CHAPTER III
RESULTS

3.1 Effect of TPA on level and distribution of PKC-alpha, delta and epsilon isoenzymes in human keratinocytes

PKC isoenzymes activation is associated with translocation from the cytosol to the membrane and it was found that human keratinocytes expressed PKC-α, -δ, -ε, -η and ζ isoenzymes. Therefore, the translocation of PKC-α, -δ and -ε isoenzymes which are TPA-responsive isotypes were evaluated in this study. The 80% confluent human keratinocytes ($3x10^6$ cells/ml) from 3 subjects were incubated for 1 h with 160 nM TPA for 0,1,2 and 18 h, then cytosolic (C) and the membrane (M) fractions were analyzed as described in the Materials and Methods. Figures 15, 17 and 19 show the Western blot analysis of PKC-α (82 KD), -δ (76 KD) and -ε (93 KD) isoenzymes level using isoenzyme-specific monoclonal and polyclonal antibodies. Tables 6, 7 and 8 reveal the densitometric analysis of developed bands which represent the level of PKC-isoenzymes -α, -δ and -ε respectively. The relative levels of PKC in the cytosolic and membrane fractions are also represented as histogram pictures (Figure 16,18,20). The keratinocytes were treated with TPA for 1 and 2 h, about 70-85 % of PKC -α and -ε isoenzymes were decreased from the cytosol associated with increased in the membrane. They were indicated that TPA induced translocation of PKC-α and -ε isoenzymes from the cytosol to the membrane in human keratinocytes. However, TPA treated cells for 18 h, induced complete downregulation (i.e., loss >99%) of PKC- α and -ε . For PKC- δ, it was found that no significant reproducible changes in either cytosolic and membrane fraction in response of TPA-treated cells (Figure 17, 18 and Table 7). Therefore from this experiment it can be concluded that in human keratinocytes there were two PKC isoenzymes, α and ε, which were activated by 160 nM TPA, and demonstrated the specificity of PKC-isoenzyme in response to TPA.

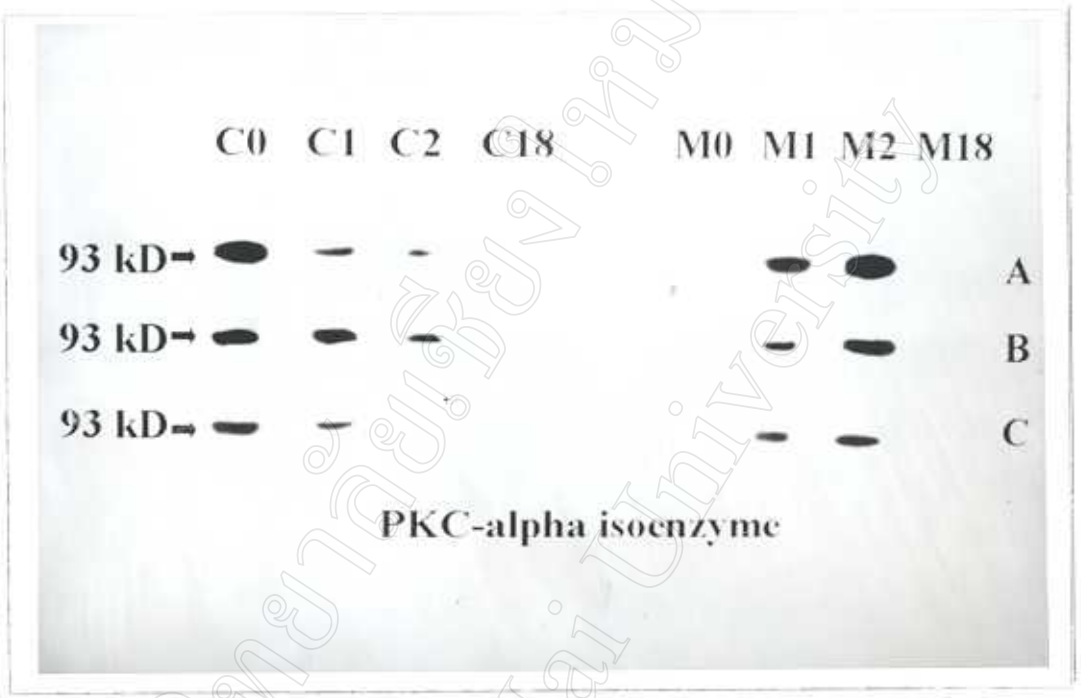


Figure 15. Western blot analysis of PKC-alpha isoenzyme in human keratinocytes in response to TPA. The Figure represents Western blot analysis of PKC-α protein expression using anti PKC-α mouse monoclonal antibody. The cytosolic (C) and membrane (M) fractions (30 μg protein/35 μl) were obtained from 3 subjects. A, Abdominal skin (cesarean) 27 years old; B, abdominal skin (cesarean) 22 years old and C, juvenile foreskin (circumcision) 4 years old were subjected to SDS-PAGE and immunoblotting as described in the Materials and Methods. Co and Mo, cytosolic and membrane fractions of DMSO-treated cells (control) ;C1 and M1, C2 and M2, C18 and M18, cytosolic and membrane fractions of TPA-treated cells for 1, 2 and 18 h respectively.

Table 6. Densitometric analysis of the Western blots shown in Figure15.

Samples	Specific activity [a] (arbitrary units/ 30 μg protein)			Relative PKC level (%)[b]
	Subject 1	Subject 2	Subject 3	
Co	23.07	23.01	22.14	100.00
C1	7.66	9.41	2.36	28.49
C2	2.35	7.52	0.08	14.57
C18	0.01	0.03	0.03	0.11
Mo	0.03	0.15	0.01	0.28
M1	16.09	14.53	20.02	74.23
M2	21.14	17.29	22.09	88.70
M18	0.06	0.03	0.04	0.18

[a] : Values were obtained by the densitometry of the Western blots shown in Figure15

[b] : Values are relative to cytosolic PKC-alpha of control cells (C0) which were set to a value of 100, and are representative of three experiments that yielded similar results

C0, M0 : Cytosolic and membrane fractions of DMSO-treated cells (control)

C1, C2, C18 : Cytosolic fractions of 160 nM TPA-treated cells for 1,2 and 18 h respectively

M1, M2, M18 : Membrane fractions of 160 nM TPA-treated cells for 1,2 and 18 h respectively

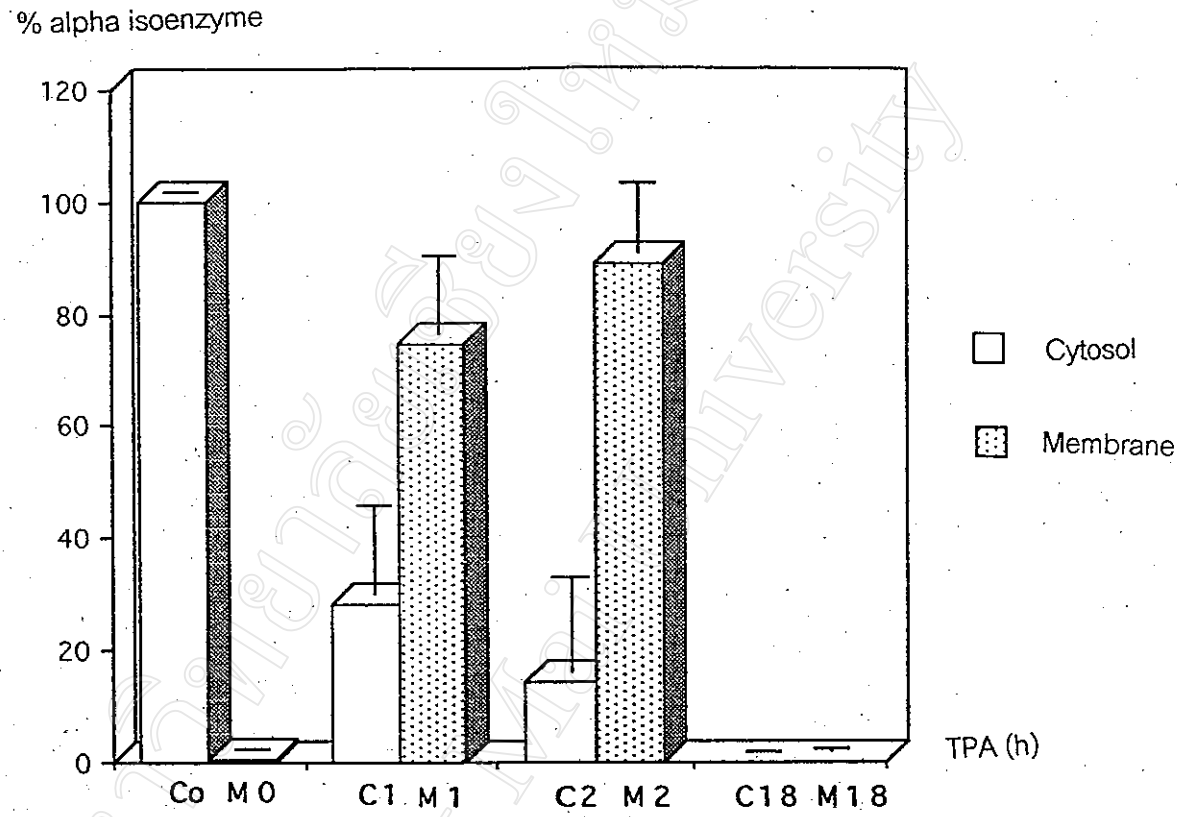


Figure 16. Translocation and down regulation of PKC-alpha in human keratinocytes.

The histogram represents the relative levels of PKC-alpha in 30 µg protein/35 µl of cytosolic (C) and membrane (M) fractions which were set the control cells (Co) to a value of 100 (Table 6). Co and Mo, cytosolic and membrane fractions of DMSO-treated cells (control) ; C1 and M1, C2 and M2, C18 and M18, cytosolic and membrane fractions of TPA-treated cells for 1, 2 and 18 h respectively.

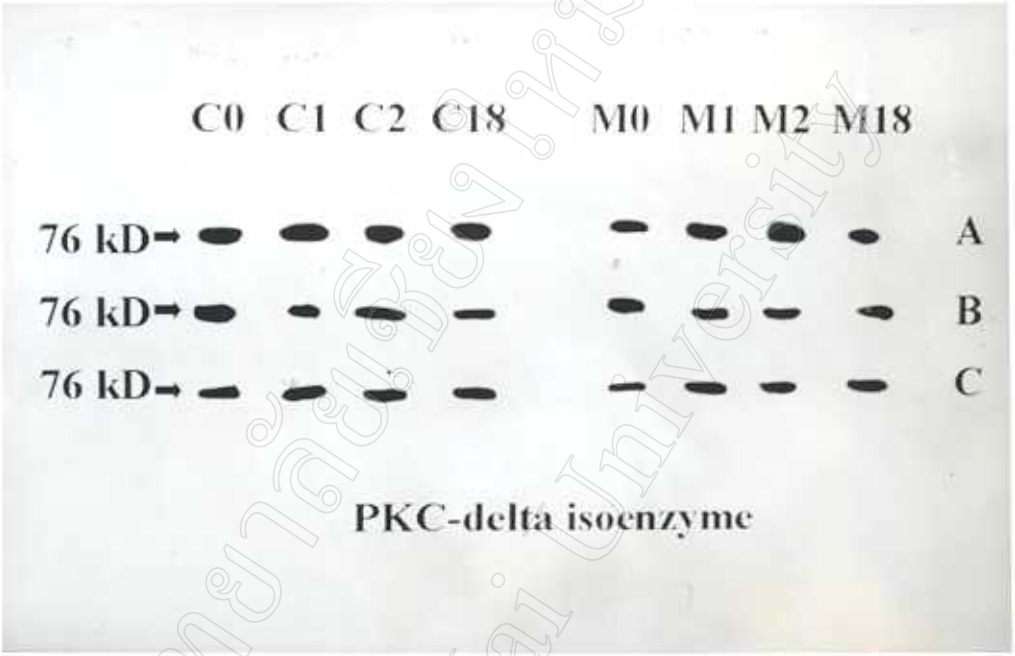


Figure 17. Western blot analysis of PKC-delta in human keratinocytes in response to TPA. The Figure shows Western blot analysis of PKC-δ isoenzyme expression using anti PKC-δ rabbit polyclonal antibody. The cytosolic (C) and membrane (M) fractions (30 μg protein/35 μl) were obtained from 3 subjects. A, Abdominal skin (cesarean) 27 years old ;B, abdominal skin (cesarean) 22 years old and C, juvenile foreskin (circumcision) 4 years old were subjected to SDS-PAGE and immunoblotting as described in the Materials and Methods. Co and Mo, cytosolic and membrane fractions of DMSO-treated cells (control) ; C1 and M1, C2 and M2, C18 and M18, cytosolic and membrane fractions of TPA-treated cells for 1, 2 and 18 h respectively.

Table 7. Densitometric analysis of the Western blots shown in Figure 17.

Table 7. Densitometric analysis of the Western blots shown in Figure 17.

samples	Specific activity [a] (arbitrary units/ 30μg protein)			Relative PKC levels (%)[b]
	Subject 1	Subject 2	Subject 3	
Co	21.59	19.76	19.04	100.00
C1	22.64	18.65	20.94	104.71
C2	21.80	19.96	20.77	103.55
C18	22.59	20.00	18.72	101.95
Mo	23.59	20.09	17.83	100.04
M1	22.63	19.20	18.23	102.52
M2	23.59	19.80	18.81	100.40
M18	23.94	19.59	19.05	103.34

[a] : Values were obtained by the densitometry of the Western blots shown in Figure17

[b] : Values are relative to cytosolic PKC-delta of control cells (C0) which were set to a value of 100, and are representative of three experiments that yielded similar results

C0, M0 : Cytosolic and membrane fractions of DMSO-treated cells (control)

C1, C2, C18 : Cytosolic fractions of 160 nM TPA-treated cells for 1, 2 and 18 h respectively

M1, M2, M18 : Membrane fractions of 160 nM TPA-treated cells for 1, 2 and 18 h respectively

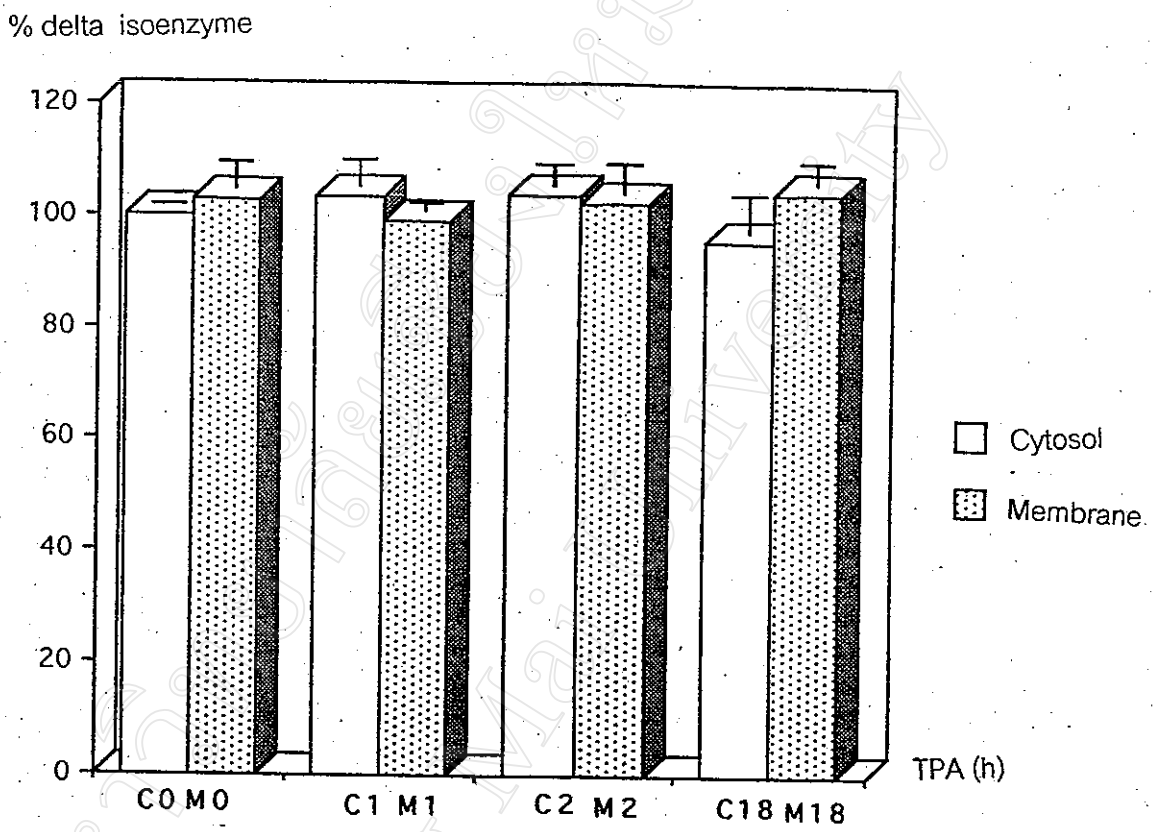


Figure 18. Effect of TPA on level and distribution of PKC-delta in human keratinocytes. The histogram represents the relative levels of PKC-delta in 30 μg protein/35 μl of cytosolic (C) and membrane (M) fractions which were set the control cells (Co) to a value of 100 (Table 6). Co and Mo, cytosolic and membrane fractions of DMSO-treated cells (control); C1 and M1, C2 and M2, C18 and M18, cytosolic and membrane fractions of TPA-treated cells for 1, 2 and 18 h respectively.

Figure 19. Western blot analysis of PKC-epsilon in human keratinocytes in response to TPA. The Figure represents Western blot analysis of PKC-epsilon isoenzyme expression using anti PKC-ε rabbit polyclonal antibody. The cytosolic (C) and membrane (M) fractions (30μg protein/35 μl) were obtained from 3 subjects. A, Abdominal skin (cesarean) 27 years old; B, abdominal skin (cesarean) 22 years old and C, juvenile foreskin (circumcision) 4 years old were subjected to SDS-PAGE and immunoblotting as described in the Materials and Methods. Co and Mo, cytosolic and membrane fractions of DMSO-treated cells (control); C1 and M1, C2 and M2, C18 and M18, cytosolic and membrane fractions of TPA-treated cells for 1, 2 and 18 h respectively.

Table8. Densitometric analysis of the Western blots shown in Figure 19.

Samples	Specific activity [a] (arbitrary units/ 30µg protein)			Relative PKC levels
	Subject 1	Subject 2	Subject 3	(%)[b]
Co	23.19	20.17	22.99	100.00
C1	6.84	10.54	2.72	30.28
C2	2.35	7.43	1.41	16.87
C18	0.02	0.03	0.04	0.14
Mo	0.03	0.02	0.01	0.09
M1	16.15	10.12	19.64	69.18
M2	20.95	12.72	21.87	83.69
M18	0.03	0.03	0.02	0.12

[a] : Values were obtained by the densitometry of the Western blots shown in Figure 19

[b] : Values are relative to cytosolic PKC-epsilon of control cells (C0) which were set to a value of 100, and are representative of three experiments that yielded similar results

C0, M0 : Cytosolic and membrane fractions of DMSO-treated cells (control)

C1, C2, C18 : Cytosolic fractions of 160 nM TPA-treated cells for 1, 2 and 18 h respectively

M1, M2, M18 : Membrane fractions of 160 nM TPA-treated cells for 1, 2 and 18 h respectively

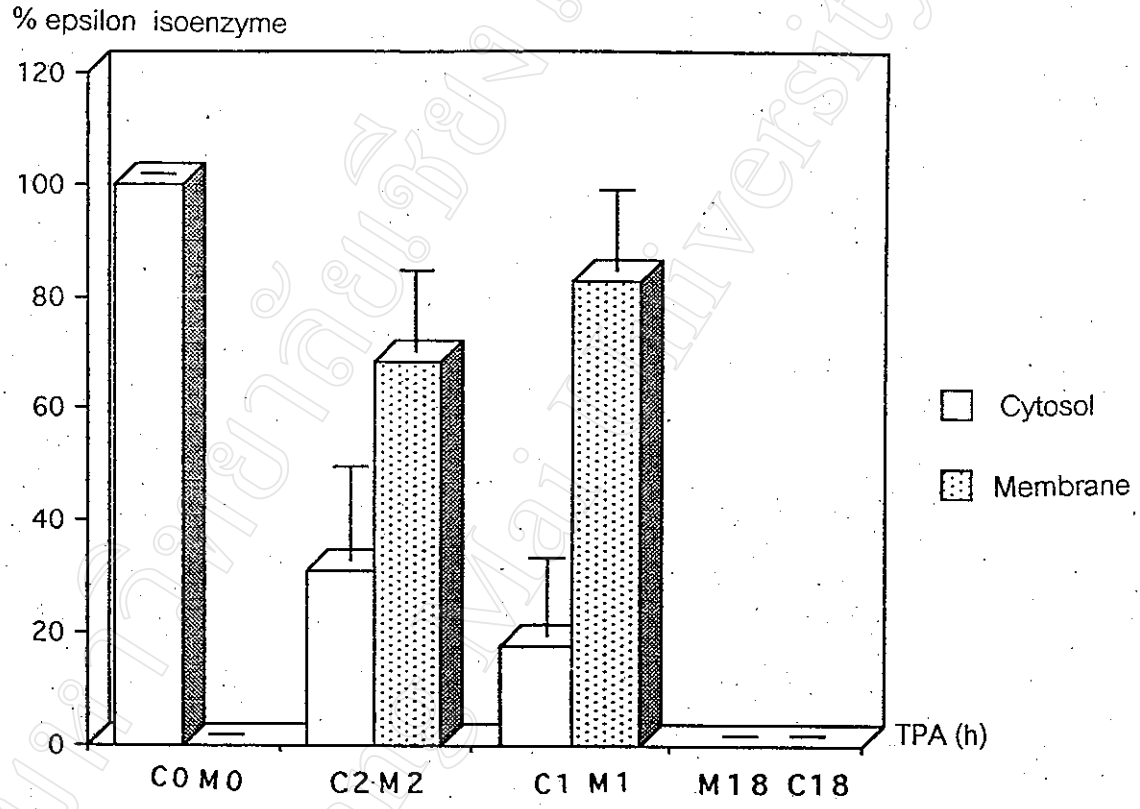


Figure 20. Effect of TPA on level and distribution of PKC-epsilon in human keratinocytes. The histogram represents the relative levels of PKC-epsilon in 30 μg protein/35 μl of cytosolic (C) and membrane (M) fractions which were set the control cells (Co) to a value of 100 (Table 6). Co and Mo, cytosolic and membrane fractions of DMSO-treated cells (control); C1 and M1, C2 and M2, C18 and M18, cytosolic and membrane fractions of TPA-treated cells for 1, 2 and 18 h respectively.

3.2 Effect of TPA on the enzyme content of the PKC-epsilon in human keratinocytes

The activation of TPA may increase the total enzyme content of PKC-epsilon isoenzyme in cytosolic or membrane fractions. To examine this possibility, cytosolic and membrane fractions from 80% confluent human keratinocytes ($3x10^6$ cells/ml) treated with 160 nM TPA for 0,1,2 and 18 h were pooled and then assayed by Western blot .The results which are represent total content of the PKC-epsilon are indicated in Figure 21.No significant difference among the band intensities of the control and TPA treated keratinocytes were observed. They were consistent with the densitometric analysis, Table 9, and the histogram Figure 22. However, TPA treatment for 18 h induced completely down regulation of PKC-epsilon. Thus this study indicated that TPA did not change the total level of PKC-epsilon in human keratinocytes.

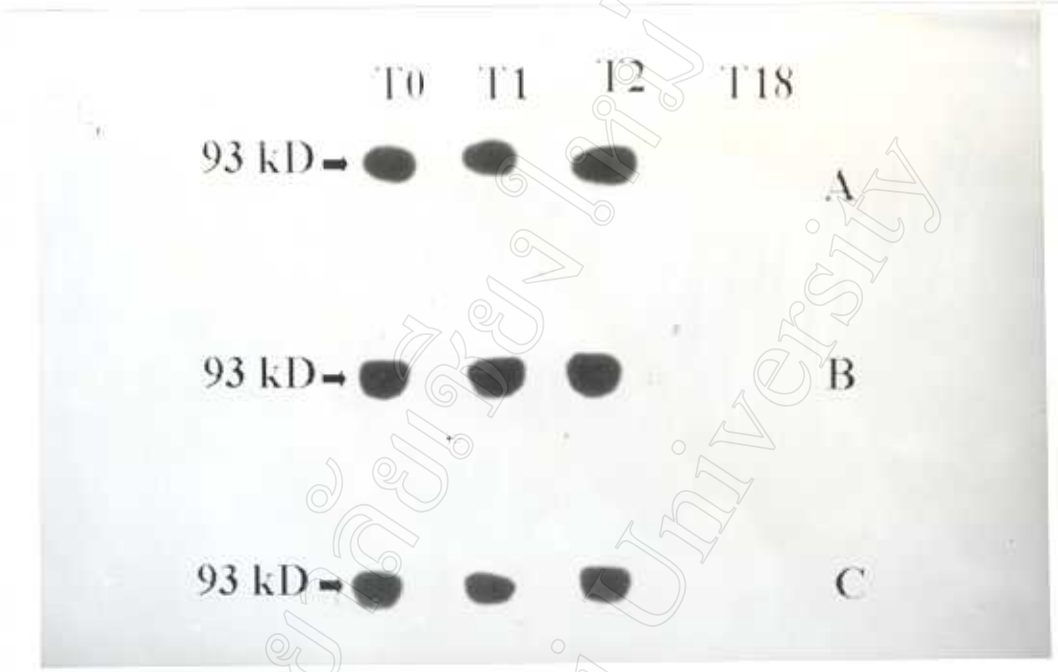


Figure 21. Western blot analysis of total level of PKC-epsilon in human keratinocyte in response to TPA. The cytosolic and membrane fractions (30 μg protein/35 μl) were obtained from 3 subjects of keratinocytes treated with 160 nM TPA for 1, 2 and 18 h were pooled and then assayed for PKC-epsilon isoenzyme by Western blot as described in Materials and Methods. A, B, Abdominal skin (cesarean) 27 and 22 years old. C, juvenile foreskin (circumcision) 4 years old. T0, total fraction (cytosol + membrane) of DMSO-treated cell (control) ; T1,T2 and T18, total fractions (cytosol + membrane) of 160 nM TPA-treated cells for 1, 2 and 18 h respectively.

Table 9. Densitometric analysis of the Western blots shown in Figure 21.

	Sample	Specific activity [a] (arbitrary units/ 30μg protein)			Relative PKC (%)[b]
		Subject 1	Subject 2	Subject 3	
PKC-ε isoenzyme	T0	20.86	23.31	19.86	100
	T1	19.25	22.67	18.18	93.87
	T2	19.46	23.25	19.27	96.90
	T18	0.47	0.08	3.06	5.62

[a] : Values were obtained by the densitometry of the Western blots shown in Figure 21.

[b] : Values are relative to total fraction of control cells (T0) which were set to a value of 100 and are representative of three experiments that yielded similar results.

T0 : Total fractions of cytosolic and membrane of DMSO –treated cells (control)

T1,T2,T 18 : Total fractions of cytosolic and membrane of TPA -treated cells for 1, 2 and 18 h.

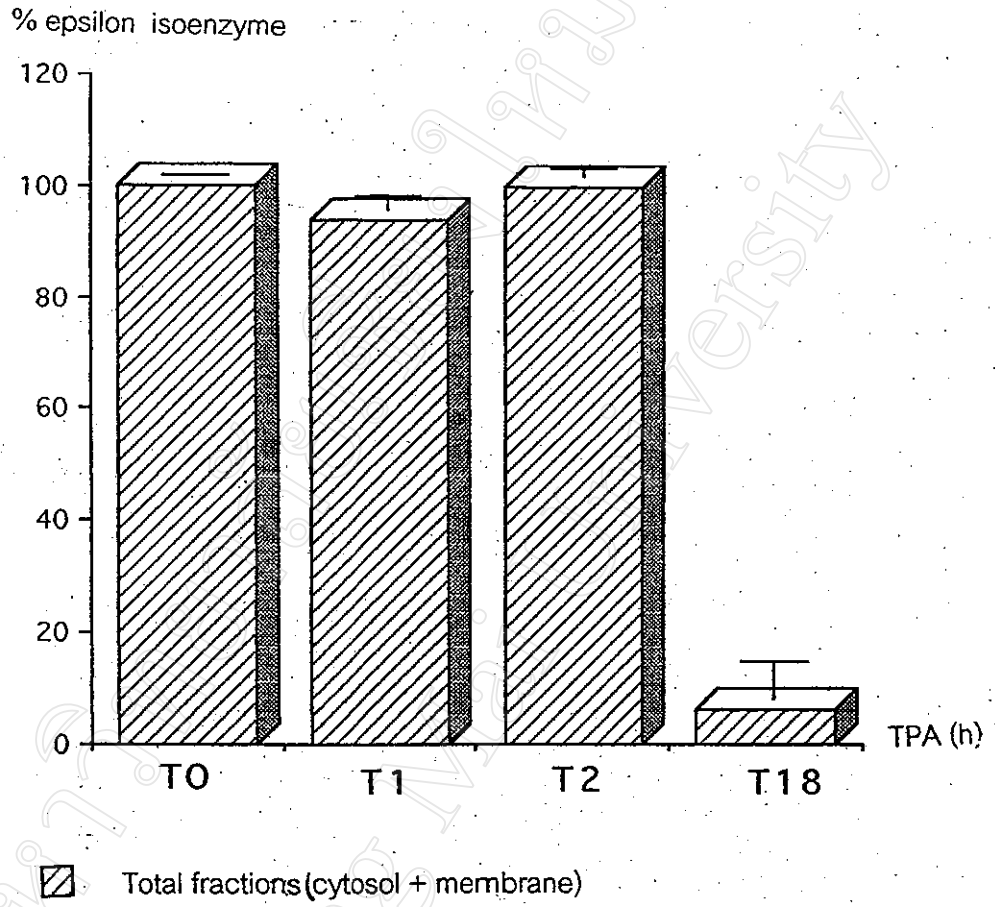


Figure 22. Effect of TPA on total level of PKC-epsilon content in human keratinocytes. The histogram shows the densitometric analysis of 30 μg protein/35μl of cytosolic and membrane fractions of keratinocytes treated with 160 nM TPA for 1, 2 and 18 h which were set T0, DMSO-treated cell (control), to a value of 100 (Table 9). T0, total fraction (cytosol + membrane) of DMSO-treated cell (control) ; T1,T2 and T18, total fractions (cytosol + membrane) of 160 nM TPA-treated cells for 1, 2 and 18 h respectively.

3.3 Effect of curcumin on level and distribution of PKC-epsilon in human keratinocytes

To explore whether curcumin itself affects on level and distribution of PKC -ε in human keratinocytes, three subjects of human keratinocytes which were obtained from abdominal skin (cesarean) 22 (A), 24 (B) and 29 (C) years old were incubated at 37 °C for 1 h at different concentration of curcumin (0,20,40,50 μM). It was found that only cytosolic fractions were detected the bands and there were no significant differences among control and curcumin treated cells (Figure23). The result of the band intensities were correlated with the densitometric analysis (Table10) and the relative level of PKC as shown in the histogram Figure 24. Moreover, there were no found PKC -ε isoenzyme bands detected in the membrane fractions. Thus, it can be suggested that curcumin itself did not affect or induce translocation of PKC -ε isoenzyme from the cytosol to the membrane in human keratinocytes.

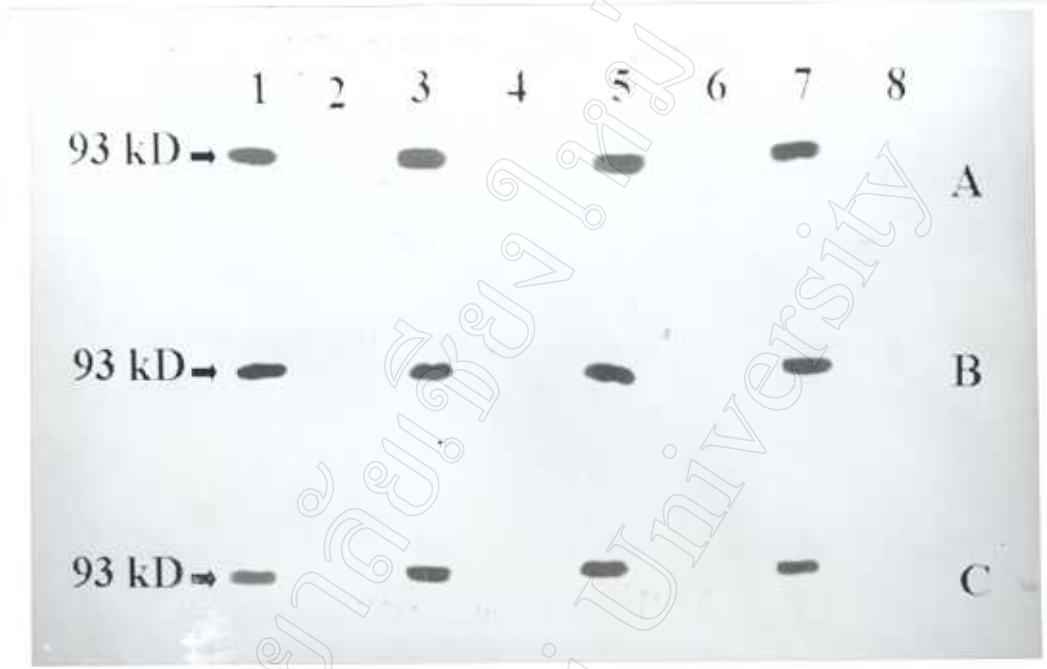


Figure 23. Western blot analysis of PKC-epsilon in human keratinocytes in response to curcumin. Human keratinocytes preparing from 3 subjects, abdominal skin (cesarean) 22 (A), 24 (B) and 29 (C) years old were incubated at 37 °C for 1 h with curcumin 0, 20, 40 and 50 μM. Then 30 μg protein/35 μl of cytosolic (C) and membrane (M) fractions were analyzed for PKC-epsilon isoenzymes by SDS-PAGE and electroblotted onto nitrocellulose paper as described in Materials and Methods. Lane 1, 3, 5, 7; cytosolic fractions. Lane 2, 4, 6, 8; membrane fractions. 1, 2; cytosolic and membrane fractions of DMSO-treated cells (control). 3, 4;cytosolic and membrane fractions of 20 μM curcumin -treated cells. 5, 6; cytosolic and membrane fractions of 40 μM curcumin-treated cells. 7, 8; cytosolic and membrane fractions of 50 μM curcumin -treated cells.

Table 10. Densitometric analysis of the Western blots shown in Figure 23.

Samples	Specific activity [a] (arbitrary units/ 30μg protein)			Relative PKC level (%)[b]
	Subject 1	Subject 2	Subject 3	
C0	21.27	18.96	20.14	100
C20	21.77	17.50	20.05	97.81
C40	21.57	18.22	19.58	97.87
C50	20.32	17.66	20.00	95.59
M0	0.02	0.03	0.03	0.12
M20	0.01	1.54	0.43	3.28
M40	0.03	0.79	0.06	1.45
M50	1.30	1.12	0.21	4.33

[a] : Values were obtained by the densitometry of the Western blots shown in Figure23

[b] : Values are relative to cytosolic PKC-epsilon of control cells (C0) which were set to a value of 100, and are representative of three experiments that yielded similar result

C0,M0 : Cytosolic and membrane fractions of DMSO treated cells (Control)

C20,C40,C50 : Cytosolic fractions of curcumin -treated cells for 20, 40 and 50 μM respectively

M20,M40,M50 : Membrane fracion of curcumin-treated cells for 20, 40 and 50 μM respectively

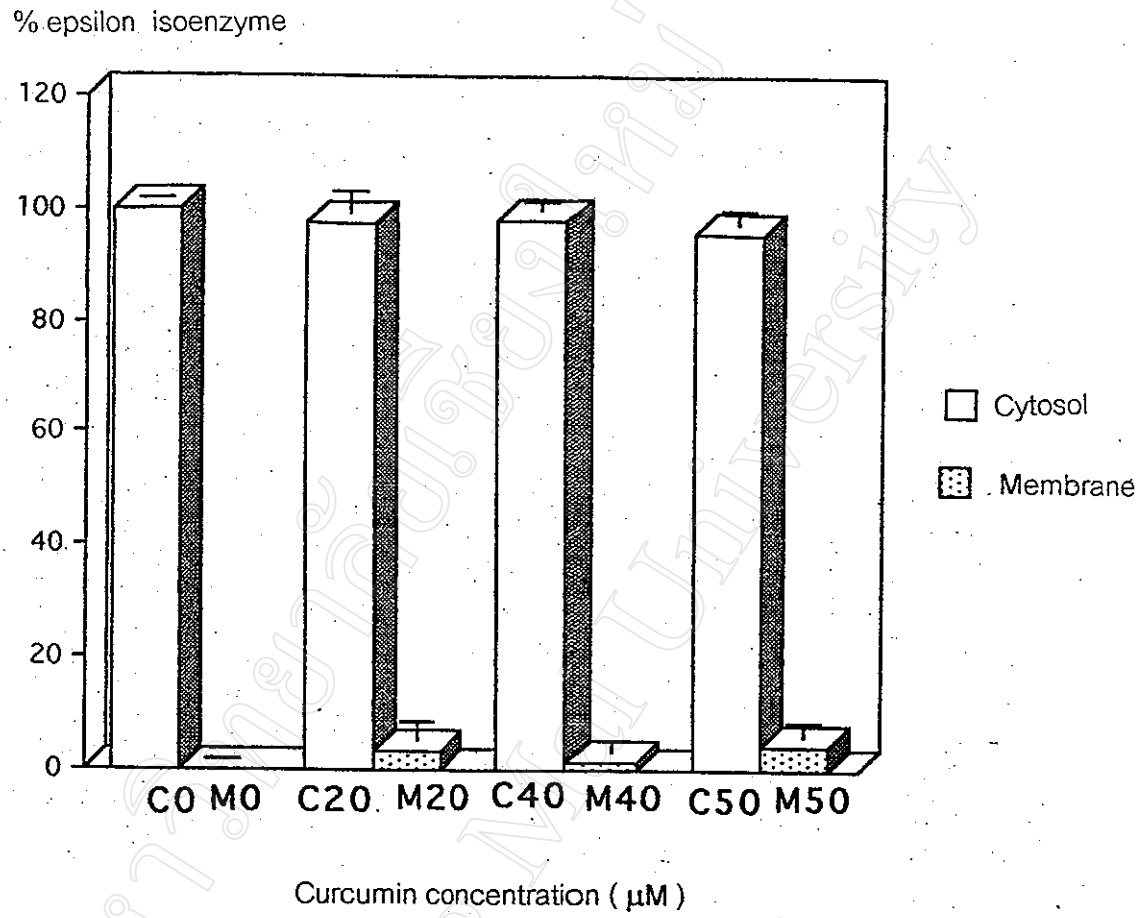


Figure 24. Effect of curcumin on level and distribution of PKC-epsilon in human keratinocytes. The historam represents relative levels of PKC-epsilon which were set the control cells (Co) to a value of 100 (Table 10). C0, M0 : cytosolic and membrane fractions of DMSO treated cells (control). C20, C40, C50: cytosolic fractions of curcumin -treated cells for 20,40 and 50 μM. M20, M40, M50 : membrane fracions of curcumin-treated cells for 20,40 and 50 μM respectively.

3.4 Effect of curcumin on level and distribution of TPA -induced PKC-epsilon in human keratinocytes.

Curcumin was reported to inhibit 98 % of TPA-induced tumor promotion on mouse skin. However the molecular mechanism of this inhibition remained to be explored. In addition, it has been reported that the tumor-promoting ability of TPA lay in its ability to bind to and activate the PKC. Therefore this study was examined the effect of curcumin on level and distribution of TPA-induced PKC-epsilon and roughly determined the molecular mechanism of curcumin in its effect on TPA-induced PKC epsilon in human keratinocytes which is not yet been reported from other studies.

Figure 25. demonstrates the Western blot analysis of PKC-epsilon level in cytosolic and membrane fractions of curcumin and/or TPA treated cell. The 93 kDa protein was detected in the presence of PKC epsilon. The band intensity of cytosolic control fraction is (lane 1) higher than cytosolic TPA-treated cells fraction (lane 2) which associated with lower intensity is found in the membrane fractions of the control than the TPA-treated cells (lane 6 versus 7). Therefore, TPA induced translocation of PKC epsilon from the cytosol to the membrane fraction (Figure 25, 26, Table 11). However, preincubation cells with curcumin 20, 40 and 50 μM for 1 h (lane 3-5; cytosolic, 8-10; membrane) decreased the translocation of PKC- epsilon from the cytosol to the membrane induced by TPA (3, 4, 5 versus 2 and 8, 9, 10 versus 7). Thus, this experiment revealed that the translocation of TPA-induced PKC-epsilon from the cytosol to the membrane was inhibited by curcumin in dose dependent manner.

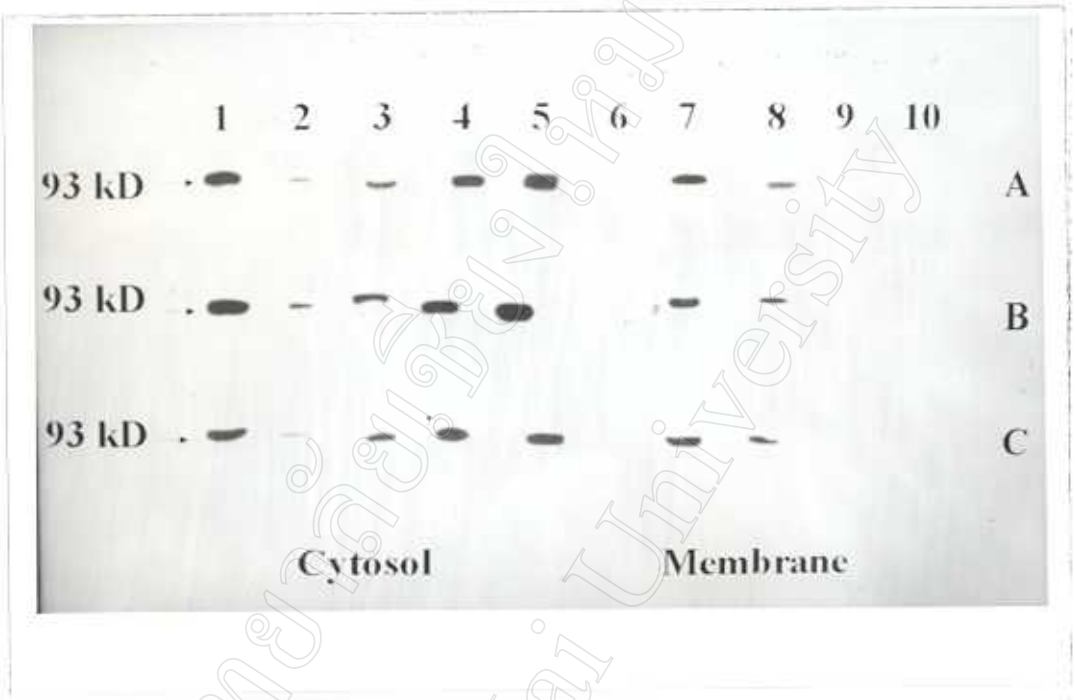


Figure 25. Western blot analysis of PKC-epsilon in human keratinocytes in response to curcumin and/or TPA.The cytosolic (lane 1-5) and membrane fractions (lane 6-10) (30μg protein/35 μl) were obtained from three subjects, A, abdominal skin (cesarean) 22 ; B, 24 and C ,29 years old, were analysed by Western blot as described in Materials and Methods. The blots were examined for immunoreactivity to anti-PKC-ε rabbit polyclonal Antibody. The arrows indicate the dark band of 93 kD of PKC-ε. Lane 1-5 ; cytosolic fractions , lane 6-10 ; membrane fractions.Lane (1, 6) ; control, lane (2,7) ; TPA –tread cells for 1 h ,lane (3, 8) ; preincubated with 20 μM curcumin, lane(4,9) ; preincubated with 40 μM curcumin, lane (5,10) ; preincubated with 50 μM curcumin.

Table 11. Densitometric analysis of the Western blot shown in Figure 25.

Samples	Specific activity [a] (arbitrary units/ 30μg protein)			Relative PKC (%)[b]
	Subject 1	Subject 2	Subject 3	
C0	21.87	22.68	20.16	100.00
C1	6.46	6.70	5.95	29.53
C20	14.72	15.26	13.57	67.29
C40	19.99	20.73	18.43	91.42
C50	21.87	22.68	20.16	99.98
M0	0.00	0.00	0.00	0.01
M1	15.40	15.97	14.20	70.43
M20	7.77	8.05	7.16	35.51
M40	1.95	2.02	1.80	8.92
M50	0.02	0.02	0.02	0.08

[a] : Values were obtained by the densitometry of the Western blots shown in Figure 25

[b] : Values are relative to cytosolic PKC-epsilon of control cells (C0) which were set to a value of 100, and are representative of three experiments that yielded similar result

C0, M0 : Control (DMSO treated cells)

C20, C40, C50 : Cytosolic fractions of curcumin treated cells for 20,40,50 μM

M20, M40, M50 : Membrane fractions of curcumin treated cells for 20,40,50 μM

C1, M1 : Cytosolic and membrane fractions of TPA-treated cells for 1 h

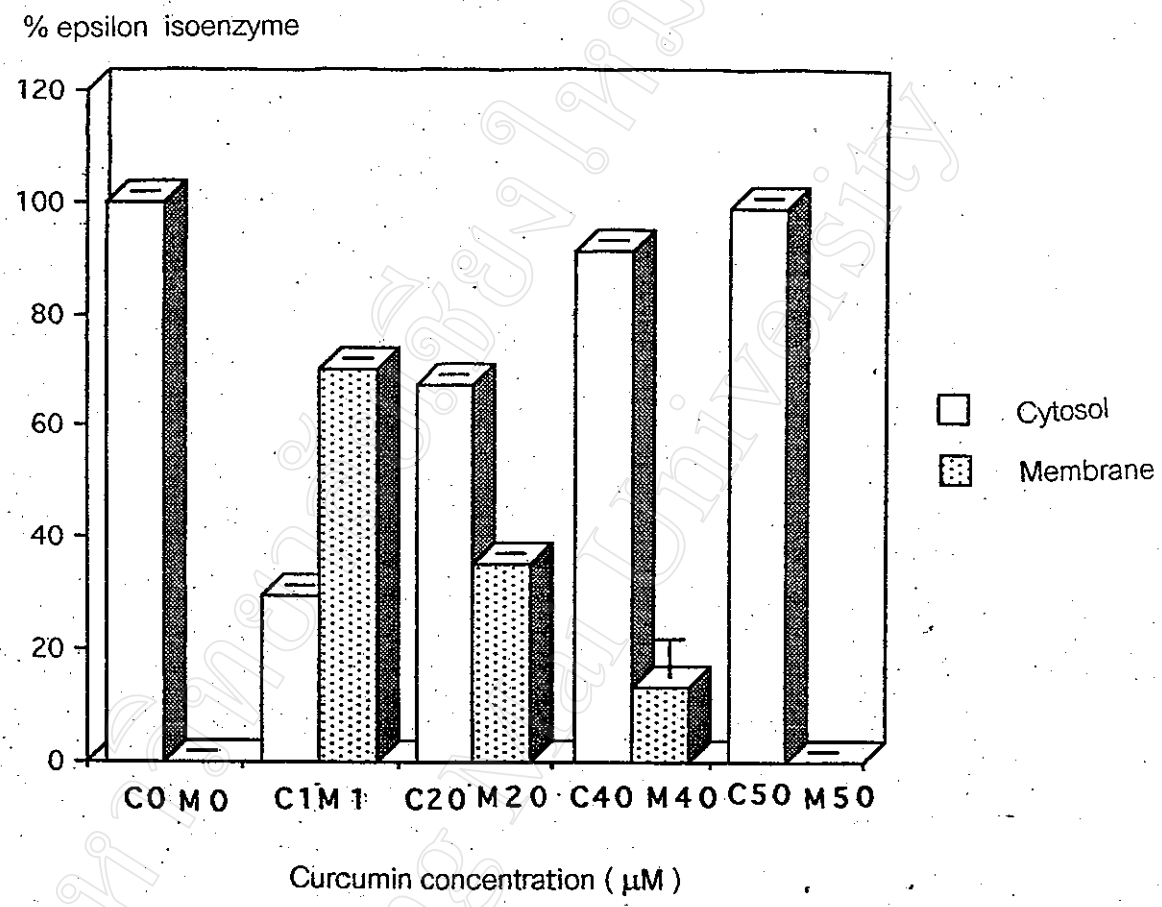
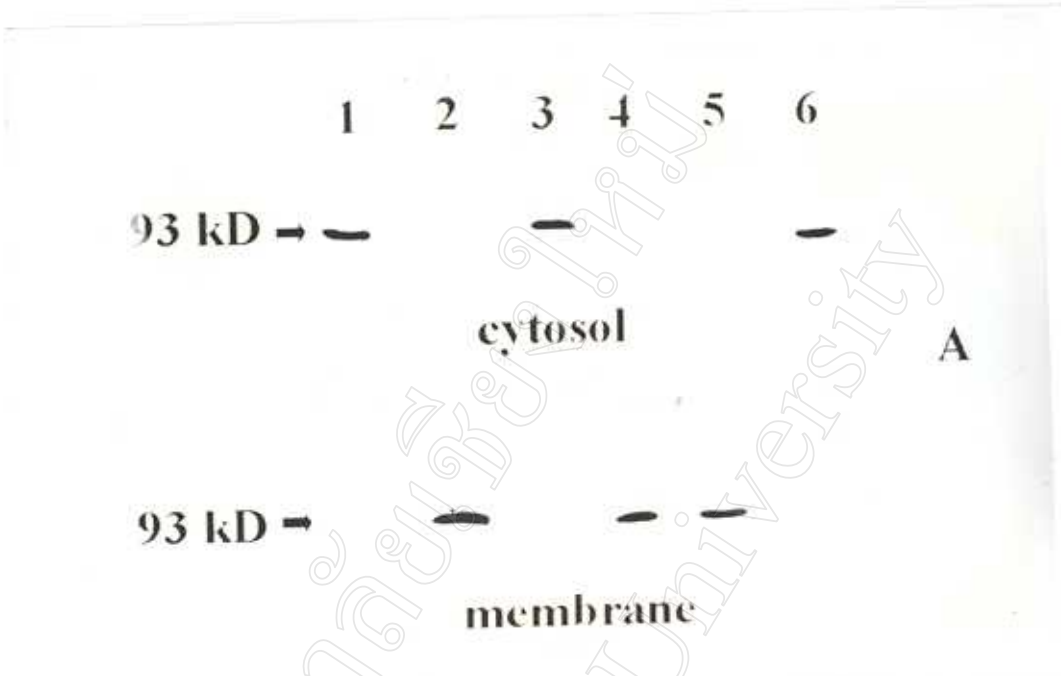
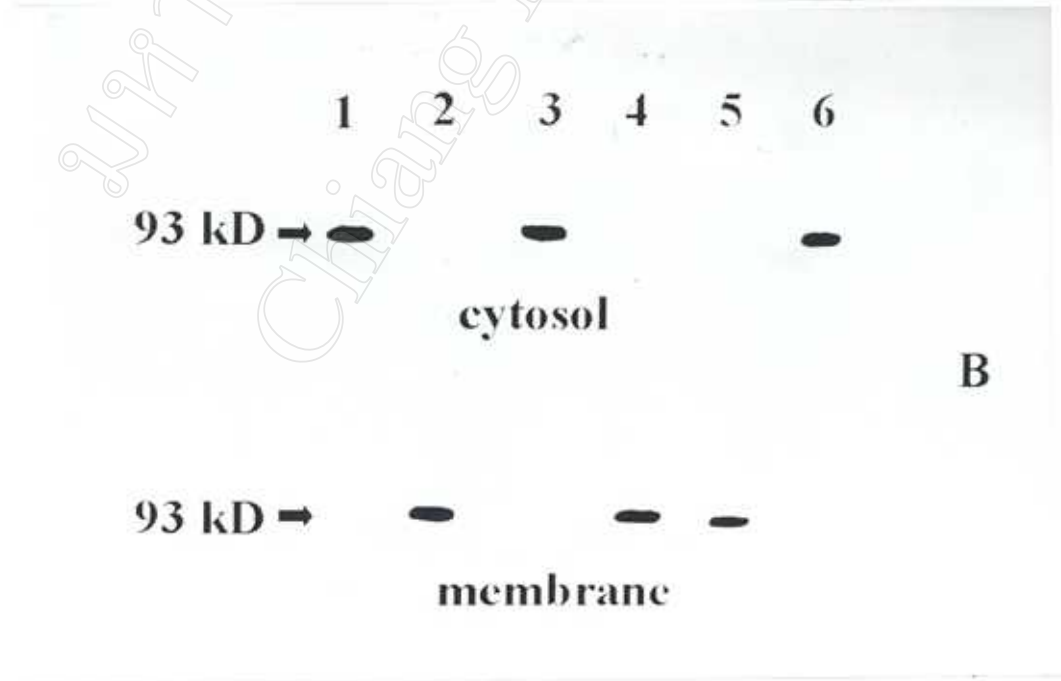


Figure 26. Dose response of inhibition of TPA-induced PKC-epsilon activation by curcumin. The historam represents the relative value of PKC-epsilon which were set the cytosolic fraction of control cells (C0) to a value of 100 (Table 11), C0, M0 : control (DMSO treated cells), C20, C40, C50: cytosolic fractions of curcumin treated cells for 20, 40, 50 μM, M20, M40, M50: membrane fractions of curcumin treated cells for 20, 40, 50 μM, C1, M1: cytosolic and membrane fractions of TPA-treated cells for 1 h.

3.5 Effect of time on curcumin addition on TPA-induced PKC-epsilon in human keratinocyes

It is apparent that the action of curcumin on molecular level is quite complicate because the targets of its action are varied from DNA to RNA and protein level (enzyme level). Accordingly, this study roughly examined the molecular inhibition of curcumin on TPA-induced translocation of PKC-ε from cytosol to membrane. Primary culture of human keratinocytes ($3x10^6$ cells/ml) were treated with 50 μM curcumin for 1 h before (pre-treatment), at the same time (co-treatment) and after (post-treatment) the addition of 160 nM TPA for 2 h. It was found that TPA activation was inhibited only when cells were pretreated with curcumin (Figure 27). The co-treatment and post-treatment were not effective. Therefore, this study demonstrated that curcumin inhibited the effect of TPA in the signal transduction cascade before TPA induced the translocation or downregulation of PKC-epsilon.





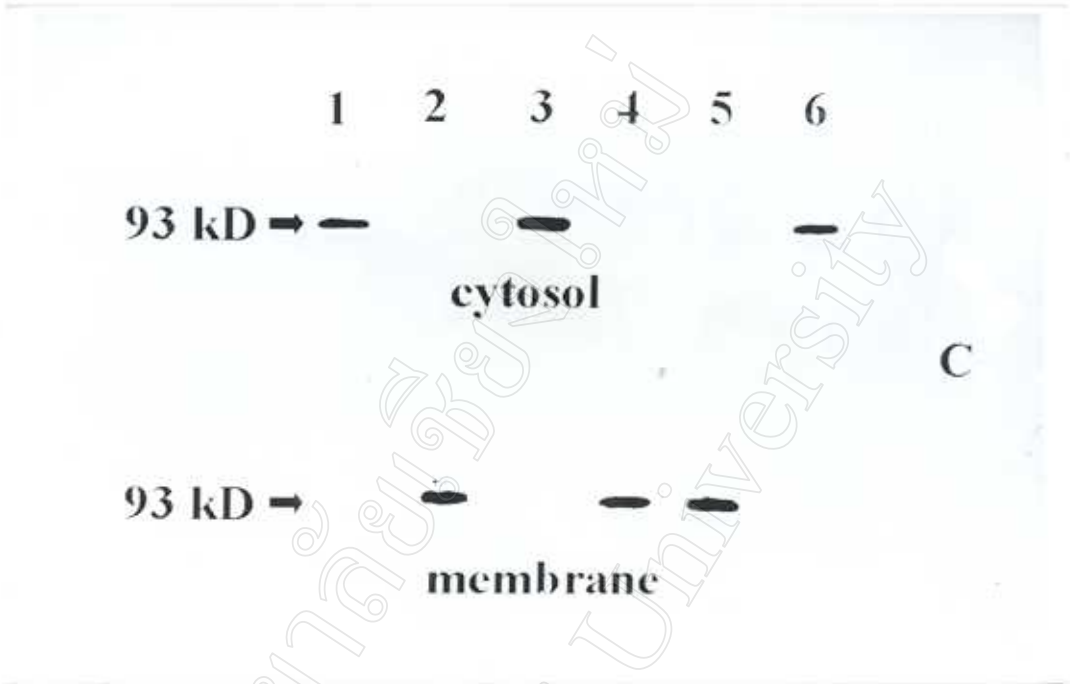


Figure 27. Western blot analysis of the effect of time on curcumin addition on TPA-induced PKC-epsilon in human keratinocytes. Human keratinocytes ($3x10^6$ cells/ml) were preincubated at 37°C with 50 μM curcumin for 1 h at different period of time (pre-, co- and post –treatment with 160 nM TPA for 2 h).Then 30 μg protein/35μl of cytosolic and membrane fractions were analyzed by Western Blot and immunodetected as described in Materials and Method. Lane 1, DMSO-treated cells (control); lane 2, TPA treated cell for 2 h (TPA); lane 3, preincubated cells with 50 μM curcumin (curcumin/TPA); lane 4, incubated cells 50 μM with curcumin at the same time with TPA (curcumin + TPA) ; Lane 5, incubated cells with 50 μM curcumin after TPA treatment (TPA/curcumin); Lane6, incubated cells with50 μM curcumin only for 2 h (curcumin). Subjects; A, abdominal skin (cesarean) 24; B, 28 and C, 29 years old. Dark bands indicate 93 kD of PKC-ε isoenzyme.

Table 12. Densitometric analysis of the Western blots shown in Figure 27.

Samples	Specific activity [a] (arbitrary units/ 30μg protein)			Relative PKC level (%)[b]
Cytosolic fractions	Subject 1	Subject 2	Subject 3	
C0 (DMSO)	19.41	22.34	21.62	100.00
C2 (TPA)	1.97	0.48	0.24	4.25
Pre-treatment (curcumin/TPA)	19.06	20.56	20.60	95.41
Co-treatment (TPA+curcumin)	1.81	0.08	0.34	3.51
Post-treatment (TPA/curcumin)	1.41	0.03	0.47	3.01
C50 (curcumin)	18.43	20.79	20.64	94.48
Membrane fractions				
M0 (DMSO)	0.44	0.06	0.29	1.24
M2 (TPA)	17.72	18.74	21.62	91.68
Pre-treatment (curcumin/TPA)	0.41	0.39	0.34	1.80
Co-treatment (TPA+curcumin)	15.11	20.20	21.46	89.60
Post-treatment (TPA/curcumin)	15.56	18.55	19.30	84.30
M50 (curcumin)	0.51	0.08	0.53	1.76

[a] : Values were obtained by the densitometry of the Western blots shown in Figure 27.

[b] : Values are relative to cytosolic PKC-epsilon of control cells (C0) which were set to a value of 100, and are representative of three experiments that yielded similar results.

C , cytosolic fraction ; M , membrane fraction. C0, M0,DMSO treated cells(Control); Pre-treatment, 50 μM curcumin treated cell for 1 h before adding 160 nM TPA ; Co-treatment, 50 μM curcumin treated cells at the same time with adding 160 nM TPA; Post -treatment, 50 μM curcumin treated cell after adding 160 nM TPA; C2, M2, cytosolic and membrane fractions of TPA-treated cells for 2 h ; C50,M50 ; cytosolic and membrane fractions of 50 μM curcumin treated cells for 2 h.

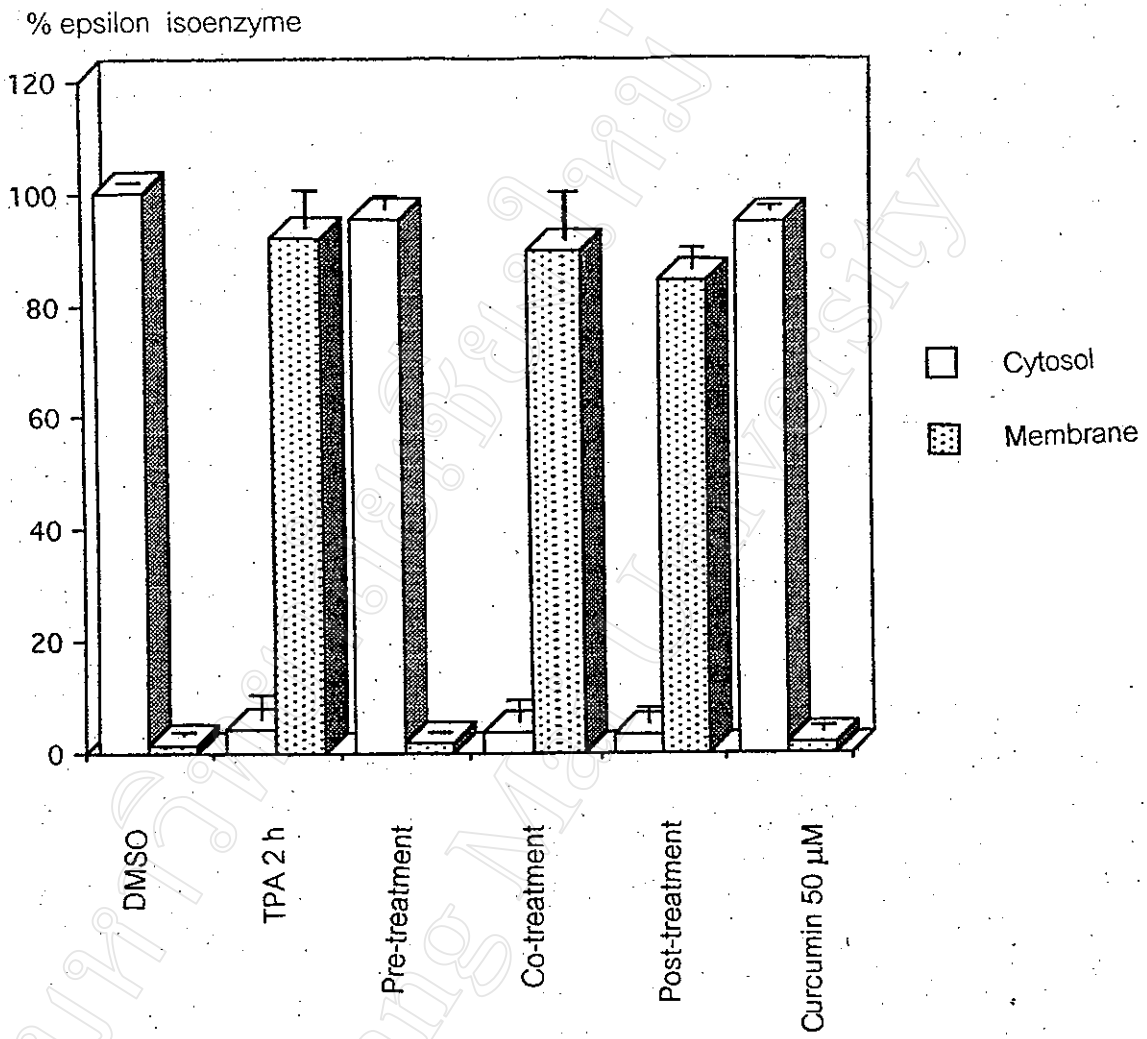


Figure 28. Effect of time on curcumin addition on TPA-induced PKC-epsilon in human keratinocytes. The histogram represents the relative value of PKC-epsilon which were set cytosolic PKC-epsilon of control cells (C0) to a value of 100 (Table 12).C, cytosolic fraction; M, membrane fraction. C0, M0, DMSO treated cells (Control); Pre-treatment, 50 µM curcumin treated cell for 1 h before adding 160 nM TPA ; Co-treatment, 50 µM curcumin treated cells at the same time with adding 160 nM TPA; Post -treatment, 50 µM curcumin treated cell after adding 160 nM TPA; C2, M2, cytosolic and membrane fractions of TPA-treated cells for 2 h ; C50, M50 ; cytosolic and membrane fractions of 50 µM curcumin treated cells for 2 h.