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Review article

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MUSCARINIC RECEPTOR SUBTYPES IN THE ALIMENTARY TRACT

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Acetylcholine is a transmitter in preganglionic autonomic and postganglionic parasympathetic nerves and a non-neuronal paracrine mediator in the alimentary tract. Acetylcholine is involved in the control of almost any function within these organ systems, and almost every cell type expresses multiple muscarinic receptor subtypes. Although muscarinic receptors at non-neuronal effector cells commonly are of the M3 subtype, the population usually consists of a mixture of muscarinic receptor subtypes often co-acting postsynaptically. However, the pattern of heterogeneity of varies between different tissues. The population in gland parenchymal tissue often consists of a mixture of M1 and M3 receptors, smooth muscle tissue of the gut of M2 and M3, blood vessels of M1, M3, M4 and M5 and neuronal cells of M1 and M4. Nitric oxide production, effects on inflammation and proliferation may involve M1, M3 and M5 receptors. Muscarinic receptors expressed on nerve terminals may indirectly modulate the responses by inhibition or facilitation of neuronal transmission in the autonomic nervous system. The present review describes signalling mechanisms, expression and functional effects of muscarinic receptors in salivary glands and in the gastrointestinal tract.

Keywords: *muscarinic receptor, secretion, vasodilatation, contraction, salivary glands, gastrointestinal tract*

INTRODUCTION

Muscarinic receptors are commonly expressed in the digestive tract and are of utter significance for organ function (1-3). The tissues and cell types expressing the receptors are numerous and include salivary glands, smooth muscle and mucosal cells in the stomach and the intestine. Orthodoxy, peripheral muscarinic receptors were regarded as a homogeneous receptor group evoking either smooth muscle contraction or glandular secretion. Today the muscarinic receptors are considered to comprise five subtypes - muscarinic M1, M2, M3, M4 and M5 receptors (4, 5). The intronless genes encoding the receptor subtypes have been cloned from several species and show a high sequence homology of the subtypes in all species so far examined (6-8).

Originally, the muscarinic receptors mediating the metabotropic effects of acetylcholine at non-neuronal effector cells were thought to be of the muscarinic M3 receptor subtype (9, 10). Although it has been well recognised for a long period of time that other subtypes of the receptor can be found on glandular as well as on smooth muscle cells when examined morphologically, the functional significance of the different receptor subtypes has not been fully unravelled. The subtypes of the receptor population interact on neuronal as well as on non-neuronal cells in regulation of autonomic responses (11, 12). However, lately muscarinic receptors have been suggested to be implicated in the control of inflammation, cell growth and proliferation also (13-18).

MUSCARINIC RECEPTOR SUBTYPES

Muscarinic receptors belong to the family of G-protein-coupled receptors. The G-proteins are heterotrimeric guanine nucleotide-binding proteins that regulate second messengers and ion channels (19). They consist of one α-, β- and γ-subunit, and on the basis of the α-subunits primary sequence homology, the G-proteins are characterized into Gαs, Gαi/o, Gαq and Gα12 (20). Receptor activation splits the heterotrimeric G-protein into α- and β/γ-subunits, of which the Gα subunits primarily regulate intracellular responses. The subunits of G-proteins activate distinct cellular pathways and the muscarinic receptor subtypes couple differentially to the G-proteins. Whereas muscarinic M2 and M4 receptors preferentially couple to Gαi/o, muscarinic M1, M3 and M5 receptors couple to Gαq/11. The Gβγ subunit is a pathway by which at least the muscarinic M2 receptor, in addition to the M3-receptor, may activate phospholipase Cβ and modulate ionic conductances (21). Also, muscarinic M2 and M4 receptors may inhibit adenylate cyclase activity, prolong the opening of potassium as well as that of non-selective cation channels and of transient receptor potential channels (22). Muscarinic M1, M3 and M5 receptors, on the other hand, increase intracellular calcium by mobilizing phosphoinositides that generate inositol 1,4,5-trisphosphate (InsP3) and 1,2-diacylglycerol (DAG (23, 24)). However, the muscarinic M1, M3 and M5 receptors differ in their coupling to Gsα. While the muscarinic M5 receptor activates down-stream enzymes less efficiently than the muscarinic M3 receptor, the M3 receptor activates the G-protein less efficiently than the muscarinic M1 receptor (25). Even so, all three subtypes have in common a production of InsP3 and DAG as a result of the activation of phosphoinositide-specific phospholipase Cβ. In addition, the muscarinic receptors regulate a number of other signalling pathways that appear to participate in muscarinic receptor control of inflammation, cell growth and proliferation (23). These pathways include both Gαi/o- and Gαq/11-coupled molecules, but may also involve the Ras homology family of small GTPases (RhoA; (26, 27)). RhoA may mediate inhibitory effects on myosin phosphatase, involving Rho-kinase and a myosin phosphatase inhibitor phosphoprotein (protein kinase C (PKC) potentiated inhibitor protein-17 kDa; CPI-17) (28-30). Furthermore, phosphoinositide-3 kinases, non-receptor tyrosine kinases and mitogen-activated protein (MAP) kinases (extracellular-signal-related kinase 1 and 2; ERK1, ERK2) have been discussed in the context of muscarinic receptor intracellular mechanisms (31-33). A pathway for activation of RhoA and the transcription factor, serum response factor, involving tyrosine kinases has been suggested to be activated uniquely by muscarinic M1 receptors and not to be shared by muscarinic M3 receptors (34). A signalling molecule activated by muscarinic receptors is sphingosine kinase (35). Sphingosine kinase metabolises sphingosine into sphingosine-1-phosphate (S1P) and the intracellular S1P then mediates rise in intracellular calcium. S1P may be a factor linking calcium store depletion to downstream calcium entry (36-38). *Fig. 1* indicates possible pathways by which muscarinic receptors may interact in inducing physiological, inflammatory and proliferatory responses.

Neuronal muscarinic receptors

Neuronal muscarinic receptors are widely expressed in the peripheral nervous systems (5, 39, 40). While antagonists with selectivity on M2/M4 over M1/M3/M5 receptors increases cholinergic overflow by reducing autoreceptor inhibitory function, antagonists with the reversed selectivity profile may decrease overflow, thus reflecting blockade of facilitator receptors (1, 12). The prejunctional inhibitory muscarinic receptor was for long considered to be of the M2 subtype (41-44). In some organs, binding studies have indicated the best correlation to the muscarinic M4 receptor and not the muscarinic M2 receptor (45-48). The same conclusions have been made out of morphological and functional observations in salivary glands (49). Furthermore, facilitator muscarinic receptors have been reported in salivary glands, in the urinary bladder and in the gastrointestinal tract (50-54). Thus, the presence of two distinct subtypes on prejunctional terminals is consistent with inhibitory/facilitator autoreceptor roles in several peripheral tissues, including smooth muscle and glandular tissue (55, 56).

Presynaptic muscarinic M2/(4) receptors may affect neuronal function *via* a G-protein-linked pathway, by which terminal K^+ conductance could be increased and thereby indirectly limiting presynaptic depolarization and Ca^{2+} influx necessary for release (57-59). Muscarinic M1 receptors have in a similar way been shown to cause a slow membrane depolarization *via* inhibition of K^+ currents (17, 60). However, both muscarinic M1 and M2 receptor subtypes could alternatively modulate transmission *via* non-Ca^{2+}, non-K^+ channel-linked mechanisms (61, 62). Accordingly, the receptors may modulate transmitter release by coupling to serine-threonine kinases PKC and protein kinase A (PKA (63, 64)). Both kinases seem to stimulate transmitter release, though in normal synaptic function only PKA is active. When the balance of the two receptors is altered, that is between muscarinic inhibitory M2/M4

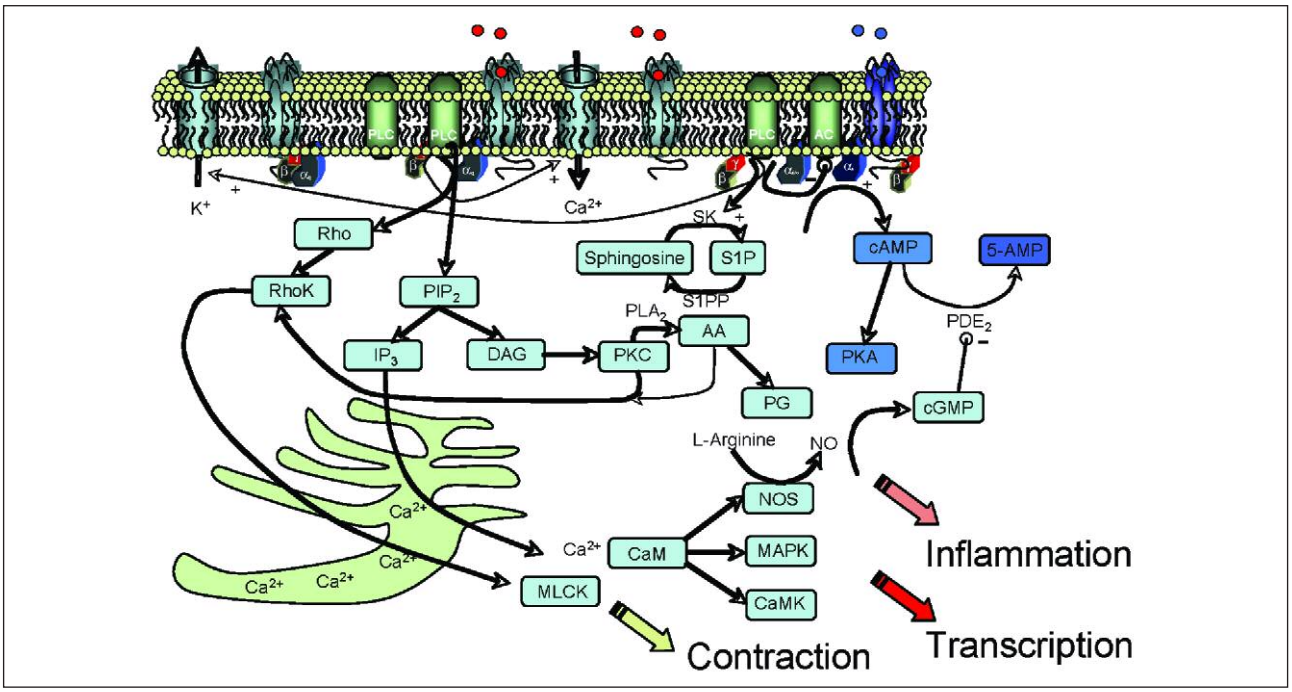

Fig. 1. Principal intracellular pathways for excitatory (M1/3/5; *via* Rho, PIP2 or S1P) and inhibitory (M2/4; *via* K^+ or AC inhibition) muscarinic receptors. The figure indicates possible inflammatory (PG, NO), proliferatory (MAPK) and contractile (MLCK, CaM; indirect *via* cAMP inhibition) effects. Stimulatory effects are indicated by arrows and inhibitory effects by lines with a round ending.

and facilitator M1 receptors, a M1-mediated increased PKC activity-dependent potentiation of release or an M2-mediated decreased PKA activity-dependent reduction of release may occur (64). Under normal conditions, the prejunctional muscarinic receptors seem to perform an inhibitory function on the release.

Non-neuronal muscarinic receptors

1. Physiological effects

It has generally been agreed that muscarinic M3 receptors mediate most postjunctional effects. In the digestive and lower urinary tracts they evoke contraction of smooth muscle and secretion from glands (65-67). However, postjunctional muscarinic M2 receptors also occur, commonly co-localised with the muscarinic M3 receptor. Studies in several species, including man, indicate synergistic effects of M2 and M3 receptors in controlling smooth muscle contraction (68, 69). Inhibitory G-proteins activated by muscarinic receptor agonists may modulate calcium-acvitated K^+-channels in the smooth muscle cells, which counteract any hyperpolarising stimulus (70). Inhibitory muscarinic M2 receptors may also open non-selective cation channels by which a sustained influx of sodium and calcium ions occurs, and further, inhibit adenylate cyclase activity. However, this effect of muscarinic M2 receptors seems to require a concomitant stimulation (*e.g.,* by M3-receptors) of the InsP3-pathway and the intracellular release of calcium (71). Also, the regulation of ion fluxes may involve synergistic effects *via* the TRPC-encoded (transient receptors potential canonical) proteins and calcium permeable cation channels (22, 72).

Possible interactions between muscarinic M2 and M3 receptors may also occur by other intracellular mechanisms concerning contractile effects. In smooth muscle, muscarinic M2 (and possibly M3) receptors may increase S1P and by that activating store-operating Ca^{2+}-channels (35, 73). In the presence of specific inhibitors of sphingosine kinase, muscarinic-induced contraction can be attenuated in smooth muscle preparations (38). Muscarinic M2 receptors also affect the contractile smooth muscle response *via* activation of RhoA. This results in a calcium sensitisation enhancing smooth muscle contraction. Moreover, muscarinic M2 receptors may activate cation channels and thereby increasing $[Ca^{2+}]_i$ (74-76). Sakamoto *et al*. demonstrated three distinct pathways mediating a muscarinic cationic current (MI_{Cat}) generation. Either of M2 and M3 receptors activates two of these pathways, whereas the third requires both M2 and M3 receptors to be active. The M2/M3 pathway was the major mediator of whole-cell MI_{Cat} and potently depolarized the membrane. The definition of a M2/M3 pathway is consistent with the existence of a signalling complex involving the M2-G_o system, the M3-PLC system and a cationic channel system mediating MI_{Cat}.

In glandular tissues, synergistic effects between muscarinic M1 and M3 receptors occur and activation of both subtypes of receptor may be a pre-requisite for maximum responses in salivary glands (77). Although the muscarinic M1 and the M3 receptors show resemblance according to intracellular pathway activation, differences occur (34, 78, 79). To examplify, TRPC-encoded proteins are implicated in glandular secretory responses (80, 81) and activation of TRPC6 channels is correlated with the formation of a multiprotein complex including muscarinic M1 receptors and PKC (82). Even though all the subtypes of excitatory muscarinic receptors increase $[Ca^{2+}]_i$ (23), they seem to affect Ca^{2+} channels differently; *i.e.,* muscarinic M3 and M5 receptors activate T-type calcium channel, which muscarinic M1 receptors do not (83).

Inhibitory muscarinic receptor intracellular pathways could possible evoke smooth muscle relaxations directly. However, muscarinic receptors usually evokes relaxation indirectly *via* paracrine substances, such as nitric oxide (NO) and prostaglandins (84-91). The effects *via* NO can be exerted by induction of different isoforms of NO synthases (92-98). Muscarinic M1, M3 and M5 receptors evoke NO formation Ca^{2+}-dependently *via* soluble guanylyl cyclase and cyclic guanosine monophosphate (cGMP) (19). However, not only the smooth muscle function may be affected by muscarinic stimulation of NO generation, but also glandular secretion (99-106).

2. Pathophysiological effects. Inflammation, cell growth and proliferation

Acetylcholine has been shown to mediate effects influencing inflammation within different organs (107, 108). So, have muscarinic M3 receptors been reported to induce release of prostanoids and inflammatory mediators from epithelial cells (*e.g.,* phospholipase A2 activation and prostaglandin E2 release) (109), muscarinic M1 receptors to stimulate neutrophil and monocyte chemotactic activity (110) and muscarinic M3 and M5 receptors to stimulate differentiation of cultured inflammatory cells into monocytic/macrophagic cells (111). The role of muscarinic receptor effects is ambiguous according to inflammation. While pro-inflammatory effects, such as increase in the release prostanoids, may be stimulated by acetylcholine, inhibition has been shown according to other, such as tumour necrosis factor (TNF) (108). Muscarinic receptors seem to participate in remodelling processes known to occur in chronic inflammatory diseases (112). In cancer cells, the muscarinic M3 receptor has been linked to cellular proliferation (113, 114) and acetylcholine seems, at least partly *via* muscarinic receptors, to be involved in the control of epithelial cell adhesion, cell–cell interactions and proliferation of epithelial cells (115-121). Muscarinic M1, M3 and M5 receptors may all posses inhibition of apoptopic cell death (122). In addition to the inflammatory and proliferatory effects by muscarinic M2 receptors involving S1P (38), the receptor may induce promotor mutagenesis by a signal transducer and activator of transcription element (123). Even though muscarinic receptor stimulation by its own causes no proliferation, it may induce cell growth and proliferation when acting together with other stimuli. So, together with either epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), which mediate proliferative stimuli themselves, muscarinic stimulation enhances the proliferative effect (124, 125). A transactivation of EGF regulatory pathways has been suggested for acetylcholine (126) and both muscarinic M1, M2 and M3 receptors have been shown to be possible candidates for exerting the effect (127-129).

SALIVARY GLANDS

The secretion from salivary glands fulfils different functions such as rinsing, protection including antimicrobial and moistening functions, remineralisation and also digestion. The regulation of the amount and quality of saliva is dependent on constant fluid delivery provided by the blood flow and on the type of stimulation of glandular activity. Muscarinic receptors play a key role in most events in salivary glands and the involvement of muscarinic receptors in the control of these different events is discussed below.

Salivary gland innervation

The control of salivary secretion depends mainly on nerve reflex impulses that involve the parasympathetic and sympathetic secretomotor and vascular nerves and the autonomic nerves reach most cell types in salivary glands (100, 130-132). While parasympathetic activity evokes a copious secretion relatively poor in protein, activity within the sympathetic innervation evokes sparse but protein-rich saliva. Secretion and blood flow are thus controlled by acetylcholine and noradrenaline but are also regulated by neuropeptides, such as vasoactive intestinal peptide (VIP; reviewed in (133, 134)). Vasodilatation in the salivary glands caused by acetylcholine and VIP release from parasympathetic nerves (135-137) also involves NO modulator mechanisms and possibly other endothelium-derived hyperpolarizing factors (87, 138-140). Blood flow is not a secretion-limiting

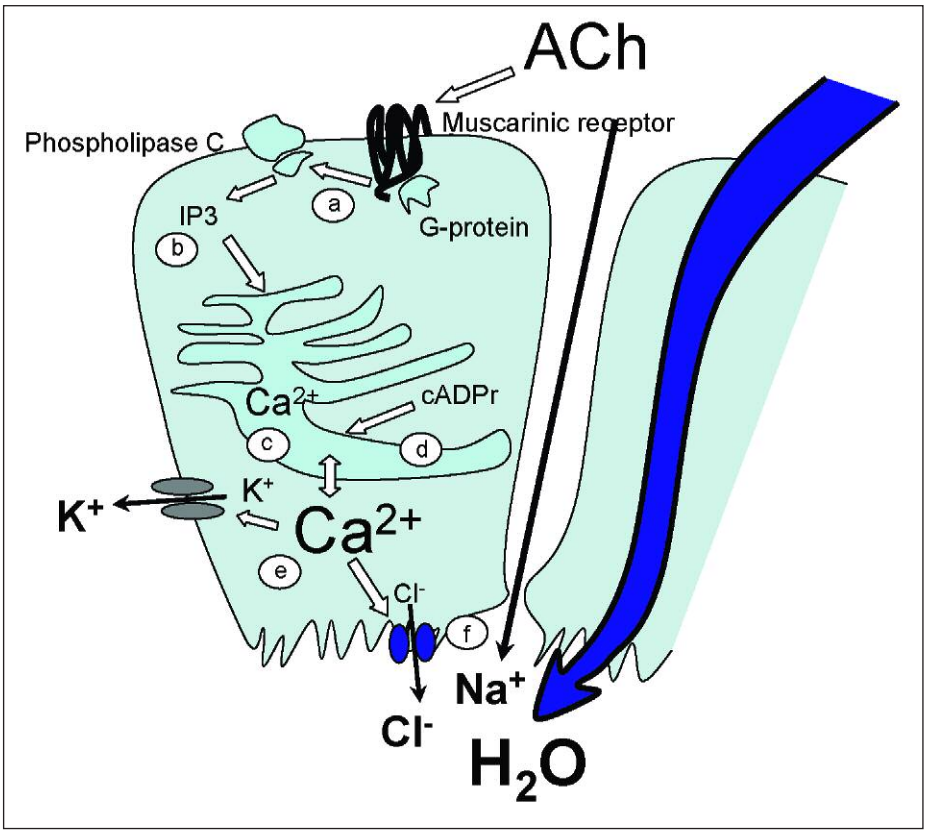

Fig. 2. Acetylcholine control of fluid secretion in salivary acinar cells. Acetylcholine (ACh) binds to the G protein–linked M3 muscarinic ACh receptor (a), which causes phospholipase C to generate inositol 1,4,5-trisphosphate (InP3) (b). InP3 binds to and opens the InP3 receptor on the endoplasmic reticulum, which releases Ca^{2+} (c). This release of Ca^{2+} stimulates Ca^{2+} induced Ca^{2+} release *via* the InP3 receptor and the ryanodine receptor (d; cADPr cyclic ADP ribose). Increased $[Ca^{2+}]_i$ activates the apical membrane Cl^- channel (e) and the basolateral K^+ channel. Efflux of Cl^- into the acinar lumen draws Na^+ across the cells, and the osmotic gradient generates fluid secretion (f).

factor initially, since the interstitial fluid will preserve the response (141, 142). However, in short, because of increase in intravascular oncotic pressure, salivation will cease unless the blood flow increases (143, 144).

Salivary gland secretion

The increase in salivary flow evoked by muscarinic agonists has generally been attributed to activation of muscarinic receptors solely of the M3 subtype (9, 145). This concept has been supported by findings obtained in studies using subtype-specific antisera as well as by functional studies on rat parotid glands (146) and in a parotid cell line PAR-5 (147). However, binding and molecular experiments on rat, ferret and ovine submandibular glands indicate the expression of muscarinic M1 receptors, occasionally accompanied by muscarinic M5 receptors, in addition to the M3 receptors (148-153). The same observations have been made in human labial glands (154, 155). Functional significance of muscarinic M1 receptors for the secretory response has been reported, *in vivo* as well as *in vitro*, in the rabbit and ovine submandibular gland (49, 156), and in the rat sublingual (77, 102) and submandibular glands (102, 157). In this latter gland, muscarinic M5 receptors seem to contribute as well. Results from the rat sublingual and ovine submandibular glands indicate that concomitant activation of the different muscarinic receptor subtypes (M1 and M3) are a necessity for glandular maximum responses (49, 77), but the muscarinic M1 receptor seems to be of particular significance at low intensity of stimulation (49). Studies of knockout-mice support the observation that both M1 and M3 receptors contribute to the secretion evoked by cholinergic stimulation (158, 159). As mentioned, muscarinic M1 and M3 receptors generate InsP3 and causes calcium release from the endoplasmic reticulum inducing the secretory process (145); *Fig. 2* indicates intracellular mechanisms in the secretion. However, diverse cellular effects by the two receptors are indicated by findings in muscarinic receptor knockout-mice. Here muscarinic M1 receptor-induced calcium signalling seems not to occur ubiquitously in submandibular acinar cells, whereas M3 receptor signalling seems to do (159). Experiments on mice knockouts suggest that muscarinic M4 and M5 receptors contribute to secretion also (160, 161). Thus, other subtypes of the muscarinic receptor than the principal secretory M3 subtype contribute to the response.

Nerve transmission in the parasympathetic innervation of salivary glands may be modulated by prejunctional muscarinic receptors (54, 156, 162). In rat salivary glands, muscarinic M1 receptors normally facilitate transmitter release during short, intense nerve activity. At low frequencies, on the

other hand, muscarinic M2 receptors, or possibly muscarinic M4 receptors (49), inhibit cholinergic as well as peptidergic transmission, but only after some delay. Furthermore, it was first described in the feline submandibular gland that stimulation of the parasympathetic innervation in a burst pattern at high frequencies causes a conspicuous enhancement of vasodilatation and secretion in comparison with continuous stimulation (163, 164). These observations have subsequently been confirmed in salivary glands of other species (54, 162, 165, 166). The phenomenon has been attributed to the release of neuropeptides, which preferentially occurs at high stimulation frequencies (164, 165), and to a short-lasting stimulation activating prejunctional facilitator and not inhibitory receptor mechanisms (54, 162). The impact of prejunctional inhibitory muscarinic receptors can be elucidated by the fact that blockade of muscarinic autoreceptors may increase fluid responses to auriculotemporal stimulation in the rat by 200% (54).

In the autoimmune disease Sjogren's syndrome that affects salivary and lacrimal glands, a significant characteristic is salivary gland hypofunction causing xerostomia and severe effects on the oral health (167, 168). Autoantibodies against muscarinic receptors have been suggested in the disease etiology (169-171) and in animal models, such antibodies have been shown to inhibit secretion (172). Interestingly, the acinar expression of M3 receptors is increased in Sjogren's syndrome (173) and this has been suggested to be an effect of long-term receptor blockade (174, 175). The up-regulation seems also to include the expressions of muscarinic M4 and in particular M5 receptors (155). This latter kind of subtype has been observed to be up-regulated in states of inflammation (96).

Salivary gland blood flow

While acetylcholine acting on muscarinic receptors is the principal stimulation for evoking fluid responses in salivary glands irrespectively of stimulation intensity (176), the cholinergic component has the greatest impact on the vasodilator response at low frequency stimulation (134, 177, 178). All five muscarinic receptor subtypes except the M2 have been described in vascular beds of salivary glands (49, 155), but the specific muscarinic receptor mediating vasodilatation in salivary glands has not been fully characterized. In glands of rats and sheep, endothelial cells express mainly muscarinic M1 and possibly M3 and M4 receptors, while vascular smooth muscle cells express M3 receptors (49, 155, 179). Muscarinic M5 receptors also occur, however, non-ubiquitously distributed both in the endothelium and in the smooth muscle layer. Glandular veins differ from arteries. While glandular veins express muscarinic M1 receptors in the smooth muscle, glandular arteries do not.

In the rat parotid gland, cholinergic vasodilatation is mediated, at least in part, by muscarinic M3 receptors (54). In the rat submandibular gland, muscarinic M3 and M1 receptors seem to mediate the cholinergic vasodilation (179), of which a large part is NO- and endothelium-dependent (139). In the ovine submandibular gland, morphological and functional findings indicate a possible muscarinic M5 receptor involvement beside the functional muscarinic M3

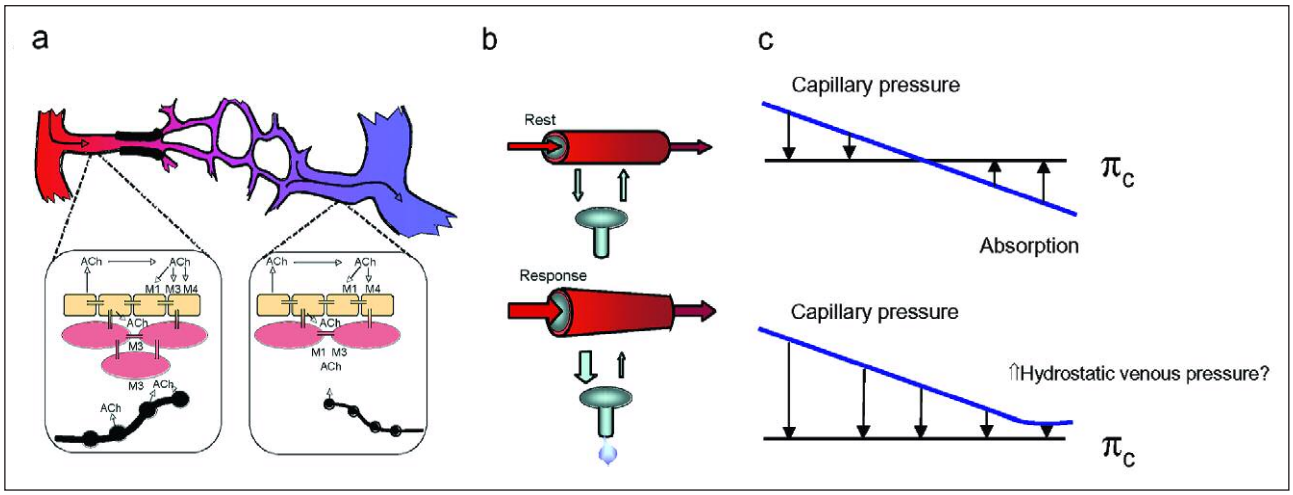

Fig. 3. Acetylcholine control of blood flow in a salivary gland. The schematic drawing indicates an intrinsic, intimal cholinergic system responding to local, luminal stimuli and an extrinsic, adventitial cholinergic system activated by perivascular nerve fibres (a), overall effect on glandular vessels during rest and response (b) and balance between hydrostatic and oncotic (π_C) pressures during rest (upper panel) and response (lower panel; c).

receptor (49). A greater inhibitory effect of muscarinic antagonists on methacholine-induced than on parasympathetic nerve-evoked vasodilatation, could possibly be interpreted in favour of an intrinsic regulatory system (49, 155) (*Fig. 3*). Acetylcholine mostly evokes vasodilatation, at least in arterial blood vessels. In the venous vasculature of some organs, the transmitter may evoke constriction (180, 181), and in rat submandibular veins muscarinic M1 receptors evoke such a response (179). Muscarinic M1 receptors have been suggested to support extravasation by raising the venular hydrostatic pressure by an autocrine cholinergic mechanism (179). The extravasation has been suggested to be supported by myoepithelial cell function also. When the myoepithelial cells are stimulated, possibly induced by muscarinic stimulation (132), the tissue surrounding the cells undergoes conformation, and by the low compliance of the gland, the interstitial fluid pressure is reduced (182). Therefore, venous muscarinic M1 receptors may be of particular significance in spontaneous secreting glands, in which myoepithelial cells are not active (183).

Inevitably, electrical stimulation of the parasympathetic nerve at low intensity induces vasodilatation that is largely dependent on acetylcholine (49, 87, 178). In view of the absence of reports visualizing cholinergic nerve fibres in proximity to the endothelium, the cholinergic nerve-evoked influence has been a matter of debate. However, data have accrued over recent years that other sources of acetylcholine exist besides the neuronal (117, 184, 185) and also from endothelial cells of blood vessel (186). These findings, and that all essential elements of the cholinergic system (choline acetyltranseferase (ChAT) and vesicular acetylcholine transporter) exist in the endothelium (187), could thus indicate an indirect parasympathetic vascular regulation *via* a non-neuronal origin of acetylcholine. Kummer and Haberberger (186) suggested, based on immunohistochemical, biochemical and functional studies, two separate cholinergic systems in the arterial vascular wall. One, an intrinsic, intimal cholinergic system serves as a regulator of basal vascular tone responding to local, luminal stimuli, whereas the other, the perivascular nerve fibres, *i.e.,* the extrinsic, adventitial cholinergic system, acts on top of this basal tone by providing fine tuning in response to reflex activation due to systemic demands (see *Fig. 3a*). Parasympathetic denervation reduces ChAT activity in salivary glands (by 95% in parotids) (188), which would indicate very low amounts of non-neuronal origin of acetylcholine in the gland and that the synthesis occurs in extraglandular vessels.

THE GASTROINTESTINAL TRACT

The gastrointestinal tract is provided with several different types of cells to fulfil its function to digest and absorb nutrients. To exemplify, the acid secretion of the stomach represents a potential threat to the epithelial layers of the stomach and the duodenum. This threat is minimized by a release of mucus and bicarbonate ions from specialized cells. The involvement of muscarinic receptors in the nervous control of these different cells is reviewed below.

Gastric acid secretion

Acid secretion from the parietal cell represents a major function of the stomach. The secretion is controlled in a complex manner by at least three different gastric cells, the enterochromaffinlike (ECL) cells producing histamine, the G cell releasing gastrin and the D cell releasing somatostatin. Furthermore, there exists a cholinergic vagal control, which is exerted on all the mentioned cells as well as directly on the parietal cell. Histamine, gastrin and vagally released acetylcholine influence directly the production of acid from the parietal cells. Somatostatin inhibits gastrin and histamine release and, hence, acid production.

Muscarinic receptors exist on all the three mentioned endocrine cells and can functionally stimulate G cells and inhibit D cells secretion (189-191). With regard to the ECL cells acetylcholine causes the release of histamine (192-194). However, whereas all ECL cells respond to gastrin with histamine release, only 10-30% of the cells respond to acetylcholine. It may reflect that only a part of the ECL cell population is vagally innervated. Functional data suggest the muscarinic receptor to be of the M1 subtype (195). Nevertheless, a muscarinic receptor activation by the release of acetylcholine from vagal nerves thus mainly leads to release of gastrin and inhibition of somatostatin release, which together with the direct muscarinic effect on the parietal cell, increase gastric acid production.

Muscarinic receptors located on the parietal cells and mediating acid secretion are of the muscarinic M3 subtype possibly accompanied by the M5 subtype (196-198). The intracellular second messenger system mediating the cholinergic effect is of the “classical” type, *i.e.*, an activation of phospholipase C and subsequent formation of InsP3 and DAG (see above). It seems less likely that the muscarinic receptors of the D cells are of the M3 subtype, since muscarinic influence on this cell inhibits the exocytosis of somatostatin. The receptor subtype involved in this muscarinic

response has not been the subject of any detailed investigation. Sachs *et al.* (194) proposed the muscarinic receptor involved to be of the muscarinic M2 or M4 receptor subtype. The involvement of muscarinic M3 receptors in gastric acid secretion has for instance been investigated in knock-out mice (199). These animals exhibited the expected attenuation of acid secretion in response to acetylcholine. Furthermore, the mice had high plasma gastrin levels. Despite of the mucosal hypertrophy not being evident, it was suggested that the trophic effects of gastrin was mediated *via* a muscarinic M3 receptor.

Gastric pepsinogen secretion

The chief cells, located in the crypts of the gastric corpus, produce a proteolytic proenzyme, pepsinogen. In an acid environment pepsinogen is activated to pepsin by the spontaneous cleavage of a small N-terminal fragment. Pepsin is important for the breakdown of protein in the ingested food. The secretion of pepsinogen is controlled, among other things, by a cholinergic, nervous influence. Vagal fibers as well as enteric nerves release acetylcholine to activate muscarinic receptors of the M1 and M3 type on the chief cells (200, 201). However, observations indicate that stimulation of vagal cholinergic nerves alone cannot evoke a pepsinogen release. Only in the presence of an acid gastric content will vagal activation lead to an enzyme release (202). In most cases the nervously evoked release of pepsinogen is accompanied by an increased gastric acid secretion.

Gastrointestinal smooth muscles

The autonomic nervous system was earlier believed to directly control the gastrointestinal smooth muscles *via* a release of neurotransmitter *en passage* from the vesicles contained in the nervous varicosities of an autonomic ground plexus. The change of membrane potential evoked by the released transmitter was proposed to spread to other smooth muscle cells *via* low resistant intercellular bridges (gap junctions). This has turned out to be a simplified description of the mechanisms for gastrointestinal neuromuscular control (203). Another type of cells, the interstitial cells of Cajal (ICC), has been shown to play a crucial role in the nervous control of motility, not the least for the muscarinic control (204). ICC are partly located between the circular and longitudinal muscle layers at the level of the myenteric plexus. This part of ICC is named ICC-MY. Other ICC, named ICC-IM, are located within the gastric smooth muscle layer in an intimate relationship with enteric nerve terminals. In the small intestine the ICC are also located at the deep muscular plexus, named ICC-DMP, which seems to correspond to ICC-IM in the stomach. Ultrastructural and biochemical studies have demonstrated synapse-like specializations (so called membrane densifications) between enteric nerve terminals and ICC-IM/ICC-DMP (205). In all probability, these structures mediate the nervous influence on ICC and, thus, the nervous motility control. They are localized between enteric nerves and ICC, but not between enteric nerves and smooth muscle cells.

ICC are of importance in integrating intestinal motor responses such as peristalsis. The propulsive effect of this rather complex nervous motor reflex is evoked by a contraction at the site of the food bolus and a relaxation distal to it. ICC-IM and ICC-DMP are essential for the cholinergic and tachykinin excitatory motor control of gastrointestinal smooth muscles and seem also to be provided with receptors for VIP and to be sensitive to NO, the inhibitory transmitters of peristalsis (206, 207). A picture thus emerges in which the receptors located on ICC-IM/ICC-DMP may be more important than the receptors at the cell membrane of the smooth muscle cells. Thus, ICC integrate on the gastrointestinal smooth muscles the ongoing influence of the different neurotransmitters underlying peristalsis.

The importance of ICC for the cholinergic motility control has been studied in some detail. Using W/W^v mice lacking ICC in the gastric muscle layer, it was convincingly demonstrated that cholinergic control of gastrointestinal smooth muscles cannot occur in the absence of ICC (206, 208). Similar results were obtained when disrupting ICC-DMP by treating neonatal rats with antibodies to Kit, a tyrosine kinase receptor (209, 210). The subtype of muscarinic receptor involved in the contractile response of the gastrointestinal smooth muscle has been investigated both at the mRNA and protein level. The studies revealed that both M2 and M3 receptors are present (211, 212). Furthermore, activation of muscarinic M1 receptors evokes a gastric smooth muscle relaxation in M3 knock-out mice *via* a NO-mediated mechanism (213, 214).

Muscarinic receptors are also indirectly involved in the sympathetic control of gastrointestinal smooth muscles (215). Early physiological studies indicated that the gastrointestinal muscle layers were innervated by parasympathetic cholinergic excitatory and sympathetic adrenergic inhibitory nerve fibers. By the use of histochemical studies, developed around the middle of the 20th century, it was shown that the direct sympathetic innervation of

gastrointestinal smooth muscles was very scarce (216-218). Most of the adrenergic nerve fibers made contact with the neurons of the myenteric and submucous plexuses. Hence, a pre-requisite for a sympathetic inhibitory influence on gastrointestinal smooth muscle function is that there is an ongoing activity in cholinergic parasympathetic neurons influencing muscarinic receptors.

Gastrointestinal bicarbonate secretion

The gastroduodenal epithelium secretes bicarbonate ions, which together with mucus form an alkaline layer of great importance for the protection of the epithelium from the acid contents in the stomach and in the duodenum. A major part of the studies reviewed below was performed on the duodenal bicarbonate secretion. The presence of a muscarinic receptor control of bicarbonate secretion has been demonstrated repeatedly. Intravascularly or subcutaneously administered muscarinic agonists increase bicarbonate release into the intestinal lumen (219) a response blocked by muscarinic antagonists (218, 220). Furthermore, giving atropine during "resting" conditions attenuated bicarbonate secretion in most studies (221, 222), indicating an ongoing "background" muscarinic activation of bicarbonate secretion. Most of the studies of the nervous bicarbonate control have been performed in three different experimental situations. First, electrical stimulation of vagal fibers augments bicarbonate secretion (221). Muscarinic receptors may be involved in this response but the experimental evidence is contradictory. Jonson *et al.* (222) failed to influence the vagal effect with atropine, whereas Glad *et al.* (220, 223-225) reported an inhibition. Second, bicarbonate secretion both in the stomach and in the duodenum is increased after sham feeding (223). There are conflicting results as to what extent this response is mediated by muscarinic receptors. According to Forssell *et al*. (220) the sham feeding response is diminished by a muscarinic receptor antagonist. Ballesteros *et al.* (220) failed to confirm this observation. Both studies were performed on humans. Third, exposing the duodenal mucosa to acid evokes a nervously mediated bicarbonate secretion. This is not influenced by atropine (219). To summarize, there is a nervous control of bicarbonate secretion but the experimental evidence for an involvement of muscarinic receptors is not yet clearly demonstrated.

Three reports have attempted to determine which type of muscarinic receptor that is localized to the bicarbonate secreting cells utilizing different pharmacological blockers. Takeuchi *et al.* (218) and Safsten *et al.* (226) working on rats proposed that the receptor was of the M2 and the M1 subtype, respectively. Larsen *et al.* (227, 228) using human material concluded that the receptor was of the M3 subtype. The different results probably reflect that the different receptor antagonists used are not specific enough to provide a clear answer and species differences may also exist.

Intestinal fluid transport

Intestinal fluid transport is of great physiological and pathophysiological importance. A net fluid secretion can be life threatening. Absorption of water occurs across the villus epithelium and is driven by a hyperosmolar compartment in the villus lamina propria mainly made up by sodium chloride (229). Fluid is secreted from the crypts presumably mediated by an active chloride secretion (230).

The nervous control of fluid transport is directed towards a control of electrolyte and fluid secretion in the intestinal crypts. Immunohistochemical investigations have revealed a large number of mucosal nerves containing established and putative neurotransmitters including acetylcholine (231). Experimental observations have demonstrated that both extrinsic sympathetic and parasympathetic as well as intrinsic enteric nerves influence fluid transport. Stimulation of the sympathetic nerves to the gut increases intestinal fluid uptake by attenuating crypt secretion (230). Nerves containing ChAT and, hence, presumably also acetylcholine, are abundant in the gastrointestinal tract. More than 40% of the efferent submucosal neurons contain ChAT (232-234). A vagal, secretory influence on fluid transport in the small intestine exists but it seems not to involve acetylcholine as a neurotransmitter (235) but probably VIP (236, 237). On the other hand, pelvic nerve stimulation causes a secretion from the cat colon, which can be blocked by atropine (238, 239). In line with this, muscarinic receptors have been demonstrated on colonic enterocytes (240-242).

Although stimulation of extrinsic nerves to the small intestine fails to evoke a muscarinic secretory response, muscarinic receptors on enterocytes have been demonstrated also in the small bowel (243). Furthermore, experimental observations *in vitro* clearly suggest that acetylcholine is a transmitter at the secreting enterocyte in the small intestine. Thus, atropine markedly attenuates the increase of short circuit current (SCC) across the intestinal wall evoked by electrical field stimulation *in vitro*. Furthermore, muscarinic agonists mimic this response (for references, see (232)). Finally, atropine enhances fluid uptake from the cat small intestine *in vivo* or turns spontaneous secretion to fluid

absorption (244). Taken together these observations indicate that certain intramural nerves may control the muscarinic receptors involved in fluid transport. In line with this, Mellander *et al*. (245) reported that the fluid secretion accompanying the migrating myoelectric complex might be mediated *via* muscarinic receptors. It is likely that a net secretion of chloride ions in the intestinal crypts drives the cholinergic fluid secretion. On the other hand, in extensive studies of different types of acute diarrhoea in anesthetized rats (*e.g.,* cholera toxin and bile salt) atropine failed to attenuate the evoked fluid secretion although the agents clearly cause secretion *via* enteric nerves (for references see, (246, 247)).

A few studies exist on which subtype of muscarinic receptor is involved in the control of colonic crypt secretion. In pharmacological analyses of which muscarinic receptor subtypes involved in regulation of colononic secretion, studies have been performed on the cholinergic control of enterocyte $[Ca^{2+}]_i$. The studies indicate that the muscarinic M3 receptor is probably the most important receptor type (248-254). In line with this, we have shown that muscarinic M1, M3 and M5 receptors are present on enterocytes, whereas we were unable to demonstrate the existence of muscarinic M2 and M4 receptors (Lundgren *et al.*, unpublished observations, 2008).

Intestinal mucus secretion

A mucus layer is covering the intestinal epithelial cells to protect the cells from the luminal contents. Mucus is produced by and secreted from the so-called goblet cells that are found both on villi and in crypts. Goblet secretion is influenced by a cholinergic mechanism as shown by muscarinic agonists and antagonists (255). In the colon there seems to be a cholinergic control of mucus secretion in the crypts but not on the mucosal surface (249).Whereas electrical field stimulation evokes a release of mucus (256), stimulation of the parasympathetic (vagal) nerves to the small intestine fails to do so (251, 252). These observations suggest, as in the case of intestinal fluid transport, that intrinsic enteric cholinergic nerves control goblet cells. Several observations support this conclusion. Cholera toxin in the intestinal lumen evokes mucus secretion *via* nerves. This effect is abolished by neonatal administration of capsaicin suggesting that the nervous cholera toxin effect is exerted *via* a so-called axon reflex (257, 258), as also proposed for the cholinergic control of intestinal stem/progenitor cells.

To our knowledge, no investigation has been published of which subtype of muscarinic receptor that is present on intestinal goblet cells. In the conjunctiva the goblet cells are provided with muscarinic receptors of the M1, M2 and M3 subtypes (254), possibly implying that the same receptors are present on the intestinal goblet cells. Activation of the muscarinic receptor in the conjunctiva transactivates the EGF receptor leading to an activation of MAP kinase (259), as has also been proposed for the nervous control of intestinal stem/progenitor cells.

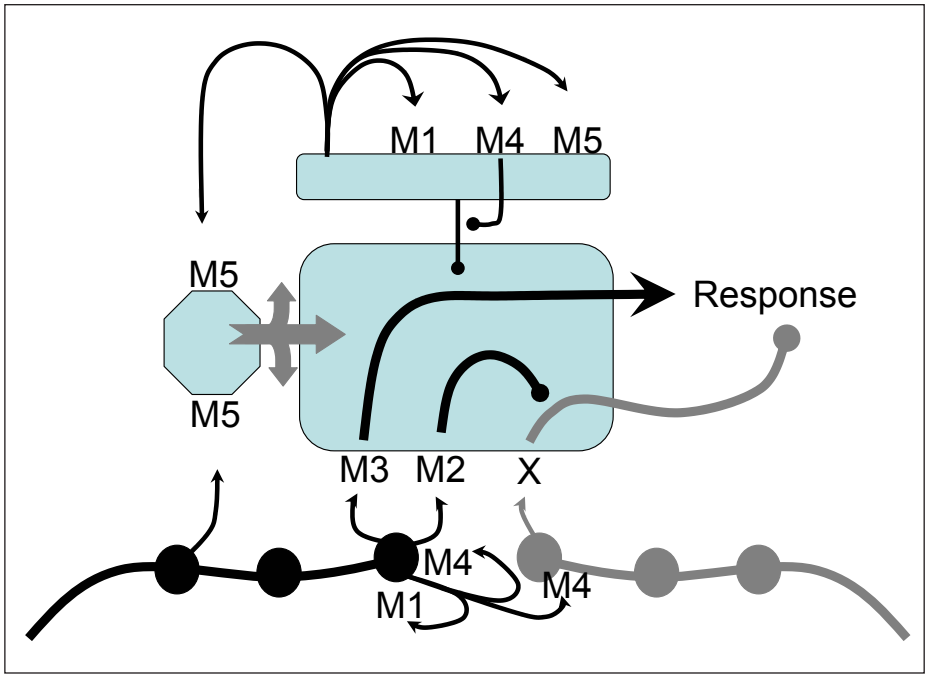

Fig. 4. Suggested principal effects by muscarinic receptor subtypes. Postjunctionally, muscarinic M3 receptors activate intracellular pathways evoking secretion and contraction, while muscarinic M2 receptors inhibit counteracting stimuli. Prejunctionally, muscarinic M1 and M4 receptors facilitate and inhibit transmitter release, respectively. Indirect effects, often by non-neuronal and intrinsic systems, often include effects by muscarinic M1, M4 and M5 receptors. Stimulatory effects are indicated by arrows and inhibitory effects by lines with a round ending.

Intestinal stem cells

The renewal rate of the intestinal epithelium is very fast, being 2-5 days. This is accomplished by means of the epithelial stem/progenitor cells located deep in the intestinal crypts, constantly reproducing themselves. A nervous control of rate of cell renewal has been inferred by Bjerknes & Cheng (260) based on the observation that the increasing effect of glucagon-like peptide 1 on the intestinal stem/progenitor cells was blocked by tetrodotoxin. Recent observations by Lundgren *et al.* (manuscript submitted) clearly indicate, however, that neurons controlling the stem/progenitor cells are cholinergic. The muscarinic receptor involved has not been definitely established but the observations indicate that it is not of the M1 or M4 subtypes.

Paneth cells

Paneth cells are located at the very bottom of the intestinal crypts. During physiological conditions they are only found in the small intestine. The cells produce bacteriocide peptides often collectively named defensins. It is known since long that cholinergic agonists cause a degranulation of Paneth cells (261-264). More recent investigations have confirmed the muscarinic control of defensin release (265), showing a blocking effect of atropine. The subtype of muscarinic receptor involved is not known.

CONCLUDING REMARKS

In the alimentary tract, almost every function involves muscarinic receptor effects (*Fig. 4* indicates a principle scheme of cholinergic signalling). However, the pattern of heterogeneity of the receptor population varies in tissues. The population in glandular parenchymal tissue often consists of a mixture of M1 and M3 receptors, in smooth muscle tissue of the gut of M2 and M3 subtypes, in blood vessels of M1, M3, M4 and M5 and on neuronal cells of M1 and M4 subtypes. NO production, effects on inflammation and proliferation may involve muscarinic M1, M3 and M5 receptors.

In salivary glands of different species including man, muscarinic M1 receptors seem to occur co-localized with muscarinic M3 receptors on secretory cells, preferentially in sero-mucous/mucous glands, and co-activation of M1 and the M3 receptors may be a pre-requisite for maximum responses. Vasodilatation may be affected by muscarinic receptors as well. While muscarinic M1, M3 and possibly M5 receptors cause vasodilatation NO-dependently, venous muscarinic M1 receptors may promote fluid recruitment from blood vessels into glandular tissue by venous contractile effects.

In the gastrointestinal tract, acetylcholine evokes gastric acid secretion *via* muscarinic M3 receptors, while both M1 and M3 receptors may be involved in pepsinogen secretion. Muscarinic M2 receptor may interact positively with M3 receptors in the contractile intestinal responses, particularly during inflammation, while M1 receptors evoke relaxation by a NO-dependent mechanism. Muscarinic M3 but also M1 receptors may be involved in the secretory responses. In addition to the contractile and secretory effects, muscarinic receptors may have an important role in nervous control of renewal rate of the intestinal epithelium. The subtypes affecting

Abbreviations	
adenosine-5´-triphosphate (ATP)	choline acetyltransferase (ChAT)
1,2-diacylglycerol (DAG)	enterochromaffin-like (ECL) cells
epidermal growth factor (EGF)	extracellular-signal-related kinase (ERK)
guanosine monophosphate (cGMP)	inositol 1,4,5-trisphosphate (InsP3)
interstitial cells of Cajal (ICC)	irritable bowel syndrome (IBS)
mitogen-activated protein (MAP)	muscarinic cationic current (MI_{Cat})
nitric oxide (NO)	platelet-derived growth factor (PDGF)
protein kinase A (PKA)	protein kinase C (PKC)
protein kinase potentiated inhibitor protein-17 kDa (CPI-17)	short circuit current (SCC)
sphingosine-1-phosphate (S1P)	transient receptors potential canonical (TRPC)
tumour necrosis factor (TNF)	vasoactive intestinal peptide (VIP)

epithelial stem/progenitor cells activation are likely to be of M3 or/and M5 subtype.

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