Evidence that DHPG-induced nociception depends on glutamate release from primary afferent C-fibres

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We examined whether enhanced glutamate release contributes to the expression of persistent spontaneous nociceptive behaviours (SNBs) in rats induced by intrathecal (i.t.) administration of the selective group I mGluR agonist, (RS)-3,5-dihydroxyphenylglycine ((RS)-DHPG). Pretreatment with drugs that have been shown to inhibit glutamate release, including a group II metabotropic glutamate receptor (mGluR) agonist (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate ((2R,4R)-APDC), a group III mGluR agonist L-2-amino-4-phosphonobutyrate (L-AP4), or the use-dependent sodium channel blockers 3,5-diamino-6-(2,3-diclorophenyl)-1,2,4-triazine (lamotrigine) and 2-amino-6-trifluoromethoxybenzothiazole (riluzole), produced dose-dependent reductions in (RS)-DHPG-induced SNBs. We have also shown that incubation of rat lumbar spinal cord slices with (RS)-DHPG potentiates 4-aminopyridine-evoked (4-AP) release of glutamate. Furthermore, we found that destruction of unmyelinated primary afferent C-fibres by neonatal capsaicin treatment significantly reduced (RS)-DHPG-induced SNBs in adult rats. Together, these results suggest that (RS)-DHPG-induced nociception is dependent on spinal glutamate release, probably from primary afferent C-fibres. *NeuroReport* 11:1631–1635 © 2000 Lippincott Williams & Wilkins.

Key words: Capsaicin; Excitatory amino acids; Metabotropic glutamate receptors; mGluR; Pain

INTRODUCTION

Metabotropic glutamate receptors (mGluRs), in particular group I mGluRs (mGluR1 and mGluR5), have been implicated in persistent nociception. Previously, we have shown that i.t. administration of the selective [1] and potent [2] group I agonist ((RS)-3,5-dihydroxyphenylglycine (RS)-DHPG) produces persistent thermal hyperalgesia and mechanical allodynia [3], as well as spontaneous nociceptive behaviours (SNBs) that are observed for prolonged periods [4]. Further studies have shown that application of group I mGluR antagonists to rat spinal cord attenuates dorsal horn neuronal activity associated with knee joint inflammation [5] or cutaneous application of mustard oil [6], and reduces mechanical and cold allodynia associated with sciatic nerve injury [7]. In contrast to group I mGluR agonists, i.t. application of mGluR group II and III agonists do not have any nociceptive effects [4], and have been shown to decrease mechanical and cold allodynia in a rat model of neuropathic pain [8].

Recent evidence suggests that group I mGluRs may influence synaptic activity by facilitating excitatory amino acid (EAA) release in the CNS [9,10]. Thus, it is expected that the nociceptive effects of spinal (RS)-DHPG may depend, at least partly, on an enhanced release of glutamate in the spinal cord dorsal horn. In contrast, activation of the group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6–8) mGluRs has been shown to decrease EAA release in the CNS [11,12]. Thus, it is possible group II and III mGluRs may be effective at alleviating persistent nociception by inhibiting glutamate release in the spinal cord dorsal horn.

To test the above hypotheses, we examined the effects of i.t. pretreatment with the selective group II agonist (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (2R,4R)-APDC)) [13] and the selective group III mGluR agonist (L-2-amino-4-phosphonobutyrate (L-AP4)) [14] on (RS)-DHPG-induced SNBs. To further implicate the involvement of glutamate release in mediating DHPG-induced SNBs, we also examined the effects of two sodium channel blockers known inhibit the release of glutamate, lamotrigine and riluzole on DHPG-induced SNBs. Lamotrigine and riluzole inhibit the release of glutamate, as well as other transmitters, by producing a use-dependent block of Na^+ channels [15].

We further sought to confirm neurochemically that application of (R,S)-DHPG was able to facilitate glutamate release in the rat spinal cord. In this experiment, we examined whether incubation of rat spinal cord slices with (RS)-DHPG potentiated basal or 4-aminopyridine (4-AP)-evoked glutamate release. Also, to delineate the mechanisms underlying (RS)-DHPG-induced SNBs, we sought to

assess the source of the glutamate release. It has been previously shown that the release of glutamate in the rat spinal cord evoked either by chemical or electrical stimulation [16,17] is significantly reduced in rats treated neonatally with capsaicin. Since neonatal capsaicin produces a near complete destruction of primary afferent C-fibres [18], these results have been interpreted as indicating that the source of stimulus-evoked release of glutamate is mainly from primary afferent C-fibres. We used a similar method to examine whether (RS)-DHPG-induced nociception is dependent on glutamate release from primary afferent C-fibres. Thus, we treated rats neonatally with capsaicin, and examined its effects on (RS)-DHPG-induced nociception in adult rats.

MATERIALS AND METHODS

Pharmacological studies: In the first experiment, 105 male Long–Evans rats (250–400 g; Charles River) were treated i.t. with either 42 mM NaOH/saline vehicle (pH 8.0), L-AP4 (6, 25 or 100 nmol) or (2R,4R)-APDC (25, 100 or 400 nmol), or i.p. with either 45% cyclodextrin/saline vehicle, lamotrigine (2, 10 or 50 mg/kg) or riluzole (6, 9 or 12 mg/kg), 15 min prior to an i.t. injection of 50 nmol (RS)-DHPG.

All mGluR agonists or the NaOH/saline vehicle were injected in a 30 µl volume by lumbar puncture between the L4 and L5 spinal vertebrae while rats were under brief halothane anesthesia. Lamotrigine, riluzole or the cyclodextrin/saline vehicle were injected i.p. to conscious rats. (RS)-DHPG and L-AP4 were obtained from Tocris Cookson (St. Louis, MO) and (2R,4R)-APDC was graciously donated by the Eli Lilly Co. (Indianapolis, IN). Riluzole was supplied by Research Biochemicals (Natick, MA), and lamotrigine was obtained from our hospital pharmacy.

Behavioural testing: Following i.t or i.p. drug pretreatment and i.t. administration of (RS)-DHPG, each rat was placed in a $30 \times 30 \times 30$ cm Plexiglas testing box, and 5 min later the following categories of SNBs were separately timed over a 60 min period: (1) elevating, shaking or stamping hind paws; (2) licking or biting hind paws; (3) elevating or whipping tail; (4) licking or biting tail. At the end of the 60 min testing period, the total time that each rat spent exhibiting SNBs was obtained by adding together the time spent exhibiting SNBs in each of the four categories.

Neurochemical studies: Male Long Evans rats (250–275 g; Charles River, Quebec, Canada) were decapitated and their spinal cords quickly removed by hydro-propulsion. Spinal cords were immediately placed in ice-cold Krebs–Heinseleit bicarbonate buffer containing (mM) 111 NaCl, 26.2 $NaHCO_3$, 4.7 KCl, 1.2 NaH_2PO_4, 1.8 $CaCl_2$, 1.2 $MgSO_4$ and 11 glucose, gassed with 95% oxygen/5% carbon dioxide to maintain a pH of 7.4. Blood vessels and dura mater were carefully pulled off the cord with tweezers prior to slicing the lumbar spinal cord with a McIlwain tissue chopper to obtain 400 µm slices.

The lumbar spinal cord slices were placed on a filter paper supported by a nylon mesh screen on a cylindrical platform. The slice perfusion chamber was a closed system with a volume of 0.75 ml. Slices were perfused at 37°C from top to bottom with a Krebs-Heinseleit buffer at a flow rate of 0.75 ml/min. Slices were equilibrated for 30 min prior to collection of 10 serial 2 min fractions. Three initial fractions were collected to determine basal glutamate release, then the superfusion buffer was switched to a buffer containing either 4-AP (10 µM), DHPG (50 µM) or DHPG + 4-AP for 10 min followed by a wash-out period. Following collection, slices were removed from the tissue baths for determination of wet tissue weights.

For determination of glutamate release, superfusate samples were deproteinated with $ZnSO_4$ and $Ba(OH)_2$ then stored at −20°C prior to analysis. Glutamate was assessed by reverse-phase HPLC as described previously [19]. Briefly, a Beckman two pump solvent delivery system equipped with a LiChrospher-100 RP-18 5 µm column was used for analysis. Samples were derivitized immediately prior to injection in a 100 µl loop. Derivitization was accomplished by addition of a borate buffer prepared from 1 M boric acid solution titrated to pH 4.2 with NaOH and 9-fluoronymethyl chloroformate (Fmoc-Cl) in acetone. The mixture was vortexed and incubated at room temperature for 3 min before extraction of organic solvents with 100 µl ethylacetate/400 µl pentane (v/v 20/80). The aqueous phase containing derivitized amino acids was analyzed. The elution was carried out using a linear gradient from 10% acetonitrile-methanol-acetic acid to 90% acetonitrile-acetic acid mobile phase at a flow rate of 1.3 ml/min. The elute was monitored by a Varian fluorescence detector (330 nm inference filter for emission). Data handling and quantification were accomplished with a DS 604 chromatographic data system (Varian Instruments). An internal standard (Fmoc-Ala) was added to each sample prior to derivitization for quantification by peak height and identification of glutamate was accomplished by comparison of retention times to glutamate standards.

Lastly, we compared the amount of evoked glutamate release from 4-AP (10 µM), DHPG (50 µM) or 4-AP + DHPG to the basal glutamate release levels.

C-fibre neurotoxin study: In the third experiment, neonatal male rats (born from female pregnant Long–Evans rats received from Charles River) were injected s.c. with capsaicin (dissolved in 60% dimethylsulfoxide (DMSO) in 0.9% saline) 24 h (25 mg/kg), 48 h (50 mg/kg) and 72 h (50 mg/kg) post-natally. When capsaicin-treated rats weighed ~250 g each they were weight-matched with male Long–Evans naive rats (Charles River). Nine animals from each group were injected i.t. with 50 nmol (RS)-DHPG and were tested using the same behavioural protocol as the pharmacological studies. A capsaicin eye-wipe test was performed with eight additional animals from each group (capsaicin-treated and control rats) in order to assess the effectiveness of C-fibre destruction in the capsaicin-treated animals. For this test, a 10 µl volume of dilute capsaicin (1%, dissolved in the 60% DMSO solution) was dropped into one of the eyes of each rat, and the time that each animal spent wiping the affected eye with the forepaws or hindpaws was recorded for 1 min.

RESULTS

In experiment 1, we assessed the effects of pretreatment with the group II mGluR agonist (2R,4R)-APDC and the group III mGluR agonist L-AP4 on (RS)-DHPG-induced

SNBs. Analysis of variance (ANOVA) indicated significant main effects of pretreatment for (2R,4R)-APDC ($F(3,22) = 6.3$, $p < 0.01$) and L-AP4 ($F(3,22) = 6.0$, $p < 0.01$). *Post-hoc* comparisons (Dunnett's *t*-test) revealed that, compared with vehicle control animals, rats pretreated with the 100 or 400 nmol, but not the 25 nmol, dose of (2R,4R)-APDC spent significantly less time exhibiting SNBs during the 60 min period (Fig. 1a). Compared with the vehicle controls, rats pretreated with the 25 or 100 nmol, but not the 6 nmol, dose of L-AP4 also spent significantly less time exhibiting nociceptive behaviours (Fig. 1b).

Second, we assessed the effects of pretreatment with the sodium channel blockers lamotrigine or riluzole on (RS)-DHPG-induced SNBs. ANOVA indicated significant main effects of pretreatment for lamotrigine ($F(3,23) = 6.6$, $p < 0.01$) and riluzole ($F(3,22) = 19.2$, $p < 0.001$). Results from *post-hoc* comparisons (Dunnett's *t*-test) revealed that, compared with vehicle controls, rats pretreated with the 10 or 50 mg/kg, but not the 2 mg/kg, dose of lamotrigine spent significantly less time exhibiting SNBs (Fig. 2a). Also compared with vehicle controls, rats pretreated with the 6, 9 and 12 mg/kg doses of riluzole spent significantly less time exhibiting nociceptive behaviours (Fig. 2b). It should be noted that locomotor behaviour was decreased in rats receiving the two higher doses of riluzole, although righting, placing and grasping reflexes were not affected.

In experiment 2, we assessed the effects of 4-AP, (RS)-DHPG, and (RS)-DHPG + 4-AP on the release of glutamate in the rat spinal cord. One-way ANOVA revealed a significant main effect of treatment ($F(3,15) = 48.2$, $p < 0.001$).

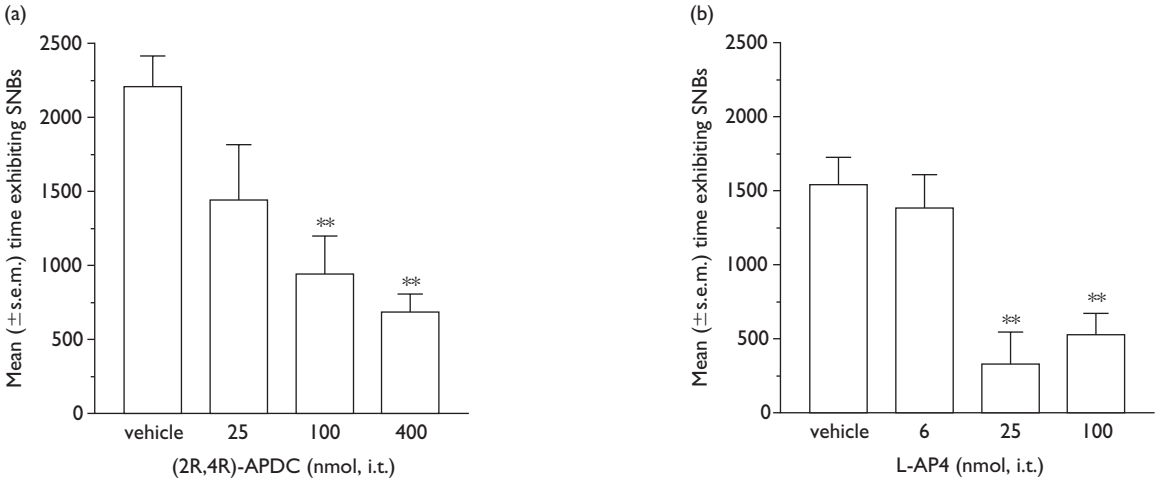

Fig. 1. Mean (± s.e.m.) time(s) spent exhibiting SNBs over a 60 min period for rats treated with 0 nmol (NaOH/saline vehicle; $n = 6$), 25 nmol ($n = 6$), 100 nmol ($n = 8$) or 400 nmol ($n = 6$) (2R,4R)-APDC (**a**), or with 0 nmol (NaOH/saline vehicle; $n = 8$), 6 nmol ($n = 6$), 25 nmol ($n = 6$) or 100 nmol ($n = 6$) L-AP4 (**b**), 10 min prior to the (RS)-DHPG (50 nmol) injection. *Post-hoc* analyses (Dunnett's *t*-test) indicate significant differences between the experimental and control groups. ** $p < 0.01$.

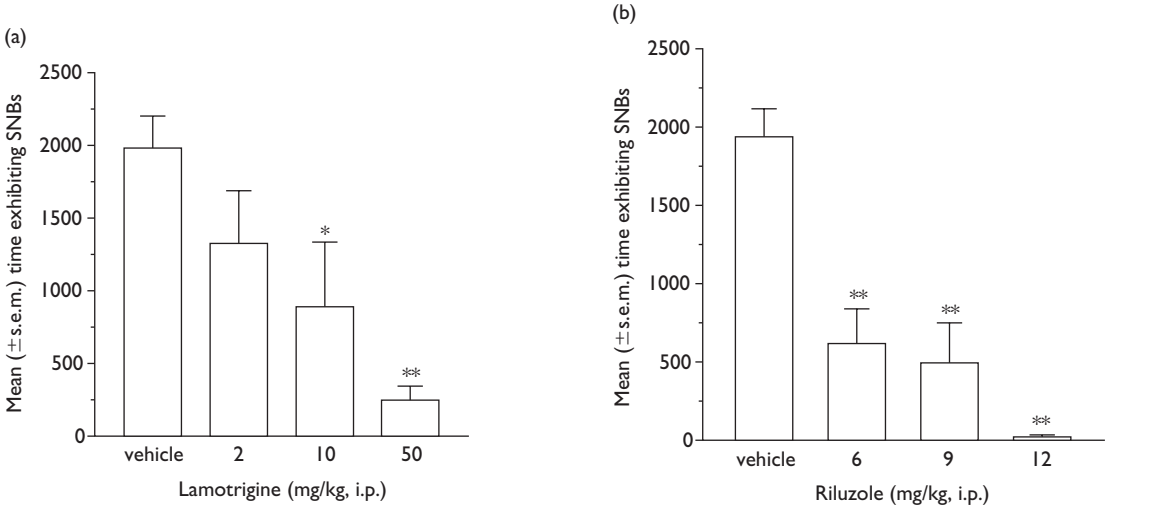

Fig. 2. Mean (± s.e.m.) time(s) spent exhibiting SNBs over a 60 min period for rats treated with 0 mg/kg (45% cyclodextrin/saline vehicle; $n = 9$), 2 mg/kg ($n = 6$), 10 mg/kg ($n = 6$) or 50 mg/kg ($n = 6$) lamotrigine (**a**) or with 0 mg/kg (45% cyclodextrin/saline vehicle; $n = 9$), 6 mg/kg ($n = 6$), 9 mg/kg ($n = 6$) or 12 mg/kg ($n = 5$) riluzole (**b**), 15 min prior to the (RS)-DHPG (50 nmol) injection. *Post-hoc* analyses (Dunnett's *t*-test) indicate significant differences between the experimental and control groups. * $p < 0.05$, ** $p < 0.01$.

Multiple comparisons (Fisher LSD test) revealed that (RS)-DHPG potentiated the release of glutamate-evoked by 4-AP (Fig. 3a). Slices that were incubated with (RS)-DHPG + 4-AP had significantly greater levels of glutamate release than the basal glutamate release, or that evoked by (RS)-DHPG or 4-AP alone ($p < 0.01$). The release evoked by (RS)-DHPG or 4-AP alone did not differ significantly from the basal glutamate release ($p > 0.05$).

In experiment 3, we assessed the effects of neonatal treatment with capsaicin on (RS)-DHPG-induced SNBs in adult rats. A *t*-test ($t(14) = 5.0$, $p < 0.01$) revealed that capsaicin-treated animals spent less time exhibiting the nociceptive behaviours during the 60 min period, compared with weight-matched control animals (Fig. 3b). A *t*-test ($t(16) = 4.6$, $p < 0.01$) also revealed that, following a drop of dilute capsaicin into one of the eyes, capsaicin-treated animals spent significantly less time wiping the affected eye than did control animals, indicating that the neonatal capsaicin treatment produced significant destruction of the unmyelinated primary afferent fibres.

DISCUSSION

Recently, group I mGluRs have become the focus of much attention in neuronal processing. Evidence suggests that this mGluR subtype plays a crucial role in various types of synaptic plasticity in the CNS [20]. The present study provides evidence that selective activation of group I mGluRs with i.t. (RS)-DHPG administration produces persistent SNBs in rats, which depends on increased glutamate release, most likely from primary afferent C-fibres.

We have demonstrated that i.t. pretreatment with the selective group II mGluR agonist, (2R,4R)-APDC, or the selective group III mGluR agonist, L-AP4, dose-dependently reduced (RS)-DHPG-induced SNBs in rats. Specifically, we found that, compared with vehicle, pretreatment with 100 or 400 nmol (2R,4R)-APDC, and 25 or 100 nmol L-AP4 significantly reduced the amount of time rats spent exhibiting SNBs. We believe the observed anti-nociceptive effects of the group II or group III mGluR agonists are attributable to their well established ability to decrease glutamate release in various CNS structures [11,12]. In support of this, group II and III mGluR agonists have been found to reduce synaptic excitation in models associated with enhanced glutamate release. Thus, pretreatment with (1S,3S,4S)-α-carboxycyclopropylglycine (L-CCGI; group II agonist) or L-AP4 attenuates limbic seizures induced by administration of (RS)-DHPG into the thalamus of mice [21], while both and (2R,4R)-APDC and L-AP4 reduce mechanical and cold allodynia in neuropathic rats [8].

It was also demonstrated that pretreatment with the sodium channel blockers, lamotrigine or riluzole, dose-dependently reduced (RS)-DHPG-induced SNBs in rats. Compared with vehicle, i.p. pretreatment with 2, 10 and 50 mg/kg lamotrigine produced ~40, 66 and 90% reductions in SNBs, respectively. No side-effects were observed following pretreatment with any dose of lamotrigine. Pretreatment with 6, 9 and 12 mg/kg riluzole produced ~66, 75 and 99% reductions in SNBs, respectively. However, the highest doses of riluzole produced side-effects that may have interfered with the rats' ability to express SNBs. Although motor reflexes remained intact, pretreatment with 9 and 12 mg/kg riluzole decreased spontaneous locomotor activity. It has previously been shown that riluzole produces a loss of the righting reflex at doses > 15 mg/kg ($ED_{50} = 25.6$ mg/kg) [22].

Lamotrigine has been demonstrated to have anti-nociceptive properties in other models of pain. Systemic administration has been shown to reduce mechanical hyperalgesia associated with streptozotocin-induced diabetes or subplantar prostaglandin E_2 injection [23] and can reduce cold allodynia associated with chronic constriction of the sciatic nerve and spinal nerve ligation in rats [24]. Furthermore application of lamotrigine to the dorsal horn of the rat spinal cord inhibits electrically-evoked release of

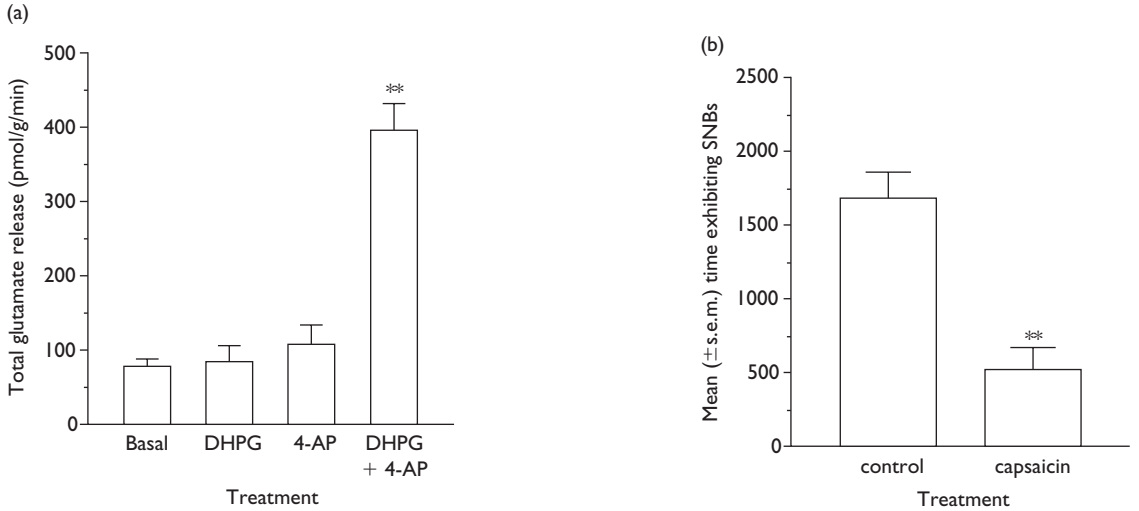

Fig. 3. (**a**) Mean (± s.e.m.) total basal glutamate release ($n = 6$) and mean total glutamate released by (RS)-DHPG (50 μM; $n = 6$), 4-AP (10 μM; $n = 4$) or the combination of both agents ($n = 6$) from lumbar spinal cord slices. *Post-hoc* analyses (Dunnett's *t*-test) indicate a significant increase in glutamate release compared to basal release. ** $p < 0.01$. (**b**) Mean (± s.e.m.) time(s) spent exhibiting SNBs induced by i.t.(RS)-DHPG (50 nmol), over a 60 min period for rats treated neonatally with capsaicin ($n = 9$) and for weight-matched controls ($n = 9$). A Student's *t*-test ($t(16) = 5.01$, $p < 0.01$) indicates significant differences between the two groups. ** $p < 0.01$.

glutamate and aspartate [25]. This gives further evidence that lamotrigine's ability to reduce the nociceptive effects induced by (RS)-DHPG is attributable to its inhibition of glutamate release. Little is known about riluzole's potential antinociceptive or anti-hyperalgesic effects.

Interestingly, sodium channel blockers like lamotrigine and riluzole produce a use- and voltage-dependent blockage of Na^+ channels. Although as sodium channel blockers they can inhibit the release of various neurotransmitters, it has been suggested that due to their use-dependence they have the ability to reduce excessive release of glutamate without interfering with normal neurotransmission in the CNS [15]. This property has been proposed to mediate the anti-convulsant and anti-ischemic effects of these so-called glutamate release inhibitors in animals, and may be useful in explaining their antinociceptive effects in persistent pain models [23,24].

Previously it has been shown that local application of the selective group I mGluR agonist, (RS)-DHPG produces an increase in glutamate release in rat parietal cortex as measured by *in vivo* microdialysis [9]. In the present study, we are the first to demonstrate that (RS)-DHPG facilitates 4-AP evoked-release of glutamate from rat lumbar spinal cord slices. Our results support the previous observation that the active isomer (S)-DHPG potentiates 4-AP-evoked release of [^{3}H]glutamate release from rat cerebrocortical synaptosomes [10].

In the third experiment, animals that were treated neonatally with capsaicin exhibited fewer SNBs following i.t. administration of (RS)-DHPG compared with weight-matched control animals. These results support the previous finding that neonatal capsaicin treatment attenuates the release of glutamate in the spinal cord of adult rats evoked either by chemical or electrical stimulation [16,17]. Since neonatal capsaicin treatment is known to permanently destroy unmyelinated primary afferent fibres in rats [18], these results provide evidence of the necessity of intact primary afferent C-fibres in mediating (RS)-DHPG-induced persistent nociception. This suggests a presynaptic locus of group 1 mGluRs on primary afferent C-fibre terminals. A presynaptic locus of group I mGluRs in the rat spinal cord is supported by the recent demonstration that there is a dramatic reduction in mGluR1α staining in lamina I–II of the spinal cord following dorsal rhizotomy [26].

CONCLUSION

Evidence presented here suggests that i.t. DHPG-induced nociceptive behaviours are dependent on an enhancement of spinal glutamate release. Thus, we have found that i.t. DHPG-induced nociceptive behaviours are attenuated by i.t. pretreatment with group I and II mGluR agonists, which have been shown to inhibit glutamate release in various CNS regions. We have also demonstrated that i.t. DHPG-induced nociceptive behaviours are attenuated by i.p. pretreatment with the sodium channel blockers lamotrigine and riluzole, which are known to inhibit glutamate release in the CNS. We have further shown that DHPG potentiates the 4-aminopyridine-evoked release of glutamate from spinal cord slices, providing neurochemical support for our interpretation. Finally, we provide evidence for a role of glutamate release from primary afferent C-fibres by demonstrating that i.t. DHPG-induced nociceptive behaviours are attenuated in rats treated neonatally with capsaicin.

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