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The Non-Competitive mGlu$_5$ Receptor Antagonist, 2-Methyl-6-Styryl-Pyridine (SIB1893), Depresses Glutamate Release through Inhibition of Voltage-Dependent Ca^{2+} Entry in Rat Cerebrocortical Nerve Terminals (Synaptosomes)

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Running title: mGlu$_5$ Receptor Modulation of Glutamate Release

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Abbreviations: 4AP, 4-Aminopyridine; DAG, diacylglycerol; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; PDBu, phorbol dibutyrate; PKC, protein kinase C; SIB1893, (E)-2-methyl-6-styryl-pyridine; VDCC, voltage-dependent Ca^{2+} channel

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Abstract

The effect of (E)-2-methyl-6-styryl-pyridine (SIB1893), a selective metabotropic glutamate (subtype 5) receptor ($\mathrm{mGlu_5R}$) antagonist, on glutamate release from isolated nerve terminals (synaptosomes) was examined. SIB1893 caused a potent inhibition of the Ca^{2+}-dependent release of glutamate evoked by 4-aminopyridine (4AP). That the implied $\mathrm{mGlu_5R}$-mediated modulation was contingent on diacylglycerol stimulation of protein kinase C (PKC) was indicated by PKC activator, phorbol dibutyrate (PDBu), and PKC inhibitor, RO32024, respectively superceding or suppressing the inhibitory effect of SIB1893. The inhibitory action of SIB1893 was not due it decreasing synaptosomal excitability or directly interfering with the release process at some point subsequent to Ca^{2+} influx, because SIB1893 did not alter the 4AP-evoked depolarization of the synaptosomal plasma membrane potential or Ca^{2+} ionophore ionomycin-induced glutamate release. Rather, examination of the effect of SIB-1893 on cytosolic $[Ca^{2+}]$ revealed that the diminution of glutamate release could be attributed to a reduction in voltage-dependent Ca^{2+} 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 Ca^{2+} channel blockers, ω-conotoxin GVIA and ω-agatoxin IVA. Together, these results suggest that non-competitive antagonism of $\mathrm{mGlu_5Rs}$ using SIB1893 effects a decrease in PKC activation, which subsequently

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attenuates the Ca^{2+} entry through voltage-dependent N- and P/Q-type Ca^{2+} 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.

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Metabotropic glutamate receptors (mGluRs) are a family of seven-transmembrane region (TM7), 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 catagorized into three groups: Group I receptors ($mGlu_{1+5}R$) being positively coupled to phospholipase C (PLC), with groups II ($mGlu_{2+3}R$) 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 $mGlu_{1+5}R$ 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 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

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excitatory synapses may contribute to a number of pathological states including, ischaemic brain damage, epilepsy, 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 excitoxicity therefore invokes the utility of antagonists of these receptors as neuroprotective agents. Indeed antagonists of $mGlu_5Rs$ 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 $mGlu_5R$ blockade specifically is subject of debate given that the relative contributions of the two group I mGluRs ($mGlu_1R$ and $mGlu_5R$) to the facilitation of glutamate remains contentious (Sistiaga et al., 1998;Reid, Toms, Bedingfield, and Roberts, 1999;Fazal, Parker, Palmer, and Croucher, 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

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newer sub-type selective receptor antagonists for mGluRs, particularly those possessing non-competitive activity at $mGlu_5Rs$, 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 non-competitive $mGlu_5R$ antagonist, on the 4-aminopyridine (4-AP)-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 appears to be through a reduction of voltage-dependent Ca^{2+} channel (VDCC) activity and subsequent decrease of Ca^{2+} influx into nerve terminals, rather than any upstream effect on nerve terminal excitability (Herrero, Miras-Portugal, and Sanchez-Prieto, 1992;Herrero et al., 1994). We conclude the presence of a SIB1893-sensitive $mGlu_5R$ activity in nerve terminals which, through a mechanism involving PKC, modulates VDCCs coupled to glutamate exocytosis.

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Materials and Methods

Materials

(E)-2-methyl-6-styryl-pyridine (SIB1893) and 2-methyl-6-(phenylethynyl)-pyridine (MPEP) were obtained from Tocris Cookson (Bristol, USA). Fura-2-acetoxymethyl ester and DiSC$_3$(5) were obtained from Molecular Probes (Eugene, OR, U.S.A.). Percoll was obtained from Pharmacia. Glutamate dehydrogenase and all other reagents were obtained from Sigma (Poole, U.K.) or Merck (Poole, U.K.).

Synaptosomal preparation

Synaptosomes were purified as described previously (Sihra, 1997). Briefly, the cerebral cortex from two-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 3,000 x g for 2 min (at 4°C), and the supernatant was centrifuged again at 14,500 x g 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 32,500 x g 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 NaHCO$_3$, 1 mM MgCl$_2$·6H$_2$O, 1.2 mM Na$_2$HPO$_4$, 10 mM glucose, 10 mM HEPES (pH

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7.4) before centrifugation at 27,000 x g 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 3,000 x g for 10 min (at 4°C). The supernatants were discarded, and the synaptosomal pellets stored on ice and used within 4-6 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 16µM bovine serum albumin (BSA) and incubated in a stirred and thermostated cuvette maintained at 37°C in a Perkin-Elmer LS-50B spectrofluorimeter. $NADP^+$ (2 mM), glutamate dehydrogenase (GDH; 50 units/ml) and $CaCl_2$ (1 mM) were added after 3 min. After 10 min of incubation, 4-aminopyridine (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 nm and 460 nm respectively) due to NADPH being produced by the oxidative deamination of released glutamate by GDH. 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 used to calculate released glutamate, expressed as nmol glutamate per mg synaptosomal protein (nmol/mg). Values quoted in the text represent levels of glutamate cumulatively release after 4 min depolarization i.e ″nmol/mg/4 min″,

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unless indicated otherwise. Cumulative data were analysed using Lotus 1-2-3 and MicroCal Origin. Statistical analysis was performed by two-tailed Student's t-tests.

Membrane potential measurement using DiSC$_3$(5)

Synaptosomes were preincubated and resuspended as described for the glutamate release experiments. After 3 min incubation, 4 μM DiSC$_3$(5) was added and allowed to equilibrate before the addition of $CaCl_2$ (1 mM) after 4 min incubation. 4AP was added to depolarize the synaptosomes at 10 min, and DiSC$_3$(5) fluorescence was monitored at excitation and emission wavelengths of 646 nm and 674 nm, respectively, and data accumulated at 2-s intervals. Cumulative data were analysed using Lotus 1-2-3 and results are expressed in fluorescence units.

Cytosolic Ca^{2+} 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 $CaCl_2$ 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 5,000 x g. The synaptosomal pellets were resuspended in HBM with BSA and the synaptosomal suspension stirred in a thermostatted cuvette in a Perkin-Elmer LS-50B spectrofluorimeter. $CaCl_2$ (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 nm and 380 nm (emission wavelength 505 nm)

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and data accumulated at 3-s intervals. Calibration procedures were performed as described by previously (Sihra et al., 1992), using 0.1% sodium dodecyl sulphate to obtain the maximal fluorescence with Fura-2 saturation with Ca^{2+}, followed by 10 mM EGTA (Tris buffered) to obtain minimum fluorescence in the absence of any Fura-2/Ca^{2+} complex. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$, nM) was calculated using equations described previously (Grynkiewicz et al., 1985). Cumulative data were analysed using Lotus 1-2-3 and MicroCal Origin. Statistical analysis was performed by two-tailed Student’s t-tests.

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Results

Nerve terminals were depolarized with the K^+ channel blocker 4-aminopyridine (4AP) which, by increasing the excitability of the synaptosomal plasma membrane, increases the opening of voltage-dependent Ca^{2+} channels (VDCCs) to elevate cytoplasmic free Ca^{2+} concentration and thus induces the release of vesicular glutamate (Tibbs et al., 1989). To examine the effect of $mGlu_5R$ blockade on Ca^{2+}-dependent glutamate release evoked by 4AP, the potent and selective $mGlu_5R$ 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 Ca^{2+} was 18.8 ± 0.7 nmol glutamate/mg protein/5 min (Fig. 1A; n = 8). 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 Ca^{2+}-independent release of glutamate attributable to cytosolic efflux via the reversal of the glutamate transporter (Nicholls and Sihra, 1986). Fig. 1B shows that, compared to a total 4AP evoked release of 18.2 ± 1.0 nmol/mg/4 min in the presence of added $CaCl_2$ (1 mM) (Fig. 1A, Total release), the secretagogue effected

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5.8 ± 0.5 nmol/mg/4 min in the presence of calcium-free medium containing 200 μM EGTA (Fig. 1B, Ca-independent release; n = 5), with the calculated net Ca^{2+}-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 (5.4 ± 0.6 nmol/mg/4 min; n = 5; Fig. 1B), while evincing a decrease of the net Ca^{2+}-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 $mGlu_5R$ 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 (3mM)-evoked glutamate release (n = 5; $P < 0.01$ Fig. 2) comparable to 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 superceded if the $mGlu_5R$-mediated activation of PLC is by-passed 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 following PDBu treatment had negligible effect on glutamate release (22.3 ± 0.9 nmol/mg/4 min; n = 9; Fig. 3A), there being no effective

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inhibition produced by SIB1893 in the presence of PDBU (Fig. 3B). To confirm that an mGluR5/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, RO32024 (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, Herrero, Sihra, Sanchez-Prieto, and Nicholls, 1994). Crucially however, in the presence of RO32024, SIB1893 addition had no further effect on 4AP-evoked glutamate release (10.3 ± 0.7 nmol/mg/4 min; n = 5; p < 0.01); the antagonist therefore failing to produce any additional inhibition compared to 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 $DiSC_3(5)$ to determine the effect of SIB1893 on the synaptosomal plasma membrane potential. 4AP (3 mM) caused an increase in $DiSC_3(5)$ fluorescence of 0.89 ± 0.06 fluoresence units/3 min. Preincubation of synaptosomes with 50 μM SIB1893 for 5 min before 4AP addition had no significant effect on the 4AP-mediated increase in $DiSC_3(5)$ fluorescence (0.93 ± 0.07 fluoresence 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.

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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 μM SIB1893 (Fig. 4, inset).

Downstream of membrane depolarization, presynaptic inhibition of neurotransmitter release can be mediated by a reduction of Ca^{2+} 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 Ca^{2+} influx, we used the Ca^{2+} indicator, Fura-2, to assess the effect of SIB1893 on the 4AP-evoked increase of $[Ca^{2+}]_c$. 4AP (3 mM) caused a rise in $[Ca^{2+}]_c$ to a plateau level of 178.4 ± 7.9 nM (Fig. 5). This 4AP-evoked rise in $[Ca^{2+}]_c$ was decreased by 22.8 nM with 50 μM SIB1893 preincubation ($n = 5$; $p < 0.01$; Fig. 5).

The data point to SIB1893 acting to affect voltage-dependent Ca^{2+} influx rather than upstream loci in the stimulus-release cascade. Glutamate release is supported by the entry of Ca^{2+} 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, 1995;Vazquez and Sanchez-Prieto, 1997). To establish which of these Ca^{2+} channel activities

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is involved in the SIB1893-mediated inhibition, we examined glutamate release in the presence of Ca^{2+} channel blockers. ω-CgTX (2 μM) reduced 4-aminopyridine-evoked glutamate release by 22.5 ± 3.3 % (n = 7; P < 0.01) by selectively blocking N-type Ca^{2+} channels (Fig. 6). In synaptosomes pretreated with ω-CgTX (2 μM), SIB1893 (50 μM) reduced glutamate release by a further 19.4 ± 2.4 % (Fig. 6). Application of ω-AgTX (200 nM) reduced 4-aminopyridine-evoked glutamate release by 48.7 ± 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 μM) was still able to reduce glutamate release by a further 21.9 ± 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 μM) and ω-AgTX (200 nM) together reduced 4AP-evoked glutamate release by 60.4 ± 3.9 % (n = 5; P < 0.01) (Fig. 6 and inset). In the combined presence of the Ca^{2+} channels inhibitors, application of SIB1893 (50 μM) only reduced glutamate release by a further 2.7 ± 2.4 % (n = 5), indicating significant reduction compared to that obtained when SIB1893 was applied alone (33.7 ± 3.2 %; n = 8; P < 0.01) (Fig. 6 and inset).

Although the foregoing data indicate a correlation of the inhibitory effect of SIB1893

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on glutamate release with a suppression of VDCCs, there remains the possibility that $mGlu_5R$ activation could affect targets downstream of Ca^{2+}-entry to also facilitate glutamate release. In order to determine whether $mGlu_5R$ 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 μM), which causes a direct increase in intrasynaptosomal Ca^{2+} levels without previous depolarization and VDCC activation (Sihra, Bogonez, and Nicholls, 1992), evoked the release of 24.6 ± 0.7 nmol/mg/4 min (Fig. 7). Preincubation of synaptosomes with 50 μM 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).

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Discussion

The data presented demonstrate that the selective mGlu$_5$R 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 mGlu$_5$Rs implicated appear 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 mGlu$_5$ subtype functioning to facilitate glutamate release from cerebrocortical nerve terminals.

The activation of Group I mGlu receptors has been shown to facilitatory at nerve terminals, an effect likely to be the result of DAG production and PKC-activation (Coffey, Herrero, Sihra, Sanchez-Prieto, and Nicholls, 1994;Herrero, Miras-Portugal, and Sanchez-Prieto, 1992;Lu et al., 1997;Reid, Toms, Bedingfield, and Roberts, 1999;Thomas, Jane, Harris, and Croucher, 2000). In the present study, we found that SIB1893-mediated inhibition of glutamate was reduced from 19.8 ± 1.5% to 3.0 ± 1.8% and 4.7 ± 1.5% in the presence of PDBu and RO32024 respectively. Thus, PDBu, by by-passing mGluR$_5$R-mediated PLC activation and DAG production, effectively superseded any effects SIB1893 acting at the level of the mGluR and its GPCR transduction machinery. Furthermore,

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occlusion of mGlu$_5$R inhibition by the prior suppression of PKC activity itself using RO32024, 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 mGlu$_5$R-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 non-competitive antagonist capable of acting in the absence of agonist (Gasparini, Kuhn, and Pin, 2002) and in this regard perhaps suggests the presence of an “intrinsic” or “constitutive” mGlu$_5$R activity in the synaptosomal preparation, even in the nominal absence of agonist. Given the facilitation of glutamate release by a mGlu$_5$R/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 Ca^{2+} channel and/or a direct effect on some component of the release machinery. The first of these possibilities,

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that mGlu$_5$R activation may effect synaptosomal plasma membrane hyperpolarization or decrease in synaptosomal excitability, is untenable on the basis of two observations: (i) 4AP-evoked membrane potential depolarization, measured with a membrane-potential sensitive dye, DiSC$_3$(5), was unaffected by the addition of SIB1893. (ii) 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 mGlu$_5$R 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, Miras-Portugal, and Sanchez-Prieto, 1992). Notably however in these previous studies, the use of non-selective agonists for mGlu$_5$R and mGlu$_1$R may have contributed to additional modes of modulation being invoked. Indeed, the relative roles of mGlu$_1$R and mGlu$_5$R in the regulation of glutamate release is subject of debate, with earlier studies using cerebrocortical synaptosomes indicating modulation with “mGlu$_1$R-like” pharmacology (Reid, Toms, Bedingfield, and Roberts, 1999), while more recent studies using cerebrocortical mini-slices evince regulation by mGlu$_5$R (Fazal, Parker, Palmer, and Croucher, 2003). Notwithstanding this, with regard to the mechanism of modulation, consistent with the lack of role mGlu$_5$R in modulating nerve terminal excitability indicated by the current work, the study by Reid et al. looking at the facilitation of radiolabelled glutamate release from

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synaptosomes by type 1 mGluR activation, also concluded modulation of membrane potential to be absent (Reid, Toms, Bedingfield, and Roberts, 1999).

If it isn't the modulation of synaptosomal excitability, then the locus of action of the mGlu$_5$R 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 Ca^{2+} 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 Ca^{2+} influx stimulated by 4AP. Moreover, while SIB1893-induced inhibition of glutamate release persisted after individual blockade of N- or P/Q-type Ca^{2+} channels, combined blockade of both channel types abrogated the effect of the inhibitor. These results are consistent with whole-cell patch clamp studies showing mGlu$_5$R-mediated potentiation of VDCC activity (Chavis et al., 1995) and support the hypothesis that facilitation of N- and P/Q-type Ca^{2+} channel activities together, potentially underlie the direct or indirect influence of mGlu$_5$R activation on glutamate release. Contrary to this, a previous synaptosomal study addressing this issue found no effect of mGluR activation on ^{45}Ca^{2+} influx, leading to the suggestion that modulation may occur downstream of Ca^{2+} entry (Reid, Toms, Bedingfield, and Roberts, 1999). However, as the latter study pharmacologically invoked an mGlu$_1$R-like rather than mGlu$_5$R activation, we directly examined whether the mGlu$_5$R invoked in the current study

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affected potential loci downstream of VDCC activation to modulate glutamate release. Our observation that SIB1893 did not affect release evoked by Ca^{2+} ionophore, ionomycin (which directly introduces Ca^{2+} into synaptosomes without previous depolarization and VDCC activation), rules out any significant effect of $mGlu_5R$ activation at steps downstream of Ca^{2+} entry and at the level of the coupling of Ca^{2+} to the exocytotic release machinery itself.

An $mGlu_5R$-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 Ca^{2+}-channel sub-type and subunit being addressed (Catterall, 2000;Dolphin, 1995). Indeed, all of the known VDCC subtypes appear 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 previously reported (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 $mGlu_5R$/PLC/DAG/PKC signaling cascade facilitating voltage-dependent Ca^{2+}-entry. Whether this modulation of synaptosomal VDCCs by PKC reflects modification of intrinsic channel activity itself or, implies modification of inhibitory GPCR modulation of

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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 aetiology of several neurological disease states (Lipton and Rosenberg, 1994) including ischaemic 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 (Bruno et al., 1999;Gong et al., 1995;O'Leary et al., 2000;Strasser et al., 1998), with the potential use of non-competitive antagonists particularly coming to the fore in recent years (Gasparini, Kuhn, and Pin, 2002). Non-competitive antagonists of $mGlu_5Rs$, such as MPEP and SIB1893, are structurally unrelated to glutamate and are suggested to operate at the G-protein transduction interface of the TM7 regions of the mGluRs. As such, they appear to be able to inhibit “constitutive” receptor activity occurring independently of the presence of ligand in heterologous expression systems (Gasparini, Kuhn, and Pin, 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 non-competitive antagonists as “inverse agonists” to inhibit the activated states of $mGlu_5R$ in nerve terminals. The situation may be more complicated however, given recent data suggesting that SIB1893 and MPEP may afford neuroprotection

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through the positive allosteric modulation of inhibitory $mGlu_4R$ (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 SIB1983 and MPEP.

In conclusion, our study supports the notion that non-competitive $mGlu_5R$ 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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Figure Legends

Figure 1. SIB1893 inhibits the Ca^{2+}-dependent release of glutamate evoked by 4AP. Rat synaptosomes (0.3 mg per ml) were preincubated for 3 min before the addition of 1 mM $CaCl_2$. 4AP (3 mM) was added after a further 7 min to effect depolarization (arrow). Ca^{2+}-independent release was assayed by omitting $CaCl_2$ and adding 200 μM EGTA, 30 s prior to depolarization. Glutamate release was assayed by on-line fluorimetry. Total release (A), Ca^{2+}-independent release (B) and Ca^{2+}-dependent release (C) were calculated in the absence or presence of SIB1893 50 μM, added 5 min prior to depolarization. Ca^{2+}-dependent glutamate release was calculated (total glutamate release minus Ca^{2+}-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 (2s), but error bars are only shown every 10 s for clarity. ** Total glutamate release and Ca^{2+}-dependent glutamate release, in the presence of SIB1893, were significantly different from control release; $p< 0.01$, two-tailed Student's t-test.

Figure 2. MPEP inhibits the release of glutamate evoked by 4AP. Rat synaptosomes (0.3 mg per ml) were treated as described in the legend to Figure 2 and glutamate release assayed in the absence or presence of MPEP (50 μM), added 5 min prior to depolarization. Data

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represent means ± S.E.M. of five independent synaptosomal preparations. Means ± S.E.M. was obtained for each data point (2s), 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.

Figure 3. SIB1893-mediated inhibition of 4AP-evoked glutamate release is disrupted by the PKC activator or PKC inhibitor. Glutamate release evoked by 4AP (3 mM) in (A) 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 RO32024 (1 μM). PDBu and RO32024 were added 10 min prior to the addition of SIB1893 50 μM. Columns are the means ± 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 (%) significantly different from that obtained with SIB1893 alone; P<0.05, two-tailed Student's t-test.

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Figure 4. SIB1893 does not change the synaptosomal membrane potential. Synaptosomal membrane potential monitored with DiSC$_3$(5) in the absence (Control) or presence of 50 μM SIB1893, before and after depolarization with 3 mM 4AP. Inset: SIB1983 modulation of 30mM 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 ± 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

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

Figure 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 μM), ω-conotoxin GVIA (2 μM) + SIB1893 (50 μM), ω-agatoxin VIA (200 nM), ω-agatoxin VIA (200 nM) + SIB1893 (50 μM), ω-conotoxin GVIA (2 μM) + ω-agatoxin VIA (200 nM) and ω-conotoxin GVIA (2 μM) +

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ω-agatoxin VIA (200 nM) + SIB1893 (50 μM). Data represent means ± 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 μM) or ω-AgTX IVA (200 nM) + ω-conotoxin GVIA (2 μM) + SIB1893 (50 μM) showing complete occlusion of the effect of SIB1893 following VDCC blockade.

Figure 7. SIB1893 fails to affect ionomycin-induced glutamate release. Glutamate release was induced by ionomycin (5 μM) in the absence (Control) or presence of SIB1893 (50 μM). Data represent means ± 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 μM was not significantly different from that in control.

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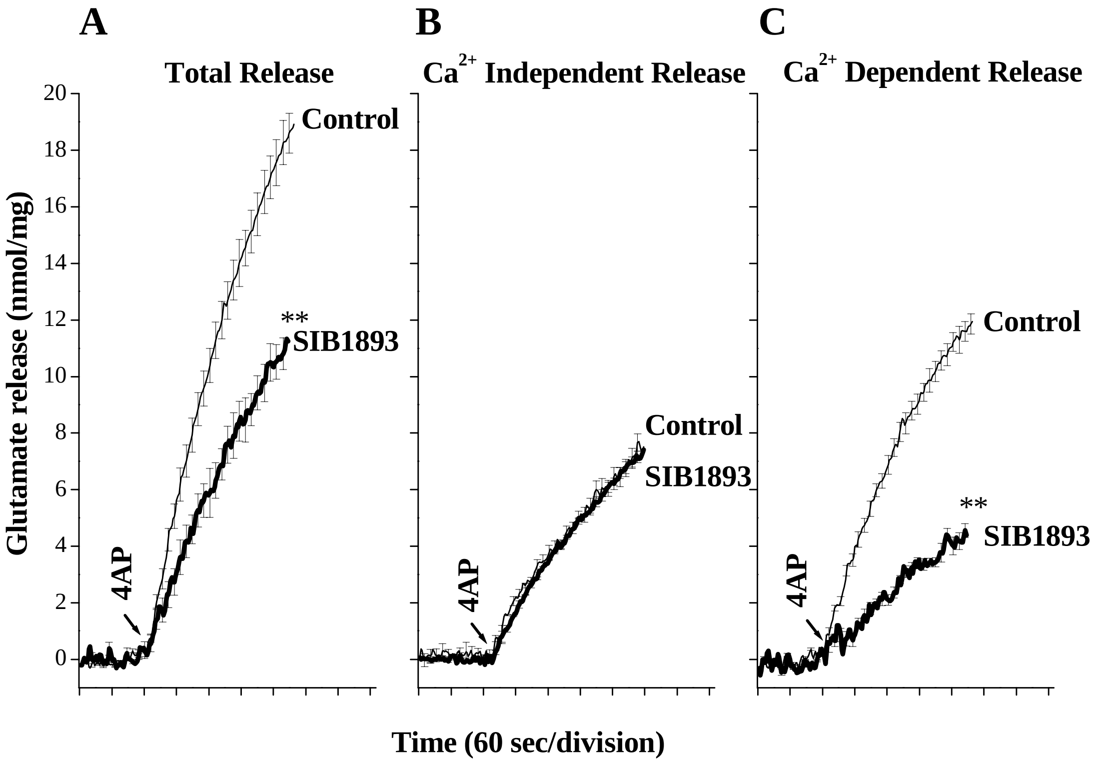

Figure 1. Wang and Sihra.

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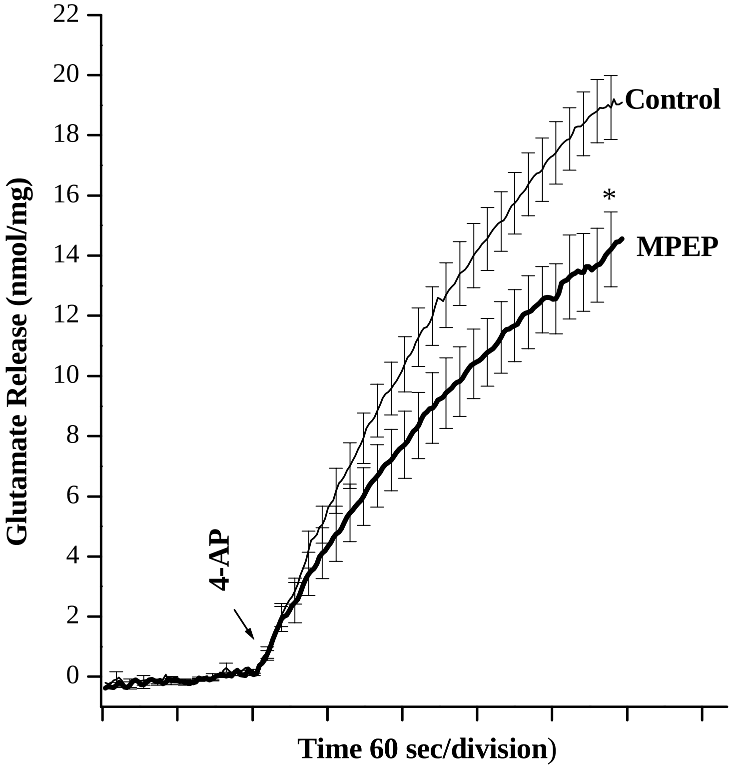

Figure 2. Wang and Sihra.

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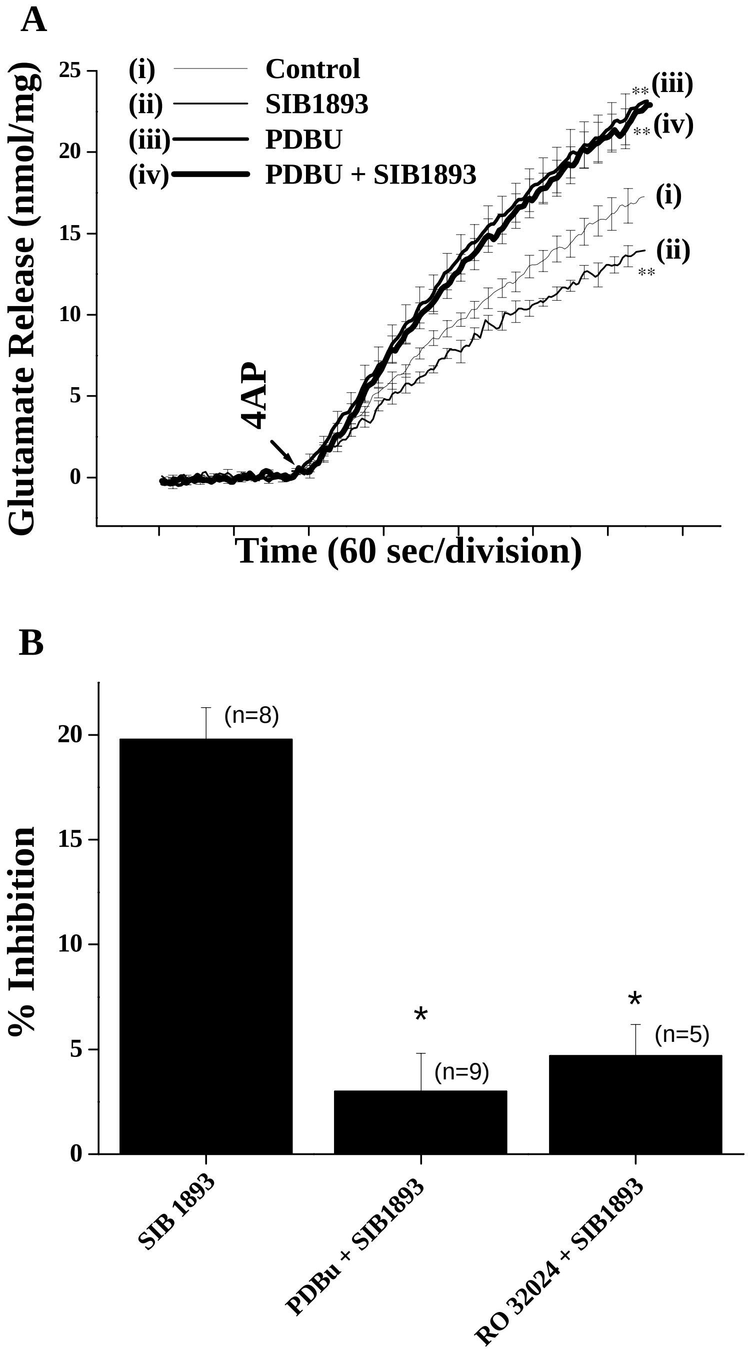

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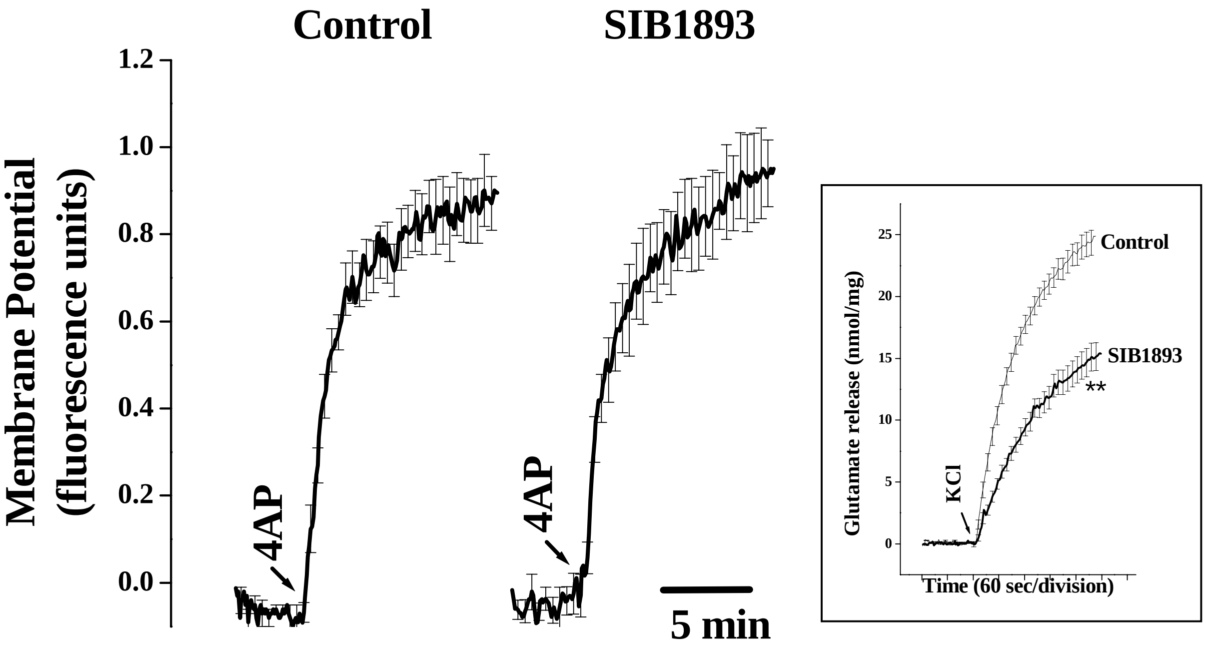

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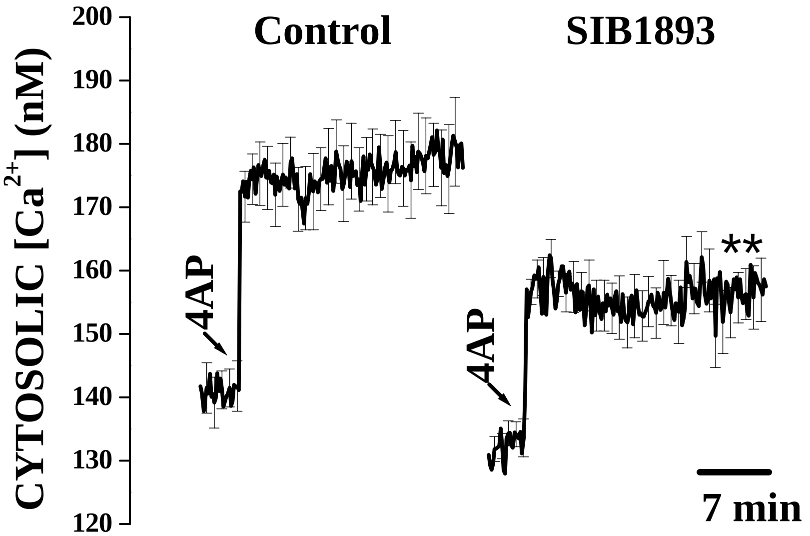

Figure 5. Wang and Sihra.

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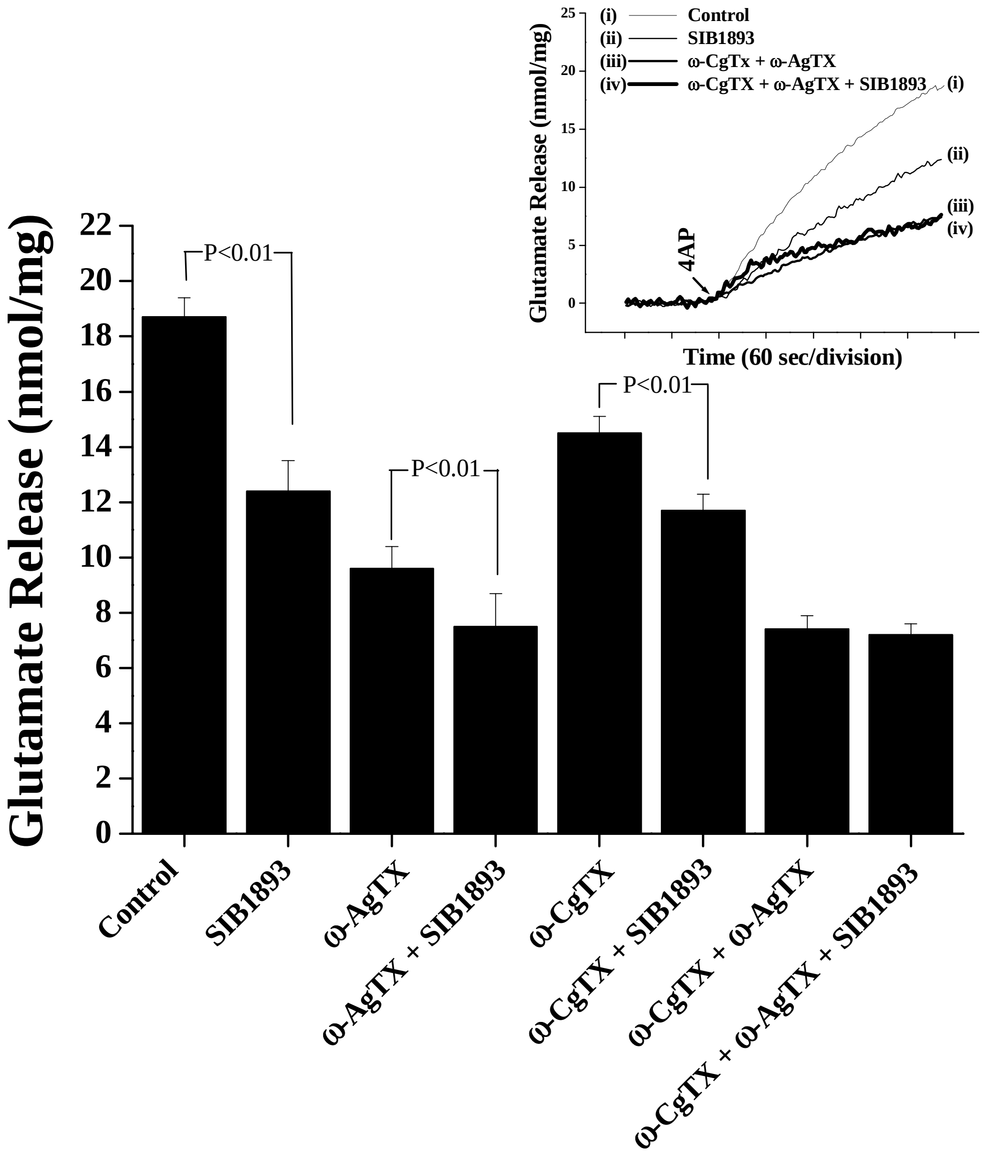

Figure 6. Wang and Sihra.

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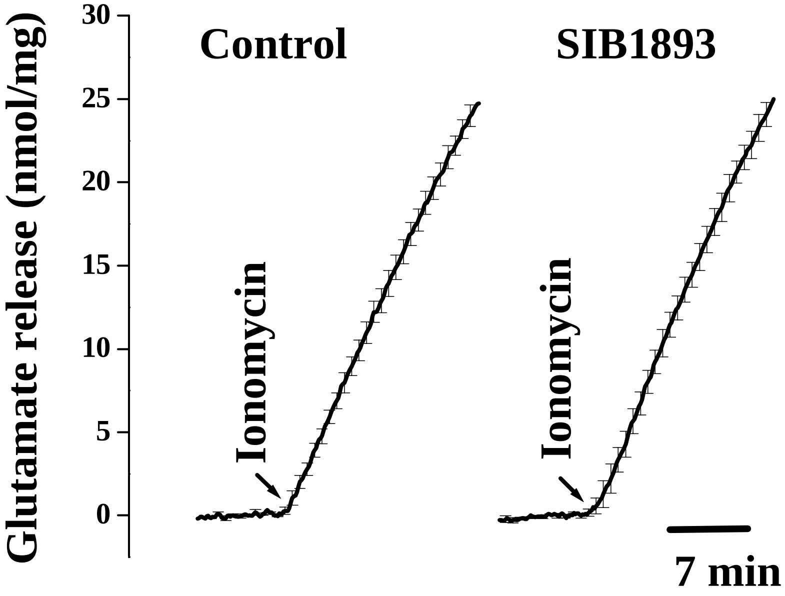

Figure 7. Wang and Sihra

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