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

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Abstract

It has been shown that analgesics such as morphine, lidocaine and clonidine increase the release of spinal acetylcholine. Acetylcholine may therefore play an important role in the regulation of spinal pain threshold. Since behavioral as well as in vitro studies have shown a clear involvement of GABA (γ-amino butyric acid) receptors in the regulation of spinal nociceptive mechanisms, the present study focused on the role of GABA receptors for spinal acetylcholine release control. GABA receptor agonists and antagonists were infused via a spinal microdialysis probe and acetylcholine release was measured. The $GABA_A$ receptor agonist muscimol decreased acetylcholine release and the antagonist bicuculline increased acetylcholine release. The $GABA_B$ receptor agonist baclofen decreased acetylcholine release whereas the antagonist saclofen did not change acetylcholine release. The results suggest that both GABA receptor subtypes have an inhibitory role on spinal dorsal horn acetylcholine release and that the $GABA_A$ receptors are tonically regulating acetylcholine release.

Keywords: GABA; GABA receptor; Acetylcholine; Spinal antinociception; Spinal pain transmission

1. Introduction

Administration of well-known analgesics such as morphine (Bouaziz et al., 1996), lidocaine (Abelson and Höglund, 2002a,b) and clonidine (Klimscha et al., 1997) as well as less established analgesics such as oxotremorine, nicotine and epibatidine (Höglund et al., 2000; Kommalage and Höglund, 2003); (Kommalage and Höglund, 2004) produces an increase in 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 acetylcholine may play an important role in the regulation of pain threshold at the spinal cord level.

Several mechanisms have been suggested for cholinomimetic-induced antinociception including decreased release of primary afferent fibre transmitters such as substance P (Smith et al., 1989) and glutamate (Li and Zhuo, 2001) and modulation of the release of transmitters in the descending pain inhibitory pathways (Meyer et al., 2000; Rogers and Iwamoto, 1993). Perhaps the most plausible mediator of acetylcholine-induced antinociception may, however, be GABA (γ-amino butyric acid) since previous behavioral as well as in vitro studies have shown a clear involvement of GABA in the regulation of spinal nociceptive mechanisms. Thus, spinal GABA levels increased with inflammatory pain (Viggiano et al., 2004) and decreased presynaptic GABA levels were demonstrated after chronic constriction injury and spared nerve injury (Moore et al., 2002). Also, an increased $GABA_B$ gene expression was shown in dorsal root ganglions following peripheral formalin injections (McCarson and Enna, 1999).

Since a strong co-localization of cholinergic and GABAergic neurons exist in the superficial lamina of the dorsal horn where the predominant nociceptive processing is taking place (Kosaka et al., 1988; Li et al., 2002; Todd, 1991), it is not surprising that muscarinic M2, M3 and M4 receptors, suggested to be localized on GABA neurones, increase the excitability of inhibitory interneurons and enhance GABA release when activated (Baba et al., 1998; Zhang et al., 2005). The activation of muscarinic receptors would thus indirectly inhibit the release of glutamate from primary nociceptive nerve fibres and produce antinociception. Further evidences for a cholinergic regulation of GABA release were shown by Li et al. (2002) who, in their spinal slices

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preparation, found that the frequency of $GABA_A$ receptor-mediated miniature inhibitory postsynaptic currents was significantly increased by acetylcholine and inhibited by atropine.

The suggestion that GABA mediates the antinociceptive effect of cholinergic agonists is strengthened by the observation that the $GABA_A$ receptor antagonist bicuculline significantly blocked the analgesic effects of the nicotinic agonists, (−) nicotine and (+)epibatidine (Rashid and Ueda, 2002). Further, Takeda et al. (2003) suggested that presynaptic nicotinic receptors directly contribute to the increase of GABA release in substantia gelatinosa.

Although it seems to be clear that GABA is a mediator of cholinergic antinociceptive mechanisms, a feedback regulation of acetylcholine release by GABA may be present in the spinal cord. After examining the role of $5\text{-}HT_{1A}$ receptor agonists (5-HT−5-hydroxytryptamine) on acetylcholine release in a previous study, we suggested that $5\text{-}HT_{1A}$ receptors are located on GABAergic neurons modulating $5\text{-}HT_{1A}$ receptor agonist-induced acetylcholine release (Kommalage and Höglund, 2005). Using our previously described in vivo intraspinal microdialysis technique (Höglund et al., 2000), the present study tested the hypothesis that $GABA_A$ and $GABA_B$ receptors are involved in the spinal regulation of acetylcholine release. We found that the $GABA_A$ receptor, but not the $GABA_B$ receptor, regulates acetylcholine release tonically in the dorsal horn.

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 and 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 at 6 am to 6 pm) at 20±1 °C for one week before use.

2.2. Drugs and chemicals

Neostigmine bromide, acetylcholine chloride, choline, muscimol hydrobromide (muscimol), bicuculline methiodide, baclofen, and saclofen were purchased from Sigma Sweden AB (Stockholm, Sweden). 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 tracheas of rats were intubated and connected to a Harvard (Harvard Apparatus Inc., 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, and 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 on-line 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 use 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. One rat was given only one dose of substance and was used only in one experiment.

Four concentrations of each receptor agonist and antagonist 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 μM and 1 mM. In control experiments, Ringer's solution was administered throughout the experiment.

The concentrations of both receptor agonists and receptor antagonists used were in accordance with previous studies where these substances were administered intrathecally and in dialysis perfusion. (Anderson et al., 1993; DeBoer and Westerink, 1994; Materi and Semba, 2001).

2.5. Statistics

All statistical analyses were performed using SPSS, version 10.0.5 (SPSS Inc., Chicago, Illinois, USA). The effect of the various substances was expressed as percent

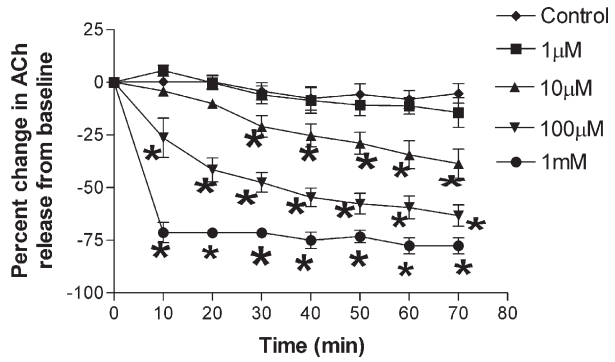

Fig. 1. 10 μM (n=6), 100 μM (n=4), and 1 mM (n=4) muscimol decreased acetylcholine release significantly (P<0.05) from baseline (*) and from control (n=5).

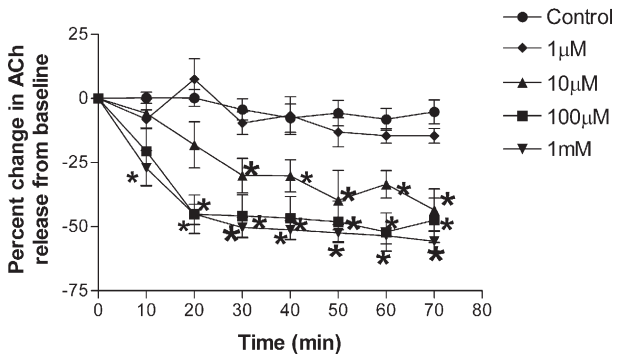

Fig. 3. 10 μM (n=5), 100 μM (n=5), and 1 mM (n=6) baclofen decreased acetylcholine release significantly (P<0.05) from baseline (*) and from control (n=5).

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. One-way 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 different doses of muscimol, bicuculline, baclofen and control.

3. Results

The $GABA_A$ receptor agonist muscimol (10 μM, 100 μM and 1 mM concentrations) decreased acetylcholine release significantly from baseline (Fig. 1) with a maximum decrease of 80.2% from basal level whereas the $GABA_A$ receptor antagonist bicuculline methiodide (100 μM, 500 μM 750 μM and 1 mM concentrations) increased acetylcholine release significantly from baseline (Fig. 2) with a maximum increase of 173.0% from basal level. The $GABA_B$ receptor agonist baclofen (10 μM, 100 μM and 1 mM concentrations) decreased acetylcholine release significantly from baseline (Fig. 3) with a maximum decrease of 55.6% from basal level whereas the $GABA_B$ receptor antagonist saclofen did not change acetylcholine release significantly from baseline (Fig. 4). Muscimol (10 μM, 100 μM and 1 mM concentrations, with F values 12.6, 67.6, and 125.2, respectively), bicuculline methiodide (100 μM, 500 μM, 750 μM and 1 mM concentrations with F values 18.8, 38.9, 148.9 and 53.6, respectively) and baclofen (10 μM, 100 μM and 1 mM concentrations with F values 18.6, 23.0, and 76.9, respectively) also showed significant changes in acetylcholine release from control (saline) experiments.

Dose–response curves were made for muscimol, bicuculline methiodide, and baclofen concentrations against the average acetylcholine release at 60 and 70 min after start of administration. Calculated EC_{50} values were 158.9 and 52.1 μM (goodness of fit for sigmoidal dose–responses were 0.98 and 0.96) for the $GABA_A$ receptor agonist muscimol and the $GABA_B$ receptor agonist baclofen, respectively. Calculated EC_{50} value was 1.812 mM (goodness of fit for sigmoidal dose–responses was 0.88) for $GABA_A$ receptor antagonist bicuculline methiodide. As we used a microdialysis probe for drug administration, tissue drug concentration would be lower than the dialysed concentrations. The concentration difference in tissue and in the infusion will result in higher EC50 values than the value obtained from receptor binding studies.

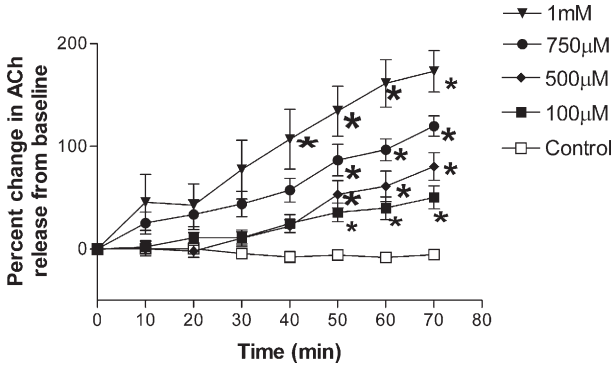

Fig. 2. 100 μM (n=5), 500 μM (n=4), 750 μM (n=4), and 1 mM (n=6) bicuculline methiodide increased acetylcholine release significantly (P<0.05) from baseline (*) and from control (n=5).

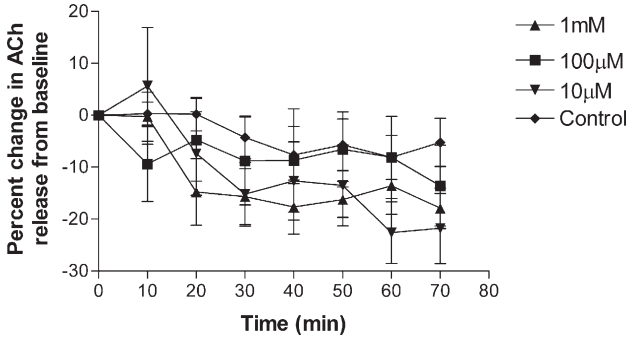

Fig. 4. 10 μM (n=5), 100 μM (n=5), and 1 mM (n=5) saclofen did not change acetylcholine release significantly (P<0.05) from baseline or from control (n=5).

4. Discussion

The $GABA_A$ receptor agonist, muscimol, inhibited acetylcholine release and the $GABA_A$ receptor antagonist bicuculline methiodide increased acetylcholine release concentration dependently. These findings suggest that $GABA_A$ receptors are tonically regulating spinal dorsal horn acetylcholine release.

The $GABA_B$ receptor agonist baclofen also decreased acetylcholine release in a concentration-dependent manner. The results suggest that $GABA_B$ receptors are also involved in the regulation of spinal dorsal horn acetylcholine release and direct or indirect stimulation of $GABA_B$ receptors decreases acetylcholine release. However, contrary to the observation after bicuculline methiodide administration, saclofen did not increase acetylcholine release. This suggests that $GABA_B$ receptors are not tonically regulating the spinal dorsal horn acetylcholine release.

The inhibitory role of $GABA_A$ receptors on acetylcholine release has earlier been reported in pontine reticular formation (Vazquez and Baghdoyan, 2004), basal forebrain (Vazquez and Baghdoyan, 2003) and cortex (Giorgetti et al., 2000; Materi and Semba, 2001). More corroborating to our findings are the results from Anderson et al. (1993), DeBoer and Westerink (1994), and Ikarashi et al. (1999) who, in the striatum, found that the $GABA_A$ receptor agonist muscimol inhibited acetylcholine release and the $GABA_A$ receptor antagonist bicuculline methiodide increased acetylcholine release. They also found that the $GABA_B$ receptor agonist baclofen inhibited acetylcholine release in the striatum without an effect from the $GABA_B$ receptor antagonist saclofen, suggesting a tonic regulation of acetylcholine release by $GABA_A$ receptors but not by $GABA_B$ receptors.

There are several earlier studies which demonstrated inhibitory properties of spinal GABA receptors on nociceptive transmission in dorsal horn. Malan et al. (2002) showed that the $GABA_A$ receptor antagonist bicuculline methiodide produced tactile allodynia and thermal hyperalgesia in normal rats and suggested that a loss of tonic inhibition of $GABA_A$ receptors resulted in the effect. The $GABA_B$ receptor agonist baclofen decreased Aδ and C fibre evoked responses of spinal dorsal horn (Sokal and Chapman, 2003) suggesting an inhibitory nature of $GABA_B$ receptors on primary afferents.

Although both $GABA_A$ and $GABA_B$ receptors have inhibitory effects on primary afferent neurons in spinal dorsal horn, the density of these receptors changes differently after peripheral nerve injuries which indicates a difference in functional localisation. Thus, $GABA_A$ receptor binding was decreased after sciatic neurectomy, whereas $GABA_B$ receptor binding was increased (Castro-Lopes et al., 1995). There were no changes in receptor binding sites for baclofen after peripheral nerve injury, although a loss of total GABA-immunoreactivity was shown in the spinal dorsal horn suggesting that the density of $GABA_B$ receptors is constant after partial nerve injury contrary to $GABA_A$ receptors (Ibuki et al., 1997; Moore et al., 2002; Smith et al., 1994).

The different responses of GABA receptor agonists and antagonists on acetylcholine release observed in this study may also indicate differences in GABA receptor localization in the dorsal horn. The present data provide evidence that $GABA_A$ receptors may be located on cholinergic neurons. This observation together with previous findings by Baba et al. (1998), Li et al. (2002), and Zhang et al. (2005) who showed that GABA neurons are under tonic cholinergic control suggests that a feedback tonic control exists between these transmitter systems in the spinal cord mediated by $GABA_A$ and muscarinic receptors. Such a feedback control would maintain a stable concentration of both GABA and acetylcholine. It is possible that an alteration of this balance changes the spinal pain threshold.

$GABA_B$ receptors may be located on non-cholinergic neurons, as reported in striatum (Ikarashi et al., 1999), or function as autoreceptors on GABAergic interneurons as suggested by Quevedo et al. (1992) (Iyadomi et al., 2000). Todd et al. showed that subpopulations of GABA neurons in spinal cord laminae I–III contain, among other transmitters, acetylcholine. Our finding that baclofen decreased the release of acetylcholine may be explained if $GABA_B$ receptors function as autoreceptors on GABA neurons that co-release acetylcholine.

$GABA_C$ receptors are also reported to be located in the spinal cord, to which muscimol also have affinity. Thus, we cannot exclude that the observed effects of muscimol are related to an effect on $GABA_C$ receptors. Further studies are needed to elucidate this possibility.

In conclusion, the present results show that dorsal spinal acetylcholine release is under control of both $GABA_A$ and $GABA_B$ receptors, where $GABA_A$ receptors are suggested to regulate acetylcholine release tonically. These findings suggest further that intraspinal acetylcholine release may play an important role in the regulation of pain threshold at the spinal cord level via GABA neurons control.

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