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RESEARCH PAPER

Smooth muscle relaxation and activation of the large conductance Ca^{++} – activated K^+ (BK_{Ca}) channel by novel oestrogens

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BACKGROUND AND PURPOSE

Oestrogens can interact directly with membrane receptors and channels and can activate vascular BK_{Ca} channels. We hypothesized that novel oestrogen derivatives could relax smooth muscle by an extracllular effect on the α and β_1 subunits of the BK_{Ca} channel, rather than at an intracellular site.

EXPERIMENTAL APPROACH

We studied the effects of novel oestrogens on the tension of pre-contracted isolated rat aortic rings, and on the electrophysiological properties of HEK 293 cells expressing the hSloα or hSloα+β_1 subunits. Two of the derivatives incorporated a quaternary ammonium side-chain making them membrane impermeable.

KEY RESULTS

Oestrone, oestrone oxime and Quat DME-oestradiol relaxed pre-contracted rat aorta, but only Quat DME-oestradiol-induced relaxation was iberiotoxin sensitive. However, only potassium currents recorded in HEK 293 cells over-expressing both hSloα and hSloβ_1 were activated by oestrone, oestrone oxime and Quat DME-oestradiol.

CONCLUSION AND IMPLICATIONS

The novel oestrogens were able to relax smooth muscle, but through different mechanisms. In particular, oestrone oxime required the presence of the endothelium to exert much of its effect, whilst Quat DME-oestradiol depended both on NO and BK_{Ca} channel activation. The activation of BK_{Ca} currents in HEK 293 cells expressing hSloα+β_1 by Quat DME-oestradiol is consistent with an extracellular binding site between the two subunits. The binding site resides between the extracellular N terminal of the α subunit and the extracellular loop between TM1 and 2 of the β_1 subunit. Membrane-impermeant Quat DME-oestradiol lacks an exchangeable hydrogen on the A ring obviating antioxidant activity.

Abbreviations

BK_{Ca}, large conductance calcium-activated potassium channel; DMAEC HCl, 2-dimethylamine ethyl chloride; DMF, dimethylformamide; hSloα, the alpha subunit of the human BK_{Ca} channel; hSloβ_1, the beta1 subunit of the human BK_{Ca} channel; IBTX, iberiotoxin; IK_{Ca}, intermediate-conductance calcium-activated potassium channels; SK_{Ca}, small-conductance calcium-activated potassium channels

Introduction

The large conductance calcium-activated potassium channel, also called BK_{Ca}, maxi-K or $K_{Ca}1.1$, (Alexander *et al.*, 2009) responds to both changes in membrane potential and increases in intracellular-free calcium; consequently, the BK_{Ca} channel is able to regulate membrane potential in a variety of smooth muscle cells (del Corsso *et al.*, 2006; Layne *et al.*, 2008; Werner *et al.*, 2008). This channel acts as a negative feedback mechanism because it hyperpolarizes and relaxes

smooth muscle following calcium entry (McManus *et al.*, 1995; Brenner *et al.*, 2000). In smooth muscle, the BK_{Ca} channel is composed of α pore-forming subunits and β_1 regulatory subunits. A reduction of BK_{Ca} channel expression and function is associated with ageing arteries (Nishimaru *et al.*, 2004), and a reduction of β_1 regulatory subunit expression is associated with hypertension (Brenner *et al.*, 2000; Pluger *et al.*, 2000; Pluznick *et al.*, 2003). The β_1 regulatory subunit confers increased calcium sensitivity on the BK_{Ca} channel, shifting the current voltage relationship to potentials that are more negative and slows the activation of the BK_{Ca} current. It was the discovery of these regulatory subunits (β_1-β_4) that explained the variation of BK_{Ca} channel behaviour in differing tissues. The β_1 regulatory subunit is, therefore, a potential target for pharmacological intervention, as it co-assembles with the α subunit in arteriole smooth muscle and is responsible for the observed behaviour.

Oestrogens such as 17β-oestradiol are known to relax vascular smooth muscle. A number of mechanisms have been proposed for this effect, including increasing NO release by endothelial cells (Broughton *et al.*, 2010), as well as direct effects on smooth muscle, such as inhibition of calcium channels (Cairrao *et al.*, 2012), elevating cyclic GMP (Rosenfeld *et al.*, 2009), activating oestrogen receptors (Han *et al.*, 2006), activating nNOS (Royal *et al.*, 2011) and finally activating BK channels (White *et al.*, 2002).

We have shown that 17β-oestradiol is able to activate BK_{Ca} channels when BK_{Ca} channels are inserted into planar lipid bilayers. This activation is stereospecific, 17α-oestradiol being inactive, and requires the presence of the β_1 subunit. We have also shown that in order to activate the channel in planar lipid bilayers (De Wet *et al.*, 2006; de Wet *et al.*, 2009), at least two β_1 subunits are required to be associating with a functional channel. This work is consistent with those of other investigators who demonstrated that 17β-oestradiol requires the β_1 subunit to activate the BK_{Ca} channel (Valverde *et al.*, 1999a,b). Deletion of the N terminal of the S0 domain removes the ability of 17β-oestradiol to activate the channel and shift the G–V relationship to negative potentials, but does not remove the ability of the β_1 subunit to slow the activation of the channel (Morrow *et al.*, 2006). What these studies imply is that β_1 subunit interaction with the α subunit is subtle, with more than one point of interaction between the subunits. These studies also imply that the binding site for 17β-oestradiol is extracellular, as the N-terminal amino acids of BK_{Ca} are extracellular (Liu *et al.*, 2010).

Other oestrogens and xenoestrogens are able to regulate the behaviour of a variety of channels; both steroidal oestrogens and non-steroidal antioestrogens have been reported to activate BK_{Ca} channels (Dick and Sanders, 2001; Dick *et al.*, 2001; Dick, 2002). However, non-steroidal antioestrogens, such as tamoxifen and toremifene, have been shown to modulate a variety of ion channels (Allen *et al.*, 1998; 1999; 2000; Sahebgharani *et al.*, 2001). We have synthesized membrane impermeable derivatives of tamoxifen, such as ethyl bromide tamoxifen, to investigate the binding site of these compounds and have demonstrated that, for some channels, the binding site is extracellular, while for others, it lies deep within the membrane or on an intracellular component of the channel (Sahebgharani *et al.*, 2001).

The present study has investigated modified steroidal oestrogens and their actions on the BK_{Ca} channel. We have synthesized oestrogens, which incorporate some of the features of tamoxifen and its membrane impermeable analogue, ethyl bromide tamoxifen, in order to generate novel BK_{Ca} channel activators. If the binding site for oestrogens is on the extracellular domains of the channel, then both steroidal oestrogens and the membrane impermeable analogues will activate the channel; if the binding site for these hormones is between the α and β_1 subunits, then the ligand should only activate if the BK_{Ca} channel is co-expressed with the β_1 subunit.

Methods

Cell culture

HEK 293 cells, stably expressing hSloα-V5-His6, were cultured in MEM supplemented with FBS 10% and 5 $\mu g \cdot mL^{-1}$ blasticidin to maintain selection. HEK 293 cells, stably co-expressing hSloα-V5-His6 and hSloβ_1-myc-His6, were cultured in MEM supplemented with FBS 10%, 5 $\mu g \cdot mL^{-1}$ blasticidin and 1 $mg \cdot mL^{-1}$ G418 (Invitrogen, Paisley, UK) for selection (De Wet *et al.*, 2006).

Prior to patch clamp recordings, 5×10^4 cells were seeded on to a 35 mm tissue culture dish and incubated for another 48 h at 37°C in 5% CO_2.

Whole cell patch-clamp recordings

Macroscopic currents were recorded from whole cells by use of standard patch clamp techniques using a PC501A patch clamp amplifier (Warner Instruments, Hamden, CT, USA). Patch electrodes were fabricated from borosilicate glass tubing and, when filled with the pipette solution detailed later, had resistances of 2–5 MΩ. Cells were viewed through an inverted microscope and continuously superfused with an extracellular solution containing in mM: 136 NaCl, 2.6 $CaCl_2$, 2.4 KCl, 1.2 $MgCl_2$, 15 HEPES, 10 glucose, titrated with 3 M NaOH to pH 7.4. Perfusion of the experimental chamber was at a rate of approximately 1 mL per minute, the volume of fluid within the chamber being held constant by continuous aspiration and the temperature of the perfusion fluid was maintained at 21°C. Patch electrodes were filled with a solution containing in mM: 110 KCl, 3.0 $MgCl_2$, 40 HEPES, 3 EGTA, titrated with 3 M KOH to pH 7.4. Atomic absorption spectroscopy revealed total Ca^{++} to be 7.04 μM, which is an equivalent free concentration of approximately 0.2 nM at room temperature and pH 7.35. $CaCl_2$ was added to this solution to adjust the free Ca^{++} concentration, where necessary, and the free concentration calculated using Maxchelator software (Pacific Grove, CA, USA).

Voltage-gated BK_{Ca} currents were recorded from cells clamped at a holding potential of −40 mV. Outward BK_{Ca} currents were evoked by stepwise changes in membrane potential lasting for 50 ms to a range of potentials (−30 to +60 mV). Raw output currents were filtered with a low band pass filter at 5 kHz, and digitized at 25 kHz, using a CED 1401 (Cambridge, UK) interface connected to a PC computer running winWCP (v4.08) patch clamp software (Strathclyde Electrophysiological Software, University of Strathclyde, Glasgow, UK).

For some of the analysis BK_{Ca} conductance was determined from whole cell current according to:

$$G_K = \frac{I_K}{V_m - V_K}$$

Where G_K is the conductance and I_K is the K channel current and V_m and V_K are the membrane potential at which the current was measured and the current reversal potential (–97 mV) respectively. The computed conductance was normalized to allow better comparison among cells of different sizes.

Preparation of isolated rat thoracic aorta rings

Adolescent male Sprague-Dawley rats, supplied by Charles River (London, UK; weight approximately 180–240 g), were killed by cervical dislocation. Thoracic aortae were removed and cleared of periadvential fat and then dissected into four rings (3–4 mm width). To obviate the effects of NO release, the endothelial layer was removed mechanically in half of the rings by gently rubbing the intimal surface of the artery lumen with a matchstick. All rings were then mounted in organ baths (AD Instruments, DMT, Oxford, UK) which were filled with warmed (37°C) and gas-equilibrated (95% O_2, 5% CO_2) Krebs' solution containing (in mmol·L^{-1}) $CaCl_2$ 1.6, $MgSO_4$ 1.17, EDTA 0.026, NaCl 130, $NaHCO_3$ 14.9, KCl 4.7, KH_2PO_4 1.18 and glucose 11. Isometric tension was measured with isometric transducers, digitized using a MacLab A/D converter (AD Instruments), stored, and displayed on a Macintosh computer. A pre-load tension of 7.5 mN was applied, and the rings were equilibrated for 60 min, changing the Krebs' solution fully every 15 min. Relaxation is expressed as a percentage of the steady-state tension (100%). Phenylephrine (1 nM–100 μM) was used to induce vasoconstriction of the aortic rings, and dose–response curves were generated. Rings were pre-contracted with a sub-maximal concentration of phenylephrine (1 μM). Following pre-contraction, cumulative concentrations (0.3, 1.0, 3.0, 10.0 and 30.0 μM) of oestrone or its derivatives were added at 8 min intervals. The pre-contraction in denuded and intact rings was not significantly different. The artery endothelium viability and integrity were confirmed by dilatory response of the ring to acetylcholine (1 nM–100 μM). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010).

Statistical analysis

Data were analyzed using GraphPad Prism version 5.03. Data are presented as the mean +/– the SEM unless otherwise stated.

Concentration response relationships for rubbed and unrubbed aortic rings were compared using two-way ANOVA. Where possible, sigmoidal curve fitting (Y = 100/(1 +

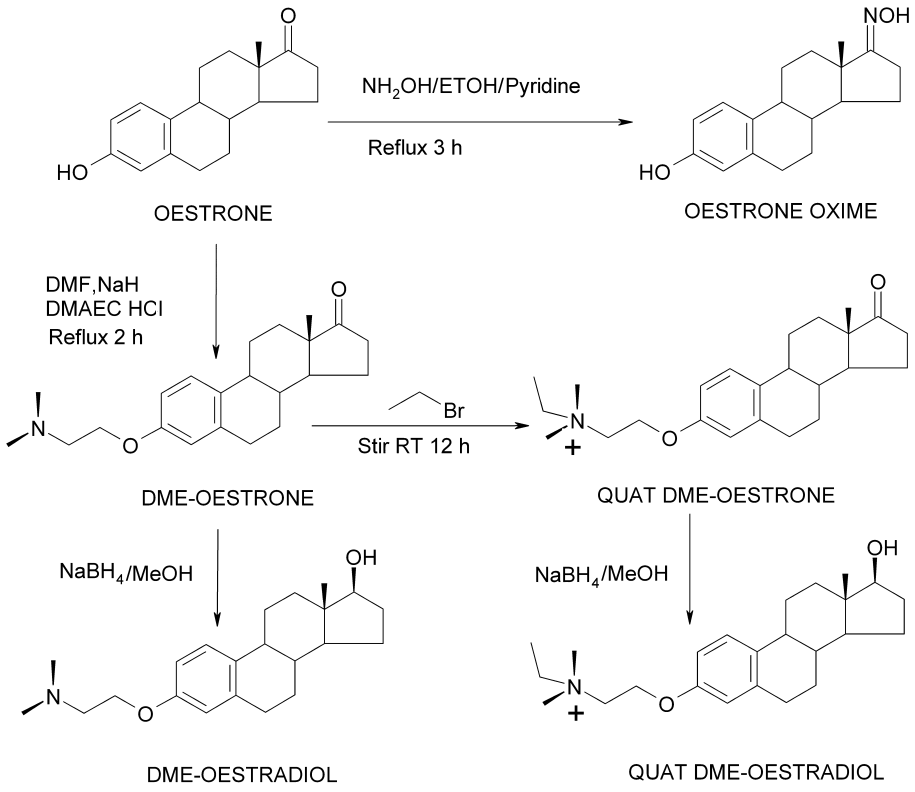

Figure 1

The synthesis of oestrogen derivatives from the parent compound oestrone. The derivatives contain the tamoxifen side chain. Structure and purity were confirmed by NMR, elemental analysis and IR spectroscopy.

$10^{((LogEC50-X)*Hill\ Slope)}$) was performed using GraphPad Prism software version 5.03 for Windows (GraphPad Software, La Jolla, CA, USA).

The effects of oestrogen derivatives on normalized peak current over time were compared with control recordings using a Kruskal–Wallis test followed by a Dunns multiple comparison test.

The conductance voltage data (G–V data) were fitted with the Boltzmann function using GraphPad Prism 5.03. The rate at which BK_{Ca} currents developed were determined by fitting a single exponential to the rising phase of the BK_{Ca} current using winWCP (v4.08) acquisition software.

Materials

Oestrogen derivatives were synthesized in the laboratories of the University of Brighton, the starting material was oestrone (Figure 1). DME-oestrone and DME-oestradiol contained the tamoxifen side chain whilst Quat DME-oestrone and Quat DME-oestradiol contained the ethyl bromide tamoxifen (EBT) side chain (Allen *et al.*, 2000; Sahebgharani *et al.*, 2001). TLC, elemental analysis, ^{1}H, ^{13}C NMR and IR spectroscopy confirmed structures and purity.

Results

The effect of novel steroidal oestrogens on isolated rat thoracic aorta rings

The novel oestrogens were tested for their ability to relax pre-contracted aorta. Aortic rings, with or without intact endothelium, contracted when exposed to phenylephrine

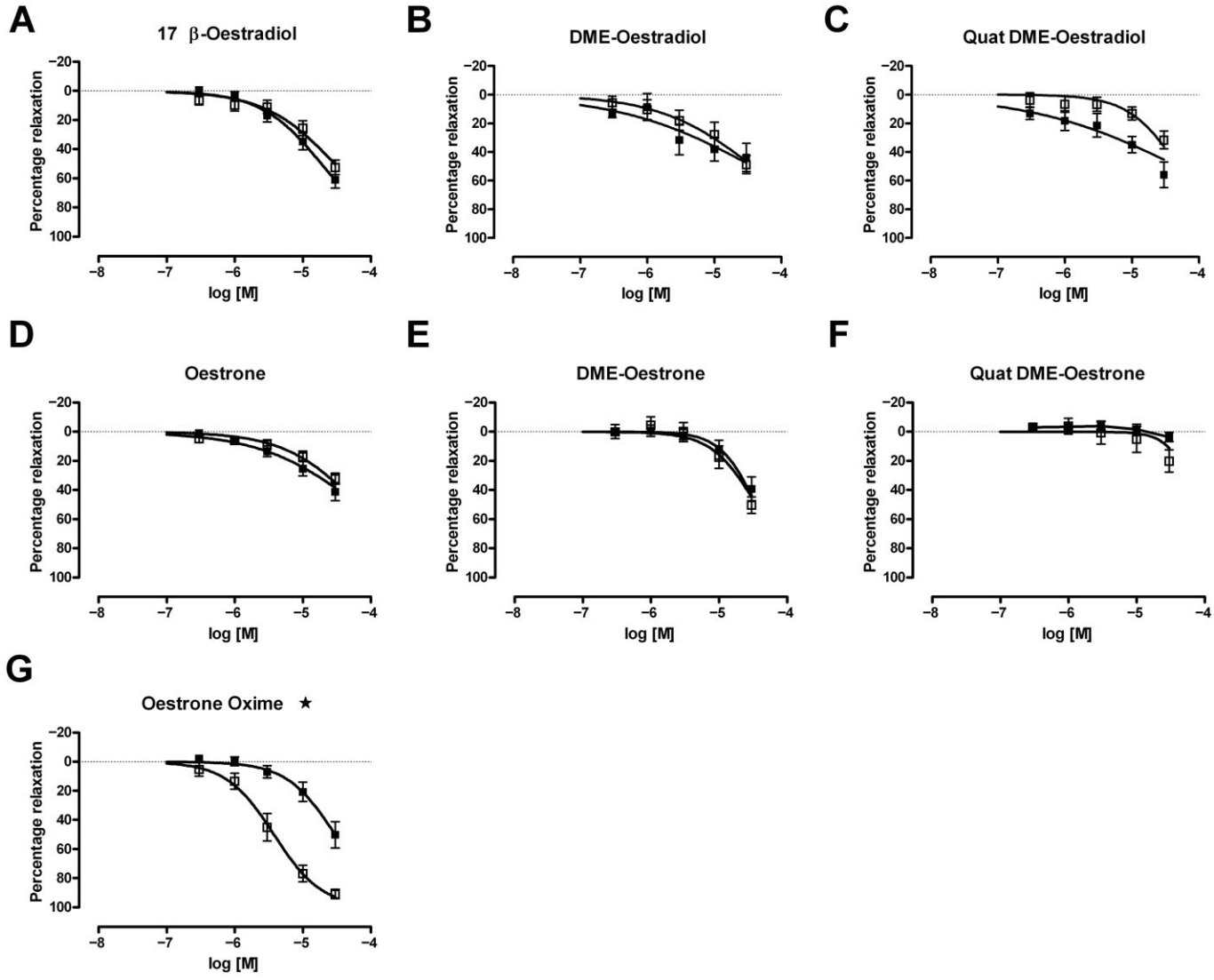

Figure 2

The effect of novel steroidal oestrogens on isolated rat thoracic aorta rings. Cumulative concentration response curves were constructed in rubbed (●) and unrubbed (□) aortic rings. Ligands tested were (A) 17β-oestradiol ($n = 12$), (B) DME-oestradiol ($n = 6$), (C) Quat DME-oestradiol ($n = 7$), (D) Oestrone ($n = 11$), (E) DME-oestrone ($n = 6$), (F) Quat DME-oestrone ($n = 6$), (G) Oestrone oxime ($n = 7$). Rubbed versus unrubbed rings were compared by two-way ANOVA ($^{*}P < 0.05$).

(1 nM–100 μM). Removing the endothelium made the aorta more sensitive to phenylephrine, shifting the pEC_{50} from 6.7 ± 0.15 to 7.71 ± 0.56. Most of the oestrone derivatives could produce a relaxant response in aortic rings pre-contracted with 1 μM phenylephrine (Figure 2). Oestrone oxime appeared to be the most potent analogue of oestrone, but this potency was dependent on the presence of an intact endothelium. The pEC50 was 5.42 ± 0.06 in aortic rings with an intact endothelium and was 4.53 ± 0.07 in aortic rings with the endothelium removed. Quat DME-oestrone appeared to lack relaxant activity, but Quat DME-oestradiol, DME-oestradiol and DME-oestrone were able to relax aorta in an endothelium independent manner. In our hands, only the relaxation by Quat DME-oestradiol showed reversibility with 100 nM iberiotoxin (IBTX) (Figure 3A), implying that the other analogues can induce relaxation independent of BK_{Ca} channels. The effects of oestrogens on arterial smooth muscle are known to involve both endothelium-dependent and endothelium-independent mechanisms, and it has been proposed that 17β-oestradiol can directly relax arteries by inducing nNOS activity within arterial smooth muscle. While reversibility of response by IBTX implies BK_{Ca} channel involvement, we wanted to be certain that any responses observed were direct rather than indirect, involving gaseous signalling molecules, such as NO. We therefore investigated the relaxant effects of Quat DME-oestradiol in aortic rings with endothelium removed and in the presence of a NOS inhibitor. These experiments demonstrated that 200 μM L-NAME partially reduced the relaxant effects of Quat DME-oestradiol, implying that Quat DME-oestradiol relaxations are at least in part due to nNOS activation (Figure 3B, C, D).

In summary, oestrone, DME-oestrone, oestradiol, DME-oestradiol and Quat DME-oestradiol caused relaxation of the aortic rings that were endothelium-independent. The exception was the oxime derivative of oestrone, which was more potent in unrubbed rings; this analogue, therefore, is likely to require endothelium to generate relaxing factors, such as NO. In contrast, Quat DME-oestrone was ineffective. IBTX reversed the relaxant effects of Quat DME-oestradiol, but the other derivatives were insensitive to IBTX, implying that these compounds primarily target non-BK_{Ca}-dependent relaxation mechanisms. This meant that Quat DME-

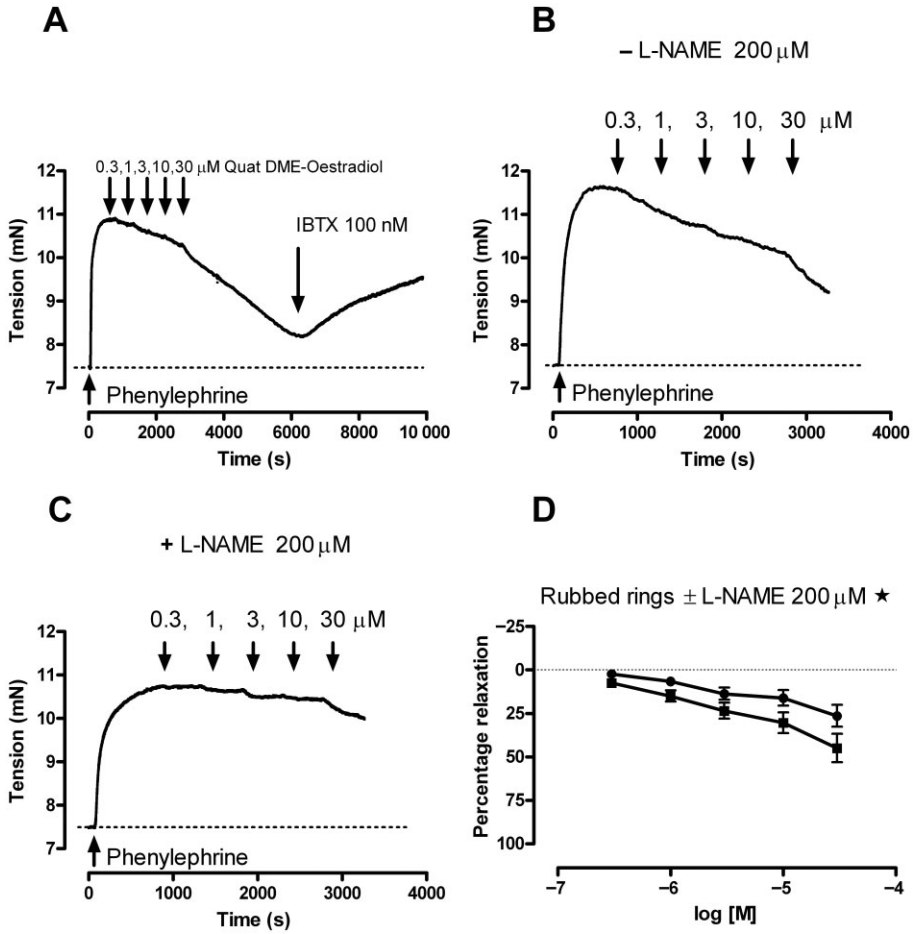

Figure 3

The effects of Quat DME-oestradiol on rat aortic rings. (A) An example of cumulative additions of Quat DME-oestradiol, as indicated, on pre-contracted aortic rings followed by 100 nM of IBTX. (B, C) An example of cumulative addition of Quat DME-oestradiol in the absence of L-NAME (B) and the presence of LNAME (C). L-NAME (●) reduced the response to Quat DME-oestradiol (■), and this is illustrated in (D). Relaxations in the presence and absence of L-NAME were compared by two-way ANOVA (*$P < 0.05$, $n = 8$).

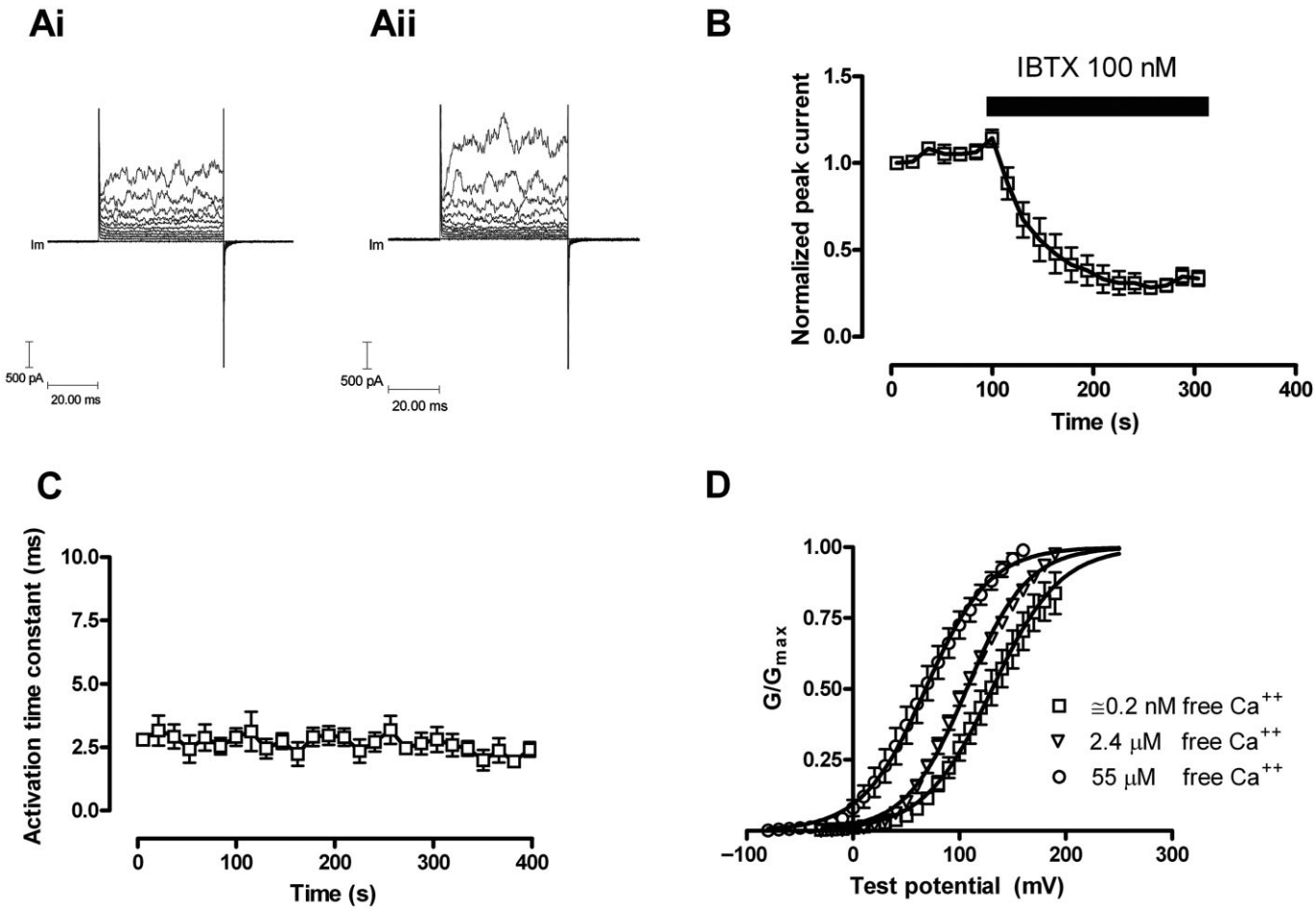

Figure 4

The change in evoked BK_{Ca} currents with time in HEK 293 cells expressing the α subunit (hSloα) of the BK_{Ca} channel. HEK 293 cells were voltage clamped at –40 mV and currents evoked by changing the voltage to a range of test potentials (–40 mV to 60 mV). (Ai) illustrates evoked currents in a HEK 293 cell at the beginning of the recording period; (Aii) illustrates evoked currents after 350 s of recording. (B) The blocking properties of IBTX on BK_{Ca} channels formed from α subunits alone. Currents were evoked at +60 mV from a holding potential of –40 mV (n = 5). (C) The activation time constant for currents evoked by changing the potential to +60 mV; the activation of the BK_{Ca} current could be fitted to a single exponential (n = 5). The free intracellular calcium ion concentration in panels Ai, Aii, B and C was 0.2 nM. (D) The effects of free intracellular Ca^{++} on the hSloα G–V relationship. Conductance was calculated from current and voltage, normalized to the maximum, and plotted error bars represent SEM. Solid curves represent fits to the Boltzmann function. V(1/2) for 0.2 nM, 2.4 μM and 55 μM free calcium were 130.9 ± 1.8 mV, 107 ± 0.7 mV and 68.3 ± 1.6 mV, respectively.

oestradiol was the derivative most likely to directly activate BK_{Ca} channels. However, additional experiments with the nNOS inhibitor suggest an indirect as well as a direct mechanism. To investigate the direct effects of our oestrogen derivatives on BK_{Ca} channels, we turned from a smooth muscle preparation to HEK 293 cells over-expressing BK_{Ca} channel subunits.

HEK 293 cells overexpressing the BK_{Ca} α subunit

HEK 293 cells over-expressing the α subunit of the BK_{Ca} channel were capable of generating robust BK_{Ca} currents (Figure 4Ai; Aii). This current was evoked by stepping the voltage to positive values, the current activated rapidly, did not appear to inactivate and was characteristically noisy.

Functional expression of BK_{Ca} channels in HEK 293 cells was also confirmed by estimating the single channel conductance underlying the evoked current. This was done by fluctuation analysis (Chung and Pulford, 1993), which is a statistical analysis comparing the mean and variance of the current and enables the estimation of the underlying unitary current. Using this method, the underlying single-channel current was estimated to be 35.6 ± 8.5 pA (n = 4), which corresponds to a single channel conductance of 223 pS, close to values reported previously (Lippiat *et al.*, 2000). BK_{Ca} currents are sensitive to internal free calcium ion concentration, which proved to be true in our HEK 293 cells expressing the α subunits of the BK_{Ca} channel. Raising the free intracellular concentration of Ca^{++} ions from approximately 0.2 nM to 55 μM shifted the $V_{0.5}$ for the voltage con-

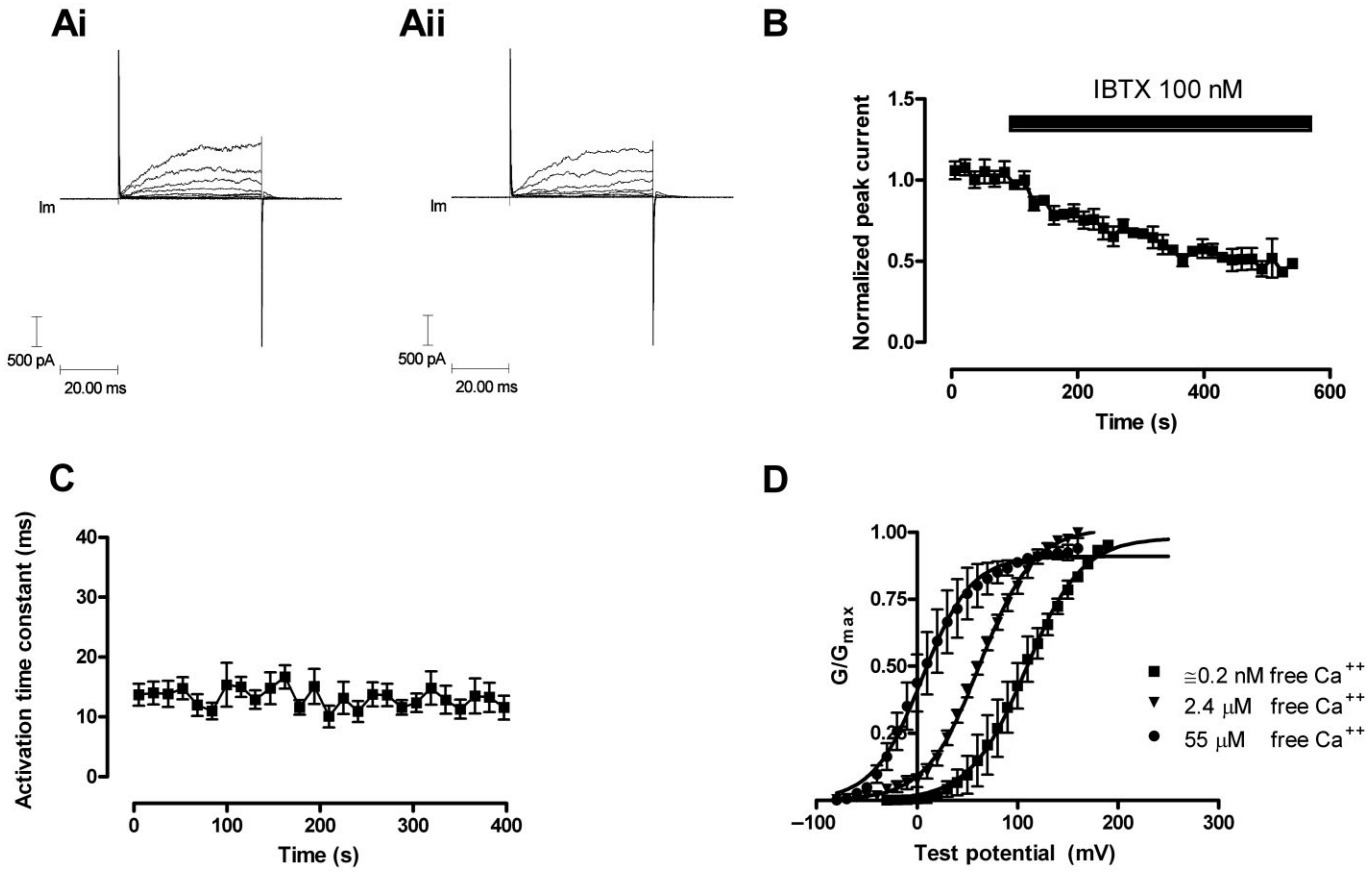

Figure 5

The change in evoked BK_{Ca} currents with time in HEK 293 cells expressing the α and β_1 subunit (hSloα + β_1) of the BK_{Ca} channel. HEK 293 cells were voltage clamped at –40 mV and currents evoked by changing the voltage to a range of test potentials (–40 to 60 mV). (Ai) illustrates evoked currents in a HEK 293 cell at the beginning of the recording period; (Aii) illustrates evoked currents after 350 s of recording. (B) The blocking properties of IBTX on BK_{Ca} channels formed from α + β_1 subunits. Currents were evoked at +60 mV from a holding potential of –40 mV. (n = 6). (C) The activation time constant for currents evoked by changing the potential to +60 mV, the activation of the BK_{Ca} current could be fitted to a single exponential (n = 5). The β_1 subunit has slowed macroscopic current kinetics at the subnanomolar [Ca^{++}] employed during these experiments. The free intracellular calcium ion concentration in panels Ai, Aii, B and C was 0.2 nM. (D) The effects of free [Ca^{++}] on the hSloα+β_1 G-V relationship. Conductance was calculated from current and voltage, normalized to the maximum, and plotted error bars represent SEM. Solid curves represent fits to the Boltzmann function. V(1/2) for 0.2 nM, 2.4 μM and 55 μM free calcium were 108.4 ± 4 mV, 62.6 ± 1.3 mV and 5.1 ± 3.1 mV respectively.

ductance curve approximately 60 mV in the hyperpolarizing direction (Figure 4D). In our hands, the BK_{Ca} currents tended to run up over time, but we saw no appreciable change in the activation rates of the BK_{Ca} currents (Figures 4C, 6A). Finally, peak BK_{Ca} currents were sensitive to inhibition by 100 nM IBTX, the time course of which could be fitted to a single exponential with a rate of block equal to (2.5 ± 0.48) × $10^5 \cdot M^{-1} \cdot s^{-1}$ (Figure 4B).

HEK 293 cells over-expressing the BK_{Ca} α and β_1 subunit

HEK 293 cells over expressing the α and β_1 subunits of the BK_{Ca} channel were also capable of generating robust BK_{Ca} currents (Figure 5). This current was similarly evoked by stepping the voltage to positive values, did not appear to inactivate and was characteristically noisy. The β_1 subunit slowed the macroscopic current kinetics at the subnanomolar [Ca^{++}] employed during these experiments (Figure 5Ai, Aii, C), which is similar to that reported with mSlo (Cox and Aldrich, 2000). Raising the free intracellular concentration of Ca^{++} ions from approximately 0.2 nM to 55 μM shifted the $V_{0.5}$ for the voltage conductance curve approximately 100 mV (Figure 5D), and at all concentrations of calcium the α and β_1 expressing HEK 293 cells displayed a $V_{0.5}$ more negative than their α alone expressing counterparts. In these experiments, the BK_{Ca} currents ran down with time (Figure 7A), which was different from that observed with HEK 293 cells only expressing the α subunits. Finally, peak BK_{Ca} currents were sensitive to 100 nM IBTX. Currents were inhibited by the toxin, the time course of which could be fitted to a single exponential with a rate of block equal to (2.18 ± 0.65) × $10^4 \cdot M^{-1} \cdot s^{-1}$. This is appreciably slower than the block in HEK 293 cells only expressing α subunits, but in line with that reported by other investigators (Garcia-Valdes *et al.*, 2001).

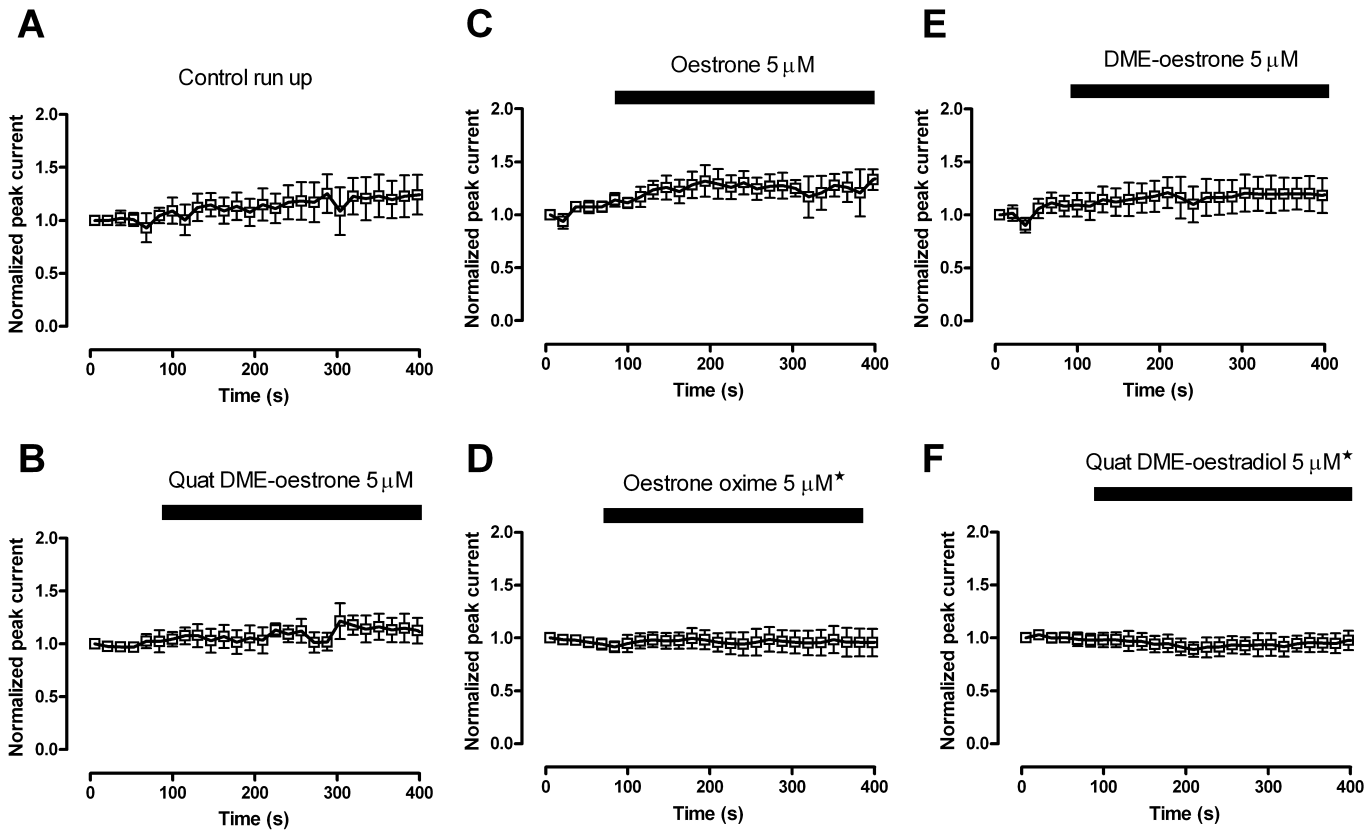

Figure 6

The effect of oestrone derivatives on BK_{Ca} currents evoked in HEK 293 cells over-expressing α subunits of the BK_{Ca} channel. The normalized peak currents over time curves were compared with control and found not to be significantly different apart from Oestrone oxime and Quat DME-oestradiol, which demonstrated a small but significant inhibition. The currents were evoked by stepping the membrane potential from –40 mV to +60 mV. (*P < 0.5 compared with control data, Kruskal–Wallis test followed by a Dunns multiple comparison test).

The effect of oestrogen derivatives on BK_{Ca} currents in HEK 293 cells expressing BK_{Ca} α subunits

BK_{Ca} channels were studied in whole cell recordings from HEK 293 cells expressing the α subunit. None of the ligands tested increased the whole cell currents (Figure 6). These currents were tested with very low intracellular calcium (≈0.2 nM free [Ca^{++}]), as oestrogens have been reported to be better activators of BK_{Ca} channels when the free [Ca^{++}] is low and the open probability for the channel is small. This finding was expected as we, and others, have reported previously that the β_1 subunit is required for BK_{Ca} activation (Valverde *et al.*, 1999a; De Wet *et al.*, 2006).

The effect of oestrogen derivatives on BK_{Ca} currents in HEK 293 cells expressing both BK_{Ca} α and β_1 subunits

Whole cell recordings from cells expressing both α and β_1 subunits responded differently. Three compounds (oestrone, oestrone oxime and Quat DME-oestradiol), seem to activate the BK_{Ca} current, the rest being largely inactive (Figures 7 & 8). Interestingly, oestrone oxime and Quat DME-oestradiol were able to activate BK_{Ca} currents, even though, in HEK 293 cells only expressing the α subunit, these compounds were weak inhibitors (Figure 6).

Further experiments showed that while these ligands could activate BK_{Ca} currents, they were unable to change activation rates (Figure 8).

Discussion and conclusions

The acute effects of 17β-oestradiol have been studied on vascular smooth muscle (Ruehlmann *et al.*, 1998; Nakaya *et al.*, 2007; Asano *et al.*, 2010; Zhang *et al.*, 2010), and a vasorelaxant effect over a large range of concentrations (10 pM–1 mM) (Tep-areenan *et al.*, 2003) was demonstrated.

We studied the acute effects of novel oestrogen derivatives as vasorelaxants in order to obtain a better understanding of their mode of action and selectivity for BK_{Ca} channels. We found that most of our oestrogen derivatives can relax pre-contracted aorta with or without an intact endothelium. Oestrone oxime had a particularly potent endothelium-dependent effect, and further studies will be required in order to determine the mechanism of action of this derivative. Only Quat DME-oestradiol caused a relaxation that was sensitive to 100 nM IBTX, suggesting that most of these compounds have other relaxant effects. These properties were not unexpected, as it has been shown that oestradiol can inhibit L-type calcium channels in vascular smooth muscle

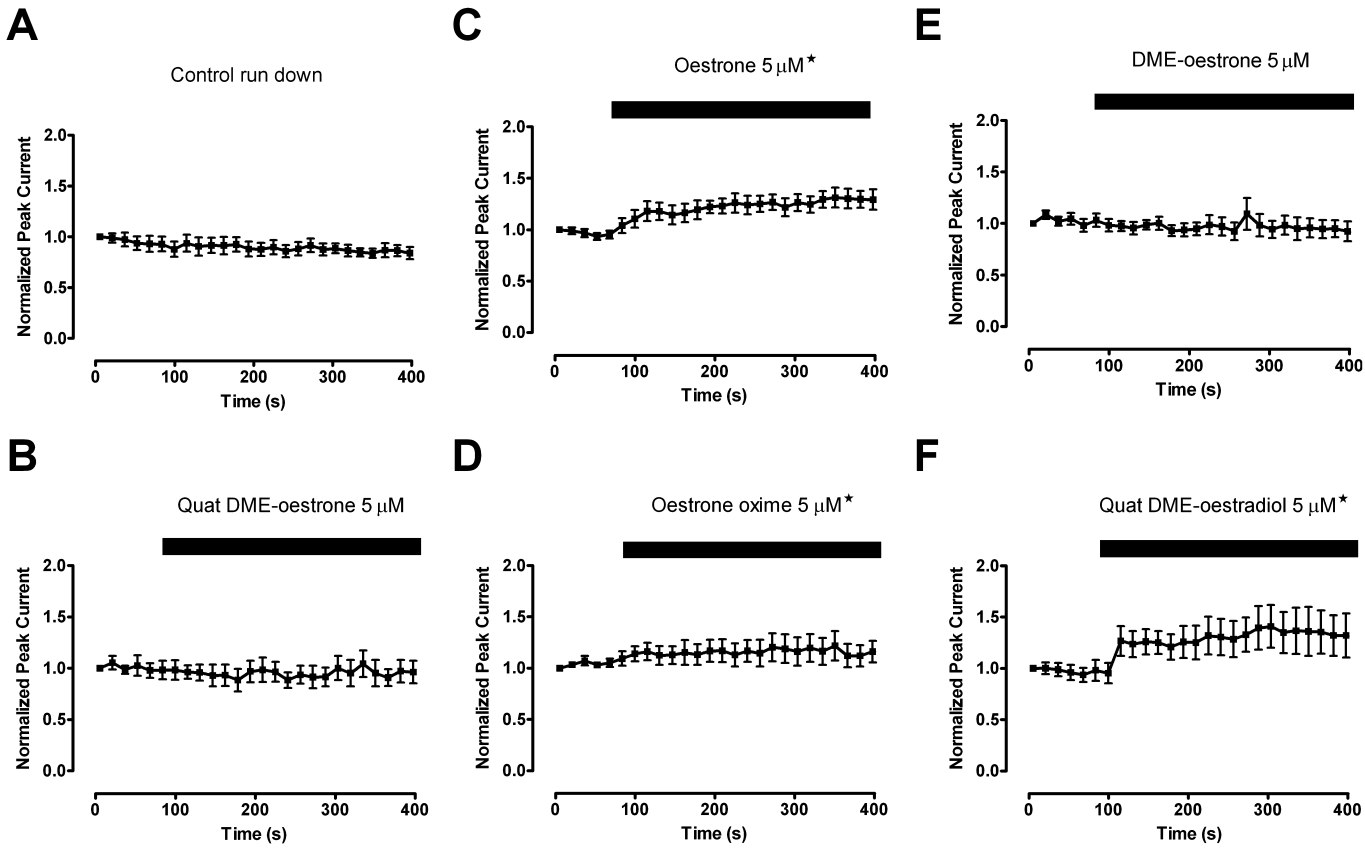

Figure 7

The effect of oestrone derivatives on BK_{Ca} currents evoked in HEK 293 cells over expressing α and β_1 subunits of the BK_{Ca} channel. The normalized peak currents over time curves were compared to control and found not to be significantly different, apart from oestrone, Oestrone oxime and Quat DME-oestradiol, which demonstrated a significant enhancement in peak current. The currents were evoked by stepping the membrane potential from –40 mV to +60 mV. (*$P < 0.5$ compared with control data, Kruskal–Wallis test followed by a Dunns multiple comparison test).

(Nakajima *et al.*, 1995; Ruehlmann *et al.*, 1998; Cairrao *et al.*, 2012) and modulate endothelial NOS (Haynes *et al.*, 2000; Broughton *et al.*, 2010; Batenburg *et al.*, 2012) and second messengers, such as cGMP (White *et al.*, 1995; 2002), all of which generate a complicated picture and explain the lack of a correlation between the ability of oestrogens to relax aortic rings and activate BK_{Ca} channels. Recently, it has been suggested that oestrogens can regulate nNOS within arterial smooth muscle (Han *et al.*, 2007; Royal *et al.*, 2011) and in support of this the relaxant effects of Quat DME-oestradiol were reduced by L-NAME, a non-specific NOS inhibitor. Because Quat DME-oestradiol is membrane impermeant, these data suggest targeting of membrane receptors that modulate nNOS within aortic smooth muscle. Since BK_{Ca} channels can be activated by NO (Ahern *et al.*, 1999) and regulated by second messengers, such cGMP (Zhou *et al.*, 2001), it cannot be assumed that relaxant reversal by IBTX is proof of a direct action of these ligands on the BK_{Ca} channel. Consequently, we turned to the simpler HEK 293 cell expression system to study the direct action of these compounds on the BK_{Ca} channel, as these cells have little, if any, NOS activity (Schmidt *et al.*, 2001; Fang and Silverman, 2009), and do not express ERα, ERβ (Leung *et al.*, 2006; Chantzi *et al.*, 2011) or GPR30/GPER receptors (Filardo *et al.*, 2007).

Previously, we have shown that 17β-oestradiol can activate BK_{Ca} channels in planar lipid bilayers (De Wet *et al.*, 2006; de Wet *et al.*, 2009). This activation requires the presence of the β_1 subunit and indeed, Bayesian analysis demonstrates that each channel requires at least two β_1 subunits for this to occur. While these experiments provided information about the stoichiometry of α and β_1 subunits required for oestrogen activation, they did not provide information about the binding site. We postulate that the putative binding site for oestrogens would be at the interface between the β_1 subunit and the α subunit. In support of this, Liu *et al.* (2010) have suggested that the S0 domain of the α subunit is in close proximity to the TM2 of the β_1 subunit, and TM1 of the β_1 subunit is associated with S1 and S2 domains of the α subunit (Liu *et al.*, 2010). Furthermore, Morrow *et al.* (2006) have demonstrated that deletion of the extracellular N terminal of the murine α subunit did not prevent the α and β_1 subunits interacting. The β_1 subunit could still slow the activation and deactivation of BK_{Ca} channel gating, but N terminal deletion did effect BK_{Ca} modulation by 17β-oestradiol (Morrow *et al.*, 2006).

If our hypothesis is correct, and the binding site for oestrogens resides between the extracellular N terminal of the α subunit and the extracellular loop between TM1 and 2 of the

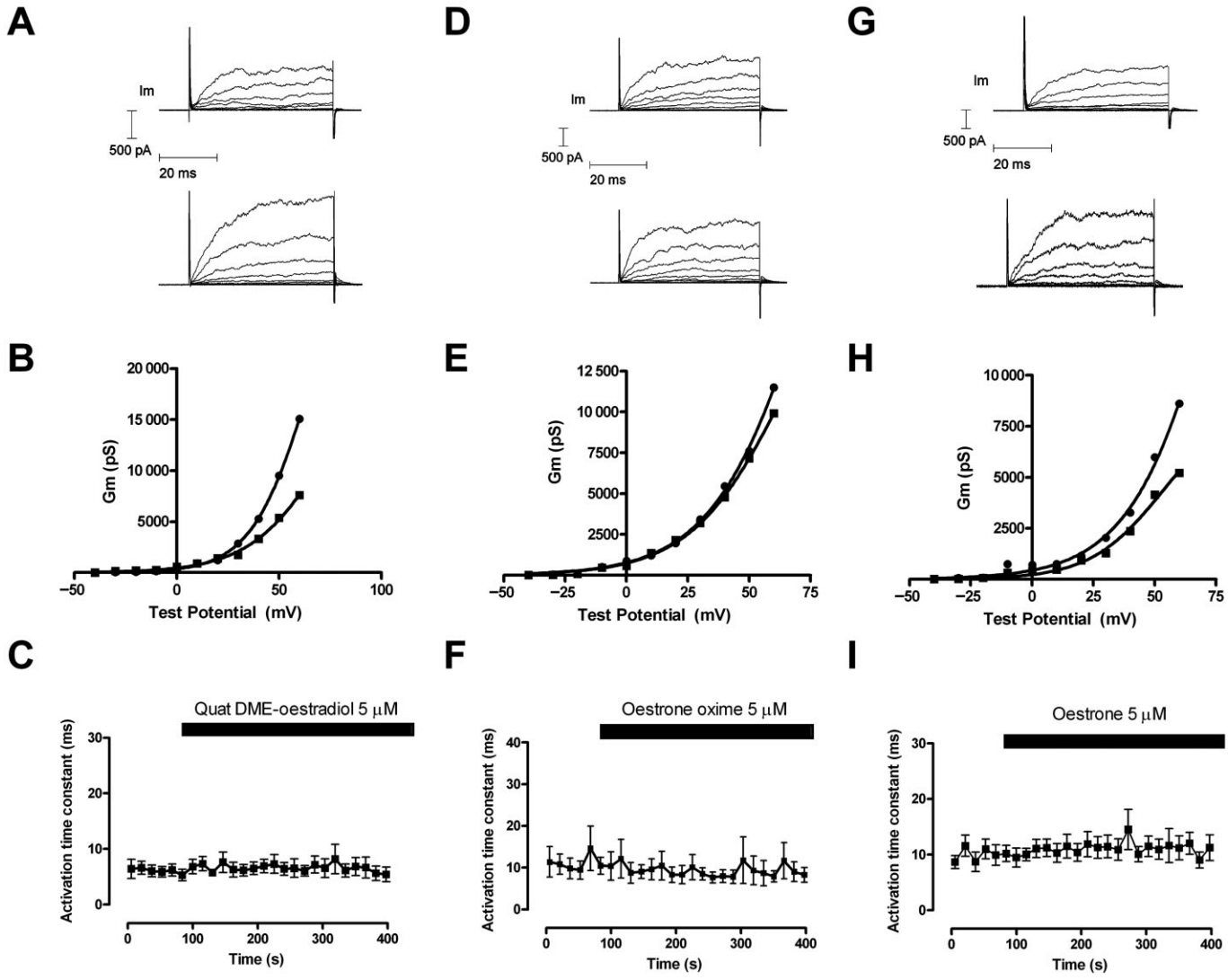

Figure 8

The effect of Quat DME-oestradiol (A), oestrone oxime (D) and oestrone (G) on superimposed evoked currents from HEK 293 cells over-expressing BK_{Ca} α and β_1 subunits. Cells were held at −40 mV and currents evoked by changing the potential to a range of voltages up to +60 mV. The top trace represents evoked currents before the application of oestrogen (5 μM), the trace immediately below represents evoked currents after application oestrogen. The pipette solution was nominally Ca^{++} free. The conductance voltage relationship for currents evoked in A, D, and G are shown in B, E and H respectively. (■) represents the control G–V relationship, while (●) represents the G–V relationship in the presence of 5 μM ligand. The activation time constant for the evoked currents at +60 mV, was fitted to a single exponential and did not change during the application of Quat DME-oestradiol (C) Oestrone oxime (F) and oestrone (I).

β_1 subunit (Figure 9), then membrane-impermeable, as well as membrane-permeable oestrogens will be able to activate BK_{Ca} channels. In our hands, Quat DME-oestradiol is, indeed, able to activate channels in HEK cells expressing α and β_1 subunits, but does not alter the macroscopic gating kinetics. These data agree with the investigation by Morrow *et al.*, who postulated that the β_1 subunit ability to confer oestrogen sensitivity is separate from its ability to alter the macroscopic gating kinetics of BK_{Ca} currents. Further, steroidal bile acids, such as lithocholate, are also postulated to bind to β_1 subunits prior to the activation of BK_{Ca} channels (Bukiya *et al.*, 2008a,b).

Oestrogens are effective activators of the BK_{Ca} channel complex when the free intracellular calcium concentration is low (Valverde *et al.*, 1999a; De Wet *et al.*, 2006). At the intracellular concentrations of free calcium employed in these experiments (<0.2 nM), the BK_{Ca} complex of α and β_1 subunits is thought to be functionally unligated by Ca^{++} (Meera *et al.*, 1996; Nimigean and Magleby, 2000), and the channel is reported to be purely voltage activated at calcium ion concentrations less than 0.1 μM. Consequently, it appears that the BK_{Ca} channel is sensitive to oestrogen activation even in the absence of free intracellular calcium. This has important implications, as BK_{Ca} activators based on oestrogens would

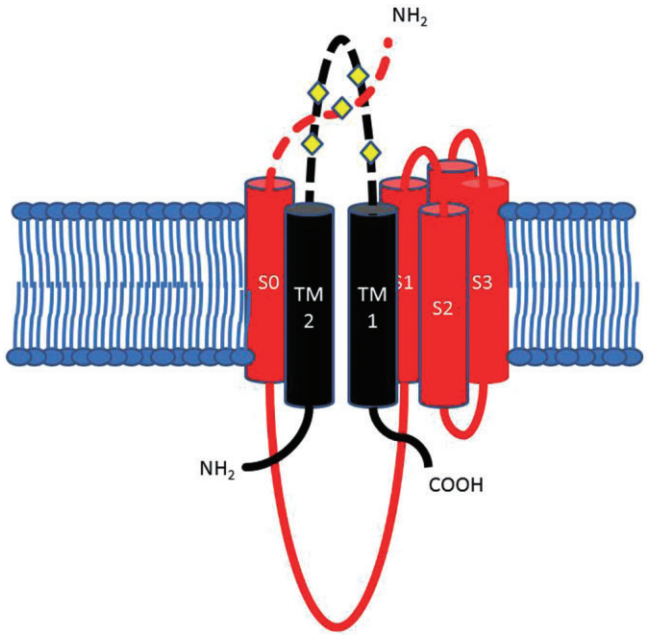

Figure 9

The potential extracellular binding site between the extracellular loop of the β_1 subunit and the Nterminal of S0 domain of the α subunit. β_1 TM1 and TM2 are black, with TM2 next to S0. Residues 16–20 of the extracellular N-terminal segment preceding S0 have been modelled as a random coil, crossing over the S3–S4 loop. This diagram represents the potential interface and binding site between one of the β_1 subunits and one of the four α subunits making up a BK_{Ca} channel. The diamonds represent the extracellular cysteines.

otherwise require high levels of intracellular Ca^{++} to activate the channel.

The cysteines (C430 and C911) within the BK_{Ca} α subunit seem to show redox sensitivity, and this is responsible for observed run down in isolated patches (Zhang *et al.*, 2006). In addition, hydrogen peroxide virtually eliminates physiological activation of the channel by targeting a cysteine residue near the Ca^{++} bowl of the BK_{Ca} α subunit (Tang *et al.*, 2004). Also, antioxidants such as Tempol (Xu *et al.*, 2005; 2006) are known to activate BK_{Ca} channels composed of α subunits. Finally, BK_{Ca} β subunits have a cysteine-rich extracellular loop connecting the two transmembrane segments (Figure 9), while the extracellular loops of the β_3 subunits are involved in a redox-sensitive gating (Zeng *et al.*, 2003). Consequently, it is likely that these extracellular loops are a redox-sensitive region for all four β subunits and can confer additional redox sensitivity to the BK_{Ca} channel.

Oestrogens can act as antioxidants by capturing a ·OH hydroxyradical to produce a phenoxyl radical. Further scavenging of a OH produces a quinol, which can be converted back to the parent oestrogen by NADPH-dependent enzymes (Prokai *et al.*, 2005; Prokai-Tatrai *et al.*, 2008). Consequently, it is possible that some, if not all, of the effects of oestrogen could be due to an antioxidant effect in the region of the β_1 and α subunit interface. These antioxidant properties could account for the effects of phenolic oestrogens, such as oestrone and 17β-oestradiol, but cannot account for the effects of oestrogens substituted in the three position, such as Quat DME-oestradiol, as these compounds cannot form quinols and act as antioxidants. It appears unlikely, therefore, that the BK_{Ca} effects of oestrogens are due to oestrogenic antioxidant activity.

Our studies, to date, reveal that the 17β position on D ring of steroidal oestrogens is important. Previous work has shown that 17β-oestradiol, which possesses an axial secondary alcohol in the 17β position, is a BK_{Ca} activator (Valverde *et al.*, 1999a; De Wet *et al.*, 2006; Morrow *et al.*, 2006), whereas the stereoisomer 17α-oestradiol, which possesses an axial secondary alcohol pointing in the opposite direction, is inactive (De Wet *et al.*, 2006). Oestrone has an equatorial or planar carbonyl group and has weak BK_{Ca}-activating properties. Thus, it appears that as oxygen moves from an axial 17α position through a planar configuration to an axial 17β position, activity increases. Consistent with this, oestrone oxime, which possesses a planar oxime group, is the weakest activator of BK_{Ca} currents. This compound produced marginal activation only noticeable at +60 mV, a potential that cells hardly ever experience. Paradoxically, this fits with the aortic ring data, which demonstrated that oestrone oxime was the most potent relaxant, but that this relaxation was endothelium dependent and was not reversed by IBTX; in short, the relaxation is not a BK_{Ca} effect.

In summary, we have synthesized novel oestrogen derivatives, some of which are membrane impermeable. Most of these compounds relax aortic smooth muscle through an endothelium-independent mechanism or induction of NOS in smooth muscle. A number of these derivatives directly activate BK_{Ca} currents in HEK 293 cells expressing both α and β_1 subunits, the putative smooth muscle channel complex, and this contributes to muscle relaxation. The putative binding site is between the N terminal of the α subunit and the extracellular loop of the β_1 subunit. An antioxidant mode of action seems unlikely.

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Conflict of interest

The authors declare no conflicts of interest.

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