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 cell response to small changes in the concentration of mGluR6 agonist. A novel role for calcineurin (CaN420), the calcium-dependent aminophenoxy-N,N,N,N-tetraacetic acid (BAPTA). However, when cells were dialyzed with BAPTA and a Ca2+ chelator, depression of the photocurrent was prevented. Similarly, CaN420 induced depression of NMDA glutamate release by Ca2+-independent form of calcineurin (CaN420), and neuronal activity to antibodies against Gα subunit 1088 (Nawy 1999). Also, mice lacking the Gα subunit do not have functional 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.

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.

In a recent study of 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...
All chemicals were obtained from Sigma (St. Louis, MO), except for °C, and dissolved in pipette solution immediately before use. Peptides were aliquoted, stored at Ballwin, MO), and the calcineurin inhibitory peptide 281 containing (in mM) 108 NaCl, 2 CaCl2 , 2.5 KCl, 1.2 MgCl2 , 10 glucose, 10 HEPES, 0.1 picrotoxin (pH 7.6 with NaOH). Solution was transfused to the recording chamber while remaining submerged, and the recording chamber was adjusted to 20° before use. Dilutions of 200 m M free Ca2+ were made from 20 mM BAPTA and by adding 3.87 mM HEPES, 10 glucose, and 0.1 picrotoxin (pH 7.6 with NaOH). Solution was perfused continuously through the recording chamber at a rate of 20 m l/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 M free Ca2+ (calculated with the program MAXCHELATOR) was made by replacing the EGTA with 1 mM BAPTA and by adding 3.87 mM Ca2+. For tight buffering of Ca2+, 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 inhibitory 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. Calcineurin inhibitor420, 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 m M glutamate was added. The tubes were mounted to a computer-controlled piezobimorph (Morgan-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 (600 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 Ca2+

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 as 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). The decay of 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 Ca2+ through the nearly continuously open cation channel (Nawy 2000). To fully activate this Ca2+-dependent depression, we supplemented endogenous Ca2+ with a pipette solution containing 1 M free Ca2+ (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 Ca2+ to the pipette solution in the present study.

Our results are consistent with a model in which Ca2+ effectively reduces the amplitude of the glutamate response by down-regulating the cation channels. An alternative model is that Ca2+ 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\(^{2+}\)-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\(^{2+}\)-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 Fig. 2A, top, which compares two responses to application of glutamate, obtained 1 and 15 min after beginning the recording. Overall, inclusion of cyclosporin A in the internal solution

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**Fig. 1.** Elevation of intracellular Ca\(^{2+}\) depresses glutamate-evoked current ($I_{\text{glu}}$) by inhibiting the underlying cation conductance. A: recording of an ON bipolar cell dialyzed with 1 μM free Ca\(^{2+}\). The record is a composite of 18 15-s epochs showing the response to a 5-s pulse of glutamate. Not shown is an additional 15-s interval between each epoch, during which glutamate was not applied. Dialysis with 1 μM Ca\(^{2+}\) resulted in a depression of the response to glutamate ($I_{\text{glu}}$) and a shift in the baseline (dashed line) that we attribute to a decrease in a constitutive cation current. Inset: comparison of the amplitude and baseline of the initial response to glutamate (thin line) and the last response (thick line). Scale is 10 pA and 2 s. B: summary of the time course of the glutamate response in cells dialyzed with 1 μM Ca\(^{2+}\) (filled symbols, $n = 7$), and in cells dialyzed with no added Ca\(^{2+}\) and 20 mM bis-(o-aminophenoxy)-N,N',N'-tetraacetic acid (BAPTA, open symbols, $n = 10$). Continuous line is an exponential fit with a time constant for 2.10 min.

**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 time points. The cell was dialyzed with cyclosporin A, which prevented depression of the glutamate response over the duration of the recording. Holding potential was −40 mV. Bottom: comparison of the time course of the mean ± SE of $I_{\text{glu}}$ amplitude in cells dialyzed with cyclosporin A (open symbols, $n = 23$), or gluconate and 0.1% DMSO (closed symbols, $n = 8$). C: summary of the time-dependent changes in $I_{\text{glu}}$ obtained with dialysis with an internal solution containing 1 mg/ml of calcineurin inhibitory peptide (CIP, open symbols, $n = 13$) or a peptide of similar size that does not inhibit calcineurin (closed symbols, $n = 6$).
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 (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 current 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.

**Ca\(^{2+}\)**-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 autacoidate-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 \(\mu\)M, 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 cation channel.

**FIG. 4.** Calcineurin can induce depression of \(I_{\text{glu}}\) when the cation channel is held closed; this normally prevents depression. A: glutamate application protocol used for this series of experiments. Glutamate was applied continuously through a flowpipe positioned near the cell. At 2-min intervals, cells were bathed with a glutamate-free solution in a second flowpipe for 5 s. B, top: example of the depression of the response to application of glutamate-free solution in a cell that was dialyzed with the standard internal solution. The amplitude of the response was essentially unchanged for the duration of the recording. Bottom: example of the depression of the response in a cell dialyzed with 100 nM CaN420. C: summary of the time-dependent depression of the glutamate response in cells recorded with normal internal solution (open symbols, \(n = 9\)) and a solution containing CaN420 (filled symbols, \(n = 5\)).

**FIG. 5.** An inhibitor of CaMKII does not prevent Ca\(^{2+}\)-dependent depression of \(I_{\text{glu}}\). A: dialysis of an ON bipolar cell with the CaMKII inhibitor autocamtide-2 related CaMKII inhibitory peptide (AIP, 10 \(\mu\)M) does not prevent depression of \(I_{\text{glu}}\) over a period of 15 min. Note the change in baseline associated with the decrease in the amplitude of \(I_{\text{glu}}\). Holding potential was −40 mV. B: summary of the effects on \(I_{\text{glu}}\) of dialysis with an internal solution containing 1 \(\mu\)M Ca\(^{2+}\) and AIP (closed symbols, \(n = 9\)). The pipette solution contained 1 \(\mu\)M Ca\(^{2+}\) to ensure that Ca\(^{2+}\) was sufficiently high to activate CaMKII. C: comparison of \(I_{\text{glu}}\) in cells dialyzed with the glucuronate-based internal solution and cyclosporin A (1 \(\mu\)M), and cyclosporin A along with the CaMKII inhibitor 281–309 (2 \(\mu\)M, \(n = 5\)). Amplitudes obtained after 15 min of recording were normalized to the initial amplitude for each cell, and the results were then pooled. Inhibition of CaMKII depressed \(I_{\text{glu}}\), suggesting that CaMKII may help to support \(I_{\text{glu}}\), but this effect was unmasked only during inhibition of calcineurin. The difference was significant at \(P < 0.05\) (unpaired t-test).
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. The family of response is plotted in Fig. 6B 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-ms application of 1 mM CPPG was always sufficient to produce a saturating response (i.e., generate the maximum amount of I_{max}). 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 found. The data in Fig. 7A, bottom, were fitted to the Hill equation with a K_{1/2} of 15.3 ms and a coefficient of 1.3.

The largest depression was 58.5 ± 4.1 pA (n = 16), compared with 35.4 ± 3.0 pA

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**FIG. 6.** Dose-dependence of focal application of the mGluR6 antagonist CPPG. A: responses to variable durations of puffs of 1 mM CPPG in an ON bipolar cell bathed in external solution containing 2 μM L-APB. Arrow indicates onset of CPPG application. Puff durations were 5–50 ms, in 2.5-ms increments, followed by a puff of 200 ms. Several of the responses to puffs between 12.5 and 45 ms have been omitted for clarity. Holding potential was −20 mV. B: plot of the data in A. Continuous line is the Hill equation with a K_{1/2} of 15.3 ms and a coefficient of 1.3. Responses to the 5- to 12.5-ms applications are replotted in the inset and fitted with a line of slope 2.9 (R^2 = 0.91), indicating that for brief applications, the response increased by 2.9 pA/ms CPPG application in this cell.
(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 Ca\(^{2+}\)-regulated phosphatase calcineurin is a downstream effector of the 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 Ca\(^{2+}\) entry through the glutamate-regulated cation channel or by Ca\(^{2+}\) added directly to the pipette solution. In addition, introduction of a Ca\(^{2+}\)-independent form of calcineurin through the pipette depressed \(I_{\text{glu}}\) in the absence of 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 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 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 dissection 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 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 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 Ca\(^{2+}\) influx is minimal, or under light-adapted conditions, when the channels are largely open and influx of Ca\(^{2+}\) is more substantial. An intriguing explanation for the apparent differences in mechanisms of 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.

**FIG. 7.** CaMKII inhibition has no effect on sensitivity. A, left: 2 responses to a 200-ms puff of 1 mM CPPG in an ON bipolar cell that was bathed in 2 \(\mu\)M L-APB and recorded with an internal solution that did not contain AIP. Black trace, obtained after 1 min of recording; gray trace, obtained 12 min later. Middle: responses from another cell under the same conditions except that the internal solution contained 10 \(\mu\)M AIP. Right: effect of AIP on depression of the response to 200-ms CPPG application in each individual cell. Bottom: summary of the effects of CaMKII inhibition on the maximum (200-ms puff) response to CPPG. B, left: composite of responses to CPPG applications of 5, 7.5, 10, and 12.5 ms at the times indicated. CPPG was applied once every 10 s, which, for these durations of applications, was sufficient for recovery to baseline. Five cells were dialyzed with internal solution that contained 10 \(\mu\)M AIP and 3 with solution that contained 20 \(\mu\)M AIP. Bottom: responses from individual cells were normalized to the 200-ms CPPG response obtained during the same time window. The results were then pooled and plotted. Slope of the data obtained early in the recording was 0.0245/ms CPPG application (\(R^2 = 0.99\)). Slope after 10–20 min was 0.0247/ms (\(R^2 = 0.92\)). We conclude 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 \( I_h \) (Burrone 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 (Burrone 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 effect 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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**REFERENCES**


