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REVIEW

New Aspects of the Pathophysiology and Treatment of Secretory Diarrhoea

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Summary

This review presents recent findings regarding the physiological and pathophysiological extra- and intracellular mechanisms of secretory diarrhoea. Putative interventions directed towards counteracting the mechanisms causing fluid loss, especially in relation to the enteric nervous system, intracellular mediators, and localization of fluid and electrolyte transport, are discussed. The enteric nervous system regulates the complex process of transmural fluid and electrolyte transport by controlling the function of the mucosa, the motility, and the microcirculation in both health and disease. Most of the processes, leading to secretory diarrhoea, involve activation of the enteric nervous system, with local release of neurotransmitters and other endogenous effectors, which induce chloride secretion. A new therapeutic approach is based on stimulation of absorption and inhibition of secretion by using receptor agonists and antagonists, and modulators of intracellular signal transduction. A physio-pharmacological review of serotonin and the antisecretory factor as modulators of intestinal fluid and electrolyte transport is given.

Key words

5-hydroxytryptamine – Antisecretory factor – Chloride secretion – Enteric nervous system – Intestinal secretion – Serotonin – Intracellular mediators – Second messengers

1. Introduction

Diarrhoea is one of the most common maladies facing medicine today. The loss of fluids from the intestine results in hypovolaemia and decreased circulation to the vital organs, which causes high morbidity and mortality, especially of malnourished children, the elderly, and patients with conditions of immunodeficiency.

During health the gastrointestinal (GI) tract is adapted for the absorption of nutrients and water, but when the gut is inflamed or invaded by toxins of noxious organisms, its functions are coordinated as a defence mechanism to expel the released contents. This protective clearance process involves vomiting, increased propulsion, hypersecretion of electrolytes, water and mucus, increased mucosal blood flow, immunological and inflammatory responses, and epithelial restitution (Neutra and Forstner 1987, Read 1991).

In accordance with new knowledge of the pathophysiology of diarrhoea, new putative, specific therapeutic approaches have emerged. One of these new important interventions is aimed to counteract the mechanisms causing fluid loss and to stimulate absorption and inhibit secretion by using agonists and antagonists of receptors in the enteric nervous system (ENS) and on enterocytes. Furthermore, the identification of the intracellular signal pathway(s) and mediators, offers an additional potential for antidiarrhoeal therapy. Because the development of therapeutic agents is mainly targeted towards man,

species differences in physiology and receptor pharmacology are important for defining appropriate animal models for the development and testing of such agents. With respect to ENS, the mean density of enteric neurones is about four times higher in large mammals and humans than in small rodents (rat and guinea-pig) (Timmermans *et al.* 1993), and the electrophysiological properties are also significantly different (Bornstein *et al.* 1994, Thomsen *et al.* 1995). These species differences have also been confirmed in physiological (Miller and Ullrey 1987) and pathophysiological studies (Schwörer *et al.* 1992, Munck *et al.* 1994, Hansen and Skadhauge 1994, Hansen 1994). At the present, pig small intestine seems to be an appropriate animal model for the human small intestine.

This review will focus on the physiology and pathophysiology of intestinal transmural fluid and electrolyte transport in relation to the ENS, the intracellular mediators, and the localization of NaCl absorption and Cl^- secretion. Readers requiring more detailed coverage of recent work are referred to the reviews quoted.

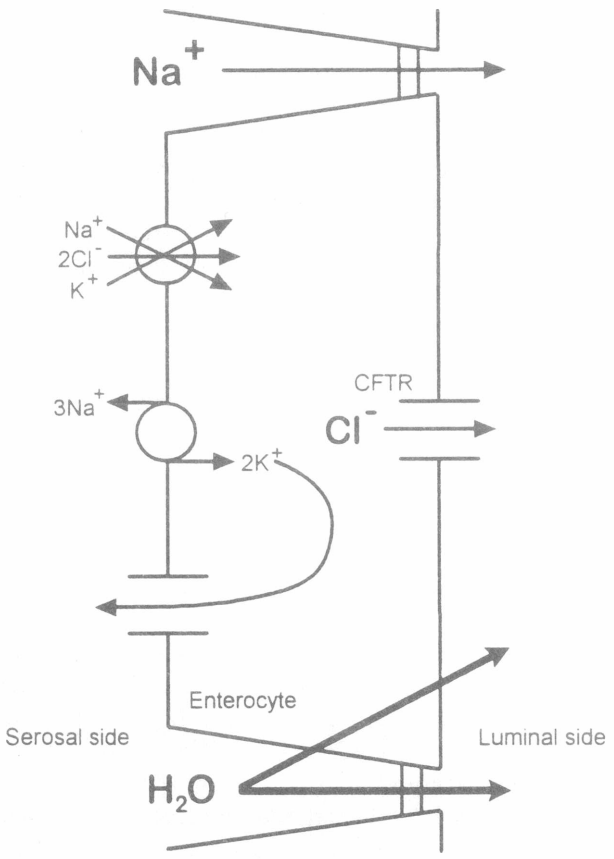

Fig. 1
Cell model for intestinal salt and water secretion. (Reproduced by courtesy of Tyge Tind Tindholdt).

2. Intestinal fluid and electrolyte transport

In the normal human small intestine, secretions and absorptions average normally about seven and ten liters each day, respectively. This illustrates the large secretory and the equally great reabsorptive capacity of the small intestine. Diarrhoea follows if the delicate balance of secretion and reabsorption is not maintained.

Under normal physiological conditions, electrolyte and fluid absorption is achieved by the energy-requiring basolateral Na^+/K^+ ATPase pump, which ensures low intracellular Na^+ and high K^+ concentration and a negative transmembrane potential (see Fig. 1). These properties allow Na^+ to be transported across the apical membrane down its electrochemical gradient. The Na^+ absorption in the small intestine is coupled in symport and antiport to a variety of electrolytes (Cl^-, H^+) and nutrients (amino acids and monosaccharides), which use this electrochemical gradient to facilitate their entry/exit *via* a number of cotransporters (Field *et al.* 1989).

Fluid and electrolyte secretion is accomplished mainly by the serosa-to-lumen transcellular transport of the anions Cl^- and HCO_3^-. Cl^- secretion occurs as a result of an increased permeability of the apical membrane (see Fig. 1). The driving force is maintained by activation of the basolateral bumetanide-sensitive Na^+, K^+, $2Cl^-$ cotransporter, which increases the intracellular level of Cl^- above its electrochemical equilibrium. K^+ exits through K^+ channels in the basolateral membrane activated to maintain the intracellular electronegativity necessary for the Cl^- secretion. Na^+ presumably follows the transcellular movement of Cl^- mainly by passing through the tight junctions *via* the paracellular spaces. The secretion is in turn stimulated by impulses from the submucous and mucosal neurones: their activity is regulated by a number of peptide and non-peptide hormones and transmitters (Frizzell *et al.* 1979, Cooke 1987, Parsons *et al.* 1992).

Control of intestinal absorption and secretion is complex, as each side of the epithelium is exposed to a different environment. On the blood-side, the basolateral membranes are exposed to stimuli coming from endocrine, immunological and neural systems (Perdue and McKay 1994). Compounds present in the intestinal lumen interact with the luminal membrane of the enterocyte and thereby modify the function of the enterocyte. This is the case for water-soluble, food-derived molecules that stimulate Na^+ absorption by means of a co-transport system located in the luminal membrane. It also applies to bacterial toxins, which bind to receptors and in turn release endogenous secretagogues, which lead to hypersecretion (Cooke 1991).

The microcirculation of the gut plays an important role in the delivery of fluids for secretion (Lundgren 1988). Vascular perfusion of the intestinal mucosa increases during digestion and diarrhoea, mostly due to increased blood flow in the submucosal arterioles (Surprenant 1994). Local mechanical and chemical stimulation of the mucosa produces the sympathetic (noradrenergic) and parasympathetic (cholinergic) controlled reflex vasoconstriction and vasodilation, respectively, in the submucosal vascular bed. It appears that adenosine triphosphate (ATP) is the sole sympathetic vasoconstrictor, while nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) seem to be the sole mediators of the cholinergic vasodilatation in submucosal arterioles (Surprenant 1994).

3. Localization of normal NaCl absorption and induced chloride secretion

Today the mechanisms of intestinal ion transport are well known at the level of the individual cell, with both channels and co-transporters being studied by advanced electrophysiological and molecular biological techniques. The only major uncertainty is the exact mechanism of the coupling between the ions during electrogenic transport, i.e. the co-ion movement of Cl^- during normal Na^+ absorption and that of Na^+ during induced secretion of Cl^- (and other ions).

In contrast to this advanced state of knowledge, the problem of the exact location of the cells which transport, particularly along the crypt/villus axis, is far from settled. The conventional view (Field *et al.* 1989) is that small/large intestinal secretion occurs from crypts, and sodium absorption from villus/surface cells. Direct evidence for enterotoxigenic secretion coming solely from crypt cells is scarce, however. The question of the location of normal absorption and induced secretion is important both at the crypt/villus level and along the length of the gut. Better knowledge is essential for the understanding of the complete mechanisms of action of oral rehydration solutions (ORS) and for further developments of prophylaxis and treatment of secretory diarrhoea (Desjeux *et al.* 1994).

For instance, if secretion were to occur solely from crypts with the villi absorbing normally, ORS-induced compensation could be induced locally just by raising the glucose and amino acid concentrations to effectively saturate the luminal Na^+/glucose co-transporter (SGLT1) and similar co-transporters. If, on the other hand, compensation had to occur more distally, including the colon, in sections of the intestinal tract less or not affected by the bacterial toxins, ORSs would have to be formulated to secure high glucose/amino acid concentrations in the ileum, and substances might be included to stimulate colonic absorption, i.e. by production of short-chain fatty acids (SCFA). It is surprising that these aspects, after more than 30 years of use of ORS, have not been more thoroughly investigated, although it is possible to use unabsorbable water markers *in vivo* as exemplified by the porcine gastroenteritis study by Argenzio *et al.* (1984).

Given the importance of absorption/ secretion in the normal and the diseased intestine this survey will focus particularly on the evidence for secretion from villi/surface and absorption from crypts.

Secretion from villus/surface

The activation of absorption and secretion from the single intestinal cell can be studied by different experimental principles:

i) Use of impalement with microelectrodes along the villus-crypt axis to measure changes of electrical potential difference (PD) and electrolyte concentrations induced by stimulation.

ii) Patch-clamp studies of single ion channels of known membrane fragments from individual enterocyte populations (a similar way of localizing individual channels is by *in situ* hybridization and immunocytochemistry).

iii) Intracellular concentration changes of messengers of secretory events such as cyclic adenosine monophosphate (cAMP).

iv) Extracellular recording by the vibrating microprobe. Some examples of the results obtained with these techniques are outlined below.

Stewart and Turnberg (1989) studied the apical membrane PD response to secretagogues along the villus/crypt axis of rat small intestine, both jejunum and ileum. A PD fall of 8 mV or more was observed after PGE_2 stimulation regardless of the site. Giraldez *et al.* (1988) found a cAMP-activated apical Cl^- conductance in *Necturus* enterocytes.

Diener *et al.* (1989) observed single Cl^- channels from both the surface and crypt tissue of rat colonic enterocytes. Trezise and Buchwald (1991) determined CFTR messenger RNA in rat tissues by *in situ* hybridization and found the CFTR gene expressed all the way along the crypt/villus axis and the proximal/distal axis although decreasing gradients were observed in both cases. De Jonge (1975) observed similar concentrations of cAMP in the crypt and villus epithelium of the small intestine of the rat and guinea-pig after cholera toxin (CT) stimulation.

Extracellular recording with the vibrating microprobe was pioneered by Jaffe and Nuccitelli (1974). This technique permits registration of voltage differences of a few μV (instead of mV as with conventional microelectrodes) in the medium above an *in vitro* preparation of an epithelium. The increase in sensitivity is thus almost three orders of magnitude higher. Holtug *et al.* (1991) applied this technique in Jaffe's laboratory to study the localization of Na^+

absorption and Cl^- secretion in a colon-like intestinal epithelium with villi. Following the August Krogh principle, the chicken coprodeum was used, as this tissue is medium-tight developing a spontaneous transmural PD of almost 60 mV and exhibiting a fully amiloride-suppressible large Na^+ absorption (10–20 μEq per cm^2/h) (Clauss *et al.* 1988). After amiloride and theophylline treatment, Cl^- secretion of approximately one third of the previous Na^+ transport emerges (Arnason and Skadhauge 1991, Clauss *et al.* 1988). Coprodeum has crypts from which the villus cells originate (Elbrønd *et al.* 1993), but only one tenth of the crypt density of the mammalian colon. This epithelium was therefore ideal for an ultralocalization study. Furthermore, it could be stretched to a flat sheet which is better for the probe measurements. Holtug *et al.* (1991) observed currents around 40 $\mu A/cm^2$ 50 μm above both villi and crypts, inward in the former, outward in the latter case. They decayed exponentially to near zero at 300 μm with similar length constants over crypts and villi (Fig. 2). After short-circuiting the villus current was doubled, and nearly abolished after amiloride treatment, and in both cases crypt currents were abolished. This is compatible with inward currents being due to Na^+ absorption at the villi, and the crypts being current sinks. Induction of Cl^- secretion after the amiloride treatment resulted in villus currents of one third of the previous magnitude and in the same direction. Quantitative estimates of crypt number and opening diameter in conjunction with isotopic measurements of active and PD driven ion fluxes demonstrate that only 4 % of the PD driven co-ion transport occurs at the crypts. This indicates, that nearly all Cl^- secretion comes from the normally Na^+ absorbing villus area. Calculations showed beyond reasonable doubt that Cl^- secretion could not occur solely from the crypts. Should that be the case, the current over the crypts (see Fig. 2) should have been in the opposite direction and 10 000 fold larger!

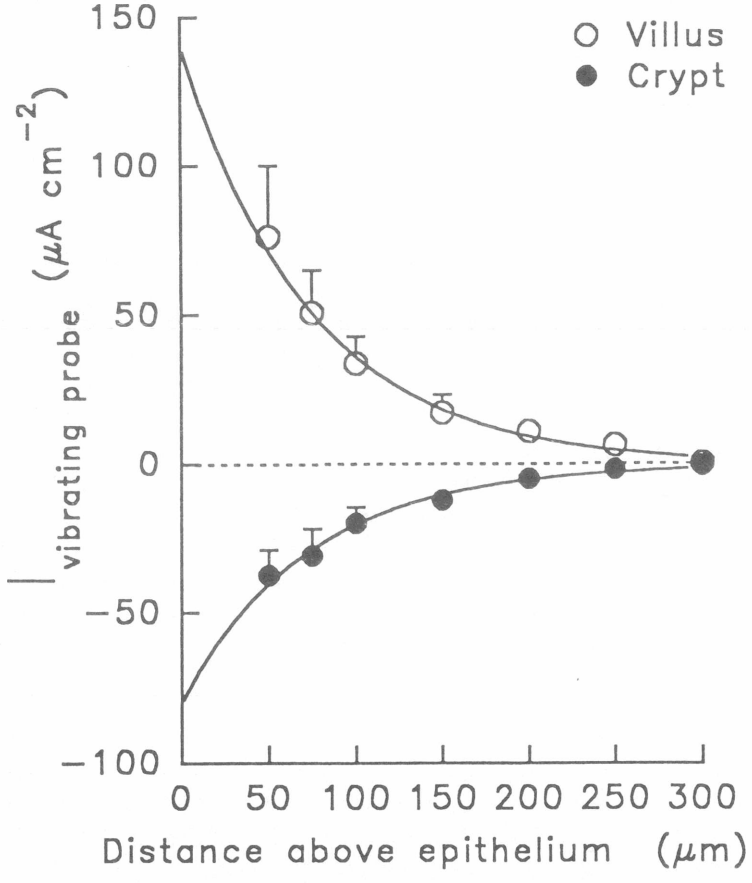

Fig. 2
The figure shows the near-exponential decay of the currents (as functions of distance) in the Ringer-fluid vertically above the tissue. It will appear that the currents flow towards the epithelium over the villi, outwards over the crypts. The figure shows clearly that it is possible with the vibrating microprobe to measure electrical signals separately from crypts and villi. (From Holtug *et al.* 1991).

The experimental results of Holtug *et al.* (1991) are strongly substantiated by the electrostatic theory (as outlined with quantitative calculations in their paper). Totally compatible with their experimental findings is a model simulation assuming that the crypt cells secrete/absorb just as the surface epithelium. This is emphasized in this review as Holtug and co-workers' work has mistakenly been quoted for excluding crypt secretion in a recent paper (Köckerling and Fromm 1993). A somewhat different vibrating microprobe technique was used by Köckerling *et al.* (1993) to monitor voltage deflections over the rat distal colon surface epithelium and over the crypts induced by an external AC current of ±75 $\mu A/cm^2$. By

calculating the local conductances and applying amiloride and aldosterone the authors could monitor the response of surface epithelium *versus* crypts. As with the vibrating microprobe that picks up voltage changes induced by spontaneous currents, this technique required only a fraction of the external current necessary to monitor spatial differences by conventional microelectrodes. For example, Frömter and Diamond (1972) applied 2800 $\mu A/cm^2$ to prove the lateral intracellular spaces as current sinks in *Necturus* gallbladder epithelium. Köckerling and co-workers found the surface conductance increased after aldosterone stimulation *in vitro* by 2.5 fold, whereas amiloride treatment reduced surface epithelium conductance to the basal value. No change was observed over the crypts. The authors have thus observed positive evidence of amiloride-sensitive electrogenic absorption from the surface epithelium. In a following study Köckerling and Fromm (1993) extended their measurements not only to the distal colon but also to the rat ileum. The authors' conclusion of the conductance changes induced by theophylline and other secretagogues is that the cAMP-dependent Cl^- secretion is not confined to crypts but is evenly performed also by surface cells in the distal colon. Their preliminary data suggest a similar distribution in the small intestine.

Absorption from colonic crypts

Patch-clamp studies give ample evidence that mammalian colonic crypts are able to secrete apical Cl^- and basolateral K^+, as was to be expected (Böhme *et al.* 1991, Diener and Scharrer 1994). While there is no doubt that colonic crypts will secrete Cl^- with Na^+ (and water) when stimulated (Welsh *et al.* 1982), Naftalin (1994) has marshalled considerable evidence to indicate that crypts of the descending colon may perform important roles in absorption. The major technical advancement of Naftalin's group is the use of fluorescent high molecular dextrans as ultrastructural markers of solvent flow from lumen into the crypts. This flow was documented by the use of confocal fluorescence microscopy in comparative studies on rat, ovine, and bovine colonic crypts (Pedley and Naftalin 1993). It is hypothesized that the increase in osmotic pressure, at least 200 mOsmol, necessary to generate mammalian faeces of only one third water content is produced not as the property of a local osmotic mechanism in the surface cells but as special trapping of ions (particularly Na^+) due to cryptal absorption. A local region of up to 600 mOsmol around the crypt is suggested. Naftalin's very stimulating studies corroborate, firstly, that mammalian colonic crypts do absorb as was recently shown directly in a perfusion study using single crypts from the rat colon (Singh *et al.* 1994). Secondly, the comparative aspect is important. The crypt "condensation" of the dye is much more pronounced in a species that produces dry faeces such as the sheep than in cattle producing much wetter faeces. Studies of the osmolality of the colonic absorbates in these two species (McKie *et al.* 1991) support the conclusion. Although caution should be expressed towards *in vitro* experiments demonstrating pericryptal ion accumulation, as the blood flow *in vivo* will diminish the gradients, the work is also very stimulating in relation to mechanisms of constipation.

Naftalin (1994) noted that bile salts reduce solute-linked water flow. It may be postulated that the increased concentration of bile salts during the passage of faeces along the large intestine and rectum may be a major system inducing self-limitation on absorption thereby naturally preventing constipation. These basic physiological mechanisms may be of clinical importance. Naftalin's work is interesting for comparative physiology as it offers a hypothesis of the mechanism behind the ability to form dryer or wetter faeces, so far only thought to be a consequence of the general Na^+ absorbing capacity of the colon. Mammals which produce very dry faeces (down to 45 % water content has been reported in East African antelopes such as the Dik-Dik) (Skadhauge *et al.* 1980)) should be studied.

4. The enteric nervous system

The effector systems in the gut (i.e. mucosa, visceral smooth muscle and the vasculature) are controlled by the three divisions of the autonomic nervous system (sympathetic, parasympathetic and enteric). Convincing work has demonstrated that the ENS ("the little brain of the gut") controls most of the intestinal effector systems (Cooke 1989, Gaginella 1990, Surprenant 1994).

Neurotransmitters in the ENS affect the mucosal function, in both health and disease, by activating submucosal afferent (sensory) and efferent (secretomotor) neurones that innervate the epithelial cells (Cooke 1989). The growing recognition of the importance of the different neurotransmitters within the ENS has led to the identification of novel targets for new therapeutic drugs, such as receptor agonists and antagonists.

The ENS consists of sensory, inter-, secretomotor and vasodilator neurones that are synaptically connected into complex microcircuits (Mihara 1993, Wood 1994). These microcircuits process information and incorporate reflex circuits for initiating and sustaining digestive and interdigestive patterns of motility, secretion, absorption and local blood supply to the specialized regions found along the digestive tract. Myenteric microcircuits mainly program motility patterns, whereas submucosal microcircuits program mucosal secretion, absorption and local blood flow (Wood 1994).

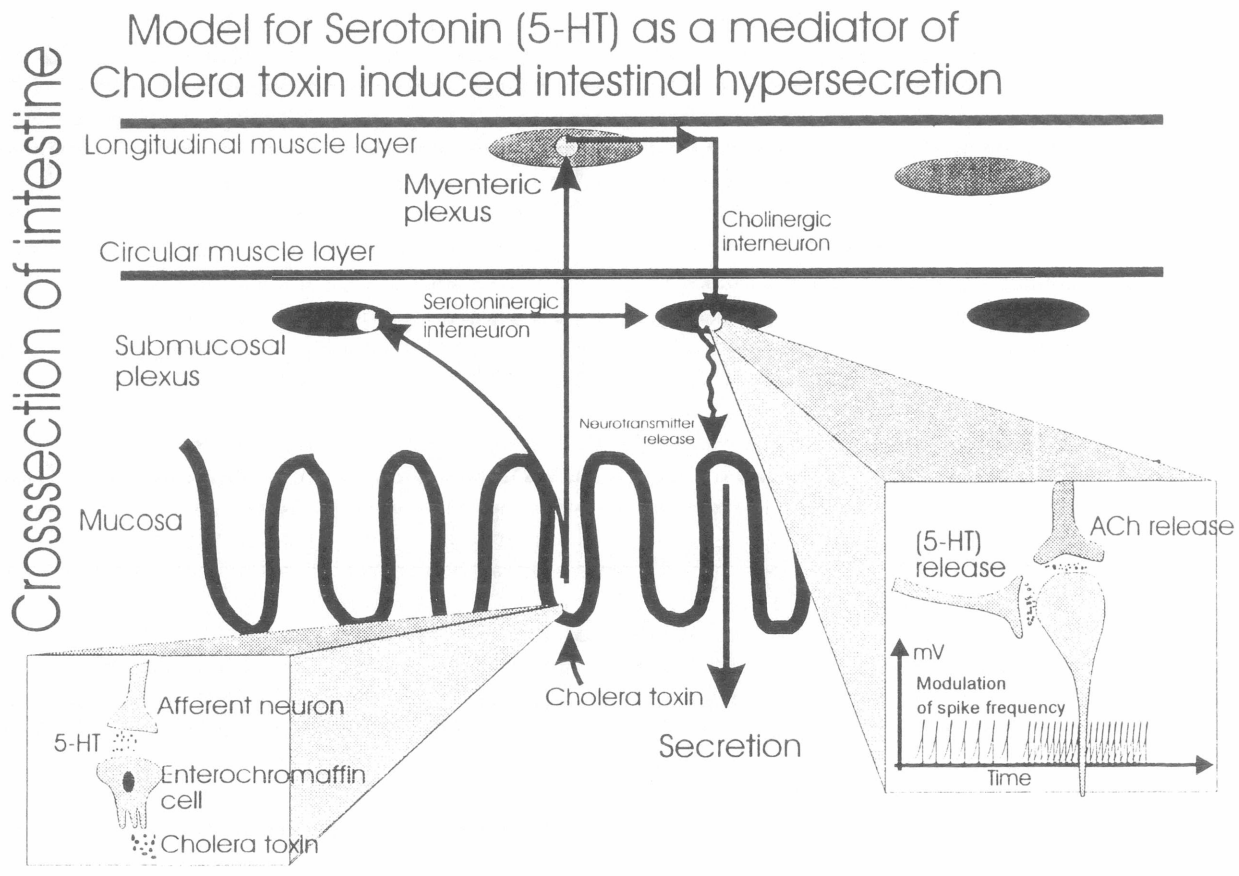

Fig. 3
Cholera toxin-induced intestinal secretion is partly mediated by the release of serotonin (5-HT) from the enterochromaffin cells. 5-HT evokes secretion directly by activating the enterocytes and indirectly by activating the submucosal and myenteric plexus through cholinergic and non-cholinergic reflex circuits. (Reproduced by courtesy of Lars Thomsen).

The ENS consists of two major parts: myenteric plexus and submucosal plexus (Fig. 3). The myenteric plexus is located between the smooth muscle layers. The submucosal plexus is found within the submucosa and consists of two different ganglionic neural networks, the plexus submucosus internus (plexus Meissneri) and the plexus submucosus externus (plexus Schabadaschi) (Timmermans *et al.* 1990). The myenteric and submucosal plexus are interconnected to form a syncytial unit. The majority of intrinsic mucosal nerve fibres arise from cell bodies in the submucosal ganglia, and are so closely connected to the submucosal nerve fibres in general, at least in the small intestine, that they functionally can be considered as one plexus (Timmermans *et al.* 1990). Extrinsic nerve fibres also supply the intestinal mucosa, since there is a sympathetic supply *via* T5 and T6 spinal segments and a parasympathetic supply mainly from the vagus nerve, which originates from the floor of the fourth ventricle of the brainstem (Dhasmana *et al.* 1993, Keast 1987).

Information within the ENS is passed from cell to cell by at least 25 different neurotransmitters, located in about 16 subtypes of entero-endocrine cells and neurones (Fig. 4, Wood 1994, Cooke 1989). The neurotransmitter systems have been defined according to their chemical classification: (1) amino acids, (2) other classical transmitters and (3) neuropeptides. The transmitter substances are released into the synaptic cleft between adjacent cells and, depending on the particular synapse, interact with presynaptic and postsynaptic receptors. The postsynaptic actions of transmitter substances are mediated *via* excitatory postsynaptic potentials (EPSP) or inhibitory postsynaptic potentials (IPSP), and can be modulated by the interaction of neurotransmitter substances at receptors in the presynaptic membrane (Cooke 1989, Wood 1994).

SECRETORY	ABSORPTIVE
Serotonin	Pancreatic polypeptide
Chylecystokinin	Peptide YY
Secretin	Enkephalins
Gastric inhibitory peptide	Somatostatin
Glucagon	Mineralocorticoids
Motilin	Glucocorticoids
Neurotensin	Angiotensin II
Substance P, Tachykinins	Norepinephrine
Atrial natriuretic peptide	Neuropeptide Y
Acetylcholine	Antisecretory factor
Vasoactive intestinal polypeptide	
Calcitonin gene-related peptide	
Gastrin-releasing peptide	
Nitric oxide	
Histamin	

Fig. 4
Endogenous effectors of intestinal fluid transport.

Enteric neurones have been characterized and classified according to their electrophysiological, neurochemical and morphological properties. It seems generally accepted that the synaptic transmission can be classified according to the following electrophysiological criteria. Fast EPSPs (less than 0.5 s duration, depolarization and hexamethonium-sensitive) appear to be mediated mainly by acetylcholine (ACh) acting at nicotinic receptors. Slow EPSPs (2–5 s duration and depolarization) seem mainly to be mediated by serotonin, histamine, tachykinins (e.g. substance P (SP) and neurokinin A), VIP, cholecystokinin, calcitonin gene-related peptide, and ATP, while IPSP's (1–5 s duration, hyperpolarization) seem mainly to be mediated by catecholamines, ACh, serotonin, opiod peptides and somatostatin (Surprenant 1994, Cooke 1987). An intermediate EPSP (0.5–2 s duration) has been reported in rodents (Mihara 1993). Serotonin seems to be the transmitter for this response, which is d-tubocurarine- and tropisetron-sensitive (Mihara 1993).

According to the classification by Wood (1994), the two main types of enteric neurones are type 1/S (S for receiving synaptic input) and type 2/AH (AH for after-hyperpolarization) cells. Additional type 3 and 4 cells have been proposed. Type 1/S cells account for about 60 % in the myenteric plexus and 90 % in the submucosal plexus, while type 2/AH cells almost account for the rest in rodents, such as the guinea-pig (Mihara 1993). Activation of type 1/S neurones evokes tetrodotoxin (TTX)-sensitive fast EPSP's, while activation of type 2/AH neurones gives TTX-insensitive but calcium-dependent, conotoxin-sensitive, slow EPSPs (Lundgren et al. 1989, Bornstein *et al.* 1994). Species differences again seem to be of importance, since more than 20 % of the neurones in the submucosal plexus of the pig jejunum are type 2/AH with some synaptic inputs and mediating predominantly fast EPSPs (Thomsen *et al.* 1995).

The type 1/S cells mostly seem to have a secretomotor (efferent) function and the morphology of Dogiel type I cells (i.e. uniaxonal), while the type 2/AH cells seem mainly to be sensory (afferent) and with the morphology of Dogiel type II cells (i.e. multiaxonal) (Lundgren *et al.* 1989, Bornstein *et al.* 1994).

The classification of enteric neurones, the understanding of the synaptic pathways in ENS and the role of ENS in regulating the function of the mucosa are still not settled and need to be further studied in the process of developing specific antidiarrhoeal drugs.

5. Intracellular signal transduction

Changes in the levels of intracellular mediators (i.e. second messengers) produce changes in intestinal ion transport (Field *et al.* 1989, Brown and Miller 1991). A new approach in treating secretory diarrhoea could thus be drug-induced alterations of the levels of intracellular mediators. This is a well established approach for diseases in other organ systems.

The major intracellular mediators are cAMP, cyclic guanosine monophosphate (cGMP), calcium (Ca^{2+}), phosphoinositides (e.g. phosphoinositol triphosphate, IP_3 and diacylglycerol, DG) and arachidonic acid (AA) metabolites (e.g. prostaglandin E_2, PGE_2) (Cooke 1989). Emerging data suggest NO and ATP to be minor intracellular mediators of intestinal fluid and electrolyte transport (MacNaughton 1993).

With respect to cyclic nucleotides, cAMP levels are elevated by several neurohumoral agents such as VIP and secretin (Beubler 1981). Furthermore, some bacterial toxins, such as CT and *E. coli* heat-labile toxin (LT), have also been shown to stimulate intracellular cAMP production (De Jonge 1975, Beubler *et al.* 1989), while others, such as *E. coli* heat-stabile toxin (STa) and *Yersinia enterocolitica* toxin cause an increase in the cGMP level and the intestinal secretion by a similar mechanism (Eklund *et al.* 1986, 1987, Brown 1987, Beubler *et al.* 1992). With respect to IP_3, several intestinal neurotransmitters and hormones, including serotonin, ACh, SP and neurotensin, have been demonstrated to control fluid and electrolyte transport by releasing IP_3 (Chang *et al.* 1986, Eklund *et al.* 1987, Hansen and Jaffe 1993), and by regulating the intracellular levels of Ca^{2+} and opening of the Ca^{2+}-sensitive ion (e.g. K^+) channels (Brown and Miller 1991, Petersen 1992).

Individual receptor subtypes reveal characteristic coupling to different intracellular mediator signalling systems. As it has been demonstrated for several biogenic amines (e.g. cholinergic, dopaminergic, adrenergic and serotonergic), classification of the functional receptor subtypes can be made by their linkage to these intracellular mediator systems. These studies allow definition of a drug as either an agonist or an antagonist, and characterize the drug's potency and the intracellular mediator system(s). This has led to the discovery and characterization of new receptor subtypes, although some receptors are linked to multiple systems (Hoyer and Schöeffter 1991).

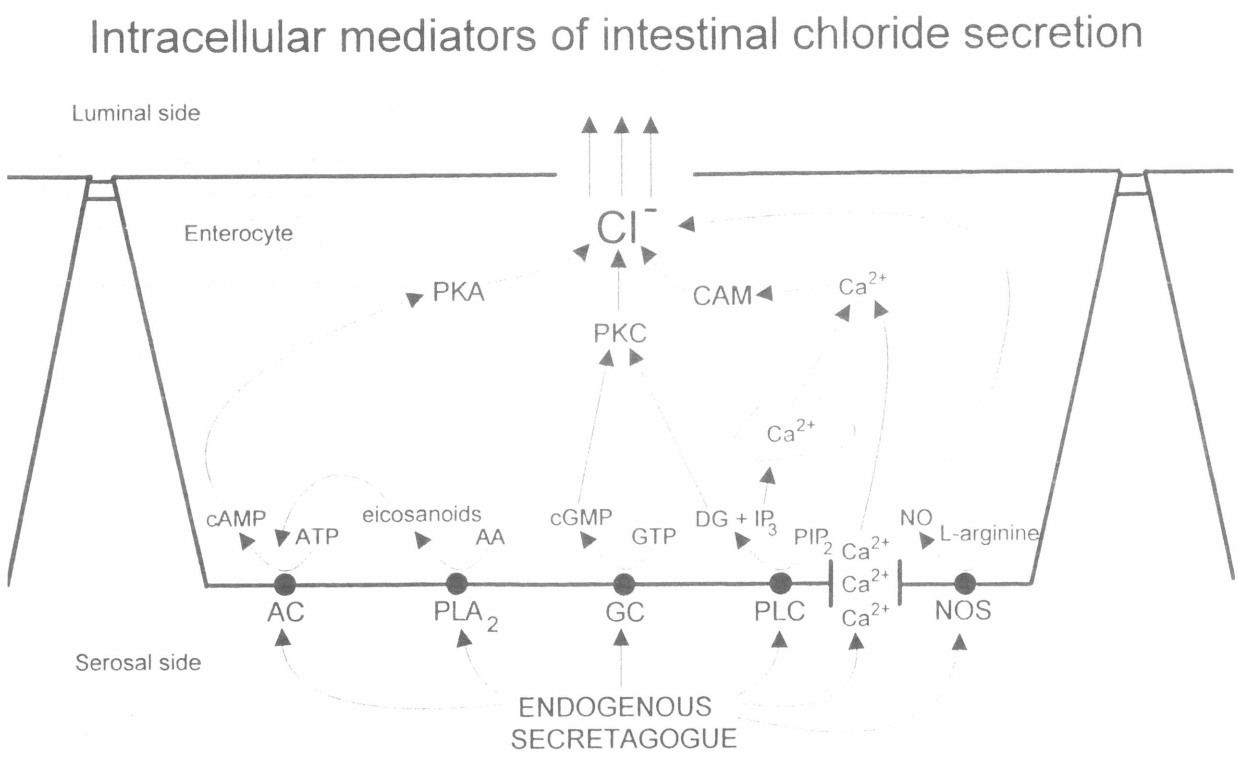

Fig. 5
Model for the intracellular mediators (second messengers) of the endogenous secretagogues mentioned in Fig. 4. (Reproduced by courtesy of Tyge Tind Tindholdt).

Different enzymes are activated in the process of receptor stimulation to changes in the level of intracellular mediators. The regulation of these enzymes, such as adenylate cyclase (AC), phospholipase A_2 (PLA_2) and phospholipase C and D (PLC, PLD), all occur with the obligatory participation of guanine nucleotide (G) proteins (Birnbaumer *et al.* 1990). Furthermore, phosphatidylinositol (PI) hydrolysis, release of AA, stimulation of several types of K^+, Na^+ or Cl^- channels, and the voltage-gated Ca^{2+} channels are also regulated by G proteins (North 1989, Birnbaumer *et al.* 1990). G proteins are

multifunctional heterotrimers that couple with at least 85 different receptors. G protein-coupled receptors are formed from single polypeptide chains folded so as to transverse the membrane, mostly seven times (i.e. 7 trans-membrane-domains) (Strader *et al.* 1994). The G proteins are classified into groups, based on the differences in the subunits. In general, Gs and Gp are responsible for stimulation, while Gi inhibits activity (Mihara 1993). In general, the G protein coupled receptors produce slow responses and desensitize slowly, while the ligand-gated receptors produces fast responses and desensitize rapidly (De Vivo and Maayani 1986, Birnbaumer *et al.* 1990, Hoyer 1991).

Protein kinases (PKs) are also involved in the intracellular cascade regulating intestinal fluid and electrolyte transport (Chang *et al.* 1985). PKs are a class of enzymes that catalyze the transfer of a phosphate group of ATP to residues of another protein to form a phosphomonoester bond. PKs are composed of dissociable regulatory and catalytic subunits. The regulatory unit binds the nucleotide and causes conformational changes resulting in activation of the PK (Chang *et al.* 1985, Aidley 1989). Protein kinase A (PKA) is present in all intestinal epithelia examined, while protein kinase C (PKC) shows an uneven tissue distribution (Chang *et al.* 1985, Aidley 1989). PKA, PKC and calmodulin (CaM) either directly phosphorylate channels or carriers or alternatively phosphorylate regulatory proteins separate from, but associated with the membrane transport proteins. cGMP, cAMP, Ca^{2+}, CaM, and G proteins have also been demonstrated to have phosphorylation-independent effects on ion permeabilities and on activities of enzymes, such as phospholipases and structural proteins (Chang *et al.* 1985, Aidley 1989, De Jonge and Rao 1990).

The combined data suggest that four major intracellular signal pathways dominate in secretory diarrhoea (Fig. 5): (1) stimulation of PKA, evoked by an increase in cAMP and eicosanoids, mainly activating Cl^- secretion, (2) stimulation of PKC, evoked by an increase in cGMP and DG, mainly inhibiting Na^+ absorption, (3) Ca^{2+} mobilization seems to be involved in the action of both and in increased levels of most phosphoinositols, such as IP_3, and (4) others, including NO and ATP. According to experimental studies (Hansen and Jaffe 1993, Hansen *et al.* 1994a), and the present knowledge on the intracellular signal transmission in states of intestinal hypersecretion, there appears to be an antidiarrhoeal drug potential in developing drugs, which alter the level of intracellular mediators, involved in the secretory cascade.

6. Serotonin

Considerable experimental research and clinical attention has been given to the important endogenous secretagogue, serotonin (5-hydroxytryptamine, 5-HT), in the process of developing new therapeutic drugs for GI disorders, such as diarrhoea and dysmotility. This section presents the current physio-pharmacological knowledge of 5-HT with respect to intestinal fluid and electrolyte transport.

5-HT is an amine, which is synthesized from tryptophan, and stored in vesicles, to be held prior to release. 5-HT, that is not stored, is rapidly metabolized to 5-hydroxyindoleacetic acid (5-HIAA) and excreted in the urine (Boadle-Biber 1993).

5-HT is widely distributed in the human GI tract (Griffith and Burnstock 1983). In the intestine, about 90 % of the whole-body content of 5-HT is present in the enterochromaffin (EC) cells, the neurones, and the mast cells in the lamina propria (Forsberg and Miller 1983, Powell 1991), although there exists pronounced species-dependent differences (Timmermans *et al.* 1990, Gershon *et al.* 1991). 5-HT is co-localized with several neuromodulators in both the myenteric and submucosal plexus (Ekblad *et al.* 1988, Lundgren *et al.* 1989, Timmermans *et al.* 1990). Firing of a neurone results in the release of 5-HT, as of other neurotransmitters, from the nerve terminals into the synaptic cleft. Termination of the effects of 5-HT in the synaptic cleft occurs by reuptake and by catabolism. The reuptake of 5-HT takes place at the presynaptic terminals, and can be blocked by a number of selective 5-HT reuptake inhibitors (Gershon and Jonakait 1979, Hyttel *et al.* 1984, Ohuoha *et al.* 1993). These drugs thereby potentiate the action of 5-HT in the gut (Hansen *et al.* 1994b).

Small amounts of 5-HT are continuously produced in the intestinal mucosa and released into the gut lumen (by spilling over ?) and into the portal circulation (Racke and Schwörer 1991). The quantity released is directly related to the intraluminal pH, to the mechanical pressure on the mucosa, and to the level of peristalsis (Blum *et al.* 1992). Furthermore, cytotoxic drugs (e.g. cisplatin), stimulation of the splanchnic nerves and increase of the tonicity of intraluminal contents have been demonstrated to increase the release of 5-HT (Larsson *et al.* 1980). The release of 5-HT from the neurones and the EC cells is regulated by a complex pattern of neuronal and humoral inputs. These 5-HT stores are endowed with several different inhibitory, as well as stimulatory receptors, again dependent on the species (Racke and Schwörer 1992, Gebauer *et al.* 1993).

5-HT exerts its physiological and pathophysiological effects by binding to receptors. Multiple receptors for 5-HT exist (Humphrey *et al.* 1993). This creates many targets for 5-HT-related drug development.

The physio-pharmacology of 5-HT in the intestine is complicated due to the wide array of 5-HT receptor subtypes, which are distributed on both the neuronal, muscular and epithelial structures. Only some of the 5-HT receptor subtypes have specific

agonists and antagonists, while the rest have only selective-agents with additional low affinity for other receptor types (Humphrey *et al.* 1993). The lack of specific agents is currently a problem in characterizing and classifying intestinal 5-HT receptor subtypes, although some progress has been made (e.g. 5-HT_3 antagonists). The distribution of 5-HT receptors, their cellular localization, and segmental or species differences are elucidated by radioligand binding, quantitative autoradiography, *in situ* hybridization, immunocytochemistry, and functional studies (Kilpatrick *et al.* 1991, Fozard 1992, Bonhaus *et al.* 1993). Finally, both the affinity, potency, and intrinsic activity of agonists and antagonists have to be taken into consideration, since they vary depending on the age and the intestinal segment of the species (Pácha 1993, Hansen et al. 1994b, Grøndahl *et al.* 1995).

Most 5-HT antagonists are competitive antagonists, which in increasing concentrations (with a fixed concentration of 5-HT) progressively inhibit the 5-HT response (Leff and Martin 1988, Hansen 1994). Conversely, sufficiently high concentrations of 5-HT can completely surmount the effect of a given concentration of the antagonist, giving a similar maximal efficacy (i.e. E_{max}) for the agonist at any fixed concentration of an antagonist. Partial 5-HT agonists produce a lower response (E_{max}), at full receptor occupancy than do full agonists. Furthermore, partial 5-HT-agonists competitively inhibit the response produced by 5-HT (Leff and Martin 1988, Hansen 1992).

The gut contains inhibitory as well as excitatory 5-HT receptor subtypes, by which the pattern of fluid and electrolyte transport (and motility) seems to be mediated, especially through the microcircuits in the ENS (Gershon *et al.* 1990). 5-HT excites in the guinea-pig mostly the 2/AH neurones but also type 1/S neurones (Gershon *et al.* 1991, Mihara 1993). The 5-HT-containing neurones form tight synapses to the effector cells in the gut (Boadle-Biber 1993).

The 5-HT_{1A} receptors have been demonstrated on neurones (Gershon *et al.* 1990, Tack *et al.* 1992). These receptors are responsible for inhibitory actions, mediating presynaptic inhibition of the release of ACh at nicotinic synapses as well as postsynaptic hyperpolarization (Cooke *et al.* 1991). Conversely, the pre- and postsynaptic 5-HT_4 receptors are believed to attenuate and enhance, respectively, the response of 5-HT by altering the release of ACh from cholinergic interneurones (Cooke *et al.* 1991, Hansen 1994). 5-HT_{1P} receptors are responsible both for presynaptic inhibition and for postsynaptic excitation. The excitatory response mediated by 5-HT_{1P} is slow (i.e. slow EPSP) (Mawe *et al.* 1989), while the 5-HT_3 receptor mediates fast EPSPs (Gershon *et al.* 1991, Tack *et al.* 1992). All 5-HT receptors, except for the ligand-gated 5-HT_3 subtype, are coupled *via* G proteins to their effectors.

5-HT appears to modulate most GI functions in both health and disease (Cooke *et al.* 1991, Mizutani *et al.* 1992). In diseases, such as enterotoxin-induced diarrhoea and chronic watery secretory diarrhoea of carcinoid patients (Cooke 1991, Ahlman *et al.* 1992), intestinal hypersecretion is evoked both by direct and indirect stimulation of the enterocytes (Wood 1994). The direct effect is controlled by increasing intracellular levels of mediators in cells of the intestinal wall (Castro *et al.* 1987, Beubler *et al.* 1992). The indirect mechanism includes intramural and luminal release of 5-HT and other regulators, such as VIP, ACh and tachykinins (Fig. 3, Cassuto *et al.* 1982).

Exogenous 5-HT causes intestinal fluid, water, and electrolyte (Cl^-, Na^+, K^+, and possibly HCO_3^-) secretion in all studied species and intestinal segments (Hansen and Bindslev 1989a, Hansen and Jaffe 1994, Hansen *et al.* 1994a). Depending on the age, the species, and the segment of the gut, the secretory response (sensitivity) to exogenous 5-HT has been demonstrated to vary (MacNaughton 1993, Hansen and Jaffe 1994, Grøndahl *et al.* 1995). 5-HT evokes intestinal secretion by complex activation of several receptor-operated pathways, again depending on the species and gut segment (Hansen and Bindslev 1989a, Beubler and Horina 1990, Hansen *et al.* 1994c, Hansen 1994). In general (see Fig. 6), disregarding species and segmental differences, the secretory pathways can be divided into a cholinergic and a non-cholinergic route. The cholinergic pathway includes at least activation of the granisetron-sensitive 5-HT_3, the tropisetron-sensitive 5-HT_4 (Hansen *et al.* 1994c, Hansen 1994, 1995), the muscarinic and the nicotinic receptors (Hansen 1994). This involves a neural reflex, by which the submucosal and the myenteric plexus are connected and activated (Cooke 1987, Franks *et al.* 1993). The non-cholinergic pathway includes activation of the enterocytic ketanserin-sensitive 5-HT_2 subtype (Hardcastle *et al.* 1984, Siriwardena *et al.* 1993, Hansen and Jaffe 1994, Hansen *et al.* 1994b, 1994c). An additional submucosal and intramural co-release of peptidergic and non-peptidergic effectors most likely takes place (Lawson and Powell 1987, Ekblad *et al.* 1988, Parsons *et al.* 1992).

5-HT mediates intestinal secretion by various intracellular mediators (see Fig. 6), again depending on the species and the gut segment. The cholinergic pathway is dominated by a mechanism *via* the cation-gated channels (Yau *et al.* 1990, Chandan *et al.* 1991, Hansen 1994), while the non-cholinergic pathway includes PGE_2, cAMP, cGMP, IP_3 and Ca^{2+} (Chang *et al.* 1986, Hansen and Bindslev 1989b, Brown *et al.* 1992, Zifa and Fillion 1992, Hansen and Jaffe 1993, Hansen *et al.* 1994a) as mediators in a complex manner.

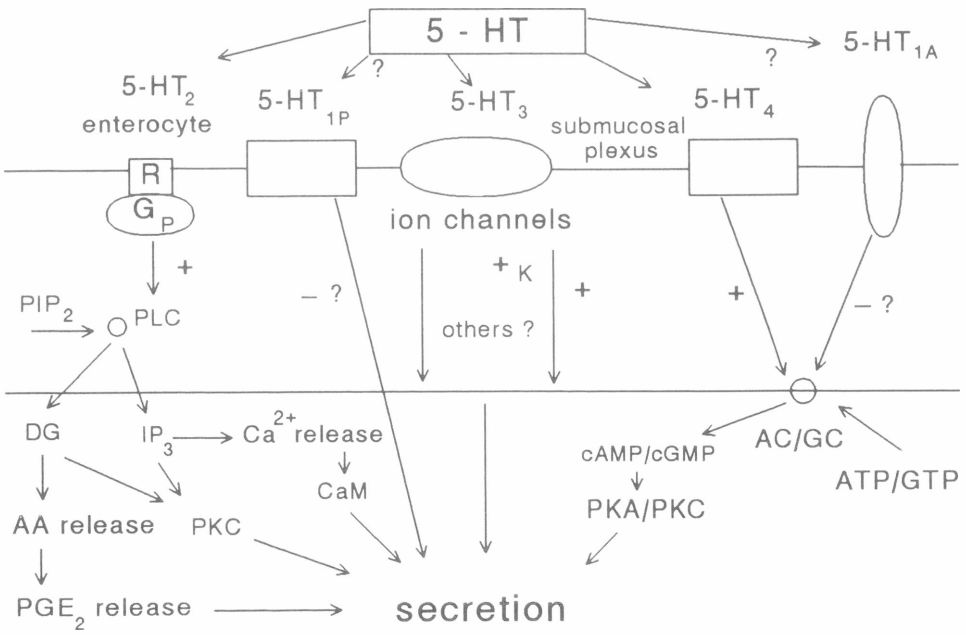

Fig. 6
Signal pathways for activation of 5-HT receptors in the submucosal plexus and mucosa of 5-HT-induced fluid and electrolyte secretion. Special abbreviations: +, stimulatory and secretory effect; –, inhibitory and anti-secretory effect; R and G_P, G-protein regulating unit.

7. The antisecretory factor

Origin, assay, and chemical composition

In 1984, a naturally occurring antisecretory factor (ASF) was described, which inhibited CT-induced intestinal secretion (Lönnroth and Lange 1984). ASF is a heat-labile acidic protein with a molecular weight of about 60 000 (Lönnroth and Lange 1986). Peroral CT immunization of rats and pigs induces the appearance of ASF in the CNS and in the intestinal mucosa (Lönnroth *et al.* 1988a). Other bacteria such as *Clostridium difficile* (Torres *et al.* 1991) and oral intake of glucose or amino acids (Lönnroth and Lange 1987) will also induce ASF-like proteins. In a pig *in vivo* study using the loop test, Lange *et al.* (1987a) demonstrated strong inhibition of porcine LT-induced intestinal hypersecretion and somewhat smaller inhibition to CT. This study also demonstrated that the pituitary gland content of ASF was small in 3- and 5-week-old pigs whereas it increased considerably in pigs aged six months or more than 2 years. After intra-intestinal instillation of CT the pituitary ASF content in the 5-week-old pigs increased within 24 hours close to the level observed in six-month-old pigs. This observation might partly explain why enterotoxigenic *E. coli* diarrhoea does not develop in older pigs, and explain the diminished CT response in 14-week-old pigs observed by McEwan *et al.* (1990a) as compared to 14-day-old piglets.

Mechanisms of action

In order to clarify the mechanisms of ASF action, Skadhauge *et al.* (1986) tested a porcine preparation of ASF on the "stripped" pig jejunum in the Ussing chamber. No effect of ASF was, however, observed on short-circuit current and Na^+ and Cl^- unidirectional fluxes, nor was any effect of ASF on these parameters observed when Cl^- secretion was induced by PGE_2 or theophylline. Similar findings were made on chicken coprodeum (Lönnroth *et al.* 1988a). Skadhauge *et al.* (1986) concluded accordingly, that the pronounced inhibition on CT and LT induced hypersecretion *in vivo* was most likely caused by a neural effect of the compound. In fact, the circumstantial evidence for a nervous mediation in the production and action of ASF fits entirely into the

model of cholera secretion put forward by Lundgren's group (Eklund *et al.* 1988). This model assumes that peptides and other mediators inhibit an intramural secretory reflex induced by the toxins possibly involving both the myenteric and the submucosal plexus (Fig. 3).

The hypothesis of an effect of ASF on nerve cells is corroborated by Lange *et al.* (1987b) who demonstrated that ASF significantly attenuated the Cl^- permeability (^{36}Cl efflux) of the isolated plasma membrane of isolated Deiters' nerve cells in rabbits. ASF was further observed to reduce the gamma amino butyric acid effect on Cl^- permeability in the same preparation (Rapallino *et al.* 1989). Since decreased Cl^- permeability increases the excitability of nerve cells, the observed effect leads to antisecretion provided ASF acts on an intestinal inhibitory neurone in a circuit, which has been activated by the secretagogue.

An alternative mode of action for ASF would be a somatostatin-like effect. This hormone hyperpolarizes the submucous neurones in the guinea-pig by opening K^+ channels (Mihara *et al.* 1987). The functional consequence is a decreased secretory activity.

Direct studies aiming at elucidating ASF effects on submucous neurones, which is now possible in the pig (Thomsen *et al.* 1994), should be carried out. The former of the two suggested modes of action is most likely, since it does not influence, as has been observed, normal absorption (Lönnroth *et al.* 1988a), which is augmented by somatostatin in the pig jejunum *in vitro* (unpublished data).

The effect of ASF in relation to age was further studied by McEwan *et al.* (1991) who investigated the effect of CT and *E. coli* STa enterotoxin-induced fluid secretion in the jejunum of pigs *in vivo* at the age of 2 and 8 weeks. The CT-induced fluid secretion was reduced by ASF by up to 90 % in the 8-week-old animals as observed by Lange *et al.* (1987a), but no effect was found in 14-day-old piglets suggesting that there is a minimum age before ASF is effective. ASF had no significant effect on net fluid transport after STa challenge in pigs from either age group. The predominant action of STa is, however, to inhibit absorption and this would not be affected by ASF, which only affects induced secretion.

Although the available evidence points to a neural effect of ASF, there is one observation suggesting a more direct action on the enterocyte as McEwan *et al.* (1990b) discovered an effect on pig jejunal acid microclimate. As in the rat and man, the pH at the mucosal surface, measured by miniaturized pH electrodes *in vivo*, is also more acid in the pig jejunum than the pH of the bulk phase of the superfused fluid (McEwan *et al.* 1990c). This luminal acidification is largely caused by an apical Na^+/H^+ exchange. McEwan *et al.* (1990b) observed that STa induced an alkalinization of the pig jejunal mucosal surface pH, which was increased from 6.3 to 6.9. This alkalinization was halved by ASF. This suggests ASF action on the enterocyte during the early stages of STa action prior to stimulation of guanylate cyclase.

Clinical effects

Clinical correlation in the pig suggests a role of ASF in enterotoxigenic diarrhoea, as a correlation was found between presence/absence of diarrhoea and the ASF level of sow's milk (Lönnroth *et al.* 1988b) and plasma levels of ASF (Lange *et al.* 1993). Furthermore, addition of glucose and some amino acids is claimed to induce ASF-like factors in the plasma, to decrease the incidence of diarrhoea and to promote better growth in newly weaned piglets (Göransson *et al.* 1993). It is difficult to judge whether the effect of these dietary manipulations is specific or might largely be caused by the general effect of a higher fraction of glucose in the diet. Concerning the putative effects at the enterocyte level, an anti-VIP or NPY-like effect should be studied.

8. Concluding remarks

Antiserotonergic drugs and the ASF are definitely new potentially important compounds for prevention and treatment of enterotoxigenic diarrhoea in human and veterinary medicine. They deserve further study of mechanisms of action, particularly effects on the intestinal nerve plexus, and clinical trials involving enterotoxigenic challenge, such as *E. coli*, *Salmonella* and *V. cholerae*.

In future intestinal studies, the pig should be used as a model for man instead of rodents. The general drug strategy should be to use agents with selectivity for the intestinal receptor subtypes, and to combine low doses of more than one selective agent, since high doses of 5-HT antagonists, for example, tend to have intrinsic agonistic activity at various receptors, thereby causing unpredictable and unwanted effects, such as enhanced intestinal hypersecretion (Hansen and Jaffe 1994, Hansen *et al.* 1994b) or reduced motility. Studies of the functional properties and the cellular location of the receptor agonists and antagonists, together with computer models of the interaction of ligand and receptor, seem to be a promising approach for the design of new drugs with higher affinity and selectivity for the intestinal receptor subtypes. Using gene targeting techniques together with experimental functional studies, may enable the construction of a detailed ligand-binding pocket for every receptor and to change specificities or modify the drugs *ad libitum* (Weydert 1993). A challenge for the future lies in further understanding the relationship between regulation of transmitter stores, neuronal firing, the release of the neurotransmitters, and their function under physiological and pathophysiological conditions.

Finally, caution should be expressed concerning the interpretation of lack of *in vitro* effects of a putative antagonist on secretion induced by theophylline or PGE_2. These secretory stimuli may simply be too strong and overwhelm the inhibiting effects of compounds that might be effective in a clinical situation. Conversely, even a pronounced antisecretory effect observed in tied-off loop tests may not ensure the clinical applicability of the drug if, e.g. an accompanying constipation worsens the bacterial infection (unpublished data).

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