Voltage-sensitive oxonol dyes are novel BK channel activators
selective for β1 and β4 but not for β2 subunits

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a) Running title: DiBAC4(3) is a novel β-subunit-selective BK channel opener

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c) Number of text pages: 29
Tables: 1 Figures: 9
References: 40 Words in the Abstract: 245
Introduction: 717 Discussion: 1383
d) Abbreviations
BK channel: large-conductance Ca2+-activated K+ channel, SK channel: small-conductance Ca2+-activated K+ channel, Kv channel: voltage-gated K+ channel, K_ATP channel: ATP-sensitive K+ channel, VDCC: voltage-dependent Ca2+ channel, AE: anion exchanger, mUBSMCs: mouse urinary bladder smooth muscle cells, HEK293 cells: human embryo kidney 293 cells, BKα: BK channel α subunit, BKβ: BK channel β subunit, BKαβ1: BK channel α plus β1 subunits, BKαβ2: BK channel α plus β2 subunits, BKαβ4: BK channel α plus β4 subunits, hBKβ2-IR: human BK channel β2 subunit without the inactivation domain, HEK-rBKα: HEK293 cells expressing rat BKα, HEK-rBKαβ1: HEK293 cells expressing rat BKαβ1, HEK-rBKαβ2: HEK293 cells expressing rat BKαβ2, HEK-rBKαβ4: HEK293 cells expressing rat BKαβ4, HEK-rSK2: HEK293 cells expressing rat SK2 channel, HEK-mSK4: HEK293 cells expressing mouse SK4 channel, HEK-rKv1.1: HEK293 cells expressing rat Kv1.1 channel, HEK-rKv4.3: HEK293 cells expressing rat Kv4.3 channel, [Ca2+]: intracellular Ca2+ concentration, Po: open probability, NPo: number of channels (N) times open probability (Po), FLIPR: fluorometric imaging plate reader, GFP: green fluorescent protein, DMSO: dimethyl sulfoxide, DHS-I: dehydrosoyasaponin-I, BA: barbituric acid, DMBA: 1,3-dimethylbarbituric acid, DETBA: 1,3-diethyl-2-thiobarbituric acid, DiBAC: bis(1,3-dialkylbarbituric acid)oligomethine oxonol, DiSBAC: bis(1,3-dialkylthiobarbituric acid)oligomethine oxonol, DiBAC4(3): bis(1,3-dibutylbarbituric acid)trimethine oxonol, DiBAC4(5): bis(1,3-dibutylbarbituric acid)pentamethine oxonol, DiSBAC4(3): bis(1,3-dibutylthiobarbituric acid)trimethine oxonol, DiSBAC4(5): bis(1,3-dibutylthiobarbituric acid)pentamethine oxonol, DiSBAC2(3): bis(1,3-diethylbarbituric acid)trimethine oxonol, DiSBAC2(5): bis(1,3-diethylthiobarbituric acid)pentamethine oxonol, DiSBAC2(1): bis(1,3-diethylthiobarbituric acid) methine oxonol, DiSBAC2(5): bis(barbituric acid)pentamethine oxonol, DiSBAC10(3): bis(1,3-didecylthiobarbituric acid)trimethine oxonol, Oxonol 595: bis(3-cyano-1-ethyl-4-methyl-2,6-dideoxy-1,2,5,6-tetrahydropyridine)trimethine oxonol, Oxonol V: bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol, Oxonol VI: bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol
Abstract

The large-conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channel is activated by both the rise of intracellular Ca\(^{2+}\) concentration and membrane depolarization. The BK channel plays crucial roles as a key molecule in the negative feedback mechanism regulating membrane excitability and cellular Ca\(^{2+}\) in various cell types. Here, we report that a widely used slow-response voltage-sensitive fluorescent dye, DiBAC\(_{4}(3)\) (bis(1,3-dibutylbarbituric acid)trimethine oxonol), is a potent BK channel activator. The application of DiBAC\(_{4}(3)\) at concentrations of 10 nM and higher significantly increased whole-cell BK channel currents in HEK293 cells expressing rat BK channel \(\alpha\) and \(\beta1\) subunits (rBK\(\alpha\beta1\)). In the presence of 300 nM DiBAC\(_{4}(3)\), the activation voltage of the BK channel current shifted to the negative direction by approximately 30 mV, but the single-channel conductance was not affected. DiBAC\(_{4}(3)\) activated whole-cell rBK\(\alpha\beta1\) and rBK\(\alpha\beta4\) currents in the same concentration range but partially blocked rBK\(\alpha\beta2\) currents. The BK channel \(\alpha\) subunit alone and some other types of K\(^{+}\) channels examined were not markedly affected by 1 \(\mu\)M DiBAC\(_{4}(3)\).

Structure-activity relationship analyses revealed that a set of oxo- and oxoanion-moieties in two 1,3-dialkylbarbituric acids, which are conjugated by oligomethine, is the novel skeleton for the \(\beta\)-subunit-selective BK channel opening property of DiBAC\(_{4}(3)\) and related oxonol compounds. This conjugated structure may be located stereochemically in one plane. These findings provide a molecular and structural basis for understanding the regulatory mechanism of BK channel activity by an auxiliary \(\beta\) subunit and will be fundamental to the development of \(\beta\)-selective BK channel openers.
The large-conductance Ca\textsuperscript{2+}-activated voltage-gated K\textsuperscript{+} (BK) channel is activated by both the increase in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) and membrane depolarization (Vergara et al., 1998). BK channels are functionally expressed in a wide variety of excitable cells as key molecules in the negative feedback mechanisms in [Ca\textsuperscript{2+}]\textsubscript{i} regulation. BK channels are composed of tetrameric sets consisting of a pore-forming α subunit and an auxiliary β subunit (Wallner et al., 1996). The α subunit has a characteristic extracellular N-terminal region coupled with the β subunit, seven transmembrane segments, and a long intracellular C-terminal region essential for Ca\textsuperscript{2+} sensing and tetramerization (Schreiber and Salkoff, 1997; Quirk and Reinhart, 2001). The pore-forming α subunit (BKα) is a member of the slo family of potassium channels (KCNMA1) originally identified in Drosophila (Elkins et al., 1986). Only one major type of BKα and several splice variants are ubiquitously expressed in a wide variety of tissues except heart (Xie and McCobb, 1998; Zarei et al., 2001). In contrast, four different β subunits with tissue-specific distribution have been identified (KCNMB1-4) (Knaus et al., 1994; Wallner et al., 1999; Uebele et al., 2000; Brenner et al., 2000a). These β subunits share a prototypic topology of two transmembrane domains with intracellular N- and C-terminals. Co-expression of β subunits with α subunits dramatically alters the biophysical and pharmacological properties of the α subunit BK channel, such as apparent Ca\textsuperscript{2+} sensitivity, voltage dependency, gating kinetics, and pharmacological sensitivity, and it contributes to the diversity in BK channel function (McManus et al., 1995; Xia et al., 1999, 2000; Weiger et al., 2000; Meera et al., 2000; Orio et al., 2002; Zeng et al., 2003).

The β1 and β4 subunits are predominantly expressed in smooth muscles and neurons, respectively (Meera et al., 2000; Weiger et al., 2000; Petkov et al., 2001). The smooth-muscle-specific β1 subunit (KCNMB1) increases the BK channel’s apparent Ca\textsuperscript{2+}/voltage sensitivity, and its disruption in mice increases arterial tonus and phasic contractions of urinary bladder by the reduction of functional coupling between Ca\textsuperscript{2+} sparks and BK channel activation (Brenner et al., 2000b; Petkov et al., 2001). A deficiency of the α
subunit (KCNMA1) in mice can cause not only diseases related to smooth muscle dysfunctions, such as elevated blood pressure, overactive bladder, urinary incontinence, and erectile dysfunction, but also cerebellar ataxia, hearing loss, and hyperaldosteronism (Meredith et al., 2004; Rüttiger et al., 2004; Sausbier et al., 2004, 2005). Therefore, BK channel openers selectively targeting the β1 subunit may effectively treat overactive smooth muscle disorders, with minimal side effects. This is supported by the fact that β-estradiol reduces susceptibility to cardiovascular disease in premenopausal women, and by the fact that it activates the BK channel only in the co-presence of the β1 subunit (Valverde et al., 1999; Ohya et al., 2005). The gain-of-function variant of the β1 subunit (the E65K mutation) protects against diastolic hypertension in aging women (Fernandez-Fernandez et al., 2004). However, despite the tissue-specific distribution of β subunits, few BK channel openers targeting selective β subunits have been identified whereas various chemical and endogenous BK channel openers have been characterized as BKα openers (Imaizumi et al., 2002; Nardi et al., 2003).

DiBAC₄(3), a slow-response voltage-sensitive fluorescent dye, and related oxonol derivatives are generally used to screen K⁺ATP channel modulators by FLIPR (González et al., 1999; Whiteaker et al., 2001) or to assess bacterial viability by flow cytometry (Jepras et al., 1997). Previously, we used DiBAC₄(3) at low concentrations to screen BK channel openers (Yamada et al., 2001) and demonstrated that pimaric acid and related pimarane compounds activate the BK channel by acting on the α subunit (Imaizumi et al., 2002). At that time, we could not determine the opening effect of DiBAC₄(3) on the recombinant BK channel, since 50 nM DiBAC₄(3) was used as a voltage indicator. In recent preliminary studies, we found that the BKαβ1-opening effects of pimaric acid and NS-1619 could not be clearly detected in the presence of DiBAC₄(3) at higher concentrations (> 1 µM), presumably because BK channels were extensively activated by DiBAC₄(3). In this study, we report the pharmacological effects of DiBAC₄(3) and selectivity among the β subunits and as well as selectivity for other K⁺ channels by patch-clamp techniques. Using DiBAC₄(3) analogs and
structurally related compounds, we determined the essential site in the bis-oxonol structure for β-subunit-dependent activation of BK channels.
Experimental Procedures

Cell isolation  Mouse urinary bladder smooth muscle cells (mUBSMCs) were isolated using a previously described method (Morimura et al., 2006). In brief, the urinary bladder was dissected out from male C57BL/6 mice (8-12 weeks of age), and mUBSMCs were obtained by enzymatic isolation using 0.2% collagenase (Amano Enzyme, Nagoya, Japan) for 11-13 min at 37 °C.

Transfection and cell culture  HEK293 cells were maintained in minimum essential medium (Invitrogen Corp., Carlsbad, CA, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Cell Culture Technologies, Buhnrain/Zurich, Switzerland), 100 units/ml penicillin (Wako Pure Chemical Industries, Osaka, Japan), and 100 µg/ml streptomycin (Meiji Seika, Tokyo, Japan) at 37 °C in 5% CO₂ atmosphere. We used HEK293 cell lines that stably expressed rBKα, rSK2, mSK4, rKv1.1, and rKv4.3 (HEK-rBKα, HEK-rSK2, HEK-mSK4, HEK-rKv1.1, and HEK-rKv4.3) and that were generated in previous studies by transfecting the cDNAs encoding rBKα, rSK2, mSK4, rKv1.1, and rKv4.3 in pcDNA3.1(+) (Invitrogen) (Yamada et al., 2001; Imaizumi et al., 2002; Hatano et al., 2004; Sakamoto et al., 2006). HEK293 cell lines stably co-expressing both rBKα and either rBKβ1 or rBKβ4 (HEK-rBKαβ1 and HEK-rBKαβ4) were also generated in the previous studies. HEK293 cells transiently co-expressing both rBKα and rBKβ2 (HEK-rBKαβ2) were generated by co-transfecting the cDNAs encoding both rBKα in pcDNA3.1(+) and rBKβ2 in pTracer-CMV2 (Invitrogen); the transfected cells were identified by GFP signals derived from pTracer-CMV2 after 24-96 hr of cultivation. Transfected cells were maintained on small pieces of cover glass in culture dishes, and then used for electrophysiological experiments.

Electrophysiology  Whole-cell and inside-out patch-clamps were applied to single cells using CEZ-2200, CEZ-2300, and CEZ-2400 amplifiers (Nihon Kohden, Tokyo, Japan) and an EPC-7 amplifier (List Electronik, Darmstadt, Germany) in the manner previously reported.
(Imaizumi et al., 2002). The procedures of electrophysiological recordings and data acquisition/analysis for whole-cell recording were performed by using two programs, Data-Acquisition and Cell-Soft, developed at the University of Calgary. Single-channel current analyses were done using PAT V7.0C software developed at the University of Strathclyde. The pipette resistance was 2 to 5 MΩ for the whole-cell and 10 to 15 MΩ for inside-out patch configurations when filled with the pipette solutions.

**Solution** For whole-cell recording from isolated mUBSMCs or transfected HEK293 cells, a standard HEPES-buffered solution comprised of (in mM) 137 NaCl, 5.9 KCl, 2.2 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 14 D-glucose (pH 7.4 by NaOH) was used as a bath solution. Whole-cell patch-clamp recordings in mUBSMCs were obtained using a 300 nM free Ca²⁺ internal pipette solution containing (in mM): 140 KCl, 4.5 CaCl₂, 3 MgCl₂, 10 EGTA, 10 HEPES, and 2 ATP (pH 7.2 by KOH) and the standard HEPES-buffered solution including 100 µM Cd²⁺. Whole-cell patch-clamp recordings in HEK-rBKα, HEK-rBKαβ1, and HEK-rBKαβ4 were obtained using a 300 nM free Ca²⁺ internal pipette solution containing (in mM): 140 KCl, 3.3 CaCl₂, 1 MgCl₂, 5 EGTA, 10 HEPES, and 2 ATPNa₂ (pH 7.2 by KOH). Whole-cell patch-clamp recording in HEK-rBKαβ2 was performed using a 1 µM free Ca²⁺ internal pipette solution containing (in mM): 140 KCl, 4.3 CaCl₂, 4 MgCl₂, 5 EGTA, 10 HEPES, and 2 ATPNa₂ (pH 7.2 by KOH). Whole-cell patch-clamp recordings in HEK-rSK2 and HEK-mSK4 were performed using a 1 µM free Ca²⁺ internal pipette solution containing (in mM): 138 K-aspartate, 8.6 CaCl₂, 2 MgCl₂, 10 EGTA, 10 HEPES, and 1 ATPK₂ (pH 7.2 by KOH). Liquid junction potentials were measured and corrected for −14 mV. Whole-cell patch-clamp recordings in HEK-rKv1.1 and HEK-rKv4.3 were performed using a nominally Ca²⁺-free (~10 nM) internal pipette solution containing (in mM): 138 KCl, 0.58 CaCl₂, 2 MgCl₂, 10 EGTA, 10 HEPES, and 1 ATPK₂ (pH 7.2 by KOH). For single-channel recording under an inside-out configuration, HEPES-buffered solution comprised of (in mM) 140 KCl, 2.2 CaCl₂, 1.2 MgCl₂, 5 EGTA, 10 HEPES, and 14 D-glucose
(pCa 7.0, pH 7.2 by NaOH) was used as bath and pipette solutions. The free Ca$^{2+}$ concentration was calculated using the WEBMAXC program (http://www.stanford.edu/~cpatton/webmaxcS.htm).

**Chemicals**

DiBAC$_4$(3): bis(1,3-dibutylbarbituric acid)trimethine oxonol was obtained from Dojindo (Kumamoto, Japan); DiBAC$_4$(5): bis(1,3-dibutylbarbituric acid)pentamethine oxonol and DiSBAC$_2$(3): bis(1,3-diethylthiobarbituric acid)trimethine oxonol were obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A.); DiSBAC$_2$(5): bis(1,3-diethylbarbituric acid)pentamethine oxonol was obtained from AnaSpec, Inc. (San Jose, CA, U.S.A.); DiSBAC$_2$(1): bis(1,3-diethylthiobarbituric acid) methine oxonol was obtained from Specs, Inc. (Delft, Netherlands); BA: barbituric acid was obtained from Wako Pure Chemical Industries; DiSBAC$_4$(3): bis(1,3-dibutylthiobarbituric acid)trimethine oxonol; DiSBAC$_0$(5): bis(barbituric acid)pentamethine oxonol; Oxonol 595: bis(3-cyano-1-ethyl-4-methyl-2,6-dioxo-1,2,5,6-tetrahydropyridine)trimethine oxonol; Oxonol V: bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol; DMBA: 1,3-dimethylbarbituric acid; DETBA: 1,3-diethyl-2-thiobarbituric acid and penitrem A were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.); Oxonol VI: bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol was obtained from Fluka (Buchs, Switzerland); and DiSBAC$_{10}$(3): bis(1,3-didecylthiobarbituric acid)trimethine oxonol was obtained from Cayman (Ann Arbor, MI, U.S.A.). DiBAC$_4$(3), DiBAC$_4$(5), DiSBAC$_2$(3), DiSBAC$_4$(3), DiSBAC$_0$(5), DiSBAC$_2$(1), Oxonol 595, Oxonol V, Oxonol VI, and penitrem A were diluted from a 10 mM stock in DMSO; and BA, DMBA, and DETBA were diluted from a 100 mM stock in DMSO and stored at -20 °C. Less soluble compounds were prepared for use in the experiments by sonication to dissolve in a bath solution for almost a minute.

**Stereochemical optimization of chemical structure and calculations of pKa and interatomic distance**
The most stable stereochemical structures of oxonol compounds were determined by calculations in the anion state (except for DiSBAC\textsubscript{0}(5), which forms a dianion by tautomerization) using the density-functional theory (DFT) methods in Spartan '04 software for Windows version 1.0.3. (Wavefunction, Inc., Irvine, CA, U.S.A.) and displayed by UCSF Chimera software. Interatomic oxygen-oxygen distances were measured from the most stable stereochemical structures. The pKa values, determined by the degree of ionization of the molecules, were calculated using Solaris V4.76 software (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada).

**Statistics**  Data are expressed as means ± S.E.M. Statistical significance between two groups and among multiple groups was evaluated using the Student’s t-test and Scheffé’s test after the F test or one-way analysis, respectively. In the figures, * and ** indicate statistical significance at p values of 0.05 and 0.01, respectively.
Results

DiBAC$_{4}(3)$ activates BK current in isolated mUBSMCs

In the first part of this study, we examined the effects of DiBAC$_{4}(3)$ on outward currents elicited by depolarization in single smooth muscle cells isolated from mouse urinary bladder (mUBSMCs) by patch-clamp techniques. Whole-cell currents in mUBSMCs upon depolarization were measured in the standard HEPES-buffered solution containing 100 µM Cd$^{2+}$ to block the voltage-dependent Ca$^{2+}$ channel (VDCC) and with the use of a pipette solution, in which the Ca$^{2+}$ concentration was fixed at pCa 6.5 with Ca$^{2+}$-EGTA buffer (see Methods). The outward currents elicited by depolarization from a holding potential of -60 mV to +40 mV for 150 ms (closed circles in Fig. 1A) were markedly enhanced by application of 300 nM DiBAC$_{4}(3)$, and then were blocked by the addition of 1 µM penitrem A, a selective BK channel blocker (Fig. 1A). The outward currents elicited by depolarization in the range of -60 mV to +60 mV in 10 mV steps for 150 ms (open circles in Fig. 1A) were recorded (Fig. 1B) and plotted as the relationship between current density and test potentials (Fig. 1C). The peak outward current densities at +60 mV in the control, in the presence of DiBAC$_{4}(3)$ and after the addition of penitrem A, were 16.9 ± 0.4, 28.8 ± 1.3 (p<0.01 vs. the control), and 6.6 ± 1.6 pA/pF (p<0.01 vs. in the presence of DiBAC$_{4}(3)$; p<0.05 vs. the control), respectively (n = 4 for each). These results suggest that DiBAC$_{4}(3)$ potently activates BK current in mUBSMCs, where BK channels consist of combinations of α and β1 subunits (Petkov et al., 2001).

DiBAC$_{4}(3)$ is a novel BK channel opener

The effects of DiBAC$_{4}(3)$ on BK channels were further examined in a heterologous expression system, where rat BK channel α subunit (rBKα) alone or both rBKα and either rat BK channel β1, β2, or β4 subunit (rBKβ1, rBKβ2, or rBKβ4) were stably or transiently expressed in HEK293 cells (HEK-rBKα, HEK-rBKαβ1, HEK-rBKαβ2, and HEK-rBKαβ4). First, whole-cell currents in HEK-rBKαβ1 were measured in the standard solution and by
using a pipette solution of pCa 6.5. The outward currents elicited by depolarization from a holding potential of -60 mV in 10 mV steps for 150 ms in HEK-rBKαβ1 were markedly enhanced at potentials positive to -40 mV by application of 300 nM DiBAC₄(3), and then were completely blocked by the addition of 1 µM penitrem A (Fig. 2A). The relationships between current density and test potentials are shown in Fig. 2B. The peak outward current densities at +60 mV in the control, in the presence of DiBAC₄(3) and after the addition of penitrem A, were 142.8 ± 14.6, 219.1 ± 35.8 (p<0.01 vs. control), and 2.4 ± 2.0 pA/pF (p<0.01 vs. the control and in the presence of DiBAC₄(3)), respectively (n = 3 for each). The cumulative application of DiBAC₄(3) in the concentration range of 10 nM to 1 µM increased the outward currents concentration-dependently at +20 mV in HEK-rBKαβ1. The outward currents were then almost completely blocked by the further addition of 1 µM penitrem A (Fig. 2C). Concentration-response relationships for DiBAC₄(3) in HEK-rBKαβ1 were summarized as the relative amplitude normalized by the peak outward current before the application of DiBAC₄(3) (Fig. 2D). The relative amplitude at +20 mV was concentration-dependently increased by DiBAC₄(3) at 10 nM (1.440 ± 0.242; p< 0.05 vs. 1.0) and higher concentrations. The effect of DiBAC₄(3) at lower concentration (<100 nM) was removed by washout within a few minutes and that at higher concentration (>300 nM) was removed more slowly (not shown).

Single-channel currents in HEK-rBKαβ1 were measured at +20 mV in the inside-out patch configuration in symmetrical 140 mM K⁺ conditions (Fig. 3A). The pCa in the bath and pipette solutions was 7.0 (see Methods). Relative open probability (Pₒ) was normalized by that before the application of DiBAC₄(3). Cumulative application of DiBAC₄(3) from 100 nM to 3 µM increased the relative Pₒ of rBKαβ1 channels in a concentration-dependent manner (Fig. 3, A and B). The increase in relative Pₒ of rBKαβ1 channels by application of DiBAC₄(3) could be quickly and completely removed by washout. The concentration-response relationships of DiBAC₄(3) on relative Pₒ are summarized in Fig. 3C. The unitary current amplitude at +50 mV and single-channel conductance were 12.37 ± 0.07
pA, 260.2 pS, and 12.76 ± 0.24 pA, 263.4 pS, (p>0.05 vs. the control, respectively; n = 5 for each) in the absence and presence of 300 nM DiBAC4(3), respectively (Fig. 3D). The P0 was increased by application of 300 nM DiBAC4(3) at any potential examined, and was well fitted by a Boltzmann relationship:

\[ P_0 = \frac{1}{1 + \exp\left(\frac{V_{1/2} - V_m}{S}\right)} \]

where \( V_{1/2}, V_m, \) and \( S \) are the voltages required for half-maximum activation, membrane potential, and slope factor, respectively (Fig. 3E). The values of \( V_{1/2} \) and \( S \) were 96.3 ± 2.7 mV and 11.4 ± 2.2 mV in the absence of DiBAC4(3), and 74.8 ± 8.4 mV (p<0.01 vs. the control) and 17.3 ± 4.3 mV (p>0.05 vs. the control) in the presence of 300 nM DiBAC4(3), respectively (n = 5 for each). The fitted line was shifted to negative potentials by approximately 30 mV in the presence of 300 nM DiBAC4(3).

To examine whether the enhancement of rBKαβ1 channel activity by DiBAC4(3) can be observed in different cytosolic Ca2+ concentration, the channel activity was also measure in inside-out configuration at pCa 6.0 and 9.0. The application of 300 nM DiBAC4(3) increased the P0 at potentials examined in pCa 6.0 solution; at -30 mV, P0 was increased from 0.042 ± 0.021 to 0.226 ± 0.113 (n = 3, p<0.05). At pCa 9.0, the P0 also tended to be increased by 300 nM DiBAC4(3) at potentials examined (from 0.185 to 0.364 at +170 mV, n=2). The enhancement of rBKαβ1 channel activity by DiBAC4(3) was observed in wide range of pCa in the bathing solution in inside-out configuration.

Similarly, whole-cell and single-channel currents in HEK-rBKα alone were measured under the same sets of conditions as used for HEK-rBKαβ1. Relative P0 in the inside-out patch configuration was normalized by P0 before the application of DiBAC4(3) (Fig. 4A). The relative P0 of rBKα channels was not markedly changed by cumulative application of DiBAC4(3) in the range of 10 nM to 3 μM (Fig. 4, A and B). Concentration-response relationships between relative P0 of rBKα channels and DiBAC4(3) are summarized in Fig. 4C. Application of 300 nM DiBAC4(3) did not affect significantly the outward currents in HEK-rBKα under the whole-cell voltage-clamp configuration (Fig. 4D). The addition of 1
µM penitrem A abolished the outward currents. The relationships between current density and test potentials are shown in Fig. 4E. The peak outward current densities at +60 mV were 152.8 ± 70.2, 145.5 ± 67.2 (p>0.05 vs. the control), and 6.8 ± 3.3 pA/pF (p<0.01 vs. the control and in the presence of DiBAC₄(3)) in the absence or presence of 300 nM DiBAC₄(3) and after the addition of penitrem A, respectively (n = 3 for each).

Selectivity of DiBAC₄(3) for subtypes of BK channel β subunits

In the next series of experiments, the selectivity of DiBAC₄(3)-induced enhancement for subtypes of BKβ was examined using HEK-rBKαβ2 and HEK-rBKαβ4 in addition to HEK-rBKαβ1. Whole-cell currents were measured in HEK-rBKαβ2 in the standard solution with the pipette solution of pCa 6.0. Under these conditions, rapidly inactivating outward currents, which have been reported as BKαβ2 current (Wallner et al., 1999), were detected in the voltage range from +20 to +80 mV. The outward currents, which were elicited by depolarization from a holding potential of -80 mV in 20 mV steps for 1000 ms in HEK-rBKαβ2, were rapidly inactivated at potentials positive to +20 mV (Fig. 5A). Unlike in HEK-rBKα and HEK-rBKαβ1, the inactivating outward currents in HEK-rBKαβ2 were not affected and were significantly blocked by application of 3 µM DiBAC₄(3). Further addition of 1 µM penitrem A completely blocked the currents (Fig. 5A). The relationships between current density and test potentials are shown in Fig. 5B. The peak outward current densities at +80 mV in the control, in the presence of 3 µM DiBAC₄(3) and after the addition of penitrem A, were 199.1 ± 35.6, 135.0 ± 26.0 (p>0.05 vs. the control), and 7.8 ± 2.3 pA/pF (p<0.01 vs. the control and in the presence of DiBAC₄(3)), respectively (n = 3 for each). The relative amplitudes at +80 mV were 0.878 ± 0.090 (p>0.05) and 0.627 ± 0.069 (p<0.01) in 1 and 3 µM DiBAC₄(3), respectively (n = 5 for each) (Fig. 5C).

The whole-cell currents in HEK-rBKαβ4 were also measured under the same conditions, except for pCa in the pipette solution (pCa 6.5). The outward currents in HEK-rBKαβ4 were markedly enhanced at potentials positive to -20 mV by application of 300
nM DiBAC_4(3), and then were completely blocked by the addition of 1 µM penitrem A (Fig. 6A). The relationships between current density and test potentials are shown in Fig. 6B. The peak outward current densities at +60 mV in the control, in the presence of DiBAC_4(3), and after the addition of penitrem A were 127.6 ± 17.9 (n = 6), 230.5 ± 36.1 (n = 6; p<0.01 vs. the control), and 9.4 ± 2.3 pA/pF (n = 4; p<0.01 vs. the control and in the presence of DiBAC_4(3)), respectively. The cumulative application of DiBAC_4(3) in the range of 10 nM to 3 µM concentration-dependently increased the outward currents at +20 mV in HEK-rBKαβ4. The outward currents were then completely blocked by the further addition of 1 µM penitrem A (Fig. 6C). The concentration-response relationships for DiBAC_4(3) in HEK-rBKαβ4 are summarized in Fig. 6D, together with those for HEK-rBKαβ1, which are shown in Fig. 2D. It is notable that the enhancement of rBKαβ4 currents by DiBAC_4(3) in the concentration range of 10 nM to 3 µM was not apparently different from that of corresponding rBKαβ1 currents. The relative amplitude of rBKαβ4 current at +20 mV in the presence of 10 nM DiBAC_4(3) was 1.322 ± 0.096 of the control (p<0.05 vs. 1.0), which was not significantly different from that of rBKαβ1 (1.440 ± 0.242; p>0.05 vs. BKαβ4).

Selectivity of DiBAC_4(3) for other K^+ channels

In the next series of experiments, the selectivity of DiBAC_4(3) for other K^+ channels, such as small-conductance Ca^{2+}-activated K^+ (rSK2, mSK4) channels and voltage-gated K^+ (rKv1.1, rKv4.3) channels, was examined by patch-clamp techniques using HEK293 cells stably expressing one of these channel α subunits (HEK-rSK2, HEK-mSK4, HEK-rKv1.1, and HEK-rKv4.3).

Whole-cell currents in HEK-rSK2 and HEK-mSK4 elicited by a ramp pulse from -160 mV to +20 mV for 250 ms were measured in the standard solution by use of a pipette solution, in which Cl^- was replaced by aspartate^- and pCa was set at 6.0. Whole-cell currents in HEK-rSK2 were slightly reduced by application of 10 µM DiBAC_4(3) and were blocked by the addition of 10 nM UCL 1684, a selective SK1-3 channel blocker (Fig. 7A). Whole-cell
currents in HEK-mSK4 were not changed by application of 10 µM DiBAC₄(3) and were blocked by the addition of 1 µM clotrimazole, a SK4 channel blocker (Fig. 7B).

Whole-cell currents in HEK-rKv1.1 and HEK-rKv4.3, elicited by depolarization from holding potential of -80 mV to +20 mV for 1000 ms, were measured with the pipette solution of pCa 8.0. Whole-cell currents in HEK-rKv1.1 were not changed by 10 µM DiBAC₄(3) and were blocked by the addition of 100 nM margatoxin, a Kv1.x channel blocker (Fig. 7C). Whole-cell currents in HEK-rKv4.3 were also not affected by 10 µM DiBAC₄(3) (Fig. 7D).

Figure 7E summarizes data on the effects of 10 µM DiBAC₄(3) on these channel currents. The K⁺ current amplitude in HEK-rSK2 and HEK-mSK4 was normalized by the control current at -50 mV, corresponding to the calculated reversal potential of Cl⁻ in each experiment. The K⁺ current amplitude in HEK-rKv1.1 and that in HEK-rKv4.3 were normalized by the peak outward current in the control at +20 mV in each cell. The relative amplitudes in the presence of 10 µM DiBAC₄(3) versus the control were 0.754 ± 0.052 (p<0.05 vs. 1.0) in HEK-rSK2, 1.051 ± 0.057 (p>0.05) in HEK-mSK4, 1.010 ± 0.013 (p>0.05) in HEK-rKv1.1, and 0.942 ± 0.055 (p>0.05) in HEK-rKv4.3, respectively (n = 4 for each). These results strongly suggest that DiBAC₄(3) is selective for BK channels, more than the other Ca²⁺-activated or voltage-gated K⁺ channels examined here.

Structure-potency relationship of DiBAC₄(3) and related oxonol compounds as BK channel openers

Based on a distinctive structure of DiBAC₄(3), in which two 1,3-dialkylbarbituric acids were connected and conjugated by the oligomethine, we performed structure-activity relationships using 16 compounds of DiBAC₄(3) and its analogs to determine the structure-potency relationship for BK channel activation and the essential moiety in the molecular structure of DiBAC₄(3). The generic names and structures of DiBAC₄(3) and its analogs used here are listed in Fig. 8A. Figure 8B shows the effects of 100 nM and 1 µM DiSBAC₂(5) (left panel), as well as of 1 and 10 µM DiSBAC₂(1) (right panel), on the
amplitude of whole-cell outward current elicited by depolarization from −60 mV to +20 mV in HEK-rBKαβ1. Application of 1 µM DiSBAC\textsubscript{5} or 10 µM DiSBAC\textsubscript{2} obviously or slightly enhanced rBKαβ1 currents, respectively. Similar experiments were performed using the listed compounds to determine their potencies (not shown). The peak amplitude of BK currents in the presence of the test compound was normalized by that before the application and is shown in Fig. 8C.

Whole-cell currents in HEK-rBKαβ1 were enhanced by about two-fold by the application of 100 nM DiSBAC\textsubscript{4}(3) (2.415 ± 0.390, n = 5). This was the same degree of enhancement as achieved by 100 nM DiBAC\textsubscript{4}(3) (2.262 ± 0.096, n = 7; p>0.05 between them), indicating that the replacement of the carbon-oxygen (C = O) bonds in DiBAC to carbon-sulfur (C = S) bonds in DiSBAC did not affect BK channel activation ability. Moreover, the currents were also enhanced by about two-fold by 100 nM DiSBAC\textsubscript{3}(3) (2.044 ± 0.32, n = 4; p>0.05 vs. DiBAC\textsubscript{4}(3) and DiSBAC\textsubscript{4}(3)). In contrast, the effect of the extension of alkyl side chains in the 1,3-positions was not clear, since extension of the side chain makes the oxonol compounds hydrophilic. For example, DiSBAC\textsubscript{10}(3) was too hydrophobic to dissolve in water even with methyl acetate, so the effects of DiSBAC\textsubscript{10}(3) on BK channels could not be determined exactly (data not shown). DiSBAC\textsubscript{5}(3) at concentrations of 1 and 10 µM did not show potency (1.017 ± 0.01, n = 5; p>0.05, vs. 1.0 and 1.003 ± 0.006, n = 5; p>0.05, vs. 1.0, respectively). Also, under the inside-out configurations, DiSBAC\textsubscript{5}(3) did not increase channel NP\textsubscript{o} at 1 µM and significantly inhibited it at 10 µM. The relative NP\textsubscript{o} were 1.180 ± 0.217 (n = 6; p>0.05, vs. 1.0) and 0.422 ± 0.146 (n = 4; p<0.05, vs. 1.0) in the presence of 1 and 10 µM DiSBAC\textsubscript{5}(3), respectively. Therefore, the lack of the side chains may markedly decrease efficacy. From these results it can be suggested that the alkyl side chains in the 1,3-positions are essential for the BK channel opening property, but that the length between C2 (ethyl) and C4 (butyl) appears to be not very critical to efficacy.

To reveal the importance of the two barbituric rings, which are conjugated by an
oligomethine chain in DiBAC$_4$(3), we examined the effects of barbiturates, clinically used as hypnotics (hexobarbital, barbital), hydantoin anticonvulsants (phenytoin), and barbituric/thiobarbituric acids (BA, DMBA, DETBA) on whole-cell currents in HEK-rBK$\alpha\beta$1. The results revealed no ability to activate the BK channel when 10 $\mu$M hexobarbital, barbital, or phenytoin were applied (data not shown). Application of 10 $\mu$M BA, TMBA, or TETBA likewise did not affect BK currents in HEK-rBK$\alpha\beta$1 (Fig. 8C). The relative amplitudes were $0.996 \pm 0.039$ (n = 5; p>0.05 vs. 1.0), $0.938 \pm 0.028$ (n = 4; p>0.05 vs. 1.0), and $1.125 \pm 0.096$ (n = 4; p>0.05 vs. 1.0) in the presence of 10 $\mu$M BA, TMBA, and TETBA, respectively. These results strongly suggest that the two barbituric rings conjugated by an oligomethine chain are essential for the BK channel opening efficacy of DiBAC$_4$(3).

To determine whether or not the length of the oligomethine chain is an important factor in determining potency, the effects of DiBAC$_4$(3) and DiSBAC$_2$(3) were compared with those of corresponding compounds having longer pentamethine chains, DiBAC$_4$(5) and DiSBAC$_2$(5), respectively. Moreover, the effects of DiSBAC$_2$(1), in which the chain is just methine, were also examined. Interestingly, the relative amplitudes were $1.257 \pm 0.025$ (n = 5; p<0.01 vs. 1.0) and $1.293 \pm 0.033$ (n = 4; p<0.01 vs. 1.0) in the presence of 100 nM DiBAC$_4$(5) and DiSBAC$_2$(5), respectively, and $1.918 \pm 0.287$ (n = 5; p<0.05 vs. 1.0) and $2.107 \pm 0.053$ (n = 4; p<0.01 vs. 1.0) at 1 $\mu$M DiBAC$_4$(5) and DiSBAC$_2$(5), respectively. The potency of DiSBAC$_2$(1) was not detected at 1 $\mu$M (1.044 ± 0.050, n = 4; p>0.05 vs. 1.0) but was at 10 $\mu$M (1.252 ± 0.063, n = 4; p<0.05 vs. 1.0) (Fig. 8C). Thus, “trimethine” is the best length of the oligomethine chain. These results suggest that the length of conjugating oligomethine is a key factor in determining the potency of bis-barbituric acid oxonol compounds as BK channel openers.

To reveal the number of oxo-moieties and the locations in the molecule that would endow potency, the effects of Oxonol 595, Oxonol V, and Oxonol VI were examined. Oxonol 595 has a structure similar to that of DiBAC$_4$(3), whereas oxo-moieties do not exist in the 4-position of pyrimidine. Oxonol V and Oxonol VI have a 5-isoxazolone structure.
Surprisingly, Oxonol 595 did not show potency at all (1.002 ± 0.025, n = 4; p>0.05 vs. 1.0 and 1.071 ± 0.078, n = 5; p>0.05 in the presence of 1 and 10 µM Oxonol 595, respectively). In contrast, both Oxonol V and Oxonol VI act as BK channel openers, but the potency of Oxonol V was about 10-fold higher than that of Oxonol VI (Fig. 8D). Therefore, a set of oxo- and oxoanion-moieties conjugated with oligomethine may be the structure least likely to have potency. Based on the acidic pKa, oxonol must almost completely form an oxoanion at pH 7.4 (Table 1). It was mysterious, however, that Oxonol 595 did not show any potency as a BK channel opener even at 10 µM.

**Stereochemical analyses of essential moiety in the molecular structure of DiBAC₄(3) for BK channel activation**

To elucidate two questions, 1) why was Oxonol 595 not potent and 2) what is the most effective length of oligomethine between oxo- and oxoanion-moieties in a molecule, we performed stereochemical analyses by calculating the most stable stereochemical structures of oxonol compounds using the density-functional theory (DFT) methods in Spartan (see Methods).

Figure 9A shows top and side views of the most stable stereochemical structures of the 10 oxonol compounds examined in this study. Table 1 shows the interatomic distance between oxo- and oxoanion-moieties in each side of the oligomethine chain in a molecule and the potency as a relative enhancement of whole-cell current in HEK-rBKαβ1. It is notable that the negatively charged oxygen atoms indicated by arrows in Fig. 9A and the oligomethine chain are the most stable in a single plane, based on the side views of these molecules. The most striking finding here is that the oxo- and oxoanion-moieties in Oxonol 595 are located on different sides of the conjugated oligomethine chain (sky blue and pink arrows in Fig. 9A). Judging from the potency and the distance between oxo- and oxoanion-moieties in Oxonol V and Oxonol VI, it may be essential for potency that at least one set of oxo- and oxoanion-moieties, which are conjugated by oligomethine, should be on one side of the
methine chain in the stereochemical structure (Fig. 9B). The calculated interatomic oxygen-oxygen distances on each side of the oligomethine chain in the DiBAC/DiSBAC compounds were 4.1 Å (short) and 6.7 Å (long) in trimethine oxonols (DiBAC₄(3), DiSBAC₄(3), and DiSBAC₂(3)); 6.7 Å (short) and 9.1 Å (long) in pentamethine oxonols (DiBAC₄(5) and DiSBAC₂(5)); and 2.7 Å (short) and 4.7 Å (long) in methine oxonol (DiSBAC₂(1)) (Table 1).
Discussion

The selectivity of DiBAC₄(3) for regulatory β subunit and speculation on the binding site on BK channel

In this study, we clearly demonstrated that DiBAC₄(3) selectively activates the BK channel only when the regulatory rBKβ₁ or rBKβ₄, but not rBKβ₂, is co-expressed with rBKα in HEK293 cells. The finding that DiBAC₄(3) markedly enhanced BK current in mUBSMCs, where BK channels are composed of BKα and BKβ₁ (Petkov et al., 2001), indicates that DiBAC₄(3) can work as a potent BKαβ₁ channel opener in native cells. Although the BKβ [DELETE: subunit] is a potential target of drug development, very limited information is available about chemicals that selectively act on BKβ as BK channel openers. Most BK channel openers that have been developed act on the α subunit (Imaizumi et al., 2002, Nardi et al., 2003), and only a few compounds have been reported as β-subunit-selective BK channel openers: β-estradiol (Valverde et al., 1999), tamoxifen (Dick et al., 2001), and dehydrosoyasaponin-I (DHS-I) (McManus et al., 1995; Giangiacomo et al., 1998).

DHS-I activates the BK channel only in the presence of the β₁ subunit by intracellular application. This is due to the low membrane permeability and to the negative charge in the structure at physiological pH. DHS-I also activates the BK channel in the presence of hBKβ₂ without the inactivation domain (hBKβ₂-IR) by intracellular application (Wallner et al., 1999). Although the effects of β-estradiol and tamoxifen on BKβ₂ have not been reported, they are effective on both BKβ₁ and BKβ₄. It is therefore noteworthy that DiBAC₄(3) enhanced the currents through rBKαβ₁ and rBKαβ₄ but not through rBKαβ₂. DiBAC₄(3) did not modify the single-channel conductance but enhanced markedly the voltage sensitivity in the rBKαβ₁ channel. These properties of DiBAC₄(3)-induced activation of the rBKαβ₁ channel appeared to be shared with the rBKαβ₄ channel (not shown). Moreover, the concentration-response relationship for DiBAC₄(3)-induced enhancement of the rBKαβ₄ current was comparable to that of the rBKαβ₁ current. These results may suggest that the
DiBAC$_4$(3) binding site is in the amino acid sequences that are common between BKβ1 and BKβ4 but not with BKβ2. Based on the sequence analysis, rBKβ1 shares only 23% sequence homology with rBKβ4 but 41% with rBKβ2. Detection of the exact location of the DiBAC$_4$(3) binding site in BKβ1 and BKβ4 will provide new insight into the functional coupling of BKβ with BKα.

**Selectivity of DiBAC$_4$(3) for BK channel versus other channels**

Small-conductance Ca$^{2+}$-activated K$^+$ (rSK2, mSK4) channels and voltage-gated K$^+$ (rKv1.1, rKv4.3) channels were not affected by 10 µM DiBAC$_4$(3). Since DiBAC$_4$(3) has been used for the screening of K$_{ATP}$ channel modulators at a concentration of 5 µM (Whiteaker et al., 2001), we guess that the K$_{ATP}$ channel is also unaffected by DiBAC$_4$(3). In our preliminary study, 1 µM DiBAC$_4$(3) did not affect the voltage-dependent Ca$^{2+}$ channel (VDCC) in mUBSMCs (unpublished observation by Morimoto). However, it has been reported that DiBAC$_4$(5) inhibits a volume-sensitive Cl$^-$ channel (Arreola et al., 1995) and anion exchangers (AE) such as the AE1 Cl$^-$/HCO$_3^-$ exchanger in red blood cells and AE2 in HL-60 cells (Alper et al., 1998). In our previous study, in which we assayed pimaric acids and related compounds as BK channel openers, we successfully measured membrane potential changes in HEK-rBKαβ1 by using DiBAC$_4$(3) at a concentration of 50 nM (Yamada et al., 2001; Imaizumi et al., 2002) but in the present study we did not detect any changes at 1 µM (not shown). Based on these results, DiBAC$_4$(3) as a voltage indicator should be used at low concentrations (< 100 nM) to avoid artifacts due to the direct action on ion channels. It is also noteworthy that DiBAC$_4$(3) binds to cytosolic proteins, presumably in a nonspecific fashion, and this elongates markedly the fluorescence lifetime following excitation (González et al., 1999). This means that DiBAC/DiSBAC dyes can bind many types of ion channel proteins, regardless of whether or not they express specific effects. Considering the possibility that DiBAC$_4$(3) may also bind to peptide BK channel blockers, iberiotoxin and charybdotoxin, we prefer to use small molecules such as penitrem A to block BK channels.
Essential moiety in the molecular structure of DiBAC₄(3) for BK channel activation

Barbiturate derivatives are rather old hypnotics and have been used at relatively high plasma concentrations. Since the DiBAC₄(3) molecule includes two barbituric acids, we at first considered the possibility that barbituric acid and its derivatives may work as BK channel openers. The present results, however, showed clearly that this is not the case; none of the barbituric acid-related compounds used in this study had sufficient potency. In addition, DiSBAC₄(3) possessed high potency, which was comparable to that of DiBAC₄(3). Therefore, the chemical structure of two barbituric/thiobarbituric acids conjugated with a certain length of oligomethine chain appears to be essential for DiBAC/DiSBAC compounds to have a BK channel-opening property. The length of the oligomethine chain modified substantially the potency of the molecule as a BK channel opener. The potency of DiBAC₄(3) was reduced to 1/10 in pentamethine oxonols (DiBAC₄(5) and DiSBAC₂(5)), and to 1/1000 in methine oxonol (DiSBAC₂(1)), indicating the order of potency was methine (C₁) << pentamethine (C₅) < trimethine (C₃). The length of the alkyl chain in 1,3-positions of barbituric/thiobarbituric acid also affected potency, since the potency of DiSBAC₂(3) (1,3-diethyl) was comparable to that of DiSBAC₄(3) (1,3-dibutyl), while the lack of a side chain in the 1,3-positions (DiSBAC₀(5)) abolished the potency.

The most important finding to elucidate the fundamental moiety as a BK channel opener in DiBAC/DiSBAC compounds was that Oxonol 595 did not show potency. Based on this finding, we considered that two sets of oxo- and oxoanion-moieties at the 4,6-positions in two barbituric acid rings are essential for potency. To confirm this assumption, we examined the effects of Oxonol V and Oxonol VI, which we supposed would be ineffective. Unexpectedly, however, they had potency. These results indicate that one set of oxo- and oxoanion-moieties that are each conjugated with an oligomethine are enough for potency as a minimum requirement. The stereochemical optimization of all the compounds used in this study, including Oxonol 595 and Oxonol V/VI, by the density-functional theory (DFT)
methods (see Methods), revealed the mechanism underlying these unexpected results. The molecular images in the most stable stereochemical structures of these compounds indicate that the oxo- and oxoanion-moieties in Oxonol 595 are located on each side of the oligomethine, while those in Oxonol V/VI were on the same side, as indicated by sky blue and pink arrows in Fig. 9A. Moreover, the two barbituric/thiobarbituric acid rings and the connecting oligomethine in DiBAC/DiSBAC compounds must be in the same plane (side views in Fig. 9A). This is also the case in Oxonol V, in which the two rings are 5-isoxazolone. This may be essential for the effective electron transfer coupling between negatively charged plane oxo-groups and the connecting oligomethine chain. The molecular image of DiSBAC2(1), which did not show potency, includes a twist between two thiobarbituric acid rings. Oxonol VI, which showed substantially lower potency than Oxonol V, also has a slight twist in the oligomethine in the most stable stereochemical structure (Fig. 9A).

The stereochemical analyses also allow us to calculate the interatomic distance between two oxygen atoms in the two sets of oxo- and oxoanion-moieties on each side of the oligomethine in DiBAC4(3) as approximately 4 and 7 Å, respectively. In DiBAC/DiSBAC compounds, the two oxygen atoms in oxo- and oxoanion-moieties at a short distance (4 Å), which were conjugated by trimethine, would give the highest potency, presumably by the most effectively conjugated π electron transfer.

Together these findings suggest the minimum requirement of a stereochemical structure for BK channel openers may be as follows. One set of oxo- and oxoanion-moieties on the same side, which are conjugated by tri- or penta-methine groups, should form an electron transfer coupling structure in a single plane, as illustrated in Fig. 9B.

**Conclusion**

Our finding that DiBAC4(3) and related oxonol compounds are β-subunit-selective BK channel openers is extremely important; not only will these compounds serve many uses as
pharmacological tools and templates for the design of β-subunit-selective BK channel openers, but they will also reveal the functional coupling mechanism between pore-forming α and regulatory β subunits. This study provides a molecular and structural basis for BK channels and contributes to the understanding of the regulatory mechanism of BK channel activity by the auxiliary β subunit.
Acknowledgments

We thank Prof. Naoki Miyata and Dr. Takayoshi Suzuki for calculations of the most stable stereochemical structures by using Spartan’04 software, as well as for their helpful advice.
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Footnotes
This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (18059029) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and by a Grant-in-Aid for Scientific Research (B) (17390045) from the Japan Society for the Promotion of Science to YI. This work was also supported by a Grant-in-Aid for Research on Health Sciences focusing on Drug Innovation (KH11001) from the Japan Health Sciences Foundation to YI.
Figure Legends

Figure 1. Effects of DiBAC$_4$(3) on whole-cell BK currents in mUBSMCs.

Whole-cell BK currents in mUBSMCs were measured in physiological K$^+$ gradient conditions (5.9 mM K$^+$ outside and 140 mM K$^+$ inside) with 300 nM free Ca$^{2+}$ in the pipette solution (pCa 6.5).  

A: Each cell was depolarized from a holding potential of −60 mV to +60 mV in 10 mV steps (○) or to +40 mV (●) for 150 ms at 15 s intervals. Whole-cell BK currents were measured in the control, in the presence of 300 nM DiBAC$_4$(3), and after the addition of 1 µM penitrem A. The peak amplitude of outward current was plotted against time. Application of 300 nM DiBAC$_4$(3) and addition of 1 µM penitrem A are indicated by horizontal bars, respectively.  

B: Recordings of the outward currents activated by 10 mV steps in “A” (○) are shown.  

C: The current-voltage relationships were obtained in the control (■; n = 4), in the presence of 300 nM DiBAC$_4$(3) (●; n = 4), and after the addition of 1 µM penitrem A (▲; n = 4).

Figure 2. Effects of DiBAC$_4$(3) on whole-cell rBK$\alpha$β1 currents in HEK293 cells.

Whole-cell rBK$\alpha$β1 currents were measured in physiological K$^+$ gradient conditions (5.9 mM K$^+$ outside and 140 mM K$^+$ inside) with 300 nM free Ca$^{2+}$ in the pipette solution (pCa 6.5).  

A: Each cell was depolarized from a holding potential of −60 mV to +60 mV in 10 mV steps for 150 ms at 15 s intervals. Whole-cell rBK$\alpha$β1 currents were measured in the control, in the presence of 300 nM DiBAC$_4$(3), and after the addition of 1 µM penitrem A.  

B: The current-voltage relationships were obtained in the control (■; n = 3), in the presence of 300 nM DiBAC$_4$(3) (●; n = 3), and after the addition of 1 µM penitrem A (▲; n = 3).  

C: DiBAC$_4$(3) was applied cumulatively in the concentration ranges of 10 nM to 1 µM, and then 1 µM penitrem A was further added. Each cell was depolarized from a holding potential of −60 mV to +20 mV (○) for 150 ms at 15 s intervals. The peak outward current amplitude was plotted against time. Original current traces at different concentrations are superimposed and shown in the inset.  

D: Concentration-response relationships of
DiBAC$_4$(3) on whole-cell rBKαβ1 currents (■) were obtained from experiments typically shown in C. The relative amplitudes were obtained by normalizing the peak outward current by that before the application of DiBAC$_4$(3). The numbers of individual experiments performed are indicated in parentheses.

Figure 3. Effects of DiBAC$_4$(3) on single-channel rBKαβ1 currents in HEK293 cells.

A: Single-channel rBKαβ1 currents were measured under the inside-out patch configuration in symmetrical 140 mM K$^+$ conditions with 100 nM free Ca$^{2+}$ in the bath and pipette solution (pCa 7.0), recorded at +20 mV. DiBAC$_4$(3) was applied cumulatively in the concentration ranges of 100 nM to 3 µM, and then washed out. Relative open probability (P$_o$) was normalized by P$_o$ before the application of DiBAC$_4$(3). C, O1, O2, and O3 indicate closed and open state levels of BK channels, respectively. The increase in relative P$_o$ of rBKαβ1 channels (○) by application of DiBAC$_4$(3) was removed by washout. B: Original traces and amplitude histograms in the control, in the presence of 300 nM or 3 µM DiBAC$_4$(3), and after washout were obtained from the recording shown in A. The arrowhead indicates the closed state level of BK channels. C: Concentration-response relationships of DiBAC$_4$(3) on relative P$_o$ of rBKαβ1 channels (●; n = 4) were obtained from experiments shown in A. D: Single-channel rBKαβ1 currents were recorded in inside-out patches at several potentials in the range of 0 mV to +50 mV in the absence (○; n = 5) and presence of 300 nM DiBAC$_4$(3) (●; n = 5). The single-channel conductance was obtained from the fitting of each set of data with a linear line. E: The relationships between the relative P$_o$ and the membrane potential were obtained from single-channel rBKαβ1 currents in inside-out patches. The relative P$_o$ was determined at several potentials in the range of +30 mV to +130 mV in the absence (○; n = 5) and presence of 300 nM DiBAC$_4$(3) (●; n = 5). The data were fitted by a Boltzmann relationship.

Figure 4. Effects of DiBAC$_4$(3) on single-channel and whole-cell rBKα currents in
HEK293 cells.

A: Effects of DiBAC$_4$(3) on single-channel current of rBK$_{\alpha}$ alone were examined. Single-channel rBK$_{\alpha}$ currents were measured under the inside-out patch configuration in symmetrical 140 mM K$^+$ conditions with 100 nM free Ca$^{2+}$ in the bath and pipette solution (pCa 7.0) at a holding potential of +40 mV. DiBAC$_4$(3) was applied cumulatively in the concentration range of 10 nM to 3 $\mu$M, and then washed out. Relative $P_o$ was normalized by $P_o$ before the application of DiBAC$_4$(3). C, O1, and O2 indicate closed and open state levels, respectively. Unlike in the case of rBK$_{\alpha\beta}$1 channels, the relative $P_o$ of rBK$_{\alpha}$ channels (○) was not changed by cumulative application of DiBAC$_4$(3). B: Original traces and amplitude histograms in the control, in the presence of 300 nM or 3 $\mu$M DiBAC$_4$(3), and after washout were obtained from the recording shown in A. The arrowhead indicates the closed state level of BK channels.

C: Concentration-response relationships of DiBAC$_4$(3) on relative $P_o$ of rBK$_{\alpha}$ channels (○; n = 3) were obtained from experiments shown in A. Concentration-response relationships of DiBAC$_4$(3) on relative $P_o$ of rBK$_{\alpha\beta}$1 channels (●; n = 4) shown in Fig. 3C were re-plotted for comparison. The inset shows the scaled concentration-response relationships of DiBAC$_4$(3) on relative $P_o$ of rBK$_{\alpha}$ channels.

D: Whole-cell rBK$_{\alpha}$ currents were measured in physiological K$^+$ gradient conditions (5.9 mM K$^+$ outside and 140 mM K$^+$ inside) with 300 nM free Ca$^{2+}$ in the pipette solution (pCa 6.5). Each cell was depolarized from a holding potential of –60 mV to +60 mV in 10 mV steps for 150 ms at 15 s intervals. Whole-cell rBK$_{\alpha}$ currents were measured in the control, in the presence of 300 nM DiBAC$_4$(3), and after the addition of 1 $\mu$M penitrem A. E: The current-voltage relationships were obtained in the control (■; n = 3), in the presence of 300 nM DiBAC$_4$(3) (●; n = 3), and after the addition of 1 $\mu$M penitrem A (▲; n = 3).

Figure 5. Effects of DiBAC$_4$(3) on whole-cell rBK$_{\alpha\beta}$2 currents in HEK293 cells.

Whole-cell rBK$_{\alpha\beta}$2 currents were measured in physiological K$^+$ gradient conditions (5.9 mM K$^+$ outside and 140 mM K$^+$ inside) with 1 $\mu$M free Ca$^{2+}$ in the pipette solution (pCa 6.0). A:
Each cell was depolarized from a holding potential of –80 mV to +80 mV in 20 mV steps for 1000 ms at 15 s intervals. Whole-cell rBKαβ2 currents were measured in the control, in the presence of 1 and 3 µM DiBAC4(3), and after the addition of 1 µM penitrem A. B: The current-voltage relationships were obtained in the control (■; n = 3), in the presence of 3 µM DiBAC4(3) (●; n = 3), and after the further addition of 1 µM penitrem A (▲; n = 3). C: Concentration-response relationships of DiBAC4(3) on whole-cell rBKαβ2 currents were obtained. Each cell was depolarized from a holding potential of –80 mV to +80 mV for 1000 ms at 15 s intervals. The relative amplitude was obtained by normalizing the peak outward current by that before the application of DiBAC4(3). The numbers of individual experiments performed are indicated in columns. **p<0.01 vs. 1.0.

Figure 6. Effects of DiBAC4(3) on whole-cell rBKαβ4 currents in HEK293 cells.
Whole-cell rBKαβ4 currents were measured in physiological K+ gradient conditions (5.9 mM K+ outside and 140 mM K+ inside) with 300 nM free Ca2+ in the pipette solution (pCa 6.5). A: Each cell was depolarized from a holding potential of –60 mV to +60 mV in 10 mV steps for 150 ms at 15 s intervals. Whole-cell rBKαβ4 currents were measured in the control, in the presence of 300 nM DiBAC4(3), and after the addition of 1 µM penitrem A. B: The current-voltage relationships were obtained in the control (■; n = 6), in the presence of 300 nM DiBAC4(3) (●; n = 6), and after the addition of 1 µM penitrem A (▲; n = 4). C: DiBAC4(3) was applied cumulatively in the concentration range of 10 nM to 3 µM, and then 1 µM penitrem A was further added. Each cell was depolarized from a holding potential of –60 mV to +20 mV (○) for 150 ms at 15 s intervals. The peak outward current amplitude was plotted against time. Original current traces at different concentrations are superimposed and shown in the inset. D: Concentration-response relationships of DiBAC4(3) on whole-cell rBKαβ4 currents (■) were obtained from experiments typically shown in C. The relative amplitudes were obtained by normalizing the peak outward current by that before the application of DiBAC4(3). Concentration-response relationships of
DiBAC$_4$(3) on whole-cell rBK$\alpha\beta$1 currents (●) shown in Fig. 2D were re-plotted for comparison. The numbers of individual experiments performed are indicated in parentheses.

**Figure 7. Effects of DiBAC$_4$(3) on other K$^+$ currents in HEK293 cells.**

Whole-cell K$^+$ currents were measured in physiological K$^+$ gradient conditions (5.9 mM K$^+$ outside and 140 mM K$^+$ inside). **A** and **B**: Effects of 10 μM DiBAC$_4$(3) on type 2 small-conductance Ca$^{2+}$-activated K$^+$ channel (rSK2) (**A**) and type 4 (mSK4) (**B**) were examined. Each HEK293 cell stably expressing rSK2 or mSK4 channels was held at -90 mV and depolarized by a ramp pulse from -160 mV to +20 mV for 250 ms. The pipette solution contained 1 μM free Ca$^{2+}$ (pCa 6.0) and aspartate$,^-$, which was replaced with Cl$.^-$ SK2 and SK4 currents were identified as the currents susceptible to 10 nM UCL 1684 (**A**) or 1 μM clotrimazole (**B**), respectively. **C** and **D**: Effects of 10 μM DiBAC$_4$(3) on voltage-gated K$^+$ channel currents, rKv1.1 (**A**) and rKv4.3 (**B**). Each cell stably expressing voltage-gated K$^+$ (rKv1.1, rKv4.3) channels was depolarized from a holding potential of -80 mV to +20 mV for 1000 ms. Whole-cell rKv1.1 currents were identified as the currents suppressed by 100 nM margatoxin (**C**). Whole-cell rKv4.3 current was identified as the rapidly inactivating current (**D**). **E**: Summarized data were obtained from experiments shown in **A-D**. The relative amplitudes were obtained by normalizing the outward current at -50 mV corresponding to the Cl$^-$ reversal potential in rSK2 and mSK4 channels or the peak outward current at +20 mV in rKv1.1 and rKv4.3 channels by that before the application of 10 μM DiBAC$_4$(3). The numbers of individual experiments performed are indicated in columns. *p<0.05 vs. 1.0.

**Figure 8. Structure-potency relationships of DiBAC/DiSBAC and related compounds.**

**A**: The chemical structures of DiBAC$_4$(3) and its analogs including barbituric acid and related compounds. **B**: Whole-cell rBK$\alpha\beta$1 currents were measured in physiological K$^+$ gradient conditions (5.9 mM K$^+$ outside and 140 mM K$^+$ inside). The pipette solution contained 300
nM free Ca$^{2+}$ in (pCa 6.5). Each cell stably expressing rBKαβ1 was depolarized from a holding potential of −60 mV to +20 mV (○) for 150 ms at 15 s intervals. The time-course of changes in BK current amplitude in the absence and presence of 100 nM and 1 µM DiSBAC$_2$(5) (left) and 1 and 10 µM DiSBAC$_2$(1). (right). C: DiBAC$_4$(3) or analogs were applied at concentrations of 100 nM (■), 1 µM (■), and 10 µM (□). The relative amplitudes were obtained by normalizing the peak outward current by that before the application of DiBAC$_4$(3) or its analogs. The numbers of individual experiments performed are indicated in columns. *p<0.05 and **p<0.01 vs. 1.0. D: The relationship between the concentration of Oxonol V (●), Oxonol VI (▲), and Oxonol 595 (□) and the relative amplitude of rBKαβ1 currents are shown. Dotted lines indicated as C1, C3, and C5 were re-plotted from the results shown in C. The numbers of individual experiments performed are n = 4-7.

Figure 9. The most stable stereochemical structures of oxonol compounds.
The most stable stereochemical structures of oxonol compounds were calculated by using the density-functional theory (DFT) methods in Spartan software. Top and side views by UCSF Chimera software are shown. Hydrogen, carbon, nitrogen, oxygen, and sulfur atoms in oxonol compounds are shown by white, gray, red, blue, and yellow, respectively. A: Two sets of oxo- and oxoanion-moieties on each side of oligomethine are indicated by sky blue and pink arrows, respectively. The distances between oxygen atoms indicated by the sky blue and pink arrows are long and short, respectively. The oxo- and oxoanion-moieties in Oxonol V and Oxonol VI were located on the same side of the oligomethine, and those in Oxonol 595 were separated one by one on either side. B: The minimum requirement of stereochemical structure for a BK channel opener.
Table 1. The interatomic distance between two oxygen atoms and the potency of oxonol compounds as BK channel openers.

The pKa value and interatomic distance between two oxygen atoms on one side of the oligomethine in DiBAC₄(3) and its analogs. The potency to enhance the whole-cell BK channel currents in HEK293 cells in which rBKαβ₁ was stable is expressed.

<table>
<thead>
<tr>
<th>generic name</th>
<th>pKa (Å)</th>
<th>oxygen-oxygen distance (Å)</th>
<th>potency on whole-cell current in HEK-rBKαβ₁</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>short</td>
<td>long</td>
</tr>
<tr>
<td>DiBAC₄(3)</td>
<td>2.17 ± 0.60</td>
<td>3.81</td>
<td>6.61</td>
</tr>
<tr>
<td>DiSBAC₄(3)</td>
<td>1.72 ± 0.60</td>
<td>4.18</td>
<td>6.71</td>
</tr>
<tr>
<td>DiSBAC₂(3)</td>
<td>1.72 ± 0.60</td>
<td>4.18</td>
<td>6.70</td>
</tr>
<tr>
<td>DiBAC₅(5)</td>
<td>3.96 ± 0.60</td>
<td>6.74</td>
<td>9.07</td>
</tr>
<tr>
<td>DiSBAC₅(5)</td>
<td>3.53 ± 0.60</td>
<td>6.73</td>
<td>9.08</td>
</tr>
<tr>
<td>DiSBAC₀(5)</td>
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</tr>
<tr>
<td>DiSBAC₂(1)</td>
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<td>2.65</td>
<td>4.69</td>
</tr>
<tr>
<td>Oxonol 595</td>
<td>n.d.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oxonol V</td>
<td>5.18 ± 0.20</td>
<td>-</td>
<td>9.67</td>
</tr>
<tr>
<td>Oxonol VI</td>
<td>5.52 ± 0.20</td>
<td>-</td>
<td>9.53</td>
</tr>
</tbody>
</table>

n.d.; not determined, *; p<0.05, **; p<0.01 vs. 1.0 (n = 4-7)
Figure 6

A

B

C

D

Molecular Pharmacology Fast Forward. Published on January 5, 2007 as DOI: 10.1124/mol.106.031146
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Figure 7

A

Control
10 µM DiBAC₄(3)
+ 10 nM UCL1684

B

Control
10 µM DiBAC₄(3)
+ 1 µM clotrimazole

C

Control
10 µM DiBAC₄(3)
+ 100 nM margatoxin

D

Control
10 µM DiBAC₄(3)

E

Relative Amplitude

Ca²⁺-activated

rSK2 mSK4 rKv1.1 rKv4.3 Voltage-gated

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Figure 9

A

Top view

Side view

DIBAC₃(3)  DIBAC₅(5)  DISBAC₃(1)  Oxonol V

Top view

Side view

DIBAC₃(3)  DIBAC₅(5)  Oxonol 595  Oxonol VI

Top view

Side view

DIBAC₃(3)  DIBAC₅(5)

B

Top view

Side view

\[
\begin{align*}
&\text{Top view} \\
&\text{Side view} \\
\end{align*}
\]