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Regulation of the Spontaneous Augmentation of $Na_V1.9$ in Mouse Dorsal Root Ganglion Neurons: Effect of PKA and PKC Pathways

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Abstract: Sensory neurons in the dorsal root ganglion express two kinds of tetrodotoxin resistant (TTX-R) isoforms of voltage-gated sodium channels, $Na_V1.8$ and $Na_V1.9$. These isoforms play key roles in the pathophysiology of chronic pain. Of special interest is $Na_V1.9$: our previous studies revealed a unique property of the $Na_V1.9$ current, *i.e.*, the $Na_V1.9$ current shows a gradual and notable up-regulation of the peak amplitude during recording ("spontaneous augmentation of $Na_V1.9$"). However, the mechanism underlying the spontaneous augmentation of $Na_V1.9$ is still unclear. In this study, we examined the effects of protein kinases A and C (PKA and PKC), on the spontaneous augmentation of $Na_V1.9$. The spontaneous augmentation of the $Na_V1.9$ current was significantly suppressed by activation of PKA, whereas activation of PKA did not affect the voltage dependence of inactivation for the $Na_V1.9$ current. On the contrary, the finding that activation of PKC can affect the voltage dependence of inactivation for $Na_V1.9$ in the perforated patch recordings, where the augmentation does not occur, suggests that the effects of PMA are independent of the augmentation process. These results indicate that the spontaneous augmentation of $Na_V1.9$ was regulated directly by PKA, and indirectly by PKC.

Keywords: Na^+ channel; tetrodotoxin; dorsal root ganglion; patch clamp; PKA; PKC

1. Introduction

Voltage-gated sodium channels (Na^+ channels) mediate the transient increase in Na^+ conductance that underlies the action potential of neurons and other excitable cells. In mammals, a family of Na^+ channel α subunits (designated $Na_V1.1$-$Na_V1.9$) exhibit unique patterns of anatomical expression and varied functional and pharmacological properties [1,2].

Unmyelinated C-fibers originate from small primary afferent neurons of the dorsal root ganglion (DRG), and transmit nociceptive information to the central nervous system. Na^+ currents expressed in small DRG neurons can be classified into two categories on the basis of their relative sensitivity to tetrodotoxin (TTX); the first, TTX-sensitive (TTX-S) Na^+ currents and the second, TTX-resistant (TTX-R) Na^+ currents. TTX-R Na^+ currents can be further subdivided into the current mediated by $Na_V1.8$, known as SNS [3–6], and the current mediated by $Na_V1.9$, known as NaN [7–11].

The $Na_V1.9$ current is characterized by a low activation threshold of about -60 mV and the depolarized steady-state inactivation (h_∞) curve [7,11–13]. From these observations, the $Na_V1.9$ current may mainly regulate subthreshold excitability of small DRG neurons [7,10,11,13]. Our previous studies have demonstrated gradual and notable increase-decrease of the peak amplitude of the $Na_V1.9$ current during whole-cell patch clamp recording (referred to as 'spontaneous augmentation of $Na_V1.9$'). The spontaneous augmentation of $Na_V1.9$ was not observed in the presence intracellular ATP (3 mM) and by using nystatin-perforated patch clamp recording. These results suggest possible involvement of the intracellular environment in the spontaneous augmentation of $Na_V1.9$ [11].

It is well known that protein kinases A and C (PKA and PKC), and phosphatases, as well as other proteins such as Ca^{2+}-calmodulin-dependent kinase, growth-factor-dependent receptor tyrosine kinases, extracellular signal-regulated kinases and heterotrimeric G proteins, also modulate Na^+ channels [14–16]. At present, endogenous factors that regulate the spontaneous augmentation of $Na_V1.9$ have not been identified. In sensory neurons, activation of PKA and PKC affects the biophysical properties of Na^+ channels [1]. As a first step to the above issue, we focused on the possible involvement of the PKA and PKC pathways in the spontaneous augmentation of $Na_V1.9$. For this purpose, in this study we examined the effects of activators and inhibitors of PKA and PKC on the spontaneous augmentation of $Na_V1.9$, using $Na_V1.8$ knock-out (KO) mice [5,11,13] to record the $Na_V1.9$ current in isolation.

2. Results and Discussion

2.1. Characterization of the $Na_V1.9$ current

The detailed properties of the $Na_V1.9$ current was already described in our previous reports [11,13,17]. A typical example of the $Na_V1.9$ currents is shown in Figure 1. Currents were evoked by a 200 ms test pulse (V_T) from a holding potential (V_H) of -80 mV. TTX-sensitive (TTX-S) Na^+ currents were completely eliminated by 200 nM TTX contained in the external solution. The $Na_V1.9$ current had an activation threshold at about -60 mV (Figure 1a), which was more negative than thresholds for Na^+ currents mediated by $Na_V1.8$ and other TTX-S Na^+ currents (about -40 mV) [13]. Time courses of activation and inactivation of the $Na_V1.9$ current were extremely prolonged, particularly at lower

activation voltages (Figure 1b). Figure 1c illustrates the current-voltage relationship for the currents shown in Figure 1b.

> **Figure 1.** Typical examples of the tetrodotoxin-resistant (TTX-R) Na$^+$ current mediated by Na$_V$1.9. The Na$_V$1.9 current was recorded from the small (diameter < 25 μm) dorsal root ganglion (DRG) neuron prepared from the Na$_V$1.8 knock-out (KO) mouse. Currents were elicited by 200 ms test pulse (V_T) from a holding potential (V_H) of -80 mV. (a) The activation threshold for the Na$_V$1.9 current. The Na$_V$1.9 current could be activated at -60 mV, whereas V_T to -70 mV failed to elicit. (b) A family of superimposed Na$_V$1.9 currents evoked by V_Ts between -70 and +10 mV in 10-mV increments from V_H of -80 mV. The external solution contained 200 nM TTX. Voltage labels attached to traces indicate V_Ts. (c) The current-voltage curve for the Na$_V$1.9 current shown in panel (b). Peak amplitudes of the currents were plotted against V_T.
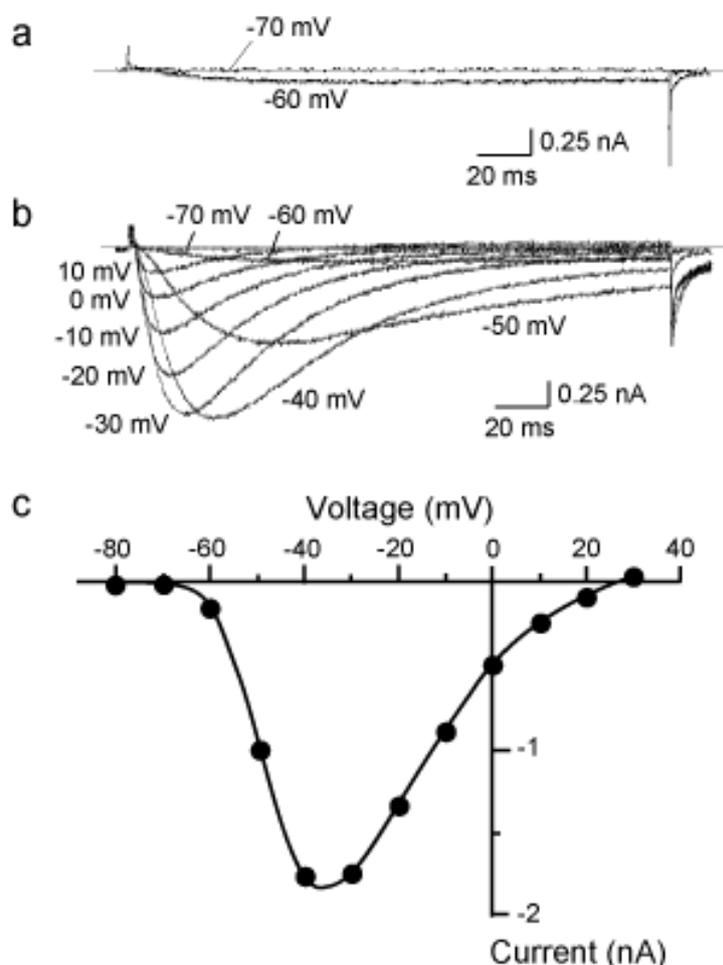
2.2. Modification of the spontaneous augmentation of Na$_V$1.9 by PKA and PKC

As previously described [11,17], the peak amplitude of the Na$_V$1.9 current remarkably increased during conventional whole-cell recording with control pipette solution. As shown in Figure 2, the peak amplitude of the Na$_V$1.9 current gradually increased up to 18.7 ± 5.0-fold of the initial value (n = 10), and then diminished towards the initial value. The total duration of the spontaneous augmentation of

$Na_V1.9$ was 12.4 ± 1.7 min (n = 10). In addition, activation and inactivation kinetics of this current did not change during the spontaneous augmentation of $Na_V1.9$ (Figure 2c).

Figure 2. (a) Time courses of the $Na_V1.9$ currents recorded with normal pipette solution. The $Na_V1.9$ current was evoked by V_T to -10 mV from V_H of -80 mV, and command pulses were applied every 30 s. Then, selected traces were superimposed. (b) Typical example of the spontaneous augmentation of $Na_V1.9$. Peak amplitudes of the $Na_V1.9$ current were plotted as a function of time after onset of the spontaneous augmentation of $Na_V1.9$. Numbers indicate the traces shown in panel (a). (c) Traces of the $Na_V1.9$ currents shown in panel (a) were normalized and then superimposed. (d) Time courses of the $Na_V1.9$ currents in all cases examined.
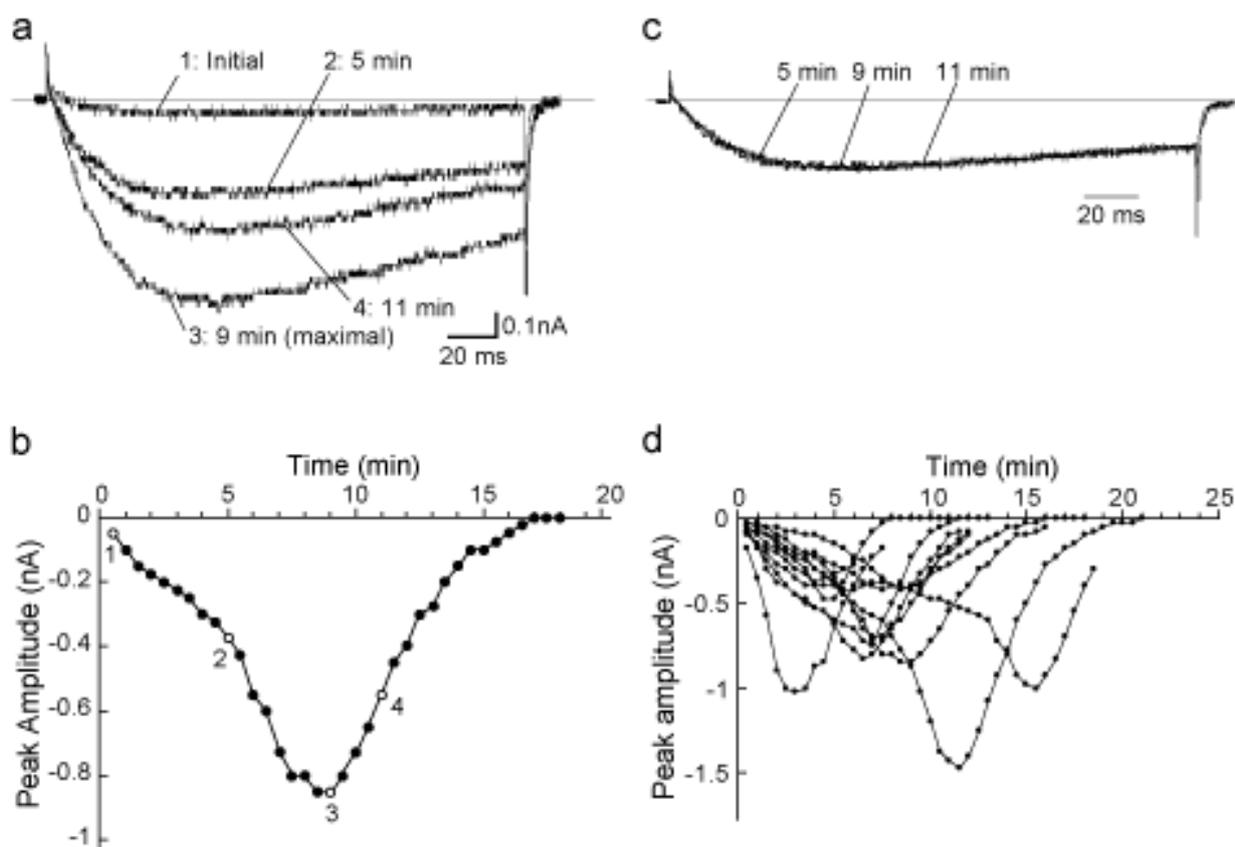

Figure 3 summarizes the intensity changes of the spontaneous augmentation of $Na_V1.9$, which were induced by compounds added to the pipette solution. In the presence of a PKA activator, forskolin (10 μM), the intensity of the spontaneous augmentation of $Na_V1.9$ was significantly attenuated (2.1 ± 0.5-fold: n = 10). Although a PKA inhibitor, H-89 (10 μM). alone had no detectable effect on the intensity of the spontaneous augmentation of $Na_V1.9$ (21.1 ± 7.3-fold: n = 10), the effect of forskolin was significantly inhibited by treatment with H-89 (17.3 ± 3.5-fold: n = 10). A PKC activator, PMA (100 nM), had significant suppressive effect on the intensity of the augmentation of $Na_V1.9$ (2.0 ± 0.3-fold: n = 10). In the presence of a PKC inhibitor, calphostin C (100 nM), the effect of PMA on the augmentation of $Na_V1.9$ was significantly inhibited (14.2 ± 2.8-fold: n = 10). Similarly to H-89, calphostin C alone had no discernable effect on the spontaneous augmentation of $Na_V1.9$ (11.3 ± 4.4-fold: n = 10).

Figure 3. Modification of the spontaneous augmentation of Na$_V$1.9 by addition of the PKA activator forskolin and the PKC activator PMA to the pipette solution. The ordinate shows the ratio of maximal amplitude during recording to the initial amplitude of the Na$_V$1.9 current ($I_{maximal}/I_{initial}$). A ratio > 1 indicates the occurrence of the spontaneous augmentation of Na$_V$1.9, whereas a ratio = 1 indicates no change or reduction of the peak amplitude of the Na$_V$1.9 current. The plots are shown on a logarithmic scale. Circles indicate the ratios obtained for individual trials. Filled circles, control; open circles, treated groups. Horizontal bars indicate the mean values of each group. **P < 0.01 *versus* control, ††*P* < 0.01 *versus* forskolin, and §*P* < 0.05 *versus* PMA.
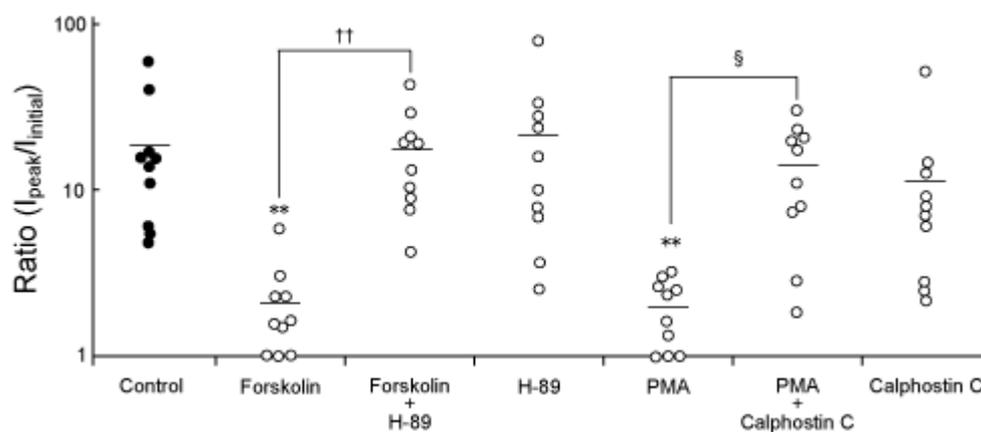
2.3. Possible involvement of PKA and PKC pathways in the voltage dependence of inactivation for the Na$_V$1.9 current

We next examined the effects of forskolin and PMA on the voltage dependence of inactivation for the Na$_V$1.9 current (Figure 4). The voltage dependence of inactivation was determined by measuring peak amplitudes of the Na$_V$1.9 currents evoked by V_T to -10 mV from V_{HS} of -70 mV (I_1) and -120 mV (I_2) intermittently (every 1 minute), and then the ratio of I_1/I_2 obtained from each group was compared. Just after commencing recording, there were no significant differences between each group (data not shown). 10 minutes after commencing recording, the presence of forskolin (10 μM) alone, or the co-presence of forskolin and H-89 (10 μM), did not significantly change the ratios of I_1/I_2 (1.00 ± 0.08: n = 8, and 0.99 ± 0.06: n = 6, respectively) *versus* control (0.99 ± 0.07: n = 8). On the contrary, 100 nM PMA significantly reduced the ratio of I_1/I_2 (0.75 ± 0.07: n = 8) as compared to control. Calphostin C (100 nM) inhibited PMA-induced reduction of I_1/I_2 (0.91 ± 0.03: n = 5).

Figure 4. The change of the voltage-dependence of inactivation for the $Na_V1.9$ current by activation of PKC. The $Na_V1.9$ currents were evoked by the pulse protocol shown in the diagram. We previously confirmed that the duration of V_H for 3 s was sufficient to achieve steady state availability for $Na_V1.9$ [11]. Peak amplitudes of the $Na_V1.9$ currents (I_1 and I_2) were monitored for 10 min after commencing recording, and the resulting I_1/I_2 ratios were compared. *$P < 0.05$ *versus* control.
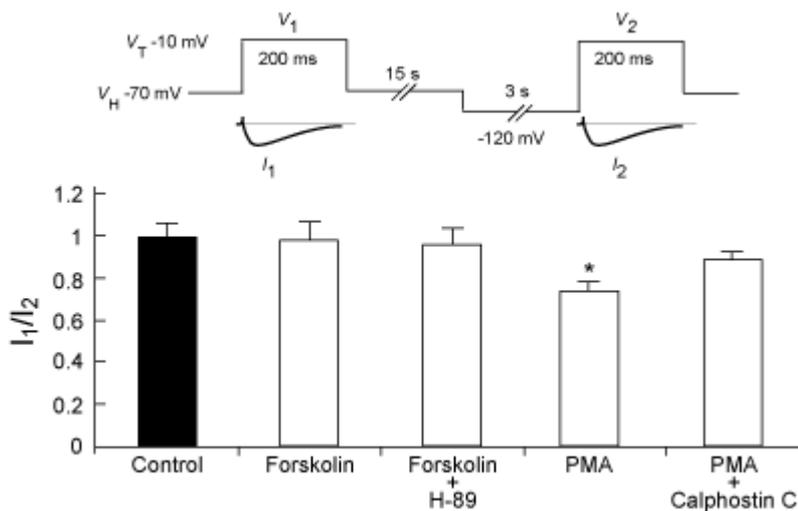

2.4. The effect of PMA on the h_∞ curve for the $Na_V1.9$ current is reproducible under nystatin-perforated patch clamp recording

The spontaneous augmentation of $Na_V1.9$ was prevented by using nystatin-perforated patch clamp recording [11]. Therefore, we further examined the effect of forskolin and PMA on the h_∞ (voltage dependence of steady-state inactivation) curve for the $Na_V1.9$ current in such a situation. The $Na_V1.9$ current was evoked by V_T to 0 mV after various V_{PRES} steps ranging from -120 mV to 10 mV in 10-mV steps for 3 s (see diagram of Figure 5). Figure 5 shows h_∞ curves for the $Na_V1.9$ current before (control) and after application of 100 nM PMA or 10 μM forskolin for 10 min. The h_∞ curves were fitted to the Boltzmann equation:

$$I/I_{max} = 1/\{1+\exp[(V_{PRE}-V_{1/2})/k]\} \tag{1}$$

Where I is the peak amplitude of the $Na_V1.9$ current obtained by V_T, I_{max} is the peak amplitude of the $Na_V1.9$ current evoked by V_C, $V_{1/2}$ is the half-maximum inactivation voltage, and k is the slope factor. We found significant differences ($P < 0.05$) between parameters obtained after PMA application (n = 5, $V_{1/2}$ = -52.1 ± 5.4 mV, k = 5.17 ± 0.44 mV) *versus* control (n = 4, $V_{1/2}$ = -44.7 ± 6.4 mV, k = 3.75 ± 0.15 mV). On the contrary, there was no significant change between values obtained after forskolin application (n = 5, $V_{1/2}$ = -44.6 ± 2.1 mV, k = 4.44 ± 0.51 mV) from the control.

Figure 5. The h_∞ curve for the Na$_V$1.9 current under nystatin-perforated patch clamp recording. The diagram illustrates the pulse protocol. Two identical pulses to 0 mV for 200 ms were applied 15 s prior (V_C) and immediately subsequent (V_T) to the conditioning pre-pulse (V_{PRE}) of -120 to 10 mV for 3 s from V_H of -80 mV. V_C was delivered to minimize the error arising from possible fluctuation of the current (see experimental section). The pulse protocol was applied every 40 s. The peak amplitude of the current (I_T) evoked by V_T was divided by the peak amplitude of the current (I_C) evoked by V_C. The ratio of I_T/I_C was normalized and plotted against V_{PRE}. The h_∞ curve for Na$_V$1.9 current was determined, before (control, filled circle) and 10 min after bath application of PMA (open circle) or forskolin (open square).
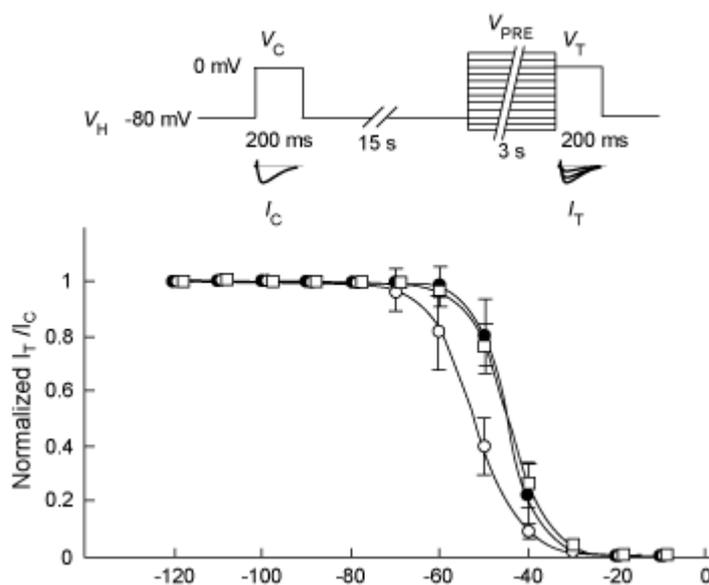
2.5. Discussion

The expression of the Na$_V$1.9 current is confined to the subpopulation of primary afferent neurons with a small cell-body diameter similarly to the Na$_V$1.8 current [4–8,10,11,18]. This observation suggests that Na$_V$1.9 plays an important role in nociception, similar to Na$_V$1.8. On the other hand, the electrophysiological properties of the Na$_V$1.9 current differ notably from those of the Na$_V$1.8 current, *i.e.*, the Na$_V$1.9 current has a more hyperpolarized activation threshold, much slower activation and inactivation kinetics, and a more depolarized h_∞ curve [11,13]. These observations suggest that the role of Na$_V$1.9 in nociception may be distinct from that of Na$_V$1.8.

It was reported that the sustained membrane depolarization (over several hundreds of milliseconds), which is resistant to TTX, is observed in myenteric ganglia [12]. In addition, we previously observed similar sustained membrane depolarization mediated by Na$_V$1.9 in small DRG neurons [13]. The Na$_V$1.9 current may have facilitatory functions in the generation of action potential. On the other hand, h_∞ curves for other Na$^+$ currents are more depolarized than that of Na$_V$1.9 [11,13]. Taking into consideration these properties, sustained activation of Na$_V$1.9 may decrease the availability of other Na$^+$ channels, and then conversely act to inhibit generation of action potential. Thus, the regulation of membrane potential by Na$_V$1.9 may be facilitatory or inhibitory to generating an action potential, in a context-dependent manner.

In addition, we observed a sporadic and remarkable increase of the peak amplitude of the $Na_V1.9$ current followed by decrease of the peak amplitude during whole-cell patch clamp recording ("spontaneous augmentation of $Na_V1.9$") [11]. We previously reported that the spontaneous augmentation of $Na_V1.9$ was inhibited by recording with the nystatin-perforated patch clamp technique, and in the presence of intracellular ATP [11,17]. These results suggest a possible involvement of the intracellular environment in the spontaneous augmentation of $Na_V1.9$.

Analyses of brain Na^+ channels have shown that the cytoplasmic loop between domains DI and DII of Na^+ channels possesses several shared PKA and PKC phosphorylation sites [19–20]. In addition, the inactivation gate has a unique site for PKC phosphorylation [22,23]. In fact, activation of PKA reduces the peak amplitude of brain Na^+ channels without the shift of the steady-state properties [20,24]. On the other hand, activation of PKC has regulatory effects on skeletal muscle Na^+ channels [25], brain Na^+ channels [26] and peripheral nerve Na^+ channel expressed in *Xenopus* oocytes [27]. However, the effects of PKA and PKC on the spontaneous augmentation of $Na_V1.9$ in sensory neurons have not been investigated.

First, we focused on the effect of PKA on the spontaneous augmentation of $Na_V1.9$. The spontaneous augmentation of $Na_V1.9$ was significantly suppressed in the presence of forskolin, and H-89 significantly inhibited this suppressive effect of forskolin (Figure 3). These results indicate that the spontaneous augmentation of $Na_V1.9$ is suppressed by activation of PKA. It is well known that the amplitude of Na^+ current is strongly affected by steady-state inactivation of the channel. However, forskolin did not change the h_∞ curve parameters for the $Na_V1.9$ current in comparison with the control (Figures 4 and 5). This suggests that inhibition of the spontaneous augmentation of $Na_V1.9$ by PKA activation was not due to the hyperpolarizing shift of the h_∞ curve for the $Na_V1.9$ current.

Next, we focused on the effect of PKC on the spontaneous augmentation of $Na_V1.9$. PMA significantly reduced the intensity of augmentation, and the effect of PMA was significantly suppressed by Calphostin C (Figure 3). However, application of PMA induced the hyperpolarizing shift of the voltage dependence of inactivation for the $Na_V1.9$ current. Thus, apparent suppression of the spontaneous augmentation of $Na_V1.9$ by PMA was largely due to hyperpolarizing shift of the h_∞ curve for the $Na_V1.9$ current.

A similar phenomenon has also been reported by Baker *et al.,* who showed that intracellular GTP [28] or activation of PKC [29] induces the up-regulation of the $Na_V1.9$ current. There are distinct dissimilarities between the up-regulation reported by Baker *et al.* and the spontaneous augmentation of $Na_V1.9$ described in the present study and also in our previous reports [11,13,17]. Namely, (1) the up-regulation reported by Baker *et al.* was observed in the presence of intracellular ATP. On the contrary, the spontaneous augmentation of $Na_V1.9$ in our experiments occurred only in the absence of intracellular ATP. (2) the up-regulation reported by *Baker et al.* was mediated by intracellular GTP or activation of PKC. On the other hand, we find that intracellular GTP has no effect on the spontaneous augmentation of $Na_V1.9$ (unpublished data). (3) *Baker et al.* showed only an increment of peak amplitude of the $Na_V1.9$ current, and did not show the entire time course of the phenomenon [28,29]. The spontaneous augmentation of $Na_V1.9$ was composed of an increase and a subsequent decrease of peak amplitude of the $Na_V1.9$ current. From these observations, the up-regulation reported by *Baker et al.* may be distinct from the spontaneous augmentation of $Na_V1.9$ in our studies.

A recent behavioral study showed that the PKA inhibitor, H-89, suppresses bee venom-induced mechanical hyperalgesia in rats [30], and the activation of PKA is conductive to the inflammatory mechanical hyperalgesia [31,32]. In addition, activity of specific PKC isozymes is increased in inflammatory-pain models in rats [33–36]. From these observations, the $Na_V1.9$ channel may be regulated not to increase the amplitude of the $Na_V1.9$ current, *i.e.*, the spontaneous augmentation of $Na_V1.9$ under pathological condition by PKA and/or PKC activation.

Recently, it has been reported that $Na_V1.9$ underlies nociceptive behavior after peripheral inflammation in $Na_V1.9$ KO mice [37]. In addition, histochemical study on $Na_V1.9$ channel proteins in axons of normal and complete Freund's adjuvant-inflamed rats showed significant decrease of the proportion of $Na_V1.9$-labeled unmyelinated axons, and no change in the proportion of labeled myelinated axons following inflammation [18]. Taken together, the $Na_V1.9$ current contributes to pain signaling, and may alter under pathophysiological conditions.

3. Experimental Section

3.1. Isolation of single DRG neurons and cell culture

The protocols were approved by Hiroshima University Animal Ethics Committee. Dissociation of single DRG neurons and their culture was described previously. [11,13]. Briefly, adult mice were sacrificed by cervical dislocation under ethylcarbamate anesthesia (3 mg/g; Wako Pure Chemicals, Osaka, Japan). DRGs from all segments of the spinal cord were dissected from $Na_v1.8$ KO mice [5] and desheathed in ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS(-)). The isolated DRGs were incubated sequentially in PBS(-) containing 0.2% collagenase (Wako Pure Chemicals) and 0.1% trypsin (Sigma, St. Louis, Mo., USA), each for 20 min at 37 °C. DRGs were then dissociated by trituration with fire-polished Pasteur pipettes in culture medium composed of Dulbecco's modified Eagle medium 10% (vol./vol.) heat-inactivated fetal calf serum.

The dissociated cells were plated on 35 mm plastic tissue-culture dishes pre-coated with 0.01% poly-L-lysine (Sigma) and maintained in culture medium supplemented with penicillin (100 IU/mL) and streptomycin (100 μg/mL). All cultured cells were maintained at 37 °C in 5% CO_2/95% air. Cells were used for experiments after short-term culture (4–12 h after plating). At this time in culture, neurite outgrowth was not observed. We defined DRG neurons that were smaller than 25 μm in diameter as small neurons [13,28], and small neurons thus defined were used throughout the study.

3.2. Electrophysiology

Voltage clamp recordings were performed using an Axopatch 200A amplifier (Axon Instruments, Union City, CA, USA). The Na^+ currents were recorded by using either the conventional whole-cell patch clamp technique [38] or the nystatin-perforated patch clamp technique [39] at room temperature (22–24 °C). Data were low-pass-filtered at 5 kHz with a four-pole Bessel filter and sampled digitally at 25–100 kHz. In some experiments, capacitive and leakage currents were subtracted digitally using the P-P/4 procedure [4].

The pipette (internal) solution contained 10 mM NaCl, 110 mM CsCl, 20 mM tetra-ethylammonium (TEA)-Cl, 2.5 mM $MgCl_2$, 5 mM 4-2-hydroxylethyl-1-piperazine-ethanesulfonic acid (HEPES) and

5 mM ethylene glycol tetraacetic acid (EGTA). The pH of the pipette solution was adjusted to 7.0 with CsOH. Osmolarity was adjusted to 290 mosmol/kg with glucose. A part of the experiments were performed with the pipette solution containing phorbol 12-myristate 13-acetate (PMA), calphostin C, forskolin and H-89 (Sigma). For the pipette solution used in nystatin-perforated patch recordings, a stock solution containing 10mg/mL nystatin (Wako) was prepared and added to the pipette solution to final concentration of 500 µg/mL. Patch clamp pipettes were made from borosilicate capillary by using a PP-83 puller (Narishige, Tokyo, Japan) and heat-polished with MF-83 microforge (Narishige). The DC resistance of patch electrodes was 1–1.5 MΩ for the conventional whole-cell patch and 3–5 MΩ for the nystatin-perforated patch, respectively.

The external solution contained 100 mM NaCl, 30 mM TEA-Cl, 5 mM CsCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM $CdCl_2$, 5 mM HEPES, 25 mM glucose, 5 mM 4-aminopyrimidine (4-AP). The pH of the external solution was adjusted to 7.4 with HCl. TEA-Cl and 4-AP were added to abolish K^+ currents and $CdCl_2$ was added to abolish Ca^{2+} currents [4]. In addition, TTX (Sanko, Tokyo, Japan) was added to eliminate TTX-S Na^+ currents. Osmolarity was adjusted to 290 mosmol/kg with glucose. A part of the experiments were performed with the external solution containing PMA and forskolin (Sigma). Liquid junction potentials between pipette and external solutions were compensated by adjusting the zero current potential to the liquid junction potential. Only cells showing an adequate voltage and space clamp [11] were used.

When measuring ion channel kinetics, it generally take a considerable time to execute the protocol, since a sufficient recovery period for the channel must be allowed between each test pulse (V_T). For various reasons, the amplitudes of Na^+ currents are not always constant during recording, e.g., due to run-down of the current or to instability of the seal condition. This can be checked by applying a control pulse (V_C) before each V_T. To avoid a possible time-dependent fluctuation of the analysis, V_C to a fixed voltage as applied 15 s before V_T or conditioning pre-pulse (V_{PRE}). The amplitude of the current (I_C) evoked by V_C served as a calibrator. The results are given as mean ± standard error of the mean (S.E.M.). Statistical significance of differences was determined using Wilcoxon *t*-test and Mann-Whitney *U*-test. Differences were considered significant if $P < 0.05$.

4. Conclusions

The spontaneous augmentation of the $Na_V1.9$ current was significantly suppressed by activation of PKA, whereas activation of PKA did not affect the voltage dependence of inactivation for the $Na_V1.9$ current. On the contrary, the finding that activation of PKC can affect the voltage dependence of inactivation for $Na_V1.9$ in the perforated patch recordings, where the augmentation does not occur, suggests that the effects of PMA are independent of the augmentation process. These results indicate that the spontaneous augmentation of $Na_V1.9$ was regulated directly by PKA, and indirectly by PKC.

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