Effect of Tamoxifen on Carbachol-triggered Intracellular Calcium Responses in Chicken Granulosa Cells

Paul Morley1 and James F. Whitfield

Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

ABSTRACT

The effect of the nonsteroidal antiestrogen tamoxifen on carbachol (CCh)-triggered intracellular Ca²⁺ surges was determined in granulosa cells from the two largest preovulatory follicles of laying hens. The intracellular calcium ion concentration ([Ca²⁺]i) was measured in cells loaded with the Ca²⁺-responsive fluorescent dye fura-2. Resting [Ca²⁺], was 96 ± 5 nM (n = 20), and CCh (1 mM) triggered a large initial [Ca²⁺]i spike to 600–800 nM, due to the mobilization of Ca²⁺ from internal stores. Following the spike, the [Ca²⁺]i dropped to a lower, suprabasal level with superimposed oscillations, which depended on Ca²⁺ influx, and returned to the resting level by 2 to 4 min. Tamoxifen (10 μM) did not by itself affect [Ca²⁺]i, but pretreating granulosa cells with tamoxifen (10 μM) prolonged the CCh-triggered [Ca²⁺]i surge and oscillations by as much as 10 to 30 min. Pretreatment with much higher concentrations of tamoxifen (e.g., 0.5 mM) also had no effect by themselves, but caused a prolonged rise in [Ca²⁺], following CCh (1 mM) stimulation. The effect of tamoxifen on CCh-triggered [Ca²⁺], responses was mimicked by the tamoxifen metabolite 4-hydroxytamoxifen (10 μM), but not by the structurally related antiestrogens nafodixone (10 μM) or clomiphene citrate (10 μM). The tamoxifen effect on the CCh-triggered [Ca²⁺], response was not mediated through estrogen receptors since pretreating granulosa cells with 17β-estradiol (10⁻⁶ M) did not mimic the tamoxifen response. The effect of tamoxifen was inhibited by pretreating granulosa cells with the Ca²⁺ channel blocker, lanthanum (1 mM), or by incubating the cells in Ca²⁺-free medium. Tamoxifen did not affect [Ca²⁺], surges triggered by 17β-estradiol (10⁻⁶ M) or dimethyl sulfide (1%) which mobilize Ca²⁺ from internal stores. Pretreating granulosa cells with tamoxifen (10 μM) or 4-hydroxytamoxifen (10 μM) before inducing Ca²⁺ influx through voltage-dependent Ca²⁺ channels by depolarizing the cells with 45 mM external K⁺, caused a prolonged rise of [Ca²⁺], with oscillations, similar to the CCh response. These studies demonstrate that tamoxifen affects the activation of chicken granulosa cell Ca²⁺ channels by CCh or by raising the external K⁺ concentration, resulting in a prolongation of the sustained [Ca²⁺], elevation and oscillations, which result from the influx of extracellular Ca²⁺. These observations suggest that tamoxifen interacts with open Ca²⁺ channels in chicken granulosa cells and keeps them open for prolonged periods of time.

INTRODUCTION

Tamoxifen is a nonsteroidal triphenylethylene estrogen receptor antagonist which is widely used to treat breast cancer (1, 2). A number of biochemical mechanisms have been proposed to explain the antineoplastic actions of tamoxifen. Tamoxifen is thought to compete with estrogen for binding to the cytoplasmic/nuclear estrogen receptor of neoplastic cells (3–6), which results in a partial activation and nuclear translocation of the receptor, but without the growth-stimulating properties of estrogens (7). Tamoxifen may inhibit estrogen-dependent mammary tumor cell proliferation by reducing the secretion of stimulatory growth factors and enhancing secretion of the inhibitory growth factor TGF-β(8, 9). However, TGF-β may not inhibit the proliferation of mammary tumor cells in vivo (10). But tamoxifen is not just an antiestrogen. For example, it can stimulate TGF-β secretion in human fetal fibroblasts which do not express conventional estrogen receptors (11). In fact, it, and related triphenylethylenes, bind to key parts of the cellular signalling apparatus. They bind to dopamine (12), histamine (13, 14), and muscarinic cholinergic (15) receptors. They bind to signalling enzymes such as phospholipase A₂ (16) and the protein kinase Cs (17–20). In fact, the protein kinase Cs have specific high-affinity binding sites for triphenylethylenes which potently inhibit these very important enzymes that control many cell activities (17–20).

Tamoxifen also variously affects Ca²⁺ signalling independently from the conventional estrogen receptor. The drug binds to calmodulin in a Ca²⁺ -dependent manner and thus inhibits the many functions that are activated by this Ca²⁺ -binding signal protein (21–23). Because of its ability to bind to calmodulin, tamoxifen can increase cyclic AMP signal surges by inhibiting cyclic AMP hydrolysis by Ca²⁺-calmodulin-dependent cyclic nucleotide phosphodiesterase (21, 22, 24, 25). Tamoxifen is a potent spasmylocyt for rat uterine myometrium, probably because of its ability to bind calmodulin (26). It can also amplify signal-induced [Ca²⁺], surges by inhibiting both Ca²⁺-calmodulin-dependent membrane ATPase Ca²⁺ pumps and the calmodulin-independent Ca²⁺ uptake by cytoplasmic Ca²⁺-sequesterings vesicles (27). However, this amplification of [Ca²⁺], signal surges is opposed by the ability of tamoxifen to reduce Ca²⁺ influx into the cell by directly inactivating Ca²⁺ channels (26, 28, 29).

In this communication we provide more evidence of the ability of tamoxifen to target key Ca²⁺ signalling mechanisms by showing that the drug dramatically enhances Ca²⁺ influx through the Ca²⁺ channels of chicken granulosa cells which have first been opened by K⁺-induced membrane depolarization or the activated receptor for the muscarinic cholinergic agonist, carbachol.

MATERIALS AND METHODS

Reagents and Hormones. CCh, clomiphene citrate, collagenase (Type 1A), DMSO, 17β-estradiol, La³⁺, nafodixone, and tamoxifen were purchased from Sigma Chemical Co., St. Louis, MO. Ionomycin was purchased from Calbiochem, La Jolla, CA, and EGTA was from Fluka, Ronkonkoma, NY. Fura-2 AM was purchased from Molecular Probes, Inc., Eugene, OR. Fetal bovine serum, M199, RPMI 1640 medium, and Dulbecco’s minimum essential medium were purchased from Gibco Laboratories, Grand Island, NY. 4-Hydroxytamoxifen was generously provided by les Laboratoires Besins-Iscover, Paris, France. Tamoxifen, 4-hydroxytamoxifen, and nafodixone were dissolved in 95% ethanol, and clomiphene citrate was dissolved in methanol. The final concentration of ethanol never exceeded 0.95% and that of methanol never exceeded 1%. These solvent concentrations did not affect [Ca²⁺], in any experiment.

Chicken Granulosa Cell Isolation. White Leghorn hens in their first year of reproductive activity were caged individually in a windowless, air-conditioned room with a 14-h light, 10-h dark light cycle. Granulosa cells were isolated as described by Asem et al. (30). Briefly, birds were killed by cervical dislocation 10–14 h before the expected time of ovulation. The granulosa cell layer from the two largest preovulatory follicles (F₁ and F₂) was separated, and...
the cells were dispersed by incubation in M199 containing 0.2% (w/v) collagenase for 10 min in a shaking water bath at 37°C. The M199 was supplemented with 25 mM HEPES, 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.625 μg/ml Fungizone. The cells were then washed and resuspended in M199 without collagenase.

**Islet Cell Culture.** Islet cell cultures were prepared from 6- to 8-week-old male C57BL/6J mice obtained from The Jackson Laboratory, Bar Harbor, ME. The cultures were prepared as described previously (31). Briefly, the splenic portion of the pancreas was removed and the islets were prepared by collagenase digestion and then individually hand picked. The cells of these islets were dispersed by aspiration through a 20-gauge needle and seeded on glass coverslips in 35-mm plastic Petri dishes (Nunc, Roskilde, Denmark) which were placed in a humidified air incubator at 37°C. Each dish contained 2 ml of a complete medium consisting of 95% RPMI 1640 medium, 5% fetal bovine serum, 11.1 mM glucose, penicillin (100 units/ml), streptomycin (100 units/ml), and 10 mM HEPES buffer (pH 7.2).

**[Ca2+]i Measurement.** To determine [Ca2+], 100,000 granulosa cells, or the cells from 5–7 pancreatic islets, were cultured on 24-mm glass coverslips (Canadawide Scientific, Ottawa, Ontario, Canada) and incubated in 1 ml of minimum essential medium containing 2.2 g/liter sodium bicarbonate, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.625 μg/ml Fungizone, and 10% fetal bovine serum at 37°C in an atmosphere of 5% CO2 in air. Granulosa cells were used for the determination of [Ca2+]i, from 4–36 h after plating, and islet cells 14 days after plating. [Ca2+]i was determined by measuring the fluorescence signal from the Ca2+-sensitive indicator fura-2. Granulosa cells were loaded with fura-2 by incubating them for 30 min, and islet cells for 1 h, at 37°C in a normal buffer solution (140 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.1 mM MgCl2, 2.6 mM dextrose, and 10 mM HEPES), containing 2.5 μM fura-2/AM. Experiments were conducted at room temperature on single cells or on small groups of 4 to 8 cells with a CM3 cation measurement spectrofluorimeter (Spx, Inc., Newark, NJ). Measurements were performed by using 350- and 380-nm excitation wavelengths. Background fluorescence was subtracted from the raw data corrected for lamp intensity fluctuations.

The concentration of [Ca2+]i was reflected in the ratio of the fluorescence intensities of fura-2 emission at 505 nm induced by the alternating excitation wavelengths (350 and 380 nm) according to the formula (32):

\[
[Ca^{2+}]_i = K_d F_{\text{max}} R_{\text{max}} - R_{\text{min}} - R_{\text{max}} - R_{\text{min}}
\]

where \( K_d \), \( R_{\text{max}} \) and \( R_{\text{min}} \) are the fluorescence ratios recorded during the experiment (R) and during calibration tests on unlysed cells, using 4 μM ionomycin in normal buffer solution (\( R_{\text{max}} \)), followed by 10 mM EGTA addition at pH 8.2 (\( R_{\text{min}} \)). \( F_{\text{max}} \) and \( F_{\text{min}} \) are the corresponding fluorescence intensities for the 350- and 380-nm excitation, and \( K_d \) is the Fura-2 dissociation constant at room temperature (135 nm).

**RESULTS**

As we have reported previously (33–36), the resting [Ca2+]i of chicken granulosa cells was 96 ± 5 nm (n = 20), and CCh (1 mM) triggered a [Ca2+]i surge which consisted of two phases (Fig. 1), the first of which was due to the release of Ca2+ from internal stores and the second of which was due to the influx of Ca2+ (33). The [Ca2+]i, first rose to a peak of 600 to 800 nm by 10 s and then dropped to a lower, but still suprabasal, level where it stayed for 2 to 4 min. Superimposed on the second, sustained influx phase were Ca2+ oscillations. [Ca2+]i typically oscillated 4 to 8 times before finally subsiding to the resting level. Adding tamoxifen (10 μM) alone to granulosa cell cultures did not affect [Ca2+]i, however, when tamoxifen (10 μM) was added 2 min before CCh (1 mM), the second phase of the CCh-triggered [Ca2+]i response and its oscillations were prolonged for as long as 10 to 30 min (Fig. 2). Two patterns of prolonged [Ca2+]i oscillations were observed. Either [Ca2+]i, dropped only to a suprabasal plateau level [Ca2+]i, (Fig. 2A) or it returned to the resting level between spikes (Fig. 2B). Tamoxifen (10 μM) also triggered prolonged oscillations when added during the second phase of the CCh-triggered [Ca2+]i response (data not shown). A higher concentration of tamoxifen (0.5 μM) also prolonged the [Ca2+]i response to CCh (1 mM), but did not by itself affect basal [Ca2+]i (Fig. 3).

Since tamoxifen weakly activates the conventional estrogen receptor (3, 4), cells were treated with tamoxifen (TX; 10 μM) for 2 min before adding CCh (1 mM). Arrowheads indicate when TX and CCh were added. Panels show tracings of representative experiments: similar results were obtained from four or five other cell cultures.

Since tamoxifen weakly activates the conventional estrogen receptor (3, 4), cells were treated with tamoxifen (TX; 10 μM) for 2 min before adding CCh (1 mM). Arrowheads indicate when TX and CCh were added. Panels show tracings of representative experiments: similar results were obtained from four or five other cell cultures.

The related triphenylethylene antiestrogens nafoxidine (10 μM; Fig. 4A) but this pretreatment did not affect the influx phase of the [Ca2+]i response to subsequently added CCh (1 mM) (Fig. 4A). The related triphenylethylene antiestrogens nafoxidine (10 μM; Fig. 4B)
other cell cultures. Tamoxifen (10 μM) and CCh (1 mM) in Ca²⁺ medium containing 2 mM EGTA (a selective Ca²⁺ chelator). In this Ca²⁺ medium from internal stores, but the tamoxifen could not prolong the second phase of the CCh-triggered [Ca²⁺]i surges. Granulosa cells were treated with 1% etha-
nol (EtOH; left) and 10⁻⁸ M 17β-estradiol (E₂; left), nafoxidine (NAF; 10 μM; right), or 4-hy-
droxytamoxifen (4-OHTX; 10 μM; bottom) before adding CCh (1 mM). Arrow and arrowheads indicate when the various agents were added. The results shown are tracings from representative experiments: similar results were obtained with cells from three to six other cell cultures.

It followed from these results that tamoxifen specifically affected the second phase of the CCh-induced [Ca²⁺]i response, that is, the influx of Ca²⁺ through membrane Ca²⁺ channels (33). This was confirmed by the inability of tamoxifen (10 μM) to affect the immediate [Ca²⁺]i surge induced by 10⁻⁸ M 17β-estradiol as we have reported previously (34) or 1% DMSO (Fig. 6), which were due entirely to the release of Ca²⁺ from internal stores (34, 36).

Chicken granulosa cells have voltage-sensitive, T- and L-type Ca²⁺ channels (39). These channels were activated, and a [Ca²⁺]i surge was triggered, when granulosa cells were depolarized by 45 mM K⁺ (Fig. 7A). To determine whether tamoxifen affected these voltage-dependent Ca²⁺ channels, tamoxifen (10 μM) was added to cultures before the external K⁺ concentration was raised to 45 mM. The drug prolonged the K⁺-induced [Ca²⁺]i oscillations just as it did in CCh-stimulated cells (Fig. 7B).

To find out whether tamoxifen affected Ca²⁺ channels and [Ca²⁺]i responses in other cells, we tested the effects of tamoxifen pretreatment on the [Ca²⁺]i responses of mouse pancreatic islet cells to CCh, glucose, and 45 mM K⁺. Islet cells responded to CCh (1 mM) with a transient [Ca²⁺]i surge which promptly dropped to the basal level (Fig. 8A), to 45 mM K⁺ with a [Ca²⁺]i spike which peaked and then dropped to a suprabasal plateau level (Fig. 8A), and to 20 mM glucose which triggered a typically small initial drop in [Ca²⁺]i, followed by a surge which persisted for at least 8 min (Fig. 8C). Pretreating the islet cells with 10 μM tamoxifen did not significantly affect the [Ca²⁺]i responses to any of these agents as it did the [Ca²⁺]i responses to CCh and 45 mM K⁺ in the chicken granulosa cells (Fig. 8, B and D).

DISCUSSION

The mechanism of the antiestrogenic and antitumor actions of tamoxifen are not fully understood. Although most work has concentrated on the interaction of tamoxifen with estrogen receptors, the lack of correlation between the binding affinity of the drug to the conventional estrogen receptor and antitumor activity suggested that something else besides estrogen receptor antagonism is involved in its actions. That something else is likely to be changes in Ca²⁺ and associated Ca²⁺-elevating signal mechanisms.
We have shown previously (33) that activation of muscarinic receptors of chicken granulosa cells rapidly triggers the release of Ca\(^{2+}\) from intracellular stores which is followed by a sustained elevation of \([\text{Ca}^{2+}]_i\) with large, superimposed oscillations which are caused by inward surges of extracellular Ca\(^{2+}\). The results of this study indicate that tamoxifen, at a concentration of 10 \(\mu\)M, by itself did not affect \([\text{Ca}^{2+}]_i\) but dramatically affected the oscillating CCh-triggered \([\text{Ca}^{2+}]_i\) surge in chicken granulosa cells by specifically prolonging the secondary external Ca\(^{2+}\) influx without affecting the initial release of Ca\(^{2+}\) from internal stores.

The serum and tissue concentrations of tamoxifen in patients being treated with the drug for breast cancer often exceed 1 \(\mu\)M (40, 41), and thus approach the concentrations used in this study. The concentrations at which tamoxifen prolonged CCh-triggered \([\text{Ca}^{2+}]_i\) surges (i.e., 10 \(\mu\)M) were similar to the functionally effective concentrations used in other \textit{in vitro} studies (5, 18, 19, 21, 24, 26–28). These concentrations are also similar to those at which tamoxifen competes with estrogen for binding to estrogen receptors (3–6). Although the classical antiestrogenic action of tamoxifen has long been thought to be mediated through binding to the conventional estrogen receptor, it is unlikely that the effect of tamoxifen on \([\text{Ca}^{2+}]_i\) oscillations in granulosa cells results from an interaction between tamoxifen and the conventional cytoplasmic/nuclear estrogen receptors, because pretreating the cells with estrogen did not affect the influx phase of the Ca\(^{2+}\) response to CCh. The biochemical mechanism(s) by which tamoxifen affects Ca\(^{2+}\) oscillations is not known.

Since the tamoxifen effect on CCh-triggered \([\text{Ca}^{2+}]_i\) surges in granulosa cells was abolished by pretreating the cells with the universal Ca\(^{2+}\)-channel blocker La\(^{3+}\), or by incubating the cells in EGTA/Ca\(^{2+}\)-free medium, the drug probably acts on Ca\(^{2+}\)-channels. The tamoxifen effect would appear to require that the Ca\(^{2+}\) channels be already activated because tamoxifen, even at concentrations as high as 0.5 mM, did not by itself affect the inactive Ca\(^{2+}\) channels in otherwise untreated granulosa cells, but it did act after the Ca\(^{2+}\) channels were activated by agonists such as CCh or by K\(^+\)-induced membrane depolarization.
It is interesting that the $[Ca^{2+}]_i$ surges in CCh-treated granulosa cells were unaffected by nafoxidine and clomiphene citrate which are related to tamoxifen (1). The ineffectiveness of these relatives strongly suggests that the effects of tamoxifen on the CCh- and K$^+$-triggered $[Ca^{2+}]_i$ surges are not nonspecific responses. Tamoxifen has been shown to be extensively metabolized in vivo (40, 42). One of its metabolites is 4-hydroxytamoxifen which reportedly has the same or even higher affinity for the conventional estrogen receptor as estradiol itself (3, 43, 44) and potently inhibits estrogen action. Therefore, this metabolite could be an important player in tamoxifen action (44, 45). Although we have not studied the metabolism of tamoxifen in our in vitro system, 4-hydroxytamoxifen did dramatically prolong the second phase of the $[Ca^{2+}]_i$ response to CCh in the chicken granulosa cells. However, 4-hydroxytamoxifen is not essential for tamoxifen action because tamoxifen is not metabolized by tamoxifen targets such as cultured MCF-7 cells (3) and chick oviduct (46).

There are several possible mechanisms and functions for $[Ca^{2+}]_i$ oscillations (47-49). However, it seems likely that different cell functions are regulated by the amplitudes and/or frequencies and shapes of the $[Ca^{2+}]_i$ transients (47-49). A direct effect of tamoxifen on $Ca^{2+}$ channels is strongly indicated by the ability of the drug to prolong the $[Ca^{2+}]_i$ response to a high external K$^+$ concentration which activates only $Ca^{2+}$ channels by depolarizing the cell membrane. Moreover, tamoxifen did not affect the $[Ca^{2+}]_i$ responses to DMSO and 17β-estradiol, which trigger the release of $Ca^{2+}$ from internal stores without affecting $Ca^{2+}$ channels (34, 36). However, whatever the tamoxifen target might be in the $Ca^{2+}$ channels of the granulosa cells, it is not in all cells, because the drug did not affect the $[Ca^{2+}]_i$ responses in pancreatic islet cells to CCh, glucose, or 45 mM K$^+$.

Several previous studies have demonstrated that tamoxifen reduces $Ca^{2+}$ influx into cells by binding to $Ca^{2+}$ channels (26, 28, 29). This must profoundly affect a variety of cell functions. It has been suggested that the inactivation of $Ca^{2+}$ channels and $Ca^{2+}$ influx by tamoxifen might directly inhibit cell growth or the release of autocrine growth factors, which are known to be produced by breast cancer cells (50). However, tamoxifen obviously did not inactivate $Ca^{2+}$ channels and reduce $Ca^{2+}$ influx in our chicken granulosa cells.

The functions of the muscarinic receptors and the $[Ca^{2+}]_i$ surges they trigger in chicken granulosa cells are unknown. They might stimulate the secretion of various nonsteroidal granulosa cell products needed for follicular maturation and function. The tamoxifen-enhanced $[Ca^{2+}]_i$ surges triggered by CCh or 45 mM K$^+$ are strong signals which might be expected to elicit a wide variety of cellular responses. They undoubtedly modulate the activities of K$^+$, Cl$^-$, and other channels (51), and may directly, or indirectly, through $Ca^{2+}$ binding signal proteins such as calmodulin, stimulate the secretion of autocrine and paracrine factors (e.g., transforming growth factor α (52), proliferation (53), and reduce the gap junctional communications between granulosa cells as well as between granulosa cells and oocytes that are essential for the promotion of oocyte growth and maturation (54).

Tamoxifen is a relatively selective antiestrogen with apparently low toxicity, which reduces the appearance of new tumors in early-stage breast cancer patients (1, 2). Large randomized, prospective trials are currently under way in England and have been proposed in the United States to evaluate tamoxifen as a safe, specific chemopreventive agent for mammary carcinoma (55). However, the results of the present study add to the growing evidence that this drug variously and profoundly affects $Ca^{2+}$ signaling and thus may affect the functions of various normal tissues as well as prevent the growth of mammary cancer cells.

ACKNOWLEDGMENTS

White Leghorn hens were generously provided by Dr. S. K. Ho, Centre for Food and Animal Research, Agriculture Canada, Ottawa, Ontario, Canada. We thank Cecilia Dias and Geoff Mealing for excellent technical assistance.

REFERENCES


21. Lam, H-Y. P. Tamoxifen is a calmodulin antagonist in the activation of cAMP

22. Lopes, M. C. F., Vale, M. G. P., and Carvalho, A. P. Ca2+-dependent binding of


Effect of Tamoxifen on Carbachol-triggered Intracellular Calcium Responses in Chicken Granulosa Cells

Paul Morley and James F. Whitfield


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/1/69

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.