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PKCε Mediates Substance P Inhibition of $GABA_A$ Receptors-Mediated Current in Rat Dorsal Root Ganglion

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Summary: The mechanism underlying the modulatory effect of substance P (SP) on GABA-activated response in rat dorsal root ganglion (DRG) neurons was investigated. In freshly dissociated rat DRG neurons, whole-cell patch-clamp technique was used to record GABA-activated current and sharp electrode intracellular recording technique was used to record GABA-induced membrane depolarization. Application of GABA (1–1000 μmol/L) induced an inward current in a concentration-dependent manner in 114 out of 127 DRG neurons (89.8 %) examined with whole-cell patch-clamp recordings. Bath application of GABA (1–1000 μmol/L) evoked a depolarizing response in 236 out of 257 (91.8%) DRG neurons examined with intracellular recordings. Application of SP (0.001–1 μmol/L) suppressed the GABA-activated inward current and membrane depolarization. The inhibitory effects were concentration-dependent and could be blocked by the selective neurokinin 1 (NK_1) receptors antagonist spantide but not by L659187 and SR142801 (1 μmol/L, n=7), selective antagonists of NK_2 and NK_3. The inhibitory effect of SP was significantly reduced by the calcium chelator BAPTA-AM, phospholipase C (PLC) inhibitor U73122, and PKC inhibitor chelerythrine, respectively. The PKA inhibitor H-89 did not affect the SP effect. Remarkably, the inhibitory effect of SP on GABA-activated current was nearly completely removed by a selective PKC_ε inhibitor epilon-V1-2 but not by safingol and LY333531, selective inhibitors of PKC_α and PKC_β. Our results suggest that NK_1 receptor mediates SP-induced inhibition of GABA-activated current and membrane depolarization by activating intracellular PLC-Ca^{2+}-PKC_ε cascade. SP might regulate the excitability of peripheral nociceptors through inhibition of the "pre-synaptic inhibition" evoked by GABA, which may explain its role in pain and neurogenic inflammation.
Key words: peripheral nervous system; substance P; $GABA_A$ receptor; protein kinase C; dorsal root ganglion

GABA functions as an inhibitory neurotransmitter in the spinal cord and can act pre-synaptically to reduce release of neurotransmitters from primary afferent terminals, termed "pre-synaptic inhibition"[1]. The function of $GABA_A$ receptor chloride channel complex is regulated by phosphorylation and dephosphorylation[2]. $GABA_A$ receptor is down-regulated by direct phosphorylation via protein kinase C (PKC)[3–6]. Biochemical studies have also suggested that phosphorylation of $GABA_A$ receptor by various protein kinases inhibits $GABA_A$ receptor function[7].

PKC is a family of serine/threonine kinases that are divided into three groups based on calcium and diacylglycerol dependence. These different PKC isozymes function as key signal transducers in cells allowing them to regulate a number of cellular functions including differentiation, proliferation, cell migration, and apoptosis making them attractive therapeutic targets for a host of human diseases[8]. The α, βI, βII, and γ isozymes are calcium- and diacylglycerol-dependent and are termed conventional (c) PKCs. The δ, ε, η, and θ isozymes are calcium-independent but diacylglycerol-dependent and are termed the novel (n) PKCs. The ξ and λ/ι isozymes are calcium- and diacylglycerol-independent and termed the atypical (a) PKCs[8]. cPKCs (α, βI, βII, γ), nPKCs (ε, δ) and aPKC (ξ) are expressed in the brain of rats[9–13]. PKC α, βI, βII, δ, ε, and ξ isozymes have also been identified in primary afferents that transmit nociceptive signals from the peripheral site of injury to the superficial dorsal horn[14]. Within the superficial laminae of the dorsal spinal cord, an area that has been implicated in pain processing, PKC α, βI, βII, and γ isoforms have been identified[15]. *In vitro* electrophysiology suggests that PKC is involved in modulating opioid and $GABA_A$ receptor function. Both cholecystokinin and substance P (SP) decrease inhibitory GABA-activated currents via PKC-dependent phosphorylation of the $GABA_A$ receptor[5, 6, 16]. These findings suggest that PKC can attenuate inhibitory neurotransmission in the pain pathway. PKC activation

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also enhances tetrodotoxin-resistant Na^+ currents[17], thus increasing action potential propagation to the central terminals of afferent neurons. One potential mechanism by which pre-synaptic PKC activity augments neurotransmitter release is PKC-dependent sensitization of voltage-dependent L-type Ca^{2+} channels[18].

SP is a pain transmitter and can modulate noxious responses in spinal cord dorsal horn[3, 19]. Application of SP depolarizes dorsal root ganglion (DRG) neurons and activates an inward current, thereby increasing the excitability of sensory nociceptors[5, 20–24]. Growing evidence supports the existence of SP receptors in the membrane of rat DRG neurons which might also express $GABA_A$ receptors[20–22, 25]. Furthermore, electrophysiological studies show that PKC activation potentiates capsaicin-induced depolarization in afferent neurons[26], which correlates with enhanced capsaicin-induced SP release from spinal cord slices[27]. While enhanced release of neurotransmitters may be secondary to PKC-mediated events at the peripheral terminal, there is evidence that local PKC activity at the pre-synaptic terminal also contributes. In afferent neurons, PKC activation alone can increase SP and CGRP release as well as potentiate potassium- and capsaicin-stimulated release of these neuropeptides[27–29]. More specifically, inhibition of PKCε decreases capsaicin-induced release of glutamate and CGRP in isolated spinal cords[30].

In the present study, whole-cell patch-clamp technique and intracellular recordings were performed to explore the role of PKC_ε in SP inhibition of the $GABA_A$ receptor-mediated responses in rat DRG neurons.

1 MATERIALS AND METHODS

1.1 Study Design

A randomized and controlled animal experiment was performed at the Department of Physiology, Shihezi University Medical College, China from August 2010 to November 2012. Healthy Sprague-Dawley rats were provided by the Experimental Animal Center of Xinjiang Medical University, China (license No. SCXK 2003-0001). Rats were housed in separate cages with a specific pathogen-free level barrier environment at 24±3°C, relative humidity of 40%–70%, 12-h dark/12-h light cycle (100–120 lx), and allowed free access to food and water. The protocol was conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China.

1.2 DRG Preparations

Irrespective of sex, 2- to 3-weeks-old Sprague-Dawley rats were used in this experiment[4, 16]. The rats were anaesthetized with ether followed by laminectomy at L_4 or L_5. The DRGs with attached dorsal roots and spinal nerves were dissected out, the fibrous sheath surrounding the DRGs was torn off carefully under a stereoscope, then the isolated preparation was transferred into recording chamber (0.25 mL volume), and perfused with oxygenated balanced salt solution (BSS) at room temperature. BSS contained (mmol/L): NaCl 140, KCl 5, $MgCl_2$ 1, glucose 5 and Tris-HCl 5 (pH 7.4). The flow rate was 3–5 mL/min. The preparation was pinned with tiny steel pins onto a silicone gum block, which was placed on the bottom of the chamber. The sciatic nerve was placed onto a pair of platinum stimulating electrodes in the neighboring compartment.

1.3 Intracellular Recording

Intracellular recordings were obtained by using glass microelectrode filled with 2 mol/L KCl and 1 mol/L potassium acetate on DRG preparation, the direct current (DC) resistance of which was in the range of 25–60 MΩ. The membrane potentials were amplified with a microelectrode amplifier (MEZ-8301, Japan) and membrane depolarization was filtered at 20 Hz. The data were recorded with a pen recorder (XWTD-264, China). The values of resting membrane potentials used in the preparations were stable at least for 10–20 min[4, 16].

1.4 Isolation of DRG Neurons

Irrespective of sex, rats, aged 8–10 weeks, weighing 250–280 g, were decapitated. The thoracic and lumbar segments of vertebrae column were dissected and longitudinally divided into two halves along the median lines on both dorsal and ventral sides. The DRGs together with dorsal and ventral roots and attached spinal nerves were taken out from the inner side of each half of the dissected vertebrate and transferred into Dulbecco's Modified Eagle's Medium (DMEM: 13.84 g/L, NaCl 2.64 g/L, Sigma, USA) at pH=7.4, 340 mOsmol/kg, immediately. After the removal of attached nerves and surrounding connective tissues, the DRGs were minced by using iridectomy scissors and incubated with enzymes including trypsin (type Ⅲ, Sigma, USA) 0.5 g/L, collagenase (type IA, Sigma, USA) 1 g/L and DNase (type Ⅳ, Sigma, USA) 0.1 g/L in 5 mL DMEM at 35°C in a shaking bath for 40 min. To stop the enzymatic digestion, soybean trypsin inhibitor (type Ⅱ-S1, Sigma, USA) 1.25 g/L was added. The isolated neurons were transferred into a 35-mm culture dish and kept still at least for 30 min[5, 22].

1.5 Whole-cell Patch Clamp Recordings of DRG Neurons

Whole-cell patch clamp recordings were carried out at room temperature (25–30°C) using a whole-cell patch clamp amplifier[5, 22]. Currents were recorded from single dorsal root ganglion neurons *in vitro* using an Axon 700B amplifier (Axon, USA) and the pCLAMP 10.2 (Axon, USA). The micropipettes were filled with internal solution containing (mmol/L): KCl 140, $MgCl_2$ 2.5, HEPES 10, EGTA 11 and ATP 5. The pH was adjusted to 7.2 with KOH, and osmolarity was adjusted to 310 mOsm/L with glucose. The cells were bathed in an external solution containing (mmol/L): NaCl 150, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, HEPES 10, D-glucose 10. Osmolarity was adjusted to 340 mOsm/L with glucose, and pH was adjusted to 7.4 with NaOH. The resistance of the recording pipette was in the range of 2–5 MΩ. A small patch of membrane underneath the tip of the pipette was aspirated to form a gigaseal, and then a negative pressure was applied to rupture it, thus a whole-cell configuration was formed. The adjustment of capacitance compensation and series resistance compensation (compensates about 70%) was carried out before the membrane currents were recorded. The holding potential was set at –60 mV, except when indicated otherwise. Membrane currents were filtered at 10 kHz[5, 22].

1.6 Harvest of Single DRG Neuron and Its Immunocytochemical Study

Soon after the whole-cell patch-clamp recordings had been done, a neuron was sucked into a glass pipette, whose tip was about 40 μm in diameter, by means of a gentle suction and transferred onto a gelatine-smeared slide by a puff. All procedures including the following immunocytochemical staining were performed and

monitored under an inverted microscope[31]. The cells were fixed in 4% paraformaldehyde dissolved in 0.1 mol/L phosphate buffer for 3 h. After washes for 10 min three times in phosphate-buffered saline (PBS), the following immunoperoxidase staining procedures were done: the neuron was incubated overnight at 4°C with polyclonal anti-SP antibody (product of China Academy of Traditional Chinese Medicine) at 1:1000 to 1:3000 dilution, and then affinity-purified goat anti-rabbit IgG (1:50) for 1–3 h and a polyclonal rabbit peroxidase anti-peroxidase complex (1:50) for 1–2 h at room temperature (RT). Three 10-min washes in PBS were done after incubation with antibodies. A 20-min pre-incubation in 10% buffered saline/PBS preceded the application of secondary and tertiary antibodies. All peroxidase reaction was run for 2–10 min in 0.05% 3,3'-diaminobenzidine (Sigma, USA) and 0.01% H_2O_2 in 0.05 mol/L Tris buffer (pH=7.4). The preparations were dehydrated and coverslipped. The secondary and tertiary antibodies were bought from Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China.

1.7 Drug Application

GABA, muscimol, bicuculline, SP, spantide, L659187, SR142801, chelerythrine, H-89, epilon V1-2, safingol, LY333531, BAPTA-AM and U73122 were from Sigma (USA). In whole-cell patch clamp recordings, all the drugs were dissolved in the external solution and applied by gravity flow from a row of tubules (outside diameter/internal diameter=500 μm/200 μm), respectively, through a connection with a series of independent reservoirs. The distance from the mouth of the tubule to the cell examined was around 100 μm. This rapid solution exchange system was manipulated by shifting the tubules horizontally with a micromanipulator. In intracellular recordings, all drugs were dissolved in BSS.

1.8 Statistical Analysis

Data were analyzed with SPSS 13.0 software (SPSS, USA) and the values of GABA-activated currents and induced-depolarization were presented as $\bar{x}\pm s_{\bar{x}}$. A homogeneity test for variance was performed followed by one-way analysis of variance, and two-group comparison was conducted using the least significant difference *t*-test. A $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 GABA-activated Inward Currents in Rat DRG Neurons

Experiments were carried out on 127 freshly-isolated DRG neurons, the diameters of which were in the range of 15–60 μm. GABA (1−1000 μmol/L) activated an inward current in the majority of the cells examined (89.8%, 114/127) in a concentration-dependent manner. The selective $GABA_A$ receptors agonist muscimol (100 μmol/L) mimicked GABA-activated response (n=11). Both GABA (100 μmol/L) and muscimol (100 μmol/L)-activated currents were completely and reversibly blocked by bicuculline (10–100 μmol/L, n=9), a specific antagonist of $GABA_A$ receptor (fig. 1B and 1C). The threshold concentration for GABA was 0.1 μmol/L, the maximum response was activated by 1000 μmol/L GABA and the K_d (dissociation constant) value was about 10 μmol/L from the concentration-response curve (fig. 2A and 2C).

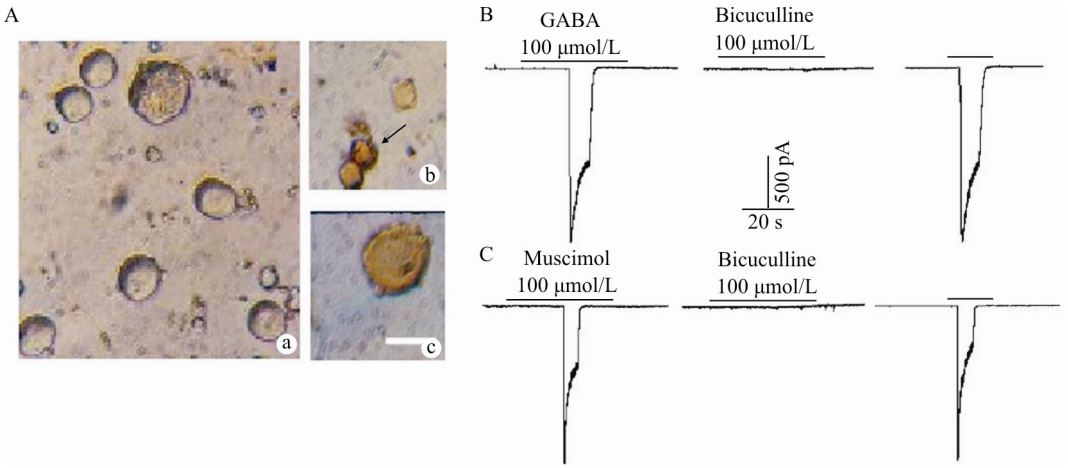

Fig. 1 Immunocytochemical results of harvested neurons and $GABA_A$ receptor-mediated inward current

A: The photomicrograph shows single freshly-isolated neurons obtained from rat DRG by means of enzymatic and mechanical treatment, and the diameters of these neurons were in the range of 20–58 μm (a). The photomicrographs represent tree cells after immunocytochemical procedures, in which SP-immunity reactivity was revealed in the two small cells whose SP-activated currents had been identified (24–26 μm) (b). The big neuron (54 μm) in the microphotograph was a negative control whose SP-activated currents were also observed (c) (scale bar=50 μm). B: The blockade of GABA (100 μmol/L)-activated inward current by $GABA_A$ receptor antagonist bicuculline (100 μmol/L), neuron from fig. 1Ab (indicated by an arrow). C: The blockade of muscimol (100 μmol/L)-activated inward current by $GABA_A$ receptor antagonist bicuculline (100 μmol/L), neuron from fig. 1Ac

2.2 Immunocytochemical Results of Harvested Neurons

Bath application of SP (0.001–10 μmol/L) activated a small and dose-dependent inward currents in the majority of the cells examined (90.9%, 40/44). The average amplitude of SP (10 μmol/L)-activated current was 236±91 pA (n=11)[5].

Freshly-isolated neurons were obtained from rat DRG by means of enzymatic and mechanical treatment; round-shaped cells with clear border and appearance

could be seen (fig. 1Aa). We focused our study on pain-sensing sensory neurons, i.e. the small and medium sized neuron. The diameters of 19 isolated neurons were in the range of 24–37 μm, and all of the neurons responding to application of SP (0.01–10 μmol/L) with an inward current were chosen for immunostaining (fig. 1Ab and 1B). Ten of these neurons appeared to be positive in SP-immunity reactivity (IR) (10/19, 52.6%), other nine of neurons was SP-IR negative (9/19, 47.4%) (fig. Ab). The cell in the fig. 1Ac was a negative control whose membrane was also found electrophysiologically to be endowed with SP receptor without SP-IR in the cell (54 μm) (fig. 1Ac and 1C).

2.3 Inhibitory Effect of SP on GABA-activated Current

Pre-application of SP (0.001−1 μmol/L) for 30 s prior to application of GABA markedly suppressed the GABA-activated current in most of the neurons examined (84.5%, 49/58). SP (0.1 μmol/L) shifted the concentration-response curve of GABA (100 μmol/L)-activated current downward and the estimated K_d value was around 10 μmol/L in the presence of SP (0.1 μmol/L). There were no significant differences in the threshold concentration, the maximum responsive concentration and the K_d value between GABA-activated current with and without pre-application of SP (fig. 2B and 2C). Inhibitory effect of SP started at least 30 s after pre-application of SP and there was no detectable change in GABA-activated current if the duration was less than 30 s. The maximal inhibition took place around 4 min after pre-application of SP and the ratio of inhibition was 49.8%±7.2% (n=7, P<0.01). It took almost 12 min to get a full recovery from SP inhibition (fig. 3).

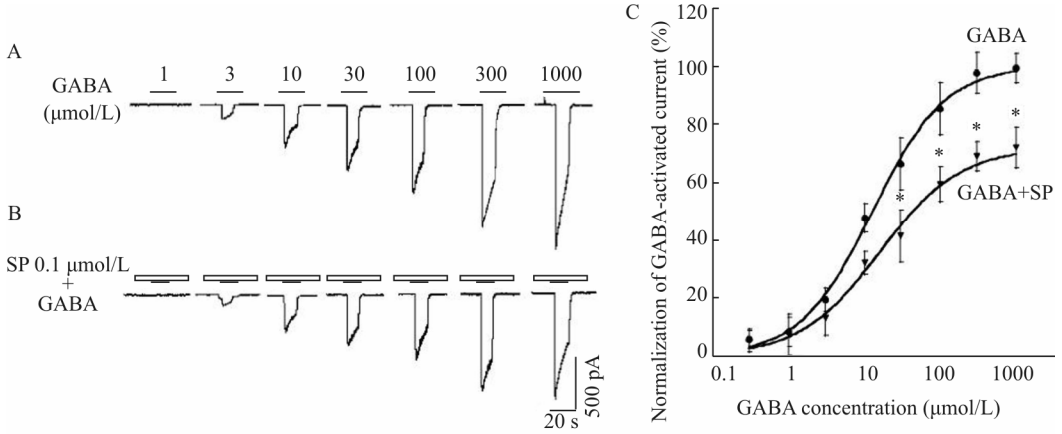

Fig. 2 The concentration-response curve for GABA in the absence or presence of SP on DRG neurons

A: the records of membrane inward current in response to different concentrations of GABA (1–1000 μmol/L) obtained from one neuron. B: the records of membrane inward current in response to different concentrations of GABA (1–1000 μmol/L) obtained after application of SP (0.1 μmol/L) in one neuron. C: the concentration-response curve for GABA (1–1000 μmol/L)-activated inward current without and with pre-application of SP (0.1 μmol/L). The curve was a good fit for the data to the logistic equation $Y=E_{max}/[1+(K_d/C)^n]$, in which C is the concentration of GABA (about 10 μmol/L), K_d is the dissociation constant of GABA-AR, and the Hill coefficient (n) is assumed to be 1. The curve for GABA with treatment of SP was drawn obviously as compared with that for GABA alone ($^*P$<0.05, paired t test).

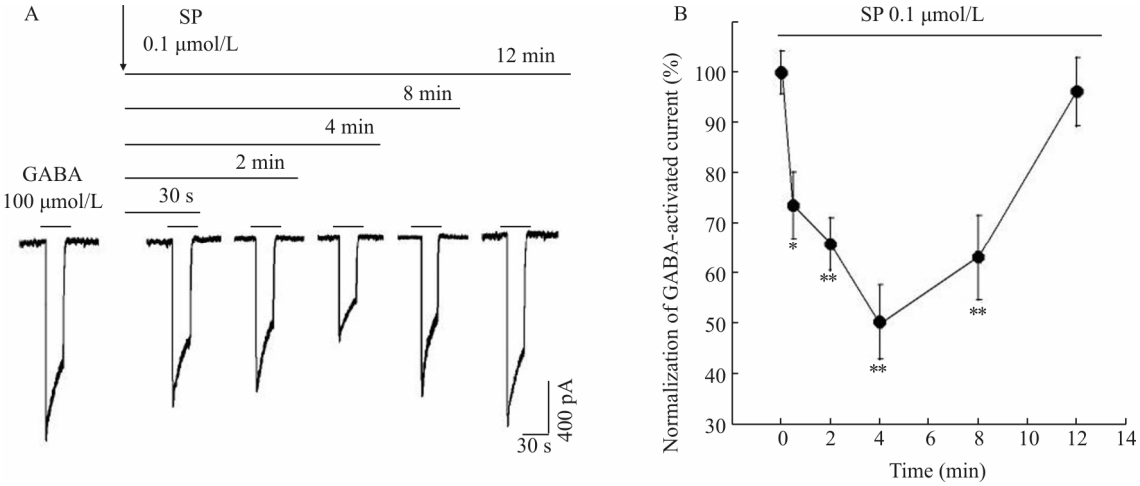

Fig. 3 SP-induced inhibition on GABA-activated currents at different intervals

A: The different intervals between pretreatment with SP (0.1 μmol/L) and application of GABA (100 μmol/L) could induce different inhibition ratio of SP-induced inhibition on GABA-activated currents. B: The inhibition ratio of SP (0.1 μmol/L)-induced inhibition on GABA (100 μmol/L)-activated currents was 0.0%±4.3%, 26.4%±6.7%, 34.2%±5.3%, 49.8%±7.4%, 36.9%±8.4% and 3.8%±6.8% when the different intervals between pretreatment with SP and application of GABA were 0, 0.5, 2, 4, 8 and 12 min (n=7–9, $^*P$<0.05, $^{**}P$<0.01, paired t test), respectively.

The SP (0.1 μmol/L)-induced inhibition on GABA-activated currents was abolished by the selective neurokinin 1 (NK_1) receptor antagonist spantide (20 μmol/L, *n*=7), but not by the selective NK_2 and NK_3 antagonist L659187 (1 μmol/L, *n*=7) or SR142801 (1 μmol/L, *n*=7) (fig. 4A and 4C). The inhibitory effect of spantide (5, 20, and 40 μmol/L) was concentration-dependent, while that of L659187 (1, 10, and 20 μmol/L) and SR142801 (1, 10, and 20 μmol/L) was not (fig. 4D).

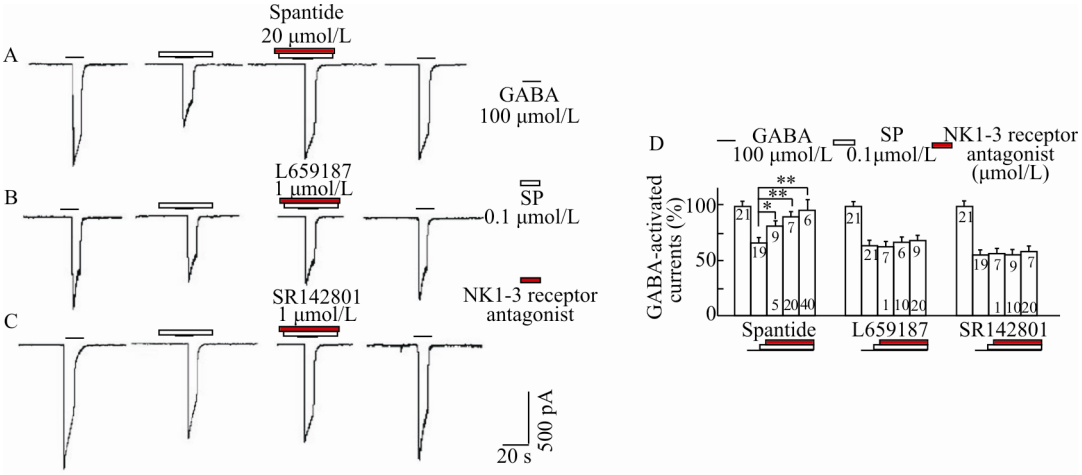

Fig. 4 Effects of NK_1, NK_2 and NK_3 receptor antagonists on the inhibition of GABA-activated inward current by SP

A–C: The SP (0.1 μmol/L)-induced inhibition could be completely suppressed by the selective NK_1 receptors antagonist spantide (20 μmol/L) (A), but not by the selective NK_2 and NK_3 antagonist L659187 (1 μmol/L) (B) and SR142801 (1 μmol/L) (C). D: The inhibition ratio of SP (0.1 μmol/L)-induced inhibition on GABA-activated inward current with pre-incubation of different concentrations of spantide, L659187 and SR142801. The inhibition ratio of SP-induced inhibition on GABA-activated membrane inward currents was 26.3%±3.4%, 17.7%±3.1%, 6.6%±6.1% (spantide, 5, 20, and 40 μmol/L), 41.9%±3.3%, 40.2%±5.5%, 38.8%±6.2% (L659187, 1, 10, and 20 μmol/L) and 48.3%±3.2%, 49.7%±5.3%, 47.9%±4.% (SR142801, 1, 10, and 20 μmol/L) (*n*=6–9, $^{*}P<0.05$, $^{**}P<0.01$, paired *t* test), respectively.

2.4 GABA-induced Depolarization

During intracellular recordings, both application of GABA (1–1000 μmol/L) evoked membrane depolarization in a concentration-dependent manner in 236 out of 257 DRG neurons (91.8%) examined. The averaged amplitude of membrane depolarization induced by 100 μmol/L GABA was 12.3±3.6 mV (*n*=27). The threshold was about 1 μmol/L and the maximal response was achieved by 300 μmol/L GABA. The K_d value was about 30 μmol/L deduced from the concentration-response curve (data not shown). A selective $GABA_A$ receptors antagonist bicuculline (100 μmol/L, *n*=8) strongly suppressed GABA (100 μmol/L)-evoked responses (*n*=12) (fig. 5A).

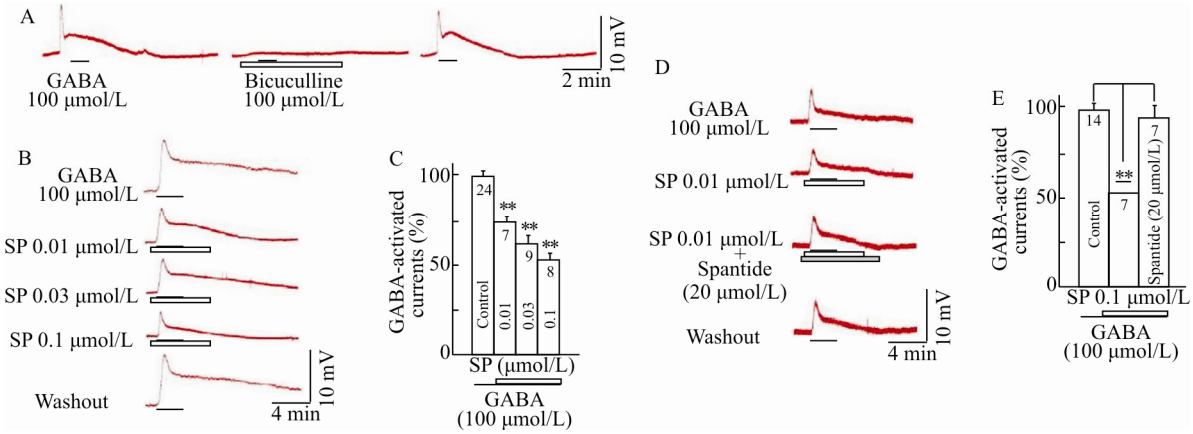

Fig. 5 Effect of $GABA_A$ receptor antagonist, different-concentrations of SP and NK_1 receptor antagonist on the GABA-induced depolarization

A: the blockade of GABA (100 μmol/L)-induced membrane depolarization by $GABA_A$ receptor antagonist bicuculline (100 μmol/L) (resting membrane potential: –57 mV). B: the records of GABA (100 μmol/L)-induced depolarization in different concentrations of SP (0.01, 0.03 and 0.1 μmol/L) (resting membrane potential: –63 mV). C: The inhibition ratio of SP on GABA-induced depolarization was suppressed by 28.1%±4.2%, 38.7%±4.6% and 43.3%±4.7% by 0.01, 0.03 and 0.1 μmol/L SP (*n*=7–9, $^{**}P<0.01$ *vs.* control, paired *t* test), respectively; D: The SP (0.1 μmol/L)-induced depolarization inhibition could be completely suppressed by the selective NK_1 receptors antagonist spantide (20 μmol/L) (resting membrane potential: –62 mV). E: the inhibition ratio of SP (0.1 μmol/L)-induced inhibition on GABA (100 μmol/L)-induced depolarization with pre-incubation of spantide (20 μmol/L). The inhibition ratio of SP-induced inhibition on GABA-activated membrane inward currents was 9.8%±7.4% (*n*=7, $^{**}P<0.01$, paired *t* test).

2.5 Inhibition of GABA-induced Depolarization by SP

When SP was pre-incubated for 2 min prior to application of GABA, the GABA-induced depolarization was attenuated markedly in most of the neurons examined (87.5%, 84/96). Inhibition of GABA-induced response by SP was concentration-dependent and increased gradually with the increase in SP concentration. The depolarization induced by GABA (100 μmol/L) was suppressed by 28.1%±4.2%, 38.7%±4.6% and 43.3%±4.7% by 0.01, 0.03 and 0.1 μmol/L SP (n=7–9), respectively (fig. 5B, 5C). It took almost 16 min to get a full recovery from SP-induced inhibition. Consistent with the above patch-clamp results, the SP (0.1 μmol/L)-induced inhibition on GABA-induced depolarization was abolished by the selective NK_1 receptor antagonist spantide (20 μmol/L, n=7) (fig. 5D, 5E).

2.6 Intracellular Signal Transduction Mechanisms Underlying SP-induced Inhibition of GABA-activated Current

To investigate the intracellular signal transduction mechanisms underlying SP inhibition of GABA-activated current, we used BAPTA-AM (a highly selective calcium chelating reagent), U73122 (a selective PLC inhibitor), chelerythrine (a selective PKC inhibitor) and H-89 (a selective PKA inhibitor). The SP-induced inhibition of GABA-activated current was strongly suppressed by BAPTA-AM (100 μmol/L, n=5), U73122 (1 μmol/L, n=6) or chelerythrine (1 μmol/L, n=9). However, H-89 (1 μmol/L, n=8) had no effect on SP inhibition of GABA-activated current (fig. 6A–6D). The inhibitory effect of BAPTA-AM, U73122, chelerythrine was concentration-dependent (fig. 6E).

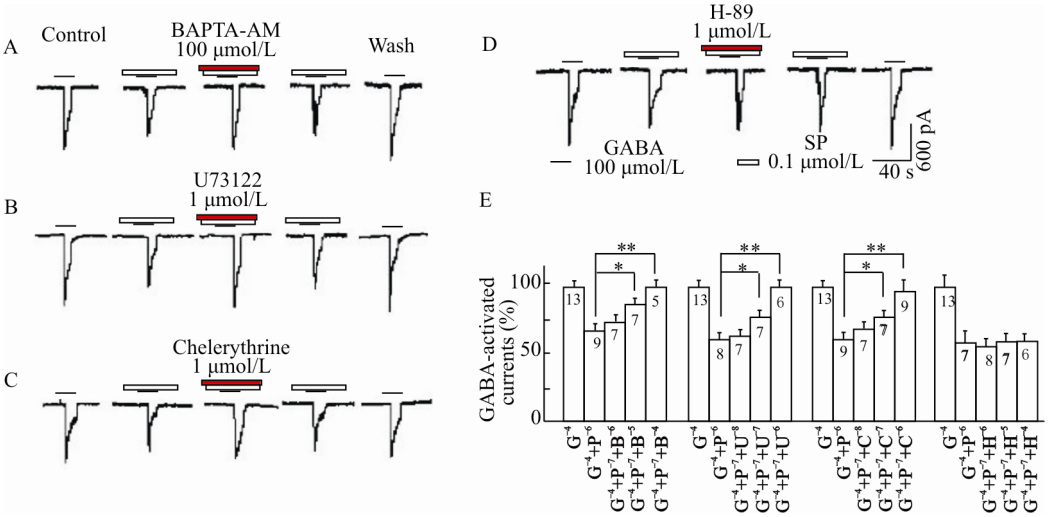

Fig. 6 Analysis of intracellular signal transduction mechanisms underlying the inhibition of GABA-activated inward current by SP

A–D: The SP (0.1 μmol/L)-induced inhibition on GABA (100 μmol/L)-activated inward current was almost completely removed after application of BAPTA-AM (100 μmol/L), U73122 (1 μmol/L) and chelerythrine (1 μmol/L), respectively, but H-89 (1 μmol/L) could not remove that. E: The inhibition ratio of SP (0.1 μmol/L)-induced inhibition on GABA (100 μmol/L)-activated inward current with pre-incubation of different concentrations of BAPTA-AM (1–100 μmol/L), U73122 (0.01–1 μmol/L), chelerythrine (1–100 μmol/L) and H-89 (1–100 μmol/L), (n=6–13, $^{*}P$<0.05, $^{**}P$<0.01, paired t test). G^{-4}: GABA 10^{-4} mol/L; P^{-6}: SP 10^{-6} mol/L; B^{-4}, B^{-5}, B^{-6}: BAPTA-AM 10^{-4}, 10^{-5}, 10^{-6} mol/L; U^{-6}, U^{-7}, U^{-8}: U73122 10^{-6}, 10^{-7}, 10^{-8} mol/L; C^{-6}, C^{-7}, C^{-8}: chelerythrine 10^{-6}, 10^{-7}, 10^{-8} mol/L; H^{-4}, H^{-5}, H^{-6}: H-89 10^{-4}, 10^{-5} 10^{-6} mol/L

2.7 PKC_{ε} Mediated Inhibition of GABA-activated Current by SP

To investigate which PKC subtype(s) mediates SP inhibition of GABA-activated current, we used epilon V1-2 (1 μmol/L, selective PKC_{ε} inhibitor), safingol (10 μmol/L, selective PKC_{α} inhibitor) and LY333531 (10 nmol/L, selective PKC_{β} inhibitor). The SP-induced inhibition on GABA-activated current was strongly suppressed by epilon V1-2 (1 μmol/L, n=6) but not by safingol (10 μmol/L, n=7) or LY333531 (10 nmol/L, n=7) (fig. 7A–7C). The inhibitory effect of epilon V1-2 was concentration-dependent (fig. 7D).

To exclude the possibility that membrane permeability precludes the translocation of PKC inhibitors, we used the re-patch technique[5]. We first patched the cells with regular internal solution and recorded SP (0.1 μmol/L) inhibition of GABA (100 μmol/L)-activated current (fig. 8A–8D, left). We next withdrew the recording pipette and re-patched the same cells with regular internal solution containing epilon V1-2 (1 μmol/L), safingol (10 μmol/L) or LY333531 (10 nmol/L). As expected, blockade of PKC_{ε} but not PKC_{α} or PKC_{β} significantly attenuated the inhibition of GABA-activated currents induced by SP (P<0.01, fig. 8E).

3 DISCUSSION

Combining electrophysiological method with immunhistochemical detection, McCarthy and Lawson[32] demonstrated that SP-IR was seen in 50% of C-fiber neurons and 20% of Aδ neurons. In our study, it has been shown that 41.2%–52.6% of rat DRG cells display positive SP-IR, most of which are of small size while others are of intermediate size[22]. In the present study and in our previous paper[22, 24], we have demonstrated that about 90% of DRG neurons were endowed with SP receptor. In rat DRG neurons, $GABA_A$ receptor mediated GABA-evoked membrane depolarizing response and inward current[4, 5]. Here we showed that pre-incubation of SP suppressed the GABA-evoked membrane depolarization and GABA-activated inward current markedly in most of the neurons examined. The inhibitory effect of

SP was mediated by the NK_1 receptors because the selective NK_1 antagonist spantide but not the selective NK_2 and NK_3 antagonists L659187 and SR142801 nearly abolished the inhibitory effect of SP. Therefore, activation of SP (NK_1) receptor can down-regulate the function of $GABA_A$ receptor in rat sensory neurons, like that in bullfrog DRG neurons[6].

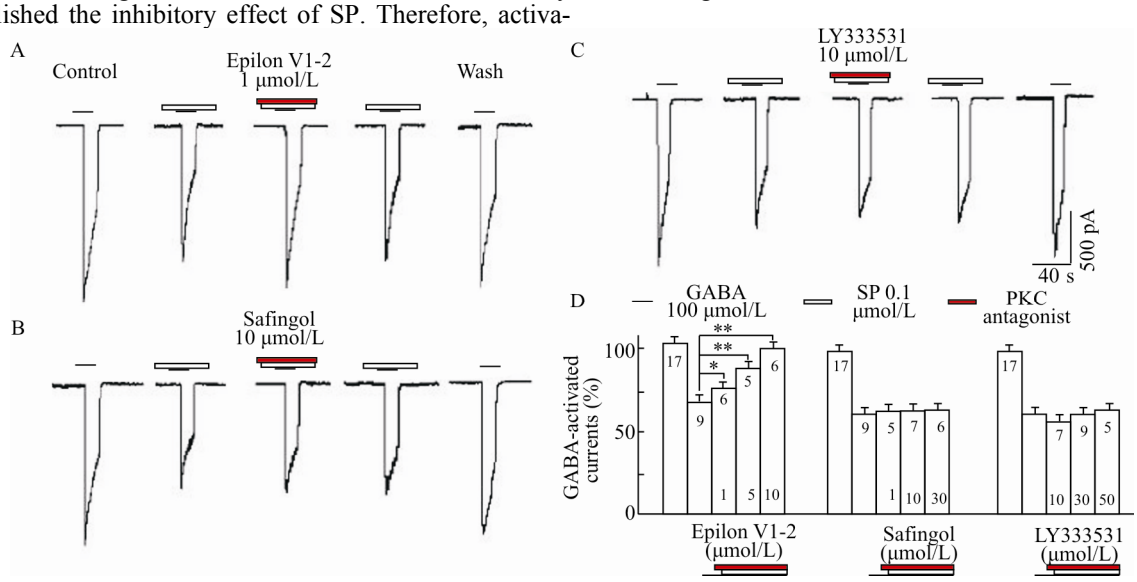

Fig. 7 Effects of PKC subtype antagonists on the inhibition of GABA-activated inward current by SP

A–C: The SP (0.1 μmol/L)-induced inhibition could be completely suppressed by epilon V1-2 (1 μmol/L), but safingol (10 μmol/L) and LY333531 (10 nmol/L) had no effect on it. D: The inhibition ratio of SP (0.1 μmol/L)-induced inhibition on GABA-activated inward current with pre-incubating different concentrations of epilon V1-2 (1, 5, and 10 μmol/L), safingol (1, 10, and 30 μmol/L) and LY333531 (10, 30, and 50 nmol/L), respectively (n=6–17, $^*P$<0.05, $^{**}P$<0.01, paired t test).

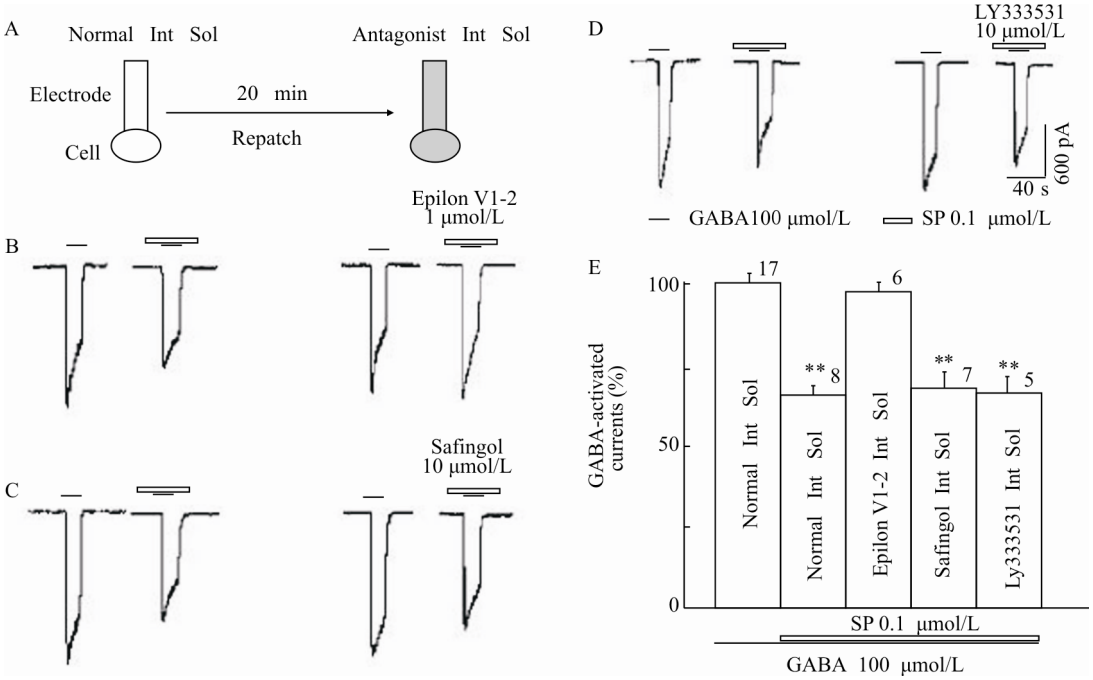

Fig. 8 Effect of protein kinase C inhibitors, epilon V1-2, safingol and LY333531, on inhibition of GABA-activated currents by SP with re-patch technique

A: the schematic drawing of re-patch technique operation; B–D: the normal internal solution GABA 100 μmol/L-activated currents were inhibited by 0.1 μmol/L SP (left). The pipette filled with epilon V1-2 (1 μmol/L), safingol (10 μmol/L) and LY333531 (10 nmol/L) containing internal solution, and there was no or only a slight decrease in the amplitude of GABA-activated currents (right). E: The inhibition ratio of SP (0.1 μmol/L)-induced inhibition on GABA-activated inward current with the re-patch pipette internal solution containing epilon V1-2 (1 μmol/L), safingol (10 μmol/L) and LY333531 (10 nmol/L) (n=5–17, $^*P$<0.05, $^{**}P$<0.01, paired t test).

NK_1 receptor is a G-protein coupled receptor[33, 34], activation of which leads to suppression of both M-current and the voltage-independent background K^+ channels in DRG neurons[20, 23, 24]. It has been shown that pertussis toxin (PTX) insensitive G-proteins mediate the inhibition of $GABA_A$ receptor function in bullfrog DRG neurons[6]. The binding of SP to NK_1R activates several possible intra-membrane and or intracellular signaling pathways and results in closure of Cl^- channels and thus

a decrease in Cl^- efflux in DRG neurons through phosphorylation of the $GABA_A$ receptor[2].

PKC_α and PKC_ε appear to be involved in peripheral nociception while PKC_γ is important to central nociception[8]. Our results suggest that stronger inhibition with increased pre-incubation time of SP up to 2–4 min implies that intracellular transduction mechanisms might be involved in the modulatory effect instead of direct interaction of SP with $GABA_A$ receptor[5]. The long-lasting inhibitory effect of SP (up to 12–16 min) also supports the involvement of intracellular signal molecules. The SP-induced inhibition of GABA-activated current was strongly suppressed by epilon V1-2 while safingol and LY333531 had no effect, which strongly suggests the involvement of PKC_ε in the NK_1R downstream signaling pathways. It is tempting to speculate that binding of SP to the NK_1 activates G-proteins which in turn activate PLC and subsequently mobilize intracellular calcium which activates PKC_ε and phosphorylates $GABA_A$ receptor[2]. This proposal is also supported by our findings that pre-incubation of BAPTA-AM, U73122 or chelerythrine reduced SP-induced inhibitory effect. It is not known how the phosphorylation of $GABA_A$ receptor recovers in sensory neurons. Previous studies showed that activated calcineurin (CaN) could dephosphorylate $GABA_A$ receptor via the direct binding of CaN catalytic domain to the second intracellular domain of the $GABA_A$ receptor $\gamma 2$ subunits[35].

What is the physiological and pathophysiological significance for SP inhibition of the responses mediated by GABAA receptor? It is well known that SP in small DRG neurons may serve as a pain transmitter or as a modulator of noxious transmission in the dorsal horn of the spinal cord[36]. There also exists SP auto-receptor in the presynaptic sensory terminals, activation of which could enhance the release of glutamate (Glu) and SP via positive feedback mechanism from primary afferent terminals[22]. NK_1 acceptor antagonist compound has already been widely applied to treat many diseases clinically[37, 38], including pain[39]. GABA, an inhibitory neurotransmitter, exerts its effect by binding to the $GABA_A$ receptor and is involved in formation of primary afferent depolarization (PAD), an effect known as "pre-synaptic inhibition". This action of GABA results in a decrease of release of SP and Glu from primary afferent terminals[5]. Therefore, if SP depresses GABA-evoked response at the central terminal of primary afferent neurons and leads to a dis-inhibition of the "presynaptic inhibition", it would result in an enhancement of nociceptive signaling in the spinal cord.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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