0026-895X/02/6204-836–846$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 62:836–846, 2002

Vol. 62, No. 4
1665/1014937
Printed in U.S.A.

Molecular Basis of Pimarane Compounds as Novel Activators of Large-Conductance Ca^{2+}-Activated K^+ Channel α-Subunit

YUJI IMAIZUMI, KAZUHO SAKAMOTO, AKI YAMADA, AYA HOTTA, SUSUMU OHYA, KATSUHIKO MURAKI, MASANOBU UCHIYAMA, and TOMOHIKO OHWADA

Departments of Molecular and Cellular Pharmacology (Y.J., K.S., A.Y., A.H., S.O., K.M.), and Organic and Medicinal Chemistry (M.U., T.O.), Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan; and Laboratory of Organic and Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, the University of Tokyo, Tokyo, Japan (M.U., T.O.)

Received February 11, 2002; accepted July 16, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Effects of pimaric acid (PiMA) and eight closely related compounds on large-conductance K^+ (BK) channels were examined using human embryonic kidney (HEK) 293 cells, in which either the α subunit of BK channel (HEKBKα) or both α and β1 (HEKBK$\alpha\beta$1) subunits were heterologously expressed. Effects of these compounds (10 μM) on the membrane potential of HEKBK$\alpha\beta$1 were monitored by use of $DiBAC_4(3)$, a voltage-sensitive dye. PiMA, isopimaric acid, sandaracoisopimaric acid, dihydropimaric acid, dihydroisopimaric acid, and dihydroisopimarinol induced substantial membrane hyperpolarization. The direct measurement of BK$\alpha\beta$1 opening under whole-cell voltage clamp showed that these six compounds activated BK$\alpha\beta$1 in a very similar concentration range (1–10 μM); in contrast, abietic acid, sclareol, and methyl pimarate had no effect. PiMA did not affect the charybdotoxin-induced block of macroscopic BK$\alpha\beta$1 current. Single channel recordings of BK$\alpha\beta$1 in inside-out patches showed that 10 μM PiMA did not change channel conductance but significantly increased its open probability as a result of increase in sensitivity to Ca^{2+} and voltage. Because coexpression of the β1 subunit did not affect PiMA-induced potentiation, the site of action for PiMA is suggested to be BKα subunit. PiMA was selective to BK over cloned small and intermediate Ca^{2+} activated K^+ channels. In conclusion, PiMA (>1 μM) increases Ca^{2+} and voltage-sensitivity of BKα when applied from either side of the cell membrane. The marked difference in potency as BK channel openers between PiMA and abietic acid, despite only very small differences in their chemical structures, may provide insight into the fundamental structure-activity relationship governing BKα activation.

Large conductance Ca^{2+} activated K^+ (BK) channels are expressed in many different types of excitable cells and have significant physiological roles in the regulation of frequency of firing, action potential repolarization, and/or afterhyperpolarization (for reviews, see Vergara et al., 1998; Kaczorowski and Garcia, 1999). In addition, BK channels are often a key factor for the negative feedback control of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and save cells from Ca^{2+} overload during pathophysiological conditions (Lawson, 2000). Excess intracellular Ca^{2+} is a major cause of neuronal cell death in the setting of brain ischemia after stroke. The activation of BK channels by elevation of $[Ca^{2+}]_i$ after Ca^{2+} influx from outside and/or Ca^{2+} release from endoplasmic reticulum hyperpolarizes the cell and down-regulates the activity of voltage-dependent Na^+ and Ca^{2+} channels in neuronal cells. In smooth muscle cells, activation of BK channels by spontaneous Ca^{2+} release (Ca^{2+} sparks) from sarcoplasmic reticulum (Nelson et al., 1995; Jaggar et al., 2000) is thought to be an essential regulator of resting membrane potential. In vascular smooth muscle of spontaneous hypertensive rats, the expression of BK channel is enhanced (Liu et al., 1997) and the deficiency of BK channel activity in β1 subunit gene-lacking mice results in systemic hypertension (Brenner et al., 2000b). BK channel activation by the Ca^{2+}-induced Ca^{2+} release during excitation-contraction coupling also significantly contributes to action potential repolarization/afterhyperpolarization in smooth muscle cells (Imaizumi et al., 1998; Ohi et al., 2001).

For these reasons, agents that enhance BK channel activity (BK channel openers) may be effective in protecting neu-

This work was supported by grant-in-aid for scientific research by Japan Society for the Promotion of Sciences and also by grant-in-aid for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation (to Y.I.).

ABBREVIATIONS: BK, large-conductance Ca^{2+}-activated K^+ channel; 1-EBIO, 1-ethyl-2-benzimidazolinon; BKα, BK channel α subunit; BKβ1, BK channel β1 subunit; BK$\alpha\beta$1, BK channel α plus β1 subunit; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ChTX, charybdotoxin; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid)trimethine oxonol; DMSO, dimetyl sulfoxide; HEK, human embryonic kidney; HEKBKα, HEK293 cells expressing BKα; HEKBK$\alpha\beta$1, HEK293 cells expressing BK$\alpha\beta$1; IK, intermediate Ca^{2+}-activated K^+ channel; PiMA, pimaric acid; P_o, open probability; RT-PCR, reverse transcription-polymerase chain reaction; SK, small-conductance Ca^{2+}-activated K^+ channel; TEA, tetraethylammonium.

rons from damage after an ischemic stroke and/or suppressing excess activity of smooth muscle tissues (Lawson, 2000; Shieh et al., 2000). Many compounds have been reported to be BK channel openers; dehydrosoyasaponin-I, maxikdiol, L-735,334, NS-4, NS-1608, NS-1619, niflumic acid, cromakalim, nitrendipine, BMS-204352, NS-8, CGS-7181, CGS-7184, etc. (for review, see Starrett et al., 1996; Coghlan et al., 2001). 17β-Estradiol (Valverde et al., 1999) and epoxyeicosatrienoic acids (Fukao et al., 2001) may be endogenous BK channel openers. Moreover, some transmitters and hormones can also enhance BK channel activity via activation of cAMP and cGMP dependent protein kinases (for review, see Vergara et al., 1998).

BK channels consist of channel forming α subunits and accessory β subunits arranged in tetramers (Vergara et al., 1998). Each β subunit interacts with N-terminal region of the α subunit (Wallner et al., 1996) and regulates the activity of α subunit by changing Ca^{2+} and voltage sensitivity and/or channel kinetics. Although only one major type of α subunit with splice variants has been described, several subtypes of β subunit have been cloned and are suggested to be responsible for the differential characteristics of BK channels in various tissues (Brenner et al., 2000a; Uebele et al., 2000; Xia et al., 2000). Synthetic openers such as NS-1619 and BMS-204352 are activators of BKα subunit, whereas dehydrosoyasaponin-I (Giangiacomo et al., 1998), 17β-estradiol (Valverde et al., 1999), and tamoxifen (Dick et al., 2001) act on BKβ subunit. Most of these BK channel openers, including BMS-204352, require concentrations higher than 300 nM to increase the macroscopic BK channel current and are therefore not highly potent activators. Terpenoids derived from natural products (i.e., dehydrosoyasaponin-I, maxikdiol, and L-735,334) are impermeable to cell membrane and effective only when applied to cytoplasmic side of BK channels (Starrett et al., 1996; Kaczorowski and Garcia, 1999).

The present study was undertaken to identify new molecules that are potent BK channel openers. Our goal was to find and characterize natural products and chemically modified derivatives with a novel assay system that we developed using recombinant BK channels in human embryonic kidney (HEK) 293 cells and voltage-sensitive dye (Yamada et al., 2001). Novel compounds were discovered from terpenoids, which have chemical structures similar to that of maxikdiol, a moderate BK channel opener, obtained from fermentation broth of an unidentified coelomycite (Singh et al., 1994). We found that pimaric acid (PiMA) and related compounds are potent BK channel openers, whereas isomeric abietic acid was not, even though it has a chemical structure very similar to that of PiMA.

Methods

Vector Constructs, Cell Culture, and Transfection. Restriction enzyme-digested DNA fragments of BKα (*Kpn*I/*Xba*I-double digested) and BKβ1 (*Eco*RI/*Xba*I-double digested) were ligated into mammalian expression vectors, pcDNA3.1(+) and pcDNA3.1/Zeo(+) (Invitrogen, Carlsbad, CA), respectively, using the TaKaRa ligation kit Ver. 1 (TaKaRa; Yamada et al., 2001). HEK293 cells were obtained from Health Science Research Resources Bank (HSRRB, Tokyo, Japan) and maintained in minimum essential medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (JRS Biosciences, Lenexa, KS), penicillin (100 units/ml, Wako, Osaka), and streptomycin (100 μg/ml, MEIJI SEIKA, Tokyo). Stable expression of BKα and BKβ was achieved by using calcium phosphate coprecipitation transfection techniques. G418 (1 mg/ml, Invitrogen)- and G418/Zeocin (0.25 mg/ml, Invitrogen)-resistant cells were selected as those which were BKα-expressing and BKα β1-coexpressing, respectively. Expression of BKα and BKβ transcripts was confirmed by RT-PCR. It was also confirmed by RT-PCR that neither BKα nor BKβ1 subunit mRNA was detected in native HEK293 cells. Transfected cell lines were maintained in minimum essential medium supplemented with 10% fetal calf serum and G418 (0.5 mg/ml). The functional expression of BKα was verified using inside-out patch-clamp recordings and detected in ~90% of cells examined. In addition, the expectation of higher activity of BK$\alpha\beta$1 than that of BKα alone was confirmed in approximately 30 cells in five separate culture dishes (Yamada et al., 2001). Functional expression of BK$\alpha\beta$1 was observed in approximately 80% of cells prepared as stable HEK$\alpha\beta$1 in this study.

Small- and intermediate-conductance Ca^{2+}-activated K^+ channels of the rat, which are encoded by SK2 and SK4 (GenBank accession numbers U69882 and NM_023021), respectively, were also subcloned into pcDNA3.1(+) and were transfected into HEK293 cells for stable expression in the same manner as BK subtypes. The expression of SK2 and SK4 in HEK293 cells was confirmed by RT-PCR using subtype-specific primers and was verified functionally by membrane hyperpolarization induced by chlorzoxazone, which was then blocked by apamin and clotrimazole, respectively. The functional expression was observed in approximately 70 and 30% of cells transfected with SK2 and SK4, respectively.

Solutions. The HEPES-buffered solution for electrophysiological recording had an ionic composition of 137 mM NaCl, 5.9 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 14 mM glucose, and 10 mM HEPES. The pH of the solution was adjusted to 7.4 with NaOH. The pipette solution contained 140 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 2 mM Na_2ATP, and 5 mM EGTA. Adding $CaCl_2$ and KOH adjusted the pCa and pH of the pipette solution to 6.5 and 7.2, respectively. During recordings of single BK channel current in the inside-out patch-clamp configuration, the pipette solution contained the HEPES-buffered solution and the bathing solution contained 140 mM KCl, 1.2 mM $MgCl_2$, 14 mM glucose, 10 mM HEPES, and 5 mM EGTA. Selected pCa of the bathing solution was obtained by adding adequate amount of $CaCl_2$ and the pH was adjusted to 7.2 with NaOH as described previously (Imaizumi et al., 1996). The perfusion solution consistently contained 0.03% dimethyl sulfoxide (DMSO), which was used for the solvent of pimarane compounds, regardless of the absence or presence of these compounds, in all experiments in this study.

Electrophysiological Experiments. Whole-cell and inside-out patch-clamp were applied to single cells using a CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan) and EPC-7 amplifier (List Electronik, Darmstadt, Germany), respectively. The procedures of electrophysiological recordings and data acquisition/analysis for whole-cell recording have been described previously (Imaizumi et al., 1996). Whole-cell currents were recorded from each single cell and leakage currents at potentials positive to −60 mV were subtracted digitally, assuming a linear relationship between current

and voltage in the range of −90 to −60 mV. Single-channel current analyses were done using the software PAT V7.0C, developed by Dr. J. Dempster (University of Strathclyde, Glasgow, UK). The resistance of the pipette was 2 to 5 MΩ for whole-cell and 10 to 15 MΩ for inside-out patch configurations when filled with the pipette solutions. The series resistance was partly compensated electrically under whole-cell voltage clamp. Whole-cell and single-channel recordings were carried out at room temperature (24 ± 1°C).

Membrane Potential Measurements by Voltage-Sensitive Fluorescent Dye. The measurement of membrane potential changes by $DiBAC_4(3)$, which is a bis-barbituric acid oxonol dye with excitation maxima at approximately 490 nm, was performed as described previously (Yamada et al., 2001). Before the fluorescence measurements, cells were incubated with 50 or 100 nM $DiBAC_4(3)$ in HEPES-buffered solution for 20 min at room temperature. Experiments were carried out in the constant presence of $DiBAC_4(3)$. The fluorescence emission was collected from cell clusters using a dichroic mirror (505 nm) and a BA filter (>520 nm). Hyperpolarization results in the extrusion of the dye from cells and a subsequent decrease in fluorescence intensity. The decrease in fluorescence intensity by 1% corresponded to approximately 0.5-mV hyperpolarization in the membrane potential range of −20 and −70 mV (Yamada et al., 2001). Data collection and analyses were performed using imaging system (ARGUS-HiSCA; Hamamatsu, Hamamatsu City, Japan). The sampling interval of $DiBAC_4(3)$ fluorescence measurements was 10 s.

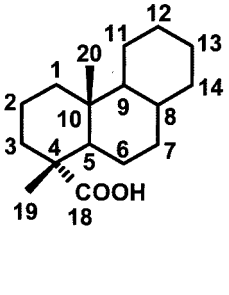
Calculation of Stable Conformations of Compounds. The most stable stereochemical structures of PiMA, abietic acid, and maxikdiol were calculated by using geometry optimization with the SYBYL (molecular mechanics; Tripos, St. Louis, MO)-PM3 (semiempirical MO calculation) methods, manipulated in the computer program Spartan 4.1.2. (SGI version; Wavefunction, Inc., Irvine, CA).

Chemicals. Most of pharmacological agents were obtained from Sigma-Aldrich (St. Louis, MO). Tetraethylammonium chloride (TEA) was from Tokyo Kasei (Tokyo, Japan), Bis-(1,3-dibutylbarbituric acid)-trimethine oxonol [$DiBAC_4(3)$] was from Molecular Probes. Inc. (Eugene, OR), and charybdotoxin was obtained from Peptide Institute Inc. (Osaka, Japan). PiMA and related natural products contained in rosin were obtained from Helix Biotech (New Westminster, BC, Canada). Methyl pimarate was prepared by methyl esterification of PiMA with ethereal diazomethane in methanol. Dihydroisopimarinol was obtained by reduction of dihydroisopimaric acid in diethyl ether with lithium aluminum hydride at reflux. Dihydropimaric acid was obtained by partial hydrogenation of PiMA over 5% Pd on carbon in methanol. The test compounds were dissolved with DMSO. The final concentration of DMSO was 0.03% or lower.

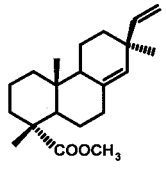
Statistics. Data are expressed as means ± S.E.M. in the text. Statistical significance between two and among multiple groups was evaluated using Student's t test and Scheffé's test after F test or one-way analysis, respectively. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively.

Results

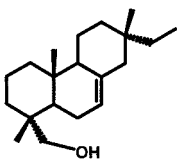
Identification of BK Channel Openers Based on Natural Products Using a Novel Assay System. Because some terpenoids and steroids, such as dehydrosoyasaponin-I, maxikdiol, and 17β-estradiol, exhibit BK channel opening activity, a survey of several types of natural terpenoids and chemically modified derivatives was undertaken to identify a novel BK channel opener. The assay techniques that we recently developed were applied. This is based on a decrease in fluorescence intensity of $DiBAC_4(3)$ corresponding to membrane hyperpolarization by BK opening, measured in HEK293 cells in which BKαβ1 had been heterologously and stably expressed. The expression of BKαβ1 was detected in more than 80% of cells when examined by inside-out patch clamp (n >70; see below). Based on their intuitive similarity of chemical structure to that of maxikdiol, nine resin acids shown in Fig. 1 were examined in this series of experiments.



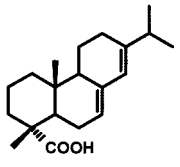
C7-C8	C8-C14	C13	Compounds
C-C	C=C		pimaric acid
C=C	C-C		isopimaric acid
C-C	C=C		sandaracopimaric acid
C-C	C=C		dihydropimaric acid
C=C	C-C		dihydroisopimaric acid



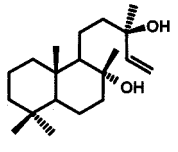
methyl pimarate



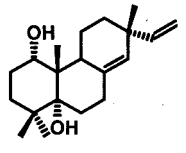
dihydroisopimarinol



abietic acid



sclareol



maxikdiol

Fig. 1. Chemical structures of compounds selected for testing; resin acids contained in pine rosin and their derivatives and maxikdiol.

PiMA and derivatives and abietic acid are known to be composites of pine rosin.

Figure 2A shows changes in relative fluorescence intensity of $DiBAC_4(3)$ in HEKBK$\alpha\beta$1 when either 10 μM PiMA (Fig. 2A, a) or abietic acid (Fig. 2A, b) was applied. The fluorescence intensity was markedly reduced by application of PiMA and it recovered partially after the addition of 5 mM tetraethylammonium (Fig. 2A, a), suggesting that the reduction was caused by membrane hyperpolarization induced by activation of BK channels. Application of 10 μM abietic acid either did not change or slightly increased the fluorescence intensity (Fig. 2A, b). Effects of other compounds listed in Fig. 1 were tested in a manner similar to that shown in Fig. 2A. Figure 2B summarizes the results obtained from native HEK293 cells (□) and HEKBK$\alpha\beta$1 (■). Application of 10 μM isopimaric acid, sandaracopimaric acid, dihydropimaric acid, dihydroisopimaric acid, and dihydroisopimarinol each induced a marked decrease in fluorescence intensity in HEKBK$\alpha\beta$1. In contrast, 10 μM methyl pimarate, abietic acid, and sclareol induced much smaller changes in fluorescence intensity in HEKBK$\alpha\beta$1. Isopimaric acid, methyl pimarate, dihydroisopimarinol, abietic acid, and sclareol induced small but statistically significant changes even in native HEK293 cells, suggesting artifacts. The responses to isopimaric acid and dihydroisopimarinol in HEK$\alpha\beta$1 were, however, much larger than those in native HEK.

Effects of PiMA and Related Compounds on Macroscopic BK Channel Currents. Effects of test compounds on BK channel currents were examined in single HEKBK$\alpha\beta$1 under whole-cell voltage clamp. The Ca^{2+} concentration in the pipette solution was fixed at pCa 6.5 using a Ca^{2+}-EGTA buffer. Depolarization from −60 to + 30 mV induced outward currents in both native HEK and HEKBK$\alpha\beta$1, but the current density was much higher in the latter (the current density at the peak: 11.0 ± 1.8 and 95.2 ± 20.9 pA/pF, $n = 5$ and 6, respectively, $p < 0.05$). Application of PiMA in a concentration range of 1 to 10 μM increased outward currents in a dose-dependent fashion in HEKBK$\alpha\beta$1 (Fig. 3A) but did not change in native HEK (data not shown). This enhancement of outward current by PiMA could be removed completely by washout. Effects of other test compounds were also examined in this manner. The peak amplitude of outward current at +40 mV in the presence of 1, 3, and 10 μM resin acids was measured relative to the value taken just before the application (Fig. 3B). PiMA, sandaracopimaric acid, isopimaric acid, dihydroisopimaric acid, and dihydroisopimarinol showed significant potentiating effects on outward current at concentration of 1 μM and higher (n =

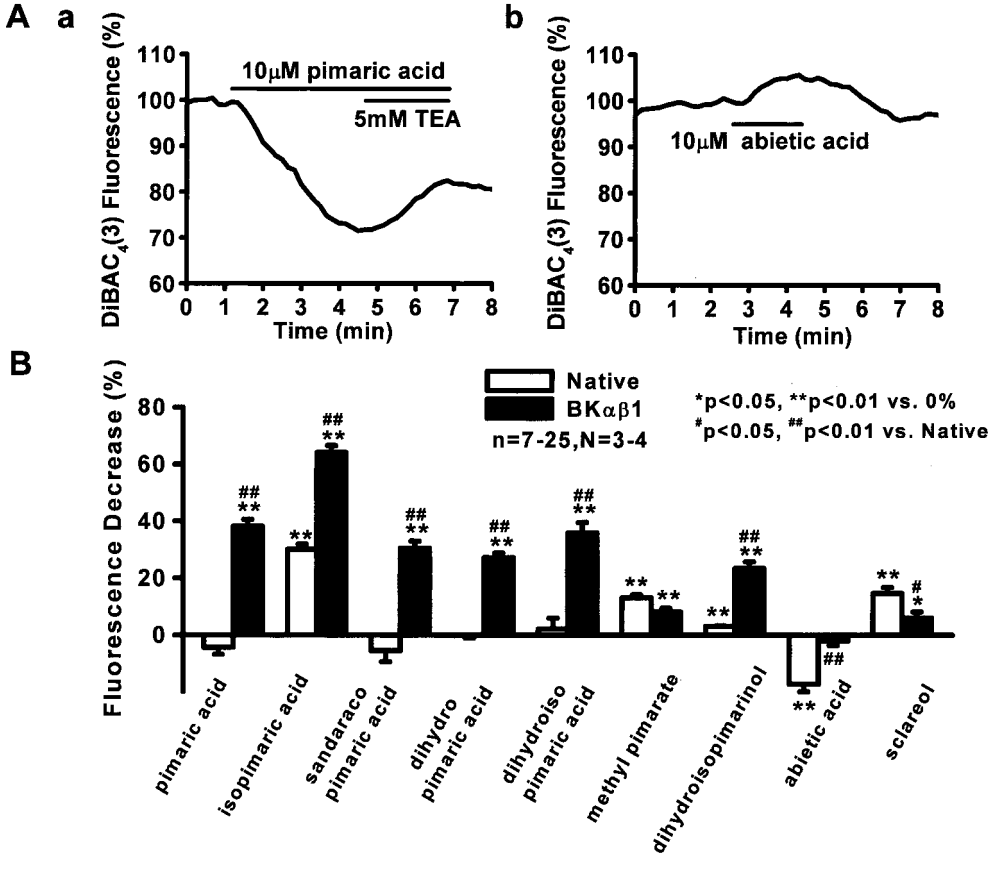


Fig. 2. Measurements of $DiBAC_4(3)$ fluorescence in HEK293 cells heterologously expressing BK channel α and β1 subunits (HEK$\alpha\beta$1) and effects of nine compounds shown in Fig. 1. A, fluorescence intensity of $DiBAC_4(3)$ was measured from clusters of HEKBK$\alpha\beta$1 in the continuous presence of 50 or 100 nM dye after loading for 20 min. PiMA (a) and abietic acid (b) were applied at the concentration of 10 μM. Each cluster included 10 to 30 cells. The fluorescence intensity measured at 1 min before the application of test compounds was taken as 100%. The perfusion solutions contained 0.1% DMSO throughout. When a test compound decreased fluorescence, 5 mM TEA was added (a). B, Summarized data demonstrating the relative decrease in fluorescence intensity of $DiBAC_4(3)$ after application of nine test compounds. Each of these compounds was also applied to native HEK293 cells as the control. □, relative decrease in fluorescence intensity in native HEK; ■, relative decrease in fluorescence intensity in HEKBK$\alpha\beta$1. Mean values and S.E.M. are depicted as columns and vertical bars. Data were accumulated from 7 to 25 clusters (n) from 3 to 4 separate culture dishes (N) for each column. **, $p < 0.01$ versus 0% in each column. #, $p < 0.05$; ##, $p < 0.01$ versus native HEK in a pair of each compound.

4–5, $p < 0.05$ versus 1.0). Abietic acid, methyl pimarate, and sclareol showed no significant effects even at 10 μM (n = 2–5), confirming the results obtained by using $DiBAC_4(3)$. No increases in outward current were observed in the presence of 5 mM TEA.

To determine whether PiMA changes the binding of charybdotoxin (ChTX) to BK channels, the concentration-dependent block of macroscopic currents by ChTX was measured in HEKBK$\alpha\beta$1 in the absence and presence of 10 μM PiMA (Fig. 4). The outward currents elicited by depolarization from −60 mV in 10 mV steps were enhanced at potentials positive to −20 mV. The current at +40 mV was increased from 1214 ± 153 to 2527 ± 448 pA ($n = 5, p < 0.05$). The addition of 100 nM ChTX significantly reduced the outward current to levels lower than those under the control conditions (Fig. 4, A and B). The remaining components in the presence of 100 nM ChTX include native delayed rectifier K^+ currents and unblocked BK$\alpha\beta$1. The current at +40 mV in the presence of 10 μM PiMA was reduced from 2527 ± 448 pA to 499 ± 90 pA ($p < 0.01$ versus before the application of and in the presence of 10 μM PiMA). Cumulative addition of ChTX in the range of 3 to 100 nM reduced outward current in a concentration dependent manner (Fig. 4C). A concentration-response relationship for ChTX-induced block was also obtained in the absence of PiMA; the current amplitude at +

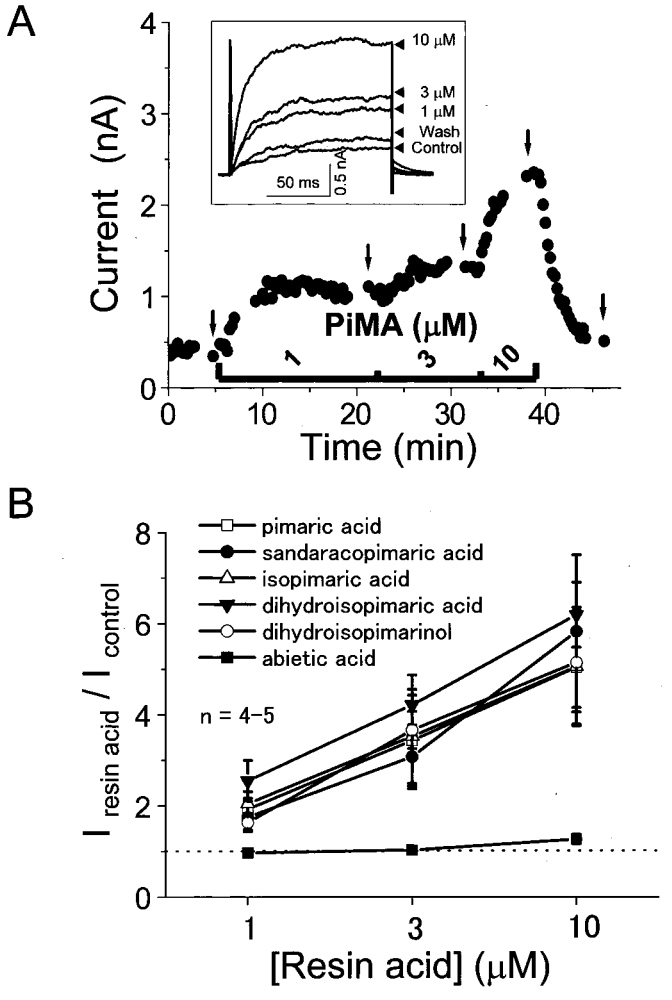


Fig. 3. Effects of PiMA and related compounds on macroscopic membrane currents in HEKBK$\alpha\beta$1 cell. A, single HEKBK$\alpha\beta$1 cell was depolarized from −60 to + 30 mV for 150 ms under whole-cell voltage clamp. PiMA was applied cumulatively in a concentration range of 1 and 10 μM. Original current recordings at different concentrations were superimposed and shown in the inset. The peak outward current amplitude at +30 mV was measured and plotted against time. The original traces were obtained at the time indicated by vertical arrows in the time course. B, the concentration-response relationships for several resin acids. Effects of PiMA (□), sandaracopimaric acid (●), isopimaric acid (△), dihydroisopimaric acid (▼), dihydroisopimarinol (○), and abietic acid (■) were examined in experiments identical to that shown in A for PiMA. The relative amplitude of peak outward current at +30 mV in the presence of test compounds ($I_{resin\ acid}/I_{control}$) was determined taking the amplitude in the absence of them as unity (dotted line). Symbols and vertical bars show mean values and S.E.M., respectively; there were 4 to 5 experiments for each compound.

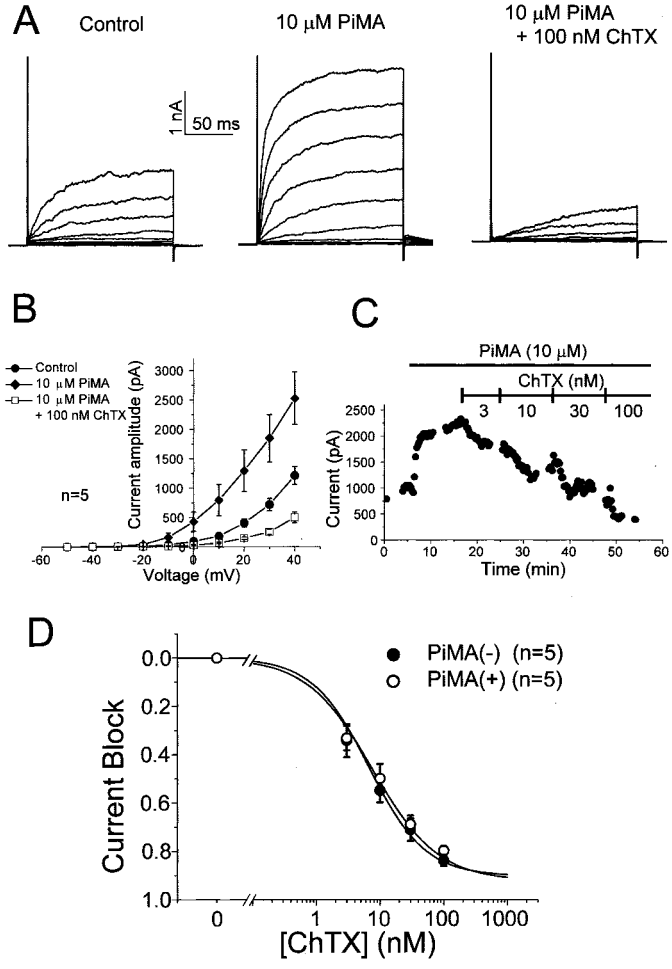


Fig. 4. Effects of PiMA on the blockade of macroscopic currents by charybdotoxin (ChTX) in HEKBK$\alpha\beta$1 cell. A, each single HEKBK$\alpha\beta$1 cell was depolarized from −60 mV by 10 mV step for 150 ms under whole-cell voltage clamp. Application of 10 μM PiMA and then 100 nM ChTX enhanced and then markedly reduced outward currents, respectively. B, the current-voltage relationships were obtained in the control (●), in the presence of PiMA (♦), and after the further addition of 100 nM ChTX (□) in experiments such as illustrated in A; five examples. C, the time course of effects of PiMA and ChTX on peak outward currents elicited by depolarizations from −60 to +40 mV applied once every 15 s. ChTX was added cumulatively at concentrations of 3, 10, 30, and 100 nM in the presence 10 μM PiMA. D, the relationship between ChTX and the block of outward currents obtained from experiments shown in C. The relative amount of block was defined as the relative amplitude of the current reduced by ChTX versus the amplitude before addition of ChTX in each cell in the absence (●) or presence (○) of 10 μM (n = 5 for each). The data were fitted with Hill equation modified for the concentration-block relationship. The fitted lines were drawn based on the following values of K_d, Hill coefficient and the constant; 7.66, 0.847, and 0.771 in the control and 6.72, 0.982, and 0.0965 in the presence of 10 μM PiMA, respectively.

40 mV was reduced from 1233 ± 226 to 205 ± 46 pA by addition of 100 nM ChTX ($n = 5$, $p < 0.01$). The normalized data were well fitted with the Hill equation modified for the concentration-response relationship. $I_{block} = (1 - C)/\{1 + (K_d/[ChTX])^{n_H}\}$, where K_d is the apparent dissociation constant of ChTX, [ChTX] is the concentration of ChTX, n_H is the Hill coefficient, and C is the constant. The K_d obtained from the best fitting were 7.66 ± 2.66 and 6.72 ± 2.26 nM ($n = 5$, $p > 0.05$), in the absence and presence of PiMA, respectively. The n_H values, 0.847 ± 0.034 and 0.982 ± 0.161 ($p > 0.05$), respectively, suggest one-to-one binding of ChTX to BK$\alpha\beta$1. The C values, which may correspond to the native K^+ currents insensitive to ChTX, were 0.077 ± 0.0226 and 0.0965 ± 0.0388 ($p > 0.05$), respectively. The data in Fig. 4D show that the relationship in the presence of 10 μM PiMA was almost identical with that in its absence. The half-maximum concentrations of ChTX for the block of BK channel current were almost identical each other in the absence and presence of PiMA (7.66 and 6.72 nM). These results suggest that PiMA does not affect the binding of ChTX to BK channels.

Effects of PiMA on Single BKα Channel Currents. Effects of PiMA on single BKα channel currents were examined in inside-out patch configuration. The pCa in the bathing solution and holding potential were 7.0 and +40 mV, respectively. The K^+ concentration in the bathing and pipette solutions was 140 mM (140 mM K^+, symmetrical conditions). The unitary current amplitude and open probability (P_o) at +40 mV was 10.1 ± 0.2 pA and 0.00362 ± 0.00099 ($n = 6$), respectively. Cumulative application of 0.3, 1, 3, and 10 μM PiMA increased channel opening events in a concentration-dependent manner (Fig. 5A). Note that PiMA was effective on BKα even when applied to cytosolic phase, as well as when applied from outside as shown in Fig. 3. The effect of PiMA was completely removed by washout. The P_o was measured from the event histogram versus current amplitude (Fig. 5B). The number of channels in a patch was determined by elevating Ca^{2+} concentration from pCa 7.0 to 4.0. PiMA significantly increased the P_o at concentrations of 1 μM and higher (Fig. 5C). The relative P_o was determined by taking P_o in the control as unity: 1.66 ± 0.19, 3.92 ± 0.78, and 13.1 ± 2.3 in the presence of 1, 3, and 10 μM PiMA, respectively ($n = 6$). It was confirmed that 0.03% DMSO, which was consistently added to the perfusion solution, did not affect P_o of BKα (at +40 mV, pCa7.0; P_o = 0.0050 ± 0.0017 and 0.0036 ± 0.0010, $n = 8$ and 6, $p > 0.05$ in the absence and presence of 0.03% DMSO, respectively).

The single-channel conductance of BKα was measured in inside-out patch configuration at pCa 6.0 and in 140 mM K^+ symmetrical conditions (Fig. 6). The conductance was 270.1 ± 6.1 and 270.1 ± 6.0 pS in the absence and presence of 10 μM PiMA, respectively ($n = 7$, $p > 0.05$), indicating that PiMA does not affect BKα channel conductance.

BK channel activity is strongly voltage-dependent except when cytosolic Ca^{2+} concentration is very high (> 10 μM). The voltage-dependence of P_o was examined in inside-out patch configuration at pCa 7.0 in 140 mM K^+ symmetrical conditions (Fig. 7A). Under control conditions, the increase in P_o was voltage dependent in the range of + 30 and + 110 mV and well fitted by a Boltzmann relationship (Fig. 7B): $P_o = 1/[1 + \exp\{(V_{1/2} - V_m)/S\}]$, where $V_{1/2}$, V_m, and S are the voltage required for half-maximum activation, membrane potential, and slope factor, respectively. Under the control conditions, the values of $V_{1/2}$ and S are 106.0 ± 5.5 mV and 13.3 ± 1.7 mV ($n = 6$), respectively. In the presence of 10 μM PiMA, the P_o was increased at any potentials examined and the fitted line was shifted to negative potentials by approximately 20 mV (Fig. 7). $V_{1/2}$ and S in the presence of 10 μM PiMA are 85.6 ± 6.4 mV ($p < 0.01$ versus control) and 11.8 ± 1.7 mV ($p > 0.05$), respectively ($n = 6$). The ratio of P_o in the presence and absence of 10 μM PiMA was calculated and plotted against test potentials in Fig. 7C. These results demonstrate that the lower the P_o in the control conditions, the larger the enhancement by PiMA.

Effects of PiMA on Ca^{2+} sensitivity of BKα were examined at 0 mV in 5.9/140 mM asymmetrical conditions. When Ca^{2+}

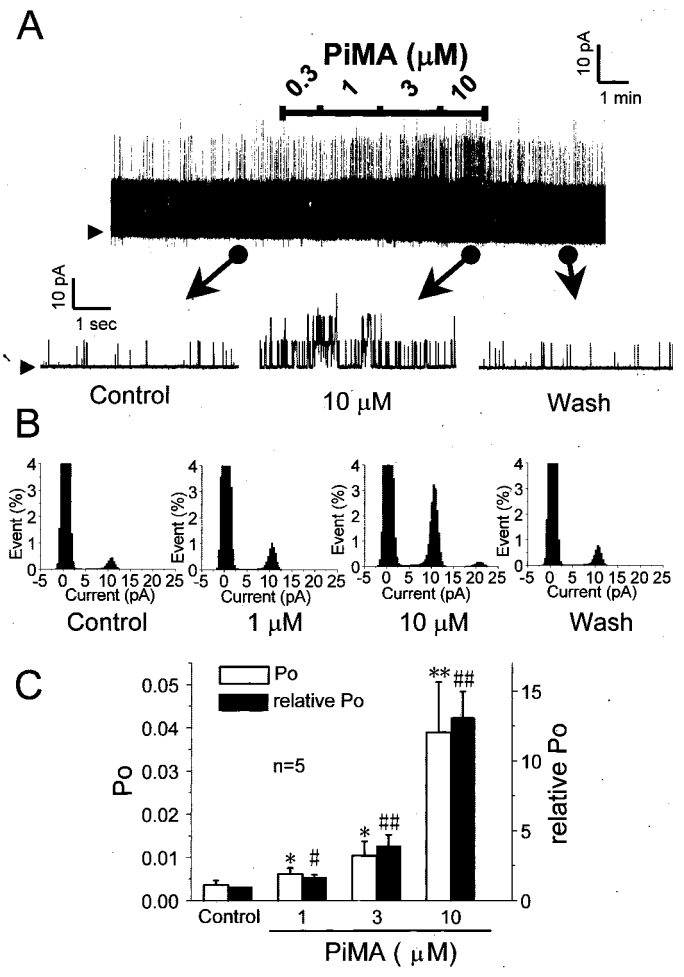


Fig. 5. Effects of PiMA on single BKα channel currents recorded using the inside-out patch configuration. A, single channel currents were recorded at +40 mV in a patch from HEKBKα using symmetrical 140 mM K^+ conditions. The Ca^{2+} in the bathing solution was adjusted to pCa 7.0. PiMA was applied cumulatively at 0.3 to 10 μM. Selected parts of the trace indicated by arrows are shown as a faster time scale. ▶, zero current level. B, amplitude histograms in the control, in the presence of 1 or 10 μM PiMA and after washout obtained from the recording shown in A. The ordinate expresses the relative time (%) spent at the corresponding amplitude in each bin (1 pA). C, summarized data demonstrating the relationship between concentrations of PiMA and open probability (P_o) of BKα obtained from experiments shown in A. P_o was calculated from the histogram shown in B as the relative time spent at open state, based on the total number of BKα channels in patches, which was determined by elevating Ca^{2+} concentration to 4.0 (□). Only the data from patches including less than 5 channels were analyzed. The relative P_o is obtained taking the P_o in the absence of PiMA as unity (■); there were 6 examples. * and **, $p < 0.05$ and $p < 0.01$ versus control.

concentration in the bathing solution was elevated in a range of pCa7.0 and 5.0, P_o was increased in a concentration-dependent manner (Fig. 8, A and B). The relationship between Ca^{2+} concentrations and the open probability (P_o) of BKα was fitted by $P_o = (1-C)/\{1 + (K_d/[Ca^{2+}])^{n_H}\}$, where K_d is the apparent dissociation constant of Ca^{2+}, $[Ca^{2+}]$ is the pCa in the bathing solution, n_H is the Hill coefficient, and C is the constant. The K_d, n_H, and C obtained from the best fitting were pCa 5.88 ± 0.03, 4.15 ± 0.42, and 0.152 ± 0.029 ($n = 5$), respectively, under the control conditions. In the presence of 10 μM PiMA, the P_o was increased at any pCa examined and the half-maximum P_o was obtained at pCa 5.98 ± 0.03 ($n = 5$, $p < 0.05$ versus the control K_d). The Hill coefficient (3.65 ± 0.29, $p > 0.05$ versus control), which indicates four Ca^{2+} ions to each α subunit in the tetramer, was not significantly affected by PiMA. The value for C was 0.102 ± 0.008 ($p > 0.05$ versus control) and was not affected by PiMA. When the P_o in the absence of PiMA was low, the relative increase in P_o by PiMA was marked (Fig. 8C).

Comparison of PiMA-Induced Effects on BKα and Those on BK$\alpha\beta$1. The results obtained using BKα clearly indicate that PiMA enhances Ca^{2+} and/or voltage-sensitivity of BKα subunit. To determine whether PiMA acts also on BKβ1 subunit, the increase in P_o by PiMA in BK$\alpha\beta$1 was compared with that in BKα in inside-out patches. It has been established that coexpression of β1 subunit with BKα increases Ca^{2+} and voltage sensitivity of BKα (Wallner et al., 1996; Cox and Aldrich, 2000). Accordingly, the P_o of BKα at pCa 7.0 was markedly increased by coexpression with β1 in 140 mM K^+ symmetrical conditions (from 0.00023 ± 0.00014 to 0.00359 ± 0.00076 at +20 mV, $n = 9$ and 8, respectively, $p < 0.01$ and from 0.00396 ± 0.00079 to 0.02084 ± 0.00529 at +40 mV, $n = 8$ and 7, respectively, $p < 0.01$) (Fig. 9A). The increase in P_o by PiMA depended upon the P_o under the control conditions as shown in Fig. 6 and 7. The P_o at +40 mV in BKα was close to that at + 20 mV in BK$\alpha\beta$1 ($p > 0.05$). Based on this observation, effects of 10 μM PiMA on BKα at

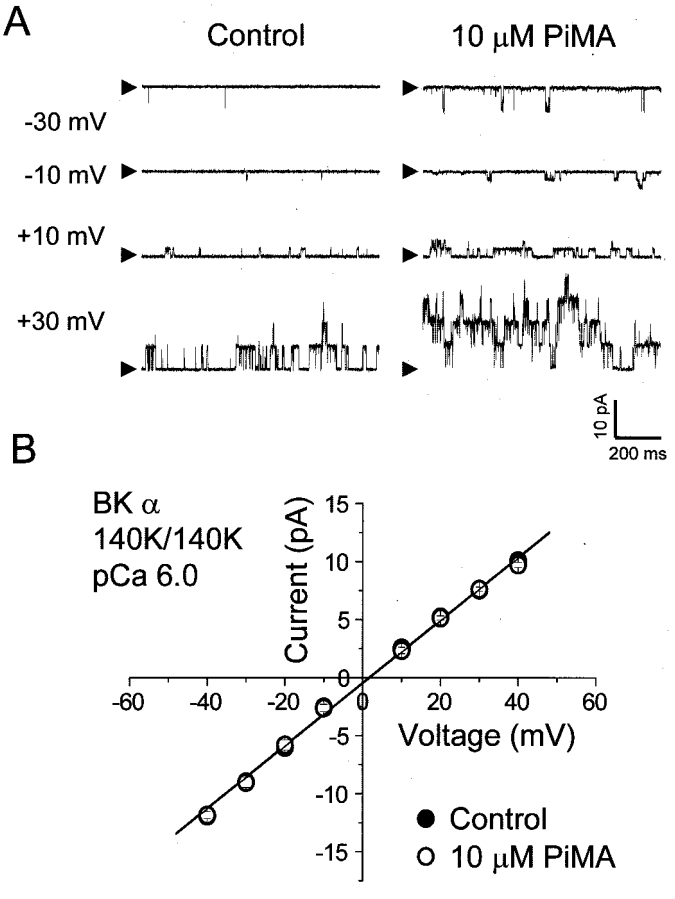


Fig. 6. Effects of PiMA on single-channel conductance of BKα. A, single-channel currents of BKα in inside-out patches were recorded at several potentials in the absence and presence of 10 μM PiMA. All conditions except applied potentials were the same as shown in Fig. 4. B, the current-voltage relationships obtained from the measurements shown in A. The relations in the absence (●) and presence of 10 μM PiMA (○) were fitted to linear lines, and the single channel conductance was determined from the slope. The number of observations was seven for each in the absence and presence of PiMA.

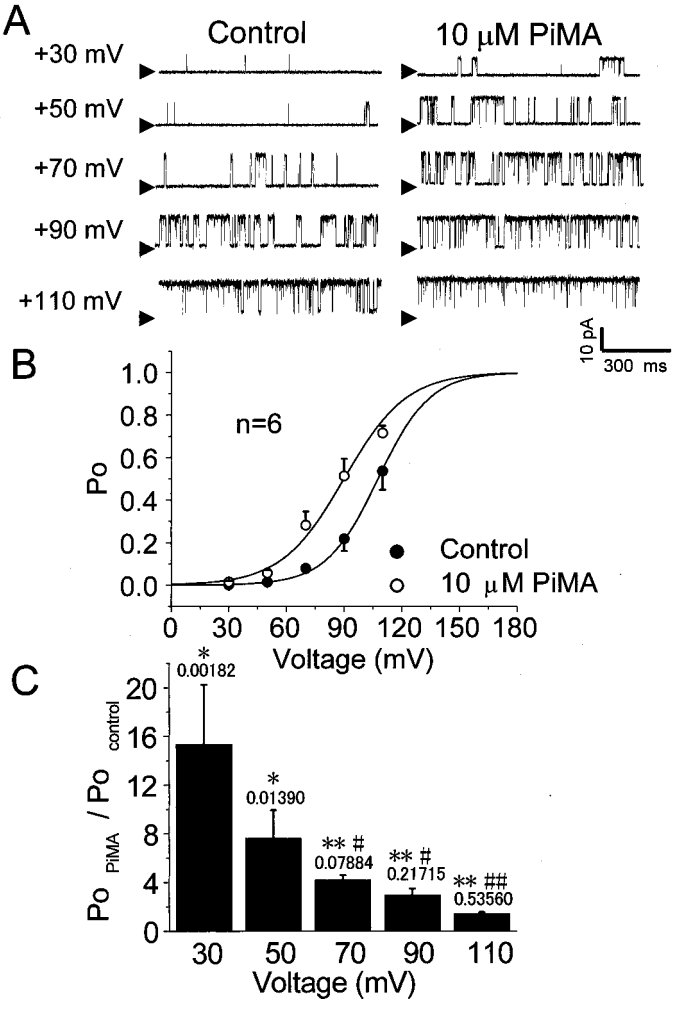


Fig. 7. Effects of PiMA on voltage dependence of BKα. A, single-channel currents of BKα were recorded in inside-out patch configuration at pCa 7.0 in symmetrical 140 mM K^+ conditions. Recordings were obtained at several test potentials in the range, +30 and +110 mV, in the absence and presence of 10 μM PiMA. B, the relationships between P_o and test potentials were obtained in the absence (●) and presence of 10 μM PiMA (○). Number of examples was six for each. The data were fitted using the Boltzmann relationship. The fitted lines are based on the following values of $V_{1/2}$ and slope factor; 106.0 and 13.3 mV, in the absence and 85.6 and 11.8 mV in the presence of 10 μM PiMA, respectively. C, the relative increase in P_o after application of 10 μM PiMA was revaluated from the data shown in B. The relative P_o in the presence of 10 μM PiMA ($P_{o\ PiMA}$) versus in its absence ($P_{o\ control}$) was illustrated against the test potentials. The numbers on the columns are the mean P_o in the absence of PiMA. *, $p < 0.05$; **, $p < 0.01$ versus unity. #, $p < 0.05$; ##, $p < 0.01$ versus $P_{o\ PiMA}/P_{o\ control}$ at +30 mV.

+ 40 mV was compared with that on BK$\alpha\beta$1 at +20 mV (Fig. 9B). The application of PiMA increased P_o from 0.00396 ± 0.00079 to 0.03297 ± 0.00629 ($n = 8$, $p < 0.01$) in BKα and from 0.00359 ± 0.00076 to 0.03821 ± 0.01389 in BK$\alpha\beta$1 ($n = 7$, $p < 0.05$). The ratio of P_o in the presence and absence of PiMA was 10.50 ± 2.07 in BKα and near that in BK$\alpha\beta$1 (9.68 ± 3.01, $p > 0.05$), suggesting that coexpression of β1 does not significantly affect the enhancement of BKα by PiMA.

Selectivity of PiMA on BK Channel versus Small- and Intermediate-Conductance Ca^{2+}-Activated K^+ Channels. To examine whether the opening action of PiMA is selective to BK channels over other Ca^{2+} activated K^+ channels, effects of 10 μM PiMA on membrane potential in HEK293 cells expressing rat small (rSK2) or human intermediate conductance Ca^{2+}-activated K^+ channels (hSK4; hIK) (Vergara et al., 1998; Kaczorowski and Garcia, 1999). It has been reported that rSK2 and hIK are similarly activated by chlorzoxazone and related compound such as 1-ethyl-2-benzimidazolinon (1-EBIO) but that they are blocked selectively by apamin and clotrimazole, respectively (Jensen et al., 1998; Cao et al., 2001). The functional expression of rat SK2 or SK4 in each cell examined was first verified as fluorescence decrease by 15 to 30% in response to the addition of 100 μM chlorzoxazone or 30 μM 1-EBIO (data not shown). After washout of chlorzoxazone or 1-EBIO, application of 10

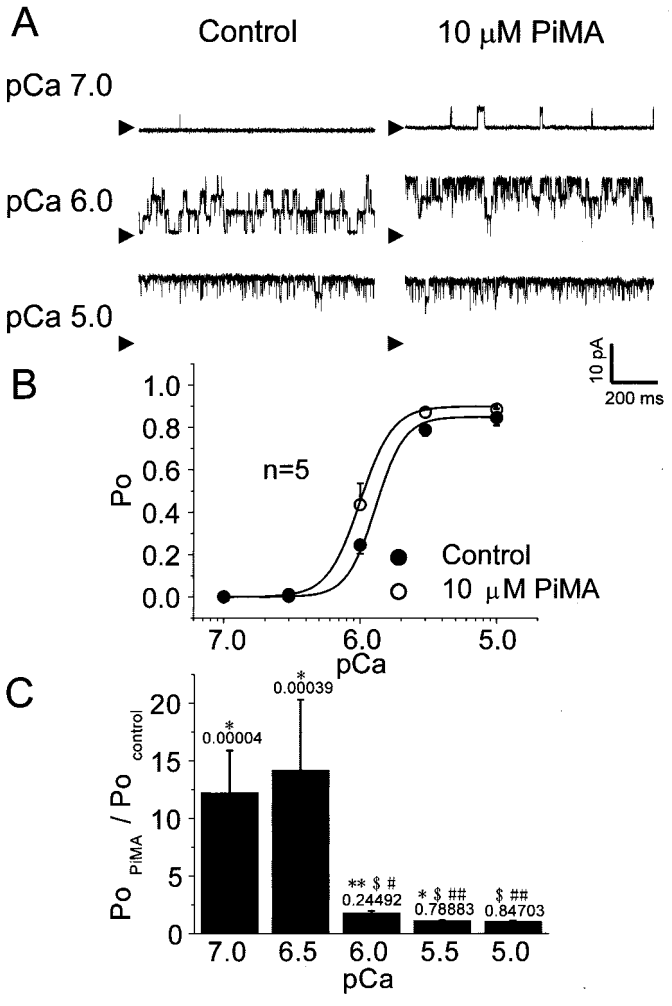


Fig. 8. Effects of PiMA on Ca^{2+} dependence of BKα. A, single-channel currents of BKα were recorded in inside-out patch configuration at +40 mV in asymmetrical K^+ conditions (5.9 and 140 mM K^+). Recordings were obtained at pCa 7.0, 6.0, and 5.0 in the absence and presence of 10 μM PiMA. B, the relationships between P_o and pCa in the bathing solutions were obtained in the absence (●) and presence of 10 μM PiMA (○). Number of examples was five for each. The data were fitted with the Hill equation. The fitted lines correspond to the following values of K_d, Hill coefficient, and the constant; 5.88, 4.15, and 0.153 in the absence and 5.98, 3.65, and 0.102 in the presence of 10 μM PiMA, respectively. C, the relative increase in P_o by application of 10 μM PiMA was revaluated from the data shown in B. The P_o in the presence of 10 μM PiMA ($P_{o\ PiMA}$) versus in its absence was illustrated against pCa. The numbers on the columns are the mean P_o in the absence of PiMA. *, $p < 0.05$; **, $p < 0.01$ versus unity. $, $p < 0.05$ versus $P_{o\ PiMA}/P_{o\ control}$ at pCa 7.0. #, $p < 0.05$; ##, $p < 0.01$ versus $P_{o\ PiMA}/P_{o\ control}$ at pCa 6.5.

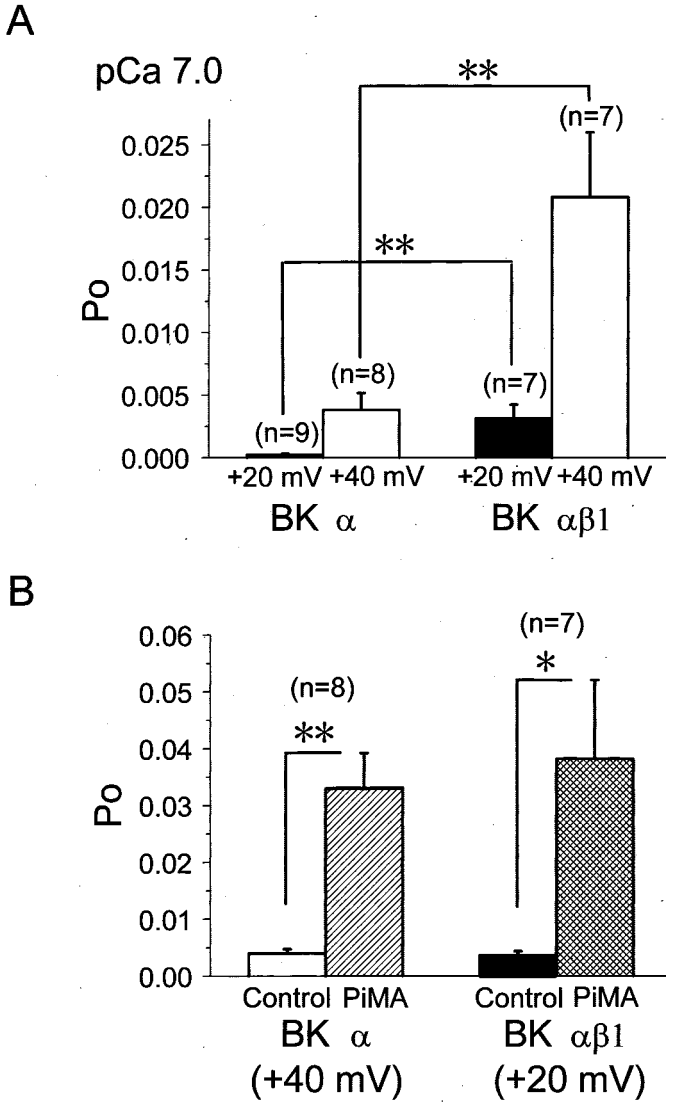


Fig. 9. Comparison of effects of PiMA on single-channel currents caused by BKα or BK$\alpha\beta$1. A, P_o was obtained from inside-out patches recording from HEKBKα and HEKBK$\alpha\beta$1 under following conditions: pCa 7.0, the holding potential of +20 or +40 mV and symmetrical 140 mM K^+ solutions. The P_o at +20 and +40 mV in BK$\alpha\beta$1 were significantly higher than that in BKα (**, $p < 0.01$). Number of examples was six for each. B, the effect of 10 μM PiMA on P_o of BK$\alpha\beta$1 was compared with that of BKα. The P_o of BKα and BK$\alpha\beta$1 in the absence of PiMA were 0.00346 ± 0.00079 at +20 mV and 0.00382 ± 0.00104 at +40 mV, respectively ($p > 0.05$). The P_o were increased to 0.03297 ± 0.00629 and 0.03061 ± 0.01237 in BKβ and BK$\alpha\beta$1 by application of 10 μM PiMA, respectively. Number of examples was six for each. *, $p < 0.05$; **, $P < 0.01$ versus the corresponding control.

μM PiMA did not change significantly the fluorescence intensity in these SK2- or SK4-expressing cells ($p > 0.05$; n = 15–20 for each). The functional expression of SK2 or SK4 verified by fluorescence decrease (> 15%) in response to SK/IK openers was observed in approximately 70 and 30% of cells, respectively. In some experiments, cells were challenged first by 10 μM PiMA and then by 100 μM chlorzoxazone or 30 μM 1-EBIO. Application of 10 μM PiMA did not change the fluorescence intensity by more than 5% in any cells examined, regardless of SK2 or SK4 expression (data not shown). The addition of chlorzoxazone or 1-EBIO in the presence or absence of PiMA did induce the decrease in fluorescence intensity by 15 to 30% in cells, which presumably expressed SK2 or SK4. The decreased fluorescence was recovered completely or even converted to the increase by further addition of 100 nM apamin or 1 μM clotrimazole, respectively (10–15 cells, for each of SK2 and SK4). The fluorescence intensity of $DiBAC_4(3)$ in HEKBKα and HEKBK$\alpha\beta$1 was not significantly affected by 100 μM 1-EBIO (n = 6–7 for each).

A Comparison of Structure of PiMA, Abietic Acid, and Maxikdiol. The most stable structures for each PiMA, abietic acid, and maxikdiol were obtained by geometry optimization based on SYBYL (Molecular Mechanics) and PM3 (Semiempirical MO Calculation) methods in the Spartan suite of programs. Comparison of structures between PiMA and maxikdiol indicates that the overall shapes of these molecules are similar each other (Fig. 10). When the tricyclic frameworks were superpositioned, the hydrophobic groups attached to C13 are located in similar spatial positions in either PiMA and maxikdiol molecules as indicated by dotted circles and a line in the side views (Fig. 10, side view A). It can be also assumed from the side views that the oxygen functionality of the carboxyl groups at C4 of PiMA and the tertiary alcohol at C5 of maxikdiol interact with the same hydrogen-bonding amino acid residue in BKα protein. Another interesting comparison can be made by assuming oxygen atom functionality of maxikdiol and PiMA with superimposed view shown in Fig. 10 (side view B). The alkenyl group of PiMA and methyl and alkenyl groups of maxikdiol are assumed to occupy the same hydrophobic pocket of the channel receptor. The structure of PiMA was also compared with that of abietic acid. Although the shapes of these molecules are similar (i.e., the carboxylic acid and ring structures are well superposed), the top and side views indicate the apparent difference in the direction and extension of the hydrophobic moieties at C13 of these two compounds. In particular, the alkenyl group of PiMA and the methyl groups of abietic acid are critical. Although the exact binding domain of these compounds to BKα protein is not known at present, we note that the hydrogen-bonding functionality around C4 and the hydrophobic region around C13 are crucial for the interaction with the channel protein α subunit.

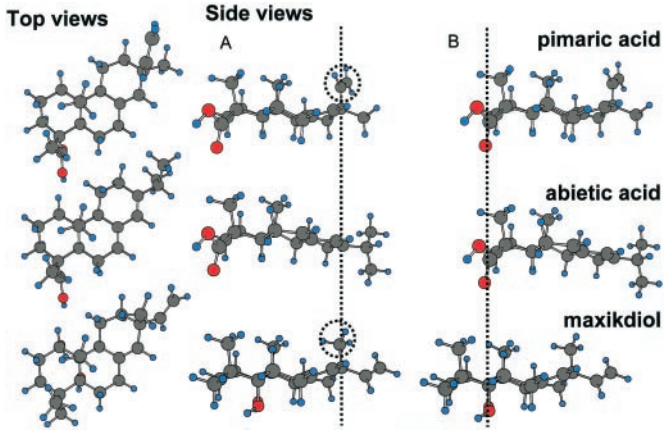


Fig. 10. Stereoscopic structures of pimaric acid, abietic acid, and maxikdiol.

Discussion

The results of this study clearly demonstrate that PiMA, a common resin acid contained in pine rosin, increases P_o of reconstituted BK channels by changing the voltage- and/or Ca^{2+} dependence of α subunit without affecting the single-channel conductance. Because PiMA and a number of other closely related compounds activated BK channels at 1 μM but isomeric abietic acid did not, new information regarding the molecular basis of the structure-function relationship is revealed.

BK channel α subunits encoded by KCNMA1 and β1 subunits encoded by KCNMB1 are the combination expressed predominantly in smooth muscles and both were cloned from smooth muscles (Garcia-Calvo et al., 1994; Knaus et al., 1994). BKα, the channel forming subunit, consists of seven transmembrane domains with a characteristic extracellular N terminus and a long intracellular C-terminal region (Meera et al., 1997). The so-called Ca^{2+} bowl in intracellular C-terminal domain (Schreiber and Salkoff, 1997) and S5 transmembrane domain (corresponding to S4 in Kv channels) are responsible for Ca^{2+}- and voltage-sensitivity of the α subunit, respectively (for review, see Vergara et al., 1998). The β1 subunit increases Ca^{2+}- and voltage-sensitivity of α subunit (McManus et al., 1995; Cox and Aldrich, 2000). Resent publications have demonstrated that tissue-dependent diversity of BK channel current characteristics (such as inactivation) is mainly due to the differential expression of β subunit subfamily. Four different subtypes have been cloned at present (Brenner et al., 2000a; Uebele et al., 2000; Xia et al., 2000). The β1 subunit is highly expressed in smooth muscle tissues but not in brain. The knockout of the gene encoding β1 subunit in mice results in the increased tone of arteries and thereby contributes to systemic hypertension (Brenner et al., 2000b). The β4 Subunit is highly expressed in brain and is responsible for the low sensitivity of brain BK channels to IbTx (Meera et al., 2000).

This diversity of BK channels, as a function of their tissue-specific distribution of β subunits, and the substantial distribution of this channel in tissues ranging from central nervous system to vascular smooth muscle offers important opportunities to develop new therapeutic agents. BK channel openers have emerged as targets for drug research and development (Starrett et al., 1996; Lawson, 2000). To date, therapeutic application for BK channel openers has focused on treatment of stroke and urinary bladder overactivity. Other aspects of hyperactivity of smooth muscles (e.g., in the setting of asthma, hypertension and gastric hypermolitily have also been targeted). Benzimidazolone derivatives such as NS-4 and NS-1619, biarylureas (NS-1608), arylpyrrole (NS-8), and indole-3-carboxylic acid esters (CGS-7181 and CGS-7184) have been described and characterized as BK

channel openers (Coghlan et al., 2001). BMS-204352 (developed from benzimidazolone) has been evaluated in clinical trials for stroke therapy, and a reduction in brain infarct size, presumably because of its neuroprotective effects, has been detected in the rat stroke model (Gribkoff et al., 2001).

In addition to these synthetic compounds, many natural products have been evaluated as BK channel openers. Terpenoids such as dehydrosoyasaponin I (McManus et al., 1993), maxikdiol (Singh et al., 1994), and L-735,334 (Lee et al., 1995) have been identified as BK channel openers by use of ^{125}I-ChTX binding assay. Despite the structural dissimilarity, these three terpenoids share biological profiles; they can displace ^{125}I-ChTX, but not fully, and activate BK channel only when applied intracellularly in electrophysiological experiments. The very low cell membrane permeability of these terpenoids limits their utility as therapeutic and pharmacological tools. Maxikdiol is a component derived from fermentation broth of an unidentified coelomycite. It activates BK channel in inside-out configuration at threshold concentration of 1 μM and has a significant effect at 3 to 10 μM (Singh et al., 1994). In this study, we chose PiMA, its isomers, and closely related compounds for the assay of BK channel opener, in part, because of their close structural similarity to maxikdiol. In contrast to maxikdiol, however, PiMA activates BK channels in HEKBK$\alpha\beta$1 when applied externally as well as when applied to 'internal phase' in inside-out patches. Its potency seems to be slightly higher than that of maxikdiol in inside-out patch recording, because significant activation was observed at 1 μM. Dehydrosoyasaponin-I (Giangiacomo et al., 1998), 17β-estradiol (Valverde et al., 1999), and tamoxifen (Dick et al., 2001) interact with β subunits of BK channels to increase the channel activity. On the other hand, NS-1619 (Ahring et al., 1997), epoxyeicosatrienoic acid (Fukao et al., 2001), and Evans blue (Yamada et al., 2001) act on α subunit. Our results clearly demonstrate that PiMA interacts with α subunit but may not with β1 subunit. We also found that the concentration-response relationship of ChTX for the inhibition of macroscopic BK$\alpha\beta$1 channel currents was not affected by the presence of 10 μM PiMA. This finding indicates that PiMA does not affect the ChTX binding to BK$\alpha\beta$1, although effects of PiMA on ^{125}I-ChTX binding were not examined in this study.

An important negative result in this study is that 10 μM abietic acid did not show BK channel opening action despite its chemical structure similar to PiMA as the isomer. The comparison of the stable conformations suggests that the major difference between PiMA and abietic acid is an extension of the hydrophobic moieties at C13 (Fig. 1). Thus, only a small difference in chemical structures at C13 markedly affects the potency. It is also of interest that sandaracopimaric acid, the diastereoisomer of PiMA with respect to C13 was equipotent as PiMA. The double bond in the substituent at C13 did not affect the potency, because dihydropimaric acid was equipotent as PiMA. Although the substitution of carbonic acid at C4 in dihydroisopimaric acid with alcohol (i.e., dihydroisopimarnol) did not significantly change the potency, the lack of this moiety, such as in sclareol or the substitution by methyl ester abolished the activity as a BK channel opener. These results indicate that appropriate carboxylic acid or alcohol functionality, conferred by a strong hydrogen bonding region at C4, is essential for potent activity of these BK channel openers. This is consistent with the slightly higher potency of PiMA than maxikdiol, which includes "OH" group at C5 instead of C4 (Singh et al., 1994).

Several compounds which activate BK channel, such as terpenoids, have been discovered using ^{125}I-ChTX binding assay (for review, see Kaczorowski and Garcia, 1999). One of the limitations of this method is that the screening paradigm includes compounds, which act only from cytoplasmic phase of the channel but may be impermeable to cell membrane. More importantly, this method may or may not detect indirect action of openers, which acts on a site in β subunit apparently distinctive from ^{125}I-ChTX binding site in α subunit. A direct measurement of BK channel activity is required for the high-throughput screening of putative BK channel openers. Application of membrane potential recording techniques using fluorescence imaging plate reader and a recombinant expression system is preferential (Gonzalez et al., 1999). Our previous study demonstrated that BK channel opening action of some agents is detected with the system (Yamada et al., 2001). In the present study, the membrane hyperpolarization induced by test compounds was again detected with the same techniques. The fact that active compounds were correctly detected demonstrates the usefulness of the assay system. The measurements, however, can include small but significant artifacts; isopimaric acid, methyl pimarate, dihydroisopimaric acid, abietic acid, and sclareol changed the fluorescence intensity even in native (untransfected) HEK cells. Slow response voltage-sensitive dyes, including $DiBAC_4(3)$, are oxonol derivatives that are lipophilic and negatively charged (Plasek and Sigler, 1996). Depolarization of cells enhances the accumulation of negatively charged dyes from extracellular solution. It has been suggested that the quantum yield of the dye fluorescence is increased markedly by its binding to unidentified cytosolic proteins (Epps et al., 1994). Artifacts may possibly be caused by changes induced by the test compounds in the binding of $DiBAC_4(3)$ to cytosolic proteins.

It is clear that PiMA is specific to BK channel over SK and IK channels as an opener and may not block them either. PiMA did not change the membrane potential in HEK293 cells expressing SK2 or SK4, which markedly hyperpolarizaied by SK/IK channel openers, chlorzoxazone, and 1-EBIO (Jensen et al., 1998; Cao et al., 2001). Genetically and even functionally in some aspects, KCNMA (BK) is closer to voltage-dependent K^+ (Kv) channels rather than KCNN (SK and IK), because of its voltage-sensitive domain (Vergara et al., 1998). Ca^{2+}-sensing mechanisms of BK channels are also different from SK/IK channel (Shieh et al., 2000). It is reasonable, therefore, that BK channel openers reported so far are selective over SK and IK channels (Kaczorowski and Garcia, 1999; Coghlan et al., 2001) and is also the case for PiMA. Effects of PiMA on cloned Kv channels and other ion channels such as voltage-dependent Ca^{2+} channels remain to be determined.

In conclusion, our results provide the first direct evidence that PiMA and related compounds abundant in pine rosin have novel BK channel opening actions. In contrast to maxikdiol, PiMA was effective from either side of cell membrane. PiMA acts on α (but not β1) subunit to increase Ca^{2+} and voltage-sensitivity of this K^+ channel complex. The fact that abietic acid, which has close structural similarity to active pimarans, does not have a channel opening action demonstrates that a very small and well-defined change of the

moiety, presumably at C13, markedly affects the potency as a BK channel opener. The present results provide important molecular information regarding the mechanisms involved in agonist induced opening of BKα. This information is being used in our program aimed for synthesis of a novel opener.

Acknowledgments

We thank Dr. Wayne Giles (University of Calgary, Calgary, Canada) for providing data acquisition and analysis programs for macroscopic current analyses and also for critical reading of this manuscript. We also thank Dr. John Dempster (University of Strathclyde, Glasgow, UK, 1987–1994) for providing data acquisition and analysis programs for single channel analyses. C-DNA of rSK2 was supplied by Dr. H. Taniguchi (Tanabe Seiyaku Co. Ltd. Toda, Saitama, Japan), to whom we are also grateful.

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Address correspondence to: Yuji Imaizumi, Ph.D., Department of Molecular and Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedori, Mizuhoku, Nagoya 467-8603, Japan. E-mail: yimaizum@phar.nagoya-cu.ac.jp