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Evidence for Regulation of Interaction of Endogenous Protein Kinase C (PKC) Substrates with Plasma Membrane by PKC Down-Regulation in K562 Cells

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In the particulate fraction obtained from PKC-down regulated K562 cells by treatment for 24 h with 200 nM TPA, phosphorylation of two proteins with molecular weight, 100 kDa and 23 kDa (designated p100 and p23, respectively) was depleted and addition of exogenous purified PKC to this fraction failed to restore their phosphorylation. However, in the soluble fraction, all of phosphoproteins abolished by long-term treatment with TPA were restored by exogenously added PKC. Phosphorylation of two proteins was increased by short-term treatment (20 min), and diminished with the persistant exposure to TPA as well as at a concentration as low as 1 nM. When K562 cells were treated with 1 nM and 200 nM TPA for 24 h, phosphorylation of p100 was restored with or without exogenous PKC on 2-3 day and 6 day after removal of treated TPA, respectively. Two-dimensional electrophoresis of phosphoproteins revealed that phosphorylated p100 (pI=5.9) and a p66 species were completely absent from the particulate fraction of K562 cells treated with 200 nM TPA for 24 h. These results suggest that the interaction of sensitive endogenous substrates, p100 and p23 with the plasma membrane might be regulated by PKC-down regulation without their displacement to the cytosol and the interaction of p100 with the membrane might be reversible.

Key words : Protein kinase C substrate, K562 cell, TPA, PKC down-regulation

PKC, originally defined Ca^{2+}-activated and phospholipid-dependent protein kinase, plays a crucial role in intracellular signal transduction (Nishizuka, 1992). PKC is the cellular receptor for the tumor promoting agent, 12-O-tetradecanoylphorbol 13-acetate (TPA) and related compounds (Ashendel, 1985) showing to elicit a variety of biological responses in tissue and cultured cells. Enzymatic studies and molecular cloning analyses have revealed that PKC molecules consist of a protein family which can be classified into three groups: conventional PKCs (cPKC), novel PKCs (nPKC) and atypical PKCs (aPKC) (Nishizuka, 1992; Ohno *et al.*, 1988). Structure features of PKC include a kinase domain and a regulatory domain with one or two zinc-finger-like structure and a basic pseudosubstrate prototope (Kikkawa *et al.*, 1989). All members of the family require phospholipid for activation of their kinase activity, while requirement for Ca^{2+} could only be shown for cPKC (α, βI, βII and δ). High homology among isozymes can be observed in three of the four "constant domains", the phospholipid-binding domain, C1, as well as in the kinase domain, C3 and C4. The nPKC isoenzymes were identified comprising the PKC δ, ε, L(η) and θ isoforms. This novel group of PKC isozymes exhibits Ca^{2+}-independent activity that presumably reflects the abscence of the calcium-binding C2 domain (Ono *et al.*, 1988; Osada *et al.*, 1990; Akita *et al.*, 1990; Mischak *et al.*, 1991; Koide *et al.*, 1992; Ogita *et al.*, 1992). On the other hand, the aPKC enzymes consist of ζ and λ isoforms, which have only one cystein-rich zinc finger-like motif and not affected by diacylglycerol, phorbol ester and Ca^{2+} (Ono *et al.*, 1989; Nishizuka, 1992; Ways *et al.*, 1992).

Each isoform showes a different tissue distribution and in many cases a distinct subcellular location, supporting the concept that individual isoforms perform specific roles in cellular signaling (Huang *et al.*, 1986; Osada *et al.*, 1990; Bacher *et al.*, 1991).

Alteration in the state of phosphorylation of a number of intracellular proteins by PKC activation *in vivo* mediates responses of central importance to PKC-dependent signaling. Although progress has been made in understanding the functional properties of the different PKC isoforms, relatively little is known about their *in vivo* substrate specificity and their specific

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roles in intracellular signaling. Furthermore the mechanisms through which PKC mediates these cellular responses remain largely unkown, it is essential to characterize the physiological substrate of this kinase family.

The MARCKS protein (myristoylated alanin-rich C kinase sbstrate; formely known as the 80/87 kDa protein) is one of prominent cellular substrates and specific *in vivo* substrates for PKC in wide variety of cells (Rozengurt *et al.*, 1983; Stumpo *et al.*, 1989). Activation of PKC results in the displacement of MARCKS from the plasma membrane to the cytosol (Thelen *et al.*, 1991), and down-regulated its mRNA (Brooks *et al.*, 1991). It has reported that TPA induced a rapid down-regulation of MARCKS, which was suppressed by staurosporine, indicating that the down-regulation of MARCKS can be suppressed by inhibiting its phosphorylation (Lindner *et al.*, 1992).

The present study showed that down-regulation of PKC might regulate the interaction of endogenous PKC substrates, p100 and p23, with membrane and the interaction of p100 with the membrane might be reversible.

MATERIALS AND METHODS

Materials

Human chronic myelogenous leukemia K562 was perchased from American Type Culture Collection (Rockville, MD); media and supplies for cell culture were from GIBCO (Grand Island, NY); [γ-^{32}P]ATP was from ICN Radiochemicals (Irvine, CA); TPA was from LC services (Woburn, MA); various reagents were from Sigma Chemical Co. (St, Louis, MO); purified pig brain PKC was kindly provided by Dr. Kuo (Girard *et al.*, 1985) at Emory University, Atlanta, Georgia.

Cell culture

K562 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, antibiotics (50 units/ml of penicillin and 50 μg/ml of streptomycin), and 2 mM glutamine in a humidified incubator at 37°C in 95% air/5% CO_2. Cells at the mid-log phase were used in all experiments. The cells were incubated with 1 nM or 200 nM TPA for 24 h to down-regulate PKC.

Preparation of soluble and particulate fractions

The cells (5×10^7 in 50 ml) obtained under various experimental conditions were homogenized in 3 volume of the homogenization buffer (0.34 M sucrose, 50 mM Tris/HCl, pH 7.5, 2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 50 mM 2-mercaptoethanol), and centrifuged for 1 h at 30,000×g to obtain the soluble (cytosol) and particulate (membrane) fractions. The particulate fraction was taken up in 3 volume of the homogenization buffer containing 0.2% Triton X-100, kept in ice for 1 h with occasional stirring, and finally centrifuged at 30,000×g for 1 h to yield solubilized particulate fraction. Proteins were assayed with the method described by Bradford (1976) using bovine serum albumin as a standard.

Endogenous protein phosphorylation

The phosphorylation of endogenous proteins (30-80 μg) in soluble and particulate fractions was performed in a 200 μl reaction mixture containing 25 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 5 μg of phosphatidylserine (PS) and 500 μM $CaCl_2$ in the presence or absence of 1.2 μg of pig brain PKC or 100 nM TPA. The reaction was initiated by addition of 10 μM [γ-^{32}P]ATP ($0.8\text{-}1.5\times10^7$ cpm) to reaction mixture, and incubated for 5min at 30°C. To terminate the reaction, 50 μl of SDS sample buffer (5x concentration) was added and boiled for 4 min. Phosphoproteins were analyzed in 10% or linear gradient (5-12%) polyacrylamide-SDS gels as described by Laemmli (1975). Autoradiographs of the dried gels were prepared using Kodak X-Omat XAR-5 film between intensifying screens.

Two-dimensional electrophoresis

Two-dimensional electrophoresis using non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension was carried out according to the method of O'Farrell *et al.*, (1977) with a slight modification. The first-dimension NEPHGE gels contained 9.2 M urea, 2% Nonidet P-40, 4% acrylamide/bis acrylamide and 2% ampholine pH 3.5-10. The anode was the top of the gel with 0.01 M H_3PO_4 as anolyte and 0.05 M NaOH was used as catalyte. The first-dimension gels were run for 6 h at 400 V. After electophoresis the first-dimension gels were equilibrated with SDS sample buffer and placed on the second-dimension of SDS gel containing 10% acrylamide. Gels were dried and subjected to autoradiography as described above.

RESULTS

Cell fractions from PKC-down regulated K562 cells by treatment with TPA were phosphorylated in the presence of Ca^{2+} and PS (Fig. 1). Also phosphorylation was conducted with addition of 100 nM TPA or exogenous purified pig brain PKC to the cell fractions. In the soluble fraction, whereas phosphorylation of p130 was increased, that of several proteins (notably p80, p35, p32) was decreased. Addition of exogenous PKC to the cytosolic fraction de-

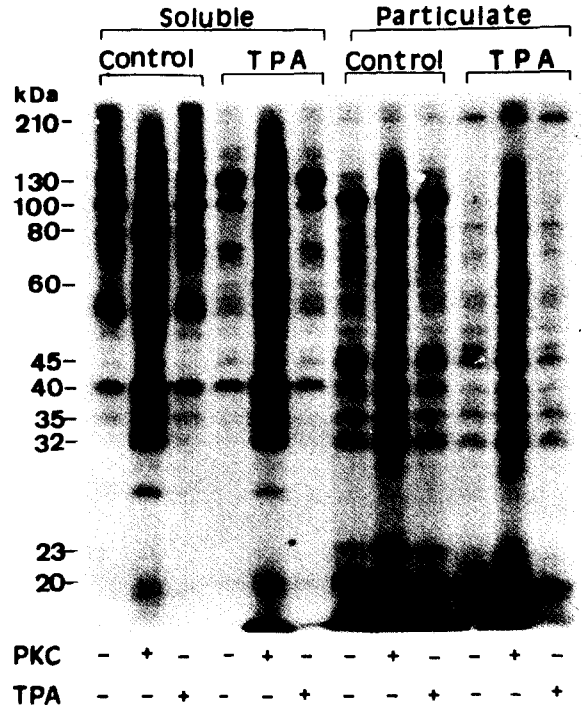

Fig. 1. Phosphorylation of endogenous proteins in soluble and particulate fractions obtained from K562 cells treated with TPA. The cells (5×10^7 in 50 ml) were treated for 24 h with 0.01% DMSO or 200 nM TPA, homogenized and separated into subcelluar fractions. Aliquots of cellular fractions were phosphorylated (30°C, 5 min) by endogenous PKC in the presence of 5 μg of PS and 500 μM Ca^{2+} with or without 100 nM TPA or 1.2 μg of the purified pig brain PKC.

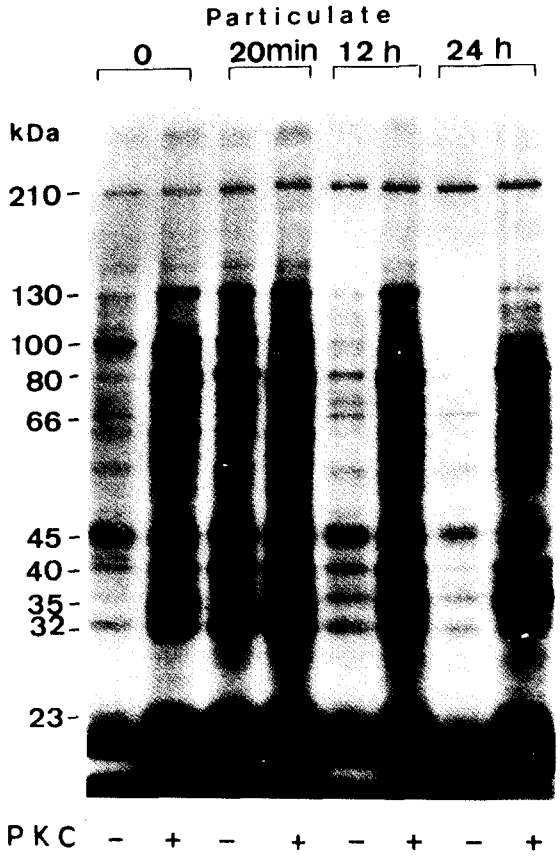

Fig. 2. Time-course of endogenous protein phosphorylation in particulate fractions from K562 cells treated with TPA. The particulate fractions from cells treated with 200nM TPA for indicated time were incubated in the reaction mixture as described in Fig. 1. The phosphorylation pattern of protein was analyzed using a linear gradient (5-12%) polyacrylamide-SDS gels.

pleted PKC by treatment with TPA restored phosphorylation of not only p80 but also that of p35 and p32. It remained to be determined whether the p80 species was the autophosphorylated PKC or the ubiquitous 80 kDa PKC substrate MARCKS (Blackshear *et al.*, 1986; Stumpo *et al.*, 1989). In the particulate fraction, treatment with TPA resulted in selective disappearance of phosphorylation of p100 and p23, and that of these endogenous substrates was not restored by addition of exogenous PKC to the particulate fraction in which PKC level was down-regulated. Addition of 100 nM TPA to both cell fractions from the control as well as TPA-treated cells had no effect on phosphorylation, it is thought that phosphorylation *in vitro* was maximized under the condition in the presence of Ca^{2+} and PS.

To know changes of phosphorylation with treated time, K562 cells were treated with 200 nM TPA for various times. As shown in Fig. 2, phosphorytion of a number of proteins (notably p130, p100, p80, p45, p 40, p32, p23) in the particulate fraction was enhanced by treatment with TPA for 20 min and subsequently phosphorylation of p130, p100 and p23 was disappeared with a longer treatment. However, depleted phosphorylation of p100 by treatment for 24 h was not restored by added PKC as seen in Fig. 1.

As shown in Fig. 3, in the soluble fraction from cells treated as described in Fig. 2, phosphorylation of p80, p40, p35, p32 and p30 was persistently reduced following treatment of TPA and exogenously added PKC was restored all of phosphoryaltion of these proteins (Fig. 3). It seemed worth noting that enhanced phosphorylation in the particulate fraction from cells treated with TPA for 20 min coincided with marked translocation of cytosolic PKC to membrane and depleted cytosolic PKC level by treatment with TPA resulted in decreased protein phosphorylation in the soluble fraction as mentioned (Kraft and Anderson, 1983). The phosphorylation of p130, p100 and p23 was dependent on the presence of Ca^{+2}/PS and there was no change of protein patterns of p100 and p23 in both cell fractions obtained from TPA-treated cells (data not shown).

These results suggest that PKC down-regulation due to long-term stimulation of PKC might change the in-

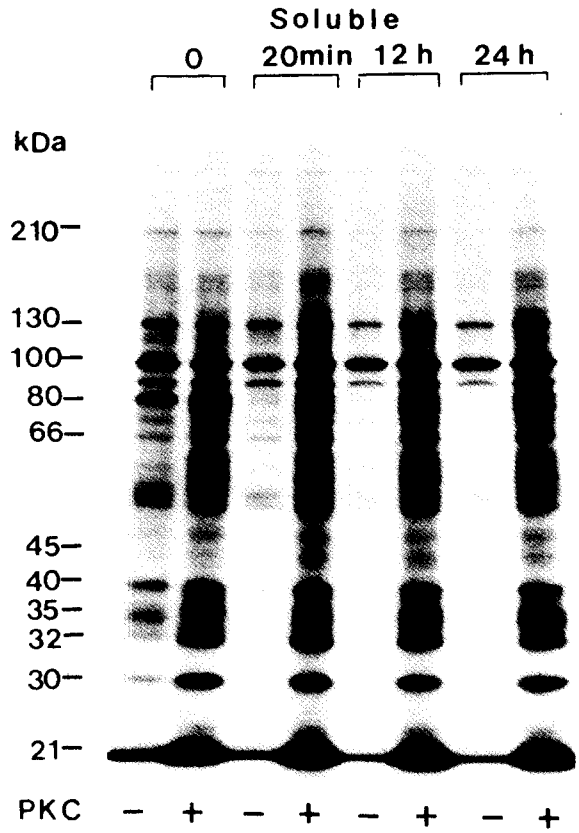

Fig. 3. Time-course of endogenous protein phosphorylation in soluble fractions from K562 cells treated with TPA. The soluble fractions from cells treated with 200 nM TPA for indicated time were incubated in the reaction mixture as described in Fig. 2.

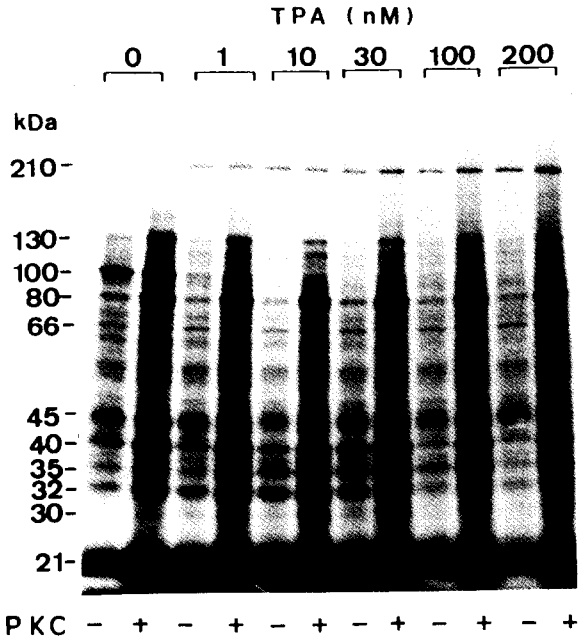

Fig. 4. Endogenous protein phosphorylation in particulate fractions from K562 cells treated with various TPA concentrations. The cells were treated for 24 h with TPA and the detailed procedures were described in Fig. 2.

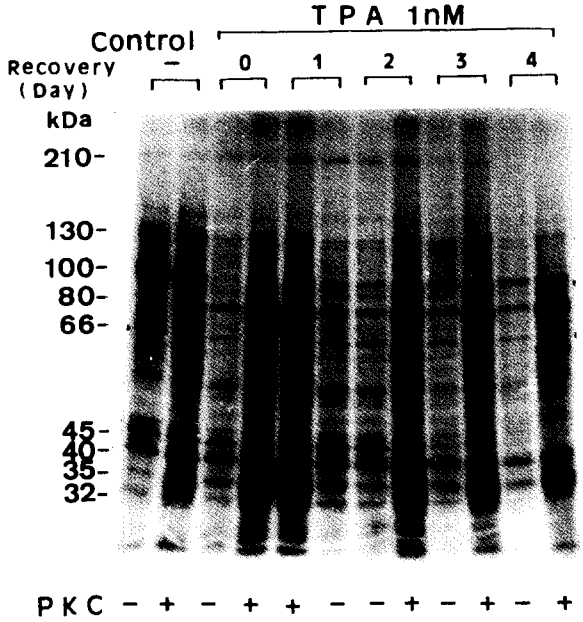

Fig. 5. Recovery of endogenous protein phosphorylation in particulate fractions from K562 cells treated with TPA. The cells were treated for 24 h with 1 nM TPA, washed with PBS to remove TPA, and incubated for 4 days. Protein phosphorylation was assayed on 0, 1, 2, 3 and 4 day after removal of TPA as described in Fig. 2.

teraction of p100 and p23 with the plasma membrane but might not displace these proteins to the cytosol, thus exogenously added PKC no more phosphorylate these endogenous substrates in PKC-depleted particulate fraction and no increase their phosphorylation in PKC-depleted cytosol fraction.

As shown in Fig. 4, when K562 cells were treated for 24 h with various concentrations of TPA, phosphorylation of p100 was depleted at a concentration as low as 1 nM, which was not restored with added PKC, indicating long-term treatment with TPA, even though the dose is low, might be essential for a change of its interaction with membrane.

In order to examine reversible interaction of these substrates with plasma membrane, K562 cells were treated with 1 nM or 200 nM TPA for 24 h and then cultured several days after removal of TPA (Fig. 5 and 6). Phosphorylation of p100 was appeared on 3 day clearly (Fig. 5) and on 6 day (Fig. 6) after removal of TPA under the basal condition (i.e., in the presence of Ca^{+2} and PS) as well as in the presence of exogenous PKC. Therefore, these results suggest that the interaction of p100 with plasma membrane might be reversible and time required for its reinteraction might be dependent on the dose of treated TPA.

Two-dimensional electrophoresis of phosphoproteins in the particulate fractions revealed that phosphorylated p100 with a pI (pH=5.9) was increas-

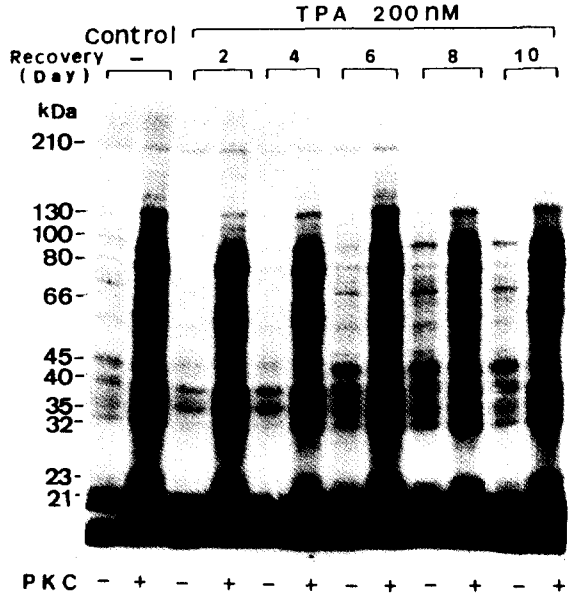

Fig. 6. Recovery of endogenous protein phosphorylation in particulate fractions from K562 cells treated with TPA. The cells were treated for 24 h with 200 nM TPA, washed with PBS to remove TPA, and incubated for 10 days. Protein phosphorylation was assayed on 2, 4, 6, 8 and 10 day after removal of TPA as described in Fig. 2.

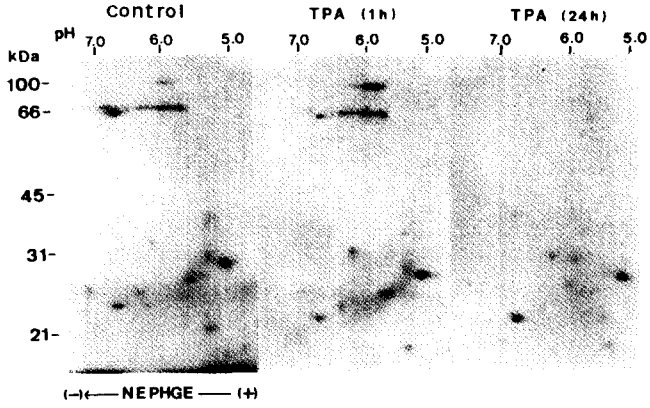

Fig. 7. Two-dimensional electrophoretic analysis of phosphoproteins in particulate fractions from K562 cells treated with TPA. The cells were treated for 1 h or 24 h with 200 nM TPA. Phosphorylation of endogenous proteins was assayed as described in Fig. 1. and analyzed by two-dimensional NEPHGE/SDS-PAGE (10% acrylamide concentration).

ed by treatment with TPA for 1 h, which was consistent with the increase by the short-term treatment, 20 min in Fig. 2. Phosphorylated p100 and p66 consisted of three migrating species of pI=5.7 to 6.5, pI= 6.8 and pI=6.9 were completely absent from K562 cells treated with TPA for 24 h.

DISCUSSION

Activation of PKC by TPA is known to involve translocation of the enzyme from the cytosol to the membrane and can result in its down-regulation via proteolytic degradation. It has been suggested that proteolysis of PKC to M-kinase may be a mechanism by which PKC mediates signal transduction. This process may also result in irreversible inactivation of PKC depending on the nature of the protease and susceptibility of the PKC isoform to proteolysis (Huang *et al.*, 1989; Melloni *et al.*, 1987; Pontremoli *et al.*, 1986).

It seems that p130, p100 and p23 are sensitive substrates for certain types of PKC in the particulate fraction of K562 cells, since these phosphoproteins were increased by translocation of PKC to the membrane, and then disappeared with down-regulation of PKC. Regarding the results that although p100 and p23 still existed in the membrane fraction, exogenously added PKC *in vitro* failed to restore their phosphorylations which were diminished with long-term treatment with TPA at a concentration as low as 1 nM, it is likely that down-regulation of PKC results in a change of the interaction of endogenous substrates, p100 and p 23 with the plasma membrane without concominant their displacement to the cytosol.

MARCKS is associated with plasma membranes (Graff *et al.*, 1989a). It bind actin (Thelen *et al.*, 1991) and calmodulin (Graff *et al.*, 1989b) and colocalizes with vinculin, talin, and PKC (Rosen *et al.*, 1990). PKC-dependent phosphorylation removes it from the plasma membrane (Aderem *et al.*, 1988; Thelens *et al.*, 1991). The cycles of membrane attachment and detachment of MARCKS are thought to provide a PKC-sensitive, reversible link between the actin cytoskelecton and plasma membrane (Thelens, *et al.*, 1991).

It is thought that PKC-dependent phosphorylation might render p100 and p23 acessible for a change of their interaction with plasma membrane. Endogenous substrate, p100 or p23, might interact their binding proteins like MARCKS, and the interaction of p100 or p23 with the membrane might be regulated by a mechanism involved in PKC-dependent phosphorylation and PKC down-regulation.

It has been shown that prolonged activation of PKC by TPA is essential for long-term cell responses such as cell proliferation and differentiation (Nishizuka, 1992). In this process, substrate phosphorylation changed by enzyme translocation and subsequent down-regulation might play specifc roles.

In this study, it is suggested that especially phosphoprylated p100 and p23 might play an important role in cellular signaling by PKC and an evidence for regulation of interaction of endogenous substrates with the membrane by PKC down-regulation.

It is attractive to investigate whether phosphoprylated p100 (pH=5.9) would be found *in vivo* as well as

in vitro, and whether protease inhibitors are able to suppress that prolonged activation of PKC depletes phosphorylation of p100 in the particulate fraction of K562 cells, since protease inhibitors can suppress PKC down-regulation due to proteolysis of PKC.

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