Regulation of BK channel by tungstate and its relevance for the control of vascular tone and intracellular signalling

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POMPEU FABRA

O *bicho*, Aroa, a miña irmá pequena
aínda que realmente é moi grande.

Pero sobre todo ós meus pais, Maribel e Antonio,
non hai verbas dabondo para agradecer o voso
apoio. Grazas a vós todo é posible.

"…tienes el cerebro carcomido por los gérmenes de la ignorancia…"

Grazas avó

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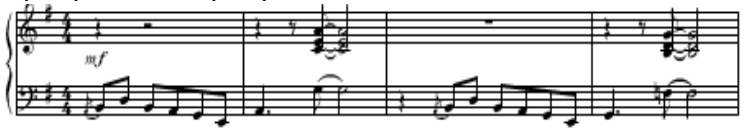
A Fly, por haber volado conmigo y por seguir ahí.

A tu, pel temps que vola… per parlar en el silenci. Merci.



A todos vosotros que habéis compartido la experiencia conmigo,

¡gracias! & welcome to my electric world...

ABSTRACT

The large-conductance Ca^{2+}- and voltage-gated K^+ (BK) channel containing the pore-forming α and the regulatory β_1 subunits play a pivotal role in the control of arterial tone and modification of channel function is associated to changes in blood pressure in both animal models and humans. Tungstate, a compound with antidiabetic and antiobesity properties, also reduces blood pressure in experimental animal models of both hypertension and metabolic syndrome, although the underlying mechanisms are not completely understood. This Thesis evaluates the effect of tungstate on BK channel function and its relevance for both the regulation of vascular resistance and intracellular signaling. Our results show that tungstate activates BK channels in a β- and Mg^{2+}-dependent manner and induces vasodilatation only in mouse arteries that express the BK β_1 subunit. Our functional and comparative structural analysis suggest that, although the tungstate interaction site is located in the BK α subunit, its positive effect on the channel requires residues of the β_1 subunit extracellular loop that stabilize the active configuration of the voltage sensor. In addition, we have found that tungstate-induced, $G_{i/o}$ protein-mediated ERK phosphorylation is enhanced by BK$\alpha\beta_1$ channels.

RESUMEN

El canal de potasio (K^+) de alta conductancia dependiente de voltaje y Ca^{2+} (BK) y compuesto por la subunidad α (formadora del poro) y la subunidad reguladora β_1, tiene un papel fundamental en el control del tono arterial. Cambios en la función del canal tienen una relación directa con modificaciones en la presión sanguínea tanto en modelos animales como en humanos. El tungstato, un compuesto con propiedades antidiabéticas y antiobesidad, también reduce la presión sanguínea en modelos animales de hipertensión y síndrome metabólico, aunque los mecanismos subyacentes no son del todo conocidos. Esta Tesis, evalúa el efecto del tungstato sobre la función del los canales BK y su relevancia en la regulación de la resistencia vascular y la señalización intracelular. Nuestros resultados demuestran que el tungstato activa los canales BK en una manera β- y Mg^{2+}-dependiente, induciendo vasodilatación de arterias murinas que expresan la subunidad β_1. Nuestros análisis funcionales junto a estudios estructurales comparativos sugieren que, aunque el sitio de interacción del tungstato está situado en la subunidad α, su efecto positivo sobre el canal requiere residuos pertenecientes al lazo extracelular de la subunidad β_1 implicados en la estabilización del sensor de voltaje del

canal BK en su configuración activa. Además, hemos observado que la fosforilación de ERK inducida por el tungstato y mediada por proteínas $G_{i/o}$ está potenciada en presencia de canales BK$\alpha\beta_1$.

PREFACE

Ion channels and the electric properties conferred to cells makes them being involved in every physiological characteristic that make us humans. Every perception, thought, movement and heartbeat depends on electrical signals generated by the activity of ion channels. Ultimately, most of these "*magical*" properties that animate us arise from the ability of channels to facilitate the movement of selected ions across membranes and the regulation of their activity by multiple gating signals. The understanding of these properties and processes can help us not only in the comprehension of different human physiological events and pathological conditions, but also in the future development of innovative and more efficient therapeutic tools.

One of the most ubiquitously expressed potassium (K^+) channels in mammals is the calcium (Ca^{2+}) and voltage-gated K^+ channel, known as Maxi K or BK ("Big K") channel due to its very large single channel conductance. BK channels have been implicated in a variety of physiological processes, which include hearing, microbial killing in leukocytes, modulation of hormone and neurotransmitter release, repolarization of action potentials, shaping of the

dendritic Ca^{2+} spikes, and regulation of smooth muscle tone. Furthermore, BK channel openers have emerged as potentially useful agents in the therapy of various diseases associated to BK channels in relation to both the central nervous system and smooth muscle, such as acute stroke, epilepsy, psychoses, erectile dysfunction, asthma, bladder hyperactivity, and arterial hypertension. Although a variety of small synthetic molecules and natural product compounds have been identified as selective BK channel openers, very little is known about the site(s) of interaction of openers of these channels and most of them have serious safety and efficacy problems for clinical use.

In the present Thesis, you will find a new activator of BK channels, the transition metal tungstate, which opens a new field in the search for BK channel agonists. In this respect, this Thesis is an attempt to enlighten us on both, the molecular processes underlying the modulatory action of tungstate on BK channels and also the potential therapeutic use of the tungstate-mediated BK channel regulation as antihypertensive treatment along with its surprising role as a new metabotropic pathway, that controls intracellular signalling.

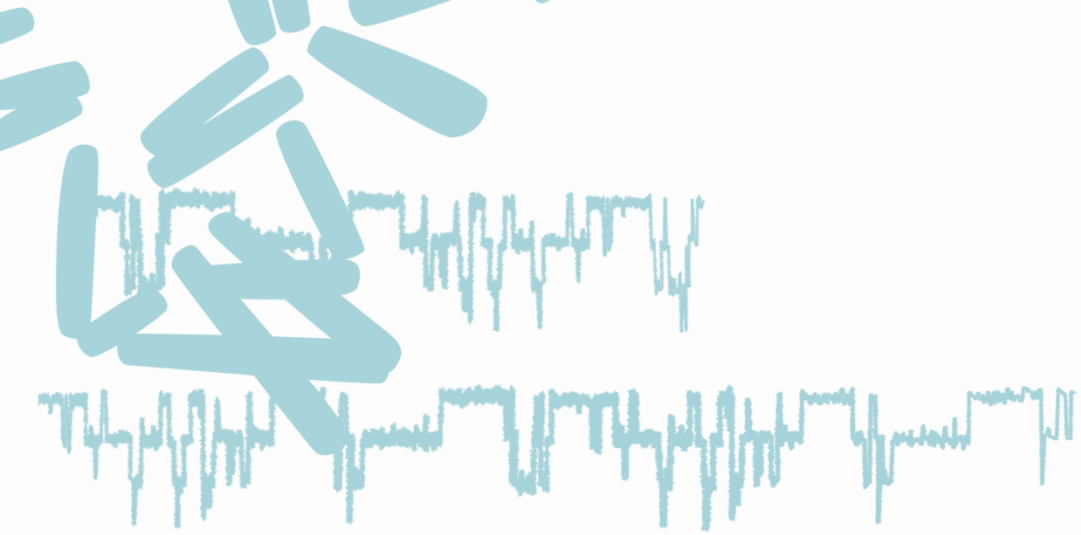
ABREVIATIONS

AA	Arachidonic Acid
ATP	Adenosine Triphosphate
BK	Large(Big)-conductance Ca^{2+}- and voltage-dependent K^+ (potassium) channel
cAMP	cyclic Adenosine MonoPhosphate
cGMP	cyclic Guanosine MonoPhosphate
CHO	chinese hamster ovary
ChTX	Charybdotoxin
CO	Carbon monoxinde
COOH	C-terminal tail
CTD	C-terminal domain
DHS-I	Dehidrosoyasaponin-I
EET	Epoxyeicosatrienoic acid
ER	endoplasmic reticulum
ERK	Extracellular-signal Regulated Kinase
GLUT	Glucose transporter

GPCR	G-protein coupled receptor
GSK3β	Glycogen synthase kinase 3β
HEK	Human embrionic kidney
IbTX	Iberotoxin
IK	Intermediate-conductance Ca^{2+}- and voltage-dependent K^+ channel
JAK	Janus Kinse
JNK	c-Jun N-terminal kinase
KCNMA1	gene encoding the α subunit of the BK channel
KCNMB1	gene encoding the β_1 subunit of the BK channel
KCNMB2	gene encoding the β_2 subunit of the BK channel
KO	knockout
LRRC	Leucine-rich repeat containing protein
mRNA	messenger RNA
NH_2	N-terminal tail
NO	Nitric oxide
PGD	Pore Gate Domain
PGI-2	Prostacyclin-2

PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PLC	Phopholipase C
P_o	Open probability
PTX	Pertussis Toxin
RCK	Regulatory domain associated with Conduction of K^+
ROS	Reactive oxygen species
SHR	spontaneously hypertensive rats
SK	Small-conductance Ca^{2+}- and voltage-dependent K^+ channel
SM	smooth muscle
STOCs	Spontaneous transient outward K^+ currents
TEA	Tetraethylammonium
TM	transmembrane domain
TRP	transient receptor potential

$V_{1/2}/V_{50}$	voltage for half-maximal current activation
VDCCs	voltage dependent Ca^{2+} channels
VSD	Voltage Sensor Domain
VSMC	vascular smooth muscle cells
WT	wild type
WKY	Wistar Kyoto Rats
XO	Xanthine Oxidase



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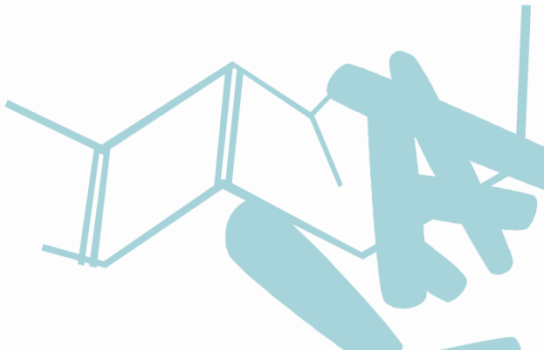

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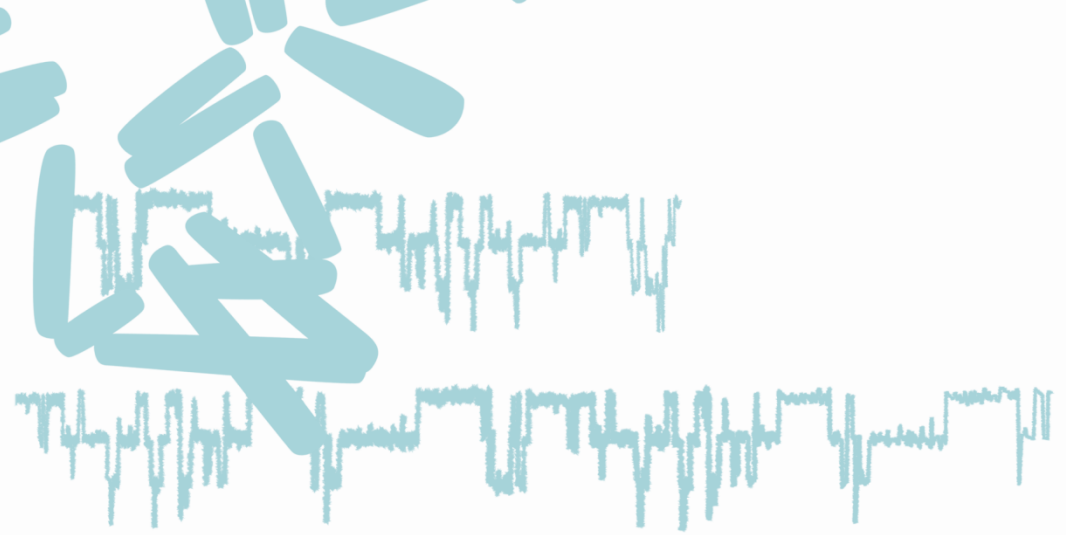


I.INTRODUCTION

The large conductance calcium (Ca^{2+})- and voltage-gated K^+ (BK) channel is the first member of the Slo family[1]. Indeed, others members have been described: Slo2 and Slo3 (reviewed in[2]).

Cloned for the first time in 1991 from Drosophila Melanogaster [3,4], years later BK channel was also cloned from several mammalian species and described in a physiological environment as a Ca^{2+}-dependent K^+ current present in vascular smooth muscle cells that hyperpolarize and dilate pressurized arteries[5]. Since these first experiments, a vast number of studies have been performed about this channel.

1. The BK channel. Molecular and structural properties.

BK channels, as many other channels, consist of two distinct subunits: α and β, arranged in a 1:1 stoichiometry[6,7]. A functional channel is composed by four ion conducting α subunit alone[8-10] or in combination with four regulatory β subunits[6,11]. The pore-forming α subunit, is encoded by a single gene (*Slo*, KCNMA1) located in the chromosome 10 (10q22.3) (Gene ID: 3778) and consists of 11 hydrofobic domains (S0-S10) divided in 7 transmembrane domains (S0-S6) and the intracellular tail (S7 -S10) **(Figure 1)**.

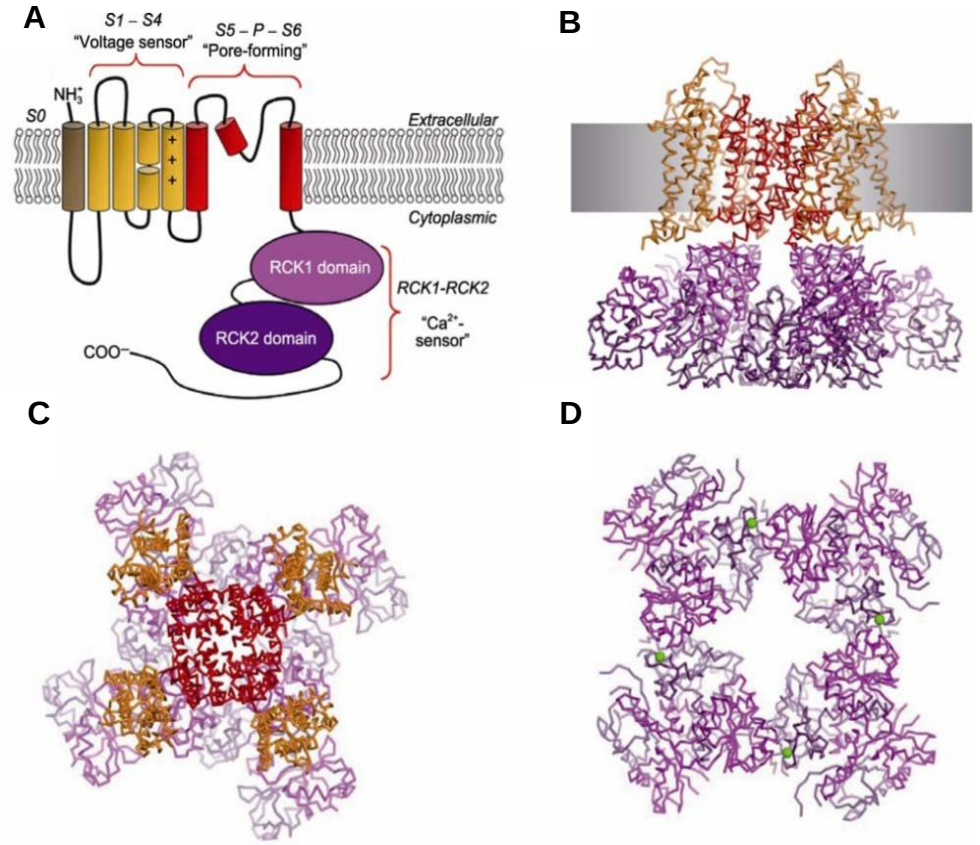


Figure 1. Molecular architecture of the BK channel. (A) Schematic diagram and topology of the α BK channel subunit. Each subunit contains a S0 helix close to the transmembrane voltage sensor domain (VSD) (S1-S4 helices orange) and pore domain (S5-P-S6 helices, red), and two tandem cytoplasmic domains (RCK1 light purple and RCK2 dark purple). The RCK1 domain contains the S7 and S8 helices and the RCK2 domain contains the S9 and S10. **(B)** Model of the human BK channel, based on the alignment of the pore domain from the crystal structure of the Kv1.2-Kv2.1 chimera and MthK channel. The voltage-sensing domain, pore domain, and RCK domains have been colored according to the diagram in A, to illustrate the hypothetical three-dimensional arrangement of the domains. The approximate location of the plasma membrane is shaded

gray. **(C)** BK channel model from part B viewed from above the extracellular side, illustrating the four-fold symmetrical arrangement of the domains about the central K^+ conduction pathway. **(D)** Crystal structure of the Ca^{2+}-bound "gating ring" of RCK domains. Ca^{2+} ions are shown as green spheres (for more details see[12]).

The *Slo* gene undergoes extensive alternative splicing, which contributes to BK channel diversity providing differences in kinetics, Ca^{2+} sensitivity and channel modulation by intracellular signalling molecules.

Most splicing sites are located in the COOH terminus of the Slo1 protein, and several sites reside between hydrophobic domains S8 and S9 **(Figure 2)**[13,14]. In terms of the molecular composition, BK channels are unique in the voltage-dependent potassium channels group. They contain an extra transmembrane domain, the S0, located in the N-terminus of each α subunit. Its localization is flanqued by the S3-S4 loop very close to the S1 and S2 helixes[15,16]. It has been reported that its function is related with the α-β subunits interaction[17,18] but also it has been described a relation with the voltage-dependent gating of the channel. An EF-hand domain for Ca^{2+} binding has been described in the S0-S1 loop which could be participating in the Ca^{2+} sensitivity of the channel[19]. Studies of single mutations in very conserved residues of this domain suggest also its involvement in the voltage-dependent activation of the channel **(Figure 3)**[20,21]. However is not clear

how this S0 domain could be involved in the movement of voltage sensor domain during the voltage-induced activation.

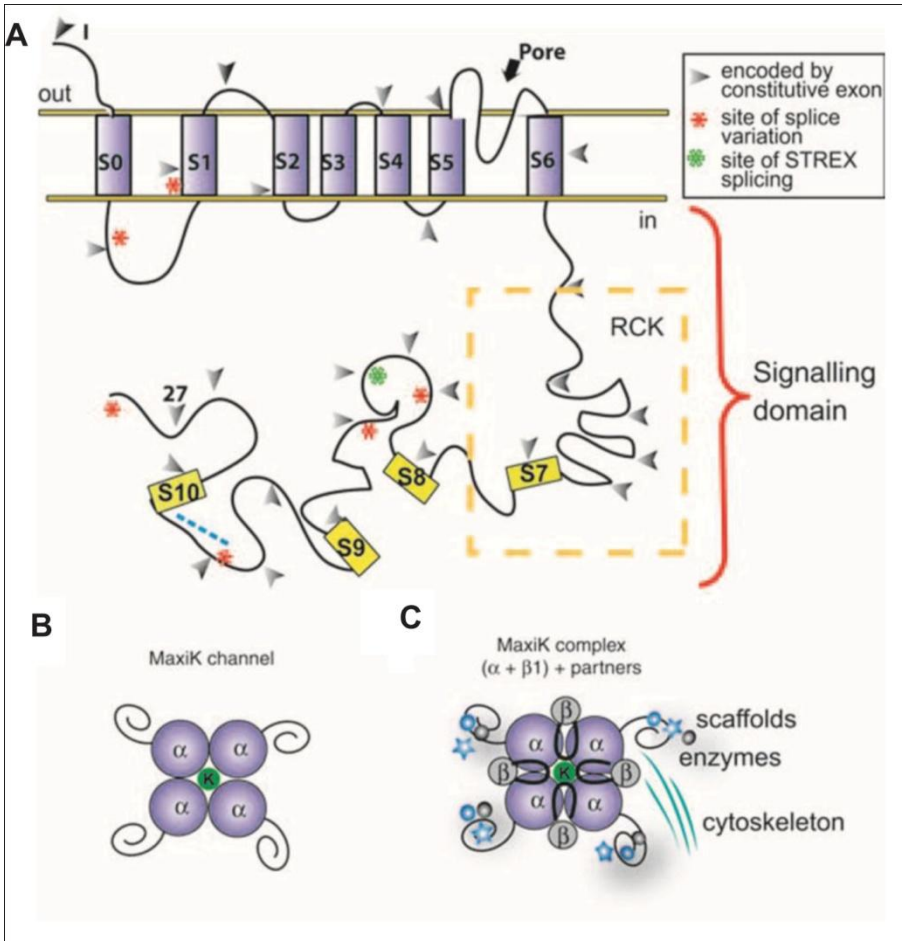


Figure 2. Alternative splicing sites in the BK α subunit. **(A)** Protein topology of the BK α subunit. Arrows mark boundaries of translated constitutive exons; asterisks, sites of splice variation; dashed blue line, string of aspartate residues forming the Ca^{2+} bowl; dashed square, the RCK domain. **(B)** and **(C)** model (top view) of tetrameric assembly showing the pore with and without β-subunits and proteins interacting at the C-terminus (extracted from[22]).

As a voltage-dependent channel, BK channel contains a voltage sensor domain (VSD) which senses the voltage of the membrane. In these channels the VSD involves from S1 to S4. The S4 segment is the actual trigger for BK activation[13].

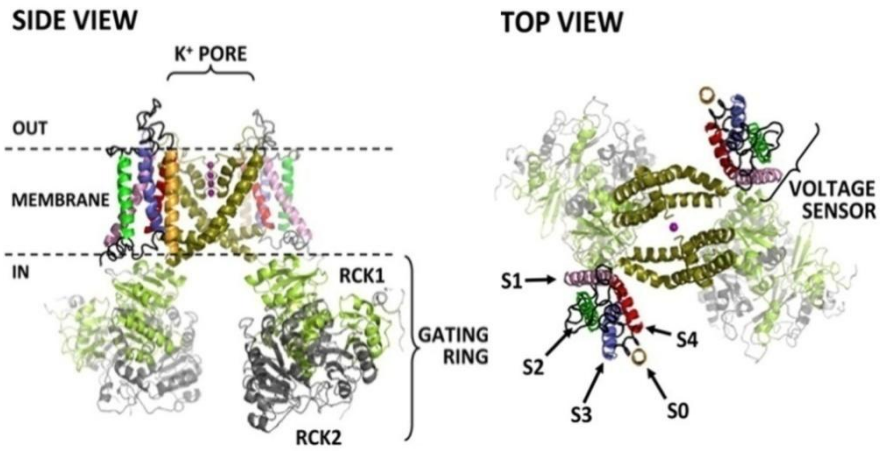


Figure 3. Side and top view of the putative structure of the BK channel. Only 2 of 4 subunits are shown for clarity. Each α subunit consists of seven transmembrane segments (S0-S6) and a large intracellular ligand binding domain. Segments S1-S4 constitute the VSD, whereas S5-S6 from all the α subunits contribute to the central K^+ selectivity pore (K^+ ion occupying the pore is shown as purple spheres). Each subunit also contributes an intracellular RCK1/RCK2 heterodimer, which assembles in the hetero-octameric gating ring superstructure. The structure shown for domains S1–S6 is from the atomic structure of the Kv1.2-2.1 chimera[23]. S0 was modeled as an ideal α helix. Note its close association with the voltage-sensing segments S3 and S4, as suggested by Liu *et al.*[15].The intracellular domain structures[24] were manually docked

on the 2R9R structure of a chimaeric voltage-gated K^+ channel (extracted from[21]).

Indeed, the BK channel contains 3 positively charged Arginine (Arg) residues in this segment, which are highly conserved in the Kv channels and when mutated affect the voltage dependence of the channel (reviewed in[25]), although only one of the positive charges (Arg213)[26] has been shown to contribute to voltage sensing.

Nevertheless charged residues are present also in the S2 (D153, R167) and S3 (D186) domains, contributing actively and in a similar way to the voltage sensing of the BK channels. In case of the BK channel all the segments of the VSD move together and repack during the voltage activation of the channel, in contrast with the movement of S4 alone proposed for Kv channels[21,26-29].

Depolarization by itself produces conformational changes in the VSD leading to pore opening. The movement of these charged residues, is reflected in the measurable "gating currents" [30,31] but also in fluorescence signal changes after labelling the S3-S4 linker with a fluorophore[32].

The mechanisms of how voltage changes can activate the channel, increasing the open probability (P_o) of a single BK channel[33,34] has been proposed by Horrigan and Aldrich as an allosteric model of interaction where the VSD can be activated either at the closed or at the open state. Being more favoured

the activation of the VSD in the open state, the activation of the voltage sensors however promotes channel opening[35,36]. In this model of interaction, Ca^{2+} plays also a role favouring the transition from the closed to the open state (see below for further details).

1.1. The pore domain as a binding site for blockers.

The conformational change produced in the VSD open and closes the pore of the channel. The pore gate domain (PGD) is located between the S5-S6 segments[8,37] and contains the selectivity signature for the K^+ selectivity filter (TVGYG)[13]. All the K^+ channels show a selectivity sequence of $K^+ \approx Rb^+ \rangle Cs^+$, with K^+ being, at least 10^4 times more permeant than Na^+. They also share very similar ion permeability characteristics and use diverse gating mechanisms.

The structure of the K^+ pore has been proposed to be as an "inverted teepee shape", based in X-ray crystallography of the KcsA channel, with the selectivity filter located at its wide end. The narrow selectivity filter is 12 Å long, whereas the remainder of the pore is wider and has a relatively inert hydrophobic lining. A large water-filled cavity and helix dipoles help to overcome the high electrostatic energy barrier facing a cation in the low dielectric membrane center[38] **(Figure 4)**.

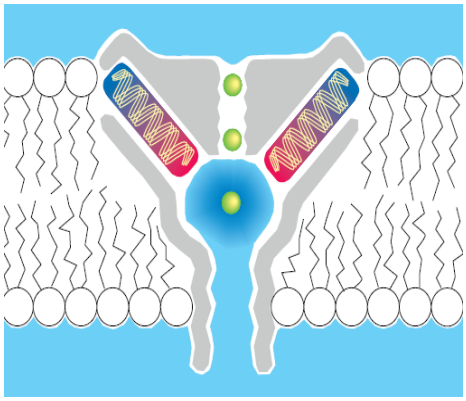


Figure 4. Cartoon of a K^+ channel pore. Two mechanisms by which the K^+ channel stabilizes a cation in the middle of the membrane. First, a large aqueous cavity stabilizes an ion (green) in the hydrophobic membrane interior. Second, oriented helices point their partial negative charge (carboxyl end, red) towards the cavity where a cation is located (extracted from[38]).

But why the conductance of BK channels is so high? The inward vestibule of BK channels contains a ring of eight negative charges. Among them, residues Glu386 and Glu389 (in the RCK1 domain) that modulate channel conductance through an electrostatic mechanisms are not present in K^+ channels with lower conductance[39]. The ring of charges formed by Glu386 and Glu389 together with another two aminoacids residues in the extracellular loop of the BK channels, Asp326 and Glu329 submerged into the external solution, form the ring of charges inducing the electrostatic potentials in the BK channel pore[40]. This situation is analogous to the large electrostatic effect of the pore α helices of the KcsA channel induced by the surrounding low dielectric constant medium[41].

In this case the dipoles of the α helices are able to stabilize a K^+ ion positioned in the pore central cavity. Also, another very

well conserved residue (Glu322) located at the S5 domain, inside de membrane (embedded in a hydrophobic environment close to the lipid-solution interface), has a strong electrostatic effect on the selectivity filter. The long–range electrostatic effect of this residue is due to its close proximity to the pore helixes and the negative residues located in the vestibule, to amplify the effect induced by the low dielectric bilayer environment. Actually, BK channels double their conductance at negative potentials by using this ring of four negative charges located near the external entrance of the selectivity filter without much effect on channel gating[40].

Finally, the mechanism for the large BK channel conductance is not completely understood, the rings of negative charges located at the inner and outer mouth contribute to it, but only partially. However it is clear that the electrostatic potential of the internal vestibule of BK in the neighbourhood of amino acids Glu386 and Glu389 is ~140 mV more negative than in Kv1.2 channels[40]. Another factor that may improve the BK channel conductance is the larger dimensions of its internal vestibule compared with other K^+ channels. A larger inner vestibule would imply a smaller access resistance and, therefore, permeation of K^+ ions may be less restrictive by the inner pore approaching the diffusion limit[42,43].

The pore region of the BK channel also bears the receptor for channel blockers, including the non specific K^+ channel

blocker tetraethylammonium (TEA)[44] or the more specific Charybdotoxin (ChTX), Paxilline and Iberiotoxin (IbTX).

Extracted from scorpion *Leiurus quinquestriatus* var. *Hebraeus*[45] ChTX is a 37-amino-acid pepide[46], policationic molecule, which interacts from the outside of the channel, in the external pore region[47]. Interaction with the BK channels has been described as a process where a single ChTX molecule causes inhibition (at nanomolar concentrations) by binding to a site on the channel either in the open or the closed conformation[48]. Channel blockade depends on the applied voltage in an apparently complex way. Whereas the association rate is sensitive to the channel's gating conformation, the dissociation rate depends on voltage. Also, the apparent affinity of ChTX is lowered by increasing the ionic strength[48]. The exact interaction site for the ChTX is not clear but some mutational studies in the Shaker channel suggest that the pore loop residue Glu422 influences the binding energy in the outer mouth of the channel[46]. Indeed, in the case of the BK channel it has been suggested the importance of some residues in the extracellular loop of the BK accessory-regulatory β_1 subunit for the ChTX specific interaction[49] (for more details see below: *The great partners: regulatory β subunits*). It has been shown that ChTX can interacts also with other K^+ channels, such as IK channels (intermediate-conductance Ca^{2+}- and voltage-dependent K^+ channels, Kv1.3)[50].

IbTX is a 37 amino acid polypeptide isolated from the venom of the scorpion *Buthus tamulus*[51]. IbTX shares a high sequence identity (around 68%) with ChTX[52]. For this reason, they may show a similar mechanism for block of the BK channel. For the mechanisms of interaction between the channel and the toxin, IbTX blocks the BK channel from the external side at nanomolar concentrations causing the appearance of long, nonconducting silent periods when the ions cannot pass through the channel which are interrupted by periods of apparently normal channel activity **(Figure 5)**.

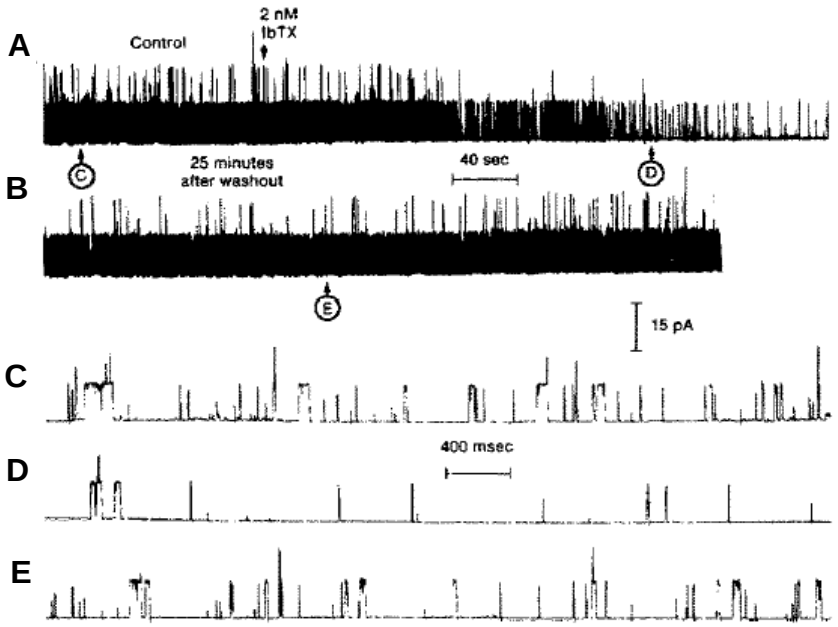


Figure 5. Inhibition of Ca^{2+}-activated K^+ channels in cultured bovine aortic smooth muscle cells by IbTX. **(A)** After recording control channel activity, an outside-out membrane patch from a bovine aortic smooth muscle cell was exposed to 2 nM IbTX and allowed to equilibrate. **(B)**

channel activitv recorded after the chamber was perfused with toxin-free medium. **(C)**, **(D)**, and **(E)**, expanded time scale recordings of channel activity before addition of toxin **(C)**, in the presence of 2 nM IbTX **(D)** and after washing away toxin for 25 min **(E)** (extracted from[51]).

However the BK channel kinetics apparently are not affected. The experiments, performed in bovine aortic smooth muscle, suggest an IbTX reversible binding to the channels. The rate of association of IbTX with the channel also decreased as the external ionic strength was increased (as observed for ChTX).

This behavior can be explained by proposing that the rate of association of a positively charged IbTX with a negatively charged channel mouth is promoted by electrostatic interactions. IbTX binds near the pore of BK channels and in a competitive mode with TEA but also with ChTX. Comparing both ChTX and IbTX, even though they share the 68% sequence homology and they share a common blockade mechanism, there are some quantitative differences in the blocking kinetics. In symmetric 150 mM KCl and +40mV, ChTX produces mean blocked times of about 64 s for BK channels from bovine aortic smooth muscle, while IbTX produces longer mean blocked times about 840s. The structural differences between IbTX and ChTX may explain this difference between toxins[52].

Paxilline, a fungal mycotoxin with alkaloid nature, obtained from fungi of the genera *Penicillium*, has been shown also to

block BK channel (at nM-µM concentration) more effectively from the inner side. The nature of the binding is allosteric and often happens to be a reversible phenomenon. Chemical modifications of paxilline indicate a defined structure-activity relationship for channel inhibition[53].

1.2. The intracellular tail.

1.2.1. Ca^{2+} interaction sites and channel gating.

The intracellular tail of BK channel is a very large domain which comprises two thirds of the total length of the primary amino acid sequence[17,54]. It also contains three of the hydrophobic regions described (S7-S10) involved in the two **R**egulator of **C**onductance sites for $\mathbf{K}^+$ (RCK)[55]. This domain contains multiple regulatory sites, such as the "calcium bowl" motif, interaction sites for divalent cations, the tetramerization region, or multiple phosphorylation sites for cAMP- and GMP-dependent kinases, protein kinase C and tyrosine kinases[56-58].

BK channel regulation is complex, with both voltage and divalent cations, intracellular Ca^{2+} at the micromolar range[59,60] and Mg^{2+} at the milimolar range[61-64], activating the channel. Ca^{2+} can increase the P_o of the channel by itself, in a concentration-dependent manner, as shown in **(Figure 6)**[65]. The mechanism proposed to underly the Ca^{2+} effects on the

channel and its relation with the other important player on the activation of the BK channel, the voltage, are described below.

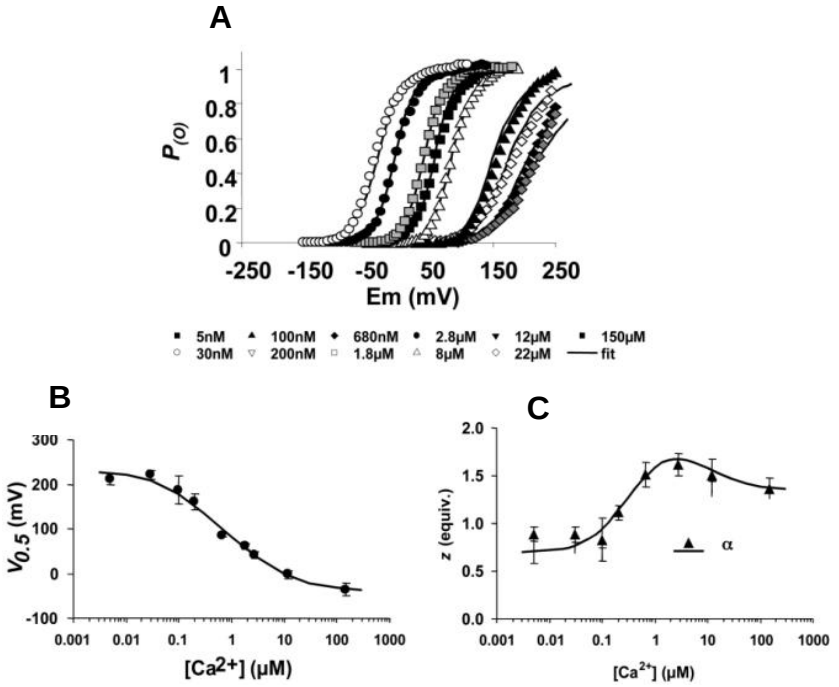


Figure 6. Voltage and Ca^{2+} dependence of the BK channel. (A) Averaged P_o-V curves at the indicated Ca^{2+} concentrations. Lines are the best fit to a Boltzman distribution **(Equation 1)**. Fitted parameters $V_{0,5}$ (or $V_{1/2}$) and z, plotted against Ca^{2+} concentration, are shown in **(B)** and **(C)**, respectivelly (extracted from[66]).

Functional studies and mutagenesis have allowed the identification of two Ca^{2+} binding sites in the C-terminal domain. One of them is the "calcium bowl", located in the RCK2 domain, corresponding to a sequence of consecutive aspartate (Asp) residues at the positions 895-901

(DQDDDDDPD) highly conserved among species[67-70] and with high affinity for Ca^{2+} binding (micromolar range).

Equation 1

Boltzman fitting equation

$$\frac{G}{Gmax} = \frac{1}{1 + exp[-ze(V - V\ 1/2act\ )/KactT]}$$

G is the conductance

z, is the valence of equivalent charge

e, is the elementary charge

$V_{1/2act}$, is the voltage of activation where the conductance is half maximum

k**,** is the Boltzman's constant

T*,* is the absolute temperature

The other site has been described in the region corresponding to the RCK1 domain, the residues Asp362 and Asp367[71,72] (reviewed in[25]) with lower affinity for Ca^{2+} binding (millimolar range). Moreover, another Ca^{2+} interaction site has been described in the RCK1 domain involving the Glu399 and defined as a low affinity binding site. This site is a suitable binding site for other divalent cations[72]. However, it has been

found that removing the entire C-terminus of the BK channel, just after the S6 domain (position 323), the channel, although presenting a poor expression, it shows a near wild-type conductance, voltage and Ca^{2+}-dependent gating at intracellular micromolar Ca^{2+} levels[73]. X-ray crystallography studies of the C-terminal structure (CTD) shows the two RCK domains folding tightly one against each other **(Figure 7A)** and linked by an "flexible interface", as in the MthK dimer, dominated by helix-turn-helix connectors[74] **(Figure 7B)**.

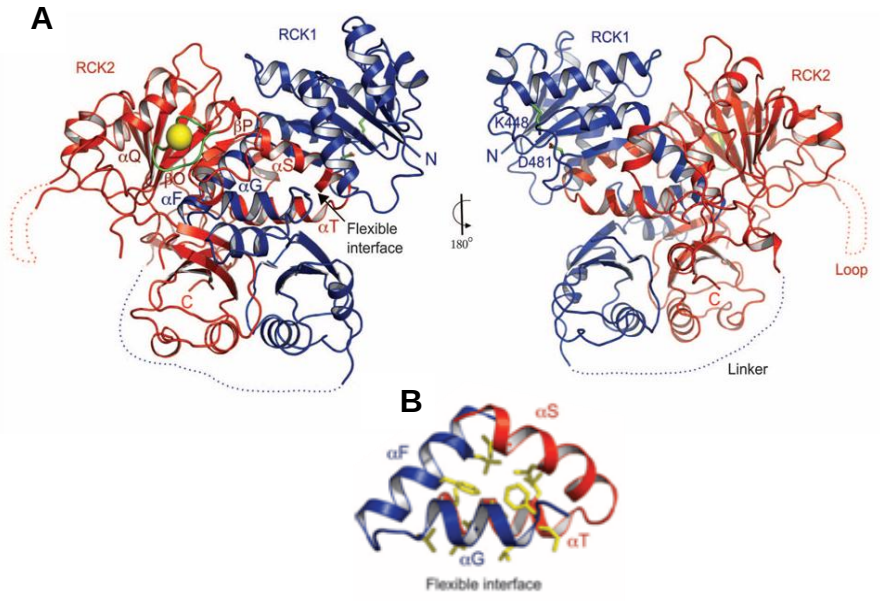


Figure 7. Structure of the BK CTD. (A) Ribbon representations at front and back views of the BK CTD showing RCK1 in blue and RCK2 in red. The Ca^{2+} ion is shown as a yellow sphere. Large disordered segments are indicated as dashed lines. **(B)** Close-up view of the αF-αG/αS-αT flexible interface. Side chains of hydrophobic residues in the interface are shown as sticks (extracted from[55]).

The Ca^{2+} bowl is coordinated in the RCK2 domain to bind Ca^{2+} by the interaction of the side chains from Asp895 and Asp897 and the carbonyl chain of Glu889 and Asp892, being the Asp894 also involved (although it is not in direct contact with Ca^{2+}, it forms salt bridges for the stabilization of the "Ca^{2+} bowl"). Nevertheless, in this structure it was not identified the second Ca^{2+} interaction site[55] **(Figure 8)**.

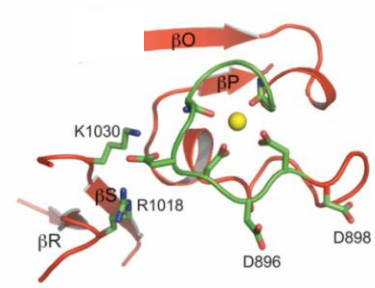


Figure 8. The Ca^{2+} bowl. Structure of the Ca^{2+} bowl, showing key residues coordinating the Ca^{2+} ion (yellow sphere) (extracted from[55]).

The model of tetramerization of the four C-terminal tails of every channel α subunit are consistent with the one identified in the "gating ring" of the MthK channel[74] **(Figure 9)**, supporting the idea of gating rings in the BK channels with slight differences[16,75].

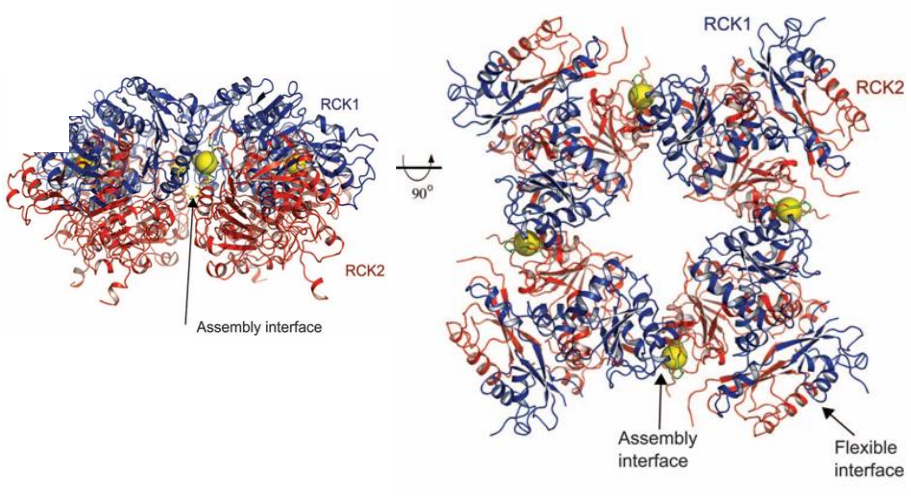


Figure 9. The BK gating ring solution. Orthogonal views of the tetrameric gating ring structure using 6.0 Å diffraction data from the homologous chicken Slo2.2 CTD. (Right) View is down the fourfold symmetry axis, with RCK1 in blue and RCK2 in red. The Ca^{2+} ions are shown as yellow spheres. The flexible and assembly interfaces are labeled, and a close-up view of the assembly interface is also shown (extracted from[55]).

Mutations in the gating ring affect channel function. Accordingly, a group of residues in the surface of this region, forming part of the RCK1 domain, are facing the VSD of the channel at a very close distance[55] **(Figure 10)**.

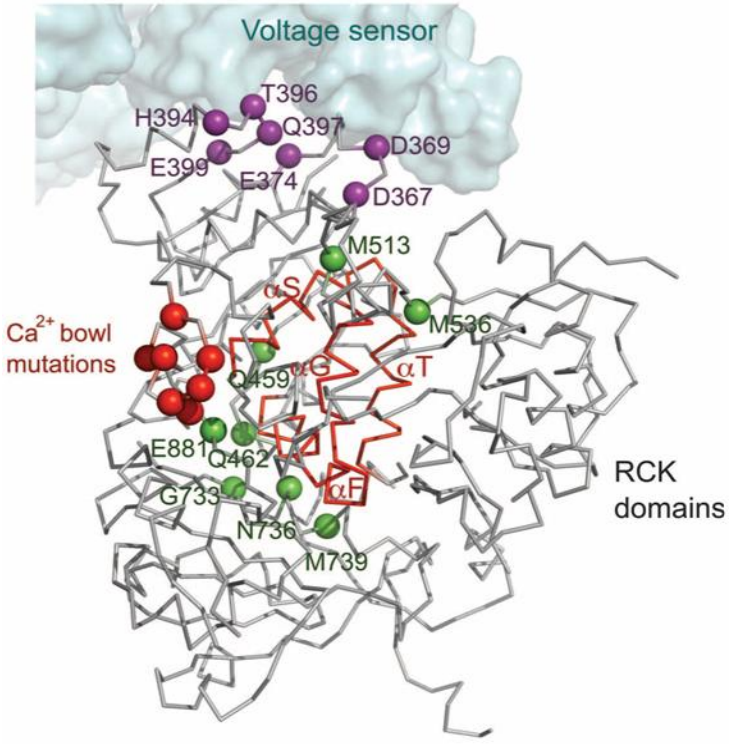


Figura 10. The gating ring and the voltage sensor modules. Sensitive functional mutations from the CTD (colored spheres) were mapped onto a single subunit from the BK model. Three distinct groups of mutations are distinguished by using the following color code: Ca^{2+} bowl mutations are colored in red; mutations near the αF-αG/αS-αT flexible interface (backbone highlighted in red) are colored in green; and mutations in the RCK1 domain facing the voltage sensor domain are colored in purple. The voltage sensor domain is shown as a surface representation (extracted from[55]).

Interactions between the channel and Ca^{2+} can be explained also by an allosteric model[76,77]. The coupling between voltage sensor movements and channel openings in the BK channel is particularly suitable for Ca^{2+}.

In the model of Horrigan and Aldrich, **(Figure 11)** they proposed that, rather than there being just a single voltage-dependent step between open and closed states, voltage sensors in each subunit move rapidly in response to changes in the membrane voltage regardless of whether the channel is open or closed, and that this movement, in an allosteric manner favours, but not requires channel opening[35,78].

This idea fits with the assumption of a weak voltage dependence associated with the central opening conformational change represented by a 10 states model **(Figure 11B)**. Horizontal transitions represent voltage sensor movement with an X indicating a voltage sensor in its active conformation, and vertical transitions represent channel opening. The model proposed in **Figure 11C** can account for

macroscopic gating and ion currents in the essential absence of Ca^{2+}, and they proposed that this scheme, coupled with the allosteric mechanism by which Ca^{2+} affects channel gating in the previous 10 states model **(Figure 11A)** may also account for BK gating at higher Ca^{2+} concentrations. The model proposed in **Figure 11C** is based in the idea that both Ca^{2+} and voltage allosterically influence a central closed-to-open conformational change. The large number of states arises naturally as a property of gating systems regulated by the two stimuli[79]. Despite its many states, the equilibrium behaviour of the model presented in **Figure 11C** is governed only by 7 parameters:

L(0) the open-to-closed equilibrium constant when no voltage sensors are active and no Ca^{2+} binding sites are occupied.

Q, the gating charge associated with this equilibrium.

Vh_C, the voltage at which a single voltage sensor is half the time active when the channel is closed.

Vh_O, the voltage at which a single voltage sensor is half the time active when the channel is open.

***Z*,** the equivalent gating charge associated with each voltage sensor's movement.

***K_C*,** the open Ca^{2+} dissociation constant.

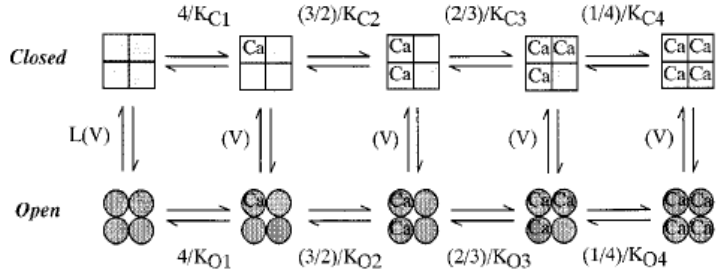
K_O, the closed Ca^{2+} dissociation constant.

P_{open} from the model in **Figure 11C** is given by the following function of Ca^{2+} concentration and voltage[79]:

Equation 2

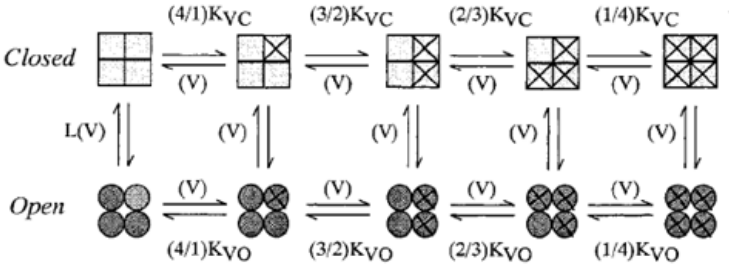
$$P_{open} = \frac{1}{1 + \left\{\frac{(1+[Ca]/K_C)}{(1+[Ca]/K_O)}\right\}^4 \left[\frac{(1+e^{ZF(V-Vh_c)/RT})}{(1+e^{ZF(V-Vh_o)/RT})}\right]^4 L(0)e^{\frac{-QFV}{RT}}}.$$

A



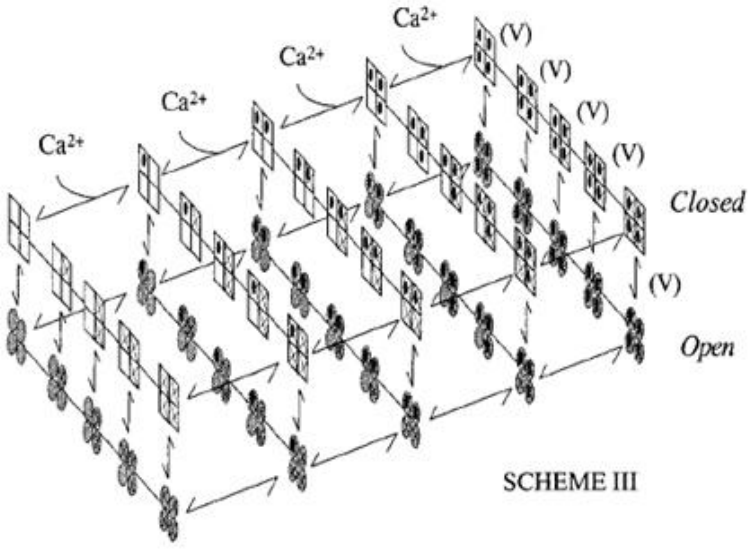
SCHEME I

B



SCHEME II

C



SCHEME III

Figure 11. (A) Scheme I, two-tiered gating scheme. Those states in the top tier are designated closed. Those in the bottom tier are designated open. The central conformational change is voltage dependent with gating charge *Q*, and equilibrium constant *L*(0)=[closed]/[open]. K_{C1}, K_{C2}, K_{C3}, and K_{C4} represent Ca^{2+} dissociation constants in the closed conformation. K_{O1}, K_{O2}, K_{O3}, and K_{O4} represent Ca^{2+} dissociation constants in the open conformation. When K_{C1}= K_{C2} = K_{C3} = K_{C4} and K_{O1} = K_{O2} = K_{O3} = K_{O4}, Scheme I represents a voltage-dependent version of the Monod-Wyman-Changeux model of allosteric proteins (for more details see[80]). **(B) Scheme II**, allosteric model. Horizontal transitions represent voltage sensor motion in each of four subunits with K_{VC} and K_{VO} representing the forward microscopic equilibrium constants for these transitions for the closed and open channel, respectively. Vertical transitions represent the conformation change by which the channel opens. All transitions are hypothesized to be voltage dependent. **(C) Scheme III**, Allosteric model to account for both the Ca^{2+}- and voltage-dependent properties of *mSlo* gating. This model represents the simplest combination of Schemes I and II. Transitions along the long horizontal axis represent Ca^{2+} binding and unbinding. Transitions along the short horizontal axis represent voltage sensor movement. Transitions from top to bottom represent channel opening. Implicit in this scheme and **Equation 2** are the assumptions that voltage sensors and Ca^{2+} binding sites in each subunit are identical and act independently, and that voltage-sensor movement does not directly influence Ca^{2+} binding, and vice versa (extracted from[79]).

At voltages where the voltage sensors are in the resting state, an increase of intracellular Ca^{2+} concentration enhances P_o of the channel[36,81,82], meaning that Ca^{2+} binding can open the channel without voltage sensor activation. Ca^{2+} can bind to both the closed and the open states, but with a higher affinity

when the channel is open. Therefore Ca^{2+} binding shifts the equilibrium between the closed and open states towards the open. Thus, voltage and Ca^{2+} together promote BK channel opening and because both, voltage sensor activation and Ca^{2+} binding are favoured when the channel is open, Ca^{2+} binding shifts the voltage dependence of the channel opening towards less positive voltages, while depolarization enhances the apparent affinity and cooperativity of Ca^{2+} binding[83].

Therefore, voltage sensor movements are modulated by Ca^{2+} and vice versa. One affects the other directly through an allosteric connection via channel opening[36,81]. This could probably occur by an interaction between the VSD and the C-terminal domain (Ca^{2+}-binding sites). This dual allosteric mechanism reproduce the steady-state behaviour of mSlo1 over a wide range of conditions with the assumption that activation of individual Ca^{2+} sensors or voltage sensors additively affect the energy of the closed to open (C-O) transition and that a weak interaction between Ca^{2+} sensors and voltage sensors occurs independently of channel opening[36].

1.2.2. Mg^{2+} interaction site and channel regulation.

Physiological concentrations of Mg^{2+} can modulate the BK channel function in an interesting way. Mg^{2+} exerts a dual

effect on the BK channel. In the one hand, Mg^{2+} can "block" BK channels in a voltage-dependent manner by reducing the single-channel conductance[63]. Although the mechanism is not clear, it has been suggested a possible binding of Mg^{2+} inside the pore[84-87]. In the other hand, Mg^{2+} increases the P_o of the channel[61] and shifts the G/V curves of the channel to more depolarizing voltages, producing a similar $V_{1/2}$ shift either in the presence or in the absence of Ca^{2+} (meaning an activation of the channel independent of cytosolic Ca^{2+} levels) **(Figure 12)**.

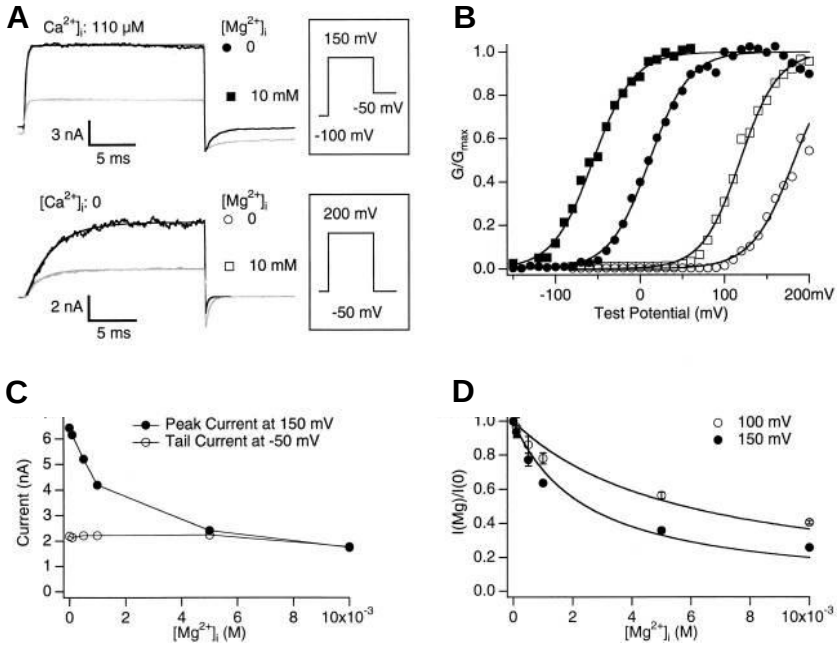


Figure 12. Intracellular Mg2+ blocks and activates mSlo1 channels. **(A)** mSlo1 currents recorded from an inside-out patch with 0 (dark traces) or 10 mM (light traces) $[Mg^{2+}]_i$ at $[Ca^{2+}]_i$ of 0 (bottom) and 110 µM (top). The

voltage protocols are schematically displayed next to the current traces. Smooth lines are exponential fits to current traces. **(B)** G-V relations of mSlo1 channels with 0 (circles) or 10 mM (squares) $[Mg^{2+}]_i$ at $[Ca^{2+}]_i$ of 0 (open symbols) and 110 μM (closed symbols). Corresponding symbols are also shown in **(A)**. The smooth lines are fits with the Boltzmann function (**Equation 1** of this Introduction). **(C)** The response to $[Mg^{2+}]_i$ of the peak current and the instantaneous tail current at the specified voltages. **(D)** Mg^{2+} block of the peak current. The ratio of the current with internal Mg^{2+} to that without internal Mg^{2+} (for more details see[63]).

Mg^{2+} binds preferentially to the open state, since it does not affect the activation kinetics but slows down the deactivation kinetics. Also, it appears to enhance the transition from closed-to-open states of the channel[63,64] (reviewed in[66]).

As mentioned before, in the C-terminus tail of the BK channel there are low-affinity (milimolar range) interaction sites for divalent cations such as Ca^{2+}, Mg^{2+} and others. Since this site binds Mg^{2+} at its physiological range, it is called the "Mg^{2+}-binding site"[61-64]. This site is unique due to its configuration. It combines residues from the VSD (Asp99 and Asn172)[88] but also from the C-terminal tail (Glu374 and Glu399)[71,89] **(Figure 13)**. This shows functionally the close proximity between the VSD and the RCK domain (in the C terminus) of two adjacent subunits, as shown in the electron microscopy solved solution[16] but also in the X-ray crystallography structure of the C terminal domain[55] (reviewed in[25]) **(Figure 10** of this Introduction**)**.

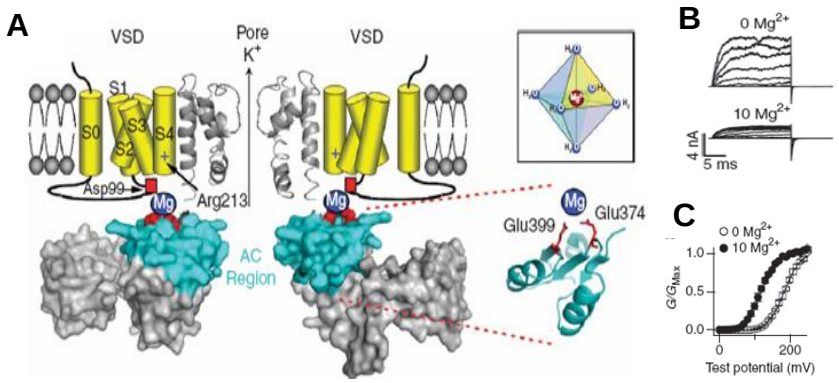


Figure 13. Mg^{2+} coordinates in the cytoplasmic domain of the mouse Slo1 channel. **(A)** BK channel model. The pore domain and the cytoplasmic RCK1 domain are based on the MthK crystal structure. Transmembrane S0-S4 segments are depicted as cartoons. Only two opposite subunits are shown for clarity. Two putative Mg^{2+} binding residues (Glu374 and Glu399, red spheres) are located in the AC region (cyan). Inset, the Mg^{2+} binding site is predominantly formed by six oxygen-containing ligands with an octahedral geometry. **(B)** Representative macroscopic current traces and **(C)** mean G-V relationship for WT channels in 0mM and 10mM $[Mg^{2+}]_i$. The smooth curves in **(C)** represent Boltzmann fits (**Equation 1** of this Introduction) (for more details see[88]).

The Mg^{2+} interaction with the VSD of the channel has an electrostatic nature. There are many evidences supporting this. The first experiments linking the VSD with the Mg^{2+}-dependent activation of BK channels were single mutations of Arg213, which abolished the Mg^{2+}-induced activation of BK channels[90]. Since it is the only charge residue in S4 contributing to gating charge[26], these results suggest that Mg^{2+}

might alter the voltage sensor movements by an electrostatic interaction. Other experiments based in ionic strength of the intracellular solution show that Mg^{2+}-dependent activation diminishes with the increasing ionic strength[91]. Also conserving the electrostatic environment close to the Mg^{2+} coordination site is important for the interaction. Thus Glu399[71,89], is important for the Mg^{2+} electrostatic interaction with Arg213 because both charges can interact [91].

The electrostatic repulsion between Mg^{2+} and Arg213 in S4 slows down the return of the voltage sensor from the activated to the resting state. Gating currents studies show that Mg^{2+} has little effect on the “on-gating” currents associated with the movement of the voltage sensor from the resting to the active state in response to depolarizing pulses, but, it prolongs and reduces the amplitude of the “off-gating” currents, which reflects the return of the voltage sensor from the activated to the resting state again[91] **(Figure 14)**. In BK channels, the voltage sensor is allosterically coupled with the activation gate and voltage sensor movements can be detected when the gate is either closed or open[35]. This effect of slowing down the off-gating currents is bigger when the channel is open rather than when the channel is closed, suggesting that channel opening enhances the interaction between Mg^{2+} and the VSD, slowing its deactivation (reviewed in[25]).

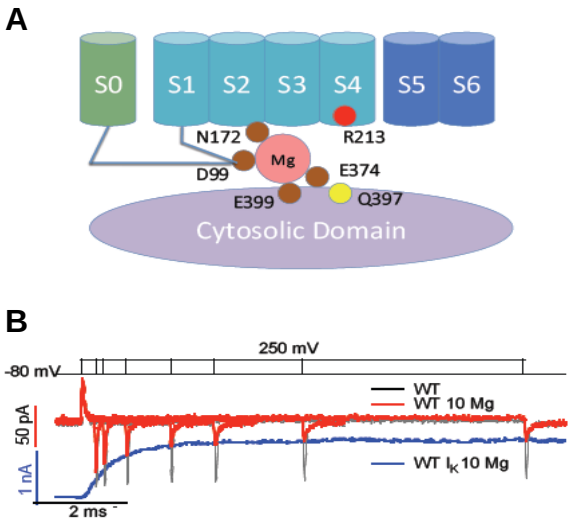


Figure 14. Mg^{2+} modulation of the voltage sensor function in BK channels. (A) Cartoon showing amino acid residues involved in Mg^{2+} binding and interaction with the voltage sensor domain. **(B)** Gating currents in the absence and presence of 10 mM Mg^{2+} (black and red traces) in response to various lengths of voltage pulses from -80 mV to +250 mV (top). The ionic current (blue trace) in response to a similar depolarizing pulse in 10 mM Mg^{2+} is aligned with gating currents to show the correlation in time courses between channel opening and the decay in off-gating current amplitude in the presence of Mg^{2+} (adapted from[91]) (for more details see[25]).

In summary, to give a general view for the Ca^{2+}- and Mg^{2+}-interaction sites and the interactions between the structural domains of the α subunit of the channel, it is very illustrative the homology model (reviewed in[92]) built based on the

available crystal structures of Kv1.2, Kv2.1 and MthK channels, the crystal structure of the C- terminal tail of the BK channel, and sequence alignments **(Figura 15)**.

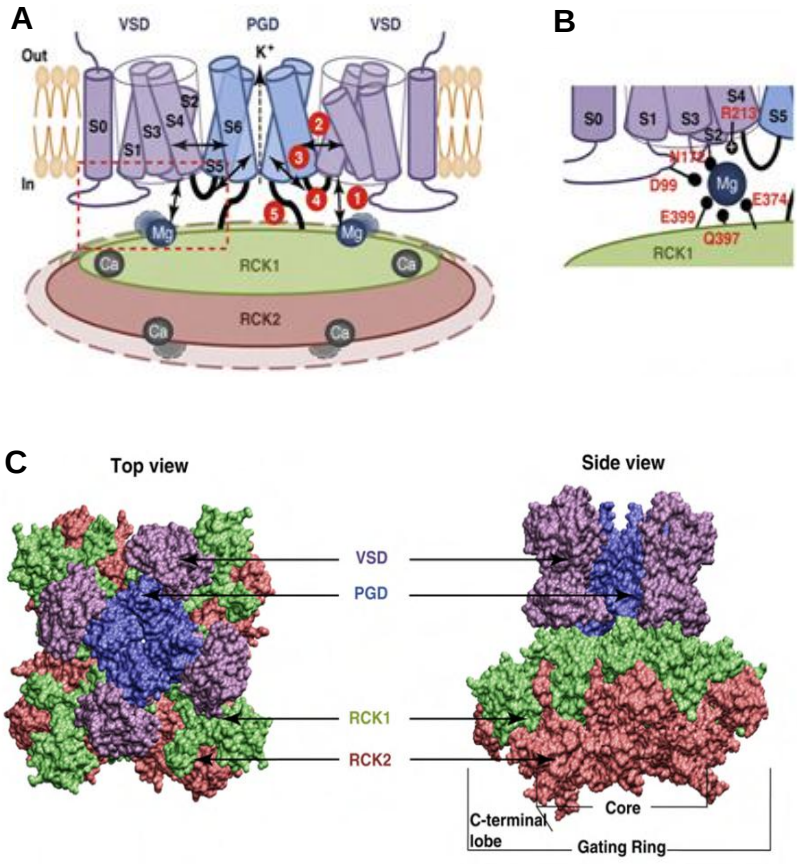


Figure 15. Interactions between structural domains in BK channels. **(A)** Sketch of the BK channel. Mg^{2+} and Ca^{2+} show metal binding sites. Black arrows and heavy set lines are used to indicate the following interactions: (1) between VSD and RCK1 in Mg^{2+} dependent activation; (2) between VSD and PGD through S4 and S5; (3) between S4–S5 linker and S6; (4) the tug of the S4–S5 linker; and (5) between PGD and cytosolic domain through the peptide C-linker. The gating ring formed by RCK1 and

RCK2 is shown to undergo expansion during channel gating similar to the MthK channel from *Methanobacterium thermoautotrophicum*. The structure in the dashed box is shown in more detail in **(B)**. **(B)** Sketch showing residues involved in Mg^{2+} dependent activation. **(C)** BK channel structure. Similar color scheme as in **(A)** and **(B)** is used to identify different structural domains except for S0, which has the same color as the VSD (for more details see[92]).

In addition to Mg^{2+} and Ca^{2+}, other divalent cations have been shown to bind and functionally interact with the BK channel. After Ca^{2+}, the most effective divalent cations were Cd^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+}. They were able to enhance the activation and increase the Hill coefficient of BK channels already activated by Ca^{2+} [62]. These other divalent cations bind to the "low affinity" interaction site, in the RCK1 domain and in close distance to the VSD. Punctual mutation studies point to residues Asp362 and Asp367 as the ones involved in the interaction with the largest cations, such as Ca^{2+}, Cd^{2+} and also Sr^{2+} (which is without effect on channel function)[62]. Residues Glu374 and Glu399 seem to contribute to the interaction site for the smallest cations (Ni^{2+}, Co^{2+} and Mn^{2+}). Interestingly they all show a voltage-dependent blockade of the pore as it has been shown before for Mg^{2+} [72]. Moreover, it has been recently described that Zn^{2+} can also activate BK channels sharing kinetic properties with Mg^{2+}. It also binds close to the environment of Glu399 (in the His365), and unless is also presenting a voltage-dependent block of the pore[62], its

binding is not affecting the activation kinetics but it slows the deactivation kinetic of channel opening[93].

Finally, another divalent cation with a modulatory effect over BK channel activity is barium (Ba^{2+}), which acts as a pore blocker of the channel[94-98]. Recently, it has been shown that Ba^{2+} can activate selectively the BK channel by binding to the "Ca^{2+} bowl", an effect that arises at negative potentials, without the confounding influence of the Ba^{2+}-induced blockade observed during depolarizations[99].

2. Channel phosphorylation by kinases: tuning BK channel function.

Not only changes in the intracellular Ca^{2+} concentration or membrane potential result in modulation of BK channel activity, there is evidence of changes in the channel activity due to phosphorylation/dephosphorylation by protein kinases/phosphatases (such as PKA, PKC, PKG and phosphatase 1)[56,100-102], cyclic nucleotides (cAMP or cGMP), G proteins and G-proteins coupled receptors[103-107] or endothelium–derivate vasoactive substances (reviewed in[2]).

In smooth muscle cells, PKA and PKG generally increase BK channel open probability (by shifting the voltage-dependent activation to more depolarized voltages, without affecting the channel conductance), whereas PKC has an inhibitory

effect[101]. The phosphorylation produced by both PKA in Ser922 and PKG in Ser1134 can be inhibited by pretreatment with phosphatases. BK channel splice variants containing these residues or not, present distinct regulation by the different kinases. The role of PKC seems to modify the PKA and PKG interaction with BK[56]. In most studies, cAMP and cGMP at physiological concentrations are without direct effect on BK channel function, although these cyclic nucleotides can influence channel activity following the activation of the corresponding protein kinase[101]. In this respect, it has been shown that, in guinea-pig aorta, increased levels of cAMP by Prostaglandin-2 (PGI-2), or its analogue Beraprost, activate BK channels to promote the relaxation of pre-contracted arteries. In such regulatory process induced by PGI-2, a cAMP-independent component has been also described, which seems to involve the direct action of the stimulatory G protein of the adenylyl cyclase (G_s) on the BK channel[108].

Cofactors such as cAMP, cGMP or MgATP need the active kinase and the phosphorylation sites available in the BK channel for the correct phosphorylation of the channel[101]. ATP in contrast has been reported to inhibit BK channels, however this result was attributed to a chelator effect over the cytosolic Ca^{2+} [109]. The inhibitory role of PKC depends on the unconditional and conditional phosphorylation of the C-terminal residues Ser1151 and Ser695, respectively. The

inhibition of BK channel conductance by PKC is due to a decrease in the channel P_o, without changes in the unitary current amplitude, the voltage dependence or the Ca^{2+} sensitivity of the BK channel. Indeed, the phosphorylation of the BK channel by PKC abolishes the stimulatory effects of PKA and PKG. BK channel mutant at Ser1151 was insensitive to both PKC and PKG induced-modulation. It was instead activated by PKA[56]. Therefore, phosphorylation and dephosphorylation of Ser1151 determines channel ability to respond to either PKA or PKG. The phosphorylation of the Ser695 depends on the preceding phosphorylation of Ser1151 or the presence of a negative charged aminoacid at position 695[102]. Balance between phosphorylation by PKC and dephosphorylation by phosphatase1 may vary in different cell types and may therefore explain the divergent results reported in the literature on BK channel regulation by PKG and PKA[102].

In other tissues such as rabbit distal colon epithelium, where BK channels play an important role in K^+ and Na^+ homeostasis, they present an increased Ca^{2+}-sensitivity due to PKA activation, which may be promoted by aldosterone simulation and the subsequent rises of cAMP levels[100,110]. Recently in experiments performed in hair cells from chickens, it has been shown that an increase in PKC phosphorylation rate enhances (by indirect mechanisms) the BK α subunit expression in the hair cell surface[111].

3. Modulation by endogenous signalling molecules.

Acute modulation of BK channels expands their functional repertoires allowing the channels to contribute to a multitude of physiological and pathophysiological processes. Besides the regulatory processes mentioned above, another molecules, which I will shortly describe below, are also involved in BK channel modulation **(Figure 16)**.

Hormons including estrogens[112], testosterone[113] and glucocorticoids[114] regulate BK channel activity through both, genomic (by regulating the expression levels and alternative splicing)[22] and non genomic pathways[115]. Regarding the later, acute activation of BK channels seems to require the presence of regulatory β subunits. It has been suggested that strogen and xenoestrogens (in the μM range) activate BK channels through direct binding to an external site available in the regulatory β_1 subunit. Still, the exact site of interaction remains to be elucidated. 17β-estradiol and tamoxifen seems to increase the activity of BK$\alpha\beta_1$ channels by direct interaction with the β extracellular loop[112,116]. Besides, 17β-estradiol is also a potent activator of BK channels containing either β_2 or β_4 subunits[117]. Finally, lithocholate (a cholane-derived steroid) specifically increase the activity of BK channels containing the β_1 subunit, although in this case the interaction site was

suggested to comprise polar residues at the β_1 transmembrane segments[118].

Phospholipids also regulate BK channels. It has been described that phosphatidylinositol 4,5-biphosphate (PIP_2) increases the P_o of BK channels by direct binding in presence of β_1 subunit[119]. Arachidonic acid (AA) has been shown to increase BK channel P_o in the presence of β_2 or β_3, but not β_4 subunits[120]. AA metabolites such as epoxyeicosatrienoic (EET) and dihydroxyeicosatrieonic acids, also modulates BK channels (reviewed in[121]).

Reactive oxygen species and nitrogen species has been reported to produce a myriad of effects on BK channels. They are capable of modifiying multiple amino acid residues, including cysteine (Cys), methionine (Met), histidine (His), tryptophan (Trp) and tyrosine (Tyr). Furthermore some oxidation reactions are critically dependent on multivalent cations such as Fe^{2+}/Fe^{3+}. H_2O_2 reactive species decrease P_o of BK channels as it happens for O_2^- and peroxynitrite. For example oxidation of Met by the addition of an oxygen atom changes the nature of the amino acid and therefore it has functional effects on the channel (reviewed in[121]).

Carbon monoxide (CO), as nitric oxide (NO), binds to the heme iron center of soluble guanilyl cyclase and increases its activity leading to PKG activation and the subsequent activation of the BK channels[122]. Besides, CO by itself

enhances BK channel P_o even at saturating concentrations of Ca^{2+} (reviewed in[121]).

Heme group, independently of the redox status of their iron center, can regulate BK channel activity by binding to the linker region between the cytoplasmic RCK1 and RCK2 in the CKACH sequence[123]. Such interaction results either in an increase or a decrease in BK channel P_o at negative or positive voltages, respectively (reviewed in[121]). Yet, the relevance of this modulatory pathway is unclear.

Finally, the regulation of BK channel by intracellular protons (H^+) is very controversial. Contradictory results have been published, ranging from a robust channel activation[124] to a decrease in the ion currents through BK channels in native tissue[125] (reviewed in[121]).

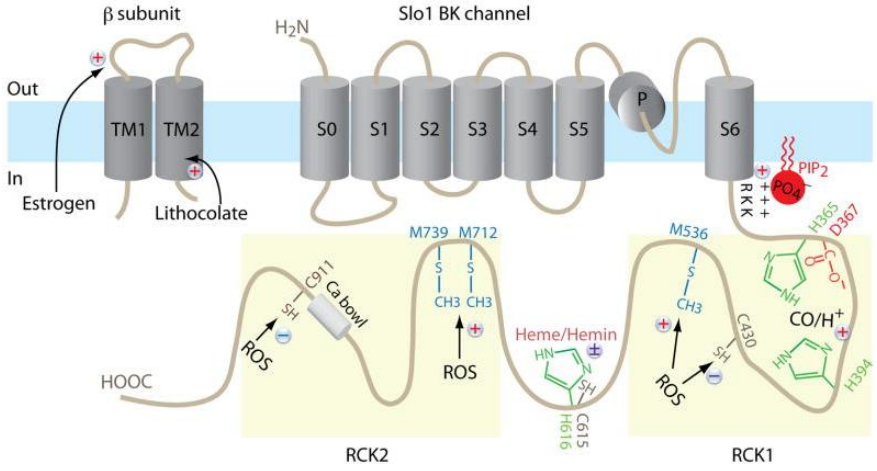


Figure 16. Schematic diagram showing the regions and the key amino acid residues involved in modulation of BK_{Ca} channel by intracellular

messengers including estrogens, cholane-derived steroids, H^+, heme/hemin, CO, ROS, and PIP_2. Stimulatory modulators are indicated by "+", inhibitory modulators are indicated by "–" and mixed-effect modulators are indicated by "±". The residue numbers are according to NP 002238 (extracted from[121]).

4. The great partners: regulatory "β" subunits.

Depending on the tissue, BK α subunits associates with different auxiliary β subunits (β_1-β_4) **(Table 1)**, modifying BK subcellular distribution, gating properties and pharmacological features. In fact, β subunits constitute the main source for the functional diversity of BK channels, beyond the existence of alternative splice variants or the above described modulatory mechanisms[126]. Altogether, they finely tune BK channels to perform their physiological functions.

Table 1. Summary of principal functions and tissue expression of BK β subunits.

β-Subunit	Gene	Binds to	Tissue expression	Functions
$BK_{Ca}\beta1$	KCNMB1	$BK_{Ca}\alpha$	Smooth muscle, trachea, aorta coronary	Increases Ca^{2+} sensitivity in Ca^{2+} >300 nM; decreases voltage dependence; high affinity by ChTx, but IbTx sensitivity is reduced
$BK_{Ca}\beta2$	KCNMB2	$BK_{Ca}\alpha$	Chromaffin cells, ovary, and brain	Confers inactivation; increases Ca^{2+} sensitivity; low ChTx affinity
$BK_{Ca}\beta3$	KCNMB3	$BK_{Ca}\alpha$	Testis, pancreas, and spleen	Confers inactivation; induces current rectification; speeds activation kinetics
$BK_{Ca}\beta4$	KCNMB4	$BK_{Ca}\alpha$	Brain	No effect in ChTx/IbTx sensitivities Decreases ChTx and IbTx binding; in low $[Ca^{2+}]$, decreases Ca^{2+} sensitivity; in high Ca^{2+}, increases Ca^{2+} sensitivity

(Adapted from[126])

4.1. The β_1 subunit.

When the first beta subunit was cloned, it was described as a membrane protein with two transmembrane domains connected by an extracellular loop, with the N-terminus (NH_2) and the C-terminus (COOH) oriented towards the cytoplasm[6]. The other β subunits cloned and described later, all share these topological properties (reviewed in[2]) (see **Figure 19B** for an schematic cartoon). A functional BK channel is thought to result from the assembly of four α and four β subunits in a 1:1 stoichiometry. Nevertheless, functional BK channels with less than four β subunits are also feasible. Thus, it has been described that, in heterologous expression systems, voltage dependence of channel gating and inactivation properties can be proportionally adjusted according to the fractional occupancy of each α subunit with one β_1 or β_2 subunit[127].

β_1 subunit is very abundant in smooth muscle like small intestine, colon, uterus, aorta and coronary artery (which are also tissues with higher α subunit mRNA expression[128]), but scarce in lymphatic tissues brain and liver. Consistent with this tissue-specificity the expression of the gene encoding for the β_1 subunit (*KCNMB1*) is under the regulation of muscle-specific enhanced factors-1 and -2[129].

The first β subunit identified was described in vascular smooth muscle cells and it was called β subunit until 1999, when other members of this family were described. Then, it was renamed as β_1. Co-expression of β_1 subunit with the pore-forming α subunit confers an apparent high Ca^{2+} sensitivity to the BK channel, meaning a shift towards more negative potentials of the P_o-V curves potentiated at higher concentrations of Ca^{2+} (>1μM) **(Figure 17)**.

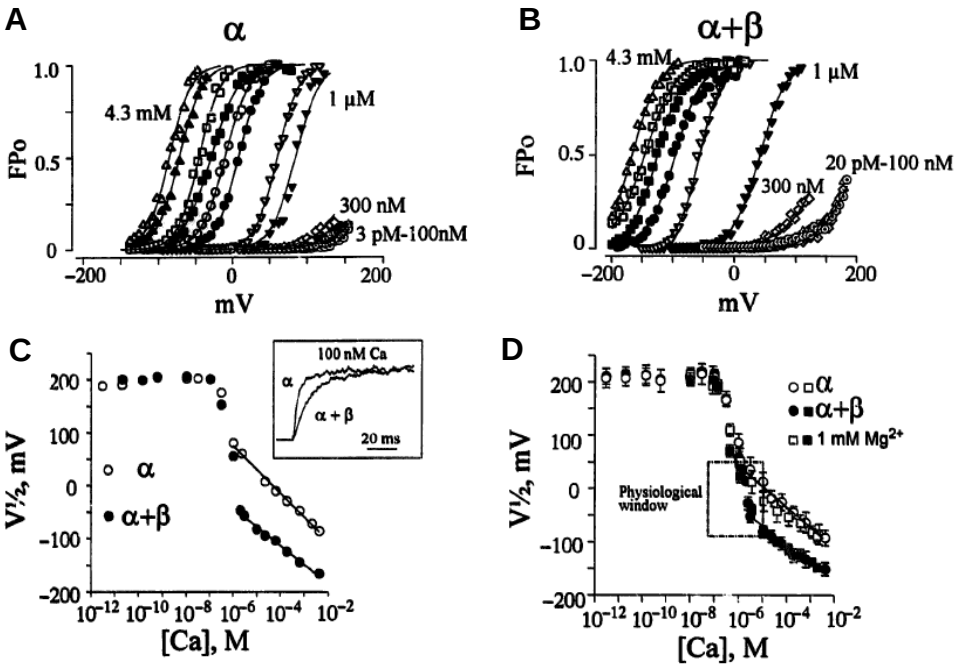


Figure 17. Ca^{2+}-dependent shift of P_o-V curves for BK channels in the absence or presence of the regulatory β_1 subunit. FP_o as a function of voltage at different $[Ca^{2+}]_i$ (from 3 pM to 4.3 mM) in single inside-out

patches containing either BKα channels **(A)** or BK$\alpha\beta_1$ channels **(B)**. Continuous lines are the best fit to Boltzmann distributions **(Equation 1)**. **(C)** $V_{1/2}$ vs. $[Ca^{2+}]_i$ plot from **(A)** and **(B)**. The inset shows normalized currents to illustrate the slower activation kinetics of BK channes in the presence of β_1. **(D)** Mean $V_{1/2}$ values vs. $[Ca^{2+}]_i$ (for more details see[59]).

Although the presence of the β_1 subunit has been reported to produce a small increase in the real affinity of the α subunit for Ca^{2+} binding[79,130], the apparent increase in Ca^{2+} sensitivity upon co-expressing $\alpha+\beta_1$ BK channel subunits is mainly due to the stabilization of the voltage sensor in its active conformation (either in the presence or in the absence of cytosolic Ca^{2+}) that facilitates the transition from the closed to the open state[131]. When Ca^{2+} is present, BK channel gating is enhanced because the work that Ca^{2+} binding must do to open the channel is decreased at all voltages[130], in agreement with the allosteric model of interaction created by Cox and Aldrich **(Figure 18)**.

Even though β_1 enhances the apparent Ca^{2+} sensitivity of BK channel[59], the coupling between α and β_1 did not require the Ca^{2+} presence. Thus, BK channel P_o is increased due exclusively to the presence of the β_1 subunit and independent of cytosolic Ca^{2+}[132].

Besides the changes in the voltage/Ca^{2+} sensitivity of the α subunit, the β_1 subunit also alters the gating kinetics of the channel[133,134] and its pharmacological properties (see below). Indeed, the presence of the β_1 slows down BK channel activation and deactivation kinetics, an effect probably related to the transition among closed and open states of the channel and not related to an action on the voltage sensor itself, as not changes in the kinetics of gating currents are produced by the regulatory subunit[131].

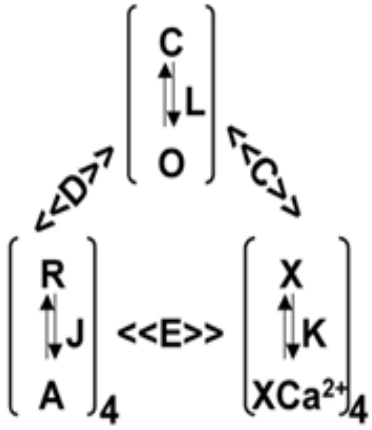


Figure 18. Activation model for BK channel. The C-O transition corresponds to the closed-open equilibrium. The R-A transition corresponds to the resting-active equilibrium of a single voltage sensor. The X·Ca^{2+} transition is Ca^{2+} binding to a single Ca^{2+} sensor, with equilibrium constant $K=[Ca^{2+}]/K_d$. These three equilibriums are related to each other by the allosteric factors *C*, *D*, and *E*. When there are *n* voltage sensors active, the C-O equilibrium constant is LD^n. Conversely, when the channel is open, the R-A equilibrium constant is *JD*. The same applies for the allosteric factors *C* and *E* (for more details see[66]).

It has been proposed that the extracellular N-terminus of the BK channel α subunit together with the first transmembrane domain (the S0 domain) are required for the functional

interaction with the regulatory β_1 subunit[18,135]. Nevertheless, the physical association among α and β_1 subunits requires the S1, S2 and S3 transmembrane helices and their associated interhelical loops contained in the pore-forming α subunit[135]. Several structural determinants at the β subunit have been shown to play a relevant role in the functional coupling between α and β subunits. Thus, studies with β_1-β_2 chimeric subunits show the importance of the transmembrane domains, the intracellular carboxyl-terminus and mainly the cytosolic amino terminus of the β_1 and β_2 subunits in the specific modulatory actions that each of them exerts on the activity of BK channel[136]. Also, there are several evidences suggesting that the large extracellular loop connecting the two putative TM segments of the β_1 subunit is, at last in part, responsible of its regulatory action on the voltage sensor activation and gating of the BK channel, beyond its involvement in channel modulation by steroids (as explained before). In this respect, point mutations in this loop tune either up (E65K)[137] or down (Y74A, S104A, Y105A and I106A)[138] the positive β_1-induced modulation of the BK channel activity. Indeed, the single alanine substitutions of several residues placed either at the highly conserved segments A (Y74A) or B (S104A, Y105A and I106A) of the β_1 extracellular loop **(Figure 19)** destabilize open-channel voltage sensor-activation either in the absence or presence of Ca^{2+} binding to the BK channel. The role of

these residues might be explained by an indirect action, related to their association with other channel regions involved in the maintenance of the correct functional interaction between α and β_1 subunits. An alternative explanation would be the direct participation of these β_1 amino acids in the modulation of BK channel activity due to their close proximity to the external mouth and, perhaps, the selectivity filter and gate of the channel, as suggested by several authors[139-141].

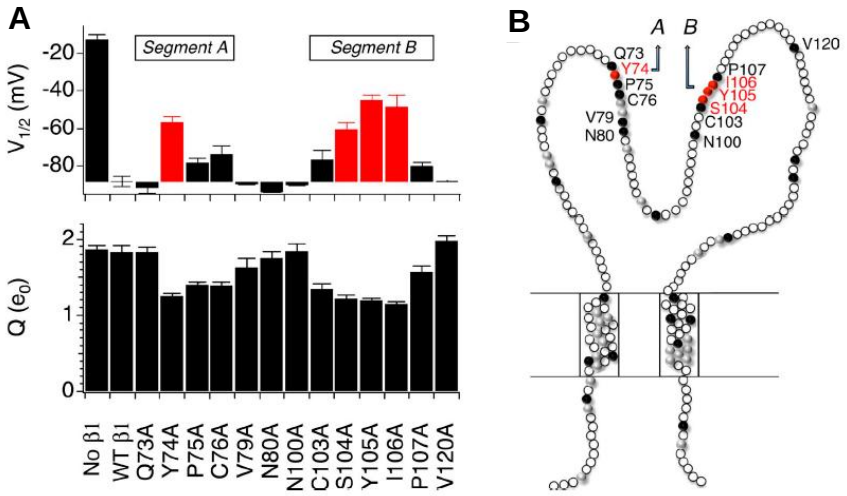


Figure 19. Steady-state effects of mutations on β_1 function. (A) Summarized steady-state effects of 13 alanine substitutions measured in 60 μM Ca^{2+}. Average $V_{1/2}$ (top) and Q (bottom). Error bars represent SEM. **(B)** Positions of key segments A and B on a schematic cartoon of β_1. Based solely on amino acid sequence, the protein has been postulated to have cytoplasmic NH, and COOH termini, two transmembrane segments, and one large extracellular domain. Residues mutated to alanine are labeled

with their respective amino acids. Mutated residues having relatively large and small effects are represented by closed red and black circles, respectively. Identical and non identical but conserved residues in other positions are represented by closed black and gray circles, respectively (for more details see[138]).

In this sense, residues placed either at the segment A (Y74A) or the segment B (S104A, Y105A and I106A) of the β_1 extracellular loop modulate not only the activation of the voltage sensor but also the intrinsic gating of the BK channel, pointing to a possible role of these particular β_1 regions in the coupling among the activated voltage sensor and the channel gate[138].

Finally, regarding the relative position of the β_1 subunit versus the pore-forming α subunit to conform a functional BK channel, it has been shown that the second β_1 transmembrane domain (TM2) is next to the S0 segment of the α subunit and the first β_1 transmembrane domain (TM1) is next to TM2, in such a way that each β_1 would lay interposed between the voltage-sensing domains of two adjacent α subunits, whit the possibility of interact with them[142] **(Figure 20)**.

The role of the β subunits is not only related with the modulation of biophysical properties of the BK channel, but

also with channel trafficking[126]. Both β_1 and β_2 subunits can reach the plasma membrane when expressed alone in HEK293 cells[143,144]. When co-expressed with the α subunit, β_1 reduces the steady-state surface expression of the pore-forming subunit by enhancing its internalization. Single-nucleotide mutation studies suggest a role of a putative endocytic signal at the β_1 C-terminus in this modulatory action[143]. Furthermore, a splice variant of the pore-forming BK α subunit (which is fully retained in the endoplasmic reticulum (ER)) shows a dominant-negative action on the surface expression of functional BK channels containing the β_1 subunit by trapped them in the ER[145].

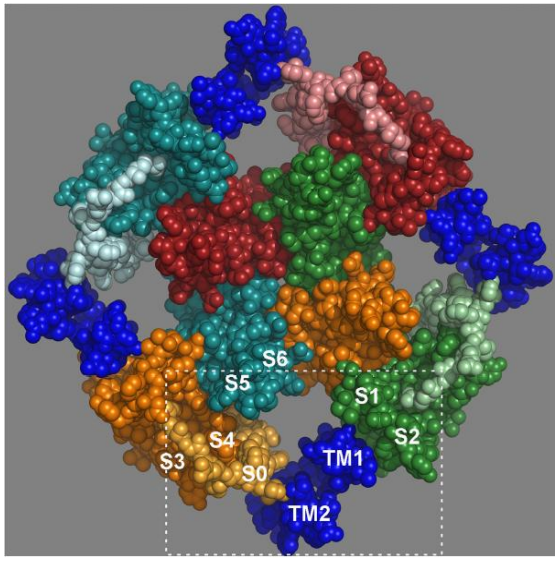


Figure 20. Model for the relative position among BK α and β_1 subunits. S1-S6 (built by homology modeling based on the crystal structure of the Kv1.2/Kv2.1 chimera) of each BK α subunit has a unique color and the color of S0 is a lighter shade of the S1-S6 color. β_1 TM1 and TM2 domains are dark blue (for more details see [142]).

To finish, it has been largely showed that the presence of the β_1 is required for some pharmacological features of BK channels. Besides its role in the already mentioned functional interaction with steroids and derived molecules, β_1 contributes to the extracellular binding site of charybdotoxin in the BK channel. Indeed, a few β_1 residues placed at the extracellular loop are determinants of the high BK channel affinity for the toxin: Leu90, Tyr91, Thr93 and Glu94[49,139]. Also, dehydrosoyasaponin-I (DSH-I, a triterpene glycoside present in the folk medicinal herb *Desmodium adscendes*, which is used in Ghana as a remedy against asthma and other diseases associated with smooth muscle dysfunction) is an effective agonist of the BK channels but only when applied to the cytosolic face of the channel containing the regulatory β_1 subunit[10,146,147].

4.2. The β_2 subunit.

The other cloned β-subunits were identified by searching homologues to the β_1-subunit in human expressed sequence tag (EST) data bases[2]. The β_2 subunit, encoded by the *KCNMB2* gene, shows 43% protein sequence identity and 53% sequence similarity when compared with β_1[148]. It is expressed preferentially in chromaffin cells, ovary and

hippocampal neurons in the brain **(Table 1)**. In these cells, it was observed a mixed current: the typical non inactivating current described previously for the BK channel, together with an inactivating current[149] **(Figure21, top right panel)**.

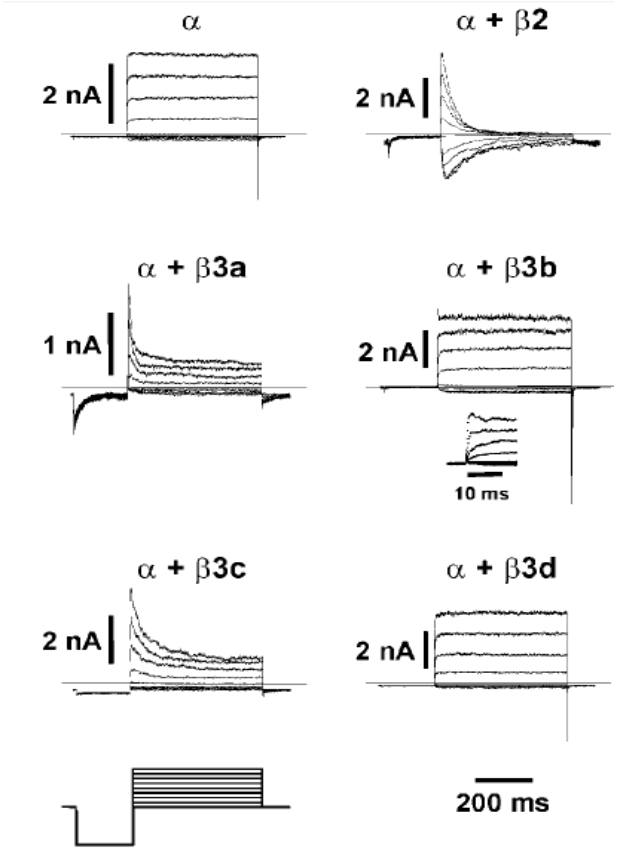


Figure 21. Differential inactivation properties conferred by β subunits to the BK channel. Current traces in response to depolarizing pulses obtained from inside-out patches expressing BK α subunit alone or with the indicated regulatory β subunit. Rapid and complete channel inactivation is only observed for channels containing the β_2 subunit (for more details see[150]).

The inactivation of the current is mediated by the cytosolic N-terminal of the β_2 regulatory subunit[148]. In comparison with the other regulatory β subunits, the β_2 subunit is distinguished by an N-terminal extension of 31 amino acids, a slightly longer extracellular loop and five additional amino acids at the C-terminus[148]. From the 31 extra amino acids described in the N-terminus of the β_2 subunit, only the first 19 aminoacids are sufficient to produce the inactivating phenotype[148]. This sequence shares the typical features of other inactivating peptides that can occlude the conduction pathway of different voltage-gated channels: it contains a hydrophobic region followed by charged residues[148].

As shown for the β_1 subunit, β_2 also increases the apparent Ca^{2+} sensitivity of the channel channels by stabilizing the voltage sensor in the active configuration (albeit to a lesser extent than β_1)[131] and leads to the slowing of current activation kinetics, an effect that becomes much more evident upon removal of inactivation by deletion of the N-terminus[148].

At the structural level, based in co-immunoprecipitation studies and transmembrane protein-protein interaction assays, it has been proposed that the first transmembrane domain of the β_2 subunit binds to the S1 transmembrane segment of the pore forming α subunit, without the involvement in the interaction

among the two subunits of neither the extracellular loop nor the second transmembrane domain of β_2[151].

The pharmacology of the BK channel in presence of the β_2 subunit is altered. Compared to BK channels composed only by the α subunit, channels containing also β_2 show a lower ChTX sensitivity and increased sensitivity to both DHS-I (in the nM range as found for BK$\alpha\beta_1$ channels)[148] and steroid hormones[117].

4.3. The β_3 subunit.

The β_3 subunit, detected in testis, pancreas and spleen[152] **(Table 1)**, is evolutionary more related to β_2 than β_1[150]. This subunit presents four splice variants (β_{3a-d}) which only differs in their cytosolic N-terminal region. They confers inactivation properties to the BK channel in different ways: β_{3a} and β_{3c} variants similarly induce strong but partial inactivation of the channel, whereas in the presence of the β_{3b} subunit BK current inactivation is faster, very small and only evident at large depolarization. Whether the β_{3d} variant interacts with the α subunit is unclear, since coexpression of both subunits together does not alter Ca^{2+} activation curves or gating kinetics of the BK channel[150] **(Figure 21)**. In the absence of

inactivation, β_3 subunits have been reported to slightly accelerate channel activation kinetics at all Ca^{2+} concentrations[133,150].

Unlike the β_1 or β_2 subunits, none of the β_3 splice variants increases the apparent volage/Ca^{2+} sensitivity of the BK channel[133,150]. In fact, the opposite effect (a significant shift in channel activation to more depolarized voltages at high (>10 μM) cytosolic Ca^{2+} levels) was observed in the presence of either β_{3a} or β_{3c} subunits[150]. More recently, measurements of BK gating currents in the presence of the β_{3b} variant reveal no effects of this subunit on voltage sensor equilibrium[131]. All these results are in conflict with the ones published by Xia et al. (2000) suggesting that β_3 indeed favours BK channel gating at high cytosolic Ca^{2+}, but in particular increases the sensitivity of the channel to sub- or low-μM Ca^{2+} (<10 μM)[152].

4.4. The β_4 subunit.

β_4 is expressed mainly in brain tissue **(Table1)**. Its functional coupling with the α subunit has been suggested to be regulated by phosphorylation in serine/threonine residues of the regulatory subunit[153]. The presence of the β_4 subunit changes BK channel kinetics, slowing down the activation (in a

similar manner than β_1) and with little or no effect on the deactivation (which remains fast as observed for the BK channels lacking β subunits)[154]. β_4 has a Ca^{2+} concentration-dependent effect on BK channel gating. It decreases the apparent Ca^{2+} sensitivity of the channel at low Ca^{2+} levels but increases the apparent sensitivity at high Ca^{2+} concentrations. Thus, β_4 coexpression shifts the G-V curves to positive potentials in low Ca^{2+} (<10 μM) and to negative voltages at high Ca^{2+} (>10 μM)[133] **(Figure 22)**.

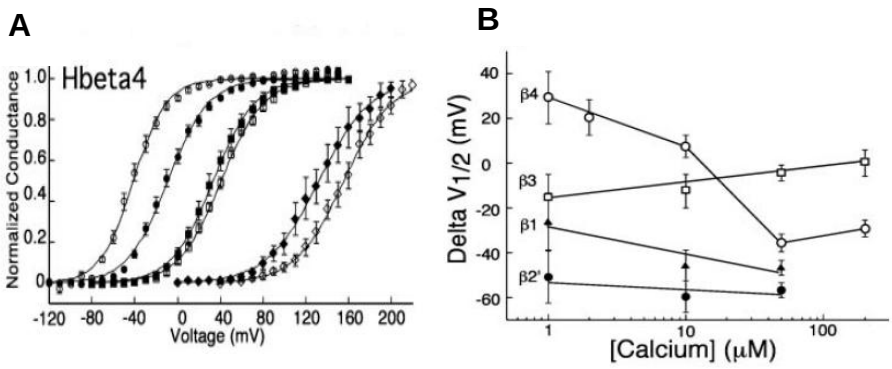


Figure 22. β_4 subunit effects on BK conductance-voltage relations. The differential effect of β_4 on BK channel G-V curves **(A)** and the corresponding $V_{1/2}$ values **(B)** in function of the cytosolic Ca^{2+}. $V_{1/2}$ *versus* Ca^{2+} concentration is shown in comparaison with either BK channels lacking β subunits or containing one of the other regulatory β subunits (for more details see[133]).

All these effects of β_4 on the biophysical properties of the BK channel can be explained by the fact that this subunit not only

stabilizes the active conformation of the voltage sensor (although in a smaller degree than β_1 or β_2 subunits) but also reduces the number of gating charges per sensor[131].

Crosslinking studies have been performed to determine the location of the two transmembrane helices (TM1 and TM2) of the β_4 subunit in relation with the seven transmembrane domains of the BK α subunit. Based in the three-dimensional structures of Kv1.2 channels as a template[155-157], the results shows that β_4 TM2 is close to α S0 transmembrane segment and that β_4 TM1 is near to both S1 and S2 segments of the pore-forming subunit, in both the open and closed states of the channel[158]. These results **(Figure 23, model 3)** support the idea developed by Liu et al. one year later regarding the location of the β_1 subunit in a functional BK channel, which suggest that the regulatory β subunit would stay inserted among the voltage-sensing domains of two adjacent α subunits, with the option of interact with them and modulate their equilibrium between inactive and active states.

The β_4 subunit alters BK channel pharmacology. β_4 strongly decreases the binding strength of ChTX and IbTX to BK channels, making them resistant to nM concentrations of the toxins. Furthermore, the generation and further analysis of β_1 and β_4 channel chimeras (that exchange their extracellular

loop), reveals that this precise extracellular region of the β subunit determine toxin binding properties and suggests that the external $\beta_{1/4}$ loop is in close proximity to the external mouth and possibly the selectivity filter and gate of the BK channel[159].

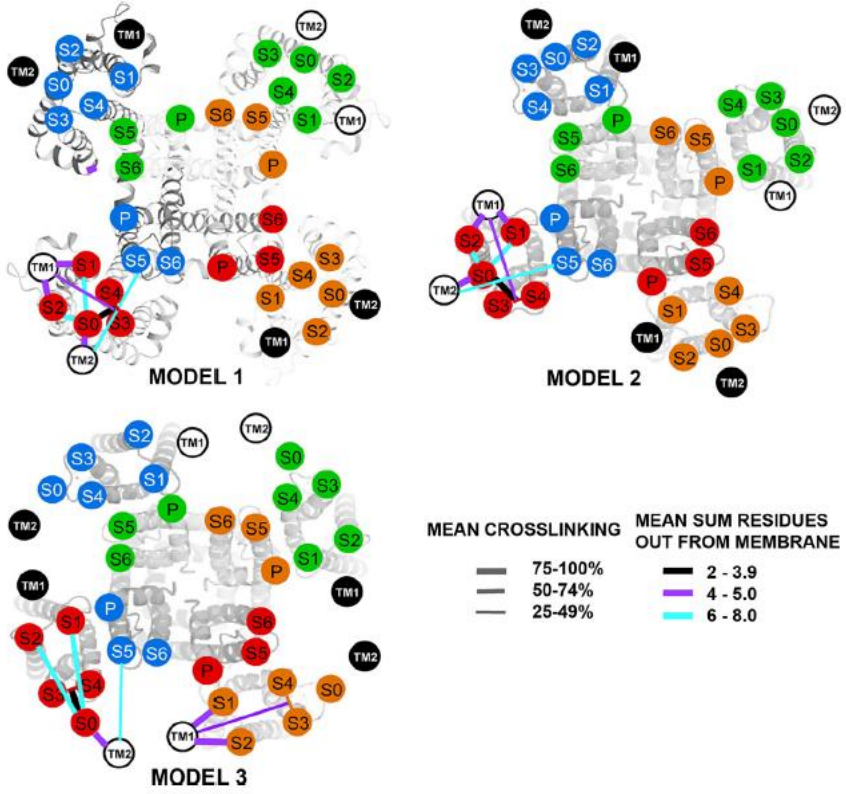


Figure 23. Locations of the extracellular ends of β_4 TM1 and TM2 relative to the extracellular ends of α S0–S6. Model 1, Kv1.2 in the closed state[156] is the template for locating the extracellular ends of BK α S1–S6. Models 2 and 3, The template is the crystal structure of Kv1.2 in the open state[155] (for more details see[158]).

As stated before, the presence of the β_4 subunit makes the BK channel sensitive to activation by 17β-stradiol, in a very similar way that β_1 subunit[117,160]. β_4 also confers to the BK channel strong sensitivity to other steroids hormones, allowing in particular a big and dosis-dependent activation by glucocorticoids such as corticosterone[117].

5. Another family of regulatory subunits: the gammas.

Recently, it has been described a new family of BK regulatory subunits, the gammas (γ). They have been found in a non excitable cancer cell line (LNCaP) where BK channels could be activated at resting potentials and without an increase of intracellular Ca^{2+} levels. The responsible for these unusual characteristics was a new auxiliary protein called LRRC26[161].

It was initially identified as a potential cancer marker protein, as it is highly expressed in prostate, breast, bone and glioma tumors. In normal human tissues, the LRRC26 mRNA has been only found in prostate and salivary gland, and at lower level of expression was detected in colon, small intestine, stomach, testis and fetal brain[161].

Four LRRC26 paralogues proteins have been described: γ_1 (LRRC26), γ_2 (LRRC52), γ_3 (LRRC55) and γ_4 (LRRC38),

expressed in different tissues and with different grades of modulation on activation voltage dependence, gating and Ca^{2+} sensitivity of BK channels[161,162].

6. BK channels in physiology and pathophysiology. How these channels works...

All in life is about balance. Intracellular Ca^{2+} signalling is one of the most important processes in mammalian physiology. In particular, Ca^{2+} entry into the cells through voltage-dependent Ca^{2+} channels is essential for life. Among other processes, it triggers neurosecretion, smooth muscle contraction, and the development of hearing. Nevertheless, some compensatory systems must be turned on to regulate Ca^{2+} influx, either to prevent or to lessen the physiological consequences driven by the increase in cytosolic Ca^{2+} levels. In many cases BK channels are in charge of this task. They are activated by membrane depolarization or increase in cytoplasmic Ca^{2+}, allowing the efflux of K^+ that is sufficient to hyperpolarize the membrane potential, providing a negative feedback mechanism that limits membrane depolarization and/or the events leading to cytosolic Ca^{2+} elevation (adapted from[2]).

As mentioned before, depending of the cell type, distinct combinations of BK channel α and β subunits are expressed, generating functional differences that mainly affect Ca^{2+}

sensitivity, macroscopic kinetics and pharmacological properties of the BK channel. In fact, their physiological impact is mostly due to the properties conferred by the particular β subunit that coexpresses with the pore-forming subunit in a given tissue[2].

6.1. ...in the vascular smooth muscle: the regulation of cell contractility.

Arterial resistance, which is mainly controlled by the basal tone of the vascular smooth muscle cells (VSMC), determines the rate of blood flow to tissues. Both circulating factors and those resident at the vessel wall participate in the establishments of this tone and their malfunction is typically associated to functional and structural vessels' alterations in patients suffering from essential hypertension[163]. Among the factors controlling vascular tone, voltage-gated ion channels mediating Ca^{2+} entry into the VSMC and their regulation by BK channels are central to this process.

The BK channel expressed in the smooth muscle is formed mostly by the pore-forming α subunit and the regulatory β_1 subunit[164], which, as described above, increases the apparent voltage/Ca^{2+} sensitivity of the BK channel[10,77] by stabilizing the active state of the voltage sensor[131]. In VSMC, wide and global elevation in intracellular Ca^{2+} levels, mainly achieved by

the activation of voltage-gated L-type Ca^{2+} channels following membrane depolarization, induces maintained contraction along with the opening of a cluster of ryanodine receptor Ca^{2+} channels in the sarcoplasmic reticulum adjacent to the cell membrane. The later produces local Ca^{2+} transients ("Ca^{2+} sparks" that reach concentrations around 10-100µM) that in turn activate a group of nearby BK channels, without major repercussion on total intracellular Ca^{2+} levels[5,165] **(Figure 24)**.

Such BK channel activation generates spontaneous transients outward K^+ currents (STOCs) that hyperpolarizes the membrane potential by 10 to 20 mV[165-167] (reviewed in[2]), preventing a large influx of Ca^{2+} via the depolarization-activated L-type Ca^{2+} channels and buffering smooth muscle contraction[165,168]. Accordingly, the blockade of the BK channels or ryanodine receptors in arterial smooth muscle causes depolarization, an elevation of arterial wall Ca^{2+} concentration and vasoconstriction[5,169]. Due to its positive action on BK channel function, the presence of the regulatory β_1 subunit makes the negative feedback loop more efficient in the regulation of vascular resistance[170].

In view of that, the expression of the β_1 subunit is relevant to smooth muscle contraction induced by vasoactive peptides and the regulation of arterial tone and blood pressure in both animal models of hypertension and human (see below for details).

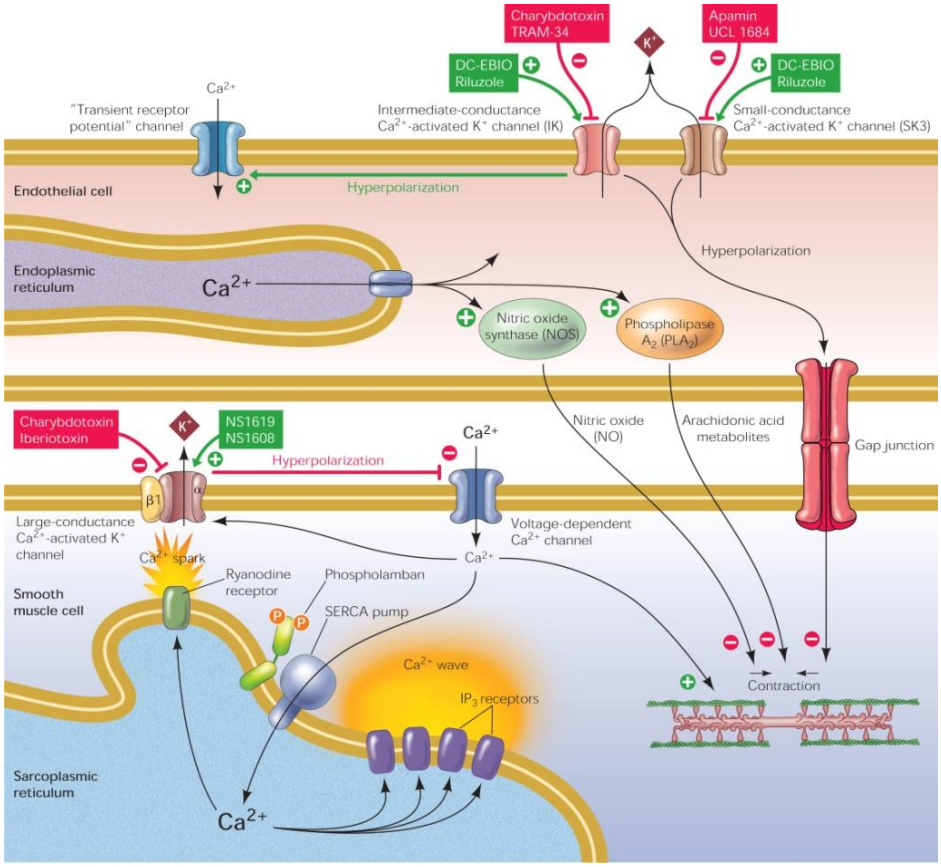


Figure 24. Roles of Ca^{2+} activated K^+ channels in the regulation of vascular function. Elevation of intravascular pressure leads to membrane potential depolarization, activation of voltage-dependent Ca^{2+} channels (VDCCs), an elevation of $[Ca^{2+}]_i$, and vasoconstriction. This vasoconstriction process is opposed by stimulation of "Ca^{2+} sparks", which activate BK channel in vascular smooth muscle (SM). The activation of small-conductance SK3 channels and intermediate-conductance IK channels by a rise in intracellular $[Ca^{2+}]_i$ can lead to vasodilation by hyperpolarizing the membrane potential of endothelial cells. This elevates the driving force for Ca^{2+} entry, possibly through transient receptor potential (TRP) channels, favouring an increase in $[Ca^{2+}]_i$ that would elevate nitric oxide (NO) and production of arachidonic acid metabolites

[prostacyclin (PGI2), epoxyeicosatrienoic acids (EETs)], which can hyperpolarize the adjacent SM. Endothelial membrane potential hyperpolarization could also be transmitted through myoendothelial gap junctions to hyperpolarize the SM, which by decreasing Ca^{2+} entry through VDCCs leads to vasodilation (for more details see[168]).

6.1.1. BK channels and the modulation of blood presure: animal models and human epidemiology.

The importance of the arterial β_1 subunit expression in physiology is patent in β_1-knockout (KO) mouse models. Disruption of the gene encoding the β_1 subunit (*KCNMB1*) leads to a BK functional impairment, due to the decrease in its apparent Ca^{2+} sentivity. As a consequence, in β_1-KO mice Ca^{2+} sparks are functionally uncoupled from BK channel activation (STOCs) leading in turn to membrane potential depolarization, vasoconstriction, elevation of blood pressure and left ventricular hypertrophy[164,170,171]. Also there is an increased aortic contractility in response to vasoconstrictors (norepinephrine or depolarizing high extracellular K^+) when comparing β_1-KO to WT mice[171]. Studies with WT and KO mice for the β_1 subunit also shows differences in the regulation of the arterial wall Ca^{2+} levels and diameter in cerebral arteries[172].

In commonly used animal models of hypertension, including spontaneously hypertensive rats (SHR) and rats made hypertensive by chronic infusion of the vasoconstrictor peptide angiotensin II, elevated blood pressure is associated with a downregulation of the β_1 subunit, but not the α subunit of the BK channel[173,174]. Besides, and more interesting in the context of human disease, it has been reported a gain-of-function BK channel β_1 subunit variant (E65K) protecting against moderate-to-severe diastolic hypertension and "combined cardiovascular disease" (myocardial infarction and stroke) [137,175], while an unrelated genetic variant of the gene coding for the α subunit (*KCNMA1*) was associated with a higher risk of hypertension[176]. Also in the context of human diseases, another SNPs mapped in the intron 3 and exon 4 of the gene encoding for β_1 subunit (*KCNMB1*) show an association between the β_1 gene and parameters relevant for the baroreflex activity, particularly parasympathetic tone, in a twins study[177].

Estradiol has also been shown to activate BK channels probably through the binding to the β_1 subunit[112]. Similar results have been obtained using xenoestrogens and cholane-derived steroids[116,118]. Finally, as mentioned before, hormones can regulate BK channel function. At the genomic levels, 17-β estradiol has been shown to upregulate the expression of the BK α subunit (but not the β_1 subunit) in guinea pig aorta[178].

This action of estradiol, together with its direct non-genomic induced-activation of BK channels containing the regulatory β_1 subunit[112], may contribute to gender differences in basal vascular tone and myogenic responses and may explain some of the cardioprotective effects of estrogens in women. However, definition of the main mechanisms employed by estrogens to regulate the vascular tone is still a matter of debate. Several investigations also indicate that estrogens can elicit vasodilatation by inducing NO release from endothelial cells[164].

6.2 ...in intracellular signalling.

BK channel α subunit has been shown to interact with multiple signalling proteins in smooth muscles or other cell types, beyond the already mentioned regulatory subunits (β and γ). The plasticity of these interactions, the subcellular localization and their specific function could be important to understand the intricate relationships of BK channels with signalling cascades and its possible metabotropic role as signal transducer **(Table 2)**[22]. It has been shown that the BK channel can be modulated by intracellular signaling proteins such as G proteins (in a protein kinase independent manner) following the activation of G proteins coupled receptors (GPCR), such as β-adrenergic receptors and muscarinic receptors, in smooth muscle from airways, coronary arteries, myometrium

or adrenal chromaffin cells[103-106]. It has been proposed that G proteins affect the transitions between the long and medium closed states and drive the gating reaction. G proteins seems to increase the binding constant for Ca^{2+} by stabilizing the open state of the channel[106].

MaxiK α partner proteins	MaxiKα association motif	Type of association	Tissue*	References
Transmembrane partners				
β1-subunit§	S0	?	Aorta, trachea, *in vitro*	(Giangiacomo *et al*. 1995; Knaus *et al*. 1994; Wallner *et al*. 1996)
β2-adrenergic receptor#	?	?	Myometrium, lung, bladder and aorta	(Chanrachakul *et al*. 2004; Liu *et al*. 2004)
L-type Ca^{2+} channels#	?	Requires β2-AR	Bladder and brain	(Liu *et al*. 2004; Grunnet & Kaufmann, 2004)
T-type Ca^{2+} channels	?	?	Brain	(Chen *et al*. 2003)
Intracellular partners				
PKA#	Leucine zipper 1, LAELKLGFIAQSCLAQGL STMLANLFSMRSFIKIE	Indirect	*in vitro*, brain	(Tian *et al*. 2003; Liu *et al*. 2004)
	dSlo C-terminus (922–956 aa)	Direct	*Drosophila* brain	(Wang *et al*. 1999; Zhou *et al*. 2002)
Src	?	RequiresY552/ Y976	*in vitro*	(Wang *et al*. 1999)
PyK2	?	?	*in vitro*	(Ling *et al*. 2004)
SyK	ITAM, YGDLFCKALKTYNML	Requires Y (emboldened)	Osteosarcoma cells (MG63)	(Rezzonico *et al*. 2002)
FAK	?	?	Osteoblasts	(Rezzonico *et al*. 2003)
Hemoxygenase-2	?	?	*in vitro*	(Williams *et al*. 2004*a*)
γ-glutamyl transpeptidase	?	?	*in vitro*	(Williams *et al*. 2004*b*)
Caveolin 1 and 2	?	?	Myometrium, vascular endothelium	(Brainard *et al*. 2005; Wang *et al*. 2005)
Actin	?	?	Myometrium	(Brainard *et al*. 2005)
Microtubule-associated protein 1A	Partial C-terminus (746–1144)	Direct	Brain, Purkinje cells	(Park *et al*. 2004)
Syntaxin 1A	C-terminus (minus 12 aa after S6)/S0–S1 loop	?	Brain	(Ling *et al*. 2003; Cibulsky *et al*. 2005)
β-Catenin	S10 hydrophobic segment	Direct	Chicken hair cells	(Lesage *et al*. 2004)
Cereblon	C-terminus (encompassing S6-S9 before the Ca^{2+} bowl)	Direct	Brain hippocampal neurones	(Jo *et al*. 2005)
AKAP79/150#	β2AR expression required (*in vitro*)	*in Vitro*, brain		(Liu *et al*. 2004)
ANKRA	C-terminal end	Direct	Brain	(Lim & Park, 2005)
Slob	C-terminus	Direct	*Drosophila* brain	(Schopperle *et al*. 1998)
dSLIP1	C-terminus	Direct	*Drosophila* brain	(Xia *et al*. 1998)
14-3-3 protein	Indirect	Via Slob	*Drosophila* neuromuscular junctions	(Zhou *et al*. 1999)

Table 2. Maxi K α partner proteins

*Associations have been mostly detected with co-IP in tissues or co-immunolabelling of cells in culture. *In Vitro*, refers to studies in heterologous expression systems or biochemical assays. Studies are in mammalian tissues unless otherwise stated. ?, unknown. §, β_2–β_4 subunits associate functionally with MaxiKα. #, signalling complex formed by MaxiKα, β2AR, AKAP79, PKA and L-type Ca^{2+} channel would facilitate β2AR receptor signalling[179] (extracted from [180]).

Recently, the molecular mechanisms underlying the relationship between BK channels and vasoconstrictor receptors have been described. The vasopressive thromboxane A2 receptor (a GPRC) can intimately interact with both BK α[107] and β_1 subunits, forming a tripartite mosaic[181]. Such direct interaction facilitates the G-protein-independent inhibition of BK channels after the stimulation of thromboxane A2 receptors, which would be expected to promote vasoconstriction[107].

Finally, the idea of ion channels acting as metabotropic transducers has been already described for several ion channels, including the voltage-gated Ca^{2+} channel at vascular smooth muscle cells. This L-type Ca^{2+} channel can mediate Ca^{2+} release from the sarcoplasmic reticulum to induce cell contraction without need of an extracellular Ca^{2+} influx, via the activation of a G protein and the phospholipase C (PLC) pathway. This signalling process involves also the RhoA/ROCK pathway activation in normal conditions of extracellular Ca^{2+} [182-187]. A plethora of K^+ channels have been involved in the control of proliferation of multiple cell types.

Classically, it was thought that by hyperpolarizing the cells, these channels would increase the driving force for Ca^{2+} entry, enhancing Ca^{2+}-dependent signalling pathways that would lead to cell proliferation. However, a new role of K^+ channels as direct transducer of intracellular signals is now emerging. Thus, it has been suggested that intermediate-conductance Ca^{2+}- and voltage-dependent (IK) K^+ channels can promote cell proliferation independent of K^+ conductance, by direct interaction with ERK1/2 and JNK signalling pathways[188].

7. BK channel as a therapeutical target: the hunt and design of activators.

Due to the pivotal and specific role that BK channels play in different physiological and pathological processes, they have a high potential therapeutic interest and represent very innovative and attractive drug targets. This applys to disorders affecting both the central nervous system and smooth muscle, such as acute stroke, epilepsy, psychosis, erectile dysfunction, asthma, bladder hyperactivity and arterial hypertension. Regarding the last, medications already exist that target different mechanisms involved in blood pressure regulation[189], although its normalization is not always satisfactory and none of them target BK channels. Compared to currently used hypertension treatments, such as Ca^{2+} antagonists, BK channel openers are expected to potentiate the existing

physiological regulatory system that buffers vascular smooth muscle contraction (as explained above[168]).

Many molecules have been identified as modulators of this channel. However, BK channel reflects per excellence the dilemma of the molecular target driven drug discovery process: after 15 years of intense research effort in both academia and industry, scientists have yet to witness the approval of a single BK channel modulator for clinical use (adapted from[190]).

A variety of small synthetic molecules has been developed by the industry as fairly selective and potent BK openers (for example, NS1619, NS11021, BMS204352 (tested in clinical trials for neuroprotection after ischaemic stroke), NS8 (which even advanced into Phase-2 clinical trials for overactive bladder)). Besides, natural product compounds (such as the already mentioned DHS-I, unsaturated fatty acid, arachidonic acid and decosahexaenoic acid (ω-3 fatty acids)) are also positive gating modulators for the BK channel (reviewed in[190-192]). However, little is known about their site(s) of interaction and most have serious safety and/or efficacy problems for clinical use.

NS1619 along with other benzoimidazolones and derivative chemicals (BMS204352 and NS8) have been extensively used in the research of BK channel functions, but it has become apparent thar their therapeutic usefulness is rather limited by

low potency, inadequate selectivity (which worsens their safety profile) and/or lack of efficacy when entered clinical trials (reviewed in[190-192]). Similarly, while DHS-I is an effective activator of the vascular BK$\alpha\beta_1$ channel when applied at the channel intracellular side, its medical application is limited by a poor membrane permeability[190].

8. Tungstate (WO_4^{2-}).

Tungsten (W) is a transition metal found in biological systems[193], even though it is a minor component in the Earth when compared with iron, copper and zinc. At biochemical level, W and molybdenum (Mo) are widely used transition metals in the modulation of different enzymes and biological processes. The relative abundance of these metals increases dramatically at the level of the marine hydrothermal vents, a niche that favours the evolution of tungstoenzymes[194]. Both Mo and W enzymes are present in all forms of life from ancient archaea to human, catalyzing a wide range of reactions in carbon sulfur and nitrogen metabolism[193]. At neutral pH, W occurs to appear predominantly as the soluble monomeric oxyanion tungstate (WO_4^{2-})[194] **(Figure 25)**.

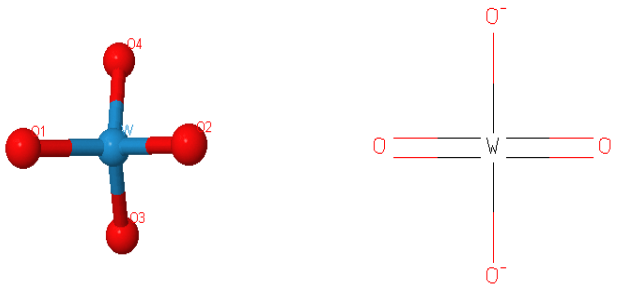


Figure 25.**Structural model of the tungstate (WO_4^{2-}) molecule.**

Tungstate has a low toxicity profile in animals and humans. Yet, the toxicological effects of W in organisms are still poorly understood compared with other metals. Pharmacokinetic studies indicate that W is rapidly and thoroughly absorbed orally and rapidly eliminated in urine (50% in 24 h) but less quickly in feces (50% in 4 days). Although these data suggest that there is little bioaccumulation of W, the data also indicate that orally ingested W is distributed throughout the body, with sufficient time to affect organ systems. Liver is one of the major sites for W distribution in the body possibly because of the high concentration of Mo-dependent enzymes (e.g., xanthine oxidase (XO)), with which W competes for the binding site, rendering the enzymes inactive. General toxicity information (e.g., LD50) is available for W (ATSDR, http://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=806&tid=157), but the site(s) and mode(s) of action of W toxicity are still uncertain[195].

Despite the fact that there is not much information about the molecular mechanisms underliying WO_4^{2-} actions, there is a considerable amount of data regarding its pharmacological and its metabolic effects. Thus, the antidiabetic and antiobesity properties of sodium tungstate have been widely reported. It has been shown that WO_4^{2-} salts are able to mimic the effects of vanadate, an insulin analogue on glucose metabolism. Like vanadate, WO_4^{2-} normalizes hepatic carbohydrate metabolism in several animal models of type-1 or type-2 diabetes when administered orally without causing hypoglucemia[196-198]. Besides, WO_4^{2-} stimulates insulin secretion and regenerates pancreatic β-cell population[199] and increases the production and translocation of the insulin-regulated glucose transporter GLUT4 in muscle[200]. Also, WO_4^{2-} has been found to favour thermogenesis and lipid oxidation in adipose tissue[201]. Finally, this compound modulates hypothalamic gene expression by activation of the leptin-signalling pathway responsible for the regulation of food intake and energy expenditure[202]. Regarding the exact molecular mechanisms mediating these effects, it has been suggested that activation of several kinases (ERK1/2 and JAK2) by WO_4^{2-} can lead to some of is antidiabetic and antiobesity actions[200,202,203]. Indeed, tungstate stimulates ERK phosphorylation in different cell types, including CHO cells, Leydig cells, neurons and hepatocytes, where it finally leads to the inactivation of the glycogen synthase kinase-3β (GSK3β) that in turn modulate cell function[203-205]. Although the nature of

tungstate targets upstream of ERK phosphorylation is not fully known, it has been recently proposed the involvement of a non-canonical pathway involving PTX-sensitive G proteins, at least in CHO and liver cells[206].

The efficacy of sodium tungstate in grade I and II human obesity has been already tested in a proof-of-concept clinical trial (TROTA-1)[207]. Contrary to the results obtained in rodent models of obesity, the data obtained from the clinical trial did not sustain WO_4^{2-} as a pharmacological tool in the treatment of human obesity, showing not significant changes in glucose/lipid metabolism. The reasons for the discrepancy in the WO_4^{2-} actions between rodents and humans remain to be elucidated.

WO_4^{2-} has been reported to reduce blood pressure in experimental animal models of both hypertension (including spontaneously hypertensive rats (SHR)[208,209]) and metabolic syndrome[210] (a clustering of cardiovascular risk factors, whose major characteristics include insulin-resistance, lipid abnormalities and hypertension), but not in normotensive animals **(Figure 26)**. The increase in arteriolar tone occurs in a variety of tissues (such as skeletal muscle, mesentery and skin) and thus serves as a major mechanism that determines elevation of systemic blood pressure. While the detailed mechanisms for the increasing tone are not very clear, it is known that activity of xanthine oxidase (XO), an oxidant-producing enzyme expressed in endothelium, is increased and

plays an important role in the elevated arteriolar tone in SHR by an augment in the level of microvascular oxygen radicals. By comparaison with the effect of known XO inhibitors and measurements of oxidative levels, the antihypertensive action of WO_4^{2-} has been associated to the inhibition of the increased XO activity in endothelial cells of hypertensive animals[208].

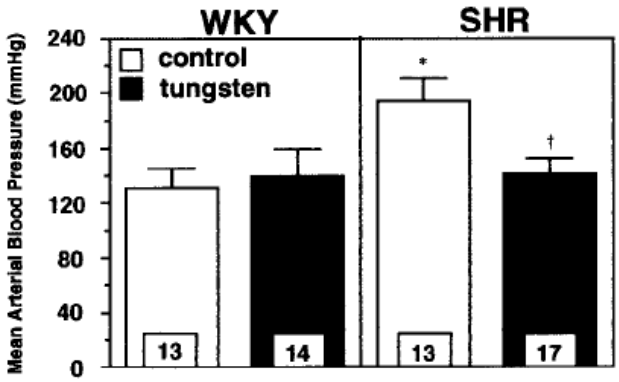


Figure 26. Mean arterial blood pressure measurements. In spontaneous hypertensive rats (SHR), a significant elevation of mean arterial blood pressure was observed relative to that in WKY (normotensive) rats. After intake of the tungsten-enriched diet, the elevated mean arterial blood pressure characteristic of SHR was significantly blunted. The number at the bottom of each column indicates the number of animals (for more details see[208]).

II.OBJECTIVES

Large-conductance, Ca^{2+}- and voltage-dependent K^+ (BK) channels, are involved in most of the physiological processes occurring in mammals. In particular, BK channels containing the pore-forming α and the regulatory β_1 subunit play a pivotal role in the control of vascular tone and modification of channel function is associated to changes in blood pressure in both animal models and humans. Therefore, the search of modulators for these channels is an interesting field of research, due to their potential therapeutic use. However the results until the moment were not successful.

Tungstate is a transition metal with antidiabetic, antiobesity and antihypertensive properties. Despite considerable knowledge on the pharmacological and metabolic effects of tungstate, little information exists regarding its molecular mechanisms of action and the nature of its primary targets, in particular those responsible for blood pressure reduction induced by tungstate. Given that BK channels are key elements in the control of arterial tone, the main objective of this Thesis is to evaluate whether BK channel targeting by tungstate can contribute to its antihypertensive effect and other metabolic actions induced by this transition metal, along with the analysis of the underlying molecular mechanisms.

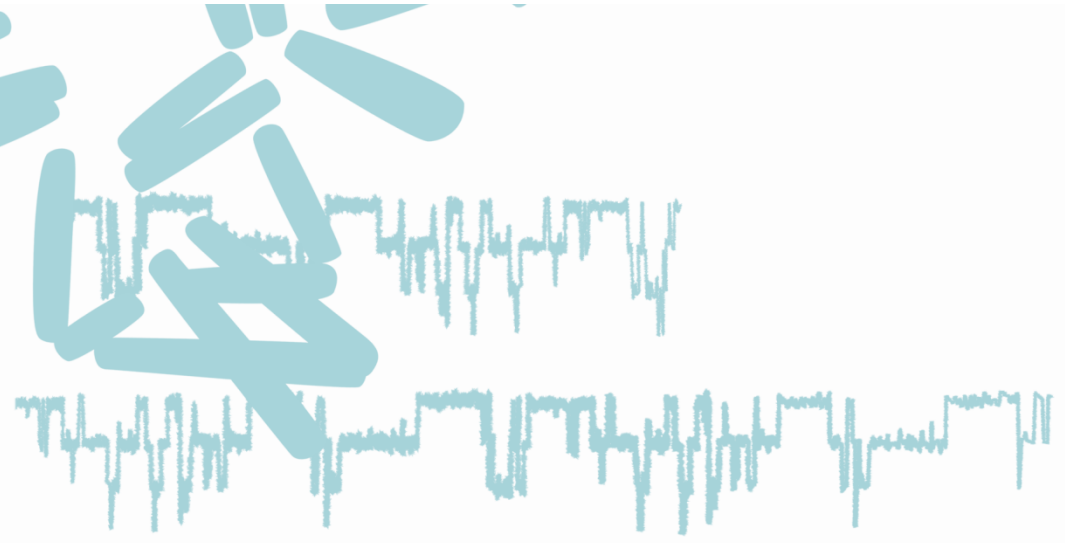
The specific objectives are:

1. To characterize the possible role of tungstate in the modulation of BK channels formed by different combinations of α and regulatory $\beta_{1\text{-}4}$ subunits.

2. To evaluate the relevance of tungstate-mediated BK channel modulation in the regulation of the vascular tone in arteries from WT and β_1-knockout mice.

3. To identify the structural determinants from both α and β subunits (with a main focus on the smooth muscle expressed β_1 subunit) responsible for the tungstate-mediated BK channel regulation.

4. To study the possible role of BK channels in the tungstate-induced activation of the intracellular signaling ERK pathway.



III. RESULTS

1. CHAPTER I

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

Ana I. Fernández-Mariño, Cristina Porras-González, Patricia González-Rodríguez, Jana Selent, Manuel Pastor, Juan Ureña, Antonio Castellano, Miguel A. Valverde, and José M. Fernández-Fernández

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2. CHAPTER II

BK channel activation by tungstate requires the β_1 subunit extracellular loop residues that are critical for the modulation of voltage sensor function and channel gating.

Ana I. Fernández-Mariño, Miguel A. Valverde and José M. Fernández-Fernández*

Pflugers Arch. 2013 Oct 26. Epub ahead of print.

3. CHAPTER III

Tungstate-induced, $G_{i/o}$ protein-mediated ERK phosphorylation is enhanced by BK channels containing the regulatory β_1 subunit.

Ana I. Fernández-Mariño, Delia Zafra, Laura Nocito, Jorge Domínguez, Miguel A. Valverde, José M. Fernández-Fernández and Joan J. Guinovart.

Tungstate-induced, $G_{i/o}$ protein-mediated ERK phosphorylation is enhanced by BK channels containing the regulatory β_1 subunit.

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Words: 5100.

Running title: BK channel-mediated ERK phosphorylation.

Abstract

Tungstate is a transition metal with antidiabetic, antiobesity and antihypertensive actions. Although there is substantial knowledge regarding its metabolic effects, the information on the primary target/s of tungstate is scarce. Tungstate triggers the activation of extracellular signal-regulated kinases (ERK) in different cell types, a process that requires the participation of an upstream G_i protein. The antihypertensive effect of tungstate depends on the activation of the vascular smooth muscle large conductance, voltage- and Ca^{2+}-activated K^+ (BK) channel. The vasorelaxant effect of tungstate requires the presence of the pore-forming α (KCNMA1) and the regulatory β_1 (KCNMB1) subunits. We now show that tunsgtate-induced activation of BK$\alpha\beta_1$ channels promotes the activation of PTX-sensitive G_i proteins to enhance the tungstate-mediated phosphorylation of ERK.

Keywords: tungstate; BK channel; KCNMB1 (β_1) subunit; PTX-sensitive $G_{i/o}$ proteins; ERK phosphorylation.

Introduction

Tungstate has antidiabetic and antiobesity actions in several animal models: 1) tungstate treatment normalizes hepatic carbohydrate metabolism[1, 2]; 2) stimulates insulin secretion and regenerates pancreatic β-cell population[3]; 3) mimics the effect of insulin on hepatocytes (but in a insulin receptor independent manner), increasing glycogen synthesis and deposition[4]; 4) increases the production and translocation of the insulin-regulated glucose transporter GLUT4 in muscle[5]; 5) favors thermogenesis and lipid oxidation in adipose tissue[6]; and 6) modulates hypothalamic gene expression by activation of the leptin-signalling pathway responsible for the regulation of food intake and energy expenditure[7]. In addition, tungstate also reduces blood pressure in experimental animal models of both hypertension[8, 9] and metabolic syndrome[10].

Despite all this knowledge on tungstate effects, there is a poor understanding of the underlying molecular mechanisms. In this respect, it has been suggested that activation of several kinases (extracellular signal-regulated kinases (ERK) 1/2 and JAK2) by tungstate can lead to some of its antidiabetic and antiobesity actions[4, 5, 7]. Indeed, tungstate stimulates ERK phosphorylation in different cell types, including CHO cells, Leydig cells, neurons and hepatocytes, leading to the phosphorylation (inactivation) of the glycogen synthase kinase-3β that in turn modulate cell function[4, 11, 12]. Although the nature of tungstate targets upstream of ERK

phosphorylation is not fully known, it has been recently proposed the involvement of a non-canonical pathway that requires PTX-sensitive $G_{i/o}$ proteins, at least in CHO and liver cells[13].

Regarding tungstate antihypertensive effect, it seems to be achieved by inhibition of the endothelial xanthine oxidase[8] and activation of the large conductance voltage- and Ca^{2+}-activated K^+ (BK) channel at the vascular smooth muscle cells[14]. Vascular BK channels are mostly formed by tetramers of the pore-forming α subunit (encoded by a single gene, *KCNMA1*)) along with the regulatory β_1 subunit (encoded by the *KCNMB1* gene). This accessory β_1 subunit favours BK channel activation by voltage and Ca^{2+} [15-17], improving the efficiency of the channel in the modulation of vascular resistance[18, 19]. Besides, the presence of β_1 is also required for channel modulation by different compounds[20-24], including the tungstate-induced channel activation and the subsequent vasodilation of pre-contracted arteries[14].

Growth factors contained in serum, such as insulin, IGF, and EGF, stimulate cell proliferation in various cell types upon binding to and activation of their cognate receptors. Thus, they transmit mitogenic signals that in many cases are amplified by the phosphorylation of ERK1/2[25], which in turn regulate multiple substrates involved in growth, differentiation and metabolic changes[26]. It has been suggested that activation of K^+ channels may be an upstream modulator of the growth

factor-mediated ERK pathway[27, 28]. Classically, it was thought that by hyperpolarizing the cells, these channels would increase the driving force for Ca^{2+} entry, enhancing Ca^{2+}-dependent signalling pathways that would lead to cell proliferation. However, a new role of ion channels as direct transducer of intracellular signals, beyond their conducting function, is emerging[29]. Thus, as an example, it has been suggested that intermediate-conductance Ca^{2+}- and voltage-dependent (IK) K^+ channels can promote cell proliferation independent of K^+ conductance, by direct interaction with ERK1/2 and JNK signalling pathways[30].

Given that tungstate can activate both the ERK1/2 pathway (in a $G_{i/o}$ protein-dependent manner) and BK$\alpha\beta_1$ channels, here we have evaluated the possible involvement of these channels in the tungstate-induced ERK phosphorylation.

Methods

Reagents

Sodium tungstate, Pertussis Toxin (PTX), GDPβs, Noradrenaline (NA) and EGF were from Sigma-Aldrich. Iberiotoxin (IbTX) was from Alomone Labs Ltd. (Jerusalem Israel). Tissue culture media and supplements were from Sigma and Invitrogen. Fetal calf serum (FCS) was from Gibco®. Phospho-ERK (Thr202/Tyr204) antibody for Western Blot was purchased from Cell Signalling TECHNOLOGY®. For PTX experiments, transfected cells were incubated with 100-500 ng/ml PTX in the culture medium, at 37 ºC and for 24-28 hours before electrophysiological recording or western blot assays.

cDNA constructs

Human β_1 subunit (KCNMB1) of the BK channel cloned into pcDNA3 was a gift from Ligia Toro (University of California – Los Angeles, Los Angeles, California, USA). Human α (KCNMA1) of the BK channel cloned into pcDNA3 were supplied by Ramón Latorre, (Centro de Neurociencias de Valparaíso, Valparaíso, Chile). Rat Gα subunit tagged with YFP, human Gβ subunit tagged with CFP and adrenergic α_{2A} receptor (α_{2A}-AR) cloned into pcDNA3 were kindly supplied

from Professor Moritz Büneman (Department of Pharmacology and Toxicology, University of Würzburg – Germany).

Cell transfection

For electrophysiological analysis, HEK293 cells were transfected using polyethylenimine ExGen 500 (Fermentas Inc., Hanover, MD, USA), following the manufacturer's instructions [seven equivalents of polythylenimine per 3.3μg of cDNAs (cloned into pcDNA3 vector) expressing the human BK α subunit together with the human β_1 subunit (1:2 ratio) and the transfection reporter pEGFPN1].

For FRET studies, HEK293 cells, HEKα cells (expressing constitutively the human α subunit of the BK channel) and HEK$\alpha\beta_1$ cells (expressing constitutively the bovine α and β_1 subunit of the BK channel) were transfected also with polyethylenimine ExGen 500(Fermentas Inc., Hanover, MD, USA) with cDNAs (cloned into pcDNA3 vector) expressing the rat Gα-YFP fusion protein (with YFP inserted between position 91 and 92), the human CFP-Gβ fusion protein (with CFP fused to the N-terminus) and the Gγ subunit. For control FRET experiments, the cDNA corresponding to α_{2A}-AR was also co-expressed in HEK293 cells.

Electrophysiology

Inside-out K^+ currents were recorded in macropatches from EGFP-positive cells, 2-3 days after transfection. Borosilicate glass patch pipettes had 2-3 MΩ resistance and were filled with a solution containing (in mM): 140 KCl, 1.2 $MgCl_2$, 0.15 $CaCl_2$, 5 EGTA, 10 HEPES (300 mOsm/l, pH 7.35). 0 Ca^{2+} solution (nominal 0 Ca^{2+}) bathing the cytoplasmic face of the patch membrane, to which 1 mM WO_4^{2-} was added after some control recordings, contained (in mM): 140 KCl, 0.7 mM Mg^{2+}, 5 EGTA, 10 HEPES (300 mOsm/l and pH 7.25). When used, GDPβs was added to the 0 Ca^{2+} bath solution before starting the electrophysiological experiments.

For current-activation studies, membrane macropatches were clamped at 0 mV, pulsed for 150 milliseconds from –100 mV to +200 mV in 10-mV steps, and repolarized to –80 mV for 20 milliseconds. 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 conductance-voltage (G-V) relationship was fitted with the following Boltzmann equation:

$$G/Gmax = 1/(1+\exp(-(V-V_{1/2\ act})/k_{act})$$

where G is the value of the instantaneous tail current at each test voltage, Gmax is the maximum obtained tail current, V is the test voltage applied to the membrane, $V_{1/2\ act}$ is the voltage

for half-maximal current activation, and k_{act} (an index of the minimum number of elementary charges that move through the electric field to gate the channel) is the slope factor of the Boltzmann term. Showed G-V curves represent averages from a set of patches at the indicated experimental condition.

pClamp8, PatchMaster and FitMaster softwares were used for pulse generation, data acquisition and subsequent analysis. Currents were acquired at 10 kHz and low-pass-filtered at 1 kHz.

Western blot analysis

Cells were grown to confluence and then deprived of FCS overnight. Tungstate and IGF/EGF treatments were performed in serum-free medium. Plates were flash frozen in liquid nitrogen and processed for protein extract preparation. Protein concentration was measured using the BCA Protein Assay (Pierce, USA). Proteins were separated by SDS–PAGE loading 20 µg of total protein per lane, transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and immunoblotted with selected antibodies. The immunoblots were developed using an enhanced chemiluminescence detection system (GE Healthcare, UK).

FRET experiments

Ratiometric FRET measurements between Gα-YFP and CFP-Gβ were recorded from 5 to 10 minutes at room temperature. Leica-SP5 Images of CFP and YFP emission were recorded simultaneously by using a similar procedure. Ratiometric FRET was obtained by excitation (458nm) of the CFP and emission (514nm) of YFP in a Leica TCS SP5 confocal microscope with a 63x Oil objective and analyzed using ImageJ software. All experiments were carried at room temperature and the cells were bathed in a solution containing (in mM): 140NaCl, 1 $MgCl_2$, 1,2 CaCl2, 10 HEPES, 5 Glucose, 0,5 EGTA (300 mOsm/l and pH 7.2-7.3). Noradrenaline was added to this bath solution in order to determine agonist-induced changes in FRET from HEK293 cells transfected with cDNAs for Gα-YFP, CFP-Gβ, Gγ and α_{2A}-AR.

Statistics

Data are presented as the means ± S.E.M. Statistical tests included Student's *t* test, Mann-Whitney test, One Way Analysis of Variance (ANOVA) followed by a Tukey posthoc test, or Nonparametric ANOVA (Kruskal-Wallis Test) followed by a Dunn posthoc test, as appropriate. Differences were considered significant if $P < 0.05$.

Results

BKαβ_1 channels play a role in the $G_{i/o}$ protein-dependent ERK1/2 phosphorylation induced by tungstate.

We have previously reported that tungstate only promotes the activation of heterologously expressed BK channels in the presence of regulatory β_1 or β_4 subunits[14]. In order to evaluate the possible role of BK channels in the tungstate-mediated activation of the ERK pathway, we analysed ERK1/2 phosphorylation using phospho-specific antibodies. Western blot analysis of phosphorylated ERK1/2 was carried out in untransfected HEK293 cells, HEKα (constitutively expressing the human α subunit of the BK channel) and HEK$\alpha\beta_1$ cells (constitutively expressing the bovine α and β_1 subunits of the BK channel) treated with tungstate. Tungstate increased the phosphorylation of ERK1/2 in all HEK293 cell lines, although to significantly higher levels only in HEK$\alpha\beta_1$ cells ($P < 0.001$) (Figure 1A, 1C, 1E and 1B (average data)). Such enhanced ERK phosphorylation induced by tungstate in HEK$\alpha\beta_1$ cells, was prevented by pretreatment with either the $G_{i/o}$ protein inhibitor pertussis toxin (PTX, 100ng/mL) (Figure 1C, 1D) or the specific BK channel blocker iberiotoxin (IbTX, 100 nM) (Figure 1E, 1F). Both toxins were without effect on the EGF/IGF-induced ERK phosphorylation that we used as control for ERK activation.

Tungstate-induced activation of BKαβ_1 channels is not mediated by $G_{i/o}$ proteins.

Since the BKαβ_1 channel-dependent enhancement of tungstate-induced ERK phosphorylation is prevented by inhibition of $G_{i/o}$ proteins, we next evaluated whether the BK channel is placed upstream or downstream of the G proteins. For that purpose, we analyzed the effect of tungstate on heterologously expressed BKαβ_1 channels in the presence of GDPβs (500μM) (which locks the Gα protein subunit in its inactive (GDP-bound) state or after preincubation of the transfected cells with PTX (500 ng/mL). Figure 2 (A, C) shows representative BKαβ_1 currents, recorded before (control) and after the addition of 1 mM tungstate (WO_4^{2-}) to a nominally Ca^{2+}-free bath (intracellular) solution containing 0.7 mM Mg^{2+} (an intracellular cation required for the tungstate-induced activation of BK channels[14]). Changes in BKαβ_1 channel activity were analyzed plotting the G-V relationships of the measured BK tail currents, before and after exposure to tungstate[14] (Figure 2B, 2D). From these curves the voltage for channel half-activation ($V_{1/2\ act}$) (Figure 2E) can be estimated. This is a convenient parameter to study the effect of BK channels modulators since it is directly related to the energy required to open the channel. We found that even in the absence of G-protein activation, tungstate still decreases by ~17-25mV the $V_{1/2\ act}$ for BKαβ_1 channels, favouring their activation by voltage. This action of tungstate on BKαβ_1

channels is similar to the one we have previously reported (a decrease of $V_{1/2\ act}$ by ~22 mV), under identical experimental conditions but without interfering with the activation of G proteins[14]. Thus, these results ruled out the involvement of G proteins in the tungstate-induced activation of BK$\alpha\beta_1$ channels.

Tungstate targeting of the BK$\alpha\beta_1$ channel promotes G_i protein activation.

These results suggest that the effect of tungstate on BK$\alpha\beta_1$ channels did not require the participation of $G_{i/o}$ proteins, while the tungstate activation of the ERK pathway does. These observations also suggest that the BK-mediated and $G_{i/o}$-dependent phosphorylation of ERK1/2 induced by tungstate may involve the activation of $G_{i/o}$ proteins downstream of tungstate interaction with BK. To give further support to this idea, we evaluated whether G_i protein activation by tungstate was related to the presence of BK$\alpha\beta_1$ channels. G protein activation was evaluated by measuring the Fluorescence resonance Energy Transfer (FRET) between α_i and β subunits of the heterotrimeric G protein tagged with the yellow fluorescent protein (YFP) and the cyan fluorescent protein (CFP) respectively[31]. FRET measurements were carried out on HEKα and HEK$\alpha\beta_1$ cells before and after the addition of tungstate (1 mM). G protein subunits undergo a molecular

rearrangement during activation (rather than a complete dissociation). Thus, when the CFP was fused to the N-terminus of the Gβ subunit, activation of the G protein following stimulation of G_i protein-coupled adrenergic α_{2A} receptors (α_{2A}-ARs) with noradrenaline (NA) results in an increase in FRET (measured as an elevation of the ratio between YPF and CFP fluorescence emission (F_{YFP}/F_{CFP})) while if the CFP is fused to the C-terminus of the Gγ subunit a decrease in FRET is produced by the same stimulus[31].

$G\alpha_i$-YFP and CFP-Gβ subunits (along with the Gγ subunit) were heterologously expressed in different HEK293 cell lines, expressing or not BK α and β_1 channel subunits. HEK293 cells also co-expressing α_{2A}-ARs were used as positive control. Cells with a reinforced membrane fluorescence pattern (Figure 3A), indicating the colocalization of the expressed fluorescent G protein subunits in the cellular membrane, were selected for FRET measurements. Addition of tungstate (1 mM) only increased FRET in HEK$\alpha\beta_1$ but not in HEKα cells expressing $G\alpha_i$-YFP and CFP-Gβ subunits (Figure 3B (magenta and blue traces, respectively) and 3C). As previously reported[31], addition of NA (1 μM) to HEK293 cells cotransfected with the cDNAs of G protein subunits and the α_{2A}-AR, results in a increase of the FRET signal (elevated F_{YFP}/F_{CFP} ratio) (Figure 3C). No increase in FRET after addition of NA was seen when the cDNA of the α_{2A}-AR was omitted in the cell transfection process (Figure 3C). The tungstate-induced elevation of the

FRET signal in HEK$\alpha\beta$ cells was about 56% of that produced by NA in HEK293 cells co-expressing the α_{2A}-AR (Figure 3C). Furthermore, as observed for the enhanced tungstate-induced phosphorylation of ERK found in HEK$\alpha\beta_1$ cells (Figure 1E, 1F), the increase in FRET among G protein subunits after tungstate application is abolished by preincubating the HEK$\alpha\beta_1$ cells with IbTX (Figure 3B (dark purple trace) and 3C).

Discussion

Tungstate is a transition metal that exerts antidiabetic[2, 32], antiobesity[6] and antihypertensive actions[8-10] in several experimental animal models. Despite considerable knowledge on the pharmacological and metabolic effects of tungstate, little information exists regarding its molecular mechanisms of action. In this sense, it is known that tungstate triggers intracellular signalling pathways related to the activation of extracellular signal-regulated kinases (ERK) in several cell types[4, 11, 12]. This signalling action of tungstate mimics the effect of insulin in hepatocytes, by increasing glycogen deposition but in an insulin receptor-independent manner. Tungstate activates PTX-sensitive G_i proteins, that in turn activates the small GTPase Ras to produce the phosphorylation of ERK, the subsequent phosphorylation of p90rsk and glycogen synthase kinase-3β and the activation of glycogen synthase leading to glycogen deposition[4, 13]. The antihypertensive effect of tungstate has been associated to both the inhibition of the endothelial xanthine oxidase[8] and the activation of the large conductance voltage- and Ca^{2+}-activated K^+ (BK) channel at the vascular smooth muscle cells[14]. Vascular BK channels are formed by the pore-forming α (KCNMA1) and the regulatory β_1 (KCNMB1) subunits. Indeed, we reported previously that tungstate only activates BK channels containing either the β_1 or the β_4 (but no β_2 or β_3) subunits[14].

Here, we provide evidences suggesting that targeting of BK channels containing the β_1 subunit by tungstate, promotes the activation of PTX-sensitive G_i proteins to enhance the tungstate-induced phosphorylation of ERK. First, we observed significant higher levels (~40-44%) of ERK phosphorylation after tungstate treatment (by 10 minutes) in HEK293 cells expressing both BK channel α and β_1 subunits (HEK$\alpha\beta_1$ cells) than in cells not expressing BK channels (HEK293 cells) or expressing the BK pore-forming α subunit alone (HEK□ cells). Second, the fact that such enhancement of the tungstate-induced activation of the ERK pathway found in HEK$\alpha\beta_1$ cells is prevented by either PTX or IbTX, supports the involvement of both $G_{i/o}$ proteins and BK channels in this tungstate-mediated signalling process. Third, $G_{i/o}$ proteins are not upstream of the tungstate-induced activation of BK$\alpha\beta_1$ channels, since tungstate-induced BK channel activity remains unaltered even in the presence of the G protein inhibitors PTX or GDPβs. This observation is in agreement with previous functional data and comparative structural analysis suggesting that tungstate modulates BK channel activity by direct binding to a site located at the BK α subunit[14]. Finally, BK$\alpha\beta_1$ channels seems to be upstream in the tungstate-induced, $G_{i/o}$ protein-mediated ERK phosphorylation pathway. Thus, tungstate only activated heterologously expressed G_i proteins (indicated by an increase in FRET among Gα_i-YFP and CFP-Gβ subunits) in HEK$\alpha\beta_1$ cells, but no in HEK□ cells, an effect that was

prevented by the blockade of BK$\alpha\beta_1$ channels with IbTX. These results altogether suggest that BK$\alpha\beta_1$ channels might well act as tungstate receptors to trigger the activation of the ERK pathway.

How the targeting of BK$\alpha\beta_1$ channels by tungstate leads to the activation of $G_{i/o}$ proteins is an issue that deserves further research. The fact that this signalling process is abolished in the presence of the BK channel IbTX, might suggest that the conduction of K^+ ions through the channel activated by tungstate is involved in G protein activation. IbTX shares a high sequence identity (around 68%) with charybdotoxin (ChTx) and a similar mechanism for BK channel blockade has been suggested[33]. The exact interaction site for the toxins is not clear but the involvement of residues around the outer mouth pore of the channel has been proposed[34]. Furthermore, some residues in the extracellular loop of the BK regulatory β_1 subunit are responsible of the high BK channel affinity for ChTx[20, 35]. These β_1 loop amino acids are in close proximity to the external mouth and, perhaps, the selectivity filter and gate of the channel[35-37]. Therefore, we cannot rule out the possibility that toxin binding to the channel might also modify the structural changes related with the activation of the voltage sensor and channel gating. Then, toxin impairment of the conformational changes in the BK$\alpha\beta_1$ channel produced by tungstate might affect the coupling of channel gating to G protein activation without the need of K^+ conduction. Whether

BK α and/or β_1 channel subunits can directly interact with G proteins is unknown at present. However, there are evidences that support the existence of a direct protein-protein crosstalk among BK channels and some G-protein coupled receptors, such as μ-opioid[38] or thromboxane A2 receptors[39].

Given that the BK β_1 channel subunit is primarily found in vascular smooth muscle cells (VSMC), where it improves channel function for a more efficient regulation of the vascular tone[18], the functional relationship between BK$\alpha\beta_1$ channels and the $G_{i/o}$ protein-ERK signaling cascade might also have physiological and/or pathological relevance in the vascular beds. In this sense, VSMC change their contractile phenotype toward a more proliferative phenotype during the pathogenesis of vascular diseases, such as hypertension or hyperlipidemia[40]. Such phenotypic remodeling has been associated to changes in the expression and distribution of voltage-gated K^+ channels[41]. Also, dedifferentiated VSMC can gradually regain contractile functions in a process mediated by PTX-sensitive G proteins (in particular, Gβγ dimmers) that involves the activation of the ERK pathway[42, 43]. Therefore, targeting of vascular BK channels by tungstate to enhance ERK phosphorylation might also help in this redifferentiation process.

In summary, our data provide evidences to consider BK channels as another member of the growing list of voltage-gated channels directly involved in the transduction of

intracellular signals, beyond their conducting function[29]. Interestingly, other member of the Ca^{2+}- and voltage-dependent K^+ channel family (IK1 or KCNN4) has been also related to the direct activation of the ERK signalling pathway in a way that is independent of its capability to conduct K^+ ions[30].

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Conflict of interest: none declared.

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Figures

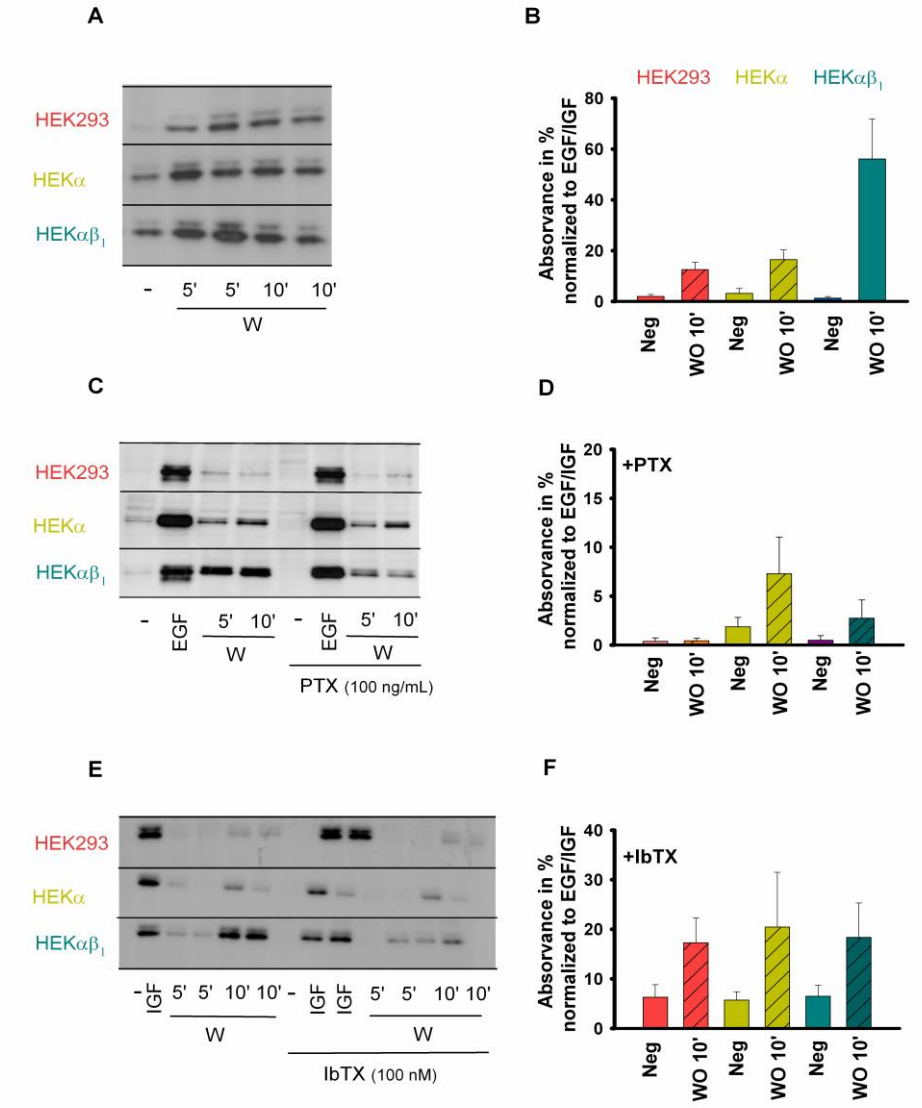

Figure 1. BKαβ$_1$ channels potentiates tungstate-induced ERK1/2 phosphorylation in a G$_{i/o}$ protein-dependent manner. Phosphorylation of ERK1/2, was analyzed by

western blot using phospho-ERK-specific antibodies. Total ERK, was used as loading control (data not shown). Protein expression and phosphorylation were quantified by densitometry of the corresponding Western blot signal. Representative western blots obtained from HEK293, HEKα and HEK$\alpha\beta_1$ cells for ERK1/2 phosphorylation levels, without treatment (-), after treatment with 100ng/ml EGF (during 10 minutes) (EGF) or after treatment with 1 mM tungstate (W) (during 5 and 10 minutes, as indicated) in the absence (A, C (left panel) and E (left panel)) or presence of PTX (C, right panel) or IbTX (E, right panel) (see Methods for further details). Average relative density (phosphorylated versus total ERK) normalized afterwards respect the inner control (EGF/IGF of each condition, which was considered as 100%), corresponding to the different experimental conditions above mentioned, in the absence (B) or presence of PTX (D) or IbTX (F) (n=4-12 in each experimental group). Error bars represent S.E.M.

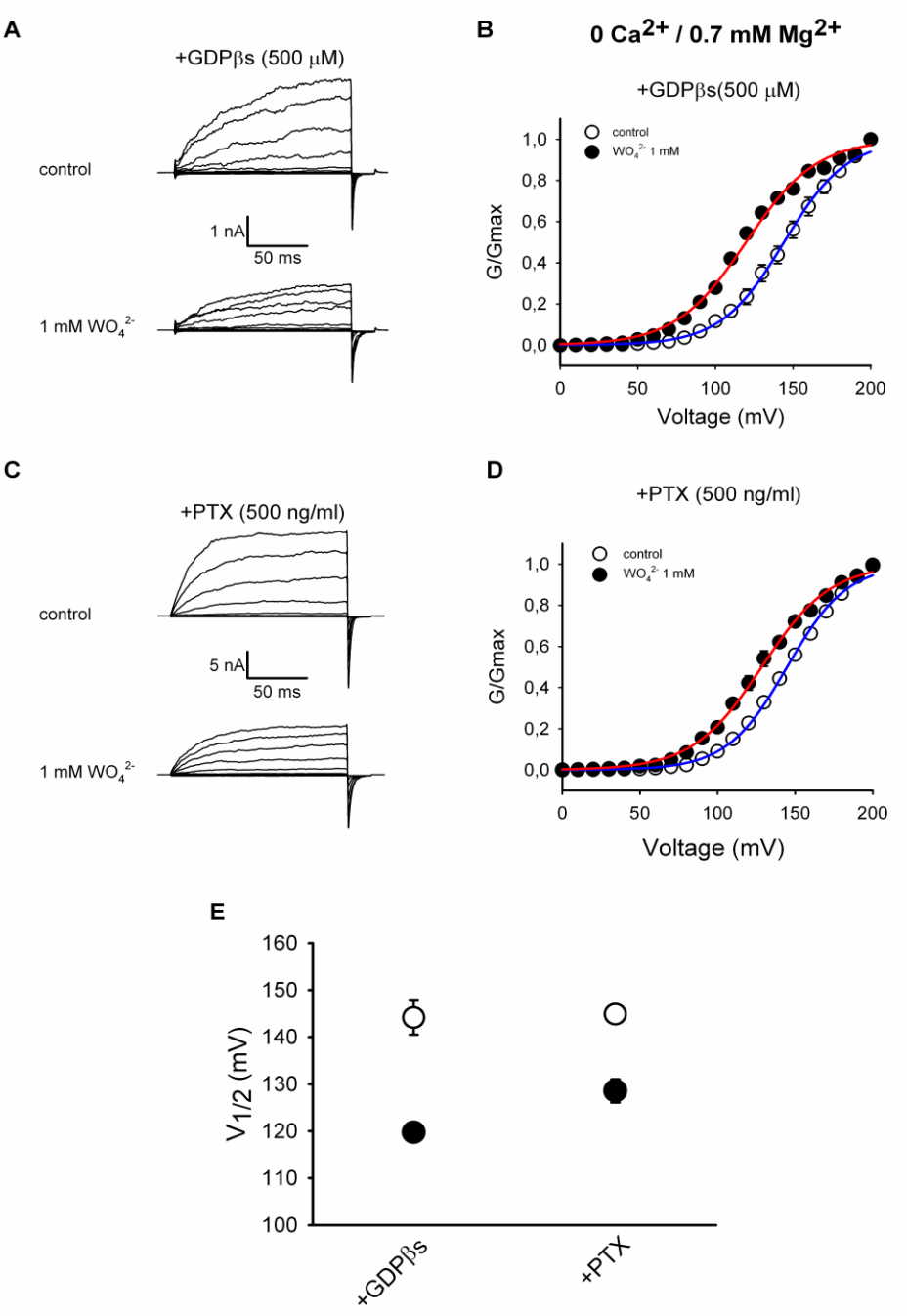

Figure 2. Tungstate-mediated activation of BK$\alpha\beta_1$ channels is independent of G proteins. Representative

currents recorded from excised inside-out macropatches obtained from HEK293 cells expressing the BK$\alpha\beta_1$ channels in the presence of 500μM GDPβs (added to the bath solution) (A) or from transfected HEK293 cells preincubated with PTX (500ng/ml, 24 hours) (C). Currents were recorded at cytsolic 0 Ca^{2+} and 0.7mM Mg^{2+} before (control, top panels) and after cytosolic application of 1mM tungstate (WO_4^{2-}, bottom panel). The voltage protocol was as described in the Methods. (B), (D) Average G-V curves for BK$\alpha\beta_1$ channels under the experimental conditions above mentioned. Solid curves were obtained by fitting the normalized conductance to the Boltzmann equation (see Methods). E, Voltage for half maximal activation ($V_{1/2act}$) of BK$\alpha\beta_1$ channels before (control, open circles) and after addition of tungstate (1mM WO_4^{2-}, filled circles) obtained for the indicated experimental conditions (+GDPβs, n=4; +PTX, n=6). Note that, as previously reported, 1 mM WO_4^{2-} also reduced substantially the K^+ current amplitude in the absence of cytosolic Ca^{2+} for all BK channels studied (A, C) (an effect that, contrary to the WO_4^{2-}-induced reduction of $V_{1/2\ act}$, has been shown to occurs either in the absence or presence of Mg^{2+} and different regulatory β subunits (β_1-β_4)[14].

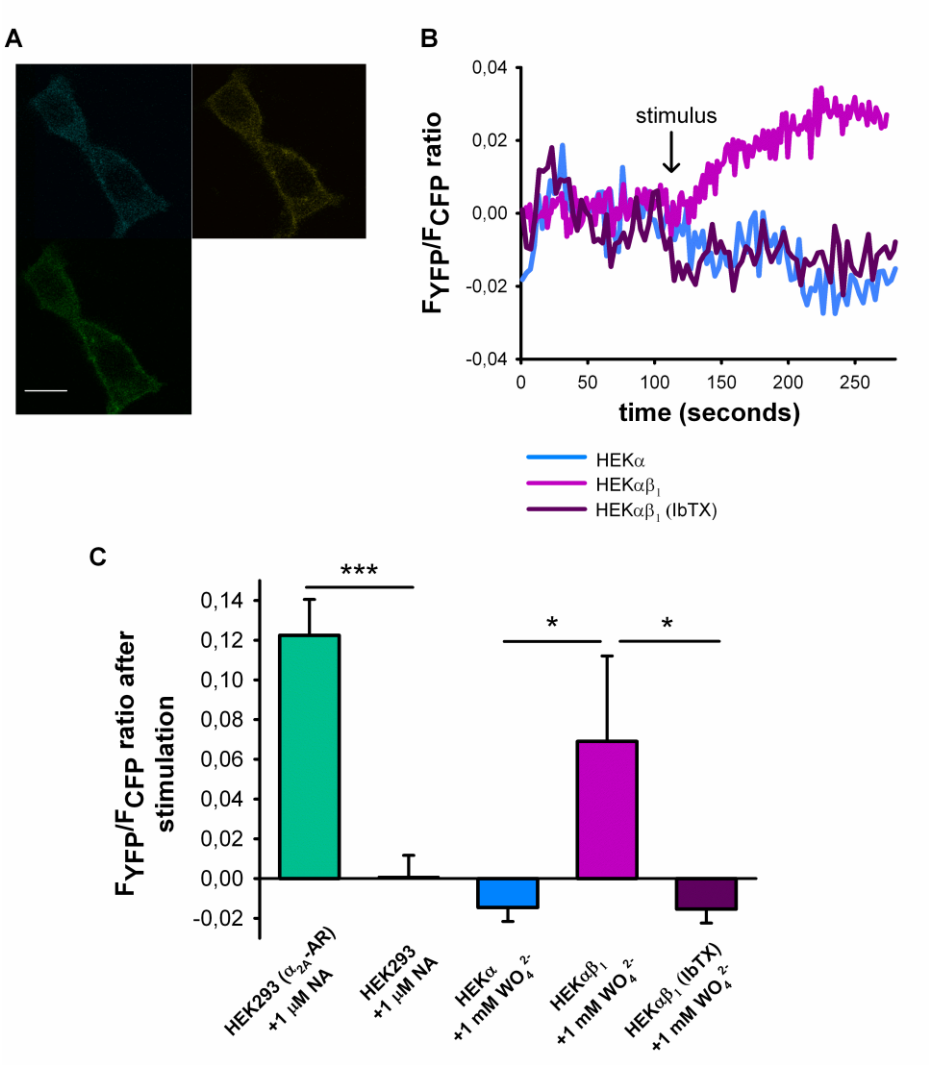

Figure 3. Tungstate-induced activation of heterologously expressed Gi/o proteins is mediated by BKαβ$_1$ channels. A, Example of the reinforced membrane fluorescence pattern and emission levels from CFP channel (up-left), YFP (FRET channel) (up-right) and the merge channels (bottom-left) from HEK293 heterologously expressing Gα$_i$-YFP and CFP-Gβ.

Fluorescence microscopy images were recorded by using confocal microscopy (for more details see Methods). FRET signal was determined by using donor ratiometric parameters (458/514) after excitation in the CFP frequency and registering in the YFP emission frequency. B, Representative FRET changes from HEK293, HEKα or HEK$\alpha\beta_1$ cells transfected with the cDNAs of G protein subunits, in response to 1 mM tungstate (in absence or presence of IbTX),(stimulus), as indicated. C, Average FRET changes for the different experimental conditions illustrated in B (n=5-9). *P < 0.05, ***P < 0.001.

IV. DISCUSSION

Large conductance, Ca^{2+}-dependent potassium (BK) channels are of paramount relevance in the control of vascular tone[168]. 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 where the BK channels are located[5]. Such activation results in an efflux of K^+ that is sufficient to hyperpolarize the membrane potential by 10-20 mV[165,166] providing a negative feedback mechanism that limits membrane depolarization and cell contraction[165,5]. 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[5,169]. Furthermore, a wide variety of vasodilators exert their actions through activation of BK channels[211-213]. The β_1 subunit is primarily found in smooth muscle cells[129,170], where it increases the apparent voltage- and Ca^{2+}-sensitivity of the BK channel α subunit by stabilizing the voltage sensor in the active configuration[79,130,131]. Thus, the presence of this regulatory subunit enhances the compensatory system that opposes vasoconstriction[10,165,170,171]. Hence, in β_1-knockout (KO) mice Ca^{2+} sparks are functionally uncoupled from BK channel activation leading to membrane potential depolarization, vasoconstriction, elevation of blood pressure and left ventricular hypertrophy[170,171]. Also, 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 downregulation of the β_1 subunit, but not the α subunit, of the BK channels[173,174]. Besides and more interesting in the context of human disease, a gain-of-function BK channel β_1 subunit variant has been reported to protect against moderate-to-severe diastolic hypertension and "combined cardiovascular disease" (myocardial infarction and stroke)[137,175] while an unrelated genetic variant of the gene coding for the α subunit (*KCNMA1*) was associated with a higher risk of hypertension[176]. Finally, estradiol has also been shown to activate BK channels probably through the binding to the β_1 subunit (see below)[112], an effect that may account for the well-characterized gender differences in basal vascular tone and myogenic responses. Similar results have been obtained by using other steroids (as xenoestrogens) and cholane-derived steroids[116,118]. Altogether provides a considerable amount of evidences pointing out at the BK$\alpha\beta_1$ channel as an interesting target for the development of new antihypertensive therapeutic agents.

Looking for therapeutic BK channel openers

An extensive variety of molecules has been already described as BK channel openers with vasodilatory actions, although in general little is known about their site(s) of interaction.

In the one hand, there are several natural compounds including dehydrosoyasaponin I (DHS-I), unsaturated fatty acids such as arachidonic acid and decosahexaenoic acid (DHA) found in the ω-3 fatty acids (reviewed in[190-192]). Precisely, long-chain polyinsaturared ω-3 fatty acids (DHA) (found abundantly in oily fish) was recently described to activate vascular BKαβ_1 but also neuronal BKαβ_4 channels, lowering blood pressure levels in anesthesied WT but not β_1-KO mice. These results suggest that the presence of the β subunits is necessary for the action of fatty acids on the BK channel. Thus, two residues in the N-terminus and the first transmembrane domain of both β_1 and β_4 BK channel subunits seems to be responsible of the specific action of these compounds[214,215]. While DHS-I is an effective activator of the vascular BKαβ_1 channel when applied at the channel intracellular side, its medical application is limited by a poor membrane permeability[190].

In the other hand, diverse small synthetic molecules have been also developed by the industry. Among them, NS1619, NS11021, BMS20435 were tested in clinical trials for

neuroprotection after ischaemic strok and NS8 even advanced into phase-2 clinical trials for overactive bladder treatment. NS1619, along with other benzoimidazolones and derivative chemicals (BMS304352 and NS8) have been also used in the research of BK channel functions. However, the therapeutic application of these compounds are limited by different factors: low potency, inadequate selectivity (important for their safety profile) or the lack of efficacy when finally entered clinical trials (reviewed in[190-192,216])

Tungstate is a compound with antidiabetic and antiobesity actions in several animal models. In this sense, tungstate treatment: 1) normalizes hepatic carbohydrate metabolism[196,198]; 2) stimulates insulin secretion and regenerates pancreatic β-cell population[199]; 3) increases glycogen synthesis and deposition in epatocytes, mimicking the effect of insulin (but in an insulin receptor independent manner[203]); 4) increases the production and translocation of the insulin-regulated glucose transporter GLUT4 in muscle[200]; 5) favours thermogenesis and lipid oxidation in adipose tissue[201]; and 6) modulates hypothalamic gene expression by activation of the leptin-signalling pathway responsible for the regulation of food intake and energy expenditure[202]. In addition, tungstate also reduces blood pressure in experimental animal models of both hypertension[208,209] and metabolic syndrome[210]. Despite considerable knowledge on the pharmacological and metabolic effects of tungstate, little information exists

regarding its molecular mechanisms of action and the nature of its primary targets. In this respect, it has been suggested that activation of several kinases (ERK1/2 and JAK2) by tungstate (see below for further details) can lead to some of its antidiabetic and antiobesity actions[200,202,203]. Also, it has been proposed that tungstate antihypertensive effect may be achieved by inhibition of endothelial xanthine oxidase (XO)[208].

Tungstate effect on BK channels

Our results show a dual action of tungstate on BK channels with relevance to the control of vascular resistance, highlighting a new tungstate target with potential therapeutic interest.

On the one hand, millimolar tungstate reduced the amplitude of K^+ currents through heterologously expressed BK channels (either in the absence or presence of different regulatory β subunits, β_1-β_4). Since BK channel blockade enhances glucose-dependent insulin secretion in both mouse and human pancreatic β-cells[217,218], such tungstate-induced decrease of BK current amplitude might also contribute to the tungstate antidiabetic action. On the other hand, tungstate selectively favoured voltage-dependent activation (by ~ 20 mV) of BK channels containing either the β_1 or β_4 (but no β_2 or β_3) subunits, an effect that required the Mg^{2+}-sensitivity of

the channel. At the single channel level, such β-dependent tungstate-induced activation of vascular BK channels involves an increase in the channel open probability (NP_o), which was only observed in inside-out patches obtained from myocytes of WT mice but not in those from β_1-KO mouse myocytes **(see Annex Figure)**. The decrease in BK current magnitude by tungstate (1 mM) may explain its constriction effect on arterial rings from both WT and β_1-KO mice. However, at micromolar levels (0.1 mM), tungstate still was able to promote (by ~ 10 mV) voltage-dependent activation of the vascular (β_1 subunit-containing) BK channel without neither lessening BK current amplitude nor constricting mice arterial rings. In agreement with such positive and β-dependent effect on BK channel function, micromolar tungstate induced vasodilation of wild-type but not β_1-knockout mouse arteries precontracted with endothelin-1. This vasodilatory action was not related to the reported tungstate-induced inhibition of the endothelial XO[208] as it was not replicated by the XO blocker allopurinol. It has been previously reported that sodium tungstate (0.1 mM) pretreatment for 10 minutes of rat mesenteric branch arteries was without effect on the Ca^{2+}-induced relaxation over the phenylephrine-induced contraction[219]. Differences regarding the experimental protocol and/or the biological material under study might account for the different results obtained between these studies.

Where is the tungstate interaction site in the BK channel?

It has been shown that the BK channel can be modulated by G proteins (in a protein kinase independent manner) following the activation of G proteins coupled receptors (GPCR), such as β-adrenergic receptors and muscarinic receptors, in smooth muscle from airways, coronary arteries, myometrium or adrenal chromaffin cells[103-106]. It has been proposed that G proteins affect the transitions between the long and medium closed states and drive the gating reaction, most probably by increasing the binding constant for Ca^{2+} and stabilizing the open state of the channel[106]. Tungstate has been shown to activate G proteins to trigger intracellular signalling pathways of relevance for its antidiabetic properties (see below for further details[206]). However, our results indicate that G proteins are not upstream of the tungstate-induced activation of vascular BKαβ_1 channels, since this process remains unaltered even in the presence of the G protein inhibitors pertussis toxin (PTX) or GDPβs.

Instead, the Mg^{2+}-dependence of BK channel activation by tungstate along with the analysis of available structural data on tungstate-protein complexes, suggest that tungsate directly binds to a site located at the human BK channel α subunit which might involves aspartate-lysine/lysine-aspartate (DK/KD) residues around those composing the

Mg^{2+} binding site, located in the voltage-sensor domain (VSD) and the first cytoplasmic regulator of conductance for K^+ (RCK1) domain. Thus, mutagenesis of residues in these regions (D99A, N172A, E374A, E399A) have an impact on channel activation induced by either Mg^{2+} or tungstate (as shown in this thesis and by Yang et al.[88]). Furthermore, both VSD and RCK1 domain contain a DK/KD motif within or next to most of the mutated residues, susceptible of forming a binding pocket for available Mg^{2+}-tungstate complexes **(Figure 27)**. For instance, in site 1, located in the cytoplasmic region between S0 and S1, the mutated D99 amino acid is part of a KD motif ([93]WMTSV**K(D99A)**WAGVM[104]). In site 2, located in the cytoplasmic region between S2 and S3, a DK motif is found in the vicinity of the mutated N172 residue ([171]A(N172A)**DK**[174]). Also, site 3 in the RCK1 domain ([356]VSNFL**KD**FLH**KD**R**DD**VNV(E374)I[375]) has two of the KD motifs near to E374, which also contributes to the Mg^{2+} binding site involved in BK channel activation[88].

Why the Mg^{2+}-dependent tungstate-induced activation of BK channels requires specifically the presence of either β_1 or β_4 subunits? Our research regarding the role of the BK β_1 subunit in BK channel modulation by tungstate, reveals that residues in two highly conserved segments (named A and B) of the large β_1 extracellular loop that promote β_1-dependent

estabilization of the BK channel voltage sensor in its active state, also play a key role in channel activation by tungstate.

It has been shown that single alanine substitutions of several residues placed either at the segment A (Y74) or the segment B (S104A, Y105A, I106E) of the β_1 extracellular loop **(Figure 27)**, reduce the left-shift of the BK G-V curves produced by the regulatory β_1 subunit at high (60 µM) cytosolic Ca^{2+} in a nonadditive way. Besides, at least Y74A, Y105A or I106A mutations destabilize open-channel voltage sensor-activation even in the absence of Ca^{2+} binding to the BK channel[138]. We have now found that β_1 mutations Y74A or S104A abolished or substantially decreased, respectively, the left-shift of the BK$\alpha\beta_1$ G-V curves induced by tungstate, therefore severely impairing channel activation by this compound. Such mutations effect was observed both in the presence of 10 µM cytosolic Ca^{2+} (which is within the physiological window of Ca^{2+} levels that are reached in the cytosol of smooth muscle cells during contraction) or in the absence of cytosolic Ca^{2+} (nominal 0 Ca^{2+}). On the contrary, mutation Q73A, which was reported not to alter the shift towards more negative potentials of the BK G-V curves promoted by the regulatory β_1 subunit at high (60 µM) cytosolic Ca^{2+}, did not prevent the tungstate-induced activation of BK$\alpha\beta_1$ channels. We have also observed that the activation of BK$\alpha\beta_1$ channels by tungstate in the presence of 10 µM cytosolic Ca^{2+}, is yet fully dependent on

the Mg^{2+}-sensitivity of the BK α channel subunit, as it is prevented by the D99A mutation in its VSD which abrogates BK channel activation by Mg^{2+}. These results are similar to those we have obtained in the absence of cytosolic Ca^{2+}. Furthermore, the impaired activation by tungstate of $BK\alpha\beta_{1Y74A}$ and $BK\alpha\beta_{1S104A}$ mutant channels is not due to a decrease in their Mg^{2+}-sensitivity, which remains similar to that of the $BK\alpha\beta_{1WT}$ channel. Altogether, our findings support the idea that the putative binding site for tungstate that mediates BK channel activation is indeed located at the BK α channel subunit, around those residues of the VSD and the RCK1 domain that coordinate the binding of Mg^{2+}. Still, residues in the segments A and B of the β_1 extracellular loop are also required for a proper tungstate-induced activation of BK channels. The role of these residues can be explained by an indirect action, related to their involvement in the maintenance of a particular three-dimensional structure of the BK α and/or the BK β_1 channel subunits that allows the binding of tungstate to the α VSD and RCK1 regions and the subsequent activation of the channel. An alternative explanation would be the direct participation of these β_1 amino acids in the modulation of BK channel activity due to their close proximity to the external mouth and possibly the selectivity filter and gate of the channel, as suggested by several authors[138-141]. In this sense, residues placed either at the segment A (Y74) or the segment B (S104A, Y105A, I106E) of the β_1 extracellular loop modulate not only the

activation of the voltage sensor but also the intrinsic gating of the BK channel, pointing to a possible role of these particular β_1 regions in the coupling among the activated voltage sensor and the channel gate[138]. Therefore, by affecting this process, mutations in segments A and B of the β_1 extracellular loop might, in turn, impair the intramolecular transduction mechanism by which tungstate interaction with the BK α subunit activates the channel.

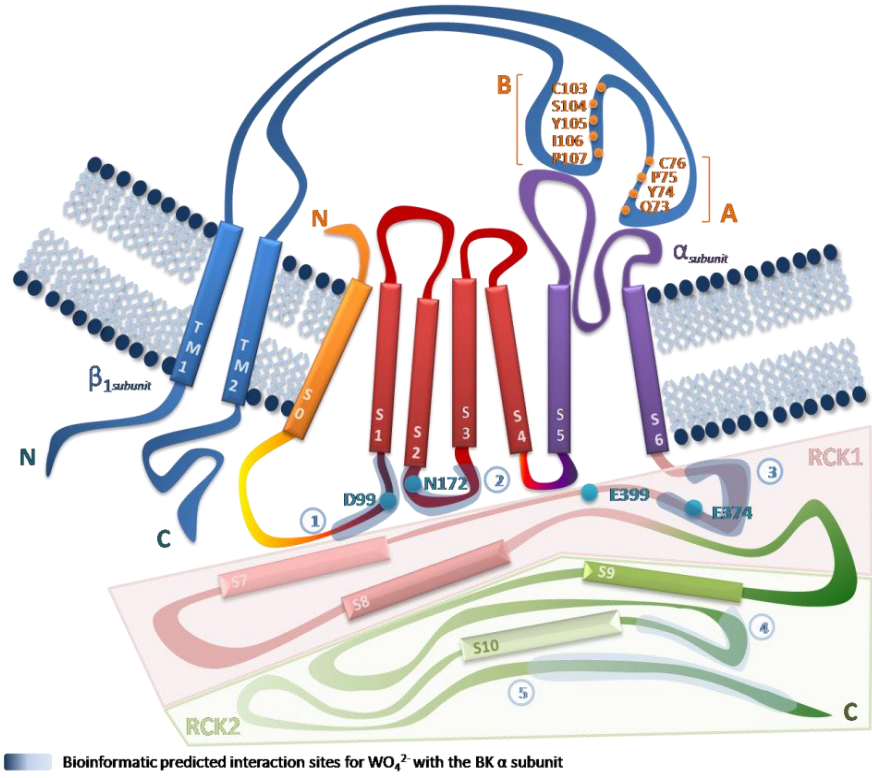

Figure 27. BK$\alpha\beta_1$ channel model. In the α subunit, the different transmembrane domains are shown in different colors based on their

different contribution to channel function (S0 in orange, VSD (S1-S4) in red, PD (S5-S6) in purple and the RCK1-2 domains in pink and green, respectively. Blue boxes in the α subunit C-tail indicate the predicted interaction sites for WO_4^{2-} based on bioinformatic structural analysis. Blue circles designate the important residues for the Mg^{2+} interaction with the α subunit. The residues at the segments A or B of the β_1 regulatory subunit extracellular loop that are important for voltage sensor activation and channel gating are shown in orange.

There are previously reported evidences signifying the relevance of the β_1 extracellular loop in the modulation of BK channel activity. In the one hand, it has been suggested that steroids activate BK channels through direct binding to an external site available in the presence of the regulatory β_1 subunit, although the exact site of interaction remains to be elucidated. Thus, 17β-estradiol (in the µM range) seems to increase the activity of BK$\alpha\beta_1$ channels by direct interaction with the β extracellular loop, as channel activation was also triggered by estradiol conjugated to a membrane-impenetrable carrier protein[112]. Similar results have been obtained using the xenoestrogen tamoxifen[116]. In the other hand, it has been shown that human β_1 polymorphisms placed at the extracellular loop, have both functional and clinical relevance. The β_1 E65K variant has been reported to increase the voltage- and Ca^{2+}-sensitivity of the BK channel[137] and the R140W variant to decrease channel

openings[220]. Consistent with such gain (induced by E65K) and loss (produced by R140W) of channel function along with the key role of the BK channel in the control of smooth muscle tone both in the vasculature[165] and in the airways[221], E65K has been found to protect against moderate-to-severe diastolic hypertension and "combined cardiovascular disease" (myocardial infarction and stroke)[137,175,] , whereas R140W has been linked to reduced pulmonary function[220].

Although not all regulatory β subunits change the biophysical properties of the BK channel in a similar way (reviewed in[126]), gating current measurements show that the stabilization of the active configuration of the voltage sensor induced by β_1 is a trait shared with β_2 and β_4, but not β_3, subunits[131]. Thus, both β_2 and β_4 subunits induce a reduction in the standard free energy difference among the active and resting states of the voltage sensor, albeit to a lesser extent than that produced by the β_1 subunit[131]. Interestingly, segments A and B of the β_1 extracellular loop, containing key residues for the modulation of voltage sensor function and gating of the BK channel, are the longest sequences of consecutive residues that are also identical in both β_2 and β_4 subunits[138]. Therefore, such high degree of conservation among β_1, β_2 and β_4 subunits of segments A and B at the extracellular loop, may lie beneath both the stabilization of the active voltage sensor induced by these three regulatory β subunits and the β-dependent effect of tungstate on BK channels.

Regarding the later, tungstate promotes the activation not only of BK$\alpha\beta_1$, but also of BK$\alpha\beta_4$ channels. The fact that we did not found a positive effect of tungstate on the activity of BK$\alpha\beta_2$ channels might be due to the specific modulatory actions that β_2 subunit (but not β_1 or β_4) have on BK channels. Indeed, β_2 induces fast and complete inactivation of the BK channel mediated by the interaction of its N-terminus with a receptor site in the open α subunit[148,222,223,]. In addition, β_2 produces an instantaneous outward rectification in which the β_2 external loop has been involved[224].

In summary, our results support the idea that tungstate activate BK channels by interacting with the pore-forming α channel subunit in a region involving the Mg^{2+} binding site, thus explaining the Mg^{2+}-dependence of such tungstate action. Our data also suggest that the β-dependence of BK channel activation by tungstate rely on how the different β subunits interact with the BK α channel subunit to modulate its activity, with the essential role of residues located at the β extracellular loop that have an important participation in the regulation of voltage sensor activation and gating of the BK channel.

BK channels as metabotropic transducers.

As mentioned above, there is not much information regarding the nature of tungstate targets leading to the well known antidiabetic[197,198], antiobesity[201] and antihypertensive actions[208-210] of this compound. However, it has been demonstrated that tungstate triggers intracellular signalling pathways related to the activation of extracellular signal-regulated kinases (ERK) in several cell types[203-205]. This signalling action of tungstate mimics the effect of insulin in hepatocytes, by increasing glycogen deposition but in an insulin receptor-independent manner. In these cells, tungstate activates PTX-sensitive G_i proteins, that in turn activates the small GTPase Ras to produce the phosphorylation of ERK, the subsequent phosphorylation of p90rsk and glycogen synthase kinase-3β, to finish with the activation of glycogen synthase and therefore glycogen deposition[203,206]. Now, the results presented in this thesis suggest that the antihypertensive effect of tungstate can be associated not just to the inhibition of the endothelial XO[208], but also to the activation of the large conductance Ca^{2+}- and voltage-activated K^+ (BK) channel at the vascular smooth muscle cells containing the regulatory β_1 subunit.

Interestingly, we now provide evidences to consider BK channels as another member of the growing list of voltage-

gated channels directly involved in the transduction of intracellular signals, beyond their conducting function[225]. Our data suggest that targeting of BK$\alpha\beta_1$ channels by tungstate, promotes the activation of PTX-sensitive G_i proteins to enhance the tungstate-induced phosphorylation of ERK. First, we observed significant higher levels (~40-44%) of ERK phosphorylation after tungstate treatment (by 10 minutes) in HEK293 cells expressing both BK channel α and β_1 subunits (HEK$\alpha\beta$ cells) than in cells not expressing BK channels (HEK293 cells) or expressing the BK pore-forming α subunit alone (HEKα cells). Second, the fact that such enhancement of the tungstate-induced activation of the ERK pathway found in HEK$\alpha\beta$ cells is prevented by either PTX or IbTX, supports the involvement of both $G_{i/o}$ proteins and BK channels in this tungstate-mediated signalling process. Third, as indicated above in this Discussion section, the activation of $G_{i/o}$ proteins is not required for the tungstate-induced activation of BK$\alpha\beta_1$ channels, since such modulatory effect of tungstate remains unaltered even in the presence of the G protein inhibitors PTX or GDPβs. This observation is consistent with a direct binding of tungstate to the BK α subunit. Finally, BK$\alpha\beta_1$ channels seems to be upstream in the tungstate-induced, $G_{i/o}$ protein-mediated ERK phosphorylation pathway. Thus, tungstate only activated heterologously expressed G_i proteins (indicated by an increase in FRET among Gα_i-YFP and CFP-Gβ subunits) in

HEK$\alpha\beta_1$ cells, but no in HEKα cells, an effect that was prevented by the blockade of BK$\alpha\beta_1$ channels with IbTX. These results altogether suggest that BK$\alpha\beta_1$ channels may well act as tungstate receptors to trigger the activation of the ERK pathway.

How the targeting of BK$\alpha\beta_1$ channels by tungstate leads to the activation of $G_{i/o}$ proteins is an issue that deserves further research. The fact that this signalling process is abolished in the presence of the BK channel blocker IbTX, might suggest that the conduction of K^+ ions through the channel activated by tungstate is involved in G protein activation. IbTX shares a high sequence identity (around 68%) with charybdotoxin (ChTX) and a similar mechanism for BK channel blockade has been suggested[52]. The exact interaction site for the toxins is not clear but the involvement of residues around the outer mouth pore of the channel has been proposed[46]. Furthermore, some residues in the extracellular loop of the BK regulatory β_1 subunit are responsible for the high BK channel affinity for ChTX[49,139]. As already commented, these β_1 loop amino acids are in close proximity to the external mouth and, perhaps, the selectivity filter and gate of the channel[139-141]. Therefore, we cannot rule out the possibility that toxin binding to the channel might also modify the structural changes related with the activation of the voltage sensor and channel gating. Then, toxin impairment of the

conformational changes in the BK$\alpha\beta_1$ channel produced by tungstate might affect the coupling of channel gating to G protein activation without the need of K^+ conduction. Whether BK α and/or β_1 channel subunits can directly interact with G proteins is unknown. However, there are evidences that support the existence of a direct protein-protein crosstalk among BK channels and some G-protein coupled receptors, such as μ-opioid[226]or thromboxane A2 receptors[107].

BK channels would not be the only Ca^{2+}- and voltage-dependent K^+ channel involved in ERK phosphorylation. IK1 (or KCNN4) channels have been also related to the direct activation of the ERK signalling pathway in a way that is independent of its capability to conduct K^+ ions[188].

Targeting of BK channels by tungstate as therapeutical strategy?

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 been already reported[207]. 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

normolipidemic and normotensive. The results obtained did not reveal significant changes neither in glucose/lipid metabolism nor in blood pressure after the active treatment with tungstate[207].

The reasons for the discrepancy in the tungstate actions between rodents and humans remain to be elucidated. The lack of efficacy of tungstate in humans may be attributed to the duration of the active treatment being not long enough, to the dose of sodium tungstate being too low or to alterations in the expression of enzymes involved in the regulation of energy homeostasis[207]. Although in one parameter, there was agreement between the laboratory animals and humans: tungstate does not affect blood pressure in normotensive subjects[207,208]. This observation, together with the fact that the vasodilatory concentration of tungstate used in this thesis (100 μM) is only slightly higher than the tungstate levels measured in the plasma of both treated rodent models and humans (~5-20 μM)[198,207] , also invites to continue with future studies in order to test the utility of tungstate as antihypertensive agent in humans, either by itself or in combination with other therapeutic tools.

Besides the role of BK channels in the regulation of the vascular tone, the functional relationship between BK$\alpha\beta_1$ channels and the $G_{i/o}$ protein-ERK signaling cascade might

also have physiological and/or pathological relevance in the vascular beds. In this sense, VSMC change their contractile phenotype towards a more proliferative phenotype during the pathogenesis of vascular diseases, such as hypertension or hyperlipidemia[227]. However, these dedifferentiated VSMC can gradually regain contractile functions in a process mediated by PTX-sensitive G proteins (in particular, Gβγ dimmers) that involves the activation of the ERK pathway[228,229]. Therefore, targeting of vascular BK channels by tungstate to enhance ERK phosphorylation might also help in this redifferentiation process.

Finally, given the potential therapeutic use of BK channel openers, we believe that our analysis of the molecular mechanisms underlying the positive modulatory action of tungstate on the BK channel may also help to improve the design of new channel opening drugs.

V. CONCLUSIONS

1. Tungstate has a dual effect on BK channel. In the one hand, millimolar tungstate reduced the amplitude of K^+ currents through heterologously expressed BK channels (either in the absence or presence of different regulatory β subunits, β_1-β_4). In the other hand, tungstate selectively favoured voltage-dependent activation of BK channels containing either the β_1 or the β_4 (but no β_2 or β_3) subunits, an effect that required the Mg^{2+}-sensitivity of the channel.

2. At micromolar levels (100 μM), tungstate promotes voltage-dependent activation of the vascular (β_1 subunit-containing) BK channel without lessening BK current amplitude.

3. The Mg^{2+}- and β_1-dependent activation of BK channels by tungstate was enhanced at 10 μM cytosolic Ca^{2+}, corresponding to Ca^{2+} levels reached in the cytosol during myocyte contraction. Still, this enhanced BK channel activation at high Ca^{2+} is fully dependent on the Mg^{2+}-sensitivity of the BK α channel subunit.

4. The decrease in BK current magnitude by 1 mM tungstate may explain its constriction effect on arterial rings from both WT and β_1-knockout mice.

5. In agreement with its positive and β_1-dependent effect on BK channel function, 100 μM tungstate induced vasodilation of wild-type but not β_1-knockout mouse arteries precontracted with endothelin-1, an action that was not related to the inhibition of the endothelial xanthine oxidase.

6. The requirement of BK channel Mg^{2+}-sensitivity of the tungstate-mediated channel, along with the comparative analysis of available structural data on tungstate-protein complexes, suggest that the putative tungstate binding site may consist of aspartate-lysine/lysine-aspartate motifs placed at the BK channel α subunit, close to amino acidic residues of both the voltage sensor and the RCK1 domains delimiting the binding site for Mg^{2+}.

7. Alanine substitution mutagenesis studies indicate that Y74 and S104 residues in two conserved segments (named A and B) of the large β_1 extracellular loop, which promote β_1-dependent stabilization of the BK channel voltage sensor in its active state, also play a key role in channel activation by tungstate without being involved in the Mg^{2+}-sensitivity of the channel.

8. Our results suggest that the β-dependence of BK channel activation by tungstate rely on how the different β subunits interact with the BK α channel subunit to

modulate the regulation of voltage sensor activation and channel gating.

9. Targeting of BK channels containing the β_1 subunit by tungstate, promotes the activation of PTX-sensitive G_i proteins to enhance the tungstate-induced phosphorylation of ERK.

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