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Activation of Postsynaptic Metabotropic Glutamate Receptors by *Trans*-ACPD Hyperpolarizes Neurons of the Basolateral Amygdala

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Glutamate has traditionally been regarded as an excitatory neurotransmitter. Synaptic activation of ionotropic glutamate receptors mediates fast EPSPs in the CNS. Moreover, activation of metabotropic glutamate receptors (mGluRs), which are coupled to second messenger effector systems via GTP-binding proteins (G-proteins), results in the expression of slow EPSPs. We have now examined the response of basolateral amygdala (BLA) neurons to activation of postsynaptic mGluRs. In approximately 78% of BLA neurons examined, activation of postsynaptic mGluRs results in membrane hyperpolarization and an associated decrease in membrane input resistance or a hyperpolarization followed by a depolarization associated with an increase in input resistance. The purpose of this study was to address the mechanisms underlying the membrane hyperpolarization. Here, we report that the ACPD-induced hyperpolarization is insensitive to TTX, is dependent on extracellular K^+ concentrations, and has a reversal potential (−84 mV) close to that estimated from the Nernst equation for an increase in a K^+ conductance. In addition, the ACPD response is resistant to (1) intracellular chloride loading, (2) the $GABA_B$ receptor antagonist CGP55845A, (3) the ACh receptor antagonist atropine, and (4) the ionotropic glutamate receptor antagonists CNQX and APV. These data suggest that the hyperpolarization results from a direct activation of postsynaptic mGluRs on neurons of the BLA. Furthermore, we performed studies that suggest that the hyperpolarization is G-protein mediated and results from activation of a TEA-sensitive, calcium-dependent potassium conductance. The sensitivity of this conductance to thapsigargin further suggests that this response requires the release of calcium from intracellular stores. In summary, these data suggest a role for glutamate as an inhibitory transmitter in the BLA during periods of metabotropic glutamate receptor activation. In nuclei such as the BLA that are exquisitely sensitive to seizure induction, an inhibitory response to glutamate may act to delay the onset of epileptogenesis.

[Key words: metabotropic glutamate receptor, ACPD, amygdala, K conductance, Ca^{2+}-dependent K^+ conductance, brain slice, IP_3, glutamate, hyperpolarization, epilepsy]

Glutamate is a major excitatory amino acid (EAA) neurotransmitter within the CNS. Glutamatergic synaptic transmission is mediated by activation of multiple glutamate receptor subtypes. Generally, glutamate receptors can be divided into two main categories termed ionotropic and metabotropic receptors (Monaghan et al., 1989). The ionotropic receptors can be further subdivided into the NMDA, kainate (KA), and 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subtypes according to their agonist profiles. Molecular cloning of ionotropic glutamate receptors has shown that these receptors contain receptor cation-specific channel complexes (Hollmann et al., 1989; Bettler and Boulter, 1990; Sakimura et al., 1990). In contrast, metabotropic glutamate receptors (mGluRs) are coupled to intracellular second messenger cascade systems via G-proteins (Sladeczek et al., 1985; Schoepp and Johnson, 1988). The mGluRs are activated by quisqualate (QUIS), ibotenate (IBO), and glutamate but not by NMDA, KA, or AMPA. These receptors are, however, more specifically activated by two conformationally restricted analogs of glutamate, *trans*-1-amino-cyclopentane-1,3-dicarboxylic acid (*trans*-ACPD; Palmer et al., 1989; Desai and Conn, 1990) and L-isomer of α-carboxycyclopropyl-glycine (L-CCG-I; Ishida et al., 1990). Molecular cloning and PCR techniques have revealed the existence of at least seven subtypes of mGluR, mGluR1–mGluR7 (for review, see Nakanishi, 1992; Okamoto et al., 1994). Furthermore, alternative RNA splicing results in variations of the mGluR1 receptor protein, mGluR1α, mGluR1β, and mGluR1c, respectively (Pin et al., 1992; Tanabe, 1992), while an evolutionally conserved variant of the mGluR5 subtype termed mGluR5β has also been described (Minakami et al., 1994).

Sequence homology of amino acids in the mGluR proteins suggests that the mGluRs can be divided into three subgroups: mGluR1 and mGluR5, mGluR2 and mGluR3, and mGluR4 and mGluR6 (Houamed et al., 1991; Abe et al., 1992; Tanabe et al., 1992). Activation of either the mGluR1 or mGluR5 subgroup is typically coupled to inositol triphosphate (IP_3) formation and intracellular Ca^{2+} mobilization, whereas the other two subgroups are associated with inhibition of cAMP (Tanabe et al., 1992).

We have previously demonstrated that low-frequency stimulation of the stria terminalis, an afferent input to the basolateral amygdala (BLA), results in postsynaptic activation of both NMDA and KA/AMPA ionotropic glutamate receptors on BLA neurons (Rainnie et al., 1991a) and that a long-lasting increase

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in glutamatergic excitatory synaptic transmission occurs in the BLA following kindling-induced epileptogenesis (Rainnie et al., 1992). Kindling has been reported to cause a prolonged increase in phosphoinositide (PI) hydrolysis in the amygdala/pyriform cortex (Akiyama et al., 1989, 1992; Yamada et al., 1989) suggesting a possible role for the mGluR1, and/or mGluR5 subgroup of glutamate receptors in epileptogenesis. Indeed, local infusion of 1*S*,3*R*-ACPD into the hippocampus leads to seizure expression *in vivo* (Sacaan and Schoepp, 1992). At present the mGluR subtypes mediating this action are unknown. It is possible, however, that several mGluR subtypes may be expressed by neurons in the same amygdaloid nuclei and subserve different physiological functions. This hypothesis is supported by the observation that the amygdala is immunoreactive against the antibody for the mGluR1α receptor protein (Martin et al., 1992), and expresses mRNA for both mGluR1 and mGluR2 receptor proteins (Shigemoto et al., 1992; Ohishi et al., 1993). Moreover, low micromolar concentrations ($\leq$50 μM) of *trans*-ACPD act presynaptically to reduce glutamatergic transmission in the BLA with no apparent effect on membrane potential (Rainnie and Shinnick-Gallagher, 1992). In the present report we have extended our investigation of metabotropic glutamate receptor responses by examining the effects of activation of postsynaptic mGluRs mediating a membrane hyperpolarization in BLA neurons.

Materials and Methods

Male Sprague–Dawley rats (110–150 gm) were decapitated and the brains rapidly removed and placed in cold oxygenated artificial cerebrospinal fluid (aCSF) solution. The brain was then hemisected and cut transversely posterior to the first branch and anterior to the last branch of the superior cerebral vein. The resulting section was glued to the chuck of a Vibroslice tissue slicer (Campden Instruments). Transverse slices of 500 μm thickness were cut and the appropriate slices placed in a beaker of oxygenated aCSF at room temperature for at least 1 hr prior to recording. The aCSF was composed of (in mM) NaCl, 117; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; and glucose, 11; and was bubbled with 95% O_2, 5% CO_2 (pH = 7.4). In low Ca/high Mg solutions, Ca was reduced to 0.2 mM and Mg was raised to 10 mM. For the Cd experiments, HEPES (10 mM) was substituted for bicarbonate and the phosphates were removed; this solution was bubbled with 100% O_2.

The slice was fully submerged in the recording chamber and maintained at 32°C ± 2°C with continuously superfused aCSF. Microelectrodes were pulled from fiber-filled capillary tubing of borosilicate glass with a Flaming- Brown micropipette puller (Sutter Instruments, model P-80). The resistance of the microelectrodes filled with 4 M K-acetate ranged between 70 and 150 MΩ. On- and off-line data acquisition and analysis was performed using an Axolab 1100 interface (Axon Instruments) between an Axoclamp-2A preamplifier and a Dell 310 personal computer utilizing PCLAMP 5.5.1 software programs (Axon Instruments). Analog signals were also stored for later analysis on video cassettes using an adapted video recorder (A. R. Vetter Co., model 420B) as well as on a Gould (model 3400) chart recorder. Intracellular recordings were considered acceptable if neurons exhibited overshooting action potentials and showed stable membrane potentials more negative than −60 mV in the absence of a DC holding current. The bridge balance was carefully monitored throughout the experiments and adjusted when necessary. All data are expressed as mean ± standard error of the mean; in all cases, n = number of neurons. Statistical analyses used in these studies were the paired one-tailed Student's t test and where appropriate ANOVA with Bonferroni post hoc t tests. Unpaired Student's t tests were used only when indicated. Statistical significance was determined at the level of $p \leq 0.05$.

Unless otherwise stated, all drugs were applied by superfusion in the aCSF and introduced into the recording chamber by means of a three way stopcock. For drop application a single, 10 μl or 50 μl drop was applied using an Oxford micropipette to the recording chamber just "upstream" from the BLA. Stock solutions were stored at −20°C and recording solutions prepared fresh on the day of each experiment. The drugs used in this study were (±)*trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*trans*-ACPD; 20 μM to 20 mM), (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD; 20 μM to 20 mM), 4-carboxy-3-hydroxyphenylglycine (4C3HPG; 20 μM to 20 mM), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), from Tocris Neuramin, Essex, UK; (3-*N*[1-(*S*)-(3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-*P*-benzyl-phosphonic acid (CGP55845A; 10 μM), from Ciba-Geigy, Basel, Switzerland; (DL)-2-amino-5-phosphonovaleric acid (APV; 50 μM) and thapsigargin (2.5 μM), from Research Biochemicals Inc., Natick, MA; bicuculline methiodide (BMI; 30 μM), guanosine 5′-*O*-3-thiotriphosphate (GTP-γ-S; 10 mM), guanosine-5′-*O*-2-thiodiphosphate (GDP-β-S; 10 mM), atropine (5 μM), 4-amino-3-[4-chlorophenol]-butanoic acid (baclofen; 1 mM drop), and tetraethylammonium (TEA; 1 mM), from Sigma Chemicals, St. Louis, MO; and bis-(*O*-aminophenoxy)-*N,N,N′,N′*-tetraacetic acid (BAPTA; 200 mM).

After recording responses to ACPD, biocytin (2%) was iontophoresed into the neurons for ≥10 min. Only one neuron was filled per slice. Slices were treated with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.2), rinsed in PBS, and sectioned (75 μM). Slices were further incubated in 0.3% hydrogen peroxide in PBS and 0.4% Triton X-100 and rinsed. The slices were then incubated with the avidin-biotin-horseradish peroxidase complex (Elite ABC kit, Vector Labs) in 0.4% Triton X-100 PBS, washed, and reacted with diaminobenzidine tetrahydrochloride (0.1 M) and 0.02% hydrogen peroxide in 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 7.2). Slices were rinsed in PBS, mounted sequentially on gelled slides, and dried. The slices were dehydrated in alcohol, defatted with Histo-clear (National Diagnostics), mounted in Permount (Fisher Scientific), and coverslipped. Photomicrographs were taken with Tmax 100 (Eastman Kodak) film in a camera mounted on a Nikon Diaphot microscope (Nikon) using Hoffman optics (Modulation Optics). Photographs were scanned (U Max Data Systems) and printed on a Laserjet IV printer.

Results

Effects of trans-*ACPD and 1*S*,3*R*-ACPD on membrane properties of BLA neurons*

Intracellular recordings were obtained from 126 neurons of the basolateral amygdala *in vitro* and the effect of mGluR activation examined. As previously reported, application of the specific mGluR agonist *trans*-ACPD at low micromolar concentrations (<50 μM) had no effect on resting membrane properties of BLA neurons (Rainnie and Shinnick-Gallagher, 1992). In higher concentrations, ACPD affected membrane potential in 78% of BLA neurons. Application of *trans*-ACPD (100–200 μM) or its active isomer 1*S*,3*R*-ACPD (100 μM) evoked either membrane hyperpolarization (54 of 98 neurons) alone (Fig. 1*A,B*) or a hyperpolarization and subsequent depolarization (44 of 98 neurons) (Fig. 1*C*). In an additional 28 neurons ACPD either had no effect (n = 6) or induced only a membrane depolarization (n = 22).

The hyperpolarization was associated with a decrease in membrane input resistance and the depolarization associated with an increase. In all cases, bath application of *trans*-ACPD evoked a membrane hyperpolarization that far outlasted the period of drug application. A typical response is shown in Figure 1*A*; here *trans*-ACPD (100 μM) evoked a prolonged hyperpolarization and subsequent depolarization. The long duration of action of the mGluR agonist (>10 min) made repeated testing with bath application difficult. Consequently, we applied *trans*-ACPD and 1*S*,3*R*-ACPD by drop application (10 or 50 μl, 20 μM to 20 mM, respectively). Using this method, reproducible responses could be obtained with repeated application. We calculated an approximate 1:20 dilution factor for the 50 μl drop and 1:100 dilution for the 10 μl drop application. A concentration-dependent increase in membrane hyperpolarization resulted from topical application of both *trans*-ACPD and 1*S*,3*R*-ACPD over

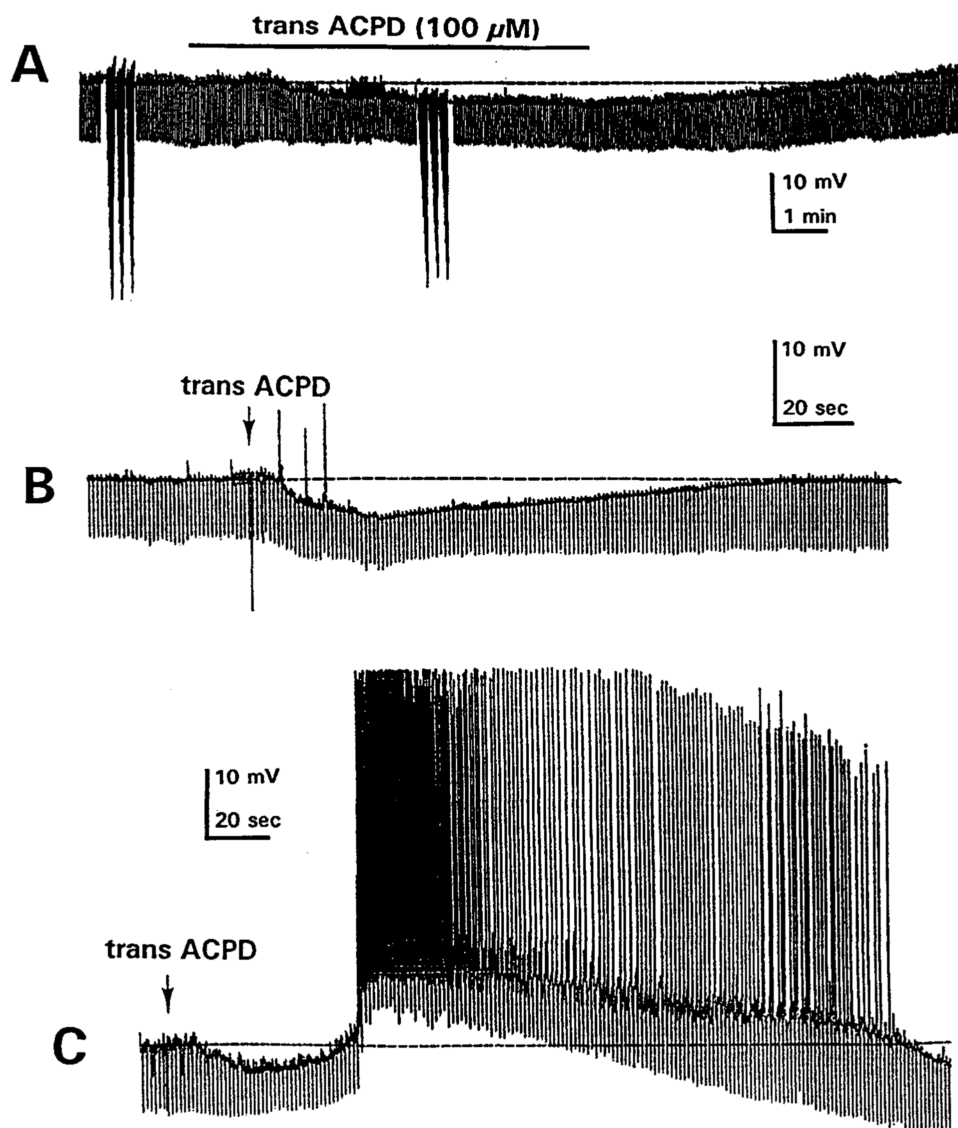

Figure 1. *Trans*-ACPD induces a membrane hyperpolarization and concomitant decrease in input resistance in neurons of the BLA. *A*, Superfusion of *trans*-ACPD (100 μM) induced a membrane hyperpolarization (5 mV) and concomitant decrease in input resistance that persists for the duration of the superfusion (8 min). The effect was reversible on washout. *B*, Drop application of *trans*-ACPD (100 μM) induced a membrane hyperpolarization (6 mV) and decrease in input resistance of similar magnitude to that induced by *trans*-ACPD superfusion. Both the membrane potential and the input resistance gradually returned to the predrug values. *C*, In another neuron application of *trans*-ACPD induced an initial membrane hyperpolarization followed by a depolarization and spontaneous action potential generation. The depolarization was often associated with an increase in input resistance. Membrane potential in *A–C* was −65 mV. Downward deflections are electrotonic potentials elicited in response to injecting 200 msec current pulses of 0.1 nA, across the membrane in *A–C*. In *A*, three series of current pulses of varying magnitude were tested in control and in the presence of *trans*-ACPD as a measure of membrane resistance.

the range of 20 μM to 200 μM. A typical hyperpolarizing response to drop application of *trans*-ACPD is shown in Figure 1*B*. In another neuron, drop application of *trans*-ACPD evoked a hyperpolarization followed by a pronounced depolarization resulting in action potential generation (Fig. 1*C*). The hyperpolarization evoked by bath superfusion of 1*S*,3*R*-ACPD (100 μM) from a holding potential of −65 mV was −4.8 ± 1.8 mV (n = 6). Similarly, drop application of *trans*-ACPD (100 μM) or 1*S*,3*R*-ACPD (50 μM) evoked membrane hyperpolarizations of −4.2 ± 0.4 mV (n = 20) and −3.8 ± 0.5 mV (n = 17), respectively. In all subsequent experiments, 1*S*,3*R*-ACPD or *trans*-ACPD was applied by drop except where noted; numbers in brackets reflect the final bath concentrations. Preliminary data suggest that the membrane hyperpolarization can be mimicked by glutamate (500 μM; n = 3), an effect that can be recorded in the presence of D-APV (50 μM) and CNQX (30 μM, n = 1; Arvanov, Holmes and Shinnick-Gallagher, unpublished observations). In the hippocampus the depolarizing response to mGluR activation is attributed to a reduction of potassium currents (Baskys et al., 1990; Charpak et al., 1990; Gerber et al., 1992). In this study, however, we were concerned with the mechanisms underlying the hyperpolarizing response and so did not address the mechanisms mediating the depolarization.

The ACPD-induced membrane hyperpolarization was observed in 78% of all BLA neurons recorded. Morphologically, neurons of the rat BLA consist of a heterogeneous population and have been divided into two main classes: class I, pyramidal and stellate spiny neurons, and class II, multipolar spine-sparse neurons. Moreover, the electrophysiological characteristics of identified BLA cells have been reported (Washburn and Moises, 1992; Rainnie et al., 1993). We first tested whether the hyperpolarizing response to ACPD was present in the different classes of BLA neurons. Application of 1*S*,3*R*-ACPD induced a membrane hyperpolarization in class I pyramidal (Fig. 2A_1,A_2) and multipolar spiny neurons (not shown) and class II multipolar aspiny neurons (Fig. 2B_1,B_2). Thus, the hyperpolarizing action of ACPD was not confined to a particular neuronal cell type.

The ACPD-induced hyperpolarization does not result from an indirect release of secondary neurotransmitter

The delayed depolarizing response to ACPD seen in some BLA neurons suggested that the hyperpolarizing response to *trans*-

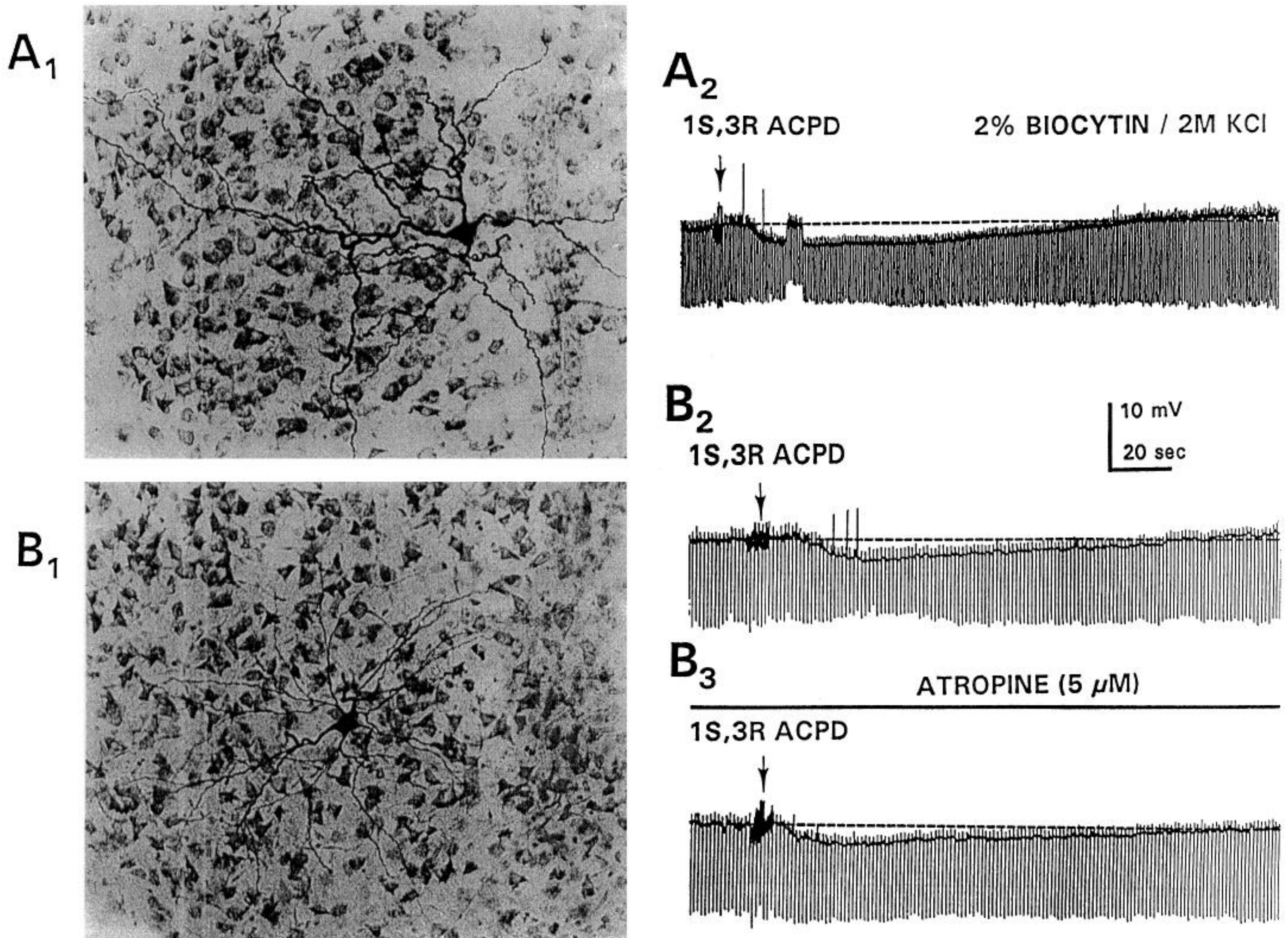

Figure 2. The 1*S*,3*R*-ACPD (100 μM)–induced hyperpolarization can be recorded in BLA neurons of differing morphology, is unaffected by chloride loading, and is resistant to the muscarinic receptor antagonist atropine. A_1, Biocytin-filled class I spiny pyramidal neuron of the BLA; the surrounding cells were counterstained with 1% neutral red. A_2, Preloading the neuron shown in A_1 with chloride during biocytin injection did not affect the hyperpolarizing response to 1*S*,3*R*-ACPD application. Resting membrane potential, −68 mV. B_1, Biocytin-filled class II aspiny multipolar neuron of the BLA. B_2, 1*S*,3*R*-ACPD also caused a hyperpolarization and decrease in input resistance in this class II neuron. B_3, Superfusion with the muscarinic antagonist atropine (5 μM) did not block the hyperpolarizing response to 1*S*,3*R*-ACPD in the same neuron. Resting membrane potential, −67 mV. Downward deflections represent electrotonic potentials generated in response to injecting 200 msec, 0.1 nA current pulses across the membrane as a measure of membrane resistance.

ACPD and 1*S*,3*R*-ACPD could be due to release of an inhibitory transmitter "upstream" from the recorded neuron. Consequently, we tested whether the ACPD hyperpolarization could be due to release of another transmitter by examining ACPD action in the presence of TTX. The ACPD-evoked hyperpolarization (3.0 ± 0.3 mV), however, was resistant to prior superfusion with TTX (0.5 μM; 2.9 ± 0.2 mV, $n = 4$, $p > 0.05$), suggesting that indirect, action potential–dependent release was not responsible for the recorded response.

Activation of feedforward inhibitory transmission in the BLA could result in membrane hyperpolarization that is sensitive to the ionotropic glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) and 2-amino-5-phosphonovalerate (APV; 50 μM) (Rainnie et al., 1991). Furthermore, the *cis*-configuration of ACPD does show some affinity at NMDA receptors; however, mGluR activation is resistant to ionotropic glutamate antagonists (Schoepp, 1993). We therefore examined the effect of pretreatment with CNQX and APV on the response to *trans*-ACPD (Fig. 3). In this neuron, both *trans*-ACPD and 1*S*,3*R*-ACPD evoked a membrane hyperpolarization (Fig. 3A_1,A_2), while superfusion of CNQX and APV blocked spontaneous PSPs but did not block the *trans*-ACPD–induced hyperpolarization (Fig. 3A_3). Although the antagonists depressed the *trans*-ACPD response in this neuron, this effect was not statistically significant across cells. (control, 3.7 ± 0.6 mV; treatment, 3.6 ± 0.8 mV; $n = 5$, $p > 0.05$). The 1*S*,3*R*-ACPD hyperpolarization increased in amplitude when the membrane potential was held at more depolarized levels (Fig. 3*B*). Moreover, during the 1*S*,3*R*-ACPD–induced hyperpolarization, spontaneous EPSP activity was reduced ($n = 5$; Fig. 3*C*).

In the BLA most spontaneous and evoked IPSPs are mediated by activation of either $GABA_A$ or $GABA_B$ receptors, resulting in an increase in either a chloride or potassium conductance, respectively (Rainnie et al., 1991b). Moreover, postsynaptic mGluR activation in hippocampal inhibitory interneurons results in an increase in spontaneous chloride-mediated IPSPs

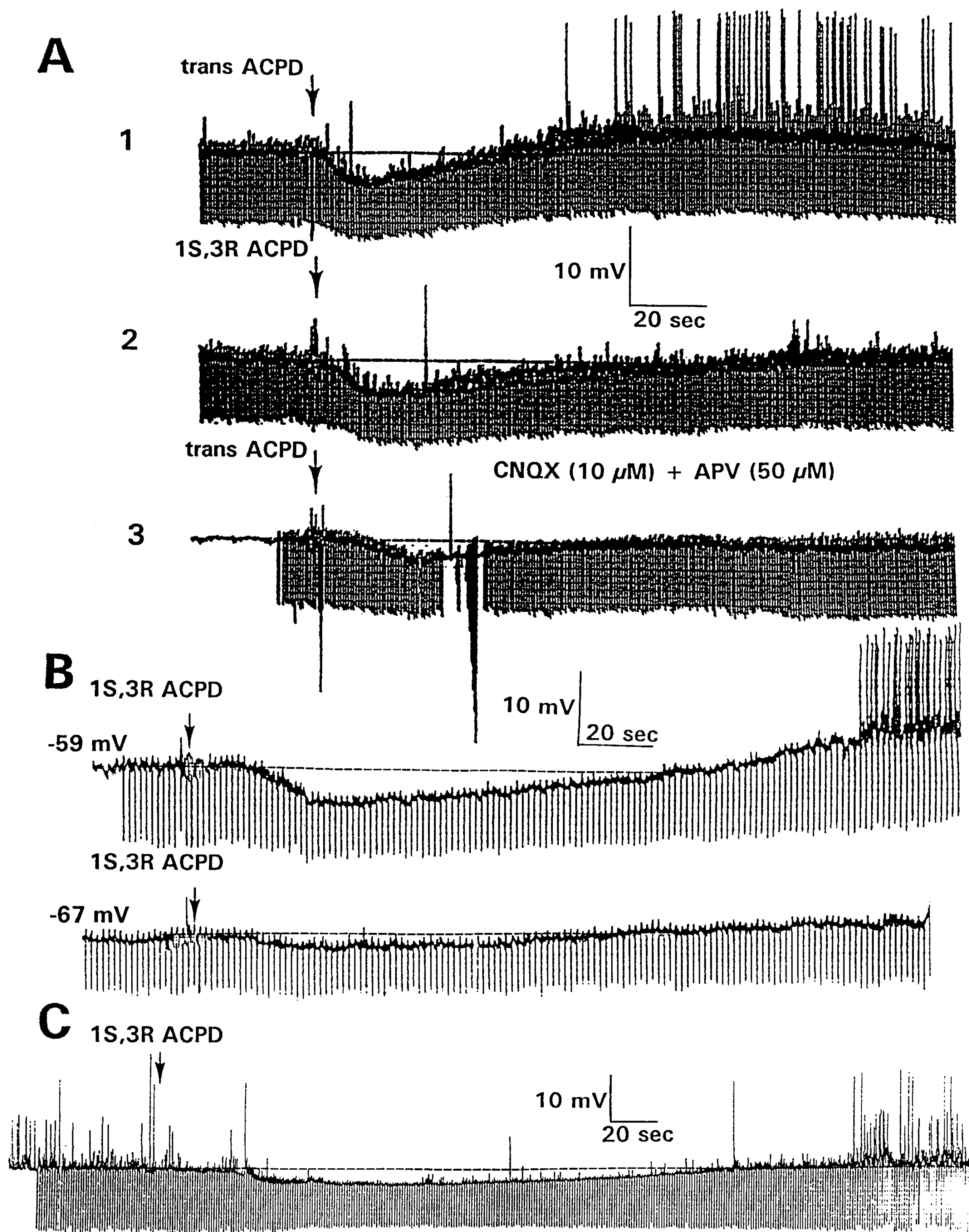

Figure 3. Pharmacology, voltage dependence, and physiological significance of the hyperpolarizing response to ACPD. *A*, The effect of *trans*-ACPD is mimicked by the specific metabotropic receptor agonist 1*S*,3*R*-ACPD and is not blocked by ionotropic glutamate receptor antagonists. A_1, Topical application of *trans*-ACPD (80 μM) in this neuron induced a membrane hyperpolarization and concomitant decrease in input resistance followed by a depolarization that was associated with an increase in input resistance. A_2, In the same neuron, topical application of the active

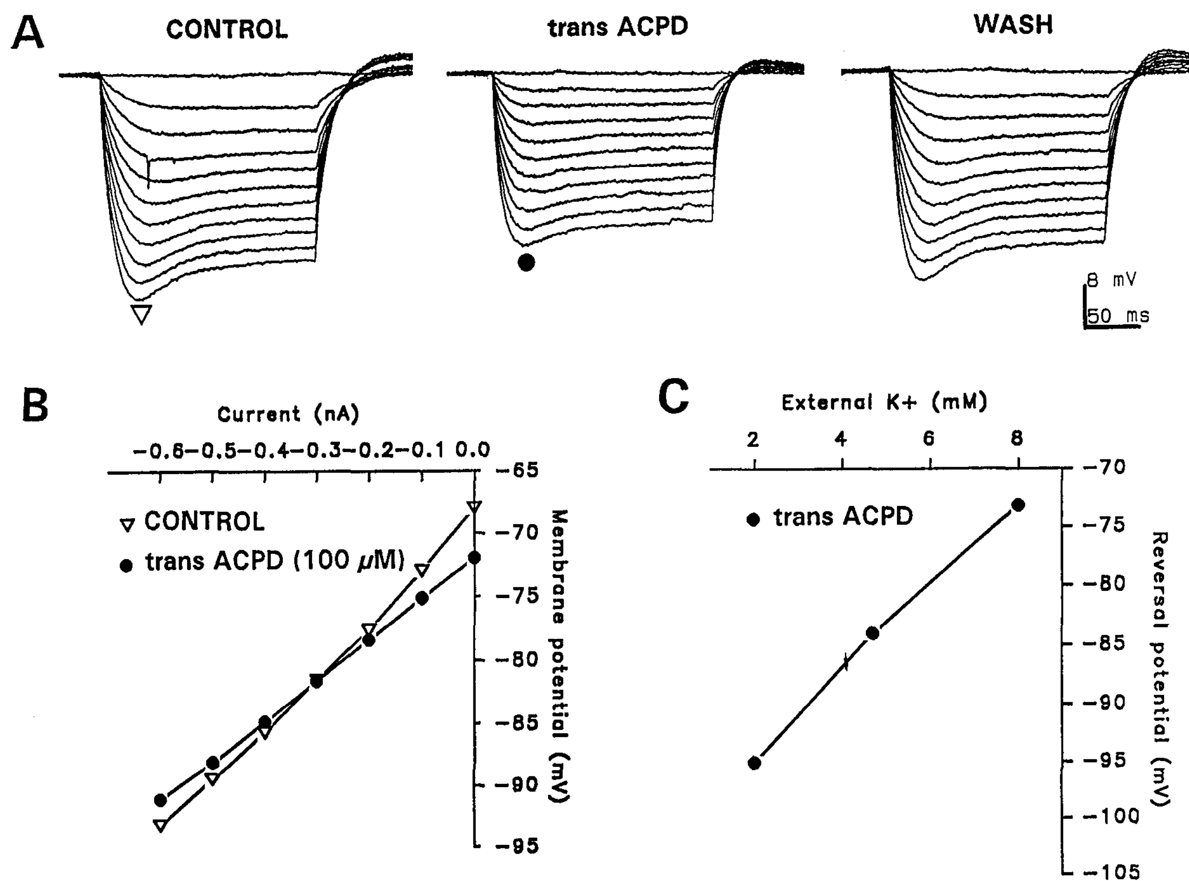

Figure 4. The reversal potential for the *trans*-ACPD–induced hyperpolarization approximates the equilibrium potential for K^+, $[E_K]$, and shifts in a Nernstian manner with changes in extracellular K^+. *A*, Electrotonic potentials (*triangle*) generated by transient (200 msec) hyperpolarizing current steps of increasing intensity (−0.05 to −0.5 nA; not shown) before, during, and after *trans*-ACPD (100 μM) superfusion. Note the decrease in the peak voltage deflection during *trans*-ACPD application (*circle*). *B*, A plot of the current–voltage relationship for the neuron shown in *A*, before (*triangles*) and during (*circles*) *trans*-ACPD (100 μM) application, indicated a reversal potential of −82.5 mV. *C*, In another neuron, extracellular $[K^+]_o$ is plotted as a function of the reversal potential for the *trans*-ACPD–induced hyperpolarization ($E_{t\text{-ACPD}}$); the $E_{t\text{-ACPD}}$ shifted in a manner predicted by the Nernst equation for a response mediated by a K^+ conductance.

recorded from pyramidal neurons (Miles and Poncer, 1993). Consequently, we examined the effects of chloride preloading on ACPD-mediated responses. When KCl-filled electrodes were used for recording, bicuculline-sensitive PSPs were depolarizing at the resting membrane potential −66 ± 1.3 mV (n = 8). However, the hyperpolarizing response to drop application of 1*S*,3*R*-ACPD (50 μM) was unaffected by chloride loading (Fig. 2*A*,*B*; see Fig. 8). In all neurons recorded with a KCl electrode, 1*S*,3*R*-ACPD evoked a membrane hyperpolarization (−3.7 ± 0.6 mV, n = 8), from a holding potential of −65 mV. In addition, the ACPD-mediated hyperpolarization persisted in the presence of bicuculline (30 μM) and CGP55845A (10 μM; control, 3.7 ± 1.2 mV; treatment, 3.8 ± 0.8 mV; n = 3, $p > 0.05$), concentrations previously demonstrated to block postsynaptic $GABA_A$ receptor activation in the BLA (Rainnie et al., 1991b) and $GABA_B$ receptors in hippocampal neurons (Davies et al., 1993; Jarolimek et al., 1993).

Washburn and Moises (1992) have reported a TTX-insensitive hyperpolarization and subsequent depolarization to occur within BLA neurons with the muscarinic agonist, oxotremorine-M. These responses were reduced by increasing extracellular potassium concentrations and were blocked by atropine (1 μM). Because of both the similarity in the BLA responses to oxotremorine-M and *trans*-ACPD, and dense innervation of the BLA by cholinergic fibers (Ben-Ari et al., 1977), we examined the effect of atropine (5 μM) on the ACPD-mediated response (Fig. 2*B*). Atropine had no effect on the hyperpolarizing response to 1*S*,3*R*-ACPD (50 μM; control, 3.8 ± 0.2 mV; atropine, 3.7 ± 0.3 mV; n = 3, $p > 0.05$) recorded at a membrane potential of −65 mV.

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enantiomer of *trans*-ACPD, 1*S*,3*R*-ACPD (50 μM), also induced a membrane hyperpolarization and decrease in input resistance. A_3, Subsequent superfusion of the ionotropic glutamate receptor antagonists CNQX (10 μM) and APV (50 μM) appeared slightly reduced but did not block the hyperpolarizing response of the BLA neuron to topical application of *trans*-ACPD. *B*, Amplitude of the 1*S*,3*R*-ACPD responses is dependent on membrane potential. In another cell, the amplitude of the 1*S*,3*R*-ACPD–induced hyperpolarization is greatly enhanced by holding the membrane at a more depolarized level (−59 mV vs −67 mV). *C*, 1*S*,3*R*-ACPD (100 μM) inhibits spontaneous EPSPs. In some cells, the conductance change induced by 1*S*,3*R*-ACPD was sufficient to inhibit spontaneous excitatory potentials. Membrane potentials = −67 mV in *A* and *C*. Downward deflections are electrotonic potentials resulting from injecting 0.2 nA, 200 msec current pulses across the membrane as a measure of membrane resistance.

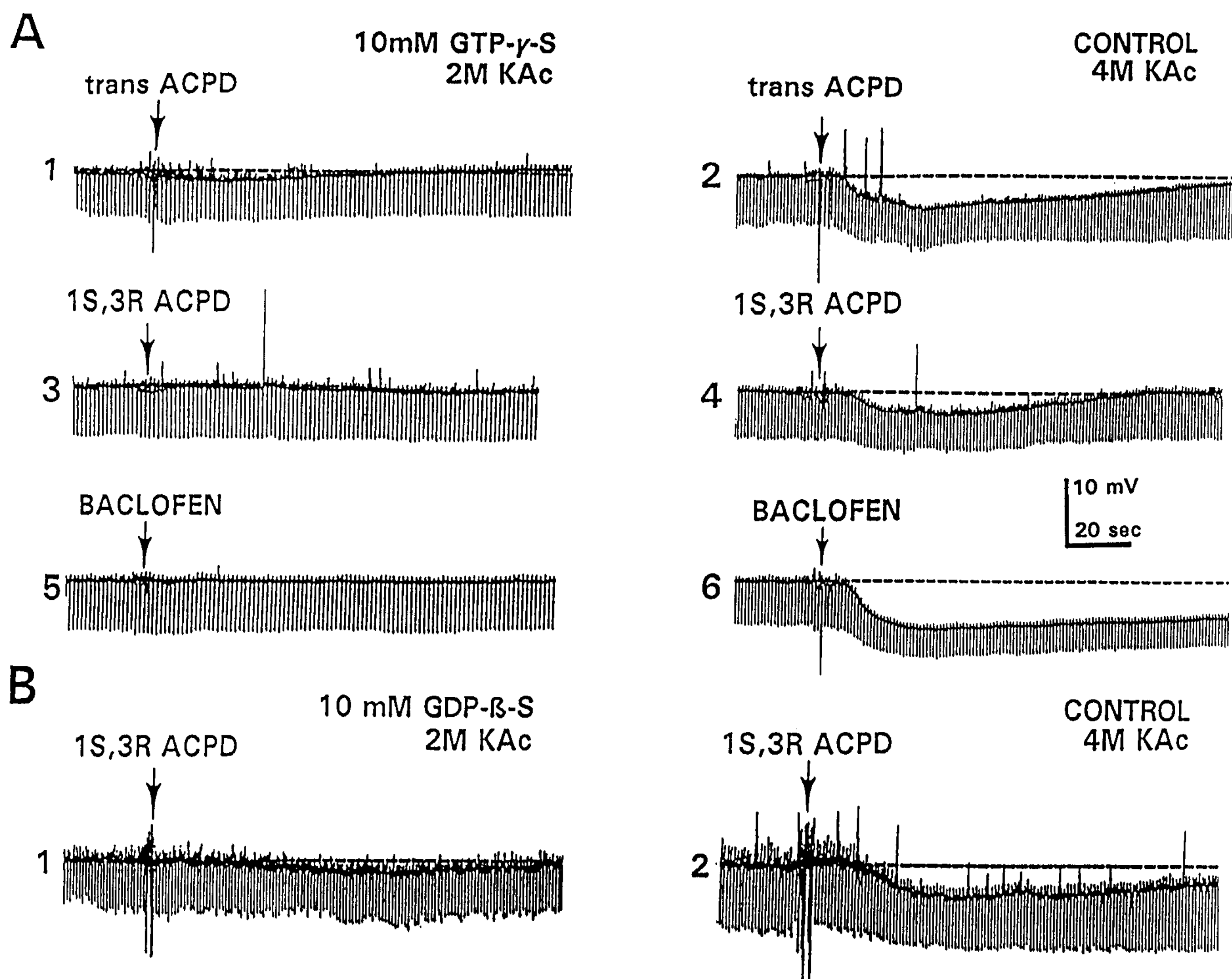

Figure 5. Intracellular injection of GTP-γ-S occludes and GDP-β-S reduces the hyperpolarizing response to *trans*-ACPD (100 μM) and 1*S*,3*R*-ACPD (100 μM). *A*, Intracellular injection of the nonhydrolyzable analog of GTP, GTP-γ-S (10 mM), occludes the response to topical application of both *trans*-ACPD (*1*) and 1*S*,3*R*-ACPD (*3*). In a slice from the same animal, a BLA neuron recorded with a conventional microelectrode containing 4 M K-acetate showed characteristic responses to topical application of *trans*-ACPD (*2*) and 1*S*,3*R*-ACPD (*4*). Topical application of baclofen (50 μM) causes a G-protein–dependent hyperpolarization of BLA neurons recorded with K-acetate containing microelectrodes (*6*). In the same neuron as in *1* and *3*, GTP-γ-S also occluded the response to baclofen (*5*). *B*, Intracellular injection of the nonhydrolyzable analog of GDP, GDP-β-S (10 mM), reduced the response to topical application of 1*S*,3*R*-ACPD (*1*). In a slice from the same animal, a BLA neuron recorded with a 4 M K-acetate–containing microelectrode showed a characteristic hyperpolarizing response to topical application of 1*S*,3*R*-ACPD (*2*). Downward deflections are electrotonic potentials generated in response to 200 msec, 0.1 nA current pulses passed across the membrane. Membrane potential was held at −65 mV with DC current injection in *A* and *B*.

The ACPD-induced hyperpolarization is mediated by an increase in K conductance

The ACPD-induced hyperpolarization was consistently associated with a decrease in membrane input resistance and corresponding increase in membrane conductance (control, 19.1 ± 1.7 nS; drug, 26.3 ± 2.2 nS; $n = 11$, $p < 0.0001$). A typical response is shown in Figure 4*A*. Here, a series of electrotonic potentials evoked in response to transient hyperpolarizing current pulses of increasing amplitude (200 msec, −0.1 to −0.6 nA) are shown before, during, and after *trans*-ACPD application. *Trans*-ACPD and 1*S*,3*R*-ACPD consistently reduced the amplitude of both the peak and steady state electrotonic potentials. Current–voltage relationships constructed before and during *trans*-ACPD application (Fig. 4*B*) show the reversal potential (E_{ACPD}) of the agonist-induced hyperpolarization to be −84.3 ± 3.0 mV ($n = 10$), which is close to that of potassium (−87 mV) estimated by the Nernst equation for an intracellular $[K^+]_i$ of 140 mM and extracellular $[K^+]_o$ of 4.7 mM. Furthermore, E_{ACPD} was dependent on extracellular potassium concentration (Fig. 4*C*). Hence, an increase in extracellular potassium shifted E_{ACPD} to a more depolarized level whereas decreased potassium shifted E_{ACPD} to more hyperpolarized levels. The shift in E_{ACPD} from 2 mM to 8 mM K was calculated by the Nernst equation to be 34.9 mV, whereas the measured value was about 22 mV. These data indicate that the ACPD-induced hyperpolarization is due primarily to an increased K conductance.

mGluR proteins are coupled to secondary cellular transduction mechanisms via G-proteins, and hence are sensitive to intracellular manipulations of GTP or GDP (Nakanishi, 1992).

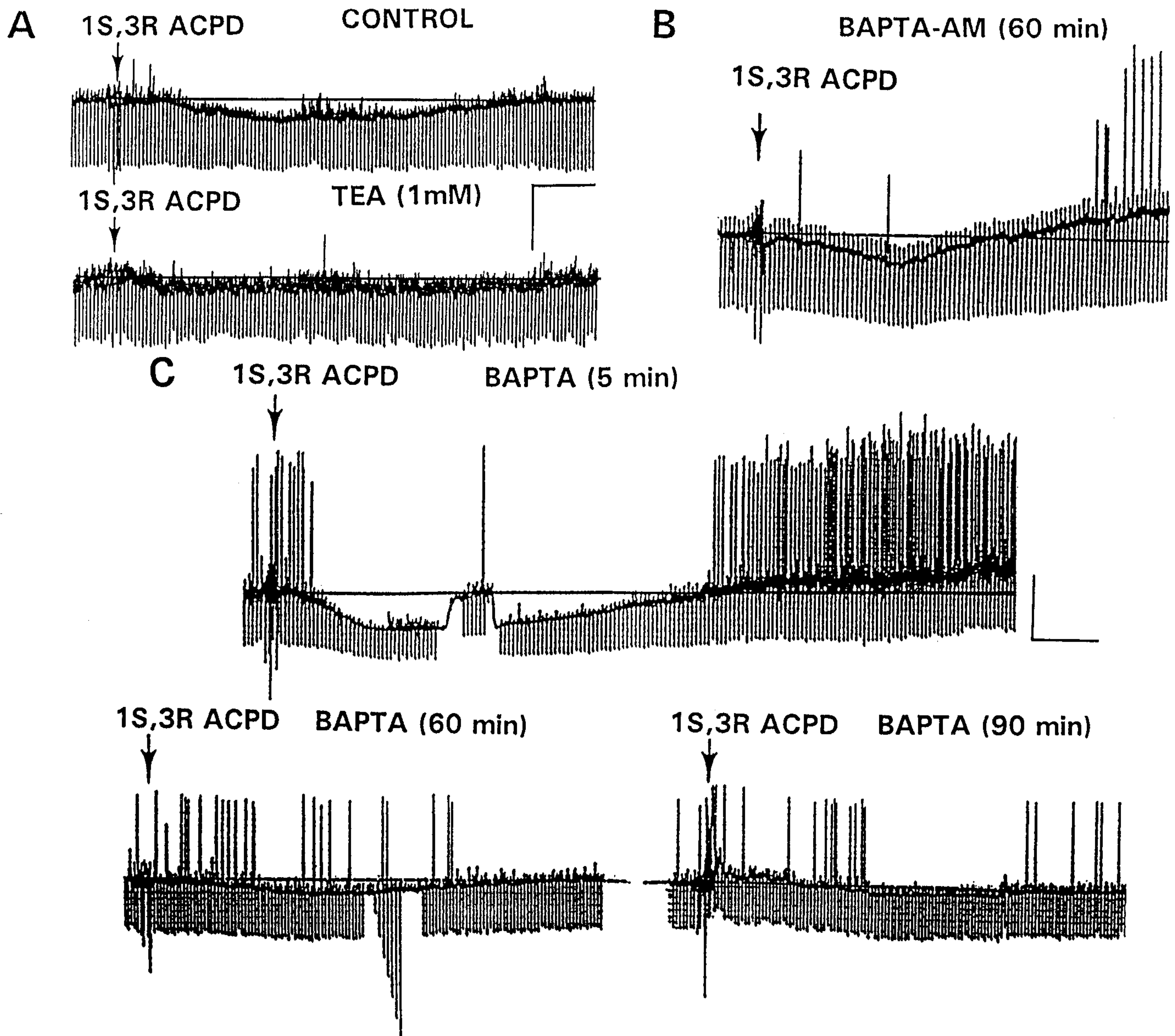

Figure 6. Effect of TEA and BAPTA on 1*S*,3*R*-ACPD (100 μM)–induced hyperpolarization. *A*, Effect of TEA (1 mM) on the ACPD hyperpolarization. Superfusing TEA (1 mM) blocked the ACPD response. *B*, Preincubation of slices for 60 min in aCSF containing the membrane-permeable form of BAPTA, BAPTA-AM (30 μM), failed to inhibit the hyperpolarization and subsequent depolarization induced by topical application of 1*S*,3*R*-ACPD in this neuron. *C*, Intracellular injection of the membrane impermeable calcium chelator BAPTA (200 mM) caused a time-dependent blockade of the 1*S*,3*R*-ACPD–induced hyperpolarization. Injection of BAPTA for 5 min had no effect on the 1*S*,3*R*-ACPD–induced hyperpolarization and subsequent depolarization. At this time point, action potential half-width was increased and the slow afterhyperpolarization following repetitive firing was abolished. In the same neuron, after 60 min of BAPTA injection, the hyperpolarization was significantly reduced, and after 90 min the hyperpolarization and membrane conductance change are abolished. Downward deflections are electrotonic potentials elicited in response to injection of 200 msec, 0.1 nA current pulses, as a measure of membrane resistance. Calibration: 10 mV, 20 msec.

We examined the sensitivity of the hyperpolarizing response to internal dialysis with nonhydrolyzable analogs of GTP (GTP-γ-S; 10 mM) and GDP (GDP-β-S; 10 mM) contained in the recording electrode. GTP-γ-S dialysis depressed the *trans*-ACPD and, subsequently, completely occluded the 1*S*,3*R*-ACPD–induced hyperpolarization (0.3 ± 0.1 mV, $n = 7$) as shown in Figure 5, A_1 and A_3. In contrast, a significant hyperpolarizing response to 1*S*,3*R*-ACPD was observed in other neurons from the same slices (4.0 ± 0.6 mV, $n = 6$; ANOVA, $p < 0.0001$; post hoc Bonferroni, control vs GTP-γ-S, $p < 0.001$), when recorded with an electrode filled only with 4 M K-acetate (Fig. 5A_2,A_4). Baclofen responses (0 mV, $n = 3$) were used as a control for G-protein activation by GTP-γ-S (Fig. 5A_5,A_6; post hoc Bonferroni: baclofen vs GTP-γ-S, $p > 0.05$; control vs baclofen, $p < 0.001$) since we have previously shown that the baclofen response in the BLA is inhibited by this procedure (Asprodini et al., 1992). Dialysis with GDP-β-S (Fig. 5*B*), however, only partially inhibited 1*S*,3*R*-ACPD–mediated responses (control, −3.5 ± 0.3 mV; GDP-β-S, −1.03 ± 0.03 mV; $n = 3$; $p < 0.001$, unpaired *t* test). GTP-γ-S (10 mM) is a tetralithium salt whereas GDP-β-S (10 mM) is a trilithium salt. It is unlikely that the effects of these compounds are due to a direct action of the

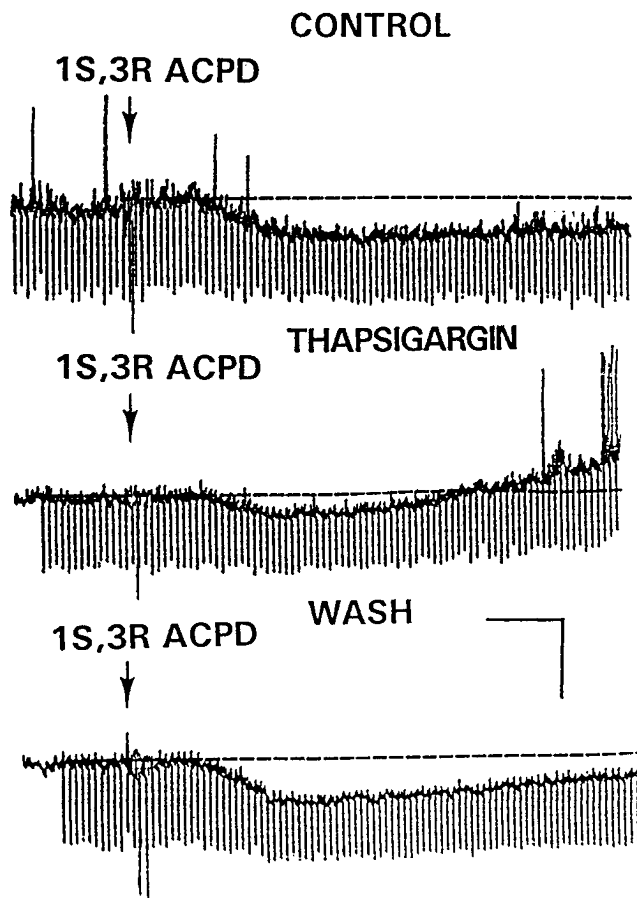

Figure 7. Effect of thapsigargin (2.5 μM) on the 1*S*,3*R*-ACPD (100 μM) hyperpolarization. In control (*upper trace*), topical application of 1*S*,3*R*-ACPD caused a membrane hyperpolarization accompanied by a decrease in input resistance. *Middle trace*, Thapsigargin slightly reduced input resistance and depressed the ACPD-induced membrane hyperpolarization after 20 min of superfusion. The reduction in input resistance caused by thapsigargin could not account for the depression of the ACPD response. *Lower trace*, The effect of thapsigargin was reversible. Downward deflections are electrotonic potentials elicited by passing 200 msec, 0.1 nA current pulses across the membrane as a measure of membrane resistance. Calibration: 10 mV, 20 msec.

lithium salt on phosphotidylinositol turnover since 1*S*,3*R*-ACPD–induced responses ($n = 2$; 4 mV) were observed when the electrodes were filled with K-acetate (2 M) and lithium (40 mM, a concentration equivalent to that of the GTP-γ-S salt). Furthermore, 1*S*,3*R*-ACPD responses were completely blocked in all but one neuron with a GTP-γ-S electrode whereas the responses persisted when recorded with a GDP-β-S–containing electrode.

Effect of alterations in the availability of intracellular Ca^{2+} on the 1S,3R-ACPD–mediated hyperpolarization

Apart from studies on the retina (Hirono and MacLeish, 1991), only one previous report has linked activation of metabotropic glutamate receptors within the CNS to enhancement of K conductance (Fagni et al., 1991). In that report, a *trans*-ACPD–activated outward potassium current was described in cultured cerebellar granule cells that was blocked by external tetraethylammonium (TEA; 1 mM). Therefore, we first examined the sensitivity of the 1*S*,3*R*-ACPD–mediated hyperpolarizing response in BLA neurons to pretreatment with TEA (Fig. 6*A*). In all neurons tested ($n = 4$), TEA (1 mM) significantly reduced the response to agonist application (control, 3.1 ± 0.4 mV; TEA, 0.3 ± 0.3 mV; $n = 4$, $p < 0.001$).

Fagni et al. (1991) also reported that ACPD activated a large-conductance calcium-dependent potassium channel, which was blocked by the intracellular calcium chelator BAPTA (11 mM). The sensitivity of the ACPD-mediated hyperpolarization to 1 mM TEA in our preparation suggested that activation of a similar channel may occur in BLA neurons. At μM concentrations 1*S*,3*R*-ACPD potently stimulates intracellular calcium mobilization in cerebellar neurons (Irving, 1990). We therefore examined the effect of BAPTA, and depletion of intracellular calcium with thapsigargin that releases and subsequently depletes intracellular Ca by inhibiting the Ca^{2+} pump (Thastrup et al., 1990). Treatment of the preparation with BAPTA-AM (30 μM), an ester of BAPTA, had little effect on the 1*S*,3*R*-ACPD–induced hyperpolarization even after 90 min of superfusion (Fig. 6*B*; $n = 4$; control, 2.75 ± 0.6 mV; BAPTA-AM, 3.0 ± 0.4 mV; $p > 0.05$, unpaired *t* test). A possible explanation for this lack of effect of BAPTA-AM is that BLA neurons do not possess sufficient esterases to cleave off the BAPTA within the cell. In contrast, however, intracellular dialysis by including BAPTA (200 mM) in the recording electrode caused a time-dependent reduction of the 1*S*,3*R*-ACPD–evoked hyperpolarization (Fig. 6*C*). In those neurons in which a stable recording lasted for greater than 90 min ($n = 4$) the 1*S*,3*R*-ACPD response was blocked, whereas in control cells at 90 min a full hyperpolarization was elicited by 1*S*,3*R*-ACPD (control, 3.0 ± 0.4 mV; BAPTA, 0.8 mV ± 0.3 mV; $p > 0.001$, unpaired *t* test; ANOVA, $p > 0.007$; post hoc Bonferroni: control vs BAPTA, $p < 0.05$; vs BAPTA-AM, NS; BAPTA vs BAPTA-AM, $p < 0.05$). Furthermore, the 1*S*,3*R*-ACPD–evoked hyperpolarization was also reduced by pretreatment with 2.5 μM thapsigargin (control, −5.2 ± 0.6 mV; thapsigargin, −2.2 ± 0.6 mV; $n = 3$, $p < 0.006$; Fig. 7), a concentration previously reported to deplete intracellular calcium stores and block the induction of LTP in hippocampal CA1 neurons (Harvey and Collingridge, 1992). In this cell thapsigargin had no effect on membrane potential or conductance; however, in two of five cells thapsigargin (2.5 μM) itself hyperpolarized the neurons (2.5 mV, $n = 2$). We also tested the effects of low Ca/high Mg and Cd (200 μM), a calcium channel blocker, on the 1*S*,3*R*-ACPD–induced response. The 1*S*,3*R*-ACPD hyperpolarization was reduced in low Ca/high Mg (control, 3.0 mV; treatment, 0.5 mV; $n = 2$) or Cd (control, 3.3 ± 0.3 mV; Cd^{2+}, 1.17 ± 0.6 mV; $n = 3$, $p > 0.05$) at a time when synaptic transmission was completely blocked. The response, although reduced, still persists in these solutions supporting the conclusion that 1*S*,3*R*-ACPD hyperpolarization is postsynaptic and requires, in part, release of intracellular calcium stores. Furthermore, these data also suggest that voltage-gated calcium channels may contribute to the 1*S*,3*R*-ACPD–induced hyperpolarizations.

Effect of putative metabotropic glutamate receptor antagonists

To characterize the metabotropic glutamate response, pharmacologically, we examined the effect of putative mGluR antagonists 2-amino-phosphonopropionic acid (L-AP3; Schoepp and Johnson, 1989) and 4-carboxy-3-hydroxyphenylglycine (4C3HPG; Birse et al., 1993). In the majority of neurons, superfusing L-AP3 (200 μM) hyperpolarized the membrane (Fig. 8*A*, second trace; 2.0 ± 0.8 mV; $n = 4$) whereas one cell was

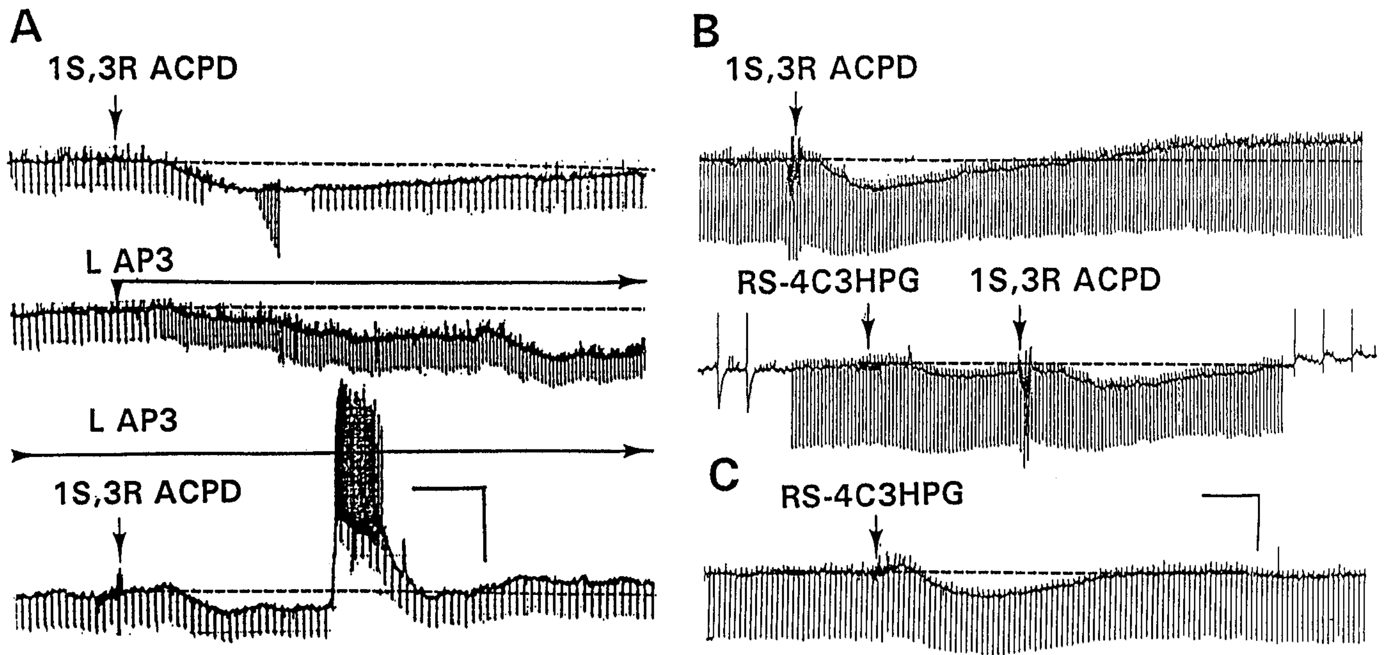

Figure 8. L-AP3 and *RS*-4C3HPG (50 μM) do not block 1*S*,3*R*-ACPD (100 μM) responses. *A*, Response to ACPD. Spontaneous EPSPs and IPSPs are inhibited during ACPD hyperpolarization (*upper trace*). Superfusion of L-AP3 (1 mM) hyperpolarizes the membrane with little change in membrane conductance (*middle trace*). *Lower trace*, ACPD response in presence of L-AP3 (1 mM) in the same cell as *A* and *B*. Membrane potential was returned to control levels by DC current injection prior to ACPD application. Downward deflections are electrotonic potentials recorded in response to injecting 0.2 nA hyperpolarizing current pulses; a series of current pulses of various magnitudes were tested during 1*S*,3*R*-ACPD application as a measure of membrane resistance (upper trace). Calibration: 10 mV, 10 sec; *middle trace*: 20 msec. RMP, −66 mV. *B*, Application of 1*S*,3*R*-ACPD hyperpolarizes the membrane (*upper trace*). *Lower trace*, Application of *RS*-4C3HPG (50 μM) hyperpolarized the membrane and decreases membrane resistance as measured by hyperpolarizing electrotonic potentials generated in response to passing 200 msec, 0.1 nA current pulses across the membrane; a subsequent response to 1*S*,3*R*-ACPD is occluded. Afterhyperpolarizations following a 100 msec, 1 nA depolarizing current injection are recorded prior to and after drug application. After 1*S*,3*R*-ACPD the afterhyperpolarization is replaced by an afterdepolarization. *C*, In another neuron, *RS*-4C3HPG (50 μM) hyperpolarizes the membrane and decreases membrane resistance. Calibration: 10 mV, 20 msec.

depolarized by L-AP3. In the presence of L-AP3, the hyperpolarizing response to 1*S*,3*R*-ACPD was unaffected (control, 2.3 ± 0.4; L-AP3, 2.4 ± 0.5 mV; $n = 5$; ANOVA, $p > 0.8$); the burst response observed in this neuron was not typically recorded in the presence of L-AP3. Drop application of 4C3HPG (100 μM) induced a membrane hyperpolarization and occluded the response to 1*S*,3*R*-ACPD (Fig. 8*B*, second trace). In fact, drop application of 4C3HPG appeared to mimic the ACPD hyperpolarizing response (Fig. 8*C*; 3.3 ± 0.4 mV, $n = 5$). This agonist action of 4C3HPG precluded its further use as an mGluR antagonist.

Discussion

The data presented here demonstrate for the first time that activation of postsynaptic metabotropic glutamate receptors produces a hyperpolarizing response at the resting potential in neurons of the BLA. The reversal potential of −84 mV for the ACPD-mediated hyperpolarization, its sensitivity to extracellular K^+ concentrations, and its blockade by GTP-γ-S and low concentrations of TEA and reduction by intracellular BAPTA, thapsigargin, and low Ca/high Mg further suggest that this hyperpolarizing response is mediated by G-proteins and involves intracellular release of calcium and subsequent activation of large-conductance calcium-dependent potassium channels.

Glutamate plays an important role in neurotransmission in both vertebrates and invertebrates. For the most part glutamate, in the vertebrate nervous system, has been assumed to have a purely excitatory role in transmission. Unlike vertebrate glutamate receptors, however, previous reports from the invertebrate system have shown that activation of postsynaptic glutamate receptors can also be inhibitory. In the sea hare *Aplysia californica* and freshwater mollusk *Planorbarius corneus*, glutamate and quisqualate activate second messenger–mediated increases in a potassium conductance (Yarowsky and Carpenter, 1976; Bolshakov et al., 1991). More recently, the glutamate-mediated increase in potassium conductance observed in *Aplysia* was mimicked by ACPD (Katz and Levitan, 1993). In the vertebrate retina glutamate-activated outward currents have been associated with both a conductance decrease (Nawy and Jahr, 1990) in slices and increase (Hirano and MacLeish, 1991) in isolated cells, having reversal potentials close to zero and to the equilibrium potential for potassium, respectively. It would seem, therefore, that increases in potassium conductance in response to glutamate agonists in invertebrate nervous systems, isolated retina cells, and mammalian brain neurons of the amygdala suggest that activation of postsynaptic metabotropic glutamate receptors may, in part, express the inhibitory responses to glutamate.

To date few researchers have reported membrane hyperpolarizations or outward currents in response to *trans*-ACPD in vertebrate preparations, and of these, none characteristically resemble the response in the BLA. East and Garthwaite (1992) recorded in cerebellar granule cells extracellular population responses to 1*S*,3*R*-ACPD in which the hyperpolarizations usually followed the depolarizations. In cultured cerebellar granule cells, however, *trans*-ACPD activated a large-conductance Ca-

dependent K channel but no change in the amplitude of single channel currents was recorded under cell-attached conditions; moreover, the threshold for the *trans*-ACPD effect on macroscopic K^+ current was −20 mV (Fagni et al., 1991). The authors conclude that these data were not consistent with a membrane hyperpolarization at rest potential, unlike the hyperpolarization in the amygdala that is observed at resting potential. In Purkinje cells of the cerebellum, an outward current has been recorded following an inward current; this outward current, however, had a reversal potential positive to 0 mV and was accompanied by a decrease in conductance (Staub et al., 1993) whereas in the BLA the ACPD hyperpolarization reversed at −84 mV and was accompanied by an increase in conductance. The ACPD outward current in Purkinje cells is not comparable to the ACPD hyperpolarizing response in the BLA. The actions of ACPD in cerebellar neurons are clearly different from those recorded in the BLA and so the 1*S*,3*R*-ACPD–induced membrane hyperpolarization in the BLA appears to be a novel response.

At low micromolar concentrations TEA specifically blocks large-conductance calcium-dependent K^+ channels (BK channels) but not small-conductance calcium-dependent K^+ channels (SK channels; for review, see LaTorre et al., 1989). Similar to the ACPD-mediated inhibitory response observed in this study, the inhibitory response to glutamate in *Planorbarius* (Bolshakov et al., 1991) was also blocked by low micromolar concentrations of TEA, suggesting that these two responses may share a common mechanism of action, that is, activation of a large-conductance Ca-dependent K channel activity, possibly BK.

In the present study, the reduction but not complete block of the ACPD response either by (1) intracellular injection of the calcium chelator BAPTA, (2) superfusing with low Ca/high Mg or Cd solution, or (3) the depletor of intracellular calcium stores, thapsigargin (Thastrup et al., 1990) further suggests that release of calcium from intracellular stores may contribute to activation of BK channels. The difference in time required for the BAPTA-mediated blockade of the ACPD response compared to that of GTP-γ-S may result from the differences in cytosolic concentration of each drug needed to affect their target regions of the cascade system. Hence, much higher intracellular levels of BAPTA may be needed to chelate cytosolic calcium than are required for GTP-γ-S to affect receptor–effector coupling. As both drugs diffuse from the microelectrode into the cytosol, it might be expected that a longer impalement may be required to observe a full BAPTA blockade of the ACPD response. However, this time factor may also be due to differing rates of diffusion of the two substances. Recently, Takagi et al. (1992) have suggested that the metabotropic glutamate receptors mediating glutamate responses are located on the dendrites of Purkinje cells. If the mGluRs mediating the ACPD-mediated hyperpolarization are also located on the dendrites of BLA neurons, this additional spatial barrier may further contribute to delay of the full expression of BAPTA blockade.

In the vertebrate CNS, activation of either mGluR1α, mGluR1β, or mGluR5 receptors mediate the stimulation of inositol triphosphate (IP_3) metabolism (Sugiyama et al., 1987; for review, see Nakanishi, 1992; Schoepp, 1993) that can subsequently regulate intracellular calcium mobilization (Murphy and Miller, 1988). Although we did not examine the source of calcium or related mechanisms by which mGluR activation results in calcium-dependent K conductance, mRNA for mGluR1 receptors (Shigemato et al., 1992) is expressed in moderate levels in the BLA, and activation of metabotropic glutamate receptors stimulates IP_3 formation (Aramori and Nakanishi, 1992). In addition, cytosolic IP_3 receptors have been identified immunohistochemically in this nucleus (Nakanishi et al., 1991). It is possible that in the BLA the ACPD-induced membrane hyperpolarization may be mediated by release of calcium from IP_3 stores that activates a K conductance. Alternatively, another second messenger system may be involved since mRNA for the mGluR2 receptor is found abundantly in the basolateral amygdala (Ohishi et al., 1993).

In the present study, pharmacological differentiation of the specific receptors mediating the ACPD hyperpolarization was hindered since the putative antagonists of metabotropic glutamate receptors L-AP3 and 4C3HPG had agonist actions. In hippocampal neurons, L-AP3 (1 mM) has been shown to increase IP_3 formation itself; it has furthermore been suggested that the L-AP3 block of 1*S*,3*R*-ACPD–induced IP_3 formation is due to desensitization of the receptor (Lonart et al., 1992). More recently, L-AP3 was shown to mimic various 1*S*,3*R*-ACPD effects including inhibiting cAMP formation (Schoepp and Johnson, 1993). Agonist actions of 4C3HPG have also been reported. In cerebral cortical slices of rat brain, 4C3HPG acts as an agonist in stimulating phosphoinositide hydrolysis and potentiating cAMP formation (Winder et al., 1993). 4C3HPG has also been shown to mimic metabotropic glutamate receptor–mediated responses in hippocampal slices (Gerber and Gahwiler, 1992).

In their patch-clamp study of isolated cerebellar granule cells, Fagni et al. (1991) noted that the ACPD-mediated activation of BK channels was mimicked by the cholinergic agonist carbachol. In *Aplysia* neurons ACh, dopamine, and histamine evoke potassium conductances that, although acting at different receptors, may be mediated by activation of the same ion channel (Ascher and Chesnoy-Marchais, 1982). It is possible that in the vertebrate CNS multiple metabotropic receptors may also activate a common ion channel. Indeed, the similarity between responses mediated by the muscarinic subclass of ACh receptors and those mediated by the metabotropic subclass of glutamate receptors is striking (see Bonner, 1989; Nakanishi, 1992). In the BLA, Washburn and Moises (1992) have reported a biphasic potential, hyperpolarization–depolarization, evoked in response to exogenous application of the muscarinic receptor agonist oxotremorine-M that was almost identical to the biphasic ACPD response seen in this study. These authors, however, concluded that the hyperpolarization was due to an indirect release of GABA from inhibitory interneurons. In our study the ACPD-evoked hyperpolarization (1) was resistant to atropine, bicuculline, and CGP55845A, (2) was unaffected by intracellular chloride loading, was (3) resistant to TTX, (4) changed reversal potential with alterations in extracellular K^+ concentrations, and (5) was blocked by 1 mM extracellular TEA. We therefore conclude that the ACPD-mediated hyperpolarization results from a direct increase in a postsynaptic K^+ conductance, possibly through large-conductance calcium-dependent K channels, but not from either an indirect release of ACh or GABA.

In summary, neurons in the BLA appear to possess a unique hyperpolarizing response to metabotropic glutamate receptor activation mediated through a calcium-dependent K conductance, the functional role of which may be inhibitory.

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