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5-HT potentiation of the $GABA_A$ response in the rat sacral dorsal commissural neurones

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1 The modulatory effect of 5-hydroxytryptamine (5-HT) on the γ-aminobutyric $acid_A$ ($GABA_A$) response was investigated in the neurones freshly dissociated from the rat sacral dorsal commissural nucleus (SDCN) using the nystatin perforated patch recording configuration under the voltage-clamp conditions.

2 5-HT potentiated GABA-induced Cl^- current (I_{GABA}) without affecting the reversal potential of I_{GABA} and the apparent affinity of GABA to its receptor.

3 α-Methyl-5-HT mimicked the potentiation effect of 5-HT on I_{GABA} while ketanserine blocked it. 1-Oleoyl-2-acetyl-glycerol (OAG) potentiated I_{GABA}, and the effect of 5-HT on I_{GABA} was occluded by OAG pretreatment. In the presence of chelerythrine, 5-HT failed to potentiate I_{GABA}, suggesting that protein kinase C (PKC) is involved in the pathway through which the activation of the $5\text{-}HT_2$ receptor potentiates the I_{GABA}.

4 The facilitatory effect of 5-HT on I_{GABA} remained in the presence of BAPTA-AM. LiCl also had no effect on 5-HT-induced potentiation of I_{GABA}.

5 H-89, genistein, okadaic acid and pervanadate all had no effects on 5-HT potentiation of I_{GABA}. Pertussis toxin treatment for 6–8 h did not block the facilitatory effect of 5-HT on I_{GABA}.

6 The present results show that $GABA_A$ receptor in the rat SDCN could be modulated *in situ* by 5-HT, one of the major transmitters involved in the supraspinal control of nociception, and that the phosphorylation of $GABA_A$ receptor by PKC may be sufficient to support such modulation. The results also strongly support the hypothesis that the cotransmission by 5-HT and GABA has an important role in the spinal cord.

Keywords: Sacral dorsal commissural nucleus; $5\text{-}HT_2$ receptor; $GABA_A$ receptor; cross-communication; second-messengers; protein kinase C; antinociception

Introduction

5-HT (5-hydroxytryptamine) is one of the major transmitters involved in the supraspinal control of nociception (for review, see Fields & Basbaum, 1994). It is well known that the rat spinal dorsal horn is heavily innervated with 5-HT-like immunoreactive fibres, and that 5-HT-containing neurones sending descending projections to the spinal dorsal horn are localized in the rostral ventromedial medulla (RVM) and caudal pons, including the nucleus raphe magnus, nucleus reticularis paragigantocellularis, and ventral portions of the nucleus rectilaris gigantocellularis (Jones & Light, 1990; Kwiat & Basbaum, 1992). Axon terminals of descending 5-HT fibres from the RVM to the spinal cord make axosomatic and axodendritic synapses with both spinothalamic projection neurones and local circuit neurones in the spinal dorsal horn of the monkey (Basbaum *et al*., 1986) and cat (Light & Kavookjian, 1985). Direct contacts of 5-hydroxytryptaminergic axon terminals on nociceptive projection neurones in the caudal spinal trigeminal nucleus of the rat have also been demonstrated recently (Li *et al*., 1997).

Activation of RVM neurones by electrical stimulation or glutamate microinjection depresses the activity of nociceptive neurones in the spinal dorsal horn, and produces behavioural antinociception (Basbaum *et al*., 1976; Jensen & Yaksh, 1989). Several possible mechanisms have been suggested to lead to such depression: a direct postsynaptic action of 5-HT through an increase of K^+ conductance (Grudt *et al*., 1995), a presynaptic inhibition of the primary afferent terminals by 5-HT (Hentall & Fields, 1983), an inhibitory action on neuronal response to excitatory amino acids (Murase *et al*., 1990), an increased inhibitory action through the activation of the local glycinergic interneurones (Grudt *et al*., 1995) or endogenous opiate-like substances (Yang *et al*., 1994), and a 5-hydroxytryptaminergic enhancement of postsynaptic glycinergic transmission (Xu *et al*., 1996).

It has been determined that γ-aminobutyric acid (GABA) and 5-HT coexist in the neurones of medulla oblongata projecting to the spinal cord (Millhorn *et al*., 1987). The extensive GABAergic innervation of spinal dorsal horn by fibres descending from RVM has been demonstrated recently (Antal *et al*., 1996). And recent immunohistochemical experiments have shown that the major $GABA_A$ receptor subtypes detected in the brain are also present in the spinal cord (Bohlhalter *et al*., 1996; Todd *et al*., 1996). On the other hand, although the glycinergic mechanism for the inhibitory neurotransmission in the spinal cord has received much emphasis in the past, there is growing pharmacological and physiological evidence that a GABAergic mechanism also contributes to the inhibitory influences on the spinal cord (McGowan & Hammond, 1993; Sorkin *et al*., 1993; Lin *et al*., 1994; Yoshimura & Nishi, 1995). The above evidence suggests that 5-HT and GABA could be coreleased and act as cotransmitters in the spinal dorsal horn and that the functional cross-communication between 5-HT and $GABA_A$ receptors

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might be one of the modulatory processes of spinal nociception.

In this study, the effect of 5-HT on $GABA_A$ receptor-mediated response was investigated in the neurones freshly dissociated from rat sacral dorsal commissural nucleus (SDCN) using the nystatin perforated patch recording configuration under voltage-clamp conditions. The SDCN is localized in the dorsal part of the central canal of the lower lumbar and sacral spinal cord of the rat and is known to be implicated in nociceptive transmission (Xu *et al*., 1996). In particular, we characterized the role of protein kinase C (PKC) in the intracellular transduction pathway through which 5-HT induces the enhancement of $GABA_A$ receptor-mediated inhibitory response.

Methods

Preparation

The sacral dorsal commissural nucleus (SDCN) neurones were acutely dissociated as described elsewhere (Xu *et al*., 1996). Briefly, two-week-old Wistar rats were decapitated under pentobarbitone-Na anaesthesia. A segment of lumbo-sacral (L_6-S_2) spinal cord was dissected out and sectioned with a vibratome tissue slicer (Dosaka, DTK-1000) to yield several transverse slices 400 μm thick containing the SDCN region. The slices were preincubated in well-oxygenated incubation solution (see below for the composition) for 50 min at room temperature (22–25°C). Thereafter, slices were treated enzymatically in well-oxygenated incubation solution containing 1 mg pronase/6–8 ml for 20 min at 31°C followed by exposure to 1 mg thermolysin/6–8 ml for another 15 min in the same condition. After the enzyme treatment, the slices were kept in enzyme-free incubation solution for 1 h. Then a portion of SDCN region was micro-punched out with an electrolytically polished injection needle and transferred into a culture dish filled with standard external solution (see below). Neurones were mechanically dissociated by fire-polished Pasteur pipettes with visual guidance under a phase contrast microscope (Nikon, TMS-1). Dissociated neurones adhered to the bottom of the dish within 20 min, allowing the electrophysiological studies to be conducted. The neurones that retained their original morphological features such as the dendritic processes were used for the experiments.

Solutions

The composition of incubation solution was (mM): NaCl 124, $NaHCO_3$ 24, KCl 5, KH_2PO_4 1.2, $CaCl_2$ 2.4, $MgSO_4$ 1.3 and glucose 10, aerated with 95% O_2/5% CO_2 to a final pH of 7.4. The normal external standard solution was (mM): NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) 10 and glucose 10. The pH was adjusted to 7.4 with tris-hydroxymethyl aminomethane (Tris-base). The patch-pipette solution for nystatin perforated patch recording was (mM): CsCl 150 and HEPES 10. The pH was adjusted to 7.2 with Tris-base. A nystatin stock solution dissolved in acidified methanol at a concentration of 10 mg ml^{-1} was prepared and stored at −20°C. The stock solution was added to the patch-pipette solution in a final nystatin concentration of 200 μg ml^{-1} just before use. When the current-voltage (*I*-V) relationship for GABA-induced Cl^- current was examined, 0.3 μM tetrodotoxin (TTX) and 10 μM $CdCl_2$ were added to the standard external solution.

Electrical measurement

Electrical measurements were carried out by using the nystatin perforated patch recording configuration under the voltage-clamp condition at room temperature (22–25°C). Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (Narishige, PB-7). The resistance between the recording electrode filled with pipette solution and the reference electrode was 4–6 MΩ. Series resistance checked every 10 min was 10–30 MΩ. The change of series resistance through recording was less than 10%. The current and voltage were measured with a patch-clamp amplifier (Nihon Koden, CEZ-2300), filtered at 1 KHz (NF Electronic Instruments, FV-665), and monitored on both a storage oscilloscope (Iwatsu Electronic, 5100A) and a pen recorder (Nippondenki San-ei, Recti-Horiz-8K21). Data were simultaneously stored on a video tape after digitization at a rate of 44 KHz (Nihon Koden, PCM 501 ESN). The membrane potential was held at −50 mV throughout the experiment, except when the *I*-V relationships were being examined. Measurements were started after the stabilization of the GABA response (15–25 min after cell attachment).

Chemicals

Drugs used in the present experiments were: thermolysin, nystatin, 5-HT, GABA, bicuculline and muscimol from Sigma; (±)-8-OH-hydroxy-2-*N*, *N*-dipropylaminotetralin (8-OH-DPAT), α-methyl-5-HT, ketanserine, genistein, okadaic acid and chelerythrine from Research Biochemicals International; pronase and 1-oleoyl-2-acetyl-glycerol (OAG) from Calbiochem; (**S**)-5-fluoro-8-hydroxy-2-(dipropylamino)-1,2,3,4-tetrahydronaphthalene hydrochloride ((**S**)-UH-301) was kindly provided by Yoshitomi Pharmaceutical Industries Ltd.; *N*-[2(methylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride (H-89) from Seikagaku corporation; 1,2-bis-(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM) from Dojin; pertussis toxin (IAP) from Kaken Seiyaku and TTX from Sankyo. Stock solutions of (S)-UH-301, OAG, genistein and okadaic acid were prepared in dimethyl sulphoxide (DMSO) and diluted to final concentrations in the external standard solution. The final concentration of DMSO was always less than 0.01%, and it did not induce any ionic current and had no effect on GABA response at the concentrations used. Sodium pervanadate was prepared freshly by mixing 1 part 500 mM H_2O_2 with 50 parts 10 mM sodium orthovanadate in saline; the solution was incubated for 15 min at room temperature to reduce H_2O_2 (Catarsi & Drapeau, 1997). Other drugs were either first dissolved in distilled water and then diluted to the final concentration in the external solution just before use or dissolved directly in the external solution. Drug solutions were applied by the 'Y-tube' method (Xu *et al*., 1996) throughout the experiment. This system allows a complete exchange of external solution surrounding a neurone within 20 ms.

Statistical analysis

Michaelis-Menten equation using a least-squares fitting was applied for evaluation of the half-maximal effective concentration (EC_{50}) of GABA in the GABA concentration-response relationships.

$$I = I_{max}.C^{n_H}/(C^{n_H} + K_d{}^{n_H})$$

where I is current, I_{max} is maximum response, n_H is Hill

coefficient and C is the concentration of GABA and K_d is the dissociation constant.

Experimental values are shown as mean ± s.e.mean. Student's t test was used when two groups were compared. P and n represent the value of significance and number of cells, respectively.

Results

Potentiation of $GABA_A$ response by 5-HT

With extra- and intracellular Cl^- concentrations ($[Cl^-]_o$ and $[Cl^-]_i$) of 161 and 150 mM, respectively, 3×10^{-6} M GABA elicited an inward current (I_{GABA}) in all SDCN neurones tested at a holding potential (V_h) of -50 mV. The I_{GABA} was completely inhibited by adding 10^{-5} M bicuculline (Figure 1Aa). Muscimol (Mus, 10^{-6} M), a $GABA_A$ receptor agonist, also caused an inward current (I_{Mus}) with the current amplitude similar to that produced by 3×10^{-6} M GABA. Baclofen, which is a $GABA_B$ receptor agonist, evoked no response at concentrations up to 10^{-3} M. These results suggested that the inward current produced by GABA is mediated by the $GABA_A$ receptor in the rat SDCN neurones. In the present experimental conditions, the pretreatment with 5-HT alone induced no noticeable current at concentrations up to 10^{-4} M in the rat SDCN neurones (Xu *et al*., 1996). However, after the continuous application of 10^{-6} M 5-HT for 5 min, the simultaneous administration of GABA and 5-HT reversibly potentiated the I_{GABA} in the majority of the neurones examined (82/100 cells), as shown in Figure 1Ab. I_{Mus} was also enhanced by 5-HT (10^{-6} M) application (Figure 1Ac). Figure 1B summarizes these experimental results. 5-HT caused a 1.38 ± 0.09 ($n = 10$) and 1.37 ± 0.1 ($n = 5$) times increase of I_{GABA} and I_{Mus}, respectively.

Concentration-response curve and current-voltage relationship of I_{GABA}

The amplitude of I_{GABA} increased in a concentration-dependent manner (Figure 2a). The GABA concentrations for threshold, half-maximal (EC_{50}) and maximal response were 3×10^{-7}, 5.2×10^{-6} and 3×10^{-4} M, respectively. Similarly the concentration-response curve of GABA in the presence of 10^{-6} M 5-HT demonstrated that the GABA concentrations for threshold, EC_{50} and maximal response were also 3×10^{-7}, 5.2×10^{-6} and 3×10^{-4} M, respectively. The results indicate that these values are not affected by the absence or presence of 10^{-6} M 5-HT (Figure 2a), and that 5-HT does not alter the apparent affinity of GABA to its receptor.

Figure 2b shows the current-voltage (I-V) relationships for GABA with or without 10^{-6} M 5-HT. The reversal potentials (E_{GABA}) of GABA responses were -0.07 ± 0.02 mV ($n = 5$) in the control and 0.05 ± 0.03 mV ($n = 4$) with 10^{-6} M 5-HT. These E_{GABA} values were close to the Cl^- equilibrium potential (E_{Cl}) of -1.8 mV calculated by the Nernst equation, indicating that 5-HT increased the GABA-induced Cl^- current without affecting other ionic channels. In addition, the potentiation induced by 5-HT showed no voltage-dependence.

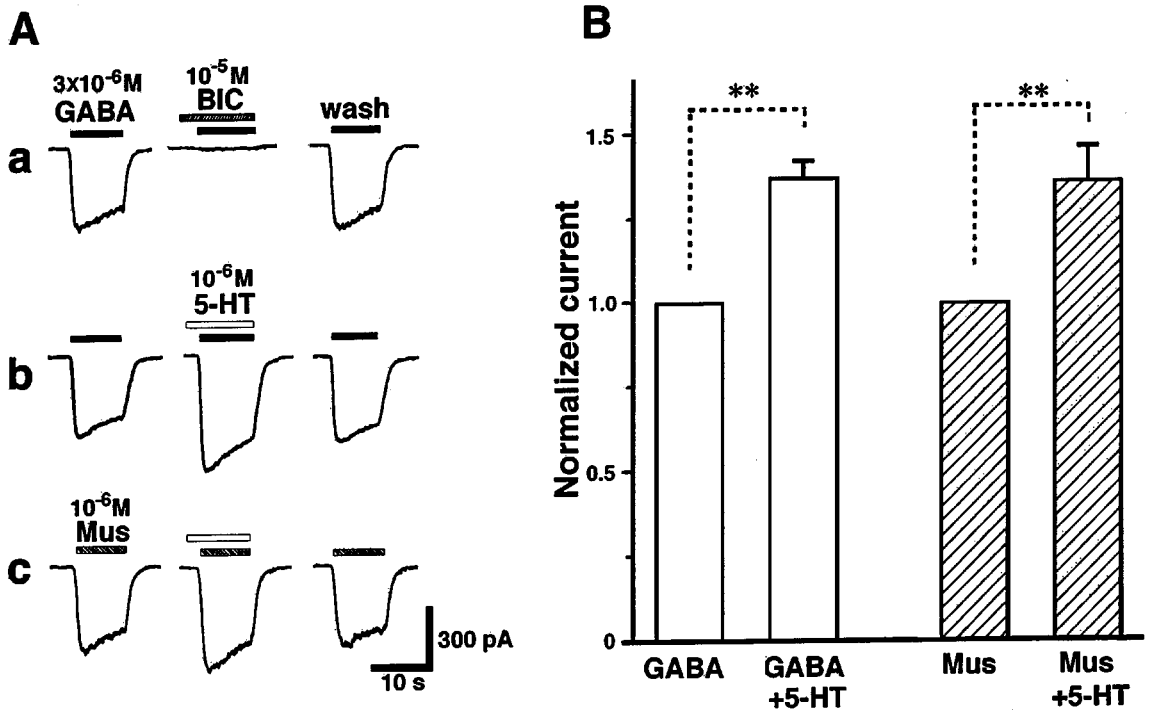

Figure 1 5-HT potentiated the $GABA_A$ response in SDCN neurones. The holding potential (V_h) was -50 mV. (Aa) GABA-induced inward current (I_{GABA}) was almost completely suppressed by 10^{-5} M bicuculline (BIC). The pretreatment time of BIC was 30 s. (Ab) 5-HT (10^{-6} M) reversibly increased 3×10^{-6} M I_{GABA} in a SDCN neurone. However, 5-HT itself induced no current. (Ac) Muscimol (10^{-6} M)-induced inward current (I_{Mus}) was also potentiated by 10^{-6} M 5-HT. (a–c) Obtained from different neurones. In (b) and (c), the pretreatment with 5-HT was for 5 min, and then 5-HT and GABA or muscimol were applied simultaneously. Right-hand records were obtained 10 min after the removal of 5-HT. (B) The potentiation of I_{GABA} and I_{Mus} by 5-HT. Ordinate scale indicates the normalized amplitude of $GABA_A$ responses where the control responses were taken before 5-HT application. In this and subsequent figures the horizontal bars indicate the periods of drug applications and the vertical lines indicate s.e.mean. $^{**}P < 0.01$.

5-HT receptor subtypes responsible for the potentiation of I_{GABA}

To explore which 5-HT receptor subtypes mediate the potentiation of I_{GABA}, the effects of several selective agonists and antagonists of 5-HT_1 and 5-HT_2 receptors were examined. 5-HT_1 agonist, 8-OH-DPAT (10^{-6} M), had no significant effect on I_{GABA}. However, α-methyl-5-HT (10^{-6} M), a 5-HT_2 selective agonist, mimicked the effect of 5-HT (Figure 3A). Furthermore, when ketanserine (3×10^{-6} M), a 5-HT_2 selective antagonist was coadministered with 10^{-6} M 5-HT, it prevented the facilitation of I_{GABA}. On the other hand, (S)-UH-301 (10^{-5} M), a 5-HT_1 selective antagonist, had no significant effect on the potentiation of I_{GABA} by 5-HT (Figure 3B). These results indicate that activation of the 5-HT_2 receptor is mainly responsible for the potentiation of I_{GABA} by 5-HT.

Involvement of protein kinase C

Several laboratories have shown that second messenger regulated protein kinases modulate $GABA_A$ receptor function (for review, see Smart, 1997). Activation of 5-HT_2 receptor stimulates phosphoinositide hydrolysis by activating phospholipase C (PLC) in neurones (Martin & Humphrey, 1994). The phosphoinositide hydrolysis produces two second messengers, inositol-1,4,5-trisphosphate (IP_3) which results in Ca^{2+} release from intracellular Ca^{2+} pools, and diacylglycerol (DAG), an activator of protein kinase C (PKC). Since previous experiments have demonstrated that the enhancement of glycine response in the rat sacral dorsal commissural neurones by 5-HT is mediated by intracellular PKC (Xu *et al*., 1996), we therefore tested whether a similar modulation of I_{GABA} by PKC is conserved in the present preparation. 1-Oleoyl-2-acetylglycerol (OAG) (3×10^{-6} M), a membrane-permeable DAG analogue, mimicked the 5-HT effect on I_{GABA}. In the presence of OAG, 5-HT did not potentiate I_{GABA} any more (Figure 4Aa, B). After neurones had been loaded with 3×10^{-6} M chelerythrine, a membrane-permeable PKC inhibitor, application of 10^{-6} M 5-HT also failed to enhance I_{GABA} (Figure 4Ab, B). Both OAG and chelerythrine themselves did not elicit any current in the present experimental conditions. The results suggest that 5-HT and PKC share a common pathway in facilitating I_{GABA} in SDCN neurones.

To determine the role of intracellular Ca^{2+} on the 5-HT-induced potentiation of I_{GABA}, the effect of BAPTA-AM, a membrane-permeable Ca^{2+} chelator, was examined. When the SDCN neurones were loaded with 3×10^{-6} M BAPTA-AM for 2 h, the potentiation of I_{GABA} by 5-HT (138.1±9.0%, $n=6$, $P<0.01$) remained almost the same as that observed in the control neurones without BAPTA-AM treatment. In the presence of LiCl, which has been shown to increase intracellular IP_3 concentration by inhibiting 1-phosphatase (Xu *et al*., 1996), 5-HT did not induce further facilitation of I_{GABA} (95.4±4.6% of that before LiCl application, $n=4$, $P>0.05$, Figure 4Ac, B).

Effects of H-89, genistein, okadaic acid and pervanadate on I_{GABA}

To examine the possible contributions of cyclic AMP-dependent protein kinase A (PKA), protein tyrosine kinases (PTKs) and protein phosphatases to the 5-HT-induced increase of I_{GABA}, SDCN neurones were pretreated with either H-89 (10^{-6} M), a membrane-permeant PKA inhibitor (Xu, 1997), or genistein (5×10^{-5} M), a membrane-permeant PTKs inhibitor (Wan *et al*., 1997), or okadaic acid (2×10^{-6} M), a membrane-permeant inhibitor of phosphatases 1, 2A and 2B (calcineurin) (Cheng & Wong, 1995). The facilitatory effect of 5-HT on I_{GABA} was not affected by these drugs (Figure 5A).

Recently, it has been shown that 5-hydroxytryptaminergic stimulation of PKC activates a tyrosine phosphatase activity which associated with channels in leech pressure-sensitive neurones (Catarsi & Drapeau, 1997). Therefore, the possible contribution of dephosphorylation of the $GABA_A$ receptor-Cl^- chanel complex by tyrosine phosphatase to 5-HT-induced potentiation of I_{GABA} was examined. When the SDCN neurones were exposed to 10^{-4} M sodium pervanadate, a membrane-permeable form of vanadate which inhibits tyrosine

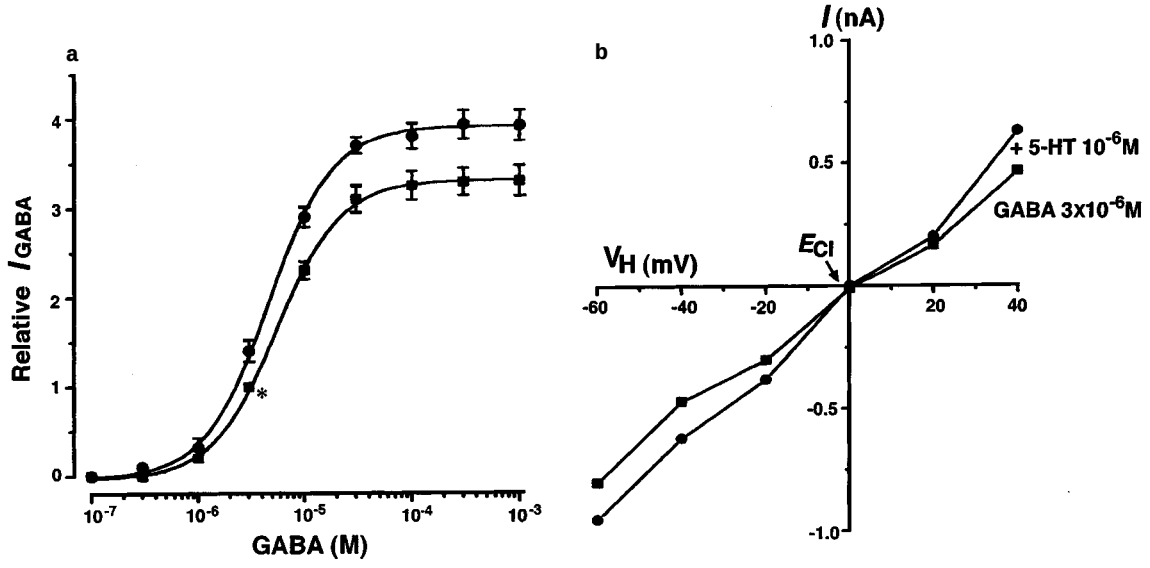

Figure 2 Effect of 5-HT on the concentration-response curve and *I*-V relationship for GABA. (a) The concentration-response curves for GABA in the presence and absence of 10^{-6} M 5-HT. All responses were normalized to the peak current induced by 3×10^{-6} M GABA alone (*). The Michaelis-Menten equation using least-squares fitting was used to draw the curves. Each point is the mean of four to six cells. (b) The *I*-V relationships for GABA in the presence and absence of 5-HT reversed near the E_{Cl} (arrow). Data were from the same neurone.

phosphatase (Catarsi & Drapeau, 1997), the 5-HT potentiation of I_{GABA} remained almost the same as that observed in control neurones without vanadate treatment (Figure 5A). Thus, PKA, PTKs, protein phosphatases and tyrosine phosphatase are unlikely to be involved in mediating the 5-HT potentiation of I_{GABA} in the SDCN neurones.

Properties of G-protein

To examine whether G-protein is involved in the 5-HT action on I_{GABA}, the dissociated SDCN neurones were incubated in standard external solution with or without pertussis toxin (IAP) (300 ng ml^{-1}) for 6 to 8 h at room temperature. To elucidate the effectiveness of IAP treatment, the disappearance of IAP-sensitive suppression of Ca^{2+} channel current by 5-HT was confirmed in the IAP-treated preparations (Xu *et al.*, 1996). In the control neurones without IAP treatment, 10^{-6} M 5-HT enhanced the I_{GABA} by $137.3 \pm 7.3\%$ ($n=4$; $P<0.01$, Figure 5Ba, c). Even in the IAP-treated neurones, in which 5-HT suppression of Ca^{2+} channel current disappeared, the application of 10^{-6} M 5-HT caused almost an equal potentiation of I_{GABA} by $138.3 \pm 7.9\%$ ($n=4$; $P<0.01$, Figure 5Bb, c). These results suggest that the IAP-insensitive G-protein is coupled to the potentiation of I_{GABA} by 5-HT.

Discussion

Cross-communication between 5-HT and $GABA_A$ receptors

Receptors that activate complex intracellular signal transduction cascades and produce changes in the levels of second messengers such as Ca^{2+}, cyclic AMP, cyclic GMP, DAG, and

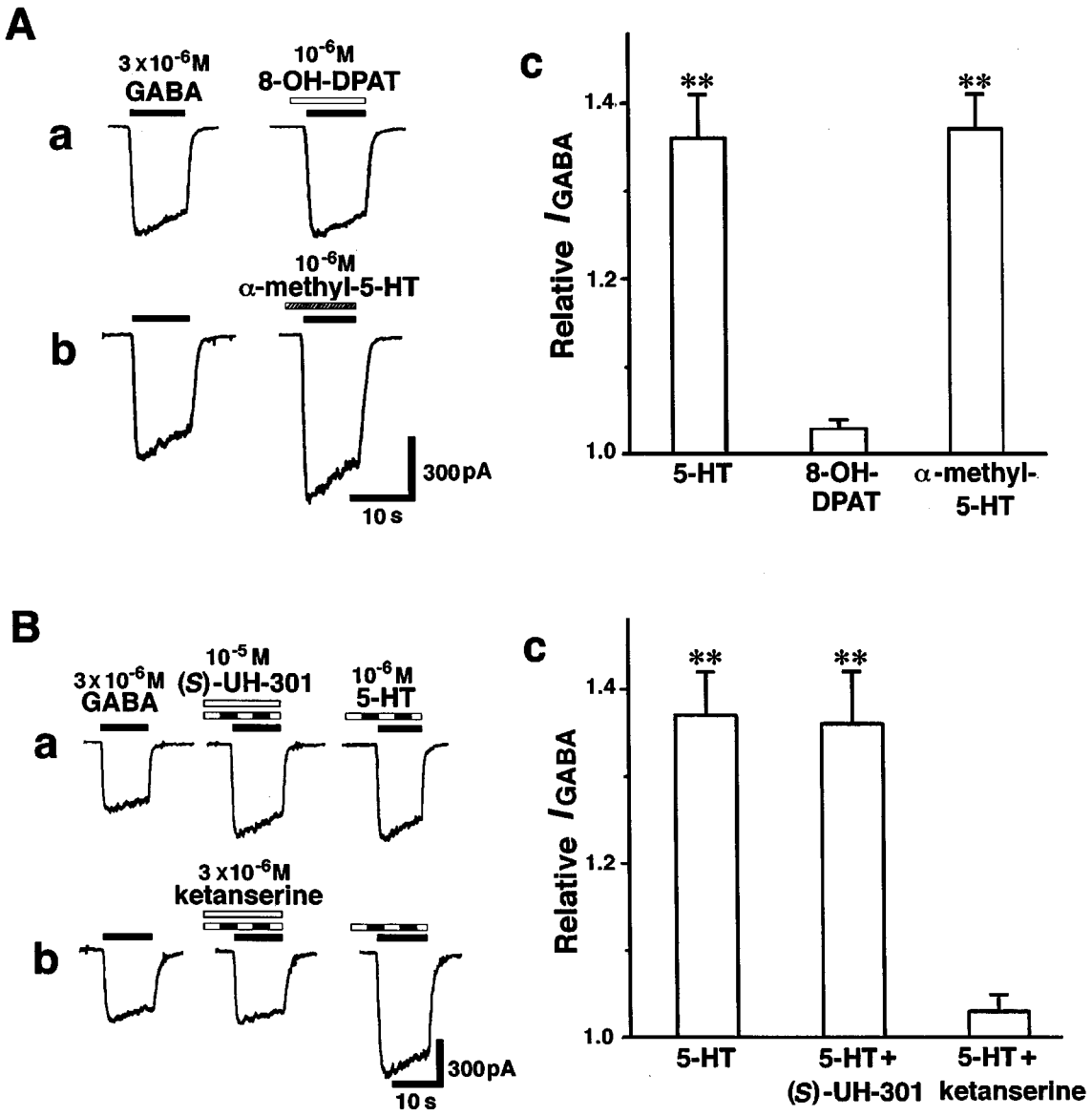

Figure 3 Effects of 5-HT receptor agonists and antagonists. (A) The effects of 5-HT agonists. I_{GABA} was augmented by 10^{-6} M α-methyl-5-HT (b) but not by 10^{-6} M 8-OH-DPAT (a). The pretreatment time of the agonists was 5 min; (c) shows the average values obtained from five neurones. Compared with I_{GABA} in the absence of agonists, $^{**}P<0.01$. (B) In the presence of 10^{-5} M (S)-UH-301, 5-HT potentiated I_{GABA} (Ba); 5-HT failed to potentiate I_{GABA} in the presence of 3×10^{-6} M ketanserine (Bb). Each antagonist was simultaneously applied with 10^{-6} M 5-HT. (c) Statistical data showing the effects of 5-HT antagonists. The potentiating response was completely blocked by ketanserine but was not affected by (S)-UH-301. Each column is the average of four to six neurones. $^{**}P<0.01$.

IP_3, are known to regulate the activity of numerous target enzymes, which in turn, could modulate the function of the $GABA_A$ receptors (for review, see Smart, 1997). G-protein-coupled 5-HT receptors are one of the most important family of regulatory receptors in the central nervous system (CNS). Several studies suggest that $GABA_A$ and 5-HT receptors, and

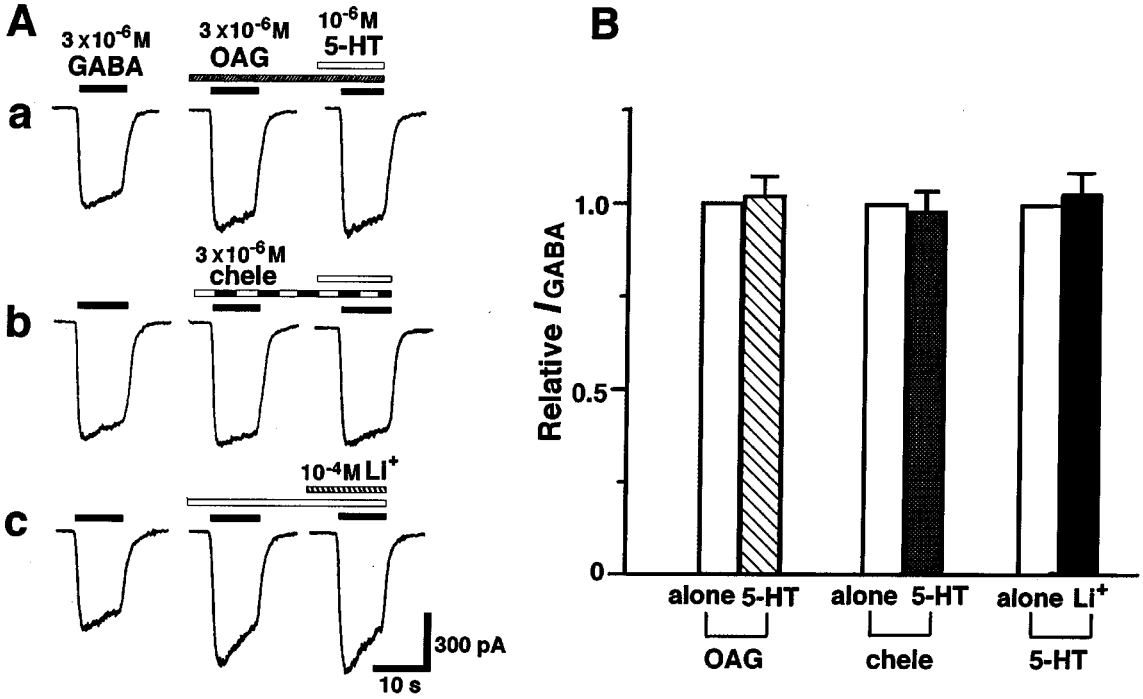

Figure 4 PKC-mediated potentiation of I_{GABA} by 5-HT. (Aa) OAG (3×10^{-6} M) facilitated I_{GABA}. During the potentiation, 5-HT produced almost no further enhancement of the I_{GABA}. (Ab) 5-HT failed to enhance the I_{GABA} in the presence of 3×10^{-6} M chelerythrine (chele). (Ac) After the pretreatment of 10^{-4} M LiCl for 2 min, the administration of 5-HT induced no further enhancement of I_{GABA}. (B) The effects of OAG, chelerythrine and LiCl. Each column was obtained from five neurones.

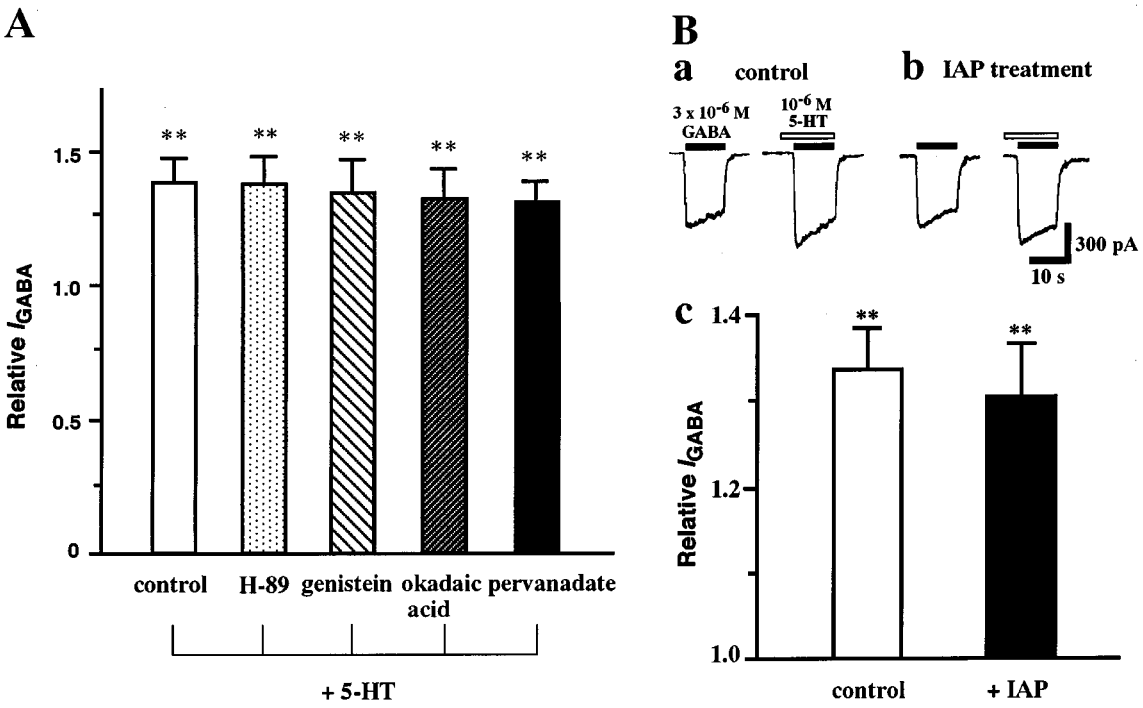

Figure 5 Effects of H-89, genistein, okadaic acid, pervanadate and IAP on the 5-HT-mediated potentiation of I_{GABA}. (A) Each modulator was applied for at least 5 min before the simultaneous application with 5-HT. Left column indicates the 5-HT-induced potentiation of I_{GABA} in intact SDN neurones. The other columns show the effects of 10^{-6} M 5-HT on I_{GABA} in neurones treated with 10^{-6} M H-89, 5×10^{-5} M genistein, 2×10^{-6} M okadaic acid or 10^{-4} M pervanadate. Each column was obtained from five neurones. $^{**}P < 0.01$. (B) 5-HT (10^{-6} M) facilitated I_{GABA} even after the treatment with IAP for 6 to 8 h (Bb), as well as in the control (Ba). The data from five neurones are summarized in (Bc). $^{**}P < 0.01$.

the neurotransmitters GABA and 5-HT, colocalize in some neuronal populations. Double immunostaining for 5-HT and glutamic acid decarboxylase, the enzyme responsible for the conversion of glutamic acid into GABA, is detected in the raphe nuclei of the rat medulla oblongata (Millhorn *et al*., 1987). 5-HT affects the frequency of $GABA_A$-receptor-dependent inhibitory postsynaptic currents in neurones from ventral tegmenta area and substantia nigra (Pessia *et al*., 1994), modulates muscimol-induced inhibition of the activity of cerebellar Purkinje cells (Strahlendorf *et al*., 1991), and facilitates GABAergic inputs to cat dorsal lateral geniculate relay cells (Funke & Eysel, 1995). Moreover, 5-HT inhibits the $GABA_A$ receptor-mediated response in the rat suprachiasmatic neurones by stimulation of the $5\text{-}HT_7$ receptor subtype (Kawahara *et al*., 1994) and the $GABA_A$ receptors in *Xenopus* oocytes coexpressing the $5\text{-}HT_{2C}$ receptor (Huidobro-Toro *et al*., 1996). The present study has shown that 5-HT facilitates the $GABA_A$ receptor response in the rat SDCN neurones and that the 5-HT potentiation of I_{GABA} is mediated by activation of the $5\text{-}HT_2$ receptor. Given that SDCN is implicated in nociceptive transmission (Lu *et al*., 1995) and receives projections for the descending 5-hydroxytryptaminergic pathway (Xu *et al*., 1996), the results provide a potential mechanism for the regulation of these spinal neurones by 5-hydroxytryptaminergic antinociceptive inputs. Furthermore, the present results together with the above observations extend the hypothesis that GABA and 5-HT could act as cotransmitters in some regions of the CNS and cross-communication between 5-HT and GABA receptors may be an important modulatory process of spinal nociceptive signals, such as pain.

Intracellular mechanisms of 5-HT potentiation of I_{GABA}

A large number of studies have demonstrated that PKC inhibits $GABA_A$ receptor function (for review, see Smart, 1997), and this effect appears to be attributable to phosphorylation of the β and $\gamma 2$ subunits on serine residues (Krishek *et al*., 1994). However, on recombinant $GABA_A$ receptors, PKM (a constitutively activated form of PKC) enhances GABA responses; this effect is abolished by mutation of selected serine residues (Lin *et al*., 1996). It is unclear whether the apparent differential modulation of $GABA_A$ receptors by PKC is attributable to the use of different expression systems or due to the heterogeneity of the subunit combinations of the receptors in different preparations. Modulation of I_{GABA} need not have arisen either in entirety or in part from the direct phosphorylation of receptor proteins. Therefore, a more substantive difference is the different mechanisms after PKC activation that modulate such GABA responses, such as PKC itself, PKA (Sugita *et al*., 1997) and Ca^{2+}/CaMKII (Browning & Dudek, 1992), PTKs (Kawakami *et al*., 1995) and tyrosine phosphatase (Catarsi & Drapeau, 1997), in different preparations. In the present SDCN neurones, mechanisms other than the interactions with PKA, Ca^{2+}/CaMKII, PTKs, or tyrosine phosphatase might be involved in mediating the 5-HT potentiation of I_{GABA}, since neither H-89, the PKA inhibitor, nor BAPTA AM, the Ca^{2+} chelator, nor genistein, the PTKs inhibitor, nor pervanadate, the tyrosine phosphatase inhibitor affected the 5-HT potentiation of I_{GABA}. Dephosphorylation of $GABA_A$ receptors by protein phosphatases (Chen & Wong, 1995) may also not be involved in this process, because okadaic acid, the protein phosphatase inhibitor, had no effect on 5-HT potentiation of I_{GABA}.

The finding of Ca^{2+}-dependent regulation of $GABA_A$ receptors has led to the speculation that cellular events causing an increase in neuronal Ca^{2+} will suppress $GABA_A$ receptor function (Akaike *et al*., 1992). In a recent study, Harris and colleagues (Huidobro-Toro *et al*., 1996) found that $5\text{-}HT_{2C}$ receptors inhibit $GABA_A$ receptors by a Ca^{2+}-dependent, but phosphorylation-independent mechanism. One may expect that all $5\text{-}HT_2$ receptor subtypes (i.e. $5\text{-}HT_{2A-2C}$) have the ability to produce inhibition of the $GABA_A$ response, given that all $5\text{-}HT_2$ receptors seem to share the same signal transduction pathways, including mobilization of intracellular Ca^{2+} (Martin & Humphrey, 1994). However, in the present study, the manipulation of intracellular Ca^{2+} and IP_3 concentrations did not affect the 5-HT potentiation of I_{GABA}, indicating that neither Ca^{2+} nor IP_3 are involved in 5-HT-induced potentiation of I_{GABA} in the rat SDCN neurones. Thus, the cascade for signal transduction in the SDCN neurones would be that activation of the $5\text{-}HT_2$ receptor leads to activation of a IAP-sensitive G protein, resulting in activation of PLC. Hydrolysis of inositol phospholipids by PLC results in the activation of DAG, which causes PKC activation that might directly phosphorylate the $GABA_A$ receptor on serine residues.

Functional implications

As mentioned before (see Introduction), activation of RVM neurones by electrical stimulation or glutamate microinjection depresses the activity of nociceptive neurones in the spinal dorsal horn, and produces behavioural antinociception (Basbaum *et al*., 1976; Jensen & Yaksh, 1989). It has generally been assumed that inhibition of spinal nociception evoked by RVM stimulation is due to the activation of a spinally projecting cell population that releases 5-HT. However, a growing body of experimental evidence suggests that RVM is not a homogeneous population of 5-HT cells, and that multiple neurotransmitters are possibly involved when raphe-spinal neurones are activated (Antal *et al*., 1996). Alternatively, it is quite likely that neurotransmitters other than 5-HT also participate in RVM-produced antinociception and are released following stimulation in addition to 5-HT. Indeed, the extensive 5-hydroxytryptaminergic and GABAergic innervation of the spinal dorsal horn by fibres descending from RVM has been demonstrated recently and the spinal corelease of 5-HT and GABA, in some case probably together with glycine, is likely to be crucial in RVM-spinal synaptic transmission (Antal *et al*., 1996). Furthermore, the finding of colocalization of GABA, glycine, and their receptors at the synapses in the rat spinal cord has led to the speculation that GABA and glycine could act as cotransmitters in the spinal cord (Todd *et al*., 1996). Thus, the results presented in this paper and a previous paper by the same authors (Xu *et al*., 1996) have important functional implications concerning the descending control of spinal nociceptive signal processing. Although speculative, it is possible that volleys in RVM-spinal tract may evoke the release of 5-HT, GABA and/or glycine from RVM terminals, and they may then act as cotransmitters to inhibit nociceptive spinal interneurones and supraspinally projecting cells through $GABA_A$ and/or glycine receptors at a site postsynaptic to terminals of primary afferent fibres, resulting in both analgesia and attenuation of pain behaviour (Sorkin *et al*., 1993; Lin *et al*., 1994). If the descending antinociceptive system in man operates similarly, the simultaneous activation of descending RVM fibres and the potentiation of GABAergic and glycinergic neurotransmission within spinal dorsal horn may turn out to be a useful alternative to addictive opioid analgesics. Thus, a more exhaustive understanding of GABAergic and glycinergic mechanisms in pain perception may lead to the development of new non-opioid, non-addictive analgesics.

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