The rabbit cornea lacks cholinergic receptors

Janet S. Olsen and Arthur H. Neufeld

Cholinergic receptors were studied in membranes prepared from rabbit cornea, iris-ciliary body, and retina, using $^3$H-quinuclidinyl benzilate ($^3$H-QNB) to identify muscarinic receptors and $^{125}$I-$\alpha$-bungarotoxin ($^{125}$I-BGT) to identify nicotinic receptors. Muscarinic cholinergic receptors were not found in the cornea. As a positive control, muscarinic cholinergic receptors were characterized in preparations of the iris-ciliary body. Specific binding of $^3$H-QNB to iris-ciliary body membrane preparations was saturable, with a $K_d$ of 1.3 nM QNB. Specificity of the assay for muscarinic receptors was confirmed by the relative abilities of the following compounds to displace $^3$H-QNB: atropine $>$ pilocarpine $>$ hexamethonium. Nicotinic cholinergic receptors were not found in the cornea. As a positive control, nicotinic cholinergic receptors were characterized in preparations of the retina. Specific binding of $^{125}$I-BGT to retinal membrane preparations was saturable with both high and low affinity receptors ($K_d$ values of 1.0 nM and 93 nM BGT, respectively). Specificity of the assay for nicotinic receptors was confirmed by the relative abilities of the following compounds to prevent $^{125}$I-BGT binding: curare $\geq$ nicotine $>$ hexamethonium $>$ atropine. The lack of cholinergic receptors in the cornea, which has high levels of acetylcholine and related enzymes, suggests either an extraordinary use or a lack of function for acetylcholine in this tissue.

Key words: $^3$H-quinuclidinyl benzilate, $^{125}$I-$\alpha$-bungarotoxin, nicotinic, muscarinic, cholinergic, receptors, cornea, iris-ciliary body, retina, rabbit

Of all the tissues in the body, the cornea has the highest concentration of acetylcholine. In addition, the cornea contains choline acetyltransferase, required for the synthesis of acetylcholine, and acetylcholinesterase, required for its hydrolysis. Biochemical and/or histochemical studies have localized these components of the cholinergic system primarily to the corneal epithelium, with much lower activities reported for stroma. Although there has been no definitive demonstration of the function of the cholinergic system in the cornea, suggestions have included a role in ion transport, maintenance of corneal transparency, afferent or efferent transmission of neural information, or some adaptation to the change in corneal environment after birth.

Implicit in any role of acetylcholine as a transmitter is interaction at a receptor site on the membrane of the target cell. Although cholinergic receptors have been demonstrated in many tissues, the presence of these receptors in the cornea has not been investigated.

Cholinergic receptors have been classified...
pharmacologically into two types, nicotinic and muscarinic, according to their relative affinities for various cholinergic agonists and antagonists. The development of specific radioligands has made possible the in vitro study of cholinergic receptors on membranes isolated from tissue preparations. As demonstrated for brain,\textsuperscript{10–13} retina,\textsuperscript{14} ileum,\textsuperscript{15} and iris,\textsuperscript{16} quinuclidinyl benzilate (QNB) is a cholinergic antagonist that binds specifically to muscarinic receptors. As shown for skeletal muscle,\textsuperscript{17–20} brain,\textsuperscript{13, 21–23} and retina,\textsuperscript{24} the snake venom extract, α-bungarotoxin (BGT), is an irreversible\textsuperscript{25} cholinergic antagonist that binds specifically to nicotinic receptors.

We used commercially available tritium-labeled QNB (\(^{3}H\)-QNB) and BGT labeled with iodine-125 (\(^{125}\)I-BGT) to determine whether muscarinic and/or nicotinic cholinergic receptors are demonstrable in membranes prepared from the rabbit cornea. A preliminary report of our results has been presented.\textsuperscript{26}

### Materials and methods

**Preparation of membranes.** Eyes were obtained either frozen from Pel-Freez Biologicals (Rogers, Ark.) or by enucleation of 3 kg male albino New Zealand rabbits sacrificed with an overdose of sodium pentobarbital (Nembutal, Abbott Labs) intravenously. Eyes were immediately placed in ice-cold 150 mM saline, and all subsequent tissue preparation was carried out at 4° C.

Membranes from cornea, iris-ciliary body, and retina were prepared similarly. Each tissue was dissected as quickly as possible and pooled. For studying muscarinic cholinergic receptors, the tissue was homogenized in 32 mM sucrose, 50 mM NaH\(_2\)PO\(_4\) (pH 7.5) with a Brinkmann Polytron. The material that passed through two layers of cheesecloth was further homogenized with a glass/Teflon homogenizer and centrifuged at —40,000 \(\times\) g at 4° C for 10 min. The pellet was washed twice in 50 mM NaH\(_2\)PO\(_4\) (pH 7.5) by centrifugation. The final pellet was resuspended in phosphate buffer, and the protein concentration was determined by the method of Lowry and associates\textsuperscript{27} using bovine serum albumin (BSA) as the standard. Membranes not assayed the same day were stored at —70° C.

For studying nicotinic cholinergic receptors, several types of preparations were used. Membranes were prepared from whole corneas, scraped corneal epithelium, or neural retina as described above, except that the buffer was 50 mM Tris (pH 7.4) and the Polytron and cheesecloth were used only for whole corneas. In addition, a crude tissue homogenate was prepared by homogenizing (glass/Teflon) corneal epithelium or neural retina in Tris buffer.

**Assay for \(^{3}H\)-QNB binding.** A slight modification of the method of Yamamura and Snyder\textsuperscript{10} was used. Standard conditions were as follows: in a final volume of 200 \(\mu\)l of 50 mM NaH\(_2\)PO\(_4\) (pH 7.5), membranes (0.3 mg protein/ml) were incubated for 15 min at 35° C with 2 nM \(^{3}H\)-QNB in the presence or absence of antagonist. The incubation was stopped by addition of 2 ml of 50 mM NaH\(_2\)PO\(_4\). Membranes were collected by filtration onto Whatman GF/C filters and washed with 10 ml of phosphate buffer three times. Filters were dried at 60° C, solubilized in 0.5 ml of Protosol, and counted in 10 ml of Econofluor.

Specific binding was defined as the difference between the amount of \(^{3}H\)-QNB bound in the absence (total binding) and the presence (nonspecific binding) of 10 mM oxotremorine. There was essentially no binding of \(^{3}H\)-QNB to the filters in the absence of membranes.

**Assay for \(^{125}\)I-BGT binding.** A slight modification of the method of Vogel and Nirenberg\textsuperscript{24} was used. Standard conditions were as follows: in a final volume of 200 \(\mu\)l of 50 mM Tris (pH 7.4), 0.15M NaCl and 0.2 mg BSA, membranes (1.0 mg protein/ml) were preincubated for 15 min at 34° C in the absence or presence of antagonist; \(^{125}\)I-BGT was then added (final concentration, 5 nM), and incubation was continued for 2 hr. The incubation was stopped by addition of 2 ml of the Tris/NaCl/BSA solution. Membranes were collected by fil-

### Table I. Binding of \(^{125}\)I-BGT to Millipore cellulose acetate filters

<table>
<thead>
<tr>
<th>Condition</th>
<th>fmole (^{125})I-BGT bound/filter</th>
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<tbody>
<tr>
<td><strong>A. Total binding</strong></td>
<td></td>
</tr>
<tr>
<td>(10 nM (^{125})I-BGT)</td>
<td>6.9 ± 1.7*</td>
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<tr>
<td><strong>B. Nonspecific binding</strong></td>
<td></td>
</tr>
<tr>
<td>(10 nM (^{125})I-BGT + 2 mM curare)</td>
<td>4.1 ± 0.9</td>
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<tr>
<td><strong>Specific binding</strong></td>
<td></td>
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<td>(A — B)</td>
<td>2.8</td>
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*Mean ± SD (n = 5).
Fig. 1. Dependence on incubation time of the binding of $^3$H-QNB to membranes prepared from cornea. Total (○) and nonspecific (●) binding of the radioligand and the assay conditions are described in the Methods section. Each point is the mean of two or three determinations.

Fig. 2. Dependence on protein concentration of the binding of $^3$H-QNB to membranes prepared from iris-ciliary body. Total (○) and nonspecific (●) binding of the radioligand and the assay conditions are described in the Methods section. Binding to the filter was subtracted at each concentration. Each point is the mean of two or three determinations.

tration onto Millipore EGWP filters (0.2 μm pore diameter) which had been presoaked in Tris/NaCl/BSA. Filters were washed once with 10 ml, then five times with 5 ml of the Tris/NaCl/BSA, air dried, and counted in 10 ml of Beckman Ready-Solv HP. For high protein concentrations, membranes were washed by centrifugation.

Specific binding was defined as the difference between the amount of $^{125}$I-BGT bound in the absence (total binding) and the presence (nonspecific binding) of 2 mM curare. Addition of NaCl to the buffer was necessary to eliminate "curare-sensitive" binding of $^{125}$I-BGT to the Millipore filters (Table I).

Reagents. The $^3$H-QNB (29.4 Ci/mmole), $^{125}$I-BGT (124.8-151.8 Ci/mmole), Protosol, and Econofluor were obtained from New England Nuclear Corp., Boston, Mass.; oxotremorine from Aldrich Chemical Co., Milwaukee, Wis.; d-tubocurarine HCl, nicotine, hexamethonium bromide, pilocarpine HCl, atropine sulfate, and Tris (Trizma base) from Sigma Chemical Co., St. Louis, Mo.; and Ready-solv from Beckman Instruments, Inc., Irvine, Calif.

Results

Muscarinic receptors. As shown in Fig. 1, membranes prepared from the cornea did not specifically bind $^3$H-QNB. There was some binding of the radioligand that was not displaced by oxotremorine.

Membranes from iris-ciliary body, assayed in parallel with the cornea as a positive control, did specifically bind $^3$H-QNB. To demonstrate the specificity of this assay for the muscarinic receptor, and therefore the absence of this receptor from the cornea, we pursued the characteristics of the binding of $^3$H-QNB to membranes prepared from iris-ciliary body. Binding of $^3$H-QNB increased linearly with increasing protein concentration (Fig. 2). Nonspecific binding was ~10% of total binding. There was no difference in specific binding between membranes prepared from fresh eyes and those prepared from previously frozen (Pel-Freez) eyes. As shown in Fig. 3, specific binding of $^3$H-QNB was complete by 15 min. The specific binding
of $^3$H-QNB was dependent on the concentration of QNB (Fig. 4). Using Scatchard analysis, the $K_d$ was 1.3 nM QNB and the maximal binding of the radioligand was 600 fmole QNB/mg protein.

Fig. 5 demonstrates the relative abilities of cholinergic agonists and antagonists to displace $^3$H-QNB from membranes prepared from iris-ciliary body. Using 10 mM oxotremorine to define maximal specific binding, the order of potency was: atropine > pilocarpine > hexamethonium.

We consistently found no specific binding of $^3$H-QNB to membranes prepared from fresh or previously frozen rabbit corneas, regardless of the concentration of $^3$H-QNB, incubation time up to 1 hr, or incubation temperature. Thus the rabbit cornea does not have muscarinic cholinergic receptors (Table II).

**Nicotinic receptors.** As shown in Fig. 6, membranes prepared from cornea did not specifically bind $^{125}$I-BGT. There was some binding of the radioligand that was not displaced by curare or nicotine.

Membranes prepared from retina, assayed in parallel as a positive control, did specifically bind $^{125}$I-BGT. To demonstrate the specificity of this assay for the nicotinic receptor, and therefore the absence of this receptor from the cornea, we pursued the characteristics of the binding of $^{125}$I-BGT to membranes prepared from retina. Binding of $^{125}$I-BGT to retinal membranes increased with increasing protein concentration (Fig. 7). Nonspecific binding was <5% of total binding. As shown in Fig. 8, the specific binding of $^{125}$I-BGT to receptors on retinal membranes was a slow process, requiring more than 6 hr for completion. The specific binding was dependent on the concentration of BGT, and the crude homogenate of retina appeared to have both high and low affinity receptors for the radioligand (Fig. 9). Using Scatchard analysis, the high affinity receptors had a $K_d$ of 1.0 nM BGT with maximal binding of 38 fmole BGT/mg protein, and the low affinity receptors had a $K_d$ of 93 nM BGT with maximal binding of 880 fmole BGT/mg protein. Storage of retinal membranes at
Fig. 5. Inhibition of specific binding of \(^{3}H\)-QNB to membranes prepared from iris-ciliary body. Drugs: atropine (o), pilocarpine (x), and hexamethonium (A). The ability of each agent to displace \(^{3}H\)-QNB was compared to 100% inhibition of specific binding by 10 mM oxotremorine. Specific binding and the assay conditions are described in the Methods section. Each point is the mean of two to four determinations.

Fig. 6. Dependence on incubation time of the binding of \(^{125}\)I-BGT to membranes prepared from cornea. Total (o) and nonspecific (•) binding of the radioligand and the assay conditions are described in the Methods section. Each point is the mean of two or three determinations.

\(-70^\circ C\) for as long as 9 weeks did not decrease the specific binding of \(^{125}\)I-BGT.

Fig. 10 demonstrates the relative abilities of cholinergic agonists and antagonists to prevent the specific binding of \(^{125}\)I-BGT to the retinal membranes. Using 2 mM curare to define maximal specific binding, the order of potency was: curare \(\geq\) nicotine > hexamethonium > atropine. Pilocarpine did not prevent \(^{125}\)I-BGT binding. The displacement curve shown for each drug was generated using our standard incubation conditions, i.e., 15 min preincubation with the drug followed by 2 hr incubation with \(^{125}\)I-BGT, in the presence of the drug. Because \(^{125}\)I-BGT binds irreversibly, however, shorter incubations with \(^{125}\)I-BGT yield data indicating higher affinities for the test drugs. For example, when \(^{125}\)I-BGT competed with curare for 2 hr, the \(K_i\) for curare was 200 \(\mu\)M, whereas with a 30 min incubation the \(K_i\) was 5 \(\mu\)M, a value in close agreement with that reported for retina by others.\(^{24, 28}\)

We consistently found no specific binding...
of $^{125}$I-BGT to membranes prepared from fresh or previously frozen whole rabbit corneas, stroma, or scraped corneal epithelium, regardless of the concentration of $^{125}$I-BGT used, incubation time up to 6 hr, or incubation temperature. Thus the rabbit cornea does not have nicotinic cholinergic receptors (Table II).

### Discussion

The enigma of the cholinergic system of the cornea has attracted the interest of many investigators. Rabbit and bovine corneas contain very high concentrations of acetylcholine$^{1,2}$ as well as choline acetyltransferase$^{3,4}$ and acetylcholinesterase.$^{2,6,7}$ The acetylcholine and associated enzyme activities have been localized to the epithelial cell layer, but...
whether the acetylcholine is located in the nerve endings, the epithelial cells, or both has not been firmly established. Fitzgerald and Cooper showed that almost all of the acetylcholine in the epithelial cell layer was lost after local denervation, indicating an association of acetylcholine with neural elements. In contrast, the early work by von Brücke and associates implied that much of the corneal acetylcholine is present in the epithelial cells, because trigeminal denervation by postganglionic denervation decreased acetylcholine levels by only 35% to 50%. Gnadinger and associates demonstrated that cultured epithelial cells contain 50% of the choline acetyltransferase activity of normal scraped epithelium, suggesting that the epithelial cells are capable of synthesizing acetylcholine. Furthermore, denervation of the cornea by retrobulbar injection of alcohol does not alter choline acetyltransferase levels, and denervation by local freezing (Cintrón, Goldberg, and Neufeld, unpublished results) does not decrease the acetylcholine levels in the epithelial layer of the cornea. Thus we can conclude that much and perhaps all of the acetylcholine is present in the epithelial cells. In addition, the acetylcholines- terase is found on the membranes of both nerve and epithelial cells.

The results of our investigations indicate that the rabbit cornea lacks cholinergic receptors. In experiments in which preparations of membranes from iris-ciliary body repeatedly yielded $^3$H-QNB binding data characteristic of muscarinic cholinergic receptors, assays of corneal preparations consistently yielded negative results. Membranes prepared from iris-ciliary body specifically bound $^3$H-QNB, which was displaced by oxotremorine and atropine > pilocarpine > hexamethonium. Such results are compatible
Fig. 10. Inhibition of specific binding of $^{125}$I-BGT to membranes prepared from retina. Drugs: curare (•), nicotine (○), hexamethonium (▲), atropine (○), and pilocarpine (▲). The ability of each drug to prevent binding of $^{125}$I-BGT was compared to 100% inhibition of specific binding by 2 mM curare. Specific binding and the assay conditions are described in the Methods section. Each point is the mean of three to six determinations.

with previous studies of muscarinic receptors from other tissues as well as bioassay and analysis of receptors of the guinea pig iris. Similarly, in experiments in which preparations of membranes from retinas repeatedly yielded $^{125}$I-BGT binding data characteristic of nicotinic cholinergic receptors in this tissue, assays of corneal preparations consistently yielded negative results. Membranes prepared from retinas specifically bound $^{125}$I-BGT which was displaced by curare ≥ nicotine > hexamethonium > atropine. Such results are compatible with previous studies of the nicotinic receptors in retinal tissue.

At the 1978 spring meeting of ARVO, we presented preliminary data that we interpreted to indicate specific binding of $^{125}$I-BGT to corneal membranes. At the time, we were unaware of "curare-sensitive" binding of $^{125}$I-BGT to the Millipore filters we used in our assay (Table 1). Once this problem was eliminated by adding NaCl to the buffer and presoaking the filters, we no longer detected specific binding of $^{125}$I-BGT to corneal membranes. It is not clear to what extent this background binding to the filters has contributed to values for specific binding of $^{125}$I-BGT reported for other tissues.

The relationship between the binding sites for BGT and the receptors for acetylcholine is controversial. Electrophysiologic and binding studies of cultures of sympathetic neurons indicate that the binding sites for BGT may be separate from the binding sites for acetylcholine. Therefore a tissue might have one type of receptor but not the other, and hence the absence of specific binding of $^{125}$I-BGT to corneal membranes does not unequivocally rule out the presence of nicotinic cholinergic receptors in this tissue. Nevertheless, for every mammalian tissue studied to date that has nicotinic cholinergic receptors, binding sites for BGT have been demonstrated. We therefore conclude that, using currently available techniques, cholinergic receptors are undetectable in the cornea.

Attempts to demonstrate the physiologic relevance of acetylcholine in the corneal epithelium have not been successful. Investigations have centered around hypothesized roles of acetylcholine in sensory information processing, regulation of ion transport and dehydration, or modulation of changes in mitotic rate associated with wound healing. However, all of these functions are based on examples from other tissues and require interaction of acetylcholine with mus-
carinic or nicotinic receptors to mediate the response. Our findings that the cornea lacks such receptors suggest that acetylcholine does not act as a transmitter in the cornea and that the components of the cholinergic system that are present may either have some unconventional role or are the remnants of a vestigial pathway.

REFERENCES