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Muscarinic excitatory and inhibitory mechanisms involved in afferent fibre-evoked depolarization of motoneurones in the neonatal rat spinal cord

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1 The involvement of acetylcholine and muscarinic receptors in spinal synaptic responses evoked by electrical and noxious sensory stimuli was investigated in the neonatal rat spinal cord *in vitro*.

2 Potentials were recorded extracellularly from a ventral root (L3–L5) of the isolated spinal cord, spinal cord-cutaneous nerve, and spinal cord-skin preparations of 1- to 4-day-old rats. Spinal reflexes were elicited by electrical stimulation of the ipsilateral dorsal root or the cutaneous saphenous nerve, or by noxious skin stimulation.

3 Single shock stimulation of supramaximum intensity of a dorsal root induced a mono-synaptic reflex in the corresponding ventral root. Bath-application of the muscarinic agonists, muscarine (0.3–30 μM) and (+)-*cis*-dioxolane (0.1–100 μM), produced an inhibition of the mono-synaptic reflex and a depolarization of motoneurones. Other muscarinic agonists, arecoline (10 nM–10 μM) and oxotremorine (10 nM–1 μM), inhibited the mono-synaptic reflex with little or no depolarization of motoneurones. Repetitive stimulation of the saphenous nerve at C-fibre strength induced a slow depolarizing response lasting about 30 s of the L3 ventral root. This slow ventral root potential (VRP) was also inhibited by arecoline (10 nM–10 μM) and oxotremorine (10 nM–1 μM).

4 In the spinal cord-saphenous nerve-skin preparation, a slow VRP was evoked by application of capsaicin (0.5 μM), bradykinin (3 μM), or noxious heat (47°C) to skin. This slow VRP was depressed by the muscarinic agonists, arecoline (3 μM) and oxotremorine (1 μM).

5 Of the (+)-*cis*-dioxolane-induced inhibition of mono-synaptic reflex and motoneurone depolarization, the M_2 antagonists, AF-DX 116 (0.1–1 μM) and methoctramine (100–300 nM), preferentially blocked the former response, whereas the M_3 antagonists, 4-DAMP (3–10 nM) and *p*-F-HHSiD (0.3–3 μM), preferentially blocked the latter response. AF-DX 116 (0.1–1 μM) and methoctramine (100–300 nM) also effectively antagonized the arecoline- and oxotremorine-induced inhibition of the slow VRP. The pA_2 values of AF-DX 116 and methoctramine against the arecoline-induced inhibition of the mono-synaptic reflex were both 6.79, and that of 4-DAMP against the (+)-*cis*-dioxolane-induced motoneurone depolarization was 8.16.

6 In the spinal cord-cutaneous nerve preparation, the saphenous nerve-evoked slow VRP was augmented by the anticholinesterase, edrophonium (5 μM). AF-DX 116 (1 μM) and methoctramine (100 nM) also potentiated the slow VRP, whereas 4-DAMP (10 nM) depressed the response. 4-DAMP (5–10 nM) depressed the capsaicin-induced slow VRP in the spinal cord-skin preparation.

7 Oxotremorine (0.3 μM) and arecoline (1 μM) markedly depressed the depolarization of motoneurones evoked by application of capsaicin (3 μM) to the spinal cord, whereas they depressed only slightly the depolarization induced by substance P (10 nM).

8 The present study suggests that both excitatory (via M_3-type receptors) and inhibitory (via M_2-type receptors) muscarinic mechanisms are involved in afferent fibre-evoked nociceptive transmissions in the neonatal rat spinal cord.

Keywords: Muscarinic receptor subtypes; nociceptive transmission; spinal cord

Introduction

Cholinergic muscarinic mechanisms have been shown to modulate pain sensation and associated behaviours at the level of the central nervous system in man and experimental animals (for review, see Green & Kitchen, 1986). A number of studies have shown that administration of cholinomimetic drugs to the spinal cord *in vivo* induces antinociceptive effects that can be blocked by muscarinic antagonists, which suggests the presence of a muscarinic analgesic mechanism in the spinal cord (Taylor *et al.*, 1982; Dirksen & Nijhuis, 1983; Yaksh *et al.*, 1985; Gillberg *et al.*, 1989; Hartvig *et al.*, 1989; Smith *et al.*, 1989). However, the precise neuronal mechanisms and the muscarinic receptor subtypes involved are not clear.

Previous studies have shown that in the isolated spinal cord of the neonatal rat cholinoceptor agonists evoke both excitatory and inhibitory muscarinic responses: a depolarization of motoneurones (Evans, 1978; Jiang & Dun, 1986; Newberry & Connolly, 1989; Yoshioka *et al.*, 1990b) and an inhibition of the mono-synaptic reflex (Newberry & Connolly, 1989; Yoshioka *et al.*, 1990b). These responses are presumably mediated by different types of muscarinic receptors (Newberry & Connolly, 1989; Yoshioka *et al.*, 1990b), but their pharmacological properties have not been fully characterized. In isolated spinal cord preparations, activation of primary afferent fibres by either electrical stimulation or peripheral noxious stimulation evokes a depolarization of slow time course in ventral roots (Yanagisawa *et al.*, 1982; Akagi *et al.*, 1985; Otsuka & Yanagisawa, 1988; Nussbaumer *et al.*, 1989; Yanagisawa *et al.*, 1992). There is evidence that this depolarization, hereafter referred to as the slow ventral root potential (VRP), represents a C-fibre-evoked nociceptive response in which tachykininergic primary afferents are

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involved (Otsuka & Yanagisawa, 1987). Evidence that the slow VRP involves excitatory amino acid transmitter(s) is also accumulating (Dray & Perkins, 1987; King et al., 1990; Kurihara et al., 1991). Furthermore, Yoshioka et al. (1990b) recently found in an isolated spinal cord-peripheral nerve preparation that conditioning stimulation of the saphenous nerve at C-fibre strength induced a prolonged inhibition of the muscle nerve-evoked mono-synaptic reflex. They suggested that tachykininergic primary afferents contained in the cutaneous nerve excite spinal cholinergic neurones and as a result the released acetylcholine inhibits the mono-synpatic reflex. In support of this, tachykinins have been shown to release acetylcholine from the spinal cord (Kobayashi et al., 1991) and cholinoceptor agonists applied to the spinal cord inhibited the monosynaptic reflex (Newberry & Connolly, 1989; Yoshioka et al., 1990b). Whether the transmitter acetylcholine released by tachykinins also influences the slow VRP, however, is not known.

In this paper we investigated the possible involvement of acetylcholine and muscarinic receptors in spinal nociceptive transmission. To this end, we examined the effects of muscarinic drugs on the slow VRPs evoked by electrical or noxious stimuli and the pharmacological characteristics of the muscarinic receptors that mediate excitatory and inhibitory muscarinic responses in the neonatal rat spinal cord.

Preliminary results of this study have been presented elsewhere (Yoshioka et al., 1990a,b; 1991).

Methods

Preparations

In this study we used the following three types of preparations isolated from Wistar rats aged 1–4 days.

Isolated spinal cord preparation Under ether anaesthesia, the spinal cord below the middle thoracic level together with spinal nerve roots (L3–L5) was isolated, hemisected, and placed in a recording chamber of 0.3 ml volume (Otsuka & Konishi, 1974). The chamber was perfused with artificial cerebrospinal fluid (CSF) at a rate of 2.5 ml min^{-1}. The composition of artificial CSF was as follows (mM): NaCl 138.6, KCl 3.35, $NaHCO_3$ 20.9, glucose 10.0, $CaCl_2$ 1.25 and $MgCl_2$ 1.15. The solution was equilibrated with a gas mixture of 95% O_2:5% CO_2 and the temperature of the solution in the chamber was kept at 27°C. A tight-fitting suction electrode was used for extracellular recording from the ventral root (L3–L5).

Another suction electrode was used for electrical stimulation of the dorsal root of the same segment (single shocks with square pulses of 500 μs in duration and 20–30 V in amplitude, supramaximum for mono-synaptic reflex; positive voltage was applied to the inside of the electrode). Potential changes were recorded on a pen recorder and spinal reflexes of fast time course were stored in a transient memory device and then recorded on the pen recorder with an expanded time-scale.

Isolated spinal cord-saphenous nerve preparation The hemisected spinal cord below the middle thoracic level was isolated together with the attached L3–L5 ventral roots and dorsal roots, the latter remaining connected with the dorsal root ganglia and the femoral and saphenous nerves (Nussbaumer et al., 1989). The saphenous nerve was stimulated supramaximally with one to five pulses of 500 μs duration and 30–40 V intensity at 50 Hz and the potential changes were recorded from the L3 ventral root on a pen recorder. The duration of the saphenous nerve-evoked slow VRP was taken as the time required for the depolarized potential to decay from the peak to 10% of the peak amplitude and the magnitude of the slow VRP was expressed as the integrated area of the depolarization on the chart record. To assess the effects of drugs on the slow VRP, the averaged magnitude of three responses before and after administration of the drugs were compared.

Isolated spinal cord-saphenous nerve-skin preparation This preparation consisted of a hemisected spinal cord that remained connected to the saphenous nerve (see above) and a piece of skin (approximately 5 × 5 mm) of the hind limb (Yanagisawa et al., 1992). The recording chamber was made from Sylgard and consisted of two wells, which were independently perfused at a rate of 2.5 ml min^{-1}. The spinal cord was placed in one well (0.3 ml volume) and the skin was placed with the outside surface upwards in the neighbouring well (0.1 ml volume). The saphenous nerve was led through a break in a thin septum (1 mm width) into the skin well. The break in the septum was sealed with Vaseline. Drugs were either applied to the skin by perfusing the skin well with solutions containing the drugs or injected into the perfusion solution with short pressure pulses as described previously (Otsuka & Yanagisawa, 1988). A heat stimulus was applied by perfusing the skin well with heated solution such that the maximum temperature in the skin well became 47–48°C. The temperature rose soon after starting the perfusion of heated solution (about 5 s), reached maximum in less than 15 s and fell to the original temperature within 30 s after stopping the heat stimulus. Capsaicin and the heat stimulus were applied to the skin at intervals of 40 min while bradykinin was applied at intervals of 1 h to avoid tachyphylaxis. The evoked responses were recorded from the L3 ventral root on a pen recorder. The magnitude of each depolarizing response was estimated as the integrated area as described above. In the experiments using capsaicin as a stimulus the skin was pretreated with prostaglandin E_1 (1 μM) for 3 min to enhance the slow VRP (Yanagisawa et al., 1992).

Esimation of pA_2 values of muscarinic antagonists for inhibition of the mono-synpatic reflex

The pA_2 values of muscarinic antagonists were determined against the arecoline-induced inhibition of the mono-synaptic reflex. Mono-synaptic reflexes were elicited by dorsal root stimulation every 30 s in the isolated spinal cord preparations. Forty to sixty min after perfusing the spinal cord with normal solution, arecoline was applied by perfusion at increasing concentrations in a cumulative manner. A 10 min exposure to each concentration of arecoline was sufficient to obtain a steady inhibitory effect on the mono-synaptic reflex. Then, the spinal cord was perfused with three increasing concentrations of an antagonist with 0.5 logarithmic concentration steps. The spinal cord was allowed to equilibrate for 40–60 min with each concentration of the antagonist and then arecoline was applied as described above. The amplitude of the mono-synaptic reflex at each concentration of arecoline was compared to the average of about six control responses before application of arecoline and concentration-inhibition curves were constructed. The pA_2 values were determined from Arunlakshana-Schild plots by the least square regression analysis (Arunlakshana & Schild, 1959).

Concentration-response curves for the muscarinic depolarization of motoneurones

(+)-*cis*-Dioxolane (Ehlert et al., 1980) was used to evoke a muscarinic depolarizing response of motoneurones in the presence of 0.3 μM tetrodotoxin (TTX). Increasing concentrations of (+)-*cis*-dioxolane were applied for 1 min duration to the isolated spinal cord preparations at intervals of 30–40 min to avoid desensitization. The magnitude of each response was estimated as the integrated area of depolarization. Concentration-depolarization curves were constructed in the absence of an antagonist and then in the presence of increasing concentrations of the antagonist. The spinal cord

was allowed to equilibrate for 40–60 min with each concentration of the antagonist before (+)-*cis*-dioxolane was applied.

Drugs

The following drugs were used: AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido [2,3-b] [1,4]benzodiazepine-6-one) was kindly donated by Dr K. Thomae, Biberach, Germany; (+)-*cis*-dioxolane, 4-DAMP (4-diphenylacetoxy-*N*-methyl-piperidine methiodide), *p*-F-HHSiD (*para*-fluoro-hexahydrosiladiphenidol hydrochloride). McN-A-343 (4-[*m*-chlorophenyl carbamoyloxy]-2-butynyltrimethylammonium chloride), and methoctramine tetrahydrochloride were purchased from Research Biochemicals Inc., arecoline hydrobromide, atropine sulphate, capsaicin, gallamine triethiodide, oxotremorine, and pirenzepine dihydrochloride were purchased from Sigma Chemicals; bradykinin and substance P (SP) were purchased from Peptide Institute, Inc., Osaka, Japan; edrophonium chloride was obtained from Kyorin, Tokyo, Japan; protaglandin E_1 was kindly provided by Ono Pharmaceutical Co., Osaka, Japan; TTX was purchased from Sankyo Co., Ltd., Tokyo, Japan. All drugs were dissolved in artificial CSF and applied by superfusion.

Results

Effects of muscarinic agonists in the neonatal rat spinal cord

We examined the effects of muscarinic agonists on the dorsal root-evoked mono-synaptic reflex in the isolated spinal cord preparation and the saphenous nerve-evoked slow VRP in the spinal cord-saphenous nerve preparation. The duration of this slow VRP was 32.0 ± 0.66 s when the saphenous nerve was stimulated with 4 pulses of 30 V intensity ($n = 3$).

Bath-application of muscarinic agonists to the spinal cord evoked two distinct types of response. One type of response was an inhibition of the mono-synaptic reflex (Figure 1) as well as the slow VRP (Figure 2) and the other was a depolarization of motoneurones (Figure 1). Of several muscarinic agonists, (+)-*cis*-dioxolane (0.3–100 μM) and muscarine (0.1–30 μM; data not shown) evoked both types of response, whereas arecoline (10 nM–10 μM) and oxotremorine (10 nM–1 μM) inhibited the mono-synaptic reflex and the slow VRP but induced little or no depolarization of motoneurones (Figure 2). The concentration of arecoline and oxotremorine required to inhibit the mono-synaptic reflex to half of the control were 0.97 ± 0.06 μM ($n = 16$) and 0.32 ± 0.05 μM ($n = 5$), respectively. Both types of response to these muscarinic agonists were completely antagonized by atropine (100 nM; data not shown).

McN-A-343 (1–100 μM), a putative M_1-selective agonist (Hammer & Giachetti, 1982), did not evoke a depolarization of motoneurones. Although it depressed the mono-synpatic reflex at a relatively high concentration (100 μM), this effect was not antagonized by atropine (100 nM; data not shown).

Effects of muscarinic agonists on the slow VRP evoked by noxious skin stimulation

In the spinal cord-saphenous nerve-skin preparation, application of capsaicin (0.5 μM), bradykinin (3 μM), or noxious heat to the skin produced depolarizing responses of motoneurones of slow time course (Figure 3). These responses were markedly depressed by arecoline (3 μM) or oxotremorine (1 μM). Oxotremorine (1 μM) reduced the capsaicin-, bradykinin- and heat-induced responses by 95.3 ± 2.24%, 94.6 ± 1.48%, 91.2 ± 3.35% of the control responses, respectively ($n = 3$).

Effects of muscarinic antagonists on the responses to muscarinic agonists

Muscarinic receptors are pharmacologically classified into M_1, M_2 and M_3 subtypes (for review see Hulme *et al.*, 1990). The M_1 receptor is characterized by a high affinity for pirenzepine (Hammer *et al.*, 1980). The receptors with lower affinities for pirenzepine are further divided into M_2 and M_3 subtypes: the former is sensitive to AF-DX 116 (Giachetti *et al.*, 1986), gallamine (Riker & Wescoe, 1951) and methoctramine (Melchiorre *et al.*, 1987), whereas the latter is sensitive to 4-DAMP (Barlow *et al.*, 1976; Brown *et al.*, 1980) and *p*-F-HHSiD (Lambrecht *et al.*, 1988).

Figures 1 and 2 illustrate the effects of muscarinic antagonists on the excitatory and inhibitory responses to muscarinic agonists. The M_2 antagonists, AF-DX 116

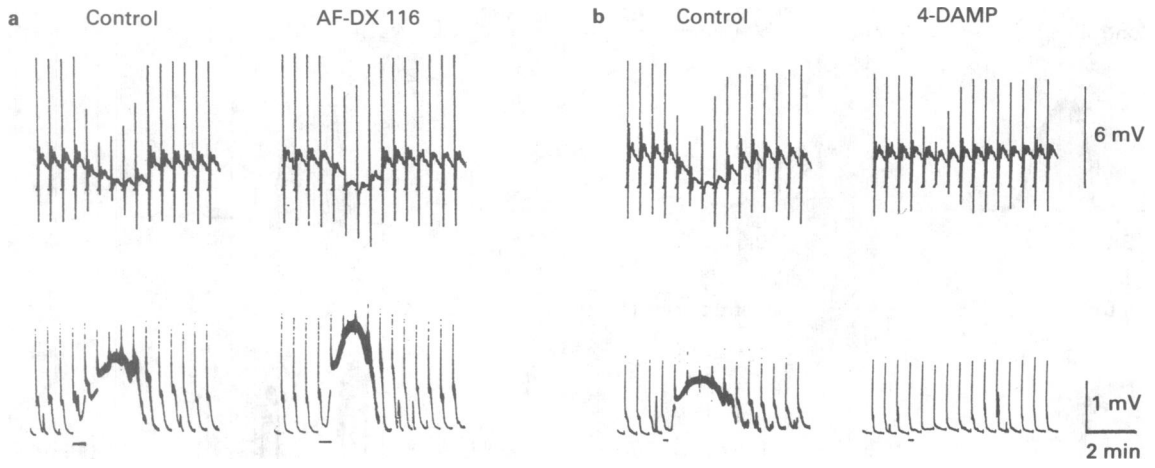

Figure 1 Effects of muscarinic antagonists on (+)-*cis*-dioxolane-evoked responses. Effects of AF-DX 116 (1 μM) (a) and 4-DAMP (5 nM) (b) in normal artificial CSF. (+)-*cis*-Dioxolane was applied at 3 μM for 30 s in (a) and at 10 μM for 10 s in (b) during the periods indicated by bars. Single-shock stimuli of supramaximal intensity were applied to the L4 dorsal root every 30 s and the resulting reflexes were recorded from the ipsilateral ventral root of the same segment. The upper traces show the records of the fast reflex responses during 52 ms post-stimulus periods, which were stored in a memory device in a.c. mode and then recorded on a pen-recorder on a 1000 times expanded time-base. Initial sharp spikes represent the mono-synaptic reflexes. The lower traces show continuous chart records of the d.c. potential. Records in (a) and (b) were taken from different preparations.

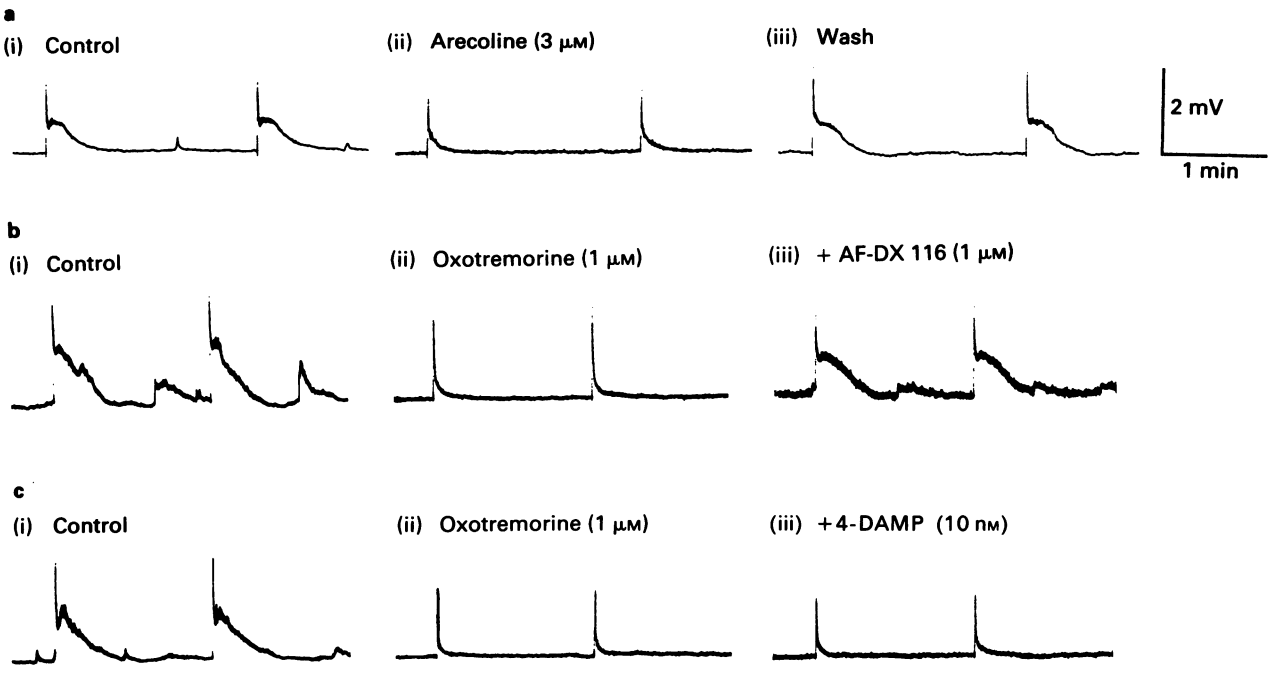

Figure 2 Effects of muscarinic agonists and antagonists on saphenous nerve-evoked slow VRP. (a) Effect of arecoline on the slow VRP evoked by saphenous nerve stimulation. (i) Control responses; (ii) in the presence of arecoline (3 μM); and (iii) after removal of arecoline. (b) Effect of oxotremorine and AF-DX 116 on the slow VRP. (i) Control responses; (ii) in the presence of oxotremorine (1 μM); and (iii) after adding AF-DX 116 (1 μM) in the continued presence of oxotremorine. (c) Effect of oxotremorine and 4-DAMP on the slow VRP. (i) Control responses; (ii) in the presence of oxotremorine (1 μM); and (iii) after adding 4-DAMP (10 nM) in the continued presence of oxotremorine. The saphenous nerve was stimulated every 2 min with 4 shocks in (a) and every 90 s with 2 shocks in (b) and (c) of supramaximal intensity. Records in (a), (b) and (c) were taken from different preparations.

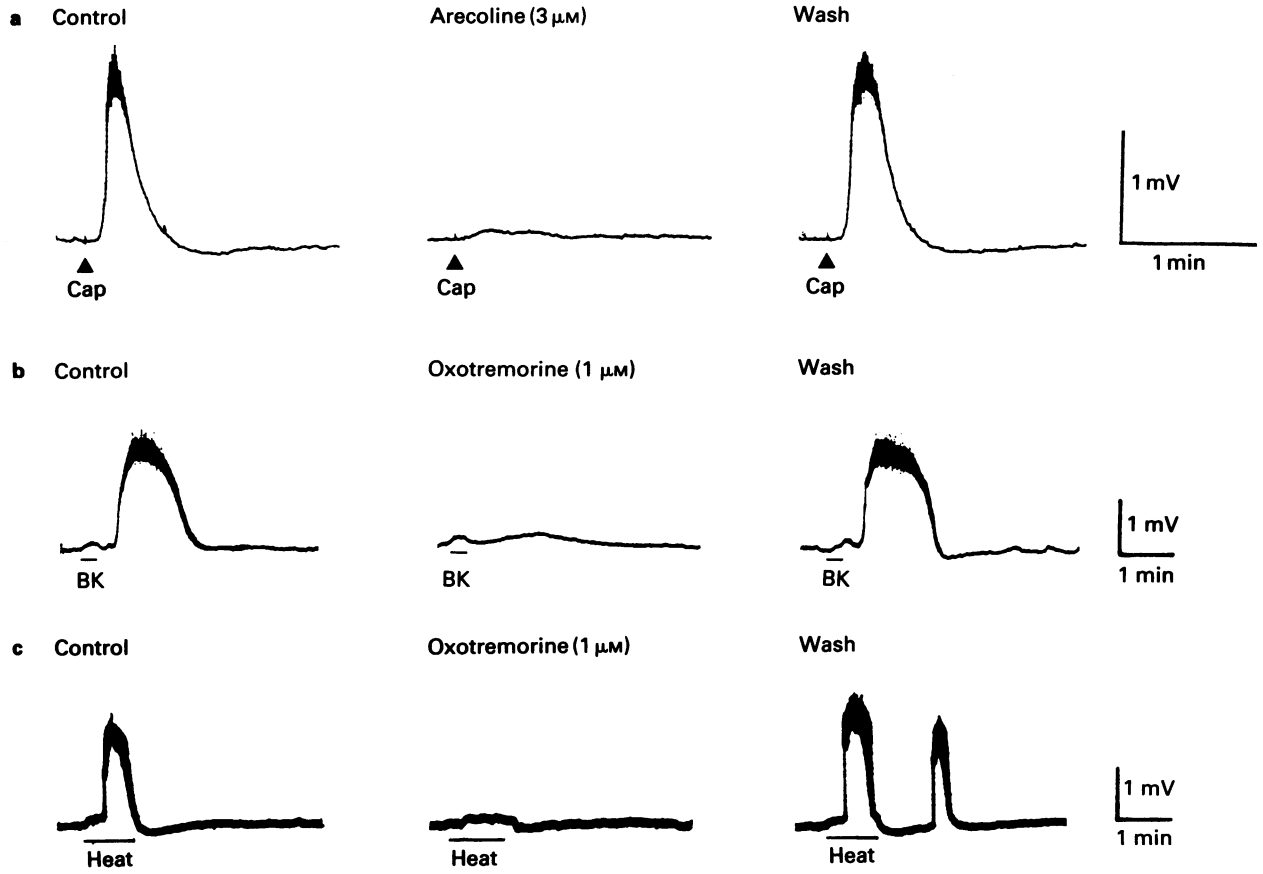

Figure 3 Effects of arecoline and oxotremorine on the slow VRP evoked by noxious skin stimulation in the isolated spinal cord-saphenous nerve-skin preparations. (a) Capsaicin (Cap, 0.5 μM) was applied to the skin with a pressure pulse of 0.45 s duration at (▲). The skin was perfused with artifical CSF containing prostaglandin E_1 (1 μM). (b) Bradykinin (BK, 3 μM) was applied during the periods (20 s) indicated by bars. (c) Heat (47°C) was applied during the periods (1 min) indicated by bars. The records in the middle column were taken after pretreatment of the spinal cord with arecoline or oxotremorine for 30 min. Records in (a), (b), and (c) were taken from different preparations.

(0.1–1 μM; Figures 1a and 2b), methoctramine (100–300 nM; data not shown), and gallamine (5–30 μM; data not shown), reduced the muscarinic inhibition of the mono-synaptic reflex and slow VRP induced by (+)-*cis*-dioxolane, oxotremorine or arecoline. The M_2 antagonists at these concentrations did not reduce the depolarization of motoneurones evoked by (+)-*cis*-dioxolane but often potentiated the response (Figure 1a). On the other hand, the M_3 antagonists, 4-DAMP (3–10 nM; Figures 1b and 2c) and *p*-F-HHSiD (0.1–3 μM; data not shown), did not affect or only slightly reduced the muscarinic inhibition of the monosynaptic reflex and slow VRP. In contrast, these antagonists markedly depressed the (+)-*cis*-dioxolane-evoked motoneurone depolarization (see also Figure 6).

Effects of edrophonium and muscarinic antagonists on the slow VRPs

In the spinal cord-saphenous nerve preparation, the anticholinesterase, edrophonium (5 μM) potentiated the slow VRP evoked by saphenous nerve stimulation (Figure 4a). This potentiating effect became evident within 10 min after starting perfusion of the drug and the slow VRP returned to the control size within 30–60 min after wash-out of the drug.

The M_1 antagonist, pirenzepine (10 nM) had little effect on the slow VRP. However, the M_2 antagonists, AF-DX 116 (1 μM; Figure 4b) and methoctramine (100 nM; data not shown), potentiated the slow VRP. In contrast, the M_3 antagonist 4-DAMP (10 nM, Figure 4c) depressed the slow VRP. Table 1 summarizes the changes of size of the slow VRP produced by edrophonium (5 μM) and the muscarinic antagonists in the presence of edrophonium. The effects of these antagonists could be observed in experiments in normal artificial CSF, but became more pronounced in the presence of edrophonium.

In the spinal cord-saphenous nerve-skin preparation, the M_3 antagonist 4-DAMP (5–10 nM; Figure 4d) also significantly depressed the motoneurone depolarization evoked by application of capsaicin to the skin (−47.9 ± 14.8, % change of the response compared to the control, $n = 3$, $P < 0.05$ by Student's *t* test). 4-DAMP (5–10 nM) also

Table 1 Changes of saphenous nerve-evoked slow VRP by muscarinic drugs

Drugs		*% change*	n
Edrophonium	(5 μM)	77 ± 4**	17
Pirenzepine	(10 nM)	11 ± 1*	4
AF-DX 116	(1 μM)	75 ± 9*	5
4-DAMP	(5 nM)	− 50 ± 9*	3

The areas of depolarization (mV × min) were measured on chart records, and the averages of 3–5 consecutive responses were determined. Each value is the percentage change of the responses induced by each muscarinic drug compared with the control responses. The effects of muscarinic antagonists were tested in the presence of edrophonium (5 μM). Details of the experiments are the same as in Figure 4. Each value represents the mean ± s.e.mean ($n = 3$–17).
*$P < 0.05$; **$P < 0.01$ in comparison with the control responses by Student's *t* test.

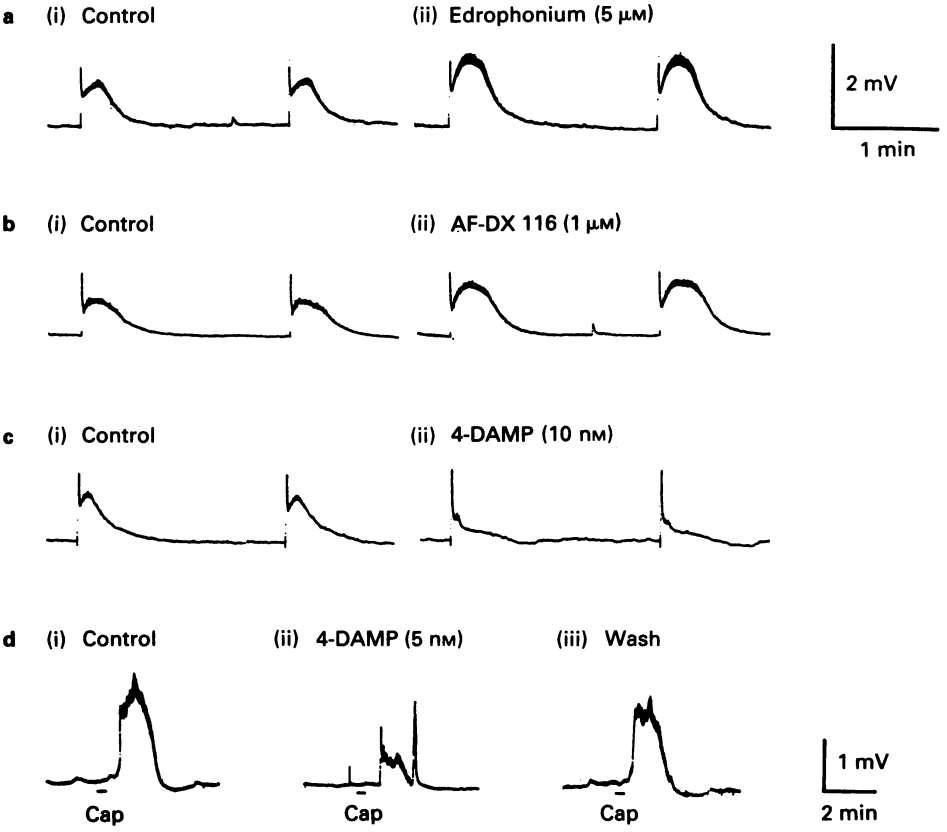

Figure 4 Effects of edrophonium and muscarinic antagonists on the slow VRPs. (a) (i) In normal artificial CSF and (ii) in the presence of edrophonium (5 μM). (b) and (c) (i) In normal artificial CSF containing edrophonium (5 μM) and (ii) after adding AF-DX 116 (1 μM) and 4-DAMP (10 nM), respectively. The saphenous nerve was stimulated every 2 min with 4 shocks in (a) and 2 shocks in (b) and (c) of supramaximal intensity. (d) (i) In normal artificial CSF; (ii) in the presence of 4-DAMP (5 nM); and (iii) after removal of 4-DAMP. Capsaicin (Cap; 0.3 μM) was applied to the skin for 20 s as indicated by bars. Records in (a), (b) and (c) were taken from spinal cord-saphenous nerve preparations and (d) was taken from spinal cord-saphenous nerve-skin preparation.

depressed spontaneous depolarizing activities of motoneurones (data not shown).

Pharmacological analyses of muscarinic responses

Figure 5 illustrates representative concentration-response curves and the Arunlakshana-Schild plot for the arecoline-induced inhibition of the mono-synaptic reflex in the absence or presence of AF-DX 116. The antagonist caused parallel shifts to the right of the concentration-response curve. Similar parallel shifts were obtained with methoctramine (30–300 nM, data not shown), pirenzepine (0.1–3 μM; data not shown), and 4-DAMP (10–100 nM; data not shown). Table 2 summarizes the results of Arunlakshana-Schild plot analysis. The plots were linear within the concentration ranges of the antagonists and the slopes of the regression lines were not significantly different from unity except for 4-DAMP ($P<0.05$ by Student's t test). The variance of 4-DAMP slope about the mean was much less than that of the other antagonists.

Muscarinic receptor-induced depolarization of motoneurones was also analyzed. Figure 6 shows concentration-depolarization curves for (+)-*cis*-dioxolane and the effects of muscarinic antagonists. The experiments were done in the presence of TTX (0.3 μM) to suppress trans-synaptic actions of drugs. 4-DAMP potently inhibited the effect of (+)-*cis*-dioxolane (Figure 6a; $n=4$), but there was a tendency towards a reduction of the slopes of the curves with increasing concentrations of the antagonist. The potency of another M_3 antagonist *p*-F-HHSiD was weak. Furthermore, increasing the concentration of *P*-F-HHSiD beyond 1 μM did not cause a further appreciable shift of the curve to the right (Figure 6b; $n=3$). The effect of the M_1 antagonist, pirenzepine, was also weak (Figure 6c; $n=6$). The M_2 antagonist AF-DX 116 considerably shifted the curve at 0.1 μM but the effect tended to be saturated at 0.3–1 μM (Figure 6d; $n=3$). Owing to the non-parallel shifts of the concentration-response curves it was not possible to obtain pA_2 values for the antagonists.

Effects of arecoline and oxotremorine on substance P- and capsaicin-evoked depolarizations

To obtain information about the mechanisms of the inhibitory muscarinic action on the slow VRPs, effects of oxotremorine and arecoline on the ventral root depolarizations induced by bath-applications of capsaicin (3 μM for 10 s) and SP (10 nM for 10 s) to the spinal cord were examined. In this experiment a relatively high (nearly maximal) concentration of capsaicin and a low (submaximal) concentration of SP were used. At these concentrations the depolarizations induced by these agents were mainly due to trans-synpatic action (Yanagisawa *et al.*, 1980; Yanagisawa & Otsuka, 1990). Oxotremorine (0.3 μM) (Figure 7b) markedly depressed the capsaicin-evoked depolarization (9.80 ± 0.72% of the control response, $n=3$), whereas the SP-evoked depolarization was much less depressed (68.7 ± 4.85% of the control response, $n=3$). Similar results were obtained for the arecoline (1 μM)-induced depression of capsaicin- and SP-evoked depolarizations (data not shown).

Table 2 pA_2 values and slopes of Arunlakshana-Schild plots for muscarinic antagonists against arecoline-induced inhibition of mono-synaptic reflex

Antagonist	*pA_2*	*Slope*	n
Pirenzepine	6.59 ± 0.08	1.07 ± 0.12	4
AF-DX 116	6.79 ± 0.06	1.06 ± 0.15	3
Methoctramine	6.79 ± 0.12	1.04 ± 0.18	3
4-DAMP	8.16 ± 0.19	1.16 ± 0.01	3

Each value represents the mean ± s.e.mean ($n=3$–4). The slopes of regression lines of the Arunlakshana-Schild plots are not significantly different from unity except for 4-DAMP ($P<0.05$ by Student's t test).

Discussion

In this study we showed the existence of two pharmacologically distinct, excitatory and inhibitory, muscarinic receptor-mediated responses in the neonatal rat spinal cord and provided evidence that both types of muscarinic mechanisms are involved in spinal nociceptive transmissions.

Previous studies have shown that the slow VRPs induced by both saphenous nerve stimulation and application of capsaicin to skin were markedly depressed by a tachykinin antagonist spantide (Nussbaumer *et al.*, 1989; Yanagisawa *et al.*, 1992). There is evidence that these slow depolarizing responses represent a component of nociceptive spinal reflexes (Nussbaumer *et al.*, 1989; Yanagisawa *et al.*, 1992).

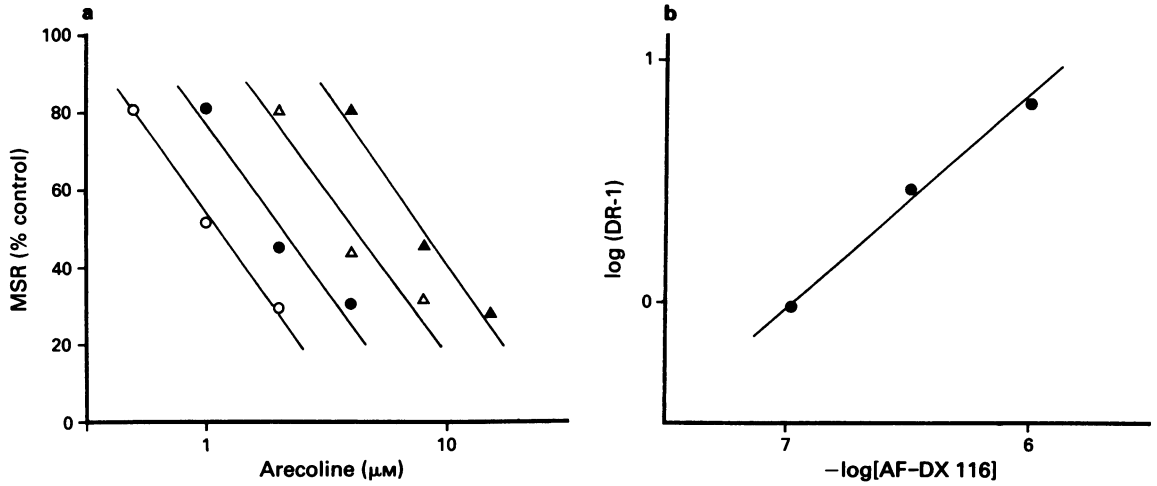

Figure 5 Antagonism by AF-DX 116 of arecoline-induced inhibition of mono-synaptic reflex. (a) Concentration-inhibition curves to arecoline in the absence (○) and in the presence of AF-DX 116 at 0.1 μM (●), 0.3 μM (△) and 1 μM (▲). Ordinate scale: amplitude of mono-synaptic reflex expressed as a percentage of the control reflex amplitude. Abscissa scale: logarithmic concentration of arecoline (see Methods for details). (b) Arunlakshana-Schild plot of log {dose-ratio (DR) − 1} versus − log concentration of AF-DX 116. Each point was derived from the results shown in (a). Data were derived from a single typical experiment but similar results were obtained in 2 other experiments.

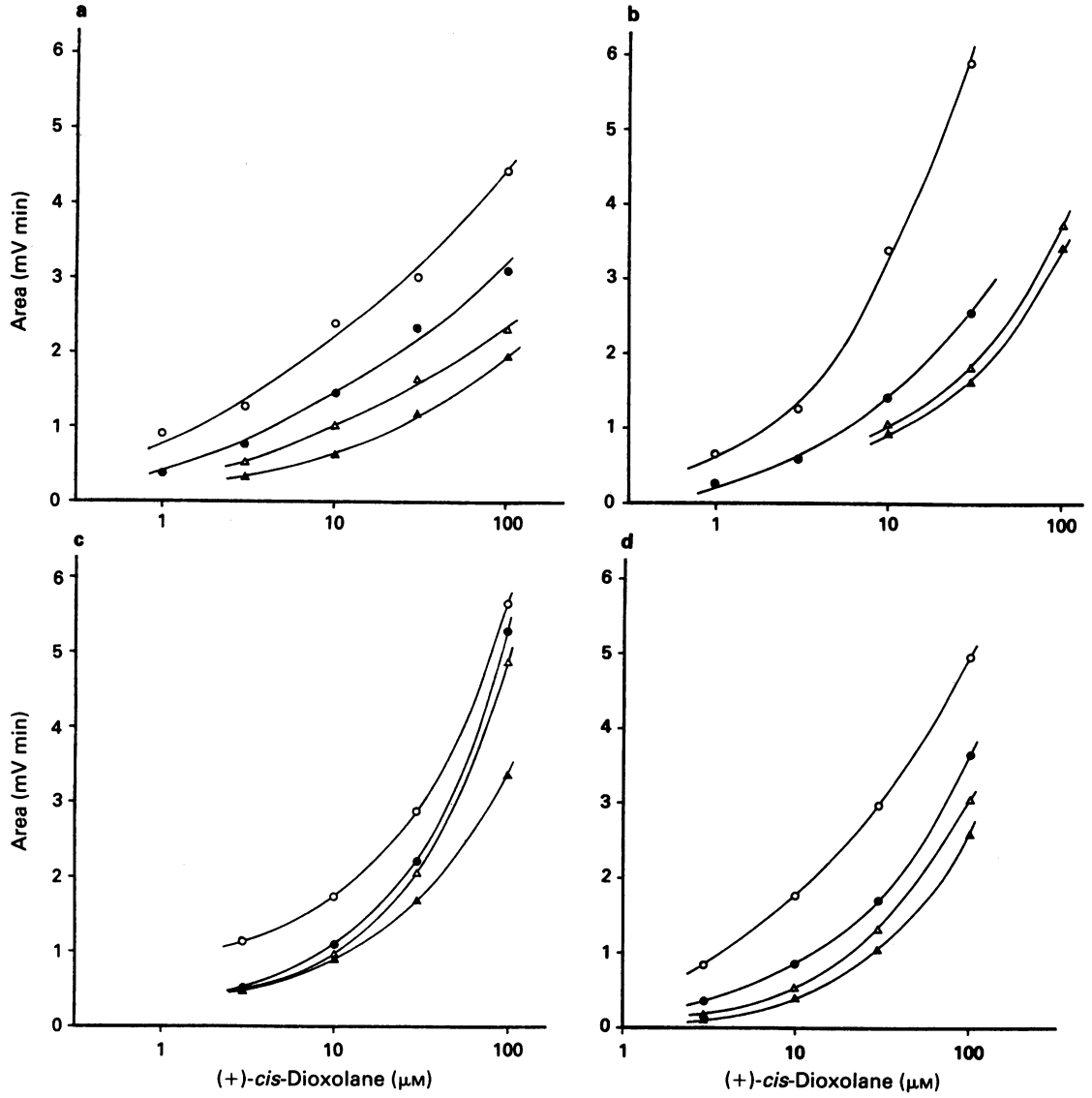

Figure 6 Antagonsim by muscarinic antagonists of (+)-*cis*-dioxolane-induced motoneurone depolarization. Effects of 4-DAMP (a), *p*-F-HHSiD (b), pirenzepine (c) and AF-DX 116 (d). Concentration-depolarization curves to (+)-*cis*-dioxolane were obtained in the presence of tetrodotoxin (TTX, 0.3 μM) and then the effect of each antagonist was examined in the continued presence of TTX. (○) Control in (a), (b), (c) and (d). In (a) 4-DAMP: (●) 1 nM; (△) 3 nM and (▲) 10 nM. In (b) *p*-F-HHSiD: (●) 0.3 μM; (△) 1 μM and (▲) 3 μM. In (c) pirenzepine: (●) 10 nM; (△) 30 nM and (▲) 100 nM. In (d) AF-DX116: (●) 0.1 μM; (△) 0.3 μM and (▲) 1 μM. Ordinate scale: area of depolarization (mV min). Abscissa scale; logarithmic concentration of (+)-*cis*-dioxolane (see Methods for details). Data were derived from a single typical experiment but similar results were obtained in 2–5 other experiments.

In this study we found that the slow VRPs were depressed by oxotremorine and arecoline, which presumably act as M_2 agonists. Furthermore, the saphenous nerve-evoked slow VRP was potentiated by the M_2 antagonists, AF-DX 116 and methoctramine. These results suggest that cholinoceptor inhibitory mechanism, via M_2-type receptors, is involved in the slow VRP and the nociceptive transmissions in the neonatal rat spinal cord. The pA_2 values of muscarinic antagonists against the muscarinic inhibition of the slow VRPs were not obtained since it was difficult to maintain stable responses of the slow VRPs for the period necessary to construct concentration-response curves (about 5–6 h). However, the qualitative analyses suggested that the muscarinic receptors were identical with the M_2 receptors mediating the inhibition of the mono-synaptic reflex.

Gillberg *et al.* (1989) observed that intrathecal application of carbachol to rats induced analgesia and suggested that both M_1 and M_2 receptors were involved since the effect of carbachol was antagonized by similar doses of AF-DX 116 and pirenzepine. At present, however, evidence of the involvement of M_1 receptors in the muscarinic inhibitory responses has not been obtained. Although the putative M_1 agonist McN-A 343 induced an inhibition of the mono-synaptic reflex, this effect was not antagonized by atropine and is therefore probably an action not mediated by muscarinic receptors. Although the M_1 antagonist, pirenzepine, at 0.1–1 μM antagonized the muscarine-evoked inhibition of the mono-synaptic reflex (Yoshioka *et al.*, 1990b), these concentrations would be sufficiently high to block other types of muscarinic receptors.

In an attempt to examine the synaptic site of the muscarinic inhibition of the slow VRPs we compared the depressant effect of oxotremorine on the capsaicin-induced depolarization with that on the SP-induced depolarization. Previous studies have shown that capsaicin applied to the neonatal rat spinal cord produces a depolarization of ventral roots and that the release of tachykinins from primary afferent terminals is involved in the depolarization (Theriault

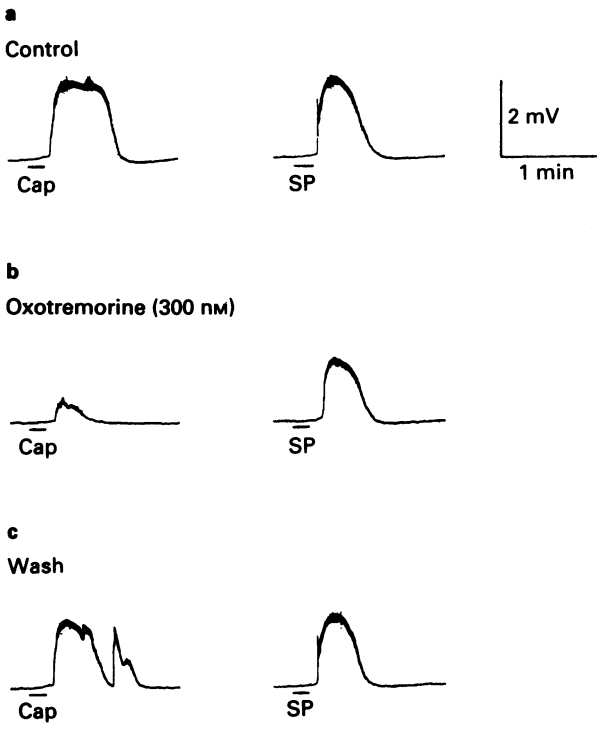

Figure 7 Effects of oxotremorine on the depolarizations evoked by application of capsaicin (Cap) and substance P (SP) to the spinal cord. (a) In normal artificial CSF. (b) In the presence of oxotremorine (300 nM). (c) After removal of oxotremorine. Potentials were recorded from L3 ventral root of an isolated spinal cord preparation. Capsaicin (3 μM for 10 s) and SP (10 nM for 10 s) were applied to the spinal cord alternately at intervals of 30 min.

et al., 1979; Yanagisawa *et al.*, 1980; Yoshioka *et al.*, 1990b). The finding that oxotremorine preferentially inhibited the effect of capsaicin over that of SP suggested that the inhibitory action is primarily due to an inhibition of the release of transmitters including tachykinins from primary afferents. In accord with this notion, Yoshioka *et al.* (1990b) have provided electrophysiological evidence suggesting that the muscarinic inhibition of the mono-synaptic reflex is through a presynaptic mechanism. Furthermore, morphological studies have shown that the dorsal horn of the rat spinal cord contains a dense plexus of choline acetyltransferase-immunoreactive axons and varicosities particularly in lamina III (Barber *et al.*, 1984; Borge & Iversen, 1986; for review see Gillberg *et al.*, 1990), where some cholinergic interneurones form axoaxonic synapses with primary sensory fibres (Ribeiro-da-Silva & Cuello, 1990). Autoradiographic studies have also demonstrated that the substantia gelatinosa of rat spinal cord contains a high density of muscarinic receptors, some of which seem to be localized on terminals of primary sensory fibres (Gillberg & Wiksten, 1986; Gillberg & Askmark, 1991; for review see Gillberg *et al.*, 1990).

The slow VRP induced by saphenous nerve stimulation was potentiated by the anticholinesterase, edrophonium and depressed by the M_3 antagonist, 4-DAMP. Furthermore, the depolarizing response of the ventral root evoked by application of capsaicin to skin was depressed by 4-DAMP. These results suggest that, in addition to M_2 inhibitory muscarinic receptors, excitatory M_3 muscarinic receptors are involved in the nociceptive transmissions.

It is not known how excitatory cholinergic neurones are involved in the neural circuits for the slow VRPS. These cholinergic neurones might be directly activated by tachykininergic C-fibres since tachykinins have been shown to release acetylcholine from the neonatal rat spinal cord (Kobayashi *et al.*, 1991). In addition, however, the neuronal pathways for the slow VRPs seem to contain interneurones releasing excitatory amino acid transmitter(s), since we and other groups have shown that excitatory amino acid antagonists reduce the slow depolarisation evoked by peripheral nerve stimualtion (Dray & Perkins, 1987; King *et al.*, 1990; Kurihara *et al.*, 1991).

The concentration-response curve for arecoline to inhibit the mono-synaptic reflex was shifted in parallel by muscarinic antagonists. The slopes of the Arunlakshana-Schild plots for the antagonists were close to unity. These results suggest that the inhibition of the mono-synaptic reflex was mediated by a homogeneous population of muscarinic receptors. The pA_2 values of pirenzepine (6.59), AF-DX 116 (6.79), 4-DAMP (8.16), for this action are comparable with those for the chronotropic muscarinic action in the guinea-pig atrium, i.e., 6.60, 6.49, and 7.90, respectively (Clague *et al.*, 1985; Eglen & Whiting, 1987). However, the pA_2 value of methoctramine for the monosynaptic reflex inhibition (6.79) is much lower than the value for the atrial chronotropism (7.73) (Melchiorre *et al.*, 1987). These results suggest that the muscarinic receptors mediating inhibition of the mono-synaptic reflex in this preparation are similar to, but not identical with the cardiac type M_2 receptors.

In contrast, the muscarinic antagonists produced non-parallel shift of the concentration-response curves for (+)-*cis*-dioxolane to induce the motoneurone depolarization, suggesting the involvement of multiple muscarinic receptors in the depolarizing action. Newberry & Connolly (1989) also reported that atropine flattened the concentration-response curves for cholinergic agonists to induce motoneurone depolarization. Although the M_1 antagonist, pirenzepine (10 nM) and M_2 antagonist, AF-DX 116 (100 nM) slightly depressed the depolarization, the effects tended to be saturated with higher concentrations of these antagonists. The contribution of M_1 and M_2 receptors to the depolarization therefore seems to be small if any. In contrast, the potent antagonistic effect of 4-DAMP at 1–10 nM on the depolarization is comparable with its effect on the M_3 receptor response of the guinea-pig ileum (pA_2 value: 9.0) (Clague *et al.*, 1985; Eglen & Whiting, 1987). However, the concentrations of another M_3 antagonist *p*-F-HHSiD needed to antagonize the depolarization were high (100 nM–3 μM), when compared with its concentrations of action on the ileal M_3 receptor (pA_2 value: 7.8) (Lambrecht *et al.*, 1988). Furthermore, arecoline and oxotremorine, which exhibit a full agonist activity on the ileum (Clague *et al.*, 1985), had a very weak depolarizing action on motoneurones in the neonatal rat spinal cord. These results suggest that the motoneurone depolarization is mainly mediated by receptors similar to, but not identical with the ileal M_3-type receptors.

Excitatory muscarinic postsynaptic actions on neurones have been reported in a variety of areas in the central nervous system (e.g. hippocampus, olfactory cortex, neocortex, thalamus, and neostriatum) and it has been suggested that the receptor subtypes involved in these actions may be either M_1- or M_2-type (see Nicoll *et al.*, 1990). The present study suggests the existence of an excitatory muscarinic response of central neurones mediated by M_3-type receptors.

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