REVIEW

Muscarinic receptor functioning and distribution in the eye: molecular basis and implications for clinical diagnosis and therapy

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The role of neurotransmitters has generated considerable interest over the last decade. Dale's first description in 1914 of the muscarinic and nicotinic components of the cholinergic system[1] provided an explanation for the effects of various cholinergic active drugs on the eye. Parasympathetic cholinergic input to the human iris sphincter muscle comes from neurons whose axons make up the ciliary nerve, a branch of the third cranial nerve. Acetylcholine is released by these neurons onto their target cells, the smooth muscle surrounding the pupil. Muscarinic acetylcholine receptors on the surface of the muscle cells transduce the chemical signal into a muscle contraction which constricts the pupil. It has also been shown that muscarinic cholinergic receptors exist in the mammalian iris dilator muscle, once thought to receive only noradrenergic input from the sympathetic system.[2] This double reciprocal innervation of the iris sphincter follows the general pattern of innervation: stimulation of the parasympathetic nervous system (cholinergic muscarinic), which functions through the polyphosphoinositide signalling pathway, leads to contraction. Relaxation is a result of the activation of the sympathetic nervous system (beta-adrenergic), which functions through the cAMP system.

The active secretion of aqueous humour is carried out by the ciliary epithelium and is therefore a key target for regulation by endogenous regulators and anti-glaucoma drugs. Histological evidence indicates that the ciliary processes receive innervation by both sympathetic and parasympathetic nerves. Further on, ciliary epithelial cells have been demonstrated to contain both adrenergic and cholinergic receptors.[3] Interactions between the two second messenger systems are important in regulation of smooth muscle tone and are an important focal point for pharmacological manipulation.[4] Besides these well-established functions of the ocular receptor interplay a vast number of receptors (cholinergic, adrenergic and others) have been found in all types of ocular tissue: the functional consequence of their activation remains elusive but is currently being investigated with great zeal. Glaucoma patients are still being treated with pilocarpine almost 40 years[5] after its introduction to western medicine in 1875.[6] The development of specific muscarinic agonists (acetyl-β-methylcholine) and antagonists (scopolamine) followed. Cholinesterase was discovered in 1926[7] and named in 1932.[8] At the same time carbachol was synthesised,[9] a drug resistant to cholinesterases with a suitable specificity for glaucoma treatment.[10,11] Over the years various compounds have been investigated regarding their potential to lower intraocular pressure (IOP), the most prominent pathophysiological feature of glaucoma. The majority of these substances do not affect the muscarinic system of the eye and intervene at different receptor sites.

The ciliary muscle, focus of intense investigation, contracts through activation of muscarinic receptors. Due to its insertion into the trabecular meshwork it increases aqueous outflow facility, thereby reducing IOP.[12,13] A variety of drugs can also reduce IOP, yet by very different mechanisms of action. This indicates the pathogenetic complexity of glaucoma, with its multiple possible causes; however, IOP regulation through the muscarinic signalling system appears to be an important component. The ocular muscarinic receptor system is not dedicated solely to the maintenance of pressure homeostasis though. A wide distribution of these receptors in the human eye has been found. Muscarinic signalling is involved in signal transduction functions of the retina,[14] possibly in reparative functions in the corneal and lenticular tissue,[15,16] and appears to play a major role in the embryonic and postnatal development of the eye.[17] The main distinction between

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muscarinic and nicotinic receptors, though noted very early, hardly explained their distinct roles in cholinergic signalling. The past decade, however, has seen the molecular cloning of both nicotinic[18] and muscarinic[19] acetylcholine receptors in Numa's laboratory,[20] leading to greatly expanded understanding of these systems. In addition, new research techniques such as patch clamping[21] and single channel recording[22] have provided additional insights into the functioning of the cholinergic signalling system.

We now know that, although acetylcholine is the physiological agonist on both nicotinic and muscarinic receptors, they are completely different entities: the first a multi-subunit, ligand-gated ion channel (i.e. an ionotropic receptor), the second a single-subunit, G protein-coupled receptor (i.e. a metabotropic receptor). It appears likely that all muscarinic receptor subtypes have now been cloned, allowing development of specific antibodies,[23] detailed mapping of tissue distribution, and synthesis of subtype-specific agonists and antagonists (Table 1a).[24–26] It has become clear that muscarinic signalling plays an important role in multiple locations of the eye, and that ocular cholinergic drugs interfere significantly with this system. This article will focus on the molecular basis of these findings. It will show that the complex distribution of muscarinic receptors in the eye is only a part of many interacting signalling systems, all resulting in the development and maintenance of vision. Following a brief summary of the molecular biology of muscarinic receptors, their distribution and function in the human eye will be described. A description of the clinical implications of these signalling pathways and their interactions in pathological processes will be outlined.

Molecular biology of muscarinic signalling

The first muscarinic receptor was cloned in 1986.[19] In the 13 years that have passed, a remarkable amount of information has been gathered about the molecular biology of muscarinic signalling. Not only have (presumably) all subtypes of muscarinic receptors been cloned, but detailed information on their structure-activity relationship is available, which will prove useful in the development of new, highly selective agonist and antagonist drugs.

Muscarinic receptors belong to the G protein-coupled receptor superfamily

When the DNA encoding the muscarinic receptor had been isolated, it was compared with previously cloned sequences, and its closest relative was found to be the visual pigment rhodopsin.[19] Although at first this may appear to be an unusual relationship, the sequence similarity relates to the fact that similar intracellular systems transduce the signals generated by these molecules. In both cases, a GTP-binding protein (G protein) forms an intermediate between membrane receptor and intracellular second messenger. More than a thousand receptor types have now been shown to belong to the G protein-coupled receptor (GCR) superfamily, of which the muscarinic receptors form a small but distinguished cluster.

GCRs all show the same molecular signature in their amino acid sequence: most are around 500 amino acids in length and include seven stretches of approximately 20 hydrophobic amino acids each. These domains are thought to form α-helices traversing the membrane, leading to the designation of these proteins as seven-transmembrane, or, more fancifully, serpentine or heptahelical receptors (Fig. 1).

The G proteins stimulated by receptor activation control a number of intercellular systems. Best described are G proteins stimulating (G_s) and inhibiting (G_i) adenylate cyclase, with corresponding changes in cAMP levels. Phospholipase C, activated by G_q or G_o, generates inositol trisphosphate (IP_3, which releases Ca^{2+} from intracellular stores) and diacylglycerol (which activates protein kinase C). In addition, G proteins can activate ion channels, as in the case of G_k (a G_i subtype), which closes a neuronal potassium channel in response to muscarinic stimulation.

Five muscarinic receptor subtypes have been cloned

Once the DNA sequence of one muscarinic receptor was known[19] other subtypes were isolated in rapid succession. Thus far, five muscarinic receptor have been cloned,[28] designated m1, m2, m3, m4 and m5. The existence of this many subtypes was surprising, as pharmacological studies suggested initially only two (M1 and M2). Glandular M2 receptors were designated M3. (Names with a capital 'M' indicate pharmacologically defined subtypes, whereas those with a small 'm' indicate clones.) Four pharmacological subtypes have now been defined (M1, M2, M3, M4).[29,30] This apparent excess of subtypes is typical for GCRs, and presumably allows finer regulation of receptor expression. The five subtypes fall into two groups – the 'odd' (m1, m3, m5) and the 'even' (m2, m4) – based on sequence homology and second messenger signalling. The odd group signals primarily through intracellular Ca^{2+} ; the even group through decreases in cAMP production. In the brain or retina, where signalling systems eventually have to transduce their actions through changes in membrane potential, m1 and m3 inhibit a G protein-coupled potassium current (I_M) and activate a Ca^{2a}-activated potassium current ($I_{K(ca)}$), a whereas m2 and m4 receptors inhibit the I_{Ca} current through voltage-activated Ca^{2+} channels (Fig. 2).[31] Although the clones were numbered in the order they were identified, the m1 clone happens to show most of the properties of the pharmacological M1 type, and the m2 clone those of the M2 type; similar correspondences exist with the m3/M3 and m4/M4 combinations. The presence of the m5 subtype in several brain regions and in the eye is documented, although its function and pharmacological profile remain to be established.

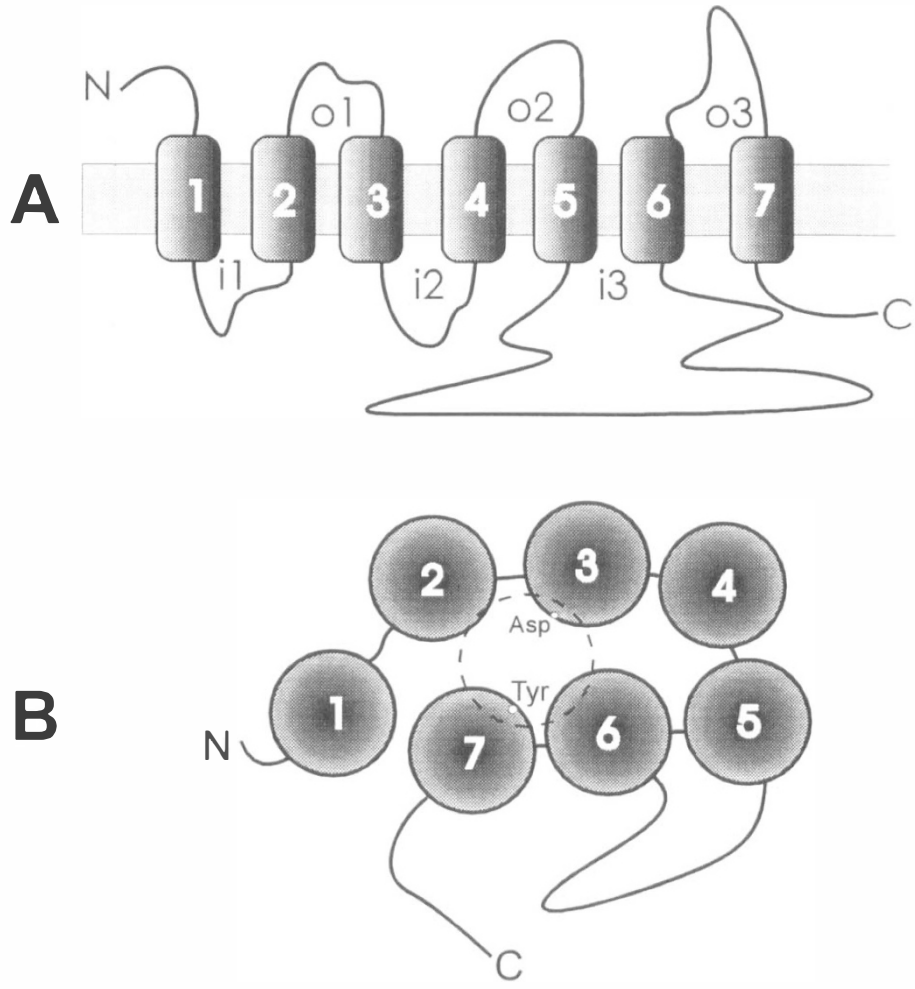

Fig. 1. *Model of a muscarinic acetylcholine receptor. (A) Linear model. The whole molecule is approximately 500 amino acids long. Seven hydrophobic stretches of approximately 20 amino acids are present, presumably forming α-helices that pass through the cell membrane, thus forming seven transmembrane domains (t1–t7). Extracellularly the aminoterminus (N) and three outside loops (01–03) are found; intracellularly there are similarly three loops (i1–i3), and the carboxyterminus (C). (B) Top-down view. Although in (A) the molecule is pictured as a linear complex, the transmembrane domains are thought to be in close proximity, forming an ellipse with a central ligand-binding cavity (indicated by a dashed circle). Asp and Tyr refer to two amino acids important for ligand interaction. G protein binding takes place at the i3 loop and the carboxyterminus. From Durieux.*[27]

Muscarinic receptor functions are related to molecular domains

The cloned muscarinic receptor subtypes and other members of the superfamily have been used to determine the intramolecular sites involved in ligand binding and G protein coupling. As the muscarinic subtypes show 89–98% amino acid sequence identity in mammalian species, specificity of ligand binding and G protein coupling must depend on relatively small changes in structure. In agreement with their functional grouping, the odd and even receptors show particularly high within-group similarity. There is, however, a remarkable lack of sequence similarity in the third intracellular loop (i3, Fig. 1), with the exception of the first and last 15 to 20 amino acids. Studies of bacteriorhodpsin (a related molecule for which the three-dimensional structure has been established) and adrenergic receptors have demonstrated that ligand binding takes place in a pocket, primarily consisting of the second, third and seventh transmembrane regions (t2, t3, t7),[32] whereas the i3 loop and the carboxyterminus (C) are involved in G protein binding[33] and regulation through phosphorylation.[34] In muscarinic receptors, the G protein binding specificity has been mapped to a remarkably small domain of approximately 20 amino acids in the i3 loop.[35] As in adrenergic receptors,[36] agonist binding in muscarinic receptors is initiated by contact with a specific aspartate residue in t3.[37] Exchange of (part of) t6, i3, t7 and i4 between the m2 and m3 subtypes resulted in a change in G protein coupling and subtype-selective ligand binding.[38] Mutation studies have shown a series of threonine and tyrosine residues in t3, t5, t6 and t7 that are of importance in agonist, but not antagonist binding,[39] again demonstrating the role of transmembrane domains for ligand binding. Thus, the functional domains of these receptors are well established and the importance and potential of highly selective ocular drugs for these domains can be foreseen.

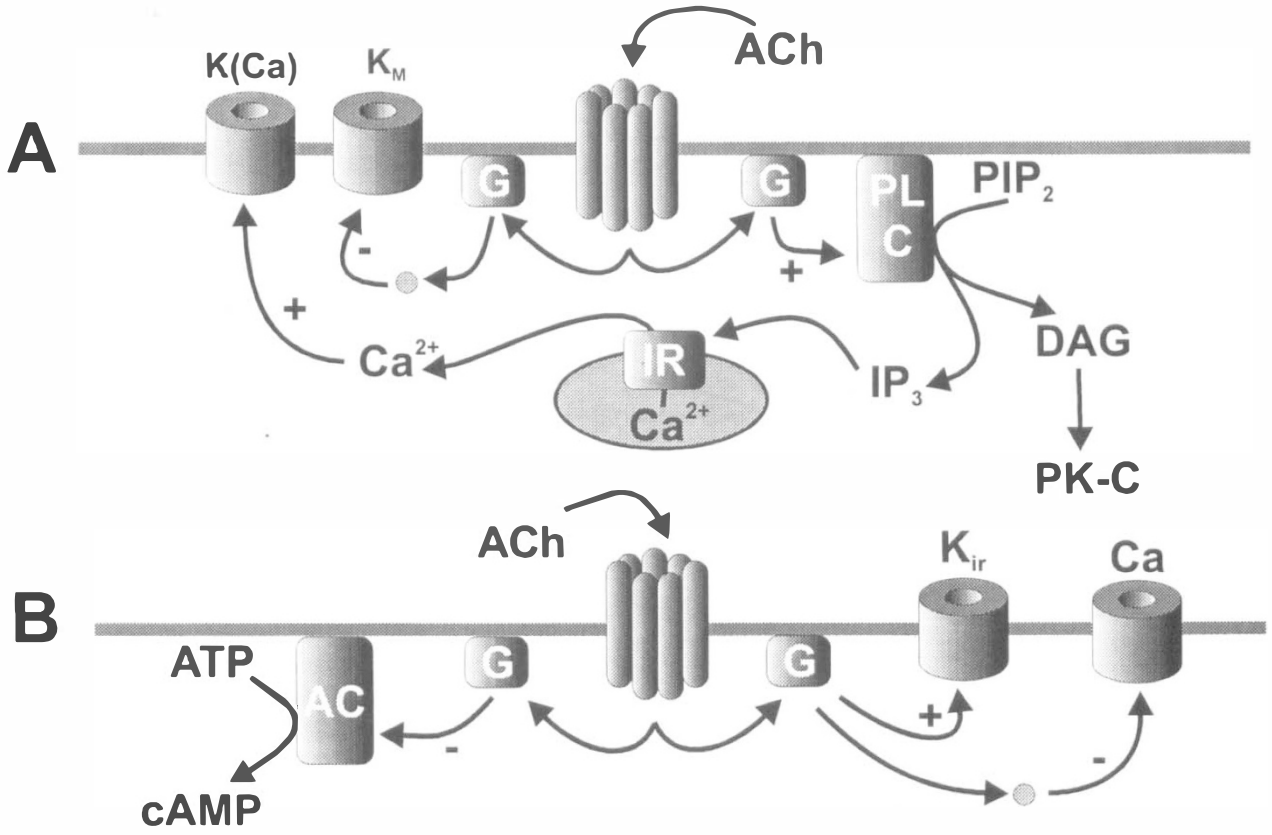

Fig. 2. *Intracellular signalling by muscarinic receptors. A composite illustration of the intracellular signalling pathways employed by muscarinic receptors. (A) Signalling through a receptor of the 'odd' group. The receptor (indicated by a stylised 7-transmembrane model) is activated by acetylcholine (ACh) and stimulates two main classes of G protein (G). One class, consisting of members of the G_0 and G_q, families, activates phospholipase C (PL-C). This results in the breakdown of phosphatidylinositol bisphosphate (PIP_2) to inositol trisphosphate (IP3) and diacylglycerol (DAG). IP_3 acting through its own receptor (IR) releases Ca^{2+} from internal stores, which can activate Ca-activated K channels ($K_{(Ca)}$). However, in neurons, $I_{K(Ca)}$ is often inhibited by muscarinic stimulation via unclear pathways. DAG activiates protein kinase C (PK-C). The other G protein, presumably $G_{q/11}$, closes K channels (K_m) in neuronal membranes through an as yet unidentified intermediary. (B) Signalling through a receptor of the 'even' group. Again several G proteins are involved. One, presumably a member of the G_i class, inhibits adenylate cyclase (AC) resulting in a decrease in the conversion of ATP to cAMP, and thus decreased cAMP levels. Another G protein, probably G_o, inhibits an N-type Ca channel (Ca) through an unidentified intermediary. In cardiac tissue (and possibly in neurons), activation of G_k directly opens a K_{ir} channel. Specific types of G proteins have not been indicated in the figure, as most have not been formally identified in studies. Not all cells expressing muscarinic receptors will show all signalling pathways indicated. From Durieux.*[27]

Pharmacology of muscarinic signalling

Until the first cloning of a muscarinic receptor was achieved in 1986,[19] investigators depended on pharmacological tools, primarily selective antagonists, to define the several subtypes of this receptor family. Unfortunately, none of the known antagonists is completely selective, so that subtypes had to be defined by measuring the binding properties of several compounds. Thus, equilibrium binding studies with pirenzipine initially indicated the existence of two classes of cerebral muscarinic receptors, named M1 and M2.[26,40] Kinetic studies allowed differentiation of three subtypes[41] and with the development of novel antagonists this number was expanded to four (M1–M4).[42] Tables 1a and 1b indicate the relative selectivity of the commonly used muscarinic antagonists, and relate the pharmacologically defined types to the cloned receptor genes. An excellent recent review of this subject is available.[24] Whereas many useful muscarinic antagonists have been developed, drugs with selective agonist activity are not as widely available. Acetylcholine and most of the classical parasympathomimetic drugs (carbachol, arecoline, muscarinic and pilocarpine) are non-selective. In functional studies several experimental compounds have displayed some selectivity for M1[43] and M2[44] receptors. However, these substances exhibit a functional selectivity for the receptor subtypes only and show no or only limited selectivity in terms of affinity.[45] So far even modestly selective agonists for the M3 and M4 subtypes are not available. Overall, since the cloning of the first muscarinic receptor a remarkable amount of information has been gathered about the molecular biology of muscarinic signalling. Not only have the main classes of muscarinic receptor subtypes been cloned, but detailed information on their structure-activity relationship is available, which has already proved useful in the development of new, highly selective agonist and antagonist drugs.

Table 1a. *An overview of differences in selectivity of various muscarinic antagonists*[a]

cDNA:	m1	m2	m3	m4	m5
Subtype:	M1	M2	M3	M4	
Pirenzepine	++	+	+	++	+
AFDX 116	+	++	+	+	+
Himbacine	+	++	+	++	+
Methroctamine	++	++	+	++	+
4-DAMP	+	+	++	++	++
pFHHSiD	+	+	++	++	++

[a]Based on potencies from functional or radioligand binding experiments on muscarinic receptors.
+, relatively low affinity; ++, relatively high affinity. AFDX 116, [11-2[[2-(9-diethylamino)methyl]-1-piperidnl]acetyl]-5,11-dihydro-6H-pyrido[2,3-*b*][1,4]benzodiazepin]-6-one, 4-DAMP, 4-diphenylacetoxylmethyl piperidine methiodide; pFHHSiD, *para*-fluorohexahydrosiladifenidol.

Intracellular pathways

As stated earlier, Ca^{2+} and cAMP are the best-described intracellular second messengers of the 'odd' and 'even' receptor groups, respectively. In the eye, with its primary function of electrical signalling, muscarinic systems also transduce their actions through changes in membrane potential. Several ion conductances, mainly in neuronal cells, have been shown to be affected by muscarinic stimulation, and the effects are most easily classified as depolarising (stimulatory) or hyperpolarising (inhibitory).[24,31] The best-known depolarising effect is by inhibition of a non-inactivating voltage-gated K^+ channel.($I_{K(m)}$) that clamps the membrane at its resting potential.[46] Stimulation of (primarily) M1 receptors inhibits this channel, resulting in a neuron more likely to fire when depolarised by other agonists. This effect has been studied in some detail, and has been shown to be mediated through the $G_{q/11}$ G protein.[47] A second depolarising influence of muscarinic signalling is through inhibition of a Ca^{2+}-dated K^+ current ($I_{K(Ca)}$), which normally hyperpolarises the cell when an action potential leads to influx of Ca^{2+} through voltage-activated Ca^{2+} channels.[48] M1 receptors seem to be the primary subtype involved, which is surprising, because their stimulation leads to increases in intracellular Ca^{2+} and therefore activation rather than inhibition of $I_{K(Ca)}$ would be expected. Such is indeed seen in transfected cells[49] but it has not been observed in neurons. The mediator involved has not been defined.

Inhibitory effects of muscarinic signalling are found in many neurons, and the best-defined pathway is the muscarinic effect on voltage-activated Ca^{2+} currents ($I_{(Ca)}$). This appears to be mediated by m2 or m4 receptors activating G_0 proteins.[50] The N-type Ca channel involved is sensitive to the Ca-channel blocker Ω-conotoxin GVIA but not to dihydropyridines. Another inhibitory effect of muscarinic signalling, even though only documented in cardiac atrial cells, is the activation of inwardly rectifying K channels (K_i) through M2 stimulation. This is responsible for the cardiac side effects of topically applied anticholinergic ophthalmic drugs, and has been shown to result from direct activity of stimulated G_i proteins on the channel. Dimming of vision, especially reported after use of carbachol,[51] might be a direct effect on retinal muscarinic signalling. A recent review on the subject is available.[52] Much interest has been generated by findings that the G protein β-subunit, traditionally considered inactive, appears to play an important role in this effect.[53,54] Although most data have been obtained in atrial cells, there is evidence that similar pathways exist in retinal[14,55] and cerebral signal transduction.[56] Fig. 2 summarises the intracellular pathways involved in muscarinic signalling. This area is the subject of active investigation, and several recent, more extensive reviews exist.[24,57,58]

Table 1b. *The selectivity ratios of different muscarinic antagonists*[a]

	Selectivity[b]		
Antagonist	M1 vs M2	M1 vs M3	M2 vs M3
Atropine	2.1*	2.6*	1.2
Telenzepine	26.3*	4.6*	0.17*
QNX	4.0*	0.45*	0.11*
Pirenzepine	37.2*	11.2*	0.30*
4-DAMP	18.6*	1.8*	0.10*
Procyclidine	15.8*	4.3*	0.27*
Dicyclomine	34.7*	2.6*	0.074*
AFDX 116	0.24*	6.2*	25.6*

From Doods.[29]
QNX, *RS*-(±)-quinuclidinyl xanthene-9-carboxylate hemioxalate hydrate.
*Significantly different from 1 ($p < 0.05$).
[a]In *in vivo* radioligand binding studies of muscarinic binding sites in hippocampus, atrium and submandibular gland.
[b]M*x* vs M*y* = $K_{i(y)}/K_{i(x)}$.

Conclusions

The investigations that followed cloning of the muscarinic receptors have provided first insights into the complex action they play in normal physiology and in the diseased eye. Ocular pharmacologically active substances can not be seen as having one receptor subtype at one tissue site within the eye – in the case of muscarinic agents several short-term and long-term effects must be considered. Besides the widely present muscarinic receptors, many classes of other GCRs are present in the eye. Structural studies, as well as determination of interactions with their secondary signalling mechanisms, will serve in the understanding and evaluation of phenomena such as elevated IOP or visual disturbances and alterations induced by various drugs. These findings can not be explained alone by the structural features of these substances and their affinity towards one receptor type. The interaction of muscarinic signalling with other signalling systems and the influence on slow pace growth promotion, smooth muscle activation and fast neuronal signalling are woven together in a complex pattern. Side effects and sometimes surprisingly beneficial observations can therefore be explained when muscarinic agonists and antagonists are used in ophthalmology. In the last 15 years highly selective drugs for certain muscarinic receptor subtypes have been discovered. Their clinical usefulness is in

many cases still elusive. The understanding of the ocular muscarinic system is therefore of great interest for the clinical ophthalmologist, since the selectivity of today's drugs requires a far more detailed understanding for their optimal application.

Localisation and function of muscarinic receptors in the eye

Understanding the functional importance of muscarcinic receptor signalling in the eye requires knowledge of the exact localisation of the receptors and subtype composition. The indication that a muscarinic receptor subtype is expressed in a certain part of the eye is taken as putative evidence that a functional role for this certain subtype exists. Not only anatomical curiosity, but also the search for reliable drugs for glaucoma and other disorders (e.g. myopia) drive efforts in mapping the quantity and types of ocular muscarinic receptors. From the observation that cholinergic drugs have effects on miosis, refraction and visual acuity it was deduced that muscarinic signalling must play a role in these processes. In addition, the development of new and more specific agonists and antagonists is of special interest for the treatment and management of ocular hypertension. Side effects developing from the long-term administration of these substances make it desirable to develop, if possible, drugs that do not have many of the undesired effects of many anticholinergics currently in use.

Separate from the functions of the mechanical apparatus are those of retinal signal transduction. Neurochemical processes important to signal transduction of visual information are believed to be modulated by a wide variety of expressed receptors in various defined structures of the retina and optic nerve. These may require more prolonged efforts in mapping and characterisation than the rest of the optic system. For the detection of receptors various methods exist, each with a variety of benefits and disadvantages, that have to be considered in their interpretation. Three different methods of investigation are highlighted here: binding studies, the use of monoclonal antibodies and the detection of mRNA encoding the specific receptor subtype. Using these techniques, a reasonably complete picture of ocular muscarinic receptor distribution can be drawn (Table 2).

Binding studies have historically been the most prominent type of investigations of muscarinic receptors. Several specific muscarinic agonists and antagonists exist, with which the distribution of muscarinic receptors in the eye has been defined. The major disadvantage of the method is that the specificity of these substances is only modest in most cases. Substances used to identify muscarinic receptor subtypes (M1, M2) do not necessarily bind with similar affinity to the cloned receptor subtypes (m1, m2).[73] However, careful comparison of binding studies using several antagonists can reveal consistent patterns of receptor distribution. An overview of the most commonly used antagonists used in binding studies is given in Tables 1a, together with their specificity on the different muscarinic receptor subtypes and their binding ratios between the M1 to M3 subtypes (Table 1b). Muscarinic receptor research has been hindered by the lack of antagonists with high affinity for one receptor subtype coupled with very low affinity for the other four receptor types. This results in the necessity to define a particular subtype with dissociation constants for a range of selective antagonists.[24]

Complementary nucleic acid sequences that are able to hybridise with parts of muscarinic receptor mRNA, either in Northern blots from tissues or directly in tissue sections, as *in situ* hybridisation, have proved another powerful tool in receptor mapping in the human eye. The presence of mRNA, when found in *in situ* tissue hybridisations, is usually a very strong indicator for the expression of the receptor molecule itself. Since many studies have been performed on isolated cell cultures from eye tissue, especially ciliary cells,[60] divergent results were reported regarding the expression of receptor subtypes in these tissues. This is not surprising, since cultured cells are isolated and are devoid of the interactive intercellular communication processes regulating receptor expression. Other reasons for, in particular, muscular diversity of muscarinic receptor expression are that the cell line derived from ocular tissue can have differential activation capacities for expression and that different rates of transcription exist. This has been described within the family of muscarinic receptors previously.[74] Therefore, results from cultured tissues are not always comparable with the status *in vivo*, even if more elegant examination methods in cultured tissues exist.

Monoclonal antibodies against various muscarinic subtypes have been introduced and their use in localisation studies will eventually give the most reliable insight into receptor expression. Most antibodies have been raised against peptide sequences of the third intracellular loop (i3) of each receptor, since this area has the least sequence homology among subtypes,[23] or against peptides of the carboxy end (e.g. of the m3 sequence).[75] Subtypes have been described in various organs and parts of the central nervous system but no subtype-specific investigations with monoclonal antibodies in the human eye have yet been performed. This leaves a wide gap in the knowledge we have so far achieved from binding and expression studies. The necessity actually to identify the expressed receptor subtypes is crucial, since their assignment to functional effects has already been studied with selective muscarinic drugs. Resolution problems, combined with low sensitivity, are presumably the main obstacles that have hindered antibody investigations in ocular tissues. Additionally, the absence of a definitive classification of subtypes might not be surprising, since many studies experience difficulties in conclusively designating a specific subtype of receptor with *in situ* hybridisation, antibodies and functional pharmacological studies.

Table 2. *The distribution of muscarinic receptor subtypes in distinct anatomical spaces of the human eye and in that of other species*

Tissue	Direct protein detection	m1–m5 mRNA *in situ* hybridisation	m1–m5 mRNA northern blot	M1–M4 radioligand binding
Cornea				QNB binding:
Epithelium	m3[m] m4[m] m5[m] m1 or m2[m]	m3 +[a] m3 +[a]		subtypes not specified[n,o]
Endothelium	m3[m] m4[m] m5[m]			
Lens				
Anterior epithelium		m3 +[a]		
Trabecular meshwork		m3 +[a]		
Ciliary non-pigmented epithelium/process		m3 +[a]	m2 +[f] m3 +++[f] m4 +[f]	M1 +++[k] M1 ++[c] M2 ++[c,k] M3 ++[j] M3 +++[l]
Cililary muscle	m3 +++[p]	m3 ++[a]	m3 +[a]	M2 +[c]
Whole muscle	m1 +[p] m2 +[p] m4 +[p] m5 +[p]			M3 ++[a,d,e]
Longitudinal		m2 +[b] m3 +[b] m5 ++[b]	m1 +[b] m2 +[b] m3 +[b] m4 +[b] m5 +[b]	M1 ++[c] M2 +++[c]
Circular		m2 ++[b] m3 +++[b] m5 +[b]	m1 +[b] m2 +[b] m3 +[b] m4 +[b] m5 +[b]	M1 ++[c] M2 +[c]
Iris		m3 ++[a]	m3 +[a]	M2 ++[c] M3 ++[c]
Sphincter			m2 +[f] m3 +++[f] m4 +[f]	M3 +++[a,d,f] M1 ++[c]
Epithelium				M3 +[a] M1 ++[c]
Retina	m1 to m5[h]			M1 ++[c] M2 ++[c,g,i] M3 +[a]
Sclera				M1 ++[p]

The comparison of different kinds of investigative tools (radioligand binding, mRNA detection and direct protein detection with monoclonal antibodies) shows a distribution pattern at some anatomical sites. When comparisons of levels of expression were made in a study these are represented as + for present to +++ for predominant.
References: a;*, 59; b*, 60; c*, 61; d*, 62; e*, 63; f, 64; g, 65; h, 66; i, 67; j, 68; k, 69; l, 70; m, 15; n, 16; o, 71; p, 72. An asterisk indicates investigations made in human tissue.
QNB, quinuclidinyl benzylate.

Muscarinic signalling in the retina

Autoradiographic binding sites for muscarinic agents in the retina have been difficult to allocate, since the spatial resolution of this technique was not satisfactory.[76] Higher-resolution studies and more advanced emulsion techniques, however, revealed the existence of M1 and M2 receptors in rat, human and monkey,[77–79] as well as in calf,[80] avian[14,81] fish[82] and rabbit[83] retina, where they are mainly found in the inner plexiform layer from early stages onwards in the developing eye. These findings impressively demonstrate the crucial role of muscarinic signalling in embryonic development and in the adult eye, since multiple patterns of expression appear to guide the layout of retinal structures and later participate in visual function throughout ocular growth. A number of possible mechanisms for the development generation of neuronal networks have been postulated on the basis of changing receptor densities and appearances in the embryogenesis of the eye.[84] The development of retinal structures appears to be greatly influenced by the expression of muscarinic receptors.[85] In different stages

of embryological and postnatal development, the subtypes, number and distribution of the muscarinic receptor proteins change during retinal synaptogenesis.[66] In embryonic maturation, muscarinic signalling seems to influence formation of retinal structures primarily through intracellular Ca^{2+} release,[14,86,87] and muscarinic signalling is predominantly responsible for the incurvation of the early embryonic neural retina.[17,88] Since precursors of ganglion and amacrine cells possess muscarinic receptors,[81,89] the concomitant emergence of different functional cholinergic receptor subtypes with differentiation *in vivo* suggests that acetylcholine plays diverse and temporarily regulating roles in the developing retina.[87,90]

The subtype composition of muscarinic receptors in the retina can not be interpreted at present. 4-DAMP labelling revealed binding to muscarinic receptors in the retina and blocking of M1 receptors with pirenzepine presumably indicated the concomitant presence of M3 receptors in human retina as well.[59] Stimulation experiments for GTPase activity revealed that the major site for muscarinic stimulation in bovine retinal membranes is pharmacologically similar to M2 receptor sites[67] and in the rat retina phosphoinositide hydrolysis and adenylate cyclase inhibition were mainly found to be induced by M1 subtypes.[91] However, the accompanying presence of molecularly defined m4 and m5 receptors can not be excluded from these findings, since affinities of the employed antagonist for these receptor subtypes exist. The m4 and m5 clones in the retina can not be defined at present with specific antagonists, so that care is needed in interpreting binding studies and their true correlation with the molecular subtypes. Investigations with monoclonal antibodies or mRNA hybridisations of these subtypes, except in the developing ferret retina,[66] are not available and will ultimately determine exact locations of these receptor subtypes. However, the role of cholinergic neurotransmission by muscarinic as well as nicotinic receptors is evident, though investigations of these complex patterns of signal transduction are yet to be performed. Physiological evidence suggests that muscarinic binding sites in the inner plexiform layer are associated with amacrine and/or ganglion cells,[92,93] although from other studies it appears that association with bipolar and horizontal cells is also possible.[79] Markers for cholinergic synapases are concentrated in the inner plexiform layer were acetylcholine is possibly being released by discrete populations of amacrine, displaced amacrine and inner bipolar cells.[93,94]

Functional correlations regarding the transmission of chromatic information, patterns or even whole visual images have not been defined but the presence of multiple receptor populations and their interactions are documented.[79] The release of acetylcholine from displaced amacrine cells under the influence of light in rabbits has been well documented[95] and effects of acetylcholine from these cells on the inner plexiform layer appear to play a role in subsequent signal transduction.[96,97]

Ciliary muscle function is regulated by muscarinic receptors

A second important and extremely well investigated site of muscarinic signalling in the human eye is the ciliary muscle.[98] The ciliary body and the trabecular meshwork have been in the initial focus of interest regarding muscarinic signalling, since they are crucial for accommodation and aqueous outflow. It has become evident that in ciliary muscle a diversification of receptor distribution exists.[99,100] The general presence of muscarinic receptors in the ciliary muscle complex was soon established[70,101,102] but further identification of the receptor subtypes seemed desirable for the explanation of accommodative and aqueous flow mechanisms. In the hope of finding an agonist of muscarinic signalling for the control of IOP, precise mappings of muscarinic subtypes through binding studies and molecular genetics have been undertaken.[100] The most prominent effect of muscarinic drugs in the eye due to constriction of the ciliary muscle is miosis, associated with accommodation and an increased outflow of aqueous humour.[13,103,104] Since the ciliary muscle can be mechanically divided into a circular portion of muscle fibres responsible for changes in accommodation and a longitudinal portion mainly responsible for changes in outflow facility, the question was soon asked whether differences in receptor distribution were responsible for this distinctive behaviour. Oxotremorine, a selective M2 agonist, binds specifically to sites on the longitudinal ciliary muscle.[61] The circular muscles, responsible for accommodation, have lower affinity, and it seems possible to influence outflow only by activating especially the longitudinal ciliary muscle fibre subtype. Similar findings were reported with the muscarinic agonist aceclidine when compared with the non-selective pilocarpine.[105–107] Dose-effect relationships for intracamerally applied doses of aceclidine to determine total outflow and accommodative amplitude were carried out in cynomolgus monkey eyes *in vivo*. The results showed a significantly stronger effect of aceclidine on outflow than on accommodation, giving further evidence for a dissociation between the accommodative and outflow facility functions of the ciliary muscle based on muscarinic activity.[108] Additionally, in the monkey eye longitudinal ciliary muscle fibres differ ultrastructurally and histochemically from fibres in other regions of the ciliary muscle,[109] providing further evidence for a specialised task in regulating humour dynamics. As previously mentioned, aceclidine, a cholinomimetic, has been used therapeutically for IOP reduction in glaucoma[110] and is known to have far less effect on accommodation than pilocarpine.[111–113] A specific receptor subtype for aceclidine action was postulated with a site predominantly on the longitudinal portion of ciliary muscles.[13,105,108] This led in the ensuing period to an intense investigation of muscarinic receptor populations in ciliary muscle tissue and in the trabecular meshwork. Additionally a number of functional studies were added to determine precise mechanisms of

muscarinic receptor interplay.[13,106,114–117] Radioligand binding studies revealed that oxotremorine as a weak M2 agonist binds selectively to the longitudinal fibres of the ciliary process whereas no binding was seen in the iris or ciliary epithelium. These results suggest that oxotremorine, by binding selectively to receptors on the longitudinal ciliary muscle and inducing its contraction, may modulate outflow facility independently from accommodation and miosis via the M2 subtype.[61] When bovine iris and whole ciliary body were investigated regarding expression of muscarinic subtypes, the ratio of m3 to m4 subtype mRNA expression was found to be 13:1. Absence of m1 mRNA in the ciliary process and the iris sphincter was noted, but small quantities of m4 mRNA were expressed in the ciliary process.[64] It is evident that the predominant muscarinic receptor in ciliary structures is the m3/M3 subtype, both in human ciliary epithelium[3] and ciliary muscle[59,60,118] as well as in other species.[119,120]

The complex innervation features of the ciliary muscle, however, make a solitary responsibility of muscarinic receptors for the distinction between outflow and accommodation improbable.[121,122] Since the ciliary muscle, like the iris smooth muscle, is innervated by nerves of the sympathetic, parasympathetic and sensory nervous system, activation or blockade of prejunctional receptors may have an additional influence on ciliary muscle tone since not only postjunctional muscarinic effects are responsible for ciliary muscle tone. Therefore, it can be difficult to predict what overall effect an agonist has, because it may differentially affect various parts of the nervous system simultaneously. Sympathetic nerve terminals in the anterior uvea, for example, contain prejunctional muscarinic receptors that, upon activation by agonists, inhibit the neural release of noradrenaline. When the prejunctional effects of muscarinic agents on evoked secretion of noradrenaline in iris-ciliary body segments were investigated, the M2 type was found to be the primary subtype present, the M1 and M3 subtypes playing a minor role.[123]

When mRNA expression studies in native and cultured tissue from the human eye were performed a clearer picture evolved: human ciliary muscle definitely expresses the mRNA of subtypes m2, m3 and m5 and may also express the mRNA of m1 and m4. Differences in expression level of the m2, m3 and m5 subtypes were observed between the circular and longitudinal portions of the ciliary muscle, but quite pronounced expression of all three subtypes of muscarinic receptors by both portions shows that a differential distribution probably is not solely responsible for the dissociation between outflow facility and accommodation that is seen under certain conditions.[60] The employment of subtype-specific antibodies will be the ultimate confirmation of these findings, since recent experiments with primates indicate that muscarinic receptor subtype distribution plays a minor role in facilitating outflow and lowering IOP.[105]

Echothiophate-induced modulation of functional cholinergic sensitivity in the parasympathetically innervated, in contrast to denervated, ciliary muscle has been shown to occur by a muscarinic-receptor-mediated process.[114,115,124] Therefore, it is probable that muscarinic receptors also play a role in mediating the inhibitory effects of parasympathetic nerve stimulation or cholinomimetic drugs on ocular sympathetic neurotransmission, indicating their crucial role in ciliary muscle cholinergic sensitivity. The trabecular meshwork, even though not a part of the ciliary muscle, is additionally involved in outflow regulation, as biomechanical studies in the monkey have shown. Here it was noted that pathophysiological changes in the outflow apparatus induced by echothiopate are in part mediated by anterior segment muscarinic receptors as well as mechanical factors.[125] The M3 subtype appears to be predominant in cultured[126] and native[59] human trabecular meshwork cells. However, it has to be mentioned that here again muscarinic signalling is only partially responsible for drug effects. Functional muscarinic, α-adrenergic and β-adrenergic receptors in bovine trabecular meshwork and ciliary muscle are differentially modulated by various drugs: cholinergic and α-adrenergic agonists induce contraction, whereas β-agonists induce relaxation.[120]

Muscarinic receptors of the ciliary epithelium

In addition to fluid transport through capillary walls, about 95% of aqueous humour is formed by a secretory process of the cells of the ciliary epithelium. This secretory mechanism and its regulation are only faintly understood and it has become one of the key targets for regulation by endogenous mediators and anti-glaucoma drugs. Multiple methodologies and conflicting results, depending on the animal species used, have created a complex picture of the role of muscarinic receptors in this process.[127] When the effects of cholinergic agents on vasoactive intestinal peptide(VIP)-stimulated cAMP accumulation were investigated in the rabbit ciliary epithelium, an inhibition of stimulation was found, indicating that the cholinergic system – via muscarinic receptor stimulation and subsequent inhibition of the ciliary epithelial adenylate cyclase – interferes with humour formation.[128] It remains open as to the biological significance of muscarinic receptors in regulating ciliary epithelial transport, and what their contributions are to intraocular responses to cholinergic drugs. Functional studies with subtype-specific agonists and mapping of the various subtypes are scarce. In human non-pigmented ciliary epithelium the carbachol-specific stimulation of inositol phosphates was significantly inhibited by 4-DAMP (M3 antagonist), showing a distinguishable predominance of M3 receptor subtypes, with a large variety of other receptors triggered by neurotransmitters and neuropeptides.[70] In the rabbit non-pigmented epithelium a predominance of muscarinic receptors is seen, in contrast to the pigmented part, which contains mainly α1-adrenergic receptors.[128] These muscarinic receptors appear to signal via the turnover of membrane phosphoinoitides, generating the second messengers diacylglycerol and IP_3,[129] and via the

inhibition of adenylate cyclase,[128] suggesting that at least two of the five molecularly defined subtypes are present in the ciliary epithelium: one of the 'odd' group (m3/M3 as shown[59]) and one of the 'even' group, the identity of which remains unknown. The crosstalk between cAMP- and IP_3-dependent Ca^{2+} generation has been summarised in a recent review and provides clearer insight into the complicated patterns of signal transduction in the human eye.[4] The physiological significance of muscarinic receptors in the ciliary epithelium, however, remains to be established. Complex interference with or modulation of ciliary epithelial muscarinic receptors by other hormones, neurotransmitters and ocular hypotensive drugs makes the muscarinic system a promising target for the development of IOP-lowering substances,[130,131] and current data support the strong involvement of muscarinic cholinergic receptors in IOP regulation.[3,70,132]

Muscarinic receptors influence iris sphincter function

The characteristics of muscarinic receptors mediating relaxation and/or contraction have been intensely investigated since the iris is an ideal model for innervation from the sympathetic, parasympathetic and sensory nervous systems.[121] It is in addition one of the few smooth muscle organs which can be parasympathetically denervated,[133] making it generally an interesting model for interaction studies of pre- and postjunctional receptors, not only of the muscarinic type, whose general, non-subtype-specific presence in the iris has been documented in a variety of species.[134–139]

The first report of putative muscarinic receptors by non-subtype-specific binding studies in the human iris soon followed, showing high densities of muscarinic receptors in the iris sphincter muscle and lower ones in the dilator muscle, matching with the well-known non-specific pharmacology of atropine[2,140] or carbachol.[141,142] In the rabbit iris sphincter, however, pilocarpine is known as a very weak partial agonist and also behaves as an antagonist,[134] which was explained by the combination of a small number of spare receptors and a threshold phenomenon.[143] Indeed, it was shown that pilocarpine causes miosis *in vivo* by indirectly decreasing the iris dilator tone via prejunctional inhibition of noradrenaline release in the dilator.[144] An atypical muscarinic receptor subtype exists in the rabbit iris,[145] different from the pharmacologically and molecularly defined subtypes, and has accounted for these differences, which were at first attributed to the presence of M3 receptors.[146,147] The postjunctional M3 receptor subtype appears to be mostly prevalent in guinea pig,[148] bovine[64] and human[123] iris sphincter and rat dilator muscle.[149–151] Functionally this makes sense, since electrically evoked release of noradrenaline in human iris preparations revealed that prejunctional muscarinic receptors in the human iris–ciliary body correspond to the M2 subtype mediating the inhibitory effects of parasympathetic nerve stimulation or cholinomimetic drugs on ocular sympathetic neurotransmission, whereas the postjunctional M3 subtype is responsible for contraction of the sphincter muscle in humans[123,148] and in rats.[150] When the potencies of several muscarinic receptor antagonists in blocking either the autoinhibition of acetylcholine release or the muscarcinic contraction of the sphincter muscle upon acetylcholine release were investigated in the guinea-pig iris, no involvement of the M1 receptor was noted. The results were consistent with the idea of M2 receptors mediating autoinhibition of acetylcholine release and M3-like receptors inducing the contraction of the sphincter muscle in guinea pigs,[148,152] in contrast with M2-mediated IP_3 accumulation and subsequent contraction of the sphincter smooth muscle of the rabbit iris.[153,154]

Binding studies of the specific M2 agonist oxotremorine revealed that no M2 subtypes are present in the human iris muscle, in contrast to specific binding of M1 and M3 antagonists.[59] These findings are supported by the presence of m3 muscarinic receptor subtype mRNA in native[61] and cultured[155] human iris. Investigations in dogs and cats show that the signalling pathways by M3 receptors in the iris smooth muscle involves both intracellular and extracellular Ca^{2+} mobilisation and subsequent stimulation of cAMP production, and that M3 receptors are coupled to the activation of both phospholipase C and adenylate cyclase.[156,157] Contraction of the iris sphincter smooth muscle in rabbits in contrast seems to be primarily mediated by IP_3 only.[158]

In conclusion, the understanding of the overall complex nature of muscarinic receptor distribution on various prejunctional and postjunctional sites in the iris is complicated by investigations in different species and inhomogeneous results. Not all species appear to have a similar receptor interplay, making the search for a valid animal model for drug screening problematic. The direct determination of subtypes in humans has been undertaken with radioligand studies; however, their known limitations should be borne in mind.

Investigations of mRNA content or direct protein assays with subtype-specific monoclonal antibodies have revealed that in the human iris sphincter the predominant subtype, as stated before, is the m3 type accounting for 60–75% of the muscarinic receptors. Lower levels of between 5% and 10% were recently found for the m1, m2, m4 and even the m5 subtype.[100]

Presence of muscarinic receptors in the cornea

Among the various mammalian tissues that have been studied for acetylcholine content, the corneal epithelium contains the highest concentrations.[159,160] Even though corneal epithelium is rich in nerve endings, the enormous concentration of acetylcholine in these cells is not in accordance with the level usually found in junctional tissues where it serves as a neurotransmitter. Suggested functions of acetylcholine might involve regulation of water and ion transport into corneal epithelium.[160] Denervation of corneal epithelium has led to a 87–100% reduction in corneal acetylcholine and is

associated with mitotic (growth) inhibition, suggesting a reparative function of muscarinic receptors in the corneal epithelium.[161,162] Initial investigations regarding muscarinic receptors showed a lack of binding in rabbit broken cornea preparations,[138] in contrast with other studies which showed the presence of muscarinic receptors in cultured rabbit corneal cells by QNB binding.[71] Today the wide presence of muscarinic receptor subtypes in corneal tissues has been documented and a picture of their multiple functions is evolving. Considering that every contact of the cornea with an extraocular object causes a destruction of sensitive corneal epithelium two physiological roles may explain the extensive amount of acetylcholine: first the sensory transmission of the damaging cause and secondly the induction of repair mechanisms, via muscarinic signalling, of an easily damaged thin epithelial layer.

Following these thoughts, a cGMP-mediated stimulatory role of cholinergic receptors in corneal epithelial growth regulation established that activated muscarinic receptors in the cornea are a main signalling step in this procedure.[16,163,164] After the presence of β-adrenergic, prostaglandin E_1 and muscarinic receptors in cultured corneal epithelial cells of the rabbit was established,[71] the interplay between these receptors was investigated. Hereby it was found that the intercellular cAMP/cGMP ratio was essential for epithelial proliferation and regrowth. The activation of prostaglandin and β-adrenergic receptors increased cAMP, inhibited regrowth and increased basement membrane production after initial injury to the corneal epithelium. Activation of muscarinic receptors led to increased cGMP-mediated regrowth of the corneal epithelium. The receptor site remained ambiguous and it was assumed that parts of the receptor protein are localised inside the cell, possibly inside the nucleus itself.[165] Recent investigations in highly purified nuclei of rabbit epithelial and endothelial cell lines have indeed demonstrated the presense of muscarinic receptors inside the nucleus.[15] Additional whole protein investigations of the molecular subtypes revealed that m3, m4 and m5 receptors are present in epithelial and endothelial cells and that m1 and m2 receptors are present in epithelial cells only. The majority of these receptors are attached to membranes; the m5 subtype, however, seems likely to regulate nuclear functions, along with an uncharacterised 47 kDa receptor-like protein. This is particularly interesting since the functional role of m5 is the least understood of all the subtypes.[15] The intranuclear presence of muscarinic receptors in corneal epithelial and endothelial cells (and other cell types as well) suggests the existence of a functional, possibly G-protein-dependent, nuclear signalling system, induced by muscarinic acetylcholine receptor (or related receptor-like) proteins in response to intracellular acetylcholine, employing pathways specific for intranuclear signalling.[166–169]

Lenticular sites of muscarinic signalling

The lens, despite its lack of innervation and the fact it is an organ of entirely epithelial origin, has high levels of acetylcholinesterase activity.[170] Initially this activity was thought to be a defensive mechanism based on the high concentrations of acetylcholine generated by such nearby sources as the iris and the ciliary apparatus.[171] But soon the functional role of acetylcholine emerged and, interestingly, interference with acetylcholine homeostasis in the lens has been shown to have a remarkable effect on its clarity. A well-known side effect of anticholinergic drugs is their ability to induce cataract in humans,[172] in *in vitro* experiments[170,173] and in monkeys.[174] In isolated epithelial cell preparations of humans, acetylcholine induced intracellular release of calcium from endoplasmic reticulum in these cells. This signalling pattern goes together with an activation of m1, m3 or m5 receptors on the cell surface. Indeed, further electrophysiological investigations in human[175] and rabbit whole lens preparations revealed that muscarinic, but not nicotinic, receptor activation induces the release of intracellular calcium. So far the only direct determination of muscarinic receptor subtypes in the human lens revealed the presence of m3 mRNA and positive radioligand binding with the M3 antagonist 4-DAMP, results matching the physiological evidence.[59] Still, the elusive role of muscarinic receptors in lens function has to be clarified. Since there is no innervation of the lens, a regulatory effect of muscarinic receptors on cell homeostasis, rather than on neuronal signal transduction, seems probable. Here again an understanding of the interplay with other receptors will finally reveal the precise functions of the muscarinic lenticular system.

Muscarinic receptors influence scleral growth

One of the most interesting approaches of the long-term application of antimuscarinic drugs is their influence on scleral growth. Especially in the prevention of myopia in humans, the long-term use of antimuscarinic drugs, such as atropine, appears promising.[176] Experimental studies in chicks have shown a marked reduction of myopic progression, accompanied by ocular elongation, when these eyes were treated with atropine.[177,178] This elongation stimulus has been shown to be present even in the absence of a connection between the retina and the brain.[179] It is now believed that a direct stimulus from retinal cells on scleral chondrocytes via the muscarinic system is present. In recent investigations M1 muscarinic receptors have been found to be primarily present in chick scleral chondrocytes when the growth of these cells was monitored in culture.[72] Several other cell types in humans are growth stimulated by muscarinic acetylcholine receptor agonists and the results of pending clinical trials for the reduction and prevention of myopia through early onset of muscarinic agonists in prone patients are promising.

Conclusions

The omnipresence of muscarinic receptors with their various expression patterns in the human eye is a startling finding. The methods employed to determine subtypes have become more sophisticated over recent years and ultimately the quantitative determination of receptor protein expression will yield definitive insights into subtype interactions. In addition to the long-known role of muscarinic receptors on outflow and accommodation regulation in the ciliary apparatus, a wide range of diverse functions has been found. These include the multijunctional sites of neurotransmission in the retina and iris, whose subtype composition is still under investigation. More exploration will be required to clarify the presence of muscarinic signalling in the lens and cornea, where these receptors most likely play a role in the nutritious efforts of these bradytrophic tissues. Here muscarinic signalling seems to be important for growth and regeneration control between cells and less important for fast pace neuronal transmission of signals. The detection of muscarinic signalling in the cellular nucleus is particularly exciting, since gene expression mechanisms might additionally be influenced by intracellular G protein coupled receptor mechanisms.

The overall picture of the muscarinic system of the eye is continuing to evolve. From the extensive investigations that have been performed it is probable that the ratio and relationship of receptor subtype expression in the eye and other body sites will help to determine their functional roles in a given system.

A new range of muscarinic receptor agonists and antagonists is being tested at present in animal and human models. Their aims are various. Prevention of myopia by muscarinic antagonists is an exciting new aspect. However, fears from toxic side effects of long-term use of substances such as atropine are pushing investigations further into the class of more selective muscarinic antagonists. The complex distribution and expression of all known muscarinic receptor subtypes in the eye can not make us believe in the solely accommodative influence of muscarinic drugs. Reparative functions in the corneal epithelium are enhanced by muscarinic agonists and retinal neurotransmission is influenced by substances interacting with muscarinic receptors. Glaucoma therapy has one of its major strongholds in using indirect muscarinic drugs and here again a more selective influence on uveoscleral outflow is hoped to be gained by increased subtype selectivity of new drugs – and the selectivity towards one receptor subtype will be needed as the picture evolves of an eye densely populated with muscarinic receptors.

The five molecular subtypes of muscarinic receptors are all present in the eye. In distinct structures a different pattern of receptor subtype composition can be found. The role of most of them are not yet understood, which is not surprising since they were first distinguished only just over 10 years ago. Atropine (including its various enduring derivatives) and pilocarpine might soon be accompanied by highly selective muscarinic subtype agonists and antagonists in their clinical applications. The knowledge of the composition of the ocular muscarinic system and its interplay with other receptor signalling systems will therefore help the ophthalmologist in choosing the optimal treatment in the future, since the limits for empirical drug use and development are beginning to be reached.

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