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Molecular and pharmacological characteristics of transient voltage-dependent K^+ currents in cultured human pulmonary arterial smooth muscle cells

[1]Haruko Iida, [1]Taisuke Jo, [1]Kuniaki Iwasawa, [1]Toshihiro Morita, [2]Hisako Hikiji, [2]Tsuyoshi Takato, [1]Teruhiko Toyo-oka, [1]Ryozo Nagai & *,[1,3]Toshiaki Nakajima

[1]Department of Cardiovascular & Respiratory Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; [2]Department of Oral and Maxillofacial Surgery, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan and [3]Department of Ischemic Circulatory Physiology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

1 The A-type voltage-dependent K^+ current (I_A) has been identified in several types of smooth muscle cells including the pulmonary artery (PA), but little is known about the pharmacological and molecular characteristics of I_A in human pulmonary arterial smooth muscle cells (hPASMCs). We investigated I_A expressed in cultured PASMCs isolated from the human main pulmonary artery, using patch-clamp techniques, reverse transcriptase–polymerase chain reaction (RT–PCR), quantitative real-time RT–PCR and immunocytochemical studies.

2 With high EGTA and ATP in the pipette, the outward currents were dominated by a transient K^+ current (I_A), followed by a relatively small sustained outward current (I_K).

3 I_A was inhibited by 4-aminopyridine (4-AP) concentration-dependently, and could be separated pharmacologically into two components by tetraethylammonium (TEA) sensitivity. A component was sensitive to TEA, and the second component was insensitive to TEA.

4 I_A was inhibited by blood depressing substrate (BDS)-II, a specific blocker of $K_V3.4$ subunit, and phrixotoxin-II, a specific blocker of $K_V4.2$ and 4.3.

5 Flecainide inhibited I_A concentration-dependently, but it inhibited it preferentially in the presence of TEA (TEA-insensitive I_A).

6 Systematic screening of expression of K_V genes using RT–PCR showed the definite presence of transcripts of the I_A-encoding genes for $K_V3.4$, $K_V4.1$, $K_V4.2$ and $K_V4.3$ as well as the I_K-encoding genes for $K_V1.1$, $K_V1.5$ and $K_V2.1$. The real-time RT–PCR analysis showed that the relative abundance of the encoding genes of I_A α-subunit and K_V channel-interacting proteins (KChIPs) was $K_V4.2 > K_V3.4 > K_V4.3$ (long) $> K_V4.1$, and KChIP3 $\gg$ KChIP2, respectively.

7 The presence of $K_V3.4$, $K_V4.2$ and $K_V4.3$ proteins was also demonstrated by immunocytochemical studies, and confirmed by immunohistochemical staining using intact human PA sections.

8 These results suggest that I_A in cultured hPASMCs consists of two kinetically and pharmacologically distinct components, probably $K_V3.4$ and K_V4 channels.
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Abbreviations: 4-AP, 4-aminopyridine; BDS-II, blood depressing substance-II; CTX, charybdotoxin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DTX, dendrotoxin; hPASMCs, human pulmonary arterial smooth muscle cells; I_A, transient outward current; I_{Ca}, voltage-dependent Ca^{2+} channels; KChIP, K_V channel-interacting protein; K_V, voltage-dependent K^+ channel; PA, pulmonary artery; PASMCs, pulmonary arterial smooth muscle cells; RT–PCR, reverse transcriptase–polymerase chain reaction; TEA, tetraethylammonium

Introduction

Voltage-dependent K^+ channels (K_V) are important in the regulation of membrane potential and the maintenance of vascular tone in vascular smooth muscle cells including pulmonary arterial smooth muscle cells (PASMCs) (Nelson & Quayle, 1995; Yuan, 1995; Turner & Kozlowski, 1997; Gurney *et al*., 2003). Activation of K_V increases K^+ efflux, resulting in membrane hyperpolarization, which leads to closure of the voltage-dependent Ca^{2+} channels (I_{Ca}), reduced Ca^{2+} entry and subsequent vasodilation. Inhibition of K_V causes depolarization of the membrane to a threshold that opens I_{Ca}, increases Ca^{2+} entry, and causes vasoconstriction. Therefore, the normal function and expression of K_V are essential to maintain the vascular tone of PASMCs. Changes in K_V expression and function are linked to many patho-

*Author for correspondence at: Department of Ischemic Circulatory Physiology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; E-mail: masamasa@pb4.so-net.ne.jp

physiological conditions. Hypoxia and drugs such as fenfluramine affect the function and expression of K_V (Weir *et al*., 1996; Patel *et al*., 1997; Wang *et al*., 1998; Hulme *et al*., 1999; Perez-Garcia *et al*., 2000; Yuan, 2001; Patel & Honore, 2001; Platoshyn *et al*., 2001). In addition, dysfunction of K_V is known in the PASMCs obtained from patients with primary pulmonary hypertension (Yuan JX *et al*., 1998).

K_V currents can be divided into two types in smooth muscle cells including PASMCs: delayed rectifier K^+ current (I_K) and transient outward current (I_A). The diversity of subunits underlying K_V allows the formation of channels with different properties (Stuhmer *et al*., 1998; Coppock & Tamkun, 2001; Davies & Kozlowski, 2001). I_K shows delayed activation and slow inactivation, and is involved in modulating membrane potential and vascular tone in vessels such as the pulmonary artery (PA) (Osipenko *et al*., 1997; Turner & Kozlowski, 1997; Archer *et al*., 1998; Gurney *et al*., 2003). On the other hand, I_A shows rapid activation and inactivation upon depolarization. I_A is not ubiquitous in smooth muscles, but affects membrane excitability, which has been identified in various PASMCs including human (Okabe *et al*., 1987; Clapp & Gurney, 1991; James *et al*., 1995; Yuan, 1995; Amberg *et al*., 2002). Several K_V subunits have been cloned that can form I_A (Baldwin *et al*., 1991; Pak *et al*., 1991; Rudy *et al*., 1991; Schroter *et al*., 1991; Stuhmer *et al*., 1998) including $K_V4.1$, $K_V4.2$ and $K_V4.3$ of Shal, $K_V3.3$ and $K_V3.4$ of Shaw and $K_V1.4$ of Shaker. Additionally, K_V1 may form rapidly inactivating K^+ channels when bound to accessory β-subunits (Rettig *et al*., 1994; Heinemann *et al*., 1996). By reverse transcriptase–polymerase chain reaction (RT–PCR), Western blotting and immunohistochemistry, both gene and protein expression for various K_V α-subunits have been identified in rat PASMCs (Archer *et al*., 1998; Yuan XJ *et al*., 1998; Davies & Kozlowski, 2001; Coppock & Tamkun, 2001; Yuan, 2001). RT–PCR has detected I_A-related genes ($K_V1.4$, $K_V4.1$, $K_V4.2$ and $K_V4.3$) in freshly isolated and primary cultured rat PASMCs (Yuan XJ *et al*., 1998; Davies & Kozlowski, 2001; Platoshyn *et al*., 2001; Yuan, 2001), but the molecular and pharmacological diversity of I_A-related K_V α-subunits and K_V channel-interacting protein (KChIP) (An *et al*., 2000; Bahring *et al*., 2001), an accessory subunit of K_V4 series, has not been investigated in human PASMCs (hPASMCs).

The present study investigated the molecular and pharmacological characteristics of I_A in cultured hPASMCs, using patch-clamp techniques, RT–PCR, quantitative real-time RT–PCR and immunocytochemical studies.

Methods

Cell preparation

Cultured cells isolated from normal human main pulmonary artery (hPASMCs) were purchased from Clonetics Corporation (San Diego, U.S.A.). The cells used for this study were obtained from six donors. The cells were cultured in 78.5 cm^2 flasks, in culture medium supplemented with 5% fetal calf serum, human epidermal growth factor (0.5 $\mu g\,ml^{-1}$), insulin (5 $mg\,ml^{-1}$), human fibroblast growth factor (1 $\mu g\,ml^{-1}$), gentamicin (50 $\mu g\,ml^{-1}$) and amphotericin B (0.05 $\mu g\,ml^{-1}$) (SmGM-2 Buffer-Kit, Clonetics) in an atmosphere of 5% CO_2 and 95% air at 37°C. At confluence, cells obtained from 78.5 cm^2 flasks were passaged using 0.25–0.5% trypsin in 0.02% EDTA. The medium was replaced twice weekly. Cells just at confluence of passages 3–8 were detached from the culture flasks with 0.25–0.5% trypsin in 0.02% EDTA, and used for later experiments. The cells were identified as smooth muscle cells, by staining α-actin, but not fibroblast growth factor, by immunostaining with biotin-conjugated antibody. All experiments were performed at 35–37°C.

Solutions and agents

The composition of control extracellular Tyrode solution was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5 and HEPES–NaOH buffer 5.5 (pH 7.4). The patch pipette contained (in mM): KCl 140, EGTA 10, $MgCl_2$ 2, Na_2ATP 3, guanosine-5′-triphosphate (GTP, sodium salt, Sigma) 0.1 and HEPES–KOH buffer 5 (pH 7.2). 4-Aminopyridine (4-AP), tetraethylammonium (TEA), charybdotoxin (CTX), dendrotoxin (DTX) and clofilium were purchased from Sigma (St Louis, MO, U.S.A.). Blood depressing substrate (BDS)-II and phrixotoxin-II were purchased from Alomone Ltd (Jerusalem, Israel). Flecainide was obtained from Eisai Company (Tokyo, Japan).

Recording technique and data analysis

Membrane currents were recorded with glass pipettes under whole-cell clamp conditions (Hamill *et al*., 1981; Nakajima *et al*., 1999), using a patch-clamp amplifier (EPC-7, List Electronics, Darmstadt, Germany). The heat-polished patch electrode had a tip resistance of 3–5 MΩ. The series resistance was compensated, and the raw data were subtracted by leakage currents. All data were acquired, stored and analyzed using a Power Macintosh 7100/80 with the PULSE + PULSEFIT software (HEKA Electronic) and Igor PRO (Wave Metrics, Lake Oswego, OR, U.S.A.) as described previously (Terasawa *et al*., 2002).

The steady-state inactivation of I_A was estimated using the double-pulse protocol. Conditioning voltage pulses (500 ms duration) to various membrane potentials between −80 and +10 mV were applied from a holding potential of −80 mV. At 10 ms after the end of each conditioning pulse, a test pulse to +40 mV (400 ms duration) was applied to activate I_A. The ratio of I_A amplitude with and without conditioning pulses was plotted against each conditioning voltage. The steady-state activation curve was obtained from the conductance (G_K), determined by dividing the peak current amplitude at each membrane potential (V_m) by the driving force for K^+ ($V_m - E_K$), where E_K is the K^+-equilibrium potential. The time course of recovery from inactivation was measured by double-pulse protocols. The first (PI, 400 ms) and the second pulse (PII, 400 ms) with variable interpulse intervals were applied from −80 to +40 mV.

Data were expressed as mean ± s.e.m. Student's *t*-test was used for statistical analysis and $P<0.05$ was considered significant.

RNA extraction and RT–PCR

Total cellular RNA was extracted using Gene Elute™ Mammalian Total RNA Miniprep Kit (Sigma). For RT–PCR, cDNA (complementary DNA) was synthesized from 1 μg of total RNA with RT with random primers (Toyobo,

Osaka) (Oonuma *et al*., 2002). The reaction mixture was then subjected to PCR amplification with specific forward and reverse oligonucleotide primers for 35 cycles consisting of heat denaturation, annealing and extension. PCR products were size fractionated on 2% agarose gels, and visualized under UV light. Primers were chosen on the basis of the sequences of human K_V1.1-6, K_V2.1-2, K_V3.1-4 and K_V4.1-3 as shown in Table 1. Total RNA from the human fetal brain (Toyobo, Osaka) was used as a positive control.

Real-time quantitative RT–PCR was performed with the use of real-time Taq-Man technology and a sequence detector (ABI PRISM® 7000, Applied Biosystems, Foster City, CA, U.S.A.). Gene-specific primers and Taq-Man probes were used to analyze transcript abundance. The 18S ribosomal RNA level was analyzed as an internal control and used to normalize the values for transcript abundance of K_V α-subunit family genes and KChIPs family genes. We performed six independent experiments.

Immunocytochemistry

Immunocytochemical analysis for the presence of K_V used polyclonal antibodies against K_V3.4. (Alomone Labs, Jerusalem), K_V4.2 (N-15) and K_V4.3 (C-17) (Santa Cruz Biotechnology Inc., CA, U.S.A.). HPASMCs were cultured on Lab-Tek Chamber Slide Glass (Nalge Nunc International, Naperville), fixed with 2% paraformaldehyde for 45 min, rinsed in phosphate-buffered saline (PBS), then blocked in 0.2% Triton X-100 (Sigma), 1% H_2O_2 in PBS. The cells were rinsed in PBS, and incubated in Block Ace (Dainippon Seiyaku, Osaka, Japan) for 30 min at 37°C. The hPASMCs were then incubated with the primary antibodies overnight at 4°C. For control sections, cells were incubated with Block Ace without primary antibody. Cells were then rinsed in PBS, incubated in biotinylated anti-rabbit IgG or anti-goat IgG (Vector Lab. Inc.) for 30 min at room temperature, rinsed in PBS, incubated in VECTASTAIN ABC kit (Vector Lab. Inc., Burlingame) for 20 min at room temperature, and rinsed in PBS. 3,3′-Diaminobenzidine, tetrahydrochloride (DAB, Dojin, Kumamoto) with 0.06% H_2O_2 in PBS was used to form a colored reaction product. Cells were dehydrated, and cover slides were placed on the slides, and viewed using an Olympus BH-2 microscope (Tokyo).

Immunohistochemistry

Immunohistochemical analysis for the presence of voltage-gated K^+ channels used polyclonal antibodies against K_V3.4, K_V4.2 and K_V4.3. Paraffin-embedded human pulmonary artery sections on glass slides (Human Adult Normal Arteriae Pulmonalis Tissue Slide for main PA and Human Adult Normal Urinary & Respiratory System Multi-Tissue Slide for small PA) were purchased from Biochain Institute Inc. (Hayward, CA, U.S.A.). The glass slides were deparaffinized and rehydrated, and the following process was the same as described for immnocytochemistry.

Results

4-AP-sensitive I_A in cultured hPASMCs

Figure 1a shows typical original current traces recorded with 3 mM ATP and 10 mM EGTA. The cells were held at −70 mV,

Table 1 PCR primers used for amplification of voltage-dependent K^+ channel genes

	Size (bp)		*Sequence ('5-'3)*
Kv1.1	352	Sense	ACC GAG ATA GCT GAG CAG GA
		Antisense	CGA TCT TGC CTC CAA TTG TC
Kv1.2	538	Sense	AGA CCA CGA GTG CTG TGA GA
		Antisense	GGA ATA GGT GTG GAA GGT CA
Kv1.3	457	Sense	TTC GGT GTC CCT ACC CTG TA
		Antisense	GGA AAC ATG GGT TGC TAT GG
Kv1.4	506	Sense	GCT TCC CTC ATT GCT CTG AC
		Antisense	AAA CTT CAA CAG GGC CTC CT
Kv1.5	685	Sense	GTG TAA CGT CAA GGC CAA GAG CAA C
		Antisense	AGA CAG AGG CTT GGA GAC ACA GGA A
Kv1.6	590	Sense	CAA TGG TGG TGT GAG TCG AG
		Antisense	AAT CGT CAT CGT CAG CCT CT
Kv2.1	641	Sense	GTC TCT GGG CTT CAC TTT GC
		Antisense	TGT CTT CCA ACT GCT GAA CG
Kv2.2	245	Sense	CTG GAA GTG TGC GAC GAC TA
		Antisense	TCT CGC CTC AGT TCT TCG TT
Kv3.1	198	Sense	CTG GTC TCC ATC ACC ACC TT
		Antisense	GAA GAT GAC ACG CAT GAG GA
Kv3.2	255	Sense	GTA CCC CCA AAC ATG GTC AG
		Antisense	TTG CCC AGA CAT GTG TCA CT
Kv3.3	308	Sense	CCC AGA CAA GGT GGA GTT TC
		Antisense	CAA TGC GCT CAG CGT AGT AA
Kv3.4	346	Sense	AGA GAC AGA GCC CAT CCT GA
		Antisense	CAG GGC CAG GAA GAT GAT AA
Kv4.1	410	Sense	GGC TCT TTG TGT CAG GAA CC
		Antisense	TGC TGA TAA TGG CAG CTA CG
Kv4.2	301	Sense	GCC TTC TTC TGC TTG GAC AC
		Antisense	GCA AGA AGC CCA ATT CTG AG
Kv4.3	359	Sense	ATC TTC ACC GGG GAG TAC CT
		Antisense	GGG ATG CTT GTG AAC TTG CT

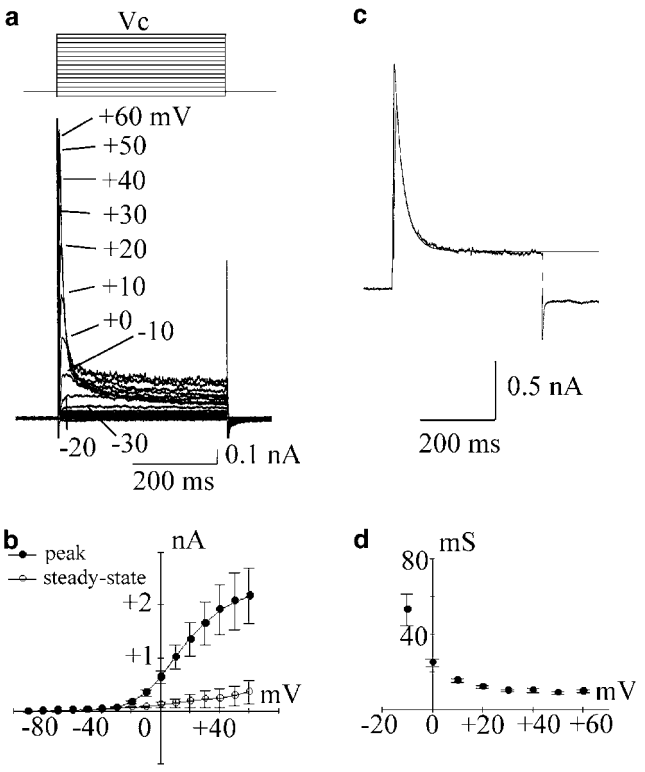

Figure 1 (a) Membrane currents measured with patch pipettes containing 3 mM ATP and 10 mM EGTA. (b) The current–voltage (*I–V*) relationships measured at the peak (close circles) and steady state (open circles). The data were obtained from six different cells. (c, d) Time courses of inactivation of I_A. The time courses of inactivation of I_A were fitted approximately by single exponential function (c). In (d), the mean ± s.e.m. values obtained from three different cells are presented.

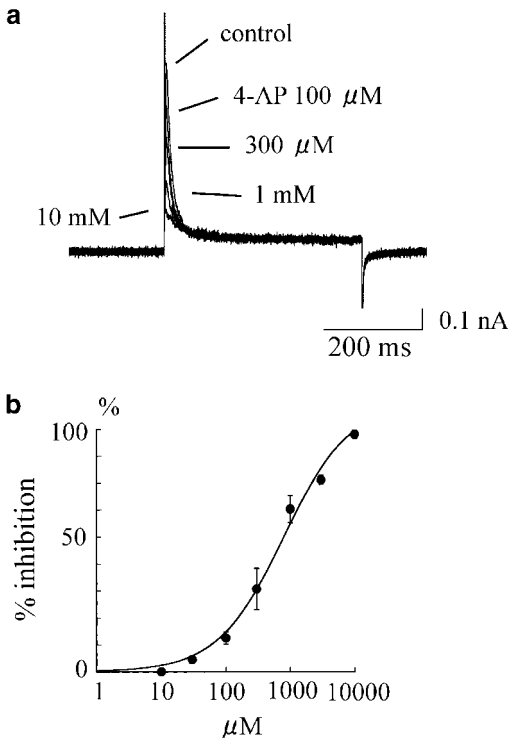

Figure 2 Effects of 4-AP on I_A. (a) Effects of various concentrations of 4-AP. The currents traces are shown in control and in the presence of various concentrations of 4-AP. The cells were held at −80 mV, and command voltage pulses to +40 mV were applied. (b) Concentration-dependent inhibitory effects of 4-AP. Data are shown as mean ± s.e.m. ($n = 3$–5), and fit by a Hill equation: % inhibition $= 100/\{1 + (IC_{50}/[4\text{-AP}])^n\}$, where n represents Hill coefficient, and IC_{50} is 50% inhibitory concentration for 4-AP. The data were best fit with an IC_{50} value of 794 μM and n of 0.8.

and the command voltage pulses to various membrane potentials were applied. During depolarizing pulses, the outward currents with a threshold potential of approximately −40 mV were activated. The currents were rapidly activated, and then rapidly declined to a relatively low steady-state level. Figure 1b shows the current–voltage (*I–V*) relationships of the outward currents measured at the peak and the steady state. The transient outward current (I_A) and the steady-state outward current (I_K) both increased with depolarization. Figure 1c and d illustrate the time courses of inactivation of I_A. We calculated τ by fitting the I_A decay with a single exponential, and typical data are shown in Figure 1c. The mean ± s.e.m. values ($n = 3$) are plotted in Figure 1d against each command potential.

Figure 2 shows the effects of 4-AP on membrane currents. 4-AP inhibited I_A concentration-dependently (Figure 2a), and the half-maximal inhibitory concentration (IC_{50}) was 794 μM (Figure 2b, $n = 3$–5).

RT–PCR and quantitative real-time RT–PCR analysis of K_V α-subunit and KChIP mRNA expression

The above results show the existence of 4-AP-sensitive I_A in cultured hPASMCs. Therefore, we investigated the systematic screening of the expression of K_V genes using RT–PCR (Figure 3). Definite expression of the transcripts of I_A α-subunit-encoding genes (K_V3.4, K_V4.1, K_V4.2 and K_V4.3) as well as I_K-encoding genes (K_V1.1, K_V1.5 and K_V2.1) was observed. However, no definite expression of K_V1.4 and K_V3.3 mRNA was observed. The identity of all K_V homologs seen by RT–PCR was performed by sequencing the relevant band excised from gel to confirm the identity of the product obtained. The quantitative expression of I_A α-subunit-encoding genes (K_V1.4, K_V3.3, K_V3.4, K_V4.1, K_V4.2 and K_V4.3) was investigated by real-time RT–PCR. Transcript levels were normalized to 18S ribosomal housekeeping gene. K_V4.3 appears to be alternatively spliced (Ohya *et al*., 1997), and the expression of K_V4.3 (long) and K_V4.3 (short) was investigated. As shown in Figure 4a, the relative abundance of the encoding genes of I_A α-subunit was $K_V4.2 > K_V3.4 > K_V4.3$ (long) $> K_V4.1$ with a ratio of 1.00 : 0.55 : 0.20 : 0.09. However, no definite expression of K_V3.3 and K_V4.3 (short) was detected. Thus, it is likely that I_A consists of K_V3.4 and K_V4 currents.

The expression of KChIP, an accessory subunit of K_V4 series, was also investigated by real-time RT–PCR analysis (Figure 4b). KChIP was mainly composed of KChIP3. Neither KChIP1 nor KChIP4 was detected significantly.

Immunocytochemical and immunohistochemical detection of K_V3.4. and K_V4 proteins

Figure 5 shows typical immunocytochemical images obtained from cultured hPASMCs containing I_A in electro-

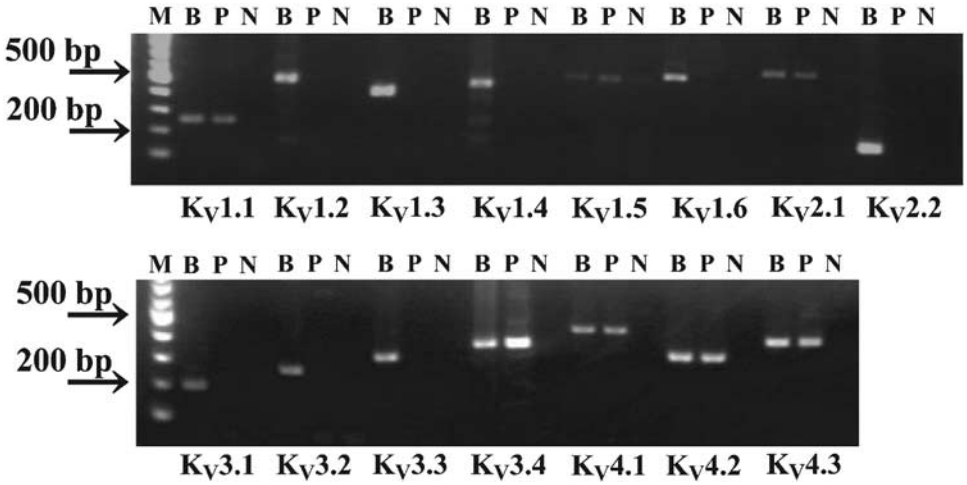

Figure 3 Analysis of K_V mRNA by RT–PCR. Ethidiumbromide-stained gel of RT–PCR products for K_V1.1-6, K_V2.1-2, K_V3.1-4 and K_V4.1-3 mRNA. M, marker; N, negative control; B, human brain; P, cultured hPASMCs.

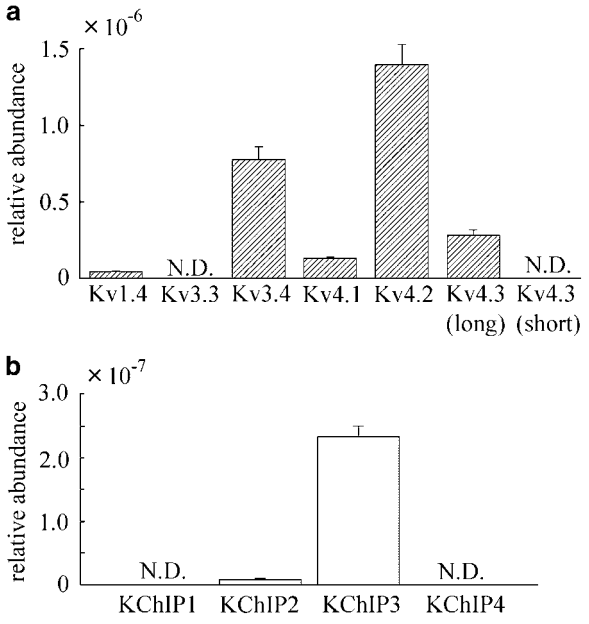

Figure 4 Expression of I_A α-subunit-encoding mRNA (a) and KChIPs mRNA (b) in cultured hPASMCs. The expression levels of I_A α-subunit-encoding genes (K_V1.4, K_V3.3, K_V3.4, K_V4.1, K_V4.2 and K_V4.3 (long and short)) and KChIPs (KChIP1, KChIP2, KChIP3 and KChIP4) genes were normalized to those of the 18S ribosomal RNA levels. Data are means ± s.e.m. from six independent samples.

physiological studies. The immunocytochemical studies showed that the cells were immunostained positively with anti-K_V3.4 (Figure 5a). Expression of K_V4.2 and K_V4.3 (Figure 5a) protein was also detected in cultured hPASMCs. Similar results were obtained from three different experiments in each case.

Immunohistochemical studies using hPA sections revealed K_V3.4, K_V4.2 and K_V4.3-like immunoreactivity in intact hPASMCs, as shown in Figures 5b (main PA) and Figure 5c (small PA).

Effects of various K^+ channel blockers on I_A

To investigate the pharmacological characteristics of I_A, the effects of various K^+ channel blockers on I_A were examined. Figure 6A shows the effects of low concentration of TEA (1 mM), which preferentially inhibits K_V3.4 compared with K_V4 currents. The cells were held at −80 mV, and command voltage steps to +40 mV were applied. TEA (1 mM) markedly reduced the amplitude of I_A, but higher concentrations of TEA (10 mM) only caused a slight further reduction. The *I–V* relationships measured at the peak and the steady state were plotted in control ($n=6$, Figure 6B, open circle and square) and in the presence of TEA (10 mM, closed circle and square). I_A consisted of a TEA-sensitive component (71.8 ± 6.4% of control I_A in a cell), and a TEA-insensitive component (28.2 ± 6.4%, $n=12$) (Figure 7). Approximately 10% of the cells tested had only TEA-sensitive I_A.

Figures 6C, D and 7 show the effects of BDS-II, a specific blocker of K_V3.4 (Diochot *et al*., 1998), and phrixotoxin-II, a specific blocker of K_V4.2 and K_V4.3 (Chagot *et al*., 2004). BDS-II (3 μM) markedly reduced I_A by 67.0 ± 4.7% ($n=3$, Figures 6Ca and 7). It failed to inhibit I_A recorded in the presence of TEA (10 mM, $n=3$, Figure 6Cb) significantly, suggesting that BDS-II selectively inhibited TEA-sensitive I_A in cultured hPASMCs. On the other hand, phyrixotoxin-II (1 μM) reduced it by 36.5 ± 2.0% ($n=3$, Figure 6Da and 7), but it failed to inhibit I_A recorded in a cell containing only TEA-sensitive I_A ($n=3$, Figure 6Db). DTX (100 nM) and CTX (100 nM) inhibited I_A by only 3.0 ± 0.6% ($n=4$) and 1.8 ± 0.6% ($n=4$), respectively (Figure 7). Clofilium (10–50 μM, Figure 7) inhibited I_A by only 2.6 ± 1.1% at 10 μM, and 6.3 ± 1.4% at 50 μM ($n=4$).

The effects of flecainide on I_A are shown in Figure 8. The current traces are shown for the control (Figure 8a) and in the presence of flecainide (10–100 μM, Figure 8a). The *I–V* relationships (Figure 8b, $n=6$) measured at the peak and the steady state are indicated in control and in the presence of flecainide. Flecainde (100 μM) decreased the amplitude of I_A at all command voltages, and inhibited it concentration-dependently. The IC_{50} value of flecainide on control I_A was 113 μM

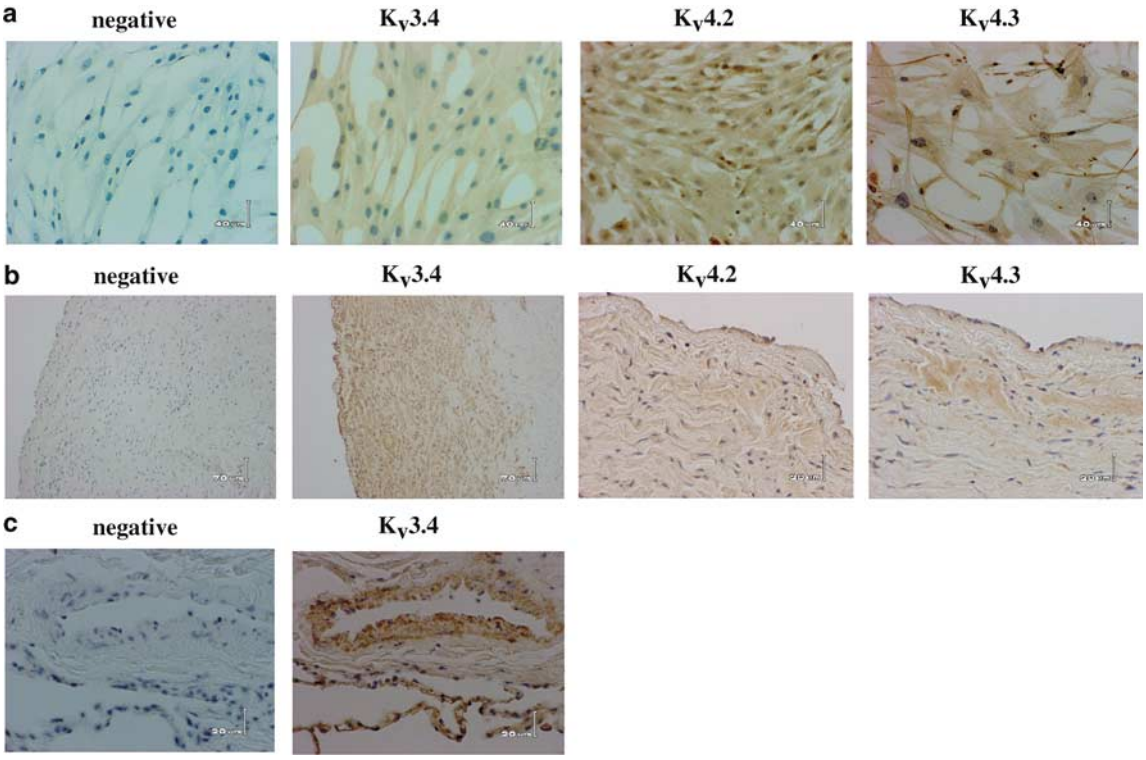

Figure 5 Immunocytochemical and immunohitochemical detection of $K_V3.4$, $K_V4.2$ and $K_V4.3$ protein in cultured hPASMCs (a) and intact human main PA (b) and small PA (c). (a) Expression of $K_V3.4$, $K_V4.2$ and $K_V4.3$ protein in cultured hPASMCs. In the negative control, cells were counterstained with hematoxylin in the absence of anti-$K_V3.4$. (b, c) Immunohistochemical detection of $K_V3.4$, $K_V4.2$ and $K_V4.3$ protein in intact human main PA (b) and small PA (c). Negative controls are shown in each case.

($n=5$). In addition, to compare the effects of flecainide on both TEA-insensitive and TEA-sensitive I_A separately, the effects of flecainide on each component of I_A were examined in a cell containing only TEA-sensitive I_A, and a cell bathed with TEA (10 mM). Flecainide inhibited it with an IC_{50} value of 30 μM in the presence of TEA (10 mM, Figure 8c, $n=4$, closed circles) and with an IC_{50} value of 160 μM in a cell containing only TEA-sensitive I_A (Figure 8c, $n=3$, closed squares).

Kinetics and voltage dependence of two different types of I_A

To clarify the characteristics of the two different types of I_A, the voltage dependence of inactivation of I_A was determined by two-step voltage pulses. Figure 9Aa and Ab show the data from a cell containing only TEA-sensitive I_A, and a cell bathed with TEA (10 mM). The peak amplitude of I_A at each test pulse was normalized to the maximal amplitude of I_A, and the normalized I_A was plotted against the conditioning voltages. The normalized values were fitted to Boltzmann equation using the least-squares methods:

$$I/I_{max} = 1/(1+\exp[(V_m - V_h)/k])$$

where I gives the current amplitude and I_{max} is its maximum, V_m is the potential of the prepulse, V_h is the half-maximal inactivation potential, and k is the slope factor. The TEA-sensitive I_A showed a mean voltage at half inactivation of -23.2 mV, and k of 6.8 ($n=6$, Figure 9Ba), whereas the TEA-insensitive I_A showed values of -54.5 mV, and 7.3 ($n=4$, Figure 9Bb), respectively. The steady-state activation curves were obtained from the conductance as described in Methods, and also fitted to Boltzmann equation (Figure 9B):

$$G_K/G_{K.Max} = 1/(1+\exp[-(V_m - V_h)/k])$$

where $G_{K.Max}$ is the maximal chord conductance, G_K is the chord conductance calculated at the membrane potential (V_m), V_h is the potential at which the conductance is one-half maximally activated and k is the slope factor. The TEA-sensitive I_A showed a mean voltage at half activation of -1.6 mV, and a slope factor of 6.9 ($n=5$, Figure 9Ba), whereas the TEA-insensitive I_A showed values of -2.4 mV and 17.8 ($n=6$, Figure 9Bb), respectively.

The time course of recovery of I_A from inactivation was investigated by the double-pulse protocol (Figure 9C). Figures 9Ca and Cb show the typical data recordings obtained from a cell containing only TEA-sensitive I_A, and a cell bathed with TEA (10 mM). The reactivation time course could be approximately fitted to a single exponential function (Figure 9D). The reactivation time constant was 1521.6 ± 101.4 ms ($n=5$) for the TEA-sensitive I_A, and 238 ± 30 ms ($n=3$) for the TEA-insensitive I_A.

Discussion

The present study showed that I_A in cultured hPASMCs includes two different types of I_A based on the pharmacological and electrophysiological characteristics. Systematic

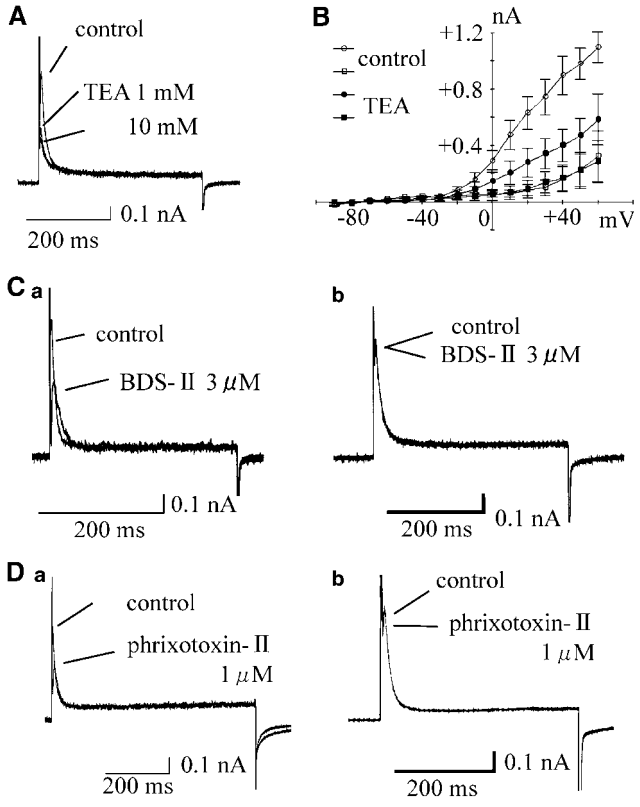

Figure 6 Effects of various K^+ channel blockers on I_A. (A) Effects of TEA (1–10 mM) on I_A. The cells were held at −80 mV, and command voltage pulses to +40 mV were applied at 0.2 Hz. (B) The *I–V* relationships measured at the peak (open and close circles), and the steady state (open and close squares) in control and in the presence of TEA (10 mM). The data are shown as mean±s.e.m. values ($n=6$). (C) Effects of BDS-II on I_A recorded in a control cell (a) and in a cell treated with TEA (10 mM, b). (D) Effects of phrixotoxin-II on I_A recorded in a control cell (a) and in a cell containing only TEA-sensitive I_A (b). Each datum is representative of three different cells.

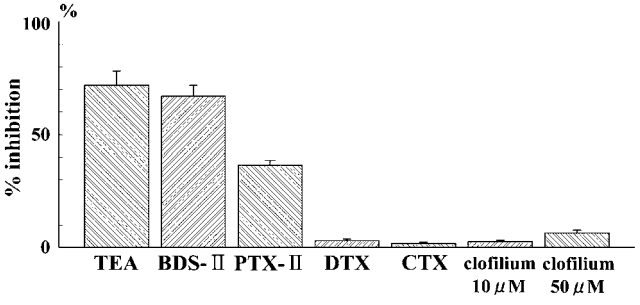

Figure 7 The percent inhibition of various K^+ channel blockers on I_A in cultured human PASMCs. TEA (10 mM, $n=12$), BDS-II (3 μM, $n=3$), phrixotoxin-II (PTX-II, 1 μM, $n=3$), dendrotoxin (DTX, 100 nM, $n=4$), charybdotoxin (CTX, 100 nM, $n=4$) and clofilium (10–50 μM, $n=4$). The cells were held at −80 mV, and command voltage pulses to +40 mV were applied at 0.2 Hz.

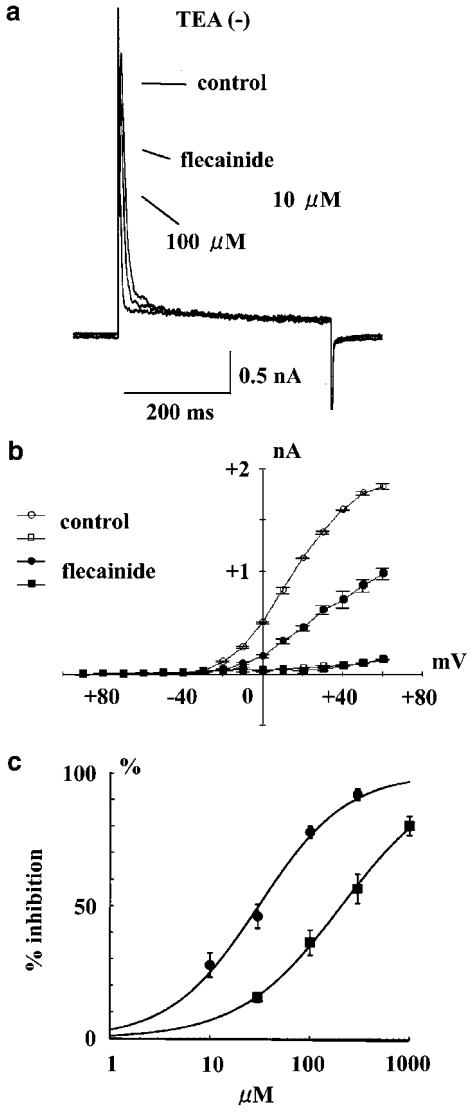

Figure 8 Effects of flecainide on I_A. (a) Effects of flecainide on I_A. The typical current traces are shown in control and after the application of flecainide (10–100 μM). The cells were held at −80 mV, and command voltage pulses to +40 mV were applied. (b) The current–voltage relationships measured at the peak and steady state in control (open circles and squares) and after the application of flecainde (100 μM, closed circles and squares). The data are shown as mean±s.e.m. values ($n=6$). (c) Concentration-dependent inhibitory effects of flecainide on I_A recorded in a cell containing only TEA-sensitive I_A (closed squares) and a cell bathed with TEA (10 mM, closed circles). Data are shown as mean±s.e.m. ($n=3$), and fitted by a Hill equation: % inhibition $= 100/\{1+(IC_{50}/[\text{flecainide}])^n\}$, where n represents Hill coefficient, and IC_{50} is 50% inhibitory concentration for flecainide. The data were best fit with an IC_{50} value of 160 μM in a cell containing only TEA-sensitive I_A, and an IC_{50} value of 30 μM in a cell bathed with TEA (10 mM).

screening of the expression of I_A-coding genes using RT–PCR detected the transcripts of the genes encoding for $K_V3.4$, $K_V4.1$, $K_V4.2$ and $K_V4.3$, but not $K_V1.4$ or $K_V3.3$. The detailed quantitative RT–PCR analysis confirmed that the relative abundance of I_A-encoding α-subunit expression was $K_V4.2 > K_V3.4 > K_V4.3$ (long), and KChIP, an accessory subunit of K_V4 series, was mainly composed of KChIP3. The electrophysiological, pharmacological and molecular analyses suggest that I_A in cultured hPASMCs consists of $K_V3.4$ and K_V4 plus KChIP3, which were also confirmed by immunocytochemical studies in cultured hPASMCs and intact PA sections.

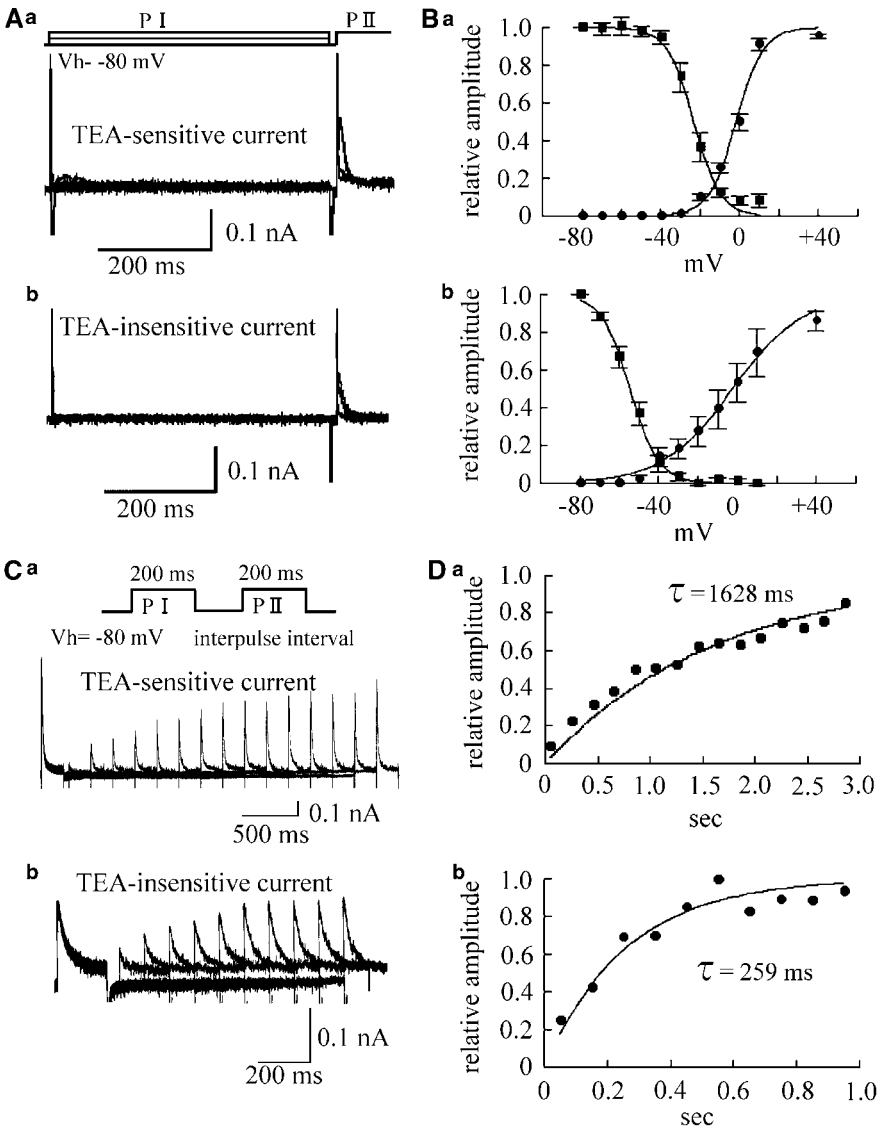

Figure 9 Two components of I_A. (A, B) Steady-state inactivation and activation curves for I_A. Using the double pulse protocol, the steady-state inactivation parameter for I_A was obtained from a cell (Aa), in which only TEA-sensitive current was present, and a cell bathed with TEA (Ab, 10 mM). The typical data were fitted by Boltzmann equation as shown in (B). The steady-state activation curves were also indicated in (B). The mean ± s.e.m. values obtained from four to six different cells. (C, D) Recovery from inactivation of I_A. The data in (C) were obtained from a cell (Ca), where only TEA-sensitive current was present and a cell (Cb) bathed with TEA (10 mM). The fitted data are shown in (D). Each datum is representative of three to five different experiments.

K_V is involved in membrane excitability, and regulates $[Ca^{2+}]_i$ and vascular tone (Nelson & Quayle, 1995). 4-AP, a K_V channel blocker, caused depolarization of the membrane and increased $[Ca^{2+}]_i$ in cultured hPASMCs (data not shown) as found in several types of PASMCs including rat (Yuan, 1995; Weir *et al*., 1996; Archer *et al*., 1998). Molecular and electrophysiological studies have shown that I_K-encoding genes such as $K_V2.1$, which are oxygen-sensitive K_V, are involved in hypoxic vasoconstriction of rat PASMCs (Osipenko *et al*., 1997; Turner & Kozlowski, 1997; Archer *et al*., 1998; Patel & Honore, 2001; Gurney *et al*., 2003). Our RT–PCR analysis also detected the I_K-encoding genes ($K_V1.5$ and $K_V2.1$) in cultured hPASMCs. However, in physiological studies, depolarizing pulses elicited I_A, followed by relatively small steady-state K^+ currents (I_K), as reported previously in cultured hPASMCs (James *et al*., 1995). Therefore, the density of I_K appears to decrease during repetitive subculture as described in rat PASMCs (Yuan *et al*., 1993). However, the small I_K facilitated the detailed discrimination and investigation of I_A in the present study.

The present study demonstrated the presence of two different types of I_A. 4-AP inhibited I_A with an IC_{50} value of 794 μM, and I_A could be divided into two different components by the sensitivity to TEA. One component was sensitive to low concentrations of TEA. These pharmacological properties were similar to the cloned $K_V3.4$ channel (Rudy *et al*., 1991; Schroter *et al*., 1991). Additionally BDS-II, a specific blocker of $K_V3.4$ (Diochot *et al*., 1998), inhibited I_A, which also supports the existence of $K_V3.4$ channel. The other component of I_A was resistant to these agents, and inhibited by phrixotoxin-II, a selective blocker of $K_V4.2$ and $K_V4.3$ (Chagot *et al*., 2004), suggesting that this component consists of K_V4

currents. In addition, flecainide inhibited I_A, but it preferentially inhibited TEA-insensitive I_A with an IC_{50} of 30 M in comparison with TEA-sensitive I_A. I_A formed by K_V4 currents has been reported to be more sensitive to inhibition by flecainide ($IC_{50} < 20\,\mu M$) (Yamashita *et al*., 1995; Yeola & Snyders, 1997), suggesting that $K_V3.4$ current is resistant to flecainide compared with K_V4 currents, and I_A in cultured hPASMCs is composed of these two different types of channels.

Two components of I_A could also be distinguished by the kinetic properties of the channels (voltage of half inactivation and time courses of recovery from inactivation). Based on the expression system of $K_V3.4$ and K_V4 ($K_V4.1$, $K_V4.2$, $K_V4.3$) (Pak *et al*., 1991; Schroter *et al*., 1991; Coetzee *et al*., 1999), $K_V3.4$ and K_V4 have the mean voltages at half inactivation of -20 to -32 mV and -50 to 69 mV, and K_V4 shows the fast recovery from inactivation compared with $K_V3.4$, which are consistent with our proposal that I_A consists of two different types of I_A, $K_V3.4$ and K_V4.

RT–PCR analysis identified the I_A-related genes (Baldwin *et al*., 1991; Pak *et al*., 1991; Rudy *et al*., 1991; Schroter *et al*., 1991) encoding for $K_V3.4$, $K_V4.1$, $K_V4.2$ and $K_V4.3$, but not $K_V1.4$ or $K_V3.3$, irrespective of passage numbers (3 and 8). The detailed quantitative real-time RT–PCR analysis provided evidence that the relative abundance of I_A-encoding α-subunit expression was $K_V4.2 > K_V3.4 > K_V4.3$ (long) $> K_V4.1$ with a ratio of 1.00 : 0.55 : 0.20 : 0.09. K_V1 coexpressed with accessory β-subunits can also form I_A (Rettig *et al*., 1994; Heinemann *et al*., 1996). However, DTX, a blocker of $K_V1.1$ and $K_V1.2$, CTX, a blocker of $K_V1.2$ and $K_V1.3$ (Grissmer *et al*., 1994) and clofilium, a blocker of $K_V1.5$ (Malayev *et al*., 1995), did not inhibit I_A. Therefore, the channel of K_V1 does not seem to be involved in forming I_A in hPASMCs. These observations suggest that I_A consists of two different types of I_A, $K_V3.4$ and K_V4. RT–PCR has shown the presence of $K_V3.4$, $K_V4.1$, $K_V4.2$ and $K_V4.3$ mRNA in rat PASMCs (Yuan XJ *et al*., 1998; Davies & Kozlowski, 2001; Platoshyn *et al*., 2001; Yuan, 2001), but the present study provided the direct evidence in cultured hPASMCs based on electrophysiological and molecular analyses. The immunocytochemical findings of $K_V3.4$ corresponded well with the results of RT–PCR. The presence of $K_V3.4$ was also confirmed by the immunohistochemical studies using intact hPA sections of the main and small PA. RT–PCR also showed the presence of $K_V4.1$, $K_V4.2$ and $K_V4.3$ mRNA, and the real-time RT–PCR analysis clearly provided the evidence showing that the relative abundance of K_V was $K_V4.2 > K_V4.3$ (long) $> K_V4.1$. In this study, we showed that transcripts encoding $K_V4.2$ and $K_V4.3$ were 11- and two-fold more abundant than $K_V4.1$ transcripts, respectively. The immunocytochemical findings also showed that $K_V4.2$ and $K_V4.3$ were expressed in cultured hPASMCs, and in intact hPA sections. We could not rule out the possible involvement of $K_V4.1$ on I_A, because anti-$K_V4.1$ antibody was not commercially available. Further studies are needed to clarify this possibility, but K_V4 is likely to be the major component of TEA-insensitive I_A in cultured hPASMCs. KChIPs, which interact with the NH_2 terminus of K_V4 proteins, enhance surface expression and modulate the kinetics of the channels (An *et al*., 2000, Bahring *et al*., 2001). It has been reported that KChIP1 is predominant in murine colonic myocytes (Amberg *et al*., 2002) and KChIP1 and KChIP3 are extensively expressed in mouse gastrointestinal myocytes (Ohya & Horowitz, 2002). The present study showed that KChIP expressed in cultured hPASMCs is mainly composed of KChIP3. From these observations, we conclude that K_V4, in association with KChIP3, is the major molecular determinant of I_A in cultured hPASMCs.

The functions of I_A in hPASMCs remain unsettled. Both components of I_A (K_V4 and $K_V3.4$ currents) were activated at potentials more positive than -40 mV, which suggests that it may not play an important role in forming membrane potential in hPASMCs. Instead, it is likely that I_A inhibits membrane excitability and prevents depolarizing stimuli such as hypoxia under the pathophysiological conditions (Osipenko *et al*., 1997; Turner & Kozlowski, 1997; Archer *et al*., 1998; Patel & Honore, 2001; Gurney *et al*., 2003). In addition, $K_V3.4$ appears to be modulated by oxidant stress (Serodio *et al*., 1994; Duprat *et al*., 1995), proposing that $K_V3.4$ may play a modulatory role in excitability of hPASMCs under the various pathophysiological conditions such as hypoxia. However, further studies are needed to clarify the physiological significance of I_A in hPASMCs.

In conclusion, I_A in cultured hPASMCs consists of two kinetically and pharmacologically distinct components, possibly $K_V3.4$ and K_V4 plus KChIP3.

Limitations of our study

The present study used cultured hPASMCs instead of freshly isolated cells because it is difficult for us to obtain human tissues. The presence of a heterogeneous population of smooth muscle cells in the PA has been reported (Smirnov *et al*., 2002), but expression of $K_V3.4$ and K_V4 was detected in cultured hPASMCs irrespective of passage number, and was confirmed by immunohistochemical analysis using PA preparations. Therefore, the findings obtained from the present study are likely to hold in native human PA, but further studies using freshly isolated hPASMCs are needed.

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