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Effects of anti-oestrogens and β-estradiol on calcium uptake by cardiac sarcoplasmic reticulum

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1 Tamoxifen and a group of structurally similar non-steroidal, triphenolic compounds inhibit the oestrogen receptor. In addition to this action, these anti-oestrogens are known to inhibit some types of plasma membrane ion channels and other proteins through mechanisms that do not appear to involve their interactions with the estrogen receptor but could be the result of their effect on membrane lipid structure or fluidity.

2 We studied the effects of β-estradiol and three anti-oestrogens (tamoxifen, 4-hydroxytamoxifen and clomiphene) on Ca^{2+} uptake into sarcoplasmic reticulum (SR) vesicles isolated from canine cardiac ventricular tissue.

3 The antiestrogens all inhibit SR Ca^{2+} uptake in a concentration-dependent manner (order of potency: tamoxifen > 4-hydroxytamoxifen ⩾ clomiphene). Although these compounds rapidly inhibit net Ca^{2+} uptake they do not have a similar rapid effect on the ATPase activity of the SR Ca pump. β-estradiol has no effect on Ca^{2+} uptake nor does it alter the inhibitory action of tamoxifen on the SR.

4 The differences in the effects of β-estradiol and the anti-oestrogens on cardiac SR Ca^{2+} uptake do not correlate with differences in the ways in which these compounds have been reported to interact with membrane lipids. Our results are consistent, however, with direct effects on a membrane protein (possibly an SR Cl^- or K^+ channel).
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Abbreviations: $[Ca^{2+}]_{50\%}$, concentration of Ca^{2+} at half-maximal velocity; $[Ca^{2+}]_{free}$, concentration of free Ca^{2+}; $[Ca^{2+}]_{total}$, total Ca^{2+} concentration; n_H, Hill coefficient; PKC, protein kinase C; SR, sarcoplasmic reticulum; V_{max}, maximum velocity of Ca^{2+} uptake

Introduction

Tamoxifen, its metabolite, 4-hydroxytamoxifen, and clomiphene are non-steroidal, triphenolic compounds that function as mixed oestrogen receptor agonists/antagonists (Jordan, 1984; Osborne, 1998; Ruenitz *et al*., 1983). The antagonistic effects of these drugs on the oestrogen receptor are believed to be responsible for their ability to inhibit tumour growth in breast tissue and has led to the use of tamoxifen in the treatment of breast cancer (Jordan, 1984; Osborne, 1998). It has become clear, however, that oestrogens and anti-oestrogen compounds can affect cellular function through mechanisms that are independent of their interaction with oestrogen receptors (Jordan, 1984; Love *et al*., 1991; Osborne, 1998; Song *et al*., 1996; Trump *et al*., 1992; Zhang *et al*., 1995). Such actions may be responsible for some of the side-effects associated with their use. Tamoxifen therapy has been associated, for example, with the development of cardiac abnormalities (Trump *et al*., 1992) and with an increased risk of cataract development in the lens of the eye (Song *et al*., 1996; Zhang *et al*., 1995). The latter effect appears to be mediated by the tamoxifen-dependent inhibition of a Cl^- channel (Zhang *et al*., 1994; 1995). Although the mechanisms by which tamoxifen affects the heart are not well understood, it has been suggested that they could involve a tamoxifen-dependent inhibition of protein kinase C or the inhibition of calcium channels (Trump *et al*., 1992). The inhibitory action of tamoxifen on protein kinase C (PKC) may involve an alteration of the interaction of PKC and membrane phospholipids (O'Brian *et al*., 1985). Tamoxifen has also been shown to block volume-regulated Cl^- currents and delayed-rectifier K^+ currents in cardiac myocytes (Duan *et al*., 1997; Liu *et al*., 1998), gap junctional communication in cultured neonatal cardiac myocytes (Verrecchia & Hervé, 1997a,b) and Ca^{2+} uptake into the cardiac sarcoplasmic reticulum (SR) (Kargacin *et al*., 2000). In the latter study, we showed that tamoxifen inhibits cardiac SR Ca^{2+} uptake through a mechanism that does not involve a direct action on the SR Ca^{2+} pump or a significant increase in the Ca^{2+} permeability of the SR membrane. These results led us to conclude that the most likely means by which tamoxifen inhibits SR uptake is by inhibiting the movement of ions across the SR membrane through Cl^- and/or K^+ channels. This ion flux is believed to be required to balance the net positive charge moved into the SR by the SR Ca-ATPase (reviewed in Kargacin *et al*., 2000; Tada & Kadoma, 1995).

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In addition to its effects on cardiac muscle and lens cells, tamoxifen has also been found to block some plasma membrane Cl^-, K^+ and Ca^{2+} channels (Dick *et al*., 1999; Hardy & Valverde, 1994; Morley & Whitfield, 1994; Song *et al*., 1996; Szücs *et al*., 1996; Valverde *et al*., 1995; Vandenberg *et al*., 1994; Voets, 1995; Waldegger *et al*., 1996) and to inhibit cyclic AMP phosphodiesterase activity and other calmodulin-dependent enzymes through mechanisms that may involve an effect on calmodulin or calmodulin binding sites (Fanidi *et al*., 1989; Lopes *et al*., 1990). Although the effects of 4-hydroxytamoxifen and clomiphene on cellular function have not been as extensively studied, 4-hydroxytamoxifen has been reported to block volume-regulated Cl^- channels (Zhang *et al*., 1994) and clomiphene has been reported to block gap junctional communication in cultured rat cardiomyocytes (Verrecchia & Hervé, 1997a,b). Clomiphene also inhibits nitrendipine binding to dihydropyridine-sensitive Ca^{2+} channels in urinary bladder and myometrial tissue (Batra, 1990).

The mechanisms by which oestrogens and anti-oestrogens affect cell protein function independent of their binding to oestrogen receptors are not well understood. Because these drugs are lipophilic, it has been suggested that they could alter the interaction of membrane lipids and proteins by changing the fluidity or structure of lipid membranes (see Custódio *et al*., 1993a,b; 1996; O'Brian *et al*., 1985; Song *et al*., 1996; Verrecchia & Hervé, 1997a,b). It is also possible that they could interact directly with proteins (see Custódio *et al*., 1996; Hardy & Valverde, 1994; Lopes *et al*., 1990; Song *et al*., 1996; Waldegger *et al*., 1996). In our experiments with cardiac SR (Kargacin *et al*., 2000), we did not determine if the inhibition of SR Ca^{2+} uptake by tamoxifen was due to a direct effect on an SR protein or was mediated indirectly through an interaction of tamoxifen with the SR membrane lipid. Evidence for or against a lipid-dependent mechanism of action might be obtained by comparing the effects of similar compounds with those of tamoxifen. 4-hydroxytamoxifen has a higher affinity for the oestrogen receptor than does tamoxifen and clomiphene a lower affinity (reviewed in Ruenitz *et al*., 1983). It has also been shown that 4-hydroxytamoxifen and tamoxifen partition differently into lipid bilayers (including SR membranes) and that they appear to associate with different regions of the bilayer (Custódio *et al*., 1991; 1993a,b). If tamoxifen alters the function of an SR membrane protein indirectly by affecting its lipid environment, then differences between the effects of tamoxifen on the SR and those of other oestrogens or anti-oestrogens would be expected to be correlated with their effects on lipid bilayers. In the work reported here, we examined the effects of tamoxifen, 4-hydroxytamoxifen, clomiphene and β-estradiol on Ca^{2+} uptake into canine cardiac SR vesicles and on the ATPase activity of the SR Ca^{2+} pump.

Methods

Materials

Aristar grade KCl, and sucrose were purchased from BDH Ltd. (Edmonton, Alberta, Canada). Analytical grade HEPES (K salt), oxalic acid, and $MgCl_2$ were purchased from Fluka (Ronkonkoma, NY, U.S.A.). K_2ATP, $CaCl_2$, creatine phosphate, creatine phosphokinase, NADH, pyruvate kinase, phosphoenolpyruvate, histidine, tamoxifen, 4-hydroxytamoxifen, clomiphene and β-estradiol were purchased from Sigma (St. Louis, MO, U.S.A.). Lactate dehydrogenase was purchased from Worthington (Freehold, NJ, U.S.A.). Fura-2 (free acid) and 4-BrA23187 were purchased from Molecular Probes (Eugene, OR, U.S.A.).

Preparation of membrane vesicles

SR vesicles were prepared from canine cardiac ventricular tissue as described by Chamberlain *et al*. (1983) except that the sucrose gradient centrifugation step was omitted. Prepared vesicles were stored at -80°C until used in a storage buffer containing 0.29 M sucrose, 0.65 M KCl, 10 mM histidine; pH 6.7.

Measurement of SR Ca^{2+} uptake

Ca^{2+} uptake into cardiac SR vesicles was measured with fura-2 as described previously (Kargacin *et al*., 1988; 1994; 1998a,b) using a SPEX CMX fluorimeter (Edison, NJ, U.S.A.) with excitation monochrometers set at 340 and 380 nm and the excitation wavelength alternated every 0.3 s. Fluorescence emission was measured at 510 nm; 340/380 ratios were determined every second. SR Ca^{2+} uptake was measured in uptake buffer containing (mM): KCl 100, oxalate 10, K-HEPES 20, K_2ATP 1, creatine phosphate 1, 1.6 units ml^{-1} creatine phosphokinase, and fura-2 free acid 2.9 μM; pH 7.0 (Kargacin *et al*., 1988; 1994; 1998a,b; 2000). Measurements were made in a 3 ml cuvette containing 2 ml of buffer. Tamoxifen, 4-hydroxytamoxifen, clomiphene and β-estradiol were dissolved and further diluted in 95% ethanol so that the same fluid volume (2 μl) was added to the cuvette for each experiment; 2 μl of 95% ethanol were added to the uptake buffer in control experiments (without the drugs). Before each experiment background fluorescence and light scatter at 510 nm were measured for 30 s in uptake buffer containing SR vesicles (25 μg total protein) and all chemical components except fura-2, ATP and the ATP regenerating system. Fura-2, ATP and the ATP regenerating system were added to the cuvette and allowed to equilibrate for 30 s prior to the addition of Ca^{2+} (from a 2.5 mM stock solution). The solutions in the cuvette were constantly stirred.

Calculations

The fura-2 340/380 fluorescence ratio (R) versus time curves, obtained experimentally, were corrected for background fluorescence and light scatter. Measurements of fluorescence spectra in the presence of the anti-oestrogens or β-estradiol showed that the drugs had no direct effects on fura-2 fluorescence.

The concentration of free Ca^{2+} ($[Ca^{2+}]_{free}$) and the total calcium concentration ($[Ca^{2+}]_{total}$) in the extravesicular solution in the cuvette were determined at each time point from the corrected 340/380 fluorescence measurements as described previously (Kargacin *et al*., 1988; 1994; 1998a,b; 2000). Calcium uptake velocity (μmol min^{-1} mg^{-1}) was calculated at each time point from the negative derivative

of the $[Ca^{2+}]_{total}$ versus time curves using the following equation:

$$\text{velocity} = A\frac{-d[Ca^{2+}]_{total}}{dt} \quad (1)$$

where A ($=v\ w^{-1}$) is the volume of solution in the cuvette divided by the amount of SR protein in the cuvette. The velocity values were then plotted against the corresponding $[Ca^{2+}]_{free}$ values. Maximum uptake velocity (V_{max}), $[Ca^{2+}]$ at half maximal velocity ($[Ca^{2+}]_{50\%}$) and the Hill coefficient (n_H) of Ca^{2+} uptake were determined from these plots by a fit to the Hill equation using a curve fitting program (Sigma Plot; Jandel Scientific, Corte Madera, CA, U.S.A.). V_{max} is dependent on the amount of Ca-ATPase relative to the total amount of protein present in a vesicle preparation and can therefore vary for different vesicle preparations. To compensate for these differences, V_{max} values are given as a percentage of the average V_{max} value for control experiments from the same vesicle preparation. This allows results from different vesicle preparations to be compared. In the control experiments reported here V_{max} values obtained from different vesicle preparations ranged from 0.426 to 0.715 μmol min^{-1} mg^{-1}.

Measurement of the ATPase activity of the SR Ca^{2+} pump

The ATPase activity of the cardiac SR Ca^{2+} pump was measured using an enzyme coupled assay as described previously (Kargacin *et al*., 2000; Karon *et al*., 1995). The assay couples ADP production by the SR Ca^{2+} pump to a change in NADH fluorescence through the following reactions:

$$\begin{aligned} &CaATPase + ATP \leftrightarrow CaATPase - P + ADP \\ &ADP + PEP^{\leftarrow PK\rightarrow} pyruvate + ATP \\ &NADH + pyruvate^{\leftarrow LDH\rightarrow} NAD^{+} + lactate \end{aligned} \quad (2)$$

where PEP is phosphoenolpyruvate, PK is pyruvate kinase and LDH is lactate dehydrogenase. The rate of decline in NADH fluorescence (excitation at 350 nm; emission at 490 nm) measures the rate of ATP hydrolysis by the SR Ca pump. For the assay, cardiac SR vesicles were added to a cuvette containing 2 ml of buffer (KCl 100 mM, $MgCl_2$ 4 mM, free-Ca^{2+} 3 μM, NADH 150 μM, PK 0.21 u ml^{-1}, PEP 460 μM, LDH 2.2 u ml^{-1}, 4-bromo A23187 3.3 μM, HEPES 20 mM; pH 7.0). Background fluorescence (measured in the presence of all components except NADH) and background changes in NADH fluorescence (measured in the presence of all components except ATP) were determined and used to correct the measurements of the ATPase activity of the SR Ca^{2+} pump. Calibration curves for converting fluorescence intensity to NADH concentration were obtained by adding known concentrations of NADH to uptake buffer and recording the resulting fluorescence intensity (350 nm excitation; 490 nm emission). In previous experiments (Kargacin *et al*., 2000), we showed that 80% of the ATPase activity in our cardiac SR vesicle preparations could be attributed to the SR Ca^{2+} pump. None of the drugs used in our experiments had a detectable direct effect on NADH fluorescence.

All experiments were performed at 22°C. The results are expressed as ± 1 s.e.mean.

Results

Inhibition of SR Ca^{2+} uptake by tamoxifen, 4-hydroxytamoxifen and clomiphene

In previous work (Kargacin *et al*., 2000), we showed that tamoxifen inhibited cardiac SR Ca^{2+} uptake in a concentration-dependent manner at concentrations between 0.5 and 12 μM with half maximal inhibition at approximately 5 μM. The highest concentrations of tamoxifen reduced SR Ca^{2+} uptake to approximately 5% of control. The inhibitory actions of tamoxifen of cardiac SR Ca^{2+} uptake were confirmed in the present experiments. Figure 1A,B shows 340/380 fluorescence ratios and $[Ca^{2+}]_{free}$ plotted as a function of time for a control experiment and an experiment done with 5 μM tamoxifen present in the uptake buffer. In this experiment, V_{max} was inhibited by 77%.

4-Hydroxytamoxifen is a tamoxifen metabolite that has an affinity for the oestrogen receptor that is more than 100 fold greater than that of tamoxifen (reviewed in Ruenitz *et al*., 1983). In the experiment shown in Figure 1C, 5 μM 4-hydroxytamoxifen inhibited cardiac SR Ca^{2+} uptake but to a lesser extent than an equivalent concentration of tamoxifen (47% inhibition for 4-hydroxytamoxifen versus 77% for tamoxifen).

Figure 2A,B shows the concentration dependence of the inhibitory effects of tamoxifen and 4-hydroxytamoxifen on the V_{max} of cardiac SR Ca^{2+} uptake. The tamoxifen (Figure 2A) result is very similar to that obtained previously (Kargacin *et al*., 2000). In these experiments half-maximal inhibition of uptake velocity was seen at approximately 2 μM tamoxifen. As suggested by the results in Figure 1, 4-hydroxytamoxifen proved to be less potent than tamoxifen as an inhibitor of SR Ca^{2+} uptake at concentrations between 2 and 10 μM (half maximal inhibition at approximately 7.5 μM) although, at the highest concentration used (25 μM), 4-hydroxytamoxifen inhibited uptake to the same extent as that seen at the highest concentration of tamoxifen (10 μM). V_{max} was $15.4 \pm 3.4\%$ ($n=5$) of control in the presence of 10 μM tamoxifen; V_{max} was $10.8 \pm 2.8\%$ ($n=6$) of control in the presence of 25 μM 4-hydroxytamoxifen. We did not detect any significant changes in $[Ca^{2+}]_{50\%}$ or n_H for SR Ca^{2+} uptake in the presence of either tamoxifen or 4-hydroxytamoxifen. Neither tamoxifen nor 4-hydroxytamoxifen appeared to be able to completely inhibit cardiac SR Ca^{2+} uptake in our experiments; however, it should be noted that tamoxifen precipitated in our uptake buffer at concentrations above ~ 15 μM and 4-hydroxytamoxifen precipitated at concentrations above ~ 25 μM. Thus, we cannot rule out the possibility that either of these agents is capable of completely stopping SR Ca^{2+} uptake at higher concentrations.

Clomiphene is a non-steroidal, triphenolic compound that, like tamoxifen and 4-hydroxytamoxifen, functions as a mixed estrogen receptor agonist/antagonist (Jordan, 1984; Ruenitz *et al*., 1983). As shown in Figure 2C, clomiphene also inhibited cardiac SR Ca^{2+} uptake in a concentration-dependent manner at concentrations between 7.5 and 25 μM. At concentrations higher than 25 μM, clomiphene precipitated in the uptake buffer. At the highest clomiphene concentration used (25 μM), the V_{max} of uptake was $20.2 \pm 3.8\%$ ($n=6$) of the control value; half maximal

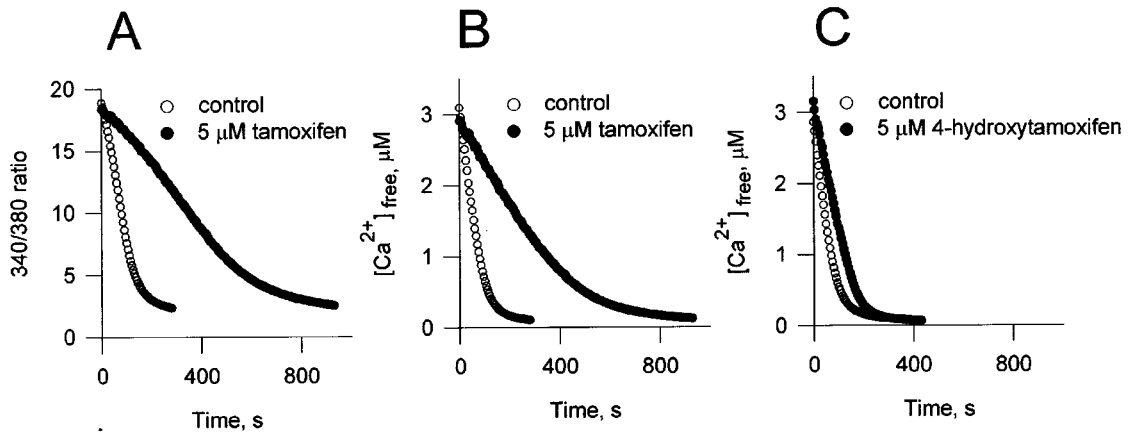

Figure 1 Inhibition of cardiac SR Ca^{2+} uptake by tamoxifen and 4-hydroxytamoxifen. (A) 340/380 fluorescence ratio as a function of time after correction for light scattering and background fluorescence in a control experiment (open symbols) and in the presence of 5 μM tamoxifen (closed symbols). Note: because extravesicular $[Ca^{2+}]$ is measured in these experiments, uptake of Ca^{2+} into the SR is shown by a decrease in 340/380 fluorescence. (B) Extravesicular $[Ca^{2+}]_{free}$ as a function of time for the experiment in (A). (C) Extravesicular $[Ca^{2+}]_{free}$ as a function of time for a control experiment (open symbols) and an experiment done in the presence of 5 μM 4-hydroxytamoxifen (closed symbols). The V_{max}, for uptake was determined from velocity versus $[Ca^{2+}]_{free}$ curves as described in Methods and were: for the experiment in (A) and (B), control $V_{max}=0.534$ μmol min^{-1} mg^{-1}, V_{max} in tamoxifen $=0.122$ μmol min^{-1} mg^{-1}; for the experiment in (C), control $V_{max}=0.586$ μmol min^{-1} mg^{-1}, V_{max} in 4-hydroxytamoxifen $=0.312$ μmol min^{-1} mg^{-1}. Note: in (A–C) only every 5th data point is plotted for clarity; 25 μg of SR vesicle protein was used for each experiment.

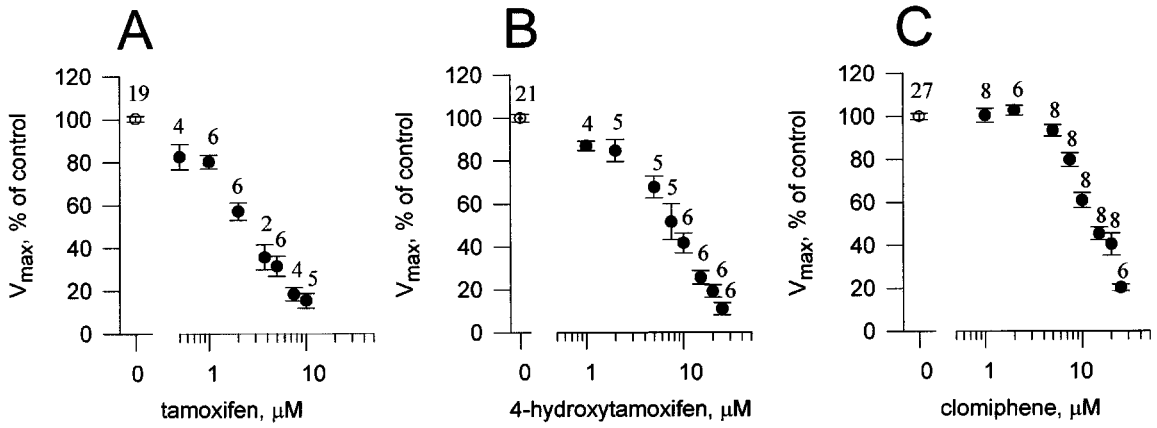

Figure 2 Effects of tamoxifen, 4-hydroxytamoxifen and clomiphene on the V_{max} of cardiac SR Ca^{2+} uptake. (A) Inhibition of V_{max} of cardiac SR Ca^{2+} uptake by tamoxifen. (B) Inhibition of V_{max} by 4-hydroxytamoxifen. (C) Inhibition of V_{max} by clomiphene. Results in (A–C) are expressed as per cent of the mean uptake velocity for control experiments (shown by the open circles at the left in each plot) conducted in the absence of the drugs. Uptake was measured with 25 μg of SR vesicle protein in the cuvette; the number above each point indicates the number of experiments.

inhibition was seen at approximately 10 μM. As was the case with tamoxifen and 4-hydroxytamoxifen, we did not detect any significant differences in $[Ca^{2+}]_{50\%}$ or n_H for SR Ca^{2+} uptake in the presence and absence of clomiphene.

In the experiments described thus far, the anti-oestrogens were added to uptake buffer and allowed to equilibrate with the SR vesicles for 1 min before Ca^{2+} was added to initiate SR uptake. To determine if there were any differences in the time required for tamoxifen, 4-hydroxytamoxifen and clomiphene to affect SR Ca^{2+} uptake, additional experiments were conducted in which these agents were added during uptake. Figure 3 shows that the addition of tamoxifen, 4-hydroxytamoxifen or clomiphene during SR Ca^{2+} uptake had a rapid ($\leqslant$ the time resolution of our measurements; $\approx$1 s) inhibitory effect on uptake. Inhibition of uptake was not accompanied by net release of Ca^{2+} from the SR.

ATPase activity of the cardiac SR Ca^{2+} pump in the presence of 4-hydroxytamoxifen or clomiphene

In previous work (Kargacin *et al*., 2000), we showed that tamoxifen has no detectable effect on the ATPase activity of the cardiac SR Ca^{2+} pump. To determine whether or not this was also true for 4-hydroxytamoxifen and clomiphene, we measured the ATPase activity of the pump in membrane vesicles using the enzyme coupled assay described in Methods. As shown in Figure 3, the addition of tamoxifen (5 μM), 4-hydroxytamoxifen (15 μM) or clomiphene (22 μM) to cardiac SR vesicles during Ca^{2+} uptake had an immediate inhibitory effect on the rate of uptake. However, when the ATPase activity of the SR Ca^{2+} pump was measured in similar experiments, there was no detectable change in the

rate of ATP hydrolysis by the pump when the anti-oestrogens were added at the same concentrations as those used in the uptake experiments shown in Figure 3. The results from measurements of the ATPase activity of the SR Ca^{2+} pump for a control experiment and for an experiment in which 4-hydroxytamoxifen was added during the course of the ATPase measurement are shown in Figure 4. In experiments similar to those shown in Figure 4B, the mean ATPase rate after the addition of tamoxifen was $106 \pm 14\%$ of the rate prior to the addition ($n=3$); the mean ATPase rate after the addition of 4-hydroxytamoxifen was 98% of the rate prior to the addition (two experiments, range = 4%); the mean ATPase rate after the addition of clomiphene was $103 \pm 2\%$ of the rate prior to the addition ($n=3$).

Effect of β-estradiol on SR Ca^{2+} uptake

Tamoxifen, 4-hydroxytamoxifen and clomiphene are all anti-oestrogens and compete with β-estradiol for the oestrogen receptor. As noted above, there is a substantial body of evidence indicating that anti-oestrogens can also affect plasma membrane proteins through mechanisms that appear to be unrelated to their interaction with oestrogen receptors. The oestrogen receptor agonist, β-estradiol, has also been

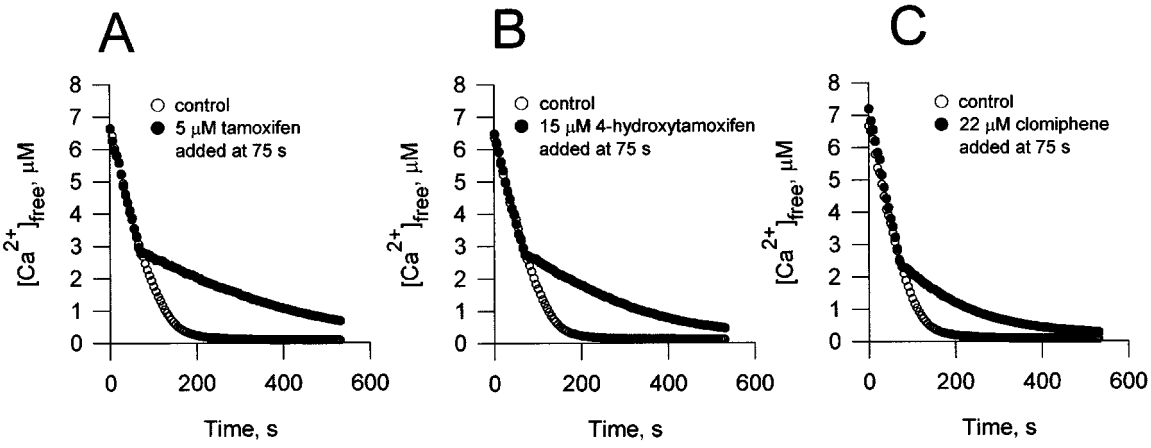

Figure 3 Addition of tamoxifen, 4-hydroxytamoxifen or clomiphene to cardiac SR vesicles during Ca^{2+} uptake. Five μM tamoxifen (A), 15 μM 4-hydroxytamoxifen (B) or 22 μM clomiphene (C) were added to SR vesicles after Ca^{2+} uptake was started. The traces in (A–C) are superimposed results of control experiments (open symbols) and experiments in which the drugs were added 75 s after uptake started (closed symbols). The drug concentrations used were chosen because they inhibited SR Ca^{2+} uptake to approximately the same extent (based on the results shown in Figure 2).

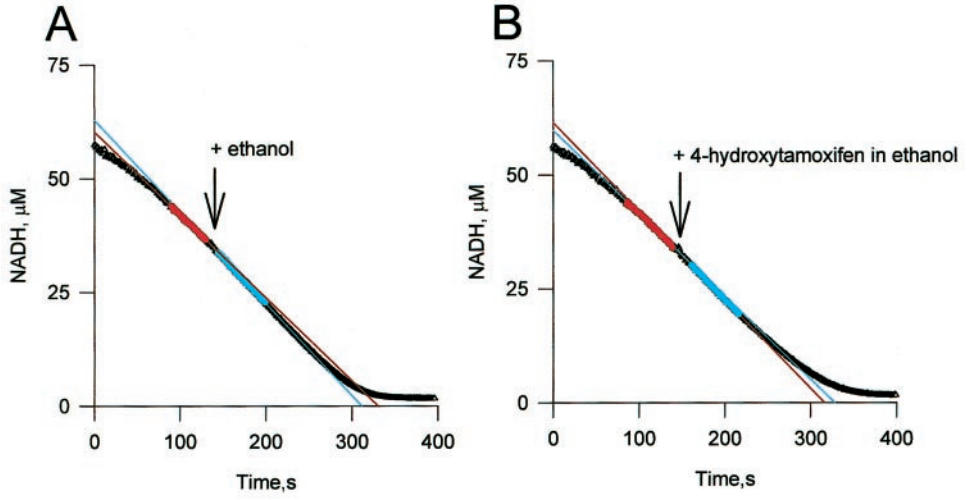

Figure 4 Measurement of the ATPase activity of the cardiac SR Ca^{2+} pump in the presence of 4-hydroxytamoxifen. (A) NADH concentration as a function of time for a control experiment in which ethanol (final concentration 0.9 μl ml^{-1}) was added to the SR vesicles (downward arrow) 150 s after the experiment started. (B) As in (A) except that 4-hydroxytamoxifen in ethanol (final 4-hydroxytamoxifen concentration 15 μM; final ethanol concentration 0.9 μl ml^{-1}) was added 150 s after the experiment started. The lines in (A) and (B) show the slopes of the curve determined by linear regression from the data points prior to (red lines and red symbols) and after (blue lines and blue symbols) after the addition of ethanol (A) or 4-hydroxytamoxifen (B) to the buffer. For the experiment in (A), the rate of change in [NADH] was 0.182 μM s^{-1} before the addition of ethanol and 0.201 μM s^{-1} after the addition; for the experiment in (B), the rate of change of [NADH] was 0.190 μM s^{-1} before the addition of 4-hydroxytamoxifen and 0.195 μM s^{-1} after the addition. Sixty μg of SR vesicle protein was used for the experiments in (A) and (B).

shown to have some actions that are unlikely to be attributed to its effects at the nucleus. Waldegger *et al*. (1996) reported that β-estradiol inhibited K^+ currents induced by $_{min}K$ in *Xenopus* oocytes. This current was also inhibited by tamoxifen. Hardy & Valverde (1994) found that β-estradiol prevented the activation of a Cl^- current in NIH-3T3 fibroblasts by tamoxifen and toremifene. We, therefore, examined the effects of β-estradiol on cardiac SR Ca^{2+} uptake. In these experiments, we did not see a detectable effect of β-estradiol on cardiac SR Ca^{2+} uptake at concentrations of 1 and 2 nM (not shown) or at higher concentrations between 1 and 25 μM (Figure 5A) nor did we find any effect of β-estradiol on the inhibition of SR Ca^{2+} uptake by tamoxifen (Figure 5B).

Discussion

In previous work (Kargacin *et al*., 2000) we showed that tamoxifen inhibited Ca^{2+} uptake into the cardiac sarcoplasmic reticulum. This occurred without a direct effect on the ability of the SR Ca-pump to hydrolyze ATP or a significant increase in the Ca^{2+} permeability of the SR membrane. As a result of these findings, we concluded that the most likely action of tamoxifen on the cardiac SR was to inhibit the movement of either Cl^- or K^+ across the SR membrane. This ion movement is thought to occur during SR Ca^{2+} uptake to compensate for the net positive charge moved into the SR by the SR Ca pump (reviewed in Tada & Kadoma, 1995). The results of the present study show that other anti-oestrogens, 4-hydroxytamoxifen and clomiphene, are also able to inhibit cardiac SR Ca^{2+} uptake (order of potency: tamoxifen > 4-hydroxytamoxifen $\geqslant$ clomiphene) without altering the ability of the SR Ca pump to hydrolyze ATP. In our previous studies (Kargacin *et al*., 2000), when we added tamoxifen to cardiac SR vesicles after they were loaded or nearly loaded with Ca^{2+} we did not detect a substantial net release of Ca^{2+} from the SR indicating that tamoxifen did not greatly increase in the leakiness of the SR membrane to Ca^{2+}. We did not see evidence for net Ca^{2+} release when 4-hydroxytamoxifen or clomiphene were added to Ca^{2+}-loaded (result not shown) or partially-loaded (Figure 3) SR vesicles in our present experiments. Our results thus suggest that tamoxifen, 4-hydroxytamoxifen and clomiphene all act in a similar manner on the cardiac SR. In our previous work (Kargacin *et al*., 2000) we suggested that the most likely action of tamoxifen on cardiac SR Ca^{2+} uptake was to inhibit the anion or cation movement through the SR membrane that is thought to occur during Ca^{2+} uptake. The hypothesis that tamoxifen, 4-hydroxytamoxifen and clomiphine inhibit SR Ca^{2+} uptake by blocking Cl^- or K^+ channels in the SR membrane is consistent with their known actions on other ion channels. Tamoxifen inhibits some types of plasma membrane chloride channels (Duan *et al*., 1997; Szücs *et al*., 1996; Voets *et al*., 1995; Vandenberg *et al*., 1994; Zhang *et al*., 1994; 1995), some types of K^+ channels (Liu *et al*., 1998; Dick *et al*., 1999), L-type Ca^{2+} channels in vascular smooth muscle (Song *et al*., 1996) and cell-to-cell communication through gap junctions (Verrecchia & Hervé, 1997a,b). 4-hydroxytamoxifen has been shown to block Cl^- currents in lens cells from the bovine eye (Zhang *et al*., 1994). These effects of tamoxifen and 4-hydroxytamoxifen on ion channels occur over the same concentration ranges as those used in our experiments. Half maximal inhibition of volume regulated Cl^- channels by tamoxifen has been reported to occur between 0.3 and 15 μM (Dick *et al*., 1999; Hardy & Valverde, 1994; Song *et al*., 1996; Szücs *et al*., 1996; Verrecchia & Hervé, 1997a,b; Voets *et al*., 1995; Zhang *et al*., 1994); in our experiments, half maximal inhibition with tamoxifen occurred at approximately 2 μM. Our results

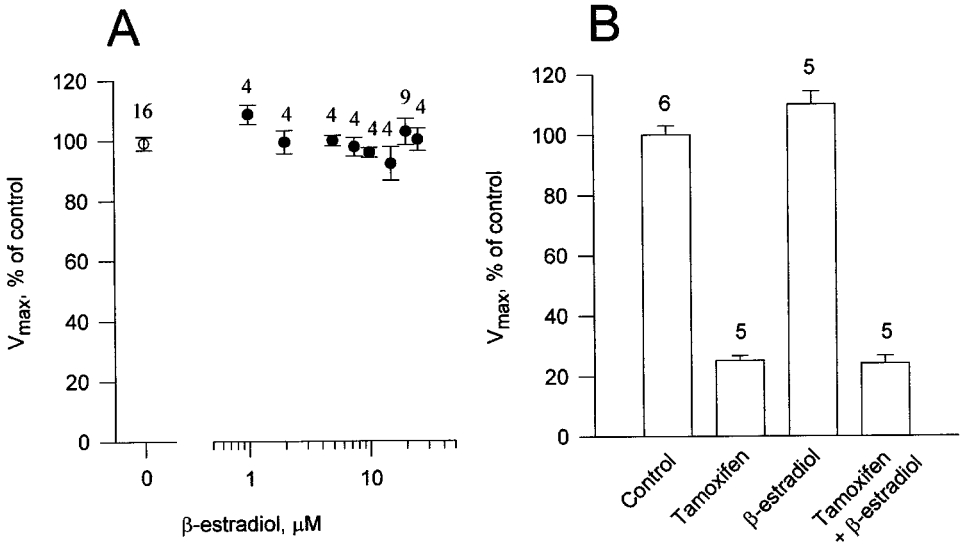

Figure 5 Cardiac SR Ca^{2+} uptake in the presence of β-estradiol. (A) V_{max} for cardiac SR Ca^{2+} uptake in the presence of β-estradiol at concentrations between 1 and 25 μM. Results are expressed as per cent of the mean V_{max} for control experiments (shown by the open symbol to the left) conducted in the absence of β-estradiol. Uptake was measured with 25 μg SR vesicle protein in the cuvette; the number above each point indicates the number of experiments. (B) β-estradiol (20 μM) had no detectable effect on tamoxifen-dependent inhibition of cardiac SR Ca^{2+} uptake (tamoxifen concentration 5 μM). Although the mean V_{max} in the presence of β-estradiol was slightly higher than that of the control, this difference was not significant ($P=0.086$). The numbers above the bars indicate the number of experiments.

showing that 4-hydroxytamoxifen is slightly less potent than tamoxifen in inhibiting SR Ca^{2+} uptake is similar to the results obtained by Zhang *et al*. (1994) when the effects of tamoxifen and 4-hydroxytamoxifen on ion currents were compared. Clomiphene and tamoxifen (at 10 μM) were shown to be equally effective blockers of cell-to-cell communication through gap junctions in cultured neonatal rat cardiac myocytes (Verrecchia & Hervé, 1997a,b). In contrast, in our experiments, higher concentrations of clomiphene were required to inhibit SR Ca^{2+} uptake to the same extent as that seen with tamoxifen.

The anti-oestrogens used in our experiments all incorporate into lipid bilayers (see Custódio *et al*., 1991; 1993a,b; Jordan, 1984; Paoutová *et al*., 1988). Thus, as noted above, there are at least two possible mechanisms by which these compounds could affect the function of membrane-associated proteins: (1) anti-oestrogens could alter membrane lipid structure or fluidity (Custódio *et al*., 1991; 1993a,b; Dicko *et al*., 1999; Luxo, *et al*., 1996; 1999) which then indirectly modifies protein function by changing the interaction of proteins with the membrane (see discussions in Custódio *et al*., 1993a,b; 1996; Song *et al*., 1996; Verrecchia & Hervé, 1997a,b). It has been suggested, for example, that inhibition of PKC by tamoxifen occurs as a result of a tamoxifen-induced alteration of the interaction of PKC and phospholipids (O'Brian *et al*., 1985); (2) anti-oestrogens could affect proteins by directly interacting with the proteins themselves (see Custódio *et al*., 1996; Hardy & Valverde, 1994; Song *et al*., 1996; Waldegger *et al*., 1996). In support of this mechanism, it has been reported that tamoxifen can bind to calmodulin (Lopes *et al*., 1990) and could inhibit calmodulin-dependent enzymes in this manner (Fanidi *et al*., 1989; Lopes *et al*., 1990). In our experiments, we have shown that, at all concentrations between 2 and 10 μM, tamoxifen inhibits cardiac SR Ca^{2+} uptake to a greater extent than does 4-hydroxytamoxifen (see Figure 2). The partition coefficient for the incorporation of tamoxifen into biological membranes (including SR membranes) decreases with concentration between 5 and 10 μM whereas that of 4-hydroxytamoxifen increases (Custódio *et al*., 1991). At 5 μM and 24°C, the partition coefficient for tamoxifen in dimyristoylphosphatidylcholine membranes was found to be approximately four times that of 4-hydroxytamoxifen; at 10 μM, the partition coefficient for tamoxifen was approximately 0.4 that of 4-hydroxytamoxifen (Custódio *et al*., 1991). Thus, at 10 μM we might expect the inhibition of SR Ca^{2+} uptake to be greater in the presence of 4-hydroxytamoxifen than in the presence of tamoxifen. The fact that tamoxifen, at 10 μM, inhibits cardiac SR Ca^{2+} uptake to a greater extent than 4-hydroxytamoxifen, therefore, does not correlate with the expected differences in the partitioning of the drugs into the SR membrane. 4-hydroxytamoxifen appears to interact with lipid bilayers more strongly in the outer region of the bilayer whereas tamoxifen interacts more strongly with the membrane interior (Custódio *et al*., 1993a,b). One might, therefore, argue that, even though at a concentration of 10 μM, less tamoxifen partitions into the SR membrane than 4-hydroxytamoxifen, it nevertheless exerts a greater lipid-mediated effect on an SR protein because of its stronger interaction with the membrane core. If this were the case, one would expect β-estradiol, which has effects similar to those of tamoxifen on the membrane core (Dicko *et al*., 1999) to also inhibit cardiac SR Ca^{2+} uptake. Comparison of Figures 2 and 5 indicate that this is clearly not the case. Thus, it appears unlikely that tamoxifen, 4-hydroxytamoxifen and clomiphene inhibit cardiac SR Ca^{2+} uptake by affecting membrane fluidity or structure. Our results are, therefore, more consistent with the interpretation that the anti-oestrogens directly act on an SR membrane protein.

The results obtained in this study of cardiac SR differ from those published by Custódio *et al*. (1996) using skeletal muscle SR. In the latter report, the authors presented evidence showing concentration-dependent effects of tamoxifen and 4-hydroxytamoxifen on the ATPase activity of the skeletal SR Ca^{2+} pump. At 10 μM, both tamoxifen and 4-hydroxytamoxifen inhibited ATPase activity by approximately 15%; at 20 μM, 4-hydroxytamoxifen inhibited the ATPase activity by approximately 35%. Custódio *et al*. (1996) also reported that 4-hydroxytamoxifen decreased the rate of SR Ca^{2+} uptake to a greater extent than tamoxifen. The differences between our results and those of Custódio *et al*. (1996) might be due to physiological differences in skeletal and cardiac muscle SR or differences in the methods used to measure SR Ca^{2+} uptake and ATPase activity (measurements with ion-sensitive electrodes in the case of Custódio *et al*. (1996); measurements with fluorescent probes in our case). It should also be noted that our ATPase and SR Ca^{2+} uptake measurements were made at extravesicular free Ca^{2+} concentrations less than 10 μM, whereas those of Custódio *et al*. (1996) were made at concentrations ranging from 20 to 200 μM.

Based on our results on cardiac SR Ca^{2+} uptake and those of others (Duan *et al*., 1997; Liu *et al*., 1998; Murphy & Khalil, 1999; Verrecchia & Hervé, 1997a,b) showing significant effects of tamoxifen and other anti-oestrogens on plasma membrane ion channels in cardiac myocytes, it is surprising that serious alterations in cardiac function are not consistently seen in patients taking tamoxifen therapeutically. From the serum and tissue levels of tamoxifen ($\sim$100 ng g^{-1} serum and 8–70 times higher in tissue) in patients undergoing tamoxifen treatment in a study published by Lien *et al*. (1991), serum concentrations of 0.3 μM and tissue concentrations of 2.4–21 μM can be calculated. Thus tissue concentrations within the range of those shown to affect cardiac myocytes are possible *in situ*. The fact that serious cardiac abnormalities are not seen clinically suggests that compensatory mechanisms may be in place that allow patients to adapt to high tissue tamoxifen levels. Further experimental work will be important for understanding such mechanisms.

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