Spatial heterogeneity and function of voltage- and ligand-gated ion channels in retinal amacrine neurons

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The spatial distribution of ion channels within amacrine cells of the tiger salamander retina was studied using patch recording in the retinal slice preparation. By focally puffing kainate, GABA and glycine at amacrine cell processes in the inner plexiform layer, it was determined that the cell's glutamate receptors were located in a confined region of the processes near the soma, while glycine and GABA receptors were located throughout the processes. Likewise, similar techniques in conjunction with voltage steps demonstrated that voltage-gated sodium channels were located throughout the cell and were shown to generate sodium-dependent spikes, while only the processes contained voltage-gated calcium channels. These results suggest that this form of transient amacrine cell collects its excitatory synaptic inputs in a region confined to a central annular region near the soma, that the signal is actively propagated throughout its processes by voltage-gated sodium channels and that calcium-dependent neurotransmitter release of glycine from this neuron can occur throughout its processes. Thus, excitatory signals are collected in the processes near the soma, inhibitory signals throughout the processes and excitation is probably propagated throughout the processes of the amacrine cell.

Keywords: retina; soma; processes; tiger salamander

1. INTRODUCTION

A vast array of retinal amacrine cell types exist. They were originally defined as axonless neurons by Cajal (1983). However, recent evidence has suggested that amacrine cells may have polarized processes (Famiglietti 1992) and, indeed, may have axons (Vaney 1990). One type of amacrine cell, the transient amacrine cell (Werblin & Dowling 1969; Kaneko 1970), displays several morphological varieties, including the wide-field (Maguire *et al.* 1989a), glycinergic type (Yang *et al.* 1991) which responds to lights on and lights off with brief excitatory postsynaptic currents (EPSCs) (Dixon & Copenhagen 1992) and classical tetrodotoxin (TTX)-dependent multiple spikes (Maguire 1998a). Because this cell exhibits a morphology that includes a proximal tufted region of processes, with a few relatively long, thin distal processes extending from the tufted region (Maguire *et al.* 1989a) and because a general feature of most neurons is the localized, immobile state of voltage-gated channels (Caldwell *et al.* 1986; Weiss *et al.* 1986; Roberts *et al.* 1990) and ligand-gated channels (Flucher & Daniels 1989; Velazquez *et al.* 1989; Srinivasan *et al.* 1990), an exploration of this cell's processes to determine whether they were functionally polarized into axonal and dendritic regions was begun. The results of the present study suggest that the central region of the neuronal processes is a receptive field for glutamate-mediated excitatory inputs, surrounded by an annular receptive and transmitting field for inhibitory synaptic interactions with other neurons.

2. MATERIAL AND METHODS

Larval tiger salamanders (*Ambystoma tigrinum*; Kon's Scientific, Germantown, WI, USA) were maintained in aquaria with continuous filtration at 12 °C under a 12 L:12 D cycle. The animals were decapitated, double pithed and the eyes removed. All experiments in this study were approved by the Animal Care Committee of the University of California, San Diego and conformed to the guidelines of the National Institutes of Health on the Care and Use of Laboratory Animals. Retinal slices were prepared as described by Werblin (1978) and single amacrine cells were patch recorded using whole cell methods as described by Hamill *et al.* (1981). No enzymes were used in the procedure. Slices were viewed under $\times 400$ Hofmann modulation contrast or differential interference contrast (DIC) using a $\times 40$ water immersion lens. Only those amacrine cells exhibiting intact morphologies, light responses and transmitter-gated responses could be used in the present study. The patch pipette was always placed on the soma and the 'puffer pipette' was oriented so that the initial spray of solution from the tip targeted the appropriate portion of the cell, but so that the resulting cloud of solution was formed beyond the targeted amacrine cell and eventually dissipated into the bulk solution. The procedures have been previously described by Maguire *et al.* (1989a, 1990). Briefly, whole cell recordings were made using an Axopatch 200 amplifier with P-clamp software. Recording pipette tip lumens were pulled to a size of *ca.* 3 μm . Seal resistances were typically 20 G Ω , with input resistances of *ca.* 0.5–3.0 G Ω . Series resistance estimates of 4–8 m Ω (50% compensation) and the electrode tip potential were corrected. Solutions consisted of a patch pipette

with 12 mM KCl, 104 mM K-gluconate, 1 mM EGTA, 4 mM HEPES and 0.1 mM CaCl_2 brought to pH 7.4 with KOH. The bathing solution consisted of 120 mM NaCl, 2 mM KCl, 3 mM CaCl_2 , 1 mM MgCl_2 , 4 mM HEPES and 3 mM glucose brought to pH 7.5 with NaOH. Lucifer yellow (potassium salt) was included in the recording pipette (0.5% solution). All chemicals in this study were purchased from Sigma Chemical Co.

The stimuli were background luminance of 15 mW cm^{-2} and the light step was full-field, white light of luminance 18 mW cm^{-2} . Puff application of drugs was through a pipette with a tip lumen diameter of less than $0.5 \mu\text{m}$ delivered for 50 ms at a pressure of 15 lb inch^{-2} using a Picospritzer II (General Valve, NY, USA) and P-clamp software control.

3. RESULTS

The general characteristics of transient and sustained amacrine cells in the slice have been reported previously (Maguire *et al.* 1989a; Yang *et al.* 1991; Maguire 1998).

(a) *Glutamate receptors are spatially constrained to the processes located near the wide-field cell's soma*

Kainate was focally applied through a micropipette to the processes of the wide-field cell in the presence of nifedipine, an L-type calcium channel blocker known to block L-type channels in retinal bipolar cells (Maguire *et al.* 1989b) and/or the absence of external calcium in the bathing solution to block synaptic transmission. In this way, the direct effects of kainate on the processes of the recorded cell were measured instead of possible polysynaptic effects, as would be the case if the kainate had been applied in normal Ringer's solution. In a wide-field, transient amacrine cell that had processes extending more than 300 μm in either lateral direction from the soma, kainate elicited an inward current with a reversal potential near +5 mV. This current was of greatest amplitude when elicited from the processes directly under the cell's soma (figure 1a). With movement of the kainate-containing pipette in the lateral direction, the amplitude of the current was increasingly diminished as the pipette was moved further from the soma. The current was abolished when the pipette was moved to 100–120 μm laterally ($n=6$). Punctate regions where the response was large, surrounded by regions of little or no response, were found within this 100–120 μm region, suggesting that the glutamate receptors were clustered within the processes, possibly at synaptic sites. Puffing kainate at the soma and away from the processes of the amacrine cell failed to elicit a current, suggesting that the soma does not express functional kainate receptors.

To help rule out the possibility of a poor space clamp and electrotonic decay of the kainate-elicited current as the reason for its diminished amplitude with increases in the distance of the kainate application from the soma (recording site), potassium was pressure ejected along the processes of these cells in the same manner as was done for kainate (figure 1b; $n=5$). Although the potassium-elicited signal is diminished somewhat with application at increasing distances from the soma, the signal is still apparent at greater than 300 μm from the soma. The small diminution of the signal with increasing distance probably indicates that, at greater distances from the

Figure 1. Ligand-gated currents measured as a result of the direct application of (a) kainate or (b) potassium to the processes of the wide-field amacrine cell being recorded. To preclude the possibility of indirect synaptic effects contaminating the measurement of ligand-gated currents, the slice was bathed in a solution containing zero calcium and/or nifedipine to block synaptic transmission. (a) Response of the wide-field, transient amacrine cell to the direct application of kainate to its processes. The pipette was moved laterally at the distances indicated under each trace, with zero marking that distance where the tip of the puff pipette was placed in the inner plexiform layer, directly beneath the cell's soma. The kainate response fell to zero at *ca.* 100 μm . (b) Response of the wide-field, transient amacrine cell to the direct application of potassium to its processes. Lateral displacement of the puff pipette loaded with potassium is designated below each trace. Although there was diminution of the response with increasing distances between the recording site (soma) and stimulus site (pipette location), the response to potassium was still evident at 300 μm lateral to the soma.

soma, potassium was applied to processes that became fewer in number and many times less accessible to the potassium because the process coursed down into the neuropil. These arguments assume uniform K^+ channel density within the processes.

(b) *Distribution of glycine and GABA receptors in the processes of wide-field, transient amacrine cells*

GABA and glycine were also loaded into micropipettes and focally applied to the processes of the wide-field cell as described for the aforesaid kainate and potassium experiments. The profiles for responsivity to GABA (figure 2a) and glycine (figure 2b) are broader than that

Figure 2. Ligand-gated currents measured for the direct application of GABA or glycine into the inner plexiform layer. (a) GABA was focally applied through a micropipette to the processes of the wide-field, transient amacrine cell similar to that described in figure 4. The response to GABA gradually diminished with increasing lateral displacement of the pipette (lateral displacement is indicated below each trace), but even with the pipette placed 300 μm lateral to the soma, there was still a large response. (b) Glycine was applied as in (a) and the response to glycine was also apparent when it was applied 300 μm lateral to the soma.

of kainate, but similar to that of potassium. In all cases, the limit of lateral displacement of the ejection pipette was 300 μm, so the distance at which the potassium, GABA and glycine response profiles fell to zero could not be determined. In the sustained cells, with processes extending laterally up to 100 μm in either direction from the soma, when the pipette was moved laterally along the cell's processes, the processes had nearly uniform response profiles for all of the neuroactive agents tested. In five of the cells tested, the amplitude of the currents elicited by potassium, GABA and glycine did not vary by more than 20% over the entire range of the processes. Thus, the sustained cells fit the classical description of an amacrine cell because they display no polarity of their processes.

(c) *Distribution of voltage-gated sodium and calcium channels in the wide-field cells*

To measure the voltage-gated currents at specific sites within the cell, the neuron was bathed in zero sodium or zero calcium. Then, to measure the sodium current at a site, sodium was pressure ejected onto a given region of the cell and the membrane voltage stepped to a depolarized level after a brief time following the puff of sodium. Similarly, for the measurement of the calcium current, I_{Ca} , at a given site, calcium was focally puffed onto a region of the neuron prior to a depolarizing command.

Thus, the ions were available for current flow only in the region of the neuron receiving focal application of the ion. This is similar to a technique first described by MacDermott & Westbrook (1986).

Voltage-gated sodium currents arising from the proximal, tufted region of processes close to the soma (top trace) and from the distal 'axonal' region (bottom trace) are shown in figure 3a. The cells were bathed with sodium-free Ringer's solution (choline replaced by sodium) and then a normal Ringer's solution (containing 115 mM sodium) was puffed onto the processes locally at either the distal or proximal processes. The current elicited at the tufted processes ($X = 80 \pm 15$ pA and $n = 5$) was always larger than that elicited at the distal processes ($X = 50 \pm 10$ pA and $n = 5$), probably because the total area of membrane being surrounded by sodium was larger in the tufted region than in the distal axonal region. This current could also be measured by puffing at the soma and in amacrine cells which had their processes removed and which were bathed in normal Ringer's solution. It was blocked by TTX (300 nM) and activated near -30 mV, similar to the sodium current reported in other amacrine cells (Maguire 1998), ganglion cells (Lukasiewicz & Werblin 1988) and interplexiform cells (Maguire *et al.* 1990). That the voltage-gated sodium currents in the processes underlie spike propagation in the processes is suggested by the experiment in figure 3c. Here the transient amacrine cell was recorded under current clamp, bathed in sodium-free Ringer's solution, sodium Ringer applied focally to the cell's processes and current injected into the cell. Under these conditions the cell was able to generate a regenerative sodium-dependent spike, whereas in the control condition, without sodium applied to the processes, no regenerative event was detected. Similarly, when sodium-free solution was puffed onto the processes and the membrane then depolarized, no regenerative event was detected. These data suggest that sodium-dependent action potentials can be generated within the processes and the soma.

Voltage-gated calcium currents arising from the proximal tufted region and the distal axonal area are shown in figure 3d and 3e, respectively. Cells were bathed in calcium-free Ringer's solution and then a normal Ringer's solution containing either calcium or barium was puffed onto the distal or proximal regions. The amplitude of the calcium current was consistently greater when elicited from the tufted region ($X = 65 \pm 10$ pA and $n = 4$) compared to that elicited from the distal region ($X = 40 \pm 5$ pA and $n = 4$). There was no current elicited when the calcium was puffed only at the cell's soma.

The profiles for the peak responses of all of the ionic currents are plotted in figure 4a. These plots reveal that glutamate receptors and probably excitatory synaptic inputs are localized near the cell's soma, probably in the tufted processes (which have similar dimensions to the glutamate puff profile) and that inhibitory synaptic inputs mediated by glycine and GABA occur throughout the cell. Pressure ejection of potassium served as the control for simple spatial decrement of the signal as the reason for diminished signals from the processes. Potassium signals recorded at the soma were evident from as far out in the processes as 300 μm.

Figure 3. Distribution of voltage-gated sodium and calcium channels in the wide-field amacrine cell. (a) Voltage-gated sodium channels are present in the proximal processes. Sodium (115 mM) was puffed onto the proximal processes of a wide-field amacrine cell and then, under whole cell voltage clamp, the holding potential of the cell was stepped from -70 mV to -10 mV. This was repeated for a step from -70 mV to 0 mV and the resultant current is the larger of the two traces. (b) As in (a), sodium was applied to the distal processes and the holding potential stepped from -70 mV to -10 mV and then repeated from 70 mV to 0 mV. These data indicate that voltage-gated sodium channels are present in both the proximal and distal processes. These currents could also be recorded by applying sodium to only the soma. (c) The distal processes are able to generate sodium-dependent spikes. The cell was whole cell patch clamped and recorded under current clamp to measure voltages. Current was injected into the cell (bottom trace) while the cell was bathed in zero extracellular sodium. This resulted in a voltage deflection with no regenerative events. When the same experiment was performed, but this time sodium (115 mM) was focally applied through a micropipette to the cell's distal processes, a regenerative sodium current was elicited. This suggests that the distal processes of the wide-field amacrine cell can generate action potentials. (d) Proximal processes possess voltage-gated calcium channels. The cell was bathed in zero calcium, 40 mM tetraethylammonium, 300 nM TTX and the recording pipette

4. DISCUSSION

(a) *Functional polarization of the wide-field, transient amacrine cell*

While others have reported amacrine cell processes exhibiting a morphology consistent with axonal and dendritic functions (Vaney 1990; Famiglietti 1991, 1992), this is the first study to demonstrate axons and dendrites in amacrine cells functionally. As shown in figure 4b, the tufted processes of the wide-field, transient amacrine cell, which are located near the soma, are the only regions in the cell sensitive to glutamate or its analogues and, thus, are the only regions of the cell capable of mediating EPSCs because glutamate is the predominant excitatory transmitter at the bipolar to amacrine cell synapse (Ehinger *et al.* 1988; Marc *et al.* 1990; Massey & Maguire 1997). Voltage-gated sodium channels are located throughout the cell, including its soma and processes and underlie the cell's ability to generate classical multiple spikes (Maguire 1998). The excitatory signal generated in the region immediately surrounding the soma can be propagated to the distal tips of the axonal processes (Cook & Werblin 1994). Because the processes contain voltage-gated calcium channels and generate action potentials and neurotransmitter release in amacrine cells is calcium dependent (Schwartz 1986), calcium-dependent neurotransmitter release from these neurons is probable throughout the processes. Previous work has demonstrated that these neurons are probably glycinergic (Yang *et al.* 1991). Thus, the cell can transmit an inhibitory glycinergic signal over a region of up to *ca.* 500 μ m (the greatest lateral dimension of the cell in either direction from the tip of its processes to the farthest point of its excitatory receptive field) following excitation near the soma at the tufted processes. The propagation of signals throughout the processes of the amacrine cell (Cook & Werblin 1994), which are themselves often either glycinergic or GABAergic (Yang *et al.* 1991), is likely to account for the broad spatial dimensions of the inhibitory input found in retinal ganglion cells (Lukasiewicz & Werblin 1990).

(b) *Inhibitory inputs*

Glycine and GABA are the major inhibitory neurotransmitters in the retina (Yazulla 1985; Marc 1988) and both

Figure 3. (Cont.) contained caesium. Under these conditions a voltage pulse from -70 mV to 0 mV elicited no inward currents. However, when an application of 10 mM barium or calcium through a micropipette onto the proximal processes of the transient amacrine cell preceded the voltage pulse, an inward current was elicited. The puff, 50.0 ms in duration, was initiated 1.0 ms before the initiation of the voltage pulse. Previous studies (Maguire *et al.* 1989) suggest that this puff forms a cloud of the solution with spatial dimensions of *ca.* 25 – 50 μ m in the form of a 'tear drop', lasting for the 50 ms duration of the pressure pulse. The currents were not leak subtracted, but capacitance artefacts were removed. (e) The same experiment as in (d) was performed, but this time barium was applied to the distal processes and again a current was elicited, albeit smaller in amplitude than that from the proximal processes. No inward current was elicited when barium or calcium was applied to the soma, suggesting that the calcium channel currents arise from the processes and not the soma.

Figure 4. Summary of the spatial distribution of neurotransmitter receptors (or chemical receptive field zone) in the wide-field amacrine cell. (a) Glutamate receptors are confined to the proximal processes, whereas GABA and glycine receptors are located throughout the cell's processes. The peak responses elicited by the drugs were normalized and then plotted as a function of the distance from the zero recording site in the inner plexiform layer directly below the soma, where the puff pipette was placed and, hence, the spatial location of where the drug elicited the current. The kainate-elicited currents fell to 20% of their peak value at a distance of 100 μm, whereas the GABA and glycine currents fell to only 35% of their original amplitude at 300 μm. The data for each condition were pooled from four cells, each cell having similar morphology, as determined by filling each cell with Lucifer yellow for subsequent morphological analysis. (b) The region of high kainate sensitivity correlated closely with the wide-field, transient amacrine cell's proximal, tufted region of processes, while the distal processes contained regions of GABA and glycine sensitivity. These data suggest that the wide-field, transient amacrine cell in this study possesses an inner region of kainate sensitivity; the only region in the cell where EPSCs are elicited, surrounded by a region of inhibitory inputs and, presumably, glycinergic inhibitory outputs. The output region is inferred from the data of this study which suggest that spikes can be generated in the processes, leading to a depolarization of the voltage-dependent calcium channels in the processes, which, as shown in other studies, may lead to calcium-dependent glycine release at synaptic sites along the distal processes.

transmitters evoke large inhibitory currents throughout the transient amacrine cell. Thus, even in the distal processes, which lack excitatory inputs, the transient cell apparently receives both GABAergic and glycinergic inputs where they could serve to oppose the regenerative sodium currents emanating from the core region of the neuron. At or near the soma, the GABA and glycinergic inhibitory postsynaptic currents (IPSCs) can also oppose the EPSCs.

(c) Implications for retinal function

Glycine has been shown to mediate transient inhibition in ganglion cells (Belgium *et al.* 1984). A likely source of

this inhibition is the neuron described in the present study that has been shown to be glycinergic (Yang *et al.* 1991) and to respond transiently to lights on and lights off. This cell is ideally suited for mediating lateral inhibition over great distances in the visual field because its lateral arm, the distance between its input and output, can extend up to 500 μm which is a large area in visual space considering the salamander's extremely small eye size. Other transient amacrine cell types exist in the salamander retina, including other wide-field types (Maguire *et al.* 1989a) and narrow-field GABAergic types (Yang *et al.* 1991; Maguire 1998), which need further study to define their synaptic input and output sites.

The exact nature of the inhibitory outputs of this cell will require additional studies, including a histochemical analysis of the cell's neurotransmitter receptor distribution and of its synapses. Another type of retinal amacrine cell in rabbit, the starburst amacrine cell, may possess specialized regions for receiving and transmitting synaptic signals (Famiglietti 1991, 1992). The wide-field amacrine cells of the salamander are thought to be involved in detecting the direction of a moving target (Werblin *et al.* 1988) using a lateral inhibitory arm that vetoes excitation (Barlow & Levick 1965). A general scheme for the neural implementation of the lateral arm in a number of different retinal neurons and in vertebrates in general could be through the separation of input and output sites within a neuron, as has been suggested to occur in wide-field amacrine cells in the present study and in the starburst amacrine cells described by Famiglietti (1991).

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