Expression of endothelial nitric oxide synthase in human and rabbit gastrointestinal smooth muscle cells

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Expression of endothelial nitric oxide synthase in human and rabbit gastrointestinal smooth muscle cells. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G342–G351, 1998.—The aim of this study was to identify the nitric oxide synthase (NOS) isoform expressed in freshly dispersed rabbit gastric smooth muscle cells and in cultured rabbit gastric, human intestinal, and guinea pig taenia coli smooth muscle cells. RT-PCR products of the predicted size (354 bp) were obtained with endothelial NOS (eNOS)-specific primers, but not neuronal NOS (nNOS)- or inducible NOS (iNOS)-specific primers, in all smooth muscle preparations except guinea pig taenia coli. Control RT-PCR studies showed absence of the endothelial markers, platelet endothelial cell adhesion molecule-1 (PECAM-1) and vascular endothelial growth factor receptor (VEGFR), and the interstitial cell marker, c-kit, from cultures of smooth muscle cells. Cloning and sequence analysis showed that the predicted amino acid sequence (117 residues) in rabbit and human smooth muscle cells differed by only one residue from that of human eNOS. Northern blot analysis, using the PCR-generated and cloned eNOS cDNA from rabbits and humans as probes, demonstrated the expression of eNOS mRNA (4.4 kb) in both species. eNOS, but not nNOS or iNOS, transcripts were localized by in situ RT-PCR in single, freshly dispersed rabbit gastric smooth muscle cells; expression was evident in the majority of cells in each preparation. We conclude that eNOS is selectively expressed in rabbit gastric and human intestinal smooth muscle cells. The results confirm functional evidence for the existence of a constitutive NOS in smooth muscle cells of the gut in different species, except for guinea pig taenia coli.

in situ reverse transcription-polymerase chain reaction; gastric smooth muscle; intestinal smooth muscle

The two constitutively expressed, Ca2+/calmodulin-dependent nitric oxide synthase (NOS) isoforms first identified in neurons (nNOS or NOS-1) and endothelial cells (eNOS or NOS-3) are now known to be more widely distributed (2, 3, 24). eNOS is found in epithelial cells (19, 22, 38), cardiac myocytes (1, 8), human platelets (37), and various neurons, particularly pyramidal neurons of the hippocampus, where it is coexpressed with nNOS (7). nNOS is found in the cytoskeleton of fast-contracting skeletal muscle fibers (33). A third NOS isoform, inducible NOS (iNOS or NOS-2), is absent in the resting, unactivated state but can be induced in many cells on exposure to endotoxins and cytokines. This isoform, first identified in macrophages (45), can also be induced in other cell types, including hepatocytes, vascular and visceral smooth muscle cells, and fibroblasts (9, 34). iNOS is expressed in much greater abundance than the constitutive isoforms, and its activity is not dependent on an increase in cytosolic Ca2++.

Both nNOS and eNOS are expressed in the gut. nNOS is present in interneurons and motoneurons of the enteric nervous system, where it is usually colocalized with vasoactive intestinal polypeptide (VIP) or pituitary adenylate cyclase-activating peptide (PACAP). eNOS is present in endothelial cells. In dogs but not in humans, a constitutive NOS isoform that immunostains with antibodies to eNOS and nNOS has been reported in interstitial cells of Cajal, a distinctive population of large stellate cells usually found at the boundaries of the inner, circular smooth muscle layer (13, 46).

A constitutive, Ca2+/calmodulin-dependent NOS has also been functionally identified in smooth muscle cells from various regions of the gut in several species but has not been detected by immunohistochemical techniques, presumably because it is not abundantly expressed (5, 11). Smooth muscle NOS is highly sensitive to inactivation by protein kinase C (14, 29) and, like eNOS, is membrane bound (27). The enzyme is coupled to pertussis toxin-sensitive GTP-binding proteins (G1 and G12) and is selectively activated by VIP and PACAP (31). The cascade is initiated by an increase in Ca2+ influx that leads to activation of the enzyme and formation of NO (28, 32). The increase in NO leads to activation of a soluble guanylyl cyclase, formation of cGMP, and activation of cGMP-dependent protein kinase. During nerve stimulation, NO generated by the activity of nNOS in nerve terminals regulates the release of VIP and PACAP and diffuses to muscle cells to participate in muscle relaxation (10, 18). In turn, VIP and PACAP act on smooth muscle cells to regenerate NO. The NO formed in muscle cells constitutes the predominant component (60–80%) of NO formed during nerve stimulation (18).

The identity of the NOS isoform in smooth muscle of the gut is unknown. In this study, we have used molecular approaches to determine the expression and characterize the isoform of NOS in freshly dispersed and cultured rabbit gastric smooth muscle cells and cultured human intestinal smooth muscle cells.
MATERIALS AND METHODS

Preparation of Dispersed Muscle Cells

Muscle cells were isolated from the circular muscle layer of rabbit stomach, human small intestine, and guinea pig taenia coli as described previously (6, 16, 28, 31). Segments of human jejunum (~5 cm long) were obtained from patients undergoing bypass surgery for morbid obesity, according to a protocol approved by the Institutional Committee on the Conduct of Human Research. Muscle strips were incubated for 20 min at 31°C in a HEPES medium containing 0.1% collagenase (type II) and 0.1% soybean trypsin inhibitor. The medium consisted of (in mM) 115 NaCl, 5.8 KCl, 2.1 KH₂PO₄, 0.6 MgCl₂, 25 HEPES, and 14 glucose, as well as 2.1% essential amino acid mixture (pH 7.4). The partially digested tissues were washed with 50 ml of enzyme-free medium, and the muscle cells were allowed to disperse spontaneously for 30 min. The cells were harvested by filtration through 500-µm Nitex mesh and centrifuged twice at 350 g for 10 min to eliminate broken cells and organelles. Previous studies have shown that centrifugation eliminates contaminant neural membranes, as shown by the absence of [³H]saxitoxin binding (31).

Preparation of Cultured Smooth Muscle Cells

Human intestinal, rabbit gastric, and taenia coli smooth muscle cells were cultured as previously described for guinea pig gastric smooth muscle cells (6). The dispersed smooth muscle cells were resuspended in DMEM containing penicillin (200 U/ml), streptomycin (200 µg/ml), gentamycin (100 µg/ml), amphotericin B (2.5 µg/ml), and 10% FCS (DMEM-10). The muscle cells were plated at a concentration of 5 x 10⁵ cells/ml and incubated at 37°C. DMEM-10 medium was replaced every 3 days for 2–3 wk until confluence was attained. The muscle cells in confluent primary cultures were trypsinized (0.5 mg trypsin/ml), replated at a concentration of 2.5 x 10⁵ cells/ml, and cultured under the same conditions. All experiments were done on cells in first passage. The purity of the cultures was determined by staining with monoclonal antibody HM19/2 for smooth muscle-specific γ-actin (Fig. 1) using a modification of the method of Vollmer et al. (44), as previously described (6, 21).

Human umbilical vein endothelial cells in primary culture were obtained from Clonetics (San Diego, CA) and recultured as follows. The cells were plated at a concentration of 2.5 x 10⁵ cells/ml and cultured in MCD-131 medium containing 2% FCS, 10 ng/ml human epidermal growth factor, 1 µg/ml hydrocortisone, 12 µg/ml bovine brain extract, 50 µg/ml gentamicin, and 50 µg/ml amphotericin B. Cells were maintained at 37°C in 10% CO₂ until full confluence was reached. After trypsinization, the cells were plated at the same density and recultured under the same conditions. Endothelial cells from the first or second passage were used in these studies. Unlike cultured rabbit and human smooth muscle cells, cultured endothelial cells did not stain with γ-actin antibody.

RT-PCR

Total RNA was isolated from freshly dispersed rabbit gastric smooth muscle cells, from cultured rabbit gastric, human intestinal, and guinea pig taenia coli smooth muscle cells, and from cultured human umbilical vein endothelial cells using the PERFECT RNA isolation kit. One microgram of total RNA from each preparation was reverse transcribed in a reaction volume of 20 µl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3.0 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.5 mM dNTPs, 2.5 µM random hexamers, and 200 U RT. The reaction was carried out at room temperature for 10 min and at 42°C for 50 min and terminated by heating at 70°C for 15 min. The reverse-transcribed cDNA (3 µl) was amplified in a final volume of 50 µl by PCR (35 cycles) under standard conditions (2 mM MgCl₂, 125 µM dNTP, 2.5 U Taq polymerase) with specific primers for human eNOS, nNOS, and iNOS as designed by Sase and Michel (37) based on the sequences of the three isoforms (9, 24, 33). The primers and experimental conditions for RT-PCR are summarized in Table 1.

Control RT-PCR was performed to determine the expression of two endothelial cell markers, platelet endothelial cell adhesion molecule-1 (PECAM-1) and vascular endothelial growth factor receptor (VEGFR), in freshly dispersed and cultured smooth muscle cells and in cultured human umbilical vein endothelial cells. The VEGFR- and PECAM-1-specific primers based on the sequence of human VEGFR (12) and PECAM-1 (39) and the experimental conditions for RT-PCR are listed in Table 1. RT-PCR for smooth muscle-specific γ-actin was performed in freshly dispersed gastric smooth muscle cells and cultured endothelial cells (Table 1).

For each experiment, a parallel control without RT was processed. Cloned cDNAs for human and bovine eNOS, human and rat nNOS, rat iNOS, and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as positive controls for PCR under the same experimental conditions. RT-PCR for GAPDH was performed with each experiment. The amplified PCR products in all experiments were analyzed on a 1–1.5% agarose gel containing 0.1 µg/ml ethidium bromide.
Southern Blot Analysis

PCR products obtained using eNOS-specific primers were fractionated by electrophoresis in 1% agarose gel and transferred in 0.4 N NaOH to a nylon membrane. The products were hybridized with a nested eNOS oligonucleotide probe (5'-AGCGGAAGGGGAGCGAGGGAGGACG-3'; 5 pmol/ml) tailed with digoxigenin-11-dUTP at the 3' end in 6x SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 20 mM Na2HPO4 (pH 7.0), 1X Denhardt's reagent, 0.5% SDS, and 10 µg/ml poly(A) for 4 h at 54°C and washed to a final stringency of 0.2X SSC and 0.1% SDS at 50°C. Binding to antidigoxigenin antibodies and color detection were performed using a digoxigenin detection kit.

Cloning and DNA Sequencing

The PCR products obtained from dispersed rabbit gastric smooth muscle cells and cultured human intestinal smooth muscle cells using eNOS-specific primers were purified by 1% agarose gel electrophoresis and cloned into pCR II vector (Invitrogen). The nucleotide sequences were determined for cDNA inserts from two positive clones (dispersed rabbit gastric muscle cells) and one positive clone (cultured human intestinal smooth muscle cells) and then sequenced by 10.220.33.4 on July 7, 2017 http://ajpgi.physiology.org/ Downloaded from

Table 1. Primers and experimental conditions

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eNOS, endothelial nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; PECAM-1, platelet endothelial cell adhesion molecule-1; VEGFR, vascular endothelial growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; S, sense; AS, antisense.

Cell fixation. Freshly dispersed rabbit gastric muscle cells were fixed in 10% buffered Formalin for 20–24 h at room temperature. The cells were then pelleted by centrifugation at 1,000–2,000 rpm for 5–10 min and washed once with distilled water. The cells were resuspended in distilled water at a concentration of 10^6 cells/ml. Three aliquots (a test aliquot and two control aliquots, 10–20 µl each) were applied to each in situ PCR glass slide and allowed to dry at room temperature.

Protease digestion. The cells in each spot were digested by trypsin (2 mg/ml) for 60–80 min at room temperature and washed with 10 ml of RNase-free water. The cells were dehydrated for 1 min and then air-dried.

DNase and RNase digestion. RNase-free DNase I (10 U) was applied to the test spot and to one of the control spots to degrade nuclear DNA, and then washed with RNase-free water for 1 min. The spots were dehydrated with 100% ethanol and then air-dried.

Reverse transcription. The SuperScript II system was used for reverse transcription of RNA using an eNOS-specific 3'-downstream primer (1 µM) (Table 1). The slides were covered by AmpliCover discs and clips and placed in the in situ PCR system. The reaction was carried out only on the test spot and was first performed at room temperature for 10 min and then at 42°C for 50 min and at 70°C for 15 min.

PCR. Each cell spot was overlaid with 50 µl of PCR mixture consisting of 2.5 mM MgCl2, 225 µM each of dATP, dCTP, and dGTP; 125 µM dTTP; 5 µM digoxigenin-11-dUTP; eNOS-specific primers (1 µM); and 6 U Taq polymerase. Amplification was under the conditions optimized for solution-phase PCR: 95°C for 2 min, then 30 cycles each at 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min, followed by two washes in buffer consisting of 0.1 M Tris–HCl and 0.15 M NaCl (Tris-buffered saline (TBS); pH 7.5) at room temperature and one wash in the same buffer at 37°C.

Digoxigenin detection. The spots were incubated with blocking reagent for 30 min and with alkaline phosphatase-labeled antidigoxigenin antibody (1:100) for 60 min at room temperature. The spots were then washed twice for 15 min with TBS and for 5 min with buffer containing 0.1 M Tris–HCl, 0.1 M NaCl, and 50 mM MgCl2. The spots were treated with alkaline phosphatase substrates (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) that were

In Situ RT-PCR

In situ RT-PCR was performed by a modification of the method of Nuovo (35) to localize the eNOS transcripts in single muscle cells.
enzymatically transformed into purple precipitate at the site of the amplified PCR product after incubation in the dark for 1–2 h. The number of positively stained cells was determined from every 100 cells in the test spot.

Control studies. Identical procedures for in situ RT-PCR were followed using nNOS-specific and iNOS-specific primers to serve as controls.

Western Blot Analysis of NOS

Western blot analysis of eNOS was determined as described previously for G proteins (30). Freshly dispersed rabbit gastric smooth muscle cells (2 × 10^6 cells/ml) and cultured rabbit and human smooth muscle cells were homogenized in a medium containing 50 mM Tris·HCl (pH 7.5), 1 mM EGTA, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM DTT, and 2 µg/ml leupeptin and centrifuged at 12,000 g for 5 min. The supernatant was subjected to SDS-PAGE, and the separated proteins were electrophoretically transferred at 4°C to nitrocellulose membranes. The blots were incubated for 1 h at room temperature in TBS containing 150 mM NaCl, 50 mM Tris·HCl (pH 7.5), and 5% nonfat dry milk to block nonspecific antibody binding. After three to four washes in TBS, the blots were incubated for 12 h at 4°C with NOS antibody (1:1,000), washed again, and incubated for 1 h with anti-rabbit IgG conjugated with horseradish peroxidase. The bands were identified by enhanced chemiluminescence reagents.

Immunoblot Analysis of NOS

Immunoblot analysis was determined as described previously for endothelial cells and cardiac myocytes (8). Freshly dispersed gastric smooth muscle cells were washed in PBS twice, pelleted by centrifugation, and suspended in a medium containing 50 mM Tris·HCl (pH 7.4), 0.1 mM EGTA, 2 mM β-mercaptoethanol, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF at a concentration of 5–6 × 10^6 cells/ml. The cell suspension was diluted fourfold in 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) buffer containing 50 mM Tris·HCl (pH 7.4), 20 mM CHAPS, 125 mM NaCl, 2 mM DTT, 0.1 mM EGTA, 4 µM tetrahydrobiopterin, 1 mM L-arginine, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF. Cells were lysed by sonication and centrifuged at 1,000 g for 10 min. The soluble cell lysate (∼600 µg protein) was incubated with 10 µg of eNOS antibody for 1 h at 4°C with gentle shaking and for an additional 1 h with 50 µl protein A Sepharose. Bound immune complexes were washed six times with CHAPS buffer and once with 50 mM Tris·HCl (pH 7.4) containing 4 µM tetrahydrobiopterin and 1 mM L-arginine. The pellet was suspended in 30 µl of SDS-PAGE sample buffer and loaded onto 10% SDS-PAGE gel. The separated proteins were treated as described above for Western blot.

Materials

Human umbilical vein endothelial cells and culture medium (MCDB-131) were obtained from Clonetics; Superscript II RT, trypsin, and the random primer labeling system were from Life Technologies (Grand Island, NY); and monoclonal antibody HM19/2 for γ-actin, terminal transferase, dipoxigenin-11-dUTP, and RNase-free DNase I were obtained from Boehringer Mannheim (Indianapolis, IN). AmpliTaq DNA polymerase and ampiclovor discs and clips were obtained from Perkin Elmer (Foster City, CA). QuickHyb hybridization solution was from Stratagene (La Jolla, CA), and Perfect RNA isolation kit was from 5′−3′ (Boulder, CO). Primers for eNOS, nNOS, iNOS, PECAM-1, VEGFR, γ-actin, and GAPDH were synthesized by Integrated DNA Technologies (Coralville, IA). Polyclonal and monoclonal antibodies to eNOS, nNOS, and iNOS were obtained from Transduction Laboratories (Lexington, KY). All other reagents were obtained from Sigma (St. Louis, MO). The cDNA for rat INOS was obtained from Alexis (San Diego, CA). The cDNA for rat nNOS was a gift from Dr. Solomon Snyder, Johns Hopkins University, the cDNA for human nNOS was a gift from Dr. Masaki Nakane, Abbott Laboratories, and the cDNA for human eNOS was a gift from Dr. Philip Marsden, University of Toronto. The cDNA for bovine eNOS was provided by T. Michel (22).

RESULTS

Identification of eNOS Expression in Smooth Muscle by RT-PCR and Southern Blot Analysis

Using eNOS-specific primers, distinct RT-PCR products of the predicted size (354 bp) were obtained from freshly dispersed and cultured rabbit gastric smooth muscle cells (Fig. 2, lanes 3 and 5) and from cultured human intestinal smooth muscle cells (Fig. 2, lane 7). A PCR product of similar size was obtained with bovine eNOS and human iNOS primers (Fig. 2, lane 2). No PCR product was obtained with human nNOS-specific primers (Fig. 2, lane 4) or in absence of RT (−RT). No PCR product was obtained with human eNOS- or iNOS-specific primers (lanes 6 and 8). A PCR product of similar size was obtained with human eNOS and iNOS primers (Fig. 2, lane 7). A PCR product of similar size was obtained with human eNOS and iNOS primers (Fig. 2, lane 7).
eNOS cDNA as template (Fig. 2, lane 2). Under similar experimental conditions, there was no detectable PCR product with nNOS-specific or iNOS-specific primers. PCR products were obtained with rat nNOS cDNA (456 bp) and rat iNOS cDNA (294 bp) as templates (Fig. 2, lane 2). Parallel experiments without RT did not yield PCR products with eNOS-, nNOS-, and iNOS-specific primers (Fig. 2, lanes 4, 6, and 8).

There was no detectable PCR product with eNOS-, nNOS-, or iNOS-specific primers from guinea pig cultured taenia coli smooth muscle cells (Fig. 3). Functional studies in taenia coli have previously shown that it is devoid of NOS activity (16).

The identity of the amplified eNOS-specific PCR products was further explored by Southern blot analysis (Fig. 4). Using labeled eNOS cDNA as probe, a single band with digoxigenin-dependent staining corresponding to the eNOS PCR product (354 bp) was detected in freshly dispersed gastric smooth muscle cells (Fig. 4, lane 5) and cultured gastric (Fig. 4, lane 3) and intestinal (Fig. 4, lane 7) smooth muscle cells. No band was obtained in parallel experiments without RT (Fig. 4, lanes 4, 6, and 8).

Experiments with specific primers for PECAM-1 and VEGFR were designed to detect the presence of contaminant endothelial cells in freshly dispersed and cultured smooth muscle cells. Using specific primers for PECAM-1, a RT-PCR product of the predicted size (296 bp) was amplified from cultured human endothelial cells (Fig. 5, lane 2) but not from freshly dispersed rabbit gastric smooth muscle cells (Fig. 5, lane 4), cultured human intestinal smooth muscle cells (Fig. 5, lane 6), or cultured guinea pig taenia coli smooth muscle cells (Fig. 5, lane 7). Using specific primers for VEGFR, however, a RT-PCR product of the predicted size (209 bp) was amplified from cultured human endothelial cells (Fig. 5, lane 2) and freshly dispersed rabbit gastric smooth muscle cells (Fig. 5, lane 4) but not from cultured rabbit gastric, human intestinal, or taenia coli smooth muscle cells (Fig. 5, lanes 5–7).

Using specific primers for γ-actin, a RT-PCR product of the predicted size (80 bp) was amplified from freshly dispersed gastric smooth muscle cells but not from cultured endothelial cells.

Using two sets of c-kit-specific primers based on the sequences of human and bovine c-kit (36, 43), no RT-PCR product was amplified from either cultured rabbit gastric or human intestinal smooth muscle cells. A PCR product of the predicted size (534 bp) was obtained with human and bovine c-kit cDNA as template.

With all primers, no PCR product was obtained from endothelial or smooth muscle cells in the absence of RT.

Cloning and Sequence Analysis of eNOS-Specific RT-PCR Product

The eNOS-specific product cloned into pCR II vector and sequenced in both directions yielded 354-bp sequences from freshly dispersed rabbit gastric smooth muscle cells (Fig. 5, lane 6), cultured human intestinal smooth muscle cells (Fig. 5, lane 7), and cultured guinea pig taenia coli smooth muscle cells (Fig. 5, lane 2). Using specific primers for VEGFR, however, a RT-PCR product of the predicted size (209 bp) was amplified from cultured human endothelial cells (Fig. 5, lane 2) and freshly dispersed rabbit gastric smooth muscle cells (Fig. 5, lane 4) but not from cultured rabbit gastric, human intestinal, or taenia coli smooth muscle cells (Fig. 5, lanes 5–7).

Using specific primers for γ-actin, a RT-PCR product of the predicted size (80 bp) was amplified from freshly dispersed gastric smooth muscle cells but not from cultured endothelial cells.

Using two sets of c-kit-specific primers based on the sequences of human and bovine c-kit (36, 43), no RT-PCR product was amplified from either cultured rabbit gastric or human intestinal smooth muscle cells. A PCR product of the predicted size (534 bp) was obtained with human and bovine c-kit cDNA as template.

With all primers, no PCR product was obtained from endothelial or smooth muscle cells in the absence of RT.

Cloning and Sequence Analysis of eNOS-Specific RT-PCR Product

The eNOS-specific product cloned into pCR II vector and sequenced in both directions yielded 354-bp sequences from freshly dispersed rabbit gastric smooth muscle cells and cultured human intestinal smooth muscle cells. The nucleotide sequence of the cloned cDNA in cultured human intestinal smooth muscle cells differed by one nucleotide from the corresponding nucleotide sequence in human eNOS (99.7% similarity), whereas the sequence of the cloned cDNA in
freshly dispersed rabbit gastric smooth muscle cells differed by 23 nucleotides (93.5% similarity). The predicted amino acid sequence (117 residues) in either human or rabbit smooth muscle differed by only one amino acid residue from the corresponding sequence in endothelial cells (Fig. 6). The location of the single amino acid residue was different in humans and rabbits. Similarity of the human and rabbit eNOS nucleotide sequence with the corresponding human nNOS sequence (71.9% and 69.9%) and human iNOS sequence (65.7% and 64.9%) was lower.

Northern Blot Analysis
A single mRNA species of ~4.4 kb was detected on Northern blots using the PCR-generated and cloned eNOS cDNA from rabbits and humans as probes (Fig. 7). The mRNA from freshly dispersed and cultured rabbit gastric smooth muscle cells appeared to be more abundant than that from cultured human intestinal smooth muscle cells.

DISCUSSION
The present study demonstrates the expression of eNOS in freshly dispersed and cultured rabbit gastric smooth muscle cells and in cultured human intestinal smooth muscle cells. RT-PCR products were obtained in these cells with eNOS-specific primers but not with nNOS- or iNOS-specific primers; the results with eNOS-specific primers were confirmed by Southern blot analysis. Expression of eNOS mRNA (4.4 kb) was demonstrated by Northern blot analysis in both rabbit and human smooth muscle cells. Cloning and sequence analysis showed that the nucleotide sequences derived from eNOS-specific cDNA products in human intestinal and rabbit gastric smooth muscle cells were closely similar (93.5%–99.7%) to the previously reported human eNOS sequences (24, 37). Similarity to human nNOS and iNOS was distinctly lower (~70%). The predicted amino acid sequence (117 residues) in either human or rabbit smooth muscle differed by only one amino acid residue from the corresponding sequence in human endothelial cells. The location of the single amino acid residue was different in humans and rabbits. Decisive evidence for expression of eNOS in freshly dispersed single smooth muscle cells was obtained by in situ RT-PCR. Although some cells appeared to be damaged by the treatment, the majority (~65%) of cells exhibiting the spindle-shape morphology of smooth muscle cells were positive.
Muscle cells expressed eNOS; none were shown to express nNOS or iNOS.

Earlier immunohistochemical studies in smooth muscle tissues had failed to detect eNOS protein in smooth muscle cells (46). In the present study as well, eNOS protein could not be detected by immunoblot analysis, suggesting that the enzyme is not abundantly expressed in gastric and intestinal smooth muscle. As discussed below and shown in earlier functional studies, the enzyme is coupled to specific G proteins (Gi₁ and Gi₂), which greatly amplify its activity and thus offset the requirement for abundant expression of the enzyme (31).

A number of control studies were done to confirm that the amplified eNOS cDNA sequence was derived from RT of smooth muscle cell mRNA. In each experiment, both positive controls (i.e., PCR using NOS cDNA as template) and negative controls (i.e., PCR in the absence of RT) were included. The faint bands corresponding to eNOS in cultured muscle cells observed with RT-PCR were more evident when the same bands were probed for Southern blot analysis (Fig. 4). No bands were detected by RT-PCR or Southern blot analysis in the absence of RT, implying that the bands represented amplification of reverse-transcribed eNOS cDNA. Furthermore, cultures of taenia coli smooth muscle cells previously shown in functional experiments to be devoid of NOS activity (16) did not express eNOS, nNOS, or iNOS. Two endothelial cell markers, PECAM-1 and VEGFR, were used to exclude the possibility that eNOS was derived from endothelial cell contaminants. Both markers were detected by RT-PCR in endothelial cell cultures but not in rabbit gastric or human intestinal smooth muscle cell cultures. However, VEGFR but not PECAM-1 was detected in freshly dispersed gastric smooth muscle cells, suggesting the presence of endothelial cell contaminants; these disappeared in cultured gastric smooth muscle cells, which nonetheless maintained their ability to express eNOS.

Interstitial cells of Cajal located at the boundaries of the circular smooth muscle layer in vivo and making up less than 1% of the cells in this layer appear to express a constitutive NOS, possibly eNOS, in dogs but not in humans (13, 46). Cultures of rabbit gastric and human intestinal smooth muscle cells did not express c-kit, a marker for interstitial cells, implying that smooth muscle cell cultures from both species were devoid of interstitial cells and further confirming that the eNOS transcripts amplified by RT-PCR were derived from smooth muscle cells.

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</table>

![Fig. 6. Comparison of amino acid sequences predicted from nucleotide sequences in rabbit gastric and human intestinal SMC with corresponding sequences of eNOS from human umbilical vein endothelial cells and human platelets. The sequences (117-amin acid residues) in rabbit gastric and human intestinal muscle cells and in human platelets differed by one amino acid residue, each at a different location (asterisk) from the sequence in human endothelial cells.](http://ajpgi.physiology.org/)

![Fig. 7. Identification of eNOS transcripts in rabbit and human SMC by Northern blot analysis. Total RNA was fractionated and hybridized with PCR-generated and cloned eNOS cDNA probe. A 4.4-kb transcript corresponding to eNOS was detected from freshly dispersed rabbit gastric SMC (lane 1), cultured rabbit gastric SMC (lane 2), and cultured human intestinal SMC (lane 3).](http://ajpgi.physiology.org/)
A recent report by Chakder et al. (4) claimed that nNOS was expressed in smooth muscle cells of the internal anal sphincter in the opossum. The authors did not study expression of eNOS, even though a report of our results on eNOS expression had already been published in abstract form (40). The authors based their claim mainly on RT-PCR and Northern blot analysis of primary muscle cultures from the internal anal sphincter. However, the derivation of the RT-PCR product was not confirmed by control experiments without RT. Furthermore, muscle cell suspensions prepared from opossum internal anal sphincter are known to be highly susceptible to contamination by neuronal ganglia, which survive during primary culture of smooth muscle cells and are probably responsible for the nNOS signal.

The expression of a constitutive, membrane-bound, Ca<sup>2+</sup>/calmodulin-dependent NOS selectively activated by VIP and PACAP is supported by a large number of functional studies (e.g., see Refs. 28 and 32). The enzyme exhibits similar properties in smooth muscle cells from various regions of the gut (stomach, intestine, colon, and lower esophageal and internal anal sphincters) in different species (human, dog, rabbit, guinea pig, opossum, rat, and mouse) (5, 11). The unique properties of eNOS when expressed in gastrointestinal smooth muscle and the derivation of NO from muscle cells are summarized below.

These properties include the ability of VIP, PACAP, and atrial natriuretic peptide (ANP) to activate NOS in smooth muscle cells. The activation results from high-affinity interaction of all three peptides with the natriuretic peptide clearance receptor (NPR-C), which is coupled via G<sub>13</sub> and G<sub>12</sub> to activation of membrane-bound NOS and stimulation of Ca<sup>2+</sup>-influx (17, 20, 31).

Our recent studies have confirmed the ability of VIP, ANP, and the selective NPR-C agonist, cANP-(4—23), to activate NOS in COS-1 cells cotransfected with eNOS and NPR-C (42). When expressed in gastrointestinal smooth muscle, eNOS is highly susceptible to inactivation by protein kinase C, which explains why Ca<sup>2+</sup>-mobilizing agonists do not activate smooth muscle NOS (29).

The derivation of NO from smooth muscle cells is confirmed by studies with oxyhemoglobin. Relaxation and cGMP formation induced by VIP and PACAP in dispersed smooth muscle cells are unchanged in the presence of oxyhemoglobin, which neutralizes NO derived from nonmuscle cell elements. cGMP formation in the presence of oxyhemoglobin is abolished by the NOS inhibitor N<sup>Г</sup>-nitro-L-arginine, whereas relaxation is partly inhibited; the residual relaxation reflects the ability of VIP to interact with VIP<sub>2</sub>/PACAP<sub>3</sub> receptors coupled to activation of adenylly cyclase (28, 32, 41). Furthermore, NO formed in muscle tissue during nerve
stimulation is largely derived from smooth muscle cells; VIP receptor antagonists inhibit neurally stimulated NO formation by 60–80% in different species, implying that the predominant component of NO formed during nerve stimulation is derived from smooth muscle cells (18). Recent studies in knockout mice lacking either nNOS or eNOS confirm that NO responsible for neurally induced relaxation is dependent on activation of both isozymes (25, 26).

The functional significance of eNOS in smooth muscle of the gut resides in the ability of this isoform to amplify NO-dependent inhibitory neurotransmission. In vascular smooth muscle, NO diffuses from adjacent endothelial cells, whereas in smooth muscle of the gut NO is released initially at neuromuscular junctions concurrently with the inhibitory neurotransmitters, VIP and PACAP. The relatively minor NO component derived from nNOS activity in nerve terminals regulates the release of VIP and PACAP; interaction of the latter with smooth muscle receptors (NPR-C) stimulates the formation of the predominant NO component in smooth muscle.

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