Tungstate activates BK channels in a β subunit- and Mg\(^{2+}\)-dependent manner: relevance for arterial vasodilatation

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Received 7 October 2011; revised 15 March 2012; accepted 28 March 2012; online publish-ahead-of-print 2 April 2012

Time for primary review: 17 days

Aims
Tungstate reduces blood pressure in experimental animal models of both hypertension and metabolic syndrome, although the underlying mechanisms are not fully understood. Given that the large-conductance voltage- and \(Ca^{2+}\)-dependent \(K^{+}\) (BK) channel is a key element in the control of arterial tone, our aim was to evaluate whether BK channel modulation by tungstate can contribute to its antihypertensive effect.

Methods and results
Patch-clamp studies of heterologously expressed human BK channels (α + β1–4 subunits) revealed that cytosolic tungstate (1 mM) induced a significant left shift (~20 mV) in the voltage-dependent activation curve only in BK channels containing \(\alpha\beta_1\) or \(\alpha\beta_4\) subunits, but reduced the amplitude of \(K^{+}\) currents through all BK channels tested. The \(\beta_1\)-dependent activation of BK channels by tungstate was enhanced at cytosolic \(Ca^{2+}\) levels reached during myocyte contraction, and prevented either by removal of cytosolic \(Mg^{2+}\) or by mutations rendering the channel insensitive to \(Mg^{2+}\). A lower concentration of tungstate (0.1 mM) induced voltage-dependent activation of the vascular \(\alpha\beta_1\) channel without reducing current amplitude, and consistently exerted a vasodilatory action on wild-type but not \(\beta_1\)-knockout mouse arteries pre-contracted with endothelin-1.

Conclusion
Tungstate activates BK channels in a β subunit- and Mg\(^{2+}\)-dependent manner and induces vasodilatation only in mouse arteries that express the BK \(\beta_1\) subunit.

Keywords
BK channel • Tungstate • Artery • Vasodilatation • Hypertension

1. Introduction
Arterial resistance, which is mainly controlled by the basal tone of the vascular smooth muscle cells (VSMCs), determines the rate of blood flow to tissues. Both circulating factors and those resident at the vessel wall participate in the establishment of this tone, and their malfunction is typically associated with functional and structural alterations in the vessels of patients suffering from essential hypertension.\(^1\) It is therefore important to identify the molecular and genetic elements participating in the control of vascular tone and their possible use as targets with therapeutic potential.

Among the factors controlling vascular tone, voltage-gated ion channels mediating \(Ca^{2+}\) entry into the VSMCs and their regulatory mechanisms are central to this process.\(^2\) One such key regulatory mechanism involves the large-conductance voltage- and \(Ca^{2+}\)-activated \(K^{+}\) (BK) channel. The BK channel couples local increases in intracellular \(Ca^{2+}\) to augmented channel activity, membrane hyperpolarization (preventing the activation of L-type \(Ca^{2+}\) channels), and vascular relaxation.\(^3\) Medications already exist that target different mechanisms involved in blood pressure regulation,\(^4\) although its normalization is not always satisfactory, and none of them targets BK channels. Compared with currently used hypertension treatments,
such as Ca\(^{2+}\) antagonists, BK channel openers are expected to potentiate an existing physiological regulatory system that acts as a negative feedback mechanism to limit membrane depolarization. L-type Ca\(^{2+}\) channel opening, and the subsequent VSMC contraction.\(^5\)

BK channels, formed by \(\alpha\) subunit tetramers, are encoded by a single gene, SLO (KCNMA1), and are activated by membrane depolarization and binding of intracellular Ca\(^{2+}\) and Mg\(^{2+}\), the effects of which are additive and involve independent pathways.\(^5\,6\) Accordingly, distinct binding sites for physiological concentrations of Ca\(^{2+}\) (in the micromolar range) and Mg\(^{2+}\) (in the millimolar range) have been identified in the pore-forming \(\alpha\) channel subunit.\(^6\,8\) Mg\(^{2+}\) blocks the pore of BK channels in a voltage-dependent manner.\(^6\,9\) but also enhances BK channel activation by stabilizing the open states. This results in a shift of the conductance–voltage (G–V) relationship to a more negative voltage range, an effect that is independent of voltage-sensor movement or Ca\(^{2+}\) binding to the channel.\(^6\,10\)

Channel activation by Mg\(^{2+}\) requires a divalent cation binding site that consists of amino acid side-chains from both the voltage-sensor domain (VSD; RCK1) domain (E374 and E399) of the BK \(\alpha\) channel subunit.\(^8\)

Depending on the tissue, BK \(\alpha\) subunits associate with different auxiliary \(\beta\) subunits (\(\beta_{1a}-\beta_{4}\)), modifying BK subcellular distribution and gating, thereby contributing to channel diversity and involvement in different physiological processes.\(^11\) The molecular composition of the vascular BK channel includes the regulatory \(\beta_{1}\) subunit, whose expression increases channel sensitivity to Ca\(^{2+}\), favouring its activation by low Ca\(^{2+}\) sparks.\(^12\) Moreover, the presence of the \(\beta_{1}\) subunit is relevant to smooth muscle contraction induced by vasoactive peptides and the regulation of arterial tone and blood pressure both in animal models of hypertension and in humans.\(^13\,–\,18\) Altogether, this makes the vascular BK\(\alpha\)\(\beta_{1}\) channel an attractive target for the development of new antihypertensive therapies.

Tungstate, a compound with antidiabetic\(^19\) and antiobesity properties,\(^20\) has been reported to reduce blood pressure in experimental animal models of both hypertension\(^21\,–\,23\) and metabolic syndrome.\(^23\)

The antihypertensive action of tungstate has been associated with inhibition of the increased xanthine oxidase activity in endothelial cells of hypertensive animals.\(^24\) Given the key role of BK channels in arterial tone control and the reported antihypertensive effect of tungstate, we assessed the effect of tungstate on BK channel activity and arterial contractility.

2. Methods

2.1 Cell transfection

HEK 293 cells were transfected using polyethylenimine ExGen 500 (Fermentas Inc., Hanover, MD, USA), following the manufacturer’s instructions [seven equivalents of polyethylenimine per 3.3 \(\mu\)g of complementary DNAs expressing the wild-type (WT), D99A or the N172A BK channel human \(\alpha\) subunit alone or with one of the four different human \(\beta\) subunits (\(\beta_{1a}-\beta_{4}\); 1:2 ratio), together with the transfection reporter pEGFPN1].

2.2 Electrophysiology

Inside-out ionic currents were recorded in macropatches from EGFP-positive cells, 2–3 days after transfection.\(^27\) For current-activation studies, membrane macropatches were clamped at 0 mV, pulsed for 150 ms (or 20 ms for expressed BK channels containing the inactivating \(\beta_{2}\) regulatory subunit) from \(-100\) to \(+200\) mV in 10 mV steps, and repolarized to \(-80\) mV for 20 ms. Experiments were performed at room temperature (22–26 °C).

Relative conductance was determined by measuring tail current amplitudes at \(-80\) mV. For each patch, the G–V relationship was fitted with the following Boltzmann equation:

\[
\frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp(-(V - V_{1/2\text{act}})/k_{\text{act}})}
\]

where \(G\) is the value of the instantaneous tail current at each test voltage, \(G_{\text{max}}\) is the maximal obtained tail current, \(V\) is the test voltage applied to the membrane, \(V_{1/2\text{act}}\) is the voltage for half-maximal current activation, and \(k_{\text{act}}\) (an index of the minimal number of elementary charges that move through the electric field to gate the channel) is the slope factor of the Boltzmann term. The G–V curves shown in the figures represent averages from a set of patches in the indicated experimental conditions.

2.3 Putative tungstate binding sites at the human pore-forming \(\alpha\) subunit of the BK channel

To obtain some insight into structural features of tungstate–protein interactions, we analysed the residue composition in the tungstate binding site of 4S available X-ray structures (see Supplementary material online for a complete list of the protein data bank identifiers (PDB IDs) and Supplementary material online, Table S1). These studies supported the role of divergent cations as cofactors for tungstate binding, and they allowed us to suggest putative tungstate binding sites at the human BK\(\alpha\) subunit based on an identified aspartate–lysine/lysine–aspartate (DK/KD) motif. Interestingly, the two BK\(\alpha\) regions that include the Mg\(^{2+}\) binding site\(^8\) contain a DK/KD motif within or next to the relevant D99, N172, and E374 residues (Supplementary material online, Figures S1–S3 and Table S2).

2.4 Arterial tension measurements

Femoral arteries were isolated from adult male, age-matched (18- to 24-week-old) (+/+) (WT) and \(-/\)– BK\(\beta_{1}\)-knockout) C57BL/6 mice. Arterial rings were obtained after surgical procedures from animals weighing 26–28 g killed by intraperitoneal administration of a lethal dose of sodium thiopental (200 mg/kg). Arteries were cleaned of connective tissue, cut into rings, and mounted on a small-vessel myograph (Danish Myo Technology, Aarhus, Denmark) to measure tension, connected to a digital recorder (AciKnowledge 3.8.1; Biopac System Inc., Goleta, CA, USA). All experiments were conducted in accordance with the Spanish legislation on protection of animals, approved by the local animal care committee (Comité Ético de Experimentación de la Universidad de Sevilla), and conformed to the Directive 2010/63/EU of the European Parliament.

2.5 Statistics

Data are presented as the means \pm\ SEM. Statistical tests included Student’s paired or unpaired t-test, Mann–Whitney U-test, one-way ANOVA followed by Tukey’s post hoc test, or non-parametric ANOVA (Kruskal–Wallis test) followed by Dunn’s post hoc test, as appropriate. Differences were considered significant if \(P < 0.05\).

See also Supplementary material online for further details of experimental procedures.

3. Results

3.1 Effect of tungstate on BK channels of different molecular composition

To evaluate whether tungstate modulates BK channel activity, we expressed the human \(\alpha\) subunit alone or with one of the four human regulatory \(\beta\) subunits (\(\beta_{1a}-\beta_{4}\)) in HEK 293 cells. Figure 1(A, C, E, G, and I) shows representative BK currents, recorded before and
Figure 1: Effect of tungstate on BK channels composed of different combinations of α and β subunits. Representative currents were recorded from excised inside-out macropatches obtained from HEK 293 cells expressing the BK α subunit alone (A), α + β₁ subunits (C), α + β₂ subunits (E), α + β₃ subunits (G) or α + β₄ subunits (I). Currents were recorded at cytosolic 0 Ca²⁺ and 0.7 mM Mg²⁺ before (top panels) and 5–10 min after cytosolic application of 1 mM tungstate (WO₄²⁻; bottom panels). The voltage protocol was as described in the Methods (this applies also to Figures 2–5). In addition, average G–V curves for the different combinations of BK channel subunits are shown as follows: α subunit only (B; n = 8); α + β₁ subunits (D; n = 7); α + β₂ subunits (F; n = 5); α + β₃ subunits (H; n = 5); and α + β₄ subunits (J; n = 5). Normalized conductance and continuous curves obtained by fitting to the Boltzmann equation are shown in this figure and in Figures 2–5. (K) Reduction of the BK peak current at the test potential of +200 mV (expressed as a percentage) produced by 1 mM WO₄²⁻ for BK channels constituted of different α/β combinations, as indicated. No significant differences were found among the distinct BK channels studied (P = 0.67, ANOVA). (L) Voltage for half-maximal current activation (V₁/2) plot for the different BK channels tested, before (control, open circles) and after tungstate application (1 mM WO₄²⁻, filled circles). The numbers of experiments for each set of experimental conditions are as indicated for the G–V curves. Differences in V₁/2 before and after tungstate application were tested by Student’s paired t-test: BKα channels, P = 0.24; BKαβ₁ channels, P < 0.0001; BKαβ₂ channels, P > 0.91; BKαβ₃ channels, P = 0.64; and BKαβ₄ channels, P = 0.0025. (M) No significant differences were found for average slope factor (kact) of the fitted G–V curves, before (control, open circles) and after tungstate application (1 mM WO₄²⁻, filled circles) in the presence of cytosolic 0 Ca²⁺ and 0.7 mM Mg²⁺ (tested by Student’s paired t-test: BKα channels, n = 8, P = 0.6; BKαβ₁ channels, n = 7, P = 0.66; BKαβ₂ channels, n = 5, P = 0.46; BKαβ₃ channels, n = 5, P = 0.11; and BKαβ₄ channels, n = 5, P = 0.87).
after the addition of 1 mM tungstate to a nominally Ca$^{2+}$-free bath (intracellular) solution containing 0.7 mM Mg$^{2+}$ (a physiological concentration found in most mammalian cells$^{24}$). Tungstate reduced (by 29–39%) the amplitude of K$^{+}$ currents through all BK channels tested (Figure 1K). Changes in BK channel activity are best evaluated from the voltage for channel half-activation ($V_{1/2}$) which is a convenient parameter to study the effect of BK channel modulators because it is directly related to the energy required to open the channel. We found that tungstate produced a significant decrease of the $V_{1/2}$ values (by 22 mV) only for $\beta_1$- or $\beta_2$-containing BK channels, favouring their activation. Tungstate did not change the slope factor of the fitted $G-V$ curves ($k_{act}$), suggesting no interference with the voltage-sensing process of BK gating (Figure 1M).

We also studied whether tungstate-induced activation of BK$\beta_1$ channels was modified by increasing [Ca$^{2+}$]i up to values detected near BK$\beta_1$ channels during VSMC contraction (10 $\mu$M). The inhibitory effect of tungstate (1 mM) on BK$\beta_1$ current amplitude was significantly smaller ($P < 0.05$) at 10 $\mu$M Ca$^{2+}$ (Figure 2A and C).

**Figure 2** Effect of tungstate on BK$\alpha_1\beta_1$ channels in the presence of physiological cytosolic Ca$^{2+}$ levels. (A) BK$\alpha_1\beta_1$ currents recorded in the presence of cytosolic 10 $\mu$M Ca$^{2+}$ and 0.7 mM Mg$^{2+}$, before (top panel) and 5–10 min after cytosolic application of 1 mM WO$_4^{2-}$ (bottom panel). (B) Average $G-V$ curves for BK$\alpha_1\beta_1$ currents in the presence of 10 $\mu$M Ca$^{2+}$ and 0.7 mM Mg$^{2+}$, before (control, open circles) and after tungstate application (1 mM WO$_4^{2-}$, filled circles; n = 3). (C) Reduction of the BK$\alpha_1\beta_1$ peak current at +200 mV (expressed as a percentage) produced by 1 mM WO$_4^{2-}$ in the presence of 500 nM or 10 $\mu$M Ca$^{2+}$, as indicated. Significant differences were found when comparing the 10 $\mu$M Ca$^{2+}$ conditions with the other experimental situations ($P < 0.05$, ANOVA followed by Tukey’s post hoc test; data for 0 Ca$^{2+}$ are replotted from Figure 1 for comparison). (D) Average left shifts of the BK$\alpha_1\beta_1$ $G-V$ relationship induced by 1 mM tungstate in the absence (n = 7) and in the presence of 500 nM (n = 6) or 10 $\mu$M cytosolic Ca$^{2+}$ (n = 3). Differences in $V_{1/2}$ before and after addition of 1 mM WO$_4^{2-}$ were tested by Student’s paired t-test, as follows: 0 Ca$^{2+}$ condition, $P < 0.0001$; 500 nM Ca$^{2+}$ condition, $P < 0.01$; and 10 $\mu$M Ca$^{2+}$ condition, $P < 0.01$. Control $V_{1/2}$ values (in millivolts) at different cytosolic Ca$^{2+}$ levels (before tungstate application) were in the range of those previously reported for BK$\alpha_1\beta_1$ channels$^{17}$ as follows: 0 Ca$^{2+}$ condition, 138.76 ± 2.46; 500 nM Ca$^{2+}$ condition, 115.64 ± 5.31; and 10 $\mu$M Ca$^{2+}$ condition, −9.41 ± 5.72.

The BK$\alpha_1\beta_1$ $V_{1/2}$ was significantly lower at 10 $\mu$M Ca$^{2+}$ (as previously reported), and was further reduced by tungstate (~40 vs. ~20 mV at 0 Ca$^{2+}$; $P < 0.05$; Figure 2B and D).

### 3.2 Tungstate-induced activation of the BK$\alpha_1\beta_1$ channel depends on its Mg$^{2+}$ sensitivity

The analysis of available structural data on tungstate–protein complexes supported the role of divalent cations as cofactors for tungstate binding (see Methods). Given the effect of tungstate on BK channel $V_{1/2}$ in the absence of cytosolic Ca$^{2+}$, we can discard this divalent cation as an essential cofactor for tungstate action. The other divalent cation of physiological relevance in the modulation of BK channel activity is Mg$^{2+}$; therefore, we investigated its possible role as a tungstate cofactor.

We observed a dual modulation of human BK$\alpha_1\beta_1$ channels by Mg$^{2+}$ in the absence of cytosolic Ca$^{2+}$ similar to that reported for murine BK channels$^{6,10}$ Millimolar concentrations of cytosolic Mg$^{2+}$ reduced the current amplitude in a dose-dependent manner at positive voltages (Figure 3A and G, open bars). Mg$^{2+}$ also left shifted the
Figure 3 Activation of BKαβ1 channels by tungstate requires physiological levels of cytosolic Mg2+. (A) BKαβ1 currents were recorded from the same patch at cytosolic 0 Ca2+ and 0 (left panel), 0.7 (middle panel), or 5 mM Mg2+ (right panel). (B) Average G−V curves for BKαβ1 currents in the presence of increasing concentrations of cytosolic Mg2+ (n = 9; open circles, 0 Mg2+; triangles, 0.7 mM Mg2+; and inverted triangles, 5 mM Mg2+). Representative currents were recorded at 0 Ca2+ and 0 Mg2+ (C) or 5 mM Mg2+ (E), before (top panels) and 5–10 min after cytosolic application of 1 mM WO42− (bottom panels). Average G−V curves are shown for BKαβ1 currents at 0 Mg2+ (D; n = 7) and 5 mM Mg2+ (F; n = 3), before (control, open circles) and after tungstate application (1 mM WO42−, filled circles). (G) Reduction of the BKαβ1 peak current at +200 mV (expressed as a percentage) produced by 0.7 (n = 5) and 5 mM Mg2+ (n = 9; open bars) or by 1 mM WO42− in the presence of the indicated levels of cytosolic Mg2+ (filled bars; 0 Mg2+, n = 5; 0.7 mM Mg2+, n = 7; and 5 mM Mg2+, n = 3). The current amplitude reduction by 5 mM Mg2+ was larger than the one induced by 0.7 mM Mg2+ (P < 0.05, Student’s unpaired t-test). No significant differences were found when comparing the effect of tungstate in the different Mg2+ conditions [P = 0.47, non-parametric ANOVA (Kruskal–Wallis test); data for current amplitude reduction by tungstate obtained at 0.7 mM Mg2+ are replotted from Figure 1 for comparison]. (H) V1/2act plot for the BKαβ1 channel at different cytosolic Mg2+ levels, before (control, open circles) and after tungstate application (1 mM WO42−, filled circles; 0 Mg2+, n = 7; 0.7 mM Mg2+, n = 7; and 5 mM Mg2+, n = 3). Differences in V1/2act before and after addition of 1 mM WO42− were tested by Student’s paired t-test or Mann–Whitney U-test, as appropriate, as follows: 0 Mg2+ condition, P = 0.53; 0.7 mM Mg2+ condition, P < 0.0001; and 5 mM Mg2+ condition, P = 0.6 (0.7 mM Mg2+ data are replotted from Figure 1 for direct comparison). (I) No significant differences were found for average slope factor of the fitted G−V curves (kact) among the different Mg2+ concentrations in control conditions (tested by one-way ANOVA: n = 9, P = 0.89), or before (control, open circles) and after tungstate application (1 mM WO42−, filled circles; tested by Student’s paired t-test or Mann–Whitney U-test, as appropriate, as follows: 0 Mg2+ condition, n = 7, P = 0.68; 0.7 mM Mg2+ condition, n = 7, P = 0.66; and 5 mM Mg2+ condition, n = 3, P = 0.73).
G–V curve of the human BKα channel (Figure 3B), thereby reducing the channel $V_{\text{act}}$ without affecting the curve slope (Figure 3H and I, open circles). Tungstate (1 mM) produced a similar reduction of BKα current amplitude in the absence or presence of cytosolic Mg$^{2+}$ (Figure 3C, E, and G, filled bars). However, removal of cytosolic Mg$^{2+}$ prevented the tungstate-induced decrease of BKα channel $V_{\text{act}}$ (Figure 3D and H). In the presence of 5 mM Mg$^{2+}$, tungstate was not able to produce a further $V_{\text{act}}$ reduction beyond the one induced by the divalent cation (Figure 3F and H).

The Mg$^{2+}$ requirement for the tungstate effect was further tested on human BKα channels containing mutations in residues of the α subunit VSD that contribute to Mg$^{2+}$ co–ordination. The D99A mutation abolishes and the N172A mutation substantially reduces the Mg$^{2+}$-induced activation of mSlo channels. Supplementary material online, Figure S3 shows the position of these mutations and the alignment of human and mouse BK channel VSD linkers containing the mutations. In accordance with previous reports on the mouse BK channel, the introduction of the D99A and N172A mutations slightly modified the $V_{\text{act}}$ for human BKα channels in the absence of divalent cations (Supplementary material online, Figure S4E), and the addition of 0.7 or 5 mM cytosolic Mg$^{2+}$ still reduced the amplitude of K$^+$ currents through mutant D99A and N172A human BKα channels at...
positive voltages without significant effects on the $G-V$ curves (Supplementary material online, Figure S4). This is consistent with the idea that Mg$^{2+}$-induced blockade and activation of BK channels occur via different binding sites. When exposed to 1 mM tungstate, both D99A and N172A human BK$\alpha_1$ channels presented a reduction in current amplitude similar to that observed for WT channels (Figure 4A, C, and E), but lost the tungstate-induced channel activation (Figure 4B, D, and F).

### 3.3 Tungstate-induced vasodilatation of pre-contracted mouse arteries relies on the expression of the BK channel $\beta_1$ subunit

To determine the impact of BK channel modulation by tungstate on vascular function, we tested the action of tungstate on the contractility of femoral arterial rings from both WT and BK $\beta_1$-knockout mice. Application of 1 mM tungstate induced similar constriction of arterial rings from both WT and $\beta_1$-knockout mice (Supplementary material online, Figure S5), an effect consistent with the considerable degree of BK current inhibition produced by this concentration of tungstate, independently of the presence of the $\beta_1$ subunit (see Figure 1A, C, and K). However, lowering the tungstate concentration to 0.1 mM did not significantly affect the basal tension (Supplementary material online, Figure S5). Therefore, we evaluated whether 0.1 mM tungstate could yet modulate heterologously expressed BK channels. In the absence of $\beta_1$, 0.1 mM tungstate produced a small reduction of BK currents (Figure 5A and E), but was ineffective in left shifting the $G-V$ relationship (Figure 5B) or the $V_{1/2}$act (Figure 5F). In the presence of $\beta_1$, 0.1 mM tungstate did not inhibit BK currents (Figure 5C); moreover, there was a slight increase in the overall current (Figure 5E) and a significant left shift in the $G-V$ curve (Figure 5D), resulting in a $\sim$10 mV decrease of $V_{1/2}$act (Figure 5F). Thus, 0.1 mM tungstate activates BK$\alpha_1\beta_1$ channels without negatively altering BK$\alpha_1$ current amplitude.

Next, we tested the effect of 0.1 mM tungstate on femoral arterial rings. Exposure of femoral arterial rings to K$^+$ depolarizing solutions produced marked and reversible contractile responses in both WT (Figure 6A) and $\beta_1$-knockout arteries (Figure 6B). Arterial rings were then pre-contracted with endothelin-1 (ET-1; 50 nM), an efficacious
vasoconstrictor peptide produced by endothelial cells that increases cytosolic Ca^{2+} in VSMCs and has been related to the pathogenesis of hypertension (reviewed in 26). No significant difference (P = 0.21, Student’s unpaired t-test) was found in the contractile response to ET-1 between WT (10.35 ± 0.73 mN, n = 21) and β1-knockout arteries (11.79 ± 0.79 mN, n = 12). Consistent with the observed β1- dependent induced activation of BK channels, 0.1 mM tungstate significantly reduced arterial tension after 5 min of application on pre-contracted arterial rings from wild-type mice, a vasodilatory effect that was maximal after 20 min of tungstate exposure (Figure 6A and C). This effect of tungstate was reversed by the addition of the BK channel blocker paxilline (Figure 6A), and was not mimicked by the xanthine oxidase inhibitor allopurinol (100 μM; Figure 6C). No vasodilatory action was observed for tungstate in arterial rings obtained from β1-knockout mice (Figure 6B). In fact, a vasocontractile response was observed within 15–20 min of tungstate (0.1 mM) application in the pre-contracted arteries of β1-knockout mice (Figure 6C), probably owing to its inhibitory effect on BKα current amplitude (see Figure 5A and E).

4. Discussion

Large-conductance, Ca^{2+}-dependent potassium (BK) channels are important for the control of vascular tone. BK channels are activated in vascular smooth muscle by local Ca^{2+} transients (Ca^{2+} sparks) caused by the opening of a cluster of ryanodine receptors in the sarcoplasmic reticulum membrane adjacent to the cell membrane. Such activation results in an efflux of K^{+} that is sufficient to hyperpolarize the membrane potential, providing a negative feedback mechanism that limits membrane depolarization and contraction. Accordingly, the blockade of BK channels or ryanodine receptors in arterial smooth muscle causes depolarization, an elevation of arterial wall Ca^{2+} concentration, and vasoconstriction. Furthermore, a wide variety of vasodilators exert their actions through activation of BK channels. The presence of the regulatory β1 subunit (highly expressed in smooth muscle, but not in other tissues) has been shown to increase the apparent voltage and Ca^{2+} sensitivity of the BK channel pore-forming α subunit, making the negative feedback loop more efficient in the regulation of vascular resistance.
Thus, in β1-knockout mice Ca2+ sparks are functionally uncoupled from BK channel activation, leading to membrane potential depolarization, vasoconstriction, elevation of blood pressure, and left ventricular hypertrophy. In commonly used rat models of hypertension, including spontaneously hypertensive rats and rats made hypertensive by chronic infusion of a vasoconstrictor peptide, elevated blood pressure is associated with a down-regulation of the β1 subunit, but not the α subunit, of the BK channel. Besides, and more interesting in the context of human disease, a gain-of-function BK channel β1 subunit variant has been reported to protect against moderate to severe diastolic hypertension and ‘combined cardiovascular disease’ (myocardial infarction and stroke), while an unrelated genetic variant of the gene coding for the α subunit was associated with a higher risk of hypertension. Finally, estradiol has also shown to activate BK channels, probably through the binding to the β1 subunit, an effect that may contribute to gender differences in basal vascular tone and myogenic responses. Similar results have been obtained using xenoestrogens and cholesterol-derived steroids. Altogether, this evidence provides considerable incentive for the development of new antihypertensive therapeutic agents that target the vascular BKαβ1 channel.

A variety of small synthetic molecules (for example, NS1619) and natural compounds (such as dehydrosoyasaponin-I (DHS-I)) have been identified as BK channel openers. However, little is known about how the different β subunits interact with and modulate the pore-forming α subunit of the BK channel. In the present study and 8) or tungstate (present study) contain a DK motif (93WMTSVK(D99A)WAGVM104). In site 2, located in the cytoplasmic region between S2 and S3, a DK motif is found in the vicinity of the mutated N172 (171A(N172A)KD174). Also, site 3 in the RCK1 domain (536YVSNLKFKDLF1KDRD1DVN(E374)) has two of the KD motifs near to the E374, which also contributes to the Mg2+-binding site involved in BK channel activation. The fact that such Mg2+-dependent tungstate-induced activation of BK channels specifically requires the presence of either β1 or β2 might rely on how the different β subunits interact with and modulate the pore-forming α subunit of the BK channel. However, we cannot rule out the possibility that β1/β2 subunits somehow contribute to the binding site for tungstate.

Tungstate has a low toxicity profile in animals and humans, and the results of the first proof-of-concept clinical trial (TROTA-1) on the efficacy of sodium tungstate in grade I and II human obesity have already been reported. Contrary to the results obtained in rodent models of obesity, the data obtained from the clinical trial did not sustain sodium tungstate as a pharmacological tool in the treatment of human obesity. The subjects included in the trial, besides being moderately obese, were non-diabetic and mostly normolipidaemic. The results obtained did not reveal significant changes in glucose/lipid metabolism or in blood pressure after the active treatment with tungstate. The reasons for the discrepancy in the actions of tungstate between rodents and humans remain to be elucidated. The lack of efficacy of tungstate in humans may be attributed to an inadequate duration of the active treatment, to the dose of sodium tungstate being too low, or to alterations in the expression of enzymes involved in the regulation of energy homeostasis. In one parameter, however, there was agreement between the laboratory animals and humans; tungstate does not affect blood pressure in normotensive subjects. This observation, together with the fact that the vasodilatory concentration of tungstate used in the present study (100 μM) was only slightly higher than the tungstate levels measured in the plasma of both treated rodent models and humans (~5–20 μM), also encourages future studies in order to investigate the molecular mechanisms of action of tungstate further and to test the utility of tungstate as an antihypertensive agent in humans, either by itself or in combination with other therapeutic tools.
Acknowledgements

We thank Dr. O. Pongs for making available the β1-knockout mice, Dr. C. Fandos for help with the generation of the mutant hSlo constructs, Dr. M. Fernández-Tenorio for assistance in the analysis of results, and Dr. J. Guinovart for helpful discussions.

Conflict of interest: none declared.

Funding

This work was supported by Spanish Ministry of Science and Innovation, Fondos Europeos de Desarrollo Regional (FEDER) Funds, and Plan E (grants SAF2009-13182-C03-02 and SAF2009-09848), Fondo de Investigación Sanitaria (Redes HERACLES RD06/0009 and RECAVA RD06/ 0020), Marató de TV3 (grant 080430), and Generalitat de Catalunya (grant 2009SGR1369). M.A.V. is the recipient of an ICREA Academia Award (Generalitat de Catalunya).

References