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ω-Agatoxin IVA blocks spinal morphine/clonidine antinociceptive synergism

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Abstract

Involvement of P-type voltage-dependent Ca^{2+} channels in spinal morphine- or clonidine-induced antinociception and in the synergistic interaction between morphine and clonidine was examined in the present studies. Coadministration of the selective P-type antagonist, ω-agatoxin IVA (25 ng) intrathecally (i.t.) to mice along with morphine or clonidine enhanced the tail flick antinociception of each agonist 5–6-fold. The greater-than-additive (synergistic) interaction that occurred when morphine and clonidine were coadministered i.t. decreased to an additive interaction in the presence of ω-agatoxin IVA. In mice pretreated with pertussis toxin (10 ng) to inactivate G proteins, ω-agatoxin IVA did not alter the morphine/clonidine synergism. Surprisingly, ω-agatoxin IVA reversed the additive morphine/clonidine interaction that occurs in morphine-tolerant mice back to synergism. These results suggest that functional P-type Ca^{2+} channels play an essential role in the antinociceptive synergism between spinal morphine and clonidine.

Keywords: Ca^{2+} channel, P-type; Antinociception, spinal; Morphine; Clonidine; Tolerance; G-protein

1. Introduction

Neuronal Ca^{2+} currents are inhibited by both opioid and α_2-adrenoceptor agonists in a G protein-dependent manner (North, 1993; Surprenant et al., 1990). Activation of opioid receptors inhibits both N- and P/Q-, but not L-type voltage-dependent Ca^{2+} channels in isolated cells from the nucleus tractus solitarii (Rhim and Miller, 1994) and in dorsal root ganglion neurons (Moises et al., 1994; Rusin and Moises, 1995; Schroeder et al., 1991) and N-type channels in neuroblastoma SH-SY5Y cells (Seward et al., 1991) and neuroblastoma × glioma NG108-15 hybrid cells (Eckert and Trautwein, 1991; Morikawa et al., 1995). The inhibitory effects of a κ-opioid receptor agonist on Ca^{2+} uptake by brain synaptosomes and on Ca^{2+} current in cerebellar Purkinje neurons are inhibited by a P-type Ca^{2+} channel antagonist (Kanemasa et al., 1995).

The antinociceptive activity of opioids is enhanced in the presence of doses of L-type or N-type Ca^{2+} channel antagonists, which have little or no antinociceptive activity on their own (Antkiewicz-Michaluk et al., 1993; Basilico et al., 1992; Contreras et al., 1988; DelPozo et al., 1987; Omote et al., 1996, 1993; Quijada et al., 1992; Spampinato et al., 1994; Vaupel et al., 1993). Recently, a similar enhancing effect by L-type and N-type antagonists has been shown for antinociception induced by the α_2-adrenoceptor agonist, clonidine (Wei et al., 1996). A spinal site of action has been proposed for this enhancing effect on opioid activity in the tail flick antinociceptive test (Omote et al., 1996, 1993).

Spinal co-administration of opioid agonists and α_2-adrenoceptor agonists produces greater-than-additive (synergistic) antinociception (Hylden and Wilcox, 1983; Meert and DeKock, 1994; Omote et al., 1991; Ossipov et al., 1990; Plummer et al., 1992; Roerig and Fujimoto, 1989; Sherman et al., 1988). The synergistic interaction between spinal morphine and clonidine decreases to an additive interaction in mice that are tolerant to subcutaneous morphine (Roerig, 1995). This alteration in morphine/clonidine synergism may be part of the mechanism involved in development of tolerance to morphine.

Recent studies have shown that in mice pretreated with spinal pertussis toxin (which decreases G protein activity), the spinal morphine/clonidine synergism decreases to an additive interaction in the presence of an N-type Ca^{2+} channel antagonist, but not an L-type antagonist (Wei et al., 1996). Neither pertussis toxin treatment alone nor the Ca^{2+} channel antagonists alone have any effect on the morphine/clonidine synergism. The L-type Ca^{2+} channel agonist 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoro-

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methyl)-phenyl]-3-pyridine carboxylic acid methyl ester (Bay K 8644) also has no effect on the morphine/clonidine interaction.

Although opioids also affect the activity of the P-type Ca^{2+} channel (Rhim and Miller, 1994; Rusin and Moises, 1995), the involvement of P-type channels in opioid-induced antinociception is uncharacterized. Also, the possible role of P-type channels in spinal morphine/clonidine synergism is unknown. To test whether P-type channels are involved in the synergism between spinal morphine and clonidine, the present studies were performed using the selective P-type channel antagonist ω-agatoxin IVA (Mintz et al., 1992). In order to assess the possible role of G proteins in P-type channel function, studies were also performed in mice which had been pretreated with pertussis toxin.

In consideration of the similarity of findings between two different treatments, pertussis toxin pretreatment plus N-type Ca^{2+} channel antagonist and morphine pellet treatment (in both conditions the morphine/clonidine synergism decreases to an additive interaction), the effects of ω-agatoxin IVA on morphine and clonidine antinociception following chronic morphine treatment were also examined. Previous studies have shown that L-type Ca^{2+} channel antagonists attenuate development of morphine dependence (Antkiewicz-Michaluk et al., 1993; Ramkumar and El-Fakahany, 1988; Tokuyama et al., 1995) and clonidine dependence (Barrios et al., 1993). Cortical binding sites for both L- and N-type Ca^{2+} channel ligands increase after chronic morphine treatment (Ramkumar and El-Fakahany, 1984; Suematsu et al., 1993) and binding of an L-type ligand increases after either chronic morphine or intracerebroventricular pertussis toxin pretreatment (Ohnishi et al., 1990). Also, chronic spinal opioid treatment or acute spinal pertussis toxin treatment both appear to uncouple the opioid receptor from G proteins (Wong et al., 1992). Thus, additional studies were performed in mice which had been implanted with morphine pellets to induce development of opioid tolerance.

2. Materials and methods

2.1. Animals

Male ICR mice (25–35 g) purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA) were used in all experiments. They were housed 5 per cage in 12 h/12 h light/dark cycles with free access to food and water. At least 2 days (usually 3 or more) were allowed for adaptation before they were used in experiments. Each animal was used only once.

2.2. Antinociceptive assay

Antinociception was measured using the radiant heat tail flick method (D'Amour and Smith, 1941) with the lamp intensity adjusted to obtain baseline tail flick latencies of 2–4 s. A 10 s cut-off time was allowed as the maximal antinociceptive response. Tail flick times were determined before and 10 min after drug administration, the time of peak drug effect as determined in preliminary studies (data not shown). The percentage maximal possible effect (% MPE) of each drug dose in each animal was calculated using the formula (Dewey et al., 1970): %MPE = [(Post-drug time − Pre-drug time)/(10 − Pre-drug time)] × 100.

2.3. Drug administration

All drugs (dissolved in sterile water) were administered in a volume of 5 μl into the lumbar spinal, intrathecal space of unanesthetized mice (Hylden and Wilcox, 1980). Morphine sulfate, morphine pellets and placebo pellets were obtained from the National Institutes on Drug Abuse. Clonidine HCl was purchased from Sigma (St. Louis, MO, USA). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA, USA). ω-Agatoxin IVA was generously provided by Pfizer Research (Groton, CT, USA).

The antinociceptive effects of morphine and clonidine administered separately or concurrently were tested. When morphine and clonidine were coadministered, equieffective dose ratios were used, based on the ED_{50} values obtained when the agonists were administered separately, as previously described (Roerig, 1995). For example, when the ED_{50} value for morphine alone was 149 pmol and the ED_{50} value for clonidine alone was 516 pmol (about a 1:3 ratio, see Table 1), the combined agonists were given in a 1:3 ratio so that the combined ED_{50} values also have a 1:3 ratio. This protocol allows the analysis of the morphine/clonidine interaction using the isobolographic method (Tallarida et al., 1989). In some experiments, ω-agatoxin IVA (25 ng) was given concurrently with the morphine and/or clonidine. At least four drug doses and 8–10 animals per dose were used to determine each ED_{50} value.

2.4. Pertussis toxin treatment

To inactivate G_i/G_o proteins, mice were given a single i.t. injection of 10 ng pertussis toxin and 14 days later, other drugs were administered for the tail flick studies. Previous studies have shown that this dose of pertussis toxin decreases the pertussis toxin-substrate G proteins in spinal cord without producing noticeable adverse effects in the animals (Wei et al., 1996).

2.5. Chronic morphine treatment

Mice were implanted s.c. with a single pellet (150 mg) containing either binding agents (placebo pellet) or 75 mg morphine base (morphine pellet). After 3 days, pellets

were removed and intrathecal drugs were administered 75 min later as described previously (Roerig, 1995).

2.6. Data analysis

Antinociceptive ED_{50} values were calculated from dose response curves using the Graded Dose Response Method (Tallarida and Murray, 1987). When morphine and clonidine were coadministered, the doses used for each drug were used to calculate the respective ED_{50} value. Interactions between morphine and clonidine were analyzed isobolographically and theoretical additive ED_{50} values were calculated (Tallarida et al., 1989).

3. Results

When morphine and clonidine were co-injected intrathecally, the anticipated synergistic interaction for tail flick antinociception was observed. The ED_{50} values for morphine and clonidine in combination were lower than the ED_{50} values for the agonists administered alone (Table 1). Isobolographic analysis of these values indicated that the interaction was greater-than-additive, or synergistic (Fig. 1). Also, both the morphine and clonidine ED_{50} values in combination were lower than the calculated theoretical additive ED_{50} values (Table 1), confirming that the interaction between the agents was synergistic.

In initial studies with ω-agatoxin IVA, various doses of the Ca^{2+} channel antagonist were coadministered with 100 pmol morphine, a dose which alone produced about a 40% MPE. A 10 ng ω-agatoxin IVA dose had no effect on the morphine-induced response (MPE = 45.7 ± 11.7%, $n = 5$), a 25 ng dose enhanced the morphine-induced response (MPE = 87.9 ± 11.8%, $n = 5$), and 50 ng ω-agatoxin IVA caused the mice to scratch repeatedly at the injection site. The 25 ng dose was selected to use in further studies because other Ca^{2+} channel antagonists had also been shown to enhance the morphine-induced effect.

Intrathecal administration of 25 ng ω-agatoxin IVA alone did not produce a change in tail flick latency times. Before drug injection the latency time was 3.0 ± 0.3 and 10 min later the latency time was 3.4 ± 0.35 ($n = 5$). Testing at 5 and 20 min after ω-agatoxin IVA also showed no change in latency times (data not shown). When the ω-agatoxin IVA was coinjected with either morphine or clonidine, the dose response curves of both agonists shifted to the left and the ED_{50} values decreased, 6- and 5-fold, respectively (Table 1). Also, the interaction between morphine and clonidine decreased to an additive interaction in the presence of ω-agatoxin IVA (Table 1 and Fig. 1). The theoretical additive ED_{50} values were not different from the experimentally determined ED_{50} values. Interestingly, the experimentally determined combination ED_{50} values were not different between control and ω-agatoxin IVA-treated animals. The decreased ED_{50} values produced by ω-agatoxin IVA when the agonists were administered separately were not accompanied by a further decrease in the ED_{50} values of the combined agonists in the presence of the toxin.

Table 1
Effect of ω-agatoxin IVA on the antinociceptive interaction between spinal morphine and clonidine in mice

Added drug	Agonist	ED_{50} (pmol) (95% confidence interval)			Interaction
		Agonist alone	Agonist in combination	Theoretical additive	
None	Morphine	149 (105–210)	11 (9–13)	74 (61–89)	Synergistic
	Clonidine	516 (290–918)	38 (31–46)	258 (214–310)	
ω-agatoxin IVA [a]	Morphine	25 (19–33)	10 (6.8–15)	12 (11–14)	Additive
	Clonidine	97 (60–159)	51 (34–76)	49 (42–57)	
H_2O [b]	Morphine	61 (40–92)	13 (7.3–21)	30 (25–37)	Synergistic
	Clonidine	312 (175–559)	62 (37–106)	156 (128–190)	
Pertussis toxin [c]	Morphine	269 (232–313)	18 (13–27)	134 (115–158)	Synergistic
	Clonidine	17454 (9853–30918)	1201 (813–1774)	8727 (7438–10238)	
Pertussis toxin [c] + ω-agatoxin IVA [a]	Morphine	180 (155–210)	23 (16–33)	90 (81–100)	Synergistic
	Clonidine	386 (268–555)	46 (32–66)	193 (174–214)	

[a] 25 ng ω-agatoxin IVA coinjected i.t. with agonists. [b] 5 μl water injected i.t. 13 days before agonists. [c] 10 ng pertussis toxin injected i.t. 14 days before agonists.

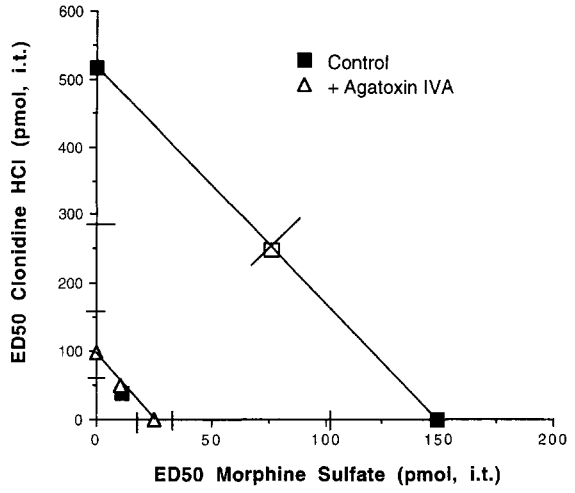

Fig. 1. Isobolographic analysis of the interaction between spinal morphine and clonidine. Points on the abscissa and the ordinate represent ED_{50} values for morphine sulfate and clonidine HCl respectively, when administered separately in the absence (squares) or presence (triangles) of ω-agatoxin IVA. Bars crossing the axes indicate the 95% confidence intervals for these ED_{50} values. The solid lines connecting the separate drug ED_{50} values represent the additive interaction lines. The square below the upper additive line represents ED_{50} values obtained when morphine and clonidine were coinjected in the absence of ω-agatoxin IVA. The triangle on the lower additive line represents ED_{50} values obtained when morphine and clonidine were coinjected in the presence of ω-agatoxin IVA. The 95% confidence intervals for the combined agonist ED_{50} values were too small to be represented in this figure. The open square drawn on the upper additive interaction line represents the calculated theoretical additive ED_{50} values for control animals and the 95% confidence intervals for those values are shown by the diagonal line through this point. Experimental values below the additive interaction line, and lower than the theoretical additive values, indicate a synergistic interaction between the drugs. Experimental values on the additive interaction line, and not different from the theoretical additive values, represent an additive interaction between drugs.

In mice injected intrathecally with water 13 days before morphine and/or clonidine were administered, the ED_{50} for morphine was lower than that found in untreated animals, but the clonidine ED_{50} remained unchanged (Table 1). There is no apparent explanation for the altered response to morphine, but pretreatment of mice with pertussis toxin increased the ED_{50} values for both morphine and clonidine when compared to either water-pretreated or untreated conditions. The increase for clonidine was much greater than that for morphine, 56-fold compared to 4-fold (pertussis toxin vs water pretreatment). However, even though the responses to the individual agonists was diminished, the interaction between the agonists remained synergistic (Table 1). This inability of pertussis toxin pretreatment to alter the spinal morphine/clonidine synergism has been shown previously (Wei et al., 1996).

When ω-agatoxin IVA was coinjected with morphine and clonidine after pertussis toxin pretreatment, the ED_{50} value for clonidine returned to control (or water-pretreated) values. The morphine ED_{50} value remained slightly greater than that in water-pretreated mice, but was the same as that in untreated animals. Thus, ω-agatoxin IVA reversed the increase in ED_{50} values produced by pertussis toxin pretreatment. The morphine/clonidine interaction remained synergistic (Table 1) even in the presence of ω-agatoxin IVA, a different result from that found in control animals.

Previous studies have shown that the spinal morphine/clonidine synergism decreases to an additive interaction in morphine pellet-implanted mice (Roerig, 1995). Data from these studies are included in Table 2 to facilitate comparison of results obtained when ω-agatoxin IVA was co-injected with morphine and clonidine in morphine pellet-implanted animals. As seen in Table 2, in the presence of ω-agatoxin IVA, the morphine ED_{50} was similar to that in the absence of ω-agatoxin IVA, but the clonidine ED_{50} decreased when co-injected with ω-agatoxin IVA. In addition, the morphine/clonidine interaction remained synergistic after morphine pellet treatment in the presence of ω-agatoxin IVA, similar to the unchanged

Table 2
Effect of ω-agatoxin IVA on the antinociceptive interaction between spinal morphine and clonidine in morphine tolerant mice

Treatment	Agonist	ED_{50} (pmol) (95% confidence interval)			Interaction
		Agonist alone	Agonist in combination	Theoretical additive	
Placebo pellet [b]	Morphine [a]	110 (95–130)	13 (11–17)	55 (45–68)	Synergism
	Clonidine [a]	720 (345–1500)	54 (43–68)	360 (290–440)	
Morphine pellet [c]	Morphine [a]	200 (140–290)	100 (87–120)	100 (88–110)	Addition
	Clonidine [a]	38 (27–55)	21 (18–25)	19 (27–55)	
Morphine pellet [c] + ω-Agatoxin IVA [d]	Morphine	203 (160–258)	16 (10–23)	100 (89–115)	Synergism
	Clonidine	6.5 (4.3–9.8)	0.49 (0.32–0.74)	3.2 (2.9–3.7)	

[a] Data are from Roerig, 1995. [b] Placebo pellets were implanted s.c. 3 days before i.t. agonist administration. [c] Morphine pellets were implanted s.c. 3 days before i.t. agonist administration. [d] 25 ng ω-agatoxin IVA was coinjected i.t. with the agonists.

(from control) interaction observed in pertussis toxin-treated animals.

4. Discussion

In the present studies, ω-agatoxin IVA, a P-type Ca^{2+} channel antagonist, when coadministered spinally with both morphine and clonidine, enhanced the agonist-induced tail flick antinociception, in agreement with previous findings for N- and L-type Ca^{2+} channel antagonists (Wei et al., 1996). The mechanism for this enhancement is not well understood (see Omote et al., 1996). Antinociception produced by spinal opioids may involve inhibition of release of substance P (Cahill et al., 1995; Yaksh et al., 1980) or other nociceptive transmitters. Both N- and P-type Ca^{2+} channels, but probably not L-type channels, are associated with neurotransmitter release in the central nervous system (Dunlap et al., 1995). However, acute spinal administration of low doses of N-type (Wei et al., 1996; Omote et al., 1996) or P-type (this study) antagonists does not produce significant antinociception in the tail flick test. If Ca^{2+} channel activity and neurotransmitter release are involved in the expression of tail flick antinociception, there may be a threshold level of neurotransmitter release that is required for inhibiting the tail flick response. For example, at higher doses the N-type antagonist ω-conotoxin GVIA does produce tail flick antinociception (Omote et al., 1996). A combination of Ca^{2+} channel antagonist and opioid (or clonidine)-induced inhibition of Ca^{2+} channel activity may lower the threshold to produce enhanced antinociception. Alternatively, because L-type antagonists also enhance morphine and clonidine effects, a mechanism not involving neurotransmitter release may also be involved.

In contrast to studies using L-type and N-type Ca^{2+} channel antagonists, the present studies show that the P-type antagonist decreased the morphine/clonidine synergism to an additive interaction. The morphine/clonidine synergism is a robust response that is not easily blocked. In fact, only two examples of the synergism blockade have been reported thus far, one example is the interaction change that is observed with the N-type (but not the L-type) antagonist only in mice that had been pretreated with pertussis toxin to inactivate G proteins (Wei et al., 1996). Thus, the first difference observed between N-type and P-type antagonists was that the ω-agatoxin IVA did not require the inactivation of G proteins in order to alter the morphine/clonidine synergism.

The finding that inhibition of P-type channels alone blocked the synergism while synergism of blockade by the N-type channel antagonist also required G protein inactivation may provide an important clue about the mechanism of the morphine/clonidine synergism. Little is known about the regulation of P-type Ca^{2+} channel activity by opioids and clonidine, but there is little evidence to show that there is any difference between the agonists' effects on P-type and effects on N-type channels (Rhim and Miller, 1994; Rusin and Moises, 1995). It is possible that one Ca^{2+} channel type is more involved in release of a peptide/neurotransmitter than another channel and this differential activity is reflected in the present findings. This putative peptide/neurotransmitter could be involved in the mechanism of morphine/clonidine synergism. A possible peptide candidate for this role is the anti-opioid peptide, neuropeptide FF, which, paradoxically, is released by morphine from spinal cord slices and may be part of the facilitatory/inhibitory nociceptive system (Devillers et al., 1995a). The major portion of neuropeptide FF release is attributed to activity of P-type, rather than N- or L-type Ca^{2+} channels (Devillers et al., 1995b). It is intriguing to speculate that inhibition of neuropeptide FF release by inhibition of P-type channel activation, could be involved in the blockade of the morphine/clonidine synergistic interaction. On the other hand, inhibition of P-type channels to inhibit release of the anti-opioid peptide may be the mechanism responsible for the ω-agatoxin IVA-induced enhancement of morphine-induced antinociception. Further investigations are warranted in this area.

A second possible neurotransmitter that could participate in spinal morphine/clonidine synergism is glutamate. Facilitated nociceptive processing in the spinal cord has been shown using the formalin test to involve both N- and P-type, but not L-type channels (Malmberg and Yaksh, 1994). Blockade of P-type, rather than N-type channels appears to be similar to the actions of NMDA receptor antagonists (Vaccarino et al., 1993). This parallel activity suggests that glutamate release could be involved in the response of the P-type Ca^{2+} channel antagonist (Malmberg and Yaksh, 1994). Antagonists for the NMDA receptor have been shown to block development of tolerance to morphine (Trujillo and Akil, 1991, 1994) at spinal sites (Gutstein and Trujillo, 1993) as do Ca^{2+} channel antagonists (Contreras et al., 1988, also see discussion below). NMDA receptor antagonists also potentiate spinal morphine-induced antinociception (Wong et al., 1996). Thus, the difference between N-type and P-type channel antagonists on the morphine/clonidine synergism could involve the actions of glutamate. Interestingly, glutamate activation of spinal NMDA receptors also induces release of neuropeptide FF (Devillers and Simonnet, 1994).

Another putative neurotransmitter that could be involved in the morphine/clonidine synergism is adenosine. Morphine-induced release of spinal adenosine is proposed to mediate morphine-induced antinociception (Sawynok et al., 1989) and this adenosine release is likely due to effects on N-type, but not L-type Ca^{2+} channels (Cahill et al., 1993). The involvement of P-type channels was not tested in these studies. Other studies have shown that intrathecal clonidine-induced antinociception is enhanced by coadministration of an adenosine receptor agonist (DeLander and Hopkins, 1987). Thus, adenosine may be involved in the morphine/clonidine synergism, but the role of P-type Ca^{2+} channels in this interaction is not known.

The second clue about the mechanism for morphine/clonidine synergism is derived from results obtained for N-type versus P-type channel antagonists after pertussis toxin treatment. Inactivation of pertussis toxin-sensitive G proteins changed the effects of both N-type and P-type antagonists on the morphine/clonidine synergism, but in opposite directions. Pertussis toxin pretreatment changed the morphine/clonidine synergism observed in the presence of the N-type antagonist to addition, while pertussis toxin changed the morphine/clonidine additive interaction observed in the presence of P-type antagonist to synergism. The reason for this difference is not clear but could involve a difference in coupling between the receptor and the G protein, or the G protein and the Ca^{2+} channel. Although there is convincing evidence of G protein-dependent coupling between cloned μ- and κ-opioid receptors and N-type Ca^{2+} channels (Morikawa et al., 1995; Tallent et al., 1995), similar studies have not been reported for opioid or α_2-adrenoceptors and P-type channels. Thus, although G proteins are likely involved in the activity of both channel types, it is not clear how the agonist receptors, the G proteins and the Ca^{2+} channels interact to produce the final expression of morphine/clonidine interactions.

The other recognized example of a decrease in spinal morphine/clonidine synergism to an additive interaction is found in mice which have been implanted with morphine pellets and are tolerant to subcutaneous morphine (Roerig, 1995). Possible involvement of Ca^{2+} channels in morphine tolerance is suggested by the finding that chronic morphine treatment increases binding sites for L-type and N-type Ca^{2+} channel ligands in cerebral cortical membranes (Suematsu et al., 1993). Also, concurrent administration of L- or N-type Ca^{2+} channel antagonists with opioids attenuates the development of tolerance and physical dependence to the opioid (Antkiewicz-Michaluk et al., 1993; Basilico et al., 1992; Contreras et al., 1988; Dierssen et al., 1990, Ramkumar and El-Fakahany, 1988; Tokuyama et al., 1995). However, other studies show that binding of the L-type antagonist [^{3}H]nitrendipine is not altered in brainstem or spinal cord membranes after chronic morphine treatment (Bernstein and Welch, 1995). In the present studies, ω-agatoxin IVA restored the synergistic interaction between morphine and clonidine that had been decreased to an additive interaction with development of tolerance to morphine. These results indicate that P-channel function may be involved in development of morphine tolerance, however, to date, there is no information about how P-type channel function or number may change with chronic morphine treatment. Further studies are needed to establish the role of these channels in development of tolerance to morphine.

In morphine-tolerant mice the P-type antagonist, ω-agatoxin IVA did not enhance morphine-induced antinociception, in contrast to the observations in naive mice. Others have shown that L-type antagonists enhance opioid-induced antinociception in both naive and tolerant conditions (Antkiewicz-Michaluk et al., 1993; Dierssen et al., 1990). The difference between the present results and previous findings may be due to the type of Ca^{2+} channel involved. However, in the presence of ω-agatoxin IVA the clonidine ED_{50} decreased 15-fold in the morphine pellet-implanted mice. This differential effect of ω-agatoxin IVA on morphine and clonidine was unexpected because the effects of Ca^{2+} channel modifying agents on morphine and clonidine-induced antinociception are usually similar (Wei et al., 1996 and Table 1). Interestingly, a similar situation occurred in the pertussis toxin-pretreated mice, ω-agatoxin IVA greatly enhanced clonidine-induced antinociception, but had little effect on morphine's antinociception.

After either pertussis toxin or morphine pellet treatment, ω-agatoxin IVA failed to decrease the morphine/clonidine synergism to addition, as it did in untreated animals. The difference between pertussis toxin treatment and morphine pellet implantation was that after pertussis toxin pretreatment, the morphine/clonidine synergism was not altered, while morphine pellet implantation did change the synergism. A previous report has shown that both pertussis toxin pretreatment and chronic morphine treatment increase binding sites for the L-type Ca^{2+} channel antagonist nitrendipine in cerebral cortical membranes (Ohnishi et al., 1990). Pertussis toxin treatment after chronic morphine treatment does not further enhance the binding. Although others have shown that chronic morphine decreases expression of specific G proteins in specific brain regions (Nestler et al., 1989), similar changes have not been seen in spinal cord (unpublished observations). Thus, the results obtained from chronic morphine versus pertussis toxin pretreatment may not be based on changes in G protein expression per se, but may be due to receptor-G protein uncoupling (Wong et al., 1992). Taken together, these results suggest a complex interaction between receptors, G proteins and Ca^{2+} channels that is altered with chronic morphine or pertussis toxin treatment.

The ω-agatoxin IVA characteristics of antinociception enhancement and synergism restoration in morphine-tolerant animals may have clinical utility. The L-type Ca^{2+} channel antagonists have been shown to enhance morphine-induced analgesia without enhancing morphine-induced respiratory depression (Pereira et al., 1993; Vaupel et al., 1993). Also, preliminary studies have shown that nimodipine (an L-type antagonist) enhances morphine analgesia in cancer patients who are tolerant to morphine (Santillan et al., 1994). The use of selective P-type antagonists such as ω-agatoxin IVA may prove to be even more beneficial than the L-type antagonists, particularly in opioid-dependent/tolerant patients.

In summary, the selective P-type Ca^{2+} channel antagonist ω-agatoxin IVA enhanced both morphine- and clonidine-induced antinociception and decreased the morphine/clonidine synergism to an additive interaction.

The ω-agatoxin IVA also restored the morphine/clonidine synergism after chronic morphine treatment. These results suggest that the functional P-type Ca^{2+} channel may have a primary role in the spinal morphine/clonidine synergistic interaction.

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