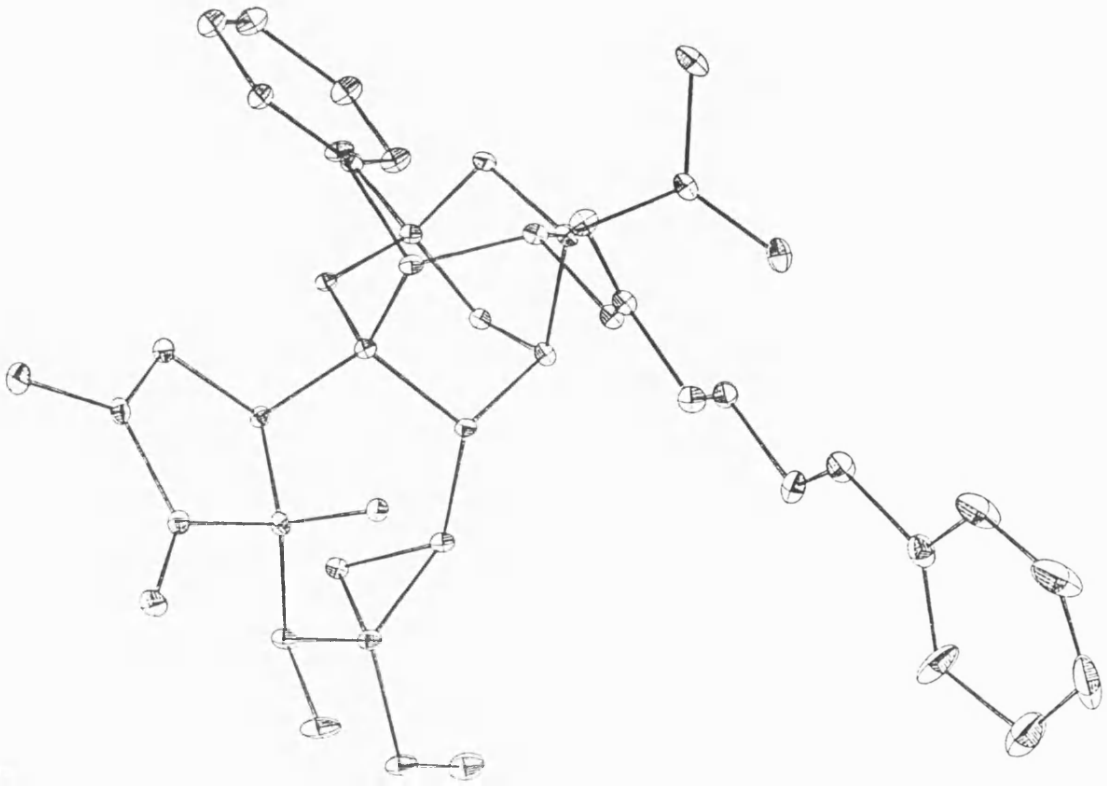

Figure 3.5.12. Structure of mezerein (A) obtained with CAMM and (B) from x-Ray crystalography (425).

Computer assisted molecular modelling

Using computer assisted molecular modelling (CAMM) on SYBYL 6.1 (R 3000) molecular modelling software, mezerein and seven additional phorbol esters were analysed. All compounds were energy-minimised for 1000 itterations and Table 3.5.4. shows the computer-generated minimum free energy values for these compounds (for structures see p. 94 and 195). Energy minimised structure obtained for mezerein was in agreement with the one obtained from X-ray crystallography experiments (425-427) (Figure 3.5.12 A. and B., p. 213). The software was used to construct Van der Waals volumes of mezerein and TPA (Figure 3.5.13., p. 216) and to calculate O-O. O-H and H-H interatomic distances. These distances for O and H atoms from functional groups (C3, C4, C9, C12, C13) that are thought to be important for activity. For mezerein and TPA, they are presented in Figure 3.5.14. A. nd B. Using PrologD software (ver. 1.0, CompuDrug Chemistru Ltd.), logD values of the same phorbol esters vere calculated and presented in Table 3.5.5., p. 215. logD is a parameter describing hydrophobicity, of a molecule containing basic or acidic groups, in an aquous environment (with more hydrophobic molecules having higher values for logD).

Table 3.5.4. Computer-generated minimum free energy values of different phorbol esters.

Phorbol ester	Minimum free energy kcal mol^{-1}	Percentage relative to TPA
TPA	142.074	100.00
DOPP	137.300	96.64
DOPPA	136.507	96.08
Sap A	136.726	96.24
Thy A	153.907	108.33
Mezerein	155.322	109.13
Rx	37.002	26.04

Table 3.5.5. logD values of different phorbol esters.

Phorbol ester	logD	Percentage relative to TPA
TPA	7.35	100
PdBu	3.72	50.6
DOPP	2.80	66.3
DOPPA	3.70	66.1
Sap A	2.71	36.9
Thy A	3.80	51.7
Mezerein	4.76	64.8
Rx	5.22	71.0

Activation of PKC isotypes α, β_1, β_2, γ, δ, ε and ζ

Using the phosphatdylserine / Triton X-100 mixed micellar assay as described before (section 2.2.4.) we investigated the ability of mezerein to activate purified PKC isotypes α, β_1, β_2, γ, δ, ε *and* ζ. Results of this study are given in Table 3.5.6., p. 218, and presented in Figure 3.5.15. A-G, pgs. 221-226. Mezerein was found to have a similar activation profile as TPA (Table 3.5.7., p. 218) for PKC isotypes α, β_1, β_2 and γ. However in the case of novel PKC isotypes δ and ε mezerein was a weaker activator than TPA (see Table 3.5.6. and 3.5.7. and Figure 3.5.15. A-G). As in the case of TPA, mezerein was not able to activate atypical PKC ζ.

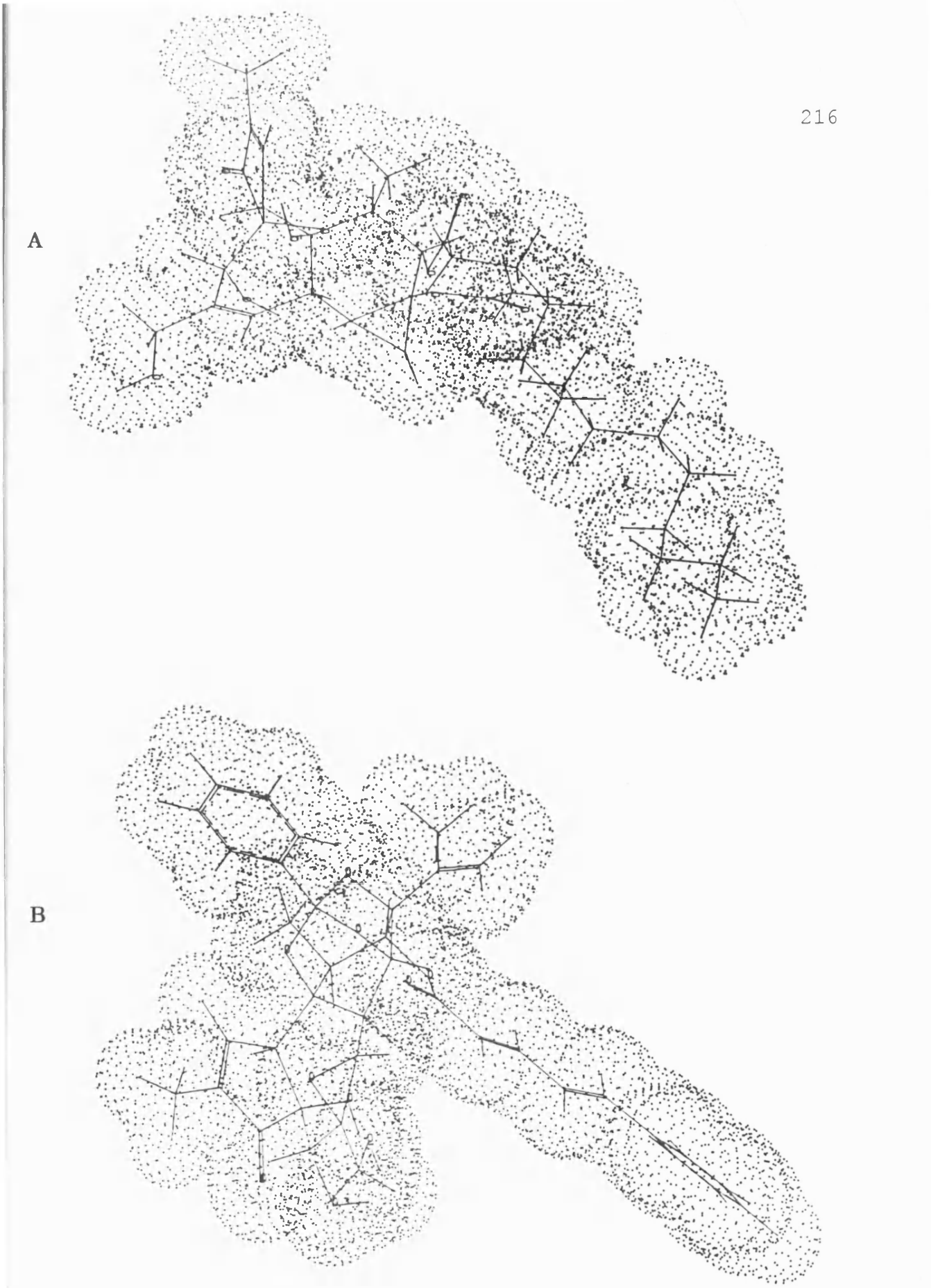

Figure 3.5.13. Van der Waals volumes of TPA (A) and Mezerein (B).

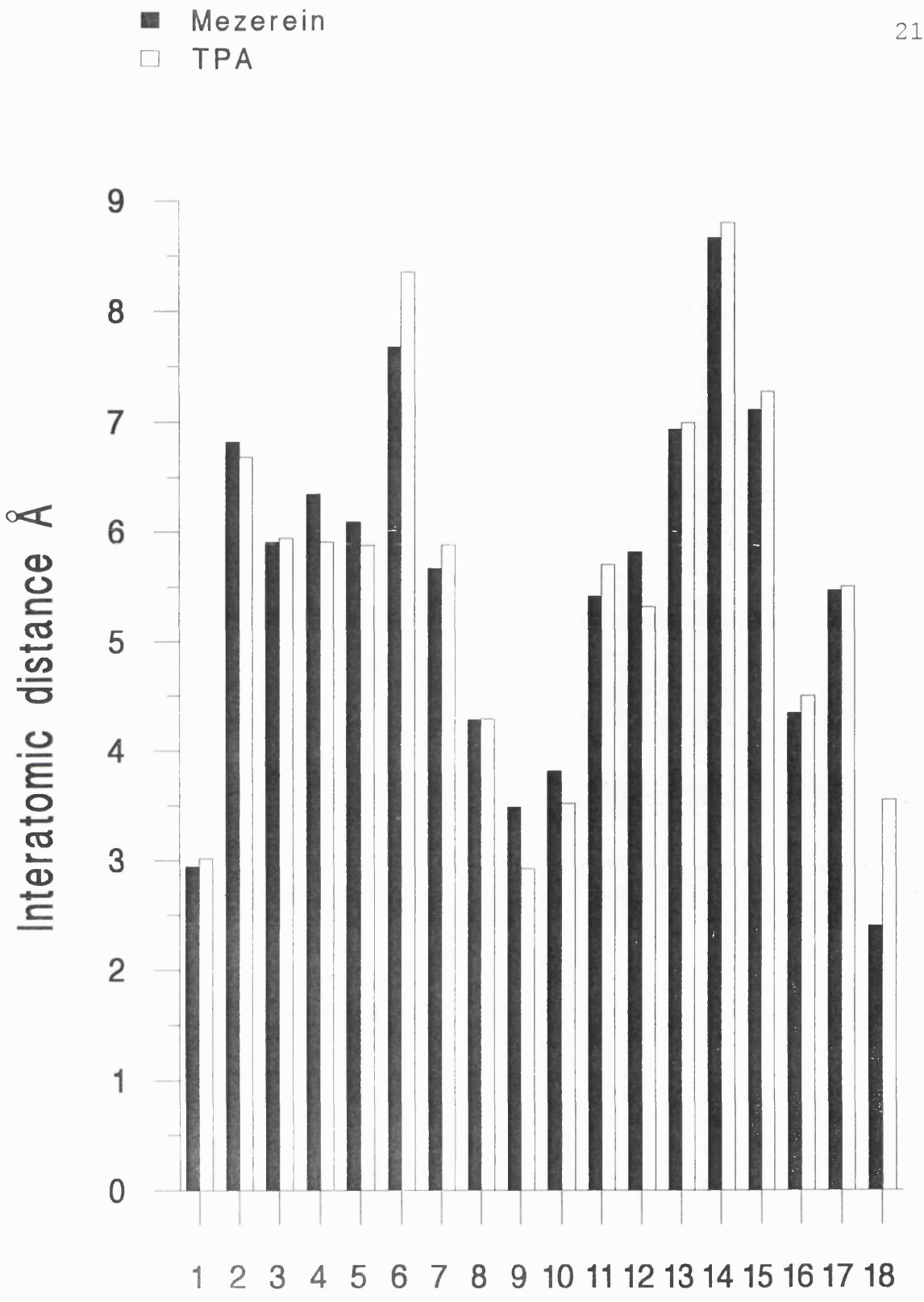

Figure 3.5.14. Interatomic (O-O) distances in TPA and Mezerein. 1, C3-C4; 2, C3-C20; 3, C3-C9; 4, C3-C12-O-; 5, C3-C12=O, 6, C3-C13-O-; 7, C4-C20; 8, C4-C9; 9, C4-C12-O-; 10, C4-C12=O, 11, C4-C13-O-; 12, C20-C9; 13, C20-C12-O-; 14, C20-C12=O, 15, C20-C13-O-; 16, C9-C12-O-; 17, C9-C12=O, 18, C9-C13-O-.

Table 3.5.6. Activation of PKC izotypes α, β_1, β_2, γ, δ and ε with Mezerein.

PKC isotype	Activation (% TPA max.)		Max 50 (nM)**	
	+ Ca	- Ca	+ Ca	- Ca
α	104	53	*	*
β_1	94	62	15	300
β_2	108	87	25	160
γ	95	53	5.5	1500
δ	73	78	790	650
ε	66	64	300	300

*PKC α showed low sensitivity on phorbol ester stimulation so Max 50 could not be determined ** Concentration required to stimulate 50% of the maximal activity observed with TPA (+ 100 μM Ca^{2+}) .

Table 3.5.7. Activation of PKC izotypes α, β_1, β_2, γ, δ and ε with TPA.

PKC isotype	Activation (% TPA max.)		Max 50 (nM)**	
	+ Ca	- Ca	+ Ca	- Ca
α	100	59	*	*
β_1	100	57	13	700
β_2	100	75	40	170
γ	100	48	4	6000
δ	100	100	24	24
ε	100	92	37	37

*PKC α showed low sensitivity on phorbol ester stimulation so Max 50 could not be determined ** Concentration required to stimulate 50% of the maximal activity observed with TPA (+ 100 μM Ca^{2+}) .

Table 3.5.8. Ratio of Max 50 values for mezerein and TPA.

PKC isotype	Max 50 Mez./Max 50 TPA	
	+ Ca	- Ca
α	*	*
β_1	1.15	0.43
β_2	0.62	0.94
γ	1.37	0.25
δ	32.92	27.08
ε	8.11	8.11

Mean values from Table 3.5.6. and 3.5.7. were used to calculate the ratio of Max 50 values for mezerein (Max 50 Mez.) and Max 50 values for TPA.

* See legend to the Table 3.5.6. and 3.5.7., p. 218.

3.5.4. Discussion

Mezerein is a member of the daphnane diterpenoid group and is the 12β-(5-Phenyl-2E,4E-pentadienoyl) ester of 12-Hydroxydaphnetoxin (Figure 3.5.3., p. 195 and 3.5.12.,p. 213). Structurally mezerein is characterised with the presence of an orthoester benzoate moiety which bridges C-9, C-13 and C-14 of ring C, 6-7 epoxide group and an isopropenyl side chain at C-13.

A number of compounds with the daphnane structure have been isolated from plants of the *Euphorbiaceae* and *Thymeleaceae* plant families(5,428-433) and references cited therein). 12-Hydroxydaphnetoxin diterpenoids have been isolated from a number of *Euphorbiaceae* and *Thymeleaceae* species (5,428-433 and references therein).

Table 3.5.9. Minimum free energy valyes of different phorbol esters, contribution of different intrinsic energies*.

Energy [kcal mol^{-1}]	TPA	Mezerein	DOPP	DOPPA	Sap A	Thy A	Rx
Bond stretch	3.516	4.469	2.348	2.372	2.644	4.483	2.322
Angle bending	120.473	130.324	114.505	115.178	116.302	129.908	19.989
Torsional	27.923	26.678	25.113	25.986	27.202	26.557	23.251
Out of plane bending	0.062	0.115	0.045	0.056	0.050	0.107	0.100
1-4 van der Waals	4.612	2.480	2.491	2.666	3.021	2.298	4.314
van der Waals	- 14.512	- 8.744	- 7.202	- 9.751	- 12.493	- 9.444	- 12.974
Total	142.074	155.322	137.300	136.507	136.726	153.907	37.002

* All compounds were energy-minimised for 1000 itterations and Table 3.5.4.,p. 214, shows the computer-generated minimum free energy values for these compounds (computer assisted molecular modelling using SYBYL 6.1, R 3000 molecular modelling software).

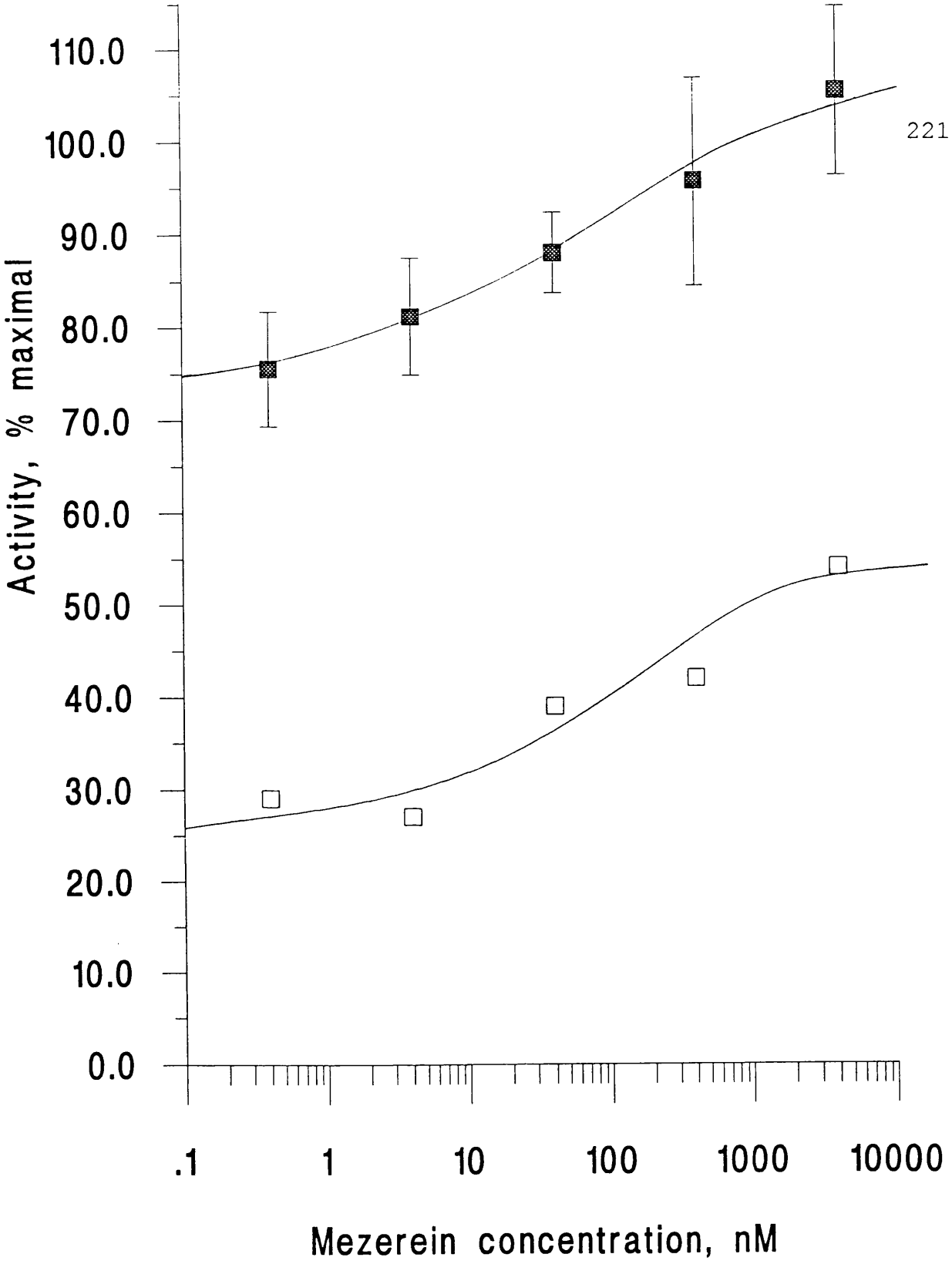

Figure 3.5.15. A. Mezerein activation of PKC α. Mezerein at final concentrations indicated was tested for the ability to activate purified PKC α in the presence (■) or absence (□) of 100 μM free calcium. At each point the phosphate incorporation into a substrate was determined and compared with the maximum incorporation with TPA as activator. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

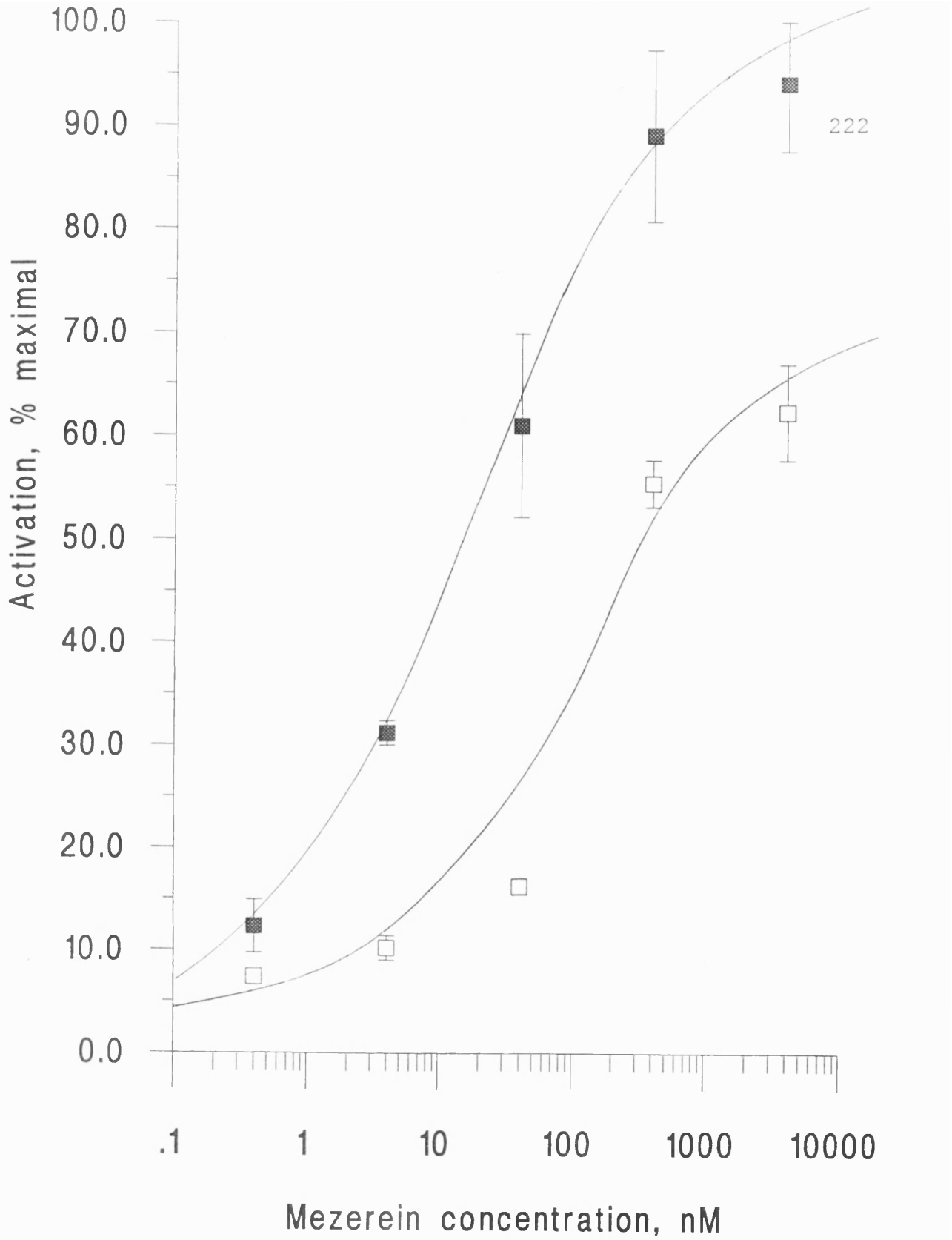

Figure 3.5.15. B. Mezerein activation of PKC β_1. Mezerein at final concentrations indicated was tested for the ability to activate purified PKC β_1 in the presence (■) or absence (□) of 100 μM free calcium. At each point the phosphate incorporation into a substrate was determined and compared with the maximum incorporation with TPA as activator. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

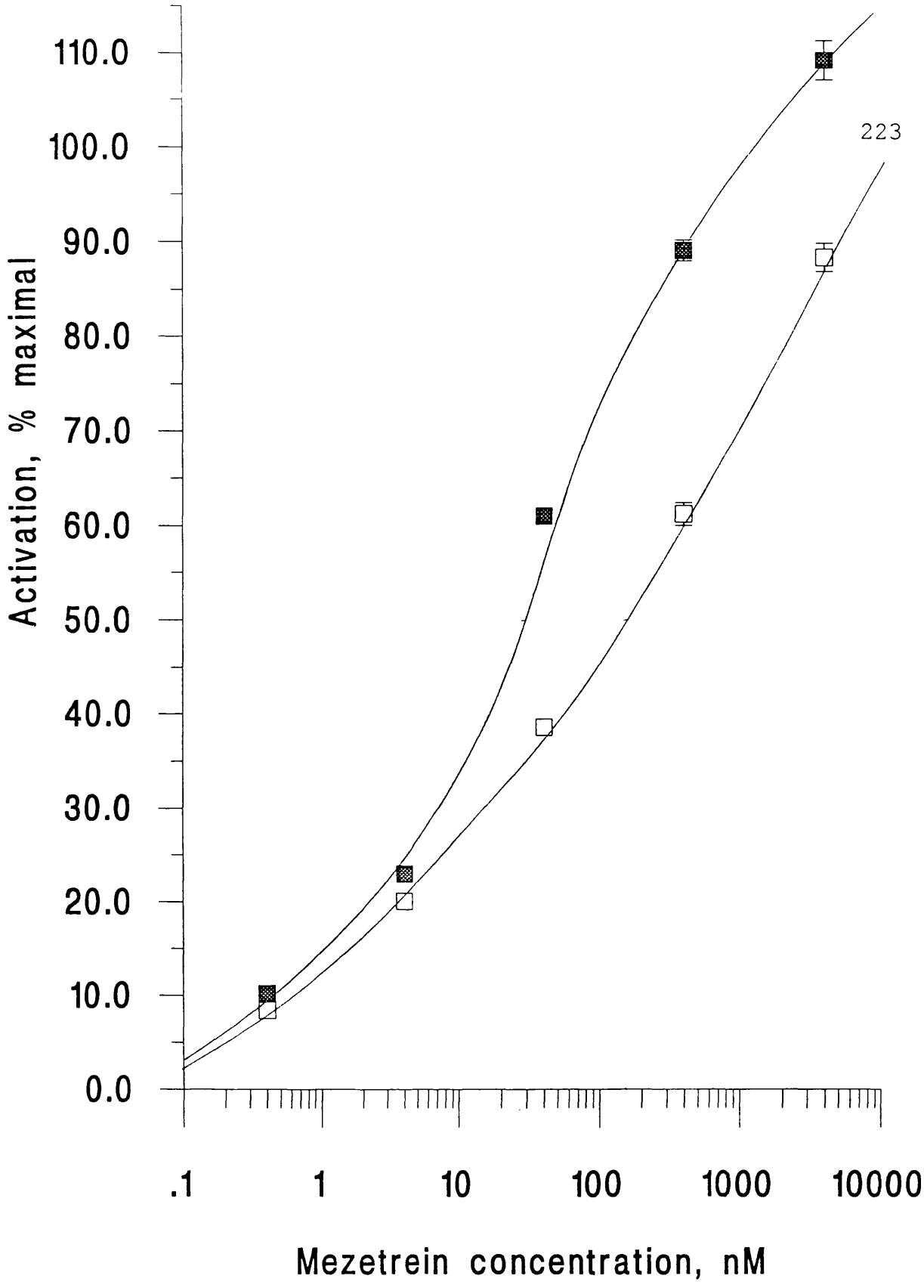

Figure 3.5.15. C. Mezerein activation of PKC β_2. Mezerein at final concentrations indicated was tested for the ability to activate purified PKC β_2 in the presence (■) or absence (□) of 100 μM free calcium. At each point the phosphate incorporation into a substrate was determined and compared with the maximum incorporation with TPA as activator. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

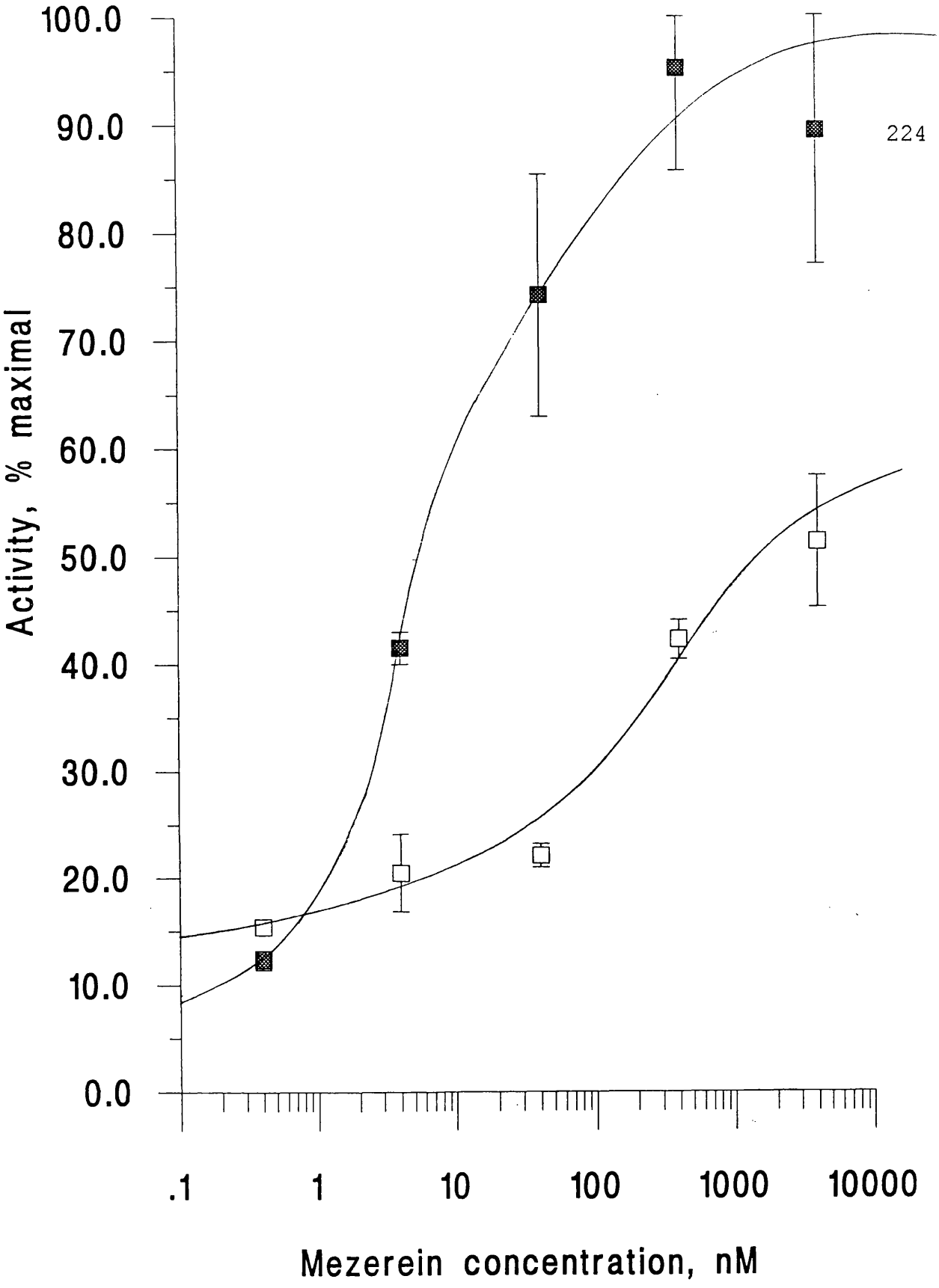

Figure 3.5.15. D. Mezerein activation of PKC γ. Mezerein at final concentrations indicated was tested for the ability to activate purified PKC γ in the presence (■) or absence (□) of 100 μM free calcium. At each point the phosphate incorporation into a substrate was determined and compared with the maximum incorporation with TPA as activator. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

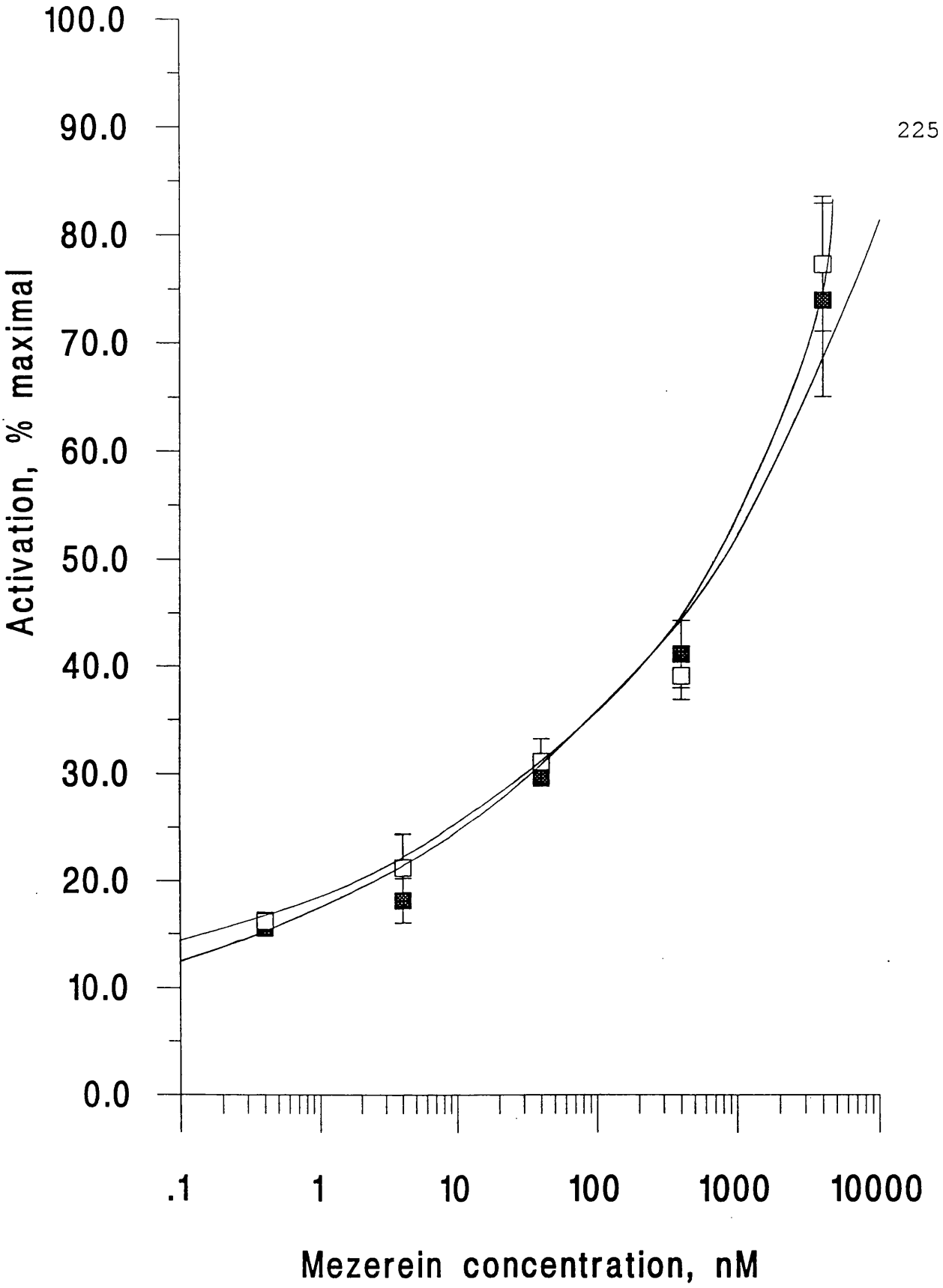

Figure 3.5.15. E. Mezerein activation of PKC δ. Mezerein at final concentrations indicated was tested for the ability to activate purified PKC δ in the presence (■) or absence (□) of 100 μM free calcium. At each point the phosphate incorporation into a substrate was determined and compared with the maximum incorporation with TPA as activator. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

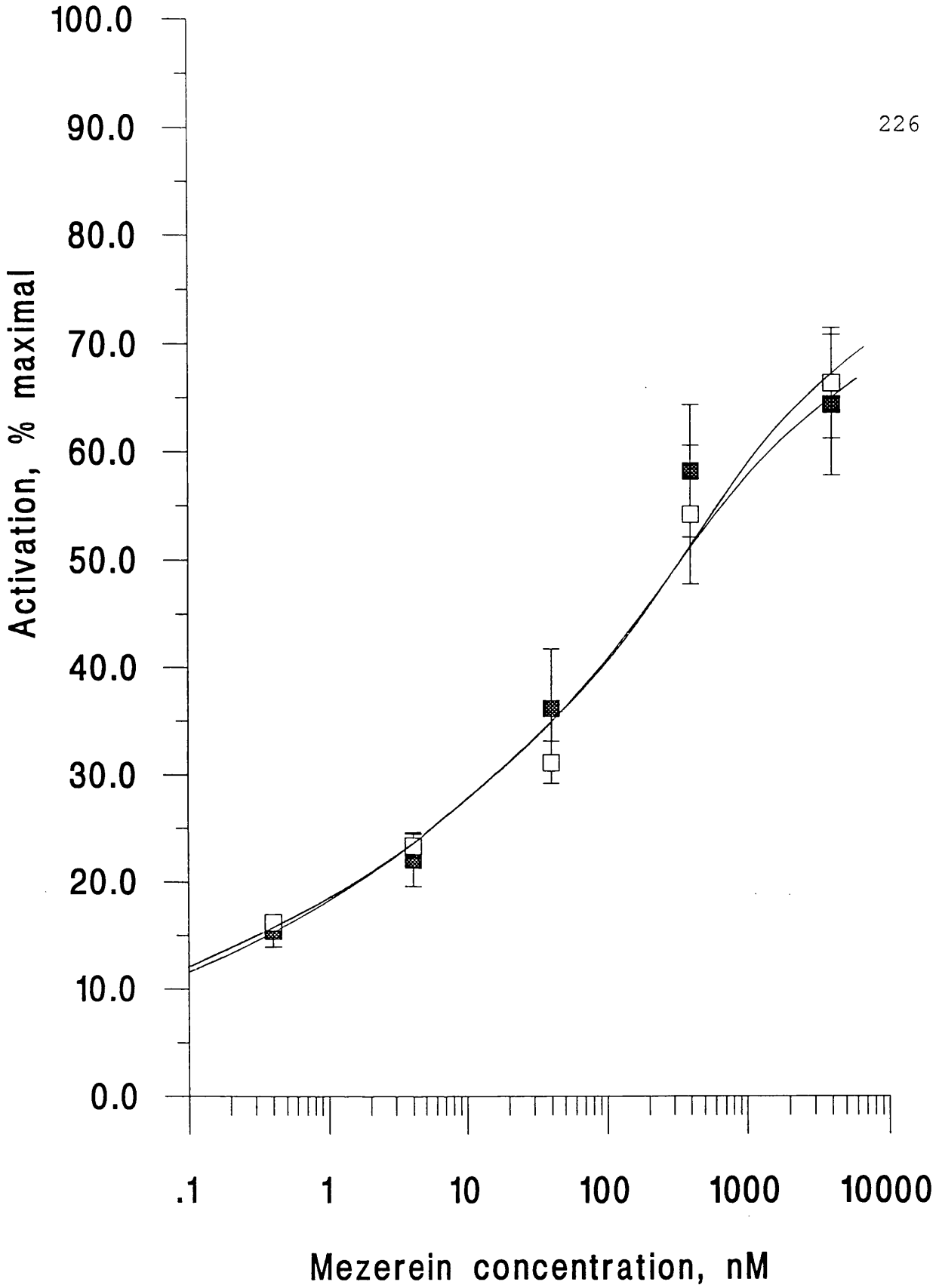

Figure 3.5.15. F. Mezerein activation of PKC ε. Mezerein at final concentrations indicated was tested for the ability to activate purified PKC ε in the presence (■) or absence (□) of 100 μM free calcium. At each point the phosphate incorporation into a substrate was determined and compared with the maximum incorporation with TPA as activator. The mean values and S.E.M. range is shown for triplicate determinations of a representative experiment.

Mezerein has been isolated so far only from the seeds of *Daphne mezereum, Thymelaeaceae* (424,426). To our knowledge this is the first report of mezerein isolation from another *Daphne* species (or any other plant species) and in addition a first report on isolation and characterisation of chemical constituents of *D. blagayana* Freyer, *Thymelaeaceae.*

The structure of mezerein was elucidated by Ronlán *et al* (424) and the absolute configuration was determined by X-Ray crystallography (425,427).

Molecular structure deduced from these study appeared to be compact, except for the side chain, with the fused ring system being approximately spherical in shape (Figure 3.5.12. A. and B., p. 213, and 3.5.13. B., p. 216). the six-membered ring is in a slightly twisted boat conformation and the seven-membered ring can be regarded as a basal plane of five carbon atoms, with the two bonds shared with the five-membered and six-membered rings at an angle of 110^0 to this plane (425,427).

However, to our knowledge, no detailed ^1H NMR and ^{13}C NMR studies of mezerein have been reported to date.

400 MHz ^1H NMR together with 2D ^1H-^1H NMR experiments (COSY and NOESY) enabled assignment of signals to corresponding protons (Table 3.5.2., p. 210, and 3.5.3., p. 212, Figures 3.5.8.-3.5.11, pgs. 207-209, 211) and confirmation of the mezerein structure and stereochemistry as reported earlier (424-427). It was possible to identify the 12-acylate as 5-Phenyl-2E,4E-pentadioate (trans double bonds configuration judged from coupling constants $H_{2"3"}=H_{4"5"}=15$ Hz, and coupling of H-4" and H5" with aromatic protons H-7" and H11" from COSY and NOESY). Additionally, for the first time ^{13}C NMR was obtained and assignment of signals has been done in comparison with data obtained recently for compounds of similar structure (5,428-433).

Other spectral data (MS and UV) were also in agreement with previously reported data (424,426).

In addition we conducted Computer Assisted Molecular Modelling (CAMM) using SYBYL 6.1 (R 3000) molecular modelling software. The energy-minimised structure obtained for mezerein (Figure 3.5.12. A., p. 213) matched the one obtained from X-Ray crystallography experiments (Figure 3.5.12 A. and B., p. 213, and

references 425,427). This result enabled the same CAMM procedure to be applied on other diterpenoids, that were used during our studies. Computer-generated minimum free energy values (Table 3.5.4., p. 214) calculated for TPA, PdBu, DOPP, DOPPA, Sap A, Thy A, Mezerein and Rx were similar (exept for Rx) and in the range between 135 to 155 kcal mol^{-1}. No large difference was found between different contributing intrinsic energies (bond stretching, angle bending, torsional, out of plane bending and Van der Waals) (Table 3.5.9., p. 220). The non promoting and strongest irritant resiniferatoxin, showed significantlly lower free energy (36.5 kcal mol^{-1}) which was almost entirely due to reduction in the angle bending energy (20 kcal mol^{-1} as compared with 120 kcal mol^{-1} for TPA, Table 3.5.9.). PrologD software allowed us to calculate logD, which is a measure of the hydrophobicity of the molecule that can exchange protons (therefore changing polarity) in an aqueous environment. As could be seen in Table 3.5.5., p. 215, the most potent tumor promoter TPA was found to have much higher logD, and therefore higher hydrophobicity, while other compounds were found to be much less (50-60% relative to TPA) hydrophobic (exept Rx) in aqueous solution. Since *in vivo* (cells in culture or whole animals) and in vitro (micellar or lyposomal models), experiments, involving phorbol esters, are conducted in more or less aqueous environments, this parameter (logD) is of great importance for estimation of phorbol ester distribution within the experimental system. More hydrophobic compounds (higher logD) would, theoretically, be able to penetrate more into the cellular membranes and to exert their activity if structural requirements are satisfied (see below).

A recent study on identification of pharmacophores in a range of structurally different tumour promoters suggested that C3 ketone, C20 primary and C9 tertiary hydroxylic groups form a pharmacophore responsible for the effect of different tumour promoting compounds, together with spatially corresponding hydrophobic moieties which ensure membrane solubility (30). The occurance of secondary interaction(s) with other hydrophylic atoms in potentially tumour-promoting structures was also postulated as a possible factor in this pharmacophore. Being in agreement with experimentaly observed structure-activity requirements, this prompted us to investigate and compare spatial arrangement of pharmacophore groups in different

phorbol esters. In Figure 3.5.14., p. 217, a comparison of interatomic distances between these groups of TPA and mezerein is presented. As could be seen from Figure 3.5.14. stereochemical profiles of TPA and mezerein for investigated atomic groups appear to be very similar. This may, at least partly, explain similarity in PKC activation profiles *in vitro* of these two compounds. However, small differences in their structure could still contribute to the more selective action of mezerein with respect of n PKC isozymes.

Mezerein was originally isolated as a toxic and antileukaemic principle from seeds of *Daphne mezereum* (424,426). Later, mezerein was described as a weak tumour promoter (435) and a second stage tumour-promoter (40) in a mouse skin two stage tumour promotion model (434), since it was only effective if the animals were first treated briefly with a complete tumour promoter such as TPA (see Section 1.1.).

Additionally, mezerein was found to inhibit TPA tumour promotion when applied simultaneously with TPA (438). However, mezerein exhibits many of the biochemical and pharmacological effects as TPA (inflammation, epidermal hyperplasia, ornithine decarboxylase induction, granulocyte-macrophage colony-stimulating factor production). On the other side mezerein differs from TPA in toxicity, induction of epidermal dark cells proliferation, induction of chronic hyperplasia and its effect on formation of reactive oxygen species (ROS) and formation of oxidised DNA bases (436-438). These differences were thought to result from the presence of polyunsaturation in the acyl residue on C-12, since compouds structurally similar to mezerein, with unsaturation in the C-12 acyl chain (e.g. Thymeleatoxin A, phorbol-12-retionate-13-acetate and phorbol-12-tetradeca-2,4,6,8-tetraenoate-13-acetate) were also found to be second-stage promoters (109,439,440). Additionally, octahydromezerein (OHM) an saturated analog of mezerein was found to be an effective complete tumour promoter (73).

Since the discovery of its activity as a tumour-promotor, mezerein has been widely used as a probe in multistage tumour promotion research, together with other diterpenoid esters of the tigliane and daphnane group. However the biochemical basis for the functional differences between complete and second-stage promoters in skin remains unresolved .

Since PKC is considered as a major receptor for phorbol esters and related compounds, the discovery that mezerein is able to bind and stimulate PKC activity *in vitro,* was not unexpected (51,73,209,362,365,441). Although it has been suggested earlier that mezerein might "differentially recognise" phorbol ester receptor "sub-classes" (from the differences in binding kinetics and differences in potency for different biological responses), few studies on mezerein interaction with separate PKC isotypes have been reported (209,362,443-445). In this study we report on selectivity in mezerein potency to activate different PKC isotypes (Figure 3.5.15. A.-F., pgs. 221-226, and Table 3.5.6., 3.5.7., p. 218, and 3.5.8., p. 219). It is a less potent activator of n PKCs δ and ε as compared with the complete tumour promotor TPA.

Major differences in biochemical effects, between TPA as a complete tumour promoter and mezerein as an incomplete promoter, *in vivo* (cells in a culture and whole animal skin) was in induction of reactive oxygen species (ROS) production (438 and references therein). On the other hand the relationship between oxidative stress, DNA damage and chemical carcinogenesis has been well established (438 and references therein) suggesting that first stage and complete-promoter activity of a compound is linked with its ability to induce ROS production.

The role of PKC isotypes in oxidative stress has been reported (130,442), suggesting that novel PKCs (e.g. δ and ε) could have a more significant (if not exclusive) role in ROS production. Additionally, the recent study of Kazanietz *et al* (362) demonstrated that mezerein was selectively less able to compete with [^{3}H] PdBu binding to nPKC isozymes δ and ε than to cPKCs α, β, and γ.

Together with previous observations (see above) our present investigation, showing that mezerein was selectively a weaker activator of n PKCs δ and ε, could implicate these effects as an explanation for at least a part of the biochemical mechanism responsible for tumour promotion. In this mechanism, TPA a non-selective PKC agonist could activate all PKC isotypes including novel PKCs leading to a high production of ROS and eventually to complete tumour promotion. On the other hand mezerein (or other second stage promoters) being a weak activator of n PKCs could not induce ROS levels high enough to cause a complete tumour promotion process.

The complex mechanism of phorbol ester- PKC interaction (involving penetration of a molecule into the lipid bilayer of cellular membrane, binding to PKC isozyme(s) and finally possible activation of PKC kinase activity), imposes multiple requirements for an effective PKC agonist to be fulfilled. Ionization status and apparent hydrophobicity (see above) of a molecule, will determine its ability to penetrate lipid membranes in the first instance. In a second step, binding to the PKC molecule would be allowed only if certain structural requirements are fulfilled (e.g. presence and relative spatial organisation of certain functional groups), allowing possible change in function (activation or inhibition) of PKC in the final step. Alternatively only binding, without change in kinase activity of the respective PKC isozyme(s), i.e. blocking, could occur. In the case of mezerein, inspite of the similarity in stereochemistry with a complete tumour promoter like TPA, the reduced hydrophobicity (as compared with TPA) could decrease its effective concentration available for binding with PKC and hence contribute to lower potency of mezerein. Additionally, unsaturation in the hydrophobic tail of the acid residue in position C12 could influence PKC interaction with membrane phospholipids and, together with small conformational differences, significantly decrease interaction with more selective isozyme(s) (e.g. n PKCs) causing different signalling effects.
However, the existance of other pathways involved in skin tumour promotion, shared or selectively targeted by compounds with different promotion properties, must not be dismissed (443).

CHAPTER 4. SUMMARY

The development of the concept of tumour promotion by the use of repeated applications of croton oil (31) resulted in intensive research to identify the biologically active constituents of the plant families Euphorbiaceae and Thymelaeaceae. Approximately twenty five years later E. Hecker (4), elucidated the structure of TPA and its parent alcohol, phorbol. Subsequent to this discovery, many laboratories isolated bio logically active diterpene esters based upon tigliane, daphnane and ingenane hydrocarbon nuclei from the plant families Euphorbiaceae and Thymelaeaceae (1). The phorbol esters had been shown to possess a large spectrum of biological activities, but it was the discovery of high affinity receptor sites (448) which were shown by several laboratories to co-purify with protein kinase C (PKC) (13), that enabled the identification of the biochemical receptor site of the phorbol esters. This established a unique use of phorbol esters as probes in research of cellular communication and regulatory signals in both physiological and pathological conditions.

At the present mammalian PKC family is known to comprise a multigene family of isozymes consisting of 11 different polypeptides. The isozymes differ in their enzymatic properties (cofactor and activator dependancy and substrate affinity), tissue and intracellular distribution. This, together with complexity of cell-signalling pathways encompassing PKC enzymes, has further hindered the understanding of how PKC and phorbol esters exert their ultimate biological effects.

Different biological effects elicited by different phorbol esters in mammalian cells is thought to be due mainly to their differential interaction with, and modification of, the cellular pool of PKC isozymes.

However, in most of investigations only TPA, as the most potent tumor-promoting phorbol ester, was used. In spite of observations that other phorbol esters exert more specific biological effects *in vivo*, relatively few studies have been reported so far (347,380-382) on their interaction with individual PKC isozymes *in vitro* or *in vivo*.

In vivo interaction of different phorbol esters with PKC involves penetration of the phorbol ester molecule into the inner layer of cellular membrane and binding

to the regulatory domain of PKC followed by redistribution of the PKC pool from cytosolic (soluble) to the membrane bound (particulate) fraction (342,372-374). It is thought that PKC redistribution ("translocation") is followed by an activation step and phosphorylation of substrate(s).

Complex mechanism of phorbol ester-PKC interaction imposes multiple requirements for an effective PKC agonist to be fulfilled. Ionization status and apparent hydrophobicity (see Section 3.5.) of a molecule, will determine its ability to penetrate lipid membranes in a first instance. In a second step, binding to the PKC molecule would be allowed only if certain structural requirements are fulfilled (e.g. presence and relative spatial organisation of certain functional groups), allowing possible change in function (activation or inhibition) of PKC in a final step. Alternatively only binding, without change in kinase activity of the respective PKC isozyme(s), i.e. blocking, could occur.

The objective of this present study was to investigate, *in vitro*, different aspects of interaction (*i.e.* binding and activation) of a range of phorbol esters with separate PKC isozymes, and to correlate them with *in vivo* observations.

Our study revealed qualitative and quantitative differences in ability of different PEs to interact with separate PKC isozymes. The ability of phorbol esters investigated, to bind, induce activation and translocation of PKC isozymes seem to parallel each other, with TPA, PdBu, DOPP and Sap A being generally very potent, Thy A showing weaker but more selective effects and Rx and DOPPA being almost inactive (see Section 3.1., 3.2, and 3.3.).

A recently proposed model for PE-PKC interaction, based on X-ray crystalographic studies, suggested that C_3=O, C_4-OH and C_{20}-OH groups of the PE molecule are involved in binding to the PKC regulatory domain. Our results obtained for TPA, PdBu, DOPP, DOPPA, Thy A and Rx are in agreement with this binding model. However, Sap A, a PE that lacks C_4-OH, was found to be a potent PKC agonist (binding, activation and translocation). This is in support (together with rest of our results) of an earlier PE-PKC interaction theory (30), according to which C_9-O- instead of C_4-OH group of PE molecule, is involved in PKC-PE interaction.

The ability of DOPP and Sap A to potently interact (bind, activate and induce "translocation") with PKC isozymes, although in agreement with recent reports on their ability to activate PKC isozymes *in vivo,* did not offer an explanation for their inactivity as tumour promoters. Additionally, we were not able to confirm an earlier report from our laboratory (347) on selective activation of the PKC β isozyme by DOPPA *in vitro,* although some selective binding of DOPPA to PKC β isozymes *in vitro* was observed. This again, was not in agreement with observed nonselective activation of PKC isozymes by DOPPA *in vivo* (380,382).

In addition, for the first time activation of novel PKC-η with phorbol esters was presented here. This is of particular importance, regarding the predominant localisation of this isozyme in skin (162,398), a model-organ used for investigation of tumor-promoting properties of different phorbol esters. Results presented in Section 3.3., suggest that activation of this isozyme by DOG and a range of phorbol esters is not dependent on calcium, as expected for a member of the nPKC subfamily. Activation with TPA, PdBu, DOPP, DOPPA, Sap A and Rx showed comparably similar profiles as with other n PKCs. However, Thy A, a second stage tumor promotor, appeared to be a comparably a much weaker activator(~ 70 and 50 x as compared with TPA, for + and - Ca^{2+} respectively) of PKC-η (Table 3.3.1. C., p. 156, Section 3.3.). This suggests the possible use of Thy A as a selective non-activator of this PKC isozyme *in vitro* and a possible explanation (at least partly) for its distinct effects *in vivo.* Unfortunately no data on Thy A effect on PKC-η isotype *in vivo* is available at the present, precluding any further speculation on the mechanism of Thy A action *in vivo.* A DOG showed to be relatively (as compared with TPA) better activator of PKC-η than of other isozymes investigated, implying possible selectivity in activation of different PKC isozymes with physiological activators (DAGs) *in vivo.* The ability of non-promoting phorbol esters , Sap A and DOPP to potently activate PKC-η could not support an explanation for their inactivity as tumor-promoters *in vivo,* leaving still open the possibility that their selective interaction with other, yet uninvestigated or undiscovered, PKC isozymes (or some other unknown receptors) could explain their specific biological effects.

To study the ability of some PEs to induce association of PKC isozymes with cellular membranes (i.e. "translocation"), membrane fraction obtained from HL-60 cells were used, in order to approach *in vivo* conditions. Although the ability of investigated PE to induce "translocation" of PKC isozymes corresponded to their ability to induce PKC activation, the ability of micromolar Ca^{2+} concentrations to induce membrane association of n-PKCs, ε and δ, was not in agreement with our activation results and current theory of Ca^{2+} independency of n-PKC isozymes. However, the ability of non-promoting Sap A and DOPP to induce translocation of PKC isozymes did correlate with their ability to induce PKC translocation *in vivo* (380,382).

The use of standardised preparation of cellular membranes has the advantage over the use of the artificial-membrane models (mixed phospholipid vesicles or phospholipid/detergent mixed micelles) because it has a higher similarity to the situation in intact cells. It would be of great importance to investigate PKC-PE interaction (binding and activation), using the same cell-membrane model. Additionaly, selective use of different cellular fractions, under the same experimental conditions, could lead to identification of postulated intracellular "factors", that are involved in expression of PE effects *in vivo*.

Discrepancies between our observations and recent reports from our and other laboratories, lead to the conclusion that constitution of artificial membranes, used *in vitro* PKC assays to emulate cellular membranes, and the source of PKC isozymes (purified from different tissues or from different cell systems after expression of recombinant enzymes), significantly influence the results (Kd values for binding, Ca^{2+} effect and PE-PKC activation profiles).

Observed distinct biochemical properties of the PKC isozymes *in vitro* are consistent with the hypothesis that each isoform may have distinct roles in the signal transduction process. However, the specific roles of distinct PKC isozyme in different cellular responses and signal transduction pathways *in vivo* await further elucidation and experimental evidence is limited and frequently conflicting. On the other hand, because of discrepancies between *in vivo* and *in vitro* results, an important question has arisen, as to whether or not these *in vitro*

characteristics can be extended to *in vivo* situations. In this respect, the use of phorbol esters of different biological activity as probes in both *in vivo* and *in vitro* investigations of any particular biological system, is of crucial importance. Nevertheless, comparison of *in vitro* results (using different experimental conditions) and their extrapolation to *in vivo* conditions, where existance of as yet undiscovered "factors" must be considered, has to be done critically and cautiously.

An additional aspect of *in vivo* phorbol ester-action was investigated in the study of metabolic deacetylation of DOPPA to DOPP by HL-60 promyelocytic leukaemia cells. Using HPLC coupled with photodiode array detection, it was possible to asses quantitatively the hydrolysis of C_{20} ester function in the DOPPA molecule, and generation of DOPP in cultures of HL-60 cells. In cell culture, appearance of DOPP was observed within 2h, while in media alone (fresh or preincubated with cells) only 5 % of DOPPA was hydrolysed by 48h, suggesting intracellular metabolism of DOPPA. This necessitated changes in the interpretation of results obtained in the study of DOPPA and DOPP effects on HL-60 differentiation, suggesting that the observed differentiation and inhibition of thymidine incorporation by HL-60 cells induced by DOPPA, is due to appearance of DOPP in the HL-60 cultures (351). This observation implies that the possibility of metabolic transformation (e.g. hydrolysis of ester function) of phorbol esters to a metabolite of different biological activity, and must be considered during long term (e.g. several hours) *in vivo* experiments.

As a part of ongoing screening of Euphorbiaceae and Thymelaeaceae plant families for the presence of biologically active constituents, we have investigated the endemic plant *Daphne blagayana*. Phytochemical investigation of this plant species is reported here for the first time. Our investigation resulted in isolation of mezerein, a daphnane diterpenoid, that has previously been isolated only from *Daphne mezereum*, making this only the second report on the occurance of mezerein in plants.

Lack of comprehensive NMR study of mezerein, prompted us to undertake

a detailed NMR (^{1}H, ^{13}C, COSY and NOESY) investigation, allowing confirmation of previously reported structure of mezerein.

As an activator of PKC isozymes, mezerein was a less potent activator of n-PKC isozymes δ and ε (~30 and ~8 times respectively), as compared with c-PKCs. On the other hand, n-PKCs are thought to have significant (if not an exclusive) role in production of reactive oxygen species (ROS), an event thought to be responsible for complete and first stage tumour-promotion (see Section 3.5.). This allowed us to suggest a possible mechanism of action of mezerein as a second stage tumour promoter, being unable to induce sufficient ROS through activation of n-PKCs. However, additional *in vivo* investigation of the ability of mezerein to activate different PKC isozymes and induce production of ROS will be necessary for this theory to be confirmed.

This part of our research provided a classic example of the isolation of a pharmacologically active constituent from a plant source and its use as a tool in biochemical research. In this respect it is our strong belief, that systematic phytochemical, pharmacological and biochemical investigation of plant species, will in future provide medicine and pharmacy with new research tools and ultimately with new therapeutic agents. This establishes, firmly, the role of pharmacognostic research in contemporary medicine and pharmacy.

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