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LABORATORY INVESTIGATION

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Rapid increase in cytosolic calcium ion concentration mediated by acetylcholine receptors in cultured retinal neurons and Müller cells

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Abstract ● Purpose: This study was conducted to detect the presence of muscarinic or nicotinic receptors in cultured retinal neurons and Müller cells. ● Methods: Pure Müller cell cultures and cocultures of retinal neurons and Müller cells were used; the former, obtained from adult rabbit retinas, and the latter, retinal neurons from neonatal rats, were cocultured with Müller cells. Intracellular calcium ion concentration ($[Ca^{2+}]_i$) following the administration of acetylcholine, a cholinesterase inhibitor (trichlorfon), nicotine or muscarinic agonist with or without a receptor antagonist was monitored using the calcium ion indicator, fura-2. ● Results: Acetylcholine and trichlorfon induced rapid increase in $[Ca^{2+}]_i$ in half of either cell type. Trichlorfon induced positive response in coculture but not in the pure Müller cell cultures. This positive response was blocked only partially in the presence of atropine. Approximately 30–40% of neurons responded to nicotine at 5 μM, which was significantly blocked by α-bungarotoxin at 50 nM. No response to nicotine could be detected in Müller cells. Approximately 50% of neurons responded to muscarine at 50 μM, but 500 μM was required for the formation of calcium transients in 50% of Müller cells. The muscarine inducement of rapid increase in $[Ca^{2+}]_i$ was blocked by atropine. The agonist of M1 (a muscarinic receptor subtype), McN-A-343, at 0.5 μM induced the most significant and rapid increase in $[Ca^{2+}]_i$ both in neurons and Müller cells. McN-A-343 administration at 0.05 μM induced positive response in half the neurons, but only in approximately 10% of Müller cells. Such positive response was not observed following preincubation with the M1 antagonist, pirenzepine, at 50 μM. ● Conclusions: Cocultured retinal neurons enhance the release of acetylcholine following anticholinesterase administration, and approximately half the neurons were found to possess muscarinic and nicotinic receptors. However, Müller cells appeared to possess only the less sensitive muscarinic receptor. Muscarinic receptor subtypes on either type of cell contained at least M1.

Introduction

Retinal Müller cells have various physiological and pathophysiological functions, such as retinal support, transmitter uptake and the regulation of ionic microenvironments [6, 16, 17]. There is considerable evidence indicating the expression of neurotransmitter receptors not only by neurons but also glial cells [5, 8, 9, 18, 23]. Acetylcholine receptors on brain astrocytes have been studied extensively [12, 15, 19]. As with astrocytes in the brain [1], Müller cells may be also involved in signaling from neuron to glia, from glia to neuron or between glial cells mediated by transmitter receptors [13]. However, little attention

has been paid to acetylcholine receptors on Müller cells. To detect receptors, fluorometric techniques using calcium imaging are presently in use [5, 8, 18]. The intracellularly loaded indicator, fura-2, converts calcium ions into fluorescent signals that are recorded by video microscopic apparatus. This study used cocultures of retinal neurons and Müller cells and a pure culture of Müller cells to determine whether acetylcholine receptors, and if so, which subtypes, are expressed in cultured retinal cells.

Materials and methods

Cell cultures

The Müller cell culture was prepared as previously reported [20]. In brief, in Hank's medium solution containing 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO4, 1 mM KH_2PO_4 and 22 mM glucose, the eye of an adult New Zealand White rabbit was cut 2 mm away from the limbus and the anterior portion and vitreous were then removed. The retina was carefully detached and the avascular nonmedullated portion was removed so as to prevent contamination by astrocytes and oligodendrocytes. The residual retina was cut into 0.25×0.25 mm pieces under a biomicroscope and the fragments were centrifuged at 150 *g* for 5 min. The tissue pieces were suspended on culture plates containing Dulbecco minimum essential medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). Seven days after incubation in 5% CO_2 in air at 36.5° C, the suspended tissue was centrifuged, made into pellets and resuspended on coverslips in fresh medium. Nearly all the explants adhered to the surface of the coverslips within 5 days. Previous immunocytochemical and electron-microscopic studies had confirmed the high purity of Müller cell cultures [20, 21]. In the present experiments on pure Müller cell cultures, 5- to 20-day in vitro periods were used. At 3–5 days before coculturing with retinal neurons, confluent cultured Müller cells were again subcultured onto glass coverslips at an approximate density of 3×10^5 cells/cm^2.

Rat retinal neurons were cultured as previously reported [22], using fetal Wistar rats at gestation day 21, or neonatal rats within 2 days after birth. The anterior portion of the eyes was incised to remove the lens and vitreous body. The retina was isolated in Hanks' medium solution, cut into small pieces with a scalpel and centrifuged at 150 *g* for 5 min. The supernatant was discarded. Trypsin (0.25%) was added to the pelleted tissue which was then passed through a 27-gauge needle, pipetted and centrifuged again. The supernatant containing trypsin was discarded, and fresh medium was added to deactivate any remaining trypsin. Finally, the tissue was suspended on glass coverslips with low-density cultures Müller cells as above. Retinal neurons and Müller cells were then cocultured for 7–14 days for use in subsequent experiments. The approximate ratio of neurons and glial cells was 2:1 to 5:1. In the coculture of neurons and Müller cells, neurons easily differentiate from Müller cells as evident from their size and their dendrites under the phase-contrast microscope [5].

Recording cytosolic calcium concentration

The cytosolic calcium concentration ($[Ca^{2+}]_i$) was measured with an Argus 100/CA (Hamamatsu Photonics, Hamamatsu, Japan) as described previously [5, 8, 23]. Cells on the coverslips were washed with KRH solution containing 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KHPO_4$, 0.5 mM CaCl, 6 mM glucose and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid (HEPES) and incubated with 5 mM fura-2-acetoxymethylester (fura-2AM, Dojin Chemical, Kumamoto, Japan), an indicator of free calcium ions, dissolved in KRH solution at room temperature for 30 min. All coverslips were then washed with KRH for removal of excess fura-2AM and examined with an inverted microscope (Olympus IMT-2, Tokyo, Japan). The dye was excited at 340 nm and 380 nm with epifluroescence using a filter exchanger (Olympus OSP-exch). Fluorescence signals were stored and analyzed in a fluorescence microscope/video-camera system equipped with an intensified charged-coupled camera (C-2400-08H, Hamamatsu Photonics) mounted on an Argus 100/CA. Video images were expressed as the ratio of fluorescence intensity during excitation at 340 nm to that during excitation at 380 nm (F340/F380). Rapid change in calcium ion concentration was measured by a computer-assisted system (intracellular calcium ion analyzer U3390-02, version 2, Hamamatsu Photonics).

The cultured cells were then treated with an agent containing the cholinesterase inhibitor, trichlorfon ((2,2,2-trichloro-1-hydroxyethyl)-phosphoric acid dimethyl ester), acetylcholine, muscarine and an acetylcholine receptor agonist at various concentrations with or without preincubation of the receptor antagonist. Acetylcholine chloride, (−)-nicotine, (+)-muscarine and atropine sulfate were purchased from Sigma Chemical Company (St Louis, USA), α-bungarotoxin (nicotinic antagonist), McN-A-343 (an agonist of M1 muscarinic receptor subtype), pirenzepine dihydrochloride (an antagonist of M1 muscarinic receptor subtype) and methoctramine tetrahydrochloride (an antagonist of M2 muscarinic receptor subtype), from Research Biochemicals International (Natick, USA), trichlorfon (cholinesterase inhibitor) from Wako Pure Chemical Industries (Osaka, Japan). The agonist was usually administered by puffing 25 µl onto each coverslip with or without preincubation of each antagonist for 10 min.

Measurement of $[Ca^{2+}]_i$ was made over 30- to 120-s periods at 3- to 5-s intervals. Cells showing more than 110% increase in F340/F380 were considered "positive response cells". The ratio of these to total cells was determined by fixed-field observation. Each experiment was conducted using at least five coverslips.

Results

Response of retinal cells to acetylcholine

The calibration curve for calcium ion concentration was obtained as previously described [7]. $[Ca^{2+}]_i$ increased rapidly in cultured retinal neurons following acetylcholine administration at 0.5 or 5 mM. Figure 1 shows pseudocolor change in intracellular calcium concentration before and after administration of 0.5 mM acetylcholine. $[Ca^{2+}]_i$ increased within 3 s after administration. In the pure Müller cell culture, slight but definite increase in $[Ca^{2+}]_i$ was observed in 30–40% of the cells in each experiment following either administration. Acetylcholine-induced positive response could not be abolished completely by preincubation with 3 µM atropine or 20 nM α-bungarotoxin and there was no positive response in cells subsequent to 0.05 mM acetylcholine administration. The numbers of cells with rapid increase in $[Ca^{2+}]_i$ following acetylcholine administration are shown in Fig. 2. The cholinesterase inhibitor, trichlorfon, at 4 µM induced rapid increase in $[Ca^{2+}]_i$ in nearly all cocultured neurons and Müller cells. After 0.4 µM trichlorfon administration, $[Ca^{2+}]_i$ increased primarily in the neurons (Fig. 3a vs Fig.

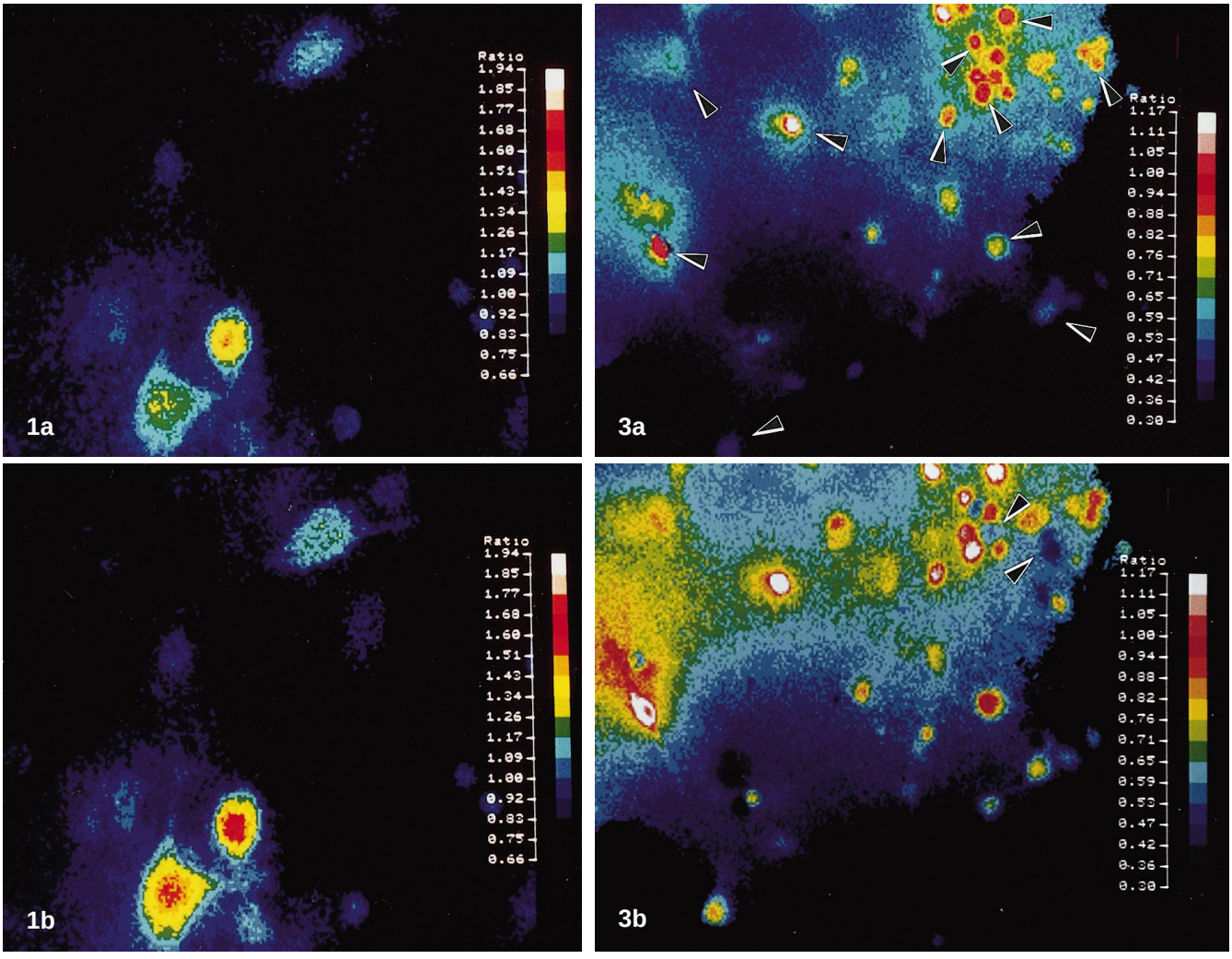

Fig. 1a, b Fluorescence in the pseudocolor image (340/380 nm) ratio. Before (**a**) and 4 s after (**b**) administering 0.5 mM acetylcholine to cultured retinal neurons, the ratio increased significantly in the lower two neurons, while no significant increase is evident in other cells. Magnification ×450

Fig. 3a, b Fluorescence in the pseudocolor image (340/380 nm) ratio. Before (**a**) and 4 s after (**b**) administering 0.4 μM trichlorfon to the coculture of retinal neurons (*triangles* in **a**) and Müller cells. Significant increase in this ratio was noted primarily in neurons, but also in surrounding Müller cells. Reduced intensity in the upper right of the figure (*triangles* in **b**) may possibly derive from an artifact caused by contaminants. Magnification ×200

3b). No positive response could be detected in the pure Müller cell cultures. The numbers of positive Müller and neuronal cells in the coculture after trichlorfon administration at various concentrations are shown in Fig. 4. Trichlorfon (0.4 μM)-induced positive response was completely blocked by preincubation with atropine at 3 μM. Rapid increase in $[Ca^{2+}]_i$ following 4 μM trichlorfon administration persisted even with preincubation with atropine at 3 μM.

Response of retinal cells to nicotine

Nicotine at 0.5–50 μM induced rapid increase in $[Ca^{2+}]_i$ in some cultured neurons (Fig. 5). Nicotine at 5 μM induced positive response in approximately 30–40% of the neurons, but in less than 10% with preincubation of α-bungarotoxin at 50 nM. There was no response by Müller cells.

Response of retinal cells to muscarine and a muscarinic receptor agonist

There was rapid increase in $[Ca^{2+}]_i$ in neurons and Müller cells following muscarine administration. Approximately 50% of the neurons responded to muscarine at 50 μM, but 500 μM was requisite for calcium transient increase in 50% of the Müller cells (Table 1). Muscarine-induced positive response was blocked for the most part following preincubation with 0.3 μM atropine. Following the preincubation of the M1 antagonist, pirenzepine at 50 μM, muscarine (500 μM)-induced positive response was evi-

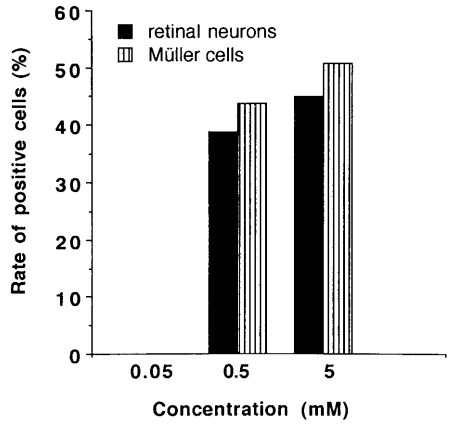

Fig. 2 Rate of positive response to acetylcholine by neurons and Müller cells. Total numbers of cells examined subsequent to administration of acetylcholine at 0.05, 0.5 and 5 mM were 89, 121 and 106 for retinal neurons and 164, 188 and 85 for Müller cells, respectively

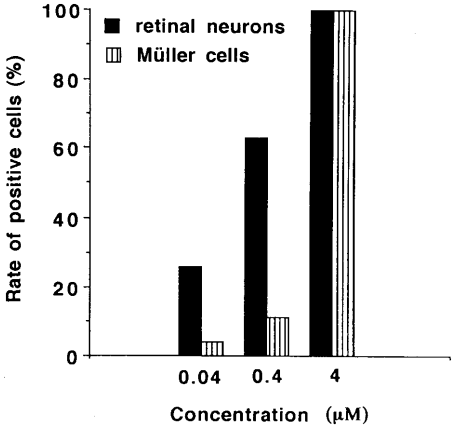

Fig. 4 Rate of positive response to trichlorfon by neurons and Müller cells. Total numbers of cells examined subsequent to administration of trichlorfon at 0.04, 0.4 and 4 μM were 153, 145 and 66 for retinal neurons and 185, 230 and 134 for Müller cells, respectively

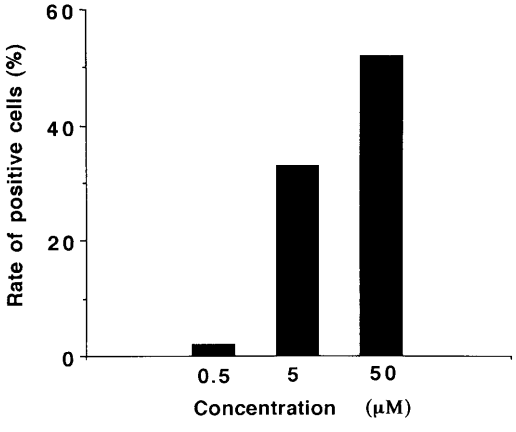

Fig. 5 Rate of positive response to nicotine by retinal neurons. Total numbers of cells examined subsequent to administration of nicotine at 0.5, 5 and 50 μM were 233, 120 and 108

Table 1 Rate of positive response to muscarine (*mus*) and McN-A-343 (*MCN*). *atr* Atropine, *met* methoctramine, *pir* pirenzepine

Administration (μM)	A: Total cells counted	B: Positive cells	B/A (%)
Retinal neurons			
mus (5)	233	5	2
mus (50)	120	64	53
mus (500)	108	90	83
mus (50)+atr 0.3 μM	121	6	4
mus (50)+met 5 μM	51	7	14
MCN (0.05)	64	30	47
MCN (0.5)	92	47	51
MCN (5)	148	94	64
MCN (0.5)+pir 50 μM	70	0	0
Müller cells			
mus (5)	51	0	0
mus (50)	60	1	2
mus (500)	123	60	49
mus (500)+atr 0.3 μM	58	2	3
mus (500)+pir 50 μM	45	3	7
mus (500)+met 5 μM	147	35	24
MCN (0.05)	86	10	12
MCN (0.5)	141	96	68
MCN (5)	125	90	72
MCN (0.5)+pir 50 μM	99	0	0

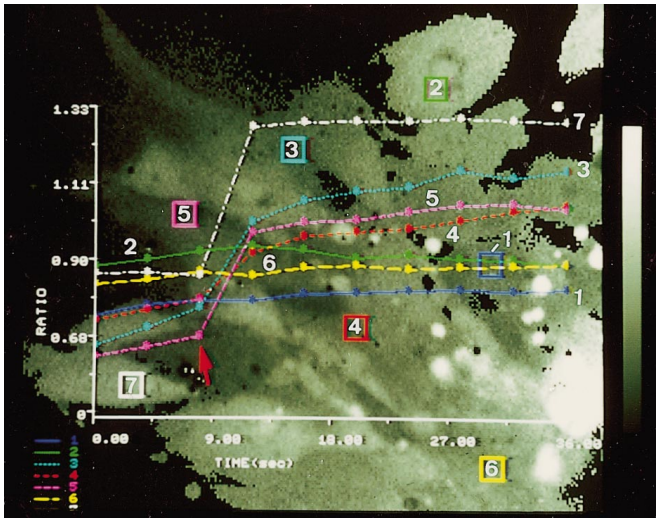

Fig. 6 McN-A-343 administration at 0.5 μM to Müller cells. Cell image is superimposed on the diagram to demonstrate temporal change in $[Ca^{2+}]_i$. Temporal change in cells indicated by squares *1–7* is shown by lines with the same numbers in the diagram. Magnification ×450

dent only in 7% of Müller cells. However, the positive response was still evident in 24% of Müller cells following the preincubation of the M2 antagonist, methoctramine, at 5 μM, with only slight increase in $[Ca^{2+}]_i$.

The M1 agonist, McN-A-343, at 0.05 μM induced positive response in half the neurons, but in only 12% of the Müller cells (Table 1). McN-A-343 at 0.5 and 5 μM in-

duced significant rapid increase in $[Ca^{2+}]_i$ in more than half the Müller and neuronal cells. The response was heterogeneous for the most part in all cells, as evident from Fig. 6, but there were more cells with positive response to McN-A-343 at 0.5 and 5 μM, especially Müller cells, than to muscarine. McN-A-343-induced positive response was completely blocked by the preincubation of pirenzepine at 50 μM.

Discussion

The present study using retinal cell cultures indicates that muscarinic acetylcholine receptors apparently are present not only on retinal neurons but also Müller cells. The adult rabbit retina is the most suitable to obtain pure Müller cell cultures, as discussed in the previous study [20], while retinal neurons may be easily cultured from fetal or neonatal rat retina. However, certain problems should be solved before interpreting the results. In the coculture, the ratio of neuronal to Müller cells was regulated only approximately and there is the possibility that rat Müller cells may be mixed with rabbit Müller cells. So that the effect of such mixing would be as minimal as possible, the present data for Müller cells were obtained only from experiments using pure Müller cell cultures and those for neurons, from the coculture. There are various types of retinal neurons in vivo, while neuronal cells in the culture cannot be clearly distinguished, though still unpublished data show approximately 70% of neurons with neurites to be Thy-1-positive cells in the same culture. The primary objective of this study was to confirm the presence of acetylcholine receptors on Müller cells but not on neuronal cells. The in vivo presence of acetylcholine receptors in retinal neurons has been clearly demonstrated [14]. Therefore, the data presented here for neurons could also be proposed as a positive control.

Among neurotransmitters of amacrine cells, acetylcholine is present, the receptors of which are also expressed in many other retinal neurons such as retinal ganglion cells and bipolar cells [14]. In the embryonic, neurophysiologically immature chick retina, muscarinic and nicotinic acetylcholine receptors may be found [2, 11, 25]. Acetylcholine apparently inhibits neurite outgrowth in the retina [7] and slows axonal transport [4]. Thus, acetylcholine receptors in the retina may have functions in addition to those of neural transmission in the retina. This gives rise to the question as to whether retinal Müller cells also possess the acetylcholine receptor since they appear essential for retinal outgrowth and wiring. In the present study, rapid increase in $[Ca^{2+}]_i$ in cultured Müller cells as well as retinal neurons was induced only at relatively high concentration (0.5 mM). The lack of a micro-injection system to regulate local drug concentration at target cells may have excluded positive results for lower concentration. The exact concentration at which acetylcholine would be functional in cells in assumedly less than that administered in our experiments because acetylcholinesterase may inactivate acetylcholine during diffusion.

Cell response to the strong anticholinesterase, trichlorfon, was induced more efficiently than to acetylcholine itself. Trichlorfon at 0.4 μM appeared to cause the release of acetylcholine exclusively from neurons, since release was noted only in the coculture, not the pure Müller cell culture. Significant inhibition by atropine indicates intrinsic acetylcholine release. However, positive response of neuronal and Müller cells to 4 μM trichlorfon was not completely blocked by atropine. This response may be mediated by nicotinic receptors or direct toxic action of trichlorfon. Nicotine was found to actually induce rapid increase in $[Ca^{2+}]_i$ in neurons, and preincubation with α-bungarotoxin eliminated positive response primarily, indicating the nicotinic receptor to likely be present in cultured retinal neurons. α-Bungarotoxin is a nicotinic receptor antagonist specific for neuromuscular junctions, but α-bungarotoxin-sensitive nicotinic receptors have recently been found abundantly present in the retina [2]. The use of α-bungarotoxin as a nicotinic antagonist would be justified by its ability to block the response to nicotine in most retinal neurons. Nicotinic receptors have been shown present in cultured brain astrocytes [3], but not in retinal Müller cell cultures.

Muscarine-induced rapid increase in $[Ca^{2+}]_i$ was not completely blocked by the M2 antagonist, methoctramine, in retinal neurons or Müller cells, suggesting that other muscarinic receptor subtypes also contribute to the muscarinic response. A response to the M1 agonist, 0.5 μM McN-A-343, was clearly evident in approximately half the neurons and even 68% of Müller cells. An M1 receptor subtype would thus appear present in both cell types. These findings are consistent with previous studies demonstrating the muscarinic M1 or M2 receptor to be present in mammalian retina [10, 24].

Whether muscarinic receptors on Müller cells have physiological or pathological functions remains unknown. Approximately 40% of the neurons responded to muscarine at 50 μM, but 500 μM was required for calcium transient increase in 30% of the Müller cells, suggesting muscarinic receptors in Müller cells have a function at high or pathologic concentration of acetylcholine.

The presence of muscarinic receptors in Müller cells may indicate functions other than neural transmission. Clarification of characteristic differences in acetylcholine receptors for retinal neurons and Müller cells may provide better understanding of the specific muscarinic receptor functions of Müller cells.

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