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Translocation of Protein Kinase C Isozymes in the Breast Cancer Cell Line

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Abstract

Protein Kinase C (PKC) activators, phorbol 12-myristate 13-acetate (PMA), bryostatin, and dioctanoyl glycerol (DiC8), induce translocation of PKC isozymes from cytoplasm to plasma membrane or into nucleus. The activated PKC negatively modulates growth of human breast cancer cells. Antiproliferative effect and translocation of PKC were investigated in MCF-7 cells. The translocation of activated PKC isozymes by PMA, bryostatin and DiC8 was occurred at the various different regions in MCF-7 cell. PKC α and β could be translocated to the nucleus or the nuclear membrane, and PKC δand ε to cell membrane by PMA while DiC8 and bryostatin induced the translocation of PKC α and β to the nucleus or plasma membrane, respectively.

In the antiproliferative effect of PKC activators, PMA (IC_{50} values of 1.2±0.3nM) and DiC8 (IC_{50} values of 5.0± 1.1μM) inhibited the cell growth. Bryostatin also inhibited the cell growth but to a much less degree than one observed with PMA : 16% growth reduction by 100nM bryostatin. However, PMA treated with bryostatin induced growth inhibition, but PMA with DiC8 at 10μM was not effective. These results suggest that each PKC isozyme is translocated to various specific sites, and that especially, PKC α isozyme plays an important role in control of antiproliferaive function of cell growth.

Key words : Protein kinase C isozyme, PKC activator

Introduction

The PKC isozymes were detected in the human breast cancer cells such as MCF-7[1] and MCF-7/Dox cells[2]. It has been reported that the six PKC isozymes in PC-3 cells[3] are involved in signal transduction pathway. Activation of PKC α, β, δ, ε, and ζ in MCF-7/Dox cells[4] and expression of the five PKC isozymes α, δ, ε, η, and μ in PC-3 and DU145 cells[5] were observed. Translocation of the PKC isozymes by PKC activators occurs in various types of cells. For example, some PKC isozymes nslocated to the various regions of platelets[6] by phorbol ester, and down-regulation of PKC α in hepatoma cells[7] by phorbol ester may be against production of erythropoietin. According to Girad and co-workers[8], the PKC activator phorbol ester has the direct effect on the expression level of and translocation of PKC isozymes in HL 60, K562, CHO, and E7SKS cells. Choi and Ahn[3] observed the translocation of PKC α and ε to the nucleus by PMA and that of PKC α to the nucleus and PKC ε to the plasma membrane or the nucleus by bryostatin-2 in prostate adenocarcinoma PC-3 cell line. Ahn and others[4]

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are tradetected that the six PKC isozymes, except for PKC γ, were localized in cytoplasm of MCF-7/Dox cells, but that when treated with PMA, they were translocated differently : PKC α to the plasma membrane or nucleus and PKC β to the nuclear membrane and cytoplasm, PKC δ to the nuclear membrane, and PKC ε to the nucleus.

The expression of PKC α, δ, ε, η, and μ was also observed in TPA-resistant LNCap, PC-3 and DU145 cells by de Vente and co-workers[5]. They suggested that PKC α and μ were translocated to the cell membrane with the TPA-treated. Others[9] reported the activation of cytoskeleton and inhibitory effect on tumor cell growth by translocation of PKC isozymes[10]. The PKC activator bryostatin[11] appeared to inhibit the cell growth of MCF-7 cells and consequently induced translocation[12] and down-regulation of individual PKC isozymes[7]. For instance, in SENCAR mouse skin tissue, the tumor promotion was repressed by bryostatin and phorbol esters[13], in MCF-7 cells by bryostatin-1, and in hepatoma[14] and glioma cells[15] by phorbol ester. The growth inhibitory effects by bryostatin-1 and TPA in human leukemia and tumor cells[16,17], in breast cancer cells by bryostatin-1 and phorbol ester[11,18,19], and in MCF-7 cells by DiC8 and phorbol ester were investigated in other studies[12].

Among the PKC isozymes, PKC α and β are responsible for growth inhibition of MCF-7 cells[11]. In MCF-7-PKC α cells, expressed by unique antisense oligonucleotide of PKC isozymes, PKC α was proposed to prevent tumor promotion by the PKC activator TPA by de Vente and co-workers[5]. It was reported that the cyclic AMP-generating systems in NIH3T3 cells, where PKC α was expressed, were suppressed[20], and that the expression of estrogen receptors and apoptosis were resulted in MCF-7 cells when treated with phorbol ester[21].

The purpose of study is to observe the translocation of PKC isozymes by PKC activators in MCF-7 cells and the antiproliferative effects of the PKC activators such as bryostatin, DiC8, and PMA and in-combination of bryostatin or DiC8 with PMA.

Materials and Methods

Phorbol 12-myristate 13-acetate (PMA), bryostatin, 1,2-dioctanoyl glycerol (DiC8), biotinylated secondary antibody, rabbit anti-mouse IgG, Texas Red-antibody conjugate and other chemicals were purchased from Sigma (St. Louis, MO) ; PKC antibodies α, β, δ, ε and ζ oligodeoxynucleotide probes for respective PKC isozyme messages, and mRNA isolation kit were from Gibco-BRL (Gaithersburg, MD). Human breast cancer, MCF-7 cell lines were obtained from NIH.

Cells were cultured in RPMI 1640 containing 5% fetal bovine serum (FBS). For cell proliferation assay, cells (200 cells in 0.2ml media per well) were placed in RPMI 1640 with 5% FBS in 96-well microculture plates and allowed to attach for 5 hours. After attachment, various concentrations of PKC activators alone or in combination were added to the microcultures. After the plates were incubated at 37℃ for 3 days, the cells were fixed by gentle addition of 50μℓ SRB solution (0.4% SRB in 1% acetic acid) to wells for 10 min. The plates were then quickly washed five times with 1% acetic acid to remove unbound dye and allowed to air dry. Bound dye was solubilized with 10mM Tris buffer (pH 10.5) prior to reading plates. The absorbancies at 570 nm were read from a Bio-Pharm Tech microplate reader (model CERES 900C) interfaced with an IBM computer.

For immunocytochemical analysis of Protein Kinase C, monolayered MCF-7 cells (100 cell/well) on culture slide were treated with PKC activators, then fixed with 3% formaldehyde, and rinsed with PBS three times at room temperature. After permeabilizing cell membranes with ice-cold 0.5% Triton X-100 for 10 min, they were incubated for 1 hr at room temperature in blocking solution containing PBS, 1% normal goat serum and 0.3% Triton X-100. The cells were treated with PKC isozymic

antibody (1-10μg/ml) in the blocking solution and incubated overnight at 4℃ in a humidified chamber. After washing 5 times with PBS, the cells were incubated with biotinylated secondary antibody, goat anti-rabbit IgG for 2 hr at room temperature, and then reacted with streptavidine Texas Red-conjugated anti-rabbit IgG antibody in PBS for 2 hr in the dark at room temperature, following washing 5 times with PBS. After rinsing 5 times with PBS and once with deionized water, the mounted specimen with 50% glycerol in PBS was observed under a fluorescent microscope (Nikon, Japan) at ×400.

Northern blot analysis of Protein Kinase C, total cellular RNAs and Poly(A)RNAs from MCF-7 cells were purified by using a mRNA purification kit as recommended by the manufacturer. 2μg of mRNA were loaded onto each lane of 1% agarose gel containing formaldehyde and electrophoretically resolved. The RNA was transferred to a nitrocellulose membrane (Hybond-N, Amersham, IL) by capillary transfer and the blot was then probed with 3×10^6 cpm/ml random-primed ^{32}P-radiolabeled oligonucleotides 40-base oligonucleotides. The synthetic oligodeoxynucleotides (Gibco-BRL, Gaithersburg, MD) were used for probes of PKC α, β, γ, δ, ε and ζ isozyme messages. These probes were complementary to a portion of the V3 region cDNA sequences of their respective isozymes except the ζ probe which is complementary to a sequence of the 3'-untranslated region of the ζ isozyme message. Autoradiography was carried out by exposing the membrane for up to 7 days. Membranes were washed at in 0.1% SSC containing 0.1 % SDS and exposed to X-ray film (Kodak, AR-2) for 72 hr at -70℃ with an intensifying screen.

Result

For the signal translocation of the activated PKC isozymes by stimulation of some activators such as phorbol 12-myristate 13-acetate (PMA), bryostatin, and dioctoanoyl glycerol (DiC8), monoclonal antibodies against the six PKC isozymes α, β, γ, δ, ε, and ζ were used. In normal MCF-7 cells, each PKC isozyme besides PKC γ was detected in the cytoplasm (Fig. 1 and 2, control ⓐs).

When MCF-7 cells treated with 10nM PMA, PKC α was translocated from cytosol to the nuclear membrane and the nucleus (Fig. 1 PKCα-ⓑ) and PKC β to the nucleus but mainly to the nuclear membrane (Fig. 1 PKCβ-ⓑ). Although PKC δ exists in much less amount than other PKC isozymes in cytoplasm (Fig. 2 PKCδ-ⓑ), it was translocated rather into the nuclear membrane than to the nucleus. Like PKC δ, PKC ε also existing in small amount was not translocated to the nucleus but usually to the cytoplasm at low degree and to the nuclear membrane (Fig. 2 PKCε-ⓑ). PKC ζ was often present in the cytoplasm similar to the control and was translocated to the cell membrane (Fig. 2 PKCζ-ⓑ).

Like PKC α in cytoplasm treated with PMA, in MCF-7 cells treated with bryostatin, it was translocated to the nucleus but at less degree than the PMA-treated (Fig. 1 PKCα-ⓒ). PKC β was usually translocated to the nuclear membrane as in PMA treatment (Fig. 1 PKCβ-ⓒ). PKC δ was less translocated to the nuelear membrane than that of PMA-treated (Fig. 1 PKCδ-ⓒ). Although PKC ε and ζ were present in cytoplasm, they were not translocated to the nuclear membrane and to the nucleus, respectively (Fig. 2 PKCε and ζ-ⓒ).

When the cells were treated with DiC8, PKC α was translocated from cytosol to the nucleus, but its signal level was the smallest (Fig. 1 PKCα-ⓓ). PKC β was usually distributed in cytoplasm but sometimes translocated to the nuclear membrane and the nucleus (Fig. 1 PKCβ-ⓓ). PKC ε was distributed in cytoplasm as well, but no translocation occurred to the nucleus (Fig. 2 PKCε-ⓓ). PKC ζ, mainly distributed in cytoplasm like PKC ε, was not translocated (Fig. 2 PKCζ-ⓓ). In consequence, PMA induced translocation of PKC α, β, δ, and ε whereas DiC8 and bryostatin translocated PKC α

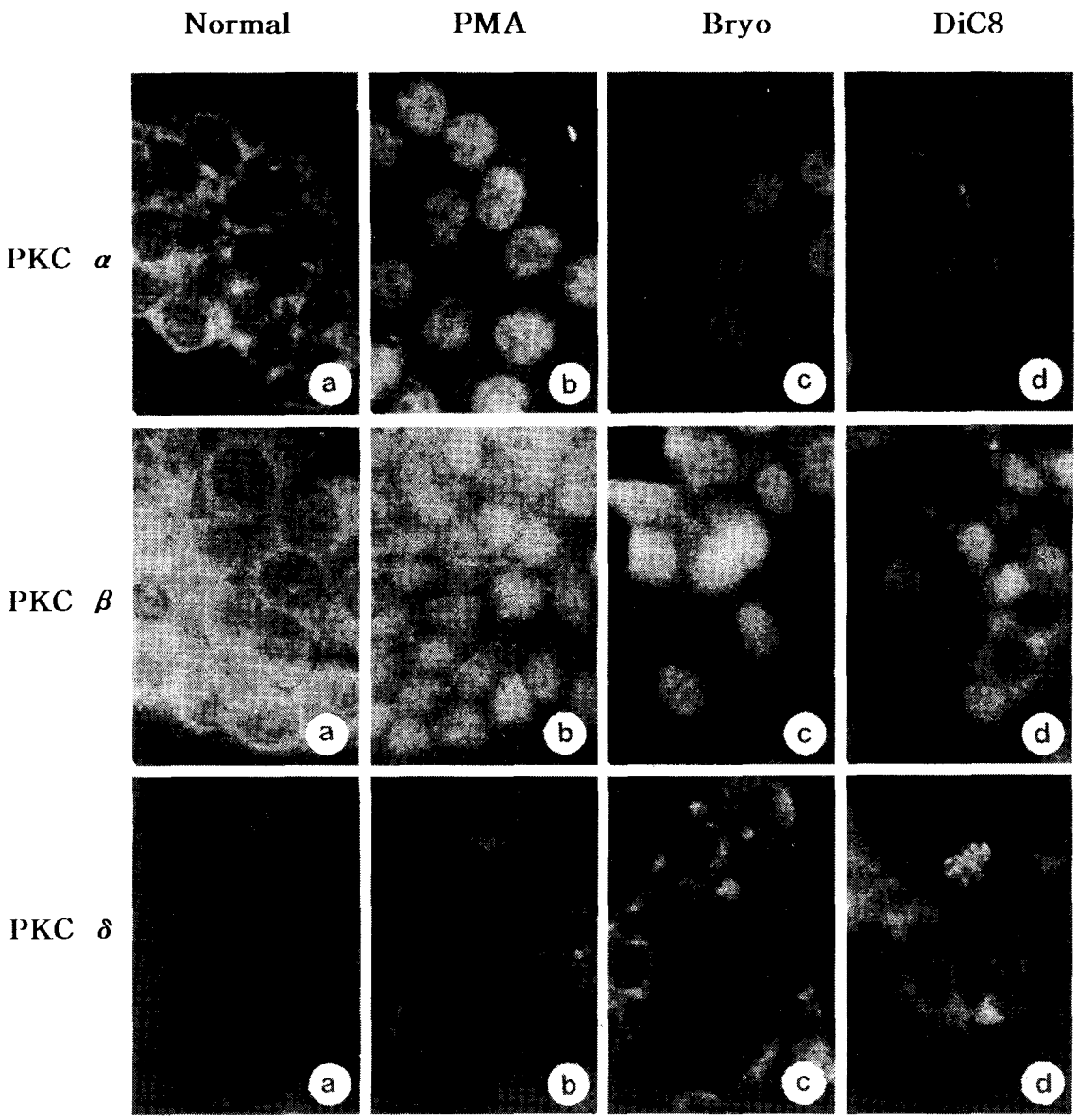

Fig. 1. Immunofluorescent detection of PKC isozymes in treatment of several activators to MCF-7 cells. Photographs of upper, middle, and lower rows were indicated localization of PKC isozyme α, β, δ, type, respectively. a. control ; b. 10nM PMA ; c. 10nM bryostatin ; d. 1μM DiC8.

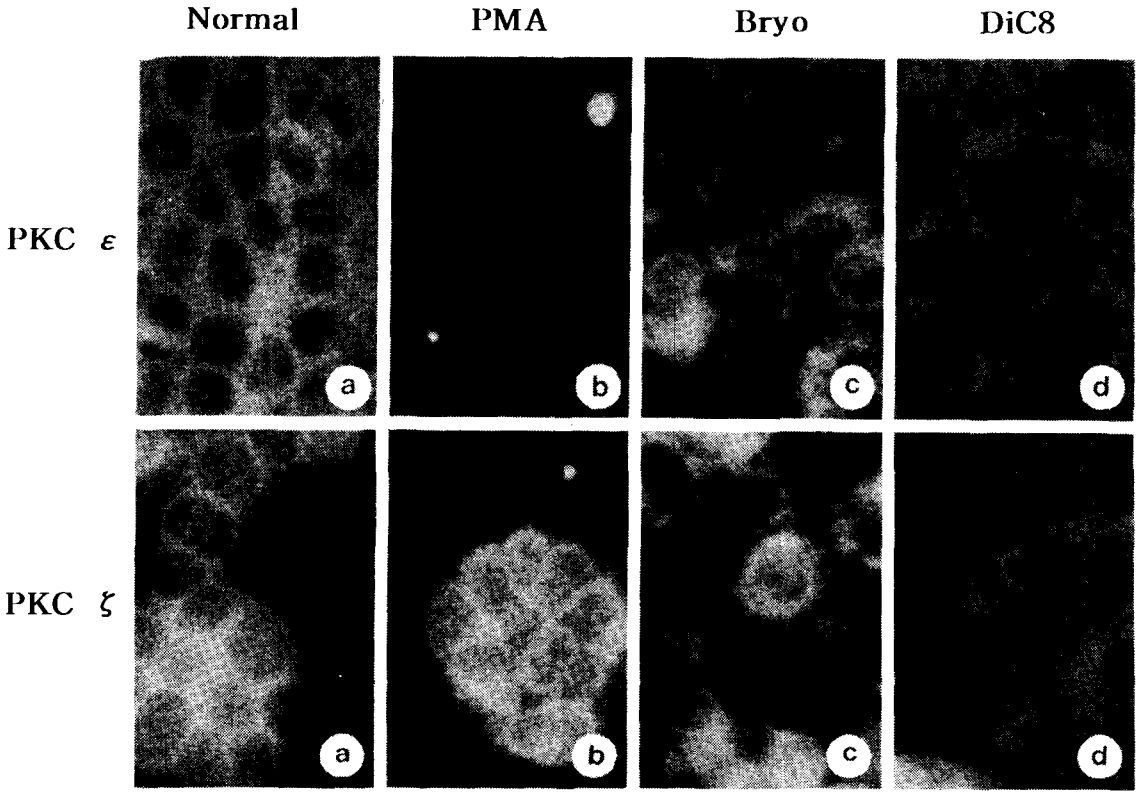

Fig. 2. Immunofluorescent detection of PKC isozymes in treatment of several activators to MCF-7 cells. Photographs of upper, and lower rows were indicated localization of PKC isozyme ε, ζ, type, respectively. a. control ; b. 10nM PMA ; c. 10nM bryostatin ; d. 1μM DiC8.

and β to the nucleus and nuclear membrane.

Using northern blot analysis, the levels of mRNAs of the PKC isozymes α, β, δ, ε, and ζ, except for γ, were detected in normal MCF-7 cells. mRNA of PKC γ was not detected ; mRNAs of PKC α, δ, ε, and ζ were expressed in high amounts but PKC β in low amount. Among the expressed PKC isozymes, protein level of PKC α, β, δ, and ε was increased, but there was no significant change in PKC ζ as shown in Figure 3.

To examine antiproliferative effect of MCF-7 cells by PKC activators, PKC activators alone at different concentrations for MCF-7 cells were measured at the absorbance of 570 nm from a microplate reader. When the cells were treated with PMA only, the antiproliferative

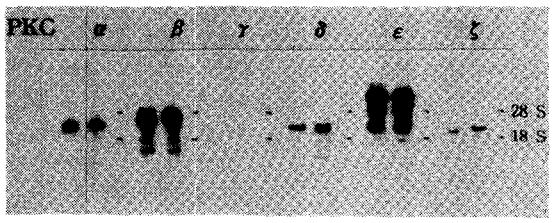

Fig. 3. Morthern blot analysis of the PKC isozymes in MCF-7 cells. The positions of rRNAs (18S and 28S) are indicated by arrowheads on the right.

effect was at maximum of 86±5% (10nM) and 84% (100nM) reduction in cell growth. Compared to the control, 10nM PMA repressed the proliferation of MCF-7 cells to IC_{50} values of 1.2±0.3nM. When the cells were

treated with 10nM and 100nM bryostatin, the growth level was moderately inhibited. The cells treated with 1 μM DiC8, growth inhibition of MCF-7 cells was expressed as IC_{50} values of 5.0±1.1μM (Fig. 4).

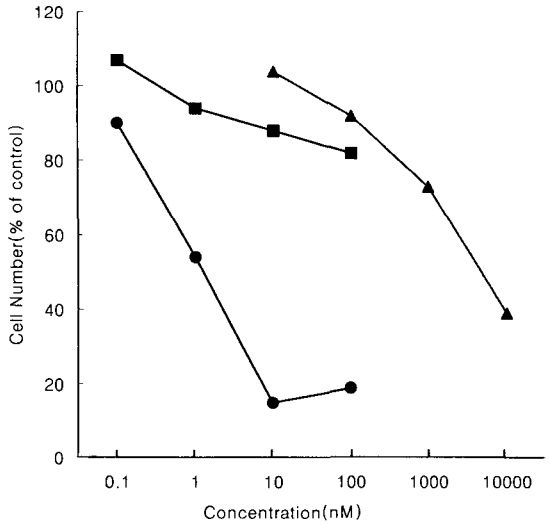

Fig. 4. Effects of PMA (●), bryostatin (■) and DiC8 (▲) on the growth of MCF-7 cells. Results are expressed as percentage of control cell growth.

The antiproliferative effect was seen in the MCF-7 cells treated with bryostatin (10 or 100nM) and PMA (10 or 100nM) at their same concentrations. The MCF-7 cells treated with bryostatin and PMA simultaneously at the same concentration (10 or 100nM) had their growth of 43±2% (10nM) and 54±4% (100nM) of the control. The antiproliferative effect of PMA was partially reversed by bryostatin. But the antiproliferative effect after treatment of DiC8 (10μM) with PMA did not change (Fig. 5).

The antiproliferative effect by PMA was partially reduced by bryostatin. Bryostain repressed the effect of PMA on proliferation of MCF-7 cells. On the other hand, the combination of DiC8 with PMA did not cause antiproliferative effect by PMA. In addition, the antiproliferative effect by PKC activators was seen that PKC

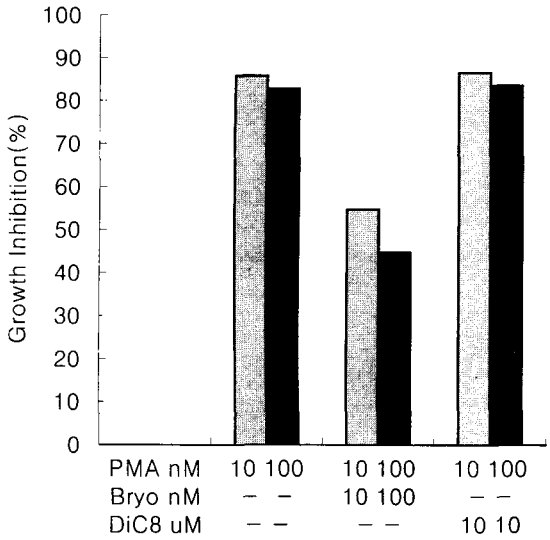

Fig. 5. Effects of bryostatin and DiC8 on the growth response of MCF-7 cells to PMA. Results are expressed as percentage of control cell growth.

α and β had correlation with growth inhibition of MCF-7 cells.

Discussion

Generally PKC activators such as PMA, bryostatin, and DiC8 have been reported to be cancer-inducing chemicals[14-17]. However, the activated PKC[22-26] inhibited the growth of breast cancer cells but also other tumor cells and especially played an important role in signal transduction for regulating proliferation of MCF-7 cells. Our results show that the PKC activators, PMA, bryostatin, and DiC8, translocated the PKC isozymes to various sites of MCF-7 cells. There are at least eleven subtypes of PKC[32] : conventional PKCs (α, βI, βII, and γ), new PKCs (δ, ε, η, μ, and θ), and atypical PKCs (ζ, λ, and ν).

The roles of PKC have been recognized by many researchers. The PKC activators induce expression of PKC isozymes and function in growth control of MCF-7 cells [27-31]. Beckman and researchers[32] identified the five ex-

pressed PKC isozymes α, β, δ, ε, and ζ by western blot and Stanwell and co-workers[1] the three PKC isozymes α, ε, and ζ. Choi and Ahn[3] demonstrated the signal transduction of the six PKC isozymes α, β, γ, δ, ε, and ζ, and these isozymes were detected in MCF-7/Dox cells by Ahn and co-workers[4]. But our results show that only the five were detected but not PKC γ. The translocation of PKC α isozyme by the PKC activators PMA and bryostatin in prostate adenocarcinoma PC-3 cell line and its translocation to the nucleus by PMA, bryostatin-1 and DiC8 in MCF-7/Dox cell were observed by Ahn and co-workers[4].

In our study, by 10nM bryostatin, the growth of MCF-7 cells was inhibited as Kennedy and co-workers [11] observed the antiproliferative effect by bryostatin-1 at the high concentration of 100nM in the MCF-7 cells. McSwin and co-workers[33] observed that HL-60 cells treated with PMA had the antiproliferative effect of IC_{50} 20±6nM, whereas our result showed as IC_{50} 1.2±0.3 nM on MCF-7 cells. The cell growth inhibitory function of the PKC activators may be mediated by PKC α isozyme[18,30]. It was reported that antisense RNA specific to PKC α in MCF-7 cells over-expressing PKC α caused growth inhibition and cell death by phorbol ester[21,34]. Thus, the antiproliferative effect of MCF-7 cells by PKC activators has the correlation with expression of PKC α, and PKC α isozyme is essential in regulating the antiproliferative effect in MCF-7 cell growth. Our investigators have suggested the involvement of PKC isozymes α and ε in growth inhibition of MCF-7 cells.[1,11] On contrary in this study, byrostatin unlike PMA did not induce translocation of PKC ε into a nucleus nor involve in growth inhibition, and thus PKCα is involved in regulating in growth of MCF-7 cells.

PKC β involved in cell growth inhibition in cytosol of MCF-7 cells as shown by Kennedy and co-workers[11]. Treatment with PMA induced translocation of PKC δ and ε into perinuclear membrane but almost no effect with bryostatin and DiC8. PKC ζ had no effect by all the three PKC activators, PMA, bryostatin, and DiC8.

However, when bryostatin and DiC8 simultaneously were added, the antiprotiferative effect of PMA was partially reduced by bryostatin. Bryostatin repressed the effect of PMA on proliferation of MCF-7 cells. But the combination of DiC8 with PMA did not affect on antiproliferative effect by PMA. These result suggest that bryostatin has two characteristics : antiproliferative and partial proliferative effects on MCF-7 cells. The different responses of bryostatin and DiC8 on PMA activity may be related to different cellular translocations of PKC isozymes and their activation pathways.

Consequently, these studies described the immunofluorescent detection of PKC isozymes of MCF-7 cells for the isozyme-specific differential response to bryostatin, DiC8 and PMA. These PKC activators inhibited growth of MCF-7 cells. Especially PKC α isozyme can play an important role in control of antiproliferative function and cell growth in nuclear membrane or nucleus of MCF-7 cells.

Acknowledgments

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Translocation of Protein Kinase C Isozymes in the Breast Cancer Cell Line

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초록 : 유방 암세포에서 Protein Kinase C 동위효소의 전위

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Phorbol 12-myristate 13-acetate (PMA), bryostatin, dioctanoyl glycerol (DiC8)과 같은 Protein Kinase C (PKC)의 활성제는 세포질로부터 막이나 핵으로 PKC 동위효소의 전위를 유도한다. 활성화된 PKC는 일반적으로 암을 유발시키는 역할을 하지만 그와 반대로 사람유방암세포의 성장을 약화시키는 기능을 가지고 있다. PKC의 항증식효과와 전위가 MCF-7 세포에서 조사되었다. PMA, bryostatin, DiC8로 활성화된 PKC 동위효소의 전위는 MCF-7 세포의 여러 장소에서 나타났다. PMA는 PKC α 와 β는 핵이나 핵막 그리고 PKC δ와 ε은 세포막으로 일부 전위시켰고, 반면 DiC8과 bryostatin은 PKC α와 β를 각각 핵과 핵막으로 전위를 유도하였다.

PKC 활성제의 항증식 효과에 있어서 PMA (IC_{50} values of 1.2±0.3nM)와 DiC8 (IC_{50} values of 5.0±1.1μM)는 세포의 성장을 억제시켰다. Bryostatin 역시 세포의 성장을 억제시켰지만, PMA로 관찰된 것보다는 낮은 수준이었다. 즉 100nM bryostatin에 의해 16% 정도 성장이 감소되었다. 그러나 PMA는 bryostatin과 함께 처리하였을 때 PMA의 항증식 효과는 낮았으나, 10μM DiC8과 함께 처리하였을 때는 효과가 없었다. 이러한 결과들은 각 PKC 동위효소들이 다른 특이한 위치로 전위되었으며, 특히 PKC α 동위효소가 세포성장의 항증식 기능을 조절하는데 중요한 역할을 함을 시사한다.