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Group I metabotropic glutamate receptor-induced Ca^{2+}-gradients in rat superficial spinal dorsal horn neurons

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Abstract

Here, we investigated changes in the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), induced by the pharmacological activation of metabotropic glutamate receptors (mGluRs), in nociceptive neurons of the superficial spinal dorsal horn. Microfluorometric Ca^{2+} measurements with fura-2 in a lumbar spinal cord slice preparation from young rats were used.

Bath application of the specific group I mGluR agonist (*S*)-3,5-dihydroxyphenylglycine ((*S*)-3,5-DHPG) resulted in a distinct increase of $[Ca^{2+}]_i$ in most of the neurons in superficial dorsal horn. In contrast, activation of groups II or III mGluRs by DCG-IV or L-AP4, respectively, failed to evoke any significant change in $[Ca^{2+}]_i$. The effect of (*S*)-3,5-DHPG was mediated by both group I subtypes mGluR1 and mGluR5, since combined pre-treatment with the subtype antagonists (*S*)-4-CPG and MPEP was necessary to abolish the $[Ca^{2+}]_i$ increase. Depleting intracellular Ca^{2+} stores with CPA or inhibiting IP_3-receptors with 2-APB, respectively, reduced the (*S*)-3,5-DHPG-evoked $[Ca^{2+}]_i$ increase significantly. Inhibition of voltage-dependent L-type Ca^{2+} channels (VDCCs) by verapamil or nicardipine reduced the (*S*)-3,5-DHPG-induced $[Ca^{2+}]_i$ rise likewise.

Thus, in rat spinal cord, (*S*)-3,5-DHPG enhances Ca^{2+} signalling in superficial dorsal horn neurons, mediated by the release of Ca^{2+} from IP_3-sensitive intracellular stores and by an influx through L-type VDCCs. This may be relevant to the processing of nociceptive information in the spinal cord.

Keywords: Metabotropic glutamate receptors; (*S*)-3,5-DHPG; Intracellular Ca^{2+}; Voltage-dependent L-type Ca^{2+} channels; Spinal cord; Nociception; Rat

1. Introduction

Synapses between primary afferent Aδ- and C-fibres, and second order neurons in the superficial dorsal horn of the spinal cord may undergo long-lasting changes in synaptic strength. The underlying mechanisms may account for some forms of hyperalgesia, allodynia and analgesia (Moore et al., 2000; Sandkühler, 2000). Use-dependent synaptic long-term potentiation (LTP) as well as long-term depression (LTD) in superficial dorsal horn has been shown in vivo (Azkue et al., 2003; Sandkühler and Liu, 1998) and in vitro (Heinke and Sandkühler, 2005; Ikeda et al., 2003, 2006).

Free cytosolic calcium plays a key role as a ubiquitous second messenger and is a crucial determinant in many forms of synaptic plasticity. Moreover, the magnitude and the kinetic profile of the post-synaptic Ca^{2+} rise may determine the direction of plasticity (Ismailov et al., 2004; Sjöström and Nelson, 2002). The $[Ca^{2+}]_i$ increase is often mediated by activation of ionotropic glutamate receptors (especially NMDA-receptors) and/or metabotropic glutamate receptors (mGluRs). Indeed, some forms of long-term plasticity (LTP and LTD) at the spinal cord level depend on activation of mGluRs (Azkue et al., 2003; Gerber et al., 2000; Heinke and Sandkühler, 2005). The important role of mGluRs in acute and persistent spinal

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nociception has been underlined by a growing body of behavioural and electrophysiological data (for reviews see: Neugebauer, 2002; Varney and Gereau, 2002).

Vertebrate mGluR subtypes are encoded by eight known genes and are classified into three groups based on amino acid sequence similarities and pharmacology: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8) (Conn and Pin, 1997). Most of the genes for mGluRs are spliced and generate variants which differ in their C-terminal domain. mGluRs are coupled through different G-proteins to several intracellular second messenger cascades. Groups II and III mGluRs are negatively coupled to adenylyl cyclase through G_i/G_o proteins. In contrast, group I mGluRs couple via $G_{q/11}$ proteins to phospholipase C and activate hydrolysis of phosphoinositides (PI) to generate diacylglycerol and inositol triphosphate (IP_3) with the subsequent release of intracellular Ca^{2+} (Conn and Pin, 1997). Remarkably, there is evidence that at least group II mGluRs may be also positively coupled to PLC and PLD pathways and can stimulate IP_3-receptor mediated intracellular Ca^{2+} mobilization (Otani et al., 2002).

Expression of mGluR subtypes in rat spinal cord has been addressed by several studies using immunocytochemical and molecular approaches. Group I mGluR1 and mGluR5 are most abundant, with mGluR5 being concentrated in superficial dorsal horn. Both, group II mGluR2/3 and group III mGluR4 and mGluR7 are also present, but at much lower level (Alvarez et al., 2000; Berthele et al., 1999; Jia et al., 1999; Karim et al., 2001; Tang and Sim, 1999; Valerio et al., 1997).

The contribution of mGluRs to Ca^{2+} signalling in superficial spinal dorsal horn is unexplored. Here, we investigated how activation of mGluR subtypes may increase cytosolic Ca^{2+} concentration at/near the first synapse in nociceptive pathways.

2. Methods

2.1. Preparation of spinal cord slices

Transverse slices were obtained from young Sprague–Dawley rats of both sexes (18- to 24-day-old). Under deep ether anaesthesia, lumbar spinal cord was exposed by laminectomy. The segments of the lumbosacral spinal cord (L4–S1) were excised. Transverse slices were cut at 400–500 μm thickness using a vibrating microslicer (DTK-1000, Dosaka EM, Kyoto, Japan). After cutting, slices were allowed to recover for half an hour at 33 °C. The incubating solution (also used for cutting) was gassed with carbogen (5% CO_2 and 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 mosmol/kg.

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. All efforts were made to minimise animal suffering and to reduce the number of animals used.

2.2. Electrophysiological recordings

For patch-clamp recordings, single slices were transferred to the recording chamber (volume 1.0 ml) mounted on an upright microscope (Olympus BX50WI, Olympus, Japan) equipped with a 40×, 0.80 water-immersion objective. Dorsal horn neurons were visualized with Dodt-infrared optics and a video camera system (PCO, Kelheim, Germany). Superficial dorsal horn was identified as a translucent band across the dorsal horn. Slices were continuously superfused at a rate of 3–4 ml/min with a recording solution (equilibrated 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. Ca^{2+}-free/EGTA solution was prepared by omitting $CaCl_2$ from recording solution and adding 1 mM EGTA. Picrotoxin (100 μM) and strychnine (4 μM) were always included in the bathing solution. Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) on a horizontal puller (P-87, Sutter Instruments, Novato, CA, USA). Pipettes were filled with a solution composed of (in mM): κ-gluconate, 120; KCl, 20; $MgCl_2$, 2; HEPES, 10; Na_2ATP, 2; NaGTP, 0.5; EGTA, 0.5; pH 7.28 adjusted with KOH; measured osmolarity ~300 mOsm, resulting in a tip resistance between 3 and 5 MΩ. Recordings were made with an Axopatch 200B patch-clamp amplifier (Molecular Devices, Union City, CA, USA) at a sampling rate of 10 kHz using a low-pass Bessel filter of 2 kHz. The software package pCLAMP 9 (Molecular Devices) was used for data acquisition and subsequent off-line analysis. Standard whole-cell patch-clamp recordings under 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.

2.3. Ca^{2+}-imaging

Fluorometric measurements of free cytosolic Ca^{2+} concentrations of single superficial dorsal horn neurons were performed by loading the cells in the recording chamber via the patch pipette for at least 10 min by replacing EGTA in the standard pipette solution with the cell-impermeant dye fura-2 pentapotassium salt (100 μM; Molecular Probes, Eugene, Oregon, USA).

For recording fluorescence signals of non-dialyzed cells, spinal cord slices were bulk-loaded by incubating them for 45 min at 35 °C in gassed incubation solution supplemented with the membrane-permeable form of the Ca^{2+}-sensitive dye fura-2 acetoxymethyl-ester (fura-2 AM, 10 μM; Molecular Probes) and pluronic F-127 detergent (0.02%; Molecular probes) dissolved in dimethylsulfoxide (DMSO, Sigma, Deisenhofen, Germany). Slices were then washed in the incubation solution without dye for 30 min to ensure complete cytoplasmatic dye de-esterification. After the loading procedure, single slices were placed in the recording chamber and continuously superfused with the recording solution containing tetrodotoxin (TTX, 0.5 μM; Alexis, Grünstadt, Germany) throughout the experiment to prevent discharges of action potentials in order to block indirect actions of mGluR activation via synaptically driven Ca^{2+} transients.

Slices were illuminated with a monochromator and images were captured at 2 Hz with a cooled CCD camera (TILLvisION Imaging system, TILL Photonics, Gräfelfing, Germany). Consecutive paired exposures to 340 and 380 nm were used to achieve digital fluorescence images. Calculations were made off-line. For measuring, a small region of interest was placed in the centre of the cell soma. $[Ca^{2+}]_i$ was calculated by ratiometric (F_{340}/F_{380}) fluorescence and no conversion to concentrations was made.

Changes in Ca^{2+} concentration of cells in bulk-loaded slices were evaluated by drawing regions of interest over every intact neuron in the analyzed area. Neuronal nature of the cells was assessed at the end of each experiment by exposing the slices to the standard recording solution containing a high K^+ concentration by replacing 50 mM NaCl isoosmotically with KCl (Crawford et al., 2000). Astrocytes may express voltage-dependent Ca^{2+} channels in culture, but patch-clamp recordings failed to reveal any voltage-dependent Ca^{2+} currents in acute brain slices (Carmignoto et al., 1998). Fluorescence signals of all neurons in a given slice, showing a rapid and distinct depolarization-induced Ca^{2+} rise, were summed for further analysis.

In all experiments, one drug effect was tested per slice.

All experiments, except slice preparation and dye loading procedure, were performed at room temperature.

2.4. Application of drugs

All drugs were dissolved in recording solution, gassed with carbogen, at known concentrations. Drugs and their sources were as follows: (*S*)-3,

5-dihydroxyphenylglycine ((*S*)-3,5-DHPG; 50 μM; Tocris, Köln, Germany), (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG; 1 mM; Tocris), (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV; 5 μM; Tocris), L-(+)-2-amino-4-phosphonobutyric acid (L-AP4, 30 μM; Tocris), (*S*)-4-carboxyphenylglycine ((*S*)-4-CPG; 2 mM; Tocris), 2-methyl-6-(phenylethynyl)-pyridine (MPEP; 30 μM; Tocris), picrotoxin (100 μM; Tocris), strychnine (4 μM; Sigma), cyclopiazonic acid (CPA; 30 μM; Calbiochem, Bad Soden, Germany), 2-aminoethoxydiphenyl borate (2-APB; 100 μM; Calbiochem), nicardipine (50 μM; Sigma), verapamil (50 μM; Sigma), cadmium(II)chloride ($CdCl_2$, 200 μM; Sigma).

(*S*)-3,5-DHPG, DCG-IV and verapamil were dissolved in an aqueous stock solution diluted in perfusing salt buffer just before use. CHPG, L-AP4 and (*S*)-4-CPG were dissolved in 1 eq. NaOH as stock solution. The solvent for TTX was acidic buffer (pH 4.8). All other drugs were dissolved in DMSO (maximal final concentration in recording solution 0.25%, v/v).

2.5. Data analysis

Analysis of the data was performed using SigmaStat 3.1 (Systat Software GmbH, Erkrath, Germany). Values are given as mean ± one standard error of the mean (SEM). After testing for normality (Kolmogorov–Smirnov test), analysis of variance (ANOVA) followed by a Tukey's post hoc test was performed for statistical comparison. A paired *t*-test was used to evaluate the DHPG-effect on membrane potential ($P < 0.05$ was considered to be statistically significant).

3. Results

3.1. Cell dialysis prevents DHPG-induced $[Ca^{2+}]_i$ rise

To quantitatively measure changes in $[Ca^{2+}]_i$ in neurons of superficial dorsal horn, we loaded individual neurons with the membrane-impermeable form of the Ca^{2+}-sensitive dye fura-2 via patch pipettes. Under these conditions, application of the specific agonist of group I mGluRs (*S*)-3,5-DHPG (Ito et al., 1992) in the superfusing solution (50 μM for 3 min) had no effect on $[Ca^{2+}]_i$ in any of the eight cells tested. The mean Ca^{2+} signal was 98 ± 1% of baseline values (expressed as the ratio between the intensity at 340 nm and at 380 nm excitation wavelength; data not shown). Probably, an essential diffusible compound of the downstream signalling cascade of group I mGluR activation was lost during the loading procedure. In order to prevent this in all subsequent experiments, we loaded the intact cells by incubating the slices with the membrane-permeant form fura-2 AM.

3.2. DHPG induces $[Ca^{2+}]_i$ rise in superficial dorsal horn neurons

Fig. 1 illustrates an example of fluorescence image and $[Ca^{2+}]_i$ records taken from different cells in one fura-2 AM "bulk"-loaded spinal cord slice. The video image of superficial dorsal horn neurons loaded with fura-2 by slice incubation for 45 min shows somata of these neurons on a dark background, measured at an excitation wavelength of 360 nm (Fig. 1A, left). Using the 40× objective, the dorsoventral extension of the displayed window corresponded to a portion of superficial spinal dorsal horn as shown in Fig. 1A on the right-handed side. Changes in Ca^{2+} concentration were analyzed by measuring the somatic fluorescent ratio changes of

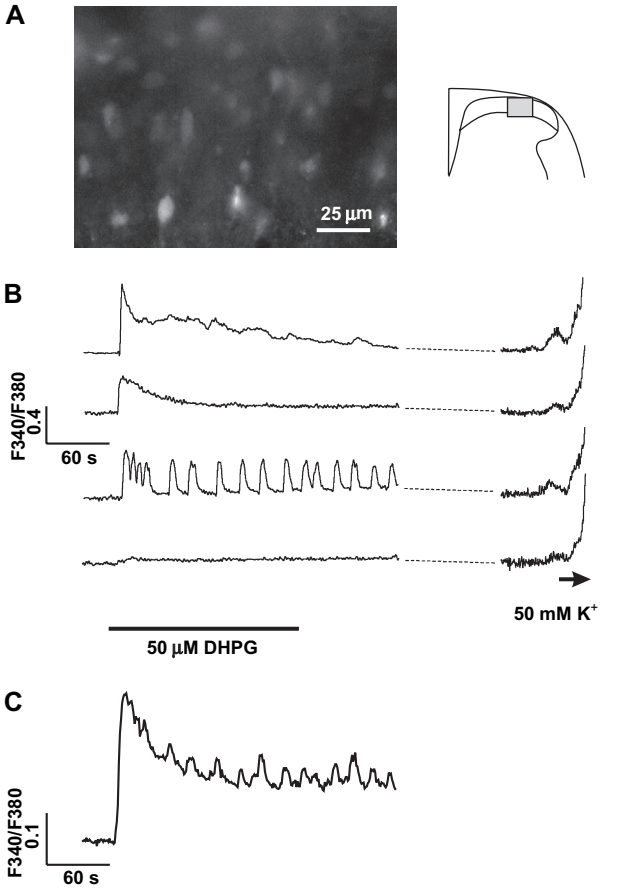

Fig. 1. Selective activation of group I mGluRs with (*S*)-3,5-DHPG-induced a $[Ca^{2+}]_i$ rise in the somata of "bulk"-loaded superficial spinal dorsal horn neurons. (A) Fluorescence intensity image of the substantia gelatinosa taken by a cooled CCD camera from a fura-2 AM "bulk"-loaded (10 μM) spinal cord slice, prepared from a 21-day-old rat (left). The right drawing displays position and extension of this imaged area in superficial dorsal horn. (B) Typical time courses of somatic fluorescent ratio changes recorded from four different neurons of the selected image are shown in Fig. 1A. Bath application of the specific agonist of group I mGluRs (*S*)-3,5-DHPG (50 μM for 3 min) induced a distinct increase of $[Ca^{2+}]_i$ in superficial dorsal horn neurons, showing different amounts and different kinetics. During all experiments, TTX (0.5 μM) was included in the recording solution. Cells were verified to be neurons by a rapid $[Ca^{2+}]_i$ rise in response to a high depolarizing K^+ concentration in the bathing solution (right traces). (C) Fluorescence ratio changes of all neurons of the imaged area, identified by the K^+ depolarization, were added up (18 cells in the exemplified slice) and the resulting Ca^{2+} peak was measured to quantify the DHPG-effect.

all neurons in the imaged area of superficial spinal dorsal horn. Analysis was restricted to neurons which were identified by a $[Ca^{2+}]_i$ rise in response to a high K^+ concentration in the bathing solution (Crawford et al., 2000) (Fig. 1B, right). To assess the action of a drug on cytoplasmatic Ca^{2+} level the somatic fluorescent ratio of all identified neurons were added up (Fig. 1C), including those which showed almost no Ca^{2+} rise (e.g. last trace in Fig. 1B). The effect was quantified as percent change of the resulting Ca^{2+} peak from baseline level.

In fura-2 AM "bulk"-loaded spinal cord slices, we investigated the effect of mGluR agonists on Ca^{2+} mobilization in superficial dorsal horn neurons. Application of the specific agonist of group I mGluRs (*S*)-3,5-DHPG added to the

recording solution (50 μM for 3 min) induced a distinct increase of $[Ca^{2+}]_i$, showing different kinetics in different superficial dorsal horn neurons (see examples in Fig. 1B). In the slice, shown in Fig. 1, the $[Ca^{2+}]_i$ peak of all neurons within the image window reached 201% of baseline. The mean Ca^{2+} signal in all slices tested changed to 184 ± 9% of the baseline ratio after (*S*)-3,5-DHPG treatment ($n = 17$ slices, including 222 cells; Fig. 2).

We next examined the effects of subtype-specific antagonists on (*S*)-3,5-DHPG-induced $[Ca^{2+}]_i$ rise. The low affinity mGluR1 antagonist (*S*)-4-CPG (Brabet et al., 1995; Hayashi et al., 1994) was applied at a concentration of 1 mM 15 min prior to and during (*S*)-3,5-DHPG application. This treatment reduced the $[Ca^{2+}]_i$ increase by (*S*)-3,5-DHPG to 140 ± 7% of baseline ($n = 4$; $P < 0.05$ vs. DHPG-effect; Fig. 2). Similar results were obtained with MPEP (30 μM), a specific mGluR5 subtype antagonist (Gasparini et al., 1999). Pre-incubation with this compound reduced (*S*)-3,5-DHPG-induced Ca^{2+} peak to 142 ± 8% of baseline ($n = 5$; $P < 0.05$ vs. DHPG-effect; Fig. 2). Combined application of both subtype antagonists nearly abolished (*S*)-3,5-DHPG-induced $[Ca^{2+}]_i$ increase (114 ± 5% of baseline, $n = 5$; $P < 0.001$ vs. DHPG-effect; Fig. 2). Thus, increase of $[Ca^{2+}]_i$ in superficial dorsal horn neurons by (*S*)-3,5-DHPG requires activation of both, mGluR1 and mGluR5 subtypes.

Selective activation of mGluR5 subtypes by application of CHPG (2 mM) induced a $[Ca^{2+}]_i$ rise to 192 ± 22% of baseline ratio ($n = 5$; Fig. 2). This effect was not significantly different from the (*S*)-3,5-DHPG-effect and could be prevented by pre-incubation with the mGluR5 antagonist MPEP (106 ± 4% of baseline $n = 5$; $P < 0.01$ vs. CHPG-effect; Fig. 2).

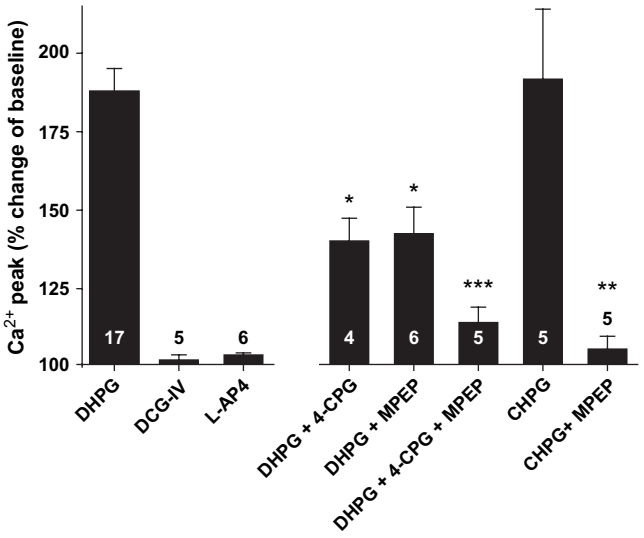

Fig. 2. $[Ca^{2+}]_i$ rise in superficial dorsal horn neurons induced by (*S*)-3,5-DHPG application is mediated by mGluR1 and mGluR5 subtypes. Bar graph representation of the mean changes of the peak amplitude of $[Ca^{2+}]_i$ rise in percent of the baseline values. Ca^{2+} signal was measured in response to specific activation of group I (50 μM (*S*)-3,5-DHPG), group II (5 μM DCG-IV) or group III (30 μM L-AP4) mGluRs. To verify the subtypes involved in the (*S*)-3,5-DHPG-effect, slices were pre-treated with the specific antagonists (*S*)-4-CPG (1 mM) and MPEP (30 μM) alone or in combination. Activation of mGluR5 subtype by CHPG (2 mM) induced Ca^{2+} peak was antagonized by pre-incubation with the specific blocker MPEP. Digits indicate number of slices (including different numbers of neurons per slice, ranging from 7 to 21). Statistical significance (one-way ANOVA followed by a Tukey's post hoc test) is indicated by $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

3.3. Role of intracellular Ca^{2+} stores and VDCCs for DHPG-induced $[Ca^{2+}]_i$ rise

We next investigated the sources of Ca^{2+} mobilization in superficial dorsal horn neurons induced by group I mGluR activation.

When the slices were superfused with nominally Ca^{2+}-free solution ($CaCl_2$ was removed from the recording solution and 1 mM EGTA was added) for 15 min, (*S*)-3,5-DHPG still evoked significant rises in $[Ca^{2+}]_i$ in superficial dorsal horn neurons (132 ± 9% of baseline values, $n = 6$; $P < 0.01$ vs. DHPG-effect; Fig. 3). Thus, a substantial part of DHPG-induced rise in $[Ca^{2+}]_i$ is mediated by influx of Ca^{2+} ions from extracellular space.

Ca^{2+} may be released from IP_3-sensitive intracellular stores upon group I mGluR activation. To examine the role of Ca^{2+} release from intracellular stores by (*S*)-3,5-DHPG, the sarco/endoplasmic reticulum Ca^{2+}/ATPase (SERCA) pump inhibitor cyclopiazonic acid (CPA), which depletes intracellular stores of Ca^{2+} by blocking Ca^{2+} re-uptake into the stores (Seidler et al., 1989), was applied to slices 15 min prior to and during (*S*)-3,5-DHPG application. Superfusion with CPA (30 μM) reduced the Ca^{2+} signal induced by (*S*)-3,5-DHPG to 143 ± 11% of baseline ($n = 5$; $P < 0.05$ vs. DHPG-effect; Fig. 3). To test, if Ca^{2+} release from internal stores was mediated by the activation of IP_3-receptors, we used the membrane-permeable antagonist of IP_3-activated Ca^{2+} release channels 2-APB (Maruyama et al., 1997). When washed into the slice, 2-APB (100 μM for 15 min) reduced the (*S*)-3,5-DHPG-evoked Ca^{2+} peak to 132 ± 4% of baseline ($n = 11$; $P < 0.001$ vs. DHPG-effect; Fig. 3). There was no significant difference between CPA and 2-APB effect.

To measure the contribution of voltage-dependent Ca^{2+} channels to the group I mGluR-mediated $[Ca^{2+}]_i$ increase in superficial dorsal horn 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 (*S*)-3,5-DHPG-induced Ca^{2+} peak to 114 ± 2% of baseline ratio ($n = 4$; $P < 0.001$ vs. DHPG-effect; Fig. 3). Since (*S*)-3,5-DHPG-induced Ca^{2+} influx through L-type Ca^{2+} channels has been described recently in striatal neurons (Mao and Wang, 2003) and in lamprey spinal cord neurons (Kettunen et al., 2002), we pre-treated the slices with L-type Ca^{2+} channel blockers. Application of verapamil (50 μM for 15 min) resulted in a reduction of the (*S*)-3,5-DHPG-evoked $[Ca^{2+}]_i$ increase to 131 ± 3% of baseline ($n = 4$; $P < 0.01$ vs. DHPG-effect; Fig. 3). Bath application of another inhibitor of L-type Ca^{2+} channels, the dihydropyridine nicardipine (50 μM for 15 min), also reduced (*S*)-3,5-DHPG-evoked increase in $[Ca^{2+}]_i$ to 120 ± 8% of baseline ($n = 3$; $P < 0.01$ vs. DHPG-effect; Fig. 3).

Suppressing Ca^{2+} release from intracellular stores and Ca^{2+} influx from extracellular space through VDCCs by

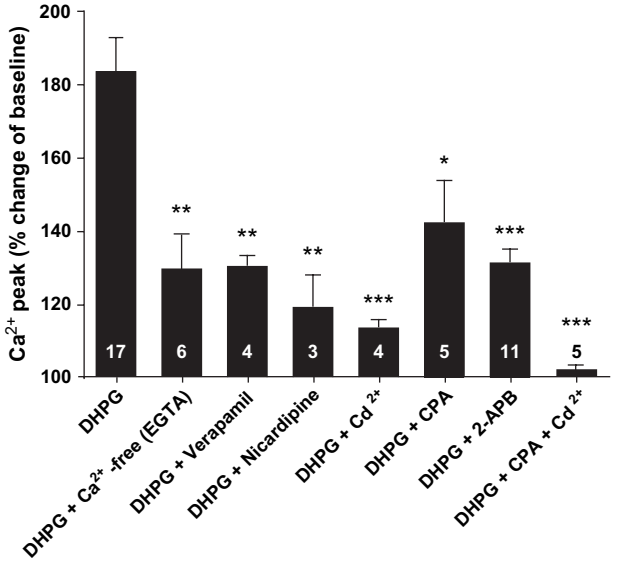

Fig. 3. (*S*)-3,5-DHPG-induced $[Ca^{2+}]_i$ rise is mediated by Ca^{2+} influx through VDCCs and by the release from IP_3-sensitive stores. Bar graph represents the mean changes of the peak amplitude of $[Ca^{2+}]_i$ rise in percent of the baseline values induced by the application of (*S*)-3,5-DHPG alone or after pre-incubation with different modulators of Ca^{2+} signalling. Using a Ca^{2+}-free buffer (including 1 mM EGTA), blocking of all VDCCs by Cd^{2+} (200 μM), of L-type channels by verapamil and nicardipine (50 μM, respectively), depleting intracellular stores by bath application of cyclopiazonic acid (30 μM) or inhibition of IP_3-receptors by 2-APB (100 μM) reduced (*S*)-3,5-DHPG-evoked $[Ca^{2+}]_i$ rise to different extent. Digits indicate number of slices (including different numbers of neurons per slice, ranging from 6 to 21). Statistical significance (ANOVA followed by a Tukey's post hoc test) is indicated by $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

combined pre-treatment of the slices with CPA and Cd^{2+} abolished (*S*)-3,5-DHPG-induced $[Ca^{2+}]_i$ rise in spinal superficial dorsal horn neurons ($102 \pm 1\%$ of baseline, $n = 5$; $P < 0.001$ vs. DHPG-effect; Fig. 3).

3.4. DHPG induces a membrane depolarization in superficial dorsal horn neurons

Bath application of (*S*)-3,5-DHPG (50 μM for 2 min) caused a reversible membrane depolarization of spinal superficial dorsal horn neurons, measured by the whole-cell patch-clamp technique in current-clamp mode (Fig. 4). In one neuron, (*S*)-3,5-DHPG-induced depolarization was large enough to elicit action potential firing (Fig. 4A left trace). The depolarizing response to bath application of (*S*)-3,5-DHPG was similar in the presence of TTX (0.5 μM), ruling out the involvement of both a TTX-sensitive release of glutamate and of TTX-sensitive voltage-dependent Na^+-channels (Fig. 4A right trace). The mean (*S*)-3,5-DHPG-evoked depolarization of spinal superficial dorsal horn neurons was 9 ± 2 mV ($n = 5$; $P < 0.01$; Fig. 4B).

3.5. Neither groups II nor III mGluR activation affects $[Ca^{2+}]_i$ in superficial dorsal horn neurons

Pharmacological activation of groups II or III mGluRs failed to evoke any significant rise in $[Ca^{2+}]_i$ in all of the neurons tested. Application of the specific group II mGluR agonist DCG-IV (5 μM for 3 min) had no effect on mean Ca^{2+} signal ($102 \pm 2\%$ of the baseline ratio ($n = 5$; Fig. 2). Administration of the specific group III mGluR agonist L-AP4 (30 μM for 3 min) also had no effect ($104 \pm 1\%$ of the baseline; $n = 6$; Fig. 2).

4. Discussion

Here we characterized mGluR-induced Ca^{2+} signalling in neurons of the superficial spinal dorsal horn of the rat. Activation of group I, but not groups II or III mGluRs enhanced intracellular Ca^{2+} concentration. This effect is mediated by both group I subtypes (mGluR1 and mGluR5). Group I mGluR-mediated rise in $[Ca^{2+}]_i$ is due to release of Ca^{2+} from IP_3-sensitive intracellular stores and to an influx through L-type voltage-dependent Ca^{2+} channels.

4.1. Group I but not groups II or III mGluR activation increases cytoplasmatic Ca^{2+} concentration

Activation of group I mGluRs by DHPG-induced $[Ca^{2+}]_i$ rise in rat spinal dorsal horn neurons which is in line with the Ca^{2+} mobilizing effect of DHPG in other regions of the central nervous system of rodents, e.g. in CA1 region of a hippocampal slice preparation (Rae and Irving, 2004), in the cerebellum (Tempia et al., 2001), or in substantia nigra (Guatteo et al., 1999).

In contrast, selective activation of groups II or III mGluRs had no effect on cytosolic Ca^{2+} concentration in superficial dorsal horn neurons. At a first glance, this is not surprising, since these G-protein coupled receptors are usually linked to inhibition of cAMP formation. However, at least group II mGluR activation has been shown to increase $[Ca^{2+}]_i$ in the prefrontal cortex (Otani et al., 2002) or in hippocampal neurons (Maiese et al., 1999). We therefore tested the contribution of all mGluR subtypes in superficial dorsal horn. Besides the general coupling of the receptors to G_i/G_o proteins, another reason for the lacking effect of groups II or III mGluR activation may be the synaptic expression of these receptor subtypes. In the superficial laminae of the rat spinal cord (in contrast to deeper laminae), group II mGluR2 or GluR3 and group III mGluR4 and mGluR7 are localized predominantly on presynaptic glutamatergic and GABAergic terminals (Azkue et al., 2001; Carlton et al., 2001; Jia et al., 1999; Tang and Sim, 1999). Since measurements were performed in somata and proximal dendrites of superficial dorsal horn neurons in the present study, the evoked changes in $[Ca^{2+}]_i$ should be mediated by postsynaptically localized receptors (i.e. group I mGluRs).

4.2. Ca^{2+} rise is mediated by both group I subtypes mGluR1 and mGluR5

Our results suggest that both group I subtypes are involved in mGluR-mediated Ca^{2+} signalling in superficial spinal dorsal horn, since combined pre-treatment with the subtype

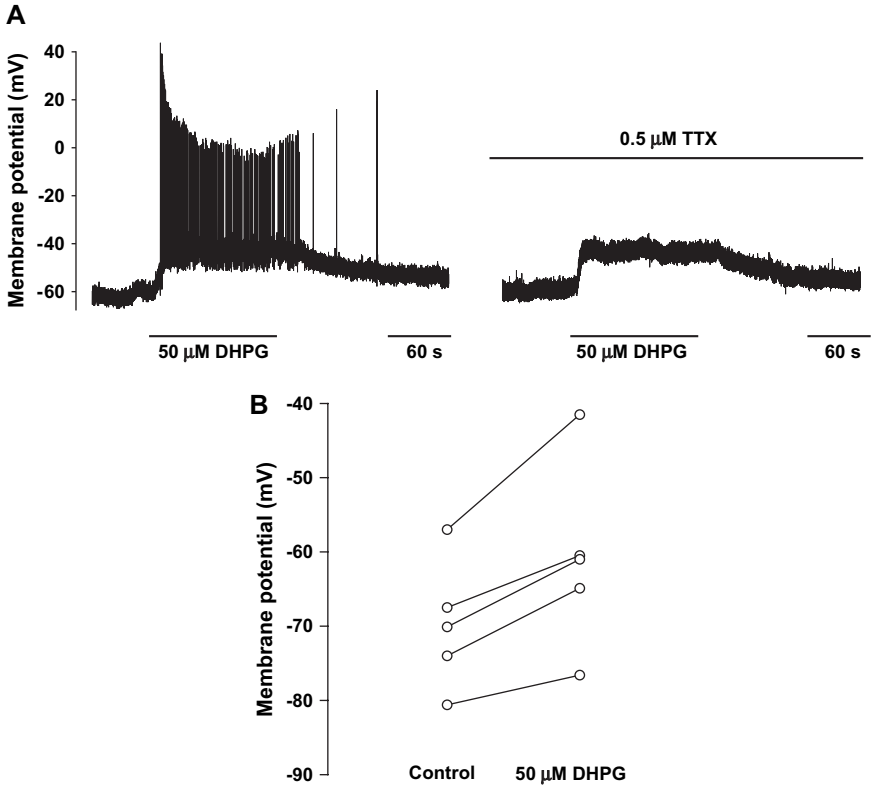

Fig. 4. Membrane depolarization of superficial dorsal horn neurons by (*S*)-3,5-DHPG application. (A) In a superficial dorsal horn neuron, (*S*)-3,5-DHPG-induced a membrane depolarization associated with spontaneous action potential firing (left trace). In the presence of TTX (0.5 μM) the depolarization was not modified (right trace). (B) Plot summarizing the depolarizing effect of (*S*)-3,5-DHPG in five experiments (one neuron per slice).

antagonists 4-CPG (Brabet et al., 1995; Hayashi et al., 1994) and MPEP (Gasparini et al., 1999) is necessary to abolish DHPG-induced increase in $[Ca^{2+}]_i$. The residual component of the DHPG-evoked Ca^{2+} rise in the combined presence of both mGluR1 and mGluR5 antagonists might be either due to a different sensitivity of the drugs for mGluR1 or mGluR5 splice variants, or due to a displacement of the antagonists by (*S*)-3,5-DHPG.

Surprisingly, activation of mGluR5 by the agonist CHPG produced a similar rise in $[Ca^{2+}]_i$ as the application of DHPG. Recently, the selectivity of CHPG has been questioned by different groups, showing that even at high concentrations CHPG did not discriminate between mGluR1 and mGluR5 (Nakamura et al., 2000; Rae and Irving, 2004). Here, lack of selectivity is unlikely, because the selective mGluR5 antagonist MPEP nearly abolished the CHPG-induced increase in $[Ca^{2+}]_i$. The DHPG concentration used (50 μM of the active enantiomer (*S*)-3,5-DHPG) was in the upper range, known to be effective on intracellular Ca^{2+} concentrations in neuronal slice preparations (Guatteo et al., 1999; Rae and Irving, 2004). Even though it is unlikely, that this was below the maximally effective concentration, it cannot be ruled out. But the most likely explanation is that DHPG has been shown to be only a partial agonist on mGluR1 as well as on mGluR5 (Brabet et al., 1995).

In situ hybridisation and immunolabelling studies have demonstrated the presence of mGluR1 and mGluR5 splice variants in the superficial laminae of the rat spinal cord (Alvarez et al., 2000; Berthele et al., 1999; Jia et al., 1999; Tang and Sim, 1999). The present data indicate that both subtypes are functionally co-expressed on the same neurons. Immunohistochemical and electrophysiological evidence for the coexistence of both group I mGluR subtypes in a single neuronal subtype has been provided in different regions of the central nervous system (Marino et al., 2001; Pisani et al., 2001; Poisik et al., 2003; Rae and Irving, 2004). Similar distribution of mGluR1 and mGluR5 indicates a co-expression on the same neurons also in superficial spinal dorsal horn (Jia et al., 1999).

Co-expression of members of the same group of mGluRs in the same cell is not indicative of a redundancy in the function of these receptors, as it has been assumed until recently. There is now a growing body of evidence that in many cases a clear separation of function exists between mGluR1 and mGluR5 when expressed in the same neuronal population (for review: see Valenti et al., 2002).

Compelling behavioural and electrophysiological evidence suggest the functional relevance of both group I mGluR subtypes for acute and persistent spinal nociception (for review see: Neugebauer, 2002; Varney and Gereau, 2002). Intrathecal pre-treatment with specific mGluR1 or mGluR5 antagonists or anti-rat antibodies both significantly attenuated DHPG-induced spontaneous nocifensive behaviours in mice (Karim et al., 2001) and rats (Fundytus et al., 1998) in vivo. Additionally, systemic or intrathecal administration of MPEP, a potent and selective mGluR5 antagonist, prevents, reduces or even

effectively reverses thermal hyperalgesia and/or mechanical hyperalgesia in a wide variety of rodent nociceptive and hypersensitivity models including CFA- or carrageenan-induced inflammatory pain, capsaicin-induced hypersensitivity, formalin-induced pain, post-operative hypersensitivity and different neuropathic pain models (Fisher et al., 2002; Soliman et al., 2005; Varty et al., 2005; Walker et al., 2001; Zhu et al., 2004, 2005). Antagonists of mGluR1 subtype, intrathecally applied before capsaicin treatment or chronic constriction injury of the sciatic nerve, also reduced development of mechanical hypersensitivity in these pain models (Fisher et al., 2002; Soliman et al., 2005). Interestingly, in inflammation-evoked nociception in mice, where antagonists of both group I mGluR subtypes produce antinociceptive effects, the mGluR1 antagonist appeared to produce stronger analgesia than the mGluR5 antagonist (Karim et al., 2001). Taken together, both group I mGluR subtypes seem to play important roles in spinal nociceptive mechanisms of inflammatory and neuropathic pain (Neugebauer, 2002; Varney and Gereau, 2002).

4.3. DHPG-induced Ca^{2+} rise is mediated by IP_3-receptor activation

DHPG-induced activation of group I mGluRs leading to cytoplasmatic Ca^{2+} rise in superficial spinal dorsal horn was mediated in part by Ca^{2+} release from IP_3-sensitive reticular stores. Since group I mGluRs are positively coupled to PLC activation, this is consistent with an increase in $[Ca^{2+}]_i$ mediated by IP_3-generation following hydrolysis of phosphoinositides as the underlying mechanism. However, other mechanisms as direct coupling of mGluR1 or mGluR5 subtypes to IP_3-receptors via linkage by post-synaptic density scaffold proteins Homer and/or Shank, which has been described in the rodent cerebellum (Xiao et al., 2000) and hippocampus (Sala et al., 2005), respectively, may also play a role.

4.4. Intracellular Ca^{2+} mobilization requires a diffusible compound

A significant DHPG-induced Ca^{2+} rise in superficial dorsal horn neurons could be seen only in cells which are loaded with the membrane-permeable form of fura-2, thereby preventing cell dialysis by the patch pipette. In previous studies we and others showed that LTD of synaptic strength between Aδ-fibres and neurons in the superficial spinal dorsal horn could be induced by DHPG application only in perforated patch-clamp recordings or recordings using sharp microelectrodes, respectively, (Chen et al., 2000; Gerber et al., 2000; Heinke and Sandkühler, 2005), which both minimise the washout of diffusible molecules. In cultured rat astrocytes, DHPG failed to induce a Ca^{2+}-dependent K^+ current in the conventional whole-cell patch-clamp configuration, but was effective in the gramicidin-perforated-patch configuration (Chen et al., 1997). Thus, one or more diffusible components of the DHPG-induced signalling cascade seem to be necessary to increase cytosolic Ca^{2+} concentration, but the nature of these compounds remains unknown.

4.5. DHPG-induced depolarization and Ca^{2+} influx through L-type VDCCs

In spinal superficial dorsal horn neurons, DHPG application induced Ca^{2+} influx from extracellular space through L-type VDCCs, as has been described in other neurons, e.g. in the striatum (Mao and Wang, 2003). Furthermore, DHPG-induced $[Ca^{2+}]_i$ oscillations in lamprey spinal cord neurons are dependent on Ca^{2+} influx through L-type channels (Kettunen et al., 2002). To activate high-threshold L-type VDCCs substantial membrane depolarization is a prerequisite. Such a depolarization may be induced by DHPG in superficial dorsal horn neurons (our study and Zhong et al., 2000).

Membrane depolarization by DHPG can be strong enough to open L-type VDCCs, as has been shown in hippocampal pyramidal cells (Bianchi et al., 1999) and in striatal neurons (Mao and Wang, 2003). Additionally, agonists of group I mGluRs facilitate L-type Ca^{2+} channels in different cell types, for example in neurons of the nucleus tractus solitarius of rats (Endoh, 2004) and in catfish horizontal cells (Linn, 2000). Thus, DHPG may induce sufficient depolarization of the cell membrane to reach the threshold for opening of facilitated L-type VDCCs.

4.6. Proposed mechanisms leading to DHPG-induced $[Ca^{2+}]_i$ rise in superficial spinal laminae

Our results suggest that Ca^{2+} released from intracellular stores and Ca^{2+} influx from extracellular space through voltage-dependent L-type Ca^{2+} channels both contribute to the rise in $[Ca^{2+}]_i$ induced by group I mGluR activation in superficial spinal dorsal horn neurons. In other cell types, mGluR dependent Ca^{2+} entry through L-type VDCCs has been shown to be triggered by Ca^{2+} released from IP_3- and/or ryanodine-sensitive stores (Fagni et al., 2000; Perrier et al., 2002). Here, such a mechanism is unlikely, because even with depleted intracellular Ca^{2+} stores, DHPG caused a substantial $[Ca^{2+}]_i$ rise in superficial dorsal horn neurons. Thus, L-type Ca^{2+} channels are activated in these cells by DHPG-induced membrane depolarization independent from a rise in $[Ca^{2+}]_i$, as has been demonstrated for example in hippocampal pyramidal cells (Bianchi et al., 1999). Activation of group I mGluRs may depolarize cells by activation of cation channels or more likely here, inhibition of K^+ channels. This has been shown for M-type K^+ channels directly coupled to group I mGluRs by Homer proteins (Kammermeier et al., 2000), or for G-protein-gated inwardly rectifying K^+ channels (GIRK) by PLC-mediated PKC activation (Sharon et al., 1997). GIRK1 and GIRK2 subunits are expressed in superficial layers of the dorsal horn and they are involved in modulation of nociceptive processing in spinal cord (Marker et al., 2004).

5. Conclusion

The presently described rise in $[Ca^{2+}]_i$ in superficial spinal dorsal horn neurons by activation of group I mGluRs likely contributes to activity-dependent long-term changes in

nociception and to pharmacological action of group I mGluR receptor agonists applied to spinal cord.

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