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Nitric oxide sensitivity in pulmonary artery and airway smooth muscle: a possible role for cGMP responsiveness

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Taniguchi, Miwa, Young Lan Kwak, Keith A. Jones, David O. Warner, and William J. Perkins. Nitric oxide sensitivity in pulmonary artery and airway smooth muscle: a possible role for cGMP responsiveness. *Am J Physiol Lung Cell Mol Physiol* 290: L1018–L1027, 2006. First published December 9, 2005; doi:10.1152/ajplung.00402.2005.—We aimed to assess intrinsic smooth muscle mechanisms contributing to greater nitric oxide (NO) responsiveness in pulmonary vascular vs. airway smooth muscle. Porcine pulmonary artery smooth muscle (PASM) and tracheal smooth muscle (TSM) strips were used in concentration-response studies to the NO donor (*Z*)-1-[*N*-2-aminoethyl-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO). PASM consistently exhibited greater relaxation at a given DETA-NO concentration (NO responsiveness) than TSM NO responsiveness, with DETA-NO log EC_{50} being -6.55 ± 0.11 and -5.37 ± 0.13 for PASM and TSM, respectively ($P < 0.01$). We determined relationships between tissue cGMP concentration ($[cGMP]_i$) and relaxation using the particulate guanylyl cyclase agonist atrial natriuretic peptide. Atrial natriuretic peptide resulted in nearly complete relaxation, with no detectable increase in $[cGMP]_i$ in PASM and only 20% relaxation (10-fold increase in $[cGMP]_i$) in TSM, indicating that TSM is less cGMP responsive than PASM. Total cGMP-dependent protein kinase I (cGKI) mRNA expression was greater in PASM than in TSM (2.23 ± 0.36 vs. 0.93 ± 0.31 amol mRNA/μg total RNA, respectively; $P < 0.01$), but total cGKI protein expression was not significantly different (0.56 ± 0.07 and 0.49 ± 0.04 ng cGKI/μg protein, respectively). The phosphotransferase assay for the soluble fraction of tissue homogenates demonstrated no difference in the cGMP EC_{50} between PASM and TSM. The maximal phosphotransferase activity indexed to the amount of total cGKI in the homogenate differed significantly between PASM and TSM (1.61 ± 0.15 and $1.04 \pm$ pmol·min^{-1}·ng cGKI^{-1}, respectively; $P < 0.05$), suggesting that cGKI may be regulated differently in the two tissues. A novel intrinsic smooth muscle mechanism accounting for greater NO responsiveness in PASM vs. TSM is thus greater cGMP responsiveness from increased cGKI-specific activity in PASM.

cGMP-dependent protein kinase

AIRWAY SMOOTH MUSCLE IS LESS responsive to exogenously administered nitric oxide (NO) and NO donors than the pulmonary arterial circulation (3, 22). In addition, concentrations of inhaled NO or of intravenously administered NO donors, which dramatically reduce pulmonary vascular resistance, have little beneficial effect on airway constriction (14, 18, 21). Our group (22) recently demonstrated that pulmonary artery smooth muscle (PASM) was more sensitive to relaxation by NO than tracheal smooth muscle (TSM) and that this was associated with greater intracellular cGMP levels ($[cGMP]_i$) at a given NO donor concentration. This finding was correlated with greater expression and activity of the enzyme responsible for NO-induced cGMP production, soluble guanylyl cyclase (sGC), in PASM (22). An additional mechanism that could contribute to these observations, an increased cGMP responsiveness in PASM vs. TSM, could not be addressed in those studies because >90% of NO-induced relaxation took place with no detectable increase in steady-state $[cGMP]_i$.

$[cGMP]_i$ plays a variety of roles in the smooth muscle cell, most of them mediated by cGMP-dependent protein kinases (cGK), with the salient cGMP-cGK-mediated mechanism for smooth muscle relaxation related to reductions in myoplasmic Ca^{2+} (10) and in the sensitivity of myofilament contractile proteins to Ca^{2+} (31, 33, 38). Two genes, coding for cGK type I (cGKI) and type II (cGKII), have been identified in mammals (8). cGKII is a membrane-bound protein that is abundantly expressed in lung (16, 43), although its presence in smooth muscle from lung has not been determined. cGKI is a soluble protein that largely exists as a homodimer in tissues and catalyzes transfer of the γ-phosphate from ATP to a consensus sequence found on a variety of proteins (24). The NH_2 terminus of cGKI is encoded by an alternatively spliced exon 1 (30), giving rise to the two cGK isoforms known to exist in smooth muscle, cGKIα and cGKIβ, which have the COOH-terminal 583 amino acids in common (34, 39, 44). The two cGKI isoforms are maximally activated to the same extent by cGMP, but cGKIβ requires 10-fold higher [cGMP] than cGKIα to achieve this degree of activation (23, 36).

Comparison of the cGMP responsiveness between two different tissues using cGK agonists is challenging because the contribution of variable tissue permeability to relaxant effects between tissues is difficult to ascertain. Particulate guanylyl cyclases, such as the natriuretic peptide receptor A, are another enzyme system that increases $[cGMP]_i$ and account for the relaxant effect of atrial natriuretic peptide (ANP) in smooth muscle (9). Previous work has suggested that, although ANP significantly increases $[cGMP]_i$ in TSM, it results in only modest relaxation (33). This suggests the possibility that an endogenous rather than exogenous cGMP source may be used to further explore the possibility of different cGMP responsiveness between PASM and TSM.

In the present study, we determined the effect of ANP on $[cGMP]_i$ and isometric force during continuous contraction of PASM and TSM. We tested the hypothesis that the cGMP responsiveness of PASM is greater than that of TSM because

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of greater cGKI expression and phosphotransferase activity in the former tissue. In the course of these studies, total cGKI mRNA and protein expression and the cGMP concentration dependence of phosphotransferase activity were semiquantitatively determined in these tissues for the first time.

MATERIALS AND METHODS

Tissue preparation. After Institutional Animal Care and Use Committee approval was received, pigs (domestic crossbreds, weight 35–77 kg) were anesthetized with intravenous pentobarbital sodium (100 mg/kg) and exsanguinated by bilateral transection of the carotid arteries. The extrathoracic trachea and lungs were excised and immersed in chilled physiological salt solution with a composition (in mM) of 110.5 NaCl, 25.7 $NaHCO_3$, 5.6 dextrose, 3.4 KCl, 2.4 $CaCl_2$, 1.2 KH_2PO_4, and 0.8 $MgSO_4$. Third-generation pulmonary artery was dissected from the lung parenchyma, cut into rings, and cleaned of adventitia under microscopic observation; the endothelium was then removed by gentle rubbing of the luminal surface with a moist cotton swab. The fat, connective tissue, and the epithelium of TSM were removed with tissue forceps and scissors under microscopic observation to make muscle strips. For isometric force measurements, strips of each type of smooth muscle were prepared of 0.1–0.2 mm width, 1 cm length, and 0.2–0.3 mg wet wt. The NO responsiveness of first- to fourth-generation pulmonary arteries was not significantly different (unpublished observations), and third-generation pulmonary artery was used in these studies because of ease of preparation and better definition of myocyte orientation. Higher generation airways were not used because of difficulties in separating epithelial cells from smooth muscle cells, which was undesirable in the biochemical and expression studies.

Mechanical measurements. Strips were suspended in 5-ml tissue baths filled with physiological salt solution (37°C) aerated with 94% O_2-6% CO_2, pH 7.4. One end of the strips was anchored to a metal hook at the bottom of the tissue bath; the other end was attached to a calibrated force transducer (model FT03D; Grass Instruments/Astro-Med, West Warwick, RI). During a 3-h equilibration period, the strips were repeatedly contracted isometrically using 40 mM KCl and then relaxed. The length of the strips was increased after each contraction-relaxation cycle until active force was maximal (optimal length). All strips were subsequently maintained at optimal length. Relaxed PASM strips were then contracted with norepinephrine (1 μM), and the absence of endothelium was verified by failure of acetylcholine (1 μM) to cause relaxation. Strips were then relaxed again until commencement of a study. Before concentration-response studies, all strips were incubated with 10 μM indomethacin to prevent the formation of prostanoids. In previous studies, contraction of TSM and PASM during incubation with indomethacin had no effect on tissue $[cGMP]_i$ (17).

Concentration-response curves. The concentration responses of TSM and PASM to the NO donor were performed 15 min after an isometric contraction with isotonic KCl. KCl was used rather than receptor agonists to avoid possible differences in the mechanism of contraction associated with different receptor agonist as a contributing factor to differences in NO responsiveness. The NO-donor (*Z*)-1-[*N*-2-aminoethyl-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) was selected because it slowly releases authentic NO (19, 20), giving rise to stable relaxations at a given [DETA-NO] and does not require biotransformation to release NO (27). In another set of experiments, the concentration response to the natriuretic peptide receptor A agonist ANP was determined in both TSM and PASM after KCl-induced contraction. A KCl concentration sufficient to result in a submaximal contraction was used in all cases. This was determined by initially contracting the strip to maximal isometric force with KCl (~40 mM), reducing the bath KCl to 23 mM, which resulted in isometric force that was 50 ± 10% of the maximal value (see Fig. 2). The concentration responses were cumulative for all but ANP in TSM. This was because of the transient nature of the ANP-induced relaxation in TSM contracted with submaximal KCl. Accordingly, each TSM strip received a single concentration of ANP (10^{-10} to 10^{-6} M in single log steps), and the maximal extent of relaxation for a given ANP concentration was recorded.

cGMP measurements. At the specified condition, PASM and TSM strips were frozen with liquid nitrogen and stored in a −70°C freezer. Strips were then homogenized, and the soluble extract was assayed for cGMP using a commercially available RIA kit (Amersham Biosciences, Piscataway, NJ) as previously described (32). The protein concentration in the tissue homogenate was determined by the method described by Lowry et al. (26), with BSA dissolved in 1 N NaOH as the standard. $[cGMP]_i$ was expressed in picomoles per milligram protein.

Time course for the effects of ANP on isometric force and $[cGMP]_i$. Eight strips were prepared from the same tissue (PASM or TSM) and mounted in tissue baths. One strip was not contracted and served as an untreated control. The other seven strips were contracted with submaximal KCl as described above to achieve stable contractions. One strip was not exposed to ANP and served as the contracted, untreated control. The other six strips were exposed to the ANP concentration, resulting in ~90% relaxation (10^{-9} M for PASM, 10^{-6} M for TSM). The effect of 100 μM β-phenyl-1, N^2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate (*Rp*-isomer), a membrane-permeant, phosphodiesterase resistant cGK inhibitor, on relaxation to ANP in PASM and TSM was determined in three strips. Strips were flash-frozen for subsequent cGMP measurements at 3, 5, 10, 15, 20, and 40 min for PASM and at 0.5, 1, 2, 5, 7.5, and 10 min for TSM, a time course based on previous observations (33).

cGKI mRNA sequencing. Total RNA was obtained from PASM and TSM treated with RNAlater (Ambion, Austin, TX), which was then flash frozen and ground under liquid nitrogen and then suspended in guanidinium isothiocyanate; total RNA was then isolated by sedimentation through CsCl) (5). RNA quality was assessed by RNA electrophoresis on an agarose gel. Only samples that had ratios of 28S to 18S ribosomal RNA bands that were at least >1.2, with little evidence of laddering below or between these bands, were studied. We isolated mRNA from total RNA using Micro-Fast Track (Invitrogen) and then reverse transcribed using avian myeloblastosis virus reverse transcriptase (Clontech, Mountain View, CA). The resulting cDNA was probed with rapid amplification of cDNA ends (Clontech), using adapter sequences and a sequence that is present in both cGKI isoforms expressed in smooth muscle (30), cGKIα (40), and cGKIβ (45). The two cGKI isoforms present in smooth muscle are alternative splice variants sharing all but exon 1. A probe designed against the 3′ end of either cGKI isoform will detect mRNA for both cGKI isoforms. This sequence for cGKIα, nucleotides 1596–1623 of the human sequence (40) (accession no. D45864), was used as the initial probe for touchdown PCR. The resulting cDNA band was purified from an agarose gel and subcloned into pCR2.1 vector by using TOPO TA cloning kit (Invitrogen). We tested positive *Escherichia coli* colonies for the insert with colony PCR using M13 forward and reverse primers. Plasmid containing the insert was purified from a broth culture of a positive colony using Wizard plasmid miniprep (Promega, Madison, WI). The sequence of the insert was initially characterized with M13 forward and M13 reverse primers by using the Sanger dideoxy method (35) at the Mayo Rochester DNA Sequencing and Synthesis Core Facility (Rochester, MN). These sequences were used to generate new primers to extend the sequence until the complete open frame was obtained. Overlapping sequences were used to design gene-specific sequencing primers for obtaining the full-length open reading frame sequence for porcine cGKIα in triplicate. The resulting cGKI sequence was submitted to the National Center for Biotechnology Information (accession no. DQ119109). Oligonucleotide probes and primers were synthesized at the Mayo Rochester Core Facility. Oligonucleotide primers designed on the basis of a published se-

quence were also used to probe for cGKII isoform (43) (accession no. L12460) in PASM and TSM.

Measurement of cGKI mRNA expression. cDNA was prepared from total RNA from PASM and TSM taken from separate animals and stored at −20°C. Quantitative RT-PCR was used for this determination (1, 11). The exogenous internal standards (mimes) were derived from pBR322 and composite primers (Table 1) for PCR amplifying these were synthesized. The pBR322 sequence was selected to have a melting point by nearest neighbor estimation (Oligo Software, Cascade, CO) within 2°C of the cGKI sequence being amplified. The respective sizes of the mime/cGKI amplicon sequences were 347/229 bp. The amplified mime sequences were purified and subcloned into pCR2.1 plasmid by using TOPO TA cloning (Invitrogen), and colonies containing the mime insert were selected as described above in the DNA sequencing section. Plasmid was purified from the *E. coli* pellet with QIAfilter plasmid maxi kit (Qiagen, Valencia, CA). Confirmation of insertion was obtained by sequencing the plasmid and testing for amplification using cGKI primers that would detect total cGKI (cGKIα + cGKIβ). We used 260-nm optical density to quantitate plasmid and mime concentrations. Mime cDNA concentrations for determination of cGKI cDNA concentration in total RNA from TSM and PASM were 0.05, 0.1, 0.25, 0.5, and 0.75 amol. This mime concentration range was determined in preliminary studies and resulted in cDNA sample band intensities that lay within the range of band intensities resulting from these mime concentrations. Samples underwent PCR for 30 cycles with the following cycling conditions: 30-s initial denaturation at 94°C, followed by 25 cycles of 10-s denaturation at 94°C and 2 min 30 s annealing and extension at 68°C with a final extension at 72°C for 3 min. The PCR products were run on a 2.5% composite agarose gel [1.5% low melting point ultrapure agarose + 1% ultrapure agarose (Invitrogen)] containing 500 ng/ml ethidium bromide for staining. We used an AlphaInnotech ChemImager 400 imaging system to digitize gels and analyzed the resulting images by using Optiquant software (Perkin Elmer, Boston, MA). We determined the amount of gene-specific mRNA using linear regression analysis of a plot of log (intensity mime/intensity sample) vs. log (mol mime in PCR reaction). The point at which the regression line crosses zero on the *y*-axis determines the number of moles of cGKI mRNA present in the original total RNA sample. The quantity of gene-specific mRNA is expressed in attomoles per microgram total RNA.

Measurement of cGK protein concentrations. Strips were flash-frozen by rapid immersion in liquid nitrogen and stored at −70°C until proteins were to be extracted. Then they were placed in a chilled mortar on dry ice and pulverized, and the resulting powder was suspended in extraction buffer of (in mM) 10 KH_2PO_4, pH 7.0, 1 DTT, 1 EDTA, 1 PMSF, 5 NaF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. The sample was centrifuged at 4,000 *g* for 10 min to pellet insoluble material. Protein concentration in the extraction buffer supernatant was determined with the Bradford method (2); 2.0 μg of soluble protein from the tissue extracts were loaded into wells, and 0.1, 0.3, 0.67, 1, 1,67, and 3 ng of purified cGKIα (Promega) were loaded into adjacent lanes to generate a standard curve. Proteins were separated by electrophoresis in SDS-7.5% polyacrylamide precast minigels (Bio-Rad) with the use of Tris-glycine-SDS buffer. The proteins were transferred to a polyvinylidene difluoride membrane for 45 min at 100 V, and the membrane was subsequently washed with 10 mM Tris-buffered saline containing 5% (wt/vol) BSA for 15 min (25°C). The membrane was then treated overnight with Tris-buffered saline containing 0.2% Tween 20 and 1:20,000 dilution of cGKI polyclonal rabbit antibody directed against the COOH terminus shared by both cGKIα and cGKIβ (StressGen Biotechnology, San Diego, CA). After membranes were washed, they were treated with 1:10,000 anti-rabbit horse radish peroxidase-conjugated IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min and then washed again. Membranes were then treated with enhanced chemiluminescence Western blotting reagents (ECL; Amersham) for 1 min, apposed to Kodak BioMax film, and developed for a period of time that avoided saturation of any bands. The film was scanned and digitized with the use of Adobe Photoshop and subsequently analyzed with Optiquant software (Packard Instruments).

Measurement of cGKI activity. cGKI activity in tissue extract was determined by measuring phosphate transfer from ATP to a cGK-selective substrate, bovine lung cGMP-binding cGK-specific phosphodiesterase NH_2-terminal peptide (BPDEtide; RKISASEFDRPLR) (7, 41). Strips were frozen in liquid nitrogen and pulverized while on dry ice. The resulting powder was suspended in ice-cold homogenization buffer composed of (in mM) 10 sodium phosphate, pH 7.0, 1 EDTA, 1 DTT, 250 sucrose, 1 PMSF, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin for 30 min. The suspension was then centrifuged for 20 min at 12,000 *g* and 4°C. The supernatant was saved, and the protein concentration was determined with the Bradford assay (2). For phosphotransferase activity measurements, 10 μl of the ~1 mg/ml tissue extract were added to 50 μl of phosphotransferase assay mixture of (in mM) 0.2 ATP, 10 magnesium acetate, 20 Tris, pH 7.4, 0.5 isobutylmethylxanthine, 10 DTT, 10 sodium fluoride, 1 μM $PKI_{5–24}$, ~250 cpm/pmol [γ-^{32}P]ATP, with 60 μM BPDEtide as the cGK-selective substrate. The [cGMP] in the assay mixture was varied from 0.01 to 100 μM in half-log steps; 1 μM synthetic peptide inhibitor of cAMP-dependent protein kinase (4), $PKI_{5–24}$ (TTYADFIASGRTGRRNAIHD), was also added to the reaction mixture. After a 10-min reaction time at 30°C, the reaction was terminated by transfer of 25 μl of the assay mixture to 1-in.^{2} section of Whatman P-81 phosphocellulose. The phosphocellulose sections were washed in 800 ml of 75 mM phosphoric acid three times and then once with 95% ethanol. Dried phosphocellulose sections were placed in scintillation vials and counted by Cerenkov radiation on a Beckman LS6000IC counter. All data were obtained in triplicate and averaged. Calculated phosphotransferase activity was reported (in pmol P_i transferred·min^{-1}·mg^{-1} protein and pmol P_i transferred·min^{-1}·ng^{-1} cGKI) in the tissue extract used in the cGKI assay. To examine the extent to which the phosphotransferase reaction was selective for cGKI, a set of experiments was performed in the presence or absence of 10 μM cGMP, 100 μM *Rp*-isomer, a membrane-permeant, phosphodiesterase-resistant cGK inhibitor (Alexis Biochemicals, Lausen, Switzerland), or 10 μM cAMP.

Materials. The NO-donor DETA-NO was purchased from Alexis Biochemical (Ann Arbor, MI). BPDEtide and $PKI_{5–24}$ were synthesized by the Mayo Protein Core Facility. Unless otherwise specified in the text, all other drugs and chemicals were purchased from Sigma (St. Louis, MO). All drugs and chemicals were dissolved in distilled water, except for the cGK inhibitor *Rp*-isomer, which was dissolved in 50% DMSO in water.

Table 1. *Oligonucleotide composite primer sequences*

	Sequence	Nucleotide No. cGKIα	Nucleotide No. pBR322
Sense	AAGGGGGATTGACATGATAGAATTTCC**GATCGTTGTCAGAAGTAAGTTGG**	1930–1956	3734–3756
Anti	CGAACACAGACACCCACTACGTTGTG**GGGTTACATCGAACTGGATCTC**	2158–2133	4028–4007

Composite primer sequences showing sequences used to probe for cGMP-dependent protein kinase I (cGKI) subunit cDNA and mime constructed from cGKI subunit sequence (accession no. DQ119109) + specified pBR322 sequence (bold).

Statistical analysis. Data are expressed as means ± SE; *n* represents the number of pigs. The effects of ANP on isometric force and $[cGMP]_i$ were assessed by repeated-measures ANOVA with post hoc analysis using Student-Newman-Keuls method. cGKI mRNA expression, cGKI protein expression, and phosphotransferase activities between PASM and TSM were compared by unpaired Student's *t*-test. A P value < 0.05 was considered statistically significant. Concentration-response curves were compared by nonlinear regression analysis, as described by Meddings et al. (29). In this method, force (F) at any concentration of drug (C) is given by the equation $F = F_mC/(EC_{50} + C)$, where F_m represents the maximal isometric force and EC_{50} represents the concentration that produces half-maximal isometric force for that drug. Nonlinear regression analysis was used to fit values of F_m and EC_{50} to data for F and C for each condition studied. This method allows comparison of curves to determine whether they are significantly different and whether this overall difference can be attributed to differences in F_m, EC_{50}, or both parameters. A P value <0.05 was considered statistically significant. The apparent V_{max} and EC_{50} for cGMP in the phosphotransferase studies were determined in the same manner.

RESULTS

Physiological studies. In both PASM and TSM, after an initial submaximal contraction with ~23 mM KCl, the relaxation to DETA-NO was stable (data not shown) at each concentration and was complete at the highest concentration. The log EC_{50} (M) for DETA-NO was significantly less for PASM than with TSM (Fig. 1) (-6.55 ± 0.11 and -5.37 ± 0.13, respectively, $P < 0.01$), a result similar to that previously reported for canine PASM and TSM (22).

Addition of 10^{-9} M ANP in PASM submaximally contracted with KCl resulted in a stable reduction in force (Fig. 2*A*). Addition of 10^{-6} M ANP resulted in an initial decrease in isometric force in TSM that partially recovered (Fig. 2*B*). ANP produced concentration-dependent relaxation of both PASM and TSM (Fig. 3), but both sensitivity and maximal responses were much greater in PASM than in TSM (EC_{50} of 0.066 ± 0.027 and 13.2 ± 2.3 nM, respectively; $P < 0.001$).

The relaxation caused by 10^{-9} M ANP in PASM was generally associated with no significant increase in steady-state, $[cGMP]_i$, with the exception of the measurement at 15 min after ANP administration (Fig. 4*A*). By 40 min, $[cGMP]_i$ was indistinguishable from baseline (pre-ANP) values, despite persistent ~80% relaxation. In contrast, the modest relaxation

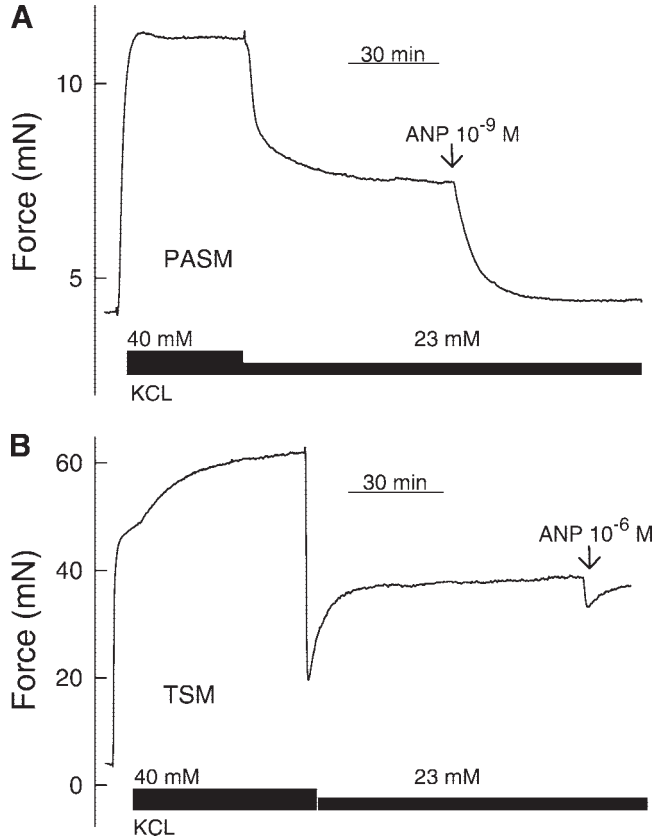

Fig. 2. Isometric force tracings for PASM (*A*) and TSM (*B*), illustrating the protocol used to determine the effect of atrial natriuretic peptide (ANP) on isometric force. Strips were initially contracted to near maximal contraction with 40 mM KCl and then contracted to ~50% maximal with 23 mM KCl. After this contraction reached a plateau value, the specified amount of ANP was added to the tissue bath. The relaxation to ANP was sustained in PASM but was biphasic in TSM.

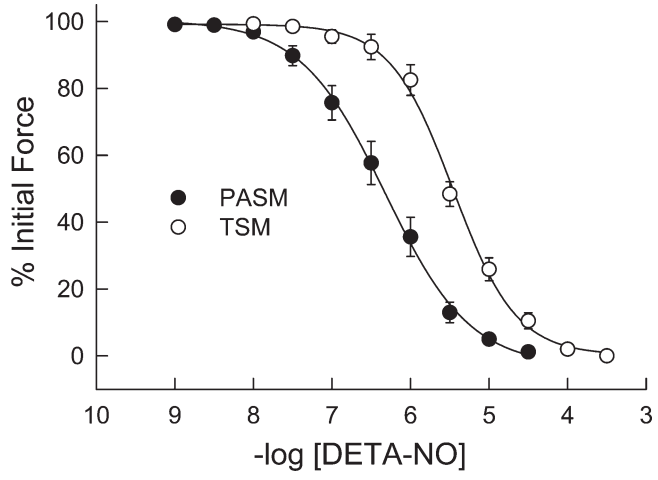

Fig. 1. Effects of the nitric oxide (NO) donor (*Z*)-1-[*N*-2-aminoethyl-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) on isometric force in pulmonary artery smooth muscle (PASM) and tracheal smooth muscle (TSM). Strips were contracted to half-maximal isometric force with KCl. Data are means ± SE; $n = 6$ pigs.

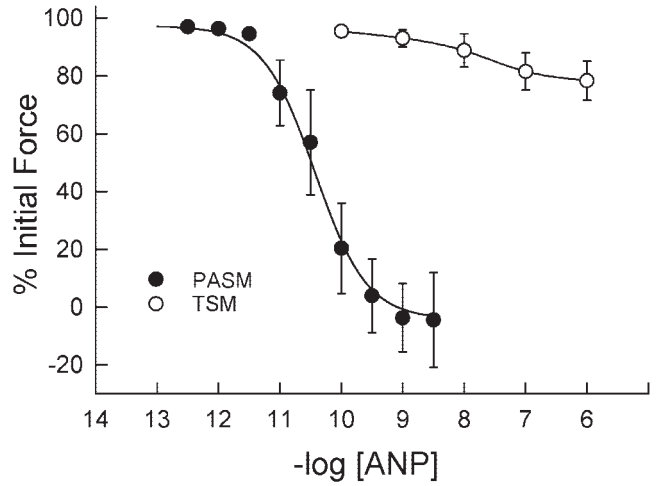

Fig. 3. Effects of ANP on isometric force in PASM and TSM. Strips were contracted to 50% of maximal isometric force with ~23 mM KCl, and the concentration response to ANP was determined in a cumulative fashion for PASM and the specified individual concentrations applied to TSM. Data are means ± SE; $n = 5$ pigs.

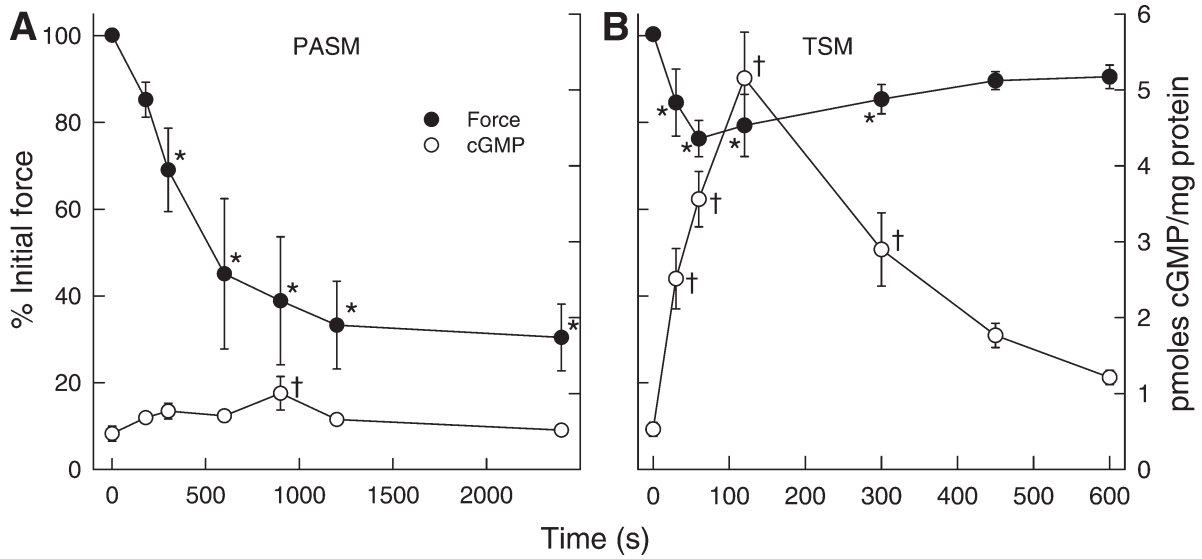

Fig. 4. Time course of the effects of ANP on isometric force and on tissue [cGMP] in PASM (*A*) and TSM (*B*). Data are means ± SE; $n = 6$ pigs. *Isometric force differed significantly from the initial control at time = 0 s. †Tissue cGMP differed significantly from the initial control at time = 0 s.

produced by 10^{-6} M ANP in TSM was accompanied by an increase in $[cGMP]_i$ that reached a maximum at 2 min after ANP administration, increasing by ~10-fold over baseline values (Fig. 4*B*). Although the increase in $[cGMP]_i$ was greater in TSM than in PASM, the extent of relaxation was significantly lower in TSM. After the peak increase in TSM, $[cGMP]_i$ decreased and was accompanied by an increase in isometric force; 100 μM cGK inhibitor decreased relaxation to the above specified concentrations of ANP in PASM and TSM by 92 ± 9 and 95 ± 15%, respectively ($n = 3$).

Expression studies. The mRNA sequence was determined for cGKIα in porcine trachea and was 96% homologous to cGKIα mRNA expressed in human vascular (40) and bovine tracheal (44) smooth muscle (accession nos. D45864 and X16086, respectively). The inferred porcine cGKIα amino acid homology and identity were 98 and 99.5% compared with both the human inferred and known bovine amino acid sequences. The predicted molecular mass of porcine cGKIα was 76,372 Da. Quantitative competitive RT-PCR successfully amplified both the constructed mime cDNA and the sample cDNA (Fig. 5*A*, *inset*). Serial dilutions of mime produced linear changes in band intensity that bracketed the intensity of cGKI-selective bands (Fig. 5*B*). The expression of total cGKI was determined by this means because all cGKI isoforms have identical mRNA and amino acid sequences in the region sampled by the primer pair used in these studies (Table 1). cGKI mRNA expression relative to total RNA (Fig. 5*B*) was greater in PASM than in TSM (2.23 ± 0.36 vs. 0.93 ± 0.31 amol mRNA/μg total RNA, respectively; $P < 0.01$).

An example of an SDS-PAGE result in which tissue homogenate was loaded into lanes adjacent to a set of six concentrations of purified cGKIα is shown in Fig. 6*A*, *inset*. Although both cGKI isoforms could be detected by the COOH-terminal-

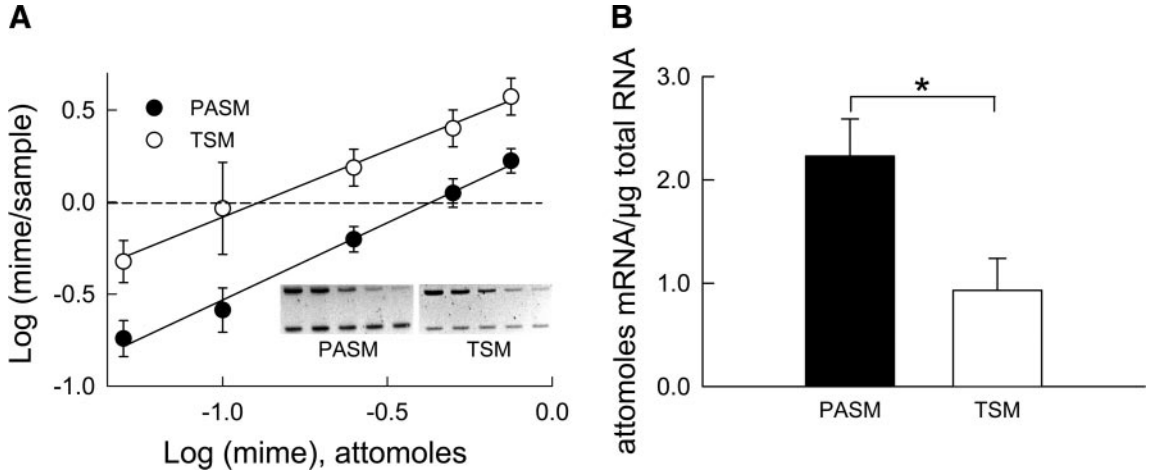

Fig. 5. Total cGMP-dependent protein kinase I (cGKI) mRNA expression in PASM and TSM using quantitative competitive RT-PCR. *A*: method for determining the amount of cGKI found in PASM and TSM using a known amount of mime cDNA sequence as an internal standard. Solid lines, linear least squares fit with $r^2 = 0.99$. Data are means ± SE, $n = 6$ pigs. Dashed line, point in the curves at which mime is equal to the sample; this value is determined for each sample by interpolation. *Inset*: example of an ethidium bromide agarose gel with mime cDNA bands above sample bands. *B*: semiquantitative measurement of total cGKI mRNA present in PASM and TSM. *Amount of cGKI is greater in PASM ($P = 0.018$). Data are means ± SE; $n = 6$ pigs.

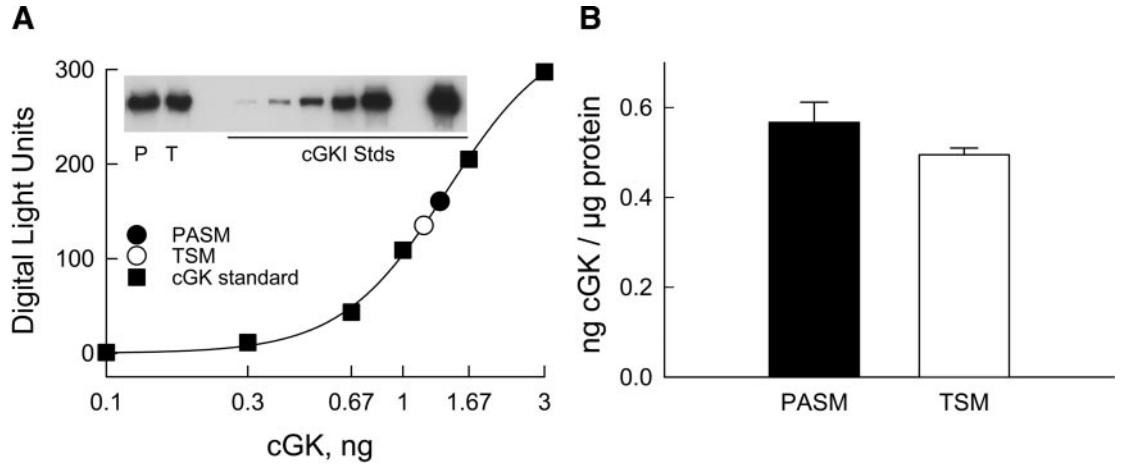

Fig. 6. Total cGMP-dependent protein kinase I (cGKI) protein in the soluble fraction of tissue homogenates from PASM and TSM using a semiquantitative immunoblotting method. *A*: representative data using known amounts of purified bovine lung cGKIα to create a standard curve and 2 μg of homogenate from each tissue. *Inset*: representative experiment of an immunoblot used to generate the standard curve and tissue data points (P, PASM; T, TSM). *B*: semiquantitative measurement of total cGKI in PASM and TSM. Data are means ± SE; $n = 6$ pigs.

directed antibody used, only one band is observed, running in the same location as purified cGKIα. A plot of arbitrary intensity units vs. the concentration of purified cGKIα resulted in a nonlinear standard curve best fit with a sigmoid curve. Equal loading of tissue homogenate protein resulted in nearly identical values for cGKI protein in both PASM and TSM in the example shown in Fig. 6*A*. A summary of the results from six experiments indicated no significant difference in the amount of cGKI protein present in the soluble fraction of tissue homogenates from PASM and TSM (0.56 ± 0.07 and 0.49 ± 0.04 ng cGKI/μg protein, respectively).

Phosphotransferase enzyme activity studies. In the initial studies, basal phosphotransferase activity obtained in the presence of a potent cGK inhibitor was 101 ± 25 pmol·min^{-1}·mg^{-1} protein in the soluble fraction of PASM tissue homogenate (Fig. 7). This increased to 880 ± 10 pmol·min^{-1}·mg^{-1} in the presence of 10 μM cGMP; 100 μM *Rp*-isomer, a potent cGKI inhibitor, decreased the phosphotransferase activity and inhibited the cGMP-induced increase in this activity by >70%; 10 μM cAMP significantly increased phosphotransferase activity but much less so than equimolar cGMP.

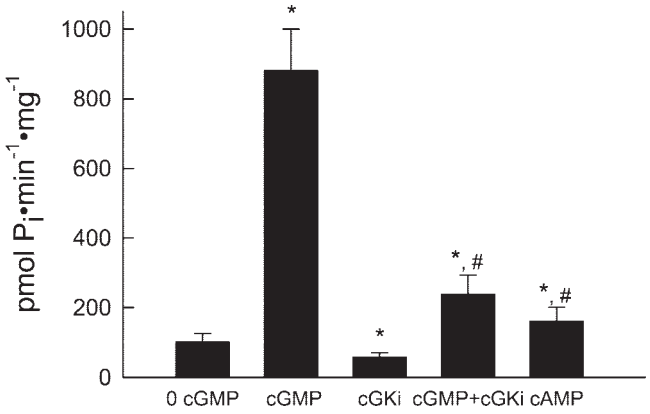

Fig. 7. Phosphotransferase activity in a soluble PASM homogenate. 0 cGMP, no cGMP added to the assay mix. cGMP indicates 10 μM cGMP; cGKi indicates 100 μM β-phenyl-1,N^2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate (*Rp*-isomer), a cGMP-dependent protein kinase inhibitor; cGMP + cGKi indicates 10 μM cGMP + 100 μM cGK inhibitor; cAMP indicates 10 μM cAMP. *Activity differs significantly from the 0 cGMP condition ($P < 0.025$). #Activity differs significantly from that obtained in the presence of 10 μM cGMP alone ($P < 0.01$). Data are means ± SE; $n = 4$ pigs.

Phosphotransferase activity was not significantly different between PASM and TSM in the absence of added cGMP. This activity was increased with increasing [cGMP], with an apparent EC_{50} of 1.2 ± 0.2 μM for homogenates from both tissues (Fig. 8*A*). Maximal phosphotransferase activity, however, was significantly greater in PASM compared with TSM (899 ± 78 vs. 462 ± 76 pmol·min^{-1}·mg^{-1}; $P < 0.001$). When normalized to the amount of cGK present in the tissue homogenates used for these assays, the maximal phosphotransferase activity of cGKI was also significantly higher in PASM than in TSM (1.61 ± 0.15 and 1.04 ± pmol·min^{-1}·ng cGKI^{-1}; $P < 0.05$) (Fig. 8*B*). There was no significant difference in phosphotransferase activity indexed to cGKI protein in PASM and TSM when no cGMP was added to the assay mix. The maximal phosphotransferase activity obtained for PASM and TSM was ~50% and 30% that of purified cGKIα. The apparent cGMP EC_{50} for purified cGKIα was significantly lower than that obtained in tissue samples (0.17 ± 0.04 μM; data not shown).

DISCUSSION

In agreement with a previous study in canine PASM and TSM, the NO donor DETA-NO caused complete relaxation in porcine PASM and TSM after a standardized contraction to KCl, resulting in ~50% of maximal isometric force (22). PASM was nearly an order of magnitude more sensitive to this NO donor than TSM. The increased NO sensitivity was previously related, in part, to increased cGMP production at a given NO donor concentration because of greater expression and activity of sGC in PASM. Greater relaxation at a given $[cGMP]_i$ is another mechanism that could contribute to a greater NO responsiveness in PASM than in TSM. This was not addressed in our previous study (22) because in both tissues, increases in steady-state $[cGMP]_i$ were not detected with up to 90% relaxation. There are, however, two additional methods by which cGMP sensitivity may be assessed in different tissues: use of cGK agonists and use of an alternative

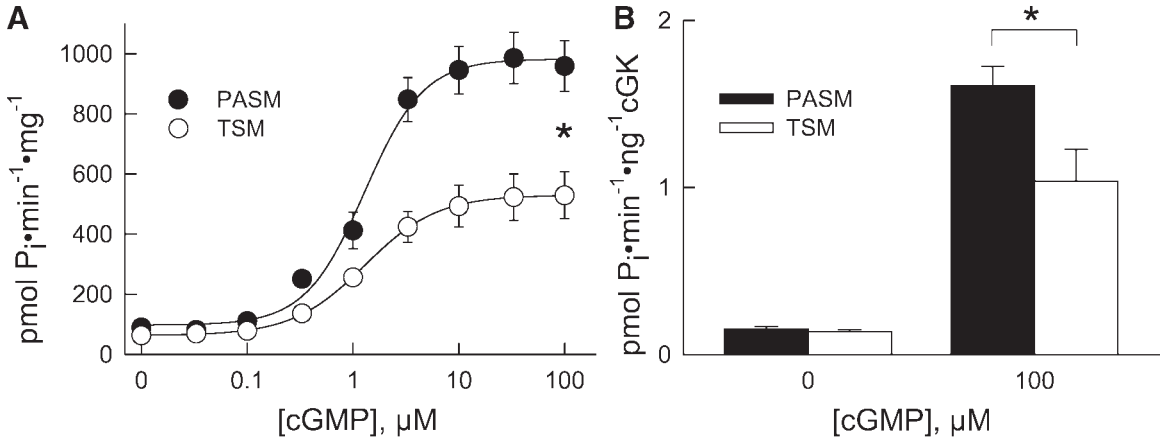

Fig. 8. Phosphotransferase activity in tissue homogenates from PASM and TSM. *A*: effect of added cGMP on the phosphotransferase activity normalized to the amount of total protein in the tissue homogenates. Data are means ± SE; $n = 6$ pigs. *TSM phosphotransferase activity is less than that obtained for PASM at cGMP ≥ 1 μM ($P < 0.01$). *B*: phosphotransferase activity at the specified cGMP concentration normalized to the amount of cGKI present in the tissue homogenates assayed. *Phosphotransferase activity in PASM is greater than in TSM ($P < 0.05$).

endogenous system for production of cGMP, the particulate guanylyl cyclases.

cGMP is inefficiently taken up by tissues and is subject to rapid breakdown by intracellular phosphodiesterases. Variably membrane-permeable cGMP, phosphodiesterase-resistant cGK agonists have been developed to facilitate assessment of cGMP responsiveness in tissue samples. Interpretation of concentration-response data between tissues with these agents must still account for the possibility of differences in tissue penetration and membrane permeability as an explanation for any concentration-response differences observed. This adds an additional complication in describing the relationship between cGK agonist and isometric force in a given tissue, namely, that, although the bath concentration of the cGMP analog is known, its intracellular concentration is not.

Particulate guanylyl cyclases are an alternative system for increasing $[cGMP]_i$ in smooth muscle independent of NO. The natriuretic peptide receptor A activated by ANP is present in both pulmonary artery (42) and airway smooth muscle (12), which is reflected by ANP-induced increases in $[cGMP]_i$ in both tissues (13, 15, 42). In the present study, the relationship between $[cGMP]_i$ and relaxation differed significantly between the two tissues. In PASM, 10^{-9} M ANP resulted in nearly complete relaxation without a significant increase in the steady-state $[cGMP]_i$ at all but one time point, a finding similar to that obtained with an NO donor in a previous study (22). As in that study, despite there being no significant increase in steady-state $[cGMP]_i$, prevention of ANP-induced relaxation with a cGK inhibitor indicates that such relaxation is cGMP mediated. In TSM, however, despite a nearly 10-fold increase in $[cGMP]_i$ above baseline values, treatment with 10^{-6} M ANP resulted in only incomplete relaxation to ANP. Similar findings of minimal relaxation to ANP in TSM, despite significant increases in $[cGMP]_i$, have been previously reported (15, 33). Of interest, in prior work, we found that when an NO donor is used to induce relaxation in TSM, it does so without a significant increase in $[cGMP]_i$ (22, 33). Thus relaxation to cGMP in TSM apparently depends on the location of the guanylyl cyclase producing the cGMP. It is very sensitive to cGMP when it is produced by soluble and insensitive when produced by membrane bound guanylyl cyclases. Although the mechanism for this is unclear, it does indicate that, under the circumstances used in this study, the cGMP sensitivity of TSM is significantly less than that of PASM. We cannot explain the biphasic nature of the relaxation to ANP in TSM, which may be accounted for by phosphodiesterase activity, rapid receptor desensitization or cGKI target dephosphorylation. The first possibility seems unlikely, since initial recovery of force occurs at a time when $[cGMP]_i$ is increasing.

If cGMP sensitivity were to play a role in the lower NO sensitivity of TSM vs. PASM, several predictions would be made. The first is that cGMP responsiveness in TSM would be lower than in PASM. Ideally, this would be examined by establishing a relationship between the relaxant effect of an NO donor and its concurrent effect on $[cGMP]_i$. This is not possible for the reasons given above. However, the cGMP responsiveness of TSM is lower than that of PASM when ANP is used to stimulate cGMP production by particulate guanylyl cyclase. A second prediction would be that expression and activity of cGK would be lower in TSM than in PASM, since cGK is the primary enzyme transducing the cGMP signal.

No cGKII mRNA was found in either PASM or TSM, a result that is somewhat surprising given the abundance of its expression in lung (43). Its association with gastrointestinal epithelium and not intestinal smooth muscle (28) suggests that cGKII in lung is probably associated with the lung epithelial cell layers. Total cGKI mRNA and protein expression were determined using cDNA probes and antibodies selective for portions of the cGKI sequence shared by both cGKIα and cGKIβ. Total cGKI mRNA expression was significantly higher in PASM than in TSM. The amount of total cGKI mRNA expression is very similar to the values obtained for sGC mRNA expression in TSM and PASM (22), on the order of 4 and 2 amol mRNA/μg total RNA, respectively. This level of expression does not reflect a high abundance of mRNA for either signaling protein, but neither are they particularly rare. The levels of cGKI mRNA expression measured in these studies are the first semiquantitative report of their absolute rather than their relative expression in smooth muscle tissues. The quantitative RT-PCR method used in these studies has the advantage of having an internal standard, but may be limited in accuracy by variability in the quality of the mRNA in each

preparation, the use of a cDNA rather than an RNA mime and differences in the PCR efficiency between the mime insert sequence and the cDNA produced by reverse transcription of mRNA in a total RNA sample. Variability in the quality of RNA was assessed before the reverse transcription step and RNA samples showing evidence of noticeable degradation were not reverse transcribed. The mime sequence in pBR322 was selected to have similar melting and annealing characteristics to that of the sGC subunit sequence being probed. The semiquantitative assessment of mRNA for each sGC subunit demonstrated low variability across multiple total RNA preparations. The possibility of systematic errors from use of a cDNA mime and reverse-transcribed mRNA from tissue total RNA exists and cannot be addressed with the methods used. The values for total cGKI mRNA expression reported here are thus relatively precise lower limits for the actual amount of cGKI mRNA expressed in PASM and TSM.

Very little is known about the quantitative relationship between mRNA and protein expression for cGKI within any tissue, let alone across tissues. Total cGKI protein expression in soluble homogenates from PASM and TSM were not significantly different, indicating an apparent discordance between levels of cGKI expression at the mRNA and protein levels. This apparent discrepancy may be due to either a systematic error in one or both of the semiquantitative measurements or may reflect a difference in gene transcription, mRNA stability, or cGKI translation and turnover between the two tissues. Of the two methods used for measuring cGKI expression, the quantitative competitive RT-PCR method makes more assumptions and may amplify any errors in initial quantitation of the total RNA used in the reverse transcription. The immunoblotting method used in this study is less susceptible to such errors, although differences in extraction efficiency and in protease activity between tissues could exist. The efficiency of soluble protein extraction between the two tissues, however, was similar because nearly equal protein concentrations were obtained in tissue homogenates from suspensions of equal weights of both tissues suspended in equal volumes of extraction buffer (data not shown). Arguing against differences in protease activity between tissues is the observation that the cGKI homogenates from both PASM and TSM give similar semiquantitative results over storage times of more than 1 mo (data not shown). In further support of the general accuracy of the semiquantitative immunoblotting methods used in this study, the level of cGKI protein expression is of the same magnitude as that previously reported in cultured rat aortic smooth muscle cells (37). Thus it is likely that the expression of cGKI in PASM and TSM is similar and a difference in protein expression does not account for differences in cGKI activity. Finally, the assumption that the same quantitative relationship between cGKI mRNA and protein expression exists either in the same tissue under different conditions or across tissues under the same conditions (i.e., that mRNA expression is an adequate surrogate for protein expression) may be an incorrect one and needs to be examined in all cases.

The presence of a single-band cGKI band on the immunoblots of tissue homogenates and the fact that they run in a position identical to commercially purified cGKIα reported in this study should not be taken to indicate that both cGKIα and cGKIβ are not present in PASM and TSM. The calculated molecular mass of these proteins differs by only 1,500 Da, and resolution of these two >75-kDa proteins under the conditions used in these studies was not expected.

Although the levels of cGKI and sGC mRNA expressions were similar in these tissues, there is nearly 10-fold more cGKI than sGC protein (22) expressed in both PASM and TSM. The functional significance of higher cGKI (primary cGMP-responsive protein) vs. sGC (NO-linked cGMP production) protein expression remains to be established.

The phosphotransferase assay used in this study (6, 7) demonstrated a marked elevation in activity with 10 μM added cGMP that was inhibited by a cGK inhibitor; 10 μM cAMP also increased phosphotransferase activity significantly above baseline values but to a much lesser extent (only ~20%) than the same concentration of added cGMP. The slight activation with cAMP probably does not indicate that cAMP-dependent protein kinase remains active in these homogenates, since cAMP also activates purified cGKI, but requires ~10-fold higher concentrations to maximally activate the enzyme. Together, these data indicate that the tissue homogenate phosphotransferase assay used in these studies reflected cGK activity in the homogenate. The phosphotransferase assay demonstrated the expected increase in activity with increasing cGMP in both tissues. The ~1 μM EC_{50} for cGMP was not significantly different between PASM and TSM but was greater than expected for a tissue containing a significant amount of cGKIα. With the use of the same phosphotransferase assay as for the tissue homogenates, commercially purified cGKIα had a nearly 10-fold lower cGMP EC_{50} (data not shown). Previous reports (23, 36) indicated that cGKIβ has a cGMP EC_{50} similar to that found for the phosphotransferase assay results from PASM and TSM homogenates. However, cGKIα is present in both porcine PASM and TSM, as attested by its successful use to establish a complete open reading frame cGKI sequence in these tissues. A simple explanation for the cGMP EC_{50} results obtained in this study is that cGKIβ is the predominant isoform expressed in both PASM and TSM. This is apparently at odds with previous reports in which cGKIα is thought to be the predominant isoform in vascular smooth muscle (25, 45). In the absence of a significant difference in the phosphotransferase activity cGMP EC_{50} between PASM and TSM, it is unlikely that differences in the proportion of cGKIα and cGKIβ could account for differences in cGMP responsiveness between the tissues.

The V_{max} for the two cGKI isoforms are the same (23, 36). Any differences in V_{max} between PASM and TSM must therefore be accounted for by either differences in the amount of total cGKI present in the two tissues or by differences in the activity indexed to the amount of cGKI in each. The amount of cGKI found in PASM and TSM did not differ significantly. However, when the maximal phosphotransferase activity was indexed to the amount of total cGKI, TSM had ~60% the activity of PASM. This difference in apparent cGKI specific activity suggests that the enzyme is regulated differently in the two tissues and would decrease the cGMP responsiveness of TSM relative to PASM. The biochemical basis of such a difference in cGKI-specific activity is unclear but might involve differences in the degree of cGKI autophosphorylation between tissues (36). Use of the same extraction procedure and the fact that both PASM and TSM samples were freshly obtained and processed at the same time make differences in

treatment of the tissue homogenates an unlikely explanation for this finding.

In summary, the present study demonstrates that the response to NO is greater in porcine PASM than in TSM. A significant difference in the responsiveness of the contractile system to the effects of cGMP, cGMP responsiveness, between these tissues was demonstrated using the NO-independent particulate guanylyl cyclase system, activated by ANP. Whereas no detectable increase in $[cGMP]_i$ was observable with nearly complete relaxation in PASM, a 10-fold increase in $[cGMP]_i$ gave rise to only modest relaxation in TSM. Although total cGKI mRNA expression was greater in PASM, total protein expression was not significantly different between the tissues. The apparent cGKI specific activity, however, was significantly lower in TSM than in PASM, a novel finding that would be expected to result in decreased cGMP responsiveness in TSM. The difference in response to NO between these tissues may thus be accounted for by both lower cGMP production (22) and cGMP responsiveness in TSM. It is possible that these differences in the NO-cGMP signaling system may play a significant role in reported differences in the therapeutic response to NO between airways and the pulmonary arterial circulation.

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