Regulation of the Retinal Bipolar Cell mGluR6 Pathway by Calcineurin

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Snellman, Josefina, and Scott Navy. Regulation of the retinal bipolar cell mGluR6 pathway by calcineurin. J Neurophysiol 88: 1088–1096, 2002. 10.1152/jn.00955.2001. Glutamate produces a hyperpolarizing postsynaptic potential in ON bipolar cells by binding to the metabotropic receptor mGluR6 and subsequently closing a cation-selective channel. It has been proposed that Ca2+-mediated depression requires activation of calcineurin, a Ca2+/calmodulin-regulated phosphatase. We measured glutamate-evoked currents (I_{glu}) with whole cell recordings of ON bipolar cells in light-adapted retinal slices. Depression of I_{glu} by Ca2+ was prevented by inhibitors of calcineurin or by tightly buffering Ca2+ with bis-(o-aminophenoxy)-N,N,N’,N’-tetraacetic acid (BAPTA). However, when cells were dialyzed with BAPTA and a Ca2+-independent form of calcineurin (CaN420), depression of I_{glu} was restored. Similarly, CaN420 induced depression of I_{glu} during continuous glutamate application, a protocol that ordinarily prevents depression. Analysis of changes in the amplitude of the cation-selective current (I_{cat}) of cells that were dialyzed with high Ca2+ (1 μM), or with BAPTA and CaN420, indicates that Ca2+ depresses I_{glu} by reducing I_{cat} and that calcineurin acts via the same mechanism. Ca2+-mediated depression of I_{glu} was not found to involve CaMKII, as inhibitors of CaMKII did not prevent this depression nor did they affect the sensitivity of the channel. It has been proposed that Ca2+ functions to antagonize metabotropic receptor mGluR6 and subsequently closing a cation-selective channel. It has been proposed that Ca2+ acts via the same mechanism. Ca2+ entry through the cation channel activates a phosphodiesterase, which hydrolyzes cGMP (Nawy and Jahr 1990; Shiells and Falk 1990). However, a more recent study from this laboratory demonstrated that hydrolysis of cGMP is not necessary for channel closure, suggesting that the channel is not gated by cGMP. Instead it was proposed that cGMP plays a modulatory role in the pathway (Nawy 1999). An alternative hypothesis for transduction is that the G protein interacts directly with the channel, perhaps via beta-gamma subunits. However, the precise transduction mechanism of mGluR6, as well as other group III metabotropic receptor, remains elusive.

Recent evidence suggests that the mGluR6 transduction pathway is strongly regulated by Ca2+. Entry of Ca2+ through the cation channel leads to a depression of the mGluR6 pathway. Buffering with bis-(o-aminophenoxy)-N,N,N’,N’-tetraacetic acid (BAPTA) prevents this depression as does lowering external Ca2+ (Nawy 2000). The loss of the response to glutamate is also prevented by keeping the cation channels closed with exogenous glutamate, thus mimicking darkness, when synaptic levels of glutamate would be highest (Nawy 2000). In the dark-adapted retina, buffering cells with BAPTA appears to disrupt an adaptive process that allows cells to recover to their dark resting potential during sustained illumination (Shiells and Falk 1999), and it has been proposed by these investigators that Ca2+ entry through the cation channel serves an adaptive purpose, closing channels that have opened as a result of a drop in transmitter levels during sustained illumination of photoreceptors.

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INTRODUCTION

In the vertebrate retina, photoreceptors synapse onto two types of bipolar cells designated as ON and OFF cells. Glutamate, released in darkness, depolarizes ON cells via AMPA and kainate receptors (DeVries and Schwartz 1999), while it hyperpolarizes OFF cells (Shiells et al. 1981; Slaughter and Miller 1981) by closing a cation channel via activation of a metabotropic G-protein-coupled receptor (Nawy and Jahr 1990; Shiells and Falk 1990). The receptor, mGluR6 (Nakajima et al. 1993), is a group III metabotropic receptor (Pin and Duvoisin 1995) that is believed to be expressed exclusively by ON bipolar cells. Several lines of evidence suggest that the receptor probably signals through the Gα subunit of G proteins. Immunoreactivity to antibodies against Gα(alph) and mGluR6 is colocalized in ON bipolar cell dendrites (Vardi 1998), and injection of Gα(alph) disrupts mGluR6 signaling (Nawy 1999). Also, mice lacking the Gα(alph) subunit do not have functional ON bipolar cell synaptic transmission, as judged by the absence of the b-wave in the electroretinogram (ERG) (Dhingra et al. 2000).

Although there is strong evidence implicating the type of G protein in this pathway, the downstream mechanism by which Gα couples to the synaptic cation channel is controversial. Primarily because application of cGMP through the recording pipette greatly potentiated the response to glutamate, it was originally proposed that the channel was gated by cGMP and closed when the G protein activated a phosphodiesterase, which hydrolyzed cGMP (Nawy and Jahr 1990; Shiells and Falk 1990). However, a more recent study from this laboratory demonstrated that hydrolysis of cGMP is not necessary for channel closure, suggesting that the channel is not gated by cGMP. Instead it was proposed that cGMP plays a modulatory role in the pathway (Nawy 1999). An alternative hypothesis for transduction is that the G protein interacts directly with the channel, perhaps via beta-gamma subunits. However, the precise transduction mechanism of mGluR6, as well as other group III metabotropic receptors, remains elusive.

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In a recent study of ON bipolar cells in the rod-dominated retina of the dogfish, Shiells and Falk (2000) suggested that activation of the Ca2+/calmodulin-regulated kinase CaMKII is
responsible for depression of synaptic responses in ON bipolar cells. It is unclear whether this mechanism is confined to rod-driven ON bipolar cells or if it can account for the Ca\(^{2+}\)-mediated depression of postsynaptic responses in the salamander retina, where many, if not most ON bipolar cells receive input from cones as well as rods (Wu et al. 2000). Accordingly, we looked for a potential role of CaMKII, as well as calcineurin, a neuronal form of the type 2B phosphatase that is activated by Ca\(^{2+}\)/calmodulin. Our primary finding is that Ca\(^{2+}\) depresses the mGluR6 pathway via a calcineurin-dependent mechanism not via activation of CaMKII. Our findings suggest that visual information in the rod and cone pathways may be processed differently in ON bipolar cells.

**METHODS**

**Preparation of slices and solutions**

Slices of retina from larval tiger salamanders (Charles Sullivan, Nashville, TN) were prepared as described previously (Nawy 1999; Walters et al. 1998). Briefly, salamanders were anesthetized with 3-amino-benzoic acid ethyl ester and decapitated, and the eyes were enucleated. Whole retinas were isolated and placed on a 0.65-μm cellulose acetate/nitrate membrane filter (Millipore, Bedford, MA) that was secured with vacuum grease to a glass slide adjacent to the recording chamber. For the experiments presented in Figs. 6 and 7, animals were dark-adapted for 1 h prior to dissection, and the eyes were removed under dim red or infrared illumination and then dissected in room light in solution supplemented with 2 μM L-2-amino-4-phosphono-butyric acid (L-APB). Slices were then cut to a thickness of 150–200 μm with a tissue slicer (Stoelting, Wood Lane, IL), transferred to the recording chamber while remaining submerged, and viewed with a Zeiss (Thornwood, NY) Axioskop equipped with a water-immersion ×40 objective with Hoffman modulation contrast (Modulation Contrast, Greenvile, NY). Slices were bathed in solution containing (in mM) 108 NaCl, 2 CaCl\(_2\), 2.5 KCl, 1.2 MgCl\(_2\), 10 HEPES, 10 glucose, and 0.1 picrotoxin (pH 7.6 with NaOH). Solution was perfused continuously through the recording chamber at a rate of approximately 1 ml/min. The pipette solution was composed of (in mM) 85 K\(^+\) gluconate, 10 KCl, 10 HEPES, 10 EGTA, 4 MgATP, and 1 LiGTP (pH 7.4 with KOH). Pipette solution containing 1 μM free Ca\(^{2+}\) (calculated with the program MAXCELATOR) was made by replacing the EGTA with 1 mM BAPTA and by adding 3.87 μM Ca\(^{2+}\). For tight buffering of Ca\(^{2+}\), the pipette solution contained 20 mM BAPTA and 65 mM K\(^+\) gluconate but was otherwise unchanged. All chemicals were obtained from Sigma (St. Louis, MO), except for (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG) (Tocris, Ballwin, MO), and the calcineurin inhibitor peptide 281–309, and autacamide-2 related inhibitory peptide, which were obtained from Calbiochem (San Diego, CA). Peptides were aliquoted, stored at −20°C, and dissolved in pipette solution immediately before use. Calcineurin420, which lacks an autoregulatory domain [a gift of Dr. Brain Perrino (Perrino et al. 1995)], was dialyzed to remove glycerol and then aliquoted and stored at −20°C for more than 2 wk before use.

**Electrophysiology and drug application**

Patch pipettes were fabricated from borosilicate glass (WPI, Sarasota, FL) using a two-stage vertical puller (Narishige, Sea Cliff, NY) and were fire-polished to resistances of 2–3 MΩ. Whole cell recordings were obtained with an Axopatch 200A or Axopatch 1D amplifier (Axon Instruments, Foster City, CA) and had input and series resistances of approximately 1 GΩ and 10–19 MΩ, respectively. ON bipolar cells were identified by their position in the slice and by their characteristic outward responses to glutamate. Cells were discarded if the series resistance exceeded 20 MΩ, the holding current changed suddenly, or the holding current during the first application of agonist exceeded −20 pA (i.e., current measured while the sustained inward current was suppressed) at −40 mV. Holding potentials were corrected for the liquid junction potential, which was measured to be 10 mV with the K\(^+\) gluconate pipette solution. Data were acquired with Axobasic software and the Digidata 1200 interface or Axograph software and the Digidata 1322A interface (Axon instruments) and analyzed with Kaleidagraph (Synergy Software, Reading PA).

Drugs were applied via two polymer-coated fused silica tubes (OD: 350 μm, ID: 250 μm, Polymicro Technologies, Phoenix, AZ) positioned close to the cell. One tube contained control bathing solution and the other contained bathing solution to which 1 mM glutamate was added. The tubes were mounted to a computer-controlled piezobimorph (Mornag-Matroc, Bedford, OH). Glutamate was applied every 30 s for a duration of 5 s beginning 30 s after breaking into the cell. In experiments using the continuous glutamate application protocol, glutamate was applied for 115 s and removed for 5 s to examine the amplitude of the response. The glutamate-free solution in this experiment contained the metabotropic receptor antagonist CPPG (60 μM) to ensure complete removal of glutamate from the mGluR6 binding sites. In some experiments, a picospritzer (General Valve, Fairfield, NJ) was used to apply drugs to ON bipolar cells by pressure ejection (typically 1–2 psi) from an unpolished pipette positioned close to the dendrites.

**RESULTS**

**Inhibition of the glutamate response by intracellular Ca\(^{2+}\)**

Glutamate-evoked responses were recorded in whole cell mode from ON bipolar cells in light-adapted slices of tiger salamander retina. At 30-s intervals, cells were exposed for 5 s to a stream of 1 mM glutamate. Glutamate elicits a response (Iglu), that appears to be an outward current. In fact, this response is not due to the generation of a true outward current but rather to the suppression of an inward current (Icat). Thus, Iglu is therefore Icat current that is suppressed by glutamate. Figure 1A shows an example of the time-dependent depression of Iglu over the course of 18 consecutive applications of glutamate. We have previously demonstrated that this protocol of brief and intermittent glutamate application reveals a time-dependent depression of Iglu, and we have postulated that this depression is due to the influx of Ca\(^{2+}\) through the nearly continuously open cation channel (Nawy 2000). To fully activate this Ca\(^{2+}\)-dependent depression, we supplemented endogenous Ca\(^{2+}\) with a pipette solution containing 1 μM free Ca\(^{2+}\) (see METHODS). In the cell illustrated in Fig. 1A, depression of the response was associated with an outward shift in the baseline, indicating a reduction in Icat. The inset shows an overlay of the first and last individual trace, highlighting the difference in amplitude and the shift in baseline. On average, Iglu decayed exponentially to 38 ± 2.8% of the initial response after 12 min of recording, with a time constant of 2.10 min (Fig. 1B, closed symbols). This rate is faster than the τ of 7.5 min that we have observed previously (Nawy 2000) probably because of the addition of Ca\(^{2+}\) to the pipette solution in the present study.

Our results are consistent with a model in which Ca\(^{2+}\) effectively reduces the amplitude of the glutamate response by down-regulating the cation channels. An alternative model is that Ca\(^{2+}\) might reduce coupling of the receptor to the channel. According to this model, the baseline holding current should remain unchanged throughout the experiment, but the size of...
the response to glutamate application should be progressively reduced, reflecting the inability of glutamate to suppress $I_{\text{cat}}$, a result that we did not observe. When we dialyzed cells with a nominally Ca$^{2+}$/H$^{11001}$-free solution that contained 20 mM BAPTA and no added Ca$^{2+}$, the amplitude of $I_{\text{glu}}$ remained stable for the duration of the recording. After 14 min of recording, the mean amplitude of $I_{\text{glu}}$ was 98.84% of the initial response (Fig. 1B, open symbols).

As the next step in defining the role of Ca$^{2+}$ in the regulation of $I_{\text{glu}}$, we wanted to identify potential intracellular targets of Ca$^{2+}$/calmodulin whose activation could cause a depression of the response. We focused our efforts on two Ca$^{2+}$/calmodulin-dependent proteins, Ca$^{2+}$/calmodulin-dependent kinase II (CaMKII) and the Ca$^{2+}$/calmodulin-dependent phosphatase calcineurin.

**Depression of the agonist response is prevented by inhibition of calcineurin**

Calcineurin is the neuronal form of type 2B phosphatases, characterized by the requirement for Ca$^{2+}$/calmodulin for activation (Klee et al. 1988). We found that the addition to the pipette solution of 500-1000 nM cyclosporin A, an inhibitor of calcineurin, prevented depression of $I_{\text{glu}}$. An example is shown in the next section.

**FIG. 2. Evidence that inhibition of calcineurin prevents depression of glutamate-evoked current.**

A. top: individual traces of an ON bipolar cell obtained at the indicated times points. The cell was dialyzed with cyclosporin A, which prevented depression of the glutamate response over the duration of the recording. Overall, inclusion of cyclosporin A in the internal solution
prevented Ca\(^{2+}\)-induced depression of \(I_{\text{glu}}\) (Fig. 2A, bottom; 124 ± 14.3% of the initial amplitude of the response), while an internal solution that contained 0.1% DMSO had no effect on the depression (32 ± 6.8% of initial response). High intracellular phosphate can inhibit the actions of phosphatases, including calcineurin (Jones and Westbrook 1997). Dialyzing cells with a solution in which gluconate was replaced by phosphate completely prevented Ca\(^{2+}\) depression of \(I_{\text{glu}}\) (Fig. 2B). 106 ± 13.4% of initial response. In contrast, \(I_{\text{glu}}\) declined to 66 ± 10.1% of the initial current in cells dialyzed with a gluconate-based internal solution. Finally, we repeated the experiment with another highly selective and mechanistically distinct calcineurin inhibitor that prevents the binding of calmodulin to calcineurin. Dialysis with this calcineurin inhibitory peptide (CIP, 10 \(\mu\)M), also prevented a loss of the response (Fig. 2C, 122 ± 16%) compared with a control peptide that does not inhibit calcineurin function (44 ± 9.4% of initial \(I_{\text{glu}}\)).

All three inhibitors of calcineurin prevented depression of \(I_{\text{glu}}\), suggesting the presence of a negative feedback pathway whereby the entry of Ca\(^{2+}\) through synaptic cation channels leads to the activation of calcineurin and subsequent down-regulation of the channel.

**Introduction of exogenous calcineurin induces depression of \(I_{\text{glu}}\) that is similar to the Ca\(^{2+}\)-dependent form of depression**

Additional evidence for a role of calcineurin in the mGlur6 pathway was obtained by dialyzing cells with a form of calcineurin (CaN420) that lacks the autoinhibitory domain, rendering it constitutively active (Perrino et al. 1995). If Ca\(^{2+}\) normally depresses \(I_{\text{glu}}\) by activating calcineurin, then introduction of CaN420 into the cell should bypass the requirement for Ca\(^{2+}\) and induce depression in cells dialyzed with an intracellular solution in which Ca\(^{2+}\) is tightly buffered. In fact, this is what we observed. Figure 3A (top) shows two traces from a recording of an ON bipolar cell obtained with the high BAPTA/low-Ca\(^{2+}\) intracellular solution. As expected, there is no depression of \(I_{\text{glu}}\). On the other hand, the depression was reinstated when cells were dialyzed with the same intracellular solution, supplemented with Ca\(^{2+}\) insensitive CaN420, as is illustrated by traces that were obtained after 1 and 10 min of recording (Fig. 3A, bottom). Comparison of these two traces suggests that calcineurin reduced \(I_{\text{glu}}\) by decreasing available \(I_{\text{cat}}\), indicated by an upward shift in the baseline. A shift in the baseline was observed in seven of nine cells. Thus both Ca\(^{2+}\) and calcineurin appear to primarily depress \(I_{\text{glu}}\) by regulating the cation channel rather than the receptor-channel coupling. In a total of nine cells, CaN420 reduced \(I_{\text{glu}}\) to 43 ± 9.3% of its initial value (Fig. 3B), similar to values observed in cells dialyzed with EGTA or 1 \(\mu\)M Ca\(^{2+}\). For comparison, the average \(I_{\text{glu}}\) from cells dialyzed with the nominally Ca\(^{2+}\)-free pipette solution alone (Fig. 1B) is replotted here.

We have previously shown that the depression of \(I_{\text{glu}}\) is use-dependent (Nawy 2000). When the channels were closed by the continuous application of glutamate (protocol illustrated in Fig. 4A), as would occur in darkness, the amplitude of \(I_{\text{glu}}\) remained constant over time. Records from a cell subjected to this protocol are shown in Fig. 4B (top). When ON bipolar cells were dialyzed with CaN420 and glutamate was applied using the same protocol, the depression of the response was reinstated (Fig. 4B, bottom). Figure 4C summarizes the time-dependent changes in \(I_{\text{glu}}\) for five cells (39 ± 11% of initial response, \(\tau = 2.23\) min) and compares them to the averaged \(I_{\text{glu}}\) of nine cells that were subjected to the same glutamate application protocol but were not dialyzed with CaN420. Thus CaN420 induced depression of \(I_{\text{glu}}\) under two conditions that were normally unfavorable for \(I_{\text{glu}}\) depression, either when intracellular Ca\(^{2+}\) was highly buffered or when Ca\(^{2+}\) entry through the cation channel was prevented.

**A Ca\(^{2+}\)-independent form of calcineurin is sufficient to induce depression in the absence of Ca\(^{2+}\).**

A, top: example of glutamate-elicited currents recorded from an ON bipolar cell with a pipette containing 20 mM BAPTA (low-Ca\(^{2+}\) solution). The amplitude of the response remained essentially unchanged over time. Bottom: example of glutamate-elicited currents recorded from another ON bipolar cell with a pipette containing the low-Ca\(^{2+}\) solution and 100 nM CaN420 (a Ca\(^{2+}\)-independent form of calcineurin). The response was depressed, with a concomitant shift in the baseline, suggesting that CaN420 might downregulate the cation current, as has been proposed for Ca\(^{2+}\). B: summary and comparison of the effect on \(I_{\text{glu}}\) of the low-Ca\(^{2+}\) solution alone (open symbols, \(n = 10\)) and with CaN420 (closed symbols, \(n = 13\)).

**Calcineurin-mediated depression of \(I_{\text{glu}}\) does not require CaMKII**

Another group has recently suggested that CaMKII depresses responses to light stimulation in ON bipolar cells (Shiells and Falk 2000). Accordingly, we added autacamtide-2 related inhibitory peptide (AIP), a CaMKII inhibitor, to the pipette along with 1 \(\mu\)M free Ca\(^{2+}\), a concentration that is sufficient for CaMKII activation (Klee 1991), to investigate the possibility that CaMKII could similarly depress responses to the application of exogenous glutamate. Examples of individual traces after 1 min and at 15 min of recording are shown in Fig. 5A. In this cell, the time-dependent depression of \(I_{\text{glu}}\) was not prevented by inclusion of AIP. In six of eight cells, depression of \(I_{\text{glu}}\) was not significantly different in the
presence of AIP (Fig. 5B; 46 ± 6.7% of initial response) compared with controls (Fig. 1, 38 ± 2.8% of initial response). However, in two of eight cells that were dialyzed with AIP, no significant rundown occurred. These two cells had small responses even when the recording was initiated, and it is possible that the Ca$^{2+}$-sensitive component of $I_{\text{glu}}$ was absent in these cells. Interestingly, when calcineurin activity was blocked with cyclosporin A, inhibition of CaMKII reinstated the depression of $I_{\text{glu}}$ (Fig. 5D; 53.9 ± 15.2 of initial response; $P < 0.05$). These data are consistent with a previous report showing that inhibition of CaMKII in the presence of phosphatase blockers depresses $I_{\text{glu}}$ (Walters et al. 1998). Under our recording conditions, it seems that calcineurin-mediated dephosphorylation predominates over CaMKII-mediated phosphorylation. Only when calcineurin was inhibited could an effect of CaMKII be observed.

Experiments described thus far were performed on light-adapted slices. To pharmacologically mimic release of transmitter in the dark-adapted retina, we first dark-adapted salamanders and then removed the eye under infrared light. All subsequent manipulations were carried out in L-APB, a potent mGluR6 agonist, at a concentration of 2 mM, which we have found to be saturating at this synapse (Nawy and Jahr 1991). To simulate responses to light, which decreases glutamate release from photoreceptors, the type III metabotropic receptor antagonist CPPG (1 mM), an antagonist at the mGluR6 receptor (Awatramani and Slaughter 2000), was pressure ejected from an unpolished patch pipette positioned near the dendrites of ON bipolar cells. The amplitude of the response to “puffs” of CPPG could be controlled by varying the duration of the application. An example of a family of responses generated this way is shown in Fig. 6A. The currents are inward because CPPG displaces bath L-APB, resulting in the opening of the
calcium channel. The family of response is plotted in Fig. 6A and could be fitted with a Hill plot with a $k_{1/2}$ of 15.3 ms.

For brief applications of CPPG, there was a linear relationship between the amplitude of the response and the duration of the application (Fig. 6B, inset). Generally this was in the range of 5–12.5 ms puffs and corresponded to ≤30% of the maximum response. In this range, the sensitivity of the cell to changes in agonist concentration is given by the slope of the linear regression. For the cell illustrated in this figure, the sensitivity was 2.9 pA/ms CPPG application.

We found that a 200-ns application of 1 mM CPPG was always sufficient to produce a saturating response (i.e., generate the maximum amount of $I_{\text{calc}}$). Over the 15-min course of our recordings, there was no time-dependent depression of the response to CPPG. This is to be expected if L-APB in the bath kept the channels closed in between puffs of CPPG and prevented Ca$^{2+}$ from entering the cell through the cation channel. A representative example is shown in Fig. 7A, top left. Dialysis with 10 μM CaMKII AIP produced variable effects on the maximum response to CPPG. In most cells, such as the one illustrated in Fig. 7A (middle), AIP produced a modest depression over a period of 15 min. In Fig. 7A, right, the distribution of effects of AIP is presented. In seven of eight cells, AIP depressed the response, while in one cell, a robust potentiation was observed. In four of eight cells, the depression was less than 20% of the initial response. Overall, there was a slight depression of the response to CPPG, compared with control cells but also a large amount of variability, as indicated by the size of the error bars (Fig. 7A, bottom).

Another group (Shiells and Falk 2000) has suggested that CaMKII decreases ON bipolar cell sensitivity to dim flashes of light in the all rod dogfish retina. We therefore used brief applications of CPPG to simulate the application of dim flashes. If CaMKII decreases response sensitivity in the salamander retina, then inhibitors of CaMKII, such as AIP, would be expected to potentiate responses to brief applications of CPPG. We therefore examined the effect of AIP on the linear range of the CPPG dose response curve. Figure 7B (top) shows the composite average response of eight cells to 5-, 7.5-, 10-, and 12.5-ms puffs of 1 mM CPPG. Traces were obtained during the first 1–3 min of recording and after 10 min of dialysis with 10 μM AIP. The peptide produced a slight depression of the response to brief puffs, but the mean sensitivity under these two conditions was nearly identical when the amplitude of individual responses was scaled to the maximum response obtained during the same time period (Fig. 7B, bottom). Our results show no effect of CaMKII on response sensitivity under these conditions, suggesting that this kinase is not involved in the regulation of adaptation to light flashes within the linear range. We also considered the possibility that AIP might have acted during the brief period of time before we initially measured response sensitivity, but close agreement in the sensitivity of cells treated with AIP (2.6 ± 0.25 pA/ms; n = 8) and control cells (2.7 ± 0.35 pA/ms; n = 9) would suggest that this is not the case.

In summary, under conditions that mimic the presentation of light to the retina (i.e., the rapid removal/antagonism of transmitter agonist from the synapse), inhibition of CaMKII with AIP had no discernible effect on brief applications of CPPG that mimic dim flashes of light.

**Discussion**

**Calcineurin versus CaMKII**

In darkness, ON bipolar cells are hyperpolarized by the synaptic release of glutamate from photoreceptor terminals. This unusual effect of glutamate is due to the unique expression, on the dendrites of ON bipolar cells, of a metabotropic glutamate receptor (mGluR6) that negatively couples to a cation channel. Our laboratory has previously demonstrated that this cation channel is permeable to Ca$^{2+}$ and that Ca$^{2+}$ influx through this channel initiates a negative feedback loop, resulting in a depression of the response to glutamate (Nawy 2000). Consequently, nearly continuous exposure to glutamate (Nawy 2000) or to the metabotropic receptor agonist L-APB (Fig. 7, this study) prevents depression of $I_{\text{ glu}}$ during the recording period because there is not sufficient Ca$^{2+}$ influx or accumulation to activate this feedback loop. Importantly, this finding is not an artifact of whole cell recording or our buffering of Ca$^{2+}$, as the amplitude of $I_{\text{ glu}}$ measured just after break-in is also dependent on the levels of agonist in the bath prior to establishing the recording. The mean amplitude of $I_{\text{ glu}}$ in experiments where L-APB was added to the bath solution was 58.5 ± 4.1 pA (n = 16), compared with 35.4 ± 3.0 pA.
(n = 27) in nominally agonist-free solution. However, in these experiments, glutamate was delivered to the cells through large diameter tubes (see METHODS), and this resulted in a significant accumulation of glutamate in the bath. When glutamate was delivered through a puffer pipette to avoid contamination of the bath with glutamate, the initial amplitude of $I_{\text{glu}}$ was significantly smaller than in nominally agonist-free solution (26.6 ± 3.3; n = 17; P < 0.02) or solution containing L-APB (P < 0.001)

Several lines of evidence support our hypothesis that the $\text{Ca}^{2+}$-regulated phosphatase calcineurin is a downstream effector of the $\text{Ca}^{2+}$-mediated negative feedback loop in ON bipolar cells of the salamander retina. Inhibitors of calcineurin prevented depression of $I_{\text{glu}}$ induced by $\text{Ca}^{2+}$ entry through the glutamate-regulated cation channel or by $\text{Ca}^{2+}$ added directly to the pipette solution. In addition, introduction of a $\text{Ca}^{2+}$-independent form of calcineurin through the pipette depressed $I_{\text{glu}}$ in the absence of $\text{Ca}^{2+}$. In support of our findings, there is recent immunological evidence that calcineurin is heavily expressed in both the inner and outer plexiform layer of the retina (Nakazawa et al. 2001). As the outer plexiform layer contains the dendrites of ON and OFF cells, as well as horizontal cells, double-labeling experiments will be necessary to confirm that the labeling observed in the outer plexiform layer is due to staining of ON bipolar cell dendrites. We also examined the potential role of CaMKII as a mediator of the effects of $\text{Ca}^{2+}$ on the mGluR6 pathway. Several recent reports suggest that inhibition of CaMKII decreases light-evoked responses in ON bipolar cells of the all-rod dogfish retina (Shiells and Falk 2000, 2001). We found that introduction of these same peptide inhibitors into salamander ON bipolar cells did not prevent $\text{Ca}^{2+}$-dependent depression of the response to exogenously applied glutamate. In fact, CaMKII inhibitors had the opposite action, reinstating depression in cells that were also dialyzed with inhibitors of calcineurin. In a second set of experiments, we added L-APB to the dissecting solution and external recording solution to keep the cation channels closed prior to recording. We then elicited responses to puffs of CPPG, an mGluR6 antagonist (Awatramani and Slaughter 2000), in much the same way that light can be used to suppress glutamate release from photoreceptors in a dark-adapted retina. Under these conditions, we observed weaker and more variable effects of CaMKII inhibitors. Because the influx of $\text{Ca}^{2+}$ through cation channels that are opened only briefly by CPPG is apparently insufficient to activate calcineurin, it seems unlikely that it is sufficient to substantially activate CaMKII, which requires higher levels of $\text{Ca}^{2+}$ for activation than does calcineurin (Klee 1991). Thus we did not find evidence that CaMKII potentiates $I_{\text{glu}}$ under conditions that mimic dark-adaptation, when the cation channels are mainly closed and $\text{Ca}^{2+}$ influx is minimal, or under light-adapted conditions, when the channels are largely open and influx of $\text{Ca}^{2+}$ is more substantial. An intriguing explanation for the apparent differences in mechanisms of $\text{Ca}^{2+}$ regulation of ON bipolar cells is that inhibition of CaMKII produced a modest depression of the overall response to CPPG but had no additional effect on sensitivity in the linear range.
bipolar cells in salamander and dogfish may be the type of input that bipolar cells receive in these two species. The retina of the dogfish is an all rod retina. In salamander, on the other hand, most types of ON bipolar cells receive a substantial cone and rod input, while very few are driven purely by rods (Wu et al. 2000).

**Does the calcineurin-mediated depression of \( I_{\text{glu}} \) result from a loss of \( I_{\text{cat}} \) or receptor-channel coupling?**

We cannot yet determine the precise target(s) of calcineurin, but our experiments with BAPTA and CaN420 provide some insight into this question. CaN420 lacks the regulatory calmodulin binding domain, and its requirement for \( Ca^{2+} \) is greatly reduced (Perrino et al. 1995). The addition of CaN420 in the presence of BAPTA allowed us to examine the effects of calcineurin without potential complications from other \( Ca^{2+} \)-mediated mechanisms. Introduction of CaN420 to the pipette solution resulted in a time-dependent depression of \( I_{\text{glu}} \). The depression of \( I_{\text{glu}} \) was associated with an upward shift in the baseline indicating a decrease in the amplitude of \( I_{\text{cat}} \). Therefore our results suggest that, at least in part, the effect of calcineurin on \( I_{\text{glu}} \) is due to a downregulation of \( I_{\text{cat}} \).

Our conclusion that \( I_{\text{cat}} \) is downregulated by calcineurin is inferred from changes in the baseline holding current. Clearly, changes over the same time period in other conductances contributing to the overall holding current would complicate the interpretation of the data. At \(-40 \) mV, the holding potential for most of the CaN420 experiments, there should be little contribution from voltage-gated \( K \) or \( L \)-type \( Ca^{2+} \) channels, or from the inward rectifier \( I_h \) (Burron and Lagnado 1997; Kaneko and Tachibana 1985; Lasater 1988). \( Ca^{2+} \)-activated \( K \) currents (\( I_{\text{KCa}} \)) might possibly contribute to the resting conductance, and a large conductance variety of \( I_{\text{KCa}} \) is known to be present in ON bipolar cells (Burron and Lagnado 1997). In addition, a \( Ca^{2+} \)-activated \( Cl^- \) conductance, has also been tentatively identified in ON bipolar cells (Protti et al. 2000). Our preliminary data suggest that charybdotoxin, a blocker of the large conductance \( I_{\text{KCa}} \), does not significantly affect changes in the baseline associated with depression of \( I_{\text{glu}} \). Furthermore we have shown previously that the change in baseline is accompanied by a conductance decrease that reverses near 0 mV (Nawy 2000), making it unlikely that a reduction in a \( Cl^- \) or \( K^- \) conductance could be responsible for this change. We therefore conclude that the shift in baseline observed in our experiments is not due to other conductances in the bipolar cell.

**Physiological significance of \( Ca^{2+} \)-dependent regulation of \( I_{\text{glu}} \) in ON bipolar cells**

In darkness, the release rate of glutamate from the presynaptic terminals of photoreceptors is high, and the cation current in ON bipolar cells is largely suppressed. This ensures that the input resistance of ON bipolar cells is high, preventing shunting of currents evoked by the opening of a few channels (Shiells and Falk 1994). As ambient illumination increases, the photoreceptor synaptic terminal hyperpolarizes, and the release of glutamate is decreased. This reduction in synaptic glutamate increases the size of the sustained cation current, lowering input resistance and depolarizing the ON bipolar cell. Results from this study suggest that the increased influx of \( Ca^{2+} \) through the open cation channels could provide a signal to close the channels, helping to restore the dark membrane potential and input resistance. Consistent with this view is a recent report showing that buffering \( Ca^{2+} \) with BAPTA reduces adaptive changes in the light response of ON bipolar cells (Shiells and Falk 1999). Regulation of the mGluR6 pathway by \( Ca^{2+} \) would provide the retina with an adaptive mechanism for coping with changes in ambient light, in addition to the photoreceptor-dependent mechanisms. Such forms of neural, photoreceptor-independent adaptation have been described previously (Dowling 1987), but their cellular mechanisms are not yet well understood.

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