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Acute actions of 3α-hydroxy-tibolone on factors influencing contraction in guinea-pig ventricular myocytes

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Abstract

We investigated whether one of the main estrogenic metabolites of the postmenopausal agent, tibolone (Org OD14), exerts direct cardiac actions in a similar way to 17β-estradiol. 3α-OH-tibolone (40 μM) decreased both cell shortening and Ca^{2+} transient amplitude of field-stimulated (1 Hz, 37 °C) guinea-pig ventricular myocytes. These effects were still observed in cells that had been incubated with the specific estrogen receptor antagonist, ICI 182,780 ($C_{32}H_{47}F_5O_3S$), suggesting an estrogen receptor-independent mechanism of action. In addition, 3α-OH-tibolone inhibited the L-type Ca^{2+} current and shortened action potential durations (APD). This mechanism may contribute to a potential cardiovascular action of tibolone.

Keywords: Action potential; Cell shortening; Estrogen receptor; Ca^{2+} current, L-type; Myocyte

1. Introduction

The synthetic steroid tibolone (Org OD14) has long been used as an alternative to conventional hormone replacement therapy in the treatment of climacteric symptoms and prevention of osteoporosis in postmenopausal women (Kicovic et al., 1982; Bjarnason et al., 1996). Increasing evidence from animal studies suggests that tibolone may also exert beneficial cardiovascular actions, although the precise mechanisms involved remain poorly understood. Tibolone has been shown to possess atheroprotective properties by preventing the deposition of cholesterol in the arterial walls of cholesterol-fed, ovariectomized rabbits (Zandberg et al., 1998; Sanjuan et al., 2003). In contrast, unlike hormone replacement therapy, tibolone was found to have no beneficial effects on endothelium-mediated dilatation in ovariectomized monkeys fed an atherogenic diet, despite reducing the incidence of myocardial ischaemia (Williams et al., 2002). Although this latter study is suggestive of direct beneficial actions of tibolone on the heart, the mechanisms underlying any such interactions are unknown.

The unique estrogenic, androgenic and progestogenic properties of tibolone can be largely attributed to the actions of its metabolites. After oral administration, tibolone is rapidly metabolized into two metabolites with estrogenic properties (3α- and 3β-hydroxy-tibolone) and a Δ4-isomer with progestogenic/androgenic properties (Kloosterboer, 2001). 3α-OH-tibolone [(3α,7α,17α)-7-methyl-19-norpreg-5(10)-en-20-yne-3,17-diol] binds with a much greater affinity to the estrogen receptor than its parent compound, but does not bind to progesterone or androgen receptors, unlike the parent compound (Kloosterboer, 2001). It is therefore likely that this metabolite is responsible for effecting many of the estrogen receptor-mediated actions of tibolone.

The mammalian estrogen, 17β-estradiol, has been demonstrated to exert direct electrophysiological actions on isolated cardiomyocytes (Jiang et al., 1992; Meyer et al., 1998). In the present study, we investigated whether 3α-OH-tibolone directly affects cardiomyocyte contraction and electrophysiological parameters in a similar way to 17β-estradiol.

2. Materials and methods

2.1. Cell isolation and indo-1 loading

Left ventricular myocytes were isolated from adult male guinea pigs (550–750 g) by enzymatic digestion as previ-

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ously described (MacLeod and Harding, 1991). Myocytes were stored in Dulbecco's modified Eagle's medium solution at room temperature and loaded with 10 μM of the acetoxymethyl ester form of the Ca^{2+}-sensitive fluorescent dye, indo-1 (Molecular Probes, Eugene, OR) for 25 min. The investigation was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Cell shortening and intracellular Ca^{2+} measurements

Myocytes were placed on the coverslip base of a bath chamber on the stage of an inverted microscope (Nikon) and superfused with normal Tyrode, NT (containing in mM: NaCl 140, KCl 6, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, HEPES 10, pH adjusted to 7.4 using NaOH). Cells were electrically field stimulated at a rate of 1 Hz using a pair of platinum electrodes positioned on either side of the chamber and shortening followed at one end with a video edge-detector system. Ultraviolet light (365-nm wavelength) was used to excite the fluorescent dye in the cells, allowing the indo-1 ratio (i.e. ratio of signals emitted at 405- and 485-nm wavelengths) to be determined. The change in indo-1 ratio during field stimulation, Δindo-1 ratio, was taken as a measure of Ca^{2+} transient amplitude. Cell shortening and Ca^{2+} transient amplitudes were recorded simultaneously and values in control solution were compared with equivalent parameters in the presence of 3α-OH-tibolone (10 and 40 μM) within the same cell. This avoided the need to calibrate indo-1 ratio values into absolute Ca^{2+} concentrations and thereby removed the potential errors that can result from dye compartmentalization during calibration experiments (Spurgeon et al., 1990).

In order to avoid potential residual effects of incomplete washout of the compound, each bath of cells was changed following every exposure to 10 or 40 μM 3α-OH-tibolone. All experiments were performed at 37 °C.

2.3. Effects of ICI 182,780

We used the specific estrogen receptor antagonist ICI 182,780 ($C_{32}H_{47}F_5O_3S$, Tocris, UK) to determine whether the effects of 3α-OH-tibolone on cell shortening and the Ca^{2+} transient were mediated via the estrogen receptor. Indo-1-loaded cells were incubated with 10 μM ICI 182,780 at room temperature for 1 h before being placed in the superfusion chamber. Cell shortening and the indo-1 ratio were measured in the continuing presence of 10 μM ICI 182,780 before (control) and after the addition of 40 μM 3α-OH-tibolone. We have previously found this protocol and concentration of ICI 182,780 to be effective in inhibiting estrogen receptor-mediated actions of other estrogenic compounds in cardiomyocytes and coronary artery rings (Figtree et al., 1999; Liew et al., 2003).

2.4. Electrophysiology

Electrophysiological parameters were measured using single electrode voltage-clamp (discontinuous switch mode with the switching rate set between 4–6 kHz). Myocytes were impaled with high resistance microelectrodes (20–30 MΩ) filled with solution containing: KCl 2 M; EGTA, 0.1 mM; HEPES, 5 mM, pH 7.2.

Action potentials were elicited under current-clamp and action potential durations (APD) at 50% and 90% repolarization (APD_{50} and APD_{90}, respectively) determined. Ca^{2+} currents were recorded under voltage-clamp with myocytes held at −40 mV and test pulses (200-ms duration, ranging from −45 to +50 mV) imposed. $I_{Ca,L}$ was determined by subtracting the trace in the presence of 100 μM cadmium from the original trace at each voltage. Steady-state activation parameters of $I_{Ca,L}$ could be obtained from the relationship between membrane conductance and the imposed potential. Steady-state inactivation parameters were analyzed with double-pulse protocols. Conditioning pulses (200-ms duration) ranging from −55 to +50 mV were imposed from a holding potential of −50 mV. Five milliseconds after the end of each conditioning pulse, a test pulse to +5 mV (200-ms duration) was applied to elicit the Ca^{2+} current. Data were recorded using Axoclamp-2B system and analyzed with pCLAMP software (Axon Instruments, Foster City, CA).

2.5. Drugs

3α-OH-tibolone (Org 4094) was a gift from N.V. Organon (The Netherlands). All drugs were analytical grade and dissolved in dimethyl sulfoxide (DMSO) to make a 100-mM stock solution. The maximum final concentration of DMSO used (0.05%) was found to have no significant effect on the parameters measured.

2.6. Statistical analysis

Results are expressed as mean ± S.E.M. and analyzed using the Student's *t*-test and one-way analysis of variance (ANOVA) with Bonferroni post-test as appropriate. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Effects on cell shortening and Ca^{2+} transient amplitude

At a concentration of 40 μM, 3α-OH-tibolone decreased cell shortening and Ca^{2+} transient amplitude by 26 ± 4% ($n = 13$, $P < 0.01$) and 11 ± 4% ($P < 0.05$), respectively. The compound had no significant effect on these parameters when tested at a lower concentration of 10 μM, Fig. 1A. The inhibitory actions of 40 μM 3α-OH-tibolone took effect

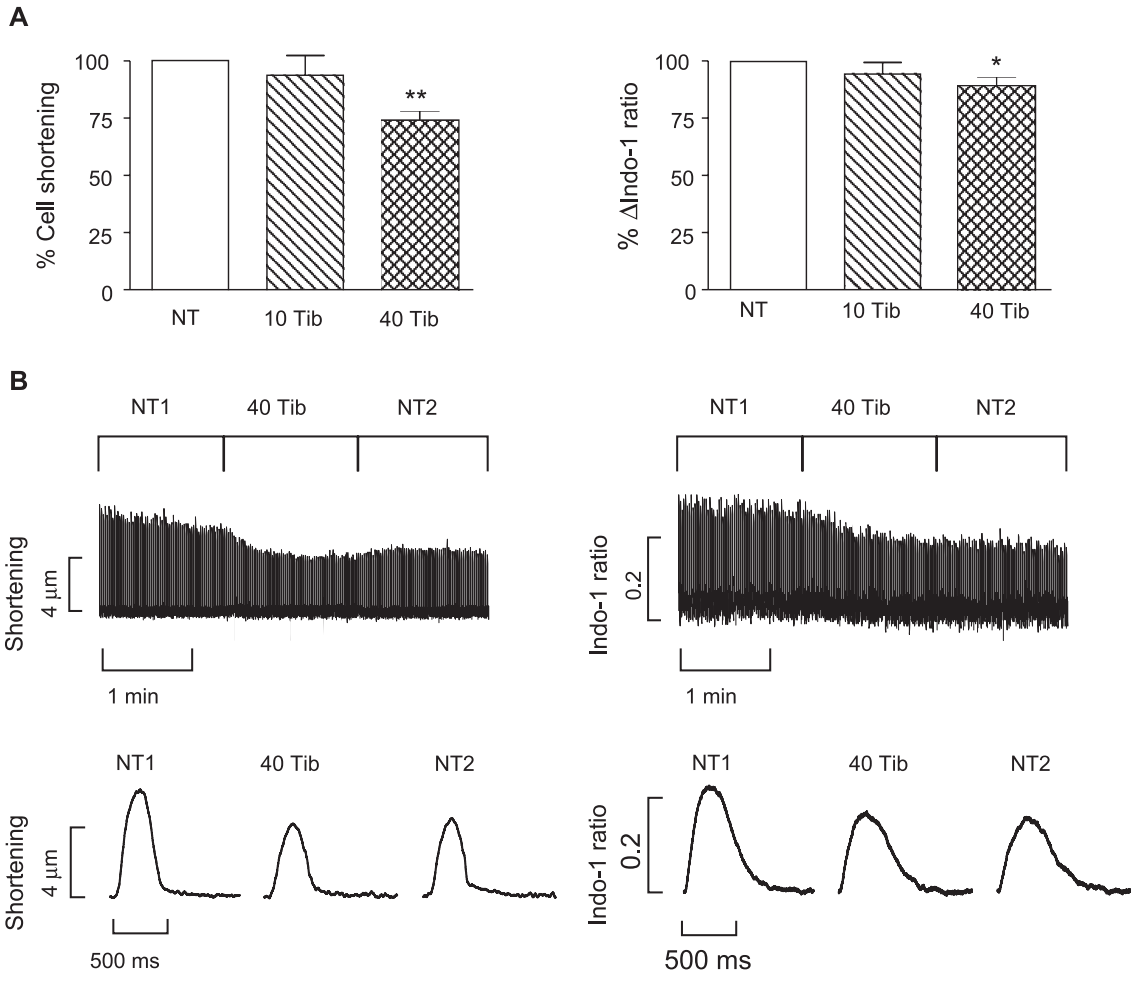

Fig. 1. Acute effects of 3α-OH-tibolone on cell shortening and Ca^{2+} transient amplitude. (A) Bar graphs showing effects of 10 and 40 μM 3α-OH-tibolone (Tib) relative to control values in normal Tyrode (NT). (B) Continuous recordings of cell shortening and the indo-1 ratio before (NT1), during (40 Tib) and after (NT2) the application of 40 μM 3α-OH-tibolone. Corresponding enlarged traces of cell contractions and Ca^{2+} transients (averaged from eight twitches) are shown below. (*$P<0.05$, **$P<0.01$).

within 1 min and were only partially reversible upon washout, Fig. 1B. The presence of ICI 182,780 did not prevent 3α-OH-tibolone (40 μM) decreasing cell shortening and Ca^{2+} transient amplitude, which declined by $14 \pm 6\%$ ($n=11$, $P<0.05$) and $15 \pm 3\%$ ($P<0.001$), respectively, of control values.

3.2. Effects on action potential waveform

Fig. 2A illustrates the effects of 3α-OH-tibolone on action potential waveform. 3α-OH-tibolone (40 μM) decreased APD_{50} from 365 ± 34 to 307 ± 26 ms ($n=15$, $P<0.05$) and APD_{90} from 431 ± 33 to 348 ± 25 ms ($P<0.01$). In addition, maximum upstroke velocity (dV/dt_{max}) was slower (43.6 ± 2.0 V/s compared with 48.3 ± 1.8 V/s in control, $P<0.05$) and the time to peak action potential depolarization prolonged (8.7 ± 0.5 ms compared with 7.5 ± 0.6 ms in control, $P<0.05$) in the presence of 3α-OH-tibolone. There was no significant change in the resting membrane potential (results not shown).

3.3. Effects on L-type Ca^{2+} current

3α-OH-tibolone (40 μM) significantly decreased peak $I_{Ca,L}$ from -7.2 ± 0.5 to -2.5 ± 0.3 pA/pF ($n=8$, $P<0.001$), i.e. inhibited $I_{Ca,L}$ by 65%, Fig. 2B and C. This occurred over the whole range of potentials tested and took effect within a minute following application of 3α-OH-tibolone.

We further investigated whether 3α-OH-tibolone had any effects on voltage-dependence of Ca^{2+} channel activation or inactivation. The relationships between membrane potentials and L-type Ca^{2+} channel conductance were fitted with the Boltzmann function:

$$G = G_{min} + \frac{(G_{max} - G_{min})}{1 + \exp\left(\frac{V_{50} - X}{K}\right)}$$

where G is the conductance at membrane potential X, G_{min} and G_{max} are the minimum and maximum conductances,

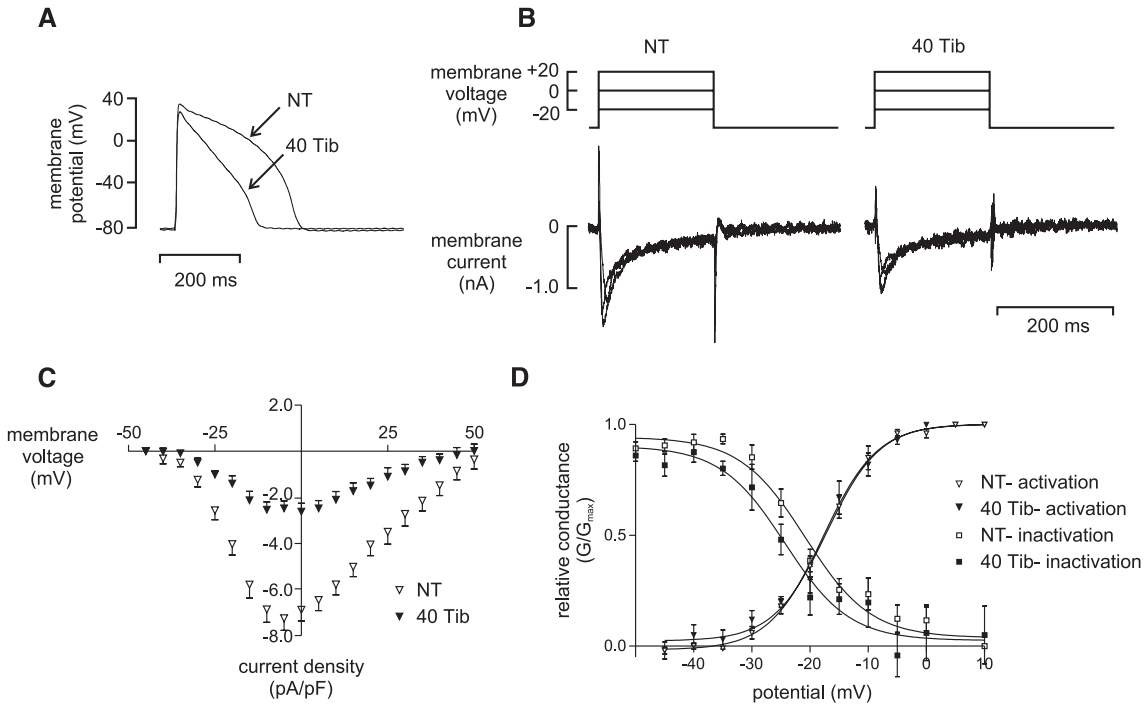

Fig. 2. Effects of 3α-OH-tibolone on action potential profile and L-type Ca^{2+} current. (A) Sample recording of the membrane potential during steady-state stimulation at 1 Hz in normal Tyrode (NT) and in the presence of 40 μM 3α-OH-tibolone (40 Tib). (B) Representative $I_{Ca,L}$ traces elicited at three depolarizing potentials in NT and in the presence of 40 Tib. (C) Current–voltage plot showing the mean peak $I_{Ca,L}$ in NT (open triangle) and 40 Tib (closed triangle) over the whole range of potentials tested. (D) Boltzmann plots of steady-state activation and inactivation parameters in NT and 40 Tib.

respectively, and K is the slope factor. This allowed the membrane potential, V_{50}, when half the channels are activated (d_∞ variable) or inactivated (f_∞ variable) to be determined in the presence and absence of 40 μM 3α-OH-tibolone. We found that 3α-OH-tibolone had no significant effect on voltage-dependent activation parameters (V_{50} or K; $n=8$). However, the compound decreased V_{50} of inactivation from -19.7 ± 2.0 to -25.4 ± 1.6 mV ($n=7$, $P<0.05$), thereby producing a leftward shift in the inactivation curve, Fig. 2D. This was not accompanied by any change in K of inactivation.

4. Discussion

The unique pharmacological profile of tibolone accounts for cardiovascular actions that are both similar to and different from those of endogenous estrogens (Kloosterboer, 2001). Differences, such as a lowering of high density lipoprotein (HDL) cholesterol and triglycerides with tibolone, may be due to the progestogenic/androgenic properties of tibolone and the Δ4-isomer. In the present study, we chose to study one of the main estrogenic metabolites, 3α-OH-tibolone, which does not possess progestogenic/androgenic properties.

Our novel finding of a negative inotropic effect of 3α-OH-tibolone is similar to that previously described for 17β-estradiol (Jiang et al., 1992; Meyer et al., 1998). Decreased cell contraction and Ca^{2+} transient amplitude observed with both compounds can be explained by inhibition of $I_{Ca,L}$, the main trigger to cardiac excitation–contraction coupling. The leftward shift in steady-state inactivation curve produced by 3α-OH-tibolone is also similar to that described with 17β-estradiol (Nakajima et al., 1999) and provides further evidence for a common mechanism of Ca^{2+} channel antagonism. That we could only partially reverse the effects of 3α-OH-tibolone on cell contraction and Ca^{2+} transient amplitude during washout in NT (despite times in excess of 10 min) suggests a semi-permanent mechanism of action or very long duration of action of the compound. Consequently, a cumulative concentration–response relationship could not be obtained, and therefore, a different cell in a fresh bath was used after each exposure to 3α-OH-tibolone.

The decrease in maximum upstroke velocity of the action potential observed in the presence of 3α-OH-tibolone suggests an inhibitory action of this compound on Na^+ currents or Na^+-channel gating. This appears to differ from the effects of 17β-estradiol on the action potential, which, to the best of our knowledge, has not been shown to affect Na^+ currents. The shortened APD_{50} and APD_{90} produced by 3α-OH-tibolone may be secondary to $I_{Ca,L}$ inhibition, although additional effects on K^+ currents cannot be discounted.

The finding that 3α-OH-tibolone continued to decrease cell shortening and Ca^{2+} transient amplitude in cells that had been incubated with ICI 182,780 suggests an estrogen receptor-independent mechanism of action. This contrasts

with the estrogen receptor-dependent vascular effects of tibolone, which are blocked by ICI 182,780 (Zoma et al., 2001; Simoncini and Genazzani, 2000). It seems likely that the acute cardiac actions of 3α-OH-tibolone described here are due to nonspecific, direct actions on a number of ion channels. However, the mechanism through which 3α-OH-tibolone interacts with ion channels and other proteins is unknown and warrants further investigation.

The concentrations of 3α-OH-tibolone used in our experiments are several orders of magnitude greater than known plasma levels of tibolone following oral administration (Zandberg et al., 1998). It is therefore questionable whether our findings are relevant in vivo. However, tissue levels of tibolone metabolites, which are of greater significance than plasma levels, are unknown. Evidence from studies of similar estrogenic compounds, such as tamoxifen, suggests that tissue levels may be up to 70 times greater than plasma concentrations due to slow accumulation and washout (Lien et al., 1991).

In summary, we have shown that the 3α-hydroxy metabolite of the widely used postmenopausal agent tibolone, directly affects cardiomyocyte function through effects on cell contraction and nonspecific inhibition of ion currents. The actions described may contribute to a possible cardioprotective role of tibolone, although further mechanistic studies and clinical trials are required before this can be confirmed or refuted.

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