Muscarinic Receptor Agonists and Antagonists: Effects on Ocular Function

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Abstract Muscarinic agonists act mainly via muscarinic M_3 cholinoceptors to cause contraction of the iris sphincter, ciliary muscle and trabecular meshwork as well as increase outflow facility of aqueous humour. In the iris dilator, the effect of muscarinic agonists is species dependent but is predominantly relaxation via muscarinic M_3 receptors. In the conjunctiva, muscarinic agonists stimulate goblet cell secretion which contributes to the protective tear film. Muscarinic M_2 and M_3 receptors appear mainly involved. In the lens muscarinic agonists act via muscarinic M_1 receptors to produce depolarization and increase $[Ca^{2+}]_i$. All five subtypes of muscarinic receptor are present in the retina. In the developing retina, acetylcholine appears to limit purinergic stimulation of retinal development and decrease cell proliferation. In the adult retina acetylcholine and other muscarinic agonists may have complex effects, for example, enhancing light-evoked neuronal firing in transient ON retinal ganglion cells and inhibiting firing in OFF retinal ganglion cells. In the lacrimal gland, muscarinic agonists activate M_3 receptors on secretory globular acinar cells to stimulate tear secretion and also cause contraction of myoepithelial cells. In Sjögren's syndrome, antibodies to the muscarinic M_3 receptor disrupt normal gland function leading to xerophthalmia although the mechanism of action of the antibody is still not clear. Atropine and pirenzepine are useful in limiting the development of myopia in children probably by an action on muscarinic receptors in the sclera, although many other muscarinic receptor antagonists are not effective.

Keywords Muscarinic agonist • Muscarinic antagonist • Muscarinic cholinoceptor • Ocular function • Sjögren's syndrome • Myopia

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1 Introduction

Drugs affecting muscarinic receptors, both agonists and antagonists, have long been used in the eye to aid in the diagnosis of neurological abnormalities affecting pupil diameter such as the Holmes–Adie syndrome. Agonists, such as pilocarpine and aceclidine, lower intraocular pressure in the treatment of glaucoma and antagonists, such as homatropine and tropicamide, are used as mydriatics and cycloplegics to facilitate ophthalmic examination. All of these uses involve the activation or inhibition of muscarinic receptors associated with cholinergic parasympathetic innervation of structures within the eye.

Additionally, there is growing evidence that acetylcholine (ACh) may have a non-neuronal location in some tissues such as epithelia and immune cells and be involved in regulation of cellular function (Kawashima and Fujii 2008; Wessler et al. 2003) and this may also apply to structures within the eye (Duncan and Collison 2003).

Epithelial cells of the cornea synthesize and store ACh (Mindel and Mittag 1976; Williams and Cooper 1965) and there are muscarinic receptors on the isolated nuclei of rabbit corneal endothelial and epithelial cells (Lind and Cavanagh 1993, 1995) despite the cornea having only a sensory innervation.

The lens is a non-innervated structure without a blood supply, receiving nutrients from the surrounding aqueous and vitreous humours. Muscarinic receptors are present on the epithelial cells of the human lens throughout life (Thomas et al. 1997) and the lens surface contains acetylcholinesterase (Michon and Kinoshita 1968).

In this review, the effects of muscarinic agonists and antagonists and their receptors in various regions of the eye and the associated lacrimal gland are discussed along with their role in some ophthalmic disorders, including myopia and the ophthalmic aspects of Sjögren's syndrome (SS).

2 Iris Sphincter

Muscarinic M_3 receptors appear primarily responsible for the contraction of the iris sphincter muscle induced by muscarinic agonists although other subtypes may be present in the tissue. Mice lacking the M_3 receptor gene showed a more dilated pupil than either wild-type mice or heterozygotes bred from crossing mutant mice lacking the M_3 receptor gene with wild-type mice and pilocarpine had little effect on pupil size in the homozygotes or heterozygotes (Matsui et al. 2000). However, full mydriasis in all the mice was only observed after installation of atropine 1% into the eye, suggesting that other muscarinic receptor subtypes were involved in pupillary constriction as well as the M_3 subtype. Subsequent studies showed $M_2-/-$ mice had pupils of normal size, but paradoxically, $M_2-/-$ $M_3-/-$ mice had smaller pupils than the $M_3-/-$ mice, suggesting that M_2 receptors activated

mydriasis rather than miosis (Matsui et al. 2002). Recently, in the iris/ciliary body of the tree shrew, *Tupaia belangeri*, mRNAs for the five muscarinic receptor subtypes were detected (McBrien et al. 2009).

In human iris, using quantitative reverse transcription-polymerase chain reaction (RT-PCR), Collison et al. (2000) found that mRNA for the M_3 receptor subtype comprised 77.5% of the total. Values for the other subtypes were M_1, 11.8%; M_4, 10.4%; M_5, 0.4%; M_2, undetected. Similarly, antibody immunoprecipitation (Gil et al. 1997) or immunofluorescent antibody studies on human iris sphincter (Ishizaka et al. 1998) showed the highest immunoreactivity for the M_3 receptor subtype-specific antibody (74%) with 5–12% levels for the other four subtypes. Studies with several subtype-preferring antagonists in cultured human iris sphincter cells (WoldeMussie et al. 1993) or human iris sphincter muscle (Ishikawa et al. 1998) indicated that the functional receptor was the M_3 subtype.

In rat iris, only mRNAs encoding the $M_{2–4}$ subtypes were detected using RT-PCR for the five subtypes (Furuta et al. 1998). Interestingly, while the amino acid sequence for the M_2 and M_4 receptors was identical with those in rat brain, the iris M_3 subtype showed minor differences from the brain M_3 subtype, differing by four amino acids at position 165 and 184 at the edge of the second intracellular loop and at positions 337 and 406 in the centre of the i3 loop. Mutations in the latter region of the M_1 receptor have been shown to affect receptor down-regulation and sequestration without affecting G protein coupling (Lameh et al. 1992).

In vivo studies in the anaesthetized rat using pirenzepine, telenzepine, AF-DX 116, 4-DAMP and hexahydrosiladiphenidol indicated that the muscarinic M_3 subtype was involved in mediating miosis (Hagan et al. 1988) [NB: the authors termed the receptor "M_2 (ileal)" as the term "M_3 receptor" was not in common usage at that time]. The potency of topical pirenzepine and telenzepine on the iris was increased up to 60-fold at low pH. In the case of pirenzepine, this was attributed to protonation of the second amine group on the piperazine ring, which facilitated corneal penetration (Hagan et al. 1988).

Early investigations into the nature of the muscarinic receptor mediating contraction of the rabbit iris, inositol trisphosphate (IP_3) accumulation and myosin light chain phosphorylation, using subtype-preferring antagonists, suggested that it was similar to the M_3 subtype (termed "M_2" or "$M_{2\beta}$" in early studies) (Akhtar et al. 1987; Honkanen and Abdel-Latif 1988), although the affinity of some antagonists such as pirenzepine (Bognar et al. 1989) and hexahydrodiphenidol enantiomers (Fuder et al. 1989) did not fully agree with that suggestion.

Bognar et al. (1992) found evidence that the muscarinic receptor mediating contraction of the isolated rabbit iris sphincter had an affinity profile for several subtype-preferring muscarinic receptor antagonists that was different from that for $M_{1–4}$ subtypes. Choppin et al. (1998) were unable to confirm these findings and concluded that the pharmacologically defined M_3 subtype was involved, although they pointed out the involvement of M_5 receptors remained a possibility, due to the lack of a suitable selective antagonist to discriminate between M_3 and M_5 subtypes.

In the dog iris sphincter, low concentrations of carbachol (CCh), <5 μM, produced contraction, increased IP_3 production and inhibited cAMP formation but at

higher concentrations (>5 μM) CCh, after an initial transient contraction, produced relaxation, inhibited IP_3 production and increased cAMP production (Abdel-Latif et al. 1992). This concentration-dependent effect is unusual as in other species, such as the cat, ox and rabbit iris, CCh produces contraction and an increase in IP_3 over the whole concentration range (1–100 μM) (Abdel-Latif et al. 1992). Influx of Ca^{2+} through Ca^{2+} channels as well as intracellular Ca^{2+} appeared to be involved, as nifedipine and the intracellular Ca^{2+} chelator, BAPTA, inhibited CCh-induced cAMP production in the dog iris (Abdel-Latif et al. 1992; Tachado et al. 1994).

Pharmacological evaluation of the receptor subtype involved in the CCh-induced contraction in the dog iris suggested that the M_3 muscarinic cholinoceptor was involved as 4-DAMP (pA_2; 9.0) was a more potent antagonist than pirenzepine (pA_2; 7.1) and AF-DX 116 was ineffective (Tachado et al. 1994). 4-DAMP was also more potent than pirenzepine at inhibiting the CCh-induced increase in IP_3 production and increased cAMP formation suggesting that the same muscarinic receptor subtype was involved in production of both second messengers in the tissue.

More recently, zamifenacin, a potent muscarinic M_3 receptor antagonist, was reported to have lower affinity (pA_2; <6.0) for the muscarinic receptor mediating contraction in the canine iris than for canine ileal M_3 receptor (pA_2; 8.6) suggesting that the M_3 receptor subtype in the iris is atypical (see Eglen et al. 1996).

Muscarinic receptors are also present on sympathetic nerve endings in the human iris–ciliary body or whole iris of rabbit, guinea pig and rat. When activated by muscarinic agonists, electrically stimulated release of ^{3}H-noradrenaline was inhibited (Bognar et al. 1988, 1989; Fuder et al. 1989; Jumblatt and Hackmiller 1994). Studies with various "subtype-preferring" antagonists have shown that this prejunctional inhibitory muscarinic receptor is the M_2 subtype (also termed "$M_{2\alpha}$" in early studies).

3 Iris Dilator

While the main parasympathetic innervation of the iris is directed to the circular sphincter muscle surrounding the pupil there is also parasympathetic innervation of the iris radial dilator muscle.

Narita and Watanabe (1982) found the rat isolated iris dilator relaxed to ACh at low concentrations (1 nM–1 μM) but contracted at higher concentrations (>1 μM). Both responses were enhanced by eserine and antagonized by atropine. Electrical stimulation produced a relaxation at frequencies ≤10 Hz and contraction at 30 Hz, both responses being abolished by tetrodotoxin. Also, the relaxation was abolished by atropine and the contraction was abolished by the adrenoceptor antagonist, phentolamine.

Methacholine and CCh also produced relaxation of the rat iris dilator muscle, but contracted the muscle in higher concentrations, ≤1 μM (Shiraishi and Takayanagi 1993; Masuda et al. 1995). However, arecoline and bethanecol produced mainly relaxation with only a small contraction (<35% ACh maximum) and McN-A-343 or

pilocarpine produced only relaxation (Masuda et al. 1995). Atropine inhibited both responses indicating their muscarinic nature. Ciliary ganglionectomy or pertussis toxin treatment selectively abolished the relaxant response to ACh (Hasegawa et al. 1988; Masuda et al. 1995; Yamahara et al. 1995). Pilocarpine and McN-A-343 produced a small contraction (<30% of ACh maximum) after denervation.

Studies with pirenzepine, AF-DX 116, himbacine and 4-DAMP suggested that the M_3 subtype was involved in both relaxant and contractile responses to muscarinic agonists (Shiraishi and Takayanagi 1993; Masuda et al. 1995; Yamahara et al. 1995). While the investigators at that time did not consider the M_5 receptor as a possibility, a comparison of the pK_B values obtained with representative literature values for the M_1, M_3 and M_5 subtypes with the above antagonists by the current author showed that the best correlations were still obtained for the M_3 subtype.

While the four "subtype-preferring" muscarinic receptor antagonists above produced similar pA_2 values against both responses, methoctramine was an exception in that it inhibited the relaxation (pA_2; 6.4) but was relatively ineffective against the contraction in pertussis toxin-treated preparations (threefold shift at 10 μM) (Yamahara et al. 1995) and completely ineffective after ganglionectomy (Masuda et al. 1995).

It was concluded that the same muscarinic receptor subtype could be responsible for both relaxation and contraction, utilizing different G proteins for each response (Masuda et al. 1995; Yamahara et al. 1995). Furthermore, relaxation required a pertussis toxin-sensitive G protein, the integrity of which required an intact parasympathetic innervation.

In the human (Yoshitomi et al. 1985), canine (Yoshitomi and Ito 1986) and bovine dilator muscle (Suzuki et al. 1983), CCh or ACh produces relaxation over the whole concentration range investigated and the relaxation was blocked by atropine. The relaxant response to CCh in the dog iris dilator did not involve an effect on the resting membrane potential of the muscle (Yoshitomi and Ito 1986) indicating pharmacomechanical, rather than electromechanical, coupling. Relative immunoreactivity of the five muscarinic receptor subtypes in the human iris dilator muscle was lower than in the sphincter muscle, with only M_1 and M_5 immunoreactivity observed at low intensity and inconsistently, with little or no staining for $M_{3/4}$ receptors, and none for M_2 receptors (Ishizaka et al. 1998).

Nerve stimulation of the dilator iris in these three species also provided evidence of an inhibitory cholinergic innervation mediated through muscarinic receptors. In human (Yoshitomi et al. 1985) or canine isolated iris dilator muscle (Yoshitomi and Ito 1986), electrical stimulation produced an initial phasic contraction followed by a prolonged relaxation. Tetrodotoxin abolished both phases of the response while atropine selectively abolished the relaxation. Guanethidine or phentolamine inhibited the contraction.

In bovine iris dilator, electrical stimulation gave rise only to a pronounced relaxation that was enhanced by physostigmine and abolished by tetrodotoxin or atropine (Suzuki et al. 1983). The relaxations produced by ACh or CCh were not affected by adrenoceptor antagonists indicating that their action was not dependent on sympathetic tone.

In the rabbit iris dilator muscle, pilocarpine inhibited noradrenaline release induced by nerve stimulation as well as the resulting contractile response. It was suggested that this action of pilocarpine was responsible for the miotic effect of the partial agonist, as in the sphincter muscle it produces only a small contraction per se and inhibited the contraction produced by CCh (Bognar et al. 1988; Takayanagi et al. 1993).

4 Ciliary Muscle

Studies on cultured human ciliary smooth muscle cells by several groups using variously: pirenzepine, 4-DAMP, pFHHSiD, HHSiD, methoctramine and AF-DX 116 indicated that the functional muscarinic receptor was the M_3 subtype for contraction, phospholipase C activity and intracellular Ca^{2+} accumulation (Matsumoto et al. 1994; Pang et al. 1994; WoldeMussie et al. 1993). Relative receptor protein concentrations, using antibody immunoprecipitation, also showed 73.5% of M_3 receptor; other receptor protein concentrations were M_1 0.8%, M_2 5.4%, M_4 4.9% and M_5 2.4% (Gil et al. 1997).

There are both circular and longitudinal muscles present in the ciliary muscle, the circular ciliary muscle being responsible for changes in accommodation and the longitudinal muscle alters outflow facility. While there are differences between the circular and longitudinal muscles in the levels of mRNA expression for the individual muscarinic receptor subtypes, Zhang et al. (1995a) suggested this could not explain the dissociation observed between accommodation and outflow reported with some muscarinic agonists.

Ishikawa et al. (1998) determined pK_B values for pirenzepine, methoctramine, pFHHSiD and tropicamide in both human isolated circular and longitudinal ciliary muscle and found similar values in the two tissues, concluding that M_3 (or M_4) receptors were not excluded in the contractile response of both muscles. Their data appears more compatible with literature values for the M_3 receptor than for any other of the five receptor subtypes. Similarly, the M_3 subtype appears to mediate contraction in both circular and longitudinal ciliary muscles in the rhesus monkey (Poyer et al. 1994) and the contraction of bovine ciliary muscle induced by transmural electrical stimulation of cholinergic nerves (Masuda et al. 1998).

5 Ciliary Process/Body

CCh increased phosphatidylinositol turnover in the rabbit ciliary process with oxotremorine and pilocarpine acting as partial agonists (Mallorga et al. 1989). All five muscarinic receptor subtypes are present in the tissue, but using antibody immunoprecipitation, the major receptor protein was the M_3 receptor (57.6%) with $<10\%$ for each of the other four subtypes (Gil et al. 1997). Similarly, all

five muscarinic receptor subtypes were expressed in the ciliary body of the tree shrew (McBrien et al. 2009). In the bovine ciliary process, mRNA encoding for the muscarinic M_3 receptor predominated with minor amounts for the M_2 and M_4 receptors; mRNA for the M_1 receptor was not detected (Honkanen et al. 1990). Chick muscarinic receptor subtypes, $M_{2–4}$ were detected in the ciliary body by Fischer et al. (1998a) although only nicotinic receptors are involved in accommodation in the chick (McBrien et al. 1993).

6 Trabecular Meshwork

The trabecular meshwork is now considered to be actively involved in the passage of aqueous humour rather than being a passive structure for outflow (Wiederholt et al. 2000). The tissue contains contractile α-isoactin filaments and various compounds have been demonstrated to contract or relax the isolated tissue. Muscarinic agonists, aceclidine, ACh, CCh and pilocarpine, were found to produce atropine-sensitive contractions of the trabecular meshwork, with pilocarpine producing only 60% of the maximal response to CCh (Lepple-Weinhues et al. 1991; Wiederholt et al. 2000).

Studies have also demonstrated outflow regulation by muscarinic agonists in perfused anterior segments of bovine eye containing the trabecular meshwork but devoid of ciliary muscle (Wiederholt et al. 1995). CCh impeded outflow through the mesh in similar concentrations to that causing contraction of the isolated trabecular meshwork and pilocarpine was a partial agonist, producing ~50% of CCh maximum (Wiederholt et al. 1995).

Muscarinic agonists, aceclidine, CCh, oxotremorine-M and pilocarpine induced an increase in intracellular Ca^{2+} and CCh also increased phosphoinositide production in cultured trabecular meshwork cells (Shade et al. 1996). Both effects of CCh were inhibited by atropine, pirenzepine, 4-DAMP and pFHHSiD; the inhibition profile of the antagonists suggested involvement of muscarinic M_3 receptors for both effects, although it should be noted that no M_2 subtype-preferring antagonist was included in the study.

ACh and CCh have also been shown to activate L-type Ca^{2+} channels in the meshwork (Steinhausen et al. 2000) but this appears to be less important for contraction than in ciliary muscle as the trabecular meshwork can still contract in the absence of extracellular Ca^{2+} (Wiederholt et al. 2000). Contraction appears to involve protein kinase C (PKC). The PKC antagonists H7 and chelerythrine partly reduced the contraction produced by CCh in the trabecular meshwork, while not affecting the action of CCh in the ciliary muscle (Wiederholt et al. 2000). It is of interest that the trabecular meshwork contains PKCε which is a Ca^{++}-independent protein kinase (Thieme et al. 1999). H7 has also been shown to increase outflow facility in the trabecular meshwork by a mechanism independent of the ciliary muscle (Tian et al. 1999).

Rho-associated coiled coil-forming protein kinase (ROCK) may be a regulator of actomyosin-based contractility in the trabecular meshwork. The ROCK inhibitor, YM-39983, lowered intraocular pressure and caused a dose-dependent inhibition of CCh-induced contraction of monkey trabecular meshwork (Nakajima et al. 2005). YM-39983 was also found to be much less effective at relaxing ciliary muscle, raising the possibility of selectively inhibiting the contractile action of cholinomimetics in the trabecular meshwork.

While contraction appears to involve M_3 receptors primarily, there is a small population of M_1 receptors in the tissue (Wiederholt et al. 2000) and there may also be a contribution to contraction from M_2 receptors. Immunoblotting in both human and bovine trabecular meshwork showed the presence of M_2 muscarinic receptors and in the bovine trabecular meshwork, methoctramine (0.1–5 μM) induced a relaxation of a CCh-induced maximal contraction, even in the presence of M_1 and M_3 receptor antagonists (Thieme et al. 2001).

Thus in summary, the contraction of the ciliary muscle by muscarinic agonists to cause opening of the trabecular meshwork with a resulting increase in outflow facility is opposed by the action of the agonists on the meshwork itself and any beneficial effect on intraocular pressure in glaucoma will be the resultant of two opposing actions (Wiederholt et al. 2000).

7 Cornea

The cornea is innervated but the nerves appear to be only sensory, at least in the rabbit (ten Tusscher et al. 1988). Nevertheless, the corneal epithelium contains choline acetylase, cholinesterase and a high content of ACh (Gnädinger et al. 1967, 1973; Mindel and Mittag 1976; Williams and Cooper 1965), which has led to the suggestion that it has a role in the tissue other than as a neurotransmitter (Williams and Cooper 1965).

Furthermore, for a number of species, muscarinic receptors have now been detected in the corneal epithelium and endothelium (Colley and Cavanagh 1982; Lind and Cavanagh 1995; Socci et al. 1996; Walkenbach and Ye 1991; Walkenbach et al. 1993) despite earlier investigators (Olsen and Neufeld 1979; Gupta et al. 1994) reporting a lack of evidence for cholinoceptors in human or rabbit cornea.

For example, Walkenbach and Ye (1991) using cultured human corneal epithelial cells found specific ^{3}H-QNB binding displaced by atropine, CCh and high concentrations of nicotine. However, binding was decreased 90% if the cultured cells were homogenized and fractionated prior to assay.

Using ^{3}H-propylbenzilylcholine mustard, which forms a covalent bond with muscarinc receptors, Lind and Cavanagh (1995) found evidence of muscarinic receptors in epithelium and endothelium layers of fresh-frozen rabbit cornea as well as in cultured rabbit epithelial and endothelial corneal cells and their nuclei. The finding of muscarinic binding sites on the cell nuclei supported a more general regulatory role for ACh in the cells (Lind and Cavanagh 1995). It was hypothesized

that muscarinic receptors on cell nuclei can regulate cyclic nucleotide-dependent protein kinases and stimulate mitotic activity of the corneal epithelium (Cavanagh and Colley 1989; Colley and Law 1987a).

In rabbit cultured corneal epithelial cell homogenates and subcellular fractions, CCh has been shown to increase guanylyl cyclase activity, cGMP levels, cGMP binding, cGMP-dependent protein kinase activity and to decrease cGMP-phosphodiesterase activity in the nuclear and some other fractions (Cavanagh and Colley 1982; Colley and Cavanagh 1982; Colley and Law 1987a; Colley et al. 1985, 1987a, b; Walkenbach and Ye 1991). These effects were inhibited by atropine. The proportion of nuclear to total cGMP-dependent protein kinase activity also increased, suggesting that nuclear translocation of the enzyme may be enhanced by CCh (Colley and Law 1987b). In these studies, cAMP binding and cAMP-dependent kinase activity was decreased and cAMP-phosphodiesterase activity was increased in the nuclear fraction also, without affecting adenyl cyclase activity.

CCh also increased RNA and DNA polymerase activity in the purified nuclear fraction of rabbit corneal epithelial cell cultures by an atropine-dependent mechanism (Colley et al. 1985). In corneal resurfacing studies on rabbits in vivo, following acid burn defects, CCh was shown to elevate V_{max} of both RNA and DNA polymerases and to specifically increase the apparent affinity of RNA polymerase II for UTP and the apparent affinity of the DNA polymerases α and β for dTTP (Colley et al. 1987b).

The subtypes of muscarinic cholinoceptor found in the cornea are subject to considerable speculation due to different findings obtained by various groups. Shepard and Rae (1998) found evidence only for the muscarinic M_4 receptor in rabbit cornea. Freshly isolated mRNA from rabbit corneal epithelium and endothelium was used to construct cDNA libraries, determined by PCR with gene-specific primers for the five muscarinic receptor subtypes. In contrast, CCh was found to increase the accumulation of ^{3}H-IP$_3$ in rabbit corneal epithelial cells (SV40-adenovirus transformed), an effect inhibited by atropine and 4-DAMP, but less effectively by pirenzepine, suggesting muscarinic M_3 rather than M_1 receptor involvement (Zhang et al. 1995b).

Muscarinic cholinoceptors in bovine corneal epithelial cells were characterized by their ligand specificity, cell signalling pathways and gene transcripts (Socci et al. 1996). Pirenzepine and AF-DX 116 were more effective in displacing ^{3}H-NMS binding than 4-DAMP, suggesting the presence of muscarinic M_1 and/or M_4 receptor and M_2 receptor subtypes. CCh increased intracellular Ca^{2+} levels and this response was inhibited by pirenzepine or atropine. CCh also inhibited cAMP accumulation induced by isoprenaline, an effect inhibited by AF-DX 116 or pertussis toxin. However, RT-PCR revealed only the presence of the transcript for the M_2 receptor, but not that for M_1, M_3 or M_4 receptors.

Recently, all five subtypes of muscarinic cholinoceptor were found in human corneal cells in the epithelium, endothelium and the cytoplasm using anti-muscarinic receptor subtype specific antibodies (Liu et al. 2007). Immunofluorescence for the M_4 subtype was more intense on the corneal epithelium while the other four subtypes were evenly distributed between the cell membranes and cytoplasm.

8 Limbus

The limbus is a narrow specialized ring of innervated tissue surrounding the cornea, at the inner edge of the conjunctiva. The limbic epithelial cells of the rabbit are similar to those in the cornea and are a different lineage to the epithelial cells in the conjunctiva (Wei et al. 1996). Corneal epithelial stem cells are located preferentially in the limbus and are now used for limbal stem cell transplantation surgery to restore severely damaged corneal epithelium (Sun and Lavker 2004). The limbus contains parasympathetic nerves as well as sympathetic and sensory nerves.

All five subtypes of muscarinic cholinoceptor were found in cultured human limbal epithelium using RT-PCR (Liu et al. 2007). While M_2, M_3 and M_4 muscarinic receptor subtype transcript levels varied by less than sixfold from the M_1 transcript level, there was an 11-fold higher level of the M_5 transcript.

9 Conjunctiva

The conjuctiva plays an important role in contributing to the protective tear film on the surface of the eye. Parasympathetic nerves, innervating goblet cells in the conjunctival epithelium, increase mucin and other protein secretion into the tear film (Dartt 2002). Stratified squamous cells which contribute fluid to the tear film are stimulated by sympathetic nerves, but appear to be unaffected by parasympathetic nerves.

Nevertheless, muscarinic M_1 receptors were detected, using antibody immunofluorescence, in the stratified squamous cells from day 17 and in the adult animal (Ríos et al. 1999, 2000). M_2 receptors were detected in the stratified squamous cells from day 9 onwards and in goblet cells from day 17 onwards. M_3 receptors were present in the stratified squamous cells on day 9 and 13 but then subsequently declined in number as their presence in goblet cells became established (Ríos et al. 1999, 2000).

While only muscarinic M_3 receptor immunofluorescence was detected in rat cultured goblet cells (Shatos et al. 2001), $M_{1–3}$ receptors were found on human, mouse and rat conjunctival goblet cells by Diebold et al. (2001) using immunofluorescence microscopy on conjunctival tissue cryosections. In the mouse, muscarinic receptors on goblet cells were located above the nuclei and sub-adjacent to the secretory granules. In human stratified squamous epithelium, the three receptor subtypes were detected occasionally, along with intense fluorescence for M_2 and M_3 receptors in the basement epithelium (Diebold et al. 2001). In mouse and rat stratified squamous epithelium M_1 and M_2 receptors were detected, but more uniformly than in the human, while the bulk of the M_3 receptors were on goblet cells.

Motterle et al. (2006) detected M_1 and M_2 receptors over the full thickness of the conjunctival epithelium in biopsy samples obtained from patients during cataract

surgery whereas M_3 receptor immunoreactivity was restricted to the epithelial basal layer. In another study, immunoreactivity to muscarinic $M_{1–3}$ receptors was detected in all conjunctival epithelial cells (Enríquez de Salamanca et al. 2005).

Also in the IOBA-NHC cell line, derived from normal human conjunctiva, muscarinic M_2 and M_3 receptors were detected on cell membranes and in the cytosol (Enríquez de Salamanca et al. 2005). The M_1 receptor was detected only in the cytosol.

More recently, all five subtypes of muscarinic cholinoceptor were found in cultured human conjunctival epithelium and fibroblast cells, using RT-PCR (Liu et al. 2007). The abundance of each of the five subtypes in the two types of conjunctival cells varied. While the level of M_1 transcript was similar in the fibroblasts and epithelium, there were higher levels of $M_{2–5}$ transcripts in the fibroblasts. The epithelial cells had a 24-fold higher level of the M_5 transcript, relative to the M_1 transcript. Other transcripts varied from the M_1 transcript level by less than sixfold. In fibroblasts, the level of M_5 transcript was 44-fold greater, and the M_2 14-fold greater, than the M_1 transcript.

CCh (1–100 μM) caused secretion of glycoconjugate from the goblet cells in the adult animal which was abolished by atropine (10 μM). The subtype-preferring antagonists, 4-DAMP, gallamine and pirenzepine, all at 10 μM, inhibited the response to the agonist (100 μM) by 72, 69 and 54%, respectively (Ríos et al. 1999). It was concluded that parasympathetic nerves acting via M_2 and M_3 receptors could be involved in conjunctival secretions from goblet cells when the eyelids open into adult life (Ríos et al. 1999, 2000). The location of M_2 and M_3 receptors on membranes above the cell nucleus close to the secretory granules of the goblet cells could decrease the latency between receptor activation and apocrine secretion (Dartt 2002).

In rat conjunctiva, goblet cell secretion induced by CCh is dependent on an increase in intracellular Ca^{2+}, presumably due to IP_3 production, release of endoplasmic reticulum Ca^{2+} stores, followed by the influx of extracellular Ca^{2+} and exocytosis of mucin (Dartt et al. 2000). Activation of PKC may also be involved in the action of cholinomimetics but Ca^{2+}/calmodulin-dependent protein kinases do not appear to be important in the secretory process (Dartt et al. 2000; Dartt 2002). CCh also transactivated the EGF receptor through activation of Pyk2 and p60Src in the rat conjunctiva, leading to MAPK activation, which in turn also increased goblet cell mucin secretion (Dartt 2002; Kanno et al. 2003). This effect of CCh was blocked by either pirenzepine, gallamine or 4-DAMP (all at 10 μM) leading to the suggestion that $M_{1–3}$ receptors were all involved (Kanno et al. 2003).

However, it should be appreciated that the subtype preferences of pirenzepine and 4-DAMP, while greater than that of gallamine, are limited. Both pirenzepine and 4-DAMP have lowest affinity at the M_2 receptor, but at 10 μM there will be a receptor occupancy of 90–98% and 99.6–99.9%, respectively, based on a representative range of literature $pK_{B/I}$ values for pirenzepine at the M_2 receptor of 6.0–6.8 and for 4-DAMP, 7.6–8.4.

Activation of MAPK by CCh in cultures of human conjunctival goblet cells, obtained during ocular surgery, appeared similar to that in cultures of rat

conjunctival goblet cells (Horikawa et al. 2003). The ~1.6-fold increase in MAPK induced by CCh (100 μM) had a similar time course in both species; it peaked at 5 min and declined to baseline by ~30 min. The increase in MAPK was also abolished by 4-DAMP (10 μM) or by the EGF receptor antagonist, AG1478, in both species.

CCh also stimulated cell proliferation and p42/44 MAPK activation in the conjunctival epithelial cell line, IOBA-NHC, both effects being inhibited by the MAPK inhibitor, U0126 (Liu et al. 2007). The effect of CCh (150 μM) on cell proliferation was also completely inhibited by atropine (150 μM). This finding was used by Liu et al. (2007) in support of the contention that CCh was acting via muscarinic receptors, but at this high concentration atropine will also inhibit some subtypes of nicotinic receptor (Shirvan et al. 1991; Verbitsky et al. 2000). Furthermore, the three subtype-preferring antagonists, pirenzepine, AF-DX 116 and 4-DAMP all produced only ~42% inhibition of CCh-induced cell proliferation when used at a concentration of 10 μM. A tenfold higher concentration of 4-DAMP (100 μM) produced only ~60% inhibition, although this concentration of the antagonist will occupy >99.9% of all five muscarinic receptor subtypes.

Stratified squamous cells secrete chloride ion leading to fluid secretion, but the basal level of ion secretion is unaffected by ACh (Dartt 2002). Goblet cells can also secrete chloride ion leading to fluid secretion but this property of goblet cells has not been studied (Dartt 2002).

10 Vernal Keratoconjunctivitis

Muscarinic receptor populations in the conjunctiva may be altered in disease states. Vernal keratoconjunctivitis is a rare allergic condition characterized by a non-specific hyper-reactivity to environmental antigens, leading to severe inflammation (Leonardi et al. 2008). In patients with the condition, conjunctival epithelial M_1 receptors were significantly decreased while M_2 and M_3 receptors became more irregularly distributed throughout the epithelium (Motterle et al. 2006). In the conjunctival stroma, M_1, M_2 and M_3 receptors were increased significantly and present throughout the tissue instead of being restricted to a few scattered cells. Motterle et al. (2006) suggested that the decline in epithelial M_1 receptors was consistent with a change to the more viscous secretion of mucus seen in the condition, as M_2 and M_3 receptors located on goblet cells are the prime activators of mucous secretion with the M_1 receptors having a more minor role, perhaps increasing the water content of the secretion. The stromal cells with muscarinic receptors found in vernal keratoconjunctivitis were not positively identified but were suggested to include mast cells and other immune cells. Connective tissue mast cells release histamine when activated by muscarinic agonists; they are present in conjunctival stroma, and markedly increased in vernal keratoconjunctivitis (Irani et al. 1990).

11 Lens

The lens is not innervated but possesses muscarinic receptors (Williams et al. 1993). ACh, CCh and pilocarpine produced depolarization and increased intracellular calcium in the anterior cells of the lens of human and rat (Collison and Duncan 2001; Thomas et al. 1997; Williams et al. 1993). Release of Ca^{2+} from endoplasmic reticulum stores and entry of Ca^{2+} through capacitative Ca^{2+} channels may both occur and Ca^{2+} cycling is important for modulating growth of the lens (Duncan et al. 1996). An increase in $[Ca^{2+}]_i$ has been associated with increased light scatter and localized cortical cataract formation (Duncan et al. 1994; Williams et al. 2001) possibly due to activation of a calcium-dependent protease, such as calpain (Duncan et al. 1994). The lens surface contains high levels of acetylcholinesterase (Michon and Kinoshita 1968) which may offer an explanation for the development of cataracts with the chronic use of anticholinesterases in the treatment of glaucoma (see Duncan and Collison 2003; Thomas et al. 1997). The source of endogenous ACh to act as a substrate for the enzyme is not known, but may possibly arise from cholinergic neurons in the ciliary process or iris (see Williams et al. 1993).

The major subtype of muscarinic receptor mRNA in native human lens epithelium is the M_1 subtype comprising 88.1% of the total with M_2 9.2%; M_3 0.6%; M_4 0.1% and M_5 receptor 6.6% (Collison et al. 2000). Pharmacological studies of the inhibition produced by pirenzepine (pK_B, 8.1) and AF-DX 384 (pK_B, 7.2) on ACh-induced Ca^{2+} mobilization in these cells also suggested that the M_1 subtype was involved.

However, cultures of human lens cells show a different pattern with mRNA for the M_3 receptor subtype predominating. In the human lens epithelial cell line, HLE-B3, the mRNA subtype for the M_3 receptor was 98.8% of the total and in lens primary cultures, 90.6% (Collison et al. 2000). The response to Ca^{++} mobilization by ACh (10 μM) in the HLE-B3 cell lines was delayed in onset and more prolonged than in the native cells, suggestive of a second component in the response. The pK_B values for antagonists were pirenzepine, 6.6; AF-DX 384, 7.4; 4-DAMP, 9.0 and methoctramine, 6.2 which correlated best with tabulated values for the M_3 receptor (Collison et al. 2000).

Shepard and Rae (1998) provide further evidence that lens tissue cultures may have a dissimilar muscarinic receptor profile to native lens tissue. They used freshly isolated mRNA from a single human lens epithelial tissue culture to construct a cDNA library. Only the M_5 muscarinic receptor, out of the five receptor subtypes, was detectable in the library. In the mouse epithelial lens cell line, αTN4, no muscarinic receptor subtype was detected in the cDNA library.

12 Choroid

All five subtypes of muscarinic receptor were detected in the choroid of the tree shrew (McBrien et al. 2009) but only three chick muscarinic receptor subtypes, $M_{2–4}$ were detected in the chick choroid by Fischer et al. (1998a).

13 Sclera

Cell lines of fibroblasts from human sclera expressed mRNA for the five subtypes with greater expression for the M_1 and M_3 receptors, while Western blot analysis detected receptor protein for the five subtypes (Qu et al. 2006). Also, all subtypes were detected in frozen scleral sections by immunohistochemistry. Using quantitative RT-PCR, Collison et al. (2000) found that mRNA for the M_3 receptor subtype in the human sclera comprised 76% of the total. Percentages for the other subtypes were M_1, 23; M_2, 0.0007; M_4, 0.06; M_5, 0.7. Similarly, all five subtypes were detected in the sclera of the tree shrew (McBrien et al. 2009). The muscarinic receptors appeared to be localized to fibroblast processes in both species. The effect of muscarinic receptor antagonists on the sclera is discussed below under *Myopia*.

14 Retina

The only cholinergic cells in the adult retina are starburst amacrine cells but muscarinic receptors are found on amacrine, bipolar, horizontal and ganglion cells in the retina (Fischer et al. 1998a; McBrien et al. 2009; Strang et al. 2010; Townes-Anderson and Vogt 1989; Wong 1995; Yamada et al. 2003).

Using quantitative RT-PCR, Collison et al. (2000) found mRNA for all five muscarinic receptor subtypes in the human retina, and that for the M_3 receptor comprised 86% of the total. Values for the other subtypes were M_1, 2.1%; M_2, 0.07%; M_4, 8.5%; M_5, 3.8%.

McBrien et al. (2009) detected all five muscarinic receptor subtypes in the retina of the tree shrew. M_1 receptors were mainly localized to the outer and inner plexiform layers; M_2 receptors were in the outer segments of photoreceptor layer, plexiform and nerve fibre layers. The M_3 receptors were localized, consistent with Müller cell expression. $M_{4–5}$ receptors had a more diffuse distribution.

ACh plays a role in early retinal development (see Martins and Pearson 2008). Stimulation of muscarinic receptors by ACh, CCh or muscarine in embryonic chick retina was found to release Ca^{2+} from intracellular stores to increase free $[Ca^{2+}]_i$ (Yamashita et al. 1994). The response was maximal at E3 and then declined until E8. ACh (10 μM) was ~tenfold more potent than either of the other agonists, despite the detection of cholinesterase as early as E4 (Yamashita et al. 1994). In vitro ACh was shown to induce curving of the neural retina of E3 chick embryo, at the stage of optic cup formation (Yamashita and Fukuda 1993).

In the chick retina, the subtype of muscarinic receptor varies with the developmental stage; the M_4 subtype is predominant early but later M_2 and M_3 receptors increase (McKinnon and Nathanson 1995; Nadler et al. 1999) whereas in the rabbit neonate, muscarinic M_1 receptors appear transiently to increase $[Ca^{2+}]_i$, in cells of the ventricular zone of the retina, disappearing shortly before the eye opens (Wong 1995). Similarly, ACh, muscarine or McN-A-343 increased $[Ca^{2+}]_i$ in foetal or rat pup retinal neurons, activating M_1 receptors (Wakakura et al. 1998).

Pearson et al. (2002) have proposed that muscarinic receptor activation limits purinergic stimulation of retinal development. In chick embryos at E6, retinal progenitor cells from the ventricular zone, adjacent to the retinal pigment epithelium (RPE), were exposed to CCh. The muscarinic agonist released Ca^{2+} from intracellular stores, frequently causing oscillations in $[Ca^{2+}]_i$ and inhibited mitosis. The effect of CCh was antagonized by pirenzepine (25 μM). Pirenzepine per se also caused an increase in mitosis and increased eye growth, supporting the suggestion that endogenous ACh was modulating retinal development.

dos Santos et al. (2003) found that in vitro exposure of 1–2-day-old rat pup retinal cells to CCh or oxotremorine over 24–48 h decreased cell proliferation. This effect was mediated by muscarinic M_1 receptors activating PKC and was dependent on phosphatidyl inositol 3-kinase and tyrosine kinase activity. Polypeptide release was also involved, possibly BDNF, as the effect of CCh was inhibited by a TrkB receptor antagonist.

In the newborn rabbit retina, after day P3, muscarinic M_1 and M_3 receptor activation was found to propagate spontaneous excitatory waves arising from amacrine starburst cells (Zhou and Zhao 2000). In the foetus, nicotinic receptors were responsible.

Recently, Strang et al. (2010) mapped the location of muscarinic receptors in the rabbit retina and explored the muscarinic effects of choline. All five muscarinic receptor subtypes were detected in subsets of amacrine, bipolar and ganglion cells, revealing a complex organization of muscarinic receptors. For example, cholinergic amacrine cells expressed all five receptor subtypes suggesting multiple feedback mechanisms. Co-localization of muscarinic receptors with the glycine transporter was limited and restricted to M_1, M_2 and M_4 receptors, suggesting muscarinic receptors were localized on GABAergic rather than glycinergic amacrine cells. Sustained OFF, transient OFF, transient ON and ON–OFF retinal ganglion cells were found to have atropine-sensitive responses to choline. Choline enhanced responses to light in transient ON and ON–OFF ganglion cells and inhibited light responses in OFF cells. The findings suggested that choline as well as ACh could contribute to muscarinic modulation of retinal neuronal activity.

Müller cells are a form of glial cell that regulates the extracellular mileau of retinal neurons. Their apical processes extend into the photoreceptor layer and their basal processes end at the inner retina. In Müller cell cultures prepared from 7–10-day-old mice, RT-PCR revealed mRNA only for muscarinic M_1 and M_4 receptors (Da Silva et al. 2008). CCh produced an increase in $[Ca^{2+}]_i$ that was inhibited by atropine or pirenzepine. The sources of the cation were IP_3-sensitive intracellular stores of Ca^{2+} and an influx of extracellular Ca^{2+} through TRP channels. High concentrations of muscarine or McN-A-343 have also been shown to increase $[Ca^{2+}]_i$ in ca 50–70% of rabbit or rat Müller cells, an effect inhibited by pirenzepine or atropine (Wakakura et al. 1998). However, the role of muscarinic receptor activation in the physiological operation of Müller cells is not clear. Hyoscine did not block the transient increase in $[Ca^{2+}]_i$ in the cells induced by exposure of the rat retina to a constant or a flickering light, rather ATP appeared to be the mediator (Newman 2005).

Glycinergic amacrine cells are part of a negative feedback loop for cholinergic amacrine cells in the rat and rabbit retina (Neal and Cunningham 1995). Exposure of the retina to low frequency (3 Hz) flickering light evokes the release of ACh from the cholinergic amacrine cells. These amacrine cells are believed to be innervated by "ON" bipolar cells. The release of ACh can be modulated by activation of inhibitory glycinergic cells to release glycine to act back directly on the cholinergic cell, or alternatively, on another excitatory input to the cholinergic cell. Muscarine was shown to activate muscarinic receptors on the glycinergic cells to potentiate K^+-evoked release of glycine, thereby limiting release of light-evoked release of ACh by 54% (Neal and Cunningham 1995). The glycinergic amacrine cell, containing M_2 muscarinic receptors, is now proposed to be the DAPI-3 cell (Zucker et al. 2005).

CCh can activate muscarinic M_1/M_3 receptors to stimulate nitric oxide synthase (NOS) activity, cGMP production and nNOS mRNA expression in rat retina (Borda et al. 2005). Pirenzepine or 4-DAMP, but not AF-DX 116, blocked the CCh-induced increase in NOS and cGMP, with 4-DAMP producing the greater inhibition. The effect of CCh could also be reduced by blocking phospholipase C (PLC) or Ca^{2+}/calmodulin but not PKC.

In salamander retina, oxotremorine activated M_2 receptors on GABAergic amacrine cells of the inner retina to increase cGMP production via nitric oxide which in turn stimulated GABA release (Cimini et al. 2008). Muscarinic M_4 receptors were also detected in the outer nuclear layer and on bipolar cells, but were not involved in cGMP production.

15 Retinal Pigment Epithelium

In rat RPE [^{3}H]-QNB binding could be detected at postnatal day 5, increasing to a maximum from day 12 to 40 and then declining by ca 50% to adult levels by day 60 (Salceda 1994). Muscarinic receptor activation by CCh in rat RPE led to an increase in IP_3 production and an increased rate of phagocytosis of rod outer segments (Heth et al. 1995) but the latter finding could not be confirmed by Hall et al. (1996).

Naruoka et al. (2003) found ACh or muscarine produced an increase in $[Ca^{2+}]_i$ in explants of RPE from embryonic chick retina (E4–E5). The muscarinic receptors appeared to be the $M_{1/3}$ subtypes rather than the M_2 as muscarine was inhibited by pirenzepine or 4-DAMP, but not by gallamine.

The RPE of the bluegill fish (*Lepomis macrochirus*) expresses only M_5 muscarinic receptors although the retina expresses both muscarinic M_2 and M_5 receptors (Phatarpekar et al. 2005). The M_5 receptor was found to share 65.3% amino acid identity with human M_5 receptor. In the RPE, CCh activated a muscarinic receptor that led to pigment granule dispersion. Dispersion of the granules into the long apical processes of RPE cells interdigitated between rod photoreceptors protects the latter from bleaching in bright light. The effect of CCh was inhibited by a phospholipase C inhibitor or an IP_3 receptor antagonist. Only the muscarinic M_1 and M_3

receptor-preferring antagonists, telenzepine (pIC_{50}, 8.5) and pFHHSiD (pIC_{50}, 7.2), respectively, inhibited the action of CCh, methoctramine (10 μM) was ineffective. No selective M_5 antagonist was available but the order of potency of antagonists that have been tested was 4-DAMP > pirenzepine > telenzepine > pFHHSiD (González et al. 2004; Phatarpekar et al. 2005), supporting the suggestion of an M_5 receptor involvement. However, it should be borne in mind that fish muscarinic receptors may have different affinities to the corresponding mammalian subtypes for subtype-preferring antagonists. For example, the muscarinic M_2 receptor subtype in the zebra fish has been shown to have a high affinity for pirenzepine (pK_i, 7.18) (Hsieh and Liao 2002).

Fischer et al. (1998a) detected $M_{2–4}$ receptors in the chick RPE using specific antibodies. In chick RPE cells, ACh activated muscarinic receptors to raise intracellular Ca^{2+} levels by releasing intracellular stores of Ca^{2+}, an effect abolished by atropine or thapsigargin (Sekiguchi-Tonosaki et al. 2009). These cells have the ability to de-differentiate via intermediates into neuronal or lens cells or back into pigmented epithelial cells and offer the possibility for lens or retinal tissue regeneration. When these cells de-differentiated, ACh was also able to raise intracellular Ca^{2+} by opening L-type Ca^{2+} channels via a nicotinic receptor, as well as retaining the muscarinic response.

16 Lacrimal Gland

Cholinergic nerves passing to the lacrimal gland activate muscarinic receptors to cause lacrimal secretion (Mauduit et al. 1993). The vital role of muscarinic receptor activation can be appreciated by the effects observed on denervation or chronic blockade of muscarinic receptors. Parasympathetic denervation of the rat lacrimal gland leads to severe dry eye, corneal ulceration and dissolution of lacrimal gland structure with the development of pro-inflammatory genes in the gland (Nguyen et al. 2006). The effect occurs without any change in expression of the muscarinic M_3 receptor although it was speculated that loss of muscarinic function was responsible. Cholinergic activation of tear secretion also appears to be necessary to maintain the integrity of the conjunctival surface. Chronic administration of transdermal scopolamine to mice led to a decrease in both tear secretion and the corneal epithelial barrier function of the conjunctiva (Dursun et al. 2002). Exposing the animals to a desiccating environment via a continuous airflow blower exacerbated the condition, leading to a decrease in goblet cell density and an increase in proliferating epithelial cells; changes resembling those occurring in keratoconjunctivitis sicca.

In rat lacrimal gland CCh, oxotremorine, methacholine and pilocarpine stimulated ^{3}H-inositol phosphate production, the latter three being partial agonists for the response. The action of CCh was inhibited competitively by atropine, 4-DAMP, pirenzepine and AF-DX 116 with "functional K_B" values of 0.56, 1.8, 56 and 664 nM, respectively, indicative of M_3 subtype involvement (Mauduit et al.

1993). Also, the antagonists displaced ^{3}H-NMS, from a single site with a similar rank order of potency from lacrimal gland membranes and Northern blot analysis of lacrimal gland mRNA indicated a uniform population of muscarinic M_3 receptors.

There are muscarinic receptors located on secretory globular acinar cells and on stellate myoepithelial cells (Lemullois et al. 1996). The latter cells contract in response to CCh and may play a facilitatory role, assisting in the movement of secretion from the acini into the tear ducts (Satoh et al. 1997).

CCh induced an increase in intracellular Ca^{++} in both cell types but only the response of the myoepithelial cells to CCh was inhibited by lowering extracellular Ca^{++} (Satoh et al. 1997). Activation of secretion by CCh in the acinar cells involved PLCβ activation to generate IP_3, with subsequent release of intracellular Ca^{++} from stores by IP_3 with PKCα/ε and PKCδ activation (see Dartt 2004; Zoukhri et al. 2000). There was also influx of extracellular Ca^{++} through capacitative Ca^{++} entry, stimulated by the depletion of the intracellular Ca^{++} stores (Berridge 1995). Thus, an initial peak in intracellular Ca^{++} levels due to release of Ca^{++} from intracellular stores was followed by a plateau as capacitative influx occurred (Zoukhri et al. 1997a, b, 2000).

Phospholipase D (PLD) in rat lacrimal glands was also activated by CCh (1 mM), independent of both PKC and Ca^{2+}, to increase phosphatidic acid twofold and this effect was prevented by atropine (10 μM) (Zoukhri and Dartt 1995). PLD hydrolyses phosphatidylcholine preferentially to yield phosphatidic acid which may release Ca^{2+} and can be converted by a specific phosphohydrolase to diacylglycerol (DAG), a PKC activator.

In the lacrimal gland acinar cell, CCh does not transactivate the EGF receptor but the agonist activated p42/p44 MAPK by a pathway utilizing Ras, Raf, MEK, c-Src, Pyk2, PKC and Ca^{2+} (Dartt 2004; Hodges et al. 2006; Ota et al. 2003). MAPK, in turn, can inhibit CCh-induced protein secretion (Ota et al. 2003).

Aquaporins (AQPs) are apical plasma membrane water channels found in lacrimal glands, salivary glands and other tissues and facilitate the movement of water across cell membranes (Ishikawa et al. 2006). In mouse lacrimal gland, pilocarpine increased tear secretion 2.5-fold over control and increased the immunoreactivity of AQP5 on the intracellular side of the apical membranes of acinar and duct cells, but not on the extracellular side (Ishida et al. 1997). AQP4, located in the basolateral membrane, showed no change in immunoreactivity to antibody after pilocarpine.

17 Ocular Vascular System

All five muscarinic receptor subtypes were detected in mouse ophthalmic arteries with real-time PCR, the mRNA levels being higher for M_1, M_3 and M_5 subtypes than for the other two subtypes. ACh and CCh produced concentration-dependent vasodilatation in normal wild-type mice, following precontraction with phenylephrine. In receptor subtype-knockout mice, the $M_5-/-$ mice reacted to the agonists as for

the wild type. However, ACh and CCh were almost inactive in $M_3-/-$ mice whereas responses to non-cholinergic vasodilators were unaffected indicating that M_3 receptors mediate the vasodilation to muscarinic agonists (Gericke et al. 2009).

In rat uveal vortex veins, electrical stimulation of the superior salvitory nucleus activated ocular parasympathetic nerves to increase blood flow (Steinle and Smith 2000). This was mediated by nitrergic nerves as the flux response was inhibited by a selective neuronal NOS inhibitor and was unaffected by atropine. Following acute (2 days) or chronic (6 weeks) sympathectomy, the flux increase on stimulation of the parasympathetic nerves was unaltered, but atropine selectively abolished the flux after chronic sympathectomy. It was postulated that sympathectomy led to a down-regulation of nitric oxide release and development of a compensatory cholinergic facilitation. Thus, the effect of atropine was due to blockade of a prejunctional facilitatory muscarinic receptor on the nitrergic nerves. In support of this explanation, bethanecol enhanced parasympathetically mediated vasodilation only in the chronically sympathectomized animals and not in the acutely sympathectomized without affecting basal flow.

It was concluded the muscarinic receptor(s) involved after chronic sympathectomy were both M_3 and M_5 receptor subtypes as 4-DAMP reduced the increased flux by 30% and atropine reduced it further, whereas methoctramine and tropicamide were ineffective. Pirenzepine increased the flux 40% beyond control suggesting the possibility of inhibitory prejunctional M_1 receptors also being present.

In rat retinal vessels, CCh stimulated iNOS activity and iNOS-mRNA gene expression to increase vascular diameter, by activation of muscarinic receptors (Berra et al. 2005). The responses were inhibited by atropine, pirenzepine and 4-DAMP, but not by AF-DX 116 suggesting involvement of M_1/M_3 receptors. The effect of CCh was also reduced by inhibition of PLC or PKC but not by inhibition of Ca^{2+}/calmodulin.

18 Glaucoma

Glaucoma is a condition in which there is excessive intraocular pressure, leading to impairment of the blood supply to the retina, with ensuing blindness. It develops when the outflow of aqueous humour from the eye is impaired. The aqueous humour is produced in the ciliary process, being secreted into the anterior chamber in front of the lens and behind the iris. It flows through the pupillary opening and exits the anterior chamber mainly through the trabecular meshwork in the angle between the front of the iris and the back of the cornea. There is an alternative outflow route, the uveoscleral outflow. This occurs by seepage of the aqueous humour, through an incomplete endothelium on the ciliary body, into the interstitial spaces between the ciliary muscle fibres and then ultimately into the episcleral tissues. In the anaesthetized monkey, the uveoscleral outflow constitutes ca

30–50% of the total aqueous outflow, whereas in humans it is only ca. 5–20% (see Sugrue 1989).

The rationale for the use of muscarinic agonists, such as aceclidine and pilocarpine, in the two main types of glaucoma differs. In narrow angle glaucoma, which constitutes ca 10% of cases, the underlying problem is a narrow angle between the front of the iris and the back of the cornea which limits the access of aqueous humour to the base of the angle where the trabecular meshwork and the canal of Schlemm are situated. This anatomical defect may be exacerbated in conditions leading to pupillary dilatation resulting in the iris being crowded back into the narrow angle. While the ultimate treatment of this condition involves surgical or laser treatment to insert an opening in the iris thereby improving access of aqueous humour to the meshwork, muscarinic agonists may be used acutely to constrict the pupil, causing miosis, drawing the iris out of the angle, thereby lowering the intraocular pressure.

In open-angle glaucoma, the most common form of the condition, there is an obstruction to outflow of aqueous humour within the trabecular meshwork. Muscarinc agonists administered topically can be used to lower intraocular pressure by causing contraction of the ciliary muscle. This results in a conformational alteration of the associated trabecular meshwork, reducing the resistance to outflow of the aqueous humour through the meshwork towards the canal of Schlemm. Studies in cynomolgus monkeys have shown that section of the ciliary muscle's attachment to the region containing the trabecular meshwork and reinsertion of the muscle further back on the inner scleral wall still allowed the ciliary muscle to contract to pilocarpine but without the marked increase in outflow facility seen in the contralateral eye (Kaufman and Bárány 1976; Lütjen-Drecoll et al. 1977).

Recently, Erickson and Schroeder (2000) found that low concentrations (1 nM–1 μM) of the muscarinic agonists, aceclidine, CCh and pilocarpine, were able to increase outflow facility in isolated, perfused, anterior segments of human eye with the lens and ciliary muscle removed. Experiments were conducted on eyes, enucleated within 9 h and dissected within 15.5 h *post-mortem*. The greatest increase in outflow facility (~100%) was obtained with aceclidine, followed by CCh (~50%) and pilocarpine (~30%). High concentrations of the agonists (100 μM–10 mM) were ineffective. In contrast, Kiland et al. (2000) found that low concentrations of pilocarpine (0.1 nM−1 μM) perfused into the anterior chamber in vivo failed to increase outflow facility, accommodation or miosis in the cynomolgus monkey. All three parameters required a concentration of 10 μM to obtain a significant response. It was concluded that the outflow facility would only be increased effectively by concentrations of pilocarpine that contracted the ciliary muscle.

It was concluded that the increase in outflow facility, as well as contraction of the sphincter pupillae and the ciliary muscle, by pilocarpine in the rhesus monkey involved the muscarinic M_3 receptor, based on studies with 4-DAMP, pirenzepine and AF-DX 116 (Gabelt and Kaufman 1992).

CCh has also been reported to inhibit the Na, K-ATPase involved in the production of aqueous humour in the bovine ciliary body by a mechanism utilizing

nitric oxide (NO), soluble guanylate cyclase and cGMP (Ellis et al. 2001) but whether this also contributes to the reduction in intraocular pressure with aceclidine or pilocarpine does not appear to have been tested. However, pilocarpine (0.5%) has been reported to have no practical effect on the production of aqueous humour in humans (Nagataki and Brubaker 1982).

It may be possible to separate the activity of the ciliary muscle on accommodation and on outflow facility since contraction of the circular division of the ciliary muscle is responsible for accommodation while contraction of the longitudinal division influences outflow. Erickson-Lamy and Schroeder (1990) showed that the muscarinic agonist, (±)-aceclidine, produced a greater effect on outflow relative to its effect on accommodation, than did pilocarpine. However, the reason for this finding is not readily apparent. (±)-Aceclidine was found to have EC_{50} values of 25 μM in the human longitudinal ciliary muscle, 20 μM in the circular ciliary muscle, being a partial agonist producing ~85% of the response to CCh in both tissues (Ishikawa et al. 1998). An EC_{50} value of 0.9 μM (E_{max}; 96%) was obtained in the human iris sphincter muscle. Subtype-preferring antagonists had similar affinities in the iris and the two ciliary muscles also. The S-(+)- enantiomer is ca two- to fourfold more potent at all five types of muscarinic receptor than the R-(−)-enantiomer, the latter also being a partial agonist at all subtypes (Ehlert et al. 1996, Griffin et al. 2007). Relative to carbachol, each enantiomer shows ca threefold preference for M_1 and M_2 receptors over M_3 receptors (Ehlert et al. 1996).

It is known that muscarinic agonists increase outflow facility in normal as well as glaucomatous eyes, but because of homeostatic influences there is little change in intraocular pressure in normal eyes (see Potts 1965). Recent findings suggest that there may be a greater cholinergic influence on outflow regulation in glaucomatous eyes. In a rat model of congenital glaucoma, treatment with botulinum toxin A to block cholinergic nerve function, led to an increase in intraocular pressure, not observed in the normotensive control (Gatzioufas et al. 2008).

Clinically, the use of pilocarpine in humans to lower intraocular pressure is not affected by the age-related decline in ciliary muscle mobility (Croft et al. 1996) or the use of argon laser trabeculoplasty (Teus et al. 1997). The use of pilocarpine to treat glaucoma has declined with the increase in the number of alternative drugs to lower intraocular pressure with a lower incidence of side effects, such as the β-adrenoceptor antagonists and prostaglandin $F_{2\alpha}$ analogues. The effect of pilocarpine or latanoprost in combination with timolol has been compared with latanoprost monotherapy in three multicentre, randomized, clinical trials in Europe (Diestelhorst et al. 2002). Latanoprost alone, and the combination of timolol and latanoprost, were both more effective and better tolerated than the combination of timolol and pilocarpine.

Latanoprost lowers intraocular pressure primarily by increasing uveoscleral outflow, whereas pilocarpine increases outflow through the trabecular meshwork and reduces uveoscleral outflow, at least in the cynomolgus monkey (Bill and Wålinder 1966; Bill 1967), probably because it contracts the ciliary muscle. Nevertheless, a review of four published clinical trials showed the effect of latanoprost was not reduced by pilocarpine and the combination of the two drugs

produced a greater decrease in intraocular pressure than either alone (Toris et al. 2002).

19 Myopia

Myopia (near-sightedness) is the state of refraction where parallel rays of light are brought to a focus in front of the retina of a resting eye. The condition is caused by elongation of the eyeball so that the eye has difficulty in focusing on distant objects. In Europe and the USA, the incidence is ~25% whereas in Singapore and other East Asian countries, the incidence may be 70% or more (Saw et al. 1996; Seet et al. 2001). The incidence can vary between ethnic groups within the same community and be influenced by the amount of close work undertaken (Saw et al. 1996, 2006).

Several clinical trials have shown that atropine, 0.05 or 1%, (Chua et al. 2006; Fan et al. 2007; Lee et al. 2006) or pirenzepine (Siatkowski et al. 2008; Tan et al. 2005) is effective in limiting the development of myopia in children. A 2-year study in a group of Asian children found atropine (1% topical ointment daily) almost completely suppressed axial elongation and decreased the rate of progression of low or moderate myopia (Chua et al. 2006). Atropine appeared to be non-toxic to retinal function, producing no effect on multifocal electroretinograms in a 2-year study in children (Luu et al. 2005) and the benefit of atropine was still apparent, 1 year after cessation of the drug (Tong et al. 2009).

Similarly, myopia progressed less in children treated over 1 or 2 years with pirenzepine (2% gel, topical twice daily) than in the placebo groups (Siatkowski et al. 2008; Tan et al. 2005). The drug was well tolerated, only 11% of patients on pirenzepine discontinued treatment over the first year in both trials. Pirenzepine ($\leq$2% gel) produces little mydriasis or effects on accommodation in myopic children (Bartlett et al. 2003; Siatkowski et al. 2008; Tan et al. 2005).

Recently, it was reported that the S2 and S4 polymorphisms of the muscarinic M_1 receptor gene *CHRM1* are associated with susceptibility for developing high myopia and that S1, S2 and S4 *CHRM1* had a cooperative association with high myopia (Lin et al. 2009). No association was found for S3 *CHRM1*.

The mechanism of action of atropine or pirenzepine in myopic eyes has been the subject of several animal studies since Wiesel and Raviola (1977) demonstrated that myopia could be induced experimentally by occlusion of one eye ("form-induced myopia"). Other techniques have included use of a black contact lens in monkeys (Tigges et al. 1999) and use of translucent diffusers in the chick (Diether et al. 2007; Vessey et al. 2002), guinea pig (Qiong et al. 2007) or tree shrew (Cottriall and McBrien 1996; McBrien et al. 2009). Wearing negative lens is another technique ("lens-induced myopia") (Diether et al. 2007; Cottriall and McBrien 1996).

Atropine was shown to inhibit experimental myopia in the rhesus monkey (Tigges et al. 1999) and chick (Stone et al. 1991) and pirenzepine in the tree shrew (Cottriall and McBrien 1996) and chick (Stone et al. 1991). The fact that

pirenzepine as well as atropine is effective in preventing myopia may indicate that the muscarinic M_1 receptor is the important subtype involved. However, while pirenzepine was effective in preventing myopia in the chick, M_1 receptors could not be detected in chick ocular or brain tissue (Yin et al. 2004). Furthermore, the dose of pirenzepine (17.7 μmol daily) was calculated to have produced concentrations of 100 μM in the choroid and sclera, 10 μM in the retina and 1 μM in the vitreous humour. These concentrations would block a substantial percentage of all subtypes of muscarinic receptor in the choroid, sclera and retina, given that pirenzepine has lowest affinity for the M_2 subtype in the reported range of pK_i/pK_b values, 6.0–6.8.

Himbacine (ED_{50} 480 μg, injected intravitreally) inhibited vitreous chamber elongation in the chick leading to a suggestion that muscarinic M_4 receptors also may be important in the development of myopia (Cottriall et al. 2001b) but the subtype preference of himbacine is not as great as pirenzepine and other subtypes could be involved.

Early studies found little or no direct evidence for the involvement of cholinergic nerves or muscarinic receptors. Steady state choline and ACh levels in the retina were not altered during development of experimental myopia in the chick or tree shrew (McBrien et al. 2001). Muscarinic receptors in the chick retina and choroid were not altered in myopia (Vessey et al. 2002) and cholinergic amacrine cells in the retina were not required for the development of form-deprived myopia or its suppression by atropine in the chick (Fischer et al. 1998b). In the rhesus monkey, topical application of atropine or pirenzepine led to a small increase in muscarinic receptor density in the ciliary body and iris, but not in the retina (Tigges et al. 1999). No changes in gene expression or protein expression for the five muscarinic receptor subtypes were detected in the retina, choroid or sclera in form-deprived myopia in the tree shrew (McBrien et al. 2009).

Luft et al. (2003) studied a number of muscarinic receptor antagonists injected intravitreally in Leghorn cockerels and found that only atropine, pirenzepine and oxyphenonium were fully effective at inhibiting form-deprived myopia; AF-DX 116, 4-DAMP, dexetimide, HHSiD, pFHHSiD, propantheline, QNB, scopolamine and tropicamide were partially effective, while dicyclomine, gallamine, methoctramine, mepenzolate and procyclidine were all ineffective or were toxic in the concentrations used. Propantheline and 4-DAMP also produced retinal damage. The antagonists were all used in high concentrations ranging from 0.1 to 10 mM, except for methoctramine (0.5 μM).

Dopamine is another neurotransmitter with a possible involvement in myopia. Both dopamine and its metabolite, DOPAC, were found to be decreased in myopic eyes of the tree shrews and chicks (McBrien et al. 2001). Atropine, injected intravitreally, increased the release of dopamine from the retina in form-deprived myopia in the chick and also produced a spreading depression of light-induced potentials in an in vitro retina-pigmented epithelium-choroid preparation (Schwahn et al. 2000). It was suggested that the spreading depression enhanced neurotransmitter release from tissue stores, inhibiting some presumed retinal signal controlling eye growth and thus, myopia.

It is of interest that the organophosphorus anticholinesterase, dyflos (DFP), injected intravitreally in the form-deprived chick eye over an 8-day period also reduced myopia 58% compared with the contralateral control eye (Cottriall et al. 2001a). Open eyes injected with DFP showed no refractive errors or increase in vitreous chamber depth compared to the contralateral control eye. A single intravitreal injection of DFP also raised dopamine levels 36% and ACh 54% in retinal tissue of the form-deprived eye compared to the control eye. The effect on dopamine was observed 1.5 h after injection of DFP but had returned to control levels by 3 h. Injection of a dopamine D_2 receptor antagonist, spiperone, reduced the inhibitory effect of DFP on myopia development suggesting that retinal dopamine release was an important factor in controlling myopia development. Since DFP produces irreversible inhibition of cholinesterases, it could be acting via a desensitization or down-regulation of muscarinic receptors to influence dopamine release.

Atropine, in high concentrations, was found to inhibit cellular proliferation and extracellular matrix production in chick sclera and along with pirenzepine and 4-DAMP was found to inhibit sulphate incorporation into glycosaminoglycans in isolated scleral chondrocytes (Lind et al. 1998), offering a possible explanation for the effectiveness of atropine and pirenzepine in myopia. Pirenzepine was also ~sevenfold more potent in myopic eyes than in normal eyes. Diether et al. (2007) found that atropine countered both lens-induced myopia and form-induced myopia in the chick, and also suggested that atropine had a direct inhibitory action on scleral chondrocytes. However, Truong et al. (2002) found pirenzepine caused only a transient reduction in glycosaminoglycan synthesis in chick cartilaginous sclera, 2 h after administration, but this was not evident at 6 h. The effect was present in both non-occluded and occluded eyes and was not observed in fibrous sclera. Also, there was no change in scleral DNA at any time point, excluding a toxic effect. The change in glycosaminoglycan synthesis could also be produced by exposing an occluded eye to brief periods of unoccluded vision, a technique that also prevents experimental myopia.

Recently, Qiong et al. (2007) found mRNA for the five subtypes of muscarinic receptor in the retina, choroid, sclera and iris–ciliary body of the guinea pig eye. After induction of form-deprived myopia, over 21 days, there was a significant increase in the posterior sclera for mRNA expression of the M_1 (+18.7%) and M_4 receptor subtype (+26.5%) as well as corresponding protein expression (+24.7%; +49.1%, respectively) with no significant changes in the other regions. These findings contrast with those obtained in the tree shrew by McBrien et al. (2009) where no change was observed in muscarinic receptor gene expression in the sclera, retina or choroid during myopia induction over 5 days.

Thus overall, there is no uniform agreement as to why atropine and pirenzepine are clinically usefully in myopia and produce similar effects at equivalent doses in experimental myopia in the rhesus monkey. The concentrations of muscarinic receptor antagonists required to inhibit RNA and glycosaminoglycan synthesis in chick scleral chondrocytes are high (Lind et al. 1998) and do not equate with the effective clinical doses and furthermore, chondrocytes are not present in the sclera

of monkeys (Tigges et al. 1999). The finding of selective increases in muscarinic M_1 and M_4 receptor protein only in the sclera of the guinea pig with experimental myopia (Qiong et al. 2007) and the transient changes in cartilaginous scleral glycosaminoglycan synthesis induced by pirenzepine in the chick, similar to that induced by brief periods of non-occlusion (Truong et al. 2002) suggest that further investigation of scleral tissue may yield an explanation.

20 Sjögren's Syndrome

SS is an autoimmune disorder characterized by xerophthalmia and xerostomia (Vitali et al. 2002) but may be accompanied by other autonomic symptoms such as mydriasis and bladder hyper-reactivity. These first two symptoms are considered to arise because disruption of the lacrimal and salivary exocrine glands follows lymphocyte infiltration, release of cytokines, inflammation and glandular destruction.

Patients with SS have numerous antibodies in their serum but there is no clear correlation between sicca symptoms of dry eye and dry mouth and the antibody titre and many of these antibodies occur in other autoimmune disorders without sicca. However, Bacman et al. (1996, 1998, 2001) reported that autoantibodies to the M_3 muscarinic receptor were present in the serum IgG of primary and secondary SS patients. These antibodies were detected using the M_3 muscarinic receptor in rat lacrimal and parotid gland.

In both glands the antibodies in IgG could displace [^{3}H]-QNB, non-competitively, from the muscarinic M_3 receptor. In the lacrimal gland the antibodies acted like CCh to increase NOS activity and cGMP production (Bacman et al. 1998). Atropine and 4-DAMP inhibited the action of the antibodies on NOS activity (Bacman et al. 1998) and on phosphoinositide turnover (Bacman et al. 1996). There was one difference between CCh and the antibodies; the PKC inhibitor, staurosporine, partially reduced the effect of CCh on NOS activation but did not alter that of the antibodies. The PLC inhibitor, neomycin, and the Ca^{2+}/calmodulin inhibitor, trifluoperazine, inhibited NOS activation by CCh or the antibodies. These findings suggest that the antibodies used only the calcium/calmodulin-dependent pathway for NOS activation, whereas CCh also used a pathway involving PKC.

It was suggested that continuous NO production by the antibodies may be cytotoxic to the lacrimal gland in line with a previous conclusion of Konttinen et al.(1997) or there may be receptor desensitization, internalization and/or degradation leading to a progressive receptor blockade and dry eye (Bacman et al. 1998).

In contrast, Waterman et al. (2000) suggested that the serum of some patients with SS contains autoantibodies that act as antagonists of muscarinic M_3 receptors. They demonstrated that serum and the purified IgG fraction from patients with primary or secondary SS contained antibodies that maximally inhibited responses

to CCh in mouse isolated bladder by ca 50%, whereas the muscarinic receptor antagonist 4-DAMP abolished the response. Endogenous ACh released by parasympathetic nerve stimulation appeared to be completely inhibited by the antibodies in that the residual response to nerve stimulation could be almost abolished by desensitization of the tissue to the purine receptor ligand α,β-methylene ATP with the subsequent addition of 4-DAMP causing no further reduction. Such a difference in the ability of a compound to inhibit various muscarinic agonists is typical of an allosteric modulator rather than a competitive antagonist. It was also reported, but not commented on in their discussion, that 60% of the sera from primary SS patients (3/5) and 33% of that from secondary SS patients (2/6) produced a contraction of the bladder which could be prevented by prior treatment of the tissue with 4-DAMP. The response when it occurred waned over the course of several minutes after which responses to CCh were inhibited. Thus, in these cases the serum contained antibodies that exhibited initial muscarinic agonist activity, similar to that observed by Bacman et al. (1996, 1998). Perhaps the autoantibodies function as a bitopic agonist (like McN-A-343) having both agonist and antagonist binding sites on the receptor (Valant et al. 2008) or that the tissue is readily desensitized by persistent binding of the antibody which occurs at variable rates such that an underlying agonist action is not always observable. One further complication was that the mouse bladder experiments were conducted in the presence of hexamethonium (C_6) (100 μM) to inhibit nicotinic ganglionic receptors. However, C_6 can interact at muscarinic receptors in the high concentrations employed (Leung and Mitchelson 1982) and therefore may be modifying the action of the antibody with its binding sites on the muscarinic receptor.

As other antibodies to GPCRs couple to epitopes on the extracellular loops of the receptor it has been considered that those in SS may bind similarly. Bacman et al. (2001) reported that serum from SS patients reacted with a 25-mer peptide corresponding to what was thought to be the second extracellular loop of the human muscarinic M_3 receptor, but was in fact the M_4 receptor due to an incorrect entry in GenPept (see Cavill et al. 2002). These findings could not be replicated by Cavill et al. (2002). However, they were able to show that antibodies raised in rabbits against the second extracellular loop of the human muscarinic M_3 receptor mimicked the action of autoantibodies from the serum of SS patients in antagonizing the contractile response to CCh on mouse isolated colon (Cavill et al. 2004).

In a mouse model of SS, the MRL/MpJ-*Fas*^{lpr} mouse, conjunctival changes occur between the 9th and 16th week which are less pronounced than the changes in the lacrimal gland but also involve T cell invasion without tissue destruction. The conjunctival goblet cells remain filled which was attributed to a decreased expression of muscarinic M_3 receptors (Diebold et al. 2007).

Clinically muscarinic agonists, pilocarpine (5 mg qid) and cevimeline (20–30 mg tid) have been shown to alleviate xerophthalmia in trials on SS patients (Vivino et al. 1999; Petrone et al. 2002; Ono et al. 2004) but cevimeline has not been successful in all trials (Leung et al. 2008).

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