CHAPTER IV
DISCUSSION

Previous study from our labarotory indicated that topical application of curcumin had marked inhibitory effect on TPA-induced tumor promotion in mouse skin (77) which was consistent with the findings of Conney *et al* (6). Moreover the study also reported the molecular level inhibition of curcumin. It was found that curcumin could inhibit TPA induced *c-fos* oncogene expression in mouse skin (8,77). Since a considerable amount of evidence reported that fos protein is one of the PKC substrates (19, 77,78) and PKC is a receptor of TPA (79,80), then this study proposed that curcumin might block a certain point on the signal transduction pathway leading to fos oncogene expression. Furthermore, recently our laboratory (81) has shown that curcumin inhibited TPA-induced PKC activity in mouse skin. Those findings were consistent with the result of Lui and Lin (9) who found that pretreatment 15-20 μM curcumin for 15 min could inhibit 0.1 μM TPA -induced PKC activity in membrane fraction of NIH3T3 cells by 25 or 60% ,respectively. These results strongly provided some insight into a possible mechanism of curcumin action *via* PKC.

In order to further investigate the mechanism by which curcumin causes inhibition, this study paid most attention to the effect of curcumin on the level and distribution of TPA induced PKC -isoenzyme in primary human keratinocytes, which is a model very closely related to human skin system and which has not yet been reported the mechanism of curcumin. This is also meant to avoid the difficulties in identifying the relevant cells, in manipulating their behavior in a controlled manner, and in separating the effects due to the interactions among the various cell types present in the organism, which is something that always occurs when studying animal models and which also makes it impractical to study the human body directly. The findings of this study demonstrate the chemopreventive potential of curcumin on TPA-induced PKC-ε isoenzyme, i.e., the isoenzyme which is a

molecular target for tumor promoting phorbol esters in skin chemical carcinogenesis models (82-83).

Human keratinocytes are the main cell type (>95%) of the epidermis which expressed both mRNA and protein of PKCα, δ, ε, η and ζ isoenzymes (69). Among the first signaling protein, it had been shown to change their sub-cellular localization upon activation (84-86). This study found that 160 nM TPA-treament for 1 and 2 h caused PKC-α and -ε isoenzyme to translocate from cytosol to membrane. The result was consistent with the finding of Nicholas and his coworkers,1994 (69) which reported the translocation of PKC-α and -ε from cytosol to membrane upon activation of human keratinocytes with 50 nM TPA for 5 min. In addition, this study also showed translocation at 2 h higher than 1 h and almost nearly complete. That result is similar to that of Sarah A,1997 (87) which found that in Swiss 3T3 cells for which the concentration of TPA treatment was varied for a fixed time, there was activation in the cells with 1,10,100,500 and 1000 nM TPA for 20 min. The control sample of both PKC-α and ε isoenzyme were found predominantly in the cytosolic fraction but with the addition of 1 nM TPA, there was a decrease in the amount of cytosolic PKC corresponding increase in the membrane fraction. Moreover, at a TPA concentration of 10 - 1000 nM, the amount of cytosolic PKC was reduced to undetectable levels and all immunoreactivity was detected in the membrane.

Consequently, our study combined with the observations from other investigators suggested that longer time or higher concentration of TPA treatment leads to an enhancement in PKC activation, which was associated with the translocation from cytosol to membrane then, bound to TPA as a TPA receptor. For a long time, it had been proved that the binding site of TPA on PKC isoenzymes was C1 domain whose membrane affinity is increased dramatically upon binding to TPA (88,89). This could explain the reason why the level of PKC-α and - ε isoenzyme on the cell membrane were higher in 2 h TPA exposure than 1 h . It was synchronized with the case of TPA treatment 10-1000 nM higher than 1 nM (87). However, although the molecular mechanism by which the binding

of TPA to C1 domains activates PKC has been fairly well characterized *in vitro*, it is also found that the regulation of PKC *in vivo* must depend on other macromolecule interaction (90). The precise determinants of sub-cellular localization and their potential importance in defining the function of PKC isoenzyme in human keratinocyte have not yet been fully characterized. There is thus a need to further study this in order to distinguish the myriad signals transduced by members of PKC-isoenzymes *in vivo* from their sub-cellular localization. Such a study should also focus on the specific proteins or enzymes regulating PKC-isoenzymes which may be identified by overlay assay (a modification of Western immunoblotting that uses protein kinase C rather than an antibody to probe protein bands), by interaction cloning (in which a cDNA expression library is probed with PKC), or using the yeast two-hybrid genetic screen for protein-protein interactions.

From the past few years of data it is possible to roughly summarize the regulation model of PKC as well as the interactions that control sub-cellular localization of PKC as shown in Figure 29.

In addition, this study found that PKC-α and -ε isoenzymes underwent down-regulation after treatment with 160 nM TPA for 18 h. (Figure 15, 17,19 ; Table 6, 7, 8). The result was consistent with the finding of Nicholas JR. *et al.*, (69) who also reported the downregula-tion of -α and -ε isoenzymes after long treatment (18 h) with 50 nM TPA. Almost nothing is known about the molecular mechanism(s) and *in vivo* determinants of the process of the down-regulation through proteolytic of PKC. Likewise little is known of the events involved in the down-regulation and proteolytic degradation of PKC. However, one protease cleavage site has been mapped to the border of the hinge region and catalytic domain of the PKC isoenzymes. There is evidence that the proteolytic process may be caused by neutral Ca^{2+} dependent proteinase which was found inserted in the membrane resulting into two fragments of PKC, 50 KD protein which was released into the cytoplasm and displayed a phospholipid and Ca^{2+} independent protein kinase C activity. Protein kinase M (PKM) and other fragment is a 35 kDa protein which retained TPA binding activity.

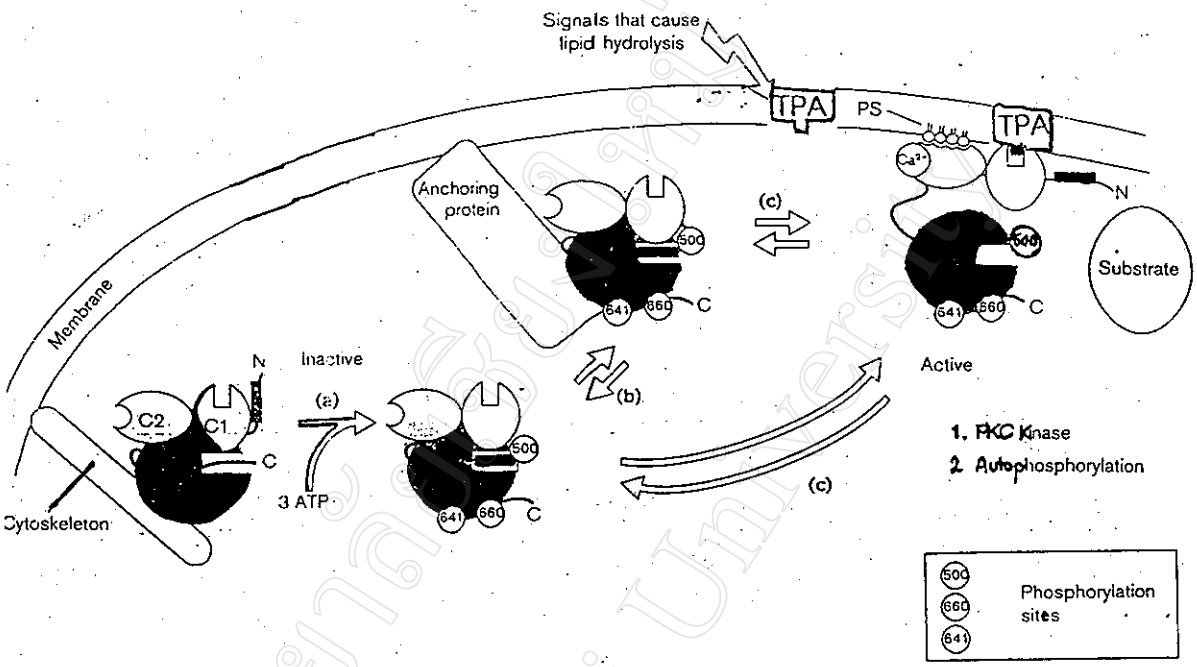

Figure 29. Model for the regulation of PKC (41).

Newly synthesized PKC associated with the cytoskeletal (far left) in a conformation that exposes the activation loop (top right point of active site, which is represented by a gap in C4) and tethers the carboxyl terminus C near the active site. (a) Phosphorylation by a putative PKC kinase on the exposed activation loop (Thr 500) correctly aligns residues for catalysis, allowing autophosphorylation at two carboxyl-terminal positions (Thr 641,Ser 660). Phosphorylation at the first of these (Thr 641) locks the kinase in a catalytically competent conformation, an event that may involve removal of the carboxyl terminus for the active site and insertion, instead of the pseudosubstrate (black rectangle). Subsequent phosphorylation at the second carboxy-terminal site (Ser 660) releases mature protein kinase C into the cytosol. (b) Specific anchoring proteins, whose role is to poise specific PKC isoenzymes at their required sites of action(C), may regulate isoenzyme-specific targeting of mature PKC. Binding of DAG or TPA to C1 domain and Ca^{2+} may or may not require, or be facilitated by step (b). Although PKC can bind to membranes by either the C1 or the C2 domain alone, both must be membrane-bound for the high-affinity interaction that results in pseudosubstrate release.

The role 35 kDa protein in gene regulation has been considered (91-92). Therfore this study combined with preliminary information can suggests that activation of PKCs is usually associated with their translocation from the cytosol to the membrane. Concomitantly, there is cleavage of the catalytic domain from the regulatory domain by proteinase which may occur at the border of the hinge region and catalytic domain. Then activated PKC (catalytic domain) functions at the membrane or may translocate to specific targets such as the nucleus to function (93, 94). However, exactly how PKC translocates to specific targets has not yet been clearly understood. However, it has been reported that eventually, the activated PKC undergoes further breakdown. Thus, it could be observed that continuous exposure to activators lead to activation and down-regulation of PKC and it is possible that either activation or down-regulation is responsible for the biological effects.

Since there was no evidence reported that the subcellular domains and proteolytic degradation sites of isoenzymes expressing in human keratinocytes, then further study it may provide valuable information on the effects of various domains of the isoenzymes on its conformation and sub-cellular localization and proteolytic by utilizing transfected human keratinocytes that over expressed different domain fragments of PKC isoenzymes. An epitope tag may add to the COOH termini of the mutant protein to allow uniform detection by an antibody recognizing the tag peptide. These over-expressed recombinant polypeptides may then be used to identify regions of the molecular possibly involved in determining its sub-cellular distribution and translocation. The finding of the study could be a key component in determining the functional and regulatory specificity of each PKC-isoenzyme in human keratinocytes.

It was found that in human keratinocyte, the roles of PKC -α and ε isoenzymes linked to epidermal differentiation program and carcinogenesis respectively (10-12). However, it has been reported that the other crucial role of PKC isoenzyme in human

keraticytes is apoptosis. A recent study revealed that in normal human keratinocyte PKC-δ isoenzyme correlated with the induction of apoptosis by UV exposure, which is distinct pathway with TPA activation. Those findings were consistent with this study because this study found that with either short-term TPA treatment (TPA 160 nM,1 and 2 h) or long-term treatment (160 nM,18 h) PKC-δ level remain relative unchanged. Again, study is also consistent with the studying of Nicholas JR. (69), who found that short-term treatment with 50 nM TPA, PKC-δ as well as PKC-ζ isoenzyme were unchanged and were still present even following long treatment (18 h). The elimination of ultraviolet (UV) radiation-damaged keratinocytes via apoptosis, an important mechanism for the protection of the skin from sunlight. This additional information provides the other specific and pivotal role of PKC isoenzyme in human keratinocytes.

Since this study found that PKC-α and ε isoenzymes are the molecular targets of TPA in human keratinocytes therefore we decided to strongly further examine these isoenzymes. However we eventually devoted our focus to PKC-ε only given that previous studies had shown that in human keratinocytes PKC-α isoenzyme was involved with the differentiation program at higher concentration of calcium (1.2 mM) (61). But, the concentration of calcium used in this study was 0.09 mM so the differentiation process could be ignored. Therefore, this study further determine only PKC-ε isoenzyme.

This study found that TPA did not affect on the total level of PKC -ε isoenzymes-a fact which may suggest that TPA has no effect on new PKC synthesis, and providing the information that TPA activation is directly involved with the translocation from the cytosol to the membrane.

Curcumin, an the active ingredient of the rhizome of the plant turmeric, is a commonly used spice has been shown to prevent cancer in laboratory animal tumor models. This study showed that curcumin completely inhibited the translocation of PKC-ε isoenzyme induced by TPA (Figure 25, Table11) in a dose dependent manner. However, there was a slight difference between 40 and 50 μM, which might have resulted from the

action of curcumin depending on the saturation concentration on the TPA receptor site that is C1 domain of PKC. This accords with the findings of Jer Yuh Lin in NIH 3T3 (9) which reported that curcumin also inhibits TPA-induced PKC activity in dose dependent manner. In addition, this study reveals that curcumin at any concentration (20, 40, 50 μM) does not affect the translocation or down-regulation of PKC-ε isoenzyme (Figure 23, 24 ; Table 10). Therefore, curcumin has no mitogenic activity on human keratinocytes, unlike the TPA actions.

Moreover it was found that TPA induced translocation of PKC-ε isoenzyme was inhibited only in case of pretreatment with curcumin (Figure 27, 28 Table 12). Co- or post-treatment was not affected. This result was consistent with the known effect of curcumin on NF-κB activation (70) which was also found that the inhibitory effect of curcumin was accomplished only in case of pre-treatment, whereas co- or post- did not. This result, combined with the characteristic structure of curcumin which is exceedingly nonpolar, suggests that curcumin may be inserted into membrane and bound to cytosolic PKC at C1 domain. It would then inhibit the translocation of PKC-ε isoenzyme by filling the TPA binding site on the PKC molecule. However, this study was showed that the affinity of TPA is much higher than curcumin, and therefore the inhibition effect was observed only in pretreatment.

This proposal was consistent with the results from the experiment of Lui and Lin (9) which showed that the inhibitory effect of curcumin could be reduced by the presence of mercaptoethanol (20 mM) or dithiothretol (1mM). Accordingly, the penetration of curcumin may assure by the result of curcumin on aryl hydrocarbon receptor and cytochrome P450 1A1A in MCF-1 human breast carcinoma which was found that curcumin was able to compete with the prototypical AhR ligand 2,3,7,8-tetracholorodibenzo-p-dioxin for binding to the AhR which was normally found in the cytosol (95,96). However this proposal should be proved in the further study by using specific tag to follow the localization of curcumin.

In conclusion, we could rather suggested that curcumin is the potent inhibitor of PKC- ε isoenzyme and that combined with previous data, it could extend the model for TPA -induced fos expression in carcinogenesis model by PKC- ε isoenzyme activation as shown in the Figure 30. However, it should be proved the inhibitory effect of curcumin on PKC- ε isoenzyme activation directly *in vivo*, i.e., to examine the inhibitory effect of curcumin on tumor development in nude mice which are subcutaneously inoculated with curcumin and human keratinocytes overexpressing PKC-ε isoenzyme.

Since the finding of this study shows only the earlier signal in signal transduction cascade, then in order to complete the signal cascade which resulted the activation of PKC-ε isoenzyme through *c-fos* oncogene expression or cell proliferation, further experimentation should be done to investigate the downstream cascade of this regulation pathway such as raf and MAPK pathway or what changes the activator to others which has most profound effect on keratinocyte proliferation such as KGF (Keratinocyte growth factor). This is in part because recently, it has been demonstrated that activation of involucrin expression which is a marker of keratinocyte terminal differentiation, involves a pathways that includes PKC-α isoenzyme, Ras, EKK1, MEK3 and p38/RK. The findings will demonstrate that KGF involved with keratinocyte proliferation via PKC-ε isoenzyme activation. Moreover, the study further points to the signal cascade which is responsible for proliferation regulation such as ERK pathway (Raf, MEK1, 2 and ERK 1, 2). In addition, other food phytochemicals which also have almost universal distribution in food and are phenolic compound like curcumin ; for instances, flavonoids (quercetin, rutin, etc.), tannins (ellagic acid and its more complex cousins), catechins, and other simple phenols, such as chlorogenic acid, should be studied.

Yet, additional studies are still required to shed more light on the mechanism which causes inhibition on other PKC isoenzymes such as PKC δ. But given that UV directly activates it, then the activator should be UVA or UV B, after which one would have to identify whether curcumin inhibited this effect. The finding may provides crucial information

for using curcumin in the protection of the skin from UV or sunburn.

Furthermore, one of the most interesting study is to determine the specific antisense of PKC-isoenzymes, which modulate drug resistance, given that there is increasing evidence that PKC is involved in drug resistance and that modulation of PKC activity may increase the cytotoxicity of number of commonly used anticancer drugs (97-98). A number of genes implicated in drug resistance (e.g. glutathione transferase and metallothioneins) have been shown to have TPA responsive elements in their promoters and are induced by TPA, cell stress and toxic agents. Preliminary evidence suggests that cytotoxic drugs may induce drug resistance by the activation of TPA responsive elements, a process that may be prevented by PKC inhibitors. However, one of the best studied mechanism for drug resistance with respect to PKC activity is multidrug (MDR), a phenomenon by which cells become resistant to several classes of structurally and functionally diverse drugs after exposure to a single cytotoxic agent. One form of MDR is associated with the overexertion of a 170-kDa plasma membrane phosphoglycoprotein called the p-glycoprotein, which is encoded by the mdr-I gene and is an energy-dependent membrane pump that reduces the accumulation of drugs inside the cell. It has been reported that PKC-mediated induction of MDR could be part of general stress response to different types of cellular damage (97). Therefore, modulation of PKC activity may be part of treatment strategies that would prevent the emergence of drug resistance during cancer therapy with cytotoxic drugs.

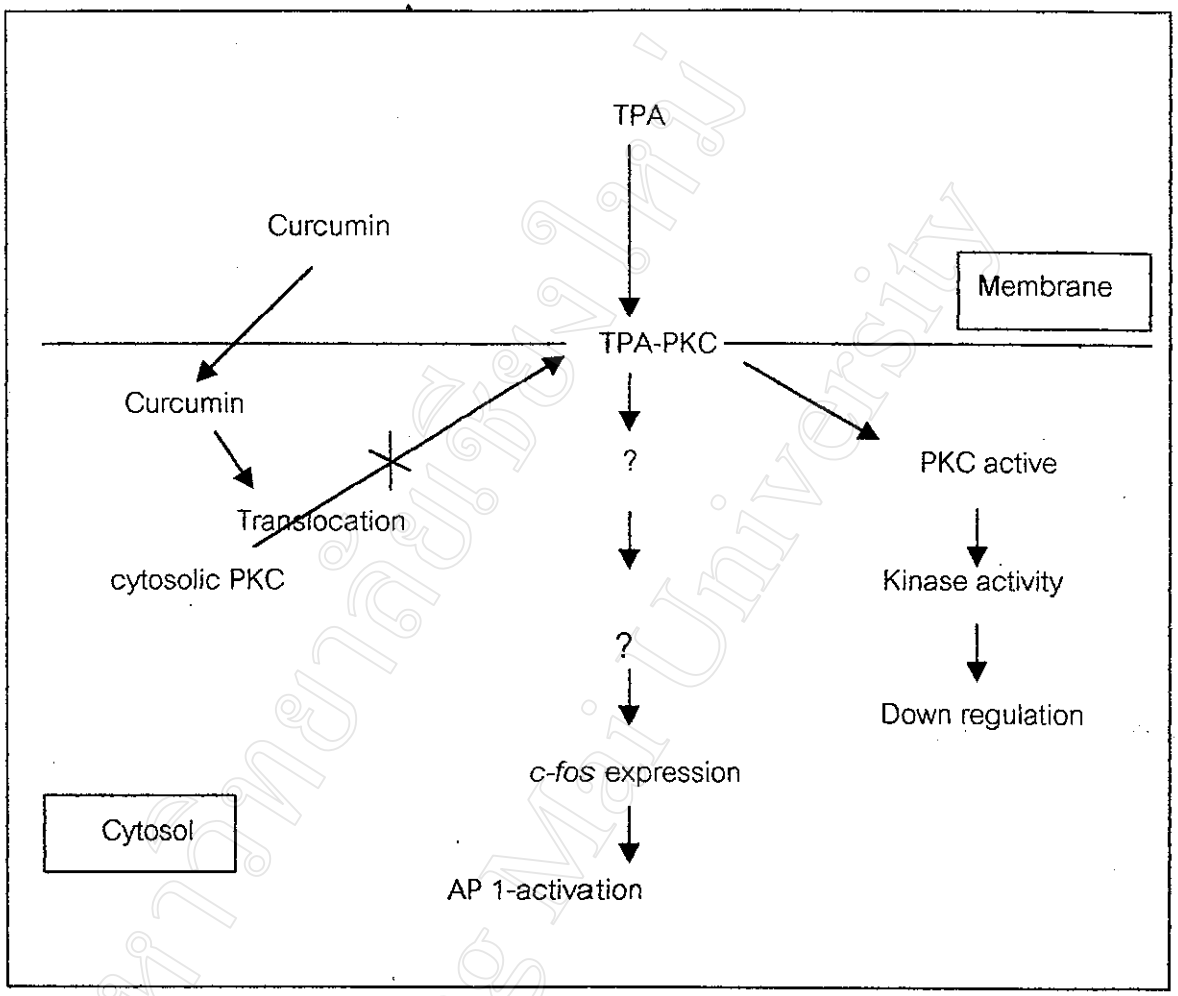

Figure 30. Model for TPA-induced fos expression in carcinogenesis and the possilble site of curcumin action