Cholecystokinin-8S-Induced Intracellular Calcium Signaling in Acutely Isolated Periaqueductal Gray Neurons of the Rat

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Many behavior studies indicate that cholecystokinin (CCK) is related to nociception and anxiety/panic actions in the midbrain periaqueductal gray (PAG). We previously reported that a sulfated form of CCK octapeptide (CCK-8S) produced excitatory effects at both pre- and postsynaptic loci in PAG neurons using slice preparations and whole-cell patch-clamp recordings. Here, we further examined the detailed mechanism of CCK-8S in acutely isolated PAG neurons of the rat using fura-2-based imaging of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and whole-cell patch-clamp recordings. Application of 1 μM CCK-8S produced an increase of $[Ca^{2+}]_i$, and its effect did not desensitize. This CCK-8S-induced $[Ca^{2+}]_i$ increase was inhibited by the CCK_2 receptor antagonist L-365260 but not by the CCK_1 receptor antagonist L-364718. In addition, the effect of CCK-8S was eliminated by removing extracellular Ca^{2+}, but not by an addition of the intracellular Ca^{2+} reuptake inhibitor thapsigargin. When simultaneous recordings of $[Ca^{2+}]_i$ imaging and whole-cell patch-clamp were performed, CCK-8S-induced $[Ca^{2+}]_i$ increase was significantly reduced at a membrane holding potential of −60 mV while CCK-8S-induced inward current was still observed. Current-voltage plots revealed that CCK-8S-induced inward current reversed near the equilibrium potential for K^+ ions with a decreased membrane conductance. However, CCK-8S produced a significant inhibition on high-voltage-activated Ca^{2+} channel currents. These results suggest that CCK-8S can excite PAG neurons by inhibiting K^+ channels, and CCK-8S-induced $[Ca^{2+}]_i$ increase occurs secondary to depolarization. The evidence presented here expands our understanding of cellular mechanisms for CCK-mediated anti-analgesic and anxiogenic actions in the PAG.

Key words cholecystokinin ($CCK)_2$ receptor; periaqueductal gray; Ca^{2+} channel; depolarization; fura-2; patch-clamp recording

The midbrain periaqueductal gray (PAG) plays a crucial role in the integration of an animal's behavioral, somatic, and autonomic responses to threat, stress, and pain.[1,2] In particular, the PAG is a major site for modulation of nociception and a part of descending antinociceptive systems that relay *via* the rostral ventromedial medulla (RVM) to the spinal cord.[3,4] In addition, stimulation of dorsal PAG in several species produced defense reaction, anxiety, and fear, and lesions of the PAG produced anxiolytic effect.[5,6] Cholecystokinin (CCK) is a bioactive peptide that functions as a gastrointestinal hormone and a neuropeptide in both the peripheral and central nervous systems. As one of the most abundant neuropeptides in the brain, CCK has also been known to influence a variety of behaviors such as satiety, nociception, and anxiety/panic action in the brain.[7—9] Immunocytochemical studies have demonstrated that CCK is a major neurotransmitter or modulator in the PAG, and CCK receptors are heterogeneously distributed within the PAG.[10,11] Microinjection of CCK or CCK receptor antagonists into the PAG antagonized or potentiated the acute antinociceptive effect of microinjected morphine, respectively.[12—15] In addition, Netto *et al.*[16] reported that microinjection of CCK into the PAG produced an anxiogenic effect through CCK_2 receptors using an animal model of anxiety. Based on these behavioral studies, the PAG could be a key site of anti-analgesic and anxiogenic actions of CCK, and it is possible that these effects of CCK are mediated through an alteration of neuronal activities in the PAG.

CCK exerts its physiological effects *via* two subtypes of receptors, CCK_1 and CCK_2 receptors.[8,9] Both CCK receptors belong to a superfamily of G protein-coupled receptors (GPCR).[7,17,18] Direct coupling of CCK_1 and CCK_2 receptors onto $G\alpha_q$ to activate phospholipase C (PLC) and cause intracellular Ca^{2+} signaling has been well demonstrated in pancreatic acinar cells[19—21] and several cell lines.[22—24] However, this effect is not clearly demonstrated in the central nervous system (CNS). We recently reported that CCK excited PAG neurons at both pre- and postsynaptic loci *via* the activation of CCK_2 receptors using slice preparations and whole-cell patch-clamp recordings.[25] Therefore, in order to examine whether CCK could modulate intracellular Ca^{2+} mobilizations in PAG neurons, we examined the effects of a sulfated form of CCK octapeptide (CCK-8S) using fura-2-based intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and whole-cell patch-clamp recordings in acutely isolated PAG neurons of the rat.

MATERIALS AND METHODS

Acute Isolation of PAG Neurons PAG neurons with synaptic boutons were acutely isolated using the technique modified from Hahm *et al.*[26] Briefly, 12 to 21-d-old Sprague-Dawley rats were anesthetized and decapitated in accordance with the NIH Guide for the Care of Use of Laboratory Animals (revised 1996). The brains were quickly removed and immersed in an ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 26.2 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.5 $MgSO_4$, 10 glucose, and 10 sucrose gassed with 95% O_2 and 5% CO_2. Transverse midbrain slices (400 μm thick) containing the PAG were cut with the use of a Vibratome tissue slicer. Slices were preincubated in ACSF that had been well saturated with 95% O_2 and 5% CO_2 at 32 °C for *ca.* 1 h before a mechanical dissociation. For the mechanical isolation of PAG neurons, slices were transferred into a 35-mm culture dish (Primaria 3801, Becton Dickinson,

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NJ, U.S.A.), and a fire-polished glass pipette was lightly touched onto the dorsolateral region of the PAG with 1—2 mm of working distance. The tip of the glass pipette was horizontally vibrated at *ca.* 20—50 Hz for *ca.* 2 min. Slices were removed, and the mechanically dissociated neurons were allowed to settle for 20 min for intracellular Ca^{2+} imaging and electrophysiological studies.

Intracellular Ca^{2+} Imaging The acetoxymethyl-ester form of fura-2 (fura-2/AM) was used as the fluorescent Ca^{2+} indicator. Cells were incubated for 40—60 min at room temperature with 5 μM fura-2/AM and 0.001% pluronic F-127 in a HEPES-buffered solution composed of (in mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH. Cells were illuminated using a xenon arc lamp, and excitation wavelengths (340, 380 nm) were selected by a computer-controlled filter wheel (Sutter Instruments, CA, U.S.A.). Emitter fluorescence was reflected through a 515 nm long-pass filter to a frame transfer cooled CCD camera, then the ratios of emitted fluorescence were calculated using a digital fluorescence analyzer and converted to intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) using the Eq. 1

$$[Ca^{2+}]_i = K_d \beta (R - R_{min})/(R_{max} - R) \quad (1)$$

where K_d is the dissociation constant for fura-2, R_{min} is the 340/380 fluorescence ratio in the presence of zero Ca^{2+}, R_{max} is the ratio in the presence of saturating concentrations of Ca^{2+}, and β is the ratio of fluorescence during 380-mm excitation at zero and saturating Ca^{2+} concentrations. All imaging data were collected and analyzed using Universal Imaging software (West Chester, PA, U.S.A.). Saline with 50 mM KCl was made by replacing an equivalent amount of NaCl.

Electrophysiological Recordings Electrophysiological recordings were achieved using whole-cell patch-clamp techniques at room temperature (22—25 °C). For Ca^{2+} currents recordings, patch electrodes with resistance of 3—4 MΩ were filled with an internal solution containing (in mM): 110 CsCl, 5 Mg-ATP, 0.2 Na-GTP, 10 EGTA, 2 $CaCl_2$, and 10 HEPES (pH 7.3). The external solution contained (in mM): 140 TEA-Cl, 4 $BaCl_2$, 2.5 CsCl, 10 HEPES, and 10 glucose, pH-adjusted to 7.4 with TEA-OH. High-voltage-activated (HVA) Ca^{2+} currents were evoked every 15 s by a 200 ms depolarizing voltage step from −60 to 0 mV. For recordings of CCK-induced inward currents, the nystatin perforated patch-clamp method was employed at a holding potential (V_h) of −60 mV. Patch electrodes were filled with an internal solution containing (in mM): 120 K-gluconate, 20 NaCl, 2 $MgCl_2$, and 10 HEPES (pH 7.4). Nystatin was prepared as a stock solution (25 mg/ml) in dimethylsulphoxide (DMSO), diluted to a concentration of 250 μg/ml using the internal solution, and back-filled into the pipette after the tip of the pipette was initially filled with the nystatin-free solution. The external solution contained (in mM): 150 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose. All experiment recordings were obtained with an EPC-9 amplifier (filtered at 10 kHz) and digitized at a sampling frequency of 1 kHz.

Data Analysis All data were analyzed using Pulse/Pulsefit (HEKA, Germany) and Prism (GraphPad Software Inc., CA, U.S.A.) software. All data were expressed as the mean±S.E. Statistical significance was determined by using a paired or unpaired Student's *t*-test. Statistical significance was set at $p<0.05$ level.

Drugs Stock solutions of all drugs were made in distilled water, except CCK receptor antagonists (made in DMSO). These were diluted to a final concentration using ACSF and applied to the perfusate. Cholecystokinin octapeptide (CCK-8S) was purchased from Tocris Cookson (Bristol, U.K.); fura-2/AM from Molecular probes (OR, U.S.A.); L-364718 and L-365260 were a gift from ML Laboratories (Warrington, U.K.). All other chemicals were purchased from Sigma (MO, U.S.A.).

RESULTS AND DISCUSSION

CCK-8S Increases $[Ca^{2+}]_i$ in Acutely Isolated PAG Neurons We previously reported that CCK produces excitatory effects at both pre- and postsynaptic loci in most PAG neurons tested using slice preparations and whole-cell patch-clamp recordings.[25] These results suggest that CCK is a main neurotransmitter to modulate neuronal activities in the PAG and also indicate possible CCK-mediated modulation on intracellular Ca^{2+} signaling *via* $G\alpha_q$/PLC pathways in the CNS as has occurred in pancreatic cells[19—21] and several cell lines.[22—24] In the present study, we examined the effects of CCK on $[Ca^{2+}]_i$ in acutely dissociated PAG neurons using a sulfated form of CCK octapeptide (CCK-8S), the most abundant and endogenously active form of CCK in the brain. Application of 1 μM CCK-8S induced an increase of $[Ca^{2+}]_i$ in most PAG neurons tested (Fig. 1A). This CCK-8S-induced response peaked within 30—50 s after the drug application and returned to the basal level about 2—5 min after washout. We examined more than 160 PAG neurons ($n=168$) and found that *ca.* 91% cell of them produced an increase of $[Ca^{2+}]_i$ by CCK-8S. The mean increase of $[Ca^{2+}]_i$ was 151.3±6.3 nM by 1 μM CCK-8S ($n=153$). In order to confirm that the response of CCK-8S was recorded from PAG neurons and not non-neuronal cells such as glia, we checked the responsiveness of a high concentration of KCl (50 mM) to cells at the end of each experiment. In a separate set of experiments, we observed that CCK-8S-induced $[Ca^{2+}]_i$ increase was reproducible and occurred in a concentration-dependent manner. The degree of CCK-8S-induced $[Ca^{2+}]_i$ increase was not significantly changed by a second application of the drug (103.3±3.8% of first application, $n=14$, Fig. 1B). This result suggests that CCK-8S-induced $[Ca^{2+}]_i$ increase does not desensitize. As shown in Fig. 1C, application of CCK-8S between 1 nM to 10 μM produced an increase of $[Ca^{2+}]_i$ in a concentration-dependent manner with the EC_{50} value of 156.0 nM.

To evaluate which of the subtypes of CCK receptors is involved in CCK-8S-induced $[Ca^{2+}]_i$ increase, two selective CCK receptor antagonists, the CCK_2 receptor antagonist L-365260 and the CCK_1 receptor antagonist L-364718, were used. As shown in Fig. 1D, CCK-8S-induced $[Ca^{2+}]_i$ increase was significantly reduced by the CCK_2 receptor antagonist, L-365260 (56.2±12.1% of control, $n=9$, $p<0.01$) while it was not affected by the CCK_1 receptor antagonist L-364718 (89.8±4.9%, $n=8$) at the concentration of 100 nM. Furthermore, the blockade of L-365260 on CCK-8S-induced $[Ca^{2+}]_i$ increase occurred in a concentration-dependent manner. The mean percentage values by 1 μM and 10 μM L-365260 were 34.7±9.6% ($n=8$) and 23.0±4.6% of control ($n=18$), re-

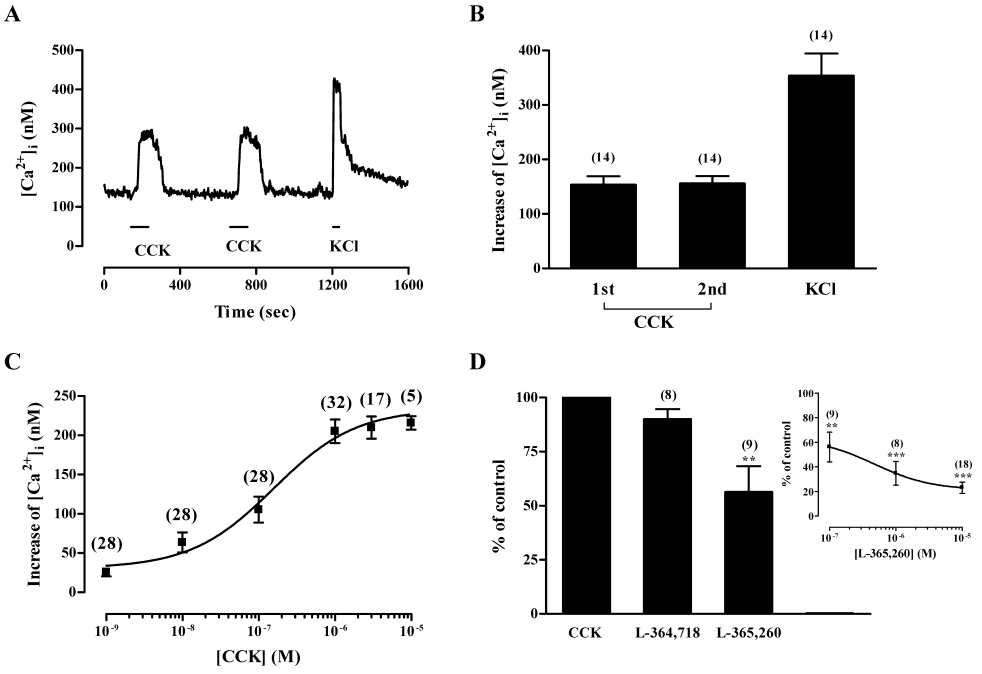

Fig. 1. CCK-8S-Induced $[Ca^{2+}]_i$ Increase in Acutely Isolated PAG Neurons of the Rat

(A) Application of CCK-8S (1 μM) for 1.5 min repeatedly produced an increase of $[Ca^{2+}]_i$ in an acutely isolated PAG neuron. At the end of each experiment, a high concentration of KCl (50 mM) was applied to a cell for 30 s in order to confirm the cell tested is a PAG neuron, not a non-neuronal cell. (B) Pooled results illustrating the mean increases of $[Ca^{2+}]_i$ by 1st, 2nd application of 1 μM CCK-8S, or 50 mM KCl. (C) Concentration-dependent curve for CCK-8S-induced $[Ca^{2+}]_i$ increase. The change of $[Ca^{2+}]_i$ is plotted as a function of CCK-8S concentration. The solid line connecting the points represents the computer fit of the data to a logistic equation, $y=I_{min}+(I_{max}-I_{min})/(1+10^{(LogEC50-X)})$, where I_{min} and I_{max} are the minimum and maximum $[Ca^{2+}]_i$, respectively, X is the logarithm of CCK-8S concentration. The EC_{50} value of CCK-8S on increasing $[Ca^{2+}]_i$ is 156.0 nM. (D) The effects of selective CCK receptor antagonists on 1 μM CCK-8S-induced $[Ca^{2+}]_i$ increase. CCK-8S-induced $[Ca^{2+}]_i$ increase was significantly reduced by the CCK_2 receptor antagonist L-365260 but not by the CCK_1 receptor antagonist L-364718. Each antagonist was used at 100 nM. Inset, concentration-dependent blockade of L-365260 on CCK-8S-induced $[Ca^{2+}]_i$ increase from 100 nM to 10 μM. ** $p<0.01$, *** $p<0.001$.

spectively. These results suggest that CCK-8S produces an increase of $[Ca^{2+}]_i$ *via* the activation of CCK_2 receptors in the PAG.

The Role of Extracellular Ca^{2+} in CCK-8S-Induced $[Ca^{2+}]_i$ Increase To determine sources of $[Ca^{2+}]_i$ increased by CCK-8S, the experiments with Ca^{2+}-free environment or the intracellular Ca^{2+} reuptake inhibitor thapsigargin were performed. First, when 1 μM CCK-8S was applied in Ca^{2+}-free HEPES buffered solution, CCK-8S-induced $[Ca^{2+}]_i$ increase was completely inhibited as shown in Fig. 2A (3.1±2.6% of control, $n=27$, $p<0.001$). In contrast, pre-treatment with thapsigargin (2 μM, 5 min) in order to deplete intracellular Ca^{2+} stores did not significantly affect CCK-8S-induced $[Ca^{2+}]_i$ increase (101.5±13.2%, $n=12$, Fig. 2B). These results suggest that the main source for CCK-8S-induced $[Ca^{2+}]_i$ increase is influx of Ca^{2+} from the extracellular space and exclude any involvement of intracellular Ca^{2+} stores in the PAG. However, CCK_2 receptors have been known to activate PLC, thereby triggering an increase in inositol 1,4,5-trisphosphate (IP_3) production *via* a $G\alpha_q$ protein in pancreatic cells and clonal cell lines.[19—22,24] To test any possibility of dysfunction in the coupling system between GPCRs and PLC/IP_3 pathways due to isolation processes of PAG neurons, we examined the effect of substance P (SP) which is also bound to $G\alpha_q$ protein-coupled SP receptors. As shown in Fig. 2C, application of 1 μM SP induced an increase of $[Ca^{2+}]_i$ in PAG neurons (131.9±12.9 nM, $n=41$) and still produced an increase of $[Ca^{2+}]_i$ in Ca^{2+}-free HEPES buffered solution (89.3±6.0% of control, $n=12$) in contrast to CCK-8S. Furthermore, SP-induced $[Ca^{2+}]_i$ increase was completely inhibited by the PLC inhibitor U73122 (10 μM, 4.9±1.5% of control, $n=25$, $p<0.001$, Fig. 2D). These results suggest that the coupling system between $G\alpha_q$-protein-coupled receptors and PLC/IP_3 pathways is properly operated in the acutely isolated PAG neurons which we used, and CCK-8S produces $[Ca^{2+}]_i$ increase *via* influx of Ca^{2+} from the extracellular space, but not intracellular Ca^{2+} stores coupled to PLC-dependent pathway in the PAG.

Simultaneous Recordings of CCK-8S-Induced $[Ca^{2+}]_i$ Increase and Membrane Currents To examine whether CCK-8S-induced $[Ca^{2+}]_i$ increase is caused by a membrane depolarization which we observed in our previous study recorded in midbrain slice preparations,[25] simultaneous recordings of $[Ca^{2+}]_i$ imaging and whole-cell patch-clamp were performed in acutely isolated PAG neurons. The PAG neurons subjected to the measurement of $[Ca^{2+}]_i$ imaging were recorded at a holding potential (V_h) of −60 mV using perforated whole-cell patch-clamp techniques in the presence of the Na^+ channel blocker tetrodotoxin (TTX, 300 nM). Under this condition, 1 μM CCK-8S produced an inward current (12.4±3.8 pA, $n=7$), which is comparable with that obtained by sole patch-clamp recording (17.8±1.4 pA, $n=13$). However, CCK-8S-induced $[Ca^{2+}]_i$ increase was significantly reduced by voltage-clamp recordings when membrane potential was clamped at −60 mV (Fig. 3A). The mean increases of $[Ca^{2+}]_i$ without and with voltage-clamp recordings were 151.3±6.3 nM and 45.8±6.3 nM, respectively ($n=7$, $p<0.01$, Fig. 3B). These results suggest that CCK-8S-induced $[Ca^{2+}]_i$ increase occurred secondary to a membrane depolarization in acutely isolated PAG neurons. What types of ion channels are

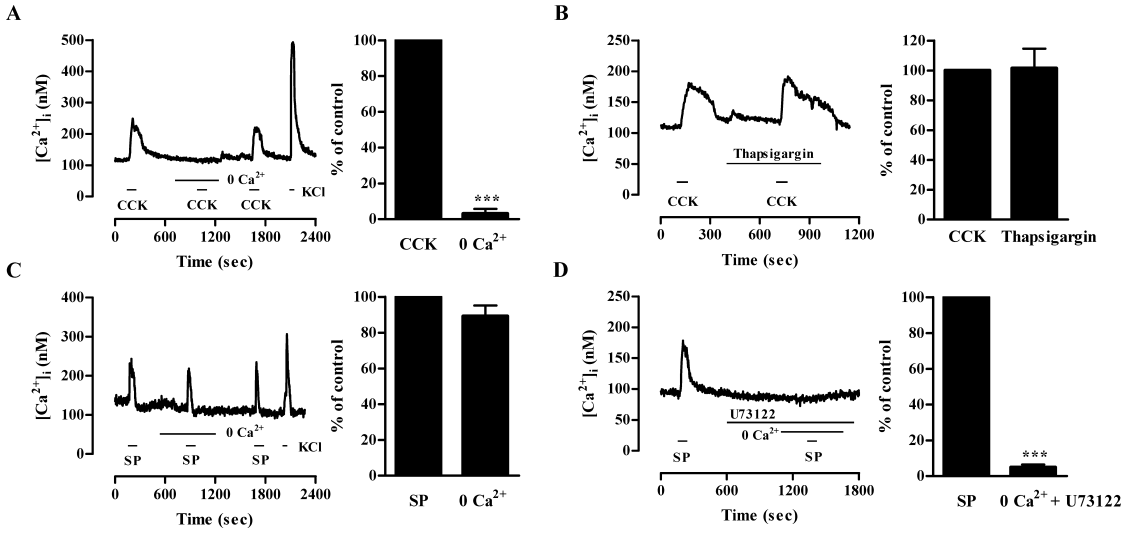

Fig. 2. The Role of Extracellular Ca^{2+} on CCK-8S-Induced $[Ca^{2+}]_i$ Increase

(A) CCK-8S-induced $[Ca^{2+}]_i$ increase was significantly inhibited in Ca^{2+}-free HEPES buffered solution (0 Ca^{2+}, 3.1±2.6% of control, n=27). (B) shows that CCK-8S-induced effect was not inhibited by pre-treatment (5 min) with the intracellular Ca^{2+} reuptake inhibitor thapsigargin (2 μM, 101.5±13.2%, n=12). (C) and (D) Application of substance P (SP, 1 μM) also induced an increase of $[Ca^{2+}]_i$ in an acutely isolated PAG neuron. This SP-induced $[Ca^{2+}]_i$ increase was still observed in Ca^{2+}-free bath solution (89.3±6.0% of control, n=12) but completely inhibited by treatment with the phospholipase C (PLC) inhibitor U73122 (10 μM, 4.9±1.5% of control, n=25). *** $p<0.001$.

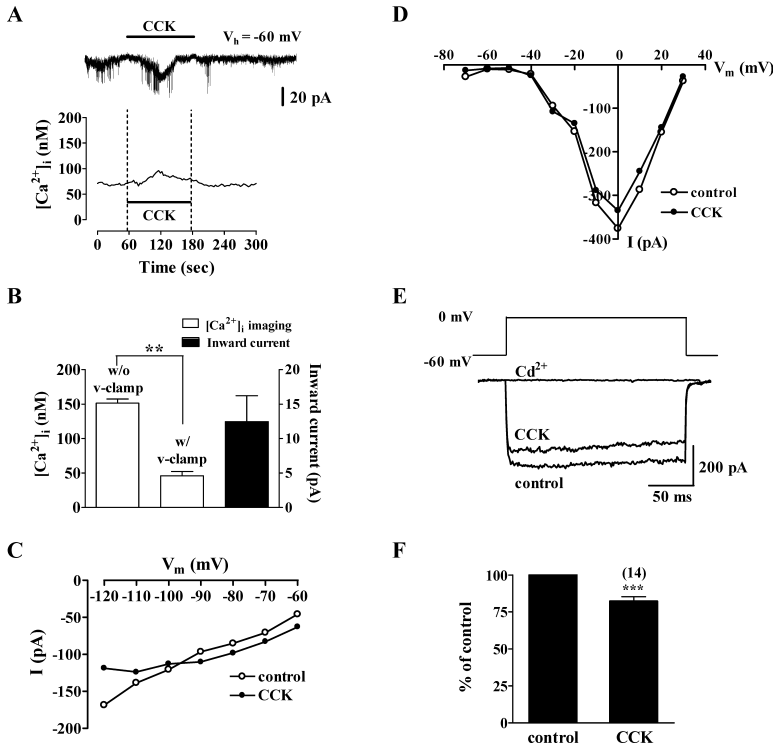

Fig. 3. Effects of CCK-8S on Membrane Holding Currents and Ca^{2+} Channels under Voltage-Clamp Conditions

(A) The simultaneous recording of 1 μM CCK-8S-induced $[Ca^{2+}]_i$ increase and membrane inward currents. Membrane currents of a neuron subjected to $[Ca^{2+}]_i$ imaging were simultaneously recorded using perforated whole-cell patch-clamp techniques. (B) The mean values of CCK-8S-induced $[Ca^{2+}]_i$ increase without and with voltage-clamp recordings were 151.3±6.3 nM and 56.8±12.3 nM (n=7), respectively. The mean amplitude of CCK-8S-induced inward current was 12.4±3.8 pA (n=7) when membrane potential was clamped at −60 mV. (C) The steady-state current–voltage (I–V) relationship prior to (○) and in the presence of 1 μM CCK-8S (●) for an acutely isolated PAG neuron. The mean value of E_{rev} is −95.7±3.2 mV (n=6) when 2.5 mM extracellular K^+ concentration was used. Cells were held at V_h of −60 mV and given square pulses from −130 to −60 mV with 10-mV steps. (D) The peak I–V relationship for whole-cell Ca^{2+} currents prior to (○) and in the presence of 1 μM CCK-8S (●) in an acutely isolated PAG neuron. Ca^{2+} currents were evoked at V_h of −60 mV by depolarization to the test potentials from −70 to +30 mV with 10-mV steps. CCK-8S moderately inhibited high-voltage-activated (HVA) Ca^{2+} currents without changing low-voltage-activated (LVA) Ca^{2+} currents. (E) 1 μM CCK-8S partially reduced HVA Ca^{2+} currents. HVA Ca^{2+} currents were evoked every 15 s by a 200 ms depolarizing voltage step from −60 to 0 mV and completely blocked by 100 μM Cd^{2+}. (F) Illustrates the summarized results of CCK-8S on HVA Ca^{2+} currents. CCK-8S significantly inhibited HVA Ca^{2+} currents from 14 cells tested (82.2±3.1% of control). *** $p<0.001$.

involved in CCK-8S-induced inward currents in order to modulate intracellular Ca^{2+} signaling? As shown in Fig. 3C, the current–voltage (I–V) relationship with or without CCK-8S indicated that CCK-8S caused a decrease in the whole-cell slope conductance with a reversal potential (E_{rev}) of −95.7±3.2 mV (n=6), which was similar to the E_{rev} of K^+

ion predicted by the Nernst equation when an extracellular K^+ concentration is 2.5 mM (predicted $E_{rev} = -97.8$ mV). All together, these results suggest that CCK-8S induced an increase of $[Ca^{2+}]_i$ by K^+ channel-mediated depolarization of membrane potentials in acutely isolated PAG neurons.

Inhibition of Ca^{2+} Currents by CCK-8S From the involvement of both K^+ channel-mediated depolarization and extracellular Ca^{2+}, we next directly investigated any involvement of voltage-dependent Ca^{2+} channels in CCK-8S-induced $[Ca^{2+}]_i$ increase. When whole-cell Ca^{2+} currents were acquired for 200 ms depolarized pulses from -70 to $+30$ mV at V_h of -60 mV with 10-mV voltage increments, the *I–V* relationship was examined. Figure 3D illustrates the peak *I–V* relation for a neuron in the absence and presence of 1 μM CCK-8S. CCK-8S moderately inhibited high-voltage-activated (HVA) Ca^{2+} currents with no significant change on low-voltage-activated (LVA) Ca^{2+} currents which activated around -30 mV. When the effect of CCK-8S was further examined on HVA Ca^{2+} currents which were evoked every 15 s by a 200 ms depolarizing voltage step from -60 to 0 mV, 1 μM CCK-8S partially inhibited HVA Ca^{2+} currents (Fig. 3E), and this effect was reversible (data not shown). Figure 3F illustrates the pooled results showing the effects of CCK-8S on HVA Ca^{2+} currents from 14 cells tested. Although the degree of inhibition was small, CCK-8S produced a significant inhibition on HVA Ca^{2+} currents ($82.2 \pm 3.1\%$ of control, $n = 14$, $p < 0.001$). Under this condition, application of 100 μM $CdCl_2$ abolished the inward currents elicited by a 200 ms depolarization pulse to 0 mV, indicating most of the inward currents are HVA Ca^{2+} currents. Taken together, these results suggest that CCK-8S partially, but significantly, inhibited HVA Ca^{2+} currents in the PAG.

In our previous study, we examined the actions of CCK-8S at the cellular level and identified two prominent effects in rat PAG neurons using slice preparations; CCK can excite PAG neurons at both pre- and postsynaptic loci *via* the activation of CCK_2 receptors.[25] Specifically, CCK-8S produced a membrane depolarization or an inward current accompanied by increased spontaneous synaptic activities and increased the frequency of miniature excitatory postsynaptic currents (mEPSCs). Based on these observations, we further examined whether CCK-8S could modulate intracellular Ca^{2+} signaling in the PAG as reported in other systems. In acutely isolated PAG neurons using fura-2-based $[Ca^{2+}]_i$ imaging and whole-cell patch-clamp recordings, the main findings of the present study are as follows: (1) CCK-8S produces an increase of $[Ca^{2+}]_i$ *via* the activation of CCK_2 receptors. (2) The sources of $[Ca^{2+}]_i$ increased by CCK-8S are not $G\alpha_q$/PLC-mediated intracellular Ca^{2+} stores, but influx of Ca^{2+} from the extracellular space. (3) CCK-8S-induced $[Ca^{2+}]_i$ increase is significantly reduced under voltage-clamp conditions. (4) CCK-8S partially, but significantly, inhibits HVA Ca^{2+} currents.

Intracellular Ca^{2+} plays an important role in Ca^{2+}-regulated neuronal functions such as membrane excitability, neurotransmitter release, and synaptic plasticity.[27,28] It is not only controlled by the release of Ca^{2+} from intracellular stores, but also by influx of Ca^{2+} into the cell through voltage-dependent Ca^{2+} channels. Although it has been well established that pancreatic cholecystokinin (CCK) receptors induce an increase of $[Ca^{2+}]_i$ from the release of intracellular Ca^{2+} stores *via* G_q/PLC/IP_3 pathways,[19—21] the events which follow an activation of CCK receptors in the CNS have not received much attention. It has also been shown in several cell lines, including CHO and COS-7, that CCK receptors activate PLC, triggering an increase in inositol 1,4,5-trisphosphate (IP_3) production *via* a G_q protein.[22—24] In rat taste receptor cells, it has been reported that CCK produced Ca^{2+} elevations mediated *via* the activation of CCK_1 receptors and intracellular Ca^{2+} store-dependent pathways.[29] Therefore, it is highly possible that the signal of the liganded CCK receptor is mediated through heterotrimeric G proteins of the G_q family to activate PLC/IP_3 pathways and release of intracellular Ca^{2+} and finally increases synaptic release of glutamate at presynaptic loci in the PAG. This fact is very important because it would provide a detailed cellular mechanism to support CCK-8S-mediated excitatory effects observed *in vivo* and *in vitro* electrophysiological recordings in this midbrain structure.[11]

However, the present study clearly demonstrated that CCK-8S *via* the activation of CCK_2 receptors induces an increase of $[Ca^{2+}]_i$ by influx of Ca^{2+} from the extracellular space but not from intracellular Ca^{2+} stores in acutely isolated PAG neurons. These observations are in agreement with other previous studies recorded in the CNS, but not in pancreatic cells and clonal cell lines, suggesting that it is highly possible that CCK-8S modulates intracellular Ca^{2+} differently in the CNS. Based on the present results, we suggest the detailed mechanism of CCK-8S-induced $[Ca^{2+}]_i$ increase in acutely isolated PAG neurons under the following circumstances. First, the present study showed that CCK-8S-induced $[Ca^{2+}]_i$ increase occurred not by direct modulation of Ca^{2+} channels because CCK-8S inhibited Ca^{2+} channels in the PAG. Although previous studies showing CCK-8S-induced $[Ca^{2+}]_i$ increase by influx of extracellular Ca^{2+} emphasized the involvement of L-type Ca^{2+} channels in myenteric neurons[30] or N-type Ca^{2+} channels in striatal neurons,[31] voltage-dependent Ca^{2+} channels are not directly activated by application of CCK-8S. If the direct coupling of CCK receptors to voltage-dependent Ca^{2+} channels is essential, the increment of Ca^{2+} channels by CCK-8S should be observed. However, the inhibition of Ca^{2+} channels by CCK-8S was noticed in the current study, and it is consistent with the previous reports recorded in the CNS such as hippocampus, motor, and sensory neurons.[32—34]

Secondly, an inhibition of K^+ channels is a main cause for CCK-8S-induced $[Ca^{2+}]_i$ increase *via* the activation of CCK_2 receptors. When simultaneous recording of Ca^{2+} imaging and whole-cell voltage-clamp on the same neuron was performed, CCK-8S-induced $[Ca^{2+}]_i$ increase was significantly decreased, and CCK-8S-induced inward current was reversed at -95.7 ± 3.2 mV ($n = 6$) with a decreased whole-cell slope conductance. This value of E_{rev} is similar to the of K^+ ion predicted by the Nernst equation when an extracellular K^+ concentration is 2.5 mM (predicted $E_{rev} = -97.8$ mV), suggesting CCK-8S-induced $[Ca^{2+}]_i$ increase is indirectly induced by a membrane depolarization due to an inhibition of K^+ channels. Attributed to prominent roles of CCK as a main neuropeptide modulating various neuronal functions such as satiety, nociception, and anxiety/panic behaviors, the nature of CCK-targeted ion channels has been examined in various neurons. For example, application of CCK has been

known to produce a membrane depolarization or an inward current in the hippocampus, nucleus accumbens, and thalamus.[32,35,36] In most studies, including our previous one,[25] CCK-induced inward current was accompanied with a decreased membrane conductance and intersected near the K^+ ion reversal potential. All together, these results suggest that CCK produced excitatory effects through an inhibition of K^+ channels. Although further investigation is necessary to identify the types of K^+ channels affected by CCK and its detailed mechanism for CCK-induced $[Ca^{2+}]_i$ increase, the evidence presented here expands our understanding of the CCK-mediated excitatory action in the brain including the PAG.

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