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Bisphenol A alters the function and expression of BK channels in vascular smooth muscle through membrane and nuclear signals

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ABSTRACT

Bisphenol A (BPA) exposure has recently become a public health concern, and the safety of the utilization of BPA has attracted the attention of the scientific community, politicians, and the general population. Although BPA is banned from baby products in a few countries because infants and young children are particularly susceptible to endocrine disruption, recent studies have consistently indicated the association between urinary BPA concentration and cardiovascular diseases in adults. Therefore, adult exposure to this xenoestrogen is becoming a healthcare issue that cannot be ignored. We selected large conductance Ca^{2+}/voltage sensitive potassium (BK) channels as one of the target proteins for BPA effects because the function and expression of BK channels are regulated by estrogen. Additionally, BK channels play an important role in vascular function and changes in BK expression are associated with disease. We hypothesized that BPA would differently affect BK channel function and expression via separate non-genomic and genomic mechanisms. The goals of our studies were 1) to characterize a novel, membrane-permeable, and economical antagonist called penitrem A to facilitate the study of BK channels, 2) to determine the mechanisms by which BPA increases BK channel activity and, 3) to determine the mechanisms by which BPA reduces vascular BK channel expression.

Current from patches of HEK 293 cells transfected with *hSlo* α or α + β1 were blocked >95% by penitrem A (IC_{50} 6.4 vs. 64.4 nM; $p<0.05$). Further, penitrem A inhibited BK channels in inside-out and cell-attached patches,

whereas iberiotoxin could not. Inhibitory effects of penitrem A on whole-cell K^+ currents were equivalent to iberiotoxin in canine coronary smooth muscle cells. Penitrem A enhanced sensitivity to K^+-induced contraction in canine coronary arteries by 23 ± 5% (p<0.05) and increased the blood pressure response to phenylephrine in anesthetized mice by 36 ± 11% (p<0.05).

In cultured canine coronary artery smooth muscle cells (CASMC), acute BPA (10 μM) exposure increased BK channel currents 179 ± 8% (p<0.05). Inside out recordings from native canine CASMCs showed that acute BPA increased BK channel open probability (P_o) from 0.11 ± 0.03 to 0.47 ± 0.09 at +40 mV (p<0.05). HEK cells transfected with BK α subunits alone were insensitive to acute BPA (currents at +100 mV were 101 ± 13% of control) while acute BPA increased BK channel currents by 53 ± 12% (p<0.05) when cells were transfected with α and β1 subunits. Similarly, single channel recordings indicated NP_o was 123 ± 20% of control in the BK α subunits alone with BPA, however, acute BPA increased NP_o 201 ± 41% in patches from cells expressing BK α and β1 subunits. Thus, BK β1 subunits facilitated BPA-induced BK channel activation and the effects of BPA were non-genomic, as these effects were observed in cell-free patches.

In contrast to the acute effects of BPA on BK channel activity, 48-72 hrs of BPA (10 μM) exposure decreased BK channel expression in A7r5 cells and cultured rat aorta. Western blot data indicated that BK α subunit protein content was 33 ± 8% of control in BPA-treated aorta (p<0.05). Patch clamp recording showed that whole cell current densities at +100 mV were 57 ± 7% lower in aortic

SMC treated with 10 μM BPA (p<0.05) while whole cell current densities from aortic SMC treated with BPA + ICI 182780 (100 nM; an estrogen receptor blocker) were not different (101 ± 14% of control). Penitrem A-sensitive current densities at 100 mV were 53.9 ± 14.8, 12.0 ± 3.0, and 48.4 ± 10.1 pA/pF in DMSO, BPA and BPA + ICI 182780, respectively (p<0.05). Thus, chronic BPA exposure decreased BK channel expression by estrogen receptor mediated signaling.

Our data indicate: 1) penitrem A is an economical alternative to iberiotoxin for BK channel analysis; 2) acute BPA exposure activates BK channel activity and BK β1 subunits play a role in BPA induced BK channel activation; 3) chronic BPA exposure decreases vascular BK channel expression via estrogen receptor-dependent mechanisms. Although acute BPA exposure activates the BK channel through a non-genomic mechanism that could be beneficial for heart due to coronary dilation, longer BPA exposure decreases the number of functional BK channels through a genomic mechanism that could be detrimental to the heart. Thus, the overall effect of BPA exposure on vascular function may be harmful due to disruption of BK channel-mediated vascular regulation. Taken together, these results indicated BPA induced altered BK channel function and expression may be one of the mechanisms for the association between BPA exposure levels and cardiovascular diseases seen in epidemiological studies.

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SPECIFIC AIMS

Scientific and technological advances provide a variety of chemicals for tremendous benefit; however, concerns have been raised regarding environmental exposure to xenoestrogens that disrupt endocrine function. Recent data suggest that the effect of estrogen on cardiovascular physiology is far more complicated than previously envisioned. Because we do not fully understand the function of estrogen in cardiovascular health and disease, it is difficult to predict the effects of xenoestrogens and endocrine disruptors on cardiovascular physiology. One estrogenic endocrine disruptor, bisphenol A (BPA), holds enormous financial and practical importance for plastic manufacturers, but issues surrounding its safety create concern. A recent epidemiological study associated urinary BPA levels with cardiovascular diseases including angina, atherosclerosis, and myocardial infarction(Lang *et al.*, 2008). Mechanistic data regarding effects of BPA on the cardiovascular system are scarce; therefore, we do not yet understand how BPA could be linked to coronary heart conditions. BPA acts on nuclear estrogen receptors (ER) to mediate genomic effects, while non-genomic effects of BPA appear to originate at the cell membrane. Membrane targets for BPA remain unclear, but include the G protein-coupled estrogen receptor (GPER30; (Thomas & Dong, 2006)) and ion channels (Choi *et al.*, 2007). Importantly, there are no data available regarding the effect of BPA on the function and expression of ion channels in vascular smooth muscle cells (VSMC), a cell type involved intimately in angina, atherosclerosis, and myocardial infarction.

Large conductance Ca^{2+}/voltage-sensitive K^+ (BK) channel is an excellent model for studying non-genomic effects of BPA, because its activity is estrogen-sensitive (Valverde *et al.*, 1999; Dick & Sanders, 2001). The BK channel is also an excellent model for studying genomic actions of BPA, because the underlying gene, *KCNMA1*, is regulated by estrogen (Kundu *et al.*, 2007). BK channels are highly expressed in VSMC, possess the regulatory β1 subunit (Tanaka *et al.*, 1997), and regulate coronary vascular tone (Ledoux *et al.*, 2006; Borbouse *et al.*, 2009). Our long-range goal is to identify molecular mechanisms by which estrogenic chemicals affect vascular function. Our objective in this application is to determine mechanisms by which BPA modulates BK channel expression and function. The central hypothesis is that BPA alters BK channel expression and function through separate genomic and non-genomic mechanisms. The rationale is that receptors for BPA exist at both the cell membrane and in the nucleus. To test the central hypothesis and achieve the objective, we propose 2 specific aims:

1. Determine the non-genomic mechanism by which BPA increases BK channel activity in VSMC. The working hypothesis is that BPA increases BK channel activity by binding to an extracellular site on the channel that is comprised, at least in part, of the regulatory β1 subunit. Patch clamp studies will be performed on VSMC using a derivative of BPA that we have synthesized. BPA-monosulfate (BPA-MS) is permanently charged, membrane-impermeable, and will be used to determine on which side of the membrane the binding site

resides. The role of the β1 subunit will be determined using cloned BK channels composed of α or α + β1 subunits.

2. Determine the genomic mechanism by which BPA decreases BK channel expression in VSMC. The working hypothesis is that BPA reduces BK channel expression by activating nuclear ER. Western blots will be used to determine the expression level of BK channels in VSMC treated with BPA. The pure ER antagonist Fulvestrant (ICI 182,780) and BPA-MS will be used to determine the involvement of nuclear and membrane ER in BPA-induced reductions of BK channel protein expression.

We expect, at the completion of this project, to define mechanisms by which BPA increases BK channel activity and reduces BK channel expression in VSMC. Knowing the mechanisms of action of BPA on VSMC will aid interpretation of correlative studies regarding BPA exposure and human health. The proposed work is innovative because it addresses both genomic and non-genomic effects of BPA related to ion channels, VSMC, and cardiovascular disease.

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CHAPTER 1 Background and significance

Overview of BPA

BPA exposure and health concerns

BPA is used to manufacture products including polycarbonate plastic, the resin inside of cans, dental sealants, and as a non-polymer additive to other plastics. Since BPA is a versatile material, over 6 billion pounds are produced each year, and over 100 tons are disposed of on the earth (Vandenberg *et al.*, 2009). BPA, which possesses estrogen mimetic properties, has recently become the subject of much interest and attention. BPA is public health concern due to exposure in the environment, and a possible association with several pathological conditions. According to the National Health and Nutrition Examination Survey (NHANES) conducted during 1988-1994, BPA was detected in over 95 % of population (Calafat *et al.*, 2005). Newer data collected during 2003-2004 also supports the initial finding of widespread human BPA exposure (Calafat *et al.*, 2008). Notably, the geometric mean of BPA exposure from 2003-2004 NHANES (2.6 μg/L) was twice as large as the one from 1988-1994 (1.33 μg/L). These data may suggest that BPA exposure is continuously increasing over the years.

Currently, the U.S. Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency (EPA) state the reference dose; a daily exposure to the human population that is likely to be without an appreciable risk of deleterious effect during a lifetime, of BPA is 50 μg kg^{-1} day^{-1}. Importantly, there is continuing controversy whether this current acceptable daily intake level

is an appropriate estimate for the safe level. A number of recent studies are suggesting the possible low dose effect of BPA, which may involve totally newly mechanisms that alter cellular functions. Furthermore, many studies have detected BPA in urine, blood and tissue; however, the levels of BPA exposure are controversial. BPA has been shown to accumulate in adipose tissue (Nunez *et al.*, 2001; Fernandez *et al.*, 2007) so that the local BPA concentration in tissue could be higher than the values reported in total blood BPA concentration. Furthermore, cumulative BPA exposure levels may play a role in BPA associated pathologies. Additionally, this reference dose was calculated based on a toxicological experiment to determine the lowest observed adverse effect level (LOAEL) in the 1980's. Thus, there is a desperate need for updated data regarding BPA's toxicological aspects.

Although there is no clear consensus of adverse health effects from BPA, the large body of evidence suggested that BPA possesses endocrine disrupting activities in a tissue specific manner. Specifically, BPA may be associated with altered fetal/neonatal development (Vandenberg *et al.*, 2007), reproductive function (Savabieasfahani *et al.*, 2006), metabolic homeostasis (Hugo *et al.*, 2008) and carcinogenesis (Wetherill *et al.*, 2002; Wetherill *et al.*, 2005). Thus, the U.S. Food and Drug Administration (FDA) and National Toxicology Program (NTP) at the National Institute of Health state that they have " some concerns regarding the potential effect of BPA on the brain, behavior, and the prostate gland in fetuses, infants and young children (http://www.fda.gov/newsevents/publichealthfocus/ucm064437.htm).

Toxicodynamics / toxicokinetics of BPA

BPA is a lipophilic molecule that contains 2 phenol rings, which resembles other toxic chemicals such as diethylstilbestrol (DES) and dichlorodiphenyltrichloroethane (DDT) (Fig. 1-1). These compounds were banned due to their endocrine disrupting properties and have been associated with an increased risk of tumor development (Vandenberg *et al.*, 2009). Similarly, one of the well-known chemical properties of BPA is an estrogenic property that interacts with estrogen receptors (ERs) to activate estrogen signals. Conventionally, it is known that endocrine disrupters act via nuclear estrogen receptors as 17 β-E_2 acts on ERs, however, the mechanisms of non-genomic effects such as the involvement of membrane receptors or ion channels are not well established. Indeed, xenoestrogens were reported to be much less potent than 17 β-E_2 (Krishnan *et al.*, 1993; Kuiper *et al.*, 1997). Recent studies have demonstrated that the nongenomic action of BPA may be associated with altered intracellular Ca^{2+} homeostasis (Nadal *et al.*, 2000; Alonso-Magdalena *et al.*, 2005; Walsh *et al.*, 2005). Yet, the exact mechanisms of BPA induced alterations remain unknown. Given that BPA has a low affinity for binding with classical nuclear receptors; it is possible that non-genomic effects of BPA may play an important role in BPA associated altered cellular function in addition to classical genomic ER signaling disruption.

In terms of BPA toxicokinetics, studies have been shown that the half-life of BPA in the blood range from 4 to 6 hours in humans (Volkel *et al.*, 2002; Volkel

et al., 2005). Considering the studies demonstrating that the half life of BPA in the blood is a relatively short time, and the studies showing the detection of BPA from urine samples in over 90% of population (Calafat *et al.*, 2005; Calafat *et al.*, 2008), the environmental BPA exposure to humans must be continuous. Alternatively, it is possible that BPA can be stored in the body and constantly released into the blood. Indeed, toxicological studies have shown that BPA accumulates in brown adipose tissue in rats (Nunez *et al.*, 2001) and BPA and its derivatives were detected in the adipose tissue of women (Fernandez *et al.*, 2007). Since there are no doubts about the prevalence of BPA in our body, further research is needed to explore the pharmacokinetic/dynamics of BPA and how it is related to the BPA associated pathologies seen in epidemiological studies.

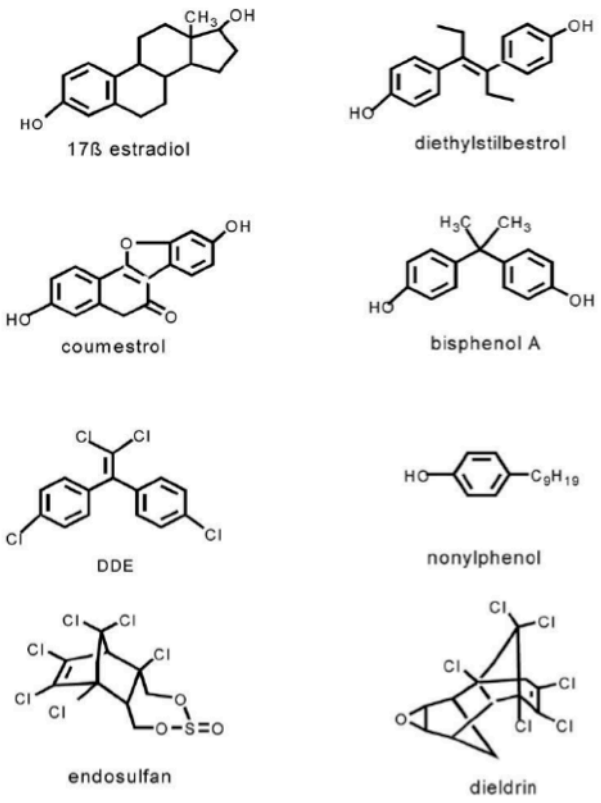

Figure 1 1 Chemical structures of E2 and xenoestrogens and related compounds.

Structures of 17β-estradiol and endocrine disruptors are shown. The structure of BPA is very similar to Dichlorodiphenylene (DDE) and Diethylstilbestrol (DES). DDE is a metabolite of DDT, which was banned as a pesticide due to its carcinogenic properties. DES is a pharmaceutical estrogen that was also banned (Watson *et al.*, 2007)

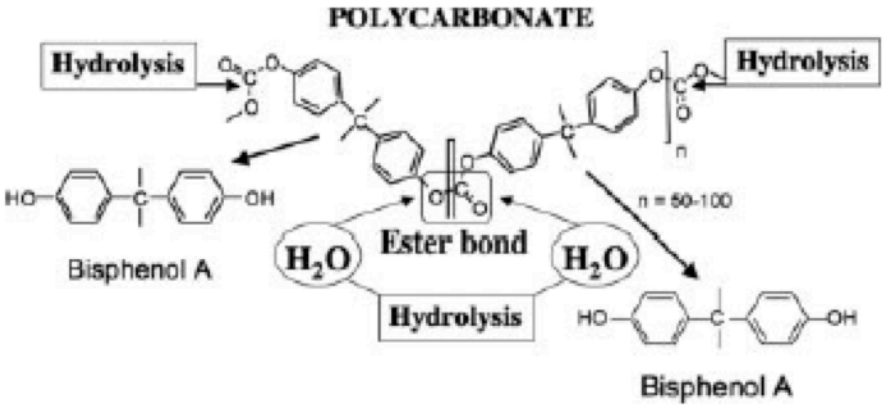

Figure 1 2 Chemical reactions to release BPA from polycarbonate products.

BPA molecule is released from polycarbonate products by the hydrolysis of the ester bond. The chemical reaction is facilitated during certain conditions, including high temperatures and acidic or basic environments. (Welshons *et al.*, 2006)

Sources of BPA

Although BPA has been detected in a variety of sources including food, beverages, air, soil, and house dust, the major BPA exposure source was considered through dietary ingestion (Miyamoto & Kotake, 2006; Wilson *et al.*, 2007). Particularly, BPA has been shown to leach from polycarbonate compounds such as plastic containers and canned food. The initial report of BPA being released was in an accidental experiment showing that an estrogenic substance was suspected of leaching from autoclaving solution in polycarbonate flasks (Krishnan *et al.*, 1993). As in all general chemical reactions, hydrolysis of ester bonds in polycarbonate products is facilitated at high temperature and pH levels (Fig. 1-2). Examples commonly seen in our lives may be washing a plastic container with harsh detergent, or heating it, or storing acidic/basic food in it. Indeed some of these conditions are shown to release BPA (Brotons *et al.*, 1995; Kang *et al.*, 2003).

Interestingly, a recent study reported that the BPA levels did not decline with fasting conditions in human subjects (Stahlhut *et al.*, 2009). This study may suggest that the oral route may not be the only route of BPA exposure. Additionally, BPA has been shown to be stored in adipose tissue (Nunez *et al.*, 2001; Fernandez *et al.*, 2007), therefore, cumulative BPA levels may also need to be considered when the source of BPA exposure needs to be determined. Taken together, there are no doubts about human BPA exposure; however, the exact source of BPA is not known. It is likely to be multiple sources responsible for human BPA exposure.

BPA and cardiovascular diseases

In 2008, an initial epidemiological study demonstrated a significant relationship between urinary BPA concentration and cardiovascular diseases in the general adult population from the United States (Lang *et al.*, 2008a). This cross-sectional analysis utilized data from the National Health and Nutrition Examination Survey 2003-2004 and the data indicated higher BPA concentrations were associated with cardiovascular diagnosis, diabetes, and altered liver enzyme levels. Later, newer data from the NHANES 2005-2006 also appeared consistent with an association between urinary BPA concentration and cardiovascular disease (Melzer *et al.*, 2010). Additionally, high levels of BPA in serum were associated with atherosclerotic plaques in the aged population (Lind & Lind, 2011). Occupational levels of BPA exposure also have been associated with an increased risk of male sexual dysfunctions such as erectile dysfunction (Li *et al.*, 2010). Although government agencies have concerns due to BPA's potential harmful effects on fetuses, infants and young children, these studies may suggest that BPA toxicity is not limited to the specific population but rather, a global effect to all populations.

All taken together, BPA has become a public health concern only in the last few years, and many controversies exist about routes of exposure, the quantity we are exposed to, mechanisms of BPA action, and its associations with diseases (Vandenberg *et al.*, 2009). Since constant BPA exposures are suspected and therefore this phenomenon will continue to intensify, early and conclusive studies for safety are extremely important to prevent public health

hazards like the ones that have been created by the use of materials such as asbestos and dichlorodiphenyltrichloroethane (DDT).

Overview of ion channels in VSM

Ion channel and toxicological compounds

Ion channels are macromolecular protein complexes that form pores in cell membranes, which regulate the movement of ions. The membrane potential determined by these ion channels are critical, since excitable cells utilize electricity for communication between the extracellular side and intracellular side to further propagate intracellular signal transduction to trigger a cellular response. (i.e. Ca^{2+} flux). Thus, ion channels located in the membrane make very convenient targets for altering cellular function. As shown in nature, many toxins (i.e. tetrodotoxin, iberiotoxin, charybdotoxin) are designed to alter ion channel functions, and are used as predators' weapons to establish a dominant world where the strong prey upon the weak. Furthermore, the disruption of membrane integrity by forming transmembrane pores (gramicidin, amphotericin, nystatin,) is also a great example of how important the ion channel is in the membrane to regulate ionic gradients between extracellular and intracellular spaces. In other words, ion channels may be susceptible to toxicological chemicals.

Potassium channels in VSMC

The primary function of VSMC is contraction, as in other muscle types such as skeletal and cardiac muscle. Calcium dependent muscle contraction

obeys the basic principle of excitation-contraction coupling (ECC) which explains the membrane excitability and intracellular Ca^{2+} concentration that regulates muscle contraction. As in other muscles, the two key players in ECC are voltage dependent L-type Ca^{2+} channel (Cav1.2) and ryanodine receptor (RYR) in the VSMC. Similar to other muscles, voltage dependent L-type Ca^{2+} channels are activated by membrane depolarization resulting in Ca^{2+} flux through extracellular component to increase global intracellular Ca^{2+} concentration (Moosmang *et al.*, 2003). Therefore, ion channels contributing to the dynamic regulation membrane potential (Em) are key factors for VSMC contraction (Nelson *et al.*, 1990). Particularly, K^+ channels are important molecules in negative feedback mechanisms to set the resting membrane potential since the equilibrium potential of K^+ are more negative than the resting membrane potential. Multiple types of K^+ channels are identified in VSMC: 1) Inward rectifying K channel (K_{ir}), 2) ATP sensitive K^+ channel (K_{ATP}) 3) Voltage dependent K^+ channels (K_V), and 4): Calcium dependent K^+ channels (K_{Ca}) (Holdefer *et al.*, 1976; Nelson & Brayden, 1993; Quayle *et al.*, 1997; Dick & Tune, 2010). These potassium channels in VSMC, that provide hyperpolarizing currents, play a critical role in maintaining the resting membrane potential, and each type of K^+ channel has its own unique biophysical properties.

K_{ir} and K_{ATP} channels

Both K_{ir} and K_{ATP} channels are categorized in the same phylogenetic tree and have been shown to express in vascular smooth muscle (Kubo *et al.*, 2005).

The structure of the K_{ir} family is composed of 2 transmembrane domains termed M1 and M2 with pore forming H5 regions that serves as ion selectivity filters (Bichet *et al.*, 2003). Functional properties of K_{ir} currents in VSMC were characterized by patch clamp recording (Edwards & Hirst, 1988; Edwards *et al.*, 1988). Patch clamp recordings demonstrated strong inward rectifying barium sensitive currents and increasing extracellular $[K^+]$ increased the conductance. In terms of the physiological role of K_{ir} channels in VSM, moderate increases in the extracellular $[K^+]$ (3-20 mM) have been shown to cause vasodilation through a mechanism depending on K_{ir} channel activation in cerebral arteriole (Girouard *et al.*, 2010). In VSMC, $K_{ir}2.1$ transcript was detected, but not $K_{ir}2.2$ and $K_{ir}2.3$ from rat cerebral, coronary and mesenteric arteries (Bradley *et al.*, 1999). To support this gene expression data further, functional data also indicated that the cerebral artery from $K_{ir}2.2$ KO mice responds to extracellular K^+, but not the cerebral artery from $K_{ir}2.1$ KO mice (Zaritsky *et al.*, 2000). Thus, this molecular and functional evidence suggests that vascular K_{ir} channels are composed of $K_{ir}2.1$.

On the other hand, K_{ATP} channels are composed of $K_{ir}6.1$ and $K_{ir}6.2$ with unique subunits known as sulfonylurea receptors (SUR). As in the $K_{ir}2.1$ subunit, $K_{ir}6.1$ and $K_{ir}6.2$ subunits are composed of 2 transmembrane domains, M1 and M2, without voltage sensor domain such as S4 segment, thus K_{ATP} channel currents are not voltage dependent. Nonetheless, SUR subunits serve as the intracellular ATP binding site to regulate K_{ATP} channel. Although human coronary endothelial K_{ATP} channel has been shown to express as a heteromeric complex of $K_{ir}6.1$, $K_{ir}6.2$ and SUR2B, it was not demonstrated in human coronary artery

smooth muscle cells (Yoshida *et al.*, 2004). Indeed, human coronary artery smooth muscle cells expressed $K_{ir}6.1$, $K_{ir}6.2$ and SUR2B mRNAs, however, a heteromeric complex of $K_{ir}6.1$ and $K_{ir}6.2$ was not confirmed as in the human coronary endothelial cells. In contrast, another study showed only $K_{ir}6.1$ and SUR2B mRNA expression (Miura *et al.*, 2003). These molecular biological data are further supported by the functional data that demonstrate that the aortic vascular responses to K_{ATP} channel agonists/antagonist (pinacidil, diazoxide and glibenclamide) are not different between wild type and $K_{ir}6.2$ KO mice (Suzuki *et al.*, 2001). Thus, this molecular and functional evidence suggests that the vascular K_{ATP} channel is composed of $K_{ir}6.1$ and SUR2B. In terms of the physiological role of K_{ATP} channel in VSM, it has been suggested that K_{ATP} channel plays an important role in ischemic vasodilation (Dart & Standen, 1995). This is because the SUR regulatory subunits serve as the sensor for intracellular ATP levels and the K_{ATP} channel is generally inhibited due to sufficient levels of ATP under normal physiological conditions. K_{ATP} channel is also activated by the G-protein mediated cAMP dependent protein kinase (PKA) (Standen & Quayle, 1998). For instance, adenosine induced vasodilation involves adenylyl cyclase-cAMP-PKA pathways to activate K_{ATP} channel.

K_V channels

K_V channels are one of the most diverse potassium channels. Although they are classified into K_V1- K_V12 subfamilies and each subfamily contains several isoforms, the expression of K_V1, K_V2, K_V3, K_V4, K_V7 and K_V9 channel

subfamilies have been confirmed in vascular smooth muscle. (Gutman *et al.*, 2003; Wulff *et al.*, 2009). K_V channel is composed of pore forming alpha subunits that can be homotetramers or heterotetramers of different families. The α subunit contains 6 transmembrane domains and the S4 segment serves as a voltage sensor. The S5-S6 linker and P-loop form the outer funnel and pore in K_V channels. The K^+ selective filter allows K^+ ions go through the pore using the highly selective dehydration of K^+ ions (MacKinnon, 2003). In addition, K_V channels have been suggested to contain auxiliary β subunits that can regulate the channel function. Although the exact molecular composition of K_V channel is not clear because the composition of K_V channels seem to be different depending on the vascular beds, K_V channels are likely to be composed of heteromultimeric K_V α subunits with diversity of β subunits that enables the formation of a variety of K_V channels to response to appropriate physiological demand in the vessels (i.e. conduit *vs* resistance). For instance, K_V α subunits (K_V1.1, K_V1.2, K_V1.4, K_V1.5, K_V1.6, K_V2.1 and K_V9.3) are expressed in rat pulmonary arterial smooth muscle cells (Yuan *et al.*, 1998), while K_V α subunits (K_V1.1- K_V1.8 except K_V1.4) are expressed in canine coronary artery (Dick *et al.*, 2008). Interestingly, within the diverse family of K_V genes, some K_V genes are particularly important in vascular function. Specifically, a mutant K_V1.5 gene overexpression caused endogenous functional K_V channels to switch dysfunctional K_V channels due to the incorporation of mutant K_V1.5 (Chen *et al.*, 2006). This dominant negative approach resulted in altered myogenic response and membrane potential in the vessels.

BK channel

Large conductance Ca^{2+}/voltage- activated K^+ (BK) channels are expressed in variety of tissues and involved in variety of cellular process including circadian rhythms (Meredith *et al.*, 2006) vascular regulation (Nelson *et al.*, 1995; Brenner *et al.*, 2000), hearing function (Pyott *et al.*, 2007). BK channels are composed of 4 α subunits encoded by *KNCMA1* gene with auxiliary β subunits encoded by *KCNMB1-4* genes. Although a single gene encodes the α subunit, it undergoes alternative splicing processes to yield several splicing variants that have diverse channel characteristics (Butler *et al.*, 1993; Tseng-Crank *et al.*, 1994). As a schematic BK channel structures are shown in Fig. 1-3, the α subunit contains 7 transmembrane segments (S0 to S6) with N-terminus located at the extracellular side and C-terminus located at the intracellular side. Similar to other voltage sensitive K channel families, S4 segment serves as a voltage sensor to regulate BK channel opening/closing states (Cui & Aldrich, 2000).

In addition to the voltage regulation, there are Ca^{2+} sensor sites similar to EF hand motif located at cytoplasmic C-terminal tail side. Furthermore, currently, there are 4 auxiliary β subunits encoded by *KCNMB1-4* genes identified and each β subunit provides further unique BK channel properties. Specifically, first β subunits are isolated from smooth muscle membrane and identified as β1 subunit (Knaus *et al.*, 1994a). The role of the β1 subunit is allosteric regulations of BK channel by improving the sensitivity to voltage and intracellular Ca^{2+} (Fig. 1-4) (Carl & Sanders, 1989; Cox *et al.*, 1997; Cox & Aldrich, 2000). Thus, BK channel

β1 subunits have been suggested to play a key role in regulation of vascular tone (Brenner *et al.*, 2000) and urinary bladder function (Petkov *et al.*, 2001). It also regulates the functional surface expression of α subunits (Toro *et al.*, 2006). Furthermore, β1 subunit-dependent BK channel activation was reported with steroid hormones (Valverde *et al.*, 1999; Lovell *et al.*, 2004; King *et al.*, 2006; Bukiya *et al.*, 2007). β2 subunits are expressed inn adrenal gland and hippocampal neuron and it uniquely causes inactivation of BK channel currents (Solaro & Lingle, 1992; Hicks & Marrion, 1998; Wallner *et al.*, 1999). The expression of β3 subunit is confirmed in testis, pancreas and spleen and it causes a rapid inactivating BK channel currents (Xia *et al.*, 2000). β4 subunits are primary expressed in brain and presence of β4 subunits make BK channel insensitive to iberiotoxin (Meera *et al.*, 2000). In summary, β subunits alter Ca^{2+}, voltage and chemical agonists/antagonists sensitivities.

The primary role of BK channel has been suggested to act as negative feedback mechanism to regulate the opening of L-type voltage gated Ca^{2+} channels in vascular smooth muscle cells by voltage and intracellular Ca^{2+} levels (Fig. 1-5). Ca^{2+} spark, local increase in subsarcolemmal Ca^{2+}, is coupled with BK channel activity, causing hyperpolarizing membrane potential to counter vasoconstriction (Nelson *et al.*, 1995). Although BK channel mediate relaxation of arterial smooth muscle by Ca^{2+} sparks is evident in cerebral artery (Nelson *et al.*, 1995), recent data suggest the heterogeneity in the contribution of BK channels in vasculature tone in vascular beds (Yang *et al.*, 2009). Thus, the physiological

role and the mechanism of BK channel activation may be far more complex and important than previously envisioned.

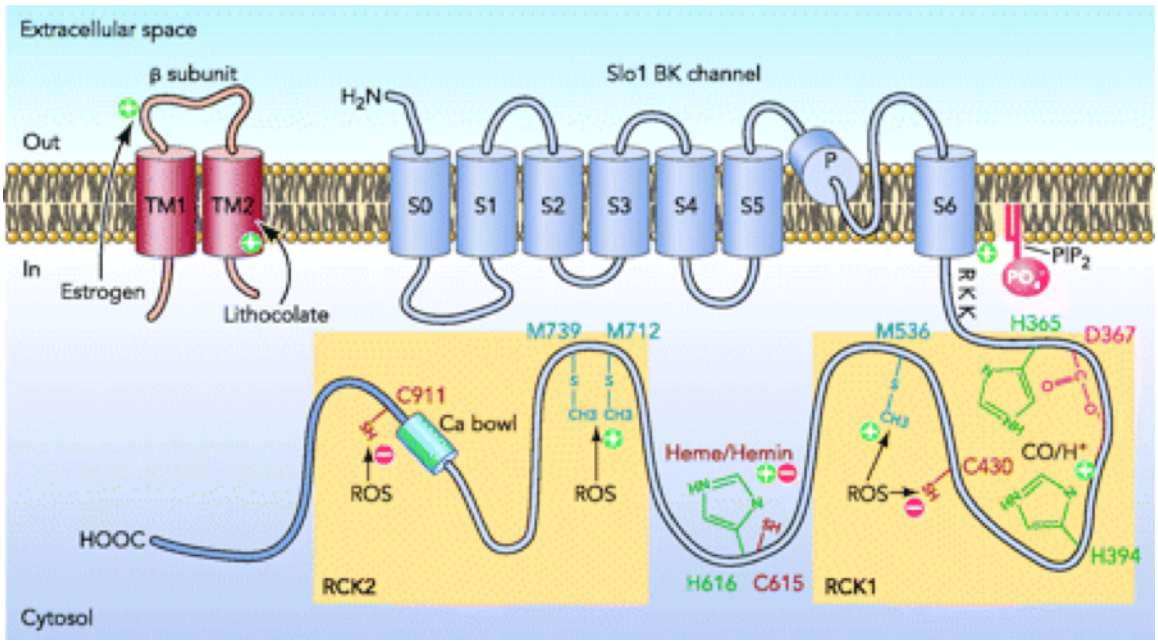

Figure 1 3 Molecular structure of BK channel α subunit and β1 subunit.

The schematic diagram shows the key amino acid residues involved in modulation of BK channel. + indicates activation of BK channel and – indicates inhibition of BK channel. (Hou *et al.*, 2009)

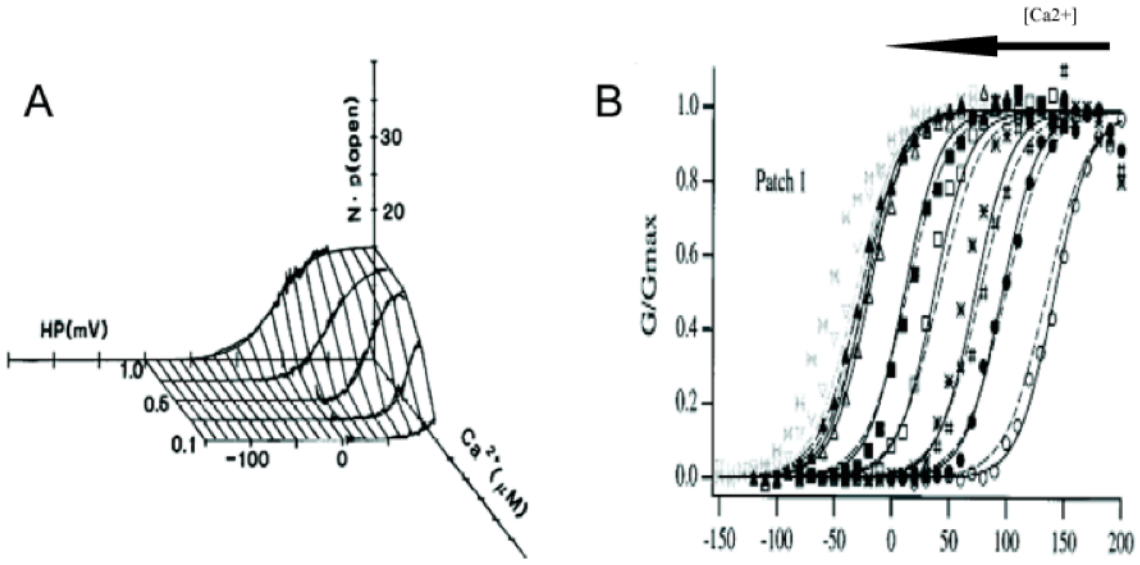

Figure 1 4 Dual regulation by Ca^{2+} and voltage on BK activity.

(A) BK channel activity is plotted against voltage and Ca^{2+} concentration. (B) G-V relationship shifts toward the left as intracellular Ca^{2+} concentration increases (Carl & Sanders, 1989)

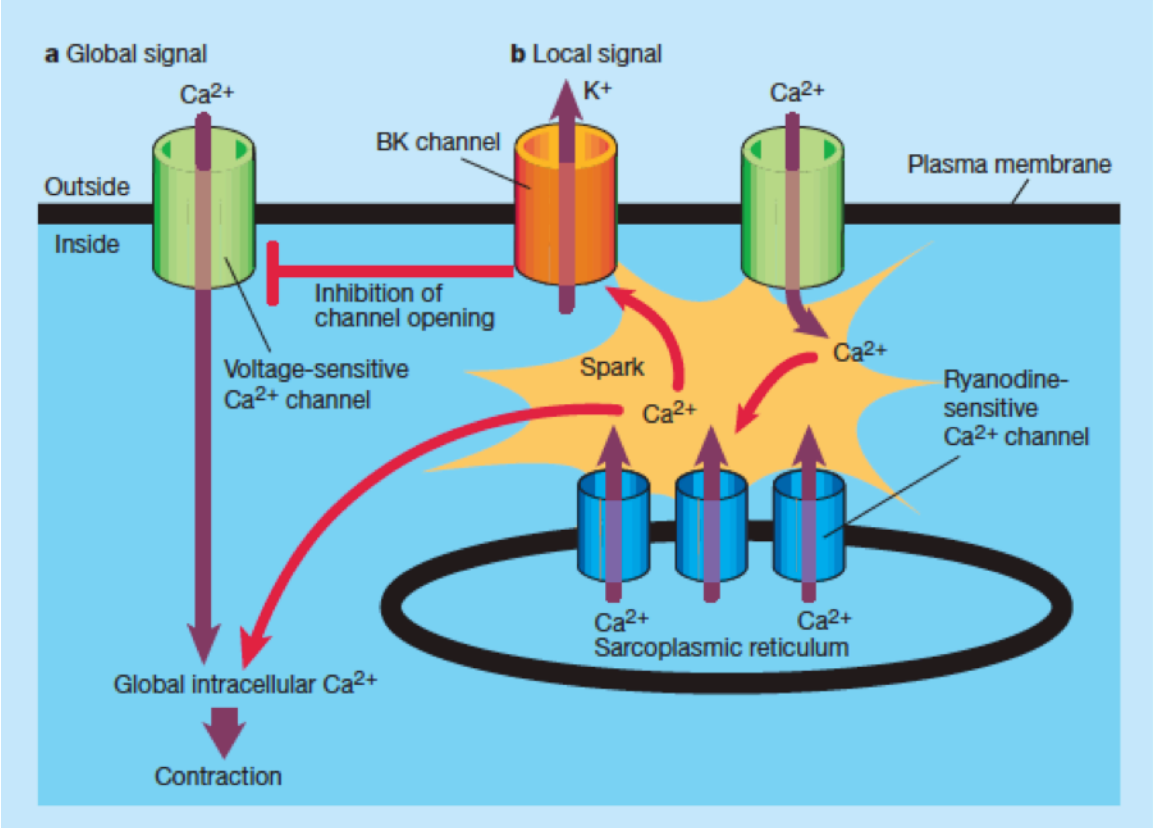

Figure 1 5 The function of BK channel in vascular smooth muscle cells.

Ca^{2+} spark activates BK channel and causes membrane hyperpolarization due to K^+ efflux. This membrane hyperpolarization blocks L-type Ca^{2+} channel to decrease the global Ca^{2+} concentration levels in cytosol. BK channel regulates intracellular Ca^{2+} levels using this negative feedback system. (Standen, 2000)

BK channel and vascular pathologies

Aging, hypertensive and diabetic vascular BK channels

In vascular smooth muscle cell, local intracellular Ca^{2+} increase can paradoxically induce smooth muscle relaxation. This is explained by the activating of the vascular BK channel that results in membrane hyperpolarization (Nelson *et al.*, 1995). Therefore, BK channel has been recognized as one of the key molecules for vascular tone in a variety of vessels. Indeed, alterations in BK channel expression in vascular smooth muscle is associated with hypertension, aging, and diabetic related altered vascular functions in variety of vascular beds (Liu *et al.*, 1997; Liu *et al.*, 1998; Marijic *et al.*, 2001; Lu *et al.*, 2005; McGahon *et al.*, 2007; Lu *et al.*, 2008; Borbouse *et al.*, 2009)

Specifically, cerebral artery smooth muscle cells from genetically hypertensive rats exhibited over 4-fold higher BK channel current density and BK α subunits expression compared to Wistar-Kyoto rat cerebrovascular smooth muscle cells (Liu *et al.*, 1998). Similarly, hypertension-induced increased BK channel has been demonstrated in aortic smooth muscle cells from spontaneously hypertensive rats (Liu *et al.*, 1997). Hypertension induced increased BK channel expression is considered compensatory mechanism to counteract the increased cerebral vascular tone.

On the other hand, age associated altered vascular ion channel remodeling indicated decreased BK channel function as age increased (Toro *et al.*, 2002). Specifically, both rat and human coronary artery smooth muscle cells have been shown to decrease in BK current density and α subunits protein

content over time (Marijic *et al.*, 2001). Additionally, age associated reduced BK channel β1 subunits and the mechanisms of aged associated reduced BK channel current density were reported later (Nishimaru *et al.*, 2004a). Interestingly, a study has demonstrated that age associated decreased BK channel function has been attenuated by exercise training (Albarwani *et al.*, 2010). Therefore, age associated BK channel dysfunction is not an irreversible phenomenon, and therapeutic benefits can be expected from exercise or pharmacological approaches. Surprisingly, decreased BK channel expression with aging was not evident in aged cerebral artery smooth muscle cells (Nishimaru *et al.*, 2004b). Thus, BK channel remodeling mechanisms compensate for age associated dysfunctional vasculature depending on the particular vascular bed, and they could be great pharmacological targets.

Diabetes and metabolic syndrome related to vascular dysfunction is associated with altered BK channel function (Dimitropoulou *et al.*, 2002; Lu *et al.*, 2005; Zhou *et al.*, 2005; Burnham *et al.*, 2006; McGahon *et al.*, 2007; Borbouse *et al.*, 2009). Some studies indicate that alterations in signaling mechanisms, such as arachidonic acid mediated signaling, result in impaired BK channel function, but not BK channel expression (Dimitropoulou *et al.*, 2002; Lu *et al.*, 2005). Other studies demonstrate that BK channel biophysical properties including channel kinetics, sensitivities to voltage, and Ca^{2+} are impaired with diabetic vessels (Burnham *et al.*, 2006; Lu *et al.*, 2008). On the other hand, altered BK channel expression in diabetes has also been reported (McGahon *et al.*, 2007; Borbouse *et al.*, 2009). Based on these multiple levels of observation,

diabetes and metabolic syndrome associated altered vascular BK channel function is associated with the combination of altered BK channel upstream signaling, biophysical properties of BK channel and BK channel expression.

Issues in studying BK channels

BK channel antagonists/agonists

In addition to wide expression of BK channel in human tissue, the numbers of chemical factors including ethanol (Dopico *et al.*, 1998), hormones (Valverde *et al.*, 1999; Lovell *et al.*, 2004; King *et al.*, 2006) and xenoestrogens (Dick *et al.*, 2001; Coiret *et al.*, 2007) have been shown to modulate BK channel function. Particularly, ethanol is often used as a solvent for a variety of drugs and it requires careful control for assessment of BK channel function when ethanol is used for vehicle of the drug. Common experimental approaches to assess the role of BK channel are pharmacological and genetic manipulation, however, each approach possess a limitation.

A traditional pharmacological approach has been utilized to study the role of BK channel in physiological function before the gene disruption technology was established. Since the identification of peptide toxin including charybdotoxin (ChTX) and iberiotoxin (IBTX) (Miller *et al.*, 1985; Galvez *et al.*, 1990) from scorpion venom, both peptide toxins have been used to study the role of K^+ channels in physiological function. Particularly, IBTX has been used as the gold standard to elucidate the physiological role of BK channel as well as the mechanism of gating because IBTX is highly selective to BK channel where

ChTX is sensitive to other type of K^+ channels (Galvez *et al.*, 1990). Although IBTX is recognized as a gold standard for BK channel inhibitor, several limitations exist. Originally, the potency of IBTX to BK channel was reported as IC_{50} pM to nM ranges (IC_{50} 250 pM: (Galvez *et al.*, 1990)), (IC_{50} 1.7 nM:(Kaczorowski *et al.*, 1996), however, a recent study has shown the IC_{50} of IBTX is much higher (371 nM) (Lippiat *et al.*, 2003) than the concentrations commonly used to assess the physiological role and biophysical properties of BK channels. Furthermore, IBTX is prohibitively expensive and its membrane impermeable characteristics make IBTX difficult to use in whole animal studies.

Recently, a group of indole diterpenes has been reported as potent BK channel inhibitors (Knaus *et al.*, 1994b); which gave a hope to overcome some of the limitations of IBTX, however, the pharmacological properties of each compound seem to be distinctive and have not been fully explored. For instance, some of these mycotoxins are tremorgenic indole alkaloids, but some are nontremorgenic such as paspalinine (Knaus *et al.*, 1994b). Within a group of these mycotoxins, inhibitory effects of paxillne in BK channel have been relatively well characterized (Sanchez & McManus, 1996). In the original report of the effect of indole diterpenes on BK channel (Knaus *et al.*, 1994b), it was mentioned that paxilline weakly blocks delayed rectifier potassium channels in mouse pancreatic β cells. Furthermore, paxilline has been reported to block Ca^{2+} activate Cl^- channels (Sones *et al.*, 2009). Penitrem A is also one of a group of tremorgenic mycotoxins that have been shown to affect the central nervous system by altering the release of neurotransmitters (Norris *et al.*, 1980). The

ingestion of penitrem A has been associated with neurological disorders such as tremor, ataxia and convulsions in dogs and humans (Eriksen *et al.*; Lewis *et al.*, 2005), However, in terms of the effect on BK channels, very little data regarding the effect of penitrem A on BK channel and on vasculature tone are available.

A few BK channel agonists have been identified and characterized by patch clamp experiments. For instance, NS004 (1-(2-hydroxy-5-chlorophenyl)-5-trifluoromethyl-2- benzimidazolone) has been shown to increase open probability of BK channel and its open dwell time (Olesen *et al.*, 1994b). Additionally, the effects of NS 1619 on BK channel have been well characterized (Edwards *et al.*, 1994; Olesen *et al.*, 1994a) and utilized as well accepted BK channel opener in a variety of studies. Although NS 1619 has been utilized as a BK channel agonist, the issues in the selectivity of NS 1619 have been reported (Holland *et al.*, 1996). It has been shown that NS 1619 not only activates BK channel, but also inhibits L-type Ca^{2+} channel activity. Therefore, it requires careful interpretation in vasodilation response when NS1619 is used for the analysis of BK channel function.

BK channel KO mice

As a recent subject of experimental popularity, the genetic modification approach has become the major experimental design; however, there are certain limitations to this approach, such as compensatory alteration to accommodate a gene disruption (Faraci & Sigmund, 1999). Indeed, constitutive BK α subunit KO mice and smooth muscle specific BK α subunit KO mice exhibit different degrees

of compensatory proteomic adaptions that resulted in more severe disruption in urinary bladder function from smooth muscle specific BK α subunit KO mice (Sprossmann *et al.*, 2009). Furthermore, inter-laboratory variability exists in experimental results using other transgenic mice. For instance, BK β1 subunit KO mice were originally reported as hypertensive (Brenner *et al.*, 2000; Pluger *et al.*, 2000) but a recent study contradicted these original reports (Xu *et al.*, 2011). Thus, although the utilization of BK channel KO mice in experiments is increasing, a combination of traditional pharmacological approaches to inhibit or activate BK channel to elucidate a role of BK channel in physiological and pathophysiological conditions is critical.

Significance of study

Although there is no clear consensus on the adverse health effects of BPA, evidence suggests that BPA may be associated with variety of pathologies including carcinogenesis, altered reproductive function, and developmental issues (Wetherill *et al.*, 2005; Savabieasfahani *et al.*, 2006; Vandenberg *et al.*, 2007; Hugo *et al.*, 2008). Fetuses, infants, and children are more susceptible to effects of BPA; however, a recent epidemiologic study revealed that BPA exposure might be related to cardiovascular disease in adults (Lang *et al.*, 2008b). Because BPA exposure is increasing and these phenomena will continue to intensify, early and conclusive studies on safety and mechanism are needed to prevent public health hazards like the ones created by asbestos and DDT. This project will generate meaningful data useful to the Food and Drug

Administration and the National Toxicology Program in assessing BPA toxicity and providing regulatory guidance. Since possible links between BPA and disease have appeared relatively recently, controversies and confusion exist regarding its health effects. There is not enough scientific evidence to draw an accurate conclusion as to whether BPA exposure is linked to cardiovascular disease; however, alterations of BK channel function and expression are associated with coronary dysfunction related to hypertension (Brenner *et al.*, 2000; Amberg & Santana, 2003), diabetes (Lu *et al.*, 2008; Borbouse *et al.*, 2009), and aging (Nishimaru *et al.*, 2004a). Our contribution will be a detailed analysis of cellular and molecular effects of BPA on BK channels of vascular smooth muscle cells. We will elucidate molecular pharmacological and toxicological effects of BPA on BK channels; specifically, we will identify genomic and non-genomic mechanisms of BPA effect on BK channels in vascular smooth muscle cells.

Summary

In conclusion, although BPA has been utilized in industry because original toxicological studies conclude that the level of exposure to human is below the levels of observed adverse effect, recent studies raised concerns regarding the association with BPA and a number of pathologies. **We hypothesize that BPA can alter BK channel function and expression via genomic/non-genomic mechanisms.** With regard to our hypothesis, we also raised the issues in studies assessing the function of BK channel. Thus, we will characterize the new BK

channel inhibitor, penitrem A, and utilize penitrem A to identify the novel mechanisms of BPA induced altered BK channel function. These alterations may explain the link to the BPA exposure and BPA associated increased risk of cardiovascular diseases.

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CHAPTER 2 Penitrem A as a tool to understand the role of BK channels in vascular function

As reviewed in JPET

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BK channel, large conductance Ca^{2+}/voltage-sensitive K^+ channel; K_V channel, voltage-dependent K^+ channel; K_{ATP}, ATP-dependent K^+ channel

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Abstract

Large conductance, Ca^{2+}/voltage-sensitive K^+ (BK) channels are well characterized, but physiological roles, often determined through pharmacological manipulation, are less clear. Iberiotoxin is considered the "gold standard" antagonist, but cost and membrane-impermeability limit usefulness. Economical and membrane-permeable alternatives could facilitate the study of BK channels. Thus, we characterized the effect of penitrem A, a tremorgenic mycotoxin, on BK channels and demonstrate utility for studying vascular function *in vitro* and *in vivo*. Whole-cell currents from HEK 293 cells transfected with *hSlo* α or α + β1 were blocked >95% by penitrem A (IC_{50} 6.4 vs. 64.4 nM; $p<0.05$). Further, penitrem A inhibited BK channels in inside-out and cell-attached patches, whereas iberiotoxin could not. Inhibitory effects of penitrem A on whole-cell K^+ currents were equivalent to iberiotoxin in canine coronary smooth muscle cells. As for specificity, penitrem A had no effect on native delayed rectifier K^+ current in canine coronary smooth muscle, cloned human $K_V1.5$ channels, or pinacidil-induced relaxation of canine coronary arteries. Penitrem A enhanced sensitivity to K^+-induced contraction in canine coronary arteries by 23 ± 5% ($p<0.05$) and increased the blood pressure response to phenylephrine in anesthetized mice by 36 ± 11% ($p<0.05$). Our data indicate that penitrem A is a useful tool to study the role of BK channels in vascular function and is practical for cell and tissue (*in vitro*) studies as well as anesthetized whole animal (*in vivo*) experiments.

Introduction

Large conductance, Ca^{2+}/voltage-sensitive (BK) channels, composed of pore forming α and auxiliary β1 subunits, may be key regulators of arterial tone via negative feedback (Nelson *et al.*, 1995; Jaggar *et al.*, 2000). Indeed, altered α or β1 expression is associated with vascular dysfunction in hypertension, diabetes, and aging (Liu *et al.*, 1998; Brenner *et al.*, 2000; Amberg *et al.*, 2003; Borbouse *et al.*, 2009). Genetic deletion of α or β1 subunits produces hypertension; however, in addition to presumed changes total peripheral resistance, the role of adrenal BK channels to regulate aldosterone production must also be considered (Cysewski *et al.*, 1975; Sausbier *et al.*, 2005). In contrast to the original report (Brenner *et al.*, 2000), a recent study indicated that β1 knockout mice are normotensive (Xu *et al.*, 2011). Thus, much remains to be determined about the physiological roles of BK channels and/or compensations in response to BK channel gene deletion.

Adding complexity to our understanding of BK channel function is heterogeneity between vascular beds (Yang *et al.*, 2009). While BK channels appear extremely important in regulating cerebral vascular tone (Brayden & Nelson, 1992; Knot *et al.*, 1998), these channels are arguably less important in skeletal muscle arteries (Kotecha & Hill, 2005; Yang *et al.*, 2009) or coronary arteries (Rogers *et al.*, 2006; Borbouse *et al.*, 2010a; Borbouse *et al.*, 2010b). Clearly, the regulation of BK channels is extremely complex and governed by multiple endogenous signaling molecules (Hou *et al.*, 2009). Thus, understanding the physiological roles and mechanisms of BK channel activation will require

additional research using a combination of genetic models and pharmacological tools.

Since the identification and characterization of peptide toxins in scorpion venom, iberiotoxin has become the “gold standard” for pharmacological study of BK channels due to selectivity and potency (IC_{50} 0.25 nM) (Galvez *et al.*, 1990). However, a recent study determined the IC_{50} of iberiotoxin is much higher (33-371 nM) than the concentrations commonly used (Lippiat *et al.*, 2003). Additionally, iberiotoxin is prohibitively expensive for many isolated organ and whole-animal studies and its membrane-impermeability creates difficulty for cell-attached or inside-out patch clamp experiments. It would be beneficial to identify an economical and membrane-permeable alternative to facilitate physiological and pharmacological studies of BK channels. Penitrem A is one such candidate molecule (Knaus *et al.*, 1994) and has been used previously as an inhibitor of BK channels in smooth muscle (Cotton *et al.*, 1997; Borbouse *et al.*, 2009; Asano *et al.*, 2010).

Little, however, is known about the basic pharmacological properties of penitrem A. For example, the IC_{50} for block of BK channels has not been published, nor are there reports of its specificity in regard to other K^+ channels. Our study was designed to characterize some pharmacological properties of penitrem A as a BK channel antagonist and to demonstrate utility in determining the physiological role of BK channels in vascular smooth muscle. Experiments were performed at the cell, tissue, and whole animal level. We used patch clamp techniques to assess the inhibitory effect, determine whether the β1 subunit

influenced block, and illustrate advantages of a membrane-permeable inhibitor. We measured the isometric tension of coronary arteries and the blood pressure of anesthetized mice to determine whether penitrem A altered vascular reactivity *in vitro* or *in vivo*. We conclude that penitrem A, compared to iberiotoxin, is an antagonist of BK channels that is comparable in potency and efficacy, but is more useful and less expensive.

Methods

Animal models

All animal procedures and protocols were approved by institutional committees and followed guidelines set forth in the Guide for the Care and Use of Laboratory Animals (National Academy Press, 2011). Male C57BL/6 mice 10-12 weeks of age were used for *in vivo* blood pressure experiments. As described below, mice were anesthetized to a surgical plane of anesthesia prior to experiments and euthanized thereafter. Coronary arteries from male mongrel dogs were used for *in vitro* isometric tension experiments as described previously (Rogers *et al.*, 2006). In brief, anesthetized dogs were used for unrelated experiments involving a thoracotomy; the heart was fibrillated and removed to isolate coronary arteries. For patch clamp experiments on native vascular smooth muscle cells, arteries were obtained from a variety of different species (rat, mouse, dog, and pig). Arteries (cerebral, coronary, femoral, and aorta) were taken from animals euthanized following unrelated experiments.

Cell culture and transfection

HEK 293 cells (Stratagene, La Jolla, CA) were grown in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and culture flasks were incubated in a 5% CO_2 incubator at 37 °C. Plasmids encoding *hSlo* α and *hSlo* α + β1 were kindly provided by Dr. Jonathan Lippiat (Lippiat *et al.*, 2003) and *hK$_V$1.5* plasmid was a generous gift from Dr. Jeffrey R Martens (McEwen *et al.*, 2007). Cells were transiently transfected with

*p*IRES-*hSlo*α or *p*IRES-*hSlo*$\alpha\beta$*1* and *pmaxGFP* (AMAXA, Gaithersburg, MD) using Lipofectamine LTX with PLUS reagent (Invitrogen, Carlsbad, CA). Cells at 50-70% confluence in 35 mm dishes were transfected with 0.5-2.5 mg of DNA and currents were recorded from GFP positive cells 1-3 days later. Transfected cells were selected in media supplemented with 0.5 mg/ml G418 (Invitrogen, Carlsbad, CA), 1% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Electrophysiology

BK channel currents were recorded at room temperature from inside-out and whole-cell patches as previously described (Asano *et al.*, 2010). The bath flowed ~2-3 ml/min into a ~0.2-0.3 ml chamber throughout the recordings. For whole-cell recordings, bath solution contained (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, 1 Mg-ATP, 0.1 Na-GTP and 5 Tris; pH 7.1. Inside-out recordings were made in symmetrical (mM) 140 KCl, 1 $MgCl_2$, 1 EGTA, 10 HEPES, 5 Tris; pH 7.1; pCa 7 (Maxchelator; http://www.stanford.edu/~cpatton/maxc.html). Stock solutions of penitrem A (MP Biomedicals, Solon, OH) were prepared in DMSO and diluted for 1:10,000 for experiments. Iberiotoxin (AnaSpec, San Jose, CA) was dissolved in water and diluted 1:1000 for experiments.

Isometric tension studies

Isometric tension was measured from segments of canine left circumflex coronary arteries as described previously (Rogers *et al.*, 2006). Arteries were denuded of endothelium and the lack of a relaxant response to acetylcholine (10 μM) in pre-contracted arterial tissue confirmed the functional loss of endothelium. For the pinacidil concentration-response experiments, arteries were pre-contracted with 1 μM U46619, a thromboxane A_2 mimetic, and the relaxation elicited by cumulative additions of pinacidil in the presence or absence of 1 μM penitrem A. For the K^+ concentration-response experiments, KCl was added cumulatively in the presence or absence of 1 μM penitrem A.

Blood pressure measurements

Mean arterial pressure was measured as described previously (Ohanyan *et al.*, 2011). Mice were anesthetized with sevoflurane gas (supplemented with O_2) and placed on a heating table to maintain body temperature at 37 °C. A polyethylene catheter was placed into the jugular vein for bolus administrations of heparin (50 U/ml) and hexamethonium (5 mg/kg), a ganglionic blocker. A micro-tip transducer (Millar Instruments, Houston, TX) was placed in the femoral artery to measure mean arterial pressure and heart rate. Data were recorded with a Power Lab acquisition system (ADInstruments, Colorado Springs, CO). Phenylephrine (0.5 μg/kg/min) and penitrem A (100 μg/kg/min) were administered intravenously. Stock solutions of penitrem A were dissolved in ethanol and subsequently diluted in lactated Ringer's solution; phenylephrine was diluted in lactated Ringer's solution.

Statistics

Data are presented as the mean and standard error of n number of samples (*e.g.*, number of patches, cells, dogs, or mice as described in the text and figure legends). When two values were compared, paired or unpaired *t*-tests were used as appropriate. When three values were compared, one-way analysis of variance (ANOVA) was used. Current-voltage relationships and concentration-response curves were analyzed by two-way ANOVA. Bonferroni post hoc tests followed ANOVA to determine differences. In all tests, $p<0.05$ was considered significant.

Results

Penitrem A inhibits BK channels in a concentration-dependent manner: influence of the β1 subunit.

Whole-cell patch clamp techniques were used to determine the effect of penitrem A on cloned BK channels (α or α + β1) expressed in HEK 293 cells. Currents at +100 mV were recorded under control conditions (in the presence of vehicle; 0.01% DMSO) and after addition of penitrem A (Fig. 1A & B). In cells expressing BK channels composed solely of α subunits, inhibition of current by 100 nM penitrem A was 93 ± 1% (n = 13 cells; Fig. 1A&C). In contrast, when BK channels contained the regulatory β1 subunit, block by 100 nM penitrem A was only 41 ± 9% (n = 9 cells; Fig. 1B&C). Over several log orders of penitrem A concentrations, BK channels composed of α subunits alone were more sensitive to block than BK channels containing the β1 subunit (Fig. 1C). The half-maximal inhibitory concentration (IC_{50}) of penitrem A for BK α was 6.4 nM (log IC_{50} = -8.20 ± 0.08; n = 7-16 cells). The IC_{50} of penitrem A on BK α + β1 subunits was 64.4 nM (log IC_{50} = -7.19 ± 0.07; n = 4-15 cells; $p<0.05$). Hill slopes were equivalent (-0.91 ± 0.12 vs. -1.07 ± 0.15). In addition to reduced sensitivity, the time course of channel block by penitrem A was slowed by the β1 subunit (Fig. 1D & E). The inhibitory effect of penitrem A on BK channel current was, for all practical purposes, irreversible (Fig. 2). When current in cells expressing BK channels composed of α + β1 subunits was inhibited by 1 μM penitrem A, 10 or more min of washing was insufficient time for appreciable reversal (Fig. 2A). After 2 min of penitrem A (1 μM) exposure, current was inhibited 91 ± 2% (n = 5; Fig. 2B). Cells

were studied in a ~0.2-0.3 ml chamber that was perfused at ~2-3 ml/min; however, washing cells for 14 min with penitrem A-free bath solution did not allow BK current to recover (Fig. 2B; current at the 20 min mark was only 6 ± 2% of the control level). Similar irreversibility was observed in native smooth muscle cells (e.g., rat aorta) with lower concentrations of penitrem A (e.g., 50 nM; Fig. 2C & D).

Penitrem A, but not iberiotoxin, inhibits BK channels in inside-out patches. Iberiotoxin blocks BK channels by "plugging" the pore from the extracellular side (Giangiacomo *et al.*, 1992); therefore, one cannot use bath application of iberiotoxin to block BK channels in cell-attached or inside-out patches. A common strategy to overcome this limitation is to backfill patch pipettes with iberiotoxin, thus allowing some time for control measurements while iberiotoxin diffuses to the tip. A membrane-permeable BK channel antagonist could strengthen experimental design and allow more time for control measurements and/or interventions prior to BK channel block. We compared the effects of iberiotoxin (100 nM) and penitrem A (100 nM) on BK channels in cell-attached and inside-out patches (Fig. 3). As expected, bath application of iberiotoxin to inside-out patches of had no inhibitory effect on BK channel activity. In contrast, bath application of penitrem A inhibited BK channel activity in inside-out patches. Mean NP_o of BK α alone under control conditions, with iberiotoxin, and with penitrem A was 0.077 ± 0.040, 0.103 ± 0.076, and 0.002 ± 0.001, respectively. (p<0.05 for penitrem A, n = 7-10). Similarly, mean NP_o of BK α + β1 under control

conditions, with iberiotoxin, and with penitrem A was 0.401 ± 0.158, 0.564 ± 0.235, 0.015 ± 0.014 (p<0.05 for penitrem A, n = 7-13). Like the whole-cell recordings, BK channel block by penitrem A was not readily reversible with extensive washing. In contrast to the whole-cell recordings where 100 nM penitrem A inhibited BK α + β1 by less than half (Fig. 1), 100 nM penitrem A inhibited BK α + β1 in inside-out patches by 97 ± 1%. This increased inhibitory effect at the cytoplasmic face was also noted in the original description of penitrem A (Knaus *et al.*, 1994).

Specificity of penitrem A: block of BK, but not K_V or K_{ATP} channels.

The effect of penitrem A to inhibit K^+ current in coronary artery smooth muscle cells is equivalent to iberiotoxin (Fig. 4). In coronary myocytes, penitrem A (300 nM) inhibited BK current significantly, and the remaining current appeared to be mediated by K_V channels (Fig. 4A & B). After penitrem A treatment, iberiotoxin (100 nM) had no further inhibitory effect on current at +100 mV (Fig. 4C); when iberiotoxin was added first, penitrem A had no further inhibitory effect (Fig. 4D). It is unclear what K_V channel type(s) mediate the delayed rectifier K^+ currents in smooth muscle, but K_V1.5 has been suggested as a component (Chen *et al.*, 2006; Dick *et al.*, 2008). Thus, we determined whether penitrem A could inhibit cloned K_V1.5 channels (Fig. 5A & B). Whole-cell recordings of K_V1.5 current were made from HEK 293 cells transfected with *hK_V1.5* plasmid (McEwen *et al.*, 2007). Penitrem A (1 μM) had no effect on K_V1.5 channels; current at +60 mV in the presence of penitrem A was 105 ± 6% of control (n = 5). We tested the effect of

penitrem A on total outward K^+ current in a variety of other native vascular smooth muscle cells (Supplemental figure). In each species and tissue, penitrem A (1 mM) inhibited BK current (i.e., strongly outwardly rectifying current at positive potentials) and appeared to have little or no effect on native delayed rectifier channels.

K_{ATP} channels are another major type of K^+ channel in coronary smooth muscle (Dart & Standen, 1995); therefore, we determined whether penitrem A could inhibit K_{ATP} channels. Dog coronary arteries were contracted with U44619 (a thromboxane A_2 mimetic; 1 μM) and relaxed with pinacidil, a K_{ATP} channel opener. These experiments were performed in the presence or absence of penitrem A (1 μM). Pinacidil-induced relaxation was unaffected by penitrem A (Fig. 5C & D; arteries from n = 3 dogs; maximum relaxation 91 ± 5 vs. 94 ± 2% and log IC_{50} = -5.94 ± 0.11 vs. -5.85 ± 0.07). We performed patch clamp experiments to measure directly the effects penitrem A on pinacidil-induced K_{ATP} current activation. Whole-cell current in mouse aortic myocytes (n = 6) was measured at -50 mV in symmetrical 140 mM K^+. Pinacidil (10 μM) activated 165 ± 49 pA of inward current. Penitrem A (1 μM) had no effect on pinacidil-activated current (87 ± 6% of control); however, glibenclamide (10 μM) inhibited pinacidil-activated current 80 ± 7% ($p < 0.05$).

Penitrem A augments smooth muscle contractility in vitro and in vivo.

To test penitrem A as a functional inhibitor of smooth muscle BK channels *in vitro*, K^+ concentration-response curves were constructed for coronary arteries in

the presence or absence of 1 μM penitrem A. Inhibition of BK channels produced a leftward shift in the K^+ concentration-response curve (Fig. 6). Contractions with 20, 30, and 40 mM K^+ were increased 79 ± 12, 26 ± 5 and 12 ± 4% by blocking BK channels with 1 μM penitrem A (p<0.05; arteries from n = 8 dogs). To test penitrem A as a functional inhibitor of smooth muscle BK channels *in vivo*, blood pressure responses to phenylephrine, an a_1 adrenergic agonist, were measured in anesthetized mice. Hexamethonium (5 mg/kg i.v.), a nicotinic receptor antagonist, was used to eliminate reflex responses to blood pressure changes. Phenylephrine (0.5 μg/kg/min i.v.) increased blood pressure to a steady state level and then addition of penitrem A (100 μg/kg/min) increased blood pressure further (Fig. 7A). In 5 mice (Fig. 7B), phenylephrine increased mean blood pressure from 57 ± 2 to 77 ± 1 mmHg (p<0.05), while the addition of penitrem A further increased blood pressure to 84 ± 3 mmHg (p<0.05 vs. control and phenylephrine).

Discussion

The objective of this study was to better characterize penitrem A as an inhibitor of BK channels (Knaus *et al.*, 1994). Penitrem A has been used previously as inhibitor of BK channels in smooth muscle (Cotton *et al.*, 1997; Borbouse *et al.*, 2009; Asano *et al.*, 2010). Importantly, however, employment of penitrem A as a BK channel antagonist has been without a complete understanding of its pharmacological properties. For example, the IC_{50} was not known and it was unclear whether BK channel subunit composition might affect the block. We show that penitrem A inhibits BK α and α+ β1 with IC_{50} values of 6.4 and 64.4 nM (Fig. 1) Additionally, the use of penitrem A to study BK channels has been, until recently (Borbouse *et al.*, 2009; Borbouse *et al.*, 2010a; Borbouse *et al.*, 2010b), limited to *in vitro* studies, particularly patch clamp experiments. Thus, we further investigated penitrem A as a BK channel inhibitor to assess vascular function *in vitro* and in *in vivo*. We show that penitrem A enhances smooth muscle contraction *in vitro* (Fig. 6) and increases total peripheral resistance *in vivo* (Fig. 7). Information regarding the specificity of penitrem A against other ion channels was limited to Ca^{2+}-activated Cl^- channels (Sones *et al.*, 2009), where it had very little effect. Here we demonstrate no effect of penitrem A on native delayed rectifier, cloned $K_V1.5$, or K_{ATP} channels (Figs. 4 and 5 and Supplement). Iberiotoxin and penitrem A are roughly equivalent in potency and efficacy (Fig. 4 and Table 1), but penitrem A is less than 0.1% of the cost on a per mole basis. We conclude that penitrem A is comparable to

iberiotoxin in potency, efficacy, and selectivity and, further, that penitrem A can be a more useful and economical alternative.

BK channel subunit knockout mice have become a popular experimental model for studying smooth muscle reactivity and a great deal of important information has been gleaned; however, this approach comes with certain limitations. For example, deletion of K^+ channel genes elicits phenotypic compensations that may compromise interpretation (Nerbonne *et al.*, 2008). Such changes may explain variable observations regarding blood pressure in BK channel subunit knockout mice. For instance, BK β1 subunit knockout mice were originally reported as hypertensive (Brenner *et al.*, 2000; Pluger *et al.*, 2000), but a recent study contradicted this (Xu *et al.*, 2011). The earlier studies included acute measurements of blood pressure in conscious mice with arterial catheters, whereas the later study used radiotelemetry to monitor blood pressure continuously for 1 wk. Both approaches are valid and it remains unknown whether differences in the method of blood pressure measurement or the response to stress could explain the discrepancies; however, compensatory changes in other K^+ channels or physiological mechanisms might be responsible for observations of normal blood pressure in BK β1 subunit knockout mice. An alternative explanation is that BK channels may play a minimal role in regulating total peripheral resistance. Thus, the power of traditional pharmacological approaches to inhibit BK channels in order to elucidate a mechanism of action should not be underestimated, as we (Borbouse *et al.*, 2009; Borbouse *et al.*, 2010a; Borbouse *et al.*, 2010b) and others (Node *et al.*, 1997) have previously

demonstrated little, if any role, for BK channels in regulating coronary vascular tone using penitrem A, charybdotoxin, or iberiotoxin. No comparable studies of organ blood flow are available for BK channel subunit knockout mice; this is likely due to the difficulty of instrumenting small animals. Studies of BK channels in regional and systemic hemodynamics might be made more readily in larger animals by employing suitable pharmacological tools such as paxilline, lolitrem B, and penitrem A (Table 1). It must be kept in mind, however, that penitrem A was first reported in the literature due to its tremorgenic effects; therefore, its use *in vivo* may best be limited to anesthetized animals because of potential neurological side effects and convulsant properties.

Some characteristics of BK channel block by penitrem A are compared to those of other BK channel antagonists in Table 1. It is important to note that while experimental details such as patch potential and free Ca^{2+} are not identical, a fairly consistent picture emerges. Moreover, our data were generated using the plasmids of Lippiat and colleagues (2003), allowing a relatively direct comparison. Penitrem A appears to be one-half to one log order more potent than iberiotoxin, while the presence of β1 subunits reduces sensitivity to either agent by a factor of 10. All of the pharmacological tools characterized in Table 1 block BK channels with similar efficacy (>95%). Penitrem A can inhibit BK channels whether it is applied from intracellular or extracellular side of the membrane; this property is shared by paxilline and lolitrem B, but not iberiotoxin. We found that β1 subunits decrease BK channel sensitivity to penitrem A. This is different from paxilline or lolitrem B, which block BK α, α + β1, and α + β4 with

similar potency (Imlach *et al.*, 2008). Specificity for BK channels is another issue surrounding the available antagonists. Paxilline has been reported as an inhibitor of Ca^{2+}-activated Cl^- channels, whereas penitrem A and iberiotoxin have much less effect (Sones *et al.*, 2009). In the original report (Knaus *et al.*, 1994), it was mentioned that paxilline weakly blocks delayed rectifier K^+ channels in mouse pancreatic β cells, but we show here that penitrem A has no effect on native delayed rectifier, cloned $K_V1.5$, or K_{ATP} channels (Fig. 4 and 5). More studies will be necessary to determine possible off-target interactions of penitrem A; these, if any, may be expected to arise from introducing penitrem A into complex systems such as isolated organ or whole animal experiments.

Importantly, however, the effects of penitrem A we observed in isolated arteries *in vitro* and on blood pressure *in vivo* were relatively straightforward and can be interpreted as specific effects on BK channels (Figs. 6 and 7). For example, in Fig. 6, the effect of penitrem A to increase smooth muscle contraction is consistent with what has been reported previously for iberiotoxin (Brayden & Nelson, 1992; Bratz *et al.*, 2005). We assessed the effect of penitrem A on smooth muscle tone *in vivo* by measuring blood pressure in anesthetized mice (Fig. 7). C57BL/6 mice are a common background for engineered mice; therefore, these data may be a reference for those interested in using penitrem A in knockout or transgenic mice. A recent study demonstrated that lolitrem B had no effect on blood pressure, but decreased heart, in mice (Imlach *et al.*, 2010). Our first impression was that these results could be explained by a reflex adjustment of heart rate, and thus cardiac output, and ultimately blood pressure

in response to an increase in total peripheral resistance. However, Imlach et al. went on to demonstrate an inhibitory effect of lolitrem B on heart rate in Langendorff-perfused hearts, where neural input is removed. The mechanism(s) by which lolitrem B reduces heart rate are unknown; however, it is important to note that they are not shared by penitrem A. We used hexamethonium to block autonomic responses and heart rate was unchanged during penitrem A-induced increases blood pressure (Fig. 7).

We demonstrated the efficacy and selectivity of penitrem A on BK channel function *in vitro* and *in vivo*; however, our study has several limitations that need to be addressed. First, penitrem A is well known to cause muscle tremor, thus, the use of penitrem A to study the role of vascular BK channels might best be limited to anesthetized animals for *in vivo* experiments. Second, in addition to vascular smooth muscle BK channels, systemic penitrem A administration will inhibit BK channels in other cells and tissues that might affect the regulation of vascular tone and blood pressure. For instance, a recent study indicated that BK channels in astrocytes are key regulators of neurovascular coupling (Girouard *et al.*, 2010). Additionally, BK channels in endothelium may regulate membrane potential, Ca^{2+} influx, and the production of relaxing factors (Sandow & Grayson, 2009). Since BK channel expression is ubiquitous, *in vivo* experiments with penitrem A will require careful design and interpretation.

In conclusion, we have further characterized penitrem A as an inhibitor of BK channels, particularly BK a + b1 channels that are generally found in smooth muscle. This information could be put to important use determining the roles of

BK channels in vascular function and serve as a complement to studies performed in BK channel subunit knockout mice. We demonstrate that penitrem A is a useful and economical alternative for studying the role of BK channels *in vitro* and *in vivo*. That is, we show that that the use of penitrem A is not limited to patch clamp experiments, rather this agent is practical for tissue, organ, and whole animal studies.

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Authorship Contributions

Participated in research design: Asano, Tune, Bratz, and Dick

Conducted experiments: Asano, Bratz, Berwick, Tune, and Fancher

Contributed new reagents or analytic tools: None

Performed data analysis: Asano and Dick

Wrote or contributed to the writing of the manuscript: Asano and Dick

Footnotes

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Legends for figures

Fig. 1 Presence of the β1 subunit decreases the sensitivity of BK channels to penitrem A. Representative traces of BK current at +100 mV in HEK 293 cells transfected with *hSlo* α (Panel A) or *hSlo* α + β1 (Panel B). Whole-cell current was measured before (control) and after addition of penitrem A (100 nM). Group data (Panel C; n = 4-16 cells at each concentration) show the effect of BK channel subunit composition on sensitivity to block by penitrem A. Asterisks indicate $p<0.05$ between the two groups as determined by two-way ANOVA. Group data (n = 3-9) show time- and concentration-dependent block of BK a (Panel D) and α + β1 (Panel E) by penitrem A. Note that, compared to a, a 10x higher concentration of penitrem A is required to achieve a similar time-dependent block in α + β1.

Fig. 2 Irreversible block of BK channels by penitrem A. Current traces are shown from a representative HEK 293 cell expressing BK channels composed of α + β1 subunits (Panel A). The voltage template used is shown below the current. Penitrem A (1 μM) abolished current within 1-2 min; however, 10 min of washing in penitrem A-free bath solution was not sufficient time for current to recover appreciably. (Panel B) Group data (n = 5 cells) illustrate the practical irreversibility of a 2 min exposure to penitrem A; BK current at +100 mV is plotted vs. time. Current traces are shown from a representative rat aortic smooth muscle cell (Panels C and D). The voltage template was same as above. Penitrem A (50 nM) reduced native BK current and was practically irreversible.

Fig. 3 Penitrem A blocks BK channels in inside-out and cell-attached patches. Recordings of channel activity from representative inside-out patches are shown for BK α (Panel A) or α + β1 (Panel B). Patch potential was +40 mV and solutions were symmetrical 140 mM K^+ with 100 nM free Ca^{2+}. Channel activity was recorded under control conditions and with bath application of iberiotoxin (100 nM) or penitrem A (100 nM). (Panel C) Penitrem A blocks BK channel (α + β1) activity in a cell-attached patch. Channel activity was recorded under control conditions and with bath application of penitrem A (100 nM). (Panel D) Group data (n = 23 patches) show that the exposure of the cytosolic side of inside-out patches to penitrem A, but not iberiotoxin, inhibits BK channel activity. Asterisk indicates $p<0.05$ vs. control by one-way ANOVA.

Fig. 4 Iberiotoxin and penitrem A block the same current in canine coronary smooth muscle cells. (Panel A) Families of current traces from a representative dog coronary artery smooth muscle cell are shown under control conditions, with penitrem A (300 nM), and with iberiotoxin (100 nM). These experiments were performed with a bath solution containing 0.01% bovine serum albumin to limit nonspecific binding of iberiotoxin to plastic. (Panel B) The I-V relationships from traces in A are shown. Penitrem A and iberiotoxin block the same current (inset). (Panel C) Group data (cells from n = 5 dogs) demonstrate that penitrem A blocks iberiotoxin-sensitive current; current at +100 mV is shown. (Panel D) Similarly,

iberiotoxin blocks penitrem A-sensitive current at +100 mV. Asterisks indicate *p<0.05* vs. control by one-way ANOVA.

Fig. 5 Penitrem A has no effect on $K_V1.5$ or K_{ATP} channels. (Panel A) Whole-cell recordings were made from HEK 293 cells transfected with *hKV1.5.* Representative $K_V1.5$ currents in the absence or presence of 1 μM penitrem A. (Panel B) Group data (n = 5 cells) show that penitrem A (1 μM) has no effect on $K_V1.5$ current. (Panel C) Representative isometric tension recordings show that penitrem A (1 μM) does not inhibit pinacidil-induced relaxation. (Panel D) Group data (arteries from n = 3 dogs) show that penitrem A (1 μM) has no effect on K_{ATP} channels. Arteries were contracted with 1 μM U46619 and relaxed with cumulative additions of pinacidil.

Fig. 6. Penitrem A selectively inhibits BK channel to alter vascular contraction from *in vitro* vascular functional experiments.
Panel A) Representative traces for KCl induced contraction with/without penitrem A are shown. (Panel B) Group data showed a leftward shift in contraction response with penitrem A. Asterisks (*) indicate significant difference ($p< 0.05$) from control by two-way ANOVA.

Fig. 7 Block of BK channels by penitrem A augments phenylephrine-induced blood pressure responses in mice. (Panel A) Mean arterial pressure and heart rate are shown for a representative mouse. The mouse received

phenylephrine (0.5 μg/kg/min i.v.), which increased pressure. There was no reflex change in heart rate, as the ganglionic blocker hexamethonium (5 mg/kg i.v.) was injected prior to the experiment. Penitrem A (100 μg/kg/min i.v.) was added and increased pressure further. (Panel B) Group data (n = 5 mice) illustrate effect of penitrem A to augment phenylephrine-induced pressor responses. Asterisk and dagger indicate significant difference (p<0.05) from control and phenylephrine, respectively, by one-way ANOVA.

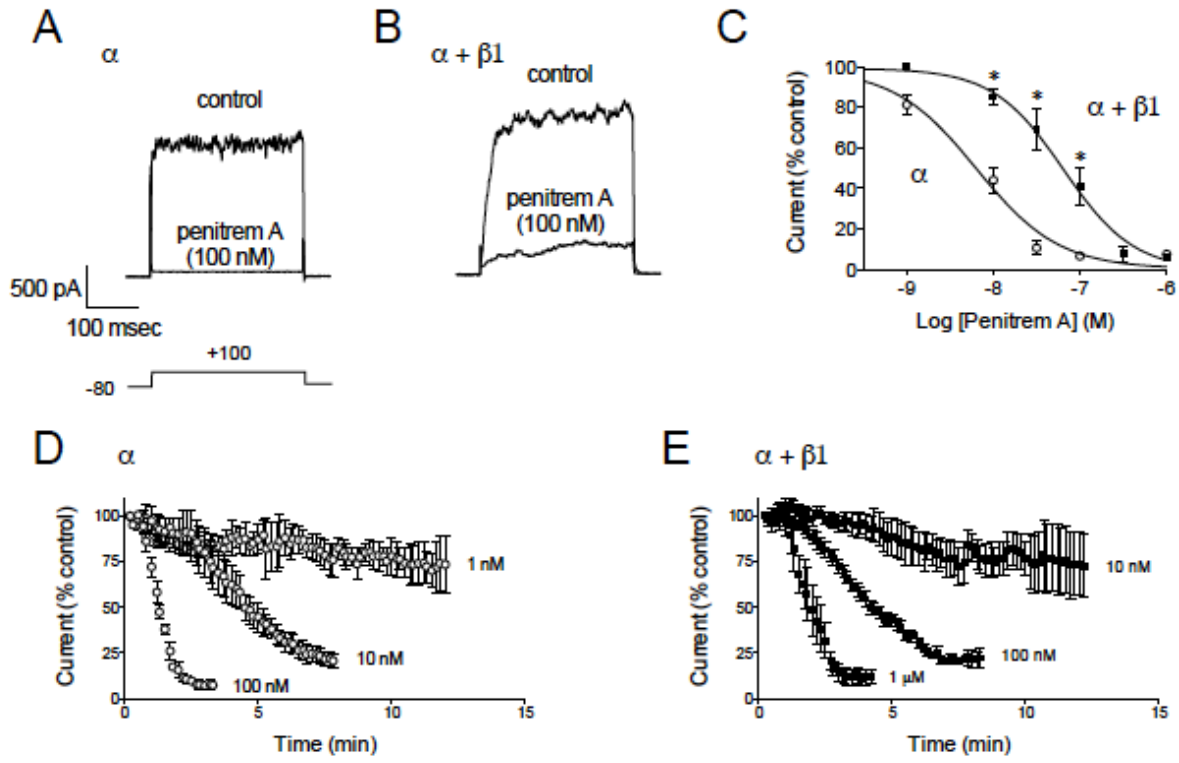

Figure 2 1 Presence of the β1 subunit decreases the sensitivity of BK channels to penitrem A

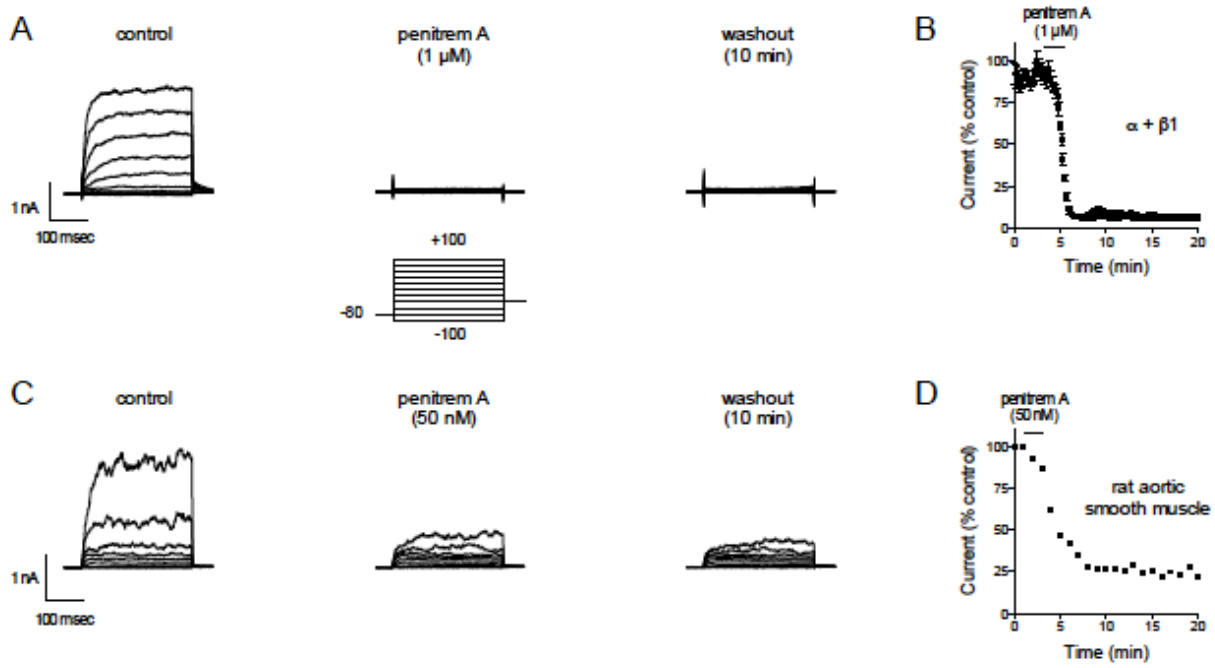

Figure 2 2 Irreversible block of BK channels by penitrem A

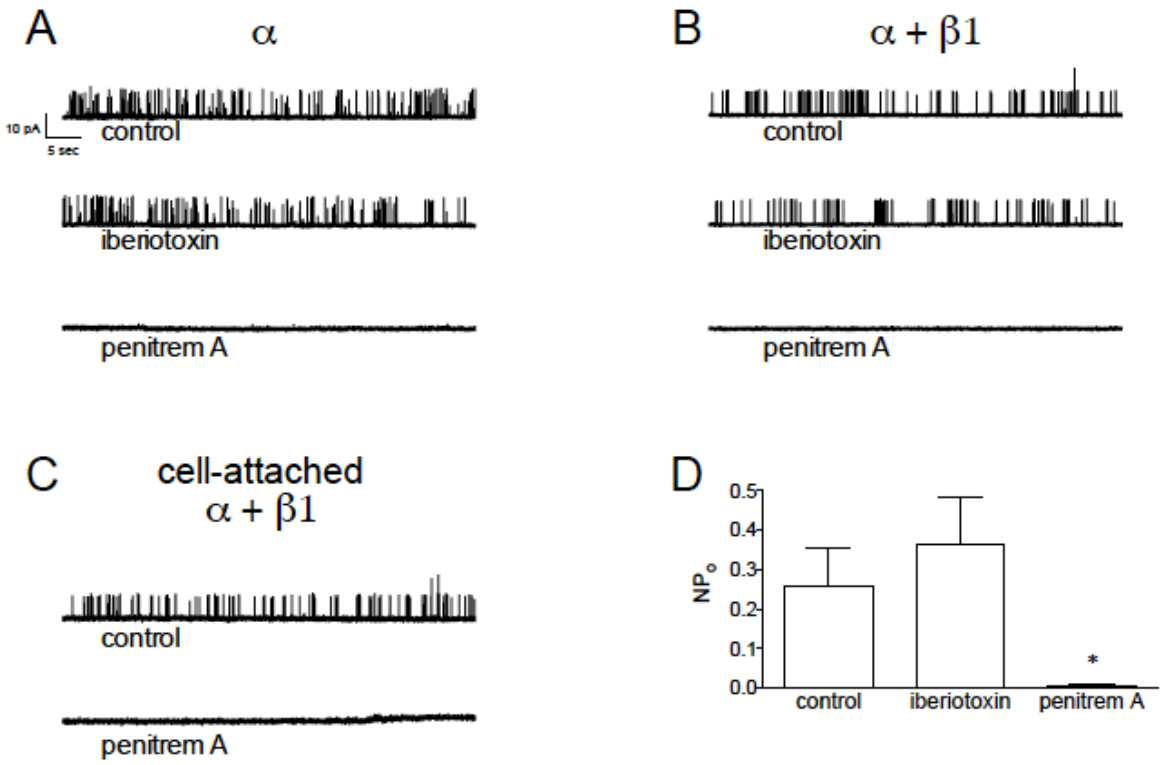

Figure 2 3 Penitrem A blocks BK channels in inside-out and cell-attached patches.

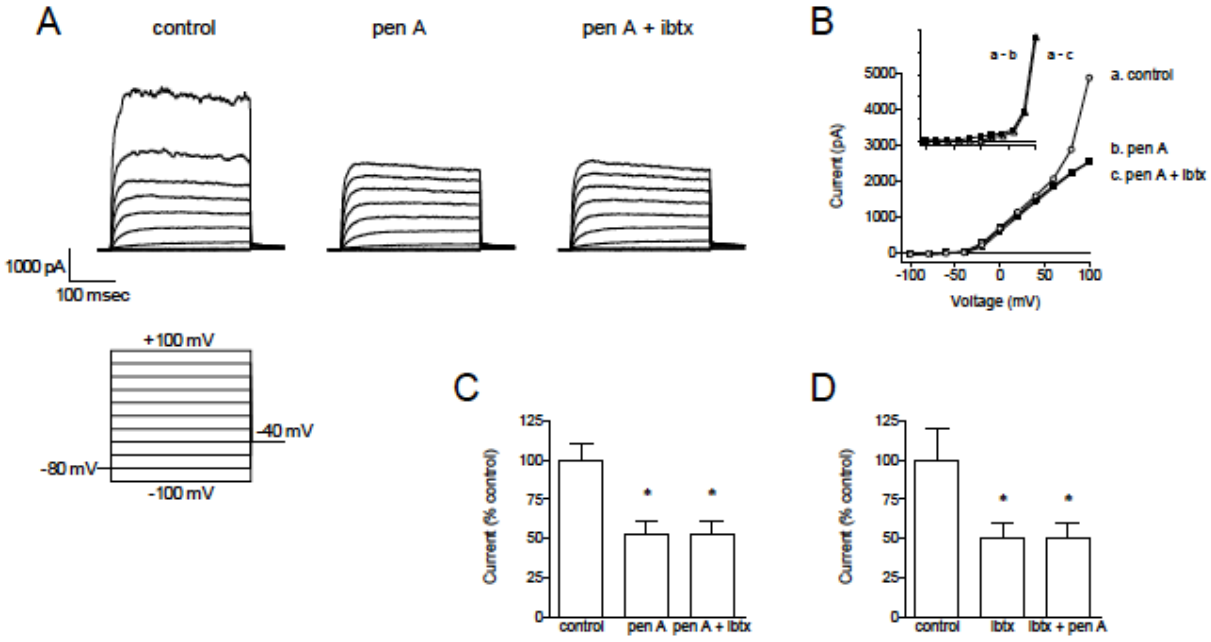

Figure 2 4 Iberiotoxin and penitrem A block the same current in canine coronary smooth muscle cells.

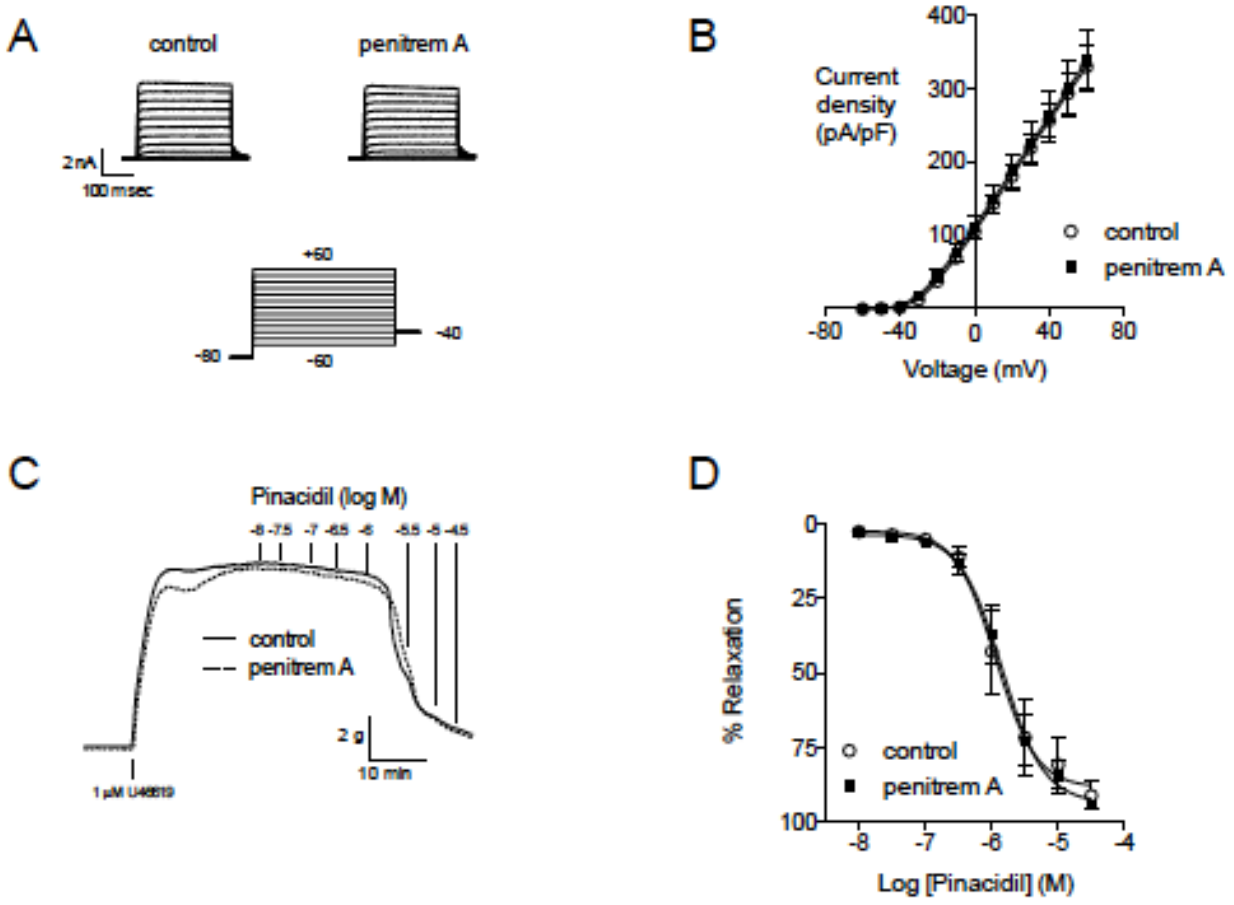

Figure 2 5 Penitrem A has no effect on $K_V1.5$ or K_{ATP} channels.

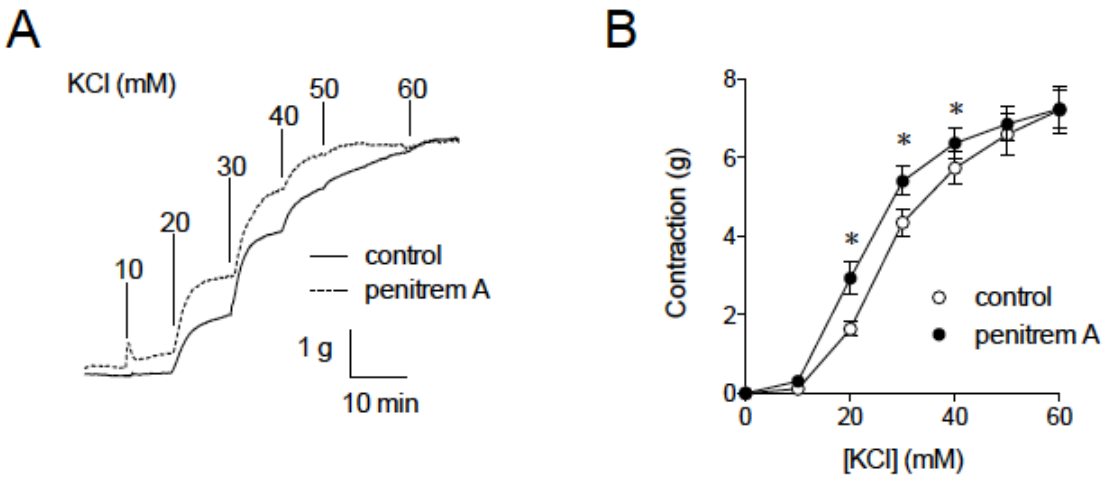

Figure 2 6 Penitrem A selectively inhibits BK channel to alter vascular contraction from *in vitro* vascular functional experiments

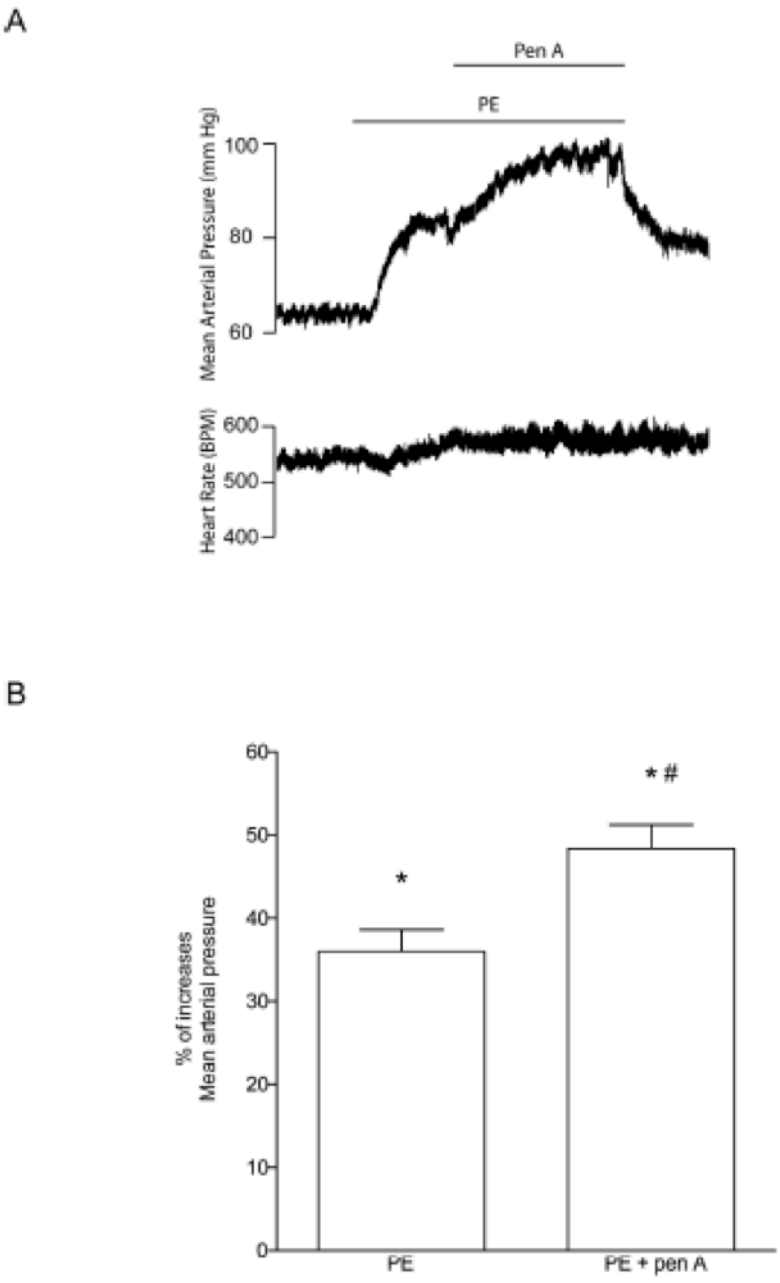

Figure 2 7 Block of BK channels by penitrem A augments phenylephrine-induced blood pressure responses in mice

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Table 2 1 Characteristics of BK channel blockers

	Reference	Extracellular block	Intracellular block	IC_{50} (nM) α (or undefined b*)	α + β1	α + β2	α + β3	α + β4
Iberiotoxin	Galvez et al. (1990)	Yes	No	0.25*	—	—	—	—
	Lippiat et al. (2003)	Yes	—	33	371	—	39	no block
Paxilline	Knaus et al. (1994)	Yes	Yes	<10*	—	—	—	—
	Sanchez and McManus (1996)	—	Yes	2	—	—	—	—
	Imlach et al. (2008)	—	Yes	22	23	—	—	18
Lolitrem B	Dalziel et al. (2005)	—	Yes	4	—	—	—	—
	Imlach et al. (2008)	—	Yes	4	5	—	—	2
Penitrem A	Knaus et al. (1994)	Yes	Yes	<10*	—	—	—	—
	this study	Yes	Yes	6	64	—	—	—

Numbers for Knaus et al. (1994) are estimations based on one dose (10 nM) that inhibited more than 50% of the current

Supplemental Data

We determined the effect of penitrem A on K^+ currents in a variety of native vascular smooth muscle cells. The point of these experiments is to demonstrate that penitrem A (1 μM) inhibits the same type of strongly outwardly rectifying current without effect on native delayed rectifiers. Panels A-E show currents in smooth muscle cells isolated from rat middle cerebral artery (n = 3), rat femoral artery (n = 3), mouse aorta (n = 3), pig coronary artery (n = 5), and dog coronary artery (n = 7). The voltage template (shown in A) was the same for all experiments. Solutions for whole-cell currents are described in the Methods. Panel F contains data showing that penitrem A 1 μM inhibits the a subunit cloned from cow (courtesy of Dr. Michael Davis, University of Missouri) and expressed in HEK 293 cells (n = 3).

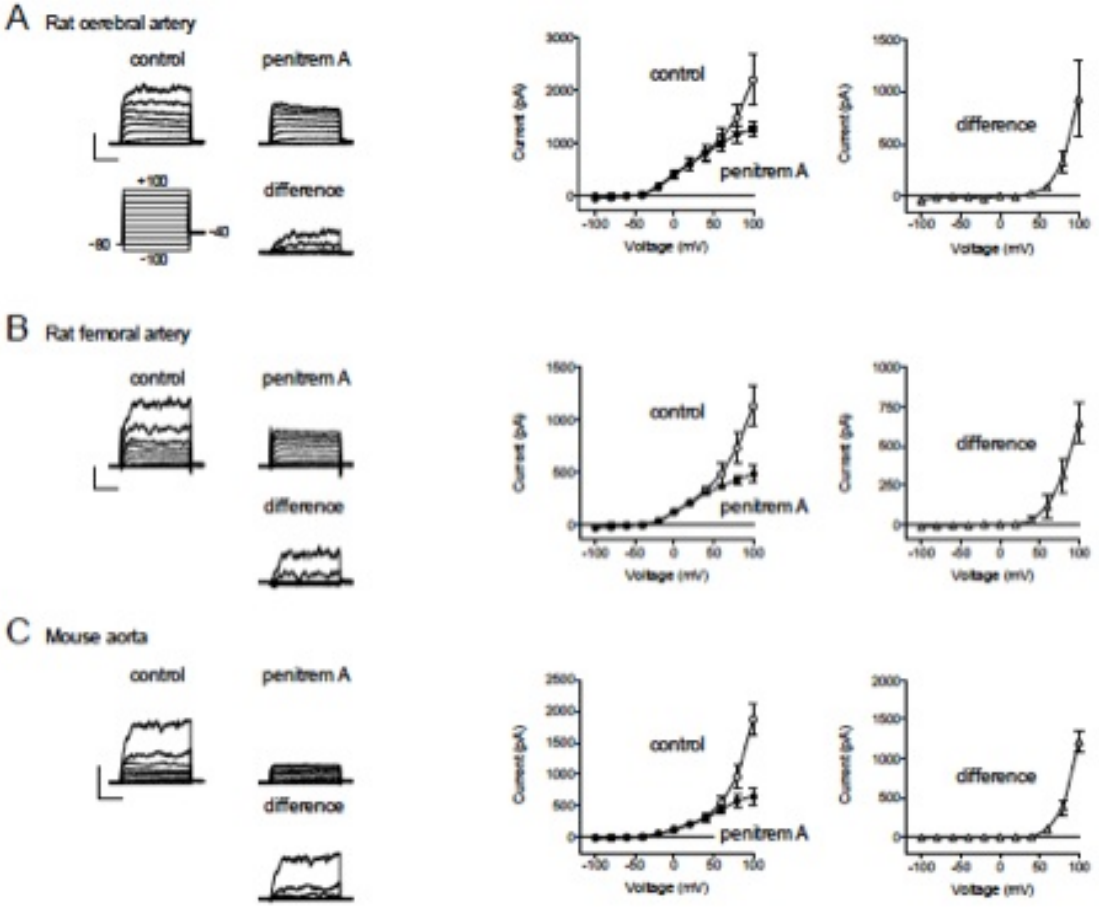

Figure 2 8 Supplemental Figure1 Penitrem A on K^+ currents in a variety of vascular smooth muscle cells

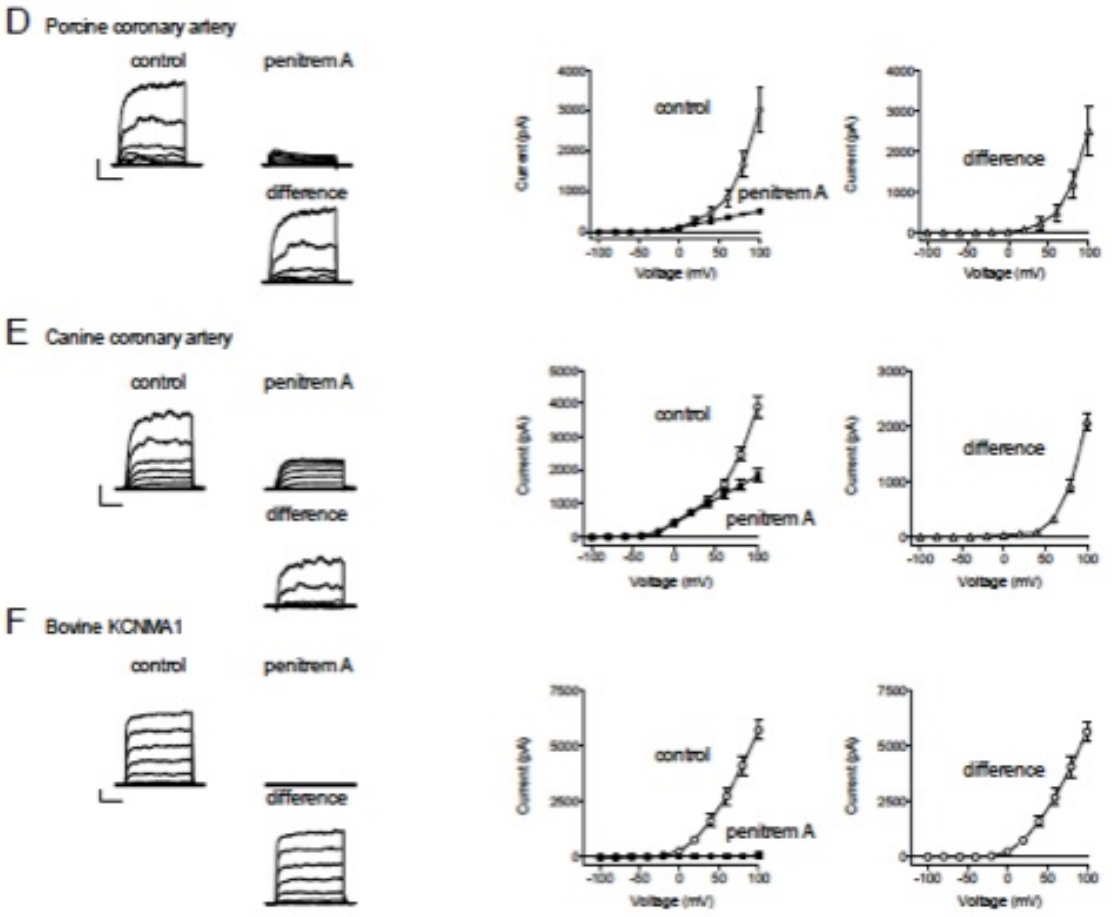

Figure 2 9 Supplemental Figure2 Penitrem A on K^+ currents in a variety of vascular smooth muscle cells

CHAPTER 3 Bisphenol A activates maxi-K channels in coronary smooth muscle

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Running head: BPA activates Maxi-K

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Abstract

Background and purpose: Bisphenol A (BPA) is used to manufacture plastics, including food containers into which it leaches. High levels of exposure to this estrogenic endocrine disruptor are associated with diabetes and heart disease. Estrogen and estrogen receptor modulators increase the activity of large conductance Ca^{2+}/voltage-sensitive K^+ (Maxi-K) 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: These data indicate BPA increases the activity of Maxi-K channels and may represent a basis for some potential toxicological effects.

Introduction

Although there is no clear consensus on detrimental health effects of 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; Wetherill *et al.*, 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.

BPA interacts with nuclear estrogen receptors, although much less potently than 17β-estradiol (Krishnan *et al.*, 1993; Kuiper *et al.*, 1997). BPA also exerts nongenomic effects, independent of nuclear estrogen 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*. Maxi-K channels offer themselves as an excellent model to test for interactions with BPA, as they are estrogen-sensitive (Valverde *et al.*, 1999).

Maxi-K channels, identified as $K_{Ca}1.1$ in the Guide to Receptors and Channels (Alexander *et al.*, 2008), are formed by 4 pore-forming α subunits encoded by *KCNMA1* (Butler *et al.*, 1993); when β1 subunits are present,

sensitivity to estrogenic substances is conferred (Valverde *et al.*, 1999; Dick *et al.*, 2001; Dick & Sanders, 2001; Dick *et al.*, 2002; Duncan, 2005; Coiret *et al.*, 2007). 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 analyzed in smooth muscle cells and in AD-293 cells expressing α or α + β1 subunits.

Methods

Smooth muscle cells. Three coronary artery smooth muscle preparations were used; these include cells: a) freshly isolated from the canine left anterior descending (LAD) artery; b) cultured from the canine LAD; and c) cultured from human hearts. Canine LAD arteries were collected from dogs euthanized under surgical anesthesia for unrelated experiments. Protocols were approved and followed guidelines set forth in the Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996). 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 (mg ml^{-1}) 2 type II collagenase and 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). 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) were cultured in Medium 231 with growth and differentiation supplements, the latter added at ~80% confluence.

Transient transfection. AD-293 cells (Stratagene) were grown in DMEM supplemented with 10% FBS, 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 GFP, pmaxGFP, was purchased from AMAXA. 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 FBS 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; product number 133027) and penitrem A (MP

Biomedicals) 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) 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). Series resistance and capacitance were compensated as much as possible using the amplifier circuitry (PC-505B; Warner Instruments). 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; 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'-AGCTCG GGATGTTTAGCAGA-3'; product size 119 bp) and *KCNMB1* (NM_004137; 5'-GCCGGG AAGACTAAATGATC-3' and 5'-TGGGAT GTAGGAGCACTG-3'; product size 357 bp). PCR amplification was performed in 50 µl reactions containing 1x 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) was used. PCR products were separated by electrophoresis on 2% agarose with ethidium bromide and images captured using a GeneFlash unit (Syngene).

Statistics. Data are presented as the mean and standard error of n number of cells or membrane patches. When two values were compared, a paired t-test was used. When more than two values were compared, one-way ANOVA was used. Current-voltage relationships were analyzed 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 recording revealed prominent Maxi-K current in smooth muscle cells cultured from the canine LAD coronary artery (Fig. 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. Current density at +100 mV was 35 ± 12 pA pF^{-1} (n = 5). Whole-cell current increased with the application of 10 μM BPA (Fig. 1A, middle; current density at +100 mV was 64 ± 24 pA 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 (Fig. 1A, right; current density at +100 mV was 3 ± 2 pA 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 (Fig. 1B; current density at +100 mV was 33 ± 10, 6 ± 3, and 4 ± 2 pA 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 Fig. 1C. Group data for both experiments in cultured canine coronary smooth muscle cells are shown in Figs. 1D and E. Cultured canine coronary smooth muscle cells were derived from 3 different dogs and a characterization of the smooth muscle phenotype of these cells is available in the Supplement.

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; $p < 0.05$; Fig. 2A & B). Single channel conductance was not affected by 10 μM BPA (100 ± 3% of control; Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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% (Fig. 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 Fig. 3F & 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 (Fig. 4A & C). NP_o was 123 ± 20% of control in the presence of 10 μM BPA (Table 1; n = 6; *p* = N.S.). In contrast, in Maxi-K channels containing the β1 subunit, 10 μM BPA increased NP_o (Fig. 4B & 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 (Fig. 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 (Fig. 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; $p < 0.05$, 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) (Fig. 5C). 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 (Fig. 6). The smooth muscle phenotype of these cells is demonstrated in the Supplement. The lower concentrations of BPA (1 and 10 μM) did not increase Maxi-K current; however, 100 μM BPA increased current significantly (Fig. 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 (Fig. 5), we investigated whether cultured human coronary smooth muscle cells expressed the β1 subunit. Thus, RT-PCR was used to analyze Maxi-K channel subunit mRNA expression. Cultured human coronary smooth muscle cells expressed *KCNMA1* mRNA, but mRNA for *KCNMB1* was undetectable (Fig. 6C). A positive control, using human brain cDNA, however, demonstrated the applicability of the *KCNMB1* primers (Fig. 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 due to a lack of *KCNMB1* expression (Yang *et al.*, 2009).

Discussion

These studies demonstrate that BPA, an estrogenic endocrine disruptor, increases Maxi-K channel current via a nongenomic 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 upheld only partially by our studies. Specifically, the β1 subunit played a compulsory 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 simply enhances 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 document the activation of Maxi-K channels by estrogenic substances, including 17β-estradiol (Valverde *et al.*, 1999). Thus, to find that BPA activates Maxi-K channels may not seem surprising at first glance. However, the lack of a relationship between the estrogenic nature of a compound and its effect on Maxi-K channels makes such presumptions difficult. For example, tamoxifen, an estrogen receptor modulator with mixed agonist and antagonist properties, activates Maxi-K channels more potently and efficaciously than 17β-estradiol (Dick *et al.*, 2001). Moreover, the pure anti-estrogen ICI 182,780 also activates Maxi-K channels (Dick, 2002). Studies generally agree, however, that the β1 subunit confers sensitivity upon Maxi-K channels to estrogens and xenoestrogens (Valverde *et al.*, 1999; Dick *et al.*, 2001; Dick & Sanders, 2001). 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 4 known types, differs depending upon the agonist. For example, the β1 subunit is important for Maxi-K channel activation by 17β-estradiol (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 impact not only the interaction of 17β-estradiol with nuclear receptors (Krishnan *et al.*, 1993; Kuiper *et al.*, 1997), but also initiate nongenomic signaling mechanisms. For instance, BPA increases intracellular Ca^{2+} in GH3/B6 pituitary tumor cells (Watson *et al.*, 2005; Wozniak *et al.*, 2005; Watson *et al.*, 2007) 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 signaling 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 signaling 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 estrogen (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; Bukiya *et al.*, 2008a; Bukiya *et al.*, 2008b). Bukiya and colleagues 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 target of BPA and β1 subunits enhance BPA binding to the α subunit. In support of this idea, Korovkina and coworkers have demonstrated that the Maxi-K channel α subunit itself functions as a binding site for 17β-estradiol, and that β1 subunits enhance binding by approximately three-fold (Korovkina *et al.*, 2004). Assuming that BPA may bind to the same site, their observations may explain why the β1 subunit, while not requisite 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 suggesting that the tamoxifen binding site is located on the Maxi-K α subunit and that β1 subunits facilitate or stabilize the interaction (Perez, 2005). The possibility remains, however, that BPA interacts with an unknown molecule to transmit a nongenomic 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. Worldwide, annual BPA production exceeds 6 billion pounds and hundreds of tons of BPA enter the atmosphere. Thus, it is surprising that data regarding BPA exposure levels remain scarce. Free unconjugated BPA in human serum ranges from approximately 0.9-90 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 are estimated to be 0.017-1.7 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 obtained with micromolar concentrations of BPA appear to be higher than what most humans are exposed to; however, certain occupational populations are at much higher risk (Hanaoka *et al.*, 2002). Much remains to be resolved about human exposure to BPA and its effects; however, we present the first evidence demonstrating that BPA affects ion channel function directly.

In conclusion, we demonstrate that BPA activates Maxi-K channels in a nongenomic 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 increases the BPA-sensitivity of Maxi-K channels; however, the α subunit alone is 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 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.

Acknowledgement

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Figure legends

Fig. 1 BPA increases Maxi-K current in smooth muscle cells cultured from the canine coronary artery. (Panel A) Representative currents are shown under control conditions, with the application of 10 μM BPA, and with 1 μM penitrem A. (Panel 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. (Panel C) The voltage template used to elicit currents in this and subsequent figures. (Panel D) Group data (n = 5) demonstrate the BPA- and penitrem A-sensitivity of currents in cultured coronary artery smooth muscle cells. (Panel E) Penitrem A prevented BPA-induced increase in Maxi-K current (n = 4). Asterisks indicate $p < 0.05$ vs. control by one-way ANOVA.

Fig. 2 BPA increases Maxi-K channel NP_o in smooth muscle cells freshly isolated from the canine coronary artery. (Panel 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. (Panel B) Group data (n = 10 cells from 4 dogs) demonstrate that 10 μM BPA increases NP_o. Asterisk indicates $p < 0.05$ by paired t test. (Panel C) Group data (n = 10) show that BPA has no effect on single conductance.

Fig. 3 Maxi-K β1 subunit confers sensitivity to 10 μM BPA. Currents were measured from AD-293 cells transfected with GFP (Panel A) and Maxi-K channels composed of α (Panel B) or α + β1 (Panel C) subunits. Voltage template used is shown in Fig. 1C. Very little whole-cell current was observed in cells expressing GFP only. Cells transfected with the α subunit demonstrate large outward currents with fast activation and little or no inactivation. Cells expressing α + β1 subunits demonstrate large outward currents with slower activation. (Panel D) Records from a representative cell transfected with the Maxi-K a subunit show that current is unaffected by 10 μM BPA, but blocked by 1 μM penitrem A. (Panel 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. (Panels F & 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. Asterisks indicate $p < 0.05$ vs. control by two-way repeated measures ANOVA.

Fig. 4 BPA (10 μM) increases NP_o of 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 (Panel A) or α + β1 (Panel B) subunits before and after exposure to 10 μM BPA. Patch potential was +40mV in symmetrical 140 mM K^+ solutions containing 100 nM free Ca^{2+}. (Panels C & D) contain all-points amplitude histograms from 1 min recordings of patches shown in Panels A & B. BPA increased the NP_o of Maxi-K channels containing the β1 subunit and no effect on single channel conductance.

Fig. 5 The activation of Maxi-K channels by BPA is concentration-dependent and reversible. (Panel 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. Asterisks indicate differences between α and α + β1 by two-way ANOVA. (Panel 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). (Panel C) Time course for the activation and the reversibility was normalized to the maximal activation of each value.

Fig. 6 BPA, at a concentration of 100 μM, increases Maxi-K current in human coronary artery smooth muscle cells. (Panel A) Representative records demonstrate that BPA (100 μM) increased whole-cell current in smooth muscle cells cultured from human coronary arteries. (Panel B) contains group data (n = 3-7) for effect of three concentrations of BPA on Maxi-K current. (Panel 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.

Table 3 1 β1 subunit determines the response of Maxi-K channels to 10 μM BPA

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

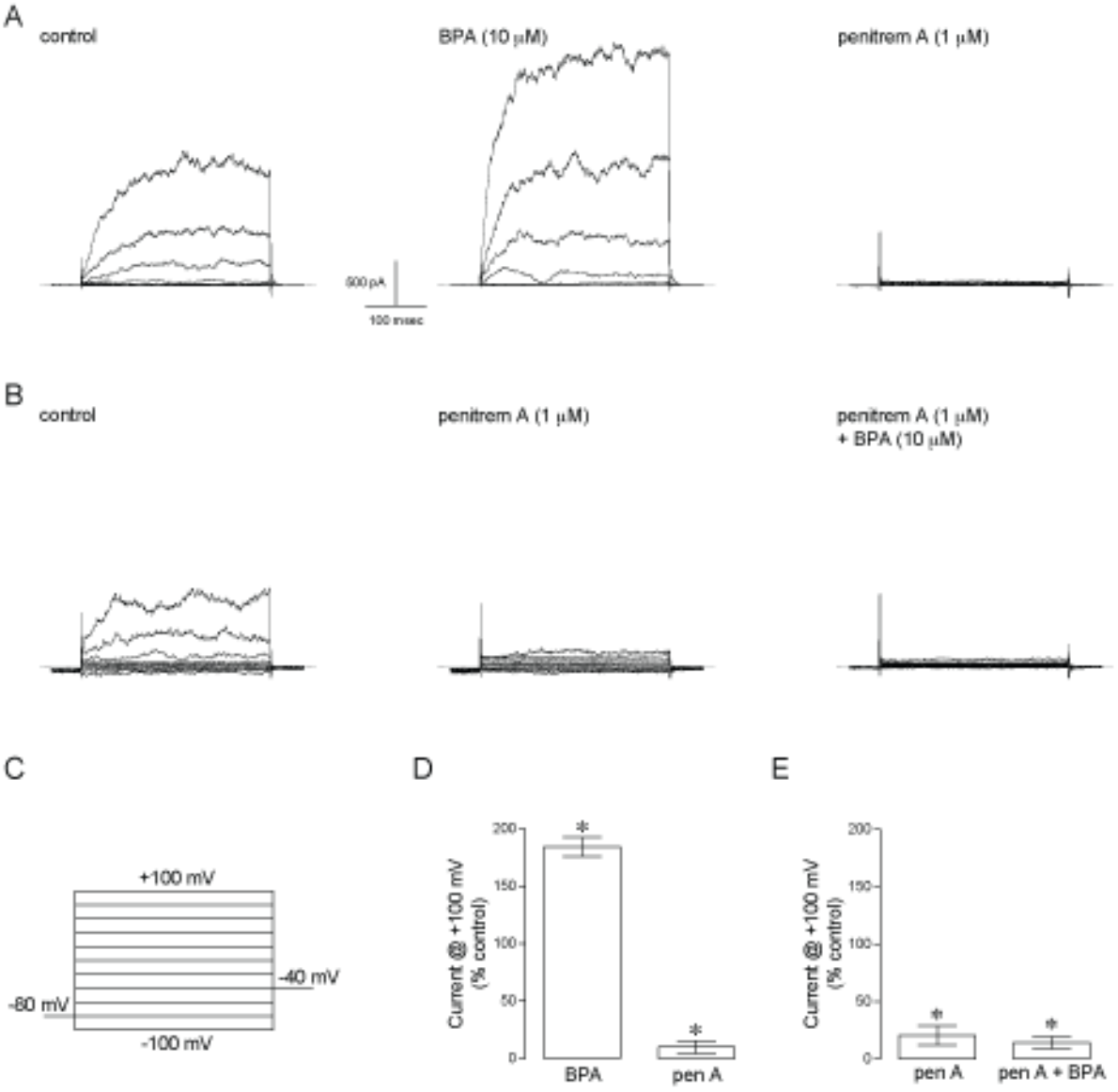

Figure 3 1 BPA increases Maxi-K current in smooth muscle cells cultured from the canine coronary artery.

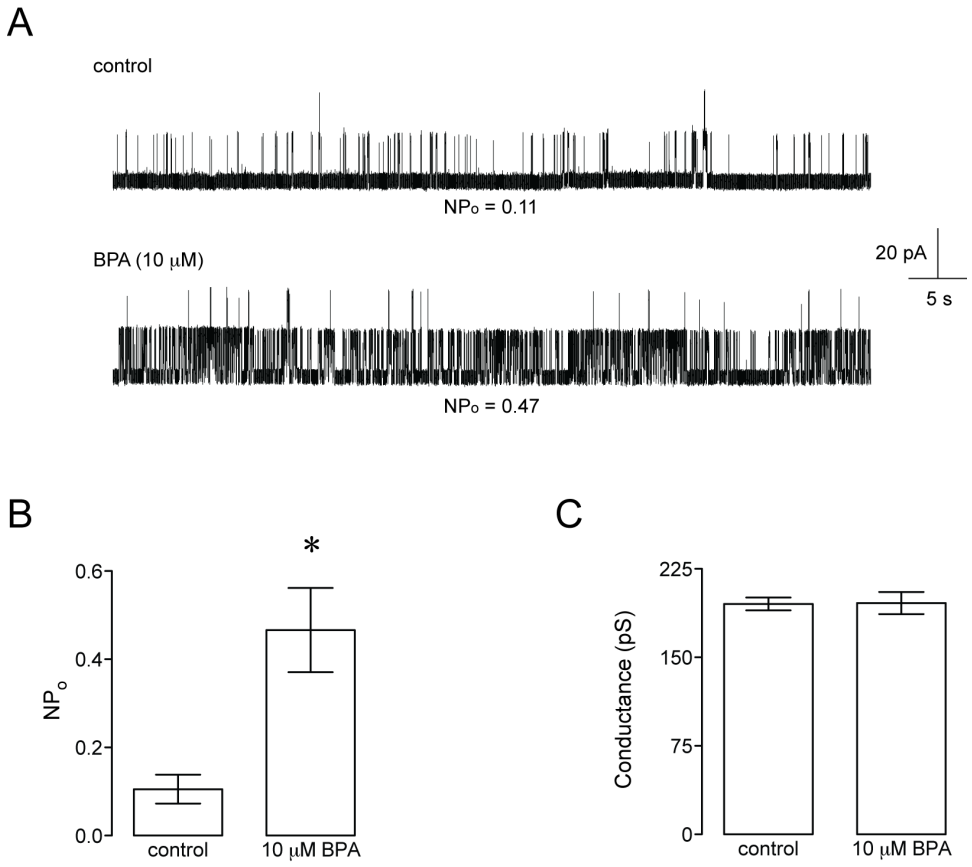

Figure 3 2 BPA increases Maxi-K channel NP_o in smooth muscle cells freshly isolated from the canine coronary artery

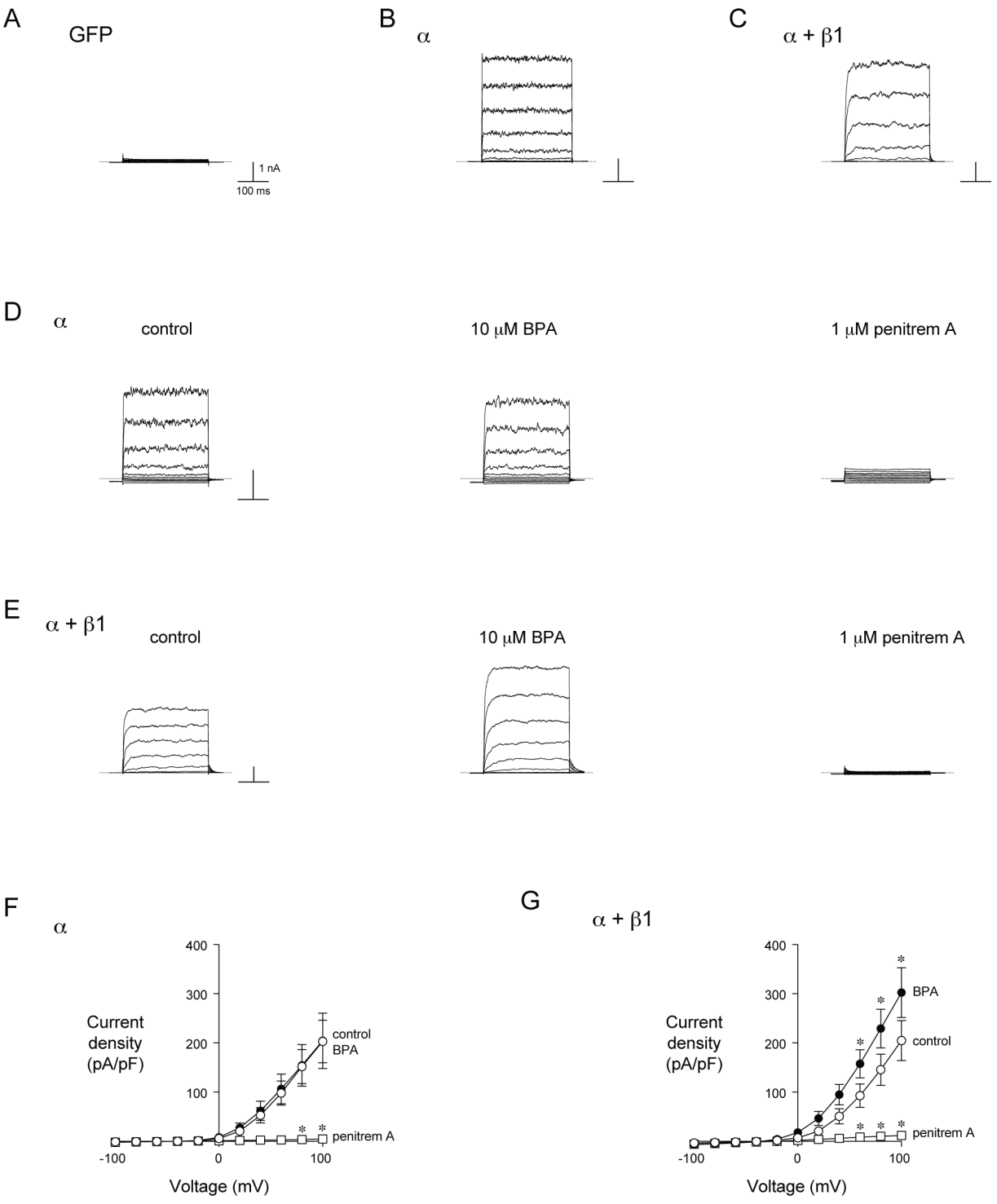

Figure 3 3 Maxi-K β1 subunit confers sensitivity to 10 μM BPA

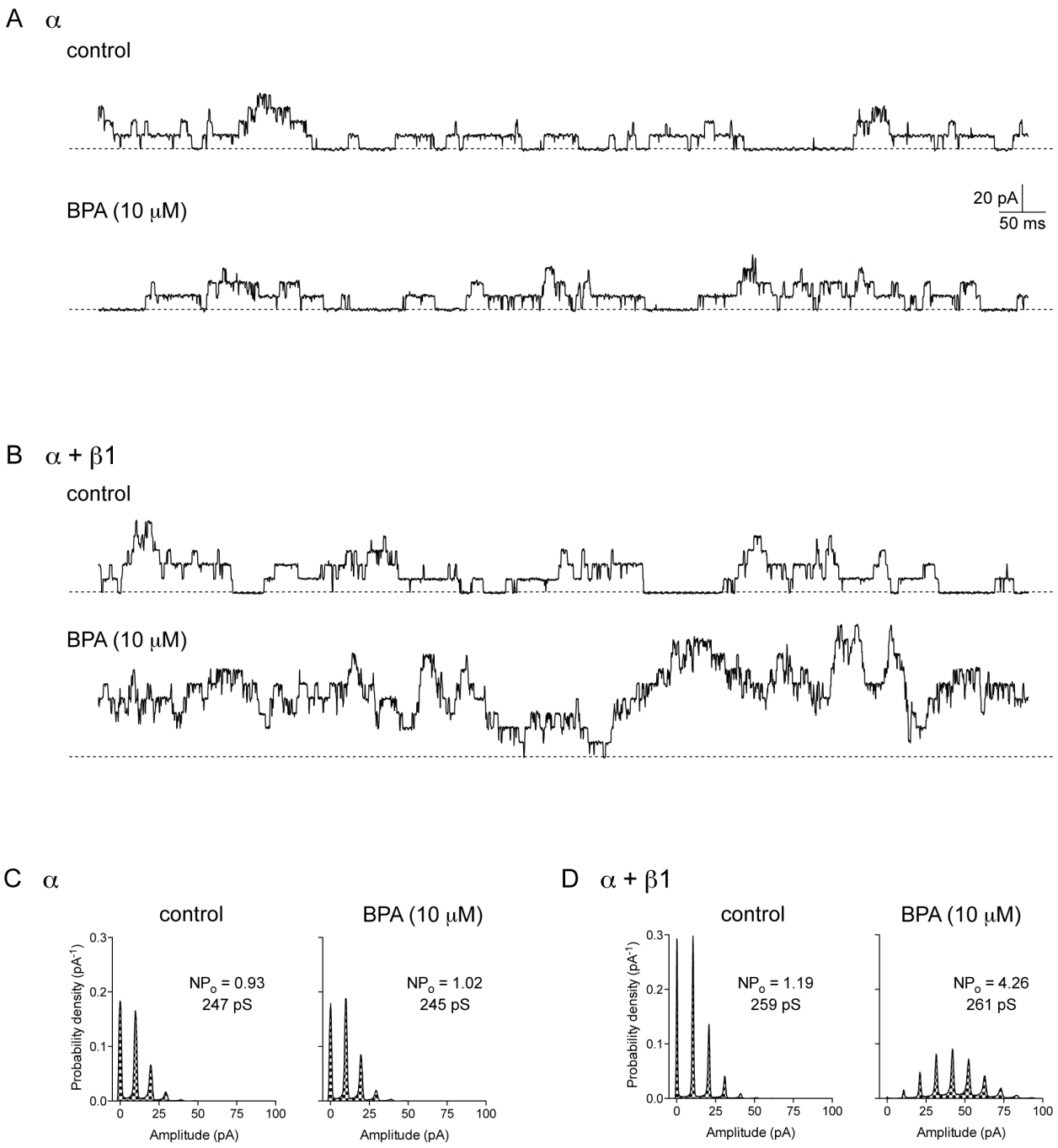

Figure 3 4 BPA (10 μM) increases NP_o of Maxi-K channels containing the β1 subunit

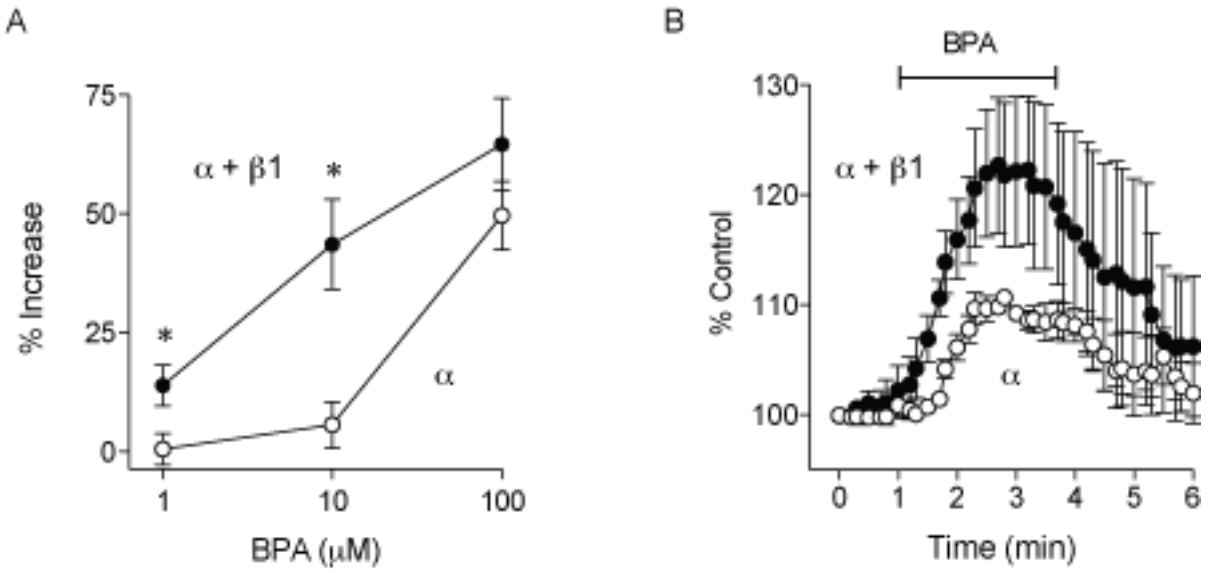

Figure 3 5 The activation of Maxi-K channels by BPA is concentration-dependent and reversible.

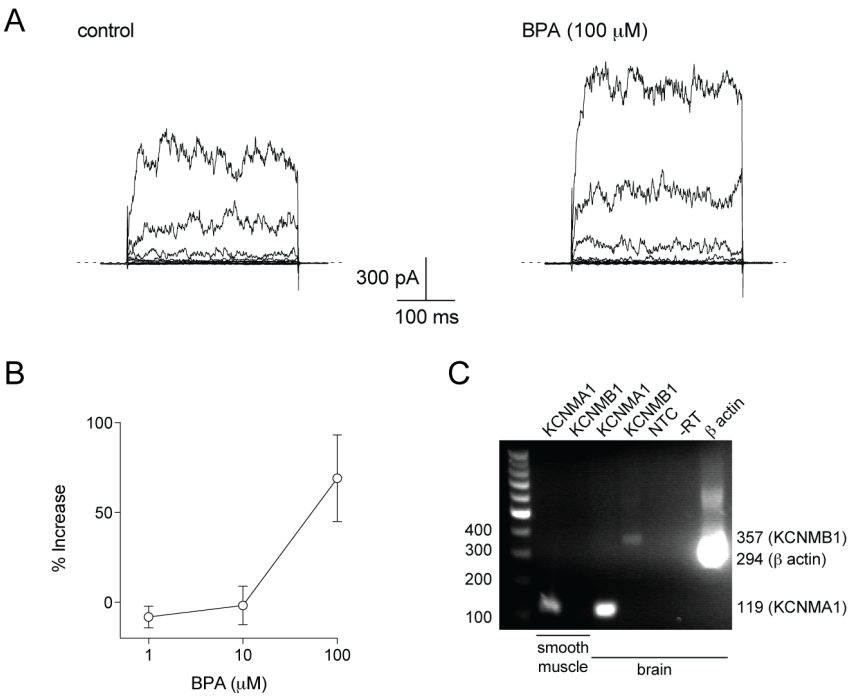

Figure 3 6 BPA, at a concentration of 100 μM, increases Maxi-K current in human coronary artery smooth muscle cells

Supplemental information

Cells from the canine coronary artery were cultured and differentiated into smooth muscle. Cells took ~4-5 days to start effectively dividing and grew to confluence in ~7 days. Cells no longer grew after passage ~10. Confluent cells exhibited a “hill and valley” pattern under phase contrast (Supplemental Fig. 1A). Immunoblots indicated the expression of smooth muscle-specific a actin and myosin heavy chain in differentiated cells (Supplemental Fig. 1B). Phase contrast images and smooth muscle-specific a actin and myosin heavy chain immunoreactivity for cultured human smooth muscle cells are also shown in Supplemental Figs. 1A & B. Smooth muscle cells were trypsinized and plated on cover glass for patch clamp experiments. These cells retained the smooth muscle phenotype, as shown by immunocytochemistry for smooth muscle-specific α actin (Supplemental Fig. 2A). After treatment with differentiation media for 1 or 2 weeks, 75 ± 4 and 90 ± 3% of the canine cells retained the smooth muscle phenotype (n = 3, Supplemental Fig. 2B). Immunocytochemistry and the percent of cells with a smooth muscle phenotype are also shown in Supplemental Figs. 2A & B. Thus, canine coronary artery smooth muscle cells demonstrated morphology and immunoreactivity consistent with those observed by others in various species (Teng *et al.*, 2006; Christen *et al.*, 199. Taken together, we conclude that the majority of cultured cells possessed a smooth muscle phenotype and were appropriate for study.

METHODS

Western blots. Cultured cells were washed two times with PBS and harvested in RIPA lysis buffer supplemented with (mM) 2 PMSF, 1 Na_3VO_4, 1 DTT and 1x protease inhibitor cocktail solution (Santa Cruz Biotechnology). Samples were sonicated on ice, incubated for 15 min on ice, and centrifuged at 10,000 g at 4 °C for 10 min. The supernatant was removed and stored at -80°C until use. Protein concentrations were determined by BCA method using BSA as a standard (Pierce). Samples were diluted to 1 mg/ml in Laemmli sample buffer and boiled at 70 °C for 15 min. Equivalent amounts of protein were separated by SDS-PAGE, followed by electroblotting onto nitrocellulose membranes. Membranes were blocked with 5% nonfat milk (Bio-Rad) in TBST for 1 h at room temperature, washed 3 times in TBST, and incubated overnight at 4 °C in primary antibodies against smooth muscle α actin (1:500; Sigma), smooth muscle myosin heavy chain (1:500; Biomedical Technologies) and b tubulin (1:1000; Santa Cruz Biotechnology). After washing 3 times in TBST, membranes were incubated with anti-species secondary antibody conjugated with HRP and HRP-linked anti-biotin secondary antibodies for a biotinlyated ladder (1:5000; Cell Signaling Technology) for 1 h at room temperature and then washed again 3 times with TBST. An enhanced chemiluminescence detection system (ECL Advance, Amersham Biosciences) was used to detect the antibodies and the lanes were analyzed using a G:Box system (Syngene).

Immunocytochemistry. Differentiated cells were plated on cover glass and allowed to adhere overnight. Cells were washed with PBS and fixed in 4% formaldehyde in PBS, pH 7.4 for 30 min at room temperature. After three washes

in PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 2 min. Cells were treated 30 min in blocking solution (2% goat serum, 3% BSA in PBS) and then incubated with anti-smooth muscle-specific α actin (1:400; Sigma) for 1 h at room temperature in a humidified chamber. Following three washes with PBS, cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Invitrogen) for 1 h at room temperature in a humidified chamber. After a final wash with PBS, cover glass was placed on mounting medium (Santa Cruz Biotechnology) on a slide. Images were obtained with an epifluorescence microscope and a SPOT RT camera (Diagnostic Instruments).

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Supplemental Fig. 1 Cells cultured from the canine LAD coronary artery revealed typical smooth muscle morphology and expressed smooth muscle phenotypic markers. (Panel 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. (Panel 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.

Supplemental Fig. 2 Smooth muscle cells plated for patch clamp experiments continue to express phenotypic markers. (Panel A) Immunofluorescence of smooth muscle-specific α actin (green) during differentiation; nuclei are stained blue. Images from canine and human cells are shown. (Panel B) The percentage of cells in culture that express smooth muscle-specific α actin increases over time in differentiation medium (n = 3). Asterisks indicate $p < 0.05$ vs. day 0.

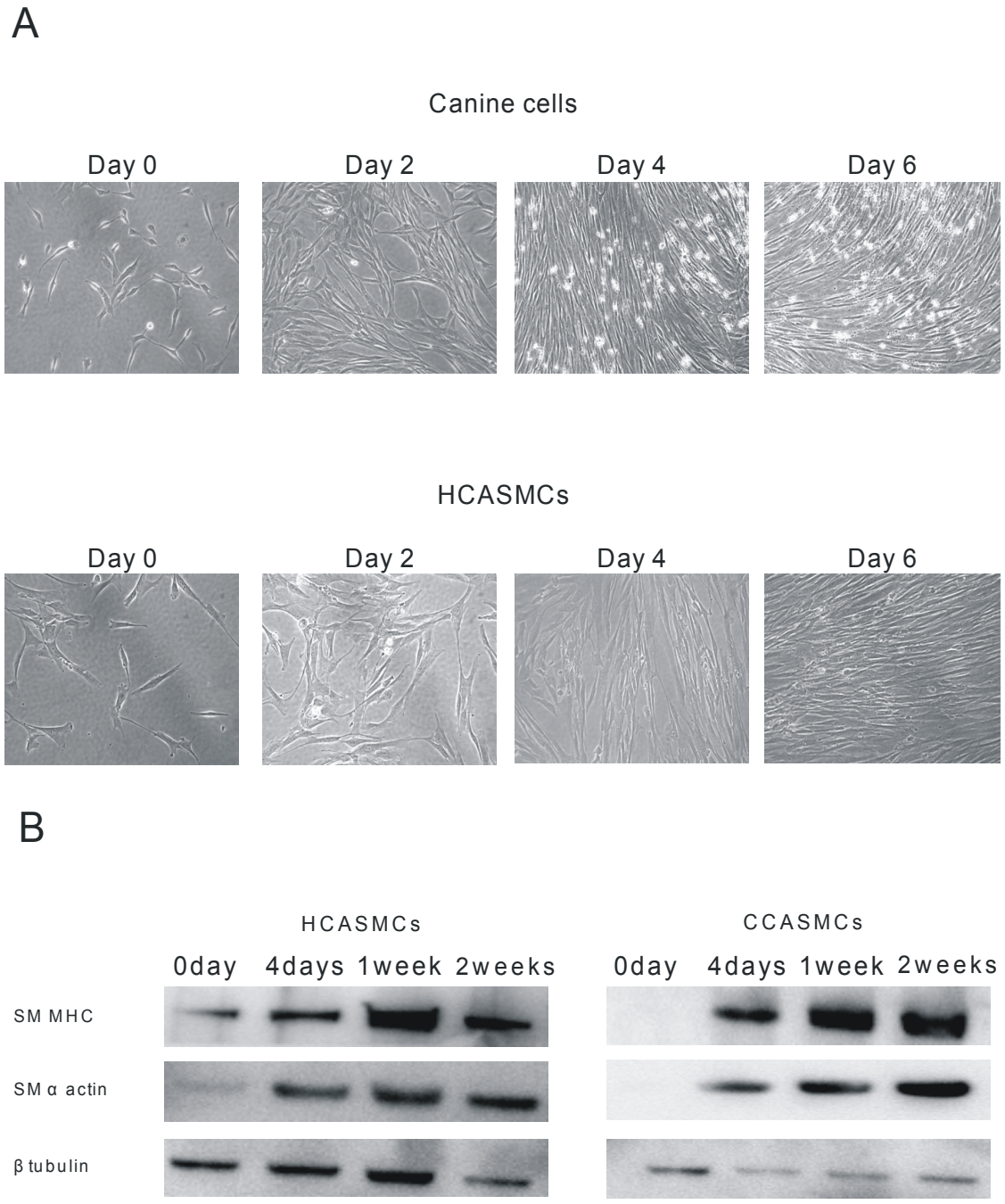

Figure 3 7 Supplemental Figure 1. Cells cultured from the canine LAD coronary artery revealed typical smooth muscle morphology and expressed smooth muscle phenotypic markers

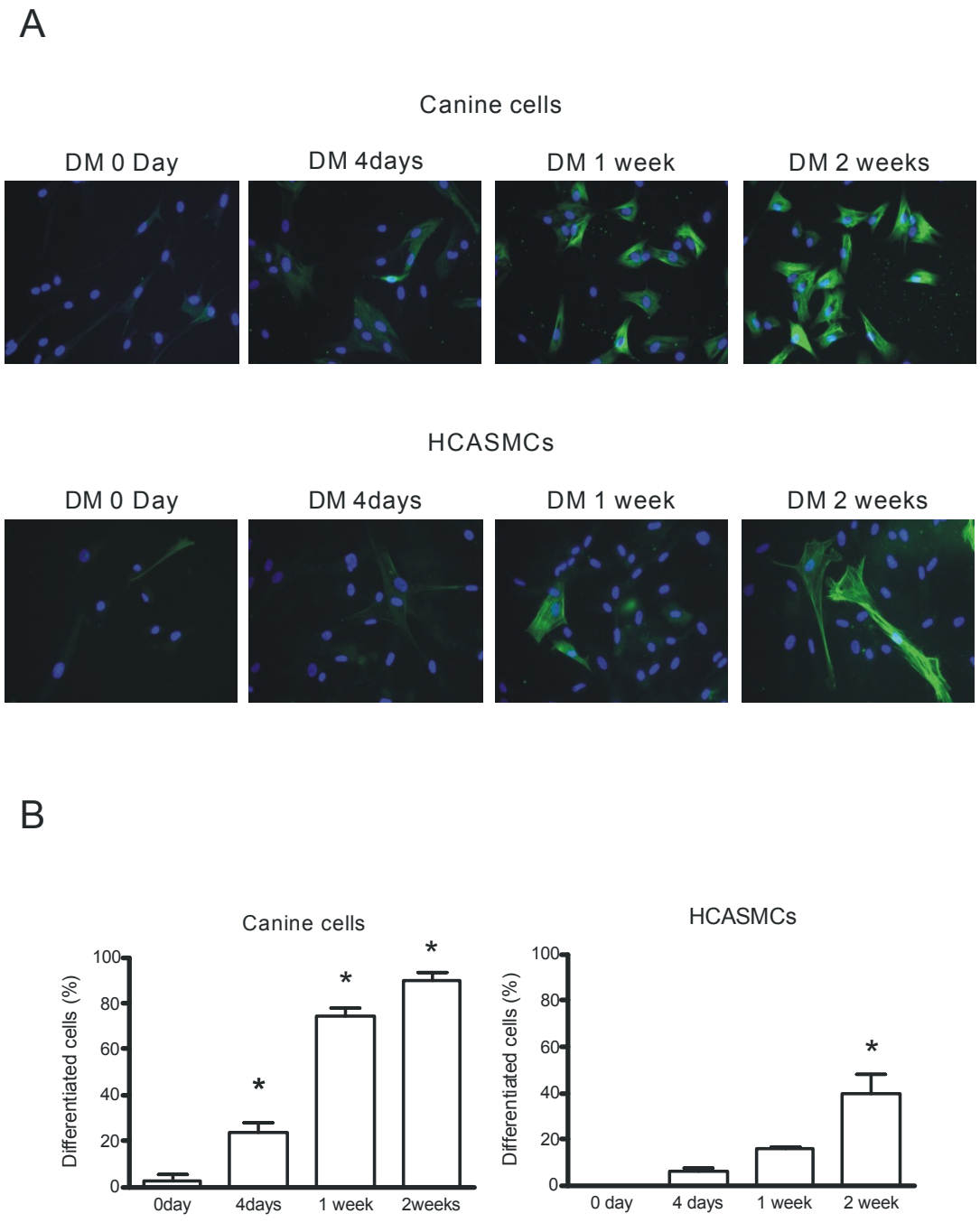

Figure 3 8 Supplemental Figure 2. Smooth muscle cells plated for patch clamp experiments continue to express phenotypic markers

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CHAPTER 4 Bisphenol A decreases BK channel expression in rat aortic smooth muscle via estrogen receptor mediated mechanisms

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Short title: BPA negatively regulates KCNMA1

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Abstract

Background and purpose: Bisphenol A (BPA) is an estrogenic endocrine disruptor implicated in the development cardiovascular disease. We previously demonstrated that BPA activates smooth muscle BK ($K_{Ca}1.1$) channels non-genomically (Asano et al. Br J Pharmacol. 160(1):160-70, 2010). This finding is seemingly inconsistent with BPA-induced cardiovascular disease. Importantly, however, estrogen also activates BK channels, but negatively regulates KCNMA1, the gene encoding BK channels. It is possible that BPA might have a similar effect; therefore, we tested the hypothesis that BPA reduces BK channel expression though a genomic mechanism.

Experimental approach: Rat aortae were placed in tissue culture for a 48-72 hour treatment with BPA (10 μmol/L). BK channel expression was determined by RT-PCR, Western blot, and whole-cell patch clamp. Experiments repeated with BPA in the presence of ICI 182,780 (1 μmol/L), an estrogen receptor antagonist.

Key Results: BPA decreased the expression of BK α subunit mRNA and protein. Penitrem A-sensitive BK current was reduced in smooth muscle cells from BPA-treated aortae. ICI 182,780, an estrogen receptor antagonist, blocked the effect of BPA to reduce BK channel mRNA, protein, and current.

Conclusions and implications: These data indicate BPA decreases BK channel expression via activation of nuclear estrogen receptors. Further, our findings represent one potential mechanism by which BPA exposure may possibly be linked to cardiovascular disease.

Keywords: estrogen; xenoestrogen; KCNMA1; $K_{Ca}1.1$; penitrem A; polycarbonate plastic; cardiovascular disease

Introduction

Bisphenol A (BPA) is used to manufacture polycarbonate plastic, resin lining of cans, and dental sealants. Further, BPA is used as a non-polymer additive to other plastics. A number of recent studies support the idea that BPA may be associated with carcinogenesis, altered reproductive function, and developmental issues due to its estrogen mimetic properties (Wetherill *et al.*, 2005; Savabieasfahani *et al.*, 2006; Vandenberg *et al.*, 2007; Hugo *et al.*, 2008). Thus, the FDA has some concerns regarding the effect of BPA, particularly in susceptible populations, such as fetuses, infants, and children. Recent epidemiological studies have revealed a significant relationship between urinary BPA concentration and cardiovascular diseases in the American adult population (Lang *et al.*, 2008; Melzer *et al.*, 2010). Furthermore, there is an association between erectile dysfunction and urinary BPA concentration in BPA-exposed workers (Li *et al.*, 2010a; Li *et al.*, 2010b). These studies suggest BPA-related pathologies may affect a much greater population, and altered vascular function with BPA exposure could be suspected, nonetheless; the mechanism of BPA action in vasculature remains unclear.

We demonstrated that acute BPA exposure could activate large conductance, voltage/Ca^{2+}-sensitive K^+ (BK; $K_{Ca}1.1$) channels in vascular smooth muscle via a non-genomic mechanism (Asano *et al.*, 2010). This non-genomic effect of BPA on BK channels was similar to that of estrogen and tamoxifen (Valverde *et al.*, 1999; Dick *et al.*, 2001). Estrogen has relevant genomic mechanisms as well, as it alters K^+ channel expression in variety of tissues (Drici

et al., 1996; Roepke *et al.*, 2007; Saito *et al.*, 2009). BK channel expression in uterine smooth muscle was decreased in late pregnancy, when estrogen is highest (Song *et al.*, 1999). Furthermore, long-term exposure to estrogen has been shown to directly interact with BK channels and down regulate BK channel expression in a heterologous expression system and human coronary artery smooth muscle (Korovkina *et al.*, 2004). These studies demonstrate that BK channel is an estrogen-sensitive protein and it may be associated with BPA-related cardiovascular pathologies. However, the effect of continuous exposure to BPA on functional BK channel expression vascular smooth muscle cells is not known. Therefore, this study examined whether BPA modulates BK channel expression in vascular smooth muscle cells and sought to determine the mechanisms. We hypothesized that BPA would alter BK channel expression through a mechanism that is dependent on interaction with classical estrogen receptors. To test this hypothesis, functional BK channel expression was analyzed in A7r5 cell lines and rat aorta.

Methods

Cell culture. A7r5 cells were purchased from American Type Cell Culture Collection (ATCC) and were cultured in DMEM supplemented with antibiotic (100 U/ml penicillin G, 100 µg/ml streptomycin) and 10% fetal bovine serum. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator. The growth medium was replaced with phenol free DMEM (Invitrogen) supplemented antibiotic (100 U/ml penicillin G, 100 µg/ml streptomycin) and 10% fetal bovine serum.

Tissue preparation and culture. The method previously described to culture arteries was utilized (Kleppisch *et al.*, 1996). Briefly, aortae were collected from male Sprague Dawley rats (age 9-12 weeks) euthanized following unrelated experiments. Thoracic aorta was dissected and opened along its longitudinal axis in 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. The aorta was cleaned of adventitia under the dissecting microscope and endothelial cells were removed by rubbing the lumen with cotton swab. After cleaning the tissue, aorta was cut into 3 pieces. These pieces of aortae were randomly placed in 35 mm dishes filled with phenol-free DMEM supplement with 0.5% BSA and antibiotics (100 U/ml penicillin G, 100 ug/ml streptomycin). Cultured aortae were treated with 0.1% DMSO, 10 µM BPA, and 10 µM BPA + 100 nM ICI 182780 for 2 days for mRNA expression assessment and for 3-4 days for protein function/expression. BPA (Sigma-Aldrich), estradiol (MP Biomedicals) and ICI 182,780 (Sigma-Aldrich) were dissolved in DMSO and diluted 1:1000 for experiments.

Electrophysiology. Whole-cell patch clamp recordings were performed on single smooth muscle cells isolated from cultured rat aorta as described previously. In brief, solutions used in the recording conditions were a bath 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, 1 ATP, 0.1 GTP and 5 Tris and free Ca^{2+} 100 nM; pH 7.1. After the successful whole-cell configuration, ramp recordings from -100 to +100 mV were performed until steady state whole-cell currents were observed.

Immunoblotting*,* Western blot was performed using anti-ER alpha (1:250; NeoMarker RM-9101), ER beta (1:250; abcam ab3577), and BK channel α subunit (1:500; alomone lab) antibodies. The details of procedures were described previously (Asano *et al.*, 2010). Rat ovary was used for the positive control for ERs in the western blot. For BK α subunits positive control, HEK cells were transfected with a plasmid encoding *hSlo* $\alpha\beta$1 (kindly provided by Dr. Lippiat from University Leeds) and these cell lysates were used as positive control.

Reverse transcriptase polymerase chain reaction (RT-PCR)*.* RT-PCR was performed as described previously (Asano *et al.*, 2010). Details of primers and PCR condition are described in Table 1.

Data Analysis*.* Results are presented as mean ± SEM. Data were analyzed using Prism software (GraphPad; San Diego, CA). Significant level was set at $p<0.05$.

Results

Estrogen receptors in rat aorta. The mRNA and protein expression of ERs have been identified in varied types of vascular smooth muscle cells including different vascular beds, cultured, and species (Orimo *et al.*, 1993; Karas *et al.*, 1994; Freay *et al.*, 1997). However, expression in our models (A7r5 cells and cultured rat aorta) is unknown. We performed RT-PCR to conform ER expression in A7r5 cells and cultured rat aorta (Fig.1 A&C). Interestingly, different degree of expression of ER α and β were observed in A7r5 cell and cultured rat aorta. In order to further confirm the ER expression in our study model, we examined protein expression level by immunoblotting (Fig 1 B&D). Using rat ovary as a positive control, anti ER α antibody (NeoMarker RM-9101) detected an immunoreactive band around 64 kDa in A7r5 and cultured rat aorta. Similarly, anti ER β antibody (abcam ab3577) detected a prominent band around 52 kDa in these cells. These findings were consistent with other studies utilizing pharmacological approach to identify ERs in vascular smooth muscle cells (Bolego *et al.*, 2005). Our RT-PCR and Western blot data indicated that A7r5 cell and cultured rat aorta express both ERα and β.

Effect of BPA on BK channel expression. E_2 has been shown to alter mRNA expression of BK channel α subunits (Song *et al.*, 1999; Benkusky *et al.*, 2000; Eghbali *et al.*, 2003; Jamali *et al.*, 2003). RT-PCR data showed BPA altered BK channel α subunits mRNA levels in A7r5 cells (Fig2 A). To determine whether BPA altered the expression of BK channel α subunit protein content in rat vascular smooth muscle cells, immunoblot using Anti-BK channel α subunit

was performed. Using HEK cells overexpressed human BK αβ1 subunit as positive control, a prominent band around 125 kDa was observed in cultured rat aorta (Fig 2 A). Group data (n=7) indicated that BK α subunits content was reduced to 33.3 ± 7.9% of control in BPA-treated aortae (p<0.05). We tried several different anti- BK β1 subunits antibodies to examine whether BK β1 subunit expression was similarly reduced by BPA, however, those commercially available anti BK β1 subunits antibodies could not detect endogenous level of BK β1 subunits expression (data not shown). Taken together, BPA decreased BK α subunit protein expression in rat aorta.

ER antagonist prevents BPA-induced reduction in BK channel expression. In order to further examine the effect of BPA on the functional expression of BK channels, whole cell patch clamp recording were performed on vascular smooth muscle cells. Whole cell recordings from cultured rat aortic smooth muscle cells showed voltage dependent outward rectifying currents (Fig. 3). Compared to aortic SMC treated with vehicle, whole cell current densities at 100 mV were 57.2 ± 6.5 % lower in aortic SMC treated with 10 μM BPA (n=9 from 3 different rats, p< 0.05) while whole cell current densities at 100 mV from aortic SMC treated with BPA + ICI were not different. (100.7 ± 13.9 % of control, n=14 from 5 different rats). Penitrem A (1 μM), BK channel antagonist, blocked 66.6 ± 6.0 % of outward currents at 100mV in vehicle (0.1% DMSO) treated aortic SMCs with intracellular Ca^{2+} 100 nM (n= 9 from 6 different rats, p< 0.05). In BPA (10 μM) treated aortic SMCs, outward currents were blocked 40.0 ± 13.7 % by penitrem A (n=4 from 3 different rats, p< 0.05). Outward currents were

blocked by 68.8 ± 7.7 % by penitrem A from BPA+ ICI (100 nM) treated aortic SMCs (n=6 from 4 different rats, $p < 0.05$). Although we detected BK channel mRNA (Fig. 2 A) and protein (Fig. 2 B) in A7r5 cells and functional BK channel expression was reported in a previously (Si *et al.*, 2006), we were unable to show any functional BK channel expression in A7r5 cells (thus no patch clamp data are shown). To determine the mechanism by which BPA reduced BK channel expression, we utilized a specific ER antagonist to test our hypothesis that BPA decreases BK channel expression via a genomic mechanism. Patch clamp whole cell recording indicated the mean penitrem A sensitive current density at 80 mV was 30.9 ± 5.61 pA/pF, 22.2 ± 2.47 pA/pF, 45.6 ± 8.29 pA/pF in DMSO, 10 μM BPA and BPA + 100 nM ICI, respectively ($p < 0.05$).

Discussion

The purpose of this study was to examine the effects of long term BPA exposure on functional BK channel expression in vascular smooth muscle cells. Major findings of this study were: 1) BPA significantly decreased BK channel mRNA/protein expression in A7r5 cells and cultured rat aorta; 2) penitrem A-sensitive BK channel currents in rat aortic smooth muscle were significantly diminished by BPA treatment; 3) BPA reduces BK channel expression through a mechanism depending up classical ER mediated signaling.

The mechanisms by which BPA is associated with cardiovascular diseases are far beyond our understanding since the association between human BPA exposure and cardiovascular disease is a relatively recent public health concern. However, recent studies are attempting to shed light on some of the potential mechanisms. For instance, BPA in the nanomolar range can promote cardiac arrhythmias due to increased SR Ca^{2+} leak via mechanisms depending on different ERs based on gender (Yan *et al.*, 2011; Belcher *et al.*, 2012). Additionally, BPA in the micromolar range can alter atrial contractility via NO-cGMP mediated mechanisms (Pant *et al.*, 2011). Similarly, previous works by our laboratory have shown that the acute micromolar range of BPA exposure activates BK channel in canine and human coronary artery via non-genomic mechanisms (Asano *et al.*, 2010). Finding that BPA was a BK channel opener did not readily fit with the idea that BPA is associated with cardiovascular disease; therefore, we decided to investigate longer exposure. In this study, we demonstrated 48-72 hr exposure to BPA decreased BK channel expression in

vascular smooth muscle via ER-mediated signaling. Taken together, these works may represent some of the possible explanations for the BPA associated altered vascular function seen in epidemiological studies (Lang *et al.*, 2008; Melzer *et al.*, 2010; Melzer *et al.*, 2012).

Estrogen/BPA on BK channel expression. The functions of estrogen are quite complex, in that it is involved not only in classical reproductive function and cell growth, but also in other systems such as the cardiovascular system. Particularly, after the results of a long period of trial and error, the current perspective of hormone replacement therapy (HRT) is that it increases the risk of cardiovascular diseases. Therefore; HRT is no longer recommended for the prevention of cardiovascular diseases (Mosca *et al.*, 2001; Rossouw, 2006). The mechanisms by which HRT increases the risk of cardiovascular diseases are not fully understood. One possibility may be E_2 induced altered ion channel expression, which has been seen in a number of other studies (Ranki *et al.*, 2002; Kundu *et al.*, 2007; O'Mahony *et al.*, 2007; Kundu *et al.*, 2008). Of those we were particularly interested in the BK channel since it plays a crucial role in vascular function (Nelson *et al.*, 1995).

Currently, there are several possible proposed mechanisms of alteration in BK channel current density via long-term E_2 treatment (and presumably BPA exposure). One of them is that E_2 causes alternative splicing to BK transcripts that causes fundamental changes to the BK channel's properties such as Ca^{2+} and voltage sensitivity. This mechanism is supported by studies from England's group showing that E_2 and pregnancy induced up-regulation in both mRNA and

protein expression in mouse uterine smooth muscle cells, but diminished BK channel currents (Benkusky *et al.*, 2000; Holdiman *et al.*, 2002). In contrast to these data, a study by Toro and Stefani's groups demonstrated E_2 down-regulates both BK channel mRNA and protein expression in rat uterine smooth muscle (Song *et al.*, 1999). Furthermore, E_2 regulates BK channel expression by different mechanisms depending on the species. Specifically E_2 regulates BK channel surface expression by transcriptional regulation in rat myomerium, whereas membrane trafficking of BK channel is the major mechanism in regulation of BK channel surface expression in mice myometrium (Eghbali *et al.*, 2003). A final proposed mechanism is that the BK channel itself serves as an E_2 receptor and that E_2 binding to BK channels triggers proteasomal degradation to decrease BK channel expression in human coronary artery smooth muscle cells (Korovkina *et al.*, 2004). These data typify the complex effects of E_2 on the regulation of BK channel expression, however, all these studies agree on one point; that long-term treatment of E_2 decreases functional BK channel expression (Wang *et al.*, 1998; Song *et al.*, 1999; Benkusky *et al.*, 2000; Holdiman *et al.*, 2002; Eghbali *et al.*, 2003; Korovkina *et al.*, 2004). Assuming BPA may regulate BK channel expression in the same way as E_2 does due to its estrogenic properties, our results agree with these previous reports that demonstrated reduced BK channel current density with E_2 exposure. Our data showed that the BPA-induced decreases in BK channel currents were most likely due to decreased protein expression in rat aorta.

It is reasonable to speculate that BPA acts similar to E_2, as it is estrogenic; nevertheless, it is also possible that BPA uses totally different mechanisms that as of yet, have not been identified. Although our data demonstrate that a pure estrogen receptor antagonist (ICI 182, 780) reversed the BPA-induced decrease in BK channel function (suggesting ER-mediated signaling), we cannot rule out other possibilities such as membrane-initiated estrogen signaling (e.g. GPER activation) may regulate BK channel expression. BPA may also activate GPER mediated cell signaling, and addressing these membrane initiated signaling will require additional experiments. Further studies are needed to determine how other cell signaling pathways are involved in BPA-induced decreased in BK channel expression in vascular smooth muscle.

Estrogen up-regulates BK channel function in uterine artery.

In contrast to our data, E_2 has been shown to reduce myogenic tone by mechanisms that depend on the up-regulation of BK β1 subunit, but not in α subunits in pregnant sheep uterine artery (Nagar *et al.*, 2005; Hu *et al.*, 2011). A similar study using uterine vascular smooth muscle from ewes showed that E_2 increased both BK α and β1 subunits mRNA/protein (Rosenfeld *et al.*, 2009). We do not have data to explain the controversy, however, expression of BK channels by E_2 is regulated differently in various species (Eghbali *et al.*, 2003). Furthermore, some authors have mentioned that different mechanisms of BK channel α or β1 subunits up-regulation exist based on factors as seemingly trivial as branch orders of uterine arteries. Again, these results highlight the complex

effects of E_2 on BK channel expression. In our experiments, we have tried several BK β1 subunit antibodies to analyze the β1 subunits expression levels, however, while those commercially available β1 subunit antibodies were able to detect overexpressed *hSlo* β1 and *bSlo* β1, they were inadequate for detection of endogenous BK β1 subunit expression. Therefore we do not know the effect of BPA on BK channel β1 expression in rat aorta. Based on this, it may be possible that several factors such as different vascular beds, sizes and species play a role in E_2/BPA induced altered BK channel expression.

A link between BPA exposure and BPA associated pathologies. Many studies have detected BPA in urine, blood and tissue, but the levels of BPA exposure are controversial. BPA has been shown to accumulate in adipose tissue (Nunez *et al.*, 2001; Fernandez *et al.*, 2007) so that the local BPA concentrations could be higher than the values reported in total blood. Furthermore, cumulative BPA exposure levels may play a role in BPA associated pathologies. Although these studies make it difficult to assess exact blood BPA levels, the BPA concentration we used (10 μM) is likely to be applicable to the occupational BPA exposure studies showing high levels of BPA exposure (Hanaoka *et al.*, 2002; Li *et al.*, 2010a). Interestingly, there was an over 4-fold increase in erectile difficulty that may suggest vascular dysfunction in BPA exposed workers than non-exposed workers (Li *et al.*, 2010a). According to our findings, BPA-induced decreased functional BK channel expression in vascular smooth muscle may be the one of plausible mechanisms.

Although it is beyond the scope of this study, much attention has been given to the effects of BPA exposure on the development of fetuses, infants and young children. It has been demonstrated that *Drosophilia* slowpoke (*dSlo;* homologous to human *KCNMA1*) gene expression is precisely regulated based on specific cell types and developmental periods for the normal development (Brenner & Atkinson, 1996; Chang *et al.*, 2000). Whether BPA alters BK channel expression during development periods is not determined yet. Additional experiments regarding BPA toxicity on ion channels will add to our understanding of the link between BPA exposure and BPA associated pathologies.

In summary, we demonstrate that BPA reduces BK channel expression in vascular smooth muscle. Given that estrogen regulates BK channel expression, this effect of BPA on BK channel expression is likely to be an estrogenic property of BPA. The mechanisms by which BPA reduced BK channel expression involved in classical ERs and nuclear signaling pathways. The findings are novel because they represent the firs known effects of BPA on ion channel expression in any tissue. Additional studies designed to examine in vivo effects of BPA on BK channel expression; as well as to explore other potential ion channel targets will be necessary to increase our understanding of the clear explanation of the association of urinary BPA levels and cardiovascular diseases.

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Figure legends

Fig. 1. Cultured rat aorta and A7r5 cells express estrogen receptors. A.B) mRNA expression levels of ERs from A7r5 cells and cultured aortas were analyzed by RT-PCR. C,D) Protein expression levels of ERs were determined by immunoblotting. A rat ovary was used as a positive control for RT-PCR and Western blot.

Fig. 2. BPA reduces BK channel expression. A) A picture of RT-PCR data from different doses of BPA treated A7r5 cells. B) Whole cell lysates obtained from A7r5 cells treated with different doses of BPA were analyzed by immunoblotting with Anti-BK channel α subunit antibody. Group data normalized to SM α-actin showed BPA decreased BK channel expression from A7r5 cells in a concentration-dependent manner (n=8). C). Proteins isolated form cultured rat aortae were analyzed by immunoblotting with Anti-BK channel α subunit antibody. Group data indicated 10 μM BPA decreased BK channel expression (n =7). An asterisk (*) indicates significant difference from vehicle treated sample. $P < 0.05$.

Fig. 3. BPA decreases functional BK channel expression in cultured rat aorta. Functional BK channel expressions from BPA treated cultured aorta were analyzed by patch clamp whole cell recordings. Currents were elicited by using the voltage template shown. Penitrem A, a selective BK channel antagonist, was utilized to identify BK channel currents. A~C) Representative current traces and

IV relationship from vehicle (0.1% DMSO), 10uM BPA, and 10uM BPA +100nM ICI (ERs antagonist) treated rat aorta. An asterisk (*) indicates significant difference from penitrem A ($P < 0.05$).

Fig. 4. ER antagonist (ICI 182,780) abrogates effect of BPA on BK channel expression A). Representative penitrem A-sensitive currents from each treatment. Group data (n=4 each group) from penitrem A sensitive currents demonstrated BPA significantly decreased BK channel currents and pretreatment with an ER antagonist prevented BPA induced decreased BK channel expression. $P < 0.05$. B). Western blot data indicated BPA decreased BK channel protein contents and that ICI pretreatment blocked this effect. An asterisk (*) indicates significant difference from vehicle treated sample. $P < 0.05$.

Table 4 1 Primers used for the RT-PCR analysis

mRNA	Forward primer	Reverse Primer	Size (bp)
Esr1 NM_012689	5'-CATCGATAAGAACCGGAGGA-3' Exon 4	5'-AAGGTTGGCAGCTCTCATGT-3' Exon 5	190
Esr2 NM_012754	5'-TTCCCGGCAGCACCAGTAAC C-3' Exon 2	5'-TCCCTCTTTGCGTTTGGACTA-3' Exon 3	262
KCNMA1 (NM_031828)	5'-TCGACATGGCTTTCAACGTG-3' Exon 13	5'-GTATACAGACACAAACACGGG G-3' Exon 14	140
Actb NM_031144	5'-CCTGAAGTACCCCATTGAACAC -3'	5'-GAGTCCATCACAATGCCAGTG -3'	267

Exon 3	Exon 4

Some of these primer sets have previously used by others (Zancan *et al.*, 1999; Amberg *et al.*, 2003).

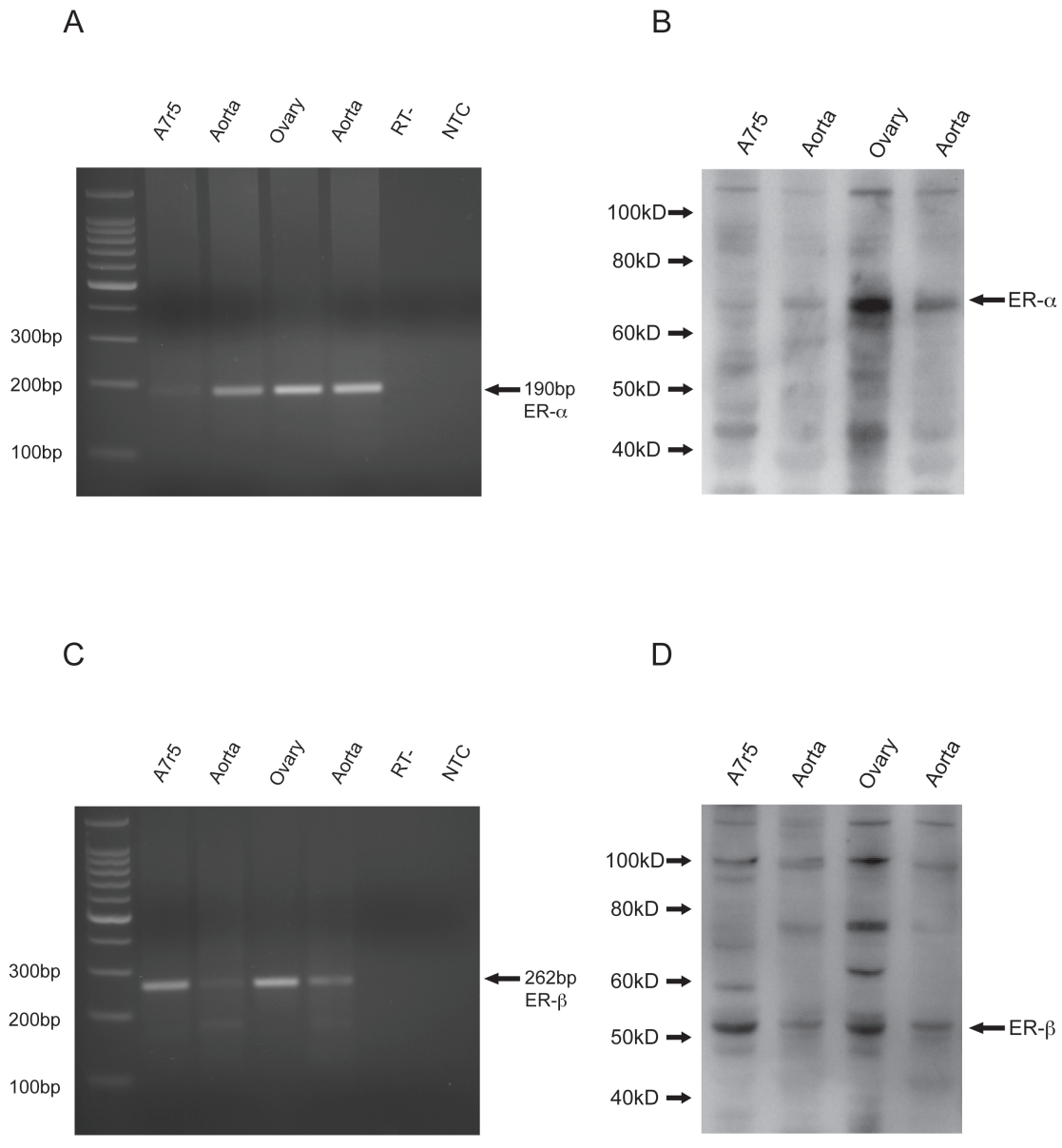

Figure 4 1 Cultured rat aorta and A7r5 cells express estrogen receptors

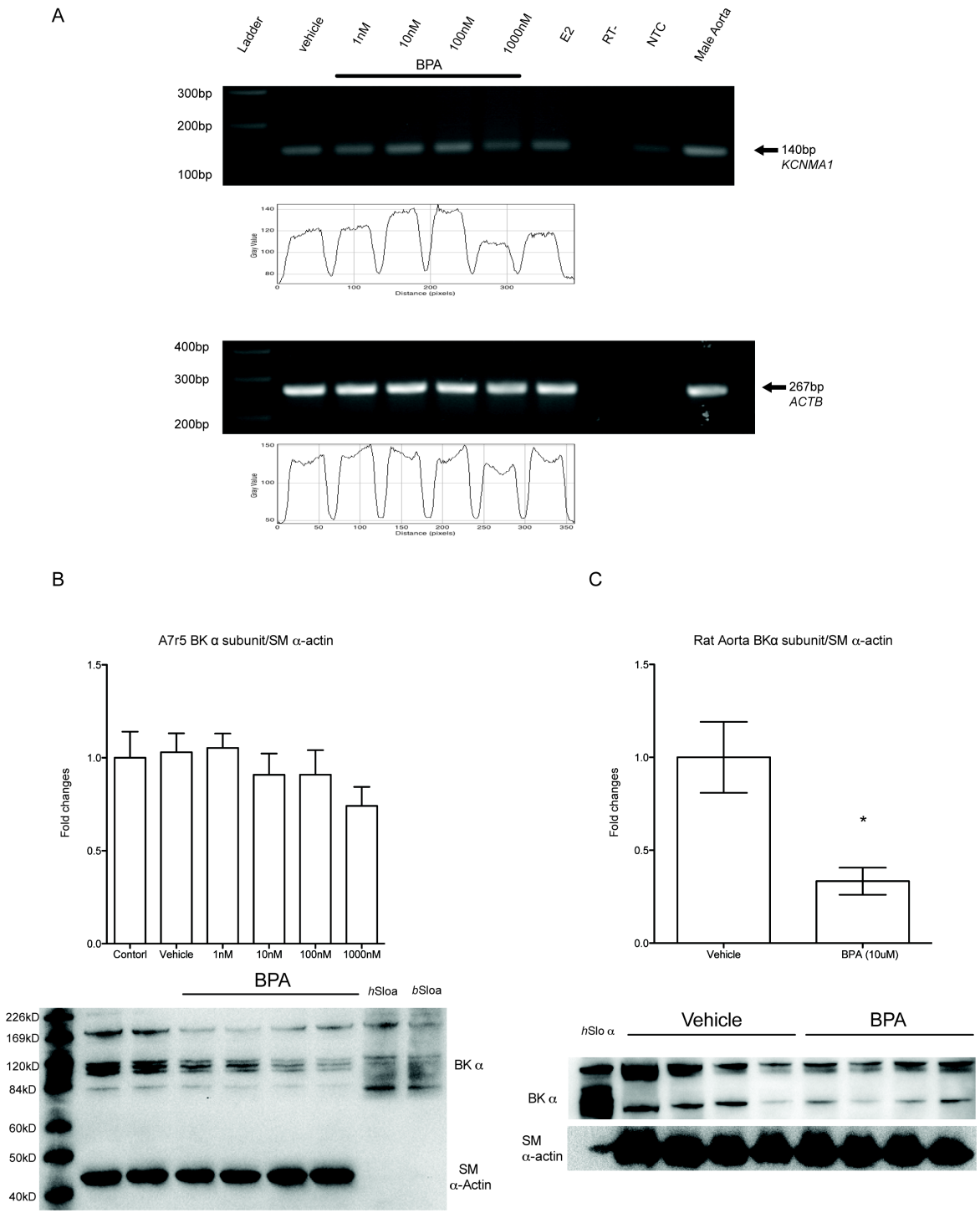

Figure 4 2 BPA reduces BK channel expression

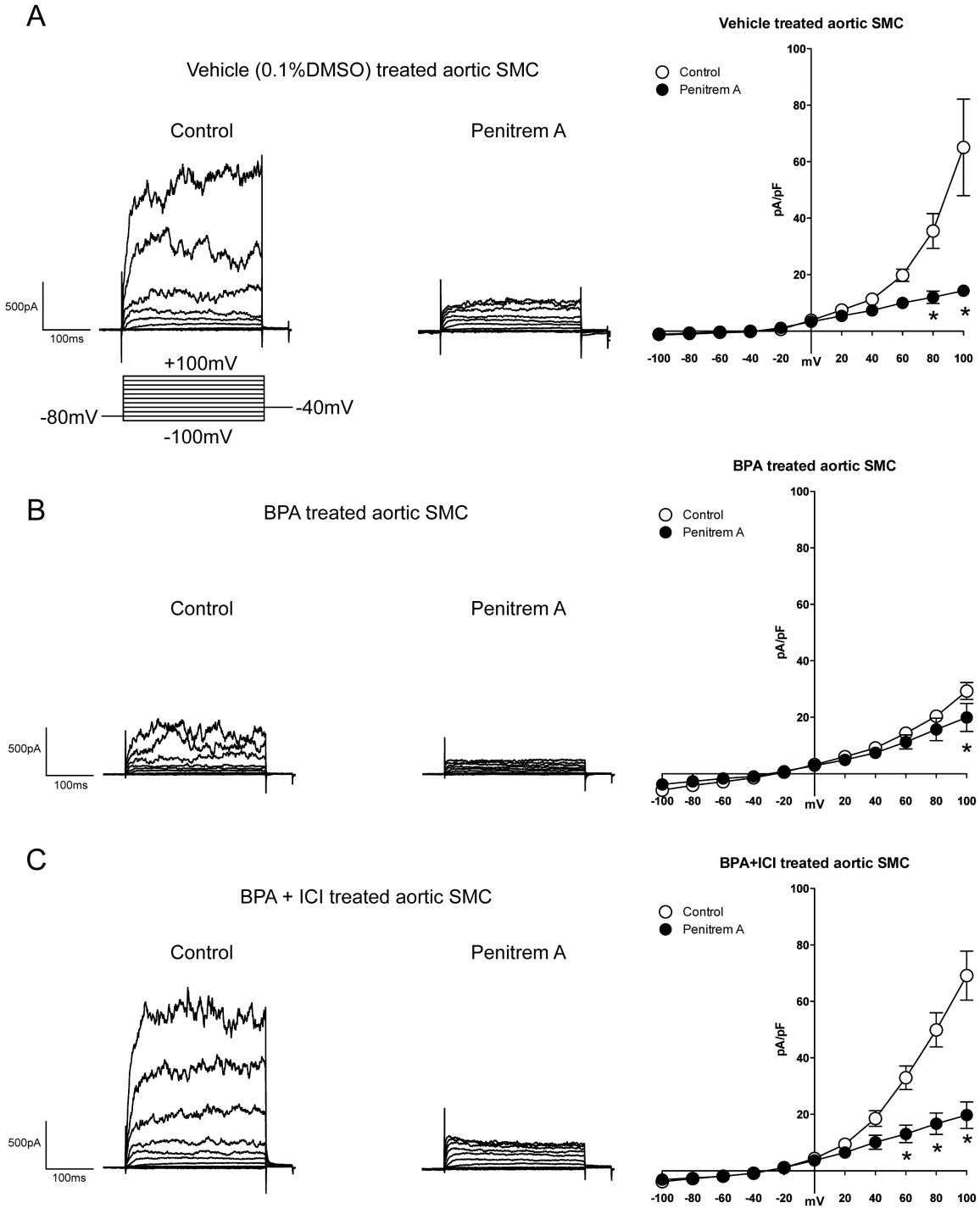

Figure 4 3 BPA decreases functional BK channel expression in cultured rat aorta

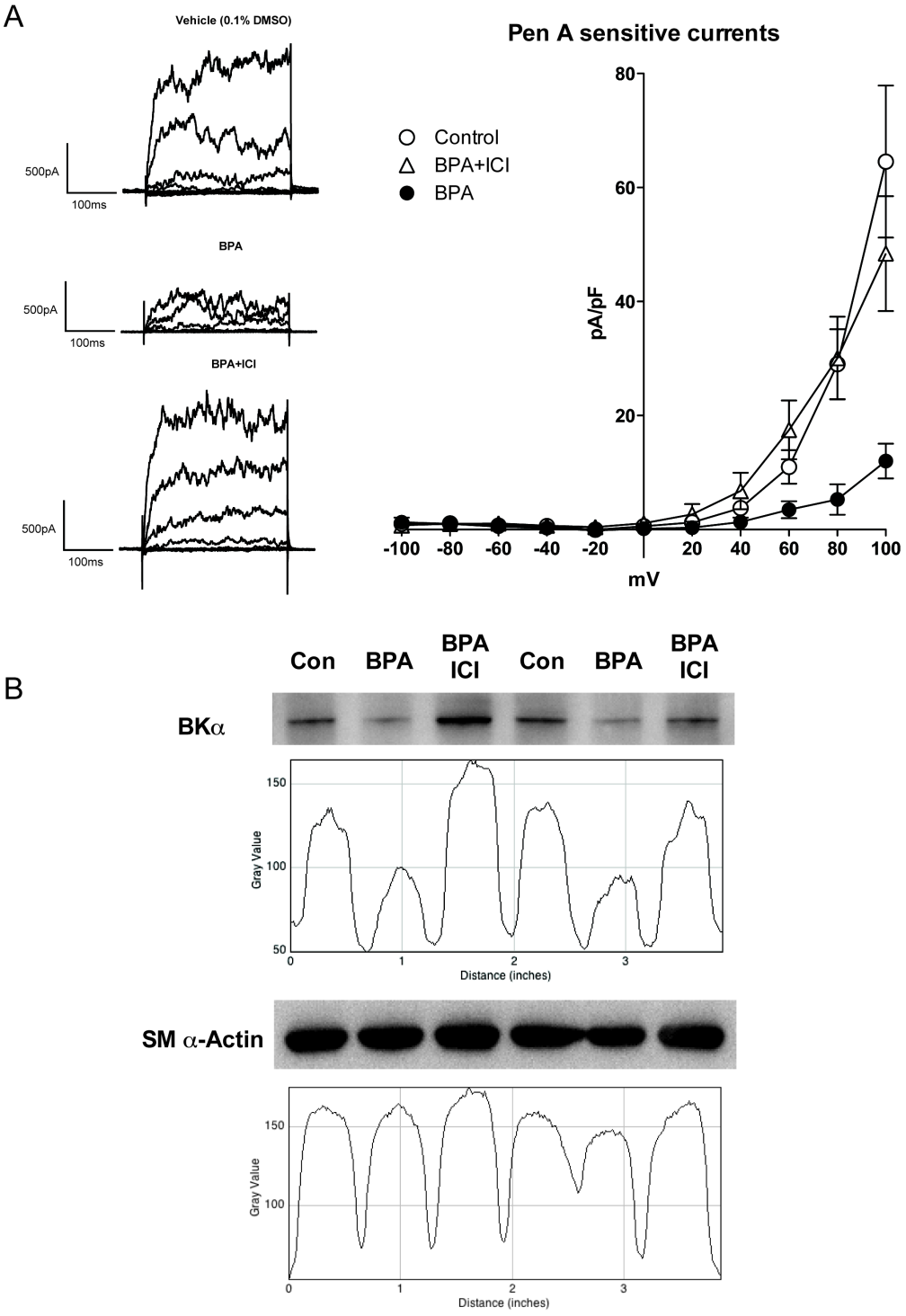

Figure 4 4 ER antagonist (ICI 182,780) abrogates effect of BPA on BK channel expression

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CHAPTER 5 General Discussion

Conclusion

The purpose of this dissertation was to determine whether BPA modulates vascular BK channel function and expression. Furthermore, we wanted to determine the mechanisms of BPA-induced reduction in BK channel function and expression. Our long-range goal was to identify molecular mechanisms by which estrogenic chemicals affect vascular function. Our objective was to determine mechanisms by which BPA modulates BK channel expression and function. The central hypothesis was that BPA alters BK channel expression and function through separate genomic and non-genomic mechanisms. The rationale was that receptors for BPA exist at both the cell membrane and in the nucleus.

Human BPA exposure and cardiovascular disease became public health concerns relatively recently; furthermore, we do not understand whether a cause and effect relationship exists in the association of BPA exposure and cardiovascular diseases seen in epidemiological studies. We chose ion channels for the possible target protein for the effects of BPA. Specifically, we selected BK channels as a target molecule since it is expressed in a variety of tissues and involved in a variety of cellular functions (Ahluwalia *et al.*, 2004; Ruttiger *et al.*, 2004; Meredith *et al.*, 2006; Pyott *et al.*, 2007; Grimm *et al.*, 2009) and plays an important role in vascular tone. Since the gold standard BK channel antagonist, IBTX, has experimental limitations such as cost and membrane impermeability, we reasoned that the characterization of an economical pharmacological alternative would facilitate the study of BK channels. Thus, our initial experiments have systematically characterized the effect of penitrem A, a tremorgenic

mycotoxin, on BK channels and demonstrate the role of BK channels in the vascular function of mice (Chapter 2). Our data demonstrated that whole cell currents from HEK 293 cells transfected with *hSlo* α or α + β1 were effectively blocked by penitrem A (IC_{50} 7 vs. 107 nM; $p<0.05$). The inhibitory effects of penitrem A (50.4 ± 9.38 %) and IBTX (49.7± 9.44 %) on K^+ currents were the same degree in coronary artery smooth muscle cells. As for specificity, penitrem A had no effect on Kv1.5 currents and pinacidil-induced vasodilation. Finally, *in vivo* hemodynamic response indicated that penitrem A significantly augmented phenylephrine induced increases in mean arterial blood pressure by blocking BK channel in mice ($p<0.05$). Taken together, our data indicated that penitrem A is an excellent tool to study BK channel in vascular function and is not only practical for cell/tissue *in vitro* studies, but also for whole animal *in vivo* vascular functional studies.

Next, we wanted to determine the effect of BPA on BK channels using penitrem A. Our data showed that BPA increased BK channel currents and BK channel β1 subunits facilitated BK channel's sensitivity to BPA (Chapter 3). This was determined by whole cell recording showing greater outward current with acute BPA exposure in cultured CCASMCs and HCASMCs. Single channel recording from freshly isolated canine LAD revealed that BPA increased NPo of BK channel. Based on these findings from cultured cells and freshly isolated cells, we next performed the patch clamp experiments on BK channels exogenously expressed on the cells that do not express BK channels endogenously. In these experiments, cells transected with BK α and β1 subunits

revealed a significant increase in BK channel current and greater NPo when cells were exposed to 10 μM BPA. In contrast, cells with BK channel α subunits alone showed no significant increase in BK outward currents or NPo. Given that several steroid hormones activate BK channel via β subunits (Valverde *et al.*, 1999; Lovell *et al.*, 2004; King *et al.*, 2006), it is possible that xenoestrogens also may be associated with β subunit dependent BK activation. These findings are important as they should serve as additional evidence that the xenoestrogen, BPA, activates BK channels and the role of BK β subunits on steroid hormones and xenoestrogens induced BK activations. Non-genomic effect of BPA on BK channel is summarized in Fig. 5-1.

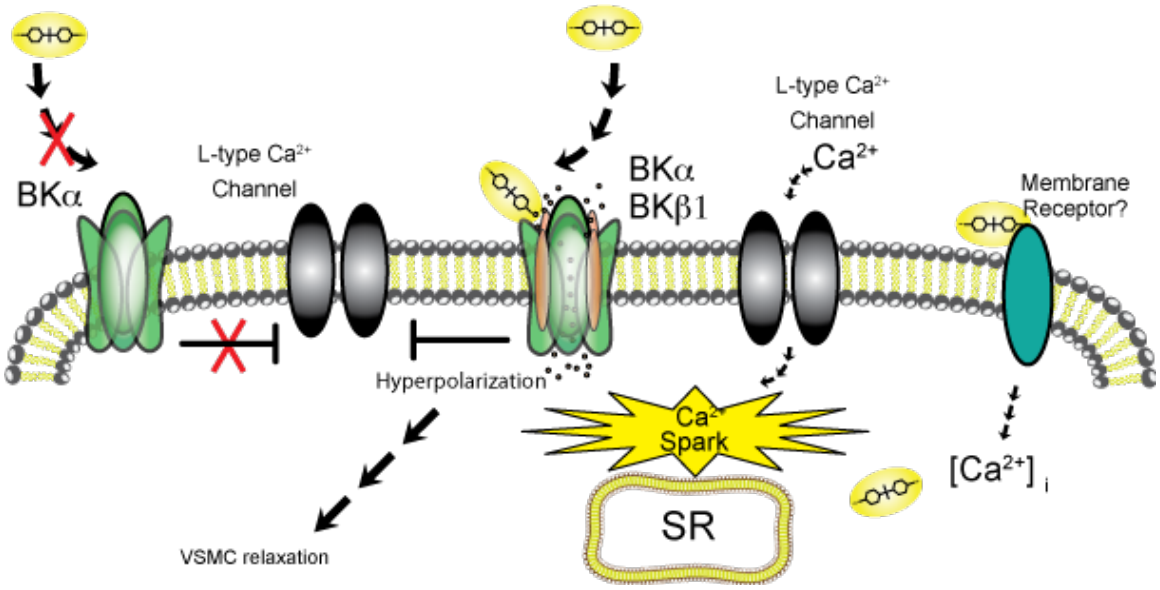

Figure 5 1 Non-genomic effect of BPA on vascular BK channel.

BPA can activate BK channel independent of changing intracellular Ca^{2+} levels.

BK β1 subunits favors BPA induced BK channel activation.

The effects of long-term exposure to BPA on BK channel expression were assessed on A7r5, rat aortic cell lines, and cultured rat aorta. In this part of the study (Chapter 4), we hypothesized that BPA would decrease BK channel expression through estrogen receptor dependent signaling. The principle behind this hypothesis was that BK channel gene expression and functional protein expression from uterine smooth muscle cells are known to be estrogen-sensitive. (Wang *et al.*, 1998; Song *et al.*, 1999; Benkusky *et al.*, 2000; Holdiman *et al.*, 2002; Eghbali *et al.*, 2003; Korovkina *et al.*, 2004). Given that BPA possesses estrogenic properties, we tested whether BPA altered BK channel expression in rat aorta. Our data indicated that BPA altered BK channel transcript from A7r5 cells and decreased protein expression levels in A7r5 cells and cultured rat aorta. The mechanisms of BPA induced decreased BK channel expression depended on classical estrogen receptor signaling, as confirmed by an estrogen receptor antagonist that inhibits BPA's effects on BK channels. Taken together, our data suggested that long term BPA exposure decreased BK channel expression via genomic mechanisms. The genomic effect of BPA on vascular BK channel expression is summarized in Fig. 5-2.

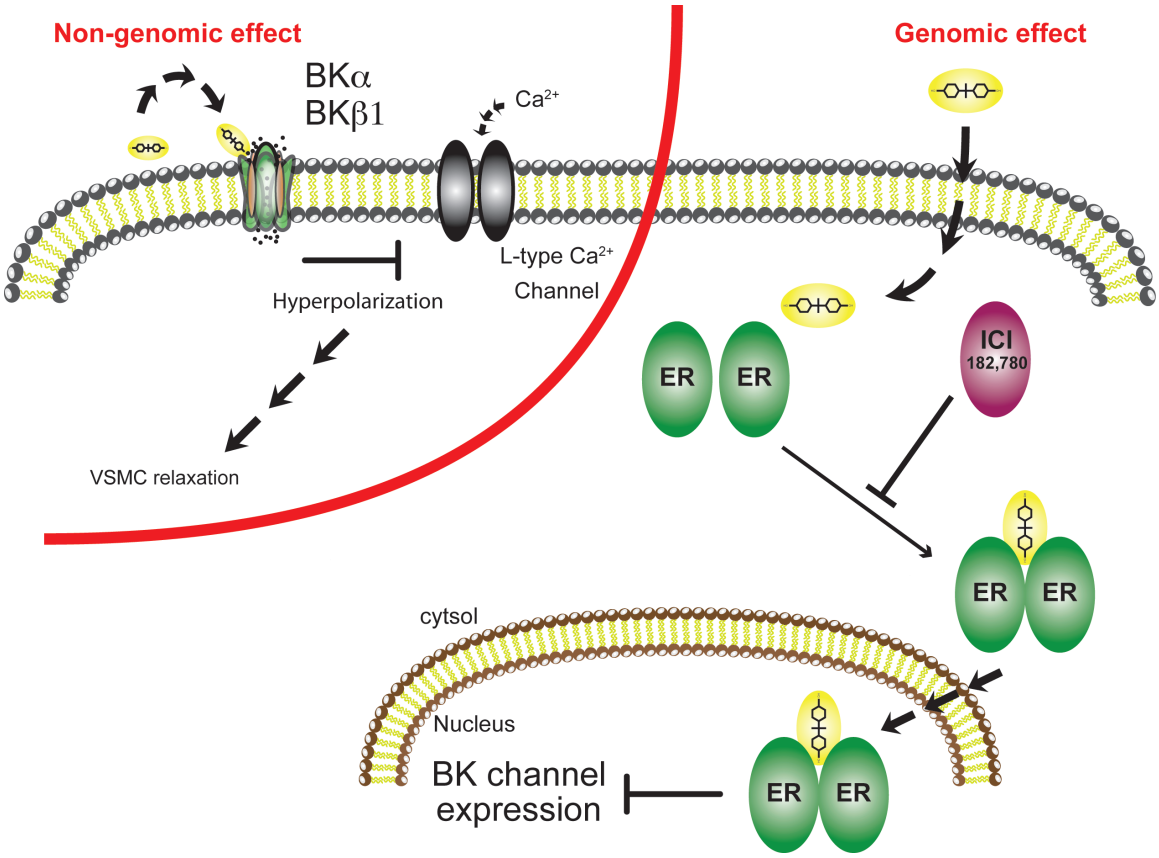

Figure 5 2 Genomic effect of BPA on vascular BK channel.

BPA can activate BK αβ1 channels at the plasma membrane. Additionally, BPA can interact with ERs to regulate BK channel α subunit expression. ER antagonist, ICI 182, 780 can prevent the effect of BPA to reduce BK channel α subunit expression.

It is important to mention some limitations of this study. In order to assess the genomic and nongenomic effects of BPA on the BK channel, multiple model cells were utilized. Specifically, nongenomic pharmacological properties of BPA were elucidated using cultured and native vascular smooth muscle cells, as well as cloned BK channels, including: cultured canine coronary smooth muscle cells, cultured human coronary artery smooth muscle cells, cloned bovine BK channels expressed in HEK 293 cells, and native canine coronary artery smooth muscle cells. The reason we used so many different cell types over time was basically due to comments from grant and manuscript reviewers (e.g., previous results were in canine cells, so reviewers asked us to establish a canine cell culture model). Further, we chose human coronary cells for relevance, but abandoned the model due to cost. We realize that having this number of model cells can be distracting; however, similar results from multiple cell types strengthened our conclusion that BPA activated BK channels through a non-genomic mechanism depending on the β1 subunit. The genomic effects of BPA on BK channels were tested in A7r5 cells (a neonatal rat aortic cell line) and smooth muscle from tissue cultured rat aorta. Given that the expression of BK channel by estrogen is regulated in different ways based on species, we limited our model system to rats for the genomic studies. It is possible that the effect of BPA on BK channel expression may be different in other species. Although our study demonstrate that BPA decreased BK channel expression via ERs, it is possible that BPA may utilize other mechanisms in reducing BK channel expression (e.g., proteosomal degradation). Since the genomic and nongenomic mechanisms of BPA's effects

on BK channel were elucidated in different models, a criticism could be that each mechanism is model-specific. Importantly, however, we demonstrated both genomic and non-genomic mechanisms of BPA in rat aorta, suggesting that the results can be generalized to other smooth muscle. Whether both genomic and non-genomic effects are relevant to human BPA exposure needs to be examined in future studies.

Given that BPA is detected in over 95 % of the US population and similar findings have been reported world wide, there is little doubt that some level of BPA is in virtually everyone's system. One important question that needs to be addressed is whether the levels of BPA exposure to the general population are significant for cardiovascular disease. Table 1 was derived from a review study by Vandenberg LN et al (Vandenberg *et al.*, 2007). The majority of epidemiological studies indicated geometric mean urinary BPA ranges from 1.12~27.3 ng/ml. Based on these studies, the current consensus regarding human BPA exposure levels is in the parts per billion levels (vom Saal *et al.*, 2007). Recent data of urinary BPA levels from US NHANES (2003/2004 and 2005/2006) are also within these ranges (2.6 ug/L (Calafat *et al.*, 2008), 2.49 ng/ml, (Melzer *et al.*, 2010)) and data from the European Prospective Investigation of Cancer (EPIC) were median urinary BPA 1.3 ng/ml (Melzer *et al.*, 2012).

Interestingly, there is a BPA pharmacokinetic study on human subjects that directly measured both blood and urinary BPA levels (Volkel *et al.*, 2002). The study demonstrated that peak blood d_{16}-BPA-glucuronide was approximately

6-fold higher than urinary d_{16}-BPA-glucuronide after consuming radiolabeled BPA (5 mg/person). Furthermore, local BPA concentration could also be higher because BPA can be stored in adipose tissue and it has been suggested that it leaches from adipose tissue (Nunez *et al.*, 2001; Fernandez *et al.*, 2007b). Thus, it is difficult to assess how accurately these urinary BPA levels reflected blood BPA levels. On the other hand, in factory workers who are exposed BPA because of their occupation, median urinary BPA concentrations were 84.6 μg/g Cr (He *et al.*, 2009), 57.9 μg/g Cr (Li *et al.*, 2010) and this population had over 20-30 times more exposure compared to the general population (2.6 μg/g Cr, US NHANES) (Calafat *et al.*, 2008)

BPA levels in human urine

Authors	Year	Detection method	Sensitivity (ng/ml)	Subjects	Glucuronidase/ sulfatase treatment?	Detection rate	Levels found [ng/ml (ppb), mean ± S.E.M.]			Unit if not ng/ml	Estimated daily intake	Other chemicals examined
							Unconjugated BPA	BPA–glucuronide	BPA–sulfate	Total BPA		
Brock et al. [120]	2001	GC–MS	0.12	Five specimen pools from at least five people	Glucuronidase	5/5 pools	Below level of detection			Range 0.11–51		
Ouchi and Watanabe [36]	2002	HPLC–FCD with column switching	0.2	Morning samples from 48 women students	Glucuronidase	1/48 (free BPA); 100% BPA–glucuronide	Range ND – 0.2	Range 0.2–9.1			0.6–1.4 μg/day	
Kim et al. [35]	2003	RP-HPLC/FD	0.28	Fifteen male Korean volunteers	Glucuronidase and sulfatase		0.58 ± 0.14	2.34 ± 0.85	0.49 ± 0.27	2.82 ± 0.73		
				Fifteen female Korean volunteers			0.56 ± 0.10	1.00 ± 0.34	1.20 ± 0.32	2.76 ± 0.54		
Matsumoto et al. [37]	2003	HPLC	1.7	Fifty university students in 1992	Glucuronidase and sulfatase	82% detection					10 μg/g creatine	
				Fifty-six university students in 1999		61% detection						
Tsukioka et al. [121]	2003	NCI-GC/MS	0.1	Six urine samples	Glucuronidase	100% detection				Range 0.2–0.8, mean 1.6		
Yang et al. [106]	2003	HPLC/FD	0.012	Seventy-three Koreans with various SULT1A1 polymorphisms	Glucuronidase	75% detection				Range 0.68–86.14, mean ~ 9.5		
Calafat et al. [33]	2005	GC–MS	0.1	Reference population – 184 American males	Glucuronidase	96% detection				1.63	(μg/g creatinine)	4-Nonylphenol
				Reference population – 210 American females		94% detection				1.12	(μg/g creatinine)	
Liu et al. [122]	2005	HPLC with ECD	0.5	Nine girls	Glucuronidase	89% detection				Range 0.04–6.6, median 2.4		Daidzein, genistein and enterolactone
				Twenty-four adults		52% detection				Range ND – 2.24, median 0.47		
Völkel et al. [105]	2005	HPLC–MS/MS	1.14	Six subjects orally administered 25 μg BPA	Glucuronidase	2/6 samples	Below LOD	Below LOQ				
Ye et al. [123]	2005	Online SPE–HPLC–MS/MS	0.3	Thirty demographically diverse volunteers	Glucuronidase and sulfatase	97% detection	Range ND – 0.6, mean below LOD	Range ND – 19.0, mean 3.1	Range ND – 1.8, mean 0.5	Range ND – 19.8, mean 3.2		
Joskow et al. [72]	2006	GC–MS	0.1	Urine prior to dental sealant application – 14 men	Glucuronidase					2.41 ± 0.33		
				Urine immediately after Delton sealant application	Glucuronidase					27.3 ± 13.03		
				Urine 1 h after Delton sealant application	Glucuronidase					7.34 ± 1.44		
				Urine immediately after Helioseal sealant application	Glucuronidase					7.26 ± 6.04		
				Urine 1 h after Helioseal sealant application	Glucuronidase					2.06 ± 0.47		
Yang et al. [45]	2006	HPLC/FD	0.026	One hundred seventy-two Koreans with various SULT1A1 polymorphisms	Glucuronidase	97.5% detection				Range 0.03–2.4, median 7.86		
Wolff et al. [34]	2007	HPLC–MS/MS	0.36	Ninety young girls, aged 6–9 years	Glucuronidase	94% detection				Range ND – 54.3, mean 2.0		Phytoestrogens, phalates and eight other phenols

Table 5 1 Studies measured urinary BPA

Based on these studies regarding the level of human BPA exposure, our studies using a micro molar range of concentration were applicable to the occupationally exposed populations, but probably not to the general population. Similar to our study, the micro molar range of BPA exposures has been shown to decrease atrial contractility in rat heart due to the alteration of NO-cGMP signaling (Pant *et al.*, 2011). Our studies examined only BK channels based on the estrogen sensitive properties of BK channels, however, it is possible that other proteins may be particularly susceptible to BPA, and may alter cardiovascular function. Indeed, recent studies indicated that exposure levels relevant to the general population, in the nano molar range of BPA, have been shown to alter Ca^{2+} handling in cardiac myocytes, promoting arrhythmic events in rodent hearts (Yan *et al.*, 2011; Belcher *et al.*, 2012). In order to address the link between BPA exposure and cardiovascular events, further investigation is required to answer the molecular mechanisms of BPA's effect.

Whether these BPA-induced changes in vascular BK channel function and expression directly link to the association of BPA exposure and cardiovascular diseases are currently unclear, our data are the first evidence to describe that BPA modulates vascular BK channel function and expression via genomic and non-genomic mechanisms. Further studies are needed to determine if BPA affects other ion channels that have roles in regulating vascular function.

Future directions:

Multiple aspects regarding the mechanism of action for BPA toxicology will be opened to scientists in this field. First, it would be interesting to determine whether there is a critical time or point of exposure that is associated with the development of cardiovascular disease. Does prenatal exposure eventually lead to cardiovascular disease in later life? How much exposure and over what time period is required to see the development of cardiovascular disease? Does in vivo exposure to BPA reduce BK channel expression in VSMC and alter vascular reactivity? Second, is it possible that BPA modulates the function and expression of other ion channels? Since steroid hormones alter Ca^{2+} channel expression and function (Er *et al.*, 2007; Ullrich *et al.*, 2007; Sarkar *et al.*, 2008) it will be interesting to determine whether BPA possesses similar properties. Lastly, are there population-specific effects of BPA? Are females more susceptible to BPA-associated disease due to the importance of estrogen signaling? Do endogenous estrogen levels influence the toxicological effects of BPA? Additionally, since BPA is lipophilic and accumulated in adipose tissue, (Fernandez *et al.*, 2007a), is the obese population affected by a higher level of BPA? These interesting studies will require an initial characterization and a certain level of understanding of BPA's interaction with other molecules. Our study provided novel evidence that BPA modulates vascular BK channel via a non-genomic and genomic mechanisms.

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