

Noncompetitive Metabotropic Glutamate₅ Receptor Antagonist (*E*)-2-Methyl-6-styryl-pyridine (SIB1893) Depresses Glutamate Release through Inhibition of Voltage-Dependent Ca²⁺ Entry in Rat Cerebrocortical Nerve Terminals (Synaptosomes)

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ABSTRACT

The effect of (*E*)-2-methyl-6-styryl-pyridine (SIB1893), a selective metabotropic glutamate (subtype 5) receptor (mGlu₅R) antagonist, on glutamate release from isolated nerve terminals (synaptosomes) was examined. SIB1893 caused a potent inhibition of the Ca²⁺-dependent release of glutamate evoked by 4-aminopyridine (4AP). That the implied mGlu₅R-mediated modulation was contingent on diacylglycerol stimulation of protein kinase C (PKC) was indicated by PKC activator phorbol dibutyrate and PKC inhibitor Ro 32-0432 (bisindolylmaleimide XI), respectively, superceding or suppressing the inhibitory effect of SIB1893. The inhibitory action of SIB1893 was not due to it decreasing synaptosomal excitability or directly interfering with the release process at some point subsequent to Ca²⁺ influx, because SIB1893 did not alter the 4AP-evoked depolarization of the synaptosomal plasma membrane potential or

Ca²⁺ ionophore ionomycin-induced glutamate release. Rather, examination of the effect of SIB1893 on cytosolic [Ca²⁺] revealed that the diminution of glutamate release could be attributed to a reduction in voltage-dependent Ca²⁺ influx. Consistent with this, the SIB1893-mediated inhibition of glutamate release was completely prevented in synaptosomes pretreated with a combination of the N- and P/Q-type Ca²⁺ channel blockers, ω -conotoxin GVIA, and ω -agatoxin IVA. Together, these results suggest that noncompetitive antagonism of mGlu₅Rs using SIB1893 effects a decrease in PKC activation, which subsequently attenuates the Ca²⁺ entry through voltage-dependent N- and P/Q-type Ca²⁺ channels to cause a decrease in evoked glutamate release. These actions of SIB1893 and related agents may contribute to their neuroprotective effects in excitotoxic injury.

Metabotropic glutamate receptors (mGluRs) are a family of seven-transmembrane region, G protein-coupled receptors (GPCRs) having a variety of modulatory functions in neuronal excitability, neurotransmitter release, and synaptic plasticity in the central nervous system (CNS) (Pin and Duvoisin, 1995). Based on pharmacological profiles and signal transduction mechanisms, eight different mGluR subtypes have been categorized into three groups: group I receptors (mGlu₁₊₅R) being positively coupled to phospholipase C (PLC), with groups II (mGlu₂₊₃R) and III (mGlu_{4/6/7+8}R),

receptor both evincing negative coupling to adenylate cyclase (Conn and Pin, 1997). Group I mGluR activation leads to facilitation of excitatory synaptic neurotransmission in the CNS (McBain et al., 1994), an effect thought to be mediated by modulation of the probability of glutamate release through presynaptic mGluR activity (Conn and Pin, 1997). This is consistent with the presynaptic location of mGlu₁₊₅R immunoreactivity in several brain regions such as cerebral cortex and hippocampus (Shigemoto et al., 1993; Romano et al., 1995), as well as functional studies showing an enhancement of glutamate release with group I mGluR activation (Herrero et al., 1992; Reid et al., 1999; Thomas et al., 2000; Fazal et al., 2003). The mechanism underlying this facilitation likely involves protein kinase C (PKC) activation by diacylglycerol (DAG) produced by receptor stimulated-PLC

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; GPCR, G protein-coupled receptor; CNS, central nervous system; PLC, phospholipase C; PKC, protein kinase C; DAG, diacylglycerol; SIB1893, (*E*)-2-methyl-6-styryl-pyridine; 4AP, 4-aminopyridine; VDCC, voltage-dependent Ca²⁺ channel; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; DiSC₅(3), 3,3'-dipropylthiadicarbocyanine iodide; HBM, HEPES buffer medium; BSA, bovine serum albumin; [Ca²⁺]_c, cytosolic free Ca²⁺ concentration; PDBu, phorbol dibutyrate; Ro 32-0432, bisindolylmaleimide XI; ω -CgTX, ω -conotoxin GVIA; ω -AgTX, ω -agatoxin IVA.

activity; however, the precise molecular targets of PKC in the nerve terminal underlying this regulation remain to be elucidated.

Occurring inappropriately, group I mGluR autoreceptor-mediated facilitation at excitatory synapses may contribute to a number of pathological states, including ischemic brain damage, epilepsy, and neurodegenerative disorders. Consistent with this, a number of studies have been shown that group I mGluR activation can exacerbate neuronal injury both in vitro and in vivo (Agrawal et al., 1998; Bruno et al., 1995; Mukhin et al., 1997) and thus may be implicit in the pathogenesis of neuronal cell death produced by excessive glutamate release (Choi, 1992; Lipton and Rosenberg, 1994). This potential for group I mGluR-mediated excitotoxicity therefore invokes the utility of antagonists of these receptors as neuroprotective agents. Indeed, antagonists of mGlu₅Rs have recently been proposed to protect cultured cortical neurons against excitotoxic neuronal death (Bruno et al., 2000) and exert anticonvulsant activity in an in vivo model (Chapman et al., 2000). Whether the inhibition of glutamate release from nerve terminals underlies this neuroprotective action of mGlu₅R blockade specifically is subject of debate given that the relative contributions of the two group I mGluRs (mGlu₁R and mGlu₅R) to the facilitation of glutamate remain contentious (Sistiaga et al., 1998; Reid et al., 1999; Fazal et al., 2003). Another connected issue arises from studies with isolated cerebrocortical nerve terminals (synaptosomes) reporting remarkably high PKC activity even in unstimulated conditions and in the effective absence of ligand (Coffey et al., 1994). This latter observation begs the question as to whether the high basal PKC activity reflects a basal, "constitutive" activity of mGluRs reported in the absence of ligand (Gasparini et al., 2002). The advent of newer subtype selective receptor antagonists for mGluRs, particularly those possessing noncompetitive activity at mGlu₅Rs, have therefore led us to examine these issues.

The isolated nerve terminal preparation is a well established model for studying the presynaptic regulation of neurotransmitter release by drugs in the absence of any complication of interpretation produced by concomitant postsynaptic effects. Using an established method for looking at endogenous glutamate release (Nicholls and Sihra, 1986), in the present study we characterized the effect SIB1893, a selective and noncompetitive mGlu₅R antagonist, on the 4-aminopyridine (4AP)-evoked release of glutamate from cerebrocortical synaptosomes. We found that SIB1893 potently inhibits glutamate release in the absence of any endogenous or exogenous ligand. This effect seems to be through a reduction of voltage-dependent Ca²⁺ channel (VDCC) activity and subsequent decrease of Ca²⁺ influx into nerve terminals, rather than any upstream effect on nerve terminal excitability (Herrero et al., 1992; Herrero et al., 1994). We conclude the presence of a SIB1893-sensitive mGlu₅R activity in nerve terminals, which through a mechanism involving PKC, modulates VDCCs coupled to glutamate exocytosis.

Materials and Methods

Materials. (*E*)-2-Methyl-6-styryl-pyridine (SIB1893) and 2-methyl-6-(phenylethynyl)-pyridine (MPEP) were obtained from Tocris Cookson Inc. (Bristol, UK). Fura-2-acetoxymethyl ester and DiSC₃(5) were obtained from Molecular Probes (Eugene, OR). Percoll was obtained from Pharmacia (Peapack, NJ). Glutamate dehydrogenase

and all other reagents were obtained from Sigma Chemical (Poole, Dorset, UK) or Merck (Poole, Dorset, UK).

Synaptosomal Preparation. Synaptosomes were purified as described previously (Sihra, 1997). Briefly, the cerebral cortex from 2-month-old male Sprague-Dawley rats was isolated and homogenized in a medium containing 320 mM sucrose, pH 7.4. The homogenate was centrifuged at 3000g for 2 min (at 4°C), and the supernatant was centrifuged again at 14,500g for 12 min (at 4°C). The pellet was gently resuspended in 8 ml of 320 mM sucrose, pH 7.4. Two milliliters of this synaptosomal suspension was placed into 3-ml Percoll discontinuous gradients containing 320 mM sucrose, 1 mM EDTA, 0.25 mM DL-dithiothreitol, and 3, 10, and 23% Percoll, pH 7.4. The gradients were centrifuged at 32,500g for 7 min (at 4°C). Synaptosomes sedimenting between the 10 and the 23% Percoll bands were collected and diluted in a final volume of 30 ml of HEPES buffer medium (HBM) consisting of 140 mM NaCl, 5 mM KCl, 5 mM NaHCO₃, 1 mM MgCl₂·6H₂O, 1.2 mM Na₂HPO₄, 10 mM glucose, and 10 mM HEPES (pH 7.4) before centrifugation at 27,000g for 10 min (at 4°C). The pellets thus formed were resuspended in 3 ml of HBM, and the protein content was determined by the Bradford assay. Aliquots of 0.5 mg of synaptosomal suspension were diluted in 10 ml of HBM and spun at 3000g for 10 min (at 4°C). The supernatants were discarded, and the synaptosomal pellets were stored on ice and used within 4 to 6 h.

Glutamate Release Assay. Glutamate release was assayed by on-line fluorimetry as described previously (Nicholls and Sihra, 1986). Synaptosomal pellets were resuspended in HBM containing 16 μM bovine serum albumin (BSA) and incubated in a stirred and thermostated cuvette maintained at 37°C in a PerkinElmer LS-50B spectrofluorimeter. NADP⁺ (2 mM), glutamate dehydrogenase (50 units/ml), and CaCl₂ (1 mM) were added after 3 min. After 10 min of incubation, 4AP (3 mM) or ionomycin (5 μM) was added to stimulate glutamate release. Glutamate release was monitored by measuring the increase of fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively) due to NADPH being produced by the oxidative deamination of released glutamate by glutamate dehydrogenase. Data were accumulated at 2-s intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment, and the fluorescence response was used to calculate released glutamate, expressed as nanomoles of glutamate per milligram of synaptosomal protein (nanomoles per milligram). Values quoted in the text represent levels of glutamate cumulatively release after 4-min depolarization, i.e., nanomoles per milligram per 4 min, unless indicated otherwise. Cumulative data were analyzed using Lotus 1-2-3 and MicroCal Origin. Statistical analysis was performed by two-tailed Student's *t* tests.

Membrane Potential Measurement using DiSC₃(5). Synaptosomes were preincubated and resuspended as described for the glutamate release experiments. After 3-min incubation, 4 μM DiSC₃(5) was added and allowed to equilibrate before the addition of CaCl₂ (1 mM) after 4-min incubation. 4AP was added to depolarize the synaptosomes at 10 min, and DiSC₃(5) fluorescence was monitored at excitation and emission wavelengths of 646 and 674 nm, respectively, and data accumulated at 2-s intervals. Cumulative data were analyzed using Lotus 1-2-3, and results are expressed in fluorescence units.

Cytosolic Ca²⁺ Measurements using Fura-2. Synaptosomes (0.5 mg/ml) were preincubated in HBM with 16 μM BSA in the presence of 5 μM Fura-2-acetoxymethyl ester and 0.1 mM CaCl₂ for 30 min at 37°C in a stirred test tube. After Fura-2 loading, synaptosomes were centrifuged in a microcentrifuge for 30 s at 5000g. The synaptosomal pellets were resuspended in HBM with BSA, and the synaptosomal suspension was stirred in a thermostated cuvette in a PerkinElmer LS-50B spectrofluorimeter. CaCl₂ (1 mM) was added after 3 min, and further additions were made after an additional 5 min, as described in the legends to the figures. Fluorescence data were accumulated at excitation wavelengths of 340 and 380 nm (emission wavelength 505 nm) and data accumulated at 3-s inter-

vals. Calibration procedures were performed as described previously (Sihra et al., 1992), using 0.1% sodium dodecyl sulfate to obtain the maximal fluorescence with Fura-2 saturation with Ca²⁺, followed by 10 mM EGTA (Tris buffered) to obtain minimum fluorescence in the absence of any Fura-2/Ca²⁺ complex. Cytosolic free Ca²⁺ concentration ([Ca²⁺]_c, nanomolar) was calculated using equations described previously (Grynkiewicz et al., 1985). Cumulative data were analyzed using Lotus 1-2-3 and MicroCal Origin. Statistical analysis was performed by two-tailed Student's *t* tests.

Results

Nerve terminals were depolarized with the K⁺ channel blocker 4AP, which, by increasing the excitability of the synaptosomal plasma membrane, increases the opening of VDCCs to elevate cytoplasmic free Ca²⁺ concentration and thus induces the release of vesicular glutamate (Tibbs et al., 1989). To examine the effect of mGlu₅R blockade on Ca²⁺-dependent glutamate release evoked by 4AP, the potent and selective mGlu₅R antagonist SIB1893 was used. In the nerve terminals from rat cerebral cortex, the total release of glutamate evoked by 3 mM 4AP in the presence of Ca²⁺ was 18.8 ± 0.7 nmol glutamate/mg protein/5 min (*n* = 8; Fig. 1A). Addition of 50 μM SIB1893 caused an inhibition of 4AP-evoked release of about 40% to 11.3 ± 0.6 nmol glutamate/mg/5 min (*n* = 8; *P* < 0.01; Fig. 1A). This concentration of antagonist produced a maximal effect judging from a steep dose-response of the drug with respect to 4AP-evoked glutamate release (18.1 ± 0.3, 18.5 ± 0.5, 11.3 ± 0.6, and 11.7 ± 0.9 nmol/mg/5 min glutamate release in the presence of 10, 30, 50, and 100 μM SIB1893, respectively).

We next investigated whether the effect of SIB1893 on total release reflected an effect on physiological exocytotic vesicular release or on the Ca²⁺-independent release of glutamate attributable to cytosolic efflux via the reversal of the glutamate transporter (Nicholls and Sihra, 1986). Figure 1B shows that, compared with a total 4AP-evoked release of 18.2 ± 1.0 nmol/mg/4 min in the presence of added CaCl₂ (1 mM) (Fig. 1A, total release), the secretagogue effected 5.8 ± 0.5 nmol/mg/4 min in the presence of calcium-free medium

containing 200 μM EGTA (*n* = 5; Fig. 1B, Ca-independent release), with the calculated net Ca²⁺-dependent glutamate component therefore being 10.8 ± 0.8 nmol/mg/4 min (*n* = 5) (Fig. 1C). Preincubation of synaptosomes with 50 μM SIB1893 for 5 min before 4AP had no significant effect on the calcium-independent component of 4AP-evoked glutamate release (*n* = 5; 5.4 ± 0.6 nmol/mg/4 min; Fig. 1B), whereas evincing a decrease of the net Ca²⁺-dependent glutamate component of release to 4.0 ± 0.3 nmol/mg/4 min (Fig. 1C). To confirm that the effect of SIB1893 reflected a suppression of mGlu₅R activation rather than an esoteric effect of the compound, we also examined the effect of a well established mGlu₅ receptor selective antagonist, MPEP. MPEP (50 μM) produced a 33% inhibition of total 4AP (3 mM)-evoked glutamate release (*n* = 5; *P* < 0.01; Fig. 2) comparable with that produced by SIB1893.

Given coupling of group 1 mGluRs to the activation of PLC, with one potential consequence being the production of DAG and downstream activation of PKC, we next sought to examine whether the inhibitory effect of SIB1893 could be superseded if the mGlu₅R-mediated activation of PLC is bypassed using direct stimulation of PKC with phorbol ester. Control 4AP (3 mM)-evoked glutamate release was potentiated by 33.1% (to 22.9 ± 1.0 nmol/mg/4 min; *n* = 7; *P* < 0.01; Fig. 3A) in the presence of phorbol dibutyrate (PDBu; 300 nM). Addition of SIB1893 after PDBu treatment had negligible effect on glutamate release (22.3 ± 0.9 nmol/mg/4 min; *n* = 9; Fig. 3A), there being no effective inhibition produced by SIB1893 in the presence of PDBU (Fig. 3B). To confirm that an mGlu₅R/PLC/PKC cascade is being suppressed by SIB1893 in its inhibition of glutamate, we next examined whether the SIB1893-mediated effect was sensitive to PKC inhibition. Control 4AP (3 mM)-evoked release of 19.4 ± 0.5 nmol/mg/4 min was attenuated by the PKC inhibitor Ro 32-0432 (1 μM) to 10.8 ± 0.4 nmol/mg/4 min (*n* = 5; *P* < 0.01), reflecting an inhibition of the reported basal PKC activity present in nerve terminals (Coffey et al., 1994). Crucially, however, in the presence of Ro 32-0432, SIB1893 addition had no further effect on 4AP-evoked glutamate release (10.3 ± 0.7 nmol/

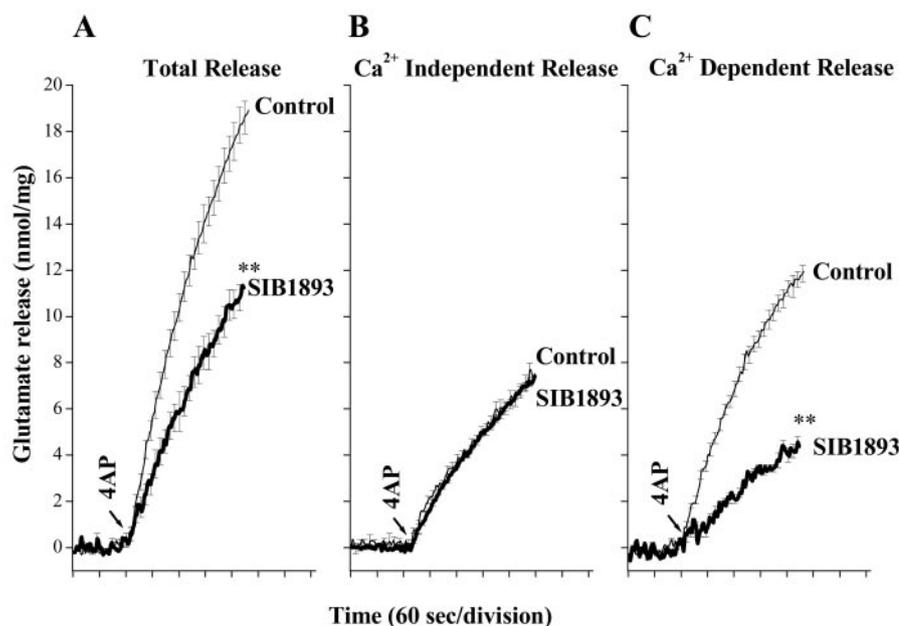


Fig. 1. SIB1893 inhibits the Ca²⁺-dependent release of glutamate evoked by 4AP. Rat synaptosomes (0.3 mg/ml) were preincubated for 3 min before the addition of 1 mM CaCl₂. 4AP (3 mM) was added after a further 7 min to effect depolarization (arrow). Ca²⁺-independent release was assayed by omitting CaCl₂ and adding 200 μM EGTA, 30 s before depolarization. Glutamate release was assayed by on-line fluorimetry. Total release (A), Ca²⁺-independent release (B), and Ca²⁺-dependent release (C) were calculated in the absence or presence of SIB1893 (50 μM), added 5 min before depolarization. Ca²⁺-dependent glutamate release was calculated (total glutamate release minus Ca²⁺-independent release) for each time point for each of the condition. Data represent means ± S.E.M. of five to eight independent synaptosomal preparations. Means ± S.E.M. was obtained for each data point (2 s), but error bars are only shown every 10 s for clarity. **, total glutamate release and Ca²⁺-dependent glutamate release, in the presence of SIB1893, were significantly different from control release; *P* < 0.01, two-tailed Student's *t* test.

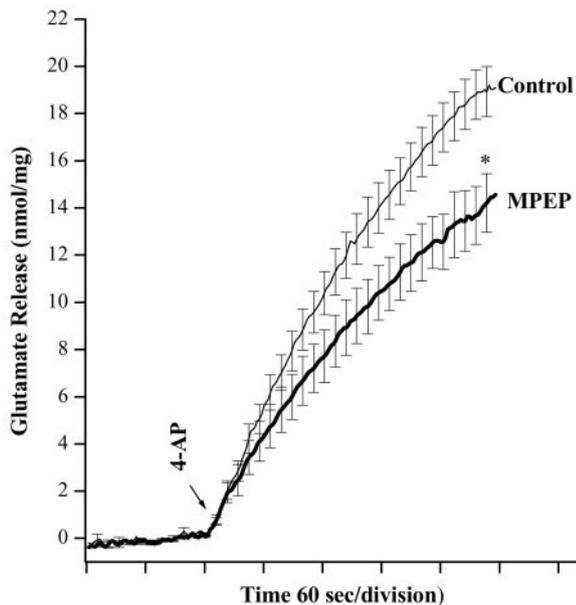


Fig. 2. MPEP inhibits the release of glutamate evoked by 4AP. Rat synaptosomes (0.3 mg/ml) were treated as described in the legend to Fig. 2, and glutamate release was assayed in the absence or presence of MPEP (50 μ M), added 5 min before depolarization. Data represent means \pm S.E.M. of five independent synaptosomal preparations. Means \pm S.E.M. was obtained for each data point (2 s), but error bars are only shown every 10 s for clarity. *, total glutamate release in the presence of MPEP was significantly different from control release; $P < 0.05$, two-tailed Student's *t* test.

mg/4 min; $n = 5$; $P < 0.01$); the antagonist therefore failing to produce any additional inhibition compared with the effect of PKC inhibitor alone (Fig. 3B).

To further understand the mechanism responsible for the SIB1893-mediated inhibition of glutamate release, we used a membrane potential-sensitive dye, DiSC₃(5), to determine the effect of SIB1893 on the synaptosomal plasma membrane potential. 4AP (3 mM) caused an increase in DiSC₃(5) fluorescence of 0.89 ± 0.06 fluorescence units/3 min. Preincubation of synaptosomes with 50 μ M SIB1893 for 5 min before 4AP addition had no significant effect on the 4AP-mediated increase in DiSC₃(5) fluorescence (0.93 ± 0.07 fluorescence units/3 min; $n = 5$; Fig. 4). This result indicates that the effect of SIB1893 on evoked glutamate release is unlikely to be due to either a hyperpolarizing effect of the antagonist on the synaptosomal plasma membrane potential or an attenuation of depolarization produced by 4-AP. Confirmation that the SIB1893 effect did not impinge on synaptosomal excitability was, however, obtained with experiments using high external [K⁺]-mediated depolarization, which "clamps" the membrane potential according to the imposed K⁺ electrochemical gradient and thereby activates VDCCs. Addition of 30 mM KCl effected a control release of 23.5 ± 0.9 nmol/mg/4 min, which was decreased to 14.1 ± 1.0 nmol glutamate/mg/4 min in the presence of 50 μ M SIB1893 (Fig. 4, inset).

Downstream of membrane depolarization, presynaptic inhibition of neurotransmitter release can be mediated by a reduction of Ca²⁺ influx into nerve terminal or by a direct interference with the neurotransmitter exocytotic process. To investigate the first of these possibilities, i.e., whether the inhibitory effect of SIB1893 on glutamate release reflected a decrease in Ca²⁺ influx, we used the Ca²⁺ indicator Fura-2 to

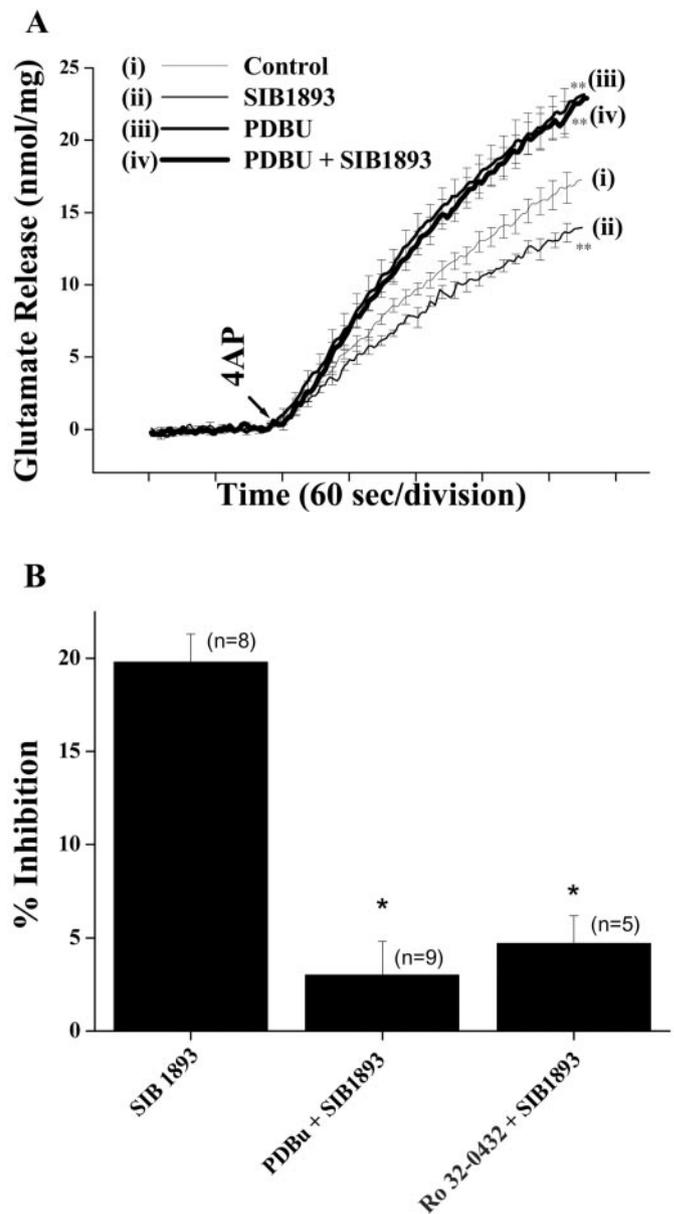


Fig. 3. SIB1893-mediated inhibition of 4AP-evoked glutamate release is disrupted by the PKC activator or PKC inhibitor. A, glutamate release evoked by 4AP (3 mM) in the absence or presence of PDBu (300 nM). B, comparison of the inhibition of glutamate release with SIB1893 alone and in the presence of PDBu (300 nM) or Ro 32-0432 (1 μ M). PDBu and Ro 32-0432 were added 10 min before the addition of SIB1893 (50 μ M). Columns are the means \pm S.E.M. of independent experiments, using synaptosomal preparations from five to nine animals. **, release significantly different from control release; $P < 0.01$, two-tailed Student's *t* test. *, inhibition (percentage) significantly different from that obtained with SIB1893 alone; $P < 0.05$, two-tailed Student's *t* test.

assess the effect of SIB1893 on the 4AP-evoked increase of [Ca²⁺]_c. 4AP (3 mM) caused a rise in [Ca²⁺]_c to a plateau level of 178.4 ± 7.9 nM (Fig. 5). This 4AP-evoked rise in [Ca²⁺]_c was decreased by 22.8 nM with 50 μ M SIB1893 preincubation ($n = 5$; $P < 0.01$; Fig. 5).

The data point to SIB1893 acting to affect voltage-dependent Ca²⁺ influx rather than upstream loci in the stimulus-release cascade. Glutamate release is supported by the entry of Ca²⁺ through the N- and P/Q-type VDCCs, which can be selectively blocked by ω -conotoxin GVIA (ω -CgTX) and by ω -agatoxin IVA (ω -AgTX), respectively (Turner and Dunlap,

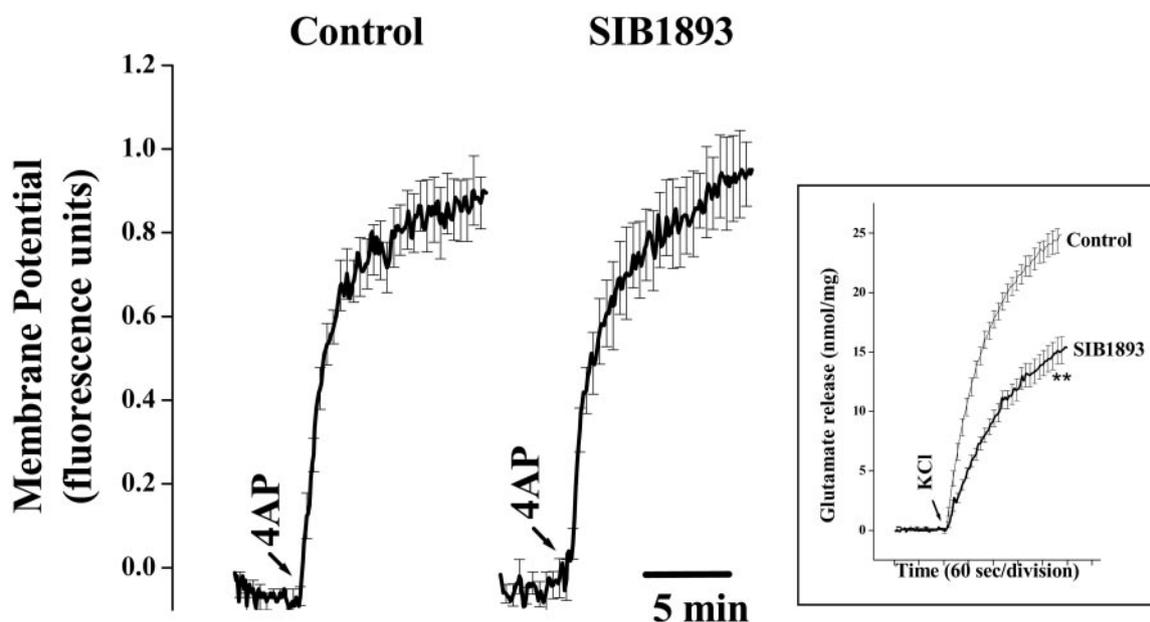


Fig. 4. SIB1893 does not change the synaptosomal membrane potential. Synaptosomal membrane potential monitored with DiSC₃(5) in the absence (control) or presence of 50 μ M SIB1893, before and after depolarization with 3 mM 4AP. Inset, SIB1893 modulation of 30 mM KCl-evoked glutamate release. Experiments were carried out as described in previous release figures except for the addition of 30 mM KCl as secretagogue instead of 4AP. Each trace is the means \pm S.E.M. of independent experiments, using synaptosomal preparations from five animals. The S.E.M. was computed for each point (2-s interval), but error bars are only shown every 10 s for clarity. **, release significantly different from control release; $P < 0.01$, two-tailed Student's t test.

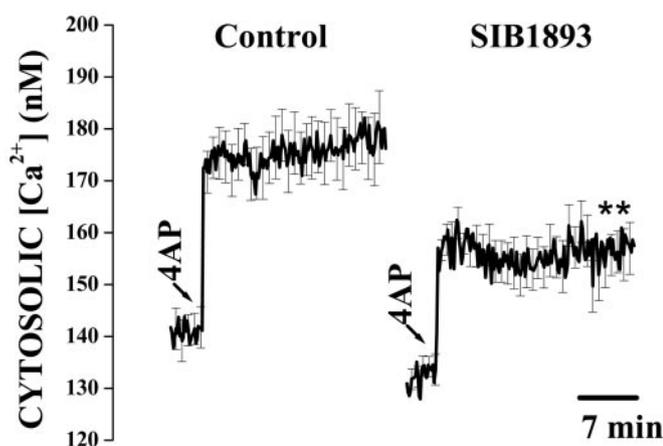


Fig. 5. SIB1893 attenuates 4AP-induced Ca^{2+} entry into nerve terminals. Synaptosomes (0.3 mg/ml) were incubated as described under *Materials and Methods*, and cytosolic $[\text{Ca}^{2+}]$ was monitored using Fura-2. Voltage-dependent Ca^{2+} influx was evoked by 4-aminopyridine in the absence or presence of SIB1893 (50 μ M). Data represent means \pm S.E.M. of five independent synaptosomal preparations. Error bars are shown every 10 s for clarity. **, synaptosomal $[\text{Ca}^{2+}]$ in the presence of SIB1893 significantly different from control; $P < 0.01$, two-tailed Student's t test.

1995; Vazquez and Sanchez-Prieto, 1997). To establish which of these Ca^{2+} channel activities is involved in the SIB1893-mediated inhibition, we examined glutamate release in the presence of Ca^{2+} channel blockers. ω -CgTX (2 μ M) reduced 4-aminopyridine-evoked glutamate release by $22.5 \pm 3.3\%$ ($n = 7$; $P < 0.01$) by selectively blocking N-type Ca^{2+} channels (Fig. 6). In synaptosomes pretreated with ω -CgTX (2 μ M), SIB1893 (50 μ M) reduced glutamate release by a further $19.4 \pm 2.4\%$ (Fig. 6). Application of ω -AgTX (200 nM) reduced 4-aminopyridine-evoked glutamate release by $48.7 \pm 4.2\%$ ($n = 6$; $P < 0.01$) through

selective blockade of P/Q-type Ca^{2+} channels (Fig. 6). After application of ω -AgTX (200 nM), SIB1893 (50 μ M) was still able to reduce glutamate release by a further $21.9 \pm 2\%$ ($n = 6$; $P < 0.01$) (Fig. 6). Thus, the individual blockade of N-type and P/Q-type by ω -CgTX and ω -AgTX did not significantly prevent the action of SIB1893 (Fig. 6). To test the possibility that SIB1893 mediates its effects through the combined inhibition of N- and P/Q-type Ca^{2+} channel modulation, the effect of SIB1893 were tested before and after the combined application of ω -CgTX and ω -AgTX. ω -CgTX (2 μ M) and ω -AgTX (200 nM) together reduced 4AP-evoked glutamate release by $60.4 \pm 3.9\%$ ($n = 5$; $P < 0.01$) (Fig. 6, inset). In the combined presence of the Ca^{2+} channels inhibitors, application of SIB1893 (50 μ M) only reduced glutamate release by a further $2.7 \pm 2.4\%$ ($n = 5$), indicating significant reduction compared with that obtained when SIB1893 was applied alone ($33.7 \pm 3.2\%$; $n = 8$; $P < 0.01$) (Fig. 6, inset).

Although the foregoing data indicate a correlation of the inhibitory effect of SIB1893 on glutamate release with a suppression of VDCCs, there remains the possibility that mGlu₅R activation could affect targets downstream of Ca^{2+} entry to also facilitate glutamate release. To determine whether mGlu₅R blockade impinged on the exocytotic machinery itself, we also examined the effect of SIB1893 on glutamate release evoked by the Ca^{2+} ionophore ionomycin. Ionomycin (5 μ M), which causes a direct increase in intrasynaptosomal Ca^{2+} levels without previous depolarization and VDCC activation (Sihra et al., 1992), evoked the release of 24.6 ± 0.7 nmol/mg/4 min (Fig. 7). Preincubation of synaptosomes with 50 μ M SIB1893 did not significantly affect ionomycin-induced release of glutamate (24.8 ± 0.6 nmol/mg/4 min; $n = 6$; $P > 0.05$; Fig. 7).

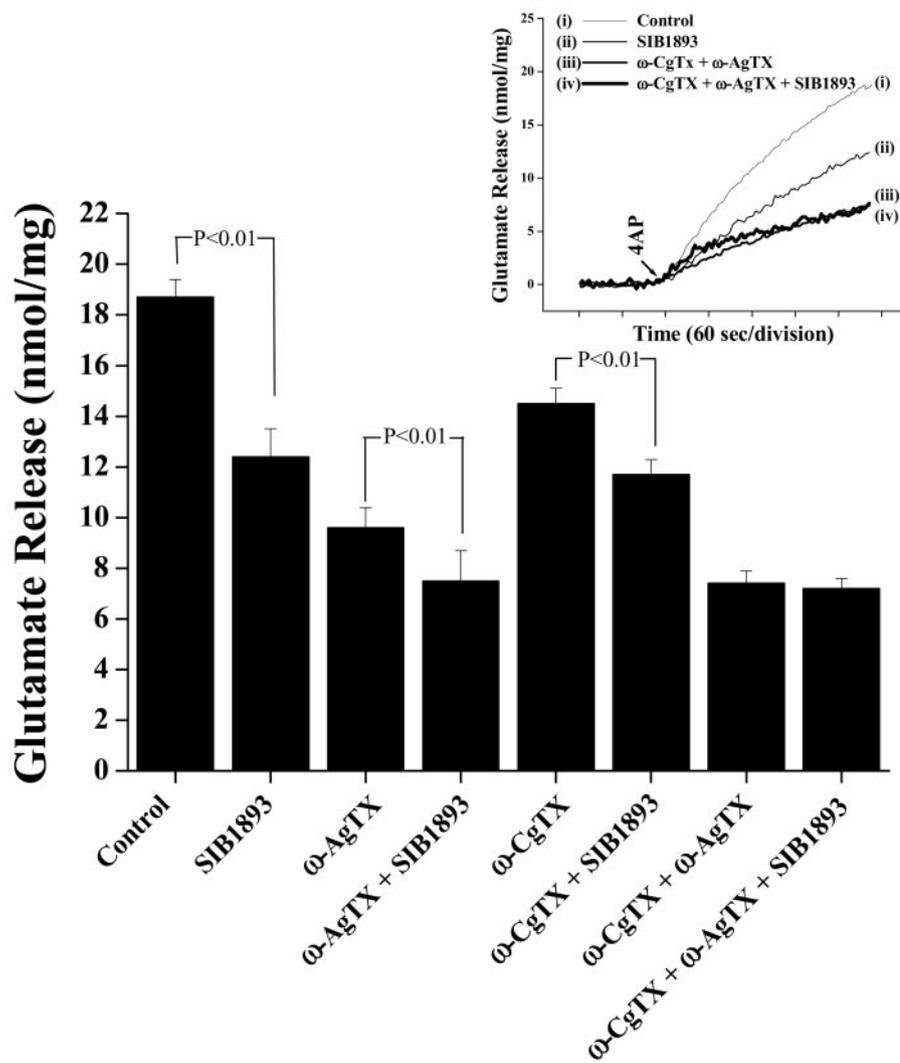


Fig. 6. Blockade of N- and P/Q-type Ca^{2+} channels abolishes SIB1893 inhibition of glutamate exocytosis. Glutamate release evoked by 4AP (3 mM) in the absence (control) or presence of SIB1893, ω -conotoxin GVIA (2 μ M), ω -conotoxin GVIA (2 μ M) + SIB1893 (50 μ M), ω -agatoxin VIA (200 nM), ω -agatoxin VIA (200 nM) + SIB1893 (50 μ M), ω -conotoxin GVIA (2 μ M) + ω -agatoxin VIA (200 nM), and ω -conotoxin GVIA (2 μ M) + ω -agatoxin VIA (200 nM) + SIB1893 (50 μ M). Data represent means \pm S.E.M. of five to seven independent synaptosomal preparations. Inset, 4AP-evoked glutamate release traces in the absence (control) or presence of ω -AgTX IVA (200 nM) + ω -conotoxin GVIA (2 μ M) or ω -AgTX IVA (200 nM) + ω -conotoxin GVIA (2 μ M) + SIB1893 (50 μ M) showing complete occlusion of the effect of SIB1893 after VDCC blockade.

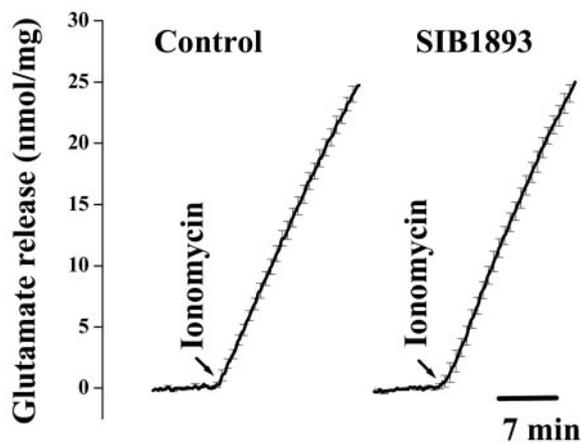


Fig. 7. SIB1893 fails to affect ionomycin-induced glutamate release. Glutamate release was induced by ionomycin (5 μ M) in the absence (control) or presence of SIB1893 (50 μ M). Data represent means \pm S.E.M. for six independent synaptosomal preparations. Error bars are shown every 10 s for clarity. Glutamate release in the presence of SIB1893 (50 μ M) was not significantly different from that in control.

Discussion

The data presented demonstrate that the selective mGlu₅R antagonist SIB1893 inhibits the 4AP-evoked release of the

Ca^{2+} -dependent, exocytotic pool of glutamate (Verhage et al., 1991) in adult rat cerebrocortical nerve terminals. The mGlu₅R_s implicated seem to be coupled to PKC activation and act to provide a positive modulatory influence on neuronal glutamate release. Furthermore, the inhibition of release by SIB1893 is seen to occur due to a reduction of Ca^{2+} influx through nerve terminal N- and P/Q-type Ca^{2+} channels. Thus, our results together provide support for the existence of functional presynaptic mGlu receptors of the mGlu₅ subtype functioning to facilitate glutamate release from cerebrocortical nerve terminals.

The activation of group I mGlu receptors has been shown to be facilitatory at nerve terminals, an effect likely to be the result of DAG production and PKC activation (Herrero et al., 1992; Coffey et al., 1994; Lu et al., 1997; Reid et al., 1999; Thomas et al., 2000). In the present study, we found that SIB1893-mediated inhibition of glutamate was reduced from 19.8 ± 1.5 to $3.0 \pm 1.8\%$ and $4.7 \pm 1.5\%$ in the presence of PDBu and Ro 32-0432, respectively. Thus, PDBu, by bypassing mGlu₅R-mediated PLC activation and DAG production, effectively superseded any effects SIB1893 acting at the level of the mGluR and its GPCR transduction machinery. Furthermore, occlusion of mGlu₅R inhibition by the prior suppression of PKC activity itself using Ro 32-0432 confirmed

this role of PKC in the mGluR facilitation of 4AP-evoked glutamate release inhibited by SIB1893.

The overall implication is that SIB1893 inhibition of glutamate release is contingent on an mGlu₅R-mediated activation of a presynaptic DAG-dependent PKC signaling cascade instrumental in glutamate release modulation. Notably in the current study, SIB1893 produced blockade of glutamate release in the absence of any added agonist and indeed without external endogenous glutamate itself being present, given that the latter is rapidly removed by virtue of the glutamate dehydrogenase added as part of our enzyme-linked assay for the neurotransmitter (Nicholls and Sihra, 1986). The effect of SIB1893 therefore reflects its property as a noncompetitive antagonist capable of acting in the absence of agonist (Gasparini et al., 2002) and in this regard perhaps suggests the presence of an “intrinsic” or “constitutive” mGlu₅R activity in the synaptosomal preparation, even in the nominal absence of agonist. Given the facilitation of glutamate release by an mGlu₅R/PLC/DAG/PKC signaling cascade, the question remains as to the effector target for this activity.

An inhibition of glutamate release by SIB1893 could be ascribed to an alteration of plasma membrane potential, a direct inhibition of the exocytosis-coupled Ca²⁺ channel and/or a direct effect on some component of the release machinery. The first of these possibilities, that mGlu₅R activation may effect synaptosomal plasma membrane hyperpolarization or decrease in synaptosomal excitability, is untenable on the basis of two observations. 1) 4AP-evoked membrane potential depolarization, measured with a membrane-potential sensitive dye, DiSC₃(5), was unaffected by the addition of SIB1893. 2) The inhibitory effect of SIB1893 was also observed when KCl was used as a depolarizing agent, conditions under which the involvement of membrane voltage-controlling K⁺ channels (transient or otherwise) is obviated. The lack of effect of mGlu₅R activation on synaptosomal excitability indicated by the data presented herein is contrary to studies suggesting mGluR agonist-mediated facilitation to occur as a result of delayed-rectifier type K⁺ channel modulation of membrane depolarization (Herrero et al., 1992). Notably, however, in these previous studies, the use of nonselective agonists for mGlu₅R and mGlu₁R may have contributed to additional modes of modulation being invoked. Indeed, the relative roles of mGlu₁R and mGlu₅R in the regulation of glutamate release is a subject of debate, with earlier studies using cerebrocortical synaptosomes indicating modulation with “mGlu₁R-like” pharmacology (Reid et al., 1999), whereas more recent studies using cerebrocortical mini-slices evince regulation by mGlu₅R (Fazal et al., 2003). Notwithstanding this, with regard to the mechanism of modulation, consistent with the lack of a role of mGlu₅R in modulating nerve terminal excitability indicated by the current work, the study by Reid et al. (1999), looking at the facilitation of radiolabeled glutamate release from synaptosomes by type 1 mGluR activation, also concluded modulation of membrane potential to be absent (Reid et al., 1999).

If it isn't the modulation of synaptosomal excitability, then the locus of action of the mGlu₅R activity suppressed by SIB1893 must lie further downstream in the stimulus-exocytosis coupling cascade. Glutamate exocytosis from mammalian CNS nerve terminals is dependent on localized Ca²⁺ influx, chiefly through N- and P/Q-type VDCCs (Turner and

Dunlap, 1995; Vazquez and Sanchez-Prieto, 1997). Using Fura-2, we directly demonstrate here that SIB1893 indeed significantly inhibited the voltage-dependent Ca²⁺ influx stimulated by 4AP. Moreover, although SIB1893-induced inhibition of glutamate release persisted after individual blockade of N- or P/Q-type Ca²⁺ channels, combined blockade of both channel types abrogated the effect of the inhibitor. These results are consistent with whole-cell patch clamp studies showing mGlu₅R-mediated potentiation of VDCC activity (Chavis et al., 1995) and support the hypothesis that facilitation of N- and P/Q-type Ca²⁺ channel activities together, potentially underlie the direct or indirect influence of mGlu₅R activation on glutamate release. Contrary to this, a previous synaptosomal study addressing this issue found no effect of mGluR activation on ⁴⁵Ca²⁺ influx, leading to the suggestion that modulation may occur downstream of Ca²⁺ entry (Reid et al., 1999). However, because the latter study pharmacologically intimated an mGlu₁R-like rather than mGlu₅R activation, we directly examined whether the mGlu₅R invoked in the current study affected potential loci downstream of VDCC activation to modulate glutamate release. Our observation that SIB1893 did not affect release evoked by Ca²⁺ ionophore ionomycin, which directly introduces Ca²⁺ into synaptosomes without previous depolarization and VDCC activation, rules out any significant effect of mGlu₅R activation at steps downstream of Ca²⁺ entry and at the level of the coupling of Ca²⁺ to the exocytotic release machinery itself.

An mGlu₅R-mediated activation of a presynaptic PKC signaling cascade leading to the regulation of VDCCs implicated by our studies is a tenable mode for neurotransmitter modulation for several reasons. Phosphorylation-dependent regulation of VDCCs has been invoked by a number of studies, with the functional effects of phosphorylation being related to the Ca²⁺ channel subtype and subunit being addressed (Dolphin, 1995; Catterall, 2000). Indeed, all of the known VDCC subtypes seem to be subject to modulation by direct or indirect phosphorylation by multiple protein kinases, but with respect to PKC, to date the most compelling evidence is for the post-translational modulation of N-type VDCCs (Jarvis and Zamponi, 2001). PKC-mediated modulation of synaptosomal VDCCs has been reported previously (Bartschat and Rhodes, 1995; Jarvis and Zamponi, 2001); thus, our observation that the capacity of SIB1893 to inhibit glutamate release depends on PKC-activity, supports an mGlu₅R/PLC/DAG/PKC signaling cascade facilitating voltage-dependent Ca²⁺ entry. Whether this modulation of synaptosomal VDCCs by PKC reflects modification of intrinsic channel activity itself or implies modification of inhibitory GPCR modulation of the VDCCs, as has been suggested (Jarvis and Zamponi, 2001), and whether indeed this type of modulation also occurs for P/Q-type VDCCs, remains to be seen.

Excessive glutamate release and activation of glutamate receptors resulting in neurotoxic cell damage have been implicated in the etiology of several neurological disease states (Lipton and Rosenberg, 1994), including ischemic brain damage (Choi, 1992) and epilepsy (Kaura et al., 1995). Antagonists of facilitatory group I mGluRs exhibit neuroprotective properties in vitro and in vivo studies (Gong et al., 1995; Strasser et al., 1998; Bruno et al., 1999; O'Leary et al., 2000), with the potential use of noncompetitive antagonists, partic-

ularly coming to the fore in recent years (Gasparini et al., 2002). Noncompetitive antagonists of mGlu₅Rs, such as MPEP and SIB1893, are structurally unrelated to glutamate and are suggested to operate at the G protein transduction interface of the seven-transmembrane regions of the mGluRs. As such, they seem to be able to inhibit "constitutive" receptor activity occurring independently of the presence of ligand in heterologous expression systems (Gasparini et al., 2002). Interestingly, in this respect, in the current study, SIB1893 produced blockade of glutamate release in the absence of any added agonist(s) and despite the effective diminution of extrasynaptosomal glutamate (as endogenous agonist). This may therefore indicate the effective use of noncompetitive antagonists as "inverse agonists" to inhibit the activated states of mGlu₅R in nerve terminals. The situation may be more complicated, however, given recent data suggesting that SIB1893 and MPEP may afford neuroprotection through the positive allosteric modulation of inhibitory mGlu₄R (Mathiesen et al., 2003). Notwithstanding this possibility, given that this latter regulation is proposed to occur through an enhancement of agonist potency and efficacy, in the current studies conducted in the effective absence of any agonist, it is unlikely to be the mechanism underlying the effects of SIB1893 and MPEP.

In conclusion, our study supports the notion that noncompetitive mGlu₅R antagonists block facilitation of glutamate release occurring through a receptor mechanism involving VDCCs. This type of intervention may provide an important template for the development of novel therapeutically useful agents for the treatment of a wide range of neurological and neurodegenerative disorders.

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