DiBAC$_4$(3) hits a “sweet spot” for the activation of arterial large-conductance Ca$^{2+}$-activated potassium channels independently of the β$_1$-subunit

Fabiana S. Scornik, Ronald S. Bucciero, Yuesheng Wu, Elisabet Selga, Cristina Bosch Calero, Ramon Brugada, and Guillermo J. Pérez

1Cardiovascular Genetics Center, Institut de Investigació Biomèdica de Girona, and Department of Medical Sciences, School of Medicine, University of Girona, Girona, Spain; and 2Masonic Medical Research Laboratory, Utica, New York

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DiBAC$_4$(3) hits a “sweet spot” for the activation of arterial large-conductance Ca$^{2+}$-activated potassium channels independently of the β$_1$-subunit. Am J Physiol Heart Circ Physiol 304: H1471–H1482, 2013. First published March 29, 2013; doi:10.1152/ajpheart.00939.2012.—The voltage-sensitive dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC$_4$(3)] has been reported as a novel large-conductance Ca$^{2+}$-activated K$^+$ (BK) channel activator with selectivity for its β$_1$- or β$_4$-subunits. In arterial smooth muscle, BK channels are formed by a pore-forming α-subunit and a smooth muscle-abundant regulatory β$_1$-subunit. This tissue specificity has driven extensive pharmacological research aimed at regulating arterial tone. Using animals with a disruption of the gene for the β$_1$-subunit, we explored the effects of DiBAC$_4$(3) in native channels from arterial smooth muscle. We tested the hypothesis that, in native BK channels, activation by DiBAC$_4$(3) relies mostly on its α-subunit. We studied BK channels from wild-type and transgenic β$_1$-knockout mice in excised patches. BK channels from brain arteries, with or without the β$_1$-subunit, were similarly activated by DiBAC$_4$(3). In addition, we found that saturating concentrations of DiBAC$_4$(3) (~30 μM) promote an unprecedented persistent activation of the channel that negatively shifts its voltage dependence by as much as −300 mV. This “sweet spot” for persistent activation is independent of Ca$^{2+}$ and/or the β$_1$-,α-, and β$_4$-subunits and is fully achieved when DiBAC$_4$(3) is applied to the intracellular side of the channel. Arterial BK channel response to DiBAC$_4$(3) varies across species and/or vascular beds. DiBAC$_4$(3) unique effects can reveal details of BK channel gating mechanisms and help in the rational design of BK channel activators.

BK channels; arterial smooth muscle; DiBAC$_4$(3); KCNMA1; KCNMB1

LARGE-CONDUCTANCE Ca$^{2+}$-activated K$^+$ (BK) channels belong to the family of ion channels with six transmembrane domains per subunit (6TM) and can be activated by both membrane depolarization and increase in intracellular Ca$^{2+}$ (14, 16). In smooth muscle as well as in several other tissues, BK channels are formed by two different types of subunits, termed α- and β-subunits. The α-subunit contains the pore-forming region, whereas the auxiliary β-subunit has regulatory functions (15, 21).

The smooth muscle β$_1$-subunit has been shown to have a crucial role as a molecular tuner for vasoregulation (3, 24). Downregulation of the β$_1$-subunit was shown to have a role in a model of acquired hypertension (1) as well as in a model of genetic hypertension (2). Conversely, a polymorphism of the β$_1$-subunit that promotes a gain of function of the BK channel is associated with a low prevalence of diastolic hypertension in humans (12). Along these lines, a gain of function of arterial BK channels was found in a model of hemorrhagic shock, associated with an increased expression of the β$_1$-subunit (36). Moreover, the β$_1$-subunit expression was found to be reduced in animal models of diabetes (17, 18). A recent report, however, found that animals lacking the β$_1$-subunit were not hypertensive, adding controversy to the role of this subunit (31). However, using the same animal model, Zheng et al. (37) did confirm more recently that β$_1$-knockout (KO) mice have elevated blood pressure. Besides these controversies, it is apparent that the tissue specificity of the β$_1$-subunit remains undisputed, what probably makes worthwhile the search for a specific BK channel β$_1$-subunit pharmacology. This emerging strategy has increased over the past years (27, 28). Among some BK channel openers, β-estradiol (29), tamoxifen (9, 10, 22), dehydrosoyasaponin-I (19), and lithocholate (5) were initially described as β$_1$-subunit-specific enhancers of BK channel activity. Morimoto et al. (20) reported that the slow response voltage-sensitive dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC$_4$(3)] has a specific selectivity for recombinant BK channels formed by α- and β$_1$- or β$_4$-subunits. DiBAC$_4$(3) was found ineffective to modify the activity of other Ca$^{2+}$-dependent 6TM potassium channels such as the small-conductance Ca$^{2+}$-dependent channels rSK2 and mSK4. DiBAC$_4$(3) did not modify the activity of the voltage-gated potassium channels rKv1.1 and rKv4.3 either. According to the same study, there is also evidence that DiBAC$_4$(3) does not affect the activity of voltage-gated Ca$^{2+}$ channels, which are members, in this case, of the 24TM ion channel family. Thus the selectivity of DiBAC$_4$(3) for ion channels appeared to be restricted to the specific subset of BK channels formed by α + β$_1$- or α + β$_4$-subunits.

In the present work we explored the effects of DiBAC$_4$(3) on single BK channels from mouse cerebral arteries, obtained from animals with targeted disruption of the gene for the β$_1$-subunit. Since BK channels from arterial smooth muscle are known to be formed by α + β$_1$ complexes, we used wild-type (WT) and transgenic β$_1$-KO mice to compare the effect of DiBAC$_4$(3) at the single-channel level in a wide range of conditions in inside-out membrane patches. We also expanded the studies to the cardiac voltage-gated sodium channel in whole cell recordings. Sodium channels belong to the branch of the 24TM ion channel family that was previously untested for DiBAC$_4$(3).

We found that DiBAC$_4$(3) can selectively enhance the activity of both WT and KO BK channels, suggesting that DiBAC$_4$(3) binding activity should reside in the α-subunit.
MATERIALS AND METHODS

Tissue and cell isolation. Wild-type C57Bl/6 (Black 6) mice (Jackson Laboratory, Bar Harbor, ME) and β1-subunit KO mice (4) were used in the experiments. The genotype of the mice was confirmed by PCR analysis of DNA obtained from mouse tails following euthanization of the animals. Murine arterial smooth muscle cells were isolated as previously described (22). Cells were kept on ice until use within 24 h. Mouse thoracic arteries for real-time PCR studies were quickly collected and stored at −80°C in RNA later solution (Qiagen, Valencia, CA) until use. Adult mongrel dogs were anesthetized with intravenous pentobarbital sodium (35 mg/kg), and their hearts were rapidly removed and placed in nominally Ca2+-free Tyrode solution. Epidural sections of the left ventricular wall containing descending branches of the circumflex coronary artery were harvested to dissect the arteries. Coronary arteries were dissected and cleaned in ice-cold low calcium solution, and smooth muscle cells were enzymatically obtained by adapting the protocol for murine cells. Only elongated cells displaying a smooth surface were used throughout the study. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, Revised 1996) and was approved by the Institutional Animal Care and Use Committee of the Masonic Medical Research Laboratory.

Heterologous expression of human cardiac sodium (Na,1.5) channels. Modified human embryonic kidney cells 293 expressing the large T-antigen of the SV-40 virus (HEK-293T) were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum (GIBCO, Invitrogen, Carlsbad, CA) in 35-mm culture dishes in a 5% CO2 incubator at 37°C. Cells were cotransfected with Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum (GIBCO, Invitrogen, Carlsbad, CA) in 35-mm culture dishes in a 5% CO2 incubator at 37°C. Cells were cotransfected with human SCN5A cDNA subcloned into the mammalian expression vector pcDNA3, and green fluorescent protein as a transfection marker.

Real-time and reverse-transcription PCR. Total RNA was obtained with RNAeasy mini kit (Qiagen) from excised membrane patches of isolated arterial myocytes in symmetrical solutions containing (in mM) 140 KCl, 10 HEPES (pH 7.2), 1 MgCl2, 5-N-(2-hydroxyethyl)ethylendiamine-N,N’N-triacetic acid (HEDTA), and typically a Ca2+-free concentration of 3 μM, unless stated otherwise. Three micromoles is a Ca2+-concentration expected to be easily reached during a calcium spark in vascular smooth muscle (23). Free Ca2+ concentrations were calculated with MaxChelator software (C. Patton, Stanford University) and adjusted with CaCl2. Final free Ca2+ concentration was measured with a Ca2+-sensitive electrode calibrated according to manufacturer instructions (World Precision Instruments, Sarasota, FL). Single-channel activity was recorded at steady potentials indicated in the text with an Axopatch 200B amplifier (Axon CNS; Molecular Devices, Sunnyvale, CA). Currents were filtered at 5 kHz and digitized at 20 kHz. All experiments were conducted at room temperature (20–22°C) and carried out in the dark because of the light-sensitive nature of DiBAC4(3). Macroscopic whole cell sodium currents (INa) were studied between 24–48 h after transfection in whole cell patch-clamp configuration. The bath solution contained (in mM) 140 NaCl, 3 KCl, 10 HEPES, 1.8 CaCl2, and 1.2 MgCl2 (pH 7.4, NaOH), and the pipette solution consisted of (in mM) 130 CsCl, 1 EGTA, 10 HEPES, 10 Na3GTP, 1 MgCl2 (pH 7.2, CsOH) and 30 μM DiBAC4(3). Osmolality was adjusted by the addition of glucose to 326 and 308 mosmol/kgH2O for bath and pipette solution, respectively. Pipettes were pulled from glass capillaries (Brand GMBH + CO KG, Wertheim, Germany), and their resistance ranged from 2.5 to 3.2 MΩ when filled with the internal solution. Resistance compensation (80–90% series) was used during whole cell measurements. We typically began the recordings after a minimum waiting time of 5 min of break-in to allow for complete exchange of the intracellular solution with the electrode solution. This waiting time was empirically judged by the stabilization of INa amplitude and its reversal potential (not shown). Membrane potentials were not corrected for junction potentials that arose between the pipette and bath solution. Data were typically filtered at 5 kHz and sampled at 20 kHz.

Chemicals. DiBAC4(3) was obtained from Invitrogen. DiBAC4(3) was dissolved in DMSO to a 25-mM stock solution, aliquoted, and stored at −20°C until use. Papain, elastase, and trypsin were obtained from Worthington (Lakewood, NJ). Pentobarbital sodium (26%; Sleepaway) was obtained from Fort Dodge Laboratories (Fort Dodge, IA). All the rest of the chemicals were obtained from Sigma Chemical (St. Louis, MO).

Data analysis and statistics. Single-channel data analysis was performed with Clampfit 9.0 (Axon CNS) using either all-point amplitude histogram or by event detection with 50% amplitude criteria. In multichannel patches, the probability (Po) of occupying each open level (k) gives rise to channel number times open probability (NPo), according to NPo = ∑kPkPo, then was determined by normalizing NPo values by channel number (N). The N number of channels present in any given excised patch was estimated from all points histograms at depolarized voltages (≥+60 mV). Although detailed current kinetic models of BK channel activity support several closed and open states, we simplified our unitary channel analysis, lumping together all closed and all open states. We consider that this assumption still provides, with limitations, a reasonable description of our data in a simplified form. Curve fitting and linear regression analyses were performed with Origin 6.1 software (OriginLab, Northampton, MA). When possible, DiBAC4(3) concentration response curves of mean data were fit to a Hill equation of the following form: 

\[ P_o = P_{o \text{ min}} + (P_{o \text{ max}} - P_{o \text{ min}})/(1 + [I]/IC_{50}^{0.5}) \]

where \( P_{o \text{ max}} \) and \( P_{o \text{ min}} \) are the maximum and minimum \( P_o \) asymptotes, respectively; \( EC_{50} \) is the concentration of DiBAC4(3) to obtain half maximal \( P_o \); \( x \) is the DiBAC4(3) concentration used, and \( N_H \) represents the slope factor of the Hill equation. Voltage activation curves for single-channel experiments were fitted with a two states Boltzmann equation of following form: 

\[ P_o = P_{o \text{ max}} + (P_{o \text{ min}} - P_{o \text{ max}})/[1 + \exp(V - V_{1/2})/V_{1/2}] \]

where \( V_{1/2} \) and \( V_{1/2} \) are the voltage at which half maximal \( P_o \) and \( P_{o \text{ max}} \) are obtained.
DiBAC₄(3) activates native BK channels from murine arterial smooth muscle in the presence or in the absence of the β₁-subunit. We studied the concentration-dependent characteristics of native BK-channel activation by DiBAC₄(3) in the presence and the absence of the β₁-subunit. Figure 1A shows single-channel activity of BK channels from WT animals at different concentrations of DiBAC₄(3). An increase in \( P₀ \) becomes manifest at submicromolar concentrations of DiBAC₄(3), whereas maximal activity is achieved at saturating concentrations (30 μM). Concentration-dependent activation can also be observed for channels from KO animals (Fig. 1C). Figure 1E summarizes the concentration-dependent effect of DiBAC₄(3) on BK channels from WT and KO animals. No significant differences were found between WT and KO at any concentration point studied (2-way ANOVA). This suggests that in native BK channels, DiBAC₄(3) acts similarly in the presence or in the absence of the β₁-subunit. Although a concentration-response relationship can be observed for both WT and KO conditions, the actual deviation of this data set from a Hill function model prevented significant fitting to the equation. High-resolution concentration-response curves with several titration points will be needed for a detailed analysis of the role of the β₁-subunit in the activation of BK channels by DiBAC₄(3). In our conditions, such extensive studies are limited by the technical hindrance of the patch stability needed for long recordings with several solution exchanges.

**RESULTS**

DiBAC₄(3) activates native BK channels from murine arterial smooth muscle in the presence or in the absence of the β₁-subunit. We studied the concentration-dependent characteristics of native BK-channel activation by DiBAC₄(3) in the presence and the absence of the β₁-subunit. Figure 1A shows single-channel activity of BK channels from WT animals at different concentrations of DiBAC₄(3). An increase in \( P₀ \) becomes manifest at submicromolar concentrations of DiBAC₄(3), whereas maximal activity is achieved at saturating concentrations (30 μM). Concentration-dependent activation can also be observed for channels from KO animals (Fig. 1C). Figure 1E summarizes the concentration-dependent effect of DiBAC₄(3) on BK channels from WT and KO animals. No significant differences were found between WT and KO at any concentration point studied (2-way ANOVA). This suggests that in native BK channels, DiBAC₄(3) acts similarly in the presence or in the absence of the β₁-subunit. Although a concentration-response relationship can be observed for both WT and KO conditions, the actual deviation of this data set from a Hill function model prevented significant fitting to the equation. High-resolution concentration-response curves with several titration points will be needed for a detailed analysis of the role of the β₁-subunit in the activation of BK channels by DiBAC₄(3). In our conditions, such extensive studies are limited by the technical hindrance of the patch stability needed for long recordings with several solution exchanges.

**Kinetic analysis reveal that DiBAC₄(3) accelerates BK channel's opening rate.** To gain mechanistic insights into the activating effect of DiBAC₄(3) on BK channels, we analyzed single-channel kinetics in membrane patches containing only one channel. Figure 2A illustrates the changes in unitary channel activity at increasing concentrations of DiBAC₄(3) recorded at 3 μM free Ca²⁺ at the indicated voltages (−20 mV for WT and 20 mV for KO). These voltages were selected to start the experiments with a similar initial \( P₀ \). Representative traces from both WT and KO conditions illustrate how micromolar concentrations of DiBAC₄(3) promote an increase in channel activity. Channel \( P₀ \) from both WT and KO, respectively, escalates from 0.06 and 0.09 at 0.1 μM DiBAC₄(3), to 0.58 and 0.68 at 10 μM DiBAC₄(3), and to 0.95 and 0.93 at saturating 30 μM DiBAC₄(3). The increased \( P₀ \) can be largely

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**Fig. 1.** The effect of bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC₄(3)] is concentration dependent and reversible. A: representative large-conductance Ca²⁺-activated K⁺ (BK) channel activity, recorded at −20 mV and 3 μM Ca²⁺, from a patch with 5 active wild-type (WT) channels showing the increase in activity with increasing concentrations of DiBAC₄(3) and the washout of the effect. B: corresponding all points histogram illustrating the change in activity and the widening of open-channel noise distribution at high concentration of DiBAC₄(3). C: knockout (KO) BK channel activity recorded from a patch with 4 active channels at −20 mV and 3 μM Ca²⁺, illustrating the change in activity with increasing concentrations of DiBAC₄(3) and its washout. Arrows indicate the zero current level. D: diary plot corresponding to C showing time course changes in open probability (\( P₀ \)) with increasing concentrations of DiBAC₄(3). E: concentration-dependent effect of DiBAC₄(3) on BK channels from WT and KO animals. Mean data points were obtained from 3 to 8 patches in WT and 12 to 18 patches in KO.
Fig. 2. DiBAC_4(3) promotes an increase in the apparent opening rate of BK channels. A: unitary channel activity at increasing concentrations of DiBAC_4(3) recorded at 3 μM free Ca^{2+} at the indicated voltages. P_o increased with DiBAC_4(3) from 0.06 and 0.09 at 0.1 μM DiBAC_4(3), to 0.58 and 0.68 at 10 μM DiBAC_4(3), to 0.95 and 0.93 at saturating 30 μM DiBAC_4(3) (WT and KO, respectively). Arrows indicate the zero current level. B: corresponding apparent mean opening (1/τ_o) and mean closing rates (1/τ_c, inset) plotted as a function of DiBAC_4(3) concentration. 1/τ_o constant was steeply DiBAC_4(3) dependent over the examined range of DiBAC_4(3) concentrations for both WT and KO channels. The points were fitted to a linear regression.

explained in both WT and KO by a decrease in the mean closed time of the channel (τ_c), most likely reflecting a shortening of inter burst time, as can be intuitively perceived from the traces. Indeed, the analysis shown in Fig. 2B supports this perception, where the apparent mean opening and mean closing rates (Fig. 2B, inset) are plotted as a function of DiBAC_4(3) concentration. The apparent mean opening rate (1/τ_o) was examined over a range of DiBAC_4(3) concentrations for both WT and KO channels. The points were fitted to a linear regression that provided very similar slopes between WT and KO (0.77 ± 0.08 vs. 0.70 ± 0.02 M^{-1}s^{-1}, WT and KO, respectively). On the other hand, the apparent mean closing rate (1/τ_c) was considerably less affected by DiBAC_4(3).

High concentrations of DiBAC_4(3) cause a robust persistent activation of BK channels. We investigated the new phenomenon of persistent activation of BK channels at high concentrations of DiBAC_4(3). At 30 μM DiBAC_4(3), arterial BK channels from either WT or KO animals display a high-P_o mode characterized by a reduction in channel’s unitary conductance and an increase in open-channel noise. The high-P_o mode prevails even at unusual hyperpolarized voltages (< −100 mV). Figure 4 illustrates these observations, showing the activity of a unitary channel recorded in control conditions during a series of voltage steps (from −80 to +80 mV, Fig. 4A), and after the application of 30 μM DiBAC_4(3) (from −45 to −235 mV, Fig. 4B). In control conditions, no channel activity can be observed for voltages more negative than −50 mV (Fig. 4A). However, after application of 30 μM DiBAC_4(3), channel activity can be recorded at hyperpolarizing potentials as low as −235 mV (Fig. 4B), a point where patch electrical stability becomes seriously compromised.

Figure 4C illustrates the changes in all points histograms of the same channel activity as in Figs. 4, A and B, before (control) and after the application of 30 μM DiBAC_4(3) at selected voltage steps (−80, −40, +40, and +80 mV). Despite the fact that DiBAC_4(3) promotes the appearance of prominent activity at the negative potentials, it can be clearly observed that open-channel amplitude distributions widen and peak amplitudes abbreviate (see values in the figure legend). The reason for this effect in channel conductance and noise will require further investigation.
We next analyzed the voltage dependence of BK channel activity in the high-\(P_o\) mode elicited by 30 \(\mu\)M DiBAC4(3). Figure 5A shows a dramatic effect of 30 \(\mu\)M DiBAC4(3), which produces a 144-mV shift in the Boltzmann activation curve, recorded in 3 \(\mu\)M free Ca\(^{2+}\) in the bath. Similarly, KO BK channels recorded in the same conditions also display a dramatic shift (~190 mV) in the Boltzmann activation curve after the treatment with DiBAC 4(3) (Fig. 5B). The estimated apparent gating charge (\(z\)) of the channels, at 20°C, remains similar for WT (1.7 to 1.5), whereas it becomes reduced to around a half of the control value (2.8 to 1.44) in KO BK channels [control vs. 30 \(\mu\)M DiBAC4(3), respectively]. Thus changes in free-energy \(\Delta G_o\) promoted by saturating DiBAC4(3) can be approximated to ~5 kcal/mol for WT BK channels and ~7.6 kcal/mol for KO BK channels. A simple interpretation of this difference is that it probably reflects the permissive work done by the \(\beta_1\)-subunit to facilitate channel opening (6). This, in turn, decreases the work that DiBAC 4(3) binding must do to open the channel.

We next explored BK channel activation with saturating DiBAC4(3) in the complete absence of cytosolic Ca\(^{2+}\). We bathed the excised patches in a zero calcium solution containing no added Ca\(^{2+}\) and 11 mM EGTA (solution contained only 140 mM KCl, 11 mM EGTA, and 10 mM HEPES). Under these extreme conditions, extreme voltages have to be applied before and after the DiBAC4(3) treatment to cover both ends of channel activity. This protocol is usually very harmful for the patch. Nevertheless, a successful experiment in these conditions is shown in Fig. 5D using a membrane patch from a canine arterial myocyte. In this case, a huge shift in the voltage dependence was observed. In the presence of 30 \(\mu\)M DiBAC4(3), \(V_{1/2}\) reaches the value of ~153 mV, providing an estimated shift of around ~300 mV, although in the absence of DiBAC4(3), data from this single experiment are too scattered to justify a Boltzmann fitting. Similar observations were also obtained in a separate experiment with canine arterial myocytes and in two additional experiments using WT mouse arterial myocytes (not shown). In the latter preparation, at 100 \(\mu\)M free Ca\(^{2+}\), we also observed the same dramatic DiBAC4(3)-dependent shift in activation (not shown). This observation suggests that DiBAC4(3) can still act in the presence of saturating Ca\(^{2+}\), implying that DiBAC4(3) does not act as a surrogate for Ca\(^{2+}\) in BK channels.

Because of the lipophilic nature of DiBAC4(3), we investigated whether the drug’s site for persistent activation of BK channel was also accessible from the extracellular side of the channel. We used excised inside-out patches bathed in 3 \(\mu\)M free Ca\(^{2+}\) with 30 \(\mu\)M DiBAC4(3) present in the pipette. Top traces in Fig. 3. BK channels from canine coronary arteries display a higher sensitivity to DiBAC4(3). A: concentration-dependent increase in BK channel activity and washout recorded at −60 mV and 3 \(\mu\)M Ca\(^{2+}\). B: concentration-dependent activation curve averaged from 5 different experiments. Membrane patches included in this curve lasted long enough to be assayed at least for 4 different concentrations of DiBAC4(3). Mean data points were fitted to a Hill function (continuous line) with an \(N_H\) of 1.5 ± 0.2 and EC\(_{50}\) of 3.4 ± 0.4 \(\mu\)M (fitting was extrapolated to 40 \(\mu\)M). C: unitary single-channel activity from canine coronary artery BK channels recorded at −40 mV and 3 \(\mu\)M Ca\(^{2+}\), illustrating the abbreviation of closed times with increasing concentrations of DiBAC4(3). D: corresponding single-channel kinetic analysis reveals that DiBAC4(3) abbreviate the mean closed time of the BK channels as shown in the logarithmic plot of the \(1/t_e\) vs. the concentration of DiBAC4(3). The linear regression fitting of the experimental \(1/t_e\) obtained at different concentrations DiBAC4(3) provides a slope of 1.5 M\(^{-1}\)·s\(^{-1}\). Arrows indicate the zero current level.

DiBAC(3) ACTIVATES ARTERIAL BK CHANNELS

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illustrate the channel activity recorded under those conditions immediately after patch excision. Subsequent traces show a moderate increase in channel activity at 5 min and further increase 40 min later. Figure 5D illustrates the dramatic activation observed with the additional cytosolic application of DiBAC4(3), strongly suggesting that the drug binding site is located at an intracellular domain of the channel protein, not readily accessed from the extracellular side. Figure 5E shows the corresponding steady-state mean current of the patch as a function of voltage. In a similar experiment, once the channels were fully activated, we added 100 μM Ba2+ to the bath (Fig. 6). This produced a block of the channels at depolarized potentials, indicating that DiBAC4(3)-activated BK channels can still be selectively blocked by Ba2+ in a characteristic voltage-dependent fashion.

High concentrations of DiBAC4(3) on voltage-gated sodium channels. It was previously reported that voltage-gated potassium and calcium channels were unresponsive to DiBAC4(3) (20). Unlike calcium channels, related 24TM sodium channels were previously untested for DiBAC4(3). To further explore the possible effect of the dye on this voltage-gated channel family, we studied the effect of intracellular DiBAC4(3) on the cardiac voltage-dependent sodium channel. We took advantage of the exquisite voltage-dependence of sodium channels to closely monitor the changes induced by intracellular DiBAC4(3). The dramatic activation of BK channels shown above is in sharp contrast, however, with the marginal changes that cytosolic DiBAC4(3) (30 μM) produces on Nav1.5 channels. Voltage and time-dependent characteristics of \( I_{Na} \) were studied in control conditions and in the presence of DiBAC4(3) in the pipette (30 μM). The analysis of DiBAC4(3) effect on Nav1.5 channels (Fig. 7) shows that DiBAC4(3) does not produce any major effect on the voltage-dependent properties of these channels. No changes were detected in current-voltage relationship between control versus DiBAC4(3) (Fig. 7A). However, we were able to detect a small hyperpolarizing shift in \( V_{1/2} \) (3.2 mV) in the presence of DiBAC4(3) (Fig. 7B) [from -32 ± 0.5 to -35.6 ± 0.6 mV, \( P < 0.0001 \), control vs. DiBAC4(3)]. Also, an even smaller depolarizing shift (2.4 mV) was produced by DiBAC4(3) in steady-state inactivation curves (Fig. 7B). Figure 7C shows that time-dependent recovery from inactivation of \( I_{Na} \) was not affected by DiBAC4(3). No
differences were detected between control and 30 μM DiBAC₄(3) in the pipette. These results suggest that DiBAC₄(3) is not effectively interacting with the gating machinery of sodium channels or altering the electric field of the membrane by nonspecific mechanisms. Nevertheless, these results emphasize the considerably selective action of DiBAC₄(3) on BK channels.

Subunit composition of DiBAC₄(3)-sensitive BK channels in mouse arterial smooth muscle. Heterologous expression studies using rat BK channel clones have shown that DiBAC₄(3) modulates these channels only when the β₁-subunit or, alternatively, the β₄-subunit was present. Therefore, we decided to measure the relative mRNA transcripts levels of Kcnma1, Kcnmb1, and Kcnmb4 genes corresponding to α-, β₁-, and β₄-subunits, using quantitative real-time RT-PCR in cDNA extracted from mouse arterial smooth muscle (thoracic aorta) from WT and KO animals. Table 1 illustrates the relative change in transcript levels of BK channel subunits between WT versus KO cDNA samples. The targeted deletion of the β₁-subunit does not affect the expression levels of the α-subunit, as the normalized values remained similar. Predictably, normalized values of Kcnmb1 transcripts were completely marginal in KO samples, whereas the expression levels of Kcnmb4 remained undetectable. These data strongly argue against the possibility of a compensatory mechanism raised in vascular beds from KO animals by alternative expression of β₄-subunits. In addition, it confirms further the previous functional finding that BK channel density was not affected by the targeted deletion of the β₁-subunit (4). This was also verified in RT-PCR analysis (not shown). The absence of detectable

Fig. 5. DiBAC₄(3) induces a dramatic hyperpolarizing shift of BK channel’s voltage-dependent activity. A: Boltzmann fit to mean data points from 3 different experiments using WT channels before and after the treatment with 30 μM DiBAC₄(3) recorded at 3 μM Ca²⁺. Voltage for half maximal activation (V½) changed from +14 ± 1.5 mV to −130 ± 1.7 mV with DiBAC₄(3). The slope factor (dV) changed, respectively, from 15.2 ± 1.5 to 17.15 ± 1.5 mV. B: Boltzmann activation curves at the same conditions as in A, fitted to mean data points from 6 different experiments using KO channels. V½ changed from +37 ± 1 in control to −153 ± 2 mV with DiBAC₄(3), dV also changed from 9 ± 1 to 17 ± 2 mV, control vs. DiBAC₄(3), respectively. C: even a more spectacular shift (~−300 mV) was observed in the absence of Ca²⁺ in a patch from a canine arterial myocyte. V½ changed from estimated approximately +150 in control to −153 mV with DiBAC₄(3), and dV was 19 mV. D: WT channel activity recorded at −40 and +40 mV from an excised patch with 30 μM DiBAC₄(3) present in the patch pipette, immediately after excision and 5 and 40 min later. Further addition of DiBAC₄(3) to the bath dramatically increased the activity as shown at 5 min after its application. Dotted line indicates the closed state of the channel. E: corresponding mean current vs. voltage plot showing changes over time and the further increase in channel activity with intracellular DiBAC₄(3) application, as indicated.
transcripts levels of $\beta_4$-subunit suggests that BK channel $\beta_4$-subunit expression in arterial smooth muscle is irrelevant to BK channel function and pharmacology in both WT and KO animals. These results were confirmed further by conventional RT-PCR analysis. Figure 8 shows the lack of transcript expression for subunits $\beta_1$, $\beta_4$, and LRCC26 ($\gamma_1$) in murine aortic cDNA (WT and KO). The $\gamma_1$ protein belongs to the newly described family of auxiliary proteins, termed BK channel $\gamma$-subunits, and was found to have a moderate expression in human aorta (32). These results show that neither $\beta_4$- nor $\gamma_1$-subunits are present in aortic smooth muscle from mice.
DISCUSSION

We have investigated the effect of the voltage-sensitive dye DiBAC$_4$(3) as a novel activator of BK channels. We have studied this activator in native murine BK channels with and without the β1-subunit, as well as in channels from canine arteries, and human Na$_v$1.5 channels, expressed in HEK-293T cells. We discovered that DiBAC$_4$(3) can activate native BK channels in a wide range of conditions including extreme hyperpolarizing voltages, the absence of Ca$^{2+}$, or in the absence of the β1-subunit.

DiBAC$_4$(3) activates native BK channels in the presence and in the absence of the β1-subunit. DiBAC$_4$(3) was originally reported as a novel (β1/4-subunit dependent) BK channel activator. The voltage dependence of DiBAC$_4$(3) activation is shown in Fig. 7. The effect of cytosolic 30 μM DiBAC$_4$(3) on human Na$_v$1.5 channels expressed in human embryonic kidney cells 293 expressing the large T-antigen of the SV-40 virus (HEK-293T). A, left: current-voltage relationship. Experimental points represent the normalized peak amplitude of sodium current ($I_{Na}$) at each given voltage for control (black circles, n = 8) and DiBAC$_4$(3) (white circles, n = 7). A, right: representative whole cell $I_{Na}$ traces recorded with 30 μM DiBAC$_4$(3) in the pipette. Currents were elicited by depolarizing potentials (inset). B, left: voltage dependence of $I_{Na}$ activation and steady-state inactivation. Conductance (G) values for the activation curves (rightmost curves) were obtained from the peak current values taken from A. Symbols represent experimental data plotted against the given depolarizing voltage values. Solid lines represent the Boltzmann fit of the experimental points. Leftmost curves show the effect of DiBAC$_4$(3) on steady-state inactivation curves, represented as fractional channel availability. B, right: representative current traces used to determine fractional channel availability, in the presence of 30 μM DiBAC$_4$(3) in the pipette. Currents were elicited by a step to −20 mV after 500 ms of preconditioning pulses to different potentials as shown in the inset. C, left: recovery from inactivation properties studied with a standard two-pulse protocol. The P2-to-P1 ratio of peak currents was plotted as a function of the recovery interval. Values were fitted to monoexponential functions (solid lines). C, right: representative current traces elicited by the double pulse protocol (inset) in the presence of 30 μM DiBAC$_4$(3) in the pipette.

Fig. 7. Effect of cytosolic 30 μM DiBAC$_4$(3) on human Na$_v$1.5 channels expressed in human embryonic kidney cells 293 expressing the large T-antigen of the SV-40 virus (HEK-293T). A, left: current-voltage relationship. Experimental points represent the normalized peak amplitude of sodium current ($I_{Na}$) at each given voltage for control (black circles, n = 8) and DiBAC$_4$(3) (white circles, n = 7). A, right: representative whole cell $I_{Na}$ traces recorded with 30 μM DiBAC$_4$(3) in the pipette. Currents were elicited by depolarizing potentials (inset). B, left: voltage dependence of $I_{Na}$ activation and steady-state inactivation. Conductance (G) values for the activation curves (rightmost curves) were obtained from the peak current values taken from A. Symbols represent experimental data plotted against the given depolarizing voltage values. Solid lines represent the Boltzmann fit of the experimental points. Leftmost curves show the effect of DiBAC$_4$(3) on steady-state inactivation curves, represented as fractional channel availability. B, right: representative current traces used to determine fractional channel availability, in the presence of 30 μM DiBAC$_4$(3) in the pipette. Currents were elicited by a step to −20 mV after 500 ms of preconditioning pulses to different potentials as shown in the inset. C, left: recovery from inactivation properties studied with a standard two-pulse protocol. The P2-to-P1 ratio of peak currents was plotted as a function of the recovery interval. Values were fitted to monoexponential functions (solid lines). C, right: representative current traces elicited by the double pulse protocol (inset) in the presence of 30 μM DiBAC$_4$(3) in the pipette.
opener (20). However, we also observed that DiBAC$_4$(3) can activate channels from transgenic KO animals that completely lack the expression of the $\beta_1$-subunit. Moreover, the increase of the apparent mean opening rate in WT mice is mirrored by its KO counterpart (Fig. 3B). This suggests a common mechanism regardless of the presence or absence of the $\beta_1$-subunit.

We decided next to explore the possible involvement of a compensatory $\beta_4$-subunit present in KO animals, which would provide KO BK channels with DiBAC$_4$(3) sensitivity. Our real-time RT-PCR results (Table 1) strongly suggest that such a mechanism is very unlikely since arterial tissue from KO animals do not produce any detectable level of Kcnmb4 or Kcnmb1 mRNAs. These results were confirmed in conventional RT-PCR studies (Fig. 8). Alternatively, some residual brain artery-specific expression of the $\beta_4$-subunit might be present in myocytes from brain arteries (25). We do not have direct molecular evidence to completely rule out this possibility since we have used thoracic aorta for results in Table 1. Nevertheless, brain arteries from KO animals are sensitive to iberiotoxin, whose blocking action is thought to be compromised if $\beta_4$-subunits were modulating BK channels (3, 4). Moreover, direct comparison of BK channel activities in the Boltzmann activation curve in KO versus WT mice. In addition, we were unable to detect $\gamma_1$-subunit expression in cDNAs from aortas taken from either WT or KO mice. Therefore, a role for $\gamma_1$-subunit in our murine vessels is unlikely, although we cannot completely rule out regional expression differences in brain arteries. Possible reasons for disparity of results might reside on the molecular diversity of the $\alpha$-subunit itself, where the exact isoforms present in native tissues might differ from the ones traditionally used in heterologous expression studies.

Indeed, Poulsen et al. (25) compiled up to 14 alternative splice sites for BK channels. This diversity may, in turn, shape BK channel’s pharmacological profile as happens to be the case for the activator Cym04 and the splice variant e9-alt (13). As a more generalized case, there seems to be a complex emergent picture of possible tissue-specific heteromeric arrangements of BK channels. This includes diverse $\alpha$-subunit isoforms, $\beta$-subunits, and perhaps $\gamma$-subunits. This provides new layers of complexity to account for the discrepancy between native versus recombinant BK channels in general. Other possible explanations for the disparities in results between native and recombinant BK channels might arise from tissue-specific posttranslational modifications, i.e., the complex phosphostatus of the channel protein (34) that might not be completely recapitulated when using heterologous expression systems. Nonetheless, our results clearly demonstrate that DiBAC$_4$(3) is a potent activator of native arterial BK channels that may act in the presence or in the absence of the $\beta_1$-subunit.

Mechanisms of DiBAC$_4$(3) activation of BK channels. We have demonstrated that DiBAC$_4$(3) can also activate canine BK channels from coronary arteries. However, these channels appear to be more sensitive to the dye than their murine counterparts. The fact that concentration-response plots behave differently among channels from different species (i.e., murine, canine) suggest that the interaction of DiBAC$_4$(3) with BK channels is specific. This specificity appears though to share a common mechanism that involves an increase in the apparent mean opening rates of the channel (Figs. 2B and 3D). Another common and very unusual feature is BK channel response at high concentrations of DiBAC$_4$(3) (10–30 $\mu$M). High concentrations of DiBAC$_4$(3) produce a dramatic activation of BK channels that is independent of Ca$_{2+}$ or the presence of the $\beta_1$-subunit. We explored the voltage-dependent characteristics of this novel high-$P_o$ mode of the channel and found that saturating DiBAC$_4$(3) concentrations produce a negative shift in the voltage-dependent activation of BK channels that far exceed the maximal effect of saturating concentrations of Ca$_{2+}$ and that of any other known activator of BK channels. To the best of our knowledge, no other compound is capable of producing changes in $\Delta V_{1/2}$ of $-170$ to $-300$ mV (Fig. 5C). Probably, the only exception to this is given by the newly identified LRRC26 auxiliary protein (or $\gamma_1$-subunit) of BK channels from prostate cancer cells (32, 33). The coexpression

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Fig. 8. RT-PCR analysis of BK channel accessory subunits in mouse aortas. Agarose gel separation of PCR products using equivalent primer pairs as the ones used for the real-time RT-PCR analysis depicted in Table 1. This experiment shows the lack of expression of $\beta_1$-subunit in cDNA from KO animals, as well as the lack of expression of $\beta_4$- and $\gamma_1$-subunit transcripts in both cDNAs (WT and KO). GAPDH was used as control. RT-PCR products were run on 2.5% agarose gels. Molecular marker (MK) lanes with corresponding base pair (bp) sizes are also indicated.
of this subunit produces changes (\(\Delta V_{\text{fs}}\), −135–160 mV) that are close in magnitude to the ones reported here. The radical alterations in the voltage dependence of BK channels produced by saturating DiBAC\(_4(3)\) also introduces relatively strong changes in free energy (from −5 to −7.6 kcal/mol; WT and KO, respectively) that are even larger than the change produced by LRRC26 (−4 kcal/mol) (33).

Since DiBAC\(_4(3)\) itself is a negatively charged lipophilic compound, it is possible that these negative charges interact from the outer leaflet of the lipid bilayer with positively charged residues of the voltage sensor in the S4 segment. This mechanism was proposed by Schmidt et al. (26) for the stabilizing interactions between arginine residues of the voltage sensor and negatively charged lipid phosphodiester groups of the cell membrane. However, high concentrations of DiBAC\(_4(3)\) applied from the extracellular side failed to fully activate BK channels (Figs. 5D and 6). This evidence argues against an effect of negatively charged DiBAC\(_4(3)\) from the outer leaflet either by interacting with positive residues of the channel protein or by a charge screening effect. Moreover, DiBAC\(_4(5)\), a closely related derivative of DiBAC\(_4(3)\), can translocate the sarcolemma in <30 ms during a depolarizing voltage step (i.e., +60 mV) (11). This evidence suggests that DiBAC\(_4(3)\) translocation will be totally accomplished in our experimental conditions of Fig. 5D. However, further full activation obtained in the same patch with 30 \(\mu\)M DiBAC\(_4(3)\) in the bath reveals a marked differential sidedness of action that is not achievable by simply translocating DiBAC\(_4(3)\) molecules across the membrane. This sidedness of action also argues against nonspecific mechanisms for channel activation that help us to exclude micellization of DiBAC\(_4(3)\) or alterations in the local environment of the channel, like membrane curvature, known to modify the activity of BK channels (35). Moreover, the effect of the dye from the intracellular side is not observed in sodium channels where their voltage and time-dependent properties remain largely unaffected by 30 \(\mu\)M DiBAC\(_4(3)\) (Fig. 7). In light of previous results showing refractoriness to this dye by \(\kappa_{\text{v}.1}\) and \(\kappa_{\text{v}.4.3}\) constructs (20), our results underscore the rather selective action of DiBAC\(_4(3)\) on BK channels. Related voltage-dependent ion channels from the 6TM family, or from the 24TM sodium channel family shown here, are largely unaffected by the dye. This also stresses the idea that DiBAC\(_4(3)\) actions are BK channel specific and not associated to membrane-related effects of the dye. It is tempting to speculate that DiBAC\(_4(3)\) might have a binding site, preferentially accessed from the cytosolic side, that directly or indirectly alters the pore-gating domain of the channel located in the S6 transmembrane domain (deep pore) of the channel. The effect of saturating DiBAC\(_4(3)\) presented here is reminiscent of constitutively active channels shown in S6 domain modification studies (7, 8, 30, 38). In addition, the observed reduction of single-channel amplitude indicates that DiBAC\(_4(3)\) binding can create a partial occlusion of the ion permeation pathway, suggesting also a possible interaction with the deep pore S6 region. Indeed, the combined high \(P_0\) and partial occlusion of the permeation pathway reported here has also been observed with some cysteine modifying agents in the S6 region of BK channels (38). Nevertheless, we cannot completely rule out at this point that the binding site can be located at an alternative distant region from the pore or even at a nearby closely associated protein different from the \(\alpha\)-subunit.

Overall, the significance of our present work is twofold. First, we provide direct evidence revealing that the \(\beta_1\)-subunit is not responsible for the activation of native arterial BK channels by DiBAC\(_4(3)\). As discussed above, this stresses the idea that the true molecular, functional, and pharmacological identity of native arterial BK channels remains incomplete. This implies that cautious interpretation is required when using recombinant models and, in particular, when these models are used to extrapolate channel subunit arrangement and pharmacology in living organisms. Second, we have discovered that arterial BK channels can be hit in a drug-accessible “sweet spot” that completely opens the channel. This has significant implications in our current understanding of BK channel function, suggesting that these channels can be shifted, with relative ease, to a new unexplored gating mode. This discovery provides, at the same time, an extraordinary opportunity for the development of suitable drugs that can massively tune a K\(^+\) conductance in arterial smooth muscle.

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Present address of F. S. Scornik and G. J. Pérez: Cardiovascular Genetics Center, IDIBGI-University of Girona, Girona, 17003, Spain.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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