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Signal transduction pathways of group I metabotropic glutamate receptor-induced long-term depression at sensory spinal synapses

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Abstract

Activation of spinal group I metabotropic glutamate receptors (mGluRs) may have antinociceptive or pro-nociceptive effects in different pain models. Pharmacological activation of group I mGluRs leads to long-term depression (LTD) of synaptic strength between Aδ-fibers and neurons in lamina II of spinal dorsal horn of the rat. Here, we studied the signal transduction pathways involved. Synaptic strength between Aδ-fibers and lamina II neurons was assessed by perforated whole-cell patch-clamp recordings in a spinal cord-dorsal root slice preparation of young rats. Bath application of the specific group I mGluR agonist (*S*)-3,5-dihydroxyphenylglycine [(*S*)-3,5-DHPG] produced an LTD of Aδ-fiber-evoked responses. LTD induction by (*S*)-3,5-DHPG was prevented, when intracellular Ca^{2+} stores were depleted by thapsigargin or cyclopiazonic acid (CPA). Preincubation with ryanodine to inhibit Ca^{2+}-induced Ca^{2+} release had no effect on LTD-induction by (*S*)-3,5-DHPG. In contrast, pretreatment with 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of inositol-1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores prevented LTD induction. Preincubation with the specific protein kinase C (PKC) inhibitors bisindolylmaleimide I (BIM) or chelerythrine, respectively, had no effect. Inhibition of L-type VDCCs by verapamil or nifedipine prevented LTD-induction by (*S*)-3,5-DHPG. The presently identified signal transduction cascade may be relevant to the long-term depression of sensory information in the spinal cord, including nociception.

Keywords: Intracellular Ca^{2+} stores; L-type VDCC; Nociception; Protein kinase C; Rat; (S)-3,5-DHPG

1. Introduction

Strength of nociceptive synapses between primary afferent Aδ- or C-fibers and second order neurons in superficial laminae of spinal dorsal horn can be modulated in an activity-dependent manner. Both, use-dependent synaptic long-term potentiation (LTP) (Azkue et al., 2003; Ikeda et al., 2003; Randic et al., 1993; Sandkühler and Liu, 1998) as well as long-term depression (LTD) (Chen and Sandkühler, 2000; Randic et al., 1993; Sandkühler et al., 1997) have been shown in vivo and in vitro. Plasticity at these synapses may account for afferent-induced hyperalgesia, allodynia (Moore et al., 2000; Sandkühler, 2000a; Willis, 2002) and analgesia (Sandkühler, 2000b). Some forms of spinal LTP and LTD depend on activation of group I metabotropic glutamate receptors (mGluRs) (Azkue et al., 2003; Chen and Sandkühler, 2000; Gerber et al., 2000). For the induction of LTD, activation of group I mGluRs is not only required, but also sufficient, since application of selective group I mGluR agonist DHPG induces LTD (so-called DHPG-LTD) between primary afferent Aδ-fibers and second order neurons in laminae I and II in vitro (Chen et al., 2000; Zhong et al., 2000). Behavioral studies also attribute group I mGluRs an important role in acute and persistent nociception at the spinal cord level (for review see: Fundytus, 2001; Neugebauer, 2002).

Group I mGluRs consist of mGluR1 and mGluR5 subtypes, which are both expressed in lamina II neurons (Alvarez et al., 2000; Jia et al., 1999; Vidnyanszky et al., 1994). Group I mGluRs are G-protein-coupled to phospholipase C (PLC) with two different downstream pathways,

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leading to activation of PKC or IP_3 (Conn and Pin, 1997; Hermans and Challiss, 2001). DHPG-dependent LTD in superficial spinal dorsal horn is mediated by PLC activation (Chen et al., 2000). PKC, but not the IP_3 pathway, may be involved in pro-nociceptive effects of spinally applied DHPG in behaving animals (Fisher and Coderre, 1998; Fundytus et al., 2001), since thermal hyperalgesia in inflammatory and neuropathic pain models depends on activation of spinal PKC (Igwe and Chronwall, 2001; Malmberg et al., 1997; Yashpal et al., 2001). In contrast, the IP_3 pathway seems not to be required for hyperalgesia, as inhibition of intracellular Ca^{2+} release by intrathecal thapsigargin had neither an effect in the formalin test (Álvarez-Vega et al., 2001) nor on hyperalgesia in diabetic mice (Ohsawa and Kamei, 1999). At present it is not clear, if the IP_3 pathway plays any role in spinal nociception and which signal transduction pathway(s) mediate(s) mGluR-induced antinociception. Therefore, we tested, which pathway(s) downstream to, or independent of the PLC cascade leads to the induction of DHPG-dependent LTD at a well-defined site of nociceptive processing at or near the first central synapse in superficial spinal dorsal horn.

2. Experimental procedures

2.1. Preparation of animals and spinal cord slices

Transverse slices were obtained from young Sprague Dawley rats of both sexes (18- to 24-d-old). Under deep ether anesthesia, lumbar spinal cord was exposed by laminectomy. The segments of the lumbosacral spinal cord (L4-S1) were excised. Transverse slices with one long (8–15 mm) dorsal root 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 in 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. A single slice was then transferred to a recording chamber (volume 1.0 ml), 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. Picrotoxin (100 μM) and strychnine (4 μM) were always included in the bathing solution.

Recordings were made from one neuron per slice at room temperature.

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

2.2. Recording techniques and data acquisition

Perforated whole-cell patch-clamp recording technique was used to record excitatory postsynaptic currents (EPSCs) in neurons of lamina II, which was identifiable as a translucent band across the superficial spinal dorsal horn. Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) on a horizontal puller (P-87, Sutter Instruments, Novato, CA, USA) and had resistances of 3–6 MΩ. For perforated patch recordings, the antibiotic gramicidin at a concentration of 50 μg/ml was used in a solution containing (in mM): potassium gluconate, 120; KCl, 20; $MgCl_2$, 2; HEPES, 20; pH 7.28 adjusted with KOH; osmolarity 300–310 mosmol/kg. Pipettes were first front-filled with gramicidin-free solution and thereafter backfilled with the same solution plus the ionophore. To detect occasional spontaneous breakthrough of the seal, we added either the fluorescent dye lucifer yellow (1 mg/ml; Sigma, Deisenhofen, Germany) or the potassium channel blocker tetraethylammonium chloride (TEA; 10 mM; Sigma) to the pipette solution. Cells were rejected, if they showed intracellular fluorescence during epifluorescence illumination or a distinct broadening of their action potential duration, respectively. Usually, series resistance started to decrease 10–20 min after sealing. After reaching stable resistances of 25–50 MΩ, cell capacitance and series resistance were compensated, and recordings were started. Mean input resistance of the recorded cells was 649 ± 69 MΩ ($n = 65$).

Neurons were 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) and an epifluorescence facility. 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 8 (Molecular Devices) was used for data acquisition and subsequent off-line analysis.

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 15 s intervals. Stimulation intensity was adjusted to supramaximal values. EPSC amplitudes ranged from 20 to 350 pA. Only the EPSCs that were produced by excitation of Aδ-fibers (calculated conduction velocity ranged between 2.5 and 15 m/s) were investigated further. A 10 Hz train of stimulation of 10 pulses was applied to the dorsal roots to test for mono- or polysynaptic input. Monosynaptic input was identified by the absence of failures of the first EPSC component in response to the stimuli and a constant latency with nearly no jitter (<0.5 ms) in responses. Six consecutive responses were averaged and synaptic strength was quantified by measuring the amplitude of the first peak of averaged responses.

The mean amplitude of six averaged test responses recorded prior to the agonist application served as controls. Significant changes from controls were assessed by measuring the amplitudes of six consecutive responses at the end of the agonist application and at 20–30 min after washout of the agonist.

2.3. 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; 100 μM; Tocris, Köln, Germany), picrotoxin (100 μM; Tocris), strychnine (4 μM; Sigma), gramicidin D (Sigma), thapsigargin (1 μM; Alexis, Grünstadt, Germany), cyclopiazonic acid (CPA; 30 μM; Calbiochem, Bad Soden, Germany), 2-aminoethoxydiphenyl borate (2-APB; 100 μM; Calbiochem), ryanodine (20 μM; Sigma), dantrolene (25 μM; Sigma), nifedipine (50 μM; Sigma), verapamil (50 μM; Sigma), bisindolylmaleimide I (BIM; GF109203X; 5 μM; Tocris) and chelerythrine chloride (5 μM; Calbiochem). (*S*)-3,5-DHPG was dissolved in an aqueous stock solution diluted in perfusing salt buffer just before use. The solvent for picrotoxin was ethanol (final maximal concentration 0.25%, v/v). All other drugs were dissolved in dimethylsulphoxide (DMSO; Sigma; maximal final concentration 0.25%, v/v). In control experiments, this DMSO concentration did no significantly affect membrane potential, membrane resistance or strength of synaptic transmission in superficial spinal dorsal horn neurons. The mean amplitude of Aδ-fiber-evoked EPSCs changed to 98 ± 5% of baseline ($n=6$, data not shown) following DMSO application.

2.4. Data analysis

Analysis of the data was performed using SigmaStat 2.03 (Systat Software GmbH, Erkrath, Germany). Values are given as means ± one standard error of the mean (SEM). If not stated otherwise, data were tested for normality (Kolmogorov–Smirnov test), and than a one way repeated measures analysis of variance (ANOVA) was performed for statistical comparison followed by an appropriate post-hoc test ($P<0.05$ was considered to be statistically significant).

3. Results

Stable recordings of up to 2 h were made from a total of 65 neurons in lamina II of the spinal dorsal horn. In all of the neurons included in this study, electrical stimulation of Aδ-fibers in the attached dorsal root evoked fast EPSCs at a holding potential of −70 mV. The majority of the Aδ-fiber-evoked EPSCs recorded was monosynaptic in nature (66%). In all figures, only results from monosynaptically evoked EPSCs are summarized. Statistical analysis was applied to the effect of (*S*)-3,5-DHPG on mono- and polysynaptically evoked EPSCs, respectively, wherever applicable, and additionally to the pooled data (summarized in Table 1). All of the neurons included in this study had membrane potentials more negative than −50 mV and the mean resting membrane potential of these cells was −59 ± 1 mV. In pilot studies, using the conventional whole-cell patch-clamp technique, pharmacological activation of group I mGluRs failed to affect synaptic transmission in lamina II ($n=4$, data not shown), probably because an essential diffusible compound was lost. This was prevented in all subsequent experiments by using the perforated patch-clamp recording technique to measure postsynaptic currents. Under these conditions, application of the specific agonist of group I mGluRs (*S*)-3,5-DHPG (Ito et al., 1992) in the superfusing solution (100 μM for 20 min) reduced the mean amplitude of monosynaptically evoked EPSCs to 65 ± 6% of control ($P<0.001$; Table 1, Fig. 1). EPSC amplitudes were still depressed to 58 ± 7% of control after washing for 20 min (long-term depression, LTD; $n=5$, $P<0.001$ vs. control). The (*S*)-3,5-DHPG-induced LTD persisted throughout the recording periods of up to 75 min after commencing washout, confirming previous results (Chen et al., 2000; Zhong et al., 2000). The mean negative holding current necessary to voltage clamp neurons at −70 mV was transiently increased 2 min after (*S*)-3,5-DHPG application from −18 ± 4 pA to −28 ± 4 pA ($n=9$, $P<0.01$, paired *t*-test) and the mean membrane resistance decreased from 731 ± 99 MΩ to 651 ± 89 MΩ ($n=9$, $P<0.05$, paired *t*-test).

The group I mGluR-mediated LTD is expressed via a PLC-initiated signaling cascade (Chen et al., 2000). Here, we examined the effects of reagents that target specific steps in this pathway. Ca^{2+} is a second messenger that is released from IP_3-sensitive intracellular stores upon group I mGluR activation. The sarco/endoplasmic reticulum Ca^{2+}/ATPase (SERCA) pump inhibitor thapsigargin depletes intracellular stores of Ca^{2+} by blocking Ca^{2+} re-uptake into the stores (Bian et al., 1991). To examine the role of Ca^{2+} release from intracellular stores by (*S*)-3,5-DHPG, thapsigargin (1 μM) was applied to slices 20 min prior to and during group I mGluR activation. Superfusion with thapsigargin had no significant effect on EPSC amplitude, but abolished the LTD-induction by (*S*)-3,5-DHPG (Table 1, Fig. 2A). Similar results were obtained with cyclopiazonic acid (CPA, 30 μM), another SERCA pump inhibitor. CPA alone did not change EPSC amplitude, but effectively prevented (*S*)-3,5-DHPG-mediated LTD-induction (Table 1, Fig. 2B).

Ca^{2+}-release from internal stores may be mediated by the activation of IP_3 receptors or ryanodine receptors. Since, both receptors have been demonstrated immunohistochemically and/or functionally in rat spinal dorsal horn neurons (Rodrigo et al., 1993; Sharp et al., 1993; Voitenko et al., 1999), we used specific receptor blockers to discriminate between the involvement of IP_3- and/or ryanodine-sensitive intracellular stores. Since we performed perforated patch-

Table 1
Effect of pretreatment of spinal cord slices with different inhibitors of Ca^{2+} signaling and of PKC, respectively, on DHPG-induced direct and long-term depression in lamina II of the dorsal horn

EPSCs	DHPG effect	No pre-treatment	Thapsigargin	CPA	2-APB	Ryanodine	Verapamil	Nifedipine	BIM	Chelerythrine
mean EPSC amplitudes (±SEM) in % of control (before DHPG application)										
Monosynaptic	Direct	65±6 (5)***	103±5 (3) n.s.	100±5 (4) n.s.	104±5 (3) n.s.	75±1 (5)***	79±4 (4)**	94±3 (6) n.s.	66±9 (6)**	61±1 (2) n.t.
	Long-term	58±7 (5)***	n.m.	n.m.	100±14 (3) n.s.	68±7 (5)***	97±5 (4) n.s.	95±4 (4) n.s.	67±10 (6)**	56±9 (2) n.t.
Polysynaptic	Direct	63±10 (4)**	98±8 (4) n.s.	95 (1) n.t.	94±3 (2) n.t.	n.m.	85±7 (2) n.t.	105 (1) n.t.	n.m.	46±3 (4)***
	Long-term	58±7 (4)**	n.m.	n.m.	89±14 (2) n.t.	n.m.	n.m.	121 (1) n.t.	n.m.	35±5 (4)***
Pooled	Direct	64±5 (9)***	100±5 (7) n.s.	99±4 (5) n.s.	100±4 (5) n.s.	75±1 (5)***	81±3 (6)**	96±3 (7) n.s.	66±9 (6)**	51±4 (6)***
			###	###	###	no	no	###	no	no
	Long-term	58±6 (9)***	n.m.	n.m.	95±9 (5) n.s.	68±7 (5)***	97±5 (4) n.s.	100±6 (5) n.s.	67±10 (6)**	42±6 (6)***
					#	no	##	##	no	no

Mean EPSC amplitudes ±SEM in % of control. Statistical significance of direct (during application for 20 min) and long-term effects (20 min after wash-out) of (*S*)-3,5-DHPG application (100 μM) on synaptic strength between Aδ-fibers and spinal lamina II neurons was assessed by one-way repeated measures ANOVA compared to pre-drug control EPSCs: * $P<0.05$, ** $P<0.01$, *** $P<0.001$ in the post-hoc test; n.s., not significant; n.m., not measured; n.t., not tested; number of tested slices in brackets. Slices were treated 20 min prior to and during group I mGluR activation with thapsigargin (1 μM) or cyclopiazonic acid (CPA, 30 μM) to deplete intracellular Ca^{2+} stores, with the IP_3 receptor blocker 2-APB (100 μM), with ryanodine (20 μM) to inhibit ryanodine receptors, with verapamil (50 μM) or nicardipine (50 μM) to block L-type Ca^{2+} channels, or with the specific PKC inhibitors bisindolylmaleimide I (BIM, 5 μM) or chelerythrine (5 μM), respectively. One way ANOVA was also used to analyze differences in the strength of inhibition between treatments: # $P<0.05$, ## $P<0.01$, ### $P<0.001$ in the post-hoc test compared to the corresponding DHPG effect (direct or long-lasting) without pretreatment; no, not significant. In addition to the pooled data, statistics were applied to mono- and polysynaptically evoked EPSCs, respectively, wherever applicable.

clamp recordings, dialyzing the neurons with heparin, a membrane-impermeable antagonist of IP_3-activated Ca^{2+}-release channels, was not possible. We therefore used the membrane-permeable inhibitor 2-APB (Maruyama et al., 1997). When washed into the slice, 2-APB (100 μM for 20 min) completely suppressed LTD-induction by subsequent application of (*S*)-3,5-DHPG (Table 1, Fig. 3A).

Ca^{2+} released from other intracellular stores may also contribute to the induction of DHPG-LTD. Superfusing the slices with both ryanodine and dantrolene, inhibitors of Ca^{2+}-induced Ca^{2+}-release had no significant effect on LTD-induction by DHPG. Using dantrolene (25 μM), the mean amplitude of EPSCs was reduced by (*S*)-3,5-DHPG to about 80% of control after 5 min, but no stable recordings could be obtained in none of five cells, since there was a continuous run-down to about 40% of control 20 min after starting wash out of (*S*)-3,5-DHPG (data not shown). Preincubation with ryanodine (20 μM) for 20 min had no effect on EPSC amplitudes. Subsequent application of (*S*)-3,5-DHPG reduced the mean amplitude of Aδ-fiber evoked EPSCs significantly and synaptic transmission remained depressed for at least further 20 min after starting wash out of (*S*)-3,5-DHPG (Table 1, Fig. 3B).

Activation of PLC may also lead to the production of diacylglycerol and activation of PKC. To evaluate the role

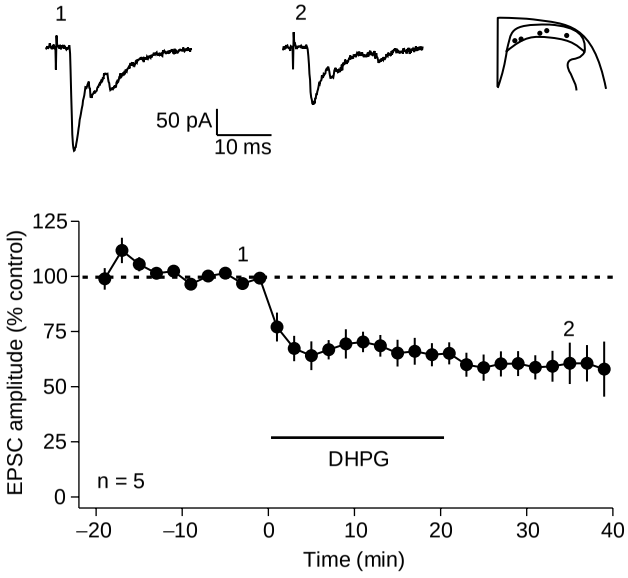

Fig. 1. Selective activation of group I mGluRs with (*S*)-3,5-DHPG induced an LTD of fast excitatory synaptic transmission in superficial spinal dorsal horn. Mean time course of immediate and long-lasting inhibition of synaptic strength between primary afferent Aδ-fibers and neurons in lamina II of spinal dorsal horn, induced by group I mGluR activation in five experiments. (*S*)-3,5-DHPG was added to the superfusion solution for 20 min (100 μM; horizontal bar). The mean amplitude of six consecutive EPSCs immediately prior to application of (*S*)-3,5-DHPG served as controls ($n=5$ monosynaptic EPSCs). Neurons were voltage clamped at −70 mV. Representative original Aδ-fiber-evoked monosynaptic (first component) EPSC recordings of one experiment prior to (*S*)-3,5-DHPG application (trace 1) and after washout of the drug (trace 2). The recording sites in lamina II for all monosynaptic experiments are shown in the inset.

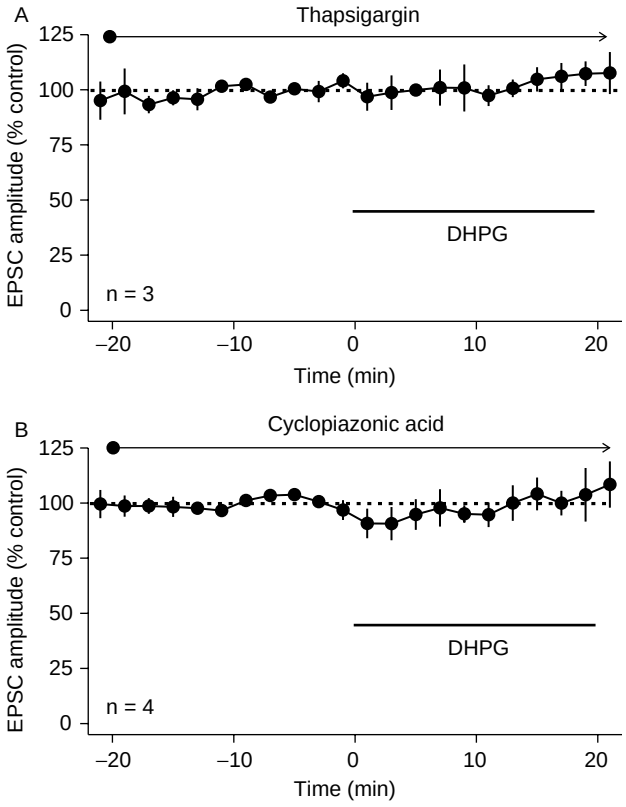

Fig. 2. Ca^{2+} release from intracellular stores is required for LTD-induction by (*S*)-3,5-DHPG application. Intracellular Ca^{2+} stores were depleted by bath application of thapsigargin (1 μM; $n=3$ monosynaptic EPSCs; (A)) or cyclopiazonic acid (CPA, 30 μM; n=4 monosynaptic EPSCs; (B)) at least 20 min before superfusing the slices with (*S*)-3,5-DHPG (100 μM), thereby preventing Ca^{2+} release and LTD-induction. The mean amplitude of six consecutive EPSCs immediately prior to application of (*S*)-3,5-DHPG served as controls.

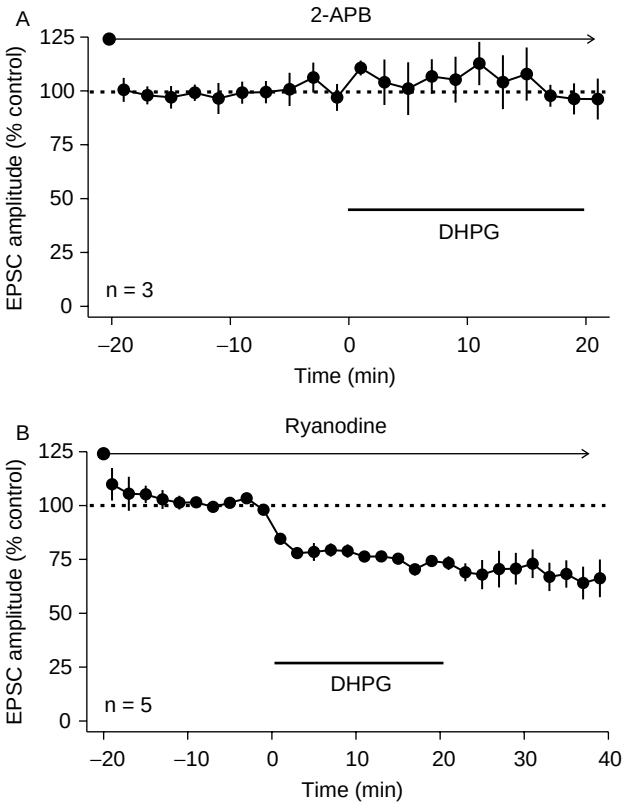

Fig. 3. LTD-induction by (*S*)-3,5-DHPG application depends on IP_3-sensitive but not on ryanodine-sensitive stores. (A) Preincubation with 2-APB (100 μM), an inhibitor of IP_3-sensitive Ca^{2+} stores, at least 20 min before the application of (*S*)-3,5-DHPG (100 μM) prevented LTD-induction of synaptic transmission by (*S*)-3,5-DHPG ($n=3$ monosynaptic EPSCs). (B) Blocking Ca^{2+}-induced Ca^{2+} release by superfusing the slices with ryanodine (20 μM) for at least 20 min failed to prevent LTD-induction of synaptic transmission by subsequent application of (*S*)-3,5-DHPG ($n=5$ monosynaptic EPSCs). The mean amplitude of six consecutive EPSCs immediately prior to application of (*S*)-3,5-DHPG served as controls.

of this pathway, we pretreated the slices with either chelerythrine (Herbert et al., 1990) or bisindolylmaleimide I (BIM; Toullec et al., 1991), respectively, both inhibiting PKC by specific interacting with the catalytic domain of this kinase. Bath application of BIM (5 μM for 20 min) had no significant effect on normal synaptic transmission, nor did it prevent the induction of DHPG-LTD (Table 1, Fig. 4A). Using chelerythrine (5 μM) as the specific PKC inhibitor gave similar results. Preincubation for 20 min had no effect on EPSC amplitudes. Subsequent administration of (*S*)-3,5-DHPG reduced the mean amplitude of Aδ-fiber evoked EPSCs significantly and synaptic transmission remained depressed for at least further 20 min after starting wash out of (*S*)-3,5-DHPG (Table 1, Fig. 4B). Thus, LTD-induction of synaptic strength between primary afferent Aδ-fibers and neurons in superficial spinal dorsal horn by group I mGluRs does not require activation of PKC.

Several forms of LTD have been found to depend on Ca^{2+} influx through L-type VDCCs (Christie and Abraham, 1994; Normann et al., 2000; Wu et al., 2001). Agonists acting on group I mGluRs may facilitate L-type Ca^{2+} channels in different cell types (Chavis et al., 1996; Svirskis and Hounsgaard, 1998). DHPG may induce Ca^{2+} influx through L-type Ca^{2+} channels in striatal neurons (Mao and Wang, 2003). Here, pretreatment with the L-type Ca^{2+} channel blocker verapamil (50 μM for 20 min) did not change normal synaptic transmission. Verapamil did, however, attenuate the reduction of the mean EPSC amplitudes during (*S*)-3,5-DHPG application (for 20 min) significantly (Table 1, Fig. 5A). Washout of (*S*)-3,5-DHPG led to a complete recovery of EPSC amplitudes to baseline within 8 min (Table1, Fig. 5A). Bath application of another inhibitor of L-type Ca^{2+} channels, the dihydropyridine nifedipine (50 μM for 20 min), also suppressed induction of DHPG-LTD, but did not prevent transient reduction of EPSC amplitudes during superfusion with (*S*)-3,5-DHPG (for 20 min; Table 1, Fig. 5B). Thus, the inhibition of Ca^{2+} influx through L-type VDCCs prevented long-term, but not direct depression of synaptic strength in spinal lamina II.

To test if there were acute and/or long-term effects of the various inhibitors to the DHPG-LTD, we performed statistical analysis (one way ANOVA) to elucidate potential

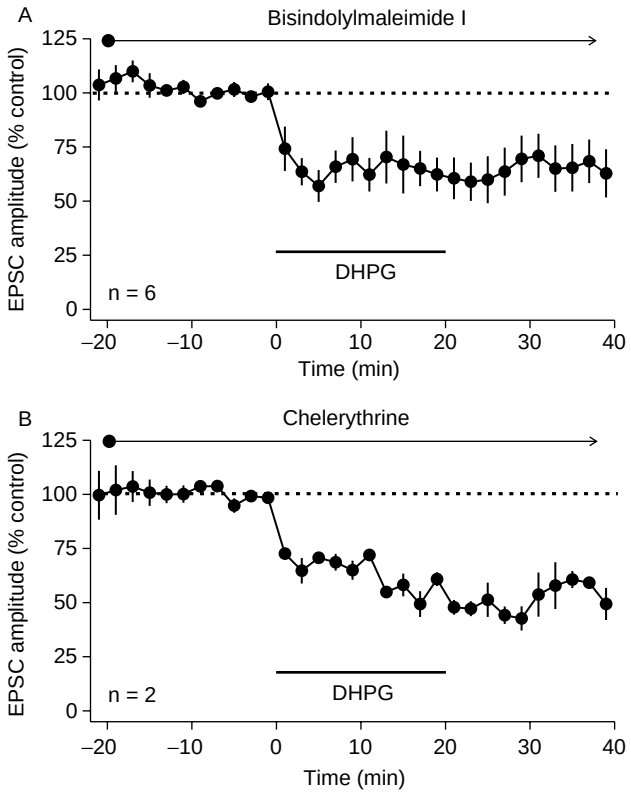

Fig. 4. Activation of PKC is not required for LTD-induction by (*S*)-3,5-DHPG application. Specific PKC inhibitors bisindolylmaleimide I (BIM, 5 μM; $n=6$ monosynaptic EPSCs; (A)) or chelerythrine (5 μM; $n=2$ monosynaptic EPSCs; (B)) were added to the bathing solution at least 20 min before the application of (*S*)-3,5-DHPG (100 μM). The presence of PKC inhibitors in the superfusate failed to prevent LTD-induction of synaptic transmission by (*S*)-3,5-DHPG. The mean amplitude of six consecutive EPSCs immediately prior to application of (*S*)-3,5-DHPG served as controls.

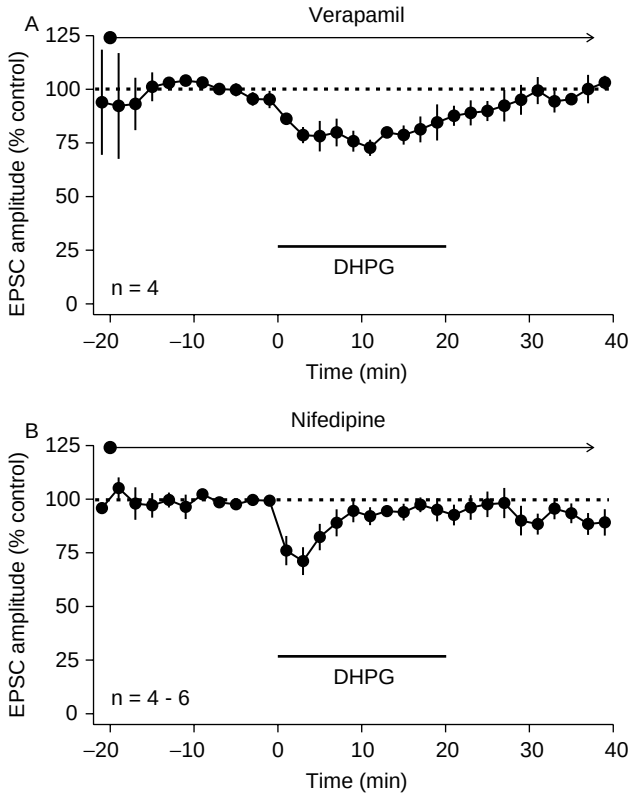

Fig. 5. Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels is required for induction of LTD by (*S*)-3,5-DHPG application. Slices were pretreated with the specific L-type Ca^{2+} channel blockers verapamil (50 μM; $n=4$ monosynaptic EPSCs; (A)) or nifedipine (50 μM; $n=6$ monosynaptic EPSCs during DHPG application and still 4 after washout of DHPG; (B)) for at least 20 min before the application of (*S*)-3,5-DHPG (100 μM). In the presence of L-type Ca^{2+} channel blockers, (*S*)-3,5-DHPG produced an acute depression but failed to induce LTD of synaptic transmission. The mean amplitude of six consecutive EPSCs prior to the application of (*S*)-3,5-DHPG served as controls.

effects of the different treatments on the strength of synaptic inhibition. No significant difference could be revealed between DHPG effect without pretreatment and pretreatment with ryanodine, verapamil, BIM or chelerythrine, respectively (Table 1). Additionally, there was no difference in the long-term effect (after DHPG washout) between these groups, except for verapamil (Table 1). In contrast, synaptic strength during DHPG application was significantly different after pretreatment with thapsigargin, CPA, 2-APB or nifedipine, respectively, from that without pretreatment. No difference could be detected between the pretreatment groups (Table 1).

4. Discussion

We have explored the signal transduction pathways leading to LTD of synaptic strength between afferent Aδ-fibers and neurons in the spinal lamina II of the rat following activation of group I mGluRs. We have previously demonstrated, that the activation of PLC is necessary for group I mGluR-induced LTD in the spinal dorsal horn (Chen et al., 2000). Here, we show that, downstream the G-protein-activated phosphoinositide turnover, it is the IP_3 branch of this cascade and not PKC that mediates the LTD of synaptic strength. In addition, Ca^{2+} influx through L-type VDCCs is a prerequisite for long-term, but not for acute depression of synaptic strength.

4.1. Pre- or postsynaptic expression of DHPG-LTD in spinal lamina II?

The locus of induction and expression of long-term synaptic plasticity is still controversial. In spinal lamina II evidence is in favor for a postsynaptic induction mechanism: (1) Dialyzing the postsynaptic neurons by conventional whole-cell recordings prevented the induction of LTD by DHPG application in the present study; (2) DHPG application increased the negative current necessary to hold the cells at −70 mV indicating a membrane depolarization in postsynaptic cells; (3) several

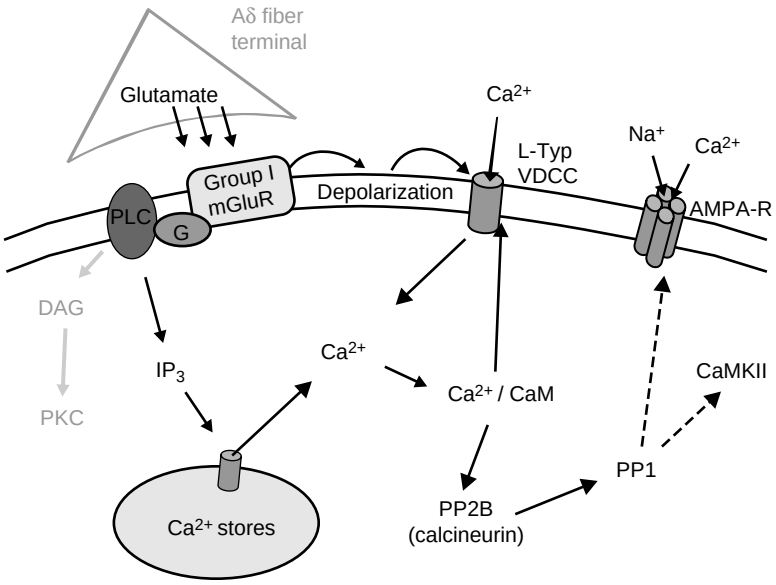

Fig. 6. Hypothetical postsynaptic mechanisms leading to DHPG-induced LTD in spinal lamina II. Glutamate activates the phospholipase C (PLC), diacylglycerol (DAG), inositol triphosphate (IP_3) pathway, which triggers the release of Ca^{2+} from intracellular stores, leading to up-regulation of L-type Ca^{2+} channels via Ca^{2+} binding to calmodulin. Depolarization of the postsynaptic membrane therefore intensifies Ca^{2+} signaling by group I mGluR activation. The resulting Ca^{2+}/calmodulin complex activates protein phosphatase 2B (PP2B), triggering the disinhibition of protein phosphatase 1 (PP1). Dephosphorylation and therefore down-regulation of AMPA receptors and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) may lead to a long-lasting depression of synaptic strength. Solid arrows, activation or facilitation; dashed arrows, inactivation (dephosphorylation). Adapted and modified from Kemp and Bashir (2001) and Perrier et al. (2002).

immunohistochemical studies failed to detect mGluR1/5 immunoreactivity in primary afferent sensory terminals and found only little evidence of immunoreactivity in other presynaptic axon terminals in spinal lamina II of the rat (Alvarez et al., 2000; Jia et al., 1999; Tang and Sim, 1999; Vidnyanszky et al., 1994), pointing to a postsynaptic expression of these receptors. However, it cannot be excluded, that the LTD in our preparation was induced postsynaptically and expressed presynaptically, mediated by a retrograde signaling mechanism.

4.2. Activation of PKC is not required for group I mGluR-induced LTD

Chelerythrine and BIM, two of the most potent and selective PKC inhibitors known (Herbert et al., 1990; Toullec et al., 1991), failed to block LTD-induction in the present study. The applied concentration (5 μM) was in the upper range of concentrations known to be effective on synaptic responses in spinal cord slice preparations (Garraway et al., 2003; Kawasaki et al., 2004). Additionally, application of 1 μm BIM prevented LTP induction in spinal lamina I neurons by conditioning stimulation of the attached dorsal root (Sandkühler and Ikeda, 2003), demonstrating the efficacy of the inhibitor in our preparation. PKC activation is also not necessary for DHPG-LTD at synapses in other regions of the central nervous system, for example in the rat dentate gyrus (Rush et al., 2002) and in the CA1 region of the hippocampus (Schnabel et al., 1999; Schnabel et al., 2001). Thus, the depressant effects of group I mGluRs apparently do not involve the PKC branch of signal transduction.

In contrast, facilitation of nociceptive processing by DHPG in some behavioral studies (Adwanikar et al., 2004; Fisher and Coderre, 1998) may depend on PKC activation. Activation of spinal PKC is necessary for the induction of hyperalgesia and allodynia in various inflammatory and neuropathic pain models (Igwe and Chronwall, 2001; Malmberg et al., 1997; Yashpal et al., 2001), including group I mGluR-dependent pain states (Fundytus et al., 2001; Guo et al., 2004).

Since, PKC activation played no role in DHPG-induced LTD in spinal lamina II in vitro (present study), dichotomy of signal transduction pathways downstream to PLC activation may explain qualitatively different effects of group I mGluR activation on nociception.

4.3. Ca^{2+} release from intracellular stores is required for group I mGluR-induced LTD

Our results show, that Ca^{2+} release from IP_3-sensitive stores is required for DHPG-induced LTD in superficial spinal dorsal horn neurons. LTD-induction, which requires Ca^{2+} release from internal stores, has also been described in the hippocampus (O'Mara et al., 1995; Reyes and Stanton, 1996; Wang et al., 1997). Some of these LTD forms required Ca^{2+}-induced Ca^{2+} release from a ryanodine receptor-gated Ca^{2+} pool (O'Mara et al., 1995; Reyes and Stanton, 1996). Our results with inhibition of ryanodine receptors suggest, however, that Ca^{2+}-induced Ca^{2+}

release is not necessary to amplify the Ca^{2+} signal initiated by IP_3 to induce DHPG-LTD in spinal lamina II neurons. Similar to the present findings, DHPG effects mediated by Ca^{2+}-release from ryanodine-insensitive internal stores, but not by PKC activation, has also been described in other systems. For example, DHPG-induced $[Ca^{2+}]_i$ oscillations in lamprey spinal cord neurons were abolished by thapsigargin, but not by ryanodine or PKC inhibitors (Kettunen et al., 2002). Inhibitory action of DHPG on membrane excitability of dentate granule neurons was blocked by IP_3 receptor inhibition, but not by PKC inhibition (Abdul-Ghani et al., 1996).

In contrast to the PKC branch of PLC signal transduction pathway, the IP_3 pathway seems not to be essential for induction of hyperalgesia. Inhibition of Ca^{2+} release from intracellular stores by intrathecal thapsigargin had no effect in the formalin test (Álvarez-Vega et al., 2001) nor on pain behavior in diabetic mice (Ohsawa and Kamei, 1999). There are contradicting results in normal mice, where pro-nociceptive (Ohsawa and Kamei, 1999) as well as antinociceptive (Bernstein and Welch, 1995) effects of intrathecally applied thapsigargin were reported. These data have to be interpreted with caution, since thapsigargin not only prevents intracellular Ca^{2+} release by depleting IP_3-sensitive stores, but also increases cytosolic Ca^{2+} concentration, at least transiently, by inhibiting ATPase-dependent Ca^{2+} re-uptake into these stores (Bian et al., 1991). In the present study, we excluded, that the resulting Ca^{2+} increase mediates the thapsigargin effect on LTD-induction by DHPG, by preincubating the slices with ATPase inhibitors for at least 20 min. This treatment had no impact on synaptic strength.

In conclusion, pharmacological activation of group I mGluRs in spinal cord may have both, pro-nociceptive effects mediated by the PKC branch of PLC pathway and long-lasting antinociceptive effects mediated by synaptic LTD which involves the IP_3 pathway.

4.4. Ca^{2+} influx through L-type VDCCs is required for group I mGluR-induced LTD

In line with other studies in the hippocampus of the rat (Christie and Abraham, 1994; Normann et al., 2000; Wu et al., 2001) we found that Ca^{2+} influx into the postsynaptic cell through L-type VDCCs is essential for LTD-induction. 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). In striatal neurons, DHPG induces Ca^{2+} influx through L-type VDCCs (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).

Membrane depolarization by DHPG can be strong enough to open L-type VDCCs (Bianchi et al., 1999; Mao and Wang, 2003). Since, we performed our recordings under voltage-clamp conditions ($V_{Hold} = -70$ mV), the question arises, how and where a sufficient depolarization may occur to activate high-threshold L-type VDCCs. The series resistance, which is achievable by the perforated patch recording technique, is three to five times higher than in conventional whole-cell recordings. This inevitably magnifies space-clamp problems especially in neurons with an extended tree of dendrites, including many neurons in lamina II of the spinal dorsal horn. Thus, it is well possible, that under nominal 'voltage-clamp' conditions DHPG may induce substantial depolarization of the cell membrane (Zhong et al., 2000) at distal dendrites, that reaches the threshold for opening of facilitated L-type VDCCs. Additionally, a contribution of these channels to the resting intracellular Ca^{2+} concentration has been described (Avery and Johnston, 1996; Magee et al., 1996). This seems to be in contrast to the voltage dependency of L-type channel activation. However, cloned L-type channels, composed of α1D (Cav1.3) subunit, can be activated at membrane potentials around -60 mV (Koschak et al., 2001; Perrier et al., 2002). This corresponds to the resting membrane potential of many spinal lamina II neurons. These class D L-type channels are expressed in the rat spinal dorsal horn (Westenbroek et al., 1998).

4.5. Hypothetical postsynaptic mechanisms leading to DHPG-induced LTD in spinal lamina II

Our results suggest, that Ca^{2+} released from intracellular stores and Ca^{2+} influx from extracellular space through voltage-dependent L-type channels is necessary to pass threshold Ca^{2+} level for induction of LTD in superficial spinal dorsal horn neurons. Presumably, group I mGluR stimulation may release Ca^{2+} from reticular Ca^{2+} stores leading to facilitation of L-type VDCCs (Perrier et al., 2002) via Ca^{2+} binding to calmodulin (Zühlke et al., 1999). According to the hypothesis of Lisman (2001), level and/or kinetic of the resulting precipitous postsynaptic Ca^{2+} rise by group I mGluR activation is sufficient to initiate a phosphatase cascade (involving calcineurin and protein phosphatase-1 (PP1)). This leads to dephosphorylation of its substrates, which may include the 'molecular switch' Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) and AMPA receptors, resulting in long-lasting synaptic weakening (Kemp and Bashir, 2001) (Fig. 6).

4.6. Functional consequences of spinal DHPG-LTD

In addition to the present results, other electrophysiological studies also demonstrate a potential antinociceptive effect of intrathecally applied DHPG on responses of spinal dorsal horn neurons to noxious stimuli, at least at higher concentrations (Neugebauer et al., 1999; Stanfa and Dickenson, 1998), even though the phenotype of the recorded neurons (excitatory or inhibitory) in these and in the present study is unclear. Some data indicate a potential

analgesic effect of spinally administered DHPG (Dolan and Nolan, 2002), but pro-nociceptive effects may predominate.

5. Conclusions

Synaptic LTP and LTD are considered to be fundamental cellular mechanisms of learning and memory in the hippocampus (Bliss and Collingridge, 1993; Bortolotto et al., 1999) and in pain pathways (Sandkühler, 2000a). Our present study elucidates signal transduction pathways leading to long-lasting depression of presumably nociceptive synaptic transmission in the rat superficial spinal dorsal horn, induced by selective activation of group I mGluRs. This may propel the targeted development of novel drugs, which may cause analgesia outlasting the period of application.

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