Differential Isoactin Gene Expression in the Sphincteric and Nonsphincteric Gastrointestinal Smooth Muscles of the Opossum (43713)

REBECCA A. LIDDELL* SUSHANTA CHAKDER,† SATISH RATTAN,† AND KIRK M. MCHUGH*,[1]

Department of Anatomy and Developmental Biology and Department of Medicine,† Thomas Jefferson University, Philadelphia, Pennsylvania 19107*

Abstract. **The sphincteric smooth muscle tissues of the gastrointestinal tract have been shown to possess mechanical properties distinct from the surrounding nonsphincteric smooth muscle tissues. Little is currently known regarding the molecular basis of this differential smooth muscle development and function. Actin is an important contractile protein whose expression has been linked to the normal development and function of smooth muscle tissues. The purpose of this study was to characterize isoactin gene expression in the sphincteric versus nonsphincteric smooth muscle tissues of the gastrointestinal tract. Northern blot analysis was performed on manometrically identified sphincteric and the flanking nonsphincteric smooth muscle tissues of the adult opossum. Quantitative analysis revealed a distinct pattern of isoactin gene expression in the sphincteric versus nonsphincteric smooth muscle tissues of the gut. The sphincteric smooth muscle tissues expressed significantly lower total quantities of isoactin mRNA than their surrounding nonsphincteric smooth muscle tissues. It is hypothesized that these differential patterns of isoactin gene expression may play a significant role in establishing the myogenic potential of the functionally distinct sphincteric and nonsphincteric smooth muscle tissues of the gut.**

[P.S.E.B.M. 1994, Vol 205]

The mature gastrointestinal tract is a complex organ system that becomes functionally compartmentalized by the development of a series of unidirectional smooth muscle sphincters (1). Prior studies (1–5) have demonstrated that gastrointestinal smooth muscle sphincters possess mechanical properties which are distinct from the surrounding nonsphincteric smooth muscle tissues. The sphincteric smooth muscles of the gut have been characterized as tonic because they possess a high resting tone and sustained response to physiological/pharmacological stimuli. In contrast, the nonsphincteric smooth muscles of the gut lack spontaneous tone, respond rapidly and transiently to stimuli, and are characterized as phasic. Several studies have suggested that the basis for this difference in resting tone is myogenic in nature (6–9).

Actin is an important cytoskeletal protein whose primary functions include maintenance of the cytoskeleton and muscle contractility. Recent studies (10–12)[1] have characterized the tissue-specific expression of the actin multigene family in the developing and mature gastrointestinal tract of the rat/mouse. These studies demonstrated that the cytoplasmic isoactins (β-cytoplasmic and γ-cytoplasmic) and smooth muscle isoactins (γ-smooth muscle and α–smooth muscle) were differentially coexpressed in the developing and

[1] To whom requests for reprints should be addressed at Department of Anatomy and Developmental Biology, Thomas Jefferson University, Philadelphia, PA 19107.

Received September 1, 1993. [P.S.E.B.M. 1994, Vol 205]
Accepted December 16, 1993.

0037-9727/94/2054–0321$10.50/0

[1] McHugh KM. *In situ* analysis of γ–smooth muscle and α–smooth muscle isoactin expression in the developing mouse embryo. In preparation, 1993.

mature gastrointestinal tract of rats/mice. The β-cytoplasmic and γ-cytoplasmic isoactins are ubiquitously coexpressed in all of the cell types of the gastrointestinal tract and are the primary actin isoforms associated with the cytoskeleton. In contrast, the expression of the γ–smooth muscle and α–smooth muscle isoactins is principally limited to developing and mature gastrointestinal smooth muscle cells. The primary role of the smooth muscle isoactins involves myofilament formation and muscle contractility. The distinct temporal and spatial patterns of isoactin gene expression observed during gastrointestinal development and maturation suggest that actin may play a critical role in defining some of the functional diversity observed in the gastrointestinal tract.

Little is currently known regarding the expression of the actin multigene family in the sphincteric and nonsphincteric smooth muscle tissues of the gut. The purpose of this present study was to determine if these functionally distinct smooth muscle tissues displayed differential patterns of isoactin gene expression. Specific sphincteric and their flanking nonsphincteric smooth muscle tissues were manometrically identified and isolated from the adult opossum gastrointestinal tract. Northern blot analysis was performed on total cellular RNA isolated from these specific smooth muscle segments using an actin-specific coding region cDNA probe. The results of this analysis demonstrated that a distinct pattern of isoactin gene expression was observed in the sphincteric versus nonsphincteric smooth muscle tissues of the gastrointestinal tract.

Materials and Methods

Northern Blot Analysis. A single adult opossum was anesthetized with pentobarbital sodium and the lower esophageal sphincter, pyloric sphincter, ileocecocolic sphincter, and internal anal sphincter were manometrically characterized as previously described (8). Briefly, manometry of different sphincters was performed after thoracotomy, laparotomy, and pelvis sectioning. Open-tip catheters perfused by a low-compliant pneumohydraulic water perfusion system were used to record the intraluminal pressures to define the limits of sphincters. The pressure was recorded on a Dynograph Recorder (Model R611; SensorMedics Corp., Anaheim, CA) via Statham pressure transducers. By establishing the anatomical relationship between the recording tip and the manometric limits of the sphincters, the boundaries of the sphincters could be identified and marked with silk sutures. The individual sphincters and their surrounding nonsphincteric tissues were carefully excised and the mucosa and adventitia/serosa were removed. In this manner, distinct smooth muscle segments were isolated from the lower esophageal sphincter and adjacent esophagus and stomach, the pyloric sphincter and adjacent stomach and duodenum, the ileocecocolic sphincter and adjacent ileum and colon, and the internal anal sphincter and adjacent rectum. The individual smooth muscle segments were immediately frozen in liquid nitrogen and stored at −70°C until RNA isolation. Each smooth muscle tissue sample was coded and Northern blot analysis was performed blindly on randomly numbered samples.

Total cellular RNA was isolated from 1.2 g or less of tissue by the methods of Chirgwin *et al.* (13) and quantitated spectrophotometrically. Quantitation values were visually confirmed by agarose/formaldehyde gel electrophoresis in the presence of 100 μg/ml ethidium bromide. Northern blot analysis was performed with 20 μg of total cellular RNA by the method of Thomas (14) using 2% agarose/formaldehyde gels and Biotrans nylon membranes (ICN Pharmaceuticals Inc., East Hills, NY). Blots were probed with the Pst 1 fragment of the full length γ–smooth muscle isoactin cDNA, pREγA-11, a previously described (15) actin-specific coding region cDNA that recognizes all six vertebrate isoactins (the α-skeletal, α-cardiac, α–smooth muscle, γ–smooth muscle, γ-cytoplasmic, and β-cytoplasmic isoactins). Prehybridization, hybridization, and wash conditions were identical to those previously described (15). Briefly, Northern blots were prehybridized/hybridized in 6× SSC (1× SSC is 0.15 *M* NaCl/0.015 *M* sodium citrate), 5× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 100 m*M* sodium phosphate, pH 6.5 and 250 μg denatured salmon sperm DNA/ml at 65°C for 2 hr. Denatured probe was added directly to this mixture and incubated with shaking at 65°C overnight. The membranes were washed three times for 5 min and one time for 20 min at room temperature in 2× SSC/0.2% SDS. The membranes were then washed one time for 20 min at room temperature and one time for 1 hr at 65°C in 0.2 × SSC/0.2% SDS. Individual blots were normalized to total RNA loaded by methylene blue staining of the membrane posthybridization (16).

Densitometry. Individual blots were exposed to Kodak (Rochester, NY) X-Omat AR film using an intensifying screen for an appropriate period of time. Each Northern blot was quantitated using an LKB Ultrascan XL Enhancer Laser Densitometer and the Gelscan XL program (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Multiple exposures were chosen to ensure that each lane scanned was in the established linear range for these studies. The quantitation values reported for each lane represent an average of at least two different scannings of the same band to eliminate band inconsistencies. Quantitation values were normalized against the highest level of isoactin expression observed in the study which was assigned a value of 1.

Results

Northern blot analysis (Fig. 1) of smooth muscle tissues isolated from the lower esophageal sphincter and the flanking esophagus and stomach, pyloric sphincter and the flanking stomach and duodenum, ileocecocolic sphincter and the flanking ileum and cecum, and internal anal sphincter and the flanking rectum, with an actin coding region probe, identified two distinct bands at 1.2 kilobases (kb) and 2.2 kb, respectively. Prior studies (15, 17, 18) have demonstrated the efficacy of using actin coding region probes to identify all six actin isoforms (α-skeletal, α-cardiac, β-cytoplasmic, γ-cytoplasmic, γ–smooth muscle, and α–smooth muscle) upon Northern blot analysis. These studies have shown that the 1.2 kb band is composed of the smooth muscle isoactins (γ–smooth muscle and α–smooth muscle) while the 2.2 kb band is composed of the cytoplasmic isoactins (β-cytoplasmic and γ-cytoplasmic). No expression of the striated muscle isoactins (α-skeletal and α-cardiac) at 1.4 kb was observed. Quantitation of total cellular RNA was verified by ethidium bromide staining of the gels before transfer (Fig. 2), and equivalent transfer was verified posthybridization by staining of the membrane with methylene blue (data not shown). Initial Northern blot analyses with the rat-specific smooth muscle isoactin cDNAs demonstrated that these isoactin-specific probes did not cross-react with opossum total cellular RNA (data not shown). No opossum-specific smooth muscle isoactin cDNAs currently exist.

Quantitative analysis (Fig. 3A–D) demonstrated that the smooth muscle isoactins were the predominant actin isoforms expressed in both the sphincteric and nonsphincteric smooth muscle tissues of the gut. A relatively consistent percentage of 72% ± 7% smooth muscle isoactins to 28% ± 7% cytoplasmic isoactins was observed in all of the smooth muscle tissues examined. A direct comparison of the sphincteric versus nonsphincteric smooth muscle tissues revealed a distinct pattern of isoactin gene expression in these functionally distinct smooth muscle tissues. The lower esophageal sphincter, pyloric sphincter, ileocecocolic sphincter, and internal anal sphincter all expressed significantly less smooth muscle and cytoplasmic isoactin mRNA than their flanking nonsphincteric smooth muscle tissues. The absolute level of smooth muscle and cytoplasmic isoactin expression appeared to be sphincter-specific, but the general pattern described above was consistently observed for all of the sphincters examined in this study.

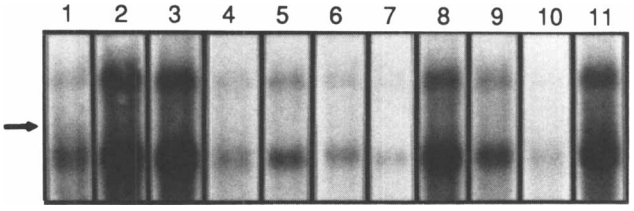

Figure 1. Northern blot analysis of isoactin gene expression in sphincteric and nonsphincteric smooth muscle tissues of the adult opossum. Twenty micrograms of total cellular RNA was blotted from adult opossum internal anal sphincter (1), rectum (2), colon (3), ileocecocolic sphincter (4), ileum (5), duodenum (6), pyloric sphincter (7), pyloric region of stomach (8), cardiac region of stomach (9), lower esophageal sphincter (10), and esophagus (11). The arrow indicates the position of the 18S standard. The lower band represents the 1.2 kb smooth muscle isoactin band while the upper band represents the 2.2 kb cytoplasmic isoactin band.

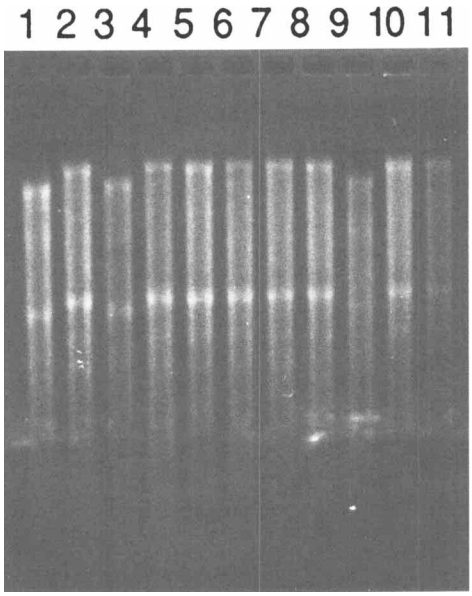

Figure 2. Control ethidium bromide stained gel of the equivalent total cellular RNA samples used for Northern blot analysis in Figure 1.

Discussion

Actin's role in muscle contractility has been well documented and recent evidence (19) suggests that actin may play an active role in modifying the contractile state of smooth muscle tissues. The smooth muscle isoactins were the predominant actin isoforms expressed in both the sphincteric and nonsphincteric smooth muscle tissues of the gut although relatively high levels of cytoplasmic isoactin expression were also observed. Similar differential patterns of isoactin gene expression have been observed in a variety of gastrointestinal tissues (10, 11). These earlier studies demonstrated that the gastrointestinal tract of the rat coordinately expressed high levels of γ–smooth muscle isoactin, moderate levels of α–smooth muscle and β-cytoplasmic isoactins, and low levels of γ-cytoplasmic isoactin. The similar patterns of smooth muscle and cytoplasmic isoactin expression observed in this study indicate that these actin isoforms are coordinately regulated in both the sphincteric and nonsphincteric smooth muscle tissues of the gut. Similar obser-

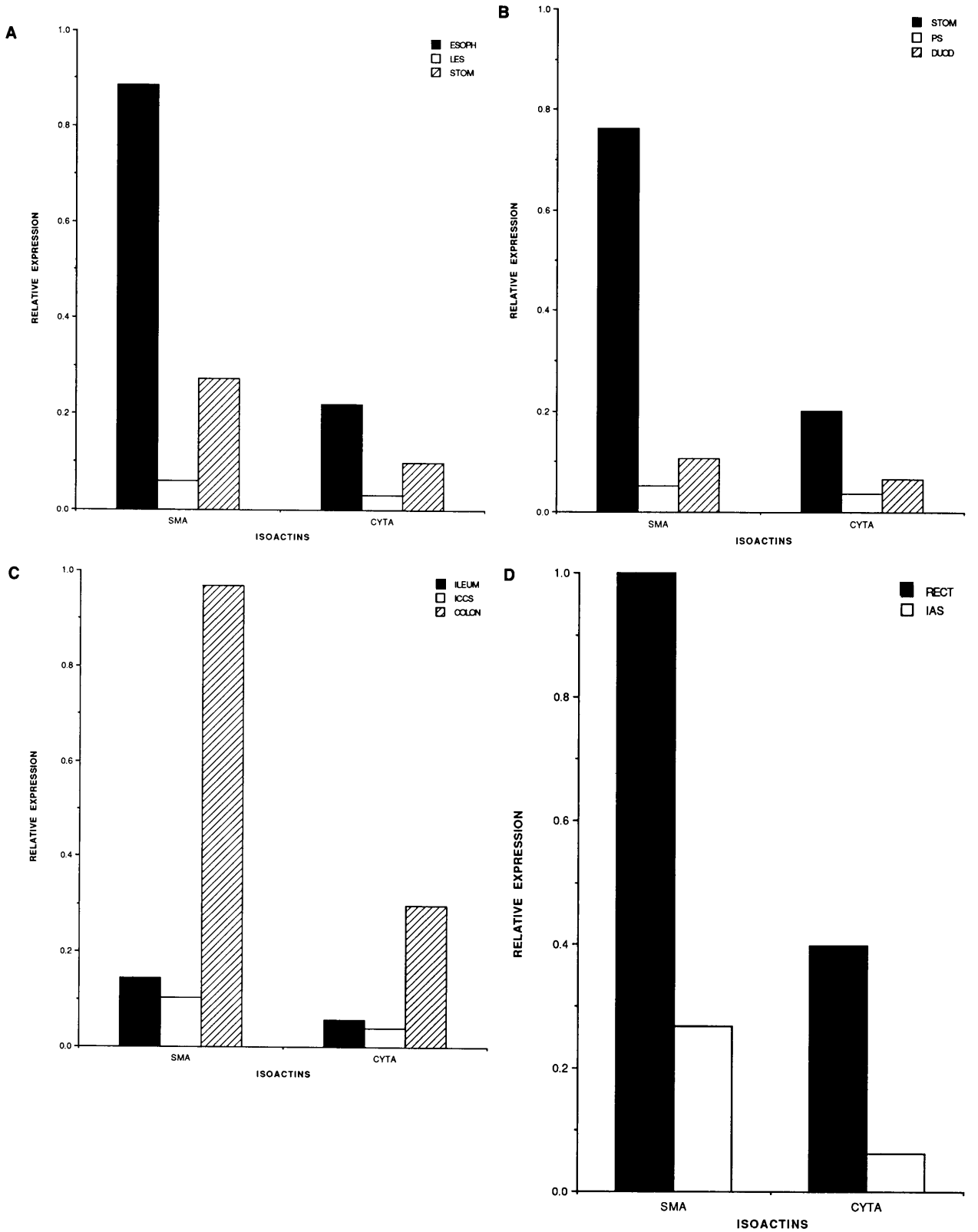

Figure 3. Relative expression of the smooth muscle (SMA) and cytoplasmic (CYTA) isoactins in adult opossum esophagus (ESOPH), lower esophageal sphincter (LES), and stomach (STOM) (A); stomach (STOM), pyloric sphincter (PS), and duodenum (DUOD) (B); ileum, ileocecocolic sphincter (ICCS), and colon (C); and rectum (RECT) and internal anal sphincter (IAS) (D). Quantitation was performed as described in Materials and Methods.

vations in previous studies have led to the suggestion that the cytoplasmic isoactins may possess an expanded functional role in smooth muscle tissues. This hypothesis is supported by recent studies by Drew *et al.* (20) which showed that smooth muscle myofilaments are composed of a random array of smooth muscle and cytoplasmic isoactin proteins. Taken together, these observations make it tempting to speculate that this mixed myofilament composition may contribute in part to the functional differences observed between various smooth muscle tissues, and that the functional significance of the various actin isoforms may reside at the level of myofilament composition rather than the specific isoactin(s) expressed.

Differential patterns of isoactin protein expression have been reported in a variety of gastrointestinal smooth muscle tissues (9, 21–23). Fatigati and Murphy (9) have also reported significant differences in the ratio of α–smooth muscle/γ–smooth muscle isoactin protein between the opossum esophagus and lower esophageal sphincter. The results of this study significantly extend these earlier observations by examining multiple sphincteric and nonsphincteric smooth muscle tissues of the gut, and demonstrating that the previously observed differential patterns of isoactin protein expression are the result of distinct differential patterns of isoactin gene expression. These observations, in conjunction with earlier studies, indicate that there is a high degree of concordance (approximately 90% or greater) between isoactin gene expression and protein production in gastrointestinal smooth muscle tissues. This data suggests that the distinct patterns of isoactin expression observed in sphincteric and nonsphincteric smooth muscle tissues are primarily regulated at the level of gene transcription/message stability.

It has been well established that the sphincteric and nonsphincteric smooth muscle tissues of the gastrointestinal tract possess distinct functional differences. However, little is currently known regarding the molecular basis for these functional differences. The results of this study demonstrate that the sphincteric smooth muscle tissues of the gut express significantly less smooth muscle and cytoplasmic isoactin mRNAs than the surrounding nonsphincteric smooth muscle tissues. Whether these low levels of isoactin message result in similar low levels of isoactin protein remains to be determined. However, the high degree of concordance between isoactin message and protein previously observed for most gastrointestinal smooth muscle tissues would strongly suggest that the sphincteric smooth muscle tissues of the gut also express significantly less smooth muscle and cytoplasmic isoactin protein. Therefore, it would appear that the sphincteric smooth muscle tissues of the gut display both quantitative as well as qualitative differences in isoactin gene/protein expression from the surrounding nonsphincteric smooth muscle tissues. The functional significance of these observations remains to be determined. However, actin's critical role in muscle contractility suggests that these differential patterns of isoactin gene expression may play an important role in establishing the myogenic potential of the functionally distinct sphincteric and nonsphincteric smooth muscle tissues of the gut.

In summary, the results of this study demonstrate that the sphincteric smooth muscle tissues of the gut appear genotypically distinct from the surrounding nonsphincteric smooth muscle tissues. The precise basis for these phenotypic/genotypic differences remains to be determined. However, a variety of factors including neurotransmitters, neuropeptides, cytokines, and growth factors have been shown to effect gastrointestinal smooth muscle differentiation, maturation, and function. For example, recent studies by Clancy *et al.* (24) have shown that nitric oxide, an important second messenger/neurotransmitter of the gastrointestinal tract (25–27), can directly alter the state of actin polymerization. Such observations, in conjunction with the results of this study, provide an exciting correlate between the regulation of gastrointestinal smooth muscle function, actin filament dynamics, and differential isoactin gene expression in the smooth muscle tissues of the gut. In conclusion, this study represents the first report of differential isoactin gene expression in the sphincteric versus nonsphincteric smooth muscle tissues of the gut, and provides a basis for future studies designed to investigate the molecular mechanisms responsible for differential smooth muscle development and function in the gastrointestinal tract.

This work was supported in part by the Foerderer Foundation and the National Institute of Health Grant HD27252 and HD00996, and the National Institute of Diabetes and Digestive and Kidney Diseases Grant DK35385.

1. Papasova M. The gastrointestinal system: Sphincteric function. In: Schultz SG, Wood JD, Rauner BB, Eds. Handbook of Physiology: Motility and Circulation. Bethesda: American Physiological Society, pp987–1023, 1989.
2. Biancani P, Zabinski M, Kerstein M, Behar J. Comparison of mechanical characteristics of the lower esophageal sphincter (LES) and pyloric sphincter (PS). In: Duthie HL, Ed. Gastrointestinal Motility. Trowbridge, UK: Esher, pp547–550, 1978.
3. Christensen J, Freeman B, Miller J. Some physiological characteristics of the esophogastric junction in the opossum. Gastroenterology **64**:1119–1125, 1973.
4. Golenhoffen K, Weiser H, Stewert R. Phasic and tonic types of smooth muscle activity in lower esophageal sphincter and stomach of the dog. Acta Hepta Gastroent **26**:227–234, 1979.
5. Rattan S, Goyal R. Free intracellular calcium ($[Ca^{2+}]_i$) in unstimulated and stimulated lower esophageal sphincter (tonic) and esophageal body (phasic) smooth muscle. Gastroenterology **91**:1064, 1986.

6. Goyal R, Rattan S. Genesis of basal sphincter pressure: Effect of tetrodotoxin on lower esophageal sphincter pressure in opossum *in vivo*. Gastroenterology **71**:62–67, 1976.
7. Goyal R, Rattan S. Effects of sodium nitroprusside and verapamil on lower esophageal sphincter. Am J Physiol **238**:G40–G44, 1980.
8. Culver P, Rattan S. Genesis of anal canal pressures in the opossum. Am J Physiol **251**:G765–G771, 1986.
9. Fatigati V, Murphy R. Actin and tropomyosin variants in smooth muscle. J Biol Chem **23**:14383–14388, 1984.
10. McHugh K, Crawford K, Lessard J. A comprehensive analysis of the developmental and tissue-specific expression of the isoactin multigene family in the rat. Dev Biol **148**:442–458, 1991.
11. Liddell R, Syms M, McHugh K. Heterogeneous isoactin gene expression in the adult rat gastrointestinal tract. Gastroenterology **105**:347–356, 1993.
12. Liddell R, Mooers S, Siegman M, McHugh K. Altered isoactin gene expression in the affected bowel segments of the lethal spotted mouse. Gastroenterology **105**:441–448, 1993.
13. Chirgwin J, Pryzbla A, MacDonald R, Rutter W. Isolation of biologically active ribonucleotide acid from sources enriched in ribonucleases. Biochem **18**:5294–5299, 1979.
14. Thomas P. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Nat Acad Sci USA **77**:5201–5205, 1980.
15. McHugh K, Lessard J. The developmental expression of the rat α-vascular and γ-enteric smooth muscle isoactins: Isolation and characterization of a rat γ-enteric actin cDNA. Mol Cell Biol **8**:5224–5231, 1988.
16. Maniatis T, Fritsch E, Sambrook J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, p206, 1982.
17. Erba H, Gunning P, Kedes L. Nucleotide sequence of the human γ-cytoskeletal actin mRNA: Anomalous evolution of vertebrate nonmuscle actin genes. Nucleic Acids Res **14**:5275–5294, 1986.
18. Gunning P, Ponte P, Okayama H, Engel S, Balu H, Kedes L. Isolation and characterization of full-length cDNA clones for human α-, β-, and γ-actin mRNAs: Skeletal but not cytoplasmic actins have an amino-terminal cysteine that is sequentially removed. Mol Cell Biol **3**:787–795, 1983.
19. Morel J, Merah Z. Muscle contraction and *in vitro* movement: Role of actin? J Muscle Cell Motility **13**:5–6, 1992.
20. Drew J, Moos C, Murphy, R. Localization of isoactins in isolated smooth muscle thin filaments by double gold immunolabeling. Am J Physiol **260**(Cell Physiol 29):C1332–C1340, 1991.
21. Vandekerckhove J, Weber K. At least six different actins are expressed in a higher mammal: An analysis based on the amino acid sequence of the amino-terminal tryptic peptide. J Mol Biol **122**:783–802, 1978.
22. Vandekerckhove J, Weber K. The complete amino acid sequence of actins from bovine aorta, bovine heart, bovine fast skeletal muscle, and rabbit slow skeletal muscle. Diff **14**:123–133, 1979.
23. Kuroda M. Change of actin isomers during differentiation of smooth muscle. Biochim Biophys Acta **843**:208–213, 1985.
24. Clancy R, Leszczynska-Piziak J, Abramson S. Nitric oxide stimulates the ADP-ribosylation of actin in human neutrophils. Biochem Biophys Res Commun **191**:847–853, 1993.
25. Stark M, Szurszewski J. Role of nitric oxide in gastrointestinal and hepatic function and disease. Gastroenterology **103**:1928–1949, 1992.
26. Rattan S, Chakder S. Role of nitric oxide in gastrointestinal and hepatic function and disease. Gastroenterology **103**:1928–1949, 1992.
27. Rattan S, Farkar A, Chakder S. Nitric oxide pathway in rectoanal inhibitory reflect of opossum internal anal sphincter. Gastroenterology **103**:43–50, 1992.