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Inhibition by TRPA1 agonists of compound action potentials in the frog sciatic nerve

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ABSTRACT

Although TRPV1 and TRPM8 agonists (vanilloid capsaicin and menthol, respectively) at high concentrations inhibit action potential conduction, it remains to be unknown whether TRPA1 agonists have a similar action. The present study examined the actions of TRPA1 agonists, cinnamaldehyde (CA) and allyl isothiocyanate (AITC), which differ in chemical structure from each other, on compound action potentials (CAPs) recorded from the frog sciatic nerve by using the air-gap method. CA and AITC concentration-dependently reduced the peak amplitude of the CAP with the IC_{50} values of 1.2 and 1.5 mM, respectively; these activities were resistant to a non-selective TRP antagonist ruthenium red or a selective TRPA1 antagonist HC-030031. The CA and AITC actions were distinct in property; the latter but not former action was delayed in onset and partially reversible, and CA but not AITC increased thresholds to elicit CAPs. A CAP inhibition was seen by hydroxy-α-sanshool (by 60% at 0.05 mM), which activates both TRPA1 and TRPV1 channels, a non-vanilloid TRPV1 agonist piperine (by 20% at 0.07 mM) and tetrahydrolavandulol (where the six-membered ring of menthol is opened; IC_{50} = 0.38 mM). It is suggested that TRPA1 agonists as well as TRPV1 and TRPM8 agonists have an ability to inhibit nerve conduction without TRP activation, although their agonists are quite different in chemical structure from each other.

1. Introduction

Plant-derived chemicals are known to activate transient receptor potential (TRP) channels such as TRP vanilloid-1 (TRPV1; [1]), TRP melastatin-8 (TRPM8; [11]) and TRP ankyrin-1 (TRPA1; [3]) channels existing in the peripheral and central terminals of primary-afferent neurons [6]. Their activations in the peripheral terminal generate a membrane depolarization, resulting in the production of action potentials (APs), while those in the central terminal lead to a barrage of the spontaneous release of L-glutamate onto superficial spinal dorsal horn or medullary neurons from there [9,17,19]. These TRP activations play a role in transmitting sensory information. On the other hand, a TRPV1 agonist capsaicin and a TRPM8 agonist menthol (which are contained in capsicum and peppermint, respectively) at high concentrations inhibit voltage-gated Na^+ channels without TRP activation and thus have an ability to inhibit AP conduction in nerve fibers [2,16]. To our knowledge, it has not yet been examined whether TRPA1 agonists have a similar action.

We have very recently reported that capsaicin and its related vanilloids [14], and also menthol and its related chemicals [5] reduce the peak amplitude of compound AP (CAP), which is fast-conducting and sensitive to a voltage-gated Na^+-channel blocker tetrodotoxin, without TRP activation in the frog sciatic nerve. In these studies, it was revealed that a difference in chemical structure among them results in a distinction in the extent of the CAP inhibition. A similar CAP inhibition in a manner dependent on the structures of the chemicals tested has been shown for opioids [4,12] and adrenoceptor agonists [8]. In order to know whether TRPA1 agonists have an ability to inhibit frog CAPs and if so this inhibition is different in extent among TRPA1 agonists having a distinct chemical structure, we examined the actions of TRPA1 agonists, cinnamaldehyde (CA, contained in cinnamon) and allyl isothiocyanate (AITC; in wasabi [15]; Fig. 1Aa, Ba), on CAPs recorded from the frog sciatic nerve by using the air-gap method. With the aim to know more about chemical structures of TRP agonists having an ability to inhibit nerve conduction, we further investigated how frog CAPs are affected by hydroxy-α-sanshool (in xanthoxylum) which activates both TRPV1 and TRPA1 channels [7], a TRPV1 agonist piperine (in black pepper) which does not have the vanillyl group [10,18], and tetrahydrolavandulol where the six-membered ring of menthol is opened.

2. Materials and methods

This study was approved by the Animal Care and Use Committee of Saga University. The method used for obtaining frog sciatic nerve preparation has been described previously [4,5,8,12,14]. In brief, either sex of frogs (*Rana nigromaculata*) was decapitated and then pithed; thereafter the sciatic nerve was dissected from

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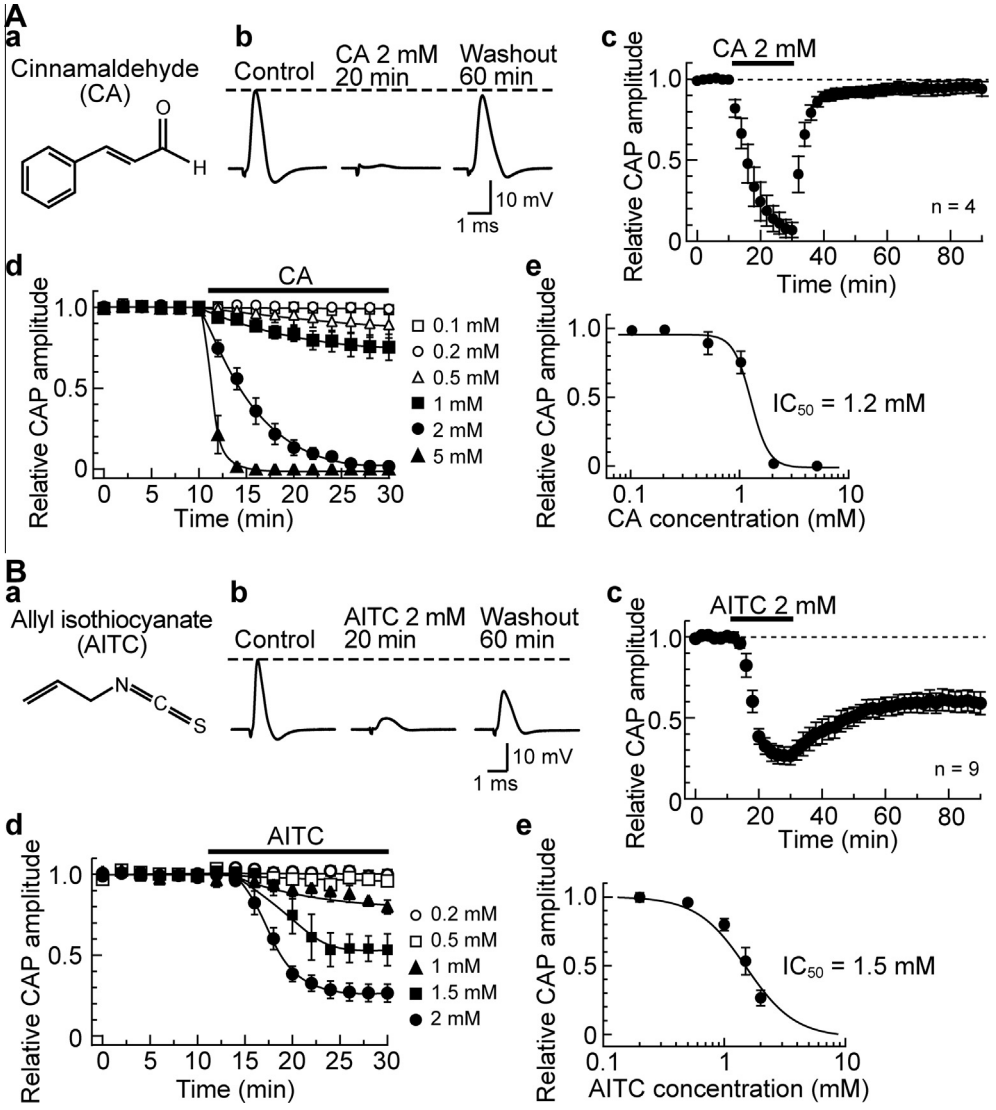

Fig. 1. TRPA1 agonists, cinnamaldehyde (CA) and allyl isothiocyanate (AITC), concentration-dependently inhibit compound action potentials (CAPs) recorded from frog sciatic nerves. (Aa, Ba) The chemical structures of CA and AITC. (Ab, Bb) Recordings of CAPs before (Control), at 20 min after exposure to CA or AITC and thereafter 60 min in the absence of CA or AITC (Washout). (Ac, Bc) Average time courses of changes in CAP peak amplitudes following exposure to CA or AITC for 20 min, relative to those before the soaking. In this and subsequent figures, *n* denotes the number of sciatic nerve examined. (Ad, Bd) Comparison in average time course among CAP peak amplitude reductions produced by CA at 0.1–5 mM (Ad; data at each concentration were obtained from 3 to 5 sciatic nerves) or by AITC at 0.2–2 mM (Bd; from 3 to 7 sciatic nerves). Here, solid lines were arbitrarily drawn. (Ae, Be) The peak amplitudes of CAPs recorded from sciatic nerve fibers treated with CA or AITC at various concentrations for 20 min, relative to control, which were plotted against its concentration. These concentration–response curves were drawn according to the Hill equation (Ae: half-maximal inhibitory concentration, IC_{50} = 1.2 mM; Hill coefficient, n_H = 5.3; Be: IC_{50} = 1.5 mM; n_H = 3.5). In this and subsequent figures, each point with vertical bars represents the mean and S.E.M.; the S.E.M. of the values without a vertical bar was within the size of symbol, and dotted line denotes the control value.

the lumbar plexus to the knee in Ringer solution. The isolated sciatic nerve was carefully desheathed under a binocular microscope and then loosely placed in five platinum wires that were glued to a Lucite plate, where the two ends of the nerve were tied to the wires by using threads. The plate was put on a beaker having Ringer solution in which the sciatic nerve was soaked. The composition of Ringer solution used was (mM): NaCl, 115.5; KCl, 2.0; $CaCl_2$, 1.8; Na_2HPO_4, 1.3; and NaH_2PO_4, 0.7 (pH = 7.0).

As performed previously [4,5,8,12,14], the Lucite plate having platinum wires attached with the sciatic nerve was moved from the beaker containing Ringer solution to a vacant one and then CAPs were recorded in air using a preamplifier. Here, two of the platinum wires were used to record CAPs, and other two were for stimulating the sciatic nerve at 1 Hz. In order not to dry the sciatic nerve in air, this procedure was quickly performed at a time interval of 2 min. When the effects of drugs on CAPs were examined, the nerve was put back into the soaking solution with drugs in between 2 measures. The data were monitored on a storage oscilloscope while being recorded on a thermal array recorder having a wave form storage module and stored on USB flash memory with a Data logger for later analyses. Stimulating the sciatic nerve produced a CAP following a stimulus artifact. The peak amplitude of the CAP was measured as a difference between baseline and CAP peak level, as done previously [4,5,8,12,14]. The peak amplitude of the CAP depended on the strength of stimulus given to the sciatic nerve in such that the CAP peak amplitude enhanced with an increase in stimulus strength and attained a maximal value. As done previously [4,5,8,12,14], we analyzed the peak amplitude of the maximal CAP unless otherwise mentioned. A conduction velocity value was determined by using the fifth electrode as an additional stimulation site. All experiments were carried out at room temperature (22–27 °C).

Concentration-dependence curve for the reduction of the peak amplitude of CAP in the sciatic nerve soaked with a drug was analyzed using the following Hill equation:

$$\text{CAP amplitude } (\%\text{ of control}) = 100/(1 + ([\text{Drug}]/\text{IC}_{50})^{n_H},$$

where [Drug] is drug concentration, IC_{50} is the concentration of drug for half-maximal inhibition and n_H is the Hill coefficient.

Data were indicated as mean ± S.E.M. and statistical significance was set at $P < 0.05$ using a paired or unpaired Student's *t*-test. In all cases *n* refers to the number of sciatic nerves studied. The peak amplitude of CAP before drug application was denoted as control.

Drugs used were CA, ruthenium red, HC-030031, piperine (Sigma–Aldrich, St. Louis, MO, USA), AITC (Wako Junyaku, Osaka, Japan), tetrahydrolavandulol (Tokyo Kasei, Tokyo, Japan) and hydroxy-α-sanshool (AdipoGen, San Diego, CA, USA). All of drugs except for ruthenium red (which was dissolved in distilled water) were first dissolved in dimethyl sulfoxide (DMSO) and then diluted to the final concentration in Ringer solution, where the concentration of DMSO was less than 1%. DMSO at 1% did not affect CAPs. The pH of Ringer solution containing drugs was adjusted to 7.0 with NaOH. Hydroxy-α-sanshool was used in a chamber filled with nitrogen and protected from light.

3. Results

Effects of drugs on fast-conducting CAPs were examined in a total of 134 sciatic nerves, and the CAPs had the averaged conduction velocity value of 27.7 ± 1.2 m/s ($n = 134$), a value comparable to those reported previously [4,5,8,12,14].

3.1. Effects of TRPA1 agonists (cinnamaldehyde and allyl isothiocyanate) on frog sciatic nerve CAPs

Soaking the sciatic nerve into CA (2 mM)-containing Ringer solution reduced the peak amplitude of the CAP, as seen from Fig. 1Ab. Fig. 1Ac demonstrates an average of the time courses of a change in CAP peak amplitude following soaking into CA solution. The CA-induced reduction in CAP peak amplitude attained a maximal effect at 20 min of the soaking, where the peak amplitude of

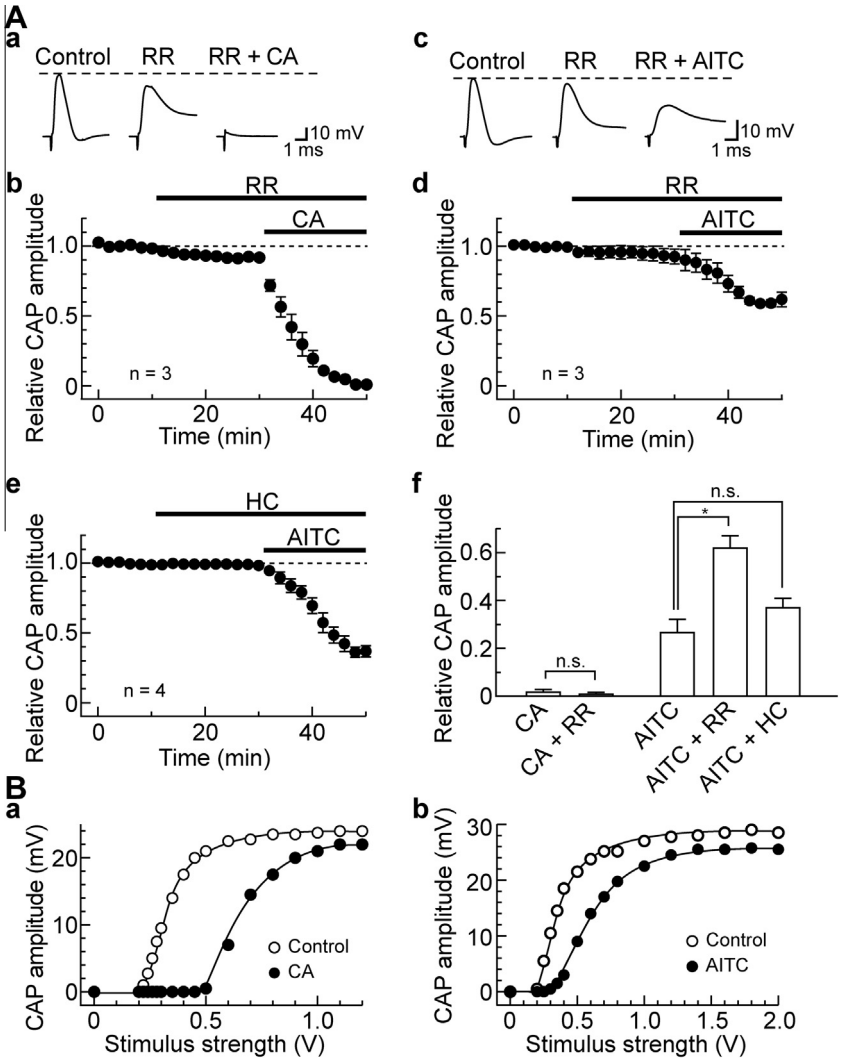

Fig. 2. CA and AITC inhibit CAPs without TRPA1 activation while having a different action on thresholds to elicit CAPs. (Aa, c) Recordings of CAPs in the control, and in the absence and presence of CA or AITC (each 2 mM) in Ringer solution containing ruthenium red (RR; 0.3 mM). (Ab, d) Average time course of changes in CAP peak amplitudes following treatment with ruthenium red (0.3 mM) alone and ruthenium red (0.3 mM) together with CA or AITC (each 2 mM), relative to that before drug treatment. (Ae) Average time course of changes in CAP peak amplitudes following treatment with HC-030031 (HC; 0.05 mM) and then both HC-030031 (0.05 mM) and AITC (2 mM), relative to that before drug treatment. (Af) CAP peak amplitudes under the action of CA or AITC (each 2 mM) in the absence and presence of ruthenium red (0.3 mM) or HC-030031 (0.05 mM), relative to that just before treatment with CA or AITC. Each column with vertical bars represents the mean and S.E.M.; $^*P < 0.05$; n.s.: not significant. (Ba, Bb) The peak amplitudes of CAPs in the absence (Control) and presence of CA (1 mM) or AITC (1.5 mM), which are plotted against stimulus strength used to elicit the CAPs. Here, solid lines were arbitrarily drawn.

the CAP was 6.8 ± 4.6% ($P < 0.05$) of control (36.7 ± 6.3 mV; $n = 4$). In nerves treated with CA and then returned to drug-free Ringer solution (washout) for up to 1 h, the CAP amplitude recovered to control level (Fig. 1Ab, c). Fig. 1Ad shows the time courses of changes in CAP peak amplitude with an increase in time after soaking the sciatic nerve into solution containing CA at various concentrations ranging from 0.1 to 5 mM. The rate of the CAP peak amplitude reduction produced by CA was enhanced in extent with an increase in its concentration. CAP amplitude reduction at 20 min of the soaking increased in magnitude with an increase in CA concentration. The concentration–response curve for the CA-induced CAP amplitude reduction obtained from many nerve trunks is given in Fig. 1Ae. Analysis based on the Hill equation showed that IC_{50} value for CA is 1.2 mM.

We next examined how another TRPA1 agonist AITC, which is quite different in chemical structure from CA, affects sciatic nerve CAPs. AITC (2 mM) inhibited CAPs in a manner similar to that of CA (Fig. 1Bb). The AITC-induced CAP peak amplitude reduction attained a maximal effect at 20 min of the soaking, where this amplitude was 26.6 ± 5.6% ($P < 0.05$) of control (24.1 ± 2.5 mV; $n = 9$). This reduction was partially reversible with a recovery to about 50% of control level (Fig. 1Bb, c). The AITC-induced CAP inhibition was concentration-dependent in the rate and extent in a range of 0.2–2 mM with the IC_{50} value of 1.5 mM (Fig. 1Bd, e). As noted from a comparison of Fig. 1Bd with Fig. 1Ad in time course, the inhibitory action of AITC was delayed in onset compared with that of CA.

As frogs, as well as mammals, express TRPA1 channels [13], we next examined whether the CA- or AITC (each 2 mM)-induced CAP inhibition is affected by a non-selective TRP antagonist ruthenium red (0.3 mM). Pretreatment with ruthenium red for 20 min reduced the peak amplitude of CAP by about 10% with a large prolongation of the duration of the CAP (Fig. 2Aa, c), as reported previously [5]. Adding CA or AITC to ruthenium red inhibited the CAP (Fig. 2Aa, c); these results obtained from several nerves were summarized in Fig. 2Ab, d. The peak amplitude after 20 min treatment with both ruthenium red and CA, relative to that just before the co-treatment, was not different from that obtained with CA only (Fig. 2Af). On the contrary, the CAP inhibition produced by AITC in the presence of ruthenium red was significantly smaller in extent than that in the absence of ruthenium red (Fig. 2Af). It is likely that ruthenium red affects voltage-gated channels involved in the production of AP as judged from CAP prolongation produced by this drug and thus that the AITC action is affected by ruthenium red at sites other than TRPA1 channels. We therefore examined how the AITC activity is affected by a specific TRPA1 antagonist HC-030031 (0.05 mM; Fig. 2Ae). Unlike ruthenium red, HC-030031 itself unaffected CAPs, as noted from Fig. 2Ae. In the presence of HC-030031, AITC (2 mM) largely reduced CAP peak amplitude; this extent was not significantly different from that in the absence of HC-030031 (Fig. 2Ae, f).

Since a membrane depolarization as a result of TRPA1 activation was expected to change a threshold to elicit CAPs, we next examined the effect of CA (1 mM) or AITC (1.5 mM) on CAPs elicited at various stimulus strengths given to the sciatic nerve. As shown in Fig. 2Ba, b, the CA and AITC activities were seen for CAPs evoked at a maximal stimulus strength, while the threshold to elicit CAPs was not affected by AITC but increased by CA. Such AITC and CA effects were obtained from 4 and 4 other sciatic nerves, respectively; CA increased the threshold by a factor of 2.0 ± 0.1 ($n = 5$).

3.2. Effects of TRP agonists (hydroxy-α-sanshool and piperine) on frog sciatic nerve CAPs

Since frog CAPs were inhibited by not only TRPV1 but also TRPA1 agonists, we next examined whether hydroxy-α-sanshool (Fig. 3Aa), which activates both TRPA1 and TRPV1 channels [7], has a similar action. As seen from Fig. 3Ab, hydroxy-α-sanshool at 0.05 mM, a maximally-soluble concentration, inhibited frog CAPs in a partially-reversible manner. Fig. 3Ac shows an average of the time courses of changes in CAP peak amplitude with an increase in time after soaking the sciatic nerve into hydroxy-α-sanshool solution. Hydroxy-α-sanshool at a lower concentration such as 0.02 mM did not affect CAPs [peak amplitude: 98.7 ± 1.5% ($n = 3$; $P > 0.05$) of control (27.8 ± 4.0 mV)].

Many vanilloids including capsaicin and zingerone (contained in ginger), which activate TRPV1 channels, inhibit sciatic nerve CAPs [14]. In order to know whether the vanillyl group is necessary for CAP inhibition, we examined how CAPs are affected by piperine which is not vanilloids (see Fig. 3Ba) while activating TRPV1 channels [10,18]. As seen from Fig. 3Bb, piperine at 0.07 mM, a maximally-soluble concentration, inhibited frog CAPs in an almost

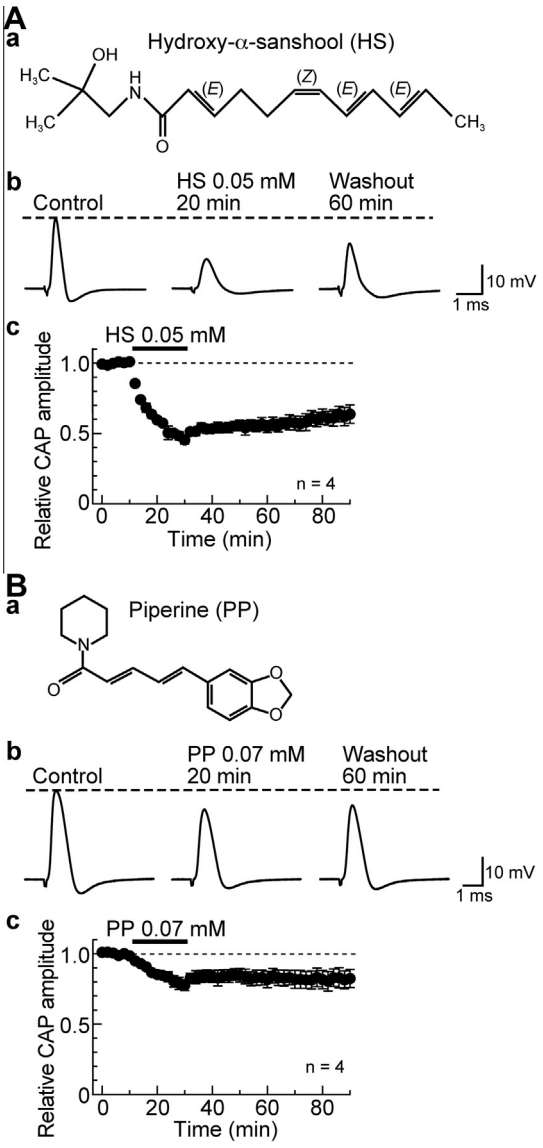

Fig. 3. Hydroxy-α-sanshool (HS; 0.05 mM) and piperine (PP; 0.07 mM) irreversibly inhibit CAPs recorded from frog sciatic nerve fibers. (Aa, Ba) The chemical structures of hydroxy-α-sanshool and piperine. (Ab, Bb) Recordings of CAPs before, at 20 min after exposure to hydroxy-α-sanshool or piperine and thereafter 60 min in the absence of hydroxy-α-sanshool or piperine. (Ac, Bc) Average time courses of changes in CAP peak amplitudes following exposure to hydroxy-α-sanshool or piperine for 20 min, relative to those before the soaking.

irreversible manner. Fig. 3Bc shows an averaged time course of the inhibitory action of piperine. This piperine action was concentration-dependent; CAP amplitudes at 20 min after soaking into solution containing piperine at 0.01 and 0.05 mM were, respectively, 99.6 ± 2.7% ($n = 3$; $P > 0.05$) and 77.3 ± 4.5% ($n = 3$; $P < 0.05$) of control.

3.3. Effect of tetrahydrolavandulol on frog sciatic nerve CAPs

TRPA1 agonists (CA and AITC), which inhibit frog CAPs, have quite different chemical structures in such that CA but not AITC has the benzene ring. Many of menthol-related chemicals inhibit sciatic nerve CAPs in a manner dependent on their chemical structures [5]. In order to know whether the six-membered ring of menthol (Fig. 4A, right) is necessary for the CAP inhibition, we examined the effect on CAPs of tetrahydrolavandulol (Fig. 4A, left) where its ring is opened. Tetrahydrolavandulol at a concentration of 1 mM reduced CAP peak amplitude in a partially-reversible manner, as seen in Fig. 4B. Fig. 4C demonstrates an average of the time courses of a change in CAP peak amplitude following soaking into tetrahydrolavandulol (1 mM) solution, relative to control, which are obtained from four sciatic nerves. Tetrahydrolavandulol exhibited a maximal effect of CAP amplitude reduction within 20 min after the soaking, where the peak amplitude reduced to 0.03 ± 0.02% ($P < 0.05$) of control (16.1 ± 2.7 mV; $n = 4$). Fig. 4D and E demonstrates the effects of tetrahydrolavandulol in a concentration range of 0.02–1 mM on CAPs. The extent and rate of the CAP peak amplitude reduction produced by tetrahydrolavandulol was enhanced with an increase in its concentration (IC_{50} = 0.38 mM).

4. Discussion

The present study demonstrated that CA and AITC, both of which are TRPA1 agonists, reduce CAP peak amplitude with the IC_{50} values of 1.2 and 1.5 mM, respectively. The CA activity was resistant to a non-selective TRP antagonist ruthenium red, indicating no involvement of TRPA1 channels. On the other hand, the AITC activity was reduced in extent in the presence of ruthenium red. Although this result indicates that AITC may share a site of action, located in TRPA1 channels, with ruthenium red, the AITC action is not affected by a specific TRPA1 antagonist HC-030031. The activity of AITC may be inhibited by ruthenium red in sites other than TRPA1 channels, e.g., voltage-gated ion channels involved in the production of AP. This idea is supported by the observation that ruthenium red prolonged the duration of CAP (see Fig. 2Aa, c), suggesting an action on voltage-gated ion channels. Thus, the inhibitory actions of CA and AITC on CAPs were not due to TRPA1 activation. However, as expected from the fact that CA and AITC have a quite different chemical structure (see Fig. 1Aa, Ba), they appear to inhibit CAPs in a manner different from each other. The AITC but not CA action was delayed in onset and partially reversible, and CA but not AITC increased thresholds to elicit CAPs. The CA-induced increase in threshold indicates that CA may change membrane potentials in sciatic nerve axons. This issue remains to be examined.

Although TRPA1 agonists as well as TRPV1 agonists inhibited CAPs, hydroxy-α-sanshool, which activated both TRPV1 and TRPA1 channels, had an ability to inhibit CAPs. This action was seen at a concentration lower than those of CA and AITC for CAP inhibition. There was not a similarity in chemical structure among TRPV1, TRPM8 and TRPA1 agonists exhibiting CAP inhibition. Hydroxy-α-sanshool, CA and AITC were not similar in structure from each other. Although TRPV1 agonists such as capsaicin and zingerone having the vanillyl group have an ability to inhibit CAPs [14], this group appears to be not necessary for CAP inhibition. A TRPV1 agonist piperine, which inhibits CAPs, does not have the vanillyl group, as seen from Fig. 3Ba. A TRPM8 agonist menthol and its related many chemicals having a ring structure inhibit CAPs [5], but the six-membered ring of menthol is not needed for CAP inhibition,

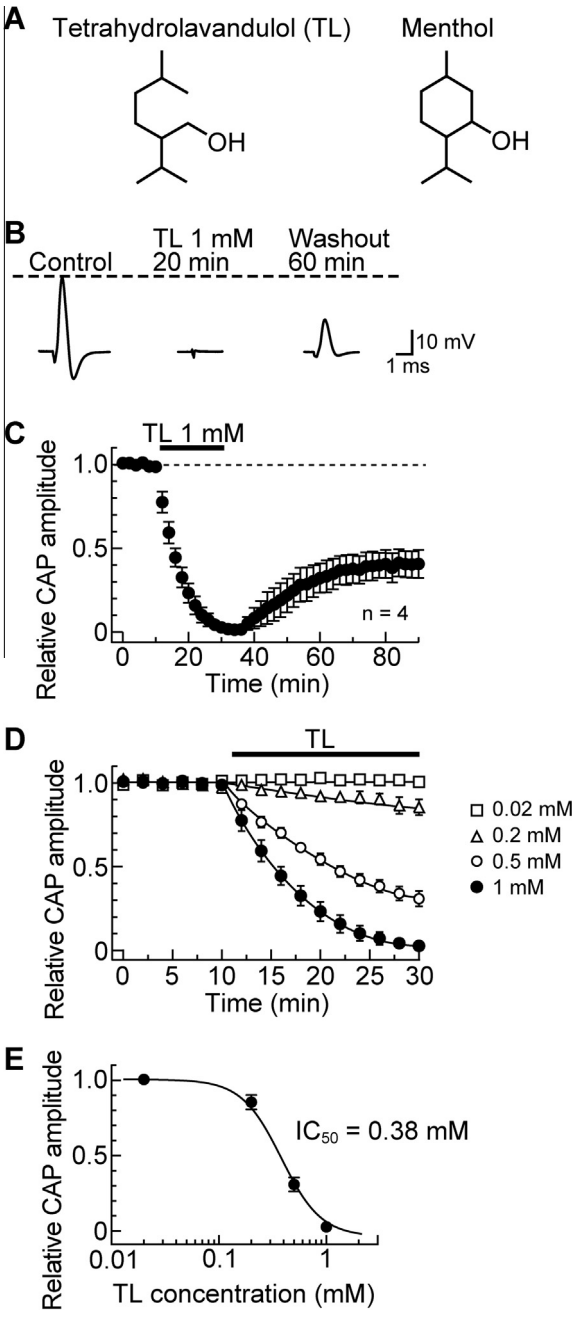

Fig. 4. Effect of tetrahydrolavandulol (TL) on CAPs recorded from frog sciatic nerve fibers. (A) The chemical structures of tetrahydrolavandulol and menthol. (B) Recordings of CAPs in the control, at 20 min after exposure to tetrahydrolavandulol (1 mM) and thereafter 60 min in the absence of tetrahydrolavandulol. (C) Average time course of changes in CAP peak amplitudes following exposure to tetrahydrolavandulol (1 mM) for 20 min, relative to those before the soaking. (D) Average time courses of CAP peak amplitude reductions produced by tetrahydrolavandulol at 0.02–1 mM; data at each concentration were obtained from 3 to 7 sciatic nerves. Solid lines in this graph were arbitrarily drawn. (E) The peak amplitudes of CAPs recorded from sciatic nerve fibers treated with tetrahydrolavandulol at various concentrations for 20 min, relative to control, which were plotted against tetrahydrolavandulol concentration. Each of the data points was obtained from 3 to 7 sciatic nerves. This concentration–response curve was drawn according to the Hill equation (IC_{50} = 0.38 mM; n_H = 2.3).

as noted from the inhibitory action of tetrahydrolavandulol on CAPs (see Fig. 4). Tetrahydrolavandulol was more effective than menthol in inhibiting CAPs; their IC_{50} values were 0.38 mM and 0.93–1.1 mM [5], respectively.

The IC_{50} values of TRPA1 agonists (CA: 1.2 mM; AITC: 1.5 mM) obtained in the present study were almost comparable to those of lidocaine and cocaine (0.74 and 0.80 mM, respectively; [4,12]), while being smaller than that of procaine (2.2 mM; [14]) and larger than those of ropivacaine and tetracaine (0.34 and 0.013 mM, respectively; [4,8]) in frog sciatic nerves. TRPA1 agonists such as CA and AITC examined here are suggested to have almost the same anesthetic effect as those of lidocaine and cocaine.

In conclusion, we demonstrated for the first time that CA and AITC inhibit CAPs in the sciatic nerve with efficacies comparable to those of several local anesthetics. Plant-derived TRPA1 agonists are suggested to have an ability to inhibit nerve conduction.

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