Acta Pharmacologica Sinica 2005 Apr; 26 (4): 428–434

Full-length article

Bradykinin potentiates 5-HT$_3$ receptor-mediated current in rat trigeminal ganglion neurons

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Key words

bradykinin; 5-HT3 serotonin receptor; regulation; patch-clamp techniques; trigeminal ganglion

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Received 2004-10-29
Accepted 2004-12-09

doi: 10.1111/j.1745-7254.2005.00074.x

Abstract

Aim: To explore the modulatory effect of bradykinin (BK) on 5-HT$_3$ receptor-mediated current in trigeminal ganglion (TG) neurons in rats. **Methods:** The whole-cell patch-clamp technique was used to record 5-HT-activated currents ($I_{5\text{-HT}}$) in neurons freshly dissociated from rat TG. Drugs were applied by rapid solution exchange. **Results:** The majority of the neurons examined responded to 5-HT applied externally with an inward current (76.3%, 74/97) that could be blocked by the 5-HT$_3$ receptor antagonist, ICS-205,930 (1×10^{-6} mol/L). In 66 of the 74 cells sensitive to 5-HT (89.2%), pretreatment for 30 s with BK (1×10^{-6}–1×10^{-10} mol/L) could potentiate $I_{5\text{-HT}}$ with the maximal modulatory effect occurring at 10^{-7} mol/L BK (71.6%±4.9%). BK shifted the 5-HT concentration-response curve upwards with an increase of 68.9%±7.2% in the maximal current response, but with no significant change in the EC_{50} value (19.1±3.2 μmol/L *vs* 20.9±3.5 μmol/L; *t*-test, $P>0.05$; $n=8$). BK potentiated $I_{5\text{-HT}}$ in a holding potential-independent manner and did not alter the reverse potential of $I_{5\text{-HT}}$. This BK-induced potentiation of $I_{5\text{-HT}}$ was almost completely blocked by Hoe 140 (5×10^{-7} mol/L), a selective B_2 BK receptor antagonist, and was removed after intracellular dialysis of GF-109203X (2 μmol/L), a selective protein kinase C (PKC) inhibitor, with the re-patch clamp. **Conclusion:** Pre-application of BK exerts an enhancing effect on $I_{5\text{-HT}}$ via a PKC-dependent pathway in rat TG neurons, which may explain the peripheral mechanism of pain and hyperalgesia caused by, for example, tissue damage and inflammation.

Introduction

Serotonin (5-HT), a major component of the inflammatory chemical milieu, is released from platelets, mast cells, or basophils that infiltrate an area of tissue damage[1]. Once released, 5-HT is free to interact with a number of molecularly distinct receptor subtypes, including the 5-HT$_3$ receptor expressed in primary afferent nociceptors[2,3], and is capable of evoking pain and hyperalgesia[4]. To date, receptors for 5-HT can be classified into seven types and can be further distinguished into at least 13 subtypes. Unlike all other known 5-HT receptor subtypes, which are G-protein coupled, the 5-HT$_3$ receptor is a member of the excitatory ligand-gated ion channel (LGIC) superfamily[5,6]. Researchers believe that 5-HT$_3$ receptors located on sensory nerve terminals are mainly responsible for 5-HT-induced pain and hyperalgesia, although other subtypes of 5-HT receptors are also involved[4,7–10]. Recently we have demonstrated the potentiation of 5-HT$_3$ receptor function by substance P and α-methyl-5-HT, an agonist of 5-HT$_2$ receptor, through a protein kinase C (PKC)-dependent pathway in primary sensory neurons[11,12].

Bradykinin (BK) is an inflammatory mediator that plays a pivotal role in pain and hyperalgesia by exciting nociceptors and sensitizing them through activation of PKC[13]. BK responses are mediated by BK receptors. There are two main types of BK receptors, B_1 and B_2. B_2 BK receptors are constitutively and abundantly expressed in primary sensory neu-

rons[14]. The B_2 BK receptor has been implicated in BK-induced nociceptor activities and nociceptive behaviors[15–17], and animals deficient in B_2 BK receptors show hypoalgesia and reduced inflammatory responses[18,19]. The cDNA of the B_2 BK receptor has already been cloned[20] and evidence for the contribution of the PKC pathway to the B_2 BK receptor-mediated algesic action of BK has accumulated[17,21]. Thus, it is highly possible that 5-HT_3 receptor function is also enhanced by BK through a PKC-dependent pathway. The present study aimed to explore whether the modulation of BK in 5-HT_3 receptor-mediated current could occur in trigeminal ganglion (TG) neurons.

Materials and methods

Isolation of TG neurons Spraque-Dawley rats, 2–3 weeks old, were anesthetized with ether and decapitated. The TG were removed and transferred immediately into Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MO, USA) at pH 7.4. After removal of the surrounding connective tissues the TG were minced with fine spring scissors and the ganglion fragments were placed in a flask containing 5 mL DMEM in which trypsin (type II-S, Sigma) 0.5 g/L, collagenase (type I-A, Sigma) 1.0 g/L, and DNase (type IV, Sigma) 0.1 g/L had been dissolved, and incubated at 35 °C in a shaking water bath for 30–35 min. Soybean trypsin inhibitor (type II-S, Sigma) 1.25 g/L was added to stop trypsin digestion. Dissociated neurons were placed into a 35 mm Petri dish and kept for at least another 30 min before electrophysiological recording. The neurons selected for the patch-clamp experiment measured 20–45 μm in diameter.

Electrophysiological recordings Whole-cell patch-clamp recordings were carried out at room temperature (22–24 ºC) using a whole-cell patch-clamp amplifier (CEZ-2400, Nihon Kohden, Tokyo, Japan). Pipettes were filled with internal solution containing (in mmol/L): KCl 140, $CaCl_2$ 1, $MgCl_2$ 2.5, HEPES 10, egtazic acid 11, and ATP 5; the pH was adjusted to 7.2 with KOH and the osmolarity was adjusted to 310 mOsm/L with sucrose. Cells were bathed in an external solution containing (in mmol/L): NaCl 150, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, HEPES 10, and *D*-glucose 10; the osmolarity was adjusted to 340 mOsm/L with sucrose and the 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 more negative pressure was applied to rupture it, thus establishing a whole-cell configuration. The adjustment of capacitance compensation and series resistance compensation was done before recording the membrane currents. The holding potential was set at -60 mV, unless otherwise indicated. Membrane currents were filtered at 10 kHz (-3 dB), and the data were stored and analyzed in a computer with data acquisition software and hardware systems (Huazhong University of Science and Technology, Wuhan, China) and recorded using a pen recorder (Nihon Kohden).

Drug application Drugs used in the experiments included: serotonin hydrochloride (5-HT, Sigma), bradykinin (BK, Sigma), 2-methyl-5-hydroxytryptamine maleate (Research Biochemicals Incorporated, Natick, MA, USA), ICS -205,930 (Research Biochemicals Incorporated), Hoe 140 (Sigma), and GF 109203X (Research Biochemicals Incorporated). All drugs except GF 109203X were dissolved in the external solution just prior to use and held in a linear array of fused silica tubes (od/id=500/200 μm) connected to a series of independent reservoirs. The distance from the tube mouth to the cell examined was approximately 100 μm. The application of each drug was driven by gravity and controlled by the corresponding valve, and rapid solution exchange could be achieved by shifting the tubes horizontally with a micromanipulator. Cells were constantly bathed in normal external solution flowing from one tube connected to a larger reservoir between drug applications. In a number of the experiments GF 109203X needed to be applied intracellularly and was dissolved in the internal solution.

Data analysis Data were statistically analyzed using Student's *t*-test or analysis of variance (ANOVA). Statistical analysis of the concentration-response data was carried out using the non-linear, curve-fitting program ALLFIT. Current values were expressed as mean±SEM.

Results

Current mediated by the 5-HT_3 receptor in rat TG neurons In our experiments neurons freshly isolated from rat TG were round or elliptic in shape under light microscopy. The majority of the cells examined responded to 5-HT applied externally with a concentration (1×10^{-3}–1×10^{-6} mol/L)-dependent inward current (76.3%, 74/97). This 5-HT-activated current ($I_{5\text{-HT}}$, 1×10^{-4} mol/L) could be mimicked by 2-methyl-5-HT (1×10^{-4} mol/L), a specific 5-HT_3 receptor agonist, and could be blocked by ICS-205,930 (1×10^{-6} mol/L), a selective antagonist of 5-HT_3 receptor, indicating that this current was mediated by the 5-HT_3 receptor (Figure 1A).

When 5-HT was applied regularly for 3-s durations with 3-min intervals, the $I_{5\text{-HT}}$ was repeated stably within at least 90 min, and the change in amplitude was within 8% (data not shown). Thus, we used this pattern of 5-HT applications in the following experiments.

Potentiation of $I_{5\text{-HT}}$ by pre-application of BK BK applied for 30 s prior to the application of 5-HT (1×10^{-4} mol/L)

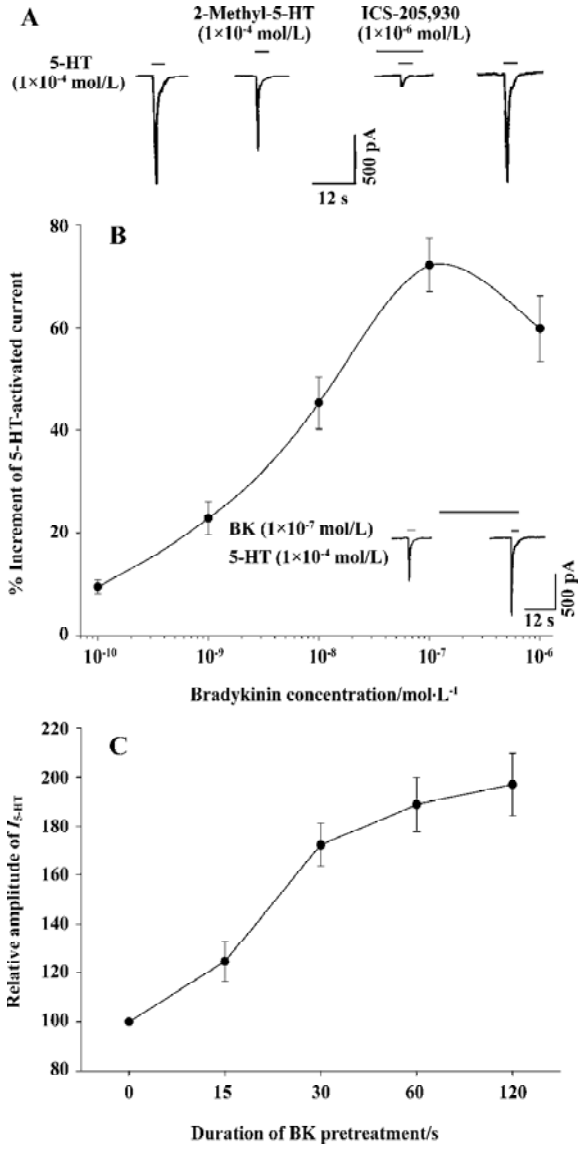

Figure 1. Potentiation of $I_{5\text{-HT}}$ by BK. (A) The current traces show that 5-HT (1×10^{-4} mol/L) activated an inward current in TG neurons, which could be mimicked by the application of 2-methyl-5-HT (1×10^{-4} mol/L), and blocked by ICS-205,930 (1×10^{-6} mol/L) (n=7). All current traces were recorded from the same neuron. (B) BK potentiated $I_{5\text{-HT}}$ in a concentration-dependent manner. The duration of BK pre-application lasted for 30 s. Each point represents the mean±SEM of 7–9 neurons. The current traces in the inset show that BK (1×10^{-7} mol/L) potentiated $I_{5\text{-HT}}$. (C) The enhancing effect of BK (1×10^{-7} mol/L) on $I_{5\text{-HT}}$ (1×10^{-4} mol/L) increased with increments in BK pre-application duration from 0 to 120 s (n=6–9).

potentiated $I_{5\text{-HT}}$ reversibly in the majority of the neurons examined (66/74, 89.2%). In 18 of the 74 (24.3%) cells sensitive to 5-HT there was also a response to BK with a very small inward or outward current (<150 pA), which showed slow desensitization (data not shown). The BK potentiation of $I_{5\text{-HT}}$ was observed irrespective of whether BK evoked an inward or outward current, or no response. The $I_{5\text{-HT}}$ was potentiated by the pre-application of BK (1×10^{-6}–1×10^{-10} mol/L) in a concentration-dependent manner. Figure 1B shows that with an increase in BK concentration from 1×10^{-10} to 1×10^{-6} mol/L, the amplitude of $I_{5\text{-HT}}$ (1×10^{-4} mol/L) increased stepwise until it reached its maximum at a concentration of 1×10^{-7} mol/L BK (71.6±4.9%). Thereafter this potentiating effect did not increase further, but rather decayed with further increases in BK concentration until 10^{-6} mol/L (Figure 1B).

Effect of the duration of BK pre-application on $I_{5\text{-HT}}$ To explore the relationship between the effect of BK on $I_{5\text{-HT}}$ and the duration of the pre-application of BK, different BK pre-application durations ranging from 15 to 120 s were tested. Figure 1C illustrates that the amplitude of $I_{5\text{-HT}}$ (1×10^{-4} mol/L) increased with increasing BK (1×10^{-7} mol/L) pre-application durations. With the duration of BK pre-application at 120 s, the amplitude of $I_{5\text{-HT}}$ increased (1.97±0.13)-fold compared with that of the control. However, there was no enhancing effect observed when 5-HT and BK were co-applied for 3 s (n=7; data not shown).

Effect of the B_2 BK receptor antagonist Hoe 140 on BK potentiation of $I_{5\text{-HT}}$ To verify whether the BK potentiation of $I_{5\text{-HT}}$ was mediated by the receptor for BK, we examined the effect of the pre-application of both BK and Hoe 140, a selective B_2 BK receptor antagonist, on $I_{5\text{-HT}}$. The pre-application of both BK and Hoe 140 abolished BK-induced potentiation of $I_{5\text{-HT}}$ significantly (Figure 2A,B, paired t-test, P<0.01, n=7).

Concentration-response relationship for 5-HT with and without BK pre-application Figure 3A demonstrates the concentration-response curves for 5-HT with or without the pre-application of BK (1×10^{-7} mol/L). The threshold concentrations of 5-HT in the two concentration-response curves for 5-HT with or without BK pre-application were similar at approximately 3×10^{-6} mol/L; and the EC_{50} values were also very similar (19.1±3.2 μmol/L and 20.9±3.5 μmol/L; t-test; P>0.05; n=8); whereas the maximal response induced by 5-HT with BK pre-application increased by 68.9%±7.2% of that without BK pre-application. The present results reveal that the concentration-response curve for 5-HT pretreated with BK shifts upwards compared with the curve for 5-HT alone.

Current-voltage (*I-V*) relationship for $I_{5\text{-HT}}$ with or without BK pre-application $I_{5\text{-HT}}$ (1×10^{-4} mol/L) with or without the pre-application of BK (1×10^{-7} mol/L) was recorded at different holding potentials. All current values from the same cell were normalized to the current response induced by

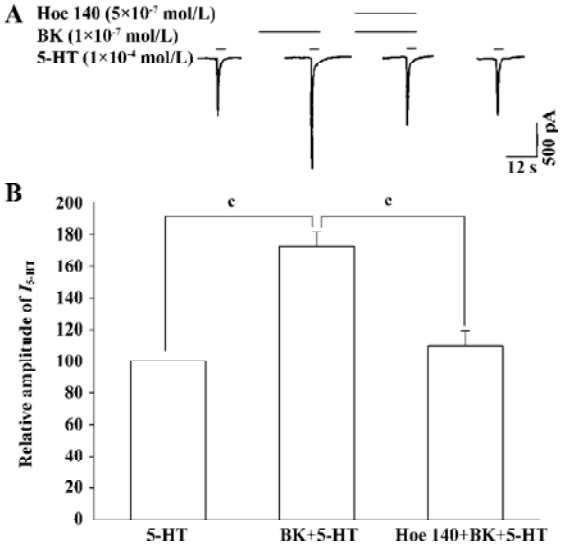

Figure 2. Blockade of BK-induced potentiation of $I_{5\text{-HT}}$ by the B_2 BK receptor antagonist Hoe 140. The current traces in (A) and the bar graph in (B) show that the potentiation of $I_{5\text{-HT}}$ by BK pre-applied alone was abolished by the co-application of BK and Hoe 140, a selective B_2 BK receptor antagonist (paired *t*-test, $^cP<0.01$ *vs* BK+5-HT).

5-HT alone at a holding potential of -60 mV when *I-V* curves were drawn (Figure 3B). The reverse potential values for the two curves were essentially the same at 0 mV. The amplitude of $I_{5\text{-HT}}$ with BK pre-application was greater than that without BK pre-application at all holding potentials from -80 to +40 mV, and the BK-induced alteration of $I_{5\text{-HT}}$ did not correlate with the change in holding potential (ANOVA; $P>0.05$, $n=8$), which suggests that the potentiation of $I_{5\text{-HT}}$ by BK occurs in a voltage-independent manner and the reverse potential of $I_{5\text{-HT}}$ is unchanged by BK.

Intracellular signal transduction mechanism underlying BK potentiation of $I_{5\text{-HT}}$ To explore whether this enhancing effect is mediated through BK-receptor-induced intracellular signal transduction, for example, activating PKC, GF-109203X, a selective PKC inhibitor[22], was included in the recording pipette for intracellular dialysis using the re-patch technique. In the control experiment with the pipette filled with normal internal solution, the BK-induced potentiation of $I_{5\text{-HT}}$ was 72.2%±5.2%. In contrast, when using a pipette filled with GF-109203X (2 μmol/L) containing internal solution the BK-induced potentiation of $I_{5\text{-HT}}$ was 13.5%±4.3%. It is evident that GF-109203X applied intracellularly removes the enhancing effect of BK on $I_{5\text{-HT}}$ (Figure 4).

Discussion

The 5-HT-activated current we recorded from TG neu-

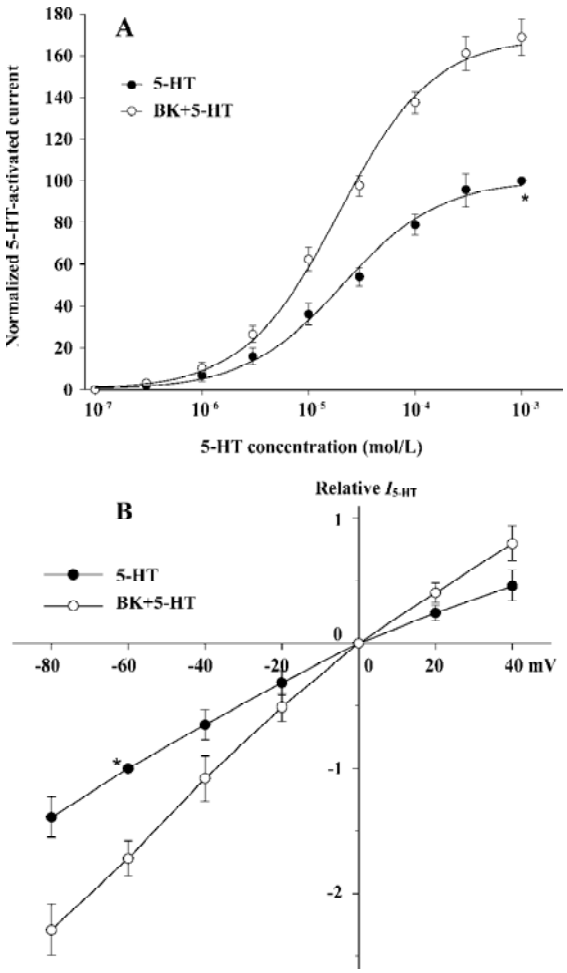

Figure 3. Concentration-response and current-voltage relationships for 5-HT with or without the pre-application of BK. (A) The concentration-response curves for 5-HT with or without BK (1×10^{-7} mol/L) pre-application, each point represents the mean±SEM of 7–11 neurons. All 5-HT-induced currents were normalized to the response induced by 10^{-4} mol/L 5-HT applied alone (marked with asterisk). The holding potential was set at -60 mV. The data for 5-HT alone is a good fit to the logistic equation $I=I_{max}/[1+(EC_{50}/C)^n]$, where C is the concentration of 5-HT, I is the normalized $I_{5\text{-HT}}$ value, and EC_{50} is the concentration of 5-HT for half maximal current response. The Hill coefficients (n) for the cases with and without BK pre-application were 0.98 and 0.96, respectively. It is evident that the curve for 5-HT with BK pre-application shifts upwards compared with the curve for 5-HT applied alone. (B) The *I-V* curves for 5-HT (1×10^{-4} mol/L)-activated current with or without BK (1×10^{-7} mol/L) pre-application. BK did not alter the reverse potential of $I_{5\text{-HT}}$ (0 mV in both cases). All current values from the same cell were normalized to the current response induced by 5-HT (1×10^{-4} mol/L) alone at the holding potential of -60 mV (marked with asterisk). BK pre-application potentiated $I_{5\text{-HT}}$ at all holding potentials from -80 to +40 mV. Each point represents the mean±SEM of 7–9 neurons. This experiment was carried out using recording pipettes filled with CsCl containing internal solution.

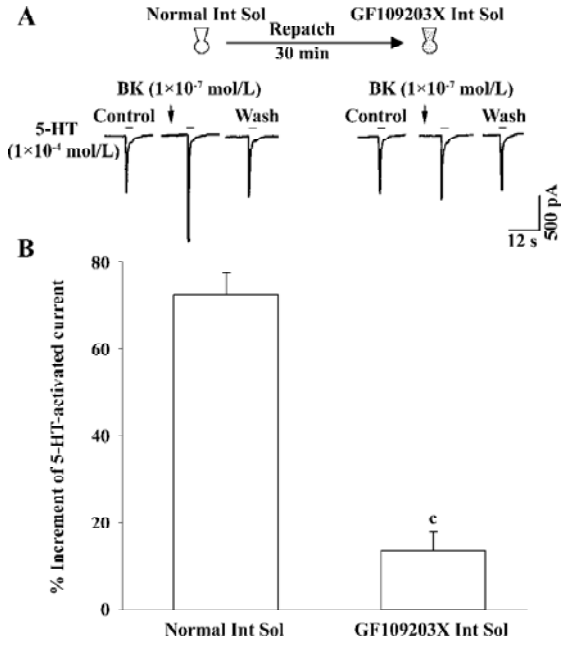

Figure 4. Reversal of the BK potentiation of $I_{5\text{-HT}}$ by intracellular dialysis of GF-109203X. The diagram in the upper row in (A) schematically shows the experiment of intracellular dialysis of GF-109203X, a selective PKC inhibitor, on an individual cell. The current traces in the lower row in (A) demonstrate that intracellular dialysis of GF-109203X (2 μmol/L) markedly reversed the potentiating effect of BK on $I_{5\text{-HT}}$. The bar graph in (B) shows the percentage increases in the $I_{5\text{-HT}}$ induced by BK pre-application with recording pipettes filled with the normal internal solution or with GF-109203X containing internal solution. n=7. Mean±SEM. (paired t-test, $^cP<0.01$ *vs* normal).

rons was mediated by the 5-HT_3 receptor, the sole ligand-gated ion channel (LGIC) in the family of 5-HT receptors, because it was blocked by ICS-205,930, a selective antagonist of the 5-HT_3 receptor (Figure 1A). There was evidence to indicate that 5-HT_3 receptors were present in rat TG neurons[23]. Similarly, B_2 BK receptors were also expressed in TG neurons[14]. In the present study we recorded both B_2 BK receptors and 5-HT_3 receptors in TG neurons, and in the majority of these neurons (89.2%, 66/74) the pre-application of BK (1×10^{-7} mol/L) potentiated $I_{5\text{-HT}}$ (1×10^{-3}–1×10^{-6} mol/L). This potentiation was mediated by B_2 BK receptors because the selective B_2 BK receptor antagonist Hoe 140 blocked this potentiating effect, obviously and reversibly (Figure 2). However, a previous study has reported that the inflammatory mediators BK, 5-HT, and prostaglandin E_2 do not cooperate to elevate intracellular calcium concentration when applied simultaneously for 10 s in cultured dorsal root ganglion neurons[24]. The distinction possibly results from different observation indices and specimens, or may be caused by the different treatment of BK. In the present experiment, there was also no enhancing effect observed when 5-HT and BK were co-applied for 3 s, whereas $I_{5\text{-HT}}$ was potentiated by the pre-application of BK for more than 15 s (Figure 1C).

It is evident from Figure 1B that the enhancement of amplitude of $I_{5\text{-HT}}$ increased gradually with incremental increases in the concentration of BK from 10^{-10} to 10^{-7} mol/L. However, when the concentration of BK increased to 10^{-6} mol/L the modulatory effect of BK on $I_{5\text{-HT}}$ did not increase further. The decrease in potentiation of $I_{5\text{-HT}}$ by BK (10^{-6} mol/L) might be a non-specific action of the agonist that emerges at high concentrations because very high concentrations of drug or ligand may block the channel and/or shelter the binding site of the receptor[25].

From the comparison between the concentration-response curves for 5-HT with and without the pre-application of BK (Figure 3A) it is clear that: (i) pre-application of BK shifted the curve upwards; (ii) the maximal response induced by 5-HT with BK pre-application increased by 68.9%, whereas the threshold concentrations of 5-HT in both cases were similar; and (iii) the EC_{50} values of the two curves were very close (19.1±3.2 μmol/L *vs* 20.9±3.5 μmol/L). This implies that the intrinsic efficacy of the 5-HT_3 receptor increases after pretreatment with BK; however, its affinity does not change.

From the *I-V* curves for $I_{5\text{-HT}}$ with and without BK pretreatment, it can be seen that the reverse potentials were the same (0 mV), indicating that there was no change in the ionic components mediating this current. This enhancement was not caused by the release of the channel blocker, as is the case in the voltage-dependent Mg^{2+} block of NMDA-gated ion channel, because the BK-induced alteration of $I_{5\text{-HT}}$ was not correlated with the change in holding potential (Figure 3B). This implies that the potentiation of $I_{5\text{-HT}}$ by BK occurs in a voltage-independent manner.

The potentiation of $I_{5\text{-HT}}$ by BK may involve intracellular signal transduction because there was no enhancing effect observed when 5-HT and BK were co-applied; nevertheless, BK applied prior to 5-HT application induced the enhancement of $I_{5\text{-HT}}$ and this effect was positively related to the duration of BK pretreatment, implying that this enhancement is a time-consuming process. Furthermore, this potentiation was blocked by Hoe 140, a selective B_2 BK receptor antagonist. B_2 BK receptors belong to the superfamily of G-protein-coupled receptors (GPCR)[20]. When activated by BK, the B_2 BK receptor is coupled to $PLC_{\beta1}$ via $G_{q/11}$ protein; which in turn catalyzes PIP_2 into secondary messengers, IP_3 and DAG. The latter activates PKC. Reports have shown

that 5-HT_3 receptor function is enhanced by the activation of PKC[26,27]. In the present experiment, the enhancing effect of BK on $I_{5\text{-}HT}$ was evidently blocked by intracellular dialysis of GF-109203X, a selective PKC inhibitor[22], indicating that potentiation occurs via a PKC-dependent pathway. How does PKC affect the function of 5-HT_3 receptors? Recently, a novel mechanism for 5-HT_3 receptor modulation by the activation of PKC was demonstrated[28]; that is, the PKC-induced potentiation of 5-HT_3 receptor mediated current in *Xenopus* oocytes and mouse NIE-115 neuroblastoma cells resulted from the enhancement of F-actin-dependent trafficking of 5-HT_3 receptors instead of direct phosphorylation of the 5-HT_{3A} receptor protein.

What is the physiological significance of this BK modulation on $I_{5\text{-}HT}$ or 5-HT_3 receptor function? In this work we used the cell body of TG neurons as a simple and accessible model to examine the characteristics of the membrane of peripheral terminals. The nerve endings of the peripheral axon of primary sensory neurons, including TG neurons, are sensitive to many inflammatory chemical mediators, of which BK and 5-HT are two potent stimulating mediators. In the case of inflammation and/or tissue damage these two substances are released. On the one hand, they exert a stimulating effect on the nerve endings and initiate nociceptive information through their corresponding receptors located on the membranes of separate nerve endings. On the other hand, in the present study we found that in the case of coexistence of B_2 BK receptors and 5-HT_3 receptors in TG neurons the inward current mediated by the 5-HT_3 receptor could be strengthened by pretreatment with BK, indicating that B_2 BK receptors and 5-HT_3 receptors may "cross-talk" in producing algesic information at nociceptors. Behavioral experiments have also demonstrated that 5-HT causes marked potentiation of BK-induced pain responses through 5-HT_3 receptors[29]. The present study may provide a hint for explaining the peripheral mechanism of pain and hyperalgesia caused by, for example, tissue damage and inflammation.

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