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RESEARCH PAPER

Bisphenol A activates Maxi-K ($K_{Ca}1.1$) channels in coronary smooth muscle

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Background and purpose: Bisphenol A (BPA) is used to manufacture plastics, including containers for food into which it may leach. High levels of exposure to this oestrogenic endocrine disruptor are associated with diabetes and heart disease. Oestrogen and oestrogen receptor modulators increase the activity of large conductance Ca^{2+}/voltage-sensitive K^+ (Maxi-K; $K_{Ca}1.1$) channels, but the effects of BPA on Maxi-K channels are unknown. We tested the hypothesis that BPA activates Maxi-K channels through a mechanism that depends upon the regulatory β1 subunit.
Experimental approach: Patch-clamp recordings of Maxi-K channels were made in human and canine coronary smooth muscle cells as well as in AD-293 cells expressing pore-forming α or α plus β1 subunits.
Key results: BPA (10 μM) activated an outward current in smooth muscle cells that was inhibited by penitrem A (1 μM), a Maxi-K blocker. BPA increased Maxi-K activity in inside-out patches from coronary smooth muscle, but had no effect on single channel conductance. In AD-293 cells with Maxi-K channels composed of α subunits alone, 10 μM BPA did not affect channel activity. When channels in AD-293 cells contained β1 subunits, 10 μM BPA increased channel activity. Effects of BPA were rapid (<1 min) and reversible. A higher concentration of BPA (100 μM) increased Maxi-K current independent of the β1 subunit.
Conclusions and implications: Our data indicate that BPA increased the activity of Maxi-K channels and may represent a basis for some potential toxicological effects.
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Keywords: oestrogen; endocrine disruptor; large conductance Ca^{2+}/voltage-sensitive potassium channel; *KCNMA1*; *KCNMB1*; $K_{Ca}1.1$; penitrem A

Abbreviations: BPA, bisphenol A; GFP, green fluorescent protein; LAD, left anterior descending artery; Maxi-K, large conductance Ca^{2+}/voltage-sensitive K^+ channel; NP_o, number of channels × open probability

Introduction

Although there is no clear consensus on detrimental health effects of bisphenol A (BPA), evidence suggests it possesses endocrine disrupting activity. BPA may affect fetal and neonatal development (Vandenberg *et al.*, 2007), reproductive function (Savabieasfahani *et al.*, 2006), metabolism (Hugo *et al.*, 2008) and carcinogenesis (Wetherill *et al.*, 2002; 2005). A recent study demonstrated an association between BPA levels and adverse cardiovascular diagnoses (Lang *et al.*, 2008). Environmental exposure through polycarbonate plastic bottles, epoxy linings of canned foods and dental sealants makes BPA a potential public health hazard.

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Bisphenol A interacts with nuclear oestrogen receptors, although much less potently than 17β-oestradiol (Krishnan *et al.*, 1993; Kuiper *et al.*, 1997). BPA also exerts non-genomic effects, independent of nuclear oestrogen receptors. For example, BPA rapidly disrupts intracellular Ca^{2+} homeostasis in a variety of cells (Nadal *et al.*, 2000; Alonso-magdalena *et al.*, 2005; Walsh *et al.*, 2005). These data suggest that BPA influences the activity of cellular transport mechanisms, perhaps including ion channels. Importantly, however, there are no data available regarding effects of BPA on the function of an ion channel *per se*. Large conductance Ca^{2+}/voltage-sensitive K^+ (Maxi-K) channels offer themselves as an excellent model to test for interactions with BPA, as they are known to be oestrogen-sensitive (Valverde *et al.*, 1999).

Maxi-K channels, also known as $K_{Ca}1.1$ (see Alexander *et al.*, 2009), are formed by four pore-forming α subunits encoded by *KCNMA1* (Butler *et al.*, 1993); when β1 subunits are also present, sensitivity to oestrogenic substances is conferred (Dick *et al.*, 2001; 2002; Dick and Sanders, 2001; Coiret *et al.*,

2007; Valverde *et al.*, 1999; Duncan, 2005). The majority of Maxi-K channels in vascular smooth muscle contain this regulatory β1 subunit, encoded by *KCNMB1* (Tanaka *et al.*, 1997), which increases Ca^{2+}- and voltage sensitivity (Mcmanus *et al.*, 1995; Meera *et al.*, 1996). Maxi-K channels (Nelson *et al.*, 1995) and the β1 subunit (Brenner *et al.*, 2000) play key roles in regulating smooth muscle excitability. The present study was designed to determine whether BPA activates Maxi-K channels and to ascertain the role of the β1 subunit. We hypothesized that BPA would activate Maxi-K channels through a mechanism that depends upon the β1 subunit. To test this hypothesis, Maxi-K channel currents were analysed in smooth muscle cells and in AD-293 cells expressing only α or α + β1 subunits.

Methods

Smooth muscle cells

All animal care and experimental protocols followed the guidelines in the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, 1996). Three coronary artery smooth muscle preparations were used; these include cells: (i) freshly isolated from the canine left anterior descending (LAD) artery; (ii) cultured from the canine LAD; and (iii) cultured from human hearts. Canine LAD arteries were collected from dogs killed under surgical anaesthesia for unrelated experiments. LAD segments were incubated for 30 min at 37°C in 20 units·mL^{-1} papain in low Ca^{2+} HEPES buffer containing (mM) 135 NaCl, 5 KCl, 0.36 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES and 5 Tris; pH 7.4. After washing, tissue was incubated for another 30 min at 37°C in low Ca^{2+} HEPES buffer containing 2 mg·mL^{-1} type II collagenase and 1 mg·mL^{-1} hyaluronidase. The solution was gently agitated with a fire-polished Pasteur pipette to disperse single cells. This suspension was filtered through 100 μm nylon mesh and centrifuged at 600× *g* for 5 min. For patch-clamp recordings, the pellet was resuspended with low Ca^{2+} HEPES buffer and kept at 4°C. Patch-clamp recordings were performed within 8 h of cell dispersion.

For primary cell culture, solutions mentioned above also contained 100 units·mL^{-1} penicillin and 100 mg·mL^{-1} streptomycin, and the pellet was resuspended in Medium 231 with smooth muscle growth supplement (Invitrogen, Carlsbad, CA, USA). Cells were plated on 1% gelatin-coated dishes and incubated in a 5% CO_2 incubator at 37°C. Media was replaced once or twice as cells grew to confluence; after subculturing, the medium was changed every 2–3 days. To induce differentiation in confluent cultures, growth medium was switched to Medium 231 containing smooth muscle differentiation supplement (Invitrogen), which was replaced every 2–3 days.

Human coronary artery smooth muscle cells (Genlantis, San Diego, CA, USA) were cultured in Medium 231 with growth and differentiation supplements, the latter added at ~80% confluence.

Transient transfection

AD-293 cells (Stratagene, La Jolla, CA, USA) were grown in DMEM supplemented with 10% fetal bovine serum, 100 units·mL^{-1} penicillin and 100 mg·mL^{-1} streptomycin. Antibiotic-free media was added 1 h prior to transfection. Plasmids encoding bovine *KCNMA1* (ENSG00000156113) and *KCNMB1* (ENSG00000145936) were kindly provided by Dr Michael J. Davis (University of Missouri; Wu *et al.*, 2008). A plasmid encoding green fluorescent protein (GFP), pmaxGFP, was purchased from AMAXA (Gaithersburg, MD, USA). Cells grown to 50–70% confluence were cotransfected with plasmids containing GFP and *KCNMA1* with or without *KCNMB1* using Lipofectamine LTX with PLUS reagent (Invitrogen). Transfection with GFP plasmid only was used as a negative control. The optimal molar ratios of *KCNMA1* to *KCNMB1* were determined to be >1:3. After incubation with DNA–lipid complexes in opti-MEM for 6 h, the medium was changed to DMEM supplemented with fetal bovine serum and antibiotic. Current recordings from GFP-positive cells were performed 1–2 days after transfection.

Electrophysiology

Freshly isolated cells were placed directly in a recording chamber on an inverted microscope, while cultured cells were plated on cover glass that was transferred to the recording chamber. The recording chamber had a volume of 0.2–0.3 mL and solutions were fed by gravity at a rate of 2–3 mL·min^{-1}. BPA (Sigma-Aldrich, St. Louis, MO, USA; product number 133027) and penitrem A (MP Biomedicals, Solon, OH, USA) were dissolved in DMSO and diluted 1:1000 or 1:10 000 for experiments. For whole-cell recordings, cells were suffused with a solution containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES and 5 Tris; pH 7.4. Pipette solution contained (mM) 140 KCl, 1 $MgCl_2$, 1 EGTA, 10 HEPES and 5 Tris; pH 7.1. The Ca^{2+} concentration of the pipette solution was brought to 100 nM by adding 281 μM $CaCl_2$ (http://www.stanford.edu/~cpatton/maxc.html). GΩ seals were made with heat-polished borosilicate pipettes (BF150-86; Sutter Instruments, Novato, CA, USA) that had tip resistances of 3–5 MΩ. The cell was ruptured with suction and membrane capacitance determined from the capacity transient (pClamp 9; Axon Instruments, Sunnyvale, CA, USA). Series resistance and capacitance were compensated as much as possible using the amplifier circuitry (PC-505B; Warner Instruments, Hamden, CT, USA). In whole-cell records, mean current during the last 80 ms of the test pulse was measured for analysis. For single channel recordings, cells were suffused with the 140 mM K^+ solution described above and pipettes had tip resistances of 5–10 MΩ. After forming GΩ seals, patches were excised for inside-out recordings in symmetrical 140 mM K^+. Channel conductance was determined from peak-to-peak differences in all-points amplitude histograms; the number of channels × open probability (NP_o) was calculated by dividing the mean current in a trace by the single channel amplitude. Currents were low pass filtered at 1 kHz and digitized at 5 kHz.

RT-PCR

Total RNA was extracted using PureLink RNA Mini kit and PureLink DNase (Invitrogen). One microgram of template was reverse transcribed with Oligo$(dT)_{20}$ primers using the SuperScript First-Strand Synthesis System (Invitrogen). PCR primers were designed against *KCNMA1* (NM_001014797;

5′-GAGGATGCCTCGAATATCA-3′ and 5′-AGCTCGGGATGTT TAGCAGA-3′; product size 119 bp) and *KCNMB1* (NM_ 004137; 5′-GCCGGGAAGACTAAATGATC-3′ and 5′-TGGGAT GTAGGAGCACTG-3′; product size 357 bp). PCR amplification was performed in 50 μL reactions containing 1× PCR buffer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.4 μM each primer, 1 μL template cDNA and 2 units Platinum Taq DNA polymerase (Invitrogen). The protocol included: denature at 94°C for 2 min, 40 cycles of 94°C for 15 s, 55°C for 30 s and 70°C for 1 min; the reaction was terminated at 4°C. As a positive control, human brain cDNA (Ambion, Austin, TX, USA) was used. PCR products were separated by electrophoresis on 2% agarose with ethidium bromide and images captured using a GeneFlash unit (Syngene, Frederick, MD, USA).

Statistics

Data are presented as the mean and standard error of *n* number of cells or membrane patches. When two values were compared, a *t*-test was used. When more than two values were compared, one-way ANOVA was used. Current–voltage relationships were analysed by two-way repeated measures ANOVA. Bonferroni *post hoc* tests followed ANOVA to determine where differences exist. In all tests, $P < 0.05$ was considered significant.

Results

The patch-clamp technique was used to measure K^+ current in coronary smooth muscle cells and determine the effect of BPA on Maxi-K current using penitrem A, a potent and selective inhibitor of Maxi-K channels (Knaus *et al.*, 1994). Whole-cell recordings revealed prominent Maxi-K currents in smooth muscle cells cultured from the canine LAD coronary artery (Figure 1A, left). Time-dependent current increased sharply at potentials positive to +40 mV and was 'noisy', indicating channels with a large unitary conductance were responsible. Mean current density at +100 mV was 35 ± 12 $pA{\cdot}pF^{-1}$ ($n = 5$). Whole-cell current increased with the application of 10 μM BPA (Figure 1A, middle; mean current density at +100 mV was 64 ± 24 $pA{\cdot}pF^{-1}$, $P < 0.05$). The effect of BPA was not voltage-dependent. Penitrem A (1 μM) blocked nearly all of the whole-cell current in smooth muscle cells cultured from the canine LAD coronary artery (Figure 1A, right; current density at +100 mV was 3 ± 2 $pA{\cdot}pF^{-1}$, $P < 0.05$). In separate experiments ($n = 4$) when 1 μM penitrem A was added first, whole-cell current was reduced and could not be increased by 10 μM BPA (Figure 1B; current density at +100 mV was 33 ± 10, 6 ± 3 and 4 ± 2 $pA{\cdot}pF^{-1}$, for control, with penitrem A, and penitrem A plus BPA, respectively; $P < 0.05$). The voltage template used to elicit currents is shown in Figure 1C. Group data for both experiments in cultured canine coronary smooth muscle cells are shown in Figure 1D and E. Cultured canine coronary smooth muscle cells were derived from three different dogs and a characterization of the smooth muscle phenotype of these cells is available in the *Supporting information*.

Excised patch recordings were used to determine the effect of BPA on Maxi-K channel NP_o in smooth muscle cells freshly isolated from the canine LAD coronary artery. Inside-out recordings were made in symmetrical 140 mM K^+ at a patch potential of +80 mV with free Ca^{2+} buffered to 100 nM. BPA (10 μM) increased Maxi-K channel NP_o 742 ± 302% ($n = 10$ cells from 4 dogs; $P < 0.05$; Figure 2A and B). Single channel conductance was not affected by 10 μM BPA (100 ± 3% of control; Figure 2C).

To determine the role of the β1 subunit in responses to 10 μM BPA, whole-cell recordings were performed on AD-293 cells transfected with GFP and α or α + β1 subunits. Cells transfected with GFP only had very small outward currents (Figure 3A; current at +100 mV was 0.04 ± 0.01 nA; $n = 3$). Cells transfected with Maxi-K α subunits demonstrated large outward currents with fast activation and little or no inactivation (Figure 3B). The current and time constant of activation at +100 mV were 5.4 ± 1.0 nA and 1.0 ± 0.3 ms respectively ($n = 11$). Cells transfected with both Maxi-K α and β1 subunits possessed large outward currents with slower activation and demonstrated little or no inactivation (Figure 3C). Current and time constant of activation at +100 mV were 5.0 ± 1.0 nA and 13 ± 7 ms respectively ($n = 7$). In cells with Maxi-K channels composed of α subunits alone, 10 μM BPA did not substantially increase current (Figure 3D; current at +100 mV was 101 ± 13% of control). When Maxi-K channels were composed of α and β1 subunits, 10 μM BPA increased current by 53 ± 12% (Figure 3E). Penitrem A (1 μM) blocked virtually all Maxi-K current, regardless of whether channels were composed of α or α + β1 subunits (93 ± 4% and 93 ± 2% inhibition respectively). Group data illustrating the effects of BPA and penitrem A on Maxi-K channels composed of α or α + β1 subunits are shown in Figure 3F and G.

Inside-out patch recordings were made to determine the effect of 10 μM BPA on Maxi-K channels composed of α or α + β1 subunits in AD-293 cells. NP_o and single channel conductance were determined at a patch potential of +40 mV in solutions containing 140 mM K^+ and 100 nM free Ca^{2+}. BPA had no significant effect on NP_o or single channel conductance in Maxi-K channels composed of α subunits alone (Figure 4A and C). NP_o was 123 ± 20% of control in the presence of 10 μM BPA (Table 1; $n = 6$; P = NS). In contrast, in Maxi-K channels containing the β1 subunit, 10 μM BPA increased NP_o (Figure 4B and D). BPA increased the NP_o of Maxi-K channels containing the β1 subunit 204 ± 41% (Table 1; $n = 6$, $P < 0.05$). BPA had no effect on conductance in channels composed of α or α + β1 subunits (99 ± 1% and 103 ± 2% of control respectively).

Experiments were performed on α and α + β1 Maxi-K channels in AD-293 cells to determine whether the effects of BPA were concentration-dependent and reversible. The response to BPA depended upon the molecular composition of Maxi-K channels (Figure 5A). When Maxi-K channels contained the β1 subunit, 1, 10 and 100 μM BPA increased current. This was sharply different from the response of Maxi-K channels composed of α subunits alone, where neither 1 nor 10 μM BPA significantly increased current. Surprisingly, however, at a concentration of 100 μM, BPA increased significantly the current in AD-293 cells expressing only the α subunit. The effect of BPA to increase whole-cell Maxi-K current was rapid and readily reversible (Figure 5B). Current through channels composed of α + β1 subunits increased dramatically during exposure to 10 μM BPA (from 4.5 ± 0.4 to 5.6 ± 0.6 nA;

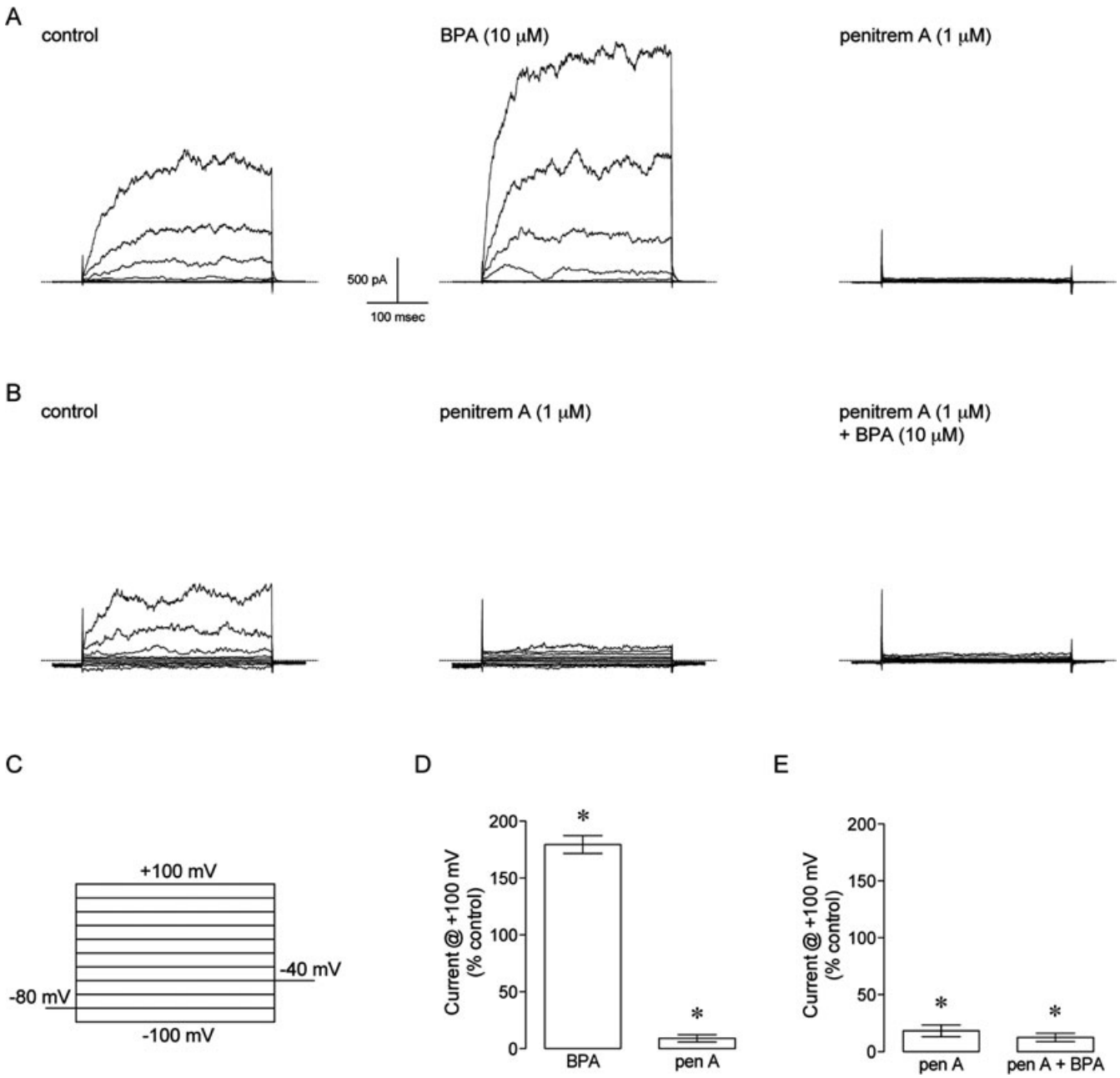

Figure 1 Bisphenol A (BPA) increases large conductance Ca^{2+}/voltage-sensitive K^+ channel (Maxi-K) current in smooth muscle cells cultured from the canine coronary artery. (A) Representative currents are shown under control conditions, with the application of 10 μM BPA, and with 1 μM penitrem A. (B) Currents from a representative cell are shown under control conditions, with 1 μM penitrem A, and with 10 μM BPA in the continued presence of penitrem A. (C) The voltage template used to elicit currents in this and subsequent figures. (D) Group data ($n = 5$) demonstrate the sensitivity of currents to BPA and penitrem A in cultured coronary artery smooth muscle cells. (E) Penitrem A prevented BPA-induced increase in Maxi-K current ($n = 4$). *$P < 0.05$ versus control by one-way ANOVA.

$P < 0.05$, $n = 4$). In cells expressing α subunits only, 10 μM BPA modestly increased current from 4.4 ± 0.3 to 4.6 ± 0.1 nA ($n = 4$). There was no difference in the time course for the onset of BPA effect between cells expressing α or α + β1 subunits (time constants of 2.0 ± 0.1 vs. 2.1 ± 0.1 min). Washing the cells with BPA-free solution rapidly returned current towards baseline.

Whole-cell patch-clamp experiments were also performed on cultured human coronary smooth muscle cells (Figure 6A). The smooth muscle phenotype of these cells is demonstrated in the *Supporting information*. The lower concentrations of BPA (1 and 10 μM) did not increase Maxi-K current; however, 100 μM BPA increased current significantly (Figure 6B). Maxi-K current at +100 mV under control conditions was 843 ± 218 pA and increased to 1446 ± 393 pA with application of 100 μM BPA ($n = 6$; $P < 0.05$). Based on concentration–response results with BPA in AD-293 cells expressing α or α + β1 subunits (Figure 5A), we investigated whether cultured human coronary smooth muscle cells expressed the β1 subunit. Thus, RT-PCR was used to analyse Maxi-K channel

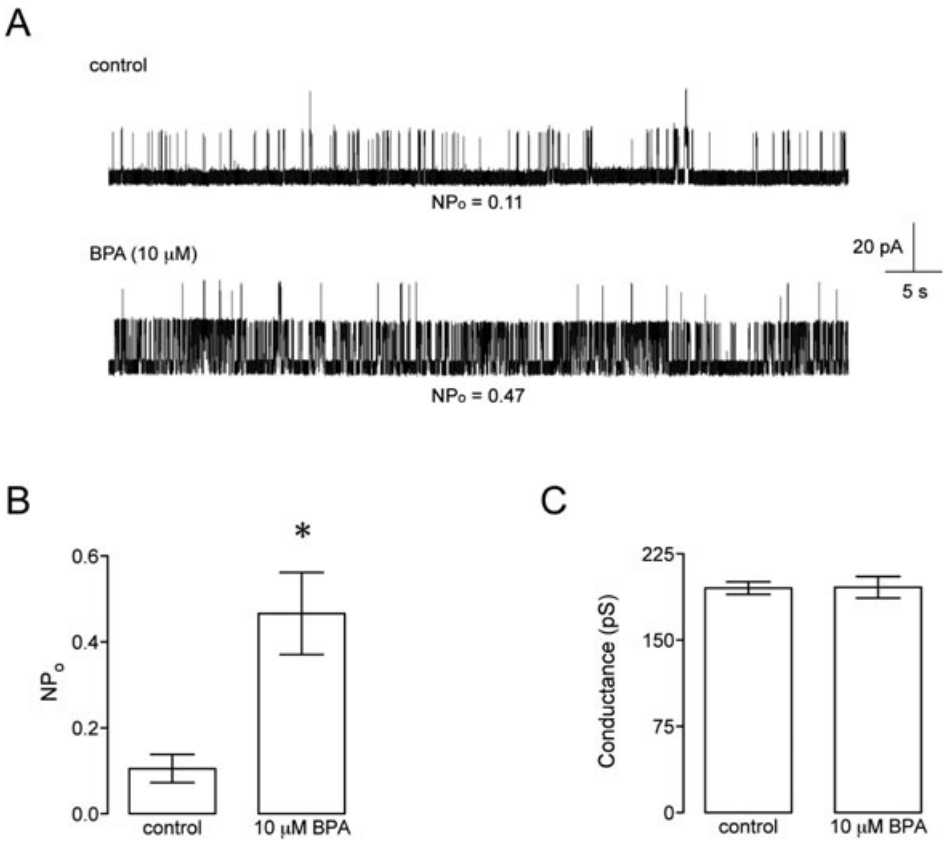

Figure 2 Bisphenol A (BPA) increases large conductance Ca^{2+}/voltage-sensitive K^+ (Maxi-K) channel the NP_o (number of channels × open probability) in smooth muscle cells freshly isolated from the canine coronary artery. (A) Representative 1 min recordings of Maxi-K channel activity in an inside-out patch before and after exposure to 10 μM BPA are shown. Patch potential was +80 mV and currents were recorded in symmetrical 140 mM K^+ with 100 nM free Ca^{2+}. BPA increased NP_o, but had no effect on single channel amplitude. (B) Group data ($n = 10$ cells from 4 dogs) demonstrate that 10 μM BPA increases NP_o. *$P < 0.05$ by paired *t*-test. (C) Group data ($n = 10$) show that BPA has no effect on single conductance.

subunit mRNA expression. Cultured human coronary smooth muscle cells expressed *KCNMA1* mRNA, but mRNA for *KCNMB1* (encoding for β1 subunits) was undetectable (Figure 6C). A positive control, using human brain cDNA, however, demonstrated the applicability of the *KCNMB1* primers (Figure 6C). Moreover, Western blots and immunocytochemistry experiments also failed to detect the β1 subunit in these cells (data not shown). Thus, the activation of Maxi-K channels in cultured human coronary smooth muscle cells by a minimum concentration of 100 μM BPA is likely to be due to a lack of *KCNMB1* expression (Yang *et al.*, 2009).

Discussion and conclusions

These studies demonstrate that BPA, an oestrogenic endocrine disruptor, increased Maxi-K channel current via a non-genomic mechanism. The activation of Maxi-K channels by BPA depends, in a concentration-dependent manner, on the presence of the regulatory β1 subunit. Several lines of evidence support these novel observations. First, BPA-induced activation of Maxi-K channels was observed in freshly isolated and cultured coronary smooth muscle cells, as well as in AD-293 cells expressing cloned Maxi-K channel subunits. Furthermore, BPA increased Maxi-K channel NP_o without affecting single channel conductance. Second, BPA-induced Maxi-K channel activation was detected in both whole-cell and single channel experiments. Moreover, results of the single channel studies clearly indicate that the effect of BPA is non-genomic, as the inside-out patches of membrane were removed from the cell. Third, the effects of BPA were rapid, reversible and concentration-dependent. While we hypothesized that BPA activation of Maxi-K channels would depend on the presence of the regulatory β1 subunit, this prediction was only partially upheld by our studies. Specifically, the β1 subunit played an essential role in activation of Maxi-K channels by BPA when concentrations of 1 and 10 μM were tested; however, at a concentration of 100 μM, BPA activated Maxi-K channels without the β1 subunit. The latter finding suggests that the α subunit itself might be the target of BPA and that the β1 subunit may simply enhance the response. The activating effect of BPA on Maxi-K channels and the transformative influence of the β1 subunit represent original observations of multifaceted mechanisms that merit additional study. Because of the potential public health impact of BPA exposure and the fact that so many cell types express Maxi-K channels, the implications of these studies may be widespread.

Previous studies have demonstrated that the activation of Maxi-K channels by oestrogenic substances, including 17β-oestradiol (Valverde *et al.*, 1999). Thus, to find that BPA

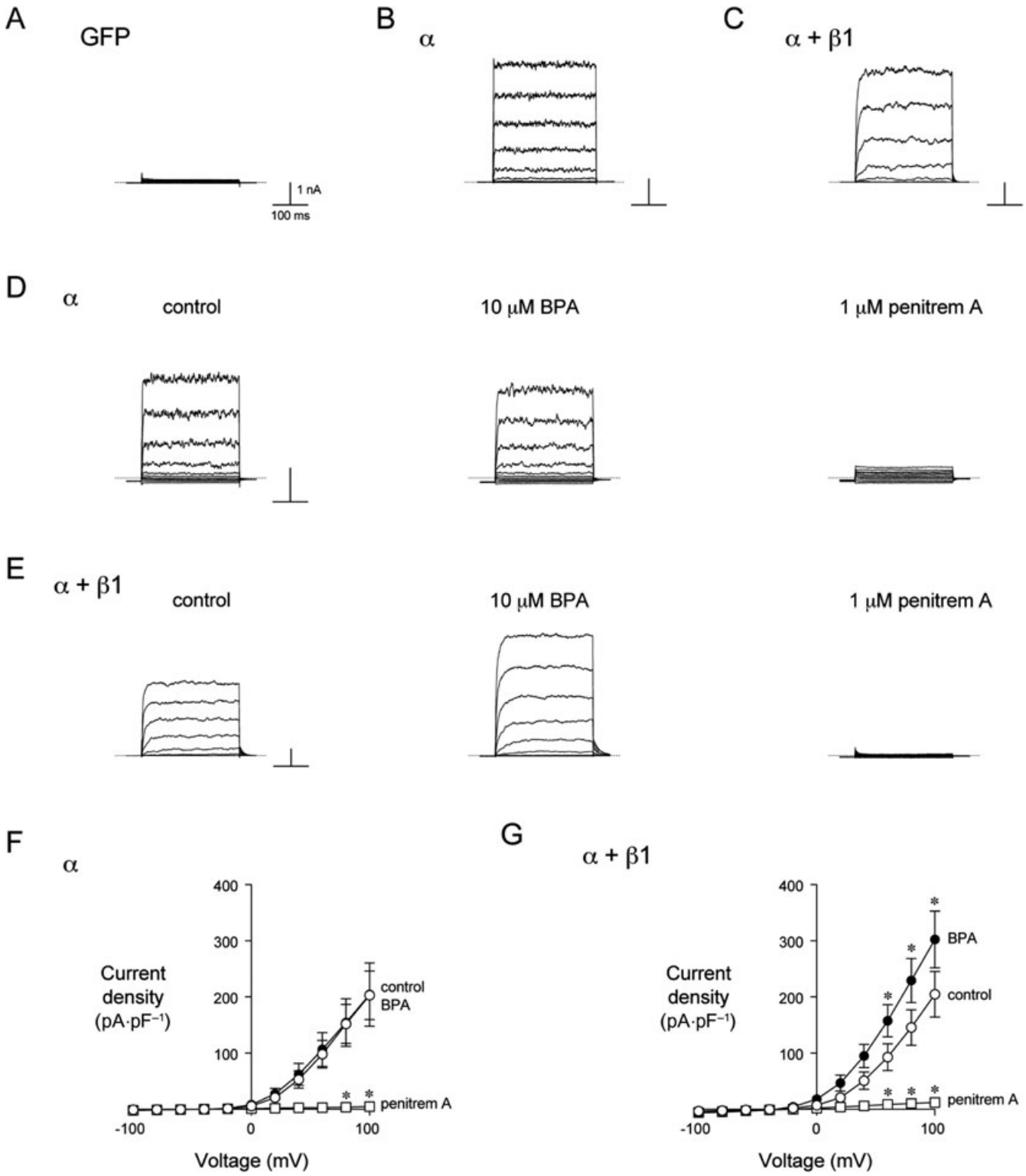

Figure 3 Large conductance Ca^{2+}/voltage-sensitive K^+ channel (Maxi-K) β1 subunit confers sensitivity to 10 μM bisphenol A (BPA). Currents were measured from AD-293 cells transfected with green fluorescent protein (GFP) (A) and Maxi-K channels composed of α (B) or α + β1 (C) subunits. Voltage template used is shown in Figure 1C. Very little whole-cell current was observed in cells expressing GFP only. Cells transfected with the α subunit demonstrated large outward currents with fast activation and little or no inactivation. Cells expressing α + β1 subunits demonstrated large outward currents with slower activation. (D) Records from a representative cell transfected with the Maxi-K α subunit show that current is unaffected by 10 μM BPA, but blocked by 1 μM penitrem A. (E) Current in a cell transfected with α + β1 subunits increased with application of 10 μM BPA and was blocked by 1 μM penitrem A. (F and G) Group data illustrate the effect of 10 μM BPA and 1 μM penitrem A on current in cells expressing α ($n = 11$) or α + β1 ($n = 7$) subunits. *$P < 0.05$ versus control by two-way repeated measures ANOVA.

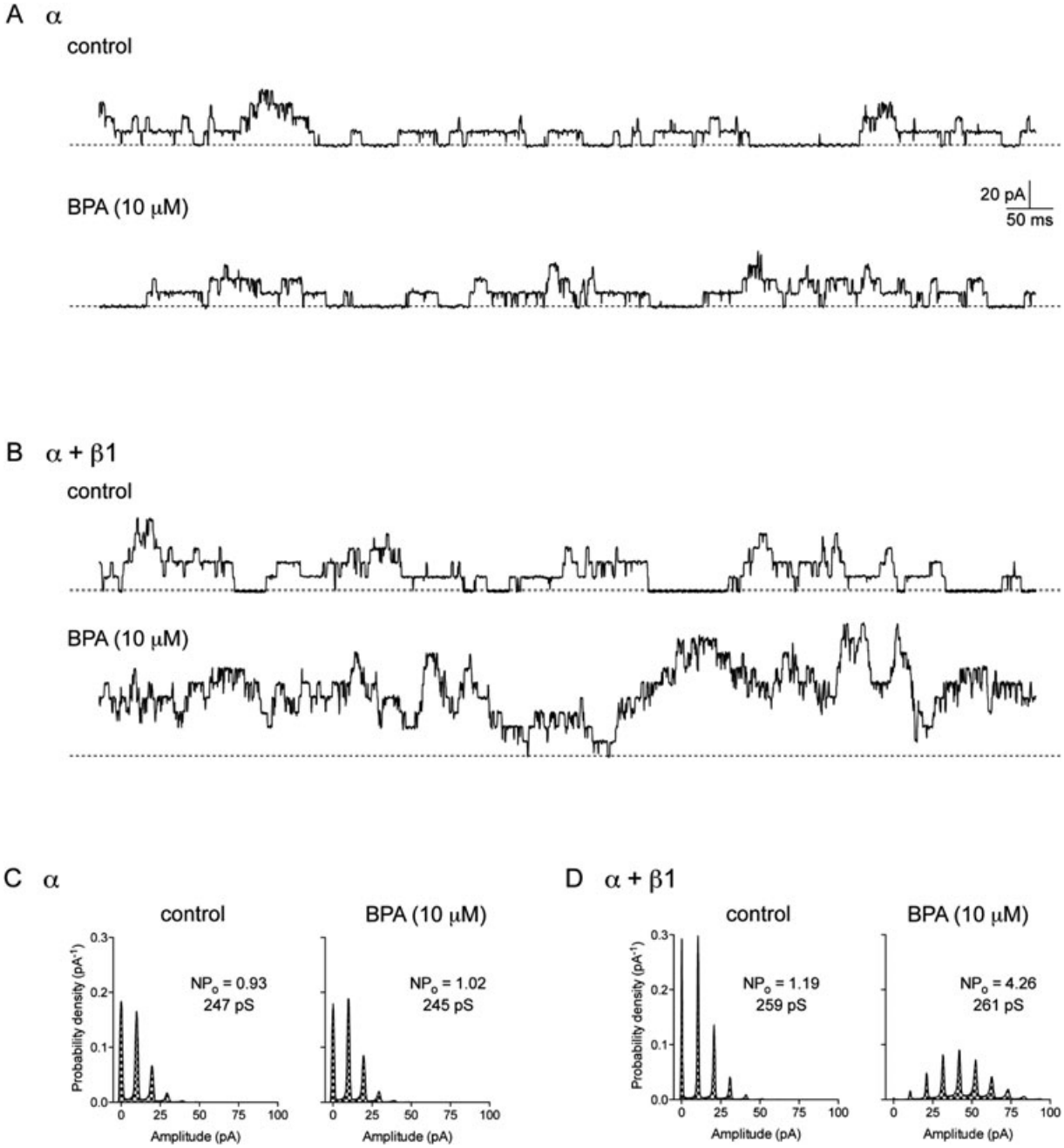

Figure 4 Bisphenol A (BPA) (10 μM) increases NP_o (number of channels × open probability) of large conductance Ca^{2+}/voltage-sensitive K^+ (Maxi-K) channels containing the β1 subunit. Representative 1 s recordings of Maxi-K channel activity in inside-out patches from AD-293 cells expressing Maxi-K α (A) or α + β1 (B) subunits before and after exposure to 10 μM BPA. Patch potential was +40 mV in symmetrical 140 mM K^+ solutions containing 100 nM free Ca^{2+}. (C and D) All-points amplitude histograms derived from 1 min recordings of patches shown in (A and B). BPA increased the NP_o of Maxi-K channels containing the β1 subunit and had no effect on single channel conductance.

Table 1 β1 subunit determines the response of Maxi-K channels to 10 μM BPA

Subunit(s)	*Condition*	*NP_o*	*Conductance (pS)*
α	control	0.75 ± 0.27	248 ± 8
(*n* = 6)	BPA	1.06 ± 0.42	246 ± 9
α + β1	control	0.96 ± 0.11	253 ± 15
(*n* = 6)	BPA	2.97 ± 0.58 (*P* < 0.05)	260 ± 13

BPA, bisphenol A; Maxi-K, large conductance Ca^{2+}/voltage-sensitive K^+ channel; NP_o, number of channels × open probability.

activated Maxi-K channels may not seem surprising at first glance. However, the lack of a relationship between the oestrogenic nature of a compound and its effect on Maxi-K channels makes such presumptions difficult. For example, tamoxifen, an oestrogen receptor modulator with mixed agonist and antagonist properties, activates Maxi-K channels more potently and efficaciously than 17β-oestradiol (Dick *et al.*, 2001). Moreover, the pure anti-oestrogen ICI 182,780 also activates Maxi-K channels (Dick, 2002). Studies generally agree, however, that the β1 subunit confers sensitivity upon

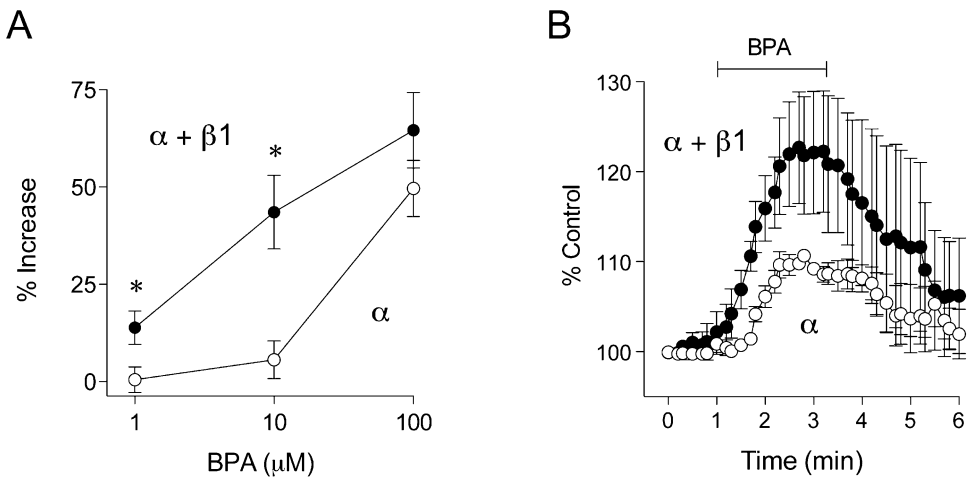

Figure 5 The activation of large conductance Ca^{2+}/voltage-sensitive K^+ (Maxi-K) channels by bisphenol A (BPA) is concentration-dependent and reversible. (A) Maxi-K currents were measured in the whole-cell configuration after stepping membrane potential from –80 to +100 mV. Cells expressing Maxi-K channels composed of α or α + β1 subunits were studied (n = 7–12) under control conditions and after exposure to 1, 10 and 100 μM BPA. Current was increased relative to control at all three concentrations in cells expressing α + β1 subunits; current was increased only with 100 μM BPA in cells expressing the α subunit alone. *Significant differences between α and α + β1 by two-way ANOVA. (B) Maxi-K (α) and Maxi-K (α + β1) currents were elicited by stepping the membrane potential from –80 to +100 mV for 300 ms every 10 s. BPA (10 μM) was applied for 2 min and washed out. Current increased rapidly with exposure to BPA and returned towards baseline with washout (n = 4).

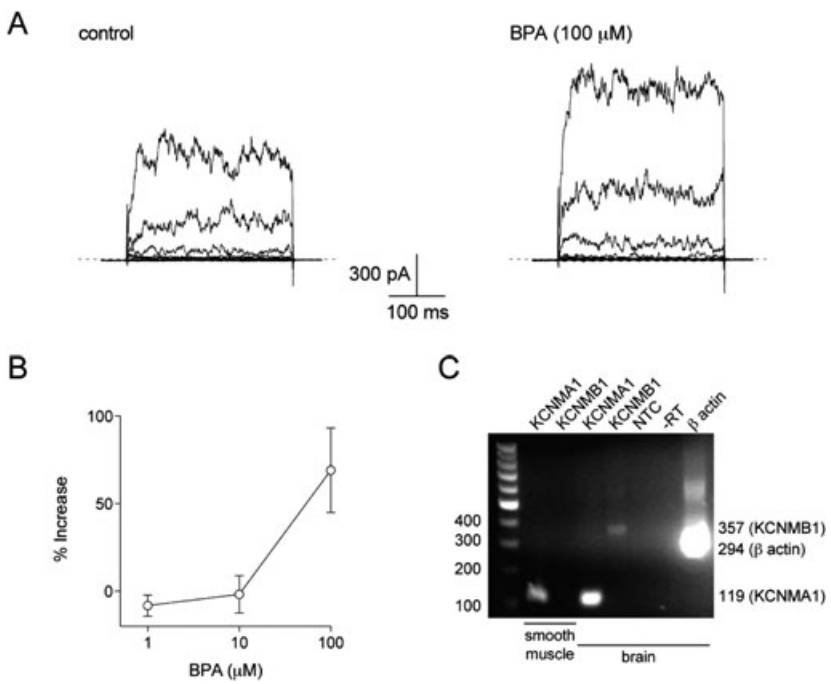

Figure 6 Bisphenol A (BPA), at a concentration of 100 μM, increases large conductance Ca^{2+}/voltage-sensitive K^+ channel (Maxi-K) current in human coronary artery smooth muscle cells. (A) Representative records demonstrate that BPA (100 μM) increased whole-cell current in smooth muscle cells cultured from human coronary arteries. (B) Contains group data (n = 3–7) for effect of three concentrations of BPA on Maxi-K current. (C) RT-PCR revealed the expression of *KCNMA1* (α subunit; 119 bp product) but not *KCNMB1* (β1 subunit; 357 bp product) in cultured human smooth muscle cells. Human brain cDNA was used as a control, and the expression of *KCNMA1* and *KCNMB1* was detected. –RT is a reaction without reverse transcriptase; NTC is a 'no template' control.

Maxi-K channels to oestrogens and xenoestrogens (Dick *et al.*, 2001; Dick and Sanders, 2001; Valverde *et al.*, 1999). Other steroid hormones, including androgens, also activate Maxi-K channels (Deenadayalu *et al.*, 2001; Han *et al.*, 2008). The role of Maxi-K channel β subunits, of which there are four known types, differs depending upon the agonist. For example, the β1 subunit is important for Maxi-K channel activation by 17β-oestradiol (Valverde *et al.*, 1999), while β2 and β4 subunits are important for responses to and discriminate between dehydroepiandrosterone and corticosterone (a stress-related adrenal androgen and a glucocorticoid respectively) (Lovell *et al.*, 2004; King *et al.*, 2006).

It is clear that xenoestrogens affect not only the interaction of 17β-oestradiol with nuclear receptors (Krishnan *et al.*, 1993; Kuiper *et al.*, 1997), but also initiate non-genomic signalling mechanisms. For instance, BPA increased intracellular Ca^{2+} in GH3/B6 pituitary tumour cells (Watson *et al.*, 2005; 2007; Wozniak *et al.*, 2005) and pancreatic β cells (Alonso-magdalena *et al.*, 2005). Elevated intracellular Ca^{2+} could explain the ability of BPA to activate Maxi-K channels; however, experiments in excised patches with solutions clamped to a known Ca^{2+} concentration eliminate the possibility of this mechanism in the present study. Additionally, while BPA activates signalling by protein kinases A and G in JKT-1 tesicular seminoma cells (Bouskine *et al.*, 2009) and these kinases are linked to Maxi-K channel activation (Kume *et al.*, 1989; Robertson *et al.*, 1993), these mechanisms seem unlikely to explain our results. This is because ATP and other cofactors necessary for kinase signalling were absent in the excised patch studies.

Thus, questions remain as to what mechanism(s) underlies the activation of Maxi-K channels by BPA. The same general uncertainty exists across the field for activation of Maxi-K channels by steroid hormones and their mimetics. At present, two types of data support the idea that an extracellular site of the β1 subunit comprises at least a part of the binding domain for these compounds. First, membrane-impermeable conjugates of oestrogen (Valverde *et al.*, 1999) and tamoxifen (Dick *et al.*, 2002) activate Maxi-K channels containing the β1 subunit from the extracellular, but not the intracellular, side of the membrane. Second, a putative binding site for steroid functional groups has been identified in the β1 subunit (Bukiya *et al.*, 2007; 2008a,b). Bukiya *et al.* show that lithocholate, a bile acid formed from cholesterol, activates Maxi-K channels containing β1, but not β4, subunits. Using a series of chimeric β1–β4 subunits, they demonstrate a region of the second transmembrane segment of the β1 subunit that may be particularly important for binding steroids. β1 Subunits, however, are unlikely to be completely responsible for Maxi-K channel responses to steroids and xenoestrogens. Our data demonstrating differential BPA sensitivity of α or α + β1 Maxi-K channels support this concept. It seems possible that the α subunit could be the actual target of BPA and that the β1 subunits enhance BPA binding to the α subunit. In support of this idea, Korovkina *et al.* have demonstrated that the Maxi-K channel α subunit itself functions as a binding site for 17β-oestradiol, and that β1 subunits enhance binding by approximately threefold (Korovkina *et al.*, 2004). Assuming that BPA may bind to the same site, their observations may explain why the β1 subunit, while not essential for BPA-induced activation, increased the BPA sensitivity of Maxi-K channels. The idea that the α subunit may be the target of BPA is further supported by the study of Perez (2005) suggesting that the tamoxifen binding site is located on the Maxi-K α subunit and that β1 subunits facilitate or stabilize the interaction. The possibility remains, however, that BPA interacts with an unknown molecule to transmit a non-genomic signal that activates Maxi-K channels. Further experiments are necessary to clarify the molecular mechanism(s) of BPA induced Maxi-K channel activation.

An important concern is relevance of the present findings in light of the levels of BPA exposure, and thus the concentrations of BPA measured, in humans. Free unconjugated BPA in human serum ranges from approximately 0.9 to 90 nM (Vandenberg *et al.*, 2007). Because free BPA is a minor fraction (5%) of the total BPA load (Csanady *et al.*, 2002), total serum concentrations could be estimated at 0.018–1.8 μM. Nonetheless, BPA became a public health concern relatively recently and many controversies exist about routes of exposure, how much we are exposed to, mechanisms of BPA action and associations with disease (Vandenberg *et al.*, 2009). Our data were obtained with micromolar concentrations of BPA and these appear to be higher than those to which most humans are exposed; however, certain occupational populations are at much higher risk (Hanaoka *et al.*, 2002). Although much remains to be resolved about human exposure to BPA and its effects, here we present the first evidence demonstrating that BPA affects ion channel function directly.

In conclusion, we demonstrate that BPA activated Maxi-K channels in a non-genomic manner. This study focused on immediate effects of BPA on Maxi-K channel activity; however, further investigation will be required to determine any long-term effects of BPA exposure on ion channel function and expression. Our data indicate that the β1 subunit increased the BPA sensitivity of Maxi-K channels; however, the α subunit alone was sufficient for the response of Maxi-K channels to BPA. The toxicology of BPA is not clear, but in cells that express Maxi-K channels, activation by BPA would be expected to hyperpolarize the membrane potential. This would decrease excitability in cells that express other voltage-dependent ion channels; however, a more negative membrane potential would also increase the driving force for Ca^{2+} entry through open Ca^{2+} channels. Based on the present findings, it would be reasonable to suggest that BPA might act as a coronary vasodilator through the opening of Maxi-K channels. Our findings represent the first known effects of BPA on an ion channel, but other types of ion channels should be studied to increase our understanding of BPA toxicity.

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Conflicts of interest

All authors declare that they have no conflicts of interest to disclose.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Cells cultured from the canine left anterior descending coronary artery revealed typical smooth muscle morphology and expressed smooth muscle phenotypic markers. (A) Phase contrast images of cells cultured from the canine coronary artery develop over time into a characteristic 'hill and valley' morphology. Human smooth muscle cell cultures are also shown. (B) Immunoblot of smooth muscle-specific myosin heavy chain and α actin in canine and human cells. The expression of these smooth muscle markers increased over time in differentiation media.

Figure S2 Smooth muscle cells plated for patch-clamp experiments continue to express phenotypic markers. (A) Immunofluorescence of smooth muscle-specific α actin (green) during differentiation; nuclei are stained blue. Images from canine and human cells are shown. (B) The percentage of cells in culture that express smooth muscle-specific α actin increases over time in differentiation medium ($n = 3$). Asterisks indicate $P < 0.05$ versus day 0.

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