Group II Metabotropic Glutamate Receptors Modulate Extracellular Glutamate in the Nucleus Accumbens

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ABSTRACT

The regulation of extracellular glutamate in the nucleus accumbens by group II metabotropic glutamate receptors (mGluR2/3) was examined in vivo. Stimulation of mGluR2/3 with 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate (APDC) or N-acetylaspartylglutamate reduced extracellular glutamate levels. Conversely, blockade of mGluR2/3 by LY143495 or (RS)-1-amino-5-phosphonoindan-1-carboxylic acid (APICA) increased extracellular glutamate, an effect antagonized by the coadministration of APDC. These effects likely involve both vesicular and nonvesicular release of glutamate, because the increase in glutamate by APICA or the decrease by APDC was prevented by blocking N-type calcium channels and the release of glutamate after potassium-induced membrane depolarization was antagonized by APDC. In addition, blockade of the cystine-glutamate exchange, a major nonvesicular source of extracellular glutamate, by (S)-4-carboxyphenylglycine blocked the effects induced by either APDC or APICA. However, blockade of Na+ channels by tetrodotoxin or Na+-dependent glutamate transporters by DL-threo-β-benzyloxyaspartate failed to affect the alterations in extracellular glutamate by APICA or APDC, respectively. Group II mGluRs are Gαi-coupled and coperfusion with the cAMP-dependent protein kinase (PKA) activator Sp-cAMPS blocked the reduction in glutamate by APDC and the PKA inhibitor Rp-cAMPS prevented the elevation in glutamate by APICA. Taken together, these data support three conclusions: 1) group II mGluRs regulate both vesicular and nonvesicular release of glutamate in the nucleus accumbens, 2) there is tonic in vivo stimulation of mGluR2/3 by endogenous glutamate, and 3) modulation of group II mGluRs of extracellular glutamate is Ca2+- and PKA-dependent.

Metabotropic glutamate receptors (mGluRs) belong to a class of G protein-coupled receptors that is comprised of eight different subtypes that have been organized into three groups based upon sequence homology and coupling to intracellular messengers. Group I receptors (mGluR1,5) are coupled to phospholipase C, whereas group II (mGluR2,3) and group III (mGluR4,6,7,8) receptors are negatively coupled to adenylyl cyclase (for review, see Conn and Pin, 1997). Group II and III mGluRs act to inhibit neurotransmitter release both as autoreceptors located on glutamatergic terminals or as presynaptic heteroreceptors. Extensive studies have emerged indicating that mGluRs play an important role in neuroplasticity (Anwyl, 1999), and various drugs targeting group II mGluRs have therapeutic potential including, protection from excitotoxicity, treatment of anxiety, Parkinson's disease, schizophrenia, and drug addiction (for review, see Conn and Pin, 1997). A possible role in addiction is indicated by the recently described involvement of glutamate transmission in the nucleus accumbens (NAcc) and the possibility that reducing glutamate transmission by group II mGluR agonists may be of therapeutic benefit (Cornish and Kalivas, 2000; Vanderschuren and Kalivas, 2000). Group II mGluRs are expressed in the nucleus accumbens (Ohishi et al., 1993a, 1993b; Testa et al., 1998). Selective activation of the group II mGluRs in the NAcc blocks amphetamine-induced locomotor behavior (Kim et al., 2000). In vitro electrophysiological studies in brain slices confirm that group II mGluRs inhibit glutamate release in the NAcc (Mazoni et al., 1997). Moreover, in vivo microdialysis studies show that group II agonists reduce extracellular dopamine in the NAcc (Hu et al., 1999).

Although the presence of group II mGluRs in the NAcc has been established, the identity and the properties of group II mGluRs in modulation of glutamate release remains unclear. For example, the basal level of extracellular glutamate is derived from both vesicular and nonvesicular sources (Timmerman and Westerink, 1997), and it is not known which

ABBREVIATIONS: mGluRs, metabotropic glutamate receptors; APDC, (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate; NAAG, N-acetylaspartylglutamate; 2-PMPA, 2-(phosphonomethyl) pentanedioic acid; APICA, (RS)-amino-5-phosphonoindan-1-carboxylic acid; (S)-4CPG, (S)-4-carboxyphenylglycine; TTX, tetrodotoxin; TBOA, DL-threo-β-benzyloxyaspartate; Sp-/Rp-cAMPS, Sp-/Rp-adenosine 3'5'-cyclic monophosphothioate triethylamine; PKA, CAMP-dependent protein kinase; ANOVA, analysis of variance; PLSD, protected least significant difference; NAcc, nucleus accumbens; NMNDA, N-methyl-D-aspartate.
glutamate pool is modulated by group II mGluRs. In addition, activation of group II mGluRs has been shown to inhibit cAMP formation in in vitro expression systems, brain slices, and neuronal cultures, but it is unknown whether cAMP signaling is also mediating the effects of group II mGluRs in vivo (for review, see Conn and Pin, 1997). Thus, the present study used in vivo microdialysis combined with mGluR2/3 immunoblotting to characterize the modulation of extracellular glutamate by direct perfusion of various group II selective agonists or antagonists into the NAcc. Experiments were also conducted to examine the involvement of various ion channels, the cystine-glutamate exchanger, glutamate transporters, and the intracellular cAMP/cAMP-dependent protein kinase (PKA) signaling cascade in mGluR modulation of glutamate release.

Materials and Methods

Animals Housing and Surgery. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The subjects were male Sprague-Dawley rats (Charles Rivers Laboratories, Inc., Wilmington, MA) weighing 250 to 275 g upon arrival and were individually housed in an American Association for Laboratory Animal Care-approved facility maintained on a 12-h light/dark cycle (lights on 7 AM). All experimentation was conducted during the light period. Using ketamine (100 mg/kg) and xylazine (3 mg/kg) anesthesia, dialysis guide cannulae (20 gauge, 14 mm; Small Parts, Roanoke, VA) were implanted over the nucleus accumbens (+1.6 mm anterior to Bregma, ±1.6 mm mediolateral, –4.7 mm ventral to the skull surface according to the atlas of Paxinos and Watson (1986)) using a 6° angle from vertical. The guide cannulae were fixed to the skull with four stainless steel skull screws (Small Parts) and dental acrylic. Surgeries were performed 5 to 7 days after arrival of the subjects, and dialysis experiments were begun 1 week after the surgical procedure.

In Vivo Microdialysis. The night before the experiment, concentric microdialysis probes (with 2 mm of active membrane) were inserted 3 mm beyond tips of guide cannulae into the nucleus accumbens. Dialysis buffer (5 mM KCl, 140 mM NaCl, 1.4 mM CaCl2, 1.2 mM MgCl2, 5.0 mM glucose, plus 0.2 mM phosphate-buffered saline to give a pH of 7.4) was advanced through the probe at a rate of 2 µl/min via syringe pump (Bioanalytical Systems, West Lafayette, IN). Beginning at 2 h after turning on the pump at 8 AM the next morning, baseline samples were collected at 20-min intervals for 100 min. After collecting the baseline samples various drugs were administered via reverse dialysis into the NAcc.

Multiple doses of each mGluR agonist or antagonist were administered alone or in combination with other drugs. Dosage ranges of the various drugs were based upon the relative EC50 or IC50 values for binding to the respective receptors. N-acetylaspartylglutamate (NAAG) was purchased from Sigma-RBI (Natick, MA), and all other mGluR agonists and antagonists, including (2R,4R)-aminopropylidene-2,4-dicarboxylate (APDC), (RS)-1-amino-5-phosphonoindan-1-carboxylic acid (APICA), LY143495, and (S)-4-carboxyphenylglycine [(S)-4CPG] were purchased from Tocris (Ballwin, MO). NAAG was dissolved with filtered dialysis buffer (see below), whereas all other mGluR compounds were initially dissolved in 0.1 N NaOH (Sigma, St. Louis, MO) and neutralized with 0.1 N HCl (Sigma) to a concentration of 10-2 M. Working concentrations were then made by diluting with filtered dialysis buffer. Diltiazem and tetrodotoxin (TTX) were purchased from Tocris, and o-conotoxin GVIA, Sp- and Rp-adenosine 3′,5′-cyclic monophosphothioate triethylamline (Sp-cAMPS, Rp-cAMPS) were obtained from Sigma-RBI. 2-(Phosphonomethyl) pentanedioic acid (2-PMPA) was a gift from Guilford Pharmaceuticals, Inc. (Baltimore, MD) and dl-threo-β-benzoyloxyaspartate (TBOA) was a gift from Dr. Keiko Shimamoto (Suntory Institute for Bioorganic Research, Osaka, Japan). All of the drugs were dissolved with filtered dialysis buffer and were freshly prepared on day of the experiment. In some experiments KCl was used to increase glutamate release and in these experiments NaCl was reduced proportionally to retain iso-osmolality.

Quantification of Glutamate. The concentration of glutamate in the dialysis samples was determined using HPLC with fluorometric detection. The dialysis samples were collected into 10 µl of 0.05 M HCl containing 2 pmol of homoserine as an internal standard. The mobile phase consisted of 13% acetyl-nitrite (v/v), 100 mM NaHPO4, and 0.1 mM EDTA, pH 6.0. A reversed-phase column (10 cm, 3 µm ODS; Bioanalytical Systems, West Lafayette, IN) was used to separate the amino acids, and precolumn derivatization of amino acids with o-phthalaldehyde was performed using a model 540 autosampler (ESA, Inc., Chelmsford, MA). Glutamate was detected by a fluorescence spectrophotometer (Linear Flour LC 305; ESA Inc.) using an excitation wavelength of 336 nm and an emission wavelength of 420 nm. The area under the curve of the glutamate and homoserine peaks was measured with ESA 501 Chromatography Data System. Glutamate values were normalized to the internal standard homoserine and compared with an external standard curve for quantification. The limit of detection for glutamate was 1 to 2 pmol.

mGluR2/3 Immunoblotting. To determine the existence of mGluR2/3 proteins in the NAcc, eight rats were decapitated, and the brains were rapidly removed and dissected into coronal sections on ice. The appropriate brain regions were sampled on an ice-cooled Plexiglas plate using a 15-gauge tissue punch, including the prefrontal cortex, parietal cortex, ventral tegmental area, dorsolateral striatum, medial nucleus accumbens (predominately medial shell), and lateral nucleus accumbens (core). Brains punches were immediately frozen on dry ice and stored at –80°C until homogenized for immunoblotting.

The dissected brain punches were homogenized with a handheld tissue grinder in homogenization medium (0.32 M sucrose, 2 mM EDTA, 1% sodium dodecyl sulfate, 50 µM phenyl methyl sulfonyl fluoride, and 1 µg/ml leupeptin, pH 7.2), subjected to low-speed centrifugation (2000g, to remove insoluble material) and stored at –80°C. Protein determinations were performed using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Samples (30 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel (8%) electrophoresis utilizing a mini-gel apparatus (Bio-Rad), transferred via semidry apparatus (Bio-Rad) to nitrocellulose membrane, and probed for the proteins of interest (1 gel/protein/brain region). mGluR2/3 was identified using a rabbit anti-rat antibody (1:3000) purchased from Upstate Biotech (Lake Placid, NY) that was made against a peptide containing the C terminus. In control experiments a peptide synthesized having the same 21 amino acid sequence on the C terminus of mGluR2/3 was used to competitively inhibit the binding of antibody to mGluR2/3. Labeled proteins were detected using an horseradish peroxidase-conjugated anti-rabbit secondary IgG diluted 1:3000 purchased from Upstate Biotech and visualized with enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL). Assurance of even transfer of proteins was evaluated with Ponceau S (Sigma) followed by de-staining with de-ionized water. Immunoreactive levels were quantified by integrating band density × area using computer-assisted densitometry (NIH Image version 1.60). The density × area measurements were averaged over three control samples for each gel and all bands were normalized as percent of the control values.

Histology. After the dialysis experiments, rats were administered an overdose of pentobarbital (>100 mg/kg i.p.) and transectionally perfused with 0.9% saline followed by 10% formalin solution. Brains were removed and placed in 10% formalin for at least 1 week to ensure proper fixation. The tissue was blocked, and coronal sec-
tions (100 μm thick) were made through the site of dialysis probe with a vibratome. The brains were then stained with cresyl violet to verify anatomical placement according to the atlas of Paxinos and Watson (1986).

**Statistical Analysis.** The StatView statistics package was used to estimate statistical significance. A one-way ANOVA with repeated measures over dose was used to determine the effect of individual drugs on extracellular glutamate levels. A two-way ANOVA with repeated measures over time or dose were used to compare between treatments. Upon identification of statistical significance, post hoc comparisons were made with a Fischer’s PLSD.

**Results**

mGluR2/3 Immunoproteins Are Highly Expressed in the Nucleus Accumbens. A high density of mGluR2/3 immunoproteins were detected in many brain regions including the shell and core of the nucleus accumbens, prefrontal cortex, ventral tegmental area, and striatum of rats. Both dimer and monomer forms were detected, and the dimer was the predominant form of mGluR2/3 in all brain nuclei examined. Figure 1 shows representative immunoblots that illustrate the two forms of mGluR2/3 proteins in the nucleus accumbens (shell and core) and prefrontal cortex. Figure 1 also shows that both the dimer and monomer forms could be completely absorbed by a synthetic peptide having the identical 21 amino acid sequence with the C terminus of mGluR2/3.

**Group II mGluRs Reduce Extracellular Glutamate Levels in the Nucleus Accumbens.** A selective agonist or antagonist for mGluR2/3 was perfused into the accumbens by reverse microdialysis and the levels of extracellular glutamate were estimated. Figure 2A shows that the mGluR2/3 agonist APDC elicited a dose-dependent decrease in extracellular glutamate levels and this effect was attenuated by the specific group II mGluR antagonist APICA (Fig. 2B). The threshold dose for producing a significant reduction was 5 μM APDC, and the reduction in extracellular glutamate was washed out by washing out the drug with dialysis buffer. Furthermore, NAAG, a mGluR3 agonist (Wroblewska et al., 1997; Schweitzer et al., 2000) elicited a dose-dependent decrease in extracellular glutamate levels in the nucleus accumbens (Fig. 2, C and D). The minimal effective dose of NAAG was 10 μM. The experiment was conducted in the presence of 500 μM 2-PMPA to inhibit the formation of glutamate derived from the metabolism of NAAG by NAALADase (Slusher et al., 1999). In the absence of 2-PMPA the capacity of NAAG to inhibit extracellular glutamate could not be demonstrated (data not shown). 2-PMPA alone did not significantly alter the extracellular levels of glutamate (Fig. 2C).

Conversely, perfusion of the mGluR2/3 antagonist APICA or LY143495 into the nucleus accumbens produced a dose-
dependent increase in extracellular glutamate (Fig. 3). Further the increase by APICA was reversed by co perfusion of the group II agonist APDC (Fig. 3B). The threshold dose for a significant response by APICA and LY143495 was 10 μM and 10 nM, respectively, and drug washout with dialysis buffer reversed the increase by the highest dose of APICA (1 mM).

**mGluR2/3 Modulation of Extracellular Glutamate is Ca^{2+}-Dependent.** Basal extracellular glutamate derives from both neuronal and glial sources, and can be derived from vesicular or cytoplasmic pools (Timmerman and Westerink, 1997). Vesicular neurotransmitter release by high K+ is predominantly Ca^{2+}-dependent (for review, see Timmerman and Westerink, 1997). To determine whether the reduction in extracellular glutamate by the group II mGluR agonists is derived from vesicular stores of glutamate, the capacity of APDC to reverse the release of glutamate by a high concentration of K+ (80 mM) was examined. Figure 4, A and B, illustrate that the high K+-evoked glutamate release was significantly inhibited by the coadministration of 50 μM APDC. In further support of a role for Ca^{2+}-dependent vesicular release of glutamate, either the L- or N-type Ca^{2+} channel blockers diltiazem or ω-conotoxin GVIA, respectively, was co infused into the NAcc with APICA. Either drug completely blocked the elevation of extracellular glutamate produced by APICA (Fig. 5A). Whereas diltiazem alone had no significant effect, ω-conotoxin GVIA alone significantly reduced the basal level of extracellular glutamate by 30 to 40%. Furthermore, coadministration of ω-conotoxin GVIA blocked the capacity of APDC to reduce extracellular glutamate (Fig. 4B). These data suggest that the reduction in basal extracellular glutamate by N-type Ca^{2+} channel blockade and mGluR2/3 stimulation were not additive and may involve the same or overlapping mechanisms.

In contrast to the involvement of extracellular Ca^{2+}, pretreatment with the voltage-dependent Na+ channel blocker TTX at a dose sufficient to nearly eliminate detectable extracellular levels of monoamine transmitters (1 μM; Timmerman and Westerink, 1997) did not block the dose-dependent increase in extracellular glutamate elicited by APICA (Fig. 5C). TTX alone did not significantly alter the basal concentration of glutamate. This result argues that mGluR2/3 directly regulates Ca^{2+}-dependent release of glutamate and is not acting indirectly via a trans-synaptic mechanism. Although Na^{+}-dependent glutamate transporters play an important role in modulating the basal level of extracellular glutamate, blockade of glutamate uptake by TBOA, a broad-spectrum glutamate uptake inhibitor (Shimamoto et al., 1998), did not attenuate the APDC-induced reduction in glutamate (Fig. 5D), suggesting that the effect of mGluR2/3 stimulation on extracellular glutamate is independent of glutamate transporters.

**mGluR2/3 Involves Cystine-Glutamate Exchange.** The basal level of extracellular glutamate measured by microdialysis is predominantly controlled by cystine-glutamate exchange, which provides the primary source of extracellular, nonvesicular glutamate (Baker et al., 2001). To determine whether group II mGluRs might reduce extracellular glutamate by negatively modulating cystine/glutamate exchange, the inhibitor of cystine/glutamate exchange (S)-4CPG (Ye et al., 1999) was infused into the NAcc. Co infusion of (S)-4CPG with APICA or APDC prevented the increase in glutamate by APICA or the decrease by APDC (Fig. 6, A and B). (S)-4CPG (5 μM) alone decreased extracellular glutamate by approximately 50% (Fig. 6A).

**Signaling through PKA Mediates Group II mGluR Reduction in Extracellular Glutamate.** Group II mGluRs are negatively coupled to adenylate cyclase and PKA via inhibitory Gα proteins (Conn and Pin, 1997; Anwyl, 1999). To evaluate a role for PKA in the capacity of mGluR2/3 to modulate extracellular glutamate levels, the PKA activator Sp-cAMPS or the PKA inhibitor Rp-cAMPS...
was perfused into the accumbens via the dialysis probe in combination with the mGluR2/3 agonist APDC or the antagonist APICA. Figure 7, A and C show the effect of increasing doses of Sp-cAMPS or Rp-cAMPS alone. Although Sp-cAMPS elevated glutamate levels at lower doses and decreased levels at higher doses, Rp-cAMPS reduced glutamate at lower doses and increased levels at higher doses. Based upon these dose-response curves a relatively low dose of each drug (5 nM) was coadministered with APDC or APICA. Figure 7B shows that Sp-cAMPS attenuated the APDC-induced decrease in extracellular glutamate. Conversely, Rp-cAMPS inhibited APICA-induced increase in extracellular glutamate (Fig. 7D). The inhibitory effect of both Sp-cAMPs and Rp-cAMPs were reversible because after wash-out with dialysis buffer, the capacity of APDC to reduce and APICA to elevate extracellular glutamate was restored.

Histology. Figure 8 depicts the dialysis probe placements in the nucleus accumbens. The majority of probe placements in the nucleus accumbens were at or medial to the anterior commissure. Placements tended to be primarily in the core of the nucleus accumbens, although a number were located at the interface between the core and either the medial or the ventral limb of the shell, and a minority of placements were primarily in the shell. In addition, some probes were partly (<30%) dorsal to the nucleus accumbens in the striatum or septal region.

Discussion

These data provide in vivo evidence that pharmacological stimulation of group II mGluRs in the nucleus accumbens reduces the basal concentration and K⁺-evoked increases in extracellular glutamate. Moreover, group II mGluRs bear significant endogenous tone because blockade of mGluR2/3 elevates extracellular glutamate levels. The effects of the group II compounds were shown to require active L- and N-type Ca²⁺ conductances, as well as functional cystine-glutamate exchange, and to be signaled through cAMP/PKA cascade. In contrast, there was no role identified for voltage-dependent sodium channels or glutamate transporters.

Group II mGluRs Act as Autoreceptors to Inhibit Presynaptic Glutamate Release in the Nucleus Accumbens. In vitro electrophysiological experiments have revealed that a prominent physiological effect of mGluR2/3 agonists in the cortex and hippocampus is to reduce glutamatergic transmission by stimulating presynaptic autoreceptors (Anwyl, 1999), which has been confirmed as well in studies examining in vitro glutamate release (Cartmell and Schoepp, 2000). Although two in vivo studies have revealed the capacity of systemically administered mGluR2/3 agonist to reduce evoked glutamate release in the prefrontal cortex and striatum (Battaglia et al., 1997; Moghaddam and Adams, 1998), the present study is the first in vivo demonstration that locally stimulating group II mGluRs lowers extracellular glutamate. Moreover, the in vivo measurements revealed the presence of substantial tone by endogenous glutamate on mGluR2/3 in the nucleus accumbens. Thus, blocking mGluR2/3 elevated extracellular glutamate, and consistent with an action on presynaptic glutamate terminals, the increase was blocked by L- and N-type Ca²⁺ channel antagonists, but not by blocking voltage-dependent Na⁺ channels. Although N- and P/Q-types of Ca²⁺ channels are thought to mediate vesicular glutamate release from nerve terminals (Anwyl, 1991, 1999), L channels are predominantly located on soma, dendrites, and/or glial cells (Anwyl, 1999; Nachman-Clewner et al., 1999). Diltiazem blocked the APICA-induced increase in glutamate, suggesting that the L-type Ca²⁺ channels and mGluR2/3 on somatodendrites or glial cells, rather than just presynaptic mGluR2/3, are playing a role in modulating vesicular and/or nonvesicular glutamate release. Also consistent with a presynaptic site of action is the inhibition of the K⁺-mediated release of glutamate by APDC.

To further determine the involvement of the subtypes of group II mGluRs, the effect of the mGluR3 agonist NAAG (Wroblewska et al., 1997; Schweitzer et al., 2000) was examined. NAAG decreased extracellular glutamate levels in the
presence of 2-PMPA, an enzyme (NAALADase) inhibitor that prevented NAAG metabolism to glutamate (Slusher et al., 1999). These data indicate that the mGluR3 contributes to the decrease in glutamate by APDC. However, no selective mGluR3 antagonist was available to further verify the role of mGluR3 in modulating endogenous glutamate release. Also, it was reported that NAAG is only 10-fold more selective for mGluR3 than mGluR2 (Cartmell et al., 1998; Schweitzer et al., 2000). Although NAAG may also acts as a weak NMDA agonist, the inhibitory effect of NAAG on glutamate release is unlikely mediated by activating NMDA receptors because previous studies have shown that NMDA receptor activation increases glutamate release in the striatum (Hashimoto et al., 2000).

**Group II mGluRs Decrease in Extracellular Glutamate May Involve Cystine-Glutamate Exchange.** The cystine-glutamate exchanger is a major nonvesicular source of glutamate. This exchanger is driven by the relative intra- and extracellular substrate gradients and typically operates to transport glutamate out and cystine into the cell (Kato et al., 1993; Warr et al., 1999). Elevation of extracellular cystine concentration increased glutamate release from brain slices (Warr et al., 1999), an effect that was blocked by the relatively selective cystine-glutamate exchanger inhibitor (S)-4CPG (Ye et al., 1999). More recently, Baker et al. (2001) used in vivo microdialysis to show that the basal, extracellular glutamate content is derived mainly from cystine-glutamate exchange, because blockade of the cystine-glutamate exchanger by homocysteic acid or (S)-4CPG lowered extracellular glutamate levels by 60 to 70%. In the present study, pretreatment with (S)-4CPG prevented the increase in basal extracellular glutamate by APICA or the decrease by APDC. The former action re-

![Fig. 5. Involvement of calcium channels, sodium channels, or glutamate transporters in mGluR2/3 regulation of extracellular glutamate. A, coadministration of L- and N-type Ca\(^{2+}\) channel blockers diltiazem (10 \(\mu\)M) and \(\omega\)-conotoxin GVIA (10 \(\mu\)M), respectively, blocked APICA-induced increase in extracellular glutamate. After collecting five baseline samples the Ca\(^{2+}\) channel antagonists were introduced into the dialysis buffer for the remainder of the experiment as indicated by the bars. A one-way ANOVA with repeated measurement over the entire dose range indicates that APICA did not increase extracellular glutamate in the presence of either diltiazem or \(\omega\)-conotoxin GVIA. B, N-type Ca\(^{2+}\) channel blocker \(\omega\)-conotoxin GVIA antagonized the APDC-induced decrease in glutamate. A two-way ANOVA with repeated measures over time indicates that the extracellular glutamate level was significantly decreased over time (drug treatment) \((F_{(19,120)} = 4.5, p < 0.001)\), but no significant treatment \(\times\) time interaction \((F_{(19,120)} = 3.5, p > 0.05)\) was observed. \(\omega\)-Conotoxin GVIA alone decreased the basal level of extracellular glutamate by around 30 to 40% beginning at 80 min after introduction into the dialysis buffer \((F_{(19,119)} = 3.39, p < 0.001)\). C, superfusion of the Na\(^{+}\) channel blocker tetrodotoxin (TTX, 1 \(\mu\)M) failed to block the APICA-induced increase in extracellular glutamate. A two-way ANOVA with repeated measures over doses (time) revealed a significant increase in extracellular glutamate \((F_{(12,120)} = 3.87, p < 0.05)\), but no significant treatment \(\times\) time interaction \((F_{(12,120)} = 3.9, p > 0.05)\). TTX alone had no significant effect on the basal level of extracellular glutamate. D, blockade of glutamate transporters with TBOA failed to alter the inhibitory effect of APDC in glutamate. A two-way ANOVA with repeated measures over time reveals a significant drug effect \((F_{(16,160)} = 4.1, p < 0.001)\) and treatment \(\times\) time interaction \((F_{(16,160)} = 5.13, p < 0.001)\). TBOA alone had no significant effect on the basal level of extracellular glutamate. #, \(p < 0.05\), comparing TBOA + APDC with TBOA alone at each collection time.
flects decreased glutamate tone by inhibiting cystine-glutamate exchange (Baker et al., 2001), while the latter suggests that cystine-glutamate exchange, at least in part, mediates the action of the group II mGluRs. Although (S)-4CPG acts as a group I mGluR antagonist, blockade of group I mGluRs does not alter extracellular glutamate (Baker et al., 2001). The mechanism by which mGluR2/3 may couple to the cystine-glutamate exchanger is unclear. However, the reversal of mGluR2/3 effects on extracellular glutamate by modulating PKA activity indicates that mGluR2/3 inhibition of PKA may be signaling changes in cystine-glutamate exchange.

PKA- and Calcium-Dependent Effects by Group II mGluRs. The most well-characterized signaling event for group II mGluRs is G<sub>i</sub>-coupled reductions in cAMP formation and the subsequent inhibition of PKA (for review, see Conn and Pin, 1997). Electrophysiological studies demonstrate that activation of the adenylate cyclase cascade increases glutamatergic transmission in striatum and hippocampal slices, and may be critical in some forms of long-term potentiation (Colwell and Levine, 1995; Trudeau et al., 1996). Furthermore, Chavis et al. (1998) showed that activation of the cAMP/PKA cascade enhances presynaptic vesicle recycling at cerebellar granule cells. Consistent with a role for this signaling cascade in the present study, coperfusion of the selective PKA activator Sp-cAMPS blocked APDC-induced inhibition of glutamate release, whereas the selective PKA inhibitor Rp-cAMPS antagonized APICA-induced increase in extracellular glutamate.

Group II mGluRs have been previously shown to be G<sub>i</sub>-coupled, negative modulators of L- and N-type Ca<sup>2+</sup> channels in electrophysiological studies using brain slices, neuronal cultures, and heterologous expression systems (Chivas et al., 1994; Schumacher et al., 2000). Similarly, in vitro and in vivo release studies reveal L- and N-type channel involvement in the inhibition of dopamine release by group II mGluRs (Hu et al., 1999). In the present study, we observed that cadministration of the Ca<sup>2+</sup> channel antagonists diltiazem (L-type) or α-conotoxin GVIA (N-type) abolished the capacity of the group II antagonist to elevate or the agonist to reduce extracellular glutamate. Surprisingly, one electrophysiological investigation reported that blockade of N-type Ca<sup>2+</sup> channels did not prevent the inhibition of the glutamate transmission induced by group II agonists in the nucleus accumbens (Manzoni et al., 1997). Possible reasons for the distinction from the present study include the use of the less selective mGluR2/3 agonist (2S,1’S,2’S)-2-(2′-carboxy-3′,3′-difluorocyclopropyl)glycine and the fact that in the present study N-type channel blockade was maintained for 1 h before administering APDC, which may have resulted in a more complete blockade of the channels. Finally, phosphorylation of presynaptic proteins by...
PKA is known to enhance transmitter release (Greengard et al., 1993) and could contribute to the Ca\(^{2+}\)-independent regulation of glutamate transmission by mGluRs observed in some studies (Scanziani et al., 1995).

**Dimerization of Group II mGluR Immunoreactive Proteins Detected in Rat Brain.** Previous anatomical studies have shown the existence of group II mGluR mRNA in the NAcc (Ohishi et al., 1993a, 1993b; Testa et al., 1998). The present study showed that there is a high density of mGluR2/3 immunoreactive proteins in the NAcc. The majority of mGluR2/3 in the NAcc, as well as in the prefrontal cortex, dorsal striatum and the ventral tegmental area appeared as a dimer. However, it is not known whether the dimer is a homodimer of mGluR2 or mGluR3 or a heterodimer of mGluR2/3, nor is the functional consequence of dimerization understood. Reports of hetero- and homodimerization of a variety of metabotropic receptors have emerged, and the functional consequences of dimerization that have been elucidated are generally consistent with promoting metabotropic receptor trafficking and signaling. For example, the hetero-dimer of GABAB receptor subtypes promotes the trafficking of active GABAB receptors into the plasmalemmal membrane (Kuner et al., 1999), and the dimerization of δ-opioid receptors stabilizes receptors in the membrane (Cvejic and Devi, 1997).

In addition to acting as autoreceptors on glutamatergic presynaptic terminals, group II mGluRs are also expressed by astrocytes (Wroblewska et al., 1998). Recent evidence reveals that glia cells can release glutamate in a Ca\(^{2+}\)-dependent fashion using presynaptic protein assemblies similar to neuronal synaptic transmission (Araque et al., 1998). Moreover, mGluR receptors can activate Ca\(^{2+}\) currents in astrocytes, although most studies attribute this action to group I mGluR stimulation (Bernstein et al., 1998). Given that Ca\(^{2+}\)-dependent release of glutamate can occur in both glia and neurons, the in vivo estimates of extracellular glutamate in the present report cannot distinguish effects of mGluR2/3 agonists on neurons versus glia. Similarly, cysteine-glutamate exchangers are present in both glia and neurons. Although the lack of effect by TTX supports a primary action on glia (which lack TTX-sensitive sodium channels), the sensitivity of the mGluR effects on glutamate to N-type Ca\(^{2+}\) channel blockade supports a role for neurons because N-type channels are absent or in very low abundance in glia (Araque et al., 2000).
Conclusions. Group II mGluRs were found to decrease both the Ca\(^{2+}\)-dependent vesicular release of glutamate and to involve the cystine-glutamate exchange, a main nonvesicular glutamate source. Moreover, the reduction in extracellular glutamate by stimulating mGluR2/3 was mediated by inhibiting PKA. Importantly, mGluR2/3 were found to bear significant in vivo glutamatergic tone because blocking mGluR2/3 elevated extracellular glutamate levels. This latter finding indicates that the extracellular pool of glutamate measured by microdialysis may regulate glutamate neurotransmission.

References

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