European Journal of Neuroscience, Vol. 19, pp. 103–111, 2004

Pre- and postsynaptic contributions of voltage-dependent Ca^{2+} channels to nociceptive transmission in rat spinal lamina I neurons

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Keywords: A-fibre, C-fibre, NMDA receptor, Sprague–Dawley rats, synapse

Abstract

Activation of voltage-dependent Ca^{2+} channels (VDCCs) is critical for neurotransmitter release, neuronal excitability and postsynaptic Ca^{2+} signalling. Antagonists of VDCCs can be antinociceptive in different animal pain models. Neurons in lamina I of the spinal dorsal horn play a pivotal role in the processing of pain-related information, but the role of VDCCs to the activity-dependent Ca^{2+} increase in lamina I neurons and to the synaptic transmission between nociceptive afferents and second order neurons in lamina I is not known. This has now been investigated in a lumbar spinal cord slice preparation from young Sprague–Dawley rats. Microfluorometric Ca^{2+} measurements with fura-2 have been used to analyse the Ca^{2+} increase in lamina I neurons after depolarization of the cells, resulting in a distinct and transient increase of the cytosolic Ca^{2+} concentration. This Ca^{2+} peak was reduced by the T-type channel blocker, Ni^{2+}, by the L-type channel blockers, nifedipine and verapamil, and by the N-type channel blocker, ω-conotoxin GVIA. The P/Q-type channel antagonist, ω-agatoxin TK, had no effect on postsynaptic $[Ca^{2+}]_i$. The NMDA receptor channel blocker D-AP5 reduced the Ca^{2+} peak, whereas the AMPA receptor channel blocker CNQX had no effect. Postsynaptic currents, monosynaptically evoked by electrical stimulation of the attached dorsal roots with C-fibre and Aδ-fibre intensity, respectively, were reduced by N-type channel blocker ω-conotoxin GVIA and to a much lesser extent, by P/Q-type channel antagonist ω-agatoxin TK, and the L-type channel blockers verapamil, respectively. No difference was found between unidentified neurons and neurons projecting to the periaqueductal grey matter. This is the first quantitative description of the relative contribution of voltage-dependent Ca^{2+} channels to the synaptic transmission in lamina I of the spinal dorsal horn, which is essential in the processing of pain-related information in the central nervous system.

Introduction

Intracellular Ca^{2+} ions control a number of neuronal functions, including transmitter release, membrane excitability, gene transcription and synaptic plasticity (Berridge *et al*., 2000). Transient increase of cytoplasmic Ca^{2+} concentration depends on Ca^{2+} influx from extracellular space through membrane channels and/or Ca^{2+} release from intracellular stores. Ca^{2+} influx occurs by three main pathways: voltage-dependent Ca^{2+} channels (VDCCs), receptor-operated Ca^{2+} channels (ROCCs), and store-operated Ca^{2+} channels (SOCCs) (Berridge *et al*., 2000). Upon membrane depolarization, opening of VDCCs gives rapid rise in intracellular Ca^{2+} concentration. Based on their voltage dependency, these Ca^{2+} currents have been traditionally divided into two groups: high-voltage activated (HVA) currents and low-voltage activated (LVA) or T-type currents. HVA channels are further classified by their pharmacological properties as L-, N-, P/Q- and R-type Ca^{2+} channels (Hofmann *et al*., 1999; Catterall, 2000).

Immunocytochemical studies and electrophysiological data have identified all subtypes of VDCCs in the dorsal horn of the spinal cord (Huang, 1989; Ryu & Randić, 1990; Westenbroek *et al*., 1998; Saegusa *et al*., 2000; Cizkova *et al*., 2002). The contribution of VDCCs to nociception, hyperalgesia and allodynia has recently attracted considerable interest (see a review for HVA Ca^{2+} channels by Vanegas & Schaible, 2000). Behavioural data from a variety of animal models indicate a physiological and/or pathophysiological role in acute and persistent pain states at least for some of these channels. Blockade of N-type channels by intrathecal application of specific blockers, or ablation of N-type channels by knocking-out the gene encoding the pore-forming α_{1B} subunit of the N-type channel, caused antinociception in acute pain models and prevented or attenuated hyperalgesia, allodynia and responses in the formalin test (Malmberg & Yaksh, 1994; Diaz & Dickenson, 1997; Vanegas & Schaible, 2000; Matthews & Dickenson, 2001b; Saegusa *et al*., 2002; Smith *et al*., 2002). For P/Q-type channels, a role in spinal nociception is established and a contribution to the second phase of the formalin response has been shown (Malmberg & Yaksh, 1994; Diaz & Dickenson, 1997; Matthews & Dickenson, 2001b; Ogasawara *et al*., 2001). Ablation of R-type channels also reduced nociceptive behaviour in the second phase of the formalin response (Saegusa *et al*., 2000). The cellular mechanisms underlying these behavioural effects remain elusive. There are hints for the synaptic distribution of VDCCs in the superficial dorsal horn (Westenbroek *et al*., 1998), but the situation for a given synapse is unclear. N- and P/Q-type channels are enriched in presynaptic nerve terminals of the superficial laminae I and II, suggesting a role for neurotransmitter release from fine primary afferents. L-type channels

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Received 12 August 2003, revised 24 September 2003, accepted 7 October 2003

doi:10.1046/j.1460-9568.2003.03083.x

seem to be present preferentially in postsynaptic membranes being more important for regulation of Ca^{2+} dependent intracellular events rather than for fast synaptic transmission. For T-type channels we recently found an essential role for the induction of long-term potentiation (LTP) at synapses between primary afferent C-fibres and lamina I projection neurons (Ikeda *et al*., 2003).

Neurons in lamina I function as the main output source of the superficial spinal dorsal horn to supraspinal sites (Craig, 2000) and receive nociceptive input from small caliber myelinated (Aδ) and unmyelinated (C) primary afferents. Recently, it has been shown that a subset of spinal lamina I neurons plays a pivotal role in the generation and maintenance of thermal hyperalgesia and mechanical allodynia in persistent neuropathic and inflammatory pain states (Nichols *et al*., 1999). We demonstrated an activity-dependent long-term potentiation of synaptic strength between nociceptive afferents and these lamina I neurons (Ikeda *et al*., 2003), which constitutes a cellular model for centrally mediated hyperalgesia (Sandkühler, 2000).

To evaluate the contribution of VDCCs to nociceptive transmission in lamina I of the spinal cord, we determined the effects of selective channel blockers on: (i) the postsynaptic Ca^{2+} influx into lamina I neurons, activated by well-tuned current injections, and (ii) the synaptic strength between primary afferent Aδ- and C-fibres and spinal lamina I neurons. Additionally, we tested the contribution of receptor-operated Ca^{2+} channels of the *N*-methyl-D-aspartate (NMDA)-type and the AMPA-type, respectively, to the activity-dependent postsynaptic $[Ca^{2+}]_i$ rise in lamina I neurons.

Materials and methods

Labelling of spinal projection neurons

Young Sprague–Dawley rats of both sexes (18- to 30-day-old) were anaesthetized with a mixture of ketamine (75 mg/kg i.p.) and xylazine (7.5 mg/kg i.p.) and placed in a stereotaxic apparatus. A hole was drilled through the skull in order to allow insertion of a 500-nL Hamilton syringe needle. The animals received a single injection of 100 nL of 1.25% 1,1-didodecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate ($DiIC_{12}$; Molecular Probes, Eugene, Oregon, USA) into the right periaqueductal grey matter (PAG) according to the atlas of Paxinos & Watson (1982) (Fig. 3A). After a 3-day survival period spinal cord slices were obtained as described below.

Slice preparation

Under deep ether anaesthesia, spinal cord was exposed by laminectomy. The segments of the lumbosacral spinal cord (L4–S1) were excised. Transverse slices with dorsal root (8–15 mm) attached were cut at 400–600 μm thickness using a vibrating microslicer (DTK-1000, Dosaka EM, Kyoto, Japan) and incubated at 33 °C for at least half an hour. The incubating solution was gassed with carbogen (5% CO_2, 95% O_2) and consisted of (in mM): NaCl, 95; KCl, 1.8; KH_2PO_4, 1.2; $CaCl_2$, 0.5; $MgSO_4$, 7; $NaHCO_3$, 26; glucose, 15; sucrose, 50; pH was 7.4, osmolarity 310–320 mOsm. A single slice was then transferred to a recording chamber (volume 1.0 mL) and was continuously perfused at a rate of 3–4 mL/min with a recording solution (gassed with carbogen), which was similar to the incubation solution except for (in mM): NaCl, 127; $CaCl_2$, 2.4; $MgSO_4$, 1.3; sucrose 0. All experiments were performed at room temperature (20–25 °C).

In all experiments, measurements were made from one neuron per slice.

All animal experiments were performed in accordance with European Communities Council directives (86/609/EEC) and were approved by the Austrian Federal Ministry for Education, Science and Culture.

Electrophysiological recordings

When visualized with Dodt-infrared optics using a × 40, 0.80 water-immersion objective on an Olympus BX50WI upright microscope (Olympus, Japan) equipped with a video camera system (PCO, Kelheim, Germany), neurons in lamina I of the spinal dorsal horn typically showed a mediolateral orientation of their proximal axon/dendrites. In contrast, the smaller lamina II neurons displayed a mainly dorsoventral orientation of proximal axon/dendrites. To detect labelled projecting neurons and nonlabelled cells, slices were illuminated with a monochromator (TILL Photonics, Gräfelfing, Germany).

Standard whole cell patch-clamp recordings under voltage-clamp (holding potential −70 mV, if not otherwise stated) and current clamp conditions were performed. Only neurons with a resting membrane potential more negative than −50 mV, measured immediately after break-through, were selected for further recording. Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) on a horizontal puller (P-87, Sutter Instruments, Novato, CA, USA). For measuring membrane currents and for Ca^{2+} imaging, pipettes were filled with a solution composed of (in mM): K-gluconate, 120; KCl, 20; $MgCl_2$, 2; HEPES, 10; Na_2ATP, 2; NaGTP, 0.5; fura-2 pentapotassium salt, 0.1 (Molecular Probes); pH 7.28 adjusted with KOH; measured osmolarity ≈300 mOsm, resulting in a tip resistance between 3 and 5 MΩ. For measuring excitatory postsynaptic currents (EPSCs) fura-2 was replaced by 0.5 mM EGTA, and lidocaine *N*-ethyl bromide (QX-314, 5 mM, Sigma) was added to the pipette solution to prevent discharges of APs.

To measure primary afferent-evoked EPSCs in lamina I neurons, dorsal root was stimulated through a suction electrode with an isolated current stimulator (A320, World Precision Instruments, Sarasota, FL, USA). After the threshold for eliciting an EPSC was determined, test pulses of 0.1 ms were given at intervals of 15 s (for Aδ-fibre stimulation) and 30 s (for stimulation of C-fibres), respectively. Stimulation intensity was adjusted to supramaximal values (0.3–3 mA). Afferent input was classified as Aδ-fibre-evoked when calculated conduction velocity ranged between 2.5 and 15 m/s and as C-fibre-evoked for conduction velocities below 2 m/s. Monosynaptic input was identified by the absence of failures in response to ten stimuli given at 10 Hz (for Aδ-fibre input) or at 1 Hz (for C-fibre input) stimulation of dorsal roots and low jitter in response latencies.

Recordings were made with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA, USA) at a sampling rate of 10 kHz using a low-pass bessel filter of 2 kHz. The software package pCLAMP 8 was used for data acquisition and subsequent off-line analysis.

Measurement of $[Ca^{2+}]_i$

Fluorometric measurements of free cytosolic Ca^{2+} concentrations were performed by loading the lamina I neurons for at least 10 min with the fluorescent dye fura-2 via the patch pipette. Cells were illuminated with a monochromator and images were captured at 5 Hz with a cooled CCD camera (TILLvisION Imaging system, TILL Photonics). Consecutive paired exposures to 340 and 380 nm were used to achieve digital fluorescence images. Calculations were made off-line. For measuring, the region of interest was placed in the centre of the cells (Fig. 1A). Cytosolic Ca^{2+} was calculated by ratiometric (F_{340}/F_{380}) fluorescence and no conversion to concentrations was made.

Experimental protocol

Ca^{2+} influx through VDCCs was induced by depolarizing current injections of 5 ms duration at a frequency of 40 Hz for 1 s in current-clamp mode. Applied currents were adjusted between 0.1 and 0.5 nA to elicit one action potential per pulse (Fig. 1A). First burst depolarization

was performed after 10 min loading time with fura-2 (first signal). The second Ca^{2+} signal was obtained 10 min later (Fig. 1B), and the resulting Ca^{2+} peaks were compared (expressed in percentage of the first signal). Drugs were washed in immediately after the first series of depolarizations.

Effects of Ca^{2+} channel blockers on synaptic strength between afferent fibres and lamina I neurons were quantified by the peak amplitudes of evoked EPSCs. Control values were obtained by comparing the mean amplitude of two series of ten test responses, respectively, recorded at intervals of 10 min (expressed in percentage of the first mean amplitude). Drugs were applied after the first series of ten test pulses and measuring the mean amplitude of ten consecutive responses 10 min later assessed significant changes from control (Fig. 3B).

NMDA-induced responses were studied in Mg^{2+}-free recording solution including 10 μM glycine and 0.5 μM TTX. The agonist NMDA (100 μM) was superfused for 10 s.

Application of drugs

All drugs were applied at known concentrations via the perfusing salt buffer, gassed with carbogen. In experiments using the Ca^{2+} channel toxins, all solutions contained 0.1 mg/mL cytochrome *c* (Sigma, Deisenhofen, Germany) to prevent unspecific peptide binding to tubing. Drugs and their sources were as follows: nifedipine (50 μM; Sigma), verapamil (50 μM; Sigma), ω-conotoxin GVIA (1 μM; Tocris, Bristol, UK), ω-conotoxin MVIIC (1.5 μM; Tocris), ω-agatoxin TK (100 nM; Alomone Laboratories, Israel), *N*-methyl-D-aspartate (NMDA, 100 μM; Sigma), glycine (10 μM; Sigma), D-2-amino-5-phosphovaleric acid (D-AP5, 50 μM, 100 μM; Alexis, Grünstadt, Germany), MK-801 maleate (10 μM; Tocris), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM; Alexis), tetrodotoxin (TTX, 0.5 μM; Alexis), cadmium(II)chloride ($CdCl_2$, 200 μM; Sigma), nickel(II)chloride ($NiCl_2$, 100 μM; Sigma). Nifedipine was dissolved in dimethylsulphoxide (DMSO; Sigma; maximal final concentration 0.25%, v/v) as stock solution. The solvent for TTX was acidic buffer (pH 4.8). All other drugs and metallic compounds were prepared as aqueous stock solutions.

Data analysis

Analysis of the data was performed using SigmaStat 2.03 (SPSS GmbH, Erkrath, Germany). Values are given as means ± one standard error of the mean (SEM). Unless stated otherwise, unpaired *t*-test was used for statistical comparison ($P < 0.05$ was considered to be statistically significant).

Results

Ca^{2+} influx through VDCCs

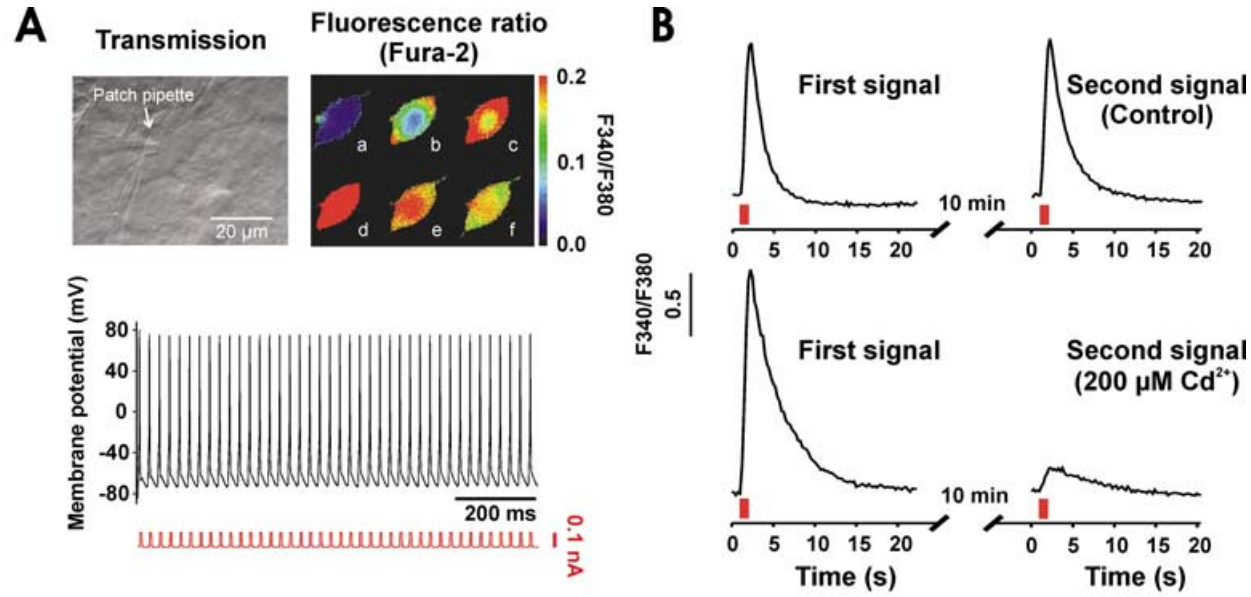
For each neuron 40 depolarizing current pulses in 1 s were finely tuned to cause discharges of an equal number of APs (Fig. 1A). This led to a transient increase of the cytosolic Ca^{2+} concentration in lamina I neurons to 234 ± 7% of baseline values (expressed as the ratio between the intensity at 340 nm and at 380 nm excitation wave length, $n = 95$). $[Ca^{2+}]_i$ returned to baseline within 10–15 s (Fig. 1B). For control, a second burst depolarization was applied 10 min after the first signal (Fig. 1B, upper trace). The resulting second Ca^{2+} peak was not significantly different from the first one (−1 ± 3%, $n = 15$; Fig. 1C). Removing Ca^{2+} ions from the bathing solution totally suppressed the depolarization-induced Ca^{2+} increase by 99 ± 0.2% ($n = 4$; $P < 0.01$, Mann–Whitney rank test) indicating that no Ca^{2+} is released from intracellular stores without Ca^{2+} influx from the extracellular space under these conditions. To measure the contribution of all voltage-dependent Ca^{2+} channels to the activity-dependent Ca^{2+} increase in lamina I neurons we applied the divalent cation Cd^{2+} at a concentration (200 μM) known to inhibit both, high- and low-voltage-activated Ca^{2+} channels. This treatment reduced the Ca^{2+} peak by 83 ± 0.2% ($n = 5$; $P < 0.001$; Fig. 1B and C). Low concentrations of the divalent cation Ni^{2+} (100 μM), known to block low-voltage-activated Ca^{2+} channels, reduced the Ca^{2+} peak by 20 ± 12%. The effect was very variable, but significant compared to control ($n = 6$; $P < 0.05$). Co-administration of Cd^{2+} and Ni^{2+} was not more effective than Cd^{2+} alone (reduction by 85 ± 5%, $n = 4$; $P < 0.01$; Fig. 1C). Superfusing the neurons with divalent cations for 10 min may not be sufficient to get the full blocking effect. We enhanced the concentration of Cd^{2+} and Ni^{2+} and/or the time for wash in, but both manoeuvres dramatically reduced the stability of the seals. Therefore, we pretreated the slices with the incubating solution containing Cd^{2+}, Ni^{2+}, D-AP5 and CNQX for at least 20 min before patch-clamping the neurons. This led to an enhanced baseline fluorescent ratio (increase by 400%) and to a complete suppression of the depolarization-induced Ca^{2+} peak ($n = 6$, data not shown).

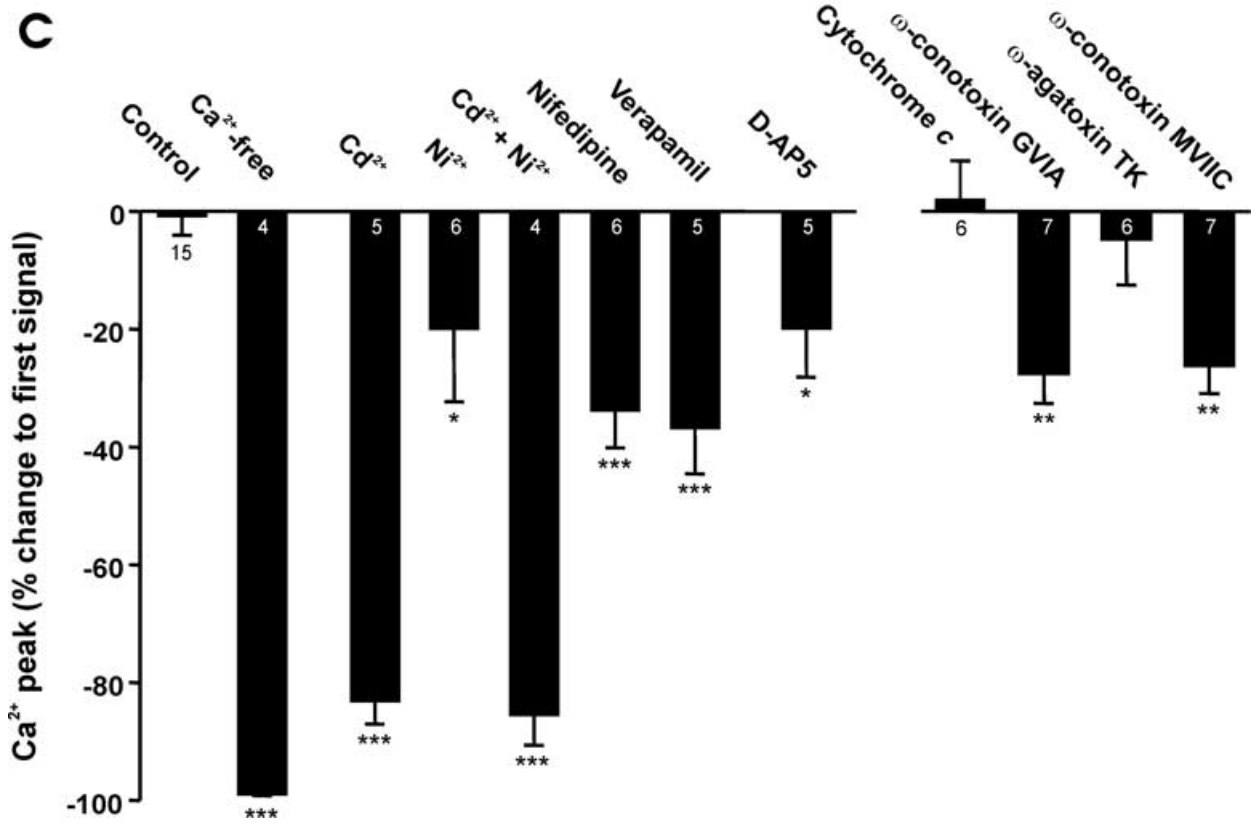
To determine the contribution of the different subtypes of high-voltage-activated Ca^{2+} channels we applied specific antagonists. The L-type channel blockers nifedipine (50 μM) or verapamil (50 μM) decreased the depolarization-induced Ca^{2+} rise in lamina I neurons by 34 ± 6% ($n = 6$; $P < 0.001$) and by 37 ± 8% ($n = 5$; $P < 0.001$), respectively (Fig. 1C). As peptide toxin blockers, specific for N-type and P/Q-type channels, have to be coadministered with cytochrome *c* to prevent unspecific peptide binding to tubing, we performed the appropriate control experiments. Cytochrome *c* (0.1 mg/mL) in the bathing solution did not change the second Ca^{2+} signal significantly, compared to the first one (+2.1 ± 6.3%, $n = 6$; Fig. 1C). Blocking N-type VDCCs by the specific inhibitor ω-conotoxin GVIA (1 μM) reduced the depolarization-induced Ca^{2+} peak by 28 ± 5% ($n = 7$; $P < 0.01$; Fig. 1C). In contrast, the P/Q-type channel blocker ω-agatoxin TK (100 nM) had no effect on the cytosolic Ca^{2+} concentration following membrane depolarization (−5 ± 8%, $n = 6$; Fig. 1C). Bath application of ω-conotoxin MVIIC (1.5 μM) depressed the $[Ca^{2+}]_i$ rise by 26 ± 5% ($n = 7$; $P < 0.01$; Fig. 1C). As this toxin inhibits both N-type and P/Q-type VDCCs and the quantitative effect was identical to the ω-conotoxin GVIA application, we conclude, that P/Q-type channels do not contribute significantly to depolarization-induced Ca^{2+} rise in spinal lamina I neurons of the rat.

Ca^{2+} influx through ionotropic glutamate receptor channels

Blocking all VDCCs by appropriate antagonists did not suppress completely the activity-dependent cytosolic Ca^{2+} increase in lamina I neurons. It is likely that the neurotransmitter glutamate is available in the spinal cord slice preparation in sufficient concentrations to potentially activate glutamate receptor channels. We tested the hypothesis, that the concomitant depolarization by current injections removes the Mg^{2+} block from NMDA receptor channels resulting in an influx of Ca^{2+} into the neurons through these channels. Bath application of the NMDA-receptor antagonist D-AP5 (50 μM) decreased the Ca^{2+} peak by 19.9 ± 8.3% ($n = 5$; $P < 0.5$; Fig. 1C). Co-administration of Cd^{2+} (200 μM), Ni^{2+} (100 μM) and D-AP5 (50 μM) reduced the depolarization-induced Ca^{2+} rise by 87 ± 2.3% ($n = 5$; $P < 0.001$; data not shown). As a subpopulation of AMPA-receptors is Ca^{2+} permeable (reviewed in Hollmann & Heinemann, 1994) and has been shown to be synaptically localized in lamina I of the dorsal horn (Engelman *et al.*, 1999), we added the specific inhibitor CNQX (20 μM) to the superfusate. This treatment had no effect on the Ca^{2+} signal ($n = 6$, data not shown).

To measure the maximal influx of Ca^{2+} ions into lamina I neurons via NMDA receptors, the slices were superfused with Mg^{2+}-free solution (to remove the voltage-dependent Mg^{2+} block) containing 10 μM glycine (as a coagonist) and 0.5 μM TTX (to inhibit action potential firing). Bath application of the agonist NMDA (100 μM) for 10 s induced a large transient inward current and a concomitant increase in the intracellular Ca^{2+} concentration (Fig. 2A). The mean inward current was -855 ± 222 pA and the mean Ca^{2+} signal reached $252 \pm 39\%$ of the baseline ratio ($n = 7$; Fig. 2B). NMDA-induced inward current and simultaneous Ca^{2+} increase could be specifically blocked by pretreating the slices with the competitive NMDA receptor antagonist D-AP5 (100 μM; -46 ± 23 pA; $106 \pm 2\%$ of the baseline ratio; $n = 3$; $P < 0.05$, Mann–Whitney rank test) and the noncompetitive antagonist MK-801 (10 μM; -33 ± 6 pA; $105 \pm 2\%$ of the





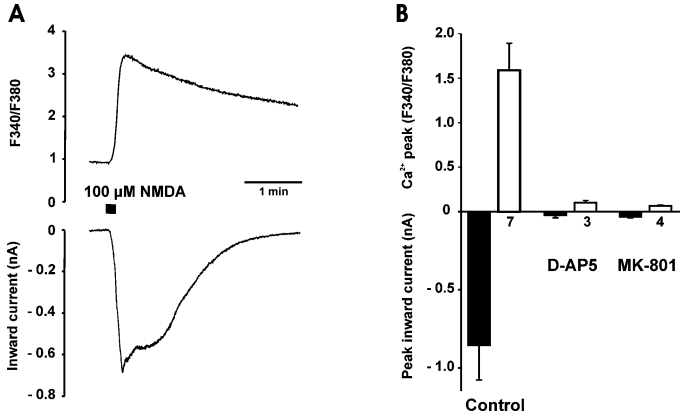


FIG. 2. NMDA-induced inward current and Ca^{2+} increase in lamina I neurons. (A) Simultaneous recordings of somatic fluorescent ratio changes (upper trace) and membrane current (bottom trace; $V_{Hold} = -65$ mV) from one experiment. NMDA (100 µM) was superfused for 10 s (black square) in a nominally Mg^{2+}-free solution containing 10 µM glycine. (B) Bar graph representation of mean peak amplitudes of NMDA-induced Ca^{2+} rises (open bars) and membrane inward currents (black bars), respectively, under control conditions and in the presence of 100 µM D-AP5 or 10 µM MK-801. Numbers of experiments are indicated (one neuron per slice).

baseline ratio; $n = 4$; $P < 0.01$, Mann–Whitney rank test), respectively (Fig. 2B).

Role of VDCCs for synaptic transmission in lamina I

Whole-cell patch-clamp recordings were performed from 75 lamina I neurons that received monosynaptic input either from primary afferent C-fibres ($n = 43$) or from Aδ-fibres ($n = 32$), respectively. The mean amplitude of two series of ten consecutive evoked EPSCs did not change significantly at intervals of 10 min (C-fibre-evoked, $+3 \pm 3\%$ of the first series, $n = 8$; Aδ-fibre-evoked, $-2 \pm 3\%$ of the first series, $n = 6$;Fig. 3C). Bath application of the unspecific Ca^{2+} channel blocker Cd^{2+} (200 µM) virtually abolished the synaptic transmission between nociceptive afferents and lamina I neurons (C-fibre-evoked EPSC amplitudes were reduced by $96 \pm 2\%$, $n = 2$; Aδ-fibre-evoked EPSC amplitudes were reduced by $97 \pm 1\%$, $n = 3$; Fig. 3C). C-fibre-evoked EPSCs were reduced during bath application of the N-type channel blocker ω-conotoxin GVIA (1 µM) by $62 \pm 6\%$ ($n = 8$; $P < 0.001$ compared to control), during application of the P/Q-type channel blocker ω-agatoxin TK (100 nM) by $24 \pm 9\%$ ($n = 7$; $P < 0.01$; Fig. 3C) and during superfusion with L-type channel blocker verapamil (50 µM) by $9 \pm 4\%$ ($n = 6$; $P < 0.05$; Fig. 3C). Aδ-fibre-evoked EPSCs were inhibited by bath application of ω-conotoxin GVIA by $79 \pm 6\%$ ($n = 5$; $P < 0.001$) and of ω-agatoxin TK by $15 \pm 3\%$ ($n = 6$; $P < 0.05$; Fig. 3C). The L-type channel blocker verapamil (50 µM) also reduced the Aδ-fibre-evoked EPSCs significantly by $12 \pm 3\%$ ($n = 6$; $P < 0.05$; Fig. 3C). To test whether there is a difference in the contribution of VDCCs on synaptic transmission between primary afferents and unidentified lamina I neurons and neurons projecting to supraspinal sites, respectively, we recorded from neurons retrogradely labelled from the PAG (Fig. 3A). No significant difference between unidentified and projection neurons concerning the effect of toxins on evoked EPSCs were observed (Fig. 3C). ω-conotoxin GVIA reduced C-fibre-evoked EPSCs in spino-PAG neurons by $53 \pm 11\%$ ($n = 5$; $P < 0.001$ compared to control) and on Aδ-fibre-evoked EPSCs by $75 \pm 9\%$ ($n = 2$; $P < 0.001$; Fig. 3C). In contrast, ω-agatoxin TK had no significant effect on C-fibre- and on Aδ-fibre-evoked EPSCs in spino-PAG neurons, respectively ($-16 \pm 9\%$, $n = 7$, and $-8 \pm 5\%$, $n = 4$; Fig. 3C).

While inhibition of P/Q-type channels had minor effects on monosynaptic transmission between nociceptive afferents and lamina I neurons, polysynaptically evoked responses were affected significantly stronger. Bath application of ω-agatoxin TK reduced C-fibre-evoked polysynaptic EPSCs by $57 \pm 7\%$ ($n = 11$; $P < 0.001$) and Aδ-fibre-evoked polysynaptic EPSCs by $56 \pm 8\%$ ($n = 5$; $P < 0.001$; Fig. 3C).

Discussion

Here, we provide a quantitative evaluation of the relative pre- and postsynaptic contributions of voltage-dependent Ca^{2+} channels to key functions of the first synapses in nociceptive pathways.

Methodological considerations

We activated the cells by 40 current injections to elicit an equal number of action potentials. Continuous depolarization was not used, because lamina I neurons respond to prolonged depolarizing pulses with different action potential firing patterns (Ruscheweyh & Sandkühler, 2002), making comparisons of activity-dependent changes of the cytosolic Ca^{2+} concentration impossible. The presently used activation pattern caused reproducible Ca^{2+} signals.

A wash in period of 10 min for the unspecific Ca^{2+} channel blocker Cd^{2+} alone and in combination with Ni^{2+} was not sufficient to completely suppress the depolarization-induced Ca^{2+} peak. As increasing the concentration and the exposure time of Cd^{2+}, respectively, led to loss of the cell, we preincubated the slices with the divalent cations before patch-clamping the neurons. This treatment abolished the cytosolic Ca^{2+} rise, but was accompanied by a strong increase of the resting Ca^{2+} concentration. One possible explanation for this Ca^{2+} increase may be the inhibition of the Na^+/Ca^{2+} exchanger by Cd^{2+} and Ni^{2+} (Iwamoto & Shigekawa, 1998), leading to an accumulation of Ca^{2+} ions in the cytosol with prolonged incubation period. Therefore, it remains unclear, whether there is a Cd^{2+} and Ni^{2+}-insensitive pathway for the activity-dependent Ca^{2+} influx into lamina I neurons, which is blocked by high intracellular Ca^{2+} levels. It is unlikely, that an insufficient wash-in time was the reason for the

FIG. 1. Effects of different Ca^{2+} channel and NMDA receptor channel blockers on depolarization-induced Ca^{2+} influx into spinal lamina I neurons. (A) Typical example of the depolarization protocol and the resulting Ca^{2+} signal. A lamina I neuron, which was filled with fura-2 (100 µM) via the patch pipette, is shown in transmission mode (left image). Bottom trace shows 40 depolarizing current injections in 1 s, eliciting 40 action potentials (upper trace). Resulting changes in somatic fluorescent ratio before (a) and 0.25 s (b), 0.5 s (c), 1 s (d), 6 s (e), 6.75 s (f) after starting the activation are shown in the right image. (B) Typical time courses of somatic fluorescent ratio changes. Under control conditions (upper traces), transient somatic Ca^{2+} increases were similar following first and second series of depolarizations. Bath application of an unspecific VDCC antagonist (200 µM Cd^{2+} right-hand bottom trace) immediately after the first burst depolarization, strongly reduced the second Ca^{2+} signal. Current injections are indicated by red bars. (C) Bar graph representation of the mean changes of the peak amplitude of the second Ca^{2+} signal in per cent of the first depolarization-induced Ca^{2+} rise under control conditions. The second Ca^{2+} signals were measured under Ca^{2+}-free conditions or in the presence of different NMDA receptor and VDCC antagonists alone or in combination (200 µM Cd^{2+}, 100 µM Ni^{2+}, 50 µM nifedipine, 50 µM verapamil, 50 µM D-AP5, 1 µM ω-conotoxin GVIA, 100 nM ω-agatoxin TK, 1.5 µM ω-conotoxin MVIIC). In additional controls for the toxin experiments 0.1% cytochrome *c* was included in the bathing solution. Numbers of experiments are indicated (one neuron per slice). Statistical significance (unpaired *t*-test, Mann–Whitney rank test) is indicated by $^{*}(P < 0.05)$, $^{**}(P < 0.01)$ and $^{***}(P < 0.001)$.

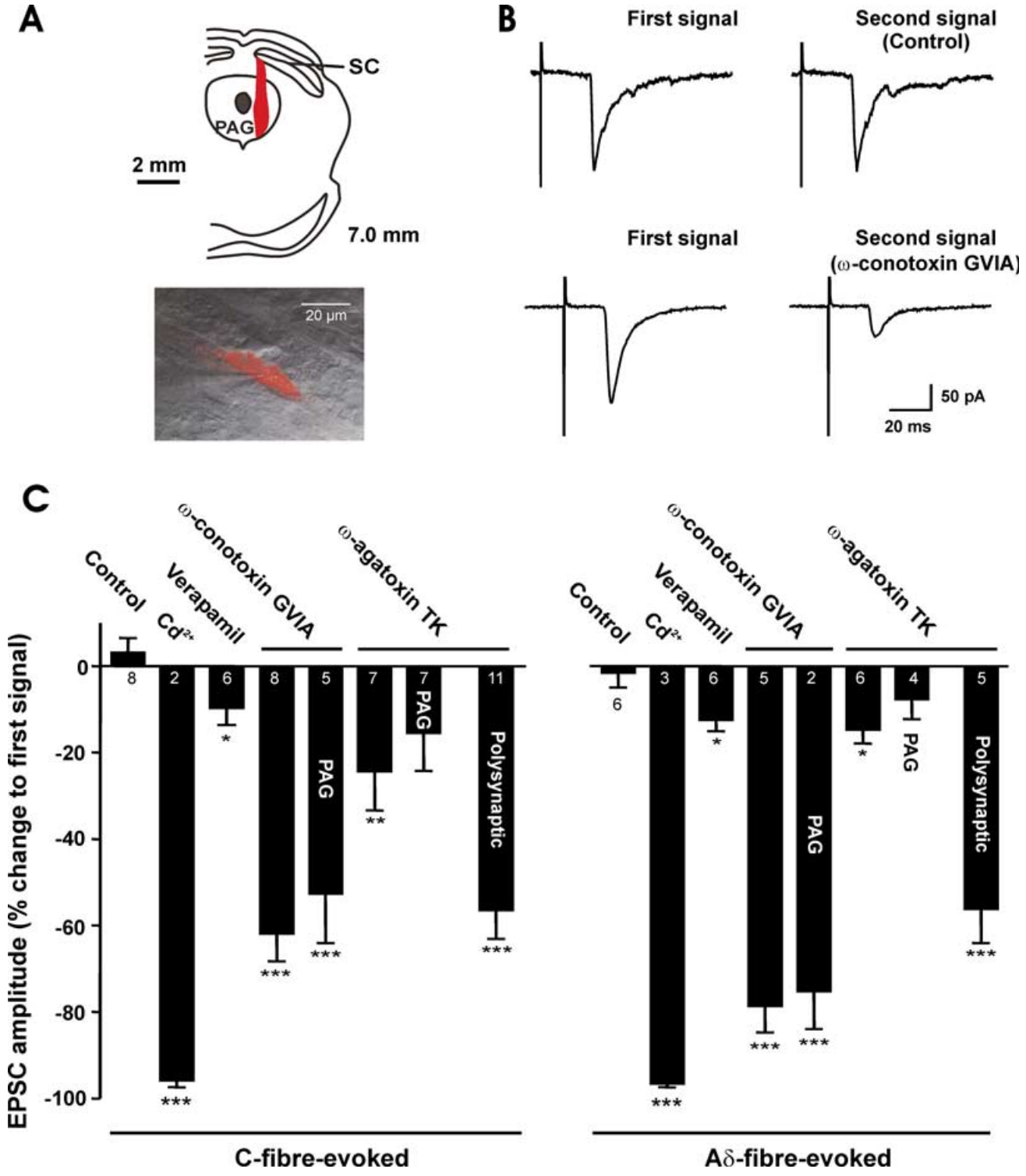


FIG. 3. Effects of VDCC antagonists on the synaptic transmission between nociceptive afferents and spinal lamina I neurons. (A) Distribution of 100 nL DiI (1.25%) injected into the right PAG in one representative animal and a retrogradely labelled lamina I neuron from a transverse lumbar spinal cord slice of the same rat three days after the injection (transmission image overlaid with the fluorescence image). PAG, periaqueductal grey matter; SC, superior colliculus. The distance posterior to bregma is given. (B) Typical recordings of EPSCs ($V_{Hold} = -70$ mV), evoked by electrical stimulation of the attached dorsal root with C-fibre intensity. Under control conditions (upper traces), EPSC peak amplitudes were similar following first and second series of stimulations (recorded at intervals of 10 min). Bath application of the specific N-type channel blocker ω-conotoxin GVIA (1 µM; right-hand bottom trace) immediately after the first series of stimulations strongly reduced the EPSC amplitudes. (C) Bar graph represents the mean changes of C-fibre-evoked (left-hand columns) or Aδ-fibre-evoked (right-hand columns) EPSC peak amplitudes under control conditions and in the presence of VDCC antagonists (200 µM Cd^{2+}, 50 µM verapamil, 1 µM ω-conotoxin GVIA, 100 nM ω-agatoxin TK). Evoked EPSC peak amplitudes (mean of ten consecutive responses) take as a reference the mean response amplitude before application of a drug (second signal/first signal × 100). There was no significant difference in the effect of toxins on evoked EPSCs between unidentified neurons and neurons projecting to the PAG. ω-Agatoxin TK had stronger effects on the polysynaptic inputs than on the monosynaptic ones. Numbers of experiments are indicated (one neuron per slice). Statistical significance is indicated by $^{*}(P < 0.05)$, $^{**}(P < 0.01)$ and $^{***}(P < 0.001)$.

incomplete effect, as the total block of the synaptic transmission in lamina I by Cd^{2+} occurred within a few minutes.

N-type Ca^{2+} channels

Our results suggest, that HVA N-type Ca^{2+} channels are the predominant Ca^{2+} channels associated with nociceptive transmission in spinal lamina I. N-type Ca^{2+} channels are present both presynaptically in afferent C- and Aδ-fibre terminals and postsynaptically in lamina I neurons. Inhibition of Ca^{2+} currents through N-type VDCCs reduced the activity of lamina I neurons both, by reduction of neurotransmitter release from the presynaptic nociceptive terminals and by postsynaptic depression of the excitability of the second order neurons.

A postsynaptic expression of N-type Ca^{2+} channels has been demonstrated by electrophysiological recordings from freshly isolated cervical dorsal horn neurons (Huang, 1989), by immunohistochemical data (Westenbroek *et al*., 1998), and by microfluorometric measurements in a spinal cord slice preparation, where depolarization-induced Ca^{2+} transients (by K^+ application) were reduced to 25% of control by ω-conotoxin GVIA in dorsal horn neurons (Voitenko *et al*., 2000). As a consequence, inhibition of N-type VDCCs at postsynaptic sites may reduce nociceptive responses of spinal lamina I neurons.

We found a major contribution of N-type Ca^{2+} channels to synaptic transmission between C-fibres as well as Aδ-fibres and spinal lamina I neurons, which is in line with a previous study (Bao *et al*., 1998). The presence of these channels on spinal terminals of fine primary afferents (see also Westenbroek *et al*., 1998) assign these channels a strategic role in the control of neurotransmitter release from nociceptive nerve fibres (Meir *et al*., 1999). Indeed, N-type Ca^{2+} channels have been shown to be colocalized with substance P (Westenbroek *et al*., 1998) and spinal release of substance P, calcitonin gene-related peptide and glutamate from primary afferents is ω-conotoxin-sensitive (Maggi *et al*., 1990; Santicioli *et al*., 1992; Gruner & Silva, 1994; Evans *et al*., 1996; Martire *et al*., 2000; Smith *et al*., 2002). Glutamate, acting on NMDA receptors, and substance P, acting on NK1 receptors, have a pivotal role for plasticity at the first synapse in nociceptive pathways (Sandkühler, 2000; Ikeda *et al*., 2003). Thus, N-type channels may modulate both, acute nociception and induction of central sensitization. The importance of spinally expressed N-type Ca^{2+} channels for the development of hyperalgesia and allodynia following nerve injury and acute or chronic inflammation is confirmed by the efficacy of N-type VDCC blockers in several animal pain models. For example, inhibition of these channels by ω-conotoxin GVIA reduced hyperalgesia and allodynia following spinal nerve ligation (Chaplan *et al*., 1994; Matthews & Dickenson, 2001b), the pain behaviour in both phases of the formalin test (Malmberg & Yaksh, 1994; Diaz & Dickenson, 1997) and hyperalgesia during knee joint inflammation (Sluka, 1998).

P/Q-type channels

We found no postsynaptic role of P/Q-type VDCCs in lamina I neurons, in agreement with immunocytochemical studies, showing a nearly exclusive localization of P/Q-type channels on nerve terminals, but not on cell bodies in spinal dorsal horn (Westenbroek *et al*., 1998). In addition, contrary to many other types of central synapses (Takahashi & Momiyama, 1993; Iwasaki *et al*., 2000), the contribution of P/Q-type channels to synaptic transmission from primary afferents terminating on lamina I was minor. The highest density of terminals labelled with antibodies against these channels was found in laminae II–VI, whereas the density of staining was much lower in lamina I (Westenbroek *et al*., 1998). In that study, the distribution of P/Q-type channels was complementary rather than overlapping with N-type channels in the superficial dorsal horn. These observations are in line with our findings, that ω-agatoxin TK had a stronger effect on polysynaptic nociceptive transmission than on monosynaptic transmission between C- and Aδ-afferents and spinal lamina I neurons, respectively. Other data demonstrated a predominant role for P/Q-type channels in mediating synaptic transmission from inhibitory interneurons in spinal dorsal horn (Takahashi & Momiyama, 1993). Possibly, fibres, equipped with P/Q-type VDCCs, synapse with interneurons in lamina II and deeper laminae of the dorsal horn, some of which may project to neurons in lamina I. This may also explain the stronger inhibition of synaptic strength by ω-agatoxin reported by Bao *et al*. (1998), as they did not differentiate between lamina I and II neurons.

It has been shown, that presynaptic Ca^{2+} channel types at central synapses undergo developmental changes (Iwasaki *et al*., 2000). The contribution of N-type VDCCs decreases and that of P/Q-type channels increases until postnatal day 13. We therefore used rats, which were at least 18-days-old to avoid confounding effects of developmental processes.

P/Q-type channels are found to be expressed in up to 50% of dorsal root ganglion neurons (Hatakeyama *et al*., 2001; Kim *et al*., 2001). The pore-forming α_{1A} subunit of the P/Q-type channel shows little colocalization with the neuropeptide transmitter substance P in the superficial dorsal horn (Westenbroek *et al*., 1998), suggesting that P/Q-type VDCCs are not of major importance for the control of transmitter release from peptidergic primary afferents. In fact, evoked release of substance P and calcitonin-gene related peptide from rat sensory neurons was not affected by ω-agatoxin IVA (Evans *et al*., 1996). This and the present finding, that the contribution of these channels to synaptic transmission in spinal lamina I is minor, may explain the weak efficacy of P/Q-type Ca^{2+} channel blockers on nociceptive behaviour, as compared to N-type Ca^{2+} channel blockers. ω-Agatoxin had no significant analgesic effect on the first phase of the formalin test, on the hot plate test (Malmberg & Yaksh, 1994), or in a chronic neuropathic pain model (Matthews & Dickenson, 2001b). It did however, reduce the responses in the second phase of the formalin test (Malmberg & Yaksh, 1994; Diaz & Dickenson, 1997).

L-type Ca^{2+} channels

Blockade of L-type Ca^{2+} channels mainly depressed the postsynaptic rise of $[Ca^{2+}]_i$ in lamina I neurons and had a small but significant effect on the synaptic transmission between C- and Aδ fibres and lamina I neurons, respectively. The expression of these channels in the membrane of dorsal horn neurons has been shown by electrophysiological and microfluorometric measurements in spinal cord slices and freshly isolated neurons (Huang, 1989; Ryu & Randić, 1990; Voitenko *et al*., 2000). L-type VDCCs may also be present on terminals of nociceptive afferents, as L-type channel blockers inhibit Ca^{2+} currents in small and medium sized dorsal root ganglion neurons (Hatakeyama *et al*., 2001; Kim *et al*., 2001), but the nifedipine-sensitive Ca^{2+} influx into synaptoneurosomes from spinal cord was only marginal (Hatakeyama *et al*., 2001), suggesting only a minor contribution of these channels to the release of neurotransmitters in spinal nerve terminals (Kim *et al*., 2001). This is supported by our findings and by others, demonstrating a minor contribution of L-type VDCCs to sensory synaptic transmission in the superficial dorsal horn (Bao *et al*., 1998). The predominant postsynaptic location of L-type Ca^{2+} channels in neurons of the dorsal horn laminae has been demonstrated by immunohistochemical staining (Westenbroek *et al*., 1998). Thus, these channels contribute to the activity-regulated Ca^{2+} influx into lamina I neurons, but not to neurotransmitter release from nociceptive afferents. Spinally delivered L-type antagonists had minimal effects in different behavioural pain studies. They caused only small or no reductions of nociceptive

responses in the formalin test (Malmberg & Yaksh, 1994) or after knee joint inflammation (Diaz & Dickenson, 1997; Sluka, 1998), in the tail flick test (Doğrul *et al*., 2001), and in the hot plate test (Malmberg & Yaksh, 1994). However, antagonists of L-type VDCCs may potentiate the analgesic action of opioids (Doğrul *et al*., 2001).

T-type Ca^{2+} channels

Reports about any role of T-type Ca^{2+} channels in spinal dorsal horn neurons are rare. LVA transient Ca^{2+} currents have been demonstrated in freshly isolated neurons and spinal cord slices (Huang, 1989; Ryu & Randić, 1990), and in the latter preparation, a slight suppression of $[Ca^{2+}]_i$ transients by Ni^{2+} could be shown (Voitenko *et al*., 2000). *In situ* hybridization studies on the spinal cord detected mRNA of these channels in the rat dorsal horn, including superficial laminae I and II (Talley *et al*., 1999). We found a high variability in the effect of Ni^{2+} on the reduction of the activity-dependent Ca^{2+} influx into lamina I neurons ranging from zero to 35%. In our previous work we demonstrated, that some lamina I neurons express T-type Ca^{2+} channels, while others do not (Ikeda *et al*., 2003). By measuring activation curves, we showed, that these Ni^{2+}-sensitive currents are indeed through LVA T-type channels and not mediated by HVA R-type channels, which are also sensitive to low Ni^{2+} concentrations (Tottene *et al*., 2000). Only a few studies are available about the functional relevance of T-type VDCCs for spinal nociception. The most serious drawback for investigating the role of these channels in animal pain models is the lack of specific channel blockers. There are drugs, showing a higher selectivity for LVA T-type currents than for HVA Ca^{2+} currents, at least in some tissues (Perez-Reyes, 2003). Intrathecal application of the antiepileptic drug ethosuximide mediated significant inhibition of electrical and natural evoked rat dorsal horn neuronal responses, but pain-related behaviour remained unaltered after neuropathy (Matthews & Dickenson, 2001a). Spinal mibefradil, an antihypertensive drug, had no effect on nociceptive responses in tail-flick test, but potentiated the analgesic effect of opioids (Doğrul *et al*., 2001).

Conclusions

The present results support key roles of voltage-dependent Ca^{2+} channels for sensory transmission within lamina I of the spinal cord. Here, we provide further evidence, that N-type Ca^{2+} channels are the predominant channel type involved in the pre- and postsynaptic processing of nociceptive information. Another major finding is the lacking difference in the relative contribution of the different VDCC types to the Aδ- and C-fibre mediated nociception, respectively. These and other electrophysiological and behavioural data suggest the importance of further systematic examination of the expression of spinal VDCCs in pain pathways, and thus may propel the targeted development of specific analgesic ligands.

Acknowledgements

This work was supported by the Austrian Fonds zur Förderung Wissenschaftlicher Forschung (FWF, P15542) and by the Deutsche Forschungsgemeinschaft (DFG; Sa 435).

Abbreviations

D-AP5, D-2-amino-5-phosphovaleric acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EPSC, excitatory postsynaptic current; HVA, high-voltage activated; LVA, low-voltage activated; NMDA, *N*-methyl-D-aspartate; PAG, periaqueductal grey matter; TTX, tetrodotoxin; VDCC, voltage-dependent Ca^{2+} channel.

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