

Inhibition of Protein Kinase C by Tamoxifen¹

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ABSTRACT

The antiestrogen drug tamoxifen inhibits rat brain protein kinase C *in vitro*, whether the enzyme is activated by Ca²⁺ and phospholipid (50% inhibitory dose, 100 μM), 12-O-tetradecanoylphorbol-13-acetate and phospholipid (50% inhibitory dose, 40 μM), or teleocidin and phospholipid. Tamoxifen does not inhibit the Ca²⁺- and phospholipid-independent phosphorylation of protamine sulfate by protein kinase C, indicating that the drug does not interact with the active site of the enzyme. The binding of [³H]phorbol dibutyrate to high-affinity membrane receptors of cultured mouse fibroblast cells is inhibited by tamoxifen (50% inhibitory dose, 5 μM). Our findings suggest that the growth-inhibitory and cytotoxic effects of tamoxifen, which have been observed at μM concentrations of the drug, may be in part due to its effects on protein kinase C.

INTRODUCTION

Tamoxifen, a synthetic nonsteroidal antiestrogen, is used in the treatment of human breast cancer (7, 10, 15). The mechanism by which tamoxifen exerts its antitumor effect is not completely understood (22). Tamoxifen competes with estrogen for binding to the estrogen receptor, and a number of the biological effects of tamoxifen can be reversed by estrogen (2, 12, 18). However, some of the biological effects of tamoxifen cannot be reversed by estrogen (18, 20, 21), suggesting that not all of its biological effects are mediated by the estrogen receptor. Tamoxifen does bind to a high-affinity site which is distinct from the estrogen-binding site of the estrogen receptor (19, 25), but it is not known whether this site mediates any biological effects of tamoxifen. It has recently been reported that tamoxifen inhibits the activation of bovine brain cyclic AMP phosphodiesterase by calmodulin and that the inhibition can be overcome by increasing concentrations of calmodulin (9).

In this paper, we report the effects of tamoxifen on PKC,³ a Ca²⁺- and phospholipid-dependent protein kinase which binds tumor-promoting phorbol esters with high affinity (8) and which can be activated by tumor promoters, including phorbol dibutyrate, TPA, mezerein, teleocidin, and aplysiatoxin (6, 13, 26). We report here that tamoxifen inhibits the activity of rat brain PKC. In addition, we show that tamoxifen inhibits specific [³H]PDBU binding to high-affinity receptors in intact C3H10T^{1/2} cells. These data provide evidence that the mechanism of growth inhibition by tamoxifen *in vivo* may include the inhibition of PKC, an enzyme which is believed to transduce a variety of growth-promoting signals.

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³ The abbreviations used are: PKC, protein kinase C; DMSO, dimethyl sulfoxide; PDBU, phorbol-12,13-dibutyrate; TCA, trichloroacetic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; IC₅₀, 50% inhibitory concentration.

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MATERIALS AND METHODS

Chemicals. [γ -³²P]ATP was purchased from Amersham. Hydrofluor was from National Diagnostics, and [³H]PDBU (specific activity, 13 μCi/mmol) was from New England Nuclear. ATP, bovine serum albumin, histone III-S, phenylmethylsulfonyl fluoride, Tris hydrochloride, DEAE-Sephacel, and tamoxifen citrate were obtained from Sigma Chemical Co. Phosphocellulose paper (Grade P81) was from Whatman, phosphatidylinositol was from Avanti Chem., Aca-34 Ultrogel was from LKB Instruments, and TPA was purchased from LC Services. All protein concentration determinations were done with the Bio-Rad protein assay solution. Leupeptin was a gift of the United States-Japan Cooperative Cancer Research program. Teleocidin was a gift from Dr. T. Sugimura (National Cancer Research Institute, Tokyo, Japan). Stock solutions of [³H]PDBU, teleocidin, and tamoxifen were made up in DMSO and stored at -20°C.

Methods. Rat brain PKC was partially purified to a specific activity of 120 nmol/min/mg, as described previously (16). The activity of PKC was enhanced 10- to 30-fold by 1 mM Ca²⁺ plus phospholipid as well as by 200 nM TPA plus phospholipid. PKC was assayed by measuring the Ca²⁺- and phospholipid-dependent phosphotransferase reaction between [γ -³²P]ATP and histone III-S in the presence of PKC. The standard reaction mixture (0.12 ml) contained 20 mM Tris-HCl at pH 7.5, 5 mM 2-mercaptoethanol, 10 mM MgCl₂, 1 mM CaCl₂ (or 1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), phosphatidylinositol (10 μg/ml) (or no phospholipid), 70 μM [γ -³²P]ATP (250 to 400 cpm/pmol), histone III-S (0.67 mg/ml) or protamine sulfate (0.67 mg/ml), 1 to 4 μg rat brain PKC, and, where indicated, tamoxifen or DMSO at the indicated concentration. In designated experiments, CaCl₂ was omitted, and either TPA or teleocidin was added at the indicated concentrations and in the presence of 1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (16). Reactions were initiated by the addition of enzyme and incubated at 30°C for 5 min. Reactions were terminated by pipeting a 40-μl aliquot onto phosphocellulose paper. Blank values were determined from samples to which enzyme was not added, and these counts were subtracted from those observed in PKC-containing samples. The papers were rinsed with three 1-liter volumes of water and counted in Aquasol. All assays were done in triplicate and varied by less than 10%. In indicated experiments, reactions were terminated by TCA precipitation. Ten ml ice-cold 20% TCA-1% PP_i were added to the reaction mixture, the precipitated solution was filtered on Millipore HA 45-μm filters, and the filters were rinsed with two 10-ml aliquots of TCA-PP_i solution and then counted in Aquasol.

High-affinity phorbol ester tumor promoter receptor sites in the mouse embryo fibroblast cell line C3H10T^{1/2} were assayed with [³H]PDBU, as described previously (24), and tamoxifen was tested as an inhibitor of the [³H]PDBU binding. C3H10T^{1/2} cells were grown in 3.5-cm-diameter tissue culture plates as described previously (24). Subconfluent cells were incubated for 1 h at 37°C in 1 ml of binding assay buffer (2 volumes of Dulbecco's medium plus 1 volume of buffered 0.9% NaCl solution (saline) containing bovine serum albumin at 1 mg/ml). Subsequently, the cells were incubated at 37°C for 30 min in binding assay buffer containing 15 nM [³H]PDBU and tamoxifen at the indicated concentration. The final concentration of DMSO in this assay system was about 1.5%. The monolayer was then rapidly washed 3 times with ice-cold binding assay buffer and then solubilized for 2 h at 37°C with 1 ml solubilizing solution (0.8% Triton X-100, 0.2% EDTA, and 0.25% trypsin in buffered saline). The plates were washed twice with 0.5 ml 1% sodium dodecyl sulfate. The pooled solubilizing solution and washings were counted in Hydro-

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fluor. Nonspecific binding of [³H]PDBU was measured in the presence of 40 μM unlabeled PDBU and was less than 10% of the total binding. Specific [³H]PDBU binding represents the difference between the total binding and the nonspecific binding. All assays were done in triplicate.

RESULTS

Since it was recently demonstrated that tamoxifen is an inhibitor of a calmodulin-dependent phosphodiesterase (9), we tested whether tamoxifen affected the activity of PKC, another Ca²⁺-interacting enzyme. We found that tamoxifen inhibits the Ca²⁺- and phospholipid-dependent activity of rat brain PKC with an IC₅₀ of approximately 100 μM, when the phospholipid concentration is 10 μg/ml (Chart 1). Tamoxifen has no effect on the small amount of protein kinase activity that our enzyme preparation displays in the absence of phospholipid and Ca²⁺ (data not shown). The TPA- and phospholipid-stimulated activity of PKC is inhibited by tamoxifen, as illustrated in Chart 2. The IC₅₀ is approximately 40 μM, when the concentration of TPA is 200 nM and that of phospholipid is 10 μg/ml. In addition, we found that tamoxifen inhibits teleocidin- and phospholipid-dependent PKC activity (Table 1).

We determined that the observed inhibition of PKC by tamoxifen was not an artifact produced by terminating assays on phosphocellulose paper, since identical effects of tamoxifen on PKC activity were observed in reactions which were terminated by TCA precipitation. We tested sodium citrate for effects on PKC activity, because our studies with tamoxifen utilized tamoxifen citrate. Sodium citrate (300 μM) had no effect on PKC activity in standard assays.

Protamine sulfate is a PKC substrate which is phosphorylated by PKC in a Ca²⁺- and phospholipid-independent reaction (23). We found that tamoxifen had no measurable effect on PKC-catalyzed protamine sulfate phosphorylation whether or not Ca²⁺ and phospholipid were present, in standard assays containing protamine sulfate (0.67 mg/ml) rather than histone III-S as the phosphoacceptor substrate (Table 2). This suggests that tamox-

ifen does not interact directly with the active site of PKC. Presumably, it interacts with hydrophobic sites on the enzyme and/or with the phospholipids (see "Discussion").

We observed that the efficacy of tamoxifen as a PKC inhibitor is decreased as the concentration of phospholipid in the reaction

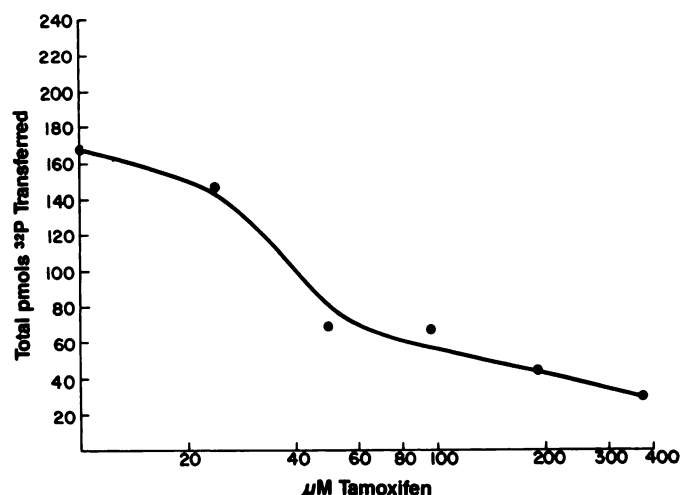


Chart 2. Inhibition of PKC by tamoxifen in the presence of added TPA and phospholipid. Rat brain PKC was assayed as described in the legend to Chart 1, except that 200 nM TPA was used in place of 1 mM Ca²⁺. Each point represents the average of triplicate assays, which agreed within 10%. This experiment was repeated in its entirety with similar results.

Table 1

Effects of tamoxifen on PKC activity at varying concentrations of phospholipid

Rat brain PKC was assayed as described in the legend to Chart 1, except that Ca²⁺, TPA, teleocidin, and phospholipid were present at the indicated concentrations. Each experimental value represents the average of triplicate assays, which agreed within 10%.

Activator	Phosphatidylinositol (μg/ml)	% of inhibition ^a by 95 μM tamoxifen
1 mM Ca ²⁺	6	87
1 mM Ca ²⁺	10	59
1 mM Ca ²⁺	40	46
1 mM Ca ²⁺	100	<10
200 nM TPA	6	73
200 nM TPA	10	74
200 nM TPA	40	52
200 nM TPA	100	<10
160 nM teleocidin	10	85
160 nM teleocidin	40	38

^a % of inhibition = 1

$$= \frac{{}^{32}\text{P incorporation into histone observed in presence of } 95 \mu\text{M tamoxifen}}{{}^{32}\text{P incorporation observed in absence of tamoxifen}} \times 100$$

Table 2

Effects of tamoxifen on PKC-catalyzed protamine sulfate phosphorylation

Standard assays were done except that the reaction mixtures contained protamine sulfate (0.67 mg/ml) rather than histone III-S as the phosphoacceptor substrate, and the reaction mixtures did not contain added Ca²⁺, TPA, or phospholipid. Each experimental value represents the mean of triplicate assays.

Tamoxifen concentration (μM)	pmol ³² P incorporated into protamine sulfate
0	638 ± 8 ^a
40	664 ± 28
80	612 ± 48
160	680 ± 29

^a Mean ± S.D.

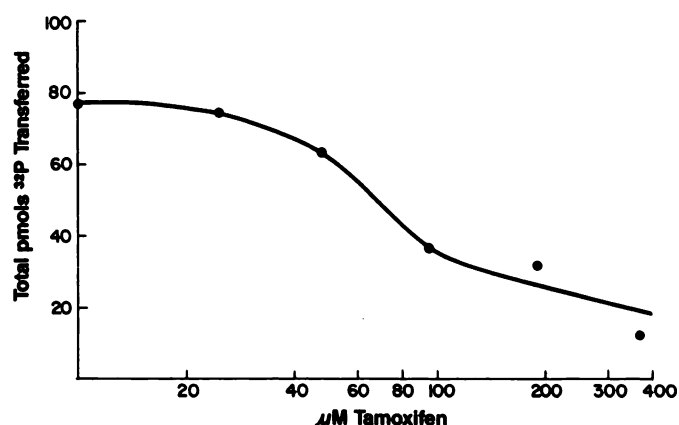


Chart 1. Inhibition of PKC by tamoxifen in the presence of added calcium and phospholipid. Rat brain PKC was assayed in the presence of Ca²⁺ and phospholipid as described in "Materials and Methods." Tamoxifen was dissolved in DMSO and added to the assay system at the indicated final concentration. Reactions were then initiated by the addition of enzyme. All reaction mixtures contained 4% DMSO. The extent of histone phosphorylation observed in PKC assays done in the presence and absence of 4% DMSO agreed within 10%. "pmol ³²P transferred," pmol ³²P transferred from [³²P] ATP to histone III-S in a Ca²⁺- and phospholipid-dependent manner. Each point represents the average of triplicate assays which agreed within 10%. This experiment was repeated in its entirety with similar results.

mixture is increased, whether PKC is activated by TPA plus phospholipid, Ca^{2+} plus phospholipid, or teleocidin plus phospholipid (Table 1). When the phospholipid concentration was 100 $\mu\text{g/ml}$, there was no detectable inhibition of PKC by 95 μM tamoxifen in the presence of either 200 nM TPA or 1 mM Ca^{2+} (Table 1). At a phospholipid concentration of 40 $\mu\text{g/ml}$, which was a saturating level for PKC activity in the absence of tamoxifen (data not shown here), tamoxifen (100 μM) caused about a 50% inhibition of enzyme activity. The observation that increasing phospholipid concentrations overcome the inhibition of PKC by tamoxifen suggests that this tamoxifen-mediated inhibition is competitive with phospholipid.

The above studies were all done in subcellular assays with partially purified PKC. In order to estimate the potency of the effect of tamoxifen on PKC in intact cells, we studied the inhibition of [^3H]PDBU binding by tamoxifen in mouse fibroblast C3H10T $^{1/2}$ cells. An IC_{50} of 5 μM was found in this system (Chart 3). Interestingly, it has been shown (18) that this concentration of tamoxifen inhibits cell growth in MCF-7 human breast cells. In order to verify that a decrease in [^3H]PDBU binding in the presence of tamoxifen was in fact due to a tamoxifen-mediated event rather than cell death of a large proportion of cells, we examined the cytotoxic effects of tamoxifen on C3H10T $^{1/2}$ cells using a standard trypan blue method (17). Subconfluent cells treated with 5 μM tamoxifen citrate with incubation times ranging from 30 to 120 min showed a viability throughout this time course above 90%. We found that concentrations higher than 40 μM gave rise to detachment of C3H10T $^{1/2}$ cells from the plate. Increasing concentrations of tamoxifen (10 to 40 μM) gave rise to a refractile appearance of the cells, which was also observed with TPA, although TPA produced this effect at nM concentrations.

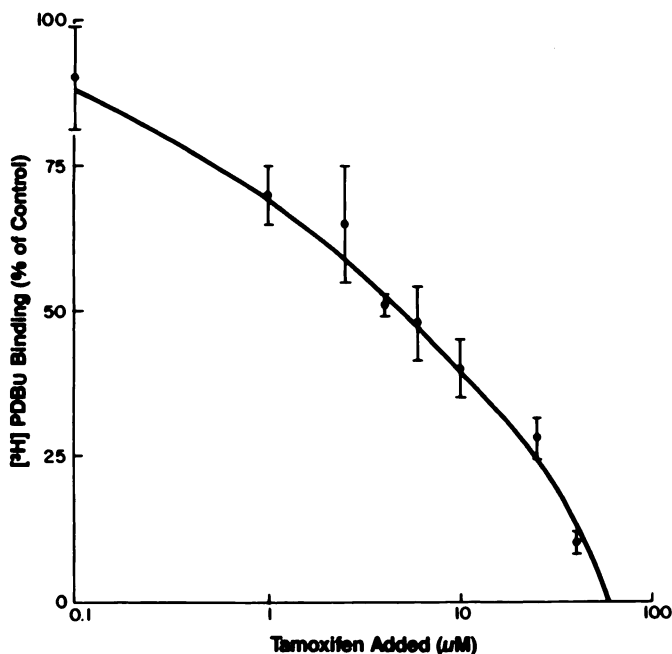


Chart 3. Inhibition of [^3H]PDBU receptor binding by tamoxifen in C3H10T $^{1/2}$ cells. Inhibition of [^3H]PDBU binding by tamoxifen was assayed as described in "Materials and Methods." Points are averages of triplicate assays and are expressed as percentage of the control value, i.e., in the absence of tamoxifen. This experiment was repeated in its entirety with similar results. Bars, SD.

DISCUSSION

In this study, we report that μM concentrations of tamoxifen inhibit rat brain PKC activity in a subcellular system and also inhibit the binding of [^3H]PDBU to specific high-affinity receptors in intact mouse fibroblasts. It has been observed that tamoxifen-treated breast cancer patients have μM levels of tamoxifen in their plasma (1, 3, 4) and an average of 25 ng tamoxifen/mg protein in their tumor tissue (3). The inhibiting activities of tamoxifen which we report here may therefore play a role in the *in vivo* tamoxifen-mediated tumor regression observed in tamoxifen-treated human breast cancer, in addition to the well-known antiestrogen effects of tamoxifen.

PKC is a Ca^{2+} - and phospholipid-dependent protein kinase which binds tumor-promoting phorbol esters with high affinity and which is activated by phorbol ester, indole alkaloid, and polyacetate tumor promoters (6, 8, 13, 26). Our results indicate that tamoxifen inhibits the Ca^{2+} -plus phospholipid-dependent, the TPA-plus phospholipid-dependent, and the teleocidin-plus phospholipid-dependent phosphotransferase activities of PKC, although it does not inhibit the phosphotransferase activity of PKC observed in the absence of activator molecules, such as Ca^{2+} and phospholipid. These data are consistent with a mechanism in which tamoxifen inhibits PKC by interacting with its regulatory domain and/or with the phospholipid, but not with the active site of the enzyme. The observed inhibition of [^3H]PDBU binding by tamoxifen may be due to a direct interaction between tamoxifen and the phorbol ester receptor or may result from indirect effects of tamoxifen on phorbol ester receptors in intact cells. We have examined the effects of TPA (1 nM to 2 μM) on the inhibition of Ca^{2+} -phospholipid-activated PKC by 100 μM tamoxifen. We find that TPA reduces the tamoxifen-mediated inhibition only at μM concentrations of TPA (data not shown). This suggests that tamoxifen does not compete for the high-affinity phorbol ester receptor site.

The phospholipid-interacting drugs chlorpromazine, dibucaine, imipramine, phentolamine, tetracaine, and verapamil all inhibit PKC (14). These drugs do not interact with the active site of the enzyme, and their inhibitory actions on PKC are overcome by increasing concentrations of phospholipid, suggesting that they inhibit PKC by competing with lipids (14). We have found that, like these phospholipid-interacting drugs, tamoxifen does not appear to interact with the active site of PKC and that the inhibition of PKC by tamoxifen is also overcome by increasing concentrations of phospholipid. These observations suggest that tamoxifen may inhibit PKC by competing with phospholipids. It is possible that the regulatory domain of PKC bears some homology to calmodulin, since several calmodulin antagonists (including trifluoperazine, chlorpromazine, W7, and, we report here, tamoxifen) also inhibit PKC activity (11, 14).

The direct activation of PKC by tumor promoters suggests that PKC activation is important in tumor promotion. Thus, the growth-inhibitory activity of tamoxifen may be due in part to the inhibition of PKC by tamoxifen. In fact, growth-inhibitory and cytotoxic effects of tamoxifen have been observed in cell culture systems at the same tamoxifen concentration range at which we observe the inhibition of [^3H]PDBU binding by tamoxifen. Thus, whereas concentrations of tamoxifen in the nM range are sufficient to inhibit the binding of estrogen at its physiological level to its receptor, inhibition of the growth of human mammary cell

lines MCF-7 and ZR-75-1 requires μM concentrations of the drug (2, 5, 18).

The importance of the interaction between tamoxifen and the estrogen receptor as a component of the mechanism of tamoxifen-mediated growth inhibition is well documented (2, 12, 18). However, it is clear that a comprehensive understanding of the action of tamoxifen as an antitumor agent requires further investigations of the effects of the drug on other cellular activities, such as those of calmodulin (9) and, as suggested by the present studies, that of PKC.

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