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# Regulatory effects of anandamide on intracellular $Ca^{2+}$ concentration increase in trigeminal ganglion neurons

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## Abstract

Activation of cannabinoid receptor type 1 on presynaptic neurons is postulated to suppress neurotransmission by decreasing $Ca^{2+}$ influx through high voltage-gated $Ca^{2+}$ channels. However, recent studies suggest that cannabinoids which activate cannabinoid receptor type 1 can increase neurotransmitter release by enhancing $Ca^{2+}$ influx *in vitro*. The aim of the present study was to investigate the modulation of intracellular $Ca^{2+}$ concentration by the cannabinoid receptor type 1 agonist anandamide, and its underlying mechanisms. Using whole cell voltage-clamp and calcium imaging in cultured trigeminal ganglion neurons, we found that anandamide directly caused $Ca^{2+}$ influx in a dose-dependent manner, which then triggered an increase of intracellular $Ca^{2+}$ concentration. The cyclic adenosine and guanosine monophosphate-dependent protein kinase systems, but not the protein kinase C system, were involved in the increased intracellular $Ca^{2+}$concentration by anandamide. This result showed that anandamide increased intracellular $Ca^{2+}$ concentration and inhibited high voltage-gated $Ca^{2+}$ channels through different signal transduction pathways.

***Key Words:*** *nerve regeneration; trigeminal ganglion; neurons; endocannabinoids; anandamide; cannabinoid receptor type 1; voltage-dependent calcium channels; vanilloid receptor; patch-clamp technique; calcium; cyclic adenosine monophosphate protein kinase; protein kinase C; NIH grant; neural regeneration*

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## Introduction

N-arachidonoylethanolamine (anandamide) is referred to as the first 'endocannabinoid' described, and anandamide signaling has been reported to be an inhibitor of axon regeneration. Anandamide acts primarily on cannabinoid receptor type 1 ($CB_1$)[1], cannabinoid receptor type 2 ($CB_2$)[2], and ionotropic receptors, including the transient receptor potential (TRP) vanilloid type 1 (TRPV1)[3-5], TRP ankyrin type 1 (TRPA1) and TRP melastatin type 8 (TRPM8) channels[6]. Anandamide can also directly modulate various other ion channels[5, 7-10].

$CB_1$ and $CB_2$ are members of the subfamily of G-protein-coupled receptors and predominantly couple to Gi/o[1, 11] to produce multiple cellular effects, such as the inhibition of adenylate cyclase and of voltage-gated calcium channels[12-14], regulation of potassium currents[15-17], and increase of $Ca^{2+}$ influx *via* $G_s$[18] and $G_q$[19]. TRPV1 is a polymodal sensor of noxious stimuli including heat, hydrogen ions and capsaicin[20]. $CB_1$[21] and TRPV1, but not $CB_2$[21] are expressed in presynaptic primary neurons such as those in the trigeminal ganglion. $CB_1$ is localized to presynaptic nerve terminals[22] and contributes to the regulation of neuronal excitability and neurotransmitter release by modulating $Ca^{2+}$ signals. TRPV1 activation causes $Ca^{2+}$ to enter into the cell and promotes neurotransmitter release. Studies have demonstrated that anandamide can activate TRPV1 by binding to cytosolic sites[23]. The co-expression and close distribution of $CB_1$ and TRPV1 in primary sensory neurons[24-27] allows cross-talk between these two receptors, which further complicates the role of anandamide in nociception and antinociception. The present study will determine which receptor contributes to the effect of low and high concentrations of anandamide in small trigeminal ganglion neurons.

It is a widely accepted general hypothesis that endocannabinoids inhibit neurotransmitter release from primary afferent neurons by reducing $Ca^{2+}$ influx *via* an inhibitory action on high voltage-gated $Ca^{2+}$ channels. This hypothesis is supported by evidence that $CB_1$ activation by anandamide

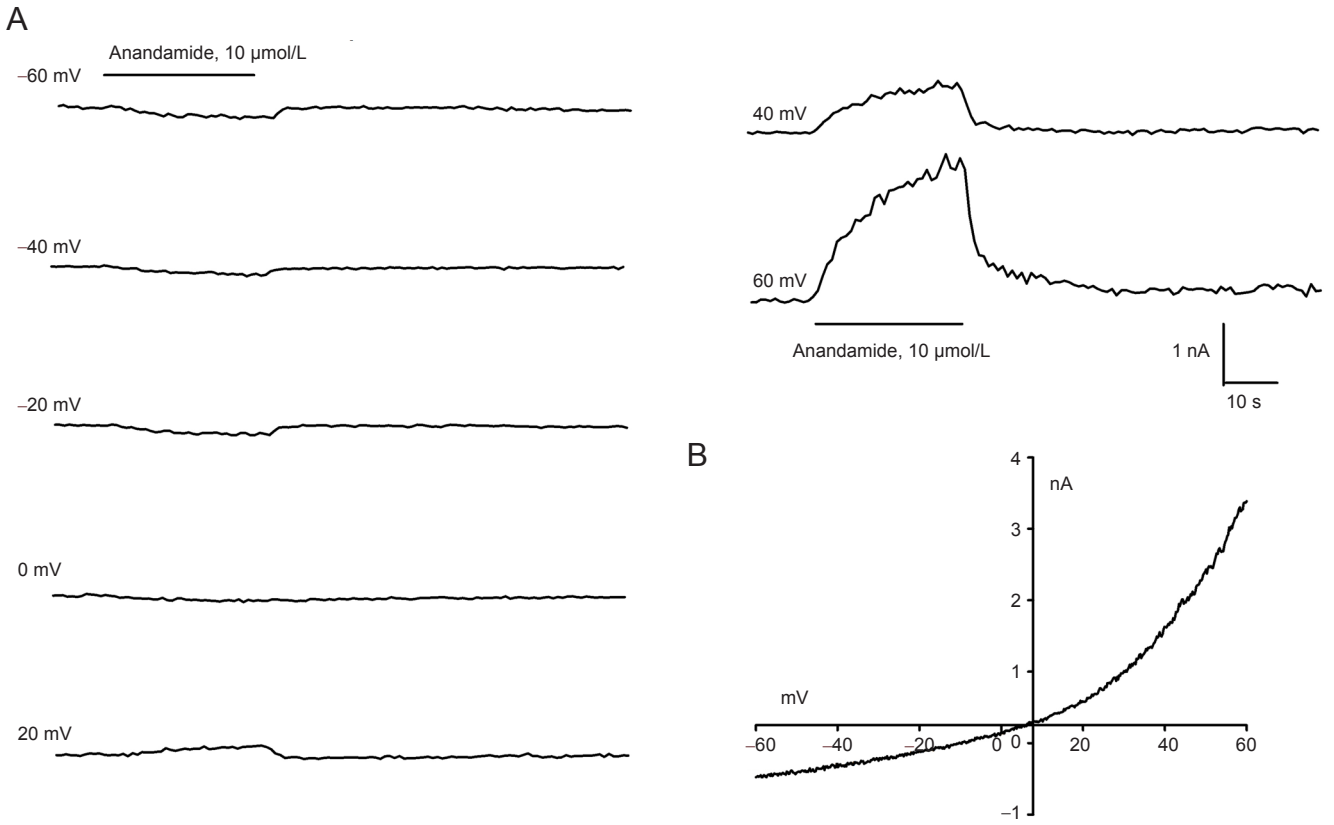


**Figure 1 Anandamide-evoked currents in rat trigeminal ganglion neurons.**
(A) Typical traces of 10 μmol/L anandamide-induced currents at different holding potentials (−60 to 60 mV) applied to a neuron. Bar indicates duration of stimulus. (B) Current-voltage relationship of 10 μmol/L anandamide-induced currents at different holding potentials. s: Second.

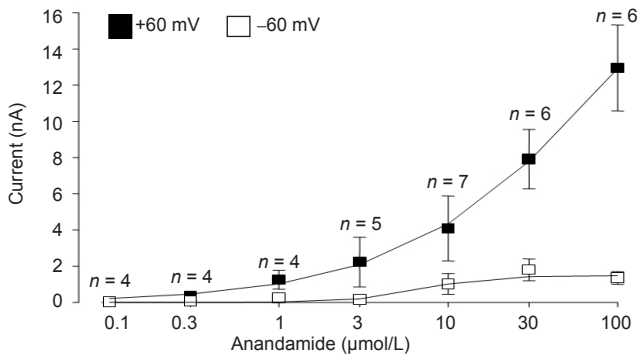


**Figure 2 Dose-dependent anandamide-evoked currents (nA) in trigeminal ganglion neurons, measured by the voltage-ramp method from −60 to +60 mV.**
*n* numbers represent individual experiments for each data point.

on primary nociceptive neurons causes antinociception by reducing high voltage-activated $Ca^{2+}$ channel activity[12-14] and $Ca^{2+}$ influx[28], thus inhibiting neurotransmission. In contrast, some studies suggest that cannabinoids can promote $Ca^{2+}$ influx and hence increase neurotransmitter release *in vitro*[19, 25, 29-31]. How endocannabinoids may cause such opposing effects in intracellular $Ca^{2+}$ concentration and neuron excitability is unclear. High voltage-gated calcium channels and ligand-gated channels, two important contributors to intracellular $Ca^{2+}$ concentration, will also be investigated. The present study tests the above hypothesis by determining the effect of anandamide on high voltage-activated $Ba^{2+}$ currents ($I_{HVA}$), $Ca^{2+}$ influx and intracellular $Ca^{2+}$ concentration, as well as the underlying mechanisms, in small trigeminal ganglion neurons.

## Results

### Anandamide-evoked inward currents

Whole-cell patch-clamp recordings were carried out in rat trigeminal ganglion neurons with bath perfusion of 0.1, 0.3, 1, 3, 10, 30 and 100 μmol/L anandamide at a range of holding potentials from −60 mV to +60 mV (Figure 1). Reversal potentials were around 0 mV. Anandamide-evoked inward and outward currents at holding potentials of −60 mV and +60 mV, respectively, were dose-dependent (Figure 2).

### Inhibition of $I_{HVA}$ by anandamide

Bath application of anandamide (0.001, 0.01, 0.1, 1, 10, 30 μmol/L) inhibited $I_{HVA}$ in a concentration-dependent manner, by 4.62 ± 0.88%, 6.24 ± 2.57%, 18.17 ± 0.99%, 31.30 ± 3.21%, 64.73 ± 1.95% and 55.19 ± 2.06%, respectively ($n$ = 4–11). The effect of anandamide was partially reversed after washout. The Hill equation was applied to the dose-response curve and revealed that the half-maximal inhibitory concentration ($IC_{50}$) of anandamide was 0.92 μmol/L. To confirm whether other $CB_1$ agonists mimicked the inhibition of anandamide, we tested the effect of WIN 55,212-2 (10 μmol/L) on $I_{HVA}$. The inhibition of $I_{HVA}$ by 10 μmol/L WIN 55,212-2 was 55.78 ± 6.07%. Anandamide at 1 μmol/L reduced current amplitudes (Figure 3A, B) but did not cause a significant shift of the activation curve (Figure 3C). However, a hyperpolarization shift of almost 14 mV ($n$ = 8; $P < 0.05$) was ob-

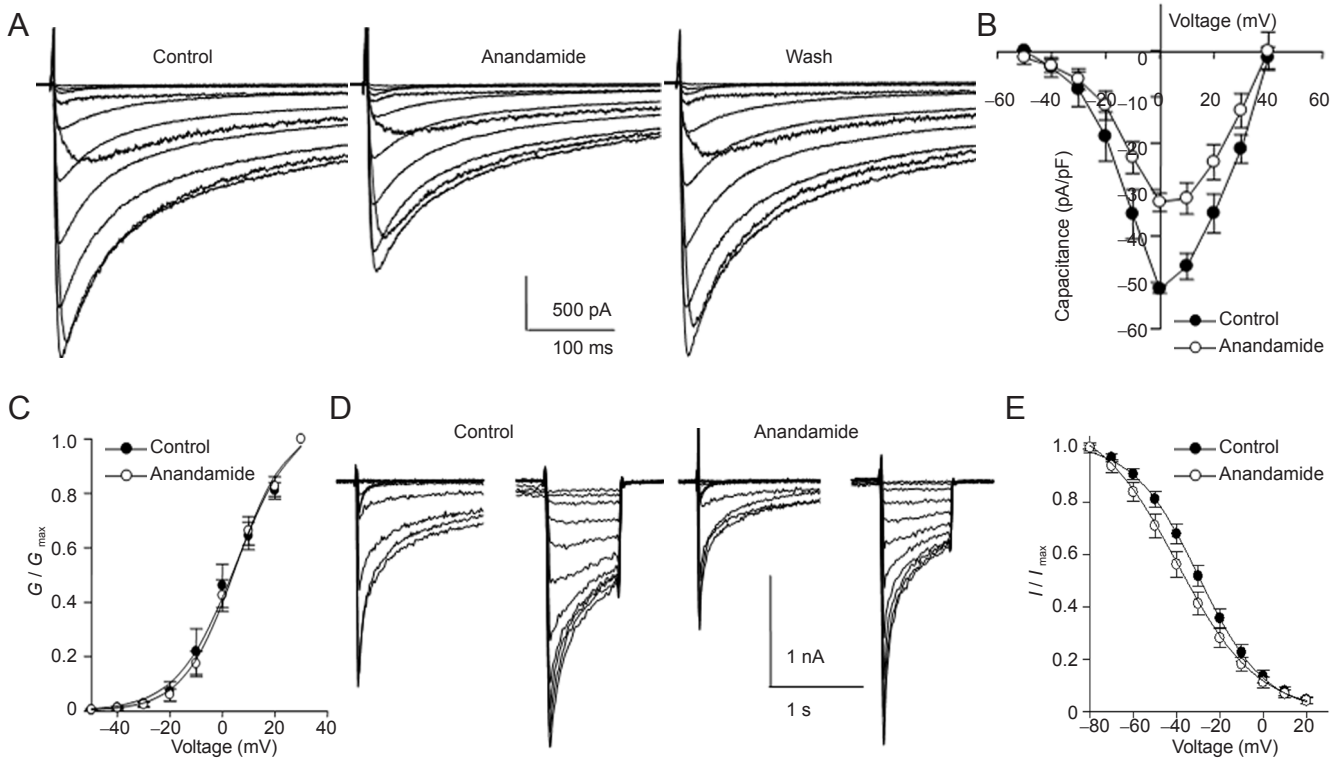


**Figure 3 Inhibition of high voltage-activated $Ca^{2+}$ currents ($I_{HVA}$) in rat trigeminal ganglion neurons by exposure to anandamide (1 μmol/L) for 3 minutes.**

(A) $I_{HVA}$ reduced from −2.42 nA to −1.67 nA, returning to −2.29 nA after 3 minutes of washout. Traces were evoked by 450 ms step depolarization from −50 mV to 40 mV in 10 mV increments. (B) Current-voltage curve of $I_{HVA}$. Anandamide perfusion for 3 minutes reduced the peak current intensity of $I_{HVA}$ from −51.50 ± 0.85 pA/pF to −32.73 ± 1.97 pA/pF (mean ± SEM, $n$ = 8; paired $t$-test, anandamide, *vs.* control, $P < 0.001$). (C) As the amplitude of $I_{HVA}$ reduced, the activation curve was not affected. The Boltzmann function was fitted to the activation curve. Before and after 1 μmol/L anandamide application, $V_{0.5}$ values were −3.73 ± 4.36 mV and −6.47 ± 3.02 mV, respectively ($n$ = 7, paired $t$-test, $P > 0.05$), and $k$ values (slope) were 8.74 ± 0.75 and 10.10 ± 0.58, respectively ($n$ = 7, paired $t$-test, $P > 0.05$). (D) Effect of 1 μmol/L anandamide on the h-infinity curve. The steady-state inactivation-voltage protocol consisted of 3 seconds of preconditioned pulses ranging from −80 mV to 20 mV followed by a 200 ms test pulse depolarizing to 20 mV. (E) Anandamide hyperpolarization shifted the h-infinity curve. After fitting the Boltzmann function, $V_{0.5}$ values were −30.93 ± 2.75 mV and −45.32 ± 5.61 mV ($n$ = 8, paired $t$-test, $P < 0.05$), and $k$ values were −15.78 ± 1.33 and −15.85 ± 2.58 ($n$ = 8, paired $t$-test, $P > 0.05$) before and after anandamide administration. s: Second.

served in the h-infinity curve (Figure 3E).

### Roles of cannabinoid and vanilloid receptors in the inhibition of $I_{HVA}$ by anandamide

Since anandamide activates TRPV1, $CB_1$ and $CB_2$ receptors, we tested whether capsazepine, AM251 and AM630, selective antagonists at the three receptors, respectively, could reverse the effect of anandamide on $I_{HVA}$. Capsazepine (10 μmol/L), AM251 (10 μmol/L), AM630 (10 μmol/L), or anandamide (1 and 10 μmol/L) were added to the bath solution during whole-cell patch clamp recordings in rat trigeminal ganglion neurons. First, we examined how pre-incubation of the cells for 3 minutes with capsazepine, a competitive antagonist at TRPV1, would affect the inhibition induced by anandamide (Figure 4A, D). Pre-incubation with capsazepine did not affect the inhibition of $I_{HVA}$ by 1 μmol/L anandamide ($n$ = 10, $P > 0.05$), but abolished the effect of 10 μmol/L anandamide ($n$ = 12, $P < 0.05$). These data indicate that TRPV1 activation is involved in the inhibition of $I_{HVA}$ by high-concentration anandamide. AM251 was co-applied with anandamide (1 or 10 μmol/L) (Figure 4B, E). Low-dose ($n$ = 7, $P < 0.05$) and high-dose ($n$ = 4, $P < 0.05$) anandamide-induced inhibition was reversed by AM251 (Figure 4G), indicating that activation of $CB_1$ is essential for the negative modulation of $I_{HVA}$ by anandamide in rat trigeminal ganglion neurons.

Pre-incubation for 3 minutes with AM630 did not affect $I_{HVA}$ inhibition induced by 1 μmol/L (Figure 4C, G; $P > 0.05$) or 10 μmol/L (Figure 4F, G; $P > 0.05$) anandamide, indicating a lack of CB2 receptors in rat trigeminal ganglion neurons[21].

### Inhibition of $I_{HVA}$ by capsaicin

As described above, inhibition of $I_{HVA}$ by high-concentration anandamide was reversed by the TRPV1 antagonist capsazepine, suggesting that TRPV1 is involved in this effect. We further tested whether $I_{HVA}$ could be directly inhibited by the TRPV1 agonist, capsaicin. Capsaicin and capsazepine were applied by bath perfusion. $I_{HVA}$ was reversibly inhibited by capsaicin in a dose-dependent manner (Figure 5). Similar to the effect observed with high-concentration anandamide, the decrease in $I_{HVA}$ by capsaicin was reversed by 10 μmol/L capsazepine. In addition, $I_{HVA}$ was not inhibited by 10 μmol/L capsazepine (Figure 6).

### Characterization of signal transduction pathways mediating the inhibition of $I_{HVA}$ *via* cannabinoid type-1 receptor activation by low-concentration anandamide

Three important signal transduction systems were examined using specific agonists and antagonists to test whether these

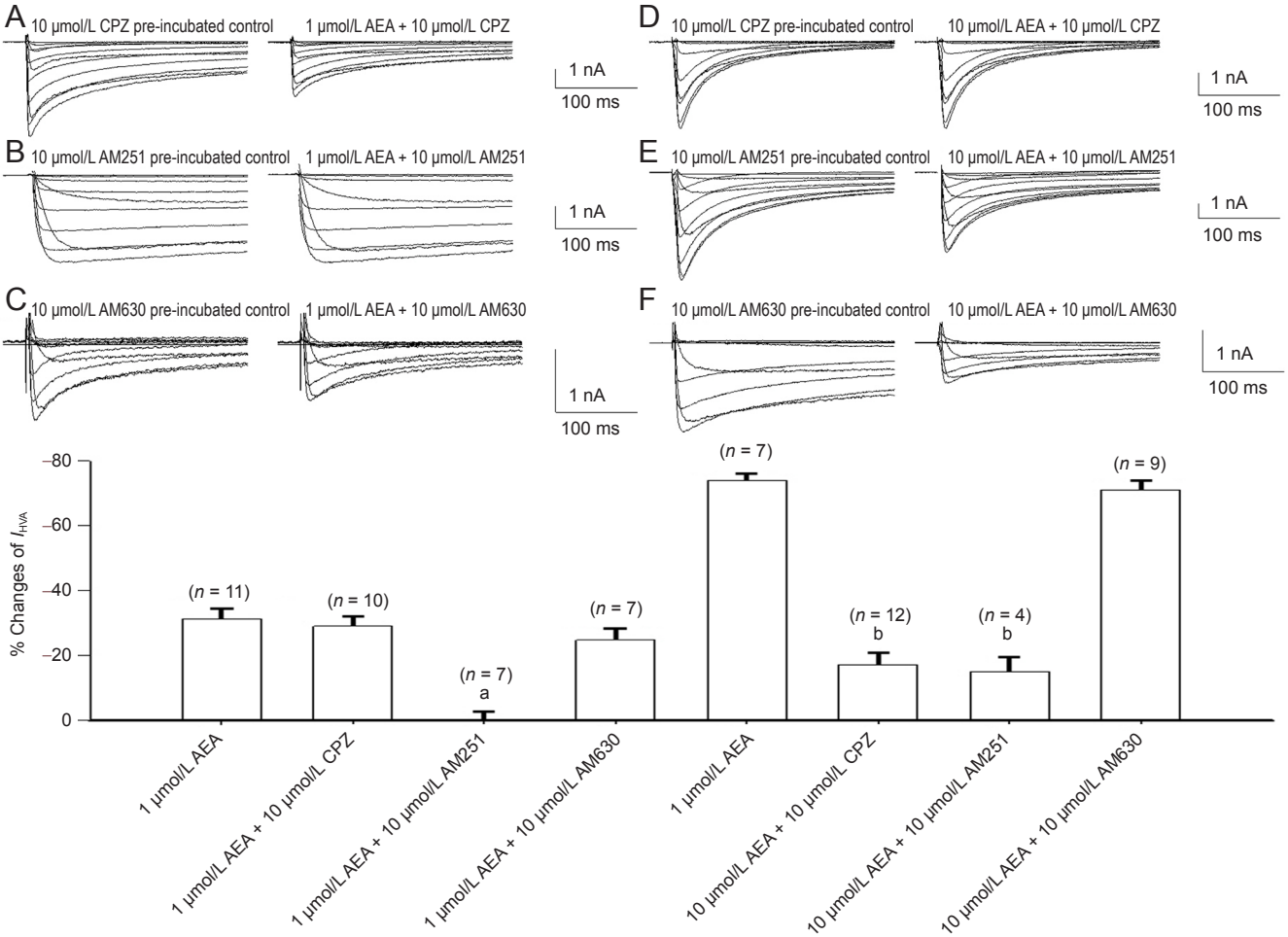


**Figure 4 Cannabinoid and TRPV1 receptor involvement in the inhibition of high voltage-activated $Ba^{2+}$ currents ($I_{HVA}$) by 1 and 10 μmol/L anandamide in rat trigeminal ganglion neurons.**

Pre-incubation with capsazepine (A) or AM630 (C) did not affect $I_{HVA}$ inhibition by 1 μmol/L anandamide. (B) Pre-incubation with AM251 abolished $I_{HVA}$ inhibition by 1 μmol/L anandamide. The decrease in $I_{HVA}$ induced by 10 μmol/L anandamide was partially attenuated by pre-infusion with capsazepine (D) or AM251 (E). Pre-incubation with AM630 (F) had no effect on 10 μmol/L anandamide-induced inhibition of $I_{HVA}$. (G) Summary data of the effect of cannabinoid and TRPV1 receptor antagonists on the inhibition of $I_{HVA}$ by 1 and 10 μmol/L anandamide. $^aP < 0.01$, *vs.* inhibition induced by 1 μmol/L anandamide (unpaired Student's *t*-test). $^bP < 0.01$, *vs.* inhibition induced by 10 μmol/L anandamide (unpaired Student's *t*-test). Data are expressed as mean ± SEM. *n*: number of neurons tested. CPZ: Capsazepine; AEA: anandamide.

systems participated in the inhibition of $I_{HVA}$ by CB1 receptors activated by 1 μmol/L anandamide.

Whole-cell patch clamp measurements in trigeminal ganglion neurons revealed that application of 1 μmol/L KT5720, an inhibitor of cyclic adenosine monophosphate -dependent protein kinase A, significantly attenuated the inhibition of $I_{HVA}$ by 1 μmol/L anandamide (Figure 7). Incubation with an antagonist of protein kinase C, bisindolylmaleimide (1 μmol/L for 10 minutes) and an inhibitor of cyclic guanosine monophosphate (cGMP)-dependent protein kinase, Rp-8-Br-cGMP (1 μmol/L for 10 minutes) also significantly attenuated anandamide-induced inhibition of $I_{HVA}$.

### Anandamide increased intracellular $Ca^{2+}$ concentration *via* a direct influx of extracellular calcium into rat trigeminal ganglion neurons

Calcium imaging was used to examine the effect of anandamide on intracellular $Ca^{2+}$ concentration in the presence of solutions containing either 0 or 2 mmol/L $Ca^{2+}$ (Figure 8A). In the absence of extracellular calcium (buffered by 10 mmol/L ethylene glycol bis(alpha-aminoethyl ether)-N,N′-tetraacetic acid to decrease extracellular $Ca^{2+}$ concentrations), the rise in intracellular $Ca^{2+}$ concentration induced by anandamide was notably decreased (Figure 8B; anandamide$_{([Ca^{2+}]_+)}$: 20.33 ± 2.10%, $n$ = 19; anandamide($[Ca^{2+}]_-$): 4.22 ± 1.06%, $n$ = 48; $P < 0.01$). Thus, the anandamide-induced rise in intracellular $Ca^{2+}$ concentration is dependent on $Ca^{2+}$ influx from the extracellular space.

### Cannabinoid / vanilloid type-1 receptor involvement in the mechanism underlying increased intracellular $Ca^{2+}$ concentration evoked by anandamide

Because AM630 had no effect on the inhibition of anandamide on $I_{HVA}$, in the present test only AM251 and capsazepine were used, to determine whether they would affect the increase of anandamide on the intracellular $Ca^{2+}$ concentration, using calcium imaging. The 1 μmol/L anandamide-induced rise in intracellular $Ca^{2+}$ concentration was abolished by co-application with 10 μmol/L AM251 (Figure 8C), while lower doses of AM251 (1 and 3 μmol/L) facilitated the in-

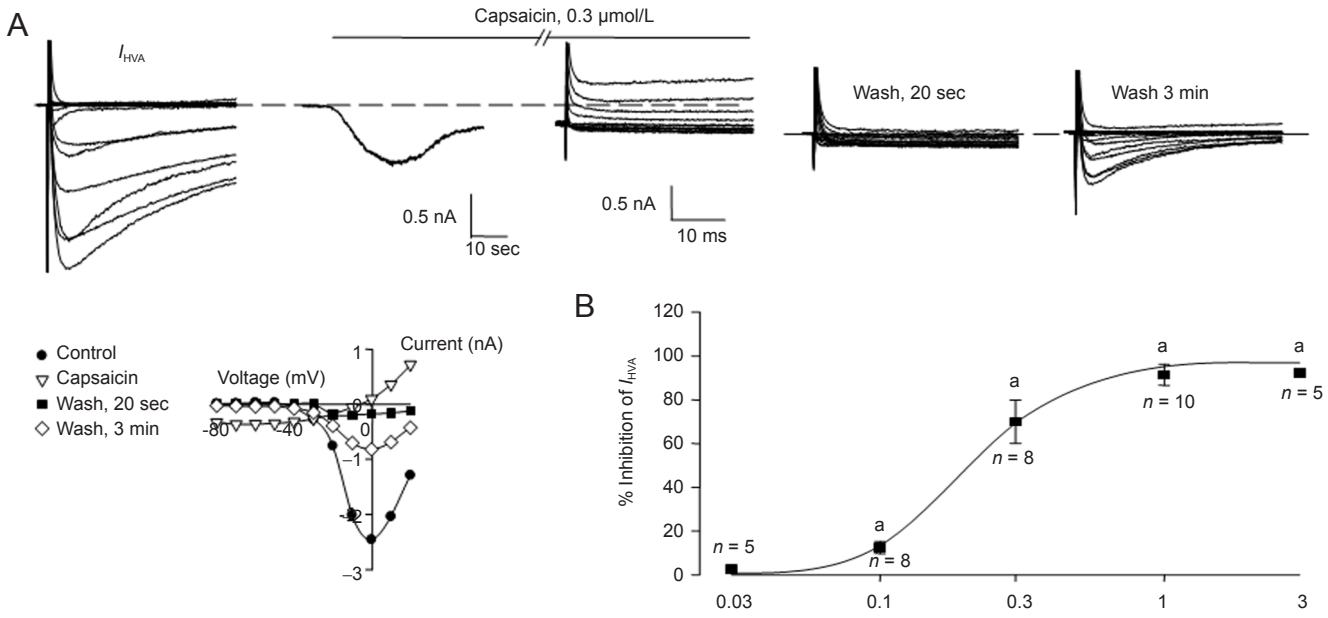


**Figure 5 Cannabinoid and transient receptor potential (TRP) vanilloid type 1 receptor involvement in the inhibition of high voltage-activated $Ca^{2+}$ currents ($I_{HVA}$) by 1 and 10 μmol/L anandamide in rat trigeminal ganglion neurons.**
(A) $I_{HVA}$ currents were generated in a neuron with a membrane resistance of 878 MΩ. Application of 0.3 μmol/L capsaicin induced a maximum inward current of 0.6 nA, which desensitized to 0.3 nA after about 60 seconds (sec). At this time, the membrane resistance was 164 MΩ and $I_{HVA}$ was reduced from 2.4 to 0.4 nA. After washing the cell for 20 seconds (sec), the capsaicin-induced inward current returned to baseline and the membrane resistance increased to 856 MΩ. $I_{HVA}$ recovered to 0.8 nA. Current-voltage curves are shown in the absence and presence of capsaicin as well as for the 20 sec and 3-minute (min) washes. (B) Dose-dependent inhibition of $I_{HVA}$ by capsaicin in capsaicin-sensitive trigeminal ganglion neurons. The Hill equation was fitted to the dose-response curve, with $IC_{50}$ = 0.21 μmol/L. *n*: Times of individual experiments carried out at each concentration. $^aP < 0.05$, *vs.* before capsaicin (one-way analysis of variance).

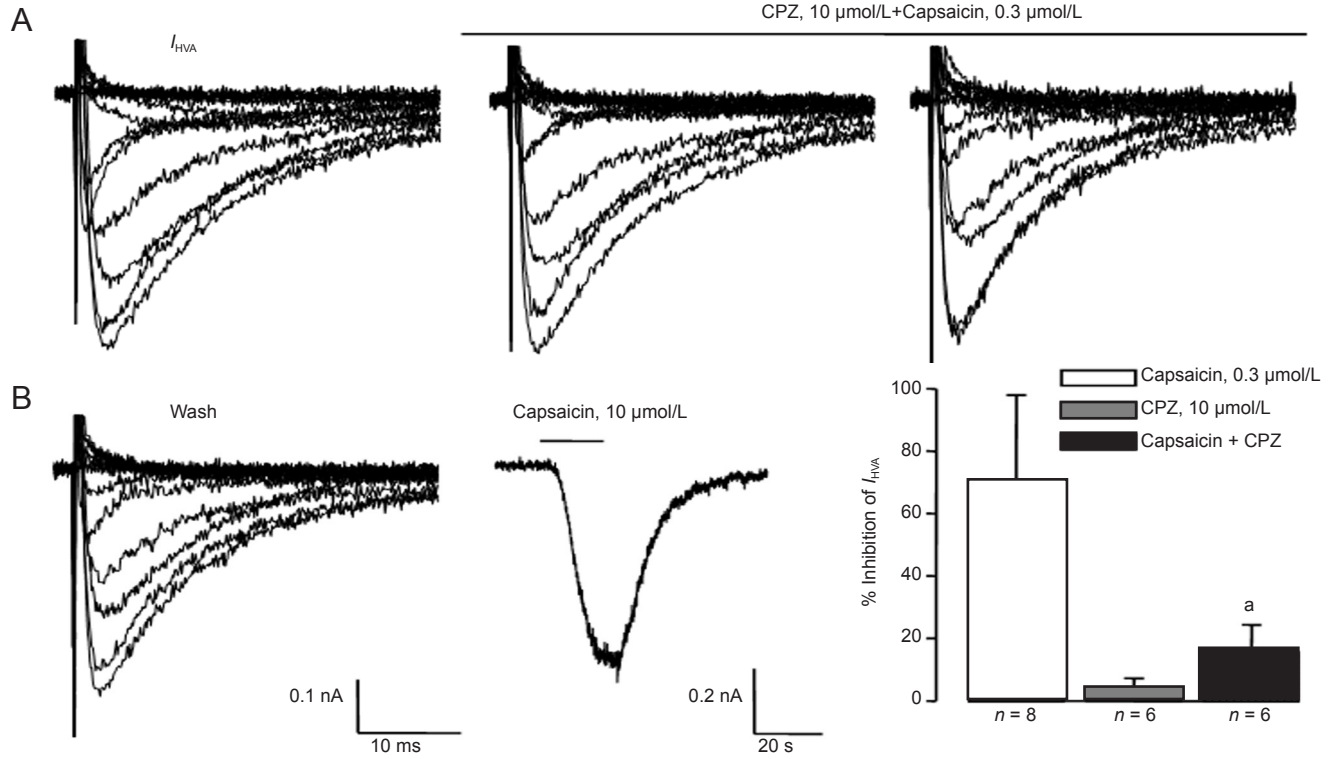


**Figure 6 Capsazepine (CPZ) inhibited the capsaicin-induced decrease of $Ca^{2+}$ current ($I_{HVA}$) in trigeminal ganglion neurons.**
(A) Representative traces showing the effects of co-application of capsazepine and capsaicin in a capsaicin-sensitive neuron. (B) Summary data of capsazepine blockade of capsaicin-induced inhibition of $I_{HVA}$. $^aP < 0.01$, *vs.* capsaicin alone by unpaired *t*-test. "*n*" indicate the times of neurons tested. After $I_{HVA}$ was obtained, neurons were incubated for 3 minutes (min) with 10 μmol/L capsazepine. Capsazepine did not affect the $I_{HVA}$. With a subsequent application of 0.3 μmol/L capsaicin plus 10 μmol/L capsazepine, and after a 3-minute wash, $I_{HVA}$ remained unchanged. An inward current was induced by 10 μmol/L capsaicin, suggesting it was a capsaicin-sensitive neuron. On average, in the presence of 0.3 μmol/L capsaicin, $I_{HVA}$ was inhibited by 70.0 ± 9.9% ($n$ = 8). In the presence of 10 μmol/L capsazepine, $I_{HVA}$ was inhibited by 2.8 ± 1.8% ($n$ = 6). In the presence of 0.3 μmol/L capsaicin plus 10 μmol/L capsazepine, the inhibition of capsaicin was reduced to 15.5 ± 8.9% ($P < 0.01$). s: Second.

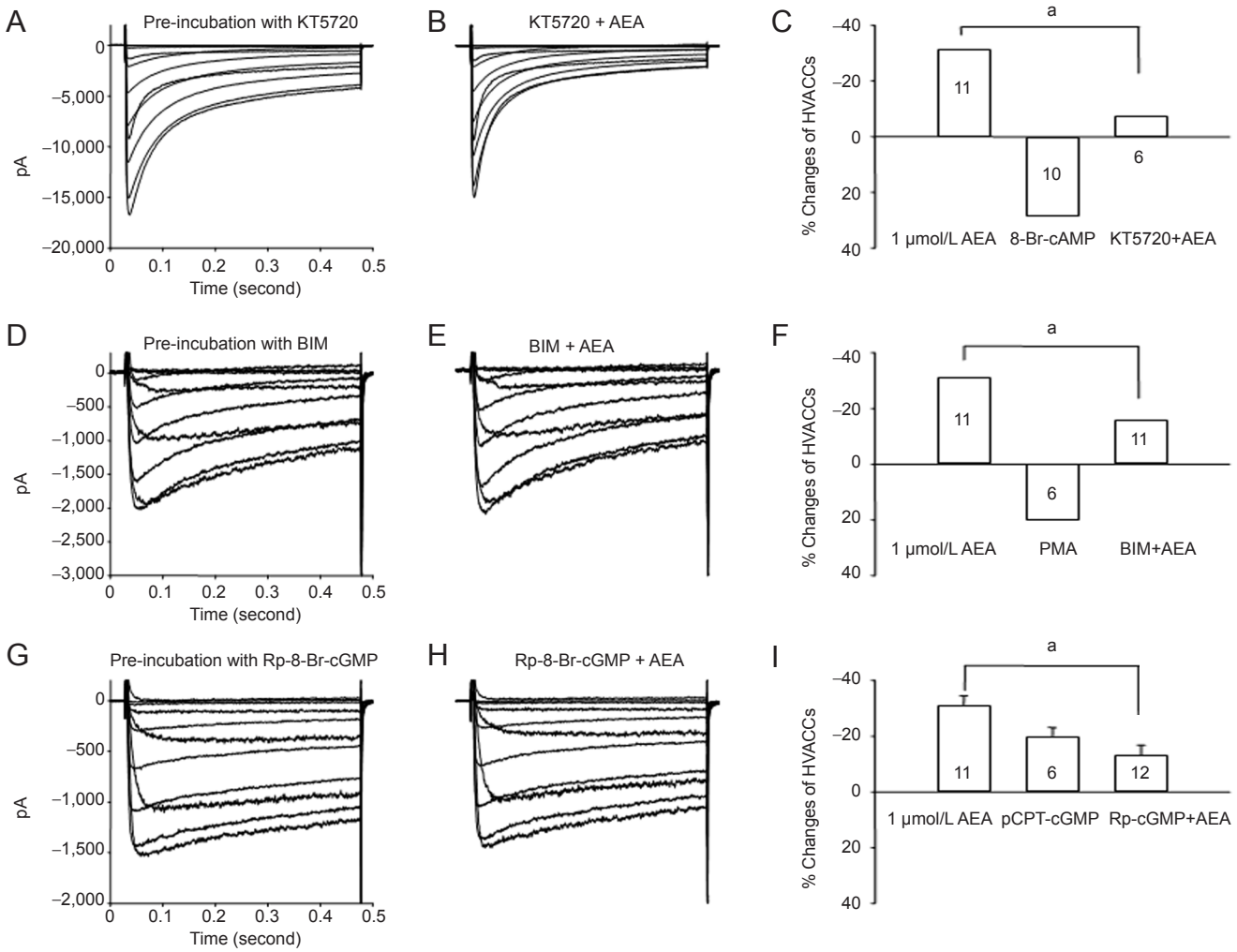


**Figure 7 The role of different signaling pathways in the inhibition of 1 μmol/L anandamide on $Ca^{2+}$ currents ($I_{HVA}$) in rat trigeminal ganglion neurons.**
Sample traces of $I_{HVA}$ currents pre-treated with KT5720 (1 μmol/L for 10 minutes in the bath solution) (A) and responding to co-application with 1 μmol/L anandamide for 3 minutes (B). (C) Averaged effects of 8-Br-cAMP alone and KT5720 co-application with 1 μmol/L anandamide on $I_{HVA}$. Typical traces of $I_{HVA}$ currents pre-treated with BIM (10 μmol/L for 10 minutes in the bath solution) (D) and responding to co-application with 1 μmol/L anandamide for 3 minutes (E, F). Typical traces of $I_{HVA}$ currents in neurons pre-treated with Rp-8-Br-cGMP (1 μmol/L for 10 minutes in the bath solution) (G) and anandamide (1 μmol/L) for 3 minutes (H). (I) Mean (± SEM) effects on $I_{HVA}$ of pCPT-cGMP alone and Rp-8-Br-cGMP co-application with anandamide (1 μmol/L). $^aP < 0.01$, *vs.* anandamide (unpaired *t*-test). Numbers in bars indicate the number of neurons tested. $I_{HVA}$: High voltage-activated $Ca^{2+}$ currents; cGMP: cyclic guanosine monophosphate; cAMP: cyclic adenosine monophosphate; AEA: anandamide.

crease of 1 μmol/L anandamide (Figure 8C). This suggests that co-incubation with 1 μmol/L anandamide and low-dose AM251 facilitates intracellular $Ca^{2+}$ influx *via* TRPV1 while at the same time inhibiting it *via* $CB_1$ receptors.

Similar to the effect of 10 μmol/L anandamide observed on $I_{HVA}$, the increase in intracellular $Ca^{2+}$ concentration was also blocked by both AM251 and capsazepine (Figure 8C), indicating that different receptors participate in the responses of intracellular $Ca^{2+}$ concentration to low and high concentrations of anandamide in rat trigeminal ganglion neurons.

### Signaling pathways mediating the increase in intracellular $Ca^{2+}$ concentration after cannabinoid receptor activation by low-concentration anandamide

Calcium imaging was used to evaluate the possible contribution of cAMP-dependent protein kinase, protein kinase C, and cGMP-dependent protein kinase systems to the observed effect on intracellular $Ca^{2+}$ concentration after CB1 activation. The effects of 1 μmol/L anandamide on intracellular $Ca^{2+}$ concentrations in trigeminal ganglion neurons pre-treated with KT5720 (an inhibitor of cAMP-dependent protein kinase), staurosporine (an inhibitor of protein kinase C) and KT5823 (an inhibitor of cGMP-dependent protein kinase) were investigated. Figure 8D showed that staurosporine (10 μmol/L for 15 minutes) had no effect on the rise in intracellular $Ca^{2+}$ concentration after anandamide application ($n = 13$, $P > 0.05$). Pretreatment with KT5720 (10 μmol/L for 10 minutes) and KT5823 (10 μmol/L for 10 minutes) markedly attenuated the response of 1 μmol/L anandamide (Figure 8D). These data suggest that the cAMP-dependent protein kinase and cGMP-dependent protein kinase systems, but not the protein kinase C system, are involved in the increase in intracellular $Ca^{2+}$ concentration with 1 μmol/L anandamide.

## Discussion

It is well established that presynaptic $CB_1$ receptor activation

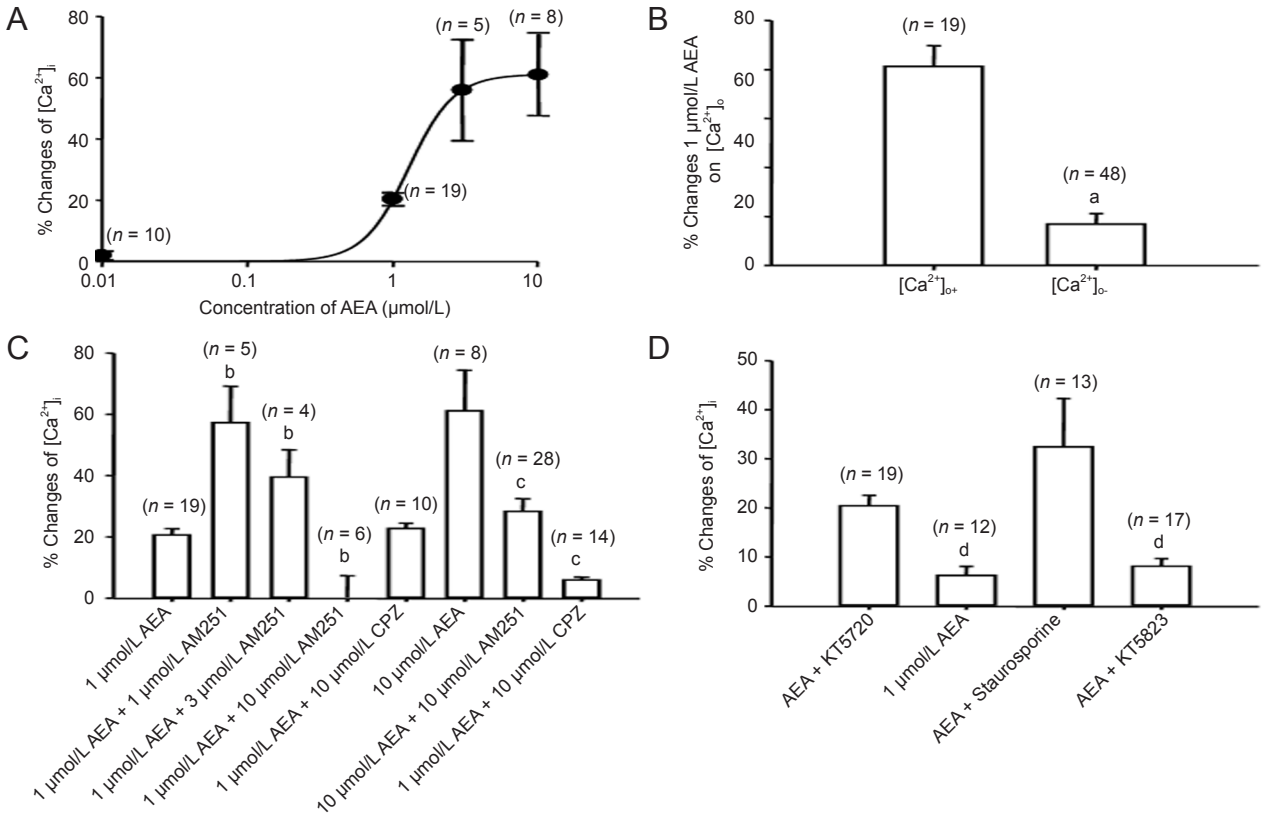


**Figure 8 Effects of anandamide on intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in rat trigeminal ganglion neurons.**
(A) Concentration-response curve for increases in ($[Ca^{2+}]_i$) with anandamide application. (B) Different responses in $[Ca^{2+}]_i$ evoked by 1 μmol/L anandamide in the presence and absence of extracellular $Ca^{2+}$ ($[Ca^{2+}]_o$). A significant effect was seen with 2 mmol/L $Ca^{2+}$ present in the extracellular environment ($n = 19$) compared with the $Ca^{2+}$-free condition ($n = 48$), $^aP < 0.01$, *vs.* presence of extracellular $Ca^{2+}$ (unpaired Student's *t*-test). (C) Responses (mean ± SEM) induced by 1 and 10 μmol/L anandamide and their co-application with AM251 and capsazepine. Pre-incubation with 10 μmol/L AM251 reversed the rises evoked by 1 and 10 μmol/L anandamide ($^bP < 0.01$, *vs.* 1 μmol/L anandamide alone; one-way analysis of variance; $^cP < 0.01$ *vs.* 10 μmol/L anandamide alone; one-way analysis of variance). 1 and 3 μmol/L AM251 facilitated the increase induced by 1 μmol/L anandamide. Pre-application of 10 μmol/L capsazepine blocked the 10 μmol/L anandamide-induced increase in intracellular $Ca^{2+}$ concentration ($n = 14$, $^cP < 0.01$, *vs.* 10 μmol/L anandamide alone), while the increase evoked by 1 μmol/L anandamide was not affected by pre-treatment with 10 μmol/L capsazepine ($P > 0.05$, *vs.* 1 μmol/L anandamide alone). (D) Mean (±SEM) responses of preperfusion with KT5720, staurosporine and KT5823 and followed by co-application of 1 μmol/L anandamide. The rise evoked by 1 μmol/L anandamide was blocked by KT5720 ($^dP < 0.01$, *vs.* 1 μmol/L anandamide alone) and KT5823 ($^dP < 0.01$, *vs.* anandamide alone) but not staurosporine ($P > 0.05$, *vs.* anandamide alone, one-way analysis of variance). Numbers in parentheses indicate the number of neurons tested. CPZ: Capsazepine; AEA: anandamide.

has an inhibitory role on high voltage-activated $Ca^{2+}$ channels, resulting in a decrease in $Ca^{2+}$ influx and an inhibition of neurotransmitter release. In contrast, results from the present study show that anandamide causes a dose-dependent elevation of intracellular $Ca^{2+}$ concentration, triggered by anandamide-evoked currents, but not modulation of high voltage-activated $Ca^{2+}$ channels by anandamide. Furthermore, anandamide-induced inhibition of the high voltage-activated $Ca^{2+}$ channels and the increase in the intracellular $Ca^{2+}$ concentration are demonstrated to be two separate events differing in signal transduction pathways but having similar effects *via* the same receptors. Thus, our data suggest that anandamide, as an endocannabinoid, might exert an excitatory effect by increasing intracellular $Ca^{2+}$ concentration, triggered by anandamide-evoked currents.

It has been well documented that the endocannabinoid anandamide, and its synthetic analogue methanandamide, have dual effects. At low concentrations, anandamide selectively activates $CB_1$ receptors, while at high concentrations it activates excitatory TRPV1 receptors[24]. In pre-contracted strips of rat hepatic artery, rat small mesenteric artery and guinea-pig basilar artery, vasorelaxation induced by high-concentration anandamide is antagonized by the TRPV1 antagonist capsazepine, but not by the $CB_1$ receptor antagonist SR141716 (0.3 μmol/L)[4]. Further evidence indicates that anandamide not only acts on transfected TRPV1 receptors to produce membrane currents and increase the intracellular $Ca^{2+}$ concentration[4, 32], but also acts on naturally-expressed TRPV1 receptors in neonatal rat dorsal root ganglia to produce membrane currents[33]. Here, we present strong evidence that anandamide selectively activates the $CB_1$ receptor at low concentrations in small trigeminal ganglion neurons. At high concentrations, anandamide activates both $CB_1$ and TRPV1 receptors, inhibiting $I_{HVA}$. To confirm that high-concentration anandamide inhibited $I_{HVA}$ *via* TRPV1 receptor activation, we compared the effects of high-concentration anandamide and the TRPV1 agonist capsaicin. We found that the inhibition induced by high-concentration anandamide and by capsaicin shared similar characteristics. First, both inhibited $I_{HVA}$ in a dose-dependent manner and were reversed by the TRPV1 receptor antagonist capsazepine. Second, like capsaicin, high-concentration anandamide induced inward currents in cultured trigeminal ganglion neurons. Similar to capsaicin-induced inward current, the

reversal potential of anandamide-induced current was also around 0 mV. Anandamide had different effects at low and high concentration *via* the activation of different receptors. By comparing the contribution of $CB_1$ and TRPV1 activation on anandamide-induced increases in intracellular $Ca^{2+}$ concentration, we conclude that anandamide-induced inhibition of high voltage-activated $Ca^{2+}$ channels and an increase in intracellular $Ca^{2+}$ concentration have the same dual effects as activation of the receptor pathway.

Inhibition of high voltage-activated $Ca^{2+}$ channels and modulation of $Ca^{2+}$ influx are important for neuronal activity and neurotransmitter release induced by endocannabinoids. However, evidence also suggests that cannabinoids can couple to $G_s$ [18] and $G_q$ [19] and activate TRPV1 receptors to increase $Ca^{2+}$ influx. In addition, the response of cannabinoids on intracellular $Ca^{2+}$ concentration differs across cell types[34]. To elucidate the complex mechanisms underlying the intracellular $Ca^{2+}$ response, we further tested the effect of anandamide on intracellular $Ca^{2+}$ concentration. We found that anandamide increased intracellular $Ca^{2+}$ and simultaneously inhibited $I_{HVA}$ in the same population of trigeminal ganglion neurons, providing strong evidence that an increase in the intracellular $Ca^{2+}$ concentration is not due to $I_{HVA}$. We found that anandamide had dual effects on the inhibition of $I_{HVA}$ and elevation of intracellular $Ca^{2+}$ concentration. However, by comparing the contribution of second messenger system, we found that cAMP- and cGMP-dependent protein kinase antagonists both reversed the anandamide-induced increase in intracellular calcium concentration and $I_{HVA}$ inhibition. Application of a protein kinase C antagonist reversed the inhibition of $I_{HVA}$ but the anandamide-induced increase in intracellular $Ca^{2+}$ concentration was not blocked. From this, we can conclude that the increase in intracellular $Ca^{2+}$ concentration and the $I_{HVA}$ inhibition observed with anandamide are separate processes that share the same receptors but differ in intracellular signal transduction pathways.

$CB_1$ receptor activation by endocannabinoids has been mostly reported to cause depression of neuronal excitability and neurotransmitter release in presynaptic primary neurons[10, 13, 25, 28, 35-36]. Accumulating evidence indicates that cannabinoids may contribute to the potentiation of neurotransmission. Endocannabinoid release potentiates synaptic transmission *via* $CB_1$ receptor activation and dopamine release in the goldfish Mauthner cell[37]. Moreover, endocannabinoids may potentiate hippocampal synaptic transmission *via* astrocytic $CB_1$ activation[30]. In the suprachiasmatic nucleus, $CB_1$ receptor activation can increase excitability of circadian clock neurons[38]. It is widely accepted that $CB_1$ receptor activation suppresses neurotransmission by inhibiting high voltage-activated $Ca^{2+}$ channels, and decreases $Ca^{2+}$ influx. In contrast to this general hypothesis, we found that endocannabinoids caused elevation of the intracellular $Ca^{2+}$ concentration in trigeminal ganglion neurons, which was triggered by endocannabinoid-evoked inward current around the resting membrane potential (−60 mV). Furthermore, endocannabinoid-induced increase in intracellular $Ca^{2+}$ concentration and decrease of high voltage-activated $Ca^{2+}$ channels were two independent events, which had the same dual effects *via* similar receptor pathways, but differed in the second messenger transduction pathway. Thus, our results suggest that presynaptic endocannabinoid release might potentiate neurotransmission *via* endocannabinoid-evoked current, increased $Ca^{2+}$ influx, and increased intracellular $Ca^{2+}$ concentration.

In summary, anandamide dose-dependently causes an increase in intracellular $Ca^{2+}$ concentration, mediated by $Ca^{2+}$ influx *via* anandamide-evoked currents, but not high voltage-activated $Ca^{2+}$ channels. In the same population of trigeminal ganglion neurons, $CB_1$ receptor activation-induced inhibition of $I_{HVA}$ and increase in high voltage-activated $Ca^{2+}$ channels are two separate processes that share the same ($CB_1$) receptors, but differ in signal transduction pathway. Thus, the effects of anandamide on high voltage-activated $Ca^{2+}$ channels ultimately depend on the balance between the increased $Ca^{2+}$ influx *via* anandamide-evoked currents and inhibition of high voltage-activated $Ca^{2+}$ channels, suggesting a complex role of anandamide on inhibitory or excitatory neuromodulation. Contrary to the general hypothesis of endocannabinoid depression on neurotransmission, this study elucidates possible mechanisms underlying endocannabinoid-induced potentiation of neurotransmission *via* $Ca^{2+}$ signaling modulation.

## Materials and Methods

### Design

A cytological *in vitro* study.

### Time and setting

All data were collected at the Departments of Pharmacology and Physiology, Tongji Medical College, *Huazhong University of Science and Technology in* China from September 2003 to April 2008. Data analysis and manuscript writing were performed at Baylor Medical College, USA, from April 2011 to October 2012.

### Materials

180 male Sprague-Dawley rats weighing 180–200 g and aged 6–8 weeks were used. All animal protocols were approved by the faculty of Laboratory Animal Science, Huazhong University of Science and Technology (license No. SYXK (E) 2009-0049). All the experiments followed the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[39].

### Methods

*Cell dissociation*

Trigeminal ganglion neurons from Sprague-Dawley rats were cultured as described previously[40]. Briefly, trigeminal ganglia were dissected aseptically and washed with cold (4°C) modified Hank's balanced salt solution containing NaCl 130 mmol/L, KCl 5 mmol/L, $KH_2PO_4$ 0.3 mmol/L, $NaHCO_3$ 4.0 mmol/L, $NaH_2PO_4$ 0.3 mmol/L, D-glucose 5.6 mmol/L, and ethylene glycol bis(alpha-aminoethyl ether)-N, N′-tetraacetic acid 10 mmol/L, hydroxyethyl piperazine ethanesulfonic acid 10 mmol/L, at pH 7.4. The ganglia were chopped into small pieces, and then incubated in 3 mL modified Hank's solution with 0.1% collagenase (type XI–S)

for 20–40 minutes at 37°C. Individual cells were dissociated by triturating them through a fire-polished glass pipette, followed by incubating with 10 μg/mL DNase I (type lV) in F12 medium (Life Technologies, Gaithersburg, MD, USA) for 10 minutes at 37°C, before centrifuging for 5 minutes at 1,000 r/min. After centrifuging three times, the cells were cultured in F12 supplemented with 10% fetal bovine serum. The cells were planted on poly-D-lysine pre-coated glass coverslips (15 mm diameter) and cultured no more than 12 hours at 37°C in a water saturated atmosphere with 5% $CO_2$.

*Patch-clamp recording*

The cells were placed in a recording chamber mounted on an inverted microscope (Leica Inc., Solms, Germany) and perfused with extracellular solution at room temperature (21–22°C). Whole-cell patch-clamp experiments were carried out using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) and the output was digitized with a Digidata 1332A converter (Axon Instruments) and program pCLAMP 9.02 (Axon Instruments). Data were acquired at a sampling rate of 2 KHz. Cell membrane capacitance and series resistance were measured and compensated (> 90%). Data obtained from neurons in which uncompensated series resistance resulted in voltage-clamp errors > 5 mV were not taken into further analysis. The cell diameters were measured with a calibrated eyepiece under phase contrast illumination.

The resistance of the microelectrode was 2–4 MΩ when filled with the pipette solution. The microelectrode was made from G85150T-4 glass pipettes (Warner Instruments Inc., Hamden, CT, USA). The external solution contained Choline-Cl 110 mmol/L, TEACl 20 mmol/L, $BaCl_2$ 10 mmol/L, $MgCl_2$ 2.0 mmol/L, hydroxyethyl piperazine ethanesulfonic acid 10 mmol/L, and D-glucose 20 mmol/L, adjusted to pH 7.4 with CsOH. $Ba^{2+}$ was used as the charge carrier when recording $I_{HVA}$. $Ba^{2+}$ was replaced by $Ca^{2+}$ when recording anandamide-evoked currents. The pipette solution contained CsCl 120 mmol/L, $CaCl_2$ 0.1 mmol/L, $MgCL_2$ 2.0 mmol/L, ethylene glycol bis(alpha-aminoethyl ether)-N, N′-tetraacetic acid 10.0 mmol/L, hydroxyethyl piperazine ethanesulfonic acid 10.0 mmol/L and Tris-ATP 5.0 mmol/L, pH adjusted to 7.2 with CsOH. Small-sized cells were selected to perform the further experiments.

The volume of the recording chamber was about 1 mL and the local superfusion rate was 1 mL/min.

*Calcium imaging*

Drug-induced changes of high voltage-activated $Ca^{2+}$ channels were measured using a confocal laser scanning imaging system (Fluoview FV500, Olympus, Tokyo, Japan). The trigeminal ganglion cells on glass cover slips were loaded with Fluo 2-AM by incubation with 1–5 μmol/L Fluo 2-AM in standard external solution and were maintained in the dark for 30–40 minutes. The standard external solution contained NaCL 140.0 mmol/L, KCL 5.0 mmol/L, $CaCL_2$ 2.0 mmol/L, $MgCL_2$ 1.0 mmol/L, glucose 10.0 mmol/L and hydroxyethyl piperazine ethanesulfonic acid 10.0 mmol/L, pH adjusted to 7.4 with NaOH. $Ca^{2+}$-free medium solution was identical except for the 2.0 mmol/L $Ca^{2+}$ and additional 10 mmol/L ethyleneglycol bis(alpha-aminoethyl ether)-N, N′-tetraacetic acid to lower extracellular $Ca^{2+}$ concentration. Small-sized trigeminal ganglion neurons (< 33 μmol/L) were selected to perform the further experiments.

*Data analysis*

The data were analyzed using pCLAMP 9.02 (Axon Instruments) and Sigmaplot 11.0 software (Systat Software Inc., San Jose, CA, USA). The amplitude of $I_{HVA}$ was calculated as the peak current. Voltage-dependent activation for the study of changes on $I_{HVA}$ was measured by a series of depolarized pulses (450 ms) from −50 mV to +40 mV, stepping by 10 mV with interval time of 5 seconds, at a holding potential of −80 mV. We fitted a Boltzmann function to the voltage-dependent activation curves, that is $G/G_{max}=1/[1+\exp(V_{0.5}-V_m)/k]$, where $G_{max}$ is the maximum conductance, $V_{0.5}$ is the membrane potential at which 50% of activation was observed, and k is the slope of the function. Voltage-dependent inactivation was measured by a two pulse protocol in which the precondition pulses (3 seconds) ranged from −80 to +20 in 10 mV increments; following test pulse (200 millsecords) was +10 mV with an internal time of 6 seconds. The Boltzmann function was also fitted to the h-infinity curve, that is, $I/I_{max}=1/[1+\exp(V_{0.5}-V_m)/k]$, where $V_{0.5}$ is the membrane potential at which 50% of inactivation was observed, and k is the slope of the function. The dose-response curve was fitted by the Hill equation, in which, $I_{peak}=I_{peakmax}/[1+(IC_{50}/C)^n]$, with $IC_{50}$ as the concentration producing 50% inhibition and *n* as the Hill coefficient.

*Statistical analysis*

Data were presented as mean ± SEM. For all experiments, data were examined for Gaussian distribution first, and then analyzed for statistical significance using the paired or unpaired *t*-test and one-way analysis of variance by using Sigma plot 11.0 software (Systat Software Inc.). A value of $P < 0.05$ was considered statistically significant.

**Author contributions:** *Liu LJ and Cao XH conceived the study and developed crucial proof-of-concept studies. Zhang Y contributed to data analysis and revised manuscript. Xie H performed calcium imaging. Lei G, Li F and Pan JP performed cell dissociation and data analysis. Liu ZG contributed to revised manuscript. Liu CJ also contributed to revised manuscript and study design. All authors approved the final version of the paper.*

**Conflicts of interest:** *None declared.*

**Peer review:** *This study systemically determines how anandamide affects $Ca^{2+}$ signaling and underlying mechanisms in small trigeminal ganglion neurons, via the effects of high voltage-activated $Ca^{2+}$ currents, $Ca^{2+}$ influx and intracellular $Ca^{2+}$ concentration, as well as the underlying mechanisms of which in small trigeminal ganglion neurons. This study will benefit the understanding and development of cannabinoids in clinical practice.*

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