

# Molecular Identification of the Role of Voltage-gated K<sup>+</sup> Channels, Kv1.5 and Kv2.1, in Hypoxic Pulmonary Vasoconstriction and Control of Resting Membrane Potential in Rat Pulmonary Artery Myocytes

Stephen L. Archer,\* Evelyne Souil,† A. Tuan Dinh-Xuan,‡ Bruno Schremmer,\* Jean-Christophe Mercier,|| Abdelhamid El Yaagoubi,‡ Lan Nguyen-Huu,‡ Helen L. Reeve,\* and Vaclav Hampel§

\*Veteran's Affairs Medical Center, Minneapolis, Minnesota 55417 and University of Alberta, Edmonton, Alberta, Canada; †Hôpital Cochin, Département de Physiologie et de Biologie Cellulaire, 75679 Paris, France; ‡Department of Physiology, Second Medical School, Charles University, 150 00 Prague 5, Motol, Czech Republic; and ||Hôpital Robert Debré, Service de Réanimation Pédiatrique, 75019, Paris, France

## Abstract

Hypoxia initiates pulmonary vasoconstriction (HPV) by inhibiting one or more voltage-gated potassium channels (Kv) in the pulmonary artery smooth muscle cells (PASMCs) of resistance arteries. The resulting membrane depolarization increases opening of voltage-gated calcium channels, raising cytosolic Ca<sup>2+</sup> and initiating HPV. There are presently nine families of Kv channels known and pharmacological inhibitors lack the specificity to distinguish those involved in control of resting membrane potential (E<sub>m</sub>) or HPV. However, the Kv channels involved in E<sub>m</sub> and HPV have characteristic electrophysiological and pharmacological properties which suggest their molecular identity. They are slowly inactivating, delayed rectifier currents, inhibited by 4-aminopyridine (4-AP) but insensitive to charybdotoxin. Candidate Kv channels with these traits (Kv1.5 and Kv2.1) were studied. Antibodies were used to immunolocalize and functionally characterize the contribution of Kv1.5 and Kv2.1 to PASMC electrophysiology and vascular tone. Immunoblotting confirmed the presence of Kv1.1, 1.2, 1.3, 1.5, 1.6, and 2.1, but not Kv1.4, in PASMCs. Intracellular administration of anti-Kv2.1 inhibited whole cell K<sup>+</sup> current (I<sub>K</sub>) and depolarized E<sub>m</sub>. Anti-Kv2.1 also elevated resting tension and diminished 4-AP-induced vasoconstriction in membrane-permeabilized pulmonary artery rings. Anti-Kv1.5 inhibited I<sub>K</sub> and selectively reduced the rise in [Ca<sup>2+</sup>]<sub>i</sub> and constriction caused by hypoxia and 4-AP. However, anti-Kv1.5 neither caused depolarization nor elevated basal pulmonary artery tone. This study demonstrates that antibodies can be used to dissect the whole cell K<sup>+</sup> currents in mammalian cells. We conclude that Kv2.1 is an important determinant of resting E<sub>m</sub> in PASMCs from resistance arteries. Both Kv2.1 and Kv1.5 contribute to the initiation of HPV. (*J. Clin. Invest.* 1998. 101:2319–2330.) Key words: volt-

age-gated potassium channels (Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kv2.1) • GIRK-1 • membrane potential • O<sub>2</sub> sensing • 4-aminopyridine

## Introduction

Hypoxia causes localized pulmonary vasoconstriction (HPV)<sup>1</sup> of the small, resistance pulmonary arteries (PA) supplying the hypoxic segment (1, 2). This is a mechanism for diverting blood flow away from poorly ventilated alveoli, thereby optimizing ventilation-perfusion matching and maintaining systemic PO<sub>2</sub>. The constrictor response to hypoxia is unique to the pulmonary circulation and hypoxia causes relaxation of smooth muscle cells (SMC) in systemic vascular beds (3). HPV occurs rapidly and, though modulated by many endothelial and humoral factors, is intrinsic to PASMCs (4). Hypoxia initiates HPV by reversibly inhibiting potassium (K<sup>+</sup>) channels causing membrane depolarization (5–7) and activation of voltage-gated calcium channels (8). Ultimately this increases calcium influx which augments the free, cytosolic calcium concentration [Ca<sup>2+</sup>]<sub>i</sub> leading to activation of the contractile apparatus. This appears to be the essence of the effector mechanism for HPV; however, the O<sub>2</sub> sensing occurs by an independent mechanism which remains controversial (although it probably involves a redox-based O<sub>2</sub> sensor [9]).

Single channel studies indicate that hypoxia inhibits a family of Kv channels (7). The goal of the current study was to determine the molecular basis of the hypoxia-inhibitable K<sup>+</sup> current (I<sub>K</sub>) and of the channel(s) underlying the resting membrane potential (E<sub>m</sub>) in PASMCs. It is challenging to dissect the whole cell I<sub>K</sub> into its component parts. However, most Kv channels have been cloned and sequenced (10). The biophysical and pharmacological attributes of a given Kv channel, though clear in expression systems where a single channel is expressed (10), are insufficiently distinct to be used in mammalian cells, which have an “ensemble” current created by the many K<sup>+</sup> channels which are simultaneously active (7). However, one can use data derived from expression of these channels in oocytes and other cell lines to define each channel's theoretical pharmacological (Table I) and electrophysiological profile (11).

Fortunately, the pharmacology and morphology of I<sub>K</sub> ac-

Address correspondence to Dr. Stephen L. Archer, Professor of Medicine, University of Alberta, Director, Division of Cardiology and Alberta Heart and Stroke Chair, 2C2 Walter C. Mackenzie Health Sciences Center, University of Alberta, Edmonton, Alberta, Canada T6G 2E7. Phone: 403-492-6353; FAX: 403-492-6452.

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1. Abbreviations used in this paper: 4-AP, 4-aminopyridine; AII, angiotensin II; CTX, charybdotoxin; E<sub>m</sub>, membrane potential; HPV, hypoxic pulmonary vasoconstriction; I<sub>K</sub>, K<sup>+</sup> current; PASMCs, pulmonary artery smooth muscle cells; TEA, tetraethylammonium.

Table I. Characteristics of Candidate  $K^+$  Channels Compared to the Basal and Hypoxia-sensitive  $I_K$  in Rat PSMCs

Current characteristic	Candidate Kv channels						CTX-sensitive channels				
	$Kv_{Em}$	$Kv_{O_2}$	$Kv_{1.1}$	$Kv_{1.5}$	$Kv_{2.1}$	$Kv_{3.1}$	$Kv_{1.2}$	$Kv_{1.3}$	$Kv_{1.4}$	$Kv_{1.6}$	$K_{Ca}$
<b>Pharmacology</b>											
Inhibited by 4-AP $< 5 \times 10^{-3}$ M	+	+	+	+	+	+	+	+	+	+	-
Inhibited by CTX $< 10^{-7}$ M	-	-	-	-	-	-	+	+	+	+	+
Inhibited by glyburide $10^{-6}$ M	-	-	-	-	-	-	-	-	-	-	-
Inhibited by TEA $< 10 \times 10^{-3}$ M	-	±	+	-	+	+	+	+	+	+	+
<b>Biophysics</b>											
Active resting at $E_m$ in normoxia	+	±	?	±	+	?	?	?	-	?	±
Outward rectifying	+	+	+	+	+	+	+	+	+	+	+
voltage-dependent and slow inactivating	+	+	+	+	+	+	+	+	-	+	+

$Kv_{Em}$ , the  $K^+$  channel(s) controlling resting membrane potential;  $Kv_{O_2}$ , the  $K^+$  channel(s) responding to hypoxia; ?, unknown in this tissue.

tive at resting  $E_m$  and the hypoxia-inhibited  $I_K$  share a profile which permits one to construct a relatively short list of the Kv channels which may be responsible (5, 7, 12) (Table I). Specifically, the whole-cell current is a slowly inactivating, delayed-rectifier type which is reduced by the preferential Kv inhibitor 4-aminopyridine (4-AP; 1–5 mM) (7, 13) but not by the inhibitor of  $Ca^{2+}$ -sensitive  $K^+$  channels ( $K_{Ca}$ ), charybdotoxin (CTX), or by glyburide, an inhibitor of ATP-sensitive  $K^+$  channels ( $K_{ATP}$ ) (7, 14). Furthermore, PSMCs depolarize in response to low dose 4-AP ( $< 5$  mM) but not tetraethylammonium (TEA,  $< 5$  mM), CTX ( $< 200$  nM), or glyburide ( $< 1$   $\mu$ M), indicating resting  $E_m$  is also likely a function of Kv, not  $K_{Ca}$  or  $K_{ATP}$  channels (7, 13–15). Quinidine has also been suggested to inhibit the hypoxia-sensitive Kv channel (16). Thus, sensitivity to 4-AP, and possibly quinidine coupled with insensitivity to CTX and glyburide constitute the profile for candidate channels involved in control of  $E_m$  or HPV. Inward rectifiers (e.g., GIRK-1, a G-protein-coupled muscarinic  $K^+$  channel [17]) and rapidly inactivating channels (e.g.,  $Kv_{1.4}$ ) are likewise unlikely candidates as their current morphology is not concordant.

Thus,  $Kv_{1.5}$  and  $Kv_{2.1}$  were selected for initial study (18).  $Kv_{2.1}$  and  $Kv_{1.5}$  are delayed rectifiers and conduct rapidly activating, slowly inactivating currents (11) which are inhibited by 4-AP (19, 20) (and by quinidine in the case of  $Kv_{1.5}$  [21]).  $Kv_{2.1}$  (22) and 1.5 (11) are insensitive to  $\geq 100$  nM CTX, although  $Kv_{2.1}$  is somewhat sensitive to TEA (23–25). Certain members of the Kv1 family were excluded from initial study based on their pharmacology (e.g.,  $Kv_{1.2}$  [11] and  $Kv_{1.3}$  and 1.6 [22] are quite sensitive to CTX, Table I). Further basis for selecting  $Kv_{2.1}$  and  $Kv_{1.5}$  for study is evidence of their presence in PASM (18, 26–28).

We examined the hypothesis that basal  $K^+$  efflux via  $Kv_{1.5}$  and/or  $Kv_{2.1}$  maintains the resistance PA in a relaxed state and that inhibition of one or both of these channels, by physiological (hypoxia) or pharmacological (4-AP) stimuli, causes membrane depolarization and vasoconstriction. We compared the electrophysiological effects of anti- $Kv_{1.5}$  and 2.1 antibodies to those of vehicle and also to an antibody directed against an inward rectifier  $K^+$  channel, GIRK-1. This antibody was chosen as a negative control because inward rectifier currents have not been detected in previous studies of PSMCs (5, 7,

29). We also used immunohistochemistry to look for these Kv channels in arteries which lack HPV (renal, cerebral, and coronary), reasoning that if these Kv channels were both the  $O_2$  sensor and effector they would be localized exclusively in the PA, whereas if they were solely effectors, they might be found in arteries which lack a constrictor response to hypoxia. We found that  $Kv_{2.1}$  and  $Kv_{1.5}$  are present in PSMCs and contribute to the  $I_K$ . Anti- $Kv_{2.1}$  depolarizes PSMCs and constricted PA rings. Anti- $Kv_{1.5}$  did not depolarize  $E_m$  but did reduce  $I_K$  and inhibit HPV. Both anti- $Kv_{1.5}$  and - $Kv_{2.1}$  reduced constriction to 4-AP in membrane-permeabilized PA rings. We conclude that  $Kv_{2.1}$  participates in setting resting  $E_m$  and confers 4-AP sensitivity to the PA.  $Kv_{1.5}$  is an effector channel involved in initiating HPV.

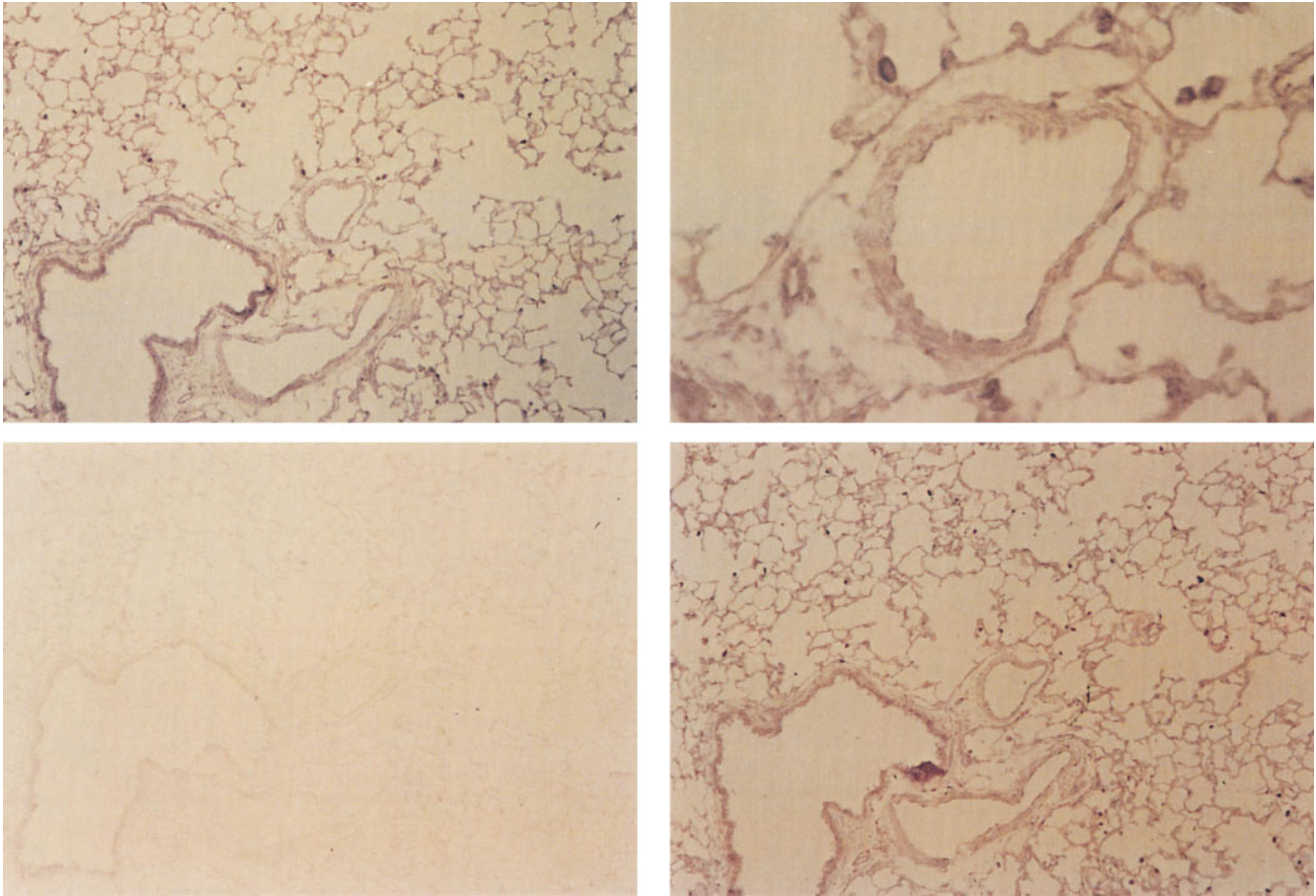
## Methods

### Verification of antibody specificity and efficacy antibodies

Polyclonal, anti-rabbit, Kv antibodies (GIRK-1,  $Kv_{1.5}$ , and  $Kv_{2.1}$ ) were obtained from Upstate Biotechnology Inc. (Lake Placid, NY).  $Kv_{1.1}$ , 1.2, 1.3, 1.4, and 1.6 antibodies were obtained from Alomone Laboratories (Jerusalem, Israel). The immunogen for the  $Kv_{1.5}$  corresponds to a GST fusion protein with the carboxyl-terminal amino acids 542–602 of rat  $Kv_{1.5}$  (distal to the pore-forming S5-S6 region). The immunogen of  $Kv_{2.1}$  is a keyhole limpet fusion protein with amino acids 837–853 of rat  $Kv_{2.1}$ .

Specificity of the  $Kv_{2.1}$  antibodies was ascertained by prior incubation of the antibody with a three times weight excess of the soluble antigen against which it was raised as well as a related but non-identical  $K^+$  channel antigen (for  $Kv_{1.5}$ ). The antigens were a generous gift from Dr. James Trimmer (SUNY, Stony Brook, NY). Preincubation with matched antigen-antibody combinations yielded inactivated antibody and thus caused no staining on immunohistochemistry, whereas mismatched combinations did not impair antigen detection (Fig. 1).

To confirm the sensitivity/specificity of anti- $Kv_{1.5}$ , the ability to detect antigen in cells expressing a single Kv channel was evaluated. A transient transfection of Chinese hamster ovary (CHO-1) cells was performed, as previously described (24) using pCMV. $Kv_{1.5}$  or pCMV. $Kv_{3.1}$  plasmids containing the Kv cDNA under a CMV promoter as well as a neomycin resistance gene (kind gifts of Dr. Koichi Takimoto, University of Pittsburgh, Pittsburgh, PA and Dr. Theresa Perney, Rutgers University, Newark, NJ). Transfected cells were fixed in paraformaldehyde 2% and then permeabilized with Tween



**Figure 1.** Kv2.1 is present in PASMCS and the antibody used is specific for its carboxyl-terminal antigen. These experiments were performed in three animals and the pictures are representative. (*Top left*) Kv2.1 is present in the media of a medium sized artery (3+) as well as in an adjoining airway ( $\times 100$ ) (4+). (*Top right*) The same artery shown  $\times 400$ , again with positive staining of the media. (*Bottom left*) Specificity was assessed by prior coincubation of the antibody with the soluble antigen against which the antibody is directed (i.e., anti-Kv1.5 + Kv1.5 antigen). This blocked the antibody and thus prevented staining (1+). (*Bottom right*) Mismatched combinations (anti-Kv2.1 coincubated with a fusion peptide containing a fragment of Kv1.5) did not affect labeling.

(0.05%) and BSA (0.8%) for 30 min. Incubation with the anti-Kv1.5 (1:100) was done overnight. Cells are washed three times and incubated with the secondary fluorescent antibody at 1:100 for 1 h, washed again, and mounted with Vectashield (Vector Laboratories Biosys, Paris, France). This technique showed that the anti-Kv1.5 detected the expected 5–10% of Kv1.5-transfected cells (Fig. 2) but did not detect Kv3.1-transfected cells (data not shown).

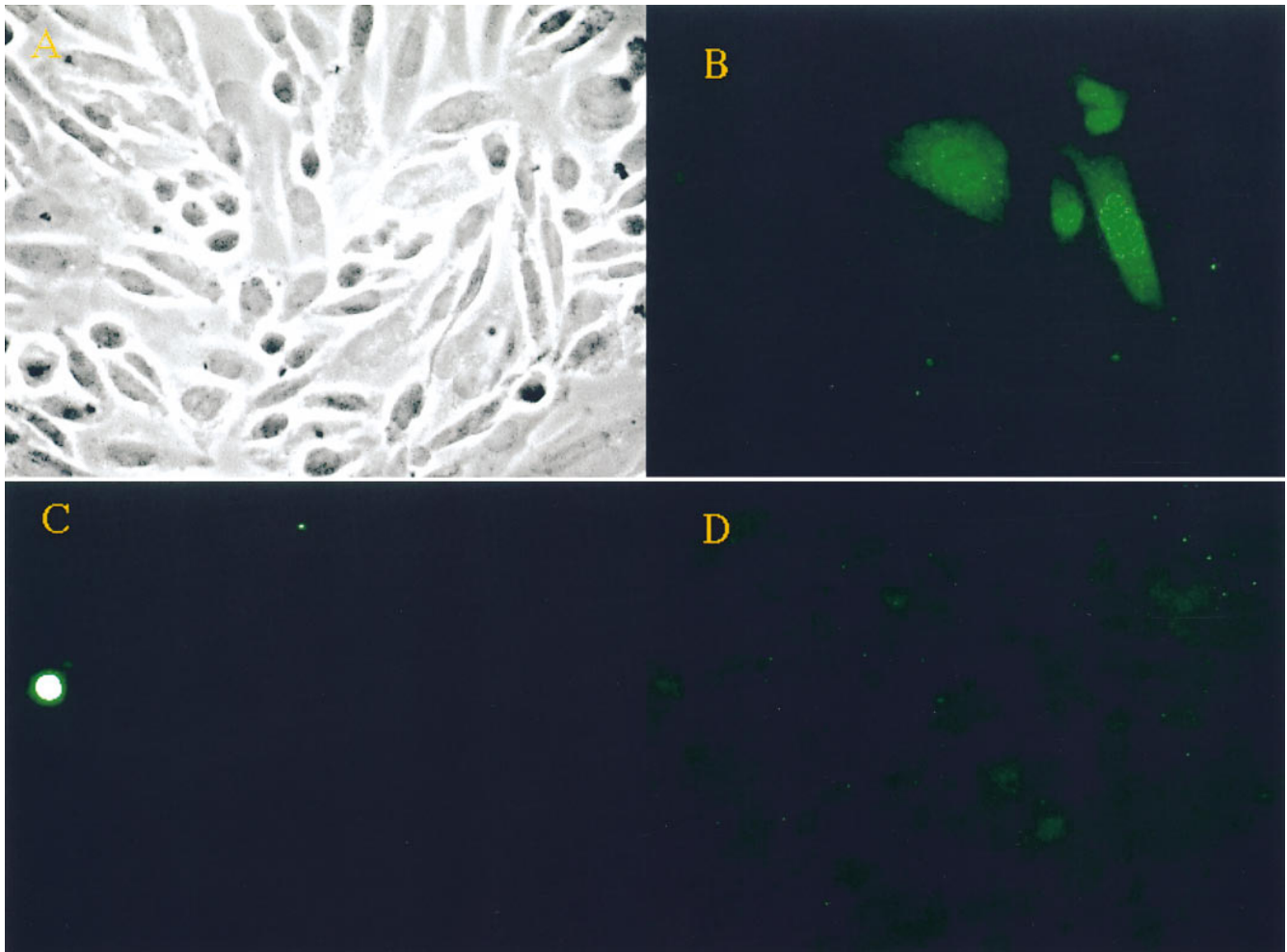
#### Immunoblotting

Specificity of the antibodies was assessed by immunoblotting for Kv1.1, 1.2, 1.3, 1.4, 1.5, 1.6, and 2.1 in rat brains, known to contain most Kv channels (25, 30–32). To further confirm that the antibodies yielded a predominant band of the predicted molecular weight, immunoblotting was also performed on a clonal pituitary line (GH3), known to contain Kv1.5 and Kv2.1 (33). Immunoblotting was also done on primary cultures of PASMCS and freshly dispersed PASMCS taken from fourth to fifth division rat PAs. Homogenates of brain were suspended in a homogenization buffer containing protease inhibitors (Sigma, Paris, France), sucrose 0.3 M, Tris 10 mM, pH 7.5, and EDTA 1 mM. A membrane purification step was then performed involving serial centrifugation first at 2,000 g (10 min) and then, after resuspension, repeat centrifugation at 45,000 g (90 min). This yielded a pellet composed of a crude membrane fraction. Membranes are

suspended in the homogenization buffer, protein is determined using the bicinchoninic acid method (BCA; Pierce Chemical Co., Rockford, IL). Freshly dispersed or primary cultured PAs were pelleted at 13,000 g and lysed in a RIPA buffer containing protease inhibitors. No membrane purification was used in cell experiments. Samples (75  $\mu$ g) were loaded onto a 4–15% gradient SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Nonspecific protein binding sites were blocked by incubation in 4% Blotto for 2 h (25°C). The membrane was probed with the primary anti-Kv antibody at a 1:100 dilution in 4% Blotto for 1 h. After washes in Blotto, membranes were incubated with a secondary anti-rabbit antibody 1:20,000 (Pierce Chemical Co.) in Blotto washed in Tween Tris-buffered saline, and finally in the substrate for enhanced chemiluminescence (Pierce Chemical Co.) for 1 min and exposed for 20 s on Fuji RX film.

#### Immunohistochemistry

Immunohistochemistry was performed on lungs, hearts, brains, and kidneys. Frozen sections and paraffin-embedded blocks were used. For frozen sections, organs ( $n = 4$ ) were isolated, flash frozen in liquid monochloro-difluoromethane ( $-40^{\circ}\text{C}$ ), and stored at  $-20^{\circ}\text{C}$ . 10- $\mu$ m sections were thaw-mounted on glass slides (Superfrost; CML, Paris, France) and immediately immersed in isotonic, paraformaldehyde (4%) in a phosphate buffer (PBS, 1 h at  $4^{\circ}\text{C}$ ), as previously de-



**Figure 2.** Chinese hamster ovary cells transfected with a eukaryotic expression plasmid containing Kv1.5. (*A* and *B*) Phase contrast shows cell confluence with several cells producing Kv1.5 as demonstrated by the intense perinuclear fluorescence. (*C*) In absence of the primary antibody there is no fluorescence. (*D*) Sham-transfected cells show a weak nonspecific fluorescence indicating that the cell line does not express Kv1.5.

scribed (34). Slides were dehydrated by serial immersion in progressively more concentrated alcohol. For paraffin inclusion, lungs were isolated and perfused with 4% paraformaldehyde, dehydrated in graded alcohol baths, then included in paraffin blocks and cut with a microtome. Slides were heated for 20 min at 80°C for increased tissue adherence to the slide, followed by an antigen unmasking step. Samples were microwaved in 3 M urea for three times 5 min, as described previously (35).

Anti-NOS3, anti-mouse antibody (Transduction Laboratories, Interchim, France) was used to identify the endothelium. Slides were warmed at room temperature and air dried for 10 min before incubation in a physiologic glycine buffer (pH 7.4, glycine 100 mM) for 1 h. Then the slides were kept in a PBS buffer containing Tween 20 (0.05%) and BSA (0.2%). After three 10-min rinses in PBS, sections were dried and incubated overnight with the primary antibody diluted in 0.1 M sodium buffer also containing Tween 20 and albumin (1:50 for the Kv channels, 1:100 for NOS3). After three 10-min rinses in PBS, sections were briefly dried and incubated with secondary antibody (for Kv channels, 1:200 anti-rabbit Vectastain; for NOS3-biotinylated anti-mouse antibodies, Elite Biosys, Paris, France). To visualize the antibodies, the slides were rinsed three times (10 min each) in PBS, briefly dried, and then incubated in Vectastain ABC (Vector Laboratories Biosys) for 1 h. Then the sections were rinsed in PBS three times (10 min each) and incubated in the appropriate peroxi-

dase substrate solution (for the Kv system, 10 min in VIP peroxidase substrate [Vector Laboratories Biosys]; for the NOS3, 10 min in Sigma Fast DAB [Sigma]). Analysis was performed by blinded observers and was qualitative, with intensity of staining scored by blinded observers on a 1–4+ scale (1+ being barely detectable, 4+ being most intense).

#### *Electrophysiology*

To specifically evaluate the effects of the antibodies on  $I_K$  and resting  $E_m$ , conventional, whole-cell patch-clamp studies were done. It was decided to administer the antibody intracellularly as this is the only way to rapidly deliver the antibody and thus clearly show acute changes in  $I_K$  and  $E_m$ . In the isolated lungs, PA rings, and cultured PASMCS, the antibody was given with a Tween/BSA vehicle to permit membrane permeabilization. This results in a gradual uptake of antibody and required several hours of exposure, a time frame unsuitable for the patch-clamp studies.

*Cell isolation.* Resistance PAs (300–500  $\mu\text{m}$  diameter, fourth or fifth division) were dissected and placed in  $\text{Ca}^{2+}$ -free Hanks' solution for 30 min at 4°C. The Hanks' solution contained (mM): NaCl 140; KCl 4.2;  $\text{KH}_2\text{PO}_4$  1.2;  $\text{MgCl}_2$  0.5; Hepes 10; EGTA 0.1 mM (pH 7.4). Arteries were then transferred to a papain solution containing 1 mg  $\text{ml}^{-1}$  papain, 0.75 mg  $\text{ml}^{-1}$  bovine albumin, and 0.85 mg  $\text{ml}^{-1}$  dithiothreitol and digested at 4°C for 15 min and then at 37°C for 10 min.

Arteries were washed thoroughly with Hanks' solution without EGTA (low-Ca<sup>2+</sup>) for at least 15 min and then maintained on ice in Hanks' solution supplemented with 1 mg ml<sup>-1</sup> glucose. This digestion protocol consistently produced high yields of viable, relaxed SMC.

**Whole-cell recordings.** Gentle trituration produced a suspension of single cells which were pipetted into a perfusion chamber on the stage of an inverted microscope for conventional whole-cell patch-clamp studies. After a brief period to allow partial adherence to the bottom of the recording chamber, cells were perfused with a solution of composition (mM): NaCl 145; KCl 5.4; CaCl<sub>2</sub> 1.5; MgCl<sub>2</sub> 1.0; Hepes 10; glucose 10 (pH 7.4 with NaOH). Patch electrodes (resistance of 1–3 MΩ) were fire polished and filled with a solution of composition (mM): KCl 140; MgCl<sub>2</sub> 1.0; Hepes 10; EGTA 5; phosphocreatinine (pH 7.2 with KOH). For experiments using antibodies, electrodes were initially dipped in antibody-free intracellular solution before back-filling with a 1:125 dilution of either Kv1.5, Kv2.1, or GIRK-1 antibody.

An initial series of experiments was performed with anti-Kv1.5 to establish an effective antibody dose and to determine whether the small amount of azide vehicle in which the antibody was supplied altered I<sub>K</sub>. Doses from 1:50 to 1:1,000 were tested and the threshold for effect (defined as a > 10% decrease in I<sub>K</sub> within 10 min) was 1:250, with a more obvious effect at 1:125. Higher concentrations of antibody caused severe current inhibition and an increase in leak. There was no difference in antibody used as provided versus antibody dialyzed to remove azide. Subsequently, experiments were performed comparing the effects on I<sub>K</sub> and E<sub>m</sub> of adding the antibodies to the patch pipette at a 1:125 dilution. In this configuration, the antibody could be rapidly administered to the cell by diffusion from the patch pipette. The baseline currents were allowed to stabilize before any recording (usually after 1 min). Vehicle controls provided time-dependent controls to detect run-down or run-up of K currents (as well as to establish the effects of hypoxia and 4-AP on normal PA cells). Aliquoted antibodies were defrosted daily to avoid degradation.

Cells were voltage-clamped at a holding potential of -70 mV and currents evoked by +20 mV steps to more positive potentials using test pulses of 200-ms duration at a rate of 0.1 Hz. Currents were filtered at 1 kHz and sampled at 4 kHz. E<sub>m</sub> was recorded using current-clamp at the resting E<sub>m</sub> of each cell. E<sub>m</sub> stability was always determined for at least 1 min before any recording. Data were recorded and analyzed using pClamp 6.02 software (Axon Instruments, Foster City, CA). Drugs were applied to the cells dissolved in the extracellular perfusate via gravity perfusion at a rate of 2 ml/min. All experiments were performed at 22° C. For both voltage-clamp and current-clamp experiments, data were recorded for a period of 10 min before application of 1 mM 4-AP.

#### Isolated perfused lungs

Adult, male, Sprague-Dawley rats were anesthetized (pentobarbital 50 mg/kg, intraperitoneally). Lungs were isolated and perfused in a recirculating manner with 25 ml of Krebs' solution containing 4% albumin, L-N<sup>G</sup> nitroarginine methylester (50 μM) and meclofenamate (17 μM) (36). Lungs were ventilated with either normoxia (20% O<sub>2</sub>, 5% CO<sub>2</sub> balance N<sub>2</sub>/PO<sub>2</sub> 120 mmHg) or hypoxia (2.5% O<sub>2</sub>, 5% CO<sub>2</sub>, balance N<sub>2</sub>/PO<sub>2</sub> 40 mmHg). The protocol consisted of six cycles of normoxia (10 min), administration of angiotensin II (AII, 0.15 μg), and, after 8 min, hypoxia (6 min). After the third cycle, when the constrictor response to hypoxia and AII was stable, the lungs received 50 μl of anti-Kv1.5, anti-Kv2.1, or vehicle (azide 10 mM), in each case dialyzed for at least 3 h to remove the azide preservative.

#### Endothelium-denuded, permeabilized PA rings

Rings were harvested from the first or second branch PAs and denuded of endothelium, as previously described (7). The effect of 4-AP, AII, and phenylephrine on these, endothelium-denuded, acetylcholine-unresponsive, rings was studied after 2 h of incubation in a 1:25 dilution of dialyzed anti-Kv1.5, anti-Kv2.1, or vehicle. Rings were permeabilized throughout the antibody incubation (Tween 20, 0.05,

and BSA, 0.2%) to facilitate intracellular uptake. Immunohistochemistry on several rings at the end of the experiments confirmed uptake of the antibody in these nonfixed tissues. Basal tension was initially 800 mg, previously determined to be optimal for proximal PA rings (7).

#### Measurement of [Ca<sup>2+</sup>]<sub>i</sub>

PASMCs from fourth division rat PAs were studied in primary cultures (7). Cells were exposed to anti-Kv1.5, anti-Kv2.1, or saline (1:400) for 1 h and then loaded with Fura 2-AM (4 μM) and studied on the heated stage of an epifluorescence microscopy system (37). Plates were superfused with Earl's solution (35°C) bubbled with either a normoxic or hypoxic gas mixture. Cells received either 4-AP (6 mM, bolus) or hypoxic perfusion (PO<sub>2</sub> ~ 40 mmHg) for 8 min. In either case, this was followed by AII (1 μg). The cells were illuminated (340 and 380 nm alternating at 60 Hz) and the ratio of the 510 nm emission was used to calculate [Ca<sup>2+</sup>]<sub>i</sub>, as previously described (37).

#### Statistics

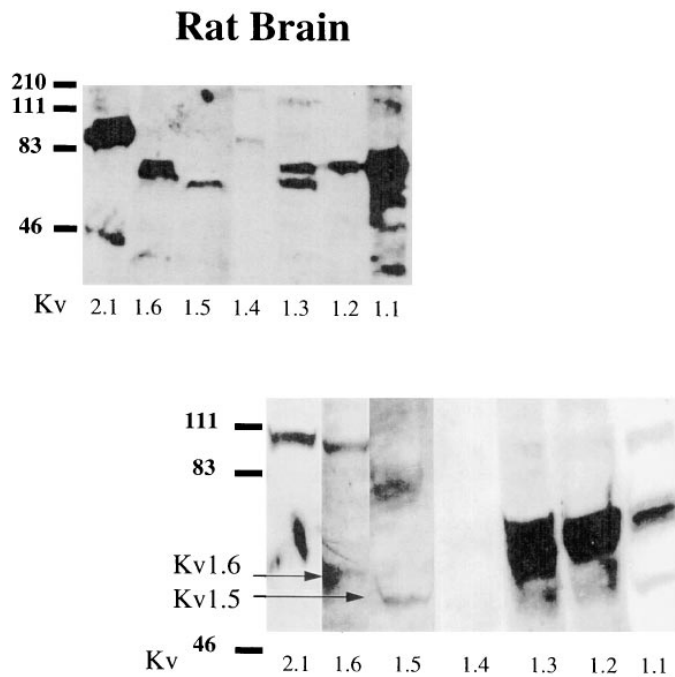
Values are expressed as the mean ± SEM. Intergroup differences were assessed by a factorial ANOVA (*P* < 0.05 is considered statistically significant). A Fisher PLSD test was used for evaluation of differences within a group.

## Results

**Specificity of anti-Kv antibodies.** Immunohistochemistry with the combination of Kv2.1 and the matching fusion protein resulted in no staining of lung tissue, indicating effective neutralization of the antibody by its antigen. Mismatching Kv1.5 with antigen for Kv2.1 and vice versa did not inhibit immunohistochemical detection of the Kv channels. In Kv1.5 transfected cells, only anti-Kv1.5 produced a fluorescent signal and this could be competed off with the Kv1.5 fusion protein. Together, these results indicate the anti-Kv1.5 and Kv2.1 are indeed specific for their antigens and do not cross-react.

**Immunoblotting and immunohistochemistry.** To evaluate the specificity of the antibodies, we assessed the antibodies in a clonal pituitary line which has been shown to have abundant Kv1.5 and a small amount of Kv2.1, the GH3 cells. The immunoblot confirmed the presence of a faint but discrete single band of Kv2.1 (110 kD) and a denser band of Kv1.5 (66 kD) (Fig. 3). As a further test of specificity in a more complex tissue, we performed immunoblots in rat brain for Kv1.1-1.6 and Kv2.1. Brain was chosen as it contains most of the Kv channels and is readily homogenized. All the Kv channels tested were present and the molecular weights (see Fig. 3) correlate well with those reported in the literature (24, 33, 38). Finally, immunoblotting confirmed the presence of all the Kv channels except Kv1.4 in the PASMCs themselves (both freshly dispersed and cultured, Fig. 3). The estimated molecular weights were similar to those obtained in brain.

In the case of Kv2.1, we found a single band, but the width of the band varied depending on the complexity of the tissue. This was not due to lack of specificity of the Kv antibodies used but rather due to posttranslational modification of the core protein. Shi and Trimmer have elegantly demonstrated that Kv2.1 is phosphorylated within minutes of synthesis in the Golgi apparatus (24). The theoretical molecular mass of Kv2.1 (~ 95.3 kD) is lower than that observed when the channel is expressed in oocytes (100 kD). Furthermore, when the Kv2.1 channel is expressed in mammalian cells the mass rises to 108 kD (24). In these expression systems the bands are, as expected, quite sharp, reflecting the simplified environment in which the protein exists. In contrast, the same group has reported molec-



**Figure 3.** Immunoblotting confirms the presence of Kv1.1, 1.2, 1.3, 1.5, 1.6, and 2.1, but not Kv1.4, in rat PSMCs. (*Top left*) Immunoblotting confirms the presence of Kv1.1–1.6 and Kv2.1 in rat brains. The antibodies produced relatively clean bands at the following molecular masses: Kv1.1, a broad band centered at 83 kD; Kv1.2, a single band at 80 kD; Kv1.3, 2 bands at ~66 and 80 kD; Kv1.4, 90 kD; Kv1.5, a single band at 66 kD; Kv1.6, a broad band at 75–80 kD; and Kv2.1, a broad band at ~110 kD. (*Top right*) GH3 cells have Kv1.5 and Kv2.1 at the predicted molecular masses. (*Bottom*) Immunoblots of freshly dissociated PSMCs from resistance PAs of several rats (combined) demonstrate the presence of the same Kv channels as in brain with the exception of Kv1.4, which is absent.

ular masses varying from ~110 to 130 in rat brain (24), similar to our results (Fig. 3). The broader band in mammalian tissues is due to posttranslational phosphorylation, glycosylation, or both (24).

Immunohistochemistry showed 3+ staining for Kv1.5 and Kv2.1 channels in PSMCs (Fig. 4). Antibodies against GIRK-1, a G-protein-coupled muscarinic Kir channel (17), labeled the SMC layer of the airways (3+) but only faintly stained PSMCs (1+, data not shown). Kv2.1 and 1.5 were strongly present in bronchial smooth muscle (4+). There were marked differences in the pattern of distribution of the channels, both in terms of their cellular and subcellular distribution. While Kv2.1 was only in the media of the PA, Kv1.5 was found in the endothelium as well (2+). Kv1.5 also had a unique pattern of staining vascular SMCs, not seen with Kv2.1. In PSMCs, as well as systemic arteries, Kv1.5 marks the nuclear membrane very intensely; in contrast, Kv2.1 has a homogeneous distribution, consistent with localization to the plasmalemma. In addition to their presence in PSMCs, both Kv channels are found in the bronchial smooth muscle, alveolar epithelium, and parenchyma (Fig. 4). Kv2.1 and Kv1.5 were also found in coronary, cerebral, and renal arteries (Fig. 5).

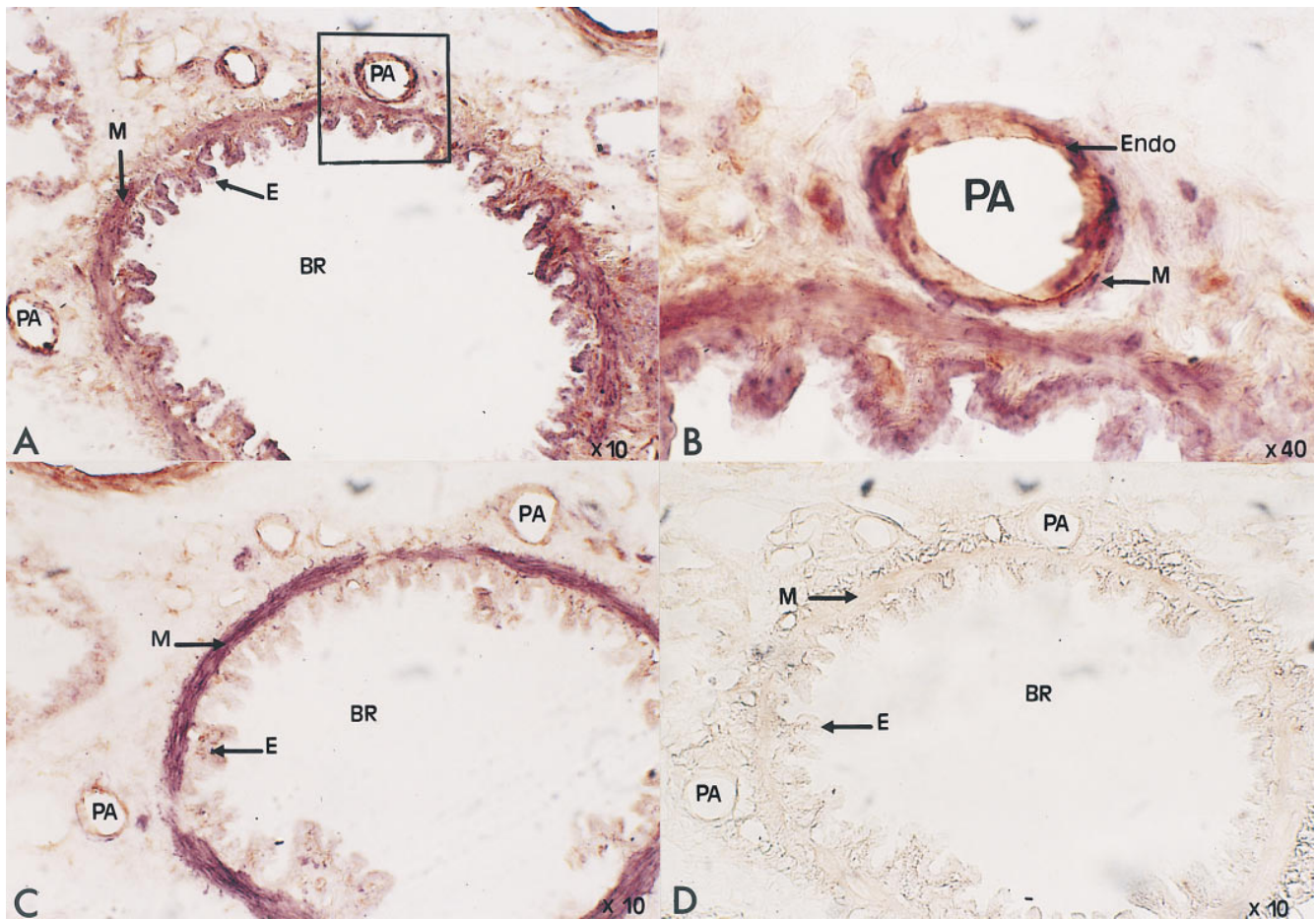
**Electrophysiology.** Anti-Kv1.5 and anti-Kv2.1 rapidly inhibited  $I_K$ , whereas anti-GIRK had no effect, compared with control cells observed for the same period of time without antibody (Fig. 6). The current inhibition onset within 2 min and was stable at ~8 min. Larger doses of antibody had more profound effects on current but could not be used since they promoted leakage of current within several minutes. The inhibition of  $I_K$  by anti-Kv2.1 was physiologically relevant, as it caused membrane depolarization (Fig. 6). Anti-Kv1.5 tended to depolarize  $E_m$  but this did not achieve statistical significance (Fig. 6).

**Effects of anti-Kv antibodies on vasoconstriction and  $[Ca^{2+}]_i$ .** Hypoxia and 4-AP caused vasoconstriction in PA

rings and isolated lungs, respectively (Fig. 7). Both stimuli caused a rapid increase in  $[Ca^{2+}]_i$  in PSMCs (Fig. 7). Incubation of endothelium-denuded, permeabilized PA rings with anti-Kv1.5 diminished 4-AP-induced constriction, without reducing phenylephrine-induced vasoconstriction or elevating basal tension (Fig. 7). Anti-Kv1.5 attenuated both hypoxia- and 4-AP-induced increases in  $[Ca^{2+}]_i$  in PSMCs, without altering the response to AII. There was a trend toward elevated basal  $[Ca^{2+}]_i$  in the anti-Kv2.1-treated cells (control  $71 \pm 5$  nM, anti-Kv2.1  $92 \pm 13$  nM, anti-Kv1.5  $87 \pm 7$  nM,  $P = 0.2$ ). The  $[Ca^{2+}]_i$  after 4-AP was higher in control and anti-Kv2.1- versus anti-Kv1.5-treated cells ( $231 \pm 22$ ,  $217 \pm 17$ , and  $115 \pm 13$  nM, respectively,  $P < 0.01$  Kv1.5 value differs from other groups,  $n = 5$ /group). A similar effect was seen with hypoxia where the  $[Ca^{2+}]_i$  was higher in control and anti-Kv2.1- versus anti-Kv1.5-treated cells ( $149 \pm 11$ ,  $178 \pm 28$ , and  $113 \pm 5$  nM,  $P < 0.05$  values differ from control,  $n = 5$ /group). In contrast, the  $[Ca^{2+}]_i$  after AII did not differ among groups:  $271 \pm 29$ ,  $286 \pm 21$ , and  $266 \pm 19$  nM, respectively.

Anti-Kv1.5 caused a progressive inhibition of HPV in isolated lungs beginning within 30 min. Constriction to AII was not altered and normoxic PA pressure did not increase (Fig. 7).

Incubation of denuded, permeabilized PA rings with anti-Kv2.1 increased basal tension and diminished the constrictor effect of 4-AP (Fig. 2). Resting tension was set at 800 mg in all rings but after 20 min it was greater in the anti-Kv2.1 group ( $871 \pm 47$  mg) than control ( $710 \pm 53$  mg) or anti-Kv1.5 ( $749 \pm 22$  mg),  $P < 0.05$ . Anti-Kv2.1 also elevated basal pressure in lungs but its effects on HPV could not be accurately assessed since most lungs went into edema within 15 min after administration of the antibody (Fig. 7). Edema was never seen with anti-Kv1.5 or vehicle, but is not infrequent with administration of 4-AP. As opposed to the patch-clamp experiments, where the antibodies were delivered to the  $K^+$  channels rapidly by dialysis of the intracellular space, in the calcium experiments the antibod-



**Figure 4.** Immunolocalization of Kv1.5 and Kv2.1 in the rat lung. Adjacent slices of isolated, perfused rat lung were stained for either endothelial nitric oxide synthase (NOS3, to identify the endothelium) and Kv1.5 or NOS3 and Kv2.1. The rose color indicates the Kv channel and the brown the NOS3. These experiments were performed in three animals and the pictures are representative. (A) Kv1.5 is apparent in PAs (3+), large and small, and bronchi (4+) (smooth muscle and epithelium). (B) Enlargement of the small artery seen in A. Note the Kv1.5 positive media with prominent staining of the nucleus. (C) Kv2.1 is detectable in PASMCS (3+) but is even more abundant in the airway SMC (4+). (D) The control shows an absence of nonspecific staining with the secondary antibody in the absence of the primary antibody. BR, Large bronchus; Endo, endothelium; E, epithelium; M, muscle. Magnification is indicated in the lower right corner of each panel.

ies entered the permeabilized cells slowly, over 1–2 h. This may account for the lack of a statistically significant elevation of  $[Ca^{2+}]_i$  in response to the antibodies.

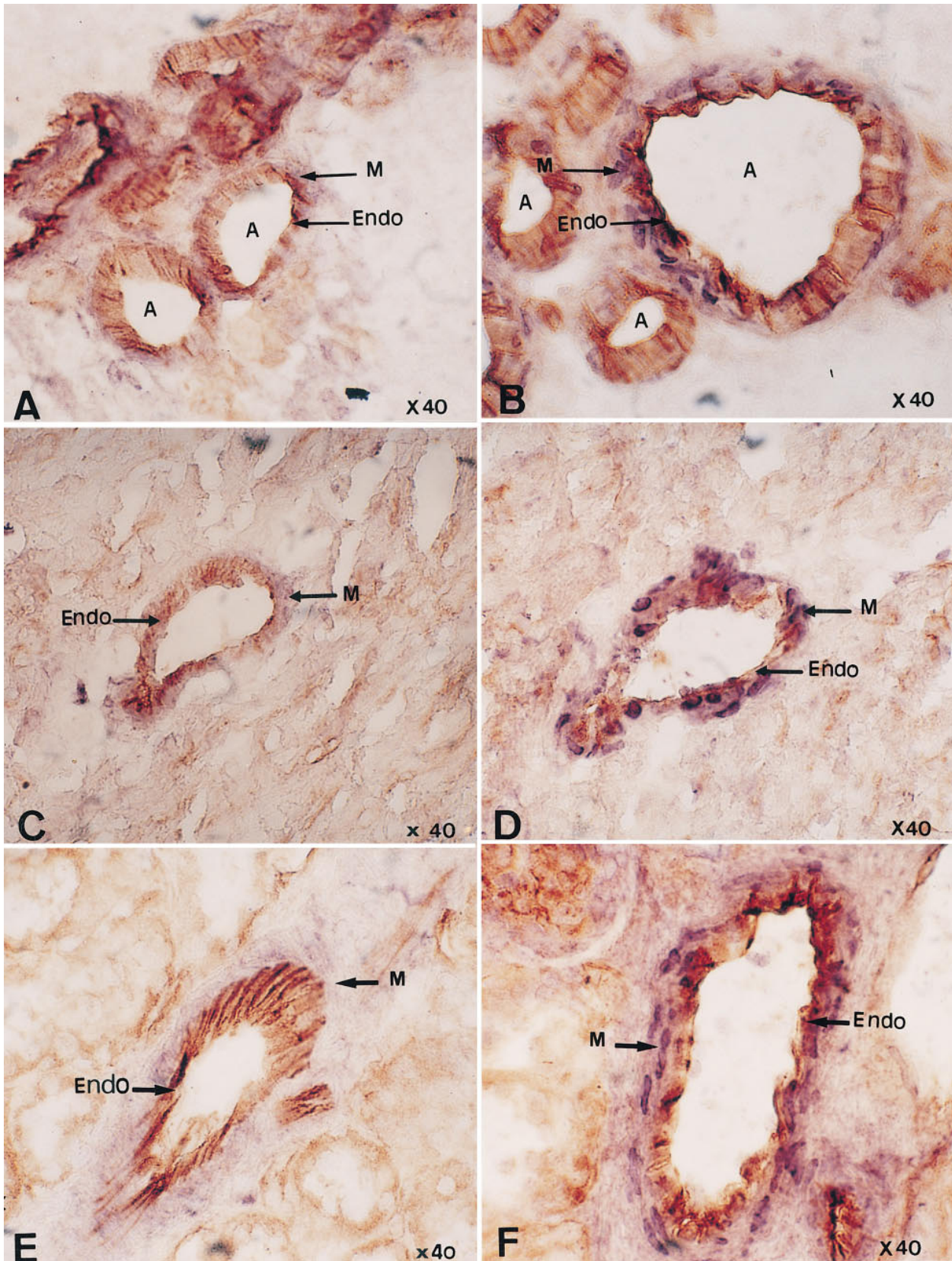
## Discussion

The primary finding of this study is that the activity of Kv2.1 and Kv1.5 contributes significantly to  $I_K$  in PASMCS from resistance arteries. Furthermore, the approach of immunological dissection of complex  $I_K$  in mammalian cells is promising as a supplement to conventional pharmacology and biophysics.

The pharmacology of adult PASMCS suggests a central role for a 4-AP-sensitive, CTX-insensitive, Kv channel(s) both in setting resting  $E_M$  and responding to hypoxia (6, 7, 13, 15, 39). This pharmacological profile is similar to the  $O_2$ -responsive Kv channels in the carotid body of adult animals (40), the neuroepithelial body (41), and the ductus arteriosus (29). Based on pharmacology of pure populations of Kv channels studied in expression systems, such as the *Xenopus* oocyte, Kv1.5 and 2.1 meet the pharmacological criteria to be candi-

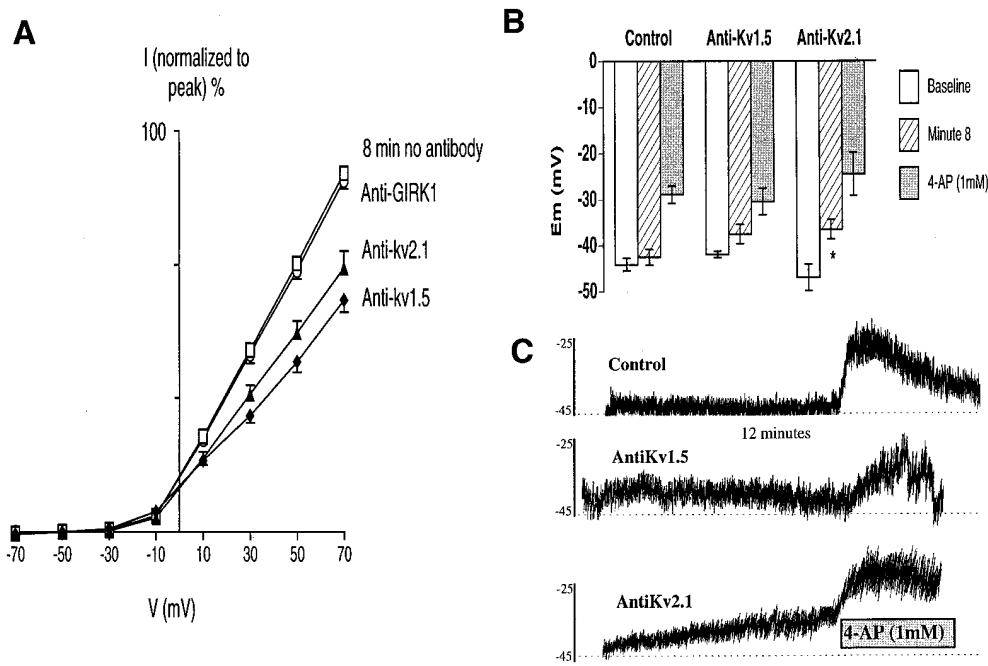
date  $O_2$ -responsive and/or  $E_m$ -regulating Kv channels. Kv2.1 is sensitive to both 4-AP and TEA, but insensitive to CTX (11, 22). Kv1.5 is also sensitive to 4-AP and to a lesser extent is inhibited by quinidine and TEA, but it too is insensitive to CTX (Table I).

*Kv2.1 controls resting  $E_m$  and thereby contributes to low PA pressure.* The molecular, electrophysiological, and physiological studies in this paper consistently indicate that Kv2.1 plays a major role in establishing the resting  $E_m$  of PASMCS from resistance arteries. The importance of a basal efflux of  $K^+$  via this channel is evident in that inhibition of the channel (by a specific antibody) rapidly inhibits  $I_K$  and causes membrane depolarization (Fig. 6). Anti-Kv2.1 also constricted membrane-permeabilized PA rings and elevated pulmonary vascular resistance in isolated lungs, as would be expected of an inhibitor of the Kv channel controlling  $E_m$  (Fig. 7). This is consistent with a recent report by Patel et al. in which they identified Kv2.1 as being central to HPV (26). In their study, Kv2.1 gene expression was found in conduit and resistance PASMCS, using RT-PCR. This is consistent with the current



**Figure 5.** Kv1.5 and Kv2.1 are present in cerebral, coronary, and renal arteries. The composite figure shows in the left column (A, C, and E) double-stained slides for Kv2.1 (rose) and NOS3 (brown). In the right column (B, D, and F) is the double staining of the adjacent section for Kv1.5 and NOS3. The staining of the media for both Kv channels was judged 3+ in all arteries. Note the diffuse nature of the staining for Kv2.1 versus the intense staining of the nucleus for Kv1.5. These experiments were performed in three animals and the pictures are representative. Endo, Endothelium; M, media. Magnification is 40-fold.





**Figure 6.** Antibodies against the Kv1.5 and Kv2.1 channels rapidly inhibit  $I_K$  and depolarize the membrane in PSMCs in resistance PAs. (A) Voltage current plot showing the effect of anti-GIRK-1 ( $n = 3$ ), anti-Kv1.5 ( $n = 4$ ), and anti-Kv2.1 ( $n = 4$ ) on  $I_K$  8 min after onset of dialysis. Control cells ( $n = 5$ ) received no antibody. Values are the mean  $\pm$  SEM.  $P < 0.05$  anti-Kv1.5 and anti-Kv2.1 inhibit  $I_K$ . (B and C) Recording of  $E_m$  for  $\sim 12$  min after onset of dialysis with anti-Kv1.5, anti-Kv2.1, or vehicle in the patch pipette. B shows the mean  $\pm$  SEM while C contains representative tracings. Anti-Kv2.1 depolarized the  $E_m$  compared with control ( $P < 0.05$ ), whereas anti-Kv1.5's effects were variable. Although some cells depolarized after anti-Kv1.5, the effect overall did not achieve statistical significance.

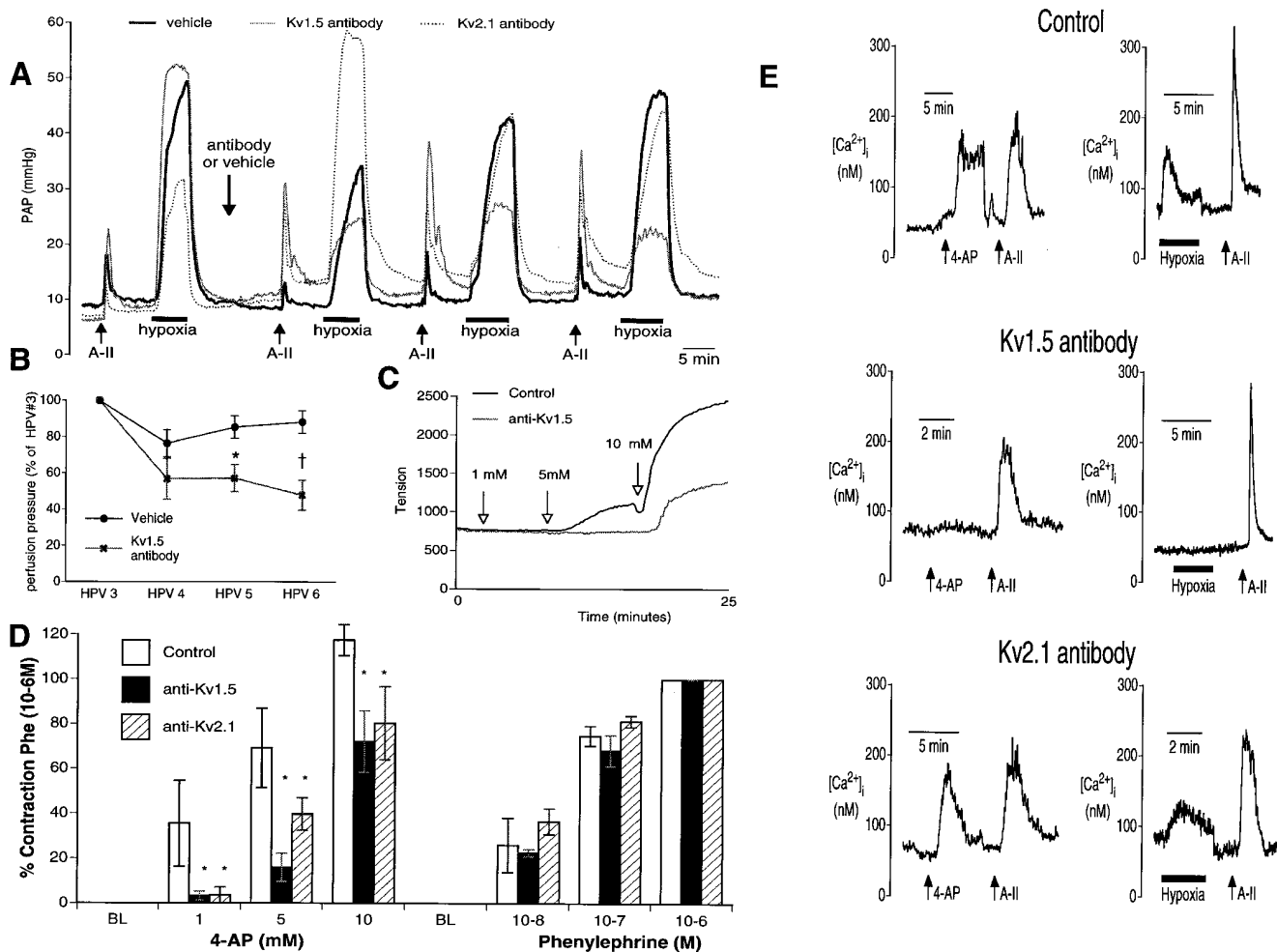
study, in which Kv2.1 protein was found in resistance and conduit PAs using immunohistochemistry and immunoblotting (Figs. 3 and 4). It is noteworthy the anorexigen dexfenfluramine, which constricts the PA in part by inhibiting a Kv channel causing membrane depolarization (42), inhibits Kv2.1 channels (26).

Anti-Kv2.1 diffusely stains the arterial media but not the endothelium. Anti-Kv1.5, present in both cell types, diffusely stains the SMC, indicating surface localization, but also intensely stains the nuclei (Figs. 4 and 5). In contrast, Kv2.1 is absent from the nuclei, consistent with the finding that cells transfected with Kv2.1 show only surface labeling with no intracellular pools of retained protein (24). The significance of the differences in the intracellular localization of the Kv1.5 channel protein is unknown. Specificity of Kv2.1 staining was proven by demonstrating that coincubation with the soluble antigen against which the antibody is directed eliminated staining, whereas mismatched antigens had no effect (Fig. 1). Immunoblotting of freshly isolated PSMCs and primary cultures of PSMCs demonstrated the expected single, 110-kD band for Kv2.1 (25). Several standards were selected for comparison of immunoblots because of the well-established presence of Kv2.1 and Kv1.5, rat brain (25), and GH3 cells (33). Both antibodies produced similar bands in the various PSMC preparations as occurred in the brain and GH3 cells (Fig. 3). This is consistent with the recent report that Kv1.5 and Kv2.1 are present in cultured PSMCs (27). In addition to the demonstrated immunological specificity, discussed above, controls in the electrophysiological studies were included to demonstrate the specific effects of the antibodies. In patch-clamp studies, the effects of anti-Kv2.1 were not mimicked by the administration of anti-GIRK-1, an inward rectifier channel which is not present in PSMCs on immunohistochemistry (Fig. 6). Furthermore, several groups have failed to find inward rectifying currents in PSMCs (5, 6). The fact that nei-

ther anti-GIRK-1 nor vehicle (dialyzed azide or saline) reduced  $I_K$  indicates the current inhibition caused by anti-Kv2.1 is not a nonspecific effect of intracellular administration of an antibody nor due to current run-down. Furthermore, even though anti-Kv1.5 inhibited  $I_K$ , it did not consistently depolarize the plasma membrane. This again indicates a special role for Kv2.1 in regulation of resting  $E_m$ . The inhibition caused by Kv2.1 was dose dependent (over the range of 1:25–1:250) and increased from 0% at 10 s to a plateau of  $\sim 50\%$  at 8 min.

Although it is beyond the scope of this study to define precisely how the binding of the antibody to the carboxyl terminus of the channel leads to channel inhibition, we hypothesize that this relates to the close proximity of the carboxyl terminus to the pore-forming regions between S5 and S6. Perhaps, the binding of a large antibody causes allosteric interference and obstruction of the pore. Certainly the anti-Kv2.1 is binding to the carboxyl terminus as coincubation of the antibody with the epitope from this region, against which it was raised, neutralizes the antibody (Fig. 1).

Since Kv2.1 was selected as a candidate channel based in part on its sensitivity to 4-AP, it would be expected that the sensitivity of the SMC to 4-AP might be diminished if the antibody occupied the carboxyl-terminal epitope. Diminished 4-AP sensitivity to PA rings was indeed noted in permeabilized, endothelium-denuded PA rings treated with anti-Kv2.1 but not the permeabilizing agents alone (Tween + BSA). The diminished sensitivity to 4-AP did not reflect toxicity of the permeabilizing regimen, as the response to phenylephrine was preserved (Fig. 7). It is also interesting that anti-Kv2.1 consistently elevated PA pressure in isolated rat lungs and lead to development of edema over 30–40 min. Although this may have been exacerbated by the use of a nitric oxide synthase inhibitor in the perfusate, anti-Kv1.5 at the same concentration did not alter basal PA pressure nor cause edema. In this regard, anti-Kv2.1 mimics the effects of 4-AP which also elevates PA pres-



**Figure 7.** Inhibitory effects of anti-Kv antibodies on vasoconstriction caused by hypoxia and 4-AP in isolated rat lungs and PA rings. (A) Representative trace showing the PA pressure in isolated lungs treated with anti-Kv1.5, anti-Kv2.1, or vehicle. Anti-Kv1.5 inhibited HPV compared with control. Unlike anti-Kv1.5, anti-Kv2.1 raises the baseline PA pressure. (B) Anti-Kv1.5 inhibits HPV. The period before and three periods after the antibody administration are shown. Values are expressed as a percentage of the magnitude of the HPV in the period just before the intervention (antibody vs. vehicle); mean  $\pm$  SEM,  $n = 4$ . \* $P < 0.05$  and  $^{\dagger}P < 0.01$ . (C) Representative traces showing a diminished sensitivity to 4-AP in membrane-permeabilized PA rings after incubation with anti-Kv1.5 versus control (rings incubated with Tween and BSA alone). (D) Mean  $\pm$  SEM of four experiments comparing the effect of anti-Kv1.5 and anti-Kv2.1 on constriction caused by 4-AP or phenylephrine versus vehicle. Both anti-Kv antibodies diminished constriction to 4-AP without impairing phenylephrine-induced constriction. Values are expressed as the mean  $\pm$  SEM of the percentage of the maximal constriction caused by phenylephrine,  $10^{-6}$  M. (E) Representative experiments (chosen from  $n = 6$ /group) showing the rise in  $[Ca^{2+}]_i$  caused by hypoxia ( $PO_2 \sim 40$  mmHg), 4-AP (6 mM), or AII ( $1 \mu g$ ) in primary culture PSMCs. Note the impaired rise in  $[Ca^{2+}]_i$  in response to hypoxia or 4-AP in the anti-Kv1.5-treated cells. Neither antibody interfered with the rise in  $[Ca^{2+}]_i$  caused by AII.

sure and tends to lead to edema at pressures well below those tolerated with other vasoconstrictors.

**Kv1.5 channels are involved in HPV.** Like Kv2.1, Kv1.5 is present in the PSMCs (Fig. 4). However, Kv1.5 is also present in the endothelium (Fig. 4). The relative importance of endothelial versus smooth muscle Kv1.5 is uncertain. However, the inhibition of the effects of hypoxia and 4-AP was demonstrated in PASM and PA rings denuded of endothelium, suggesting that the channels in the SMC are important for the  $O_2$  responsiveness. The current report of Kv1.5 in PSMCs concurs with several recent studies (27, 43), although it disagrees with the findings of Patel et al. Whereas Patel used PCR to identify the gene product, we measured the protein it-

self. The concordance among reports from several other labs (27, 28, 43) and our findings (Figs. 3 and 4) provides relatively conclusive evidence that Kv1.5 is present in PSMCs. The reason for the discrepancy with Patel et al. is unclear.

There are significant differences between the function of Kv1.5 and Kv2.1. Most notably, anti-Kv1.5 does not consistently depolarize the PASM and never constricted PA rings or raised pulmonary vascular resistance in isolated lungs. Together these indicate the Kv1.5 does not set the resting  $E_m$ . However, Kv1.5 is important in the effector response to acute hypoxia. In permeabilized cells, anti-Kv1.5 selectively inhibits the rise in  $[Ca^{2+}]_i$  caused by hypoxia and 4-AP. Anti-Kv1.5 also reduces HPV in the isolated rat lung. This is not a nonspe-

cific effect of permeabilization or antibody administration as the effects of an unrelated constrictor, AII, on tone and calcium were unaltered (Fig. 7).

It remains controversial whether there are separate sensor and effector mechanisms for hypoxia. Immunohistochemistry of kidney, brain, and heart confirms that Kv1.5 and Kv2.1 are present in the arteries of all these organs (Fig. 5). Most of these systemic vessels are either not O<sub>2</sub>-responsive or respond to hypoxia with vasodilatation. This suggests that although Kv1.5 and Kv2.1 contribute to the effector mechanism of HPV, the channel itself is not the O<sub>2</sub> sensor. With regard to the sensing of O<sub>2</sub>, it is noteworthy that Duprat et al. found Kv1.5 but not 2.1 was susceptible to redox modulation (44). Furthermore, Ospienko et al. found the O<sub>2</sub>-sensitive Kv channel in rabbit PA was sensitive to quinine (16), a property shared by Kv1.5. Thus, the Kv1.5 channel is well suited to fulfill the characteristics of an O<sub>2</sub>-sensitive Kv channel (21, 43).

How is Kv1.5 involved in HPV if it does not set the E<sub>m</sub> itself? Although this requires further study we hypothesize that Kv2.1 is primarily inhibited by hypoxia. Since Kv2.1 controls the resting E<sub>m</sub>, the hypoxic depolarization caused by Kv2.1 block shifts the E<sub>m</sub> into a range where Kv1.5 is active. Hypoxia may then inhibit Kv1.5, augmenting the inhibition of I<sub>K</sub>.

To demonstrate a link between the effects of the antibody on I<sub>K</sub> and E<sub>m</sub> and the effects on tone, we studied the ability of 4-AP or hypoxia to raise [Ca<sup>2+</sup>]<sub>i</sub> in primary cultures of PSMCs. The permeabilization with Tween and BSA, or Triton, has been demonstrated previously to allow uptake of large antibodies into the cell (24, 38, 45). The disadvantage of permeabilization is the lengthy period needed to permeabilize intact tissues (2–3 h for PA rings) and the presumably more gradual accumulation of lower quantities of antibody. These constraints are not suitable for patch-clamp studies, where study duration is limited, but are acceptable for use with PA rings and cultured cells. Because the uptake of the antibody necessitated 1–2 h of preincubation, acute effects of the antibody on basal [Ca<sup>2+</sup>]<sub>i</sub> are difficult to assess. Any elevation in [Ca<sup>2+</sup>]<sub>i</sub> resulting from depolarization, whether due to 4-AP or hypoxia, tends to roll off within minutes (Fig. 7), despite persistence of the stimulus. This probably reflects the many homeostatic mechanisms (pumps and sinks) which tightly modulate [Ca<sup>2+</sup>]<sub>i</sub>. Consequently, it is not surprising that neither antibody significantly elevated [Ca<sup>2+</sup>]<sub>i</sub> when given in the 2 h before measurement by slow permeabilization.

**Limitations.** This study did not examine all possible O<sub>2</sub>-sensitive K channels. In addition to those excluded based on sensitivity to CTX or glyburide, we did not examine several other possible channels. The CTX-insensitive channels Kv1.1 (K<sub>d</sub> for 4-AP 290 μM vs. 0.3 mM for TEA) and Kv3.1 (K<sub>d</sub> for 4-AP 29 μM vs. 0.2 mM for TEA) (11) have the requisite pharmacological profile to be considered for future examination. Furthermore, the strategy used to select candidate channels (appropriate current kinetics and pharmacological sensitivity of the channel in expression systems), although a reasonable starting point, may be incomplete. β subunits of K<sup>+</sup> channels can modify current kinetics and also inactivation (32, 46). In addition, Kv channels are tetramers and the α subunits can assemble as heterotetramers (47). These heterotetrameric channels (which could include Kv1.5 or Kv2.1 subunits) may lead to intermediate pharmacological and biophysical properties.

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