ABSTRACT

We investigated the effects of the antiestrogen tamoxifen on MCF-7 cell protein kinase C either by using the in vitro histone kinase assay or by studying the phosphorylation of its endogenous M, 28,000 protein substrate in intact cells.

In the in vitro assay, tamoxifen inhibited the enzyme competitively with respect to phospholipid, whereas estradiol and morpholinobenzyl phenoxy ethanamine, a specific ligand for antiestrogen binding sites, were considerably less efficient.

In contrast, tamoxifen did not affect phosphorylation of the M, 28,000 protein induced by the phorbol ester 12-O-tetradecanoylphorbol 13-acetate in intact MCF-7 cells. Estradiol and morpholinobenzyl phenoxy ethanamine also had no effect. At high concentration (100 μM), tamoxifen itself stimulated specific phosphorylation of this M, 28,000 protein. Estradiol and morpholinobenzyl phenoxy ethanamine neither mimicked nor interfered with this effect.

Our data suggest that the effect of tamoxifen on protein kinase C activity depends on the phospholipid environment of the enzyme, and opposite effects may be observed in intact cells to those seen in disrupted cells. The action of tamoxifen on endogenous protein phosphorylation was thought to be due to direct interaction with the phospholipid binding domain of the enzyme rather than by interaction with the estrogen receptor or the antiestrogen binding site. Nevertheless, our results do not rule out a possible activation by tamoxifen of specific protein kinase(s) and phosphatase(s). In any case, the antiproliferative activity of tamoxifen on MCF-7 cells cannot be attributed to its effects on protein kinase C.

INTRODUCTION

The triphenylethylen derivative tamoxifen is a synthetic nonsteroidal antiestrogen which is used in the treatment of human breast cancer to inhibit tumor growth (1, 2). Its mechanism of action is not yet clearly understood, although there is evidence that tamoxifen competes with estrogen at the estrogen receptor (3–6). However, several reports indicate that not all of its biological effects are mediated by the estrogen receptor (6–9). Tamoxifen and other related antiestrogens also bind specifically to a microsomal high-affinity site which is distinct from the estrogen receptor (10–16). The nature and significance of this ABS remain to be established. It has been suggested to play a role in the growth-inhibitory effect of antiestrogens (17–19), although this has not been confirmed in other studies (20, 21).

Using in vitro assays, tamoxifen has been shown to inhibit Ca²⁺- and phospholipid-dependent PKC (22–24), an enzyme which plays a key role in transmembrane signaling of a wide variety of extracellular stimuli, including growth factors, hormones, and other biologically active substances (25–27). The physiological activator of protein kinase C is DAG, which accumulates transiently because of a receptor-mediated breakdown of inositol phospholipid (25,27). Tumor-promoting phorbol esters, such as TPA, can mimic the intracellular messenger DAG by activating protein kinase C directly (28). In fact, the high-affinity phorbol ester receptor in target cells is now thought to be PKC (29, 30).

The question is whether or not tamoxifen exerts its antiproliferative action by inhibiting protein kinase C (22–24). Such an hypothesis implies that PKC plays a positive modulatory role on cell growth. However, in MCF-7 human breast cancer cells, our group (31–33) along with others (34, 35) have demonstrated that activators of PKC, such as phorbol esters and diacylglycerols, completely inhibit cell proliferation.

In previous work, we showed that the arrest of MCF-7 cell growth was correlated with the phosphorylation of an endogenous M, 28,000 protein which appears to be a specific substrate of PKC (33, 36, 37). In the present study, we investigated the effects of tamoxifen and morpho-BPE, a phenoxy-ethanamine derivative which binds to the antiestrogen binding site but not to the estrogen receptor (19), on both in vitro PKC activity and protein phosphorylation in MCF-7 cells.

MATERIALS AND METHODS

Chemicals. Histone H1, TPA, phosphatidylyserine, 1,2-diacyl-glycerol, H7, and protease V8 (from Staphylococcus aureus) were obtained from Sigma. Staurosporine was from Calbiochem. [γ-³²P]ATP (0.5 to 3 Ci/mmol), [³H]leucine (146 Ci/mmol), and [³²P]phosphoric acid were purchased from Amersham. Acrylamide and bisacrylamide were from Biorad. Tamoxifen was obtained from ICI.

Cell Culture. MCF-7 cells were adapted to grow in the absence of serum (38) at 37°C in RPMI 1640 without phenol red (Gibco), supplemented with 2 g/liter sodium bicarbonate, pH 7.3, 2 mm L-glutamine, 1 μm insulin (Novo Laboratories), and 0.1 μm transferrin.

RPh-4 variants were isolated from MCF-7 cells (39) from their resistance to the inhibitory action of TPA on cell growth and were routinely cultured in the same medium as MCF-7 cells but in the presence of 50 ng/ml of TPA.

RTx-6 variants were isolated from MCF-7 cells grown in the presence of 4% fetal serum stripped of endogenous hormones (13) and were subcultured in RPMI 1640 medium without phenol red.

Protein Kinase C Assay. Subconfluent cells (40 × 10⁴ cells) were rapidly harvested in cold PBS and homogenized in 20 mm Tris-HCl, pH 7.5, containing 0.25 mM sucrose, 2 mM EDTA, 2 mM EGTA, 100 μg/ml of leupeptin, and 5 mm β-mercaptoethanol (Buffer A).

The cell lysate was centrifuged for 1 h at 105,000 × g. The supernatant was applied to a DEAE-cellulose column (DE52, 0.8 x 3 cm) equilibrated with 20 mm Tris-HCl, pH 7.5, containing 2 mM EDTA, 1 mM EGTA, 50 μg/ml of phenylmethylsulfonyl fluoride, and 5 mM β-mercaptoethanol (Buffer B). Columns were washed with 10 ml of Buffer B. PKC activity was eluted with 4 ml of Buffer B containing 0.13 M NaCl.

PKC was assayed by measuring the incorporation of ³²P from [γ-³²P] ATP into H1 histone. The standard assay mixture (200 μl) contained...
20 mM Tris-HCl, pH 7.4, 40 μg of histone. 10 μM ATP (550 cpm/ pmol), 5 mM MgCl₂, 40 μl of sample, and either 0.5 mM EGTA or 0.5 mM CaCl₂, and various amounts of phosphatidylserine and 1,2-dioleoyl-glycerol. After incubation for 5 min at 30°C, 10% TCA was added to stop the reaction. The protein precipitate was dissolved in 1 N NaOH, and the incorporation of ³²P was measured by scintillation counting in Pico-fluo (Packard).

Protein Phosphorylation. Subconfluent cultures (1 × 10⁶ cells/35-mm dish) were washed twice in a phosphate-free Krebs-Ringer buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.3, 0.1% bovine serum albumin, and 0.2% glucose and incubated for 2 h at 37°C in 1 ml of the same buffer containing 50 μCi of [³²P]phosphoric acid. Stimuli were then added for a further 30-min period. Cells were washed twice with cold PBS, and 10% TCA was added. TCA-precipitated proteins were dissolved in 150 μl of electrophoresis sample buffer containing 0.06 M Tris-HCl, pH 6.7, 2% SDS, 8% glycerol, 2% β-mercaptoethanol, and 0.005% bromophenol blue. Samples were then boiled for 5 min at 90°C. Proteins were fractionated by electrophoresis on 4.5% and 12% (w/v) discontinuous SDS-polyacrylamide slab gel. After fixation of proteins with cold TCA and staining with Coomassie blue, the gel was dried and then exposed to Hyperfilm-MP for 48 to 72 h.

Peptide Mapping by Limited Proteolysis. One-dimensional peptide mapping was carried out according to the method of Cleveland et al. (40) using protease V8 from S. aureus. Briefly, the M, 28,000 phosphorylated band was excised from the gel, directly loaded on a second SDS-polyacrylamide (4% and 15%) slab gel, and then overlayed with protease (20 and 200 ng). Digestion proceeded directly in the stacking gel during the subsequent electrophoresis.

Protein Synthesis. Subconfluent cultures (1.5 to 3 × 10⁶ cells per 60-mm dish) were submitted to heat shock by incubation at 43°C for various times, or they were exposed to 100 μM tamoxifen for 30 min at 37°C. After washing, cells were incubated for 1 h at 37°C in 2 ml of leucine-free medium containing 100 μCi of [³H]leucine. At the end of incubation, cells were washed twice with 5 ml of cold PBS, and 10% TCA was added. TCA-precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Tamoxifen inhibited protein (histone) kinase C activity from MCF-7 cells in a dose-dependent manner (Fig. 1). The efficiency of inhibition depended on the amount of phosphatidyserine (and diacylglycerol) used. The IC50's were around 50, 60, 85, and 250 μM for 20, 40, 60, and 80 μg/ml of phosphatidylserine, respectively (and 0.75, 1.5, 2.25, and 3 μg/ml of 1,2-dioleoyl-glycerol, respectively). Tamoxifen (20 μM) inhibited significantly (by 20%) PKC activity in the presence of 20 μg/ml of phosphatidyserine, whereas 200 μM tamoxifen was required to affect the enzyme activity in the presence of 80 μg/ml of phosphatidyserine (i.e., the standard conditions for assay of PKC). Similar results were obtained when the enzyme activity was assayed with a constant concentration of 1,2-dioleoyl-glycerol (3 μg/ml) and various concentrations of phosphatidyserine (data not shown).

We also investigated the effect of the phenoxy-ethanamine derivative, morpho-BPE (Fig. 2), another inhibitor of MCF-7 cell proliferation (19), on PKC activity. In contrast to tamoxifen, concentrations of morpho-BPE up to 50 μM did not inhibit enzyme activity at any of the phospholipid concentrations tested. A dose as high as 500 μM of morpho-BPE only led to a 20% inhibition of PKC activity in the presence of 80 μg/ml of phosphatidyserine (Fig. 1). Estradiol (100 μM) did not affect PKC activity under these conditions, and only led to a 30% inhibition of enzyme activity in the presence of 20 μg/ml of phosphatidyserine (data not shown). None of the compounds tested affected basal histone kinase activity measured in the absence of Ca2+ and phospholipid (data not shown).

It can be seen from Fig. 3 that tamoxifen also inhibited PKC activity when 1,2-dioleoyl-glycerol was replaced with TPA. As in the previous experiments, inhibition kinetics was highly dependent on the concentration of phosphatidyserine (IC50 = 25, 75, 90, and 150 μM for 20, 40, 60, and 80 μg/ml of phosphatidyserine), lending further support to a competitive inhibition of the enzyme by tamoxifen with respect to phosphatidyserine.

Since the inhibitory action of tamoxifen on PKC appeared to depend on the phospholipid environment, we wondered whether tamoxifen would affect the enzyme activity in intact MCF-7 cells. Fig. 4 shows that tamoxifen (up to 100 μM) did not affect the TPA-induced phosphorylation of the M, 28,000 protein. Morpho-BPE (100 μM) and estradiol (100 μM, not shown) were also without effect on this TPA-induced protein phosphorylation.

Surprisingly, when added alone at high concentration (100 μM), tamoxifen consistently stimulated phosphorylation of the M, 28,000 protein. In contrast, at the same concentration of 100 μM, neither morpho-BPE (Fig. 4) nor estradiol (see below Fig. 7) stimulated this phosphorylation.

We have recently reported that the M, 28,000 PKC substrate may be ascribed to the stress protein family. Since cytotoxic concentrations of tamoxifen may induce stress of MCF-7 cells, we wondered if the stimulation of the M, 28,000 protein phos-
Fig. 3. Inhibition by tamoxifen of PKC activity in the presence of TPA and various concentrations of phosphatidylserine. Protein kinase C activity was assayed on MCF-7 cell cytosol following DEAE-cellulose chromatography, in the presence of calcium (10 μmol), TPA (10 ng/ml), and various concentrations of phosphatidylserine (20, 40, 60, and 80 μg/ml). Tamoxifen was dissolved in DMSO and added at the final indicated concentrations. Controls contained the same concentration of solvent DMSO (5%). Data were expressed as the percentage of controls.

Fig. 4. Effects of TPA, tamoxifen, and morpho-BPE on protein phosphorlation in intact MCF-7 cells. Subconfluent cells were incubated for 30 min in phosphate-free Krebs-Ringer buffer containing 50 μCi of [32P]phosphoric acid in the absence (Cont) or in the presence of 100 ng/ml of TPA, various concentrations (1, 10, and 100 μmol) of tamoxifen (Tam), 100 μM morpho-BPE, or combination of TPA and tamoxifen or TPA and morpho-BPE (+TPA). Tamoxifen was dissolved in DMSO. The control at the same final concentration (1%) is also shown (Cont. DMSO). Morpho-BPE was dissolved in distilled water. TCA-precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The arrow indicates the position of the M, 28,000 protein.

Fig. 5. Effects of heat shock and tamoxifen treatment on M, 28,000 protein synthesis in MCF-7 cells. MCF-7 cells were incubated at 37°C (CONT) or at 43°C for various times (HS), or they were exposed to 100 μM tamoxifen for 30 min at 37°C (TAM). After washing, cells were incubated for 1 h at 37°C in 2 ml of leucine-free medium containing 100 μCi of [3H]leucine. At the end of incubation, cells were washed twice with 5 ml of cold PBS, and 10% TCA was added. TCA-precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

phorylation caused by the antiestrogen could have been due to an increase in the cellular content of this protein. Fig. 5 shows that 100 μM tamoxifen was unable to induce synthesis of the M, 28,000 protein, whereas a 30-min heat shock did.

Fig. 6A shows that the two PKC inhibitors H7 and staurosporine markedly inhibited the TPA-induced phosphorylation of the M, 28,000 protein in MCF-7 cells, but were without influence on the antiestrogen effect. However, peptide mapping of the M, 28,000 phosphoprotein revealed identical phosphopeptides upon phorbol ester and tamoxifen stimulation (Fig. 6B).

We have recently selected variants of the MCF-7 line (RPh-4) which are resistant to the inhibitory effect of TPA on cell proliferation (39). These variants are routinely cultured in the absence of phorbol ester. We found that PKC content in RPh-4 cells depended on the duration of subculture in the absence of TPA. Recovery of enzyme activity was low (35%), intermediate (70%), and total (100%) after 8 days (RPh-4: d8), 15 days (RPh-4: d15), and 3 mo (RPh-4: Rv), respectively, of subculture in TPA-free medium.

We found that neither tamoxifen (100 μmol) nor TPA stimulated phosphorylation of the M, 28,000 protein in RPh-4: d8 cells (Fig. 7). Surprisingly, tamoxifen was also ineffective in stimulating the phosphorylation of the specific M, 28,000 PKC substrate in RPh-4: d15 and RPh-4: Rv cells, whereas it inhibited the TPA-induced phosphorylation of the M, 28,000 protein.

Furthermore, tamoxifen reduced the 32P labeling of a M,
EFFECTS OF TAMOXIFEN ON PKC AND PROTEIN PHOSPHORYLATION IN MCF-7 CELLS

Fig. 6. Effects of H7 and staurosporine on TPA- and tamoxifen-induced M, 28,000 protein phosphorylation. In A, MCF-7 cells were incubated for 30 min in the absence (CONT) or in the presence of 400 nM H7 (H7), 100 nM staurosporine (ST), 100 ng/ml of TPA (TPA), 100 µM tamoxifen (TAM), or combinations of TPA and H7, TPA and staurosporine, tamoxifen and H7, or tamoxifen and staurosporine. Phosphorylated proteins were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography. In B, the M, 28,000 protein phosphorylated after TPA and tamoxifen stimulation was excised from the gel and submitted to peptide mapping analysis by limited proteolysis with 20 ng (1) and 200 ng (2) of protease V8. Arrows indicate the position of phosphopeptides revealed after autoradiography of the second SDS-polyacrylamide gel.

Fig. 7. Effects of tamoxifen and estradiol on protein phosphorylation in intact MCF-7 and RPh-4 cells. Subconfluent cells were incubated as described in the legend to Fig. 4 in the absence (control, a) or in the presence of 100 ng/ml of TPA (b), 100 µM tamoxifen (c), 100 µM estradiol (d), 100 µM tamoxifen plus 100 µM estradiol (e), or 100 ng/ml of TPA plus 100 µM tamoxifen (f). Estradiol and tamoxifen were dissolved in DMSO (final concentration, 1% as in the control). RPh-4 cells were subcultured in TPA-free medium for 8 days (RPh-4: d8), 15 days (RPh-4: d15) or 3 mo (RPh-4: Rv) before the experiment. Arrows indicate the position of M, 28,000 and M, 20,000 proteins. The labeling of the M, 28,000 protein observed in RPh-4: d8 control was probably due to small amounts of the highly lipophilic phorbol ester remaining in the cell culture despite the period of TPA starvation. The 32P labeling of the M, 20,000 protein in TPA plus tamoxifen-treated RPh-4: d8 cells was most probably artifactual as it was not observed in two other similar experiments.

20,000 protein. Preliminary data indicate that Ca2+ ionophore and ionomycin increase slightly the degree of phosphorylation of this protein in intact MCF-7 cells, but do not affect the M, 28,000 protein (not shown). The M, 20,000 protein may thus be a substrate of calmodulin kinase. It is of interest in this respect that tamoxifen has been reported to be a possible antagonist of calmodulin (41). Alternatively, the decrease in 32P incorporation into the M, 20,000 protein in MCF-7 and RPh-4 cells as well as the lack of 32P labeling of the M, 28,000 protein in RPh-4 cells may be due to antiestrogen stimulation of specific phosphotases.

Fig. 7 also shows that estradiol did not compete with tamoxifen in stimulating phosphorylation of the M, 28,000 protein in MCF-7 cells. We have recently selected other MCF-7 cell variants (RTx-6) which are resistant to the inhibitory effect of tamoxifen on cell proliferation, and which lack antiestrogen binding sites (13). Fig. 8 shows that tamoxifen induced phosphorylation of the M, 28,000 protein in the RTx-6 as in the MCF-7 cells. Estradiol was also unable to mimic the antiestrogen effect in these cells (data not shown).

DISCUSSION

We report here opposite effects of tamoxifen on protein kinase C activity measured in vitro and protein phosphorylation observed in intact MCF-7 cells. While the triphenylethylene antiestrogen inhibited the enzyme activity competitively with respect to phospholipid concentration in the in vitro assay, it did not reduce significantly TPA-induced phosphorylation of the M, 28,000 PKC substrate in intact MCF-7 cells. In contrast, tamoxifen at high concentration (100 µmol) induced phosphorylation of the M, 28,000 protein, although it did not increase
were analyzed in duplicate by SDS-polyacrylamide gel electrophoresis and autoradiography. Absence (COAT) or in the presence of 100 ng/ml of TPA (TPA) or 100 μM phosphate-free Krebs-Ringer buffer, subconfluent cells were incubated in the fetal calf serum stripped of endogenous steroid hormones. After washing in phosphate-free Krebs-Ringer buffer, the basal protein kinase activity measured in vitro in the absence of calcium and phospholipid.

The action of this triphenylethylene antiestrogen on protein phosphorylation is unlikely to be mediated by either the estrogen receptor or the ABS for the following reasons. (a) The specific ligands estrogen and morpho-BPE did not mimic or interfere with the effect of tamoxifen on M, 28,000 protein phosphorylation. (b) Tamoxifen stimulated M, 28,000 protein phosphorylation in RTx-6 cells which lack ABS. (c) Morpho-BPE which has the same affinity for ABS as tamoxifen (19) was 6 times less potent than tamoxifen in inhibiting the enzyme activity when assayed under the same conditions (20 μg/ml of phosphatidylserine). Estradiol also appears to have little action on PKC activity in vitro.

The inhibitory effect of tamoxifen appears to be competitive with respect to phosphatidylserine and probably reflects a direct interaction with the phospholipid binding domain of the enzyme, which has been observed in studies on PKC from rat (22, 23, 42) or pig (24) brain. However, tamoxifen may interact with the catalytic domain of the enzyme (43). In our study, the lower efficiency of morpho-BPE in inhibiting PKC is in line with the weak hydrophobic nature of this amphiphilic ligand compared with tamoxifen.

Tamoxifen significantly reduced PKC activity at μM concentrations which have been reported to inhibit MCF-7 cell growth. However, in this cell type, there is increasing evidence that PKC plays a negative modulatory role on cell proliferation. Inhibition of the enzyme should, therefore, stimulate cell growth. Moreover, the inhibition at μM concentrations in vitro was only observed at low concentrations of phospholipid, which do not necessarily correspond to the true environment of the enzyme in the intact cell.

We also showed that concentrations of tamoxifen up to 100 μmol did not reduce the phosphorylation of the specific M, 28,000 PKC substrate which is induced by TPA in MCF-7 cells. This result is in disagreement with a recent report showing that tamoxifen inhibits the calcium- and phosphatidylserine-induced phosphorylation of various endogenous proteins from rat brain and ovary homogenates (24). However, disruption of cell integrity may dramatically modify the phospholipid environment of PKC.

On the other hand, the lack of effect of morpho-BPE on phosphorylation of the M, 28,000 protein is in agreement with the inefficiency of N,N-diethyl-2-[4-(phenyl methyl)phenoxy]-ethanamine-HCl, another phenoxy-ethanamine derivative, in blocking phosphorylation of the M, 47,000 protein which is induced by TPA in platelets (44). The fact that tamoxifen itself increases phosphorylation of the M, 28,000 protein is intriguing, although it is probably not linked to its cytostatic antiproliferative action as it was only observed at 100 μmol which is cytotoxic for MCF-7 cells. More surprising was the inability of tamoxifen to induce phosphorylation of the M, 28,000 protein in RPh-4 cells which contain the same amount of functional PKC as MCF-7 cells. Although MCF-7 and RPh-4 cells may contain distinct PKC isoforms with different sensitivities to tamoxifen, this result might also indicate that the M, 28,000 protein is phosphorylated by another type of protein kinase in tamoxifen-treated MCF-7 cells. The inhibition of PKC by H7 and staurosporine to block the M, 28,000 protein phosphorylation induced by the antiestrogen lends support to this idea. However, this latter finding may suggest that tamoxifen prevents the inhibitory action of both these compounds on PKC. Indeed, preliminary experiments indicate that tamoxifen, at a concentration which does not affect PKC activity, obviates the inhibitory effect of H7 under the in vitro assay.

In any case, previous work in our laboratory demonstrated that the M, 28,000 was a specific substrate for PKC. Only serine residues were found to be phosphorylated on this M, 28,000 protein (33), which was not induced by activators of either the cyclic AMP-dependent (36) or the calmodulin kinases. Moreover, permeant diacylglycerols and exogenous phospholipase C mimicked the action of phorbol esters on phosphorylation of the M, 28,000 protein (33, 36). Finally, peptide mapping of the phosphorylated M, 28,000 protein after TPA and tamoxifen stimulation suggested that these two compounds may activate the same kinase. The differences observed in the behavior of PKC in RPh-4 and MCF-7 cells after stimulation with tamoxifen may thus be due to differences in the microenvironments of the enzyme in these two sublines. This may explain why the antitestrogen inhibited the TPA-induced phosphorylation of the M, 28,000 protein in the RPh-4 but not in the MCF-7 cells. Activation of specific phosphatases in RPh-4 cells by tamoxifen cannot, however, be ruled out.

Taken together, our results indicate the importance of the phospholipid environment on the regulation of PKC activity. This may account for the differences in the effects of tamoxifen on PKC activity observed in the in vitro assay and in intact MCF-7 cells.

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Fig. 8. Effect of tamoxifen on protein phosphorylation in intact MCF-7 and RTx-6 cells. MCF-7 cells and RTx-6 variants were grown in the presence of 4% fetal calf serum stripped of endogenous steroid hormones. After washing in phosphate-free Krebs-Ringer buffer, subconfluent cells were incubated in the absence (CONT) or in the presence of 100 ng/ml of TPA (TPA) or 100 μM tamoxifen (Tam) as indicated in the legend to Fig. 4. Phosphorylated proteins were analyzed in duplicate by SDS-polyacrylamide gel electrophoresis and autoradiography.
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Opposite Effects of Tamoxifen on *in Vitro* Protein Kinase C Activity and Endogenous Protein Phosphorylation in Intact MCF-7 Cells

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