Tamoxifen Modulates Protein Kinase C via Oxidative Stress in Estrogen Receptor-negative Breast Cancer Cells*

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Nonsteroidal agent tamoxifen (Tam), a therapeutic/chemopreventive agent for breast cancer, inhibits protein kinase C (PKC), which is considered to be one of its extra-estrogen receptor sites of action. This drug is required at higher (>100 μM) concentrations to inhibit PKC in the test tube, whereas it is required at lower (1–10 μM) concentrations to induce inhibition of cell growth in estrogen receptor-negative cell types. To identify additional mechanisms of action of Tam on PKC and cell growth, studies with MDA-MB-231, an estrogen receptor-negative breast carcinoma cell type, have been carried out. Upon treatment with 5–20 μM Tam, a cytosol to membrane translocation of PKC occurred within 30 min, which was then followed by a down-regulation of the enzyme within 2 h. A transient generation of Ca²⁺/lipid-independent activated form of PKC was observed during this period. Rapidly growing cells require nearly 2–3-fold lower concentrations (2–5 μM) of Tam than do confluent cells to induce changes in PKC. Furthermore, phorbol ester binding observed with intact cells also decreased in Tam-treated cells only under the conditions PKC was inactivated. Unlike phorbol esters, Tam did not directly support the membrane association of PKC. The release of arachidonic acid correlated with the PKC membrane translocation. Studies carried out with [³H]Tam revealed that Tam partitioned into the membrane, and there was no appreciable covalent association of [³H]Tam with cellular proteins within this limited time period (2 h). Various antioxidants (vitamin E, vitamin C, β-carotene, catalase, and superoxide dismutase) inhibited all these cellular effects of Tam. Moreover, vitamin E strikingly blocked Tam-induced growth inhibition. To determine whether oxymetabolites of Tam can affect PKC permanently, OH-Tam was tested with purified PKC. In contrast to Tam, which reversibly inhibited PKC, OH-Tam permanently inactivated the enzyme by modifying the catalytic domain at lower concentrations. The vicinal thiols present within this domain were found to be required to induce this inactivation. This effect was partially blocked by various antioxidants. This is the first report showing the role of oxidative stress in mediating the actions of Tam. Taken together these results suggest that Tam, by initially partitioning into the membranes, induces a generation of transmembrane signals and an oxidative stress to elicit the membrane association of PKC, followed by an irreversible activation, and subsequent down-regulation of this enzyme, which, in part, may lead to cell growth inhibition.

Tamoxifen (Tam) is a synthetic nonsteroidal anti-estrogen that is widely used for the chemotherapy of breast cancer and is currently being evaluated for chemoprevention of this disease (1–3). The antiproliferative actions of Tam and other triphenylethylene derivatives at submicromolar concentrations in estrogen-dependent breast carcinoma cells are believed to be mediated by high affinity binding to the estrogen receptor (ER) (4–8). The effects induced by submicromolar concentrations of Tam can be overcome by a high concentration of estrogen (4–8). However, Tam also inhibits growth of ER-negative cell lines at low (1–10 μM) concentrations, which is not overcome by estrogen (8–11). Furthermore, it inhibits growth of other cell types, which have no ER, in some cases at nanomolar concentrations (11). The mechanism of such ER-independent inhibition of tumor cell growth by Tam is not clearly known. Binding to so-called anti-estrogen sites and the inhibitions of calmodulin and protein kinase C (PKC) are considered to be some known additional sites of action of Tam-related agents (12–16).

Since PKC may play a crucial role in the signal transduction that influences cell growth and transformation (17, 18), the observation of the inhibition of PKC by Tam has gained considerable attention. Purified PKC has been shown to be reversibly inhibited by Tam-related agents in the test tube (15, 16). This inhibition seems to involve a number of different mechanisms including binding of the drug to phospholipids that are required for activation, binding to the catalytic domain at ATP-binding site, and binding to the regulatory domain (15, 16, 19–21). Moreover, the modes of action have been found to be slightly different for individual types of triphenylethylene anti-estrogens tested (22). The metabolites of Tam, 4-hydroxysteroids (OH-Tam) and N-demethyltamoxifen, have also been shown to inhibit PKC in a reversible manner (23). The inhibition of PKC in the test tube requires higher concentrations of Tam (IC₅₀ 100 μM) (15), while the ER-independent cell growth inhibition requires only 1–5 μM Tam. This raises the possibility that Tam induces growth inhibition at this low concentrations by acting on cellular targets other than PKC and/or by inducing other cellular mechanism(s), which may complement its action at PKC.

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¹ The abbreviations used are: Tam, tamoxifen; OH-Tam, 4-hydroxysteroid; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-acetate; MEM, minimum essential medium; ER, estrogen receptor; FCS, fetal calf serum; SOD, superoxide dismutase; DTT, dithiothreitol.
We have been involved in studies of regulation of PKC by oxygen radicals and thiol modifying agents (24, 25). Such studies also facilitated understanding of the mechanisms of inactivation of PKC by its commonly used inhibitors, calphostin C, hypericin, and chelerythrine. These inhibitors induce an irreversible inactivation of PKC either involving oxygen radical production or alkylating mechanisms (24). PKC inhibitors are classified based on their site of action, either related to regulatory or to catalytic domains. Based on the mechanism of action, these inhibitors also can be classified as reversible inhibitors or irreversible inactivators (24). However, in order to identify irreversible inhibitors, it is not only necessary to evaluate them using an isolated enzyme in the test tube, it is also important to study them in intact cells. A reversible inhibitor in test tube could become an irreversible inactivator after metabolic activation. This is especially important for Tam, which is known to be oxidatively metabolized in the cell and can form reactive metabolites that can covalently bind to DNA and proteins (26–29).

Tamoxifen also has been shown to stimulate the Ca\(^{2+}\)/phospholipid-dependent PKC activity in test tube at high concentrations of Ca\(^{2+}\) (30). Unlike in test tubes, where Tam inhibited PKC-mediated phosphorylation of proteins, with intact cells Tam did not inhibit phorbol ester-induced phosphorylation of endogenous proteins, but instead by itself stimulated the phosphorylation of some endogenous proteins (31). Recent studies have shown that staurosporine, an inhibitor for PKC in a test tube, could function as an activator of PKC in intact cells inducing the membrane translocation of PKC (32–34). Staurosporine also induced a release of arachidonic acid, which is a common effect induced by a variety of structurally unrelated tumor promoters (32, 35). In fact, in the mouse skin carcinogenesis model, staurosporine acts as a tumor promoter (32, 33). In this context it is noteworthy that Tam acts as a chemopreventive agent in mammary carcinogenesis, while it can also induce uterine cancer in humans as well as liver cancer in rats (10, 36–38). Therefore, it is important to extend the studies of Tam to intact cells to understand whether it directly or after metabolic activation can influence PKC or the upstream signal transduction mechanisms in a bimodal manner. However, to date, such studies have not been carried out with Tam in intact cells.

In this report we show that Tam and other related agents at low (1–10 \(\mu M\)) concentrations can induce the membrane association of PKC, the irreversible association and the subsequent down-regulation of the enzyme involving the generation of transmembrane signals, and oxidative regulation in a ER-negative breast cancer cell line, MBA-MB-231. Unlike Tam, which inhibits PKC in a reversible way, at least one of its oxymetabolites, 4-hydroxytamoxifen (OH-Tam), can induce an irreversible oxidative inactivation of the isolated kinase, probably by interacting with the vicinal thiol(s) present within the catalytic domain.

**EXPERIMENTAL PROCEDURES**

Materials—Tamoxifen (trans) citrate, catalase from bovine liver, and superoxide dismutase from bovine liver were obtained from Sigma. Vitamin E (\(\alpha\)-tocopherol) and \(\beta\)-carotene were from Fluka. [N-methyl-\(^{2}\)H]Tamoxifen citrate (specific activity 84 Ci/mmol) was mass from Amer sham Corp.; [\(^{3}H\)]phorbol 12,13-dibutyrate (specific activity 20 Ci/mmol), and [\(^{32}P\)]orthophosphate (specific activity 84 Ci/mmol) were obtained from Du Pont NEN; [\(^{3}H\)]ATP (specific activity 20 Ci/mmol), [\(^{3}H\)]thymidine (specific activity 20 Ci/mmol), and \(^{45}Ca\)Cl\(_2\) (specific activity 12 mCi/mg Ca) were from ICN. 4-Hydroxytamoxifen was a generous gift from Besins Iscovesco Laboratories, Paris. Human breast carcinoma cell lines were obtained from the American Type Culture Collection.

Rabbit brain PKC (a mixture of \(\alpha\), \(\beta\), and \(\gamma\) isoenzymes) was purified as described previously (24). Unless otherwise mentioned, unfraccionated mixture of these Ca\(^{2+}\)-dependent isoenzymes of PKC was used for modification studies. In some cases individual isoenzymes separated by hydroxyapatite (39) were used. The catalytic and regulatory domains were separated after treating PKC with a low (1 \(\mu\)g/ml) concentration of trypsin (19).

**DEAE-Cellulose Chromatographic Isolation of PKC from Cells**—Cells were grown in 100-mm Petri dishes in minimal essential medium with Earl's salts (MEM) supplemented with 5% fetal calf serum. Either subconfluent (25%) or confluent cells were changed to fresh serum-free MEM and incubated with various concentrations of Tam, OH-Tam, or control vehicle (ethanol) for the indicated period of time at 37 °C. After the soluble and detergent-solubilized membrane fractions were prepared from the treated cells and subjected to DEAE-cellulose chromatography as described previously (40). The Ca\(^{2+}\)/phospholipid-stimulated PKC activity (proform) was eluted using 0.1 M NaCl (peak A) and the Ca\(^{2+}\)/phospholipid-independent and the modified PKC activity was eluted with 0.2 M NaCl (peak B).

**PKC Assay**—The assays of PKC as well as AMP-dependent protein kinase were carried out in 96-well plates with filtered phenol red membranes made of Durapore membranes (40). Briefly, PKC reaction samples containing 20 \(\mu\)l Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 0.33 mM CaCl\(_2\), 0.1 M ATP, histone H1 (0.1 mg/ml), 0.04 \(\mu\)M leupeptin, and 25 \(\mu\)l of PKC sample in a total volume of 125 \(\mu\)l were incubated at 30 °C for 5 min. PKC activity was expressed as units, where 1 unit of enzyme transfers 1 nmol of phosphate to histone H1/min at 30 °C (30). For optimal PDBu binding to purified PKC, the conditions discussed in method 1, described previously, were used (40). To determine the optimal PDBu binding to PKC fraction that was isolated by DEAE-cellulose chromatography from the crude cell extracts, the conditions standardized with the cystosolic receptor (method 2) were used (40).

**Phorbol Ester Binding with Isolated PKC**—[\(^{3}H\)]Phorbol 12,13-dibutyrate (PDBu) was used as a ligand for the determination of phorbol ester binding, using the multimodal approach (40). For optimal PDBu binding to purified PKC, the conditions discussed in method 1, described previously, were used (40). To determine the optimal PDBu binding to PKC fraction that was isolated by DEAE-cellulose chromatography from the crude cell extracts, the conditions standardized with the cystosolic receptor (method 2) were used (40).

**Phorbol Ester Binding in Intact Cells**—Cells were grown in 35-mm Petri dishes. Sets of three Petri dishes were used for determining specific and nonspecific bindings. The medium was changed to a fresh MEM (no serum), and then the cells were treated with various concentrations of Tam for a 2-h time period. Then 37.5 \(\mu\)l [\(^{3}H\]PDBu (0.25 \(\mu\)Ci) was added to the medium and cells were further incubated for 45 min. For determining nonspecific binding 10 \(\mu\)l unlabeled PDBu was included. After incubations, cells were washed four times with ice-cold saline and lysed with 0.2 M NaOH, and the radioactivity present in the cell extract was determined. The specific binding was calculated by subtracting the nonspecific binding from the observed total binding.

**PKC Membrane Binding with Homogenates and Isolated Membranes**—Whether Tam or OH-Tam induce chelator-stable membrane association of PKC in the homogenates or with isolated membrane using purified PKC was determined using previously described procedures (41). Briefly, crude cell homogenates were incubated with Tam or OH-Tam (10 \(\mu\)M) or PMA (100 nM) in the presence of 0.1 mM CaCl\(_2\), at room temperature for 10 min. Then EGTA was added to the homogenate, and the membrane and cystosolic fractions were separated to determine PKC activity associated with these fractions. Similarly, experiments were conducted using the isolated membrane and purified PKC.

**Covalent Binding of Tam to Proteins in Intact Cells**—Subconfluent cells (10–15 \(\times\) 10\(^{6}\)) were incubated with 25 \(\mu\)M \([\(^{3}H\)]Tam (3.75 \(\mu\)Ci) for a 2-h period at 37 °C in a serum-free MEM. In the absence of Tam to allow subsequent precipitation of Tam, the [\(^{3}H\)]Tam, except that was washed four times with saline and then scraped into 10 ml of saline and collected by centrifugation at 2,000 \(\times\) g for 5 min. The cell pellet was then lysed with 3 ml of cold acetone by sonication, and then the precipitate was resuspended in kinase buffer by keeping it at 10°C for 30 min. The precipitated protein was collected by centrifugation. The protein pellet was washed twice with each organic solvent, acetone, n-hexane, and methanol. The washed protein pellet was suspended in SDS-polyacrylamide gel electrophoresis sample buffer without mercapto-compounds and heated at 100 °C for 3 min to solubilize the protein, and then the radioactivity was counted. To determine a covalent binding of [\(^{3}H\)]Tam to PKC, the detergent-solubilized cell extract was prepared from the cells treated with [\(^{3}H\)]Tam and it was subjected to the centrifuge column technique to remove free Tam. The protein fraction
was incubated with rabbit polyclonal antibodies raised against PKC (mixture of α, β, and γ) at 30°C for 4 h, and then the antibody-PKC complex was isolated by incubation with protein A-Sepharose, followed by centrifugation. From the Sepharose beads, protein was extracted with SDS-polyacrylamide gel electrophoresis sample buffer and the radioactivity was counted.

Modification of Purified PKC with OH-Tam—Initially mercapto compounds present within the PKC preparation were removed using a PD-10 gel filtration column (Pharmacia Biotech Inc.). The column chromatography also facilitated the exchange of buffer in PKC preparation to buffer consisting of 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 150 mM NaCl, 1 μM leupeptin. PKC (approximately 1–1.5 units) was incubated with various concentrations of Tam or OH-Tam present in the presence of 1 mM CaCl₂ in a total volume of 0.5 ml for 10 min at 37°C. Then 25 μl of bovine serum albumin solution (10 mg/ml) and 25 μl of 0.1 M EDTA in 50 mM Tris-HCl, pH 8, were added. Tam and other low molecular weight compounds present in the treated PKC samples were removed by subjecting them to the centrifugal column technique using Sephadex G-50 (24). The used Sephadex G-50 columns were washed with 2 bed volumes of ethanol to remove the trapped Tam in the gel and then equilibrated with buffer. This ethanol wash to remove Tam that remained within the gel was required to reuse the column, which otherwise would result in a low recovery of PKC from the column upon subsequent reuse.

Arachidonic Acid Release—Cells were grown to subconfluence or confluence in a regular medium in 96-well plates. The medium was then replaced with MEM with 0.1% serum and [³H] arachidonic acid, 0.5 μCi/ml medium, and the cells were incubated for 4 h at 37°C. Then the cells were washed four times with MEM medium. The labeled cells (four wells for each point) were incubated with MEM supplemented with 0.1% serum along with various concentrations of Tam. At the indicated periods of time, the medium was transferred from the wells of the culture plate to appropriate wells in a 96-well filtration plate fitted with Durapore membrane filtration disks. Then the samples were filtered into an ordinary 96-well plate by using a minivacuum manifold (Millipore). The filters removed any radioactive labeled cells that were detached during the incubation of the cells. The radioactivity present in the filters was then counted.

[^2]: While chelating high concentrations of Ca²⁺ with EDTA, it is important to use high strength alkaline buffer (pH 8) to neutralize the acid released during this process.
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**Fig. 2.** DEAE-cellulose chromatography of native and modified forms of PKC isolated from the control and Tam-treated MDA-MB-231 breast carcinoma cells. PKC activity profile from control untreated cells (A), Tam (20 μM) treated for 1 h (B), and Tam (20 μM) treated for 2 h (C). Confluent MDA-MB-231 cells were treated with Tam. The detergent-solubilized cell extract containing total PKC (cytosol and membrane) was applied to a small (0.5 ml) DEAE-cellulose column (DE-52). The bound PKC (active form) was eluted with 0.1 M NaCl (peak A), whereas the modified form exhibiting less dependence on Ca²⁺/lipids was eluted with 0.25 M NaCl (peak B). Fractions of 0.25 ml were collected and the PKC activity present in these fractions was measured in the presence of Ca²⁺ and lipids (●) or 1 mM EGTA (○).

**Fig. 3.** Effect of PMA pretreatment on the Tam-induced inactivation of PKC in MDA-MB-231 breast carcinoma cells. Three sets of confluent MDA-MB-231 breast carcinoma cells were treated with PMA (100 nM), Tam (20 μM), or a combination of PMA and Tam for the indicated periods of time. Total PKC (cytosol and membrane) was then extracted from the treated cells with a buffer containing detergent, and PKC activity was determined.

Phosphotransferase activity that transiently elevated in the peak B was also inhibited by the pseudosubstrate peptide (PKC residues 19–31).

Cell Growth Rate and Susceptibility to Tam-induced Changes in PKC—Given the fact that cell growth inhibition occurs at concentrations (5–10 μM) lower than that of the concentrations required to inactivate PKC in confluent cells within a 2-h period, it is possible that PKC inactivation byTam may be more sensitive in rapidly growing subconfluent cells than that in confluent cells. Since the membrane-associated PKC activity was high in subconfluent cells, the extent Tam-induced cytosol-membrane translocation of PKC was less. However, subconfluent cells were 2–3-fold more sensitive to down-regulation by Tam than were confluent cells (data not shown). For example, in order to induce a 50% down-regulation of PKC, Tam was required at 35 μM with confluent cells, while it required only 5 μM with subconfluent cells. Since membrane association of PKC was high in the rapidly growing cells, it is possible that prior membrane association of PKC might have enhanced subsequent down-regulation of PKC at a lower concentration of Tam. Alternatively, it is possible that the metabolic activation of Tam may be higher in the rapidly growing cells to accelerate the process of inactivation of PKC.

PMA-induced Membrane Association of PKC Promoted Its Inactivation by Tam—To determine whether prior membrane association of PKC enhances the rate of the down-regulation of PKC by Tam, PMA was used to induce an initial membrane translocation of PKC. Since PMA by itself can induce the down-regulation of PKC with a prolonged treatment, the pretreatment with PMA was restricted to a limited time just enough to induce only the membrane association of PKC but not its down-regulation. When confluent cells were treated with Tam (20 μM) alone, there was only a 22% decrease in the total (cytosol + membrane) PKC activity within a 1-h time period (Fig. 3). However, Tam, when coadministered with PMA, inactivated PKC by 64% within this time. This suggested that PMA can enhance Tam-induced inactivation of PKC by facilitating the initial membrane association of PKC.

Indirect Action of Tam on PKC Translocation—Previous studies carried out by this laboratory have shown that PMA could induce a chelator-stable membrane association of PKC by directly binding to PKC-lipid complex (41). To determine
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Confluent MDA-MB-231 breast carcinoma cells were treated with Tam (20 \( \mu M \)) for 2 h either alone or in combination with one of the antioxidants. The following antioxidants were used: NAC, N-acetylcysteine (1 \( mM \)); vit. C, vitamin C (1 \( mM \)); vit. E, vitamin E (100 \( \mu M \)); \( \beta \)-carotene (100 \( \mu M \)); SOD (40 units/ml); catalase (1000 units/ml). PKC activity represents the total (cytosol and membrane) extractable with detergent.

Induced by Tam, whereas vitamin C, vitamin E (\( \alpha \)-tocopherol), and \( \beta \)-carotene all inhibited the down-regulation of PKC (Fig. 6). Furthermore, antioxidant enzymes, SOD, and catalase were partially effective in preventing the Tam-induced inactivation of PKC in both cell types. Conceivably, an oxidative process may be involved in the irreversible inactivation of PKC occurring in the Tam-treated cells. Inhibition of this process by different antioxidant systems suggests the formation of different reactive oxygen species in the Tam-treated cells.

Whether antioxidants inhibited PKC translocation prior to blocking the PKC down-regulation was studied using vitamin E, which produced the best protective effect. Vitamin E decreased the extent of the translocation of PKC occurring within a 30-min time period (data not shown). Therefore, it is possible that vitamin E, by decreasing the oxidative stress, prevented membrane association of PKC. Furthermore, vitamin E also decreased arachidonic acid release from the cells (Fig. 7). There is a possibility that inhibition of Tam-induced oxidative stress may prevent the subsequent activation of phospholipases \( A_2 \) and \( D \), leading to a decrease in the release of arachidonic acid from the membrane phospholipids. However, we cannot exclude the possibility that vitamin E directly inhibited phospholipase \( A_2 \) activity as has been shown by others (45).

Since vitamin E inhibits several effects of Tam, whether vitamin E inhibits the intake and retention of Tam in the cell was determined. Even at higher concentrations (100 \( \mu M \)) of vitamin E inhibited only 5–10\% \[^{3}H\]Tam retention within the cell (data not shown). Such a small effect of vitamin E on Tam retention is unlikely to be responsible for its dramatic effects on PKC and cell growth.

Vitamin E Blocks Tam-induced Cell Growth Inhibition—Since vitamin E blocks the Tam-mediated oxidative regulation of PKC, we have determined whether vitamin E can block the Tam-induced growth inhibition of the cells. Vitamin E alone had no growth inhibitory effect on the cell growth. However, when coincubated with Tam, vitamin E blocked nearly 80–90\% of the Tam-induced cell growth inhibition (Fig. 8). This strongly suggests that Tam-mediated growth inhibition might have been mediated by oxidative stress occurring in the membrane.

Irreversible Inactivation of Purified PKC by 4-Hydroxytamoxifen—Since Tam-induced inhibition of PKC activity in test tube is totally reversible, the observed irreversible inactivation of PKC occurred in intact cells treated with Tam was unlikely to be caused by a direct action of unmodified drug. It is possible that either the endogenous agents that are elevated in response to Tam treatment or the metabolites of Tam might have induced this irreversible inactivation of PKC. Among the oxymeabolites of Tam, OH-Tam was extensively studied (1). Therefore, we determined whether or not OH-Tam can induce an irreversible inactivation of purified PKC in test tube. Rabbit brain PKC (a mixture of \( \alpha, \beta \), and \( \gamma \) isoenzymes) was incubated with either Tam or OH-Tam under defined conditions, and after removing the drug from the treated PKC preparation, both kinase activity and PDBu binding were determined. Thus, at a later step during the determination of kinase activity and PDBu binding, the drug was no longer present. When incubations were carried out in the presence of 0.1 \( mM \) EGTA, there
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Identification of PKC Domain That Is Sensitive to OH-Tam—Previous studies suggested that Tam-induced inhibition of PKC caused by binding of Tam to both the catalytic and regulatory domains (15, 16, 19–21). To identify the site that was irreversibly affected by OH-Tam, the Ca²⁺/phospholipid-independent activity of the enzyme using proteamine sulfate, an indicator of the catalytic domain function independent from the regulatory domain, was determined. The proteamine phosphotransferase activity was also lost parallel to Ca²⁺/lipid-dependent histone H1 phosphotransferase activity. Furthermore, among the cofactors tested, Mg²⁺ enhanced OH-Tam mediated inactivation, while ATP/Mg²⁺ complex protected the enzyme from this inactivation (Fig. 12). Protection was also observed with H-7, an inhibitor that competitively binds at the ATP-binding site on the enzyme. Furthermore, the catalytic domain (so-called M-kinase) generated by trypsin digestion was also inactivated by OH-Tam with an IC₅₀ of 25 μM (Fig. 13). This concentration was nearly 2-fold lower than that required with the three isoenzymes (approximately 1 unit) treated with the indicated concentrations of OH-Tam as described in the legend to Fig. 9 and after removing the OH-Tam the PKC activity was determined.

The Ca²⁺ needed for this inactivation was above 50 μM and optimal at 0.8–1 mM in the absence of other regulators (data not shown). In the presence of phosphatidyserine and diolein, the concentrations of Ca²⁺ required to promote this inactivation were lower (Fig. 10). In contrast, PMA either in the presence or in the absence of Ca²⁺, even at a high (100 μM) concentration.

Fig. 9. Inactivation of purified PKC by Tam and OH-Tam. Purified PKC (approximately 1.2 units) from rabbit brain was incubated with indicated concentrations of Tam or OH-Tam along with 1 mM CaCl₂ in a total volume of 0.5 ml for 10 min at 37°C. Then 25 μl of bovine serum albumin (10 mg/ml) and 25 μl of 0.1 M EDTA in 50 mM Tris-HCl, pH 8 were added. Tam or OH-Tam was removed from the treated PKC sample by using centrifuge column technique. Then, the treated PKC sample was used to determine the residual kinase activity and PDBu binding without further addition of Tam or OH-Tam.

Fig. 10. Ca²⁺ requirement for inactivation of purified PKC by OH-Tam. Purified PKC (approximately 1.2 units) was incubated with OH-Tam (100 μM) for 10 min at 37°C either in the presence of 1 mM EGTA or defined concentration of Ca²⁺. In the absence of lipid cofactors or PMA, 1 mM CaCl₂ was used, while in the presence of diolein (0.8 μg/ml)/phosphatidyserine (1 μg/ml), PMA (100 μM)/phosphatidyserine, or PMA alone, 10 μM CaCl₂ was used. PKC activity remaining was determined after removing the OH-Tam from the treated samples. Controls were set up with either EGTA or Ca²⁺ and were used to calculate as the percentage of inactivation induced by OH-Tam in the presence of various regulators.
OH-Tam. The enzyme was incubated with OH-Tam (100 μM) under the standard conditions described in the legend to Fig. 9. To these standard conditions, one of the following ligands was added to determine its protective effect. The concentrations of ligands tested were Mg²⁺ (8 mM) and ATP (0.1 mM) either alone or in combination, H-7 (100 μM), and histone (0.1 mg/ml). The activity of PKC remained after OH-Tam treatment in the presence of these agents was expressed as the percentage of the activity observed with appropriate control set up with these ligands in absence of OH-Tam.

Fig. 12. Protection of OH-Tam-mediated inactivation of PKC by various ligands that bind to PKC. Purified PKC (approximately 1.2 units) was incubated with OH-Tam (100 μM) under the standard conditions. After removing the OH-Tam by dialysis using Spectra/Por membrane (cut-off M, 10,000), the residual protein activity for generation of phenoxyl radical from the phenolic compound.

Importance of Vicinal Thiols in the Catalytic Domain to Sensitize PKC for the Inactivation Induced by OH-Tam—Previous studies have shown that phenoxyl radicals that formed from the phenolic compounds could react with thiols to produce reactive oxygen species (46, 47). To determine whether vicinal thiols present within the catalytic domain could play a role in mediating the phenolic compound or phenoxyl radical-mediated inactivation of PKC, the enzyme was initially treated with a nitric oxide-generating agent, S-nitroso cysteine, to mask the vicinal thiols by inducing the formation of disulfide bonds(s) and then treated with OH-Tam. The treatment with S-nitroso cysteine resulted in a modification of the enzyme with a loss of PKC activity, which was reversed by a treatment with 10 mM DTT (Fig. 15). In contrast, the modification of PKC induced by OH-Tam was not reversed by DTT. Nonetheless, the pretreatment with S-nitroso cysteine and subsequent treatment with OH-Tam resulted in a lack of DTT-resistant inactivation of PKC. This suggested that masking the vicinal thiol groups present within the catalytic domain was sufficient to prevent the irreversible inactivation of PKC induced by OH-Tam.

It is important to remove S-nitroso cysteine by centrifuge column technique before incubation with OH-Tam, which otherwise resulted in inactivation of more enzyme. Nitric oxide released from S-nitroso cysteine reacted with superoxide released from the incubation with OH-Tam to form a more reactive oxidant (presumably peroxynitrite), which inactivated the enzyme.
The enzyme, while the modification of the catalytic domain leads to an inactivation of the enzyme (24). The regulatory domain contains 12 cysteine residues, which coordinate the binding of 4 zinc atoms (50), and the zinc-thiolate structure is required for binding of phospholipase C and diacylglycerol (51). This positively charged zinc-thiolate structure is more susceptible to oxidative modification by anionic oxidants than that of free thiols present within the catalytic domain (24). Such differences in the reactivity of the thiols in PKC can lead to the activation of the enzyme when the oxidants are generated at lower concentrations, and an inactivation of the enzyme by a modification of thiols in the catalytic site with an increase in the generation of oxidants.

Oxidants have been shown to activate both phospholipases A2 and D, which can release directly or indirectly arachidonate from the membrane phospholipids (52, 53). In a recent study, an activation of phospholipase D by micromolar concentrations of Tam in intact cells was reported (54). Several agents, such as nonphorbol tumor promoters, staurosporine, thapsigargin, okadaic acid, and calcycin-A, that promote the release of arachidonic acid have been shown to induce a cytosol-to-membrane translocation of PKC (55, 56). Once PKC is associated with the membrane, its susceptibility to oxidative modification may be enhanced (24). A lack of inactivation of protein kinase A under these conditions suggests certain specificity in the effects of Tam on protein kinases in intact cells.

The metabolite OH-Tam is the major circulating species formed by the hepatic biotransformation of Tam in rats (1). However, in humans, OH-Tam is a minor metabolite, and it is not known whether it is formed in sufficient concentrations in the Tam-treated cells in culture. Since Tam is a reversible inhibitor of PKC in the test tube while it is an irreversible inactivator in intact cells, the observed irreversible inactivation of PKC in the test tube by OH-Tam suggests at least one of the metabolites formed from Tam in the body is capable of irreversibly inactivating PKC. Although we are sure that PKC inactivation in the Tam-treated cells is initiated by an oxidative process, it is not clear at present whether this inactivation process is mediated directly by a metabolite formed from Tam or mediated by endogenous oxidants generated in response to Tam action. In spite of the fact that OH-Tam was a catalytic site-directed inactivator of PKC, it did not inactivate the catalytic subunit of PKA, suggesting that certain specificity may exist in the action of OH-Tam to affect limited protein kinases.

Multiple mechanisms leading to formation of oxidants may be involved in mediating the inactivation of PKC induced by Tam in the cell or OH-Tam in the test tube. One possibility is that an initial formation of phenoxyl radical could react with vicinal thiols to produce reactive oxygen species that can inactivate PKC (46, 47). The other possibility is that autoxidation of certain phenolic agents such as hydroquinone and catechol could produce superoxide and hydrogen peroxide through the redox cycle process (57). Alternately, Tam may form a carbon-centered radical and participate in inducing oxidative stress. Potential formation of carbon-centered radicals from related agents such as triphenylmethane was discussed before by others (30). Although in this study, a prooxidant role of Tam or OH-Tam has been presented, previous studies have suggested an antioxidant function for these agents (48). It is possible that depending on the conditions, Tam or its hydroxymetabolite could function as either prooxidant or antioxidant. This is not an unusual situation, since several other phenolic agents have previously been shown to influence oxidative processes in a bimodal manner depending on the conditions (58, 59).

The pharmacological interest of estrogen-related agents had originally begun with the observation of estrogen-like effects
produced by stilbene and triphenylethylene. Although the role of redox regulation and the formation of reactive oxygen species have been well documented for a stilbene derivative, diethylstilbestrol (60), redox metabolism was not studied for the metabolites of triphenylethylene derivative, Tam. Similarly, it has been well established that the carcinogenic effects of high concentrations of estradiol may involve a formation of catechol estrogens and reactive oxygen species (61). The oxidants play an important role in tumor promotion (62–65). Furthermore, drugs such as chloroquine induce retinopathy, which may involve its ability to produce oxidative stress (66). Therefore, the oxidative stress induced by Tam may have some role in producing its side effects, such as increased incidences of uterine cancer and retinopathy in humans.

In ER-positive cell lines, Tam can elicit estrogen-reversible growth inhibitory effects at a concentration range of 10–100 nM (4–7). However, at such a concentration range, Tam in ER-positive cell line (MCF-7) growing in a regular serum-containing medium did not affect PKC within a short period of time (2 h). It is difficult to interpret the changes occurring in PKC after a prolonged treatment with Tam at lower concentrations. In order to sensitize the ER-positive cell lines to submicromolar concentrations of Tam, it is necessary to grow these cells for a few days in a phenol red-free medium supplemented with dextran-coated charcoal-treated serum. Therefore, further studies in this direction are certainly needed. These studies do not exclude the role for ER in the action of Tam in ER-positive cell lines. The Tam-induced oxidative stress may complement the effects occurring through ER. Both estrogen and Tam were reported to induce peroxidase activity in target tissues such as uterus and mammary gland (67). Phenol red is known to mimic certain actions of estrogen in breast carcinoma cells in culture (68). This dye is also a good substrate for peroxidase (69).

Moreover, peroxidase has been recently shown to activate Tam (70). This dye is also a good substrate for peroxidase (69).

Inhibition of ER activity, however, may complement the action of ER in inducing cell growth inhibition. Recent studies revealed that mutations in ER appeared in only a small percentage of tumors that are resistant to Tam and may not alone contribute to the development of resistance to Tam (72). Based on the current study, it is possible that a cellular resistance to Tam could develop as a consequence of multiple factors, which may include a decrease in metabolism of the Tam, a low rate of generation of oxidants in response to Tam, and an increase in expression of antioxidant systems.

Tam also resembles phorbol ester to some extent by inducing membrane translocation and down-regulation of PKC. Simply an induction of these changes alone may not lead to tumor promotion. Bryostatin can induce PKC membrane translocation and down-regulation, but can also block some actions of PMA and has cancer therapeutic potential (73, 74). Nevertheless, bryostatin differed from TPA in inducing the extent of translocation and down-regulation of various isoenzymes (74). Such quantitative differences may explain the action of tumor promoter versus a chemopreventive agent.

Further studies in vivo are certainly required to understand the relation between Tam-induced oxidative regulation of PKC and the organospecificity involved in therapeutic action of this drug or its unwanted side effects such as induction of uterine and liver cancer or retinopathy. In the event that these ER-independent oxidative changes in PKC are related to side effects of these drugs, antioxidants, particularly of dietary origin such as vitamin E, vitamin C, and β-carotene, may be a beneficial addition to Tam therapy to reduce the risk of side effects produced by Tam.

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