Muscarnic acetylcholine receptors of the developing retina
(synapse formation/receptor localization/3-quinuclidinyl benzilate)

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ABSTRACT Six- and 13-day chicken embryo retinas contain 10 and 320 fmol per mg of protein of specific binding sites for 3H-quinuclidinyl benzilate, a ligand of muscarinic acetylcholine receptors. Most of the receptors of 13-day embryo retina were found, by autoradiography, to be localized in two sharp bands within the inner synaptic layer of the retina. In the adult, the receptors were found almost exclusively in three bands in the inner synaptic layer of the retina. A possible mechanism for generating sets of stratified or columnar neurons and relating one set to another is proposed.

The vertebrate retina provides a model system for synapse formation because synaptic circuits may be assembled with relatively few types of cells and because cultured neurons dissociated from retina form synapses in profusion in vitro (1, 2). Biochemical (3-7), histological (8, 9), and electrophysiological (10-13) evidence strongly suggests that acetylcholine (ACh) functions as a neurotransmitter in the retina. Developmental and histological studies of chicken retina acetylcholinesterase (EC 3.1.1.7 AChE) (8), ACh (14), choline acetyltransferase (EC 2.3.1.6) (2), and nicotinic ACh receptors (15, 16) have been reported.

In this report, the properties of muscarinic ACh receptors of chicken retina, the number of receptors, and their distribution within the retina during embryonic development are described.

MATERIALS AND METHODS

Homogenate Preparations. Neural retinas of White Leghorn chicken embryos were homogenized in 50 mM sodium phosphate buffer, pH 7.4 (buffer A). In some experiments, homogenates were diluted several times with buffer A and centrifuged at 17,300 X g for 20 min at 3°. The pellet was suspended in buffer A (membrane fraction). All experiments were performed with freshly prepared homogenates or membranes.

Binding Assay. (3±)-Quinuclidinyl benzilate (QNB), a gift from Hoffman-La Roche, Inc., was labeled by catalytic 3H exchange and purified as described by Yamamura and Snyder (17); the specific activity was 8.4 Ci/mmol. 3±-3H]QNB used in some experiments was obtained from Amersham/Searle (15 Ci/mmol).

[3H]QNB binding was measured by a modification of the method of Yamamura and Snyder (17). Homogenates were combined with [3H]QNB in buffer A and incubated for various periods. Each 100- to 150-μl portion of the reaction mixture (usually containing 100-200 μg of protein) then was diluted into 5 ml of ice cold buffer A, immediately filtered, and washed three times, each with 5 ml of buffer A. Binding kinetics were measured at 25° by using Whatman glass fiber GF/C filters. The concentration of (±)-[3H]QNB in the reaction mixture was 0.5-1.0 nM. When the effects of competing ligands were tested, homogenates were incubated with desired concentrations of ligands for 5-10 min and then mixed with [3H]QNB solution containing the same concentrations of the ligands. Equilibrium studies were performed at 4° with Millipore HAWP filters or, in some cases, GF/C filters (results were essentially the same). For the determination of nonspecific binding, homogenates were incubated with 0.4-10 μM atropine sulfate for 10 min in ice and then mixed with [3H]QNB solution containing the same concentration of atropine. The number of [3H]QNB binding sites was determined by Scatchard analysis in some experiments but more often was determined at one saturating concentration of (±)-[3H]QNB (6-10 nM).

[3H]QNB Autoradiography. Neural retinas were dissected in cold Dulbecco's phosphate-buffered saline with Ca2+ and Mg2+ (PBS). Pieces of retina from 13-day embryos or adult chickens were incubated for 90 min in 5 ml of PBS containing 4 or 2 nM (±)-[3H]QNB (13 Ci/mmole), respectively, and then washed eight times, each with 5 ml of PBS. In control experiments, pieces of retina were preincubated in 5 ml of PBS containing 0.4 μM atropine sulfate for 10 min, followed by incubation in 5 ml of [3H]QNB solution in PBS containing 0.4 μM atropine sulfate for 90 min. The tissue then was washed twice with 5 ml of PBS containing 0.4 μM atropine sulfate and six times with 5 ml of PBS. Samples were kept in an ice bath at each step. Both experimental and control retinas were washed for 25 min (all washes). Retinas then were sandwiched between two pieces of mouse liver and frozen quickly in liquid Freon cooled in liquid nitrogen. Frozen pieces were sectioned (12 μm thick) and thaw-mounted onto glass slides coated with Kodak NTB-2 nuclear emulsion. To minimize diffusion of [3H]QNB, mounted sections were immediately dried under a stream of nitrogen gas. Slides were stored in the dark at 4° with a desiccant. Autoradiographs were developed, fixed, and then immersed in 2.5%; glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, for 1 hr at room temperature. Some slides were stained with 0.02% toluidine blue for 5 min at room temperature.

Retina Cell Cultures. Cells were prepared from 8-day embryo and cultured in rotating petri dishes as described (1) with minor modifications: 1.5 X 105 cells in 3 ml of medium (95% Eagle's basal medium with Earle's salts and 5% fetal bovine serum) were cultured in a bacterial petri dish (35 mm, Falcon no. 1008) placed on a rotary shaker with an excursion of 2.6 cm (75-80 rpm) in a 37° incubator in a humidified atmosphere of 5% CO2/95% air. Half of the medium was replaced each day.

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; QNB, 3-quinuclidinyl benzilate; PBS, phosphate-buffered saline with Ca2+ and Mg2+.
RESULTS

Receptor Properties. The rates of $^{3}H$QNB binding to and release from receptors in homogenates of 13-day chicken embryo retina are shown in Fig. 1A. $^{3}H$QNB bound rapidly to retina membranes; in the presence of 2 nM (+)-$^{3}H$QNB, half maximal binding was achieved in 2 min and maximal binding, in approximately 15 min. Some, but not all, of the reactions are reversible. The addition of 0.38 nM oxotremorine and dilution of reaction mixtures 20-fold resulted in the dissociation of approximately 50% of the $^{3}H$QNB-receptor complex. QNB association and dissociation reactions both exhibited biphasic kinetics with fast and slow association reactions and dissociation reactions. The kinetics will be discussed elsewhere; however, the rate constants ($k$) for fast and slow QNB association reactions were estimated, by assuming bimolecular irreversible reactions as a first approximation, to be $2.7 \times 10^{9}$ M$^{-1}$ min$^{-1}$ and $1.4 \times 10^{8}$ M$^{-1}$ min$^{-1}$, respectively. Both fast and slow QNB-receptor dissociation reactions were first-order reactions with rate constants of $1.2 \times 10^{-5}$ min$^{-1}$ and $0.041$ min$^{-1}$, respectively.

The relationship between $^{3}H$QNB concentration and binding to receptors in retina is shown in Fig. 1B. The binding of the pharmacologically active isomer, (+)-$^{3}H$QNB, to retina receptors is a saturable process. In the presence of 0.4 nM atropine, relatively little nonspecific (+)-$^{3}H$QNB binding was found with homogenates prepared from 13-day chicken embryo retina; however, nonspecific $^{3}H$QNB binding was markedly increased when homogenates are prepared from >15-day chicken embryo retina or posthatched retina. The dissociation constant of specific QNB binding sites for ligands closely resembles that of muscarinic ACh receptors. The apparent Hill coefficient is 1.0 (plot not shown), which suggests that QNB binds to independent, noninteracting receptors. Although only one population of QNB binding sites was detected by Scatchard analysis, kinetics of the QNB binding to and release from receptors show that $^{3}H$QNB–receptor complexes are heterogeneous.

The effects of different concentrations of unlabeled ligands known to activate or inhibit muscarinic ACh receptors on the initial rate of $^{3}H$QNB binding to receptors in homogenates prepared from 13 day chick embryo retina are shown in Fig. 2A. $^{3}H$QNB binding was markedly decreased in the presence of antagonists of muscarinic ACh receptors such as scopolamine or atropine or receptor activators such as oxotremorine, ACh, carbamylcholine, pilocarpine, or muscarine at expected physiological concentrations. $^{3}H$QNB binding was not affected by prior incubation of homogenates with 10 nM α-bungarotoxin for 3 hr at 25°C (not shown). Thus, the specificity of QNB binding sites for ligands closely resembles that of muscarinic ACh receptors.

As shown in Fig. 2B, the apparent Hill coefficients of activators of the muscarinic ACh receptor such as oxotremorine, ACh, and carbamylcholine were 0.6 to 0.8, whereas those of receptor antagonists were approximately 1. These results agree well with those of Birdsell et al. (18). The apparent Hill coefficients of pilocarpine and muscarine were approximately 1. Pilocarpine has been shown to be both an activator and an ant-
Table 1. Apparent dissociation constants and Hill coefficients of ligands for muscarinic acetylcholine receptors of 13-day chick embryo retina

<table>
<thead>
<tr>
<th>Ligands</th>
<th>App Kd*</th>
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<tr>
<td>QNB 25°</td>
<td>0.12</td>
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<tr>
<td>QNB 4°</td>
<td>0.44</td>
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<td>Atropine</td>
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<td>Scopolamine</td>
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<td>Acetylcholine</td>
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<td>Carbachol</td>
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<td>Muscarine</td>
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<td>Pilocarpine</td>
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<td>Tetracaine</td>
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* Values at 25° except otherwise specified. The Kd values for QNB were obtained by determining the binding of [3H]QNB at equilibrium. The Kd values for the other antagonists and activators represent the concentrations that result in 50% inhibition of the initial rate of [3H]QNB binding; values were not corrected for h < 1. The K app values for local anesthetics are estimated from experiments where the retina homogenate with or without different concentrations of a local anesthetic were incubated for 60 min at 25° in the absence of [3H]QNB, then 0.50 nM (+)-[3H]QNB was added and the reaction mixtures were incubated for an additional 5 min.

Values of 0.29 and 4.4 nM (-I-QNB are the dissociation constant values determined from rate constants for slow and fast association and dissociation reactions, respectively.

Muscarinic ACh Receptors during Embryonic Development

The concentration and number of muscarinic ACh receptors in chicken embryo retina are shown in Fig. 3 A and B, respectively, as a function of developmental age. Values reported for nicotinic ACh receptors (16) also are shown for comparison. Muscarinic ACh receptors were detected in 5- to 9-day chick embryo retina, but the concentration was relatively low (10 fmol/mg of protein). Between the 6th and 14th days of embryonic development, the concentration of specific QNB binding sites increased 3-fold. In the 5- to 9-day chicken embryo retina, specific QNB binding sites accumulated exponentially with a doubling time of approximately 26 hr; in 9- to 14-day embryo retina, the doubling time was approximately 60 hr. The maximal concentration of QNB binding sites (320 fmol/mg of protein) was attained in the retina of the 13- to 14-day embryo. No further change was detected during later embryonic development; however, receptor concentrations were lower in retina 2 weeks after hatching and in adult retina. Although the concentration of receptors decreased after hatching, the number of receptors per retina increased slightly after hatching (Fig. 3B). The adult chick retina contained 1200 fmol of specific QNB binding sites per retina (7.2 × 10^11 sites per retina).

These results show that genes for muscarinic ACh receptors are expressed early in the development of the retina and suggest that some neurons synthesize muscarinic ACh receptors but not neuroblasts as reported for nicotinic ACh receptors (16). The number of muscarinic and nicotinic ACh receptors increase more than 30-fold and the receptors accumulate at similar rates between the sixth and ninth days in embryos. The maximal concentration of muscarinic ACh receptors is attained in the retina of the 13-day embryo, whereas nicotinic ACh receptors continue to increase until hatching. The concentration of specific QNB binding sites in retina of the 6- to 13-day embryo is 2- to 3-fold higher than the concentration of α-bungarotoxin binding sites; however, this ratio is reversed in the adult retina. Thus, the ratio of muscarinic to nicotinic ACh receptor changes markedly during retina development.

Cells dissociated from 8-day chicken embryo retina were cultured for various times in rotating petri dishes. At various times, homogenates were analyzed for specific binding of [3H]QNB (Fig. 3A). The concentration of QNB binding sites increased from 50 fmol of specific QNB binding sites per mg of protein in retina of the 8-day embryo to 225 fmol/mg of protein after 4 days of culture. Thus, the accumulation of muscarinic ACh receptors in cultured retina cells resembled that in the intact retina.

Receptor Distribution in Retina. The distribution of [3H]QNB binding sites in intact 13-day chicken embryo retina and adult retina is shown in Fig. 4. In 13-day embryo retina, most of the silver grains were localized in two narrow bands within the inner synaptic layer of the retina (also termed "inner plexiform layer") (Fig. 4A and B). In adult retina (Fig. 4C and...
D1, two or three bands of silver grains could be seen within the inner synaptic layer of the retina.

Histograms relating the density of silver grains on autoradiographs that had been exposed for shorter times with grain location over the retina are shown in Fig. 5. Two sharply defined bands of silver grains of equal density can be seen within the inner synaptic layer of 13-day embryo retina. Fewer silver grains were associated with the lower portion of the inner nuclear layer (cell bodies of amacrine and bipolar neurons and Müller cells) and with ganglion neuron soma and axons but were not associated with other regions of the retina. The average number of silver grains over the entire retina incubated in the absence or presence of 0.4 μM atropine (nonspecific [3H]QNB binding) was 3.83 and 0.87 grain per 100 μm², respectively.

Thus, specific QNB binding accounted for 77% of total QNB binding. In the adult retina (Fig. 5B), three bands of specific QNB binding sites were localized in the inner synaptic layer of the retina. Few, if any, specific binding sites for QNB were detected elsewhere in the retina; thus, muscarinic ACh receptors are localized to a greater extent in the adult retina than in the 13-day embryo retina. In other sections, the first band of specific QNB binding sites near the inner nuclear layer overlapped the first and second fractions of the inner synaptic layer and the demarcation between the second and third bands was less distinct than that shown. The average number of silver grains over the entire retina in the absence or presence of 0.4 μM atropine was 1.39 and 0.96 grain per 100 μm², respectively. Specific [3H]QNB binding was 31% of total QNB binding, in accord with ligand binding results. Adult retina was incubated with 2 rather than 4 nM [3H]QNB to decrease nonspecific [3H]QNB binding; however, the grain density was somewhat lower than expected.

These results show that muscarinic ACh receptors are localized in the inner synaptic layer of the retina and suggest that the receptors are present in some amacrine and ganglion neurons but not in other cell types in the retina.

**DISCUSSION**

The distribution of muscarinic ACh receptors within the inner synaptic layer of chicken embryo and adult retina is compared with previously reported distributions of nicotinic ACh receptors (16) and AChE activity (8) in Fig. 6. Muscarinic and nicotinic ACh receptors and AChE activity are localized in bands within the inner plexiform layer of chick retina. In the embryo, two bands, each with high concentrations of muscarinic ACh receptors and high AChE activity, can be seen; nicotinic ACh receptors are distributed diffusely in two broad bands throughout most of the inner synaptic layer. After hatching, the inner synaptic layer of the retina contains three muscarinic ACh receptor bands, four nicotinic ACh receptor bands (16), and four bands with high AChE activity (8). Nicotinic ACh receptors are present in the outer synaptic layer (16), but not muscarinic receptors. Most, but not all, of the ACh receptor bands are associated with AChE bands. The bands appear in...
an ordered sequence during development, with respect to
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temporal and positional relationships. The maximal concen-
trations of muscarinic and nicotinic ACh receptors are attained
on the 13th and 21st days of embryonic development. Thus,
most of the synapses mediated by muscarinic ACh receptors
probably are formed at an earlier developmental stage in retina
than those mediated by nicotinic ACh receptors. Vogel et al.
(21) have shown that nicotinic ACh receptors are localized at
sites of synapses in chicken retina. The localized bands of
muscarinic ACh receptors within the inner synaptic layer and
the apparent absence of the receptors from cell bodies and axons
of ganglion neurons in adult retina raise the possibility that
muscarinic receptors also may be localized at certain synapses.
Further work is needed to resolve this question.

Bipolar neurons and photoreceptors of retina form double
or triple synapses (ribbon synapses) wherein one cell transmits
information across one synapse simultaneously to two or three
neurons. ACh probably is the transmitter at some double sy-

napses of bipolar neurons because localized nicotinic ACh re-
ceptors have been found on the processes of one or both post-
synaptic cells (21). Because three species of ACh receptor—
muscarinic excitatory, muscarinic inhibitory, and nicotinic—are
widely distributed in the nervous system, ACh released at one
synapse may excite and/or inhibit the recipient neurons,
depending on the species of ACh receptor that are present.

The inner synaptic layer is composed predominantly of
neurites of amacrine, bipolar and ganglion neurons, and syn-
aptic connections with processes of ganglion, amacrine, or bi-
polar neurons. Five layers can be distinguished by phase-con-
trast microscopy but not by transmission electron microscopy
within the inner synaptic layer. However, Dubin (22) has shown
that three classes of synapses that can be identified by ultra-
structural features are stratified in different ways in the inner
synaptic layer of pigeon retina; stratification was not detected
in the retina of other organisms examined.

Eleven layers can be distinguished within the inner synaptic
layer of chick retina on the basis of ACh receptor concentra-
tions and AChE activity. Three additional layers rich in catechol-
amines have been identified in the inner synaptic layer of
chicken retina (5), and four or five glutamic acid decarboxylase
bands have been detected in rat retina (23). These results show
that neurites of one type sort out from those of other types. A
neuron that forms synaptic connections with two or more
neurons is, in effect, a polyanivalent crosslinking agent. Thus,
neighboring neurons that form synapses with two or more cells
of the same type, at the same stage of development, become
linked to one another and sort out from other sets of neurons.
Since a single neuron may both send and receive information
across synapses and may form multiple kinds of synapses, such
neurons may link sets of neurons that form different types of
synapses. The extent of sorting out and the relationship of one-
class of neurons to another may be determined by the number
and kinds of synapses formed by each class of neurons (both pre-
and postsynaptic connections), the sequence of synapse for-
mation, and the initial spatial relationships of the neurons.

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