Modulatory effect of substance P on GABA-activated currents from rat dorsal root ganglion[1]

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KEY WORDS patch-clamp techniques; spinal ganglia; substance P; GABA; protein kinase C

ABSTRACT

AIM: To explore the modulatory effect of substance P (SP) on GABA-activated current of dorsal root ganglion (DRG) neurons in rat. **METHODS:** The whole-cell patch-clamp technique was used to record SP- and GABA-activated currents in neurons freshly dissociated from rat DRG neurons. Drugs were applied by rapid solution exchange. **RESULTS:** Application of SP (28/41, 68.5 %) and GABA (36/41, 88.2 %) could induce concentration-dependent inward current in some cells. SP-(10 μmol/L) and GABA (100 μmol/L)-activated inward currents were (244±83) pA (n=9) and (1.8±0.5) nA (n=13), respectively. The majority of GABA-activated current had obvious three processes, the peak value (I_p), the steady state (I_{ss}) and the desensitization (I_d). The desensitization of GABA-activated current was a biphasic process, including fast and slow desensitization. However, pre-application of SP (0.001-1 μmol/L) could inhibit the GABA-activated inward current which was identified to be $GABA_A$ receptor-mediated current. The inhibitory effects were concentration-dependent. The inhibitory effect of SP on the peak value of GABA-activated current was more than the steady state of GABA-activated current. The inhibition of GABA-activated current by SP (0.1 μmol/L) was related to the time after application of SP, the inhibition of GABA-activated currents by SP reached the peak at about 4 min (49.8 %±7.2 %, n=7, P<0.01) and took about 12 min to get a full recovery. The inhibition of GABA-activated currents by SP was almost completely removed after blockade of PKC by H-7 with the re-patch clamp. **CONCLUSION:** Pre-application of SP exerts a more strong inhibitory effect on the peak value of GABA-activated current than the steady state of GABA-activated current.

INTRODUCTION

SP has been reported to serve as a pain transmitter or a modulator of noxious transmission in the dorsal horn of the spinal cord[1,2]. Application of SP to neurons of the DRG produced a depolarizing response[3-5] and activated an inward current[6,7] associated with an increase in neuronal excitability. γ-Aminobutyric acid (GABA) is the primary inhibitory transmitter in the spinal cord, which causes a reduction of the release of excitatory transmitter from primary afferent nerve terminal, termed 'pre-synaptic inhibition'[8]. It has been suggested that phosphorylation and dephosphorylation of the $GAGA_A$ receptor-chloride channel complex are involved in modulation of the GABA response[9]. $GABA_A$ receptor are downregulated by direct phosphorylation via protein kinase C (PKC)[10,11]. Biochemical studies have suggested that the phosphorylation of $GABA_A$ re-

[1] Project supported by the National Natural Science Foundation of China, No 30160026, 39860027.
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Received 2003-05-09 Accepted 2003-12-07

ceptors by various protein kinases inhibits $GABA_A$ receptor function[12].

$GABA_A$ and $GABA_B$ receptors could exist separately and/or co-exist in the membrane of rat dorsal root ganglion (DRG) neurons[13,14]. Growing evidence showed that substance P (SP) receptor existed in the membrane of rat DRG[3-7]. Since these receptors were co-expressed in the same rat DRG neuron[10,15,16], if they were activated simultaneously, an interaction might occur between the responses mediated by these receptors. We demonstrated that SP modulated $GABA_A$ and $GABA_B$-mediated membrane responses in DRG neurons of rat *in vitro* using intracellular recording technique, *ie*, SP could inhibit $GABA_A$-receptor mediated membrane depolarization and reverse the shortening of action potential duration (APD) induced by activation of $GABA_B$-receptor[17]. Activation of neurokinin-1 (NK_1) receptors by SP could down-regulate the function of the $GABA_A$ receptor in primary sensory neurons of bullfrogs through a PTX-insensitive G-protein[10] and up-regulate the glutamate-induced currents in acutely isolated rat spinal dorsal horn neurons[18].

In the present study, whole-cell patch-clamp technique was performed to explore the effects of SP on the $GABA_A$ receptor-mediated responses in neurons freshly isolated from rat DRG.

MATERIALS AND METHODS

Isolation of dorsal root ganglion neurons Two to three-week-old Sprague-Dawley rats (100-150 g), irrespective of sex, were decapitated, the thoracic and lumbar segments of vertebrate 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, Sigama: DMEM 13.84 g/L, NaCl 2.64 g/L) at pH=7.4, 340 mOsmol/kg, immediately. After the removal of attached nerves and surrounding connective tissues the DRGs were minced with iridectomy scissors and incubated with enzymes including trypsin (type III, Sigma) 0.5 g/L, collagenase (type IA, Sigma) 1 g/L and DNase (type IV, Sigma) 0.1 g/L in 5 mL DMEM at 35 ºC in a shaking bath for 40 min. To stop the enzymatic digestion, soybean trypsin inhibitor (type II-S1, Sigma) 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. Experiments were performed at room temperature (25-30 ºC)[14-16].

Electrophysiological recordings Whole-cell patch-clamp recordings were carried out using a PC-II patch-clamp amplifier (Huazhong University of Science and Technology, Wuhan, China). A micropipette was filled with internal solution, the composition was as follows (in mmol/L): KCl 140, $MgCl_2$ 2.5, HEPES 10, egtazic acid 11, ATP 5, its osmolarity was adjusted to 320 mOsmol with sucrose and pH was adjusted to 7.4 with KOH. The external solution contained (in mmol/L): NaCl 150, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, HEPES 10, *D*-glucose 10, its osmolarity was adjusted to 340 mOsmol with sucrose and pH was adjusted to 7.4 with NaOH. The resistance of recording electrodes was in the range of 1-4 MΩ. A small patch of the membrane underneath the tip of the pipette was aspirated to form a gigaohm seal (1-10 GΩ) and then a more negative pressure was applied to rupture it, thus establishing a whole-cell mode. The adjustment of series resistance and capacitance compensation was carried out before the start of experiments. Membrane currents were filtered at 1 kHz (-3dB). Data were stored and analysed in a super computer with a data acquisition software and hardware system (Huazhong University of Science and Technology, Wuhan, China) or recorded by a pen recorder (Nihon Kohden). Experiments were carried out at holding potential of -60 mV.

Drug application Drugs used in the experiments were SP (Sigma), spantide (Sigma), GABA (Shanghai No 3 Reagent Factory, China) and bicuculline (Sigma). All the drugs were dissolved in the external solution and applied by gravity flow from a row of tubules (OD/ID=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.

Statistical methods The values of SP- and GABA-activated currents were presented as mean±SD and compared with *t*-test.

RESULTS

The isolated DRG neurons had a round or oval shape with a residue of stem process, which was cut off by enzymatic and mechanical treatment. Experiments were carried out on 41 freshly-isolated DRG neurons, the diameters of which were in the range of

15-60 μm. The sensitivity to SP was 68.5 % (28/41), to GABA was 88.2 % (36/41), respectively. There were 63.2 % (26/41) DRG neurons sensitive to both SP and GABA.

SP-activated inward currents in the membrane of rat DRG neurons In the majority of the cell examined (68.5 %, 28/41), an inward current was induced by external application of SP (0.001−10 μmol/L). The SP-activated inward currents were concentration-dependent (Fig 1) and the values of the threshold, EC_{50} and maximum concentration were 0.01, 0.8, and 10 μmol/L, respectively. The amplitudes for SP-activated currents were in the range of 151.3−372.4 pA, the amplitude of SP 10 μmol/L-activated current was (244±83) pA (n=9). The SP-activated current shows no or very slow desensitization as compared with a rapid desensitizing current such as GABA- and ACh-activated currents. The SP-activated currents can be partially suppressed (blocked in two neurons) by spantide (1-100 μmol/L) (n=6), a nonspecific antagonist of SP (data not shown).

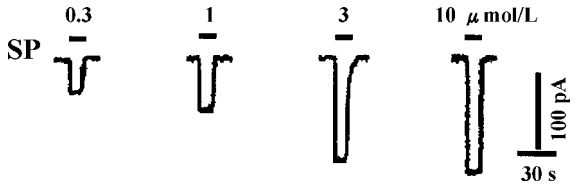

Fig 1. Records of SP-activated currents in the same DRG neuron.

GABA-activated inward currents in the membrane of rat DRG neurons The majority of the cells examined (88.2 %, 36/41) in the present experiment were sensitive to external application of GABA (0.01−1000 μmol/L) with an inward current concentration-dependently (Fig 2), the amplitudes were in the range of 0.98-2.5 nA. The amplitudes of GABA-activated currents at a concentration of 100 μmol/L was (1.8±0.5) nA (n=13) and GABA 100 μmol/L-activated current could be completely and reversibly blocked by bicuculline (10-100 μmol/L, n=7), a specific antagonist of $GABA_A$ receptor (data not shown). The difference between SP- and GABA-activated currents were that the desensitization of GABA-activated current was very apparent, ie, though GABA is continually present and its concentration remained unchanged, the amplitude of the current decayed exponentially after it reached a peak value (I_p) and then maintained at a level of steady

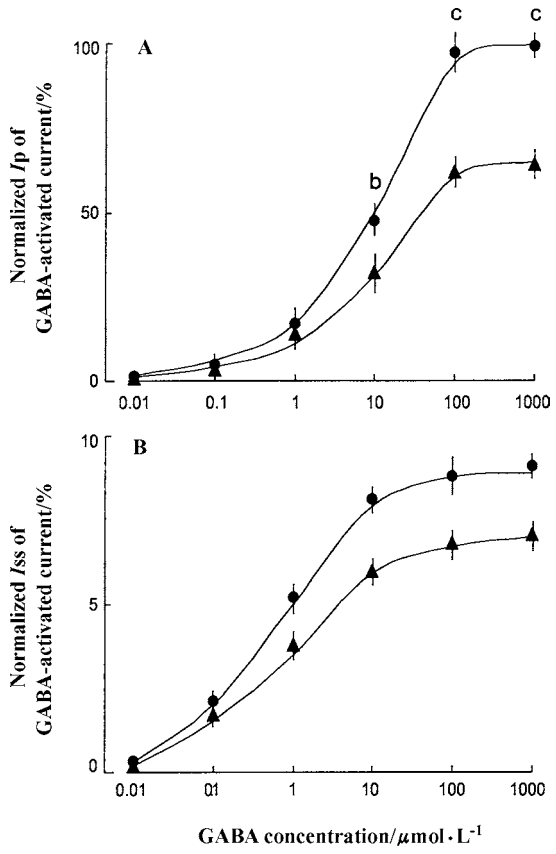

Fig 2. The concentration-response curve for GABA (0.01-100 μmol/L) with (▲) and without (●) pre-application of SP. (A) I_p; (B) I_{ss}. $^bP<0.05$, $^cP<0.01$ *vs* pre-application of SP.

state (I_{ss}) (Fig 3A). The majority of GABA-activated current included three processes, the peak value (I_p), the desensitization (I_d) and the steady state (I_{ss}) (Fig 3A). The minority of GABA-activated current (<10 %) had only two processes, I_p and I_d. The desensitization

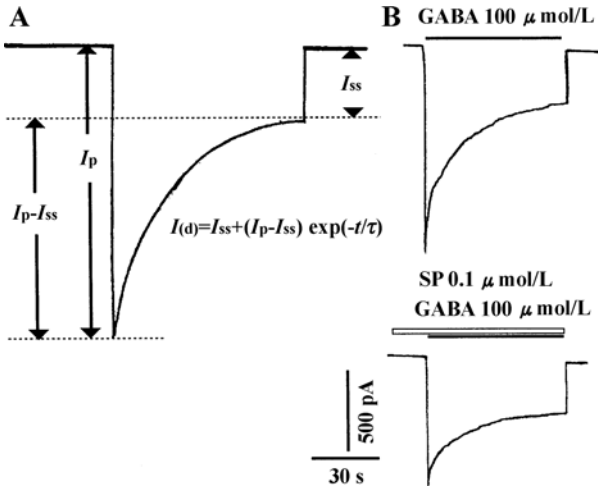

Fig 3. Three processes of GABA-activated current.

of GABA-activated current was a bi-exponential function curve, so it had biphasic process, including fast and slow desensitization (Fig 4). A concentration-response relation was derived from bath application of GABA (0.01-1000 μmol/L) (Fig 2). Each point represents the mean±SD of GABA-activated currents of 6-12 neurons. The curve is the best fit of the data to the logistic equation $Y=E_{max}/[1+(K_d/C)_n]$, where C is the concentration of GABA, Y is the fraction of the maximum value; K_d, the dissociation constant of the $GABA_A$ receptor. The Hill coefficient (n) was assumed to be 1. It could be seen that the threshold concentration was 0.1 μmol/L, the maximum responsive concentration was 1000 μmol/L and the K_d value was 10 μmol/L from the response curve for GABA-activated currents in Fig 2.

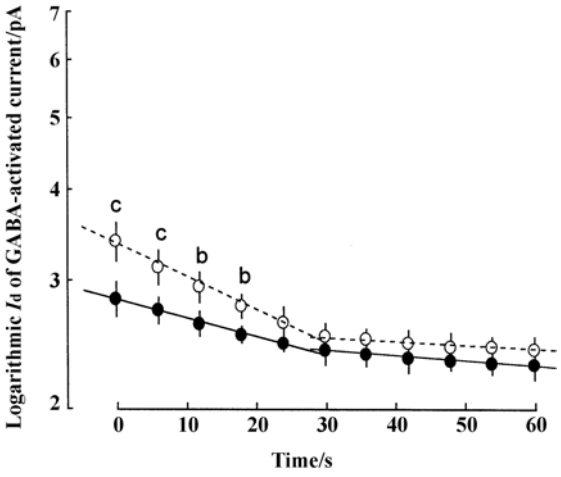

Fig 4. Fast and slow desensitization of GABA (100 μmol/L)-activated currents (I_d) with (●) and without (○) pre-application of SP (0.1 μmol/L). $^bP<0.05$, $^cP<0.01$ *vs* pre-application of SP. n=4-8 neurons.

Inhibitory effect of SP on GABA-activated current Applying SP (0.001−1 μmol/L) for 30 s prior to application of GABA, the GABA-activated current was attenuated markedly in most of the neurons examined (26/36).

Application of SP (0.1 μmol/L) shifted the concentration-response curve of GABA (100 μmol/L)-activated current downward, the estimated K_d value was around 10 μmol/L in the presence of SP (0.1 μmol/L). There was no statistical difference between the threshold concentration, the maximum responsive concentration and the K_d value for GABA obtained before and after application of SP (Fig 2). These results indicate that SP inhibits GABA-activated current in rat DRG neurons in a non-competitive manner. The inhibitory effect of SP on the peak value of GABA-activated current was more obvious than the steady state of GABA-activated current, *ie*, the desensitization of GABA-activated current was obvious accelerated (Fig 3B). The desensitization of GABA-activated current was a biphasic process, including fast and slow desensitization. After application of SP, the fast and slow desensitization of GABA-activated current were accelerated, the fast desensitization was accelerated more obviously than the slow desensitization (Fig 4).

Inhibitory effect of SP was concentration-dependent and increased gradually with the increase in SP concentration . The currents activated by GABA 100 μmol/L were suppressed by 13.1 %±2.3 %, 21.4 %±5.4 %, 48.3 %±4.7 %, and 34.3 %±5.2 % by SP 0.001, 0.01, 0.1, and 1 μmol/L, respectively. But the GABA-activated current was increased by SP 10 μmol/L (n=9, Fig 5). Current traces are sequential from left to right, obtained from one cell.

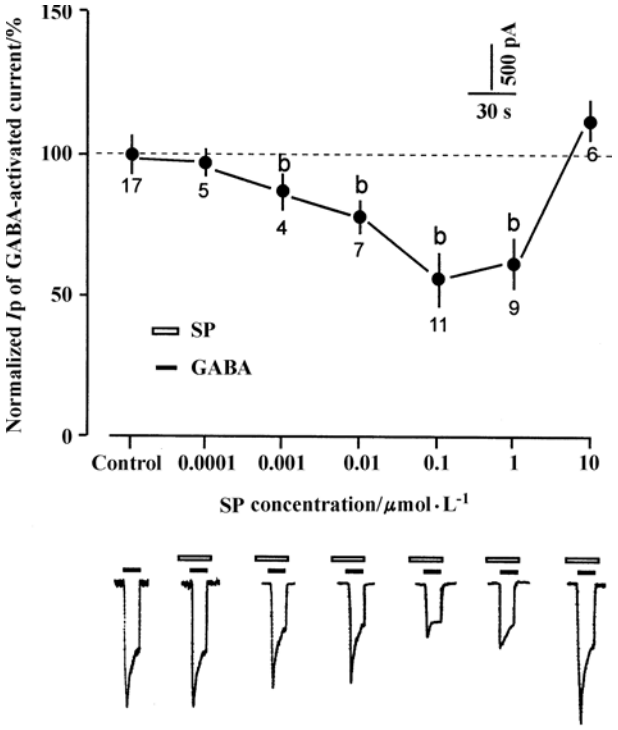

Fig 5. Inhibitory effect of SP on GABA-activated currents. $^bP<0.05$ *vs* control. Each point represents 5-10 neurons.

Time-course of inhibition of GABA-activated current by SP It took about 4 min to get a full recovery from itself of GABA-activated current (Fig 6), then the interval between the first application of GABA and

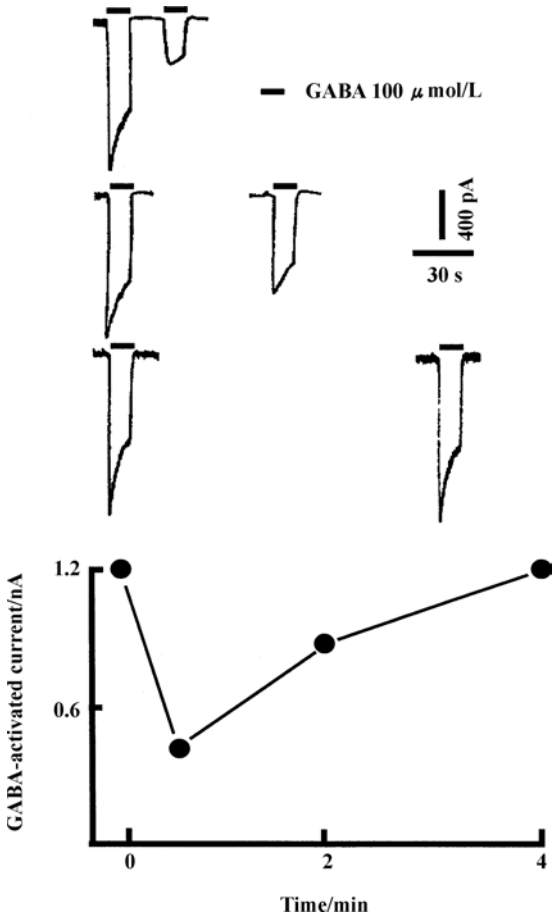

Fig 6. The time-course of recovery of GABA (100 μmol/L)-activated current.

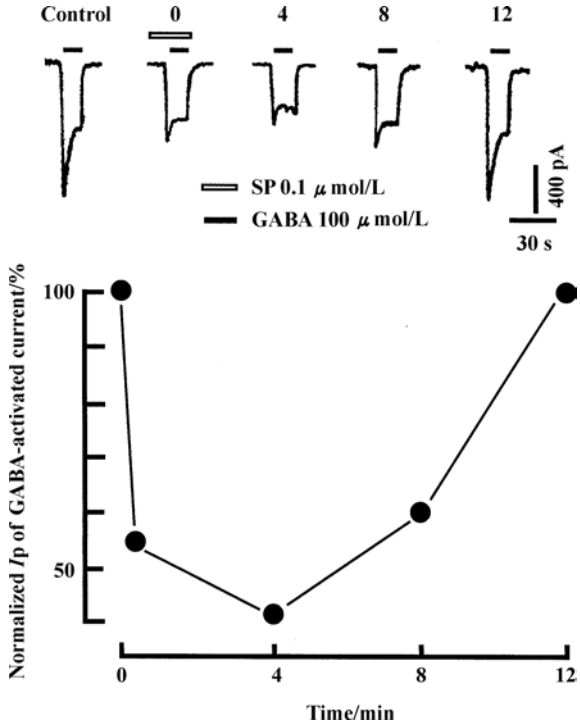

Fig 7. The time-course of SP 0.1 μmol/L-induced inhibition of GABA (10 μmol/L)-activated current.

the second application need to be longer than 4 min. GABA-activated current did not get a full recovery if the interval was less than 4 min. This kind of inhibition began at least 30 s after pre-application of SP and there was no detectable effect on GABA-activated membrane current if the duration was less than 30 s. It was evident that the maximum inhibitory effect took place at around 4 min with pre-application of SP, 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 7).

Analysis of intracellular signal transduction mechanisms underlying the inhibition of GABA-activated currents by SP To explore intracellular signal transduction mechanisms underlying SP inhibition of GABA-activated currents we used re-patch technique. In the control experiment with the normal internal solution GABA 100 μmol/L-activated currents were inhibited by SP 1.0 μmol/L (Fig 8A left). Whereas, by using the pipette filled with H-7 20 μmol/L containing internal solution there was no or only a slight decrease in the amplitude of GABA-activated currents (Fig 8A right). After blockade of PKC by H-7 with the re-patch clamp, the inhibition of GABA-activated currents by SP was almost completely removed (P<0.01, Fig 8B).

DISCUSSION

In the present study the inhibition of $GABA_A$ receptor-mediated response by SP was concentration-dependent; the desensitization of GABA-activated current was obvious inhibited, ie, the fast and slow desensitization of GABA-activated current were accelerated (Fig 3). It is convincing that activation of SP (NK_1) receptor can exert a negative modulation of the currents activated by $GABA_A$ receptor.

As we know, $GABA_A$ receptor belongs to the super-family of ligand-gated ion channel receptor. Cloning of cDNA encoding tachykinin receptor has revealed that the SP (NK_1) receptor is classified as a G-protein-coupled receptor[19,20], activation of NK_1 receptor causes suppression of both M-current and the voltage-independent background K^+ via a G-protein in DRG neurons[4,6]. It has been shown that a pertussis toxin (PTX)-insensitive G-protein couples to the NK_1 receptor to

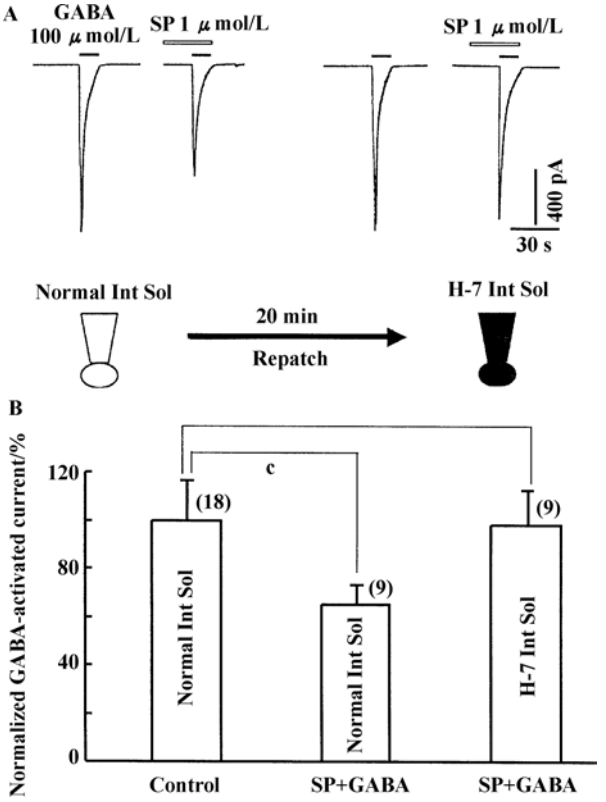

Fig 8. Effect of protein kinase C inhibitor, H-7, on inhibition of GABA-activated current by SP. $^{c}P<0.01$ *vs* control.

mediate the inhibition of $GABA_A$ receptor function in bullfrog DRG neurons[10]. The binding of SP to the NK_1 receptor binding site activates G-protein, which couples in turn to several possible intra-membrane and/or intracellular routes and results in closure of Cl^--channel and thus decrease in Cl^- efflux in DRG neurons through phosphorylation of $GABA_A$ receptor[9]. In the present study the intracellular signal transduction mechanisms of the inhibition by SP of GABA-activated current deduced as follows: SP receptor, after binding SP, couples via G protein to activate PKC, which causes the phosphorylation of GABA receptor.

It was obvious that there was no inhibition by SP of the GABA-activated current if the time of SP pre-application was less than 30 s. A relatively long latency (30 s) for SP inhibitory effect implies that intracellular transduction mechanisms, as mentioned above, may be involved in this modulatory effect. On the other hand, the long-lasting time-course for inhibition (12 min or more) may also explain this intracellular transduction (Fig 7).

GABA-activated current was increased by SP 10 μmol/L. We suggested that the phenomenon was non-physiological effect of SP, because the concentration of SP 10 μmol/L was higher than physiological concentration of SP.

What is the physiological significance for the responses mediated by $GABA_A$ 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[21]. According to the reports published recently[7,22], there exists SP auto-receptor in the pre-synaptic sensory terminals, which enhances the further release of glutamate (Glu) and SP via positive feedback mechanism from primary afferent terminals. GABA as an inhibitory neurotransmitter acts on $GABA_A$ receptor, opened the Cl^- channel and was involved in formation of primary afferent depolarization (PAD), an effect known as "pre-synaptic inhibition". This action of GABA results in the decrease of release of neurotransmitter including SP and Glu from primary afferent terminals. If SP depressed the GABA response at the central terminal of primary afferent neurons, as it did at the soma membrane, then dis-inhibition of the "presynaptic inhibition" would result in the facilitation of nociception in the spinal cord.

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