Nitric oxide synthase and soluble guanylate cyclase are involved in spinal cord wind-up activity of monoarthritic, but not of normal rats

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Abstract

While increasing evidence points to a role for the nitric oxide (NO)/cyclic guanosine 3,5-monophosphate (GMPc) cascade in hyperalgesia and allodynia, participation of the NO/GMPc pathway in synaptic processing in the spinal cord, i.e. wind-up activity, is less clear. We studied the effects of intrathecal administration of *N*ω-nitro-L-arginine methyl ester (L-NAME) and methylene blue, inhibitors of NO synthase and guanylate cyclase respectively, on wind-up activity developed in a C-fiber reflex response paradigm. 5, 10 and 20 μg i.t. of L-NAME or methylene blue did not modify spinal wind-up in normal rats, while a dose-dependent inhibition of wind-up was observed in monoarthritic rats. Results suggest that the NO/GMPc pathway plays a non-significant role in wind-up activity evoked in normal animals, while it may be essential in chronic pain processing.

Keywords: C-fiber reflex; Wind-up; *N*ω-nitro-L-arginine methyl ester; Methylene blue; Pain; Rat

There is considerable evidence implicating *N*-methyl-D-aspartate (NMDA) receptor activation in the mechanisms that underlie both short-term (wind-up) and long-term (hyperalgesia, allodynia) changes in synaptic nociceptive processing in the spinal cord dorsal horn. Many of the long-term effects of NMDA receptor activation appear to be ultimately mediated through stimulation of a nitric (NO)/cyclic guanosine 3,5-monophosphate (GMPc) cascade, as they are blocked by inhibiting the activity of either nitric oxide synthase or soluble guanylate cyclase (see reviews of Meller and Gebhart [6] and Millan [10]). In contrast, the participation of the NO/GMPc pathway in short-term synaptic potentiation in the spinal cord, i.e. wind-up activity, is less clear. In fact, despite that increased wind-up has been reported to be associated to hyperalgesia in several rat models of chronic pain (see reviews of Melzack et al. [8] and Yaksh [13]), thereby suggesting a relationship between the production of NO/GMPc and wind-up activity, it is also clear that wind-up is a synaptic potentiation phenomenon that can be evoked in normal animals [2,5]. Wind-up occurs in dorsal horn neurons in response to repetitive C-fiber inputs of around 0.5–2 Hz, which means that a time period less than 1 s is sufficient for the triggering of the molecular mechanisms underlying wind-up, a period of time hardly compatible with the functioning of a signaling pathway that includes intercellular diffusion of NO. In fact, using kinetic modeling and calculations of the diffusibility and scavenging of NO in aqueous solution based on published data, it has been demonstrated that it may take some seconds for intercellular diffusion of NO at distances ranging diameters of neuronal cell bodies (i.e. 50 μm) [4]. Since, theoretically, there would be a temporal incompatibility between wind-up development and functioning of the NO/GMPc pathway, the present investigation was designed to explore whether wind-up activity in the spinal cord of normal rats is dependent on the NO/GMPc cascade, by studying wind-up sensitiveness to intrathecal administration of both *N*ω-nitro-L-arginine methyl ester (L-NAME) and methylene blue, inhibitors of NO synthase and soluble guanylate cyclase respectively. Further, dependence of wind-up on the NO/GMPc pathway was also studied in a

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monoarthritic rat chronic pain model, where abnormally high activity in the NO/GMPc pathway at the spinal cord dorsal horn is expected to be occurring.

The experiments were carried out on normal and monoarthritic Sprague–Dawley rats weighing 200–300 g. The present studies were performed in accordance to the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals [14]. Monoarthritis was produced by intra-articular injection (0.05 ml) of complete Freund's adjuvant (60 mg of killed *mycobacterium butyricum* suspended in a mixture of 6 ml of paraffin oil, 4 ml of NaCl 0.9% and 1 ml of Tween 80) into the tibio-tarsal joint, as described by Butler et al. [1]. Rats injected with the vehicle utilized to suspend the killed mycobacteria served as normal controls. After 6 weeks, the rats were anesthetized with 1.1 g/kg i.p. urethane and the flexor reflex and wind-up activity were elicited as described previously [5]. Briefly, rectangular electric pulses of sufficient strength for supramaximal activation of C-fibers (15 mA strength, 1 ms duration, 0.2 Hz) were initially applied to the sural nerve receptive field of the left hind limb by means of two stainless steel needles inserted into the skin of toes 4 and 5 (Grass S11 stimulator with Grass CCU 1A constant current unit). The C-fiber evoked reflex activity was recorded from the ipsilateral *biceps femoris* muscle via another pair of stainless steel needles. After amplification (Grass P511 amplifier), the electromyographic responses were fed to a computerized system for on-line digitization, full-wave rectified and integrated into a time-window from 150 to 450 ms after the stimulus (Acer PC with 10 KHz sampling rate A/D converter card). Afterwards, the stimulating current was adjusted to 2-fold the intensity required to obtain threshold C reflex responses. These currents were measured in all normal and monoarthritic rats used, and afterward compared by utilizing a Student's *t*-test for independent samples. Thereafter, a train of twelve stimuli at 0.6 Hz was delivered to the toes in order to develop wind-up activity. At these stimulating parameters, wind-up saturation was obtained at around the 7th or 8th stimulus. All responses were stored in hard disk for later analysis. Least-square regression lines were fitted among experimental points showing only incremental trend (prior to wind-up saturation), discarding the remainder points (Instat 3.0 software). The slopes of the regression lines represent wind-up scores.

The experiments began with the measurement of a basal wind-up prior to saline, L-NAME or methylene blue administration. The effects of intrathecal administration of saline or 5.0, 10.0 and 20.0 μg of L-NAME or methylene blue were investigated in normal and monoarthritic rats. The intrathecal administration of saline, L-NAME or methylene blue was performed in accordance to Mestre et al. [9] by performing a direct transcutaneous intrathecal injection of the drug. Ten and 20 min after injecting the drug or saline, spinal wind-up was evaluated. Results were expressed as means of slopes ± SEM. Drug-induced effects on slopes (wind-up activity) were compared to those obtained under saline by utilizing a one-way ANOVA followed by the Tukey–Kramer post-hoc test for multiple comparisons.

Application of 12 successive constant electric pulses at 0.6 Hz induced wind-up in normal and monoarthritic rats, as revealed by the gradual but remarkable increase of the C reflex gain induced by the repetitive stimuli. Normal animals required 7.48 ± 0.53 mA to evoke threshold C reflex responses, while in monoarthritic rats the current required amounted only to 5.28 ± 0.67 mA ($P < 0.01$, Student's *t*-test). In normal animals, intrathecally administered L-NAME (Fig. 1) or methylene blue (Fig. 2) did not induce a change in the C reflex gain at any of the doses administered (5, 10 or 20 μg), as revealed by the statistical analysis of the slopes of the regression lines. In contrast, 5, 10 or 20 μg of either L-NAME (Fig. 1) or methylene blue (Fig. 2) dose-dependently depressed the wind-up activity in the monoarthritic group, the higher dose utilized being able to inhibit by about 50% the spinal wind-up.

As discussed elsewhere, electric stimulation of the sural nerve receptive field evokes a two-component reflex response in the ipsilateral hind limb flexor muscle, the fast and slow components being elicited by activation of myelinated and unmyelinated afferent fibers, respectively [11]. The slow component is known as the C-fiber evoked reflex, and involves activation of convergent dorsal horn neurons in the spinal cord. The present study confirmed that repetitive stimulation of the C-fiber population of the sural nerve evoked spinal wind-up in normal rats, as evidenced by the increasingly greater C-fiber reflex responses elicited by the 12 successive electric stimuli. The temporal pattern of the wind-up recorded here (result not shown) was completely consistent with earlier descriptions of wind-up by using both C-fiber reflex responses [5] and single-unit recordings from convergent dorsal horn cells [2].

Experimental series utilizing i.t. administration of different doses of L-NAME or methylene blue, at doses similar to those required to inhibit hyperalgesia in a neuropathic model of chronic pain [7], showed that these chemicals induced no changes in spinal wind-up activity of

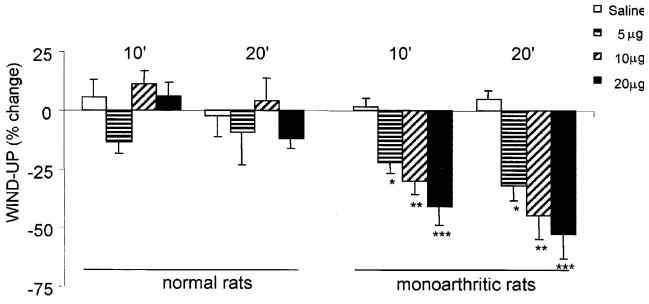

Fig. 1. Effect of 5, 10 and 20 μg i.t. of L-NAME on spinal cord wind-up activity in normal and monoarthritic rats. Values are expressed as means ± SEM of the percent changes, 10 and 20 min after L-NAME. Each bar represents the mean obtained from of six rats. Significant changes are denoted by asterisks: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, when L-NAME series were compared to the respective saline series (one-way ANOVA followed by Tukey–Kramer test).

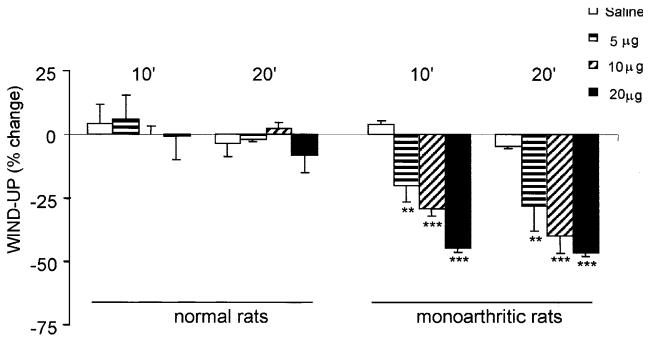

Fig. 2. Effect of 5, 10 and 20 µg i.t. of methylene blue on spinal cord wind-up activity in normal and monoarthritic rats. Values are expressed as means ± SEM of the percent changes, 10 and 20 min after L-NAME. Each bar represents the mean obtained from of six rats. Significant changes are denoted by asterisks: $^{**}P < 0.01$, $^{***}P < 0.001$, when methylene blue series were compared to the respective saline series (one-way ANOVA followed by Tukey–Kramer test).

normal rats, suggesting that the NO/GMPc pathway plays a non-significant role in electric stimulus driven wind-up activity evoked in normal animals. This result suggests that an alternative mechanism should be responsible for short-term synaptic potentiation in the spinal cord of these animals. In this respect, it has been reported that entry of calcium through NMDA receptor ion channels stimulates a calmodulin-dependent protein kinase, which is able to phosphorilate NMDA receptors, thereby allowing entry of extra calcium leading to synaptic potentiation, i.e. wind-up development [3]. Whether this pathway could constitute the sole mechanism underlying wind-up phenomena in spinal cord of normal animals remains to be elucidated, since other protein kinases also may contribute to the increased neuronal responsiveness to glutamate in the central nervous system [12].

In contrast, only 5 µg of intrathecally administered of either L-NAME or methylene blue were enough to depress spinal wind-up in monoarthritic rats, indicating an important role for the NO/GMPc cascade in wind-up modulation under a time-sustained nociceptive input. This effect is dose-dependent, and is in agreement with the significant lower stimulating current required to evoke threshold C reflex responses reported here, as well as with the proposed role of the NO/GMPc pathway in the establishment and maintenance of central hyperalgesia that occurs in conditions of chronic pain and the related increase of wind-up activity associated to the hyperalgesic status [8].

In conclusion, the present results indicate that i.t. administration of L-NAME or methylene blue did not modify wind-up activity in normal rats, while depressing wind-up in the monoarthritic ones, suggesting a role for the NO/GMPc pathway in chronic pain complaints but not in spinal cord acute pain processing.

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