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Involvement of spinal serotonin receptors in the regulation of intraspinal acetylcholine release

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Abstract

Stimulation of spinal serotonin (5-HT) receptors results in analgesia and release of acetylcholine. We investigated the involvement of 5-HT_1, 5-HT_2, and 5-HT_3 receptor subtypes in the regulation of spinal acetylcholine release. A spinal microdialysis probe was placed dorsally at about the C5 level in anaesthetized rats. The selective serotonin reuptake inhibitor citalopram was found to increase acetylcholine release when infused via the microdialysis probe. Several doses of the 5-HT receptor agonists 8-hydroxy-2-(di-*n*-propylamino)tetraline (8-OH-DPAT, 5-HT_{1A}), 1,4-dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5H-pyrrolo[3,2-*b*]pyridin-5-one dihydrochloride (CP93129, 5-HT_{1B}), α-methyl-5-hydroxytryptamine maleate (m5-HT, 5-HT_2), 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 5-HT_{2C}), and 1-(*m*-chlorophenyl)-biguanide (5-HT_3) were subsequently infused via the microdialysis probe. Only 8-OH-DPAT, CP93129, and m5-HT increased acetylcholine release dose dependently. The 5-HT_{1A} receptor selective antagonist (*S*)-*N*-*tert*-butyl-3-(4-(2-methoxyphenyl)piperazine-1-yl)-2-phenylpropanamide hydrochloride and the 5-HT_{2A} receptor selective antagonist ketanserin tartrate inhibited the 8-OH-DPAT and the m5-HT induced acetylcholine release. The results suggest that 5-HT_{1A} and the 5-HT_{2A} receptors are involved in the regulation of acetylcholine release in the spinal cord.

Keywords: Serotonin; Serotonin receptor subtype; Spinal antinociception; Acetylcholine release

1. Introduction

Administration of not only oxotremorine, nicotine and epibatidine (Höglund et al., 2000; Kommalage and Höglund, 2003, 2004), but also potent analgesics such as morphine (Bouaziz et al., 1996), lidocaine (Abelson and Höglund, 2002a,b) and clonidine (Klimscha et al., 1997) increase intraspinal acetylcholine release. In contrast, when administered in a dose that produce hyperalgesia in the tail-flick test atropine decreases the intraspinal release of acetylcholine (Abelson and Höglund, 2002a,b). These findings suggest that intraspinal acetylcholine release may play an important role in the regulation of pain threshold at the spinal cord level.

Acetylcholine may function as a mediator of the serotonin-induced analgesia since endogenous acetylcholine is necessary for the nociceptive suppression induced by spinal serotonin (5-hydroxy tryptamine, 5-HT) administration (Li et al., 1994) and since 5-HT_2 receptor agonist induced antiallodynic effects are suggested to be mediated via spinal acetylcholine release (Obata et al., 2003). Furthermore, muscarinic acetylcholine receptors are involved in 5-HT_2 receptor agonist induced antiallodynia (Obata et al., 2002) and antinociception in neuropathic pain (Sasaki et al., 2003).

5-HT fibres have been described descending from the brain stem to the spinal cord that either facilitate or inhibit transmission of nociceptive stimuli (Millan, 1997). It seems that 5-HT elicits pro- or antinociceptive responses in the spinal cord, probably dependent on the involvement of various receptor subtypes. Spinal administration of 5-HT produces antinociception in tail-flick and hotplate tests (Crisp et al., 1991; Xu et al., 1994), which can be

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counteracted by the non-selective 5-HT receptor antagonist methysergide (Yaksh and Wilson, 1979). Moreover, stimulation of some of the spinal 5-HT receptor subtypes with selective receptor agonists results in antinociception or pronociceptive responses in animal models. 5-HT_{1A}, 5-HT_{1B}, 5-HT_2, 5-HT_{2A}, 5-HT_{2C} and 5-HT_3 receptor agonists produce antinociception or antinociceptive behaviour in different behavioural models (Eide and Hole, 1991; Eide et al., 1990; Glaum et al., 1990; Millan et al., 1997; Ochi and Goto, 2000; Sasaki et al., 2001; Xu et al., 1994), whereas some studies have reported pronociceptive effects of 5-HT_{1A} and 5-HT_{2C} receptor agonists (Ali et al., 1994; Bervoets et al., 1990; Millan et al., 1997).

The present study tested the hypothesis that an increased availability of endogenous spinal serotonin influences the spinal release of acetylcholine. Furthermore, we investigated the role of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_3 receptors on acetylcholine release since these are suggested to be involved in nociceptive mechanisms in the spinal cord (Kidd et al., 1993; Marlier et al., 1991; Millan, 1997; Thor et al., 1993). Receptor agonists, alone or in combination with receptor antagonists, to 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_3 receptor subtypes were administered and intraspinal acetylcholine release measured. The present results suggest that the 5-HT_{1A} and the 5-HT_{2A} receptors are involved in the regulation of acetylcholine release in the spinal cord.

2. Materials and methods

2.1. Rats

All experiments were conducted after approval by the Animal Ethics Committee in Uppsala, Sweden. Male Sprague–Dawley rats (B&K Universal, Sollentuna, Sweden) weighing 330–400 g were provided with free access to food (R36, Ewos, Vadstena, Sweden) and tap water at all times. The animals were kept on a 12 h light/dark cycle (lights on 6 am to 6 pm) at 20±1 °C for 1 week before use.

2.2. Drugs and chemicals

Neostigmine bromide, acetylcholine chloride, choline, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), and mesulergine hydrochloride, were purchased from Sigma Sweden AB (Stockholm, Sweden). The substances 1-(*m*-chlorophenyl)-biguanide (mCPBG), 1,4-dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5H-pyrrolo[3,2-*b*]pyridin-5-one dihydrochloride (CP93129), α-methyl-5-hydroxytryptamine maleate (m5-HT), ketanserin tartrate, *N*-(1-azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1,4-benzoxazine-8-carboxamide (Y25130), isamoltane hemifumarate, (*S*)-*N*-*tert*-butyl-3-(4-(2-methoxyphenyl)piperazine-1-yl)-2-phenylpropanamide hydrochloride (WAY-100135), and 8-hydroxy-2-(di-*n*-propylamino)tetraline (8-OH-DPAT), and citalopram hydrobromide, were purchased from Tocris Cookson (Bristol, England). Some ligands such as quipazine, SB 216641, and SDZ SER 082 which are reported as 'more selective' to certain 5-HT receptors could not be used due to poor solubility in Ringer's solution. The salts NaCl, $CaCl_2$, KCl, and Na_2HPO_4, were purchased from Kebo lab (Spånga, Sweden).

2.3. Microdialysis

Anaesthesia was induced with 4.5% isoflurane (Abbott Scandinavia AB, Solna, Sweden) in 100% oxygen. The trachea of rats were intubated and connected to a Harvard (Harvard Apparatus, Holliston, MA, USA) ventilator and placed on a heated pad to maintain body temperature (perirectal temperature) at 37.5 °C. During surgery, anaesthesia was maintained with about 3% isoflurane in 100% oxygen and the end-tidal pCO_2 was kept at 4 kPa. For insertion of the microdialysis probe, a midline incision was made at the back of the skull. Neck muscles were removed carefully to expose the *cisterna magna*. The *dura* and *pia mater* were cut and a semi-rigid spinal microdialysis probe was inserted dorsally in the spinal tissue. The probe was located longitudinally with the tip at about the C5 level in the superficial dorsal horn. The probe was constructed from a hollow fibre of 300 μm outer diameter having a cut-off at 11 kDa molecular weight. The dialysis membrane was bowed to form a U-shaped loop, 12 mm long. The microdialysis probe was perfused at a flow rate of 2.5 μl/min with Ringer's solution (147 mM NaCl, 2.4 mM CaCl, 4.0 mM KCl) containing 10 μM neostigmine to prevent degradation of acetylcholine (Billard et al., 1995; Höglund et al., 2000; Roth et al., 1996). After insertion of the microdialysis probe, the isoflurane concentration was reduced to 1.5% and rats were allowed to rest for 40 min before starting the sampling of 20 μl spinal microdialysates. Acetylcholine was quantified online by High Performance Liquid Chromatography (HPLC) as described earlier (Höglund et al., 2000). In each experiment in vitro, pre- and post-recovery of the probes was assessed by dialysis of a 10 pmol standard to ensure that the probes had not been damaged during the experiment. Only data from experiments where the mean post-recovery was within three standard deviations of mean pre-recovery are presented here.

2.4. Doses of drugs

Prior to be used in an in vivo experiment, the drugs were tested in the HPLC system to ensure that they were not interfering with acetylcholine measurements. Before administration of the drugs the basal release of acetylcholine was determined by analysis of samples from five 10 min cycles with Ringer's solution as the dialysis fluid. The drugs were dissolved in Ringer's solution and administered via the dialysis probe using a syringe pump with a 2.5 μl/min flow rate.

The concentrations of citalopram and the duration of the citalopram experiments were based on previous microdialysis experiments where the drug was administrated via a microdialysis probe similarly to the present experiments (Consolo et al., 1994; Hilgert et al., 2000). Four or five concentrations of each receptor agonist were selected to be used in the dose–response relationship studies after pilot experiments in which the receptor agonists were administered in a range between 1 nM and 1 mM. In control experiments, Ringer's solution was administered throughout the experiment.

To test the inhibitory effect of receptor antagonists on 8-OH-DPAT, CP93129, and m5-HT induced increase in acetylcholine release the lowest receptor agonist concentration (10 μM) that significantly increased the acetylcholine release from baseline was used. DOI and mCPBG, which did not produce a dose-dependent increase in acetylcholine release as determined from the preceding experiments, were used in a concentration of 100 μM in these experiments. The receptor agonists were administered up to seven cycles before the receptor antagonist was added to the dialysis fluid. The receptor agonist and receptor antagonist mixture was thereafter administered for another six or seven 10 min sampling periods. The receptor antagonist concentration to be used in these studies was selected after several pilot experiments to find the lowest concentration, in a range between 1 nM and 1 mM, which inhibited the receptor agonist-induced acetylcholine release. The concentrations of both receptor agonists and receptor antagonists used were in accordance with previous studies where these substances were administered intrathecally (Bardin et al., 2000; Obata et al., 2003; Sasaki et al., 2001, 2003). In control experiments, the receptor agonists were administered alone throughout the experiment.

2.5. Statistics

All statistical analyses were performed using SPSS, version 10.0.5 (SPSS, Chicago, Illinois, USA). The effect of the various substances was expressed as percent change from baseline, defined as the mean release of acetylcholine during five 10 min sampling periods during which the microdialysis probe was perfused with Ringer's solution only. Analysis of variance (ANOVA) with Dunnett's post-hoc test was used to calculate the statistical significance of effects of individual substances, against baseline release of acetylcholine. Repeated measures analysis was used to calculate the statistical difference in intraspinal acetylcholine release observed after administration of citalopram and control, different receptor agonist doses and control as well as between receptor antagonists and control.

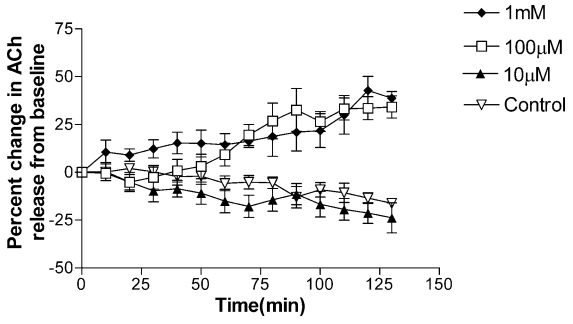

Fig. 1. Microdialysis of 100 μM (n=8), and 1 mM (n=5) citalopram increased acetylcholine release significantly (P<0.05) from baseline and from control experiments as determined by a repeated measures test (P<0.05). Average basal acetylcholine release was 304.4±34.2 nM in these experiments.

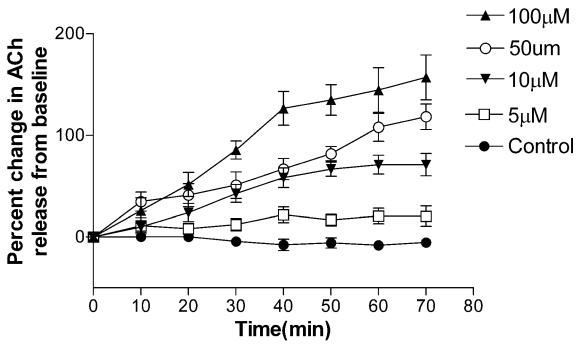

Fig. 2. Microdialysis of 10 μM (n=8), 50 μM (4), and 100 μM (n=7) 8-OH-DPAT increased acetylcholine release significantly (P<0.05) from baseline. The 5 μM, 10 μM, 50 μM, and 100 μM concentrations increased acetylcholine release significantly (P<0.05) from control experiments. Average basal acetylcholine release was 172.4±44.3 nM in these experiments.

3. Results

Citalopram 100 μM and 1 mM increased the acetylcholine release compared to the control, whereas citalopram 10 μM did not change acetylcholine release significantly (Fig. 1). The 5-HT_{1A} receptor agonist 8-OH-DPAT (10, 50 and 100 μM concentrations), the 5-HT_{1B} receptor agonist CP93129 (10, 50 and 100 μM concentrations), the 5-HT_2

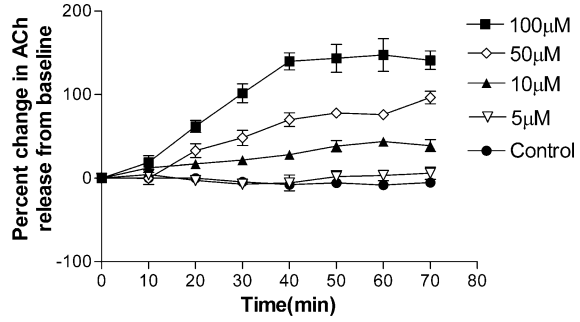

Fig. 3. Microdialysis of 10 μM (n=7), 50 μM (n=4), and 100 μM (n=4) CP93129 increased acetylcholine release significantly (P<0.05) from baseline and from control experiments. Average basal acetylcholine release was 159.8±34.1 nM in these experiments.

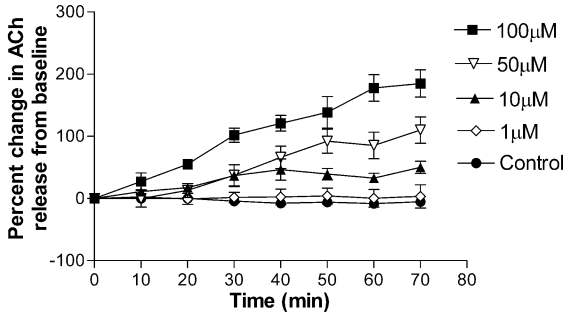

Fig. 4. Microdialysis of 10 μM (n=6), 50 μM (n=4) and 100 μM (n=5) m5-HT increased acetylcholine release significantly (P<0.05) from baseline and from control experiments. Average basal acetylcholine release was 184.7±34.9 nM in these experiments.

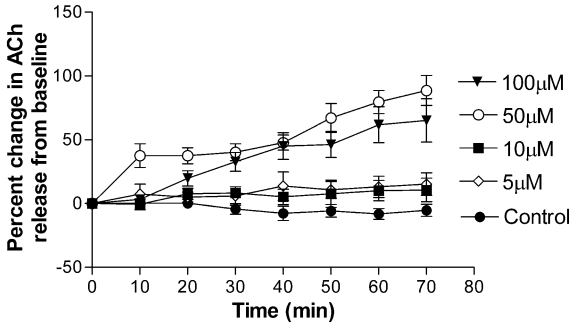

Fig. 6. Microdialysis of 50 μM (n=4) and 100 μM (n=7) mCPBG increased acetylcholine release significantly (P<0.05) from baseline and from control experiments. Average basal acetylcholine release was 207.2±53.7 nM in these experiments.

receptor agonist m5-HT (10, 50 and 100 μM concentrations), the 5-HT_{2C} receptor agonist DOI (5, 50 and 100 μM concentrations), and the 5-HT_3 receptor agonist mCPBG (50 and 100 μM concentrations) all increased acetylcholine release significantly from baseline (Figs. 2–5 and 6).

A dose–response curve was made for each of the dialyzed receptor agonist concentrations against the average acetylcholine release at 60 and 70 min after start of administration. Calculated EC_{50} values were 17.7 μM, 155 μM, and 494 μM (goodness of fit for sigmoidal dose–responses were 0.96, 0.96, and 0.97) for 8-OH-DPAT, CP93129, and m5-HT respectively. Goodness of fit for sigmoidal dose-responses for DOI and mCPBG was as low as 0.86 and 0.81 respectively why EC_{50} values were not calculated.

The 5-HT_{1A} receptor antagonist WAY100135 (100 μM) reduced the receptor agonist 8-OH-DPAT (10 μM) induced acetylcholine release significantly (Fig. 7). Similarly, the 5-HT_{2A} receptor antagonist ketanserin (100 μM) reduced the receptor agonist m5-HT (10 μM) induced acetylcholine release significantly (Fig. 8).

The 5-HT_{1B} receptor antagonist isamoltane, the 5-HT_{2C} receptor antagonist mesulergine or the 5-HT_3 receptor antagonist Y-25130 administered in concentrations up to 1 mM did not significantly affect the induced acetylcholine release observed after administration of the receptor agonists CP93129, DOI, or mCPBG respectively.

As some 5-HT receptor agonists have low selectivity, and since the 5-HT_{1B} receptor antagonist isamoltane, the 5-HT_{2C} receptor antagonist mesulergine, and the 5-HT_3 receptor antagonist Y25130 did not block the corresponding receptor agonist-stimulated acetylcholine release, it is conceivable that these receptor agonists act on other 5-HT receptors than their 'target' 5-HT receptors. Therefore, other 5-HT receptor antagonists were used to study the influence on CP93129-, DOI-, and mCPBG-induced acetylcholine release. The 5-HT_{1A} receptor antagonist WAY100135 (100 μM) was administered after CP93129, since both are 5-HT_1 family ligands. A mixture of WAY100135 (100 μM) and ketanserin (100 μM) was administered after DOI and mCPBG since it was found that stimulation of 5-HT_{1A} and 5-HT_{2A} receptors influenced the acetylcholine release.

The 5-HT_{1B} receptor agonist CP93129 (10 μM) induced acetylcholine release was reduced by administration of the 5-HT_{1A} receptor antagonist WAY100135 (100 μM) (Fig. 9).

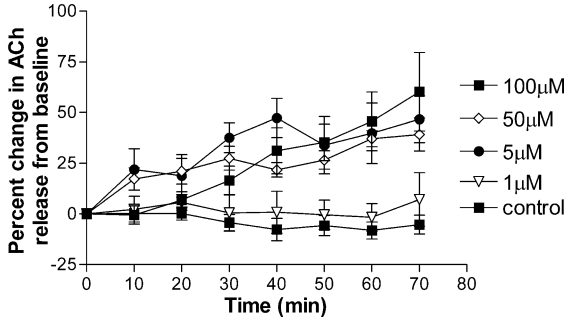

Fig. 5. Microdialysis of 5 μM (n=5), 50 μM (n=4), and 100 μM (n=9) DOI increased acetylcholine release significantly (P<0.05) from baseline and from control experiments. Average basal acetylcholine release was 199.7±52.7 nM in these experiments.

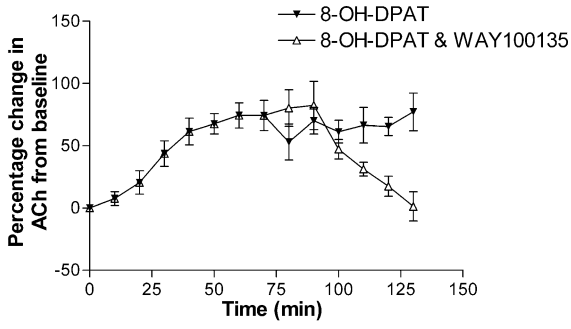

Fig. 7. The figure shows the effect of 8-OH-DPAT (10 μM, n=3) administered during thirteen 10 min periods and the effect of a mixture of WAY100135 (100 μM) and 8-OH-DPAT (10 μM) (n=4) administered during the 7th to the 13th periods. The WAY100135 (100 μM) and 8-OH-DPAT (10 μM) mixture significantly decreased the acetylcholine release in comparison to 8-OH-DPAT (10 μM) alone (P<0.05) as determined by a repeated measures test. Average basal acetylcholine release was 298.2±77.3 nM in these experiments.

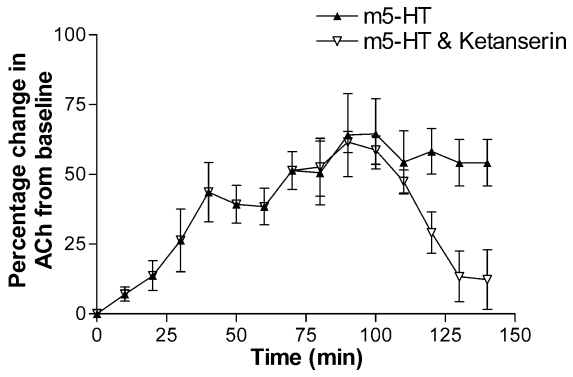

Fig. 8. The figure shows the effect of m5-HT (10 μM, n=6) administered during fourteen 10 min periods and the effect of a mixture of ketanserin (100 μM) and m5-HT (10 μM) (n=4) administered during the 7th to the 14th periods. The ketanserin (100 μM) and m5-HT (10 μM) mixture significantly decreased the acetylcholine release in comparison to m5-HT (10 μM) alone (P<0.05) as determined by a repeated measures test. Average basal acetylcholine release was 242.3±33.4 nM in these experiments.

Acetylcholine release induced by the 5-HT_{2C} receptor agonist DOI (100 μM), or the 5-HT_3 receptor agonist mCPBG (100 μM) was not significantly affected by a mixture of the 5-HT_{1A} receptor antagonist WAY100135 (100 μM) and the 5-HT_{2A} receptor antagonist ketanserin 100 μM (data not shown) (Table 1).

Table 1
Serotonin agonist (ago) and antagonist (antago) effects on ACh release

Drugs	ACh release
8-OH-DPAT(5-HIT_{1A} ago)	↑
8-OH-DPAT(5-HIT_{1A} ago) and WAY100135(5-HIT_{1A} antago)	↓
CP93129 (5-HIT_{1B} ago)	↑
CP93129 (5-HIT_{1B} ago) and Isamoltane (5-HIT_{1B} antago)	→
CP93129 (5-HIT_{1B} ago) and WAY100135(5-HIT_{1A} antago)	↓
m5-HT (5-HT_2 ago)	↑
m5-HT (5-HT_2 ago) and Ketanserin (5-HT_{2A} antago)	↓
DOI (5-HT_{2C} ago)	↑
DOI (5-HT_{2C} ago) and Mesulergine(5-HT_{2C} antago)	→
DOI (5-HT_{2C} ago) and Mixed antagonist[a]	→
mCPBG (5-HT_3 ago)	↑
mCPBG (5-HT_3 ago) and Y-25130 (5-HT_3 antago)	→
mCPBG (5-HT_3 ago) and Mixed antagonist[a]	→

The effect of a mixture of receptor antagonist and receptor agonist on acetylcholine release is relative to the receptor agonist induced acetylcholine release.

[a] Mixed antagonist means WAY100135 (5-HT_{1A} antago) and Ketanserin (5HT_{2A} antago).

4. Discussion

Citalopram is the most selective inhibitor of serotonin reuptake currently available, with minimal inhibition of norepinephrine, acetylcholine and dopamine uptake (Bezchlibnyk-Butler et al., 2000). Since citalopram administration in the present study increased the intraspinal acetylcholine release it may be concluded that increased serotonin availability in the dorsal horn stimulates acetylcholine release. In contrast to the effects of the serotonin receptor agonists, citalopram acted slowly to increase the spinal acetylcholine release. This observation is in correspondence with previous studies (Consolo et al., 1994; Hilgert et al., 2000) in which citalopram was administrated via a dialysis probe.

The results also show that the 5-HT_{1A} receptor agonist 8-OH-DPAT increased intraspinal acetylcholine release in a dose-dependent manner and that the 5-HT_{1A} selective receptor antagonist WAY100135 reduced 8-OH-DPAT-induced acetylcholine release. This suggests that 8-OH-DPAT induces spinal acetylcholine release by acting on spinal 5-HT_{1A} receptors. This finding is consistent with a previous microdialysis study of the dorsal hippocampus, which showed that the 5-HT_{1A} receptor agonist 8-OH-DPAT induced acetylcholine release (Izumi et al., 1994).

Although the 5-HT_{1B} receptor agonist CP93129 increased acetylcholine release in a dose-dependent manner, the effect of CP93129 was not reduced by the 5-HT_{1B} receptor selective antagonist isamoltane. Furthermore, we found that the 5-HT_{1A} receptor antagonist WAY100135 inhibited CP93129-induced acetylcholine release. Although CP93129 is a potent receptor agonist on 5-HT_{1B} receptors, it has low selectivity (Barnes and Sharp, 1999). On the other hand, the selectivity of WAY100135 is high for the 5-HT_{1A} subtype (Barnes and Sharp, 1999; Fletcher et al., 1993; Schoeffter et al., 1997). Therefore, it is likely that CP93129 induced spinal acetylcholine release is mediated through an action on 5-HT_{1A} receptors.

The present results show that the 5-HT_2 receptor agonist, m5-HT induced a dose-dependent acetylcholine release and

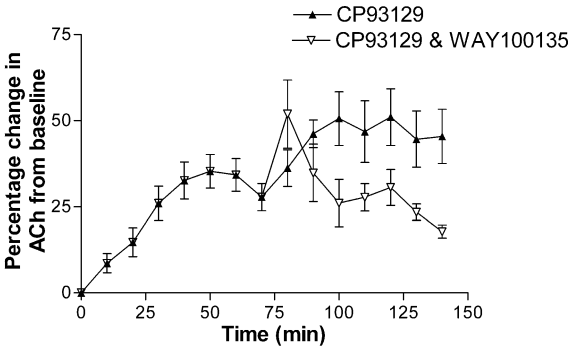

Fig. 9. The figure shows the effect of CP93129 (10 μM, n=4) administered during fourteen 10 min periods and the effect of a mixture of WAY100135 (100 μM) and CP93129 (10 μM) (n=4) administered during the 7th to the 14th periods. The WAY100135 (100 μM) and CP93129 (10 μM) mixture significantly decreased the acetylcholine release in comparison to CP93129 (10 μM) alone (P<0.05) as determined by a repeated measures test. Average basal acetylcholine release was 160.1±100.9 nM in these experiments.

that the 5-HT_{2A} receptor antagonist ketanserin reduced m5-HT-induced acetylcholine release. This suggests a 5-HT_{2A} receptor involvement in the regulation of spinal acetylcholine release. Regarding the role of the 5-HT_{2C} receptors our results are ambiguous. Although the 5-$HT_{2A/2C}$ receptor agonist DOI also increased the acetylcholine release, the effect was not dose-dependent. Considering also that the 5-HT_{2C} receptor selective antagonist mesulergine (Bardin et al., 2000; Pazos et al., 1984) and that a mixture of WAY100135 and ketanserin did not inhibit DOI-induced acetylcholine release, it is unlikely that 5-HT_{1A} or 5-HT_{2A} receptors are involved in DOI-induced acetylcholine release. It is possible that DOI produces an increase in acetylcholine release through a non-specific effect since it is well known that the 5-HT_2 receptor family agonists are non-selective, whereas the receptor antagonists are highly selective (Barnes and Sharp, 1999; Baxter et al., 1995; Cussac et al., 2002).

Thus, the present data suggest that 5-HT_{2A}, but not 5-HT_{2C} receptors, are involved in the regulation of spinal acetylcholine release. In a recent study, Obata et al. (2003) presented data corroborating our findings in that the 5-HT_2 receptor agonist induced antiallodynic effect may be mediated by 5-HT_{2A}, but not 5-HT_{2C} receptor induced spinal acetylcholine release.

In the present study, we found that the 5-HT_3 receptor agonist mCPBG increased acetylcholine release was not dose-dependent. This observation, together with the finding that the 5-HT_3 receptor antagonist Y25130 did not block mCPBG-induced acetylcholine release, suggests that mCPBG at the concentrations used does not act on 5-HT_3 receptors to induce acetylcholine release.

The 5-HT_3 receptor is structurally different from other 5-HT receptors as it is a multi-subunit, ligand-gated ion channel. Few studies are available concerning the affinity of the 5HT_3 receptor ligands, although it is well known that ligands differ appreciably in inter-species affinity (Barnes and Sharp, 1999). Y25130 was shown to be a potent and selective 5-HT_3 receptor antagonist in rat, rabbit, and guinea pig (Sato et al., 1992). mCPBG has been shown to have high affinity for 5-HT_3 receptors with large inter-species differences in affinity (Kilpatrick et al., 1990, 1991; Lummis et al., 1993). Not much is known about the selectivity of mCPBG. One study, however, showed a relatively high level of non-specific binding associated with mCPBG (Steward et al., 1993).

As administration of the 5-HT_{2C} and 5-HT_3 receptor agonists did not produce dose-dependent increases of acetylcholine release, and since the induced acetylcholine release was not inhibited by any of the receptor antagonists used, it is possible that these receptor agonists act on other 5-HT receptors than the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C} or 5-HT_3 subtypes investigated in the present study. Further studies are needed to elucidate if DOI and mCPBG increase spinal acetylcholine release through an action on the 5-HT_{2B}, 5-HT_4, 5-HT_5, 5-HT_6, or 5-HT_7 receptors that recently was proposed to be present in spinal cord (Baxter et al., 1995; Millan, 2002; Schmidt and Jordan, 2000; Wu et al., 2001).

As we use a microdialysis probe for substance administration, only a fraction of dialyzed substances diffuse into the tissue depending on the limited time for passive diffusion through the dialysis membrane. Factors such as fat solubility of the administered substances and blood flow may further alter the amount that actually reaches the receptors. Thus, EC_{50} values reported here are possibly higher that what would be obtained in a more isolated preparation of spinal cord.

In conclusion, the present study shows that 5-HT_{1A} and 5-HT_{2A} receptors are involved in the regulation of acetylcholine release in spinal cord and that the 5-HT_{1B} receptor agonist CP93129 augment acetylcholine release by acting on 5-HT_{1A} receptors. The most probable location of the 5-HT_{1A} receptors is on cell bodies of GABA neurons which inhibit the firing rate of these neurons when activated by serotonin. By inhibiting the release of GABA, one of the inhibitory transmitters in the spinal cord, serotonin indirectly would increase acetylcholine release (Baba et al., 1998). It is unlikely that 5-HT_{1A} receptors are located on cholinergic neurones as they are not inhibiting acetylcholine release contrary to the inhibitory nature of 5-HT_{1A} receptors (Millan, 2002). On the other hand, 5-HT_{2A} receptors might be located on cholinergic neurons increasing cellular excitability and hence increasing acetylcholine release, probably by activating muscarinic receptors as suggested by Obata et al. (2003).

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