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REVIEW

Cholinergic regulation of epithelial ion transport in the mammalian intestine

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Acetylcholine (ACh) is critical in controlling epithelial ion transport and hence water movements for gut hydration. Here we review the mechanism of cholinergic control of epithelial ion transport across the mammalian intestine. The cholinergic nervous system affects basal ion flux and can evoke increased active ion transport events. Most studies rely on measuring increases in short-circuit current (I_{SC} = active ion transport) evoked by adding ACh or cholinomimetics to intestinal tissue mounted in Ussing chambers. Despite subtle species and gut regional differences, most data indicate that, under normal circumstances, the effect of ACh on intestinal ion transport is mainly an increase in Cl^- secretion due to interaction with epithelial M_3 muscarinic ACh receptors (mAChRs) and, to a lesser extent, neuronal M_1 mAChRs; however, AChR pharmacology has been plagued by a lack of good receptor subtype-selective compounds. Mice lacking M_3 mAChRs display intact cholinergically-mediated intestinal ion transport, suggesting a possible compensatory mechanism. Inflamed tissues often display perturbations in the enteric cholinergic system and reduced intestinal ion transport responses to cholinomimetics. The mechanism(s) underlying this hyporesponsiveness are not fully defined. Inflammation-evoked loss of mAChR-mediated control of epithelial ion transport in the mouse reveals a role for neuronal nicotinic AChRs, representing a hitherto unappreciated braking system to limit ACh-evoked Cl^- secretion. We suggest that: i) pharmacological analyses should be supported by the use of more selective compounds and supplemented with molecular biology techniques targeting specific ACh receptors and signalling molecules, and ii) assessment of ion transport in normal tissue must be complemented with investigations of tissues from patients or animals with intestinal disease to reveal control mechanisms that may go undetected by focusing on healthy tissue only.
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Keywords: acetylcholine; enteric nervous system; intestine; ion transport; muscarinic acetylcholine receptors; nicotinic acetylcholine receptors; short-circuit current; Ussing chambers

Abbreviations: AChR, ACh receptor; ATR, atropine; BCh, bethanechol; CCh, carbachol; DMPP, dimethylphenylpiperazinium; EFS, electrical field stimulation; EGF, epidermal growth factor; HEX, hexamethonium; IFN-γ, interferon-γ; IHC, immunohistochemistry; mAChR, muscarinic acetylcholine receptor; MP, myenteric plexus; nAChR, nicotinic acetylcholine receptor; PD, potential difference; PG, prostaglandin; I_{SC}, short-circuit current; SI, small intestine; SMP, submucosal plexus; SP, substance P; TTX, tetrodotoxin; TNF, tumour necrosis factor; VIP, vasoactive intestinal polypeptide; VAChT, vesicular acetylcholine transporter

Introduction

The epithelial lining of the intestine separates two biological compartments: the gut lumen (i.e. the external environment) from the interstitium. Under normal circumstances, the polarized epithelial cells impede the movement of luminal material into the body and regulate the passage of a variety of substances – nutrients, electrolytes and water – from lumen to body and *vice versa*. The ability to secrete and absorb electrolytes and fluids is critical to maintain proper hydration of the organism – the intestine and the organs that drain into them (e.g. salivary glands) secrete ~9 l of fluid into the lumen daily, most of which (~8 l) is reabsorbed before it reaches the rectum (Keely and Barrett, 2000). Proper hydration provides the aqueous environment necessary for the processes of contact digestion and nutrient absorption and provides surface lubrication to propel intestinal contents aborally. A properly hydrated gut provides the medium for activity of antimicrobial peptides and movement of water into the lumen contributes to host defence through its ability to flush noxious substances and harmful organisms (e.g. parasites and pathogenic bacteria), thus minimizing their contact with the gut wall. Therefore, regulation of

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water movement is critical from both physiological (i.e. nutrition) and immunological (i.e. host defence) perspectives. If the process of intestinal hydration becomes dysregulated, as during acute intestinal infections or in the context of intestinal inflammation, diarrhoea or constipation can result.

It is not surprising then that water movement into and out of the intestine is highly regulated. There are three known mechanisms by which water can cross the epithelial barrier: (1) absorption along with sodium and glucose via sodium-coupled glucose transporters in the small intestine; (2) specific molecular water channels known as aquaporins; (3) passive osmosis driven by the directed transport of ions across epithelial cells. The expression and function of aquaporins are well described in the kidney, lung, eye and brain (reviewed in King *et al.*, 2004), and their expression and function in the intestinal epithelium is currently the subject of much research (Mobasheri *et al.*, 2004; Laforenza *et al.*, 2005a, b). Sodium-coupled glucose transporters and aquaporins are not discussed here, and we refer the reader to recent reviews on these subjects (Loo *et al.*, 2002; Masyuk *et al.*, 2002; Shachar-Hill and Hill, 2002; Matsuzaki *et al.*, 2004). Here, we review control of enteric epithelial ion transport by acetylcholine, drawing attention to species-specific differences, differences in tissues from naïve animals compared to those with disease and comment on putative research directions.

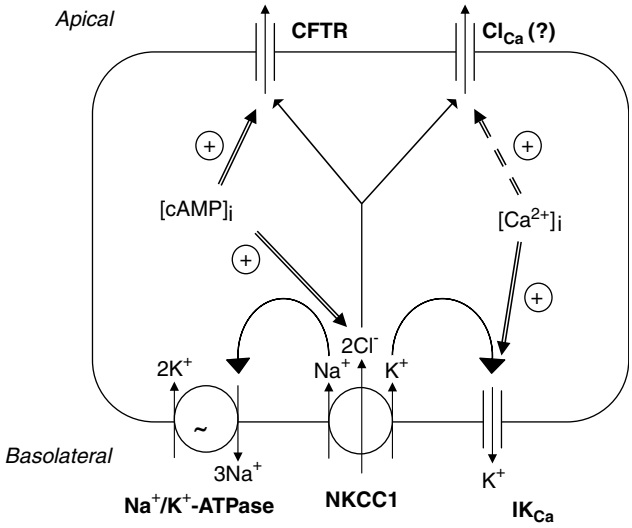

Figure 1 Schematic representation of the intestinal epithelial Cl^- secretory pathway that is upregulated by ACh. The electrochemical gradient is established by the basolateral Na/K-ATPase pump; this allows entry of Na^+, Cl^- and K^+ through the basolateral membrane via the NKCC1 cotransporter; K^+ is recycled through basolateral K^+ channels, one of which is controlled by $[Ca^{2+}]_i$, likely the intermediate conductance K^+ channel (IK), and Cl^- is shunted to the apical membrane, where it exits into the lumen via the apical CFTR Cl^- channel and possibly also through an apical Ca^{2+}-activated Cl^- channel (CaCC, designated Cl_{Ca}). For additional details, refer to Barrett and Keely (2000).

Intestinal ion transport

Ion transport is an active process driven by electrochemical gradients that are established by the Na^+/K^+-ATPase pump located in the basolateral membrane of polarized epithelial cells. The magnitude, rate and direction of ion flux are dependent on the integrated activity of specific channels, transporters and exchangers asymmetrically expressed on the apical and basolateral cell membranes (Figure 1). The activity of the ion transport machinery is regulated by a variety of compounds signalling through membrane-bound or intracellular receptors to alter levels of intracellular messengers, such as Ca^{2+} and cyclic nucleotides.

Study of intestinal ion transport has been greatly facilitated by the development of the Ussing chamber (Ussing and Zerahn, 1951). With this technique, passive flow of ions across a tissue or epithelial cell layer is eliminated by balancing electrical, osmotic, hydrostatic and chemical gradients across the preparation, such that only active ion transport is measured. In the Ussing chamber, electrodes are placed close to each side of the tissue to allow detection of the spontaneous potential difference (PD) across the epithelium, generated as a consequence of active ion transport. The voltage-clamp apparatus matched to the Ussing chamber allows for the PD to be held at a predetermined value by injecting current via two additional silver–silver chloride electrodes, creating a short-circuit current (I_{SC}). Typically, the PD is clamped at zero volts and the amount of injected current required to maintain this reflects the net active ion transport across the preparation. Using the I_{SC} and the PD values (obtained by switching intermittently to an open certain mode), tissue conductance, which indicates passive ion flow, can be calculated using Ohm's law. By substituting specific ions (e.g. isethionate and acetate for Cl^-) or using radiolabelled ions in the bathing buffers, by selectively blocking or removing specific receptors, channels, exchangers, pumps and transporters, and by blocking non-epithelial cell signalling (e.g. nerve block by tetrodotoxin (TTX), which blocks fast Na^+ channels on most, but not all, enteric nerves without affecting epithelial Na^+ channels), the contributions of these components to active electrolyte transport is determined. In the same manner, the effects of nerve stimulation and biological agents (e.g. biogenic amines, bacterial toxins) on active intestinal ion transport can be assessed in tissues or epithelial preparations mounted in Ussing chambers.

Acetylcholine

One of the most important biological regulators of intestinal ion transport is the major parasympathetic mediator acetylcholine (ACh), which is synthesized by the catalytic conversion of acetyl-CoA and choline to CoA and ACh by choline acetyltransferase (ChAT) (Hebb, 1954; Hebb and Whittaker, 1958). ACh was described as a classical neurotransmitter in the early 1920s (reviewed in Eiden, 1998). More recently, a distinction has been made between ACh as a neurotransmitter and ACh as a signalling molecule in non-neuronal tissue (e.g. blood) and in organisms that lack a nervous system (e.g. protozoa, algae, bacteria, primitive

plants) (Grando *et al*., 2003; Wessler *et al*., 2003). As a classical neurotransmitter, ACh synthesis is followed by its transport via the vesicular ACh transporter (VAChT) to synaptic vesicles, from which it is released *en masse* from nerve terminals as a result of membrane action potentials. However, in non-neuronal cells ACh is not stored in vesicles and its release, which is likely facilitated by organic cation transporters, is continuous and much slower compared to ACh released from nerves (Wessler *et al*., 2003). In all tissues, ACh is rapidly and specifically degraded by acetylcholinesterase (AChE), although in mammals a second, nonspecific cholinesterase, designated butyrylcholinesterase, which is abundant in the intestine, may also contribute to the degradation of ACh (Massoulié *et al*., 1993; Li *et al*., 2000).

As with other neurotransmitters, direct measurement of ACh can be accomplished through microdialysis followed by high-performance liquid chromatography: this method has been applied in the intestine (Furuichi *et al*., 2001). However, owing to the difficulty and invasiveness of this technique and because ACh does not lend itself to reliable fixation for detection in preserved tissues, the presence of ACh is often inferred based on expression of the enzymatic components of the cholinergic pathway (Eckenstein and Thoenen, 1982). Analyses of ChAT and VAChT are more accurate than AChE for determining the presence of ACh, as the breakdown of ACh need not occur in the same location as its production. Additionally, VAChT is only relevant to neuronal ACh, leaving ChAT as the universal surrogate for ACh. ChAT activity can be determined radiochemically in tissue homogenates and the protein localized by immunohistochemistry (IHC) with commercial antibodies (Fonnum, 1975; Eckenstein and Thoenen, 1982; Sharkey *et al*., 1998).

Neuronal cholinergic networks, as defined by IHC detection of ChAT, relating to the control of intestinal motility and, to a lesser extent, ion secretion have been extensively studied in the guinea-pig (Costa *et al*., 2000; Furness, 2000; Brookes, 2001; Bornstein *et al*., 2004). Although enteric nerve signalling is well defined in the guinea-pig (Brown and Timmermans, 2004), it is important that this information be complemented by delineation of cholinergic pathways in mammalian species commonly used as models of human intestinal (patho)physiology/disease. Table 1 details the enteric localization of ACh in several mammalian species, including humans. The divide between ACh as a neurotransmitter and ACh as a signalling molecule is especially apparent in the intestine and cholinergic pathways exhibit regional differences along the length of the intestine. Also, while several cholinergic pathways are conserved across species, there are differences between species, one notable example being the colocalization of ChAT and vasoactive intestinal polypeptide (VIP) in secretomotor neurons of the submucosal plexus (SMP) in humans (Schneider *et al*., 2001), a pattern not observed in guinea-pigs at least. Although the enteric nervous system can be considered a discrete entity, cholinergic input from extrinsic nerves that project fibres into the gut (i.e. vagus and pelvic splanchnic nerves) should be recognized as a component in the bigger picture perspective of cholinergic control of physiological events in the gut.

Acetylcholine receptors and pharmacology

ACh receptors (AChRs) fall into two categories: metabotropic G-protein-coupled muscarinic receptors, of which there are five known subtypes, M_1–M_5, and ionotropic nicotinic receptors, which are made up of five subunits that may be in homomeric or heteromeric combinations. Muscarinic (mAChRs) and nicotinic AChRs (nAChRs) are expressed on neuronal as well as on non-neuronal cells in the gut (Tables 2 and 3). Signalling pathways and functions associated with mAChRs have been reviewed in detail elsewhere (Caulfield, 1993; Lanzafame *et al*., 2003).

Briefly, odd numbered mAChRs couple to $G_{q/11}$ α subunits to activate phospholipase C (PLC), whereas even numbered mAChRs couple to the G_i α subunit to inhibit adenylate cyclase. Activation of the PLC pathway leads to degradation of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol-1,4,5 triphosphate (IP_3), which, together, lead to activation of protein kinase C (PKC) and increased levels of intracellular Ca^{2+} as the main outcome. nAChRs are allosteric ion channel proteins that are permeable to monovalent cations and, to a lesser extent, Ca^{2+}. Binding of an agonist (i.e. ACh) to nAChRs preferentially stabilizes it in an open or active conformational state. Subsequent entry of cations through the open channel depolarizes the cell membrane, thus contributing, in the case of a neuron, to the generation of an action potential. The structure and function of nAChRs has been described in detail in the context of the central nervous system and the neuromuscular junction (Hogg *et al*., 2003; Gotti and Clementi, 2004).

Several agonists of AChRs mimic the effects of ACh and are often used in its place experimentally because they are less susceptible than ACh to degradation by AChE. However, mAChR agonists, including ACh, are not highly subtype selective and this needs to be appreciated when evaluating the data from studies relying on these reagents (Hulme *et al*., 1990). Additionally, some mAChR agonists, such as pilocarpine, McN A-343 and, surprisingly, even ACh and bethanechol (BCh), may behave as partial, rather than full, agonists in some experimental systems (Yule *et al*., 1993; Sharif *et al*., 1995). Receptor subtype selectivity has also been a problem with commonly used mAChR antagonists (Hulme *et al*., 1990). However, the characterization of toxins isolated from the venom of mamba snakes has provided several compounds that are highly selective toward the M_1 and M_4 subtypes of mAChRs (see Table 4; reviewed in Karlsson *et al*., 2000). For an extensive discussion of mAChR pharmacology and associated physiology, refer to Caulfield (1993), Eglen *et al*. (1996), Caulfield and Birdsall (1998) and Eglen (2005).

nAChR pharmacology is complicated by the fact that, while at least 11 genes have been identified for mammalian non-muscle nAChR subunits (α2–α7, α9–α10, β2–β4), not all of the subunit combinations natively expressed in different tissues and species are known (Nicke *et al*., 2004). Generally, the α4, β2 and α7 subunits predominate in the central nervous system, whereas α3, β4 and α7 subunits are common in the peripheral nervous system. α7 subunit-containing receptors occur in homomeric combination, whereas α4, α3, β2 and β4 subunits occur in heteromeric combinations of

Table 1 Localization of ACh production in the mammalian gut

Species	*Tissue*	*ChAT/VAChT-positive cell types*[a]	*References*
Guinea-pig	Small intestine	*MP*: Excitatory muscle motor neurons, Ascending interneurons to excitatory circular muscle motor neurons of MP, **descending interneuron**s (three types that all are ChAT-IR – those containing ChAT/5-HT are involved in secretomotor reflex), **IPANs** (mucosal varicose processes likely release ACh), intestinofugal nerves to prevertebral ganglia *SMP*: **Secretomotor/vasodilator neurons, secretomotor (non-vasodilator) neurons, IPANs** (mucosal varicose processes likely release Ach)	McMurray *et al*. (1993), Neunlist *et al*. (1999), Costa *et al*. (2000), Furness (2000), Brookes (2001), Furness *et al*. (2004), Harrington *et al*. (2005)
	Large intestine	*MP*: Ascending neurons, **IPANs**, excitatory muscle motor neurons *SMP*: Ascending secretomotor/vasodilator neurons, **secretomotor neurons** EC cells of small intestine	
Mouse	Small intestine	*MP*: ~60–65% of neurons; including Excitatory muscle motor neurons, Descending interneurons *SMP*: ~40% of neurons; includes likely secretomotor neurons, secretomotor/vasodilator neurons	Sang and Young (1998)
	Large intestine	*MP*: ~55% of neurons; includes excitatory muscle motor neurons, descending interneurons *SMP*: ~20% of neurons; includes likely secretomotor neurons, secretomotor/vasodilator neurons *Note*: ChAT-IR EC cells observed in both SI and LI; ChAT- and VAChT-IR nerve terminals observed throughout MP, SMP, muscle layers, at the base of glands and associated with submucous blood vessels in both SI and LI	
Rat	Small and large intestine	*MP*: Nerve cell bodies and fibres (unidentified subtypes) *SMP*: Nerve cell bodies and fibres (unidentified subtypes) EC cells of small intestine	McMurray *et al*. (1993)
Pig	Small intestine	*MP*: ~80% of total traced neurons; includes IPANs, excitatory muscle motor neurons, secretomotor neurons, descending interneurons, intestinofugal neurons *OSP*: ~85% of total traced neurons; includes IPANs, excitatory muscle motor neurons, secretomotor neurons, descending interneurons, intestinofugal neurons *ISP*: ~25% of total traced neurons; includes IPANs and secretomotor neurons Nerve fibres within circular muscle layer; cell bodies and fibres at the margins of PP follicles; occasional fibres within PP follicles and domes and adjacent to submucosal blood vessels; neurons of interfollicular ganglia, *Note*: minor discrepancies between jejunum and ileum	Kulkarni-Narla *et al*. (1999), Hens *et al*. (2000), Brown and Timmermans (2004)
Human	Small and large intestine	*MP*: Excitatory muscle motor neurons, descending interneurons, ascending interneurons, IPANs *SMP*: Secretomotor neurons EC cells, T cells	Porter *et al*. (1996), Kawashima and Fujii (2000), Porter *et al*. (2002), Schemann and Neunlist (2004)

Abbreviations: EC, enterochromaffin; IPANs, intrinsic primary afferent neurons; IR, immunoreactive; ISP, inner submucosal plexus; LI, large intestine; MP, myenteric plexus; OSP, outer submucosal plexus (ISP and OSP are subdivisions of SMP found in larger mammals, i.e. pig); PP, Peyer's patch; SI, small intestine; SMP, submucosal plexus.
[a]Bold text indicates neurons believed to be involved in intestinal secretion and secretory reflexes in the guinea-pig.

both α and β subunits (Gotti and Clementi, 2004). A few compounds are able to distinguish between homomeric α7 nAChRs and non-α7-containing nAChRs, most notably the snake venom component α-bungarotoxin and methyllycaconitine. Both compounds competitively and selectively antagonize homomeric α7 nAChRs (Davies *et al*., 1999). *Conus* snail venom has also provided several potentially competitive nAChR antagonists, the α-conotoxins, which show subtype selectivity for specific known subtype combinations, although it is unknown if this subtype selectivity applies to all species (Nicke *et al*., 2004). Non-competitive antagonists of nAChRs include the archetypal ganglionic blockers mecamylamine and hexamethonium (HEX), which demonstrate relatively low affinity for nAChRs (K_i values in the mM range) (Macallan *et al*., 1988; Davies *et al*., 1999).

Table 4 lists the affinities of several major AChR agonists and antagonists for specific AChR subtypes. Table 5 lists the affinities of mAChR agonists and antagonists that have been determined in intestinal tissue from a few different species. These drugs have been instrumental in profiling mAChR subtypes involved in intestinal ion transport. Subtype characterization of nAChRs involved in intestinal ion transport has been hindered by a lack of knowledge of the precise nAChR subtypes expressed in the gut. Details of nAChR involvement in intestinal ion transport requires better characterization of the receptor subtypes expressed in the gut before pharmacology can be fully validated. It should be cautioned that, when dealing with promiscuous compounds (i.e. the majority of mAChR ligands), studies using only one compound to implicate the involvement of a specific receptor subtype are not reliable – AChR subtype characterization requires a diverse array of pharmacological tools. Thus, ACh receptor subtype involvement inferred from the use of poorly selective

Table 2 Non-pharmacological demonstration of muscarinic ACh receptors in the mammalian gut

Species	*Tissue*	*Receptor subtype detected*	*Cell type/tissue region*	*Method*	*References*
Rabbit	Ileum	M_1 (3%)[a], M_2 (69%), M_3 (4%), M_4 (12%), M_5 (0%)	ND	IP	Dorje *et al.* (1991a)
Mouse	Ileum	M_2, M_4	Circular and longitudinal smooth muscle (M_2 only); M_2 and M_4 colocalization in cell bodies and fibres of ChAT-positive myenteric neurons	IF	Takeuchi *et al.* (2005), Haberberger *et al.* (2006)
	Colon	M_1	Epithelium	RT-PCR	
Rat	Ileum	M_1 (17%), M_2 69%), M_3 (7%)	Epithelial cells (adult and fetal), including endocrine L cells	IF; IP	Wall *et al.* (1991), Levey (1993), Anini *et al.* (2002)
	Colon	M_4	Nerve fibres (likely presynaptic)	IHC	
Sheep	Ileum	M_1, M_2, M_3 M_4, M_5	Adult smooth muscle	IHC	Oyachi *et al.* (2003)
Pig	Ileum	M_2, M_3	Smooth muscle	NB	Maeda *et al.* (1988)
Human	Ileum	M_3	Mucosa (adjacent to epithelium), muscularis mucosa, submucosal blood vessels	IF	Sato *et al.* (1999), Oue *et al.* (2000), Lindqvist *et al.* (2002), Anini and
	Colon	M_1, M_2, M_3	Epithelial cells, endocrine L cells	IF	Brubaker (2003), Banks
	(juvenile)	M_2, M_3	Mucosal layer, smooth muscle, cell bodies and fibres of myenteric plexus neurons	IHC; ISH	*et al.* (2004)
	Colon	M_1	Mucosal layer	IHC	
	Blood	M_3	Crypt epithelial cells	IF	
	Cultured cell lines	M_1, M_2, M_3, M_4, M_5	MNL; *Note*: variable expression of M_1–M_3 among subjects	RT-PCR	
		M_1, M_2, M_3	NCI-H716 endocrine L cells	WB; RT-PCR	
		M_1, M_2, M_3, M_4, M_5	CEM, MOLT-3, HUT-78, HPB-ALL and Jurkat T cells; *Note*: variable expression of M_1–M_3 among cell lines	RT-PCR	
		M_1, M_2, M_3, M_4, M_5	BALL-1, Daudi and NALM-6 B cells; *Note*: variable expression of M_1–M_3 among cell lines	RT-PCR	

Abbreviations: IF, immunofluorescence; IHC, immunohistochemistry; IP, immunoprecipitation; ISH, *in situ* hybridization; MNL, mononuclear leukocytes; NB, Northern blot; ND, not determined; RT-PCR, reverse transcription-polymerase chain reaction; WB, Western blot.
[a]Percentage of total receptors precipitated (IP experiments performed in whole tissue).

antagonists and/or partial agonists should be verified using newer, more selective drugs and with molecular biology techniques, such as gene targeting strategies to delete specific AChR subtypes (as reviewed in Wess (2004) for mAChRs).

Cholinergic control of enteric epithelial ion transport

Baseline ion transport

Typically, the baseline active ion transport of normal mammalian intestinal tissue, as demonstrated by I_{SC}, reflects a negative charge on the luminal side and positive charge on the serosal side of the tissue (Field, 1974). Absorption of Na^+ and Cl^- typically accounts for most of this baseline ion transport; the relative contributions of K^+, HCO_3^- and H^+ vary according to intestinal region and species. Numerous studies indicate that baseline epithelial transport is in part due to tonic nerve activity, and this is via neurons of the SMP (Furness, 2000; Vanner and MacNaughton, 2004), with a small contribution from secretomotor neurons that project from the myenteric plexus into the mucosa (Costa *et al.*, 2000). This concept is supported by the fact that gut preparations stripped of the outer muscle layers and attendant myenteric plexuses still display positive baseline I_{SC} values.

Additional evidence in support of neural control of baseline I_{SC} is provided by consideration of the effect of the neuronal blocker TTX, which can elicit a drop in baseline I_{SC}. For instance, TTX applied to muscle-stripped segments of porcine distal jejunum produces a dramatic drop in baseline I_{SC}, although this effect is less pronounced in the proximal jejunum (Rangachari and McWade, 1986; Hildebrand and Brown, 1990; Chandan *et al.*, 1991a, b). Similarly, TTX treatment reduces the baseline I_{SC} across muscle-stripped dog, rabbit and porcine colon and full-thickness mouse jejunum and colon (Sheldon *et al.*, 1989; Biagi *et al.*, 1990; Traynor *et al.*, 1991; Sayer *et al.*, 2002). Data in favour of, and refuting, the ability of TTX to lower baseline I_{SC} in muscle-stripped human left/sigmoid colon has been presented (Hubel *et al.*, 1987; Kuwahara *et al.*, 1989), and, in support of the latter, TTX treatment does not alter baseline I_{SC} in the rat colon (Zimmerman and Binder, 1983). It is difficult to

Table 3 Non-pharmacological demonstration of nicotinic ACh receptors in the mammalian gut

Species	*Tissue*	*Receptor subunits detected*	*Cell type/tissue region*	*Method*	*References*
Guinea-pig	Ileum and Colon	$\alpha3$, $\alpha5$, $\beta4$ (?)	Nerve cell bodies and fibres – MP (more abundant staining in colon): inhibitory and excitatory motor neurons, secretomotor neurons, afferent and/or ascending interneurons; SMP: secretomotor neurons, interneurons	IF; IHC	Kirchgessner and Liu (1998), Obaid *et al.* (1999), Zhou *et al.* (2002), Glushakov *et al.* (2004)
		$\beta2$	EC cells	IF; IHC	
		$\alpha7$	SMP neurons of small intestine Nerve cell bodies and fibres of MP and SMP; especially abundant in SMP	IF; IHC	
	Cultured cells	$\alpha3$, $\alpha5$, $\beta4$ (?), $\beta2$, $\alpha7$	Primary cultured MP neurons of small intestine (only a few neurons positive for $\alpha7$); *Note*: colocalization of nAChR subunits with cholinergic and non-cholinergic neurons	IF	
Human	Ileum and Colon	$\alpha3$, ($\alpha5$, $\beta4$?)	MP and SMP neurons and fibres traversing smooth muscle; Epithelium; Lymphoid tissue; *Note*: variable distribution among subjects	ISH; IHC	Hiemke *et al.* (1996), Battaglioli *et al.* (1998), Sato *et al.* (1999),
	Blood	$\alpha3$, $\alpha4$	$CD4^+$ Lymphocytes; *Note*: variable expression among subjects	ISH; IHC	Richardson *et al.* (2001), Richardson *et al.* (2003),
		$\alpha2$, $\alpha5$, $\alpha6$, $\alpha7$, $\beta2$	MNL; *Note*: variable expression of $\alpha6$ and $\beta2$ among subjects	RT-PCR	Summers *et al.* (2003)
	Cultured cell lines	$\alpha4$, $\alpha5$, $\alpha7$, $\beta1$	HT29 colonic epithelial cell line	RT-PCR	
		$\alpha3$	Jurkatt and MOLT-4T cells	NB	
		$\alpha2$, $\alpha3$, $\alpha5$, $\alpha6$, $\alpha7$, $\beta4$	CEM, MOLT-3, HUT-78, HPB-ALL and Jurkat T cells	RT-PCR	
		$\alpha2$, $\alpha5$, $\alpha6$, $\alpha7$, $\beta2$, $\beta4$	BALL-1, Daudi and NALM-6 B cells	RT-PCR	

Abbreviations: EC, enterochromaffin; IHC, immunohistochemistry; IF, immunofluorescence; ISH, *in situ* hybridization; MNL, mononuclear leukocytes; MP, myenteric plexus; NB, Northern blot; SMP, submucosal plexus.

determine the source of variability in these studies: differences in tissue preparation, subtle region-specific differences or environmental variability (i.e. diet, resident intestinal flora) are possibilities. Moreover, use of TTX gives no indication of the phenotype of neuron, or the possibility of signalling within the ENS via interneurons that impacts upon baseline I_{SC}. Also, analyses of the role of neurons in the control of epithelial ion transport should, for example, be complemented by identification of the ion fluxes responsible for the TTX effect.

Although technically demanding, some enteric tissues (e.g. dog colon) can be stripped of both the outer muscle layers and the submucosa, leaving sheets of epithelial crypts and a supporting basement membrane. [It should be noted that the procedure to remove the muscle and mounting in the Ussing chamber have the potential to activate stretch receptors that could elicit intracellular and intercellular signalling cascades that could affect active ion transport across the enterocyte.] These preparations maintain active ion transport that is unaffected by TTX, although baseline I_{SC} values are lower than in preparations containing an intact submucosa (Rangachari and McWade, 1986; Diener *et al.*, 1989). Monolayers of human colon-derived epithelial cell lines (i.e. T84 cells, HT-29 cells, Caco-2 cells) also exhibit baseline ion transport in the absence of any neural input, demonstrating that whereas enteric nerves modulate epithelial baseline active ion transport, the enterocyte itself is primarily responsible for the generation of driving forces to support vectorial ion movements. Thus, baseline production of intracellular ATP allows for energy-dependent ionic gradients to be established while other mediators, such as cAMP, positively regulate the opening of apical and basolateral ion channels. Other non-neuronal mediators present in the mucosa (e.g. prostaglandins (PGs); Craven and DeRubertis, 1983) probably also contribute to baseline levels of active ion transport, although this has mostly been studied in the context of stimulated ion transport (see following sections).

Using atropine (ATR) as the gold standard mAChR antagonist has revealed contrasting data in terms of cholinergic input to tonic enteric epithelial I_{SC}. Thus, full thickness mouse colon, muscle-stripped rat colon and porcine jejunum respond to ATR treatment with a decrease in I_{SC} (Diener *et al.*, 1989; Chandan *et al.*, 1991b; Sayer *et al.*, 2002). Contrarily, treatment with ATR does not affect baseline I_{SC} in guinea-pig small bowel, porcine jejunum, and rabbit and human colon (Cooke, 1984; Keast *et al.*, 1985; Hubel *et al.*, 1987; Kuwahara *et al.*, 1989; Biagi *et al.*, 1990; Hildebrand and Brown, 1990; Chandan *et al.*, 1991b). Few studies have assessed the impact of nAChR antagonists on baseline I_{SC}: porcine jejunum, mouse mid-colon and rat distal colon all display small but consistent reductions in baseline I_{SC} following treatment with HEX (Hildebrand and Brown, 1990; Sun *et al.*, 2000; Sayer *et al.*, 2002), although this effect requires high (100 μM) concentrations of HEX in the porcine jejunum (Hildebrand and Brown, 1990; Chandan *et al.*, 1991a).

Collectively, the data support the contention that the enteric nervous system contributes to baseline epithelial ion transport, albeit to varying degrees between species and

Table 4 Affinity profiles of AChR agonists and antagonists for known AChR subtypes

Drug	*K_i (nM)*	*References*
mAChR agonist		
Carbachol	205 (M_1), 93.4 (M_3), 35.7 (M_5)[a]	Kukkonen *et al*. (1996)
mAChR antagonist		
AF-DX 116	1300 (M_1), 186 (M_2), 838 (M_3), 2800 (M_5)[b]	Buckley *et al*. (1989), Billard *et al*. (1995)
	344.6 (M_1), 32.4 (M_2), 198.6 (M_3), 123.5 (M_4)[b]	
AF-DX 384	30.9 (M_1), 6.03 (M_2), 66.1 (M_3), 10.0 (M_4), 537.0 (M_5)[b]	Dorje *et al*. (1991b)
Atropine	0.21 (M_1), 1.5 (M_2), 0.15 (M_3), 0.21 (M_5)[b]	Buckley *et al*. (1989), Moriya *et al*. (1999)
	0.28 (M_1), 0.76 (M_2), 0.19 (M_3), 0.13 (M_4), 0.24 (M_5)[a]	
4-DAMP, 4-diphenylacetoxyl-*N*-methylpiperidine methiodide	0.58 (M_1), 3.8 (M_2), 0.52 (M_3), 1.27 (M_4), 1.05 (M_5)[b]	Dorje *et al*. (1991b), Moriya *et al*. (1999)
	0.57 (M_1), 7.3 (M_2), 0.37 (M_3), 0.72 (M_4), 0.55 (M_5)[a]	
Hexahydro-sila-difenidol	22.4 (M_1), 131.8 (M_2), 15.5 (M_3), 31.6 (M_4), 93.3 (M_5)[b]	Dorje *et al*. (1991b)
Hexocyclium	2.3 (M_1), 23 (M_2), 1.4 (M_3), 5.5 (M_4), 3.7 (M_5)[b]	Buckley *et al*. (1989)
Himbacine	107.2 (M_1), 10.0 (M_2), 93.3 (M_3), 11.0 (M_4), 489.8 (M_5)[b]	Dorje *et al*. (1991b), Billard *et al*. (1995)
	49.2 (M_1), 4.4 (M_2), 29.6 (M_3), 5.5 (M_4)[b]	
Methoctramine	16 (M_1), 3.6 (M_2), 118 (M_3), 57 (M_5)[b]	Buckley *et al*. (1989), Dorje *et al*. (1991b)
	50.1 (M_1), 13.2 (M_2), 213.8 (M_3), 31.6 (M_4), 134.9 (M_5)[b]	
M_1-toxins:		Jerusalinsky *et al*. (2000), Karlsson *et al*. (2000)
MT7	0.1 (M_1), >2000 (M_2), >2000 (M_3), >2000 (M_4), >2000 (M_5)[b]	
MT1	22–49 (M_1), >1000 (M_2), >1000 (M_3), 29-58 (M_4), >1000 (M_5)[b]	
MT2	630 (M_1), >2000 (M_2), >2000 (M_3), 1900 (M_4), >2000 (M_5)[b]	
MT4	62 (M_1), >1000 (M_2), >1000 (M_3), 87 (M_4), >1000 (M_5)[b]	
MT5	180 (M_1), >1000 (M_2), >1000 (M_3), 540 (M_4), >1000 (M_5)[b]	
M_4-toxins:		Jerusalinsky *et al*. (2000), Karlsson *et al*. (2000)
MT3	78–1100 (M_1), >1000 (M_2), >1000 (M_3), 1.4-2 (M_4), >1000 (M_5)[b]	
MT6	190 (M_1), >425 (M_2), >425 (M_3), 3.6 (M_4), >425 (M_5)[b]	
MTα	23 (M_1), 44 (M_2), 3 (M_3), 5 (M_4), 8 (M_5)[b]	
MTβ	>1000 (M_1), >2000 (M_2), 140 (M_3), 120 (M_4), 350 (M_5)[b]	
Oxybutinin	0.66 (M_1), 13 (M_2), 0.72 (M_3), 0.54 (M_4), 7.4 (M_5)[a]	Moriya *et al*. (1999)
Pirenzepine	16 (M_1), 906 (M_2), 180 (M_3)[b]	Buckley *et al*. (1989), Dorje *et al*. (1991b), Billard *et al*. (1995), Kukkonen *et al*. (1996), Moriya *et al*. (1999)
	6.3 (M_1), 223.9 (M_2), 138.0 (M_3), 37.2 (M_4), 89.1 (M_5)[b]	
	4.6 (M_1), 211.8 (M_2), 37.8 (M_3), 21.1 (M_4)[b]	
	11.4 (M_1), 373 (M_3), 69.3 (M_5)[a]	
	7.1 (M_1), 303 (M_2), 75 (M_3), 17 (M_4), 66 (M_5)[a]	
Scopolamine	0.085 (M_1), 0.88 (M_2), 0.063 (M_3), 0.1 (M_4)[b]	Billard *et al*. (1995)
Silahexacyclium	2.0 (M_1), 35 (M_2), 1.2 (M_3), 3.2 (M_4), 2.0 (M_5)[b]	Buckley *et al*. (1989)
Tiquizium	4.1 (M_1), 4.0 (M_2), 2.8 (M_3), 3.6 (M_4), 8.2 (M_5)[a]	Moriya *et al*. (1999)
[R]-Trihexyphenidyl	0.37 (M_1), 7.08 (M_2), 2.45 (M_3), 0.83 (M_4), 5.01 (M_5)[b]	Dorje *et al*. (1991b)
Tripitramine	1.6 (M_1), 0.3 (M_2), 38 (M_3), 6 (M_4), 3.4 (M_5)[b]	Maggio *et al*. (1994)
nAChR agonist		
Anabaseine	58 (Rat α7)	Kem *et al*. (1997)
Carbachol	4130 (Bovine $\alpha3\beta4$)[c]	Free *et al*. (2003)
Cytisine	517 (Bovine $\alpha3\beta4$)[c]	Free *et al*. (2003)
1,1-Dimethyl-4-phenylpiperazinium	469 (Bovine $\alpha3\beta4$)[c]	Free *et al*. (2003)
(−)-Epibatidine	230 (Rat α7)	Gerzanich *et al*. (1995), Bunnelle *et al*. (2004)
	0.15 (Human $\alpha3\beta2$)[d], 0.031 (Human $\alpha3$)[e], 3.1 (Human α7)[e]	
(+)-Epibatidine	0.23 (Human $\alpha3\beta2$)[d], 0.00059 (Human $\alpha3$)[e], 9.8 (Human α7)[e]	Gerzanich *et al*. (1995)
(±)-Epibatidine	0.3 (Bovine $\alpha3\beta4$)[c]	Davies *et al*. (1999), Free *et al*. (2003)
	233.6 (Rat α7)	
Nicotine	400–14 000 (Rat α7)	Kem *et al*. (1997), Davies *et al*. (1999), Free *et al*. (2003), Bunnelle *et al*. (2004)
	517 (Bovine $\alpha3\beta4$)[c]	
RJR-2403	36 000 (Rat α7)	Bunnelle *et al*. (2004)
nAChR antagonist		
α-Bungarotoxin	0.7 (Rat α7)	Davies *et al*. (1999)
Methyllycaconitine	1.15 (α7)[f]	Alkondon *et al*. (1992), Maggi *et al*. (1999), Davies *et al*. (1999), Free *et al*. (2003)
	1.1–4.3 (Rat α7)	
	420 (Bovine $\alpha3\beta4$)[c]	
MG 624 (4-oxystilbene derivative)	106 (α7)[f]	Maggi *et al*. (1999)
D-Tubocurarine	1570 (α7)[f]	Maggi *et al*. (1999), Free *et al*. (2003)
	105 (Bovine $\alpha3\beta4$)[c]	

[a]Stably transfected Sf9 insect cells.
[b]Stably transfected CHO-K1 cells.
[c]Stably expressed in human embryonic kidney (HEK) 293 cells.
[d]Stably expressed in *Xenopus* oocytes.
[e]Immuno-isolated from SH-SY5Y neuroblastoma cells.
[f]Immuno-immobilized from chick optic lobe.
Note: When affinity values were reported as pK_i, the negative antilogarithm was calculated to determine K_i value; unless otherwise specified, affinity for α7 subunits is determined by displacement of ^{3}[H]-α-bungarotoxin binding.

Table 5 Affinity profiles of mAChR agonists and antagonists in intestinal tissue

Drug	*Tissue preparation*	*K_i (nM)*	*References*
mAChR agonist			
Bethanechol	Guinea-pig ileum – mucosal scrapings	53 000	Carey *et al.* (1987), Kuwahara *et al.* (1987b)
	Guinea-pig proximal colon – mucosal scrapings	53 000	
	Guinea-pig distal colon – mucosal scrapings	35 000	
Carbachol	Guinea-pig ileum – mucosal scrapings	8500	Carey *et al.* (1987), Kuwahara *et al.* (1987b)
	Guinea-pig proximal colon – mucosal scrapings	9800	
	Guinea-pig distal colon – mucosal scrapings	5500	
McN A343	Guinea-pig ileum – mucosal scrapings	180	Carey *et al.* (1987)
MAChR Antagonist			
AF-DX 116	Canine ileum – purified synaptosomal fraction from deep muscular nerve plexus	6200	Chandan *et al.* (1991a, b), Kostka *et al.* (1992), O'Malley *et al.* (1995)
	Pig jejunum – mucosa-submucosa	300	
	Rat colon – mucosa-submucosa	2189	
Atropine	Pig jejunum – mucosa-submucosa	0.4	Chandan *et al.* (1991b), O'Malley *et al.* (1995)
	Rat colon – mucosa-submucosa	0.87	
4-DAMP	Canine ileum – purified synaptosomal fraction from deep muscular nerve plexus	39	Carey *et al.* (1987), Kuwahara *et al.* (1987b), Chandan *et al.* (1991a, b), Kostka *et al.* (1992), O'Malley *et al.* (1995)
	Guinea-pig ileum – mucosal scrapings	1.8	
	Pig jejunum – mucosa–submucosa	2.5	
	Rat colon – mucosa–submucosa	5.2	
	Guinea-pig proximal colon – mucosal scrapings	3.0	
	Guinea-pig distal colon – mucosal scrapings	2.2	
Methoctramine	Canine ileum – purified synaptosomal fraction from deep muscular nerve plexus	2250	Kostka *et al.* (1992)
Pirenzepine	Canine ileum – purified synaptosomal fraction from deep muscular nerve plexus	2540	Carey *et al.* (1987), Kuwahara *et al.* (1987b), Chandan *et al.* (1991a, b), Kostka *et al.* (1992), O'Malley *et al.* (1995)
	Guinea-pig ileum – mucosal scrapings	35	
	Guinea-pig proximal colon – mucosal scrapings	100	
	Guinea-pig distal colon – mucosal scrapings	170	
	Porcine jejunum – mucosa–submucosa	87.5	
	Rat colon – mucosa–submucosa	155.0	

Note: When affinity values were reported as pK_i, the negative antilogarithm was calculated to determine K_i value.

gut regions, and on the balance of the data it seems that ACh plays a minor role. Perplexingly, however, there is little consensus on the role of specific AChRs, with studies suggesting involvement of mAChRs but not nAChRs, nAChRs but not mAChRs, both mAChRs and nAChRs, or neither receptor type. Much of this variation is attributed to species and regional differences, although tissue preparation and environmental variability may also play a role. Additionally, some of the vagaries in the field could be reconciled by a consideration of the expression profiles of specific mAChR and nAChR subtypes and the location of these receptors (i.e. on stimulatory verses inhibitory neurons).

Stimulated ion transport

The majority of studies of cholinergic regulation of ion transport have focused on stimulated ion transport, either through electrical field stimulation (EFS – causes release of ACh and other neurotransmitters) or through application of a cholinergic compound to tissues in Ussing chambers. Thus, it has long been known that ACh and cholinergic agonists stimulate fluid and Cl^- secretion in the mammalian (including human) gut (Tidball, 1961; Hubel, 1976; Isaacs *et al.*, 1976). It is also well established that the addition of carbachol (CCh) or BCh to muscle-stripped intestine (small or large bowel) results in a rapid onset, but transient ($\sim$10–15 min duration), increase in I_{SC} that is due mainly to luminally directed Cl^- efflux across the epithelium (Cooke, 1984; Chandan *et al.*, 1991b; Chough *et al.*, 1993; Strabel and Diener, 1995). Additionally, cholinergic stimulation promotes HCO_3^- secretion and may inhibit Na^+ absorption in some tissues (Browning *et al.*, 1978; Chandan *et al.*, 1991c; Geibel *et al.*, 2000).

However, species- and tissue-specific differences are commonplace when considering: (1) the contribution of cholinergic nerves to the transient (1–5 min duration) increase in I_{SC} evoked by EFS, (2) the contribution of neurons to I_{SC} responses evoked by CCh or BCh and (3) involvement of mAChR and nAChR in cholinergic control of increases in I_{SC}. For example, CCh-induced ΔI_{SC} in guinea-pig ileum is TTX insensitive (Cooke, 1984), whereas colonic responses to CCh are reduced by TTX (Kuwahara *et al.*, 1987b). In porcine proximal, but not distal jejunum, TTX pretreatment actually augments the increase in I_{SC} evoked by low concentrations of CCh (0.1–3 μM) (Chandan *et al.*, 1991a, b). Also, CCh- and BCh-evoked ΔI_{SC} in the rat small intestine are TTX insensitive (Przyborski and Levin, 1997), whereas, in the rat colon, I_{SC} responses to several AChR agonists, including ACh and CCh, are reduced by TTX (O'Malley *et al.*, 1995). Along with the species and regional differences in TTX sensitivity to cholinergically mediated ion transport, there is further

evidence that should caution against making broad conclusions based on individual studies: Prior *et al*. (2004) showed that colonic tissue from two lines of the Flinders strain of rats have different TTX sensitivities to CCh-induced increases in I_{SC}.

Although the relative contribution of nerves to the ΔI_{SC} evoked by CCh or BCh can be debated, there is little doubt that EFS elicits increases in I_{SC} that are due in large part to ACh release. Thus, EFS-induced ΔI_{SC} are typically reduced by $\sim$50% in the presence of ATR (Cooke *et al*., 1983a, b; Cooke, 1984; Kuwahara *et al*., 1987a, b; Hildebrand and Brown, 1990; Chandan *et al*., 1991a, b; Javed and Cooke, 1992). Some investigators, based on the use of HEX, have implicated a small role for nAChR in the EFS response in whole thickness tissue (Hildebrand and Brown, 1990; Chandan *et al*., 1991a, b). Thus, the majority of reports indicate that changes in epithelial ion transport as a consequence of ACh release (mimicked *in vitro* by EFS or application of exogenous cholinomimetics) are via mAChRs and pharmacological analyses do, in general, support the dogma that this is via epithelial M_3 and neuronal M_1 mAChRs (Cooke *et al*., 1983a; Cooke, 1984; Carey *et al*., 1987; Kuwahara *et al*., 1987a, b; Diener *et al*., 1989; Traynor *et al*., 1991; Chandan *et al*., 1991a, b; Javed and Cooke, 1992; O'Malley *et al*., 1995; Przyborski and Levin, 1997; Townsend *et al*., 2005). Stimulation of nAChRs does affect intestinal I_{SC}, typically in full-thickness tissue (i.e. with an intact myenteric plexus), but the magnitude of the ΔI_{SC} is significantly smaller than that evoked by CCh or BCh (Zimmerman and Binder, 1983; Diener *et al*., 1989; Hildebrand and Brown, 1990; Chandan *et al*., 1991a, b; O'Malley *et al*., 1995; Sayer *et al*., 2002). This does not trivialize the importance of nAChR input to cholinergic control of epithelial ion transport, rather it highlights the fact that high concentrations of CCh, with its mixed mAChR and nAChR effects, are unlikely to reveal nAChR effects because these are over-run by the effects of mAChR ligation (Tapper *et al*., 1978; Sayer *et al*., 2002).

Surprisingly, cholinergically mediated ion transport is poorly understood in the mouse. In full-thickness jejunal tissue from suckling and adult mice (it is extremely difficult to strip away the outer muscle layers from the mouse gut), I_{SC} responses to EFS are partially mediated by mAChRs and CCh-induced ΔI_{SC} are TTX insensitive (Carey and Cooke, 1989; Sheldon *et al*., 1989). However, I_{SC} responses following application of the Na^+ channel activator veratridine, which stimulates enteric nerves pharmacologically, rather than electrically, do not involve muscarinic receptors (Sheldon *et al*., 1990). Dimethylphenylpiperazinium (DMPP) (i.e. nAChR agonist)-induced increases in I_{SC} in the jejunum are, in accordance with other species, neuronally driven (Sheldon *et al*., 1989). CCh- and BCh-evoked increases in I_{SC} in the mouse colon are mediated in part by nerves (Sagmanligil and Levin, 1993; Carew and Thorn, 2000), although subsequent studies failed to confirm this (Sayer *et al*., 2002). The latter study found that HEX did not significantly affect ΔI_{SC} evoked by CCh, but that nicotine evoked a drop in I_{SC} that was inhibited by TTX pretreatment. Furthermore, as a striking addition to the field, mice lacking M_3 mAChRs produce I_{SC} responses to CCh or BCh of normal magnitudes, with M_1 mAChRs implicated as the compensating mechanism for the lack of the M_3 receptor subtype (Haberberger *et al*., 2006; Hirota and McKay, 2006).

There is a dearth of information regarding cholinergic regulation of ion transport in the human intestine. In muscle-stripped segments of left colon, 25–50% of the ΔI_{SC} evoked by EFS is due to cholinergic input (Hubel *et al*., 1987; Kuwahara *et al*., 1989) and responses to CCh are completely abolished by ATR (Kuwahara *et al*., 1989).

Clearly, the lack of data obtained from human tissue needs to be addressed, as does a comparison of the applicability of findings from mouse tissue, particularly as the mouse is the species of choice to model human intestinal disease. Additionally, whereas cholinergic control of enteric epithelial ion transport is, under normal circumstances, dominated by M_3 and M_1 mAChRs (lack of involvement of M_2 and M_4 mAChR subtypes is generally inferred from pharmacological data, but has never been directly proven, and M_5 mAChR involvement has not been addressed), the modulating effect of nAChRs should be better characterized, as should cholinergic neuronal interactions within the submucosa and between the submucosal and myenteric plexuses. Finally, in segments of rabbit small intestine containing Peyers's patches, CCh evoked a decrease rather than an increase in I_{SC} (Brayden and Baird, 1994) and greater involvement of nAChR has been implicated in colonic tissue from young compared to mature guinea-pigs (Powell and Reddix, 2000); thus, whereas the majority of our understanding of the control of enteric epithelial ion transport has been gleaned from analyses of tissues from adult animals, there is value in considering tissue from infants/young animals and from those with an enhanced complement of immune cells (see below).

Intracellular epithelial effects of cholinergic agonists: influence on ion transport

Assessment of rat intestine has demonstrated that ACh-driven epithelial Cl^- secretion is Ca^{2+} dependent (Hardcastle *et al*., 1984), activating a basolateral K^+ efflux from the enterocyte, which is required for the subsequent increase in I_{SC} (Hardcastle and Hardcastle, 1986). Whole-cell patch-clamp recordings from rat and human colonic epithelial cells showed that ACh (and CCh) act through mAChRs to evoke membrane hyperpolarization, an effect that is dependent on the presence of Ca^{2+} (Yada and Okada, 1984; Yada *et al*., 1989; Devor *et al*., 1990; Bohme *et al*., 1991). This hyperpolarization is attributed to K^+ efflux from the basolateral cell membrane, which, in the human T84 colonic epithelial cell line, occurs independently of cAMP-stimulated K^+ efflux, indicating a separate Ca^{2+}-activated K^+ channel in these cells (McRoberts *et al*., 1985; Dharmsathaphorn and Pandol, 1986) (Figure 1). Furthermore, microelectrode recordings in the human colon-derived HT-29 epithelial cell line show that CCh-stimulated K^+ efflux immediately follows, but is not dependent on, epithelial cell depolarization, a phenomenon attributed to Cl^- efflux from both apical and basolateral membranes (Bajnath *et al*.,

1992a, b), although the functional significance of this finding is unclear. CCh-induced increases in I_{SC} have consistently been associated with serosal-to-mucosal movement of Cl^- across epithelial cell monolayers (Dharmsathaphorn and Pandol, 1986), and most, but not all, of the studies to date favour a CCh-driven apical Cl^- conductance in T84 cells (frequently used as a model of secretory epithelia) (Dharmsathaphorn and Pandol, 1986; Cliff and Frizzell, 1990; Devor *et al*., 1990; Venglarik *et al*., 1990).

cAMP-dependent secretagogues (e.g. VIP, PGE_2 and forskolin) increase apical Cl^- conductance via the cystic fibrosis transmembrane conductance regulator (CFTR) (Mandel *et al*., 1986) and potentiate the I_{SC} response to CCh in human colonic epithelial cell lines and freshly isolated human colonic tissue (Dharmsathaphorn and Pandol, 1986; Mall *et al*., 1998). The Cl^- conductances evoked by cAMP versus Ca^{2+} are not identical, thus suggesting the existence of two distinct Cl^- channels in human intestinal epithelium (Cliff and Frizzell, 1990; Vaandrager *et al*., 1991; Bajnath *et al*., 1992a), a postulate supported by molecular analyses (Mohammad-Panah *et al*., 2001; Evans *et al*., 2004). Collectively, these studies indicate that activation of basolateral M_3 mAChRs raises intracellular Ca^{2+}, resulting in Ca^{2+}-dependent basolateral K^+ efflux, which creates the driving force for flux of Cl^- into the gut lumen, observed in the Ussing chambers as an increase in I_{SC} (Kachintorn *et al*., 1993b) (Figure 1).

In human colonic epithelial cell monolayers, CCh stimulation induces turnover of membrane phospholipids (Kopp *et al*., 1989; Dickinson *et al*., 1992). Indeed, it has been elegantly demonstrated that inositol 3,4,5,6-tetrakisphosphate (IP_4) generation, subsequent to IP_3 production, contributes to the transient nature of the epithelial response to CCh, serving to turn off Cl^- secretion (Kachintorn *et al*., 1993a; Vajanaphanich *et al*., 1994). PKC appears to have a similar role in limiting the Cl^- secretory actions of CCh (Cohn, 1990; Kachintorn *et al*., 1992), although there is also evidence that at least one PKC isoform, PKCα, contributes initially to the stimulation of Cl^- secretion, while acting to inhibit this response in the longer run, possibly through inhibition of the basolateral Ca^{2+}-activated K^+ channel (Bajnath *et al*., 1992b; van den Berghe *et al*., 1992) (Figure 2). Analyses with the T84 cell line suggest that phosphorylation of the MAP kinases, extracellular signal-regulated kinase and p38, constitute additional 'off' signals for CCh-stimulated epithelial chloride secretion (Keely *et al*., 1998; Keely and Barrett, 2003). This occurs via transactivation of the epidermal growth factor receptor (EGFr) and requires release of transforming growth factor-α (TGF-α), elevations in intracellular Ca^{2+}, activation of calmodulin, and activation and association of Src kinase and PYK-2 with the EGFr (Uribe *et al*., 1996a; Keely *et al*., 2000; McCole *et al*., 2002). Interestingly, phospholipid production has also been linked to epidermal growth factor (EGF)-induced inhibition of Cl^- secretion (Uribe *et al*., 1996b). However, the phospholipid-generating effects of EGF are dependent on phosphatidylinositol 3-kinase, whereas those of CCh are not, demonstrating that mobilization of distinct intracellular signalling pathways can culminate in a similar physiological end point.

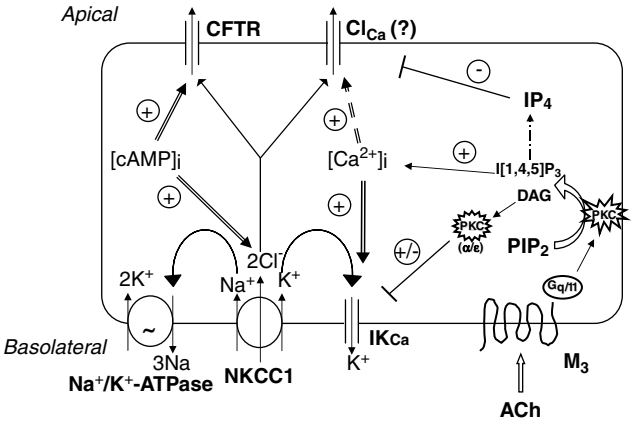

Figure 2 Intracellular regulation of intestinal epithelial Cl^- secretion by ACh (based on the secretory pathway depicted in Figure 1). $G_{q/11}$ α subunits (coupled to odd-numbered mAChRs) stimulate membrane phospholipid turnover by PLC. Inositol 1,4,5-trisphosphate (I[1,4,5]P_3) stimulates a rise in $[Ca^{2+}]_i$; eventual conversion of IP_3 products to IP_4 by phosphatidylinositol kinases turns off Cl^- secretion, possibly by inhibiting Ca^{2+}-activated Cl^- channels (Cl_{Ca}). DAG activates classical (e.g. PKCα) and novel (e.g. PKCε) isoforms of PKC; PKCα and/or PKCε may be required for initial stimulation of apical Cl^- secretion, but ultimately serve to downregulate this process through effects on ion transport molecules. Refer to the text for further details.

Cholinergic–non-cholinergic interactions: influence on ion transport

ACh does not exist in isolation and so it is not surprising that other signalling molecules from nerves, stromal or immune cells influence cholinergic regulation of epithelial ion transport. The opportunities for such interaction are immense given the diversity of biogenic amines, neuropeptides and neurotransmitters that exist in the gut (at least 25 in the enteric nervous system alone; McConalogue and Furness, 1994). As examples: in the guinea-pig small intestine, noradrenaline inhibits the secretory responses to cholinergic nerve stimulation and somatostatin inhibits ΔI_{SC} evoked by CCh and DMPP (Keast *et al*., 1986); opioid analogues can inhibit tonic ACh release from the rabbit ileal SMP (Hautefeuille *et al*., 1985); neuropeptide Y and the structurally related peptide YY reduce baseline I_{SC} and diminish BCh-induced ΔI_{SC} in mouse jejunum (Riviere *et al*., 1993; Nakanishi *et al*., 1996; McKay *et al*., 1996a); increases in I_{SC} evoked by substance P (SP) in guinea-pig jejunum are reduced in the presence of ATR, implying SP-induced release of ACh as a significant part of the Cl^- secretory response (Perdue *et al*., 1987; Goldhill and Angel, 1998). Products of arachidonic acid metabolism also influence cholinergic responses. Thus, the prosecretory effects of leukotrienes in the guinea-pig colon are partially dependent on the activity of mAChRs (Hammerbeck and Brown, 1993), PGD_2 antagonizes the effects of BCh and CCh on canine proximal colon (Rangachari and Betti, 1993), and blockade of PG synthesis with indomethacin reduces the I_{SC} response evoked by CCh by 70% in human descending colon (Mall *et al*., 1998).

The observation that other neurotransmitters modulate the cholinergic control of enteric epithelial ion transport is

intuitive, especially considering that most cholinergic nerves also synthesize and release other neurotransmitters/neuropeptides. Indeed, the implications of this cotransmission, occurring in the context of endogenous eicosanoids and cytokines (see below), for modulation of the cholinergic control of epithelial ion transport is immense, not to mention the effects of the temporal and stoichiometric relationship between the release of multiple mediators. Clearly, many outstanding issues remain: Do specific neurotransmitters simply oppose/antagonize the effect of ACh, and if so, how (e.g. activation of inhibitory neurons)? Are other neurotransmitters influencing the release of ACh under normal or pathophysiological conditions? Are specific neurotransmitters influencing expression or activity of ACh receptors or of subsequent intracellular signalling cascades? The answers to these questions will significantly enhance our understanding of the complexities of the cross-talk between the cholinergic system and other mediators in the control of enteric ion transport in health and disease.

Alterations to cholinergically mediated intestinal ion transport during pathology

Intestinal infection and inflammation profoundly effect water balance in the gut, leading to either secretory or malabsorptive diarrhoea or to constipation. However, and seemingly paradoxically, gut dysfunction is often characterized by a general hyporesponsiveness to prosecretory agents, including diminution of ΔI_{SC} evocable by cholinomimetics. Thus, tissue from diabetic rats (Perdue and Davison, 1988), rats exposed to 10 Gy radiation (Francois *et al*., 1998), rats infected with nematode parasites (Masson *et al*., 1996; Venkova and Greenwood-van Meerveld, 2006), mice with T-cell-driven enteropathies (McKay *et al*., 1999; Radojevic *et al*., 1999), aganglionic colon from children with Hirschsprung's disease (Hardy *et al*., 1993) and tissues from virtually every animal model of colitis (Goldhill *et al*., 1993; Kachur *et al*., 1995; Asfaha *et al*., 1999; Diaz-Granados *et al*., 2000; Miceli *et al*., 2002; Sanchez de Medina *et al*., 2002; Perez-Navarro *et al*., 2005a, b) display reduced I_{SC} responses to cholinomimetics. In some instances, such as pathologies accompanied by severe ulceration, such hyporesponsiveness, could simply reflect a lack of intact epithelium, although this is not an adequate explanation for most intestinal pathologies. Indeed, there is significant evidence implicating several specific mechanisms in the cholinergic hyporesponsiveness associated with intestinal infection and inflammation. In some instances, this involves altered ACh metabolism; for example, increased AChE expression and activity have been noted in the jejunal epithelium of rats infected with *Nippostrongylus brasiliensis* (Russell *et al*., 2000) and in aganglionic segments of rectosigmoid colon from children with Hirschsprung's disease (characterized by a lack of development of cholinergic enteric nerves in the hindgut) (Hardy *et al*., 1993). Tri-nitrobenzene sulphonic acid (TNBS)-induced colonic inflammation and infection with *Trichenella spiralis* both affect ACh metabolism in smooth muscle preparations as well, where decreased packaging and exocytosis of ACh from the myenteric plexus is observed (Main *et al*., 1993; Davis *et al*., 1998; Poli *et al*., 2001). In contrast, others have found either no change or an increase in intestinal ChAT expression (Palmer and Koch, 1995; Green *et al*., 2004), whereas increased levels of non-neuronal ACh have been reported in other (non-intestinal) inflammatory diseases (Wessler *et al*., 2003). It is possible that this hyporesponsiveness represents a means to limit ongoing Cl^- secretion during disease, but the full significance of these data is not clear. Moreover, should some commonality be identified in the mechanism responsible for the epithelial hyporesponsiveness to cholinomimetics; this could represent a significant advance in the development of therapies to regulate enteric water balance.

We have reported that I_{SC} responses to CCh in colonic tissue from mice with dextran sodium-sulphate (DSS)-induced colitis are not only attenuated, but reversed in direction (Sayer *et al*., 2002). The CCh-induced decrease in I_{SC} was abolished by pretreatment with TTX or inhibitors of inducible nitric oxide (NO) synthase and partially reversed by pretreatment with HEX; overall, the findings were compatible with a novel means of controlling epithelial ion transport via activation of neuronal nAChRs in the myenteric plexus leading to NO liberation from glial cells (Green *et al*., 2004). Although NO release can explain the CCh-induced drop in I_{SC}, it does not account for the lack of a CCh-induced increase in I_{SC} in colonic tissue from DSS-treated mice. We speculate that this deficiency is due to loss of mAChRs or inflammation-induced changes in epithelial Ca^{2+}-dependent secretory pathways, although proinflammatory cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) may actually increase expression of AChRs (Poea-Guyon *et al*., 2005) (see below).

The issue of direct regulation of cholinergic effects by cytokines has been taken up *in vitro* using CCh treatment of human colon-derived epithelial monolayers. Exposure to activated immune cells or recombinant IFN-γ results in diminished responses to CCh (Holmgren *et al*., 1989; Madara and Stafford, 1989; McKay *et al*., 1996b; Madsen *et al*., 1997; McKay and Singh, 1997) that have been linked to reduced expression of the Na^+/K^+-ATPase pump and the $Na^+/K^+/2Cl^-$ co-transporter (Sugi *et al*., 2001; Bertelsen *et al*., 2004), whereas TNF-α treatment appears to enhance the effect of CCh (Oprins *et al*., 2000, 2002). Treatment of colonic epithelial cells with IFN-γ leads to an increased production of TGF-α, which, through activation of the EGFr, as mentioned earlier, has been shown to inhibit Ca^{2+}-dependent Cl^- secretion (Uribe *et al*., 1996a, 2002). These studies only hint at the full influence inflammatory mediators undoubtly have on cholinergically mediated epithelial ion transport; the potential of other cytokines to alter cholinergic responses remains to be defined, and the number of cytokines/growth factors that occur constitutively or sporadically in the gut translates into a daunting myriad of possibilities. This area is especially fascinating in light of the reverse relationship, that is, the ability of the cholinergic pathway to alter cytokine responses. This relationship is designated as the cholinergic anti-inflammatory pathway, through which stimulation of the vagus nerve suppresses endotoxin-induced release of inflammatory mediators, in-

cluding TNF-α, from immune cells, an effect that is mediated by α7 nAChRs (Pavlov *et al*., 2003; Wang *et al*., 2003).

In vitro infection with the bacterial pathogen enterohemorrhagic *Escherichia coli* reduces CCh-induced secretory responses in T84 cell monolayers (Hecht and Koutsouris, 1999). Intriguingly, application of fatty acids has been postulated as another means of limiting CCh-induced Cl^- secretion (Schultheiss *et al*., 2001) and the gut commensal flora is the predominant source of fatty acids. Additionally, both viral and helminthic infections have been shown to reduce CCh-evoked ΔI_{SC} (Argenzio *et al*., 1996; Reardon *et al*., 2001). Finally, and of major health concern, diarrhoea resulting from *Vibrio cholerae* infection is potentiated by cholinergically mediated secretory events (Banks *et al*., 2004) that can be mobilized by serotonin release from enterochromaffin cells (Lundgren, 1998), which, in turn, at least in the duodenum, evokes ACh release (Tuo and Isenberg, 2003). Infections can have long-lasting effects on the host (e.g. post-infectious irritable bowel syndrome) (Farthing, 2005), and so awareness of any cholinergic contribution to the altered intestinal fluid status elicited by infections (Jodal *et al*., 1993) can be of value in not only providing a means of symptom relief/disease management but also in the evaluation of disease aetiology. These isolated examples simply illustrate the general lack of data on the impact of one of the major endogenous homeostatic control systems (i.e. the cholinergic system) on the duration of infection (and *vice versa*) and the physiological perturbations that they evoke.

Conclusion

ACh is an important regulator of the electrogenic ion fluxes that govern directional water movements in the intestine. Identification of ACh receptor subtypes involved in this process has not always been straightforward and this is partially attributable to a lack of pharmacological agonists and antagonists with high specificity for receptor subtypes. For the most part, however, it is generally accepted that cholinergic control of intestinal ion transport is accomplished predominantly via activation of M_1 and M_3 AChR on neurons and enterocytes, respectively. These fundamental pharmacological data can now be confirmed or refuted and supplemented by a molecular knockout strategy that has already been applied to the mouse and has revealed some intriguing and unexpected data (Wess, 2004; Haberberger *et al*., 2006; Hirota and McKay, 2006). Also, data from human and mouse tissue is noticeably scant; a particular concern in the context of modelling and treating human disease. Moreover, it is clear that infection and inflammation result in an altered enteric cholinergic system, a generalized hyporesponsiveness of the epithelium to cholinergic stimulation and unmasking of regulatory pathways that are either not operational, or go undetected, when tissues from healthy individuals are assessed. Furthermore, there is intriguing, but fragmented, data on how disease can uncouple mAChRs from intracellular signalling cascades and we have only a rudimentary knowledge of how other neuroimmune (and stromal)-derived factors influence the overall activity of cholinergic signalling in the gut and produce changes in active epithelial ion transport. The cholinergic system is a principal player in enteric homeostasis, and we suggest that the outstanding challenges in this field lie in unraveling the communication within the neuronal circuitry and the role of specific AChRs therein, in defining the mechanism(s) by which immune responses (i.e. disease) modulate the cholinergic system, and in assessing cholinergic signalling in the setting of a dynamic tissue in which other signalling molecules are released, either tonically or sporadically.

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Conflict of Interests

The authors state no conflict of interests.

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