Full Paper

Effects of KRN4884, a Novel K⁺ Channel Opener, on Ionic Currents in Rabbit Femoral Arterial Myocytes

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Abstract. Effects of KRN4884 (5-amino-*N*-[2-(2-chlorophenyl)ethyl]-*N'*-cyano-3-pyridine-carboxamidine), a novel K⁺ channel opener, on ionic currents were examined in rabbit femoral arterial myocytes (RFAMs). Under whole-cell clamp conditions where cells were superfused with 5.9 mM K⁺ bathing solution, KRN4884 elicited an outward current at −30 mV. KRN4884-induced current had a reversal potential of −78 mV and was abolished by application of glibenclamide (glib). KRN4884 was approximately 43 times more potent than levcromakalim in activating an ATP-sensitive K⁺ current (I_{K-ATP}). On the other hand, KRN4884 affected neither voltage-dependent Ca²⁺ nor delayed rectifier K⁺ channel currents. In the inside-out patch clamp configuration where cells were superfused with the symmetrical 140 mM K⁺ solution, KRN4884 activated 47 pS K⁺ channels in the presence of adenosine diphosphate. Similar 47 pS K⁺ channels, which were reversibly inhibited by glib, were recorded under outside-out patch conditions. Using RT-PCR analysis, we found that inward rectifier K channel 6.1 (Kir6.1) and sulfonylurea 2B (SUR2B) transcripts were predominantly expressed in rabbit femoral artery. These results indicate that KRN4884 potently activates I_{K-ATP} in RFAMs. The KRN4884-sensitive 47 pS K⁺ channel activity underlying I_{K-ATP} is a vascular type K_{ATP} channel consisting of Kir6.1 and SUR2B and has similar characteristics to those of ATP-sensitive K⁺ channels activated by K⁺ channel openers in other types of smooth muscles.

Keywords: KRN4884, ATP-sensitive K⁺ channel, femoral artery, K⁺ channel opener

Introduction

The activation of K⁺ channels in vascular smooth muscle causes the membrane to hyperpolarize toward the equilibrium potential of K⁺, resulting in closure of voltage-dependent Ca²⁺ channels (VDCCs) and hence reducing the membrane excitability in vascular smooth muscles (1–4). A number of synthetic K⁺ channel openers (KCOs) that activate ATP-sensitive K⁺ channels (K_{ATP} channels) have therefore been developed toward a clinical use for the treatment of systemic hypertension, coronary angina pectoris and congestive heart diseases (2, 5–7). Although clinical trials with KCOs such as cromakalim, pinacidil, and diazoxide were carried out, the use of these drugs has been restricted by side-effects (e.g., headache, flushing, tachycardia, and catecholamine

release (2)). Nevertheless, KCOs can potentially serve as useful therapeutics, if KCOs facilitate the opening of K_{ATP} channels in a specific organ or tissue. Quite recently, it has been shown that in mice without Kir6.1, a molecular candidate for K_{ATP} channels in the vasculature, administration with a vasoconstrictor caused ST elevation followed by cardiac death, which is a phenotype characterized by hypercontractility of coronary arteries (8).

KRN4884, 5-amino-*N*-[2-(2-chlorophenyl)ethyl]-*N'*-cyano-3-pyridinecarboxamidine, is a novel vasodilator that has a K⁺ channel opening action (9, 10). Since KRN4884 reduces vascular tone more potently than cromakalim, it might be predicted that KRN4884 has an advantage over cromakalim for clinical uses (9, 11, 12). Although it has been suggested that KRN4884 activates I_{K-ATP} in vascular smooth muscle, there has been no direct evidence of this. On the other hand, KRN4884

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can open cardiac type K_{ATP} channels (13, 14). However, since cardiac and vascular type K_{ATP} channels consist of different molecular complexes: Kir6.2/SUR2A and Kir6.1/SUR2B, respectively, the effect of KRN4884 on vascular type K_{ATP} channels has not been determined (15–17). In the present study, effects of KRN4884 on ionic currents in RFAMs were examined and the potency with which KRN4884 activates I_{K-ATP} was compared with that of levcromakalim. The K_{ATP} channel that was sensitive to KRN4884 was also identified with the single channel recording technique. Moreover, the molecular candidate for the K_{ATP} channel in RFAMs was identified in an RT-PCR study.

Materials and Methods

Cell-dispersion

All experiments were carried out in accordance with guiding principles for the care and use of laboratory animals (the Science and International Affairs Bureau of the Japanese Ministry of Education, Culture, Sports, Science, and Technology) and also with the approval of the ethics committee in Nagoya City University. Single smooth muscle cells were enzymatically dispersed from the femoral artery of the rabbit. The procedures for cell-dispersion were similar to those previously described (7, 18, 19). Briefly, male New Zealand White rabbits, weighing 1.5–2.0 kg, were anesthetized with sodium thiopental (70 mg/kg, i.v.). The isolated femoral artery (diameter: approximately 100 μ m) was cleaned of fat and connective tissues and cut open longitudinally. Two pieces of arterial segments were incubated in Ca^{2+} , Mg^{2+} -free Krebs' solution (no EGTA) for 20 min at 37°C and then placed in the Ca^{2+} , Mg^{2+} -free Krebs' solution containing 0.4% collagenase, 0.1% papain, 0.1% dithiothreitol, and 0.1% trypsin inhibitor for 15–20 min. After the solution was replaced with Ca^{2+} , Mg^{2+} -free Krebs' solution, the segments were gently agitated with a glass pipette having a fire-polished tip.

Electrical recordings

Whole-cell or single channel current recordings were made from a single arterial myocyte or a membrane patch, respectively, using an EPC-7 (List, Darmstadt, Germany) and CEZ-2300 amplifier (Nihon Kohden, Tokyo). The resistance of microelectrodes filled with the pipette solution was approximately 2–5 M Ω for whole-cell, inside-out, and outside-out patch configurations. The series resistance was partially compensated electrically under the whole-cell clamp conditions and ranged between 4 and 10 M Ω . The cell capacitance value determined from the capacity current elicited by a square hyperpolarizing voltage step from –60 to –70 mV

was 21.5 ± 1.5 pF ($n = 15$). All experiments were carried out at $25 \pm 1^\circ$ C.

Data storage and analysis

Membrane currents and voltage signals were monitored on a storage oscilloscope (VC-6041; Hitachi, Tokyo) and stored on videotapes after being digitized by a PCM-recording system (modified to get DC signal, PCM 501ES; Sony, Tokyo). The data on the tape were replayed later and loaded into a computer through an A-D converter (DT2801A; Datatranslation, Morlborough, MA, USA). Data-acquisition and analysis for whole-cell and single channel currents were carried out using AQ/Cell-soft, developed in the laboratory of Dr. Wayne Giles, University of Calgary, and a single channel analysis program, V7.0C, developed by Dr. John Dempster, University of Strathclyde, respectively. In some experiments, ramp waveform pulses were applied as a voltage-clamp command using a multi-pulse generator (FS-1915; NF Electronics, Tokyo). All current records were filtered at 1 kHz (4-pole Bessel filter, NF Electronics).

The relative open-state probability of channels (NPo) was calculated using the following equation:

$$NPo = \frac{\sum_{i=0}^N (t_i \cdot i)}{T}$$

, where “i” is the number of channels open, t_i is the time spent with “i” channels open, N is the maximum number of open channels observed in the patch and T is the sampling time. Since we did not define the total number of channels present in each patch membrane, we assumed the maximum number of unitary current levels observed in a patch to be equal to the number of active channels in the patch. The single channel data were sampled into a computer by using V7.0C. Single channel events were detected using a half amplitude criterion, and the all point amplitude histogram was fitted with the Gaussian distribution function.

Reverse Transcription (RT)-PCR amplification

RT-PCR amplification for Kir and SUR expression was performed as described previously (20, 21). For total RNA extraction from rabbit femoral arterial myocytes, we used RNeasy mini kit (Qiagen, Inc., Hilden, Germany). Oligonucleotide sequences of primers specific for Kir6.1, Kir6.2, SUR1, and SUR2AB (SUR2X) of the rabbit (sense and antisense, 5' to 3') were GGTC GAACTACGGGCATTA and TTCGGATGGAGTTG TTCTC for Kir6.1, GACCCTCATCTTCAGCAAGC and AGGATCTCGTCTGCCAGGTA for Kir6.2, ACC ATGCCTTGCCCTTCTG and TCCAGCTGGCATG

TATAAG for SUR1, and CCGTGGTCACCATAGCTC and TCACATGTCTGCACGAAC for SUR2AB. These primers yield 302, 393, 337, 336, and 162-bp for Kir6.1, Kir6.2, SUR1, SUR2A, and SUR2B PCR products, respectively. The thermal cycler program used for PCR amplification included a 0.5-min denaturation step at 94°C, a 0.5-min annealing step at 55°C, and a 0.5-min primer extension step at 72°C for 31 cycles (GeneAmp 2400; Perkin Elmer ABI, Norwalk, CT, USA). Amplified products were separated on 1.5% agarose gels in Tris acetate/EDTA buffer, visualized with 1 μ g/ml ethidium bromide, and documented on FAS 1000 (Toyobo, Tokyo).

Solutions

Ca²⁺, Mg²⁺-free Krebs' solution for cell-dispersion had the following composition: 112 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, and 14 mM glucose. The Ca²⁺, Mg²⁺-free Krebs' solution was gassed with mixture of 95% O₂ and 5% CO₂. The external solution for whole-cell and outside-out patch recording had the following composition: 137 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 2.2 mM CaCl₂, 14 mM glucose, and 10 mM HEPES. To record inward Ba²⁺ current through VDCCs (I_{Ba}), 45 mM NaCl in the external solution was replaced with 30 mM BaCl₂. In Fig. 2, high K⁺ external solution (140 mM) was used to obtain a large I_{K-ATP}. The external solution for the inside-out patch configuration had the following composition: 2.9 mM NaCl, 140 mM KCl, 1.2 mM MgCl₂, 14 mM glucose, 10 mM HEPES, and 1 mM EGTA. The pH in each external solution was adjusted to 7.4 with 10 N NaOH. The pipette solution for whole-cell and outside-out patch recordings had the following composition: 140 mM KCl, 4 mM MgCl₂, 10 mM HEPES, 1 mM ATP-2Na, and 10 mM EGTA. When delayed rectifier K⁺ currents (I_{KdS}) were recorded, concentration of ATP in the pipette solution was increased to 5 mM to reduce I_{K-ATP}. To record I_{Ba}, 140 mM KCl was replaced with equimolar CsCl and 5 mM ATP was added to the pipette solution. Pipette solution for inside-out patch configuration had the following composition: 2.9 mM NaCl, 140 mM KCl, 1.2 mM MgCl₂, 14 mM glucose, 10 mM HEPES, and 2.2 mM CaCl₂. In some experiments, to reduce the activity of large conductance Ca²⁺-dependent K⁺ channels (BK channels) or VDCCs, 100–300 nM iberiotoxin (Fig. 4), or 0.1 mM CdCl₂ (Fig. 3), respectively, was added to the pipette solution. The pH of the pipette solutions was adjusted to 7.2 using 10 N KOH.

Drugs

The following drugs were used in this study:

KRN4884 (supplied from Kirin Brewery Corp., Tokyo), levcromakalim (made by the Kirin Brewery Corp.), glibenclamide (glib) (Sigma, St. Louis, MO, USA), tetraethylammonium (TEA) (Tokyo Kasei, Tokyo), 4-aminopyridine (4-AP) (Tokyo Kasei), adenosine diphosphate (ADP) (Oriental, Tokyo), adenosine triphosphate (ATP) (Oriental and Sigma), iberiotoxin (Peptide Inc., Osaka). To construct the relationships between the relative current density and concentration of KRN4884 and levcromakalim (Fig. 2), pooled data were fitted by the following equation:

$$\text{relative amplitude} = 1 - C/[1 + (K/[D])^n]$$

, where C is a constant, K is the apparent dissociation constant of the drug, D is the concentration of KRN4884 or levcromakalim, and n is the Hill coefficient.

Statistics

Pooled data are expressed as the mean $\pm$ S.E.M., and 'n' indicates number of cells used (in text and figures). Statistical significance between two or among multiple groups was examined using Student's *t*- or Scheffé's test, respectively. Statistical significance at *P* values of 0.05 and 0.01 is indicated in figures and text by * and **, respectively.

Results

KRN4884-induced membrane current in RFAMs

In Fig. 1, effects of KRN4884 on a holding current across membrane were examined in RFAMs. Cadmium at 0.1 mM was added to the standard external solution to block VDCCs. When a RFAM was voltage-clamped at a holding potential of -30 mV, a slowly developing outward current was elicited by application of 1 μ M KRN4884 (I_{KRN4884-out}, Fig. 1A). Addition of 10 μ M glib, an inhibitor of K_{ATP} channels, to the external solution abolished the I_{KRN4884-out}. To obtain the current-voltage (I-V) relationship for I_{KRN4884-out}, triangular ramp waveform pulses were applied before and during the activation of I_{KRN4884-out} (see the inset in Fig. 1Ba). Subtracting the current obtained at the time indicated by "1" from that by "2" (Fig. 1Ba) produced I_{KRN4884-out}, which was plotted against the ramp potentials in Fig. 1Bb. I_{KRN4884-out} reversed at -78.1 ± 2.2 mV (n = 6), suggesting that the activation of a K⁺ current was responsible for I_{KRN4884-out}. In addition, the effective inhibition of I_{KRN4884-out} by glib indicates that I_{KRN4884-out} is an I_{K-ATP}.

In Fig. 2, the potency of KRN4884 in activating I_{K-ATP} was compared with that of levcromakalim. Since the amplitude of I_{KRN4884-out} under a physiological K⁺ gradient was not large enough for a quantitative comparison, the K⁺ concentration in the external solution was

Fig. 1. KRN4884-induced outward current in RFAMs. At a holding potential of -30 mV, $1 \mu\text{M}$ KRN4884 was applied, followed by $10 \mu\text{M}$ glib. The pipette was filled with a solution that mainly contained 140 mM KCl and 1 mM ATP. **A:** a typical current trace. Before and during the activation of the outward current, ramp waveform pulses (see the inset in panel **Ba**) were applied at the time indicated by “1” and “2”. A KRN4884-sensitive current was obtained by subtracting the current recorded at “1” from that at “2” (**Ba**) and plotted against the ramp potentials (**Bb**). A value shown in **Bb** indicates the mean and S.E.M. of the reversal potential obtained from six separate cells. The bar in panel **A** indicates zero current.

Fig. 2. Activation of I_{K-ATP} by levromakalim and KRN4884 in high K^+ solution. Pipette and external bathing solution contained mainly 140 mM K^+ . Levromakalim (**Aa**) or KRN4884 (**Ab**) was cumulatively applied. The holding potential was -60 mV. **B:** Concentration-response relationships of levromakalim (open triangles) and KRN4884 (closed triangles). **Ba:** Current densities obtained by dividing the current amplitude with each cell-capacitance were plotted against drug-concentrations. **Bb:** The relative current densities were normalized to the maximum. Numbers in parentheses indicate the number of cells employed.

elevated to 140 mM. Cumulative application of levcromakalim or KRN4884 at concentration ranges of 1 – 100 or 0.01 – 10 μ M, respectively, elicited inward currents in a concentration-dependent manner (Fig. 2Aa, Ab). These inward currents were abolished by the addition of 10 μ M glib, indicating that both levcromakalim- and KRN4884-induced inward currents were I_{K-ATP} (not shown). In Fig. 2Ba, concentration-response relationships of levcromakalim and KRN4884 are summarized. The current density was obtained by dividing the amplitude of the inward current with that of the cell-capacitance and plotted against drug concentrations. The maximum current density induced by levcromakalim was not significantly different from that by KRN4884 (9.3 ± 1.6 pA/pF, $n = 8$ vs 9.6 ± 2.3 pA/pF, $n = 7$, $P > 0.05$). However, the apparent EC_{50} s of levcromakalim and KRN4884 in activating I_{K-ATP} were estimated to be 3 μ M and 70 nM, respectively, indicating that KRN4884 was approximately 43 times more potent than levcromakalim (Fig. 2Bb).

No effect of KRN4884 on I_{Kd} and inward Ba^{2+} current through VDCCs (I_{Ba}) in RFAMs

In Fig. 3, effects of KRN4884 on I_{Kd} s and I_{Ba} were examined in RFAMs. In the presence of 0.1 mM Cd^{2+} , a voltage step from a holding potential of -60 to 0 mV for 300 ms elicited I_{Kd} s as shown in the inset in Fig. 3Aa. The treatment with 1 μ M KRN4884 had little effect

on I_{Kd} s, whereas application of 5 mM TEA and 4-AP markedly inhibited the I_{Kd} s. Moreover, the I-V relationship of I_{Kd} s in the presence of 1 μ M KRN4884 (open circles, Fig. 3Aa) was identical to that in its absence (closed circles). Figure 3Ab demonstrates the time course of the amplitude of I_{Ba} in the absence and presence of 1 μ M KRN4884. To measure the amplitude of I_{Ca} quantitatively, Ba^{2+} (30 mM) was used as the charge carrier. I_{Ba} elicited upon depolarization to $+10$ mV was not affected by 1 μ M KRN4884 ("1" vs "2" in the inset). Thereafter, application of 0.1 mM Cd^{2+} abolished the I_{Ba} ("3"). Figure 3B summarized the results that application of KRN4884 affected neither I_{Kd} nor I_{Ba} in RFAMs ($P > 0.05$, vs control, respectively).

Single channel current activated by KRN4884 in RFAMs

The single channel current activated by KRN4884 is shown in Fig. 4. After the inside-out patch clamp configuration was established under symmetrical high K^+ conditions (140/140 mM K^+), the channel activity was not observed when 1 mM ADP was added to the external solution ("a"). However, the simultaneous presence of KRN4884 and ADP induced a single channel current whose unitary amplitude was approximately 2 pA at -40 mV ("b" in Fig. 4). In the lower panel in Fig. 4, the channel activity was expressed as NPo against time. When both 1 μ M KRN4884 and 1 mM ADP were present, the channel activity was

Fig. 3. No effect of KRN4884 on I_{Kd} and I_{Ba} . Aa: Cells were depolarized on various potentials from -40 to $+40$ mV for 300 ms. The amplitudes of peak I_{Kd} in the absence (open circles) and presence (closed circles) of 1 μ M KRN4884 and the simultaneous presence of 5 mM TEA and 4-AP (closed triangles) were plotted against test potentials. Number of cells used was five. Ab: To record I_{Ba} through VDCCs, cells were superfused with external solution containing 30 mM Ba^{2+} . The pipette was filled with Cs^+ -rich solution to block K^+ currents. The time course of the amplitude of peak Cd^{2+} -sensitive I_{Ba} is shown. The Cd^{2+} -sensitive I_{Ba} was obtained by the difference of the current before and during application of 0.1 mM Cd^{2+} . Current traces (1–3) shown in the inset were obtained at the corresponding time indicated by the numbers in panel b. The bar in each current trace shows zero current. B: Summary of negative effects of KRN4884 on I_{Kd} and I_{Ba} .

Fig. 4. KRN4884-sensitive 47 pS K^+ channel current in RFAMs. After the establishment of the inside-out patch clamp configuration, 1 mM ADP and thereafter, 1 μ M KRN4884 was applied to the membrane-patches voltage-clamped at -40 mV. The lower panel indicates the time course of the channel activity expressed as NPo, which was calculated by the equation shown in “Materials and Methods”. The upper panel shows the single channel current traces recorded at the corresponding time indicated by “a” and “b” in the lower panel.

markedly potentiated (“b” in Fig. 4, 0.0001 ± 0.0001 ($n = 4$) vs 0.16 ± 0.08 ($n = 4$)). The recordings shown in Fig. 4 (upper panel) were obtained at the time shown correspondingly by “a” and “b” in the lower panel. The channel conductance obtained under these experimental conditions was calculated to be 46.5 ± 2.7 pS ($n = 3$).

In Fig. 5, KRN4884-sensitive 47 pS channels were recorded in the outside-out patch clamp configuration and the same experimental solutions as those in the whole-cell recording mode were used. When the outside-out patch clamp configuration was established, the channel activity was extremely low at any holding potential. Exposure to 1 μ M KRN4884, however, induced the channel activity at 0 mV as illustrated in panel A. Panel B shows a current amplitude histogram in the absence and presence of 1 μ M KRN4884. The histogram was constructed using data sampled over 30 s. When KRN4884 was present in the external solution, the channel activity whose unitary amplitude was approximately 1.1 pA was markedly potentiated. In Fig. 6, single channel current data obtained under the outside-out patch clamp configuration are summarized. Figure 6A shows the I-V relationships of the KRN4884-sensitive 47 pS channel. When the pipette contained 140 mM K^+ , change of external K^+ concentration from 5.9 to 140 mM increased the conductance from 28.8 ± 0.94 ($n = 6$) to 45.6 ± 3.8 pS ($n = 5$), which was close to that obtained under the inside-out patch clamp configuration (46.5 ± 2.7 pS under superfusion with symmetrical 140 mM K^+). In Fig. 6B, the activity of 47 pS channels, which was calculated as NPo, was summarized. When 1 μ M KRN4884 was present in the external solution, the channel activity was markedly increased in comparison with that of the control (0.0127 ± 0.0068 vs 0.446 ± 0.090 , $P < 0.05$). Moreover,

5.9K/145K outside-out patch

Fig. 5. KRN4884-sensitive 47 pS K^+ channels recorded in outside-out patch clamp configuration. K^+ concentration of the pipette and external solution was 145 and 5.9 mM K^+ , respectively. A: At a holding potential of 0 mV, single channel currents were recorded for 30 s. B: A current amplitude histogram was constructed from the recording in the absence and presence of 1 μ M KRN4884.

Fig. 6. Characteristics of KRN4884-sensitive 47 pS K^+ channels. A: plot of single-channel current amplitude against test potentials. Circle and triangular symbols indicate the current amplitude obtained in outside-out patch clamp configuration under superfusion with external solution containing 5.9 and 145 mM K^+ , respectively. The two line segments show the current and voltage relationship of 47 pS K^+ channels recorded in inside-out patch clamp configuration under symmetrical K^+ conditions (140 mM K^+). Lines were drawn using the slope obtained by linear regression. B: Summary of effects of KRN4884 and glib on the activity of 47 pS K^+ channels. NPo was calculated from data obtained at a holding potential of 0 mV for 30 s. * and ** indicate the significant difference at $P < 0.05$ and 0.01, respectively. Numbers in parentheses show the number of cells employed.

Fig. 7. Kir and SUR transcripts in RFAMs. Kir6.1 and SUR2B transcripts were predominantly detected in dispersed rabbit femoral arterial myocytes using PCR amplification (31 cycles). The arrow in SUR2X and SUR1 lanes denotes the deduced position of SUR2A and SUR1 PCR products, respectively.

the addition of 10 μ M glib significantly reduced the channel activity (0.0085 ± 0.005 , $P < 0.05$). Withdrawal of glib, however, restored the channel activity completely (0.63 ± 0.25 , $P < 0.05$).

Kir and SUR transcripts in rabbit femoral arterial myocytes

In Fig. 7, we evaluated the expression of Kir6.X and SURX mRNA in an effort to find a candidate for the K_{ATP} channel activated by KRN4884. Total RNA isolated from acutely dispersed rabbit femoral arterial myocytes was subjected to RT-PCR. The transcripts of

mRNA of Kir6.1 and SUR2B were predominantly expressed in rabbit femoral myocytes, suggesting that the K_{ATP} channel in rabbit femoral artery consists of a vascular type of K_{ATP} channel, a complex of Kir6.1/SUR2B.

Discussion

The present study clearly shows that KRN4884 activates glib-sensitive K^+ channel currents 43 times more potently than levcromakalim in RFAMs. KRN4884, however, had an effect on neither I_{Ba} nor I_{KdS} . K^+ channels activated by KRN4884 are susceptible to glib and ADP, their conductance under symmetrical 140 mM K^+ conditions is 47 pS, and Kir6.1 and SUR2B were predominantly expressed in mRNA level, strongly suggesting that KRN4884 can facilitate the opening of vascular type K_{ATP} channels whose activation then induces vasodilation.

$I_{KRN4884-out}$ in RFAMs

It had been expected that KRN4884 would open K_{ATP} channels as a KCO and hence dilates vascular smooth muscles (9, 10, 13). The KRN4884-induced vasodilation was effectively inhibited by glib and more extensive when vessels were pre-contracted with hormonal agonists than when pre-contracted with high K^+ solution

(9, 10). Additionally, KRN4884 activated a glib-sensitive K^+ current in cardiac myocytes (13, 14). In the present study, under whole-cell clamp conditions where cells were superfused with the 5.9 mM K^+ bathing solution, application of KRN4884 consistently elicited an outward current that had a reversal potential of -78 mV: close to the equilibrium potential of K^+ . In addition, the outward current was abolished by an exposure to glib. On the other hand, it has been shown that BK channels are also activated by levcromakalim (22). Activation of BK channels by KRN4884 would be, however, ruled out in the present study, because the pipette solution contained 5 mM EGTA to minimize the activation of BK channels. Furthermore, preliminary experiments suggested that 1 μ M KRN4884 did not affect the activity of BK channels under the inside-out patch clamp conditions where Ca^{2+} concentration in the bathing solution was adjusted to pCa 6.0 with Ca^{2+} -EGTA buffer (0.73 ± 0.1 and 0.78 ± 0.06 ($n=4$) in the absence and presence of KRN4884, respectively). KRN4884 affected neither I_{Kd} nor I_{Ba} through VDCC. Therefore, KRN4884 selectively activates I_{K-ATP} in RFAMs and effectively sets the membrane potential negative toward equilibrium potential of K^+ .

Potent activation of I_{K-ATP} by KRN4884 explains the greater efficacy of KRN4884 to relax vascular smooth muscles than that of levcromakalim. Vasodilation by KRN4884 in porcine coronary artery and rat aorta was approximately 10 times more potent than that of levcromakalim (9, 10). Moreover, the recovery of contraction after repeated washing following inhibition by KRN4884 was significantly slower than that after inhibition by levcromakalim, suggesting that KRN4884 is a long-lasting relaxant in vascular vessels (23). In the present study, we directly demonstrated that the maximum amplitude of the current obtained in the presence of KRN4884 or levcromakalim was similar (7–9 pA/pF), whereas KRN4884 was 43 times more potent than levcromakalim in activating I_{K-ATP} , explaining the greater efficacy of KRN4884 for vasodilation. Moreover, KRN4884 at a concentration that induced a maximum activation of I_{K-ATP} , 1 μ M, it had no effect on I_{Ba} or I_{KdS} . Contraction produced by 80 mM K^+ , which is sensitive to dihydropyridine Ca^{2+} -antagonists, was consistently not affected by KRN4884 (9).

KRN4884-sensitive single channel current

It has been clearly revealed that a vascular type of K_{ATP} channels consists of Kir6.1 channels and the sulfonylurea 2B subunit (SUR2B, 15, 16). Expression of the Kir6.2/SUR2B complex reconstitutes functional K_{ATP} channels that have a unitary conductance of 33–35 pS under symmetrical high K^+ conditions (140–

145 mM), NDP-dependent activation, and no spontaneous activation in the absence of internal ATP. The characteristics of K_{ATP} channels in native vascular smooth muscles are, however, complicated. Although the activity of the K_{ATP} channels is effectively inhibited by sulfonylureas, their unitary conductances under symmetrical high K^+ conditions (140–150 mM) fall in two groups: small/medium (20–50 pS) (24–27) and large conductance (200–260 pS) (28, 29). Moreover, the modulation of the activity of K_{ATP} channels by ATP and NDPs is substantially different depending on cells studied (24, 26, 27). On the other hand, the data obtained with K_{ATP} channels reconstituted from the Kir6.1/SUR2B complex exhibit several characteristics of the vascular type of K_{ATP} channels, but the reason for the diverse characteristics of K_{ATP} channels found in native vascular myocytes is still unclear (15–17). Moreover, knock-out mice that have no Kir 6.1 lost sensitivity to KCOs, whereas mice without Kir6.2 retained this sensitivity (8, 30). In human and rat arterial smooth muscle, transcripts for Kir6.1 and SUR2B, but not Kir6.2 were found (31). Taken together, it is likely that the 47 pS channel in RFAMs is a member of the K_{ATP} channel family, which consists of the Kir6.1/SUR2B complex, and is consistent with the observation that RT-PCR analysis showed that Kir6.1 and SUR2B transcripts are predominantly expressed in RFAMs. The contribution of Kir6.2, which is slightly detected in RFAMs, to 47 pS channel is not clear. The differences between Kir6.1/SUR2B and Kir6.2/SUR2B complex responsible for K_{ATP} channels are their conductance (approximately 35 pS and approximately 80 pS, respectively) and susceptibility to ATP (activation and inhibition, respectively). The present evidence that the 47 pS channel was activated by KRN4884 even in the presence of 1 mM ATP without NDPs (Figs. 5 and 6) suggests that there is a similarity between the 47 pS channels and Kir6.1/SUR2B channels. However, the reason for the difference of the response to ADP without KCOs between 47 pS channels (inactive) and Kir6.1/SUR2B channels (active) is not clear. Since ADP has inhibitory as well as stimulatory effects on K_{ATP} channels, the inhibition possibly masks the stimulation under the present experimental conditions (15–17). Lowering ATP significantly potentiated responses to levcromakalim in human arterial smooth muscle cells, but not in HEK293 cells transfected with Kir6.1/SUR2B even though both cell types were sensitive to levcromakalim (31). Certain regulators that are lost under the reconstituted experimental system may affect characteristics of K_{ATP} channels. Alternatively, since intracellular Mg^{2+} plays an important role in the regulation of K_{ATP} channels, slightly lower concentration of free Mg^{2+} in the present

study (approximately 0.5 mM) than those in the previous studies where NDPs themselves activated K_{ATP} channels, may result in the failure of activation of K_{ATP} channels by ADP (17, 32).

In conclusion, KRN4884 effectively activates I_{K-ATP} in RFAMs, whereas KRN4884 affects neither I_{Ca} nor I_{Kd} . The potency of KRN4884 as a KCO is significantly higher (approximately 43 times) than that of levcromakalim. The KRN4884-sensitive 47 pS K^+ channels that possibly consist of Kir6.1/SUR2B complex are susceptible to glib and ADP, therefore underlying the I_{K-ATP} observed under whole-cell clamp conditions. The opening of the 47 pS K^+ channels by KRN4884 primarily contributes to the vasodilation induced by KRN4884.

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