Effect of clonidine, nimodipine and diltiazem on the *in vitro* opioid withdrawal response in the guinea-pig ileum

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1 The effects of clonidine, nimodipine and diltiazem, on the *in vitro* withdrawal contracture induced by naloxone in the guinea-pig ileum obtained from morphine-dependent animals, were evaluated.

2 The *in vitro* incubation with clonidine (0.01, 0.1 and 1 μM), diltiazem (0.25, 0.5 and 1 μM) or nimodipine (0.05, 0.1 and 1 μM) reduced significantly the force of the contracture induced by naloxone in the morphine-dependent guinea-pig ileum.

3 The intraperitoneal administration of clonidine (0.3 mg kg^{-1}), nimodipine (5 mg kg^{-1}) or diltiazem (20 mg kg^{-1}) reduced the contractile response induced by naloxone in the morphine-dependent guinea-pig ileum.

4 It is concluded that at least part of the effect of clonidine, nimodipine and diltiazem on withdrawal contractures is mediated via a peripheral, rather than a central site of action. Even though, the mechanism responsible for the effect of the calcium channel blockers differs from that of α_2 -adrenoceptor agonists, all of the drugs tested prevented the contracture induced by naloxone in morphine-dependent guinea-pig ileum.

Introduction

After chronic morphine treatment, naloxone administration precipitates an abstinence syndrome in animals and man. During opioid withdrawal the release of numerous neurotransmitters is augmented, and specifically a direct relationship between noradrenergic hyperactivity and the intensity of the abstinence symptoms has been demonstrated (Swann *et al.*, 1983). The existence of a direct relationship between α_2 -adrenoceptors in rat central nervous system and chronic morphine administration (Hamburg & Tallman, 1981) is well known, and clonidine, a drug widely used as an α_2 -adrenoceptor agonist, has been used effectively to reduce withdrawal symptoms (Fielding *et al.*, 1978; Taylor *et al.*, 1988). Furthermore, peripheral mechanisms may be implicated in the anti-withdrawal effect of clonidine. It has been demonstrated that this drug suppresses the contracture of the opiate-dependent guinea-pig ileum elicited by administration of naloxone (Collier *et al.*, 1981). Nevertheless, the usefulness of clonidine in this regard is often limited by potential side effects such as hypotension and sedation (Washton & Resnick, 1980).

On the other hand, evidence suggesting an important role of calcium in the actions of opioid analgesics has accumulated. Chronic morphine administration is associated with a large increase in the content of calcium in the brain and during naloxone precipitated withdrawal these elevated calcium levels return toward control values (Yamamoto *et al.*, 1978). It has been shown that the number of calcium channel antagonist binding sites is greatly increased in the brain of morphine-tolerant mice (Ramkumar & El-Fakahany, 1988).

Recent studies have demonstrated the ability of the calcium entry blockers to inhibit the manifestation of the morphine abstinence syndrome in rats and mice (Bongianni *et al.*, 1986; Baeyens *et al.*, 1987) by a mechanism that differs from that of α_2 -adrenoceptor agonists.

In order to compare the effect of clonidine on the abstinence signs with that of the calcium entry blockers we have evaluated the frequency of presentation of a contractile response after *in vitro* naloxone administration to the myenteric plexus-longitudinal muscle strips (MP-LM) obtained

from guinea-pigs chronically treated with morphine. This contractile response represents a morphine withdrawal response (Ehrenpreis *et al.*, 1972; Schulz *et al.*, 1982).

Methods

Tissues were obtained from the small intestine caudal to the gastroduodenal junction of adult guinea-pigs weighing 250–400 g, fasted 24 h and killed by decapitation. MP-LM preparations were prepared from 5 cm segments (Ambache, 1954) and suspended in an organ bath containing 20 ml of Krebs solution (NaCl 118, KCl 4.75, K_2HPO_4 1.2, CaCl_2 2.52, MgSO_4 1.2, NaHCO_3 25 and glucose 11.1 mM) at 34°C, aerated with 95% O_2 and 5% CO_2 . A resting tension of 1 g was applied to each tissue. Contractions were elicited by electrical stimulation with a Grass SD9 stimulator (single square-wave pulses: 2 ms duration, 0.3 Hz and supramaximal voltage), or naloxone (1 μM) and detected with a Grass FT03 force displacement transducer and recorded on a Grass polygraph. Strips were allowed to equilibrate for 15 min before control measurements were taken.

Animals were made morphine-dependent by a single subcutaneous injection of a slow release suspension of morphine 200 mg kg^{-1} as described previously (Frederickson *et al.*, 1976). Four days after treatment the guinea-pigs were killed and the terminal ileum was removed for the *in vitro* study. Control animals were treated with the non-opiate containing vehicle. The *in vitro* withdrawal was precipitated by addition of naloxone (1 μM) to the organ bath. The naloxone effect was tested on the MP-LM strips obtained from the following groups of guinea-pigs: control animals; non-dependent animals, treated with clonidine, diltiazem or nimodipine; dependent animals; dependent animals treated with clonidine, diltiazem or nimodipine. Tissues obtained from dependent guinea-pigs were bathed in Krebs solution containing morphine (0.1 μM).

Intraperitoneal administration of clonidine (0.3 mg kg^{-1}), diltiazem (20 mg kg^{-1}) or nimodipine (5 mg kg^{-1}) were made 1 h before killing. The naloxone-induced contraction was also evaluated in morphine-tolerant preparations incubated for 15 min with clonidine (0.01, 0.1 and 1 μM), nimodipine (0.05, 0.1 and 1 μM) or diltiazem (0.25, 0.5 and 1 μM).

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Figure 1 Effect of incubation with clonidine (0.01, 0.1 and 1 μ M) diltiazem (0.25, 0.5 and 1 μ M) and nimodipine (0.05, 0.1 and 1 μ M) on the contraction induced by naloxone (1 μ M) in MP-LM strips obtained from morphine-tolerant guinea-pigs. Open column-control (non-incubated), stippled columns lowest dose, lined columns middle dose, closed columns highest dose of each drug tested. Each column is the mean value of at least 9 preparations. Vertical bars represented s.e.mean. * P < 0.05; ** P < 0.01 compared with control (non-incubated) preparations.

The drugs used were: clonidine (Boehringer Ingelheim, S.A. Barcelona, Spain), diltiazem (Esteve, S.A. Barcelona, Spain), morphine HCl (Alcaliber, S.A. Spain), naloxone (Sigma Chemical Co. St. Louis, MO, USA) and nimodipine (Bayer Leverkusen, Germany).

The force (g) of the naloxone-induced contractile response was measured in each group of guinea-pigs and compared with the observed responses in morphine-dependent preparations. Statistically significant differences were determined by Student's t test and by ANOVA test.

Results

The contractile response of the MP-LM strips induced by electrical stimulation was inhibited in a dose-dependent manner when morphine (0.1, 0.2, 0.4, 0.8 μ M) was cumulatively administered to the organ bath. When tissues were obtained from guinea-pigs treated with morphine for 4 days the inhibitory effect of added morphine was greatly reduced. The % inhibition for each dose was: in control preparations ($n = 10$): 35.7 ± 3.4 , 60.2 ± 3.9 , 75.4 ± 4.3 , 83.3 ± 2.3 and in morphine-tolerant preparations ($n = 10$): 2.8 ± 0.9 , 5.8 ± 1.6 , 16.1 ± 3.7 , 30.1 ± 5.3 , the difference being statistically significant ($P < 0.001$).

Figure 2 Contractile force of the naloxone-induced contraction in guinea-pig ileum obtained from morphine-dependent animals. Animals were pretreated with saline (control), clonidine (0.3 mg kg⁻¹), diltiazem (20 mg kg⁻¹) or nimodipine (5 mg kg⁻¹). Each column is the mean of at least 9 preparations. Vertical bars represent s.e.mean. * P < 0.05; ** P < 0.01.

The amplitude of electrically-evoked twitches did not differ between control tissues and those chronically treated with morphine.

The addition of naloxone (1 μ M) to the organ bath induced a contractile response in all MP-LM strips removed from morphine-treated guinea-pigs. Ileal segments from naive animals did not respond to naloxone.

When morphine-dependent preparations were incubated for 15 min before addition of naloxone, with clonidine (0.01, 0.1 and 1 μ M), diltiazem (0.25, 0.5 and 1 μ M) or nimodipine (0.05, 0.1 and 1 μ M) the contractile response induced by naloxone was inhibited in a dose-dependent manner (Figure 1). Clonidine, diltiazem and nimodipine did not affect electrically-evoked twitches when added to the organ bath, either in control-tissues or in dependent-tissues at the doses tested.

A decreased sensitivity to naloxone was also found in the MP-LM strips obtained from morphine-dependent guinea-pigs pretreated, 1 h before killing, with clonidine (0.3 mg kg⁻¹), diltiazem (20 mg kg⁻¹) or nimodipine (5 mg kg⁻¹) (Figure 2). The amplitude of contraction induced by electrical stimulation did not differ between control tissues and those obtained from animals to which clonidine, diltiazem and nimodipine had been administered.

Discussion

It is well known that MP-LM preparations obtained from guinea-pigs chronically treated with morphine, develop tolerance to the inhibitory effect of morphine on the electrically-induced contractions. Our results agree with previous reports (Goldstein & Schultz, 1973). Moreover, in preparations of guinea-pig small intestine tolerant to morphine, naloxone induces a withdrawal sign consisting of a contracture of the longitudinal muscle. This abrupt contracture is produced by an increased release of acetylcholine (Frederickson *et al.*, 1976; Schulz *et al.*, 1985) and substance P (Chahl, 1983). Furthermore, withdrawal-induced electrophysiological events have been recorded in myenteric neurones. The experiments of Johnson *et al.* (1987) provided direct evidence for a naloxone-induced depolarization of the neuronal membrane. It is generally accepted that the typical symptoms of morphine withdrawal are caused by a rebound neuronal hyperactivity in the central and peripheral nervous systems.

The morphine abstinence symptoms can be effectively suppressed by administering clonidine. This drug has the ability to decrease noradrenaline (NA) release in the brain (Taylor *et al.*, 1988) and it has been used in the treatment of opioid withdrawal in man (Washton & Resnick, 1980). The main effect of clonidine on the withdrawal syndrome seems to be mediated by a decrease in NA release from locus coeruleus neurones. Nevertheless, clonidine also induces peripheral effects, like the inhibition of the neural activity in the guinea-pig ileum decreasing acetylcholine release (Malta *et al.*, 1981; Ilhan & Long, 1985), and the inhibition of the contractile response induced by naloxone in the morphine-dependent MP-LM preparation (Collier *et al.*, 1981).

On the other hand, after chronic morphine treatment the binding of ⁴⁵Ca²⁺ to synaptic vesicles was increased and a large increase in the content of calcium in the brain has been described. During the naloxone-precipitated withdrawal syndrome these elevated calcium levels return toward control values (Yamamoto *et al.*, 1978). The effects of calcium channel blockers on the behavioural signs of naloxone-induced abstinence in animals have been studied previously (Bongianni *et al.*, 1986; Baeyens *et al.*, 1987); however, the mechanism underlying this has not been elucidated and it is possible that central and peripheral sites of action could be implicated. In fact, in MP-LM strips, calcium entry blockers antagonize the contractile response induced by electrical stimulation and this effect has been attributed to a decrease of calcium entry into the smooth muscle through voltage-dependent channels (Martin *et al.*, 1990). The release of acetylcholine, induced by

electrical stimulation, was not affected by nifedipine in control preparations (Gilbert & Tucker, 1989); however, these authors found, in morphine-withdrawn tissues, that nifedipine significantly attenuated the electrically-induced release of acetylcholine, and suggested that this effect may be explained by changes in the number and/or characteristics of the voltage-operated calcium channels in the nerve endings of myenteric neurones.

The present work shows that when clonidine, diltiazem and nimodipine were administered *in vivo* to morphine-dependent guinea-pigs, or when morphine-dependent tissues were incubated with them, the *in vitro* withdrawal contracture was significantly decreased and this effect was dose-dependent.

These findings provide evidence to support the peripheral anti-withdrawal effects that have been suggested (Franz *et al.*, 1982; Baeyens *et al.*, 1987). All of the drugs studied are able to

decrease the withdrawal contracture in morphine-dependent tissues, even though this decrease was associated with different mechanisms: clonidine activates α_2 -presynaptic adrenoceptors and calcium antagonists reduce the entry of calcium into the neurones by blocking voltage-dependent calcium channels. Since calcium influx is an essential factor in neurotransmitter release, the calcium antagonist effect observed could be due to a blockade of the release of acetylcholine or substance P. Therefore, our results suggest that the effectiveness of the evaluated drugs in preventing *in vitro* naloxone withdrawal could be better attributed to an action at a neural site rather than a muscular site.

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