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Depression of NMDA receptor-mediated synaptic transmission by four α_2 adrenoceptor agonists on the *in vitro* rat spinal cord preparation

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1 α_2-Adrenoceptor agonists have a spinal site of analgesic action. In the current study the synaptic depressant actions of xylazine, detomidine, romifidine and dexmedetomidine have been compared on segmental reflexes containing NMDA receptor-mediated components in the neonatal rat hemisected spinal cord preparation *in vitro*.

2 Reflexes were evoked in the ventral root following either supramaximal electrical stimulation of the corresponding ipsilateral lumbar dorsal root to evoke the high intensity excitatory postsynaptic potential (e.p.s.p.) involving all primary afferent fibres, or low intensity stimulation to evoke the solely A fibre-mediated low intensity e.p.s.p. The high intensity e.p.s.p. contains a greater NMDA receptor-mediated component.

3 Xylazine, romifidine, detomidine and dexmedetomidine all depressed both the high intensity e.p.s.p. and the low intensity e.p.s.p. giving respective EC_{50} values of 0.91 ± 0.2 μM ($n=12$), 23.4 ± 3 nM ($n=12$), 37.7 ± 7 nM ($n=8$) and 0.84 ± 0.1 nM ($n=4$) for depression of the high intensity e.p.s.p. and 0.76 ± 0.1 μM ($n=12$), 22.0 ± 3 nM ($n=12$), 24.9 ± 6 nM ($n=4$) and 2.7 ± 0.6 nM ($n=4$) for depression of the low intensity e.p.s.p., respectively. Unlike the other three drugs, the two values for dexmedetomidine, showing a greater selectivity for the high intensity e.p.s.p., are significantly different.

4 Each of these depressant actions was reversed by the selective α_2-adrenoceptor antagonist atipamezole (1 μM).

5 In contrast to previous reports of the actions of α_2-adrenoceptor agonists on the *in vitro* spinal cord preparation, at concentrations ten fold higher than the above EC_{50} values xylazine, romifidine, detomidine and dexmedetomidine depressed the initial population spike of motoneurons (MSR). This depression was not reversed by atipamezole.

6 Comparison of the rank order of the present EC_{50} values for depression of the high intensity e.p.s.p. with potency ratios from *in vivo* analgesic tests in previous studies show a close correlation between the present *in vitro* tests and analgesic potency. There is no correlation between the present data and previously obtained affinities of the agonists at non-adrenergic imidazoline binding sites.

7 The current findings therefore suggest that xylazine, romifidine, detomidine and dexmedetomidine are exerting their central analgesic actions at the spinal level principally through α-$_2$-adrenoceptors. All four agonists showed the same profile of selective depression of the NMDA receptor-mediated component of reflexes similar to that reported previously for clonidine. However dexmedetomidine, unlike the other ligands, selectively depressed the high intensity e.p.s.p.

Keywords: α_2-Adrenoceptor agonists; spinal cord; dexmedetomidine; xylazine; romifidine; detomidine

Introduction

α_2-Adrenoceptor agonists are analgesics *in vivo* (Leimdorfer & Metzner, 1949; Reddy *et al*., 1980; Yaksh & Reddy, 1981; Eisenach *et al*., 1987); they have been shown to be as efficacious as opioids but lack the side effects of opioids, such as respiratory depression and addiction (Delfs *et al*., 1994). They may also be more effective than opioids in treating some forms of pain such as neuropathic pain (Yaksh *et al*., 1995). α_2-Adrenoceptors have been shown to modulate nociceptive transmission at the level of the spinal cord (Engberg & Ryall, 1966; Fleetwood-Walker *et al*., 1985; Yaksh, 1985); these α_2-adrenoceptors may be located either presynaptically on primary afferent terminals (Kuraishi *et al*., 1985; Calvillo & Ghignone, 1986; Howe *et al*., 1987), postsyaptic to primary afferent fibres on interneurones mediating segmental transmission (Engberg & Ryall, 1966; Belcher *et al*., 1978; North & Yoshimura, 1984; Davies & Quinlan, 1985; Pertovaara *et al*., 1991), or postsynaptic to descending noradrenergic tracts from the brainstem (Yaksh, 1985). All of the drugs used in the present study are imidazol(in)es and interact with non-adrenergic imidazoline binding sites (Hieble & Ruffulo, 1995; Sjoholm *et al*., 1995). Therefore the involvement of the latter sites in the central depressant actions of these drugs is uncertain. The current study aimed to examine the synaptic depressant actions of these four different α_2-adrenoceptor agonists in order to compare their spinal synaptic depressant potencies with their reported analgesic potencies *in vivo* and with their reported affinities at non-adrenergic binding sites. The *in vitro* neonatal rat hemisected spinal cord preparation has been used for this purpose.

The reflex evoked in the ventral root following supramaximal stimulation of the dorsal root (the high intensity excitatory postsynaptic potential h.e.p.s.p. has been considered

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to be due to the activation of C fibre primary afferents and therefore to reflect a nociceptive reflex (Akagi *et al*., 1985; Garcia-Arraras *et al*., 1986). This reflex can be depressed by opioids (Yanagisawa *et al*., 1985; Faber *et al*., 1997a) and α_2-adrenoceptor agonists (Kendig *et al*., 1991; Faber *et al*., 1997a). In addition, stimulating the dorsal root at low intensities can evoke an A fibre-mediated response, the low intensity e.p.s.p. (l.e.p.s.p.), which is also sensitive to these drugs (Faber *et al*., 1997a).

The following drugs were selected for the present comparison because of their widely differing selectivity for α_2-adrenoceptors compared to non-adrenoceptor imidazoline binding sites. Xylazine (Virtanen, 1986; Nolan *et al*., 1987; Ley *et al*., 1991) and detomidine (Virtanen, 1986; Kamerling *et al*., 1988; Hamm *et al*., 1995) are used clinically as analgesics in veterinary medicine; romifidine is a novel α_2-adrenoceptor agonist which has been shown to have a sedative but not an analgesic action in horses (Hamm *et al*., 1995). Dexmedetomidine is the (+)-isomer of medetomidine, currently the most selective α_2-adrenoceptor agonist available (Virtanen *et al*., 1988; MacDonald *et al*., 1988).

Methods

Experiments were carried out as previously described (Faber *et al*., 1997a). Briefly, spinal cords with attached lumbar L4 or L5 dorsal and ventral roots were removed from unsexed neonatal Sprague-Dawley rats (aged between 3 and 6 days old, 9–14 g). The spinal cords were hemisected and set up in a chamber with the dorsal root in contact with the stimulating electrode and the ventral root in contact with the recording electrode. The hemicords were continuously perfused at 2 ml min^{-1} with artificial cerebral spinal fluid (ACSF) which consisted of (mM): NaCl 118, $NaHCO_3$ 24, glucose 12, $CaCl_2$ 1.5, KCl 3, $MgCl_2$ 1.25 and was gassed with O_2/CO_2 (95%/5%) pH 7.4 and maintained at a temperature of 25–27°C. Drugs were applied to the preparation by diluting them to a known concentration in the superfusate.

A fibre-mediated synaptic responses were evoked by a single 0.5 ms square pulse at three times threshold, where threshold was the intensity at which a discernible response first appeared in the ventral root. Stimulation at this intensity gave rise to the monosynaptic compound action potential of motoneurones (MSR, Figure 1c) superimposed on the low intensity population e.p.s.p., which has a duration of up to one second (Figure 1b). Supramaximal stimulation with a single 0.5 ms square pulse at sixteen times threshold gave rise to the long duration presumed C fibre-mediated high intensity population e.p.s.p. (Figure 1a, Akagi *et al*., 1985; Garcia-Arraras *et al*., 1986).

The actions of the α_2-adrenoceptor agonists on these evoked potentials were assessed following cumulative application of each concentration for the 25 min required to reach equilibrium. Responses were amplified, monitored and analysed by use of commercially available software (Scope v 3.3, MacLab). The results are expressed as mean ± s.e.mean. The areas under the curves of the high intensity e.p.s.p. and the low intensity e.p.s.p. and the peak amplitude of the MSR were measured. The EC_{50} values were calculated as the concentration of drug required to produce a 50% depression of the maximum effect of the drug. Apparent K_d values for atipamezole were found by calculating the dose-ratios following the reversal of the depressant actions of the α_2-adrenoceptor agonists on the high intensity e.p.s.p. by atipamezole. Some of the data have been presented previously in abstract form (Faber *et al*., 1995; 1997b).

Drugs and chemicals

Dexmedetomidine was a gift from Farmos Group Ltd. (Finland), detomidine and atipamezole were obtained from Smithkline Beecham; romifidine was obtained from Boehringer Ingelheim Ltd. and xylazine was obtained from Sigma.

Results

The effects of the four α_2-adrenoceptor agonists on the various components of segmental synaptic response are summarized in Table 1. In agreement with previous findings α_2-adrenoceptor agonists had a depressant action on the high intensity e.p.s.p. (Figures 1 and 2; Kendig *et al*., 1991; Faber *et al*., 1997a). In addition to this depressant action on the high intensity e.p.s.p., the α_2-adrenoceptor agonists also depressed the A fibre-mediated low intensity e.p.s.p. (Figures 1 and 2; Faber *et al*., 1997a).

Figure 1a–c shows the typical profile of action of the α_2-adrenoceptor agonists tested in the current study, in this case xylazine, on the synaptic responses; 10 μM xylazine maximally depressed the high intensity e.p.s.p. (Figure 1a) by 83% and

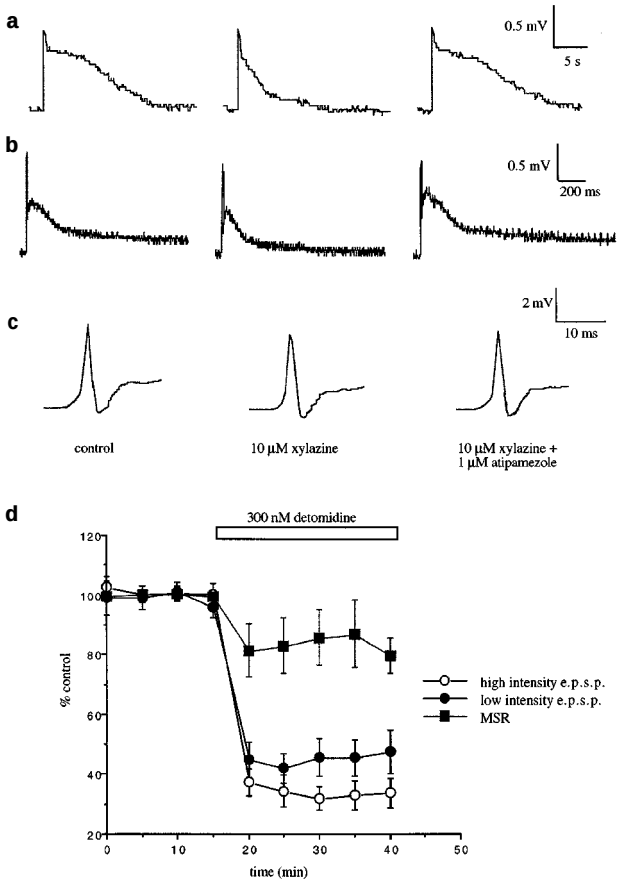

Figure 1 (a–c) The actions of xylazine on the synaptic responses. Xylazine 10 μM depressed the high intensity e.p.s.p. (a) and the low intensity e.p.s.p. (b), but had a much smaller effect on the MSR (c). The depressant actions of xylazine on the high and low intensity e.p.s.ps, but not the MSR, were reversed by atipamezole (1 μM, right panels). (d) Time course showing the depressant actions of detomidine on the synaptic responses; 300 nM detomidine depressed the high intensity e.p.s.p. by 66.4 ± 5% (n = 8), the low intensity e.p.s.p. by 52.5 ± 7% (n = 4) and the MSR by 20.3 ± 6% (n = 8). These effects reached equilibrium within 25 min.

the low intensity e.p.s.p. by 51% (Figure 1b; Table 1). The depressant action was predominantly on longer latency components of the reflexes. Romifidine, detomidine and dexmedetomidine showed the same profile of action as xylazine. At concentrations ten fold or higher than the EC_{50} levels for depression of the population e.p.s.ps the agonists had a small but significant depressant effect on the MSR (Table 1) ($P<0.05$, Mann-Whitney, non-parametric test). This is apparent for xylazine in Figure 1c, for detomidine in Figure 1d, for dexmedetomidine in Figure 2a and romifidine in Figure 2b. Figure 1d (detomidine) and 2 (dexmedetidine and romifidine) illustrate the time-course of the action (which was similar for all) of the α_2-adrenoceptor agonists. Equilibrium was reached within 25 min following each cumulative increase in concentration (Figures 1 and 2).

The depressant actions of all four α_2-adrenoceptor agonists on the high and low intensity e.p.s.ps were reversed by the selective α_2-adrenoceptor antagonist atipamezole (1 μM, Figure 1a and b, right panels). However, atipamezole failed to reverse the depressant actions of these drugs on the MSR (Figure 1c and 2). Atipamezole (applied alone) had no significant effect on any of the synaptic responses ($P>0.05$, Mann-Whitney non-parametric test, Figure 3). Reversal of the depressant actions of the α_2-adrenoceptor agonists by atipamezole (Figure 2b) yielded dose-ratios and hence apparent K_d values of 5.6 ± 3.7 nM ($n=4$) for xylazine, 4.6 ± 2.5 nM ($n=4$) for detomidine and 4.0 ± 2.8 nM ($n=4$) for romifidine (Figure 2b; Table 1). These values are not significantly different ($P>0.05$, Mann-Whitney, non-parametric test). An apparent K_d value for atipamezole in the presence of dexmedetomidine could not be calculated because only one concentration of atipamezole (1 μM) was used which fully reversed the depressant effects of dexmedetomidine.

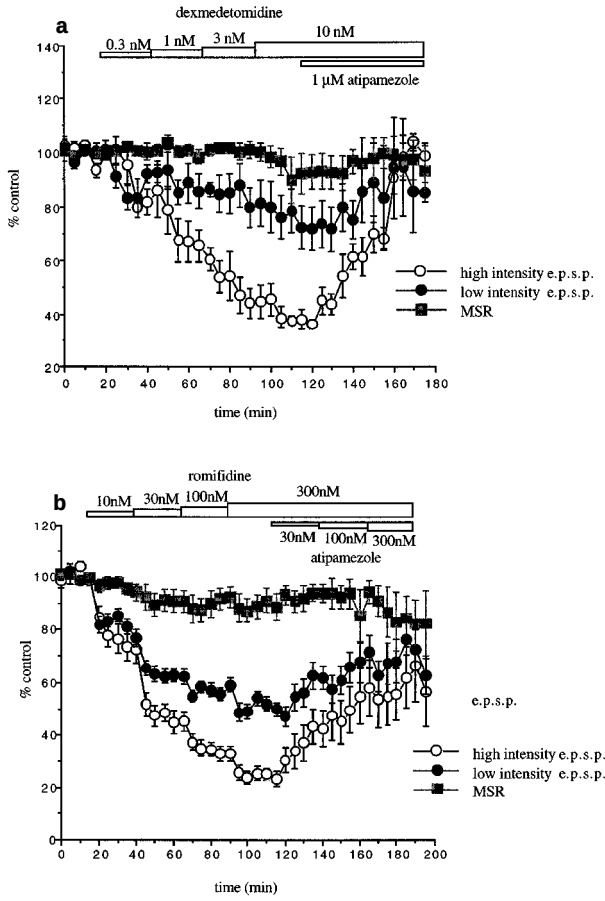

Figure 2 Time-courses showing the depressant actions of dexmedetomidine (a) and romifidine (b) on the different components of the synaptic response. Dexmedetomidine ($n=4$; a) and romifidine ($n=4$; b) depressed the high intensity e.p.s.p. and the low intensity e.p.s.p. in a concentration-dependent manner, and at higher concentrations had a small depressant action on the MSR. The depressant actions of each concentration of dexmedetomidine (a) and romifidine (b) reached equilibrium within 25 min. The depressant effects of dexmedetomidine on the high and low intensity e.p.s.ps were fully reversed by atipamezole (1 μM); reversal of the depressant effect of romifidine on the high intensity e.p.s.p. by a number of different concentrations of atipamezole (30 nM, 100 nM and 300 nM) allowed the calculation of a dose-ratio and thence an apparent K_d value.

Table 1 Comparison of the four α_2-adrenoceptor agonists on the *in vitro* preparation with *in vivo* potencies and non-adrenoceptor binding site affinities

	Xylazine	*Romifidine*	*Dexmede-tomidine*	*Detomidine*
Maximum depressant actions (%)				
MSR	17 ± 5 (8)	13 ± 5 (12)	18 ± 5 (4)	20 ± 6 (8)
LEPSP	51 ± 5 (8)	50 ± 3 (12)	28 ± 8 (4)	58 ± 5 (4)
HEPSP	82 ± 2 (12)	76 ± 2 (12)	64 ± 2 (4)	68 ± 4 (8)
Mean EC_{50} values (nM)				
LEPSP	760 ± 10 (8)	22 ± 3 (12)	2.7 ± 0.6 (4)	25 ± 6 (4)
HEPSP	910 ± 20 (12)	23 ± 3 (12)	0.84 ± 0.1 (4)	38 ± 7 (8)
Apparent K_d values of atipamezole (nM)				
HEPSP	5.6 ± 4 (4)	4 ± 3 (4)	NT	5 ± 3 (4)
Relative depressant potency[a]				
LEPSP	0.04	1.4	11	1.2
HEPSP	0.044	1.7	47	0.95
Relative analgesic potency[b,c,d]				
	0.14	–	20	0.22
Relative affinity for non-adrenoceptor binding sites[e]				
	–	–	106000	77000

Mean values are presented $\pm$s.e.mean (n). NT denotes not tested. Relative potencies have been calculated compared to clonidine (where clonidine = 1) using the EC_{50} values for clonidine taken from Faber *et al.* (1997a). The sources of analgesic, α_2-adrenoceptor and non-adrenoceptor binding site potencies are indicated by the superscripts: [a]Faber *et al.*, 1997a; [b]Idanpaan-Heikkila *et al.*, 1994; [c]Ossipov *et al.*, 1988; [d]Virtanen, 1986; [e]Sjoholm *et al.*, 1995.

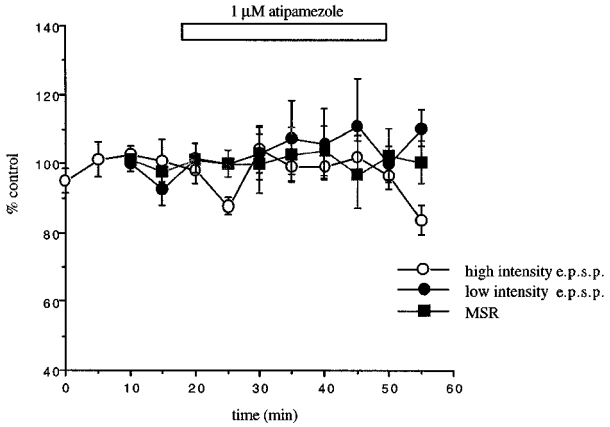

Figure 3 Atipamezole (1 μM) had no significant effect on any of the components of the synaptic response when applied for a duration similar to that required for the reversal of the depressant effects of the α_2-adrenoceptor agonists ($n=4$).

The concentration-dependence of the depressant actions of the α_2-adrenoceptor agonists is shown in Figure 4 and the mean EC_{50} values calculated from these plots are presented in Table 1. There was no significant diffference between the mean EC_{50} values for depression of the low intensity e.p.s.p. and the high intensity e.p.s.p. ($P>0.05$, Mann-Whitney non-parametric test) by xylazine, detomidine and romifidine. However, the mean EC_{50} value for depression of the high intensity e.p.s.p. by dexmedetomidine (Table 1) was approximately three fold lower than that for depression of the low intensity e.p.s.p. This difference is significant ($P<0.05$, Mann-Whitney non-parametric test).

As shown previously with clonidine (Siarey *et al*., 1992; Faber *et al*., 1997a) the present α_2-adrenoceptor agonists failed to abolish the synaptic responses. For all four agonists the mean maximum depressant action was greater on the high intensity e.p.s.p. than on the low intensity e.p.s.p. (Table 1). The difference between the mean maximal depression of the low (with the low intensity e.p.s.p. having about 30% more of a resistant component) and the high intensity e.p.s.p. by xylazine, romifidine and dexmedetomidine was significant ($P<0.05$, Mann-Whitney non-parametric test, Figure 3). However, the greater mean depressant effect of detomidine (300 nM) on the high intensity e.p.s.p. compared to the low intensity e.p.s.p. (100 nM) failed to reach statistical significance ($P>0.05$, Mann-Whitney non-parametric test; Figure 1d; Table 1). The maximal depressant effect of dexmedetomidine on the low intensity e.p.s.p. being only 28% (Figure 3b, Table 1), was significantly less than for the other three agonists ($P<0.05$, unpaired Student's t test).

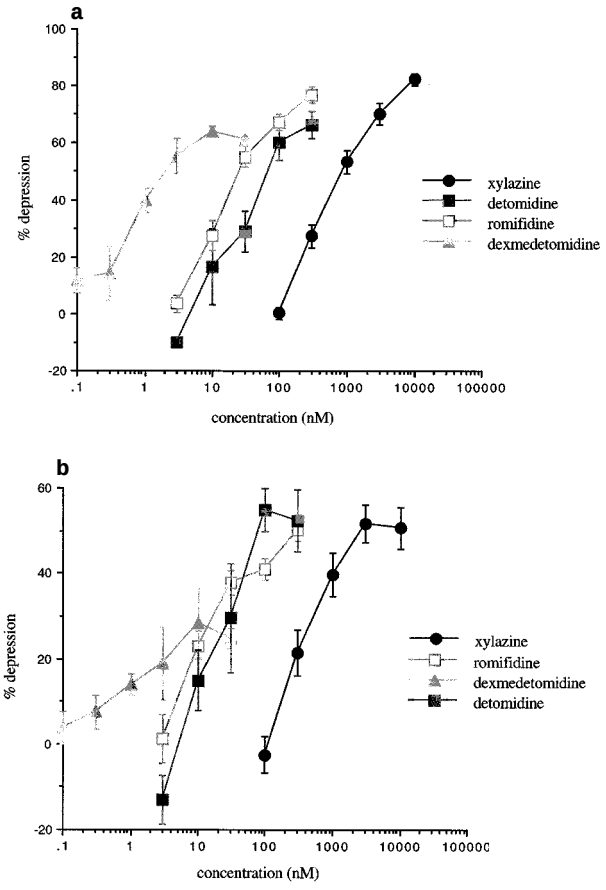

Figure 4 Concentration-effect plots showing the effects of the α_2-adrenoceptor agonists on the high intensity e.p.s.p. (a) and the low intensity e.p.s.p. (b). (a) Xylazine depressed the high intensity e.p.s.p. with an EC_{50} of 910 nM ($n=12$); romifidine with an EC_{50} of 23 nM ($n=12$); detomidine with an EC_{50} of 38 nM ($n=8$) and dexmedetomidine with an EC_{50} of 0.84 nM ($n=4$). (b) Xylazine depressed the low intensity e.p.s.p. with an EC_{50} of 760 nM ($n=12$); romifidine with an EC_{50} of 22 nM ($n=12$), detomidine with an EC_{50} of 25 nM ($n=4$) and dexmedetomidine with an EC_{50} of 2.7 nM ($n=4$).

Discussion

The findings of the present study are in agreement with previous studies which have shown a depressant action of α_2-adrenoceptor agonists on synaptic transmission in the *in vitro* spinal cord preparation (Kendig *et al*., 1991; Siarey *et al*., 1992; Faber *et al*., 1997a). Each of the α_2-adrenoceptor agonists depressed both the high intensity e.p.s.p. and the low intensity e.p.s.p. in a concentration-dependent manner (Figure 4; Table 1). In the present study it was noticed that although the dominant depressant action was on the population e.p.s.ps, at higher concentrations the drugs produced a small depression of the MSR. This weak depression of the MSR, which was not observed with morphine or clonidine (Faber *et al*., 1997a) and was not reversed by atipamezole, probably relates to the local anaesthetic action that higher concentrations of these drugs possess (Hayashi & Maze, 1993). Thus clonidine appears to have a more selective synaptic depressant action at α_2-adrenoceptors than any of the four drugs of the present study.

The present findings showing a depressant action of α_2-adrenoceptors on the A fibre-mediated low intensity e.p.s.p. contrasts with previous *in vitro* results showing that these drugs selectively depress only the C fibre-mediated components of synaptic transmission (Kendig *et al*., 1991). A train of stimuli at an intensity too low to activate C fibres can evoke a long duration synaptic response in this preparation, which is susceptible to depression by morphine and clonidine (Faber *et al*., 1997a). These data indicate that the activation of C fibres is not essential in order to see spinal depressant actions of α_2-adrenoceptor agonists; excitatory synaptic transmission appears to be mediated by common pathways regardless of the afferent fibre type activated (Faber *et al*., 1997a). In agreement with previous electrophysiological studies *in vivo* (Sullivan *et al*., 1987; 1992; Hamalainen & Pertovaara, 1995) the present study showed a greater depressant action of α_2-adrenoceptor agonists on C fibre-mediated activity than on A fibre-mediated activity. The high and low intensity e.p.s.ps, unlike the MSR, have NMDA receptor-mediated components (Faber *et al*., 1997a); it appears that centrally acting analgesics selectively depress NMDA receptor-mediated synaptic excitation (Siarey *et al*., 1992; Faber *et al*., 1997a). The low intensity e.p.s.p. has a larger non-NMDA receptor-mediated component than does the high intensity e.p.s.p. (Faber *et al*., 1997a). That would explain the greater depressant action of the α_2-adrenoceptors in the present study on the high intensity e.p.s.p. than on the low intensity e.p.s.p.

It is possible that the depressant actions of these drugs may have been mediated in part through non-adrenoceptor imidazoline binding sites. Atipamezole has been reported to bind non-adrenoceptor imidazoline binding sites with a pharmacology distinct from imidazoline I_1 and I_2 sites in the neonatal rat lung (Sjoholm *et al*., 1992; 1995). Dexmedetomidine and detomidine also showed a high affinity for these non-adrenoceptor binding sites. Table 1 shows a comparison of the affinities for non-adrenoceptor imidazoline binding sites where available (Sjoholm *et al*., 1992; 1995) with the relative depressant potencies on the present preparation. The ratios range from approximately 25 times weaker (xylazine) to 50

times stronger (dexmedetomidine) than clonidine. EC_{50} values for clonidine were obtained from our previous study (Faber *et al*., 1997a). The potency ratio of clonidine to dexmedetomidine and of clonidine to detomidine at non-adrenoceptor imidazoline binding sites (bottom row of Table 1) was approximately 100 000 (Sjoholm *et al*., 1995). Thus there is a thousand fold disparity between the relative affinities at non-adrenoceptor binding sites and the relative depressant potencies on the present preparation. A recent study by Diaz *et al*. (1997) showed that the selective I_2 imidazoline receptor agonist BU-224 depressed nociceptive responses of spinal dorsal horn neurones; these effects were not reversed by atipamezole. Thus it appears that both α_2 adrenoceptors and I_2 imidazoline receptors may mediate antinociception at the level of the spinal cord. However, the present findings suggest that imidazoline receptors are not involved in the spinal depressant actions of α_2 adrenoceptor agonists.

The reversal of the depression of the e.p.s.ps by the selective antagonist atipamezole (Virtanen *et al*., 1989) indicates that the dominant action of these drugs is at α_2 adrenoceptors. In support of α_2 adrenoceptors mediating the synaptic depressant actions of the α_2 adrenoceptor agonists are the apparent K_d values of atipamezole in the presence of these ligands (Table 1). There was no significant difference between the apparent K_d values for atipamezole in the presence of xylazine, romifidine and detomidine, suggesting a common site of action of these drugs. Furthermore, these values are comparable with the value of 2.5 nM for the antagonism by atipamezole of the action of clonidine on the rat vas deferens (Virtanen *et al*., 1989).

A spinal depressant action at α_2 adrenoceptors is believed to underlie the basis for the analgesic action of these drugs. Table 1 shows data from *in vivo* analgesic tests using the present α_2 adrenoceptor agonists where available (Virtanen, 1986; Ossipov *et al*., 1988; Idanpaan-Heikkila *et al*., 1994). It can be seen that the rank potency order of the spinal depressant actions of the α_2-adrenoceptor agonists on the present *in vitro* preparation on the high intensity e.p.s.p. is the same as their rank analgesic potencies *in vivo*; dexmedetomidine > clonidine > detomidine > xylazine (Virtanen, 1986; Ossipov *et al*., 1988; Idanpaan-Heikkila *et al*., 1994). This supports the conclusion that the actions of the α_2-adrenoceptor agonists in the present study reflect an action on synaptic pathways involved in analgesia.

The α_2-adrenoceptor subtype (Millan *et al*., 1994; Bylund, 1995) involved in the depressant actions of the α_2-adrenoceptor agonists was not characterized in the present study. However, it is probable that their actions are mediated through the α_{2A} adrenoceptor subtype since this subtype has been shown to predominate in the neonatal rat spinal cord (Savola & Savola, 1996) and the antinociceptive actions of dexmedetomidine have been shown to be mediated through the α_{2A} adrenoceptor subtype at the spinal level (Hunter *et al*., 1997).

Dexmedetomidine had a different profile of action from the other α_2-adrenoceptor agonists, in that the EC_{50} value for depression of the high intensity e.p.s.p. was significantly less than for depression of the low intensity e.p.s.p., and the maximal depression of the low intensity e.p.s.p. by dexmedetomidine was significantly less than that seen with the other ligands. Dexmedetomidine is the most selective of all the α_2-adrenoceptor agonists (Virtanen *et al*., 1988; MacDonald *et al*., 1991) and has the highest α_2-: α_1-adrenoceptor selectivity ratio (Virtanen *et al*., 1988; 1989). Thus it may be that the differences between the actions of these ligands are attributable to an action at α_1-adrenoceptors.

In summary, each of the α_2-adrenoceptor agonists selectively depressed the NMDA receptor-mediated low intensity e.p.s.p. and high intensity e.p.s.p., showing a depressant action on both A- and C primary afferent fibre-mediated synaptic transmission. These actions are most likely mediated through α_{2A}-adrenoceptors with non-adrenoceptor imidazoline binding sites unlikely to be involved. The rank order of the spinal depressant activity *in vitro* is the same as the rank order of *in vivo* analgesic potencies of these drugs, validating the *in vitro* neonatal rat hemisected spinal cord preparation as an effective predictor of analgesic potency.

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