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Kunkle, Bruce Neil, Ph.D.

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Pharmacological studies on STb heat-stable enterotoxin in pig and rat jejunum

by

Bruce Neil Kunkle

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

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INTRODUCTION

Acute diarrheal diseases are the primary cause of morbidity and mortality throughout the world in humans and farm animals (Rao and Field, 1984). Noninvasive enterotoxigenic *Escherichia coli* strains are responsible for a major portion of acute diarrheal disease (Guerrant, 1973).

Three enterotoxins have been shown to be produced by these organisms: a heat-labile toxin (LT), a heat stable, methanol soluble toxin (STa) and a heat stable, methanol insoluble toxin (STb). The enterotoxins of *E. coli* stimulate a secretory response in the small intestine characterized by fluid and electrolyte loss which may be fatal in the neonate. The economic losses associated with this syndrome have stimulated research in the areas of prevention, treatment and pathogenesis of this disease.

Considerable work has been conducted to determine the cellular events involved in the secretory mechanism of LT and STa which appears to involve the elevation of levels of cyclic AMP and cyclic GMP respectively. However, the mechanism and site of action of STb has not been determined. Investigation of the effect of drugs to augment or suppress the secretory response to STb may contribute to the knowledge of epithelial transport under the influence of this toxin.

LITERATURE REVIEW

Organization and Architecture of the Small Intestine

The wall of the small intestine is comprised of four main layers, which include the serosa, muscularis externa, submucosa, and mucosa (Cormack, 1987). The outermost coat, the serosa, consists of simple squamous epithelium (mesothelium) overlying loose connective tissue. The serosa surrounds two layers of smooth muscle that comprise the muscularis externa. The thinner outer layer is make up of longitudinally disposed fibers (longitudinal muscle layer), while the thicker inner layer is formed by circularly arranged fibers. The plasma membranes of adjacent smooth muscle cells are fused at various points forming nexi, which allow for electrical coupling among cells (Granger et al., 1985). The muscular sheath formed by the muscularis externa undergoes spontaneous and rhythmic contractions that regulate the lumen size of the bowel and produces peristaltic movements.

Between the circular and longitudinal layers of muscle is a layer of connective tissue which contains the myenteric plexus (Auerbach's plexus). The plexus is composed of nerve fibers associated with numerous ganglia. Preganglionic fibers of the parasympathetic nervous system synapse with cells of terminal ganglia. Postganglionic fibers of the sympathetic nervous system also contribute to the plexus (Cormack, 1987).

The submucosa lies between the circular muscle layer and the muscularis mucosae. The loose connective tissue of this layer contains

lymphatic vessels, large blood vessels and the submucosa plexus (Meissner's plexus) (Granger et al., 1985). The submucosa plexus consists of nonmyelinated nerve fibers of the sympathetic division of the autonomic nervous system and preganglionic fibers and their terminal ganglia of the postsynaptic nervous system (Cormack, 1987). Elastic fibers admixed with the collagen fibers within the submucosa lend a quality that allows the formation of cores of folds of the mucosa (Granger et al., 1985). The submucosa of the duodenum and upper jejunum of some species contain tubuloalveolar submucosal glands (Brunner's glands) (Stinson and Calhoun, 1987) that produce a mucous to serous secretion (species dependent) rich in glycogen and bicarbonate ions that lubricates the surface epithelium (Granger et al., 1985).

Joyce et al. (1987) recently has described a contractile cell network in the rat intestinal mucosa. This network is composed of a two dimensional network of fibroblasts subjacent to the epithelial basal lamina of villi and crypts and a three dimensional network deep within the villar lamina propria. They suggest these networks of cells may provide supportive tonus for the epithelium, force for the expulsion of secretory products and propulsion of absorption products in the lamina propria, microvasculature and lacteals of the villus.

The innermost layer, the mucous membrane, consists of three subdivisions; the muscularis mucosa, the lamina propria, and the epithelial lining.

The muscularis mucosae is generally composed of an outer longitudinal and an inner circular layer of smooth muscle fibers (Stinson and Calhoun, 1987) and varying amounts of elastic tissue

(Cormack, 1987). The muscularis mucosa is three to ten cells thick (Madara and Trier, 1987). This arrangement permits localized movement of the mucosa. Increased tonus of the circular fibers tend to throw the mucosa into circular folds. In the small intestinal mucosa where the fingerlike processes called villi are formed, one smooth muscle bundle from the muscularis mucosa goes to the tip of each villus. These bundles contain the longest smooth muscle cells in the body (Cormack, 1987). Contraction of the smooth muscle bundle causes the villus to shorten and is responsible for lateral movements as well. This action may also aid in movement of lymph out of the lacteal and into the basal lymphatic plexus (Stinson and Calhoun, 1987). The lateral movement may stir the unstirred water layer (Madara and Trier, 1987).

The lamina propria is primarily composed of loosely arranged collagen and elastic fibers that support the epithelial lining, forms the core of each villus, and surrounds the crypts (Granger et al., 1985; Stinson and Calhoun, 1987). Contained within this layer are blood capillaries, a centrally located lacteal, small unmyelinated nerve fibers, smooth muscle fibers, fibroblasts, lymphocytes, plasma cells, mast cells, macrophages, globular leukocytes, and eosinophils (Cormack, 1987; Granger et al., 1985; Stinson and Calhoun, 1987).

The central lacteal is formed by a single lymphatic vessel with a blind terminal end located at the villus tip. Chylomicrons (lipids) enter the lacteals via gaps between adjacent lymphatic endothelial cells (Stinson and Calhoun, 1987). The function of the lacteal is to transport the chylomicrons to the systemic circulation (Granger et al., 1985).

The blood supply of the lamina propria consists of a single arteriole that forms within the villus an arteriovenous loop and capillary network subjacent to the surface epithelium. The central arteriole splays apically creating a terminal capillary network that courses down the periphery of the lamina propria subjacent to the epithelial basal surface. The subjacent capillaries contain thin attenuated walls with many 0.05 to 0.1 $\mu\nu$ diameter fenestrations that face the basal surface of the epithelial cells (Madara and Trier, 1987). The capillaries conduct water soluble substances absorbed by the epithelium. The vascular network becomes engorged with blood during periods of increased digestive activity, causing the villus to lengthen. The villus shortens during contraction of the muscularis mucosae and blood and lymph is pumped out. Thus food-laden blood and lymph are moved into the general circulation through the villus pumping station (Stinson and Calhoun, 1987).

Nerve fibers arising from the submucosa plexus ascend into each villus and form networks that extend throughout the connective tissue core (Cormack, 1987).

Aggregates of lymphoid tissue are disseminated throughout the small intestine. Small lymphoid nodules are present in the upper small intestine while large organized nodules (Peyer's patches), that may extend into the submucosa, are more prominent in the ileum (Granger et al. 1985; Banks, 1986). Peyer's patches are considered to be the initiating sites of mucosal immunity. These nodules contain the precursor population of B lymphocytes that migrate to and populate the lamina propria. These same cells synthesize and secrete

immunoglobulins, primarily IgA. The lymphoid cells, lymphoid nodules, Peyer's patches, and intraepithelial lymphocytes (T cells located between cells of the epithelium) together comprise one of the major subdivisions of the immune system which is designated as gut associated lymphoid tissue (GALT) (Granger et al., 1985).

The luminal surface of the small intestine is covered by a continuous sheet of epithelial cells, one layer thick, that covers the surface of the villi and lines the crypts (Granger et al., 1985). The epithelial cells rest on a continuous basement membrane composed of a basal lamina and a deeper layer of reticular fibers, glycoprotein connective tissue and ground substance (Madara and Trier, 1987). The villi are covered by primarily absorptive cells (enterocytes) and smaller numbers of goblet cells. A few endocrine cells and the rare caveolated cell are also present (Keast et al., 1985; Madara and Trier, 1987). M cells overlie the apex of Peyer's patches (Owen and Jones, 1974). The epithelial lining of the crypts is composed of undifferentiated cells, goblet cells, endocrine cells, caveolated cells and in most species Paneth cells (Granger et al., 1985; Madara and Trier, 1987). The function of the villus epithelium is primarily digestive, and absorptive. The crypt epithelium is responsible for epithelial cell renewal of the crypt and villus along with exocrine and endocrine secretory functions (Madara and Trier, 1987). It is also thought that the crypt epithelium is involved in the secretion of immunoglobulins into the intestinal lumen.

In order to efficiently perform the required digestive and absorptive functions the small intestine must present a large surface

area to the luminal content. The great length of the small intestine contributes to the large surface area. In addition, anatomic features amplify the mucosal surface area. Circular folds referred to as the plica circulares or valves of Kerckring formed by folding of the submucosa increase the surface area by a factor of 2 to 3 (Trier, 1967). These structures are most prominent in the duodenum and jejunum, but are not present in all species. Fingerlike projections of the mucosa consisting of epithelium covered cores of lamina propria form the intestinal villi. These mucosal projections increase the surface area 7 to 14 fold (Madara and Trier, 1987). Generally the length and surface area of the villi are maximal at the beginning of the small intestine and decrease gradually to the termination of the ileum. The length and number of crypts between villi decrease along the intestine from duodenum to the ileum (Satoh et al., 1985). Finally, the absorptive surface is made still greater by the microvilli present on the apical surfaces of the epithelial cells. It has been calculated that the absorptive surface is amplified 14 to 39 times by the microvilli over that which would be present if the cells had a flat apical surface (Trier, 1967).

The mucosa undergoes continuous replacement of the epithelium. The normal replacement rate varies with species, age and location (Moon, 1983). In the small intestine of the newborn pig 7 to 10 days is required for replacement, but only 2 to 4 days is needed in the 3 week old pig (Moon, 1971). The undifferentiated crypt cells are the source of the replacement cells which mature as they migrate from the crypt to the villus.

During migration the epithelial cells can be mixed and redistributed on the villus (Moon, 1983). Some cells of the same type have a greater life span than others (Smith and Jarvis, 1978) and certain cell types have different replacement times than other specific cell types (Tsubouchi and Leblond, 1974).

The movement of cells out of the crypts is in part due to cell proliferation deep in the crypts resulting in a displacement of the cells out of the crypt and onto the villus. Active cell motility also plays a role. If proliferation within the crypt is blocked, the cells will continue to leave the crypts and ascend the villi (Cormack, 1987).

Several cell types differing in structure and function join together in forming the intestinal epithelial cell layer. The absorptive epithelial cells and their precursors, the undifferentiated crypt epithelial cells predominate. The absorptive epithelial cells are responsible for the principle functions of the epithelium, digestion and absorption. Renewal of the varied epithelial cell population and secretion of IgA synthesized by plasma cells in the lamina propria are the primary role of the undifferentiated cells (Moon, 1983).

The tall, columnar absorptive cells display 3000 to 7000 microvilli on their apical surface (Granger et al., 1985). The microvilli range in height from 0.5 to 1.5 μm (Curran and Creamer, 1963) and are 0.1 μm wide (Madara and Trier, 1987; Trier, 1967). A study conducted by T.S. Sinclair et al. (1984) demonstrated the microvillus height to be greatest in the mid-region of the villus. The plasma membrane covering the microvilli is coated by the glycocalyx, a filamentous protein-containing structure. This surface coat is well developed in man and is

approximately 0.1 to 0.3 μm thick (Trier, 1967). The coat is firmly attached to the plasma membrane and it is removed with difficulty by exposure to mucolytic and proteolytic substances. It is synthesized and extruded by the absorptive cells to which it is attached (Ito, 1965; Ito and Revel, 1964). The microvillus and its glycocalyx are considered to be the digestive-absorptive unit of the enterocyte (Granger et al., 1985). High concentration of many enzymes have been found in this structure of the cell by biochemical, histological, and immunological studies. Alkaline phosphatase, leucine aminopeptidase, adenosine triphosphatase and disaccharidases are among the enzymes noted (Trier, 1967).

It has been suggested that the glycocalyx may also have a protective role as a barrier to the entry of substances into the absorptive cell (Ito, 1964). In different regions of the small intestine receptor proteins specific for certain substances have been localized (i.e., receptors for the intrinsic factor-vitamin B_{12} complex are found on ileal, but not on jejunal microvilli). The specific role of the glycocalyx is not completely understood. It may act as a support for large hydrophilic portions of microvillus intrinsic enzymes and it may provide a bonding surface for adsorption of pancreatic enzymes (Granger et al., 1985).

The plasma membrane of the microvilli is 10 to 11 nm wide in comparison to the 7 to 9 nm wide baseolateral membrane (Farquhar and Palade, 1963; Sjostrand, 1963; Trier, 1968). An additional characteristic is its high protein to lipid ratio (1.7:1) and a distinctive lipid composition that is rich in cholesterol and

glycolipids. These two characteristics appear to contribute to the low fluidity of the apical membrane (Madara and Trier, 1987).

The high protein content of the microvillus membrane reflects the presence of digestive enzymes (disaccharidases, peptidases), nonenzymatic proteins (receptor for vitamin B_{12} & calcium) and transport proteins that are responsible for the co-transport of Na^+ and D-glucose, Na^+ and amino acids and perhaps fatty acids. In addition, the glycolipid content may play a role in the binding of cholera toxin and Escherichia coli heat labile toxin. These toxins selectively bind to monosialosyl glycolipid G_M1 ganglioside.

Significant differences in the composition of the microvillus membranes of absorptive cells at different levels of small intestine has been described. Specific activation of lactase, alkaline phosphatase, trehalase and sucrose-isomaltase are higher in the proximal small intestinal mucosa than in the distal mucosa of the rat (Madara and Trier, 1987).

The enterocytes' basolateral membrane is different from the microvillus membrane in that it is thinner (7 to 9 nm) (Farquhar and Palade, 1963; Sjostrand, 1963; Trier, 1968), has a lower protein to lipid ratio (0.5:1) (Madara and Trier, 1987) and is more permeable than the brush border membrane. Na^+-K^+-ATPase, the enzyme involved in Na^+-K^+ transport is limited to the basolateral membrane (Granger et al., 1985).

During conditions of net water and electrolyte secretion into the intestinal lumen such as occurs after exposure of cholera toxin to the mucosa, the lateral plasma membranes closely oppose each other (15-30 mm apart) along their entire length (Di Bone et al., 1974). The lateral

borders interdigitate extensively via fold-like cytoplasmic processes that intrude and indent the cytoplasm of adjacent cells (Moon, 1983). Expansion of the intercellular space occurs between cells covering the upper third to half of the villi during active net fluid absorption (Di Bone et al., 1974 Tomasini and Dobbins, 1970). Widening of the lateral intercellular space is greatest at the basal half of the cells, but can extend toward the apex to the tight junction complex. At the base of the absorptive cells the width of the intercellular space is reduced by cytoplasmic extensions that approximate those of neighboring cells and at foci where adjacent cells are joined together by desmosomes. The absence of these spaces during net fluid and electrolyte secretion and their occurrence during net water and electrolyte absorption imply the size of the intercellular space is controled, in part, by solute flow and osmotic gradients (Madara and Trier, 1987).

The apical portion of the lateral plasma membrane is specialized morphologically. The outer leaflets of adjacent plasma membranes form intercellular attachment zones or junctional complexes that are composed of three parts: a) the zonula occludens; b) the subjacent zonula adherens; and c) the basally located macula adherens (Farquhar and Palade, 1963).

The zonula occludens, also referred to as the occluding or "tight" junction is formed by an apical circumferential band varying from 100 to 600 nm in thickness in which the lateral membranes of adjacent cells are closely apposed. Electron microscopy reveals a meshwork of interconnecting strands. Each strand appears to be a chain of 10 nm particles (Knutton et al., 1978). The composition of the intramembrane

particles has not been clearly determined. The structure of the tight junction varies with the region, cell type, and the position of the cell on the villus (Madara and Trier, 1987). In primates the tight junction between ileal absorptive cells have greater depth and more strands than tight junction between jejunal absorptive cells (Madara et al., 1980). The crypt tight junction varies from the villus occluding junction by exhibiting loose aberrant strands.

In one study, primate ileal absorptive cell tight junctions averaged 6.3 strands and 0.32 μm in depth, while ileal crypt cells averaged 4.3 strands and 0.19 μm in depth (Madara et al., 1980). Crypt epithelial jostling produced by mitosis appears to be responsible for the focal disorganization and variability of crypt tight junctions. Crypt tight junction disorganization increases during increased mitotic activity while a decrease in the mitotic index produces tight junction that resembles those of absorptive cells (Tice et al., 1979). Evidence has been gathered that shows tight junction influences the paracellular flow of water and electrolytes and thus regulates epithelial permeability. Structural features such as the complexity, strand number, and depth of the tight junction, frequently correlate inversely with the permeability of the epithelia (Claude and Goodenough, 1973). There appears to be a specific relationship between junctional strand counts and paracellular resistance (Madara and Trier, 1987). Claude and Goodenough (1973) demonstrated that paracellular resistance increased logarithmically as tight junction strand count increased arithmetically.

Subjacent to the occluding junction the zonula adherens, also termed the belt desmosome, or intermediate junction is the second

component of the tight junction complex. This structure is characterized by an intercellular space (approximately 20 nm) that appears to contain finely fibrillar material (Farquhar and Palade, 1963). At the site of the zonula adherens, the cytoplasmic side of the lateral membrane exhibits a thick mesh of cytoplasmic filaments that splay into the terminal web region. This zone appears to be the main site of membrane insertion for the filaments of the terminal web (Madara and Trier, 1987).

The macula adherens or spot desmosome, the third and last component of the junctional complex, is a discontinuous, button-like structure 100 to 500 μm in diameter. The intercellular span contains a central disc of dense material that is separated from the adjacent lateral membranes by an 8 nm lucent zone (Farquhar and Palade, 1963; Madara and Trier, 1987). One end of bundles of cytoplasmic fibrils are embedded within the plaques, while the other end loops through the terminal web and attaches to other spot desmosomes (Chen et al., 1972).

The enterocyte basolateral membrane contains abundant but fewer proteins than are noted in the apical membranes. The specialized function of the basolateral membranes are reflected in its biochemical composition. Higher specific activities of Na-K-ATPase are found in the basilar membranes when compared to microvillus membranes. The localization of Na-K-ATPase to this membrane signifies its important role in water and electrolyte transport (Madara and Trier, 1987). Adenylate cyclase also is localized to the basolateral plasma membrane of absorptive cells (Murer et al., 1976).

A continuous sheet of fine fibrillar material of intermediate

electron density comprises the basal lamina upon which the basal surface of the intestinal epithelial cell rests. This 30 nm wide layer separates the epithelial cells from the lamina propria and spans the intercellular space between cells. Occasional gaps are noted within this structure (McClugage and Low, 1984). The gaps are of such dimension to permit the passage of chylomicrons and other lipoproteins from the intercellular space to the lamina propria (Tygat et al., 1971). Cells of the lamina propria, such as lymphocytes, may pass to the intercellular spaces or intestinal lumen via these gaps (Madara and Trier, 1987). Occasionally, pseudopod-like extensions of absorptive cell basal cytoplasm project through the gaps in the basal lamina and contact the underlying mesenchymal elements. These projections occur with greater frequency in late fetal and early postnatal life and are of unknown significance (Mathan et al., 1972). The function of the basal lamina is not known. It possibly serves as a barrier or has a regulatory role in the exchange of material between the epithelium with its intercellular spaces and the lamina propria (Madara and Trier, 1987)

In the absorptive cells a basally displaced elongated nucleus is noted (Banks, 1986). The absorptive cell contains abundant cytoplasm in which a scarcity of free ribosomes and an abundance of mitochondria are noted (Cormack, 1987). The rough endoplasmic reticulum and Golgi saccules are well developed (Madara and Trier, 1987). The terminal web and extensive smooth endoplasmic reticulum are noted in the apical cytoplasm. The rough endoplasmic reticulum and free ribosomes are evident in the basal part of the cell (Stinson and Calhoun, 1987).

The undifferentiated crypt cell is the progenitor for all the other

epithelial cells and is the most abundant cell type in the crypts of Lieberkuhn. The cells display sparse, short, irregular microvilli and straight lateral membranes. The glycocalyx is poorly developed and an absence of enzymes and transport proteins is evident. The tight junction complexes are shallow, less complex, and contain fewer strands (Madara and Trier, 1987). An intercellular space is not evident between undifferentiated crypt cells (Moon, 1983). The rough and smooth endoplasmic reticulum are sparse. Prominent in the apical one third of the cytoplasm are secretory granules rich in glycoprotein (Madara and Trier, 1987). Secretory antibodies, sIgA and sIgM, are some of the substances released by these cells. The secretory component produced by these cells is located on the baseolateral membrane where it acts as a receptor for the IgA and IgM produced by plasma cells in the lamina propria. The sIgA and sIgM are transported to the lumen by the undifferentiated cells (Brown, 1978; Tomasi et al., 1980). The proliferative nature of these cells is evident by the frequent observation of mitotic stages.

Goblet cells occur in both crypt and villus epithelium (Moon, 1983). These cells occur with increasing frequency toward the distal ileum (Banks, 1986). These cells have few apical microvilli and a wine goblet appearance due to the fact the apical portion is distended by mucin-containing granules (Madara and Trier, 1987; Banks, 1986). Their mucus secretion has a protective and lubricative function (Banks, 1986). Noxious agents such as _E. coli_ and _Vibrio cholerae_ enterotoxins can stimulate the release of mucus by small intestinal goblet cells (Moon et al., 1971). Also goblet cell mucus could bind intraluminal bacteria

preventing their binding to epithelial cells and thus reduce contact with detrimental strains (Madara and Trier, 1987).

Paneth's cells are located at or near the base of the crypts (Moon, 1983; Madara and Trier 1987). These pyramid shaped cells contain a basally displaced nuclei and supranuclear acidophilic granules (Banks, 1986). The function of these cells is unknown. It has been suggested that they have phagocytic and secretory properties (Erlandsen and Chase, 1972; Peeters and Vantrappen, 1975; Rodning et al., 1976). The presence of lysozymes within the cells suggests a phagocytic function (Banks, 1986). It has also been shown by immunolabeling techniques that Paneth cells contain cytoplasmic trypsin-like material (Bohe et al., 1984). These cells are reported to be present in most mammalian species, but Paneth-like cells are not observed in dogs, cats, or raccoons (Madara and Trier, 1987). Most texts report that Paneth cells are not present in the pig, but their presence has been reported by some observers (Myer, 1982; Sloss, 1954). Paneth cells increase in number from duodenum to ileum (Elmes, 1976; Taylor and Flaa, 1964).

The enterochromaffin cells are found predominantly within the crypts. These triangular shaped cells contain subnuclear granules (Moon, 1983). The narrow apex presents tufts of microvilli to the luminal surface. The microvilli are presumed to act as micro-receptors and/or chemoreceptors (Granger et al., 1985; Madara and Trier, 1987; Herzog and Farquhar, 1977). These endocrine cells produce a variety of peptides and/or amines which include somatostatin, secretin, cholecystokinin (Cormack, 1987), serotonin, catecholamine, gastrin, enteroglucagon (Moon, 1983), substance P (Granger et al., 1985),

bombensin, and neurotensin. Release of secretory granules occurs at the basal and/or lateral surface of the cell (Madara and Trier, 1987).

In the mucosal epithelium overlying Peyer's patches, M cells are interspersed among the enterocytes (Granger et al., 1985). The M cells are usually located at focal sites when multiple mononuclear cells infiltrate the epithelium lining Peyer's patches. As their name suggests the cells are shaped like the letter M. Adjacent enterocytes are bridged by the apical portion of the cell. From the apical cytoplasmic bridge, M cell cytoplasm usually extends along the lateral membrane of the adjacent epithelial cells and partially surrounds mononuclear cells. The apex of the cell is covered by a series of ridges, which displays sparse numbers of short and occasionally branched microvilli (Madara and Trier, 1987). In the apical cytoplasm, numerous endocytic vesicles are present suggesting these cells may function to process and transport antigenic substances from the lumen to the underlying lymphoid follicles (Granger et al., 1985; Madara and Trier, 1987; Moon, 1983).

The uncommon caveolated cells (tuft cells, brush cells, multivesicular cells or fibrovesicular cells) are dispersed throughout the villus and crypt region (Granger et al. 1985; Isomaki, 1973; Nabeyama and Leblond, 1974). These pear-shaped cells have a narrow apex that contacts the intestinal lumen and a broader base in contact with the basal lamina. Long thick microvilli containing long bundles of filaments that extend deep into the apical cytoplasm distinguish these cells (Cormack, 1987). Vesicles or irregular tubules called caveoli characterize this cell. The caveoli are noted in the periphery and

intermixed between the filament bundles (Madara and Trier, 1987; Nabeyama and Leblond, 1974). Their role has not been determined, but it has been suggested that they are chemoreceptor cells (Madara and Trier, 1987).

The cup cell occurs with a frequency varying from rare to occasional depending on the species examined. Cup cells are characterized by a shortening of the microvilli which forms a cup-like indentation in the brush border of the cell (Madara, 1982). This cup-like indentation is due to the variability in height of the microvilli with the shortest at the center and the longest at the periphery of the cell apex. Also the apical cytoplasmic portion of the cell is slightly concave in nature. The apical membranes of cup cells are significantly lacking in alkaline phosphatase activity. The function of cup cells has not been determined (Madara and Trier, 1987).

Lymphocytes are frequently noted within the intercellular spaces between epithelial cells. These lymphocytes are referred to as intraepithelial lymphocytes (Cormack, 1987) or theliolymphocytes (Moon, 1983). Predominantly these are of the T (thymus) cell type (Moon, 1983).

Also present in the intercellular spaces of normal and parasite-infested individuals are globule leukocytes. These are mononuclear cells containing large eosinophilic cytoplasmic granules. Their origin and function are unclear (Cormack, 1987; Moon, 1983).

The small intestine is one portion of the tube that comprises the gastrointestinal tract. The combination of the various structural components comprising the intestinal wall act as a interface between the

external environment and the circulatory system. These components work in concert to aid in the digestive and absorptive process that is needed to efficiently provide the nutrients to sustain life of the animal, yet provide a barrier to harmful elements.

The Enteric Nervous System

The enteric nervous system has been described as the third subsystem of the autonomic nervous system (sympathetic, parasympathetic, and enteric systems). Extrinsic, efferent nerve pathways (parasympathetic and sympathetic) exert an influence on the enteric nervous system, but little alteration of digestive function is noted when the extrinsic pathways are severed. Within the enteric nervous system are sensory neurons, interneurons, and motor neurons that form reflex pathways that are anatomically and physiologically independent from the central nervous system (CNS). The peristaltic reflex and intrinsic vasodilator reflex are examples of the reflexes coordinated by the enteric neuron circuitry (Furness and Costa, 1980).

The enteric nervous system contains many neuropeptides similar to those found within the CNS (Tapper, 1983). Some of the neuropeptides present in the enteric nervous system include bombensin, cholecystokinin, dynorphin, enkephalin, neurotensin, neuropeptide y, somatostatin, substance P, and vasoactive intestinal peptide (Dockray, 1987).

Neuroregulation of water and electrolyte transport by the intestinal epithelium is important in gastrointestinal function.

Studies were conducted by Wright et al. (1940) in decerebrate or decapitate cats that under went various procedures such as vagal stimulation, severing preganglionic sympathetic fibers and administration of eserine, acetylcholine, and atropine. All of these procedures, with the exception of atropine administration, resulted in increased intestinal secretion. Atropine caused cessation of secretion induced by vagal stimulation. These studies suggested that the autonomic nervous system plays a role in regulating intestinal absorption and secretion.

The gut contains as many neurons as the spinal cord, approximately 10^7 to 10^8. Ten or more different types of enteric neurons have been discovered based on electrical, pharmacological, histochemical, biochemical, ultrastructural, and functional studies (Furness and Costa, 1980). The enteric nerves form five nerve plexuses: subserous, myenteric, deep muscular, submucous and mucosal (Davenport. 1977; Furness and Costa, 1980) with the majority of neurons located within the myenteric (Auerbach's) and submucous (Meissner's) plexuses (Cassuto et al., 1981b). The plexuses consist of interconnecting nerve strands and ganglia (Cassuto et al., 1981b). Nerve fiber bundles connect the ganglia in the plexuses forming a continuous meshwork. Sympathetic and parasympathetic axons along with processes of sensory neurons enter the fiber network and connect with the intrinsic neurons. Intrinsic neuronal axons, however, make up the majority of the network fibers (Furness and Costa, 1980). The fiber network projects the length of the intestine within the plexus as well as forms interconnections between other plexuses and layers of the intestinal wall (Furness and Costa, 1980).

The presence of cholinergic nerve fibers as detected by a cholinesterase stain by Isaacs et al. (1976) was represented by dense staining especially around the crypts and also in the villi with fibers approaching close to the villous epithelial cells. Jacobowitz (1965), used a histofluorometric method for the detection of monoamine-containing fibers as markers for adrenergic neurons. In the cat, most of the mucosal adrenergic fibers ended on or near the basal portion of the glandular epithelium with some mucosal branches traced almost to the surface epithelium.

More conclusive studies were conducted in the distal jejunum of the rat by Thomas and Templeton (1981). They used a histofluorescence technique to detect fibers containing catecholamines and noted that several nerve fibers were detected in the crypt region, but only a single fiber appeared to extend toward the villous tip. Work conducted by Keast et al. (1985) using immuno-histochemical techniques to map the distribution of peptide containing neurons in the mucosa of the small and large intestines of five mammalian species demonstrated a common pattern. Varicosities were noted in most of the mucosal nerve fibers, but no fibers were observed penetrating the epithelium. Nerve fibers within the lamina propria of the small intestine formed dense networks around and below the crypts. The fibers were observed in close proximity to the epithelium. In the tips of the villi, fibers were associated with small blood vessels. The nerves, present as single fibers or bundles, occasionally ran subjacent to the epithelium and divided into finer bundles or single fibers that were located immediately beneath the epithelium.

A comparison of proximal and distal sites in the small intestine of the rat and guinea pig revealed an increase in the density of mucosal nerves distally; there was a tendency for the nerve bundle thickness and the number of observed nerves in the villous tip to increase. Other putative neurotransmitters or neuromodulatory substances found in the enteric neurons include: acetylcholine, norepinephrine, 5-hydroxytryptamine, purine nucleotides, dopamine, glycine, motilin, angiotensin, secretin, galanin, GABA, gastrin, histamine thyrotropin-releasing hormone, and prostaglandins (Wood, 1987).

In vivo experiments were conducted in cats and rats using isolated jejunal intestinal segments with intact vascular supply, and elimination of the cholinergic influence by intravenous (iv) administration of atropine in cats and total denervation of the intestinal segment in rats. Exposure of the intestinal segments to cholera toxin revealed that the enteric nervous system is involved in the secretory response to cholera toxin. Tetrodotoxin injected into the superior mesenteric artery or lidocaine added to the intestinal lumen caused a marked reduction in the rate of cholera toxin stimulated secretion which in the majority of experiments was reversed to net absorption. In control intestinal segments, tetrodotoxin and lidocaine had no effect, or in the case of lidocaine in cats, a significant reduction in absorption was noted (Cassuto et al., 1981c). Additional work showed that hexamethonium, a nicotinic ganglionic receptor blocker also decreased cholera induced secretion in the cat (Cassuto et al., 1983). In further support of the involvement of enteric nerves in cholera toxin induced secretion it has been demonstrated in cats that vasoactive intestinal

polypeptide (VIP) is released in increasing amounts from cholera toxin stimulated intestinal segments and that tetrodotoxin inhibited the secretory response and the release of VIP (Cassuto et al., 1981a).

Using fluorescence microscopy it was shown that exposure of feline jejunal segment to cholera toxin resulted in a significant decrease (70%) of serotonin (5-HT) in enterochromaffin cells both in crypts and on the villi (Nilsson et al., 1980).

Cassuto et al. (1980) demonstrated in the rat that when the animals were made tachyphylactic against serotonin or if 5-HT receptor blocking agents were given iv that cholera toxin induced secretion was inhibited and in most instances absorption was induced. These observations support the hypothesis that 5-HT is released from enterochromaffin cells by cholera toxin and stimulates adjacent dendrites. At the other end of the reflex vasoactive intestinal polypeptide may be one of the transmitters released to stimulate the enterocytes of the crypts and/or villi.

A secretory response stimulated by the heat-stable enterotoxin from a strain of E. coli producing both STa and STb was reduced in the periarterially denervated jejunal segments of rats by hexamethonium (iv) and lidocaine (applied serosally). Similar results were noted in cats by close intra-arterial administration of tetrodotoxin. The results suggest that enterotoxins may evoke a secretory response in part via the enteric nervous system (Eklund et al., 1985).

Tactile stimulation of the extrinsically denervated jejunum and ileum of dogs (modified Thiry-Vella loop preparation) caused a significant increase in fluid secretion. Atropine (subcutaneous) and

hexamethonium (iv) administered 30 minutes before tactile stimulation abolished the secretory response, which suggests the response involved intrinsic neural reflexes (Caren et al., 1974).

During sympathetic nervous stimulation an increase in net water uptake was noted in the jejunal segments of cats in which the distal ends of the divided splanchnic nerves were placed on silver ring electrodes. During the time of stimulation and increased net water uptake, an increase in blood pressure and decrease in total intestinal blood flow was observed (Brunsson et al., 1979).

Arterial and venous vessels are often accompanied by adrenergic fibers although stimulation of the sympathetic vasoconstrictor fibers does not decrease villus blood flow (Svanik, 1973).

Ion Transport in Small Intestine

The intestine is traversed by solute and water through two parallel routes, the paracellular and transcellular pathways. The paracellular pathway occurs through tight junctions and between cells. This is a major route for the passive transport of ions (Armstrong, 1987) and other water-soluble substances (Anonymous, 1983) across the intestine. In the transcellular pathway the apical cell membrane and basolateral cell membrane must be traversed. Since most water-soluble substances utilizing this route employ a specialized transport process, this pathway is also called the active transport path.

Studies on the tight junctions of the paracellular shunt pathway indicate that it is lined with negative charges, thus it displays cation

selectivity (Powell, 1981a). A comparison of the relative membrane permeability to these ions displays a selectivity pattern such that PK > PNa > PCl (Powell; 1987; Armstrong, 1987).

Net water flow across the intestinal mucosa has been established as a passive phenomenon (Armstrong, 1987) due to the coupling of net water flow to active net ion transport (Trunheim, 1984). The intestinal mucosa can transport water against an osmotic gradient (Powell, 1987). A number of models have been proposed as a mechanism by which the ion flow is coupled to the flow of water.

The double membrane or three compartment model proposed by Curran and Macintosh (1962) is represented by two membranes (A and B) that separate three compartments: I, II, and III. Movement of fluid occurs against an osmotic gradient from compartment I to compartment II when the osmolarity in compartment II is greater than in compartment I and the permeability of membrane B is greater than A. Compartment I represents the intestinal lumen, compartment II the intercellular spaces and compartment III the lumen of the capillaries and lymphatics. The epithelial cells possibly represent membrane A and the epithelial basement membrane or the endothelium of the capillaries and lymphatics may represent membrane B. An osmolality of 800 to 900 mOsm may occur in the villus tip during absorption of a glucose-saline solution. Thus compartment II, in vivo may be represented by the lamina propria between the basement membrane and the blood and lymph vessels rather than the intercellular space.

Experimental evidence has been presented for a counter current multiplier system in the villus of the dog. The occurrence of the

hyperosmolar environment in the villus tip is due to a proposed intestinal countercurrent mechanism (Lundgren, 1984; Bond et al., 1977). The arrangement of the vasculature in the villi is such that a central arteriole branches into a close meshed capillary network subjacent to the epithelial cells, with the capillaries joining venules at various levels. Solutes that easily diffuse may accumulate in the villous tip by diffusing from the subepithelial capillary network to the central arteriole. The difference in concentration between the subepithelial capillaries and the central arteriole is multiplied along the length of the villus by the flow of blood through the hairpin vessels. Either the solute then diffuses along a concentration gradient toward the central vessel or water may move in the opposite direction due to osmotic forces.

A second model, the standing osmotic gradient, has been proposed by Diamond (1977). In this model and its variants, Na is actively pumped into the lateral intercellular spaces thus raising the osmotic pressure and delivering a net positive charge to this compartment. Negatively charged ions are drawn into this space further raising the osmotic pressure. The increased osmotic gradient causes a net flow of water from the lumen into the lateral intercellular space via the tight junctions and cell membranes. This results in an increase in hydrostatic pressure in the intercellular space which drives the solute and water across the basement membrane and into the capillary. In order for this model to function, the resistance to flow through the tight junction must be of greater magnitude than the resistance across the basement membrane (Argenzio, 1984).

Some studies propose a major role for the tight junction in net

absorptive water movement (Schultz, 1979; Schultz et al., 1974). Other studies indicate the tight junctions only account for approximately 10 to 16% of net water absorption (Wright et al., 1972; Van Os et al., 1974). Thus, it remains unsettled as to whether osmotic volume transfer in the small intestine is primarily transjunctional or transcellular.

The movement of ions and other solutes across the absorptive cells of the small intestine involves two steps. The first is the entry into the cell across the apical or lumenal membrane and the second is the exit across the baseolateral membrane. Different transport processes are involved in these two steps (Armstrong, 1987).

Several transport processes have been proposed for Na and Cl absorption in the small intestine. Included among the processes are 1) uncoupled (electrogenic) Na absorption, 2) Na absorption coupled to absorption of organic solutes, 3) Neutral Na-Cl cotransport, 4) Coupled Na/H and Cl/HCO3 antiport systems, and 5) passive (convective) Na absorption.

<u>Uncoupled (electrogenic) Na absorption</u>

The interior of the cell is at least 40 mV negative with respect to the mucosal solution and the intracellular Na concentration is approximately 1/10 that in the mucosal and interstitial solutions (15 mEq intracellular vs 140 mEq surrounding solutions). Na entry into the cell is driven by simple diffusion down chemical and electrical potential gradients. Na exit from the cell is directed against steep chemical (15 mEq to 140 mEq) and electrical (-40 mV to + 3 mV) potential differences as noted in Figure 1. The driving force for Na exit is provided by the basolateral Na pump (Na-K-ATPase), which obtains its

energy from ATP hydrolysis (Granger et al., 1985). Studies utilizing serosally applied ouabain (Schultz, 1980), autoradiography and enzymatic analyses of isolated small intestinal membranes have demonstrated that the ouabain-sensitive ATPase is localized in the basolateral membranes (Schultz, 1978). The sodium pump extrudes three sodium ions for every two potassium ions it brings into the cell creating an electrogenic potential and a low intracellular sodium concentration. This provides an electrochemical driving force for Na entry. The electrical potential difference between the mucosal and serosal solutions (3-5 mV serosal solution positive) creates a driving force for the passive diffusion of Cl from mucosa to serosa (Schultz, 1980).

Na absorption coupled to absorption of organic solutes

The absorption of a number of water-soluble organic compounds is coupled to the absorption of Na as shown in Figure 2. Examples include D-hexose sugars, neutral, anionic, and cationic L-amino acids, dipeptides, tripeptides, and vitamins (in the mammalian ileum). Na is drawn into the cell down its electrochemical gradient. A carrier molecule in the apical membrane couples the entry of Na and the organic solute. Na is extruded from the cell via the basolateral Na^{+}-K^{+}-ATPase pump, thus maintaining the electrochemical gradient for Na. The energy for the movement of the organic solute against its concentration gradient is indirectly provided by the Na pump. Movement of the organic solute out of the cell may occur by carrier-mediated facilitated diffusion (Schultz, 1980).

Figure 1. Cellular model of "electrogenic" sodium transport by small intestine accompanied by passive, "electrically coupled" chloride absorption (redrawn from Schultz, 1980)

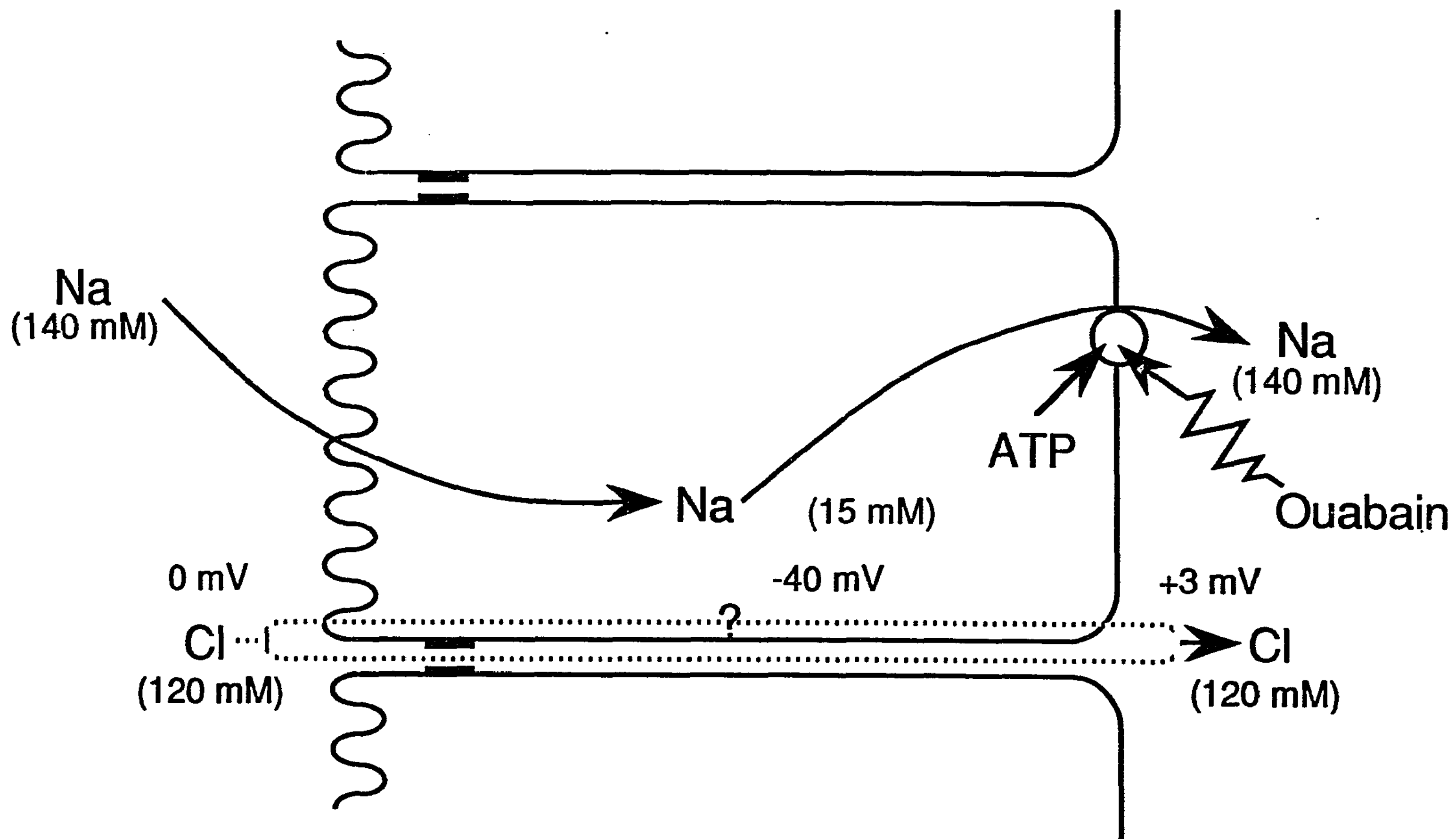
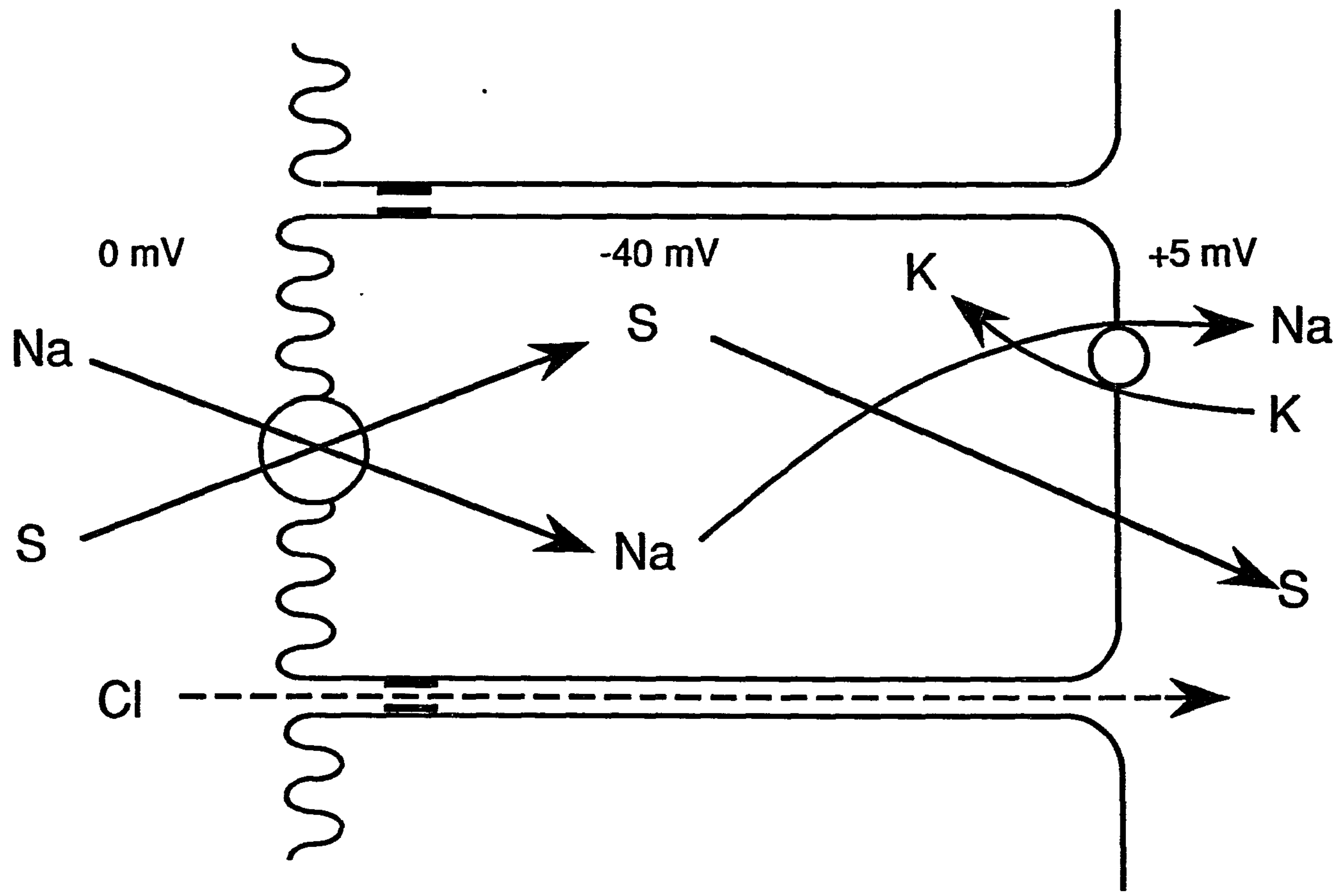

Na
(140 mM)
Na
(15 mM)
Na
(140 mM)
ATP
Ouabain
0 mV
-40 mV
+3 mV
?
Cl
(120 mM)
Cl
(120 mM)

Figure 2. Cellular model of sodium-coupled absorption of organic solutes (S) by small intestine (redrawn from Schultz, 1980)


0 mV
-40 mV
+5 mV
K
S
Na
Na
K
S
Na
S
Na
Cl

Neutral Na-Cl cotransport

This model, illustrated in Figure 3, utilizes a carrier molecule that couples the one for one, neutral entry of Na and Cl at the apical membrane. Elevated intracellular levels of cyclic AMP and calcium has been shown to inhibit this entry process (Frizzell et al., 1975; Frizzell et al., 1979; Nellans et al., 1973). The movement of Na down its electrochemical gradient energizes the movement of Cl against the uphill electrical potential difference. Na is extruded from the cell via the Na-K-ATPase activated pump thus maintaining the electrochemical gradient for Na. Chloride ions passively move out of the cell down their electrical gradient.

Countertransport

A second mechanism proposed for neutral NaCl absorption promotes the existence of two neutral antiport processes. The processes are a Na-H exchange and a Cl-HCO_3 exchange illustrated in Figure 4. In this model, carbon dioxide and water, under the influence of carbonic anhydrase, combine in the enterocyte to form carbonic acid. The carbonic acid dissociates into hydrogen and bicarbonate ions. Na moving down its electrochemical gradient into the cell provides energy for the uphill movement of hydrogen ions from the interior of the cell to the mucosal surface. Bicarbonate moving down its electrochemical gradient energizes the entry of chloride into the cell against its electrical gradient. In the lumen, the entering hydrogen and bicarbonate ions combine to form water and carbon dioxide. Thus, the downhill flow of Na energizes the uphill transport of Cl (Schultz, 1981). A one-for-one absorption of sodium and chloride ions was the net effect of this

Figure 3. Cellular model of neutral sodium chloride absorption by small intestine (redrawn from Schultz, 1980)

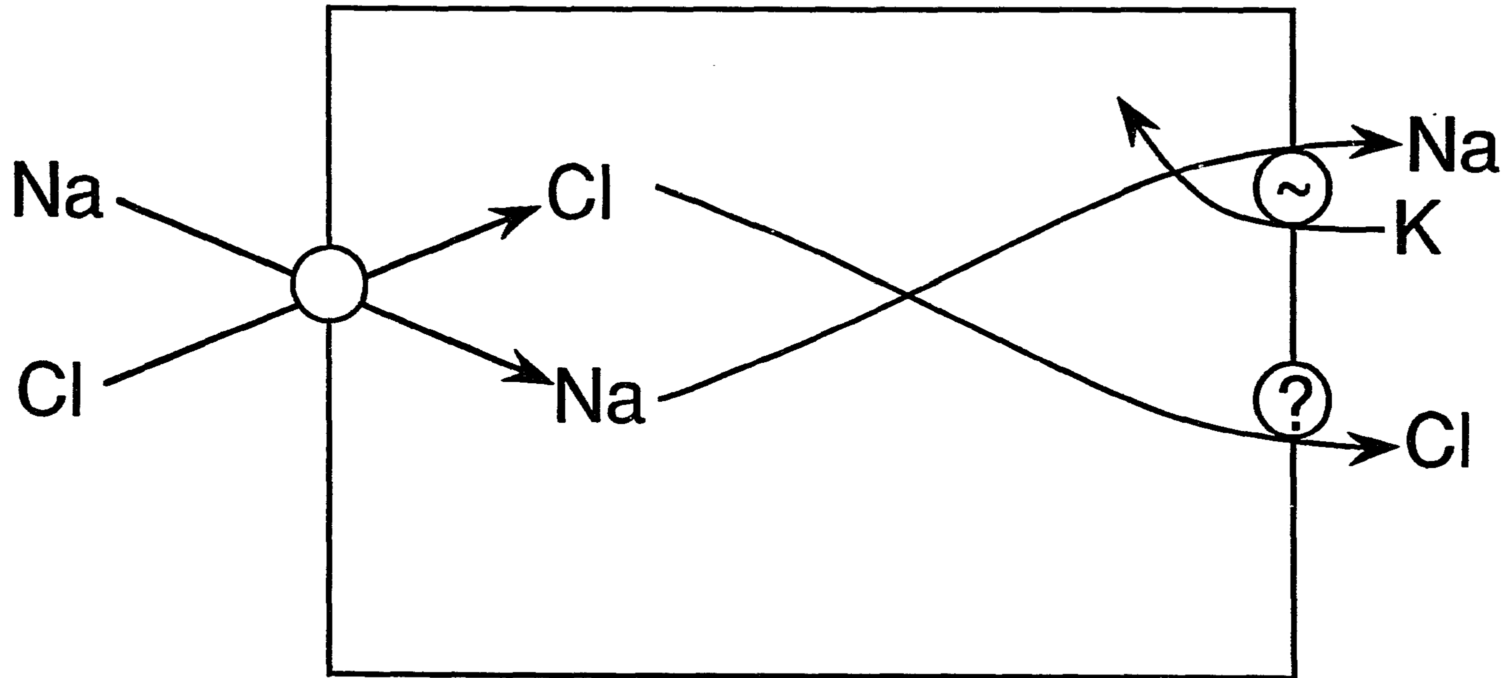
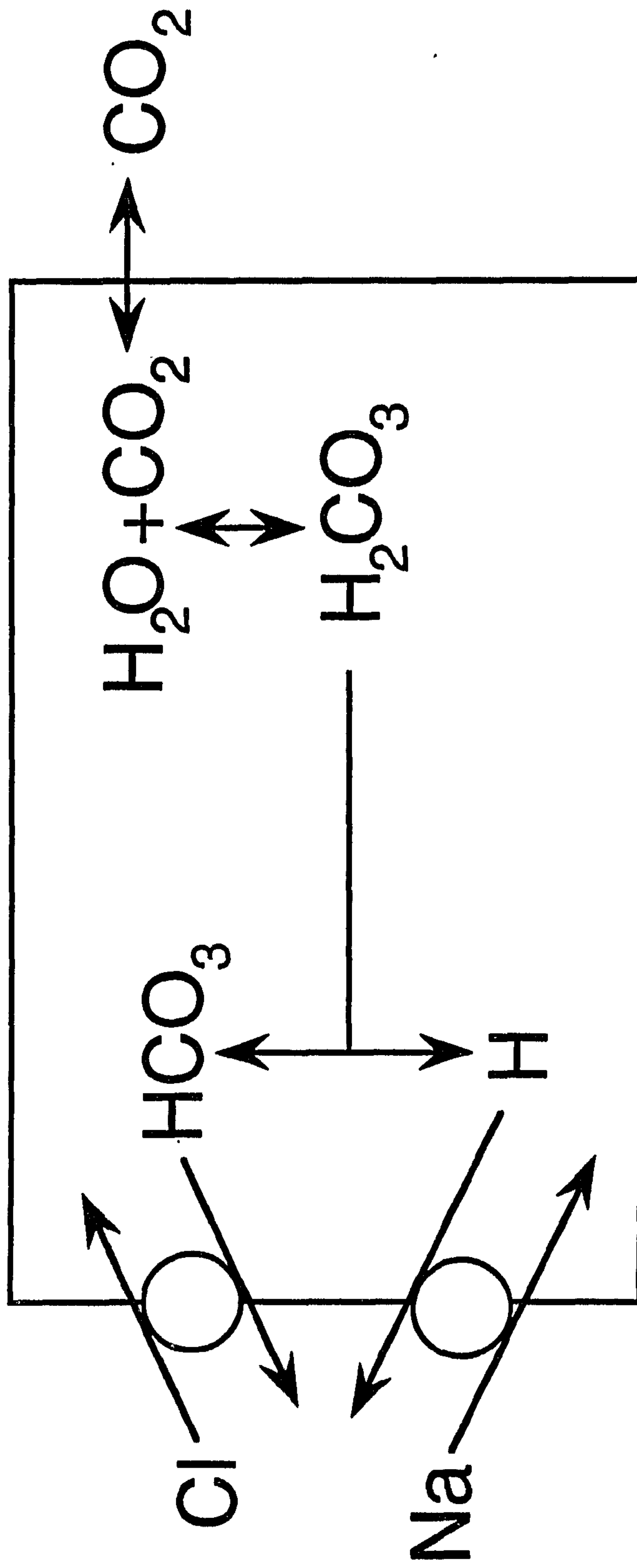

Mucosal
Solution
Cell
Serosal
Solution
Na
Cl
Cl
Na
Na
~
K
?
Cl

Figure 4. Sodium and chloride entry occur via parallel exchange mechanisms, which may be coupled through the mediation of intracellular factors (e.g., H^+) (redrawn from Armstrong, 1987)



CO2
H2O+CO2
H2CO3
HCO3
H
Cl
Na

process (Granger et al., 1985; Schultz, 1981). It has been proposed that the two antiport processes could be indirectly coupled by intracellular pH, but this remains to be supported experimentally (Schultz, 1981).

Passive (convective) Na absorption

Passive transport secondary to the bulk flow of water is another method of Na absorption. This process is of greater importance in the jejunum because of the relatively large size of intercellular pores in this tissue. As much as 85% of the increased Na transport during glucose absorption is passive. Sodium is carried along with the increased amount of absorbed water (convection) due to the active absorption of glucose. Absorption of Na passively by convection occurs through the tight junctions and lateral spaces (Granger et al., 1985).

In the small intestine, all of the mentioned mechanisms of Na absorption are present, but the relative contribution of each mechanism to the total transport of sodium differs in the jejunum and ileum. The predominant process in the jejunum is the passive transport of sodium with a smaller contribution made by uncoupled and organic solute stimulated sodium absorption. Uncoupled sodium absorption and neutral sodium chloride absorption are predominant in the ileum with minimal contributions from organic solute stimulated absorption and passive transport.

In the jejunum, active Cl absorption is reported not to occur. There is no evidence of a direct linking of Na and Cl transport nor of a Cl/HCO_3 or Cl/OH exchanger (Gunter and Wright, 1983).

In the ileum, Na and Cl transport are linked. Na/H and Cl/HCO3

exchangers are present and the Na substrate mechanisms are less prominent (Nellans et al., 1973; Nellans and Popovitch, 1981).

A cyclic AMP inhibitable Na-Cl cotransport system has only been demonstrated in the rabbit ileum. It remains to be determined if it is also present in the jejunum (Schultz, 1981).

A sodium and chloride-linked transport process, a Na-K-Cl_2 cotransporter, has been noted in the apical membrane of flounder intestine. Flounder intestine is characterized by the absence of crupts. The stripped flounder epithelium, under open-circuit conditions and in the absence of a chemical gradient, generates a negative serosal potential difference. This has not been noted in mammalian intestine. Under short-circuit conditions, the rate of chloride absorption is threefold greater than sodium. In the absence of sodium, active absorption of chloride is inhibited. Secretion of potassium by this tissue also has been observed under short-circuit conditions. It has been postulated that sodium, potassium, and chloride are transported across the apical cell membrane of the flounder enterocyte. This transport process is driven by the sodium gradient. Potassium is recycled to the lumen by a potassium conductance pathway. Sodium is pumped into the lateral intercellular space and recycles back to the lumen via a highly cation selective junctional complex. Virtually all of the chloride that enters the intercellular space is prevented from recycling to the lumen. Interestingly cholera toxin and heat stable enterotoxin had no effect on flounder intestine. Calcium, cAMP, and cGMP all caused a reduction of the transepithelial potential difference. It has been suggested that a basolateral Na-K-Cl_2 cotransport process,

inhibitable by loop diuretics, is located in the baseolateral membrane of mammalian small and large intestine, but as of yet it has not been identified (Donowitz and Welsh, 1987).

Chloride secretion

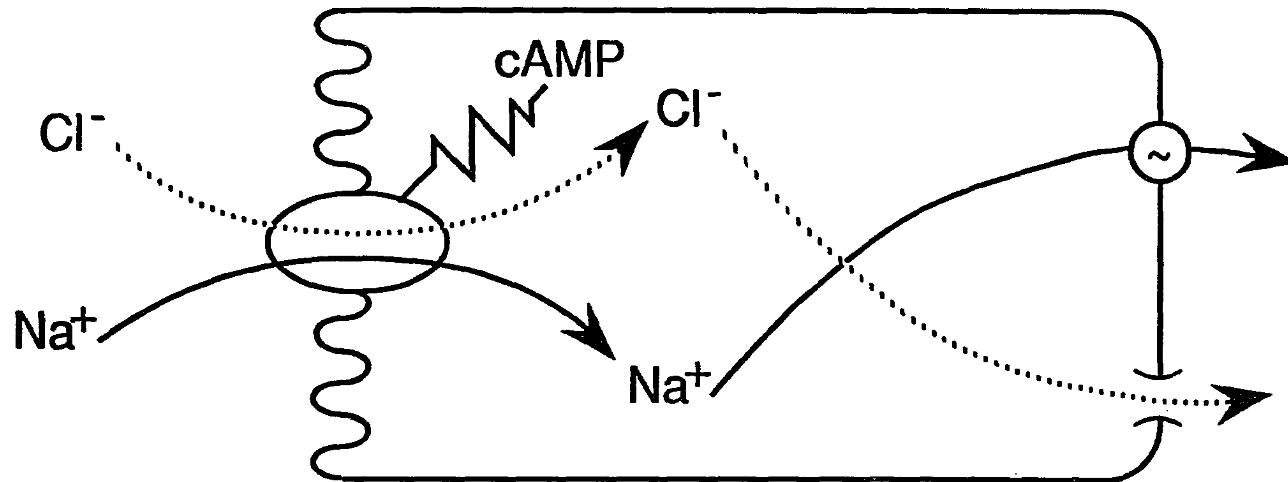
Frizzell et al. (1980) has proposed a model for the secretion of chloride as illustrated in Figure 5. The entrance of Cl across the serosal membrane is coupled to the entry of Na (serosal Na-Cl cotransport). Chloride ions accumulate intracellularly above its electrochemical equilibrium due to the driving force of Na which moves down its electrochemical gradient. The Na that enters the cell with the Cl is actively extruded by the basolateral Na-K-ATPase. Thus, the Na pump activity maintains the low Na intracellular concentration upon which the driving force of Na depends. In the resting secretory cell, all or most of the Cl entering by the basolateral Na-Cl cotransport recycles through the basolateral membrane by Na-independent permeability or by Na-Cl cotransport (Schultz, 1981). Little or no Cl leaves via the luminal membrane. The cell is "poised" for secretion awaiting an increase in apical membrane permeability due to the response elicited by a secretory agent (Field, 1981). Na pumped across the basolateral membrane is then passively secreted into the lumen across the intercellular tight junction driven by the transepithelial potential difference.

Bicarbonate secretion

Weikel and Guerrant (1985) have proposed the stimulation of bicarbonate (HCO_3) secretion by small intestinal epithelium exposed to the Escherichia coli heat stable enterotoxin STb. The mechanism

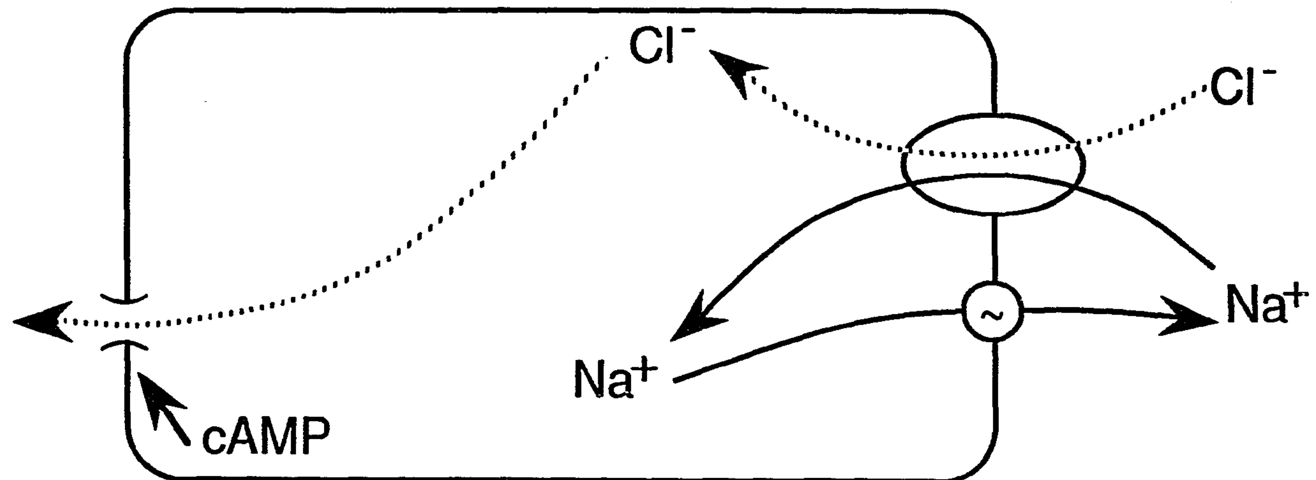
Figure 5. Model illustrating the antiabsorptive and secretory effects of cyclic AMP in the small intestine (redrawn from Field, 1980)

Villus Cell



Lumen

Crypt Cell



involved has not been defined. It is possible that the stimulated Cl secretion induced in crypt cells by secretagogues is modified by the CL/HCO_3 exchanger in the apical membrane of villus absorptive cell. Thus, this may be one facet of the HCO_3 secretory mechanism in diarrheal diseases (Field, 1981). Secretory stimuli in general increase the potential difference across the intestinal mucosa, halt Na absorption and reverse Cl absorption to secretion. In vivo they also induce HCO_3 secretion as demonstrated by cholera toxin (Rao and Field, 1983).

Localization of absorptive and secretory processes

It is generally proposed that the electrolyte absorptive and secretory processes are conducted by the villus absorptive and crypt cells, respectively. Experimental evidence such as studies demonstrating amino acid uptake only being noted in epithelial cells in the upper 30% of the villus (Nilsson et al., 1980; Paterson and Smith, 1982), and crypt secretion of water following challenge with theophylline (Nasset and Ju, 1973) supports this viewpoint. De Jonge (1975b) demonstrated that brief exposure of rat or guinea pig intestine to cholera toxin caused stimulation of villous adenylate cyclase and inhibition of salt and water absorption. Prolonged exposure stimulated crypt and villous adenylate cyclase and induced a secretory response. Other work suggests that some of the villus cells may contribute to the secretory response (Ahrens and Zhu, 1982b). Whipp et al. (1985) noted the secretory response stimulated by STa and STb was markedly reduced in the jejunum of pigs where the mature villous absorptive cells were markedly reduced and crypt hyperplasia was produced by exposure to transmissible gastroenteritis virus (TGE). In contrast, the secretory

response to PGE_1 was not decreased. This suggests that part if not most of the secretory response to STa and STb was dependent on the presence of mature villous epithelium while the secretory response to PGE_1 was mostly if not totally dependent on the undifferentiated crypt epithelium. Thus, the response to STa is consistent with the reported increasing gradient of guanylate cyclase from crypt to villous tip (Quill and Weisner, 1975). Additional evidence supporting the secretory role of absorptive epithelium was demonstrated by Donowitz and Madara (1982) in rabbit ileum. In their study, the mucosal surface was exposed to a calcium free solution containing ethyleneglycol bis(aminoethylether)tetraacetate (EGTA), causing damage only to villus epithelial cells. They noted that damage to the villous epithelium resulted in about a 50% reduction in the electrical response and a decrease in chloride secretion stimulated by theophylline. Isolated enterocyte studies were unable to demonstrate any difference in the rate of chloride efflux from villus and crypt cells. An increase in the chloride efflux rates was noted when villus and crypt cells were exposed to a cell free filtrate of culture broth containing STa and STb, but again no difference in the rates of chloride efflux in the different cell fractions was detected (Panichkriangkrai, 1982).

Intracellular Molecular Systems of Communication Involved in Small Intestinal Ion Transport

There are at least four intracellular molecular systems of communication possibly involved in secretory response of intestine.

They are 1) cyclic nucleotides; 2) calcium; 3) phosphatidylinositol metabolites; and 4) some forms of the prostaglandins. These systems may act independently or may have multiple interactions.

E. W. Sutherland and T. W. Rall (1958), the discoverers of cyclic adenosine 3,5-monophosphate (cAMP), were the first to refer to the compound as a "second messenger" and the activator of adenylate cyclase. Calcium (Ca) was suggested by Rasmussen (1970) as qualifying as a second messenger. It has been shown that the phospholipid, phosphatidylinositol, is the precursor of at least two second messengers, diacylglycerol (DG) and inositol triphosphate (IP_3) (Nishizuka, 1984). Prostaglandins although not traditionally classified as second messengers may act intracellularly or extracellularly to influence the production of other second messengers or in turn other prostaglandins.

Studies by Field (1980) with small intestine mounted in Ussing chambers show that increased cAMP caused increased sodium-dependent chloride secretion and decreased sodium chloride cotransport absorption across the brush border. One model proposes that cAMP stimulates chloride secretion primarily in crypt cells and inhibits neutral sodium chloride absorption primarily in villus cells as depicted in Figure 5.

Normally rabbit ileal epithelium absorbs Na and Cl and secretes HCO_3 and displays a serosa-positive transepithelial potential difference (Rao and Field, 1983). Studies by Field (1971a) utilizing rabbit ileum mounted in Ussing chambers noted that serosal addition of cAMP, dibutyryl cAMP or theophylline (a phosphodiesterase inhibitor) caused an increase in the potential difference and the short circuit current.

Coinciding with these electrical events an inhibition of Na absorption and a reversal of chloride absorption to secretion were also observed.

It is proposed that cAMP affects intestinal ion transport at two different sites, the villus and crypt cells. In the villus cell, cAMP affects neutral sodium chloride absorption. A low intracellular Na concentration maintained by the basolateral Na-K-ATPase (Na-pump) creates a Na gradient responsible for the uphill entry of Cl across the luminal membrane via the NaCl cotransport process. The electrically silent cotransport process has been shown to be inhibitable by the renal loop diuretic furosemide (Guandalini et al., 1982) and cAMP (Nellans et al., 1973). Similar inhibition of NaCl cotransport by cAMP has been reported in rabbit gallbladder (Frizzell et al., 1975).

The molecular mechanism involved in the inhibition of Na-Cl cotransport is unknown. Cholera toxin, heat labile E. coli enterotoxin, and prostaglandins (Rao and Field, 1983; Smith et al., 1987) can stimulate adenylate cyclase. Cyclic AMP-dependent protein kinases could be activated by the elevated intracellular level of cAMP resulting in the phosphorylation of a protein in the brush border membrane. This may result in the inactivation or closing of a NaCl carrier or channel. A second possibility is the indirect action of cAMP via another messenger system such as Ca (Berridge, 1985) which may directly affect the membrane protein, carrier or channel (Rao and Field, 1983).

Active anion secretion of Cl is stimulated in the crypt cell by cAMP. Similar mechanisms of secretion are noted in the cornea (Klyce and Wong, 1977). Chloride accumulates intracellularly above the electrochemical equilibrium via a Na-Cl cotransport mechanism located at

the base of the cell. Like its counterpart in the luminal membrane of the small intestine, the Na-Cl mechanism is inhibited by loop diuretics (Donowitz and Welsh, 1987; Mailman, 1981). Under normal nonstimulated conditions the chloride entering the cell recycles back across the baseolateral membrane. In the presence of elevated levels of cAMP the permeability of the luminal membrane to chloride greatly increased resulting in chloride secretion. An increase in potential difference and short circuit current is produced. Na is then passively moved to the lumen via the paracellular pathway. The mechanism by which cAMP increases the luminal membrane permeability to chloride is unknown. Again it may be due to membrane protein phosphorylation by cAMP-dependent protein kinase or the indirect action of cAMP via other intracellular messengers (Field, 1980).

Another cyclic nucleotide, cyclic guanosine monophosphate (cGMP), also has been shown to stimulate secretion in the small intestine. Numerous studies have shown that the heat stable _E. coli_ enterotoxin, STa, produces a secretory response in the small intestine that is associated with increased levels of mucosal cGMP (Field et al., 1978; Hughes et al., 1978). Levels of cAMP were unchanged. Paradoxically, adrenergic agonists such as epinephrine which increase absorption in the small intestine also elevate the mucosal intracellular levels of cGMP (Field and McColl, 1973; Brasitus et al., 1976). It has been proposed that there are several pools of cyclic nucleotide that can be stimulated in the intestinal mucosa (Powell and Tapper, 1979). A more detailed explanation will be found in the discussion of the enterotoxins.

Calcium, prostaglandins (Nishizuka, 1984), the heat-stable _E. coli_

enterotoxin STa (Guandalini et al., 1982; Field et al., 1978), and the hydrolytic products of phosphatidylinositol 4,5-biphosphate, diacylglycerol, and inositol 1,4,5-triphosphate (Nishizuka, 1984) all have been shown to stimulate elevated levels of cGMP.

Cyclic GMP has an effect equal to that of cAMP in reducing the Na dependent Cl influx across the intestinal brush border of rabbit ileum (Guandalini et al., 1982). However, its effect on the short circuit current is smaller than that seen with cAMP (70% of what is observed with cAMP) (Guandalini et al., 1982; Rao and Field, 1983). Correspondingly, STa stimulated Cl secretion was less than that produced by theophylline (Field et al., 1978). This may be explained by the findings of Quill and Weisner (1975) that there is a decreasing gradient of guanylate cyclase activity from the villous tip to the crypt in the rat jejunum. Alternatively, cGMP may not be as effective in elevating a chloride secretory response in the crypt cells (Field, 1980). Experiments with pig jejunum in vivo demonstrated that STa, a guanylate cyclase stimulant, induced an electrical response of smaller magnitude than that induced by theophylline, but the secretory response to STa and theophylline was equivalent (Argenzio et al., 1984). This suggests that a neutral secretory process may be involved in part of the response to STa (Whipp et al., 1985). Accordingly, it has been proposed that cGMP stimulates the Cl/HCO_3 antiport system in the luminal membrane which would provide an electrically neutral channel for the passage of Cl out of the cell (Argenzio et al., 1984). It has been demonstrated that elevated levels of cGMP stimulated by STa only inhibited net Cl absorption, but did not induce net secretion of Cl (Field et al., 1978).

As with cAMP, the molecular mechanism involved in cGMP alterations of intestinal ion transport are not known. It is possible that cGMP may activate a cGMP-dependent protein kinase that may, in a fashion similar to protein kinase activation by cAMP, phosphorylate membrane proteins that affect the activity of ionic pores and channels. Evidence bolstering this concept was observed by De Jonge and Van Dommelen (1981) and Schlatz et al. (1978, 1979). They noted that physiological concentrations of cAMP and cGMP phosphorylated a single membrane protein in the microvillus membrane and a second specific protein in the basolateral membrane of intestinal epithelial cells. Cyclic GMP may indirectly exert its activity via Ca and/or prostaglandins.

Some studies suggests that elevated levels of cGMP do not elicit any effect on intestinal ion transport. This work is reviewed in the section on STa enterotoxins.

The hypothesis that secretion occurs in the villous as well as the crypt epithelium was proposed by Naftalin and Simons (1979). They suggest that observed decreases in absorption of Na and Cl induced by cyclic nucleotides and calcium is due to the refluxing of Na and possibly some Cl in the intercellular space back through the tight junction accompanied by an increase in microvillous membrane permeability to Cl.

Supporting this hypothesis are studies with triaminopyrimidine, a polyvalent organic cation that is proposed to block the conductance of Na through the tight junction by titrating the negative charges that provide the cation selectivity to the tight junction of many epithelia (Moreno, 1975). When triaminopyrimidine is used with theophylline, the

decrease in Na and Cl absorption induced by cyclic nucleotides is blocked, but there is no alterations in the induced Cl secretion (Powell and Fan, 1983).

Calcium has been demonstrated to be a second messenger for a variety of cell functions. Secretion, contraction, phototransduction, cell division and differentiation, and alteration of K+ and Na+ permeability are a few of the processes in which calcium may play a role. The resting intracellular free calcium concentration is low in all animal cells (10^{-7} to 10^{-8} M) (Bygrave, 1978). A small change in the absolute amount of calcium within the cytosol can produce a large signal-to-background ratio. Increases in the intracellular concentration of calcium may be due to movement of calcium from the extracellular fluid across the cell membrane or from intracellular stores.

Cells must be able to detect the calcium signals. This may be accomplished by utilizing calcium-sensing molecules such as calmodulin. Calcium has been shown to act as an intracellular second messenger by binding to the mediator protein calmodulin and through protein kinase activation (Forsyth et al., 1985). The resulting calcium-calmodulin complex undergoes structural changes that translate into the alteration of activities of regulated molecules.

Secretion of fluid into the intestinal lumen can occur in response to several different diarrheogenic agents. Bile acids, hydroxy-fatty acids, Ca++ ionophores (e.g., A 23187), and bacterial enterotoxins (Field, 1980; Bolton and Field, 1977) may all stimulate a secretory process. Various intracellular mediators for these secretory agents

(e.g. cAMP, cGMP, and calcium ions) acting independently or with various degrees of cooperativity activate a secretory mechanism characterized by a net flux of water and electrolytes into the lumen of the intestine.

Second messengers associated with secretion in the intestine are considered to be capable of independent action, but this assumption has been challenged. Agents were utilized by Forsyth et al. (1979) to reduce cAMP concentrations in rabbit and weanling pig intestinal mucosa, but the net secretory effects induced by cholera toxin were not effectively reduced. The secretory response induced by cholera toxin persisted even though the concentration of cAMP was reduced by pharmacological agents. They proposed that the secretory effects of bacterial enterotoxins may not be mediated entirely through cyclic nucleotides (Forsyth et al., 1979). Studies conducted with weanling pigs demonstrated a secretory response with cholera toxin which was independent in elevation of mucosal concentration of cAMP and cGMP (Forsyth et al., 1978). Hamilton et al. (1978) found that cholera toxin and Escherichia coli heat labile toxin (LT) also failed to elevate levels of cAMP while stimulating fluid secretion in the weanling pig small intestine. Tosteson and Tosteson (1978) have demonstrated a possible role of cholera toxin as an ionophore that may be of importance in secretion. Work conducted by Thomas and Knoop (1982) suggests that the enterotoxin, ST, acts on the cell membrane to alter calcium channels leading to an increase in calcium uptake. The elevated intracellular calcium levels results in the activation of calmodulin followed by the stimulation of phospholipase A_2. Phospholipase A_2 activation leads to the transformation of membrane triglycerides to arachidonic acid which

in turn is converted to prostaglandins by cyclooxygenase and isomerase enzymes. Guanylate cyclase is then stimulated by the prostaglandins.

Studies, some of which were carried out in the rabbit ileum, show a role for calcium in the regulation of Na and Cl transport that suggest increasing intracellular concentrations of calcium decreases Na and Cl absorption and/or stimulates Cl secretion (Bolton and Field, 1977; Donowitz et al., 1981; Frizzell, 1977; Fan and Powell, 1982; Donowitz, 1983 review), while decreasing intracellular calcium levels increases Na and Cl absorption (Donowitz and Asarkor, 1982; Hubel and Callahan, 1980). The use of drugs and manipulation of the bathing solution calcium concentration were some of the methods used in these studies. Agents with calcium ionophore activity which insert into intestinal epithelial cell membranes can allow calcium ions to enter the cytosol by diffusion through a large electrochemical gradient. The calcium ionophore A23187 causes effects on Na and Cl transport that are similar, but of smaller magnitude than that noted with elevation of cAMP levels (Bolton and Field, 1977; Field, 1981; Smith and Field, 1980). The effects observed are an increase in short-circuit current, decreased absorption of NaCl or secretion of Na and Cl. These effects of A23187 on transport are calcium dependent. A23187 also caused an increase in production of prostaglandins and leukotrienes from arachidonic acid, which influences active transport of ions (Donowitz, 1983 review). Thus the changes in ion transport observed may be indirectly caused by changes in the level of intracellular calcium.

Conditions that lower intracellular calcium stimulate Na and Cl absorption and decreases short-circuit current and electrical resistance

(Donowitz and Asarkor, 1982; Hubel and Callahan, 1980). The addition of verapamil or cadmium, both calcium channel blockers, to the serosal surface of rabbit ileum causes an increase in active Na and Cl absorption (Donowitz and Asarkor, 1982) without changing the effect of cAMP or theophylline on active ion transport (Donowitz et al., 1980a).

Dantrolene is a drug that decreases free intracellular calcium (Dedman et al., 1979) by trapping calcium within intracellular stores (Desmedt and Hainaut, 1977). The serosal addition of dantrolene to rabbit ileum results in an increase in net Na and Cl absorption simulating observations noted with decreased calcium entry (Donowitz et al., 1982b).

Calmodulin is proposed to play a role in the influence of calcium on ion transport across the apical cell membrane. It is present in the soluble fraction and the microvillus core of the brush border (Glenney and Weber, 1980; Howe et al., 1980). Direct evidence of the effect of calmodulin is provided by Fan and Powell (1982) in experiments that showed a lower calcium concentration was required to reduce Na and Cl uptake into rabbit ileum microvillus membrane vesicles upon the addition of calmodulin. Experiments utilizing the phenothiazines such as trifluoperazine and chlorpromazine, inhibitors of calcium-calmodulin, have caused a decrease in the short circuit current (Smith and Field, 1980; Hubel and Callahan, 1980). Trifluoperazine blocks secretion induced by A23187 in the rabbit ileum and decreases the effect of cAMP on ion transport (Smith and Field, 1980). Since cAMP causes a increase in ^{45}Ca efflux, this may indicate increased mobilization of Ca from intracellular stores by cAMP. This may indicate that cAMP induced

alterations in ion transport utilizes calcium-calmodulin as an intermediate (Donowitz, 1983 review).

Experiments in the rabbit ileum have demonstrated that increased cAMP and calcium have redundant and nonadditive effects on active Na and Cl transport. Maximal concentrations of theophylline in combination with agents that elevate intracellular calcium did not change active ileal Na and Cl transport significantly more than theophylline alone (Donowitz et al., 1980b). Mucosal application of chloroquine, a membrane-stabilizing drug, inhibits the effect of cAMP, but not of increasing intracellular calcium on active ileal transport (Fogel et al., 1982). Also, the effects of cAMP are independent of extracellular calcium as shown by the fact theophylline causes the same alteration in ileal Na and Cl transport before and after exposure to calcium-free bathing solution (Donowitz et al., 1980a).

Calcium does not act through cyclic nucleotides to alter intestinal transport as demonstrated by the fact calcium ionophore A23187 does not change rabbit ileal cAMP or cGMP concentration (Bolton and Field, 1977). However, it is possible that an increase in mucosal calcium may mediate part of the effects of cAMP since theophylline increases the ^{45}Ca efflux from rabbit ileum and colon (Donowitz et al., 1980a). It has also been noted that in other tissues calcium is mobilized from intracellular stores by cAMP (Donowitz, 1983 review)

Calcium and cAMP appear to have similar, but nonadditive effects on ion transport in the rabbit ileum. This pattern of interaction of intracellular messengers has been referred to as redundant control (Rasmussen (1981).

It is possible that calcium exerts its effect as a second messenger through a calcium-calmodulin mechanism involving the phosphorylation of transport proteins. It has been observed that calcium-calmodulin phosphorylates peptides in microvillus membranes of rabbit ileum (Donowitz et al,, 1982a; Taylor et al., 1981). Calcium can also act by activating a calcium-phospholipid-dependent kinase. Substrates in the cell membranes and in cytosol are phosphorylated by this protein kinase (Kuo et al., 1980). The role of this protein kinase in intestinal electrolyte transport is unknown.

Studies by Fan and Powell (1982) in rabbit ileal brush border membrane vesicles demonstrated an inhibition of Na and Cl uptake by 20-30% when elevated levels of calcium were present inside the vesicle. Elevated levels of calcium outside of the vesicles had no effect. Further studies utilizing calmodulin showed that calmodulin alone displayed no effect, but when applied with calcium, Na uptake was inhibited 20% when Cl was the counter ion. Inhibition of NaCl uptake by Ca-calmodulin was abolished by trifluoperazine, a known inhibitor of calmodulin-Ca complex. Calcium-calmodulin complexes may alter electrolyte transport by direct stimulation of calcium-dependent protein kinases (Kuo et al., 1980) or they may stimulate the synthesis of prostaglandins (Powell and Fan, 1983).

Work conducted by Thomas and Knoop (1982) has implicated a role for Ca and prostaglandins in intestinal secretion stimulated by elevated levels of cGMP induced by E. coli heat stable enterotoxin. Their experiments showed a significant reduction in the secretory response induced by ST when calcium uptake was inhibited by the specific calcium

channel blockers nifedipine and diltiazem. Prostaglandins and their precursors have been shown to stimulate the synthesis of cGMP (Thomas and Knoop, 1982; Glass et al., 1977) and thus
may be involved in the intestinal secretory response to ST. Quinacrine hydrochloride, a phospholipase A_2 inhibitor and zomepirac sodium, an inhibitor of cyclooxygenase, reduced the fluid response to ST when applied simultaneously or 30 minutes prior to exposure to ST. This suggests an involvement of prostaglandins in the intestinal fluid response to ST. Further drug studies involving 8-bromo-GMP implicated that calcium uptake and prostaglandin synthesis occur prior to the activation of guanylate cyclase (Thomas and Knoop, 1982). These findings and the observations of others involving the calcium-calmodulin complexes in the ST secretory response (Abbey and Knoop 1979; Brasitus et al., 1976) led Thomas and Knoop (1982) to propose that ST induces an elevation of intracellular calcium by altering membrane calcium channels. The increased levels of calcium activates calmodulin and in turn stimulates phospholipase A_2 to convert triglycerides to arachidonic acid. Arachidonic acid is converted to prostaglandins that activate guanylate cyclase resulting in elevation of intracellular levels of cGMP.

Prostaglandins are biological compounds derived from arachidonic acid. They are synthesized in numerous tissues and modulate numerous physiological responses. The prostaglandins are not stored within cells and their effects are targeted to the cell in which they are synthesized or adjacent cells. Presently their mechanism of action is not well understood, but it appears that cAMP or calcium mediate their actions.

The release of arachidonic acid from stores within the cell may occur via three possible pathways which involve the action of a calcium-dependent phospholipase. One pathway involves phospholipase A_2 that catalyzes the release of arachidonic acid from several phospholipids. A second proposed pathway involves the conversion of phosphatidylinositol to diacylglycerol by a phospholipase C. A diglyceride lipase converts diacylglyerol to glycerol, stearic acid, and arachidonic acid. In the third pathway diacylglycerol is phosphorylated by a diglyceride kinase to form phosphatidic acid, which in turn is converted by an acid-specific phospholipase to arachidonic acid and lysophosphatidic acid (Ballou and Cheung, 1985 review).

Prostaglandins have been shown to be synthesized in the intestinal tract (Knapp et al., 1978; Le Duc and Needleman, 1979) and to induce secretion in the small intestine (Matuchansky and Bernier, 1973). Smith et al. (1982) showed that the synthesis of PGE_2 occurs primarily in the lamina propria while degradation occurs predominantly in the epithelial cells.

Matuchansky and Bernier (1973) demonstrated that intraluminal PGE_1 in human jejunum reversed net absorption of water and electrolytes which resulted in net secretion into the lumen. Glucose absorption was reduced 25%. Cummings et al. (1973) administered $PGF_{2\alpha}$ intravenously to human volunteers and demonstrated net secretion of water, sodium and chloride in the jejunum and ileum. PGE_2 serosally applied to human jejunal mucosa in Ussing chambers caused a rise in the short-circuit current, a decrease in net Na-flux and the secretion of chloride resulting in decreased conductance (Sharon et al., 1984). Konturek et

al. (1985) perfused 30 cm Thiry-Vella loops of the upper jejunum and distal ileum in dogs. PGE_2 and $PGF_{2\alpha}$ were infused intra-arterially by a catheter in the mesenteric artery resulting in a significant reduction in sodium and water absorption.

Prostaglandins have been observed to stimulate adenylate cyclase in the basolateral membrane of epithelial cells (Kimberg et al., 1971; Murer et al., 1976). Receptors for prostaglandins at this site have yet to be demonstrated. PGE_2 has been shown to induce a dose dependent elevation of cAMP in isolated rat intestinal epithelial cells and activation of adenylate cyclase in plasma membrane fractions isolated from these cells (Smith et al., 1987). The levels of PGE_2 required to significantly stimulate elevated levels of cAMP were 100 fold greater than the concentration required to cause ionic secretion in whole epithelium (Burkhave and Rask-Madsen, 1980). This suggests that mechanisms other than those involving cAMP may be also involved in prostaglandin stimulated secretion. Multiple effects are exerted on the gut by prostaglandins. In a given segment of the gastrointestinal tract different prostaglandins have different and sometimes opposite effects (Branski et al., 1986). PGE^1 and PGE_2 stimulate ion secretion (Na and Cl) in the small intestine while it is inhibited by PGI_2 (Rask-Madsen and Burkhave, 1981). PGI_2 blocks prostaglandin and cholera toxin induced enteropooling (Robert et al., 1979). Intestinal motility is stimulated by PGE_1 and $PGF_{2\alpha}$, but PGI_2 is inhibitory (Ruart and Rush, 1984). PGI_2 has been shown to stimulate the formation of cAMP in certain tissues. This contradicts the perception that the stimulation of intestinal cAMP is the mediator for enteropooling (Robert et al.,

1979).

The metabolites of lipids may also play a role as a second messenger affecting ion transport in the small intestine enterocyte. The phospholipid, phosphatidylinositol, is primarily situated in the inner leaflet of the bilayer of the cell membrane. It is converted into phosphatidylinositol 4,5 biphosphate (PIP_2) by the sequential addition of phosphate groups obtained from ATP. In response to an extracellular signal, membrane bound calcium is mobilized and phospholipase C is stimulated resulting in the hydrolysis of PIP_2 into diacylglycerol and inositol triphosphate (IP_3). IP_3 stimulates the release of calcium from intracellular pools such as the endoplasmic reticulum. The second hydrolysis product is diacylglycerol which remains in the cell membrane (Berridge, 1985). Diacylglycerol activates the calmodulin-independent protein kinase C by increasing its affinity for calcium (Nishizuka, 1984). Protein kinase C phosphorylates the serine and threonine residues on several proteins one of which is also phosphorylated by cAMP-dependent protein kinase and calmodulin-dependent protein kinases (Nishizuka, 1984).

Weikel et al. (1985) demonstrated in weaned pig jejunal loops that phorbol esters, activators of protein kinase-C, stimulated a secretory response at 5 hours post exposure with intraluminal accumulation of Na, K, Cl, and HCO_3.

Diacylglycerol may be degraded by diglyceride lipase (Ballou and Cheung, 1985 review) to glycerol, stearic acid, and arachidonic acid. Alternatively diacylglycerol may be phosphorylated by the enzyme diglyceride kinase (Bell et al., 1979) to produce phosphatidic acid.

Phosphatidic acid in turn may be acted upon by a phosphatidic acid-specific phospholipase to produce lysophosphatidic acid and arachidonic acid. It has been postulated that phosphatidic acid may act as a calcium ionophore (Marshall et al., 1980, Ballou and Cheung, 1985 review) and that the increase in intracellular calcium activates phospholipase A_2 resulting in arachidonic acid release (Ballou and Cheung, 1985 review). Marshall et al. (1980) argue against the "calcium gating" effect. They propose that the arachidonic acid release and subsequent prostaglandin synthesis produce movements of calcium which may be responsible for the cellular response or possibly calcium and prostaglandins could be acting in concert.

As the knowledge of intracellular messengers increases, it becomes evident that they do not operate independently, but have multiple interactions. Changes in the levels of one messenger influences the activities of a second and/or third messenger system. Calcium and cAMP nearly always serve together as intercellular messengers (Rasmussen et al., 1985). Calcium may regulate cAMP metabolism (Rasmussen et al., 1985) and in turn cAMP may respond in kind to modulate calcium activity (Donowitz, 1983). Increased intracellular levels of calcium can activate phopholipases. The resulting hydrolysis of phospholipids yield metabolic products that further elevate intracellular calcium levels, stimulate production of prostaglandins or other eicosanoids that may in turn modulate levels of cyclic nucleotides and intracellular calcium. The complexity of these interactions hinders the investigation of the mechanics of the second messenger systems. Another impediment to

unraveling these interacting systems is the fact that not all the second messengers signal pathways have been identified.

Enterotoxins

Acute diarrheal diseases are the major cause of morbidity and mortality in humans and farm animals throughout the world especially in developing countries. Sonme of the primary agents responsible for acute diarrheas are bacteria. Enteropathogenic bacteria may be classified as invasive or noninvasive. Invasive strains penetrate the intestinal mucosa and bring about diarrhea by destruction of enterocytes and/or stimulating an inflammatory reaction. Enterotoxins are also produced by some invasive strains. Non-invasive bacteria elicit their enteropathogenic effects by producing one or more enterotoxins (Rao and Field, 1984).

Cholera toxin (CT), produced by *Vibrio cholerae* is one of the most thoroughly studied enterotoxins that produces a secretory diarrhea. Cholera toxin is a protein with an apparent molecular mass of 84,000 daltons (Finkelstein and LoSpalluto, 1969; Finkelstein, 1973; Lonnroth and Holmgren, 1973; Van Heyningen, 1974) and is both heat and acid labile. Cholera toxin is composed of two subunits designated as A (mol. mass 27,000) and B (mol. mass 58,000) (Fishman, 1980).

The B subunit is composed of five identical polypeptide chains (Gill, 1976; Lai et al., 1977) and binds to its receptor the monosialoganglioside GM_1 (Cuatrecasas, 1973a; Cuatrecasas, 1973b; Holmgren et al., 1973) on the cell surface. A conformational change in

the structure of the toxin occurs when all five subunits are bound to a receptor. The A subunit separates from the B subunit and penetrates the cell luminal membrane (Fishman, 1980). Recently it has been proposed that the B subunit may also penetrate the cell membrane (Tsuru et al., 1982) and form a channel through which the A subunit passes (Antoine et al., 1974). After the A subunit has entered the cytoplasm, its disulfide bond is reduced and the A_1 peptide (mol. wt. 22,000) separates from the A_2 peptide (mol. wt. 5,000). The A_1 peptide catalyzes the cleavage of nicotinamide adenine dinucleotide (NAD) to nicotinamide and adenosine diphosphate ribose (ADP-ribose). The A_1 peptide then transfers ADP-ribose to the regulatory protein of adenylate cyclase. It is not known how the A_1 peptide after penetrating the luminal membrane gains access to the adenylate cyclase located on the basolateral membrane (Fishman, 1980). The regulatory protein is a GTP-binding protein whose GTPase activity is inhibited by ADP-ribosylation (Cassel and Pfeuffer, 1978; Gill and Meren, 1978; Johnson et al., 1978). The binding of GTP to the regulatory protein activates adenylate cyclase and the hydrolysis of the GTP turns off the stimulated adenylate cyclase. By blocking the hydrolysis of GTP, CT caused the permanent activation of adenylate cyclase, thus, cAMP levels are raised (Fishman, 1980; Coulson et al., 1984). After CT is applied to the mucosa of the small intestine, a lag period of approximately 30 minutes occurs before an effect on fluid transport can be detected. Maximum secretion is noted at 2 to 3 hours and continues for 8 to 10 hours (Carpenter et al., 1969). The rate limiting step appears to be the entry of the A_1 subunit into the cell (Rao and Field, 1984). The lag period may also be due to

partly the density of choleragen receptors on the cell surface and be related to the rate at which multivalent binding of the toxin to the cell occurs.

Field et al., (1968), in experiments utilizing segments of rabbit distal ileum placed in Ussing chambers, demonstrated that cAMP stimulated active secretion of chloride and possibly bicarbonate ions. Similar ionic movement was noted when cholera toxin was added to the luminal surface of the ileum. Addition of cyclic AMP or theophylline to the cholera toxin expossed ileum caused only a slight increase in ion movement. Schafer et al. (1970) noted that the exposure of canine intestine to cholera toxin increased cAMP levels 3 to 4 fold. Elevation of adenylate cyclase by cholera toxin in rabbits, guinea pigs, and man (Kimberg et al., 1971; Sharp and Hynie, 1971; Chen et al., 1971, 1972) further substantiated the link between cholera toxin and the second messenger cAMP.

Studies (Field, 1971b; Field et al. 1972) where the short-circuit current and the fluxes of Na and Cl were measured simultaneously in a isolated preparation of rabbit ileum demonstrated that cholera toxin, cAMP, and theophylline induced an increase in the short-circuit current and reduced the net sodium flux to zero. The basal flux of chloride was reversed resulting in net chloride secretion. The quantitative effects of CT were similar to those of cAMP and theophylline except that changes with cAMP occurred promptly and those due to CT occurred only after a latent period. It was concluded that CT and cAMP stimulated chloride secretion and inhibited Na reabsorption.

Experiments conducted by Hamilton et al. (1978) demonstrated that a

secretory response to CT in pigs was not accompanied by an elevation in cAMP concentrations in the mucosa. Thus intestinal secretion stimulated by CT may be accompanied by elevations in cyclic nucleotides, but it is not necessarily required.

In the 1800s it was proposed that Escherichia coli was responsible for diarrheal syndromes (Greenberg and Guerrant, 1981). Whole-broth cultures of E. coli were shown to cause a secretory response in the small intestine of rabbits in 1956 (De et al., 1956). Viable organisms were shown not to be required to elicit the intestinal secretory response in the 1960s (Moon et al., 1966; Evans et al., 1972). In the early 1970s it was found that E. coli was capable of producing at least two types of enterotoxin, a heat-labile enterotoxin (LT) and a heat-stable enterotoxin (ST) (Evans et al., 1972; Kohler, 1971; Moon and Whipp, 1971; Smith and Gyles, 1970). ST is now known to be two different types of heat-stable enterotoxins, STa and STb (Alderete and Robertson, 1978; Burgess et al., 1978; Gyles, 1979). The plasmid-encoded heat-labile and acid labile toxins vary in size, immunological reactivity and amino acid composition, but display a broad similarity to each other and CT in subunit composition and ganglioside receptor (Donta and Viner, 1975; Field, 1979; Moss et al., 1981). Others state that the LT's characterized thus far from all strains of E. coli are similar if not identical (Sack, 1980).

Heat labile enterotoxin

The structure of LT is similar to CT and consists of a subunit A of 25,500 mol. wt. and five copies of a subunit B (mol. wt. 11,500) (Dallas et al., 1979). Antisera directed against the B subunit of cholera toxin

cross-reacts immunologically with LT indicating some homology between the binding domains of each toxin (Klipstein and Engert, 1977). LT appears to bind specifically to ganglioside G_{M1}, but it has been suggested that the binding is less avid due to the fact the amount of G_{M1} required to neutralize LT biological activity is greater than for cholera (Zenser and Metzger, 1974). The biological activity of E. coli LT is not blocked to the same extent by cholera toxoid (choleragenoid) as is cholera toxin activity (Holmgren, 1973).

LT is similar to CT in that it exhibits a long duration of action and activates adenylate cyclase (Evans et al., 1972; Guerrant, 1973). The resulting elevation in cAMP results in the inhibition of coupled Na-Cl influx and active secretion of Cl resulting in reduced absorption and increased isotonic secretion (net isotonic flux). LT and CT differ in their association with the bacterial cell of origin. Some strains of Vibrio cholera release 80-90% of the toxin into the extracellular medium while 10% is present in the periplasm. Most of the LT of E. coli appear to be periplasmically located. It was proposed that LT may leak out of the periplasm as the bacterial cells grow as the result of the release of outer membrane fragments (Hirst et al., 1984).

Heat stable enterotoxins

Two other known types of enterotoxins are elaborated by E. coli in addition to or in lieu of LT. These heat and acid stable enterotoxins are designated as STa (ST I) and STb (ST II).

STa enterotoxin exists as a family of methanol soluble molecules that differ in size and amino acid composition (Burgess et al., 1978). Alderete and Robertson (1978) isolated a STa peptide consisting of 47

amino acids with a molecular weight of 4,700. So and McCarthy (1980) identified a 72 amino acid peptide with a molecular weight of 8076 which contained 7 cysteine residues and Chan and Gianella (1981) isolated an 18 amino acids peptide with a molecular weight of 1,800 and 6 cysteine residues. Robertson et al. (1983) described five different heat-stable enterotoxins (STa). The STa peptides were composed of 18 amino acid residues which contained 10 or 11 different amino acids, a high proportion of which were acidic amino acids. All contained 6 half cysteine residues.

Some of the STa family members sequenced show a homologous (or highly conserved) amino acid sequence at the carboxyl terminal (Rao and Field, 1984). The homologous C-terminal sequence of 18 amino acids is essential for its biological activity (Saeed et al., 1983; So and McCarthy, 1980; Chan and Gianella, 1981). These peptides are rich in cysteine residues indicating a complex secondary structure (Rao and Field, 1984). Preservation of the -S-S bonds is required to maintain biological activity (Aimoto et al., 1982). STa exhibits a rapid, reversible action (Field et al., 1978) and acts only on various segments of the small and large intestine (Rao et al., 1980). The C-terminal contains six half-cysteine residues partially linked by disulfide bridges. This unique structure is responsible for the heat and acid stability of the toxin and its sensitivity to reducing agents (Staples et al., 1980). Disulfide-reducing agents such as thiols have been shown to reduce the disulfide bonds in STa resulting in the loss of biological activity and reversal of the activation of guanylate cyclase (Eldeib et al., 1986).

STa stimulates particulate guanylate cyclase, but not the soluble form (Rao et al., 1980; Guerrant et al., 1980). Guanylate cyclase is localized primarily in the brush border of mature enterocytes (De Jonge, 1975a). STa stimulates a secretory response more rapidly than CT. A significant secretion of fluid is noted within 30 minutes and peak accumulation occurs within 6 hours (Hughes et al., 1978). The action of STa is reversible. Field et al. (1978) found that the electrical potential difference response in vitro was reversed by washing the mucosal surface with fresh Ringer's solution.

Hughes et al. (1978) demonstrated in rabbit intestine that culture filtrates of STa producing strains significantly elevated mucosal levels of cGMP three fold over basal levels. Cyclic AMP levels were not significantly changed. Similar results have been noted in vitro (Field et al., 1978; Rao et al., 1980). The effects of the enterotoxin STa and those of 8-bromo-cGMP, an analogue of cGMP, are similar (Field et al., 1978; Hughes et al., 1978). STa exhibits a tissue specificity in that it has no effect on cyclic nucleotide levels in tissues other than the small and large intestine (Rao et al., 1979; Rao et al., 1980; Guerrant et al., 1980).

Two models have been proposed for the coupling process between STa and guanylate cyclase. The cascade model proposes that STa binds to a protein receptor on the luminal surface of the enterocyte (Gianella et al., 1980; Thomas and Knoop, 1983) and triggers phospholipase A_2 possibly by induction of phosphoinositol turnover (Takai et al., 1981). Thus arachidonic acid is released from phospholipids or diacylglycerol is converted to endoperoxides or hydroperoxides all known to be

activators of guanylate cyclase (Spies et al., 1980 White et al., 1982).

In the second model called the direct coupling model, a toxin-receptor complex or the toxin alone combines with receptors on the guanylate cyclase and activates the enzyme by inducing a conformational change (De Jonge, 1984).

STa, like CT, appears to inhibit the Na absorptive process and reverse Cl absorption to secretion. The effects of STa or cyclic GMP on increasing net Cl secretion are similar, but not additive and of smaller magnitude to the effects observed with CT and cyclic AMP (Rao and Field, 1984; Guandalini et al., 1982). This may be due to a decreasing gradient of cyclic GMP-dependent protein kinase from villus to crypt regions (De Jonge and Van Dommelen, 1981) since guanylate cyclase is localized primarily in the brush border of mature (villus) enterocytes (De Jonge, 1975a; Quill and Weisner, 1975). Also, the secretory cells of the crypt may have fewer toxin receptors or are less responsive to cyclic GMP (Guandalini et al., 1982).

Field et al. (1978) demonstrated that STa inhibited net Cl absorption, but did not cause net secretion. They suggested that while CT's elevation of cAMP results in a inhibition of the coupled NaCl transport in the brush border and stimulation of active anion secretion, cGMP exerts only the first effect in that STa inhibited net Cl absorption, but did not cause net secretion of Cl. In contrast, the secretory response in pig jejunum induced by STa and STb was markedly reduced in pigs where the villous epithelium was decreased due to infection with transmissible gastroenteritis virus (TGE) when compared with noninfected pigs. The magnitude of this effect does not correspond

to the proposed anti-absorptive effect of STa and STb proposed by some investigators, but suggests that villus epithelium has a role in the secretory response induced by the heat-stable enterotoxin (Whipp et al., 1985).

The concept that STa elicits its effect on ion transport solely through the elevation of cGMP has been challenged. Experiments conducted with isolated enterocytes by Brasitus et al. (1976) demonstrated that epinephrine stimulated α-adrenergic receptors induced a five to fifteen fold increase in cGMP concentration. Transient increases in cGMP were also produced by cholecystokinin and insulin. Further in vitro work with rabbit ileum in Ussing chambers showed that the exposure of the tissue to epinephrine stimulated Na Cl absorption and decreased the short circuit current. Additions of cGMP or 8-bromo-cGMP did not reproduce the effect of epinephrine on Na Cl transport or short circuit current. Guandalini et al. (1982) concluded from this previous work and similar in vitro experiments that the levels of cGMP stimulated by epinephrine, cholecystokinin, and insulin were not of sufficient magnitude to influence ion transport and that the elevation of cGMP concentration occurs at a site in the mucosa other than in the enterocytes.

Work by Rao et al. (1980) demonstrated that STa when applied luminally caused an elevation of cGMP in duodenal, jejunal, ileal, cecal, and distal colonic mucosa, but stimulated short-circuit current in only in jejunal tissue. It is possible that the secretory mechanisms in the proximal and distal portions of the intestine are not cGMP-responsive or that elevated levels of cGMP are stimulated by the

enterotoxin, but do not have a casual role in the secretory process.

Studies in the suckling mouse have demonstrated that indomethacin, intragastrically administered, significantly reduced ST induced secretion, but did not affect basal secretion nor fluid secretion induced by 8-bromo-cGMP. In homogenized rat mucosal scrapings indomethacin reduced both basal and ST induced guanylate cyclase activity. These findings suggest that the action of indomethacin occurs before the activation of guanylate cyclase. Due to the finding that similar concentrations of indomethacin inhibit the effects of ST, but not the secretory response induced by 8-bromo-cGMP, it has been suggested that prostaglandin synthesis may be involved in the ST induced activation of guanylate cyclase. In the same study it was found that butylated hydroxylanisole, an antioxidant reduced ST activation of guanylate cyclase activity and fluid secretion. Basal guanylate activity and fluid secretion were not affected. In addition the secretory response to 8-bromo-cGMP was not affected. This suggests that an oxidative event may occur during ST induced secretion before the activation of guanylate cyclase (Guerrant et al., 1980).

STa binding studies conducted by Gianella et al. (1983) have shown that binding was: 1) rapid; 2) reversible; 3) saturable; 4) temperature dependent; 5) specific for STa; and 6) correlated with a biological response (production of cGMP). This data strongly supports the existence of STa receptors. Immature rats (14 and 21 days old) have 600 fold increased jejunal sensitivity to STa as compared to adult rats. This increased sensitivity is suggested to be due to an increased number of jejunal receptors in the immature rat (Cohen et al., 1986).

STb is the second heat-stable enterotoxin produced by Escherichia coli. In 1970, Moon and Whipp noted that some strains of E. coli caused secretion in pigs less than two weeks of age. A second class of E. coli produced a secretory response in piglets of all ages (Moon and Whipp, 1970). Jacks and Wu (1974) and Jacks et al. (1973) reported that they could only detect LT activity in the E. coli strain P 307 using the infant mouse assay and rabbit loop assay. Previously Gyles and Barnum (1969) and Smith and Gyles (1970) using the same strain of E. coli had detected ST activity in pig intestinal loops. Burgess et al. (1978) during initial purification of ST detected two forms of ST. The first was a methanol soluble fraction that was active in infant mice and neonatal pigs which was designated as STa. The second form, STb, was methanol insoluble, inactive in infant mice, but active in older piglets. It was proposed by Nalin et al. (1978) that certain E. coli stains produce a type of ST that is detected in the dog, but not in the infant mouse. Experiments conducted by Gyles (1979) supported the view that two forms of heat-stable enterotoxin exist, ST1 which elicits a secretory response in infant mice, ligated intestine of rabbits, and ligated intestine of weaned pigs, and ST2 which produces a response only in the ligated pig intestine.

Olsson and Soderlind (1980) demonstrated that some E. coli stains isolated from cases of porcine neonatal diarrhea failed to produce a positive response in the infant mouse assay, but were positive in intestinal loops of 3 to 7 week old pigs.

Kashiwazaki et al. (1981) examined 49 strains of E. coli isolated from piglets with or without diarrhea. All strains were negative for LT

and heat-stable enterotoxins by the Y-1 adrenal cell test, rabbit ileal loop test, and the infant mouse test. Seven strains were positive only in ileal loops of pigs. The enterotoxic component had a rapid onset of action, was of long duration, and was resistant to heating at 80°C for 20 minutes. The stains that were positive in the pig ileal loop assay were designated as "ST pig loop".

Lee et al. (1983) and Picken et al. (1983) working independently cloned the gene encoding STb. From the nucleotide sequence they deduced the amino acid sequence. They propose STb is formed as a 71 amino acid structure with a 18 to 23 amino acid signal sequence. The amino acid sequence of STb does not bear any resemblance to that of STa, but the size of the peptide is similar to some isolated STa peptides. The STb peptide was found to have four cysteine residues evenly distributed throughout the molecule. A series of hydrophobic amino acids are located at the carboxyl terminal. Utilizing a DNA fragment containing the STb gene as a probe, Lee et al. (1983) found that it only hybridized to DNA from E. coli known to produce STb. So and McCarthy (1980) demonstrated that a STa probe did not hybridize with DNA from STb producing strains.

It is generally agreed that STb is not active in the infant mouse, but is active in the weaned pig (Burgess et al., 1978; Gyles, 1979; Olsson and Soderlind, 1980; Whipp et al., 1981; Kashiwazaki et al., 1981; Kennedy et al., 1984). In 3 to 9 week old weaned pigs STb filtrates are more active than STa filtrates (Kennedy et al., 1984). During initial purification attempts Burgess et al. (1978) demonstrated activity of STb in rabbit loops using the methanol insoluble fraction of

a 10-fold concentrate of crude culture filtrate. STb has been shown by other investigators (Kashiwazaki et al., 1981; Kennedy et al., 1984; Olsson and Soderlind, 1980; Weikel and Guerrant, 1985) to be inactive in the rabbit. Kennedy et al. (1984) did not note a secretory response to STb in the rat small intestine.

Studies using DNA probes and DNA colonizing techniques showed that 74% of enterotoxigenic E. coli isolated from swine carried the gene for STb. The STa gene was present in 52% of the isolates and 31% had the gene for LT. The majority of the ETEC contained genes for more than one enterotoxin with LT-STb being the most prevalent gene combination. ETEC isolated from swine less than one week of age contained the gene for only STa 35% of the time, while ETEC from swine greater than one week of age had only the gene for STb 33% of the time. None of the enterotoxigenic isolates from swine contained the human genotype of STa (STaH) (Moon et al., 1986). Other workers also found a higher frequency of STb-positive strains among enteropathogenic E. coli isolated from swine with diarrhea (De Castro et al., 1984).

In a study conducted in Sri Racha, Thailand utilizing DNA hybridization techniques, STb-positive strains were observed in 3% of suckling, 32% of weaned and 1% of adult pigs cultured. Of the suckling pigs with STb ETEC, 21% had diarrhea, but none of the older pigs had diarrhea. STb ETEC was more frequently noted in suckling pigs with diarrhea than without diarrhea (Echeverria et al., 1984). In the same study it was noted that out of 466 people, 246 of which lived on a pig farm, ETEC carrying the gene for STb was isolated from only one individual, an asymptomatic pig farmer. Studies on E. coli strains

isolated from human diarrheal diseases failed to demonstrate that any of the strains produced STb as detected by enterotoxin activity (pig loop assay, weaned pigs 4-6 weeks of age) or by DNA hybridization with a STb gene probe. No response to STb by human ileal tissue was detected in vitro utilizing Ussing chambers. These results support the suggestion that strains of E. coli that produce STb are not a major cause of human diarrheal illness (Weikel et al., 1986b).

In vitro studies conducted by Weikel et al. (1985, 1986a) utilizing jejunum of weaned piglets in Ussing chambers, demonstrated that STb did not induce alterations in Na or Cl unidirectional or net fluxes. STb did appear to significantly increase the residual ion flux. It was suggested that the residual ion possibly was bicarbonate. The stimulation of electrogenic secretion in the small intestine by cyclic AMP is thought to involve reduced absorptive sodium chloride cotransport across the apical membrane, increased sodium chloride cotransport into the enterocyte across the basolateral cell membrane and increased chloride conductance (intracellular to lumen) through the apical membrane. Work conducted by Frizzell and Heintze (1979) evaluated the role of sodium and the effects of furosemide and ouabain on chloride secretion in the rabbit colon. When sodium was replaced by choline in the solution bathing the tissue in the Ussing chambers, the short circuit current was abolished, but electrically neutral chloride absorption was not altered. Theophylline, added to the mucosal and serosal solutions was not capable of inducing chloride secretion, but the addition of sodium to the serosal bathing solution caused stimulation of the short circuit current and chloride secretion. In

similar studies furosemide, an inhibitor of Na-Cl cotransport, was shown to abolish the secretory response when added to the serosal bathing solution. Ouabain, an inhibitor of the serosal sodium-potassium ATPase pump, also interfered with chloride secretion. These observations suggest that sodium is involved in the process of chloride secretion and the sodium-potassium-ATPase pump may link the metabolic and secretory processes. Furosemide when applied serosally did not inhibit the increased short-circuit current response induced by STb. However, furosemide did decrease the short circuit response induced by theophylline, a stimulant of chloride secretion. Ouabain, serosally applied did inhibit the response to STb. This suggests the sodium-potassium-ATPase pump may be involved in alterations in ion transport induced by STb (Weikel and Guerrant, 1985).

Further studies by Weikel et al. (1986a) conducted in vivo in small intestinal loops of weaned piglets showed that STb caused a significant rise in the bicarbonate content and a corresponding increase in the pH of the secreted fluid when compared to control loops. Secretory stimuli generally increase the potential difference, halt sodium absorption and reverse chloride absorption to secretion. In vivo, they also stimulate bicarbonate secretion (Rao and Field, 1983). STb also stimulated a significant increase in potassium concentration within the lumen. STb loops contained significantly greater total quantity of sodium and chloride (μEq/cm) than control loops, but the concentration (μEq/ml) of these ions were not significantly increased above those contained in the preinoculation filtrates.

Similar experiments conducted with STa displayed significant

secretion, but a significant increase in bicarbonate concentration accompanied by an increase in pH of the luminal fluid was not observed.

In 4-9 week old weaned pigs, STb caused significant fluid secretion within 30 minutes which was maximal at 3 to 6 hours (Kennedy et al., 1984; Greenberg et al. 1982a). Stripped porcine jejunal mucosa mounted in Ussing chambers demonstrated an immediate increase in short circuit current within two minutes of addition of STb. The response was maximal within 15 minutes and remained significant up to 55 minutes. The potential difference changes paralleled the short circuit current changes representing an increased charge across the intestinal mucosa. Interpretation of the in vitro results and the occurrence of an in vivo secretory response suggest that a net anion secretory flux is stimulated by STb. In contrast to other enterotoxins, STb caused a decrease in tissue resistance approximately 30 minutes after exposure to the toxin (Weikel and Guerrant, 1985).

Weikel et al. (1985) demonstrated an 80% reduction in secretion in ligated porcine intestinal loops in vivo and in vitro a 50% reduction in short circuit current in Ussing chambers by rinsing with a non-toxigenic culture filtrate. In comparison, the effect of STa was completely reversed by rinsing in Ussing chambers (Field et al., 1978). No morphological differences in villous and crypt epithelial cells were noted between STb and control loops in an intestinal loop assay conducted by Kennedy et al. (1984) in weaned pigs. Examination by transmission electron microscopy did not reveal any differences between STb exposed and control tissue (Rose et al., 1987). Partial villous atrophy was observed in pig jejunal loops exposed to STb (Whipp et al.,

1987; Whipp et al., 1985; Whipp et al., 1986). Ligated jejunal loops of three week old pigs exposed to STb containing crude culture filtrates displayed shorter villi, more sloughed epithelial cells within the lumen, more villous tips with disrupted epithelium, and larger numbers of villous tips covered by cuboidal epithelium. Jejunal loops exposed to nontoxigenic strains of E. coli and to a strain producing STa did not display microscopic alterations (Whipp et al., 1986). Morphometric techniques reveal a significant decrease in villous epithelial surface and mucosal volume in porcine small intestinal loops exposed to STb containing culture filtrates when compared to control loops. Histologically, the epithelium at the villous tips was observed to be cuboidal, or squamous, or absent.

The mechanism of action of STb is unknown. STb does not induce a measurable increase of cyclic GMP in the small intestinal mucosa of mice, rats, and pigs (Kennedy et al. 1984). Weikel et al. (1984) noted that STb did not activate adenylate cyclase in the pigeon erythrocyte assay method. Experiments conducted by Greenberg et al. (1982a) utilizing STb culture filtrates in weaned pig intestinal loops demonstrated, after a 6 hour incubation period, an increase in cAMP levels and an 8 fold increase in PGE_2 over control levels. No increase in mucosal cGMP levels were noted.

Experiments conducted in Ussing chambers, where calcium was removed from the mucosal and serosal bathing solutions, failed to demonstrate any inhibition of the increase in short circuit current induced by STb. Similarly mucosal and serosal addition of the calcium channel blocker, lanthanum chloride (10 mM), had no effect in Ussing chambers or in vivo

in ligated intestinal pig loops (Weikel and Guerrant, 1985).

In contrast, Greenberg et al. (1982b) demonstrated that lanthanum chloride when administered with STa intragastrically in infant mice did significantly reduce STa induced secretion. Activation of guanylate cyclase was not affected by lanthanum chloride. However, lanthanum chloride did significantly inhibit secretion stimulated by cyclic GMP. Thus it was suggested that lanthanum chloride's effect occurred late in secretion after the STa activation of guanylate cyclase. It is proposed that the steps late in the secretory pathway for STa may be different for STb.

Cholera toxin, E. coli LT and STa stimulated intestinal secretion have been noted to be inhibited by chlorpromazine (Holmgren and Greenough, 1980; Greenberg et al., 1980). Chlorpromazine has many biological properties such as the binding of calmodulin (Wolff and Brostrom, 1979), scavenging free radicals (Cohen and Heikkila, 1974), and stabilization of membranes (Greenberg et al. 1980). Its anti-secretory effects have been theorized to be due to the inhibition of the activation of adenylate and guanylate cyclase and to the inhibition of cyclic AMP and cyclic GMP stimulated secretion (Holmgren and Greenough, 1980; Greenberg et al., 1980).

Lonnroth and Munck (1980) using isolated mucosa of the chicken intestine demonstrated in the Ussing chamber that serosal chlorpromazine induced a decrease in the basal short circuit current that corresponded to increased chloride absorption. Net secretion stimulated by cholera toxin, cyclic AMP or cyclic GMP was reduced by chlorpromazine due to an increased chloride mucosal to serosal flux. The induced secretory

chloride flux was not changed.

Chlorpromazine (0.1 mM and 0.4 mM) when included with STb in the weaned piglet intestinal loop assay abolished the STb secretory response over a five hour period. Chlorpromazine applied mucosally in the Ussing chamber caused a significant decrease in baseline short circuit current. When added simultaneously and sequentially with STB, chlorpromazine decreased the STb stimulated increase in short circuit current to baseline. The authors propose the inhibition of STb in the intestinal loop assay by chlorpromazine was a non-specific reversal of the STb effect because in Ussing chambers chlorpromazine induced an equal and opposite effect on the short circuit current. These results suggest that the effects of STb and chlorpromazine were independent and support the idea that calmodulin and calcium modulation do not affect the STb secretory response (Weikel and Guerrant, 1985).

The activity of STb has been reported in some experiments to be inconsistent between pigs as well as within individual pigs (Rose et al., 1987; Whipp et al., 1987). Work conducted by Whipp (1987) in porcine jejunal loops demonstrated that when the intestinal lumen was prerinsed with saline, STB stimulated a secretory response in 60 of 60 loops. In experiments where prerinsing with saline was not conducted, STb induced secretion was observed in only 40 of 60 loops. When STb was mixed with jejunal luminal washings and then introduced into the jejunal ligated loops, STb induced secretion was not observed. The anti-STb activity of the jejunal washings passed through a 0.45 μm pore-size filter, was inactivated by heating at 100°C for 15 min., and was blocked by soybean trypsin inhibitor. Trypsin was shown to inactivate STb, when

incubated with the enterotoxin for 60 min. at 37°C. The amino acid sequence of STb deduced from the nucleotide sequence of the cloned STb gene contains arginine and lysine residues (Lee et al., 1983; Picken et al., 1983). Trypsin cleaves peptide chains on the carboxyl side of arginine and lysine residues (Stryer, 1981). It was concluded that the swine jejunum contains trypsin like activity that interferes with the response to STb (Whipp, 1987).

Bacterial enterotoxins are one of the primary causes of acute diarrheal syndromes. The stimulation of electrolyte secretion within the intestine if severe enough, can lead to systemic electrolyte imbalance and even death. Efforts to determine the structure, presence of membrane receptors and the effect on the intestinal epithelium of the enterotoxin may lead to a better understanding of the mechanism of action involved in producing the secretive response. This knowledge will greatly aid in the development of therapeutic agents to reverse or prevent the detrimental influence of these toxins.

Cholinergic Drugs

For many years the autonomic nervous system has been recognized as exerting an effect on intestinal water and ion transport (Florey et al., 1941; Miller et al., 1981; Powell and Tapper, 1979). The sympathetic system or adrenergic agents stimulate intestinal absorption and the parasympathetic system or cholinergic agents promote intestinal secretion. Studies in the dog (Tidball, 1961) and in the rat (Hubel, 1976, 1977) demonstrated that bethanechol or pilocarpine reduced water

and electrolyte absorption or induced secretion, while norepinephrine stimulated ion and water absorption. Atropine, a parasympatholytic drug, increased water and chloride absorption in dogs (Blickenstaff and Lewis, 1952). In the dog and cat, sympathetic denervation caused intestinal secretion that was reduced by atropine (Florey et al., 1941). In vitro techniques measuring short circuit current and ion fluxes support these observations. Acetylcholine (Browning et al., 1978), pilocarpine (Hubel, 1976) and bethanechol (Hubel, 1977) promote decreased absorption or increased secretion in the small intestine of the rat. The effects are inhibited by atropine (Hubel, 1976; Wright et al., 1940). In three week old pigs atropine reduced the secretion of water and electrolytes in jejunal segments perfused with E. coli heat-stable enterotoxin (1261) (Ahrens and Zhu, 1982b).

Issacs et al. (1976) and Jacobowitz (1965) applied a cholinesterase stain to sections of human jejunal mucosa and demonstrated dense staining around the crypts and the villi. The stained nerve fibers were in close proximity to the villous epithelial cells. Thus, it seems possible that cholinergic agents and the parasympathetic nervous system may be able to affect ion transport via an effect on the intestinal epithelial cells.

Historically, the cholinergic receptor was the first receptor to be designated as having subtypes, i.e., muscarinic and nicotinic (Goyal and Rattan, 1978). Recently pharmacological and ligand binding studies suggest that muscarinic receptors consist of a heterogenous population. Initially it was proposed that they should be divided into two subclasses, M_1 and M_2, which exhibit high and low affinity for the

antagonist pirenzepine respectively (Hammer and Giachetti, 1982). The discovery of other muscarinic antagonists have led researchers to propose that there are three subtypes of muscarinic receptors. Doods et al. (1987) demonstrated that pirenzepine (PZ) had a high affinity for M_1 receptors, 4-diphenylacetoxy-N-methylpiperidine methobromide (4 DAMP) and dicyclomine demonstrated a high affinity for both M_1 and M_3 receptors. AF-DX 116 (11-2[[2-(diethylamino) methl]-1-piperidinyl] acetyl]-5,11-dihydro-6-H-pyrido [2,3-b][1,4]benzodiazepin-6-one) had a high affinity for M_2 receptors.

M_1 muscarinic receptors are found in sympathetic ganglia and the CNS. They have a high affinity for pirenzepine and 4-DAMP, but intermediate affinity for AF-DX116. M_2 receptors are located in the myocardium and have low affinity for 4-DAMP (Barlow et al., 1976; Berrie et al., 1983) and pirenzepine, but has a stronger affinity for AF-DX116. M_3 receptors are found in smooth muscle and have a high affinity for 4-DAMP, intermediate affinity for pirenzepine and do not strongly bind AF-DX116. As more muscarinic agonists and antagonists are discovered and compared in different types of tissues it is possible other muscarinic receptor subtypes may be discovered.

Pirenzepine is a tricyclic compound with a high degree of hydrophobicity (Hammer and Giachetti, 1984). In man, pirenzepine causes a significant reduction of secretion of pepsinogen and gastric acid (Jaup et al., 1982). In the in vitro preparation of the isolated mouse stomach, Schiavone et al. (1983) studied the antisecretory action of pirenzepine. The results indicate that pirenzepine is a strong antagonist of gastric acid secretion induced by field stimulation and a

weaker antagonist of acid secretion produced by exogenous muscarinic agonists, such as bethanecol. Studies with rat duodenum demonstrate that pirenzepine, considered to be a selective M_1-antagonist is a potent stimulant of bicarbonate secretion. This action of pirenzepine was dependent on intact vagal innervation of the duodenum.

Binding studies have demonstrated that the interactions of muscarinic agents with muscarinic receptor binding sites on intestinal epithelial cells differs from binding sites on intestinal smooth muscle cells. The epithelial cells have sites characterized by a high affinity for pirenzepine and a lower affinity for oxotremorine, another muscarinic antagonist. The smooth muscle sites have lower affinity pirenzepine and higher affinity oxotremorine sites (Tien et al., 1985).

The muscarinic antagonist, 4-diphenylacetoxy N-methyl-piperidine (4 DAMP) methiodide or methobromide, displays a greater affinity for M_2 receptors in ileum than in atrial pacemaker cells. This suggests there may be a further subdivision of M_2 receptors.

Muscarinic receptors have been demonstrated on both intestinal muscle tissue and epithelial cells. The muscarinic ligand [3H] quinuclidinyl benzilate was used to demonstrate the existence of muscarinic receptors on jejunal, ileal, and colonic epithelial cells (Rimele et al., 1981; Zimmerman and Binder, 1982; Rimele and Gaginella, 1982) and that these same receptors are involved in mediating intestinal secretion (Rimele and Gaginella, 1982).

The knowledge of the effects of cholinergics on the secretory response of the small intestine has led to studies of their involvement in the response to enterotoxin. The discovery of selective muscarinic

receptor antagonists will possibly lead to new therapeutic agents that can be used to modify or inhibit the enterotoxin secretory response.

Adrenergic Drugs

Adrenergic nerve fibers have been noted in the intestinal mucosa with nerve endings near the crypt and villus cells (Thomas and Templeton, 1981). The crypts are surrounded by the most dense innervation (Jacobowitz, 1965; Newson et al., 1979).

Studies conducted by Field and McColl (1973) to examine the effects of catecholamines on intestinal transport showed that epinephrine and norepinephrine induced a reduction in potential difference and short circuit current when added to rabbit ileal mucosa in Ussing Chambers. The changes were greater in HCO_3-Ringers than HCO_3-free Ringers. Isoproterenol and propranolol did not affect the short circuit current suggesting the effects of epinephrine and norepinephrine are alpha-adrenergic. Epinephrine and norepinephrine altered ion transport by stimulating: (1) a net increase in Na absorption due to an increase in mucosal to serosal unidirectional flux; (2) an increase in net chloride absorption as a result of an increased mucosal to serosal flux; (3) a decrease in serosal to mucosal unidirectional fluxes; and (4) the disappearance of the residual ion flux. Most of the changes in the short circuit current were due to the third effect. In the presence of HCO_3-free Ringers solution, the effects were not observed. The authors suggest that the observed enhanced absorption of Na and Cl and the reduced short circuit current (due to decreased HCO_3 secretion) resulted

from stimulation of alpha-adrenergic receptors.

Field et al. (1975) noted that elevated levels of cAMP stimulated by cholera toxin were reversed by epinephrine or norepinephrine in rabbit ileal mucosa. Phenoxybenzamine, an alpha adrenergic antagonist, prevented the reversal. Increased levels of cAMP induced by PGE_1 were also partially reversed by epinephrine. Theophylline-induced, but not cAMP or cholera toxin-induced Cl secretion was decreased by epinephrine. However, a reduction in short-circuit current was noted in the cAMP and cholera toxin stimulated secretion.

Durbin et al. (1982) demonstrated in rabbit ileum that the action of clonidine, an α_2 receptor agonist, on the short circuit current was similar to the action of epinephrine. Yohimbine, an α_2 antagonist, blocked the action of both clonidine and epinephrine. Methoxamine, an α_1 agonist, did not affect the short circuit current and prazosin, an α_1 antagonist, did not affect the action of epinephrine. Similar results were noted by Chang et al (1982).

In order to determine if endogenous opiates are released or if opiate receptors are affected by clonidine, tissues were pretreated with naloxone and clonidine and then exposed to clonidine and naloxone respectively. Naloxone did not change the decline in short circuit current produced by clonidine nor did naloxone reverse the effect of clonidine as it did for Met-enkephalin. This suggests that opiate receptors were not involved (Durbin et al., 1982). $Alpha_2$ receptors have been demonstrated on enterocytes by competitive displacement studies of $[^3H]$ yohimbine from plasma membranes obtained from rabbit ileal enterocytes (Chang et al., 1983). Agonist binding affinities

correlated with the concentration dependence of agonist effects on the short circuit current.

Donowitiz et al. (1984) found that α_2 agonists decreased ^{45}Ca entry across rabbit ileal basolateral membrane and decreased rabbit ileal calcium content. In addition, α_2 agonists were noted to cause the same changes as low calcium bathing solutions and plasma membrane calcium channel blockers (Donowitz, 1983).

In vivo human studies using a triple lumen perfusion technique showed that isoproterenol given intravenously significantly increased absorption of sodium, chloride, and water in the jejunum and ileum and potassium in the ileum. Intravenous propranolol, a beta adrenergic antagonist, reduced jejunal electrolyte absorption and stimulated water and sodium secretion in the ileum. These results provide evidence for beta adrenergic control of intestinal transport in humans (Morris and Turnberg, 1981). This is in contrast to in vitro experiments with rabbit ileum where isoproterenol and propranolol had no effect on the short circuit current (Field and McColl, 1973).

Clonidine added to the mucosal or serosal bathing solutions bathing rabbit ileum in Ussing chambers caused a decrease in short circuit current and potential difference and an increase in tissue conductance and Na and Cl absorption. The increased net Na and Cl absorption was due to increased mucosal to serosal movement of Na and Cl and a decrease in serosal to mucosal movement of Cl. The action of clonidine and epinephrine on short circuit current was quantitatively similar. Yohimbine reversed the action of both of these adrenergics. Clonidine, administered orally, blocked diarrhea in mice induced by

caster oil, PGE_2, 5-hydroxytryptophan and bethanechol. The action of clonidine was blocked by yohimbine, but not by naloxone, propranolol, prazosin or cimetidine indicating its activity was directed to α_2 adrenergic receptors. Other α_2 agonists such as naphazoline, quanabenz, ergometrine, and alpha-methylnorepinephrine also exhibited antidiarrheal effects. Isoproterenol (beta agonist) methoxamine and phenylephrine (α_2 agonist) were not effective (Doherty and Hancock, 1983).

In vivo work in three week old pigs demonstrated that clonidine, when administered to perfused jejunal segments, caused a significant increase in absorption of water and Na from the control segments (Ahrens and Zhu, 1982a) and a significant decrease in net secretion of water, Na and Cl in ST perfused loops (1261) (Ahrens and Zhu, 1982a; Zhu and Ahrens, 1983).

Epinephrine and the α_2 agonist, clonidine, have been shown in vitro to induce absorption of Na and Cl, reduce Cl secretion in the basal state and reduce the effects of cholera toxin stimulated chloride secretion. The blockade of this action by the α_2 antagonist, yohimbine, and the lack of effect by prazosin, an α_1 antagonist, supports the proposed α_2 agonistic nature of this response. The antidiarrheal effects of these α_2 agonists in vivo further supports the nature of the antisecretory action.

Opiates

For many years the drug therapy of diarrheal diseases has been directed toward controlling propulsive hypermotility. Increased

motility and decreased intestinal transit time is a contributory factor to decreased absorption. Recently the target of drug therapy has been the modulation of electrolyte transport. More specifically its aim has been to convert intestinal secretion of water and electrolytes to absorption of the same.

The opiates have been used for over 2000 years to control diarrhea (e.g., paregoric) (Way and Way, 1987; Sandhu et al., 1974). The popular explanation of opiate action was that they caused increased rhythmic activity of gut circular muscle while inhibiting the contraction of the longitudinal muscle thus intestinal transit time was increased by the nonpropulsive muscle activity. Currently it is believed that the antidiarrheal action of opiates involves alterations in water and electrolyte transport (Powell, 1981b).

Several types of opiate receptor subtypes have been suggested to be present in the intestinal tract. They include μ, δ, ϵ and κ which are preferentially acted upon by morphine, enkephalin, B endorphin, and dynorphin respectively. The σ receptors may be acted upon by N-allylnormetazocine (Kachur et al., 1980, Kachur and Miller, 1982; Miller et al., 1985).

Morphine acts preferentially at μ-receptors while the enkephalins preferentially act at δ-receptors (Kachur et al., 1980). Naloxone blocks all types of opiate receptors, but with different affinities (e.g., $\mu > \delta$). The specificity of opiates for receptor subtypes is not complete (Miller et al., 1985). B-endorphin and etorphine have effects on both μ- and δ-receptors (Kachur et al., 1980).

The location of μ- and δ-type opiate receptors in the intestinal

tract has not been clearly determined. Kachur et al. (1980, 1982) (252, 253) characterized the opiate receptor in guinea-pig ileal mucosa using D-Ala^2-D-Leu^5-enkephalin and D-Ala^2-D-Leu^5-enkephalin as δ-receptor agonists and fentanyl and morphine as μ-receptor agonists. They noted the effects of the opiates on the transepithelial potential difference and short circuit current across guinea pig ileum stripped of one muscle layer and mounted in Ussing Chambers. They concluded that in guinea pig mucosa, the opiate receptor present that was responsible for the suppression of the net Cl secretion was similar in to the δ-receptor and the μ-opiate receptor was responsible for the effects on guinea pig intestinal smooth muscle.

Autoradiographic studies using [^{3}H]-D-Ala^2-D-Leu^5 Enkephalin and [^{3}H]-dihydromorphine were conducted by Nishimura et al. (1986) in an effort to localize the μ-and δ-type opiate receptors in the gastrointestinal tract of the rat and guinea pig. A dense distribution of μ- and δ-receptors were demonstrated through out the duodenal and ileal mucosa of the rat, but only μ-receptors were present in the submucosa plexus and muscle layers of the guinea-pig ileum. Binding sites were not observed in the mucosa. This lack of correlation with the functional studies of Kachur et al. (1980 and 1982) may suggest the occurrence of receptor subtypes other than μ and δ or the lack of the proper conditions for receptor binding.

Studies conducted in rat jejunum demonstrated strong antisecretory activity (VIP induced secretion) by the μ-agonist morphine and only negligible antisecretory activity with the δ-agonist D-Ala^2-D-Leu^5-enkephalin. The small response by D-Ala^2-D-Leu^5-enkephalin was possibly

attributed to its unstable biological activity (Coupar, 1983).

The location of opiate receptors mediating electrolyte transport is not known. It may be located on the enterocytes or on nerve endings or both. Dobbins et al. (1980) reported that tetrodotoxin blocks the action of enkephalins on the short circuit current in the rabbit ileum. In the guinea pig, opiates inhibited electrically stimulated output of acetylcholine in the myenteric plexus (Down and Szerb, 1980). These studies suggest that the site of opiate action, is at least in part, neuronal.

In vivo studies in the pig and rat jejunum has demonstrated that morphine inhibits secretion produced by heat-stable enterotoxin (Ahrens and Zhu, 1982a) carbachol and PGE_1 (Berbler and Lembeck, 1979) and VIP (Berbler and Lembeck, 1979; Coupar, 1983; Lee and Coupar, 1980) and in some cases, increases basal absorption (Ahrens and Zhu, 1982a; Berbler and Lembeck, 1979). In other studies, utilizing rabbit ileal mucosa in Ussing chambers, morphine caused an increase in basal chloride absorption (McKay et al., 1984) and a reduction in the induced net secretion of chloride by PGE_2 and acetylcholine (McKay et al., 1982). Naloxone blocked the ion transport response to morphine (McKay et al., 1984). Kachur et al. (1980), however, showed morphine was almost ineffective in reducing the short circuit current in guinea pig ileum. Their evidence suggests that morphine exerts its primary effect on μ-receptors existing on the smooth muscle cells and a minimal effect on δ-receptors on the mucosal cells that modulate intestinal electrolyte transport. In vivo studies conducted by Brown and Miller (1984) also demonstrated no significant anti-secretory effects when morphine was

administered into the right cerebral ventricle of rats with intestinal loops containing cholera toxin. In perfused jejunal intestinal segments of 3 week old pigs morphine significantly increased net water, Na, and Cl absorptive fluxes in control loops and significantly decreased net secretion of water, Na, and Cl in ST (1261) loops (Ahrens and Zhu, 1982b).

It has been proposed that the opiates mediate antidiarrheal activity by inhibition of secretory agents from enteric nerves, mediation of the peripheral release of adrenergic agents from the central nervous system, inhibition of motility via μ-receptors, and/or stimulation of intestinal sodium chloride absorption by δ-receptors (Fedorak and Field, 1987).

Enkephalins

The endogenous opioid peptides, the enkephalins, have been shown to be present within the gut. Neurons containing enkephalins are prominent in the myenteric plexus of all levels of the gastrointestinal tract while few are present in the submucous plexus and the mucosa (Furness et al., 1983). Endocrine cells of the gut mucosa also contain enkephalins. These peptides are then able to act as neurotransmitters or to have a paracrine effect to alter the activity of other neurons, smooth muscle, or the intestinal mucosa (Kachur et al., 1980). The enkephalins appear to be predominantly δ-receptor agonists (Fedorak and Field, 1987; Kachur et al., 1980; Miller et al., 1985).

Kachur et al. (1980) using D-Ala^2-D-Leu^5-enkephalin and D-Ala^2-D-

Meth^5-enkephalin demonstrated that these opioid peptides reduced the transepithelial potential difference and short circuit current of guinea-pig ileum in Ussing chambers. Etorphine, an oriparvine narcotic with affinity for δ- and μ-receptors, was also effective, but fentanyl and morphine, both μ-receptor agonists were not. Naloxone reversed the opioid effects, but exhibited no effect when added by itself. The effects of Leu^5-enkephalin and Met^5-enkephalin were transient, unlike the effects of D-Ala^2-D-Met^5-enkephalin. This was possibly due to the rapid metabolism of the former compounds. Natural analogs may be more susceptible to proteases or synthetic analogs may have greater affinity for the receptors involved (Hughes, 1975). Similar observations were made by Dobbins et al. (1980) in vitro studies with rabbit ileum. This study also demonstrated a linear correlation between the decrease in short circuit current and the change from net Cl secretion to net Cl absorption.

The addition of tetrodotoxin, a neurotoxin, to the preparation caused a decrease in the short circuit current and potential difference and prevented any additional decrease in short circuit current by D-Ala^2-Met enkephalin. The prior addition of tetrodotoxin did not prevent epinephrine from causing a further decrease in short circuit current. Thus it is suggested that D-Ala^2-Met enkephalin may be functioning as a preganglionic neurotransmitter or tetrodotoxin may block the effect of enkephalin directly on the enterocytes. Dobbins also reported that D-Ala^2-Met enkephalin did not affect the basic tissue or VIP-stimulated cAMP levels, but did slightly depress prostaglandin and theophylline-stimulated cAMP levels.

Fogel and Kaplan (1984) administered naloxone, a μ-opiate antagonist, or diprenorphine, a μ- and δ-opiate receptor antagonist intraluminally into rat jejunum and ileum and noted decreased basal water and electrolyte absorption. Administration of atropine intravenously prevented the changes in water transport produced by naloxone, but had no effect on the diprenorphine-induced changes. Naloxone (10^{-4} M) prevented the increased absorption of water by morphine, a μ-receptor agonist, but not the absorptive effect by the δ-agonist D-Ala-Met-enkephalinamide. Diprenorphine (10^{-6} M) abolished the absorptive effect of both. They propose the endogenous opiates are involved in regulating the basal intestinal absorptive processes; and that cholinergic nerves mediate the effect of naloxone, but diprenorphine acts via a different mechanism.

BW 9426, an enkephalin-like pentapeptide, promoted Na and Cl absorption in vitro in rabbit ileum and prevented the cytopathic effects of cholera toxin and E. coli heat-labile enterotoxin in the Y-1 adrenal cell assay. In rabbit ileal loop studies, the peptide inhibited the secretory response of E. coli heat-stable and heat labile toxin and cholera toxin. Similarly it abated the fluid accumulation stimulated by E. coli heat-stable toxin in the suckling mouse model (Morgan et al., 1985).

Loperamide

Loperamide is a synthetic antidiarrheal butyramide derivative (Hughes et al., 1982) that binds to opiate receptors in the longitudinal

muscle layer and myenteric plexus of the intestine (Giagnoni et al., 1983). In longitudinal muscle preparations of rabbit jejunum, loperamide inhibited the spontaneous activity as well as induced contractions stimulated by PGE_2, $PGF_{2\alpha}$ and acetylcholine It also reduced contraction in guinea-pig ileum, colon, gerbil colon, hamster and rat fundus induced by PGE_2, $PGF_{2\alpha}$, acetylcholine, and histamine (Karim and Adaikan, 1977). There are several reports indicating that loperamide can attenuate or reverse intestinal secretion stimulated by cholera toxin (Sandhu et al., 1981), E. coli heat stable and heat labile enterotoxin (Hughes et al., 1982; Watt et al., 1982) and PGE_2 (Hughes et al., 1982; Mackerer et al., 1976; Karim and Adaikan, 1977).

In experiments conducted with isolated rabbit ileal mucosa mounted in Ussing chambers, loperamide inhibited secreting mucosa stimulated by PGE_2 and E. coli heat-labile enterotoxin (Hughes et al., 1982). Another study showed that loperamide inhibited a secretory response induced in rat jejunum by PGE_2 and cholera toxin. The inhibition of the cholera toxin-stimulated secretion did not affect the increased activity of adenylate cyclase or the concentration of cAMP within the tissues (Sandhu et al., 1981). Knoop and Abbey (1981) reported that loperamide did not alter the net intestinal fluid accumulation induced by E. coli heat-stable enterotoxin in infant mice. In contrast Watt et al. (1982) observed that loperamide administered before or after the secretory response was established reduced secretion elicited by E. coli heat-stable enterotoxin in infant mice.

The increase in transepithelial short circuit current induced by the endogenous gut peptides, substance P, neurotensin, and bombensin was

reversed by loperamide in ion transport studies conducted with stripped intestinal mucosa of chinchillas and chickens. The action of loperamide and etorphine, a synthetic opiate, was not blocked by diprenorphine, a δ- and μ-receptor antagonist, suggesting that these effects did not involve enteric opiate receptors. In addition DADLE a specific δ-receptor agonist, and morphine, a μ-receptor agonist, did not alter the effect of substance P on the short circuit current (Chang et al., 1986). However, Ilundain and Naftalin (1981) demonstrated the enhanced movement of Cl from mucosa to serosa in rabbit ileum stimulated by loperamide was prevented by naloxone.

The mechanism of action of loperamide is not known. There is evidence that loperamide interacts with μ and δ opioid receptors in guinea pig ileum and mouse vas deferens respectively (Giagnoni et al., 1983). Mackerer et al. (1976) has shown that loperamide binds to opiate receptors in the brain and myenteric plexus. Chang et al. (1986) found that increased levels of intracellular calcium stimulated by substance P in isolated chicken enterocytes were blocked by loperamide. The chemical structure of loperamide is similar to calcium channel blockers such as verapamil. In addition it has been demonstrated that loperamide may act as a calcium channel antagonist (Reynolds et al., 1984).

It also has been suggested that loperamide may act to reduce intestinal secretion by binding to calmodulin. It has been shown that loperamide, diphanolylate, and chlorpromazine displace [^{3}H] trifluoperazine from calmodulin. The ability of these drugs to inhibit intestinal fluid secretion stimulated by 16,16-dimethyl prostaglandin E_2

correlate positively with their ability to displace calcium dependent [^3H] trifluoperazine binding from calmodulin (Zavecz et al., 1982).

Intragastric infusion studies in human subjects demonstrated that the antidiarrheal effect of loperamide was due to its effect on gastrointestinal motility. No effect on the absorption rate of the gastrointestinal tract as a whole could be demonstrated (Schiller et al., 1984).

Loperamide may act by blocking calcium channels in plasma membranes of enterocytes thus preventing their activation by enteric peptides such as substance P, neurotensin, and bombensin. It is also possible that loperamide may block the action of these peptides on enteric neurons and thus inhibit the secondary release of neurotransmitters such as VIP (Chang et al., 1986).

Drugs Affecting Calcium and Calmodulin

Intracellular free calcium may have a role in the regulation of ion and water movement in the intestine. Elevated intracellular levels of calcium decrease Na and Cl absorption and stimulate Cl secretion (210), but decreased intracellular calcium levels have the opposite effect (Donowitz and Asarkor, 1982; Hubel and Callahan, 1980).

Calcium channel blockers, such as verapamil and nifedipine, stimulate the basal absorption of water in vivo in the rat ileum and stimulate active Na and Cl absorption in vitro in the rat ileum (Donowitz et al., 1985). Intestinal secretion stimulated by substance

P, which acts by raising intracellular levels of Ca by opening Ca channels in the plasma membrane, is inhibited by verapamil and loperamide (Chang et al., 1984; Zinner et al., 1985).

Calmodulin is the major calcium modulating receptor in nonmuscle cells. This protein binds four molecules of calcium and the resulting calcium-calmodulin complex combines with and regulates the activities of calmodulin acceptor proteins (CAPs). The calmodulin acceptor proteins are found in transporting epithelial and secretory cells. They are considered to be transducers of the calcium-calmodulin signal (Dedman, 1984).

Drugs which are calcium-calmodulin antagonists include the phenothiazines, such as chlorpromazine, and trifluoperazine. The phenothiazines are proposed to bind to hydrophobic domains on the calcium-calmodulin complexes thus blocking their interaction with the complimentary hydrophobic site in the calmodulin acceptor proteins (Donowitz et al., 1984).

Trifluoperazine inhibited the increase in Isc and net Cl secretion in response to Ca ionophore A23187 in rabbit ileal mucosa mounted in Ussing chambers. Studies carried out on human small intestinal and rabbit ileal mucosa showed that trifluoperazine inhibited the increase in short circuit current produced by VIP. Similarly, trifluoperazine reduced the increase in short circuit current stimulated by theophylline and *E. coli* heat-stable enterotoxin. No effect on basal cAMP and cGMP concentration in rabbit ileal mucosa were noted and there was no significant alteration in the elevated levels of cAMP produced by 16, 16

dimethylprostaglandin E_2 ($dMPGE_2$) and cGMP produced by E. coli heat-stable enterotoxin (Smith and Field, 1980).

Experimental diarrhea in newborn pigs was induced by oral administration of E. coli bacteria which produce heat-labile (LT) and heat-stable (ST) enterotoxins. One hour after the onset of diarrhea, chlorpromazine was administered intramuscularly. Four hours after drug administration, the animals were killed and the fluid content of the small intestine weighed. Chlorpromazine administered at a rate of 5 mg per kg of body weight eliminated the secretory response while a dose of 1 to 2 mg per kg of body weight was not as effective, but still significantly reduced fluid content. Adenylate cyclase, proposed to mediate the cellular effects of LT, was two to threefold higher in activity in infected vs control animals. Chlorpromazine treatment reduced the activity of adenylate cyclase by 50%.

In a spontaneous outbreak of E. coli enterotoxigenic diarrhea, piglets treated with chlorpromazine (1 mg/kg IM) exhibited a significantly shorter duration of diarrhea (4.1 hr.) than controls (7.2 hr.) (Lonnroth et al., 1979).

It has been observed that chlorpromazine and trifluoperazine significantly inhibited net intestinal fluid accumulation in infant mice exposed to E. coli heat-stable (ST) enterotoxin (Knoop and Abbey, 1981; Abbey and Knoop, 1979). EGTA, a calcium chelator, also significantly inhibited net fluid accumulation. These studies suggest calcium and possibly calmodulin may have a role in ST stimulated intestinal secretion.

Research Objectives

The objectives of this research are to study the effects of Escherichia coli heat-stable enterotoxin, STb, on water and electrolyte fluxes in the jejunum of swine and rats and to note the effects of pharmacological agents on these responses.

The study will be partitioned into in vivo and in vitro investigations. The in vivo investigations will consist of perfused segments and ligated loops of pig and rat jejunum in which the effect of STb on water, sodium, potassium and chloride transport will be determined. The ability of α-adrenergic, antimuscarinics, opiates, opioids, and ganglionic blockers to modify the effects of STb will be assessed. The in vitro studies will determine the effect of STb on the influx of ^{36}Cl and ^{45}Ca and the efflux of ^{36} in isolated jejunal enterocytes.

A better understanding of the mechanism of action of STb and possibly assistance in the development of antidiarrheal therapy may be derived from these studies.

MATERIALS AND METHODS

Enterotoxin

The heat-stable enterotoxin, STb, was produced utilizing facilities and materials kindly provided by the National Animal Disease Center (NADC), Ames, Iowa. Stock cultures of the toxin producing or non toxigenic E. coli strains were kept in the dark at room temperature on Brain Heart Infusion (BHI) agar slants. The respective organisms were inoculated into 6 to 8 ml of Brain Heart Infusion broth and allowed to grow overnight (12 to 16 hours) at 37°C. A 2 L Erlenmeyer flask containing 200 ml of sterile BHI broth was inoculated with 0.2 ml of this culture. The flask was plugged with a cotton stopper and incubated at 37°C on a shaker (200 rpm) for 22 to 24 hours. The broth culture was centrifuged at 23,000 x g and then filter sterilized by passing through a cellulose acetate-nitrate filter (Millipore filter MS series) of 0.45 µm pore size under positive N_2 pressure. The filtrates were stored at 4°C. The STb producing strain and nontoxigenic strain, 1790 (O9:K+) and 123 (O43:K-:H28) respectively, were used for the ligated intestinal loop and perfusion studies. The E. coli strain 1261 (O138:K81), which produces STa and STb was used as the enterotoxigenic producing strain in some rat perfusion studies. HB 101 (K12 rough) and HB101-pRAS-1 (K12 rough) were used in the isolated enterocyte studies. The latter strain differs from the first by the fact it contains a recombinant plasmid, pRAS-1, with the cloned gene for STb. The plasmid pRAS-1 was derived from pBR322 and contains an insert which encodes for STb. The activity

of the STb and nontoxigenic broths were assayed in ligated intestinal loops of 4 to 6 week old pigs.

Pig Perfusions

Three week old, unweaned pigs were used for the perfusion experiments. The pigs were anesthetized with halothane and an incision was made on the left side just caudal to the last rib. The proximal jejunum was isolated 30 cm distal to the pylorus and the small intestine was flushed with a physiological salt solution. Two 20 cm long segments of jejunum separated by a 10 cm interloop were isolated. A Silastic Foley catheter size 14 Fr (Dow Corning Corp.) was fixed in the end of each 20 cm segment. Following the cannulation the intestine was replaced in the abdominal cavity and the abdominal incision was closed. The pigs were maintained on a heating pad. A solution containing _E. coli_ strain 123 culture broth filtrate (negative control) was perfused through one loop and a solution containing 1790 culture broth filtrate (STb) was perfused in the remaining loop. The order of the loops was alternated between experiments. The solutions were perfused at a rate of 2 ml/min. and maintained at 37°C. The solutions contained sodium, 148 mM; chloride, 120 mM; potassium, 6.5 mM; calcium, 1 mM; phosphate, 1.4 mM; polyethylene glycol 4000 (PEG),1 g/L; and [^{14}C] PEG 1.25 μCi. The pH was adjusted to 7.4 and the osmolality approximated 300 mOsm/kg. A 45 minute equilibration period preceded the four 20 minute perfusion periods. Effluent from the perfused loops were collected and saved for further analysis. The drug under examination was then added to the

control and STb solutions and the equilibration and perfusion periods were repeated. After the end of the perfusion the intestinal segments were quickly dissected out and their length determined. The collected perfusate was maintained at 4°C until analyzed for sodium, potassium, chloride, [^{14}C] PEG concentration and osmolality.

Pig Ligated Loops

Weaned pigs six weeks of age were used for the ligated loop experiments. After a 12 hour fast the pigs were anesthetized with halothane. A ventral midline abdominal incision was performed and the jejunum was isolated. Sterile saline solution (37°C) was used to flush the small intestine. Beginning 100 cm distal to the ligament of Treitz, 9 to 11 loops 15 cm in length were formed separated by interloops 2 cm in length. Each loop was injected with a total volume of 10 ml of which 6 ml was composed of either control or STb broth. A loop designated as a STb sentinel loop was located at the first and last positions. The remaining loops consisted of control, control plus agonist, control plus antagonist, control plus agonist and antagonist, STb, STb plus agonist, STb plus antagonist, and STb plus agonist and antagonist. All loops located between the STb sentinel loops were randomly assigned. The loops were placed into the abdominal cavity and the abdominal incision was closed. The body temperature of the pigs was maintained on heating pads for 2 hours. At the end of the incubation period the contents of the loops were aspirated into syringes in the order of preparation and the length of the loops was determined. The samples were centrifuged

and stored at 4°C for further analysis.

Rat perfusions

For each drug studied, 4 to 10 male rats with a mass of 250 to 350 g were used. The rats were fasted for 24 hours prior to the experimental studies. The rats were anesthetized intraperitoneally with pentobarbital (45 mg/kg) and the body temperature was maintained at 37°C on a hot water pad. A ventral midline abdominal incision was performed and the cranial jejunum was isolated 10 cm caudal to the ligament of Treitz. Three ml of perfusion solution, minus the broth culture component, was injected at this site to flush the entire length of the small intestine. This was repeated a second time.

Beginning 50 cm cranial to the ileocecal junction, two segments of the jejunum, each 10 cm long and separated by a 5 cm segment, were isolated. The anterior and posterior ends of each segment was cannulated with Intramedic Polyethylene tubing, PE-240, I.D. 1.67 mm, (Clay Adams).

The intestinal segments were randomly assigned to be perfused with toxin or control broth. Prior to the perfusion each segment was instilled with the respective toxin or control broth (full strength) and the intestinal segments were then placed into the abdominal cavity, and the incision closed. After a 30 min. incubation period, the loops were perfused (0.7 ml/min.) (Harvard Apparatus Peristaltic Pump, Harvard Apparatus Millis, Mass.) with a physiologic salt solution containing the respective toxin or control crude filtrate broth at a dilution of 1:2

and maintained at 37°C. The solutions contained sodium, 148 mM; chloride, 120 mM; potassium, 6.5 mM, bicarbonate, 26 mM; calcium, 1 mM; and phosphate 1.4 mM. In addition polyethylene glycol (PEG) 4,000, 1 g/L; [^{14}C] PEG, 1.25 μCi/L and soybean trypsin inhibitor Type II-S, 1 mg/ml (Sigma Chemical Co., St. Louis, MO) were present. The osmolality of the solutions approximated 300 mOsm/kg and the pH of the solution was adjusted to 7.4. At the start of the perfusion a 20 minute equilibration period was carried out. Following this three perfusion periods, each of 20 minutes duration were conducted. The loop effluent was collected during these periods for later analysis. The pharmaceutical agents under study were then added to each of the perfusion solutions and the equilibration and perfusion periods were repeated. After the last perfusion period the intestinal segments were isolated and their lengths determined.

Preliminary studies were carried out with cell-free broth containing the heat-stable toxins produced by the E. coli strain 1261 and with 1790 broth which had been concentrated approximately 1000 fold, both without soybean trypsin inhibitor. In these initial experiments the intestinal segments were 20 cm in length beginning 10 cm distal to the ligament of Treitz. An equilibration period of 30 minutes duration was followed by four perfusion periods of 20 minutes duration. The 1261 broth was diluted 1:5 with the physiological salt perfusion solution while the 1790 concentrated (1000 X) broth was diluted to 100 X.

Rat Ligated Loops

Male rats, 250 to 350 grams, were used for the ligated loop experiments. The rats were fasted for 24 hours. Following the intraperitoneal administration of anesthetic (pentobarbital at 45 mg/kg), a ventral midline abdominal incision was made and the cranial jejunum, 10 cm distal to the ligament of Treitz was isolated. The small intestinal tract was flushed twice with 3 ml of physiological salt solution containing soybean trypsin inhibitor (Type 1-S) (Sigma) at a concentration of 1 mg/ml.

Starting 10 cm cranial to the ileocecal junction and proceeding orally 8 intestinal loops, 5 cm in length, separated by 0.5 cm long interloops, were formed. Four control loops and four toxin loops were present in each rat. The control loops contained 0.25 ml of strain 123 filtered culture broth and 0.75 ml of the physiologic salt solution containing soybean trypsin inhibitor (1 mg/ml). The toxin loops contained 0.25 ml of strain 1790 filtered culture broth and 0.25 ml of the physiologic salt solution containing soybean trypsin inhibitor (1 mg/ml). The solutions were adjusted to a pH of 7.4 and had an osmolality of approximately 300 mOsm/kg. Three of the respective control and toxin loops contained an agonist, antagonist, or combination of the agonist and antagonist. The order of injection of the loops with the respective solutions was randomized. Each syringe used in filling the intestinal loops was weighed after filling and after injection of the respective solution. The abdominal incision was closed after the loops had been injected and the rats were maintained on a heating pad

for two hours. The contents of the loops were aspirated into pre-weighed syringes in the order of preparation and the syringes were weighed after collection. The length of the loops and interloops was determined.

Analyses

The [^{14}C] PEG concentration was determined by liquid scintillation spectrometry and the osmolality by freezing-point depression. Sodium and potassium concentrations were determined potentiometrically utilizing a Beckman Electrolyte 2 Analyzer. Chloride determination in the pig ligated intestinal loop experiments was determined colorimetrically. In the pig perfusion experiments chloride concentration was measured by the Corning 925 Chloride Analyzer (Corning Glass Works, Medfield, Mass.) utilizing the Volhard chloride estimation. Standard equations were used to calculate net water and electrolyte fluxes (Leng-Peschlow, 1980). Data for the perfusion experiments was normalized to a 20 cm length of jejunum. Experimental data from the rat and pig ligated intestinal loops was normalized to a 1 cm and 15 cm length of jejunum respectively.

Isolated Enterocyte Studies

The experimental techniques utilized in the isolated enterocyte studies were modified from procedures described by Kimmich (1970); Brown and Sepulveda (1985); Evans et al. (1971); and Sepulveda et al. (1982).

Six week old pigs were anesthetized with halothane and a ventral midline abdominal incision was made. Beginning approximately 200 cm from the ligament of Treitz, a 60 cm segment of jejunum was removed. The lumen of the intestinal segment was immediately flushed with 60 ml of sterile phosphate-buffered saline (pH 7.1 @ 4°C). One end of the intestine was fastened to a spiral steel rod which had previously been covered with cellulose dialysis tubing (Spectrapor Membrane tubing dia 20.4 mm, Spectrum Medical Industries, Inc., Los Angeles, CA) by a cable tie (Dannison Manufacturing Co. Framingham, MA). The intestine was then everted onto the steel rod and the remaining end and underlying dialysis tubing was firmly tied with a second cable tie. The steel rod was 8 mm in diameter and formed a coil 4.2 cm wide and 15 cm high. Holes were spaced along the coil to allow for air inflation. The dialysis tubing and overlying everted intestinal segment were inflated. The steel coil and everted jejunum were then placed in chilled (4°C), aerated isolation medium while being transported to the laboratory.

The isolation medium contained NaCl, 75 mM; Na HCO_3, 25 mM; $CaCl_2$, 1.3 mM; $MgCl_2$, 0.5 mM; K_2HPO_4, 0.36 mM; KH_2PO_4, 0.44 mM; KCl, 5 mM; B-hydroxybutyrate, 0.5 mM; D-mannitol, 98 mM; N-2-hydroxyethylpiperazine-N'-2-ethanesalfonic acid (HEPES), 10 mM; albumin, 1 g/L; and hyaluronidase, 1 mg/ml. After the temperature of the isolation medium was warmed to 37°C the intestinal segment was incubated for 30 minutes. The rod was attached to a vibrator (Chemapec Inc., Woodbury, NY) and vibrated for a few minutes to remove loose cells and mucus. The isolation medium was then changed to fresh medium without hyaluronidase (Isolation media II). The intestinal segment was then vibrated for 45

to 60 minutes. During the incubation and vibration periods the isolation media was continuously aerated with 95% O_2 and 5% CO_2. Since isolated cells tend to clump on contact with glass (Bradford and McGivan, 1982), plastic containers were used during the procedures. Following vibration the cells were centrifuged at 900 x g for 10 minutes at 4°C. The supernatant was discarded and the cells were washed with 30 mls of isolation media II and centrifuged at 900 x g for 3 minutes at 4°C. This was repeated a second time. The weight of the cell pellet was obtained. The cells were resuspended in 5 to 10 ml of isolation media II (depending on the concentration of the cells) and refluxed with a plastic pipette (0.2 mm ID orifice) to break up large clumps of cells into smaller sheets of cells.

Influx experiments

In the uptake experiments, two plastic 50 ml tubes each received a 5 ml aliquot of the cell suspension. One cell suspension was labeled control and received 3 ml of HB 101 filtrate broth and the other was designated STb and received an equal amount of HB 101-pRAS-1 broth filtrate. The volume in both tubes was brought up to 15 ml with incubation medium II. The final dilution of culture filtrate broth was 1:5. The tubes were incubated for 15 minutes at 37°C in a shaker water bath. The radioisotope of the electrolyte under study (^{36}Cl, 10 μCi or ^{45}Ca, 10 μCi) and [^{3}H] PEG, (6.25 μCi) which was used to determine the quantity of extracellular fluid trapped between cells in the cell pellet, were added to the cell suspensions. After rapid mixing, 0.5 ml aliquots were introduced into tared Eppendorf 1.5 ml polypropylene tubes (Brinkman Instruments, Westbury, N.Y.) that contained 0.5 ml ice cold

$MgCl_2$-Tris buffer ($MgCl_2$, 110 mM; and Tris-HCl, 2 mM; 292 mOsm; pH 7.4) layered on top of 250 μl of oil (di-n-butyl phthalate:dinonyl phtalate, 3:2) at timed intervals. The purpose of the ice cold buffer was to stop or retard the transport processes present in the cell membrane. The oil mixture separated the cell pellet from the supernatant thus preventing further exchange of electrolytes between these two components of the cell suspension. Immediately after the aliquot of cell suspension was introduced into the cold buffer, the cells were centrifuged through the oil at 15,000 x g for 10 seconds (Eppendorf centrifuge 5415 Eppendorf Geratebau Nethelert Hinz Gmbh, Hamburg, West Germany). The supernatant was retained for determination of radioisotope, and [^{3}H] PEG concentration and to determine the percentage of lactate dehydrogenase leaking out of the isolated cells. The oil overlying the cell pellet was discarded. The cells were lysed by the addition of 0.5 ml of Triton X-100 (0.5%). After vortexing, the cellular protein was precipitated with 0.5 ml trichloroacetic acid (TCA) (5%). The tubes and their contents were weighed to determine the mass of the cell pellet and after mixing they were centrifuged at 15,000 x g for one minute. The resulting supernatant was collected and analyzed later for intracellular concentration of the electrolyte studied and determination of the amount of extracellular fluid trapped within the cell pellet. The precipitate was prepared for protein analysis by the Lowry method (Lowry et al., 1951) by adding 1 ml of 1 N NaOH to each tube, mixing, and allowing the mixture to stand for 48 hours. A 10 μl aliquot of this mixture was then added to 990 μl of 0.1 N NaOH.

The amount of ^{36}Cl or ^{45}Ca within the cell pellet at designated

time intervals was determined. This was expressed as cpm/mg protein within the pellet. The cpm/mg protein was then plotted against time. The best fitting line was determined by linear regression and the slope was calculated. The slopes were compared between treatments and analyzed using analysis of variance.

Efflux experiments

The preparation of the isolated enterocytes followed the same procedure as the uptake experiments. After the cell pellet was washed, centrifuged and weighed, the cells were resuspended and refluxed in 5 ml of isolation media II. The cell suspension was divided into two 2.5 ml aliquots to which an additional 1.5 ml of isolation media II and 1 ml of control or STb broth was added. The final dilution of the control and STb culture broth was 1:5. After mixing, the suspensions were placed in a shaker water bath for 15 minutes at 37°C. After ^{36}Cl (10 μCi) was added to the cell suspension, the mixture was vortexed and incubated an additional 15 minutes. Twenty-five ml of a mixture of cold culture broth and $MgCl_2$-Tris buffer mixture, which represented a final 1:5 dilution of broth, was added to each batch of cell suspension. The suspensions were then centrifuged for 1 minute and the supernatant discarded. This procedure was repeated twice. A 1:5 dilution of culture broth and tracer free incubation medium was then added and the total mixture vortexed. At timed intervals, a 0.5 ml aliquot of each cell suspension was placed in Eppendorf tubes prepared in the same manner as described in the uptake experiments. In addition 0.25 ml aliquots were placed directly into liquid scintillation vials at the beginning and end of the timed collection period. Tubes were

centrifuged, supernatant was collected and the cell pellet was processed as previously described.

The amount of ^{36}Cl within the pellet and supernatant was determined. These values were expressed as the percent of ^{36}Cl within the cell pellet and within the extracellular fluid respectively. The percent ^{36}Cl within the cell pellet and within the extracellular fluid was plotted against time. The best fitting line was determined by linear regression and the slopes were calculated. The slopes were compared between treatments and analyzed using analysis of variance.

Dye exclusion test

The viability of the isolated enterocytes was estimated by a dye exclusion test modified from the description by Phillips (1973). A 1 ml aliquot of cell suspension was mixed with 0.4 ml of a 1 % aqueous solution of Trypan Blue. Occasionally if the concentration of cells within the suspension was high, 1 ml of a 1:2 dilution of cell suspension with isolation medium was used. Approximately 4 minutes after mixing, the chamber of a hemocytometer was filled with the cell and dye mixture. The number of stained and nonstained cells in five of the secondary squares of the center primary square were counted. The ratio (nonstained cells/total cells) X 100 was used to determine the percentage of viable cells. The total number of cells per ml of the cell suspension was also determined.

Protein determination

The method of Lowry et al. (1951) was used to determine the concentration of protein in the timed aliquots of cell suspension. Proteins complex with copper in an alkaline solution and the reduction

of phosphomolybdate-phosphotungstate salts (Folin-Ciocalteu Phenol reagent) by tyrosine and tryptophan residues in the proteins are responsible for the color formation. A Beckman Model 24 Spectrophotometer was used at 660 mu (UV) to determine the color formation against a reagent blank. Dilutions of a bovine albumin standard solution were used to form a standard curve.

Lactate dehydrogenase determination

The viability of the enterocytes was also estimated by lactate dehydrogenase (LD) determination. The lactate dehydrogenase determination was based on the spectrophotometric method of Wroblewski and La Due (1955) which is an adaptation of the serum assay described by Kubowitz and Ott (1943). Monitoring the rate at which the substrate, pyruvate, was reduced to lactate was used to determine the activity of LD. The proton donor for the reduction of pyruvate is the reduced form of nicotinamide adenine dinucleotide (NADH). Nicotinamide adenine dinucleotide has a peak absorbance at 340 nm. The reaction rate is measured spectrophotometrically by following the rate of decrease in absorbance due to the decrease in concentration of NADH. Absorbance was read on a Beckman Model 24 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 340 nm at 30 second intervals for 3 minutes versus water as a reference. A period was selected where the decrease in absorbance was linear with time. The change in absorbance per minute was calculated for this period. Lactate dehydrogenase activity was then calculated using the equation LD activity [units/ml] = change in absorbance/min. X 20,000 X Temperature Correction Factor (TCF). The LD activity in the supernatant of cells at each time period was then

expressed as a percentage of the LD activity of a sonicated cell suspension.

Statistics

The significance of the water and electrolyte flux data in perfusion and ligated loop experiments was tested by analysis of variance and by determining the least significant difference.

The rate of ^{36}Cl and ^{45}Ca influx was determined by plotting the cpm/mg cell protein of the respective isotope within the cell pellet against time. The determination of the rate of ^{36}Cl efflux was made by plotting the percent of ^{36}Cl present in the extracellular fluid against time. The best fitting line was estimated by linear regression and the slopes were compared by analysis of variance.

RESULTS

Pig Perfusions

Effect of clonidine on intestinal transport

Net water and electrolyte fluxes are shown in Figures 6 to 9. STb reduced net absorption of water (7/8 segments), sodium (7/8), and chloride (5/8) and increased net secretion of potassium (6/8) when compared with the control ($P < 0.05$). Clonidine added to the control solution caused an increase in absorption of water (5/8), sodium (6/8), and chloride (5/8), but the increase was not significant. Clonidine did reduce secretion of potassium (6/8) ($P < 0.05$). In STb-exposed loops, clonidine increased absorption of water (5/8) ($P < 0.05$) and tended to increase, but not significantly, sodium (6/8) and chloride absorption (5/8). Potassium secretion was reduced in the presence of clonidine (7/8) ($P < 0.05$).

Effect of yohimbine on intestinal transport

Net water and electrolyte fluxes are shown in Figures 10 to 13. STb reduced net absorption or induced secretion of water (4/5) ($P < 0.05$). STb also tended to decrease net absorption of sodium (3/5), and increase secretion of potassium (3/5) and chloride (4/5). These changes were not significant.

Addition of yohimbine to the control perfusion solution tended to reduce net water absorption (4/5) and chloride secretion (3/5) and increase sodium absorption (3/5) and potassium secretion (3/5), but there was no significant difference in the means.

Figure 6. Effect of clonidine on the net water flux in paired control and STb exposed perfused jejunal loops of pigs

Concentration of clonidine in perfusate was 5×10^{-7} M. Each bar represents the mean $\pm$ S.E.M. of water flux in ml / 20 cm / 20 / min. of eight pigs

* $P < 0.05$ compared with control

** $P < 0.05$ compared with STb

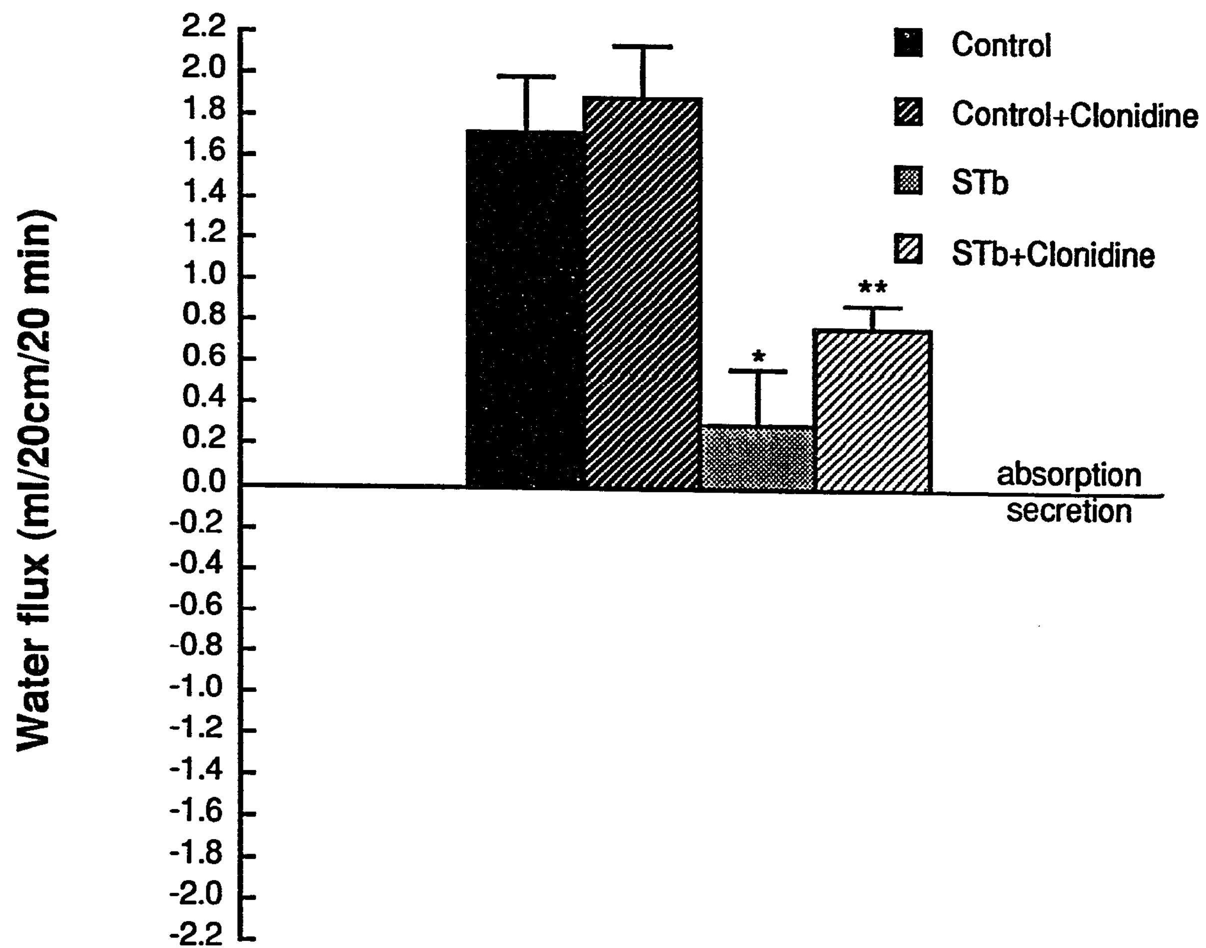

Pig Perfusion Clonidine
Water flux (ml/20cm/20 min)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
-2.0
-2.2
Control
Control+Clonidine
STb
STb+Clonidine
*
**
absorption
secretion

Figure 7. Effect of clonidine on the net sodium flux in paired control and STb exposed perfused jejunal loops of pigs

Concentration of clonidine in perfusate was 5×10^{-7} M. Each bar represents the mean ± S.E.M. of sodium flux in µEq / 20 cm / 20 / min. of eight pigs

* $P < 0.05$ compared with control

** $P < 0.05$ compared with STB

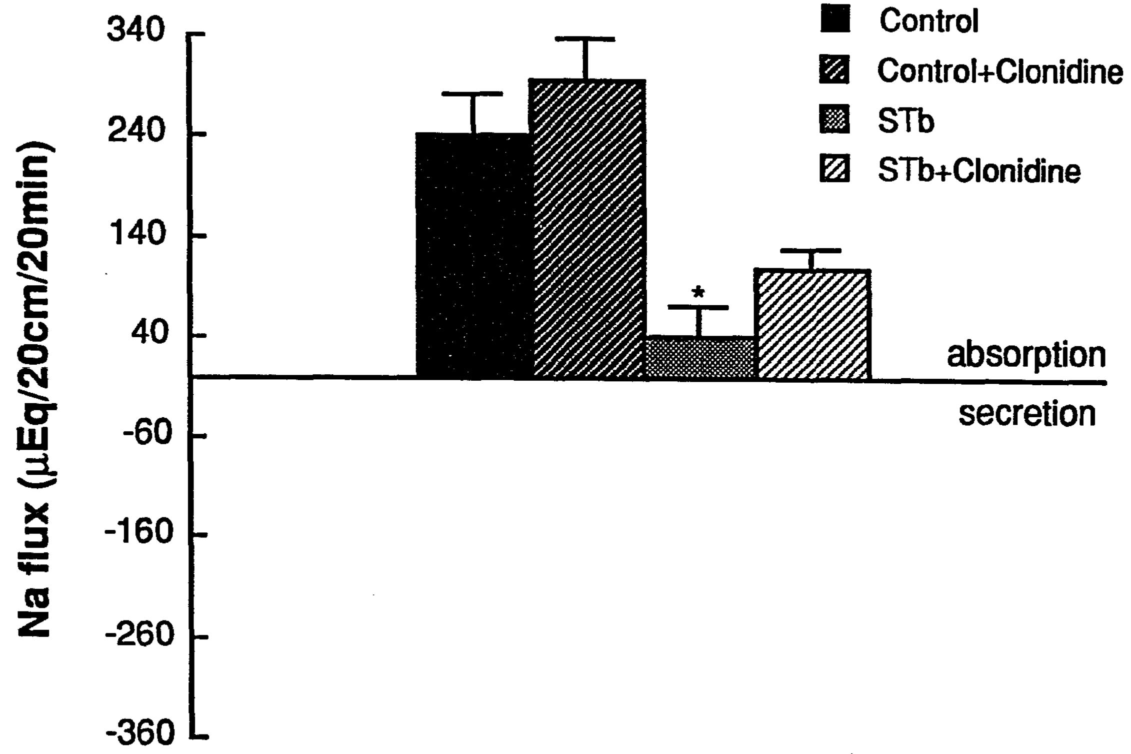
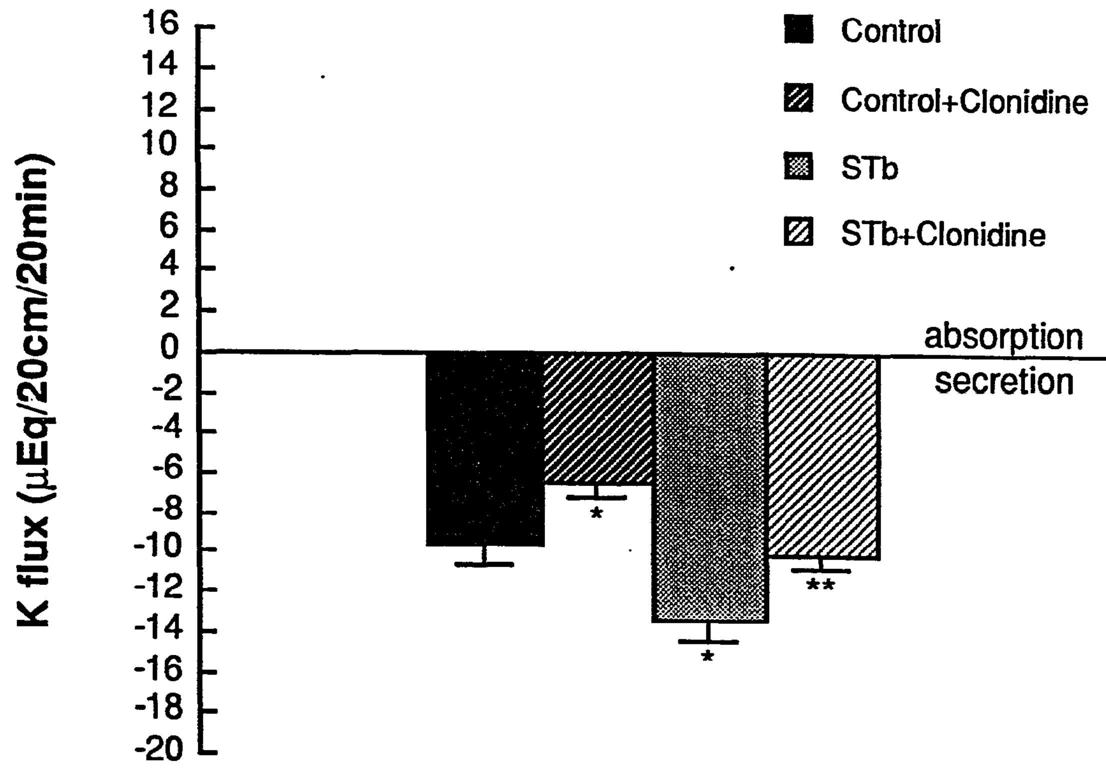

Pig Perfusion Clonidine
Na flux (μEq/20cm/20min)
340
240
140
40
-60
-160
-260
-360
Control
Control+Clonidine
STb
STb+Clonidine
*
absorption
secretion

Figure 8. Effect of clonidine on the net potassium flux in paired control and STb exposed perfused jejunal loops of pigs

Concentration of clonidine in perfusate was 5×10^{-7} M. Each bar represents the mean ± S.E.M. of potassium flux in μEq / 20 cm / 20 / min. of eight pigs

* $P < 0.05$ compared with control

** $P < 0.05$ compared with STb



Pig Perfusion Clonidine
Control
Control+Clonidine
STb
STb+Clonidine
absorption
secretion
K flux (µEq/20cm/20min)
16
14
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
*
*
**

Figure 9. Effect of clonidine on the net chloride flux in paired control and STb exposed perfused jejunal loops of pigs

Concentration of clonidine in perfusate was 5×10^{-7} M. Each bar represents the mean $\pm$ S.E.M. of chloride flux in μEq / 20 cm / 20 / min. of eight pigs
* $P < 0.05$ compared with control

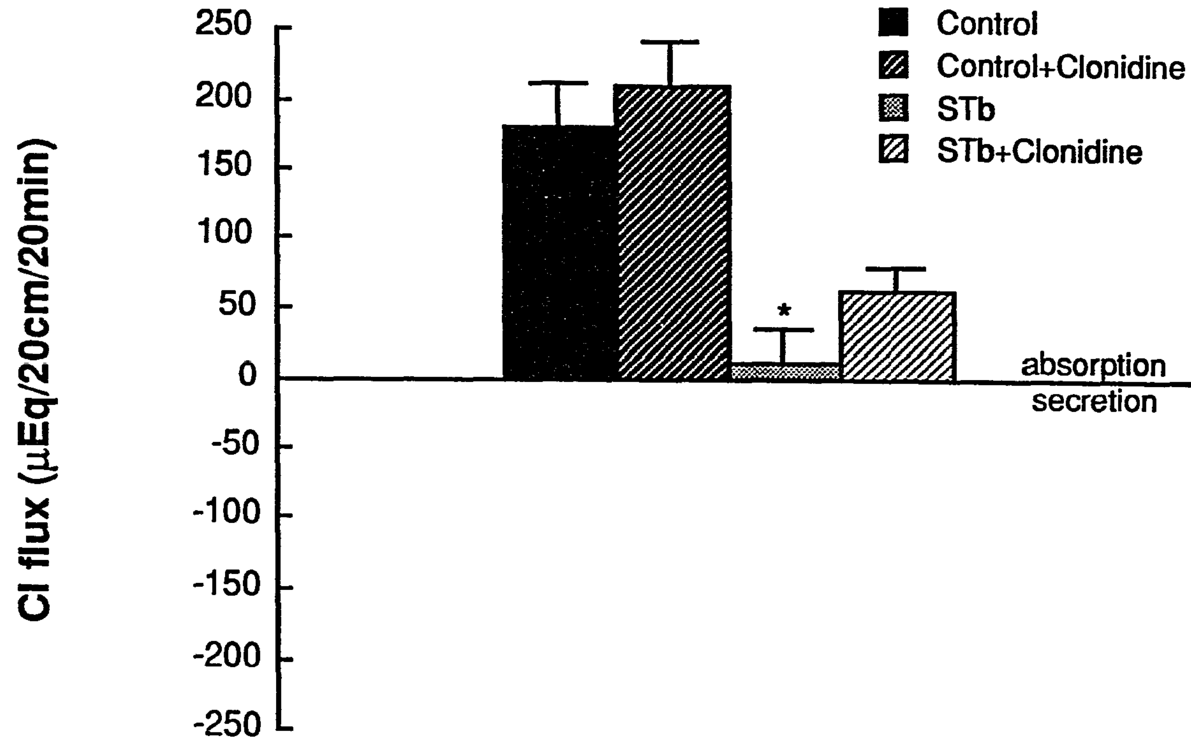

Pig Perfusion Clonidine
Control
Control+Clonidine
STb
STb+Clonidine
250
200
150
100
50
0
-50
-100
-150
-200
-250
Cl flux (μEq/20cm/20min)
*
absorption
secretion

Figure 10. Effect of yohimbine on the net water flux in paired control and STb exposed perfused jejunal loops of pigs

Concentration of yohimbine in perfusate was 1×10^{-4} M. Each bar represents the mean ± S.E.M. of water flux in ml / 20 cm / 20 / min. of five pigs

* $P < 0.05$ compared with control

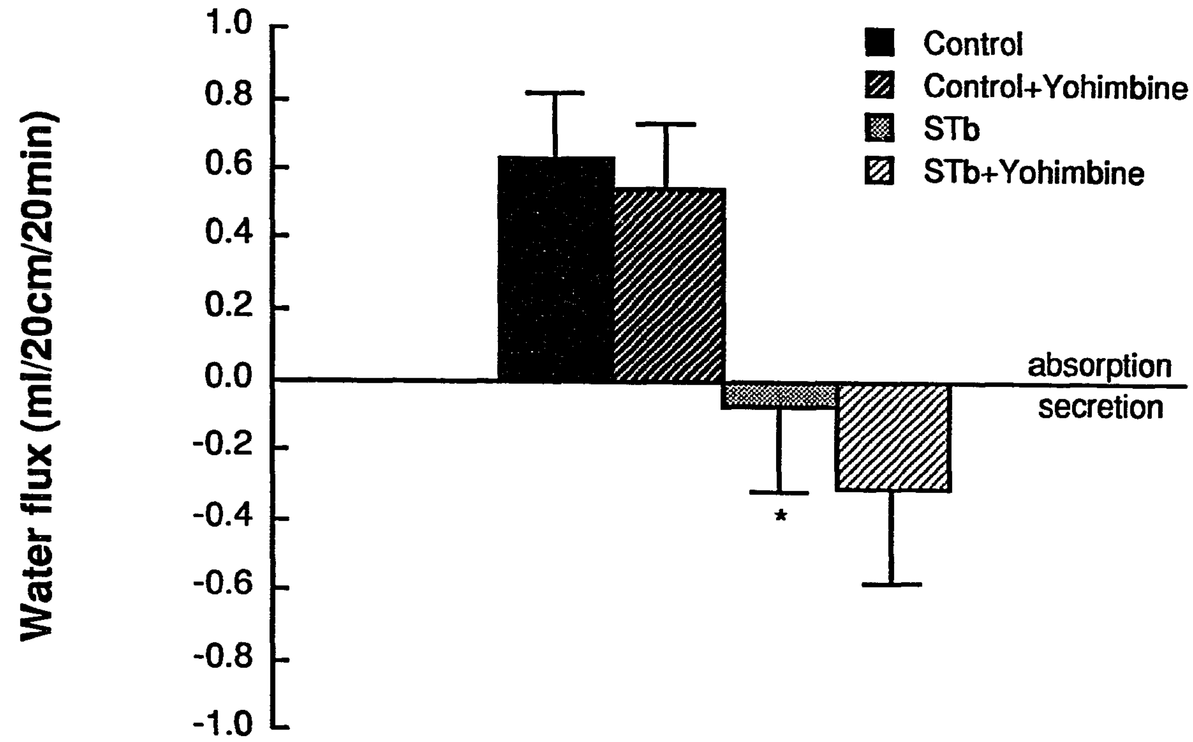

Pig Perfusion Yohimbine
Water flux (ml/20cm/20min)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Control
Control+Yohimbine
STb
STb+Yohimbine
absorption
secretion
*

Figure 11. Effect of yohimbine on the net sodium flux in paired control and STb exposed perfused jejunal loops of pigs

Concentration of yohimbine in perfusate was 1×10^{-4} M. Each bar represents the mean ± S.E.M. of sodium flux in μEq / 20 cm / 20 / min. of five pigs

* $P < 0.05$ compared with control

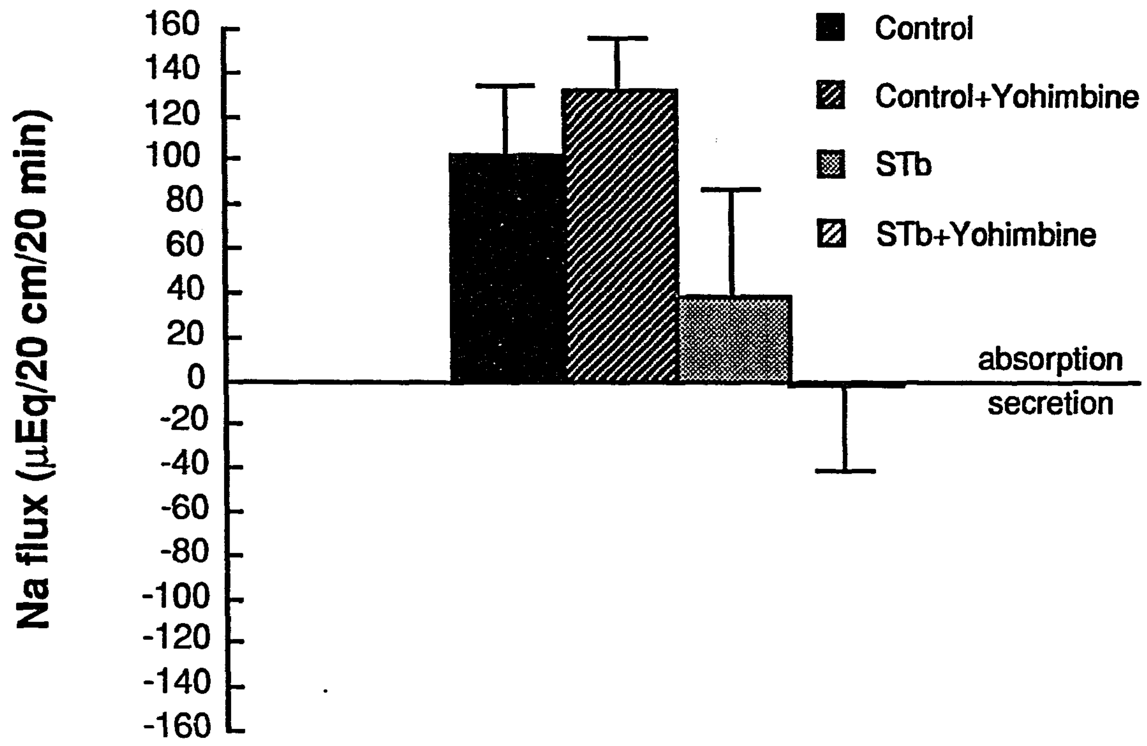

Pig Perfusion Yohimbine
Na flux (µEq/20 cm/20 min)
160
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
Control
Control+Yohimbine
STb
STb+Yohimbine
absorption
secretion

Figure 12. Effect of yohimbine on the net potassium flux in paired control and STb exposed perfused jejunal loops of pigs

Concentration of yohimbine in perfusate was 1×10^{-4} M. Each bar represents the mean $\pm$ S.E.M. of potassium flux in μEq / 20 cm / 20 / min. of five pigs
* $P < 0.05$ compared with control

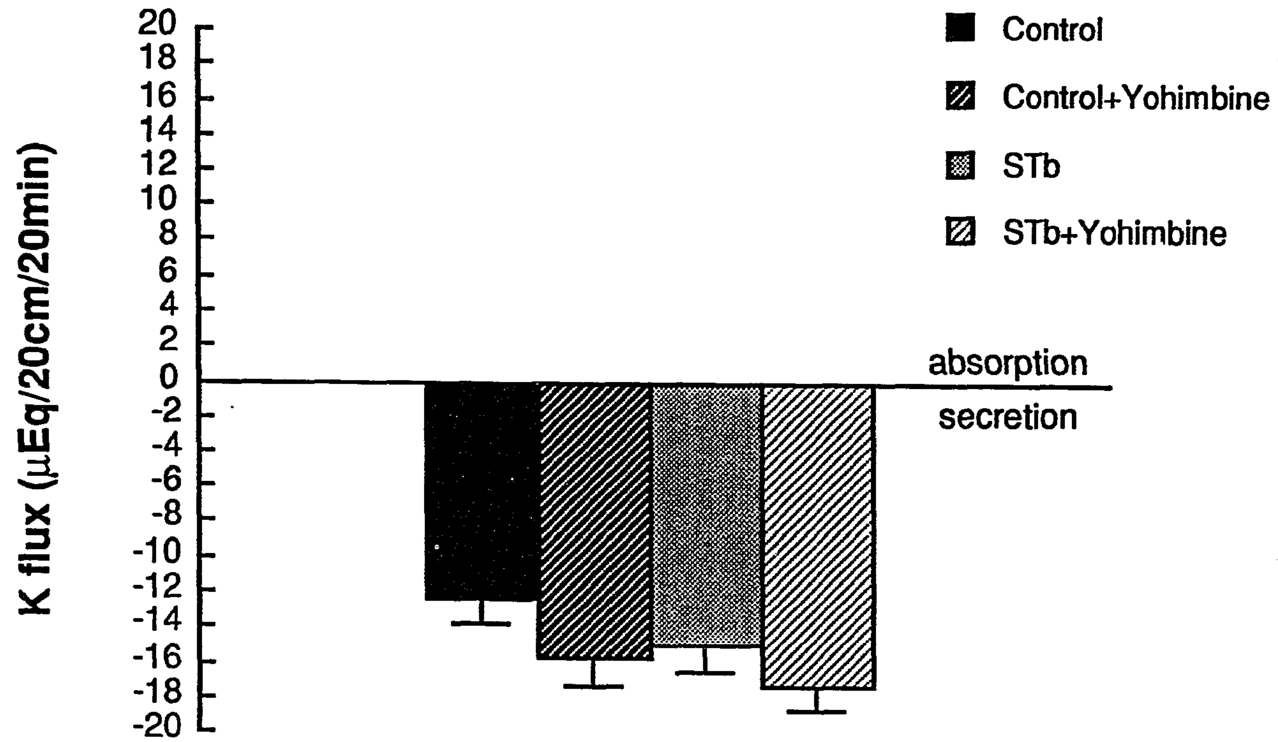
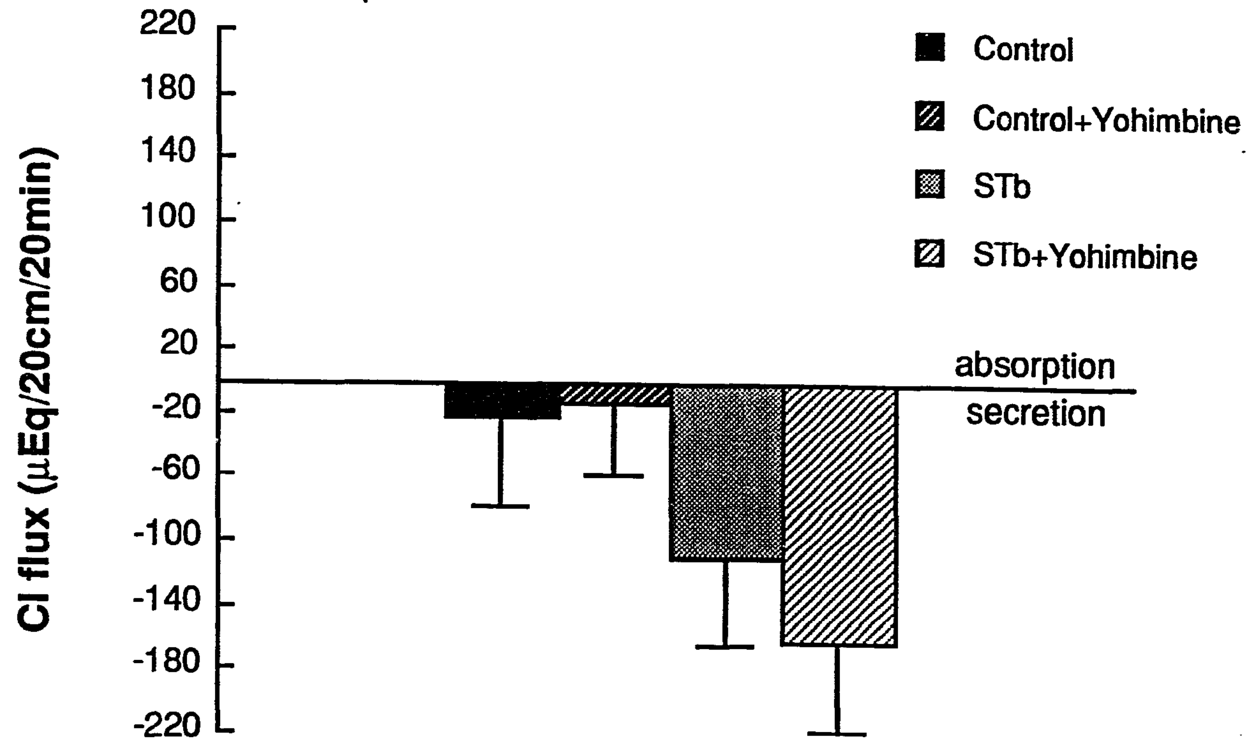

Pig Perfusion Yohimbine
K flux (µEq/20cm/20min)
20
18
16
14
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
Control
Control+Yohimbine
STb
STb+Yohimbine
absorption
secretion

Figure 13. Effect of yohimbine on the net chloride flux in paired control and STb exposed perfused jejunal loops of pigs

Concentration of yohimbine in perfusate was 1×10^{-4} M. Each bar represents the mean $\pm$ S.E.M. of chloride flux in μEq / 20 cm / 20 / min. of five pigs

* $P < 0.05$ compared with control


Pig Perfusion Yohimbine
Cl flux (µEq/20cm/20min)
220
180
140
100
60
20
-20
-60
-100
-140
-180
-220
Control
Control+Yohimbine
STb
STb+Yohimbine
absorption
secretion

When yohimbine was included in the STb perfusion solution, there was a tendency to change net sodium absorption to net secretion (2/5) and net secretion of water (2/5), potassium (4/5) and chloride (2/5) was increased. None of these changes were significant.

Pig Ligated Loops

Effect of clonidine and yohimbine on transport in control and STb-exposed jejunal loops

Net water and electrolyte fluxes are shown in Figures 14 to 17. STb elicited a net secretory response for water (9/10 loops) (-5.914 ml/10 cm/2 hr), that was significantly different from the absorptive control response (9/14) (1.847 ml/10 cm/2 hr) ($P < 0.05$). Similarly, net secretion of sodium (8/10) and potassium (10/10) was induced by STb that differed ($P < 0.05$) from the net absorption of sodium (10/14) and net secretion of potassium (7/14) noted with the control broth. Secretion of chloride was evident in both STb (5/9) and control loops (7/14), but the difference was not significant.

Clonidine when present in control loops had a tendency to enhance absorption of water (7/8) and reduce net secretion of sodium (7/8) and potassium (8/8). Similarly the presence of clonidine in the toxin loops tended to reduce sodium (8/8) and potassium (5/8) secretion and increase water absorption (6/8). Net absorption of chloride tended to be increased in both control (5/8) and toxin (7/7) loops. None of these changes were significant.

Yohimbine tended to cause a decrease in secretion of chloride

Figure 14. Effect of clonidine (3.8×10^{-6} M) and yohimbine (2.56×10^{-5} M) on net flux of water in control and STb exposed ligated jejunal loops of pigs

Each bar represents the mean $\pm$ S.E.M. of eight loops
* $P < 0.05$ compared with control

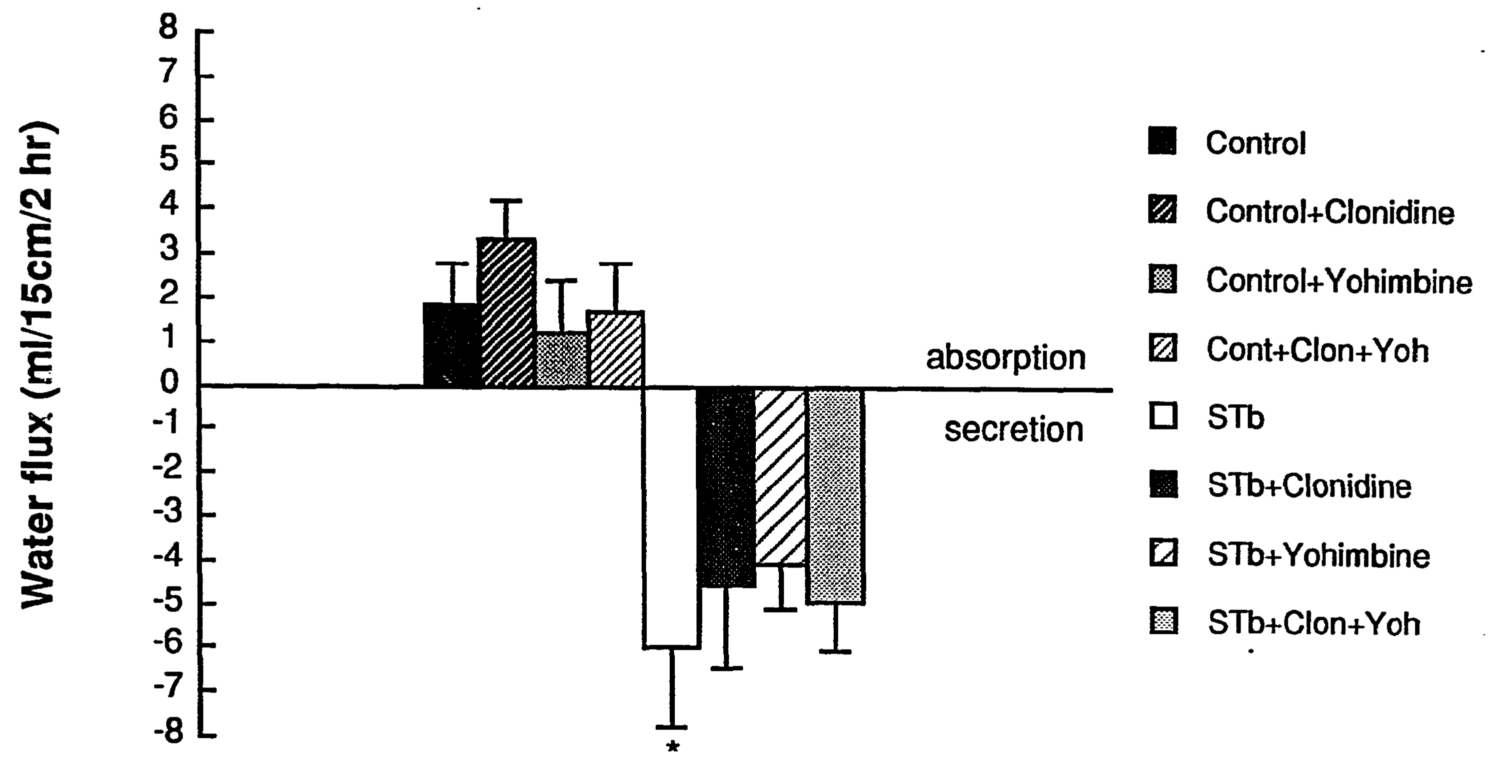
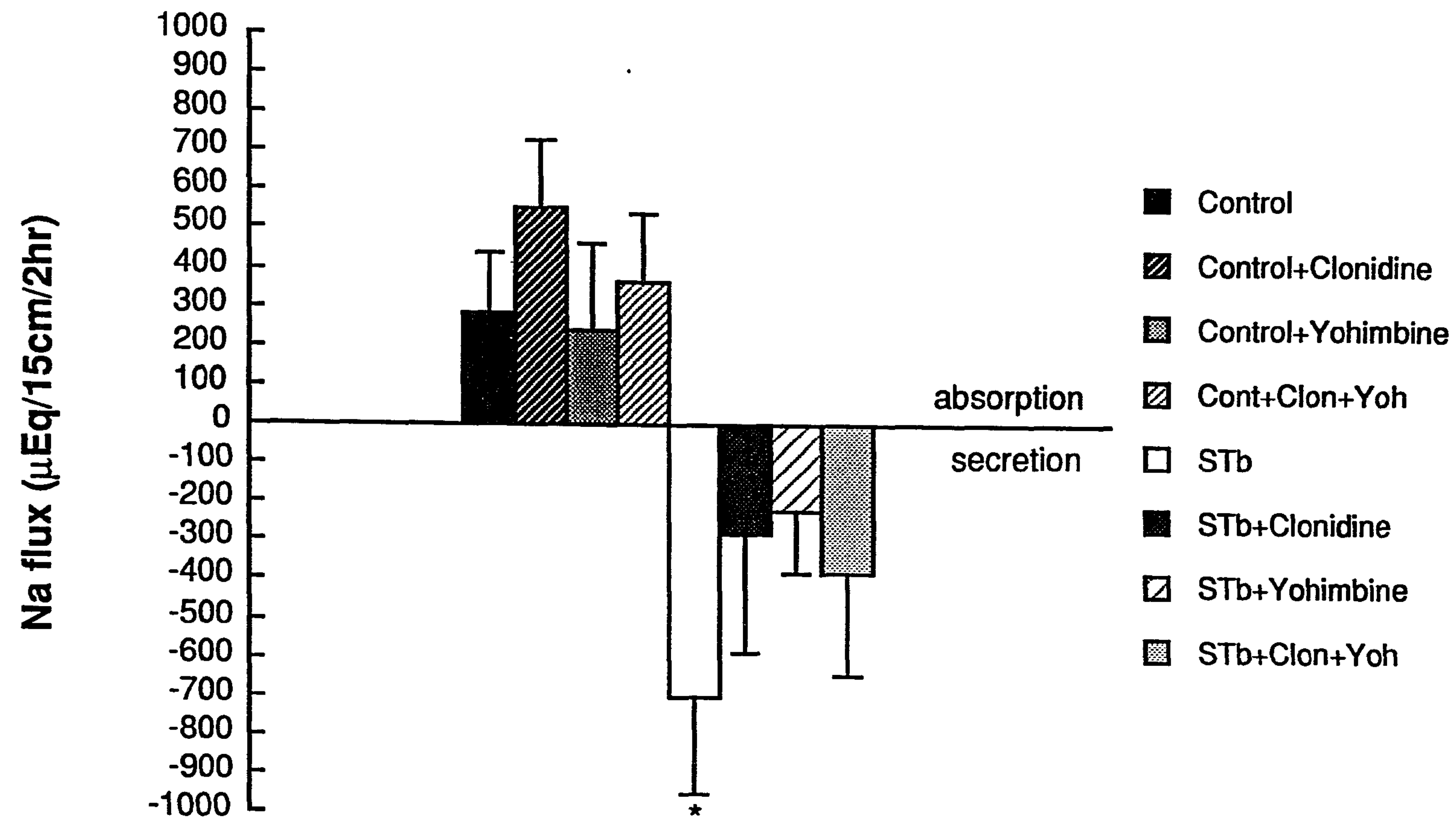

Pig Loops Clonidine & Yohimbine
Water flux (ml/15cm/2 hr)
8
7
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
absorption
secretion
*
Control
Control+Clonidine
Control+Yohimbine
Cont+Clon+Yoh
STb
STb+Clonidine
STb+Yohimbine
STb+Clon+Yoh

Figure 15. Effect of clonidine (3.8×10^{-6} M) and yohimbine (2.56×10^{-5} M) on net flux of sodium in control and STb exposed ligated jejunal loops of pigs

Each bar represents the mean ± S.E.M. of eight loops
* $P < 0.05$ compared with control



Pig Loops Clonidine & Yohimbine
Na flux (μEq/15cm/2hr)
1000
900
800
700
600
500
400
300
200
100
0
-100
-200
-300
-400
-500
-600
-700
-800
-900
-1000
absorption
secretion
*
Control
Control+Clonidine
Control+Yohimbine
Cont+Clon+Yoh
STb
STb+Clonidine
STb+Yohimbine
STb+Clon+Yoh

Figure 16. Effect of clonidine (3.8×10^{-6} M) and yohimbine (2.56×10^{-5} M) on net flux of potassium in control and STb exposed ligated jejunal loops of pigs

Each bar represents the mean $\pm$ S.E.M. of eight loops
* $P < 0.05$ compared with control

Pig Loops Clonidine & Yohimbine

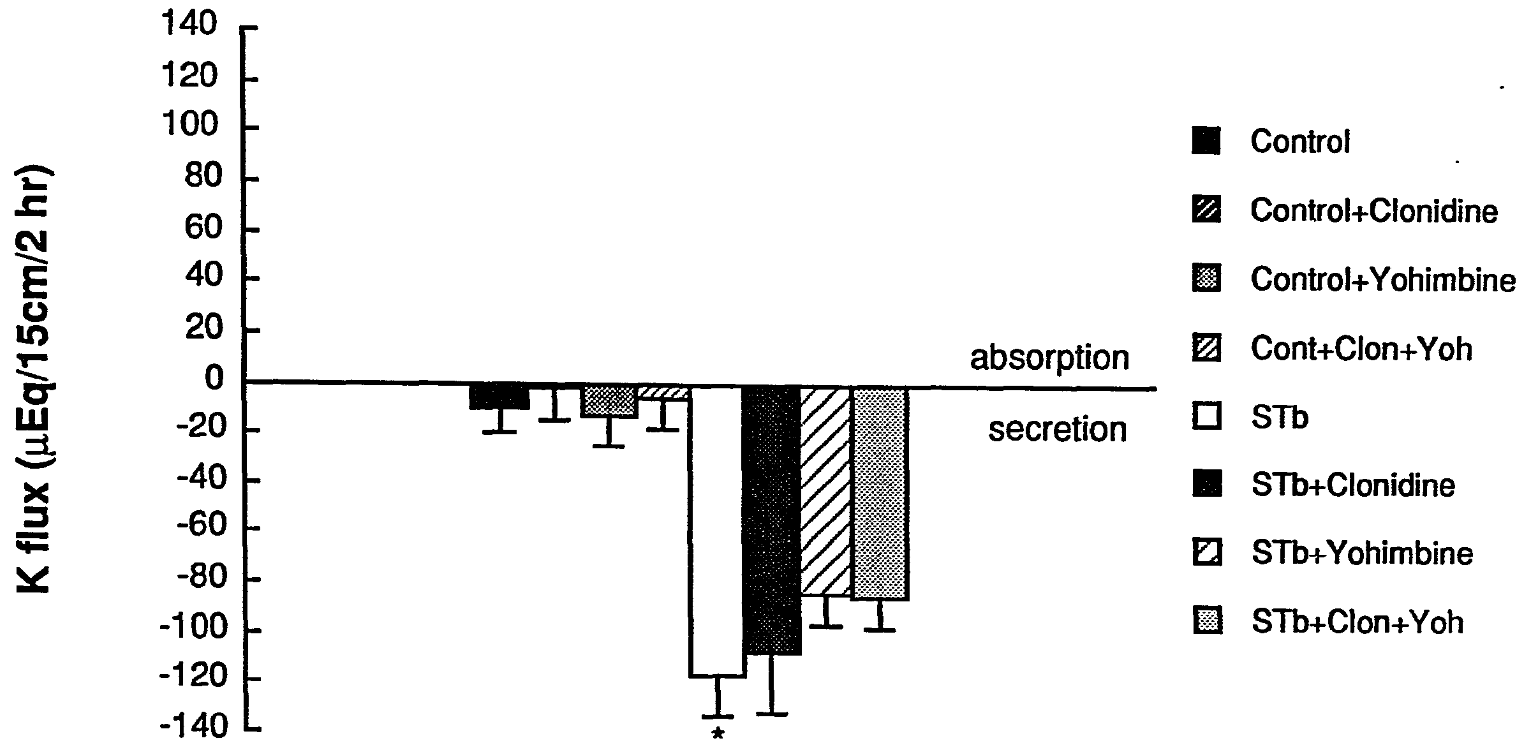
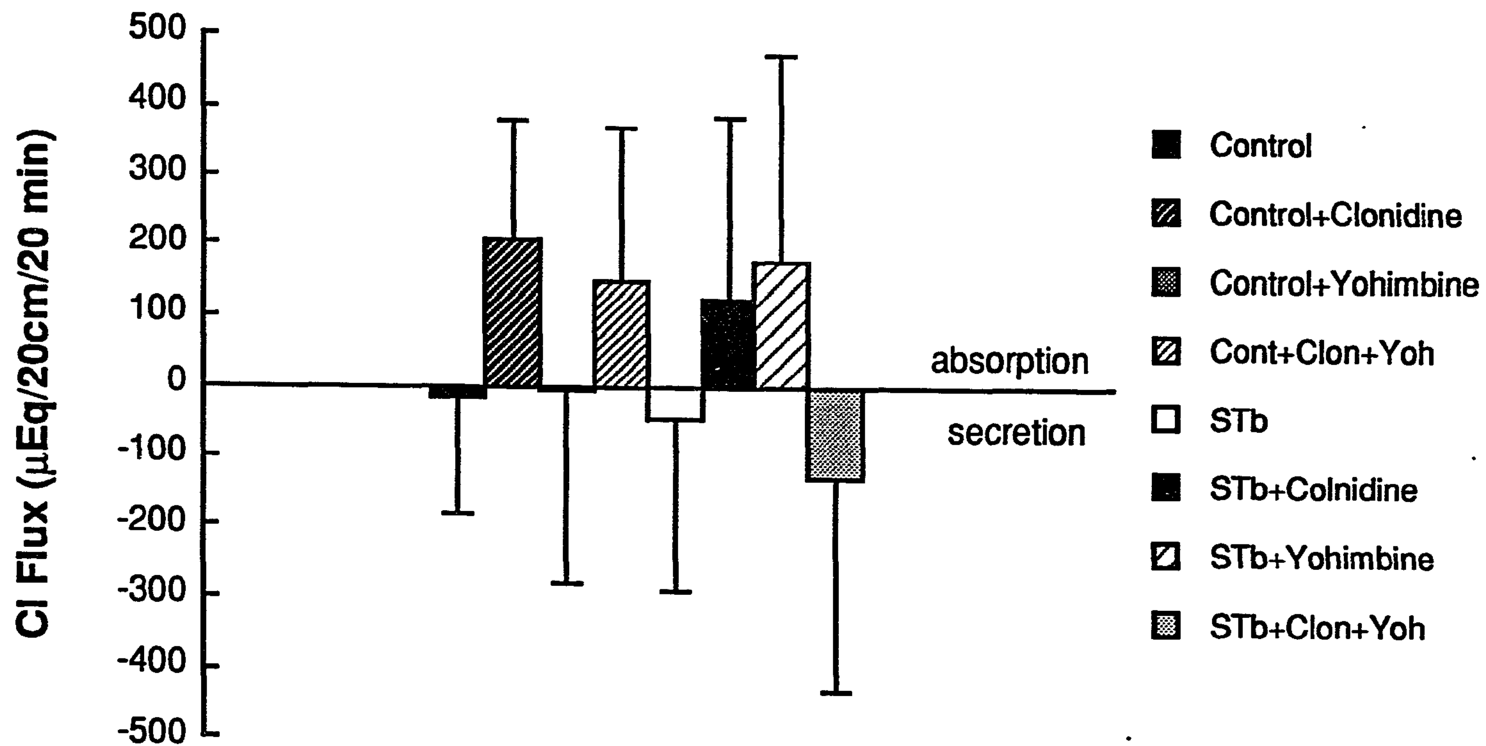


Figure 17. Effect of clonidine (3.8×10^{-6} M) and yohimbine (2.56×10^{-5} M) on net flux of chloride in control and STb exposed ligated jejunal loops of pigs

Each bar represents the mean $\pm$ S.E.M. of eight loops
* $P < 0.05$ compared with control


Pig Loops Clonidine & Yohimbine
Cl Flux (µEq/20cm/20 min)
500
400
300
200
100
0
-100
-200
-300
-400
-500
absorption
secretion
Control
Control+Clonidine
Control+Yohimbine
Cont+Clon+Yoh
STb
STb+Colnidine
STb+Yohimbine
STb+Clon+Yoh

(3/7), a slight increase in potassium secretion (5/8) and a slight decrease in absorption of water (6/8) and sodium (4/8) in the control loops. In STb loops, yohimbine tended to decrease secretion of water (4/8), sodium (6/8) and potassium (5/8), and increase absorption of chloride (8/8). These changes were not significant.

Inclusion of clonidine and yohimbine in control or STb broth caused no significant alteration in the water, sodium, chloride, or potassium flux.

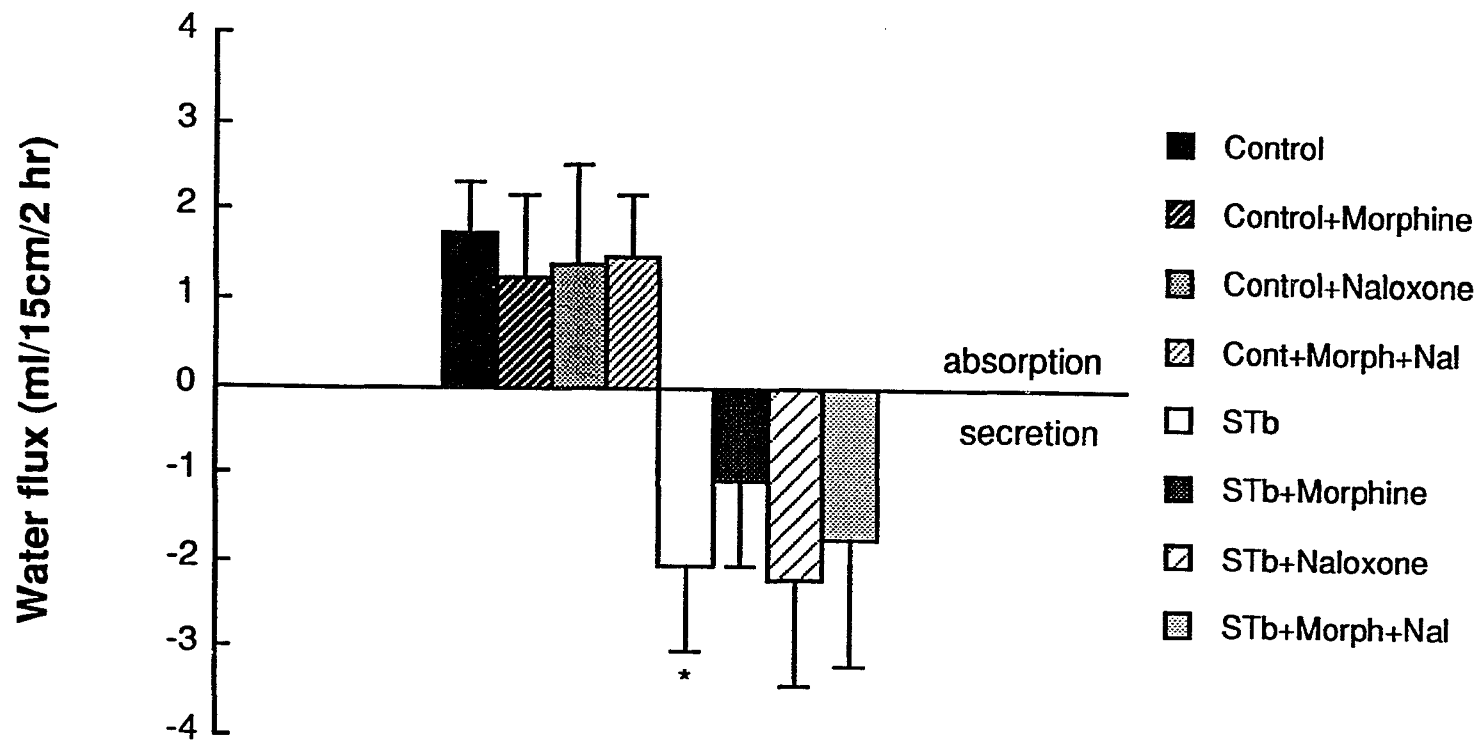
Effect of morphine and naloxone on transport in control and STb exposed jejunal loops

Net water and electrolyte fluxes are shown in Figures 18 to 21. STb stimulated a net secretory movement of water (7/12), sodium (8/13), potassium (12/12) and chloride (6/11) that was, with the exception of the chloride flux, significantly different from the absorptive responses noted in the control loops for water (14/20), sodium (19/20) and chloride (12/19) and the secretory movement of potassium (10/20). Morphine had no significant effect on control fluxes, but significantly reduced the secretion of potassium (8/10) and increased the absorptive flux of sodium (7/10) and chloride (7/10) in toxin loops ($P < 0.05$). Net secretion of water was also reduced (5/10), but not significantly.

Naloxone reduced STb stimulated secretion and increased the absorption of sodium (8/10) and chloride (4/6) ($P < 0.05$). No significant effect was induced by naloxone on the intestinal transport in intestinal segments containing control broth. The combination of morphine and naloxone reduced secretion and increased absorption of sodium (9/10) and chloride (6/7) and reduced potassium secretion (9/10)

Figure 18. Effect of morphine (1.5×10^{-5} M) and naloxone (1.5×10^{-5} M) on net flux of water in control and STb exposed ligated jejunal loops of pigs

Each bar represents the mean ± S.E.M. of ten loops
* $P < 0.05$ compared with control



Pig Loops Morphine & Naloxone
Water flux (ml/15cm/2 hr)
4
3
2
1
0
-1
-2
-3
-4
absorption
secretion
*
Control
Control+Morphine
Control+Naloxone
Cont+Morph+Nal
STb
STb+Morphine
STb+Naloxone
STb+Morph+Nal

Figure 19. Effect of morphine (1.5×10^{-5} M) and naloxone (1.5×10^{-5} M) on net flux of sodium in control and STb exposed ligated jejunal loops of pigs

Each bar represents the mean ± S.E.M. of ten loops
* $P < 0.05$ compared with control
** $P < 0.05$ compared with STb

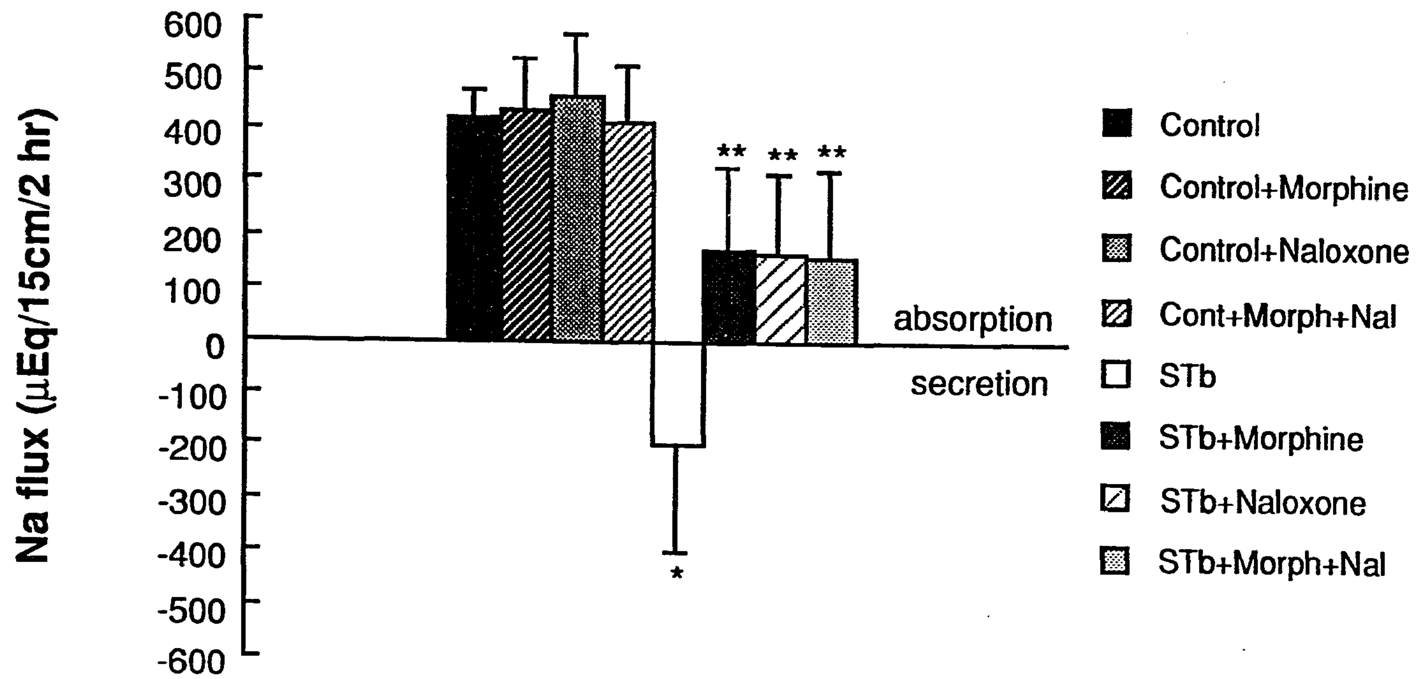
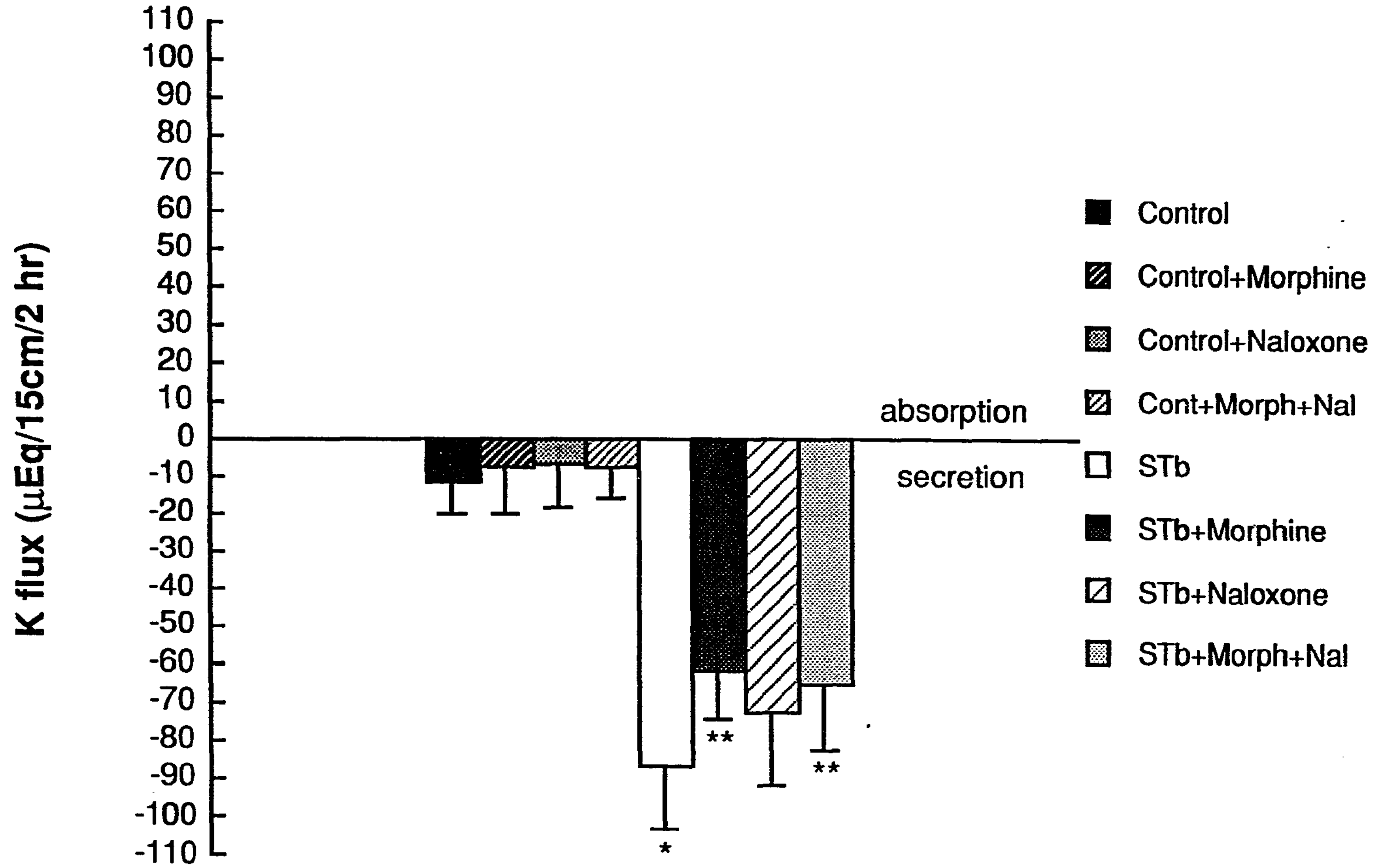

Pig Loops Morphine & Naloxone
Na flux (µEq/15cm/2 hr)
600
500
400
300
200
100
0
-100
-200
-300
-400
-500
-600
absorption
secretion
**
*
Control
Control+Morphine
Control+Naloxone
Cont+Morph+Nal
STb
STb+Morphine
STb+Naloxone
STb+Morph+Nal

Figure 20. Effect of morphine (1.5×10^{-5} M) and naloxone (1.5×10^{-5} M) on net flux of potassium in control and STb exposed ligated jejunal loops of pigs

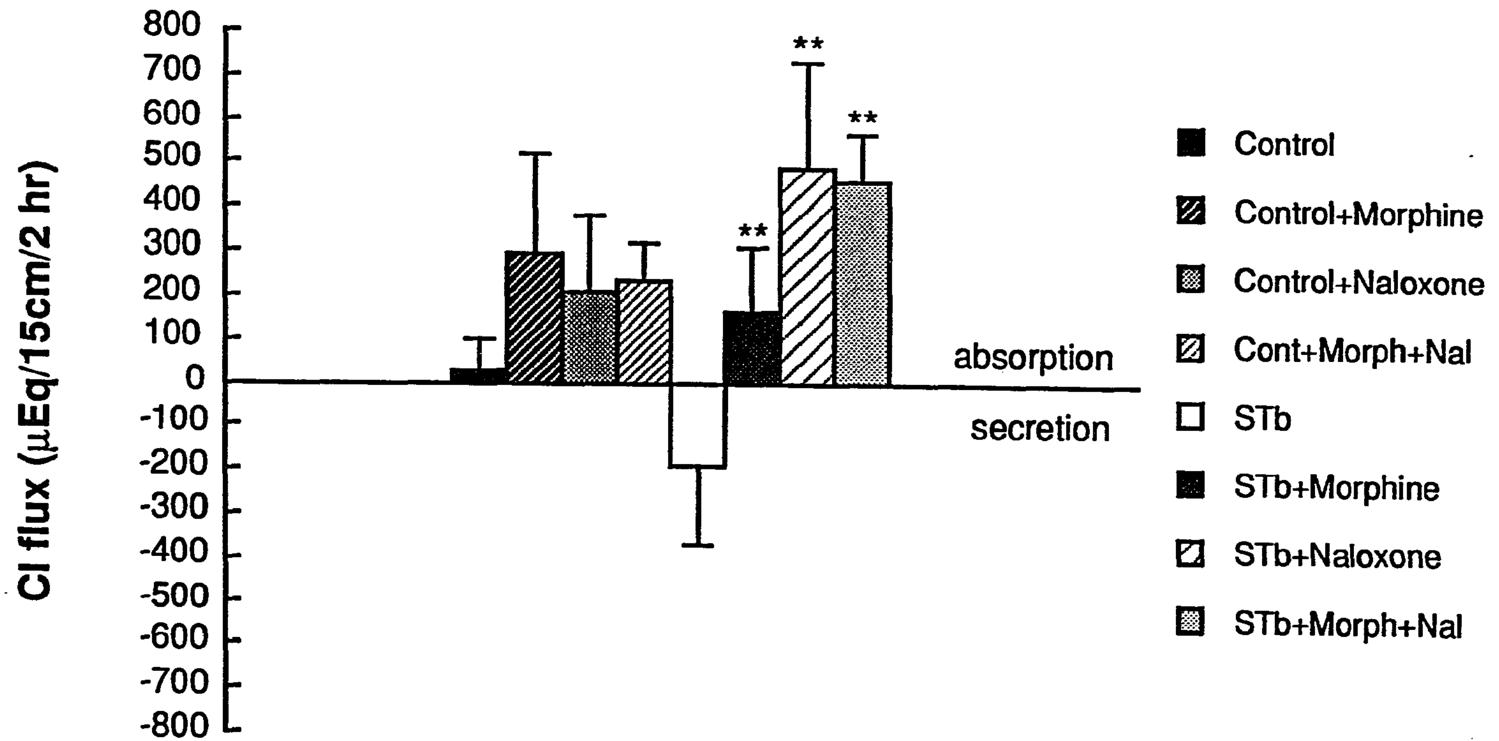
Each bar represents the mean ± S.E.M. of ten loops
* $P < 0.05$ compared with control
** $P < 0.05$ compared with STb



Pig Loops Morphine & Naloxone
K flux (µEq/15cm/2 hr)
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
absorption
secretion
*
**
**
Control
Control+Morphine
Control+Naloxone
Cont+Morph+Nal
STb
STb+Morphine
STb+Naloxone
STb+Morph+Nal

Figure 21. Effect of morphine (1.5×10^{-5} M) and naloxone (1.5×10^{-5} M) on net flux of chloride in control and STb exposed ligated jejunal loops of pigs

Each bar represents the mean ± S.E.M. of ten loops
** $P < 0.05$ compared with STb


Pig Loops Morphine & Naloxone
Cl flux (µEq/15cm/2 hr)
800
700
600
500
400
300
200
100
0
-100
-200
-300
-400
-500
-600
-700
-800
**
**
**
absorption
secretion
Control
Control+Morphine
Control+Naloxone
Cont+Morph+Nal
STb
STb+Morphine
STb+Naloxone
STb+Morph+Nal

(P < 0.05) in toxin loops. A reduction in the secretory flux of water (4/10) was noted, but it was not significant.

Effect of hexamethonium and scopolamine on transport in control and STb jejunal loops

Net water and electrolyte fluxes are shown in Figures 22 to 25. STb significantly stimulated a reversal of net absorption of water to net secretion (6/8), reduced net absorption of sodium (7/8) and increased net secretion of potassium (8/8) (P < 0.05) when compared to control. Net flux of chloride was not significantly changed.

Hexamethonium increased net absorption of chloride when added to the control (6/8) and toxin (8/8) broths (P < 0.05). Scopolamine also caused an increase in absorption of chloride in control (6/8) and toxin (8/8) loops, but the increase was only significant in toxin loops.

Rat Perfusions

1261 versus 1790 effect on intestinal water transport

Net water fluxes are shown in Figure 26. In order to evaluate the sensitivity of rat intestine to the E. coli heat stable enterotoxin, STb, the intestinal response to E. coli strain 1261 cultured broth filtrate (STa and STb) was compared with the response to E. coli strain 1790 culture broth filtrate (STb only). In both experiments the control broth was obtained by the filtration of broth from cultures of E. coli strain 123.

Intestinal segments perfused with toxin broth derived from strain 1261 developed a net secretion of water (8/9 segments) (-0.297 ml/20

Figure 22. Effect of hexamethonium (2.8×10^{-3} M) and scopolamine (2.0×10^{-4} M) on net flux of water in control and STb exposed ligated jejunal loops of pigs

Each bar represents the mean $\pm$ S.E.M. of eight loops
* $P < 0.05$ compared with control

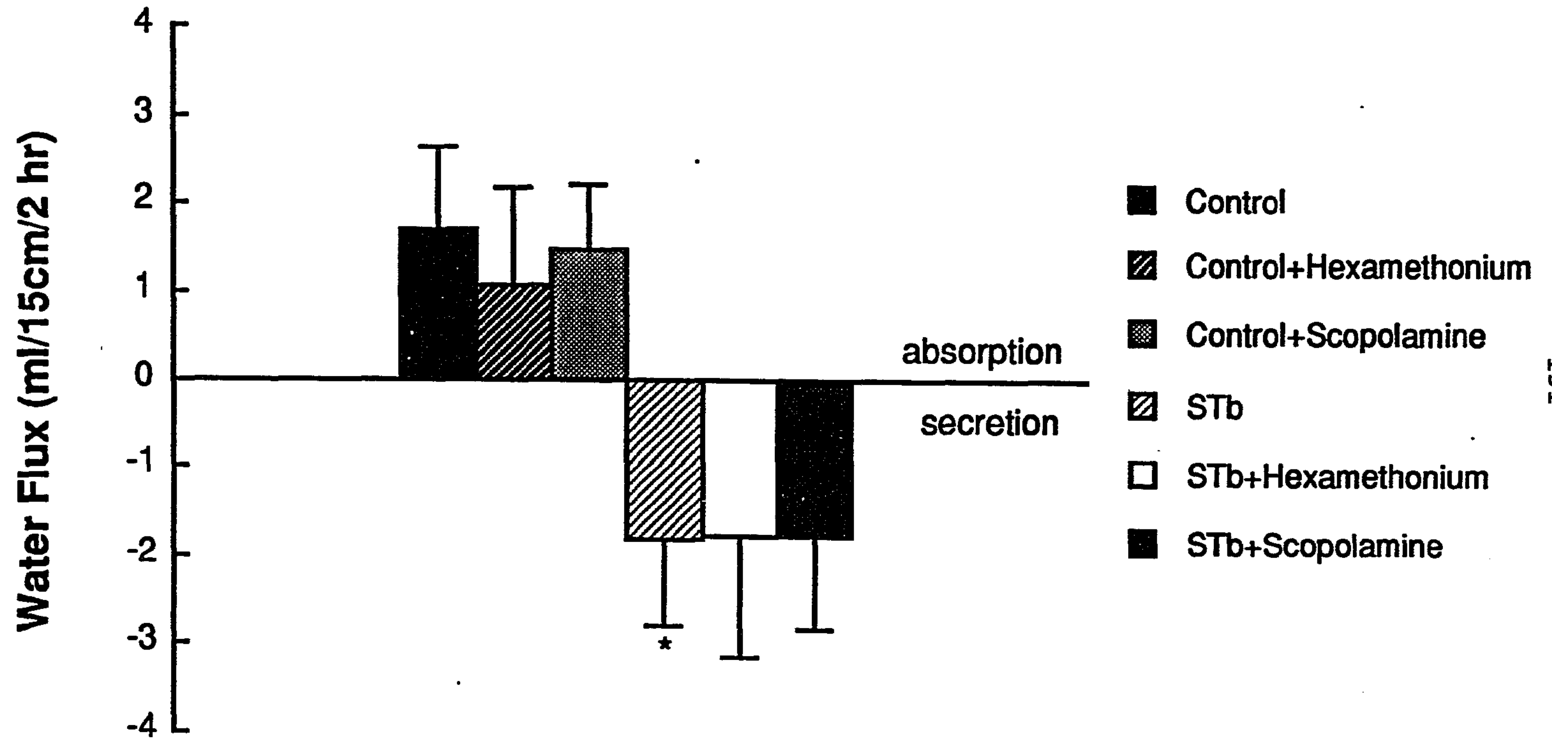

Pig Loops Hexamethonium & Scopolamine
Water Flux (ml/15cm/2 hr)
4
3
2
1
0
-1
-2
-3
-4
absorption
secretion
*
Control
Control+Hexamethonium
Control+Scopolamine
STb
STb+Hexamethonium
STb+Scopolamine

Figure 23. Effect of hexamethonium (2.8×10^{-3} M) and scopolamine (2.0×10^{-4} M) on net flux of sodium in control and STb exposed ligated jejunal loops of pigs

Each bar represents the mean ± S.E.M. of eight loops
* $P < 0.05$ compared with control

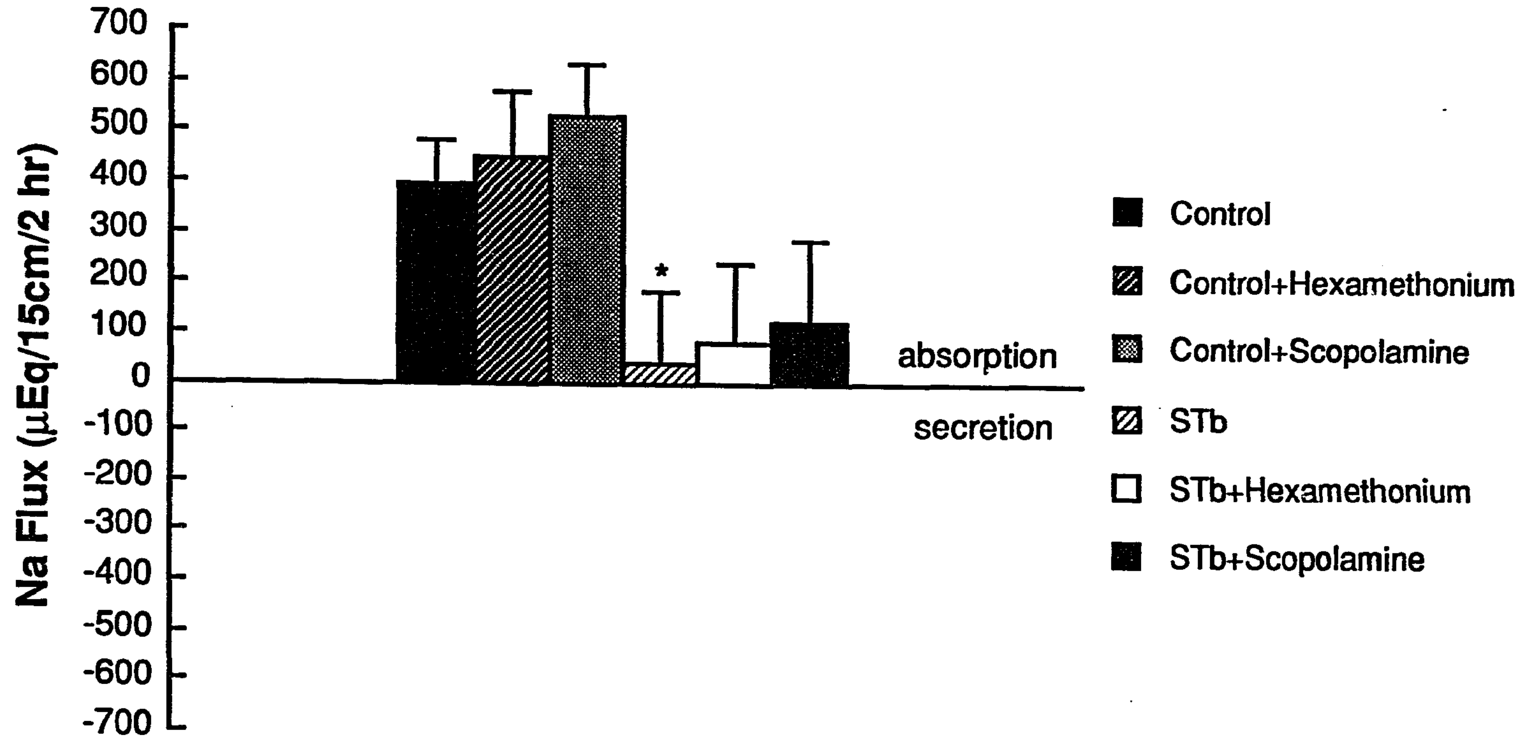

Pig Loops Hexamethonium & Scopolamine
Na Flux (µEq/15cm/2 hr)
700
600
500
400
300
200
100
0
-100
-200
-300
-400
-500
-600
-700
*
absorption
secretion
Control
Control+Hexamethonium
Control+Scopolamine
STb
STb+Hexamethonium
STb+Scopolamine

Figure 24. Effect of hexamethonium (2.8×10^{-3} M) and scopolamine (2.0×10^{-4} M) on net flux of potassium in control and STb exposed ligated jejunal loops of pigs

Each bar represents the mean $\pm$ S.E.M. of eight loops
* $P < 0.05$ compared with control

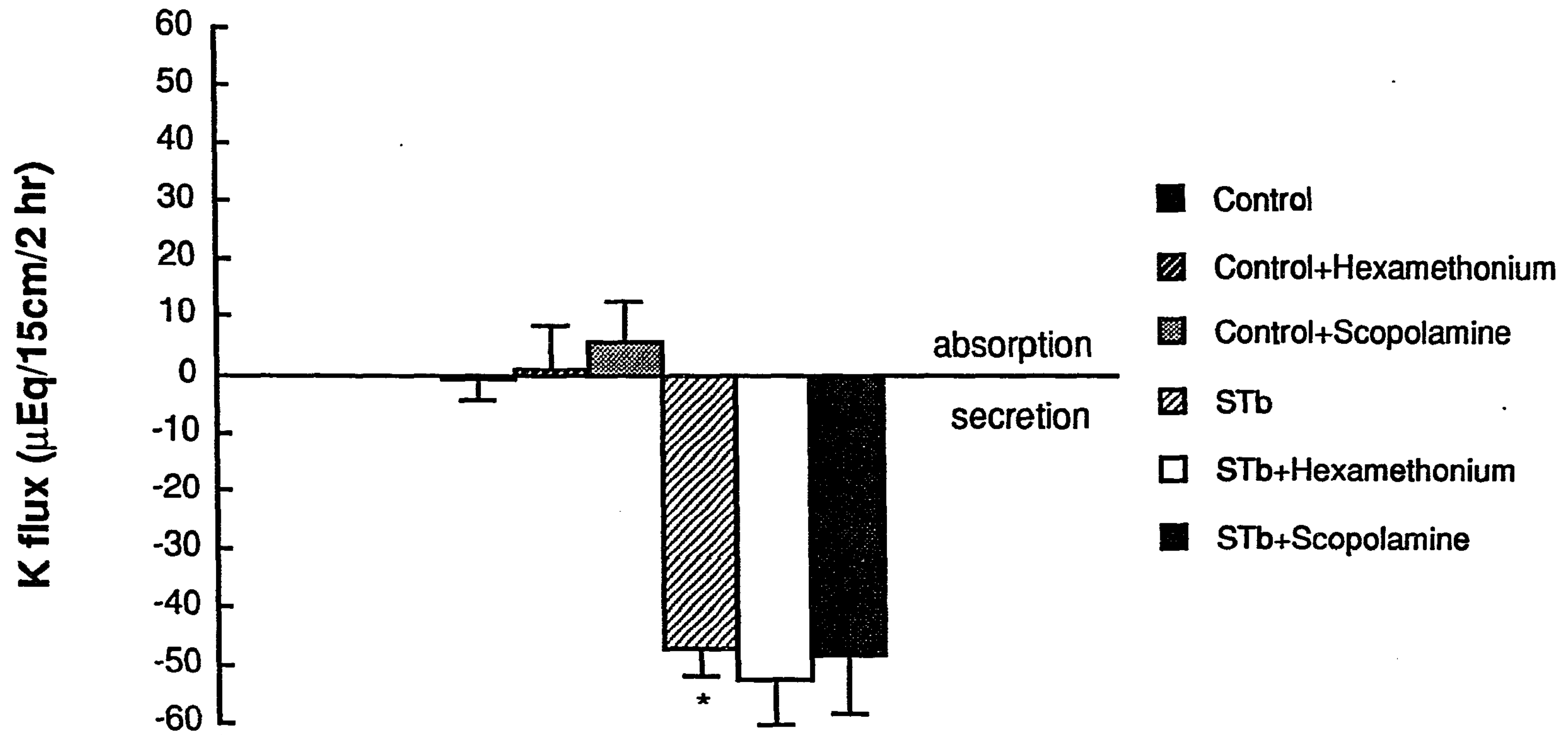

Pig Loops Hexamethonium & Scopolamine
K flux (µEq/15cm/2 hr)
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
absorption
secretion
*
Control
Control+Hexamethonium
Control+Scopolamine
STb
STb+Hexamethonium
STb+Scopolamine

Figure 25. Effect of hexamethonium (2.8×10^{-3} M) and scopolamine (2.0×10^{-4} M) on net flux of chloride in control and STb exposed ligated jejunal loops of pigs

Each bar represents the mean ± S.E.M. of eight loops
* $P < 0.05$ compared with control
** $P < 0.05$ compared with STb

Pig Loops Hexamethonium & Scopolamine

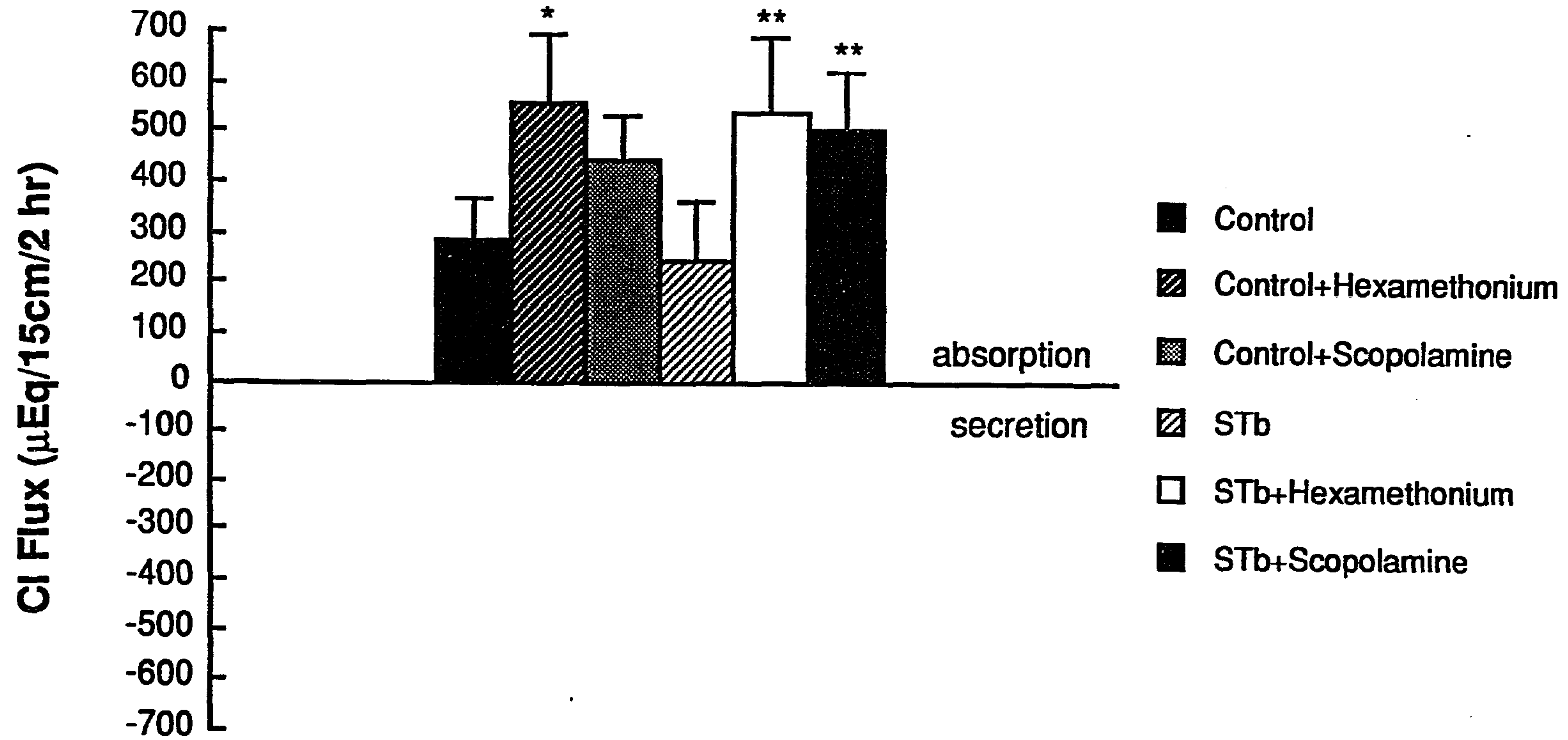


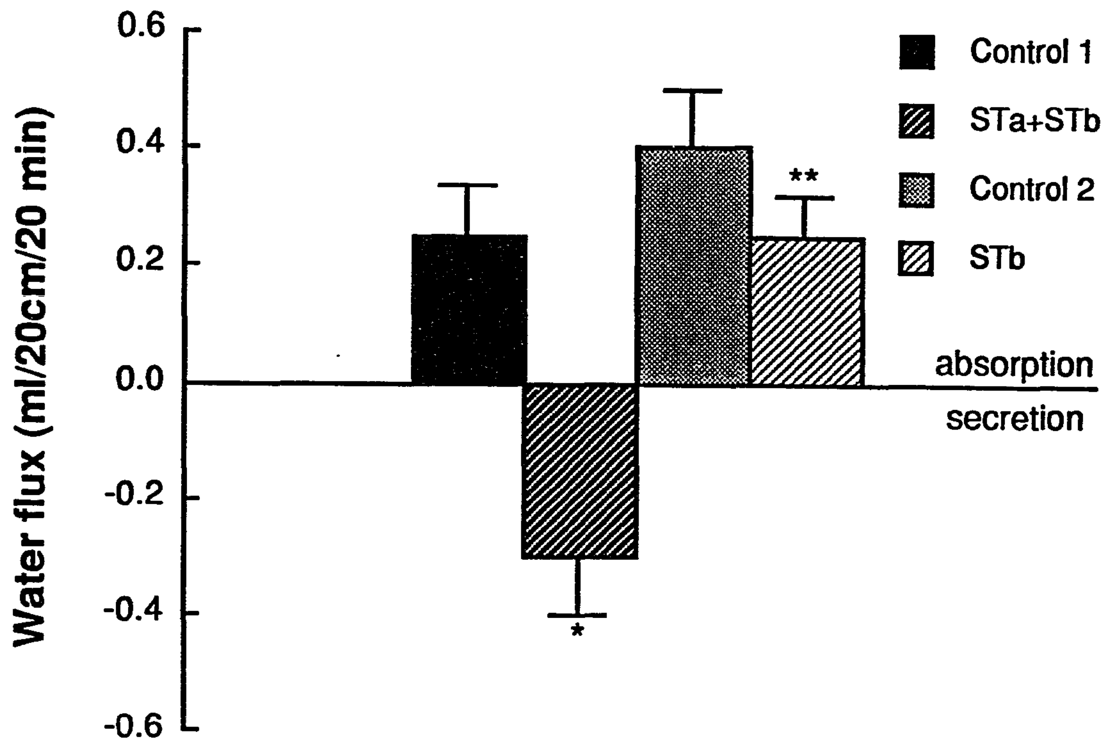
Figure 26. Effect of broth containing STa and STb (n = 7) or STb only (n = 6) vs paired control on water flux in perfused jejumal loops of rats

Each bar represents the mean (± S.E.M.) of water flux in ml / 20 cm / 20 min

* P = 0.0001 STa and STb compared with control

** P = 0.002 STb compared with control

Rat perfusion



cm/20 min.) when compared to the absorptive response present in intestinal segments perfused with control broth (7/9) (0.252 ml/20 cm/20 min.) (P = 0.0001). Intestinal segments of rats perfused with toxin broth derived from 1790 exhibited a reduction of net water absorption (5/6) (+0.253 ml/20 cm/20 min.) that was significantly different (P = 0.0020) from the absorptive response in control segments (6/6) (+0.405 ml/20 cm/20 min). The difference between the responses to the 1261 and 1790 enterotoxin preparations was not statistically significant.

Effect of soybean trypsin inhibitor on the response to STb

Net water fluxes are shown in Figure 27. Perfusions were conducted with STb and control broth solutions with and without the addition of soybean trypsin inhibitor. Perfusions with the control solution and control solution containing soybean trypsin inhibitor resulted in an absorptive response of +0.405 and +0.569 ml/20 cm/20 min. respectively. The perfused STb solution without soybean trypsin inhibitor produced a reduced absorptive response (6/6) of +0.253 ml/20 cm/20 min., while perfusion with the STb solution with soybean trypsin inhibitor resulted in a secretory response (12/14) of -0.414 ml/20 cm/20 min. Response to STb with soybean trypsin inhibitor was significantly different (P = 0.0010) than STb alone.

Effect of pirenzepine on intestinal water flux

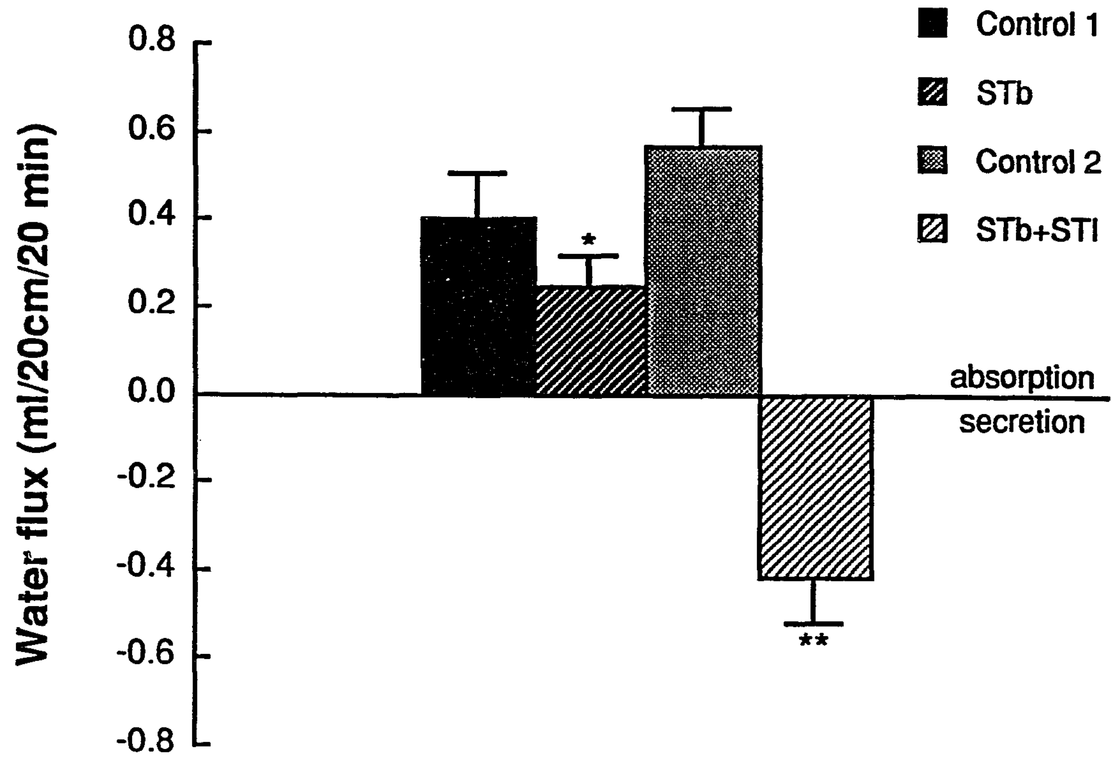
Net water fluxes shown in Figure 28. In this set of experiments the net absorptive flux of water (3/4) for the control (0.303 ml/20 cm/20 min.) was significantly different ($P < 0.05$) from the secretory response (4/4) to the toxin (-0.272 ml/20 cm/20 min.). Pirenzepine when added to perfusion solutions had no significant effect on the water

Figure 27. Effect of broth containing STb (n = 7) or STb + soybean trypsin inhibitor (n = 14) vs paired controls in perfused jejunal loops of rats

Concentration of soybean trypsin inhibitor in the perfusate was 1mg/ml. Each bar represents the mean (± S.E.M.) of water flux in ml / 20 cm / 20 min

* P = 0.002 compared with control 1

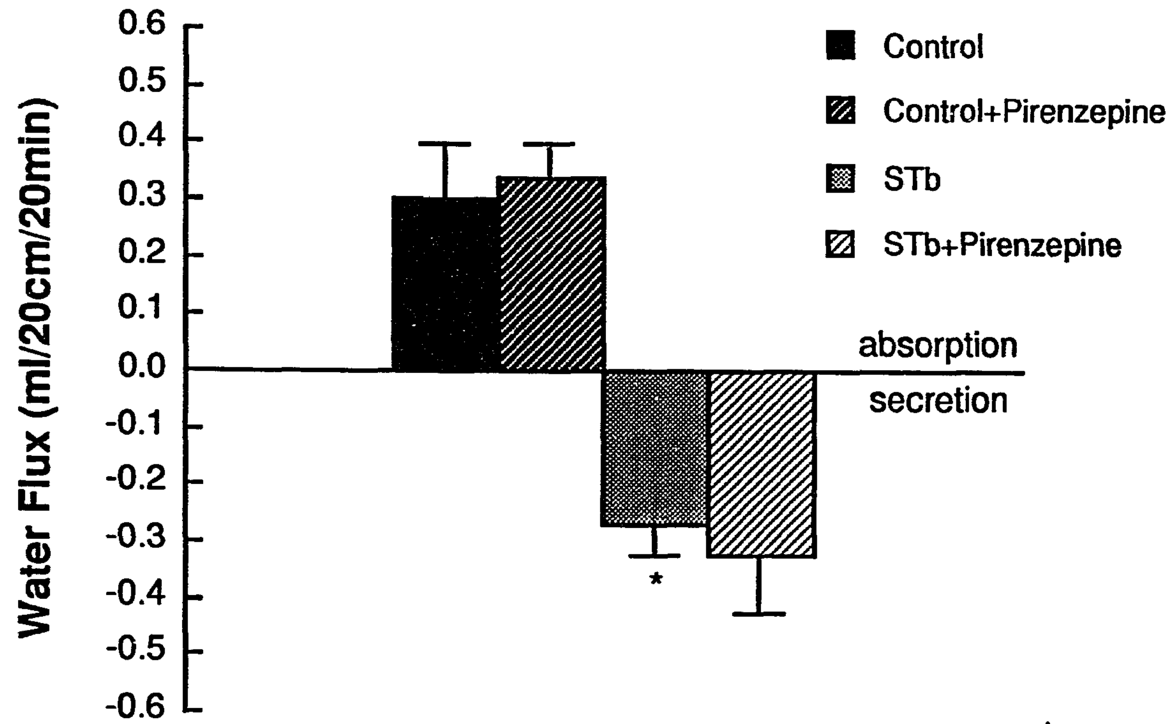
** P = 0.0001 compared with control 2; P = 0.001 compared with STb



Rat Perfusion
Control 1
STb
Control 2
STb+STI
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Water flux (ml/20cm/20 min)
*
**
absorption
secretion

Figure 28. Effect of pirenzepine on the net water flux in paired control and STb exposed perfused jejunal loops of rats

Concentration of pirenzepine in the perfusate was 1×10^{-6}. Each bar represents the mean (± S.E.M.) of four rats
* $P < 0.05$ compared with control


Rat Perfusion Pirenzepine
Water Flux (ml/20cm/20min)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
Control
Control+Pirenzepine
STb
STb+Pirenzepine
absorption
secretion
*

flux.

Effect of 4-DAMP on intestinal water flux

Net water fluxes shown in Figure 29. This group of experiments contained rats that exhibited a net secretory flux of -0.054 ml/20 cm/20 min. for the controls (5/10) and -0.897 ml/20 cm/20 min. for the toxin (9/10). These net flux rates were significantly different ($P < 0.05$). Addition of 4-DAMP to the control perfusion solution significantly reversed net water secretion to net water absorption (6/10) (0.253 ml/20 cm/20 min.). Addition of 4-DAMP to the STb containing solution significantly decreased ($P < 0.05$) the net secretory response (6/10) (-0.535 ml/20 cm/20 min.).

Rat Ligated Loops

In the series of ligated intestinal loop experiments conducted in rats, STb reversed water movement from net absorption to net secretion (42/42 loops) ($P < 0.05$). All loops contained soybean trypsin inhibitor.

Effect of clonidine and yohimbine on water transport in control and STb-exposed jejunal loops

Net water fluxes shown in Figure 30. Clonidine tended to reduce net absorption of water in control loops (5/8) and increase net secretion in toxin containing loops (5/8), but the difference between the means was not significant. Yohimbine reduced net absorption in control loops (7/8) ($P < 0.05$), but induced a nonsignificant increase in net secretion in toxin loops (6/8). When a combination clonidine and

Figure 29. Effect of 4-DAMP on the net water flux in paired control and STb exposed perfused jejunal loops of rats

Concentration of 4-DAMP in the perfusate was 1×10^{-5}. Each bar represents the mean ($\pm$ S.E.M.) of ten rats
* $P < 0.05$ compared with control
** $P < 0.05$ compared with STb

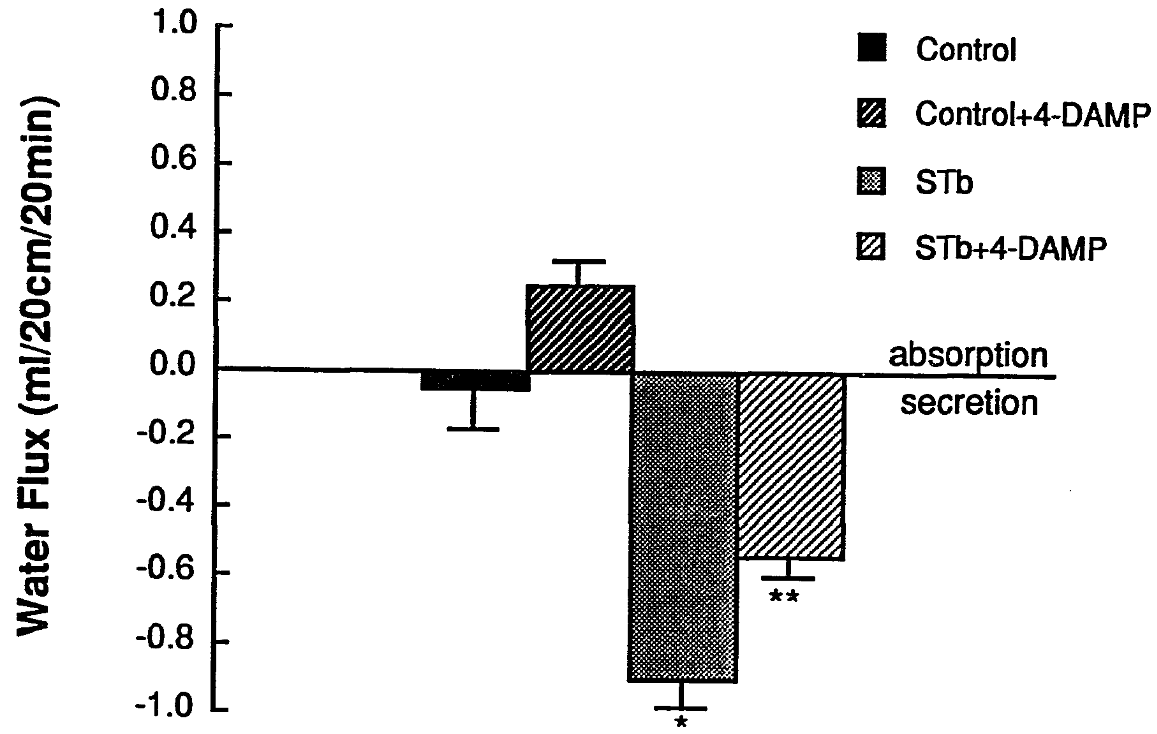
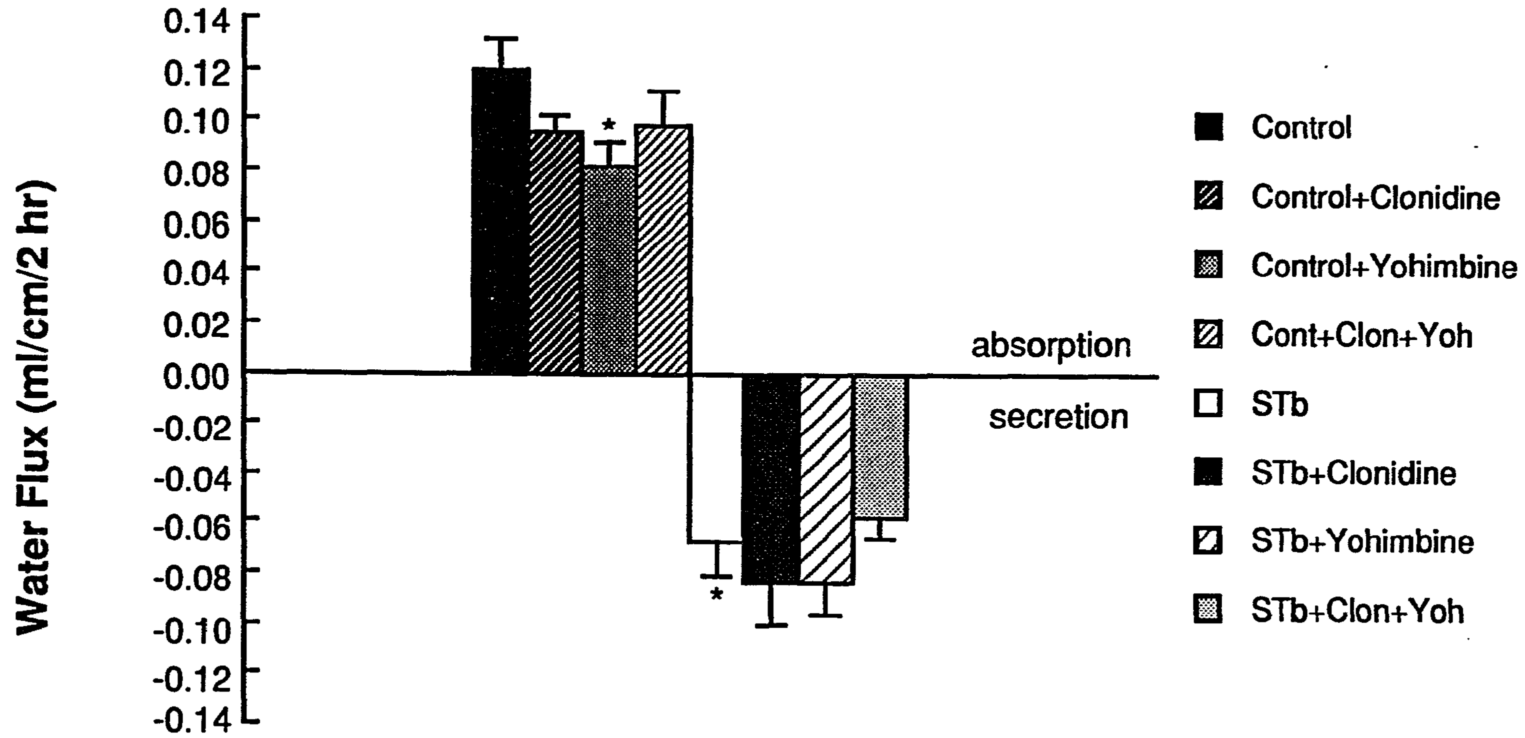

Rat Perfusion 4-DAMP
Water Flux (ml/20cm/20min)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Control
Control+4-DAMP
STb
STb+4-DAMP
absorption
secretion
*
**

Figure 30. Effect of clonidine (3.8×10^{-4} M) and yohimbine (2.6×10^{-5} M) on the net flux of water in control and STb exposed ligated jejunal loops of rats

Each bar represents the mean ± S.E.M. of eight rats
* $P < 0.05$ compared with control



Rat Loops Clonidine & Yohimbine
Water Flux (ml/cm/2 hr)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
*
*
absorption
secretion
Control
Control+Clonidine
Control+Yohimbine
Cont+Clon+Yoh
STb
STb+Clonidine
STb+Yohimbine
STb+Clon+Yoh

yohimbine was included in loop contents, the tendency for reduced water absorption was noted in control loops (6/8) while decreased secretion was observed in toxin loops (5/8). No significant difference between the means was observed.

Effect of morphine and naloxone on water transport in control and STb-exposed jejunal loops

Net water fluxes shown in Figure 31. Morphine and naloxone alone or in combination did not significantly alter water transport in control or toxin loops.

Effect of leucine enkephalin and methionine enkephalin on water transport in control and STb-exposed jejunal loops

Net water fluxes shown in Figures 32 and 33. No significant alterations in water fluxes were observed when leucine and methionine enkephalin were present in toxin and control loops.

Effect of loperamide on water transport in control and STb-exposed jejunal loops

Net water fluxes shown in Figure 34. Loperamide did not significantly alter the absorptive response of control loops nor the secretory response observed in toxin loops.

Isolated Enterocyte Studies

Microscopic examination

Examination of sections of intestine obtained after mechanical vibration revealed most areas of villi that were devoid of epithelial cells. Most of the crypts were lined by cells. The results indicated

Figure 31. Effect of morphine (1.5×10^{-5} M) and naloxone (1.5×10^{-5} M) on the net flux of water in control and STb exposed ligated jejunal loops of rats

Each bar represents the mean ± S.E.M. of eight rats
* $P < 0.05$ compared with control

Rat Loops Morphine & Naloxone

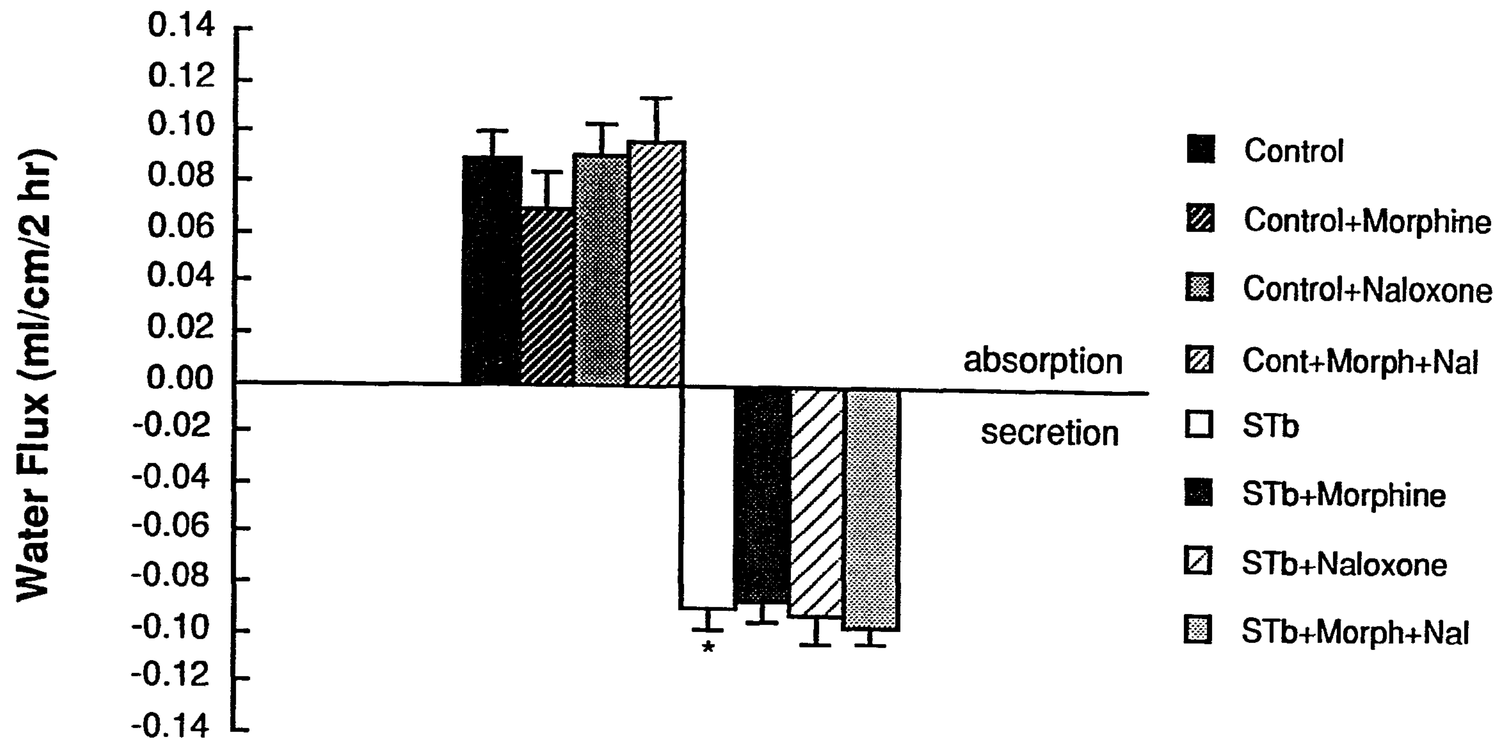
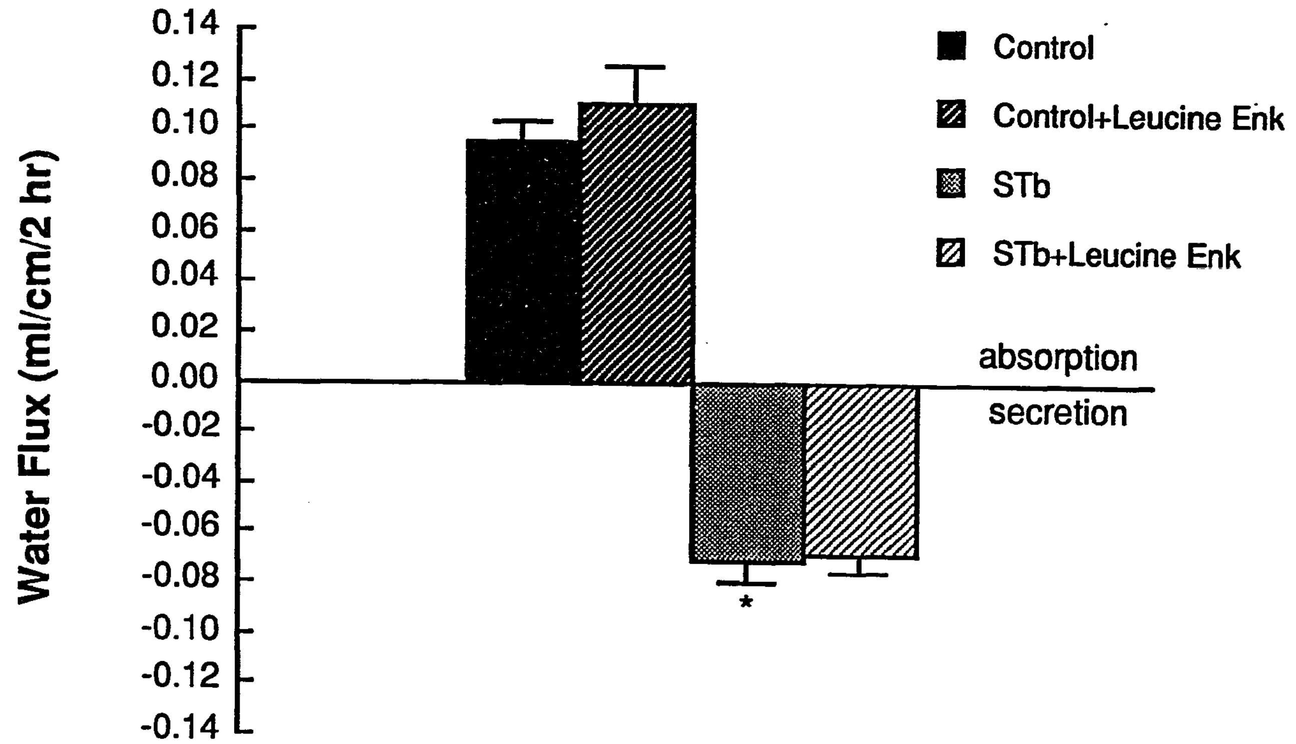


Figure 32. Effect of leucine enkephalin (1×10^{-4} M) on the net flux of water in control and STb exposed ligated jejunal loops of rats

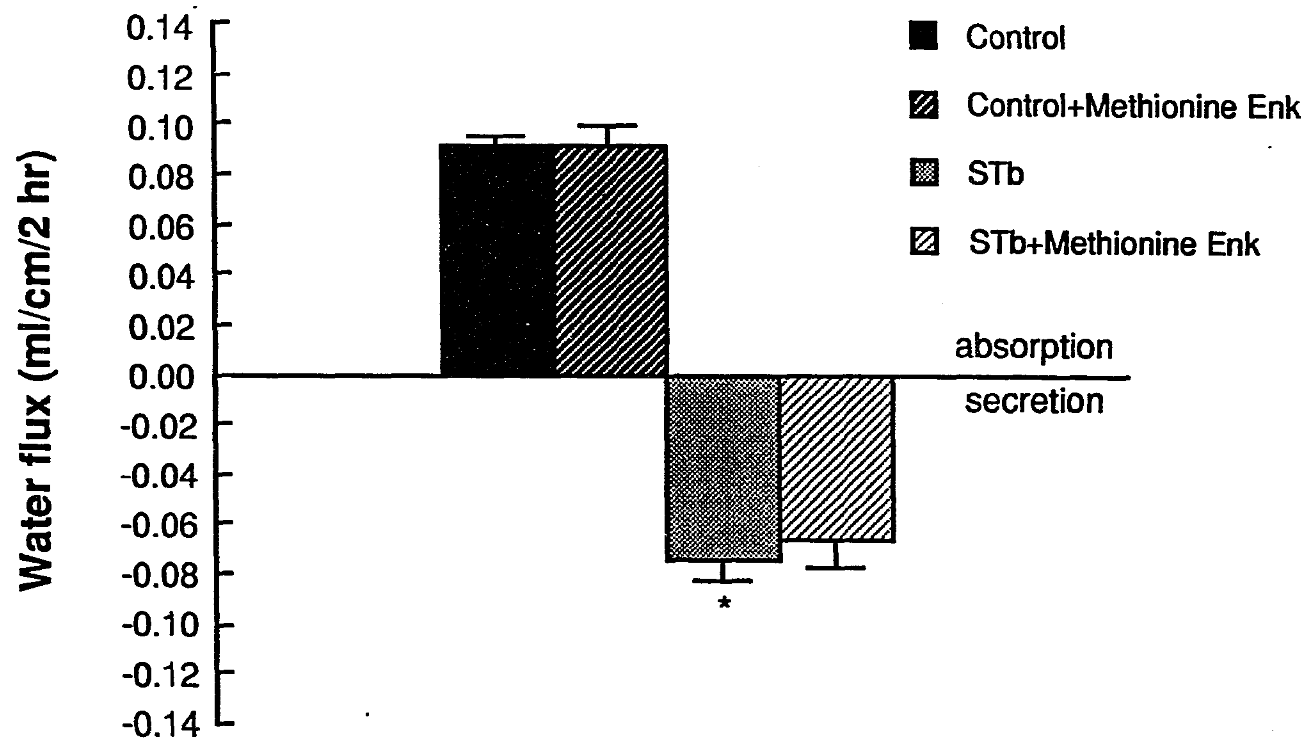
Each bar represents the mean ± S.E.M. of four rats
* $P < 0.05$ compared with control



Rat Loops Leucine Enkephalin
Water Flux (ml/cm/2 hr)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
Control
Control+Leucine Enk
STb
STb+Leucine Enk
absorption
secretion
*

Figure 33. Effect of methionine enkephalin (1×10^{-4} M) on the net flux of water in control and STb exposed ligated jejunal loops of rats

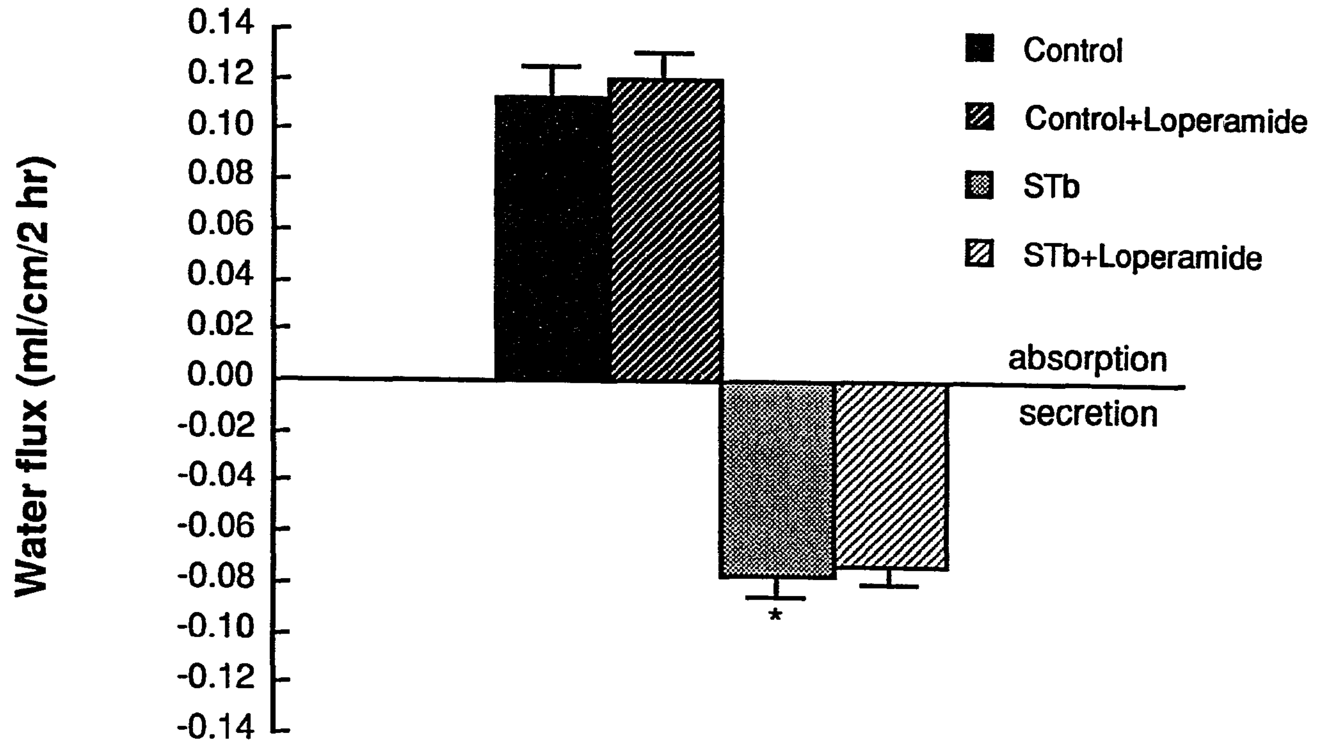
Each bar represents the mean ± S.E.M. of four rats
* $P < 0.05$ compared with control



Rat Loops Methionine Enkephalin
Water flux (ml/cm/2 hr)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
Control
Control+Methionine Enk
STb
STb+Methionine Enk
absorption
secretion
*

Figure 34. Effect of loperamide (1×10^{-5} M) on the net flux of water in control and STb exposed ligated jejunal loops of rats

Each bar represents the mean ± S.E.M. of five rats
* $P < 0.05$ compared with control



Rat Loops Loperamide
Water flux (ml/cm/2 hr)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
Control
Control+Loperamide
STb
STb+Loperamide
absorption
secretion
*

that mechanical vibration effectively removed mature, but not immature cells from the intestinal mucosa.

Trypan Blue exclusion test

At the termination of each experiment the cell suspension was mixed with Trypan Blue. More than 73 % of the isolated cells excluded Trypan Blue, suggesting a majority of cells remained viable during the duration of the experiment.

Lactate Dehydrogenase assay

Lactate dehydrogenase activity was measured at intervals in supernatants from enterocyte cell suspensions. The mean activities ranged from 23.08 % at 0.5 sec. to 28.20 % at 10 sec. The results suggest that leakage of enzyme from cells was minimal and that the majority were viable.

Efflux of ^{36}Cl

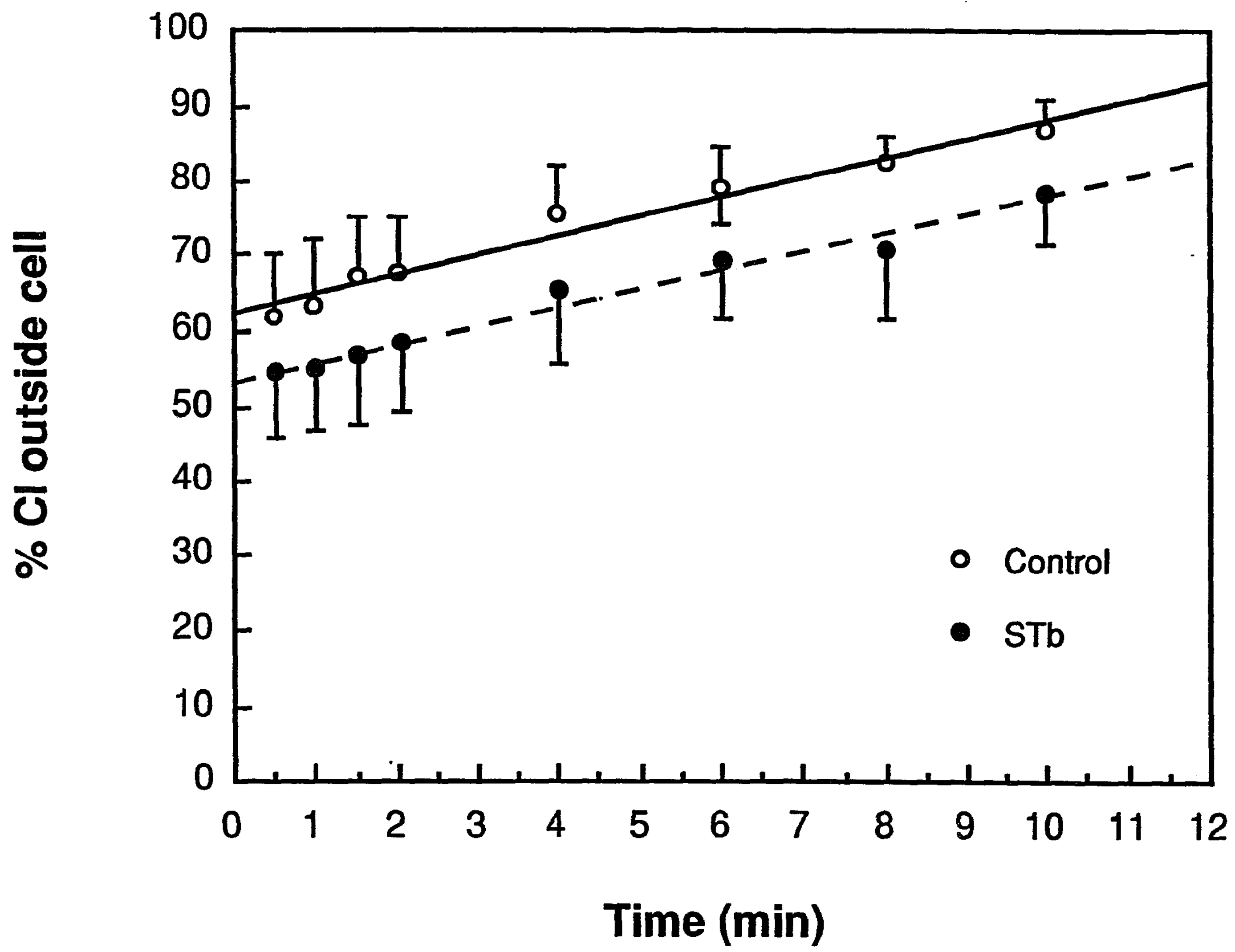
The rate of chloride efflux for control and toxin treated enterocytes are shown in Figure 35. The curve was plotted as the percent of ^{36}Cl entering the extracellular fluid versus time. The best fitting line through the points was determined and the slope of the line was calculated. The efflux rate of ^{36}Cl from isolated enterocytes under the influence of control and toxin broth was +0.043 and +0.042 for the percent of ^{36}Cl entering the extracellular fluid. No significant difference was determined.

Influx of ^{36}Cl

The influx of ^{36}Cl was determined as the amount of ^{36}Cl activity taken up per mg cell protein per unit time. No significant difference was noted between the influx slopes of control (+2.3633) and toxin

Figure 35. Comparison of chloride efflux rate constants in control and STb-exposed isolated enterocytes

The curve was plotted as the percent of ^{36}Cl entering the extracellular fluid versus time (n = 4). Slope (mean ± SEM): Control +0.043 ± 0.013; STb +0.042 ± 0.002


% Cl outside cell
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (min)
Control
STb

(+2.2157) exposed enterocytes (Figure 36).

Influx of ^{45}Ca

^{45}Ca influx was determined as the amount of ^{45}Ca activity per mg cell protein per unit time. Comparison of the slopes of control (+11.4796) and toxin (+11.6079) demonstrated no significant difference (Figure 37).

Figure 36. Comparison of chloride influx rate constants in control and STb-exposed isolated enterocytes

The curve was plotted as the number of cpm/mg cell protein of ^{36}Cl entering the cells versus time (n = 7). Slope (mean ± SEM): Control +2.363 ± 0.377; STb +2.216 ± 0.608

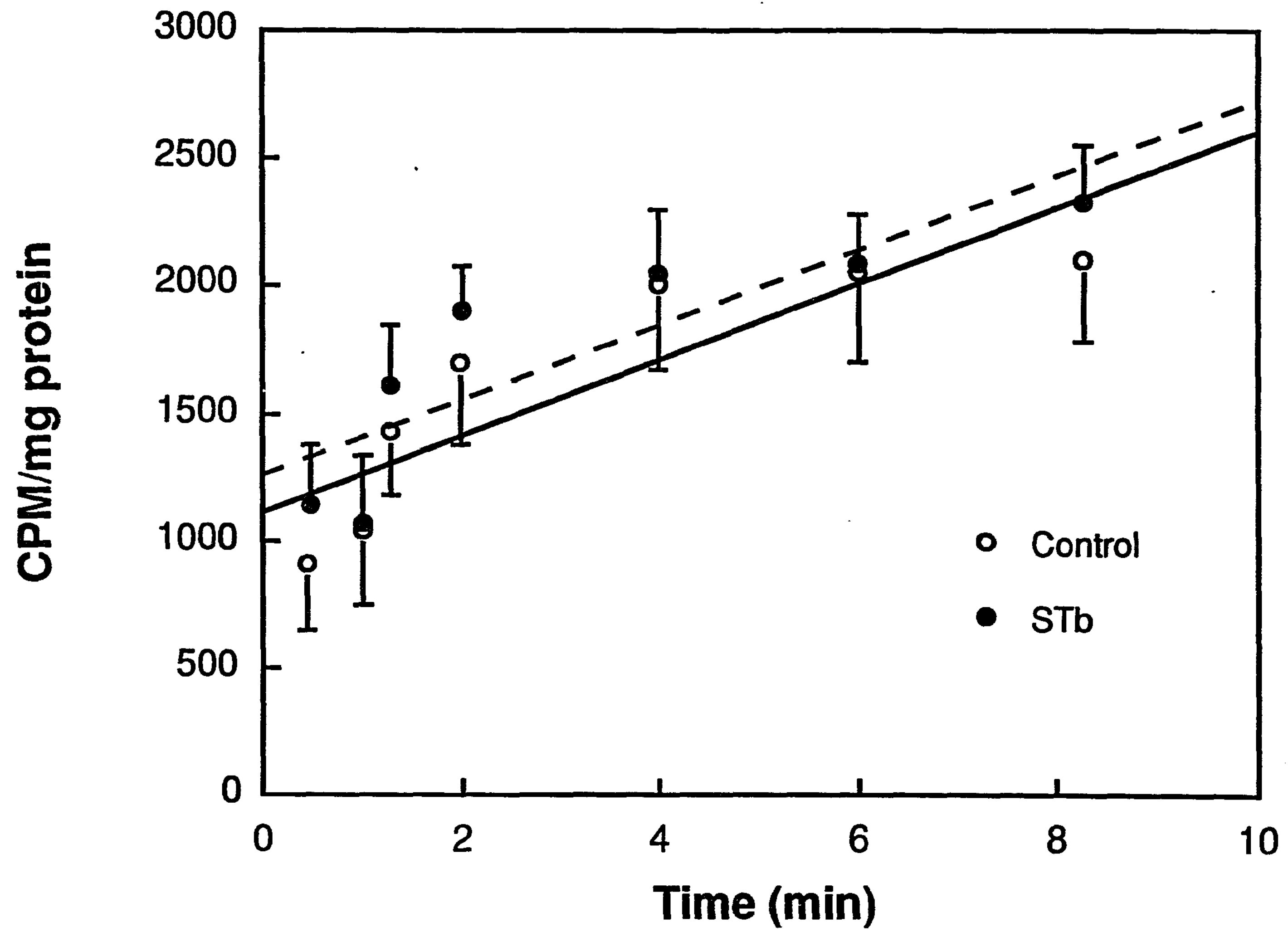
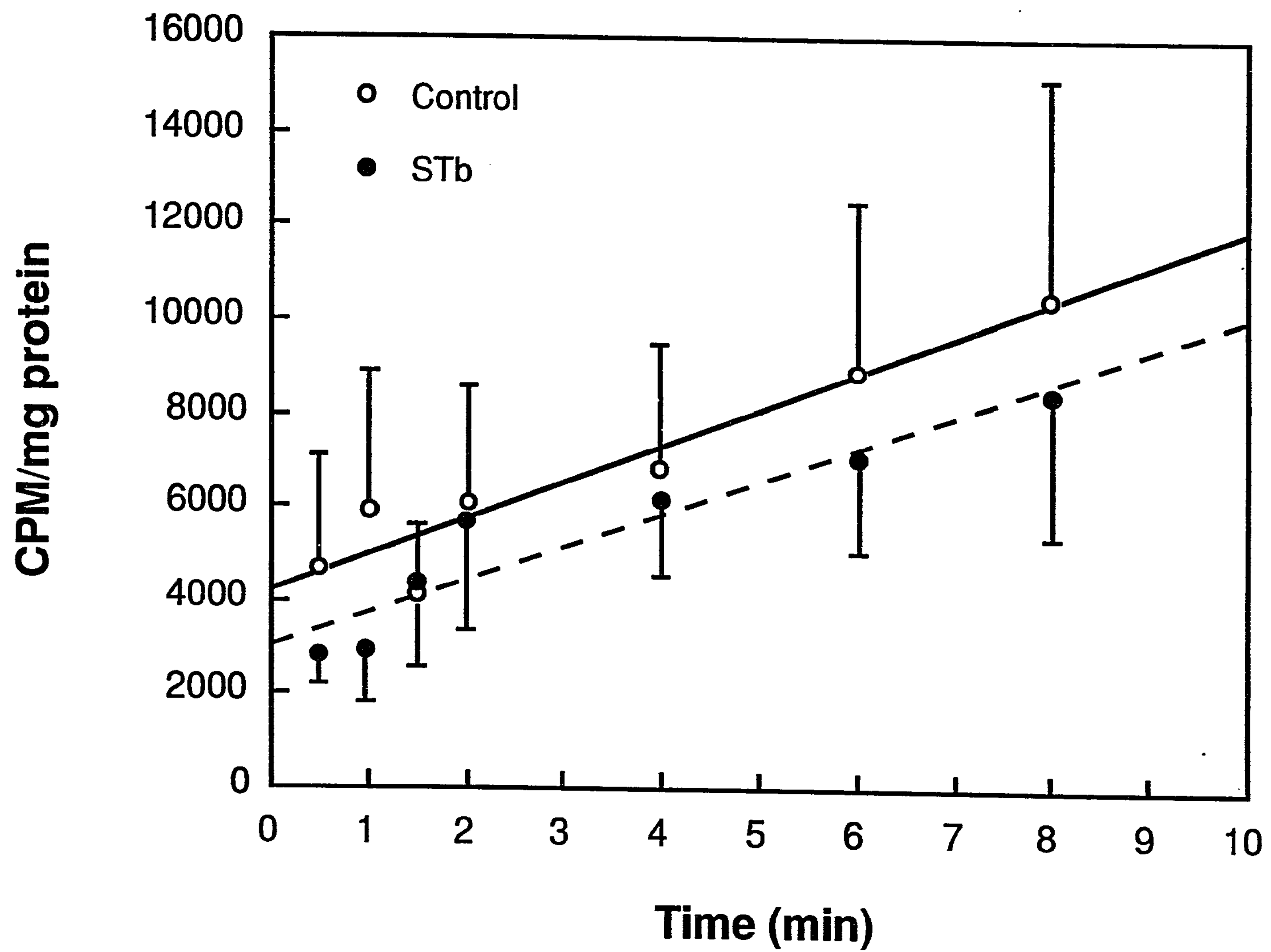

3000
2500
2000
1500
1000
500
0
CPM/mg protein
0
2
4
6
8
10
Time (min)
Control
STb

Figure 37. Comparison of calcium influx rate constants in control and STb-exposed isolated enterocytes

The curve was plotted as the number of cpm/mg cell protein of ^{45}Ca entering the cells versus time (n = 4). Slope (mean ± SEM): Control +11.480 ± 4.495; STb +11.608 ± 4.546



Control
STb
CPM/mg protein
Time (min)
16000
14000
12000
10000
8000
6000
4000
2000
0
0
1
2
3
4
5
6
7
8
9
10

DISCUSSION

Escherichia coli heat-stable enterotoxin, STb, in perfused jejunal segments of three week old unweaned pigs, produced a secretory response in 5/13 segments and decreased absorption in 6/13. In porcine ligated intestinal loops, STb exposure induced secretion in 22/30 loops. The secretory response to STb was variable as noted by others (Whipp et al., 1987; Rose et al., 1987).

Earlier perfusion studies conducted in the rat had shown that a large secretory response (8/9) was produced by the sterile filtered broth of E. coli strain 1261 (STa and STb) ($P < 0.0001$), while 1790 (STb only) caused reduced absorption (5/6) ($P < 0.002$). The difference between the response of the small intestine to the two culture filtrates was not statistically significant. The variable response observed with STb in part may be due to the smaller magnitude of the response and/or the presence of endogenous proteases..

Jejunal perfusion studies in the rat where soybean trypsin inhibitor was added to the control and toxin perfusion solutions were conducted. The secretory response to STb with soybean trypsin inhibitor was significantly different from the control and from the reduced absorptive response induced by STb without soybean trypsin inhibitor. In experiments where soybean trypsin inhibitor was included in the culture broths, 25/28 segments of rat intestine perfused with STb broth produced a secretory response and in rat ligated intestinal loop studies, 42/42 loops exposed to STB responded with a secretory response. This absorvation comfirms the observations of Whipp (1987) that

endogenous protease activity interferes with intestinal responses to STB. In addition the strong, consistent secretory response of the rat small intestine to STb in the presence of soybean trypsin inhibitor allows the rat to serve as an experimental model of STb-induced diarrhea. The inclusion of soybean trypsin inhibitor in the porcine experiments would have reduced the variable response to STb as demonstrated by Whipp, 1987.

Experiments were conducted to evaluate the effects of clonidine, an α_2 agonist, and yohimbine, an α_2 antagonist, on the secretory response to the E. coli heat-stable enterotoxin STb. In pig jejunal segments perfused with control solution clonidine significantly reduced potassium secretion and tended to increase the absorption of water, sodium and chloride. The addition of clonidine to the STb solution resulted in a significant increase in water absorption and decrease in potassium secretion. Sodium and chloride absorption also tended to increase. A similar trend was observed in porcine ligated intestinal loops exposed to clonidine, but no significant changes were observed. In contrast to the porcine studies, the addition of clonidine to solutions injected in the ligated intestinal loops of rats resulted in the tendency to reduce net absorption of water in control loops and increase net secretion in toxin loops.

Yohimbine did not have a significant effect in the perfused loops or ligated loops of pigs. However, in ligated loops of rats, yohimbine caused a significant decrease in the absorptive response in the control loops and tended to increase the secretory response in the toxin loops. The response in the toxin loops was not significant.

Perfusion studies, similar to ours, were conducted by Ahrens and Zhu (1982a) in three week old pigs using control broth and broth containing STa and STb. Clonidine, when added to the control broth, significantly increased the absorption of water and sodium. Potassium and chloride absorption was increased, but not significantly. The addition of clonidine to the toxin containing solutions caused a significant reduction of water, sodium, and chloride secretion. Clonidine induced similar effects in porcine ligated loops exposed to broth containing STa and STb (Zhu and Ahrens, 1983).

The sympathetic nervous system has been shown to be involved in intestinal transport (Wright et al., 1940; Field and McColl, 1973; Brunsson et al., 1979). Epinephrine and norepinephrine were shown by Field and McCall (1973) to cause a decrease in potential difference and short circuit current in rabbit ileum mounted in Ussing chambers. Enhanced absorption of sodium and chloride was also noted. Isoproterenol and propranolol had no effect on the short circuit current suggesting the above effects were due to α-adrenergic receptors.

Clonidine added to the mucosal or serosal solution bathing rabbit ileum in Ussing chambers caused a reduction in short circuit current and potential difference and an increase in Na and Cl absorption. The action of clonidine on the short circuit current was similar to that of epinephrine. Yohimbine, an α_2-antagonist, blocked the action of both of these adrenergics (Durbin et al., 1982).

The mechanism by which clonidine influences ion transport is unknown. Comparative displacement studies of [^{3}H-yohimbine] have demonstrated the presence of α_2-receptors on rabbit ileal enterocytes

(Chang et al., 1983). $Alpha_2$ agonists have been shown to decrease ^{45}Ca entry across the basolateral membrane of rabbit ileum. Adrenergic nerve endings have been described in close proximity to crypt and villus cells (Thomas and Templeton, 1981).

The antisecretory action in the pig of the α_2-agonist, clonidine, on STb-induced secretion and the accentuation of STb-induced secretion by the α_2-antagonist, yohimbine, suggests that the action of α_2-agonists on their receptors may reduce the secretory fluxes induced by STb. The lack of effect of clonidine and yohimbine in the rat may be due to lack of specific receptors due to species differences or effective drug concentration.

In experiments conducted to study the effect of morphine in ligated jejunal loops of pigs, morphine did not alter the control fluxes, but in the STb containing loops morphine significantly changed the net secretion of sodium and chloride to net absorption ($P < 0.05$) and reduced potassium secretion ($P < 0.05$). Net water secretion also had a tendency to be reduced, but not significantly. In ligated jejunal loops of rats morphine did not exhibit a significant effect on water transport in control or toxin containing loops.

Our results in the pig isolated loop studies were consistent with experiments conducted by Zhu and Ahrens (1983) using ligated jejunal loops in two to three week old pigs. Morphine significantly reduced the secretion of water, sodium, and chloride stimulated by a cell free broth containing STa and STb. In perfused jejunal segments (Ahrens and Zhu, 1982a), morphine increased net water, sodium and chloride absorption in control segments and reduced secretion of these same constituents in

toxin perfused loops. Net potassium fluxes were unchanged in the presence of morphine.

In contrast to our ligated loop studies in the rat, Beubler and Lembeck (1979) measured the small intestinal fluid volume in rats and reported that morphine reduced the basal intestinal fluid volume and the increase in intestinal fluid volume stimulated by PGE_1 and VIP. Coupar (1983) measured net water transport in the jejunum of rats by a recirculation technique and noted that morphine reversed the secretory phase of a VIP stimulated response, while the δ-agonist [D Ala^2 D Leu] enkephalin had negligible antisecretory activity. Thus they proposed that μ-opioid receptor stimulation mediated inhibition of VIP stimulated secretion in the rat jejunum.

In studies with rabbit ileal mucosa in Ussing chambers, morphine caused a fall in the potential difference and short circuit current (McKay et al., 1984). This was primarily due to an increase in net chloride absorption resulting from a decrease in the serosal to mucosal flux. No change in sodium transport was noted. The residual flux represented by bicarbonate secretion or hydrogen ion absorption was enhanced. In addition the electrical response induced by morphine was altered by changes in calcium concentration in the bathing medium. Lowering or removal of calcium from the serosal solution resulted in further lowering of the short circuit current by morphine. Thus, the mechanism involved in the regulation of ion transport by morphine may involve the regulation of intracellular calcium concentrations.

In other studies in rabbit ileum by McKay et al. (1982), morphine had no effect on the electrical response stimulated by PGE_2 or

acetylcholine, but did inhibit net secretion of chloride that was induced by both agents and prevented the reduction of sodium absorption induced by PGE_2. Also, morphine reduced the electrical response and the net chloride secretion induced by cholera toxin.

Kachur et al. (1980, 1982) found that morphine, primarily a μ-agonist was almost completely ineffective in reducing the transepithelial potential difference and short circuit current across stripped guinea pig ileum in Ussing chambers. Opioid peptides such as [D Ala^2 D Leu^5] enkephalin and [D Ala^2 D Met^5] enkephalin, which are primarily δ-opiate agonists were effective. They propose the antisecretory effects of opioids are mediated by δ-opiate receptors and the effects on smooth muscle are primarily mediated by a μ-opiate receptor. The difference in the antisecretory response or lack of response by the different opioids in different species may be due to interspecies differences.

Supporting this concept are autoradiographic studies of the distribution of δ- and μ-type opioid binding sites in the gastrointestinal tract of the rat and guinea pig. Species differences in the distribution of opioid receptors were noted. A dense distribution of μ- and δ-type binding sites were noted in the mucosa throughout the villi and in the submucosal plexus of the rat duodenum and ileum. No binding sites were apparent in the muscle layers, myenteric plexus or Brunner's glands. In the guinea pig ileum an absence of detectable δ-type binding was observed. The mucosa was devoid of μ-type binding, but the submucosal plexus and muscle layers displayed dense μ-ligand binding (Nishimura et al., 1986). This study,

although supporting the concept of interspecies differences, contradicts the previously mentioned findings of Kachur et al. (1980, 1982) and Coupar (1983) that suggest δ-agonists are effective in the guinea pig and μ-agonists are effective in the rat.

The reduction of STb induced water and electrolyte secretion by morphine in the pig suggests that stimulation of opiate receptors may reduce the STb secretory response. The lack of response in the rat may be due to interspecies differences involving differences in opiate receptor populations

In pig ligated jejunal loops, naloxone when present in the control loops produced no significant changes. In the toxin loops naloxone reversed sodium and chloride secretion to absorption ($P < 0.05$). When present in the control or toxin loops of rats, naloxone did not significantly affect the transport of water.

Naloxone has been shown to act as a competitive antagonist of morphine. In studies conducted with rat small intestine using a recirculation technique, naloxone caused a parallel displacement of the antisecretory dose-response lines to morphine during VIP stimulation (Coupar, 1983). Naloxone had no demonstrable effect on basal intestinal fluid volume as determined by measurement of the intestinal fluid volume of the entire small intestine, but did enhance the secretory effect of PGE_1 and VIP in the rat small intestine (Beubler and Lembeck, 1979). In contrast, intraluminal perfusion of naloxone (10^{-4} M) in rat ileum has been shown to decrease basal water, sodium, and chloride absorption. Net jejunal water transport was decreased by naloxone (10^{-4} and 10^{-5} M). Increased water absorption due to morphine was prevented by naloxone

(Fogel and Kaplan, 1984).

It has been noted that naloxone occasionally causes a increase in short circuit current across rabbit ileum mounted in Ussing chambers. This may suggest naloxone blocks the effect of endogenous opiates (Dobbins et al., 1980). It also blocked the significant fall in potential difference and short circuit current and the induced significant increase in chloride absorption attributed to morphine. The dose of naloxone was 20 times less than that of morphine (McKay et al., 1984).

In our studies the reduction of secretion in the presence of naloxone in the ligated loops of pigs containing toxin may be due to its ability to act as a partial agonist (Jaffe and Martin, 1985). Naloxone has been found to have characteristics of an antagonist-agonist. At low concentrations (3×10^{-9} to 1.5×10^{-6} M) the drug has antagonistic effects, but at higher concentrations (10^{-4} M) it displays agonistic effects (Frank, 1985 review).

The effect of muscarinic receptor antagonists on absorption and STb stimulated secretion was investigated in perfusion studies conducted in rat jejunum. Pirenzepine did not significantly alter the absorption or secretory response of the respective control and STb perfusion solutions. The muscarinic antagonist, 4-diphenylacetoxy-N-methyl piperidine (4-DAMP), however, did significantly reverse the response to STb from secretion to absorption. The control response was not significantly altered.

Carey et al. (1987) conducted in vitro receptor studies with muscle-stripped flat sheets of guinea pig ileum mounted in Ussing

chambers. They demonstrated that electrical field stimulation of the submucosal neurons caused a biphasic increase in short-circuit current. The cholinergic phase of this response was inhibited by atropine and 4-DAMP (10^{-7}) in an equipotent manner. Similarly atropine and 4-DAMP inhibited the secretory response of the muscarinic agonists carbachol and bethanechol. Pirenzepine was much less potent in its inhibitory action.

Analogous studies in guinea pig colon conducted by Kuwahara et al. (1987) demonstrated the same rank order of potency for the three muscarinic antagonists in the reduction of the short circuit current induced by carbachol bethanechol and neural stimulation.

Binding assays conducted with mucosal scrapings from guinea pig ileum (Carey et al., 1987) and colon (Kuwahara et al., 1987) showed that [^{3}H] quinuclidinyl benzilate was displaced from the mucosal scrapings by the muscarinic antagonists. The compound 4-DAMP was more potent than pirenzepine.

Subtypes of muscarinic receptors were first suggested by Birdsall et al. (1978) in studies on agonist binding. Pirenzepine, a tricyclic compound (Hammer and Giachetti, 1984), selectively inhibited vagally mediated gastric secretion in the mouse (Schiavone et al., 1983) and stimulated bicarbonate secretion in the rat duodenum (Safsten and Flemstrom, 1986). Subclassification of the muscarinic receptors has been based on the affinity of pirenzepine on the receptor subtypes. Receptors with a high affinity for pirenzepine such as those in neural tissue (autonomic ganglia and discrete areas of the brain) were designated M_1 and those with a low affinity for pirenzepine as found

mainly in peripheral organs such as the heart and ileal smooth muscle were designated M_2 (Hammer and Giachetti, 1982).

4-DAMP has been shown to have a greater affinity for the receptors of the smooth muscle of ileum than receptors of the atrium (Barlow et al., 1976). Other binding studies have demonstrated that 4-DAMP exhibits a high affinity for lacrimal gland and cerebral cortex and low affinity for the myocardium (Berrie et al., 1983).

In vitro work conducted with radioligand binding studies on rat tissues consisting of hippocampus, atria and submandibular glands suggested the existence of at least three different types of muscarinic binding sites. The proposed predominate muscarinic receptors found in these tissues were M_1, M_2 and M_3 respectively (Doods et al., 1987).

Conclusions based on the results of these and other studies are conflicting. An overview of two of these studies shows that in vivo, 4-DAMP has been described as M_2 selective (Gilbert et al., 1984), M_3 selective (Doods et al., 1987) and in binding studies has a high affinity for M_1 and M_3 and low affinity for M_2 muscarinic receptors (Doods et al., 1987). Pirenzepine as been designated in vivo as M_1 selective (Gilbert et al., 1984), a potent antagonist for M_1 and M_3 in vivo (Doods et al., 1987) and having high affinity for M_1 receptors, intermediate affinity for M_3 receptors and low affinity for M_2 receptors in binding studies (Doods et al., 1987).

The discrepancy between these studies may in part be due to the difficulty in making comparisons between binding and functional studies and in vitro and in vivo work. The potency of a drug to bind to receptors does not necessarily correspond to the strength of the

response it elicits at those same receptors. This is evident with McN A343, a muscarinic agonist, which elicits a secretory effect on the ileal mucosa via muscarinic receptors on submucosal neurons. McN A343 also exhibits more potent binding to muscarinic receptors on ileal epithelial cells than carbachol and bethanechol, but does not produce an agonistic effect (Carey et al., 1987).

Comparisons of in vitro and in vivo work are also not easily made. The equilibrium of the free and receptor bound drug cannot be assumed to occur in vivo and the concentration of the agonist and antagonist at the level of the receptors cannot be well controlled. Thus under in vivo conditions the ID_{50} values cannot be considered as a direct measure of the affinity of a drug. Other kinetic factors that affect the drugs concentration at the receptor are the blood flow through the investigated tissue and the tissue:blood partition coefficient of the drug. The ability of the antagonists to differentiate between M_1 and M_2 binding receptors was more pronounced in the radioligand binding experiments than in experiments conducted in vivo (Doods et al., 1987).

The discrepancy between the in vitro and in vivo results might be explained by the fact that the muscarinic receptor subtypes may have non identical binding sites in different tissues, the potency of the antagonist may be influenced by the pharmacokinetic properties of the drug in vivo (Doods et al., 1987) and the possibility these compounds may induce the formation of different affinity states in different tissues (Englen and Whiting, 1985).

The subclassification of muscarinic receptors in M_1 and M_2 receptors is under scrutiny. This classification in the past has rested

on a single compound, pirenzepine (Englen and Whiting, 1985).

In contrast to studies suggesting heterogenous muscarinic receptors in peripheral tissues, some studies suggest a homogenous receptor population. Szelenyi (1982) conducted studies on isolated ileum and atrium of guinea pig and the acid secretion of the isolated stomach of the mouse. Pirenzepine inhibited bethanechol induced changes in all three tissues in a dose dependent manner. The estimated pA_2 values were very similar (pA_2 = -log of concentration of the antagonist that shifts the dose curve of the agonist two fold). This suggests that pirenzepine was unable to discriminate any subtypes of muscarinic receptors and points to a homogenous receptor population.

Clague et al. (1985) used the antagonist pirenzepine in affinity studies on the receptors mediating ileal contraction and atrial chronotropic and inotropic effects. The pirenzepine data suggested that all three receptors were of the M_2 type. However, 4-DAMP displayed a significantly greater affinity for the ileal muscarinic receptors when compared with the atrial muscarinic receptors. This suggests that the subclass of muscarinic receptors, M_2, may in turn be composed of two subtypes.

Binding studies conducted by Tien et al. (1985) in the rat showed that intestinal epithelial cells had a higher affinity for pirenzepine than intestinal smooth muscle cells. Similar studies by Wahawisan et al. (1985) noted that muscarinic receptors were present on the basolateral membrane of both rat ileal villus and crypt epithelial cells. From these studies and the generally accepted muscarinic classification system it was suggested that the receptor subtype in both

types of epithelial cells was M_1 (Tien et al., 1985; Wahawisan et al., 1986). Carey et al. (1987), however, has demonstrated that the epithelial muscarinic receptor has a greater affinity for 4-DAMP than pirenzepine.

Because of conflicting studies, the classification of muscarinic receptors based on the binding and functional activity of pirenzepine does not appear to be adequate. With the discovery of new selective agonists and antagonists perhaps more definitive conclusions can be made as to what receptors are present in certain tissues and with what drugs there is preferential binding.

Studies by Carey et al. (1987) showed that 4-DAMP, when compared to pirenzepine had greater affinity and was more effective in inhibiting neurally induced secretion in guinea pig ileum. The latter comparison is supported by our findings in the STb exposed rat jejunum. These findings may be due to the fact the two muscarinic antagonists act on different epithelial cell muscarinic receptors that are not equal in functional capabilities, or that they may act on the same receptor, but are not equal in binding affinity or efficacy.

The significant reduction of the STb-induced secretory response by 4-DAMP possibly indicates that the drug and toxin are acting via the same receptors, the drug influences the mechanism of STb-induced secretion or 4-DAMP may be stimulating an absorptive response that antagonizes the STb-induced secretory response. The lack of response by pirenzepine emphasizes the difference in binding and potency of action between muscarinic receptors and the antagonists. Further investigation of the antimuscarinic drugs may lead to the development of drugs that

can specifically and efficiently alter secretion in the small intestine and/or may aid in the understanding of the secretory mechanism stimulated by STb.

Leucine and methionine enkephalin did not induce any significant changes in the absorptive and secretory response in the respective control and toxin loops of the rat jejunum.

The identification of endogenous opiates, the enkephalins, in the brain (Hughes et al., 1975) was followed by the discovery of their presence in the rabbit and guinea pig ileum (Smith et al., 1976). Enkephalins act preferentially at δ-receptors (Miller et al., 1985).

Dobbins et al. (1980) employed rabbit ileal mucosa in Ussing chambers to demonstrate that leucine enkephalin, methionine enkephalin and D Ala^2 Meth enkephalin amide caused a decrease in short circuit current and potential difference. The effect of D Ala^2 Meth enkephalin was more pronounced and prolonged. Possibly the natural analogs, leucine and methionine enkephalin, had greater susceptibility to tissue proteases or the synthetic analog had a greater affinity for the opiate receptor. D Ala^2 Met enkephalin induced an increase in sodium and chloride absorption and increased tissue conductance by 37%. A strong linear correlation between the decrease in short circuit current and an increase in net chloride absorption suggested that the chloride absorption partly accounts for the decrease in short circuit current. The synthetic analog had no effect on basal or increased cyclic AMP levels stimulated by vasoactive intestinal peptide. Naloxone blocked the effect of D Ala^2 Met enkephalin on short circuit current thus suggesting the involvement of opiate receptors. These workers also

noted that tetrodotoxin blocked the decrease in short circuit current induced by D Ala^2 Met enkephalin, but not by epinephrine. This suggests the effect of enkephalin involves neural pathways and may act as a preganglionic neurotransmitter.

Other work conducted in the rabbit ileum by McKay et al. (1984) also noted that synthetic enkephalin analogs enhanced chloride absorption due to a reduction in the serosal to mucosal chloride flux. No alterations in the sodium flux was observed. In these experiments the analog proved to be more potent than morphine.

Studies with guinea pig ileum also showed similar results with D Ala^2 D Leu enkephalin and D Ala^2 D Meth enkephalin. Morphine was noted to be ineffective and the natural enkephalins, Leu^5 and Met^5 enkephalin, exhibited transient effects (Cummings et al., 1973, Kachur and Miller, 1982).

In the rat small intestine D Ala^2 D Leu^5 enkephalin has been shown to have negligible antisecretory activity against vasoactive intestinal peptide stimulated secretion (Coupar, 1983).

The lack of antisecretory activity in the rat jejunum noted in experiments conducted with methionine and leucine enkephalin may be due to the reported rapid metabolism of these compounds by tissue proteases. The lack of significant numbers of δ-opiate receptors in the rat jejunum may be another factor in the lack of response.

In the control and toxin ligated loops of rats loperamide had no significant effect on the respective absorptive or secretory response.

Studies conducted by Karim and Adaikan (1977) demonstrated the absence of loose stools or watery diarrhea in 4 out of 5 human subjects

given PGE_2 thirty minutes after orally administered loperamide. In rats the authors reported that pretreatment with loperamide, reduced the fluid accumulation induced by PGE_2 in the small intestine by 50% to 80%. Perfusion studies conducted in rat jejunum showed that loperamide stimulated the absorption of fluid, electrolytes and glucose. In addition it reversed PGE_2 and cholera toxin induced secretion to absorption (Sandhu et al., 1981). The antisecretory activity of loperamide was not mediated by an effect on adenylate cyclase activity nor on the tissue cyclic AMP concentration. The antisecretory effect of loperamide was blocked by naloxone (Sandhu et al, 1974; 1981).

Watt et al. (1982) demonstrated that loperamide was effective in reducing the secretory response in infant mice both prior to and after exposure of the mucosa to ST. This contradicts other findings (Knoop and Abbey, 1981) that demonstrated that loperamide had no effect on the ST secretory response in infant mice.

In in vitro studies using rabbit ileum mounted in Ussing chambers, there was a dose related fall in the transepithelial potential difference and short circuit current upon the addition of loperamide. This observation was due to a reduction of the serosa to mucosa flux of chloride. In addition loperamide inhibited the secretory response stimulated by the heat-stable and heat-labile enterotoxins of E. coli. The electrical events induced by loperamide were inhibited by naloxone (Hughes et al., 1982).

Intragastric infusion of balanced electrolyte solution in humans showed that loperamide had no effect on the flow rate of rectal effluent. Loperamide also failed to reverse the decreased absorption

rate produced by intravenously administered vasoactive intestinal peptide. These studies suggest the antidiarrheal effect of loperamide is due to an alteration in gastrointestinal motor function resulting in an increase in the capacity of the gut and a delay in the passage of fluid through the intestine (Schiller et al., 1984).

Loperamide did not affect the basal short circuit current of chinchilla ileum mounted in Ussing chambers (Chang et al., 1984). In chicken and chinchilla ileum it also did not inhibit the increase in short circuit current stimulated by PGE_1, bradykinin, 5-HT, carbachol or vasoactive intestinal peptide. However, loperamide did inhibit the increased short circuit current induced by substance P, bombesin and neurotensin. The latter peptides all require extracellular calcium to be effective. In addition diprenorphine, a potent opiate antagonist did not reverse the inhibitory effects of loperamide. In isolated chicken enterocytes loperamide reversed inhibition of the Na^+/H^+ exchange caused by substance P, but not that caused by carbachol. Measurements of free intracellular calcium using the Ca-fluorescence indicator quin-2 determined that the increase in free intracellular calcium stimulated by substance P was blocked by loperamide, but the increase stimulated by carbachol was not altered. Since substance P has a requirement for extracellular calcium it was proposed that loperamide inhibits the effect of certain gut peptides by blocking agonist-activated plasma membrane calcium permeabilities. Furthermore it was deduced that it was unlikely loperamide interfered with an intracellular site for calcium action since it did not interfere with the short circuit response to Ca-ionophore A23187 (Chang et al., 1986).

An additional mechanism of action for loperamide has been proposed by Stoll et al. (1985a). Their studies noted that the antidiarrheal activity of loperamide was directly correlated to its ability to bind to calcium/calmodulin. Using brush border membrane vesicles of human ileum the calmodulin levels were measured with a commercial radioimmunoassay system. The calmodulin content in loperamide pretreated brush border membrane vesicles was significantly decreased. Intravesicular loperamide alone stimulated Cl^-/OH^- antiport and coupled Na^+/Cl^- transport in brush border membrane vesicles which was mediated by the inhibition of the intrinsic calmodulin content.

There is evidence that loperamide binds to opiate receptors (Mackerer et al., 1976, Giagnoni et al., 1983) and that the antisecretory activity of loperamide is inhibited by naloxone in perfusion (Sandhu et al., 1974, 1981) fluid accumulation (Berbler and Lembeck, 1979) and Ussing chamber studies (Hughes et al., 1982). Thus loperamide may act through opiate receptors to exert its antisecretory action.

In contrast, naloxone did not inhibit the action of loperamide in human brush border vesicles (Stoll et al., 1985b) and diprenorphine, a potent opiate antagonist did not reverse the inhibition by loperamide of the increase in short circuit current stimulated by substance P, bombensin and neurotensin (Chang et al., 1984). The observations that loperamide completely blocks calcium induced contraction of ileal smooth muscle (Reynolds et al., 1984) and prevents the influx of calcium into epithelial cells (Chang et al., 1984) suggests that loperamide may exert antisecretory effects via the blockade of calcium channels.

The lack of response of loperamide in our studies may be due to the possibility that calcium is not involved in mediating the STb secretory process.

Muscarinic antagonists are frequently employed in the treatment of diarrhea. Scopolamine (hyoscine), an atropine-like drug, is present in the shrubs Hyoscyamus niger and Scopolia carniolica. This drug is a competitive antagonist of acetylcholine and other muscarinic agonists and is readily absorbed from the gastrointestinal tract (Weiner, 1965).

Scopolamine significantly increased chloride absorption in toxin loops. Sodium and chloride absorption in control loops and sodium absorption in toxin loops were increased, but not significantly. Net water absorption was unchanged. The results suggest that muscarinic antagonists may reduce net chloride flux in secretory states. Similar results were observed in perfused pig jejunum (Ahrens and Zhu, 1982b). In their studies atropine reversed chloride secretion to absorption and increased sodium absorption in the control segments. Water and potassium absorption were also increased, but not significantly. In jejunal segments perfused with a cell free broth containing STa and STb, atropine significantly reduced the intestinal secretion of water, sodium, potassium and chloride.

Decerebrate and decapitate cats were used by Wright et al. (1940) to demonstrate that electrical stimulation of the vagus nerve or severing of the splanchnic nerves induced duodenal secretion that was inhibited by intravenous atropine.

Atropine produced a significant increase in the absorption of water and chloride from Omi fistulas in the jejunum of dogs (Blickenstaff and

Lewis, 1952). Increased fluid output induced by tactile stimulation in Thiry Vella loops of denervated dog ileum was found to be lowered by subcutaneously administered atropine. Atropine also reduced the basal rate of secretion (Caren et al., 1974).

In vitro work conducted by Issacs et al. (1976) on stripped human ileal mucosa mounted in Ussing-type Perspex half chambers showed that acetylcholine caused a rise in the mean potential difference and short circuit current. The effect was enhanced by neostigmine, an acetylcholinesterase inhibitor, and was blocked by atropine. In similar studies with rabbit ileum, atropine itself did not have an effect on the short circuit current of electrolyte transport. However, atropine did inhibit an increase in short circuit current induced by carbachol (Powell and Tapper, 1979).

Atropine caused increased absorption of sodium and bicarbonate in the jejunum and decreased bicarbonate secretion in the ileum of rats in vitro. The increase in transmural potential difference, reduced absorption of sodium, potassium, bicarbonate and water and the increased secretion of chloride in the jejunum and secretion of sodium, water and potassium and reduced absorption of chloride in the ileum induced by pilocarpine was prevented by atropine (Hubel, 1976).

In the present study, scopolamine did not significantly influence water or ion transport except for a significant increase in chloride absorption in the loops exposed to STB. This lack of effect may be due to the route of drug administration. Guerrant et al. (1980) noted in suckling mice that atropine administered intragastrically or subcutaneously had no effect on STa induced secretion. In addition

atropine had no effect on basal or STa induced guanylate cyclase activity.

In human subjects, triple lumen perfusion of the small intestine demonstrated that atropine administered intravenously caused a significant increase in chloride absorption and a insignificant increase in water and sodium absorption in the jejunum and a significant increase of water, sodium and chloride absorption in the ileum. However, when atropine was administered intraluminally, no significant changes were observed in salt and water transport (Morris and Turnberg, 1980).

Eklund et al. (1985) observed in one series of perfusion and non perfusion experiments conducted in the rat jejunum that secretion stimulated by a batch of toxin composed of STa and STb was inhibited to some extent by atropine. In a second series of experiments conducted with a second batch of toxin, atropine displayed no effect. They proposed that the two batches of toxin differed in the relative concentration of the two heat-stable toxins as suggested by the difference in the course of onset of secretion. They further suggest that only one of the toxins may mediate its effect via cholinergic muscarinic effectors. They do not state which heat-stable toxin was inhibited by atropine.

The increased absorption of chloride in the toxin exposed ligated pig loops may be the result of muscarinic receptor blockade. The absence of action by scopolamine on water, sodium and potassium ion transport may be due to the small secretory response in the pig produced by STb in the absence of soybean trypsin inhibitor. If a greater secretory response, as noted with STa or with STb and soybean trypsin

inhibitor, was induced, perhaps a more marked effect by scopolamine would be noted on water and sodium. Scopolamine is absorbed more rapidly than atropine from the lumen of the jejunum and thus it may be more rapidly removed from its site of action.

Hexamethonium, a ganglionic blocker, stimulated chloride absorption in control and toxin loops ($P < 0.05$). As with scopolamine, sodium absorption was enhanced, but the increase was not significant. Net water flux was unchanged. The results suggest that blockade of muscarinic receptors or ganglia may predominantly reduce chloride conductance from serosa to mucosa in intestinal epithelium and that drugs acting at these sites do not stimulate neutral sodium chloride influx into the cell.

Hexamethonium (C6) is a quaternary ammonium compound that inhibits ganglionic transmission by blocking sodium channels while they are open (Taylor, 1985). Tactile stimulation of denervated Thiry Vella loops in dogs stimulated an increase in fluid output. Intravenously administered hexamethonium abolished the secretory response suggesting the involvement of an intrinsic neural reflex (Caren et al., 1974). Experiments conducted in 10 to 15 cm segments of the mid jejunum of cats demonstrated a secretory response when crude cholera toxin was instilled in the intestinal lumen. The observed net fluid secretion was markedly reduced following the intravenous administration of hexamethonium. Preganglionic stimulation of the splanchnic nerve did not induce intestinal vasoconstriction suggesting the occurrence of nicotinic ganglionic receptor blockade by hexamethonium (Cassuto et al., 1983).

Hexamethonium administered intravenously in rats abolished the

secretion induced by an extract of broth in which a strain of _E. coli_ producing STa and STb had been grown. The experiments were conducted in non-perfused and perfused small intestinal segments (Eklund et al., 1985).

The absorption of hexamethonium, a quaternary ammonium compound, from the enteric tract is incomplete and not predictable. Ionized substances like hexamethonium have limited ability to penetrate the cell membrane. After absorption these substances are primarily confined to the extracellular spaces (Taylor, 1985). This may explain why we did not observe an effect on water, sodium, and potassium transport when hexamethonium was instilled in the intestinal lumen in contrast to other studies where hexamethonium was administered intravenously.

Isolated enterocyte studies were conducted to note the effect of STb on the rate of influx and efflux of chloride ions and the influx of calcium ions. STb had no demonstrable effect on these events.

Increased secretion and reduced absorption of chloride ion has been associated with the exposure of the small intestinal mucosa to enterotoxins and secretagogues. Field et al., (1968) demonstrated that cholera toxin stimulated active chloride secretion in rabbit ileum placed in Ussing chambers. Similar findings were noted by Field et al. (1972) and Hamilton et al. (1978). STa has been reported to induce chloride secretion and inhibit chloride absorption (Rao and Field, 1984; Guandalini et al., 1982). However, chloride secretion stimulated by STa was not noted by Field et al. (1978). In vitro studies conducted with pig jejunum in Ussing chambers did not demonstrate any alteration in chloride unidirectional or net fluxes in the presence of STb (Weikel et

al., 1985, 1986a). Work conducted by Ahrens and Panichkriangkrai (1985) has demonstrated that culture broth containing STa and STb stimulated an increase in chloride flux from isolated porcine enterocytes.

It has been proposed that the secretory effects stimulated by bacterial enterotoxins may not be entirely mediated by cyclic nucleotides (Forsyth et al., 1979). Secretory responses in weanling pigs stimulated by cholera toxin have been demonstrated to be independent in the elevation of the mucosal concentration of cyclic AMP and cyclic GMP (Forsyth et al., 1978; Hamilton et al., 1978). Cholera toxin has been suggested to play a possible role as a calcium ionophore involved in secretion (170). ST has been suggested to act on the cell membrane to alter calcium channels resulting in calcium influx (Thomas and Knoop, 1982).

A role for calcium in the regulation of sodium and chloride transport have been shown in several studies, some of which were conducted in the rabbit ileum. These studies suggest that increasing intracellular calcium levels decreases sodium and chloride absorption and/or stimulates chloride secretion (Bolton and Field, 1977; Donowitz et al., 1981; Frizzell, 1977; Fan and Powell, 1982; Donowitz, 1983 review). An increase in sodium and chloride absorption has been associated with a decrease in intracellular calcium levels (Donowitz and Asarkor, 1982; Hubel and Callahan, 1980).

The cells of the epithelium of the small intestine maintain their own ionic balance and transport ions across the whole epithelium. An important functional aspect of these cells is the distribution of ions. This is due to the fact that the driving force for the absorption of

many different nutrients appears to be the electrochemical difference for sodium (Sepulveda et al., 1982).

The information gained from experiments with isolated cells cannot stand alone. It has to be interpreted by incorporating it with supporting information obtained from intact tissue preparations (Kimmich, 1975).

The problem of cell viability must be addressed when conducting work with isolated cells. Any cell population whether in intact tissue or in a suspension of isolated cells contains a mixture of cells exhibiting varying degrees of viability. The viability of the isolated enterocytes was determined in order to assure the experiments were conducted with cells capable of normal cellular functions such as transmembrane ion transport. The assessment of cell viability was estimated by a trypan blue exclusion test and the determination of the amount of the cytoplasmic enzyme, lactate dehydrogenase, that leaked into the extracellular fluid. In our study greater than 73% of the isolated cells excluded trypan blue. Kimmich (1975) noted that approximately 80% of the isolated intestinal epithelial cells excluded the dye. The author reported these results were similar to values reported by others, but the metabolic and functional capability of the preparation, utilizing hyaluronidase in the isolation technique, was superior to cells isolated with other methods. The reliability of the dye exclusion test has been questioned. Various kinds of cells fixed in osmium tetrotoxide have been observed to exclude trypan blue for several hours. The dye exclusion occurred even though osmium fixation produces nonviable cells with cell membranes permeable to large molecules

(Barrett and Coleman, 1973).

A second evaluation of cell viability was undertaken by measuring the activity of the cytoplasmic enzyme, lactate dehydrogenase, that escaped into the surrounding medium and comparing it to the total activity of lactate dehydrogenase in the cell suspension. Normally in intact viable cells lactate dehydrogenase is retained within the cytoplasm. When the integrity of the membrane is lost the enzyme leaks into the surrounding environment. The activity during the last collection period measured in the cell free supernatant in our experiments was an average of 28.4% of the total suspension activity. These levels are similar to those observed by Evans et al. (1971). They found the level of lactate dehydrogenase activity that had escaped from isolated guinea pig epithelial cells was at a level of approximately 20% three to five hours after isolation. These workers noted that if the cells were continually gently shaken the rate of enzyme leakage increased. If the cells were kept at 37°C or gassed with O_2:CO_2 (95%: 5%) the rate of leakage was twice that for cells at room temperature and in equilibrium with room air. The results of the two viability evaluations in our study indicate that the viability of the isolated cell preparations was similar to those reported by others.

The lack of an observable effect of STb on the flux of chloride is consistent with the absence of an effect on the unidirectional or net fluxes of sodium and chloride noted by Weikel et al. (1985, 1986a) in pig jejunum mounted in Ussing chambers.

STb did not influence the uptake of calcium in our experiments. In vitro work by Weikel and Guerrant (1985) showed that the removal of

calcium from the mucosal and serosal bathing solution of pig jejunum in Ussing chambers produced no inhibition of the short circuit current induced by STb. Thus calcium may not play a role in STb-induced secretion. Possibly STb does not influence the transmembrane flux of chloride or calcium, but may affect other ions such as bicarbonate.

The lack of an observed influence of STb on chloride and calcium ion transport may be due to the fact STb exerts its effect via other ion transport systems or perhaps STb does not exert its influence directly on enterocytes, but may act indirectly through the enteric nervous system.

SUMMARY

The secretory response of the intestine to Escherichia coli STb enterotoxin was consistantly produced in the presence of soybean trypsin inhibitor (STI). The magnitude of net water secretion was comparable to that produced by broth containing both STa and STb. The results support the concept that trypsin-like activity is present in the intestinal lumen and interferes with STb activity. These results suggest that the addition of STI to luminal solutions of STb will allow the rat as well as the pig to serve as an experimental model of STb-induced diarrhea.

Clonidine significantly increased the absorption of water and sodium and decreased the secretion of potassium in pig jejunal segments perfused by broth containing STb. In the ligated loops of pigs clonidine reduced or reversed the response to STb toxin, but the changes were not significant. No remarkable alterations in water transport were induced by clonidine in rat ligated loops. The results in pig and rat ligated loops may have been due to the more variable response to STb in ligated loops compared with perfused loops. Alpha adrenergic receptor agonists may reduce the net secretory fluxes induced by STb under the more physiological conditions of the intact intestine.

The α_2-antagonist, yohimbine, accentuated the secretory response to STb in pig perfusion studies. Similar results were noted in rat ligated intestinal loops. These results, although not significant, when viewed along with the response to clonidine further supports the role of α_2-receptors in the reduction of STb secretion.

Morphine caused a significant reduction in the STb induced potassium secretion and reversed sodium and chloride secretion to

absorption in the ligated pig loops. Naloxone induced a significiant reversal of STb stimulated sodium and chloride secretion to absorption. In the rat intestinal loops neither drug caused any significant alterations in the STb response. The lack of the drug response in the rat may be due to species differences involving the location and types of opiate receptors in the rat small intestine. The results in the pig suggest the stimulation of opiate receptors can alter the STb secretory response. The action of naloxone, an opiate antagonist, may be due to its ability to act as a partial agonist (Jaffe and Martin, 1985). Naloxone has been found to have characteristics of an antagonist-agonist. At low concentrations (3×10^{-9} to 1.5×10^{-6} M) the drug has antagonistic effects, but at high concentrations (10^{-4} M) it displays agonistic effects (Frank, 1985 review). The action of the muscarinic antagonist 4-DAMP in the rat perfusion experiments was to reduce the water secretion stimulated by STb. Blockade of muscarinic postsynaptic receptors on enteric neurons or presynaptic receptors on the nerve terminals of sympathetic neurons may possibly reduce the effect of STb. This blockade could also occur at the level of the enterocyte. Scopolamine and hexamethonium both increased net chloride absorption in the toxin loops of ligated pig loops. This may indicate that blockade of muscarinic receptors or ganglia may predominantly reduce chloride conductance from serosa to mucosa in intestinal epithelium.

STb's secretory response was not affected by leucine and methionine enkepalin, loperamide, and pirenzepine. As with the previous drugs this may be due to the strength of the STb response, species and receptor specificity and drug concentration.

Isolated enterocyte studies did not demonstrate an effect of STb on the uptake or efflux of chloride and uptake of calcium. This may have been due to the fact STb does not influence the transmembrane transport of these ions or possibly the functional integrity of the submucosal neuron plexus is required for STb to exert its activity.

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