Phenylarsine Oxide and H₂O₂ Plus Vanadate Induce Reverse Translocation of Phorbol-Ester-Activated PKC β II

Hideyuki Takahashi, Kingo Suzuki*, and Hideo Namiki

Department of Biology, School of Education, Waseda University, Shinjuku-ku, Tokyo 169-0051, Japan

ABSTRACT. The intracellular localization of protein kinase C (PKC) is important for the regulation of its biological activity. Recently, it was reported that, whereas phorbol esters such as PMA induce prolonged translocation of PKC to the plasma membrane, with physiological stimuli, the translocation of PKC is transient and followed by rapid return to the cytoplasm. In addition, this membrane dissociation of PKC was shown to require both the kinase activity of PKC and the phosphorylation of its carboxyl terminus autophosphorylation sites. However, the detailed molecular mechanism of PKC reverse translocation remains obscure. We demonstrated that in porcine polymorphonuclear leucocytes (PMNs), phenylarsine oxide (PAO), a putative protein tyrosine phosphatase (PTPase) inhibitor, induced reverse translocation of PMA-stimulated PKC β II. Hydrogen peroxide (H₂O₂) in combination with vanadate, both of which are PTPase inhibitors, also induced reverse translocation of PKC β II. H₂O₂ or vanadate alone had little effect on PMA-induced PKC β II translocation. Furthermore, genistein and ethanol, which are inhibitors of tyrosine kinase and phospholipase D, respectively, prevented the PKC β II reverse translocation induced by the PTPase inhibitors. These results indicate, for the first time, that the tyrosine phosphorylation/phospholipase D pathway may be involved in the process of membrane dissociation of PKC.

Key words: Protein kinase C/tyrosine phosphorylation/translocation/phospholipase D/protein-tyrosine phosphatase/neutrophil

Protein kinase C (PKC), a phospholipid-dependent serine/threonine kinase, plays an important role in signal transduction, regulating a number of biological processes such as cell proliferation, differentiation, and gene expression (Nishizuka, 1992; Nishizuka, 1995). Activation of PKC is initiated by stimulation with a wide variety of agonists, including hormones, neurotransmitters, and growth factors. These agonists stimulate guanine nucleotide-binding regulatory protein (G protein)-coupled receptors, and induce the activation of phospholipase C, which leads to the generation of the two important second messengers, diacylglycerol and inositol trisphosphate. Inositol trisphosphate elevates intracellular Ca²⁺ by binding to its intracellular receptors. The increase in intracellular Ca²⁺ and membrane diacylglycerol is the key 'on' switch for PKC activation (Nishizuka, 1992;

Nishizuka, 1995; Newton, 1997).

PKC, upon activation, translocates from the cytosol to the plasma membrane (Farrar and Anderson, 1985). Since tumor-promoting phorbol esters, which are functional analogues of diacylglycerol, are long lived in the cell, for over two decades these molecules have been used as tools for studies of translocation and subcellular localization of PKC (Mochly-Rosen, 1995; Newton, 1995). However, several reports have questioned whether phorbol esters regulate PKC by the same mechanism as diacylglycerol (Kraft and Anderson, 1983; Bazzi and Nelsestuen, 1989a, 1989b; Nelsestuen and Bazzi, 1991; Kazanietz *et al.*, 1992). Recently, with the development of green fluorescent protein-conjugated PKC β II and several of its mutants, Feng *et al.* showed that translocation of PKC to the plasma membrane in response to physiological stimuli is followed by a rapid return of PKC to the cytoplasm, whereas PMA-stimulated PKC remains on the plasma membrane (Feng *et al.*, 1998). This is consistent with the results of previous biochemical studies indicating that diacylglycerol is metabolized very rapidly, whereas phorbol esters such as PMA are metabolically stable (Nishizuka, 1992; Newton, 1995, 1997). Inter-

*To whom correspondence should be addressed: Kingo Suzuki, Ph.D., Department of Biology, School of Education, Waseda University, Shinjuku-ku, Tokyo 169-0051, Japan.

Tel: +81-3-5286-1507, Fax: +81-3-3207-9694

E-mail: kingo@kurenai.waseda.jp

Abbreviations: PKC, Protein kinase C; PMA, phorbol-12-myristate-13-acetate; PMNs, porcine polymorphonuclear leucocytes; PAO, phenylarsine oxide; PTPase, protein tyrosine phosphatase; DAB, 3,3'-diaminobenzidine tetrahydrochloride dihydrate.

estingly, however, Feng and Hannun also showed that reverse translocation of PKC to the cytosol requires both the kinase activity of PKC and the phosphorylation of its carboxyl terminus autophosphorylation sites (Feng and Hannun, 1998). This evidence suggests that PKC reverse translocation is not a default step following diacylglycerol metabolism, but is instead regulated by a complex mechanism requiring both kinase activity and autophosphorylation, and that relocalized PKC may play another important role in the cytosol. However, to date, there have been few reports on the mechanism by which PKC dissociates from the plasma membrane.

To elucidate the mechanism of membrane dissociation of PKC, we hypothesized that phorbol esters lack the physiological-stimuli-specific signal(s) that cause PKC to dissociate from the plasma membrane, rather than inducing prolonged translocation of PKC. Whereas phorbol esters activate PKC by direct interaction with it, agonist-stimulated G-protein-coupled receptors not only induce PKC activation, but also induce rapid protein tyrosine phosphorylation of various signaling molecules (Molloy *et al.*, 1993; Ptasznik *et al.*, 1995; Eguchi and Inagami, 2000). Therefore, we focused on tyrosine phosphorylation as a candidate for the membrane dissociation signal, and examined the effects of protein tyrosine phosphatase (PTPase) inhibitors on PMA-induced PKC β II translocation in porcine polymorphonuclear leukocytes (PMNs), through the use of immunoblotting with antibody specific to PKC β II as previously reported (Tsao and Wang, 1997; Sawai *et al.*, 1997). Here we show that PTPase inhibitors induce reverse translocation of PMA-stimulated PKC β II to the cytosol.

Materials and Methods

Materials

PMA, PAO, genistein, and sodium orthovanadate (Na_3VO_4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PMA (500 $\mu\text{g}/\text{mL}$), PAO (25 mM), and genistein (50 mg/mL) were dissolved in DMSO and stored at -20°C . Ethanol and 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB) was supplied by Kanto Chemical Co. (Tokyo, Japan). H_2O_2 was supplied by Wako Pure Chemical Co. (Tokyo, Japan). Anti-PKC β II polyclonal antibody was purchased from Calbiochem (San Diego, CA). Anti-phosphoserine 660 antibodies and horseradish-peroxidase-conjugated anti-rabbit IgG were purchased from New England Biolabs (Beverly, MA). Peroxidase-conjugated anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). All other chemicals were of analytical grade.

Isolation of PMNs

PMNs were isolated from porcine peripheral blood (Suzuki and

Namiki, 1998a). Briefly, erythrocytes were removed by 0.2% methylcellulose sedimentation followed by hypotonic lysis. After centrifugation, cells were resuspended in PBSG (Ca^{2+} -, Mg^{2+} - free phosphate buffered saline, pH 7.4, containing 5 mM glucose), layered on Ficoll-Paque (Pharmacia Biotech, Japan). Cells were suspended in ice-cold PBSG at 6×10^7 cells/mL and used for experiments immediately. More than 95% of the isolated cells were polymorphonuclear leukocytes, as confirmed by nuclear staining with 2% acetate.

Stimulation and subcellular fractionation of PMNs

Subcellular fractionation was performed as described (Tsao and Wang, 1997; Sawai *et al.*, 1997) with modifications. PMNs (3×10^7 cells/mL) were preincubated with different reagents (as described in the figure legends) at 37°C and then stimulated with PMA for the times indicated. After centrifugation, cells were resuspended in disruption solution (1 mM phenylmethylsulfonylfluoride, 10 $\mu\text{g}/\text{mL}$ leupeptin and antipain in PBSG). After sonication, the lysate was centrifuged at $2,000 \times g$ for 30 s to remove nuclei and unbroken cells, and then further centrifuged at $30,000 \times g$ for 40 min at 4°C to yield cytosol (supernatant) and membrane (pellet) fractions.

Lysis of PMNs

For detection of tyrosine phosphorylation, PMNs were lysed on ice in lysis buffer (10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM orthovanadate, 1 mM phenylmethylsulfonylfluoride, 10 $\mu\text{g}/\text{mL}$ leupeptin and antipain). The lysate was centrifuged at $2,000 \times g$ for 30 s, and the supernatants separated by 8% SDS-PAGE followed by immunoblotting, as described below. The tyrosine phosphorylated proteins were detected with anti-phosphotyrosine antibody 4G10.

SDS-PAGE and immunoblot analysis

Protein samples from equal numbers of cells were separated by 8% SDS-PAGE, and electrophoretically transferred to ImmobilonTM membranes (Millipore, Bedford, MA). Membranes were blocked in 5% dried milk — PBS, and then incubated with primary antibodies diluted in 5% dried milk — PBS for 1 h. Membranes were washed in PBS containing 0.05% Tween 20 and incubated with HRP-labeled secondary antibodies diluted in 5% dried milk — PBS for 1 h. DAB color reaction was used for visualization of the protein bands.

Results

To investigate whether tyrosine phosphorylation regulates membrane dissociation of PKC, we examined the effects of phenylarsine oxide (PAO), a putative phosphotyrosine phosphatase (PTPase) inhibitor, on PMA-induced PKC β II translocation. PMNs were stimulated with PMA for 30 min with or without pretreatment with PAO for 10 min, and the cytosolic and membrane fractions were analyzed by immu-

noblotting using antibody specific to PKC β II. At low concentrations (2–10 μ M), PAO induced an increase of PKC β II in the cytosolic fraction (Fig. 1A). High concentrations of PAO (≥ 12 μ M) did not induce an increase in PKC β II levels in the cytosolic fraction (Fig. 1A) (see *Discussion*). Therefore, to determine whether low concentrations of PAO induced reverse translocation of PMA-stimulated PKC β II, we examined the time-course of the effect of 4 μ M PAO on PMA-stimulated PKC β II. In contrast to PMA alone, pretreatment with 4 μ M PAO caused relocalization of PKC β II to the cytosolic fraction at 30–60 min (Fig. 1B). PAO alone at 4 μ M had no effect on PKC β II localization (data not shown). To confirm that PAO induced the change of

PKC β II distribution, but not the increase of total PKC β II, the lysates after sonication were subjected to SDS-PAGE and immunoblotting, and then densitometric evaluation was performed with NIH Image version 1.61. No changes in total PKC β II were observed by treatment with 4 μ M PAO (Fig. 1C, densitometric data not shown). Furthermore, PAO induced PKC β II relocalization even after preincubation with protein synthesis inhibitor cycloheximide (data not shown). These results suggest that low concentrations of PAO induce reverse translocation of PKC β II, but not the increase of total PKC β II.

Previous studies have shown that autophosphorylation of PKC occurs during maturation (Newton, 1995, 1997) and

Fig. 1. Effects of PAO on PMA-induced translocation of PKC β II.

A) PMNs were stimulated with 500 ng/mL PMA for 30 min following pretreatment with the concentrations of PAO indicated for 10 min.

B) PMNs were pretreated with 4 μ M PAO for 10 min, and then stimulated with 500 ng/mL PMA for 0, 5, 30, and 60 min.

C) PMNs were pretreated with 4 μ M PAO for 10 min, and then stimulated with 500 ng/mL PMA for 0, 5, and 30 min.

D) PMNs were pretreated with or without 100 nM staurosporine for 1 h and pretreated with 4 μ M PAO for 10 min, followed by stimulation with 500 ng/mL PMA for 30 min.

PKC β II (A, B, C, and D upper) and the phosphorylated form of PKC β II (D lower) were detected by immunoblotting using anti-PKC β II and anti-phospho-PKC (Ser660) antibody, respectively. Results are representative of three separate experiments.

regulates the cellular localization of PKC (Newton, 1997; Edwards *et al.*, 1999). In addition, reverse translocation of PKC has been shown to require autophosphorylation of the carboxyl terminus (Feng and Hannun, 1998). Reversal has also been shown to require kinase activity of the enzyme (Feng and Hannun, 1998). Therefore, we evaluated the phosphorylation state of the PKC β II carboxyl terminus in PAO- and PMA-treated PMNs, and examined whether the PKC kinase inhibitor staurosporine affected PAO-induced PKC β II reverse translocation. PMNs were pretreated with or without staurosporine for 1 h and pretreated with 4 μ M PAO for 10 min, followed by stimulation with PMA for 30 min. The cytosolic and membrane fractions were analyzed by immunoblotting with anti-phospho-PKC (Ser660) antibody. The relocalized PKC β II maintained the phosphorylation of its carboxyl autophosphorylation site, and staurosporine completely inhibited the reverse translocation of PKC β II that had been induced by 4 μ M PAO (Fig. 1D). These results indicate that PAO-induced PKC β II reverse translocation may require both kinase activity and autophosphorylation of the carboxyl terminus, consistent with previous studies that used staurosporine and several PKC β II mutants (Feng and Hannun, 1998).

Vanadate has also been used as an inhibitor of PTPases (Gordon, 1991), and it has been proposed that H₂O₂ increases tyrosine phosphorylation of cellular proteins by inhibiting PTPases (Finkel, 1998; Rhee, 1999). Therefore, we examined whether H₂O₂ and vanadate affected PMA-induced translocation. PMNs were pretreated with 1 mM Na₃VO₄ for 10 min, followed by stimulation with PMA for 5 min and then treatment with 1 mM H₂O₂ for 25 min. Although H₂O₂ or Na₃VO₄ alone had little effect on PMA-induced PKC β II translocation, H₂O₂ in combination with Na₃VO₄ induced reverse translocation of PKC β II (Fig. 2A). In addition, the reverse translocation was dose-dependent for H₂O₂ (Fig. 2B). Staurosporine also inhibited PKC β II reverse translocation induced by H₂O₂ plus Na₃VO₄, and relocalized PKC β II maintained the phosphorylation of its carboxyl autophosphorylation site (Fig. 2C), consistent with PAO-induced PKC β II reversal. These results show that PTPase inhibitors induce reverse translocation of PMA-stimulated PKC β II, and suggest that tyrosine phosphorylation is involved in the membrane dissociation of PKC β II.

PAO forms stable ring complexes with vicinal or neighboring thiols. As a result, PAO inhibits PTPases that have a cysteine residue in their active site (Garcia-Morales *et al.*, 1990; Fischer *et al.*, 1991). H₂O₂ is also proposed to oxidize the active site cysteine residue of PTPases (Finkel, 1998; Rhee, 1999). Therefore, other vicinal-dithiol-containing proteins may be targeted by PAO or H₂O₂ in our system. To determine whether the observed effects on PKC β II cellular distribution were specifically due to PAO- or H₂O₂ plus Na₃VO₄-induced changes in tyrosine phosphorylation, and not to other possible activities of PAO or H₂O₂, the following two experiments were performed. First, protein tyrosine

phosphorylations of both PTPase inhibitors and PMA-treated PMNs were analyzed by using anti-phosphotyrosine specific antibody 4G10. PAO (4 μ M) or H₂O₂ (0.05–1 mM) in combination with 1 mM Na₃VO₄, which causes PKC β II reverse translocation, increased tyrosine phosphorylation of cellular proteins (Fig. 3), consistent with other published data (Garcia-Morales *et al.*, 1990; Yousefi *et al.*, 1994; Yamamoto *et al.*, 2000). Furthermore, the tyrosine kinase inhibitor genistein prevented PAO- or H₂O₂ plus Na₃VO₄-induced PKC β II reverse translocation (Fig. 4). These results support the suggestion that tyrosine phosphorylation is involved in PKC β II reverse translocations induced by PTPase inhibitors.

There is evidence for the involvement of protein tyrosine phosphorylation in the regulation of phospholipase D activity in several types of cell (Exton, 1999; Uings *et al.*, 1992). This is indicated by numerous observations that vanadate in combination with H₂O₂, or PAO, stimulates phospholipase D activity *in vivo* (Bourgoin and Grinstein, 1992; Kumada *et al.*, 1994; Kumada *et al.*, 1996; Min *et al.*, 1998; Taher *et al.*, 1998). Further support comes from the observations that tyrosine kinase inhibitors such as genistein, herbimycin or tyrphostin, inhibit phospholipase D activation in several cell types (Uings *et al.*, 1992; Schmidt *et al.*, 1994; Ward *et al.*, 1995; Jinsi *et al.*, 1996; Natarajan *et al.*, 1996; Ito *et al.*, 1997; Exton, 1999). These reports prompted us to investigate whether phospholipase D activity is involved in PTPase-inhibitor-induced PKC β II reverse translocations. It is known that primary alcohols such as ethanol and 1-butanol block phospholipase-D-mediated phosphatidic acid production by competing with water (English *et al.*, 1996; Sergeant *et al.*, 2001). Therefore, to investigate whether ethanol affects PTPase-inhibitor-induced PKC β II reverse translocations, PMNs were pretreated with PAO or Na₃VO₄ for 10 min in the presence of 2% ethanol, and then treated with PMA alone or with PMA and H₂O₂. Ethanol prevented PAO- or H₂O₂ plus Na₃VO₄-induced PKC β II reverse translocation (Fig. 5). One percent 1-butanol also prevented PAO- or H₂O₂ plus Na₃VO₄-induced PKC β II reverse translocation (data not shown). These results suggest that phospholipase D may be involved in these PTPase-inhibitor-induced PKC β II reverse translocations.

Discussion

Our aim was to investigate the mechanism of membrane dissociation of PKC. We hypothesized that stimulation by phorbol esters such as PMA lacks the signal required for dissociation of PKC from the membrane rather than causing prolonged translocation of PKC. Here, we provided evidence that if PTPase inhibitors are used to increase tyrosine phosphorylation, then PMA-stimulated PKC β II dissociates from the plasma membrane. These results suggest that tyrosine phosphorylation is involved in PKC membrane dissociation. This suggestion is supported by further observa-

Fig. 2. Effects of H₂O₂ and vanadate on PMA-induced translocation of PKCβII.

A) PMNs were pretreated with or without 1 mM Na₃VO₄ for 10 min, followed by stimulation with 500 ng/mL PMA for 5 min and then treatment with or without 1 mM H₂O₂ for 25 min.

B) PMNs were pretreated with 1 mM Na₃VO₄ for 10 min, followed by stimulation with 500 ng/mL PMA for 5 min, and then treatment with the indicated concentrations of H₂O₂ for 25 min.

C) PMNs were pretreated with or without 100 nM staurosporine for 1 h and pretreated with 1 mM Na₃VO₄ for 10 min, followed by stimulation with 500 ng/mL PMA for 5 min and then treatment with 1 mM H₂O₂ for 25 min.

PKCβII (A, B, and C upper) and the phosphorylated form of PKCβII (C lower) were detected by immunoblotting using anti-PKCβII and anti-phospho-PKC (Ser660) antibody, respectively. Results are representative of three separate experiments.

tions that the tyrosine kinase inhibitor genistein prevented PTPase-inhibitor-induced PKCβII reverse translocation. In addition, ethanol also prevented PTPase-inhibitor-induced PKCβII reverse translocation, suggesting that PKCβII membrane dissociation may be mediated by phospholipase D.

In our results using PMNs, the effect of PAO on reverse translocation was specific only at low concentrations. Several published data have shown that the effects of PAO are greatly changed by changes in its concentration (Yousefi *et al.*, 1994; Suzuki and Namiki, 1998b), perhaps because of its pharmacological capacity to react with vicinal dithiol in

cellular proteins (Garcia-Morales *et al.*, 1990). All vicinal-dithiol-containing proteins can be targeted by PAO. In our study, it is possible that at high concentrations, PAO non-specifically affected other vicinal-dithiol-containing proteins besides PTPases, and that the high concentrations of PAO therefore perturbed the signal transduction for reverse translocation of PKCβII. In contrast with the effects of PAO, that of H₂O₂ plus Na₃VO₄ was dose-dependent for H₂O₂. The mechanism of the difference in action of PAO and H₂O₂ plus Na₃VO₄ remains unclear. As it is known that H₂O₂ induces tyrosine phosphorylation of certain PKC isozymes (Konishi *et al.*, 1997), it is possible that high con-

Fig. 3. PAO or H₂O₂ plus vanadate induces tyrosine phosphorylation of cellular proteins in PMNs.

A) PMNs were pretreated with 4 μ M PAO for 10 min, and then stimulated with 500 ng/mL PMA for 30 min. The lysates were directly subjected to immunoblotting using anti-phosphotyrosine antibody 4G10.

B) PMNs were pretreated with 1 mM Na₃VO₄ for 10 min, followed by stimulation with 500 ng/mL PMA for 5 min and then treatment with 1 mM H₂O₂ for 25 min. The lysates were directly subjected to immunoblotting using anti-phosphotyrosine antibody 4G10.

Results are representative of three separate experiments.

centrations of H₂O₂ not only inhibit PTPases, but also induce tyrosine phosphorylation of PKC β II. Therefore, tyrosine phosphorylation of PKC by H₂O₂ may play another important role in PKC membrane dissociation.

In other studies, ceramide, a product of sphingomyelin hydrolysis by sphingomyelinase, has been demonstrated to induce translocation from the membrane to the cytosol of PKC δ and ϵ in HL-60, U937, and HBP-ALL cells (Sawai *et al.*, 1997). In addition, more recently, both exogenous and endogenous ceramide were demonstrated to induce reverse translocation of PMA-stimulated classical PKCs in Jurkat cells and MCF7 cells (Signorelli *et al.*, 2001). In these studies, ceramide may have acted, perhaps indirectly, as a

membrane dissociation signal. However, it has been reported that ceramide inhibits the activity of phospholipase D *in vitro* (Venable *et al.*, 1996) and *in vivo* (Gomez-Munoz *et al.*, 1995; Nakamura *et al.*, 1996; Mansfield *et al.*, 2002). In addition, in our system both exogenous and endogenous ceramides have been unsuccessful in inducing reverse translocation of PKC β II (data not shown). Therefore, the relationship between tyrosine phosphorylation/phospholipase D and ceramide in PKC membrane dissociation remains unclear.

With regard to physiological stimuli, PKC reverse translocation was shown to require both kinase activity and autophosphorylation of the carboxyl terminal of PKC (Feng and Hannun, 1998). We demonstrated that both PAO- and H₂O₂ plus Na₃VO₄-induced PKC β II reverse translocations were inhibited by the PKC kinase inhibitor staurosporine, and that PKC β II relocalized to the cytosol maintained its carboxyl terminus autophosphorylation. However, the reason why PKC reverse translocation requires kinase activity and autophosphorylation is not known. Min *et al.* reported that selective PKC inhibitors (Ro31-8220 and calphostin C) and down-regulation of PKC inhibited activation of phospholipase D by H₂O₂ (Min *et al.*, 1998). Therefore, it is possible that in PKC β II reverse translocations induced by PTPase inhibitors, kinase activity of PKC β II is required for activation of phospholipase D. Of course, other mechanisms are possible.

It has been reported that PMA stimulation induced a marked degradation of PKC β II in human neutrophils (Kent *et al.*, 1996). In our system, the presence of numerous ladder bands and the decrease of the major band of PKC β II were observed in the membrane fraction of PMA-treated PMNs, but not in that of PTPase-inhibitors-pretreated PMNs (data not shown). These observations suggest that membrane-bound PKC β II in response to PMA undergoes degradation by some proteinases, and that PTPase inhibitors prevented the degradation of PKC β II by some unknown mechanism. However, the relationship between the degradation and membrane dissociation of PKC β II remains unclear. This is currently under investigation in our labora-

Fig. 4. Effects of genistein on PTPase-inhibitor-induced reverse translocation of PKC β II. PMNs were pretreated with 75 μ g/mL genistein for 1 h and then treated with 4 μ M PAO or 1 mM H₂O₂ in combination with 1 mM Na₃VO₄, and 500 ng/mL PMA as described above. PKC β II was detected by immunoblotting using anti-PKC β II antibody. Results are representative of three separate experiments.

Fig. 5. Effects of ethanol on PTPase-inhibitor-induced reverse translocation of PKC β II. PMNs were pretreated with 4 μ M PAO or 1 mM Na₃VO₄ for 10 min in the presence of 2% ethanol, and then stimulated with 500 ng/mL PMA and 1 mM H₂O₂ as described above. PKC β II was detected by immunoblotting using anti-PKC β II antibody. Results are representative of three separate experiments.

tory.

In conclusion, our findings provide the first evidence that tyrosine phosphorylation and perhaps phospholipase D are important for dissociation of PKC from the membrane. To date, few studies of PKC reverse translocation in response to physiological stimuli and the role of PKC relocated to the cytosol have been performed. Elucidation of these problems is a promising area of future study. We expect that our findings and analytical method will provide a clue to the study of the mechanism of PKC membrane dissociation.

References

- Bazzi, M.D. and Nelstuen, G.L. 1989a. Differences in the effects of phorbol esters and diacylglycerols on protein kinase C. *Biochemistry*, **28**: 9317–9323.
- Bazzi, M.D. and Nelstuen, G.L. 1989b. Properties of the protein kinase C-phorbol ester interaction. *Biochemistry*, **28**: 3577–3585.
- Bourgoin, S. and Grinstein, S. 1992. Peroxides of vanadate induce activation of phospholipase D in HL-60 cells. *J. Biol. Chem.*, **267**: 11908–11916.
- Edwards, A.S., Faux, M.C., Scott, J.D., and Newton, A.C. 1999. Carboxyl-terminal phosphorylation regulates the function and subcellular localization of protein kinase C β II. *J. Biol. Chem.*, **274**: 6461–6468.
- Eguchi, S. and Inagami, T. 2000. Signal transduction of angiotensin II type 1 receptor through receptor tyrosine kinase. *Regul. Pept.*, **91**: 13–20.
- English, D., Cui, Y., and Siddiqui, R.A. 1996. Messenger functions of phosphatidic acid. *Chem. Phys. Lipids*, **80**: 117–132.
- Exton, J.H. 1999. Regulation of phospholipase D. *Biochim. Biophys. Acta*, **1439**: 121–133.
- Farrar, W.L. and Anderson, W.B. 1985. Interleukin-2 stimulates association of protein kinase C with plasma membrane. *Nature*, **315**: 233–235.
- Feng, X., Zhang, J., Barak, L.S., Meyer, T., Caron, M.G., and Hannun, Y.A. 1998. Visualization of dynamic trafficking of a protein kinase C β II/green fluorescent protein conjugate reveals differences in G protein-coupled receptor activation and desensitization. *J. Biol. Chem.*, **273**: 10755–10762.
- Feng, X. and Hannun, Y.A. 1998. An essential role for autophosphorylation in the dissociation of activated protein kinase C from the plasma membrane. *J. Biol. Chem.*, **273**: 26870–26874.
- Feng, X., Becker, K.P., Stribling, S.D., Peters, K.G., and Hannun, Y.A. 2000. Regulation of receptor-mediated protein kinase C membrane trafficking by autophosphorylation. *J. Biol. Chem.*, **275**: 17024–17034.
- Finkel, T. 1998. Oxygen radicals and signaling. *Curr. Opin. Cell Biol.*, **10**: 248–253.
- Fischer, E.H., Charbonneau, H., and Tonks, N.K. 1991. Protein tyrosine phosphatases: a diverse family of intracellular and transmembrane enzymes. *Science*, **253**: 401–406.
- Garcia-Morales, P., Minami, Y., Luong, E., Klausner, R.D., and Samelson, L.E. 1990. Tyrosine phosphorylation in T cells is regulated by phosphatase activity: studies with phenylarsine oxide. *Proc. Natl. Acad. Sci. USA*, **87**: 9255–9259.
- Gomez-Munoz, A., Waggoner, D.W., O'Brien, L., and Brindley, D.N. 1995. Interaction of ceramides, sphingosine, and sphingosine 1-phosphate in regulating DNA synthesis and phospholipase D activity. *J. Biol. Chem.*, **270**: 26318–26325.
- Gordon, J.A. 1991. Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. *Methods Enzymol.*, **201**: 477–482.
- Ito, Y., Nakashima, S., Kanoh, H., and Nozawa, Y. 1997. Implication of Ca²⁺-dependent protein tyrosine phosphorylation in carbachol-induced phospholipase D activation in rat pheochromocytoma PC12 cells. *J. Neurochem.*, **68**: 419–425.
- Jinsi, A., Paradise, J., and Deth, R.C. 1996. A tyrosine kinase regulates α -adrenoceptor-stimulated contraction and phospholipase D activation in the rat aorta. *Eur. J. Pharmacol.*, **302**: 183–190.
- Kazanietz, M.G., Krausz, K.W., and Blumberg, P.M. 1992. Differential irreversible insertion of protein kinase C into phospholipid vesicles by phorbol esters and related activators. *J. Biol. Chem.*, **267**: 20878–20888.
- Kent, J.D., Sergeant, S., Burns, D.J., and McPhail, L.C. 1996. Identification and regulation of protein kinase C δ in human neutrophils. *J. Immunol.*, **265**: 706–712.
- Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U., and Nishizuka, Y. 1997. Activation of protein kinase C by tyrosine phosphorylation in response to H₂O₂. *Proc. Natl. Acad. Sci. USA*, **94**: 11233–11237.
- Kraft, A.S. and Anderson W.B. 1983. Phorbol esters increase the amount of Ca²⁺, phospholipid-dependent protein kinase associated with plasma membrane. *Nature*, **301**: 621–623.
- Kumada, T., Nakashima, S., Miyata, H., and Nozawa, Y. 1994. Potent activation of phospholipase D by phenylarsine oxide in rat basophilic leukemia (RBL-2H3) cells. *Biochem. Biophys. Res. Commun.*, **199**: 792–798.
- Kumada, T., Nakashima, S., Nakamura, Y., Miyata, H., and Nozawa, Y. 1996. Phenylarsine oxide (PAO)-mediated activation of phospholipase D in rat basophilic leukemia (RBL-2H3) cells: possible involvement of calcium and protein kinase C. *Immunobiology*, **195**: 347–359.
- Mansfield, P.J., Hinkovska-Galcheva, V., Carey, S.S., Shayman, J.A., and Boxer, L.A. 2002. Regulation of polymorphonuclear leukocyte degranulation and oxidant production by ceramide through inhibition of phospholipase D. *Blood*, **99**: 1434–1441.

- Min, D.S., Kim, E.G., and Exton, J.H. 1998. Involvement of tyrosine phosphorylation and protein kinase C in the activation of phospholipase D by H_2O_2 in Swiss 3T3 fibroblasts. *J. Biol. Chem.*, **273**: 29986–29994.
- Mochly-Rosen, D. 1995. Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science*, **268**: 247–251.
- Molloy, C.J., Taylor, D.S., and Weber, H. 1993. Angiotensin II stimulation of rapid protein tyrosine phosphorylation and protein kinase activation in rat aortic smooth muscle cells. *J. Biol. Chem.*, **268**: 7338–7345.
- Nakamura, Y., Nakashima, S., Ojio, K., Banno, Y., Miyata, H., and Nozawa, Y. 1996. Ceramide inhibits IgE-mediated activation of phospholipase D, but not of phospholipase C, in rat basophilic leukemia (RBL-2H3) cells. *J. Immunol.*, **156**: 256–262.
- Natarajan, V., Scribner, W.M., and Vepa, S. 1996. Regulation of phospholipase D by tyrosine kinases. *Chem. Phys. Lipids*, **80**: 103–116.
- Nelsestuen, G.L. and Bazzi M.D. 1991. Activation and regulation of protein kinase C enzymes. *J. Bioenerg. Biomembr.*, **23**: 43–61.
- Newton, A.C. 1995. Protein kinase C: structure, function, and regulation. *J. Biol. Chem.*, **270**: 28495–28498.
- Newton, A.C. 1997. Regulation of protein kinase C. *Curr. Opin. Cell Biol.*, **9**: 161–167.
- Nishizuka, Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science*, **258**: 607–614.
- Nishizuka, Y. 1995. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.*, **9**: 484–496.
- Ptasznik, A., Traynor-Kaplan, A., and Bokoch, G.M. 1995. G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase/Shc adapter protein signaling complexes. *J. Biol. Chem.*, **270**: 19969–19973.
- Rhee, S.G. 1999. Redox signaling: hydrogen peroxide as intracellular messenger. *Exp. Mol. Med.*, **31**: 53–59.
- Sawai, H., Okazaki, T., Takeda, Y., Tashima, M., Sawada, H., Okuma, M., Kishi, S., Umehara, H., and Domae N. 1997. Ceramide-induced translocation of protein kinase C- δ and - ϵ to the cytosol. *J. Biol. Chem.*, **272**: 2452–2458.
- Schmidt, M., Huwe, S.M., Fasselt, B., Homann, D., Rumenapp, U., Sandmann, J., and Jakobs, K.H. 1994. Mechanisms of phospholipase D stimulation by m3 muscarinic acetylcholine receptors. Evidence for involvement of tyrosine phosphorylation. *Eur. J. Biochem.*, **225**: 667–675.
- Sergeant, S., Waite, K.A., Heravi, J., and McPhail, L.C. 2001. Phosphatidic acid regulates tyrosine phosphorylating activity in human neutrophils. *J. Biol. Chem.*, **276**: 4737–4746.
- Signorelli, P., Luberto, C., and Hannun, Y.A. 2001. Ceramide inhibition of NF- κ B activation involves reverse translocation of classical protein kinase C (PKC) isoenzymes: requirement for kinase activity and carboxyl-terminal phosphorylation of PKC for the ceramide response. *FASEB J.*, **15**: 2401–2414.
- Suzuki, K. and Namiki, H. 1998a. Phorbol 12-myristate 13-acetate induced cell death of porcine peripheral blood polymorphonuclear leucocytes. *Cell Struct. Funct.*, **23**: 367–372.
- Suzuki, K. and Namiki, H. 1998b. Phenylarsine oxide and ethanol prevent cell death of porcine polymorphonuclear leucocytes induced by phorbol myristate acetate. *Cell Struct. Funct.*, **23**: 389–393.
- Taher, M.M., Mahgoub, M.A., and Abd-Elfattah, A.S. 1998. Redox regulation of signal transduction in vascular smooth muscle cells: thiol oxidizing agents induced phospholipase D. *Biochem. Mol. Biol. Int.*, **46**: 619–628.
- Tsao, L.T. and Wang, J.P. 1997. Translocation of protein kinase C isoforms in rat neutrophils. *Biochem. Biophys. Res. Commun.*, **234**: 412–418.
- Uings, I.J., Thompson, N.T., Randall, R.W., Spacey, G.D., Bonser, R.W., Hudson, A.T., and Garland, L.G. 1992. Tyrosine phosphorylation is involved in receptor coupling to phospholipase D but not phospholipase C in the human neutrophil. *Biochem. J.*, **281**: 597–600.
- Venable, M.E., Bielawska, A., and Obeid, L.M. 1996. Ceramide inhibits phospholipase D in a cell-free system. *J. Biol. Chem.*, **271**: 24800–24805.
- Ward, D.T., Ohanian, J., Heagerty, A.M., and Ohanian, V. 1995. Phospholipase D-induced phosphatidate production in intact small arteries during noradrenaline stimulation: involvement of both G-protein and tyrosine-phosphorylation-linked pathways. *Biochem. J.*, **307**: 451–456.
- Yamamoto, T., Matsuzaki, H., Konishi, H., Ono, Y., and Kikkawa, U. 2000. H_2O_2 -induced tyrosine phosphorylation of protein kinase C δ by a mechanism independent of inhibition of protein-tyrosine phosphatase in CHO and COS-7 cells. *Biochem. Biophys. Res. Commun.*, **273**: 960–966.
- Yousefi, S., Green, D.R., Blaser, K., and Simon, H.U. 1994. Protein-tyrosine phosphorylation regulates apoptosis in human eosinophils and neutrophils. *Proc. Natl. Acad. Sci. USA*, **91**: 10868–10872.

(Received for publication, November 21, 2002

and accepted, March 4, 2003)