FUNCTIONAL AND MORPHOLOGICAL EFFECTS OF INFLAMMATION ON OPOSSUM ESOPHAGEAL SMOOTH MUSCLE CELLS

by

Ron W. Wells

A thesis submitted to the Department of Biology
in conformity with the requirements
for the degree of Master of Science

Queen's University
Kingston, Ontario, Canada
November 2001

Abstract:

Inflammation of the esophagus results in altered contractility of the smooth muscle layers. Previous studies using muscle strips showed that circular smooth muscle (CSM) becomes hypo-responsive while longitudinal smooth muscle (LSM) becomes hyper-responsive. To determine whether altered contractility is a result of changes to the surrounding tissue or due to direct alterations in smooth muscle cells, isolated esophageal smooth muscle cells were examined. Erosive esophagitis similar to human reflux disease was induced in opossum esophagi by perfusing intraluminally for 45 minutes on each of 3 consecutive days with either 0.9% saline (control; n=8) or 100 mM HCl (n=9). 24 hours after the final perfusion, the esophagus was excised and tissue 3 – 5 cm proximal to the LES was removed. Isolated CSM and LSM cells were obtained by dissection and enzymatic digestion, exposed to various concentrations of carbachol (CCh) for 30 seconds, and fixed with acrolein. Cell length was determined by video microscopy (30 cells/animal). The mean resting cell length was unchanged between controls and esophagitis animals (CSM & LSM, $p>0.05$). Resting length of the CSM cells was significantly greater that the LSM cells ($p<0.01$). CCh resulted in a dose-dependent contraction for both CSM and LSM cells, with maximal contraction occurring at 1 and 10 nM, respectively. No differences in CCh-induced contraction were noted between control and esophagitis LSM cells ($p>0.05$), however, there was marked attenuation in the contraction of CSM cells at the higher concentrations of CCh. At 1 nM CCh contraction decreased from 21.1±2.0% in control animals to 8.7±1.6% in esophagitis animals ($p<0.001$). There was significant hypertrophy of CSM cells as determined by direct ($p<0.01$) and relative ($p<0.05$) measures for cellular hypertrophy. There was a significant increase ($p<0.01$) in nuclear

size in CSM cells isolated from inflamed tissue compared to controls. However, no significant differences were observed in LSM cells. These studies suggest that the decreased contractility of CSM of the inflamed esophagus can, at least in part, be directly ascribed to alterations of the CSM cell. In contrast, the hyper-contractility demonstrated in LSM strips is most likely related to other factors in the surrounding tissue interacting with the LSM cells.

Acknowledgements:

I would like to take this opportunity to extend my deepest gratitude to everyone whom has helped me in the completion of this thesis. First and foremost I would like to thank my supervisors and mentors Dr. William Paterson and Dr. Gerald Morris who with their vast knowledge and great patience, have given me the wonderful opportunity of experiencing graduate work with the GIDRU.

I would like to extend a special thanks to Dr. Michael Blennerhassett and David Miller. Dr. Blennerhassett has helped me with the finer details of graduate work and without his help I never would have made it this far. Without the help of Super Dave Miller I never would have been able to do this. Dr. Praveen Roy and I learned the hard way just how skilled Dave is with opossums.

I would like to express my appreciation to Drs. Steven Vanner, Sandra Lourenssen, Yong Zhang, Ivan Beck, Kerri Hill, and all the members of GIDRU whom have all helped me develop my understanding and knowledge of the GI system. All of your knowledge and input have guided me, and will continue to guide me in the future.

I would like to thank my good friends Jason Kovac, Agueda Lin, David Reed, Tim Stewart and Robert White who have helped me work through the highs and lows involved in graduate work and who have helped me in the preparing and running a few clinics. To my parents, Mike and Darla, whom have always supported me, I thank you.

Finally, I am forever indebted to my lovely, vivacious fiancée Sarah. Over the past five years, she has stood by my side and always supported me in all of my endeavors. Without her love and support I would not be where I am today and I owe her for everything that I am and will become.

Table of Contents:

List of Tables:

Table #

List of figures

Figure #

Abbreviations:

#	number
%	percent
°C	degrees Celsius
μm	micometre
μM	micro-molar
AA	arachidonic acid
ACh	acetylcholine
bpm	beats per minute
BSA	bovine serum albumin
Ca^{2+}	calcium cation
cADPR	cyclic adenosine diphospho-ribose
CaM	calmodulin
CCh	carbachol
CCK	cholecytokinin
Cl^-	chloride anion
cm	centimeter
CO_2	carbon dioxide
$cPLA_2$	cytosolic phospholipase A_2
CSM	circular smooth muscle
DAG	1,2-diacylglycerol
DNBS	dinitrobenzene sulfonic acid
DTT	DL-dithiothreitol
EFS	electrical field stimulation
ER	endoplasmic reticulum
GERD	gastroesophageal reflux disease
GI	gastrointestinal
GMC	giant migrating contractions
G-protein	GTP-binding protein
GTP	guanosine 5-triphosphate

HCl	hydrochloric acid
HIS	histamine
HPSS-df	HEPES physiological salt solution-digestion formula
HPSS-if	HEPES physiological salt solution-isolation formula
IL	interleukin
IP_3	inositol-1,4,5-triphosphate
KCl	potassium chloride
kg	kilogram
LES	lower esophageal sphincter
LSM	longitudinal smooth muscle
M_1-M_5	muscarinic receptors type 1-5
MEA	myoelectric activity
mg	milligram
min	minute
ml	millilitre
MLC	myosin light chain
mM	millimolar
mm	millimetre
MMC	migrating motor complexes
n=	sample size
NANC	nonadrenergic-noncholinergic
nM	nanomolar
NO	nitric oxide
O_2	oxygen
p=	probability that the null hypothesis is true
PC	phosphatidylcholine
PC-PLC	phosphatidylcholine-specific phospholipase C
PC-PLD	phosphatidylcholine-specific phospholipase D
PG	prostaglandin
PIP	phosphatidylinositol 4-monophosphate

PIP_2	phosphatidylinositol 4.5-bisphosphate
PI-PLC	phosphatidylinositol-specific phospholipase C
PKC	protein kinase C
PLA_2	phospholipase A_2
PTX	pertussis toxin
rpm	respirations per minute
SE	standard error
SMC	smooth muscle cell
SP	substance P
SPC	spontaneous phasic contractions
$sPLA_2$	secretory type phospholipase A_2
TNF	tumor necrosis factor
UES	upper esophageal sphincter
VIP	vasoactive intestinal polypeptide
X	magnification level

Overview:

Inflammation causes many physiological and functional changes to the esophagus. Prolonged exposure to acid can cause esophagitis and might, in some cases, lead to the development of a premalignant condition known as Barrett's esophagus. Previous studies from our laboratory have shown that inflammation of the esophagus causes altered contractility of the two muscle layers. In whole organ and muscle strip studies, it was shown that the inner circular smooth muscle layer became hypo-responsive (Shirazi *et al.*, 1989), whereas the outer longitudinal smooth muscle layer became hyper-responsive (White *et al.*, 2001) to excitatory stimuli. Our laboratory has also shown that esophagitis causes an axial shortening of the esophagus due to sustained contraction of the longitudinal muscle layer (White *et al.*, 2001). However, it is not known whether these changes in contractility are a result of alterations to the muscle cells directly or a reflection of changes in inflammatory mediators within the tissue.

Studies on inflammation of intestinal smooth muscle have shown the development of altered function and morphology. Along with inflammation-induced hypo- and hyper-responsiveness of the circular and longitudinal smooth muscle tissue, respectively, there was the development of hypertrophy and hyperplasia (Blennerhassett *et al.*, 1992). While this established a cellular basis for the altered contractility in the inflamed intestine, similar studies have not been performed in the inflamed esophagus.

Most of the information on the effects of inflammation on smooth muscle contractility comes from studies done on the intact organ and/or muscle strips. Since smooth muscle strips retain intrinsic innervation, it is often difficult to distinguish between the myogenic and neurogenic effects of smooth muscle agonists. The distinction

is usually made indirectly by the use of specific antagonists or neurotoxins, such as tetrodotoxin. The study of isolated smooth muscle cells, produced by enzymatic digestion, offers an opportunity to elucidate the myogenic response of smooth muscle. Furthermore, the use of dispersed cells eliminates the effects of intercellular coupling and minimizes diffusional barriers thus facilitating the analysis of stimulus-receptor interactions. Therefore, the aim of this thesis was to use isolated smooth muscle cells, from the inflamed opossum (*Didelphis virginiana*) esophagus, to determine if the altered function is due to alterations in the smooth muscle cell or to changes in mediators in the milieu acting on the syncytium.

Overview of esophageal function:

Anatomical considerations:

The primary role of the esophagus is the transportation of food to the stomach for further digestion. This is achieved by a series of aborally propagated contractions, known as primary peristalsis. In humans the esophagus is approximately 18 – 22 cm in length, with the beginning being identified by the presence of the upper esophageal sphincter (UES) (located about 18 cm distal to the incisors) and the termination by the lower esophageal sphincter (LES) (DeMeester *et al.*, 1999; Goyal and Sivarao, 1999; Goyal and Paterson, 1989).

In humans the proximal 2 – 4 cm of the esophagus is composed entirely of striated muscle, whereas the middle section (approximately 4 – 13 cm distal to the UES) is composed of equal amounts of striated and smooth muscle. Finally, the segment of the

esophagus distal to this 13 cm region (approximately the lower 1/2 to 2/3 of the esophagus) is composed entirely of smooth muscle (Goyal and Sivarao, 1999; Christensen *et al.*, 1973). The distal 2/3 of the opossum esophagus is composed entirely of smooth muscle (Goyal and Paterson, 1989), adding to its value as model for human esophageal function in both normal and diseased states.

Like other regions of the gastrointestinal (GI) tract, the esophagus consists of a mucosa, submucosa and muscularis propria, the latter being composed of inner circular and outer longitudinal muscle layers, separated by the myenteric plexus, a thin layer of ganglionated nervous tissue (Goyal and Sivarao, 1999; Goyal and Paterson, 1989). The muscularis mucosa is a region of muscle within the submucosa, running parallel to the longitudinal muscle layer (Goyal and Sivarao, 1999). The esophageal mucosa consists of stratified squamous epithelium in all regions except the LES where this squamous epithelium joins the columnar epithelium of the stomach (Goyal and Sivarao, 1999). The serosal surface of the esophagus is bound only by a thin layer of poorly defined connective tissue, in contrast to other areas of the GI tract (Goyal and Sivarao, 1999; Goyal and Paterson, 1989).

Marking the end of the esophagus is the LES, a region that is approximately 2 – 4 cm in length (Goyal and Paterson, 1989). The pressure provided by the LES is a result of the tonic contraction of the circular smooth muscle comprising this sphincter. The longitudinal muscle within the LES has no distinctive features, with the fibres following the greater curvature of the stomach on the left and the lesser curvature on the right (Goyal and Paterson, 1989). Ultrastructural studies have shown that the smooth muscle cells of the LES have a larger diameter, more numerous mitochondria, a more developed

endoplasmic reticulum and fewer gap junctions than those of the esophageal body (Biancani *et al.*, 1987; Daniel and Posey-Daniel, 1984).

Functional innervation of the esophagus:

Control of the esophagus involves a complex integration of hierarchal neural centres. These neural networks are positioned in the brain, spinal cord, prevertebral sympathetic ganglia, and in the walls of the esophagus. Initiation of the swallowing reflex occurs through the activation of neurons within the swallowing centre, consisting of the nucleus retrofacialis, nucleus ambiguus and nucleus tractus solitarius, located in the brain stem (Goyal and Sivarao, 1999; Diamant, 1997). The transmission of information to the smooth muscle of the esophagus (and LES) occurs through nerve fibres originating in the preganglionic neurons of the dorsal motor nucleus of the vagus, which contains both excitatory and inhibitory fibres (Goyal and Paterson, 1989). These axons eventually branch to form the plexus that enters the esophagus at different levels, travelling within the esophageal wall for several centimetres before reaching the post-ganglionic neurons within the intramural plexus (Goyal and Sivarao, 1999; Goyal and Paterson, 1989; Schultz *et al.*, 1989).

The smooth muscle of the esophagus and LES also contain sympathetic innervation, arising from cell bodies of the intermediolateral columns of spinal segments T_1-T_{10} (Goyal and Paterson, 1989). These fibres travel in the greater splanchnic nerve and enter the celiac ganglia where they synapse with postganglionic neurons (Goyal and Paterson, 1989). Most preganglionic axons terminate on the myenteric and submucosal plexuses (enteric nervous system) (Goyal and Sivarao, 1999; Goyal and Paterson, 1989).

There is also a poorly defined afferent nervous system. These nerve fibres extend back to the nucleus solitarius via the vagus nerve, as well as the splanchnic and thoracic sympathetic nerves, making many projections within the brain (Goyal and Paterson, 1989). The vagal fibres have their cell bodies located in the nodose ganglion, while the sympathetic afferents travel via the dorsal root ganglia to the spinal cord (Goyal and Paterson, 1989).

The enteric nervous system of the esophagus is also capable of functioning independently of extrinsic nervous control (Wood *et al.*, 1999). The intramural neurons found in the myenteric and submucosal plexuses of the digestive tract form a nervous system with mechanisms for integrating and processing information much like those found in the brain and spinal cord (Wood *et al.*, 1999). These axons branch as they approach the smooth muscle cells, or other effector cells (such as interstitial cells of Cajal), and synaptic contact can be identified by the presence of varicosities in the nerve fibre (Goyal and Paterson, 1989). The varicosities contain neurotransmitter vesicles, and more than one vesicle type may occur in the same varicosity (Sharkey and Kroese, 2001; Goyal and Paterson, 1989). However, there are two predominant types of neurons present: those staining positively for choline acetyltransferase (Seeling *et al.*, 1984) and substance P (SP), and those that are positive for nitric oxide (NO)-synthase and vasoactive intestinal polypeptide (VIP) (Goyal and Sivarao, 1999).

Peristaltic contraction of esophageal smooth muscle is achieved by activation of both excitatory (cholinergic) and inhibitory nonadrenergic-noncholinergic (NANC) neurons. NANC neurons are responsible for the initial inhibition of circular smooth muscle, by causing membrane hyperpolarization, which is followed by rebound

depolarization and muscle contraction (Anand and Paterson. 1994). The circular muscle layer is involved in the peristaltic contractions required for bolus transport. Studies in several species. including opossums and humans. suggest that NO is the primary mediator of the NANC inhibitory neurons of the esophagus and the LES (Diamant. 1997; Paterson and Indrakrishnan. 1995; Anand and Paterson. 1994; Paterson *et al.* 1992). The excitatory cholinergic influence is most prominent in the proximal esophagus while the inhibitory influence is most prominent distally (Diamant. 1997). This leads to a progressive delay in the contractions along the esophagus providing a mechanism for the regulation of peristaltic direction and velocity (Diamant. 1997).

In the longitudinal smooth muscle layer. functional innervation is primarily cholinergic. since atropine usually blocks all esophageal shortening associated with primary and secondary peristalsis (Paterson. 1997a). However. some animals maintain a sustained. low-amplitude swallow-induced shortening following atropine. indicating that there is a noncholinergic component to the contraction of the longitudinal smooth muscle layer (Paterson. 1997a). In contrast to the circular muscle layer where NO innervation causes initial inhibition. there is excitation of the longitudinal muscle layer following NO application (Zhang and Paterson. 2001; Goyal and Sivarao. 1999; Saha *et al.*. 1993). There is no evidence to suggest the presence of functional inhibitory innervation to the longitudinal muscle layer.

Esophageal motility:

The movement of ingested substances to the stomach via primary peristalsis occurs through sequential contractions originating in the pharynx and continuing down

the esophagus to the stomach. The movement of the bolus involves the peristaltic contractions of the esophageal circular muscle and esophageal shortening through the contraction of the longitudinal muscle layer (Paterson, 1997a; Crist *et al.*, 1986). Primary peristalsis is initiated by the activation of the swallowing centre either by voluntary input from higher brain centres or involuntary input through reflex stimulation of peripheral afferents (Goyal and Paterson, 1989). The swallow centre then activates the vagal nerve, which mediates the contraction of both the striated and smooth muscle portions of the esophagus (Goyal and Paterson, 1989). While swallow-induced primary peristalsis is dependent on the activation of both the swallowing centre and vagal pathways to the esophagus (as demonstrated by vagotomy and vagal cooling), the circular smooth muscle portion possesses an intramural neuromuscular mechanism that allows for peristalsis independent of the central nervous system (Goyal and Paterson, 1989). This was identified when simultaneous electrical stimulation of all vagal efferents produced peristaltic contractions in the circular smooth muscle portion of the esophagus (Gidda and Goyal, 1983; Mukhopadhyay and Weisbrodt, 1975).

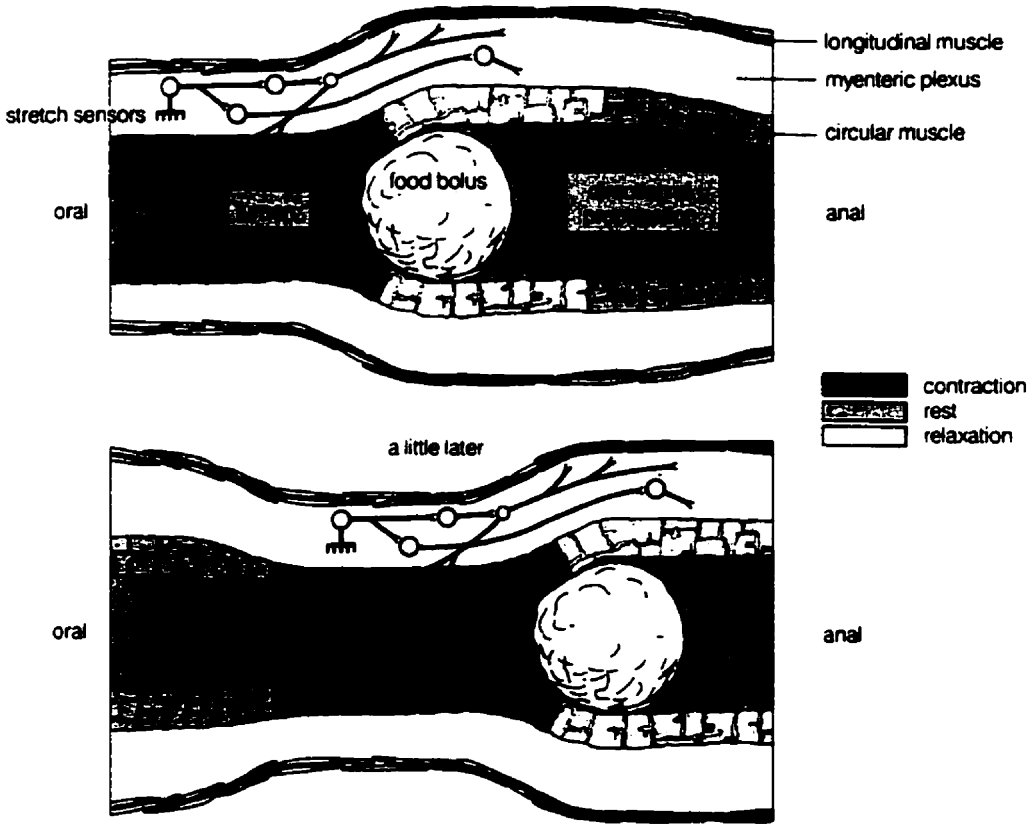
The smooth muscle cells of the esophagus respond directly to excitatory neuronal stimulation, primarily acetylcholine (ACh), with phasic contractions lasting for relatively short durations (Sohn *et al.*, 1997b; Hillemeier *et al.*, 1991; Biancani *et al.*, 1987). However, peristaltic contraction of the circular muscle layer involves a biphasic response, with an initial NO-mediated inhibition followed by contraction. Since the esophagus lacks resting tone, the initial inhibition is not apparent when recording esophageal motility using intraluminal manometry. However, if several swallows are taken within a

few seconds of each other. this inhibitory function becomes apparent as the circular contractions are inhibited until the end of the final swallow (Goyal and Paterson, 1989).

The purpose of the longitudinal muscle contraction is currently unknown, however it has been proposed that this contraction would increase the cross-sectional area of the esophagus, by shortening axial length, thus facilitating bolus transit (Paterson, 1997a; Goyal and Paterson, 1989). Studies on the opossum esophagus have demonstrated that longitudinal contraction, like that of the circular muscle layer, occurs sequentially in an aboral direction during primary peristalsis (Sugarbaker *et al.*, 1984). However, unlike the circular muscle layer, longitudinal muscle peristalsis is controlled centrally by vagal efferents. This was demonstrated when vagal electrical stimulation caused simultaneous contraction of the longitudinal muscle layer (Tottrup *et al.*, 1990; Sugarbaker *et al.*, 1984).

During peristalsis, longitudinal muscle contractions begin before, have a longer duration than, and simultaneously inhibit, the circular muscle layer (Sugarbaker *et al.*, 1984). This leads to a well-coordinated set of contractions that effectively propels a food bolus to the stomach (figure 1) (DeMeester *et al.*, 1999; Goyal and Sivarao, 1999; Paterson, 1997a; Cohen *et al.*, 1986; Crist *et al.*, 1986). The contractions of primary peristalsis are usually powerful enough to ensure that no bolus residue is left behind, and under normal circumstances the esophagus is completely cleared of the ingested food bolus within 8 – 10 seconds (Goyal and Sivarao, 1999). However, weaker contractions can sometimes leave bolus residue behind. This is removed by involuntary contractions known as secondary peristalsis, a term given to any peristaltic contraction that does not involve pharyngeal peristalsis or upper esophageal sphincter relaxation, and is localized

Figure 1: Pictorial representation of the contractions and relaxations that occur during peristalsis. The circular smooth muscle segment of the esophagus just proximal to the bolus contracts while at the same time the longitudinal smooth muscle in this same region relaxes. Simultaneously, a more distal segment of the esophagus prepares to receive the bolus through a relaxation of the circular, and contraction of the longitudinal, muscle layers. The myenteric plexus (of the enteric nervous system) lies between these two layers, ensuring that the contraction-relaxation pattern proceeds smoothly. Adapted from Smout and Akkermans, 1992.


longitudinal muscle
myenteric plexus
circular muscle
stretch sensors
food bolus
oral
anal
contraction
rest
relaxation
a little later
oral
anal

only to the esophagus (Goyal and Paterson, 1989). Secondary peristalsis is initiated by the distention produced by material left in the esophagus, such as bolus residue, or by contents refluxed from the stomach. These contractions begin just proximal to the distending substance and propel it to the stomach through peristaltic contractions similar to those observed during primary peristalsis (Goyal and Sivarao, 1999; Paterson, 1991).

Lower esophageal sphincter function:

The primary function of the LES is to prevent the acidic contents of the stomach (positive pressure) from gaining access to the esophagus (negative pressure). This occurs through myogenic tonic contraction of the circular smooth muscle cells of the LES, which creates a pressure barrier at the gastroesophageal junction. Even though the LES lacks macroscopic anatomical landmarks, it is easily identified with manometric techniques as a rise in pressure over the gastric and esophageal baseline pressures (Goyal and Sivarao, 1999; Goyal and Paterson, 1989; Schultz *et al.*, 1989). Following a swallow, NO causes LES hyperpolarization causing the inhibition of tonic contraction, therefore allowing for the passage of the bolus. During swallow-induced LES relaxation there is never a complete opening of the LES but rather the strength of the peristaltic contraction on the bolus is powerful enough to force the lumen open at the region of the LES.

The primary role of vagal efferent innervation of the LES is to induce relaxation (Matarazzo *et al*, 1976). Studies on the opossum found that bilateral cervical vagotomy caused either a transient increase in the resting LES pressure or had no effect. When electrical stimulation was applied to the vagal efferents there was a relaxation of the LES (Rattan and Goyal, 1974). The cells of the LES have muscarinic receptors, but the

application of atropine does not antagonize the resting LES pressure in opossums indicating that there is an intrinsic component to LES tonic contraction (Holloway *et al.* 1985; Dodds *et al.* 1981). However, in humans and dogs, the application of atropine decreased LES tone by approximately 50%, indicating that basal LES tone is mediated partially by cholinergic neural input (Dodds *et al.*, 1981).

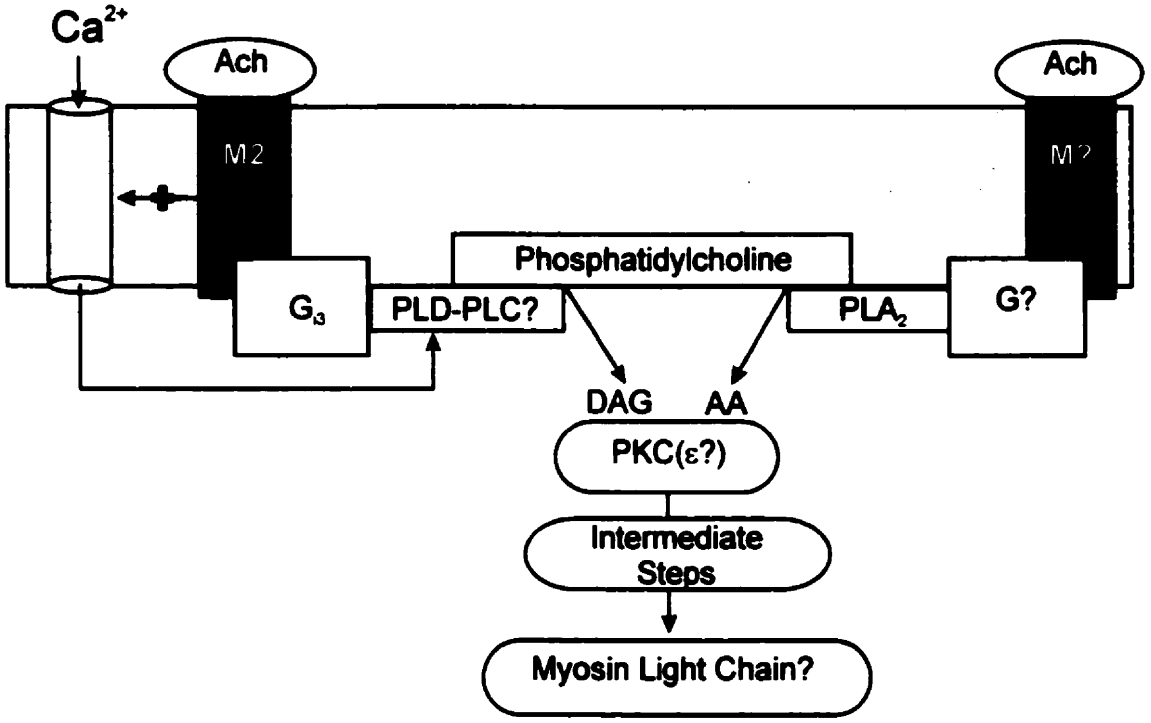
Cellular basis of smooth muscle contraction:

Biochemistry of esophageal smooth muscle contraction:

When a smooth muscle cell is exposed to an excitatory stimulant, such as ACh, a series of controlled steps initiates cellular contraction. The excitatory agonist interacts with membrane receptors linked to intracellular proteins. The activation of these intracellular proteins increases the levels of second messengers, which ultimately causes intracellular levels of Ca^{2+} to increase. The Ca^{2+} then interacts with various proteins to initiate contraction through the phosphorylation of contractile proteins such as myosin light chain (Hillemeier *et al.*, 1991).

Agonists such as ACh initiate contraction through the interaction with muscarinic receptors located on the membrane surface of the smooth muscle cell. Currently 5 types of muscarinic receptors (M_1 – M_5) have been identified and of these the first four can be selectively identified by using pharmacological blocking agents (Shi and Sarna, 1997; Barnes, 1996; Sohn *et al.*, 1993; Barnes, 1993). In the cat, evidence suggests that ACh induces contraction of esophageal circular smooth muscle through M_2 receptors (figure 2; Sohn *et al.*, 1995; Sohn *et al.*, 1993). The muscarinic receptor is the first of a triad of

Figure 2: Biochemistry of esophageal circular smooth muscle contraction in response to acetylcholine (ACh). ACh binds to muscarinic M_2 receptors causing the influx of extracellular calcium (Ca^{2+}) as well as the activation of a G_{i3}-type G-protein linked to phospholipase D (PLD) and phosphatidylcholine-specific phospholipase C (PLC). Cytosolic phospholipase A_2 (PLA_2) is also activated, but the type(s) of muscarinic receptor and G-protein responsible for activation are currently unknown. PLC and PLD produce diacylglycerol (DAG); PLA_2 produces arachidonic acid (AA). DAG and AA interact to activate protein kinase C (PKC). PKC causes contraction of esophageal muscle through intermediate steps that presently are not well understood. Adapted from Biancani *et al.* (1997).


Ca2+
Ach
M2
Ach
M2
Phosphatidylcholine
Gi3
PLD-PLC?
PLA2
G?
DAG
AA
PKC(ε?)
Intermediate Steps
Myosin Light Chain?

interacting proteins that serve to convert extracellular signals into intracellular events. The second protein in this complex is the GTP-binding protein (G-protein), which is composed of 3 sub-units (α, β and γ). The G-protein is located on the cytosolic side of the membrane, bound to a portion of the receptor that penetrates the membrane (Barnes, 1996). G-proteins can be categorized into 2 major groups, pertussis toxin (PTX)-sensitive (G_o) and PTX-insensitive (G_q) (Sohn *et al.*, 1993). PTX is a potent toxin that is known to affect only specific G-proteins through ADP-ribosylation (Abebe and Mustafa, 1998). The use of antibodies specific for the α-sub-unit of the G-proteins has shown that the PTX-sensitive G_o-G_{i3} G-protein is involved in the contraction of circular smooth muscle in the cat esophagus (Sohn *et al.*, 1995; Sohn *et al.*, 1993).

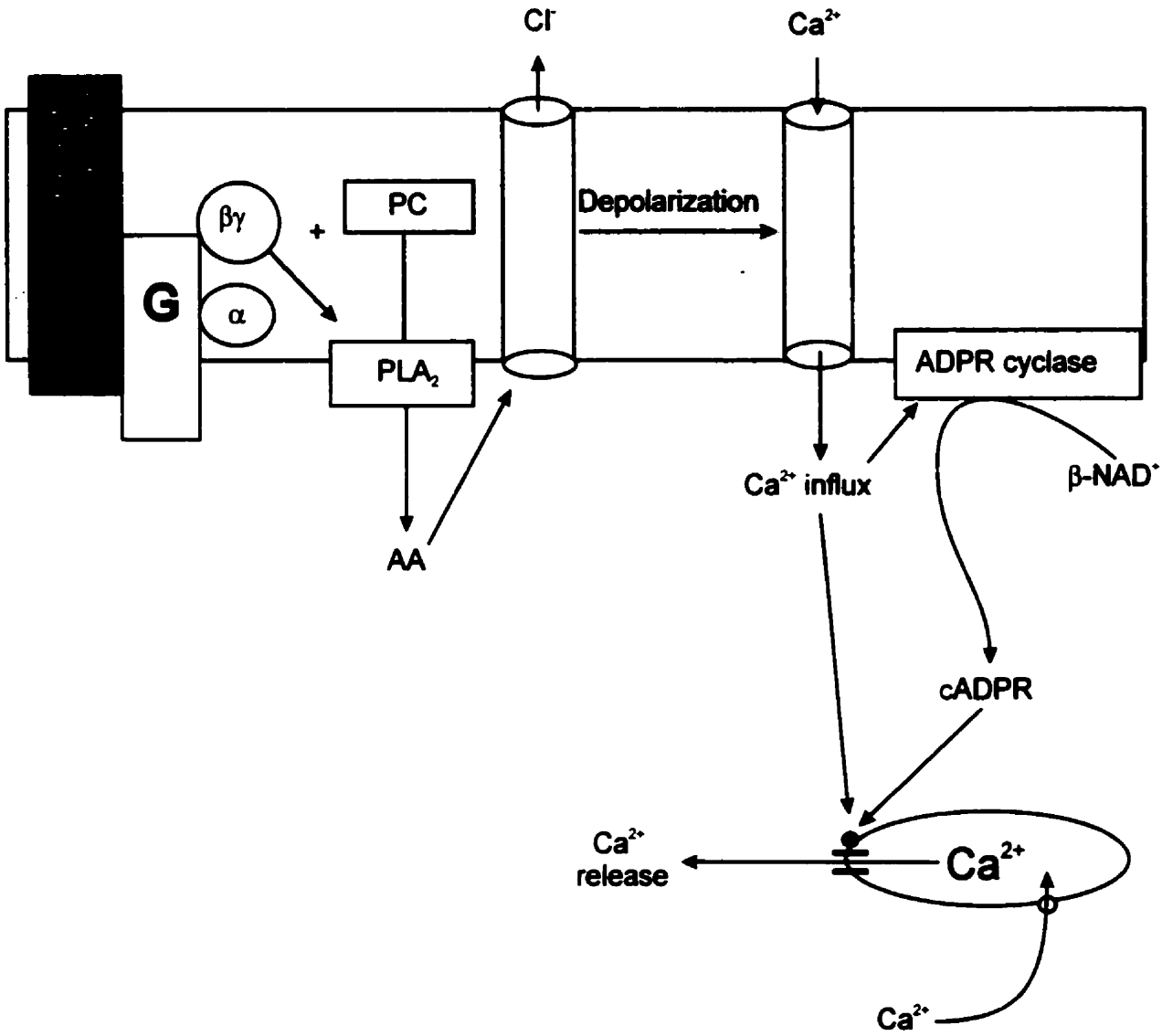
The third component of this complex is the effector proteins, which initiate the breakdown of membrane phospholipids (such as phosphatidylinositol 4,5-bisphosphate (PIP_2)) producing second messengers such as inositol-1,4,5-triphosphate (IP_3) and/or 1,2-diacylglycerol (DAG). In the cat esophagus G_o-G_{i3} is linked to phosphatidylcholine-specific phospholipase C (PC-PLC) and PC-PLD (Sohn *et al.*, 1995; Sohn *et al.*, 1993). These effector proteins hydrolyze phosphatidylcholine into DAG without the subsequent production of IP_3 (Sohn *et al.*, 1994a; Sohn *et al.*, 1993). High levels of DAG are critical in these cells because the contractility of the cell, initiated by protein kinase C (PKC), is augmented through the interaction of DAG with PKC (Abebe and Mustafa, 1998; Sohn *et al.*, 1995; Biancani *et al.*, 1994; Sohn *et al.*, 1993; Berridge and Irvine, 1989). DAG-induced activation of PKC is Ca^{2+} independent, suggesting that Ca^{2+} influx is required only to activate the phospholipase responsible for the production of DAG (Figure 2; Biancani *et al.*, 1997).

Also, during excitatory stimulation of these cells, phospholipase A_2 (PLA_2) is activated resulting in the production of arachidonic acid (AA) (Sohn *et al.*, 1994a; Haller *et al.*, 1990) which interacts synergistically with DAG to increase its affinity for the activation of PKC (Biancani *et al.*, 1997; Sohn *et al.*, 1994a). Since the reactivity of DAG becomes greatly increased, the ability of the cell to contract under minimal excitatory stimulation is elevated, causing the fast and powerful contractions found in the esophageal body.

The intracellular mechanisms involved in the contraction of esophageal longitudinal smooth muscle have not been studied. However, in the small intestine the longitudinal smooth muscle, unlike the circular, appears to not involve IP_3. The circular smooth muscle of the intestine utilizes PIP_2, whereas the longitudinal preferentially hydrolyzes PIP producing minimal amounts of IP_3 (Murthy and Makhlouf, 1991; Murthy *et al.*, 1991). Furthermore, it has been shown that IP_3 had no effect on permeabilized longitudinal smooth muscle cells, and inhibition of IP hydrolysis in longitudinal cells had no effect on agonist-induced Ca^{2+} mobilization (Kuemmerle *et al.*, 1998; Makhlouf and Murthy, 1997). PLC and PLD are not involved in longitudinal smooth muscle contraction since inhibition of their products had no effect.

Inhibition of PLA_2 completely abolished agonist-induced contraction of intestinal longitudinal smooth muscle, and the addition of AA could dose dependently restore contraction (Murthy *et al.*, 1995). It appears that AA, produced by PLA_2 activation, causes membrane depolarization through the activation of Cl^- channels, which in turn leads to the opening of voltage-sensitive Ca^{2+} channels (figure 3). However, Ca^{2+} influx is not completely responsible for cellular contraction since depletion of intracellular

Figure 3: Signalling cascade initiated by contractile agonists in intestinal longitudinal smooth muscle cells. This represents events during the initiation of contraction. Agonist-induced activation of phospholipase A_2 (PLA_2), mediated by a pertussis toxin-sensitive G-protein, hydrolyzes phosphatidylcholine (PC), producing arachidonic acid (AA). AA activates Cl^- channels, resulting in depolarization of the plasma membrane and opening voltage-sensitive Ca^{2+} channels. Ca^{2+} influx (via these channels) induces Ca^{2+} release by activating sarcoplasmic ryanodine receptor/Ca^{2+} channels. The increase in $[Ca^{2+}]_i$ activates membrane-bound ADP ribosyl (ADPR)-cyclase resulting in the formation of cADPR which enhances Ca^{2+}-induced Ca^{2+} release. Adapted from Makhlouf and Murthy (1997).


Cl-
Ca2+
βγ
+
PC
Depolarization
G
α
PLA2
ADPR cyclase
Ca2+ influx
β-NAD+
AA
cADPR
Ca2+
release
Ca2+
Ca2+

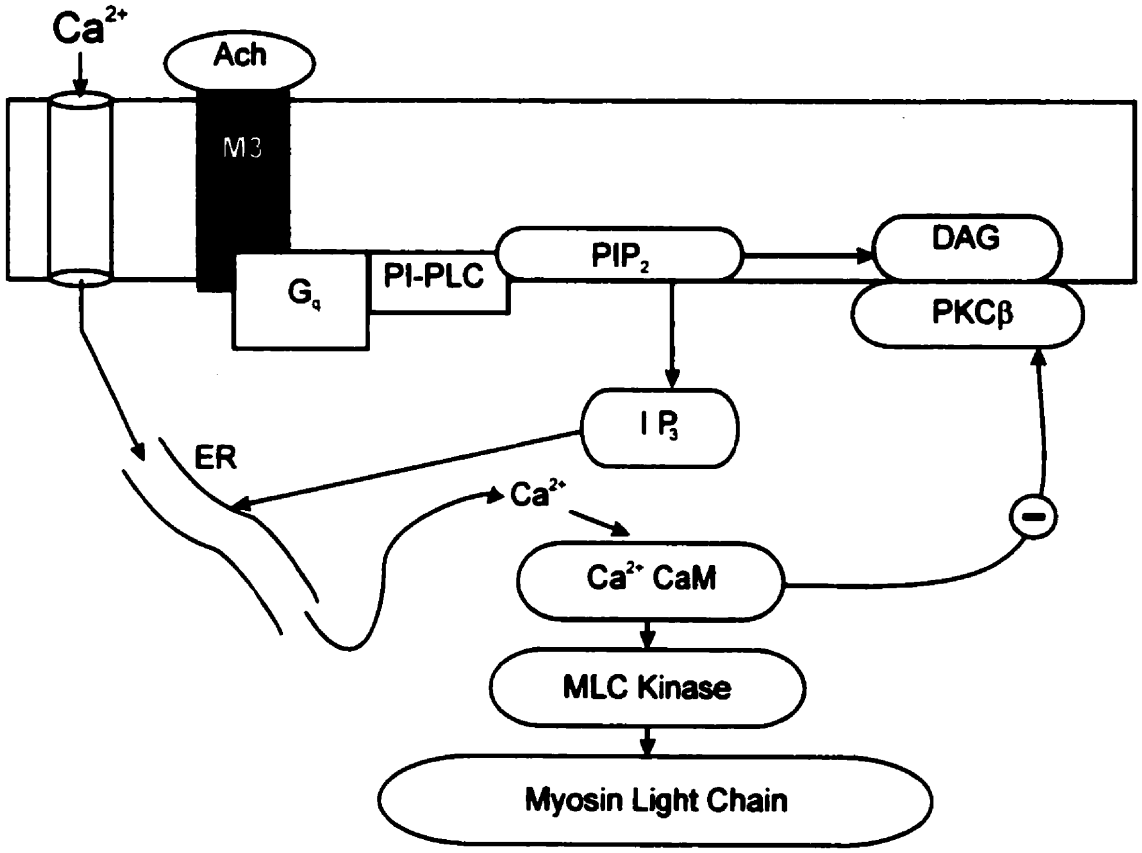
stores could block contraction by approximately 70% (Kuemmerle *et al.*, 1998; Murthy *et al.*, 1995). It has also been demonstrated that cyclic adenosine diphospho-ribose (cADPR) is involved in intestinal longitudinal, but not circular, smooth muscle contraction (Murthy *et al.*, 1995). Contraction of intestinal longitudinal smooth muscle involves Ca^{2+} influx, which causes the release of Ca^{2+} from ryanodine-sensitive, IP_3-insensitive intracellular stores, leading to the activation of cADPR, which acts synergistically with Ca^{2+} to enhance Ca^{2+} release from the stores (figure 3) (Kuemmerle *et al.*, 1998; Grider and Makhlouf, 1988). Whether similar distinct pathways are involved in longitudinal smooth muscle contraction in the esophagus have not been determined.

Biochemistry of lower esophageal sphincter contraction:

The biochemistry of agonist-induced LES contraction differs from the esophageal body. For example, ACh-induced contraction of LES cells depends on M_3 receptors linked to PTX-insensitive G_q-G_{11} G-proteins that subsequently activate phosphatidylinositol-specific PLC (PI-PLC) (figure 4). PI-PLC hydrolyzes PIP_2 into equal proportions of IP_3 and DAG (Sohn *et al.*, 1997a; Sohn *et al.*, 1995; Biancani *et al.*, 1994; Sohn *et al.*, 1994a). IP_3 binds to receptors on the intracellular Ca^{2+} stores initiating the release of Ca^{2+}. Elevated Ca^{2+} produces a calcium-calmodulin complex that eventually results in myosin light-chain phosphorylation and contraction of the LES smooth muscle cell (Sohn *et al.*, 1995; Biancani *et al.*, 1994; Haller *et al.*, 1990).

LES tone is mediated by mechanisms different than those mediating the ACh-induced contraction (Biancani *et al.*, 1997). It appears that tone is mediated through the spontaneous activity of PLC, resulting in the hydrolysis of PIP_2 into DAG and IP_3. These

Figure 4: Biochemistry of lower esophageal sphincter (LES) smooth muscle contraction in response to acetylcholine (ACh). ACh binds to muscarinic M_3 receptors and causing activation of G_q-type G-protein linked to phosphatidylinositol-specific phospholipase C (PI-PLC). PI-PLC hydrolyzes phosphatidylinositol bisphospate (PIP_2) producing 1,4,5-inositol triphospate (IP_3) and diacylglycerol (DAG). IP_3 causes the release of calcium (Ca^{2+}) from intracellular stores at concentrations (>1μM) sufficient to activate calmodulin (CaM). Ca^{2+}-CaM causes the activation of myosin light chain (MLC)-kinase and inhibition of protein kinase C (PKC), inducing contraction that is entirely CaM-dependent. Adapted from Harnett *et al.* (2000) and Biancani *et al.* (1997).


Ca2+
Ach
M3
Gq
PI-PLC
PIP2
DAG
PKCβ
IP3
ER
Ca2+
Ca2+ CaM
MLC Kinase
Myosin Light Chain

submaximal concentrations of IP_3 release low levels of Ca^{2+} from the intracellular stores. The Ca^{2+} then interacts with PKC, resulting in the tonic contraction of the LES circular smooth muscle cells (Biancani *et al.*, 1997). Calmodulin does not become activated as observed in ACh-induced LES contraction, because calmodulin requires higher concentrations of Ca^{2+}. During the relaxation of the LES, IP_3 levels are reduced decreasing the concentration of free Ca^{2+}, and thus reducing the Ca^{2+}-PKC-induced tone (Biancani *et al.*, 1997). In all species examined so far, including the opossum (Uc *et al.*, 1999; Paterson *et al.*, 1992; Yamato *et al.*, 1992), cat (Xue *et al.*, 1996) and human (Tomita *et al.*, 1997; Preiksaitis *et al.*, 1994; Oliveira *et al.*, 1992), LES relaxation appears to be mediated by NO.

The application of different agonists (ACh, bombesin and SP) to LES smooth muscle cells has demonstrated that the pathways involved in the contraction converge onto one common intracellular event. This indicates that the contractile pathways activated are muscle-type dependent rather than agonist specific (Sohn *et al.*, 1995). The common pathway for the above agonists indicates that contraction probably occurs in the same manner regardless of the agonist used. However, this has not yet been determined for much of the gastrointestinal tract, including the smooth muscle cells of the esophageal body.

Involvement of Ca^{2+} in the regulation of smooth muscle contraction:

It well known that the divalent cation calcium (Ca^{2+}) regulates contractility of the smooth muscle cell. The typical response of the smooth muscle cells to excitatory nerve stimulation is depolarization of the cellular membrane, allowing for the influx of

extracelluar ions such as Ca^{2+} (Akbarali *et al.*, 1995). For example, ACh-induced stimulation of the smooth muscle cell activates Ca^{2+}-activated potassium channels, Ca^{2+}-sensitive chloride channels and nonselective cation channels. In smooth muscle cells from the esophageal body it is these currents that cause the depolarization of the membrane, leading to the entry of Ca^{2+} from external sources via voltage sensitive Ca^{2+} channels (Goyal and Sivarao, 1999).

Contractility experiments on the circular smooth muscle of the esophageal body have indicated that external sources of Ca^{2+} are critical for smooth muscle contraction (Hillemeier *et al.*, 1991; Biancani *et al.*, 1987). When extracellular Ca^{2+} was removed with a chelating agent, or when Ca^{2+} influx was interrupted with Ca^{2+}-channel blockers, the contractility of the cat esophageal circular smooth muscle was completely abolished (Sohn *et al.*, 1994b; Sohn *et al.*, 1993; Biancani *et al.*, 1987). Similarly, de Carle *et al.* (1977) found that electrically-induced contraction of the longitudinal smooth muscle of the opossum esophagus was dependent on extracellular Ca^{2+}. Observations on the longitudinal smooth muscle of human and guinea pig intestine suggest a similar dependence on extracellular Ca^{2+} in the development of contraction (Sohn *et al.*, 1994b; Murthy *et al.*, 1992; Grider and Makhlouf, 1988).

In contrast to the esophageal body, when the cat LES is exposed to conditions where extracellular Ca^{2+} is removed or the influx of Ca^{2+} blocked, agonist-induced contraction was not impaired (Biancani *et al.*, 1987). However, contractility of the LES could be completely eliminated by incubation in Sr^{2+}, indicating that intracellular Ca^{2+} is critical for the function of LES cells but extracellular sources are not. These cells may require extracellular Ca^{2+} only to replace the gradual depletion of the internal stores, that

will eventually empty over time (Sohn *et al.*, 1993; Biancani *et al.*, 1987). Since the refilling of these stores occurs approximately 3 times faster than their depletion (Saida and van Breeman, 1984), the elimination of extracellular Ca^{2+} has little effect on the contractility of the LES over the short term (Sohn *et al.*, 1993; Biancani *et al.*, 1987).

However, recent findings indicate that LES tone in the opossum (Zhang *et al.*, 2000) and dog (Salapatek *et al.*, 1998) could be significantly inhibited or blocked by the removal of extracellular Ca^{2+} or by the addition of L-type Ca^{2+} channel blockers such as nifedipine. In addition, other researchers have found that either the application of L-type Ca^{2+} channel blockers or reduction of extracellular Ca^{2+} could reduce LES tone in the opossum (Fox and Daniel, 1979), dog (Allescher *et al.*, 1988), and human (Tottrup *et al.*, 1990). This suggests that extracellular Ca^{2+} is critical for the tonic function of the LES, which is in contrast to the findings of Sohn *et al.* (1993) and Biancani *et al.* (1994) that indicate a strong dependence on intracellular Ca^{2+}.

Impact of inflammation on smooth muscle:

Inflammation-induced changes to smooth muscle contractility:

Studies of inflammation have focused on the effects on the mucosa and sub-mucosa, but the smooth muscle is particularly sensitive to inflammation and the neuromuscular function is often altered (Table 1; Collins, 1996). Evidence from a number of animal models, as discussed by Collins (1996), shows that in general, inflammation causes the circular smooth muscle layer to become hypo-responsive to

Table 1: Typical effects of inflammation on gastrointestinal function. *In vivo* and *in vitro* (whole organ and muscle strips) studies typically show that inflammation results in an increased response of the longitudinal smooth muscle (LSM) layer to excitatory agonists and electrical field stimulation (EFS). However, the circular smooth muscle (CSM) layer displays a decrease in the ability to contract to the same excitatory stimuli, including potassium (KCl) depolarization. *In vivo* studies report conflicting data on changes of giant migrating contraction (GMC) and migrating motor complexes (MMC). Studies on isolated smooth muscle cells typically report changes in the L-type Ca^{2+} channels. Acetylcholine (ACh); cholecystokinin (CCK); carbachol (CCh); histamine (HIS); myoelectric activity (MEA); spontaneous phasic contractions (SPC).

Group:	Animal/ location:	Study type:	Inflammation-induced changes observed:
Aube *et al.* (1999)	Rat/ Colon & ileum	*In vivo*	↑ in ileal MMC; no change in ileal GMC
Sarna (1998)	Dog/ Ileum	*In vivo*	↓ in methacholine-induced contractions
Oliver *et al.* (1997)	Rat/ Colon	*In vivo*	↑ myoelectric activity
Jouet *et al.* (1995)	Dog/ Ileum	*In vivo*	↑ in GMC (↑ diarrhea & discomfort); ↓ in MMC
Fass *et al.* (1994)	Human/ Esophagus	*In vivo* manometry	↓ in CSM contractility; ↑ in bolus transport time
Cucchiara *et al.* (1986)	Human/ Esophagus	*In vivo* manometry	↓ amplitude of peristalsis; ↑ motor dysfunction
Marshall and Gerhardt (1982)	Human/ Esophagus	*In vivo* manometry	↓ frequency and amplitude of peristalsis
Kalff *et al.* (2000)	Rat & mouse/ Ileum	Organ bath	↓ in CSM contraction
De Man *et al.* (2001)	Mouse/ Ileum	Muscle strips	↑ in contractility to CCh & EFS
Gay *et al.* (2001)	Rat/ Jejunum	Muscle strips	↑ in LSM contractility to CCK
Gonzalez and Sarna (2001)	Rat/ Colon	Muscle strips	↓ in CSM contractility to ACh; SPC and GMC no change
Hierholzer *et al.* (2001)	Rat/ Jejunum	Muscle strips	↓ in CSM contractility to bethanechol
Moreels *et al.* (2001)	Mouse/ Ileum & gastric fundus	Muscle strips	↑ in ileal LSM contractility; ↓ GI transit
Bogers *et al.* (2000)	Mouse/ Ileum	Muscle strips	↑ in contractility
Parkman *et al.* (1999)	Guinea pig/ Gallbaldder	Muscle strips	↓ in CSM contractility to KCl and bethanechol
Vallance *et al.* (1999)	Mouse/ Jejunum	Muscle strips	↑ in LSM contractility
Martinolle *et al.* (1997)	Guinea pig/ Ileum	Muscle strips	↑ in CSM passive tension; ↑ in LSM contraction to CCh & His
Myers *et al.* (1997)	Rat/ Colon	Muscle strips	↓ in CSM contractility to ACh & KCl
Ali *et al.* (2000)	Dog/ Colon	Isolated cells	↓ in CSM contractility
Shi and Sarna (2000)	Dog/ Colon	Isolated cells	↓ in CSM contractility ↓ in # of L-type Ca^{2+} channels
Akbarali *et al.* (2000)	Mouse/ Colon	Patch clamp	↓ in inward Ca^{2+} currents
Liu *et al.* (2001)	Dog/ Colon	Western blot	↓ in the # of L-type Ca^{2+} channels

excitatory stimuli, while the longitudinal smooth muscle layer becomes hyper-responsive. However, there are exceptions, such as hypo-responsiveness of the longitudinal smooth muscle from the inflamed rat colon (Grossi *et al.*, 1993).

Numerous studies, both *in vivo* and *in vitro*, have examined the effects of inflammation on the function of smooth muscle (Table 1). For example, *Trichinella spiralis* infection of the rat small intestine has been shown to cause an increase in intestinal motility between the third and seventh day of infection (Vermillion and Collins, 1988; Castro *et al.*, 1976). This has subsequently been correlated with a reduction in slow wave frequency and spiking activity along with the presence of migrating action potential complexes (Palmer *et al.*, 1984). Shi and Sarna (1997) reported that inflammation of the canine ileum was associated with the suppression of spontaneous phasic contractions and increased frequency of giant migrating contractions, which produce rapid propulsion of intestinal contents contributing to diarrhea.

Contractility studies on inflamed tissue strips from the rabbit colon (Cohen *et al.*, 1986), rat colon (Myers *et al.*, 1997), rat jejunum (Crosthwaite *et al.*, 1990), canine ileum (Shi and Sarna, 1997) and guinea pig ileum, (Martinolle *et al.*, 1997; Boyer *et al.*, 1997) have shown that the circular smooth muscle layer was hypo-responsive to excitatory stimulation (such as carbachol (CCh), histamine and 5-hydroxytryptamine). In contrast, tissue from the inflamed longitudinal smooth muscle of the guinea pig ileum (Martinolle *et al.*, 1997), mouse jejunum (Barbara *et al.*, 1997) and rat jejunum (Vallance *et al.*, 1997; Khan and Collins, 1994; Blennerhassett *et al.*, 1992; Vermillion and Collins, 1988; Farmer *et al.*, 1983) was hyper-responsive to excitatory stimulation. Mitchell *et al.* (1993) reported agonist-induced hyper-responsiveness of the inflamed swine trachea,

resulting in increased maximal contractile force, shortening velocity, and shortening capacity.

Several studies have reported impaired cholinergic contraction of circular smooth muscle strips from patients with ulcerative colitis (Grossi *et al.*, 1993; Middleton *et al.*, 1993; Xie *et al.*, 1992). However, in contrast, Boyer *et al.* (1997) found that circular smooth muscle strips from individuals with ulcerative colitis did not reveal any significant differences, in CCh- or histamine-induced contraction, from that of control tissue. Vermillion *et al.* (1993) found that both the circular and longitudinal smooth muscle tissue from patients with Crohn's disease displayed increased responsiveness to CCh and histamine.

Blennerhassett *et al.* (1999b) concluded that the origin of altered contractility in tissue strips from the inflamed mouse jejunum was a result of changes to the smooth muscle cells. In a preliminary study they found that the average CCh-induced contraction of isolated longitudinal smooth muscle cells from inflamed tissue was 9% greater than cells from control tissue. In contrast, inflammation has been reported to have no impact on ACh-induced contractility of cells isolated from the cat LES (Biancani *et al.*, 1997). The direct impact of inflammation on the smooth muscle cells of the esophageal body has not yet been determined.

Hypertrophy of the smooth muscle cells:

Typically there is an increase in the total weight and thickness of inflamed tissue compared to that of controls. Following *T. spiralis*-induced inflammation of the rat jejunum, there was a significant increase in the thickness of the muscle wall which was

associated with an overall increase in the size (determined *in situ*) of the smooth muscle cells, with width and length increasing by 20% and 50%, respectively (Blennerhassett *et al.*, 1992). This was similar to preliminary findings on isolated smooth muscle cells from the same area (Blennerhassett *et al.*, 1999b).

The increase in the size of the smooth muscle cell was also associated with an increase in the amount (5-fold) and density (2-fold) of smooth muscle α-actin (Blennerhassett *et al.*, 1999a). In addition, Weisbrodt *et al.* (1986) found that inflammation was not associated with an increase in the amount of collagen. Since these increases in smooth muscle mass and actin content could exaggerate muscle contraction and amplify the effect of excitatory stimulation, hypertrophy may be an important contributor to altered gastrointestinal motility. Whether smooth muscle cell hypertrophy occurs as a result of esophagitis has not yet been determined.

Hyperplasia of the smooth muscle cells:

Hyperplasia may also contribute to the increased muscle mass present after inflammation. In the rat, 6-days after the induction of jejunitis, the low basal rate of mitotic activity (0.3+/-0.2%) had increased to 3.6% and 4.5% in the longitudinal and circular smooth muscle layers respectively, and the number of nuclei in tissue sections increased 2-fold in the longitudinal layer and 1.5-fold in the circular layer (Blennerhassett *et al.*, 1992). The observed increase in mitotic activity returned to control levels by day 23 of inflammation; however, the increased number of smooth muscle cells remained, indicating a long-term change in the jejunal smooth muscle. There was also an increase

in nuclear size, which was associated with increased mitotic activity (Blennerhassett *et al.*, 1992).

The increase in cell number and size may lead to altered motility by causing increased force production and elevated intraluminal pressure. Since hypertrophic cells occupy more space, hypertrophy often decreases the force generated per unit of area (Johansson, 1984), but there is an increase in the force produced per cell because of the increased area it occupies. Similarly, hyperplasia increased the number of smooth muscle cells present and thus increases the maximal force produced by the tissue.

Inflammation-induced alteration to neuronal function:

Inflammation has many adverse effects on myenteric neuronal function in addition to the direct loss of neurons (Sanovic *et al.*, 1999). During acute *T. spiralis* infection, there is an 80% decrease in stimulated release of both ACh (Collins *et al.*, 1989) and norepinephrine (Ruhl *et al.*, 1994; Swain *et al.*, 1991). However, since an increase in both the neuron specific uptake of choline and choline acetyltransferase activity occurred during inflammation, this indicates that there is likely an increase in ACh synthesis as a result of inflammation (Davis *et al.*, 1998). Therefore, it was suggested that the decreased ACh release might involve defects in the packaging, storage and/or exocytosis of this neurotransmitter (Davis *et al.*, 1998).

Altered neuronal function might be due to activation of pro-inflammatory cytokines such as interleukin (IL)-1β and IL-6, released from activated monocytes (Goto *et al.*, 1984). For example, *in vitro* application of IL-1β resulted in suppression of neurotransmitter release, similar to that seen during inflammation (Collins *et al.*, 1992).

In keeping with this role of cytokines in altered neuronal function, the application of IL-1 receptor antagonists to inflamed tissue reversed the impairment of neurotransmitter release (Hurst and Collins, 1993).

Prostaglandins (PG) may also play an important role in intestinal inflammation. PG synthesis is increased during inflammation and results in increased secretion and altered motility within the affected areas (Sharkey and Kroese, 2001). Evidence shows that the stimulatory effects of PG are at least partially due to direct neural activation. PG application results in an increase in the occurrence of fast excitatory postsynaptic potentials (Sharkey and Kroese, 2001). In addition, PG causes a slow membrane depolarization, accompanied by an increase in action potentials (Frieling *et al.*, 1995).

Impact of inflammation on the esophagus:

Gastroesophageal Reflux Disease (GERD):

Gastroesophageal reflux disease (GERD) is typically manifested by the presence of heartburn and regurgitation. Additional, atypical symptoms include chronic coughing, wheezing, hoarseness, and chest pain (DeMeester *et al.*, 1999; DeVault and Castell, 1994). Approximately 7 – 10% of the adult population in Western society has daily episodes of heartburn (Nebel *et al.*, 1976) while as many as 50% have intermittent symptoms (Richter, 1998). GERD accounts for approximately 75% of all esophageal pathology in the United States (Richter, 1998; Stein *et al.*, 1992).

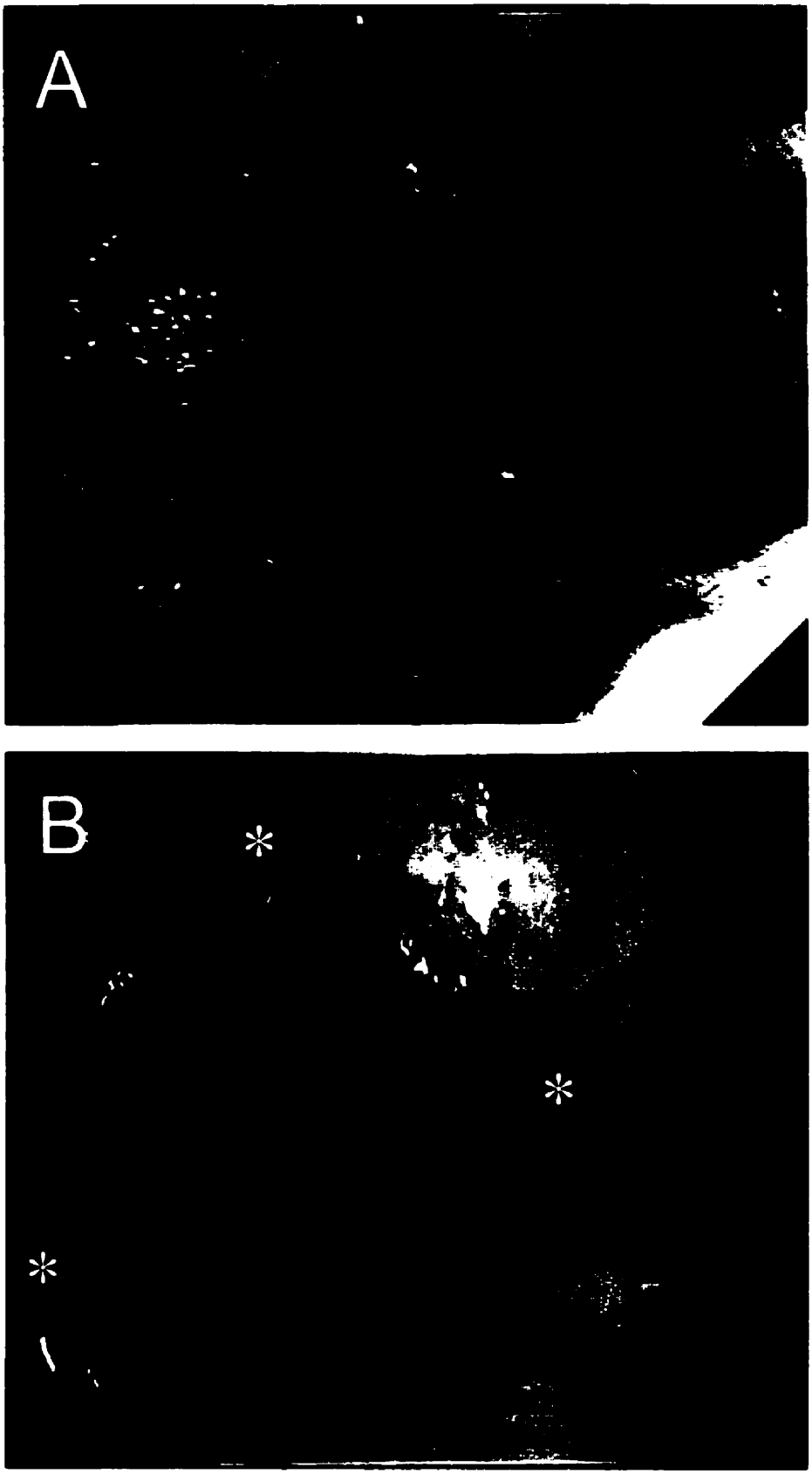
The cause of virtually every episode of gastroesophageal reflux is the loss of the resistance to the flow of gastric juice from the stomach (an environment of high pressure)

to the esophagus (an environment of low pressure) (Buttar and Falk, 2001; DeMeester *et al.*, 1999). This resistance is normally provided by the tonic contraction of the LES, which provides a high-pressure zone that prevents gastric juice from flowing into the esophagus. Evidence indicates that gastroesophageal reflux occurs during inappropriate (transient) relaxations of the LES, which can last up to 60 seconds in duration (Mittal and Balaban, 1997). Transient LES relaxation is a vagally mediated reflex, resulting from release of NO onto the smooth muscle cells via post-ganglionic neurons (Mittal and Balaban, 1997). However, transient relaxation of the LES alone is not significant for acid reflux to occur (Dodds *et al.*, 1982) since the crural diaphragm has been shown to prevent reflux in the absence of LES pressure (Mittal *et al.*, 1995). Therefore, for reflux to occur both the LES and crural diaphragm must relax. However, the mechanism for relaxation of the crural diaphragm is currently unknown (Mittal and Balaban, 1997).

Patients with GERD have more frequent transient relaxations than normal individuals. It has been suggested that gastric distention, posture and meals high in saturated fat may increase the frequency of these transient relaxations (Mittal and Balaban, 1997). In patients with severe GERD, decreased LES pressure and increased acid clearance time, due to impaired circular smooth muscle function, have been identified (Mittal and Balaban, 1997; DeMeester *et al.*, 1976).

With repeated gastroesophageal reflux, esophagitis may develop in the distal portion of the esophagus (figure 5). With the development of esophagitis, LES pressure and the force of peristaltic contraction in the distal esophagus may decrease (Goyal and Sivarao, 1999). The loss of LES tone can lead to a worsening of the inflammation

Figure 5: Images from an esophageal endoscope representing the appearance of normal distal esophagus (A) and that inflamed due to acid reflux disease (B). Normal esophageal tissue appears as a bright pink colour whereas the inflamed tissue has a swollen, reddened appearance. Inflammation due to acid reflux has also caused esophageal erosions (*, B). Images provided by Hotel Dieu Hospital and image of erosive esophagitis courtesy of R. Wells.

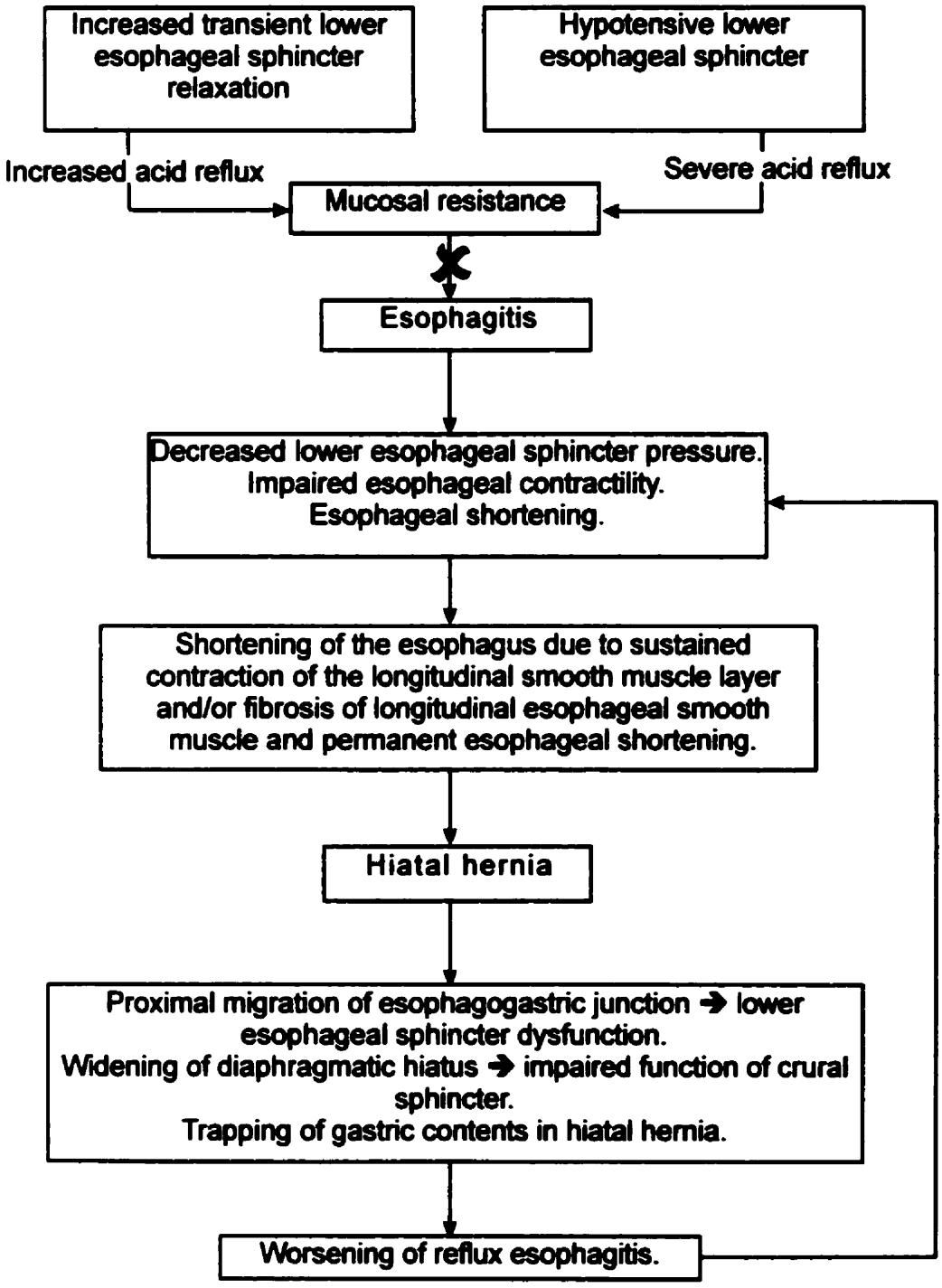

A
B

because there will be an increase in the occurrence of gastroesophageal reflux. Shirazi *et al.* (1989) have shown that the contractility of the circular smooth muscle becomes decreased during inflammation, which could result in increased acid clearance time leading to worsening of the esophagitis (Figure 6).

Acute acid exposure also causes the esophagus to contract along its longitudinal axis (White *et al.*, 2001; Shirazi *et al.*, 1989). If the esophagus is continuously challenged by acid there could be a continual and sustained contraction of the esophageal longitudinal smooth muscle. This sustained contraction of the longitudinal muscle layer could eventually pull the stomach into the thoracic cavity. Observations suggest that the majority of patients with moderate to severe reflux disease have a coexisting hiatal hernia, a condition where a portion of the stomach has migrated into the thoracic cavity through the esophageal hiatus in the diaphragm (Mittal and Balban, 1997; Paterson and Kolyn, 1994).

Chronic acid exposure may lead to the development of other serious problems, such as the formation of a pre-malignant condition known as Barrett's esophagus. This is a condition in humans where the normal squamous esophageal epithelium becomes replaced by metaplastic intestine-like epithelium containing goblet cells (DeMeester *et al.*, 1999; Fitzgerald *et al.*, 1996; DeVault and Castell, 1994; Vanner and Paterson, 1988). The transformation to this specialized epithelium has been observed in approximately 10% of patients with chronic GERD and has been associated with a 30 fold increased risk for the development of esophageal adenocarcinoma (Fitzgerald *et al.*, 1996; Vanner and Paterson, 1988). Previously, these cancers were considered rare, but in the past two

Figure 6: A model for the development of gastroesophageal reflux disease (GERD). Increased esophageal acid exposure due to transient lower esophageal sphincter (LES) relaxation (the predominant mechanism in the development of GERD) leads to the development of esophagitis. Inflammation causes esophageal dysfunction, which can lead to the development of impaired peristalsis and esophageal shortening. A subset of patients with GERD have a hypotensive LES and usually have severe reflux disease. Adapted from Buttar and Falk (2001).


Increased transient lower esophageal sphincter relaxation
Hypotensive lower esophageal sphincter
Increased acid reflux
Severe acid reflux
Mucosal resistance
Esophagitis
Decreased lower esophageal sphincter pressure.
Impaired esophageal contractility.
Esophageal shortening.
Shortening of the esophagus due to sustained contraction of the longitudinal smooth muscle layer and/or fibrosis of longitudinal esophageal smooth muscle and permanent esophageal shortening.
Hiatal hernia
Proximal migration of esophagogastric junction → lower esophageal sphincter dysfunction.
Widening of diaphragmatic hiatus → impaired function of crural sphincter.
Trapping of gastric contents in hiatal hernia.
Worsening of reflux esophagitis.

decades their incidence in the United States has increased by over 74%; a rate faster than that of any other cancer (Oberg *et al.*, 1997; Fitzgerald *et al.*, 1996). Along with the direct effect of GERD and the potential for the development of Barrett's esophagus and adenocarcinoma, there have been many extra-esophageal complications such as asthma, chronic cough, chronic bronchitis, and dental erosions correlated with the presence of this disease (Alexander *et al.*, 2000; Richter, 1998; Paterson, 1997b).

Effects of inflammation on esophageal smooth muscle function:

Esophagitis can be categorized into different degrees of inflammation. Low-grade esophagitis is characterized by superficial epithelial loss, reactive epithelial changes (such as epithelial hyperplasia), extended papillae, and a minimal infiltration of inflammatory cells (Lanas *et al.*, 1999). Early in the course of acid-induced esophageal mucosal injury, there is an increase in microvascular permeability and blood flow, which may play a fundamental role in the further development of mucosal damage (Feldman *et al.*, 1996; Zijlstra *et al.*, 1991).

High-grade esophagitis is characterized by severe epithelial injury, including the development of erosions and ulcerations, along with the infiltration of the mucosa by neutrophils, eosinophils, and lymphocytes that are confined mostly to the esophageal mucosa, lamina propria, and muscularis mucosa (Lanas *et al.*, 1999). It has also been observed that damage to the esophagus caused by repetitive gastric acid exposure results in a permanent alteration to the function of the LES (DeMeester *et al.*, 1999; Biancani *et al.*, 1992; Biancani *et al.*, 1984). Inflammation also has many adverse effects on esophageal motility, producing a variety of symptoms including chest pain and dysphagia.

In the feline model of esophagitis, histological analysis of tissue revealed that the inflammatory infiltrate did not invade the muscle layers, which appeared entirely normal (Eastwood *et al.*, 1975). However, when studies were performed on the LES, it was discovered that despite the lack of inflammatory infiltrate there was impaired muscle function (Biancani *et al.*, 1984).

White *et al.* (2000) and Shirazi *et al.* (1989) found that the deglutition (swallowing) reflex in the opossum was impaired after the induction of esophagitis. Deglutition is a component in the defense mechanism that initiates a peristaltic wave to push refluxed material back to the stomach, as well as to deliver salivary bicarbonate to aid in acid neutralization (Helm *et al.*, 1984; Helm *et al.*, 1983). This impairment in the defensive repetoir may lead to increased acid exposure and worsening of esophagitis.

After the induction of acute esophagitis in the opossum, Barclay *et al.* (1995) observed fewer stainable mast cells in histological sections, indicating that there had been mast cell degranulation. Mast cell degranulation occurred in both the subepithelial and submucosa/muscularis regions, suggesting that the acid may have penetrated deep into the tissue layers (Feldman *et al.*, 1996; Barclay *et al.*, 1995). Since the products of the mast cell can activate enteric neurons and induce smooth muscle contraction (Paterson, 1998; Collins, 1996), this could lead to the hyper-responsiveness of the esophageal longitudinal smooth muscle layer as observed in the opossum after repeated acid exposure (White *et al.*, 2001). However, it has not been determined if there are changes directly to the smooth muscle cells. Therefore, detailed studies are needed to determine the source of the altered contractility and identify the effects of acid-induced esophagitis on the smooth muscle cells of the different smooth muscle layers.

Shirazi *et al.* (1989) found a decrease in the response to electrical field stimulation in the inflamed opossum esophageal circular and longitudinal smooth muscle strips, but the longitudinal muscle developed steeper length tension curves than in the normal esophagus. White *et al.* (2001) also observed that the CCh-induced active tension, at stretches from 0 - 50%, was significantly greater in longitudinal smooth muscle strips from inflamed animals than from controls. In addition to this, the passive tension generated by these strips showed a trend towards hyper-responsiveness at increasing levels of stretch, but this did not reach statistical significance (White *et al.*, 2000).

After inflammation, the resting membrane potential of the longitudinal smooth muscle cells from the opossum esophagus was significantly depolarized relative to controls (White *et al.*, 2001). This was consistent with observations on inflamed rabbit colonic circular smooth muscle (Cohen *et al.*, 1986). In addition to the depolarization of the membrane potential, acid-induced inflammation has been shown to cause spontaneous electrical activity, unstable membrane potentials, and ongoing spike-like action potentials (White *et al.*, 2001). The mechanisms for these alterations to the smooth muscle cells are currently unknown, and could represent changes in membrane ion channel expression, or in inflammatory mediators.

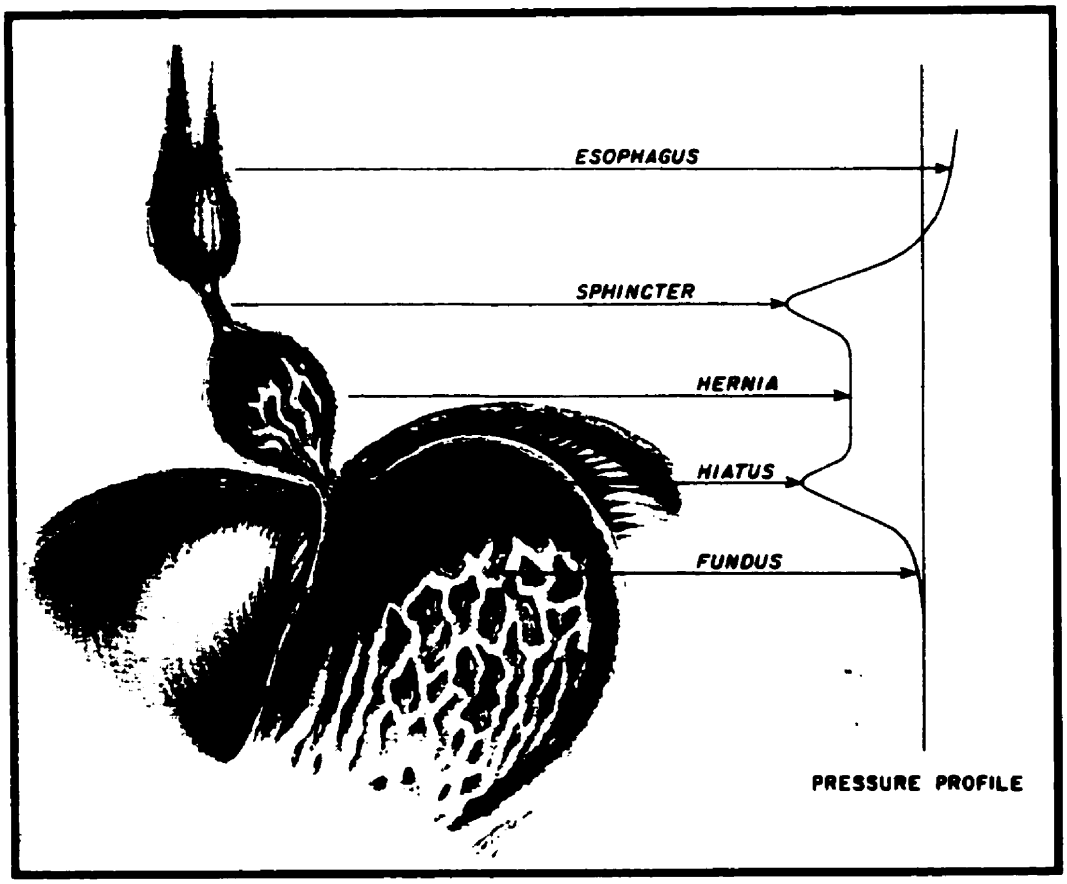
Sohn *et al.* (1997a) discovered that the cells of the feline LES had a functional phenotypic switch after inflammation. These cells switched from an ACh-induced contraction mediated by M_3 receptors linked to G_q-G_{11} that activated PI-PLC, to a contractile pathway using newly expressed M_2 receptors linked to G_o-G_{i3} that activate both PC-PLC and PC-PLD, as well as the previous pathway (Sohn *et al.*, 1997a). The expression of a new contractile pathway may represent a response to regain full cellular

function or it may also be the source of the dysfunction. Similar results have also been observed in circular muscle of the inflamed canine ileum (Shi and Sarna, 1999; Shi and Sarna, 1997). Therefore, it seems plausible that the altered contractility observed smooth muscle tissue strips (both longitudinal and circular) is a result of the inflammation-induced changes to the smooth muscle cells.

While GERD commonly coexists with a hiatus hernia, a direct cause/effect relationship has yet to be established. For many years it was believed that the presence of a hiatal hernia led to the development of GERD. However, in recent years there has been evidence suggesting that GERD leads to the development of a hiatal hernia (Paterson and Kolyn, 1994). The primary reason that this cause/effect relationship has not been identified is the fact that there are many patients with a significant hiatal hernia who do not develop GERD and patients with GERD who do not have the presence of a significant hiatal hernia (Paterson and Kolyn, 1994; Petersen *et al.*, 1991; Vanner and Paterson, 1988).

The presence of a hiatal hernia compromises several components of the esophageal defensive repertoire. Firstly, the displacement of the LES into the thoracic cavity provides a pocket where there is high pressure above (LES) and below (crural diaphragm), allowing gastric acid to pool (figure 7; Petersen *et al.*, 1991; Sloan and Kahrilas, 1991; Mittal *et al.*, 1987b). During transient LES relaxation the pool of acid has ready access to the esophagus and therefore reflux occurs more frequently. Secondly, it has been proposed that the LES and the diaphragm work together to maintain pressure (Mittal and Balaban, 1997; Mittal and Fisher, 1990; Boyle *et al.*, 1985). When the LES becomes displaced from the crural diaphragm, this pressure barrier decreases (Heine and

Figure 7: Possible mechanism for gastroesophageal reflux with the presence of a hiatus hernia. The drawing illustrates anatomic deformity of hernia. The pressure tracing demonstrates the resting pressure profile associated with hernia. The *right* tracing also illustrates the pressure differences of the gastric fundus (positive pressure) compared to the esophagus (negative pressure) relative to atmospheric pressure. Tonic LES contraction provides an anatomical pressure barrier that resists the tendency for the gastric contents to migrate into the esophagus. Adapted from Payne and Olsen (1974).


ESOPHAGUS
SPHINCTER
HERNIA
HIATUS
FUNDUS
PRESSURE PROFILE

Mittal. 1992; Mittal *et al.*, 1987a; Boyle *et al.*, 1985). Therefore, with the separation of these barriers, acid may more easily reflux into the esophagus during episodes of increased abdominal pressure. When acid comes into contact with the opossum esophagus there is a contraction in the longitudinal axis. With repeated acid exposure (45 minutes a day for 3 days) the longitudinal muscle layer maintained contraction 24 hours after the final acid exposure (White *et al.*, 2001). White *et al.* (2001) and Shirazi *et al.* (1989) found that the opossum esophagus shortened by approximately 8% and 13%, respectively, which is equivalent to approximately a 2 – 3 cm shortening of the human esophagus. The acid-induced sustained esophageal shortening observed in animal models suggests that gastroesophageal reflux may lead to hiatal hernia development. This shortening could pull a portion of the stomach above the diaphragm, creating a hiatal hernia. Observations have also shown that esophageal shortening normally associated with swallowing is not present in patients with a hiatal hernia (Kahrilas *et al.*, 1995) suggesting that these muscles may already be contracted.

With the development of a permanent hiatal hernia the esophagus would either have to be curved or become permanently shorter. A curved esophagus has not been observed to date and shortening likely depends on permanent contraction of the longitudinal smooth muscle (Paterson, 1998; Paterson and Kolyn, 1994; Shirazi *et al.*, 1989). However, it is currently unknown if the esophageal longitudinal smooth muscle cells are physically shorter after inflammation. It is more likely that esophageal shortening is a result of a sustained contraction of the hyper-responsive longitudinal smooth muscle cells.

Rationale for experimentation on isolated smooth muscle cells:

Many functional and physiological changes have been observed after inflammation in the opossum esophagus, but the basis of these changes is currently unknown. The purpose of this thesis was to determine if inflammation-induced altered contractility is due to changes in the smooth muscle cells themselves, or alternatively to changes in mediators within the tissue. To evaluate this, pure populations of smooth muscle cells were isolated from the opossum esophagus.

Goals:

1. To develop a technique for the isolation of viable individual smooth muscle cells from the different muscle layers of the esophagus.
2. To evaluate contractile responses to CCh in cells isolated from the different layers (longitudinal and circular) for both the control and inflamed esophagus.
3. To identify inflammation-induced morphological changes in the smooth muscle cells isolated from the different tissue layers

Hypothesis:

It is hypothesized that 1) isolated longitudinal smooth muscle cells will be hyper-responsive to the CCh, while the circular smooth muscle cells will be hypo-responsive and 2) there will be hypertrophy and hyperplasia of the cells of the circular smooth

muscle layer due to the closer proximity to the mucosal insult. but little or no change in the morphology of longitudinal smooth muscle.

Methodology:

Animal and tissue preparation:

Animal treatment:

Experiments were performed using adult opossums (*Didelphis virginiana*) of either sex and weighing between 3.1 – 5.3 kg (supplied by North Eastern Wildlife, South Plymouth, NY). Animals were housed at Queen's University Animal Care Services in metal rabbit cages for a minimum of 2 weeks to allow for acclimatization. Animals received antimicrobial treatment consisting of the antihelminthic agent Ivermection (0.1 ml/kg subcutaneous every two weeks) and the antibacterial agent Chloromycetin (0.1mg/kg subcutaneous every two weeks). Animals were maintained on a diet of dry cat food, canned dog food and fresh (seasonal) fruit. This protocol was approved by Queen's University Animal Care Committee, in accordance with the guidelines established by the Canadian Council on Animal Care. Animals were fasted in empty cages 10 – 12 hours prior to experimentation but allowed free access to water at all times.

Induction of esophagitis:

Experiments, as previously described by White *et al.* (2001), were performed at approximately the same time each day over a 4-day period. Briefly, opossums were anesthetized each day via dorsal tail vein injection of ketamine hydrochloride (25 mg/kg; Rogar, Montreal, PQ) and Valium (1 mg/kg; Hoffman-La Roche, Mississauga, Ontario, Canada). Buprenorphrine (0.01 mg/kg; Buprenex, Rickitt & Colman Pharma, Richmond,

VA) was then administered via tail vein to maintain analgesia, and the opossums were allowed a 30-minute stabilization period prior to experimentation. Animals were secured in a supine 30° head-up position (to minimize the reflux of esophageal perfusate) to a surgery table maintained at 35°C. An endotrachial tube was inserted and the cuff inflated to minimize the chance of aspiration of the perfusate. Maintenance doses of ketamine hydrochloride (12 mg/kg) were given via tail-vein as required.

A perfusion catheter was passed orally and placed 7 cm proximal to the LES. Either 0.9% saline or 100 mM HCl (both heated to 37°C) was perfused at a rate of 2 ml/min through the esophageal lumen for 45 minutes on each of 3 consecutive days. Rectal temperature, cardiac rate and rhythm and respiratory rate were monitored every 5 – 10 minutes. At the end of each perfusion (days 1 – 3), 100 ml of Ringer's solution (heated to 37°C) was administered subcutaneously to maintain the animals fluid and electrolyte balance. Intravenous injection of atropine sulfate (0.05 mg/kg; Abbott Laboratories Ltd., Montreal, Quebec, Canada) was given to prevent vomiting and excess secretion.

Manometry:

Isolation of the LES was accomplished using a custom-designed opossum LES sleeve assembly catheter system (Dentsleeve, Pty Ltd., Bowden, South Australia), which consisted of eight recording tubes with a 1.0 mm side-hole recording port. The tubing immediately distal to each recording port was sealed and bubble free water was perfused through each port at a rate of 0.3 ml/min. Pressure transducers (Transpack II, Sorenson

Research. Abbott. Salt Lake City, Utah) connected to recording tubes displayed esophageal pressure using Windaq/200 data acquisition software (DataQ Instruments. Akron. Ohio). The catheter was passed orally into the stomach and using a stepwise pull out technique the LES was localized by an elevation in pressure over basal gastric pressure. The location of the LES was then confirmed by several swallow-induced relaxations. The catheter was then slowly withdrawn until the LES pressure declined slightly identifying the proximal margin of the LES. The position of the LES was then marked on the catheter relative to a fixed position in the animal's mouth. Twenty-four hours after the final perfusion the LES was again located manometrically by a blinded observer and a second mark was made on the catheter. The distance between the two marks were measured and the difference was taken as a change in the LES position. The changes in LES position were then interpreted as a change in the overall length of the esophagus with proximal migration of the LES being interpreted as an overall shortening of the esophageal body (White *et al.*, 2001).

Isolation of circular and longitudinal smooth muscle cells:

On day 4 the animal was once again anesthetized and LES localized as outlined above. The esophagus was then exposed via midline sternotomy and the distal 9 – 10 cm excised and placed in Krebs' solution after first measuring the *in vivo* resting length. The animal was then sacrificed by an overdose of sodium pentobarbital (Biomedia/MTC Pharmaceutical; Cambridge. ON).

The esophagus was opened along the longitudinal axis and pinned out to its natural resting length (as determined by *in vivo* measurement) in fresh Krebs' solution

bubbled with 95%O_2/5%CO_2. The LES was located and the segment of the esophagus 3 – 5 cm proximal to the LES was removed and placed into fresh Krebs' solution. Using a dissecting microscope the tissue was pinned out in ice-cold Krebs' solution (5°C) with a slow 95%O_2/5%CO_2 bubble and the mucosa and sub-mucosa removed by sharp dissection (Zhang *et al.*, 2000). The tissue was then inverted, pinned out with slight stretch, and the connective tissue was removed from longitudinal smooth muscle layer. Special care was taken to remove as much of the serosal layer as possible as this was a limiting factor for the future use of this tissue. The tissue (now containing only circular and longitudinal smooth muscle with intervening myenteric plexus) was again inverted and pinned (circular layer up) with a slight stretch. Using fine forceps and scissors, the circular smooth muscle was removed from the longitudinal layer and placed into a separate beaker with fresh Krebs'. During the course of the tissue dissection the Krebs' solution was replaced at least every 10 minutes, and was constantly bubbled with 95%O_2/5%CO_2.

The technique for cell isolation was a modification of that described by Cao *et al.* (2000) and Sims (1992). Prior to tissue dissection, the HEPES physiological salt solution-digestion formula (HPSS-df) was prepared and cooled to 5°C. Isolated tissue layers were cut into thin strips (5 mm in width) and then cut again along one side to create a feathered effect. This increased the surface area exposed to the enzymes during the cell dissociation. Papain (0.5 mg/ml; lot #118H70091; Sigma), BSA (1 mg/ml), and DL-dithiothreitol (DTT) (1 μM; Sigma) was dissolved in HPSS-df, the pH adjusted to 7.2, and then collagenase Type-F (1 mg/ml for circular smooth muscle; 2.5 mg/ml for longitudinal smooth muscle; lot #89H8618, Sigma) was added. The tissue was placed in

the enzyme-solution, covered with parafilm and placed at 5°C overnight (approximately 14 hours).

The next morning, HPSS-isolation formula (HPSS-if) was prepared, placed in the water bath at 31°C with a steady oxygen (O_2) bubble for 30 minutes, and then the pH was adjusted to 7.4. The beakers containing the tissue were removed from the refrigerator and placed at room temperature for 30 minutes with O_2 superfused over the surface without agitating the solution. The beakers were then placed in the water bath at 31°C for 30 minutes with O_2 superfusion. The solution was then poured through a 200 μm-nylon filter and the tissue rinsed with HPSS-if (approximately 50 ml) to remove all traces of the enzyme solution. Tissue was then re-suspended in approximately 10 ml of HPSS-if solution and gently titrated until the solution became cloudy (approximately 50 titrations), which indicated that the cells had become dissociated. This solution was then filtered through a 500 μm-nylon mesh to remove the large pieces of tissue and the filtered solution was then placed on ice for approximately 15 minutes.

Measurement of carbachol-induced contraction of isolated smooth muscle cells:

CCh was dissolved in HPSS-if (without glucose added) to make stock solutions. All experiments were performed with the same stock solutions to ensure consistency of the results. CCh was added to microcentrifuge tubes, followed by addition of isolated cells for 30 seconds. The reaction was then stopped with acrolein at a final concentration of 1%. The final concentrations of CCh used in this study were 0.001-10 nM.

A small sample of the acrolein-fixed cell solution was placed on a glass slide with a cover-slip and viewed on an inverted phase-contrast microscope (Olympus IMT-2) connected to a closed circuit video camera. 30 random images (usually containing one cell per image) were taken per slide. Images were saved and coded to ensure a lack of bias. Length measurements were obtained by manually tracing down the middle of each cell using Image Pro 5 image-analysis program (Media Cybernetics, USA). The mean cell length was determined for each dose of CCh and plotted to obtain a dose response curve for the cells of each muscle layer.

Assessment of morphological changes:

Smooth muscle hypertrophy (tissue studies):

Segments of opossum esophagus 3 – 5 cm proximal to the LES were removed, opened along the longitudinal axis and placed in neutral buffered formalin (NBF) for 24 hours. The esophageal segments were then dehydrated in increasing ethanol concentrations, placed in xylene and then embedded in paraffin. Wax sections were cut (4 μm thick) using a microtome (American Optical), placed on APTEX coated slides, and allowed to air dry for 24 hours at room temperature.

Sections were de-waxed, stained with hematoxylin (to stain all nuclei), dehydrated and mounted with permount. Random images (5 images/section) were taken of each muscle layer (longitudinal and circular) at 40X magnification on an inverted bright field microscope and digitized. The area of each image was determined using the Image Pro program and the number of nuclei per image counted. The combination of these two

values allows calculation of the smooth muscle cell size. i.e., area of image/# nuclei (Blennerhassett *et al.*, 1992: Gabella, 1975). Note, however, that the relative sizes can only be compared between similar muscle layers (e.g. circular) and cannot be used to compare between muscle layers (e.g. circular to longitudinal).

Smooth muscle hypertrophy (cellular studies):

Smooth muscle cells were isolated (as described above), fixed with 1% acrolein (no agonists were applied) and placed on slides with a cover slip. 30 random images of viable-cells were taken at 20X magnification using an inverted phase contrast microscope (Olympus IMT-2). The area of the cells was determined in the control and inflamed longitudinal and circular muscle layers by manually tracing the outline of the cell using the Image Analysis program. Analysis of cellular size in using this method is a direct measure of cell size: therefore hypertrophy of the smooth muscle in the different muscle layers could be evaluated and compared.

Statistical Analysis:

All data are expressed as means ± standard error (SE). Statistical analysis was performed using one-way analysis of variance (ANOVA) with the student Newman-Keuls multiple comparison test for all contractility experiments and two-tailed students t-test for all other data comparisons. Maximal contraction was determined using a standard curve fit equation with weight on SE (Zhang and Lang, 1994). P values of <0.05 were considered statistically significant.

Results:

Acid-induced changes observed *in vivo*:

Altered LES position:

On day 1, after the 45-minute perfusion with 0.9% saline (n=7) there was a 7.1±2.2 mm aboral migration of the LES, indicating that the esophagus lengthened after saline perfusion (figure 8). Similarly, the results obtained on day 4, after 0.9% saline perfusion for 45-minutes a day for 3 consecutive days (n=7), indicated an esophageal lengthening of 6.0±1.2 mm (figure 8). The saline perfusion results (day 1 vs. day 4) were not significantly different ($p>0.05$) from each other. In contrast, esophageal perfusion with 100 mM HCl caused a significant ($p<0.0001$) oral migration of the LES by approximately 7.1±1.6 mm and 7.7±1.5 mm for both days 1 (n=8) and 4 (n=8), respectively (figure 8). There was no significant difference ($p>0.05$) between the acid perfusion results (day 1 vs. day 4).

Altered animal rectal temperature:

During the perfusion period rectal-temperature and cardiac and respiratory rates were monitored (table 2). Mean values (5 random measurements/animal) were compared for each animal, in the three categories listed above, on days 1 and 3 of the protocol. No significant differences were observed in any of the categories for animals that received saline perfusion ($p>0.05$). While no significant differences were observed between the cardiac ($p>0.05$) and respiratory rates ($p>0.05$), the animals that received esophageal acid perfusion (n=9) had a significantly greater ($p<0.001$) rectal temperature on day 1 of

Figure 8: The change in lower esophageal sphincter (LES) position after perfusion with 0.9% saline (control: n=7) or 100 mM HCl (esophagitis: n=8) on day 1 and day 4. There were no differences in the length of the esophagus between days 1 and 4 within either group (saline $p>0.05$: HCl $p>0.05$). However there was a significant difference between the saline and acid perfused groups after both the day 1 ($p<0.0001$) and day 4 ($p<0.0001$) perfusions. Positive values indicate an aboral migration of the LES (esophageal lengthening) and negative values indicate an oral migration of the LES (esophageal shortening). All values expressed as mean ± SE; **** = $p<0.0001$.

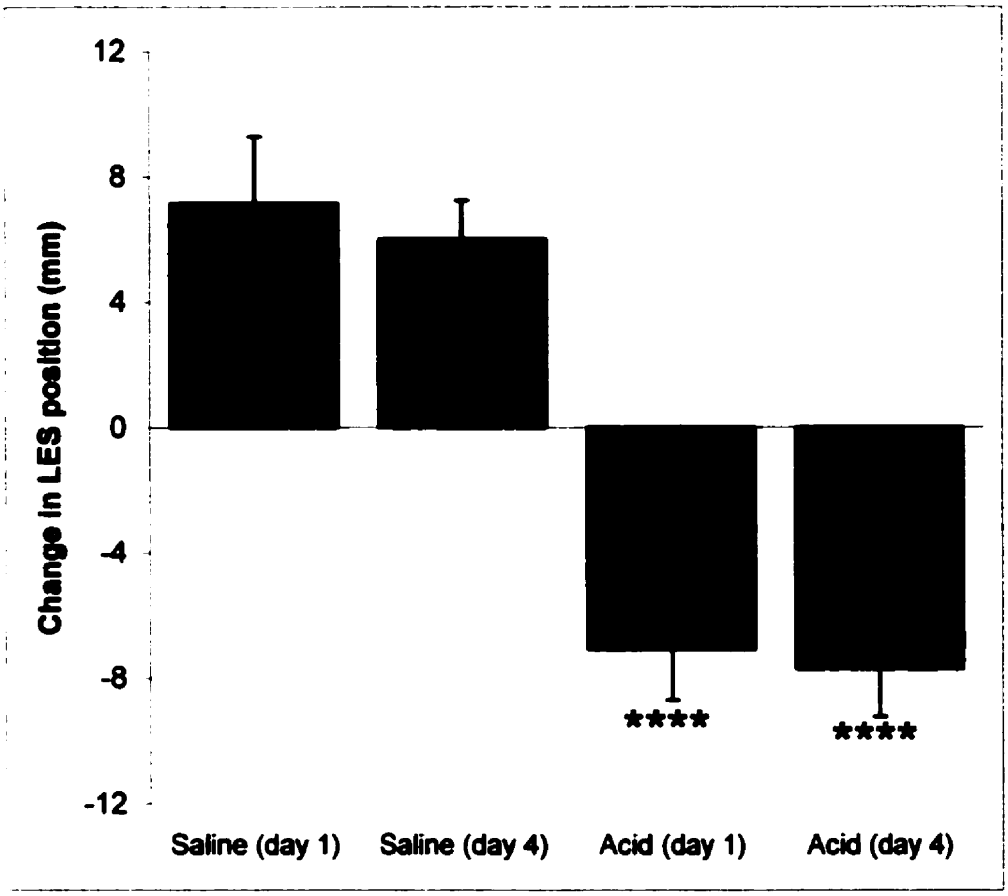

12
8
4
0
-4
-8
-12
Change in LES position (mm)
****
****
Saline (day 1)
Saline (day 4)
Acid (day 1)
Acid (day 4)

Table 2: The effects of esophageal perfusion on opossum heart and respiratory rates and body temperature. Perfusion with either 0.9% saline or 100 mM HCl did not have any effect on the heart (beats per minute) or respiratory (respirations per minute) rates at any point during the procedure. However, acid perfusion did cause a significant increase ($p<0.001$) in the rectal body temperature on day 1. This increase in temperature subsided by the end of the protocol on day 4.

	Saline perfusion		Acid perfusion	
	Day 1	Day 3	Day 1	Day 3
Cardiac rate (bpm)	156.6±7.7	152.0±4.7	149.3±5.3	153.2±5.2
Respiratory rate (rpm)	37.5±3.6	35.1±2.9	30.6±2.2	31.4±3.2
Rectal body temperature (°C)	33.3±0.4	33.1±0.3	34.0±0.2 ***	32.7±0.2

perfusion compared to day 3 (n=8; table 2).

Acid-induced changes in the smooth muscle cells:

Resting length:

Under phase contrast microscopy, viable smooth muscle cells had smooth membranes free of distortions and blebs (figure 9). The resting length of the smooth muscle cells isolated from animals receiving saline perfusion was not significantly different than cells from the acid perfused animals (64.6±7.8 μm vs. 69.0±5.6 μm ($p>0.05$) in longitudinal smooth muscle cells: 89.0±4.8 μm vs. 96.6±3.6 μm ($p>0.05$) in circular smooth muscle cells, saline vs. acid, respectively) (figure 10). Longitudinal smooth muscle cells were significantly shorter that circular smooth muscle cells from both acid ($p<0.001$) and saline ($p<0.05$) perfused tissues.

Contractility of longitudinal smooth muscle cells:

CCh caused shortening (contraction) of isolated longitudinal smooth muscle cells in a dose-dependent fashion. There were no significant differences ($p>0.05$) between the lengths of cells isolated from animals perfused with saline (n=6) or acid (n=8) at any of the concentrations of CCh used (figure 11). Similarly, there were no differences ($p>0.05$) observed in the percent contraction of the cells isolated from the two conditions to increasing concentrations of CCh. Maximal contraction occurred at 10 nM CCh, for both saline and acid perfused groups, decreasing the mean longitudinal smooth muscle cell

Figure 9: Images of typical isolated opossum esophageal smooth muscle cells (SMC). Top panels are circular SMC (A) relaxed and (B) maximally contracted with 1nM CCh. Bottom panels are of isolated longitudinal SMC (C) relaxed and (D) maximally contracted with 10 nM CCh. Note scale bar is 25 μm.

Figure 10: The resting length of isolated esophageal smooth muscle cells (SMC) perfused with either saline or acid. SMC were enzymatically isolated from tissue 3 – 5 cm proximal to the lower esophageal sphincter from the saline (circular n=8; longitudinal n=6) and acid (circular n=8; longitudinal n=9) perfused animals. The mean resting lengths from cells obtained from saline and acid perfused animals were not significantly different in longitudinal ($p>0.05$) or circular ($p>0.05$) tissue. However, the mean resting length of longitudinal and circular SMC were significantly different in cells obtained from both saline ($p<0.05$) and acid ($p<0.001$) perfused tissues. All values expressed as mean ± SE; * = $p<0.05$ & *** = $p<0.001$.

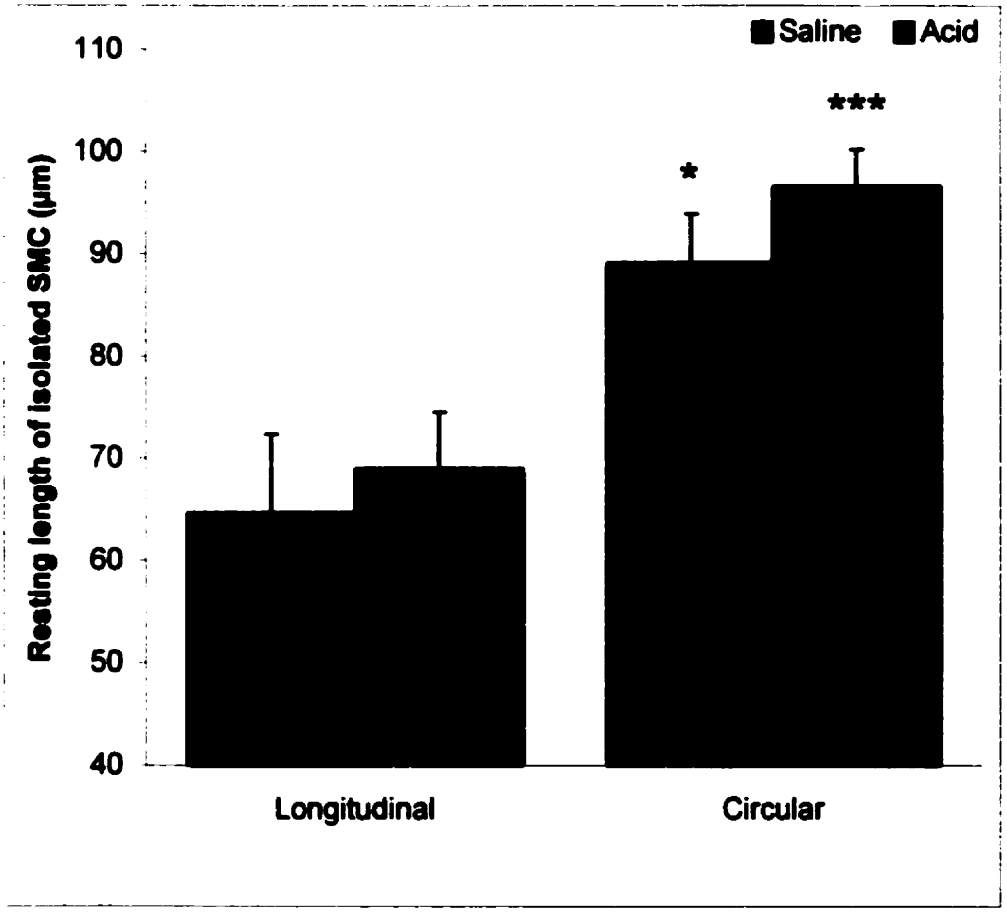
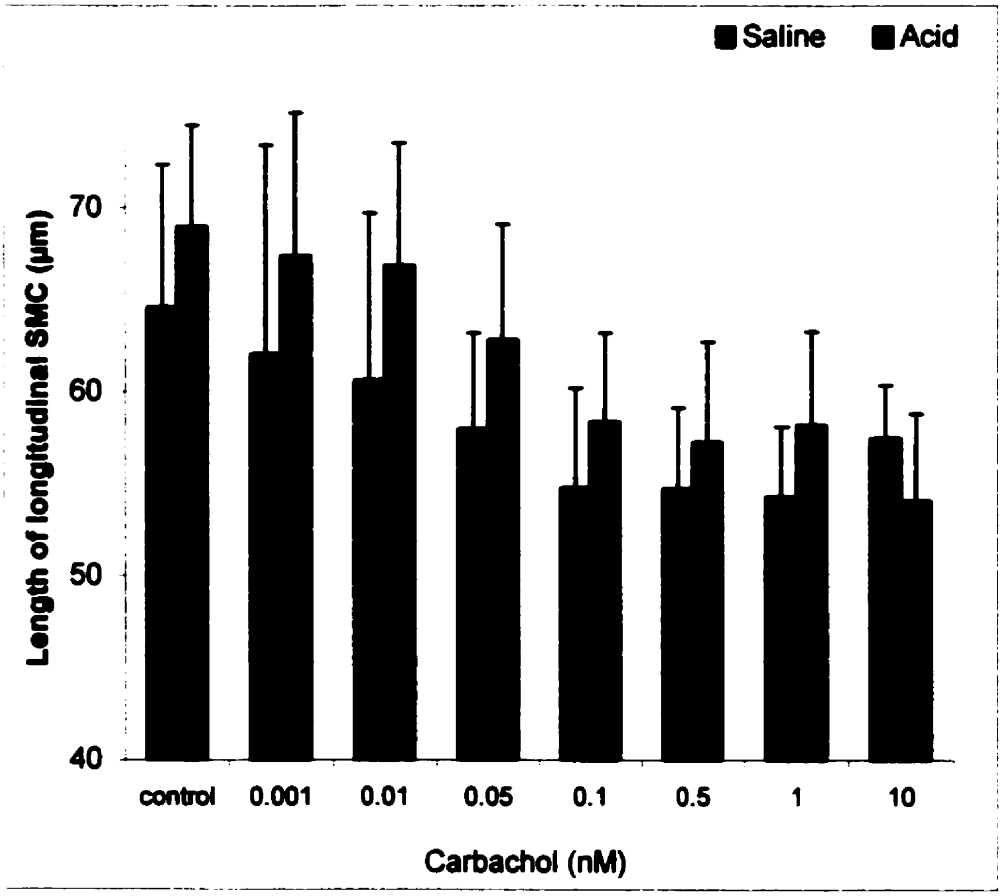

Saline
Acid
110
100
90
80
70
60
50
40
Resting length of isolated SMC (µm)
*
***
Longitudinal
Circular

Figure 11: Carbachol (CCh)-dose response of isolated longitudinal smooth muscle cells (SMC). SMC were enzymatically isolated from opossum esophagi, 3 – 5 cm proximal to the lower esophageal sphincter, perfused with either 0.9% saline or 100 mM HCl. Aliquots of isolated cells were exposed to various doses of CCh (0.001 – 10 nM) for 30 seconds and fixed with acrolein (final concentration 1%). There were no significant differences ($p>0.05$) in the contractility of cells isolated from tissue perfused with saline or acid. CCh caused a dose-dependent contraction in both groups with maximal contraction occurring at 10 nM for each group. Saline n=6 with the following exceptions: 0.001, 0.01 & 10 nM n=4; Acid n=8 with the following exceptions: 0.001 nM n=5; 0.01 & 10 nM n=6. All values expressed as mean ± SE.


Saline
Acid
Length of longitudinal SMC (µm)
70
60
50
40
control
0.001
0.01
0.05
0.1
0.5
1
10
Carbachol (nM)

length by 19.4±4.2% and 21.7±3.4% (saline and acid, respectively) of control length (figure 12).

Contractility of the circular smooth muscle cells:

CCh caused shortening (contraction) of isolated circular smooth muscle cells in a dose-dependent fashion. There were no significant differences ($p>0.05$) in the mean lengths of cells isolated from animals perfused with saline ($n=8$) or acid ($n=9$) at all concentrations of CCh used (figure 13). However, there was a significant decrease in the ability of the cells obtained from animals perfused with acid to contract at the higher concentrations of CCh (>1 nM, $p<0.01$) compared to cells isolated from saline perfused animals. Maximal contraction occurred at 1 nM CCh for saline perfused animals and 0.5 nM CCh for the acid perfused group. While the maximal contraction for the cells obtained from saline perfused animals was 21.1±2.0%, the maximal contraction of the acid treated cells was significantly reduced to only 8.7±1.6% (figure 14). This represents a 2.4 fold decrease in the contractility of the circular smooth muscle cells from acid-perfused tissue.

Figure 12: Percent shortening of isolated longitudinal smooth muscle cells (SMC) exposed to various concentrations of carbachol (CCh). Percent contraction was determined as the percentage of shortening of cells at a specific CCh concentration compared to the mean resting cell length. Smooth muscle cells were enzymatically isolated from opossum esophagi, 3 – 5 cm proximal to the lower esophageal sphincter, perfused with either 0.9% saline or 100 mM HCl. Aliquots of isolated cells were exposed to various doses of CCh (0.001 nM - 10 nM) for 30 seconds and fixed with acrolein (final concentration 1%). CCh caused a dose dependent increase in the contraction of cells isolated from both the saline and acid perfused animals with maximal contraction occurred at 10 nM for each group. There were no significant differences ($p>0.05$) found in the contractility of the cells in these two groups. Saline n=6 with the following exceptions: 0.001, 0.01 & 10 nM n=4; Acid n=8 with the following exceptions: 0.001 nM n=5; 0.01 & 10 nM n=6. All values expressed as mean ± SE.

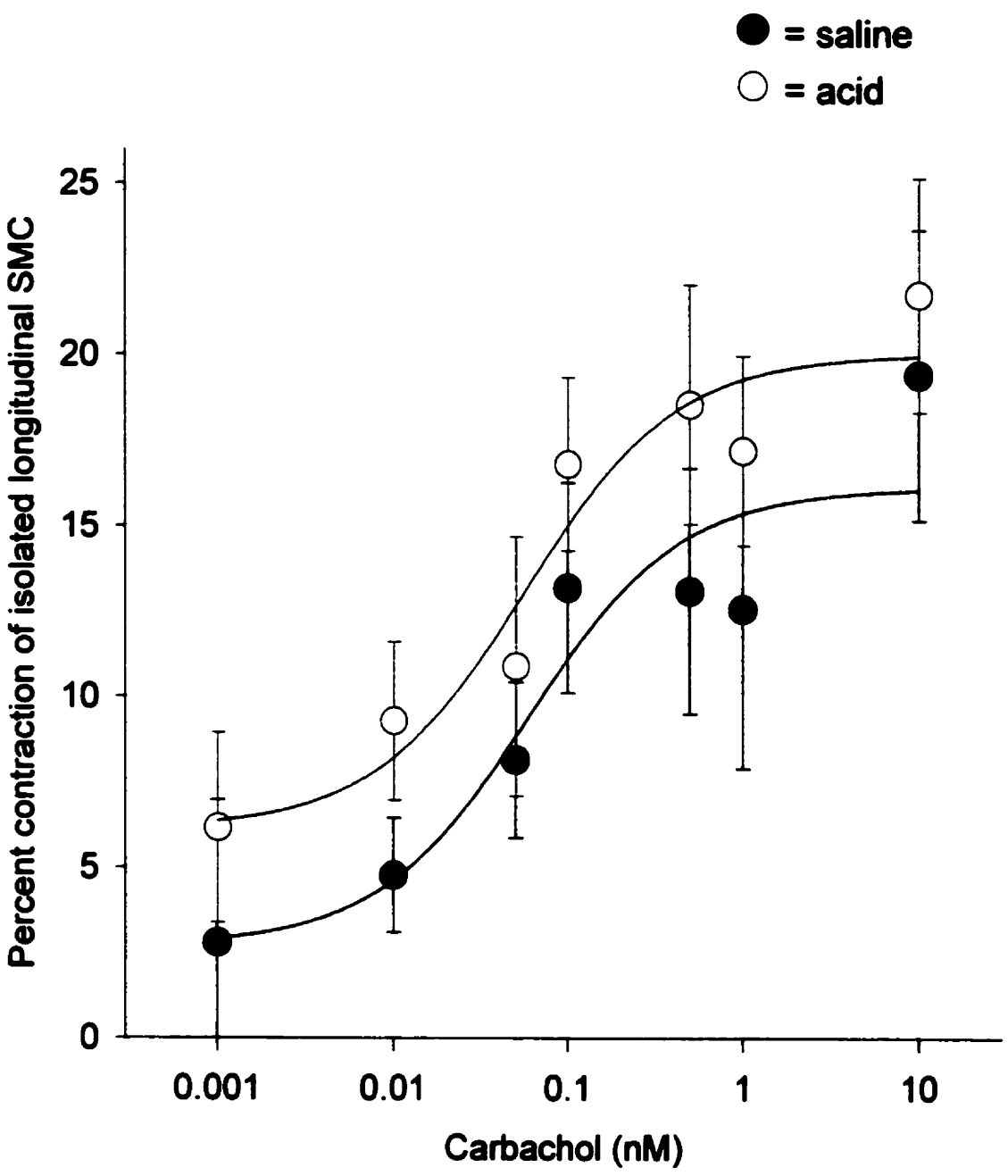

● = saline
○ = acid
Percent contraction of isolated longitudinal SMC
25
20
15
10
5
0
0.001
0.01
0.1
1
10
Carbachol (nM)

Figure 13: Carbachol (CCh)-dose response of isolated circular smooth muscle cells (SMC). SMC were enzymatically isolated from opossum esophagi. 3 – 5 cm proximal to the lower esophageal sphincter. perfused with either 0.9% saline of 100 mM HCl. Aliquots of isolated cells were exposed to various doses of CCh (0.001 nM - 10 nM) for 30 seconds and fixed with acrolein (final concentration 1%). CCh caused a dose dependent contraction in both the control and inflamed cell with maximal contraction occurring at 1 nM and 0.5 nM respectively. There were no significant differences ($p>0.05$) between the two groups. Saline n=8 with the following exceptions: 0.001 n=4 & 0.01 nM n=5: Acid n=9 with the following exceptions: 0.001 nM n=5 & 0.01 nM n=6. All values expressed mean ± SE.

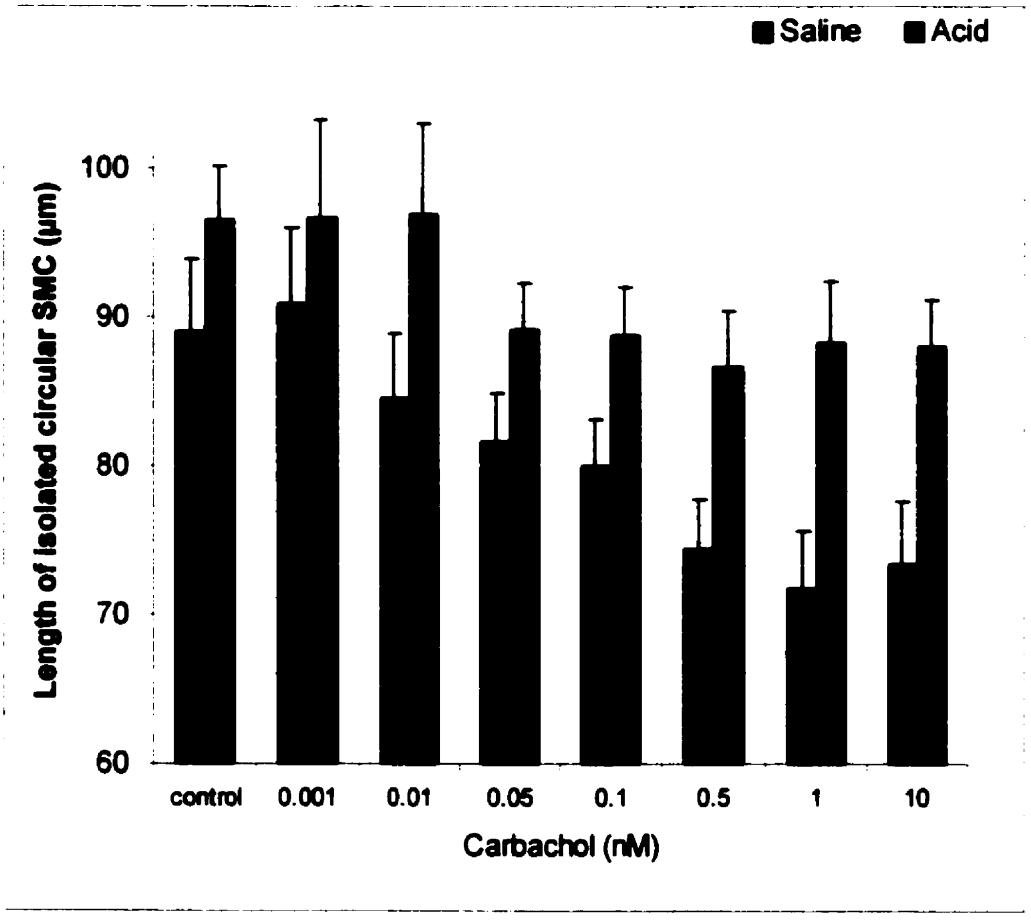
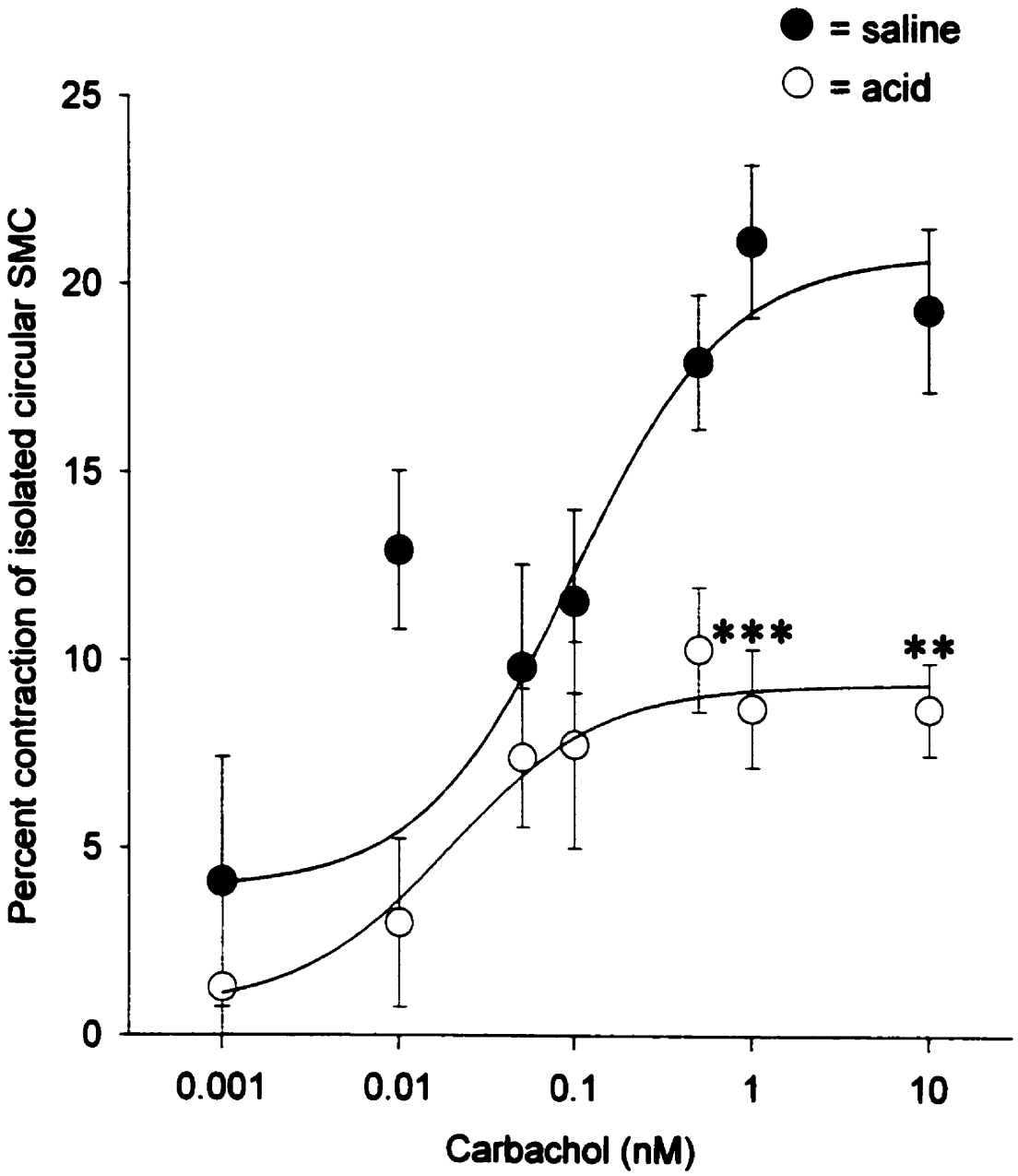

Saline
Acid
Length of isolated circular SMC (µm)
100
90
80
70
60
control
0.001
0.01
0.05
0.1
0.5
1
10
Carbachol (nM)

Figure 14: Percent shortening of isolated circular smooth muscle (SMC) to various concentrations of carbachol (CCh). Percent contraction was determined as the percentage of shortening of cells at a specific CCh concentration compared to the resting cell length. Smooth muscle cells were enzymatically isolated from opossum esophagi, 3 – 5 cm proximal to the lower esophageal sphincter, perfused with either 0.9% saline or 100 mM HCl. Aliquots of isolated cells were exposed to various doses of CCh (0.001 nM - 10 nM) for 30 seconds and fixed with acrolein (final concentration 1%). CCh caused a dose dependent increase in contraction of cells isolated from both the saline and acid perfused animals with maximal contraction occurring at 1 nM and 0.5 nM, respectively. There was a significant attenuation in the contraction of cells from the acid perfused tissue at the higher doses of CCh (1 & 10 nM, $p<0.001$ & $p<0.01$, respectively). Saline n=8 with the following exceptions: 0.001 n=4 & 0.01 nM n=5; Acid n=9 with the following exceptions: 0.001 nM n=5 & 0.01 nM n=6. ** $p<0.01$: *** = $p<0.001$. All values expressed as mean ± SE. Note: line of best fit for saline perfused animals excluded 0.01 nM value.


● = saline
○ = acid
Percent contraction of isolated circular SMC
25
20
15
10
5
0
***
**
0.001
0.01
0.1
1
10
Carbachol (nM)

Acid-induced morphological changes in esophageal smooth muscle:

Using two methods to calculate the size of the smooth muscle cells. we identified hypertrophy in the cells from the circular but not the longitudinal smooth muscle layer. The first method for the identification of hypertrophy involved measuring the area of isolated smooth muscle cells from saline and acid perfused animals. The size of the isolated longitudinal smooth muscle cells from animals perfused with acid (736.2±17.2 μm^2) was not significantly different ($p>0.05$) from cells isolated from saline (746.3±19.3 μm^2) perfused animals (figure 15).

In contrast. circular smooth muscle cells isolated from animals perfused with acid (941.1±17.0 μm^2) had a significantly larger area ($p<0.01$) than cells isolated from animals perfused with saline (875.7±17.8 μm^2: figure 15). Unlike the resting length. there was no significant difference ($p>0.05$) in the area of longitudinal and circular cells isolated from saline perfused animals. However. as expected. the area of the circular smooth muscle cells from animals perfused with acid was significantly greater ($p<0.01$) than the longitudinal smooth muscle cells.

The second method for the identification of hypertrophy involved using tissue sections to determine cell size. This was performed by identifying the number of smooth muscle cell nuclei present in a specific (known) area of the tissue section. These values could then be converted to a relative size for each smooth muscle cell present in that section (see methods). We found that there was no difference ($p>0.05$) in the size of the longitudinal smooth muscle cells between saline (623.6±139.6 μm^2) and acid (586.9±29.6

μm^2) perfused animals (figure 16). However, there was a significant increase ($p<0.05$) in the size of the circular smooth muscle cells in tissue from animals perfused with acid (1032.8±194.7 μm^2) compared to those perfused with saline (716.5±32.0 μm^2; figure 16).

In addition to the increase in cell size, it was also noted that there was a significant increase ($p<0.01$) in the mean size of the nuclei in the circular smooth muscle from animals perfused with acid (16.1±1.0 μm) compared to animals perfused with saline (13.3±1.5 μm; figure 17). There was no significant difference ($p>0.05$) in nuclear size in the longitudinal smooth muscle cells (6.0±1.0 μm and 6.1±0.5 μm, saline vs. acid, respectively). Size was determined by measuring the length through the longest part of the nucleus.

Figure 15: Direct measure of hypertrophy in isolated longitudinal (A) and circular (B) smooth muscle cells (SMC). Enzymatically isolated SMC were fixed with acrolein (final concentration 1%). 30-cells/muscle-layer/animal were viewed and digitized using video microscopy and the area of the isolated cells was determined using image pro software. There was no significant difference ($p>0.05$) between the mean areas of isolated longitudinal SMC from animals perfused with saline (n=6) or acid (n=8). However, there was a significant increase ($p<0.01$) observed in the area of circular SMC from acid (n=9) perfused animals compared to saline (n=7). All values expressed as mean ± SE. ** = $p<0.01$.

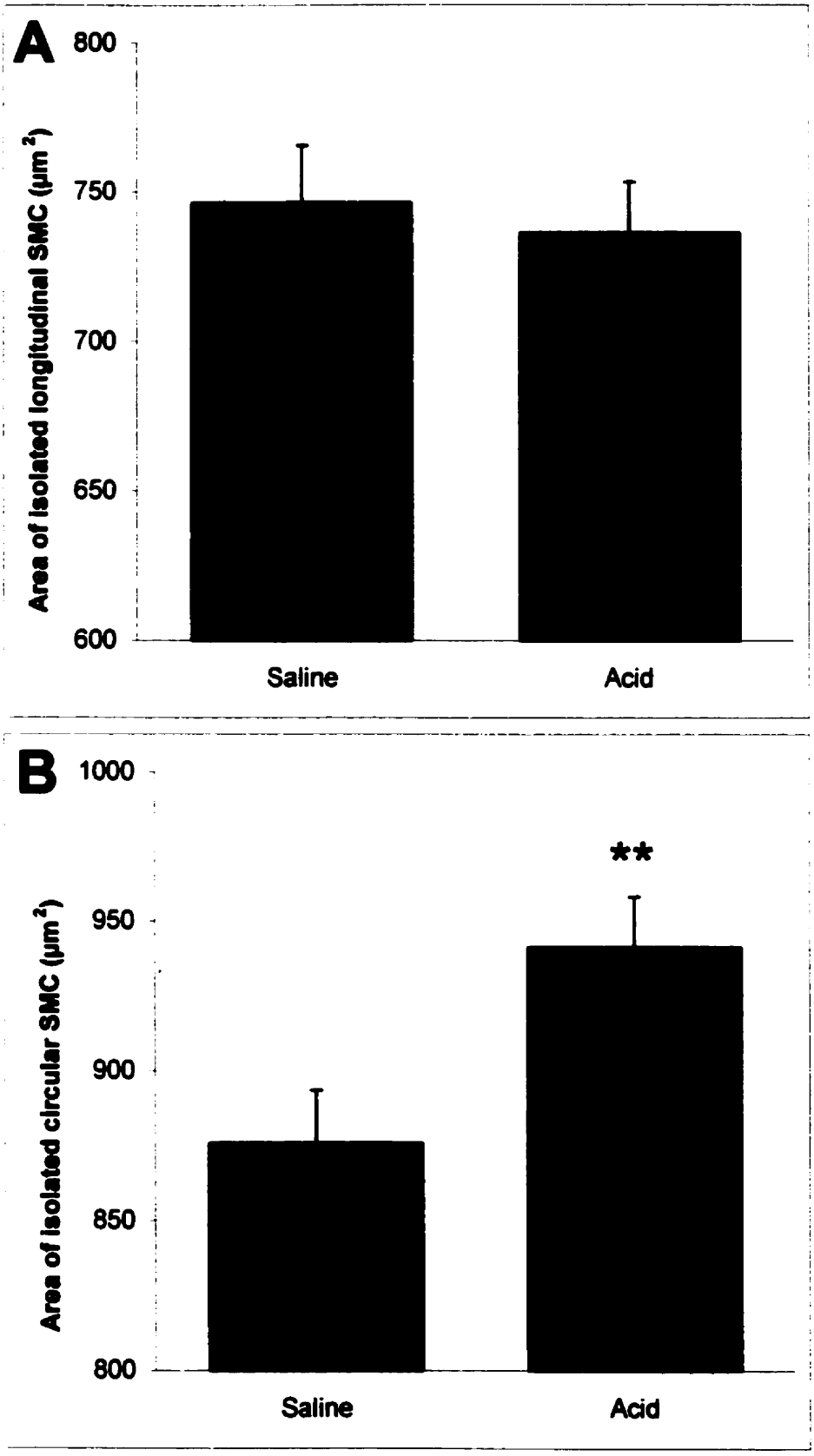

A
800
750
700
650
600
Area of isolated longitudinal SMC (μm²)
Saline
Acid
B
1000
950
900
850
800
Area of isolated circular SMC (μm²)
**
Saline
Acid

Figure 16: Relative measure of cellular hypertrophy in longitudinal (A) and circular (B) smooth muscle using tissue sections. Sections were made from opossum esophageal tissue, located 3 – 5 cm proximal to the lower esophageal sphincter, perfused with either 0.9% saline (n=3) and 100 mM HCl (n=3). Sections were stained using hematoxylin to identify nuclei. 5 random images of a known area were taken of each muscle layer (circular and longitudinal), all nuclei/image were counted, and relative cell size was determined using these two values. There was a significant increase ($p<0.05$) in the size of the circular smooth muscle cells (SMC) from animals perfused with acid compared to animals perfused with saline. In contrast there were no differences observed ($p>0.05$) in the size of the longitudinal SMC from saline and acid perfused animals. All values expressed as mean ± SE. * = $p<0.05$.

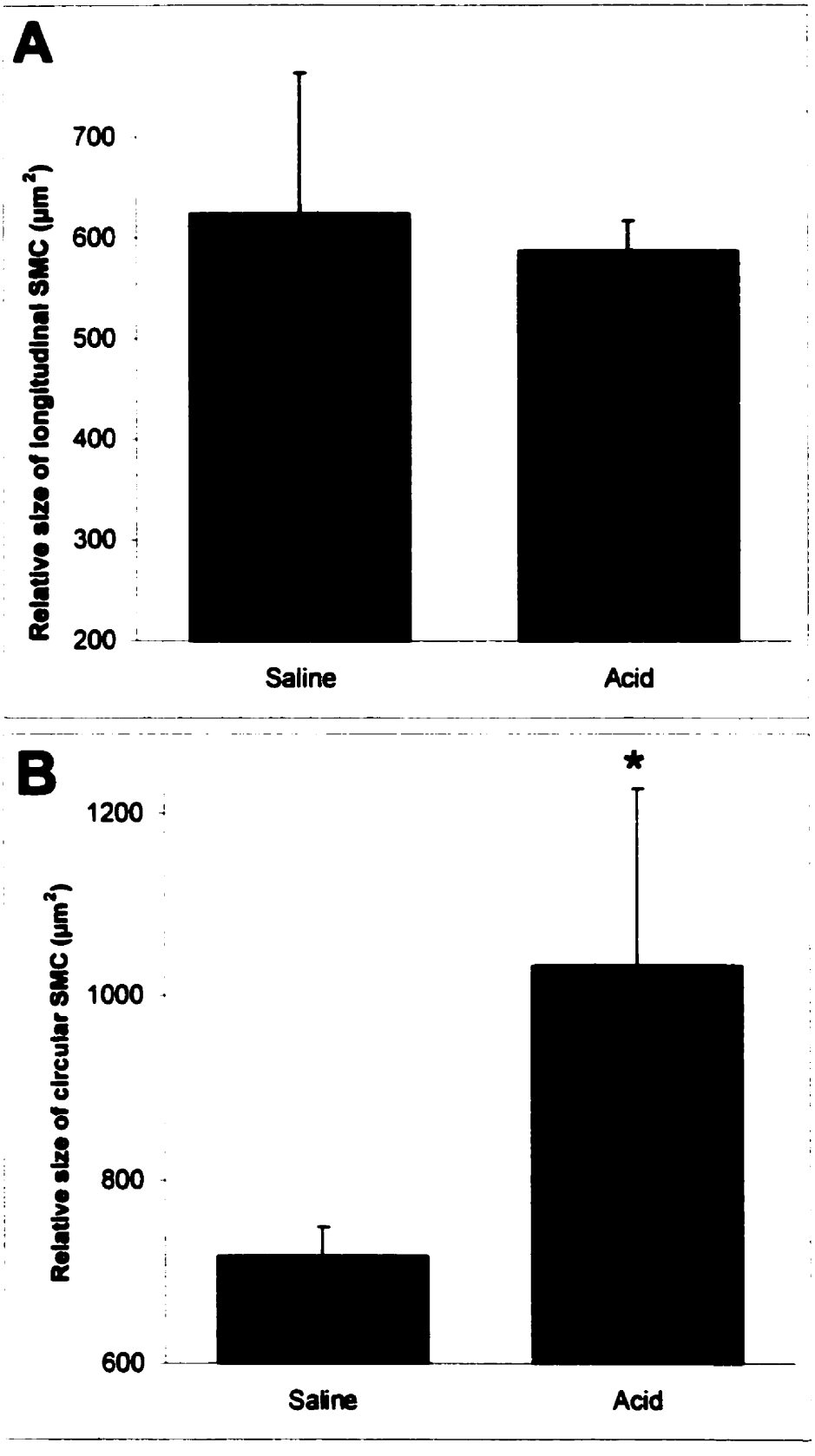
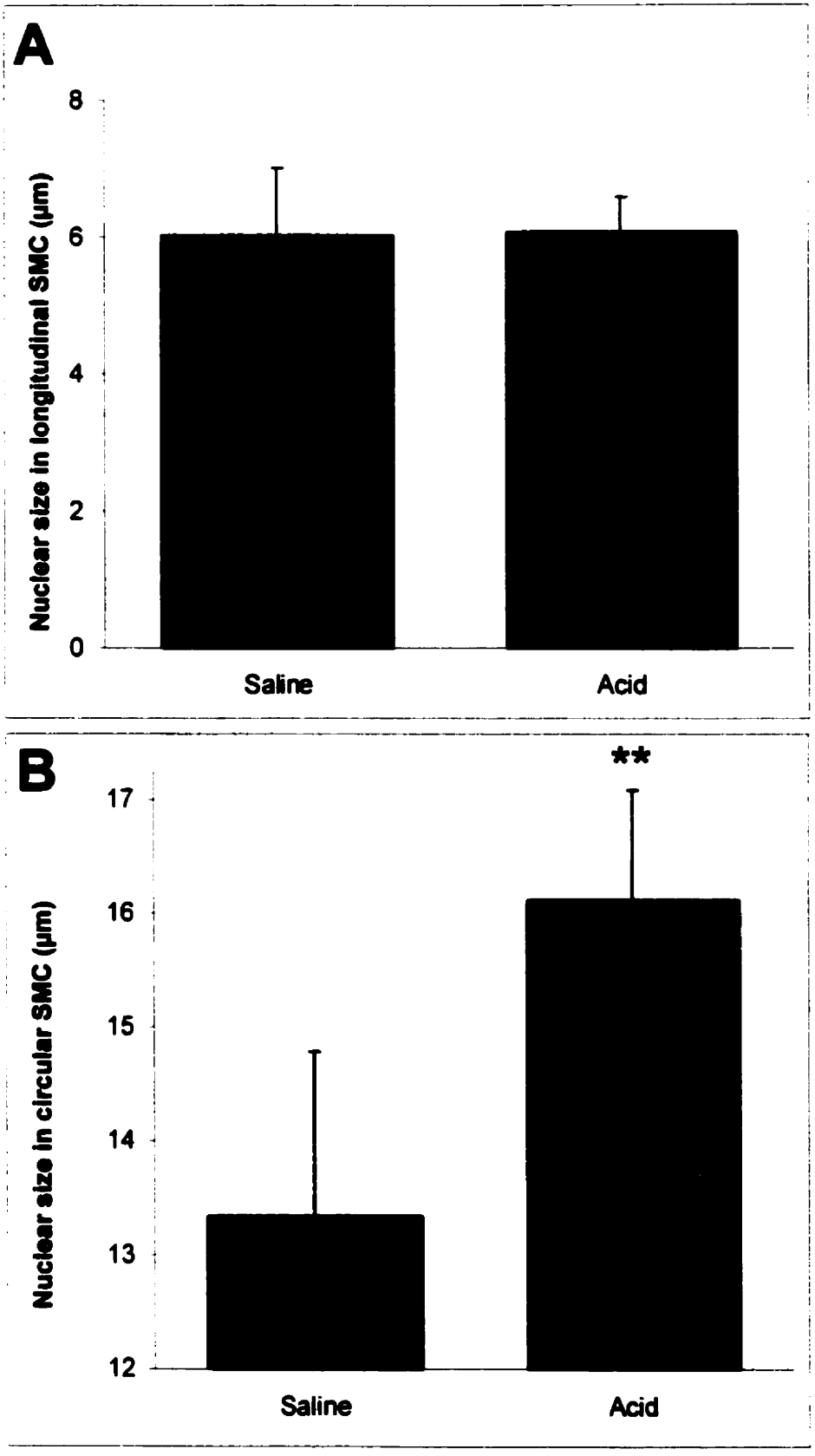

A
Relative size of longitudinal SMC (μm²)
700
600
500
400
300
200
Saline
Acid
B
*
Relative size of circular SMC (μm²)
1200
1000
800
600
Saline
Acid

Figure 17: Change in mean nuclear length as a result of acid-induced inflammation. Nuclear size was determined from longitudinal (A) and circular (B) smooth muscle tissue sections taken from the opossum esophagus, 3 – 5 cm proximal to the lower esophageal sphincter, perfused with either 0.9% saline (n=3) or 100 mM HCl (n=3). Sections were stained using hematoxylin to identify nuclei. 5 random images were taken of each muscle layer (circular and longitudinal) and the length (determined by the longest diameter present) of all smooth muscle nuclei were measured using video micrometry. There was a significant increase ($p<0.01$) in the mean length of the nuclei in the circular smooth muscle cells (SMC) from animals perfused with acid compared to animals perfused with saline. In contrast there were no differences observed ($p>0.05$) in the length of nuclei from the longitudinal SMC between saline and acid perfused animals. All values expressed as mean ± SE. ** = $p<0.01$.


A
8
6
4
2
0
Nuclear size in longitudinal SMC (μm)
Saline
Acid
B
**
17
16
15
14
13
12
Nuclear size in circular SMC (μm)
Saline
Acid

Discussion:

The results of this study provide novel insight into the pathophysiology of GERD and the etiology of hiatus hernia. Previous studies have provided evidence that acid-induced inflammation results in altered smooth muscle function. with the longitudinal smooth muscle becoming hyper-responsive and the circular smooth muscle layer becoming hypo-responsive. However. the origin of the altered esophageal contractility was unknown. There is speculation that sustained shortening and hyper-contractility of the esophagus along the longitudinal axis could be associated with hiatal hernia development (Paterson and Kolyn. 1994). We have confirmed previous studies (White *et al.*. 2001; Shirazi *et al.*. 1989) indicating that acid-induced inflammation is associated with a sustained esophageal shortening. However. we have shown for the first time that sustained esophageal shortening and altered contractility are not associated with changes (contractile or morphological) to the individual longitudinal smooth muscle cells. Rather. the functional alterations of the inflamed esophageal longitudinal smooth muscle layer are most likely a result of inflammatory mediators in the milieu interacting with the smooth muscle cells. In contrast to the longitudinal smooth muscle. we have shown that the decreased contraction of the circular smooth muscle layer can be (at least partially) attributed to direct functional and morphological changes to the circular smooth muscle cells.

Inflammation-induced esophageal shortening:

Manometry enables the identification of LES location, and recently this technique

has been adapted to determine changes in esophageal length by identifying oral or aboral migration of the LES (White *et al.*, 2001; Shirazi *et al.*, 1989). Using this technique, we found that acid perfusion caused a significant oral migration of the LES (esophageal shortening) on days 1 and 4. In contrast, saline perfusion resulted in an aboral migration of the LES (esophageal lengthening) on both days 1 and 4. The reason for the observed esophageal lengthening in the control group is unclear, but may be a result of stressors (such as food deprivation, transportation, injection, and handling) on the opossum, prior to the perfusion period. Such non-specific stress may have resulted in a slight contraction of the esophageal longitudinal smooth muscle layer, and the observed esophageal lengthening with saline perfusion may simply represent a return to resting length. By day 4, opossums may have acclimatized to the experimental protocol and therefore there may have been little or no non-specific esophageal shortening during animal preparation.

These same stress factors would have been present in the animals receiving acid perfusion. Therefore, the esophagus of animals receiving acid perfusion should respond to stress in a similar fashion as the saline perfused animals. Since the acid-perfused esophagus shortened an additional 7.1 mm, the actual acid-induced esophageal shortening would be the difference between the esophageal length in saline and acid perfused animals. Therefore, a 45-minute acid perfusion caused a mean esophageal shortening of approximately 14 mm. In addition, the overall shortening observed on day 4 was 24 hours after the final acid perfusion. Therefore, this shortening must be due to a sustained contraction of the longitudinal muscle layer. These results are similar to those observed by White *et al.* (2001) and Shirazi *et al.* (1989).

While it has been widely accepted that the presence of a hiatal hernia provides a

mechanism for increased gastroesophageal reflux, it is unknown if the hiatus hernia causes gastroesophageal reflux or if gastroesophageal reflux leads to the development of a hiatal hernia. These results suggest that hiatal hernia development may occur as a result of sustained esophageal shortening triggered by recurrent episodes of acid reflux.

The opossum esophagus is approximately 15 cm long (White *et al.*, 2001), thus the degree of shortening observed in this study represents 9.3% of the total esophageal length. Since the human esophagus is approximately 22 cm in length (Goyal and Sivarao, 1999), the shortening observed in the opossum would be equivalent to a 2.0 cm oral migration of the LES in humans. Along with providing a pocket for the acid to pool, a hiatal hernia separates the LES from the crural diaphragm. It has been postulated that these two features work together to provide an anti-reflux barrier (Mittal and Balaban, 1997; Boyle *et al.*, 1985). Therefore, with the separation of these two structures there would be a decrease in the anti-reflux barrier at the gastroesophageal junction and subsequently reflux could occur more easily.

Since the opossum LES is only 5 mm in length it is unlikely that changes in esophageal length are a reflection of measurements taken at different locations within the LES. However, to minimize this possibility several precautions were taken. First, all measurements were made, using manometric pull-through technique, on the oral side of the LES, and were confirmed at least twice. Second, a blinded observer (who did not know the composition of the esophageal perfusate) confirmed the position of the LES, thus eliminating observer bias. Third, if the size of the LES were a factor it would have been expected to increase the variance of the measurements, yet this was not the case.

It is noteworthy that on day 1 there was an initial spike in rectal temperature in animals receiving an acid perfusion. This increase in the opossum rectal temperature is likely a systemic response to the esophageal inflammation. Michel *et al.* (2001) and Michel *et al.* (1997) found that in humans, inhaled lipopolysaccharide significantly elevated body temperature (approximately 0.7°C) for up to 4 hours, after which the temperatures returned to normal. The elevated temperature observed in our animals had subsided by day 3 of the protocol. This elevation in temperature would not have significantly influenced the *in vitro* function of the smooth muscle cells for two reasons. Firstly, the fever subsided by the time the tissue was harvested and any temperature-dependent variables would no longer be present. Secondly, since the smooth muscle cells were isolated from the tissue and maintained at 35°C during isolation, these minor changes in animal body temperature would not likely affect cellular function *in vitro*.

Inflammation-induced alterations to smooth muscle cells:

CCh-induced contractility of isolated smooth muscle cells:

The resting lengths of isolated longitudinal and circular smooth muscle cells were compared between opossums that had received esophageal perfusion with either 0.9% saline or 100 mM HCl. The longitudinal smooth muscle cells were significantly shorter than the circular smooth muscle cells in both the saline and acid perfused animals. However, there was no significant difference in the resting length of cells isolated from saline or acid perfused animals in either the longitudinal or the circular smooth muscle layers.

Inflammation may result in a sustained contraction of the longitudinal smooth muscle cells, causing an esophageal shortening and possibly the development of a hiatus hernia. However, when we examined the resting length of isolated longitudinal smooth muscle cells there were no observed differences between control and inflamed tissues. This is not surprising since we have taken scrupulous measures to ensure the cells were isolated in a relaxed state. Therefore, possibly as a result of isolation techniques, the cells from inflamed tissue were the same length as control cells. This suggests that the esophageal shortening is not likely a result of permanent changes to the resting length of the longitudinal smooth muscle cells.

We have provided evidence that sustained shortening and hyper-responsiveness during esophagitis is not associated with changes (resting length and contractility) of the longitudinal smooth muscle cells. In a similar study, inflammation of the canine ileum had no effect on muscarinic receptor-induced contraction of the longitudinal smooth muscle cells (Shi and Sarna, 1999). In contrast, Blennerhassett *et al.* (1999b) found that there was a marginal yet statistically significant increase (9%) in contractility of the longitudinal smooth muscle cells, isolated from the inflamed mouse jejunum. However, this study has only been published in abstract form. Also, the standard error of their measurement was extremely small, which may have resulted in the significant difference between these marginal alterations in contraction. Finally, the cells isolated from the infected jejunum were wider and approximately twice the length of the cells isolated from control tissue (125±13μm vs. 57±12μm, respectively). However, these changes in cell size did not affect the variation suggesting that inflammation affects all the smooth muscle cells in the same manner, resulting in the same degree of hypertrophy in all

affected cells. This uniform cellular hypertrophy suggest that the inflammation induced in their model by *T. spiralis* infection produced profound changes to the entire muscularis propria, whereas in our model, changes to the longitudinal smooth muscle are more subtle. *T. spiralis* can gain access to the mucosa and submucosa, thereby causing an inflammatory response deep into the tissue. In contrast, with our model of inflammation, acid is perfused over the luminal surface, which may not initiate an inflammatory response as deep into the tissue as the parasite.

Our results suggest that other factors interacting with the longitudinal smooth muscle cells must be involved in the sustained contraction of the longitudinal smooth muscle layer and the altered contractility that has been observed. Inflammatory mediators within the tissue, such as the products of mast cells, may result in sustained acid-induced esophageal shortening (Feldman *et al.*, 1996; Barclay *et al.*, 1995). Not only has acid-induced esophageal inflammation been shown to be associated with mast cell degranulation and histamine release (Feldman *et al.*, 1996), but also mast cell stabilizers can prevent acid-induced esophageal shortening (Paterson, 1998). The mast cell mediators involved in this response are unknown. Histamine is not likely to be involved as histamine causes weak, unsustained contractions of the esophageal longitudinal smooth muscle layer *in vitro* (Paterson, 1998).

Bradykinin is a potential candidate as it has been shown to contract the opossum longitudinal smooth muscle and not the circular smooth muscle (Saha *et al.*, 1990). Bradykinins are formed by the action of proteases and are involved in inflammation by causing vasodilation and increased vascular permeability. Studies have shown that bradykinins can activate both PLC and PLA_2 (Castano *et al.*, 1998). Therefore, it is

possible that during inflammation bradykinins are released into the smooth muscle layer and cause contraction of only the longitudinal muscle layer. Upon isolation of the smooth muscle cells from the inflamed tissue, bradykinin is eliminated and the cells return to their normal resting lengths. Therefore, due to the smooth muscle cell isolation, many different mediators would be washed away, which in turn would result in resting lengths that are not different between the control and inflamed tissue.

Sustained esophageal shortening may also be a result of the effects of inflammation on other cells (such as excitatory neurons) within the tissue. In the DNBS model of colitis, it has been demonstrated that during inflammation the enteric nervous system undergoes major structural changes and early in inflammation axonal proliferation compensates for neuronal loss (Sanovic *et al.*, 1999). After nematode infection of the mouse intestine there was a 60% decrease in electrical field-induced contraction suggesting that inflammation altered the neuromuscular function (Barbara *et al.*, 1997). It has been demonstrated that acid-induced esophageal shortening could not be altered by the administration of atropine or by vagotomy (Paterson and Kolyn, 1994); however, this does not exclude a role for the products of other excitatory neurons (e.g. tachykinins)

In contrast to the longitudinal smooth muscle cells, inflammation appears to directly affect the circular smooth muscle cells resulting in impaired cellular contraction. Therefore, the hypo-responsiveness observed in smooth muscle tissue strips is likely due (at least in part) to alteration in the circular smooth muscle cells. Since the clearance of gastroesophageal reflux is dependent on the peristaltic contractions provided by the circular smooth muscle, the impaired contractility could result in prolonged esophageal

exposure to gastric acid. This would result in worsening of inflammation, possibly causing more cellular dysfunction and thus further impairing esophageal clearance.

Since the circular smooth muscle cells do not function properly as a result of the acid-induced inflammation it seems logical that particular components of the cell would be damaged. The circular smooth muscle of the cat (Cao *et al.*, 2001; Biancani *et al.*, 1992) and opossum (Muinuddin and Paterson, 2001) esophagus is dependent on extracellular Ca^{2+} for the initiation of contraction. Recent evidence suggests that, in the mouse (Akbarali *et al.*, 2000) and canine (Liu *et al.*, 2001; Shi and Sarna, 2000) colons, the L-type Ca^{2+} channels become down-regulated during inflammation, resulting in reduced Ca^{2+} influx and impaired contraction of the individual smooth muscle cells. The decreased contraction observed in the isolated circular smooth muscle cells could be a result of down-regulation of the L-type Ca^{2+} channels, resulting in insufficient levels of Ca^{2+} to induce contraction. In addition to the alterations in L-type Ca^{2+} channels, the expression of the sodium/potassium pump is also altered as a result of inflammation of the rat intestine (Muller *et al.*, 1989).

However, impaired Ca^{2+} influx may not be enough to impair contraction since intracellular Ca^{2+} stores have been shown to be sufficient in causing CCh-induced contraction (in the presence of nifedipine and tetraethylammonium) of the opossum circular smooth muscle cells (Wang *et al.*, 1996), and maintaining partial contraction of human esophageal circular smooth muscle cells (Sims *et al.*, 1997). Nevertheless, inflammation has been shown to damage the intracellular Ca^{2+} stores in the cat LES (Biancani *et al.*, 1992; Biancani *et al.*, 1984) and rat colon (Myers *et al.*, 1997). It appears that regardless of the source of Ca^{2+}, the decreased contractility observed could be

a result of damage to the Ca^{2+} handling properties of the cell. However, more work is needed to determine if this is the case in the inflamed esophagus.

Another possibility for the decrease in circular smooth muscle cell contractility could be an increase in gelsolin production. Gelsolin is involved in severing actin filaments causing a reorganization and generation of new actin filament growth (McLaughlin *et al.*, 1993). Gelsolin has also been localized on the inner surface of the plasma membrane next to the Ca^{2+} channels (Ehrilich *et al.*, 2000). In smooth muscle cells obtained from the affected muscularis propria of patients with Crohn's disease, it was demonstrated that along with altered smooth muscle contractility there was an increase in gelsolin expression (Ehrilich *et al.*, 2000). Therefore, in the inflamed esophageal circular smooth muscle cells there may be an increase in gelsolin expression resulting in decreased contraction of the smooth muscle cell as a result of actin relocation. However, this remains purely speculative and needs to be examined.

In esophageal circular smooth muscle cells, cytosolic PLA_2 ($cPLA_2$) has been shown to participate in ACh-induced contraction by releasing AA, which synergistically interacts with DAG to activate a PKC dependent pathway (Sohn *et al.*, 1994a). When antagonists were used against $cPLA_2$, contraction was decreased by approximately 50% (Sohn *et al.*, 1994a). In the circular smooth muscle of the canine colon, $cPLA_2$ was shown to be involved in ACh-induced contraction (Ali *et al.*, 2000) in a similar fashion as observed in the cat esophagus. Furthermore, during inflammation of the canine colon, the circular smooth muscle was shown to have a significant decrease in both the expression and activation of $cPLA_2$ (Ali *et al.*, 2000). Therefore, it seems plausible that during esophageal inflammation, there is altered $cPLA_2$ expression in the circular smooth muscle

cells, resulting in impaired contraction. It is interesting to note that the both inflammation and the application of a $cPLA_2$ antagonist impair contraction by approximately 50%.

However, it is not reasonable to assume that the hypo-responsiveness of the circular smooth muscle is entirely a result of changes to the smooth muscle cell itself. Changes in the surrounding tissue are likely to contribute to the altered contraction observed. As previously mentioned, the opossum longitudinal smooth muscle will contract in response to bradykinin whereas the circular smooth muscle will not (Saha *et al.*, 1990). However, bradykinins have been associated with both smooth muscle contraction and relaxation (Wang *et al.*, 1997). Since NO has been shown to cause contraction in the longitudinal smooth muscle layer and relaxation in the circular smooth muscle layer (Zhang and Paterson, 2001; Goyal and Sivarao, 1999), it is not unreasonable to believe that bradykinin might produce the same results. Therefore, a portion of the hypo-responsiveness of the circular smooth muscle layer could be due to the presence of bradykinin (or other mediators), causing suppressed contraction.

Khan and Collins (1994) found that inflammation of the rat jejunum caused an increase in interleukin (IL)-1α, IL-1β, IL-6 and tumor necrosis factor (TNF)-α expression in the deeper neuromuscular tissue. These increased cytokines might be involved in the neuromuscular disturbance that accompanies intestinal inflammation. Increased IL-1β was shown to alter neurotransmitter release (Main *et al.*, 1993) and treatment with an antagonist to IL-1 attenuated this response (Khan and Collins, 1994). In our model of esophagitis, specific cytokines could be expressed in the muscle layers resulting in the increased contractility observed in the longitudinal muscle layer. The circular muscle

layer does not display the increase in contractility possibly as a result of the inflammation-induced damage to the smooth muscle cells.

Recently, secretory PLA_2 ($sPLA_2$) has been implicated in tonic LES contraction (Cao *et al.*, 1999). $sPLA_2$ is involved in lipid digestion, cell proliferation and smooth muscle contraction (Sommers *et al.*, 1992). Application of $sPLA_2$ or metabolites of $sPLA_2$ (such as AA) induced dose-dependent contraction of smooth muscle cells isolated from the esophageal body (circular muscle layer) and LES (Cao *et al.*, 1999). Inflammation of the cat esophagus has been shown to result in a biochemical switch from only $cPLA_2$-induced AA release to include both $cPLA_2$ and $sPLA_2$ (Kim *et al.*, 1997). Therefore, it is possible that inflammation may be causing the longitudinal smooth muscle cells to produce $sPLA_2$, causing a tonic or sustained contraction of this muscle layer. During the cell isolation, $sPLA_2$ would be washed away, thus eliminating the contraction.

The resting lengths of our circular smooth muscle cells are believed to be accurate since they were similar to the resting length of circular smooth muscle cells isolated from the cat (Hillemeier *et al.*, 1991; Biancani *et al.*, 1987) and opossum (Akbarali *et al.*, 1995) esophagus (83 μm, 85 μm and 100 μm, respectively). While the resting lengths of the circular smooth muscle cells in our study were slightly shorter than those observed by Maton *et al.* (1988), Bitar and Makhlouf (1982) and Bitar *et al.* (1979), this is not thought to be significant for two reasons. Firstly, these studies reported a very large distribution of cell size ranging from 75 – 350 μm (Bitar *et al.*, 1979). Secondly they were performed on gastric smooth muscle of guinea pig (Maton *et al.*, 1988) and *Bufo marinus* (Bitar and Makhlouf, 1982; Bitar *et al.*, 1979). Hillemeier *et al.* (1991) found that gastric and

esophageal smooth muscle cells were similar in size. Therefore, the differences in length most likely exist as a result of species variation rather than regional differences.

The significant difference observed between the length of the longitudinal and circular muscle cells is likely due to physiological differences between the two muscle layers, and not a result of problems with the isolation of longitudinal smooth muscle cells. First, significant differences have been observed in the size of cells isolated from the LES and esophageal body (Hillemeier *et al.*, 1991), indicating that differences exist within an organ. Second, the mean resting length of the longitudinal smooth muscle cells isolated from the mouse jejunum was 56.7±12 μm (Blennerhassett *et al.*, 1999b), similar to the mean resting length of our longitudinal smooth muscle cells. Third, our cells could be induced to contract and had maximal contraction of approximately 20% in both groups. This was consistent with the maximal contractions observed by other investigators (Cao *et al.*, 1999; Shi and Sarna, 1999; Sohn *et al.*, 1995). These three arguments lead us to conclude that that the length of the isolated longitudinal smooth muscle cells is valid.

Since the longitudinal smooth muscle layer contained more connective tissue than the adjacent circular smooth muscle layer, a higher concentration of collagenase was required to release the smooth muscle cells from the tissue. Therefore, as a result of the cell isolation technique, the longitudinal smooth muscle cells may have been slightly contracted resulting in the size difference observed between the cells from the longitudinal and circular smooth muscle layer. In an attempt to eliminate any cellular contraction that may have occurred, a 30-minute stabilization period was employed after isolation in every experiment. This might allow elimination of any prior contraction that may have occurred from the isolation protocol. It has been noted that after a strong

contraction. smooth muscle cells relax very slowly and rarely return to their initial resting length (Bagby *et al.*, 1971). However, these observations were made on cells that had strongly contracted. If the digestion process resulted in contraction of the longitudinal smooth muscle cells it would likely have been minimal, as our cells were able to contract in response to CCh.

As previously described (Sohn *et al.*, 1994a; Sohn *et al.*, 1993), we applied the CCh to the smooth muscle cells 30-seconds prior to fixation. However, Fay and Singer (1977) reported that maximal contraction occurred at approximately 15-seconds, and at 30-seconds this contraction fell to approximately 80% of the maximal contraction. Therefore, it is possible that CCh exposure was too long and the optimal time to observe maximal shortening had passed. However, all cells received a 30-second exposure and therefore the cells should be in the same state of contraction. The only problem associated with the longer CCh exposure would be that our maximal contraction would be an underestimate. However, this is probably not the case since our percent shortening was similar to those observed by other investigators (Cao *et al.*, 1999; Shi and Sarna, 1999; Sohn *et al.*, 1995; Sohn *et al.*, 1994a). Also, Hillemeier *et al.* (1991) reported that ACh-induced contraction of isolated esophageal smooth muscle cells required 30-second exposure.

Morphological changes to circular smooth muscle cells:

Inflammation of the rat jejunum results in hypertrophy and hyperplasia of the smooth muscle cells (Blennerhassett *et al.*, 1992). In our study, two measures of hypertrophy were used. The first measured the size of isolated smooth muscle cells and

the second determined the size of the smooth muscle cells in tissue sections. Using these two independent measures for hypertrophy we found no differences in longitudinal smooth muscle cells isolated from the saline- or acid-perfused animals. In contrast both measures of hypertrophy indicated that there was a significant increase in the size of the circular smooth muscle cells after acid perfusion.

In this study we found that the contractility of the circular smooth muscle cells was significantly impaired as a result of acid-induced inflammation. However, using manometry, it has been shown that the amplitude of contraction in the circular smooth muscle layer was not significantly altered in animals subjected to an identical acid-induced injury as used in our experiments (White *et al.*, 2001). Smooth muscle cell hypertrophy is an important contributor to altered motility in inflammatory states, since increased smooth muscle mass will exaggerate muscle contraction and amplify the effect of excitatory stimulation (Blennerhassett *et al.*, 1992). Since inflammation had no detectable effect on the amplitude of circular smooth muscle contraction *in vivo*, and the smooth muscle cells display decreased contractility and hypertrophy *in vitro*, it seems plausible that hypertrophy of the circular smooth muscle layer is an adaptive response, allowing the esophagus to maintain function in the face of impaired contraction in the individual cells.

This seems to suggest that inflammation is not altering esophageal clearance and therefore the clearance time would not increase. However, in animals receiving longer acid perfusion (Shirazi *et al.*, 1989) or humans with chronic GERD (Diener *et al.*, 2001; Rakic *et al.*, 1997; Timmer *et al.*, 1994) altered peristalsis has been observed. This would suggest that any compensatory effect hypertrophy may have is overcome by prolonged

inflammation. Furthermore, acid-induced inflammation has been associated with an impaired deglutition reflex in the opossum esophagus (White *et al.*, 2000). Since deglutition initiates primary peristalsis (pushing refluxed material back into the stomach) and delivers bicarbonate (for acid neutralization) via saliva (Morris *et al.*, 1997), impairment of deglutition may contribute to more severe esophageal injury.

Finally, nuclear size was significantly larger in circular smooth muscle cells from acid-perfused as compared to saline perfused animals. In models of intestinal inflammation, such increases in nuclear size have been attributed to increased mitotic activity (Blennerhassett *et al.*, 1992) and therefore appear to reflect hyperplasia of smooth muscle. Carpizo *et al.* (1998) reported an increase in mucosal proliferation in the rabbit esophagus after a short-term acid perfusion. They concluded that esophageal acidification acted either directly or indirectly as a tissue mitogen. Therefore, the increased nuclear size in the circular smooth muscle as a result of the acid-induced inflammation is likely due to increased cell proliferation. An increase in cell number would likely contribute to exaggerated muscle contraction, further amplifying the effects of excitatory stimulation. Similar changes were not observed in the longitudinal smooth muscle layer.

Inflammation appears to directly influence both the function and morphology of the circular smooth muscle cells, possibly because the circular smooth muscle layer is closer to the mucosal injury. In contrast, inflammation appears to not have an impact on the function of isolated longitudinal smooth muscle cells. It is only when the longitudinal muscle cells are working together with the other cell types (e.g. *in vivo* or muscle strip studies *in vitro*) that functional alterations are observed. Thus, the altered function of the

longitudinal muscle layer is most likely a result of mediators within the tissue interacting with the smooth muscle cells. Whether chronic acid exposure results in direct damage to the longitudinal smooth muscle cells altering function remains to be determined.

References:

Abebe, W., and S. J. Mustafa, 1998. A_1 adenosine receptor-mediated $Ins(1,4,5)P_3$ generation in allergic rabbit airway smooth muscle. *Am. J. Physiol.* 275:L990-997.

Akbarali, H. I., C. Pothoulakis, and I. Castagliuolo, 2000. Altered ion channel activity in murine colonic smooth muscle myocytes in an experimental colitis model. *Biochem. Biophys. Res. Commun.* 275(2):637-642.

Akbarali, H. I., N. Hatakeyama, Q. Wang, and R. K. Goyal, 1995. Transient outward current in opossum esophageal circular muscle. *Am. J. Physiol.* 268:G979-G987.

Alexander, J. A., L. W. Hunt, and A. M. Patel, 2000. Prevalence, pathophysiology, and treatment of patients with asthma and gastroesophageal reflux disease. *Mayo. Clin. Proc.* 75:1055-1063.

Ali, I., W. B. Campbell, S. K. Sarna, 2000. Impaired activation of cytosolic phospholipase A(2) in inflamed canine colonic circular muscle. *Gastroenterology.* 119(1)62-70.

Allescher, H. D., I. Berezin, J. Jury, and E. E. Daniel, 1988. Characteristics of canine lower esophageal sphincter: a new electrophysiological tool. *Am. J. Physiol.* 255:G441-G453.

Anand, N., and W. G. Paterson, 1994. Role of nitric oxide in esophageal peristalsis. *Am. J. Physiol.* 266:G123-G131.

Aube, A. C., C. Cherbut, M. Barbier, J. H. Xing, C. Roze, and J. P. Galmiche, 1999. Altered myoelectrical activity in noninflamed lieum of rats with colitis induced by trinitrobenzene sulphonic acid. *Neurogastroenterol. Motil.* 11(1):55-62.

Bagby, R. M., A. M. Young, R. S. Dotson, B. A. Fisher, and K. McKinnon, 1971. Contraction of single smooth muscle cells from *Bufo marinus* stomach. *Nature.* 234:351-352.

Barbara, G., B. A. Vallance, and S. M. Collins, 1997. Persistent intestinal neuromuscular dysfunction after acute nematode infection in mice. *Gastroenterology.* 113:1224-1232.

Barclay, R. L., P. K. Dinda, G. P. Morris, and W. G. Paterson, 1995. Morphological evidence of mast cell degranulation in an animal model of acid-induced esophageal mucosal injury. *Dig. Dis. Sci.* 40(8):1651-1658.

Barnes, P. J., 1996. Neuroeffector mechanisms: The interface between inflammation and neuronal responses. *J. Allergy. Clin. Immunol.* 98:S73-S83.

Barnes, P. J., 1993. Muscarinic receptor subtypes in airways. *Life. Sci.* 52:521-527.

Berridge, M. J., and R. F. Irvine, 1989. Inositol phosphates and cell signalling. *Nature.* 341:197-205.

Biancani, P. U. D. Sohn, H. G. Rich, K. M. Harnett, and J. Behar, 1997. Signal transduction pathways in esophageal and lower esophageal sphincter circular muscle. *Am. J. Med.* 103:23S-27S.

Biancani, P., K. M. Harnett, U. D. Sohn, B. Y. Rhim, J. Behar, C. Hillemeier, and K. N. Bitar, 1994. Differential signal transduction pathways in cat lower esophageal sphincter tone and response to ACh. *Am. J. Physiol.* 266:G767-G774.

Biancani, P., G. Billett, C. Hillemeier, M. Nissensohn, B. Y. Rhim, S. Szewczak, and J. Behar, 1992. Acute experimental esophagitis impairs signal transduction in cat lower esophageal sphincter circular muscle. *Gastroenterology.* 103:1199-1206.

Biancani, P., C. Hillemeier, K. N. Bitar, and G. M. Makhlouf, 1987. Contraction mediated by Ca^{2+} influx in esophageal muscle and by Ca^{2+} release in the LES. *Am. J. Physiol.* 253:G760-G766.

Biancani, P., K. Barwick, J. Selling, and R. McCallum, 1984. Effects of acute experimental esophagitis on mechanical properties of the lower esophageal sphincter. *Gastroenterology.* 87:8-16.

Bitar, K. N., and G. M. Malhlouf, 1982. Receptors on smooth muscle cells: characterization by contraction and specific antagonists. *Am. J. Physiol.* 242:G400-G407.

Bitar, K. N., A. M. Zfass, and G. M. Makhlouf, 1979. Interaction of acetylcholine and cholecystokinin with dispersed smooth muscle cells. *Am. J. Physiol.* 237(2):E172-E176.

Blennerhassett, M. G., F. M. Bovell, S. Lourenssen, and K. M. McHugh, 1999a. Characteristics of inflammation-induced hypertrophy of rat intestinal smooth muscle cell. *Dig. Dis. Sci.* 44(7):1265-1272.

Blennerhassett, M. G., P. Vignjevic, D. L. Vermillion, and S. M. Collins, 1992. Inflammation causes hyperplasia and hypertrophy in smooth muscle of rat small intestine. *Am. J. Physiol.* 262:G1041-G1046.

Blennerhassett, P., B. A. Vallance, and S. M. Collins, 1999b. Mucosal inflammation induced changes in smooth muscle contraction are a property of the muscle cell rather than the syncitium. *Gastroenterology.* A975.

Bogers, J., T. Moreels, J. De Man, G. Vrolix, W. Jacobs, P. Pelckmans, and E. van Marck, 2000. *Schistosoma mansoni* infection causing diffuse enteric inflammation and damage of the enteric nervous system in the mouse small intestine. *Neurogastroenterol. Motil.* 12(5):431-440.

Boyer, J. C., C. Guitton, C. Pignodel, P. Cuq, P. Moussu, P. Pouderoux, M. O. Christen, J. L. Balmes, and J. P. Bali, 1997. Differential responsiveness to contractile agents of isolated smooth muscle cells from human colons as a function of age and inflammation. *Dig. Dis. Sci.* 42(11):2190-2196.

Boyle, J. T., S. M. Altschuler, T. E. Nixon, D. N. Tuchman, A. I. Pack, and S. Cohen, 1985. Role of the diaphragm in the genesis of lower esophageal sphincter pressure in the cat. *Gastroenterology.* 88:723-730.

Buttar, N. S., and G. W. Falk, 2001. Pathogenesis of gastroesophageal reflux and Barrett's esophagus. *Mayo. Clin. Proc.* 76:226-234.

Cao, W., Q. Chen, U. D. Sohn, N. Kim, M. T. Kirber, K. M. Harnett, J. Behar, and P. Biancani, 2001. Ca^{2+}-induced contraction of cat esophageal circular smooth muscle cells. *Am. J. Physiol.* 280:C980-C992.

Cao, W., V. E. Pricolo, L. Zhang, J. Behar, P. Biancani, and M. T. Kirber, 2000. Gq-linked NK_2 receptors mediate neurally induced contraction of human sigmoid circular smooth muscle. *Gastroenterology.* 119:51-61.

Cao, W. B., K. M. Harnett, Q. Chen, M. K. Jain, J. Behar, and P. Biancani, 1999. Group I secreted PLA_2 and arachidonic acid metabolites in the maintenance of cat LES tone. *Am. J. Physiol.* 277:G585-G598.

Caprizo, D. R., A. J. Reaka, W. R. Glaws, N. Pooley, L. Schmidt, A. G. Halline, J. L. Goldstein, and T. J. Layden, 1998. Acute acid exposure increases rabbit esophageal cell proliferation. *J. Lab. Clin. Med.* 131(2):157-162.

Castano, M. E., J. P. Schanstra, C. Hirtz, J. B. Pesquero, C. Pecher, J. P. Girolami, and J. L. Bascands. 1998. B2 kinin receptor upregulation by cAMP is associated with BK-induced PGE2 production in rat mesangial cells. *Am. J. Physiol.* 274:F532-F540.

Castro, G. A., F. Badial-Aceves, J. W. Smith, S. J. Dudrick, and N. W. Weisbrodt, 1976. Altered small bowel propulsion associated with parasitism. *Gastroenterology.* 71:620-625.

Christensen, J., J. L. Conklin, and B. W. Freeman, 1973. Physiologic specialization at esophagogastric junction in three species. *Am. J. Physiol.* 225(6):1265-1270.

Cohen, J. D., H. W. Kao, S. T. Tan, J. Lechago, and W. J. Snape Jr., 1986. Effect of acute experimental colitis on rabbit colonic smooth muscle. *Am. J. Physiol.* 251:G538-G545.

Collins, S. M., 1996. The immunomodulation of enteric neuromuscular function: Implications for motility and inflammatory disorders. *Gastroenterology.* 111:1683-1699.

Collins, S. M., S. M. Hurst, C. Main, E. Stanley, I. Khan, P. Blennerhassett, and M. Swain, 1992. Effect of inflammation on enteric nerves. Cytokine-induced changes in neurotransmitter content and release. *Ann. N. Y. Acad. Sci.* 664:415-424.

Collins, S. M., P. A. Blennerhassett, M. G. Blennerhassett, and D. L. Vermillion, 1989. Impaired acetylcholine release from the myenteric plexus of *Trichinella*-infected rats. *Am. J. Physiol.* 257:G898-G903.

Crist, J., J. Gidda, and R. K. Goyal, 1986. Role of substance P nerves in longitudinal smooth muscle contractions of the esophagus. *Am. J. Physiol.* 250:G336-G343.

Crosthwaite, A. I. P., J. D. Huizinga, and J. E. T. Fox, 1990. Jejunal circular muscle motility is decreased in nematode-infected rat. *Gastroenterology.* 98:59-65.

Cucchiara, S., A. Staiano, C. Di Lorenzo, R. D'Ambrosio, M. R. Andreotti, M. Prato, P. De Filippo, and S. Auricchio, 1986. Esophageal motor abnormalities in children with gastroesophageal reflux and peptic esophagitis. *J. Pediatr.* 108(6):907-910.

Daniel, E. E., and V. Posey-Daniel, 1984. Neuromuscular structures in opossum esophagus: role of interstitial cells of Cajal. *Am. J. Physiol.* 246:G305-G315.

Davis, K. A. M., J. Masella, and M. G. Blennerhassett, 1998. Acetylcholine metabolism in the inflamed rat intestine. *Exp. Neurol.* 152:251-258.

de Carle, D. J., J. Christensen, A. C. Szabo, D. C. Templeman and D. R. McKinley, 1977. Calcium dependence of neuromuscular events in esophageal smooth muscle of the opossum. *Am. J. Physiol.* 232(6):E547-E552.

De Man, J. G., T. G. Moreels, B. Y. De Winter, J. J. Bogers, E. A. Van Marck, A. G. Herman, and P. A. Pelckmans, 2001. Disturbance of the prejunctional modulation of cholinergic neurotransmission during chronic granulomatous inflammation of the mouse ileum. *Br. J. Pharmacol.* 133(5):695-707.

DeMeester, T. R., J. H. Peters, C. G. Bremner, and P. Chandrasoma, 1999. Biology of gastroesophageal reflux disease: pathophysiology relating to medical and surgical treatment. *Annu. Rev. Med.* 50:469-506.

DeMeester, T. R., L. F. Johnson, G. J. Joseph, M. S. Toscano, A. W. Hall, and D. B. Skinner, 1976. Patterns of gastroesophageal reflux in health and disease. *Ann. Surg.* 184(4):459-469.

DeVault, K. R., and D. O. Castell, 1994. Current diagnosis and treatment of gastroesophageal reflux disease. *Mayo. Clin. Proc.* 69:867-876.

Diamant, N. E., 1997. Neuromuscular mechanisms of primary peristalsis. *Am. J. Med.* 103:40S43S.

Diener, U., M. G. Patti, D. Molena, P. M. Fisichella, and L. W. Way, 2001. Esophageal dysmotility and gastroesophageal reflux disease. *J. Gastrointest. Surg.* 5(3):260-265.

Dodds, W. J., J. Dent, W. J. Hogan, J. F. Helm, R. Hauser, G. K. Patel, and M. S. Egide, 1982. Mechanisms of gastroesophageal reflux in patients with reflux esophagitis. *N. Engl. J. Med.* 307:1547-1552.

Dodds, W. J., J. Dent, W. J. Hogan, and R. C. Arndorfer, 1981. Effect of atropine on esophageal motor function in humans. *Am. J. Physiol.* 240:G290-G296.

Eastwood, G. L., D. O. Castell, and R. H. Higgs, 1975. Experimental esophagitis in cats lowers esophageal sphincter pressure. *Gastroenterology.* 69:146-153.

Ehrlich, H. P., G. M. Allison, M. J. Page, W. A. Kolton, and M. Graham, 2000. Increased gelsolin expression and retarded collagen lattice contraction with smooth muscle cells from Crohn's diseased intestine. *J. Cell. Physiol.* 182:303-309.

Farmer, S. G., J. M. Brown, and D. Pollack, 1983. Increased responsiveness of intestinal and vascular smooth muscle to agonists in rats infected with *Nippostrongylus brasiliensis*. *Arch. Int. Pharmacodyn. Ther.* 263:217-227.

Fass, J., J. Silny, J. Braun, U. Heindrichs, B. Dreuw, V. Schumpelick, and G. Rau, 1994. Measuring esophageal motility with a new intraluminal impedance device. First clinical results in reflux patients. *Scand. J. Gastroenterol.* 29(8):693-702.

Fay, F. S., and J. J. Singer, 1977. Characteristics of response of isolated smooth muscle cells to cholinergic drugs. *Am J. Physiol.* 232(3):C144-C154.

Feldman, M. J., G. P. Morris, P. K. Dinda, and W. G. Paterson, 1996. Mast cells mediate acid-induced augmentation of opossum esophageal blood flow via histamine and nitric oxide. *Gastroenterology.* 110:121-128.

Fitzgerald, R. C., M. B. Omary, and G. Triadafilopoulos, 1996. Dynamic effects of acid on Barrett's esophagus. *J. Clin. Invest.* 98(9):2120-2128.

Fox, J. E. T., and E. E. Daniel, 1979. Role of Ca2+ in genesis of lower esophageal sphincter tone and other active contraction. *Am. J. Physiol.* 6:E163-E171.

Frieling, T., C. Rupprecht, G. Dobreva, D. Haussinger, and M. Schemann, 1995. Effects of prostaglandin F2 alpha (PGF2 alpha) and prostaglandin I2 (PGI2) on nerve-mediated secretion in guinea-pig colon. *Pflugers Arch.* 431(2)212-220.

Gabella, G., 1975. Hypertrophy of intestinal smooth muscle. *Cell. Tiss. Res.* 163:199-214.

Gay, J., J. Fioramonti, R. Garcia-Villar, and L. Bueno, 2001. Enhanced intestinal motor response to cholecystokinin in post-*Nippostrongylus brasiliensis*-infected rats: modulation by CCk receptors and the vagus nerve. *Neurogastroenterol. Motil.* 13(2):155-162.

Gidda, J. S., and R. K. Goyal, 1983. Influence of successive vagal stimulations on contractions in esophageal smooth muscle of opossum. *J. Clin. Invest.* 71(5):1095-1103.

Gonzalez, A., and S. K. Sarna, 2001. Different types of contraction in rat colon and their modulation by oxidative stress. *Am. J. Physiol.* 280:G546-G554.

Goto, K., S. Nakamura, F. Goto, and M. Yoshinaga, 1984. Generation of an interleukin-I-like lymphocyte-stimulating factor at inflammatory sites: Correlation with the infiltration of polymorphonuclear leucocytes. *Br. J. Exp. Pathol.* 65:521-532.

Goyal, R. K., and D. V. Sivarao. 1999. Functional Anatomy and Physiology of Swallowing and Esophageal Motility: Chapter 1 from The Esophagus 3rd edition. Lipincott Williams and Wilkins. Philadelphia.

Goyal R. K., and W. G. Paterson. 1989. Esophageal Motility: Chapter 22 from The Handbook of Physiology Section 6: The Gastrointestinal System. Schultz S. G., J. D. Wood, and B. B. Rauner. American Physiological Society. Bethesda, Maryland.

Grider, J. R., and G. M. Makhlouf, 1988. Contraction mediated by Ca^{++} release in circular and Ca^{++} influx in longitudinal intestinal muscle cells. *J. Pharmacol. Exp. Ther.* 244(2):432-437.

Grossi, L., K. McHugh, and S. M. Collins, 1993. On the specificity of altered muscle function in experimental colitis in rats. *Gastroenterology.* 104:1049-1056.

Haller, H., J. I. Smallwood, and H. Rasmussen, 1990. Protein kinase C translocation in intact vascular smooth muscle strips. *Biochem. J.* 270:375-381.

Harnett, K. M., W. Cao, N. Kim, U. D. Sohn, H. Rich, J. Behar, and P. Biancani, 2000. Signal transduction in esophageal and LES circular muscle contraction. *Yale. J. Bio. Med.* 72:153-168.

Heine, K. J., and R. K. Mittal, 1992. Lower esophageal sphincter: how to quantitate. *Gastroenterology.* 103(1):346-347.

Helm, J. F., W. J. Dodds, L. R. Pelc, D. W. Palmer, W. J. Hogan, and B. C. Teeter, 1984. Effect of esophageal emptying and saliva on clearance of acid from the esophagus. *N. Engl. J. Med.* 310(5):284-288.

Helm, J. F., W. J. Dodds, D. R. Riedel, B. C. Teeter, W. J. Hogan, and R. C. Arndorfer, 1983. Determinants of esophageal acid clearance in normal subjects. *Gastroenterology.* 85(3):607-612.

Hierholzer, C., J. C. Kalff, A. Chakraborty, S. C. Watkins, T. R. Biliar, A. J. Bauer, and D. J. Tweardy, 2001. Impaired gut contractility following hemorrhagic shock is accompanied by IL-6 and G-CSF production and neutrophils infiltration. *Dig. Dis. Sci.* 46(2):230-241.

Hillemeier, C., K. N. Bitar, J. M. Marshall, and P. Biancani, 1991. Intracellular pathways for contraction in gastroesophageal smooth muscle. *Am. J. Physiol.* 260:G770-G775.

Holloway, R. H., E. Blank, I. Takahashi, W. J. Dodds, W. J. Hogan, and J. Dent, 1985. Variability of lower esophageal sphincter pressure in the fasted unanesthetized opossum. *Am. J. Physiol.* 248:G398-G406.

Hurst, S., and S. M. Collins, 1993. Interleukin-1β modulation of norepinephrine release from rat myenteric nerves. *Am. J. Physiol.* 264:G30-G35.

Johansson, B., 1984. Different types of smooth muscle hypertrophy. *Hypertension.* 6(suppl III):III 64-III 68).

Jouet, P., S. K. Sarna, C. Singaram, R. P. Ryan, C. J. Hillard, G. L. Telford, J. Fink, and J. D. Henderson, 1995. Immunocytes and abnormal gastrointestinal motor activity during ileitis in dogs. *Am. J. Physiol.* 296:G913-G924.

Kahrilas, P. J., S. Wu, S. Lin, and P. Pouderoux, 1995. Attenuation of esophageal shortening during peristalsis with hiatus hernia. *Gastroenterology.* 109:1818-825.

Kalff, J. C., W. H. Schraut, T. R. Billiar, R. L. Simmons, and A. J. Bauer, 2000. Role of inducible nitric oxide synthase in postoperative intestinal smooth muscle dysfunction in rodents. *Gastroenterology.* 118(2):316-327.

Khan, I., S. M. Collins, 1994. Expression of cytokines in the longitudinal muscle myenteric plexus of the inflamed intestine of rat. *Gastroenterology.* 107:691-700.

Kim, N., U. D. Sohn, V. Mangannan, H. Rich, M. K. Jain, J. Behar, and P. Biancani, 1997. Leukotrienes in acetylcholine-induced contraction of esophageal circular smooth muscle in experimental esophagitis. *Gastroenterology.* 112:1548-1558.

Kuemmerle, J. F., K. S. Murthy, and G. M. Makhlouf, 1998. Longitudinal smooth muscle of the mammalian intestine. *Cell. Biochem. Biophy.* 28:31-44.

Lanas, A., Y. Royo, J.Ortego, M. Molina, and R. Sainz, 1999. Experimental esophagitis induced by acid and pepsin in rabbits mimicking human reflux esophagitis. *Gastroenterology.* 116:97-107.

Liu, X., N. J. Rusch, J. Striessnig, and S. K. Sarna, 2001. Down-regulation of L-type calcium chanels in inflamed circular smooth muscle cells of the canine colon. *Gastroenterology.* 120(2):480-489.

Main, C., P. Blennerhassett, and S. M. Collins, 1993. Human recombinant interleukin 1β suppresses acetylcholine release from rat myenteric plexus. *Gastroenterology.* 104:1648-1654.

Makhlouf, G. M., and K. S. Murthy, 1997. Signal transduction in gastrointestinal smooth muscle. *Cell. Sig.* 9(3):269-276.

Marshall, J. B., and D. C. Gerhardt, 1982. Improvement in esophageal motor dysfunction with treatment of reflux esophagitis: a report of two cases. *Am. J. Gastroenterol.* 77(6):351-354.

Martinolle, J. P., R. Garcia-Villar, J. Fioramonti, and L. Bueno, 1997. Altered contractility of circular and longitudinal muscle in TNBS-inflamed guinea pig ileum. *Am. J. Physiol.* 272:G1258-G1267.

Matarazzo, S. A., W. S. Snape, Jr., J. P. Ryan, and S. Cohen, 1976. The relationship of cervical and abdominal vagal activity in lower esophageal sphincter function. *Gastroenterology.* 71:999-1003.

Maton, P. N., V. E. Sutliff, Z. C. Zhou, S. M. Collins, J. D. Gardner, and R. T. Jensen, 1988. Characterization of receptors for calcitonin gene-related peptide on gastric smooth muscle cells. *Am. J. Physiol.* 254:G789-G794.

McLaughlin, P. J., J. T. Gooch, H-G. Mannherz, and A. G. Weeds, 1993. Structure of gelsolin segment 1-actin and the mechanism of filaments severing. *Nature.* 364:685-692.

Michel, O., M. Dentener, F. Corazza, W. Buurman, and R. Rylander, 2001. Healthy subjects express difference in clinical response to inhaled lipopolysaccharide that are related with inflammation and with atopy. *J. Allergy. Clin. Immunol.* 107(5):797-804.

Michel, O., A-M. Nagy, M. Schroeven, J. Duchateau, J. Neve, P. Fondu, and R. Sergysels, 1997. Dose-response relationship to inhaled endotoxin in normal subjects. *Am. J. Respir. Crit. Care. Med.* 156:1157-1164.

Middleton, S. J., M. Shorthouse, and J. O. Hunter, 1993. Relaxation of distal colonic circular smooth muscle by nitric oxide derived from human leucocytes. *Gut.* 34:814-817.

Mitchell, R. W., I. M. Ndukwu, K. Arbetter, J. Solway, and A. R. Leff, 1993. Effect of airway inflammation on smooth muscle shortening and contractility in guinea pig trachealis. *Am. J. Physiol.* 265:L549-L554.

Mittal, R. K., and D. H. Balaban, 1997. The esophagogastric junction. *N. Engl. J. Med.* 336(13):924-932.

Mittal, R. K., R. Holloway and J. Dent, 1995. Effect of atropine on the frequency of reflux and transiten lower esophageal sphincter relaxation in normal subjects. *Gastroenterology.* 109:1547-1554.

Mittal, R. K., and M. J. Fisher, 1990. Electrical and mechanical inhibition of the crural diaphragm during transient relaxation of the lower esophageal sphincter. *Gastroenterology.* 99:1265-1268.

Mittal, R. K., D. F. Rochester, and R. W. McCallum, 1987a. Effect of the diaphragmatic contraction on lower oesophageal sphincter pressure in man. *Gut.* 28:1564-1568.

Mittal, R. K., R. C. Lange, and R. W. McCallum, 1987b. Identification and mechanism of delayed esophageal acid clearance in subjects with hiatus hernia. *Gastroenterology.* 92(1):130-135.

Moreels, T. G., J. G. De Man, J. J. Bogers, B. Y. De Winter, G. Vrolix, A. G. Herman, E. A. Van Marck. and P. A. Pelckmans, 2001. Effect of *Schistosoma mansoni*-induced granulomatous inflammation on murine gastrointestinal motility. *Am. J. Physiol.* 280:G1030-G1042.

Morris, G. P., M. J. Feldman, R. L. Barclay, and W. G. Paterson, 1997. Esophagitis as the outcome of progressive failures of the defensive repertoire. *Can. J. Gastroenterol.* 11:28B-36B.

Muinuddin, A., and W. G. Paterson, 2001. Initiation of distension-induced descending peristaltic reflex in opossum esophagus: role of muscle contractility. *Am. J. Physiol.* 280:G431-G438.

Mukhopadhyay, A. K., and N. W. Weisbrodt, 1975. Neural organization of esophageal peristalsis: role of vagus nerve. *Gastroenterology.* 68(3):444-447.

Muller, M. J., J. D. Huizinga, and S. M. Collins, 1989. Altered smooth muscle contraction and sodium pump activity in the inflamed rat intestine. *Am. J. Physiol.* 257:G570-G577.

Murthy, K. S., J. F. Kuemmerle, and G. M. Makhlouf, 1995. Agonist-mediated activation of PLA_2 initiates Ca^{2+} mobilization in intestinal longitudinal smooth muscle. *Am. J. Physiol.* 269:G93-G102.

Murthy, K. S., J. R. Grider, and G. M. Makhlouf, 1992. Receptor-coupled G proteins mediate contraction and Ca^{++} mobilization in isolated intestinal muscle cells. *J. Pharmacol. Exp. Ther.* 260(1):90-97.

Murthy, K. S., and G. M. Makhlouf, 1991. Phosphoinositide metabolism in intestinal smooth muscle: preferential production of $Ins(1,4,5)P_3$ in circular muscle cells. *Am. J. Physiol.* 261:G945-G951.

Murthy, K. S., J. R. Grider, and G. M. Makhlouf, 1991. $InsP_3$-dependent Ca^{2+} mobilization in circular but not longitudinal muscle cells of intestine. *Am. J. Physiol.* 261:G937-G944.

Myers, B. S., J. S. Martin, D. T. Dempsey, H. P. Parkman, R. M. Thomas, and J. P. Ryan, 1997. Acute experimental colitis decreases colonic circular smooth muscle contractility in rats. *Am. J. Physiol.* 273:G928-G936.

Nebel, O. T., M. F. Fornes, and D. O. Castell, 1976. Symptomatic gastroesophageal reflux: incidence and precipitating factors. *Am. J. Dig. Dis.* 21:953-956.

ÖBerg, S., J. H. Peters, T. R. DeMeester, P. Chandrasoma, J. A. Hagen, A. P. Ireland, M. P. Ritter, R. J. Masson, P. Crookes, and C. G. Bremner, 1997. Inflammation and specialized intestinal metaplasia of cardiac mucosa is a manifestation of gastroesophageal reflux disease. *Ann. Surg.* 226(4):522-532.

Oliveira, R. B., N. M. Matsuda, A. R. Antoniolli, and G. Ballejo, 1992. Evidence for the involvement of nitric oxide in the electrically induced relaxations of human lower esophageal shpincter and distal pylorus. *Braz. J. Med. Res.* 25(8):853-855.

Oliver, M. R., D. T. Tan, D. R. Kirk, K. P. Rioux, and R. B. Scott, 1997. Colonic *and jejunal motor disturbances after colonic antigen challenge of sensitized rat. Gastroenterology.* 112(6):1996-2005.

Palmer, J. M., N. W. Weisbrodt, and G. A. Castro, 1984. *Trichinnella spiralis*: intestinal myoelectric activity during enteric infection in the rat. *Exp. Parasitol.* 57:132-141.

Parkman, H. P., L. J. Bogar, L. L. Bartula, A. P. Pagano, R. M. Thomas, and S. I. Myers, 1999. Effect of experimental acalculous cholecystitis on gallbladder smooth muscle contractility. *Dig. Dis. Sci.* 44(11):2235-2243.

Paterson, W. G., 1998. Role of mast cell-derived mediators in acid-induced shortening of the esophagus. *Am. J. Physiol.* 274:G385-G388.

Paterson, W. G., 1997a. Studies on opossum esophageal longitudinal muscle function. *Can. J. Physiol. Pharmacol.* 75:65-73.

Paterson, W. G., 1997b. Extraesophageal complications of gastroesophageal reflux disease. *Can. J. Gastroenterol.* 11(Suppl B):45B-50B.

Paterson, W. G., and B. Indrakrishnan, 1995. Descending peristaltic reflex in the opossum esophagus. *Am. J. Physiol.* 269:G219-G224.

Paterson, W. G., 1994. Neuromuscular mechanisms of esophageal responses at and proximal to a distending balloon. *Am. J. Physiol.* 260:G148-G155.

Paterson, W. G., and D. M. Kolyn, 1994. Esophageal shortening induced by short-term intraluminal acid perfusion in opossum: a cause for hiatus hernia? *Gastroenterology.* 107:1736-1740.

Paterson, W. G., M. A. B. Anderson, and N. Anand, 1992. Pharmacological characterization of lower esophageal sphincter relaxation induced by swallowing, vagal efferent nerve stimulation and esophageal distention. *Can. J. Physiol. Pharmacol.* 70:1011-1015.

Paterson, W. G., 1991. Neuromuscular mechanisms of esophageal responses at and proximal to a distending balloon. *Am. J. Physiol.* 260:G148-G155.

Payne, S. W., and A. M. Olsen, 1974. The Esophagus. Lea and Febiger, Philadelphia.

Petersen, H., T. Johannessen, A. K. Sandvik, P. M. Kleveland, E. Brenna, H. Waldum, and J. D. Dybdahl, 1991. Relationship between endoscopic hiatus hernia and gastroesophageal reflux symptoms. *Scand. J. Gastroenterol.* 26:921-926.

Preiksaitis, H. G., L. Tremblay, and N. E. Diamant, 1994. Nitric oxide mediates inhibitory nerve effects in human esophagus and lower esophageal sphincter. *Dig. Dis. Sci.* 39(4)770-775.

Rakic, S., H. J. Stein, T. R. DeMeester, and R. N. Hinder, 1997. Role of esophageal body function in gastroesophageal reflux disease: implications for surgical management. *J. Am. Coll. Surg.* 185(4):380:387.

Rattan, S., and R. K. Goyal, 1974. Neural control of the lower esophageal sphincter: incluence of the vagus nerves. *J. Clin. Invest.* 54(4):899-906.

Richter, J. E., 1998. Extraesophageal manifestations of gastroesophageal reflux disease. *Clin. Perspect. Gastroenter.* 28-39.

Ruhl, A., S. Hurst, and S. M. Collins, 1994. Synergism between interleukins 1 beta and 6 on noradrenergic nerves in rat myenteric plexus. *Gastroenterology.* 107:993-1001.

Saha, J. K., I. Hirano, and R. K. Goyal, 1993. Biphasic effect of SNP on opossum esophageal longitudinal muscle: involvement of cGMP and eicosanoids. *Am. J. Physiol.* 265:G403-G407.

Saha, J. K., J. N. Sengupta, and R. K. Goyal, 1990. Effect of bradykinin on opossum esophageal longitudinal smooth muscle: evidence for novel bradykinin receptors. *J. Pharmacol. Exp. Ther.* 252(3):1012-1020.

Saida, K., and C. van Breemen, 1984. Characteristics of the norepinephrine-sensitive Ca^{2+} store in vascular smooth muscle. *Blood Vessels.* 21:43-52.

Salapatek, A. M. F., A. Lam, and E. E. Daniel, 1998. Calcium source diversity in canine lower esophageal sphincter muscle. *J. Pharmacol. Exp. Thera.* 287:98-106.

Sanovic, S., D. P. Lamb, and M. G. Blennerhassett, 1999. Damage to the enteric nervous system in experimental colitis. *Am. J. Pathol.* 155(4):1051-1057

Sarna, S. K., 1998. In vivo signal-transduction pathways to stimulate phasic contractions in normal and inflamed ileum. Am. J. Physiol. 274:G618-G625.

Schultz, S. G., J. D. Wood, and B. B. Rauner. 1989. Handbook of Physiology Section 6: The Gastrointestinal System Vol I & II. American Physiological Society. Bethesda, Maryland.

Seeling Jr., L. L., P. Doody, L. Brainard, J. S. Gidda, and R. K. Goyal, 1984. Acetylcholinesterase and choline acetyltransferase staining of neurons in the opossum esophagus. *Anat. Rec.* 209:125-130.

Sharkey, K. A., and A. B. A. Kroese, 2001. Consequences of intestinal inflammation on the enteric nervous system: Neuronal activation induced by inflammatory mediators. *Anat. Rec.* 262:79-90.

Shi, X. Z., and S. K. Sarna, 2000. Impairment of Ca^{2+} mobilization in circular muscle cells of the inflamed colon. *Am. J. Physiol.* 278:G234-G242.

Shi, X. Z., and S. K. Sarna, 1999. Differential inflammatory modulation of canine ileal longitudinal and circular muscles. *Am. J. Physiol.* 277:G341-G350.

Shi, X. Z., and S. K. Sarna, 1997. Inflammatory modulation of muscarinic receptor activation in canine ileal circular muscle cells. *Gastroenterology.* 112:864-874.

Shirazi, S., K. Schulze-Delrieu, T. Custer-Hagen, C. K. Brown, and J. Ren, 1989. Motility changes in opossum esophagus from experimental esophagitis. *Dig. Dis. Sci.* 4(11):1668-1676.

Sims, S. M., Y. Jiao, and H. G. Preiksaitis, 1997. Regulation of intracellular calcium in human esophageal smooth muscles. *Am. J. Physiol.* 273:C1679-C1689.

Sims, S. M., 1992. Cholinergic activation of a non-selective cation current in canine gastric smooth muscle is associated with contraction. *J. Physiol.* 449:377-398.

Sloan, S., and P. J. Kahrilas, 1991. Impairment of esophageal emptying with hiatal hernia. *Gastroenterology.* 100(3):596-605.

Smout, A. J. P. M., and L. M. A. Akkermans. 1992. Normal and Disturbed Motility of the Gastrointestinal Tract. Wrightson Biomedical Publishings. LTD. Petersfield. Hampshire.

Sohn, U. D., K. M. Harnett, W. Cao, H. Rich, N. Kim, J. Behar, and P. Biancani, 1997a. Acute experimental esophagitis activates a second signal transduction pathway in cat smooth muscle from the lower esophageal sphincter. *J. Pharmacol. Exp. Ther.* 283(3):1293-1304.

Sohn, U. D., D. Zoukhri, D. Dartt, C. Sergheraert, K. M. Harnett, J. Behar, and P. Biancani, 1997b. Different protein kinase C isozymes mediate lower esophageal sphincter tone and phasic contraction of esophageal circular smooth muscle. *Mol. Pharmacol.* 51:462-470.

Sohn, U. D., B. Han, A. H. Tashjian Jr., J. Behar, and P. Biancani, 1995. Agonist-independent, muscle-type-specific signal transduction pathways in cat esophageal and lower esophageal sphincter circular smooth muscle. *J. Pharmacol. Exp. Ther.* 273(1):482-491.

Sohn, U. D., D. K. Kim, J. V. Bonventre, J. Behar, and P. Biancani, 1994a. Role of 100-kDa cytosolic PLA_2 in ACh-induced contraction of cat esophageal circular muscle. *Am J. Physiol.* 767:G433-G441.

Sohn, U. D., T. T. Chiu, K. N. Bitar, C. Hillemeier, J. Behar, and P. Biancani, 1994b. Calcium requirements for acetylcholine-induced contraction of cat esophageal circular muscle cells. *Am. J. Physiol.* 266:G330-G338.

Sohn, U. D., K. M. Harnett, G. De Petris, J. Behar, and P. Biancani, 1993. Distinctmuscarinic receptors. G proteins and phospholipases in esophageal and lower esophageal sphincter circular muscle. *J. Pharmacol. Exp. Ther.* 267(3):1205-1214.

Sommers, C. D., J. L. Bobbitt, K. G. Bemis, and D. W. Snyder, 1992. Porcine pancreatic phospholipase A2-induced contractions of guinea pig lung pleural strips. *Eur. J. Pharmacol.* 216:87-96.

Stein. H. J., A. P. Barlow, T. R. DeMeester, and R. A. Hinder, 1992. Complication of gastroesophageal reflux disease: Role of the lower esophageal sphincter. esophageal acid and acid/alkaline exposure. and duodenogastric reflux. *Ann. Surg.* 216(1):35-43.

Sugarbaker, D. J., S. Rattan, and R. K. Goyal, 1984. Swallowing induces sequential activation of esophageal longitudinal smooth muscle. *Am. J. Physiol.* 247:G515-G519.

Swain, M. G., P. A. Blennerhassett, and S. M. Collins, 1991. Impaired sympathetic nerve function in the inflamed rat intestine. *Gastroenterology.* 100:675-682.

Timmer, R., R. Breumelhof, J. H. Nadorp, and A. J. Smout, 1994. Oesophageal motility and gastro-oesophageal reflux before and after healing of reflux oesophagitis. A study using 24-hour ambulatory pH and pressure monitoring. *Gut.* 35(11):1519-1522.

Tomita, R., Y. Kurosu, and K. Munakata, 1997. Relationship between nitric oxide and non-adrenergic non-cholinergic inhibitory nerves in human lower esophageal sphincter. *J. Gastroenterol.* 32(1):1-5.

Tøttrup, A., A. Forman, P. Funch-Jensen, U. Raundahl, and K. E. Andersson, 1990. Effects of transmural field stimulation in isolated muscle strips from human esophagus. *Am. J. Physiol.* 258:G344-G351.

Uc, A., S. T. Oh, J. A. Murray, E. Clark, and J. L. Conklin, 1999. Biphasic relaxation of the opossum lower esophageal sphincter: roles of NO. VIP. and CGRP. *Am. J. Physiol.* 277:G548-G554.

Vallance, B. A., P. A. Blennerhassett, Y. Deng, K. I. Matthaei, I. G. Young, and S. M. Collins, 1999. IL-5 contributes to worm expulsion and muscle hyper-contractility in a primary T. spiralis infection. *Am. J. Physiol.* 277:G400-G408.

Vallance B. A., P. A. Blennerhassett, and S. M. Collins, 1997. Increased intestinal muscle contractility and worm expulsion in nematode infected mice. *Am. J. Physiol.* 272:G321-G327.

Vanner, S. J., and W. G. Paterson, 1988. Gastroesophageal reflux disease. *Medicine North America.* 17:3198-3206.

Vermillion, D. L., J. D. Huizinga, R. H. Riddell, and S. M. Collins, 1993. Altered small intestinal smooth muscle function in Crohn's disease. *Gastroenterology.* 104:1692-1699.

Vermillion, D. L., and S. M. Collins, 1988. Increased responsiveness of jejunal longitudinal muscle in *Trichinella*-infected rats. *Am. J. Physiol.* 254:G124-G129.

Wang, D. Z., L. Chao, and J. Chao, 1997. Hypotension in transgenic mice overexpressing human bradykinin B2 receptor. *Hypertension.* 29:488-493.

Wang, Q., H. I. Akbarali, N. Hatakeyama, and R. K. Goyal, 1996. Caffeine- and carbachol-induced Cl- and cation currents in single opossum esophageal circular muscle cells. *Am. J. Physiol.* 271:C1725-C1734.

Weisbrodt, N. W., and R. A. Murphy, 1986. Myosin phosphorylation and contraction of feline esophageal smooth muscle. *Am. J. Physiol.* 249:C9-C14.

White, R. J., Y. Zhang, G. P. Morris, and W. G. Paterson, 2001. Esophagitis-related esophageal shortening in opossum is associated with longitudinal muscle hyperresponsiveness. *Am. J. Physiol.* 280:G463-G469.

White, R. J., G. P. Morris, and W. G. Paterson, 2000. Impairment of deglutition reflux by acid-induced esophageal mucosal injury. *Dig. Dis. Sci.* 46(4):802-807.

Wood, J. D., D. H. Alpers, and P. L. R. Andrews, 1999. Fundamentals of neurogastroenterology. *Gut.* 45(Suppl II):II6-II16.

Xie, Y. N., W. T. Gerthoffer, S. N. Reddy, F. Cominelli, V. E. Eysselein, and W. J. Snape Jr., 1992. An abnormal rate of actin myosin cross-bridge cycling in colonic smooth muscle associated with experimental colitis. *Am. J. Physiol.* 262:G921-G926.

Xue, S., D. Valdez, P. I. Collman, and N. E. Diamant, 1996. Effects of nitric oxide synthase blockade on esophageal peristalsis and the lower esophageal sphincter in the cat. *Can. J. Physiol. Pharmacol.* 74(11):1249-1257.

Yamato, S., J. K. Saha, and R. K. Goyal, 1992. Role of nitric oxide in lower esophageal sphincter relaxation to swallowing. *Life Sci.* 50(17):1263-1272.

Zhang Y. and W. G. Paterson, 2001. Nitric oxide contracts longitudinal smooth muscle of opossum oesophagus via excitation-contraction coupling. *J. Physiol.* 536(1):133-140.

Zhang, Y., D. V. Miller, and W. G. Paterson, 2000. Opposing roles of K^+ and Cl^- channels in maintenance of opossum lower esophageal sphincter tone. *Am. J. Physiol.* 279:G1226-G1234.

Zhang, Y. and R. J. Lang, 1994. Effects of intrinsic prostaglandins on the spontaneous contractile and electrical activity of the proximal renal pelvis of the guinea-pig. *Br. J. Pharmacol.* 113:431-438.

Zijlstra, F. G., T. T. Hynna-Liepert, P. K. Dinda, I. T. Beck, and W. G. Paterson, 1991. Microvascular permeability increases early in the course of acid-induced esophageal injury. *Gastroenterology.* 101:295-302.

Appendix I:

(Detailed description of methodology used for esophageal dissection):

(1) Before the dissection prepare the HPSS-df. do not add the papain. BSA. DTT. or collagenase type-F at this point. The solution can be covered with parafilm and placed at 5°C until it is needed later in the experiment.

(2) Acquire the tissue from the animal and place in cold Krebs' solution with a 95/5 bubble. Open the esophagus in the longitudinal axis. wash several times to remove blood and bolus residue.

(3) Dissect the esophagus as follows:
- Pin the tissue out with the mucosal side up.
- Remove the mucosa and submucosa with fine scissors and forceps. It is critical to remove as much of the connective tissue as possible. This step should take several hours to do correctly. Care should be taken to ensure that the underlying circular smooth muscle is not damaged (extensively) during this step.
- The tissue is then flipped over. so the longitudinal muscle layer is up and the connective tissue from the serosal side is removed. in the same manner as above.
- The tissue is flipped over again and strips of the circular muscle layer are removed. attempting to keep them relatively large (several mm in diameter). Care should be taken not to cut into the underlying longitudinal muscle layer. which could contaminate the circular muscle. To isolate CSMC the above tissue sections are placed into HPSS-df. To isolate LSMC the tissue is cut into strips and then cut again perpendicular to the first cut. This creates a feather effect allowing maximal surface area for the enzymes to react with. The circular layer must be removed from the longitudinal due to the thin nature of the longitudinal layer.

(4) Tissue is kept in Krebs' bubbled with $95\%O_2/5\%CO_2$ until it is placed in the HPSS-df. If there is going to be a long wait between dissection and digestion the tissue should be placed into a beaker with fresh Krebs'. covered with parafilm and placed at 5°C until it is needed for digestion.

Appendix II:

(Detailed description of methodology used for isolation of smooth muscle cells):

Part A: Cell Digestion

After dissecting the tissue (ensuring as much of the collagen is removed as possible) the tissue is kept in fresh Krebs' solution (with a 95%O_2/5%CO_2 bubble) until HPSS-df is prepared. If it is going to be a long time until the tissue is used it can be placed in Krebs' solution. covered with parafilm and placed in the fridge at ~5°C.

Make the HPSS (for digestion only): The HPSS-df should be prepared and placed at 5°C prior to the tissue dissection.

Have stock solutions of the following:

- EDTA	50 mM	MW: 372.2
- Na-HEPES	154 mM	MW: 260.3
- $CaCl_2$	0.5M	MW: 147.02
- $MgCl_2$	4.9M	MW: 203.31
- DTT	1M	MW: 154.2

- Na-HEPES was obtained from SIGMA: Catalog #H-0763
- Aliquot the DTT into smaller tubes (20 µl) and store at –20°C.

In a small beaker add:

-	46 ml	ddH_2O	
-	100 µl	$CaCl_2$	1 mM/L
-	3.25 ml	HEPES	10 mM/L
-	10.2 µl	$MgCl_2$	1 mM/L
-	250 µl	EDTA	250 µM/L
-	90.1 mg	Glucose	10 mM/L
-	14.9 mg	KCl	4 mM/L
-	365 mg	NaCl	125 mM/L

Let the above mixture stir for ~10 minutes.

Take 10 ml aliquot of the above mixture and place in a clean 20 ml beaker with a stir bar. Note: the solution should be cold at this point.

(1) Weigh out 5 mg of papain and dissolve in a microcentrifuge tube tube with approximately 1 ml of the above solution. Add the dissolved papain to the 10 ml aliquot of HPSS making sure to rinse the microcentrifuge tube with the solution to ensure that all of the papain is added to the mixture. Stir the solution until it becomes clear (approximately 10 minutes), if it is not clear the dissociation will not work.

- Papain obtained from SIGMA #P-4762: it is 27U/mg (if stronger/weaker then adjust the amount added).

(2) Next, add 10 mg of BSA to the HPSS. Gently place the powder directly onto the solution making sure it stays on the surface. Slowly stir the solution until the BSA dissolves if the BSA is pulled under to quickly it will form clumps and will not properly dissolve. Let spin until the BSA is dissolved (~10 minutes).

(3) Take an aliquot of the DTT from the freezer and add 10 µl to the HPSS. The frozen aliquots of the DTT are good for along time when stored at –20°C.

(4) Adjust the pH to about 7.2 (anywhere from 7.1 – 7.2 is fine).

(5) Once the pH of the HPSS has been adjusted add Collagenase type-F to the solution.

(1 mg/ml for CSM)
(2.5 mg/ml for LSM)

(6) When the solution becomes clear add the smooth muscle cover with parafilm and place at 5°C overnight.

Part B: Cell Isolation

The next day place the solution on the counter at room temperature for 30 minutes.

(1) While waiting for the tissue to warm the HPSS (isolation formula) should be made.
In a large beaker add the following solutions:

- STOCK 1	200 ml	100 ml	50 ml
- STOCK 2	40 ml	20 ml	10 ml
- STOCK 3	4.8 ml	2.4 ml	1.2 ml
- STOCK 4	1.2 ml	0.6 ml	0.3 ml
- BME	10 ml	5 ml	2.5 ml
- Glucose	500 mg	250 mg	125 mg
- STI 20 mg	10 mg	5 mg	

- Let spin for ~10 minutes.
- BME is from SIGMA #B-6766 BME Amino Acids (50X)
- STI (soybean trypsin inhibitor)

• STOCK 1:	NaCl	144 mM	8.421 g/L
	KCl	7 mM	0.534 g/L
	KH_2PO_4	2.6 mM	0.357 g/L
• STOCK 2:	Na-HEPES	154 mM	40.1 g/L (adjust pH to ~7.8)
• STOCK 3:	$CaCl_2 \cdot 2H_2O$	100 mM	14.7 g/L
• STOCK 4:	$MgCl_2 \cdot 6H_2O$	120 mM	24.896 g/L

(2) Heat the solution to 35°C and adjust the pH to 7.4.

(3) After the HPSS-if has been adjusted to pH 7.4 cover with parafilm and gas with 100% oxygen (put the gas directly into the solution to ensure the solution has an adequate oxygen saturation).

(4) After the tissue has been at room temperature for 30 minutes place the tissue in the water bath at 35°C for 30 minutes. Make sure the parafilm has a good seal. Superfuse the solution 100% oxygen ensuring the gas is not in the liquid as agitation of the liquid will result in death and contraction of cells). The tissue should still be in the solution that they were placed in the night before (with Collagenase type F in it). If the tissue is digesting too fast it is good to turn down the temperature of the water bath to slow down the digestion process.

(5) Pour the tissue through filter mesh (200 μm) and pour ~50 ml of the fresh HPSS-if over the tissue to wash off the collagenase.

(6) Pour some fresh HPSS-if into a clean beaker and place the washed smooth muscle into this solution.

(7) Cut the tip off a disposable plastic Pasteur pipette and gently titrate the tissue until the solution appears cloudy (the cloudier it looks the more cells present). Take a small sample and check under the microscope to evaluate their viability.

(8) Cover the beaker with parafilm and let the cells sit at room temperature for ~30 minutes.

(9) After the 30 minutes filter the solution with 500-μm-filter mesh to remove the large clumps of tissue. The titration of this tissue can be done again if desired. but not necessary if the cells looked good the first time. Put the cells on ice or in the fridge until the contraction studies are preformed.

Part C: Cell Contraction

To study contractility of certain agonist concentration:

(1) In a microcentrifuge tube add the volume of agonist stock needed to make the final concentration the one desired for the experiment (100 µl of stock).

(2) Swirl the beaker of cells to ensure that a random sample is taken and using a 1 ml pipette (with the tip cut off) take the appropriate volume of the solution (900 µl) and add it to the agonist. Care must be taken not to agitate the cells.

(3) The cells are allowed to react with the drug for 30 seconds after which the reaction is terminated with the application of acrolein. 110 µl of acrolein is added (final concentration of 1%) the tube to fix the cells. Acrolein must be diluted in ddH_2O to make a stock of around 10%, any salts present in the acrolein will become precipitated out of solution.

(4) The tubes are capped and placed on ice until they are ready for measurement. Cell suspensions should be good for many hours if the cells are on ice and the cap is on tight.

(5) For measurement a small sample of the cells are placed on a glass slide with a cover slip. Using phase contrast microscopy ~30 cells are digitized and measured using the image analysis package. When taking the images care is taken to ensure that the cells used have smooth looking membranes and are bright looking. If the cells are dark, have rough membranes, sharp bends in them, or generally look poor they were likely dead before the fixation. Live cells look the same just after they were isolated as they do when fixed in acrolein, therefore it is easy to identify the good cells for the study.

Appendix III:

(Detailed description of solutions used for isolation of smooth muscle cells):

Krebs' Formula:

	Molecular Weight	Final Molarity (mM):	Volume added (per Liter):	For 10X stock (per Liter):
$NaHCO_3$	84.0	24.79	2.08 g	20.8 g
NaCl	58.4	118.0	6.89 g	68.9 g
KCl	74.6	4.7	0.3506 g	3.506 g
NaH_2PO_4	142.0	1.0	0.142 g	1.42 g
$MgSO_4$	246.5	1.2	0.2958 g	2.958 g
Glucose	180.2	11.1	2.0 g	20.0 g
$CaCl_2$	111.0	2.5	0.2775 g	N/A

** Note: Do not add the $CaCl_2$ to the stock of Krebs'.

- Take 100 ml of the 10X stock, dilute it with 900 ml of ddH2O, add the $CaCl_2$ (0.2775 g) while stirring.
- Bubble the solution with 95%O_2/5%CO_2, adjust the pH and return to the fridge until needed.

HPSS-df:

	Molecular Weight	Final Molarity (mM)	Volume added (per Liter)	Volume needed for 50 ml
$CaCl_2$	147.02	1 mM	2 ml	100 µl
EDTA	372.2	250 µM	5 ml	250 µl
Na-HEPES	260.3	10 mM	62.5 ml	3.25 ml
$MgCl_2$	203.3	1 mM	204 µl	10.2 µl
Glucose	180.18	10 mM	1.802 g	90.1 mg
KCl	74.55	4 mM	298.2 mg	14.91 mg
NaCl	58.44	125 mM	7.305 g	365 mg
Taurine	125.15	10 mM	1.25 g	62.6 mg
dH_2O			920 ml	46 ml

Note: the chemicals with ** by them come from stock solutions of:

- $CaCl_2$ 0.5 M stock
- EDTA 50 mM stock
- Na-HEPES 154 mM stock
- $MgCl_2$ 4.9 M stock

Enzymes needed for HPSS-df

	Volume Needed	Stock
HEPES (above)	10 ml	
Papain	5 mg	
BSA	10 mg	
DTT	10 µl	1 M
Collagenase type-F	10 mg (CSM) 25 mg (LSM)	

- Papain is based on a lot of 27U/mg, therefore adjust amount needed for lots with different reactivity.
- Make a 1M stock of the DTT and store at -20°C
- The Collagenase F is ~150U/ml (if different adjust accordingly)
- Before the Collagenase is added the pH of the solution needs to be adjusted to ~7.2.

HPSS-if:

	Molecular Weight	For 64 ml	For 128 ml	For 256 ml
STOCK 1		50 ml	100 ml	200 ml
STOCK 2		10 ml	20 ml	40 ml
STOCK 3		1.2 ml	2.4 ml	4.8 ml
STOCK 4		0.3 ml	0.6 ml	1.2 ml
BME-amino acids		2.5 ml	5 ml	10 ml
Glucose	180.18	125 mg	250 mg	500 mg
Soybean Trypsin Inhibitor		5 mg	10 mg	20 mg

- Adjust the pH to 7.4 at 35°C and then gas with 100% O_2 for 30 minutes.

STOCK 1:

	Molecular Weight	Final Molarity (mM):	Volume added (per Liter):	For 5L of stock:
NaCl	58.4	144.0 mM	8.421 g	42.105 g
KCl	74.6	7.0 mM	0.534 g	2.67 g
KH_2PO_4	136.09	2.6 mM	0.357 g	1.785 g

STOCK 2:

	Molecular Weight	Final Molarity (mM):	Volume added (per Liter):
Na-HEPES	260.3	154.0 mM	40.1 g

- Adjust the pH to ~7.8 you will not need to adjust the pH of the HPSS-if too much later.

STOCK 3:

	Molecular Weight	Final Molarity (mM):	Volume added (per Liter):
$CaCl_2 \bullet 2H_2O$	111.0	100 mM	14.7 g

STOCK 4:

	Molecular Weight	Final Molarity (mM):	Volume added (per Liter):
$MgCl_2 \bullet 6H_2O$	203.2	120 mM	24.896 g