Cholinergic Regulation of $[Ca^{2+}]_i$ during Cell Division and Differentiation in the Mammalian Retina

Rachel O. L. Wong

Vision, Touch and Hearing Research Centre, Department of Physiology and Pharmacology, University of Queensland, Brisbane, Queensland 4072, Australia

Transmitter-like molecules are thought to influence many aspects of neuronal development, often by regulating the levels of intracellular calcium. Using the Ca^{2+} sensitive dye, fura-2, this study demonstrates that in the rabbit retina, ACh analogs stimulate a rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in many cell types, and in cells at various stages of differentiation during embryonic and neonatal development. The elevation in $[Ca^{2+}]_i$ in cells within the ventricular zone (VZ) resulted primarily from the activation of muscarinic receptors. By contrast, the cholinergic regulation of $[Ca^{2+}]_i$ of ganglion cells and amacrine cells, cell types which migrate to their final destinations early in fetal life, was largely mediated by nicotinic receptors. The muscarinic response of the VZ cells was mediated by the M1, rather than the M2-type of muscarinic receptor. This response was abolished in the absence of extracellular Ca^{2+} and in the presence of $NiCl_2$, but it was not affected by verapamil or ω -conotoxin, thus suggesting that while Ca^{2+} influx occurred, it did not involve L- and N-type voltage-gated Ca^{2+} channels. The muscarinic response in the VZ disappeared at the end of the period of cell division in the retina, just prior to eye opening. By contrast, nicotinic-induced changes in $[Ca^{2+}]_i$ in ganglion cells and amacrine cells persisted throughout development. Since previous studies have implicated that the precursors of ganglion cells and amacrine cells also possess muscarinic receptors (Yamashita and Fukuda, 1993), the concomitant emergence of different functional cholinergic receptor (AChR) subtypes with differentiation *in vivo* suggests that ACh may play diverse and temporally regulated roles in the developing retina.

[Key words: muscarinic receptors, nicotinic receptors, ventricular zone, retinal development, intracellular calcium, cell division and differentiation]

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Correspondence should be addressed to Dr. R. O. L. Wong, Department of Anatomy and Neurobiology, Washington University School of Medicine, Box 8108, 660 South Euclid, St. Louis, MO 63110.

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Neuronal cells and their progenitors depend on signals from the extracellular environment to guide their development. In recent years, many *in vitro* studies have implicated a developmental role for neurotransmitter-like molecules, even before synaptic contacts are established (Lankford et al., 1988; Mattson, 1988; Lipton and Kater, 1989; Spitzer, 1991; Komura and Rakic, 1993; Lauder, 1993). The common neurotransmitter, ACh, binds to two major subclasses of receptor, the muscarinic receptor (mAChR) and the nicotinic receptor (nAChR). Acting on mAChRs, ACh can regulate cellular proliferation *in vitro* (Ashkenazi et al., 1989; Gutkind et al., 1991). By contrast, nAChRs are involved with neurite outgrowth (Lipton et al., 1988) and pathfinding by neuronal growth cones (Zheng et al., 1994). Thus, ACh appears to influence many different aspects of development, probably because its receptors are linked to a variety of signaling pathways (Nicoll et al., 1990).

The influence of ACh on neuronal development *in vivo* is likely to depend on the subclass of AChR that is functionally expressed at each stage. However, because of the difficulty of recording from immature neurons in the intact tissue, little is known about when neuronal AChRs become functional, and which signaling pathways are associated with these receptors during development. The present study examines when cells of the developing mammalian retina become responsive to mAChR and nAChR agonists, with the aim of establishing whether muscarinic and nicotinic responses are correlated with the developmental state of the cell. The rabbit retina was used as a model because it possesses mAChRs and nAChRs (Neal and Dawson, 1985; Hutchins, 1987), and because there exists a well-defined spatial and temporal order in the generation and differentiation of its cell types (Masland, 1977; McArdle et al., 1977; Greiner and Weidman, 1982). In addition, the retina remains intact upon dissection and can be maintained *in vitro* for many hours in Ames medium (Ames and Nesbett, 1981), thus permitting the recording of cell responses over several hours, in an environment similar to that found *in vivo*.

Changes in intracellular Ca^{2+} are correlated with fundamental developmental events (Lipton and Kater, 1989; Ciapa et al., 1994). Thus, optical recording techniques and the calcium sensitive dye, fura-2, were used here, not only to determine which cells in the immature retina are responsive to cholinergic stimulation, but also to determine which of their AChRs are linked to Ca^{2+} signaling pathways during development. Such an approach also enabled a rapid assessment of the responses of a large number of immature cells, and a direct comparison of the responses of the various cell types within a single field of view (Yutse and Katz, 1991).

Figure 1. Images of the ganglion cell layer of a E25 rabbit retina labeled with fura-2, viewed during illumination with 380 nm light. During addition of the cholinergic agonist, carbachol (CCh), some cells showed a decrease in fluorescence intensity (examples marked by arrows). The cells which responded can be seen when the image at the top right (CCh present) is digitally subtracted from the image at the top left (resting levels). If the signal-to-noise ratio in the difference image (bottom left) is relatively low, the image is contrast enhanced (bottom right). The responses of cells are confirmed by playing back a movie of the sequence of images before and during application of the agonists, and also plotting their $[Ca^{2+}]_i$ change using the ratiometric method (see Materials and Methods).

In this study, the responses of retinal cells to exogenous application of AChR agonists were examined between embryonic day 20 (E20) and postnatal day 21 (P21). Cytogenesis in the rabbit retina occurs during embryonic life (gestation is 30–32 d) and ceases at the end of the first postnatal week (Stone et al., 1985). At E20, the first postmitotic cells, the ganglion cells, are present in large numbers, and by P21, the retina attains its full complement of cell types with adult-like electrophysiological responses (Masland, 1977). The muscarinic and nicotinic responses of each cell type, identified by their somata size and spatial location, were compared within the same retinal piece, and examined as a function of age.

Materials and Methods

A total of 16 fetal (E20–27), and 27 neonatal rabbit retinæ (P1–21) were examined. Fetuses were delivered by caesarean section under 4% halothane, supplemented by 60 mg of sodium pentobarbital (Nembutal). After the fetuses were removed, the mother was given an overdose of Nembutal. The fetuses and neonates were euthanized with Nembutal, and the eyes enucleated and hemisected. The retinæ were dissected from the eyecup in 4°C oxygenated Ames medium (Sigma), and mounted on a piece of black filter paper (Millipore, HABP 045). After incubation in fura-2AM (Molecular Probes, 10 μ M in 0.001% pluronic acid/Ames medium) for 1 hr, the retinæ were washed briefly in Ames medium and transferred to a temperature-controlled perfusion chamber fitted onto an inverted microscope. All recordings were performed at 30–34°C. So that comparisons of cellular responses could be made

Figure 2. Spatial patterns of CCh-induced $[Ca^{2+}]_i$ changes in the fetal and neonatal rabbit retina. Red regions indicate those in which the fluorescence intensity decreased (under 380 nm excitation) on addition of 50 μ M CCh to the perfusion bath, while those in green and blue showed no changes in fluorescence intensity (see Materials and Methods for details). *A*, The ganglion cell layer (GCL) of an E25 retina, viewed from the vitreal surface. *B*, Ventricular surface of an E25 retina. *C*, Cross-sectional view of a P1 retina. AmL, Amacrine cell layer; IPL, inner plexiform layer; VZ, ventricular zone. *D*, Cross-sectional view of a P11 retina (at eye opening). INL and ONL are inner and outer nuclear layers, respectively; arrow indicates a cell in the ONL that responded to CCh. Scale bar (for A–D, 10 μ m).

across ages, the recordings were always performed on the inferior edges of the retina, avoiding the relatively more mature visual streak (Stone et al., 1985). Pharmacological agents were either perfused (5 ml/min) into the chamber or the agonists pipetted directly into the chamber to give the final desired bath concentration. All agonists and antagonists were obtained from Sigma (St. Louis, MO) except for ω -conotoxin which was purchased from Alomone Labs (Israel).

Using IMAGE-1 FL (Universal Imaging Corp.) and a shutter system (Lambda-10, Sutter Instr.), consecutive pairs of images (each an average of 16 or 32 frames) were acquired by a low-light level camera (SIT, Hamamatsu) during 340 nm and 380 nm excitation, and stored on optical disk every 5–10 sec (Sony LVR/LVS 5000). Background measurements were taken from regions outside the retinal piece. An estimate of the $[Ca^{2+}]_i$ was obtained after converting the ratio (F_{340}/F_{380}) of the average pixel intensity of the cell at 340 nm (F_{340}) and 380 nm (F_{380}) excitation, using the calibration procedures described by Grynkiewicz et al. (1985) and Yuste and Katz (1991). (The absolute $[Ca^{2+}]_i$ values obtained from slices is subject to some error, due to the fluorescence from out-of-focus cells; see Yuste and Katz, 1991). K_d values for fura-2 ranged from 225 nM at 34°C to 250 nM at 30°C.

Cells which responded to agonist stimulation could be identified by observing the changes in the “ratio” image. Alternatively, responsive cells were identified after digital subtraction of a “380 nm” image ac-

quired at the peak of the response, from an image taken at resting Ca^{2+} levels (baseline image). The image frame at which the peak response occurred was determined by observing the changes in the “ratio” image. An example of the results of the digital subtraction procedure is given in Figure 1. In cases where the signal-to-noise ratio appeared low, the difference image was contrast-enhanced either digitally using the software of IMAGE-1, or by adjusting the contrast and brightness settings of the SIT camera prior to image acquisition.

To demonstrate responsive cells and nonresponsive cells in a single (color) image, the difference image and baseline image were transferred to a Macintosh computer and processed using Adobe PHOTOSHOP 2.0. The baseline (380 nm) image was first split into its RGB (red, green, blue) components; the “blue” and “green” components of the baseline image were then merged with the enhanced difference image which was assigned as the “red” component. The resultant RGB image shows activated cells in “red” and nonactivated cells in “blue-green.” Figure 2A is the result of using this procedure to color code the responses of the cells in the region shown in Figure 1. These images produced identical spatial distributions of responsive and nonresponsive cells as those seen in the ratio images, but provided a clearer definition of cell boundaries. Cell responses were confirmed by playing back the sequence of image frames as a movie to detect intensity changes, and plotting the $[Ca^{2+}]_i$ of each cell over time, using the software of IMAGE-1 FL (UIC).

Figure 3. CCh-induced responses of representative cells from the VZ of an E25 (*A, B*) and a P1 (*C–E*) retina. In between perfusion with the various substances (horizontal bars), the retinae were superfused with Ames medium alone. Arrows (in *A, C–E*) indicate the addition of 50 μ M CCh, which was present in the bath for a total of 60 sec. *B*, (+)-Muscarine chloride (*M*, 100 μ M) was added as indicated for a total of 60 sec. In *E*, 40 mM KCl was added for 30 sec. *PZ*, Pirenzepine dihydrochloride; *Ga*, gallamine triethiodide; *Atrp*, atropine; *VP*, verapamil; ω -CTx, ω -conotoxin.

Results

In fura-2-labeled fetal and neonatal retinae, putative ganglion cells and amacrine cells were identified by their round somata and their location within the vitreal half of the retina. In the rabbit, ganglion cells and amacrine cells differentiate and reach their respective positions within the retina early in fetal life (Greiner and Weidman, 1982; Robinson, 1991) and are found to contain transmitter-like molecules after E20 (Pow et al., 1994). Between E20 and P5, cells in the scleral half of the fura-2 loaded retinae, the ventricular zone (VZ), had oval somata that resembled undifferentiated cells (Greiner and Weidman, 1982; Robinson, 1991). The VZ has been previously found to comprise cytotlastic cells and cells which have left the mitotic cycle and are migrating to their final destinations (Rapaport et al., 1985; Robinson, 1991). Cytoblasts that are present between E20 and P5 differentiate into the inner and outer nuclear layers of the retina by eye opening, at around P10; the majority of these cells become bipolar cells, Müller glial cells, horizontal cells, or photoreceptors (Robinson, 1991).

Regulation of $[Ca^{2+}]_i$ by mAChRs

Carbachol (carbamylcholine chloride, CCh), a nonhydrolyzable cholinergic agonist, has previously been shown to activate

mAChRs on neuronal and non-neuronal cells (Ashkenazi et al., 1989; Gutkind et al., 1991; Yamashita and Fukuda, 1993). In Figure 2, cells that exhibited an increase in $[Ca^{2+}]_i$ in the presence of CCh are coded in red (see Materials and Methods). Very few cells of the fetal ganglion cell layer (GCL) responded with increased $[Ca^{2+}]_i$ in the presence of 25–100 μ M CCh (Fig. 2*A*). By contrast, virtually every cell at the ventricular surface of the fetal retina were stimulated (Fig. 2*B*). The different effects of CCh on cells in the VZ and cells in the GCL was even more apparent from recordings of retinal cross-sections. Figure 1*C* shows that in this field of view, cells in the GCL and developing amacrine cell layer (AmL) were not stimulated by CCh, but cells throughout the VZ responded with an elevation in $[Ca^{2+}]_i$. After eye opening (P11), CCh was less effective in raising the $[Ca^{2+}]_i$ of the retinal cells, especially the cells in the outer retina which by this stage, had differentiated into bipolar cells, Müller glial cells, horizontal cells or photoreceptors (Fig. 2*D*). Thus, the CCh-induced $[Ca^{2+}]_i$ rise in the VZ occurred only for a transient period of development, that is, primarily during the period of active cell division and cell migration in this region.

Ratiometric measurements indicated that the resting levels of $[Ca^{2+}]_i$ of the ventricular cells ranged from 25–50 nM, and that CCh (25–200 μ M) increased the $[Ca^{2+}]_i$ by 50–350 nM in a dose-

Figure 4. Nicotine raised the $[Ca^{2+}]_i$ of ganglion cells and amacrine cells more effectively than CCh. Red colored cells indicate stimulated cells, and blue-green colored cells indicate those which did not respond. *A*, The ganglion cell layer (GCL) of an E25 retina, seen from the vitreal surface of a whole mount, during perfusion with (–)-nicotine ditartrate (200 μ M). *B*, Cross-section of an E25 retina showing cells in the developing amacrine cell layer (AmL) and GCL responding to nicotine (200 μ M). VZ, Ventricular zone. *C* and *D*, Cross-sectional view of a P1 retina in the presence of 200 μ M nicotine (*C*) and 100 μ M CCh (*D*). IPL, Inner plexiform layer. Some cells responded to both CCh and nicotine (arrows, *C*, *D*). Scale bars, 10 μ m.

dependent manner. The increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) varied from cell to cell, with no apparent correlation with age; for example, at E25, $\Delta[Ca^{2+}]_i = 55\text{--}348$ nM (number of cells = 100), and at P1, $\Delta[Ca^{2+}]_i = 45\text{--}340$ nM (number of cells = 100).

To investigate whether the CCh-evoked responses in the developing retina resulted from mAChR activation, the effects of CCh were monitored in the presence of mAChR antagonists (Kuba et al., 1989; Hulme et al., 1990). Atropine (100–300 μ M), a general mAChR blocker, completely abolished the CCh-induced $[Ca^{2+}]_i$ of the ventricular cells (Fig. 3*A*). Pirenzepine, at low concentrations, is more selective for the pharmacologically classified M1 mAChR than the M2 mAChR (McCormick and Prince, 1986). While 2 μ M pirenzepine dihydrochloride reduced the peak rise in $[Ca^{2+}]_i$, 10 μ M of this antagonist abolished the CCh action (Fig. 3*A*). The pirenzepine blockade was rapidly reversible after washing in Ames medium, although the peak response was somewhat lower. By contrast, gallamine triethiodide, a substance that is more antagonistic to the M2 receptors,

did not block the CCh-evoked $[Ca^{2+}]_i$ rise (Fig. 3*A*). (+)-Muscarine chloride evoked an agonistic pattern of response in the ventricular cells, which was blocked by pirenzepine (Fig. 3*B*). These results suggest that the changes in $[Ca^{2+}]_i$ associated with CCh are due to the activation of mAChRs, and probably involve the pharmacologically classified M1 mAChRs rather than the M2 mAChRs. However, it is not possible to assess pharmacologically whether other mAChRs (such as m3, m4, and m5, by molecular characterization) are also activated by CCh because the specific agonists and antagonists to these receptors are not available (Nathanson, 1987; Kuba et al., 1989; Hulme et al., 1990).

The CCh-induced rise in $[Ca^{2+}]_i$ may have resulted from Ca^{2+} influx and/or the release of Ca^{2+} from intracellular stores. To determine if extracellular Ca^{2+} was necessary, the CCh responses were monitored in the presence of the Ca^{2+} -chelator, ethylene glycol-bis(*b*-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA). In the fetal and neonatal retinæ, no increase in $[Ca^{2+}]_i$

Figure 5. Line-drawings of cross-sectional views of the retina at various ages showing cells which responded (shaded in black) to 100–200 μ M nicotine with an increase in $[Ca^{2+}]_i$. In the fetal retina, differentiating cells which form the primitive ganglion cell layer (GCL) were located in the vitreal half (V) of the retina; the scleral half (S) comprises ventricular cells. At P1, cells in the ventricular zone (VZ) were still homogenous in shape and size, except for some horizontal cells, which were often difficult to identify in the fura-2 labeled retinae (when present, these cells marked the position of the forming outer plexiform layer, indicated by the asterisks). By P5, a thin outer plexiform layer separated the inner (INL) and outer (ONL) nuclear layers. Putative amacrine cells (a) and bipolar cells (b) are indicated.

was seen in the presence of CCh and Ames medium containing 5 mM EGTA (e.g., Fig. 3C). Instead, on perfusion with the EGTA-Ames solution, the resting $[Ca^{2+}]_i$ of the ventricular cells decreased, presumably because spontaneous influx of Ca^{2+} was abolished after removal of the extracellular Ca^{2+} . This observation suggests that most, if not all, of the increase in $[Ca^{2+}]_i$ evoked by CCh can be attributed to the influx of Ca^{2+} from the extracellular space. In order to confirm that Ca^{2+} influx occurred in response to CCh, the responses of the ventricular cells to CCh were examined in the presence of calcium channel blockers (Tsien and Tsien, 1990). Ventricular cells of the fetal and neonatal retina failed to respond to CCh in the presence of 1.5–5 mM $NiCl_2$ (e.g., Fig. 3D). Interestingly, the resting levels of $[Ca^{2+}]_i$ decreased on perfusion with Ni^{2+} , as with EGTA. Thus, the increase in $[Ca^{2+}]_i$ due to CCh stimulation is mainly the result of Ca^{2+} influx.

To determine whether L- and N-type voltage-gated Ca^{2+} channels were involved, the CCh responses were recorded in the presence of Verapamil, an L-type voltage gated calcium channel blocker, and ω -conotoxin, an N-type channel blocker (Tsien and Tsien, 1990). Neither blocker abolished the CCh-evoked response, even though in combination they dramatically reduced the action of KCl (Fig. 3E). These observations suggest that Ca^{2+} influx probably occurs through receptor-operated channels, rather than via L- and N-type voltage-gated Ca^{2+} channels. However, it remains possible that some Ca^{2+} entered the cells via

other voltage-gated channels, in particular, the T- and P-type channels (Tsien and Tsien, 1990). It also remains possible that Ca^{2+} entry may have caused the subsequent release of Ca^{2+} from intracellular stores (Tsien and Tsien, 1990), although it is unlikely that CCh resulted in the mobilization of Ca^{2+} from internal stores by means of other second messengers, such as inositol 1,4,5-triphosphate (IP_3) (Neher et al., 1988).

Regulation of $[Ca^{2+}]_i$ by nAChRs

Nicotine-induced elevations in $[Ca^{2+}]_i$ were observed as early as E20, when a primitive ganglion cell layer was present. In the fetal retina, almost all the cells in the ganglion cell layer showed an increase in $[Ca^{2+}]_i$ in response to 100–200 μ M (–)-nicotine ditartrate (Fig. 4A). Recordings from retinal cross-sections indicated that putative amacrine cells were also responsive to nicotine during fetal life (Fig. 4B). By contrast, few cells in the VZ responded to nicotine, although almost all of these cells were stimulated by CCh (compare Fig. 4C,D for a P1 retina). The distributions of cells that were stimulated by nicotine at the different ages studied are summarized in Figure 5.

Ratiometric measurements confirmed that ganglion cells and amacrine cells were more responsive to nicotine than to CCh (Fig. 6). Resting levels of $[Ca^{2+}]_i$ of ganglion cells and amacrine cells were similar, ranging between 40 and 100 nM at all ages studied. In the fetal and neonatal retinae, nicotine (100–200 μ M)

Figure 6. Examples of the responses of P1 ganglion cells (GC) and amacrine cells (Am) to carbachol (CCh) and nicotine that was present for the duration marked by the solid bars. Asterisks indicate spontaneous activity in two of the cells displayed here. Calcium peaks arising from spontaneous activity lasted 15–25 sec, whereas cholinergic stimulation elicited Ca^{2+} elevations that lasted over 1 min.

caused an increase in $[\text{Ca}^{2+}]_i$ of ganglion cells and amacrine cells between 100 and 300 nM ($n = 180$ at E25, 45 at P1).

Previous investigations have examined exhaustively the action of various nAChR antagonists on retinal ganglion cells (Lipton et al., 1987). The present study confirms that the nicotinic responses of the ganglion cells and amacrine cells are blocked by *d*-tubocurarine (e.g., shown in Fig. 7A for a P1 ganglion cell). Furthermore, influx of Ca^{2+} is responsible for the increase in the $[\text{Ca}^{2+}]_i$ of these cells as suggested by the Ni^{2+} blockade (Fig. 7B). By contrast to the CCh action on the progenitor cells, the nicotine-induced rise in $[\text{Ca}^{2+}]_i$ of ganglion cells and amacrine cells was significantly reduced by ω -conotoxin and nifedipine,

suggesting the participation of N- and L-type channels, respectively (Fig. 7C).

Quantitative comparison of cells responding to CCh or nicotine as a function of age

Figure 8 summarizes the percentage of cells in the GCL and the VZ, which responded to muscarinic and nicotinic receptor agonists at different ages. At all ages examined, many cells in the GCL responded with an increase in $[\text{Ca}^{2+}]_i$ in the presence of nicotine; only a few cells in this layer responded to CCh. The number of nicotine-responsive cells remained high throughout fetal and neonatal development, although there appeared to be a decrease in numbers by the second and third postnatal weeks. By contrast, a large proportion of ventricular cells between E20 and P5 were affected by CCh and only a small number of these cells (later presumed to be bipolar cells) were stimulated by nicotine. A dramatic decrease in muscarine-induced Ca^{2+} responses was observed at the time of eye opening, when cell division and migration in the retina had ceased and the INL and ONL were established.

Discussion

Spatial and temporal patterns in the appearance of mAChRs and nAChRs

Ventricular cells of the rabbit retina were transiently sensitive to mAChR agonist between E20 and P5; few of these cells responded to nicotine. Since virtually every cell in the VZ shows a muscarinic-induced $[\text{Ca}^{2+}]_i$ rise and large numbers of cells in this region can be labeled with ^3H -thymidine (Rapaport et al., 1985; Robinson, 1991), it is likely that at least some, if not all, mitotically active cells responded to the mAChR agonists. However, it could be that migrating postmitotic cells also responded to CCh and muscarine. Furthermore, because the current recordings involve bathing the entire slice with the agonist, it is possible that only a subpopulation of the cells in the VZ actually bear mAChRs. If so, then the observation that virtually every ventricular cell shows an $[\text{Ca}^{2+}]_i$ rise implies that these cells could be linked by intercellular junctions which help spread the response from cell to cell (Lo Turco and Kriegstein, 1991). Thus, whether it is only mitotically active cells and/or migrating cells that are directly stimulated by mAChRs agonists, and whether intercellular coupling exists in the retinal VZ, remain interesting issues to be resolved.

The current observations suggest that whilst mAChR agonists stimulate cells in the VZ, nicotinic agonists are more potent in eliciting an $[\text{Ca}^{2+}]_i$ increase in the early generated ganglion cells and amacrine cells. Recordings from the chick retina at the neuroepithelial stage indicate that the $[\text{Ca}^{2+}]_i$ of the precursors of ganglion cells and amacrine cells is also regulated by mAChRs, rather than nAChRs (Yamashita and Fukuda, 1993). Thus, the different spatiotemporal patterns in the appearance of functional mAChRs and nAChRs implicate an “early” and “late” developmental role, respectively, for these receptor subtypes in the retina.

Potential developmental roles for mAChRs and nAChRs

Recordings from the chick retina show that the activation of mAChRs on the neuroepithelial cells leads to a change in the retinal curvature (Yamashita and Fukuda, 1993). The current findings suggest other potential roles for these receptors. One possibility is that the mAChRs on the ventricular cells are involved in neurogenesis, since in cell culture, the M1 and M4

Figure 7. Recordings from ganglion cells (A, B; GC in C) and an amacrine cell (Am in C) from PI retinæ, in the presence of nicotine (100 μ M, added at the time marked by the arrow, and present for a total of 60 sec). Horizontal bars indicate durations for which the relevant pharmacological agents were added. ω -CTx, ω -Conotoxin; Nif, nifedipine. In C, 40 mM KCl was added into the bath at the time point indicated by the arrow, and was washed out after 40 sec.

subtypes of mAChRs have been implicated in the control of DNA synthesis and cellular proliferation (Conklin et al., 1988; Ashkenazi et al., 1989; Gutkind et al., 1991). In addition, the activity of the ACh hydrolyzing enzyme, AChE, increases as DNA synthesis in the VZ decreases (Layer and Sporn, 1987). The disappearance of the muscarine-induced Ca^{2+} response in the VZ upon the completion of mitosis in the retina raises the possibility that muscarinic stimulation might promote continual cell division, and therefore delay cell differentiation in the retina. Alternatively, if migrating postmitotic cells are responsive (directly or indirectly) to mAChR agonists, ACh might influence their behavior in a manner similar to that involving NMDA receptors on migrating granule cells in the developing cerebellum (Komura and Rakic, 1993).

Previous studies have shown that nAChRs assist in the regulation of neurite outgrowth of the ganglion cells (Lipton et al., 1988). The present results suggest that the neurite outgrowth of some amacrine cells may also depend on nicotinic stimulation and that as in ganglion cells, this may be achieved through the modulation of $[\text{Ca}^{2+}]_i$. By contrast, the optical recordings suggest that neurite outgrowth and synapse formation associated with the bipolar cells and photoreceptor cells are less likely to be dependent on ACh, since upon differentiation, the majority of these cells do not show a nicotinic response. Instead, other transmitter-like molecules, such as glutamate (Mattson et al., 1988) may regulate neurite outgrowth of the bipolar cells, whereas taurine appears more effective in regulating the further differentiation of photoreceptor cells (Altshuler et al., 1993).

Although the general trend observed from the optical record-

ings implicates mAChRs in Ca^{2+} -dependent events before cells reach their final positions, and nAChRs in events subsequent to this stage, this is not a strict rule. For example, some ganglion cells respond to both mAChR (see also Sugiyama et al., 1977) and nAChR agonists during development and at maturity (Fig. 8). In the fetal and early neonatal retinæ, however, the few cells in the VZ that are sensitive to both muscarinic and nicotinic agonists may represent those cells that are much further in the process of differentiation.

$[\text{Ca}^{2+}]_i$ regulation by retinal mAChRs and nAChRs

The activation of M1 mAChRs on the retinal ventricular cells led to Ca^{2+} influx, most likely through receptor operated channels. This was surprising, in view of past observations that M1 mAChRs are usually linked to phosphoinositide hydrolysis and the release of Ca^{2+} from IP_3 sensitive stores (Moroi-Fetters et al., 1988; Neher et al., 1988). Furthermore, studies on embryonic chick suggested that the muscarinic-induced Ca^{2+} rise results from both mobilization of Ca^{2+} from internal stores and Ca^{2+} influx (Yamashita and Fukuda, 1993; Oetting et al., 1988, 1992). In the rabbit retina, 5 mM of EGTA abolished the CCh-induced Ca^{2+} response, whereas 1 mM EGTA did not (not shown). However, because the resting levels of $[\text{Ca}^{2+}]_i$ decreased after a few minutes in 5 mM EGTA, but not in 1 mM EGTA, a depletion of Ca^{2+} from the extracellular space in the slice may require the use of relatively high levels of EGTA. Thus, the CCh response appears to be largely the result of Ca^{2+} influx, a conclusion that is supported by the observation that at concentrations that block receptor-operated channels, 1.5–5 mM NiCl_2 to-

Figure 8. Summary histograms showing the percentage of fura-2 labeled cells in the ganglion cell layer (GCL) and ventricular zone (VZ) which responded with an increase in $[Ca^{2+}]_i$ to either carbachol (CCh, 50–100 μ M) or nicotine (100–200 μ M), as determined from ratiometric wavelength measurements. Between E20 and P5, a total of three retinæ were examined for each age; n = total number of cells that were analyzed. The data were pooled for two P10 and two P21 retinæ, as poorer loading of cells in retinal whole mounts after P5 led to a smaller sample size. The asterisks indicate a cell population that included putative bipolar cells, Müller cells, horizontal cells and photoreceptors; these cells are presumed to have originated from cells in the VZ prior to P5. Cell division ceases after the first postnatal week (Stone et al., 1985). Error bars are 1 standard deviation of the mean.

tally abolished the CCh response. The current findings, however, do not rule out the possibility that IP_3 was generated during stimulation with mAChR agonists; if formed, IP_3 did not appear to mobilize Ca^{2+} from the internal stores, but it may have instead caused Ca^{2+} influx by acting directly on plasma membrane receptors (Kuno and Gardner, 1987; McDonald et al., 1993).

Not surprisingly, an influx of Ca^{2+} occurred during nAChR activation as evinced by the blockade of this response in the presence of divalent cations, and voltage-gated Ca^{2+} channel blockers. Thus, the rise in $[Ca^{2+}]_i$ evoked by mAChRs and

nAChRs in the developing retina both require the presence of extracellular calcium. But, whilst L- and N-type voltage-gated Ca^{2+} channels are involved in the nicotinic response, these channels do not contribute significantly to the Ca^{2+} influx resulting from mAChR activation. Since the expression of various early genes depends not only on changes in $[Ca^{2+}]_i$ but also on the route of Ca^{2+} entry, (Murphy et al., 1991; Bading et al., 1993), mAChRs and nAChRs are likely to be involved in different aspects of cellular differentiation in the retina.

ACh as an environmental signal during development?

The demonstration that mAChRs and nAChRs are functional early in retinal development raises the question as to which cells release ACh in the immature retina. In cultures of cells dissociated from the neonatal rat retina, ACh is released spontaneously by amacrine cells (Lipton, 1988), most likely the ACh-synthesizing amacrine cells (Masland et al., 1984). In the chick, cholinergic amacrine cells appear early in embryonic life (Spira et al., 1987). In mammals, amacrine cells are also among the first neurons to be generated in the retina (Robinson, 1991) and are found to contain a variety of transmitter-like molecules before birth (e.g., Pow et al. 1994). In rabbit, putative cholinergic amacrine cells can be identified morphologically at birth (Wong and Collin, 1989) and in the cat, ChAT-containing amacrine cells are present by this age (Dann, 1989). Thus, amacrine cells may also be the major source of ACh in the embryonic mammalian retina. However, other early sources of ACh may be transiently present, since even migrating cells (neural crest) contain ChAT (Smith et al., 1979).

Cellular differentiation in the retina is not entirely dependent on lineage but, rather, is influenced by cues from the microenvironment (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Adler and Hatlee, 1989; Reh, 1992; Watanabe and Raff, 1992). The present results suggest that cellular interactions evoked by ACh could be diverse, yet specific to the functional subclass of receptor that is expressed. Since the commitment to a particular phenotype in the retina varies with age (Reh and Kljavin, 1989; Watanabe and Raff, 1990), the transient expression of mAChRs in the VZ would permit ACh to act as a suitable temporary signal early in development.

However, the cues provided by ACh are unlikely to act alone to invoke a specific developmental decision. Instead, it is more likely that ACh acts in synergy with other environmental factors, including growth factors and extracellular matrix molecules, in order to promote each developmental step (Jessell, 1988; Reh and Radke, 1988; Reichardt and Tomaselli, 1991). The current findings, however, underscore the belief that ACh could play an important part in orchestrating many developmental events *in vivo*, and that the signals it provide may vary as development proceeds, due to the temporal regulation of the expression of its receptor subtypes.

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