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ORIGINAL PAPER

The potentiating effect of calcitonin gene-related peptide on transient receptor potential vanilloid-1 activity and the electrophysiological responses of rat trigeminal neurons to nociceptive stimuli

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Abstract Growing evidence suggests that calcitonin gene-related peptide (CGRP) participates in trigeminal nociceptive responses. However, the role of CGRP in sensitization or desensitization of nociceptive transduction remains poorly understood. In this study, we sought to further investigate the CGRP-induced up-regulation of transient receptor potential vanilloid-1 (TRPV1) and the responses of trigeminal neurons to nociceptive stimuli. Rat trigeminal ganglion (TG) organ cultures and isolated trigeminal neurons were incubated with CGRP. An increase in TRPV1 levels was observed in CGRP-incubated TG organ cultures. CGRP potentiated capsaicin-induced increase in phosphorylated CaMKII levels in the TG organ cultures. The incubation of the trigeminal neurons with CGRP significantly increased the inward currents in response to capsaicin challenge, and this effect was inhibited by co-incubation with the CGRP receptor antagonist, BIBN4068BS or the inhibitor of protein kinase A, H-89. These findings reveal that CGRP acting on trigeminal neurons may play a significant role in facilitating cellular events that contribute to the peripheral sensitization of the TG in nociceptive transmission.

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Introduction

Calcitonin gene-related peptide (CGRP) belongs to the calcitonin family [1]. Several lines of evidence have emphasized the potential role of CGRP in pathophysiological changes that occur in both the central and peripheral trigeminal nociceptive systems. The CGRP concentrations of the plasma of migraineurs are higher than those of non-migraine patients [2] and are correlated with the timing and severity of headaches during migraine attacks [3]. In the trigeminal ganglion (TG), CGRP potentially affects nociceptor function via mechanisms including enhanced P2X3 gene expression [4], increased TRPV1 levels [5] and increased endogenous CGRP mRNA levels in the trigeminal neurons [6] as well as by elevating inducible nitric oxide synthase (iNOS) [7] and the amount of PGE_2 released from trigeminal satellite glial cells [8]. CGRP can also activate nociceptive neurons in the trigeminal nucleus caudalis (TNC) [9] and spinal nociceptive neurons in the dorsal horn of the spinal cord and facilitate spinal nociceptive transmission [10]. Therapeutically, CGRP receptor antagonists display efficacy in migraine treatment and are being explored as alternatives to 5-HT1 agonists. CGRP antagonists can block vasodilation, inhibit CGRP actions in the TG [11] and limit nociceptive trigeminovascular transmission [12].

The transient receptor potential vanilloid type I (TRPV1) channel is a non-selective cation channel, and its activation results in Ca^{2+} influx and the stimulation of intracellular calcium signaling cascades. TRPV1 can be activated by acid, noxious temperatures and capsaicin and

can be phosphorylated by many protein kinases, including PKA, PKC and CaMKII. TRPV1 has important roles in peripheral pain mechanisms, including tissue injury and inflammation pain [13, 14]. In our previous study, we demonstrated the modulatory roles of CGRP in the induction of increases in TRPV1and CGRP levels in TG neurons and the activation of neurons in the TNC by observing increases in c-Fos expression [5]. While experimental and clinical studies indicate that CGRP plays a key role in the generation and maintenance of pain during migraine attacks by facilitating cellular events that contribute to the peripheral sensitization of the TG and the central activation involved in nociceptive transmission [15] information describing the underlying molecular mechanisms and electrophysiological effects produced by CGRP in the trigeminal system is lacking. Therefore, the main purpose of this study is to investigate the modulatory roles of CGRP on the activation of intracellular signaling cascades and the consequent induction of increased TRPV1 levels and activities in rat TG.

Materials and methods

Animals

Eight-week-old male Wistar rats from the National Laboratory Animal Centre, Mahidol University, Nakhonpathom, Thailand were used for all Western immunoblotting experiments. All of the experiments in this study were conducted in accordance with the NIH Guidelines for the Care and Use of Animals, and the protocol was approved by the Institute of Molecular Biosciences Animal Care and Use Committee (MB-ACUC) of Mahidol University, Thailand (COA. No. MB-ACUC 2011/002). All of the electrophysiological experiments used 6–8 weeks old male Wistar rats from the Animal Resources Center of Perth. All of the experiments were performed with protocols that were approved by the Macquarie University Animal Ethics Committee (approval 2012/058). The animals were housed in groups of up to 4, and food and water were continuously available.

Rat trigeminal ganglion organ cultures

Adult male Wistar rats were anesthetized via intraperitoneal injection (ip) of 60 mg/kg pentobarbiturate, and their TGs were collected for organ cultures. The TGs were cut rostrocaudally into two or three pieces in ice-cold phosphate-buffered saline (PBS) and transferred to Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum and penicillin/streptomycin. The TG pieces were subsequently incubated for 24 h at 37 °C in a humidified 5% CO_2 in incubator. After incubation, TG organ cultures were washed with complete media and used to study the dose- and time-dependent effects of CGRP.

Primary trigeminal neuron cultures

Adult male Wistar rats were anesthetized with isoflurane and decapitated. The TGs were excised and washed with ice-cold MgHBS containing 140 mM NaCl, 2.4 mM KCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 10 nM glucose. The ganglia were cut and incubated at 37 °C for 15 min in 3 mg/ml collagenase in Leibovitz/s L15 medium supplemented with 5 mM NaHEPES, 5 mM glucose and penicillin/streptomycin (complete L15) followed by 25 min in papain (1 mg/ml in complete L15). Enzyme activity was stopped with a trypsin inhibitor (1 mg/ml) and bovine serum albumin (1 mg/ml in complete L15). The cells were washed with complete L15 and transferred to NeuroBasal medium supplemented with B27 supplement, GLUTAMAX and penicillin/streptomycin (complete NeuroBasal). The cells were dissociated via gentle trituration through Pasteur pipettes with fire-polished tips. The cells were plated on plastic culture dishes that were precoated with poly-D-lysine and laminin and were maintained in an incubator at 37 °C and 5% CO_2 for 90 min. After the cells settled, 2 ml of complete NeuroBasal medium was added to each dish, and the cells were returned to the incubator.

The trigeminal neurons were incubated with CGRP at 10 nM, 100 nM or 1 μM in complete NeuroBasal medium for 1, 4 or 24 h in the incubator at 37 °C and 5% CO_2. The effects of the CGRP receptor antagonist BIBN4096BS and the PKA inhibitor H-89 were tested by pretreatment of the trigeminal neurons for 30 min prior to incubation with CGRP for 4 h.

Western immunoblotting

TG pieces from the organ cultures were collected and washed with PBS and then homogenized in ice-cold lysis buffer containing phosphatase and protease inhibitors. The protein content was determined according to the method of Lowry. The protein samples were denatured and separated by 10% SDS–polyacrylamide gel-electrophoresis and subsequently transferred onto a PVDF membrane. The membranes were incubated with 3% non-fat milk in 0.1% Tween-Tris-buffered saline (TBST) for 1 h at room temperature and then incubated overnight at 4 °C with goat anti-VR1 (P-19) (dilution 1:1,000; Number sc-12503, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-phospho-CaMKII (Thr286) (dilution 1:1,000; Number 12176, Cell Signaling, Beverly, MA) primary antibodies followed by HRP-conjugated donkey anti-goat IgG

(dilution 1:20,000; Number sc-2020, Santa Cruz Biotechnology) or HRP-conjugated goat anti-rabbit IgG (dilution 1:5,000, Number 7074, Cell Signaling) for 90 min. Chemiluminescence ECL Plus-Western Blotting detection reagents were used to detect the immunoblots, which were quantified by densitometry analysis with the Scion Image program (National Institutes of Health, Bethesda, MD, USA). To normalize the protein loading of the gels, the immunoblots were re-probed with a mouse anti-β-actin antibody.

Electrophysiology and inward current recording

Whole-cell patch-clamp recordings were performed with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) using AxoGraph X software (http://axographx.com). The dishes were continuously perfused with HEPES-buffered saline (HBS) that contained the following: 150 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.2 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 10 mM glucose. This solution was adjusted to a pH of 7.3–7.4 with NaOH and an osmolarity of 325–330 mOsmol. The microelectrodes were made with fire-polished borosilicate glass pipettes and filled with an internal solution that contained 120 mM CsCl, 10 mM NaCl, 20 nM HEPES, 10 mM EGTA, 2 mM $CaCl_2$, 5 mM MgATP and 0.3 mM NaGTP and was adjusted to a pH of 7.3–7.4 with CsOH and an osmolarity of 285–290 mOsmol.

The potentiating effect of CGRP on the TRPV1 activity evoked by capsaicin

Capsaicin was applied at the maximally effective concentration of 10 μM to evoke reproducible inward currents. The drug was applied using a sewer pipe above the cells. Functional responses were recorded for up to 30 min after the wash out of the CGRP-containing medium. TRPV1 basal-responsive neurons were defined as capsaicin-treated TG neurons that produced inward currents about 100 pA when the neurons were held at −60 mV. All of the current traces were independently assessed by an investigator who was blinded to the drug pretreatment. The current density was calculated by dividing the peak inward current (pA) by the membrane capacitance as read from the amplifier after capacitance compensation (pF). Cell diameters were estimated with an eyepiece graticule.

Statistical analyses

The data are expressed as the mean ± SEM. One-way analyses of variance (ANOVA) with Tukey–Kramer tests were used to analyze the differences between several groups. Bartlett's tests were used to analyze the equal variances across groups. The statistical analyses were performed using the GraphPad Prism® scientific software. Probability (p) values less than 0.05 were considered statistically significant.

Results

Effect of CGRP on the TRPV1 levels in rat TG organ cultures

To determine whether CGRP could potentiate the expression of TRPV1 in rat TGs in vitro, the levels of TRPV1 protein were determined in TG organ cultures using western immunoblotting. The TG organ cultures were incubated for 1 h with various concentrations of CGRP (0.5, 1, 2 and 4 μM). The control cultures were incubated with culture media. CGRP (1 μM) significantly increased the TRPV1 protein levels in the rat TG organ cultures (1.61 ± 0.11-fold, $n = 4$, $p < 0.05$) compared with the control cultures ($n = 4$) (Fig. 1a). Some organ cultures were incubated with 1 μM CGRP for 1 and 2 h. Control-untreated organ cultures were incubated with cultured medium for 2 h. The results of 1 μM CGRP-incubated in various times showed that after 1 and 2 h incubation, CGRP significantly increased TRPV1 protein levels (2.2 ± 0.18 and 2.2 ± 0.19, respectively) when compared to control-untreated organ cultures (1.4 ± 0.14) (Fig. 1b).

The effect of capsaicin on CaMKII phosphorylation (pCaMKII) in CGRP-incubated TG organ cultures

Evidence has consistently demonstrated that the activation of TRPV1 receptors with capsaicin causes the induction of downstream signaling cascades, including the calcium-dependent signaling pathway. We determined the levels of phosphorylated CaMKII (pCaMKII) in the CGRP-incubated TG organ cultures after challenge with capsaicin. Capsaicin alone and capsaicin challenge of the organ cultures following exposure to CGRP significantly increased the pCaMKII levels (1.40 ± 0.06-fold, $n = 4$, $p < 0.05$ compared with the control and 1.73 ± 0.12-fold, $n = 4$, $p < 0.01$ compared with the control, $p < 0.05$ compared with the capsaicin, respectively). CGRP alone had no effect on the pCaMKII levels compared with the control vehicle-treated organ cultures. However, pCaMKII levels were significantly lower within the CGRP alone (1.0 ± 0.04-fold, $n = 4$, $p < 0.01$) when compared with the CGRP plus capsaicin (Fig. 2).

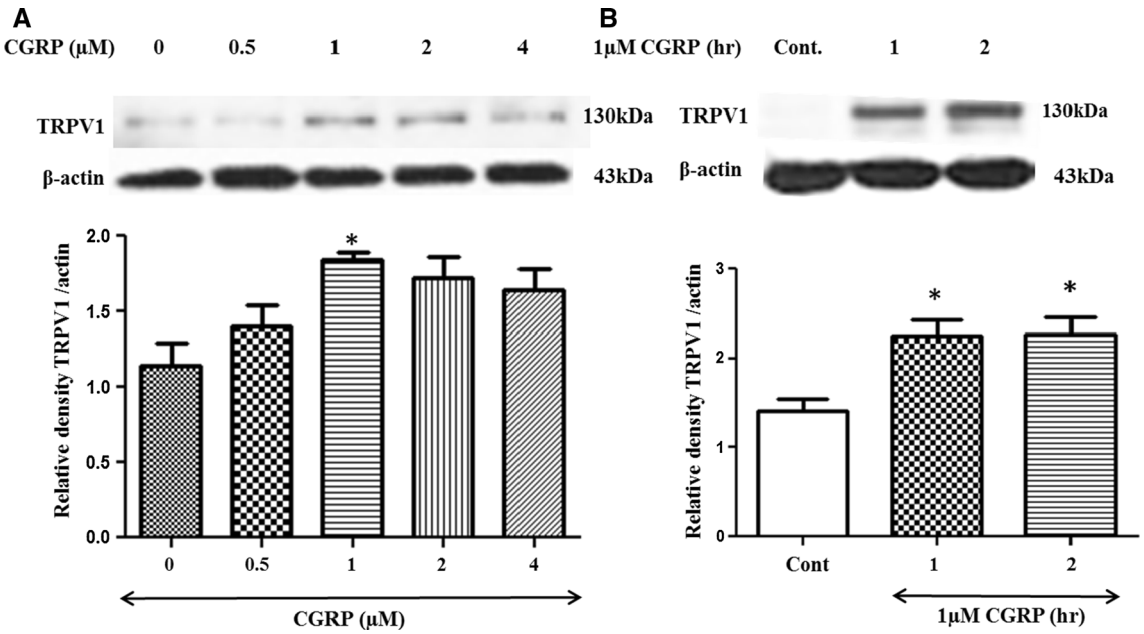

Fig. 1 Effect of CGRP on the TRPV1 levels in rat TG organ cultures. **a** TG organ cultures were incubated for 1 h with CGRP (0.5, 1, 2 or 4 μM). **b** TG organ culture were incubated with 1 μM CGRP for 1 and 2 h. The control cultures were incubated with culture media. The TG proteins were subjected to *western blot* analysis for TRPV1 (130 kDa), and the amounts were quantified (relative to β-actin). The results are expressed as the mean ± SEM of four independent experiments. Statistics was performed using ANOVA with Tukey–Kramer tests. $^{*}p < 0.05$ compared with the control

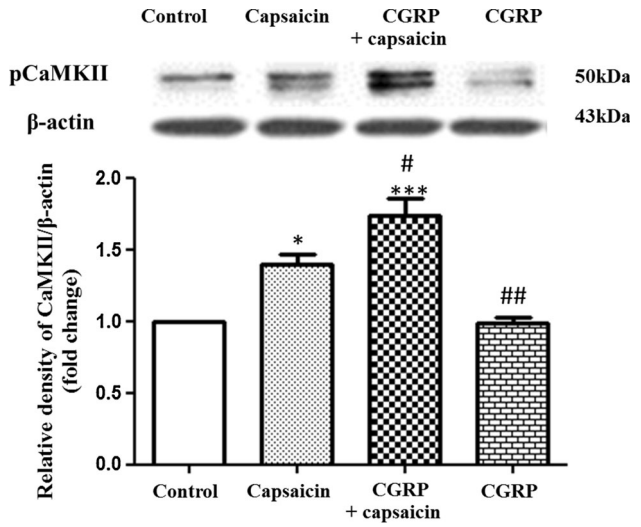

Fig. 2 The effect of capsaicin on CaMKII phosphorylation (pCaMKII) in CGRP-incubated TG organ cultures. After incubation with 1 μM CGRP for 1 h, the TG organ cultures were subjected to 30 min capsaicin (3 μM) challenges. The control cultures were incubated with culture medium for 90 min. Some cultures were incubated with 1 μM CGRP for 90 min or 3 μM capsaicin for 30 min. The proteins were isolated from the TG organ cultures and subjected to *western blot* analysis for pCaMKII (50 kDa), and the amounts were quantified (relative to β-actin). The results are expressed as the mean ± SEM of four independent experiments. Statistics was performed using ANOVA with Tukey–Kramer tests. $^{*}p < 0.05$ and $^{***}p < 0.01$ compared with the control, $^{\#}p < 0.05$ compared with the capsaicin and $^{\#\#}p < 0.01$ compared with the CGRP plus capsaicin

The potentiating effect of CGRP on the TRPV1 activity evoked by capsaicin

To test whether the up-regulation of TRPV1 in the TG by CGRP enhanced trigeminal neuron activation, the capsaicin currents were examined in isolated trigeminal neuron cultures (Fig. 3a). Trigeminal neurons with cell diameters in the range of 25–35 μm were tested by applying capsaicin (10 μM) during voltage-clamping at −60 mV. The majority of the small- to medium-sized trigeminal neurons exhibited capsaicin-stimulated currents (198/324, 61%). The trigeminal neurons with reversible capsaicin-induced inward currents about 100 pA were defined as TRPV1 basal-responsive neurons. The numbers of TRPV1 basal-responsive neurons were not different between the CGRP-incubated neurons and the control vehicle-treated neurons (data not shown). CGRP treatment of the trigeminal neurons elicited concentration- and time-dependent changes in the TRPV1 current densities. There were no appreciable changes after 1 h, but the 4 h CGRP treatments at concentrations of 100 nM and 1 μM significantly increased the current densities in capsaicin-stimulated trigeminal neurons to 11.3 ± 1.9 pA/pF ($n = 8$ neurons, $p < 0.05$) and 15.5 ± 2.7 pA/pF ($n = 8$ neurons, $p < 0.01$), respectively, compared with the control trigeminal neurons ($n = 8$ neurons, 3.8 ± 0.4 pA/pF). After 24 h of treatment, only the cells incubated with

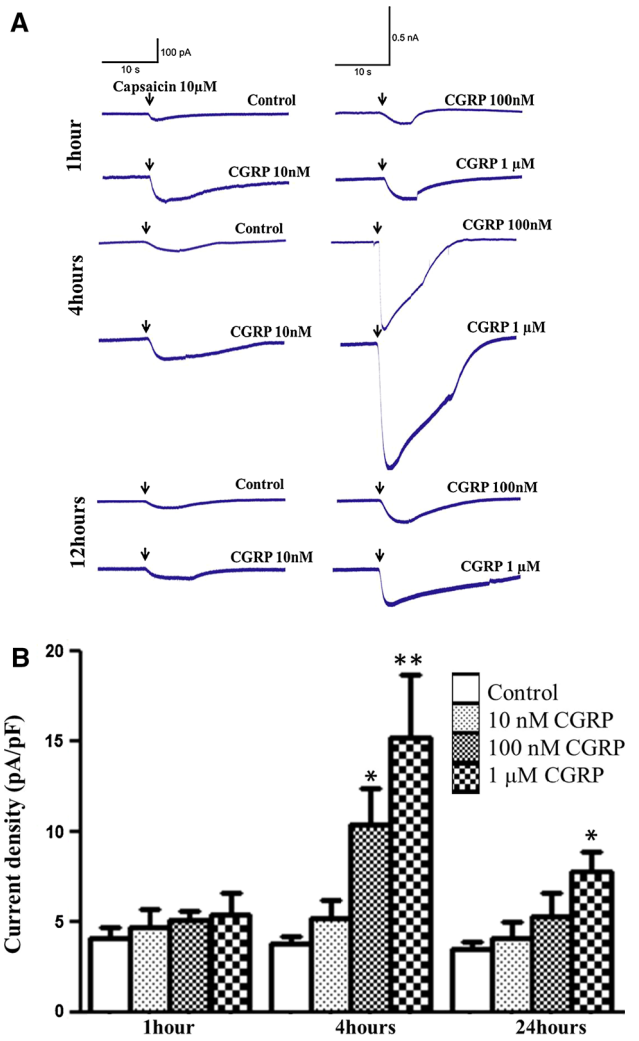

Fig. 3 The potentiating effect of CGRP on the TRPV1 activity evoked by capsaicin. Trigeminal neurons were incubated with CGRP at various doses (10 nM, 100 nM or 1 μM) or control treatment for various times (1, 4, and 24 h) before wash-out of the medium and recording of the reproducible inward currents evoked by 10 μM capsaicin. **a** The inward current responses of the trigeminal neurons evoked by 10 μM capsaicin (*arrow*). **b** The histogram summarizes the current densities (pA/pF). The results are expressed as the mean ± SEM. Statistics was performed using Bartlett's tests and ANOVA with Tukey–Kramer tests. $*p < 0.05$ and $**p < 0.01$ compared with the control; $n = 6$ for 1 and 24 h, and $n = 8$ for 4 h

CGRP at 1 μM exhibited significantly increased capsaicin current densities ($n = 6$ neurons, 7.8 ± 1.1 pA/pF, $p < 0.05$) compared with the control trigeminal neurons ($n = 6$ neurons, 3.5 ± 0.4 pA/pF) (Fig. 3b).

CGRP receptor- and PKA-dependent effects on the CGRP-induced capsaicin-stimulated current densities in the trigeminal neurons

The role of the CGRP receptor in the CGRP-induced increase in capsaicin-stimulated current density was examined in trigeminal neurons using the CGRP receptor antagonist BIBN4096BS (Fig. 4a). Exposure to 1 μM CGRP for 4 h significantly increased the capsaicin-stimulated current density in the trigeminal neurons to 23 ± 3.8 pA/pF ($n = 13$ neurons, $p < 0.01$) compared with the control neurons ($n = 13$ neurons, 8.9 ± 1.7 pA/pF). Pre-incubation with 100 nM BIBN4096BS for 30 min prior to exposure to 1 μM CGRP for 4 h significantly decreased the capsaicin-stimulated current density to 7.21 ± 1.08 pA/pF ($n = 13$ neurons, $p < 0.001$) compared with the CGRP-incubated trigeminal neurons. BIBN4096BS56 did not alter the capsaicin-stimulated current density in the trigeminal

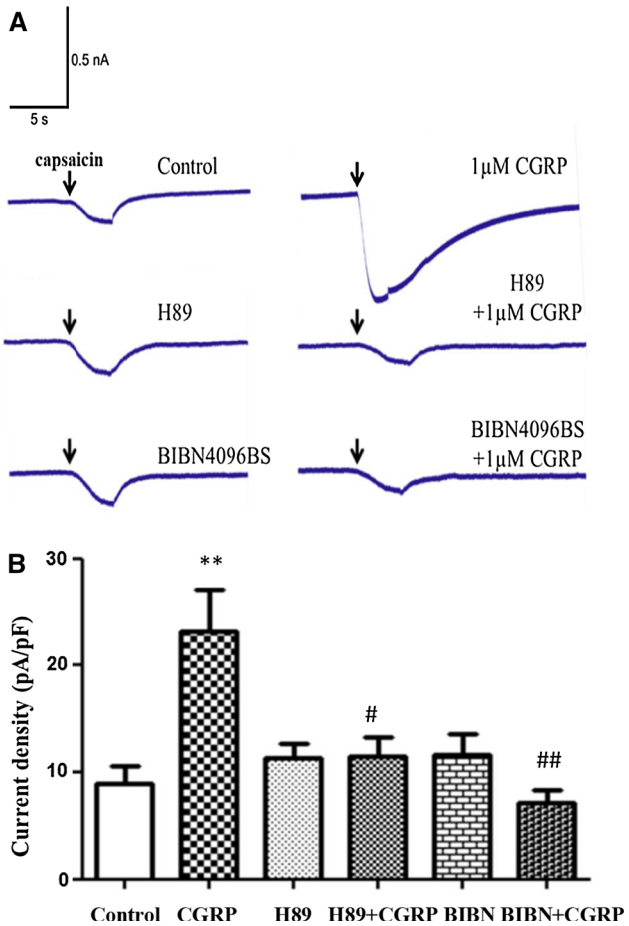

Fig. 4 CGRP receptor- and PKA-dependent effects on the CGRP-induced capsaicin-stimulated current densities in the trigeminal neurons. The trigeminal neurons were incubated with a PKA inhibitor (1 μM H89) or a CGRP receptor antagonist (100 nM BIBN4096BS) for 30 min prior to incubation with 1 μM CGRP for 4 h, and currents were evoked with 10 μM capsaicin. **a** The inward current responses of the trigeminal neurons evoked by 10 μM capsaicin (*arrow*). **b** The histogram summarizes the current densities (pA/pF). The results are expressed as the mean ± SEM of 13 independent records. Statistics was performed using ANOVA with Tukey–Kramer tests. $**p < 0.01$ compared with the control, and $^{\#}p < 0.01$ and $^{\#\#}p < 0.001$ compared with the CGRP-incubated neurons

neurons ($n = 13$ neurons, 11.7 ± 1.9 pA/pF) compared to the control neurons (Fig. 4b).

The involvement of the cAMP/PKA signaling cascade on the effect of CGRP was tested with the PKA inhibitor H89 (Fig. 4a). Pre-incubation with 1 μM H89 for 30 min prior to incubation with 1 μM CGRP for 4 h significantly decreased the capsaicin-stimulated current density (11.4 ± 1.82 pA/pF, $n = 13$ neurons, $p < 0.01$) compared to the 1 μM CGRP-incubated trigeminal neurons without H89. Incubation of the trigeminal neurons with H89 alone did not cause any change in the capsaicin-stimulated current density ($n = 13$ neurons, 11.3 ± 1.36 pA/pF) compared with the control neurons (Fig. 4b).

Discussion

CGRP is a neuropeptide that is a potent vasodilator and a mediator of neurogenic inflammation and nociceptive neurotransmission [16]. The intravenous administration of CGRP induces intracranial vessel dilation that might cause headaches in humans due to the function of CGRP receptors on the smooth muscle cells of the cerebral arteries [17]. Activated large cerebral vessels in the brain and meninges are able to enhance nociceptive signals in the TG [18]. The role of TRPV1 in the regulation of the vascular diameter is mediated through the release of CGRP and other neuropeptides [19]. We hypothesized that the up-regulation of TRPV1 levels by CGRP may affect the pain mediator-activated TRPV1 responses in TG neurons. Our previous study demonstrated that a single i.v. injection of CGRP into a rat results in a significant elevation of TRPV1 expression in the TG within 1 hour [5]. In the present study, we found that exposure to 1 μM CGRP for 1 and 2 h also significantly increased TRPV1 protein levels in the TG organ cultures. It has been demonstrated that changes in TRPV1 expression do not necessarily have to involved changes in transcription. In particular, the study of Ji RR, et al. [20] showed a sustained increased in TRPV1 protein in DRG after inflammation, but no change in TRPV1 mRNA, suggesting that there was an increase in the translation of the mRNA or possibly an increase in TRPV1 stability. The 1 μM CGRP-treated TG organ cultures was rather high concentration when compared to CGRP plasma level of about 7 nM that caused delayed migraine like headache [21]. It is likely that during a migraine attack the trigeminal nerves release CGRP, which in turn may cause the production and release of CGRP in the TG [22]. Additionally, we also demonstrated that CGRP potentiated the increase in p-CaMKII levels in capsaicin-stimulated TG organ cultures. Capsaicin is known to activate TRPV1 nociceptors to increase cytosolic free Ca^{2+} concentrations [23]. It has been reported that p-CaMKII protein levels increase in sensory neurons following capsaicin injection [24] and that this increase plays an important role in regulating the excitability of cells. Phosphorylated CaMKII is also present in the CGRP-ir and TRPV1-ir neurons of the TG [25]. The presence of p-CaMKII in presynaptic sites can facilitate the release of neurotransmitters, including glutamate, SP and CGRP [26]. Previous work has shown that the exposure of the TG to capsaicin elicits a 50% increase in p-CaMKII [27], and the activation of CaMKII, PI-3 kinase and ERK1/2 by Ca^{2+} influx has been reported to potentiate TRPV1 channel activity both in vivo and in vitro [6, 28]. The Ca^{2+}–CaM–CaMKII pathway has been shown to have an important role in the primary sensory neuronal transduction of peripheral nociception [29]. Together with our data, these findings suggest the possibility of a feed-forward loop in which the activation of TRPV1 leads to enhanced receptor activity.

The direct measurements of TRPV1 currents allowed us to determine whether the increases in TRPV1 protein were associated with larger functional TRPV1 responses. Capsaicin at 10 μM was used in this study because this dose of capsaicin has previously been shown to evoke maximal currents in sensory neurons [30], and this increase in current likely reflects greater numbers of channels in the membrane rather than changes in capsaicin potency. Our electrophysiological study showed that the capsaicin current density was significantly increased by CGRP pre-incubation for 4 h and that a lesser effect was present at 24 h. The decline in CGRP-activation at long incubation times might be attributable to the desensitization of the receptor [31]. The results of the CGRP-induced alterations in the TRPV1 levels and activities observed in the present study may indicate the presence of time-dependent CGRP activation responses in the TG. CGRP-induced increases in TRPV1 protein levels are observed 1 h after injection into the animal [5] or the incubation of TG organ cultures. In contrast, capsaicin-stimulated TRPV1-evoked inward currents in TG neurons were observed after 4 h of CGRP-incubation. The delay of the increment in the TRPV1 electrophysiological response might be explained by the post-translational modification of TRPV1 expression in the TG. Devesa et al. [32] showed that membrane expression of TRPV1 could be increased following recruitment of the channel to the cell surface from large dense core vesicles. They also showed that CGRP signaling through the CGRP receptor is necessary for this process. The functional effects of TRPV1 activation shown in the present study may occur via mobilization of TRPV1 to the cell membrane in TG neurons.

In the present study, we found that the CGRP-induced increase in capsaicin-stimulated current density was significantly attenuated by the CGRP receptor antagonist BIBN4096BS, suggesting that this effect was CGRP

receptor-dependent. Notably, CGRP-activated CGRP receptors may signal and modulate transduction processes in trigeminal sensory neuron fibers [33]. The transduction mechanisms of CGRP are primarily mediated via the activation of a Gs-protein-coupled receptor complex on the cell membrane that causes the stimulation of c-AMP-dependent and calcium-dependent signaling cascades [6]. Several lines of evidence have consistently demonstrated that CGRP is able to activate CaMKII-p-CREB, p-p38-ERK and PKA- and PKC-dependent cascades in neurons and p38-NFκB and ERK-STAT cascades in glial cells [4, 9, 34]. The activations of these cascades can lead to changes in gene transcription, including the transcription of TRPV1, and both the PKC- and PKA-dependent cascades can also be involved in the post-translational modification and trafficking of TRPV1 receptors [35]. However, in the present study, the effects of CGRP were blocked by an inhibitor of protein kinase A, H89, implying a mechanism that partially depends on an elevation in cAMP. Previous work has also reported that PKA inhibitors attenuate the effects of CGRP-induced hyperalgesia and the sensitization of neurons in the spinal dorsal horn [34] and that treatment of the dorsal root ganglion (DRG) neurons with CGRP increases cAMP production and pCREB, which in turn elicit changes in gene transcription that are inhibited by blocking PKA [36]. In conclusion, these findings establish substantial evidence for the role of the CGRP-mediated transmission of trigeminovascular nociceptive inputs via the TRPV1 complex. The monoclonal antibodies to CGRP and its receptor are currently being developed for the CGRP-based migraine therapeutics. However, behavioral studies of these proposed mechanisms of CGRP-induced increases in the levels and activities of TRPV1 and pain sensation in the trigeminovascular nociceptive system deserve further exploration.

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Author contributions The individual contribution to the article of each author is declared following: (1) DC contributed to data acquisition and analysis and preparation of the drafting manuscript. (2) MC contributed to analysis and interpretation of data. (3) AS contributed to analysis and interpretation of data. (4) BC contributed to experimental design, analysis and interpretation of data and critically revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors have no financial or other relationship that could lead to a conflict of interest. None of the authors have any competing interests.

Ethical approval All of the experiments in this study were conducted in accordance with the NIH Guidelines for the Care and Use of Animals, and the protocols were approved by the Institute of Molecular Biosciences Animal Care and Use Committee (MB-ACUC) of Mahidol University, Thailand (COA. No. MB-ACUC 2011/002) and the Macquarie University Animal Ethics Committee (approval 2012/058).

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