BIOCHEMICAL BASIS FOR INHIBITORY CONTROL OF COLON FUNCTION

By

CHIDI MEMEH

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AUTHOR	Chidi Memeh
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ABSTRACT

Direct VIP-nerve and indirect ICC-mediated inhibitory control of colon motility were studied using biochemical techniques. In order to define a basis for VIP action in colon, longitudinal muscle, ICC-poor circular muscle and ICC-rich layers separated by dissection were characterized by electron microscopy and subcellular fractionation using markers for synaptosomes, smooth muscle membrane and mitochondria. Synaptosomal, mitochondrial and smooth muscle enriched membrane fractions were isolated but a putative ICC membrane enriched fraction has the highest 5'nucleotidase, Mg^{2+}ATPase activities and density of ^{125}I-VIP binding followed by synaptosome and smooth muscle membranes. High VIP binding density can be used as a marker for ICC membrane.

Study of nitric oxide production in fractions from ICC-rich and-poor preparation by measuring nitrite levels showed that mitochondrial, synaptosome and ICC-rich membrane fractions produced nitric oxide but greater levels were seen in the ICC-membrane rich fraction. Nitric oxide synthase activity on ICC membrane was constitutive and calcium-dependent. VIP increased (2-fold) nitric oxide production in ICC-rich strips which was inhibited by L-NAME, reduced by EGTA and increased by exogenous calcium. Partial inhibition of VIP-induced nitric oxide production by ω-Conotoxin GVIA was recorded in the absence (22%) and presence (32%) of L-arginine suggesting that the source of nitric oxide was in large part non-neural, from ICC.

I conclude that direct nerve-mediated control of longitudinal and circular muscle occurs through VIP binding on membrane receptors while indirect control of circular muscle occur through VIP-nerve induced production of nitric oxide from ICC to cause smooth muscle relaxation.

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LIST OF ABBREVIATIONS.

ICC	Interstitial Cells of Cajal.
NANC	Non-adrenergic, non-cholinergic.
VIP	Vasoactive Intestinal Polypeptide.
NO	Nitric oxide.
IJP	Inhibitory junction potential.
CM	Circular muscle.
LM	Longitudinal muscle.
BSA	Bovine serum albumin.
MIC	Microsomal fraction.
P	Pellet fraction.
L-NAME	L-N^G-nitro-L-arginine methyl ester
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanidine monophosphate
ATP	Adenosine triphosphate.

CHAPTER ONE

BASIS FOR PRESENT STUDIES

1.1 Introduction:

Ultrastructural studies from our laboratory have shown that Vasoactive intestinal polypeptide (VIP)-containing nerves are close (< 40nm) to Interstitial Cells of Cajal (ICC) in the circular smooth muscle canine lower esophagus (Berezin, Allescher, Daniel, 1987). Recently this arrangement has been shown to occur on the submucosal border of (canine colon) circular muscle and on the myenteric side of colonic circular muscle (Berezin, Huizinga, Daniel, 1988; Berezin, Huizinga, Daniel, Chow, 1990). Other laboratories have confirmed the presence of ICC in the submucosal border of rat (Stach, 1972) and human colonic circular muscle (Faussone-Pellegrini, 1990) and in mouse small intestine (Thuneberg, 1982). Morphological evidence from our laboratory using immunocytochemistry and electron microscopy has demonstrated that ICC which receives inhibitory innervation in the form of VIP containing neurones are interposed between these inhibitory nerves and circular muscle; i.e. ICC are directly innervated and coupled to smooth muscle and other ICC by gap junctions. This led to the proposal that ICC forms an intermediary system between nerve and smooth muscle communication (Daniel and Daniel, 1984). Furthermore, functional studies from our laboratory have also shown that circular smooth muscle of canine colon can be directly innervated without ICC being essential intermediaries (Berezin, Huizinga, Daniel, Chow, 1990).

Comparison of electrophysiological properties of colonic circular muscle layer with ICC and without ICC has shown that ICC-rich preparations produced consistent slow wave activity with prolonged plateau potentials of about 10 seconds duration while circular muscle preparation devoid of ICC usually produced no slow waves or produced slow waves of different waveshape without plateau potentials. These latter slow waves in contrast to those from ICC are abolished by L-type calcium channel antagonists (Berezin, Huizinga, Farraway, Daniel, 1990). This study from our laboratory suggests that ICC produce slow waves. In support of that electrophysiological work it has also been demonstrated that slow wave activity can be recorded from cells positively identified as ICC using electron microscopy (Barajas-Lopez, Berezin, Daniel, Huizinga, 1989). These slow waves are abolished with methylene blue and subsequent exposure to light. Experimental results from other laboratories partially confirm these findings (Publicover, Horowitz, Sanders, 1992).

1.2 Hypotheses:

From the background synopsis, significant facts stand out in relation to signal transmission to circular muscle of canine colon. The first important issue in these studies is that ICC are innervated by VIP containing neurons. The significance of this evidence is demonstrated by the fact that VIP hyperpolarizes ICC by 9 mV when these cells were electrically isolated by 1mM

heptanol in canine colon (Berezin, Huizinga, Farraway, Daniel, 1990). Thus ICC seem to have functional VIP receptors.

The second important fact is that circular muscle of canine colon is directly innervated by inhibitory nerves which upon stimulation produce inhibitory junction potentials (ijps) in muscle cells. However, near the ICC network at the submucosal border, ICC or closely coupled smooth muscle cells demonstrate the largest ijps. In total, these findings suggest that there might be a VIP receptors on ICC and VIP is the transmitter for inhibitory junction potentials on circular muscle of canine colon. Furthermore, recent functional studies from our laboratory suggest that the effect of VIP on colonic smooth muscle is mediated in part by nitric oxide either through nerves or ICC (Huizinga, Tomlinson, Pinton-Quezada, 1992). Therefore the study is based on the following hypotheses:

i. The ICC of colon have plasma membranes which have distinct properties compared to those of smooth muscle cells and nerves. It should therefore be possible to identify and enrich fractions from canine colon in these membranes. These properties will include VIP binding sites. Membranes from ICC will be enriched in membrane fractions from ICC-rich strips of canine colon.

ii. The ICC of colon or synaptosomes associated with ICC have receptors for VIP and contain nitric oxide (NO) synthase activated by VIP. If so it will be possible to identify NO synthase biochemically in either or both ICC-membrane enriched and synaptosomal fractions.

1.3 Objectives:

Based upon the above stated hypotheses the general objectives of this study are outlined as follows:

i. To attempt to localize and characterize ICC membranes in canine colon.

ii. To localize and characterize nitric oxide (NO) synthase activity in colon ICC-membrane enriched fractions.

iii. To determine the association between VIP and nitric oxide production in ICC-rich strips.

CHAPTER TWO

BACKGROUND

2.1 Inhibitory motor control of the colon.

Earlier electrophysiological studies using microelectrodes to record from single smooth muscle cells of longitudinal and circular muscle have shown that there is inhibitory innervation of smooth muscle of colon. Stimulation of these intrinsic inhibitory neurons which are located in the walls of colonic muscle led to decreased tone found to be resistant to blockade of adrenergic nerves. Microelectrode studies in guinea-pig colon showed that stimulation of these inhibitory nerves produced inhibitory junction potentials (IJPs) in 90% of longitudinal muscle cells and in nearly all the circular smooth muscle cells. These IJPs which were preceded by brief depolarization were not inhibited by guarethidine but are affected by procaine (Furness, 1969; reviewed by Furness and Costa, 1980). This showed that IJPs are produced by non-adrenergic neurons. Following sympathetic denervation with a freezing method, stimulation of intrinsic inhibitory nerves of guinea-pig colon produced IJPs (in the absence of fluorescent noradrenergic nerves) showing evidence of colonic control by these non-adrenergic inhibitory nerves. Certain studies have shown that the production of IJPs by stimulation of inhibitory nerves was followed by rebound excitation. This rebound excitation increases as the amplitude of the IJP increases with increase in the frequency of stimulation of inhibitory nerves (Bennett, 1966).

2.2 Nature of the inhibitory neurotransmitter in the gastrointestinal system.

Many studies using guanethidine to block adrenergic nerves show that inhibitory neurotransmission persisted in the gut (Furness, 1969). In addition, rebound excitation which follows IJPs persisted in the presence of atropine (a cholinergic blocker) which has been shown to increase the amplitude of IJPs (Bennett, 1966). Altogether, these reports suggest that the inhibitory neurotransmission in the gut is non-adrenergic and non-cholinergic (NANC).

The first insight into the nature and characteristics of the NANC neurotransmitter was given by collection of perfusate from toad stomach after stimulation of inhibitory nerves. Concentration of the perfusate by freeze drying and application to paper chromatograms with subsequent elution and testing for pharmacological activity showed that the fraction which causes significant inhibition of guinea-pig taenia coli contained adenosine. This led to the proposal that adenosine and adenosine monophosphate (AMP) have been metabolized from ATP which was released from nerves. Therefore ATP or an analogue was proposed to be the inhibitory neurotransmitter substance (Satchell, Burnstock and Campbell, 1969).

The hypothesis that ATP may be the NANC inhibitory neurotransmitter in the gut was not supported by experiments which show that ATP tachyphylaxis (using 800 ug/kg i.a.) did not

affect vagal stimulated lower esophageal sphincter (LES) relaxation. In addition, 2-2'-pyridylisatogen tosylate (a selective ATP antagonist) does not antagonize vagal stimulated LES relaxation, although the intra arterial effects of ATP were effectively antagonized (Rattan and Goyal, 1980).

Several studies on the LES provided the basis for understanding the nature and character of the NANC inhibitory neurotransmitter. The first insight into the role of peptides in NANC neurotransmission came from the studies showing that VIP causes a significant dose-dependent decrease in pressure of opossum and human LES (Rattan, Said and Goyal, 1977; Domschke, Lux, Domschke et al, 1978). Similar observation were reported on the LES of awake baboons (Siegel, Brown, Castell et al, 1979). Further studies using VIP antiserum established that VIP immunoreactive nerves were localized in the esophagus of rats, cats and pigs (Uddman, Edvinsson, Hakanson and Sundler, 1978).

Evidence to support the role of VIP as the NANC inhibitory neurotransmitter in the gut came from antagonism of vagally stimulated and VIP-induced fall in LES pressure by pretreatment of opossums with a high titer of VIP antiserum (1: 12.5). This finding led the authors to propose that VIP is the inhibitory neurotransmitter in the gut (Goyal, Rattan and Said, 1980). Studies showing that stimulation of pelvic nerves to the cat colon led to a transient vasodilatation and a significant increase in VIP level in the venous effluent from colon seem to support the view that VIP

is the NANC inhibitory neurotransmitter (Fahrenkrug, Haglund, Jodal et al, 1978). Additional evidence for VIP as NANC neurotransmitter came from the finding that VIP antiserum inhibited descending relaxation of rat colon in a concentration-dependent manner (Grider and Maklouf, 1986).

Immunocytochemical studies of the rat colon showed that VIP containing neurons were found in both the myenteric plexus and submucous plexus (Ekblad, Ekman, Hakanson and Sundler, 1988). In the guinea-pig colon, VIP containing nerves are common in the submucous plexus but were found in lower density in the myenteric plexus (Jessen, Saffrey, Van Noorden et al, 1980). A similar trend was also seen in the cat colon (Du, Conklin and Hammer, 1991) and in those of man and pigs (Larsson, Fahrenkrug, De Muckadell et al, 1976).

A functional study of longitudinal and circular muscle strips of human colon indicated that VIP relaxed both muscle types at equal concentrations ($0.27 - 1.35 \times 10^{-7}$) (Couture, Mizrahi, Regoli and Devroede, 1980). In contrast, another study showed that circular muscle strips of both proximal and distal colon relaxed in response to VIP while strips of longitudinal muscle were not responsive (Burleigh, 1990). A similar pattern of response to VIP has been reported for guinea-pig colon circular muscle and longitudinal muscle. This led the authors to ask the important question: Is VIP an inhibitory transmitter in the circular but not longitudinal muscle of guinea-pig colon ?

(Bennett, Bloom, Ch'Ng et al, 1984).

2.3 Mechanism of VIP action in the gut.

Although it is known that VIP release from inhibitory nerves in the gut could lead to smooth muscle relaxation through VIP receptors (Koch, Carney, Go, Szurszewski, 1991), the mechanism involved is not so clear . Certain studies seem to favour the notion that VIP induced relaxation (as seen in LES of adult cats) is through a commensurate increase in levels of cAMP which inhibits smooth muscle contraction through inactivation of myosin light-chain kinase and through decrease of 1,4,5-inositol triphosphate which is known to cause release of calcium from intracellular stores (Szewczak, Behar, Billet et al, 1990). Further studies using rat stomach smooth muscle indicated that VIP inhibitory action may also be associated with elevation of cAMP and influence upon signal transduction processes that release calcium from its intracellular stores and also allow influx of extracellular calcium (Ohta, Ito and Ohga, 1991). Other studies showed that electrical field stimulation caused elevation of cGMP while VIP raised cAMP levels (Torphy, Fine, Burman, Barnette, Ormsbee, 1986) . Although these studies were inconclusive, they suggest that VIP alone is not the mediator. Further studies are necessary to elucidate the mechanism of VIP induced relaxation in smooth muscle cell of gastrointestinal tract.

2.4 VIP as the final mediator of NANC inhibitory transmission.

The studies cited above seem to imply that VIP may be the NANC inhibitory neurotransmitter in the gut. Studies of the opossum esophagus was used to show that neither VIP nor ATP is the NANC inhibitory transmitter in the gut. Although electrical field stimulation of these inhibitory nerves induced a transient hyperpolarization of the smooth muscle cell membrane followed by a transient depolarization, the muscle did not relax during IJP production because no tone was present. This study also showed that application of adenosine and its related adenine nucleotides in concentrations up to 10^{-3}M did not have any direct effect on the preparation while prolonged superfusion with adenosine and 6-chloroadenosine caused a decrease in the IJP, considered to be a prejunctional effect. However, exogenous application of VIP induces slight hyperpolarization followed by depolarization of smooth muscle cell membrane and spontaneous oscillations which were not sensitive to neurotoxins but all VIP effects were absent in chloride free media. Furthermore, field stimulation in the presence of VIP produced an IJP. These studies led the authors to conclude that neither a purine nor VIP is the mediator of NANC inhibitory nerves in the opossum esophageal smooth muscle (Daniel, Helmy-Elkholy, Jager and Kannan, 1983).

2.5 Nitric oxide as the final mediator of NANC inhibitory nerves in the gut.

Earlier studies using rat anococcygeus muscle (which is only loosely defined as a gastrointestinal muscle) first gave insight into understanding that the NANC inhibitory neurotransmitter may be nitric oxide (NO). The basis for this assumption came from experiments utilizing N^G-monomethyl L-arginine (L-NMMA) a competitive inhibitor of L-arginine, a substrate for nitric oxide synthesis by the enzyme NO synthase. Electrical field stimulation gave relaxation responses to NANC nerve stimulation and these responses were inhibited in a dose -dependent manner by L-NMMA in rat anococcygeus muscle (Gillespie, Liu and Martin, 1989). Further studies using fluorescent techniques showed that field stimulation led to a decreased level of cytosolic calcium levels and relaxation in the rat anococcygeus muscle (Ramagopal and Leighton, 1989). These studies gave indirect evidence that NO is the NANC inhibitory neurotransmitter. However, direct evidence for NO as the transmitter for NANC nerves in the gut was given by investigations using isolated canine ileocolonic junction (ICJ). It is known that electrical field stimulation of ICJ tissue leads to the release of a transferable factor which has a vasodilator activity like NO in the presence of atropine and guanethidine. Further evidence that the factor released was nitric oxide came from the observation that hemoglobin (Hb), which traps NO eliminated the biological activity and tetrodotoxin abolished its release.

In addition N^G-nitro-L-arginine (L-NNA) which is a known inhibitor of NO biosynthesis blocked the release of this factor. The pharmacological manipulations of NO biosynthesis and release confirm, indeed, that NO is the final mediator of NANC nerves in the gastrointestinal system (Bult, Boeckxstaens, Pelckman et al, 1990).

2.6 Are other structures other than nerves involved in NANC neurotransmission ?

Previous electron microscopic studies have shown that in addition to nerves, there are special neuromuscular cells known as the Interstitial Cells of Cajal (ICC) in the gastrointestinal system of vertebrates (Thuneberg, 1982). Insight into the role of ICC in NANC inhibitory transmission came from studies of opossum esophagus. These studies showed that in both the LES and body circular muscle (BCM) of opossum ICC had dense innervation by nerves containing small agranular vesicles and few large granular vesicles. A closer structural relationship existed between these nerves and ICC than between nerves and smooth muscle cells. The close association between nerve and ICC and presence of gap junction contacts between ICC and smooth muscle cells support the hypothesis that ICC are intercalated between nerves and muscle which mediate nerve stimulated responses (Daniel and Daniel, 1984). Furthermore, evidence for role of ICC in NANC inhibitory neurotransmission is given by ultrastructural localization of

VIP immunoreactive nerves in the distal esophagus of dogs. The highlight of that study was that ICC were densely and closely innervated by VIP-containing nerves in addition to making gap junction contacts with smooth muscle (Berezin, Allescher and Daniel, 1987). These studies led to the hypothesis in this thesis as to how ICC can modulate responses of nerves during neurotransmission. However, several studies have been performed with electronmicroscopy and histochemical methods to analyse ICC structure and function. Therefore, in addition to nerves, ICC should be considered as an important cell type which may utilize unknown mechanisms in the inhibition of gut function. Various perspectives on ICC are considered in the following section.

2.7 Historical perspective and ontogeny of ICC.

Between the end of the 19th century and the beginning of the 20th century (circa 1893-1911) a very important discovery was made by Ramon Y Cajal who described for the first time, using early staining techniques and microscopy, a special cell type in the plexus muscularis profundus (PMP) better known as the deep muscular plexus of the small intestine (Rumessen and Thuneberg, 1982). This special cell type or a subset of them later known as the ICC turned out to be a group of cells which differed from one another in their staining properties (Daniel and Berezin, 1992). These cells maintained certain common structural features although variations

were seen under microscopic examination depending on their location in the gastrointestinal system (Rumessen and Thuneberg, 1982). These differences have been highlighted in addition to species differences in ICC (Faussone-Pellegrini; 1987). However, there have been many studies since the report of Cajal and the emerging views from these investigations seem to identify certain anatomical, physiological and biochemical properties associated with ICC. However, these findings become more and more concrete since modern staining techniques are utilized in conjunction with electronmicroscopy which appears to be the ultimate in instrumentation regarding the study of ICC. Based upon these studies there seems to be a certain amount of controversy as to the anatomical or embryological origin of the ICC.

Different views have been expressed about the nature of ICC such as the suggestion that ICC may be special nerve cells which are linked to autonomic nerve endings of the intestine. This view received support from some of the early microscopists (Meyling, 1953). This controversy has not been laid to rest as further studies based upon different shapes of ICC and their staining properties led to more controversial findings which project ICC as either special neurons or connective tissue or Schwann cells (Meyling, 1953; Schofield, 1968). Further studies by various workers showed that ICC became regarded as modified fibroblasts (Richardson, 1958, Yamanuchi, 1964, Rogers and Burnstock, 1966, Gabella, 1972, Gabella ,1974, Cook and Burnstock, 1976, Taylor et

al 1977, Komuro, 1982).

A more recent and powerful emerging view is that ICC may have smooth muscle properties with a common origin with smooth muscle cells (Imaizumi and Hama, 1969, Stach, 1972, Duchon et al, 1974, Yamamoto, 1977, Faussone-Pellegrini et al, 1977, Thuneberg, 1982, Faussone-Pellegrini and Cortesini, 1983, Faussone-Pellegrini, 1985). This line of thought seems to compete with the proposal which recently reemerged that cells stained by Cajal's method were glial cells, nerves and ICC (Kobayashi et al, 1986). Although this controversy has not been resolved up to date, further studies which may show biochemical similarities or differences between ICC on the one hand and either nerve or smooth muscle on the other hand may enhance the resolution of the present puzzle. However, biochemical studies on ICC are sparse and much limited to staining for microscopical work or functional studies related to contractility of different segments of the gut.

A recent study of cytodifferentiation of ileal and colonic ICC favoured the notion that ICC have smooth muscle properties and may have a common precursor cell (Faussone-Pellegrini, 1987). Evidence (subject to dispute) in favour of the smooth muscle hypothesis was that contact areas between these two cell types were present and there were similarities in the structural features from fetal to adult life. Such evidence is not sufficient to claim that ICC is related to smooth muscle cells.

Other results from the study showing that cytodifferentiation of ICC was dependent upon innervation and type of food ingested. This was regarded as the functional basis for the localization of ICC in the gut. The study of ICC appears to be in its early stages and few studies have clearly demonstrated the relation between structure and function in the different regions in which ICC are localized.

2.8 Ultrastructure of ICC.

Several studies have established that although ICC from different animal species showed subtle differences in morphology, there might be variation in structure depending upon the region of the gastrointestinal tract in which ICC are found. However, there are certain morphological characteristics common not only to ICC of various mammalian species but to ICC of different regions of the alimentary canal. A generalized ultrastructure of ICC has been obtained by comparison of ICC of the alimentary canal of different mammals (Faussone-Pellegrini, 1987). This and other studies showed that ICC were smaller than smooth muscle cells. Although ICC appeared elongated with short and thin lateral branches their basal lamina may be continuous or discontinuous. There may also be a number of caveolae open along the plasma membrane. Gap junctions were found where ICC appose smooth muscle cells. ICC may either have a dense or clear cytoplasm with centrally located nucleus having dispersed chromatin and nucleoli. Numerous mitochondria

containing cristae were found in the cytoplasm bearing similarities to that of smooth muscle cells. The endoplasmic reticulum of ICC consisted of cisternae which either coursed along the plasma membrane or ran in a diverse pattern. Small cisternae of the rough endoplasmic reticulum and large Golgi apparatus near the nucleus were also prsent on ICC. Thin filaments that were 5-6nm thick and bundles of intermediate filaments of 8-10nm thickness were found attached to the plasma membrane and scattered in the cytoplasm. Free ribosomes exist and dense bodies may be found along the axis of the thin filaments. Glycogen particles, dense granules and lipid droplets may be found as inclusions (Faussone-Pellegrini, 1987, Berezin et al, 1988, Rumessen et al, 1982).

2.9 Function and metabolism in ICC.

Based upon certain morphological findings which resembled the structures found in various other cells of known function such as motile cells, suggestions as to the function of ICC have been made.

The connection of ICC between smooth muscle and one another in different parts of the gut is prominent in the view that ICC serves as a pacemaker in the gut by generation of spontaneous electric activity or slow waves in the intestine (Thuneberg, 1982). Evidence for this hypothesis was clearly shown by the fact that the application of methylene blue stain to the intestine damaged ICC and abolished slow waves (Thuneberg, 1983). However, an earlier

study gave insight into the metabolic aspect of the origin of slow waves as well as supported the hypothesis that ICC served as a pacemaker in the intestine. In this study ischemia reduced the frequency and changed the shape of slow waves (Kyi Kyi and Daniel, 1970). Although nerves may be affected by ischemia, ICC could be affected as well. Therefore the evidence given by Thuneberg that ICC damage by methylene blue (which is an inhibitor of guanylyl cyclase, an enzyme activated by NO to produce cyclic guanosine 3' 5'monophosphate) may have a metabolic basis and may suggest that ICC are capable of producing NO. This is in agreement with the fact that low O_2 tension causes decreased production of NO in endothelial cells (Rengasamy and John, 1991). Oxygen was shown to be an absolute requirement for NO production (Leone, Palmer, Knowles, Francis, Ashton, Moncada, 1991). These were indirect studies and no study to date has isolated ICC and shown that they were capable of producing NO. Although certain studies recently suggested this capability by the localization of NO synthase in ICC using NADPH-diaphorase method (Daniel, Jury, Cayabyab, Christinck, Kostka, Berezin, 1992). Most studies have been indirect functional studies using inhibitors of NO synthase enzyme. Constitutive NO production by ICC and the nature of its NOS remain unexplored but nothing so far relates it to the production of slow waves, although both cellular functions of ICC may be dependent on metabolism.

Slow wave type action potential has an ionic basis and could

develop by depolarization from activation of a non L-type calcium conductance which constitutes the initial phase of the upstroke potential in the colon. This initial non L-type calcium conductance contributes substiantially to L-type calcium channel opening when E_m is about 35 mV and to the plateau phase of the slow wave although sodium and chloride conductances may also contribute to the plateau potential. These observations derive from the fact that L-type calcium channel blockers reduce the rate of rise of action potentials and shorten the plateau. Activation of potassium conductance causes repolarization (Huizinga et al, 1991). The initial part of the slow wave could be isolated in a glucamine-nitrendipine Krebs solution because glucamine replaces NaCl and abolishes gradients for Na^+ and Cl- while nitrendipine blocks the L-type calcium conductance. This non L-type initial upstroke is due to a calcium conductance different from that of L-type or dihydropyridine calcium channels. Certain properties of the slow waves are not in agreement with the upstroke potential being generated by T-type calcium channels since slow waves can be activated from potentials of about -110 mV while T-type calcium channel is readily activated at negative potential with small depolarization (Huizinga et al, 1991). The initial phase of the slow wave upstroke is reduced by a decrease in extracellular calcium while it is increased by high intracellular calcium. However, cAMP concentration also decreases the frequency of this upstroke potential without any change in membrane potential.

In addition, slow waves can be generated by ICC at identical frequency over a range of -110 to-40 mV. These findings suggest that intracellular metabolic activity (probably including change in cAMP without change in voltage) activates calcium conductance associated with the generation of the upstroke potential in slow waves (Huizinga et al, 1991).

The presence of numerous mitochondria in ICC is a marker for high intracellular metabolic activity. This is shown by adenosine triphosphate (ATP) production and the generation of cAMP (which modulates the initial upstroke of slow waves) that are associated with the ICC mitochondria. Furthermore, the relation between the frequency of ICC slow wave conductances and temperature supports the hypothesis that this electrical activity may have a metabolic basis (El-Sharkawy 1983, Barajas-Lopez, Berezin, Daniel, Huizinga, 1989).

2.10. Tissue and Species distribution of ICC.

Transmission electron microscopy showed that ICC can be found associated with smooth muscle cells throughout the various segments of the digestive tract. There was a positive correlation between the morphology of ICC and their location in the alimentary canal (Faussone-Pellegrini, 1987).

A structural basis for the proposed role of ICC in neurotransmission have been demonstrated in body circular muscle and lower esophageal sphincter (LES) of oposssum (Daniel and

Daniel, 1984). Similar studies were done in the different segments of human esophagus (Faussone-Pellegrini and Cortesini, 1983). The lower esophagus of the opossum resembled that of humans because it consisted of smooth muscle unlike those of mouse, rabbit, cat and dog which were known to have striated muscle except for the sphincter (Thuneberg, 1989). In the opossum esophagus the number of ICC was about 2-4 per 100 smooth muscle cells with a higher density in the body circular muscle than in the LES (Daniel and Daniel, 1984; Faussone-Pellegrini and Cortesini, 1984). Further studies showed that canine esophageal ICC were innervated by VIP-containing neurones in circular muscle (Berezin, Allescher, Daniel, 1987) but no ICC have been found in the longitudinal muscle or myenteric plexus of opossum esophagus (Daniel and Daniel, 1984). NO has been shown to be the final mediator of NANC inhibitory nerves in the esophagus (Christinck, Jury, Cayayab, Daniel, 1991).

In the stomach, ICC was found associated with circular muscle in the gastric level of the LES, fundus and corpus. The density of ICC in the corpus was found to be higher than in other areas of the stomach (Faussone-Pellegrini, 1987). The ICC density in the corpus was about one ICC to 1000 smooth muscle cells (Daniel and Daniel, 1984).

The distribution of pyloric ICC was studied by Daniel and Allescher (1990). Their observation was that most of the pyloric ICC were similar in structure to those of circular muscle while

some resembled the ICC of the myenteric plexus. In the ileum, ICC were found located in the myenteric plexus and associated with longitudinal muscle and the deep muscular plexus between the two sublayers of circular muscle where they form a discontinuous layer in close contact with nerve cells and make gap junctions with smooth muscle cells. ICC of ileum have been described (Rogers and Burnstock, 1966; Faussone-Pellegrini and Cortesini, 1983; Faussone-Pellgrini, 1987; Thuneberg, 1989; Daniel and Berezin, 1992). ICC of ileum were found in toad (Rogers and Burnstock, 1966), bat (Yamamoto, 1977), chicken (Gabella,1985), mouse (Cook and Burnstock, 1976; Gabella, 1972; Rumessen et al, 1982), rabbit (Richardson, 1958; Cheung and Daniel, 1980), cat (Taylor et al, 1977) and man (Faussone-Pellegrini and Cortesini, 1985).

2.10.1 ICC of colon

Interstitial Cells of Cajal have been found both in the myenteric and submucosal plexuses of canine colon. In the myenteric plexus, ICC forms two networks or groups of cells with one network facing either longitudinal or circular muscle. Certain ultrastructural characteristics are common to ICC in the myenteric plexus of different species. These cells have few cisternae of smooth endoplasmic reticulum and rough endoplasmic reticulum, few thin filaments, many mitochondria and no basal lamina. ICC of submucosal plexus have bundles of thin filaments, no intermediate

a discontinuos basal lamina, many caveolae and mitochondria (Faussone-Pellegrini, 1987; Berezin et al, 1988). However, this ultrastructure varied with different species which were studied (Daniel and Berezin, 1992).

Generally, colonic ICC in cross section were slightly thinner compared with muscle cells but had a large number of caveolae. The ICC located on the submucosal side of circular muscle like those on the myenteric side usually showed circular orientation with long thin cell body and short processes enclosed by a partial basal lamina. These cells also had wide Golgi apparatus, numerous mitochondria, bundles of thin filaments, cisternae of smooth endoplasmic reticulum near the plasma membrane or close to the caveolae of the peripheral cytoplasm. Some cisternae of rough endoplasmic reticulum were also present and glycogen was widely distributed in the cytoplasm. ICC made desmosome like junctions through their lateral processes with each other in areas without a basal lamina while they were close to nerve fibers and even made contacts with them. However, smooth muscle cells were rarely in close contact with nerve varicosities nor with ICC (Faussone-Pellegrini, 1985; Berezin et al; 1988).

2.10.1.1 Distribution of ICC in canine colon

Both functional and electron microscopic studies indicated the presence of ICC in the colon. At present most investigations have established that ICC layers existed in the myenteric and

submucous areas of the colon. However, this clearly varies with species (Daniel and Berezin, 1992).

2.10.1.2 ICC in myenteric plexus of canine colon.

Structural studies showed that ICC of the myenteric plexus (ICC-MP) of canine colon were interconnected by gap junctions which existed between their primary and secondary processes. Other types of contacts such as close-apposition and the adherens-type contacts were also present between ICC-MP and their cell bodies. ICC-MP was oriented parallel to the plane of the plexus in the regions where muscle layers were close together. ICC-MP had an elongated cell body and were characterized by dark, condensed cytoplasm of the cell bodies and processes which differentiated them from macrophage-like cells and smooth muscle cells. Furthermore ICC-MP had large ovoid nuclei which were smooth with few indentations.

In ICC-MP, there were cisternae of rough endoplasmic reticulum, free ribosomes, mitochondria, microtubules, actin-like and intermediate filaments and Golgi complex, perhaps some centrioles (Berezin, Huizinga and Daniel, 1990).

ICC-MP do not make frequent contacts with smooth muscle cells. However, smooth muscle cells of the innermost subdivision of the longitudinal muscle layer extended foot-like processes towards ICC-MP and occasionally formed small gap junctions or adherens-type contacts with ICC-MP. In addition, ICC-MP made gap-junctions with outer circular muscle cells and adherens-type contacts with macrophage-like cells. (Berezin, Huizinga and Daniel, 1990). The significance of these contacts between ICC-MP and non-muscle cells remains to be determined.

Nerve varicosities containing large granular vesicles and small agranular versicles made close contacts (<20 nm) with ICC-MP, although no synapse-like configurations existed between these nerve profiles and ICC-MP plasma membrane. Furthermore, no close contacts existed between the myenteric nerves and smooth muscle cells. ICC-MP which were found within the longitudinal muscle layer were interconnected by gap-junctions and occasional close contacts were formed with nerves. Although the cell bodies of ICC-MP were not found between muscle cells of the outer circular muscle layer, the secondary processes projected into the first and second layers of muscle cells. ICC-MP at the longitudinal muscle border did not

interconnect with ICC at the circular muscle border and no ICC-MP or their processes existed in the central regions of the circular muscle bundles (Berezin, Huizinga and Daniel, 1990). The structural presentation of ICC-MP showed that it may be receiving input from nerves. However, further studies are important to clarify the role of ICC-MP in colon function.

2.10.1.3 ICC in submucous plexus of canine colon

Recent studies of the canine proximal colon using the electron microscope showed a dense network of ICC and nerve fiber around the inner border of circular muscle layer which had a thickness of 1-3 μm to 10-20μm in cross section. The ICC at the inner border of circular muscle layer (ICC-CM) had extremely branched primary processes towards circular muscle cells and secondary and smaller branches in all directions. The processes were separated by a space containing elastin and collagen fibers (Berezin, Huizinga and Daniel, 1988).

ICC-CM had similar appearance with those localized in the circular muscle of other parts of the gastrointestinal system. There were large ovoid nuclei usually having smooth contours and containing one nucleolus in the ICC-CM. The ICC-CM contain numerous nuclear pores in the nuclear envelope and the perinuclear cytoplasm contained many prominent Golgi complexes, Golgi transporting vesicles, cisternae of rough endoplasmic reticulum, mitochondria,

free ribosomes, microtubules, thin and intermediate filaments and dense bodies. A pair of centrioles may occasionally be seen (Berezin , Huizinga and Daniel, 1988). However, the role of these organelles in ICC function in response to neuronal transmission remains to be clarified.

2.10.1.4. Innervation of ICC-MP and ICC-CM by VIP neurons in canine colon

Structural studies have been the basis for assessment of innervation of ICC by peptidergic neurons. Using VIP immunoreactivity as a measure of VIP nerve profile density in the myenteric and submucous plexuses and in the circular muscle-submucosa interface, it has been recently reported that the highest proportion of VIP nerve profile (about 38%) exists in the circular muscle-submucosa interface while VIP immunoreactivity could not be identified in the nerve cell bodies of the myenteric and submucous plexuses. In addition, many nerve varicosities were interposed between branches of ICC with the circular muscle-submucosa interface. Although few nerve varicosities made close associations (< 40 nm) with ICC, there was no distinction in their morphology and relationship to ICC between VIP immunopositive and immunonegative nerve profiles. In total, it appeared that ICC was innervated by VIP containing neurons. Quantitative techniques confirmed these qualitative results and the finding suggested that less than 50% of nerve varicosities in canine colon are VIP-

immunoreactive. However, about 35-45% of total VIP immunoreactivity were present in the circular muscle-submucosa interface (Berezin, Huizinga, Farraway, Daniel, 1990). Furthermore, functional studies have shown that VIP innervation of ICC in the submucosal border of circular muscle contributed to muscle relaxation (Huizinga, Tomlinson, Pintin-Quezada, 1992).

2.10.1.5 Functional significance of VIP innervation of ICC in NANC responses.

Recent studies indicate that ICC on the submucosal border of circular muscle receive inhibitory innervation. This is shown by electrophysiological studies in which application of electrical field stimulation to ICC-rich preparations containing some attached smooth muscle cells produced ijps consistently but such inhibitory responses were not recorded from nerve stimulation of cells having a resting membrane potential of about -75mV or lower, presumably because the ijp was produced by increased potassium conductances and E_k was about -75 to -80 mV. The slow waves which followed ijps were reduced in amplitude. In addition, circular smooth muscle cells also received direct inhibitory innervation without ICC as intermediates since removal of the ICC layer did not eliminate ijps. Furthermore, ijps which were tetrodotoxin (sodium channel blocker) sensitive were also recorded from longitudinal muscle suggesting that direct or indirect inhibitory innervation of the longitudinal muscle existed (Huizinga, Berezin, Daniel and Chow,

1990). These studies did not clearly show that VIP may be acting directly on ICC, particularly those on the submucosal side of circular muscle.

Evidence from intracellular recordings obtained from superficial cells of ICC-rich preparations suggested that VIP acted on ICC. The effects of VIP on ICC have been recorded in the presence of heptanol which abolished gap junction conductance. Electrical recordings from ICC showed that VIP gave a dose-dependent hyperpolarization and reduced cell resistance at threshold concentration of $5x10^{-7}M$ (Berezin, Huizinga, Farraway and Daniel, 1990). These studies led to an important question as to how ICC transmitted inhibitory signals to circular muscle and how longitudinal muscle was controlled either directly or indirectly by VIP.

2.10.1.6 Inhibitory control of circular smooth muscle by colonic ICC.

Several functional studies have attempted to provide evidence that inhibitory control of circular smooth muscle cells may be through release of an inhibitory mediator which caused hyperpolarization and production of ijps in circular smooth muscle cells (Berezin, Huizinga, Farraway and Daniel,1990).

A recent study suggested that the control of colon circular smooth muscle cells may be through release of nitric oxide from smooth muscle by direct effect of VIP (Grider et al, 1992) or

by nitric oxide release from nerves (Dalziel et al, 1991) or from ICC (Publicover et al, 1992) which was produced by nitric oxide synthase. Another recent study suggested that VIP acted on smooth muscle to produce nitric oxide (Grider et al, 1992). Although this investigation was important in its theoretical perspective, it did not properly differentiate ICC from other cells such as smooth muscle cells or macrophage-like cells. Therefore this lack of identification of ICC casts ambiguity on the source of nitric oxide in that study. However, even more tempting are the following questions: (i) What is the site of nitric oxide synthase in ICC? (ii) What are the biochemical characteristics of this enzyme? In order to answer these questions, certain biochemical approaches are important and they form part of the work for the present study.

CHAPTER THREE

ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF INTERSTITIAL CELLS OF CAJAL RICH LAYER OF COLON.

3.1 Introduction.

In order to meet the first objective of this study which is to attempt to localize and characterize ICC membranes in canine colon, a step-wise approach involving dissection techniques (described in Methods) and isolation procedures were adopted using subcellular fractionation method developed in our laboratory. This fractionation method is essential to the present study as it provides separation of membranes of various cell types on the basis of their buoyant density and allows estimation of purity and enrichment of fractions by use of membrane markers (Kidwai, Radcliffe and Daniel, 1971, Matlib, Crankshaw, Garfield et al, 1979, Grover, Kwan, Garfield et al, 1980, Daniel, Grover and Kwan, 1982).

Experience from our laboratory has shown that the first important step to be taken towards preparation of tissue for analysis is careful dissection which would ensure little or no damage to the colonic tissue under study. This is usually confirmed by examination of specimens of dissected tissues under electron microscopy. Proper dissection would ensure that longitudinal muscle, circular muscle and ICC-rich submucosal region of circular muscle are free of unwanted tissue. These steps have been taken in the various studies involving the characterization of different fractions from various layers of canine small intestine. In all previous studies from our laboratory, the next step after dissection is homogenization of tissues in an isotonic medium,

usually, sucrose 3-(-N-morpholino-propanesulfonic acid (MOPS) buffer pH 7.4.

Homogenization is crucial to the study of membrane fractions because it makes physical separation of plasma membrane from intra- and extracellular components possible, thus allowing isolation of membrane fractions. Following homogenization, the dissected colonic tissues can be subjected to differential centrifugation using the scheme in Figure 1 which has been developed in our laboratory for the submucous region of the small intestine (Ahmad et al, 1988; Mao, Tougas, Barnett and Daniel, 1990). In this technique both low and high speed centrifugation is important at various stages of fractionation. Low speed centrifugation is intended to bring down unbroken cells or cell fragments and later mitochondria and synaptosomes. Higher speed centrifugation ensures that microsomal membranes (plasma membranes and endoplasmic reticulum) are brought down.

Careful centrifugation is important to avoid loss of materials and this is ensured by measurement of volumes of each fraction derived as supernatant and measurement of volumes of buffer used (MOPS) in resuspending pellet fractions. These volumes are recorded to facilitate calculation of recoveries for each fraction in order to ensure that the overall scheme obeys the law of mass action.

Various markers have been used in our laboratory to investigate biochemical events in canine small intestine.

5'nucleotidase and Mg^{2+}ATPase are mostly used as markers for smooth muscle plasma membrane while high affinity ^{3}H-Saxitoxin (which interacts to block neuronal sodium channel) binding and ^{125}I-ω-Conotoxin binding (which is known to bind neuronal N-type calcium channel) are used as markers for synaptosomes and the content of VIP immunoreactive material is used as a measure of intact synaptosomes (Kidwai, Radcliffe and Daniel, 1971, Wei, Janis and Daniel, 1976, Ritchie and Rogart, 1977, Ahmad, Allescher, Manaka et al, 1988, Ahmad, Rausa, Jang and Daniel, 1989).

Cytochrome c oxidase has been found to be present on the inner mitochondrial membranes, but its use requires performance of measurement on fresh membranes (Kwan, Triggle, Grover, Lee, Daniel, 1977). The levels of various markers in different fractions are used in the assessment of contamination of each fraction containing the desired membrane.

The different membrane markers mentioned above will be employed in our fractionation scheme and characterization of membranes from various colonic preparations under study. 5'nucleotidase is not a general plasma membrane marker, for example, it is not present in nerve plasma membrane except in very low activity (Ahmad et al, 1988). The same could be said of high affinity ^{3}H-Saxitoxin binding to sodium channels which are present only in synaptosomes. Such sodium channels with high affinity for Saxitoxin are not present in smooth muscle plasma membrane.

Mg^{2+}ATPase is not known to be high in synaptosomes but it exists there; its activity is higher in plasma membranes of smooth muscle cells. Because it is also present on synaptosome as well as on smooth muscle plasma membrane and even in mitochondrial membranes. It is not as valid as 5'nucleotidase as a marker for smooth muscle plasma membranes. It is unknown whether any of these markers are present on ICC-plasma membranes and thus it is unclear whether any of them can be used in the characterization of ICC membrane enriched fraction.

The morphology of fractions is important in differentiation of fractions (Daniel, 1985).

However, the working idea is to compare marker distributions in ICC-rich and poor components of circular muscle to obtain clues about ICC membrane activity. In addition VIP binding could be used as a marker for ICC enriched membrane fraction since ultrastructural and functional studies show that there might be VIP binding sites or receptors on ICC membrane. However, there may also be VIP receptors on smooth muscle and nerve membranes.

Based upon the knowledge from functional studies in our laboratory about the high mitochondrial content of ICC and the susceptibility of ICC to damage when there is hypoxia , there was the suspicion that there might be untoward effects in ICC of using our conventional techniques of suspending tissues in sucrose MOPS buffer before dissection. Therefore, I compared the conventional dissection procedure in cold sucrose MOPS to use of oxygenated

(95% oxygen, 5% carbon dioxide) Krebs solution (23-24 °C) for the various steps of dissection. However, during homogenization, tissues should be placed in our conventional buffer in order to reduce ionic strength and diminish membrane aggregation (De Duve, 1964) which interferes with membrane fractionation.

In the present study, dissection and preservation of tissue in oxygenated Krebs solution will be termed method 1 while dissection of tissue on ice and preservation in cold MOPS will be termed method 2. In both methods tissue will be dissected at ice temperature in order to minimize loss of enzyme activity and essential metabolites. Once dissection was complete all tissues were placed in sucrose MOPS for homogenization etc., so that the results of fractionation are likely to be unaffected.

An additional possible outcome of comparing these two methods of tissue preparation was to gain insight into the comparative effects of oxygenated Krebs solution compared to MOPS on structure and biochemistry of nerves, smooth muscle and ICC membrane. In order to obtain initial information on these matters, it is important to compare the effects of the two methods on structural features of canine colonic ICC-rich submucosal and ICC-poor circular muscle layers by fixing tissues after dissection and then examine them using electron microscopy. It is also important to examine the effects of these two methods on the activities of synaptosomal and plasma membrane markers in fractions, derived from

the above preparations.

3.2 Materials.

^{125}I-labelled VIP (2000 Ci\ mmol) was purchased from Amersham (Arlington Heights, IL) while ^{125}I-labelled ω-Conotoxin was similarly purchased from (New England Nuclear Research Products, Boston, Ma). Unlabelled porcine VIP was acquired from a commercial source (Peninsula laboratories). ^{3}H-Saxitoxin (20 Ci\mmol) was purchased from Amersham (Arlington Heights, IL). Tetrodotoxin (TTX), MOPS, bovine serum albumin (BSA), tris (hydroxylmethyl) aminomethane hydrochloride (Tris HCl) were commercially obtained from Sigma Chemicals (St. Louis, MI). All other salts of analar grade were obtained from either Baker or BDH Chemicals.

3.3 Methods

3.3.1 Tissue handling and Dissection

Mongrel dogs of either sex were euthanized with an overdose of pentobarbital sodium (100mg\kg i.v.). Incisions were made in the abdomen along the midline immediately after the dog was confirmed dead followed by excision of the colon. After colonic contents were carefully removed, the tissues obtained were placed in either oxygenated (95% O_2, 5%CO2) Krebs solution pH 7.4 for further processing using method 1 or placed in ice cold sucrose MOPS buffer pH 7.4 for processing using method 2. The composition of the oxygenated Krebs solution (23-24 °C) was (mM) NaCl 120.3; KCl 5.9; $CaCl_2$ 2.5; $MgCl_2$ 1.2; NaH_2PO_4 1.2; Glucose 11.5 pH 7.4. The sucrose MOPS buffer contained 0.25M sucrose, 10 mM MgCl and 20 mM MOPS.

Dissections were performed on colonic segments which had been cleared of blood vessels, nerves and fat in the mesenteric arcade. These segments were cut along the mesenteric line and pinned flat with the mucosal side facing upwards in a dissection dish filled with oxygenated Krebs solution in method 1 but in method 2 as previously stated dissection was done in a moist dish placed upon ice bucket. The mucosal layers were carefully excised using a pair of scissors with curved tips and the segments were flipped over to carefully remove the longitudinal muscle layer along with the

myenteric plexus. Then two kinds of preparations were made (i) a preparation containing circular muscle with removal of both the submucosal ICC-network and the ICC in the myenteric plexus; (ii) a preparation containing the complete submucosal network of ICC with associated nerves and associated circular muscle cells. Preparations obtained during dissection from method 1 were placed in oxygenated Krebs solution pH 7.4 while those from method 2 were placed in ice-cold sucrose MOPS buffer (pH 7.4). Strips were fixed for transmission electron microscopic examination at the beginning and end of dissection using either method. This was done to check if the contents of each preparation were as intended.

3.3.2 Homogenization and differential centrifugation.

After dissection, the preparations from each method were quickly blotted dry on filter paper, weighed and resuspended in sucrose MOPS buffer pH 7.4 (10ml\g tissue wet weight) and carefully minced (with a pair of sharpened scissors) in plastic beakers held on ice. The minced tissue preparations were placed in plastic centrifuge tubes and homogenized for 24 seconds in Polytron [Brinkman model] homogenizer (setting 5) for 3x8 seconds bursts at speed 15,000 rpm. This was followed by several steps in differential centrifugation. Homogenates in each pair of centrifuge tubes were weighed on a balance to ensure that they were equal weight. The homogenates were centrifuged for 10 minutes at 750 x g

and the supernatant obtained was termed post nuclear supernatant (PNS).

The PNS was filtered through two layers of cotton gauze and centrifuged at 4000 x g for 10 minutes and the supernatant obtained was designated S_1 while the pellet was termed P_1. The supernatant S_1 was then centrifuged at 28,000 x g for 10 minutes which led to sedimentation of pellet P_2 and the supernatant S_2 was collected. The supernatant S_2 was subjected to high speed centrifugation at 120,000 x g for 60 minutes. This gave rise to a pellet fraction (MIC 1) which was resuspended in MOPS buffer using a manual teflon coated pestle and homogenizing tube. MIC 1 was centrifuged for 10 minutes at 17,000 x g. The supernatant thus obtained was termed MIC 2 while the pellet was designated P_3. Note that throughout the centrifugation scheme sucrose MOPS buffer pH 7.4 was utilized in order to avoid aggregation of plasma membrane (De Duve, 1964). Figure 1 gives a summary of the centrifugation scheme. 2.5ml of MIC 1 from ICC-poor preparation was subjected to a discontinuous sucrose (14%,33%,40%,48%) density gradient centrifugation to obtain membranes enriched in smooth muscle plasma membrane fraction (M_2) at the interphase between 14% and 33% sucrose.

1. HOMOGENATE

   ↓ 750 x g x 10′

2. POST NUCLEAR SUPERNATANT (PNS) and PELLET

   ↓ 4,000 x g x 10′

3. SUPERNATANT (S_1) and PELLET (P_1)

   ↓ 48,000 x g x 10′

4. SUPERNATANT (S_2) and PELLET (P_2)

   ↓ 120,000 x g x 60′

5. PELLET (MIC 1) and SUPERNATANT (soluble)

   ↓ 17,000 x g x 10′

6. SUPERNATANT (MIC 2) and PELLET (P_3)

FIGURE 1. CENTRIFUGATION SCHEME.

The supernatant or pellet used for each subsequent step is indicated by the locus of the arrow.

3.3.3 Biochemical assays

3.3.3.1 Protein concentration.

This was determined in various fractions by the Folin-Phenol method (Lowry, Rosenbrough, Farr, Randall, 1951). A set of seven tubes containing 0, 10 ,20, 30 up to 70 μl of 1 mg \ml of BSA were prepared as standards to determine the standard curve while another set of tubes (pairs) containing 20 μl of membrane fractions were prepared including a pair of tubes containing 20 μl of MOPS buffer to be used as blanks. Into these tubes, distilled water was added to make up a volume of 200 μl in each tube. Following that, 3 ml of solution containing 100 volumes of 2% $NaCO_3$\ 0.1M NaOH, 1 volume of $CuSO_45H_2O$ and 1 volume of 2% Na K tartrate was added to each tube. After 15 minutes 200 μl of 1 N folin-phenol was added to each tube and the optical density was determined spectrophotometrically at 700 nm 10 minutes later. By using values obtained from the standard curve, protein concentrations were calculated for each fraction.

3.3.3.2 5'nucleotidase activity.

The activity of this enzyme was measured by the technique of Song and Bodansky (1967). Standards were prepared containig 0 to 700 μl of inorganic phosphate (1 um\ml) to which distilled water was added to a volume of 1 ml. Tubes containing 880 μl of

10 mM $MgCl_2$\ 50mM imidazole pH 7.5 and 20 μl membrane fraction were prepared and incubated for 5 minutes. 100 μl of 50mM Na adenosine monophosphate\0.1 M imidazole was added to each tube to start the reaction which was terminated by the addition of 1 ml cold 10% trichloroacetic acid (TCA) after 15 or 30 minutes (depending on the activity present). These tubes were centrifuged at maximum speed for 15 minutes in a desk top centrifuge after 1 ml supernatant was removed. 1 ml of reagent containing 1% NH_3 molybdate\1.15 NH_2SO_4 was added to each tube containing either the standard or the membrane fraction and vortexed. The optical density was read at 700 nm in the Beckman laboratory spectrophotometer (model DU-88) after tubes had sat for 20 minutes. Calculation of 5 nucleotidase activity took into account the volume of membrane fraction, incubation time and protein concentration. Activity in each fraction prepared in duplicates was expressed in umol Pi mg^{-1}.

3.3.3.3 Mg^{2+}ATPase activity.

Standards identical to those used in the 5'nucleotidase measurement were prepared. Tubes each containing 50 μl of membrane fraction or an equal volume of buffer (MOPS pH 7.4); 100 μl of 100 mM $MgCl_2$; 500 μl of 100 mM imidazole, 250 μl of distilled water and 100 μl of 50 mM Na ATPase pH 7.4 were prepared and incubated for 15 minutes. The reaction was terminated by adding 1ml of 10% TCA at 4 °C to each tube. These tubes were centriuged at maximum speed in a

desk top centrifuge. 1 ml of the supernatant was removed and mixed with 1 ml $FeSO_4\backslash NH_3$ molybdate and the optical density of reaction materials in each tube was read at 700 nm after 10-15 minutes in Beckman laboratory spectrophotometer (model DU-88). The calculation for specific activity of this enzyme was similar to that of 5'nucleotidase.

3.3.3.4 ^{3}H-Saxitoxin binding.

The binding was performed at 20^{0}C in a buffer containing 50 mM Tris HCl (pH 7.4), 0.2% BSA with 0.5-0.8 nM radiolabelled saxitoxin. This concentration is near the Kd value determined for intestinal synaptosomes. Non-specific binding was determined in the presence of 1 μM TTX. Saxitoxin binding was started by addition of membranes (30-80 μg) to the incubation tubes. After 20 minutes the reaction was stopped by addition of 3 ml ice-cold buffer. This was immediately followed by filtration and washing 3 times in the same volume of buffer over a Whatman GF/F filters using a millipore filtration apparatus. The filters were placed in vials containing 4ml scintillation fluid and the radioactivity was counted on a Beckman (LS 6800) beta counter with an efficiency of about 40%. The calculation for ^{3}H-Saxitoxin binding was done taking into account the protein concentrations, the specific activity of the ligand and the volume of membrane fraction utilized. ^{3}H-Saxitoxin binding was expressed in fmol/mg.

3.3.3.5 VIP binding

This assay involved incubation of membranes (30 μg protein/ml) in a medium containing 20 pmol ^{125}I-VIP, 10 μl of 1mg/ml aprotinin, 50 μl of membrane and 100 μl of buffer consisting of 25 mM Tris HCl (pH 7.4), 1% BSA and 2mM $MgCl_2$. The ^{125}I-VIP concentration used was near the Kd value found in intestinal synaptosomal membranes (Mao et al, 1990). Non-specific binding was determined by addition of 20 μl of 1.0 μM unlabelled VIP to the medium. The difference between total and non-specific binding was considered specific binding. Membrane bound ^{125}I-VIP was separated by filtration under vacuum using Whatman GF/F filters which had been pretreated with 1% polyethylenamine. Each of these filters were washed thrice with 3 ml ice-cold Tris HCl buffer pH 7.4 and radioactivity on the filters was measured in a gamma counter (Beckman 5500). The percentage of non-specific binding was less than 30% of total binding. ^{125}I-VIP binding was expressed in fmol/mg protein.

3.3.3.6 ^{125}I-ω-Conotoxin binding

This was performed in 50 mM Tris-HCl pH 7.4 containing 0.2% BSA. Binding was done using the Kd value found in the small intestinal deep muscular plexus membranes (Ahmad et al, 1989).
Non-specific binding was determined by addition of 10^{-6}M unlabelled ω-Conotoxin to the medium. Vacuum filtration was used to separate

membrane bound ^{125}I-`-Conotoxin using Whatman GF/B filters presoaked in 1% polythelenamine. This was followed by washing of each filter thrice in 4 ml of ice-cold Tris HCl buffer pH 7.4 and quantitation of radioactivity on the filters was determined in a gamma counter (Beckman 5500). The percentage of non-specific binding was less than 30% of total binding. ^{125}I-ω-Conotoxin binding was expressed in fmol/mg protein.

3.3.3.7 VIP immunoreactive content.

This was determined by radioimmunoassay of VIP in fractions extracted in 0.5 M acetic acid for 10 minutes in a boiling water bath, lyophilized and stored at -20°C until the assay is performed. The concentration of VIP in each centrifugation fraction was determined using anti-serum R-501 from Professor Yanaihara at a final dilution of 1: 70,000. The buffer used in this assay consisted of 0.01M phosphate, 0.14 M NaCl, 0.025 M EDTA, 0.5% BSA and 250 KIU/ml aprotinin (pH 7.4). The sample, tracer, buffer and antiserum were incubated at 4°C for 48 hours in a final volume of 700 ul. Antirabbit alpha-globulin goat serum was utilized in the separation of antibody bound and unbound tracer. The radioactivity was counted in a gamma counter (Yanaihara et al, 1977).

3.3.3.8 Other biochemical assay.

Cytochrome c oxidase activity was measured according to the method of Cooperstein and Lazarow (1951).

3.3.3.9. Electron microscopic studies

At the beginning and end of dissection strips of tissue obtained from preparations using methods 1 and 2 were fixed using 2% glutaraldehyde in 0.075 M cacodylate buffer, pH 7.4, containing 1% OsO_4 while dehydration and embedding of preparations were carried out as usual in our laboratory. Cross sections of tissues were made and stained for 2 minutes with lead citrate and examined under the transmission electron microscope. Tissues from either method 1 or 2 were examined (Rumessen, Thuneberg and Mikkelson, 1982).

3.3.3.10. Data analysis

Total protein, specific activities of 5'nucleotidase, Mg^{2+}ATPase and ^{3}H-Saxitoxin binding were compared between subcellular fractions from similar preparations subjected to methods 1 and 2 using paired t-test. Differences between similar fractions less than $p<0.05$ were considered significant.

3.4. RESULTS

3.4.1 Electron microscopic examination.

ICC-rich and -poor preparations placed briefly in each buffer before dissection were similar in their structural features using either method of dissection. Figures 2 and 3 show ICC-rich preparations while figures 4 and 5 show ICC-poor preparations at the beginning of dissection. These structural studies, however, showed a marked decrease in the number of ICC observable in the ICC-rich preparation at the end of dissection using method 2. Furthermore, the membranes of ICC observed after using method 2 appeared disrupted and damaged (Figures 6 and 7) and large lacunae were present in the cells. In contrast to the observation in the ICC-rich layer, there were no significant differences in morphologic features of smooth muscle cells in ICC-poor circular muscle preparations using either methods (Figures 8 and 9).

Figure 2 (Top). ICC-rich strip in Krebs solution at the start of dissection. Magnification = x4000; Scale 2cm=2μm. CM=circular muscle.

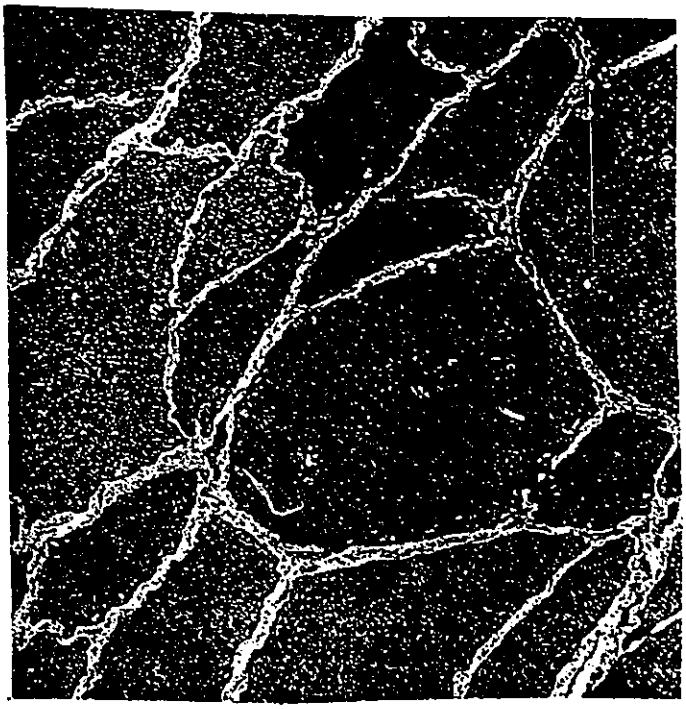
Figure 3 (Bottom). ICC-rich strip in MOPS solution at the onset of dissection. Magnification = x3000; Scale 2cm=2μm. CM=circular muscle.


ICC
ICC


ICC

Figure 4 (Top). ICC-poor preparation in Krebs solution at the start of dissection. Magnification = x4000; Scale 2cm=500nm.

Figure 5 (Bottom). ICC-poor preparation in MOPS solution at the beginning of dissection. Magnification = x4000; Scale 2cm=500nm.



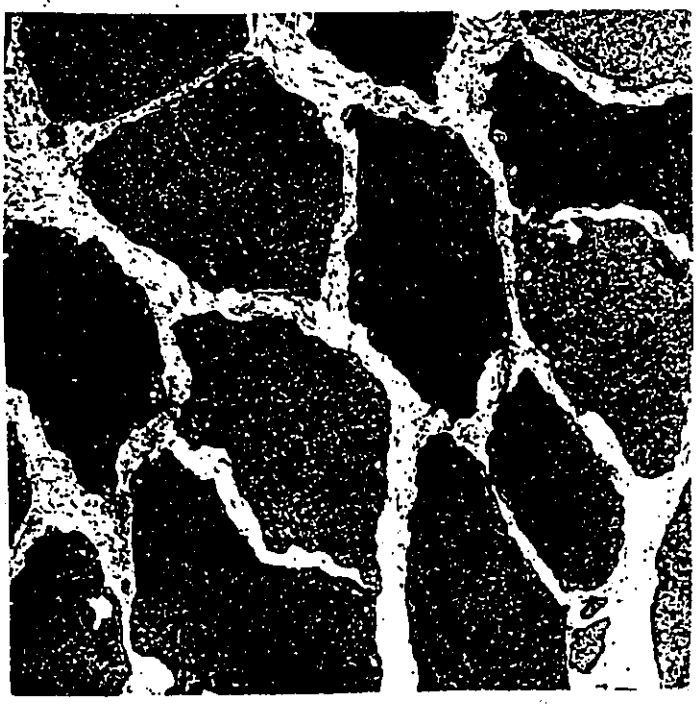


Figure 6 (Top). ICC-rich preparation in Krebs solution at the end of dissection. Magnification = x2000; Scale 1cm=2μm. CM=circular muscle.

Figure 7 (Bottom). ICC-rich strip in MOPS solution at the end of dissection. Magnification = x2000; Scale 1cm=2μm. CM=circular muscle.


ICC

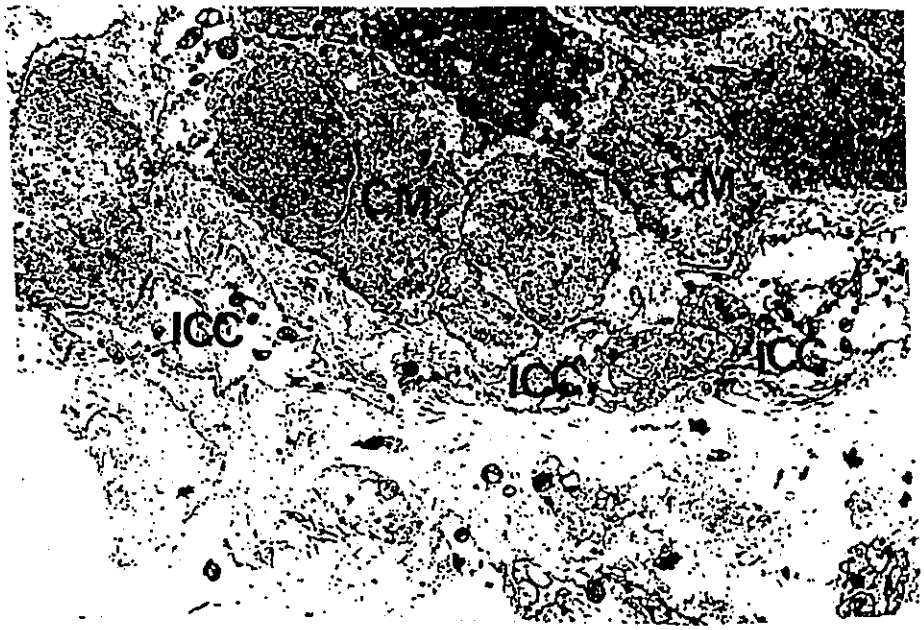
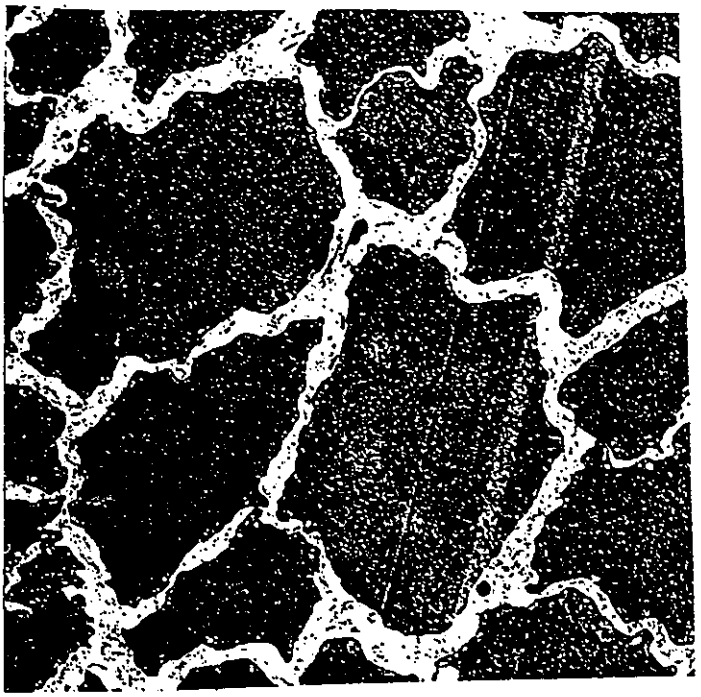
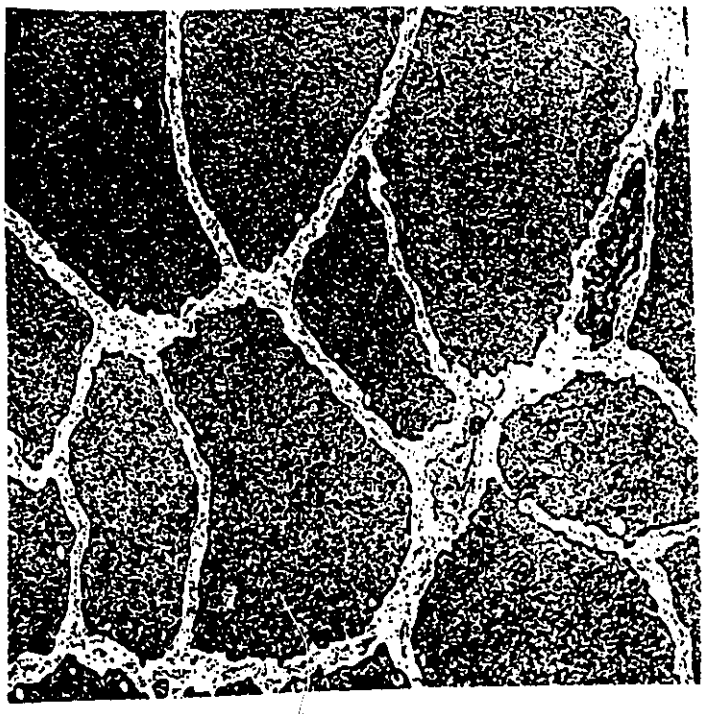

CM
CM
ICC
ICC
ICC

Figure 8 (Top). ICC-poor preparation in Krebs solution at the end of dissection. Magnification = x4000; Scale 2cm=500nm.

Figure 9 (Bottom). ICC-poor preparation in MOPS solution at the end of dissection. Magnification = x4000; Scale 2cm=500nm.





3.4.2 Fractionation

3.4.2.1 Reproducibility of fractions

The centrifugation and fractionation scheme showed that: 1) it is reliable for the purpose for which it was designed; 2) each fraction maintained a consistent position in the centrifugation scheme; 3) it is suitable for preparations from the different layers of the colon. These points are summarized in observing the similarities in the appearance of fractions from the three different layers of the colon (Figures 10-22) which sediment at a particular gravitational force spin and the differences between the appearance of fractions which sediment at various centrifugal force spin. P_1 fractions (Figure 10) contain mostly mitochondria, while P_2 fractions (Figures 11-13) contain mostly synaptosomes while MIC 1 fractions (Figures 14 and 15) give rise to two plasma membrane fractions designated P_3 (Figures 16-18) and MIC 2 (Figures 19-21).

Figure 10. P_1 fraction of ICC-rich preparation enriched in mitochondria (m) with synaptosomes (N). Magnification = x15000; Scale 2cm=500nm.

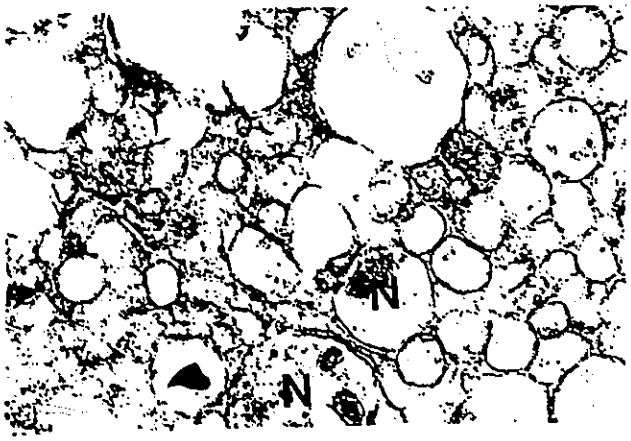

N
m
m

Figure 11 (Top). P_2 fraction of longitudinal muscle preparation showing synaptosomes (N) and some mitochondria (m). Magnification = x15000; Scale 2cm=500nm.

Figure 12 (Middle). P_2 fraction of ICC-poor circular muscle preparation containing some synaptosomes (N) and mitochondria (m). Magnification = x15000; Scale 2cm=500nm.

Figure 13 (Bottom). P_2 of ICC-rich preparation containing synaptosomes (N) and some mitochondria (m). Magnification = x15000; Scale 2cm=500nm.


N


N
N

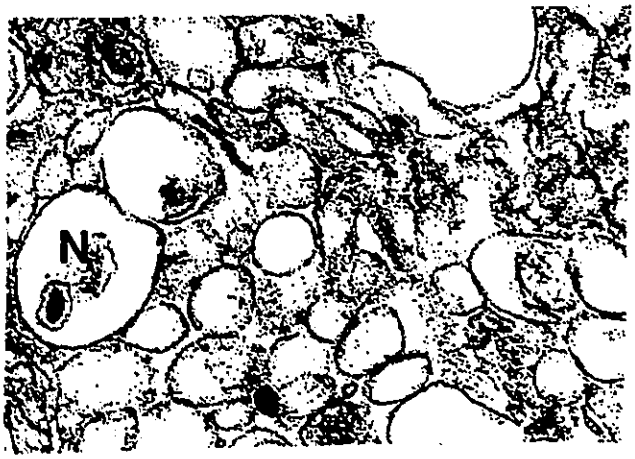
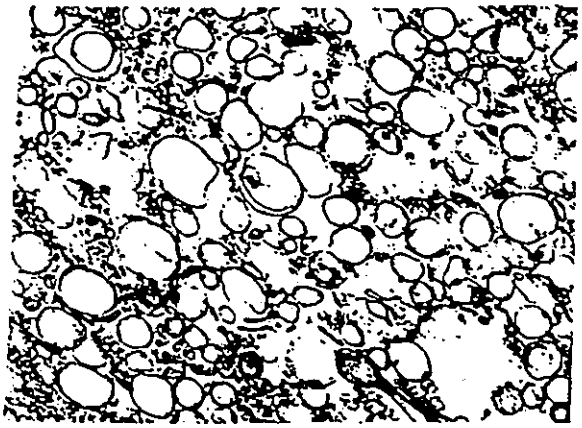

N

Figure 14 (Top). Mixed fraction (MIC 1) of ICC-poor circular muscle preparation. Magnification = x12000; Scale 2cm=500nm.

Figure 15 (Bottom). Mixed fraction (MIC 1) of ICC-rich preparation. Magnification = x15000; Scale 2cm=500nm.



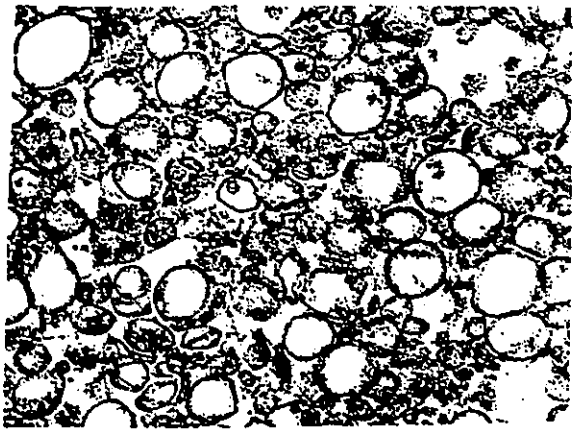
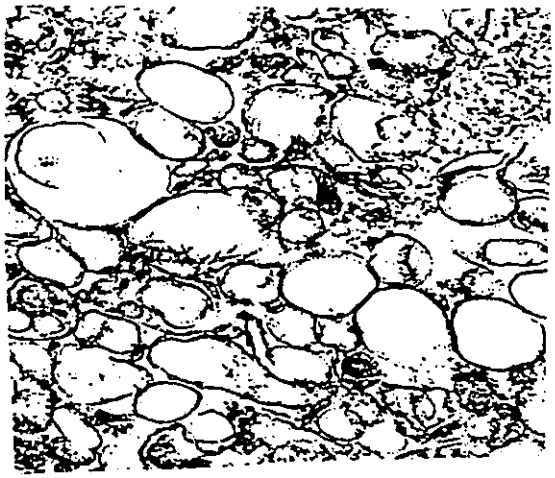
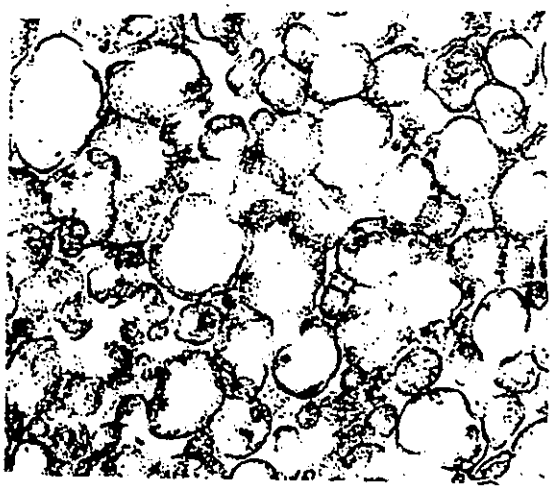


Figure 16 (Top). P_3 fraction of longitudinal muscle/myenteric plexus preparation. Magnification = x15000; Scale 2cm=500nm.

Figure 17 (Middle). P_3 fraction of circular muscle (ICC-poor) preparation. Magnification = x15000; Scale 2cm=500nm.

Figure 18 (Bottom). P_3 fraction of ICC-rich preparation. Magnification = x15000; Scale 2cm=500nm.





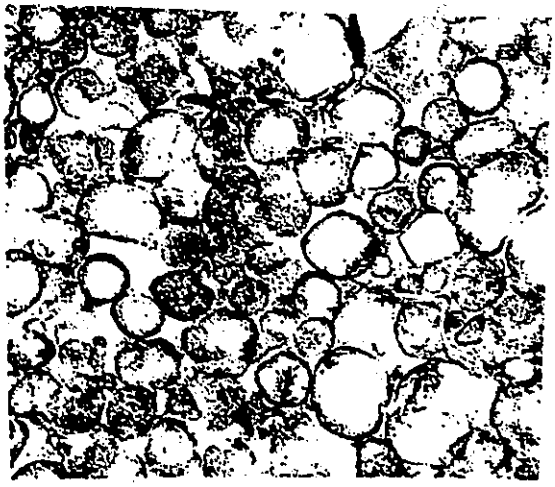
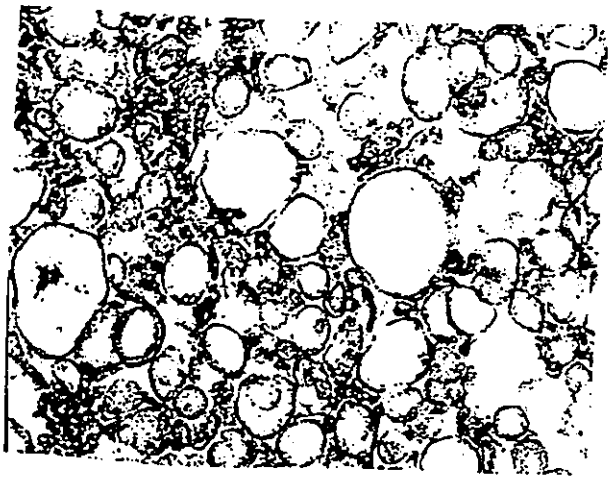
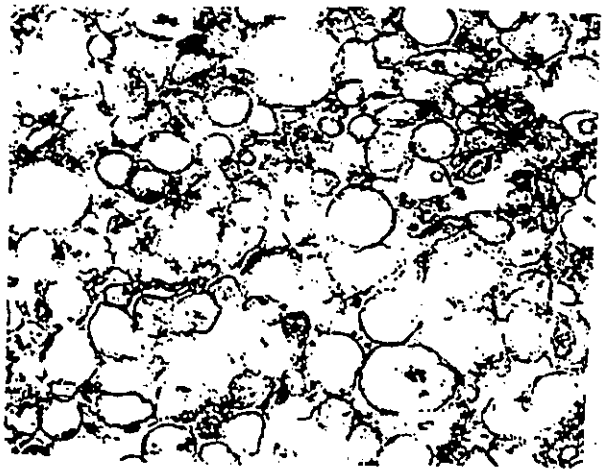


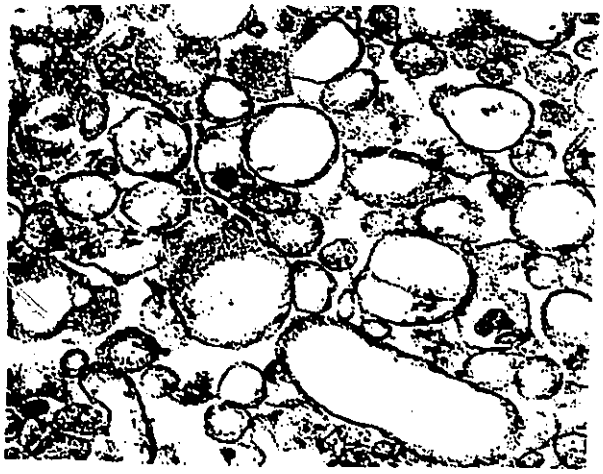
Figure 19 (Top). MIC 2 fraction of longitudinal muscle preparation. Magnification = x15000; Scale 2cm=500nm.

Figure 20 (Middle). MIC 2 fraction of ICC-poor circular muscle preparation. Magnification = x15000; Scale 2cm=500nm.

Figure 21 (Bottom). MIC 2 fraction of ICC-rich preparation. Magnification = x15000; Scale 2cm=500nm.







3.4.2.2 Total protein of ICC-rich and poor preparations

Tables 1 and 2 show total protein and recovery in relation to the starting material in the centrifugation fractions from methods 1 and 2 of ICC-rich and-poor preparations. In the ICC-rich preparation there were statistically significant differences between methods 1 and 2 in the total protein and percentage recovery in P2, P_3 and MIC 1 fractions. These values were significantly higher ($p< 0.05$) after use of method 1 compared to use of method 2. However, such differences were not seen in PNS, S_1, S_2, P_1 and MIC 2 fractions subjected to both methods. A similar trend was noted in the ICC-poor preparations together with a significantly higher ($p<0.001$) total protein in PNS of method 1 compared to that of method 2. Generally, it was found that recoveries of total protein were higher in P_2, P_3, MIC 1 and MIC 2 fractions of ICC-rich preparations irrespective of the method used.

Table 1 Comparison between total protein of methods 1 and 2 in ICC-rich preparations.

Fraction	Method 1	Method 2	t
PNS	87.00±10.50	91.50±8.25	0.6731
	(100)	(100)	
S_1	80.30±9.48	84.24±8.76	0.541
	(92.39)	(92.12)	
S_2	71.02±9.45	80.43±13.40	1.148
	(81.63)	(87.90)	
P_1	1.98±0.33	1.92±0.42	0.351
	(2.28)	(2.09)	
P_2	3.46±0.66	2.40±0.22	3.029**
	(3.69)	(2.60)	
P_3	1.67±0.45	0.96±0.33	2.629*
	(1.90)	(1.05)	
MIC 1	4.90±0.55	3.75±0.52	3.042**
	(5.63)	(3.75)	
MIC 2	1.51±0.21	1.54±0.14	0.2381
	(1.73)	(1.68)	

Values are given as mean ± SEM. n=4. total protein (mg). Percentages of total protein relative to the PNS in each fraction are given in parenthesis. *p<0.05; **p<0.025; +p<0.005; ++p<0.001

Table 2 Comparison between total protein of methods 1 and 2 in ICC-poor preparations

Fractions	Method 1	Method 2	t
PNS	316.80±12.10	260.70±16.50	5.484++
	(100)	(100)	
S_1	273.92±28.89	258.94±17.12	1.855
	(86.50)	(99.32)	
S_2	262.50±25.20	238.35±7.35	1.855
	(82.90)	(91.43)	
P_1	3.27±0.66	2.82±0.30	1.241
	(1.03)	(1.08)	
P_2	6.66±0.93	3.57±0.84	4.928+
	(2.10)	(1.36)	
P_3	2.90±0.54	0.48±0.02	8.963++
	(0.92)	(0.18)	
MIC 1	12.12±1.26	6.54±1.20	7.690++
	(3.83)	(2.51)	
MIC 2	5.02±0.28	4.16±0.49	1.286
	(1.58)	(1.80)	

Values are given as means±SEM. n=4; total protein (mg). Percentages of total protein relative to PNS in each fraction are given in parenthesis. *p<0.05; **p<0.025; +p<0.005; ++p<0.001

3.4.2.3 Location of synaptosomal and smooth muscle plasma membrane fraction

Tables 3 and 4 show smooth muscle plasma membrane markers - 5'nucleotidase and Mg^{2+}ATPase activities while table 5 shows synaptosomal marker - Saxitoxin binding in ICC-rich preparation. Corresponding markers for ICC-poor preparation are shown in Tables 6,7 and 8. In the ICC-rich preparation, it was clear that values obtained from method 1 were markedly greater in general than values from method 2. However, this was not the case in ICC-poor preparation in which the values obtained for only two fractions (PNS and P_3) from method 1 were significantly greater than values from method 2.

In the ICC-rich preparation which is not enriched in smooth muscle plasma membrane due to dissection, the highest activities and recoveries recorded for 5'nucleotidase and Mg^{2+}ATPase were in P_3 fraction followed by significantly high levels in P2 and MIC 2 fractions. However, Saxitoxin binding and recoveries were greatest in P_2 fraction followed by much lower (4-5 fold) levels in P3 and MIC 2 fractions. Therefore P_2 fraction is enriched in synaptosomes while P_3 and MIC 2 fractions from ICC-rich preparation cannot be easily characterized as smooth muscle plasma membrane fraction since there is a low smooth muscle content in the original preparation. However,these two fractions possess saxitoxin binding and recovery greater than found in usual smooth muscle plasma membrane. P_3,in particular, cannot be classified as smooth muscle

plasma membrane for these reasons in addition to the fact that it sediments differently as a pellet while smooth muscle plasma membrane is found in the supernatant at the same gravitational force spin. Based upon electron/micrographic information and additional biochemical evidence such as Saxitoxin binding, only the P_2 fraction can be confirmed as being enriched in synaptosomes while P_3 and MIC 2 fractions though having certain quantities of Saxitoxin binding (intact synaptosomes were not found in electron micrograph) cannot be classified strictly as smooth muscle plasma membrane since the enrichment and recoveries of their biochemical markers differ from previous results for such membrane enriched fraction in our laboratory.

The results obtained from ICC-poor fraction show that P_2 is the synaptosome enriched fraction as it contains high Saxitoxin binding and recoveries, although certain quantities of plasma membrane markers are present. This and electron micrographic evidence qualify P_2 fraction to be classified as a synaptosome enriched fraction. Furthermore, smooth muscle plasma membrane markers are enriched in the MIC 2 fraction while its Saxitoxin binding and recovery are extremely low. Micrographic evidence shows no intact synaptosomes in this fraction prepared from smooth muscle. Therefore MIC 2 fraction of ICC-poor preparation qualifies to be referred to as a smooth muscle plasma membrane enriched fraction. This is confirmed by previous results from our laboratory (Ahmad,

Allescher, Manaka et al, 1988) using the small intestine which shows that such fractions have similar biochemical markers as obtained in the present preparation as well as sedimenting at the same gravitational force spin.

However, it is noteworthy that the P_3 fraction, which for reasons stated above cannot be classified as a smooth muscle plasma membrane enriched fraction, shows lower activities and recoveries of plasma membrane markers in the ICC-poor preparation compared to ICC-rich preparation. Similar biochemical evidence is seen in P_3 and MIC 2 fractions from longitudinal muscle (Table 9 and Figures 11-21). In addition, electron micrographic studies and biochemical evidence (ie cytochrome c oxidase, see Table 10 and Figure 10) show that the P_1 fraction of ICC-rich preparation is enriched in mitochondria.

Table 3 Comparison between 5'nucleotidase activities of methods 1 and 2 in ICC-rich preparation.

Fractions	Method 1	Method 2	t
PNS	4.32±1.36	1.87±0.53	2.816*
	(100)	(100)	
S_1	4.06±1.40	1.97±0.33	2.944**
	(86.7)	(103.00)	
S_2	3.68±1.36	1.98±0.39	2.408*
	(69.54)	(94.60)	
P_1	4.83±1.57	2.63±0.67	2.579*
	(2.54)	(5.60)	
P_2	13.46±5.07	3.48±0.30	3.973+
	(11.50)	(7.44)	
P_3	18.04±9.52	4.12±0.86	2.912**
	(14.40)	(8.81)	
MIC 1	14.14±5.80	2.88±0.46	3.869+
	(18.43)	(10.27)	
MIC 2	13.20±5.80	2.39±0.81	3.690+
	(5.30)	(5.96)	

Values are given as mean±SEM. n=4; 5'nucleotidase umol Pi h^{-1}mg. Percentages of activity relative to PNS are given in parenthesis. *p<0.05; **p<0.025; ***p<0.02; +p<0.01; ++p<0.005; +++p<0.001.

Table 4 Comparison between Mg^{2+}ATPase activity of methods 1 and 2 in ICC-rich preparation

Fraction	Method 1	Method 2	t
PNS	20.14±2.15	16.78±2.30	2.140
	(100)	(100)	
S_1	20.21±1.75	13.64±2.93	2.925**
	(97.67)	(79.00)	
S_2	15.38±2.61	10.54±1.70	3.123***
	(68.22)	(56.11)	
P_1	58.87±5.20	38.56±6.98	3.749+
	(10.90)	(9.19)	
P_2	119.71±22.13	85.56±9.03	2.858**
	(23.74)	(20.40)	
P_3	158.82±26.61	103.93±18.72	3.373***
	(31.54)	(24.55)	
MIC 1	91.01±11.75	59.99±9.85	4.050++
	(30.13)	(23.83)	
MIC 2	95.26±12.05	52.82±9.33	5.570+++
	(22.07)	(14.69)	

Values are given as mean±SEM. n=4; Mg^{2+}ATPase activity μmol Pi h^{-1} mg protein. Percentages of activity relative to PNS are given in parenthesis. *p<0.05; **p<0.025; ***p<0.02; +p<0.01; ++p<0.005;+++p<0.001

Table 5 Comparison between ^{3}H Saxitoxin binding of methods 1 and 2 in ICC-rich preparation

Fraction	Method 1	Method 2	t
PNS	71.29±4.87	28.45±7.03	10.020+++
	(100)	(100)	
S_1	67.72±9.51	39.58±13.43	3.423***
	(92.46)	(101.70)	
S_2	18.57±0.80	9.26±1.86	9.904+++
	(23.14)	(12.95)	
P_1	412.46±18.55	92.10±5.42	33.160+++
	(23.14)	(12.95)	
P_2	690.65±92.35	308.41±31.21	7.842+++
	(38.75)	(43.36)	
P_3	179.29±12.80	49.00±17.08	12.211+++
	(10.06)	(6.89)	
MIC 1	151.16±35.33	70.97±16.11	4.129+
	(14.14)	(16.63)	
MIC 2	127.94±15.94	48.44±21.57	5.018+
	(8.37)	(7.95)	

Values are given as mean±SEM. n=4; ^{3}H Saxitoxin binding(fmol/mg protein).Percentage of binding relative to PNS is given in parenthesis. *p<0.05; **p<0.025; ***p<0.02; +p<0.01; ++p<0.005; +++p<0.001.

Table 6 Comparison between 5'nucleotidase activity of methods 1 and 2 in ICC-poor circular muscle preparation

Fractions	Method 1	Method 2	t
PNS	3.51±0.75	2.92±0.24	1.513
	(100)	(100)	
S_1	3.60±0.85	2.40±0.67	2.222
	(88.68)	(81.88)	
S_2	2.89±0.80	2.13±0.76	1.382
	(61.94)	(66.87)	
P_1	4.15±1.19	4.49±0.60	0.513
	(1.22)	(1.67)	
P_2	8.02±2.73	7.09±2.41	0.841
	(2.31)	(0.52)	
P_3	8.87±2.70	8.16±1.99	0.423
	(2.31)	(0.52)	
MIC 1	7.39±2.06	8.19±1.84	0.597
	(8.10)	(7.06)	
MIC 2	9.06±2.40	8.46±0.14	0.510
	(4.97)	(3.52)	

Values are given as mean±SEM. n=4; 5'nucleotidase(μmol Pi h^{-1}mg protein. Percentages of activity are shown in parenthesis.

Table 7 Comparison between Mg^{2+}ATPase activity of methods 1 and 2 in ICC-poor circular muscle preparation

Fractions	Method 1	Method 2	t
PNS	10.01±1.11	18.28±4.93	3.269***
	(100)	(100)	
S_1	10.04±0.71	14.67±5.79	1.597
	(97.56)	(78.63)	
S_2	8.52±0.72	9.45±8.71	0.593
	(81.25)	(49.35)	
P_1	35.19±7.26	41.52±8.70	1.117
	(9.59)	(6.19)	
P_2	63.03±28.71	81.93±18.98	1.098
	(17.17)	(12.22)	
P_3	59.95±9.05	99.40±25.36	2.931**
	(10.89)	(9.89)	
MIC 1	46.39±19.53	52.54±20.34	0.557
	(25.28)	(15.68)	
MIC 2	49.39±17.56	64.13±20.05	1.107
	(17.94)	(12.76)	

Values are given as mean±SEM. n=4; Mg^{2+}ATPase (μmol Pi $h^{-1}mg$)
Percentages of activity are shown in parenthesis.
**p<0.025; ***p<0.02.

Table 8 Comparison between ^{3}H Saxitoxin binding of methods 1 and 2 in ICC-poor circular muscle preparation

Fractions	Method 1	Method 2	t
PNS	22.89±3.42	23.83±4.93	0.596
	(100)	(100)	
S_1	18.48±2.85	18.64±4.43	0.053
	(82.31)	(77.60)	
S_2	11.89±3.88	10.99±3.98	0.281
	(50.64)	(42.16)	
P_1	158.95±15.74	123.10±45.66	1.287
	(10.27)	(10.13)	
P_2	461.50±44.42	419.24±61.28	0.968
	(30.19)	(24.09)	
P_3	62.74±4.81	55.16±11.35	1.066
	(3.46)	(0.48)	
MIC 1	29.21±7.45	26.03±5.04	0.614
	(8.81)	(2.70)	
MIC 2	9.46±1.89	10.06±3.41	0.267
	(0.67)	(0.52)	

Values are given as mean±SEM. n=4; ^{3}H Saxitoxin (fmol/mg protein) Percentage of binding relative to PNS are shown in parenthesis.

Table 9 Mg^{2+}ATPase and ^3H Saxitoxin binding in longitudinal muscle/myenteric plexus preparation.

Fractions	Mg^{2+}ATPase	Saxitoxin
PNS	24.27±2.49	64.14±10.92
S_1	23.10±3.77	45.55±28.82
S_2	15.93±0.37	26.67±6.87
P_1	71.46±4.97	289.76±30.23
P_2	117.09±5.47	832.99±201.01
P_3	120.33±9.21	74.41±6.36
MIC 1	98.49±9.31	44.11±11.82
MIC 2	100.98±7.79	37.64±28.18

Values are given as mean±SEM. n=3; Mg^{2+}ATPase (μmol Pi $h^{-1}mg$) ^3H Saxitoxin binding (fmol/mg protein).

Table 10. Cytochrome c oxidase activity in fractions from ICC-rich-preparation

Fractions	Cytochrome c oxidase
PNS	2.072±0.647
S_1	1.213±0.407
S_2	1.660±0.373
P_1	20.33±1.820
P_2	8.617±0.813
P_3	5.973±0.805
MIC 1	6.450±0.713
MIC 2	nd

Values are given as mean±SEM. d=not detectable. n=3.

3.4.2.4 Possible location of ICC-membrane enriched fraction

Evidence from morphological studies and data in Tables 3-8 leads to the hypothesis that ICC-membrane enriched fraction may be in MIC 1, P_3 and MIC 2 fractions obtained from differential centrifugation of the ICC-rich tissues. The morphological evidence that ICC membrane is damaged and ICC number reduced in method 2 appears to correspond to biochemical data showing that total protein and its recoveries in MIC 1 and P_3 are significantly reduced in method 2 compared to method 1. However, morphological evidence show that the smooth muscle cells were unaffected by the method of dissection used and this is reflected by biochemical data of the MIC 2 fraction, believed to contain smooth muscle plasma membrane, which remained unchanged in both ICC-rich and poor preparation.

The total protein of the synaptosome-enriched fraction, P_2 of ICC-rich preparation, was greater than those of P_3 and MIC 2 but like P_3 and MIC 1 this parameter was reduced when method 2 was used.

P_3, MIC 1 and 2 fractions of the ICC-rich preparation had the highest 5'nucleotidase activities. Their Mg^{2+}ATPase activities were also higher than in P_2 fractions when method 1 was used and like their total protein values these parameters were reduced in method 2 (Table 4). Compared to the ICC-rich preparation, the recoveries for 5'nucleotidase and Mg^{2+}ATPase were lower in P_3 fractions of the ICC-poor preparation while those of MIC 2 were relatively greater

suggesting that in this preparation MIC 2 received a higher proportion of smooth muscle content and lesser ICC membranes (Tables 6 and 7) than P_3.

In both ICC-rich and poor preparations, P_3 fraction had higher Saxitoxin binding and recoveries compared to MIC 2 fraction which, as in intestinal muscle preparations, has been previously known to have low Saxitoxin binding because it is a smooth muscle plasma membrane. Although, P_3 and MIC 2 were derived from one fraction (MIC 1), Saxitoxin binding in MIC 2 of ICC-rich origin was much higher than in MIC 2 of ICC-poor origin. Also Saxitoxin binding and recoveries in P_3 fraction of ICC-rich preparation were greater than those of ICC-poor preparation (Tables 5 and 8). In both preparations Saxitoxin binding and recoveries were higher in method 1 than in method 2. The cellular origins of P_2 was further tested by changing the centrifugation scheme by spinning S_1 at lower speed (24,000 x g x 10'instead of 48,000g). Tables 11 and 12 show that Saxitoxin binding migrated from P_2 to P_3 suggesting that the original centrifugation speed for S_1 fraction was better in differentiating P_2 from P_3 fractions of both ICC-rich and poor preparations. Further differentiation between P_2 and P_3 fractions was sought using two approaches : 1) determination of VIP immunoreactive content (Table 13) and comparison between Saxitoxin and ω-Conotoxin GVIA binding (Tables 14 and 15) in the various fractions of ICC-rich and poor preparations.

Results from these studies confirm that VIP immunoreactive content, Saxitoxin binding and ω-Conotoxin binding and recoveries were highest in the P_2 fraction suggesting that it contains chiefly synaptosomes but in P_3 fraction of ICC-rich preparation, ω-Conotoxin binding and recovery were nearly as high as that of P_2 fraction while Saxitoxin binding was lower than in P_2. This discrepancy suggests that N-type calcium channel usually found in nerves may reside on ICC membrane. However, low VIP- immunoreactive content in P_3 fraction suggest that few intact synaptosomes were present in it.

Furthermore, study of VIP binding (Table 16) showed that the highest binding density was in P_3 fraction from both ICC-rich and poor preparations while levels in MIC 2 were lower. The recovery of binding in P_3 of ICC-poor preparation was very low. Surprisingly, the P_3 fraction of longitudinal muscle preparation had very low VIP binding while it was highest in smooth muscle plasma membrane enriched fractions. Since it has been shown that there are more VIP nerves in the submucosal region of the colon having high density of ICC than in myenteric plexus, these observations together with the hypothesis that ICC have VIP receptors suggest like other data that ICC membranes sediment preferentially in P_3. There was also some VIP binding in P_2 fraction of all the preparations and the significance of this is explored in subsequent sections.

Although it was difficult to subfractionate MIC 1 or P_3

of ICC-rich preparation due to their low total protein contents for purposes of further purification, it was possible to subfractionate MIC 1 fraction of ICC-poor preparations to obtain a highly enriched smooth muscle plasma membrane fraction. This purification was confirmed by a higher density of VIP receptor binding sites on the M_2 fraction which contain biochemical markers that are typical of smooth muscle plasma membrane (Tables 17 and 18).

The similarity in micrographic appearance of MIC 2 fraction of ICC-poor preparation enriched in smooth muscle plasma membrane and the P_3 fraction of ICC-rich preparation suggest that both fractions may contain plasma membranes but the differences in biochemical markers and sedimentation clearly indicate that they are plasma membrane of different origins.

These data are consistent with the hypothesis that ICC membrane is enriched in the P_3 fraction of ICC-rich preparation as well as suggesting the presence of sodium and N-type calcium channels which are considered to be markers of nerve membrane. However, no clear evidence can be presented to exclude that these ion channels are derived from nerve membranes which may be closely associated with ICC. In addition, a high density of VIP receptor sites can be found in this fraction.

Table 11 Revised centrifugation scheme showing total protein, 5'nucleotidase activity and ^{3}H Saxitoxin binding in ICC -rich preparation.

Fractions	Total protein	5'nucleotidase	Saxitoxin
PNS	1.89±0.18	2.89±0.21	68.44±10.66
S_1	1.87±0.14	2.89±0.10	41.70±12.66
S2	1.88±0.24	3.20±0.34	29.51±7.73
P_1	1.33±0.13	3.38±1.01	435.73±108.47
P2	0.95±0.18	4.05±1.51	492.35±164.88
P3	1.94±0.50	6.15±1.66	492.13±93.41
MIC 1	1.96±0.20	6.13±1.46	260.53±53.29
MIC 2	0.98±0.17	8.33±0.77	86.48±16.14

Values are given as mean±SEM. n=3; Total protein (mg); 5'nucleotidase (μmol Pi h^{-1}mg); ^{3}H Saxitoxin (fmol/mg protein)

Table 12 Total protein, 5'nucleotidase and ^{3}H-Saxitoxin binding in ICC-rich preparation from revised centrifugation scheme.

Fractions	Total protein	5'nucleotidase	Saxitoxin
PNS	2.70±0.39	1.26±0.12	34.58±5.70
S_1	2.72±0.35	1.87±0.02	27.72±4.77
S_2	2.82±0.41	1.37±0.29	7.03±1.02
P_1	1.40±0.21	2.01±0.34	325.95±114.80
P_2	1.24±0.13	1.77±0.55	342.16±130.02
P_3	1.36±0.37	1.67±0.64	156.73±70.76
MIC 1	1.40±0.29	1.90±0.84	99.43±40.34
MIC 2	0.67±0.06	2.83±0.86	35.18±10.84

Values are given as mean±SEM. n=3; total protein (mg); 5'nucleotidase (μmol Pi h^{-1}mg); ^{3}H Saxitoxin (fmol/mg protein). These studies were done with fractions from the original centrifugation scheme.

Table 13 VIP immunoreactive content in colonic preparations

Fractions	LMP (n=4)	ICC-poor (n=3)	ICC-rich (n=3)
PNS	0.08±0.03	0.12±0.04	0.21±0.07
S_1	0.09±0.03	0.13±0.05	0.20±0.10
S_2	0.07±0.02	0.10± 0.03	0.21±0.12
P_1	0.33±0.06	0.74±0.37	0.55±0.20
P_2	0.45±0.11	0.93± 0.26	0.67±0.24
P_3	0.22±0.09	0.28±0.03	0.26±0.07
MIC 1	0.13±0.03	0.16±0.06	0.13±0.05
MIC 2	0.15±0.06	0.10±0.03	0.10±0.04

Values are given as mean±SEM. VIP immunoreactive content (pmol/mg protein). LMP=Longitudinal muscle/myenteric plexus preparation.

Table 14 ^{3}H-Saxitoxin and ^{125}I-ω-Conotoxin binding (fmol/mg) in ICC-rich preparations.

Fractions	Saxitoxin	Conotoxin	CTX/STX
PNS	42.1±9.1	20.9±2.6	0.50
	(100)	(100)	
S_1	55.2±12.4	24.5±3.3	0.44
	(116)	(104)	
S_2	7.6±2.1	12.7±2.1	1.67
	(15)	(54)	
P_1	323.8±108.5	38.7±4.2	0.12
	(7)	(2)	
P_2	788.9±157.9	60.3±23.2	0.19
	(48)	(8)	
P_3	301.5±93.9	114.9±23.2	0.38
	(7)	(6)	
MIC 1	191.2±54.9	29.4±6.6	0.15
	(25)	(8)	
MIC 2	79.7±18.7	28.6±5.4	0.36
	(4)	(3)	

Values are given as mean±SEM. n=3; Percentages of binding relative to PNS are shown in parenthesis. STX=Saxitoxin; CTX=Conotoxin.

Table 15 ^{3}H-Saxitoxin and ^{125}I-ω-Conotoxin binding (fmol/mg protein in ICC-poor preparations.

Fractions	Saxitoxin	Conotoxin	CTX/STX
PNS	35.2±6.4	26.1±3.3	0.74
	(100)	(100)	
S_1	23.5±2.2	23.6±2.6	1.00
	(66)	(86)	
S_2	8.1±3.9	22.3±3.8	2.75
	(28)	(74)	
P_1	279.8±27.6	24.3±8.9	0.09
	(9)	(1)	
P_2	384.4±27.6	24.4±8.9	0.06
	(25)	(5)	
P_3	61.3±30.2	32.9±16.0	0.54
	(1)	(1)	
MIC 1	37.9±15.9	15.7±4.3	0.41
	(6)	(3)	
MIC 2	23.7±5.6	19.3±12.9	0.81
	(1)	(2)	

Values are given as mean±SEM. n=3; Percentages of binding relative to PNS are shown in parenthesis. STX=Saxitoxin; CTX=Conotoxin.

Table 16 ^{125}I-VIP binding (fmol/mg) in longitudinal muscle/myenteric plexus (LM), ICC-poor circular muscle (CM) and ICC-rich (IC-R) submucosal preparations.

Fractions	LM	CM	IC-R
PNS	18.60±2.70	13.70±3.00	7.80±1.00
	(100)	(100)	(100)
S_1	14.00±6.30	7.50±0.40	4.70±1.00
	(46.70)	(91.00)	(76.20)
S_2	8.80±1.20	7.40±0.30	5.50±1.00
	(34.00)	(71.80)	(49.20)
P_1	10.40±1.40	31.50±5.00	20.30±5.90
	(0.80)	(0.10)	(11.00)
P_2	24.00±4.00	40.40±6.50	26.30±7.30
	(6.70)	(4.20)	(11.20)
P_3	14.50±7.40	79.50±15.50	78.90±6.20
	(0.1)	(1.7)	(15.70)
MIC 1	38.20±6.00	43.50±6.30	71.30±6.40
	(9.10)	(6.20)	(32.00)
MIC 2	45.30±5.50	31.20±3.60	19.10±7.10
	(8.20)	(2.80)	(8.30)

Values are given as mean±SEM. n=4; Percentages of binding relative to PNS are shown in parenthesis.

Table 17 Mg^{2+}ATPase (μmol Pi h^{-1}mg) and ^{3}H-Saxitoxin binding from profile of MIC 1 fraction of ICC-poor circular muscle preparation.

Fraction	Mg^{2+}ATPase	^{3}H-Saxitoxin (fmol/mg)
M_1	60.13±13.48	27.51±1.73
M_2	232.01±23.68	72.54±5.57
M_3	167.09±11.92	134.60±28.82
M_4	61.18±18.60	31.09±10.66

Values are given as mean±SEM. n=3.

Table 18 ^{125}I-VIP binding (fmol/mg) of fractions from gradient of MIC 1 fraction of ICC-poor circular muscle preparation.

Fraction	^{125}I-VIP binding
M_1	30.94±11.74
	(8.30)
M_2	73.24±11.56
	(40.99)
M_3	18.85±7.43
	(11.86)
M_4	36.76±4.00
	(0.12)
MIC 1	37.73±9.09
	(100)

Values are given as mean±SEM. Percentages of binding of VIP are given in parenthesis.

3.5 DISCUSSION

3.5.1. Isolation of synaptosome, smooth muscle and ICC membrane.

The isolation of membranes from synaptosomes, ICC and smooth muscle has been achieved by following the principles of tissue fractionation utilizing differential and sucrose density centrifugation techniques in addition to using enzyme markers such as 5'nucleotidase and Mg^{2+}ATPase characteristic of smooth muscle plasma membrane. Markers for synaptosomes include Saxitoxin, VIP immunoreactive content and ω-Conotoxin binding. The presence of N-type calcium channel in ICC membrane enriched fraction demands some explanation because previous localizations of this channel were restricted to synaptosomes from nerves. However, N-type calcium channel have been shown to exist in non-neural cells such as lung carcinoma and PC12 cell lines (Artalejo, Perlman and Fox, 1992). It has not been found in muscle or muscle cell lines.

At the onset of this investigation, there were no markers for ICC membrane but based upon the premise that ICC are hyperpolarized by exogenously applied VIP and innervated by VIP containing nerves on the submucosal border of canine colon (Berezin, Huizinga, Daniel, 1988) and this innervation of ICC is

less prominent on the myenteric border of colonic circular muscle (Berezin, Huizinga, Farraway, Daniel; 1990), VIP receptor binding was chosen to be a marker for ICC. This decision was made in anticipation that results from fractionation would show differences in marker distribution in ICC-rich and-poor preparations and in longitudinal muscle myenteric plexus preparations.

An attempt has been made to localize fractions enriched in synaptosomes, ICC, smooth muscle plasma membrane and mitochondria. Following the basic tenets of biochemical fractionation laid down by previous work on diverse tissue types (De Duve and Berthet, 1954; De Duve, 1964; Peters, 1976; Matlib, Crankshaw, Garfield et al, 1979; Kwan, Lee, Daniel, 1981; Kwan, Triggle, Grover et al, 1983; Daniel, 1985; Ahmad, Allescher, Manaka et al, 1988; Kinne-Saffran, Kinne; 1989), various crude fractions were isolated from colonic ICC-rich and-poor and longitudinal muscle\myenteric plexus preparations. These fractions were characterized using criteria such as specific activity of marker enzymes, enrichment factors and recoveries of markers.

The present study shows that the P_1 fraction of the colonic tissue preparations contained mostly mitochondria with some synaptosomes, while P_2 of these preparations contained chiefly synaptosomes but P_3 and MIC 2 consisted of a putative ICC membrane fraction and a smooth muscle plasma membrane fraction respectively.

Higher activities and recoveries of synaptosomal markers for sodium and N-type calcium channels found in ICC-membrane rich fraction of ICC-rich preparation compared to ICC-poor preparation suggest that ICC membrane also settled in MIC 2 fraction of ICC-rich preparation. Although this could be due to contaminations by nerves, it seems unlikely because intact synaptosomes sediment first before sedimentation of smooth muscle plasma membrane and ICC membrane at the later stages using higher force of centrifugation. Furthermore, intact synaptosomes were not found in MIC 2 fraction of ICC-rich preparation and it is not possible to differentiate between membranes of ICC, nerves and smooth muscle in electronmicrographs so no categorical statement can be made about association of nerve membranes with other membranes, although biochemical data suggest so.

At this juncture it is worth stating that activity of plasma membrane and synaptosomal markers in the colon is much less than those found in the small intestine (Ahmad, Allescher, Manaka et al, 1988), although biochemical data from the present study fit the marker profile for synaptosomes and smooth muscle plasma membrane as found in previous studies using canine ileum (Ahmad, Allescher, Manaka et al, 1988; Mao, Barnett, Coy et al, 1991).

However, this study has shown that longitudinal muscle preparation also has enrichment of synaptosomal and plasma membrane markers in respective P_2 and MIC 2 fractions. This preparation is shown to have a putative ICC-rich fraction with similar levels of markers as those of an identical fraction from the submucosal ICC-rich preparation. However, the ICC membrane-rich fraction from longitudinal muscle preparation has low VIP binding. If this is an an ICC-membrane rich fraction these data suggest that ICC of myenteric plexus have fewer VIP receptors than those at the submucous plexus.

3.5.2 VIP binding distribution and its relation to colonic function

VIP binding has been used in these studies for reasons stated already in the preceding section. However, its distribution in the present investigation is interesting.

Although VIP binding is high in synaptosome-enriched fractions of both ICC-rich and-poor preparations and longitudinal muscle\myenteric plexus preparation, significant binding of VIP to the putative ICC membrane fraction from ICC-rich preparation and to purified smooth muscle plasma membrane fractions of circular muscle preparation and an identical fraction from longitudinal muscle \ myenteric plexus preparation were observed (Table 16).

However, there is very little VIP binding on the membrane fraction derived from longitudinal muscle which corresponds to the putative ICC membrane enriched fraction. This suggests that there are differences in VIP receptor density between the myenteric plexus ICC and ICC on the submucosal border of canine colon. Differences in innervation could account for low VIP receptor density in ICC membrane rich fraction from longitudinal muscle preparation and high VIP receptor density in a similar fraction from submucosal ICC-rich preparation since submucosal ICC receive a higher density of VIP containing nerves than ICC of myenteric plexus (Berezin, Huizinga, Farraway, Daniel, 1990). In support of differences in function between longitudinal muscle ICC and submucosal ICC, a recent morphological study has shown that ICC network in the myenteric plexus of canine colon differs from that on the submucosal border (Berezin, Huizinga, Daniel, 1990). The high density of VIP receptor sites on ICC membrane from the submucosal border of circular muscle suggest that VIP may have

functional relations to ICC. Another binding study has also shown the presence of VIP receptors on ICC of colon (Starich, Mihaylova-Todorova, Mansfield, Lardinois, 1992). The functional significance of VIP receptors on ICC membrane has been demonstrated by certain studies. Recent work from our laboratory clearly show that VIP hyperpolarized ICC of submucosal border of colon by 9 mV when it is electrically isolated by 1mM heptanol (Berezin, Huizinga, Farraway, Daniel, 1990). Furthermore, recent studies from another laboratory show that 10^{-7}M VIP caused a transient delay in spontaneous calcium oscillations or slow waves in both freshly dispersed and cultured ICC (Publicover, Horowitz, Sanders, 1992).

The presence of VIP receptors on circular muscle plasma membrane is supported by a recent VIP binding study (Starich, Mihaylova-Todorova, Mansfield, Lardinois, 1992) but it is slightly at variance with another study which reports that VIP receptors cannot be found on freshly isolated colonic circular muscle cells due to damage caused by isolation procedure although they can be found in cultured cells (Ennes, McRoberts, Heyman, Snape Jr, 1992). In the present studies, VIP receptors have been found on freshly isolated membranes. This means that inhibitory control of colonic circular muscle is either through ICC or directly through VIP receptors of smooth muscle. Evidence for this assumption is given by studies showing that electrical stimulation of inhibitory nerves in the presence of atropine diminished the amplitude and duration of slow waves when the resting membrane potential is -75 mV or greater

which suggest that VIP from nerves cause inhibition of muscle directly or by acting on ICC to reduce slow waves (Berezin, Huizinga, Farraway, Daniel; 1990; Huizinga et al, 1991). In addition, the finding that in ICC-poor circular muscle preparation large amplitude inhibitory junction potentials can be recorded in the event of neural stimulation suggest that circular muscle receives direct inhibitory innervation (Huizinga, Berezin, Daniel, Chow, 1990). VIP has also been shown to relax human colonic circular muscle layer (Burleigh,1990). The correlation between biochemical findings in the present studies and functional studies suggest dual control of colonic circular muscle by VIP. One way of exercising this control is the binding of VIP to its receptors on circular muscle and the other means is to bind to its receptors on ICC membrane to release an inhibitory mediator which will cause inhibition of colonic circular muscle contractile activity. A recent study suggested that VIP causes release of NO from muscle to cause relaxation (Grider et al, 1992).

Furthermore, the present study shows that VIP receptor sites are found in longitudinal muscle membrane. This finding is supported by previously cited biochemical study showing similar results in canine colon (Starich, Mihaylova-Todorova, Mansfield, Lardinois, 1992). The fact that VIP receptors are on longitudinal muscle cell membrane suggest that VIP can directly inhibit this smooth muscle activity. Evidence for such physiological function in the canine colon is provided by a recent investigation from our laboratory showing that inhibitory neural stimulation in the presence of

atropine caused abolishment of spike action potentials in longitudinal muscle (Huizinga, Berezin, Daniel, Chow, 1990). However, other studies show that in guinea-pig colon (Bennett, Bloom, Ch'Ng et al, 1984) and human colon (Burleigh, 1990) VIP failed to relax longitudinal smooth muscle. Perhaps these differences in VIP action may be due to species differences.

3.5.3 CONCLUSION

The present study has been designed to fill the gap in previous studies which utilized autoradiography (Zimmermann, Gates, Mantyh et al, 1988; Korman, Sayadi, Bass et al, 1989) to study inhibitory control of colon function. These earlier studies did not actually localize VIP receptors on membranes of diverse cell types of the colon. In fact no mention of ICC was made in these studies. This significant omission severely limits interpretation from such investigations since the inhibitory control of colonic circular muscle involves ICC.

Biochemical fractionation has been utilized in the present study to confirm the hypothesis put forward that ICC membrane has distinct biochemical properties and contain VIP receptors. This study also suggest that VIP can act directly on receptors in longitudinal muscle and in circular muscle layers of canine colon. In addition, this study suggests that indirect inhibition of colonic circular muscle layer by VIP can occur through its binding to ICC membrane to cause release of an inhibitory mediator which is possibly nitric oxide. This possibility will be explored in the next section.

CHAPTER FOUR

NITRIC OXIDE PRODUCTION IN ICC-RICH LAYER OF CANINE COLON.

4.1.0 Nitric oxide (NO)-Discovery:

NO is a gaseous material which has been demonstrated to be of utmost importance in biological systems during the last decade. The significance of NO presence in various systems of the body cannot be overestimated. Many accounts about NO can be found in several reviews (Culotta and Koshland, 1992; Nathan, 1992; Ignarro, 1989; Ignarro, 1990) .

The discovery of the role of the physiological role of NO in the body evolved from early observations in the vascular system that a factor produced by the endothelium in response to acetylcholine (known as the endothelium-derived relaxing factor (or EDRF) is responsible for relaxation of arterial smooth muscle. This relaxation was found to be absent in strips of blood vessels without endothelium when challenged with acetylcholine (Furchgott and Zawadski, 1980).

Further studies showed that EDRF produced similar biological activity as NO and both had similar stability enhanced by superoxide dismutase, similar susceptibility to inhibition by hemoglobin and both produced increases in cyclic guanosine monophosphate (cGMP). Based upon these studies, it was suggested that NO and EDRF may be one and the same substance (Palmer, Ferridge and Moncada, 1987; Ignarro, Buga, Wood et al, 1987).

The argument that NO is responsible for the action of EDRF was not immediately accepted since experimental results from

various laboratories were not conclusive. Some workers argued that the vasorelaxant properties of EDRF more closely resemble that of S-nitrocysteine than of NO (Myers, Minor Jr, Guerra Jr et al, 1990) while others postulated that although NO may be involved in vasorelaxation, the formation of active, unstable and intermediate endogenous nitrosothiols may be the principal relaxing factors (Kowaluk and Fung, 1990). However, the general concensus at the moment is that NO is the principal factor mediating vasorelaxation.

4.1.1 Pathway for NO production:

Various studies show that NO is synthesized from an enzyme catalyzed oxidation of a terminal guanidine nitrogen of L-arginine (Palmer, Ashton, Moncada, 1988; Marletta, Yoon, Iyengar et al, 1988). NO biosynthesis seems to be a two stage process in which intermediate compounds such as N-hydroxy-L-arginine are formed by the enzyme NO synthase (EC 1.14.13.39) in the presence of molecular oxygen and NADPH. This intermediate product is then converted (by NO synthase and NADPH) to citrulline with NO being released (Fukuto, Wallace, Hzieh and Chaudhuri, 1992). Experimental evidence suggests that the conversion of L-arginine to L-citrulline is stoichiometric (Marletta, Yoon, Iyengar et al, 1988).

Furthermore, results from studies of liver microsomes suggest that cytochrome P450 may also be a catalyst for synthesis of NO

from L-arginine (Boucher, Genet, Vandon et al, 1992). However, other molecular studies showed that NO synthase (NOS) has significant amino acid sequence homology to NADPH-cytochrome P-450 reductase . This shows that neuronal NOS is clearly different but related to cytochrome P-450 reductase (Bredt, Hwang, Glatt et al, 1991). Some investigations suggest that there may be a cytochrome P-450 hemeprotein in macrophages (White and Marletta, 1992). These findings cast light upon the chemical nature of NOS. Certain reports showed that neuronal NADPH diaphorase is either a NOS (Hope, Michael, Knigge and Vincent, 1991) or is colocalized with NOS in the same neurons (Dawson, Bredt, Fotuhi et al, 1991; Bredt, Glatt, Hwang et al, 1991). Recent evidence suggest that NADPH diaphorase activity is mediated by the same enzyme NOS. However, the diverse biochemical findings from these studies suggest that there might be distinct types of NOS.

4.1.2 Types of NOS-biochemical characteristics and localization.

Various studies have shown conclusively that there are two known distinct types of NOS. One of the two NOS is a constitutive calcium/calmodulin-dependent enzyme which is involved in the catalysis of spontaneous generation of NO while the other is inducible (with cytokines and bacterial lipopolysaccharides) and is known to be calcium-independent (Leone, Palmer, Knowles et al, 1991). The latter enzyme appears to have a slower rate of NO

synthesis than the former (Xie, Cho, Calaycay et al, 1992).

Constitutive NOS has been localized using various techniques in the vascular endothelium (Palmer, Ferridge, Moncada, 1988; Palmer and Moncada, 1989; Mulsch, Bassenge, Busse, 1989; Mayer, Schmidt, Humbert, Bohme, 1989), platelets (Radomski, Palmer, Moncada, 1990), adrenal gland (Palacios, Knowles, Palmer and Moncada, 1989) and brain (Knowles, Palacios, Palmer, Moncada, 1989; Bredt and Snyder, 1990; Schmidt, Pollock, Nakane et al, 1991). Investigations have demonstrated the presence of inducible NOS in macrophages (Hibbs Jr, Taintor, Vavrin, 1987; Marletta, Yoon, Iyengar et al, 1988; Tayeh and Marletta, 1989; Stuehr, Kwon, Nathan, 1990), neutrophils (Knowles, Palacios, Palmer, Moncada,1989; McCall, Boughton-Smith, Palmer, Whittle, Moncada, 1989; McCall, Feelisch, Palmer, Moncada, 1991); liver cells (Billiar, Curran, Stuehr et al, 1990; Knowles, Merrett, Salter and Moncada, 1990) and vascular smooth muscle (Knowles, Salter, Brooks, Moncada, 1990; Rees, Cellek, Palmer and Moncada, 1990 ; Busse and Mulsch, 1990). Furthermore both types of NOS have been found in diaphragm, heart, spleen, thymus and in various sections of the gastrointestinal tract (Bredt, Hwang and Snyder, 1990; Salter, Knowles and Moncada, 1991). However, it has been shown (using antiserum to NOS) that in the small intestine NOS is present in the myenteric and deep muscular plexuses (Bredt, Hwang and Snyder, 1990) which is known to have ICC. NADPH-diaphorase studies also suggest that ICC may have NOS (Daniel and Berezin, 1992).

Pharmacological studies show that NOS can be inhibited with N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{G}-monomethyl-L-arginine monoacetate (L-NMMA), L-N^{G}-nitroarginine (L-NNA) and D-arginine (Dwyer, Bredt and Snyder, 1991; Peterson, Peterson, Archer and Weir, 1992). It is not clear how these inhibitors depress NOS activity but certain studies suggest that this occurs by modification of electron transfer through iron centers in NOS during oxidation (Peterson, Peterson, Archer and Weir, 1992). This may be one of the mechanisms of inhibition of NO synthesis since NOS has been shown to contain iron (McMillan, Bredt, Hirsch et al, 1992). Although these studies were performed using rat brain NOS, they suggest that if NOS in GI neurons or ICC are similar such inhibition of NOS by the above mentioned agents may be possible through the same mechanism.

However, investigations of NO synthesis in the brain, endothelium and macrophages show that there are differences in sensitivity to inhibition by arginine analogues. This suggests that distinct constitutive NOS enzymes may be involved in NO synthesis in different tissues (Lambert, Whitten, Baron et al, 1991).

Although many studies agree that L-arginine is the substrate for basal and stimulated release of NO (Schmidt, Zernikow, Baeblick and Bohme, 1991), other studies do not agree that all the known cofactors for NOS such as FAD, FMN and tetrahydrobiopterin are

required in the synthesis of NO by distinct NO producing cell types or distinct NOS (Stuehr, Cho, Kwon et al, 1991; Galea, Feinstein and Reis; 1992; Dwyer, Bredt and Snyder; 1991).It appears that macrophage NOS requires exogenous tetrahydrobiopterin while brain and endothelial NOS have no such requirement (Dwyer, Bredt and Snyder; 1991). It also seems that NOS of neurons of intestinal synaptosomes do not require exogenous tetrahydrobiopterin for NO synthesis (Watson and Daniel, unpublished observation).
Presently, it is agreed that the difference is in the requirement for exogenous tetrahydrobiopterin since all known forms of NOS require it among other cofactors such as FAD and calcium/ calmodulin etc.

In the cells studied so far, attempts have been made by various investigators to localize NOS in order to understand its mechanism of action. NOS activity in endothelial cells have been localized with subcellular fractionation which shows that there are both membrane-bound and cytosolic NOS enzymes (Hecker, Walsh and Vane, 1991; Pollock, Forstermann, Mitchell et al, 1991). However, it appears that most of NOS in macrophages and brain tissue are localized in the cytosol (Marletta, Yoon, Iyengar et al, 1988; Bredt and Snyder, 1990; Schmidt, Pollock, Nakane et al, 1991). Although no study has clearly distinguished the biochemical properties of particulate (membrane bound) and cytosolic NOS, it seems that in some cell types such as the brain cytosolic NOS

is calcium-dependent (Bredt and Snyder, 1990) while in macrophages cytosolic NOS is calcium-independent (Marletta, Yoon, Iyengar et al, 1988) but both particulate and cytosolic NOS from NANC containing tissue of rat anococcygeus muscle are calcium-dependent (Mitchell, Sheng, Fortermann, Murad, 1991). There may be problems associated with determination of calcium-dependency of NOS since it is not known if adequate concentration of calcium is present in tissue or medium before determination of NOS activity. Adequate levels of calcium in tissue or medium may reduce sensitivity of NOS to it.

4.1.3 Measurement of NO production in tissues/cells

Several methods have been developed to determine levels of NO production in tissues or cells. These techniques range from simple and reproducible to complex and expensive.

NO production in tissues/cells has been approached through direct and indirect measurement of NO levels in functional studies. Direct measurements attempt to determine concentrations of NO produced spontaneously by using advanced techniques such as electron paramagnetic resonance spectroscopy (Mordvintcev, Mulsch, Busse and Vanin, 1991) and chemiluminescence (Palmer, Ferridge, Moncada, 1987 ; Termin, Hoffman and Bing, 1992). However, indirect functional detemination of NO production is based upon observation of its known effect on tissue such as relaxation of vascular smooth muscle (Palmer, Ferridge, Moncada, 1987, Schmidt, Baeblich, Zernikow et al

1990) and gastrointestinal smooth muscle (Bult, Boeckxstaens, Pelckmans et al, 1990; Boeckxstaens, Pelckmans, Ruytgens et al, 1991; Christinck, Jury, Cayabyab, Daniel, 1991; Allescher, Tougas, Vergara et al, 1992). In addition, certain other techniques have been utilized in cellular studies to determine presence of NOS in non-functional studies. These methods include immunohistochemical and histochemical staining techniques to determine NOS positive and NADPH-diaphorase positive nerve fibers in the gut (Belai, Schmidt, Hoyle et al, 1992; Bredt, Hwang and Snyder, 1990; Berezin, Bredt, Snyder, Daniel, submitted). Colocalization of NOS and NADPH-diaphorase has been demonstrated in enteric neurons of canine proximal colon (Ward, Xue, Shuttleworth et al, 1992). Minor disadvantages associated with these histochemical techniques are that subcellular localization of NOS is difficult and adequate quantitation is impossible. However, these methods give qualitative results which are important in localization of NOS activity.

Biochemical techniques are also important in the determination of NO production or NOS activity. These include indirect measurement of NO production or NOS activity by quantitation of guanylate cyclase activity or cGMP level stimulated by NO (Knowles, Palacios, Palmer and Moncada, 1990) or by a difference spectrophotometric method which is based on the rapid oxidation of oxyhemoglobin to methemoglobin by NO (Feelisch and Noack, 1987,

Kelm, Feelisch, Spahr et al, 1988). Although these biochemical methods have been important in elucidation of NO function, they do not directly quantitate NO or citrulline which is a coproduct. Two important biochemical techniques have been employed in several studies to either measure NO or citrulline production. The first method measures the stable product of NO by determination of nitrite (an oxidation product of NO) levels using Greiss reagent (Bell, O'Neill, Burgison, 1963; Green, De Luzuriaga, Wagner et al, 1981; Green, Wagner, Glogowski et al, 1982; Bredt and Snyder, 1989). The second biochemical method widely adopted for the determination of NO levels produced by cells is the assay of labelled citrulline which is stoichiometrically produced from labelled arginine using cation exchange chromatography to separate the incubation mixture (Palmer and Moncada, 1989; Bredt and Snyder, 1989). These two biochemical techniques are simple and adaptable in addition to giving reproducible results although they measure different things as stated above. Both methods have been widely used in the determination of NOS activity. Furthermore, quantitative results are obtainable from their use in the measurement of NOS activity and could complement use of histochemical techniques. This is why these two biochemical methods have been adopted in the present study.

4.1.4 Basis for determination of NOS activity in colonic subcellular fractions.

It is now known that NO is the main NANC neurotransmitter in the gut (Bult, Boeckxstaens, Pelckman et al, 1990; Christinck, Jury, Cayabyab, Daniel, 1991; Dalziel, Thornbury, Ward, Sanders, 1991) but only a few studies have established clearly the structures which produce NO in the gut. Such investigations have used immunohistochemical and histochemical techniques to indicate that NOS is localized in the nerve plexuses of the ileum (Bredt and Snyder, 1990; Belai, Schmidt, Hoyle et al, 1992) but it is not certain if it is nerves alone and/or ICC that is producing NO in the nerve plexuses of the ileum. Moreover, the arrangement of ICC as intermediate structures between nerve and muscle in the colon (Berezin, Huizinga and Daniel, 1988) and the observation that VIP nerves project towards ICC and are hyperpolarized by VIP (Barajas-Lopez et al, 1989) suggest that ICC may produce NO. Also some effects of VIP on canine colon circular muscle are reduced by tetrodotoxin and L-NAME (Huizinga, Tomlinson, Pintin-Quezada, 1992).

Furthermore, studies of the proximal canine colon showing that there is colocalization of NADPH-diaphorase and NOS in enteric neurons suggest that NO is produced in the colon but this does not clearly indicate if other cell types such as ICC could produce NO (Ward, Xue, Shuttleworth et al, 1992). However, in our laboratory, a combination of immunohistochemical techniques and electron

microscopy suggest that ICC contain NOS and may produce NO (Berezin, Bredt, Snyder and Daniel, submitted). Based upon these findings, it is important to determine which structures specifically produce constitutive NOS to effect inhibition of colonic smooth muscle since the above-mentioned studies suggest that either nerves or ICC or both may produce NO.

In order to determine which specific structures are involved in NO production, it is necessary to isolate nerve or ICC enriched membrane fractions to determine if these membranes are capable of producing NO. This task could be accomplished by using the subcellular membrane fractionation scheme developed in our laboratory as (as described in Chapter 3). Subcellular fractionation not only allows separation of membranes from different cells but also allows separation and enrichment of certain subcellular organelles such as mitochondria, endoplasmic reticulum and smooth muscle plasma membrane in distinct fractions derived from tissues under investigation. For these reasons subcellular fractionation methods were employed to obtain fractions containing synaptosomes, ICC and smooth muscle plasma membranes to determine biochemically the activity of NOS in the colon.

4.1.5. Properties of ICC of colon which makes it likely to produce NO.

Electron microscopic studies portray ICC, in several species studied so far, as having a higher density of mitochondria (Thuneberg, 1982; Berezin, Huizinga, Daniel, 1988) which suggest increased metabolic function. Moreover, their intercalation between nerve and muscle (Daniel and Daniel, 1984 ; Berezin, Huizinga and Daniel, 1988 ; Berezin, Farraway, Huizinga and Daniel, 1990) suggests that they play a role in neurotransmission (Daniel and Daniel, 1984). However, functional studies of NANC nerve function in the colon suggest that hyperpolarization and subsequent relaxation of canine and rat colonic circular smooth muscle is linked to the effect of NO (Hata, Ishii, Kanada et al, 1990; Thornbury, Ward, Dalziel et al, 1991; Ward, Dalziel, Thornbury et al, 1992). Although these studies show that NO is produced in the colon, there were no indications of which structures produced NO. Up to now, it was assumed that nerves alone produce NO without serious thought being given to ICC as capable of producing NO. This assumption is not given credence by recent findings in our laboratory showing that antiserum to NOS stained ICC (Berezin, Bredt, Snyder and Daniel, submitted). Based upon this premise it is important to determine if ICC produce NO in addition to nerves in colonic preparations. Furthermore certain studies report that VIP can act on smooth muscle to cause NO production (Grider et al, 1992; Grider, 1993, Murthy, Jin, Makhlouf, 1992; Grider, 1993).

4.1.6. Is there an association between VIP and NO production in ICC-of colon ?

Earlier studies suggested that VIP may be the NANC neurotransmitter in the gut (Rattan and Goyal, 1980) but this suggestion has never been fully substantiated. Instead, experimental results now suggest that NO is the NANC neurotransmitter in the gut (Bult, Boeckxstaens, Pelckman et al, 1990; Christinck, Jury, Cayabyab, Daniel, 1991; Shuttleworth, Murphy, Furness, 1991). Only few studies have looked at an association between VIP and NO in NANC inhibitory neurotransmission. One study in the guinea-pig airway suggest that endogenous VIP and NO may modulate cholinergic neurotransmission at smooth muscle level by prejunctional inhibition of acetylcholine release from cholinergic nerve terminals (Belvisi, Mura, Stretton and Barnes, 1993). Another study from our laboratory suggests that VIP and NO are inhibitory in action in the colon but may either function independent of each other, However, L-NAME reduced VIP induced relaxation (Huizinga, Tomlinson, Pintin-Quezada, 1992). The presence of VIP receptor sites on colonic ICC membrane isolated by subcellular membrane fractionation technique (Memeh, Berezin, Cipris, Daniel, 1992) or on membrane of enzymatically isolated ICC from canine colon (Starich, Mihaylova-Todorova, Mansfield and Lardinois, 1992) may be associated with these events.

This is why it is important to examine (using appropriate, biochemical techniques) if there is a functional relationship between VIP and NO production in ICC enriched strips of colonic preparations.

4.2.0 MATERIALS:

[^{3}H] L-Arginine (specific activity, 58.4 Ci/mmol) was purchased from New England Nuclear (Boston, MA) while L-Arginine, citrulline, NADPH and L-NAME were commercially obtained from Sigma Chemicals Co (St Loius, MO). Dowex AG50X-8 was bought from BioRad (Richmond, CA) while purified (bovine brain) calmodulin was a generous gift from Dr Philip Coggins (Biomedical Sciences, McMaster University). Other reagents were commercially obtained in pure quantities from known sources.

4.3. METHODS:

4.3.1 Tissue strips and membrane fractions:

ICC-rich tissue strips and various membrane fractions were prepared following known procedure for dissection and subcellular fractionation scheme described in chapter 3. Tissue was dissected in Krebs solution (with constituents as described in chapter 3 under methods) but homogenized and centrifugated in sucrose MOPS.

4.3.2. Determination of NO synthase activity in membrane fractions.

Assay of NO synthase was done using two widely known techniques. The first method involved determination of nitrite levels as a stable product of NO oxidation in membrane fractions using Greiss reagent technique (previously described by Green, Wagner, Glogowski et al, 1982; Bredt and Snyder, 1989). Membrane fractions were incubated for 45 minutes at 37^{0}C. The other technique utilized in the determination of membrane NOS activity is based upon the protocol of Bredt and Snyder (1989) and modified in our laboratory by Kostka, Jang, Watson et al (1993).

4.3.3. Determination of NO synthase activity in ICC-rich strips.

The Greiss reagent method was also used in the determination of NOS activity in strips of ICC-rich colonic preparation. Greiss reagent traps nitrite obtained from oxidation of NO.

4.4.0 RESULTS:

4.4.1 Nitric oxide (as nitrite) production in ICC-rich and-poor preparations.

This phenomenon was investigated by determination of the preparations of the colon incubated in 200 μM L-arginine and 100 μM NADPH and 0.1 mM calcium. Results in Table 19 shows that in the ICC-rich preparation it was the ICC membrane enriched fraction, P_3, that produced the highest level of nitrite and that it was about 9-fold higher than in the PNS or starting material. This was followed by moderately high levels in mitochondria and synaptosome enriched fractions, P_1 and P_2 respectively, in that there was 6-and 5-fold higher levels of nitric oxide production than in the PNS. However, nitric oxide production was also high in the MIC 1 fraction which is a mixed fraction that yields on differential centrifugation both ICC-rich membrane fraction and the smooth muscle and ICC contaminated fraction, MIC 2. The production of nitric oxide appears to be low in the latter fraction. Nitrite levels were not detected in tubes without membranes.

In the ICC-poor preparation , the profile of nitric oxide production is different in magnitude than was seen in the ICC-rich preparation. Table 20 shows that although nitric oxide production was lower in ICC-poor preparations (as seen in the PNS) compared to the ICC-rich preparation, there was 7-fold enrichment of nitric oxide production in the ICC membrane enriched fraction, P_3, and in

the synaptosome enriched fraction, P_2.

However, in the mitochondria enriched fraction, P_1, there was a 5-fold enrichment of nitric oxide production.

Table 19 Nitrite levels (μmol/mg protein h^{-1}) in ICC-rich preparations

Fractions	Nitrite concentration.
PNS	0.355±0.051
S_1	0.359±0.091
S_2	0.356±0.103
P_1	2.051±0.197
P_2	1.779±0.211
P_3	3.081±0.870
MIC 1	2.529±0.314
MIC 2	0.383±0.067

Values are given as mean±SEM. n=3.

Table 20 Nitrite levels (μmol/mg protein h^{-1}) in ICC-poor preparations.

Fractions	Nitrite concentration
PNS	0.199±0.006
S_1	0.207±0.008
S_2	0.182±0.050
P_1	0.950±0.060
P_2	1.317±0.500
P_3	1.337±0.360
MIC 1	0.780±0.001
MIC 2	0.537±0.030

Values are given as mean±SEM. n=3.

4.4.2 Characterization of nitric oxide synthase activity in the membrane enriched fraction.

Table 21 is a summary of results obtained from the assay of nitric oxide synthase activity in the ICC membrane enriched fraction, P_3, under different experimental conditions using stoichiometric conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline. The incubation of this membrane fraction (solubilized with Triton X-100) in [^{3}H] L-arginine and supplemented with 1 mM NADPH produced [^{3}H] L-citrulline levels taken as basal since NADPH in isolated ICC membrane may be reduced. This basal level was important in the determination of calcium dependency of ICC membrane nitric oxide synthase activity. EGTA (0.1mM) a known calcium chelator caused only 12% reduction of basal nitric oxide synthase activity while in the presence of exogenous 0.1 mM calcium and 1 μM calmodulin nitric oxide synthase activity increased by only 8% from basal level. However, the difference between nitric oxide synthase activities in the presence of EGTA and in the presence of calcium and calmodulin is about 20% of the basal activity while the activity in the presence of EGTA is roughly about 88%. This suggests that the calcium-dependent component of nitric oxide synthase activity on ICC membrane was about 20% of its overall activity while the calcium-independent component is about 80%.

Table 21 Characterization of Nitric oxide synthase activity of ICC-membrane rich fraction

	DPM x 10^3/mg protein
Basal	5.336±0.885
EGTA	4.691±0.560
Calcium/Calmodulin	5.768±0.116

Values are given as mean±SEM. n=4.

4.4.3. Determination of effect of VIP on nitric oxide synthase activity in ICC-rich strips of canine colon.

This was determined by measuring nitrite levels produced by VIP in comparison with the level recorded without VIP. Results from Table 22 show that there was a 100% increase in nitric oxide synthase activity in the presence of VIP while there was only an 18% increase in the activity of this enzyme when 0.1 mM calcium and 1 μM calmodulin was present. However, the calcium-independent component of nitric oxide synthase activity was shown by the effect of EGTA. In the presence of 0.1 mM EGTA nitric oxide synthase activity returned to basal level of activity seen in the absence of VIP. The difference between the activity of nitric oxide synthase in the presence of VIP+EGTA and VIP alone suggested that calcium-free Krebs (with no EGTA) contains some contaminating calcium which is utilized by VIP to increase NO production. However, increase in exogenous calcium did not increase NO synthase activity.

1 μM L-NAME in the presence of VIP inhibited the activity of nitric oxide synthase activity to about 14% below basal level at which VIP was not present. This inhibition is about 60% when the level of activity of the synthase in the presence of VIP was compared to the level of activity recorded in the presence of VIP and L-NAME.

Table 22 Effect of 1 nmol VIP on production of nitrite levels in ICC-rich and-poor strips of colon in the presence of 1 mM L^{-1}) arginine + 1 mM NADPH.

	ICC-rich	%	ICC-poor	%
-VIP (basal)	6.11±1.27	(100)	2.96±1.04	(100)
+VIP	12.83±0.94	(210)	6.31±4.27	(213)
+VIP +EGTA	6.40±1.14	(105)	3.14±1.46	(107)
+VIP calcium /calmodulin	15.11±0.91	(247)	8.09±4.48	(273)
+VIP +L-NAME	5.30±1.21	(87)	3.52±2.01	(119)

Values are given as mean±SEM. n=3.

Calcium-free Krebs solution was used in the incubation experiments except in the calcium /calmodulin experiments in which 0.1 mM calcium was added but tissue was previously dissected in Krebs containing 2.5mM calcium. EGTA was used to remove the effect of endogenous calcium bound to tissue.

Cells were not lysed because because ionic concentration of Krebs and its pH buffers were adequate to maintain normal osmotic pressure. In addition Triton-X was not added to lyse the cells.

4.4.4. Determination of the role of N-type calcium channel in VIP-induced nitric oxide production.

The role of N-type calcium channel in VIP induced nitric oxide production from ICC-rich strips of canine colon was determined by using 1 μM omega-conotoxin which is a specific blocker of this type of calcium channel. Results from Table 23 show that 1 μM concentration of omega-conotoxin inhibited nitric oxide production by about 22% in the absence of L-arginine with VIP present. However, this inhibition seemed to increase to about 32% in the presence of arginine and VIP. These results suggest that although N-type calcium channels can participate in nitric oxide production, its role is limited and not significant.

Table 23 Effect of 1 μM ω-Conotoxin GVIA on VIP induced nitrite formation in ICC-rich strips of colon.

	Conc.(pmol/mg tissue wt h^{-1}	% inhibition	p-value
-VIP -Arg	751.60±79.02		
+VIP -Arg	1364.50±283.77		
		22.00	ns
+VIP -Arg +CTX	1064.00±247.05		
+VIP +Arg	1665.60±350.25		
		32.00	ns
+VIP +Arg +CTX	1133.40±311.96		

Values are given as mean±SEM. n=5. Arg= L-Arginine.
CTX= ω-Conotoxin. Although tissue was dissected in normal Krebs with 2.5mM calcium, incubation was done in calcium-free Krebs solution as described in Table 22.

4.5 DISCUSSION

4.5.1 Nitric oxide production in canine colon.

Previous studies have shown that nitric oxide(NO) synthesis can occur in the gut but the exact sources of NO were not known (Salter, Knowles, Moncada, 1991).

The present studies show that membranes from synaptosomes, smooth muscle and ICC of canine colon are capable of producing different levels of NO when incubated in a medium containing L-arginine and NADPH.

Substantial levels of NO made in synaptosome-rich fraction of both ICC-rich and-poor preparations suggest the presence of NO synthase (NOS) on membranes from synaptosomes. A recent study from our laboratory which showed NOS activity in synaptosomes from canine ileum (Kostka, Jang, Watson et al; 1993) supports the evidence of such activity in synaptosomes isolated from canine colon. Immunohistochemical studies showing the presence of NOS in nerve fibers from small intestinal plexuses suggest that NO is produced by nerves (Bredt, Hwang, Snyder, 1990). Other studies have shown a similar phenomenon in neural cells of rat brain (Knowles, Palacios, Palmer, Moncada, 1989; Bredt, Snyder, 1990).

There is a significant level of NO production in the mitochondrial fraction. This may reflect contamination from synaptosomes and ICC membranes. The level of NO in the mitochondrial fraction of ICC-rich preparation was greater than

that of the corresponding fraction of the ICC-poor preparation. However, the level of NO in produced in synaptosome rich fractions from both ICC-rich and-poor preparations were similar but they were also lower than the concentration of NO produced by the mitochondria-rich fraction of ICC-rich preparation. This suggests a role for NO in mitochondria from ICC but further studies are necessary to clarify this issue. In addition, low levels of NO production were seen in smooth muscle plasma membrane-rich fractions but the presence of such activity may be due to the presence of synaptosomes in this fraction. However, it has been reported that smooth muscle is capable of producing NO in response to VIP (Grider, Murthy, Jin, Makhlouf, 1992). Such findings need further confirmation.

It is interesting to find that NO synthesis is not the preserve of nerves alone but may also occur in ICC. The presence of NOS activity on ICC membrane suggest that non-neural cells in the colon are capable of producing NO. Other studies have clearly demonstrated that NO can be produced by non-neural cells such as the endothelium (Schmidt, Zernikow, Baeblich, Bohme, 1990; Lambert, Whitten, Baron et al, 1991; Pollock, Forstermann, Mitchell et al, 1991), macrophages (Marletta, Yoon, Iyengar et al, 1988; Lambert, Whitten, Baron et al, 1991), platelets (Radomski, Palmer, Moncada, 1990), adrenal glands (Palacios, Knowles, Palmer, Moncada, 1989) and smooth muscle cells (Grider, Murthy, Jin, Makhlouf, 1992). Furthermore, characterization of this ICC-membrane bound NOS shows

that its activity is reduced in the presence of EGTA which suggests a role for calcium in the synthesis of NO. This calcium dependence is, however, not substantial which shows that the constitutive membrane bound NOS of ICC is similar to that found in endothelial cells (Hecker, Walsh, Vane, 1991).

4.5.2 Association between VIP and NO production in colon.

The relationship between the presence of VIP receptors on ICC membrane and its ability to synthesize NO was tested in colonic ICC-rich preparation by incubation with exogenous VIP. Results from this study clearly show that VIP increased production of NO in colon supporting the observation made with membrane fractions. This increased NO production showed some calcium dependency because EGTA reduced its production although exogenous calcium did not change NO production. This suggests that the calcium in internal stores or bound to tissue surface is sufficient for NO production.

Increased production of NO in ICC-rich preparation of colon in response to exogenous VIP requires explanation because this preparation contains some synaptosomes in addition to ICC. Therefore, which cell type is the major source of NO when exogenous VIP is added? Although VIP receptors are found on both synaptosomes and ICC (Memeh, Berezin, Cipris, Daniel, 1992; Starich, Mihaylova-Todorova, Mansfield, Lardinois, 1992), previous reports suggest

that it is unlikely that synaptosomes produce NO in response to exogenous VIP. This is because exogenous VIP can cause prejunctional inhibition of VIP release (Grider, Makhlouf, 1987) which could cause NO production from ICC. Even if exogenous VIP causes further VIP release from synaptosomes, it is accompanied by corelease of peptide histidine isoleucine (PHI) (Yasui, Naruse, Yanaihara et al, 1987) which can also cause prejunctional inhibition of VIP release from synaptosomes (Yasui, Naruse, Yanaihara et al, 1987). Another reason for not considering synaptosomes as the source of NO in response to exogenous VIP is that VIP simulates cyclic AMP production and depolarization in nerves of both central nervous system (Ferron, Siggins, Bloom, 1985; Wang and Aghajanian, 1990; Hedlund, Dufy, Barker, 1988; Lasater, Watling, Dowling, 1983) and peripheral nervous system (Kawatani, Rutiglano, DeGroat, 1985; Ito, Kurokawa, Ohga, Ohta, Sawabe, 1990). Recent studies from our laboratory suggest that cyclic AMP inhibits NOS activity in synaptosomes from canine ileum (Kostka, Jang, Watson, Stewart, Daniel, 1993). However, other studies show that NOS activity in brain neurons is not altered by increased levels of cyclic AMP and its kinase (Brune, Lapetina, 1991; Bredt, Ferris, Snyder, 1992). These findings suggest that VIP action on neurons may not lead to release of NO. This is confirmed in the present study which shows that NO production in the ICC-rich region of colon is not inhibited in the presence of neuronal N-type calcium channel blocker, `-Conotoxin GVIA.

Therefore, NO production in ICC-rich region in response to VIP is predominantly from non-neural cells and ICC is the best candidate since its membrane has a high density of VIP receptors and NOS activity.

Although, smooth muscle has been suggested to produce NO (Grider et al, 1992), the level of NO produced is lower in ICC-poor preparations and smooth muscle plasma membrane rich fractions. Furthermore, immunohistochemical studies from our laboratory suggest the presence of inducible NOS activity in ICC (Berezin, Bredt, Snyder, Daniel, submitted).

Several lines of evidence lend credence to the hypothesis that VIP-mediated inhibition of colonic circular muscle is primarily through increasing release of NO from ICC. These evidence are as follows: 1) VIP receptors were found localized on ICC-membrane (Memeh, Berezin, Cipris, Daniel, 1992; Starich, Mihaylova-Todorova, Mansfield, Lardinois, 1992); 2) VIP nerve fibers project towards ICC localized between nerve and muscle and VIP hyperpolarized ICC by 9 mV when electrically isolated by 1mM heptanol (Berezin, Huizinga, Farraway, Daniel, 1990); 3) Other studies suggest that ICC can produce NO without extracellular calcium (Publicover et al, 1992); 4) NOS is localized on ICC. However, the mechanism through which VIP activates NOS is not presently known. Since one study (Publicover et al, 1992) suggests a role for intracellular calcium levels in NO production from ICC due to its reduction by ryanodine application, further investigation is necessary to examine the

possibility of VIP elevation of intracellular calcium to augment NO production.

4.5.3 Implications of present findings for inhibitory control of colon motility.

The main new findings of the present studies are that ICC on the submucosal border of circular muscle have a high density of VIP receptors. In addition, these ICC are capable of producing NO in response to VIP action on its membrane in a calcium dependent manner suggesting that ICC membrane bound NOS is constitutive. NO production in ICC-rich submucosal border of colonic circular muscle is predominantly from ICC because N-type calcium channel blocker, ω-Conotoxin GVIA which blocks neuronal transmitter release including NO (Dutar, Rascol, Lamour, 1989; Cohen, Jones, Angelides, 1991; Daniel et al, submitted) did not significantly inhibit VIP induced NO production. Therefore, the projection of VIP nerves to ICC suggest that VIP release from nerves can cause ICC to produce NO which inhibits circular muscle of colon. This action of VIP on ICC to produce NO which causes circular muscle relaxation is termed indirect VIP relaxation of colonic circular muscle motility.

Electron microscopic studies showed that certain areas of colonic longitudinal and circular muscle devoid of ICC are directly innervated by VIP-containing nerves (Berezin, Huizinga, Daniel,

1988; Huizinga, Berezin, Daniel, Chow, 1990). This finding coupled with the presence of VIP receptors on plasma membrane of both longitudinal and circular muscle (Memeh, Berezin, Cipris, Daniel, 1992; Starich et al, 1992) indicates that direct inhibition of longitudinal and circular muscle of colon occurs through receptor-mediated cyclic AMP mechanism, although a recent report suggests that this could also occur through VIP-stimulated NO production (Grider et al, 1993; Grider, 1993). The present biochemical studies support functional studies from our laboratory showing that inhibitory control of colon motility is through direct VIP and indirect NO mediated mechanisms (Huizinga, Tomlinson, Pintin-Quezada, 1992). The mechanism through which NO mediates ICC and colonic muscle hyperpolarization and relaxation has been suggested to be through opening of calcium-activated potassium channels (Huizinga, Berezin, Daniel, Chow, 1990; Thornbury, Ward, Dalziel et al, 1991).

The functional significance of these findings in the control of colon motility is shown by the fact that VIP is released during electrically stimulated relaxation (Grider, Cable, Said, Makhlouf, 1985). Although the inhibitory effect of VIP antiserum on colonic descending relaxation led to the belief that VIP mediates this aspect of colonic motility (Grider and Makhlouf, 1986), the advent of NO studies demonstrated that NO is the major mediator of descending relaxation of colon. This is because descending

relaxation is present even when colonic segments have been desensitized to ATP, neurotensin and VIP but is reduced in the presence of NOS inhibitor, L-NNA; an effect reversed by L-arginine- the NOS substrate (Hata, Ishii, Kanada et al, 1990). The finding in the study by Hata et al (1990) that colonic descending relaxation can occur in the absence of VIP responsiveness suggest that bursts of NO may be released from a non-neural source without VIP stimulation. This is in agreement with the present studies showing that NO is produced mainly in ICC in the absence of VIP stimulation because membrane fractions and ICC-rich preparations produced NO without addition of exogenous VIP to the incubation medium. Therefore increased levels of NO seen with VIP application suggest that VIP only serves to enhance NO release from ICC. These views indicate that VIP is an inhibitory neurotransmitter released by nerves to enhance NO release from ICC which leads to colonic circular muscle relaxation. VIP could also have a direct effect on smooth muscle to produce NO and cause relaxation (Grider et al, 1992).

In addition to production of NO by ICC, VIP could also inhibit colonic smooth muscle indirectly by having a dual effect on ICC either by elevation of cyclic AMP which reduces slow wave amplitude and frequency or by reduction of intracellular calcium oscillations which depends on extracellular calcium level (Huizinga, Farraway, Den Hertog, 1991; Publicover, Horowitz,

Sanders, 1992). It is not known if these processes are partitioned since the role of cyclic AMP in ICC-based NO production is not known at the moment. However, from present and other studies, it is believed that indirect inhibition of circular muscle occurs through VIP induced NO release from ICC. Therefore, these findings satisfy the second hypothesis that ICC of colon have receptors for VIP and contain nitric oxide synthase activated by VIP to synthesize nitric oxide.

CHAPTER FIVE

PERSPECTIVES

5.1 CONCLUSION

The biochemical basis for inhibitory control of colon function has been investigated by utilizing two methods one of which is the fractionation technique to characterize synaptosomes, ICC and smooth muscle membranes and the other method is in vitro determination of the effect of VIP on nitric oxide production in the ICC-rich preparation of canine colon. Although biochemical markers and quantitative analyses have been useful in the isolation and characterization of synaptosomes, ICC and smooth muscle membrane, there is an inherent problem of the differential centrifugation technique in not yielding very pure fractions where complete dissociation of these markers are evident. Furthermore, a large amount of tissue is required to obtain a reasonable quantity of ICC membrane for experiments. However, the fractionation technique is basic in understanding the contribution of each of these cell types to colonic inhibition. Furthermore, it has led to the understanding of appropriate medium necessary for preparation of ICC-rich and poor layers of colon. These studies are difficult to perform because they involve time-consuming and delicate dissection coupled with lengthy fractionation procedure which would be unreliable if performed the day after tissue preparation or using frozen tissue because of low enzyme yield which could result from freezing and thawing or other unknown factors. For this reason, the present studies utilized fresh colonic tissue prepared immediately after mongrel dogs were killed by overdose of

pentobarbital sodium in order to obtain optimum and near physiolological results.

However, despite these problems, the present studies have shown that both synaptosomes of ICC membrane and mitochondria of ICC-rich preparation are capable of producing high levels of nitric oxide. Furthermore, these studies have also shown that in spite of basal release of nitric oxide by ICC, VIP stimulates nitric oxide release from ICC and this can be the basis for indirect inhibitory control of colonic smooth muscle. The findings that VIP receptors are localized on both longitudinal and circular smooth muscle membranes suggest direct inhibitory control of these muscle layers by VIP. However, it is not absolutely clear whether these direct and indirect inhibtory mechanisms occur simultaneously or separately.

5.2.2 Future directions

Although studies reported here have yielded interesting results it by no means gives a total picture of biochemical events involved in the inhibitory control of colon. ATP has been suggested to be an inhibitory neurotransmitter but it is not known if it participates in conjunction with VIP in inhibitory control of colon, although it has been shown to be effective in guinea-pig colon. Apart from these puzzles certain other questions arise from this study which demands further investigation using basic techniques from this and other studies.

1. It is important to resolve the molecular characteristics of nitric oxide synthase on ICC membrane such as its molecular weight and amino acid sequence to gain further understanding of its control.

2. It is necessary to clarify whether direct and indirect ICC-mediated inhibition in the colon occur simultaneously.

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