ORIGINAL ARTICLES – Laboratory science

Muscarinic receptor M1 and M2 subtypes in the human eye: QNB, pirenzipine, oxotremorine, and AFDX-116 in vitro autoradiography

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Abstract

Muscarinic cholinergic agents are used to lower intraocular pressure in the medical management of glaucoma and subtypes of muscarinic receptors have now been recognised in many tissues including the eye. To localise muscarinic receptors and their M1 and M2 subtypes in the human eye, in vitro ligand binding and autoradiographic techniques with densitometric quantitation on postmortem eye sections were used. As ligands, [^{3}H] quinuclydinyl benzylate (QNB) (non-subtype specific muscarinic antagonist), [^{3}H]pirenzipine (M1 antagonist), [^{3}H]oxotremorine (M2 muscarinic agonist), [^{3}H]AFDX-116(11[{2[diethylaminomethyl]1-piperidinyl}acetyl]5,11dihydro-6H-pyrido [2,3b][1,4]benzodiazepine-6-one) (M2 antagonist) were studied. Specific binding sites for QNB, pirenzipine, and AFDX-116 were localised in the entire ciliary muscle, the iris, and ciliary epithelium. [^{3}H]oxotremorine localised *only* in the longitudinal portion of the ciliary muscle, and additionally, was not localised 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.

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Direct and indirect acting muscarinic cholinergic agonists have been mainstays in the medical management of primary open angle glaucoma. The stimulation of muscarinic receptors in the ciliary muscle causes its contraction, and by virtue of its insertion into the trabecular meshwork, aqueous outflow facility is increased and intraocular pressure reduced.[1–5] Subtypes of muscarinic receptors have now been recognised in many tissues and their localisation in the eye is an important step towards an understanding of the effects of subtype specific muscarinic drugs and the rationalisation of new ocular therapies based on their potentially selective actions.

Three muscarinic receptor subtypes – namely, M1, M2, and M3, have been pharmacologically defined based primarily on their affinities for pirenzipine, AFDX-116(11[{2[diethylamino) methyl]1-piperidinyl}acetyl]5,11-dihydro-6H-pyrido[2,3b][1,4]benzodiazepine-6-one), and 4-DAMP (4-diphenylacetoxy-*N*-methyl-piperidine methiodide) respectively.[6] AFDX-116, a muscarinic antagonist and oxotremorine, a muscarinic agonist, have been shown to have high affinity for M2 receptors[7 8] which appear to be a heterogeneous group.[9 10] The genes for five muscarinic receptor species have been cloned and sequenced (m1 to m5)[11] and evidence indicates that the protein products of the m1, m2, m3 clones must closely resemble the M1, M2, and M3 receptors respectively.[12]

The subtypes of muscarinic receptors involved in lowering intraocular pressure and their intraocular location is not well understood. In this study, we report the localisation and quantitation of binding sites in human eye sections for QNB, pirenzipine (M1 subtype), oxotremorine, and AFDX-116 (M2 subtypes) using in vitro ligand binding, autoradiography, and densitometric techniques as previously described.[13 14 15]

Materials and methods

Seventeen human cadaveric eyes were obtained within 12 hours after death from the Eye Bank of British Columbia (Table 1). At least seven different specimens were used for each ligand binding study, and additionally, eight different specimens were studied with all four ligands. The eyes were frozen in liquid isopentane cooled to −80°C on dry ice, and stored at −20°C until use. Sections of 20 μm thickness were cut with a cryostat (Cambridge Instruments, Nussloch, Germany) and placed on glass slides coated with 1% gelatin.

The sections were thawed at room temperature and incubation with 1 nM[^{3}H]QNB ([^{3}H]QNB:

Table 1 Human cadaveric eyes

Sex/age (years)	*Hours postmortem*	*Cause of death*
M/75	3	Glioblastoma
M/40	3·5	Myocardial infarction
M/71	4·5	Lung carcinoma
F/42	5	Cervical carcinoma
M/41	5	Multiple sclerosis
M/39	5	Myocardial infarction
F/41	5	Multiple sclerosis
M/42	6	Lung carcinoma
F/46	7	Breast carcinoma
M/34	7	Pneumonia
M/30	7	Cardiovascular accident
M/88	7	Myocardial infarction
M/61	8	Myocardial infarction
F/51	9·5	Pulmonary embolus
M/61	9·5	Drowning
M/63	11·5	Breast carcinoma
M/75	12	Diabetes

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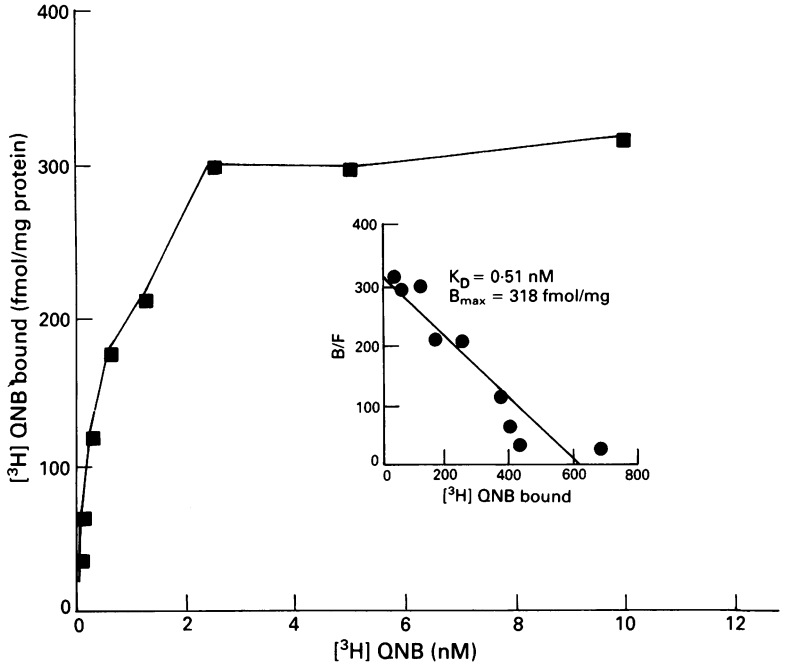

Figure 1 Scatchard analysis of [³H]QNB ligand binding studies in human iris/ciliary body tissue showing a B_{max} of 318 fmol/mg and K_D of 0·51 nM.

32·9 Ci/mmol, Amersham, Oakville, Ontario, Canada) for 60 minutes at room temperature in sodium phosphate buffer pH 7·4 was followed by two 5 minute washes in ice cold buffer. Incubation with 0·5 nM [³H]oxotremorine ([³H]oxotremorine: 87 Ci/mmol, New England Nuclear, Mississauga, Ontario, Canada) for 30 minutes in 20 nM HEPES Tris pH 7·5 buffer containing 10 mM Mg^{2+} at room temperature was followed by three successive 2 minute washes in ice cold buffer and a final rinse in deionised water. Sections were incubated with 1 nM [³H]pirenzipine ([³H]pirenzipine: 82 Ci/mmol, New England Nuclear, Mississauga, Ontario, Canada) in 20 nM HEPES Tris pH 7·5 containing 10 mM Mg^{2+} for 60 minutes and washed as described for [³H]oxotremorine.[16] Incubation with 0·5 nM [³H]AFDX-116 ([³H]AFDX-116: 97·0 Ci/mmol, New England Nuclear, Mississauga, Ontario, Canada) in Krebs buffer for 60 minutes at room temperature, was followed by three 5 minute washes. To evaluate non-specific binding, parallel experiments included 10 μM concentrations of unlabelled atropine sulphate (atropine sulphate, New England Nuclear Corp, Boston, MA, USA) (muscarinic antagonist). Sections were dried in an air stream and apposed to tritium sensitive film in the dark (LKB Ultrofilm, Amersham, Canada, Oakville, Canada). Exposure time determined empirically was as follows; [³H]QNB – 2 weeks, [³H]oxotremorine – 12 weeks, [³H] pirenzipine – 3 weeks, and [³H]AFDX 116 – 3 weeks. Original eye sections were stained with cresyl violet for Nissl substance for anatomical reference.

Grey values of autoradiograms were calibrated against optical density readings of the co-exposed density standards (autoradiographic [³H] microscales, Amersham, Canada) and mean (n=3) densitometric quantitative values were expressed in fmol/mg (SD). Image analysis was performed with Aristo light table, Nikon Micro-Nikkor 55 mm 1:28, Nikkon F-C adaptor, Panasonic Wv-BD400 CCD camera, 0·002 μF Capacitor, Sony Trinitron PVM-8200J monitor, data translation quick capture board, Macintosh IIfx computer, and Image 1.23 program (NIH).

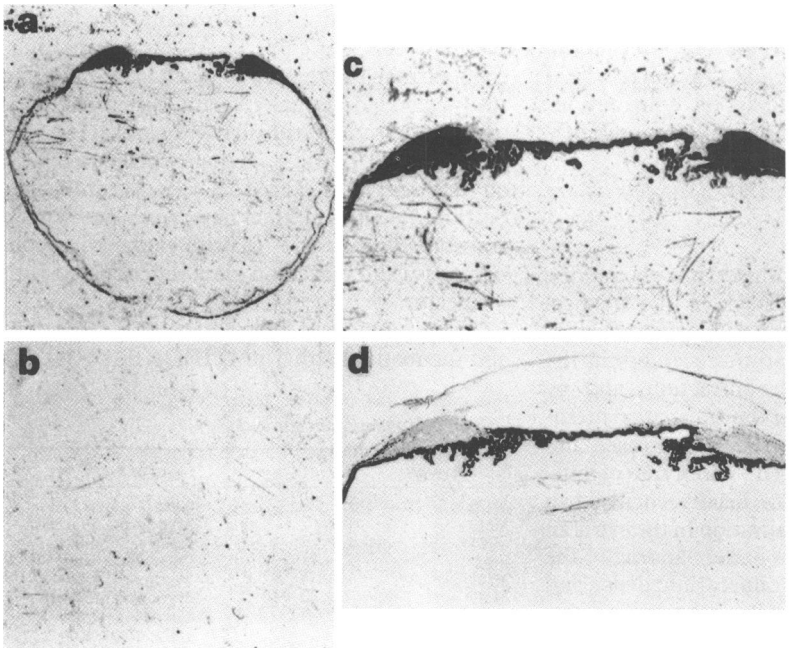

Figure 2 Autoradiographic views of [³H]QNB binding sites in eye sections from a 42-year-old woman. The binding of 1 nM [³H]QNB concentrates in the ciliary muscle, iris, ciliary epithelium, retina (detached) and retinal pigment epithelium (a). In the higher power view of the anterior segment (c), the ciliary muscle, iris, and ciliary epithelium are intensely labelled and this was completely displaced by 10 μM unlabelled atropine sulphate. (b). A Nissl stained histological section of (c) is given in (d). Findings were consistent in all 10 eye specimens studied.

Results

Characterisation of QNB binding in iris/ciliary body preparations showed a B_{max} of 318 fmol/mg and K_D of 0·51 nM by Scatchard analysis (Fig 1).

Silver grain densities showed a high concentration of specific binding sites for QNB localised in the ciliary body and iris, with moderate binding in the posterior segment of the eye (Fig 2a). The binding of 1 nM QNB could be totally displaced by addition of unlabelled atropine sulphate (Fig 2b). Anterior segment binding sites were localised in the ciliary muscle, iris, and ciliary epithelium (Fig 2c). Densitometric quantitation revealed specific binding concentrations of 363·7 (16·9) fmol/mg in the ciliary muscle, 283·6 (34·1) fmol/mg in the iris, and 159·4 (23·4) fmol/mg in the ciliary epithelium. Comparison of Figure 2c with its histological counterpart (Fig 2d) showed binding in the cornea and lens equal to non-specific background binding.

Specific binding sites for oxotremorine were localised (Fig 3a) in the anterior segment and in the retina. This binding was totally displaced by unlabelled atropine sulphate (Fig 3b) or unlabelled oxotremorine. Comparing the autoradiogram of the anterior segment in Figure 3c with its histological section in Figure 3d, highly specific oxotremorine binding sites were noted in the longitudinal ciliary muscle. The concentration of specific oxotremorine binding sites was 112·1 (9·1) fmol/mg in the longitudinal ciliary muscle. The amount of binding in the remainder of the ciliary muscle, the cornea, lens, iris, and

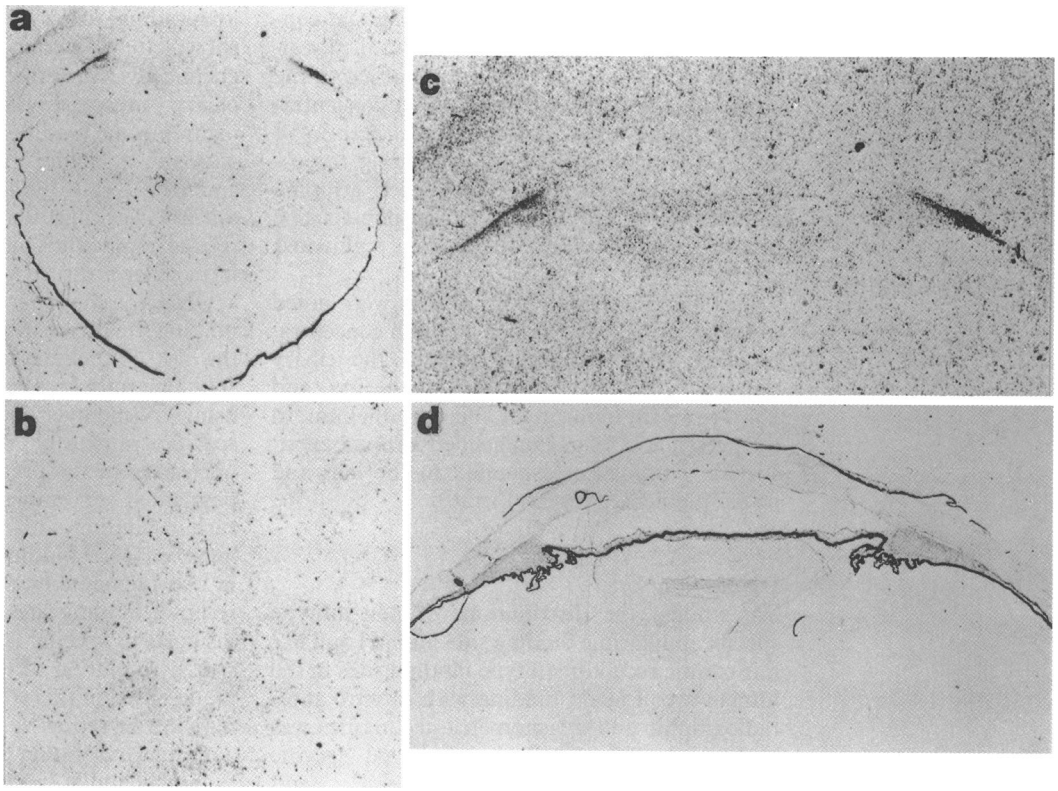

Figure 3 Autoradiographic views of [^{3}H]oxotremorine binding sites in eye sections from a 42-year-old woman. The binding of 1 nM [^{3}H]oxotremorine concentrates in the ciliary muscle, and in the retina. In the higher power view of the anterior segment (c), binding is predominantly in the longitudinal portion of the ciliary muscle, and this was completely displaced by 10 μM unlabelled atropine sulphate (b). A Nissl stained histological section of (c) is given in (d). Findings were consistent in all 11 eye specimens studied.

ciliary epithelium was equal to non-specific background binding (Fig 3b).

A moderate amount of AFDX-116 specific binding was noted in the ciliary muscle, iris, and retina (detached as the result of processing) (Fig 4a). In the ciliary muscle, AFDX-116 binding sites appeared to be more concentrated in the longitudinal portion of the ciliary muscle compared with the rest of the muscle and this was confirmed by densitometric measurements

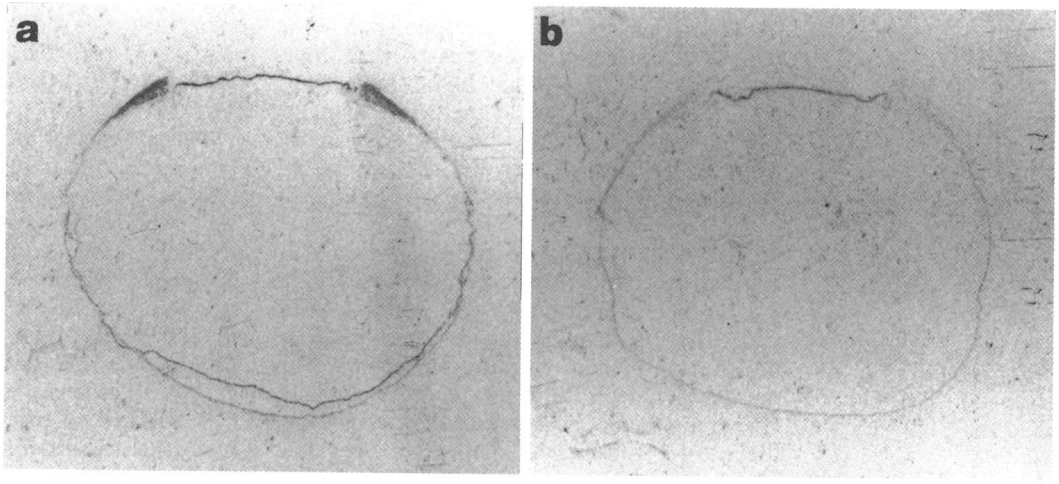

Figure 4 Autoradiographic views of [^{3}H]AFDX-116 binding sites in eye sections from a 42-year-old woman. The binding of 1 nM [^{3}H]AFDX-116 is seen in the ciliary muscle, the iris and in the detached retina (a). Binding sites in the ciliary muscle and retina were completely displaced by 10 μM unlabelled atropine sulphate, and only partially displaced in the iris (b). Non-specific binding correlated with regions of ocular pigmentation. Findings were consistent in all seven eye specimens studied.

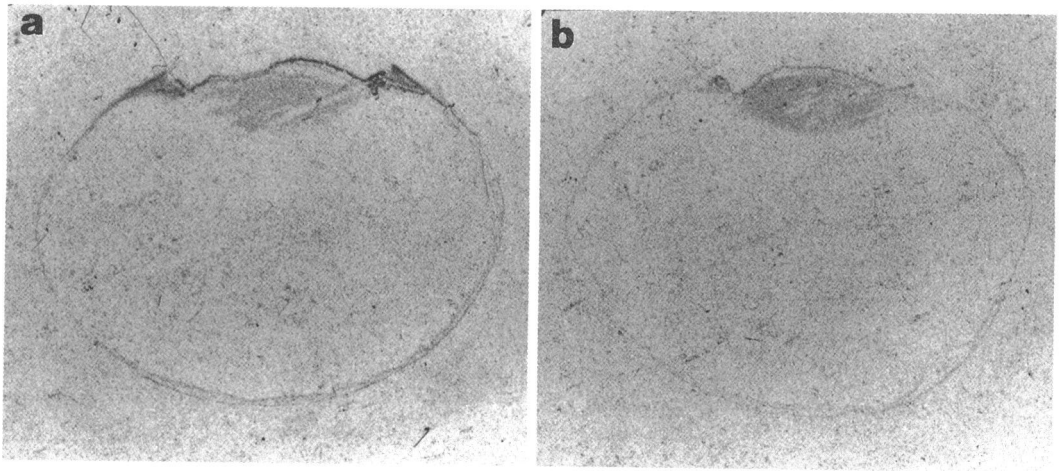

Figure 5 Autoradiographic views of [^{3}H]pirenzipine binding sites in eye sections from a 42-year-old woman. The binding of 1 nM [^{3}H]pirenzipine is seen in the ciliary muscle, the ciliary epithelium, and the iris (a). 10 μM unlabelled atropine sulphate completely displaced binding sites in the ciliary muscle, and partially displaced binding sites in the iris (b). Non-specific binding was noted in the lens and pigmented ocular structures (b). Findings were consistent in all 11 eye specimens studied.

revealing AFDX-116 specific binding site concentrations of 262·9 (11·5) fmol/mg in the longitudinal muscle and 204·1 (10·3) fmol/mg in the remaining ciliary muscle. Binding site concentrations in the iris and ciliary epithelium were 367·4 (9·5) fmol/mg and 194·2 (6·4) fmol/mg respectively. In the presence of unlabelled atropine sulphate (Fig 4b), most of the non-specific binding corresponded to ocular pigmented layers.

Specific pirenzipine binding sites were noted in the anterior segment (Fig 5a) with concentrations of 294·5 (14·7) fmol/mg in the ciliary muscle, 525·9 (16·1) fmol/mg in the iris, and 252·4 (39·7) fmol/mg in the ciliary epithelium. In the presence of 10 μM unlabelled atropine, non-specific binding corresponded to the lens and ocular pigmented layers (Fig 5b).

Discussion

We studied the distributions of non-subtype specific muscarinic binding sites and M1 and M2 muscarinic receptor subtype binding sites in the human eye. Ligand binding with in vitro autoradiographic and densitometric techniques was performed with QNB and subtype specific ligands pirenzipine, oxotremorine, and AFDX-116. Muscarinic receptor B_{max} and K_D reported are consistent with previously reported non-subtype specific muscarinic receptor properties.[5 5a] Concentrations of QNB specific binding sites were highest in the ciliary muscle, followed by the iris and ciliary epithelium. Pirenzipine binding sites in the anterior segment were most abundant in the iris, with moderate concentrations in the ciliary muscle and ciliary epithelium. AFDX-116 specific binding sites were more concentrated in the iris than in the ciliary body, and in the ciliary muscle there was a higher concentration of specific binding sites in the longitudinal portion of the ciliary muscle. Oxotremorine binding was localised specifically in the longitudinal portion of the ciliary muscle, and not in the remainder of the ciliary muscle. In addition, no specific binding was detected in the iris or ciliary epithelium. This finding is of particular interest since by its direct insertion into the trabecular meshwork, contraction of this muscle facilitates aqueous humour outflow, lowers intraocular pressure and is the basis for the use of muscarinic cholinergic agents in glaucoma therapy.[17] Agents currently used in the medical treatment of glaucoma include pilocarpine and carbachol, non-subtype specific muscarinic drugs. Unfortunately, side effects often preclude their clinical use, including painful ciliary muscle spasms particularly in young patients, and pupillary constriction, which in the presence of cataracts is visually debilitating.

The specific localisation of oxotremorine binding sites in the longitudinal ciliary muscle provides evidence that oxotremorine and/or related subtype specific muscarinic agents, have the potential selectively to increase outflow without inducing accommodation. This has been reported with the muscarinic agonist aceclidine[18] and it would be interesting to see whether aceclidine binding sites show the same pattern as oxotremorine. In addition, in the monkey eye, longitudinal ciliary muscle fibres have been shown to differ ultrastructurally and histochemically from fibres in other regions of the ciliary muscle.[19] The absence of specific oxotremorine binding sites in the iris in contrast with the moderate to high QNB, pirenzipine, and AFDX-116 binding site concentrations in the iris also suggests that oxotremorine and/or related compounds may be helpful in decreasing intraocular pressure without inducing miosis.

QNB, pirenzipine, and AFDX-116 receptor binding sites in the iris appeared to be localised in the iris epithelium, and probably in the iris sphincter muscle as has been previously reported using *N*-methylscopolomine, a non-subtype specific muscarinic antagonist.[20] It remains unclear from the present study which layer(s), pigmented or unpigmented contained muscarinic binding sites in the ciliary epithelium; however QNB binding sites have been reported in non-pigmented cells.[21] Similarly, muscarinic receptor binding sites in the retina could not be assigned to specific layers owing to the limited spatial resolution of the autoradiographic technique[15] used in this study; however, QNB binding sites in retinal sections have been reported with finer resolution emulsion techniques.[22] We found M1 and M2 receptor subtypes in the retina and they have been identified in the calf retina.[23]

Pharmacological agents used to identify muscarinic receptor subtypes (M1, M2) do not necessarily correspond exactly to the same cloned receptor subtype (m1, m2).[11] While [^{3}H]oxotremorine is a selective M2 agonist, it has been shown to bind to heterogeneous M2 sites.[9] This, in addition to differences in agonist/antagonist interaction with M2 receptors, may explain in part the different distributions of binding sites between oxotremorine and AFDX-116.

The presence of m2 muscarinic receptor subtype mRNA in cultured human ciliary muscle cells has been shown previously.[24 25] Further in situ hybridisation with cDNAs of muscarinic receptor subtypes may help to determine the genomic subtype corresponding to the oxotremorine and/or AFDX-116 binding sites identified in this study. Pharmacological evidence for M2 and M3 muscarinic receptor subtypes has been demonstrated in tissues and cell cultures of iris sphincter muscle, non-pigmented ciliary epithelium, trabecular meshwork, and ciliary muscle.[14 26 27 28 29] In vivo pharmacological studies suggest a role for at least the M3 receptor subtype in ciliary muscle function[30] and M3 receptors and m3 mRNA have been localised in human anterior segment tissues.[14 24 25] Further physiological experiments will be needed to clarify the ocular roles of M1 and M2 muscarinic receptors, and the effects of oxotremorine and other muscarinic receptor subtype specific drugs in the human eye.

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This work was conducted at the Eye Research Laboratory, Department of Ophthalmology, Faculty of Medicine, 2550 Willow Street, Vancouver, British Columbia, Canada V5Z 3N9.

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