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Modulation of mitochondrial Ca^{2+} uptake by estrogen receptor agonists and antagonists

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1 Ca^{2+} uptake by mitochondria is a key element in the control of cellular Ca^{2+} homeostasis and Ca^{2+}-dependent phenomena. It has been known for many years that this Ca^{2+} uptake is mediated by the mitochondrial Ca^{2+} uniporter, a specific Ca^{2+} channel of the inner mitochondrial membrane. We have shown previously that this channel is strongly activated by a series of natural phytoestrogenic flavonoids. We show here that several agonists and antagonists of estrogen receptors (ERs) also modulate the activity of the uniporter.

2 The specific α-ER agonist 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) was the strongest activator, increasing the rate of mitochondrial Ca^{2+} uptake in permeabilized HeLa cells by 10-fold at 2 μM. Consistently, PPT largely increased the histamine-induced mitochondrial [Ca^{2+}] peak and reduced the cytosolic one.

3 Diethylstilbestrol and 17-β-estradiol (but not 17-α-estradiol) were active at pharmacological concentrations while the β-estrogen-receptor agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) was little effective.

4 The ER modulators tamoxifen and 4-hydroxy-tamoxifen inhibited mitochondrial Ca^{2+} uptake (IC_{50} 2.5±1.5 and 2.5±1.4 μM, mean±s.d., respectively) both in the presence and in the absence of PPT, but raloxifene and the pure estrogen antagonist ICI 182,780 produced no effect.

5 Activation by PPT was immediate and inhibition by tamoxifen or 4-hydroxy-tamoxifen required only 5 min to reach maximum.

6 Tamoxifen did not modify mitochondrial membrane potential and PPT induced a slow mitochondrial depolarization at higher concentrations than those required to activate mitochondrial Ca^{2+} uptake.

7 These results suggest that some kind of ER or related protein located in mitochondria controls the activity of the Ca^{2+} uniporter by a nongenomic mechanism. This novel mechanism of action of estrogen agonists and antagonists can provide a new interpretation for several previously reported effects of these compounds.
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Abbreviations: $[Ca^{2+}]_c$, cytosolic $[Ca^{2+}]$; $[Ca^{2+}]_M$, mitochondrial $[Ca^{2+}]$; DES, diethylstilbestrol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; E2, 17-β-estradiol; ER, estrogen receptor; PPT, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole; TMRE, tetramethylrhodamine ethyl ester

Introduction

It is now well established that Ca^{2+} uptake by mitochondria is a key controller of cellular Ca^{2+} homeostasis (Duchen, 2000; Rizzuto *et al*., 2000; Parekh, 2003). The level of Ca^{2+} in the mitochondrial matrix regulates the rate of respiration and ATP production (Jouaville *et al*., 1999; Rutter & Rizzuto, 2000), and is a critical trigger for the opening of the permeability transition pore and thus for apoptosis (Bernardi *et al*., 2001; Hajnoczky *et al*, 2003; Rizzuto *et al*., 2003). On the other hand, Ca^{2+} uptake by mitochondria damps cytosolic Ca^{2+} transients. Owing to the low Ca^{2+} affinity of the mitochondrial Ca^{2+} uptake mechanism, this mitochondrial Ca^{2+} buffering is specially effective to modulate the local high-Ca^{2+} microdomains, which are responsible for many of the physiological effects of Ca^{2+} signaling (Berridge *et al*., 2003). For example, mitochondria have been shown to modulate catecholamine secretion in chromaffin cells (Giovannucci *et al*., 1999; Montero *et al*., 2000), the Ca^{2+}-dependence of voltage-dependent Ca^{2+} channels (Hernandez-Guijo *et al*., 2001) and capacitative Ca^{2+} channels (Hoth *et al*., 2000) or the rate of cytosolic Ca^{2+} waves (Boitier *et al*., 1999).

The main system responsible for mitochondrial Ca^{2+} uptake is the mitochondrial Ca^{2+} uniporter, a highly specific

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Ca^{2+}-activated Ca^{2+} channel (Bernardi, 1999; Kirichok *et al*., 2004) placed in the inner mitochondrial membrane. We have shown before that this channel can be activated by the p38 mitogen activated protein kinase inhibitor SB202190 (Montero *et al*., 2002). Although we initially suggested that this effect could be mediated by phosphorylation by this or a closely related kinase, we noticed that other p38 mitogen activated protein kinase inhibitors, with chemical structure very similar to that of SB202190, were little or no effective on the activation of mitochondrial Ca^{2+} uptake (Montero *et al*., 2002). In addition, we have later shown that the same activation can be obtained in the absence of ATP in permeabilized cells and that a series of natural flavonoid compounds were also potent activators of mitochondrial Ca^{2+} uptake with a defined structure-activity relationship (Montero *et al*., 2004). The required chemical structure included a critical hydroxyphenyl group, which was also an essential part of SB202190, as its substitution by methylsulfinylphenyl or nitrophenyl groups largely decreased the activity (Montero *et al*., 2002). In both cases, SB202190 and flavonoids, the activation was similar in magnitude (up to 20-fold maximum stimulation) and required no preincubation (Montero *et al*., 2004), suggesting a common mechanism, most probably a direct effect of these compounds on the uniporter or an associated protein.

It has been known since the 1940s that natural flavonoids possess estrogenic activity. They are all polyphenols and many of them harbor some structural similarity to natural and synthetic estrogens. These so-called phytoestrogens have rather weak estrogenic activities compared to the natural estrogens. However, because of their higher concentrations, this activity could account for some of the many biological effects attributed to them (Bravo, 1998; Middleton *et al*., 2000; Havsteen, 2002). We have tested in this work the hypothesis that the activation of mitochondrial Ca^{2+} uptake by flavonoid compounds could take place through their interaction with some kind of estrogen receptor (ER). Although these receptors are usually thought of as nuclear-located ligand-dependent transcription factors, they can also rapidly mobilize signals at the plasma membrane or in the cytoplasm (Falkenstein *et al*., 2000; Nadal *et al*., 2001; Levin, 2002; Lösel *et al*., 2003) and some of them have been localized in the plasma membrane (Collins & Webb, 1999; Li *et al*., 2003; Razandi *et al*., 2004; Song *et al*., 2004) and in mitochondria (Chen *et al*., 2004; Yang *et al*., 2004). We show here that several natural and synthetic agonists of ERs activate mitochondrial Ca^{2+} uptake, while selective ER modulators like tamoxifen and 4-hydroxy-tamoxifen inhibit mitochondrial Ca^{2+} uptake. These results suggest that some kind of ER or related protein is involved in the modulation of the mitochondrial Ca^{2+} uniporter.

Methods

Cell culture and transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. MCF-7 cells, kindly provided by Dr S. Ramos, University of Oviedo, Spain, were grown in RPMI medium supplemented with 10% fetal calf serum. The construction strategy of the mutated mitochondrially targeted aequorin chimera has been described previously (Montero *et al*., 2000). The HeLa cell clone MM5 that stably expresses mitochondrially-targeted mutated aequorin has also been described previously (Montero *et al*., 2002). Similar data were obtained using wild-type cells transiently transfected with a pcDNA3.1 plasmid containing the construct for mitochondrially targeted mutated aequorin. The construct for cytosolic aequorin, cloned into the pcDNA3.1 plasmid, has been also described previously (Montero *et al*., 2002). Transfections were carried out using Metafectene (Biontex, Munich, Germany).

$[Ca^{2+}]_M$ and $[Ca^{2+}]_c$ measurements

The Ca^{2+}-sensitive photoprotein aequorin was used to measure $[Ca^{2+}]$ in both the mitochondrial and the cytosolic compartments. In the case of the mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_M$) measurements, a mutated form of aequorin with lower Ca^{2+} affinity was used in order to reduce aequorin consumption and thus measure better the real changes in $[Ca^{2+}]_M$, avoiding the artifacts due to saturation with Ca^{2+} of aequorin. A detailed discussion of the reasons for using a low-affinity aequorin to measure $[Ca^{2+}]_M$ changes has been presented elsewhere (Montero *et al*., 2002). The HeLa cell clone MM5 was used for most of the measurements of $[Ca^{2+}]_M$. In some cases, $[Ca^{2+}]_M$ measurements were made using wild-type HeLa cells transfected with the pcDNA3.1 plasmid containing the construct for mitochondrially targeted mutated aequorin. Cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) measurements were carried out using HeLa cells transiently transfected with the plasmid for cytosolic aequorin. Cells were plated onto 13 mm round coverslips. For aequorin reconstitution, HeLa cells expressing cytosolic aequorin were incubated for 1–2 h at room temperature with 1 μM of wild-type coelenterazine, and cells expressing mitochondrially targeted mutated aequorin were incubated for 1–2 h at room temperature with either 1 μM of wild-type coelenterazine (for experiments with intact cells) or 1 μM of coelenterazine n (for experiments with permeabilized cells), in standard medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. Cells were then placed in the perfusion chamber of a purpose-built luminometer thermostatized at 37°C. For the experiments with permeabilized cells, mitochondrially targeted mutated aequorin was reconstituted with coelenterazine n in order to reduce still further its Ca^{2+}-affinity, thus allowing measurement of higher $[Ca^{2+}]_M$. Then, standard medium containing 0.5 mM EGTA instead of Ca^{2+} was perfused for 1 min, followed by 1 min of intracellular medium (130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM K_3PO_4, 0.5 mM EGTA, 1 mM ATP, 20 μM ADP, 2 mM succinate, 20 mM HEPES, pH 7) containing 100 μM digitonin. Then, intracellular medium without digitonin was perfused for 5–10 min, followed by buffers of known $[Ca^{2+}]$ between 3.5 and 7 μM, prepared in intracellular medium using HEDTA/Ca^{2+}/Mg^{2+} mixtures. In some experiments, either medium without succinate or medium without sodium (140 mM KCl, 1 mM $MgCl_2$, 1 mM K_3PO_4, 0.5 mM potassium EGTA, 1 mM potassium ATP, 2 mM potassium succinate, 20 mM HEPES, pH 7 with KOH) were used.

Measurements of mitochondrial membrane potential

Mitochondrial membrane potential was monitored using the fluorescent indicator tetramethylrhodamine ethyl ester

(TMRE). For studies with intact cells, HeLa cells were incubated for 1 min with 1 μM TMRE. Cells were then washed with standard medium and mounted in a cell chamber in the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. For studies with permeabilized cells, HeLa cells were placed in the cell chamber, permeabilized as described above and then perfused with intracellular medium containing 20 nM TMRE until a steady-state fluorescence was reached (usually about 5 min). Single-cell fluorescence was excited at 540 nm using a Cairn monochromator (200 ms excitation every 2 s) and images of the fluorescence emitted between 570 and 630 nm obtained with a 40 $\times$ Fluar objective were recorded by a Hamamatsu ORCA-ER camera. Single-cell fluorescence records were analyzed off-line using the Metafluor program (Universal Imaging). Experiments were performed at 37°C using an on-line heater from Harvard Apparatus.

Materials

Wild-type coelenterazine, coelenterazine n and TMRE were obtained from Molecular Probes, OR, U.S.A. SB202190 (4-(4-fluorophenyl)-1-(4-piperidinyl)-5-(2-amino-4-pyrimidinyl)-imidazole), 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) and tamoxifen were from Tocris, Bristol, U.K. 17-β-estradiol, 17-α-estradiol, diethylstilbestrol (DES) and 4-hydroxy-tamoxifen were from Sigma, Madrid. Other reagents were from Sigma, Madrid or Merck, Darmstadt.

Statistical analysis

Averaged data are means$\pm$s.e.m. Analysis of variance (ANOVA) was applied to see differences between groups. The level of significance is indicated as $*P<0.05$; $**P<0.005$; $***P<0.0005$. IC_{50} values were calculated using the four parameter ($logEC_{50}$) fitting routine of the BioDataFit program (Chang Bioscience, CA, U.S.A.).

Results

We have measured the rate of Ca^{2+} uptake into mitochondria using permeabilized HeLa cells expressing aequorin targeted to the mitochondria. After permeabilization with digitonin, the mitochondrial network becomes directly exposed to the perfusion medium and the rate of Ca^{2+} entry into mitochondria can be directly measured in the presence of controlled $[Ca^{2+}]$ in the perfusion buffer. Figure 1 shows the effect of different concentrations of 17-β-estradiol, DES, PPT and DPN on the Ca^{2+} uptake into mitochondria induced by perfusion of a 3.5 μM $[Ca^{2+}]$ buffer. All of them activated mitochondrial Ca^{2+} uptake, the stronger effect being that obtained with PPT. This specific α-ER agonist activated mitochondrial Ca^{2+} uptake at submicromolar concentrations and produced a maximum effect of above 20-fold stimulation at 5–10 μM (see Figure 2). 17-β-estradiol, DES and DPN also activated mitochondrial Ca^{2+} uptake, although at higher concentrations

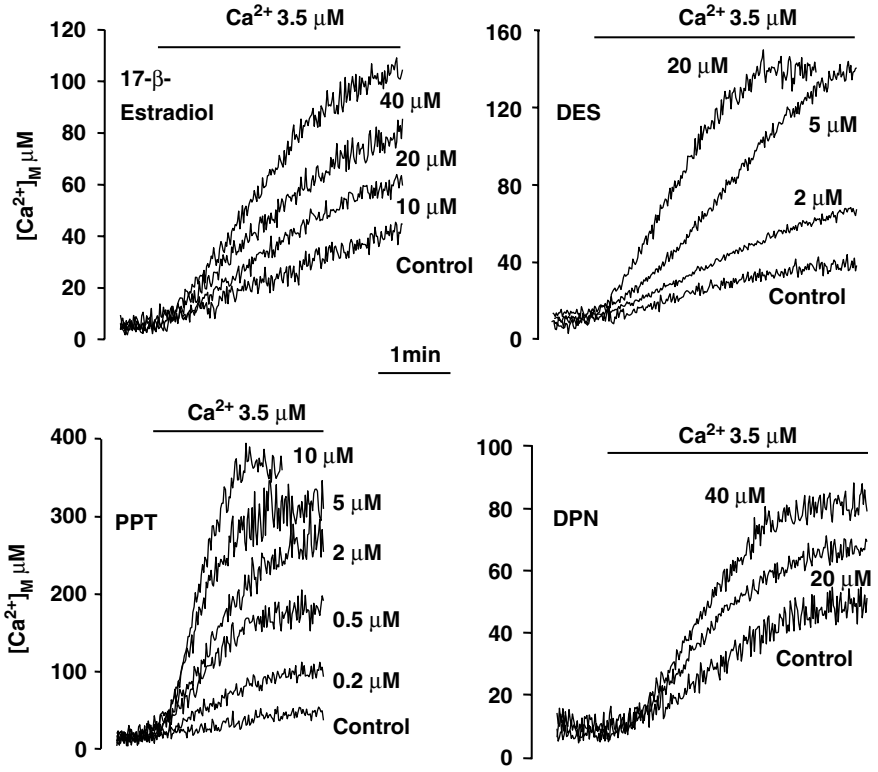

Figure 1 Effects of 17-β-estradiol, diethylstilbestrol (DES), PPT and DPN on mitochondrial Ca^{2+} uptake. MM5 cells expressing mutated mitochondrially targeted aequorin were reconstituted with coelenterazine n. Then they were permeabilized as described in Methods and a Ca^{2+} buffer containing 3.5 μM $[Ca^{2+}]$ was perfused as indicated in the figure either in the absence (control) or in the presence of different concentrations of each compound (as indicated in μM). Experiments are representative of 4–6 similar ones of each kind.

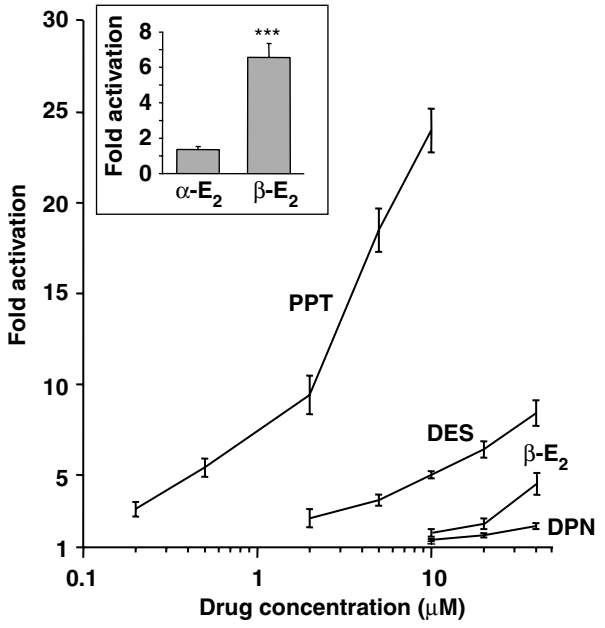

Figure 2 Dose-dependence of the activation of mitochondrial Ca^{2+} uptake by 17-β-estradiol, diethylstilbestrol (DES), PPT and DPN. The figure shows mean$\pm$s.e. data from 4 to 6 experiments of each kind similar to those shown in Figure 1. Activation of mitochondrial Ca^{2+} uptake is expressed as fold-activation with respect to the controls. The inset shows mean$\pm$s.e. data from four experiments of each kind carried out in parallel comparing the effects of 40 μM 17-α-estradiol (α-E_2) or 40 μM 17-β-estradiol (β-E_2) on mitochondrial Ca^{2+} uptake. Significance refers to the comparison of the mitochondrial Ca^{2+} uptake in the presence of 17-α-estradiol or 17-β-estradiol with that of the controls.

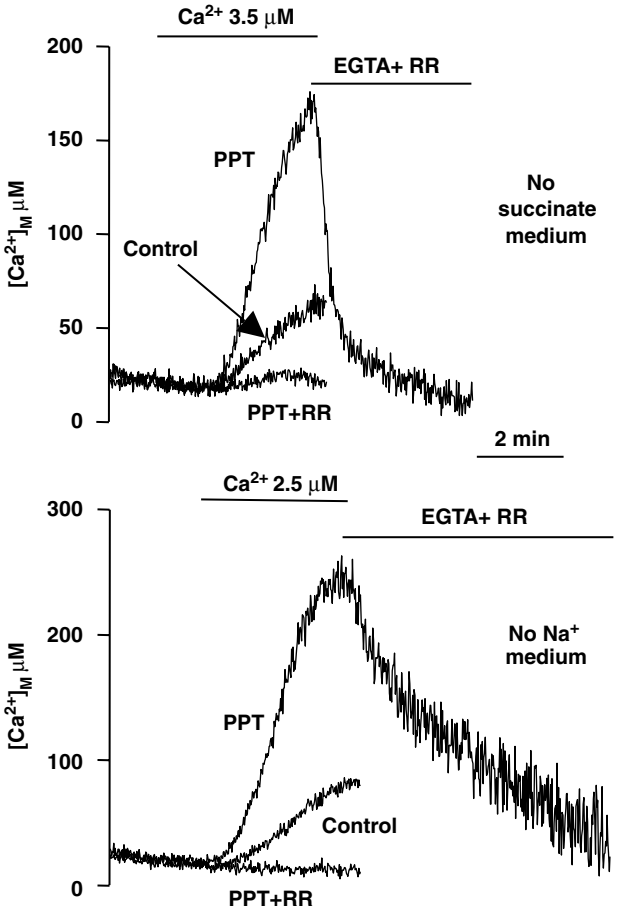

Figure 3 Effects of PPT on mitochondrial Ca^{2+} uptake in succinate-free and Na^+-free mediums. MM5 cells expressing mutated mitochondrially targeted aequorin were reconstituted with coelenterazine n. Then they were permeabilized as described in Methods either using a succinate-free (upper panel) or a Na^+-free (lower panel) intracellular perfusion medium. Then, Ca^{2+} buffers prepared in the same mediums and containing either 3.5 or 2.5 μM [Ca^{2+}] were perfused as indicated in the figure. In the traces labeled 'PPT', 2 μM PPT was added together with the [Ca^{2+}] buffer. In the traces labeled 'PPT + RR', 1 μM ruthenium red was added 1 min prior and 2 μM PPT + 1 μM ruthenium red were added together with the Ca^{2+} buffer. 'EGTA + RR' stands for perfusion of medium containing 0.5 mM EGTA and 1 μM ruthenium red. Experiments are representative of three similar ones of each kind.

and at a smaller degree. However, in spite of the high concentrations of 17-β-estradiol required, its effect was specific in the sense that it could not be mimicked by 17-α-estradiol. The inset of Figure 2 shows that 40 μM 17-β-estradiol increased mitochondrial Ca^{2+} uptake by about six-fold while the same concentration of 17-α-estradiol produced no significant effect.

The effect of these compounds developed with no measurable delay. As described in Methods, cells were permeabilized in the absence of Ca^{2+} and then perfused with medium without Ca^{2+} for 5–10 min prior to the addition of the [Ca^{2+}] buffer. The compounds were added at the same time as the [Ca^{2+}] buffer, and no further effect was obtained by previous incubation with the drug. PPT (2 μM) activated Ca^{2+} uptake by 9.4$\pm$1.1 fold (mean$\pm$s.e., $n=6$) without preincubation and by 9.0$\pm$0.8 (mean$\pm$s.e., $n=3$) when PPT was added 5 min before perfusion of the [Ca^{2+}] buffer. Figure 2 shows the dose–response curves for these compounds. It can be seen that the effect of PPT was still not saturated at 10 μM, a concentration that stimulated mitochondrial Ca^{2+} uptake by nearly 25-fold. The concentration of PPT required for full saturation was difficult to measure accurately because of the large rates of Ca^{2+} accumulation into mitochondria obtained.

To confirm that the Ca^{2+} uptake into mitochondria activated by PPT takes place through the uniporter, we have performed several control experiments. First, we have tested if the activation could be dependent on the type of mitochondrial substrate used. For this, we have taken advantage of the fact that the mitochondrial membrane potential can be created in permeabilized cells *via* reversion of the ATP synthase in ATP-containing perfusion medium. In Figure 3, upper panel, we show that PPT still activated mitochondrial Ca^{2+} uptake when a perfusion medium lacking succinate was used. In addition, this activated Ca^{2+} uptake was fully blocked by 1 μM ruthenium red. On the second place, we wanted to exclude also the possibility that the effect of PPT could be somehow mediated by the mitochondrial Na^+/Ca^{2+} exchanger. This transport system is the main responsible of the fast decrease in $[Ca^{2+}]_M$ that can be observed in Figure 3, upper panel, when we block Ca^{2+} entry using EGTA and ruthenium red (EGTA + RR). Figure 3, lower panel, shows similar experiments performed in Na^+-free medium. Owing to the inhibition of this Ca^{2+}-extrusion mechanism, we had to decrease the [Ca^{2+}] in the perfusion buffer from 3.5 to 2.5 μM to obtain comparable uptake rates. The degree of inhibition of mitochondrial Na^+/Ca^{2+} exchange can be appreciated from

the inhibition of the rate of $[Ca^{2+}]_M$ decrease after Ca^{2+} entry block with EGTA and ruthenium red (compare with the upper panel). However, in spite of the large inhibition of this transport system, PPT still activated mitochondrial Ca^{2+} uptake to the same extent, confirming that the effect of PPT is not mediated by the mitochondrial Na^+/Ca^{2+} exchange.

The specific α-ER agonist PPT potently activated also mitochondrial Ca^{2+} uptake in intact cells. In the upper panel of Figure 4, we show that PPT largely increased the histamine-induced $[Ca^{2+}]_M$ peak in the same concentration range that it activated $[Ca^{2+}]_M$ uptake in permeabilized cells (see Figure 1). The inset shows normalized mean data of 4–6 similar experiments performed at each PPT concentration. Even a PPT concentration as low as 0.2 μM was able to double (2.0 ± 0.19 fold the control values, mean ± s.e., $n = 4$) the histamine-induced $[Ca^{2+}]_M$ peak. In the lower panel of Figure 4 we show the effects of this compound on the histamine-induced mitochondrial and $[Ca^{2+}]_c$ peaks. The data shown correspond to the average of four different experiments of each kind performed in HeLa cells transiently transfected with either mitochondrially-targeted or cytosolic aequorin. The left panel shows that PPT largely increased the height of the $[Ca^{2+}]_M$ peak (from 16.4 ± 3.4 to 58.4 ± 4.3 μM, mean ± s.e., $n = 4$, $P < 0.0005$). This effect was clearly not due to any increase in the $[Ca^{2+}]_c$ peak, which was in fact reduced in the presence of PPT (from 0.92 ± 0.05 to 0.73 ± 0.04, mean ± s.e., $n = 4$, $P < 0.05$, right panel). Instead, the observed decrease in the $[Ca^{2+}]_c$ peak is most probably a consequence of the larger Ca^{2+} uptake by mitochondria, which is the primary effect of PPT.

If an ER is involved in the regulation of mitochondrial Ca^{2+} uptake, we thought that further information on the nature of such receptor could be obtained by studying the effects of selective ER modulators such as tamoxifen, 4-hydroxy-tamoxifen and raloxifene, as well as that of the pure ER antagonist ICI 182,780. Figure 5 shows that both tamoxifen and 4-hydroxy-tamoxifen inhibited mitochondrial Ca^{2+} uptake with similar affinity. IC_{50} obtained from a four parameter fitting of the data were (mean ± s.d.) 2.5 ± 1.5 μM for tamoxifen and 2.5 ± 1.3 μM for 4-hydroxy-tamoxifen. To investigate if this inhibitory effect took place *via* the same modulatory mechanism as that activated by PPT or SB202190, we tested the effects of tamoxifen on the mitochondrial Ca^{2+} uptake induced by PPT and SB202190. Figure 6 shows that tamoxifen reverted also the activation of Ca^{2+} uptake into mitochondria induced by these compounds. As we show in this figure, the inhibitory effect of tamoxifen required preincubation for at least 5 min to reach maximum potency, in contrast with the effect of PPT that did not require any preincubation (see above).

Contrarily to the effects of tamoxifen and 4-hydroxy-tamoxifen, no significant modulation of mitochondrial Ca^{2+} uptake was observed in the presence of raloxifene or ICI 182,780. The rate of mitochondrial Ca^{2+} uptake in the

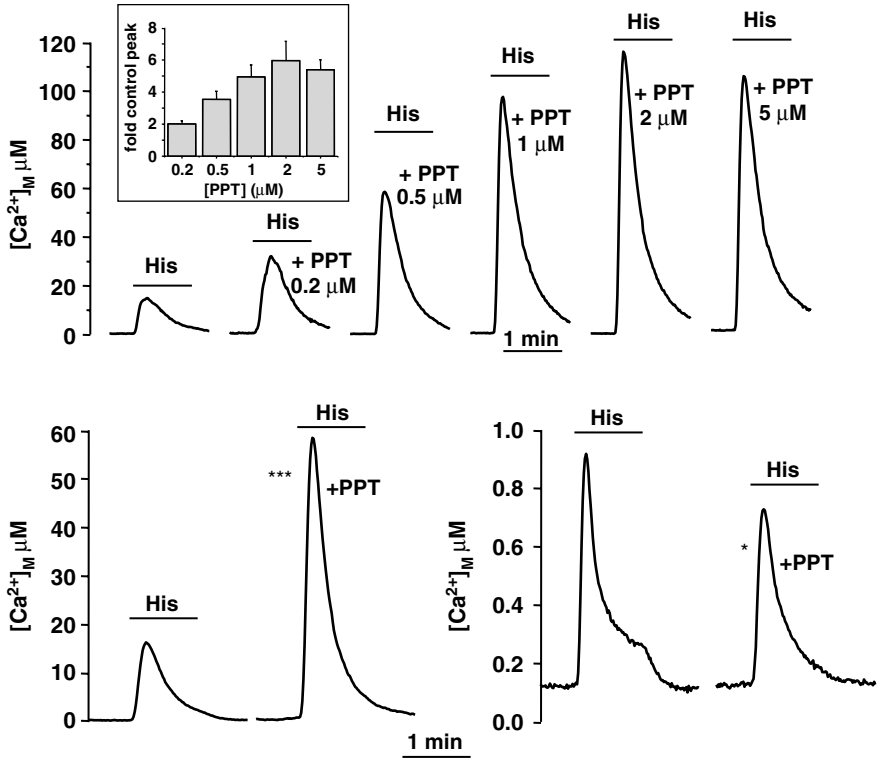

Figure 4 Effects of PPT on the $[Ca^{2+}]_M$ and $[Ca^{2+}]_c$ peaks induced by histamine. Upper panel: MM5 cells expressing mutated mitochondrially targeted aequorin were reconstituted with native coelenterazine and stimulated with 100 μM histamine in the presence or in the absence of several concentrations of PPT, as indicated. PPT was added in each case 5 min prior to the addition of histamine. The inset shows normalized (control = 1) mean ± s.e. data of 4–6 similar experiments performed at each PPT concentration. Lower panel: wild-type HeLa cells transfected with plasmids for either mutated mitochondrially targeted aequorin (left panels) or cytosolic aequorin (right panels) were reconstituted with native coelenterazine. Then, they were stimulated with 100 μM histamine either in control cells or in cells incubated with 5 μM PPT for 5 min prior and during stimulation (+PPT). Experiments shown are the mean of four different experiments.

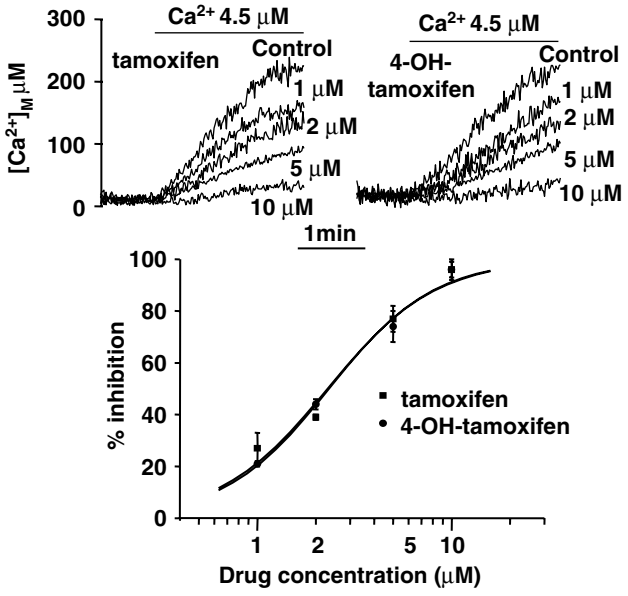

Figure 5 Effects of tamoxifen and 4-hydroxy-tamoxifen on mitochondrial Ca^{2+} uptake. MM5 cells expressing mutated mitochondrially targeted aequorin were reconstituted with coelenterazine n. Then, they were permeabilized and a Ca^{2+} buffer containing 4.5 μM [Ca^{2+}] was perfused as indicated in the figure either in the absence (control) or in the presence of different concentrations of tamoxifen (left panel, in μM) or 4-hydroxy-tamoxifen (right panel, in μM). Both tamoxifen and 4-hydroxy-tamoxifen were present in the intracellular perfusion medium 5 min prior and during perfusion of the Ca^{2+} buffer. Experiments are representative of 3–5 similar ones of each kind.

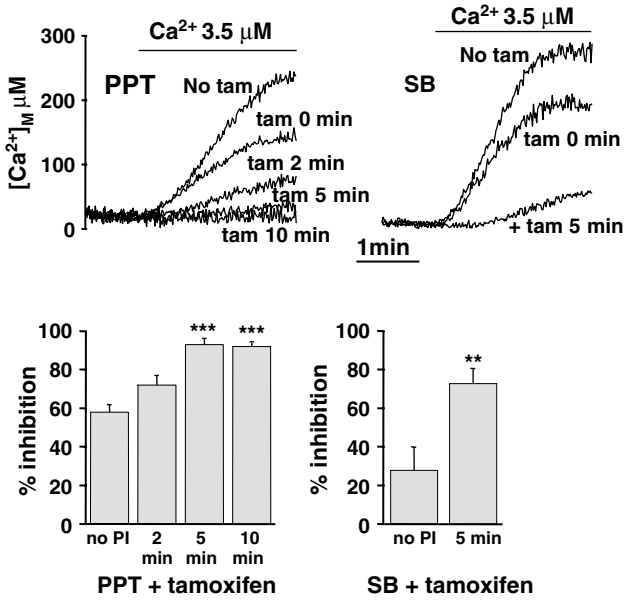

Figure 6 Effect of tamoxifen on the mitochondrial Ca^{2+} uptake activated by PPT of SB202190. MM5 cells expressing mutated mitochondrially targeted aequorin were reconstituted with coelenterazine n. Then, they were permeabilized and a Ca^{2+} buffer containing 3.5 μM [Ca^{2+}] was perfused as indicated in the figure either in the presence of 5 μM PPT (left panels) or 10 μM SB202190 (right panels). Except for the upper traces (no tam), 10 μM tamoxifen (tam) was added to the Ca^{2+} buffer and also preincubated for 0, 2, 5 or 10 min (left panels) or for 0 and 5 min (right panels) prior to the addition of the Ca^{2+} buffer, as indicated. The lower panels show statistical data of the percentage of inhibition of mitochondrial Ca^{2+} uptake obtained from 4–5 experiments of each kind similar to those of the upper panels. Significance refers to the comparison of the effects of tamoxifen after preincubation for different periods (2, 5 or 10 min) with respect to its effects in the absence of preincubation (no PI).

presence of 10 μM raloxifene or 10 μM ICI 182,780 (5 min preincubation in both cases) was 113±4% (mean±s.e., $n=4$) and 102±11% (mean±s.e., $n=4$) that of the control, respectively. ICI 182,780 did not modify either the activation of mitochondrial Ca^{2+} uptake by PPT (data not shown).

The inhibition of mitochondrial Ca^{2+} uptake by tamoxifen could be attributed to mitochondrial membrane depolarization. To exclude this possibility, we have measured the effect of tamoxifen on mitochondrial membrane potential both in intact and permeabilized cells using the mitochondrial potential sensitive dye TMRE. For these measurements, we have perfused permeabilized cells with intracellular medium containing this cationic dye, which accumulates into mitochondria thanks to the negative potential of the matrix. TMRE is often used at high concentrations in self-quenching mode so that depolarization induces an increase in mitochondrial fluorescence. Instead, we have preferred to use here very low concentrations of TMRE in the perfusion medium (20 nM), so that the shortcomings of using the dye in quenching mode are avoided (O'Reilly *et al*., 2003). Similarly, we have used a very short loading protocol in intact cells (1 min with 1 μM TMRE) to obtain conditions in which depolarization induces only decrease in TMRE fluorescence. Under these conditions, Figure 7 shows that addition of an uncoupler such as the protonophore FCCP induced a fast and complete decrease in TMRE fluorescence both in intact and in permeabilized cells. On the contrary, addition of 10 μM tamoxifen induced little changes in TMRE fluorescence either in intact or in permeabilized cells, indicating that this compound does not significantly modify mitochondrial membrane potential.

The large activation of mitochondrial Ca^{2+} uptake by PPT is more difficult to explain in terms of changes in mitochondrial membrane potential (e.g. a large mitochondrial hyperpolarization resulting in activation of Ca^{2+} influx though the channel). Anyway, we decided to test also the effect of PPT on mitochondrial membrane potential. Figure 7 shows that 2 μM PPT did not produce significant changes in mitochondrial membrane potential in intact cells, but induced a slow mitochondrial depolarization in permeabilized cells. When the PPT concentration was increased to 5 μM, the depolarizing effect in permeabilized cells was larger and some mitochondrial depolarization was also apparent in intact cells. This depolarizing effect of PPT cannot explain and is probably unrelated to the activation of mitochondrial Ca^{2+} uptake, which appears at lower concentrations, particularly in intact cells (see Figures 1 and 4).

The human cervical carcinoma HeLa cells are ER negative, because they do not express significant amounts of ERs α or β (Zhang *et al*., 1999; Brouillet *et al*., 2001). We then decided to study mitochondrial Ca^{2+} uptake in an ER positive cell line, such as the MCF-7 breast cancer cell line. Figure 8 shows that both PPT and SB202190 strongly activated also mitochondrial Ca^{2+} uptake in MCF-7 cells. In addition, this activation was sensitive to tamoxifen in the micromolar range. Therefore, the modulation of mitochondrial Ca^{2+} uptake by these com-

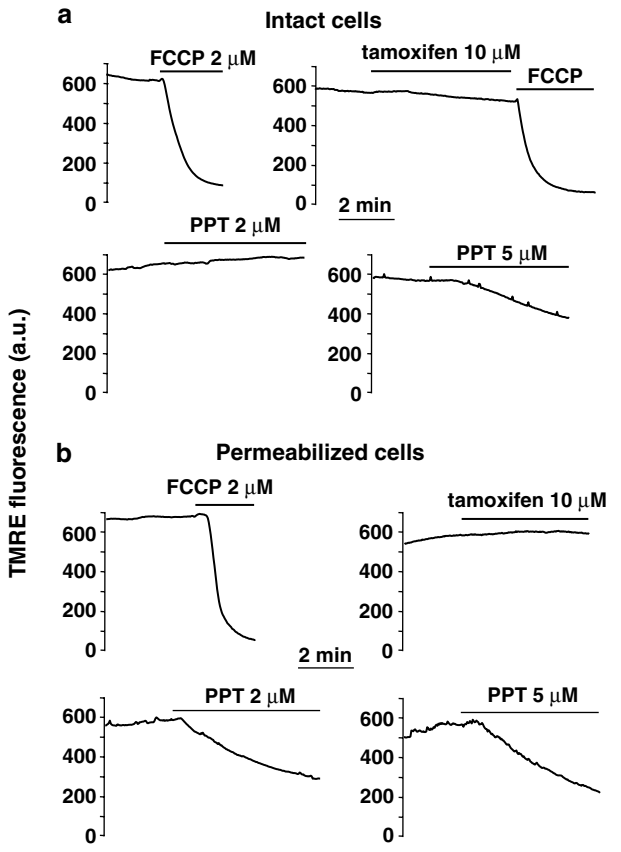

Figure 7 Effects of tamoxifen and PPT on mitochondrial membrane potential both in intact and permeabilized HeLa cells. In panel a, intact HeLa cells were loaded with TMRE (1 μM, 1 min) and then stimulated with either 2 μM FCCP, 10 μM tamoxifen or 2–5 μM PPT, as indicated. In panel b, cells were permeabilized as described in Methods and then 20 nM TMRE was perfused until a steady-state fluorescence was reached. Then, either 2 μM FCCP, 10 μM tamoxifen or 2–5 μM PPT were added as indicated in the same intracellular medium containing 20 nM TMRE. The traces shown are the mean response of all the cells present in the microscope field in each experiment (6–21), and are representative of 3–5 different experiments of each kind with a total of 30–91 cells analyzed with each protocol.

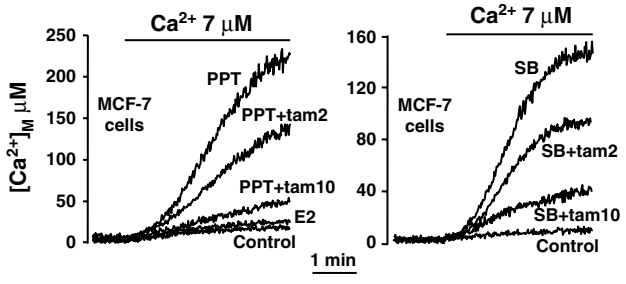

Figure 8 Effects of PPT, SB202190 and tamoxifen on mitochondrial Ca^{2+} uptake in MCF-7 cells. MCF-7 cells expressing mutated mitochondrially targeted aequorin were reconstituted with coelenterazine n. Then, they were permeabilized and a Ca^{2+} buffer containing 7 μM [Ca^{2+}] was perfused either in the presence or in the absence of the following compounds (as indicated in the figure): PPT 5 μM (PPT), tamoxifen 2 μM (tam2) or 10 μM (tam10), SB202190 (SB) 10 μM, 17-β-estradiol (E2) 1 μM. Experiments are representative of three similar ones of each kind.

pounds occurred similarly in both HeLa and MCF-7 cells. Finally, we tested also if the presence of ERs in MCF-7 cells increased the sensitivity of the activation mechanism to the natural agonist, 17-β-estradiol. This was not the case. Figure 8 shows that 1 μM 17-β-estradiol, a concentration well above the physiological values, produced little stimulation of mitochondrial Ca^{2+} uptake in MCF-7 cells.

Discussion

We show in this paper that several natural and synthetic ligands of ERs modulate the activity of the mitochondrial Ca^{2+} uniporter, the main pathway for Ca^{2+} entry into the mitochondria. Agonists of ERs such as PPT, DES, DPN and 17-β-estradiol at pharmacological concentrations activated the uniporter, while antagonists/partial agonists such as tamoxifen or 4-hydroxy-tamoxifen inhibited its activity. Activation was immediate and did not require any preincubation, while inhibition reached maximum potency after 5 min. In any case, the fast development of both effects indicates that they are nongenomic ones in nature. In addition, both effects developed in permeabilized cells, after full washing of the cytoplasmic compartment. Thus, they are most probably mediated by some kind of ER located either in the mitochondria or closely associated to this organelle.

The classic pathway for estrogen action occurs in the nucleus, where ERs bind to estrogen-responsive elements in DNA to activate transcription of a series of target genes (Beato & Klug, 2000). This pathway requires long times for full activation, typically more than 1 h. In contrast, a large number of effects of estrogen agonists have been reported that occur with very short time lags (for reviews, see Falkenstein *et al*., 2000; Nadal *et al*., 2001; Levin, 2002; Lösel *et al*., 2003). The mechanism/s of these nongenomic actions of estrogens are a source of controversy regarding the existence and identity of the receptors that mediate these responses. Several hypothesis have been proposed, including the presence in the plasma membrane of either classic α- or β-ERs, novel membrane esteroid receptors or other unrelated proteins bearing estrogen sensitivity, such as the β-subunit of the maxi-K potassium channel (Valverde *et al*., 1999) and the γ-adrenergic receptor, which mediates the closing of ATP-dependent potassium channels in pancreatic β-cells (Nadal *et al*., 2000). Consistent with these hypothesis are several reports that describe the presence of ERs in the plasma membrane (Collins & Webb, 1999; Li *et al*., 2003; Razandi *et al*., 2004; Song *et al*., 2004) and even more intriguing is the fact that classic α- and β-ERs have been detected in mitochondria (Chen *et al*., 2004; Yang *et al*., 2004), suggesting that estrogens can directly affect also mitochondrial function through a non-nuclear pathway.

Similar to the effect of estrogens on the mitochondrial Ca^{2+} uniporter described in this paper, many of the nongenomic effects of estrogens on ion channels previously described occur only at supraphysiological concentrations (Zhang *et al*., 1994; Nakajima *et al*., 1995; Yamamoto, 1995; Kitazawa *et al*., 1997; Cario-Toumaniantz *et al*., 1998; Ruehlmann *et al*., 1998; Valverde *et al*., 1999; Kim *et al*., 2000). This obviously raises questions on the physiological significance of these observations and on the possible presence of non-specific effects of high steroid concentrations, such as changes in membrane fluidity or antioxidant activities. However, high local concen-

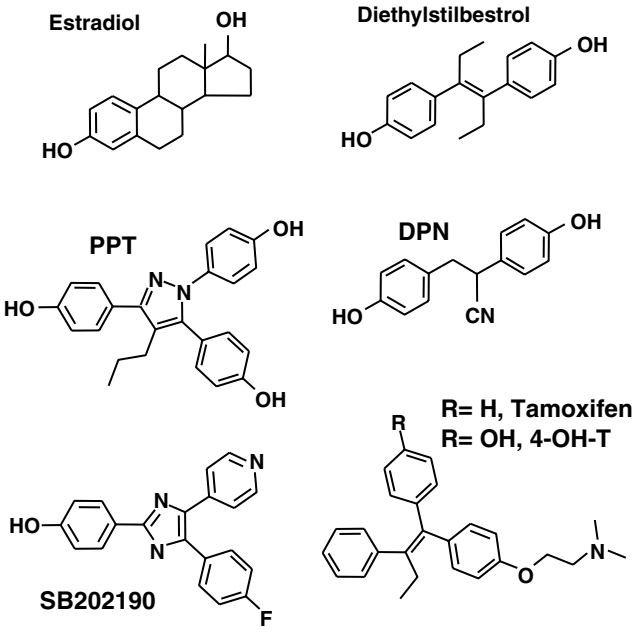

Figure 9 Chemical structures of the compounds used in this study.

trations of estrogens may be reached close to tissues producing 17-β-estradiol or in certain areas of the brain, and the hidrophobic nature of these compounds may contribute to their enrichment in the membrane region. Regarding the specificity of the effect, we have shown before the presence of large differences in activity in a series of closely related flavonoids (Montero *et al*., 2004), pointing to a defined structure–activity relationship. The lack of effect of 17-α-estradiol demonstrates also that the effect is not due to unspecific changes in membrane fluidity. Similarly, the activation of mitochondrial Ca^{2+} uptake induced by the p38 mitogen activated protein kinase inhibitor SB202190 may be attributed to its structural similarity to PPT (Figure 9). In addition, classical antioxidants like tocopherol, lipoic acid, ascorbic acid or *N*-acethyl-cysteine produced no effect on mitochondrial Ca^{2+} uptake (Montero *et al*., 2004). Finally, we show here that PPT was highly active even at submicromolar concentrations (it increased mitochondrial Ca^{2+} uptake by more than five-fold at 0.5 μM, see Figure 2), and its effect was reverted by antagonists of ERs such as tamoxifen or 4-hydroxy-tamoxifen. In conclusion, our data point to a specific interaction of these compounds with a protein receptor probably located in mitochondria, which controls mitochondrial Ca^{2+} uptake.

It has been previously reported that DES inhibits mitochondrial ATP synthase with an IC50 of 10 μM, and that under some circumstances it may behave as an apparent uncoupler acting through mechanisms different from other traditional uncouplers (McEnery & Pedersen, 1986; McEnery *et al*., 1989). More recently, it has also been shown that mitochondrial ATP synthase binds estradiol conjugated to bovine serum albumin (Zheng & Ramirez, 1999a) and that several estrogens and polyphenolic phytochemicals inhibit mitochondrial ATP synthase activity (Zheng & Ramirez, 1999b; 2000). However, the structure–activity relationship for the effect of the flavonoids was quite different to that found by us for the activation of mitochondrial Ca^{2+} uptake (Montero *et al*., 2004), the IC_{50} for estradiol was above 100 μM and no selectivity between 17-α- and 17-β-estradiol was found. Therefore, the estrogen target responsible for the activation of mitochondrial Ca^{2+} uptake is probably different from that responsible of ATP synthase inhibition. Nevertheless, that effect of estrogens on the ATP synthase could perhaps explain the mitochondrial depolarization induced by PPT, an effect that is probably unrelated to the activation of mitochondrial Ca^{2+} uptake and appears at higher PPT concentrations.

The new mechanism of action of estrogen agonists shown here could provide a novel interpretation for several previously reported effects of these compounds. For example, the concentrations of PPT, 17-β-estradiol and DPN required to induce activation of mitochondrial Ca^{2+} uptake are very similar to those needed to induce vasodilatory response in isolated small arteries (Montgomery *et al*., 2003). This vasodilatory response is known to be accompanied by a decrease in smooth muscle intracellular free Ca^{2+} (Han *et al*., 1995). That decrease is thought to be due to inhibition of L-type Ca^{2+} channels (Nakajima *et al*., 1995; Kitazawa *et al*., 1997), but stimulation of mitochondrial Ca^{2+} uptake could also play a role in that effect. Consistently, the vasodilatory effect of several flavonoids on rat aortic smooth muscle (Duarte *et al*., 1993) has the same structure–activity relationship as that described by us for the activation of mitochondrial Ca^{2+} uptake (Montero *et al*., 2004). Pharmacological concentrations of 17-β-estradiol have been also shown to inhibit catecholamine secretion in PC12 cells by reducing the depolarization-induced $[Ca^{2+}]_c$ rise (Kim *et al*., 2000). We have shown before that inhibition of mitochondrial Ca^{2+} uptake strongly potentiates depolarization-induced catecholamine secretion by increasing the cytosolic Ca^{2+} transient (Montero *et al*., 2000). Thus, activation of mitochondrial Ca^{2+} uptake by 17-β-estradiol may contribute to reduce catecholamine secretion. Finally, the larger cytosolic Ca^{2+} buffering effect of mitochondria in the presence of estrogen agonists may contribute to the neuroprotective effect of high concentrations of 17-β-estradiol or polycyclic phenols (Dykens *et al*., 2003).

In conclusion, our results point to the presence of a specific receptor in the mitochondrial membrane that mediates the activation/inhibition of the mitochondrial Ca^{2+} uniporter by estrogen agonists and antagonists at pharmacological concentrations. The nature of that receptor is unknown, but the lack of effect of ICI182,780 and the high concentrations of 17-β-estradiol required for the interaction suggest that it is not one of the classical ERs. Consistent with this view is also the similar activation of mitochondrial Ca^{2+} uptake observed in both ER-negative and ER-positive cell lines HeLa and MCF-7, respectively. Alternative possibilities include the involvement of proteins unrelated to ERs, but with estrogen sensitivity or the recently described estrogen-related receptors, a family of orphan receptors hardly sensitive to 17-β-estradiol but modulated by a series of ER agonists and antagonists (Horard & Vanacker, 2003).

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