Dopamine Modulates GABA_C Receptors Mediating Inhibition of Calcium Entry into and Transmitter Release from Bipolar Cell Terminals in Tiger Salamander Retina

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Using optical recording techniques, we directly monitored pre- and postsynaptic calcium dynamics at bipolar cell terminals while inhibiting synaptic release with applied GABA and modulating inhibition with dopamine. To monitor presynaptic activity, individual bipolar cells in the retinal slice were filled with either fura-2 or fluo-3 through a patch electrode. Calcium entry into bipolar terminals, elicited by depolarization from -60 mV to 0 mV, was reduced to 36% of control in the presence of $200 \mu\text{M}$ bath-applied GABA. Further addition of $100 \mu\text{M}$ dopamine to the bath relieved the GABAergic inhibition and nearly doubled the calcium entry. Yet dopamine alone had no apparent direct effect upon calcium entry. The relief from GABAergic inhibition could be reproduced with SKF-38393, a dopamine D1 receptor agonist, and with forskolin, an adenylyl cyclase activator, suggesting that dopamine acts through a cAMP second-messenger pathway.

To monitor transmitter release from bipolar cells, slices were loaded with fura-2AM, a membrane permeable form of the dye. Puffs of 110 mM KCl at bipolar dendrites depolarized bipolar cells and elicited calcium signals that could be monitored both at bipolar terminals and in postsynaptic cells. Consistent with the results above, GABA inhibited calcium entry at bipolar terminals and also reduced transmitter release, measured as a decrease in calcium entry in amacrine and ganglion cells. The addition of dopamine relieved this inhibition and increased transmitter release. Our results show the spatiotemporal correlation between the GABAergic inhibition of calcium entry at bipolar terminals, the resulting reduction in postsynaptic activity, and the relief of this inhibition with dopamine.

[Key words: GABA, dopamine, synaptic transmission, bipolar cell, retina, optical recording, fura-2, presynaptic calcium, presynaptic inhibition, ensemble activity, neuromodulation]

Neurotransmitter release from most neurons is critically dependent upon calcium (Ca) influx into the presynaptic terminal (Katz, 1969; Augustine et al., 1987). Mechanisms that alter pre-

synaptic Ca entry can profoundly affect the strength of synaptic transmission (Dunlap and Fischbach, 1981). Retinal bipolar cells provide a useful model for studying modulation of neurotransmission since amacrine cells feed back to bipolar terminals to inhibit synaptic release, and since direct spatiotemporal measures of presynaptic Ca entry and postsynaptic activity at bipolar terminals are now possible in the retinal slice.

Morphological evidence shows that GABAergic amacrine cells make synaptic contacts with bipolar cell terminals (Marc et al., 1978; Yazulla et al., 1987; Chun and Wassle, 1989). Consistent with the morphology, GABA application at bipolar terminals evokes a chloride current recorded at the soma (Tachibana and Kaneko, 1987; Heidelberger and Matthews, 1991; Karschin and Wassle, 1990; Suzuki et al., 1990; Lukasiewicz et al., 1994; Matthews et al., 1994). In salamander, bipolar cells mainly express the recently discovered GABA_C receptor (Feigenspan et al., 1993; Qian and Dowling, 1993; Dong et al., 1994) on their terminals (Lukasiewicz et al., 1994). Recent work by Matthews and colleagues in goldfish (Heidelberger and Matthews, 1991; Matthews et al., 1994) and by Lukasiewicz and Werblin (1994) in salamander has shown that activation of GABA receptors causes a decrease in Ca entry into bipolar terminals, probably by shunting the depolarizing signal which gates the Ca channels. In salamander, it has been suggested that the inhibition of Ca current reduces synaptic release from bipolar terminals (Lukasiewicz and Werblin, 1994).

Dopamine exerts numerous neuromodulatory effects in retina (Dowling, 1991; Witkovsky and Dearth, 1991). Dong and Werblin (1994) recently showed that dopamine reduces GABA_C receptor sensitivity in catfish horizontal cells. This opened the possibility that dopamine could also modulate the GABA_C receptors found on salamander bipolar terminals. Since activation of GABA_C receptors inhibits Ca entry into bipolar terminals, dopamine modulation of GABA_C receptors could have important effects on synaptic release from bipolar terminals.

Direct measurement of Ca current at presynaptic terminals and simultaneous measurement of pre- and postsynaptic activity are now possible with optical recording (Cohen et al., 1978; Grinvald, 1985; Ross, 1989; Delaney et al., 1991; Cinelli and Kauer, 1992; Regehr et al., 1994; Wu and Saggau, 1994), which offers several advantages over conventional electrical recording. First, activity along cellular processes, often invisible to electrical recordings from the soma, can be directly evaluated. Second, the coordinated activity of many cells and their processes can be monitored. Third, intracellular Ca dynamics, which are more relevant to synaptic transmission than electrical recording, can

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be measured. Finally, optical recording can verify that the decrease in net inward current evoked by a depolarizing step in the presence of GABA (Lukasiewicz and Werblin, 1994) is due to a decrease in Ca entry and not just an increase in outward current carried by Cl^- ions.

We applied optical recording techniques to retinal slices to directly measure the simultaneous spatiotemporal dynamics of Ca entry into bipolar terminals and the subsequent activity in the inner plexiform layer elicited by synaptic release from those terminals. We then examined how dopamine modulated the GABAergic inhibition of these events. We discovered that dopamine, by apparently downmodulating GABA_A receptor sensitivity, can relieve GABAergic inhibition of Ca entry into bipolar terminals and thereby enhance transmission from bipolar cells to third-order retinal neurons.

Materials and Methods

Preparation. All experiments were performed with slices of larval tiger salamander retina, prepared according to the methods of Werblin (1978). Briefly, a small piece of eyecup was placed vitreal side down onto a rectangular piece of Millipore filter (black, HABP02500, Millipore, Bedford, MA). The sclera and pigment epithelium were pulled away, leaving the retina adhering to the filter. The retina and filter were sliced with a tissue chopper at about 200 μm intervals. The ends of the filter paper, with attached retina, were then embedded in Vaseline rails on the bottom of the recording chamber in an orientation such that all retinal layers along the cut face were directly observable (see Figs. 2B, 11).

For those experiments designed to simultaneously observe ensemble activity in second- and third-order retinal neurons, slices were incubated in a solution of the membrane permeable form of fura-2 (fura-2AM, 10–20 μM , Molecular Probes, Eugene, OR) in 0.02% Pluronic F-127 (a detergent that facilitates AM ester solubilization) for 1–2 hr and then thoroughly rinsed. Fura-2AM was stored frozen in 50 μl , 2 mM (in anhydrous DMSO) aliquots and Pluronic F-127 (20% solution in distilled H_2O) was stored at room temperature.

Whole-cell recording. Whole-cell recordings (Hamill et al., 1981) were made from bipolar cells and amacrine cells as in Barnes and Werblin (1986) and Lukasiewicz et al. (1994). Patch pipettes were fabricated from borosilicate capillary tubing (TW150F-4, World Precision Instruments, Sarasota, FL) pulled on a horizontal puller (P-87, Sutter Instruments, Novato, CA). Pipette resistance measured in bath solution was between 5 and 10 M Ω . Bipolar cells were patch clamped at -60 mV with a List L/M EPC-7 patch clamp amplifier (Medical Systems, Greenvale, NY). Responses to depolarizing steps were filtered at 3 kHz and digitized at 1 kHz using a DT2828 data acquisition board (Data Translation, Marlboro, MA) in an IBM AT computer. Software developed in this laboratory (PATCHIT, provided by G. Grant) generated the voltage command outputs for the amplifier and puff pipette, as well as controlled data acquisition. Electrophysiological data were analyzed off line with TACK (also provided by G. Grant) and QUATTRO PRO (Borland International, Santa Cruz, CA).

All cells were unequivocally identified following their filling with a Ca-sensitive dye included in the recording pipette (see, e.g., Fig. 2A). Several images of each filled cell were saved during the course of each experiment to monitor the baseline fluorescence and for localizing bipolar terminals within the inner plexiform layer (IPL).

Electrode and bathing solutions. The standard intracellular solution contained (in mM) 96 K-gluconate, 10 KCl, 1 MgCl₂, 9 HEPES, 4 Na₂-ATP, and 0.5 GTP, buffered to a pH of 7.7 with KOH. E_{Cl} was calculated to be -58 mV. Outward K⁺ currents were predominantly observed during voltage steps to depolarizing potentials (Tessier-Lavigne et al., 1987). Since we did not want to buffer intracellular Ca in these studies, standard Ca²⁺ chelators were not included in our intracellular solution. Unless otherwise indicated, all chemicals were obtained from Sigma (St. Louis, MO). The Ca-sensitive dyes fura-2 or fluo-3 (both pentapotassium salts, Molecular Probes, Eugene, OR) were stored frozen in 100 μl , 10 mM aliquots and were diluted to 100 or 200 μM in 125 or 250 μl of intracellular solution at the beginning of each experiment. In those experiments designed to directly measure GABA-evoked currents (i.e., Fig. 1), Cl^- currents were enhanced by substituting KCl (96 mM) for an equimolar amount of K-gluconate. E_{Cl} , under these conditions,

was near 0 mV. In those experiments designed to directly measure Ca currents (i.e., Fig. 5), potassium currents were blocked by substituting CsCl (84.8 mM) and TEA-Cl (21.2 mM, Aldrich, Milwaukee, WI) for an equimolar amount of K-gluconate and KCl in the intracellular solution and 20 mM TEA-Cl for an equimolar amount of NaCl in the bathing solution.

The standard bath solution contained (in mM) 108 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES, and 3 glucose, buffered to a pH of 7.8. GABA and dopamine were added to the bathing solution without substitution by diluting 10 mM stock solutions immediately before use. Ascorbic acid was usually included with dopamine to prevent oxidation. Bathing solutions were exchanged by a simple dual syringe push-pull perfusion system. Ca currents were blocked with the L-type channel blocker nifedipine (stored as 10 mM solution in 95% ethanol), or with 30–100 μM CdCl₂, or with 0.1 mM CaCl₂, 10 mM MgCl₂ (substituted for NaCl) included in the bathing solution. Forskolin (an adenylyl cyclase activator, Calbiochem, La Jolla, CA) was diluted into the bathing solution from a 10 mM stock in DMSO, whereas ($\pm$)SKF-38393 (a dopamine D1 receptor agonist, Research Biochemicals International, Natick, MA) and SR-95531 (a GABA_A receptor antagonist, Research Biochemicals International) were freshly prepared at their final concentrations for each experiment.

Stimulation. Ca entry into single dye-filled bipolar cells was evoked with 100 msec depolarizing steps to 0 mV from a holding potential of -60 mV. Calcium currents peak in this voltage range for these cells (Maguire et al., 1989; Lukasiewicz and Werblin, 1994). The steps were timed to occur precisely between the sixth and seventh frames of a 2 sec movie (see below).

GABA-evoked currents in bipolar cells were elicited by puffing 1 mM GABA at the synaptic terminals of the recorded cell from a second pipette. GABA was ejected for 30 msec with positive pressure (4–8 psi) pulses via a computer-controlled solenoid valve.

Activity in fura-2AM loaded slices was elicited by puffing either a potassium solution (110 mM KCl substituted for NaCl in bath solution) or 1 mM glutamate (in bath solution) directly at the outer plexiform layer for 10–30 msec to depolarize bipolar cell dendrites. The resulting activity observed in the IPL was initiated by the release of transmitter from bipolar cell terminals. The puff pipette (1 or 2 M Ω) was slightly larger than a whole-cell pipette, and the pressure ranged between 4 and 8 psi. Lukasiewicz and Werblin (1990) previously showed that the spatial extent of the puff delivery is limited. Puffs of KCl or glutamate were delivered at the beginning of the third frame in a movie.

Optical recording. Real-time video imaging techniques were used to obtain the highest spatial resolution recordings from bipolar terminals and postsynaptic processes. Single wavelength excitation of fluo-3 and fura-2 was used exclusively in these studies since fluo-3 is not a ratiometric Ca-sensitive dye and since the time necessary to switch excitation filters for conventional ratiometric measures of fura-2 fluorescence precludes real-time video recording. The changes in $[\text{Ca}^{2+}]_i$ evoked by a stimulus are presented as % changes expressed as $\Delta F/F$, where F is the resting fluorescence at 480 nm or 380 nm excitation, respectively, and ΔF is the net change in F between background and experimental runs (see below). As long as the path length and dye concentration do not change during the experiment, this ratio should provide a reliable qualitative measure of the stimulus-induced change in $[\text{Ca}^{2+}]_i$. The path length did not appear to change during the stimulus, since no apparent Ca signals were observed in conditions that blocked Ca entry (see Results). Dye concentration was kept constant by waiting until fluo-3 or fura-2 dialyzed completely into the terminal region, which usually took 5–20 min after establishing access to the interior of the cell. During the recordings, very little or no change in resting F was observed at the terminals or in loaded slices. We minimized bleaching the dye by using neutral density filters and a shutter (described below). Any bleaching that did occur within a single experimental run was removed by our protocol of subtracting a background run from an experimental run (see below). When these conditions are held constant, Ca levels estimated with single wavelength and dual wavelength techniques show a close correspondence (Neher and Augustine, 1992).

The absolute level of $[\text{Ca}^{2+}]_i$ was not routinely determined, but our fura-2 measures of stimulus-induced $\Delta F/F$ could be roughly translated to $[\text{Ca}^{2+}]_i$ by Eq. 5 of Grynkiewicz et al. (1985). If we assume $[\text{Ca}^{2+}]_{\text{rest}}$ to be between 50 and 200 nM (from similar measures of $[\text{Ca}^{2+}]_{\text{rest}}$ in isolated goldfish bipolar terminals by Heidelberger and Matthews, 1991, 1992; Matthews et al., 1994), $\Delta F/F_{\text{max}} = 0.8$ and $K_D = 230$ nM, a 20% change in $\Delta F/F$ (from our Fig. 4) would translate to a 140–340 nM

increase in $[Ca^{2+}]_i$. This calculation serves only as a crude estimation, since the fluorescence and K_D of fura-2 can be affected by numerous additional factors (Neher and Augustine, 1992), but qualitative comparisons should still be valid. These estimated levels are within a non-saturating region of the relationship between $\Delta F/F$ and $[Ca^{2+}]_i$ in other preparations (Lev-Ram et al., 1992; Neher and Augustine, 1992), but are probably orders of magnitude lower than expected for the $[Ca^{2+}]_i$ just inside the membrane (see Discussion).

All experiments were performed on an upright Nikon Optiphot microscope (Nikon Instruments, NY) modified for a fixed stage (Technical Instruments, San Francisco, CA). The epi-illumination light source was a 100 W mercury arc lamp with neutral density filters. An Omega Optical (Brattleboro, VT) filter set (XF04, excitation 380 ± 15 nm, dichroic mirror 430 nm, emission 510 ± 40 nm) was used for viewing the dye fura-2, whereas a standard fluorescein filter set (Nikon, excitation 470–490 nm, dichroic mirror 510 nm, emission barrier 515 nm) was used for viewing fluo-3. A Uniblitz shutter (Vincent Associates, Rochester, NY) placed in the light path was controlled by computer to limit the exposure of the slices to the epi-illumination necessary to collect the images and to minimize dye bleaching. Fluorescence was monitored with a Dage-MTI (Michigan City, IN) series 66 SIT camera, and was acquired by an eight-bit frame grabber (FG100-AT, Imaging Technology, Woburn, MA or DT-3852, Data Translation, Marlboro, MA) in an IBM-compatible 80486 computer.

Single dye-filled bipolar cells were viewed with a long-working distance water immersion $40\times$, 0.75 NA objective (Zeiss, Germany). The gain of the camera was usually set high in order to detect the fluorescence emitted from the fine terminal processes (see Fig. 2A). At this gain, the fluorescence emitted by the soma often saturated the frame grabber; these sites are indicated in the figures by pink ellipses. Slices stained with fura-2AM were viewed over a broader field with a long working distance water immersion $20\times$, 0.4 NA objective (Nikon). In our system, each pixel corresponds to less than 1 (with $40\times$ objective) or 2 μm (with $20\times$ objective) of the field, but each pixel measures light scattered from a larger area.

Sequences of video frames (movies) were taken at 15 or 30 frames/sec and digitized at 256×256 pixels or 128×128 pixels. Movies did not begin until 300 msec after opening of the shutter to control for camera lag and subsequent movies were separated by at least 1 min. Images were saved on optical disks (DE-UH7101, Pioneer, Santa Clara, CA) and were displayed on an RGB monitor (PVM-1341, Sony, Teaneck, NJ). Images of loaded bipolar cells were digitized and processed with Global Lab Image (Data Translation). Images of loaded slices were digitized and processed with a version of imaging software originally developed in the laboratory of Dr. J. S. Kauer. Images were converted to a PCX format (Winsurfer, Image Processing Solutions, Woburn, MA or Global Lab Image) and further manipulated in COREL DRAW (Ontario, Canada) for figure presentation.

Measurements of $[Ca^{2+}]_i$ changes (ΔF) were calculated by making difference movies between baseline images and test images in one of two ways. In the case of loaded bipolar cells, each frame of a test movie was subtracted pixel by pixel from a baseline frame taken immediately before at -60 mV with no voltage steps or drug. Dye bleaching rate was estimated by measuring baseline fluorescence over 3 sec movies without a stimulus. Comparisons of the baseline fluorescence at the beginning of the movie to that measured at the end of the movie yielded an average bleaching rate of 1–1.5% per sec. Difference movies and $\Delta F/F$ plots (see below) were corrected for bleaching by subtracting this rate. The bleaching correction has only a small effect ($<0.5\%$) on peak $\Delta F/F$ measures (below), since the peaks occurred within the first 500 msec of a movie, but the correction does enhance the rate of recovery of Ca signals to baseline. Alternatively, for loaded slices, each frame of a test movie (with stimulus) was subtracted pixel by pixel from the corresponding frame of a baseline movie (without stimulus or drug). In this case, the bleaching rate was automatically subtracted out from the difference movie, assuming the rate was constant for the background and experimental runs. The background and experimental runs were usually separated by 5 sec. Data were usually collected in a single run, but records were sometimes averaged over two runs.

The difference images were normalized to background fluorescence ($\Delta F/F$) and smoothed with a low-pass 3×3 digital spatial filter. These net changes in $\Delta F/F$ recorded over a single run ranged between 5 and 20% for bipolar terminals, and will be referred to as “Ca signals” in the rest of the article. The changes in fluorescence were replaced with a color scale indicating low to high levels of $[Ca^{2+}]_i$, as blue to red,

respectively. Since fura-2 fluorescence generated at 380 nm varies inversely with increases in $[Ca^{2+}]_i$, fura-2 data were inverted to match the increase in fluorescence with an increase in $[Ca^{2+}]_i$, signalled by fluo-3. The pseudocolor images were then superimposed onto a bright-field image of the preparation for precise spatial localization of Ca signals within the IPL.

Only a few processed/averaged difference images are shown for each experiment, but the temporal changes in the Ca signal across all frames are plotted in corresponding graphs. These $\Delta F/F$ plots were calculated by averaging the pixel values within a region, manually defined, covering the entire terminal in the control frame (average area = $657 \mu\text{m}^2$) for each frame in the test movie. For loaded slices, $\Delta F/F$ plots were calculated by averaging the pixel values within a 5×5 square (area $\sim 200 \mu\text{m}^2$) across all frames at the site indicated in the difference images with a square (see Fig. 12). Peak Ca signals (i.e., Fig. 9) were calculated by first averaging the absolute F in three frames before the stimulus and in three frames at the peak of the response (usually immediately after the stimulus), subtracting those values, and finally normalizing the difference to baseline F.

The light responses of bipolar cells and of retinal slices to the 380 nm light did not contribute significantly to the responses shown here for several reasons. First, for OFF bipolar cells, depolarizing stimuli are opposite in polarity to their normal hyperpolarizing light responses. Second, for ON bipolar cells and ensemble slice studies, the effects of light were subtracted out by our experimental protocol of calculating $\Delta F/F$ from background and experimental runs. In addition, light responses recorded in ON bipolar cells usually ran down prior to adequate terminal filling and the beginning of optical recordings. Finally, the stimuli used depolarized bipolar cells and slices far more than 380 nm light. The 380 nm light generated 7.2 ± 2 pA (SEM) of inward current in 25 ON bipolar cells recorded in standard intracellular solution.

Results

Single bipolar cell recordings in slice

Dopamine reduces bipolar cell responses to GABA. Earlier studies have shown that bipolar cells are most sensitive to GABA at their terminals (Tachibana and Kaneko, 1987; Karschin and Wässle, 1990; Suzuki et al., 1990; Heidelberger and Matthews, 1991; Lukasiewicz et al., 1994). As in these earlier studies, we puffed GABA at bipolar terminals in retinal slices. Figure 1A shows that under symmetrical Cl^- conditions ($E_{Cl} = 0$ mV) and at a holding potential of -60 mV, puff application of 1 mM GABA at bipolar terminals evoked a large inward current recorded at the soma. This current could be completely blocked by 100 μM picrotoxin (not shown; cf. Lukasiewicz et al., 1994). Figure 1A also shows that the peak GABA responses were reversibly reduced by $24 \pm 1\%$ ($n = 5$, mean $\pm$ SEM) when 100 μM dopamine bathed the slice. The effect of dopamine on GABA-induced responses could be mimicked by bath application of the dopamine D1 receptor agonist ($\pm$)SKF-38393 (Fig. 1B; 250 or 500 μM , $n = 2$, 32% reduction) and the adenylyl cyclase activator forskolin (Fig. 1C; 25–50 μM , $n = 3$, $36 \pm 1\%$ reduction), suggesting that a dopamine D1 receptor acting through a cAMP second-messenger pathway may be involved.

Microfluorometric measurements of Ca entry into bipolar cells. To determine whether dopamine modulation of GABA receptor sensitivity can effect the entry of Ca into bipolar terminals, we filled single bipolar cells with either of the Ca-sensitive dyes fura-2 or fluo-3 through the whole-cell pipette. Figure 2A shows an example of a fura-2 (200 μM)-filled bipolar cell in a retinal slice. The dendritic (*d*), somatic (*s*), and terminal (*t*) regions are clearly visualized. Ca entry into dye-filled bipolar cells was evoked by depolarizing the soma through the pipette to 0 mV from a holding potential of -60 mV. The relative changes in fluorescence (ΔF), and therefore increase in $[Ca^{2+}]_i$, were then mapped onto a bright-field image of the same slice to localize the Ca signals to the retinal laminae (Fig. 2B). For sim-

Figure 1. Dopamine reduced GABA sensitivity of bipolar terminals. *A*, Local puff application of 1 mM GABA at bipolar terminals evoked large inward currents that were reversibly reduced 20–30% when 100 μ M dopamine bathed the slice. *B*, The dopamine D1 receptor agonist ($\pm$)SKF-38393 (500 μ M) and *C*, the adenylyl cyclase activator forskolin (25 μ M) mimicked the effect of dopamine. Bipolar cells were recorded under symmetrical Cl^- conditions ($E_{\text{Cl}} = 0$ mV). Puff duration indicated by the timing traces above response traces.

plicity in the remaining figures, the morphology of the cell will be represented as a cartoon tracing, and the boundaries of the outer and inner plexiform layers will be represented as dotted lines (Fig. 2C).

Figure 3 shows optical recordings of Ca signals in three different bipolar cells filled with Ca-sensitive dye under different experimental conditions. In Figure 3A, four difference images are shown sequentially in time from left to right for a representative fura-2 (200 μ M)-filled bipolar cell. Ca signals, depicted with color where red represents the highest levels of $[\text{Ca}^{2+}]_i$, were observed in the terminal following a 100 msec step depolarization to 0 mV at $t = 400$ msec. The same stimulus was used in Figure 3, *B* and *C*. Figure 4A shows the Ca signal averaged over the entire terminal plotted across 30 frames. Changes in $[\text{Ca}^{2+}]_i$ were also observed at the cell soma in other cells (not shown). While the Ca signals were usually most rapid and of greatest magnitude in the terminals, meaningful comparisons require dual wavelength measurements. The signal at the terminal consisted of a rapid rise to peak followed by a much slower decay to baseline, often lasting several seconds (i.e., Fig. 4B; see also Figs. 7, 8). This long decay presumably reflects the buffering and extrusion of Ca from the intracellular space and the slow kinetics of Ca unbinding from, and possibly rebinding to, the dye. Baseline levels of fluorescence were always allowed to recover completely before the next test movie. The circled square in Figure 4A indicates the baseline fluorescence level after 1 min following the preceding step depolarization. Similar responses were found for a total of 20 OFF bipolar cells and 64 ON bipolar cells. Of these, 13 cells were filled with fluo-3 and 71 cells were filled with fura-2. The simultaneously recorded whole-cell currents are not shown here, but outward K^+ currents characteristic of bipolar cells predominated the electrophysiological response to depolarizing steps (cf. Tessier-Lavigne et al., 1987).

Ca entry could also be evoked at the cell body in bipolar cells whose axons were severed during the slicing procedure (not shown), indicating that the Ca signal observed in the soma and

Figure 2. Localizing filled bipolar terminals within the inner plexiform layer. *A*, A fura-2 filled ON bipolar cell showing dendrites, *d*, soma, *s*, and terminals, *t*. The terminals of salamander bipolar cells are composed of many very thin processes, unlike the bulbous terminal of some goldfish bipolar cells (cf. Marc et al., 1978). This image is an average of 128 frames. The bright wedge on the left is the fura-2 filled whole-cell pipette. *B*, A negative of the fluorescence shown in *A* is overlaid onto a bright-field image of the same slice. *C*, Simplified diagram of bipolar cell morphology and retinal laminae utilized in the following figures. OPL = outer plexiform layer, IPL = inner plexiform layer.

Figure 3. Optical recordings from individual bipolar cells filled with Ca-sensitive dye. In each experiment, Ca entry was evoked by a 100 msec step to 0 mV from a holding potential of -60 mV , occurring at $t = 400 \text{ msec}$. Increases in $[\text{Ca}^{2+}]_i$ are represented as colors from blue to red with red representing the highest levels of $[\text{Ca}^{2+}]_i$. The blue to red range approximates the range of the $\Delta F/F$ measurement in the corresponding plots of Ca signals across all frames. Pink ellipses over the dendritic and somatic regions indicate areas of dye saturation (see Materials and Methods). **A**, Four selected difference images of activity from a bipolar cell filled with $200 \mu\text{M}$ fura-2 are shown sequentially in time from left to right. The temporal development and decay of the Ca signal, obtained by averaging all pixels covering the terminal, is plotted across all 30 frames and shown in Figure 4A. **B**, Three difference images of Ca entry into a fura-2 ($200 \mu\text{M}$)-filled ON bipolar cell, each at the same time but under different

Figure 4. Time course of Ca entry evoked by somatic depolarization into bipolar terminals. *A*, The Ca signal averaged across the terminals for all frames evoked in the bipolar cell illustrated in Figure 3*A*. The response rose rapidly but decayed back to baseline levels over seconds. The trace below shows the timing of the voltage step applied to the whole cell pipette. The circled square indicates the baseline fluorescence from the same terminal about 1 min after the depolarizing step. *B*, The Ca signal averaged across the terminal of an ON bipolar cell showing a near complete decay to baseline levels within 3 sec.

dendrite was not produced by passive diffusion of intracellular Ca from the terminal but by direct Ca entry at these regions. This result, suggesting that Ca channels are located on the somata of bipolar cells, is consistent with the findings of Heidelberger and Matthews (1992) but differs from the findings of Tachibana et al. (1993).

The Ca signals observed optically at bipolar terminals were generated by Ca entry through voltage-gated Ca channels. Maguire et al. (1989) suggested that the majority of Ca current in

Figure 5. Timing and pharmacology of the Ca signals. *A*, Nifedipine, 50 μM , strongly suppressed the Ca entry into the terminals of an ON bipolar cell filled with 200 μM fura-2. This cell was recorded under symmetrical Cl^- conditions ($E_{\text{Cl}} = 0$ mV). *B*, For a different ON bipolar cell filled with 200 μM fura-2, the simultaneously recorded Ca current, continuous trace, and the derivative of the optical Ca signal, squares, are shown. The derivative of the optical trace (dF/dt) was inverted and superimposed onto the Ca current to show the close correlation. The bipolar cell in *B* was recorded with the CsCl/TEA intracellular solution in a bath containing TEA ($E_{\text{Cl}} = 0$ mV).

bipolar cells entered through L-type Ca channels at the terminal region. Consistent with Maguire et al. (1989) and with recent results from Tachibana et al. (1993) and from Heidelberger and Matthews (1992), the L-type Ca channel blocker nifedipine (50 or 100 μM , $n = 5$) strongly suppressed the peak Ca signal measured optically at bipolar terminals ($67 \pm 2\%$ reduction; SEM; Fig. 5*A*). The Ca signal could also be blocked by a bathing solution containing either 30 μM CdCl_2 or 0.1 mM $\text{CaCl}_2/10$ mM MgCl_2 (not shown here). Direct electrical measures of Ca currents in salamander ON bipolar cells proved difficult, however, because of the possibilities of a long electrotonic distance from the terminal to the recording electrode, an incomplete block of K^+ currents, or the removal of variable amounts of terminal during the slicing procedure. Ca currents measured in nine ON

conditions: *Control*, under control conditions; *GABA*, in the presence of 200 μM GABA bathing the slice; and *Wash*, following the washout of GABA with standard bath solution. The Ca signals averaged across the terminals for all frames in each condition are shown in Figure 6. GABA reversibly blocks Ca entry into the terminals. *C*, Four difference images of activity, each at the same time, evoked in an OFF bipolar cell filled with 200 μM fura-2 under control conditions, *Control*, in the presence of 200 μM GABA, *GABA*, in 200 μM GABA and 50 μM dopamine, *GABA + DA*, and following a wash with standard bath solution, *Wash*. GABA inhibited Ca entry into the terminals, and dopamine partly relieved the GABAergic inhibition. The Ca signals averaged across the terminals for all frames in each condition are shown in Figure 8. All three bipolar cells were recorded with the standard intracellular solution ($E_{\text{Cl}} = -58$ mV). The somata appear larger in the cartoon than they really are because of the bright fluorescence and because the entire cell did not always fall within a single focal plane.

Figure 6. GABA inhibited Ca entry into bipolar terminals. The Ca signals recorded from the terminal of the ON bipolar cell illustrated in Figure 3B are plotted across all 30 frames. GABA reversibly inhibited Ca entry into the terminals.

bipolar cells, in 2 or 10 mM $[Ca^{2+}]_o$, were 39.6 ± 15 pA (SEM) (Fig. 5B), whereas in four OFF bipolar cells, Ca currents were 116.5 ± 15 pA (SEM). While barium (Ba) is often used as a charge carrier to enhance electrical recordings of Ca currents, Ba entry into bipolar cells did not generate any detectable change in fura-2 fluorescence ($n = 2$, not shown) with our recording system, contrary to the results of McMahon and Nicholls (1993).

We also tested whether the rate of change of the optical Ca signal at bipolar terminals was proportional to the Ca current (recorded at the soma) evoked by the step depolarization. Figure 5B shows simultaneously recorded Ca current and Ca signals from an ON bipolar cell terminal. The temporal derivative of the optical signal (dF/dt , $dt = 33$ msec, a single frame time) should reflect the rate of Ca entry at the terminals. When the values for dF/dt were inverted so that they could be superimposed upon the current trace (Fig. 5B), the time course of $-dF/dt$ was very similar to that of the electrically recorded Ca current. Therefore, these results suggest that the majority of the Ca signal we measured from bipolar terminals can be accounted for by Ca current through Ca channels and not release from intracellular stores.

GABA inhibits Ca entry into bipolar terminals. Figures 3B and 6 illustrate the effect of GABA on Ca entry into a representative ON bipolar cell. Figure 3B shows three difference images, each at the same time but under different conditions. When 200 μ M GABA bathed the slice, the Ca signal recorded at the terminal was strongly inhibited when compared to the control image. The pink ellipses on the soma and dendrite indicate saturating baseline fluorescence. The Ca signal averaged over the entire terminal is plotted across 2 sec in Figure 6 and shows that GABA reversibly inhibited Ca entry into the terminal. Similar results were found in 9 OFF bipolar cells and 24 additional ON bipolar cells. In the 34 cells tested, 200 μ M GABA reduced the peak Ca signal to $35.9 \pm 4\%$ (SEM) of control levels, consistent

Figure 7. GABA_A receptors did not mediate the GABAergic inhibition of Ca entry into bipolar terminals. The plot of the Ca signal averaged across the terminal of an OFF bipolar cell filled with 200 μ M fura-2 shows that GABA strongly and reversibly inhibited Ca entry into the terminals in the presence of SR-95531. The effectiveness of SR-95531 was confirmed in five amacrine cells (see Results). This cell was recorded with the standard intracellular solution ($E_{Cl} = -58$ mV).

with the recent results of Heidelberger and Matthews (1991), Matthews et al. (1994), and Lukasiewicz and Werblin (1994), showing that GABA inhibits Ca/Ba currents in bipolar cells. GABA had almost no effect on entry into the soma (not shown).

Bipolar cells in salamander mainly express GABA_C receptors, with a variable complement of GABA_A receptors (Lukasiewicz et al., 1994). In order to confirm this result, the sensitivity of the GABA-induced inhibition of Ca entry into bipolar terminals was tested by coapplying GABA_A receptor antagonists with GABA. Bicuculline methiodide (BMI) was not useful in these studies since the background slice fluorescence was inexplicably and permanently enhanced when BMI bathed the slice ($n = 2$), greatly reducing the fluorescent signal/background ratio. Figure 7 shows representative results from an experiment testing the effect of SR-95531 (SR), a potent GABA_A receptor antagonist, on Ca entry into an OFF bipolar cell. Coapplication of 200 μ M GABA and 40 μ M SR-95531 slightly reduced the inhibition of Ca entry into the terminals (Fig. 7). In five such experiments, SR-95531 did not significantly reverse the GABA effect: GABA alone reduced peak Ca entry to $24 \pm 7.6\%$ (SEM) and GABA in the presence of SR-95531 reduced peak Ca entry to $29 \pm 11.6\%$ (SEM) of control levels. Considering the changes in each cell individually, addition of SR-95531 with GABA enhanced the Ca signal present in GABA alone by an average of 13%, indicating that GABA_A receptors do exist on the bipolar cells tested, but in only a small proportion of total GABA receptors.

To test the effect of 40 μ M SR-95531 in our preparation, we tested its ability to block the effect of GABA on amacrine cells, which express mainly GABA_A receptors (Lukasiewicz and Werblin, 1994) whose activation also causes an inhibition of Ca entry (Wellis and Werblin, unpublished observations). In all five amacrine cells, 40 μ M SR-95531 blocked the inhibitory effects of GABA on optical Ca signals (not shown). The small effect of SR-95531 on blocking the GABAergic inhibition of bipolar

Figure 8. Dopamine relieved the GABAergic inhibition of Ca entry into bipolar terminals. The Ca signals recorded from the OFF bipolar terminal illustrated in Figure 3C are plotted for each of the four conditions illustrated in Figure 3C. GABA inhibited Ca entry into the terminals, dopamine relieved the effect, and the effects were reversible.

terminals is consistent with the results of Lukasiewicz and Werblin (1994) and Matthews et al. (1994), and indicates that GABA_A receptors are only secondarily responsible for the effects of GABA on bipolar terminals.

Previously, Maguire et al. (1989) showed that a specific subpopulation of bipolar cells whose terminals ramified in the middle of the IPL received GABAergic synaptic input at a GABA_B receptor subtype. Very few bipolar cells with this morphology were recorded in this study, and in one such cell, bath-applied GABA had no effect on Ca entry. No further tests were made on these few cells. Baclofen (a GABA_B receptor agonist, 20 or 50 μ M) had no effect on the bipolar cells whose terminals branched within the inner or outer IPL (not shown), consistent with Maguire et al. (1989).

Dopamine relieves the GABAergic inhibition. To determine if dopamine modulates the GABAergic inhibition of Ca entry into bipolar terminals, dopamine was subsequently added in the presence of GABA. We found that a several minute bath application of 100 μ M dopamine with 200 μ M GABA relieved the GABAergic inhibition of Ca entry into bipolar terminals in some cells (see below). Figure 3C shows four difference images, each at the same time but under different conditions, from a representative OFF bipolar cell. Under control conditions (Fig. 3C, control), depolarization of the soma evoked Ca entry into the terminal region. When 200 μ M GABA bathed the slice (GABA), Ca entry into the terminal was strongly inhibited. However, subsequent addition of 100 μ M dopamine to 200 μ M GABA (GABA + DA) partially relieved the inhibitory effect of GABA. The Ca signals averaged over the entire terminal and plotted across all frames (Fig. 8) illustrate that GABA strongly inhibited Ca entry at the terminal and this inhibition was much less in the presence of dopamine. For nine bipolar cells (four OFF and five ON) bathed in GABA and dopamine, the peak Ca signals averaged $67 \pm 2\%$ (SEM) of control, nearly double the averaged peak Ca signals measured in GABA alone ($35 \pm 8\%$ of control).

Figure 9. Summary of effects of GABA and dopamine on the peak Ca signals measured in bipolar cell terminals ($n = 9$ bipolar cells). GABA inhibited the peak Ca signals to about 34% of control, whereas Ca signals measured in the presence of GABA and dopamine measured about 67% of control. Addition of dopamine in the presence of GABA nearly doubled the peak Ca signal measured in GABA alone.

These results are summarized in Figure 9. Considering each of the nine cells individually, subsequent addition of dopamine enhanced the Ca signal present in GABA by an average of 85%. The relieving effect of dopamine upon GABAergic inhibition was observed in 9 out of 12 cells tested. However, in the other three cells (two ON and one OFF), the addition of dopamine in the presence of GABA had little (<10%) or no effect on the Ca signal. This may be related to the washout of Ca currents or other intracellular enzymatic activity during whole-cell recording (see Discussion). Dopamine alone had no significant enhancing effect on the Ca signal above control levels ($n = 7$; Fig. 10A).

Dopamine modulates both glutamate (Knapp and Dowling, 1987; Maguire and Werblin, 1994) and GABA (Dong and Werblin, 1994) responses in the retina via the dopamine D1 receptor subtype. The GABAergic inhibition of Ca signals reported above could also be relieved by the D1 receptor antagonist ($\pm$)SKF-38393 (500 μ M) and by forskolin (25 or 50 μ M; Fig. 10B). In three cells bathed in GABA and ($\pm$)SKF-38393, peak Ca signals averaged $36 \pm 9\%$ (SEM) of control, more than double the averaged peak Ca signals measured in the presence of GABA alone ($12.7 \pm 10\%$). In four cells bathed in GABA and forskolin, peak Ca signals averaged $62 \pm 7\%$ (SEM) of control, nearly double the average peak Ca signal measured in GABA alone ($36.7 \pm 4\%$). The results are consistent with the hypothesis that dopamine may be acting on D1 receptors that are coupled to GABA_C receptors indirectly via a second-messenger pathway involving cyclic AMP.

Ensemble recordings in slice

Correlation of modulation of Ca entry at bipolar terminals with transmission to third-order retinal neurons. Optical recording allows direct observation of the simultaneous activity of bipolar terminals and postsynaptic populations of cells and their processes. In order to begin to investigate how GABA and dopamine receptors interact at bipolar terminals to modulate synaptic release, entire slices were loaded with the membrane permeable form of fura-2, fura-2AM. Figure 11A shows a bright-field image of a representative retinal slice, with photoreceptors at the top and ganglion cells near the bottom. Figure 11B shows a fluorescent image (380 nm excitation) of the same slice labeled

Figure 10. Dopamine alone did not enhance Ca signals, but forskolin mimicked the dopamine effect in the presence of GABA. *A*, Ca signals from an ON bipolar terminal showing that dopamine (100 μ M) applied alone did not appreciably enhance Ca signals above control levels. No enhancement of the Ca signal was found when this bipolar cell was stepped to -30 mV instead of 0 mV (not shown). *B*, Effects of bath applied GABA (200 μ M) and forskolin (*Forsk*, 50 μ M) on Ca entry into an OFF bipolar cell. Forskolin almost completely relieved the inhibitory effects of GABA in this experiment, and the effects were reversible. Both bipolar cells were recorded with the standard intracellular solution ($E_{Cl} = -58$ mV).

with fura-2AM. Under our loading conditions, all photoreceptors loaded with dye but the other cell types were labeled in a selective, "Golgi"-like fashion: some cells were heavily labeled, whereas others were completely devoid of label. Figure 11, *C* and *D* reveals the labeling at higher magnification. Rod terminals (arrow, Fig. 11*C*) and the processes of amacrine cells (arrows, Fig. 11*D*) could be clearly visualized.

Synaptic activity was elicited in slices by short (10–30 msec) puffs of KCl (110 mM) or glutamate (1 mM) from a puff pipette directed at bipolar dendrites within the outer plexiform layer (see

Fig. 11*A* for Puffer position). The depolarized bipolar cells, in turn, synaptically activated amacrine and ganglion cells. Figure 12 illustrates optical recordings from two fura-2AM-loaded slices under different experimental conditions. Figure 12*A* illustrates the time course of the slice response to localized KCl stimulation at bipolar dendrites. Following a small stimulus artefact at the tip of the puff pipette (not shown), the initial Ca signal occurred within the IPL (Fig. 12*A*; $t = 133$ –264 msec), followed by a buildup in the bipolar and amacrine cell bodies. Activity also spread laterally within the IPL, and Ca signals were observed in ganglion cell somata. The Ca signal from the IPL and from amacrine and ganglion cell somata could be strongly reduced by the AMPA/kainate receptor antagonist CNQX (20 μ M, not shown). This result precludes the possibility that amacrine and ganglion cells were activated directly by a widespread leakage of KCl from the puff pipette, and indicates that the postsynaptic targets of bipolar terminals were synaptically activated by non-NMDA receptors, consistent with results from Dixon and Copenhagen (1992) and others. The CNQX results would also suggest that the contribution of NMDA receptors to the Ca signal in the IPL was minor, but high concentrations of CNQX antagonize NMDA receptor activation in salamander retina (D. R. Copenhagen, personal communication).

To determine the effects of GABA and dopamine on neurotransmitter release from bipolar cells, the pre- and postsynaptic activity generated in slices was tested under the same conditions used for imaging single bipolar terminals. Figure 12*B* shows three sets of four averaged difference images (at the same time in each of three conditions) for the same slice illustrated in Figure 12*A*. Under control conditions, a 20 msec KCl puff evoked activity in bipolar and amacrine cells and their processes (Fig. 12*B*, control). When 200 μ M GABA bathed the slice, activity within the IPL was inhibited, whereas much less change was noted at the level of the cell bodies (Fig. 12*B*, GABA). Dopamine (50 μ M), subsequently applied in the presence of GABA, relieved the GABAergic inhibition of IPL activity (Fig. 12*B*, GABA + DA). Figure 13 shows the Ca signal measured within the IPL (square in Fig. 13*B*) across all 64 frames in each condition. The general features of this plot are similar to those shown for bipolar terminals: GABA inhibited peak Ca entry into processes within the IPL, to approximately 56% of control levels, and dopamine relieved the GABAergic inhibition, increasing the peak Ca signal to about 87% of control. Consistent results were found in a total of six slices. A similar modulation of transmission from bipolar cells to third-order cells by GABA and dopamine was observed when bipolar cells were stimulated by puffing glutamate (1 mM) rather than KCl at the OPL ($n = 3$ slices). However, since dopamine also enhances glutamate receptor sensitivity on bipolar dendrites (Maguire and Werblin, 1994), specific localization of the dopamine effects to bipolar terminals was not possible in these experiments.

GABA and dopamine likely have direct effects on third-order cells as well as on bipolar terminals. We have found in a parallel series of experiments that bath-applied GABA also inhibits Ca entry into the processes of fura-2-filled amacrine cells (Wellis and Werblin, unpublished observations). Dopamine has also been recently shown to have effects on amacrine cell coupling (Hampson et al., 1992) and ganglion cell Ca currents (Liu and Lasater, 1994). In salamander retina, amacrine and ganglion cells express mainly GABA_A receptors (Lukasiewicz and Werblin, 1994) that do not appear to be modulated by dopamine (P. D. Lukasiewicz, personal communication). To rule out the possi-

Figure 11. Fura-2AM loading of retinal slices. *A*, Bright-field image of a retinal slice with photoreceptors at the top and ganglion cells below, nearest to the Millipore filter (black). The IPL was clearly visualized. A puff pipette, *puffer*, was directed at the outer plexiform layer. *B*, Fluorescent image (380 nm excitation) of the slice shown in *A*. All cell types show labeling, but not all cells are labeled. *C*, Higher magnification fluorescent image of labeled photoreceptors, their terminals (*arrow*), and less intensely labeled bipolar and amacrine cells deep to the photoreceptors. *D*, High magnification fluorescent image of a row of amacrine cells and their associated processes (*arrows*) within the IPL. Each image is a single raw frame captured with an SIT camera. Calibration in *B* also applies to *A*; calibration in *D* applies to *C*.

bility of postsynaptic effects of GABA on amacrine and ganglion cells, we bathed the slice in 40 μM SR-95531, a GABA_A antagonist. The effects of dopamine alone on overall retinal activity are less meaningful here, since dopamine may have multiple, as yet undescribed, effects on third-order cells.

Figures 12C and 14 illustrate the results of coapplying SR-95531 with GABA and dopamine. Figure 12C shows three sets of four averaged difference images from another fura-2AM stained slice. Under control conditions (Fig. 12C, control), a 30 msec puff of KCl evoked widespread activity in the slice. The GABA_A antagonist SR-95531 did not block the inhibitory effect of GABA on Ca entry in the IPL (Fig. 12C, GABA + SR), but subsequent addition of 50 μM dopamine partly relieved the inhibitory effect of GABA even when SR-95531 was present (Fig. 12C, GABA + SR + DA). Figure 14A shows the Ca signals measured within the IPL (squares in Fig. 12C) across all 16 frames. GABA almost completely inhibited Ca entry into processes within the IPL in the presence of SR-95531 to 1% of control, and dopamine relieved the GABAergic inhibition, increasing the peak response to 62% of control. Figure 14B shows the results from a similar experiment on a different slice in which the video camera gain was lowered to measure the fluorescence of a well-filled ganglion cell soma. In the presence of 40 μM SR-95531, 200 μM GABA strongly inhibited activity at the ganglion cell to <1% of control, whereas coapplication of 50 μM dopamine relieved the inhibitory effect of GABA, increasing the peak response to 22% of control. Consistent results were found in a third slice, indicating that GABA_C receptor sensitivity on bipolar terminals was consistently downmodulated by dopamine to regulate transmission from bipolar terminals to third-order retinal cells.

Discussion

We report here the first composite optical recordings of presynaptic Ca entry into bipolar cells correlated with ensemble activity within the intact circuitry of the retinal slice. GABA inhibits Ca entry into bipolar terminals and thereby inhibits transmission

from bipolar cells to amacrine and ganglion cells. In the presence of GABA, dopamine reduces GABA_C receptor sensitivity at bipolar terminals, relieves the inhibitory effects of GABA, and partly restores transmission. Thus, the modulation of transmission to third-order cells by GABA and dopamine is entirely consistent with modulation of Ca entry at bipolar terminals.

Comparison with other measurements of calcium entry into presynaptic terminals

The rapid rise of the Ca signal, whose temporal derivative is directly proportional to Ca current (Fig. 5), and the prolonged decay to baseline are consistent with Ca signals measured in isolated bipolar cells (Tachibana et al., 1993; vonGersdorff and Matthews, 1994) and in other vertebrate (Peng and Zucker, 1993; Regehr et al., 1994; Wu and Saggau, 1994) and invertebrate (Augustine et al., 1983) presynaptic terminals. These kinetics suggest a common mechanism of Ca buffering in presynaptic terminals, but may also depend upon the binding kinetics of Ca to fura-2. The variability in the decay of the Ca signals recorded here (see Figs. 4–8) clearly suggests that the Ca dynamics are more complicated in this phase of the response.

Presynaptic GABA receptors and suppression of Ca entry

Activation of the GABA_C receptor, like the GABA_A receptor, generates a chloride current (Qian and Dowling, 1993; Feigenspan et al., 1993; Dong et al., 1994; Lukasiewicz et al., 1994) and most likely reduces Ca entry by shunting the membrane toward E_{Cl} (Lukasiewicz et al., 1994). GABA likely has no direct effects on the Ca current via a GABA_B pathway since bath-applied GABA does not effect Ca currents in the presence of picrotoxin (Lukasiewicz and Werblin, 1994) and since baclofen, a GABA_B agonist, had no effect on the bipolar cells studied here. Studies from goldfish and rat bipolar cells suggest that GABA sensitivity is mediated mainly by the GABA_A receptor subtype (Tachibana and Kaneko, 1987; Suzuki et al., 1990), which would suppress Ca entry by a similar shunting mechanism. However, Matthews and colleagues (Heidelberg and Matthews, 1991;

Figure 12. Optical recordings from fura-2AM loaded retinal slices. **A**, Development of activity evoked by bipolar activation in fura-2AM-loaded slices. Sequences of averaged difference images of activity, at the indicated times, are shown from left to right following a puff of KCl (magenta star) at the outer plexiform layer (OPL). The initial response occurred in the IPL, which was followed by a buildup of $[\text{Ca}^{2+}]$ in amacrine, bipolar, and ganglion cells and a lateral spread of activity within the IPL. **B**, Three sets of four averaged difference images, one in each condition, are shown of activity elicited by locally puffing KCl at the OPL of the slice illustrated in **A**. *Control*, under control conditions, activity was evoked in bipolar, amacrine, ganglion cells, and their processes. *GABA*, activity within the IPL was inhibited when $200\ \mu\text{M}$ GABA bathed the slice, whereas less change was evident in the inner nuclear layer. *GABA + DA*, coapplication of $50\ \mu\text{M}$ dopamine with GABA, relieved the inhibitory effects of GABA. The Ca signals measured within the IPL, square, in each condition, are shown in Figure 13. **C**, Experiments similar to those illustrated in

Figure 13. Dopamine relieved the inhibitory effects of GABA on synaptic transmission from bipolar cell to third-order cells. Ca signals measured across all 64 frames in the IPL of slice activity illustrated in Figure 12B. GABA inhibited activity in the IPL and dopamine, applied with GABA, relieved the effect of GABA. The origin of the oscillation in the Ca signals is not known. The timing of the 30 msec puff of KCl is shown below.

Matthews et al., 1994) have discovered that in addition to a GABA_A-receptor activated Cl⁻ conductance, GABA also directly inhibited Ca currents in goldfish bipolar cells via an intracellular GTP-dependent mechanism, similar to that of, but not, a GABA_B receptor. Bipolar cells from different species may express different complements of GABA receptor subtypes at their terminals (Lukasiewicz et al., 1994), and each may act to reduce Ca entry and therefore synaptic transmission.

Finally, it also seems unlikely that GABA reduced Ca current via a pH-dependent mechanism. Kaila and Voipio (1987) reported in crayfish muscle that GABA can activate a bicarbonate conductance that causes an intracellular acidosis. While intracellular acidification can reduce Ca currents in catfish horizontal cells (Dixon et al., 1993), the GABA-activated bicarbonate conductance and acidosis does not occur in a HEPES-buffered bath solution (Kaila and Voipio, 1987).

Dopamine modulation of transmitter release from bipolar terminals

Dopaminergic synaptic input to salamander bipolar terminals likely originates from a subpopulation of tyrosine hydroxylase-containing amacrine cells (Yang et al., 1991). The processes of dopaminergic amacrine cells project in both sublaminae a and b of the IPL, consistent with our findings of dopamine modulation in both OFF and ON bipolar terminals.

We have shown here that dopamine relieves the inhibitory

Figure 14. The inhibitory effects of GABA were likely mediated by GABA_A receptors on bipolar terminals. *A*, Ca signals measured across all 16 frames in the IPL of the slice illustrated in Figure 12C in each of four conditions. GABA inhibited activity in the IPL and dopamine, applied with GABA, relieved the inhibitory effect of GABA. The timing of the 30 msec puff of 110 mM KCl is shown below. These effects were reversible. *B*, Ca signals measured from a ganglion cell soma in a different slice. As in the other experiments shown, GABA-inhibited bipolar transmission to the ganglion cell and dopamine, applied with GABA, relieved the GABAergic inhibition.

effects of GABA on Ca entry into bipolar terminals and on postsynaptic responses. While further descriptive pharmacology is necessary, this effect likely occurs through a dopamine D1 receptor and a second-messenger pathway involving cyclic AMP, similar to that recently suggested for catfish horizontal

Figures 12B and 13 were repeated in the presence of the GABA_A antagonist SR-95531 (SR, 40 μM) to block the GABA_A receptors on amacrine, ganglion, and bipolar cells. Three sets of four averaged difference images, one in each condition, are shown of activity elicited by local KCl puffs at the OPL of a different fura-2AM-loaded slice. *Control*, widespread activity was evoked under control conditions. *GABA + SR*, 200 μM GABA inhibited activity in the IPL in the presence of SR. *GABA + SR + DA*, coapplication of 50 μM dopamine with GABA and SR relieved the GABAergic inhibition. The Ca signals plotted in the IPL, *square*, under each condition are shown in Figure 14A.

cells (Dong and Werblin, 1994). Our hypothesis is that a cAMP-dependent protein kinase phosphorylates the GABA_C receptor and reduces the conductance of its associated channel, similar to the finding that GABA_A receptor conductance is reduced by phosphorylation (Moss et al., 1992).

Heidelberger and Matthews (1994) have recently shown that dopamine alone, probably also by a cAMP-dependent mechanism, enhanced presynaptic Ca signals in isolated goldfish bipolar terminals. We found that dopamine had little effect on salamander bipolar terminal Ca signals (Fig. 10A). While the reason for this difference is unclear, the data may reflect a species difference, since it appears that the GABA receptors present on goldfish bipolar terminals differ from those on salamander bipolar terminals.

Variability in dopamine modulation

Dopamine reliably reduced GABA-evoked currents (Fig. 1) and relieved the GABAergic inhibition of bipolar transmission in slices loaded with fura-2AM (Figs. 12–14). However, dopamine did not always relieve the GABAergic inhibition of Ca entry into individual bipolar terminals under patch clamp. There are several reasons for this discrepancy. First, the ratio of GABA_C to GABA_A receptors on an individual terminal may dictate the magnitude of dopamine neuromodulation. Salamander bipolar cells express different proportions of the two GABA receptor subtypes, and in a few cases, the GABA_A component exceeded the GABA_C component (Lukasiewicz et al., 1994). GABA_A receptors on salamander amacrine and ganglion cells do not appear to be modulated by dopamine (Lukasiewicz, personal communication). Second, the washout of Ca current or required intracellular enzymes that occurs during whole-cell recording may reduce the effectiveness of dopamine. Although ATP and GTP were included in the pipette to slow the rundown of Ca current, imaging did not begin until the terminals were observable, which varied between 5 and 20 min. Finally, the slices may have been light adapted during single bipolar terminal imaging. In these cases, the endogenous levels of dopamine could have been high (Dong and McReynolds, 1989; Weiler et al., 1989), interfering with the effects of applied dopamine. The robust effects of GABA and dopamine on ensemble recordings and on bipolar responses to puffs of GABA, however, suggest a strong and consistent interaction of GABA and dopamine at bipolar terminals.

Significance of dopamine modulation of presynaptic GABA receptors

These results add to a growing list of examples of dopamine neuromodulation of retinal circuitry. Dopamine modulation occurs within the outer retina on horizontal cell coupling (Dowling, 1991) and on receptor sensitivities of horizontal cells to glutamate (Knapp and Dowling, 1987) and GABA (Dong and Werblin, 1994). Dopamine modulation has also been described within the inner retina on amacrine cell coupling in rabbit (Hampson et al., 1994) and on Ca currents in ganglion cells in turtle (Liu and Lasater, 1994). Our results further indicate that dopamine modulates transmission from the outer to the inner retina.

These studies also provide the first evidence that presynaptic GABA receptors can be modulated by dopamine to further regulate synaptic release from those terminals. Dopamine modulation of GABA receptor sensitivity on bipolar terminals further complements the recent studies by Maguire and Werblin (1994), showing that dopamine upmodulates glutamate responses in

OFF bipolar cells, since both effects act to increase excitability and enhance synaptic release from bipolar cells. By correlating Ca activity at postsynaptic cells with activity at bipolar terminals, we were able to study the relationships between Ca entry, transmitter release, and postsynaptic patterns of activity. Further studies of the modulation of activity patterns among processes of amacrine and ganglion cells could provide new insights into functional interactions within the IPL.

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