Cellular/Molecular

Presynaptic Inhibition by α2 Receptor/Adenylate Cyclase/PDE4 Complex at Retinal Rod Bipolar Synapse

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G-protein-coupled receptor (GPCR)-mediated presynaptic inhibition is a fundamental mechanism regulating synaptic transmission in the CNS. The classical GPCR-mediated presynaptic inhibition in the CNS is produced by direct interactions between the $G_{\beta\gamma}$ subunits of the G-protein and presynaptic Ca^{2+} channels, K^+ channels, or synaptic proteins that affect transmitter release. This mode of action is shared by well known GPCRs such as the α2, $GABA_B$, and CB_1 receptors. We report that the α2 receptor-mediated inhibition of presynaptic Ca^{2+} channel and transmitter release in rat retinal rod bipolar cells depends on the G_α subunit via a G_α–adenylate cyclase– cAMP cascade and requires participation of the type 4 phosphodiesterase (PDE4), a new role for phosphodiesterase in neural signaling. By using the G_α instead of the $G_{\beta\gamma}$ subunits, this mechanism is able to use a cyclase/PDE enzyme pair to dynamically control a cyclic nucleotide second messenger (i.e., cAMP) for the regulation of synaptic transmission, an operating strategy that shows remarkable similarity to that of dynamic control of cGMP and transmitter release from photoreceptors by the guanylate cyclase/PDE6 pair in phototransduction. Our results demonstrate a new paradigm of GPCR-mediated presynaptic inhibition in the CNS and add a new regulatory mechanism at a critical presynaptic site in the visual pathway that controls the transmission of scotopic information. They also provide a presynaptic mechanism that could contribute to neuroprotection of retinal ganglion cells by α2 agonists, such as brimonidine, in animal models of glaucoma and retinal ischemia and in glaucoma patients.

Key words: **α2 receptor; Ca^{2+} channel; GPCR; PDE4; presynaptic inhibition**

Introduction

The α2 receptor is a well characterized G-protein-coupled receptor (GPCR) that regulates the release of neurotransmitters through presynaptic inhibition in the CNS (Scheibner et al., 2001; Bücheler et al., 2002; Delaney et al., 2007). The inhibition is accomplished by direct interactions between the $G_{\beta\gamma}$ subunits and presynaptic Ca^{2+} or K^+ channels, or synaptic proteins that affect neurotransmitter release (Starke, 2001; Delaney et al., 2007). This classic mode of presynaptic inhibition is shared by other well known GPCRs such as the A_1, $GABA_B$, and CB_1 receptors, which are widely involved in the regulation of neurotransmitter release in the CNS (Miller, 1998; Stephens, 2009).

The α2 receptor itself is also a drug target. For example, brimonidine (an α2 agonist)-based eyedrops are widely used to lower the intraocular pressure (IOP) of glaucoma patients. In clinical studies, brimonidine also preserves visual function in glaucoma patients, which appears to be independent of its well known IOP-lowering action (Evans et al., 2003; Krupin et al., 2011) and is interpreted to be a result of neuroprotection (Evans et al., 2003). Indeed, brimonidine protects retinal ganglion cells (RGCs) in animal models of glaucoma and retinal ischemia independent of IOP lowering (Donello et al., 2001; Dong et al., 2008). However, how brimonidine protects RGCs is incompletely understood. We previously reported that the modulation of NMDA receptor activity in RGCs by brimonidine is an important mechanism of neuroprotection in animal models of glaucoma (Dong et al., 2008). In view of its well known role in presynaptic regulation of transmitter release in the brain, we hypothesized that a similar α2 modulation of glutamate release from retinal bipolar cells may be another important mechanism for brimonidine, which is expected to limit overactivation of the NMDA receptor in RGCs under pathological conditions.

Bipolar cells are second-order neurons in the retina (Wu, 2010). Their presynaptic terminals are critically involved in spatial and temporal processing of visual information (Dong and Hare, 2003; Shields and Lukasiewicz, 2003), which is largely accomplished by the regulation of glutamate release through presynaptic ionotropic inhibitory mechanisms that involve $GABA_A$, $GABA_C$, glycine receptors, and a glutamate transporter (Veruki et al., 2006; Eggers and Lukasiewicz, 2011). Bipolar cells, particularly rod bipolar cells (RBCs), are one of the most extensively studied CNS glutamatergic relay neurons. Surprisingly, information on the modulation of synaptic transmission by GPCRs at this critical presynaptic site in the visual pathway is sparse. Specifically, it is unknown whether the α2 receptor is involved in presynaptic modulation in bipolar cells, or whether bipolar cells actually express the α2 receptor.

To further understand α2 signaling in the retina and to test our hypothesis, we examined the role of the α2 receptor in the

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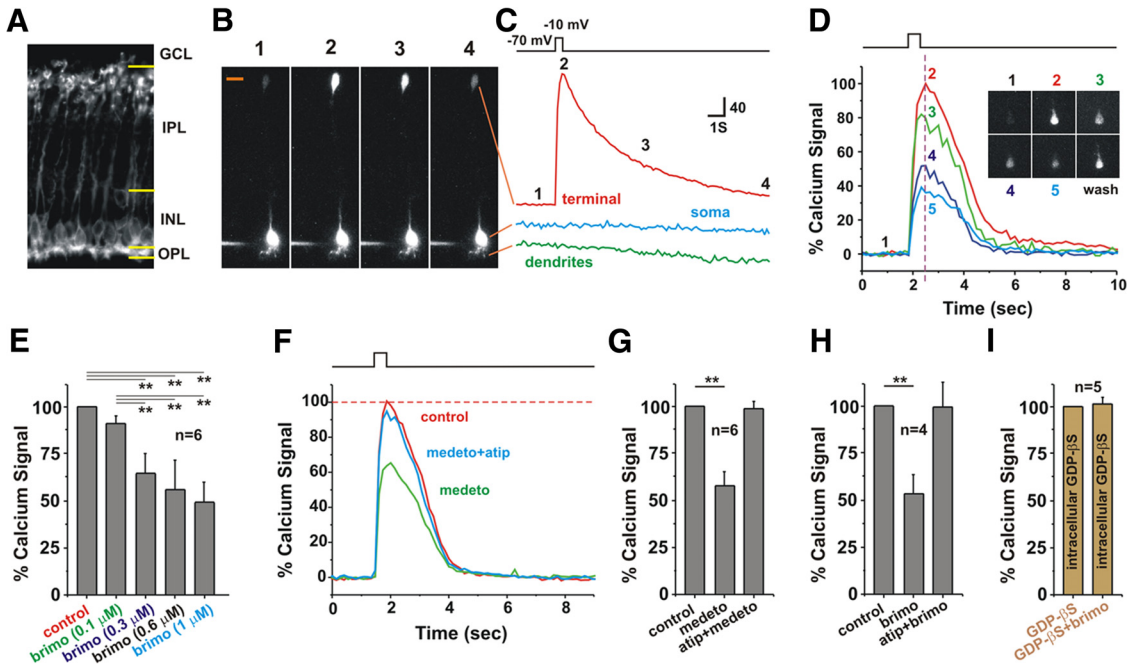

Figure 1. α2 Modulation of presynaptic Ca^{2+} signals at RBC synaptic terminals. ***A***, Anti-PKCα antibody-labeled RBCs in a cross-section of the rat retina. OPL, Outer plexiform layer; GCL, ganglion cell layer. ***B***, Confocal images (from a single focal plane) of a Fluo-4 filled *in situ* RBC taken at four different time points (corresponding to the time points marked on the traces in ***C***) before and after a 0.5 s depolarization pulse. In this RBC, the dendrites, soma, and a part of the axon happened to be visible at the same focal plane with a synaptic bouton. Scale bar, 5 μm. ***C***, Changes of Ca^{2+} signals (arbitrary units) measured at the axon terminal, soma, and dendrites of the RBC shown in ***B***. ***D***, Normalized presynaptic Ca^{2+} signals (measured at the peak) from a representative RBC synaptic bouton (inset) under control condition (red) and in the presence of 0.1 (green), 0.3 (navy blue), and 1 (blue) μM brimonidine. ***E***, The dose–response relation of the effect of brimonidine on presynaptic Ca^{2+} signals. ***F***, Medetomidine (medeto; 0.3 μM) mimics the effect of brimonidine. ***G***, ***H***, Atipamezole (atip; 5 μM) blocks the effect of both medetomidine (0.3 μM) and brimonidine (0.6 μM). ***I***, Intracellular GDP-βS (0.5 mM) abolishes the effect of brimonidine. In this and all following figures: $^*p < 0.05$, $^{**}p < 0.01$.

modulation of bipolar cell synaptic output. We report a new mode of α2 receptor-mediated presynaptic inhibition at rod bipolar cell synaptic terminals. This mode, in contrast to its classical mode of $G_{\beta\gamma}$-dependent signaling in the brain, works through the G_α cascade, and requires participation of the adenylate cyclase (AC) and type 4 phosphodiesterase (PDE4) enzyme pair. Its basic operating strategy is remarkably similar to that used by rhodopsin in phototransduction.

Materials and Methods

Preparations. Live retinal slices (for imaging presynaptic Ca^{2+} signals and whole-cell recording depolarization-induced Ca^{2+} currents in RBCs) and flat-mount isolated retinae (for imaging synaptic vesicle recycling with FM 1–43 dye) were prepared from male adult Brown Norway rats (body weight, 350–400 g). The protocols used were approved by an institutional animal care and use committee. Briefly, Brown Norway rats were deeply anesthetized using intramuscular injection of ketamine (75 mg/kg) and xylazine (10 mg/kg). Following enucleation of both eyes, animals were killed immediately with intracardial injection of Eutha-6 (120 mg/kg). Retinae were carefully isolated, and a small piece of retina (~2 × 6 mm) was placed, with the photoreceptor side down, in a dye-loading chamber (flat-mount retina). For retinal slices, the isolated retina was placed, with the vitreal side down, onto a piece of black filter paper (catalog #habp04700, Millipore). The retina and filter paper was then sliced at ~350–400 μm intervals. Retinal slices were carefully transferred to recording chambers and were securely positioned, after a 90 degree turn, by placing the filter paper to which the retinal slices were attached on two tracks of vacuum grease, so that all retinal layers, including the inner nuclear layer (INL) and the inner plexiform layer (IPL), could be viewed and imaged with a fixed-stage microscope (BX50WI, Olympus America).

Solutions. The rat Ringer's solution contained the following (in mM): 120 NaCl, 3 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 0.5 KH_2PO_4, 10 glucose, and 26 $NaHCO_3$. The Ringer's solution was bubbled continuously with 95% O_2 and 5% CO_2. The high-K^+ Ringer's solution was prepared by replacing 77 or 37 mM NaCl in the normal rat Ringer's solution with equimolar KCl, yielding a total K^+ concentration of 80 or 40 mM (for loading and unloading FM 1–43 dye in transmitter release experiments, respectively). The Ca^{2+}-free Ringer's solution was prepared by replacing $CaCl_2$ with equimolar EGTA. For Ca^{2+} imaging, the normal intracellular (patch pipette) solution contained the following (in mM): 125 $CsCH_3SO_3$, 1 $MgCl_2$, 15 TEA · Cl, 10 HEPES, 4 ATP-Mg, 0.5 GTP-Na_3, 12 phosphocreatine, and 0.1 Fluo-4 pentapotassium salt. For recording whole-cell Ca^{2+} currents, 0.1 mM Alexa Fluor 488 (replacing Fluo-4 pentapotassium salt) was added to the intracellular solution to confirm the identity of the recorded cells. In experiments in which G-protein blockade was needed, GTP-Na_3 was replaced by equimolar GDP-βS. In some experiments, tool agents, such as forskolin, Sp-cAMPS, rolipram, Ro 20–1724, and piclamilast, were also applied intracellularly by adding to the intracellular solutions.

Tool agents and their administration. Tool agents were all purchased from Sigma-Aldrich or Tocris Bioscience, except for Alexa Fluor 488 and FM 1–43, which were purchased from Invitrogen. The $G_{\beta\gamma}$-inhibiting peptide and small-molecule $G_{\beta\gamma}$ inhibitor gallein were purchased from AnaSpec (catalog #24178) and Tocris Bioscience, respectively. The agents were delivered by either multichannel bath perfusion or local perfusion systems, in combination with intracellular delivery through the whole-cell electrode.

Whole-cell recording, calcium imaging, and imaging exocytosis at RBC synaptic terminals. Conventional methods for whole-cell voltage-clamp were used. *In situ* RBCs were recorded with patch electrodes (8–10 MΩ) pulled from borosilicate glass pipettes (catalog #BF-150-86-10, Sutter Instrument) with a P87 flaming/Brown Micropipette Puller (Sutter Instrument). The current signals were amplified, low-pass filtered at 1 kHz with an Axopatch 200B amplifier, digitized at 5 kHz with a Digidata 1440A digitizer, acquired, and analyzed with pCLMP10 data acquisition software (Molecular Devices). The serial resistance was not compensated for. Ca^{2+} imaging was performed with a high-speed Yokogawa QLC-100

spinning disc confocal system (Solamere Technology Group) mounted on a fixed-stage upright microscope (BX51WI, Olympus America). The Ca^{2+} indicator (100 μM Fluo-4 pentapotassium salt) was delivered to the RBC cytosol directly via patch electrodes. Images were acquired and analyzed with QED (MediaCybernetics) and ImageJ software (National Institutes of Health). For imaging depolarization-induced exocytosis of synaptic vesicles from RBC terminals, the synaptic vesicles were first labeled by FM 1–43 dye through depolarization-induced endocytosis. Flat-mount isolated rat retinae were incubated for 10–15 min at room temperature in 80 mM high K^+ rat Ringer's solution containing 10 μM FM 1–43, 2 μM strychnine, 50 μM (TPMPA, 1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid, 10 μM SR95531, 50 μM D-AP5, 20 μM CNQX, and 50 μM DL-APB. In some experiments, high K^+ was replaced by 1 mM (*RS*)-α-cyclopropyl-4-phosphonophenylglycine (CPPG) as a depolarizing agent. While similar results were obtained, a high-K^+ Ringer's solution produced more consistent results. The depolarization step was followed with a repolarization (hyperpolarization) step by incubating in normal Ringer's solution (3 mM K^+) that contained 10 μM FM 1–43 and 100 μM DL-APB for 10 min to complete the endocytosis process (FM 1–43 loading). Thereafter, extracellular FM 1–43 was washed out with normal Ringer's solution containing 1 mM Advasep-7 and 100 μM DL-APB for 15 min. The retina was then transferred to a recording chamber and superfused with normal Ringer's solution containing various test agents, which are described in the legend of Figure 5. The FM 1–43 dye was excited with a 488 nm laser, and RBC images were collected using a 40× water-immersion objective (numerical aperture, 0.80; LUMPlanFI, Olympus America). We took a stack of 20 consecutive confocal images every minute for 12 min on the *z*-axis, which covered a depth of 16 μm in the inner most part of the ON-sublamina in the IPL (see Fig. 5*A*, between the two green lines), where RBC synaptic boutons are found. Maximum projection confocal images were produced based on these 20 image stacks (see Fig. 5*B*) and were used in analyzing the kinetics of exocytosis of FM 1–43-labeled synaptic vesicles (transmitter release) under various experimental conditions (see Fig. 5).

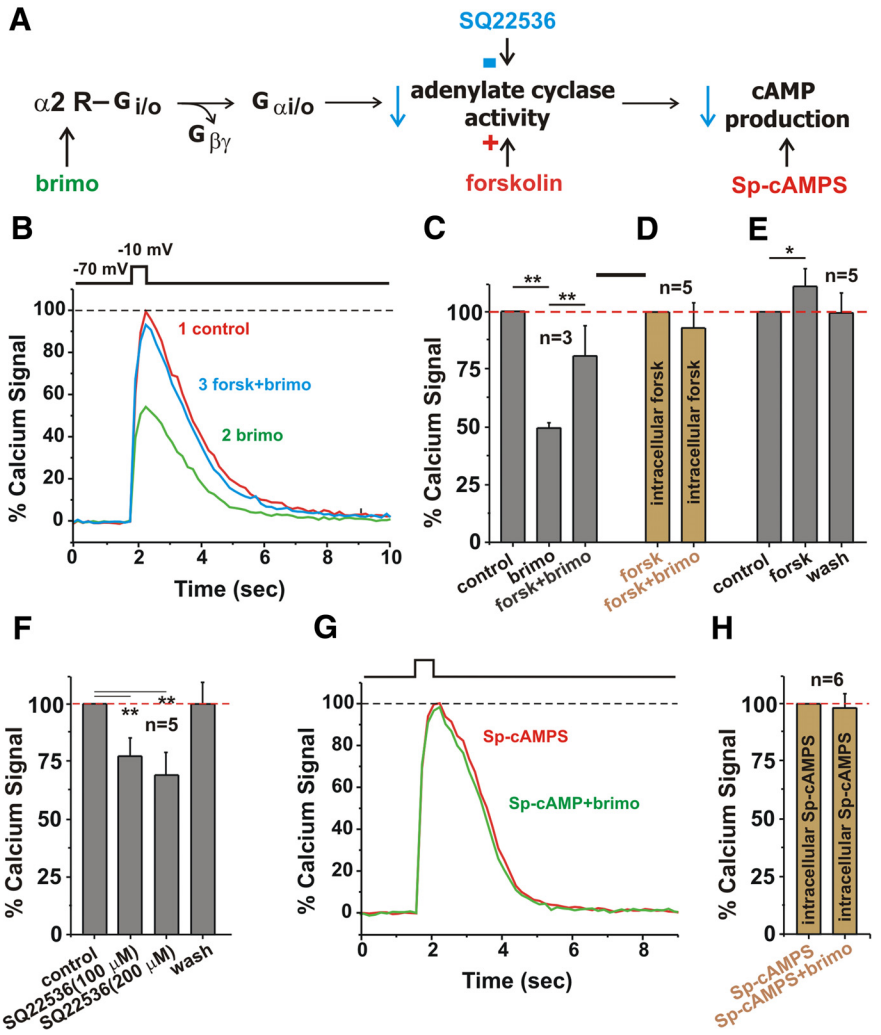

Figure 2. α2 Modulation of presynaptic Ca^{2+} signal through the $G_{\alpha i/o}$–AC– cAMP pathway. ***A***, The $G_{\alpha i/o}$–AC– cAMP pathway and the site of action of the tool agents. ***B–D***, The effect of brimonidine is blocked by both extracellular and intracellular forskolin (forsk; 10 and 5 μM, respectively). ***E***, The effect of extracellular forskolin alone reversibly produces a small, but significant ($p < 0.05$), enhancement of the presynaptic Ca^{2+} signal. ***F***, SQ22536 mimics the effect of brimonidine on the Ca^{2+} signal. ***G***, ***H***, Intracellular Sp-cAMPS blocks the effect of brimonidine.

Immunohistochemistry. Isolated rat eyecups were fixed in 4% paraformaldehyde/PBS for 1 h at room temperature. Eyes were rinsed with PBS three times and placed in 30% sucrose/PBS overnight at 4°C. Eyecups were then embedded in mounting media and frozen in liquid nitrogen. Frozen eyecups were cut into 10-μm-thick cross-sections and mounted on glass slides. Cross-sections were allowed to dry at room temperature for half an hour before storage at −80°C. Before use, frozen sections were thawed and dried at room temperature for 1 h, then rehydrated in PBS and permeabilized in 0.2% Triton X-100/PBS at room temperature for 15 min. Tissue sections were then blocked with 5% goat normal serum in 0.1% Triton X-100/PBS, followed by incubation with a primary antibody cocktail containing anti-PKCα and subtype-specific PDE4 antibodies overnight at 4°C (1:250 rabbit anti-PDE4A, Abcam; 1:250 rabbit anti-PED4B, Abcam; 1:250 rabbit anti-PDE4C, Abcam; 1:250 rabbit anti-PDE4D, FabGennix; and 1:1000 mouse anti-PKCα, Millipore). After overnight incubation, sections were washed three times at 5 min intervals with 0.1% Tween-20/PBS. A secondary antibody cocktail [a mixture of 1:250 Alexa Fluor 488 anti-mouse IgG for PKCα, Invitrogen; and 1:250 Alexa Fluor 594 goat anti-rabbit IgG for PDE4 subtypes] diluted in blocking buffer was added to the sections, followed by washing six times at 5 min intervals with 0.1% Tween-20/PBS. Slides were then coverslipped using ProLong Gold antifade reagent (Invitrogen). The omission of primary antibodies or preabsorbed antibodies was used as a negative control. Images were obtained using a Zeiss Axio Imager M2 microscope with the aid of AxioVision version 4.8.2 software.

Statistical analysis. Datasets before and after brimonidine treatment were compared by two-tailed paired *t* test. Datasets of different treatments were compared either by one-way ANOVA followed by a *post hoc* means comparison with Tukey test (for three or more datasets) or by unpaired *t* test (for two datasets). Statistical analysis was performed with Origin version 7.0383 (OriginLab). A *p* value of <0.05 was considered to be significant. Data are expressed as the mean ± SD.

Results

α2 Modulation of presynaptic Ca^{2+} channel activity at rod bipolar cell terminals

We investigated the role of the α2 receptor in the modulation of transmitter release from the synaptic terminal of *in situ* RBCs in acutely prepared rat retinal slices. Figure 1*A* shows anti-PKCα (a specific RBC marker) antibody-labeled RBCs in a retinal cross-section. In RBCs filled intracellularly with Fluo-4 Ca^{2+} indicator, a 0.5 s depolarization of RBCs from a holding potential of −70 to

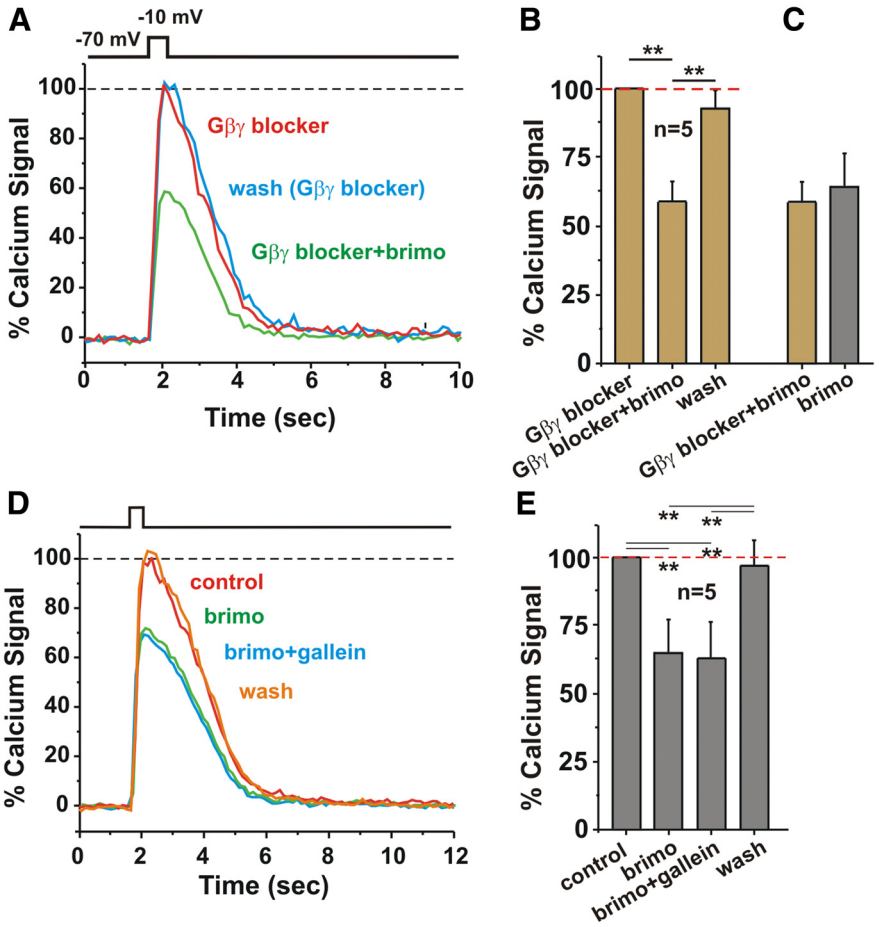

Figure 3. Specific $G_{\beta\gamma}$ blockers fail to alter the effect of brimonidine. ***A***, ***B***, The effect of intracellularly applied $G_{\beta\gamma}$ blocking peptide (1 μM) on the modulation of the presynaptic Ca^{2+} signal by brimonidine. ***C***, The $G_{\beta\gamma}$ blocking peptide has no effect on the modulation of the presynaptic Ca^{2+} signal by brimonidine. The two datasets compared are from ***B*** and ***E***, which were obtained in the presence and absence of 1 μM intracellular $G_{\beta\gamma}$ blocking peptide, respectively. ***D***, ***E***, Extracellularly applied gallein (a small-molecule $G_{\beta\gamma}$ blocker; 20 μM) has no effect on the modulation of the presynaptic Ca^{2+} signal brimonidine.

−10 mV with a whole-cell patch electrode elicited a robust Ca^{2+} signal exclusively at the presynaptic terminal (Fig. 1*B*,*C*), indicating that voltage-gated Ca^{2+} channels are predominantly expressed at the axon terminal in RBCs (Protti and Llano, 1998; Hartveit, 1999). This Ca^{2+} signal was blocked by nimodipine, a selective L-type Ca^{2+} channel blocker, and was abolished by perfusion with Ca^{2+}-free Ringer's solution (data not shown), indicating that the signal is produced by Ca^{2+} influx through the voltage-gated L-type Ca^{2+} channels located at RBC synaptic terminals.

The administration of brimonidine, a selective α2 receptor agonist, effectively inhibited the presynaptic Ca^{2+} signal (Fig. 1*D*,*E*). The effect of brimonidine was mimicked by medetomidine (Fig. 1*F*,*G*), another selective α2 agonist. Atipamezole, a highly selective α2 antagonist, blocked the action of both α2 agonists (Fig. 1*G*,*H*). The effect of brimonidine was also blocked by intracellularly applied GDP-βS, a G-protein inhibitor (Fig. 1*I*), indicating that brimonidine acted on α2 receptors in RBCs (Figs. 2*D*,*G*,*H*, 4*E*–*H*, 5*D*,*E*).

α2 Modulation of presynaptic L-type Ca^{2+} channel via the G_α–AC–cAMP pathway

The α2-mediated presynaptic inhibition of neurotransmitter release in the CNS signals through the $G_{\beta\gamma}$ subunits and is accomplished by direct interactions between the $G_{\beta\gamma}$ subunits and presynaptic Ca^{2+} channels, K^+ channels, or synaptic proteins (Starke, 2001; Delaney et al., 2007; Gilsbach and Hein, 2008). The α2 receptor can signal through either the G_α or $G_{\beta\gamma}$ subunit of the G-protein. The G_α subunit coupled to the α2 receptor is typically a $G_{\alpha i}$ or $G_{\alpha o}$ that usually causes inhibition of adenylate cyclase, leading to a reduced production of cAMP (Limbird, 1988). We found that α2 modulation of the presynaptic Ca^{2+} signal in RBCs is mediated by the G_α subunit via inhibition of adenylate cyclase (Fig. 2*A*). The inhibitory effect of brimonidine on the presynaptic Ca^{2+} signal was largely overridden by extracellular forskolin, an AC activator (Fig. 2*B*,*C*) and completely abolished by intracellular forskolin (applied through the patch electrode; Fig. 2*D*). Applying the same extracellular concentration of forskolin alone produced only a small enhancement of the presynaptic Ca^{2+} signal (Fig. 2*E*), suggesting a nearly maximal resting AC activity that is not very sensitive to further stimulation by forskolin. The small enhancement of the presynaptic Ca^{2+} signal is presumably produced by a small elevation in cAMP level due to forskolin action and is consistent with a positive role of cAMP in maintaining L-type Ca^{2+} channel activity. The effect of brimonidine was also mimicked by SQ22536, a selective AC inhibitor (Fig. 2*A*,*F*), and completely abolished by intracellular Sp-cAMPS, a cAMP analog that is resistant to PDE hydrolysis (Fig. 2*G*,*H*). These results demonstrate that after α2 receptor activation, interfering with the G_α signaling pathway at either AC level (overriding G_α-induced suppression of AC with forskolin), or downstream at cAMP level (maintaining the intracellular cAMP level with Sp-cAMPS) can both completely abolish α2-mediated inhibition of the presynaptic Ca^{2+} signal. This indicates that the inhibition is most likely through the G_α signaling pathway with negligible contribution from the $G_{\beta\gamma}$ subunits. However, in a cell transfection system in which G_β and G_γ cDNAs were cotransfected with either AC isoform I or II, it has been shown that AC activity can be directly modulated by $G_{\beta\gamma}$ subunits (Bayewitch et al., 1998). While this effect of $G_{\beta\gamma}$ subunits has not been demonstrated in neurons expressing native GPCRs, the finding raises a possibility that $G_{\beta\gamma}$ subunits may also inhibit AC directly in RBCs and thus contribute to α2 receptor-mediated inhibition of the presynaptic Ca^{2+} signal. To evaluate the contribution of $G_{\beta\gamma}$, we tested the effect of intracellular (Fig. 3*A*,*B*) and extracellular (Fig. 3*D*,*E*) application of a potent $G_{\beta\gamma}$ inhibiting peptide (Orr et al., 2002) and a small-molecule $G_{\beta\gamma}$ inhibitor (gallein; Ukhanov et al., 2011) on the modulation of the presynaptic Ca^{2+} signal by brimonidine. The application of those specific $G_{\beta\gamma}$ inhibitors did not alter the effect of brimonidine (Fig. 3*C*–*E*), which further supports our proposal that α2 modulation of the presynaptic Ca^{2+} signal is through the G_α signaling pathway.

α2 Modulation of the presynaptic Ca^{2+} signal revealed by confocal Ca^{2+} imaging is further corroborated by the effect of

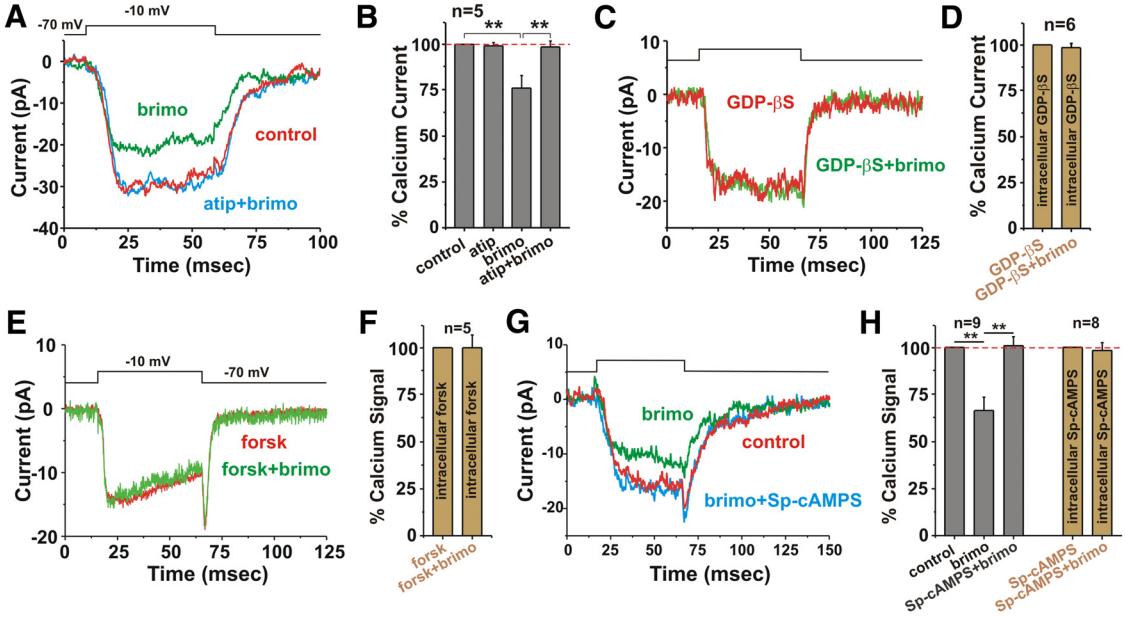

Figure 4. α2 Modulation of the whole-cell Ca^{2+} current via G_{α}–AC–cAMP pathway in RBCs. ***A***, ***B***, Modulation of the whole-cell Ca^{2+} current by brimonidine (brimo; 0.6 μM), and this effect was blocked by atipamezole (atip; 5 μM). ***C***, ***D***, The effect of brimonidine was abolished by intracellularly applied GDP-βS (0.5 mM replacing 0.5 mM GTP in the intracellular solution). ***E***, ***F***, Intracellular forskolin (forsk; 5 μM) abolished the effect of brimonidine. ***G***, ***H***, Both extracellular (100 μM) and intracellular (50 μM) Sp-cAMPS abolished the effect of brimonidine.

brimonidine, atipamezole, and intracellular GDP-βS on the depolarization-elicited whole-cell Ca^{2+} current recorded from RBCs (Fig. 4*A–D*). While recorded from the soma, the whole-cell Ca^{2+} current in bipolar cells originates from the synaptic terminals (Protti and Llano, 1998; Hartveit, 1999). Additionally, forskolin and Sp-cAMPS also blocked the modulation by brimonidine of the whole-cell Ca^{2+} current recorded from RBCs (Fig. 4*E–H*), which was similar to their effect on the modulation by brimonidine of the presynaptic Ca^{2+} signal (Fig. 2). Thus, these results further support the idea that the α2 receptor is expressed in RBCs and modulates presynaptic L-type Ca^{2+} channel activity via a G_{α}–AC–cAMP pathway with negligible, if any, contribution of the $G_{\beta\gamma}$ subunits.

PDE4 activity is required for α2 modulation of presynaptic Ca^{2+} channel activity in RBCs

Effective signaling via cyclic nucleotide second messengers may require the coordinated activities of a specific cyclase and PDE pair to dynamically and efficiently regulate the signaling molecule. This is exemplified by the guanylate cyclase/PDE6 pair in phototransduction, a sophisticated G_{α}-mediated cascade coupled to the photon receptor (Korenbrot, 2012). We hypothesized that a cAMP-specific PDE may play a critical role in presynaptic α2 receptor signaling in RBCs. PDE4 is a cAMP-specific PDE (Houslay and Adams, 2003). PDE4 isozymes are widely expressed in the CNS (Bolger et al., 1994), including the retina (Fig. 5*B*; see also Whitaker and Cooper, 2009). We therefore investigated the role of PDE4 in presynaptic α2 signaling in RBCs. Our immunostaining experiments showed colocalization of PDE4A and PKCα at synaptic boutons (Fig. 5*B*, area enclosed by the blue box) of RBCs in addition to their dendrites and soma. PDE4B, but not PDE4C or PDE4D, is also expressed in RBCs (data not shown). We found that rolipram, a selective and cell membrane-permeable PDE4 inhibitor, also completely abolished the inhibition of presynaptic Ca^{2+} signals by brimonidine when it was applied both extracellularly and intracellularly (Fig. 5*C–E*). This rolipram effect was mimicked by intracellular Ro 20–1724 and piclamilast, two other selective PDE4 inhibitors (Fig. 5*E*). These results indicate that (1) PDE4 is a necessary component in α2-mediated presynaptic signaling in RBCs; (2) coordinated actions of reduced production and active degradation of cAMP by the AC/PDE4 pair are required for effective α2 inhibition of the presynaptic L-type Ca^{2+} channel in RBCs (Fig. 5*A*); and (3) the α2-mediated presynaptic inhibition in RBCs is accomplished through the G_{α}-mediated inhibition of cAMP production, not by direct interactions between the $G_{\beta\gamma}$ subunits and presynaptic Ca^{2+} channels, K^+ channels, or synaptic proteins that affect transmitter release, as in the classic mode described in the brain (Starke, 2001; Delaney et al., 2007).

α2 Receptor modulation of exocytosis of synaptic vesicles from RBC terminals

To demonstrate α2-mediated presynaptic inhibition of transmitter release at RBC terminals, we investigated the effect of brimonidine on depolarization-induced exocytosis of FM 1–43-labeled synaptic vesicles, a process that is required for transmitter release (Betz at al., 1996; Ichinose and Lukasiewicz, 2012), in flat-mount isolated rat retinae. RBCs are ON bipolar cells (depolarized upon light stimulation due to reduced glutamate release from photoreceptors). Glutamate hyperpolarizes RBCs by closing TRPM1 channels coupled to mGluR6s on their dendrites (Shen et al., 2009; Koike et al., 2010). After FM 1–43 dye was loaded into synaptic vesicles at RBC terminals by strong depolarization-induced endocytosis with a high-K^+ saline or CPPG (a type III mGluR antagonist that blocks the hyperpolarizing effect of glutamate at mGluR6; Awatramani and Slaughter, 2001), the kinetics of dye unloading (as an indicator of synaptic release) was evaluated under various experimental conditions. APB (also known as AP4), a glutamate agonist at mGluR6 (Slaughter and Miller, 1981), was used to selectively hyperpolarize RBCs (simulating dark condition) and served as an unstimulated control. High K^+ saline or CPPG was used as a depolarizing agent (mimicking light condition) to induce transmitter release. To more accurately determine release kinetics, at each time point

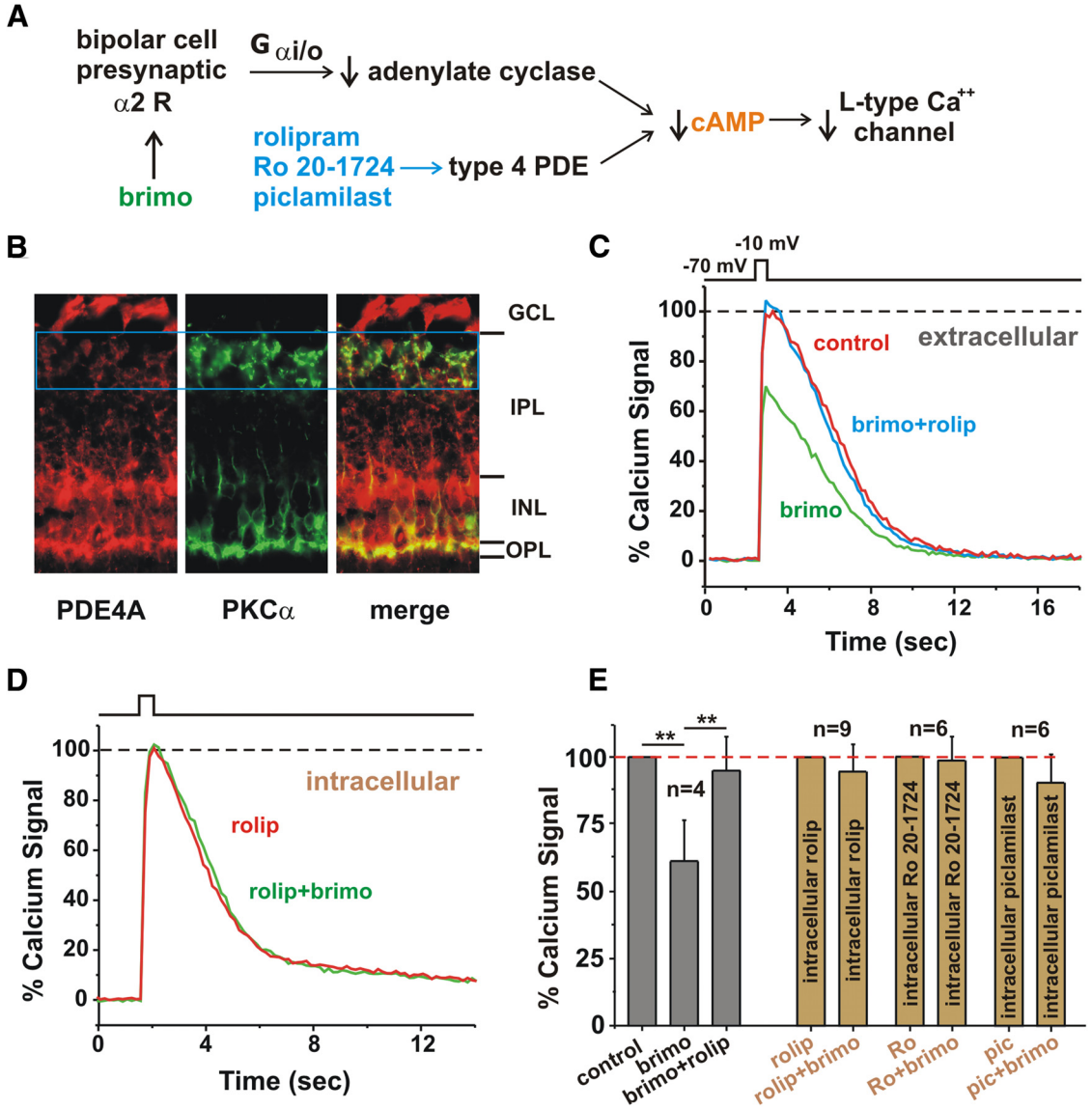

Figure 5. PDE4 activity is required for α2 modulation of presynaptic L-type Ca^{2+} channels in RBCs. ***A***, "Push–pull" regulation of intracellular cAMP level by AC and PDE4 in the α2 modulation of L-type Ca^{2+} channels and the site of action of the tool agents. ***B***, Immunohistochemistry staining shows that PDE4A is expressed in various compartments of RBCs, including synaptic boutons (in the area enclosed by the blue box). ***C–E***, The effect of brimonidine on L-type Ca^{2+} channels is abolished by both extracellularly and intracellularly applied rolipram (rolip; 5 μM). ***E***, The effect of brimonidine is also abolished by Ro 20 –1724 (Ro; 10 μM) and piclamilast (pic; 10 μM) applied intracellularly.

a maximum projection image (Fig. 6*B*) was produced based on a stack of 20 consecutive confocal images that covers ~16 μm in depth (Fig. 6*A*, between the two green lines) in the IPL, where RBC terminals are found. With a 40 mM high-K^+ saline solution, FM dye unloading was complete in ~12 min with a half unloading time of 2 min (Fig. 6*B*,*C*). The application of nimodipine blocked high K^+-induced unloading (Fig. 6*C*,*F*), confirming that exocytosis of FM 1–43-labeled synaptic vesicles (transmitter release) requires activity of presynaptic voltage-gated L-type Ca^{2+} channels at RBC terminals. Brimonidine substantially slowed exocytosis of synaptic vesicles (Fig. 6*D–F*) as expected based on the results shown in Figures 1, 2, 3, 4, and 5. Both atipamezole and rolipram abolished the effect of brimonidine (Fig. 6*D–F*). Brimonidine also significantly inhibited RBC transmitter release (exocytosis of synaptic vesicles) when 0.5 mM CPPG was used to mimic light stimulation (data not shown). These results demonstrate α2 receptor-mediated presynaptic inhibition of transmitter release from RBC terminals and an essential role of PDE4 in this α2-mediated inhibition.

Discussion

Together, our data have demonstrated a new mode of presynaptic inhibition by GPCRs in the CNS. By using the G_{α}, instead of $G_{\beta\gamma}$ subunits as in the classical mode (Starke, 2001; Brown and Sihra, 2008; Stephens, 2009; Fig. 7), the α2 receptor uses a cyclase/phosphodiesterase (AC/PDE4) enzyme pair to dynamically control the level of a cyclic nucleotide second messenger (cAMP), which in turn modulates neurotransmitter release from RBC synaptic terminals (Figs. 5*A*, 7*B*). Surprisingly, the operating strategy of this new mode shares remarkable similarities to that used by the photon receptor (visual pigment) in phototransduction, which is a sophisticated GPCR-mediated mechanism that is capable of providing fine and precise regulation of photoreceptor membrane potential under complex visual environments, as fol-

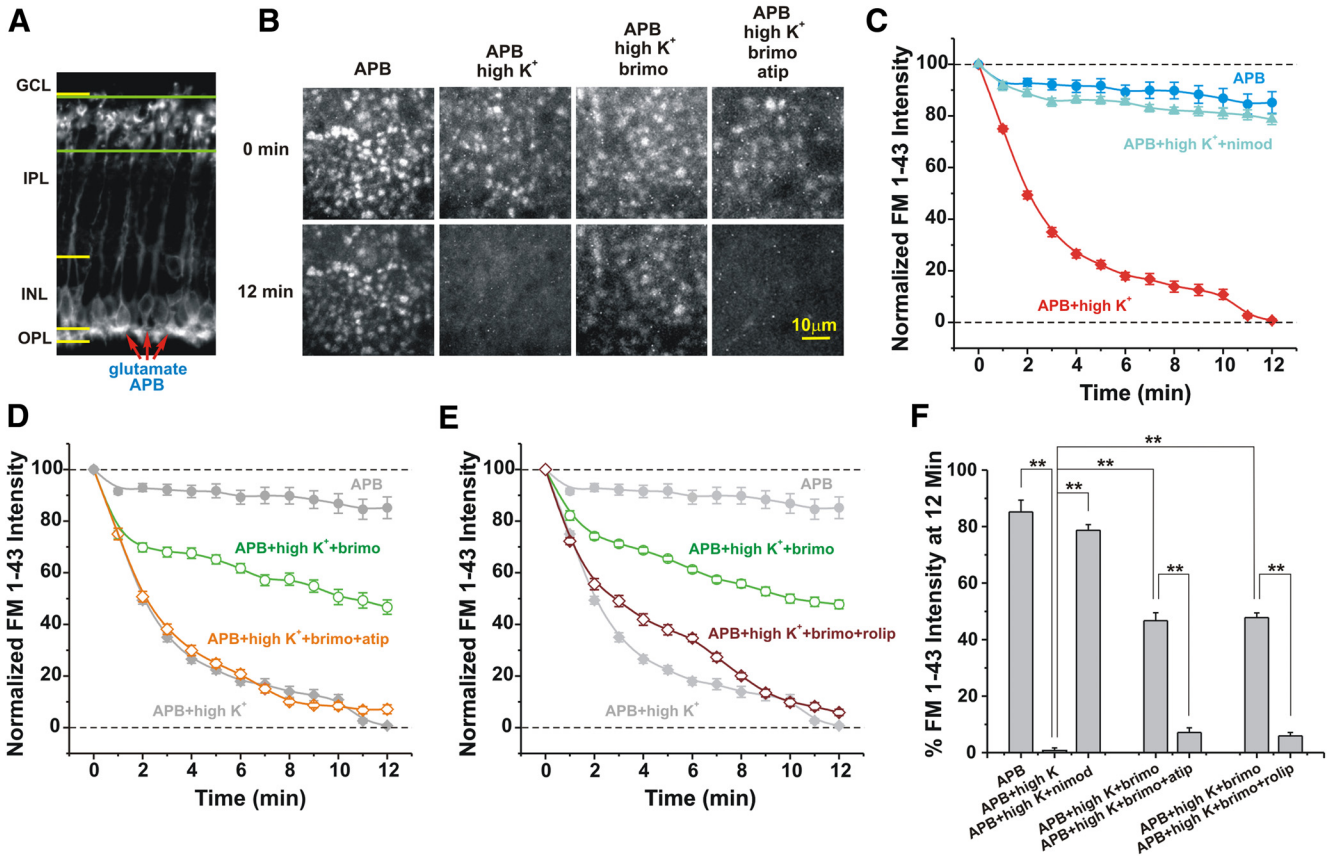

Figure 6. α2 Modulation of transmitter release from presynaptic terminals in RBCs. ***A***, PKCα antibody-labeled RBCs in a cross-section of the rat retina that show (1) the site of action of APB (an mGluR6 agonist) and (2) the depth range (between the two green lines) in the IPL where RBC synaptic boutons are found, over which a stack of 20 consecutive confocal images are taken from a flat-mount retina to produce the maximum projection images shown in ***B***. The brightly fluorescent dots in ***B*** are individual synaptic boutons filled with FM 1–43-stained synaptic vesicles. ***C***, Hyperpolarization of the RBC with DL-APB (APB; 50 μM) retards spontaneous exocytosis of synaptic vesicles and is used as a control, whereas direct depolarization with high-K^+ Ringer's solution (high K^+; 40 mM) rapidly unloads FM-1–43-labeled synaptic vesicles. This high-K^+-induced unloading is blocked by nimodipine (nimod; 20 μM). ***D***, ***E***, High-K^+-induced unloading is also partially suppressed by brimonidine (brimo; 0.6 μM), and this suppressive effect is completely blocked by atipamezole (atip; 10 μM, ***D***) or rolipram (rolip; 10 μM, ***E***). ***F***, Statistical comparisons of the percentage of exocytosis of FM 1–43-labeled synaptic vesicles between different experimental conditions at 12 min.

lows: (1) both are accomplished through a G_α-mediated cascade; (2) both are centered on dynamic control of a cyclic nucleotide second messenger (cAMP or cGMP) by a pair of production/degradation enzymes (AC/PED4 or guanylate cyclase/PDE6); and (3) both control glutamate release from an L-type Ca^{2+} channel-coupled, graded potential-driven ribbon synapse that could transmit higher rates of information than a conventional synapse (Sterling and Matthews, 2005).

There is no evidence for a significant contribution by the $G_{\beta\gamma}$ units to the observed α2-mediated presynaptic inhibition in RBCs, because the agents that interfere selectively with the G_α signaling pathway, such as forskolin (an AC activator), Sp-cAMPS (an hydrolysis-resistant cAMP analog), and rolipram (a selective membrane-permeable PDE4 inhibitor), all abolish α2-mediated presynaptic inhibition in RBCs (Figs. 2, 4, 5, 6). Furthermore, direct inhibition of AC by SQ-22536 mimics the effect of α2 agonists (Fig. 2*F*). Finally, neither intracellular application of a highly selective and potent $G_{\beta\gamma}$-blocking peptide nor extracellular application of a selective $G_{\beta\gamma}$ blocking agent alter the inhibition of the presynaptic Ca^{2+} signal by brimonidine (Fig. 3). Together, these results make the inhibition by parallel actions of both G_α- and $G_{\beta\gamma}$-mediated signaling very unlikely.

cAMP-degrading PDEs, especially PDE4 isozymes, are widely expressed in various regions of the CNS including the retina (Bolger et al., 1994; Pérez-Torres et al., 2000; Whitaker and Cooper, 2009; see also Fig. 5*B*) and have long been recognized as a target of drug therapy for neurological and psychiatric disorders (Hebb and Robertson, 2007). However, their role in synaptic transmission, especially in presynaptic regulation of neurotransmitter release, is unknown. Our results reveal a previously unknown role of PDE4, or cAMP-degrading PDEs in general, in synaptic transmission in the CNS. The requirement of PDE4 participation in a GPCR (α2 receptor)-mediated presynaptic inhibition raises a general question related to GPCR ($G_{\alpha i/o}$-coupled)-mediated, cAMP-dependent signaling in the CNS: is partnering with a cAMP-degrading PDE (e.g., PDE4) critical for those GPCRs to signal properly? GPCR activation alone only reduces the production of new cAMP, and may not be enough for effective and rapid attenuation or termination of the cAMP-dependent cellular signaling because of the existing cAMP. Active and rapid degradation of those existing cAMP molecules by cAMP-degrading PDEs may be required to achieve the intended physiological or therapeutic effect. Indeed we found that the *in vivo* neuroprotective effect of the α2 agonist is abolished in both the rat glaucoma model and the rabbit retinal excitotoxicity model (Dong et al., 2008) after coadministration of a PDE4 inhibitor.

Chemical and electrical synapses are the two cornerstones of the nervous system through which neurons communicate with each other and with cells outside the nervous system. While our

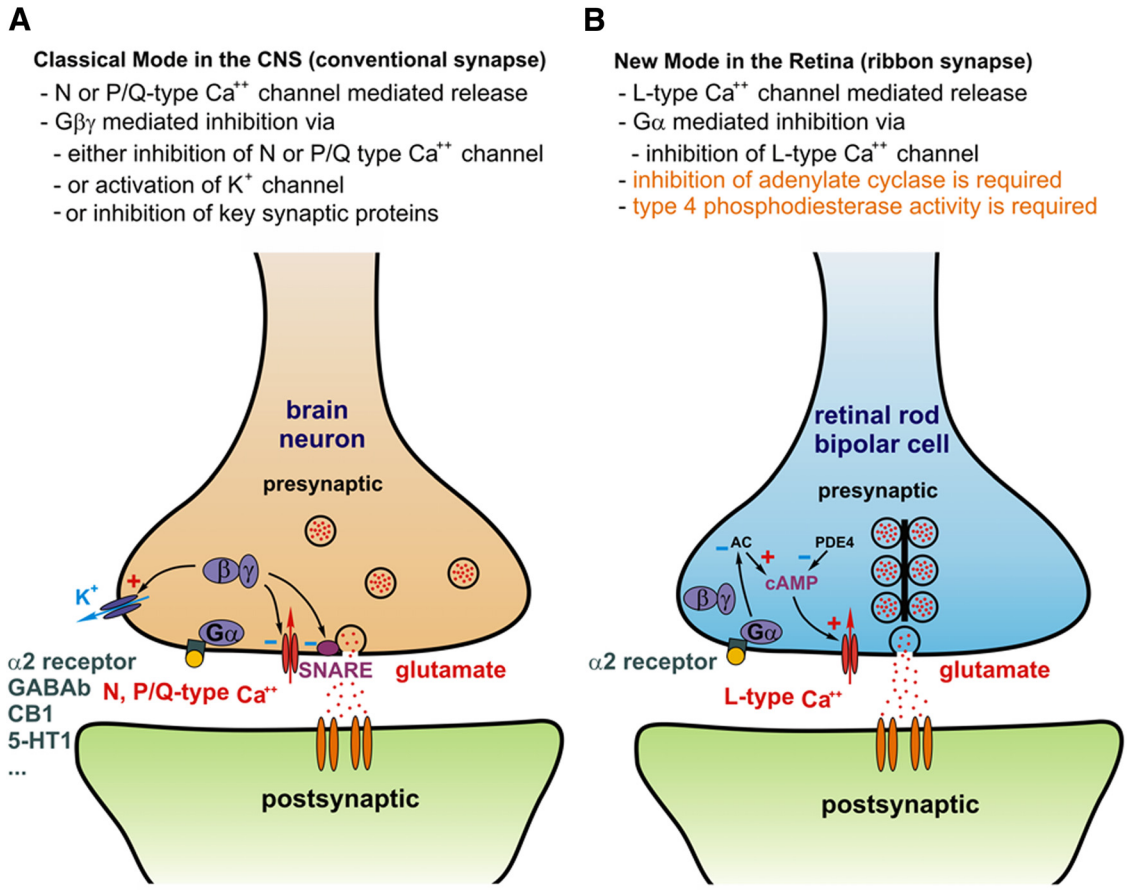

Figure 7. ***A***, ***B***, Diagram that summarizes and compares the α2 receptor-mediated presynaptic inhibition in the brain (***A***) and at the RBC synaptic terminal in the retina (***B***).

data show only the role of PDE4 in the modulation of synaptic transmission at a central chemical synapse, given well established cAMP-mediated modulation of electrical synapses between different types of CNS neurons (Hatton, 1998; Bloomfield and Völgyi, 2009), especially retinal neurons (Bloomfield and Völgyi, 2009), and widely expressed PDE4 and other cAMP-degrading PDEs in the CNS (Bolger et al., 1994), our data raise the possibility that PDE4 and other cAMP-degrading PDEs may likely play a critical role in regulating transmission at the electrical synapse as well in these CNS regions.

RBC terminals have a complex, rapid-acting ionotropic presynaptic inhibition machinery comprising $GABA_A$, $GABA_C$, glycine receptors (Shields and Lukasiewicz, 2003; Chávez et al., 2010; Eggers and Lukasiewicz, 2011), and glutamate transporter [EAAT-5 (excitatory amino acid transporter-5); Veruki et al., 2006; Wersinger et al., 2006; Ichinose and Lukasiewicz, 2012]. The GPCR (α2 receptor)-mediated presynaptic inhibition mechanism described here complements those rapid-acting ionotropic mechanisms and adds a powerful tool to the repertoire of inhibition at this critical presynaptic site that regulates the processing and transmission of scotopic information in the visual pathway (Dong and Hare, 2003; Shields and Lukasiewicz, 2003; Oesch and Diamond, 2011).

Adrenaline-producing retinal neurons have been identified in the retina (Park et al., 1986), and the action of an endogenous α2 ligand has also been demonstrated (Hadjiconstantinou et al., 1984). However, compared with other common neurotransmitter systems in the retina (such as glutamate, GABA, and dopamine), the function, circuitry, and receptor distribution of the retinal adrenergic system is much less well known. Our data not only demonstrate the presence of α2 receptors in RBCs, but also reveal a significant function of the receptor in retinal signal processing as well. However, we do not know at this point the neural circuitry that activates and regulates this mechanism. Future work is needed to address this important question.

Excessive activation of the NMDA receptor in RGCs is a major mechanism underlying their injury in acute retinal ischemia (Lagrèze et al., 1998; Lee et al., 2012) and in animal models of chronic glaucoma (WoldeMussie et al., 2002; Hare et al., 2004; Dong et al., 2008; Ju et al., 2009), regardless of what animal species is used or how IOP is elevated. Brimonidine protects RGCs in both ischemia and glaucoma models partially by postsynaptic modulation of NMDA receptor signaling in RGCs (Dong et al., 2008; Lee et al., 2012). The data presented here support our hypothesis by demonstrating a presynaptic mechanism that can protect RGCs through limiting glutamatergic excitatory drive to them in models of both acute retinal ischemia and glaucoma, and possibly in human glaucoma.

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