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Original Article

Experimental Neurobiology

Capsaicin Blocks the Hyperpolarization-Activated Inward Currents via TRPV1 in the Rat Dorsal Root Ganglion Neurons

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Capsaicin, the pungent ingredient in hot pepper, activates nociceptors to produce pain and inflammation. However, prolonged exposures of capsaicin will cause desensitization to nociceptive stimuli. Hyperpolarization-activated cation currents (I_h) contribute to the maintenance of the resting membrane potential and excitability of neurons. In the cultured dorsal root ganglion (DRG) neurons, we investigated mechanisms underlying capsaicin-mediated modulation of I_h using patch clamp recordings. Capsaicin (1 μM) inhibited I_h only in the capsaicin-sensitive neurons. The capsaicin-induced inhibition of I_h was prevented by preexposing the TRPV1 antagonist, capsazepine (CPZ). Capsaicin-induced inhibition of I_h was dose dependent (IC_{50}= 0.68 μM) and partially abolished by intracellular BAPTA and cyclosporin A, specific calcineurin inhibitor. In summary, the inhibitory effects of capsaicin on I_h are mediated by activation of TRPV1 and Ca^{2+}-triggered cellular responses. Analgesic effects of capsaicin have been thought to be related to desensitization of nociceptive neurons due to depletion of pain-related substances. In addition, capsaicin-induced inhibition of I_h is likely to be important in understanding the analgesic mechanism of capsaicin.

Key words: capsaicin, DRG neuron, hyperpolarization-activated cation current, rat

INTRODUCTION

The hyperpolarization-activatedcurrent (I_h) is a cation current activated by membrane hyperpolarization. The characteristics of I_h channels are: slow opening at negative potentials, permeability to both Na^+ and K^+ ions, and sensitivity to blockade with Cs^+ ions.Activation of I_h at negative potentials can result in a slow depolarization,such a depolarizing influence could accelerate neuronal firing discharges. Because I_h has been thought to contribute to the maintenance of the resting membrane potential [1], modulation of I_h can affect neuronal excitability. The ion channels underlying I_h have been discovered about a decade ago, these proteins were termed hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. In mammals, the HCN channel family comprises four distinct members (HCN1-4) [2]. HCN1 and HCN2 channels are largely expressed in the DRG neurons [3] that play an important role in the transmission of sensory information from periphery. Especially, Emeryet al. reported that HCN2 ion channels play a critical role in inflammatory and neuropathic pain in mice [4]. Upregulation of I_h expression in the DRG neurons were related to the neuronal hyperexcitability in the neuropathic pain and pharmacological blockade of I_h could relieve the pain [5-8]. There have been many reports that suggest the inhibition of I_h may be the mechanism of opioid analgesics [3, 9] and local anesthetics [3, 5, 10-13]. These reports suggest that inhibition of I_h

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may be responsible for the peripheral analgesia [9].

Capsaicin, the main pungent ingredient in chili peppers can excite nociceptive sensory neurons and produce transient pain in animals and humans. Capsaicin activates the transient receptor potential vanilloid subtype 1 (TRPV1), a nonselective cation channel with high Ca^{2+} permeability [14]. The TRPV1 channel is expressed in subsets of primary sensory neurons and nerve terminals and plays an essential role in detecting noxious heat and several other nociceptive stimuli. Based on the reports that TRPV1knockout mice exhibit reduced inflammatory thermal hyperalgesia, TRPV1 appears to be essential for mediating thermal hyperalgesia induced by inflammation [15, 16]. Paradoxically, prolonged or repetitive exposure to capsaicincan desensitize nociceptive sensory neurons and results in long lasting pain relief [17]. Ca^{2+}-dependent desensitization of TRPV1 themselves likely contributes to the analgesic effects of capsaicin [18].

Neuronal excitability is due to the presence of voltage-sensitive ion channels in the plasma membrane [19]. Thus, activation or inhibition of these ion channels may contribute to the excitability of the sensory neurons and capsaicin analgesia. Capsaicin blocks voltage-gated Na^+ channels in trigeminal ganglion [20] and DRG neurons [20-22], Ca^{2+} channels in the sensory neurons [23-25] and K^+ channels in the trigeminal ganglion neurons [26]. Such inhibitions of voltage-sensitive channels by capsaicin may result in decrease ofthe excitability of neurons and block the transmission of painful signal from peripheral tissues.

As stated above, inhibition of I_h may be related to peripheral analgesia. Thus, we hypothesized that inhibition of I_h in the DRG neurons may be one of the mechanisms underlying analgesic effect of capsaicin.Here, we studied the hypothesis that capsaicin could induce the inhibition of I_h in the cultured DRG neurons using patch clamp technique.

MATERIALS AND METHODS

Cell culture

Primary cultures of DRG neurons dissected from all levels of lower cervical, thoracic, and lumbar spinal cord of two-day-old neonatal rats were prepared. The dissected ganglia were collected in cold culture medium (4°C), which contained Dulbecco's modified Eagle medium/F-12 mixture (DMEM/F-12; Gibco, Invitrogen, Grand Island, NY, USA), 10% fetal bovine serum (FBS; Gibco, Invitrogen), 1 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The collected ganglia were washed with the culture medium and incubated at 37°C for 30 min in 1 mg/ml collagenase (Type II; Worthington, Freehold, NJ, USA). The ganglia were then washed three times with Mg^{2+}- and Ca^{2+}-free Hank's balanced salt solution (HBSS; Gibco, Invitrogen) and incubated in 2.5 mg/ml trypsin (Gibco, Invitrogen) at 37°C for 30 min. Subsequently, the ganglia were centrifuged at 1,000 rpm for 10 min, and the pellet was washed two or three times with the culture medium to inhibit the enzyme. The pellet in turn was suspended in the culture medium by gentle trituration with a Pasteur pipette, and the suspended cells were plated on square glass coverslips coated with poly-L-lysine (Sigma-Aldrich), which were placed in small Petri dishes. Then, 25 ng/ml nerve growth factor (Alomone Labs, Jerusalem, Israel) were added to each Petri dish. Cells were incubated at 37°C in a 95% air -5% CO_2 gas mixture and used 1~3 days after plating.

Electrophysiology

Whole-cell membrane currents were recorded from the somata of DRG neurons. Electrodes were made by pulling borosilicate glass capillaries (Harvard, Kent, UK). Tip resistances were 2~3 MΩ for whole-cell recordings. To record whole-cell current, cellmembrane was ruptured by gentle suction after the formation of a gigaohmseal. Capacitative transients were then cancelled. Whole-cell membrane currents were recorded at -60 mV using an Axopatch 200 Bamplifier (Molecular Devices, Union City, CA, USA). Membrane currents were low pass filtered at 1 kHz and sampled at 2.5 kHz with a Digidata 1,322 data acquisition system (Molecular Devices). Data were analyzed using the pClamp 8.0 software (Molecular Devices) and OriginPro 8.0 (OriginLab, Northampton, MA, USA). All electrophysiological experiments were performed at room temperature.

Solutions and chemicals

Normal Tyrode's (NT) solution contained (in mM) 140 NaCl, 3 KCl, 10 HEPES, 10 glucose, 2.5 $CaCl_2$, 1 $MgCl_2$ (pH was adjusted to 7.4 with NaOH). The recording electrodes were filled with apipette solution containing (in mM) 135 K gluconate, 1 $MgCl_2$, 10 NaCl, 10 HEPES, 2 Mg-ATP, and 0.1 Na-GTPtitrated to pH 7.4 with KOH. For intracellular Ca^{2+} chelation, 10 mM BAPTA was added to the internal solution. The nominally Ca^{2+}-free bath solution contained (in mM) 140 NaCl, 3 KCl, 10 HEPES, 10 glucose, 1 $MgCl_2$,10 EGTA (pH was adjusted to 7.4 with NaOH). To isolate I_h from other voltage-sensitive currents, 0.5 mM $BaCl_2$, 0.1 μM tetrodotoxin, 10 mM tetraethylammonium chloride, 1 mM 4-aminopyridine and 0.1 mM $NiCl_2$ were added to the bath solution throughout the experiments. Capsaicin was dissolved and stored as a 10 mM stock solution in 100% ethanol. All stock solutions were stored at -20°C and were diluted with the bath solution to the desired final concentrations at the beginning of each experiment.

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Statistical analysis

Data are presented as means±standard error of the mean (SEM). Inhibitory effects capsaicin were given as % inhibition=100×(1-($I_{h,cap}$/$I_{h,con}$)), where $I_{h,con}$ and $I_{h,cap}$ are the currents measured before and during application of capsaicin, respectively. All comparisons between means were tested for significance using Student's paired *t*-testand analysis of variance (ANOVA) followed by the *post hoc* Tukey test. p<0.05 was considered to indicate significant difference.

RESULTS

Effect of capsaicin on I_h

Hyperpolarization of DRG neurons voltage-clamped at -60 mV induced noninactivatinginward current consisting of an instantaneous and a slowly-activating component (Fig. 1A). I_h was calculated as the difference between instantaneous current (I_{inst}) at the beginning and the steady state current (I_{ss}) at the end of the 1s-voltage step, respectively. Capsaicin (1 μM) evoked inward currents ranging from 0.69±0.15 nA at -60 mV in the DRG neurons.After applying capsaicin continuously for 1~3 min, the capsaicin-evoked currents were desensitized to a near steady state value 0.38±0.05 nA (n=17). At this time, to study the effect of capsaicin on I_h, hyperpolarizing voltage pulses were applied from -130 to - 60 mV with 10 mV increments in the presence of capsaicin. Amplitudes of I_h measured at -130 mV in the presence of capsaicin were significantly reduced by 92.5±14.2% (n=16). After washing these cells for another 3~5 min, I_h was partially recovered to their pre-capsaicin levels. Capsaicin blocked I_h in a dose-dependent manner (Fig. 1C). IC_{50} was 0.68 μM and Hill coefficient was 1.13. The half-activation voltage ($V_{1/2}$) was -94.1±2.3 mV in control and -109.9±3.1 mV in capsaicin treated cell, a negative shift of about 15 mV (Fig. 1D). Inhibition magnitudes of I_h by capsaicin were not significantly different at various voltages (One-way ANOVA, F=1.57393, p=0.21659, n=19) (Fig. 1E).

Involvement of TRPV1 in I_h inhibition by capsaicin

We found that in capsaicin-insensitive cells where capsaicin did not evoke membrane currents, capsaicin had no significant effect on the amplitudes of I_h (n=3) and activation kinetics (Fig. 2). To determine whether the effect of capsaicin on I_h is mediated by TRPV1 activation, we used TRPV1 antagonist, capsazepine. As shown in Fig. 3, capsazepine (10 μM)alone did not produce any effect on I_h. In the presence of capsazepine, 1 μM capsaicin failed to induce any inward currents as well as inhibitory effects on I_h. These results suggest that capsaicin indirectly inhibited I_h through

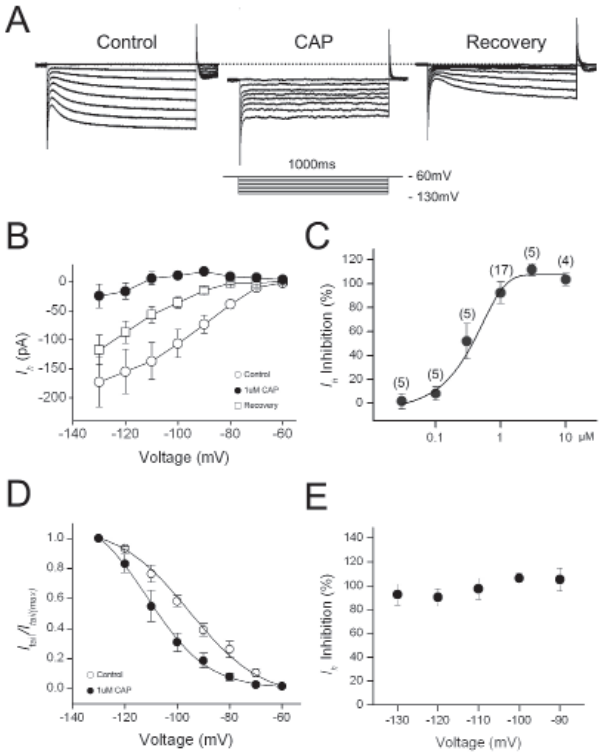

Fig. 1. Capsaicin inhibited I_h in DRG neurons. (A) Representative current responses of DRG neurons to 1-s voltage pulses injected in 10-mV increment under control condition, during application of 1 μM capsaicin (CAP) and after washout (recovery). (B) Current-voltage relationship of I_h. Amplitudes of I_h in the presence were compared with those of control I_h at the same test voltage. (C) Concentration dependence of capsaicin inhibition of I_h. The curve was fitted by Hill equation (IC_{50}=0.68 μM). (D) Effects of capsaicin on the voltage dependence of activation. Normalized tail current was plotted versus various potentials (-60 to -130 mV) and fitted with Boltzmann equation. Estimated $V_{1/2}$ was shifted to left by CAP. (E) Voltage dependence of capsaicin inhibition of I_h. Amplitudes of capsaicin inhibition of I_h in the presence of capsaicin were normalized to those in control at the same test voltages.

the activation of TRPV1.

Effect of Ca^{2+} on I_h inhibition by capsaicin

Because TRPV1 is highly permeable to Ca^{2+} [14], intracellular Ca^{2+} concentration can be greatly increased when the channel is opened. To determine the role of Ca^{2+} influx in the inhibitory effect of capsaicin on I_h, we removed extracellular Ca^{2+} from the bath solution. Amplitudes of I_h were not significantly changed by in Ca^{2+}-free bath solution. Capsaicin inhibition of I_h was not abolished in Ca^{2+}-free bath solution (Fig. 4). In the presence of intracellular BAPTA (10 mM), a rapid Ca^{2+} chelator, 1 μM capsaicin still inhibited I_h (n=6). However, the magnitude of I_h inhibition by capsaicin (62.1±10.2%) was significantly different from that obtained in pipette solution without BAPTA (n=6)

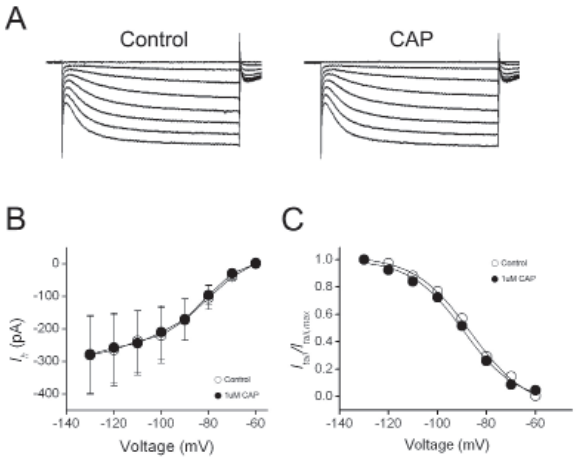

Fig. 2. Capsaicin did not inhibit I_h in the capsaicin-insensitive DRG neurons. (A) Representative current responses of DRG neurons to 1-s voltage pulses injected in 10-mV increment under control condition, during application of 1 μM capsaicin (CAP). (B) Current-voltage relationship of I_h. Amplitudes of I_h in the presence CAP were compared with those of control I_h at the same test voltage. (C) Effects of capsaicin on the voltage dependence of activation. Normalized tail current was plotted versus various potentials (-60 to -130 mV) and fitted with Boltzmann equation. Estimated $V_{1/2}$ was not significantly changed by CAP.

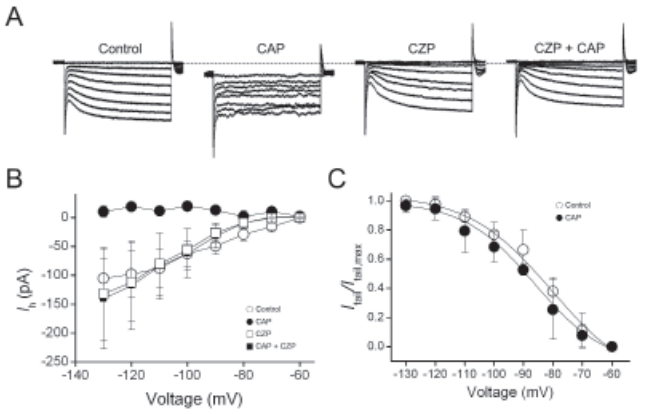

Fig. 3. Capsazepineprevented the inhibition of I_h by capsaicin. (A) Representative current responses of DRG neurons to 1-s voltage pulses injected in 10-mV increment under control condition, during application of 1 μM capsaicin (CAP), during application of 10 μM capsazepine (CZP) and subsequent addition of CAP. (B) Current-voltage relationship of I_h. Amplitudes of I_h in the presence of CAP, CZP and CAP+CZP were compared with those of control I_h at the same test voltage. (C) Normalized tail current was plotted versus various potentials (-60 to -130 mV) and fitted with Boltzmann equation. Estimated $V_{1/2}$ was not significantly changed by CAP+CZP.

(Fig. 4A).Therefore, intracellular Ca^{2+} may be required for the inhibition of I_h by capsaicin in the DRG neurons.

Rapid rise in intracellular Ca^{2+} levels may due to either Ca^{2+} influx or release of Ca^{2+} from an intracellular store. Thus, we investigated whether the inhibitory effect of capsaicin on I_h was mediated by Ca^{2+} released from the intracellular stores. To deplete intracellular Ca^{2+} stores, we pretreated DRG cells with thapsigargin (10 μM) and caffeine (10 mM) for 3 min and washed them out for at least 5 min, before applying capsaicin. The percentile inhibition of I_h by capsaicin at -120 mV were reduced to 61.9±9.9% (n=7, $p<0.05$). These results demonstrated that increase in intracellular Ca^{2+} level is partially involved in the inhibition of I_h by capsaicin.

Role of Calcineurin in I_h inhibition by capsaicin

Because the increase in intracellular Ca^{2+} appeared to be required for the inhibitory effect of capsaicin on I_h, we next determined if calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II are involved in this effect. The specific calmodulin antagonist, W-7 or the selective Ca^{2+}/Calmodulin-dependent protein kinase II inhibitor, KN-93 was included in the pipette solution. In the presence of W-7 (10 μM), capsaicin still inhibited I_h (65.1±8.3%, n=5), which was not significantly different from the effect of capsaicin on I_h recorded using pipette solution without W-7 (92.5±8.9%, n=17) (Fig. 5A). In the presence of KN-93 (10 μM), capsaicin still produced large inhibition on I_h (59.0±6.2%, n=9), which was not significantly different from the effect of capsaicin on I_h recorded using control pipette solution (Fig. 5B).

I_h can be inhibited by phosphorylation by PKC [27, 28], we determined whether PKC inhibitor can block the inhibitory effect of capsaicin on I_h. When 20 μM RO318210, PKC inhibitorwas included in the pipette solution, capsaicin decreased I_h by 69.3±9.0% (n=21), which was not significantly different from the capsaicin-induced% inhibition obtained with normal pipette solution (Fig. 5C).

Calcineurin (protein phosphatase 2B) is a Ca^{2+}-sensitive protein phosphatase and can be activated by a rise in intracellular Ca^{2+}. To determine whether capsaicin inhibited I_h through dephosphorylationmediated by calcineurin, cyclosporin A, aspecific inhibitor of calcineurin was added to the pipette solution. As shown in Fig. 5D, subsequent application of capsaicin reduced I_h amplitude measured at -130 mV by 37.7±8.7% (n=8), a significantly smaller reduction than the effect of capsaicin in the absence of cyclosporin A ($p< 0.01$).

DISCUSSION

In the present study, we demonstrate that capsaicin reversibly and voltage-independently inhibited hyperpolarization-activated cation current, I_h, in the culturedrat DRG neurons. We also found that capsaicin did not change I_h in the capsaicin-insensitive DRG neurons and TRPV1 antagonist, capsazepine eliminated the effect

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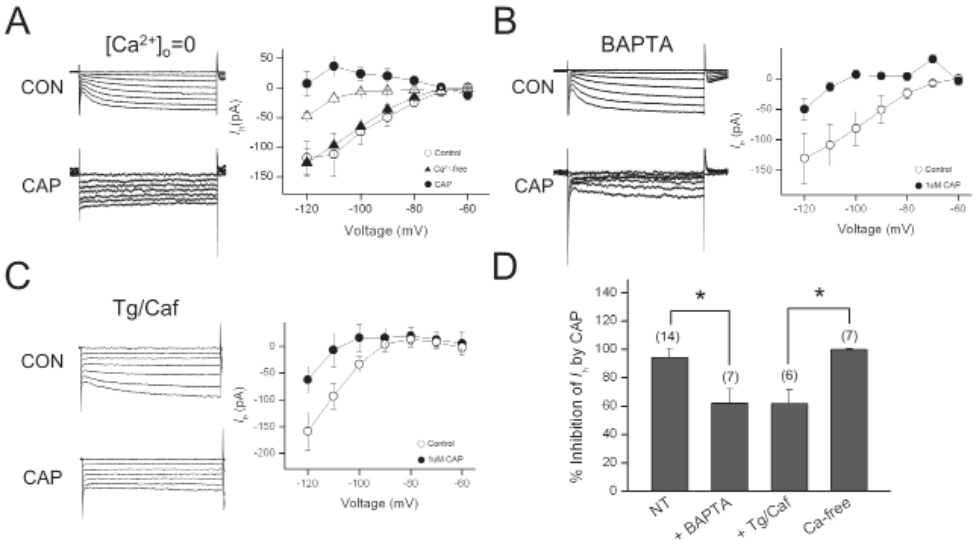

Fig. 4. Effect of Ca^{2+} on I_h inhibition by capsaicin. (A) Representative current responses of DRG neurons to 1-s voltage pulses under control (CON) condition, during application of 1 μM capsaicin (CAP) in Ca^{2+}-free bath solution (0 Ca^{2+}). Graph shows the current-voltage relationship of I_h under control and during capsaicin application in Ca^{2+}-free bath solution. (B) Representative current responses of DRG neurons to 1-s voltage pulses under control condition, during application of CAP in the presence of BAPTA in the pipette. Graph shows the current-voltage relationship of I_h under control and during capsaicin application in the presence of BAPTA. (C) Representative current responses of DRG neurons to 1-s voltage pulses under control condition, during application of 1 μMCAP after intracellular Ca^{2+} depletion bythapsigargin (Tg) and caffeine (Caf, 10 mM). Graph shows the current-voltage relationship of I_h under control and during capsaicin application after Tg/Caftretatment. (D) Bar graph summarizes % inhibitions induced by capsaicin. Values at the top of each bar represent the number of experiments. Bars represent the mean±S.E.M. Asterisks indicate a significant difference (*p<0.05).

of capsaicin on I_h. Therefore, capsaicin may indirectly modulate I_h via TRPV1 activation.

Because TRPV1, capsaicin receptor is highly permeable to Ca^{2+} [14], extracellular Ca^{2+} influx through the activated channel can influence on I_h. But, our findings that inhibition of I_h was not abolished by removal of Ca^{2+} from the bath solution revealed that extracellular Ca^{2+} influx and Ca^{2+}-induced Ca^{2+} release (CICR) were not essential for the effect of capsaicin on I_h. We found that intracellular Ca^{2+} chelation and depletion of intracellular store partially abolished the inhibitory effect of capsaicin on I_h. It has been reported that functional TRPV1 was also expressed on the endoplasmic reticulum membrane of DRG neuron [29, 30] and capsaicin could increase intracellular Ca^{2+} level in the Ca^{2+}-free condition [31]. Thus, it is likely that release of Ca^{2+} by the activation of TRPV1 in the endoplasmic reticulum could contribute to the inhibition of I_h by capsaicin to some extent.

As described in the result section, BAPTA, thapsigargin and cyclosporin A partly attenuated the inhibition of I_h by capsaicin. It is possible that additional mechanism underlies in the capsaicin-induced I_h inhibition. Remaining portion of I_h inhibition by capsaicin may be caused by decrease in driving forces that move cations into the DRG neurons. When TRPV1 is activated by capsaicin, Na^+ can enter rapidly into the neurons by concentration gradient and negative holding potential (-60 mV). It has been reported that activation of TRPV1 blocked voltage-gated Na^+ channels as a result of increase in intracellular Na^+ concentration. I_h is a mixed cation current carried by both Na^+ and K^+ [2]. Therefore, movement of Na^+ through the I_h channels might be inhibited by decreased Na^+ concentration gradient due to increase of $[Na^+]_i$ by TRPV1 activation. Therefore, amplitudes of I_h can be decreased by capsaicin as a result of decreased Na^+ movement into the DRG neuronsthrough I_h channels in addition to Ca^{2+}-dependent modulation of I_h.

Increase of intracellular Ca^{2+} concentration can trigger many intracellular events such as protein phosphorylation. Therefore, we were interested in the cellular events following the increase of intracellular Ca^{2+} level through activated TRPV1 in the modulation of I_h by capsaicin. Ca^{2+} can bindcalmodulin and desensitize TRPV1 [18]. If TRPV1 desensitization were responsible for the effect of capsaicin on I_h, inhibition of calmodulin could abolish the effect the capsaicin action. From the result thatcalmodulin antagonist, W7 failed to diminish the capsaicin- induced I_h inhibition, Ca^{2+}/calmodulin-dependent TRPV1 desensitization may not be responsible for the I_h inhibition. It has been reported that activation of PKC could inhibit I_h [27, 28], we tested the effect of PKC inhibitor in I_h inhibition by capsaicin. However, PKC was

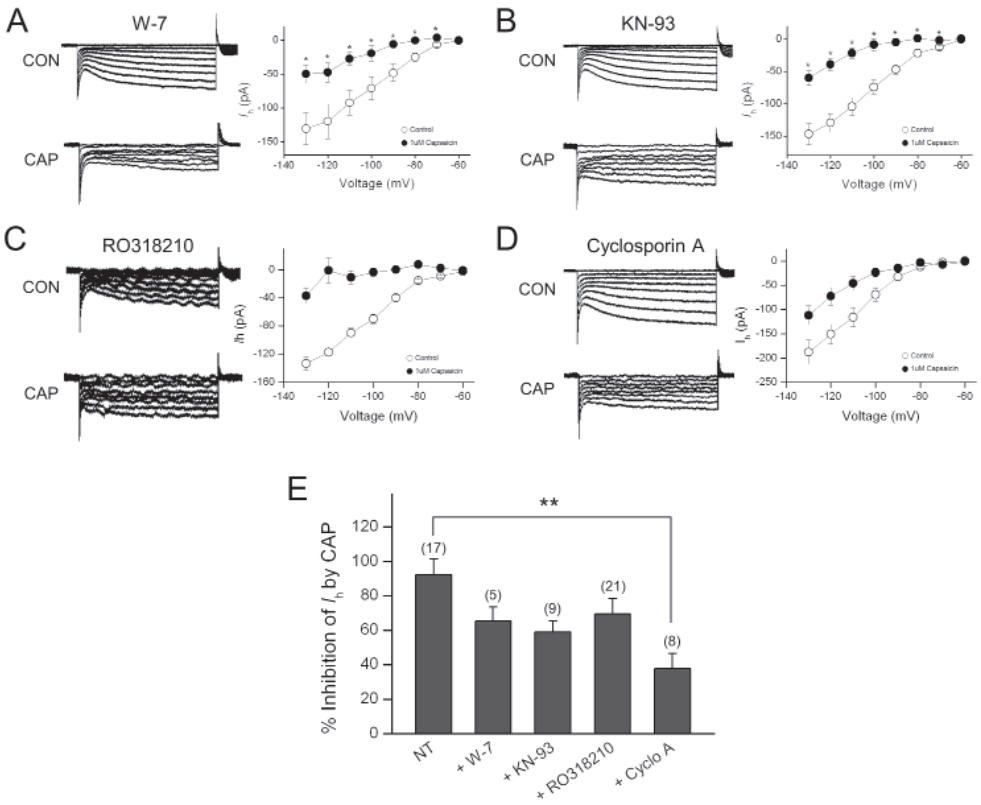

Fig. 5. Cyclosporin A partially abolished the I_h inhibition by capsaicin. (A) Representative current responses of DRG neurons to 1-s voltage pulses under control condition, during application of 1 μM capsaicin (CAP) in the presence of W-7 in the pipette. Graph shows the current-voltage relationship of I_h under control and during capsaicin application in the presence of W-7. (B) Representative current responses of DRG neurons to 1-s voltage pulses under control condition, during application of 1 μM capsaicin in the presence of KN-93 in the pipette. Graph shows the current-voltage relationship of I_h under control and during capsaicin application in the presence of KN-93. (C) Representative current responses of DRG neurons to 1-s voltage pulses under control condition, during application of 1 μM capsaicin in the presence of thapsigargin in the pipette. Graph shows the current-voltage relationship of I_h under control and during capsaicin application in the presence of thapsigargin. (D) Representative current responses of DRG neurons to 1-s voltage pulses under control condition, during application of CAP in the presence of cyclosporine A in the pipette. Graph shows the current-voltage relationship of I_h under control and during capsaicin application in the presence of cyclosporine A. (E) Bar graph summarizes % inhibitions induced by capsaicin. Values at the top of each bar represent the number of experiments. Bars represent the mean±S.E.M. Asterisks indicate a significant difference from the first bar (**p<0.01).

not involved in the inhibition of I_h in our experiment condition. In the present study, we found that calcineurin (protein phosphatase 2B) could play a role in down-regulation of I_h caused by TRPV1 activation in the DRG neurons. Calcineurin is a Ca^{2+}-dependent protein phosphatase and is responsible for the desensitization of TRPV1, either [32]. Since calmodulin antagonist failed to reverse the capsaicin effect on I_h, desensitization of TRPV1 may be irresponsible for thisinhibition of I_h.The target molecule of the dephosphorylarion by calcineurin may be not TRPV1 but I_h channel. From these results, we suggest that capsaicin may increase intracellular Ca^{2+} followed by modification of the I_h channel.

It has been reported that I_h plays an important role in the control of electrical activity in various neuronal cells. Thus, suppression of I_h by capsaicin may cause depressant effects on neuronal activity and analgesia. Topical application of capsaicin has been used to treat neuropathic pain [33, 34]. It has been thought that capsaicin causes release of substance P from C fiber afferent neurons, and repeated application depletes stores of substance P and therefore reduces pain transmission from peripheral nerve fibers to higher centers [35]. In addition to substance P depletion, inhibition of I_h may explain the analgesic effect of capsaicin. Our electrophysiological data can suggest the possible involvement of I_h inhibition in capsaicin-induced analgesia. Althoughrecent report that showed coexpression of TRPV1 and subtypes of HCNs in the sensory neurons [36] support this possibility, further animal studies will be needed to elucidate it.

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