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DAMGO, a μ-Opioid Agonist and Cholecystokinin-Octapeptide Have Dual Modulatory Effects on Capsaicin-Activated Current in Rat Dorsal Root Ganglion Neurons

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Capsaicin, a pungent ingredient of hot pepper, elicits an intense burning pain when applied cutaneously and intradermally. Activation of capsaicin-gated channel in C-type dorsal root ganglion (DRG) neurons produces nonselective cationic currents. Although electrophysiological and biochemical properties of capsaicin-activated current (I_{CAP}) were studied, the regulatory mechanism and intracellular signaling pathway are still unclear. In the present study, we investigated the modulations of I_{CAP} by DAMGO (μ-opioid agonist) and cholecystokinin octapeptide (CCK-8). In 18 out of 86 cells, the amplitude of I_{CAP} was significantly increased by DAMGO and completely reversed after washout, while I_{CAP} was decreased by DAMGO in 25 cells. In 43 cells, DAMGO had no effect on I_{CAP}. Mean action potential duration was significantly different between 'increased-by-DAMGO' group and 'decreased-by-DAMGO' group. Mean amplitudes of I_H were not significantly different between both groups. CCK-8 reversibly enhanced the amplitude of I_{CAP} (5/13). DAMGO also increased I_{CAP} amplitude significantly in the same cells. The amplitude of I_{CAP} was increased in additive manner by combined applications of DAMGO and CCK-8 in these cells. These results suggest that DAMGO and CCK-8 can either increase or decrease I_{CAP} presumably depending on the subtypes of DRG cells and classified by electrophysiological properties.

Key Words: Capsaicin, Opioids, Cholecystokinin, Dorsal root ganglion, Pain

INTRODUCTION

To characterize cellular mechanism of afferent sensory neurons, many studies have been performed in dorsal root ganglion (DRG) cells in primary culture due to technical difficulties *in vivo*. Specific receptors and ion channels in the plasma membrane of DRG neurons involved in heat, chemical (i.e.; capsaicin, proton, ATP, etc.) and mechanical stimuli have been extensively characterized (Gschossmann et al, 2000). Capsaicin-activated channel is the most well characterized one among those receptors and ion channels in DRG.

Capsaicin, a pungent ingredient of hot peppers, provokes an immediate burning pain or hyperalgesia when applied cutaneously or intradermally. Capsaicin also causes neurogenic inflammation mediated by release of neuropeptide, such as substance-P (SP) or calcitonin gene-related peptide (CGRP), from sensory nerve endings (Szolcsanyi, 1996).

A specific population of primary afferent neurons can be distinguished by their excitatory response to the capsaicin. These capsaicin-sensitive A δ- and C-fibers are believed to be involved in the processing of nociceptive information. Activation of capsaicin

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receptor (vanilloid receptor-1, VR1) generates an inward current through nonselective cationic channel that results in membrane depolarization sufficient to activate voltage-dependent Ca and Na channel (Oh et al, 1996). The opening of these channels in turn will result in the generation of action potential of primary afferent neurons.

Although electrophysiological and biochemical properties of capsaicin-activated current (I_{CAP}) have been studied extensively (Wood et al, 1988; Oh et al, 1996; Liu et al, 1999), few studies have been concerned about the regulatory mechanisms and intracellular signal transduction pathways of I_{CAP} in the process of nocicetpive modulation

With regard to modulation of nociceptive process, opioid is clearly established to suppress voltage-dependent Ca channel of primary afferent neurons (Taddese et al, 1995) and increases K conductance of dorsal horn neurons in the spinal cord (Schneider et al, 1998). These would be directly related to the reduction of the duration of action potential (APD) of primary afferent and neurotransmitter release, and excitability of dorsal horn cells, respectively. On the other hand, cholecystokinin-octapeptide (CCK-8) is known to have anti-opioid effect in many system (Benedetti, 1997). CCK-8 was reported to reverse μ- and κ-opioid receptor-mediated inhibition on voltage-dependent Ca^{2+} current (Liu et al, 1995) in rat DRG neurons, and antagonize the analgesic effect produced by μ-and κ-but not δ-opioid agonist in spinal cord (Wang et al, 1990). The mechanism of interaction of opioid and CCK, however, remains obscure.

In the present study, we investigated whether I_{CAP} observed in small-sized DRG cells in adult rat is differently modulated by DAMGO and, if so, whether such modulatory effects of DAMGO are reversed by CCK-8.

METHODS

Isolation of DRG cells

Male Spraque-Dawley rats of 6~8 weeks old were used for the experiment. Animals were sacrificed by cervical dislocation. Lumbosacral DRGs were collected from the rat spinal cord under sterile condition and placed in the cold buffer solution (4°C) containing (mM): 145 NaCl, 5 KCl, 10 HEPES and 25 D(+)-glucose. Adherent connective tissues were removed and each DRG was minced with two surgical blades.

Small pieces of DRG were incubated with gently shaking at 37°C in buffer containing 0.15% (w/v) collagenase (Worthington, type 2, USA) for 15 min and then, in 0.125% (w/v) trypsin (Boehringer mannheim, Germany) and 0.02% (w/v) DNase 1 (Sigma, type 1, USA) for 5 min. The digested DRG was triturated using a polyethylene transfer pipette, and cell suspension was centrifuged for 5 min at 1000 RPM.

The cells were then resuspended in Dulbecco's modified eagle's medium (Sigma) supplemented with v/v 10% (v/v) fetal bovine serum (Gibco BRL Life technologies, USA), 2 mM glutamine (Sigma), 1 mM pyrubate (Sigma) and 1% (w/v) penicillin-streptomycin (Sigma). The cells were plated on polyethyleneimine-coated coverslips, and then kept at 37°C in a 5% CO_2 incubator. All experiments were performed within 24 hours after plating.

Electrophysiology

Standard whole-cell configuration was employed to record the membrane current and voltage from individual DRG neurons. All experiments were performed on cell 4~24 hr after plating at room temperature (22°C). Whole-cell signals were recorded with an EPC-7 (List Medical Electronic, Germany) and filtered at 3 kHz with a low pass Bessel filter.

Whole-cell voltage clamp was controlled with the Clampex program in the pClamp6 software package (Axon instruments, USA). Continuous trace recording with repetitive applications of capsaicin or drugs was controlled with Fetchex program (Axon instruments, USA). Capsaicin-activated current, hyperpolarization-activated current (I_H) and action potential were digitized at 20 Hz, 2 kHz and 5 kHz, respectively. None of the traces in this study was leakage-subtracted. Kimble glass tubing was used to make recording electrodes by a two-step process on a Narashige PP-83 electrode puller. The holding potential in all voltage clamps experiments were −80 mV.

Solutions

Drug solutions were delivered to the cells via a continuous flow system. Volume of the bath was approximately 100 μL and perfusion rate was 1.0~1.2 mL min^{-1}. All experimental solutions were adjusted to pH 7.4 and osmolarity of ~300 mOsm. The standard external solution contained (in mM): 140

NaCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5.4 KCl, 10 HEPES, 5 glucose, 25 sucrose. The internal solution consisted of (in mM): 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 2 Mg-ATP, 0.1 Na-GTP, 10 BAPTA (bis 2-amino-phenoxy ethane-N,N,N',N'-tetraacetic acid, Calbiochem).

All reagents were purchased from Sigma except: [D-Ala^2, N-$MePhe^4$, Gly-ol^5] enkephalin (DAMGO) and sulfated cholecystokinin-octapeptide (CCK-8) from RBI. Capsaicin and capsazepine were dissolved and stored as stock solution in ethanol, which did not exert any electrophysiologic effect on cells at the concentration used as a solvent. All the other drug solutions were prepared with distilled water, aliquoted and stored at $-20°C$. On the day of the experiment, aliquots were diluted in the standard external solution.

Capsaicin was applied for 30 sec and washed out for 2 min before the next applications. Drug solutions including DAMGO were basically pretreated for 1 min before combined application of capsaicin and drugs in the experiment.

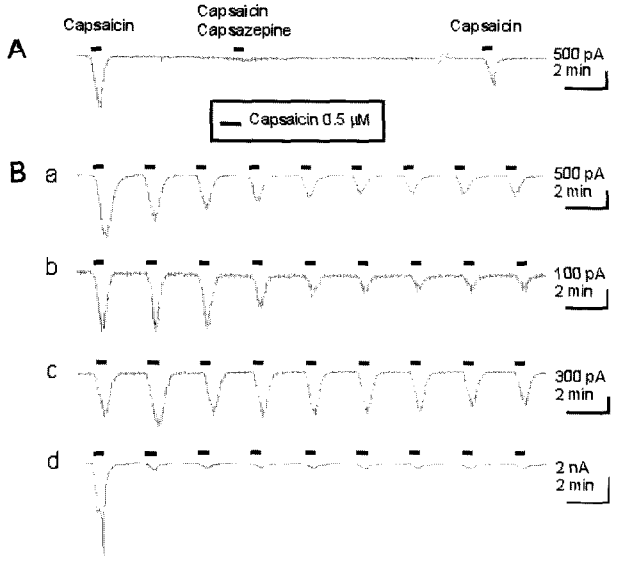

Fig. 1. Whole-cell currents activated by capsaicin (I_{CAP}) and the various tachyphylaxis patterns. (A), Capsaicin (0.5 μM) induced inward currents at holding potential of -80 mV. Capsazepine (10 μM), a competitive antagonist of capsaicin receptor, reversibly inhibited capsaicin-activated currents when it was perfused together with capsaicin. (B), Current traces in a-d were recorded from different cells. Capsaicin was applied for 30 sec and washed out for 2 min before the next applications. Note that tachyphylaxis was highly reduced from the 4th capsaicin application.

Statistics

Results are expressed as mean $\pm$ SEM. Comparison was made using Student's t-test. A difference was considered to be significant when $P<0.05$.

RESULTS

Whole-cell currents activated by capsaicin

DRG neurons around 25 μm-diameter were selected and examined for the experiment because capsaicin preferentially affects the small diameter neurons (Wood et al, 1988; Del Mar et al, 1996). Whole-cell inward currents could be evoked by 0.5 μM capsaicin in adult rat DRG neurons (Fig. 1), consistent with the previous reports (Petersen et al, 1996). Electrophysiological properties of capsaicin-activated currents were charac-

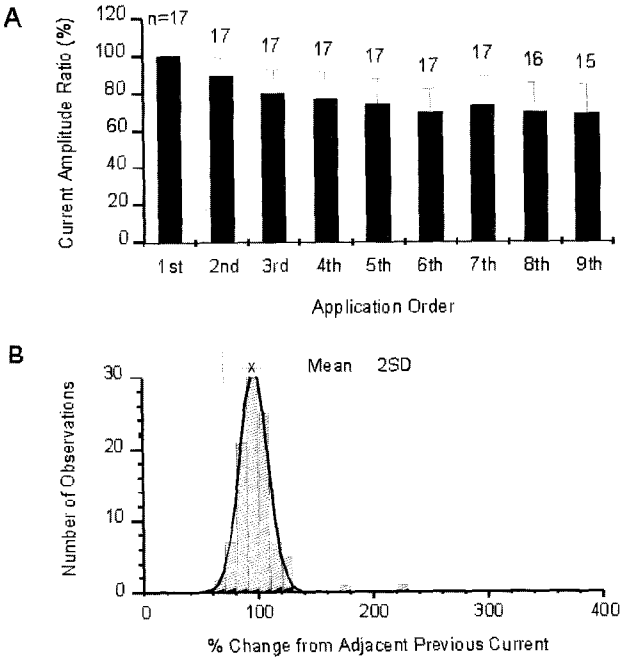

Fig. 2. Statistics of tachyphylaxis pattern of I_{CAP}. (A), Mean tachyphylaxis pattern of I_{CAP}. Relative current amplitudes compared to the first I_{CAP} were averaged from 17 cells. Since 4th application, the tachyphylaxis markedly reduced. (B), Histogram of I_{CAP} of tachyphylaxis. Each amplitude was compared to that of adjacent previous I_{CAP} (n=99). The distribution was fitted by Gaussian function. The mean of fitting function was 96.0% and the standard deviation (SD) was 12.4% (n=99). Among total population, 95.5% fall into mean $\pm$ 2SD.

terized well in DRG neurons (Oh et al, 1996).

Inward currents were not produced when cells were perfused together with 0.5 μM capsaicin and 10 μM capsazepine, a competitive antagonist of capsaicin receptor (Fig. 1A). The data suggests that capsaicin action is directly mediated by the VR-1. In some cases, capsaicin exerts some biological actions by the indirect mechanism (Bleakman et al, 1990; Choi et al, 1999).

Tachyphylaxis of I_{CAP}

Capsaicin-activated currents exhibited heterogenous pattern of tachyphylaxis, which refers to the diminution of the current amplitude observed during repeated applications of the same capsaicin concentration. Capsaicin was applied for 30 sec and washed out for 2 min before the next applications. Tachyphylaxis patterns of I_{CAP} were variable (Fig. 1B) and the variability may reflect the contribution of subtypes of capsaicin-gated channels. The existence of capsaicin receptor subtypes has previously been suggested by Liu et al, based on the rapid and slow inward currents activated by capsaicin in rat trigeminal ganglion neurons (Liu et al, 1999).

Based on the observation from 17 cells, there were general common features in tachyphylaxis pattern although they were basically heterogenous. The tachyphylaxis was markedly reduced after the 4th application (Fig. 2A). We compared each I_{CAP} after 4th application to adjacent previous I_{CAP}. The distribution of I_{CAP} was fitted by Gaussian function. In Fig. 2B, the mean value of fitting function was 96.0% and the standard deviation (SD) was 12.4% (n=99). The changes in I_{CAP} amplitude ranging from 71.2% to 120.8% (mean$\pm$2SD) were considered within normal range. DAMGO and CCK-8 were therefore applied after 4th application of capsaicin and recognized to have phar-

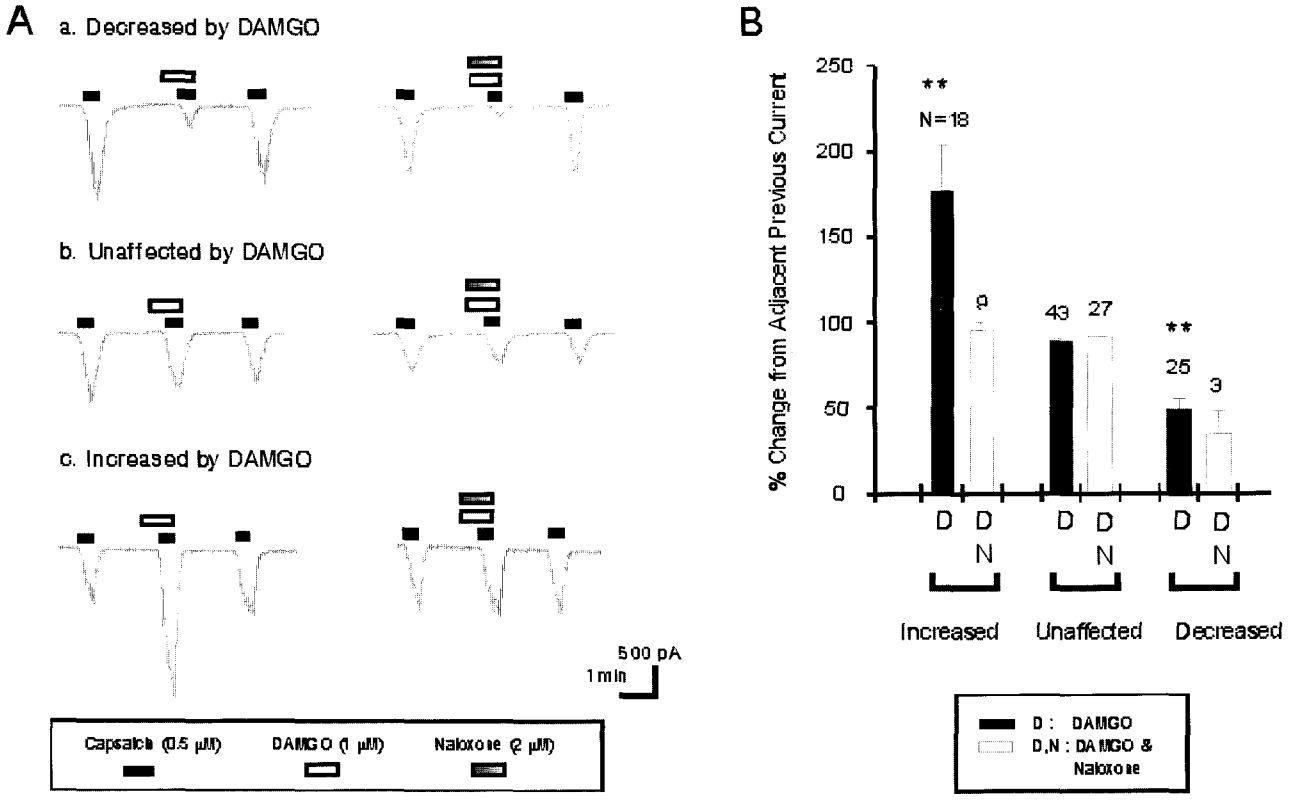

Fig. 3. Effects of DAMGO, a μ-opioid agonist, on I_{CAP}. In 25 out of 86 cells, DAMGO diminished I_{CAP} to 50.0$\pm$5.2% of adjacent previous current (**: $P<0.001$) and and the next I_{CAP} after 2 min-washout period reversed to the amplitude before DAMGO treatment. In 18 out of 82 cells, 1 μM DAMGO increased amplitude of I_{CAP} up to 177$\pm$27% of adjacent previous current (**: $P<0.001$) and the next I_{CAP} after washout reversed to the amplitude before DAMGO treatment. In 43 out of 86 cells, DAMGO had no effect on I_{CAP}. Naloxone (2 μM) reversed the opioid effect on I_{CAP} in 'increased group' while naloxone did not reverse the opioid effect on I_{CAP} in 'decreased group'.

macological effects on I_{CAP} in cases that the changes of I_{CAP} amplitude after its application were above or below normal range.

Effects of DAMGO, a μ-opioid agonist, on I_{CAP}

We examined whether DAMGO (1 μM) affect the amplitude of I_{CAP} in 86 DRG cells (Fig. 3). Typically DAMGO was pretreated for 1 min before co-application of capsaicin and DAMGO. However, there were no differences in opioid effect even if pretreatment of DAMGO was omitted before co-application of capsaicin and DAMGO.

In 25 out of 86 cells, DAMGO significantly diminished I_{CAP} to 50.0±5.2% of adjacent previous current ($P<0.001$), since the next I_{CAP} after 2 min washout period was recovered to thaet value before DAMGO treatment. We designated this group as 'decreased group'. In 18 out of 86 cells, DAMGO increased I_{CAP} up to 177±27% of adjacent previous current significantly ($P<0.001$) and the next I_{CAP} after washout reversed to that before DAMGO treatment ('increased group'). In 43 out of 86 cells, DAMGO had no effect on I_{CAP} (unaffected group).

Naloxone (2 μM) reversed the opioid effect on I_{CAP} in 'increased group' while it did not reverse the opioid effect on I_{CAP} in 'decreased group'. Naloxone itself reversibly decreased I_{CAP} to 62.6±6.0% ($P<0.05$) in 3 cells out of 7 cells examined (data not shown). Due to the effect of naloxone on I_{CAP}, it could not block the opioid effect in 'decreased group'.

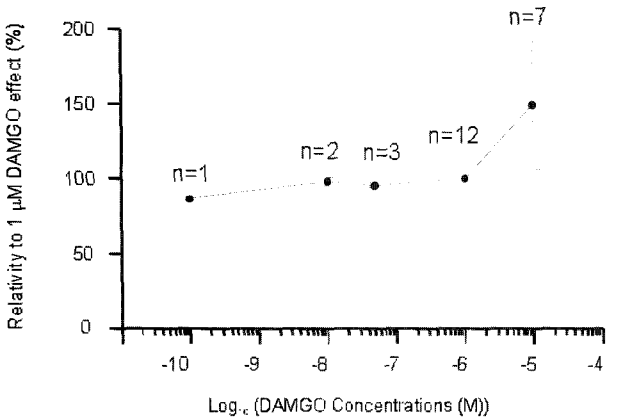

Fig. 4. Effects of DAMGO on I_{CAP} at different concentrations. Effects of DAMGO at various concentrations were compared to effects at 1 μM. The effects at all concentrations were not significantly different from effects at 1 μM.

To examined whether the effect of DAMGO on I_{CAP} is different according to concentration of DAMGO, we compared the effects of DAMGO at various concentrations were compared to those at 1 μM. The effects of DAMGO at all concentrations were not significantly different from those at 1 μM except at 10 μM, without regardless toof 'increased group', 'decreased group', and 'unaffected group' (Fig. 4).

Effects of CCK on I_{CAP}

We examined whether CCK-8 affects I_{CAP} and interacts with DAMGO on I_{CAP} in DRG cells (Benedetti, 1997). CCK-8 (20 nM) increased I_{CAP} up to 360.2± 92.5% of the adjacent previous I_{CAP} ($P<0.01$) in 5 cells out of 13 cells. CCK-8 either decreased I_{CAP} (73.7±3.1%, 3/13) or did not affect I_{CAP} (96.8± 2.6%, 5/13) in other cells (Fig. 5).

DAMGO also increased I_{CAP} (229.5±18.6%, $P<$ 0.001) significantly in the same cells in which CCK-8 enhanced I_{CAP} (5/13). The combined applications of DAMGO and CCK-8 in these cells (Fig. 6) increased amplitude of I_{CAP} (718.8±12.6%).

In some cells (2/13), both DAMGO and CCK-8 decreased I_{CAP} respectively in the same cells and combined application of DAMGO and CCK-8 also decreased I_{CAP} with no additive effect. However, the

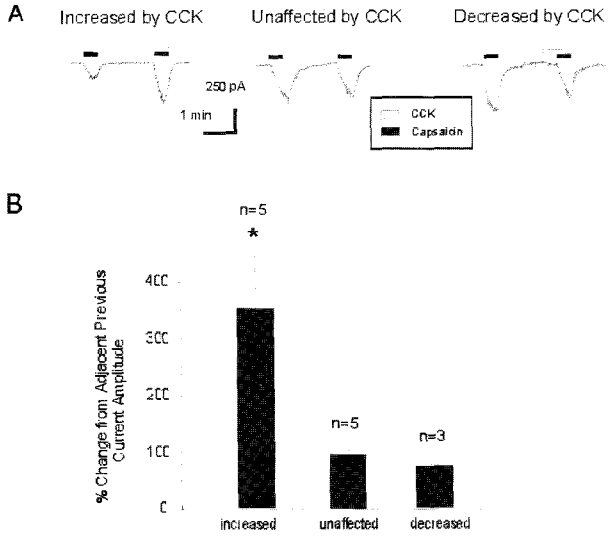

Fig. 5. Effects of CCK on I_{CAP}. CCK (20 nM) either increased I_{CAP} up to 353.0±94% significantly (*: $P<$ 0.01) in 5 out of 13 cells tried in the experiment. In other cells, CCK decreased I_{CAP} (73.7±3.1%, 3/13) or did not affect I_{CAP} (96.8±2.6%, 5/13).

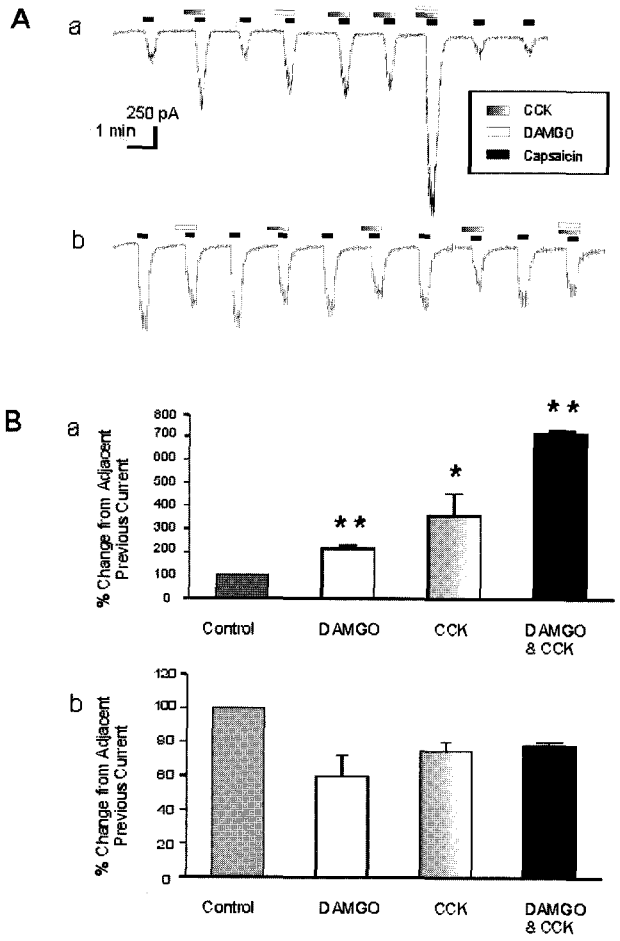

Fig. 6. Various patterns of modulating effect on I_{CAP} by DAMGO and CCK-8 in the same cell. In 5 out of 13 cells, amplitude of I_{CAP} were increased by DAMGO (**: $P<0.001$) and CCK-8 (*: $P<0.01$) respectively in the same cells. Combined application of DAMGO and CCK increased I_{CAP} in additive manner. In some cells (2/13), both DAMGO and CCK decreased I_{CAP} respectively in the same cells and combined application of DAMGO and CCK also decreased I_{CAP} with no additive effect. However, the decrease is not statistically significant.

decrease is not statistically significant. In other cells (4/13), both DAMGO and CCK-8 did not affect I_{CAP} respectively.

I_H and action potential characteristics of each group

Before testing effects of DAMGO on I_{CAP}, hyperpolarization-induced, slowly activating current (I_H) and action potential of most DRG cells were measured to see whether the mode of opioid effects on I_{CAP} correlates with the subtypes of DRG cells clas-

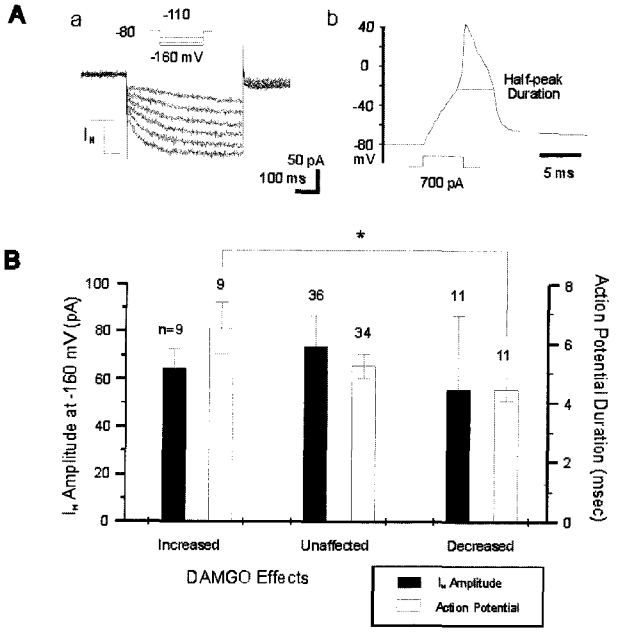

Fig. 7. I_H and action potential characteristics of each group (Aa), Representative traces of I_H were elicited at -110 to -160 mV step pulse from -80 mV holding potential. I_H was considered as amplitude difference between onset of 500 msec pulse and end of the pulse. (Ab), Action potential recorded from cell in Aa. Current of 300~900 pA was injected into current-clamped cells and the half-peak duration was measured from waveform. (B), I_H amplitudes were not significantly different when compared to each other group. However, action potential duration of 'increased group' was significantly different from that of 'decreased group' (*: $P<0.05$).

sified by electrophysiological property (Fig. 7). I_H was elicited at -110 to -160 mV step pulse from -80 mV holding potential. I_H was calculated as amplitude difference between onset of 500 ms pulse and end of the pulse. Current of 300~900 pA was injected into current clamped cells to induce action potential and the half-peak duration was measured from waveform.

I_H was 64.7 ± 8.0 pA (n=9) in 'increased group', 55.7 ± 31.3 pA (n=11) in 'decreased group', and 73.8 ± 12.8 pA (n=36) in 'unaffected group' by DAMGO. I_H were not significantly different among all groups. SD of I_H amplitudes was small in 'increased group' while I_H amplitudes fell into broad ranges in 'decreased group'.

On the other hand, APD of 'increased group' was 6.50 ± 0.86 ms (n=9) and 4.46 ± 0.38 ms (n=11) in 'decreased group', and 5.23 ± 0.42 ms (n=36) in 'unaffected group'. APD of 'increased group' was significantly longer than that of 'decreased group' ($P<0.05$).

DISCUSSION

We investigated to see if DRG cells in adult rat varyinging with different effects of DAMGO and CCK-8' effects on I_{CAP} are classified on the basis of such electrophysiological properties as APD and I_H of these cells.

Present study showed that DAMGO affects I_{CAP} in diverse manner. In 18 out of 86 cells (21% of total cells), DAMGO significantly increased the I_{CAP}, in 25 cells (29%), significantly decreased it and in remaining 43 cells (50%), DAMGO exerted no effect. One possibility for 'unaffected group' is that opioid receptors may not be expressed on the plasma membrane of affected cells. It is also possible that individual function of subtypes of receptor coupled to different G protein (i.e., Gs, Gi/o, etc.) could be nullified due to the function of opposite effector. In addition opioid receptors may not be functionally or structurally connected to capsaicin receptor (VR1) in 'unaffected group' cells.

If we exclude the 'unaffected group', then the increased and decreased effects could be attributed to the dual effect of opioids. Actually such dual effects of opioid agonists have been observed in the previous study (Crain et al, 1988). Opioid agonists (μ, δ, κ) were shown to increase the duration of action potential (APD) in low concentration ($\sim$nM), while they decrease APD in high concentration ($\sim \mu$M). It is generally known that opioid receptors fall into a superfamily of G protein-coupled receptors. Then these dual effects are assumed to be dependent on subtypes of G protein linked to opioid receptors.

Opioids are known to decrease Ca^{2+} conductance of DRG cells (Taddese et al, 1995) and increase K^+ conductance of dorsal horn cells in spinal cord when they are linked to pertussis toxin-sensitive Gi/o protein (Schneider et al, 1998). These may decrease APD of primary afferent. However, opioids exhibit the opposite effects when opioid receptors are coupled to cholera toxin-sensitive Gs protein (Fan & Crain, 1995). In this case opioids increase APD of DRG cells via Gs protein-adenylyl cyclase-cAMP-PKA signaling pathway. Overall effect of opioid agonists seems to be dependent on the subtype of G protein exerting stronger effect on the cells or tissues (Chen et al, 1989; Crain & Shen, 2000). Therefore, distribution of subtype of G protein may contribute to diverse effects of opioids on I_{CAP}.

We also examined whether CCK-8 affects on I_{CAP} and interacts with DAMGO, because it is known to have anti-opioid effect in many systems (Benbedetti, 1997). CCK-8, as well as DAMGO, also showed dual effects on I_{CAP} in the present study. It is interesting when both DAMGO and CCK-8 were perfused together to DRG cells, they increased I_{CAP} in additive manner in 5 cells. The data suggest that DAMGO and CCK-8 may employ different signaling pathways to affect I_{CAP}.

It is well established that CCK receptors (i.e, CCK-AR, CCK-BR) exerts their biological function by activation of pertussis toxin-insensitive G protein, presumably a member of Gq family coupled to phospholipase C (PLC). The subsequent cascade of this pathway involves the release of Ca^{2+} from intracellular Ca store and activation of protein kinase C (PKC) (Nozu et al, 1999). Considering action mechanism of DAMGO and CCK-8, it could becould be possible that DAMGO may employ PKA and CCK-8 may activate PKC in increasing I_{CAP} (Wank, 1995). Effects of DAMGO and CCK-8 were therefore superposed in additive manner when they were combined.

In this study, I_H and APD were investigated to see whether effects of DAMGO on I_{CAP} correlate with cell subtypes. Primary afferent cells are largely divided by conduction velocity (A α-, A β-, A δ- and C-type). A δ- and C-type nociceptors are assumed to have many subtypes as peripheral somatic sensations are various in types. They show various responses to mechanical, heat and chemical stimulation depending on cell subtypes. Since conduction velocity is not able to be measured in dissociated cells *in vitro*, several electrophysiological properties have been employed to classify subtypes of primary afferents (McLean et al, 1988; Scroggs et al, 1994; Del Mar et al, 1996; Yoshimura et al, 1996). We tried to classify effects of DAMGO on I_{CAP} based on I_H and APD. The data demonstrated APD of 'increased group' was significantly longer than that of 'decreased group'. However, I_H amplitudes were not significantly different among all groups.

In conclusion, the results demonstrated that DAMGO and CCK-8 either increase or decrease amplitude of I_{CAP} and these dual effects presumably depend on subtypes of DRG cells or signal transduction pathways, which are linked to the receptor and specific G protein subtypes. Cellular mechanisms underlying dual effects of opioids and CCK-8, and their functional implications are poorly understood and remain

to be further studied.

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