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Electrophysiologically distinct smooth muscle cell subtypes in rat conduit and resistance pulmonary arteries

Sergey V. Smirnov, Richard Beck, Paolo Tammaro, Tetsuro Ishii * and Philip I. Aaronson *

*Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY and * Centre for Cardiovascular Biology and Medicine, King's College London, New Hunt's House, London SE1 1UL, UK*

Pulmonary arteries (PAs), particularly those of the rat, demonstrate a prominent voltage-gated K^+ (Kv) current (I_{Kv}), which plays an important role in the regulation of the resting potential. No detailed characterization of electrophysiological and pharmacological properties of I_{Kv}, particularly in resistance PA myocytes (PAMs), has been performed. The aim of the present study was therefore to compare I_{Kv} in rat conduit and resistance PAMs using the standard patch clamp technique. We found that 67 % of conduit PAMs demonstrated a large, rapidly activating I_{Kv} which was potently blocked by 4-aminopyridine (4-AP; IC_{50}, 232 μM), but was almost insensitive to TEA (18 % block at 20 mM). Thirty-three percent of cells exhibited a smaller, more slowly activating I_{Kv} which was TEA sensitive (IC_{50}, 2.6 mM) but relatively insensitive to 4-AP (37 % block at 20 mM). These currents (termed I_{Kv1} and I_{Kv2}, respectively) inactivated over different ranges of potential ($V_{0.5}$ = −20.2 *vs.* −39.1 mV, respectively). All resistance PAMs demonstrated a large, rapidly activating and TEA-insensitive K^+ current resembling I_{Kv1} (termed I_{KvR}), but differing significantly from it with respect to 4-AP sensitivity (IC_{50}, 352 μM), activation rate, and inactivation potential range ($V_{0.5}$, −27.4 mV). Thus, cells from conduit PAMs fall into two populations with respect to functional I_{Kv} expression, while resistance arteries uniformly demonstrate a third type of I_{Kv}. Comparison of the properties of the native I_{Kv} with those of cloned Kv channel currents suggest that I_{Kv1} and I_{KvR} are likely to be mediated by Kv1.5-containing homo/heteromultimers, while I_{Kv2} involves a Kv2.1 α-subunit.

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Corresponding author S. V. Smirnov: Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK. Email: s.v.smirnov@bath.ac.uk

Work over the past decade has revealed that smooth muscle cells from different vascular beds vary widely in their functional expression of different types of K^+ current (Nelson & Quayle, 1995). Within this diverse spectrum, pulmonary arteries (PAs), particularly those of the rat, are distinguished by the presence of a prominent voltage-gated K^+ (Kv) current (I_{Kv}), which plays an important role in the regulation of the resting potential in this tissue (Smirnov *et al.* 1994; Yuan *et al.* 1998). It has previously been demonstrated that native I_{Kv} in PAMs is diminished by hypoxia (Post *et al.* 1992) and it was proposed that this effect may contribute to hypoxic pulmonary vasoconstriction (Weir & Archer, 1995).

In freshly isolated rat PA myocytes (PAMs) I_{Kv} appears as a rapidly activating slowly decaying delayed rectifier with no transient component (Smirnov *et al.* 1994; Smirnov & Aaronson, 1994; Patel *et al.* 1997; Turner & Kozlowski, 1997). The current is only slightly attenuated by 3–10 mM tetraethylammonium (TEA) (Smirnov *et al.* 1994; Patel *et al.* 1997), but is markedly blocked by 4-aminopyridine (4-AP) (Smirnov *et al.* 1994; Yuan, 1995; Hulme *et al.* 1999) with an apparent dissociation constant of ~200 μM (Smirnov & Aaronson, 1994).

Using RT-PCR and Western blot analysis and immunocytochemistry both gene and protein expression for various types of Kv α-subunits, including Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kv2.1, Kv9.3, Kv3.1b and Kv4.3, have previously been demonstrated in rat intact pulmonary arteries and primarily cultured PAMs (Patel *et al.* 1997; Archer *et al.* 1998; Yuan *et al.* 1998; Hulme *et al.* 1999; Osipenko *et al.* 2000; Platoshyn *et al.* 2001). Kv1.4 and Kv4.3 α-subunits, which encode rapidly inactivating A-type currents (Stühmer *et al.* 1989; McIntosh *et al.* 1997), are unlikely to be responsible for the native delayed rectifier current in freshly isolated PAMs. Recently, it has been shown that the currents through several types of cloned Kv α-subunits expressed in mammalian cell lines were reduced by acute hypoxia. Thus, Patel *et al.* (1997) showed that the properties and hypoxia sensitivity of the native current in resistance PAMs were mimicked by co-expression of cDNA for Kv2.1 and Kv9.3 (an electrically silent Kv α-subunit) in COS cells (Patel *et al.* 1997). It was subsequently found that the expression of Kv1.2, as well as the co-expression of Kv1.2 and Kv1.5 α-subunits, in mouse L cells also resulted in hypoxia-sensitive currents (Hulme *et al.* 1999). On the other hand, Osipenko *et al* (2000) reported that the expression of the Kv3.1b, but not Kv1.2, α-subunit in L929

cells resulted in the development of a hypoxia-sensitive current. These four cloned Kv channel subtypes, which encode slowly inactivating delayed rectifier currents, were therefore proposed as potential candidates for the native I_{Kv} current inhibited by hypoxia in intact PA cells (Coppock *et al.* 2001), although the exact role of Kv channels in general and each individual Kv α-subunit in particular in hypoxic pulmonary vasoconstriction remains controversial (Ward & Aaronson, 1999; Coppock *et al.* 2001).

In addition to the molecular diversity of Kv α-subunits expressed in PAMs, the distribution of K^+ channels along the pulmonary arterial tree is also not uniform. It has been shown previously that the distribution of some K^+ currents, in particular the Ca^{2+}-activated K^+ current ($I_{K,Ca}$), varies along the PA tree (Albarwani *et al.* 1995; Archer *et al.* 1996). The presence of three cell subtypes which differed in the relative proportion of $I_{K,Ca}$ *vs.* I_{Kv} components in the whole-cell current has been proposed in rat conduit PAMs (Archer *et al.* 1996). When isolated and visualized using light microscopy, these groups of cells were also found to differ with respect to their appearance. Based on this analysis the authors concluded that the relative proportion of cells containing I_{Kv} current was greater in resistance PAMs compared with conduit PAMs (Archer *et al.* 1996). No detailed electrophysiological or pharmacological characterization of the whole-cell membrane currents, in particularly I_{Kv} in resistance arteries was performed.

The multiple expression of various Kv channel subtypes in conduit and resistance PAs (Coppock *et al.* 2001) forms a potential basis for heterogeneity of the native Kv currents in various regions of the PA tree, although evidence for this has not been presented previously. The aim of the present study was therefore to compare I_{Kv} in rat conduit and resistance PAs, in order both to examine whether this current exhibits regional heterogeneity, and to determine the extent to which the characteristics of I_{Kv} correspond to those of any known Kv subtypes.

Here for the first time we present electrophysiological and pharmacological evidence which demonstrates the presence of three subpopulations of smooth muscle cells in the PA tree, each expressing a distinct I_{Kv} in a relatively homogeneous manner. The properties of these native I_{Kv} are compared with those previously described for cloned Kv channels, and the possible molecular correlates of I_{Kv} in rat PA are discussed. Part of this work has been published in abstract form (Smirnov *et al.* 2000, 2001).

METHODS

Materials

All chemicals, enzymes for cell isolation and K^+ channel inhibitors were purchased from BDH Merck (UK) or Sigma unless otherwise indicated.

Cell isolation procedure

Male Wistar rats (weight, 225–300 g) were killed by cervical dislocation in accordance with UK Home Office guidelines and the heart and lungs were removed. Conduit (main extralobar) and resistance (4th or 5th order intralobar) branches of PA were dissected, cleaned of connective tissue and cut open longitudinally. Tissues were left on ice for 30 min in normal physiological salt solution (PSS) and then transferred to nominally Ca^{2+}-free PSS containing 0.2 mM EGTA and incubated for 10 min at 37 °C. After incubation arteries were transferred into 2 ml prewarmed nominally Ca^{2+}-free PSS containing 2–3 or 1–2 mg ml^{-1} collagenase (Type XI) and 1 or 0.5 mg ml^{-1} papain for conduit and resistance PA, respectively. Dithiothreitol (DTT, 1 mM) was added to activate papain. Also, to increase the activity of collagenase, 10 μl normal PSS was added per 1 ml of the enzyme-containing solution, giving an estimated Ca^{2+} concentration of ~15 μM. Pieces of arteries were then incubated for 25 (conduit) or 15 min (resistance) at 37 °C. After incubation tissue was transferred into Ca^{2+}-free PSS containing 0.2 mM EGTA, cooled on ice for 5 min and then gently triturated in three sequential volumes (4 ml) of cooled Ca^{2+}-free EGTA-containing PSS. The last two volumes were combined and cells were spun down at 1100 *g* and then resuspended in cooled Ca^{2+}-free PSS (0.3–0.5 ml). The final volume of the cell suspension was then doubled using normal PSS. The cell suspension was stored on ice and cells were used on the same day. Single smooth muscle cells used for electrophysiological recordings had an elongated spindle shape and were able to contract reversibly upon the approach of the patch pipette filled with high K^+ solution containing ATP. For Western blot analysis conduit and resistance PAs were pooled from at least four animals to obtain a sufficient quantity of the total protein.

Electrophysiological experiments

Current recordings were made with an Axopatch 200B patch clamp amplifier and pCLAMP 8.02 software (Axon Instruments, Foster City, CA, USA) using the whole-cell configuration of the patch clamp technique. Currents were filtered at 2 kHz and sampled at 5 kHz. Experiments were performed at room temperature. Electrode resistance when filled with the pipette solution was 1–5 MΩ, which gave a mean series resistance in whole-cell mode of 11 ± 0.4 MΩ ($n = 85$) and 12 ± 1 MΩ ($n = 29$) in conduit and resistance PAMs, respectively. Since no significant difference in series resistance between the three groups of cells described in Results was found, this was not compensated. At the beginning of each experiment, the capacitative transient in response to 10 mV hyperpolarizing step depolarization (filtered at 50 kHz and sampled at 200 kHz) was recorded. The cell membrane capacitance (C_m) was then calculated from the area under the capacitative transient. Holding potential was −80 mV.

Immunoblot analysis

Cell lysates were prepared in lysis buffer containing a protease inhibitor cocktail ('Complete', Boehringer Mannheim). Tissues were homogenized using an Ultra-Turrax T25 homogenizer (Janke & Kunkel, IKA-Labortechnik, 24 000 r.p.m., 2 × 1 min, 4 °C) and then agitated slowly for 1 h at 4 °C. Debris was removed by centrifugation (2 500 *g*, 30 min, 4 °C) and supernatants were used immediately or stored at −70 °C.

Samples (20–50 μg protein) were mixed with SDS-gel loading buffer and separated using 6 or 8 % acrylamide gels. The total protein concentration was measured by the Bradford method using bovine serum albumin as a standard protein, and an equal amount of protein was loaded for each gel. Proteins were blotted

onto PVDF membranes, and then washed in phosphate buffered saline (PBS, Gibco) and PBS containing 0.05 % Tween-20 (PBST). After blocking in a 5 % (w/v) solution of dried skimmed milk in PBST for 1 h at room temperature, membranes were probed overnight (4 °C) with either Kv1.2 (1 : 1000), Kv1.5 (1 : 500), Kv2.1 (1 : 1000) or Kv3.1b (1 : 500) antibodies (Alomone Labs, Israel) in 1 % milk in PBST. Proteins were labelled with a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1 : 2000) for 1 h at room temperature. Bound antibody was detected by the ECL method (Amersham). All bands observed were due to specific binding of the corresponding anti-Kv antibody, as they were not detected when the primary antibody was pre-incubated (blocked) with the corresponding antigen (data not shown).

During preliminary work we found that the fraction of protein which is recognized by the Kv1.5 antibody was sensitive to boiling or even preheating of protein samples to 60 °C for 10 min, a standard procedure used to denature the protein before loading on gel (data not shown). This effect was also observed with Kv1.5 antibodies obtained from Upstate Biotechnology or Chemicon. Other Kv antibodies used in this study were virtually unaffected by high temperature. To minimize the possible degradation of the Kv1.5 channel protein and increase sensitivity of Kv1.5 antibodies, the samples were therefore not heated before loading.

Solutions

PSS contained (mM): 130 NaCl, 5 KCl, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 10 Hepes and 10 glucose, pH was adjusted to 7.2 with NaOH. Nominally Ca^{2+}-free solution was obtained by omitting $CaCl_2$ from PSS. The pipette solution contained (mM): 110 KCl, 10 NaCl, 5 MgATP, 10 Hepes, 10 EGTA and 0.5 $CaCl_2$ (giving an estimated free [Ca^{2+}] of 8 nM); the pH was adjusted to 7.2 with KOH. Lysis buffer had the following composition (mM): 50 Tris-Cl (pH 7.5), 250 NaCl, 5 EDTA, 5 DTT, 10 NaF and 0.1 % (v/v) Igepal.

Curve fitting and statistical analysis

Data analysis, curve fitting and presentation were performed using pCLAMP 8.02 (Axon Instruments, Foster City, CA, USA) and Origin 6.0 (Microcal Software, Northampton, MA, USA) software. The results are expressed as means ± S.E.M. Student's *t* test was used to calculate the statistical significance of the differences between two populations. Values of $P < 0.05$ were considered to indicate significance unless stated otherwise.

RESULTS

Kv channel currents in rat conduit pulmonary arteries

The voltage-gated K^+ current (I_{Kv}) was recorded in normal PSS in the presence of 1 μM paxilline and 10 μM glibenclamide in order to eliminate any residual contamination by BK_{Ca} and/or K_{ATP} channel currents, respectively. Under these conditions, it quickly became apparent that two types of I_{Kv} were present in the rat conduit PA, and that individual cells predominantly expressed either one or the other. The two types of current are illustrated in Fig. 1*A* and *B*. In the majority of cells (39/66), the current was of high amplitude (1574 ± 146 pA at +60 mV) and activated rapidly (Fig. 1*A*). In the minority of cells (27/66), the current activated more slowly, and was much smaller (308 ± 41 pA; $P < 0.0001$) (Fig. 1*B*). The difference in amplitude was not due to cell size, since the membrane capacitance was not significantly different between two groups of cells (16.5 ± 1.2 pF, $n = 39$, and 19.7 ± 1.8 pF, $n = 27$, respectively). Moreover, we found that the larger current showed very little sensitivity to TEA, with even 20 mM causing little block. On the other hand, in cells with the smaller, slower activating I_{Kv}, the current was mostly blocked by 20 mM TEA (compare Fig. 1*A* and *B*).

In Fig. 1*C*, the relationship between the percentage of block by 20 mM TEA has been plotted against current density and also $t_{½}$, the time taken for the current to attain 50 % of its maximal amplitude, in 59 cells in which all of these parameters were measured with steps to +60 mV. It is clear that the currents fall into two groups on the basis of each of these characteristics. The groups could be particularly well distinguished on the basis of their sensitivity to TEA, since currents were either > 55 % or < 35 % inhibited by 20 mM TEA; no cells were found which demonstrated an intermediate TEA block.

In the group ($n = 39$) which was more sensitive to 20 mM TEA (> 55 % block), the mean $t_{½}$ was 7.4 ± 0.4 ms, the mean current density was 17 ± 1.3 pA pF^{-1}, and the mean current amplitude was 268 ± 31 pA. In the group which was relatively insensitive to TEA (< 35 % block), the mean $t_{½}$ was 3.6 ± 0.2 ms, the mean current density was 103 ± 10 pA pF^{-1}, and the current amplitude was 1416 ± 147 pA ($n = 27$). Each of these parameters was highly significantly different in the two groups of cells ($P < 0.003$). Figure 1*D* depicts the current–voltage (*I–V*) relationship, and the effect of 20 mM TEA, in these two groups of cells. TEA blocked the current significantly in both groups ($P < 0.0001$ at +60 mV), but the extent of the block differed greatly between the groups over a wide range of potentials. Hereafter, the relatively TEA-insensitive rapidly activating currents will be referred to as I_{Kv1}, and the TEA-sensitive slowly activating currents will be termed I_{Kv2}.

Figure 2*A* and *B* illustrates the concentration dependency of the block by TEA of the current at +60 mV in a cell demonstrating I_{Kv2}. The current was approximately halved by 3 mM TEA in this cell, and recovered quickly even after the approximately 80 % block achieved at 20 mM TEA. The IC_{50} for TEA was 2.6 ± 0.2 mM in a group of 6 cells of this type.

In 26 cells, in which I_{Kv1} predominated, the current was not sensitive to a low concentration (1 mM) of TEA but was inhibited by 18 ± 2.4 % by 20 mM TEA (Fig. 2*B*, open circles, see also Fig. 1*D*). One explanation of this effect of 20 mM TEA was that some I_{Kv2} was also present in these cells and was being blocked by the drug. This possibility was supported by the fact that the mean absolute amplitude of the TEA-sensitive component was very similar, regardless of whether I_{Kv1} or I_{Kv2} predominated in a particular cell (Fig. 1*D*). Furthermore, block of I_{Kv1} by

4-AP (see below) typically revealed a small current with the pharmacological and kinetic properties of I_{Kv2} (i.e. block by TEA, inactivation over more negative potentials, a slower activation). We did not, however, attempt to characterize this current in any detail due to preliminary experiments which suggested that its properties were somewhat altered by the presence of 4-AP. Due to the presence of this current, subsequent experiments designed to characterize I_{Kv1} were carried out in the presence of 20 mM TEA to minimize a possible contamination by I_{Kv2}. It should be noted, however, that parallel experiments were also usually carried out in the absence of TEA, and always gave similar results (see below), probably because a contribution of I_{Kv2} to the net Kv current in I_{Kv1} cells was very small in comparison to that of I_{Kv1}. The converse possibility, that I_{Kv1} was significantly contaminating the current in 'I_{Kv2} cells', also seemed unlikely, as described below.

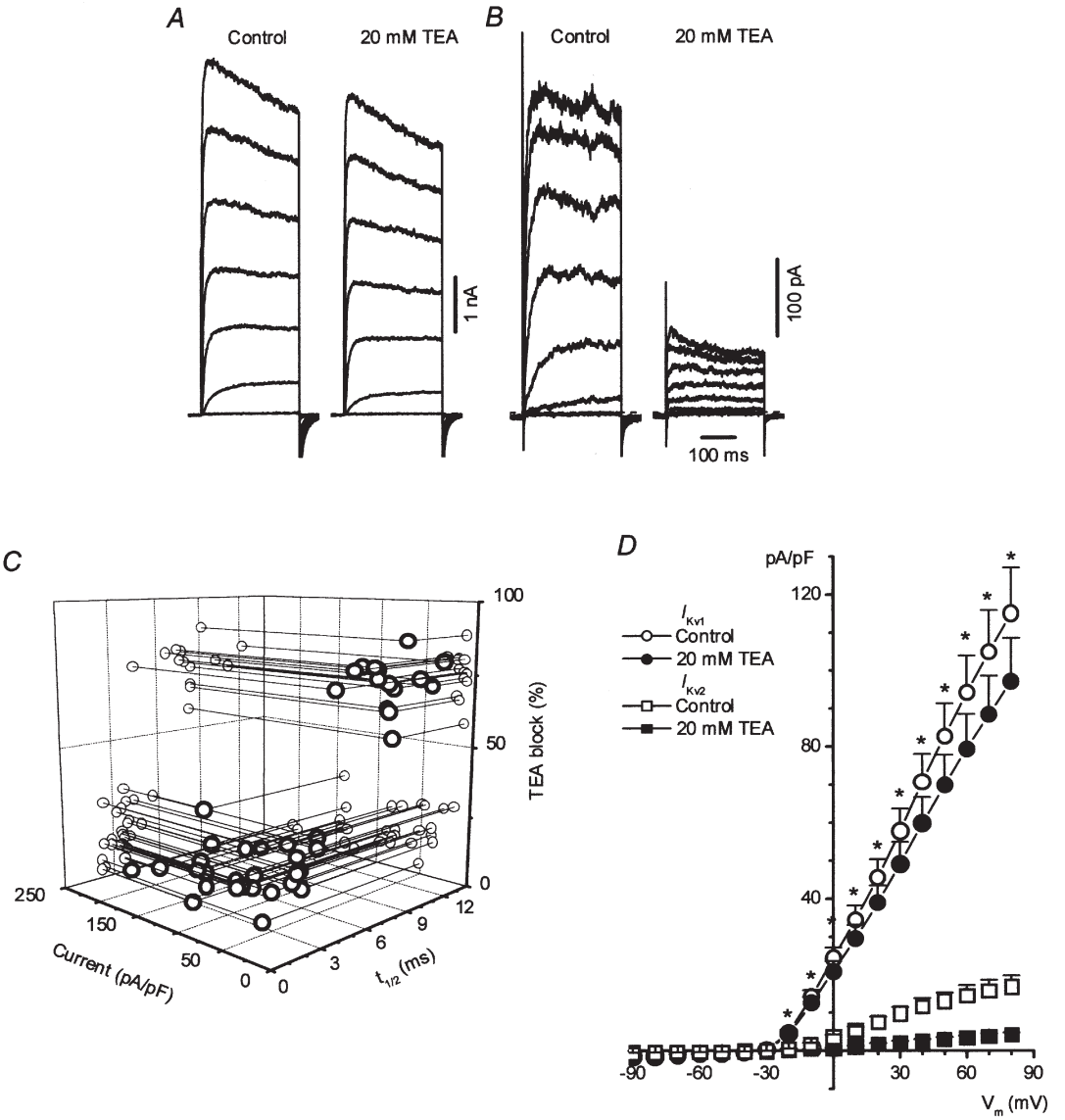

Figure 1. Two subpopulations of cells in rat conduit PA

A and *B*, families of I_{Kv} recorded from two representative cells in response to a 300 ms membrane depolarization between −40 and +80 mV in 20 mV increments and the effect of 20 mM TEA on the current. Holding potential was −80 mV. C_m = 6.3 (*A*) and 14.4 pF (*B*). Here and in subsequent figures horizontal dashed lines indicate zero current level. *C*, a relationship between the current density, percentage of TEA (20 mM) block and half-time to maximum I_{Kv} amplitude ($t_{½}$) in 59 rat conduit PAMs. All parameters were measured at the test potential of +60 mV. Lines and grey circles show the projection of individual data points on corresponding planes. *D*, *I–V* relationships for I_{Kv1} (n = 26) and I_{Kv2} (n = 7) in two groups of cells in the absence and presence of 20 mM TEA as indicated. Asterisks show significant differences between I_{Kv1} and I_{Kv2} densities under both conditions ($P < 0.003$).

Figure 3*A* shows an example of the block by 4-AP of I_{Kv2} in a cell in which this current predominated. It is apparent that I_{Kv2} was relatively insensitive to 4-AP. Figure 3*B* shows an experiment in which the effect of 4-AP on I_{Kv1} was evaluated in the presence of 20 mM TEA. 4-AP blocked I_{Kv1} with a higher efficacy and potency than it demonstrated against I_{Kv2}, as also shown for groups of cells in Fig. 3*C*. The IC_{50} for block of I_{Kv1} by 4-AP (in the presence of 20 mM TEA, open circles) was 232 ± 35 μM, while I_{Kv2} was only blocked by 37 ± 3 % ($n = 5$) at 20 mM 4-AP, the highest concentration tested (open squares). The block of I_{Kv1} by 4-AP which was observed in the absence of TEA (filled circles) was similar to that seen in its presence.

It is noteworthy that 0.1 mM 4-AP produced a substantial inhibition of I_{Kv1}, but had virtually no effect on the current in I_{Kv2} cells. This suggested that any contamination by I_{Kv1} of the current in I_{Kv2} cells was likely to be negligible.

In both I_{Kv1} and I_{Kv2} cells, the kinetics of activation of the outward current could be well fitted by a single exponential function over wide range of potentials, as shown in Fig. 4*A*. Figure 4*B* demonstrates that the resulting time constants of activation of I_{Kv2} were significantly greater than those for I_{Kv1} at all test potentials between −20 and +80 mV. Moreover, the presence of 20 mM TEA had no significant effect on the time constant for I_{Kv1} activation at any potential. In contrast to the time constant of activation, the voltage dependency of the steady-state activation of the outward current (calculated according to the Boltzmann equation described in the legend to Fig. 4) was very similar for both I_{Kv1} and I_{Kv2} cells, and was also not affected by 20 mM TEA in I_{Kv1} cells.

The voltage dependency of current availability in I_{Kv1} and I_{Kv2} cells was assessed by stepping cells to +60 mV, following 10 s steps to conditioning potentials ranging from −100 to +80 mV. Examples of typical experiments are shown in Fig. 5*A* (for I_{Kv1}) and 5*B* (for I_{Kv2}), and the mean current availabilities derived from a number of similar experiments are depicted in Fig. 5*C*. I_{Kv2} inactivated to a greater extent, and over a more negative potential range compared with I_{Kv1}. When the data were fitted with the Boltzmann function, $V_{0.5}$, the potential at which the current was half-inactivated, was −39.1 ± 2.3 mV ($n = 13$) in I_{Kv2} cells, and at the maximal extent of inactivation was 77 %. In contrast, $V_{0.5}$ was −20.2 ± 1.6 mV in I_{Kv1} ($n = 13$, $P < 0.0001$) cells, and the maximal level of inactivation was 62 % ($P < 0.0001$). Moreover, as the conditioning potential was made progressively more positive, the current in I_{Kv2} cells began to increase again. This type of U-shaped current availability profile was never observed in I_{Kv1} cells.

Kv channel currents in resistance pulmonary arterial myocytes

Myocytes isolated from resistance PAs were significantly smaller (C_m, 8.2 ± 0.5 pF, $n = 29$, $P < 0.0001$) than those from the total of 85 conduit PAMs studied with either I_{Kv1} (15.4 ± 0.9 pF, $n = 57$) or I_{Kv2} (19.9 ± 1.8 pF, $n = 28$). Figure 6*A* illustrates a typical family of voltage-gated K^+ currents in a resistance artery cell. The current (hereafter I_{KvR}) resembled that present in I_{Kv1} cells of the conduit PA

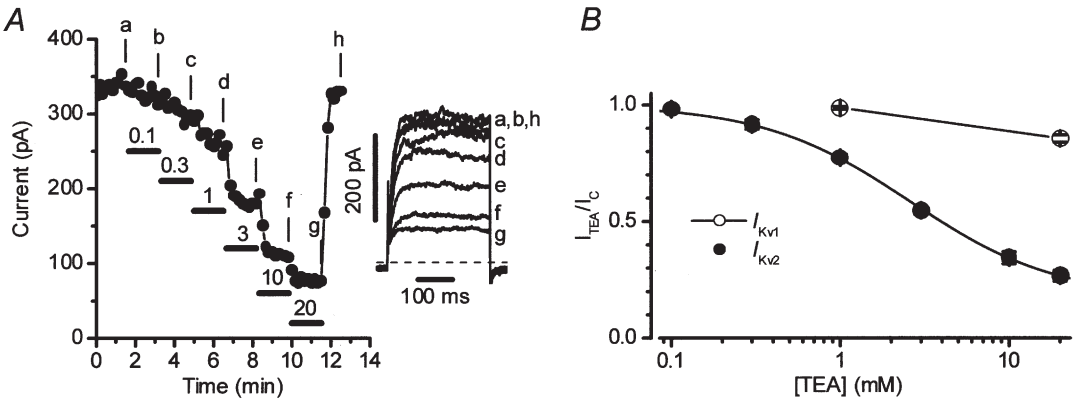

Figure 2. Effect of TEA on I_{Kv} in rat conduit PAMs

A shows the inhibition of I_{Kv2} by various external concentrations of TEA (indicated in mM above the horizontal bars). The cell was stimulated by a 300 ms voltage step to +60 mV applied at 0.1 Hz. The inset shows representative current traces at times indicated by letters in *A*. $C_m = 38.9$ pF. *B*, concentration-dependent inhibition of I_{Kv2} (filled circles, $n = 6$) and I_{Kv1} (open circle, $n = 4$ and 26 for 1 and 20 mM, respectively) by TEA. Continuous line through filled circles is drawn according to the following equation:

$$I_{TEA}/I_C = \frac{1 - A}{1 + [TEA]/IC_{50}} + A,$$

with an IC_{50} of 2.6 mM and residual component A equal to 0.17. I_{TEA}/I_C is a ratio of the current in the presence of a corresponding concentration of TEA (I_{TEA}) and control current (I_C) in the absence of the drug.

(Fig. 6*B*) both in its rapid rate of activation, and in its low sensitivity to 20 mM TEA (18 ± 2.7 %, $n = 22$ for I_{KvR} *vs.* 18 ± 2.4 %, $n = 26$, for I_{Kv1}). Similar to I_{Kv1}, I_{KvR} was not blocked by 1 mM TEA (not shown). Also, the mean I_{KvR} amplitude (0.18 ± 0.4 nA, $n = 23$) measured at +60 mV in PSS was not significantly different from that for I_{Kv1} (0.16 ± 0.15 nA, $n = 39$). The smaller cell size in the resistance arteries resulted, however, in a significant difference in the current density between I_{Kv1} and I_{KvR} both in the absence and presence of 20 mM TEA (Fig. 6*C*). No significant differences in the cell slope resistance, calculated from a linear range of *I–V* relationship between −90 and −70 mV, between I_{KvR} (11 ± 3 GΩ, $n = 23$) and I_{Kv1} (13 ± 3 GΩ, $n = 39$) or I_{Kv2} (8 ± 1 GΩ, $n = 27$) were found.

When examined more closely, in addition to its higher current density, I_{KvR} exhibited properties clearly differentiating it from I_{Kv1}. The time constant of activation of I_{KvR} was significantly larger than that of I_{Kv1} at potentials ranging from +10 to +80 mV (Fig. 6*D*), a property which was observed in both the presence and absence of 20 mM TEA. Also, as summarized in Table 1, both the activation and inactivation of I_{KvR} occurred over a potential range which was about 7 mV more negative than that found for I_{Kv1}. Finally, it also emerged that compared with I_{Kv1}, I_{KvR} was blocked by 4-AP with a significantly lower potency (IC_{50} = 352 ± 27 *vs.* 232 ± 35 μM, Table 1). In contrast to the conduit PAMs, no heterogeneity in the Kv current characteristics was found in the 29 resistance PAMs studied.

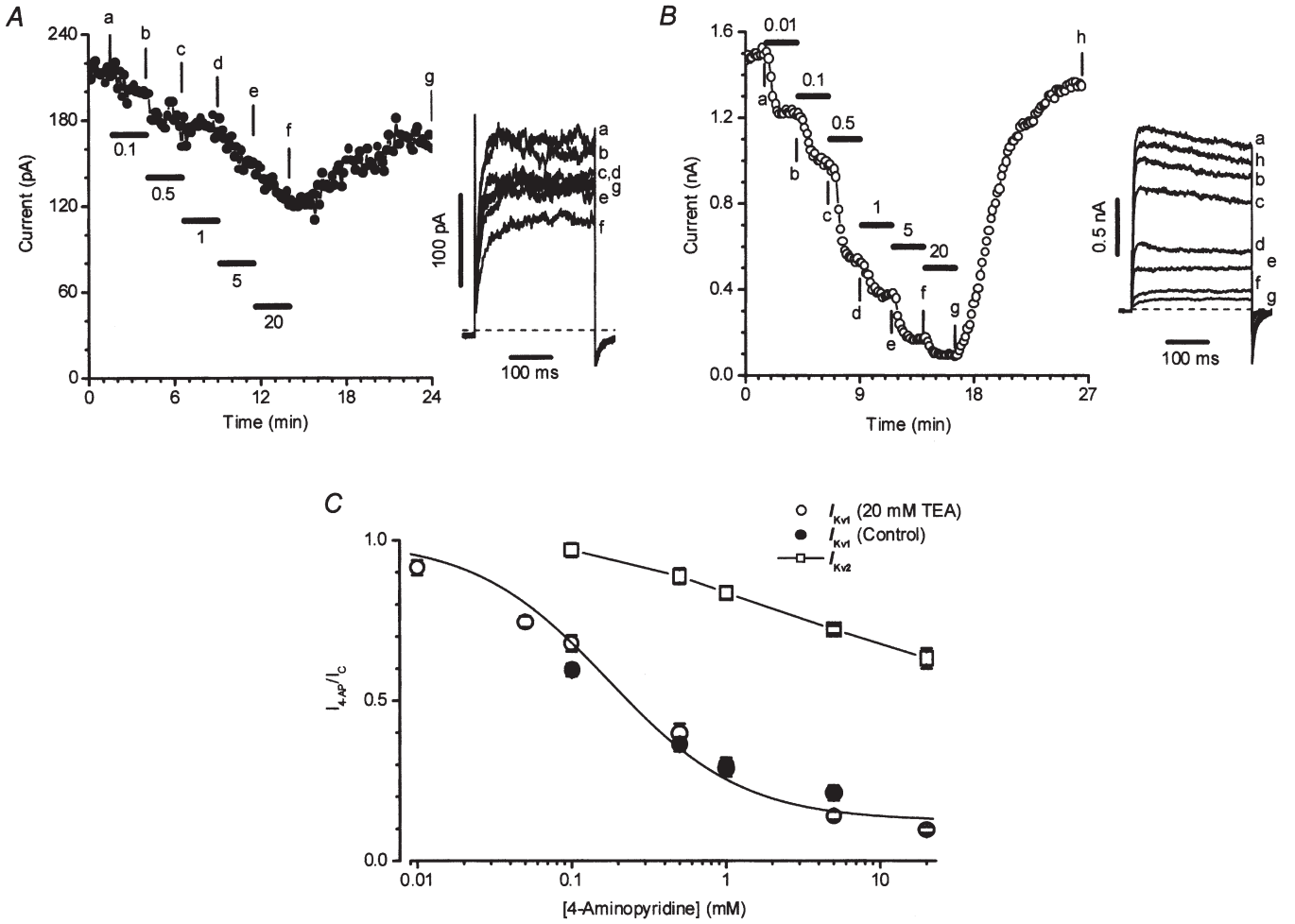

Figure 3. Inhibition of I_{Kv} by 4-aminopyridine in rat conduit PAMs

A and *B*, the effect of various concentrations of 4-AP (indicated in mM) on I_{Kv2} and I_{Kv1} in two representative cells, respectively, using the experimental protocol described in the legend to Fig. 2*A*. Insets show current traces at times indicated by the letters. C_m was 12.7 (*A*) and 12 pF (*B*). *C*, concentration dependence of the block of I_{Kv1} (circles) and I_{Kv2} (squares) by 4-AP. The continuous line through the circles is drawn according to the equation described in the legend to Fig. 2*B* with an IC_{50} of 0.17 mM and a residual component *A* equal to 0.12. Open ($n = 11$) and filled ($n = 6$) circles show normalized current in the presence and absence of 20 mM TEA, respectively. The line through the squares ($n = 6$) is drawn by eye. $I_{4\text{-}AP}/I_C$ represents a ratio of the current in the presence of the corresponding concentration of 4-AP ($I_{4\text{-}AP}$) and control current (I_C) in the absence of the inhibitor.

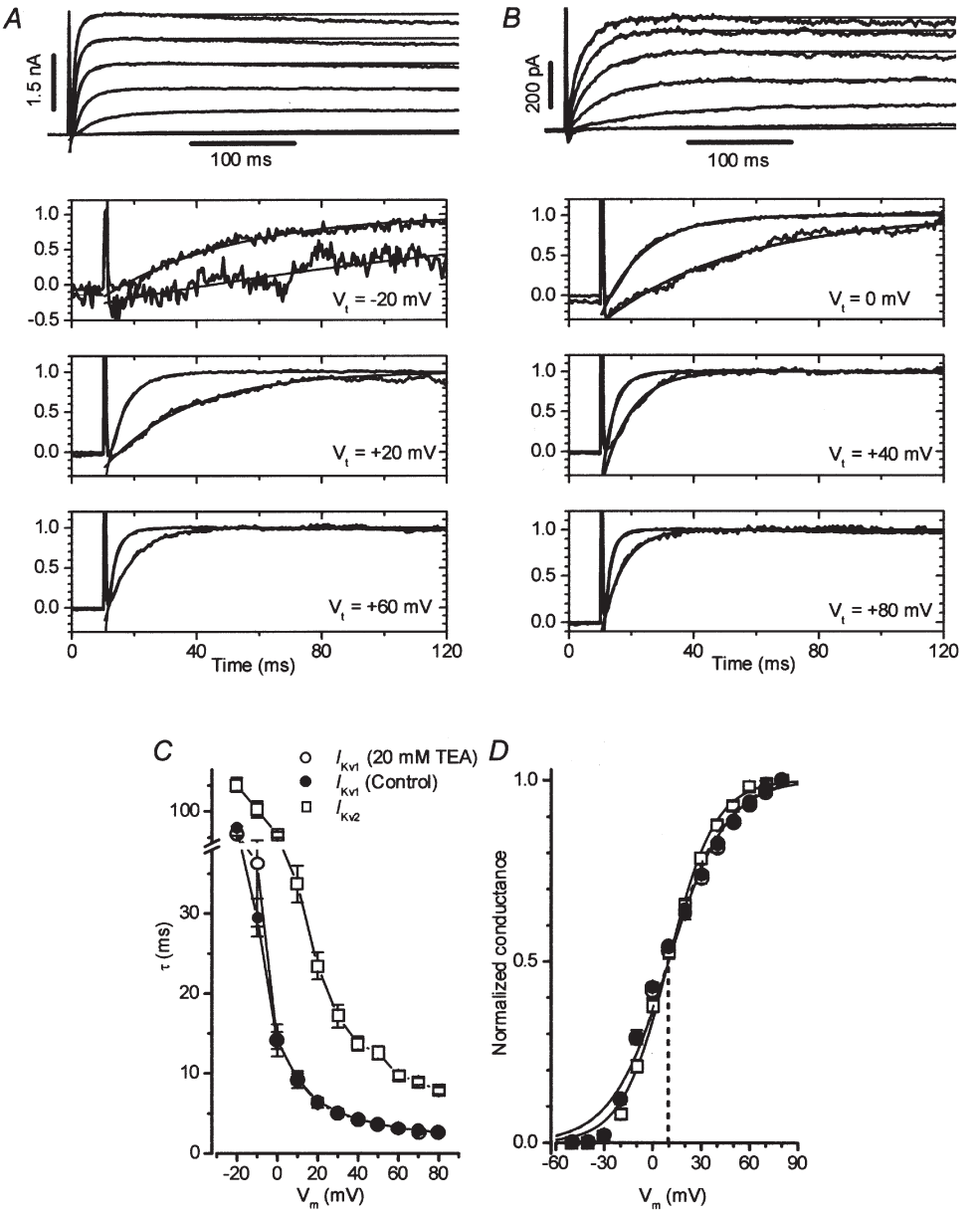

Figure 4. Activation of I_{Kv1} and I_{Kv2} in rat conduit PAMs

A and *B*, a family of I_{Kv1} and I_{Kv2}, respectively, recorded from two representative cells and fitted with a mono-exponential function (thin lines). C_m was 21 (*A*) and 19.9 pF (*B*). Bottom panels compare normalized and superimposed current traces at each membrane potential as indicated. Values of the time constant of activation (τ) were 69 and198 ms at −20 mV, 14 and 43 ms at 0 mV, 6.1 and 27 ms at +20 mV, 4 and 9 ms at +40 mV, 2.8 and 8.4 ms at +60 mV, and 2.2 and 6.7 ms at +80 mV for I_{Kv1} and I_{Kv2}, respectively. *C*, dependence of τ for I_{Kv1} in the presence (open circles, $n = 26$) and in the absence (filled circles, $n = 39$) of 20 mM TEA and I_{Kv2} (squares, $n = 27$) on the membrane potential. *D*, steady-state activation dependencies for normalized conductance (g) for I_{Kv1} and I_{Kv2} derived from the *I–V* relationships. Continuous lines were drawn according to the modified Boltzmann equation:

$$g = \frac{1}{1 + \exp((V_m - V_{0.5})/-k)},$$

with half-activation potential ($V_{0.5}$, also indicated by dashed lines) and slope factor (k) equal to 9.9 and 9.2 mV and 17.8 and 17.3 mV for I_{Kv1} in the absence and presence of 20 mM TEA, respectively, and 9.8 ($V_{0.5}$) and 14.4 mV (k) for I_{Kv2}. V_m, membrane potential.

Effect of α-dendrotoxin on Kv currents in rat conduit and resistance PAMs

It is known that α-dendrotoxin (DTX) is a potent inhibitor of number of homomultimeric Kv channels which belong to the Kv1 subfamily. Low doses of DTX block Kv channels encoded by Kv1.1, Kv1.2 or Kv1.6 α-subunits (IC_{50}, 4–20 nM), but not by the Kv1.5 α-subunit ($IC_{50} > 1$ μM) (Harvey, 2001). This selective effect of α-dendrotoxin makes this toxin a useful pharmacological tool which allows its use as a probe for the presence of Kv1.1, Kv1.2 or Kv1.6 homomultimeric currents in native cells. The effect of α-dendrotoxin on I_{KvR}, I_{Kv1} and I_{Kv2} in rat PA cells was therefore investigated (Fig. 7). As can be seen from the figures, the currents recorded in representative I_{Kv1}, I_{Kv2} and I_{KvR} cells at +60 mV in the absence and 5 min after addition of 200 nM α-dendrotoxin were practically superimposable. The average inhibition of I_{Kv} was equal to 1.5 ± 1.4 ($n = 7$), 2.5 ± 2 ($n = 5$) and 4.4 ± 4.5 % ($n = 6$) for I_{Kv1}, I_{Kv2} and I_{KvR}, respectively, suggesting that the native currents in rat PAMs are not sensitive to α-dendrotoxin.

Expression of Kv1.2, Kv1.5, Kv2.1 and Kv3.1b α-subunits in rat conduit and resistance PAs

Figure 8 illustrates immunoblots for Kv1.2, Kv1.5, Kv2.1 and Kv3.1b channels carried out on homogenates from conduit and resistance pulmonary arteries. Channel proteins for Kv1.2, Kv1.5 and Kv2.1, but not Kv3.1b, were detected in both sizes of arteries.

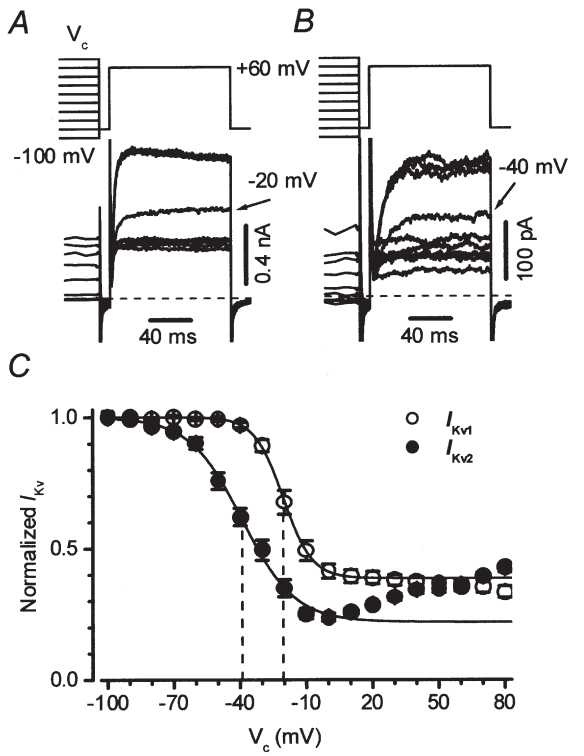

Figure 5. Inactivation of I_{Kv1} and I_{Kv2} in rat conduit PAMs

A and *B*, families of I_{Kv1} (*A*) and I_{Kv2} (*B*) recorded according to the experimental protocol indicated above with the conditioning potential (V_c) incremented 20 mV between −100 and +80 mV. V_c, duration 10 s; interpulse interval, 10 ms. C_m was 15.9 (*A*) and 22.4 pF (*B*). *C*, dependence of the normalized I_{Kv1} (open circles, $n = 13$) and I_{Kv2} (filled circles, $n = 13$) on V_c. Continuous lines were drawn according to the Boltzmann equation:

$$I_{NORM} = \frac{1 - A}{1 + \exp((V_m - V_{0.5})/k)} + A,$$

with half-inactivation potential ($V_{0.5}$, indicated by dashed lines) equal to −20.5 and −39.1 mV, slope factor of 6.5 and 11.9 mV and non-inactivating component *A* equal to 0.39 and 0.22 for I_{Kv1} and I_{Kv2}, respectively. I_{NORM} is a ratio of the current amplitude measured at the end of test pulse at the corresponding conditioning potential normalized to that at $V_c = -100$ mV.

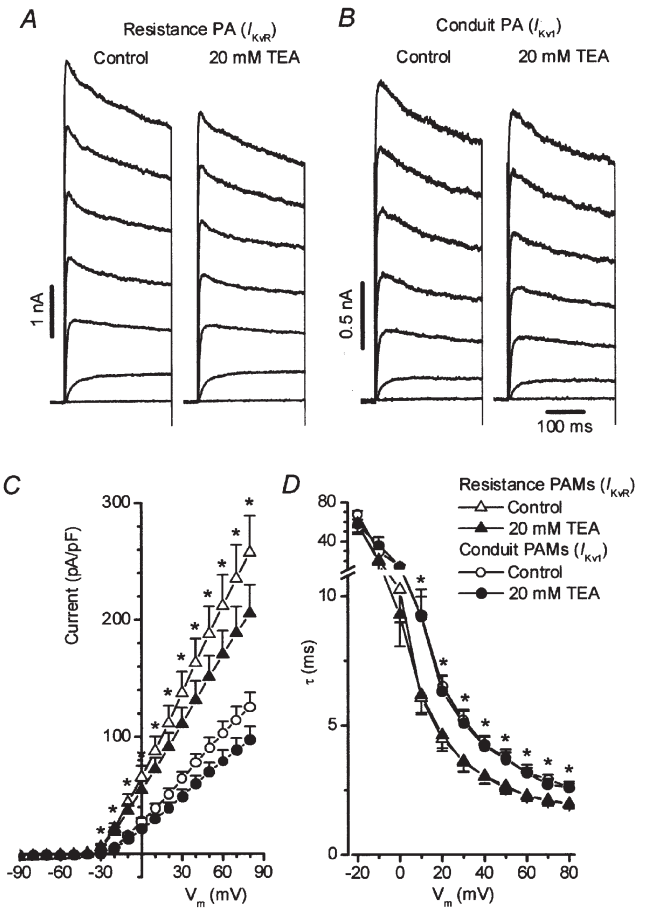

Figure 6. Voltage-gated K^+ currents in rat resistance PAMs

A, a family of I_{Kv} recorded from the cell isolated from resistance PA in the presence and absence of 20 mM TEA (C_m = 14.2 pF). For comparison I_{Kv1} recorded under similar conditions in conduit PA cell is also shown in *B* (C_m = 19.4 pF). *C* and *D* compare *I–V* relationships for I_{KvR} and I_{Kv1} densities (*C*) and the dependence of the time constant of current activation (τ) on the membrane potential (V_m, *D*) in the absence and presence of 20 mM TEA in 23 and 22 myocytes (I_{KvR}) and 39 and 26 myocytes (I_{Kv1}), respectively. Asterisks show significant differences ($P < 0.003$ in *C* and $P < 0.05$ in *D*).

Table 1. Comparison of electrophysiological and pharmacological properties of I_{KvR} and I_{Kv1}

		Resistance PAMs (I_{KvR})	Conduit PAMs (I_{Kv1})	P
Activation parameters	$V_{0.5}$ (mV)	2.9 ± 1.8	9.9 ± 1.4	< 0.003
	k (mV)	17.6 ± 0.4	17.3 ± 0.3	n.s.
		(n = 26)	(n = 26)	
Inactivation parameters	$V_{0.5}$ (mV)	−27.4 ± 2	−20.2 ± 1.7	< 0.02
	k (mV)	5.9 ± 0.6	5.7 ± 0.5	n.s.
	A	0.32 ± 0.02	0.38 ± 0.02	< 0.004
		(n = 13)	(n = 13)	
4-AP sensitivity	IC_{50} (μM)	352 ± 27	232 ± 35	< 0.009
	B	0.13 ± 0.02	0.1 ± 0.01	n.s.
		(n = 9)	(n = 11)	

Comparison was performed in the presence of 20 mM TEA to block a TEA-sensitive component of the whole-cell I_{Kv}. n.s., not significant. A is the non-inactivating component of the current. B is a residual 4-AP-insensitive component of I_{Kv}.

DISCUSSION

In this study we have for the first time demonstrated a heterogeneity of Kv channel currents in the rat PA vasculature. About 67 % of the 85 conduit PAMs studied demonstrated a large TEA-insensitive and 4-AP-sensitive current termed I_{Kv1}, whereas a relatively small TEA-sensitive and 4-AP insensitive current, designated I_{Kv2}, was recorded in the remaining 33 % of conduit PA cells. In addition to differences in pharmacological characteristics, these currents were also distinct in their kinetics of activation and voltage dependence of inactivation. In contrast, every resistance PA cell we studied contained a large current (I_{KvR}) which resembled I_{Kv1}, but which could be unambiguously distinguished from it on both pharmacological and biophysical grounds.

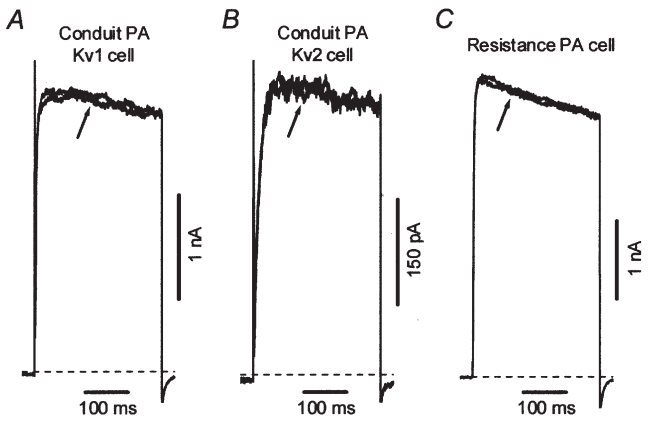

Figure 7. Effect of α-dendrotoxin on I_{Kv} in rat resistance and conduit PAMs

I_{Kv} were recorded at +60 mV in the absence and 5 min after addition of 200 nM α-dendrotoxin (at arrows) in Kv1 (*A*, C_m = 38.3 pF) and Kv2 (*B*, C_m = 21.6 pF) cells isolated from conduit PAs and in resistance PAMs (*C*, C_m = 8.6 pF).

The presence of three cell types in rat conduit PA which differ in the relative expression of I_{Kv} and $I_{K,Ca}$ were reported previously by Archer *et al.* (1996). Using TEA as a Ca^{2+}-activated K^+ current ($I_{K,Ca}$) blocker, and 4-AP as a I_{Kv} inhibitor, they found that in the majority of rat conduit PAMs (70 %, referred to as 'mixed' cells) the net outward current was partially blocked by both TEA (5–10 mM) and 4-AP (4 mM), whereas equal minorities of cells expressed

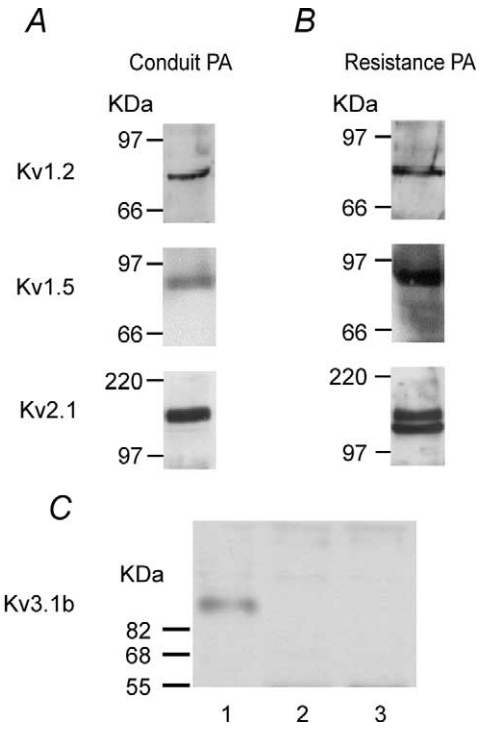

Figure 8

Expression of Kv1.2, Kv1.5 and Kv2.1 α-subunits in rat conduit (*A*) and resistance (*B*) PAs. *C* shows the Western blot analysis of the expression of Kv3.1b α-subunit in rat brain (lane 1), which was used as a positive control, in comparison to conduit (lane 2) and resistance (lane 3) PA.

Table 2. Comparison of electrophysiological and pharmacological properties of the native Kv currents in rat PAMs and cloned Kv channels

	I_{Kv1}, I_{KvR}	I_{Kv2}	Kv1.1 [a,b]	Kv1.2 [a,b]	Kv1.5 [a,b]	Kv1.6 [c]	Kv2.1 [d]	Kv3.1b [a]
Rate of activation	Rapid	Slow	Rapid	Rapid	Rapid	Rapid	Slow	Rapid
U-shape of inactivation	No	Yes	No	No	No	No	Yes [e]	No
TEA †	> 20	2.6	0.3–0.6	> 200	> 200	4	3–10	0.1–0.2
4-AP †	0.23; 0.35	> 20	0.29–1	0.6–0.8	0.17–0.5	0.3	5–100	0.03–0.1
DTX ‡	> 200	> 200	12–20	4–17	> 1000	9–25	n.d.	> 1000

Superscript letters indicate the following references: [a] Grissmer *et al.* (1994); [b] Stühmer *et al.* (1989); [c] Swanson *et al.* (1990), Grupe *et al.* (1990), Kirsch *et al.* (1991); [d] Shi *et al.* (1994), Patel *et al.* (1997); [e] Klemic *et al.* (1998), Kerschensteiner & Stocker (1999). n.d., not determined. †‡ Values are given as IC_{50} in mM or nM, respectively.

either mainly TEA-sensitive ($I_{K,Ca}$ predominant) or 4-AP-sensitive (I_{Kv} predominant) components. Cells containing only $I_{K,Ca}$ were ~3 times larger than those containing only I_{Kv}, and the 'mixed' cells were of intermediate capacitance. When isolated and visualized using light microscopy, these groups of cells were also found to differ with respect to their appearance and based on this morphological analysis the authors concluded that the relative proportion of cells containing I_{Kv} current (65 %) was greater in resistance PAMs compared with conduit PAMs (Archer *et al.* 1996). We did not, however, detect any obvious morphological dissimilarity between PAMs dominated by I_{Kv1}, I_{Kv2} or I_{KvR} and, although I_{Kv2} cells tended to be bigger than I_{Kv1} cells, this difference in C_m was not significant. Our direct electrophysiological measurement of whole-cell currents also indicated a relative homogeneity of cell population in resistance arteries, at least with respect to Kv channel currents. Moreover, our observation that I_{Kv2} was only slightly inhibited by 5 mM 4-AP, but was greatly blocked by TEA with an IC_{50} of 2.6 mM, suggests that the pharmacological approach of using high concentrations of TEA to distinguish Ca^{2+}-activated and voltage-gated K^+ currents employed by Archer *et al.* (1996) was flawed, so that cells predominantly expressing the TEA-sensitive voltage-gated K^+ current (I_{Kv2} cells in conduit PA) could have been incorrectly classified as containing 'mixed' or Ca^{2+}-activated K^+ currents.

The exact molecular identity of Kv channel genes which are responsible for the native I_{Kv} in pulmonary arteries remains inconclusive although recent evidence strongly suggest that Kv1.2, Kv1.5, Kv2.1 and Kv3.1b α-subunits are the most likely candidates (Coppock *et al.* 2001). Our immunoblot analysis demonstrated that Kv1.2, Kv1.5 and Kv2.1 α-subunits were expressed in both conduit and resistance arteries isolated from rat. In addition, comparison of the pharmacological and biophysical profile of I_{Kv1}, I_{Kv2} and I_{KvR} with those reported for the delayed rectifier types of cloned Kv channels suggests that the Kv1.5 channel profile corresponds closely to that of I_{Kv1} and I_{KvR}, whereas I_{Kv2} mimics the properties of the Kv2.1 channel (Table 2). Homomultimers of Kv1.1, Kv1.2 and Kv1.6 are unlikely to be responsible for I_{Kv1} and I_{KvR}, since these currents were insensitive to high doses of DTX (200 nM) and only slightly sensitive to TEA (20 mM), observations which are similar to those described previously for Kv currents in rat resistance PAs (Patel *et al.* 1997). The presence of the Kv1.2 α-subunit protein in both conduit and resistance arteries is consistent with the possibility that I_{Kv1} and I_{KvR} could be encoded by Kv1.2–Kv1.5 heteromultimers. It has been shown previously that Kv1.2–Kv1.5, but not Kv1.1–Kv1.5 (Hatton *et al.* 2001), heteromultimers become insensitive to DTX and also CTX (Russell *et al.* 1994; Hulme *et al.* 1999).

An important functional contribution of Kv3.1b to I_{Kv} in rat PAMs seems to be ruled out, since when expressed this current is potently blocked by micromolar doses of both TEA and 4-AP (Table 2). This pharmacological profile does not match any of the three types of currents we observed, since we found that 1 mM TEA had no effect on I_{Kv1} and I_{KvR} currents and I_{Kv2} current was relatively insensitive to 4-AP. Also, Western blot analysis showed the expression of the Kv3.1b α-subunit in rat brain, which was used as a positive control, but not in resistance or conduit arteries (Fig. 8*C*).

It is noteworthy that under conditions when $I_{K,Ca}$ was eliminated using high Ca^{2+} buffered pipette solution and the specific $I_{K,Ca}$ inhibitor paxilline, a small TEA-sensitive current component in cells expressing predominantly I_{Kv1} and I_{KvR} was present. Although, as described in Results, this current was difficult to characterize, it is possible that it represents a component of I_{Kv2} which is constitutively present in all PAMs. This suggestion is also supported by the presence of Kv2.1 α-subunit proteins in resistance PA, where no I_{Kv2} cells were detected. This question, however, needs to be further addressed with inhibitors other than 4-AP, which could selectively eliminate I_{Kv1} or I_{KvR} without alteration of their properties. The presence of this small TEA-sensitive current, however, did not affect our characterization of the properties of I_{Kv1} and I_{KvR}, since no significant differences between the electrophysiological and pharmacological properties of these currents were found in the absence and presence of 20 mM TEA.

The divergence in properties between I_{Kv1} and I_{KvR} is intriguing, and could arise from a different hetero-multimeric combination of, for example, Kv1.5 and Kv1.2 α-subunits, or the formation of more complex oligomeric channels composed of more than one channel protein (Po *et al.* 1993; Shamotienko *et al.* 1997). Alternatively, the association of different types of Kv β-subunits with Kv α-proteins in rat conduit and resistance PA could be responsible for the differences in the properties of I_{Kv1} and I_{KvR}. It has been found that co-expression of Kv β1 and Kv β2 subunits can both modulate the voltage dependence of activation and inactivation (McCormack *et al.* 1995; Uebele *et al.* 1996; McIntosh *et al.* 1997), and modify the pharmacological sensitivity (Shi *et al.* 1996) of Kv1 α-subunits. Although mRNA for all three subtypes of Kv β-subunits was shown to be expressed in rat PA cells (Yuan *et al.* 1998; Platoshyn *et al.* 2001) and the expression of Kv β proteins increased in resistance in comparison to conduit bovine PAMs (Coppock *et al.* 2001), identification of the origin of these differences will require further experimental work.

Since it was first demonstrated that Kv currents in PAMs were diminished by a reduction in P_{O_2} (Post *et al.* 1992), Kv channels have been thought to act as an oxygen sensor in PA (Weir & Archer, 1995). Although, recent work from our laboratory suggests that the contribution of I_{Kv} to acute hypoxic pulmonary vasoconstriction is limited (Robertson *et al.* 2000), I_{Kv} in rat PAMs was significantly diminished by chronic hypoxia, a condition which is associated with sustained membrane depolarization and increased cell size (Smirnov *et al.* 1994; Barnes & Liu, 1995; Osipenko *et al.* 1998). In support of these data in a recent elegant study Platoshin *et al.* (2001) using quantitative RT-PCR showed that Kv1.5, Kv2.1 and Kv9.3 gene expression and Kv1.5 and Kv2.1 α-protein expression were significantly reduced in PAs but not mesenteric arterial cells grown under hypoxic conditions in primary culture (Platoshyn *et al.* 2001). These data suggest that diminution of Kv channels could be an important contributor to long-term PA depolarization, the mechanisms of which remain in dispute (Barnes & Liu, 1995). We therefore believe that our findings could form a functional basis for elucidation of these mechanisms.

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Author's present address

R. Beck: Division of Protein Structure, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

Author's permanent address

T. Ishii: Department of Biochemistry, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba-Shi, Ibaraki-Ken 305, Japan.