

Spinal effects of the calmodulin inhibitor calmidazolium on dorsal horn neurons in the rat

Luis Menéndez, Ana Baamonde and Agustín Hidalgo

Laboratory of Pharmacology. Department of Medicine. School of Medicine, C/ Julián Clavería, 6, 33006 Oviedo, Asturias, Spain

Abstract. Drugs able to inhibit calmodulin activation can prevent some consequences of the rise in intracellular calcium. It has recently been shown that intrathecal injection of calmodulin inhibitors induce analgesia in rats. We study here the effect induced by the calmodulin inhibitor, calmidazolium, on the activity of dorsal horn neurons driven by noxious and non-noxious stimuli. Extracellular recordings of convergent ($n = 12$), low-threshold mechanoreceptive ($n = 5$) and proprioceptive ($n = 5$) units were made in the presence of calmidazolium. Calmidazolium (600 μg) reduced the noxious (50°C) heat-evoked responses obtained in convergent neurons. On the contrary, the non-noxious tactile responses obtained in low-threshold mechanoreceptive neurons as well as the joint movement-evoked responses obtained in proprioceptive units remained unmodified. We conclude that calmidazolium can block nociceptive processing in the spinal cord and that this fact can help to explain the analgesic effects that intrathecal W-7 and calmidazolium induce in behavioral tests.

Short
communication

Key words: calmidazolium, calmodulin, pain, spinal cord, nociception

Drugs able to prevent the intracellular calcium rise evoked by excitatory neurotransmitters in nociceptive spinal neurons can inhibit the transmission of nociceptive messages. Accordingly, spinal analgesia can be induced by drugs such as antagonists of NMDA (N-methyl-D-aspartate) receptors, intracellular calcium chelators or antagonists of N- and P- types of voltage-sensitive calcium channels (Cahusac et al. 1984, Coderre and Melzack 1992, Malmberg and Yaksh 1994). At the neural level, electrophysiological studies have shown the ability of NMDA antagonists and selective antagonists of the N- and P- types of voltage-sensitive calcium channels to inhibit the activation of dorsal horn convergent neurons evoked by different types of noxious stimuli (Headley and Grillner 1990, Díaz and Dickenson 1997).

Another pharmacological tool to prevent some intracellular events triggered by calcium is the inhibition of calmodulin, one of the main proteins activated when cytosolic calcium concentration increases (Gnegy 1993). We have recently shown that drugs that act as calmodulin inhibitors, (W-7 and calmidazolium), intrathecally administered, induce analgesic effects both on phasic (tail-flick) and tonic (formalin test) nociceptive tests in rats (Menéndez et al. 1996). Furthermore, the spinal administration of W-7 and calmidazolium attenuates the nociceptive behavior evoked by the intrathecal injection of NMDA or the agonist of the NK1 receptors, septide, without modifying the nociceptive responses induced by AMPA (Menéndez et al. 1997). Since agonists of the NMDA receptors induce an important calcium entry (MacDermott et al. 1986) and the tachykinin-NK1 receptor agonists induce the release of calcium from intracellular stores (Torrens et al. 1995), it seems likely that the inhibition of calmodulin could produce spinal analgesia by preventing these excitatory signals which involve calcium as a second messenger.

In order to ascertain the electrophysiological basis of the spinal analgesia induced by calmodulin inhibitors in behavioral tests, we study here the effects that the calmodulin inhibitor, calmidazolium, induces on the activity of spinal neurons in the rat. Our initial approach was to study the effect of calmidazolium on the noxious-evoked activity of nociceptive convergent neurons. Moreover, in order to investigate if calmodulin inhibition can selectively block nociceptive signals or, alternatively, induce a wider non-specific, neural depression, we have also tested the effects of this drug on the electrophysiological responses evoked in spinal non-noci-

ceptive (low threshold mechanoreceptive and proprioceptive) neurons.

Experiments were performed on 22 male Wistar rats, weighing 250-350 g, from the Animalario de la Universidad de Oviedo (Reg. 33044 13A). Experimental design was in agreement with the rules of the local research ethics committee. Rats were anesthetized with urethan (Merck; 1.5 g/kg; i.p.), a tracheotomy was performed and animals were artificially ventilated. The rate and volume of the respirator (NEMI 131) were adjusted in function of the end-tidal CO₂ that was continuously monitored (Artema MM202) and maintained at 3.5-4 %. Corneal and flexor nociceptive reflexes were controlled as indicators of the depth of the anesthesia and a supplementary dose of urethan was injected if reflexes were observed. Core temperature was maintained at 37 ± 0.5°C by a homeothermic blanket system.

Rats were placed on a stereotaxic frame (Unimecanique) and a laminectomy was performed on L1-L3 vertebrae. The area of maximal responses to a low intensity electrical stimulation from the right hindpaw was selected by moving rostrocaudally a silver macroelectrode on the surface of the dura. At this point, the dura was removed and a stainless steel microelectrode (10-12 MΩ; Phymep) was lowered into the spinal cord using a hydraulic microdrive (David Kopf). Extracellular recordings were amplified, filtered and visualized on an oscilloscope. Mechanical non-noxious stimulation of the ipsilateral hindpaw was used as a search stimulus for single spikes. Once isolated, the unit recordings were fed into a window discriminator (Digitimer) and the frequency of discharge in Hz (number of spikes discharged per second by the neuron) was continuously monitored and recorded on a polygraph (Omniscribe).

Spinal neurons were classified as either convergent, low-threshold mechanoreceptive or proprioceptive units (Wall 1967, Menetrey et al. 1977). Convergent units, also called polymodal, wide dynamic range, multireceptive or Class 2 cells (Le Bars et al. 1986) were driven by both non-noxious stimulus (light touch) and noxious thermal (50°C) or mechanical (pinch) applied to the receptive field of the unit. The noxious stimulus used for testing the effects of calmidazolium on convergent units was the immersion of the hindpaw in 50°C water during 25 s. Those units only excited by low intensity tactile stimulations in which noxious mechanical or thermal stimuli did not induce an additional increase in the response were classified as low-threshold mechanoreceptive or Class 1 cells (Wall 1967, Menetrey et al. 1977).

The light touch of the receptor field of these neurons induces a brief, short lasting, discharge. Thus, in order to obtain a maintained response, the stimulus used to test the activity of these neurons was the application of repetitive light manual touches of the receptive field at a frequency of two times per second (2 Hz) during 25 s. This repetitive stimulation was able to induce an almost continuous and reproducible neural discharge during all the testing period. Finally, neurons excited by the passive movement of an ankle or toe joint but not responding to tactile or to noxious stimuli were classified as proprioceptive or movement detection cells, also called Class 4 cells (Wall 1967, Menetrey et al. 1977). When the joint of the receptive field (either one ankle or a toe) of this type of neuron was submitted to a flexor/extensor movement at a frequency of 2-Hz (two times per second), a maintained response could be obtained. In all cases, only one neuron from each animal was tested.

The frequency of discharge (number of spikes per second) induced by the corresponding stimulus applied during 25 s, was measured every 15 min. When two consecutive similar discharge frequencies were obtained, the last one was considered as the control response and the drug was immediately administered. Calmidazolium, dissolved in 50 μ l of 10% dimethyl sulfoxide (DMSO), was directly applied onto the region of the spinal cord at which the dura was removed for recordings, allowing the direct local diffusion into the dorsal horn mimicking an intrathecal administration (Dickenson and Sullivan 1990, Raigorodsky and Urca 1990). The solvent did not induce any effect by itself and those induced by calmidazolium at each time were calculated as a percentage of the control frequency of discharge (postdrug response (Hz)/control response (Hz) \times 100). Means and standard errors of the mean were calculated at each time and comparisons between control and postdrug responses were made by an ANOVA followed by a Dunnett's t test. The level of significance was set at $P < 0.05$.

The effect of spinal calmidazolium (300 and 600 μ g) on the responses evoked in convergent neurons by noxious thermal stimulation (50°C) was studied in 12 units located at a depth of 922 ± 105 μ m (mean \pm standard error). The immersion of the receptive field in water at 50°C induced a prompt discharge in all the units tested, ranging from 20 to 114 Hz. The responses obtained in the presence of 300 μ g of calmidazolium ($n = 5$) remained unaltered at all times tested (Fig. 1B). In contrast, 600 μ g of calmidazolium reduced the neural firing evoked by

this noxious thermal stimulation in all the units tested (Fig. 1A). The comparison of the means of the inhibitory effects measured in the six units tested with this dose (Fig. 1B) show that significant reductions of nociceptive responses were obtained 45, 60 and 75 min after drug application. In a single convergent neuron, we have tested the effects of calmidazolium (600 μ g) on the neural responses induced by both non-noxious (touch) and noxious (50°C) stimuli. At each time studied (before and 15 and 30 min after the administration of calmidazolium), the stimuli were consecutively applied at a one min in-

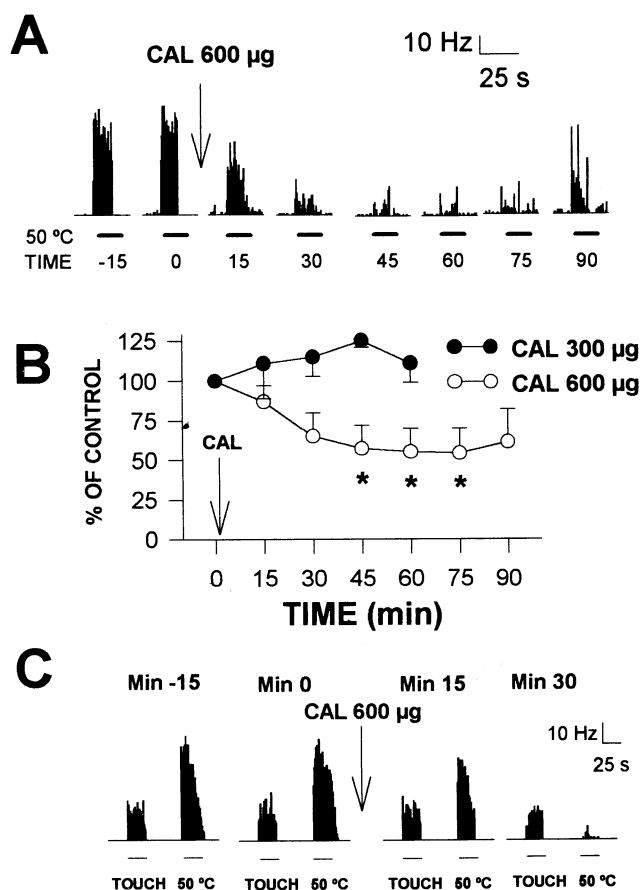


Fig. 1. Effects of calmidazolium (CAL) on the responses obtained in convergent neurons by noxious thermal stimulus (50°C) of the hindpaw. A, example of one unit in which calmidazolium produces a clear-cut inhibitory effect. B, mean values of the percentages of inhibition related to control responses obtained at each time when testing the doses of 300 μ g ($n = 5$) or 600 μ g ($n = 6$), * $P < 0.05$, as compared with the control (predrug). C, response of one neuron to tactile and thermal stimuli consecutively applied with one min interval between them, before and after the administration of calmidazolium (600 μ g).

terval. As shown in Fig. 1C, the nociceptive response was inhibited by calmidazolium 30 min after its administration, while the frequency of responses evoked by light manual touch stimulation of the same unit remained unaltered.

The effects of calmidazolium (600 μ g) were studied in five low-threshold mechanoreceptive neurons that were located at a mean depth of $720 \pm 219 \mu\text{m}$. As Fig. 2A

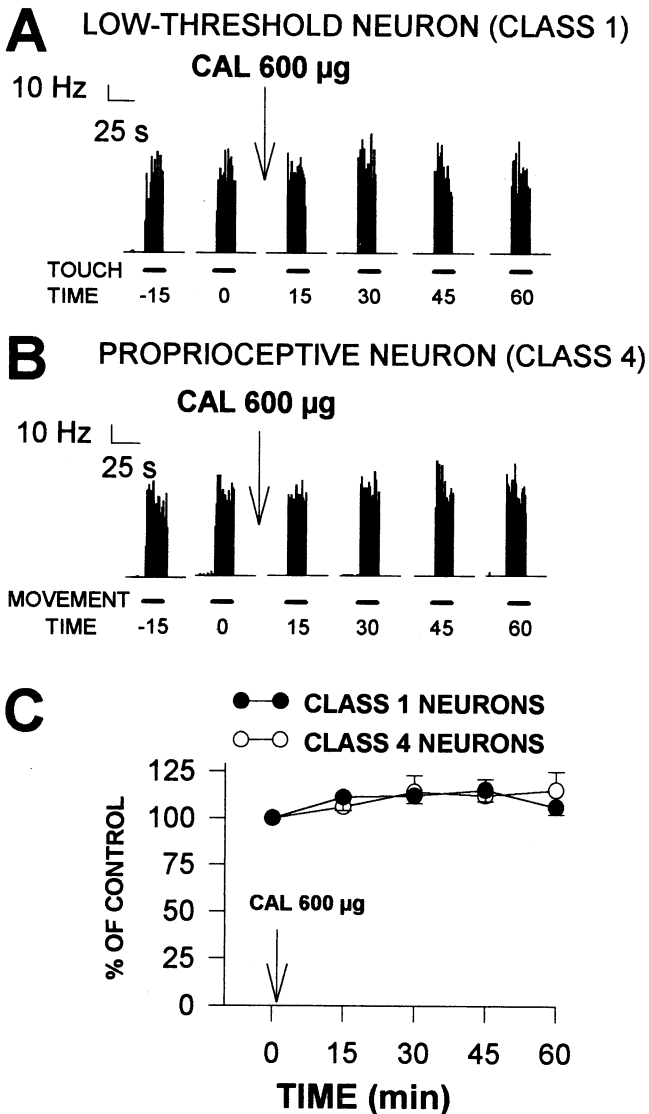


Fig. 2. Effects of calmidazolium (CAL; 600 μ g) on the responses obtained in class 1 (low-threshold mechanoreceptive neurons) and class 4 (proprioceptive) neurons to tactile and movement stimuli of the hindpaw respectively. A and B, representative examples of each type of neuron. C, mean values of the percentages of inhibition related to control responses obtained at each time in low-threshold mechanoreceptive ($n = 5$) and proprioceptive ($n = 5$) neurons.

shows, similar responses can be obtained in low-threshold mechanoreceptive neurons by the light manual touch of the receptive fields at a fixed frequency (2 Hz). The neural response tested 15, 30, 45 and 60 min after the administration of 600 μ g of calmidazolium showed no modification compared to the predrug response (Fig. 2A and C). Also, the effect of calmidazolium was tested on five proprioceptive (movement detection) units responding to joint movements ($940 \pm 114 \mu\text{m}$ of depth). The movement of the joint at a frequency of 2 Hz induced a response that was not modified by calmidazolium (600 μ g) when tested 15, 30, 45 and 60 min after its administration (Fig. 2B and C).

The present results show that the calmodulin inhibitor calmidazolium reduces the responses evoked in convergent neurons when stimulated by noxious thermal stimulation. Thus, nociceptive processing can be inhibited by drugs that modify the cellular pathway activated after an intracellular calcium rise. Furthermore, calmidazolium-mediated inhibition of convergent neurons seems to preferentially occur in nociceptive processing. In fact, in the convergent neuron in which this topic was addressed calmidazolium did not modify the frequency of responses to light manual stimulation whereas it inhibited the nociceptive-evoked response. In addition, the same dose of calmidazolium (600 μ g) failed to inhibit the neural responses observed in low-threshold non-nociceptive and proprioceptive units. These data suggest that calmidazolium is able to block nociceptive transmission without affecting other sensory modalities such as those driven by tactile or proprioceptive stimuli.

Since convergent neurons play a key role in nociceptive processing (Le Bars et al. 1986), the inhibition induced by calmidazolium on their activity can help to explain the analgesic properties of this drug after its intrathecal administration in rats. Our data suggest that spinal analgesia induced by calmidazolium can be obtained without the depression of other sensory modalities, such as touch or proprioception. The different pharmacological modulation of each type of spinal transmission could account for this result. In fact, in both nociceptive and non-nociceptive transmission AMPA receptors are involved (Cumberbatch et al. 1994) whereas in pain spinal processing other receptors able to increase intracellular calcium (i.e. NMDA and Neurokinin NK1 and NK2 receptors) also participate. This possibility is supported by the inhibition that calmidazolium induces on NMDA- and NK1-evoked behavioral responses without affecting those induced through AMPA receptors (Menéndez et

al. 1997). Overall, these results add new insights to the effects of calmidazolium on spinal nociceptive transmission and support the results that calmodulin inhibitors, such as calmidazolium and W-7, induce in behavioral tests of analgesia.

This work was supported by grants from the II Plan Regional de Investigación del Principado de Asturias (PB-SAL97-03).

- Cahusac P.M., Evans R.H., Hill R.G., Rodríguez R.E., Smith D.A. (1984) The behavioural effects of an N-methylaspartate receptor antagonist following application to the lumbar spinal cord of conscious rats. *Neuropharmacology* 23: 719-724.
- Coderre T.J., Melzack R. (1992) The contribution of excitatory aminoacids to central sensitization and persistent nociception after formalin-induced tissue injury. *J. Neurosci.* 12: 3665-3670.
- Cumberbatch M.J., Chizh B.A., Headley P.M. (1994) AMPA receptors have an equal role in spinal nociceptive and non nociceptive transmission. *Neuroreport* 5: 877-880.
- Díaz A., Dickenson T.L. (1997) Blockade of spinal N- and P-type, but not L-type calcium channels inhibits the excitability of rat dorsal horn neurons produced by subcutaneous formalin inflammation. *Pain* 69: 93-100.
- Dickenson A.H., Sullivan A.F. (1990) Differential effects of excitatory aminoacid antagonists on dorsal horn nociceptive neurons in the rat. *Brain Res.* 506: 31-39.
- Gnegy M.E. (1993) Calmodulin in neurotransmitter and hormone action. *Ann. Rev. Pharmacol. Toxicol.* 32: 45-70.
- Headley P.M., Grillner S. (1990) Excitatory amino acids and synaptic transmission: the evidence for a physiological function. *Trends Pharmacol. Sci.* 11: 205-211.

- Le Bars D., Dickenson A.H., Besson J.M., Villanueva L. (1986) Aspects of sensory processing through convergent neurons. In: *Spinal afferent processing*. (Ed. T.L. Yaksh). Plenum Publishing Corporation, New York, p. 467-504.
- MacDermott A.B., Mayer M.L., Westbrook G.L., Smith S.J., Barker J.L. (1986) NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurons. *Nature* 321: 519-522.
- Malmberg A.B., Yaksh T.L. (1994) Voltage-sensitive calcium channels in spinal nociceptive processing: blockade on N- and P-type channels inhibits formalin-induced nociception. *J. Neurosci.* 14: 4882-4890.
- Menéndez L., Hidalgo A., Baamonde A. (1997) Spinal calmodulin inhibitors reduce N-methyl-D-aspartate- and septide-induced nociceptive behavior. *Eur. J. Pharmacol.* 335: 9-14.
- Menéndez L., Pérez-Vallina J.R., Cantabrana B., Hidalgo A., Baamonde A. (1996) Calmodulin inhibitors induce spinal analgesia in rats. *Brain Res.* 731: 114-121.
- Menetrey D., Giesler G.J., Besson J.M. (1977) An analysis of response properties of spinal cord dorsal horn neurons to nonnoxious and noxious stimuli in the spinal rat. *Exp. Brain Res.* 27: 15-33.
- Raidgorodsky G., Urca, G. (1990) Involvement of N-methyl-D-aspartate receptors in nociception and motor control in the spinal cord of the mouse: behavioral, pharmacological and electrophysiological evidence. *Neuroscience* 36: 601-610.
- Torrens Y., Beaujouan J.C., Saffroy M., Glowinski J. (1995) Involvement of septide-sensitive tachykinin receptors in inositol phospholipid hydrolysis in the rat urinary bladder. *Peptides* 16: 587-594.
- Wall P.D. (1967) The laminar organisation of dorsal horn and effects of descending impulses. *J. Physiol.* 188: 403-423.

Received 1 September 1998, accepted 4 January 1999