CHAPTER I
INTRODUCTION

1.1 Statement and significance of the problem

Turmeric contains about 1-5 % curcumin, which has been identified as the major yellow pigment. It has been widely used as a spice, to color cheese and butter, as a cosmetic and in some medicinal preparations in many parts of the world (1). Curcumin (diferuloylmethane) a phenolic compound that has been identified as the major pigment in turmeric, posses both anti-inflammatory (2) and antioxidant properties (3). Consistent with its antioxidant and anti-inflammatory activity, curcumin has demonstrated chemopreventive activity during both initiation and promotion stages of cancer formation. It has been specifically shown to inhibit mutagenesis (4), carcinogenesis (5) and DNA-carcinogen adduct formation (6); decrease expression of *c-jun, c-fos* and *c-myc* (7-8), possibly through inhibition of protein kinases (9); inhibit ODC activity (10) and EGFR function (11); modify cytochrome P450 (12), enhance glutathione-S-transferase activity (13) and DNA repair (14).

Several studies have demonstrated that compounds which posses antioxidant or anti-inflammatory properties can inhibit 12-O-tetradecanoyl phorbol-13-actate (TPA)-induced tumor promotion in mouse skin (15-17). The previous data demonstrating the chemopreventive efficacy and anti-inflammatory activities of curcumin are from published studies on mouse skin. It has been shown that topical application of curcumin inhibits TPA-induced tumor promotion in mouse skin and edema of mouse ears (18). Topical application of curcumin also inhibited benzo (a) pyrene-induced DNA adducts and skin tumors as well as DMBA-induced skin tumors (6). In other studies, dietary administration of 2% turmeric in the diet inhibited DMBA-induced skin, benzo (a) pyrene-induced forestomach, and AOM-induced small and large intestinal tumors in mice. Recently, the chemopreventive efficacy of dietary curcumin (1%) on TPA-induced skin tumors in mice has been reported (19). Including with its perceived human safety following centuries lead to curcumin becomes a

potent phytochemical candidate for cancer prevention. However, how curcumin elicits this chemopreventive efficacy on the carcinogenesis is ongoing research in many laboratories. A large body of data has demonstrated that curcumin is engaged in multiple anti-tumor promoting pathways. It has been reported that curcumin inhibited a variety of biological activities of TPA, a potent skin tumor promoter, which induces several biosynthetic processes, namely enhanced expression of cellular oncogenes such as *c-jun, c-fos* and *c-myc* (8), induction of ODC (17), induction of cyclooxygenase and lipooxygenase (18) and last but not least, elevation or translocation of protein kinase C (PKC) (9).

PKC is a serine-threonine kinase enzyme family. Currently, 12 mammalian isoenzymes are known. They are classified into three families, based on activation by Ca^{2+} and phospholipid: (1) Ca^{2+} dependence (PKC α, β, γ) ; (2) Ca^{2+} independence (PKC $\delta, \varepsilon, \theta, \mu$) and (3) not requring Ca^{2+} or lipid (PKC ζ, λ) (20-22).

Among the PKC isoenzymes it has been reported that PKC -ε isoenzyme exhibited full oncogenic potential (22-23) and implicated in the regulation of other biological processes, including antiviral resistant (24), hormonal secretion, transporters regulation and golgi function modulation (25-26). Thus this study paid most attention on PKC -ε isoenzyme. The study determined whether an *in vitro* culture system could be used to predict biological activity of curcumin on TPA -induced translocation or downregulation of PKC-ε isoenzyme in human keratinocytes. The result will be discussed in terms of the selectivity in subcellular localization of TPA-induced PKC-isoenzyme in human keratinocytes. The kinetic inhibition of curcumin on TPA -induce PKC-ε isoenzyme including the possible signal cascade will be elucidated.

1.2 Literature reviews

1.2.1 Curcumin

Turmeric (Figure 1) is the spice obtained from the rhizome of herbaceous plant, *Curcuma longa* Linn. (Zingiberaceae), it contains 3-5 percents of an aromatic essential oil and 2.5-6 percent of a bright yellow pigment, curcumin, which is extensively used for coloring of food and for medicinal purposes such as prevent and relieve *rashes*, itches, chafing and mosquito bites and several studies have shown that dietary administration of curcumin effectively reduce tumor incidence (6,7,8,13,19).

1.2.1.1 Chemistry of curcumin

Curcumin used in this study is an orange crystalline powder, insoluble in water and ether, but soluble in DMSO (Dimethyl sulfoxide) and alcohol. The molecular weight is 368.39 and its structure as shown in Figure 1, the general formula is $C_{21}H_{20}O_6$ (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) (28).

Curcumin contains two molecule of ferulic acid linked at the carbon atoms of the carbonyl groups, *via* a methylene bridge, to form a β-diketone. It is not known what part of the curcumin molecule is important for its biological activity. However, the presence of two α,β-unsaturated ketone groups in the curcumin molecule may act as a specific reagent for targeting the SH-group of the enzyme (21). It has been proposed that curcumin may inhibit PKC activity by interacting with sulfhydryl group on the cysteine residues located in the regulatory domain of PKC. However, the mechanisms of modification have not been clarified (9).

1.2.1.2 Anti-inflammatory activity

Conney *et al.* (29) evaluated the effects of curcumin on TPA-and arachidonic acid-induced edema of mouse ear. Curcumin, in the amount of 1 μM inhibits TPA-induced ear edema by 99%. The application of 1 μM arachidonic acid to the ears of mice causes a rapid and substantial inflammatory response within one hour. Topical application of 5 μM curcumin 30 min before 1 μM of arachidonic acid causes a 60 % inhibition in the inflammatory response. Curcumin at 5-10 μM can inhibit epidermal metabolism of arachidonic acid to 5- and 8- hydroxyeicosatetraenoic acid (HETE) by about 50% (18). Curcumin at 5 μM inhibited lipopolysaccharide (LPS) -induced production of tumor necrosis factors (TNF) and interleukin-1β (IL-1) by a human monocytic macrophage cell line (Mono Mac 6). In addition, it demonstrated that curcumin, at the corresponding concentration, inhibits LPS-induced activation of nuclear factor kappa B and reduces the biological activity of TNF in L929 fibroblast lytic assay (30).

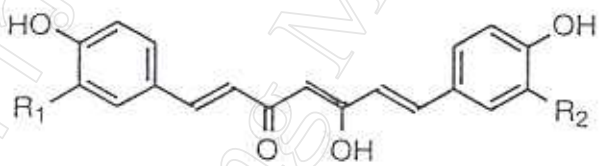
The results strongly indicated that curcumin has anti-inflammatory activity.

1.2.1.3 Anticancer activity

Intensive studies on the action of curcumin in various biological systems have indicated that this compound has engaged in the inhibition of DNA adduct formation induced by chemical carcinogens and multiple anti-tumor promoting pathways. It has been demonstrated that curcumin inhibits a versatile biological activity of TPA which induced several biosynthetic processes e.g. enhanced expression of cellular oncogenes, *c-jun, c-fos, c-myc* (7,8), induction of ODC (10), cyclooxygenase and lipoxygenase (18). It is

a)

b)



R_1	R_2	
$-OCH_3$	$-OCH_3$	Curcumin
$-H$	$-OCH_3$	Demethoxycurcumin
$-H$	$-H$	Bisdemethoxycurcumin

Figure 1. The *Curcuma longa* Linn. (Zingiberaceae) and its chemical structure.

a. Rhizome of *Curcuma longa* Linn.

b. Chemical structure of curcumin and its derivatives.

noteworthy that all of these processes are required for anabolic pathway and cell proliferation.

In conclusion, it can be listed the biochemical effects of curcumin as shown in Table 1 (31).

1.2.1.4 Antioxidant activity

Srimal RC (2) and Sharma OP (3) have shown that curcumin is a good antioxidant and a potent inhibitor of lipid peroxidation catalysed by iron in that is its chelates. The study suggested that the phenolic group of the methoxy group on the benzene ring is not important for the inhibition of iron-catalysed lipid peroxidation. The 1,3-diketone system is a potent ligand for metals such as iron. From the spectral studies it is clear that the curcumin are capable of interaction with Fe^{2+} and similar results were obtained with Fe^{3+}.

1.2.1.5 Activity on gastrointestinal system

Curcumin in diet enhanced intestinal lipase activity and also enhanced the disacharidases, sucrase and maltase of intestinal mucosa in experimental rat that are generally well recognized to stimulate digestion (32).

1.2.1.6 Activity on the cardiovascular system

Nirmala et al (33) demonstrated the effect of curcumin on lysosomal hydrolases in serum and heart by determining the activities of beta-glucuronidases, beta-N-acetylglucosaminidases, cathepsin B, cathepsin D and acid phosphatase. Rats treated with isoproterenol (30 mg/100 g body weight) showed the significant increase in serum lysosomal hydrolase activities, which were found to decrease after curcumin treatment. Isoproterenol administration to rat results in decreased stability of membranes, which was

Table 1. Biochemical effects of curcumin.

Level of action	Parameter
DNA synthesis	-Inhibition of Thymidine incorporation into DNA.
Transcription	-Suppression of *c-jun* mRNA.
Translation	-Inhibition of TRE binding by c-jun/AP-1 protein.
Enzyme	1.Inhibition of cyclooxygenase and lipooxygenase 2.Inhibition of ornithine decarboxylase 3.Inhibition of protein kinase C
Activated oxygen	- Inhibition of the formation of 8-hydroxydeoxyguanosine

reflected by the lowered activity of cathepsin D in mitochondrial, lysosomal, and microsomal fractions. Curcumin treatment increase the activity levels almost to normal showing that curcumin restores the normal function of the membrane.

1.2.1.7 Activity on hormonal system

Babu and Srivanna (34) examined the effect of feeding 0.5% curcumin diet or 1% cholesterol diet I albino rats diabetic with streptozocin injection. They found that curcumin feeding improves the metabolic status in diabetic conditions, but without effect on hyperglycemic status or the body weight, suggesting the mechanism by which curcumin improves this situation is probably by virtue of its hypercholesterolemic influence, antioxidant nature and free radical scarvenging property. However, curcumin has several activities not only in animals but also in humans. Kuttan studied curcumin as a chemopreventive substance against arterial disease. The effect of oral administration in reducing the serum levels of cholesterol and lipid peroxidases was studied in ten healthy human volunteers, receiving 500 mg of curcumin per day for 7 days. There are significant decreases in serum lipid peroxidases by 33 % and in total serum cholesterol by 11.6 % but the HDL cholesterol is increased significantly by 29 % . Since curcumin reduces serum lipid peroxidase and serum cholesterol, the study of curcumin as a chemopreventive substance against arterial diseases is warranted. On the other hand. curcumin also has an anti-HIV activity and anti-fungal activity. These findings led us to have interest more into curcumin.

1.2.1.8 Inhibitory effects of curcumin on protein kinase C

Lui and his coworkers (9) evaluated PKC activity translocated from the cytosolic to membrane fraction in mouse fibroblast cells (NIH3T3). They found that curcumin inhibited PKC activity in membrane fraction of 0.1 μM TPA treated cells which were pretreated with

15 or 20 μM curcumin for 15 min by 26 or 60% respectively. It appeared that curcumin can inhibited PKC activity in both cytosolic and membrane fractions *in vitro* by competing with phosphatidylserine. However, inhibitory effect of curcumin was reduced after preincubation with the thiol compounds. Thus they suggested that the suppression of PKC activity may contribute to the molecular inhibition mechanism of TPA -induced promotion by curcumin.

1.2.2 12-O-tetradecanoyl-13-phorbol-acetate (TPA)

1.2.2.1 Chemistry of TPA

In the first issue of *Cancer Research*, published in 1941,Berenblum (35) stated that " Croton oil causes a marked augmentation of carcinogenesis when applied at weekly intervals the skin of mice in conjunction with a very dilute solution of benzo (a) pyrene in acetone " The oil obtained from the seeds of croton (Figure 2) showed a powerful activity of tumor promotion ,and the active principle present in this croton oil was later identified as a phorbol ester by Hecker (36) and also by Van Duran (37) the most powerful phorbol ester is TPA, its structure as shown in Figure 3.

TPA, when applied to the cell, elicits a wide variety of responses that are very similar to those of hormones and neurotransmitter .A number of kinetic studies with various cell types suggest that the primary site of action of TPA is located on cell surface membranes (38).

1.2.2.2 Cells in culture respond to TPA

TPA rapidly causes inflammation and hyperplasia in the mouse skin irrespective of prior treatment with an initiating agent: 24 hr after the application of TPA, the skin is heavily

infiltrated by leukocytes. In addition, TPA causes primary rat embryo fibroblast transfected with a 'transforming' oncogene (*ras*) to grow out and to form foci. Only when unphysiologically high TPA concentrations are used, *myc*-transfected cells also form foci. Since only a combination of transforming and an immortalizing oncogene is capable of transforming primary cells, *ras* expression may substitute for the initiating carcinogen, while TPA treatment may imitate the action of one of the immortalizing oncogenes. Also in the mouse skin, the treatment with an initiating agent can be replaced by infection of the epithelial cells with Harvey- or Balb-murine sarcoma. In line with the idea that *myc* substitutes for the promoting treatment, overexpression of an exogenouse *myc*-gene leads to an increase in radiation-induced transformation of cells in culture.

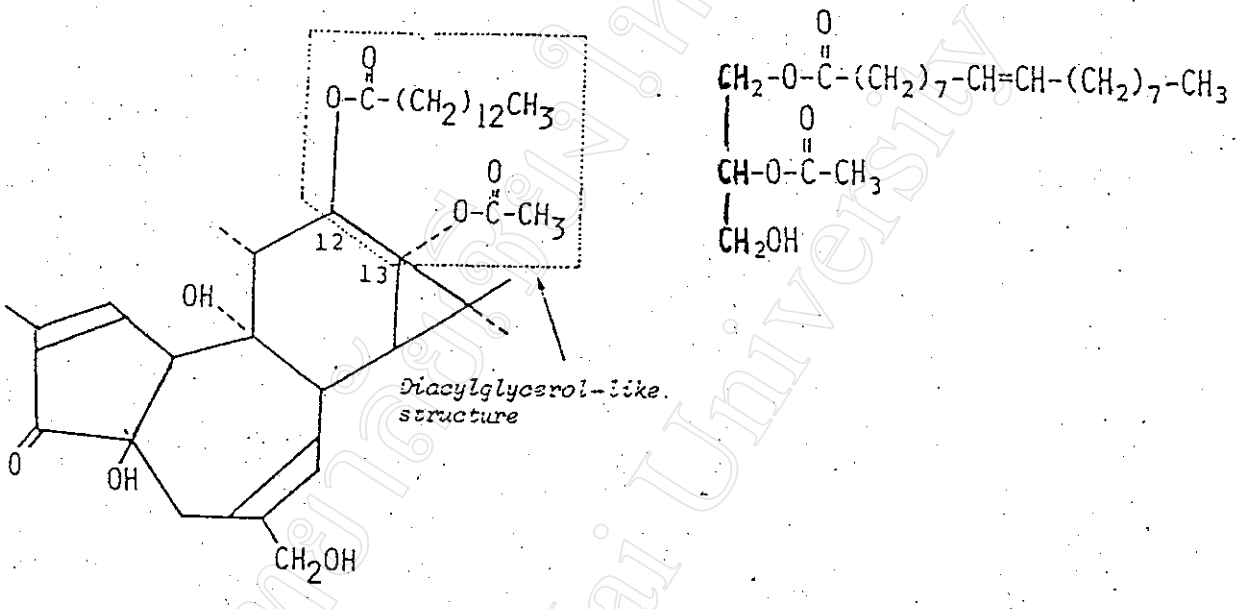
1.2.2.3 Action of TPA on gene

Genes which have been found to respond to phorbol esters are shown in Table 2 (39).Many genes are activated within minutes after treatment with TPA. Their expressions are usually transient and their induction are mostly not affected by inhibitors of protein synthesis .Some of these have attracted particular attention, especially those coding for cellular homologous of viral nuclear oncogene *c-fos, c-jun* and *c-myc* are among the immediated response gene in fibroblast .Their transcriptions after phorbol ester treatment are not simultaneous : *c-fos* mRNA is detected first (within a few minutes) followed by an increased accumulation of *c-jun* RNA and *c-myc* RNA respectively. These gene are induced by TPA either by activating specific transcription factors or by increasing their intracellular concentration.

a)

b)

Figure 2. The croton tree (a) and its seeds (b)



12-O-Tetradecanoylphorbol-13-acetate or Phorbol ester (TPA)

1-Oleyl-2-acetyl-glycerol or Diacylglycerol (DAG)

Figure 3. The chemical structure of phorbol ester,TPA ,compare with diacylglycerol (DAG)

Table 2. TPA responsive genes.

Activated gene	Cell lines	Cyclohexamide inhibition	Transcriptional regulation	Post-transcriptional regulation
Oncogenes				
c-fos	fibr,HL-60	No	Yes	
c-juns	fibr	No	Yes	
c-myc	fibr	No	Yes	Yes
c-sis	neothel cell	No	Yes	
c-rel	fibr	No	Yes	
c-fms	HL-60	No	Yes	
Other genes				
Type I procollagen	chondr	Yes	Yes	
Urokinase	fibr	No	Yes	
Actin	fibr	No	Yes	
Serine proteinase I	T-cells		Yes	
Collagenase type I	fibr	Partly (80%)	Yes	
Collagenase type 4	macrophage			

1.2.2.4 TPA induced signal transduction

In non- treated cells the transciption factors responding to TPA reside in the cytoplasm (NFkB) or in the nucleus. TPA, however, are lipid solutes, and therefore, are prodominantly dissolved in the plasma membrane. In order to influence transcription factors, obviously intermediate components are required .The finding that a Ca^{2+} and phospholip activaTable protein kinase can dissociate the cytoplasmic form of NFkB from its inhibitor seemed to indicate that this protein kinase C can replace TPA. This experiment is based on large body of previous data that suggest PKC as one of the targets of TPA. Radioactive labeled phorbol esters offered to cells will be found in the plasma membrane and bound to a protein receptor, TPA receptor. TPA receptors and PKC copurify to homogeneity. Thus one of the primary targets of TPA is PKC. TPA substitute for a physiological activator of the enzyme. The same PKC is activated following treatment of cells with several growth factors or hormones. The presumable physiologic pathway involves the splitting of phosphatidyl inositols to inositol trisphosphate and diacylglycerol; diacylglycerol is the natural activator of PKC. This finding may explain why growth factors and TPA often influence the expression of the same genes.

Evidence suggesting that TPA activate gene through activation of PKC is circumstantial : (i) In cells in which PKC has been consumed by prior TPA treatment, the responsive genes are refractory to TPA stimulation ; microinjection of PKC restores the response. (ii) cells overexpressing PKC transcribed from transected cDNA clones, change their growth behavior and express TPA inducible genes. (iii) PKC inhibitors that inhibit the activity of PKC *in vitro*. interfere with TPA induced gene activation *in vivo*. An inhibitor usjed inkmanu exxperiments belongs to the isoquinoline series. It inhibits TPA induced activation of c-fos and of collagenase, but it also interferes with cyclic AMP, ultraviolet and Ca^{2+}-ionophore induced c-fos mRNA accumulations.

1.2.3 Protein kinase C

1.2.3.1 The protein structure of protein kinase C isoenzymes

Protein kinase C (PKC) is a multigene family consisting of at least twelve distinct lipid-regulated protein serine/threonie kinases which play pivotal roles in signal transduction and growth control as shown in the Table 3 (40). Each isoenzyme posses unique structural properties, as well as distinct tissue and cell distribution, supporting the concept that individual isoenzyme perform specific roles in cellular signaling .The isoenzymes can be divided into three distinct groups : conventional protein kinase Cs (cPKCs), novel protein kinase Cs (nPKCs) and atypical protein kinase Cs (aPKCs). The cPKC isoenzymes (α,β,γ) posses four conserved region (C1-C4) and five variable (V1-V5) regions .The nPKC isoenzymes ($\delta,\varepsilon,\theta$) do not require Ca^{2+} and lack of C2 region .In contrast, the aPKC isoenzymes (ι,λ,ζ) have only one cysteine-rich region zinc finger-motif, require phosphatidylserine but not respond to diacylglycerol, phorbol ester or Ca^{2+}(41). The structure has shown in Figure 4.

Regulatory domains

The regulatory half of protein kinase Cs contains an autoinhibitory domain,that is the pseudosubstrate, and one of two membrane-targeting motifs, namely, the C1 domain, which is present in all isoenzymes, and the C2 domain, which is present in conventional (Figure 4).

The C1 domain is a cysteine-rich sequence that coordinates with Zn^{2+} atom. It folds into a globular structure with two pulled-apart β sheets forming the ligand-binding cavity. Hurley and coworkers (42) elucidated the crystal structure of the C1 domain with and without bound phorbol and found that the domain undergoes no significant conformational

change upon phorbol binding. Rather, the ligand caps the hydrophobic binding site so that the top third of the domain displays a contiguous hydrophobic surface. Thus, membrane targeting is achieved by ligand altering the surface of the protein to promote hydrophobic interactions. On the basis of structural and mutagenesis studies key residues that define the ligand binding cavity have been identified and used to classify C1 domains as typical or atypical, for those that do or do not bind phorbol esters, respectively.Elucidation of an atypical C1 domain structure revealed that the phorbol-binding site is, indeed, compromised by the lack of consensus residues that form a hydrophobic face of the pocket in typical C1 domain.

Elucidation of the structure of C2 domains and phospholipase C (43) revealed a β-sheet rich domain with a novel Ca^{2+}-binding pocket; two loops comprising sequences at the amino and carboxyl termini of the C2 core come together to form an aspartate lined mouth that coordinates Ca^{2+}.For the C2 domain studied, NMR and crystallographic data have shown that this pocket coordinates two metal ions (43). Metal ion binding produced a significant conformational change in the phopholipase Cδ C2 domain, exposing three lysine residue on the back face of the Ca^{2+}-binding site that could potentially bind acidic lipids. This conformational change supports biochemical data binding.A number of proteins without C2 domains do not bind Ca^{2+}, notably the novel protein kinase Cs and atypical PKCs sequence alignment reveals that key aspartate involved in Ca^{2+} coordination are not present in these proteins (41,44).

Table 3. Examples of proposed roles of protein kinase C in cellular responses.

Tissues and cells	Cellular responses
Endocrine systems	
Adrenal medulla	Catecholamine secretion
Adrenal cortex	Aldosterone secretion, steriodogenesis
Pancretic islets	Insulin release
Hypothalamus	Releasing hormone release
Pituitary cells	Growth hormone release, luteinizing hormone release, prolactin release, thyrotropin hormone release
Parathyroid cells	Parathyroid hormone release
Thyroid C cells	Calcitonin release
Leydig cells	Steroidogenesis
Exocrine systems	
Pancreas acinar cells	Amylase secretion
Parotid gland	Amylase and mucin secretion
Submandibular gland	Mucin secretion
Gastric gland	Pepsinogen secretion, gastric acid secretion,
Nervous systems	
Neuronal synapses	Transmitter release
Neurons	Membrane conductance
Muscular systems	
Smooth muscle	Muscle contraction
Inflammation and immune systems	
Platelets	Serotonin release, lysosomal enzyme release, arachidonic acid release,thromboxane synthesis

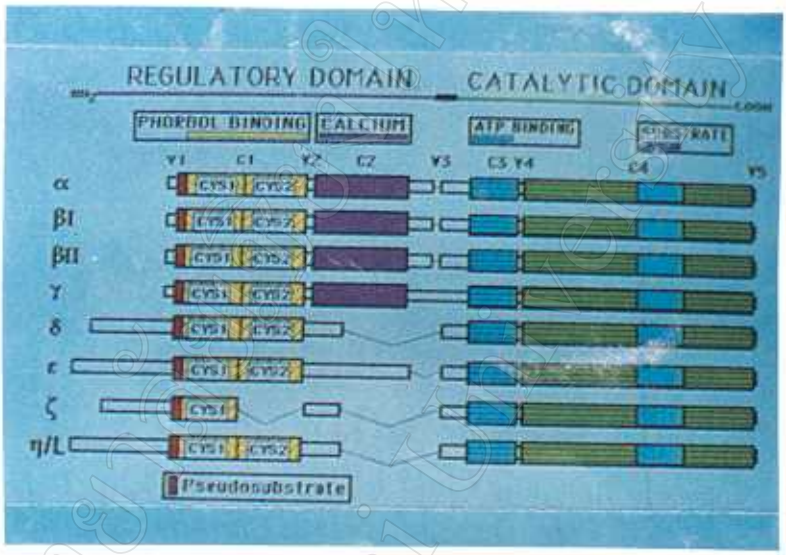


Figure 4. Domain structure of PKC- isoenzymes

Catalytic domains

The catalytic domain of protein kinase C has similar to that of other protein kinases (45).The C3 region contains the ATP-binding motif $XGXGX_2GX_{16}KX$ conserved in most protein kinases. Only PKCζ differs slightly from the consensus ATP-binding motif,it contains an alanine instead of glycine at position 264.Nevertheless,human PKCζ purified from recombinant baculovirus-infected insect cells does not show kinase activity (44).

The C4 region contains the substrate binding site and the phosphate transfer region. The central element in the phosphate transfer region is the sequence DFG. The Asp residue is thought to be responsible for the transfer of the phosphate group to substrate .In all PKC isoenymes, there is a conserved distance of 105-108 (113 in PKCζ) amino acids between the end of the ATP-binding site and the beginning of the phosphate transfer region. Again, PKC ζ exhibits a slight deviation from the consensus sequence DFG with a substitution of phenylalanine by tyrosine (44).

1.2.3.2 Biochemical regulation of protein kinase C

I. Regulation by cofactor

Activation of protein kinase C requires the removal of the inhibitory pseudo substrate domain from the active site. This conformational change is achieved by highlight specific binding of 1,2-sn-diacylglyserol and calcium to the two membrane-targeting domain, C1 and C2. Binding of ligand to either domain is sufficient to recruit the enzyme membranes by a low-affinity interaction; however, both domains must be membrane-bound for the high-affinity interaction that results in pseudosubstrate removal and maximal activation (46).

Diacylglycerol and phorbol ester

Structural, biophysical, and mutagenesis studies in the past year have resulted in much headway into the search for the molecular mechanism for the diacylglycerol/phorbol ester dependent membrane association that has served as a hallmark for protein kinase C activation *in vivo*. Complementing the structural determination showing the phorbol binding changes the surface hydrophobicity of the C1 domain. the absence of a conformational change binding measurement revealed that phorbol esters and diacylglycerol activate protein kinase C by the same mechanism. They increase protein kinase C's membrane affinity linearly with respect to the molar fraction of ligand in the membrane .The dissociation constant of 1.5 $x10^{-5}$ mol % describes the remarkably high affinity of membrane-bound protein kinase C for membrane-bound phorbol myristate acetate (47). The dissociation a constant of diacyglycerol is two orders of magnitude lower possibly because the increased flexibility of this ligand result in a greater entropic loss upon binding. Consistent with this, conformationally constrained analogs of diacylglycerol have a significantly increased affinity for protein kinase C (47).

II.Regulation by phosphorylation

Protein kinase C has recently been shown to be phosphorylated at three positions in the kinase core *in vivo* (48,49) .In protein kinase C βII, these residues correspond to Thr 500 in the activation loop,Thr 641 and Ser 660 at the carboxyl terminus. Hydroxyl -containing residues (or acidic residues at the equivalent position of Ser660 in protein Cs ζ and ι) at corresponding positions are conserved throughout the protein kinase C family, with the exception of in protein kinase C μ whose -carboxyl terminal phosphorylation sites are not apparent from sequence comparison. Protein kinase C recovered from the detergent -soluble fraction of cells (mammalian or insect) is completed phosphorylated at

the two carboxyl- terminal positions and half the population is also phosphorylated at the activation-loop site ; this protein kinase C migrates as a single band on SDS-PAGE (at proximately 80 kDa for conventional protein kinase Cs) The protein kinase C in the detergent -insoluble fraction contains faster-migrating species that correspond to nonphosphorylated enzyme or enzyme phosphorylated on the activation loop (both migrating with an apparent molecular weight of 76 kDa for conventional protein Cs) and enzyme phosphorylated on the activation loop and Thr 641 or its equivalent (migrating with an apparent molecular weight 78 kDa for conventional protein kinase Cs). Biochemical analyses of protein kinase C dephosphorylated at selective positions, coupled with analysis of phosphorylation - site mutants, revealed that each site has a specific function (50).

Carboxy-terminal phosphorylaion sites

Following phosphorylation at the activation loop protein kinase C becomes phophorylated at Thr 641. Both the proximity of the carboxyl terminus to the active site and the finding that neighboring residues are phosphorylated by an intramolecular reaction *in vitro* suggest that the reaction is an autophosphorylation. This phosphorylation appears to lock protein kinase C in a catalytic competent conformation, perhaps by anchoring the carboxyl terminus out of the active site. Subsequently selective dephosphorylation at the activation loop position results retention of catalytic activity. The final *in vivo* phosphorylation occurs at Ser 660 and is an autophosphorylation reaction. This phosphorylation correlates with release of protein kinase C in to the cytosol, suggesting that its role is to direct the subcellular localization of protein. The two autophosphorylation reactions at the carboxyl terminus appear to be triggered by phosphorylation at the activation loop; thus, phosphorylation by the putative protein kinase C kinase initiates this phosphorylation cascade (50).

Most of the protein kinase C in unstimulated cells is the triple-phosphorylated, mature form, suggesting that phosphorylation at these conserved sites is involved in the processing of the enzyme rather than in modulating the function of the enzyme in response to specific signals. However, protein kinase C's subcellular localization and function may be modulated by dephosphorylation at these key position: ceramide has recently reported to inactivate protein kinaseC α, probably by activating a phosphatase (51).

III. Regulation by localization

Immunocytochemically analysis has clearly established that different protein kianse C isoenzymes localize to different subcellular compartments. Subcellular targeting by interaction with specific proteins provides an attractive mechanism for isoenzyme-specifc regulation .A number of proteins have been identified that bind protein kinasC ;these proteins include the RACKs (rceptor for activated C kinase),a group of protein that are proposed to bind to the active conformation of protein kinase C (52). Binding site for this proteins have been localized to both conserved and variable regions in the protein kinase C. PKCs α and βII bind a multi-enzyme scaffold protein, AKAP 79(for a kinase-anchoring protein) and protein kinase ε binds to 14-3-3 protein , to cytoskeletal components *via* its hinge or pseusubstrate ,and to actin *via* a sequence between to first and second C1 domains (53). Whereas Protein kinase C βII has been reported to bind to actin, in this case *via* its carboxyl terminus (54).

1.2.3.3. Protein Kinase C and signal transduction pathway cascades

From the large body of data it might be classified the pathway for activation PKC *via* release of DAG into three pathways (Figure 5). As shown, phospholipase Cα and β is activated by many G-protein coupled ligands and released DAG and IP_3 from the

membrane phospholipid phosphatidylinositol-4,5-biphosphate (PIP_2) .Phospholipase C γ is activated by many receptor tyrosine kinases ,including IGF1, EGF,FGF and PDGF; it metabolized PIP_2 similarly to the case of phospholipase Cβ as well. DAG produced by these two main receptor pathways can then activate PKC directly. Depending on which isoenzymes are expressed, among other factors, this activation will result in nuclear signal transduction.

A third pathway for activation of PKC by lipid intermediates is *via* phospholipase D (PLD) which metabolized phosphatidylcholine to phosphatidic acid. The latter directly activate PKC or may be metabolized by phosphatidic acid phosphohydrolase to DAG (55,56).

The three pathways described above all result in DAG activation of members of the PKC family.Some members of the PKC family that are not activated by DAG (e.g. PKC ζ) are critical in mitogenic signal transduction. They may act downstream from *Ras* or may be substrate for phosphatidyl inositol -3,4,5-triphosphate .The latter PI metabolite is produced from IP-4,5 bis by phosphatidyl inositol-3-kinase,another enzyme activated by receptor tyrosine kinases.

Gene amplification for the receptor tyrosine kinases, overexpression of their ligands ,mutations in receptors, rearrangements of tyrosine kinases provide a multiplicity of mechanism by which aberrant activation of PKCs could occur for example ,expression of mutant *ras* in human epithelial cells confers a striking sensitivity to the toxic effects of phorbol ester and it was found that there has been controversy over the relative importance of PKC in transformation , and this may relate to problems in methodology and the multiple isoforms (57,58).

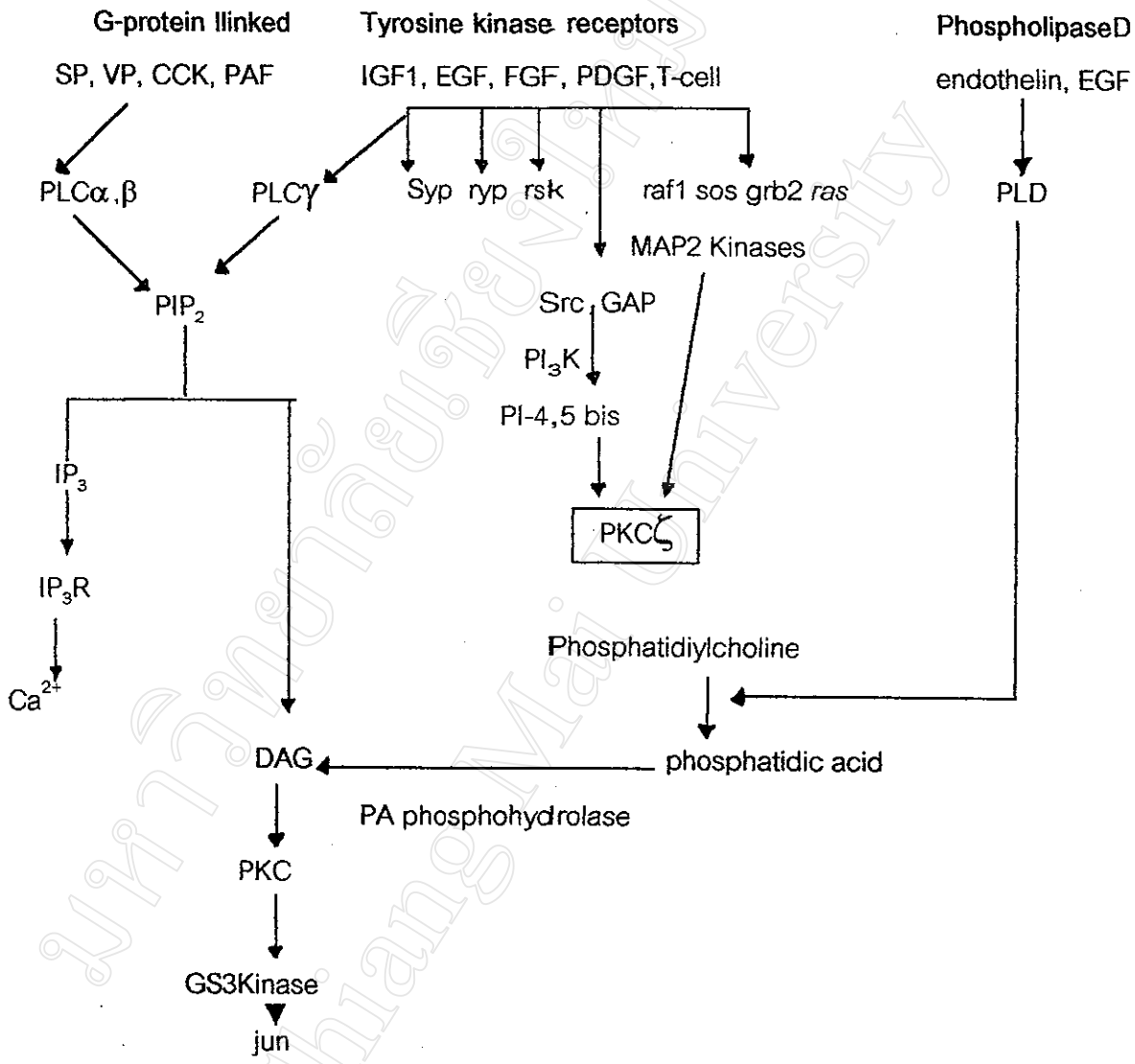


Figure 5. The possible cascades for PKC activation.

SP, substrate P; VP, vasopressin ; CCK, cholecystokinin; PAF, platelet activating factor; PLC(α, β, γ), phospholipaseC (isoforms); PLD, phospholipaseD; IP_3, inositol1, 4, 5-triphos phate; IP_3R, IP3rceptor; Ca^{2+}, ionized calcium; PIP_2, phosphatidyl inositol (4,5) biphosphate; PKC, protein kinase C; DAG, diacylglycerol ; EGF, epidermal growth factor; FGF, fibroblast growth fctor ; PDGF, platelet derived growth factor; IGF1, insulin-like growth factor1 ; GAP, GTPase activating protein; other abbreviaions are names of oncogenes and phosphoprotein involves in signal transduction.

1.2.4 Human keratinocytes

The keratinocytes is the major cell type of the epidermis. The understanding of keratinocytes biology is rapidly increasing due to the availability of cell culture models that mimic the *in vivo* differentiation process and the techniques of molecular biology. Keratinocytes cultures provide abundant source of pure kerainocytes that can be used to test the effects of proliferation- and differentiation -regulating agents on cell function and as a source for the isolation of keratinocyte proteins and genes.

1.2.4.1 The skin : A protective barrier

All organisms are continually challenged by a variety of environmental hazards. The first line of defense and protection is provided by the epidermis, the outermost skin-layer. The epidermis includes a variety of specialized cell types; however, the major cell type, and the cell type responsible for constructing the protective barrier, is the epidermal keratinocyte. The keratinocyte elaborates its protective function by undergoing a complex and carefully choreographed program of differentiation (Figure 7). The basal epidermal layer (stratum basalis) consists of a single layer of relatively undifferentiated cells that are anchored to the basal lamina *via* hemidesmosomal junctions. This layer contains the epidermal stem cell population that provides a continuos supply of new cells to repopulate the epidermis .The cells in this layer undergo a transient amplification to produce daughter cells, which then leave the basal layer to begin the 2 wk journey to the epidermal surface. The second skin layer,the spinous layer of stratum spinosum, is situated immediately above the basal layer .This layer is characterized by the presence of extensive desmosomes appears as narrow projections that can be seen in immunohistological sections that give the layer its name. Some of the early markers of keratinocytes differentiation are first expressed in this layer eg., involucrin and transglutaminase. The third layer, the stratum granulosum or

granular layer, is distinguished by the presence of granules. These granules, including the corneocyte membrane or marginal band, contained loricrin, fillaggrin, crystatin-α and lipids that are used in the assembly of the corneocyte membrane. In addition to these specialized organelles, granular layer cells contain all of the organelles and activities typically associated with intact cellular metabolic function, indicating that they are still living even as they approach the final steps in differentiation. The stratum lucidum, also called the transition zone, is the layer that seperates the dead from living epidermal layers. This is a region of the intense enzymatic activity in which cellular organelles and nucleic acids are destroyed by the action of protease, nuclease and other enzymes. However, simultaneous with this wholesale destruction of cell synthetic capacity are the final steps in assembly of the keratin intermediate filament network and the cornified envelope. The cornified cells (corneocyte) represents the terminal stage in keratinocytes differentiation and consist of a stabilized array of keratin filament continued within a covalently cross-linked protein envelope hence cells, which occupy the stratum corneum, are dead flattened polyhedrons that are uniquely adopted to provide a protective surface. Disulfide course through their interior and connect at various points to the surrounding marginal band. The cornified envelopes consist of an array of protein that are covalently connected *via* ε-(γ-glutamyl)-lysine protein-protein cross-links *via* the action of transglutaminase. Adjacent corneocytes are held together by modified desmosomes and by an interlocking system of ridges and grooves. Taken together, the composite of billions of terminal keratinocytes (cornified envelopes) forms the protective epidermal surface. Without this protection, we would quickly hydrate in wet environments, dehydrate in arid environments and be extremely susceptible to infection by phathogens. The best evidence of the importance of this barrier for survival is the heroic efforts required to assist burn victims in regulating fluid balance and remaining free of infection (59).

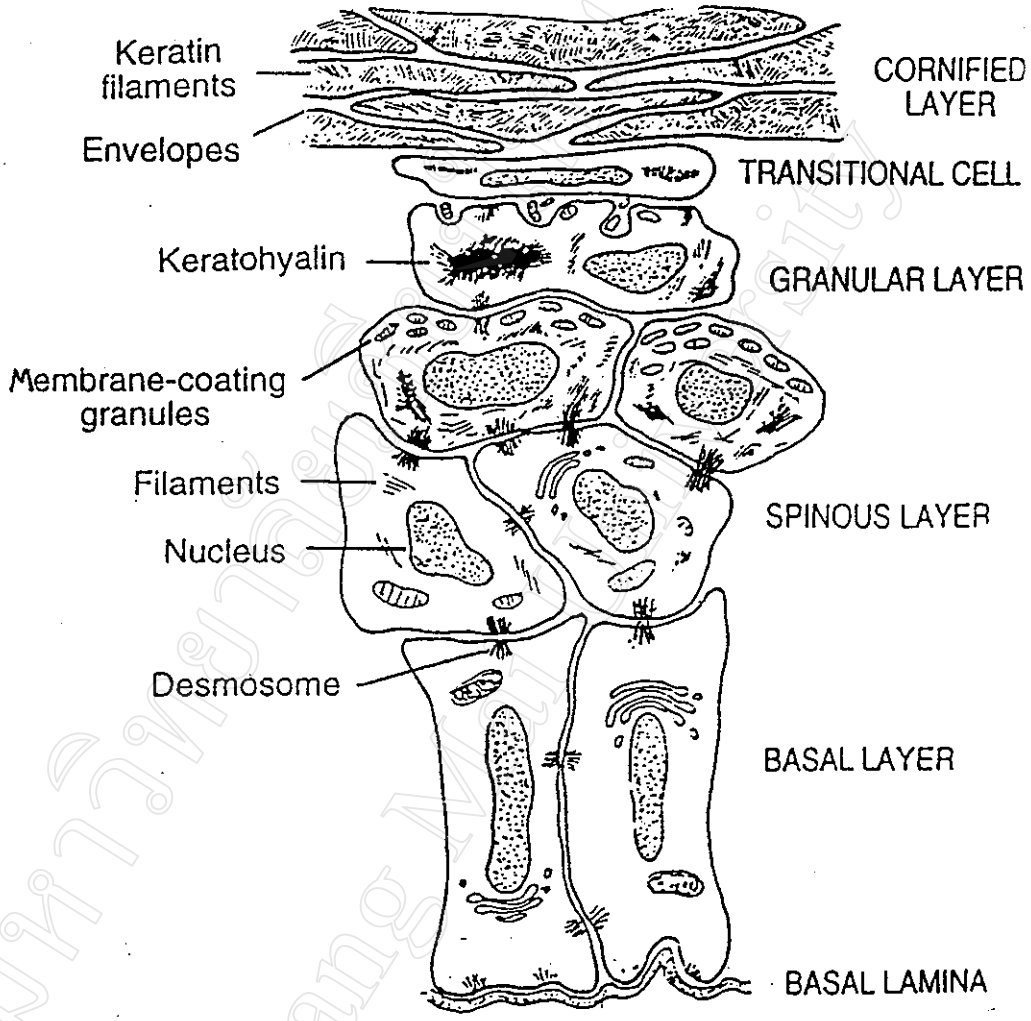


Figure 6. Morphological progression during keratinocyte differentiation showing various epidermal layers

1.2.4.2 Agents that regulate keratinocytes proliferation /differentiation

Regulation of stem cell proliferation is an important topic, since the rate of stem cell proliferation determines the rate at which differentiated cells enter the upper epidermal layers. Stem cells, which provide a continuous supply of new cells to the epidermis, reside in the epidermal basal layer and also in the bulge region of the hair bulb .The factors that trigger epithelial cell commitment and differentiation in the epidermis are still incompletely understood, however, specific agents have been identified that regulate keratinocyte proliferation as shown in Table 4 (60).

Table 4. Role of various agents that regulate keratinocytes function.

Compound	Proliferation	Differentiation
IGF-I	Enhanced	
EGF	Enhanced	
TGF-α	Enhanced	
Cholera toxin	Enhanced	
Phorbol ester	Enhanced	Enhanced
TGF-βI	Reduced	Enhanced
KGF	Enhanced	Enhanced
IFN-γ	Reduced	Enhanced
Retinoid		Reduced
Vitamin D	Reduced*	Enhanced
Hydrocortisone		Enhanced
Calcium	Reduced	Enhanced

IGF-I,insulin -like growth factor I; EGF, epidermal growth factor ; TGF, transforming growth factor; KGF, keratinocyte growth factor ; IFN, interferon. * Response depends on vitamin D concentration.

1.2.4.3 Abnormal keratinocyte differentiation and proliferation

Many disease have been described in which there is abnormal keratinocyte differentiation. Some of these involved a lesion in the regulation of keratinocytes differentiation, Some of these involve a lesion in the regulation of keratinocyte growth, and others involve a lesion in the expression of differentiation products (61-63).

Cancer

Because the cells can be readily growth in culture, the best studied epidermal cancer is SCC (squamous carcinoma cancer cell line). These cells are defective in the expression of the keratins, in the envelope precursor involucrin, in their ability to form envelope and in transglutaminase expression. They also differ from normal cells in their sensitivity to physiological agents, such as Ca^{2+}, retinoids, and hydrocortisone. In many respect these cells manifest characteristics of primitive epidermal cells. An example of this is the expression of the 40-kDa Keratin that under normal condition is only expressed in epidermal keratinocytes during early embryogenesis. This indicates that transformed keratinocytes, like other transformed cell types, express a more primitive phenotype and exhibit less differentiated function.

SCCs differ from normal cells in two ways, 1: the biochemical markers of differentiation (involucrin and transgluatmanase) are highly sensitive to factor that regulate keratinocyte differentiation, and 2: SCCs are defective in spontaneous envelope formation. In normal cultured cells the level of the differentiated products is not significantly affected by physiological agents, these cells may have altered membrane permeability properties, reducing their sensitivity to the differentiating effects of Ca^{2+}.

Hyperproliferating Diseases

Hyperproliferating disease of the epidermis are conditions where there is an enhanced rate of cell growth. The most intensive studied disease of this type is psoriasis. In psoriasis the basal cells divide at a higher than normal rate, and cells in the upper more differentiated layer are deficient in the expression of selected markers of terminal differentiation, such as the high-molecular-mass, differentiation-dependent keratins. The 48-56 kDa keratins appear to be markers for hyperproliferative disease, including psoriasis. The causative agent of psoriasis is not known, but it appears to be multifactorial disease. Again, this is an example of condition where keratinocytes differentiation is incomplete. One possible explaination of the observed changes is that the high rate of cell division within the basal layer and the concomitant higher rate of passage through the upper layers may not allow enough time for the cells to complete their differentiation before they are lost. This, however, is likely to be far too simple an explanation for an exceedingly complex disease. Other factors, such as inflammation, may also be important.

1.2.5 Role of protein kinase C signaling cascade in regulating keratinocyte proliferation and differentiation

Among the various transcription factors that have been implicated as being important in regulation of keratinocyte gene expression, AP-1 is the most heavily studied. Activator protein 1 site have been localized in a wide variety of keratinocytes genes and AP-1 proteins appear to mediate the effects of variety of regulating agents. Many of the agents that regulate AP-1 appear to function by activating protein kinase C (64,65). Protein kinase C consists of a family of 12 isoenzymes, 5 of which are expressed in epidermal keratinocytes (α, δ, ε, η, ζ) (66). Physiological activation of PKC occurs when extracellular

stimuli activate phospholipase C (PLC), which hydrolyzes membrane-localized phospatidylinositol, to produce inositol triphosphate (IP_3) and 1,2-diacylglycerol (DAG). Phosphtidylinositol is involved in mobilizing intracellular calcium, and DAG is activator of PKC. Phorbol ester such as TPA can be used to selectively activate PKC which, in turn, phosphorylates proteins at serine and threonine residues. Calcium treatment of keratinocyte influences PLC and PKC activity. Mouse keratinocytes differentiation is induced when cells are shifted from 0.05 to 0.12 mM calcium. This Change in cell phenotype is associated with modulation of PKC. Differentiation can be blocked by inhibitors of PKC function, sugessting that this pathway is required for differentiation (67). Protein Kinase C activation enhance loricrin, profilaggrin and TG1 trascript levels and suppresses the calcium-dependent induction of transcript encoding K1 and K10 (68). Furthermore, they have also been reported that PKC activation simultaneously increase ornithine decarboxylase and transglutaminase activities, the former a marker of keratinocytes proliferation and the latter a marker of differentiation.

In human keratinocytes, PKC activation increase expression of suprabasal markers of differentiation. The transcription factor AP-1 is a nuclear target of the PKC pathway and in some cases appears to mediate the last step in translocation of the PKC signal to the nucleus (64,68).

1.3 Experimental designs

Effects of curcumin on level and distribution of protein kinase C-ε isoenzyme in human keratinocytes were performed as follows.

In the first step; in order to examine the effect of TPA on level and distribution of cPKC (PKC-α) and nPKC (PKC-δ, -ε), 80 % confluent human keratinocytes ($3x10^6$ cells/ml) were placed in growth-factor-free medium (keratinocyte-SFM without hydrocortisone, insulin, BPE, and rEGF) for 48 h then treated with 160 nM TPA for 1, 2 and 18 h. Control cells received DMSO with the final concentration of 4%.Then cytosolic and membrane fractions were prepared (69,70). The cytosolic and membrane fractions (30 μg protein/35μl) were analyzed by Western blot analysis using specific anti PKC-α,δ and ε respectively (Figure 7). To further determine whether TPA increased the level or induced newly synthesis of PKC -ε isoenzyme,the cytosolic and membrane fractions (30 μg protein /35μl) of TPA- treated cells were pooled and then assayed for PKC -ε isoenzyme by Western blot compared with the control (untreated cells) (Figure 8).

In the second experimental protocol, the other three epidermis subjects of keratinocytes were treated for 1h with different concentration of curcumin (0, 20, 40, 50 μM) to investigate the effect of curcumin on level and distribution of PKC-epsilon isoenzyme (Figure 9). The cytosolic and membrane fractions (30 μg /35μl) were tested for PKC-ε isoenzyme by Western blot analysis .The third protocol was to examine the effect of curcumin on level and distribution of TPA-induced PKC-epsilon isoenzyme. The cells were preincubated for 1 hr with curcumin (0, 20, 40 ,50 μM) and then treated with TPA 160 nM for 1 h at 37 °C. The level of PKC-ε isoenzyme in cytosolic and membrane fractions (30 μg protein /35μl) were analyzed by Western blotting technique (Figure 10).

The last experiment is to explore the kinetic inhibition of curcumin on TPA-induced PKC-epsilon isoenzyme (Figure 11). Human keratinocytes were incubated at 37 °C with curcumin (50 μM) for 1 h prior to the addition of 160 nM TPA, at the same time and after

the addition of TPA. Following the treatment, cytosolic and membrane extracts were assayed for PKC-ε isoenzyme protein expression by Western analysis using specific antibody of PKC-ε isoenzyme, immunoreactive proteins were visualized using the enhance chemiluminnescence Western blotting detection system (Amersham). PKC band intensities were quantified by laser densitometer. The arbitrary units represent the relative amount of the respective bands on the blot.

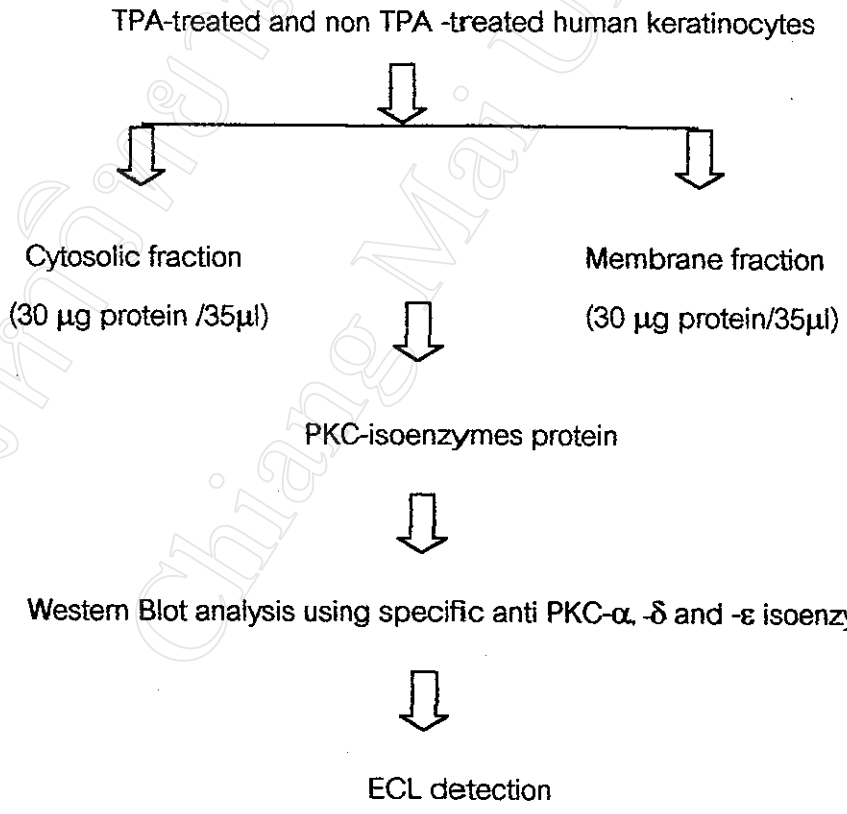


Figure 7. Experimental design for evaluation the effect of TPA on level and distribution of PKC-α,δ and ε-isoenzymes in human keratinocytes

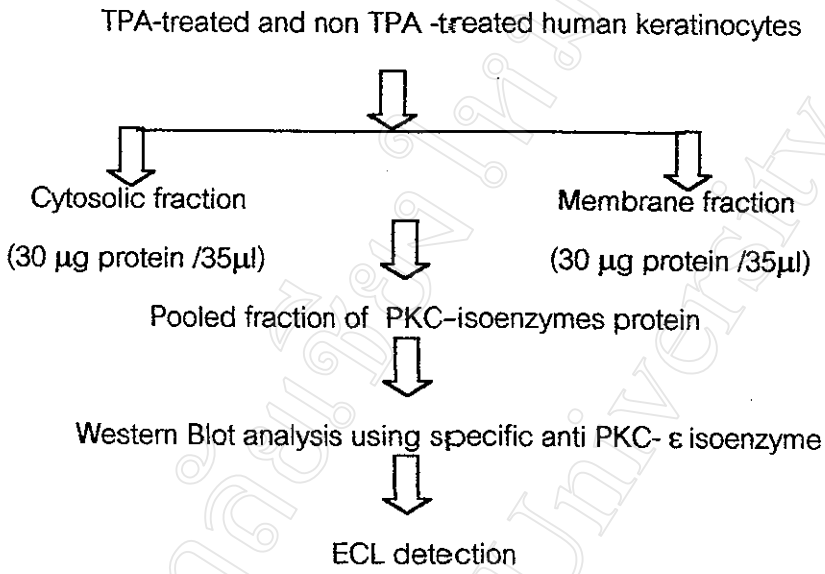


Figure 8. Experimental design for determination the effect of TPA on the enzyme content of PKC ε in human keratinocytes

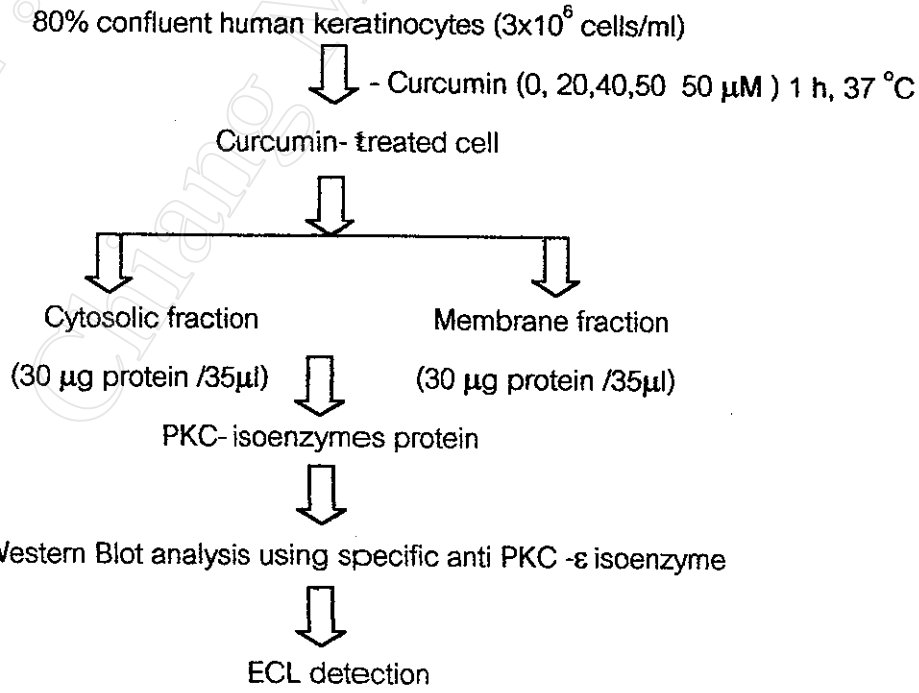


Figure 9. Experimental design for investigation the effect of curcumin on level and distribution of PKC-ε in human keratinocytes

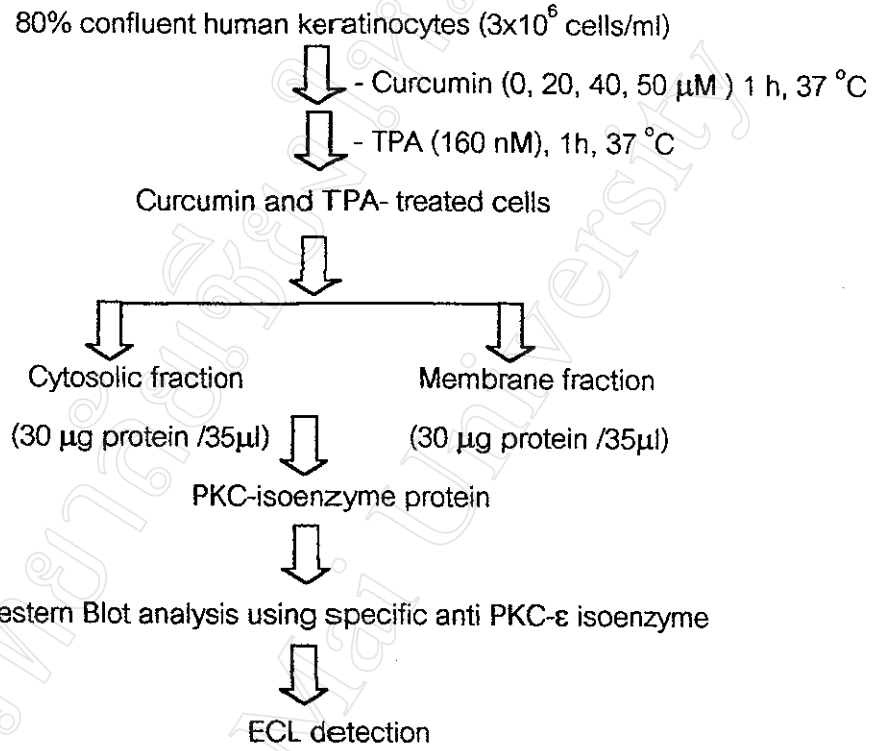


Figure 10. Experimental design for examination of the effect of curcumin pretreatment on level and distribution of TPA-induced PKC ε in human keratinocytes

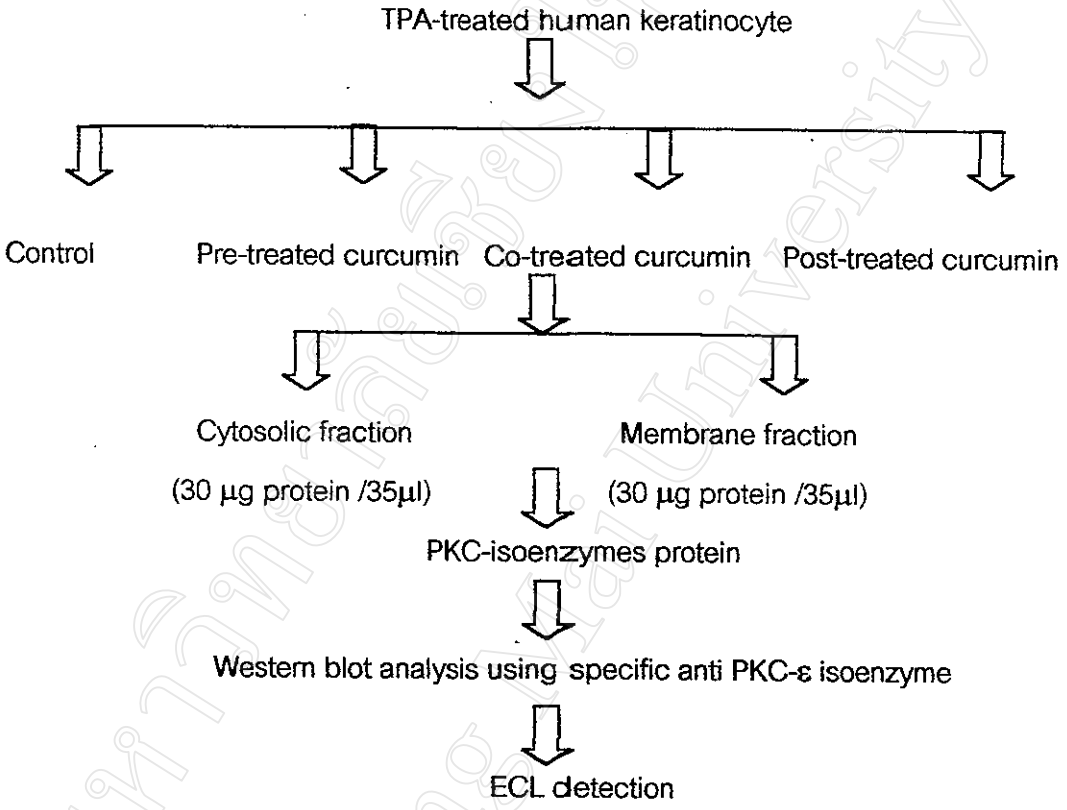


Figure 11. Experimental design for exploring the effect of time on curcumin addition on TPA- induced the translocation of PKC ε in human keratinocyte

1.4 Objectives

1. To determine the biochemical effect of TPA on level and distribution of PKC-α, δ and ε isoenzymes in human keratinocytes.
2. To examine the effect of curcumin on level and distribution on PKC-ε isoenzyme in human keratinocytes.
3. To explore the effect of curcumin on TPA-induced PKC-ε isoenzyme in human keratinocytes.