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Synergy between intrathecal ω-conotoxin CVID and dexmedetomidine to attenuate mechanical hypersensitivity in the rat

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Abstract

The analgesic effects of intrathecal (i.t.) ω-conotoxin CVID, an N-type Ca^{2+} channel antagonist, and the α_2-adrenoceptor agonist, dexmedetomidine, were tested alone and in combination following unilateral ligation of L (lumbar) 5/6 spinal nerves in rats. Mechanical allodynia was observed prior to insertion of an i.t. catheter. Effects and interactions of ω-conotoxin CVID (0.01–10 μg/kg) and dexmedetomidine (0.1–10 μg/kg) were tested on allodynic and tail flick (thermal stimulus) responses. Only dexmedetomidine increased the latency of the tail flick response. Both dexmedetomidine and ω-conotoxin CVID completely inhibited allodynia (ED_{50} 0.78±0.02 and 0.35±0.08 μg/kg, respectively; n=63, 41). Dexmedetomidine and ω-conotoxin CVID combined in dose ratios 0.7 and 1.3 (adjusted for ED_{50}) were synergistic in decreasing mechanical hypersensitivity; interaction index (γ) 0.39 (confidence interval [CI] 0.33, 0.46) and 0.3 (CI 0.23, 0.38). Despite the necessity for i.t. administration, these data suggest that the synergistic combination confers enhanced potency (lower doses) of both drugs that may avoid clinical toxicity of single drug therapy.

Keywords: α_2-adrenoceptor; ω-conotoxin CVID; Dexmedetomidine; Mechanical hypersensitivity; N-type Ca^{2+} channel

1. Introduction

Progress in the clinical management of neuropathic pain, associated with allodynia and hyperalgesia, has been limited by the toxicity and side-effects of agents acting directly on the complex neural mechanisms involved (Jensen et al., 2001). The development of allodynia and mechanical hypersensitivity after lumbar nerve root ligation in rodents depends on an intact sympathetic nervous system which activates sensory neurons in the periphery and in the dorsal root ganglion (Kim et al., 1993; McLachlan et al., 1993). However, α_2-adrenoceptor agonists such as dexmedetomidine or clonidine reverse the hypersensitivity (Malmberg et al., 2001; Yaksh et al., 1995). This effect may involve an interaction of spinal adrenergic and cholinergic neurons, specifically with the muscarinic receptor subtype M_4 (Kang and Eisenach, 2003). Endogenous inhibitory controls involving α_2-adrenoceptors also limit the development of symptoms of allodynia in the spinal nerve ligation model (Xu et al., 1999). Studies in mutant mice suggest that the antiallodynic effects of dexmedetomidine depend on α_{2A}-adrenoreceptors located in the dorsal horn of the spinal cord (Malmberg et al., 2001). Activation of these receptors decreases the release in the spinal cord of substance P and calcitonin gene-related peptide following noxious stimuli (Takano et al., 1993). The effect of α_2-adrenoreceptor agonists is maintained in chronic neuropathic pain, and epidural clonidine is effective in patients no longer responding to opioid agonists (Eisenach et al., 1995).

Intrathecal (i.t.) ω-conotoxins block spinal N-type voltage-gated Ca^{2+} channels and have been shown to inhibit mechanical hypersensitivity responses in a variety of animal studies (Bowersox et al., 1996; Malmberg and Yaksh, 1994, 1995; Scott et al., 2002; Smith et al., 2002). ω-Conotoxin MVIIA (ziconotide) is effective but has significant toxicity in patients with chronic and neuropathic pain (Atanassoff

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et al., 2000; Brose et al., 1997; Penn and Paice, 2000). A novel peptide isolated from Conus catus, ω-conotoxin CVID (AM-336), has also been evaluated and may have less toxicity (Lewis et al., 2000; Scott et al., 2002; Wright et al., 2000). Scott et al. (2002) observed tail writhing, hind limb twitching and ataxia after i.t. ω-conotoxin CVID (3 μg/kg) in rats, but the therapeutic index (ratio of dose causing moderate toxicity to dose for 50% attenuation of tactile allodynia) was 9.7 versus 2.1 for ω-conotoxin MVIIA. The ω-conotoxins may only be efficacious clinically if toxicity can be avoided by the use of low doses combined with adjunct drugs with additive or synergistic effects. Neurotoxicity has not been demonstrated with i.t. dexmedetomidine, and central administration is associated with only minor changes in autonomic vascular control (Blake et al., 2000). We have therefore investigated effects on the spinal cord of a combination of the selective α_2-adrenoceptor agonist, dexmedetomidine, with ω-conotoxin CVID. Although their primary mechanisms of action are through a receptor and a discrete calcium channel, respectively, there may be some overlap as α_2-adrenoceptor inhibition of transmitter release is mediated by reduction in the activity of N-type calcium channels (Lipscombe et al., 1989).

2. Methods

Male Sprague–Dawley rats were housed in a facility with a 12-h day–night cycle and free access to food and water. They were caged in groups until after i.t. catheter placement when they were caged separately in high–top cages but adjacent to other animals. Animals were observed regularly throughout the treatment period, and any showing signs of distress were removed from the study and humanely killed. All experiments were approved by the University of Melbourne Animal Research Ethics Committee, in accordance with the guidelines of the National Health and Medical Research Council of Australia.

2.1. Neuropathy

Production of a unilateral hind limb neuropathy was achieved using the technique of spinal nerve ligation (Kim and Chung, 1992) in male rats weighing 140–160 g. Following gaseous induction and maintenance of anaesthesia with halothane (4% decreasing to 1.5%) in oxygen, a dorsal paramedian incision was made to the left of the vertebral column at the level of the L4 to L6 vertebrae. Deep dissection, including partial resection of the transverse process of L6, allowed the L5 and L6 spinal nerves to be identified and isolated distal to the dorsal root ganglion. At this point, they were tightly ligated but not transected, using 6–0 silk sutures. The wound was then closed in layers, and the rats were allowed to recover in a warm box. A small number of animals had sham surgical procedures, identical to the above but excluding manipulation or ligation of the spinal nerves. Animals were given a week to recover from surgery before testing to confirm development of neuropathy.

2.2. Intrathecal catheters

Animals were assessed for neuropathy commencing 2 weeks following nerve ligation. Once von Frey thresholds (see below) of less than 10 g had been measured on two consecutive testing occasions, chronic i.t. catheters were implanted (typically 3–4 weeks after nerve ligation). A modification of the technique described by Yaksh and Rudy (1976) was used. Following gaseous induction and maintenance of anaesthesia, as above, the animal was supported in a stereotaxic frame, and the atlanto-occipital membrane was exposed using aseptic techniques. A 32-gauge polyimide catheter (TFX Medical, New Jersey, USA) was then threaded into the subarachnoid space and passed in a caudal direction for approximately 8 cm, to end in the region of the lumbar expansion. The external end of the catheter was connected to a length of PE-10 polyethylene tubing which was tunnelled and exteriorised over the forehead (Scott et al., 2004). The skin wound was closed, and the exteriorised catheter end was plugged after flushing with 10 μl of saline, catheter dead space being approximately 7 μl. Animals were then caged individually and allowed to recover for 3 days before treatment. Any animal developing motor impairment following catheter placement was excluded from the study.

2.3. Drugs

Drugs were dissolved in sterile saline (0.9%) and administered as a bolus via the i.t. catheter in volumes of 10–30 μl. The catheter was flushed afterwards with 10 μl saline. Dexmedetomidine (0.1–10 μg/kg) was a gift of Abbott Australasia (Kurnell, NSW), and ω-conotoxin CVID (0.01–10 μg/kg) was provided by AMRAD (Burnley, Victoria, Australia). Drug combinations were prepared with dexmedetomidine dose/ω-conotoxin CVID dose maintained in each of two fixed ratios, 0.7 and 1.3×(ED_{50} dexmedetomidine/ED_{50} ω-conotoxin CVID), to facilitate calculation of interaction indices (Tallarida, 2002).

2.4. Testing

Testing of the animals involved measuring their responses to acute pain using heat (tail flick) and detecting mechanical hypersensitivity (tactile allodynia) using touch (von Frey hairs). In addition, they were regularly weighed. All testing was done after the animals had familiarized themselves with their enclosures (usually 10–15 min) and were resting quietly. For tail flick testing, animals were housed in an enclosure with a glass floor and allowed to settle. In this way, the animals could be tested without physical restraint. A focused infra-red heat source with an in-built timer (Plantar Test, Ugo Basile, Italy) was then

directed under the tail at least 5 cm from the base, and the time was automatically measured until the tail was withdrawn, which was usually with a characteristic twitch. The heat source was adjusted to a level which resulted in a baseline latency of approximately 3 s. This required an upper limit of stimulus duration to be set at 10 s to prevent thermal injury. Three measurements were taken for each animal, with at least a 60-s rest between readings. The average of these three values was taken to be the tail flick latency, with 10 s as the maximum possible value.

The degree of mechanical hypersensitivity was measured following nerve ligation surgery. The hind paw on the operated side became sensitive to normally innocuous levels of touch, evoking a withdrawal response at a much lower pressure threshold (tactile allodynia). Von Frey hairs were used to measure the withdrawal pressure threshold (Stoelting, IL, USA; Chaplan et al., 1994). The test animals rested in a clear enclosure, the base of which was an open wire mesh such that the plantar surface of the paw was easily accessible from below. The hairs were applied sequentially, starting with 2 g of force and proceeding upwards to a maximum of 15.1 g force or downwards to a minimum of 0.4 g force. A force of 15.1 g force thus defines the 'maximum possible effect' (MPE) for this test and chosen so as to be just short of enough force to physically lift the hind paw off the mesh. Once a paw withdrawal occurred, the 50% withdrawal threshold was determined using the 'Up–down' method as published by Dixon (1980). Both hind paws were tested. Sham nerve ligation surgery, performed in 20 rats and reported previously, resulted in no significant difference between ipsilateral and contralateral hind limbs at any time during testing (Scott et al., 2002).

A total of eight i.t. injections of the test drug or drug combination were given to each rat, with an interval of 2 days between doses. The order of drug dose was randomised, but the observer performing the testing was not blinded. At the conclusion of the treatment cycle, the correct placement of the i.t. catheter was confirmed by an i.t. injection of 10 μl of lignocaine (2%). Observation of symmetric paralysis confined to the hind limbs was considered to indicate satisfactory positioning and patency of the catheter. Animals were not included in the study if a satisfactory block was unable to be demonstrated.

2.5. Data analysis

Data were analysed using analysis of variance (ANOVA) for continuous variables, with repeated measure adjustment where appropriate. Values are expressed as mean±standard error (S.E.M.) or 95% confidence intervals (CI). The average S.E.M. within rats was calculated from repeated measures ANOVA, using the pooled estimate of error from the residual mean square as (error mean square/number of rats)$^{0.5}$. Responses to drugs as measured by tactile allodynia threshold or tail flick latency were calculated as a percentage of the maximum possible effect (%MPE) using the following equation: %MPE=(Measured Value−Pretreatment value)×100/(MPE−Pretreatment value). Sigmoidal nonlinear regression curve fitting for dose–response data and estimation of log ED_{50} (allodynia) and linear regression (tail flick latency) were done using Prism 3 software (GraphPad Software, San Diego, CA, USA). For the two fixed dose ratios of dexmedetomidine and CVID studied, the interaction index (γ) was estimated at the ED_{50} (Tallarida, 2002). The index is defined as $\gamma=a/A+b/B$ where A and B are the ED_{50} doses of drug A and drug B alone and (a,b) is the combination dose causing an ED_{50}. The variance of the log ED_{50} of a theoretical additive combination of the two drugs was estimated from the variances of the log ED_{50} of each drug alone, according to the method of Tallarida. An interaction index $\gamma<1$ indicates a synergistic combination at the measured effect level. The variance of $\log(\gamma)$ is the sum of the variances of the log ED_{50} for the theoretical additive combination and of the log ED_{50} for the actual drug combination. P values <0.05 were taken as statistically significant.

3. Results

Drug administration and testing was completed in all rats within 21 days of i.t. catheter insertion. There was no behavioural evidence of neurotoxicity following i.t. doses of dexmedetomidine or with any of the combined doses of dexmedetomidine and ω-conotoxin CVID tested. Some sedation with decreased exploratory behaviour and grooming was evident with doses of dexmedetomidine above 1 μg/kg. As reported previously (Scott et al., 2002), moderate neurotoxicity, with evidence of occasional tail writhing, spontaneous twitching and ataxia, was observed in 50% of rats after 1 μg/kg i.t. ω-conotoxin CVID.

The baseline thermal tail flick latency was 4.1±0.85 s (n=42), and there was no significant effect on latency following nerve ligation. Dexmedetomidine significantly increased thermal tail flick latency (slope 17.4±5.7, P=0.003; Fig. 1, top left) to 30% MPE following 10 μg/kg. As reported previously (Scott et al., 2002), CVID did not alter tail flick latency (Fig. 1, upper right). Combinations of dexmedetomidine and CVID (in dose ratios 0.7ED_{50} and 1.3ED_{50} determined for tactile allodynia) also did not significantly alter tail flick latency (P=0.18 and P=0.8, respectively; Fig. 1).

Following nerve ligation, the mean tactile allodynia threshold in the operated left hind paw was 2.6±0.8 g. The threshold for the right hind paw did not differ significantly from the maximum force applied, 15.1 g. The allodynia remained stable over the 4-week testing period, although the patency and positioning of the i.t. catheters, as tested by the response to 2% lignocaine, varied from 2–21 days. Catheters were not used after 3 weeks because of the likelihood of fibrosis and limitation of the spread of drug in the cerebrospinal fluid.

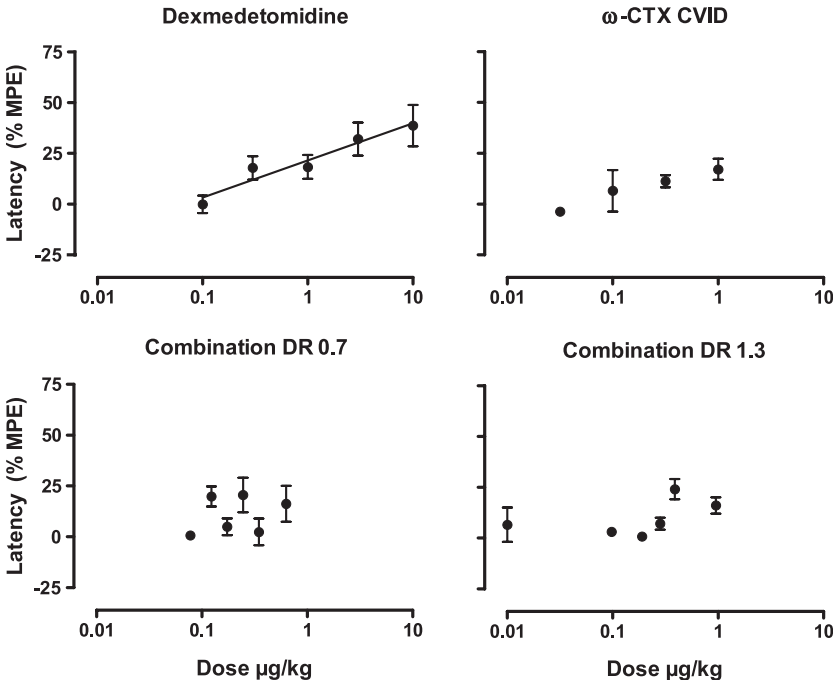

Fig. 1. Tail flick latency prolongation, expressed as percentage of maximum possible effect (%MPE) versus i.t. drug dose (µg/kg). Linear regression shown for dexmedetomidine (P=0.003 for difference of slope from 0). Error bars indicate S.E.M. for dexmedetomidine (n=16), ω-conotoxin CVID (n=11), drug combination dose ratio 0.7 (n=10) and dose ratio 1.3 (n=11). For combination dose ratios 0.7 or 1.3, dexmedetomidine dose equals dose ratio×ω-conotoxin CVID dose/relative potency for relief of allodynia.

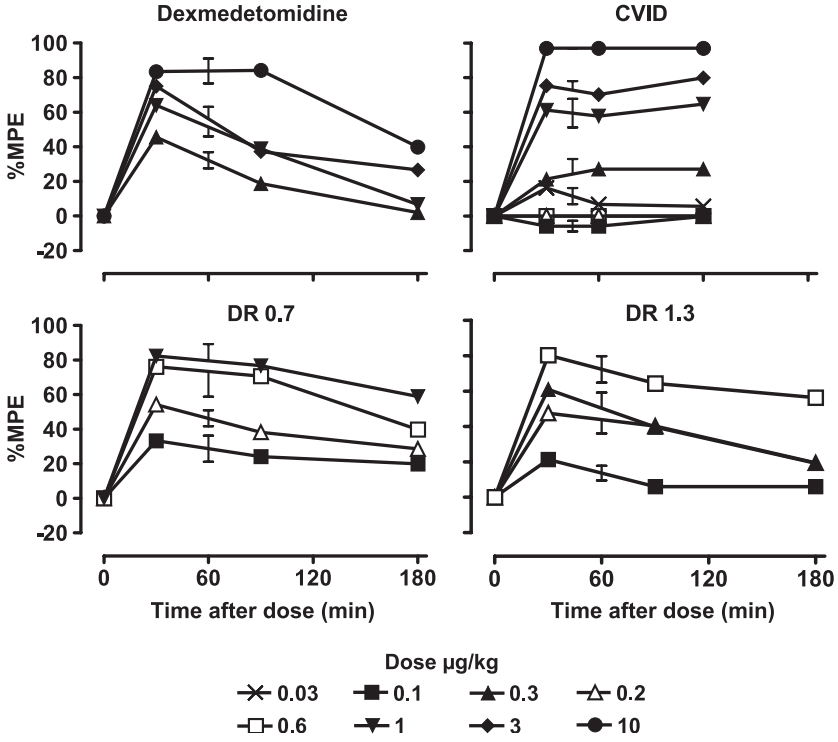

Fig. 2. Mechanical hypersensitivity, attenuation over time expressed as percentage of maximum possible effect (%MPE). Dose (µg/kg i.t.) refers to total drug dose for dexmedetomidine (n=16), ω-conotoxin CVID (CVID, n=11) and fixed dose ratio combinations (dose ratio 0.7 and dose ratio 1.3, n=7). For drug combinations, dexmedetomidine dose equals dose ratio×ω-conotoxin CVID dose/relative potency. Error bars=S.E.M. derived from analysis of variance for each dose, shown at 60 min only for clarity.

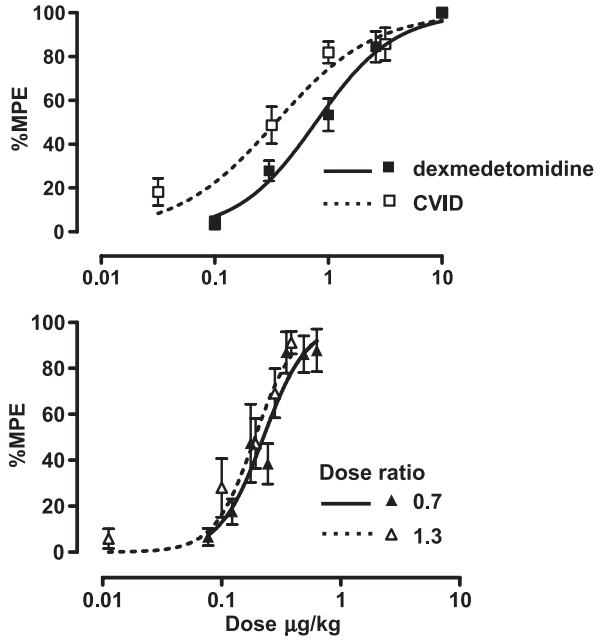

Fig. 3. Log dose–response curves for attenuation of mechanical hypersensitivity expressed as percentage of maximum possible effect (%MPE). Upper panel shows i.t. dexmedetomidine and ω-conotoxin CVID. Lower panel shows intrathecal drug combinations, dose ratios 0.7 and 1.3 where dexmedetomidine dose=dose ratio×ω-conotoxin CVID dose/relative potency. Data points are mean±S.E.M.

The time course of the antinociceptive effects of dexmedetomidine, ω-conotoxin CVID and combinations with dose ratios $0.7ED_{50}$ and $1.3ED_{50}$ are shown in Fig. 2. The effect of dexmedetomidine peaked between 30 and 90 min after i.t. injection, but the effect of ω-conotoxin CVID was sustained for at least 180 min. A sustained effect at 180 min was not seen with either of the drug combinations; however, the effect of dexmedetomidine was prolonged. Comparison of the responses after 0.35–0.6 µg/kg i.t. dexmedetomidine combined with ω-conotoxin CVID (total dose 0.6 µg/kg at dose ratios 0.7 and 1.3; Fig. 2, lower graphs) with the responses after 1.0 µg/kg i.t. dexmedetomidine alone (Fig. 2, upper left) indicates greater attenuation of the allodynia with the combined treatment at both 90 min (P=0.002) and 180 min (P<0.001).

The maximum responses for attenuation of tactile allodynia in each experiment were used to construct the dose–response curves for each drug and combination ratio experiments shown in Fig. 3. Comparison of the ED_{50} values for these curves indicated that ω-conotoxin CVID had approximately twice the potency of dexmedetomidine in this pain model (Table 1). Comparison of the observed ED_{50} for the drug combination at each dose ratio was made with an estimated ED_{50} based on an additive effect to obtain the interaction index. Interaction indices <0.4 (95% CI 0.3–0.5) indicated highly significant synergistic interactions.

4. Discussion

Previous studies have reported either minor or non-significant effects of i.t. ω-conotoxins on acute nociceptive responses such as the thermal tail flick response (Malmberg and Yaksh, 1994; Scott et al., 2002; Wang et al., 2000). Dexmedetomidine has a potent antinociceptive effect on the tail flick response in the rat, acting via α_{2A}-adrenoceptors (Takano and Yaksh, 1992), although it is less effective in other thermal responses, possibly mediated by α_{2B}-adrenoceptors (Graham et al., 1997).

Dexmedetomidine is more potent in the reversal of allodynia following nerve constriction injury than for acute antinociception, although both effects depend on actions on α_{2A} adrenoceptors in the spinal cord (Malmberg et al., 2001; Yaksh et al., 1995). Results of the present study are consistent with these findings in that the dose range for i.t. dexmedetomidine required for maximum reversal of allodynia only achieved about one third of maximum possible antinociception, and there was no significant contribution from ω-conotoxin CVID.

The relatively short duration of action of i.t. dexmedetomidine compared with CVID suggests that if the combination was used clinically for neuropathic pain, a continuous infusion would be required. In these experiments, the drug combination showed less decline in effect at 90 and 180 min when compared with similar or greater doses of dexmedetomidine alone. ω-Conotoxin CVID was found previously to have some residual effect 24 h after i.t. injection in the rat (Scott et al., 2002), which might delay dose titration and make avoidance of toxicity more difficult in an individual patient. The neurotoxicity reported with the clinical use of ω-conotoxins includes nystagmus, ataxia, confusion and auditory and visual hallucinations (Penn and Paice, 2000). In the present study, behavioural observations

Table 1
Potencies to attenuate tactile allodynia of i.t. dexmedetomidine, ω-conotoxin CVID or drug combinations

	Dexmedetomidine	ω-Conotoxin CVID	Combination dose ratio 0.7[a]	Combination dose ratio 1.3[b]
ED_{50} µg/kg (CI)	0.78 (0.60, 1.0)	0.35 (0.24, 0.49)	0.22 (0.19, 0.27)	0.19 (0.15, 0.24)
n	62	24	49	43
Relative potency to CVID	0.44	1.0	1.54	1.81
Interaction index γ (CI)			0.39 (0.33, 0.46)	0.30 (0.23, 0.38)

CI—95% confidence interval, n—number of observations; γ—interaction index<1 indicates synergistic combination.

[a] Dexmedetomidine dose=0.7×ω-conotoxin CVID dose/relative potency.

[b] Dexmedetomidine dose=1.3×ω-conotoxin CVID dose/relatively potency.

of writhing movements and ataxia indicated a corresponding neurotoxicity after i.t. injection of ω-conotoxin CVID in rats. Dexmedetomidine may limit cardiovascular reflex responses after central administration (Blake et al., 2000), but a significant synergistic interaction between dexmedetomidine and CVID may provide the best available strategy for the avoidance of neurotoxicity with N-type Ca^{2+} channel blockade. The increased selectivity of CVID for N-type versus P/Q type channels (Adams et al., 2003) may be responsible for the greater separation of antiallodynic and neurotoxic effects compared with other ω-conotoxins following i.t. doses in the rat (Scott et al., 2002). Interactions of CVID with other analgesic drugs have not previously been reported, but i.t. ω-conotoxin MVIIA (ziconotide) shows either additive or synergistic antinociception with morphine in the rat, depending on the pain model (Omote et al., 1996; Wang et al., 2000). Although tolerance to the effects of morphine rapidly develops, Wang et al. found no cross-tolerance to ziconotide antinociception, and prolonged administration of ziconotide itself did not result in tolerance with chronic pain after formalin injection or nerve constriction (Bowersox et al., 1996; Malmberg and Yaksh, 1995). Tolerance develops to the acute antinociceptive effects of α_2-adrenoceptor agonists and also a cross-tolerance with opioid agonists, probably reflecting common actions via descending inhibitory noradrenergic pathways in the spinal cord (Solomon and Gebhart, 1988). In the treatment of chronic or neuropathic pain, however, clinical reports suggest that the efficacy of α_2-adrenoceptor agonists is not limited by the development of tolerance (Eisenach et al., 1995; Siddall et al., 1994).

The synergism between α_2-adrenoceptor agonism and N-type Ca^{2+} channel blockade suggests additional as well as overlapping mechanisms inhibiting the allodynia mediated by low-threshold sensory afferents after nerve root injury. This allodynic state, maintained by sympathetic nerve activity, is resistant to the effects of i.t. morphine (Kim and Chung, 1992; Scott et al., 2002; Yaksh et al., 1995). The α_{2A}-adrenoceptors responsible for the analgesic effect of i.t. dexmedetomidine (Malmberg et al., 2001) are mainly localised on the terminals of capsaicin-sensitive substance P-containing primary afferent neurons in the rat spinal cord (Stone et al., 1998). Coupling is via G-proteins to inhibit adenyl cyclase, to enhance K^+ currents and to decrease Ca^{2+} currents (several classes of voltage-sensitive channels) and the release of neurotransmitters including substance P (Millan, 2002). The ω-conotoxins also prevent the release of key pronociceptive neurotransmitters in the dorsal horn by blocking N-type Ca^{2+} channels important for Ca^{2+} influx with persistent pain or inflammation (Bowersox et al., 1996; Diaz and Dickenson, 1997; Smith et al., 2002). Dorsal horn α_2-adrenoceptors are also important in mediating the descending inhibition from noradrenergic projections especially with peripheral inflammation or injury of the primary afferent fibres (Kontinen et al., 1998; Malmberg et al., 2001; Millan, 2002). The variety of mechanisms of α_2-adrenoceptor-mediated antinociception in the spinal cord underlies the synergistic effects of α_2 agonists with other drugs (Millan, 2002).

In conclusion, the significant synergistic interaction between the antiallodynic effects of ω-conotoxin CVID and dexmedetomidine after spinal nerve ligation suggests that dexmedetomidine may be used clinically to avoid unacceptable side-effects associated with the N-type Ca^{2+} channel antagonists. However, usefulness of the combination may be limited by the shorter duration of action of dexmedetomidine and by the need to deliver the drugs i.t. for maximum effect at the spinal cord.

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