Expression and Function of Voltage-dependent Potassium Channel Genes in Human Airway Smooth Muscle

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Patch clamp and RNA-polymerase chain reaction methods were used to determine the expression of voltage-dependent potassium channel currents and mRNAs in human airway smooth muscle cells, and tension measurements were used to examine the functional role of specific potassium channel gene products in human bronchial smooth muscle. RNA from airway smooth muscle tissue revealed the presence of Kv1.2 (11 kilobases (kb)) and Kv1.5 (3.5 and 4.4 kb) transcripts, as well as Kv1.1 mRNA (9.5 kb), which has not previously been reported in smooth muscle; transcripts from other gene families were not detected. RNA-polymerase chain reaction from cultured human myocytes confirmed that the identified transcripts were expressed by smooth muscle cells. The available voltage-dependent potassium current in human airway myocytes was insensitive to charybdotoxin (200 nM) but blocked by 4-aminopyridine. Dendrotoxin (1–300 nM; inhibits Kv1.1 and Kv1.2 channels), charybdotoxin (10 nM to 1 μM; inhibits Kᵥ1.2 channels), and glybenclamide (0.1–100 μM; inhibits Kᵥ1.5 channels) had no effect on resting tone. Conversely, 4-aminopyridine increased resting tension with an EC₅₀ (1.8 μM) equivalent to that observed for current inhibition (1.9 μM). Human airway myocytes express mRNA from several members of the Kv1 gene family; the channel that underlies the predominate voltage-dependent current and the regulation of basal tone appears to be Kv1.5.

Recent molecular studies have identified a superfamily of voltage-dependent potassium channel genes and characterized specific gene subfamilies (see Ref. 10, 11 for review). Two members of the Kv1 potassium channel gene family, Kv1.2 and Kv1.5, have been reported in gastrointestinal smooth muscle (12, 13) and aortic tissue (14). In order to determine the specific genes that are expressed in airway smooth muscle cells and to determine the specific gene products associated with the regulation of membrane potential, mRNA specific for candidate potassium channels was examined using RNA-PCR and Northern analysis. Studies were performed using RNA isolated from cultured myocytes as well as muscle segments, in order to demonstrate that channel mRNA expression occurred in smooth muscle cells. We demonstrate that airway myocytes express Kv1.2 and Kv1.5 mRNA, as well as Kv1.1 mRNA, which has not previously been demonstrated in smooth muscle cells. We also used peptide toxins and potassium channel antagonists selective for specific channel subtypes to determine the gene products that play an essential role in the control of human bronchial smooth muscle tone.

EXPERIMENTAL PROCEDURES

Preparation of mRNA and Northern Blots—Cultured cells were obtained by dissociation of dissected smooth muscle segments, as described previously (15, 16). Total RNA was extracted from equine trachealis and from subcultured human tracheobronchial myocytes (3rd passage) (17); poly(A)⁺ RNA was isolated on oligo(dT)-cellulose. For Northern analysis, 5 μg of poly(A)⁺ RNA or 15 μg of total RNA was resolved by electrophoresis through a 1% agarose gel and capillary blotted onto GeneScreen Plus membrane (DuPont NEN) with 20 × SSPE. The membranes were baked for 60 min at 65 °C and incubated at 55 °C in prehybridization solution (20% formamide, 6 × SSPE, 10 × Denhardt’s reagent, 2% SDS, 200 μg/ml sonicated salmon sperm DNA). Random 32P-labeled cDNA probes (10⁶ cpm/ml) were added and hybridized for an additional 20 h. Membranes were subsequently washed at high stringency (twice in 0.2 × SSPE, 0.1% SDS at 55 °C) and autoradiography performed at ~80 °C with Kodak X-AR film and intensifying screens.

To generate probes, unique 3’ ends of rat potassium channel clones were amplified by PCR and cloned with the Eukaryotic TA Cloning kit (Invitrogen). RK1 and RK2 clones were kindly provided by Dr. M. Tamkun (Vanderbilt University School of Medicine) and the Kv1 clone by Dr. R. Swanson (Merck Sharp & Dohme Research Labs.). Specific probe sequences are described below.

Synthetic Oligonucleotides—Synthetic oligonucleotides were prepared on a DNA synthesizer (Applied Biosystems). For Kv1.1, the sense oligonucleotide primer was 5’-ATCTTCCAA(A/G)CTCTCCGGCACTCT-CAA(A/G)GG-3’; the antisense primer was either 5’TGTGTTTTTAACTCGGT-3’ (predicted PCR fragment size 591 bp) or 5’-GATGGTCCATTCAATCTCCA-3’ (predicted size 479 bp). The unique internal probe was 5’-CTTAGCCCTGACAGTGACC-G-3’. For Kv1.2, the sense oligonucleotide primer was 5’-ATCCTGGCCGACTATCC-3’.
d oligonucleotide was 5’-TACATGGGAGATACAGG-3’; the antisense was 5’-ATATTCGTGTCTCAAATCA-3’. The probe was 5’TGCAGAACATTGCTTTTC-3’. The predicted size for the PCR fragment was 294 bp.

RNA-PCR—cDNA was made from DNase I (Boehringer Mannheim)-treated total cellular RNA with Superscript II reverse transcriptase (Life Technologies, Inc.) as recommended. As a control, duplicate RNA samples were incubated under identical conditions but without reverse transcriptase. Samples were boiled for 10 min immediately prior to PCR. Amplifications were performed in the supplied buffer and 2.0 mM MgCl2 with Taq polymerase in a DNA thermal cycler-480 (Perkin-Elmer); PCR cycle parameters were 2 min at 94°C, 1.5 min at 50°C, and 1.5 min at 72°C for 30 cycles. In some cases a second amplification was performed with 1 μl of the first reaction. PCR products were separated by electrophoresis on a 4% agarose gel (NuSieve, FMC) in 1 × TAE buffer (Tris, acetic acid, EDTA) and stained with ethidium bromide.

The RNA-PCR products were transferred by capillary blotting with 0.4 M NaOH, 1 M NaCl onto nylon membrane (GeneScreen Plus, DuPont NEN). Blots were prehybridized at 50°C (6 × SSC, 2% SDS, 5 × Denhardt’s, and 200 μg/ml sonicated salmon sperm DNA). Internal oligos (see above) were 5’-end-labeled with T4 kinase (Life Technologies, Inc.), added to prehybridization solution (106 cpm/ml), and incubated with the membranes for 18 h at 50°C. Blots were washed 2 times with 2 × SSC and 0.2% SDS at 50°C and then for 20 min in 0.2 × SSC and 0.2% SDS at 50°C.

Cell Dissociation and Electrophysiology—Tracheal and bronchial (mainstem) myocytes were isolated from lungs of organ donors obtained from International Institute for the Advancement of Medicine (Exton, PA) and National Disease Research Interchange (Philadelphia, PA) as described previously (16). Single dispersed human tracheal myocytes were voltage-clamped using the nystatin perforated-patch method at room temperature or 35°C as described previously (9). The pipette solution contained (in mM) 80 potassium aspartate, 50 KCl, 5 MgCl2, 3 EGTA, 1 CaCl2, 10 HEPES, pH 7.34 (KOH). The bath solution contained 125 NaCl, 5 KCl, 1 MgSO4, 10 HEPES, 1.8 CaCl2, pH 7.40 (NaOH).

Contraction Studies—Human lung tissue from organ donors was obtained within 24 h of removal from IIA and NDI. First to fifth generation bronchial strips were dissected free of lung tissue, placed in 10-ml organ baths containing Krebs-Henseleit solution, and force measurements made as described previously (18). Experiments were conducted in the presence of tetrodotoxin (TTX, 1 μM) and atropine (1 μM) to minimize the influence of released neurotransmitters, including acetylcholine. Responses for each tissue were expressed as a percentage of the reference contraction obtained at the start of the experiment. Geometric mean EC50 values (pD2 values) were calculated from linear regression analyses of the data. Statistical analysis was conducted using analysis of variance or two-tailed Student’s t test for paired samples where appropriate, with a probability value less than 0.05 regarded as significant.

Chemicals—Carbachol, atropine, glybenclamide, tetrodotoxin, 4-aminopyridine, and 3,4-diaminopyridine were obtained from Sigma; drotoxin I (59-amino acid peptide) was obtained from Calbiochem.

RESULTS

Voltage-dependent Potassium Channel Gene Expression in Airway Smooth Muscle—Northern analysis of mRNA from equine trachealis tissues using K+ channel subtype-specific probes revealed the presence of transcripts of the appropriate size for Kv1.1, Kv1.2, and Kv1.5 (Fig. 1). Using a unique fragment 3’ to the 56 hydrophobic domain of Kv1.1 (bp 1292–1495 of the open reading frame; accession number M26161) as a probe, a single transcript of approximately 9 kb was identified, similar to previous reports for expression in other tissues (19). Similarly, the analogous region of Kv1.2 (bp 1372–1666; accession number X16003) identified an 11-kb fragment, consistent with the size of the major transcript of that channel (14). The use of a unique Kv1.5-specific probe from the same region (bp 1886–2101; accession number M31745) resulted in the identification of two major transcripts of approximately 3.3 and 6.7 kb. Kv1.5 splice variants of these approximate sizes have previously been reported in mouse cardiac myocytes (20). Conversely, specific probes for Kv1.6 and Kv3.1 did not hybridize to the same mRNA in repeated experiments.

We confirmed that channel message is expressed by smooth muscle cells using mRNA from cultured human myocytes. As shown in Fig. 2, Northern analysis using the Kv1.5-specific probe revealed the presence of the previously identified transcripts. RNA-PCR analysis using channel subtype-specific primers for Kv1.1 and Kv1.5 and human mRNA also resulted in the amplification of the predicted size DNA (Fig. 3A); the identity of the amplified cDNA was confirmed by hybridization with internal oligonucleotide primers specific for Kv1.1 and Kv1.2 (Fig. 3B).

Using the same and other batches of mRNA from human cells or equine tissue, specific primers designed for Kv1.3, Kv1.4, Kv1.6, Kv3.1, and all known Kv2 and Kv4 family members did not result in RNA-dependent amplification. Positive controls were performed to verify that the probes identified channel transcripts in human tissue.

Voltage-dependent Potassium Channel Currents in Human Airway Myocytes—Potassium currents were studied in freshly dispersed human tracheal myocytes using the perforated patch clamp technique to determine the available current in nonadherent cells. Fig. 4A shows typical voltage-dependent potassium currents recorded from a human tracheobronchial myocyte. Depolarizing voltage steps from a holding potential of −80 mV evoked outward potassium currents with activation and inactivation characteristics quite similar to our previous measurements of delayed rectifier potassium currents in animal airway myocytes (7–9). Delayed current activation as well as time-dependent current inactivation were consistent features of the
currents (n = 23), although the extent of current inactivation varied substantially. Fig. 4B shows a current-voltage plot of conductance as a function of clamp potential for a cell recorded at 35°C. The current threshold was negative to −40 mV consistent with a role in the maintenance of membrane potential in human cells (∼45 mV (21)). The average potassium current was 20.1 ± 3.4 pA at −40 mV (35°C, n = 5); complete blockade of such a current would be expected to produce a depolarization of approximately 20 mV, given the typical input resistance of the cells (approximately 1 GΩ). The voltage dependence of conductance in human cells was quite similar to previous reports for ferret airway myocytes recorded under identical conditions (9); peak conductance for the experiment shown was 7.2 nS and the potential at which half-maximal conductance was obtained was 16.1 mV. The average current density at 40 mV for cells recorded at 35°C was 15.3 ± 1.6 μA/cm² (n = 5).

Results

The delayed rectifier current was characterized pharmacologically by the application of 4-aminopyridine (4-AP) 3,4-diaminopyridine, and charybdotoxin, which blocks large-conductance calcium-activated potassium (K<sub>Ca</sub>) channels and Kv1.2 channels (22). Fig. 5 shows an example of a cumulative dose-response experiment in which potassium currents were evoked by voltage-clamp steps during exposure to successive concentrations of 4-AP. 4-AP (0.5–6 mM) blocked outward currents in a dose-dependent fashion, with a calculated EC<sub>50</sub> for contraction (Fig. 7). The increase in tone produced by 4-AP was 1.8 mM (4-AP) at 35°C (5). Using the response to 10 mM 4-AP as 100%, the EC<sub>50</sub> for contraction was 18.2% (n = 6). In contrast to the effects of 4-AP, glybenclamide, an ATP-sensitive potassium channel inhibitor (0.1–100 μM), charbdotoxin (10 nM to 1 μM), and dendrotoxin (1 nM to 0.3 μM; inhibits Kv1.1 and Kv1.2, see Ref. 21) did not contract human bronchial muscle segments (n = 4–6; Figs. 6 and 7), whereas addition of 4-AP (10 mM) resulted in a marked contraction (Fig. 7). The increase in tone produced by 4-AP was likely due to depolarization and the opening of voltage-depend-
ent calcium channels, since tension development to 1 mM 4-AP was completely reversed by nicardipine (1 μM, Fig. 8). In four additional experiments addition of 1 μM nicardipine produced 66.7 ± 36.1% relaxation of the contraction produced by 10 mM 4-AP.

**DISCUSSION**

The electrical activity of smooth muscle cells is in large part determined by the potassium conductance of the membrane (28, 29). Thus the resting membrane potential (29, 30), the passive spread of postsynaptic potentials (31), the spiking activity of the cell (1, 32), and the outward rectification of the cell membrane (8, 23, 33) are directly associated with the membrane potassium conductance of the myocyte. An impressive diversity exists in the type of potassium channels expressed in smooth muscle, including voltage-dependent (8, 23, 24), calcium-activated (2, 34–37), ATP-sensitive (22), and inwardly rectifying channels (40, 41). Each of these potassium-selective ion channels belongs to a distinct gene family, and the selective expression of individual members of these families likely underlies the substantial diversity in electrical activity observed in different smooth muscle tissues.

The present studies were designed to determine the specific potassium channel genes expressed in airway myocytes and the degree to which these channels regulate tone in human bronchial smooth muscle. Northern analysis using subtype-specific probes revealed the presence of transcripts encoding three channels of the shaker, or Kv1, family in mRNA isolated from equine trachealis. Kv1.1, Kv1.2, and Kv1.5 mRNAs were detected in dissected airway smooth muscle segments, and confirmed with PCR in cultured human tracheobronchial myocytes. Previous studies have indicated the expression of Kv1.2 (12) and Kv1.5 (13) mRNA in gastrointestinal smooth muscle, and Northern analysis has indicated the presence of Kv1.1, Kv1.5, and Kv2.1 transcripts in DNA isolated from aorta (14). We exploited the sensitivity of Kv1.1 and Kv1.2 channels to the peptidyl snake toxin dendrotoxin (22) to examine the role of these channels in determining the resting membrane potential and hence tone in isolated human bronchial rings. Dendrotoxin (up to 0.3 μM) had no effect on the resting tone, and similar results were obtained with charbdotoxin and glybenclamide (Fig. 6). Conversely, 4-aminopyridine, which blocks Kv1.5 (as well as Kv1.1 and Kv1.2, see Ref. 22) channels, resulted in a marked contraction. Moreover, the EC50 for the production of tone in human bronchial segments (1.8 mM) was quite similar to the Ki for current block at negative membrane potentials (1.9 mM at −20 mV). The contractile response induced by 4-AP was reversed by nicardipine, indicating that it is mediated predominantly by influx of extracellular Ca2+ through voltage-dependent calcium channels.

Our results suggest that homomultimeric Kv1.1 and Kv1.2...
channels do not play an appreciable role in the maintenance of myocyte membrane potential. It should be acknowledged that heteromultimers form between α subunits within this gene family and that all four α subunits interact to form a high affinity dendrotoxin binding site (44), so that we can not exclude the participation of Kv1.1 or Kv1.2 peptides in formation of heterotetramers.

Taken together, these results suggest that Kv1.5 potassium channels regulate resting membrane potential in human bronchial myocytes and extend our previous studies demonstrating the participation of Kv1.1 or Kv1.2 peptides information associated with the regulation of human bronchial tone.

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