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NONSTEROIDAL ANTIESTROGEN INHIBITION OF PROTEIN KINASE C IN HUMAN CORPUS LUTEUM AND PLACENTA*

KATHLEEN M. EYSTER†‡ and MARTIN R. CLARK

Division of Reproductive Endocrinology and Fertility, Department of Obstetrics and Gynecology, University of North Carolina School of Medicine, Chapel Hill, NC 27599, U.S.A.

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Abstract—These studies were undertaken to determine whether nonsteroidal antiestrogens would inhibit the calcium/lipid-dependent protein kinase (protein kinase C) activity in hormonally-responsive human reproductive tissues. Cytosol was prepared from human corpus luteum and term placenta. Protein kinase C activity was examined with various antiestrogens, estrogens, and catecholestrogens. The nonsteroidal antiestrogens tamoxifen, clomiphene and Z-4-hydroxytamoxifen inhibited protein kinase C in cytosol from human corpora lutea and placentae in a concentration-dependent manner. The IC_{50} values were 35–45 μM for tamoxifen, 58–66 μM for clomiphene, and 88 μM for hydroxytamoxifen. Protein kinase C purified 600-fold from human placenta was also inhibited by tamoxifen. The estrogens, estradiol and diethylstilbestrol (DES), and the catecholestrogens, 2-hydroxyestradiol and 4-hydroxyestradiol, had no effect on protein kinase C activity, nor were they able to prevent the inhibition of protein kinase C by the antiestrogens. Inhibition of the enzyme by the antiestrogens was competitive with phosphatidylserine and 1,2-diolein. In addition, tamoxifen inhibited enzyme activity stimulated by the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). The data suggest that the action of these antiestrogens on protein kinase C was a direct inhibition of the enzyme. Furthermore, the site of interaction showed markedly different structural specificity from that of the estrogen receptor.

The calcium and lipid-dependent protein kinase (protein kinase C) is an important intracellular mediator of the hormonally-sensitive phosphoinositide pathway [1, 2]; furthermore, protein kinase C has been implicated in cell growth and differentiation [3] and in tumor promotion processes [4]. Rat [5] and bovine [6] ovarian tissues respond to gonadotropins with an increase in phosphatidylinositol turnover. Furthermore, protein kinase C activity has been demonstrated in ovarian tissue from the human [7], bovine [8], and rat [7] and in human placenta [9]. Thus, this second messenger pathway is present and hormonally responsive in ovarian tissues. Hormones which act via this pathway stimulate ovulation and have gonadotropic effects; that is, they stimulate ovulation and steroidogenesis [10, 11]. However, the precise physiological role of the phosphoinositide pathway in ovarian function has not yet been well defined.

It has been reported that the nonsteroidal antiestrogens inhibit protein kinase C in the rat brain [12, 13] and the rat ovary [13]. These drugs are widely used clinically. Tamoxifen is prescribed to treat estrogen receptor-positive breast cancer, and clomiphene is used in the treatment of infertility. In both pharmacologic situations, the major mechanism of drug action is apparently competition with estrogen for the estrogen receptor.

Since tamoxifen and clomiphene are used therapeutically in humans, and the phosphatidylinositol/protein kinase C pathway is present and active in human reproductive tissues, we believed that it was important to examine the effects of these agents on protein kinase C activity in human tissues. Thus, we chose to examine the effects of antiestrogens on human corpus luteum and placenta which contain both estrogen receptors [14, 15] and protein kinase C activity [7, 9]. The studies were also performed in rat brain tissue for comparative purposes.

MATERIALS AND METHODS

Materials. The source for phosphatidylserine was Supelco, Inc. (Houston, TX). The [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, CA). Dimethyl sulfoxide and *N*,*N*-dimethylformamide were purchased from EM Science (Gibbstown, NJ), and 1,4-dioxane and ScintiVerse II from the Fisher Scientific Co. (Springfield, NJ). Z-4-Hydroxytamoxifen was from Amersham (Arlington Heights, IL) and ethanol was purchased from the AAPER Alcohol & Chemical Co. (Shelbyville, KY). HAWP nitrocellulose filters were obtained from the Millipore Corp. (Bedford, MA). All other chemicals, *trans*-tamoxifen citrate, clomiphene citrate, and estrogens were purchased from the Sigma Chemical Co. (St Louis, MO).

Tissue preparation. One human corpus luteum tissue used in this study was obtained (with informed consent and approval of the Human Subjects Committee of the University) from a woman in early to midluteal phase, during surgery for nonendocrine indications. The corpus luteum was dissected free

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† Recipient of NIH Postdoctoral Fellowship HD-06871.

‡ Address reprint requests to: Kathleen M. Eyster, Ph.D., Department of Physiology and Pharmacology, University of South Dakota, Vermillion, SD 57069.

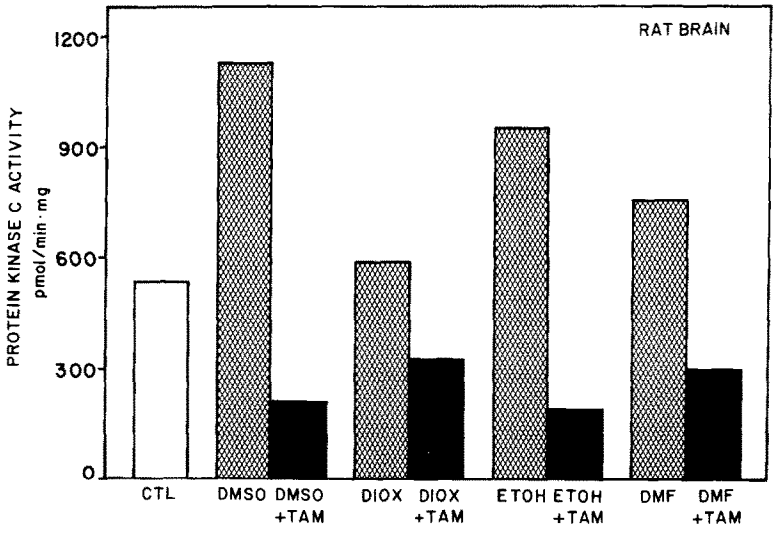

Fig. 1. Effects of various solvents ± tamoxifen on protein kinase C from rat brain. The control value (Ctl, open bar) indicates enzyme activity when there were no additions to the assay. Hatched bars represent activity in the presence of the solvents dimethyl sulfoxide (DMSO, 4%), 1,4-dioxane (Diox, 4%), ethanol (4%, ETOH), and *N*,*N*-dimethylformamide (4%, DMF). Closed bars denote enzyme activity in the presence of the various solvents (4%) plus tamoxifen (50 μM).

from the ovary, and the incision in the ovary was repaired (see Ref. 16). The corpus luteum was placed on ice and taken to the laboratory to be processed. Other human corpora lutea were obtained from the National Disease Research Interchange and were removed at ovariectomy/hysterectomy for nonendocrine indications. Human placentae were obtained from the delivery room after normal term pregnancy and delivery. The placentae were placed in physiological saline and rinsed to remove blood. Tissue was dissected from the maternal side and rinsed through several volumes of saline. The tissue was then blotted, weighed, and placed in homogenization buffer. Rat brains were obtained by dissection from normal immature female animals which were being killed for other studies.

Cytosol was prepared from the three types of tissues in the same manner, as previously described [17]. Tissues were placed in homogenization buffer at a dilution of 1:5 (w/v). Homogenization buffer contained 20 mM Tris–HCl at pH 7.5, 2 mM EDTA, 5 mM ethyleneglycol-bis-*N*,*N*,*N*′,*N*′,tetraacetic acid (EGTA), 0.25 M sucrose, and 50 mM 2-mercaptoethanol. A Wheaton Instruments Overhead Stirrer was used to homogenize the tissues. The homogenizer was used for 30 sec at full speed, followed by a 1-min cooling period, then a second 30-sec burst. The homogenate was then centrifuged for 10 min at 1000 *g*. The supernatant fraction was removed and centrifuged at 600,000 *g* minutes. The resulting supernatant fraction was retained as the cytosol preparation and was stored at −70° until used in the protein kinase assay.

Protein kinase C assay. The assay of protein kinase C activity was performed as previously described [7]. The assay mixture contained 20 mM Tris–HCl (pH 7.5), 5 mM magnesium acetate, 10 μM ATP (with 1×10^6 cpm [γ-^{32}P]ATP), 0.2 mg/ml histone III-S, 20 mM 2-mercaptoethanol, 100 mM sucrose, 0.8 mM EDTA, and 2.0 mM EGTA. Enzyme activity was measured in the absence and in the presence of 2.9 mM Ca^{2+}, 0.8 μg/ml 1,2-diolein, and 20 μg/ml phosphatidylserine. The assay was begun by the addition of 15–20 μg cytosol protein, and was stopped after 3 min at 30° by the addition of 2 ml of ice-cold 25% trichloroacetic acid (TCA). The samples were filtered through 0.45 μm Millipore HAWP filters. The filters were rinsed five times with 2-ml aliquots of 25% TCA, and then counted in ScintiVerse II liquid scintillation fluid. Enzyme activity was linear with respect to time and tissue concentration with these assay conditions as previously reported [17].

The antiestrogens (tamoxifen, clomiphene, and Z-4-hydroxytamoxifen), estrogens (estradiol and diethylstilbestrol) and catecholestrogens (2-hydroxyestradiol and 4-hydroxyestradiol) were dissolved in DMSO. Four percent DMSO was required to keep tamoxifen in solution at concentrations that yielded 50 μM or greater tamoxifen in the final assay mixture. To maintain consistency, all other compounds were dissolved in 4% DMSO as well. DMSO was not diluted out in the concentration–response curves but was maintained at 4% throughout. Controls with and without DMSO were run in each experiment.

Each data point in each experiment was tested as follows: duplicate samples in the absence and in the presence of calcium and lipid (1,2-diolein and phosphatidylserine). Phosphorylation measured in the absence of calcium and lipid was then substracted from that measured in their presence, and the resulting values represent specific calcium and lipid-dependent phosphorylation. Values for a given data point did not differ by more than 10% from the mean. Each figure in this paper is a composite of at least two experiments.

Protein content was determined by the Bradford assay [18]. The data are expressed as picomoles of

Table 1. Effect of DMSO on protein kinase C activity in rat brain and human corpus luteum and placenta

	Protein kinase C activity (pmol/min·mg)		
	Control (no additions)	DMSO (4%)	Fold-stimulation with DMSO
Human corpus luteum	354	884	2.5
Human placenta	55	152	2.7
Rat brain	812	1461	1.8

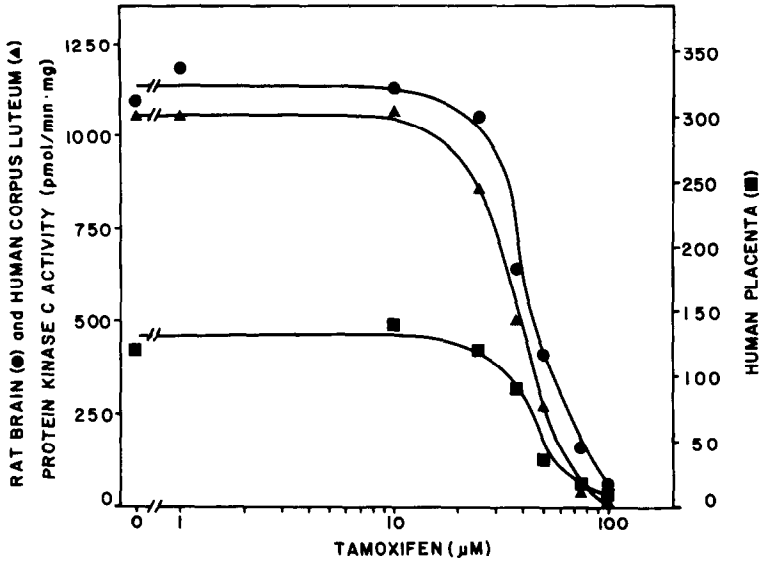

Fig. 2. Effect of tamoxifen on the activity of protein kinase C of human corpus luteum (▲), rat brain (●), and human placenta (■). The concentration of DMSO did not vary across the range of concentrations of tamoxifen (0–100 μM). Note the difference in scale of protein kinase C activity in human corpus luteum and rat brain versus enzyme activity in human placenta.

^{32}P transferred from [γ-^{32}P]ATP to histone III-S per minute times milligrams of cytosol protein (pmol/min·mg).

RESULTS

In the presence of calcium and lipid, protein phosphorylation was enhanced above the levels of phosphorylation in their absence. The increase in phosphorylation due to calcium and lipid was 2.4-fold in human corpus luteum, 2.2-fold in placenta, and 6-fold in rat brain cytosol. DMSO (final assay concentration 4%) caused a significant increase in calcium and lipid-dependent protein kinase activity, as summarized in Table 1. Protein kinase C activity was greater in the presence of 4% DMSO than in control samples in all three tissues (Table 1), and was enhanced 1.3-fold in an enzyme preparation that was purified 600-fold from human placenta (data not shown). Neither DMSO nor the antiestrogens affected phosphorylation in the absence of calcium and lipid (for control vs DMSO in human corpus luteum cytosol: 268 vs 266 pmol/min·mg in the absence of calcium and lipid, and 626 vs 1149 pmol/min·mg in the presence of calcium and lipid). Enzyme activity was enhanced 2-fold by 4% DMSO, however, when calcium but no lipid was present (data not shown).

Figure 1 illustrates that other organic solvents had a similar effect on protein kinase C from rat brain. Both ethanol and *N*,*N*-dimethylformamide caused an increase in protein kinase C activity at a concentration of 4%. Tamoxifen (50 μM) dissolved in any of these solvents inhibited protein kinase C activity below the control levels seen in the absence of solvent. In contrast, 1,4-dioxane (4%) did not cause an increase in protein kinase C activity. However, 50 μM tamoxifen dissolved incompletely in 4% 1,4-dioxane.

Tamoxifen inhibited protein kinase C activity in a concentration-dependent manner in cytosol prepared from human corpus luteum, rat brain, and human placenta, as shown in Fig. 2. The concentration–response curve is steep; the inhibitory effect occurred over a concentration range of less than one order of magnitude. The 50% inhibitory concentrations (IC_{50}) for tamoxifen were approximately 36 μM for human corpus luteum, 41 μM for rat brain, and 45 μM for human placenta. Since tamoxifen inhibition has been reported to be competitive with lipids used to activate protein kinase C [13], further analyses were performed. The inhibi-

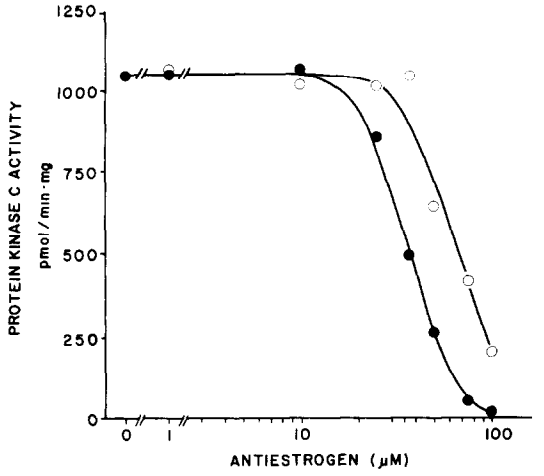

Fig. 3. Protein kinase C activity from human corpus luteum in the presence of increasing concentrations of the antiestrogens, tamoxifen (●) and clomiphene (○). Concentrations of antiestrogen ranged from 0 to 100 μM. DMSO (4%) was present at each concentration level.

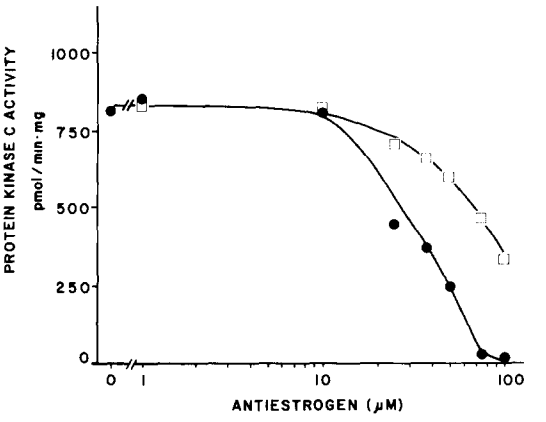

Fig. 4. Activity of protein kinase C from human corpus luteum in the presence of increasing concentrations of tamoxifen (●) and Z-4-hydroxytamoxifen (□). The concentrations of antiestrogens varied from 0 to 100 μM. DMSO was maintained at 4% across the range of concentrations.

tory constant (K_i) for tamoxifen in rat brain cytosol was determined by regression analysis of a Lineweaver–Burk plot of competition of tamoxifen for phosphatidylserine- and 1,2-diolein-activation of protein kinase C (data not shown). These experiments yielded a K_i value of 11 μM. Although the IC_{50} values were similar among the three tissues, total protein kinase C activity was much greater in the human corpus luteum and rat brain than in human placental tissue. Tamoxifen also inhibited protein kinase C activity in an enzyme preparation that was purified 600-fold from human placenta, with an IC_{50} of approximately 35 μM (data not shown).

The inhibition of protein kinase C from human luteal cytosol by a related antiestrogen, clomiphene, is depicted in Fig. 3. The concentration–response curve for tamoxifen is included as a direct comparison. Clomiphene inhibited protein kinase C in a concentration-dependent manner, with an apparent IC_{50} in human corpus luteum of 66 μM. Clomiphene also inhibited protein kinase C activity in rat brain and human placental cytosol with IC_{50} values similar to that of human luteal tissue (data not shown).

Z-4-Hydroxytamoxifen, a metabolite of tamoxifen, also inhibited protein kinase C activity in human corpus luteum in a concentration-dependent manner, as shown in Fig. 4. Again, the concentration–response curve for tamoxifen was repeated in these experiments and included for direct comparison. The apparent IC_{50} for Z-4-hydroxytamoxifen was 88 μM, twice that of tamoxifen.

Tamoxifen inhibited protein kinase C activity stimulated by the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). TPA was substituted for phosphatidylserine and 1,2-diolein in the assay. Control values for protein kinase C activity were lower when the enzyme was stimulated by 10 ng/ml TPA than when it was stimulated by phosphatidylserine and 1,2-diolein (288 vs 812 pmol/min·mg, respectively, for rat brain tissue). DMSO (4%) enhanced enzyme activity by 1.3-fold over control. In the presence of 10 ng/ml TPA, protein kinase C activity was inhibited by tamoxifen in a concentration-dependent manner. The IC_{50} values were 32 μM in rat brain cytosol and 25 μM in placenta (data not shown).

Diethylstilbestrol (DES) and estradiol, at doses of 10, 50 and 100 μM, had no effect on protein kinase C activity, whereas tamoxifen caused a dramatic inhibition of enzyme activity at these concentrations (Fig. 5). Furthermore, the addition of DES or estradiol in the presence of tamoxifen (in equimolar concentrations) did not prevent the inhibition of protein kinase C by tamoxifen (Fig. 6). The same results were found with rat brain and human placental cytosol preparations (data not shown).

Figure 7 illustrates similar results with the catecholestrogens. 2-Hydroxyestradiol (2-OH-E_2) and 4-hydroxyestradiol (4-OH-E_2) did not affect protein kinase C activity at concentrations of 10, 50, and 100 μM, nor could either catecholestrogen prevent the inhibition of the enzyme by tamoxifen when the compounds were present in equimolar concentrations (50 μM).

DISCUSSION

In contrast to previous reports [12, 13], our data indicate that DMSO and other solvents caused an increase in protein kinase C activity. The solvents clearly affected only the calcium/lipid-dependent protein kinase and not other kinases, as phosphorylation in the absence of calcium and lipid was not changed. However, the effect did occur when calcium, but not lipid, was present. The enhancement of enzyme activity was present in both cytosol and purified preparations. The presence of DMSO may alter the presentation of lipids and/or inhibitors to protein kinase C, analogous to the "mixed micelle" assay for enzyme activity [19, 20].

Our data corroborate previous reports [12, 13] that the non-steroidal antiestrogens inhibit protein kinase

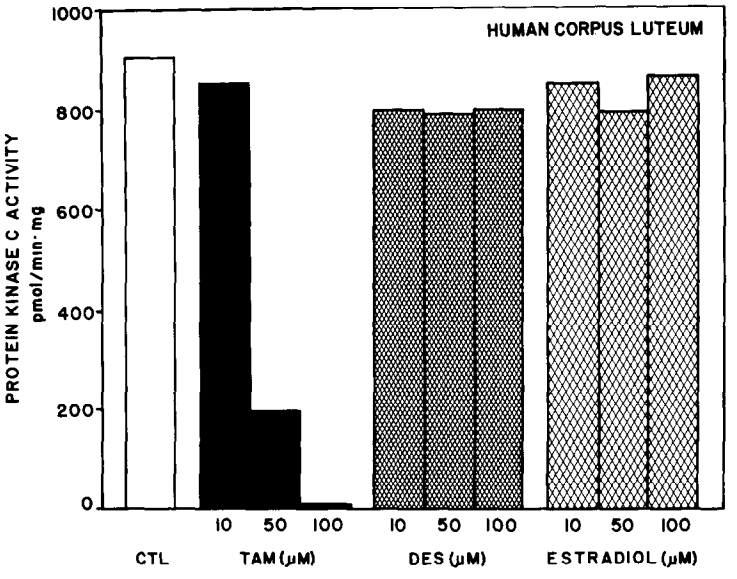

Fig. 5. Protein kinase C activity from human corpus luteum tissue in the presence of increasing concentrations (10–100 μM) of tamoxifen (TAM), diethylstilbestrol (DES), and estradiol. The open bar denotes protein kinase C activity in the presence of 4% DMSO.

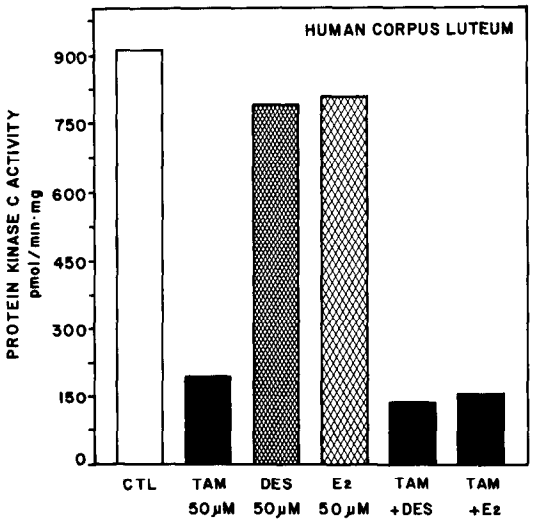

Fig. 6. Effects of DES and E_2 on the activity of protein kinase C from human luteal tissue in the presence and absence of tamoxifen. The control value (open bar) denotes enzyme activity in the presence of 4% DMSO. Activity in the presence of DES (50 μM) or estradiol (E_2; 50 μM) is shown in the shaded bars. The closed bars represent protein kinase C activity in the presence of tamoxifen (TAM, 50 μM) alone, or tamoxifen (50 μM) + DES (50 μM) or E_2 (50 μM).

C in rat brain and extend the findings to human, hormonally-responsive reproductive tissues. It is not likely that the inhibitory effects of antiestrogens on protein kinase C occur via the estrogen receptor since the affinities of the estrogen receptor and protein kinase C for antiestrogens differ. The failure of estrogens and catecholestrogens to affect protein kinase C activity, and their inability to prevent the inhibition of protein kinase C by antiestrogens indicate that the site of interaction shows markedly different structural specificity from that of the estrogen receptor. The data suggest that the effect of the antiestrogens is directly upon protein kinase C.

Although the major pharmacological effects of tamoxifen and clomiphene are believed to be mediated by competition with estrogen for the estrogen receptor, there is a body of evidence that describes the interaction of estrogens with a non-estrogen receptor site [21, 22]. A specific, saturable antiestrogen binding site has been demonstrated [23, 24] which is not competible with estrogen [23]. It has a broad distribution both within and outside of estrogen-responsive tissues [25, 26]. However, the physiological relevance of the antiestrogen binding site is unclear. There are data which suggest that some of the anti-proliferative effects of tamoxifen occur through this site [27, 28], and other data which suggest that the growth-modulatory effects of tamoxifen do not occur through the antiestrogen binding site [29]. The specificities of protein kinase C and the antiestrogen binding site for antiestrogens are grossly similar. However, the antiestrogen binding site is saturable at nanomolar concentrations [21], whereas low micromolar amounts of antiestrogens are needed for the inhibition of protein kinase C. It seems likely that protein kinase C and the antiestrogen binding site are distinctly different entities; however, a definitive statement cannot be made until direct comparative studies have been done.

It is uncertain at this time whether inhibition of protein kinase C by tamoxifen plays role in the pharmacologic action of the drug. Tumor tissue levels of tamoxifen were reported to be 25 ng/mg [30]. If one assumes a protein content of 5–10%, the concentration of tamoxifen in tumor tissue would be 3.4 to 6.7 μM [31]. The 50% inhibitory concentrations for tamoxifen at optimal lipid levels *in vitro* were 35–45 μM. However, our results cor-

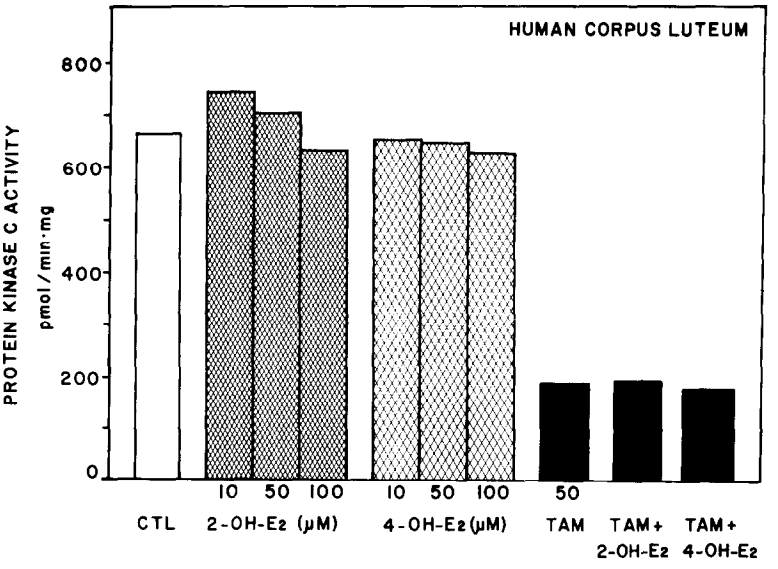

Fig. 7. Effects of the catecholestrogens, 2-hydroxyestradiol (2-OH-E_2) and 4-hydroxyestradiol (4-OH-E_2), on protein kinase C activity in human corpus luteum tissue in the absence and presence of tamoxifen. When tamoxifen and the catecholestrogens were present together, the concentration of each was 50 μM.

roborate the report [13] that the inhibition of protein kinase C by antiestrogens is competitive with phosphatidylserine, a lipid which is necessary for enzyme stimulation and is present in the protein kinase C assay. The inhibitory constant (K_i) as determined under competitive conditions for lipid was 11 μM. If one makes the reasonable assumption that lipid levels in the resting cell are less than optimal for protein kinase C activity, then it is probable that tamoxifen would inhibit protein kinase C in whole cells.

Although the role of the antiestrogen binding site in cell growth is unresolved, the activation of protein kinase C has been associated with increased cell proliferation. Tumor-promoting phorbol esters activate protein kinase C directly [32], and some oncogenes are reported to activate the enzyme through stimulation of the phosphatidylinositol pathway [4, 33]. Thus, a decrease in protein kinase C activity should lead to a decrease in cell growth. By analogy, the inhibition of protein kinase C by antiestrogens may be responsible for the antiproliferative effects of the antiestrogens. Indeed, our data demonstrate that tamoxifen inhibits phorbol ester-stimulated protein kinase C activity.

In summary, we report that the nonsteroidal antiestrogens, tamoxifen, clomiphene, and Z-4-hydroxytamoxifen, inhibited protein kinase C of the human corpus luteum and placenta in a concentration–dependent manner which was not reversible by estrogens or catecholestrogens. The antiestrogens appeared to inhibit protein kinase C directly. Furthermore, the inhibition of protein kinase C by antiestrogens may play a role in the pharmacologic action of these drugs.

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