Calmodulin Antagonism and Growth-inhibiting Activity of Triphenylethylene Antiestrogens in MCF-7 Human Breast Cancer Cells

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ABSTRACT

The triphenylethylene antiestrogen tamoxifen has been shown previously to inhibit both calmodulin and protein kinase C activities, which are involved in the control of cell proliferation. We have studied the effect of several derivatives of the triphenylethylene antiestrogens family on the inhibition of both calmodulin-dependent cyclic adenosine 3'5'-monophosphate-phosphodiesterase activity and proliferation of breast cancer cells cultured with 0.5 µM estradiol in order to prevent interaction of these drugs with the estrogen receptor. We have observed that hydroxylation of the triphenylethylene molecule significantly decreases its ability to inhibit the calmodulin-dependent phosphodiesterase activity in vitro. Furthermore, the growth-inhibiting activity of several antioestrogens and other calmodulin antagonists (R24571, trifluoperazine, N-(6-aminohexyl)-5-chloronaphthalene-1-sulfonamide, and N-(6-aminohexyl)-1-naphthalenesulfonamide) correlated with their antagonistic effects on calmodulin activity. The level of activity was determined as follows: R24571 > tamoxifen = N-demethyltamoxifen = nafodixine > 4-hydroxytamoxifen > 3,4-dihydroxytamoxifen = trifluoperazine > N-(6-aminohexyl)-5-chloronaphthalene-1-sulfonamide > metabolite A > N-(6-aminohexyl)-1-naphthalenesulfonamide. On the other hand both protein kinase C-activating and -inhibiting drugs (phorbol-12,13-diacetate and tamoxifen, respectively) have a synergistic inhibitory effect on the growth of MCF-7 cells. Our data suggest that antioestrogen interactions with calmodulin and not protein kinase C may play a role in mediating the drug-induced estrogen-independent inhibition of breast cancer cell growth.

INTRODUCTION

Triphenylethylene antioestrogens have been reported to inhibit mammmary cancer cell proliferation in vitro (1) and are widely used in the treatment of human breast cancer (2). Since these compounds are able to compete with estrogen for binding to estrogen receptor (1), it has been suggested that antioestrogens induce these effects by antagonizing the growth-enhancing activity of estradiol at the cellular level. However, the presence of specific intracellular antioestrogen binding sites distinct from the estrogen receptor (3-5) and the finding that TAM (6) inhibits the proliferation of estrogen receptor-negative breast cancer cells (6) suggest that this drug may control estrogen-independent processes involved in cell proliferation. Calcium ions have been shown to play an important role in the mechanism by which several growth factors control cell proliferation (7). Calcium is able to transduce the signals of two main pathways. In the first pathway, calcium interacts with an intracellular calcium receptor, termed CAM, which is an ubiquitous protein that is activated by calcium to regulate several enzymes and physiological cellular processes (8). There is accumulating evidence to suggest that CAM is involved in the control of cell proliferation. Not only has cellular transformation to malignancy been associated with an increase of intracellular CAM (9) but a positive correlation between CAM levels and growth rate has also been demonstrated in cancer cells (10). Moreover, CAM antagonists inhibit tumor cell proliferation in vitro (11) and in vivo (12), while they do not inhibit the growth of variant cells missing a specific CAM-binding protein (13).

In the second pathway, calcium ions have been reported to act synergistically with diacylglycerol (a product of membrane phosphatidylinositol breakdown) and with the synthetic analogue tumor promoter TPA in the activation of protein kinase C, which may act to transduce the growth-promoting signals of some growth factors (14).

TAM has been recently reported to antagonize both CAM and protein kinase C activities in vitro (15, 16). In this report we investigated whether the interaction of TAM with CAM or protein kinase C could play a role in the control of breast cancer cell proliferation. We first correlated the in vitro CAM-antagonistic activity of several triphenylethylene derivatives and other CAM antagonists with their effects on the proliferation of MCF-7 human breast cancer cells in culture. Secondly, to study the role of the protein kinase C activation in the proliferation of MCF-7 cells, we investigated the effect of TPA on the growth of this breast cancer cell line. We observed that CAM antagonism by several antioestrogen derivatives correlates with their estrogen-independent, growth-inhibiting potencies on MCF-7 cells. On the other hand, the protein kinase C-activating and -inhibiting drugs (TPA and TAM, respectively) combined have a greater inhibitory effect on the growth of MCF-7 cells than either of the drugs alone. Our data suggest that CAM rather than protein kinase C antagonism by antioestrogens mediates the estrogen-independent antiproliferative effect of these drugs.

MATERIALS AND METHODS

Chemicals. [3H]Estradiol (specific activity, 100 Ci/mmol), [3H]CAMP (specific activity, 20 Ci/mmol), and [3H]thymidine (specific activity, 2 Ci/mmol) were obtained from New England Nuclear (West Germany). Hog brain purified CAM and calmidazolium (R24571) were purchased from Boehringer Mannheim (Mannheim, West Germany). Activator-deficient bovine brain cAMP-PDE, 5’-nucleotidase, cAMP, TFP, W-7, W-5, diethylstilbestrol, estradiol, and TPA were obtained from Sigma Chemical Co. (St. Louis, MO). We have used both crude and highly purified PDE preparations from Sigma and we have observed no differences in CAM antagonism by the drugs we have studied. TAM (ICI 46474), MOHT (ICI 77307), DOHT (ICI 77307), Met A (ICI 46929), and DMT (ICI 55548), were kindly provided by ICI Pharma (England). NAF (U11100 A) was a gift from Upjohn Laboratories (Kalamazoo, MI). DEAE-cellulose, Affi-Gel-CAM, and Dowex AG
Cells and Culture Conditions. MCF-7 human breast cancer cells (kindly provided by Dr. M. E. Lippman, National Cancer Institute, Bethesda, MD) were cultured at 37°C in a humidified atmosphere of 5% CO₂-air in Eagle's minimum essential medium supplemented with 2 mM glutamine, nonessential amino acids, and antibiotic plus 5% fetal calf serum (growth medium) or the same medium plus 5% dextran-coated charcoal-treated fetal calf serum for growth experiments (experimental medium). Details of growth experiments are indicated in the appropriate figure legends.

Assay of CAM-dependent cAMP-PDE Activity. The CAM activity of MCF-7 cells was partially purified by CAM affinity chromatography according to the method described by Smoake et al. (17). MCF-7 cells were homogenized in 5 mM Tris-HCl-0.25 M sucrose buffer, pH 7.5, and centrifuged at 800 x g for 30 min. The supernatant was boiled for 4 min and centrifuged further at 3500 x g for 30 min. The supernatant was then assayed for CAM activity by evaluating the activation of PDE. CAM-dependent PDE activity was assayed as described by Teo and Wang (18), by evaluating the P₁ (19) liberated from 0.9 mM cAMP (1 ml) incubated at 30°C for 30 min with 72 mM Tris, 36 mM imidazole, 1 mM magnesium acetate, 0.1 mM CaCl₂ (pH 7.5), 8 mM PDE, 0.1 unit 5'-nucleotidase, and 0.1 μg purified hog brain CAM or MCF-7 cell extract containing an equivalent amount of CAM activity (quantitated by the comparison to a standard curve constructed with purified CAM). CAM-independent PDE activity was determined either in the absence of CAM (or cell extract) or in the presence of 1 mM EGTA. Increasing concentrations of drugs were added to the incubation mixture in order to determine the IC₅₀ obtained after subtracting CAM-independent PDE activity from total enzyme activity. Alternatively, cAMP-PDE activity was assayed by the method of Schonhofer et al. (20). Briefly, the enzyme activity was evaluated by the conversion of cAMP (10 μM cAMP plus 0.025 μCi [³H]cAMP as a tracer) to 5'-AMP followed by precipitation with Ba(OH)₂-ZnSO₄ and Dowex 50-H⁺ chromatography.

Extraction of cAMP-PDE from MCF-7 Cells. PDE from MCF-7 cells was partially purified by CAM affinity chromatography according to the method described by Sharma et al. (21). Briefly, MCF-7 cells were homogenized in 40 mM Tris-HCl (pH 7.0)-2-Mercaptoethanol (10 mM)-0.1 mM EGTA and centrifuged at 10,000 x g for 10 min. The supernatant was submitted to DEAE-cellulose chromatography in order to separate endogenous CAM from PDE. The DEAE-cellulose column was first eluted with 20 mM Tris-HCl (pH 7.0)-1 mM imidazole-1 mM EDTA-10 mM 2-mercaptoethanol-0.05 mM NaCl (buffer A) and then with the same buffer plus 0.22 mM NaCl. After dialysis against buffer B (buffer A containing 0.1 mM CaCl₂ instead of EGTA), the fractions containing the PDE activity (0.22 mM NaCl eluate) were submitted to a chromatography on a column of Affi-Gel-CAM in order to remove CAM nonbinding proteins. After the affinity column was washed with buffer B plus 0.22 mM NaCl, the CAM-dependent PDE was eluted with EGTA-containing buffer (buffer A plus 0.22 mM NaCl).

Effect of Other CAM Antagonists on MCF-7 Cell CAM Activity. To determine if the antagonism of CAM by triphenylethylenes could play a role in the estrogen-independent inhibition of cell proliferation induced by these drugs, we studied the effect of several antiestrogens on the growth of MCF-7 cells cultured in the presence of 0.5 μM estradiol in order to prevent the interaction of these drugs with the estrogen receptor. This estradiol concentration increased the cell growth (by 20%) while higher estrogen concentrations decreased MCF-7 cell number (Fig. 3A). Fig. 3B shows that the order of growth-inhibiting activity of these antiestrogens on MCF-7 cells was TAM = DMT = NAF > MOHT > DOHT > Met A (P > 0.05, > 0.05, < 0.05, < 0.05, and < 0.01, respectively, by comparing the IC₅₀ of each derivative with that of TAM by Student’s t test). The decrease in cell number induced by TAM appears to be due to a slowing of cell proliferation, since a parallel decrease in the uptake of [³H]thymidine was observed as early as 24 h after the treatment (40 ± 3 (SE)% decrease after 5 μM of TAM) while the cell viability was not affected. A decrease in the cell viability (evaluated by the trypan blue dye exclusion test) with the appearance of some cytolysis was observed only after 7-day treatments with TAM concentrations ≥ 5 μM.

Effect of Other CAM Antagonists on MCF-7 Cell CAM Activity and Growth. Fig. 4 shows that the CAM antagonists (R24571, TFP, and W-7) antagonized the activation of cAMP-PDE induced by MCF-7 cell CAM in a dose-dependent manner [IC₅₀ 0.1 ± 0.02 (SD), 17 ± 2, and 50 ± 4, respectively] while the chlorine-deficient analogue W-5 was less active [IC₅₀ > 100 μM]. These drugs were also effective inhibitors of MCF-7 cell growth, with R24571 being a much more active growth-
Effect of TPA and TAM on the Growth of MCF-7 Cells. Fig. 5 shows the effect of TPA on the uptake of \([\text{H}]\)thymidine by MCF-7 cells cultured in the presence of 0.5 \(\mu\)M estradiol. An inhibitory effect on the uptake of \([\text{H}]\)thymidine was observed 24 and 96 h after TPA treatment at concentrations as low as \(10^{-10}\) and \(10^{-11}\) M, respectively, with a maximal effect observed at doses between \(10^{-9}\) and \(10^{-7}\) M. When TAM was added together with TPA to MCF-7 cells a higher growth-inhibitory effect was observed as compared to treatment with either drug alone (Fig. 5). This decrease in the uptake of \([\text{H}]\)thymidine was paralleled by a decrease in the cell number (49 to 56% decrease) 3 days after TPA treatment (not shown), while the cell viability was not affected.

**DISCUSSION**

We observed a \(\text{Ca}^{2+}\)-dependent CAM-like activity on cAMP-PDE in MCF-7 cells. The increase of PDE activity induced by MCF-7 cell extracts was not due to the endogenous cellular PDE since it was also observed after heating, which is known to completely inactivate PDE but not CAM (25). The \(\text{Ca}^{2+}\)-dependence of the activation of cAMP-PDE by MCF-7 cell extracts also suggests that it was not induced by phospholipids which are known to be \(\text{Ca}^{2+}\)-independent activators of PDE (26). Finally, the sensitivity of MCF-7 cell CAM-like activity to several drugs is very similar to that of purified CAM obtained from hog brain. In this regard, the main structural modification affecting the ability of antiestrogens to antagonize the CAM activity is represented by hydroxylation of these compounds. This is in agreement with data reported by other authors who suggest that a decrease in hydrophobicity of the hydrophobic region of several CAM antagonists (represented by the triphenylethylene group in the compound we have studied in our work) is also suggested by the findings that these drugs do not compete with \([\text{H}]\)estradiol for the binding to MCF-7 estrogen receptor (Table 1). The concentrations of the CAM antagonists used here were not cytotoxic during the treatments described in our study. However, some cytotoxic effect was observed after prolonged treatment (7 days) with 5 \(\mu\)M R24571.

Correlation between CAM Antagonism and MCF-7 Cell Growth-inhibiting Activity. The results reported above showed that the less active antagonists of CAM activity also inhibited to a lesser extent MCF-7 cell proliferation. Therefore, we correlated the concentrations of drugs giving 50% inhibition of CAM activity with those giving 50% inhibition of cell growth. Fig. 4C shows that a positive correlation \(r = 0.98\) was found between CAM antagonism and cell growth-inhibiting potencies of the drugs we have studied.

**Effect of estradiol on cAMP-PDE in MCF-7 cells.** The increase of PDE activity induced by MCF-7 cell extracts was not due to the endogenous cellular PDE since it was also observed after heating, which is known to completely inactivate PDE but not CAM (25). The \(\text{Ca}^{2+}\)-dependence of the activation of cAMP-PDE by MCF-7 cell extracts also suggests that it was not induced by phospholipids which are known to be \(\text{Ca}^{2+}\)-independent activators of PDE (26). Finally, the sensitivity of MCF-7 cell CAM-like activity to several drugs is very similar to that of purified CAM obtained from hog brain. In this regard, the main structural modification affecting the ability of antiestrogens to antagonize the CAM activity is represented by hydroxylation of these compounds. This is in agreement with data reported by other authors who suggest that a decrease in hydrophobicity of the hydrophobic region of several CAM antagonists (represented by the triphenylethylene group in the compound we have studied in our work) results in a decrease in the CAM antagonistic potency (27). Moreover, the ability of estradiol to antagonize the CAM activity suggests that, besides the other compounds already described (Refs. 15 and 27; present study), steroid hormone structures (i.e., estrogens) may also be able to modulate CAM function.

Our study suggests that antiestrogen-CAM interactions could play a role in the control of breast cancer cell proliferation. The involvement of CAM in mediating antiestrogen-induced inhibition of MCF-7 cell growth is suggested by the positive correlation between CAM-antagonistic potencies and the estrogen-independent growth inhibiting activities of several triphenylethylene antiestrogen derivatives and other CAM-antagonistic drugs (TFP, W-7, W-5, and R24571). Inhibition of cell proliferation by TFP, W-7, and W-5 compounds has also been described in other experimental models (11, 28). Interestingly,
high doses of estradiol, which are able to antagonize CAM activity, also inhibit MCF-7 cell growth.

TAM and other CAM antagonists such as TFP, W-7, W-5, and R24571 also inhibit the activity of protein kinase C (29, 30) which is involved in the transduction of growth-promoting signals of some growth factors (14). Therefore, we have studied the effect of TPA, which activates protein kinase C, on the growth of MCF-7 cells. Although TPA has been reported to be mitogenic in most cell lines (31), we have observed that this compound inhibited the growth of MCF-7 cells. This is in agreement with the recent report of Ross et al. (32) who correlated the inhibition of the growth of MCF-7 cells by TPA with the activation of protein kinase C. A few examples of TPA-induced inhibition of proliferation of other cell lines also exist in the literature (33–35). Furthermore, we observed that the TAM plus TPA combined treatment of MCF-7 cells resulted in an augmented inhibitory effect on cell proliferation, suggesting that the previously reported in vitro inhibition of protein kinase C by TAM (16) does not play any role in the growth-inhibiting activity of this drug. The mechanism by which TPA decreases MCF-7 cell growth is unclear. However, the activation of protein kinase C has been shown to decrease the binding affinity of the EGF receptor for EGF as well as the activity EGF receptor kinase in several cell lines including MCF-7 cells (36–39). This could result in a decrease of the sensitivity of breast cancer cells to the EGF receptor-binding growth factors which have been shown to be secreted by breast cancer cells and to play a role in the proliferation of these cells (40–42). The mechanisms by which antiestrogen-CAM interactions could be involved in the control of MCF-7 cell growth remain to be elucidated. However, the antagonistic effect of
antiestrogens on CAM-activated PDE could result in an increase in the intracellular levels of cAMP which could affect cell growth. In fact, it has been described previously that the intracellular accumulation of cAMP, either endogenously generated or exogenously supplied, inhibits both in vitro and in vivo growth of mammary cancer cells (43, 44). Alternatively, antiestrogens could interfere with other CAM-regulated cellular processes, such as the assembly of microtubules (45, 46), which play a pivotal role in the mitotic cell cycle (47).

In conclusion, the data reported in this study show that CAM antagonism by several triphenylethylened derivatives and other drugs correlates with their efficiencies in inhibiting MCF-7 cell growth. This suggests that, besides interfering with estrogen receptor-regulated cell proliferation, antiestrogens could also inhibit breast cancer cell growth by affecting CAM-mediated events.

REFERENCES

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