Nitric Oxide Depresses GABA_A Receptor Function via Coactivation of cGMP-Dependent Kinase and Phosphodiesterase

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Nitric oxide (NO) is thought to play an essential role in neuronal processing, but the downstream mechanisms of its action remain unclear. We report here that NO analogs reduce GABA-gated currents in cultured retinal amacrine cells via two distinct, but convergent, cGMP-dependent pathways. Either extracellular application of the NO-mimetic *S*-nitroso-*N*-acetylpenicillamine (SNAP) or intracellular perfusion with cGMP depressed GABA currents. This depression was partially blocked by a pseudosubstrate peptide inhibitor of cGMP-dependent protein kinase (PKG), suggesting both PKG-dependent and independent actions of cGMP. cAMP-dependent protein kinase (PKA) is known to enhance retinal GABA responses.

8-Bromoinosine 3',5'-cyclic monophosphate (8Br-cIMP), which activates a type of cGMP-stimulated phosphodiesterase that hydrolyzes cAMP, also significantly reduced GABA currents. 1-Methyl-3-isobutylxanthine (IBMX), a nonspecific phosphodiesterase (PDE) inhibitor, blocked both the action of 8Br-cIMP and the portion of SNAP-induced depression that was not blocked by PKG inhibition. Our results suggest that NO depresses retinal GABA_A receptor function by simultaneously upregulating PKG and downregulating PKA.

Key words: retina; amacrine; culture; nitric oxide; guanylate cyclase; ODQ; SNAP; PKG inhibitory peptide

Nitric oxide (NO) was first identified as a factor released by endothelial cells that relaxes vascular smooth muscle (Palmer et al., 1987). Subsequently, its synthetic enzyme, nitric oxide synthase (NOS/NADPH diaphorase), has been identified in almost every region of the CNS (Dawson et al., 1991). As a gas, NO is able to cross cell membranes freely, giving rise to the notion that NO might act as a retrograde messenger at synapses from which it is released and possibly at neighboring synapses as well.

One of the best-documented targets of NO is an intracellular soluble guanylate cyclase (sGC), the enzyme that synthesizes cGMP (Schmidt et al., 1993). cGMP can bind to at least three distinct classes of proteins. First, cGMP can gate channels directly. Cyclic nucleotide-gated channels have been found in cells in both the CNS and PNS (Fesenko et al., 1985; Nakamura and Gold, 1987; Nawy and Jahr, 1990; Goulding et al., 1992; Schmidt et al., 1993; Bourgeois and Rakic, 1996; Meissirel et al., 1997). Second, cGMP can activate cGMP-dependent protein kinase (PKG) (Scott, 1991). Finally, two subtypes of phosphodiesterases (PDE) are known to be regulated by cGMP. Type II PDE (GS-PDE) is stimulated by binding cGMP, whereas type III PDE (GI-PDE) is inactivated when cGMP is bound (Beavo et al., 1971a,b).

Cyclic nucleotides are well-established modulators of ion channels in retinal neurons. Elevated levels of cAMP decrease electrotonic coupling among both horizontal and amacrine cells (DeVries and Schwartz, 1989, 1992; Mills and Massey, 1995).

Similarly, cGMP uncouples teleost horizontal cells and reduces heterologous coupling between mammalian bipolar and AII amacrine cells (DeVries and Schwartz, 1989, 1992; Mills and Massey, 1995). Although both cAMP and cGMP reduce gap junction coupling, they seem to act antagonistically in regulating the activity of glutamate receptors. It has been shown that cAMP enhances glutamate-evoked currents in horizontal cells (Knapp and Dowling, 1987; Liman et al., 1989). In contrast, cGMP reduces such currents (McMahon and Ponomareva, 1996).

In retinal neurons, cAMP also enhances GABA-gated chloride currents (Feigenspan and Bormann, 1994; Veruki and Yeh, 1994). Despite the recognized importance of NO and the observation that cAMP and cGMP act on common targets, relatively little is known concerning cGMP-dependent modulation of GABA_A receptor function. However, studies in the nucleus tractus solitarius (Glaum and Miller, 1993, 1995), cerebellar granule cells (Robello et al., 1996), and hippocampus (Zarri et al., 1994) suggest that cGMP may downregulate GABA_A receptor function. Therefore, the present study was undertaken to examine how cGMP might modulate retinal GABA_A receptor function and to elucidate which enzymes mediate its effects.

MATERIALS AND METHODS

Cell culture. Retinas were removed from newborn Long-Evans-hooded rats after cryoanesthesia and were incubated for 45–60 min at 37°C in DMEM with HEPES (Mediatech, Washington, DC), supplemented with papain (Worthington, Freehold, NJ) at 6 units/ml and cysteine at 0.2 mg/ml. Papain was inactivated by replacing the enzyme solution with medium of the following composition: DMEM plus HEPES, 0.1% mito⁺ serum extender (Collaborative Research, Bedford, MA), 5% heat-inactivated fetal calf serum (HIFCS), 0.75% penicillin–streptomycin–glutamine mix (Life Technologies), and 7.5% sterile water to lower osmolarity. Retinas were triturated through a fire-polished Pasteur pipette, plated onto glass coverslips pretreated with poly-D-lysine (0.1 mg/ml), and maintained in medium supplemented with 15 mM KCl, 2 ng/ml basic fibroblast growth factor (bFGF; Life Technologies), and 100 ng/ml brain-derived neurotrophic factor (BDNF; Regeneron/Amgen).

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At 72 hr after plating, cells were treated with the antimitotics 5-fluoro-2-deoxyuridine (0.01 mg/ml) and uridine (0.026 mg/ml) for 24 hr. Subsequently, every 3rd day, 25% of the culture medium was exchanged for fresh medium. Cells were used for recording after 10–17 d *in vitro*.

Immunocytochemistry. Coverslips containing primary cultured neurons were washed three times with D-PBS, fixed in 4% paraformaldehyde for 15 min at room temperature and with 100% methanol at -10°C for 15 min, rinsed with Dulbecco's PBS (D-PBS), and then incubated overnight at 8°C with primary antibody. Anti-HPC-1 (Sigma, St. Louis, MO), anti-GABA (Chemicon, Temecula, CA), and anti-neurofilament 145 (NF145) (Chemicon) were diluted 1:100 in D-PBS, 10% fetal calf serum, and 0.5% Triton X-100. After 12–24 hr, coverslips were rinsed twice with D-PBS, incubated for 90 min in TRITC- or FITC-conjugated secondary antibody (Chemicon) diluted 1:100 at room temperature, rinsed twice in D-PBS and sterile water, and mounted onto glass slides using Prolong AntiFade (Molecular Probes, Eugene, OR). For labeling with anti-Thy1.1 IgM (gift of Dr. David Weinstein), live cells were incubated with primary antibody diluted 1:50 in normal culture medium for 60 min at 37°C , followed by rinsing twice in D-PBS and fixing with 4% paraformaldehyde for 5 min at room temperature. Labeling with secondary antibody was as detailed above. Cells were viewed with a Zeiss Axiovert 135 microscope equipped with a 100 W mercury lamp (HBO 100).

Electrophysiology. Recordings were made using an Axopatch 1-D amplifier, Digidata 1200 data acquisition board, and Axobasic software (Axon Instruments). Patch electrodes were pulled from standard hematocrit tubing (VWR Scientific) using a Narishige PP-83 vertical two-stage puller and were fire polished (Narishige MF-83) to a resistance of 2–3 M Ω . Recordings typically had series resistances of 10–20 M Ω , and those that exceeded 20 M Ω or varied by $>10\%$ during the experiment were discarded.

The standard extracellular solution contained 160 mM NaCl, 2 mM CaCl₂, and 5 mM HEPES. The gluconate-based intracellular solution was as follows: 110 mM potassium gluconate, 40 mM KCl, 10 mM EGTA, 5 mM HEPES, 2 mM Mg-ATP, and 250 μM Na-GTP, pH 7.3. Mannitol was added to adjust the osmolarity. The phosphate-based intracellular solution contained potassium phosphate monobasic (120–140 mM), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was stored at -20°C as a 10 mM stock in DMSO. 8-Bromo-cGMP (8Br-cGMP), 8-bromoinosine 3',5'-cyclic monophosphate (8Br-cIMP), and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were stored frozen (-20°C) as a dry powder and were made up each day in the standard extracellular solution. SNAP was prepared as an 11% (w/v) stock in DMSO (i.e., 1.1 mg of SNAP/10 μl of DMSO). Sodium nitroprusside (SNP) was made every 3–5 hr. Final DMSO concentrations never exceeded 0.1%. ATP, GTP, cAMP, cGMP, and cGMP-dependent protein kinase inhibitory peptide (GKIP; Arg-Lys-Arg-Ala-Arg-Lys-Glu; Peninsula Labs) were stored at -20°C as 1000 $\times$ stocks in distilled water. 1-Methyl-3-isobutylxanthine (IBMX) and microcystin (Research Biochemicals, Natick, MA) were stored at -20°C as 1000 $\times$ stocks in dry DMSO. Drugs were delivered through a pair of parallel, fused silica flow pipes (375 or 500 μm inner diameter; Polymicro Technologies). Each flow pipe was supplied by six separate reservoirs, each with its own control valve to feed fluid through a six-to-one tubing manifold (Warner Instruments). The flow pipe apparatus was under computer control and was driven by a piezo bimorph actuator (Morgan Matroc). This apparatus is an adaptation of that used by Lester et al. (1990). With the larger flow pipes, 10–90% whole-cell solution exchanges were achieved in <2.5 msec.

In experiments in which either cGMP or microcystin was included in the internal solution, the tip of the recording pipette was filled with control solution, and the shank was filled with either the cGMP- or microcystin-containing solution. This delayed the entrance of the test compound into the cell for several minutes, during which time E_{Cl} was observed to shift from -60 to -30 mV. GABA responses in cells held at -60 mV displayed a "run-up" in amplitude during this period of chloride shifting, a consequence of an increased chloride driving force. This run-up period was succeeded by 1–3 min of stable, "baseline" GABA responses. Time 0 was defined as the last point of this baseline period.

RESULTS

Identification of amacrine cells

Amacrine cells were distinguished from ganglion and horizontal cells in culture using both morphological and physiological criteria. Virtually all of the large multipolar cells >12 μm (280 $\pm$ 17 cells/mm²; n = 6 cultures) were immunoreactive for HPC-1, an amacrine cell marker (Barnstable et al., 1985). In contrast, $<1\%$

of all cells were labeled with medium weight (145 kDa) neurofilament (NF145), a cytoskeletal protein expressed by both horizontal cells and ganglion cell axons (Shaw et al., 1984). Moreover, only 5% (15 ± 4 ; n = 6) of large (>12 μm) neurons expressed Thy1.1, an antigenic marker for ganglion and displaced amacrine cells. Fewer than one fifth of these Thy1.1 positive cells (i.e., 1% of all cells) exhibited a robust, nonpunctate staining pattern typical of ganglion cells (Taschenberger and Grantyn, 1995). Taken together, these data suggest that nearly all large cells were amacrine and not ganglion or horizontal cells. This relative homogeneity of cell type may have been the result of maintaining the cultures in a medium of high osmolarity (340–385 mOsm), which has been reported to prevent ganglion cell survival (Meyer-Franke et al., 1995).

Figure 1*A* shows a typical cell that was immunoreactive for GABA, the neurotransmitter used by amacrine cells (Versaux-Botteri et al., 1989; Wu, 1992). Cells with this morphology had membrane potentials of -30 to -40 mV and rapidly accommodating action potentials, consistent with the physiological properties of amacrine cells (Taschenberger and Grantyn, 1995). Under whole-cell voltage clamp, all cells of the type shown in Figure 1*A* (7–14 d in culture) responded to GABA (Fig. 1*B*, *overhead bar*) with a desensitizing inward current that reversed at -30 mV, the predicted E_{Cl} , and that was blocked by 100 μM bicuculline, suggesting that these cells expressed primarily GABA_A receptors.

From the fit of the dose–response relation shown in Figure 1*C*, we obtained an EC_{50} of 40 ± 2 μM (n = 8) and a Hill coefficient of 1.8 ± 0.2 . Subsequent experiments were performed using 50 μM GABA, a concentration near the EC_{50} for these receptors. The Hill coefficient is in good agreement with the value of 1.9 obtained for GABA_A currents in amacrine cells from rat retinal slice (Feigenspan and Bormann, 1994). The EC_{50} for GABA in that study was found to be 72 μM , although agents that promote phosphorylation by cAMP-dependent protein kinase (PKA) shifted the EC_{50} to 45 μM (Feigenspan and Bormann, 1994), close to the value obtained in the present study.

NO agonists depress GABA currents

We examined the potential role of NO in regulating GABA_A receptor-gated currents using two NO analogs, SNP and SNAP, which activate soluble guanylate cyclase (Bohme et al., 1984; Schmidt et al., 1993). GABA was applied for 500 msec at intervals of 20 sec, a protocol that avoids cumulative desensitization associated with longer or more frequent GABA applications. When the control solution was switched to a solution containing 50 μM SNP, the GABA response was reversibly reduced. In four cells, SNP reduced GABA responses by $29 \pm 7\%$ (Fig. 2*A*).

SNAP also depressed GABA currents (Fig. 2*B*). Application of 50 μM SNAP produced a depression of $24 \pm 4\%$, whereas 100 μM SNAP depressed GABA currents by an average of $34 \pm 4\%$. One hundred micromolar SNAP seemed to be near saturating as a fivefold higher concentration produced little further increase in depression ($37 \pm 2\%$). DMSO (0.1%) alone did not produce any consistent reduction of GABA responses. Measurement of *I*–*V* relations indicated that depression of the GABA response by SNP or SNAP was not because of a shift in the reversal potential of the response (data not shown). With continuous application of either SNAP or SNP, recovery of the GABA response was often observed. The reason for this recovery is not clear.

If NO agonists depress GABA currents by activating sGC, then direct application of cGMP should similarly depress them. GABA currents were elicited as before, and cells were perfused

Figure 1. Primary culture of postnatal day 0 rat retinal amacrine cells 7–14 d *in vitro*. *A*, A cultured neuron exhibiting the characteristic amacrine cell morphology labeled with anti-GABA antiserum. Scale bar, 12 μm. *B*, Whole-cell recording of GABA responses from a representative amacrine cell held at -60 mV. The overhead bar shows the duration of GABA (35 μM) application. As is characteristic of GABA_A responses, GABA elicited a rapidly desensitizing inward current that was blocked by the coapplication of bicuculline methiodide (100 μM). *C*, Dose–response relation for the peak GABA currents elicited by 250 msec applications of varying concentrations of GABA. The averaged data were fit with the equation $I = I_{\max} \times (1/[1 + (EC_{50}/[GABA])^N])$, with an observed EC_{50} of 40 ± 2 μM ($n = 8$) and a Hill coefficient (N) of 1.8 ± 0.2 .

with the membrane-permeant cGMP analog 8Br-cGMP (Fig. 2C). In eight cells, 1 mM 8Br-cGMP decreased the amplitude of the GABA response by an average of $41 \pm 9\%$. Thus both cGMP itself and compounds that elevate intracellular cGMP depressed GABA responses.

To test whether SNAP reduced GABA currents by activating sGC, we first preincubated cells in 1 μM ODO, a specific inhibitor of sGC (Garthwaite et al., 1995). In the presence of ODO, there was a $20 \pm 2\%$ enhancement of the GABA currents, possibly because of inhibition of a basal guanylate cyclase activity and subsequent reduction in cGMP-mediated depression. Depression of GABA current by 8Br-cGMP was not significantly affected by ODO, as would be expected if raising intracellular levels of cGMP directly circumvents the need for guanylate cyclase activity. In the presence of ODO, 8Br-cGMP depressed the GABA response by $35 \pm 4\%$ ($n = 6$; Fig. 3A). ODO significantly reduced the depression of GABA current by SNAP at all three concentrations of SNAP that were tested (Fig. 3B). The efficacies of ODO against either a submaximal SNAP concentration (50 μM, 46%) or a near-saturating concentration (500 μM, 44%) were similar. ODO is reported to block 75% of SNAP-induced sGC activity, measured biochemically, (Garthwaite et al., 1995) compared with the 45% efficacy obtained in this study. The difference suggests that there may be an additional component of SNAP-induced depression of GABA currents in amacrine cells that is independent of sGC (Pozdnyakov et al., 1993; Brune et al., 1994; Zoche and Koch, 1995).

A component of cGMP-induced depression requires PKG

One target of cGMP is PKG. To determine whether PKG activation was responsible for depression of GABA currents, we blocked the activation of PKG in individual amacrine cells by including a pseudosubstrate inhibitory peptide (GKIP) in the intracellular patch pipette solution (Glass, 1983; Glass and Smith, 1983; McMahon and Ponomareva, 1996). The average size of GABA currents in cells internally perfused with 50 μM GKIP for 10 min (867 ± 208 pA) was not significantly different than that in

untreated cells (835 ± 136 pA). However, SNAP produced only an $18 \pm 2\%$ mean depression in six cells internally perfused with GKIP, approximately half of the SNAP-induced depression observed in control cells (Fig. 4).

Similar effects of GKIP were observed when cells were internally dialyzed with cGMP (Fig. 5). Twenty-five minutes after achieving whole-cell recording, GABA responses in cells perfused with 1 mM cGMP were depressed by $58 \pm 3\%$ ($n = 8$), compared with $10 \pm 1\%$ in the control solution ($n = 11$). In the presence of GKIP, cGMP decreased GABA-evoked currents by $35 \pm 10\%$ ($n = 8$) over the same period. Regardless of whether intracellular cGMP was elevated directly by addition to the pipette or indirectly with an analog of NO, inhibition of PKG blocked $\sim 50\%$ of the cGMP-induced depression of GABA currents.

Depression of GABA currents was also observed when phosphatase activity was inhibited either with a phosphate-based internal solution (Kennelly et al., 1993; Thiebart-Fassy and Hervagault, 1993; Weiner et al., 1993; Caselli et al., 1994; Gao and Fonda, 1994; Bernardi et al., 1995) or with 1 μM microcystin-LR, an inhibitor of type 1 and 2A phosphatases (Honkanen et al., 1990). Data are summarized in Figure 6A. Both phosphate ($39 \pm 2\%$; $n = 5$) and microcystin ($43 \pm 5\%$; $n = 12$) produced a significant depression of the GABA response compared with that seen in the control ($10 \pm 1\%$; $n = 11$).

Run-down of GABA currents during phosphatase inhibition might be expected if endogenous levels of cGMP are sufficiently high to activate PKG or if basal PKG activity is sufficient to phosphorylate targets when counteracting phosphatases are inhibited. Alternatively, run-down may be caused by other factors not related to PKG. To distinguish between these two possibilities, we included the PKG peptide inhibitor in the phosphate-based internal solution. Run-down of GABA currents because of high phosphate was almost completely blocked by inhibiting PKG (Fig. 6B). The observation that inhibition of PKG was sufficient to completely prevent depression of GABA currents in response to basal, but not stimulated, levels of cGMP prompted us to consider

Figure 2. Activators of soluble guanylate cyclase depress GABA responses. *A*, Single-cell record of the peak inward currents elicited by 250–500 msec applications of GABA (50 μ M) delivered every 20 sec is shown. The overhead bar indicates the timing of the application of 50 μ M SNP. The insets are sample traces recorded at the times indicated by the numbers. The internal solution contained phosphate and 10 μ M cAMP. SNP reversibly depressed the GABA response by 33% in this cell and by $29 \pm 7\%$ ($n = 4$) on average. *B*, SNAP (500 μ M) reversibly depressed the GABA response by 38% in this cell and by $37 \pm 2\%$ ($n = 6$) on average. *C*, 8Br-cGMP (1 mM) reversibly depressed the GABA response by 42% in this cell and by $41 \pm 9\%$ ($n = 8$) on average.

the possibility that cGMP could be acting on additional targets besides PKG.

A second component of cGMP-induced depression of GABA currents requires PDE activation

cGMP is known to activate a cGMP-stimulated cAMP-phosphodiesterase (GS-PDE), an effect that is independent of

Figure 3. SNAP depressed GABA_A currents via activation of soluble guanylate cyclase. *A*, ODQ does not block cGMP-mediated depression of the GABA response. Cells were internally dialyzed with control glutamate solution and externally perfused with ODQ (1 μ M). Application of ODQ potentiated the GABA_A currents by $20 \pm 2\%$ ($n = 6$). After a 5 min perfusion with ODQ alone, cells were perfused with a combination of ODQ and 8Br-cGMP (1 mM) (overhead bar). 8Br-cGMP depressed the GABA response by $35 \pm 4\%$ from the new, elevated baseline ($n = 6$). *B*, Summary of the dose-dependent depression of the peak GABA current by SNAP, an NO agonist, is shown. At the three concentrations tested, SNAP (500, 100, and 50 μ M) depressed the GABA-evoked current by $37 \pm 2\%$ ($n = 6$), $34 \pm 4\%$ ($n = 8$), and $24 \pm 4\%$ ($n = 5$), respectively, when applied to a naive cell (control, solid bar; mean $\pm$ SEM). Immediately after a 10 min preincubation with the sGC inhibitor ODQ (1 μ M; hatched bar), SNAP depressed the GABA-evoked currents by $16 \pm 5\%$ ($n = 3$), $17 \pm 4\%$ ($n = 8$), and $11 \pm 2\%$ ($n = 5$), respectively (* $p = 0.05$, unpaired two-tailed Student's t test, compared with control in SNAP alone).

PKG activation (Beavo et al., 1994). Because cAMP and its analogs have been shown to potentiate amacrine cell GABA responses (Feigenspan and Bormann, 1994), activation of GS-PDE by cGMP could indirectly depress GABA currents by hydrolyzing intracellular cAMP. cAMP is reported to be two orders of magnitude more potent at activating GS-PDE than at activating PKG (Miller et al., 1973). We therefore tested the possibility that selective activation of GS-PDE with cAMP might depress GABA currents.

Application of a cell permeant analog of cAMP, 8Br-cAMP (250

Figure 4. Inhibition of cGMP-dependent protein kinase partially blocks SNAP-induced depression. *A*, Inward currents elicited by 250 msec application of GABA (50 μ M; overhead bars) to either a control cell or one perfused with a pseudosubstrate peptide inhibitor of PKG (GKIP; 50 μ M). The three traces presented for each cell were recorded before (*Pre*), during (*SNAP*), or after (*Recovery*) application of SNAP (500 μ M). *B*, Time course of inhibition of GABA currents by 500 μ M SNAP (timing of application indicated by overhead bar) in five cells recorded with gluconate internal solution and in six cells recorded with solution containing GKIP. GKIP blocked approximately half of the SNAP-induced depression [GKIP, $18 \pm 1\%$ ($n = 6$); control, $37 \pm 2\%$ ($n = 5$)].

μ M), depressed GABA currents by an average of $18 \pm 6\%$ (Fig. 7*B*, hatched bar; $n = 6$). The inhibition of GABA currents by 8Br-cIMP in an individual cell is shown in Figure 7*A*. When cells were internally perfused with the phosphate-based internal solution and 10 μ M cAMP to ensure maximal PKA-dependent phosphorylation (Simmons and Hartzell, 1988), 8Br-cIMP depressed the peak GABA current by $37 \pm 3\%$ (Fig. 7*B*, left gray bar; $n = 5$). The tendency for the response to relax during prolonged 8Br-cIMP exposure has also been observed for hippocampal calcium currents under similar experimental conditions (Doerner and Alger, 1988). The depression induced by 8Br-cIMP was blocked by internal perfusion of the PDE inhibitor IBMX (Fig. 7*B*, right gray bar; $n = 3$). In a separate group of cells dialyzed with IBMX and the phosphate-based internal solution, the run-down of GABA_A currents over 20 min was $27 \pm 7\%$ ($n = 5$). This

Figure 5. GKIP partially blocks cGMP-induced depression. *A*, GABA-evoked currents immediately after establishment of recording and after 20 min are shown in three cells perfused with either control solution, 1 mM cGMP, or cGMP plus 50 μ M GKIP. Overhead bars indicate timing of GABA application. *B*, Inclusion of cGMP in the recording pipette caused a $58 \pm 3\%$ ($n = 8$) decline in the GABA response compared with a $10 \pm 1\%$ ($n = 11$) decline in control cells over the course of 25 min. When cells were dialyzed with cGMP plus GKIP, GABA responses declined by $35 \pm 10\%$ ($n = 8$) over the same time period, about one-half of the reduction observed in the presence of cGMP alone. For each cell, the amplitude of the peak GABA response was normalized to the initial amplitude. Normalized amplitudes were then averaged and binned at 2 min intervals. The points plotted are mean $\pm$ SEM of each bin.

difference was not statistically significant when compared with cells perfused with phosphate-based solution alone ($28 \pm 4\%$; $n = 5$). Our results suggest that amacrine cells may contain a PDE that is inhibited by IBMX and stimulated by cIMP, resulting in a depression of GABA currents.

Inhibition of both PKG and PDE blocked most of the SNAP-induced depression that was not blocked by PKG inhibition alone. We compared the depressant effects of 100 μ M SNAP when

Figure 6. Phosphatase inhibition unmasks PKG-dependent run-down of the GABA response. *A*, Summary of normalized GABA currents recorded after 30 min in cells internally perfused with control (gluconate), phosphate, or gluconate plus 1 μ M microcystin-LR. After 30 min, GABA responses recorded in control cells were reduced by $10 \pm 1\%$ ($n = 11$) compared with reductions of $39 \pm 2\%$ in high phosphate ($n = 5$) and $43 \pm 5\%$ ($n = 12$) in microcystin, consistent with the unmasking of a kinase-dependent mechanism (* $p = 0.05$, one-way ANOVA, compared with control). *B*, Averaged time course of six cells dialyzed with GKIP (50 μ M) plus high phosphate and five cells dialyzed with high phosphate alone. GKIP prevented run-down of the GABA response in high phosphate solution. GABA currents were reduced by $12 \pm 2\%$ in cells dialyzed with GKIP plus phosphate compared with $10 \pm 1\%$ in cells with gluconate.

GKIP alone was added to the internal solution with that observed when 1 mM IBMX was added together with GKIP. We used twice the concentration of GKIP (100 μ M) used in previous experiments to help insure that PKG was maximally blocked. The averaged time course and peak inhibition of SNAP under each condition are shown in Figure 8. SNAP depressed the GABA current to a lesser degree ($21 \pm 3\%$; $n = 15$) in cells perfused with GKIP than in control cells ($34 \pm 4\%$; $n = 10$). Moreover, internal perfusion with a combination of GKIP and IBMX in 14 cells further limited the SNAP-induced depression of the GABA response to $11 \pm 2\%$.

DISCUSSION

Our findings support the following NO-mediated cascade regulating GABA_A receptors on retinal amacrine cells. NO stimulates soluble guanylate cyclase, which raises the intracellular concentration of cGMP. cGMP increases PKG-mediated phosphoryla-

Figure 7. Activation of phosphodiesterase also suppresses GABA currents. *A*, Application of 8Br-cIMP (overhead bar) to a cell perfused with phosphate plus 10 μ M cAMP produced a rapid decline in the GABA-evoked current. All currents were normalized to the amplitude of the current just before the application of 8Br-cIMP. The insets are currents evoked by 250 msec applications of 50 μ M GABA to a representative cell before, during, and after the application of 8Br-cIMP, as indicated by the numbers. *B*, Application of the GS-PDE activator 8Br-cIMP (500 μ M) depressed GABA-evoked currents by 18 $\pm 6\%$ in cells perfused with gluconate ($n = 6$; hatched bar) and by 37 $\pm 3\%$ in cells perfused with phosphate plus cAMP ($n = 5$; left gray bar). In contrast, 8Br-cIMP did not diminish the GABA current in cells perfused with IBMX, consistent with the idea that 8Br-cIMP activated PDE ($n = 3$; right gray bar; $p = 0.05$, unpaired two-tailed Student's t test).

tion and concomitantly decreases PKA phosphorylation, via stimulation of GS-PDE. These events act synergistically to depress GABA_A receptor-gated currents. With continuous application of NO agonists, recovery of the GABA response was often observed.

Of the seven known forms of PDE, two are regulated by cGMP. Although both catalyze the hydrolysis of cAMP, the type III form is inhibited by cGMP, whereas the type II form (GS-PDE) is stimulated by cGMP (Beavo et al., 1971a,b). There is precedence for a GS-PDE indirectly modulating ion channel function. Calcium currents (I_{Ca}) in cardiac myocytes (Hartzell and Fischmeister, 1986) and hippocampal CA1 pyramidal cells (Doerner and Alger, 1988) are potentiated by PKA phosphorylation, yet they are depressed by cGMP via PKG-independent activation of a GS-PDE. In these cells, cGMP stimulation of GS-PDE lowers intracellular cAMP, subsequently reducing PKA activity.

Figure 8. Inhibition of phosphodiesterase attenuates PKG-independent depression. *A*, Averaged time course of SNAP-induced depression of GABA currents in cells dialyzed with control (gluconate) internal solution, GKIP (100 μ M), or GKIP plus 1 mM IBMX. Application of 100 μ M SNAP is indicated by the overhead bar. *B*, Summary of results from *A*. SNAP depressed GABA currents by $34 \pm 4\%$ in control cells ($n = 10$), by $21 \pm 1\%$ in cells dialyzed with GKIP ($n = 15$), and by $11 \pm 1\%$ in cells dialyzed with GKIP plus IBMX ($n = 14$). The single asterisk indicates $p = 0.05$ compared with control. The double asterisks indicate $p = 0.05$ compared with both control plus GKIP; $p = 0.05$; one-way ANOVA.

Two lines of evidence support our contention that regulation of amacrine cell GABA-gated currents by GS-PDE may be analogous to regulation of calcium currents in hippocampal pyramidal cells and cardiac myocytes. First, the application of 8Br-cGMP, a selective activator of GS-PDE, produced a marked depression of GABA currents. This effect was blocked by the broad-spectrum PDE inhibitor IBMX, consistent with an action on GS-PDE (Fig. 7). Second, intracellular dialysis with IBMX also blocked a substantial fraction of the SNAP-induced depression that was not blocked by PKG inhibition alone (Fig. 8). However, because neither inhibition of sGC nor the combined inhibition of PKG and PDE completely blocked SNAP-induced depression of GABA currents, it is possible that SNAP might also inhibit GABA currents via a third mechanism that is completely independent of cGMP, perhaps by acting directly on the channel, as has been demonstrated for olfactory cAMP-gated channels (Broillet and Firestein, 1996).

When the phosphorylation-dependent component of the de-

pression was pharmacologically isolated by inhibiting phosphatases, GABA currents ran down with time. Because there was no source of exogenous cGMP in this experiment, there must have been sufficient active PKG to produce a net increase in phosphorylation while phosphatases were inhibited. In phosphate, run-down of I_{GABA} was slow, ~ 1.5 pA/min, and this may reflect a very low level of basal PKG activity. Our data also suggest that, in the absence of cGMP stimulation, GS-PDE is inactive, because inhibition of PKG by GKIP was sufficient to block nearly all of the run-down. One possible explanation for this finding is that higher concentrations of cGMP are needed to activate GS-PDE than PKG. Biochemical studies have suggested that PKG can be activated by concentrations of cGMP that are between 10 and 100 times lower than those that are required to activate GS-PDE (Martins et al., 1982; for PDE review, see Butt et al., 1993). Addition of extracellular cGMP (Fig. 4) or SNAP (Fig. 4) produced a depression of GABA currents that was only partially blocked by inhibition of PKG, presumably because the intracellular levels of cGMP were increased sufficiently to activate both GS-PDE and PKG. It seems unlikely that the partial block of SNAP-induced I_{GABA} is attributable to incomplete PKG inhibition by GKIP, because doubling the concentration of the peptide inhibitor from 50 to 100 μ M did not decrease the suppression of GABA currents by SNAP. Second, GKIP is a pseudosubstrate inhibitor, acting at the PKG catalytic site rather than at the regulatory site. Thus, it is not competitively antagonized by increasing intracellular cGMP.

Several reports have defined a mechanism by which activators of adenylate cyclase, such as dopamine, enhance GABA_A currents in mammalian retina (Veruki and Yeh, 1992, 1994; Feigenspan and Bormann, 1994). The present study suggests that activators of guanylate cyclase, such as NO, do just the opposite. The enzymes that synthesize dopamine and NO are both located in subpopulations of GABAergic amacrine cells (Wassle and Chun, 1988; Vaney and Young, 1988; Darius et al., 1995). Thus, GABAergic transmission in the inner retina may be modulated in a push-pull manner by activators of adenylate cyclase such as dopamine or vasoactive intestinal peptide on the one hand and by NO on the other.

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