Voltage-Sensitive Calcium Channels in Spinal Nociceptive Processing: Blockade of N- and P-Type Channels Inhibits Formalin-induced Nociception

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The role of spinal voltage-sensitive calcium channels (VSCC) in a behavioral model of prolonged nociception was examined in rats. Blockade of VSCC by the trivalent cations neodymium (NdCl₃) and lanthanum (LaCl₃) resulted in a dose-dependent suppression of both phases of the response to formalin. ω -Conopeptides, which selectively block N-type VSCC, also produced a dose-dependent inhibition of both the initial behavior [phase 1; ED₅₀ (nmol): SNX-111 (0.003) > SNX-185 (0.010) > SNX-239 (0.16) > SNX-159 (>0.26); SNX-199 (>0.30)] and the facilitated response [phase 2; ED₅₀ (nmol): SNX-111 (0.003) > SNX-185 (0.009) > SNX-239 (0.020) > SNX-159 (0.120) = SNX-199 (0.230)]. In contrast, SNX-231 (0.24 nmol), which is selective for a non-L/non-N site and also the L-type VSCC blockers nifedipine (24 nmol), nimodipine (29 nmol), verapamil (200 nmol), and diltiazem (220 nmol), had minimal effects on either phase of the formalin test at the highest dose examined. The P-type channel blocker ω -agatoxin IVA produced a 40% inhibition of phase 1 at the highest dose and phase 2 was suppressed in a dose-dependent fashion (ED₅₀, 0.001 nmol). The response latency to a high-threshold thermal stimulus (the 52.5°C hot plate) was moderately (20%) increased by NdCl₃ (0.30 nmol) and SNX-111 (0.008 nmol), but not verapamil (200 nmol) and ω -agatoxin IVA (0.006 nmol). High doses of the N-type VSCC produced characteristic shaking behavior, serpentine-like tail movements, and impaired coordination. However, at antinociceptive doses there was no significant motor effect, though three of the N-type antagonists produced some tail movements. These studies demonstrate that VSCC of the N- and P-type, but not L-type, are involved in facilitated nociceptive processing at the spinal level.

[Key words: voltage-sensitive calcium channels, pain, spinal cord, ω -conopeptides, ω -agatoxin IVA, formalin test, antinociception]

Calcium influx through voltage-sensitive calcium channels (VSCC) is believed to play an important role in the regulation of synaptic transmission and is essential for cellular functions such as neurotransmitter release, enzyme activity, and mem-

brane excitability. Pharmacological and electrophysiological characteristics (for review, see Miller, 1987; Bean, 1989a; Hess, 1990; Swandulla et al., 1991) suggest the existence of at least three different types of neuronal, high-voltage-activated, calcium channels (L-, N-, and P-type). The L-type calcium channel is selectively blocked by 1,4-dihydropyridines (such as nifedipine and nimodipine), phenylalkylamines (e.g., verapamil), and benzothiazepines (e.g., diltiazem) (see Porzig, 1990). The N-type channel can be distinguished from other calcium channels by the selective sensitivity to ω -conopeptides (Aosaki and Kasai, 1989; Sher and Clementi, 1991). The ω -conopeptides are 24–29 amino acid peptides found in venoms of fish-hunting marine snails belonging to the genus *Conus* (Olivera et al., 1985). Structurally related peptides have been synthesized and provide additional agents for pharmacological studies of the study of N-type VSCC (Hillyard et al., 1992; Ramilo et al., 1992). A third calcium channel, the P-type, has been described as being resistant to both ω -conopeptides and dihydropyridines (Regan et al., 1992; Hillyard et al., 1992; Mintz et al., 1992a), but sensitive to toxins found in venom from funnel web spiders (Llinas et al., 1989; Cherksey et al., 1991; Mintz et al., 1992b). In addition, VSCC can also be inhibited by polyvalent cations with an ionic radius similar to calcium but of higher valence and which bind to extracellular calcium sites in a less reversible fashion (see reviews by Weiss, 1974; Lansman, 1990; Reichling and MacDermott, 1991).

Several characteristics of N-type channels suggest that this type of VSCC may be important in spinal sensory processing in general and nociceptive transmission in particular. First, there is growing evidence for the involvement of N-type channels in regulating neurotransmitter release in different areas of the brain, and the spinal release of the primary afferent peptides calcitonin gene-related peptide (CGRP) and substance P have been demonstrated to be ω -conopeptide sensitive (Holz et al., 1988; Maggi et al., 1990; Santicioli et al., 1992). Second, agents that are known to produce potent antinociception after spinal delivery, such as opioids and α_2 -adrenergic agonists (see Malmberg and Yaksh, 1993a), reduce spinal CGRP and substance P release (Pang and Vasko, 1986; Go and Yaksh, 1987; Pohl et al., 1989; Takano et al., 1993), and act at presynaptic sites by blockade of N-type VSCC (Bean, 1989b; Anwyl, 1991; Schroeder et al., 1991). Third, spinal localization of N-type channels by the ω -conopeptide GVIA showed the highest density of binding in the superficial laminae of the dorsal horn, where primary afferents terminate (Kerr et al., 1988; Takemura et al., 1989). Recently, involvement of the P-type channel has been implicated

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in glutamate release in certain brain regions (Dickie and Davies, 1992; Pfrieger et al., 1992; Pocock and Nicholls, 1992; Turner et al., 1992). Because of the important role of glutamate-sensitive channels, in facilitated nociceptive transmission, it is possible that the P-type channels might also have a role in spinal nociceptive processes.

Spinal injection of low doses of the trivalent cations lanthanum and neodymium have been shown to produce a weak, dose-dependent, antinociceptive effect on tests of acute thermal nociception such as the hot plate and tail flick tests (Reddy and Yaksh, 1980), suggesting that VSCC may mediate certain nociceptive systems. However, this study was limited in that it did not distinguish between the different types of VSCC and employed only acute pain end-points. Recent work has indicated that repetitive C-fiber activity yields both an acute pain state and a state of facilitated pain processing (hyperalgesia) as illustrated by the formalin test (see Wheeler-Aceto et al., 1990). Subcutaneous injection of formalin into the paw results in an acute barrage of C-fiber activity followed by a low, but measurable, activity for the next 60 min (Heapy et al., 1987). Behaviorally, this is reflected by an acute flinching of the injected paw (phase 1) followed by a quiescent period and then an extended period of intense flinching behavior (phase 2). Pharmacological studies have shown that the phase 2 behavior is initiated by spinal activation of *N*-methyl-D-aspartate (NMDA) and neurokinin (NK) 1 receptors (Yamamoto and Yaksh, 1991, 1992). The role of spinal VSCC in the pain behavior produced by formalin injection is not known. L-type channel blockers have been reported to have mild antinociceptive activity at a spinal level in the formalin test (Coderre and Melzack, 1992). The contributions of N-type and P-type channels to nociceptive behaviors have not been investigated in awake rats.

In the present study, N-type, L-type, and P-type antagonists and also nonselective VSCC antagonists were injected intrathecally (IT) in order to examine potential behavioral effects and modulation of formalin-induced nociceptive behaviors. In addition, selected agents were tested for their ability to suppress the escape response evoked by high-threshold thermal stimulus (hot plate test).

Materials and Methods

Animal preparation

Rats (male Sprague-Dawley, 275–300 gm, Harlan Industries, San Diego, CA) were implanted with lumbar intrathecal catheters under halothane anesthesia according to the method described by Yaksh and Rudy (1976). Polyethylene catheters (PE-10) extended from the cisterna to the rostral edge of the lumbar enlargement. IT injection studies were started 5–7 d after implantation, and only animals with normal motor function were used. Experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the University of California at San Diego.

Behavior and motor assessment

Systematic observation of general behavior was carried out. Behavior was noted in a normal environment and after stimuli, such as handling, a hand clap from 25 cm (startle response), and toe pinching (withdrawal response). Irritability, in the form of touch-evoked agitation (TEA), was assessed by gently stroking the flank of the rat with a pencil. Motor function was examined by assessing the placing/stepping reflex, where normal behavior is a stepping reflex when the hind paws are drawn across the edge of a table. Righting and ambulation were assessed by placing the rat horizontally with its back on the table, which normally gives rise to an immediate coordinated twisting of the body to an upright position. Catalepsy was tested by placing the forepaws on a horizontal

bar kept at 4 cm from a table surface. Failure to move from the bar within 30 sec was defined as a positive cataleptic response.

Nociceptive tests

Formalin test. The formalin test was assessed as previously described (Wheeler-Aceto et al., 1990; Malmberg and Yaksh, 1992a). Rats were lightly anesthetized with halothane (3%) and 50 μ l of 5% formalin solution was injected subcutaneously into the dorsal surface of the right hind paw with a 30 gauge needle. Observation of the formalin injected paw was made with the rats individually placed in an open Plexiglas chamber with a mirror positioned on the opposite side. Pain-related behavior was quantified by counting the incidence of flinching/shaking, based on the experience that subcutaneous injection of formalin produces characteristic reliable flinching behavior without significant involvement of other behaviors (see Wheeler-Aceto et al., 1990), and that antinociceptive agents suppress the flinching behavior, without altering behaviors such as paw elevation and paw licking. The incidence of flinching was counted in 1 min periods, starting 1–2 min after formalin injection, then 5–6 min after injection, and thereafter at 5 min intervals during the interval from 10 to 60 min. After the observation period, animals were sacrificed with an overdose of barbiturate mixture (Beuthanasia, 50 mg/kg, i.p., Schering-Plough Animal Health Corp., Kenilworth, NJ).

Hot plate test. The response latency to a high-threshold stimulus was examined using the 52.5°C hot plate with a response being defined as either a hind paw lick or a jump. If there was no response within 60 sec, the trial was terminated and this value noted. Before the IT injection of the drugs, each rat was examined twice at 10 min intervals to establish a baseline value. Following drug administration the hot plate latencies were recorded at 5, 15, 30, and 60 min.

Drugs and IT injections

For IT injection studies, agents were mixed such that all doses were delivered in a total volume of 10 μ l followed by 10 μ l of saline to flush the catheter. The ω -conopeptides SNX-111 (MW 2639; peptide content, 70.7%), SNX-159 (MW 2619; peptide content, 68.3%), SNX-185 (MW 3001; peptide content, 67.9%), SNX-199 (MW 2554; peptide content, 76.5%), SNX-231 (MW 2896; peptide content, 69.7%), and SNX-239 (MW 2596; peptide content, 76.2%) were provided by Neurex Corporation (Menlo Park, CA). The ω -conopeptide sequences and their synthesis has been reported previously (Olivera et al., 1985; Bowersox et al., 1992; Hillyard et al., 1992; Ramilo et al., 1992). Nifedipine (BAYa 1040; MW 346.3) and nimodipine (BAYe 9736; MW 418.4) were provided by Miles Pharmaceutical Co. (New Haven, CT). Verapamil-HCl (MW 491.1) and diltiazem-HCl (MW 451.0) were purchased from Sigma Chemical Co. (St. Louis, MO). ω -Agatoxin IVA (MW 5202.3) was provided by Peptide Institute, Inc., Osaka, Japan. Lanthanum chloride (LaCl_3 ; MW 245.3) and neodymium chloride (NdCl_3 ; MW 358.7) were purchased from Aldrich Chemical Co. (Milwaukee, WI). All of the ω -conopeptides, verapamil, diltiazem, ω -agatoxin IVA, LaCl_3 , and NdCl_3 were diluted in perservative free physiological saline (0.9% w/v NaCl; Abbott Laboratories, North Chicago, IL), while nifedipine and nimodipine were diluted in 50% dimethyl sulfoxide (DMSO; Sigma Chemical Co.).

Data analysis and statistics

Time-response data from the formalin test are presented as the number of flinches for the period of 1–2 min, 5–6 min, and at 5 min intervals for 60 min and are expressed as mean number of flinches per min $\pm$ SEM. Statistical analyses were employed on raw data using the sum of flinches for either phase 1 or phase 2. Group comparisons were made with one-way ANOVA and post hoc testing against control was made with Dunnett's test provided that the *F* ratio gave *p* < 0.05. Vehicle-treated control animals were interspersed with drug-treated rats and statistical comparisons were made against a vehicle-treated group specific to each study. For illustrative purposes, dose-response curves are presented as the percentage inhibition of the control response for each observation period (phase 1 and phase 2). A value close to 0% thus represents a sum of flinches close to the number of flinches for control and 100% represents no flinching activity. To achieve this, the mean total number of flinches for phase 1 (0–9 min) and phase 2 (10–60 min) are determined for the control group (rats receiving IT saline). This is defined as the effect_{control}. The percentage inhibition for each drug treated

Table 1. Behavioral and motor effects of spinally administered VSCC antagonists in the rat

Drug	Dose (nmol, IT)	Severe motor dysfunction ^a	Tail movements/mild shaking ^a	Paralysis ^a
ω-Conopeptides with N-type VSCC blocking activity				
SNX-111	0.0008–0.022 ^b	0/9 ^c	0/9	0/9
	0.008	0/10	4/10	0/10
	0.08	1/4	4/4	0/4
	0.27	2/4	2/4	0/4
	0.80	2/2	2/2	0/2
	2.68	1/1	1/1	0/1
SNX-159	0.026	0/4	0/4	0/4
	0.078	0/4	1/4	0/4
	0.26	0/4	4/4	0/4
	0.78	2/2	2/2	0/2
SNX-185	0.002–0.022 ^b	0/12	0/12	0/12
	0.068	0/4	4/4	0/4
	0.22	0/2	2/2	0/2
	0.68	0/1	1/1	0/4
	2.26	1/1	1/1	0/4
SNX-199	0.030	0/4	0/4	0/4
	0.090	0/4	1/4	0/4
	0.30	0/4	1/4	0/4
	0.90	2/2	2/2	0/2
SNX-239	0.008–0.29 ^b	0/16	0/16	0/16
ω-Conopeptide blocking non-L/non-N VSCCs				
SNX-231	0.072	0/4	0/4	0/4
	0.24	0/6	0/6	4/6 ^d
	0.72	1/1	0/1	1/1
	2.40	1/1	0/1	1/1
Dihydropyridines and other L-type VSCC blockers				
Nifedipine	24	0/6	0/6	0/6
Nimodipine	29	0/6	0/6	0/6
Verapamil	200	0/6	0/6	0/6
Diltiazem	220	0/6	0/6	0/6
P-type VSCC blocker				
Agatoxin IVA	0.0002–0.006 ^b	0/18	0/18	0/18
	0.2–2	3/3	0/3	3/3
Trivalent cations blocking VSCC				
Lanthanum (LaCl ₃)	0.01–0.3 ^b	0/17	0/17	0/17
Neodymium (NdCl ₃)	0.01–0.3 ^b	0/17	0/17	0/17

^a Onset < 60 min.^b No dysfunction at any of the doses with an interval of 0.5 log units. $n \geq 4$ for each dose.^c Number of rats with indicated behavior/number of rats examined. These data reflect the animals receiving this dose 10 min before formalin injection and includes, but is not limited to, the animals used to calculate ED₅₀ (see Table 2).^d Onset of paralysis approximately 90 min ($n = 4$) after IT injection. **Figure 1.** Time-effect curve of IT SNX-111 (0.008 nmol), SNX-231 (0.240 nmol), neodymium (0.300 nmol), verapamil (200 nmol), agatoxin IVA (0.006 nmol), and saline on the formalin test. All the agents were administered IT 10 min before the formalin injection. The data are presented as the mean $\pm$ SEM (five to six rats per line) of the number of flinches per minute versus the time after the formalin injection.

by one-way ANOVA followed by Dunnett's test for individual comparisons against control.

Results

General behavior and motor effects

During the observation period (60 min), none of the agents showed significant effects upon motor function at doses that produced an antinociceptive effect in the formalin test. However, three of the N-type VSCC produced tail movement and in a few rats mild shaking behavior (Table 1) in doses close to ED₅₀. These behaviors usually appeared around 40–60 min of injection. IT injection of higher doses of N-type channel-selective ω -conopeptides induced typical behavioral effects consisting of intense whole-body shaking, coordination (walking) problems, circling behavior (often backward), and serpentine-like movements of the tail (see Table 1). The shaking behavior was intermittent and was primarily observed when the rat was moving. Such shaking behavior could also be evoked in a quiescent animal by touching it. In between these "bursts" of shaking, the rat sat quietly but often displayed serpentine-like movement of the tail. Although the high doses produced intense "shaking" syndrome, no sign of muscle weakness was apparent based on normal placing/stepping, writhing, and pinch responses. However, many rats (approximately 50%) showed increased irritability as indicated by vocalization when touched gently on the flank and back with a pencil and also a facilitated startle response. Though not systematically examined, the shaking behavior produced by the N-type VSCC antagonists were found to be reversed within 24 hr. Interestingly, while the drugs differed in their antinociceptive activity, the doses necessary for inducing motor dysfunction were relatively similar across the active agents (approximately 0.3–0.9 nmol). In contrast to the N-type ω -conopeptides, the non-L/non-N-type, SNX-231, produced a flaccid paralysis of the hind limbs. This flaccidity was observed after 30 min with an IT dose of 2.4 nmol and was followed by breathing depression and death. This effect was also observed 90 min after IT injection in four out of six rats at a dose of 0.24 nmol (Table 1). Similarly, high doses of the P-type

rat is then calculated by % inhibition = (effect_{control} – effect drug-treated rat)/(effect_{control}) $\times$ 100. This value, calculated for each rat, is then used to construct dose-response curves using a least-square linear regression. ED₅₀ (effective dose resulting in a 50% reduction of the control formalin response) and 95% confidence intervals were calculated according to formulas given by Tallarida and Murray (1987).

For the hot plate test, responses were converted to percentage of the maximal possible effect (% MPE) by % MPE = (postdrug latency – baseline latency)/(cutoff time – baseline latency) $\times$ 100. Statistical comparisons of the response latencies for the treatment groups were made

Figure 2. Formalin test dose-response curves for the trivalent cations lanthanum and neodymium administered IT 10 min before formalin injection. Data are presented as the percentage inhibition of control (saline) response of the cumulative number of formalin-evoked flinches during phase 1 (0–9 min, *top*) and phase 2 (10–60 min, *bottom*). Each dose point on the graph represents mean $\pm$ SEM for four to five rats.

blocker agatoxin IVA produced toxic effects (Table 1). Approximately 15–30 min after IT injection of high doses of agatoxin IVA (0.2–2 nmol), an intense agitation behavior was observed with sudden bursts of scratching, jumping, and vocalization followed by motor weakness, breathing depression, and death about 90 min after injection.

Formalin test

Subcutaneous injection of 5% formalin into the rat paw produced a biphasic flinching behavior of the injected paw (Fig. 1). There was no difference between the vehicle-treated animals during the study period. The trivalent cations neodymium and lanthanum (administered as chlorides) produced a dose-dependent suppression of both phases 1 and 2 of the formalin test (Fig. 2, Table 2). However, phase 1 was only suppressed to about 50% of the control response at the highest dose examined (0.30 nmol; both agents). This prevents calculation of ED_{50} because it would not represent a dose within the dose interval tested. Doses of the salts higher than 0.30 nmol IT were not examined because an earlier study demonstrated interference with HP

Figure 3. Formalin test dose-response curves for the ω -conopeptides and agatoxin IVA administered 10 min before formalin injection. Data are presented as the percentage inhibition of control (saline) response of the cumulative number of formalin-evoked flinches during phase 1 (0–9 min, *top*) and phase 2 (10–60 min, *bottom*). Each dose point on the graph represents mean $\pm$ SEM for four to six rats.

response motor function (Reddy and Yaksh, 1980). In the present study none of the doses examined produced any detectable motor dysfunction.

Intrathecal delivery of N-type VSCC antagonists resulted in a dose-dependent reduction in the magnitude of both phases of the formalin test for three out of five ω -conopeptides and all five suppressed phase 2 of the formalin test at doses that did not interfere with motor function (Fig. 3, Table 2). The order of potency for suppression of phase 2 (and ED_{50} values in nmol) was SNX-111 (0.003) > SNX-185 (0.009) > SNX-239 (0.020) > SNX-159 (0.120) = SNX-199 (0.230) (Fig. 3, Table 2). Dose-dependent inhibition of the first phase was found only for the following ω -conopeptides (ED_{50} values in nmol): SNX-111 (0.003) > SNX-185 (0.010) > SNX-239 (0.160) (Fig. 3, Table 2). The ω -conopeptide selective for a non-L/non-N-type channel SNX-231 had no significant effect on the formalin test, although the second phase was numerically less than control (Fig. 1, Table 2).

The L-type channel blockers produced only a moderate (20–30%) reduction of the formalin response (Fig. 4). Verapamil (200 nmol) and diltiazem (220 nmol) showed a statistically significant reduction of the second phase at the highest dose examined. Nifedipine and nimodipine had to be dissolved in 50%

Table 2. ED₅₀ values and 95% confidence interval (CI) for intrathecal conopeptides on phase 1 (0–9 min) and phase 2 (10–60 min) of the formalin test

		ED ₅₀ (nmol, IT) ± 95% CI	
Drug	n ^a	Phase 1 (0–9 min)	Phase 2 (10–60 min)
N-type channel antagonists			
SNX-111	13	0.003 (0.001–0.006)	0.003 (0.002–0.004)
SNX-159	12	>0.26	0.12 (0.01–1.3)
SNX-183	16	0.010 (0.007–0.014)	0.009 (0.007–0.013)
SNX-199	12	>0.30	0.23 (0.03–1.7)
SNX-239	12	0.54 (0.09–2.2) ^b	0.052 (0.02–0.23)
L-type channel antagonists			
Nifedipine	6	>29	>29
Nimodipine	6	>24	>24
Verapamil	6	>200	>200
Diltiazem	6	>220	>220
P-type channel antagonists			
Agatoxin IVA	12	>0.006 ^b	0.001 (0.0008–0.002)
Non-N/non-L-type channel antagonists			
SNX-231	10	>0.24	>0.24
Trivalent cations			
Lanthanum	13	>0.3 ^c	0.11 (0.06–0.19)
Neodymium	13	>0.3 ^c	0.06 (0.02–0.14)

^a n, Number of animals in the dose–response curve for calculation of ED₅₀.^b The highest dose produced about a 60% inhibition of the first-phase control.^c Highest dose produced a 40–50% reduction of the first-phase response.

DMSO, which restricted studies to relatively low doses. DMSO-treated rats did not show any modification of the formalin response compared to saline-treated rats, although some rats vocalized upon IT injection of 50% DMSO. Nifedipine (29 nmol) and nimodipine (24 nmol) numerically reduced the second phase of the formalin test, but this value did not differ significantly from DMSO-treated rats (Fig. 4).

The P-type channel blocker agatoxin IVA produced a mild (40% at the highest dose) suppression of phase 1 at the highest dose studied (0.008 nmol). However, as shown in Figure 3 and Table 2, agatoxin IVA produced a potent dose-dependent inhibition of phase 2 of the formalin test.

The temporal association of drug treatment in relation to the

initiation of the stimulus (formalin injection) was investigated for NdCl₃, SNX-111, verapamil, and agatoxin IVA. As shown in Figure 5, SNX-111 (0.008 nmol) treatment both before (10 min and 5 hr, but not 24 hr) and after (9 min) formalin injection produced significant suppression of phase 2 compared to control. Although pretreatment appeared to be more effective, there was no statistical difference between pre- and posttreatment. Using similar treatment regimes (treatment 9 min after formalin injection and thus between phases 1 and 2), NdCl₃ (0.30 nmol) produced a significant reduction of phase 2 of the formalin test compared to controls (saline injection 9 min after formalin), while verapamil (200 nmol) and agatoxin IVA (0.008 nmol) had no statistically significant effect (Table 3).

Figure 4. Effect of IT injection of L-channel blockers administered 10 min before the formalin test. Each histogram represents the mean ± SEM ($n = 6$ rats) of the total number of flinches during phase 2 (10–60 min). The treatment groups were compared with their appropriate control group (vehicle). Statistical analyses were made by one-way ANOVA followed by Dunnett's test (*, $p < 0.05$).

Figure 5. Effect of different IT administration times of SNX-111 (0.008 nmol) relative to formalin injection. Each histogram represents the mean $\pm$ SEM ($n = 4-6$ rats) of the total number of formalin-evoked flinches during phase 2 (10–60 min). Statistical comparisons were made by one-way ANOVA followed by Dunnett's test (*, $p < 0.05$) to compare drug-treated animals to rats receiving saline as control. Phase 1 for the group that received postinjections is not included in the graph, as it did not include any drug treatment and did not differ from control response.

Hot plate test

The dose of NdCl_3 (0.30 nmol), SNX-111 (0.008 nmol), verapamil (200 nmol), and agatoxin IVA (0.006 nmol) that produced a maximal response in the formalin test was tested for efficacy against a high-threshold stimulus. NdCl_3 and SNX-111 produced a mild increase in the latency to the response to the 52.5°C hot plate, while verapamil and agatoxin IVA were inactive (Fig. 6). The maximal increase (about a 20% increase in latency) was seen 15 min after IT injection and was short lasting, with normal response latencies observed 30 min after injection.

Figure 6. Time-response curve and comparisons of maximal effect on the hot plate test of IT SNX-111 (0.008 nmol), neodymium (0.300 nmol), verapamil (200 nmol), agatoxin IVA (0.006 nmol), and saline. The data are presented as the mean $\pm$ SEM (four rats per line) of the percentage maximal possible effect (%MPE) on the hot plate test. The inset compares the effect at the time for maximal response (15 min after IT injection) using one-way ANOVA followed by Dunnett's test (*, $p < 0.05$) to compare drug-treated animals to controls (saline).

Table 3. Effect of posttreatment (9 min after formalin injection) on the second-phase response of the formalin test

Drug	Dose (IT)	n	Total number flinches (phase 2)
SNX-111 ^a	0.008 nmol	6	85 $\pm$ 13*
Verapamil	200 nmol	5	108 $\pm$ 12 ^{NS}
Neodymium	0.30 nmol	5	57 $\pm$ 10*
Agatoxin IVA	0.006 nmol	6	123 $\pm$ 7 ^{NS}
Saline	10 μ l	4	134 $\pm$ 10

^a Data are also presented in Figure 5 and are shown here for comparison. SNX-111 was tested on one more occasion than the other two agents; thus, the control group in the table does not apply for SNX-111.

* Statistically significant inhibition of the phase 2 response compared to saline-treated rats (ANOVA, Dunnett, $p < 0.05$). NS, not significant.

Discussion

Role of VSCC in nociceptive processing

In our study we found that antagonists for VSCC administered into the lumbar intrathecal space were able to produce a potent antinociceptive effect with respect to the formalin test. The trivalent cations lanthanum and neodymium compete with calcium flux at VSCC of all classes and presumably reduce the charge otherwise carried by that channel and the elevation of intracellular calcium otherwise produced by the opening of VSCC (Weiss, 1974; Lansman, 1990; Reichling and MacDermott, 1991). These agents showed a suppression of both the acute (phase 1) and prolonged (phase 2) behavioral response to formalin injection. The modest effect on acute pain behavior is in accordance with an earlier report showing an antinociceptive effect of IT-injected lanthanum and neodymium on the acute thermal nociception measured by the hot plate test (Reddy and Yaksh, 1980). The development of the second phase of the formalin test has been shown to be dependent on the first phase (Dickenson and Sullivan, 1987). It is thus not surprising that

phase 2 was also reduced by trivalent cations. However, IT injection of neodymium between phase 1 and 2 also significantly suppressed phase 2, suggesting that VSCC are involved in both the induction and the maintenance of facilitated spinal processing.

Assessing the role of different subtypes of VSCC in spinal nociceptive processing showed that IT delivery of N-type selective ω -conopeptides produced a potent dose-dependent suppression of both phase 1 and 2 of the formalin test. The ordering of potency was observed to be SNX-111 > SNX-185 > SNX-239 > SNX-159 = SNX-199. SNX-231 was not active and produced general behavioral effects at higher doses. This ordering of activity agrees with other descriptions of an N-type channel that binds SNX-111 in a reversible manner, and binds GVIA irreversibly (Olivera et al., 1985, 1987). The relative affinities of the peptides in ligand displacement studies versus 125 I-SNX-111 carried out in rat brain synaptosomes were found to be SNX-111 > SNX-199 > SNX-239 = SNX-159 > SNX-185 = SNX-231 > SNX-230. The ω -conopeptides SNX-231 and SNX-230 (synthesized version of the ω -conopeptide MVIIC) have higher affinity for a binding site that is not recognized by SNX-111 or GVIA and characteristics of this site have previously been reported by using MVIIC (Hillyard et al., 1992). In that study, it was shown that MVIIC (SNX-230) blocked P-type channels but that this occurred only at high concentrations (mM). This is in contrast to the picomolar affinity for the high-affinity site of the peptides (Hillyard et al., 1992). However, we found no effect of SNX-231 in the formalin test. This suggests that the calcium channel sites of the non-L/non-N type and possibly non-P type that are characterized by SNX-230 and SNX-231 are not involved in spinal nociceptive processing. Taken together, these observations support the hypothesis that, for this family of the ω -conopeptides, the site of spinal action for antinociception corresponds closely with the site associated with an N-type channel.

The L-type VSCC blockers showed little effect on the formalin test. Nifedipine has previously been demonstrated to produce a moderate suppressant effect on the phase 2 of the formalin test (Coderre and Melzack, 1992). This difference may depend on the formalin test method (behavior scores vs counting of flinches) and the method of IT injection (direct lumbar puncture vs chronic IT catheters). In our study, only verapamil and diltiazem produced a modest, but significant, inhibition. These compounds are HCl salts and readily soluble in saline. In contrast, nifedipine and nimodipine could not be given in equivalent doses. Generally, the dihydropyridines have higher affinities and specificities than the other VSCC antagonists. At higher concentrations, verapamil has been shown to have nonspecific actions and inhibit other neural processes including blockade of sodium and potassium channels (see Miller, 1987). Our data therefore suggest that L-type VSCC have a limited role in nociceptive transmission at the spinal level.

The P-type selective antagonist agatoxin IVA produced only a modest effect on the acute response of formalin injection, while a potent suppression of the facilitated response was observed when the agent was injected prior to formalin injection. In contrast, injection of ω -agatoxin IVA between phases 1 and 2 failed to inhibit the second-phase response. As only a single P-type channel agent was examined, the possibility that agatoxin IVA might be acting at a non-P site cannot be excluded. The results, however, suggest that the P-type channel is involved in events that induce the second-phase response and that this channel has

a different function than the N-type channel at which SNX-111 acts.

In summary, N-type and possibly P-type, but not L-type, channel antagonists appear to exert a selective effect upon nociceptive transmission by an action limited to the spinal cord.

Mechanism of antinociceptive action of spinal N-type channels

The blockade of spinal N-type channels produces a powerful effect upon the prolonged phase of the formalin test and a modest effect upon acute thermal nociception. These models differ in several important respects. After the injection of formalin, A δ /C fiber afferents innervating the paw display a burst of activity, followed by an ongoing, low level of afferent activity (Heapy et al., 1987). The exaggerated response observed during phase 2 is disproportionate to the level of afferent activity present at this time, and it has been proposed that a facilitated state of spinal processing accounts for the magnitude of the second phase (Dickenson and Sullivan, 1987). Systematic examination of the pharmacology of this second phase indicates that agents believed to block peptide release from small primary afferents, such as μ -opioid and α_2 -adrenergic agonists (Go and Yaksh, 1987; Pohl et al., 1989), can prevent the development of phase 2 (Yamamoto and Yaksh, 1992; Abram and Yaksh, 1993). These drugs may block phase 2 by preventing the initiating barrage or block the low level of afferent input driving second-phase behavior. Other investigations of the spinal pharmacology of phase 2 have indicated that spinal pretreatment (pre-phase 1), but not post-treatment (between phase 1 and 2), with NMDA or NK-1 antagonists (Yamamoto and Yaksh, 1991, 1992) only attenuates the second-phase response. This has been taken to suggest that the NMDA site is responsible for initiating, but not sustaining, a period of facilitated processing evoked as a result of the small afferent barrage.

ω -Conopeptides, by an interaction at N-type channels, may act through several processes. Considerable evidence indicates that N-type channels play a principle role in regulating the calcium flux necessary for depolarization-evoked release of neurotransmitters (see Augustine et al., 1987; Smith and Augustine, 1988). Of particular interest is the observation that ω -conopeptides block the evoked release of small primary afferent peptides from spinal cord slices (Maggi et al., 1990; Santicioli et al., 1992). Those observations are consistent with the report that small dorsal root ganglion cells, which give rise to C-fiber afferents, are characterized by having an N-type channel calcium current (Scoggs and Fox, 1992a,b). While appealing, the possible blockade of small afferent-evoked release will not adequately explain all of the actions of the ω -conopeptides. First, if transmitter release from C fibers was uniformly reduced, then one would anticipate a clear hypoalgesia, as is produced by other agents that block spinal release such as morphine. Second, in the formalin test previous studies have shown that posttreatment with either NMDA or NK-1 antagonists will not block phase 2 of the formalin test (Yamamoto and Yaksh, 1991, 1992). This suggests that simple blockade of transmitter release, or interference with calcium flux generated by the depolarization of the membrane secondary to NMDA or NK-1 channel activation, cannot account for the ability of ω -conopeptides to act when given before or after phase 1. More recent studies have shown that the hyperalgesic state in the formalin test, initiated by direct activation of NMDA and NK-1 sites, is mediated in part by the spinal release of prostanoids and by nitric oxide (Malmberg and Yaksh, 1992b, 1993b). These agents are be-

lieved to accentuate dorsal horn neurotransmitter release (Nicol et al., 1992) and appear to represent intermediaries in the hyperalgesic state. It will be interesting to see whether this release evoked by direct NMDA receptor occupancy is blocked by the ω -conopeptides. It should be noted that while there are no data suggesting that ω -conopeptides interact directly with the NMDA receptor, lanthanum, which induces behavioral characteristics similar to those reported for ω -conopeptides, has been shown to block NMDA-evoked currents (Reichling and MacDermott, 1991). This raises the possibility that the trivalent cations may exert a functionally similar, but mechanistically distinct, action.

Mechanism of action of spinal P-type channels

As NMDA receptors are not considered to be postsynaptic to primary afferents (Davies and Watkins, 1983), blockade of glutamate release from interneurons could provide one mechanism whereby N- and P-type channel antagonists act to alter facilitated spinal processing. The role of N-type channels in glutamate release is, however, controversial. In several systems, N-type channels appear to play little role in mediating glutamate release (Pocock and Nicholls, 1992; Turner et al., 1992), while in others, ω -conopeptides result in a significant reduction in depolarization-evoked glutamate release (Terrian et al., 1990; Dickie and Davies, 1992). Such heterogeneity in location, if it occurs within the spinal cord, could account for the selectivity of ω -conopeptides in regulating a state of facilitated processing at the spinal level. In contrast, several recent studies indicate the importance of agatoxin IVA-sensitive P-type channels in regulation of depolarization evoked glutamate release (Pocock and Nicholls, 1992; Turner et al., 1992). Moreover, the characteristics of the spinal action of agatoxin IVA resemble those of spinal NMDA antagonists in that they are selective to the facilitated phase of the formalin test, with no activity on phase 1 and the hot plate test, and do not produce an effect when administered between phase 1 and 2. This suggests that P-type channel activity is involved in the initiation of a facilitated state and is not important for its maintenance. It thus seems possible that the antinociceptive effect produced by agatoxin IVA channel blockade is mediated by reduction of P-type channel-induced glutamate release.

Behavioral effects of spinal VSCC antagonists

High intrathecal doses of the N-type antagonists produced a characteristic shaking behavior as has previously been noted for GVIA (Olivera et al., 1985). The shaking behavior and the effects on motor function occurred at a comparable dose for each of several ω -conopeptides. The similarity of the relative potency inducing this motor effect across agents that vary widely in N-type channel affinity, suggests that there may be a common non-N-type site that regulates this effect on motor horn function. Although not systematically studied, at the highest doses examined, the motor and shaking response were found to be reversible within 24 hr. Doses of the N-type antagonists that produced antinociception in the formalin test had no significant effect on the rats' motor function, as indicated by normal ambulation and reflexes. However, SNX-111, SNX-159, and SNX-199 produced serpentine-like movements in the tail and in a few rats some mild shaking in ED₅₀ dose range. These effects typically appeared 40–60 min after injection. The peak nociceptive response following paw formalin injection is typically seen around 30 min after injection, and in most rats the motor effects were not apparent at this time point. It is thus unlikely

that the tail movements and mild shaking found in some rats account for the suppression of flinching behavior.

In conclusion, these studies indicate a potential role of N-type, but not L-type, VSCC in the induction of facilitated states of large and small afferent processing in the spinal cord. The association of N-type calcium channels with small primary afferent neurotransmitter release may in part account for the selective effect upon spinal nociceptive transmission, but the functional characteristics of these agents suggest a more complicated organization.

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