Non-genomic action of estradiol and progesterone on cytosolic calcium concentrations in primary cultures of human granulosa-lutein cells

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BACKGROUND: The present study examined whether the sex steroids, estradiol and progesterone, could alter cytoplasmic calcium concentrations ([Ca²⁺]cyt) in human granulosa-lutein cells. METHODS: Human granulosa cells were obtained at the time of oocyte retrieval for IVF and cultured for 3–7 days. Cells were loaded with Fura-2 AM and changes in [Ca²⁺]cyt of single cells were studied using a dynamic digital Ca²⁺ imaging system. RESULTS: Both estradiol and progesterone stimulated elevations of [Ca²⁺]cyt in Ca²⁺-containing medium within seconds of exposure of the granulosa-lutein cells to the steroid, but only estradiol caused an increase in [Ca²⁺]cyt in Ca²⁺-free medium. Both ICI-182780 and RU 486 stimulated [Ca²⁺]cyt increases and inhibited the effects of estradiol and progesterone, respectively. Tamoxifen also induced transient increases in [Ca²⁺]cyt concentrations but inhibited the effects of both estradiol and progesterone. The inhibitory effects of tamoxifen, ICI-182780 and RU 486 on [Ca²⁺]cyt responses to estradiol and progesterone could be reversed with higher concentrations of estradiol and progesterone, respectively. The [Ca²⁺]cyt effects induced with tamoxifen could not be eliminated by prior treatment with RU 486 or ICI-182780. CONCLUSION: These results provide strong evidence that both estradiol and progesterone act as the steroid antagonists, tamoxifen, RU 486 and ICI-182780, on human granulosa-lutein cells through a non-genomic mechanism.

Key words: calcium response/estradiol/granulosa cells/progesterone

Introduction

One of the earliest events of non-genomic action of steroids is an increase in intracellular uptake of calcium (Falkenstein et al., 2000). Such rapid actions have been reported for steroids in human ovarian tissue (Machelon et al., 1998) where luteinizing granulosa cells were found to respond to androstenedione with a rapid increase in intracellular Ca²⁺ concentrations ([Ca²⁺]i) by mobilization of Ca²⁺ stores from the endoplasmic reticulum and by Ca²⁺ influx through the voltage-dependent Ca²⁺ channel. A similar rapid effect on Ca²⁺ fluxes was found for dichlorodiphenylchloroethylene (DDE, p,p'-DDE) in human granulosa-lutein cells (Younglai et al., 2004). These two studies seem to be the only ones to date using human granulosa-lutein cells. This action of DDE was a novel observation for an endocrine-disrupting chemical since DDE had hitherto been known to act as an anti-androgen on the nuclear receptors (Kelce et al., 1995). Recent evidence suggest that the isomer of DDE, α,α'-DDE binds strongly to the estradiol membrane receptor on breast cancer cells (Thomas et al., 2005), lending further support for this additional mechanism of action of endocrine disruptors.

Chicken and porcine granulosa cells can respond to estradiol, but not to progesterone or androgens, with a rapid increase in [Ca²⁺]i, and the source of Ca²⁺ for these cells was shown to be exclusively intracellular (Morley et al., 1992). Granulosa cells from diethylstilbestrol-treated immature rats failed to respond to estradiol with any increase in Ca²⁺ (Morley et al., 1992). On the other hand, granulosa cells from equine chorionic gonadotrophin-treated 23-day-old rats or spontaneously immortalized rat granulosa cells do show any Ca²⁺ responses to estradiol and progesterone could be reversed with higher concentrations of estradiol and progesterone, respectively. The [Ca²⁺]cyt effects induced with tamoxifen could not be eliminated by prior treatment with RU 486 or ICI-182780. CONCLUSION: These results provide strong evidence that both estradiol and progesterone act as the steroid antagonists, tamoxifen, RU 486 and ICI-182780, on human granulosa-lutein cells through a non-genomic mechanism.
increases in $[\text{Ca}^{2+}]_\text{i}$, when stimulated with estradiol, progesterone and androstenedione (Lieberherr et al., 1999). The current study was undertaken to determine whether estradiol and progesterone can induce changes in $[\text{Ca}^{2+}]_\text{i}$ in human granulosa-lutein cells and whether these changes could be influenced by various antagonists.

Materials and methods
Unless otherwise stated, all cultures, fluorescent labelling, digital fluorescence calcium ratio imaging and experimental conditions were identical to those described by Younglai et al. (2004).

Source of granulosa cells
Patients were treated with a long luteal protocol of GnRH agonist (Lupron; Abbott Laboratories, Montreal, QC: 0.5 mg per day for 10–14 days) and recombinant FSH (12–85 ampoules, 75 IU per ampoule Gonal F; Serono Canada Ltd, Oakville, ON) followed by HCG (Profasi; Serono). After removal of oocyte–cumulus complexes, the remaining follicular aspirates were transported to the research laboratory in polypropylene tubes and the granulosa cells were isolated and cultured. After 3–7 days in culture, areas containing small luteinized cells, characterized by the cytoplasmic–nuclear ratio, were chosen for imaging.

Incubation medium conditions for imaging
Granulosa cells in culture were always exposed to 1–2 mmol Ca$^{2+}$/l except for the experiments requiring Ca$^{2+}$-free conditions, where the medium was replaced by the Ca$^{2+}$-free isotonic physiological medium containing 0.1 mmol EGTA/l immediately prior to the measurement. Although distilled and de-ionized water were used for the preparation of solutions, contaminating Ca$^{2+}$ from containers and other chemicals may contribute up to 10 μmol Ca$^{2+}$/l. Therefore, EGTA 0.1 mmol/l was always included in the Ca$^{2+}$-free medium.

Digital dynamic fluorescence ratio measurements
Changes in Ca$^{2+}$ concentration were measured using a dynamic digital Ca$^{2+}$ imaging system (Image-IFL, Universal Imaging Corporation) with a Zeiss lamp (XBO 100 W/DC) coupled to a Zeiss inverted microscope (Zeiss IM 35) with a 100x oil immersion lens and a numerical aperture of 1.25, as previously described (Low et al., 1997). Images were integrated and collected by a Pulnix camera (TM-720, maximal at 3 frames/frame) initially at a speed of 15 frames/frame. In general, 3–4 probes covering an area of five pixels each were placed on different spatial areas of cells, usually near the plasma membrane and over the nuclear region. Changes in the fluorescence ratio were recorded and the data stored. Since the probes covered small areas of interest within the cell, quantification of calcium changes was not attempted. Quantification would vary in the different regions since our previous findings suggested that there are spatial and temporal differences within the cell upon stimulation with agonists (Kwan et al., 2003; Younglai et al., 2004). Images were saved and, in the event that some areas of interest showed oversaturation of colour during processing, the sequences were rerun with new areas of interest. Cells from at least three different patients had to show a response before the results were accepted as meaningful and quoted. Representative patterns of response are shown in the figures. In some instances, the positions of the probing windows were changed from the original placements to capture the spatial changes in Ca$^{2+}$ concentrations. Ethical approval was obtained from the institutional research ethics board for this work, and patients signed a consent form agreeing to donate their excess cells for research.

Results
Effects of estradiol and progesterone
Granulosa-lutein cells were treated with steroids at concentrations of up to 1 μg/ml. Figure 1 shows the $[\text{Ca}^{2+}]_\text{cyt}$ response of granulosa cells to estradiol and progesterone. In Figure 1A, four probing windows were placed on three separate cells. Addition of estradiol caused an immediate increase in $[\text{Ca}^{2+}]_\text{cyt}$ above the baseline in all the probed cells, and the changes became oscillatory. The increase in $[\text{Ca}^{2+}]_\text{cyt}$ then declined to baseline and, over the period of observation, started to rise slowly. Addition of EGTA diminished the Ca$^{2+}$ elevation, thus terminating the experiment. In Figure 1B, three cells were probed. Progesterone caused an immediate elevation in $[\text{Ca}^{2+}]_\text{cyt}$ in all three cells, higher than that elicited with estradiol. EGTA was then added to preserve cell viability when $[\text{Ca}^{2+}]_\text{cyt}$ became excessively elevated.

In view of the Ca$^{2+}$-lowering effect of EGTA seen above, the effects of estradiol and progesterone in Ca$^{2+}$-free medium were then investigated. Figure 2A shows that estradiol induced an immediate peak in $[\text{Ca}^{2+}]_\text{cyt}$ followed by a return to baseline in Ca$^{2+}$-free medium. Addition of Ca$^{2+}$ to the medium to a final concentration of 2.5 mmol/l resulted in a slow oscillatory increase in $[\text{Ca}^{2+}]_\text{cyt}$. The response to progesterone was different, as shown in Figure 2B. There was no response to progesterone in Ca$^{2+}$-free medium, and subsequent addition of 2.5 mmol/l Ca$^{2+}$ also failed to induce an elevation of $[\text{Ca}^{2+}]_\text{cyt}$ as seen for estradiol. However, addition of a further dose of progesterone at 22 min caused a sharp increase in $[\text{Ca}^{2+}]_\text{cyt}$ which was then suppressed with EGTA. This pattern of response for progesterone in Ca$^{2+}$-free medium was observed in cells from all three different patients.

Controls
The effects of various other steroids on $[\text{Ca}^{2+}]_\text{cyt}$ were investigated in order to demonstrate that the $[\text{Ca}^{2+}]_\text{cyt}$ changes were not non-specific effects. Figure 3 shows the results with 1 μg/ml each of androstenedione, testosterone, dehydroepiandrosterone (DHEA) and estrone. Estradiol and progesterone
were the positive controls. Figure 3A is a representative of cells from four of eight patients which showed a \([\text{Ca}^{2+}]_{\text{cyt}}\) response to androstenedione, and two doses (10 µg/ml each) of progesterone were needed to elicit a \([\text{Ca}^{2+}]_{\text{cyt}}\) response. Cells from four of six patients responded to testosterone with increases in \([\text{Ca}^{2+}]_{\text{cyt}}\), and Figure 3B is a representative of one of these. There was a delay in the \([\text{Ca}^{2+}]_{\text{cyt}}\) response to progesterone. The solvent used for dissolving the steroids, DMSO, as well as DHEA and estrone, had no effects on \([\text{Ca}^{2+}]_{\text{cyt}}\) changes (Figure 3C). Pregnenolone (four patients), dihydrotestosterone (three patients) and estriol (three patients) at concentrations of 1 and 10 µg/ml also had no effects. In other experiments, a single addition of a control steroid was followed by either progesterone or estradiol alone 6–10 min later, and these control steroids also had no effect on \([\text{Ca}^{2+}]_{\text{cyt}}\). The effects of \(\text{Ca}^{2+}\)-free medium with these steroids were not investigated.

### Effects of antagonists

Steroid antagonists are known to act at the nuclear level and therefore were not expected to affect \([\text{Ca}^{2+}]_{\text{cyt}}\) changes induced by exogenous steroids. Figure 4A shows that RU 486 at 1 µg/ml, by itself induced a slight \(\text{Ca}^{2+}\) transient in two of four cells probed, but inhibited the subsequent...
ICI-182780 (Figure 4D). Tamoxifen, another anti-estrogen, markedly increased \([\text{Ca}^{2+}]_{\text{cyt}}\) (Figure 4E). In addition, it completely suppressed the \([\text{Ca}^{2+}]_{\text{cyt}}\) response to estradiol and progesterone. The \([\text{Ca}^{2+}]_{\text{cyt}}\) was shown to have a rapid effect on \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in human granulosa-lutein cells (Machelon et al., 1998) and to have an inhibitory effect on the non-genomic response of oocytes to estradiol (Tesarik and Mendoza, 1997). Thus it would appear that human granulosa-lutein cells can have non-genomic responses to a variety of steroids. Porcine granulosa cells also respond to estradiol, progesterone and androstenedione with a rapid increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) (Lieberherr et al., 1999). Both estradiol and progesterone receptors have been detected on human sperm membranes (Luconi et al., 1998, 1999), and there appears to be an interference of estradiol receptors with membrane receptors. It is possible that the steroid effects on granulosa-lutein cells are also mediated by membrane receptors.

The probes for detection of \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in individual cells each covered an area of five pixels. Up to nine probes have been used in the past to follow the progression of \([\text{Ca}^{2+}]_{\text{cyt}}\) changes through the cell membrane to the nucleus (Kwan et al., 2003). Thus changes can be detected adjacent to the cell membrane, over intracellular organelles or in the nucleus, and the magnitude of responses in individual cells would depend on the location of the probes. Usually two probes were used per cell—one near the membrane and the other near the nucleus. The differences in responses could also be due to patient variation as well as cell type. Small and large cells derived from the mural, cumulus or theca layers in the corpus luteum have different abilities to produce progesterone (Lemoin and Mauleon, 1982).

Non-specific effects of supraphysiological doses of steroids have been described (Losel et al., 2003). The concentrations of estradiol and progesterone used are consistent with levels of ICI-182780 (Figure 4D). Tamoxifen, another anti-estrogen, markedly increased \([\text{Ca}^{2+}]_{\text{cyt}}\) (Figure 4E). In addition, it completely suppressed the \([\text{Ca}^{2+}]_{\text{cyt}}\) response to estradiol and progesterone. The \([\text{Ca}^{2+}]_{\text{cyt}}\) ionophore, ionomycin, immediately elevated \([\text{Ca}^{2+}]_{\text{cyt}}\). As with RU 486 and ICI-182780, increasing the concentration of estradiol and progesterone can overcome the inhibitory effects of the antisteroids (Figure 4F). The effects of tamoxifen were not prevented by prior treatment with RU 486 or ICI-182780 (Figure 5).

Discussion

The non-genomic actions of estradiol and progesterone on reproductive tissues are well known (Revelli et al., 1998; Gerdes et al., 2000; Levin, 2001, 2002; Bramley, 2003; Sak and Everaus, 2004). Although this non-genomic action has been demonstrated in gonadal cells from several animal species such as chicken, rat and pig granulosa cells (Morley et al., 1992; Revelli et al., 1998; Lieberherr et al., 1999; Peluso et al., 2001), to our knowledge the present communication is the first report on a similar action on human granulosa-lutein cells. The non-genomic action of estradiol has been demonstrated in chicken and porcine granulosa cells (Morley et al., 1992) and that for progesterone in rat granulosa cells (Peluso et al., 2002). Androstenedione has been shown to have a rapid effect on \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in human granulosa-lutein cells (Machelon et al., 1998) and to have an inhibitory effect on the non-genomic response of oocytes to estradiol (Tesarik and Mendoza, 1997). Thus it would appear that human granulosa-lutein cells can have non-genomic responses to a variety of steroids. Porcine granulosa cells also respond to estradiol, progesterone and androstenedione with a rapid increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) (Lieberherr et al., 1999). Both estradiol and progesterone receptors have been detected on human sperm membranes (Luconi et al., 1998, 1999), and there appears to be an interference of estradiol receptors with membrane receptors. It is possible that the steroid effects on granulosa-lutein cells are also mediated by membrane receptors.

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commonly found in mature human antral follicles (Kreiner et al., 1987; Yie et al., 1995) and used for demonstrating non-genomic effects in human spermatozoa (Wennemuth et al., 1998; Luconi et al., 2001). We have also used 10-fold concentrations of non-active steroids such as estriol, which has a similar structure to estradiol, to show the absence of non-specific effects.

The genomic estrogen receptors have been located in human granulosa cells (Suzuki et al., 1994; Saunders et al., 2000; Jakimiuk et al., 2002), but not in corpus luteum which contained progesterone receptors (Suzuki et al., 1994). Genomic progesterone receptors have also been detected in human corpus luteum cells by immunohistochemistry (Maybin and Duncan, 2004). Although there have been suggestions that the genomic and membrane receptors may have some similarities (Saner et al., 2003; Sak and Everaus, 2004) and may arise from a single transcript (Razandi et al., 1999), there is no evidence to date on the nature of the human granulosa-lutein

Figure 4. Effects of steroid antagonists on intracellular Ca\(^{2+}\) changes in granulosa cells. Inserts are similar to those in Figure 2. (A) Changes in [Ca\(^{2+}\)]\(_{cyt}\) induced by RU 486 (1 \(\mu\)g/ml per application), followed by two doses of 1 \(\mu\)g/ml progesterone. This figure is representative of four different patients. (B) Cells were pre-treated with 2 \(\mu\)g of RU 486/ml for at least 1 h prior to stimulation with 1 \(\mu\)g of progesterone/ml followed 3 min later by 1 \(\mu\)g of estradiol/ml. This figure is representative of three patients with different time intervals between progesterone and estradiol. (C) ICI-182780 (two doses of 1 \(\mu\)g/ml each) was added to the medium at the times indicated, followed \(\sim\) 7 min apart by 2 \(\mu\)g of estradiol/ml and 2 \(\mu\)g of progesterone/ml. (D) Three doses of 1 \(\mu\)g of estradiol/ml for a final concentration of 3 \(\mu\)g of estradiol/ml reversed the inhibitory effect of ICI-182780. (E) Tamoxifen was used at a concentration of 5 \(\mu\)g/ml which inhibited the effects of both estradiol and progesterone. (F) The two doses of tamoxifen (0.5 \(\mu\)g/ml) resulted in a final concentration of 1 \(\mu\)g/ml, the second dose of estradiol led to a final concentration of 2.3 \(\mu\)g/ml and progesterone was added to a final concentration of 1.3 \(\mu\)g/ml. E2 = estradiol; P4 = progesterone; RU = RU 486; ICI = ICI-182780; TAM = tamoxifen; Ion = ionomycin. Inserts show the location of probes at the start of recordings.
membrane receptor for estradiol or progesterone. In addition, there may be multiple classes of proteins which can function as non-genomic steroid receptors (Watson and Gametchu, 2003), and recent evidence suggests that these membrane receptors may be G protein-like-coupled receptors (Edwards, 2005).

The different patterns of \([Ca^{2+}]_{cyt}\) responses observed with estradiol and progesterone suggest that there are distinct and separate mechanisms by which these agonists act on the granulosa-lutein cell. The \([Ca^{2+}]_{cyt}\) response to estradiol in \(Ca^{2+}\)-free medium suggests that \(Ca^{2+}\) is mobilized from intracellular stores such as the smooth endoplasmic reticulum, and \(Ca^{2+}\) influx could also occur. The \(Ca^{2+}\) response to progesterone, however, suggests that the mechanism by which progesterone acts is different from that of estradiol, in that intracellular sources are not utilized. The failure of the cells in the presence of progesterone to respond on addition of \(Ca^{2+}\) to the medium was unexpected. This suggests that the membrane factors through which progesterone acts are more sensitive to the destabilizing effect of the lack of calcium (Webb and Bohr, 1978) and that calcium is required for restoration of membrane stability (Ou et al., 1997). In porcine granulosa cells, on the other hand, progesterone triggers rapid transmembrane \(Ca^{2+}\) influx and/or calcium mobilization from endoplasmic reticulum (Machelon et al., 1996). The concentration of \(Ca^{2+}\) within the cell is controlled by two general mechanisms: (i) entry from extracellular fluid by voltage-operated channels; or (ii) release from endoplasmic reticulum by capacitative calcium entry which activates the store-operated channel permitting influx from the extracellular fluid (Brini and Carafoli, 2000). Calcium oscillations have been shown to be required for cell division (Swann et al., 2004) and it is possible that these oscillations trigger mitosis in the cultured granulosa cells. Calcium oscillations also represent a physiological mechanism to prevent a rapid rise of cytosolic \(Ca^{2+}\) concentration to toxic levels (Miyazaki, 1995; Bootman et al., 2001). Further studies with the use of inhibitors are required to elucidate the different intracellular mechanisms involved in estradiol- and progesterone-induced \(Ca^{2+}\) fluxes in human granulosa-lutein cells.

Although a previous communication revealed that human luteinizing granulosa cells did not show a \([Ca^{2+}]_{cyt}\) response to testosterone as they did to androstenedione (Machelon et al., 1998), in four of six patients we found that testosterone could induce \([Ca^{2+}]_{cyt}\) uptake in granulosa-lutein cells. In addition, cells from only four of eight patients responded to androstenedione. This may reflect patient variability or possible high levels of androgens or estrogens produced during stimulation protocols, attenuating the effects of these steroids. A similar attenuation has been found in estrogen-treated microvessels (Kakucs et al., 2001). Moreover, testosterone at a concentration of \(10^{-5}\) mol/l can induce \([Ca^{2+}]_{cyt}\) influx in chicken granulosa cells (Morley et al., 1992) as well as activated T cells (Benten et al., 1997). This observation with testosterone is validated further by the absence of \([Ca^{2+}]_{cyt}\) responses to the structurally similar steroids, DHEA and dihydrotestosterone.

Neither DMSO, estrone, estriol, pregnenolone, DHEA or dihydrotestosterone stimulated \(Ca^{2+}\) uptake in the human granulosa-lutein cells. The use of these control steroids, while not eliciting increases in \([Ca^{2+}]_{cyt}\) concentrations, raised the possibility that these treatments could enhance or inhibit the responses to estradiol and progesterone. Such a possibility is reflected in Figure 3A and B where the rapid response to progesterone is apparently delayed. This inhibition of a steroid effect by another steroid is being examined in more detail. The media used in our experiments do not exceed 0.5% DMSO. A concentration of 0.2–1% DMSO in the culture medium can induce a 2- to 6-fold increase in \(Ca^{2+}\) uptake in chicken granulosa cells (Morley and Whitfield, 1993), pointing to species variation in sensitivity of membrane receptors.

It is interesting that tamoxifen by itself had a membrane effect in stimulating an increase in \([Ca^{2+}]_{cyt}\). While this was unexpected, it has been reported that tamoxifen has a non-genomic effect in stimulating membrane-bound guanylate cyclase in porcine proximal tubular LLC-PK1 cells (Chen et al., 2003) and can stimulate an increase in \([Ca^{2+}]\) uptake in MCF-7 breast cancer cells (Chang et al., 2002). Tamoxifen has also been found to stimulate \(Ca^{2+}\) influx in human sperm and to inhibit the progesterone-induced \(Ca^{2+}\) influx (Luconi et al., 2001). In chicken granulosa cells, tamoxifen had no
effect (Morley et al., 1992). Tamoxifen, which is a selective estrogen receptor modulator, can have antagonist activity in breast cancer cells but is an agonist for endometrial growth (Hermenegildo and Cano, 2000). In our study, tamoxifen completely inhibited the effects of both estradiol and progesterone. With chicken granulosa cells, tamoxifen prolonged the carbachol-triggered [Ca^{2+}]_{cyt} surges (Morley and Whitfield, 1994).

ICI-182780 prevented the [Ca^{2+}]_{cyt} response to estradiol, but further addition of estradiol could reverse this inhibition. The relative binding of ICI-182780 is 0.89, compared with that of estradiol 1.0 (Wakeling et al., 1991), and therefore excess estradiol would be expected to reverse the non-genomic effect of ICI-182780. The inhibitory effect of ICI-182780 on the non-genomic action of estradiol has also been observed in rat astrocytes (Chaban et al., 2004). These estrogen receptor antagonists can activate large conductance, calcium-activated potassium channel (BK_{Ca}) activity in smooth muscle (Dick, 2002) just like estradiol and tamoxifen (Valverde et al., 1999; Dick et al., 2001), but in cultured endothelial cells of human coronary artery it is inhibitory for BK_{Ca} channel activity (Liu et al., 2003). Thus these two estrogen antagonists, tamoxifen and ICI-182780, act by different mechanisms at the cell membrane. However, more recent evidence suggests that tamoxifen and ICI-182780 have high affinities for the membrane receptor for estradiol in the SKBR3 breast cancer cell line (Thomas et al., 2005), confirming our observations on the non-genomic effects of these compounds. Competition between estradiol and the antagonists can explain the reversal of effects when excess steroid is added.

RU 486 has a K_d of ~10^{-9} mol/l (Cadepond et al., 1997) and has both anti-progestational and anti-glucocorticoid activities. At a concentration of 1 µg/ml used in these experiments, RU 486 showed a slight increase in [Ca^{2+}]_{cyt} uptake but inhibited the [Ca^{2+}]_{cyt} response to progesterone. Similar to the effects of ICI-182780, excess progesterone can override the inhibitory effect of RU 486. Concentrations of RU 486 used in the past have ranged from 640 nmol/l to inhibit the effects of a similar concentration of progesterone (Peluso et al., 2001) to 100 µmol/l to show an increase in apoptosis in luteinizing granulosa cells (Svensson et al., 2001). The inability of RU 486 and ICI-182780 to inhibit the [Ca^{2+}]_{cyt} responses to tamoxifen suggests that tamoxifen is acting at a different site compared with the other two antagonists. Taken together, these data suggest that the sex steroids, estradiol and progesterone, as well as the antagonists, tamoxifen, ICI-182780 and RU 486, can act as genomic and non-genomic agents.

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