# Tamoxifen Stimulates Calcium Entry Into Human Platelets

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**Abstract:** The anti-estrogenic drug tamoxifen, which is used therapeutically for treatment and prevention of breast cancer, can lead to the development of thrombosis. We found that tamoxifen rapidly increased intracellular free calcium $[Ca^{2+}]_i$ in human platelets from both male and female donors. Thus 10 μM tamoxifen increased $[Ca^{2+}]_i$ above the resting level by 197 ± 19%. Tamoxifen acted synergistically with thrombin, ADP, and vasopressin to increase $[Ca^{2+}]_i$. The anti-estrogen ICI 182780 did not attenuate the effects of tamoxifen to increase $[Ca^{2+}]_i$; however, phospholipase C inhibitor U-73122 blocked this effect. 4-hydroxytamoxifen, a major metabolite of tamoxifen, also increased $[Ca^{2+}]_i$, but other tamoxifen metabolites and synthetic derivatives did not. Three hydroxylated derivatives of triphenylethylene (corresponding to the hydrophobic core of tamoxifen) which are transitional structures between tamoxifen ($Ca^{2+}$ agonist) and diethylstilbestrol ($Ca^{2+}$ antagonist) increased $[Ca^{2+}]_i$ slightly (6% to 24%) and partially inhibited thrombin-induced $[Ca^{2+}]_i$ elevation (68% to 79%). Therefore the dimethylaminoethyl moiety is responsible for tamoxifen being a $Ca^{2+}$ agonist rather than antagonist. 4-Hydroxytamoxifen and polymer-conjugated derivatives of 4-hydroxytamoxifen increased $[Ca^{2+}]_i$, with similar efficacy. The ability of tamoxifen to increase $[Ca^{2+}]_i$ in platelets, leading to platelet activation, and its ability to act synergistically with other platelet agonists may contribute to development of tamoxifen-induced thrombosis.

**Key Words:** tamoxifen, platelets, calcium, thrombosis, thrombin, nongenomic

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## INTRODUCTION

Breast cancer is the leading newly diagnosed cancer (32% of all cancers) and the second leading cause of cancer deaths among women in the United States. Tamoxifen (Fig. 1) is the most widely used drug for the treatment of all stages of breast cancer. In 1998, the FDA approved tamoxifen for breast cancer prevention in healthy women at elevated risk of the disease aged 35 years or older. The Breast Cancer Prevention Trial demonstrated that tamoxifen treatment brings about a 50% reduction in the risk of invasive and noninvasive breast cancer among women who took tamoxifen for 4 years.[1] The number of women who would actually benefit from tamoxifen is estimated to be more than two million.[2] Unfortunately, some women experience adverse outcomes from tamoxifen use, including increased incidence of cardiovascular events such as pulmonary embolism, stroke, and deep vein thrombosis (DVT). The risk of DVT is 2 to 3 times higher in tamoxifen versus placebo group.[3] The NSABP B-14 study demonstrated increased incidence of all thrombotic events in tamoxifen users, including a 4-fold increase in DVT and pulmonary embolism. Given the growing number of women who use tamoxifen for treatment and prevention, the number of adverse thrombotic events is also likely to rise.

Despite the ongoing efforts to establish the mechanism which links tamoxifen with the higher incidence of thrombosis, the reason for this adverse effect is unknown. Reduction of protein S and antithrombin 3, as well as activated protein C resistance due to a common gene defect in Factor V Leiden were thought to cause tamoxifen-induced thrombosis,[4] but later studies did not confirm this association.[5] Interestingly, one recent study found that platelet exposure to tamoxifen and its metabolites leads to the superoxide release resulting in modest changes in platelet function.[6]

Platelets' primary function is hemostasis (cessation of bleeding). Physiological agonists that are generated at the site of vascular injury make platelets undergo shape change, shedding of microvesicles, and fibrinogen receptor expression. As a result, platelets form a blood clot, or thrombus, thereby preventing further blood loss. $Ca^{2+}$ ions play a pivotal role in platelet aggregation. Without a sufficient rise in $[Ca^{2+}]_i$ (intracellular free calcium concentration), platelet aggregation can't occur. On the other hand, agents that promote $Ca^{2+}$ entry into platelets cause platelet aggregation.

It is believed that $Ca^{2+}$ influx occurs in platelets predominantly through a store-operated $Ca^{2+}$ channels (SOCC), also referred to as capacitative $Ca^{2+}$ influx in nonexcitable cells.[7] There is also evidence that SOCC in platelets is TRPC1. The regulation of this $Ca^{2+}$ channel possibly involves the

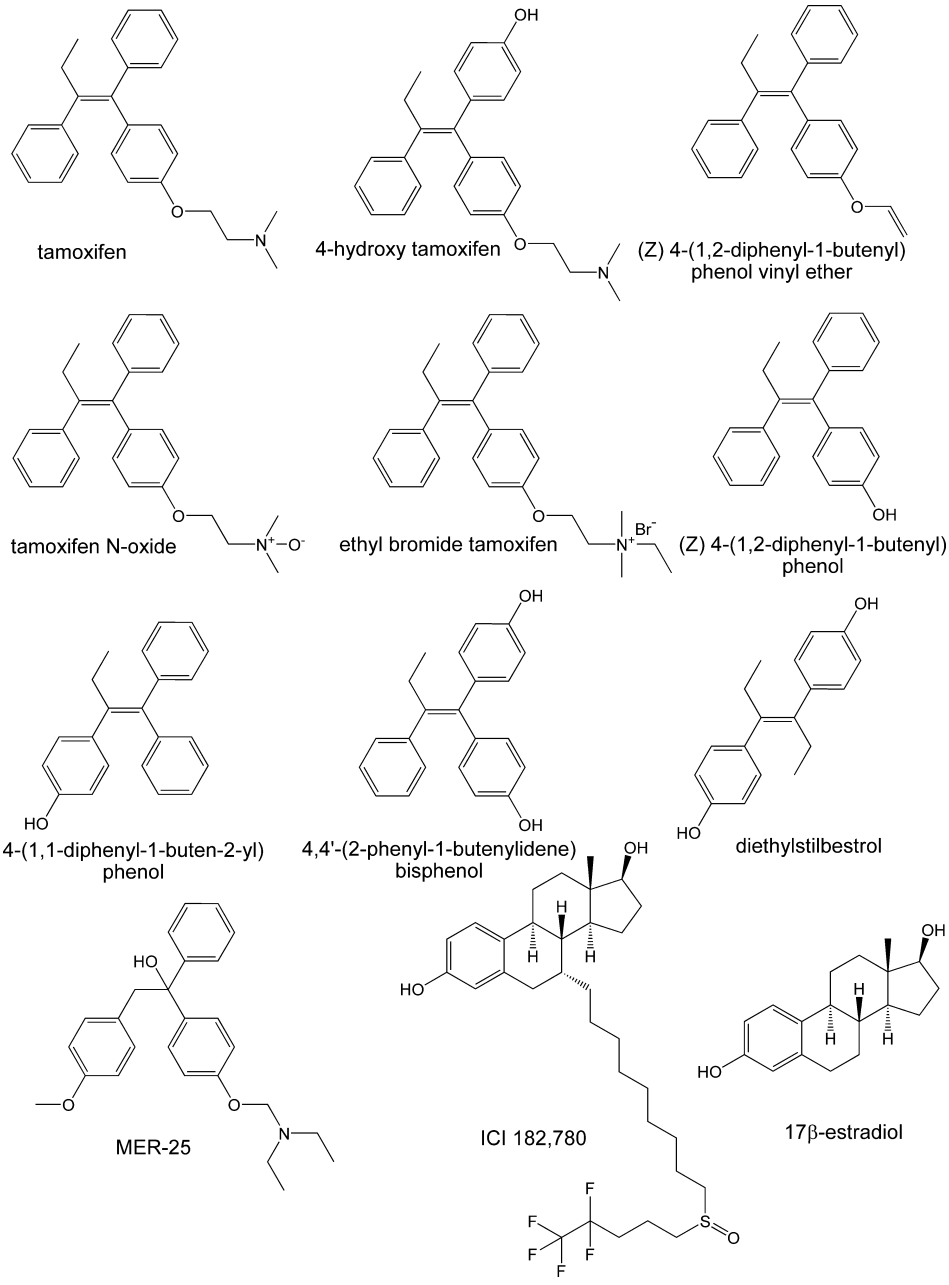


**FIGURE 1.** Structures of the various tamoxifen analogs and other steroid derivatives used in the present study.

conformational coupling of TRPC1 in the plasma membrane and the $IP_3R$ in the endoplasmic reticulum. The involvement of SNAP-25, the actin cytoskeleton, pp60src, and extracellular regulated kinase have all been implicated in the regulation of the $Ca^{2+}$ influx process in platelets.[8]

We recently demonstrated that the synthetic estrogen agonist, diethylstilbestrol (Fig. 1), inhibited thrombin- induced $Ca^{2+}$ influx in human platelets.[9] We also showed that diethylstilbestrol was a potent inhibitor of CRAC ($Ca^{2+}$-release-activated-$Ca^{2+}$) channels in several cell types.[10] We also demonstrated that other naturally occurring and synthetic non-steroidal estrogenic compounds possessing the trans-stilbene moiety, like DES, inhibited thrombin-induced $Ca^{2+}$ influx through SOCC. Such compounds include rigid analogs of DES (tetrahydrochrysenes), stilbenes (eg, trans-resveratrol), and a number of dietary polyphenolic compounds such as phytoestrogenic isoflavonoids genistein, daidzein, and flavonoids such as apigenin.[9,11,12] Tamoxifen also possesses a stilbene moiety, so we reasoned that this compound may also inhibit $Ca^{2+}$ influx in human platelets. On the contrary, tamoxifen stimulated an increase in $[Ca^{2+}]_i$ in human platelets. The ability of tamoxifen to stimulate $Ca^{2+}$ influx rather than inhibit it makes tamoxifen unique among other compounds with a trans-stilbene pharmacophore.

We performed SAR (structure activity relationship) studies of a limited set of tamoxifen analogs including

compounds with triphenylethylethylene core and novel polymer-conjugated derivatives of 4-hydroxytamoxifen. This study provides some preliminary information into the mechanism by which tamoxifen increases $[Ca^{2+}]_i$ in platelets and defines the pharmacophore responsible for tamoxifen action to stimulate $Ca^{2+}$ influx into platelets.

## MATERIALS AND METHODS

### Materials

The following were obtained from Sigma-Aldrich, St Louis, MO: EGTA, dimethyl sulfoxide (DMSO), tamoxifen, 4-hydroxytamoxifen, diethylstilbestrol, human thrombin, thapsigargin, norepinephrine, adenosine diphosphate (ADP), and arginine vasopressin. HEPES and glucose were from Fisher Scientific Co., Pittsburgh, PA. U-73122 and U-73343 were from Alexis Biochemicals, San Diego, CA. ICI 182,780 (fulvestrant) (Fig. 1) was from Tocris. MER-25 (ethamoxytriphetol) (Fig. 1) was from Merrell Dow Research Institute. Ethyl bromide tamoxifen (Fig.1) was generously provided by Dr. Gregory M. Dick, Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN.13 Fura-2/AM was from Invitrogen, Carlsbad, CA.

### Blood Donors and Platelet Preparation

All donors were healthy, nonsmoking volunteers (age, 20 to 60 years). Venous blood was collected into 1/10 volume of (74.8 mM sodium citrate, 38.1 mM citric acid, and 123 mM dextrose pH 6.4; Baxter Healthcare Corp.). The blood was centrifuged at 250 × *g* for 10 minutes at room temperature to obtain platelet rich plasma (PRP). The PRP was centrifuged at 550 × *g* for 12 minutes to sediment the platelets. The platelets were then suspended in a modified Tyrode's physiological salt solution (NaCl, 145 mM; KCl, 4 mM; $MgSO_4$, 1 mM; $Na_2HPO_4$, 0.5 mM; Na/HEPES, 10 mM; glucose, 6 mM; pH 7.4) containing 1.0 mM EGTA to prevent spontaneous aggregation during the various experimental manipulations.

### Platelet Loading With Fura-2 and Measurement of $[Ca^{2+}]_i$

Intracellular free calcium ($[Ca^{2+}]_i$) was measured using the fluorescent dye fura-2. Platelets were incubated with cell permeant fura-2/AM (2 μM) for 1 hour at room temperature. Excess fura-2/AM was removed by centrifugation (500 × *g* for 10 min), and the platelets were suspended in a modified Tyrode's buffer, without added EGTA. Aliquots of platelet suspension (0.5 mL) were added to 1.0-mL aggregometer tubes containing a Teflon-coated stirrer bar (CHRONO-LOG, Havertown, PA). To measure intracellular $Ca^{2+}$ mobilization, no $Ca^{2+}$ was added to the platelet suspension; test compounds were added at approximately 10 seconds. To evaluate $Ca^{2+}$ influx, at approximately 30 seconds before $[Ca^{2+}]_i$ measurements were performed, $Ca^{2+}$ was added to the buffer to a final concentration of 2.0 mM, then test compounds dissolved in DMSO (various concentrations in 2.5 μL) were added. In some cases, thrombin was added after test compounds were added to evaluate their ability to either inhibit or potentiate the ability of thrombin to increase $[Ca^{2+}]_i$. The measurements of $[Ca^{2+}]_i$ were performed at room temperature in a SPEX ARCM spectrofluorometer using excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 505 nm. Calibration was performed as previously described.[14] In some experiments, the data was expressed as 340 nm/380 nm ratios. The level of $[Ca^{2+}]_i$ was calculated by using the SPEX dM3000 software. To calculate the percent inhibition of thrombin or tamoxifen by different analogs, thrombin-induced or tamoxifen-induced elevation of $[Ca^{2+}]_i$ in the presence of DMSO was compared with this in the presence of the analog. DMSO up to 1% (v/v) did not affect platelet responses. None of the compounds used in the study affected fura-2 fluorescence.

### Synthetic Experimental

Some of the compounds [tamoxifen N-oxide and (Z)-4-(1,2-diphenyl-1-butenyl) phenol vinyl ether, trivial name des dimethylamino tamoxifen; (Fig. 1)] were prepared according to modified procedures published in the literature. The polymer conjugates of 4-hydroxytamoxifen were synthesized by conjugating a 4-hydroxytamoxifen analog containing a diaminoalkyl linker containing either 2 or 6 carbons[15] to a poly(methacrylic acid) polymer prepared as the N-hydroxysuccinimide—activated ester.[16] After coupling in dimethylformamide for 72 hours at 80°C, the solution was concentrated and treated with either 2 M NaOH or 2 M $NH_4OH$ for 8 hours to convert the unreacted side chains to either carboxylic acids or carboxamides, respectively. Neutralization of the base followed by extensive dialysis provided the conjugates. Percent incorporation was determined by comparing the NMR integration of the polymer methyl group peak with the peak corresponding to the methyl group on the tamoxifen analog.

Starting materials and solvents were obtained commercially from Sigma Aldrich Chemical, Milwaukee, WI. Thin layer chromatography (TLC) was performed on Analtech silica gel plates. Solvent system was ethyl acetate:methanol:$NH_4OH$, 100:10:3 by volume. Melting points were uncorrected. Proton NMR spectra were obtained on a Varian Unity 200 MHz spectrometer in solvent $CDCl_3$ as indicated. The chemical shifts are reported in δ values down field from tetramethylsilane (TMS). Singlets, doublets, triplets, and quartets are reported as s, d, t, and q. Elemental analyses were obtained from Huffmann Laboratories Inc., Golden, CO and were within 0.4% of the theoretical. Mass spectra were obtained by positive electrospray technology. Samples were dissolved in acetonitrile, an Agilent Technologies 1100LC/MSD machine was used at Hunter College Mass Spectrometry Facility, New York, NY.

### Preparation of Tamoxifen-N-oxide

Tamoxifen N-oxide (Fig. 1) was synthesized by the following procedure. Tamoxifen, (0.3715 g, 0.001 mol) was taken in 30 mL of methanol and stirred with warming at 50°C to 60 °C for 3 hours with 3 mL of $H_2O_2$ (35 % by weight) and left overnight. The reaction mixture was processed by chilling and adding water. A white precipitate (0.165 g, 43% yield) was obtained by filtration; mp 81°C to 82°C. Mass spectrum by electrospray showed a peak, m/e 388 calculated for $C_{26}H_{29}NO_2$. Proton NMR spectrum ($CDCl_3$) showed a multiplet at δ 7.32 -7.05 (10 H, aromatic); a pair of doublets were obtained, centered at 6.75 and 6.51 (J = 8.8 Hz, 4H,

para-disubstituted phenyl). Absorbances centered at 4.39 (t, J = 8.6 Hz, 2H, O-$CH_2$-), 3.55, [(distorted t, 2H-$CH_2$-$N^+O(CH_3)_2$], 3.21 [s, 6H, N-$(CH_3)_2$ ], 2.40 (q, J = 7.4 Hz, 2H, allylic -$CH_2$-), 0.88 (t, J = 7.2 Hz, 3H, $CH_3$) were observed. This compound has been described in the literature and has been recorded to have a Registry number 75504-34-6 in Chemical Abstracts.[17]

## Preparation of (Z)-4-(1,2-diphenyl-1-butenyl) Phenol Vinyl Ether

Tamoxifen-N-oxide was pyrrolized at slightly above its melting point for 3 hours under a stream of $N_2$ gas in order to perform a Cope elimination 18 (Reaction A in Fig. 2). The residue was taken up in methanol and recrystallized to give a small amount of white crystalline powder mp 103°C to 105°C. Chromatography over $SiO_2$ gel and elution with $CH_2Cl_2$ gave a small amount of white powder, which upon recrystallization with $CH_3OH/H_2O$ gave pure white crystals, mp 107°C to 108°C of (Z)-4-(1,2-diphenyl-1-butenyl) phenol vinyl ether. The mass spectrum showed base peak calculated for $C_{24}H_{22}O$, m/e 326. Proton NMR spectrum ($CDCl_3$) showed resonances 7.31-7.10 as a complex multiplet (10H, aromatic); a pair of doublets centered at 6.79 and 6.61 (J = 8–9 Hz, 4H, para-disubstituted phenyl); a four-line resonance 6.51-6.46 (two doublets J = 14 Hz and J = 6 Hz, 1H, vinylic-ether methine); a two-line absorbance at 4.65 (d, J = 13.6 Hz, 1H, vinylic); another two- line absorbance at 4.32 (d, 4.32, J = 6 Hz, 1H, vinylic). Centered at an absorbance at 2.45 (7q, J = 7 Hz, 2H, allylic $CH_2$) and centered at 0.91 a triplet (J = 7 Hz, 3H, $CH_3$).

## General Procedure for the Synthesis of Triphenylethylethylenes

The triphenylethylethylene compounds were synthesized in 1 step (Reaction B in Fig. 2) using the McMurray coupling reaction[19] under the following general reaction conditions. Zinc powder (2.0 g, 0.031 mol) was suspended in 20 mL of dry tetrahydrofuran (THF) and cooled to −10°C. After drop-wise addition of $TiCl_4$ (1.5 mL, 0.014 mol), the solution was heated at reflux for 2 h. After allowing the solution to cool to room temperature, a solution of the 2 ketones dissolved in dry THF (40 mL) was then added and the solution was heated at reflux for 12 h. After the solution cooled to room temperature, 10% aqueous potassium carbonate (30 mL) was added and the solution was filtered and extracted with diethyl ether. The organic layer was washed with brine, dried over magnesium sulfate, and concentrated under reduced pressure. Flash column chromatography (30% ethyl acetate in hexanes) afforded the final compounds. When forming alkene stereoisomers, the reaction generated a 1:1 mixture of E and Z stereoisomers that interconverted readily at room temperature in a manner similar to 4-hydroxytamoxifen.[20] As a result, it was impossible to obtain pure samples of one stereoisomer, and the samples were used as a 1:1 mixture. Details for the synthesis of each compound follows.

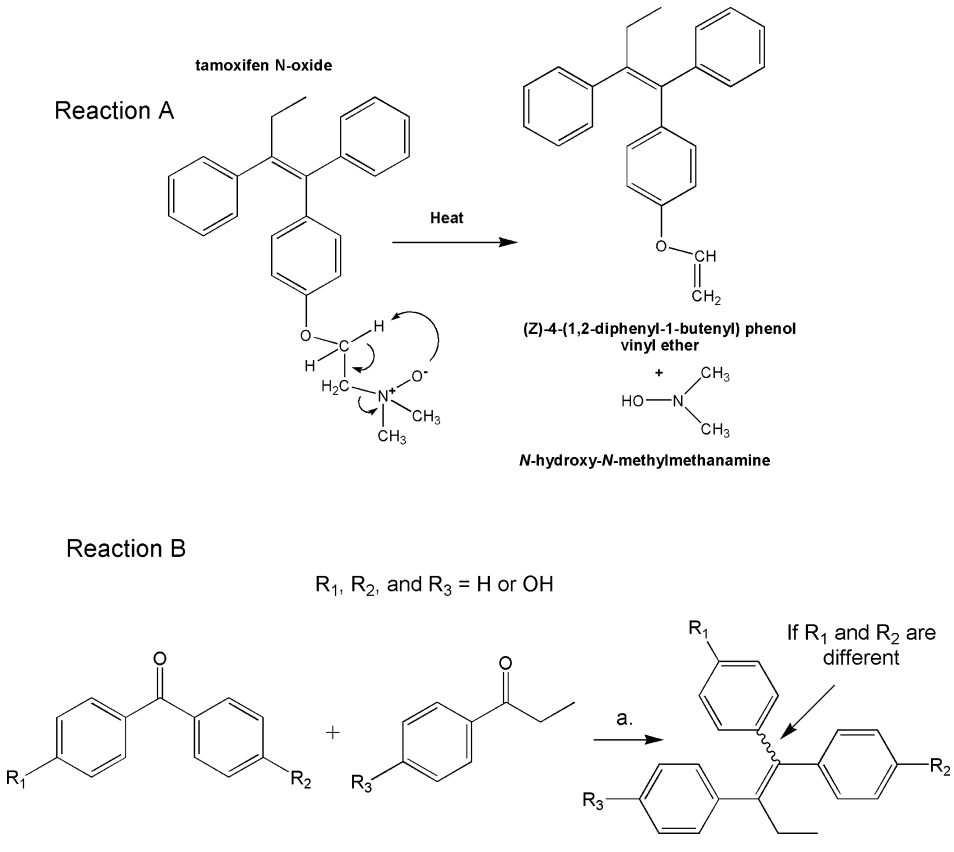


**FIGURE 2.** Reaction schemes for the synthesis of (Z) 4-(1,2-diphenyl-1-butenyl)phenol vinyl ether and triphenylethylethylenes. Tamoxifen N-oxide was pyrrolized under a stream of $N_2$ gas in order to perform a Cope elimination to produce (Z) 4-(1,2-diphenyl-1-butenyl)phenol vinyl ether (Reaction A). Reagents and conditions for the synthesis of triphenylethylethylenes (Reaction B): (a) $TiCl_4$, Zn, THF, reflux ($R_1$ and $R_2$ are either H or OH). Wavy lines indicate that the compound exists as a rapidly interconverting 1:1 mixture of E/Z stereoisomers when $R_1$ and $R_2$ are different.

### (Z) 4-(1,2-diphenyl-1-butenyl)phenol

Using the procedure above, propiophenone (1.0 g, 7.5 mmol) and 4-hydroxybenzophenone (0.49 g, 2.5 mmol) were used to produce 0.58 g of (Z) 4-(1,2-diphenyl-1-butenyl)phenol (Fig. 1) as a 1:1 mixture of interconverting E and Z stereoisomers in 84% overall yield. Spectroscopic properties matched previous reports.[21]

### 4,4′-(2-phenyl-1-butenylidene)bisphenol

Using the procedure above, propiophenone (1.0 g, 7.5 mmol) and 4,4′-dihydroxybenzophenone (0.54 g, 2.5 mmol) were used to produce 0.31 g of 4,4′-(2-phenyl-1-butenylidene)bisphenol (Fig. 1) in 39% overall yield. Spectroscopic properties matched previous reports.[22]

### 4-(1,1-diphenyl-1-buten-2-yl)phenol

Using the procedure above, 4-hydroxypropiophenone (1.0 g, 7.5 mmol) and benzophenone (0.45 g, 2.5 mmol) were used to produce 0.62 g of 4-(1,1-diphenyl-1-buten-2-yl)phenol (Fig. 1) in 83% overall yield. 1H NMR (300 MHz) ($CDCl_3$) δ 7.22-6.90 (m, 12H), δ 6.62 (d, J = 8.7 Hz, 2H), δ 4.00 (t, J = 8.9 Hz, 1H), δ 2.44 (q, J = 7.5 Hz, 2H), δ 0.93 (t, J = 7.2 Hz, 3H); 13C NMR (300 MHz) ($CDCl_3$) δ 157.7, δ 156.9, δ 141.1, δ 138.5, δ 137.2, δ 136.7, δ 135.3, δ 134.8, δ 132.5, δ 131.1, δ 130.2, δ 129.2, δ 128.7, δ 127.7, δ 127.5, δ 127.2, δ 126.4, δ 126.2, δ 115.7, δ 113.6, δ 27.1, δ 8.0.

## RESULTS AND DISCUSSION

The data in Figure 3A show the effect of 10 μM tamoxifen on $[Ca^{2+}]_i$ in human platelets. After a few second in the presence of extracellular $Ca^{2+}$, tamoxifen produced a very large increase in $[Ca^{2+}]_i$ that was comparable to that observed with thrombin. In the absence of extracellular $Ca^{2+}$, the effect of tamoxifen to increase $[Ca^{2+}]_i$ was 12 ± 5% of that in the presence of extracellular $Ca^{2+}$. The effect of thrombin to increase $[Ca^{2+}]_i$ was likewise inhibited by approximately 90% when extracellular $Ca^{2+}$ was absent (eg, see Fig. 2 in Reference 14 ). Thus, tamoxifen and thrombin both appear to increase $[Ca^{2+}]_i$ via mobilization of $Ca^{2+}$ from the endoplasmic reticulum followed by influx of extracellular $Ca^{2+}$ through SOCC where majority of $[Ca^{2+}]_i$ elevation is due to the $Ca^{2+}$ influx 8.

The dose response curve of tamoxifen-stimulated $[Ca^{2+}]_i$ elevation in the presence of extracellular $Ca^{2+}$ is shown in Figure 3B. Small increases in $[Ca^{2+}]_i$ was observed with 1.0 μM tamoxifen (24.5 ± 9%), 5.0 μM tamoxifen increased $[Ca^{2+}]_i$ by 80 ± 30% over baseline while 10.0 μM increased $[Ca^{2+}]_i$ by 175 ± 11% over baseline. We did not add higher concentrations of tamoxifen since plasma levels rarely exceed this value during tamoxifen therapy.[23]

An experiment in which tamoxifen elevated $[Ca^{2+}]_i$ synergistically with the physiological platelet agonist, thrombin, is shown in Figure 4. A lower concentration of tamoxifen (5.0 μM) produced a small elevation of $[Ca^{2+}]_i$. However when 5.0 μM tamoxifen was combined with a low concentration of thrombin (0.005 units/mL), there was a large synergistic increase in $[Ca^{2+}]_i$ that was approximately twice that obtained if both tamoxifen and thrombin responses were added together (theoretical additivity). The increase in $[Ca^{2+}]_i$ over basal $[Ca^{2+}]_i$ (108 ± 2.0 nM from 4 separate experiments) was: tamoxifen, 30.5 ± 7.4 nM; thrombin, 45.5 ± 5.9 nM; thrombin plus tamoxifen, 148.8 ± 18.5 nM. This increase was significantly higher ($P < 0.01$) than if the individual tamoxifen and thrombin responses were added together for each individual experiment, which would be 76.3 ± 11.9 nM.

We next examined combining tamoxifen with other platelet agonists that increase $[Ca^{2+}]_i$, such as ADP and vasopressin. Like thrombin, these agonists also act via

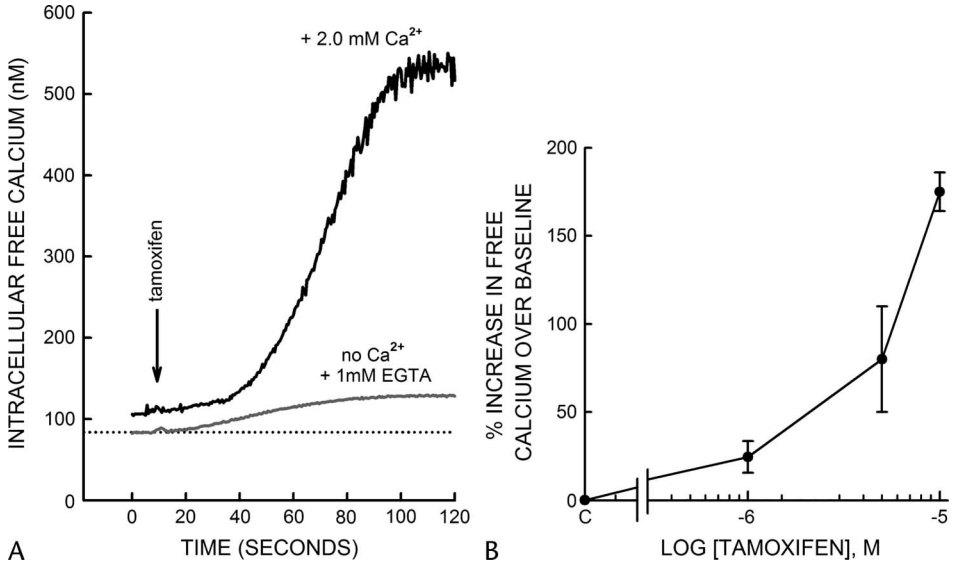


**FIGURE 3.** (A) Time course of tamoxifen to increase $[Ca^{2+}]_i$ in human platelets in the presence and absence of extracellular $Ca^{2+}$. Platelets were incubated in either the presence of 2.0 mM extracellular $Ca^{2+}$ or in the absence of extracellular $Ca^{2+}$ plus 1 mM EGTA. Tamoxifen (10 μM) was added at 10 seconds. A representative trace is shown. The response observed in the absence of extracellular calcium was 12 ± 3 % (mean ± SEM from four separate experiments) of the response seen when extracellular calcium was present. ( B) Dose response of tamoxifen to increase $[Ca^{2+}]_i$ in human platelets in the presence of extracellular $Ca^{2+}$. The peak increase in $[Ca^{2+}]_i$ was measured in platelets treated with 1.0, 5.0, or 10.0 μM tamoxifen. Platelets were incubated in the presence of 2.0 mM $Ca^{2+}$, and tamoxifen was added 10 seconds after data collection was commenced. Values shown are mean ± SEM from four separate experiments.

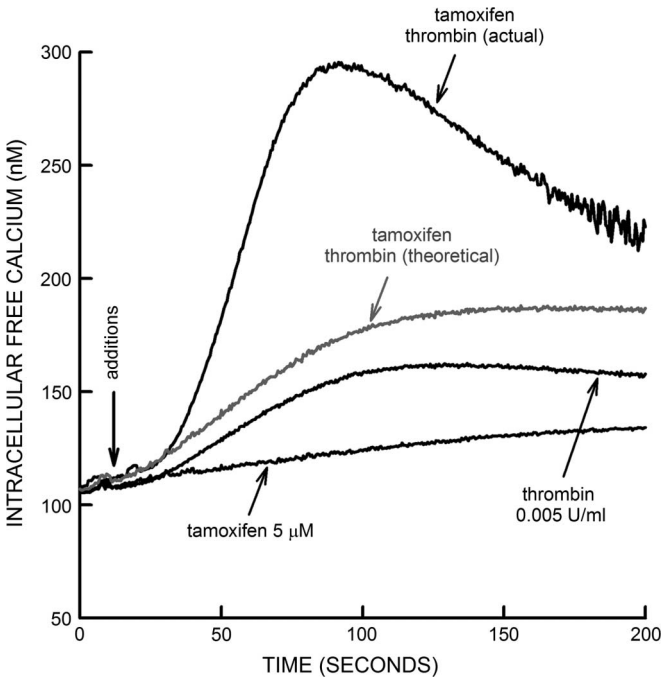


**FIGURE 4.** Effect of combining submaximal concentrations of tamoxifen and thrombin on $[Ca^{2+}]_i$ in human platelets. A submaximal concentration of tamoxifen (5 μM) and a submaximal concentration of thrombin (0.005 U/mL) were added to platelets in the presence of 2.0 mM $Ca^{2+}$ and $[Ca^{2+}]_i$ measured. These submaximal concentrations were also combined and $[Ca^{2+}]_i$ measured. The combination of submaximal concentrations of tamoxifen and thrombin (actual) produced a more than additive (theoretical) effect on $[Ca^{2+}]_i$ that was comparable to the effects observed with maximal concentrations of tamoxifen and or thrombin alone. A representative experiment of four is shown.

**TABLE 1.** Effect of Tamoxifen, Tamoxifen Plus ADP, and Tamoxifen Plus Vasopressin to Increase $[Ca^{2+}]_i$ in Human Platelets

| Agonist | $[Ca^{2+}]_i$ Elevation Above Baseline, % |
|---|---|
| Tamoxifen (μM) | 31 ± 9 |
| Vasopressin (10 nM) | 87 ± 27 |
| Vasopressin + Tamoxifen (actual) | 322 ± 0* |
| Vasopressin + Tamoxifen (theoretical) | 118 ± 27 |
| ADP (0.1 mM) | 94 ± 13 |
| ADP + Tamoxifen (actual) | 183 ± 1† |
| ADP + Tamoxifen (theoretical) | 125 ± 13 |

Platelets loaded with fura-2 were incubated in the presence of 2.0 mM extracellular $Ca^{2+}$. ADP (0.1 mM) or vasopressin (10 nM) were added to platelets in the presence or absence of tamoxifen (1 μM), and the peak increase in $[Ca^{2+}]_i$ was measured. Values shown are means ± SEM from at least 3 separate experiments. The actual measured values obtained when tamoxifen was combined with either vasopressin or ADP were significantly greater than the values obtained when individual responses were added together (theoretical).

*$P$ = 0.014 compared with theoretical.

†$P$ = 0.04 compared with theoretical.

activation of the PLC-β. ADP increases $[Ca^{2+}]_i$ by activating $G_q$ /PLC-β and lowering cAMP, 24 vasopressin also activates $G_q$/PLC-β.25 The data in Table 1 show the effect of combining tamoxifen with ADP and vasopressin. In each instance, tamoxifen at 1.0 μM potentiated the effects of the other platelet agonists to increase $[Ca^{2+}]_i$.

The unexpected finding that tamoxifen was able to rapidly and dose-dependently increase $[Ca^{2+}]_i$ (Fig. 3), leading to platelet activation, may be one of contributing factors by which tamoxifen causes thrombosis in a small number of patients.[26–29] In one study, the plasma levels of tamoxifen in patients being treated with high- dose tamoxifen was 2.94 ± 3.44 μM, with 2 patients having values of 8 and 16 μM.[23] On the basis of these plasma levels of tamoxifen and our data demonstrating the ability of tamoxifen to promote $Ca^{2+}$ influx, it is conceivable that platelet $[Ca^{2+}]_i$ may be increased sufficiently so as to promote aggregation in some patients with high serum levels of tamoxifen. Furthermore, tamoxifen is very lipophilic ($V_d$ = 50–60 L/kg)[30] and partitions into cell membranes,[31] where many proteins of the $Ca^{2+}$ signal transduction complexes reside. Thus plasma concentration may not necessarily reflect the amount of the membrane-bound drug, which can be critical for the effect on $Ca^{2+}$ homeostasis in platelets. Synergistic effects of tamoxifen and other agonists on $[Ca^{2+}]_i$ in platelets may play a role in developing thrombosis even when tamoxifen concentration is too low to promote $Ca^{2+}$ influx. In the presence of low concentrations of physiological platelet agonists, the two factors together may lead to the heightened platelet activation and aggregation.

It is known that many platelet agonists such as thrombin activate PLC to produce inositol-1,4,5-$P_3$ ($IP_3$), and this activation is inhibited by the PLC inhibitor U-73122.[32] The data in Figure 5 show that tamoxifen-induced $[Ca^{2+}]_i$ increase was substantially inhibited by U-73122 in the presence of 2.0 mM extracellular $Ca^{2+}$, whereas the inactive negative control U-73343 produced a very small inhibition. This result implies that tamoxifen was activating at least 1 form of PLC in platelets, which leads to $IP_3$ -induced $Ca^{2+}$ mobilization and SOCC activation. The PLC inhibitor U-73122 does not discriminate between PLC-β and PLC-γ, so it is not presently known which PLC isoform is activated by tamoxifen.

DES (diethylstilbestrol) is a nonsteroidal estrogen analog, structurally similar to tamoxifen (Figure 1), which inhibits SOCC in platelets and other cells.[9,10] DES does not have an effect on basal $[Ca^{2+}]_i$ in platelet by itself, however DES at 10 μM completely inhibited tamoxifen-induced $[Ca^{2+}]_i$ elevation, (100 ± 6%, Fig. 6). The pure antiestrogen ICI 182,780, [33] which is not structurally related to tamoxifen (Fig. 1), produced a small increase in $[Ca^{2+}]_i$ by itself and slightly potentiated tamoxifen-induced $Ca^{2+}$ influx (Fig. 6). No inhibition of tamoxifen effect by ICI 182,780 suggests that tamoxifen is increasing $[Ca^{2+}]_i$ by a mechanism that does not involve the classical estrogen receptor, since ICI 182,780 is a full antagonist of this estrogen receptor.

In order to investigate what structural features of the tamoxifen molecule render it an agonist rather than antagonist like the other compounds with stilbene moiety, a limited structure activity relationship (SAR) study of tamoxifen derivatives (Fig. 1) was investigated. Nevertheless, this study

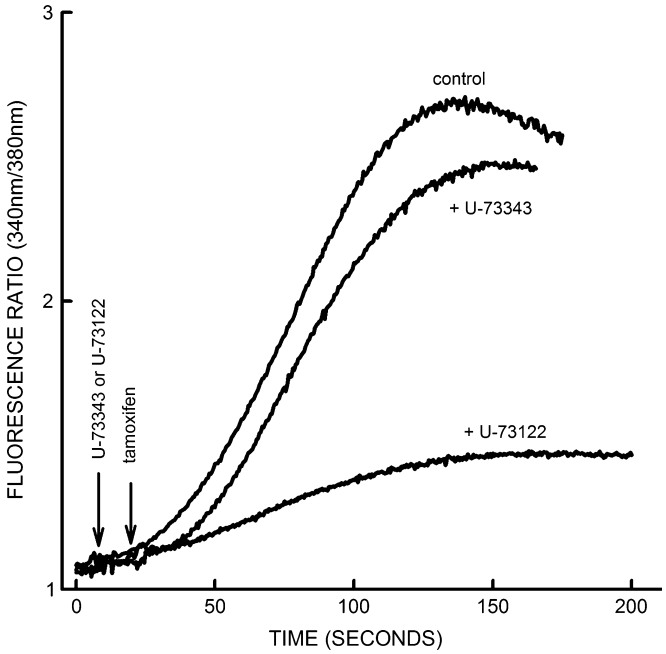


**FIGURE 5.** Effect of U-73343 and U-73122 of the ability of tamoxifen to increase $[Ca^{2+}]_i$ in human platelets. Platelets were incubated in the presence of 2.0 mM extracellular $Ca^{2+}$ similar to that shown in Fig ure 3. After approximately 10 seconds of data collection, either 5 μM U-73343 or 5 μM U-73122 was added to the platelets. At 20 seconds, 10 μM tamoxifen was added. The PLC inhibitor U-73122 produced a large inhibition of the increase in $[Ca^{2+}]_i$, whereas the inactive analog U-73343 produced a very small inhibition. A representative trace of three is shown.

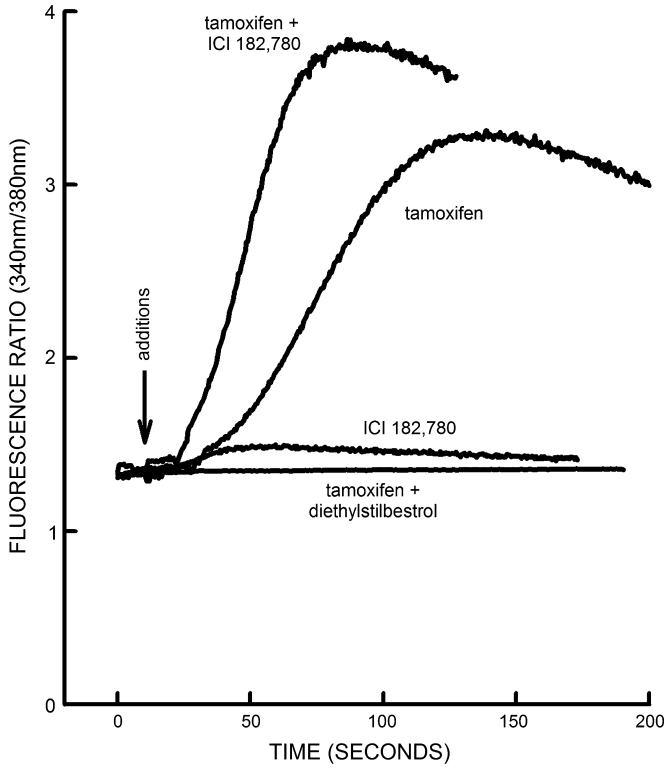


**FIGURE 6.** Effect of ICI 182,780 and diethylstilbestrol on the ability of tamoxifen to increase $[Ca^{2+}]_i$ in human platelets. The nonsteroidal estrogen analog diethylstilbestrol (10 μM) was able to inhibit the effect of tamoxifen to increase $[Ca^{2+}]_i$. The pure antiestrogen ICI 182,780 (10 μM), which increased $[Ca^{2+}]_i$ slightly by itself, was unable to inhibit the ability of tamoxifen to increase $[Ca^{2+}]_i$ (a small potentiation was observed when the compounds were combined). A representative experiment of three is shown.

points out important structural features responsible for activity of tamoxifen. Tamoxifen and to a lesser degree, 4-hydroxy-tamoxifen induced a rapid increase in $[Ca^{2+}]_i$ in platelets with a very similar time course (data not shown). Therefore the addition of a hydroxyl group to an aromatic ring leads to diminishing activity of the compound. Ethyl bromide tamoxifen and MER-25,[34] only increase $[Ca^{2+}]_i$ very slightly, and Tamoxifen N-oxide had a small inhibitory activity (Fig. 7).

Even minor modifications to the amino group have a profound effect on activity. Tamoxifen N-oxide differs from tamoxifen only in one oxygen atom, and this modification leads to a complete loss of activity to elevate $[Ca^{2+}]_i$ (11 ± 6% decrease below the basal level; see Fig. 7 and Table 2). Likewise, ethyl bromide tamoxifen, a charged, cell impermeable quaternary derivative of tamoxifen, was also practically devoid of activity to elevate $[Ca^{2+}]_i$ (12 ± 8 % increase above the basal level; see Fig. 7 and Table 2). Finally removal of dimethyl amino group of tamoxifen, which produces (Z)-4-(1,2-diphenyl-1-butenyl) phenol vinyl ether (trivial name des dimethyl amino tamoxifen), resulted in the loss of activity to increase $[Ca^{2+}]_i$ (26 ± 7% decrease from basal level; see Fig. 7 and Table 2). MER-25 (Fig. 1), which does not have a stilbene pharmacophore but has 3 phenyl rings, only possessed a weak ability to increase $[Ca^{2+}]_i$ (22 ± 11% above basal; see Fig. 7). Therefore, this limited study, summarized in Figure 7 points to the importance of the amino group, especially the lone electron pair of nitrogen. Since even subtle modifications to the amino group resulted in large changes in activity, this points towards the involvement of a specific receptor rather than to a general nonspecific membrane effect of tamoxifen.

We next tested the effect of the triphenylethylenes (Fig. 1). These compounds are structurally similar to both tamoxifen, which increases $[Ca^{2+}]_i$, and DES, the most potent inhibitor of SOCC that we have found.[10] These compounds have a triphenylethylene core and posses the stilbene moiety, but they are devoid of the dimethylamino ethanol side chain. The three triphenylethylethylenes that inhibited thrombin-induced $[Ca^{2+}]_i$ elevation (by 68 to 79%), similar to that observed with DES but with a weaker efficacy (Table 2). Like tamoxifen, triphenylethylenes possessed an agonist activity to increase $[Ca^{2+}]_i$ albeit a weak one (6 to 24% increase over basal, Table 2). Compound (Z)-4-(1,2-diphenyl-1-butenyl)-phenol, which has phenolic oxygen positioned similar to that of tamoxifen, has the highest activity to elevate $[Ca^{2+}]_i$. The 3 triphenylethylenes also almost completely inhibited (91 to 95%) the tamoxifen- induced $[Ca^{2+}]_i$ elevation, similar to that seen with DES. This is likely due to competition between the

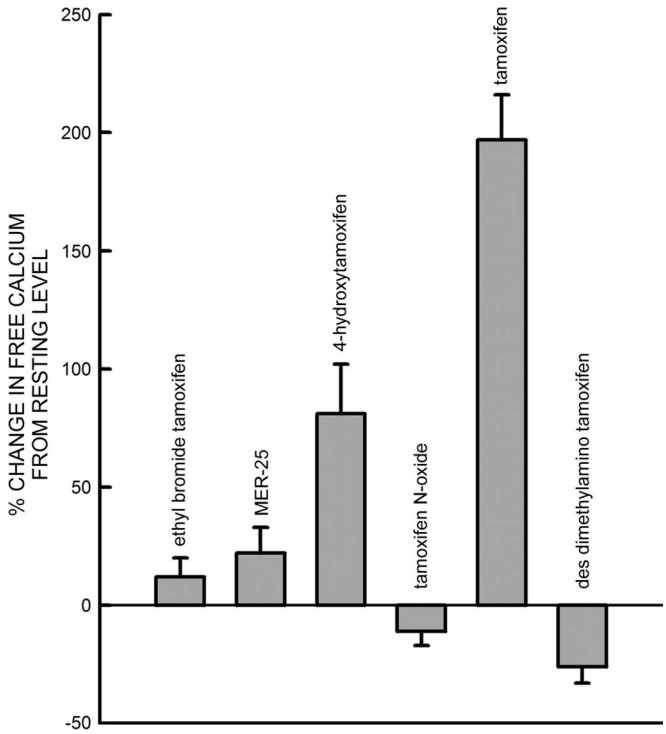


**FIGURE 7.** Summary of the effects of tamoxifen, 4-hydroxytamoxifen, tamoxifen N-oxide, ethyl bromide tamoxifen, des dimethylamino tamoxifen, and MER-25 to increase $[Ca^{2+}]_i$ in human platelets. Platelets, incubated in the presence of 2.0 mM extracellular $Ca^{2+}$, were treated with 10 μM concentrations of tamoxifen, 4-hydroxytamoxifen, tamoxifen N-oxide, ethyl bromide tamoxifen, des dimethylamino tamoxifen, and MER-25. Each agent was added at 10 seconds. Ethyl bromide tamoxifen and MER-25 caused $[Ca^{2+}]_i$ elevation by 12 ± 8% and 22 ± 11% above the baseline, respectively. Tamoxifen N-oxide and des dimethylamino tamoxifen slightly decreased baseline $[Ca^{2+}]_i$ by 11 ± 6% and 26 ± 7%, respectively. Only tamoxifen and 4-hydroxytamoxifen increased $[Ca^{2+}]_i$ significantly (by 197 ± 19% and 81 ± 21%, respectively). The data shown are means ± SEM from 4 separate experiments.

structurally similar triphenylethylenes and tamoxifen. Therefore triphenylethylenes being transitional structures have both antagonist activity in thrombin-stimulated platelets, like DES, and agonist activity (albeit small) to promote $Ca^{2+}$ influx into platelets, like tamoxifen.

To establish the cellular localization for tamoxifen action in platelets, polymer-conjugated derivatives were synthesized, where 4-hydroxytamoxifen derivatives (1 or 2; Fig. 8) were conjugated to poly methacrylic acid via a 6 carbon (poly-6C-OHT-acid), or to a 2 carbon (poly-2C-OHT-acid) diamine linker, or to poly methyl methacrylamide via a 2 carbon (poly-2C-OHT-amide) diamine linker (Fig. 8).

Compounds 1 and 2 had $K_i$ values for binding to estrogen receptor α in fluorescent competition binding assay of 30 ± 10 nM and 6 ± 4 nM, respectively, but both compounds also had a similar affinity for estrogen receptor β.[15] The 4-hydroxytamoxifen analogs 1 and 2 were then conjugated to a 20- kDa polymethacrylic acid polymer to produce poly-2C-OHT-acid, poly-2C-OHT-amide, and poly-6C-OHT-acid, respectively (Fig. 8) (Weatherman et al, manuscript in preparation).

With analogs 1 and 2, there was approximately 60% and 10% side chain conjugation respectively in the polymers. The polymer conjugates were tested for their ability to bind to purified estrogen receptor α and estrogen receptor β using a fluorescence polarization competition assay (Weatherman et al, manuscript in preparation). Both poly-2C-OHT-acid and poly-6C-OHT-acid conjugates were able to bind to both estrogen receptors with $K_i$ values in the low-to-submicromolar range. The overall binding affinities of the conjugates poly-2C-OHT-acid and poly-6C-OHT-acid were approximately 50-fold less than the binding affinity of the analogs 1 and 2. Conjugate poly-6C-OHT-acid, which has a 6-carbon linker, has approximately 5-fold higher affinity for the estrogen receptor than conjugate poly-2C-OHT-acid with the 2-carbon linker (Weatherman et al, manuscript in preparation). This difference in affinity parallels the difference seen with the nonconjugated analogs 1 and 2. It is not known if the difference is due to the fact that analog 2 is the higher-affinity ligand or if the difference between conjugates is due to the difference in ligand density on the 2 conjugates (60% versus 10%).

**TABLE 2.** Effect of Various Tamoxifen Analogs on Thrombin-induced or Tamoxifen-induced Calcium Entry

| Analog | % Change in Basal $[Ca^{2+}]_i$ by Compound Alone | % Change in Thrombin-Induced $[Ca^{2+}]_i$ Elevation | % Change in Tamoxifen-Induced $[Ca^{2+}]_i$ Elevation |
|---|---|---|---|
| Tamoxifen ethyl bromide | 12 ± 8* | 6 ± 7 | −6 ± 7 |
| Tamoxifen N-oxide | −11 ± 6* | 13 ± 16 | −17 ± 1 |
| (Z)-4-(1,2-diphenyl-1-butenyl)phenol vinyl ether | −26 ± 7* | −16 ± 5 | −41 ± 6 |
| 4-(1,1-diphenyl-1-buten-2-yl)phenol | 12 ± 7 | −78 ± 4 | −91 ± 9 |
| (Z)-4-(1,2-diphenyl-1-butenyl)phenol | 24 ± 9 | −68 ± 6 | −99 ± 5 |
| 4,4'-(2-phenyl-1-butenyllidene)bisphenol | 6 ± 2 | −79 ± 3 | −95 ± 12 |
| diethylstilbestrol | 1 ± 3 | −98 ± 3[9] | −100 ± 6 |

Platelets loaded with fura-2 were incubated in the presence of 2.0 mM extracellular $Ca^{2+}$. The appropriate tamoxifen analog was added at a final concentration of 10 μM; 10 seconds later, 10 μM tamoxifen or thrombin (0.01 units/mL) was added. Values shown are means ± SEM from at least three separate experiments. Positive value indicates elevation over the control; negative value indicates inhibition.

*From Figure 7.

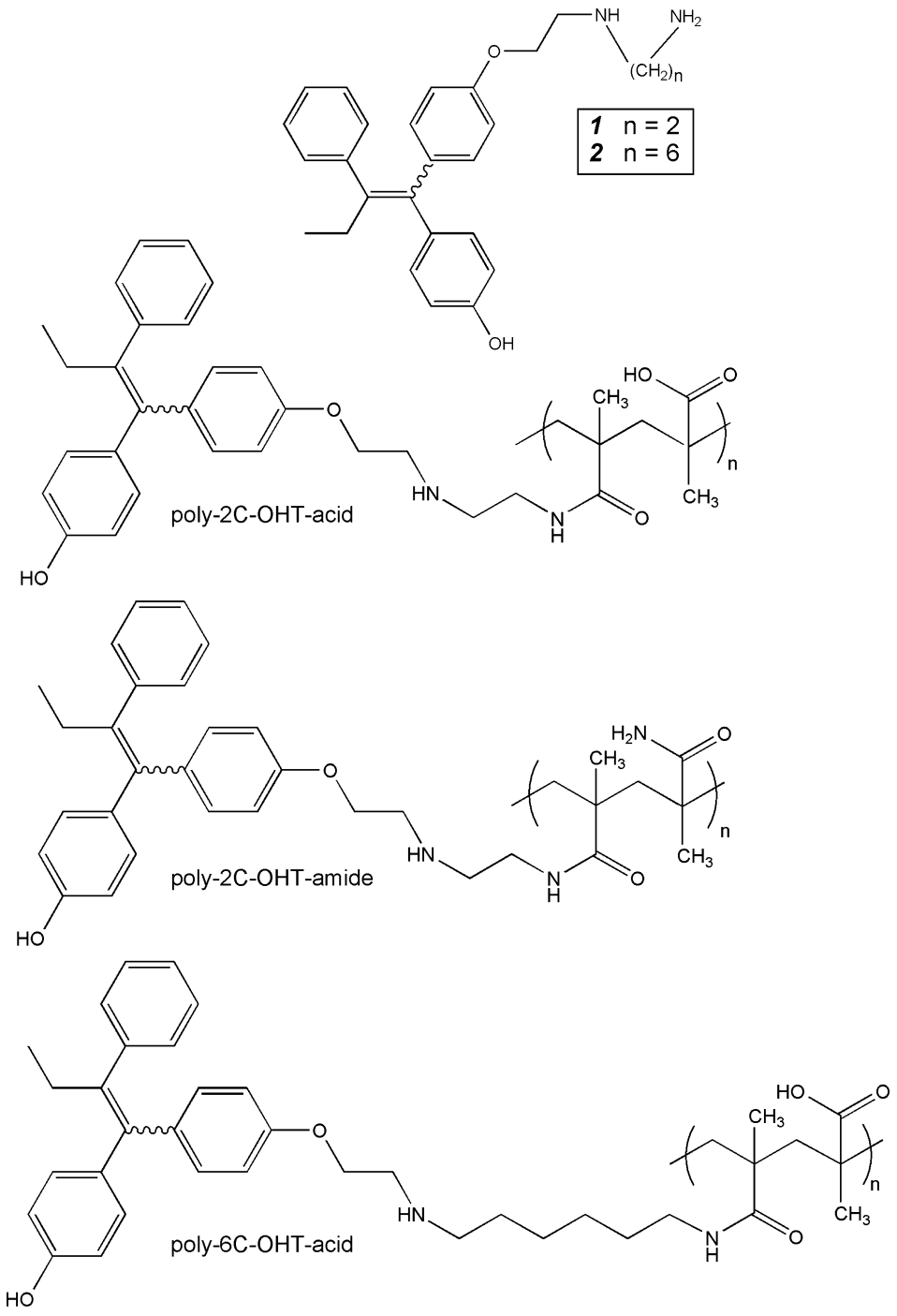


**FIGURE 8.** Structures of 4-hydroxytamoxifen diaminoalkane conjugates and conjugated derivatives of 4-hydroxytamoxifen. Wavy lines indicate that the compounds exist as rapidly interconverting 1:1 mixture of E/Z stereoisomers.

The 2 derivatives of 4-hydroxytamoxifen conjugated via 2-carbon linkers (poly-2C-OHT-acid and poly-2C-OHT-amide) caused an increase in $[2]_i$ (101 ± 25 and 57 ± 19% increases, respectively; representative traces are shown in Fig. 9), similar to that observed with 4-hydroxytamoxifen (81 ± 21% increase; see Fig. 7). The 6-carbon linker conjugate (poly-6C-OHT-acid) was inactive (0 ± 3% increase; see Fig. 9 for a representative trace). The results suggest a cell surface receptor, but specific uptake of the conjugates into platelets is still not known. The extracellular target for conjugates poly-2C-OHT-acid and poly-2C-OHT-amide in the human platelet is unlikely to be the estrogen receptor itself, because poly-6C-OHT-acid possessed a 5-fold higher affinity for the estrogen receptor than poly-2C-OHT-acid conjugate; therefore, a pharmacological profile of these compounds in respect to their action on estrogen receptor and their action on $Ca^{2+}$ in platelets is not correlated. Activity of the 4-OH tamoxifen conjugates depends so crucially on the linker length, making it

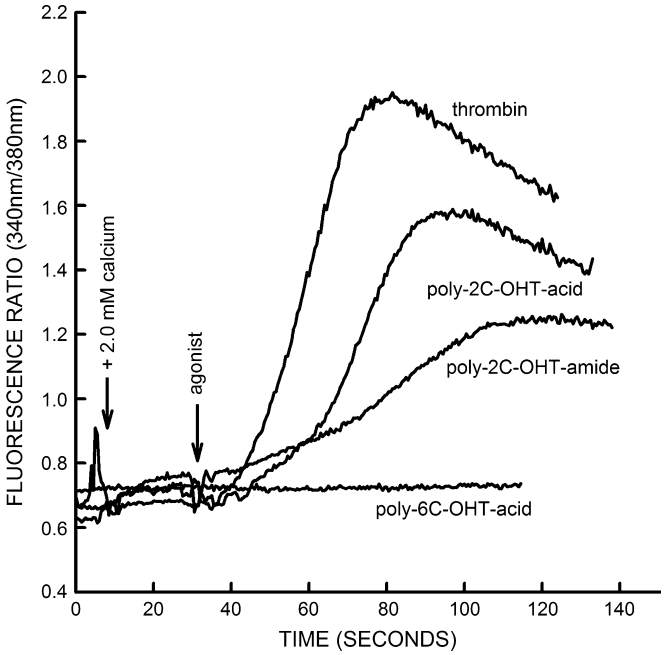


**FIGURE 9.** Effect of conjugated 4-hydroxytamoxifen derivatives on $[Ca^{2+}]_i$ in human platelets. Platelets were incubated in the absence of added $Ca^{2+}$. At 5 seconds, 2.0 mM $Ca^{2+}$ was added. At 30 seconds, the various conjugated 4-hydroxytamoxifen derivatives were added. Each conjugate was added to give a final concentration equivalent to 10 μM 4-hydroxytamoxifen. For comparison purposes thrombin (0.01 units/mL) was also added. Representative traces from three separate experiments are shown.

most likely that the 4-OH tamoxifen molecule has to be presented in a certain way to the putative cell surface receptors.

Estrogen receptors α and β have been detected in human platelets from both men and women.[35–37] The glycosylated form of the estrogen receptor β was identified in the membrane fraction of platelets.[36,37] In addition, 17β-estradiol has been shown to potentiate thrombin-induced platelet aggregation via a nongenomic mechanism.[37] The question remains whether the effects observed in our studies are due to tamoxifen and its analogs interacting with estrogen receptor β or another protein such as a $Ca^{2+}$ influx channel. The SAR studies indicate that the responses observed on $[Ca^{2+}]_i$ do not fit with the currently accepted steroid specificity of estrogen receptor β, and tamoxifen action is not blocked by the ICI 182,780. The action of 4-hydroxytamoxifen at the genomic estrogen receptor is a more potent antagonist than tamoxifen,[38] but it was less effective than tamoxifen at increasing $[Ca^{2+}]_i$ in platelets (Fig. 7). We therefore believe that the effects of tamoxifen and its analogs to promote $[Ca^{2+}]_i$ elevation in platelets is most likely mediated by a nongenomic mechanism not involving the estrogen receptor.

## CONCLUSIONS

We found that tamoxifen rapidly increased $[Ca^{2+}]_i$ in human platelets from both male and female donors. Tamoxifen also acted synergistically with thrombin, ADP, and vasopressin to increase $[Ca^{2+}]_i$. The anti-estrogen ICI 182,780 did not inhibit the effect of tamoxifen to increase $[Ca^{2+}]_i$. 4-Hydroxytamoxifen, a metabolite of tamoxifen, also increased $[Ca^{2+}]_i$, but other tamoxifen metabolites and synthetic derivatives did not. Three hydroxylated derivatives of triphenylethylene (corresponding to the hydrophobic core of tamoxifen), which are transitional structures between tamoxifen (a $Ca^{2+}$ agonist) and diethylstilbestrol (a $Ca^{2+}$ antagonist), increased $[Ca^{2+}]_i$ slightly and partially inhibited thrombin-induced increases in $[Ca^{2+}]_i$. Therefore the dimethylaminoethyl side chain moiety of tamoxifen was responsible for tamoxifen being a $Ca^{2+}$ agonist rather than an antagonist. Polymer-conjugated derivatives of 4-hydroxytamoxifen rapidly increased $[Ca^{2+}]_i$, with efficacy similar to 4-hydroxytamoxifen, this suggests that the action of tamoxifen is a nongenomic cell surface —mediated effect. The ability of tamoxifen to increase $[Ca^{2+}]_i$ in platelets, leading to platelet activation, may contribute to development of tamoxifen-induced thrombosis.

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