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NPPB structure-specifically activates TRPA1 channels

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ABSTRACT

TRPA1 channels have been found to play an important role in mammalian pain sensation, especially when the pain is caused by chemicals on site of inflammation. A large number of structurally diverse chemicals are found to activate TRPA1 channels, implicating a potential chemosensor in neuronal nociception. Identification of the channel activation by cysteine modification through covalent chemical reaction provides arguments for the diversity of the agonist structures. However, it is largely unknown how nonreactive compounds activate TRPA1 channels. Here, we report that NPPB, a classic Cl^- channel blocker, potently activated human TRPA1 channels overexpressed in mammalian HEK-293 cells. This effect was confirmed in Ca^{2+} imaging assay, patch clamp whole cell and single channel recordings. The NPPB response was quick, fully reversible and replicable, contrary to the effect of covalent modification by AITC. The mutagenesis studies revealed a refreshed look at several mutations known to be critical for the actions of AITC and menthol. The blocking profile of NPPB on these mutants showed that the NPPB activation was similar to that of FTS and different from AITC and menthol. The results indicated a possible close interaction between S5 and N-terminal domains of the channel. We also tested a group of NPPB analogs on TRPA1 channel activities. The results demonstrated that NPPB activation was tightly associated with chemical structure. None of the single chemical group was sufficient to activate the channel, indicating that NPPB activated TRPA1 through a structure-specific mechanism.

1. Introduction

Transient receptor potential (TRP) channel family consists of 27 gene members in human that can be divided into 6 subfamilies by structure homologies [1–3]. TRP channels are nonselective cation channels that directly respond to various internal or external chemical and/or physical stimuli with no or little voltage dependence. These channels play key roles in signal reception and transduction for biological integrations at cell, organ and system levels. Structurally, TRP channels possess six-transmembrane domain topology, similar to voltage gated potassium channels and cyclic nucleotide gated channels despite little or no sequence homology with these channels. TRP channels are activated by highly diverse signals, including temperature, pH, mechanical stretch, naturally occurring chemical products, signal transduction components and cellular metabolites such as lipid metabolites and reactive oxygen species (ROS) [4,5]. Although extensive studies have been directed to elucidate activation mechanism of TRP channels, little is understood about the molecular basis for TRP channel activation.

One of the most interesting TRP channels is TRPA1, an ankyrin repeat channel that is expressed predominantly in sensory neurons. TRPA1 coexists with other sensory channels such as TRPV1 and TRPM8 in nerve terminals that are known to be responsible for temperature sensing and nociception [6]. A number of pungent chemicals and noxious compounds have been found to activate TRPA1 channels, suggesting that this channel may serve as chemical nocisensor especially for inflammatory nociception. It has been demonstrated that the reactive pungent natural products, such as allyl isothiocyanate (AITC) from mustard, cinnamaldehyde from cinnamon [7,8], allicin from garlic [9,10] and some endogenous α,β-unsaturated aldehydes such as 4-hydroxy-2-nonenal (HNE) [11], as well as the exogenous aldehyde, formalin [12], activate TRPA1 channels through reversibly covalent modification of cysteine residues [13,14]. These compounds are electrophilic, reacting with certain cysteine residues to induce conformational changes of the channel proteins. The activation is based on chemical reactivity, rather than chemical structure, contrary to the classic structure-specific mechanism. As well as these reactive chemicals, numerous nonreactive compounds are

Abbreviations: NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; TRP, transient receptor potential; ROS, reactive oxygen species; AITC, allyl isothiocyanate; FTS, farnesyl thiosalicylic acid; CFTR, cystic fibrosis transmembrane conductance regulator; FLIPR, Fluometric Imaging Plate Reader.

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found to activate TRPA1. These include menthol [15–17], farnesyl thiosalicylic acid (FTS) [18], clotrimazole [19], nifedipine [20], and flufenamic acid [21]. Because of the nonreactive chemical property, it is not anticipated that these compounds activate the channel through cysteine modification. The molecular mechanism of TRPA1 activation by these nonreactive compounds still remains largely unknown.

5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) has been widely used as a chloride channel blocker [22]. It is a negatively charged molecule that voltage-dependently blocks cystic fibrosis transmembrane conductance regulator (CFTR) channel [23] and other Cl^- permeable channels. It is generally considered as a selective blocker and often used in the studies that involve Cl^- channel function. Here we report a novel action of NPPB as a potent TRPA1 channel agonist. Using patch clamp recording, we demonstrated that NPPB activated TRPA1 channel at both whole cell and single channel levels. The mutagenesis studies revealed possible interactions of amino acid residues for channel activation by NPPB and other agonists. We further examined a number of NPPB analogs and confirmed a structure-specific mechanism for NPPB action on TRPA1 channels.

2. Materials and methods

2.1. Generating TRPA1 and TRPV1 expressing cells

Human TRPA1 (accession number: BC153003) and TRPV1 (accession number: AM393687) full length cDNAs were purchased from Fisher Scientific (Huntsville, AL, USA) and subcloned into pCDNA-DEST40 vector (Invitrogen, Carlsbad, CA, USA). Site-directed mutagenesis was performed using QuikChange® protocol (Agilent Technologies, Santa Clara, CA, USA). For transient expression, HEK-293 cells were split into 100 mm culture dish at 3.5–4 × 10^6 cells and cultured overnight for transfection. Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was used for DNA transfection according to the manufacturer protocol. Five hours later, transfection medium was replaced and the cells were incubated overnight at 37 °C in 95/5% air/CO_2. The transfected cells were then harvested and replated into a 96-well cell-culture plate at a density of 50,000 cells/well. After 24 h of incubation, the plate was used for FLIPR Ca^{2+} assay. The HEK-293 cell line stably expressing human TRPA1 channel was obtained as described previously [24]. The stable cells used for FLIPR assay were prepared by the way as described above. For patch clamp recording, cells were grown in 35 mm culture dish and trypsinized at 80–90% confluence. The isolated suspension was kept at room temperature and used for the experiment within 4 h.

2.2. FLIPR calcium assay

FLIPR calcium assay was performed in accordance with the manufacturer's protocol (Calcium4® assay, Molecular Devices, Sunnyvale, CA, USA). Briefly, 96-well cell plates were loaded with 150 μl of assay buffer containing 1× Calcium4® dye component A. The assay buffer contains the following components (in mM): 135 NaCl, 2 KCl, 1 $CaCl_2$, 1.17 $MgSO_4$ and 10 HEPES (pH 7.4). After 40-min incubation at room temperature, the plates were placed on the platform of FLIPR384 (Molecular Devices, Sunnyvale, CA, USA) and the fluorescence was read with excitation/emission at 488/540 nm. During the recording time, 50 μl of the assay buffer containing 4× desired concentration of agonists was added into the wells to induce channel activation. Baseline subtraction was conducted and the relative fluorescent unit (RFU) was defined as difference in fluorescence intensity compared with time 0. The maximal RFU within 90 s after addition of agonist was the measure for channel activity and used to construct concentration–response curve.

2.3. Electrophysiological recording

Patch clamp recording was performed in both whole cell and single channel configurations. Cells were loaded into the recording chamber and allowed to settle down in 5–10 min. The bath solution contains the same components as used for FLIPR assay except that $CaCl_2$ was omitted. The Ca^{2+}-free condition was used for both whole cell and single channel recording unless Ca^{2+} was added as otherwise indicated. High resolution current recordings were acquired with a computer-based patch clamp amplifier system (EPC-10, HEKA, Lambrecht, Germany). Patch pipettes were made with electrical resistance between 2 and 5 MΩ after filling with the pipette solution containing (in mM) 140 CsCl, 4 $MgCl_2$, 10 EGTA, 2 Mg-ATP and 10 HEPES (pH 7.2). After establishing whole-cell configuration, the membrane potential was held at −20 mV. A 1-s ramp voltage protocol from −60 to 60 mV was applied repetitively in an interval of 4 s to evoke channel currents. Liquid junction potential, capacitive currents and series resistance were determined and corrected using the automatic compensation of EPC-10. The current amplitudes at the voltages of −60 and 60 mV were used to plot time-dependent response. For single channel recording, the patch pipettes were coated with Sylgard184® (Dow Corning, Midland, MI, USA) before fire polish. As soon as seal was established, cell-attached recording was started. The inside-out patches were acquired by retracting the pipette from the cell. Sometimes, a second pipette with larger opening might be needed to immobilize the cell. As soon as the connection between the cell and the recording pipette was ruptured, the pipette was exposed to air shortly and returned to the bath chamber containing the pipette solution. During the experiment, compounds were delivered through a gravity driven perfusion system. The current signal was digitized at 10 kHz and stored into computer hard drive.

2.4. Chemical and compound preparation

NPPB, AITC, menthol, ruthenium red, capsaicin and the chemicals used to prepare assay buffers were purchased from Sigma–Aldrich (St. Louis, MO, USA). FTS was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). NPPB analogs were obtained from the chemical repository library at Wyeth Research. All compounds were prepared in dimethyl sulfoxide (DMSO) stock and the final DMSO concentrations in assay buffer did not exceed 0.2%.

2.5. Data analysis

The concentration-dependent response from FLIPR assay was normalized to the maximal value and fitted using normal Hill equation to generate EC_{50}. All data analyses including graphing, calculating and fitting were performed using the software Origin® 6.1 (Origin Lab Corporation, Northampton, MA, USA). Statistical data are presented in average with standard error (mean ± SE). Student's *t*-test was performed for analysis of significant difference between two data groups.

3. Results

3.1. NPPB evoked cytoplasmic Ca^{2+} elevation in TRPA1 expressing cells but not in TRPV1 cells

FLIPR Ca^{2+} assay is a sensitive and high throughput approach for the detection of cellular Ca^{2+} concentration and is often employed in assessing Ca^{2+} channel activities. We first conducted FLIPR assay to examine the effect of NPPB on human TRPA1 channels stably expressed in HEK-293 cells. As shown in Fig. 1A, NPPB induced a concentration-dependent increase in Ca^{2+} response. The effect was

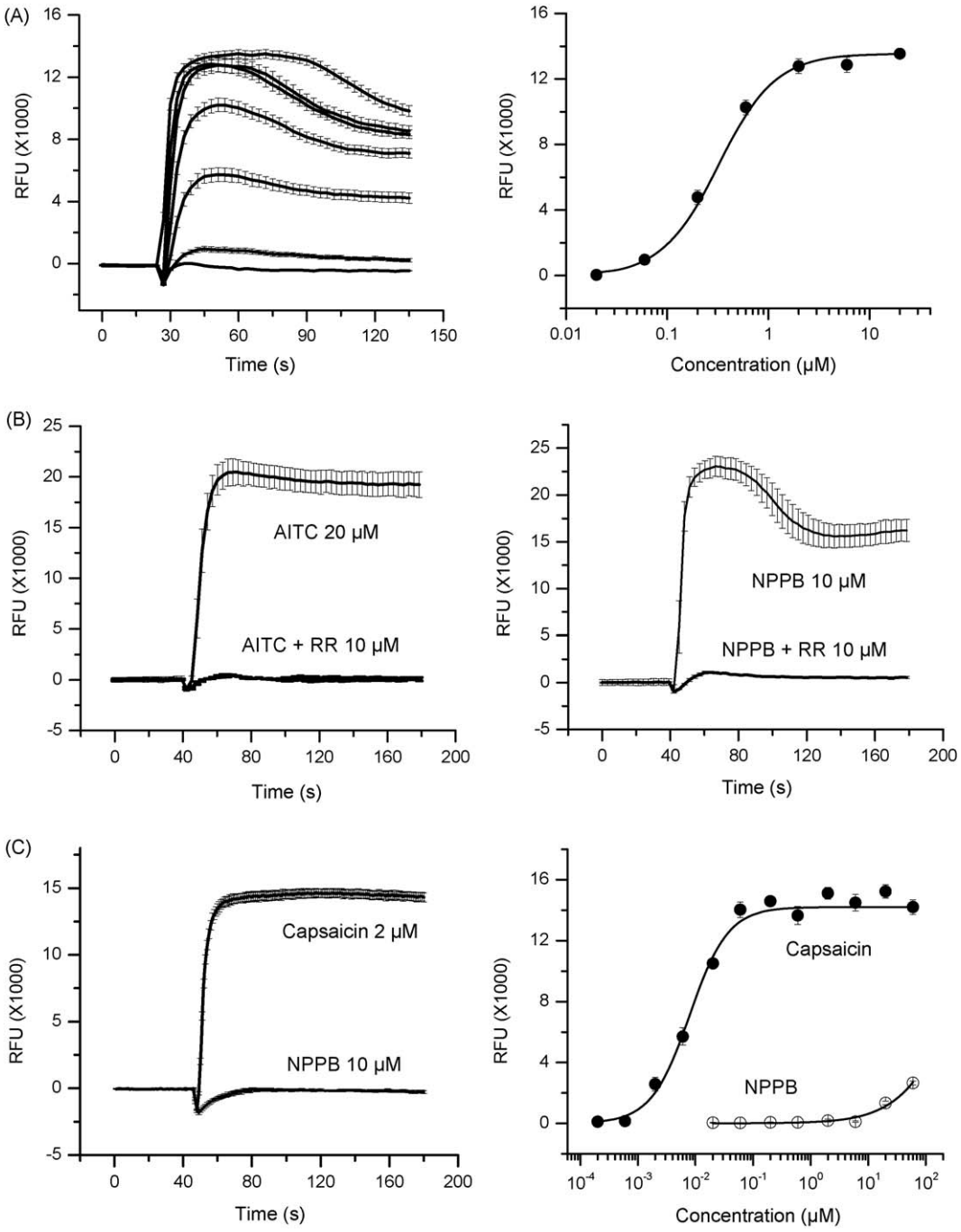

Fig. 1. Effect of NPPB on TRPA1 and TRPV1 channels in FLIPR Ca^{2+} imaging assay. (A) NPPB induced a concentration-dependent response in TRPA1 cells. The assay was performed in a 96-well plate with 8 concentrations of NPPB. NPPB was added at 20-s time point. The fluorescent traces are shown in averages of 12 individual wells. The maximal response within 90 s after addition of NPPB was used to construct the concentration–response curve shown on the right side. The curve fitting with Hill equation generated EC_{50} of 0.32 μM. (B) The assay on TRPA1 cells was performed with ruthenium red (RR) as an antagonist to the activation evoked by AITC and NPPB (n = 12). RR was pretreated 10 min before the assay was started on FLIPR. (C) Effects of NPPB and capsaicin on TRPV1 cells were tested in one 96-well plate. For each data point, n = 4–6.

not observed in HEK-293 parental cells (data not shown), suggesting that NPPB acted on TRPA1 channels. The concentration–response curve indicates that the NPPB effect has an EC_{50} of 0.32 μM. This is so far one of the most potent compounds as known TRPA1 agonist. Similar to the effect of AITC on Ca^{2+} response, NPPB elicited response was completely abolished by pretreatment of 10-μM TRPA1 channel blocker ruthenium red (Fig. 1B). To examine the specificity of the NPPB effect on TRP channels, we tested NPPB in HEK-293 cells transiently expressing TRPV1 channels using FLIPR assay. As shown in Fig. 1C, NPPB at 10 μM induced no changes in Ca^{2+} response whereas the TRPV1 agonist capsaicin at 2 μM elicited a dramatic increase in fluorescence intensity. The EC_{50} of capsaicin was 7.9 nM, consistent with the earlier report (~10 nM [25]). In comparison, NPPB only induced a weak increase at concentrations above 20 μM, indicating the specificity of NPPB effect on TRPA1 channels.

3.2. NPPB activated TRPA1 channels in patch clamp whole cell and single channel recordings

We next conducted patch clamp recording to characterize the effect of NPPB on whole cell membrane currents. The experiment

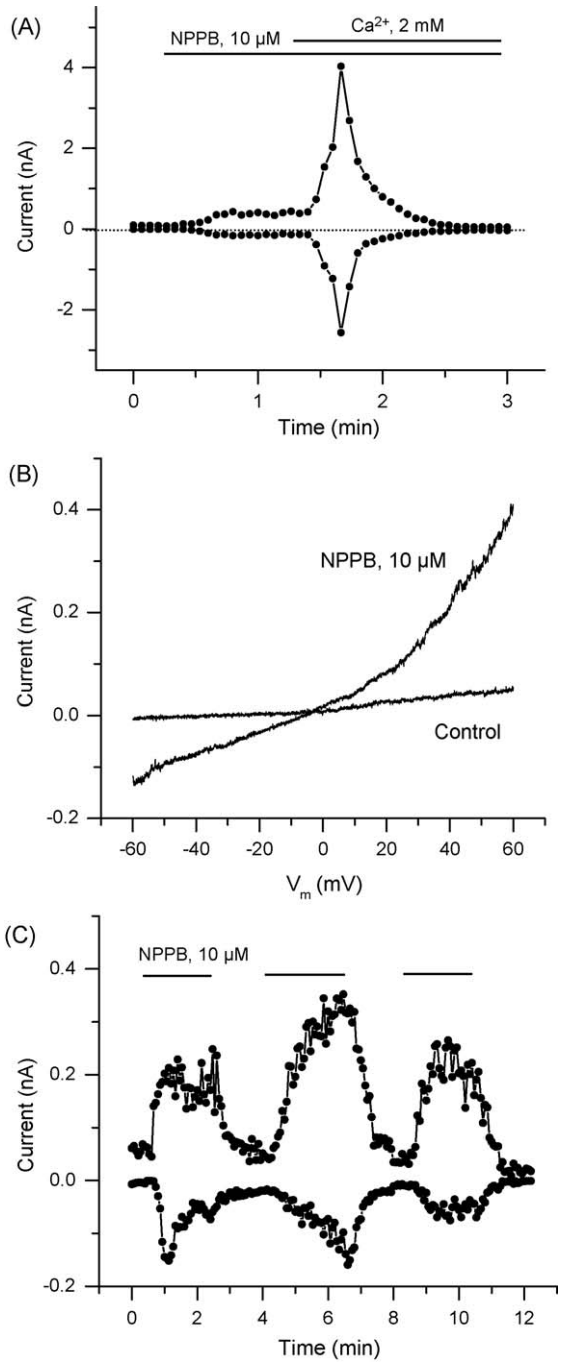

Fig. 2. Activation of TRPA1 channel by NPPB in whole cell patch clamp recording. (A) HEK-hTRPA1 cell was recorded with the holding potential at −20 mV in Ca^{2+}-free external solution. A ramp protocol was used to elicit both inward and outward currents. The current amplitude at −60 and 60 mV was used to construct the plot. The solid horizontal bars indicate the durations of the compound or Ca^{2+} application. (B) The current traces elicited by a 1-s ramp protocol before (Control) and after application of 10 μM NPPB were plotted against the membrane voltage. The current recording for NPPB was depicted from the plateau region in (A). (C) The current traces were recorded from a representative cell to which NPPB was repetitively applied.

was first conducted in the absence of Ca^{2+} in external solution. As shown in Fig. 2A, NPPB at 10 μM significantly evoked both inward and outward currents. The response to NPPB was relatively quick, reaching a plateau in about 30 s. No significant inactivation in the presence of NPPB was observed. As soon as Ca^{2+} was added into external solution, the current was drastically amplified, reaching the peak amplitude in seconds, and then suddenly decreased until completely abolished. This phenomenon has been described as "Ca^{2+} potentiation and inactivation", a unique property of TRPA1 reported in earlier studies [8,26,27]. In the absence of NPPB, the current evoked by voltage ramp protocol was insignificant (Fig. 2B). NPPB induced a current with weak voltage dependence. The average plateau currents at −60 and 60 mV were -94 ± 15 and 403 ± 86 pA ($n = 8$), respectively. The reversal potential was -3.7 ± 2.5 mV ($n = 8$), indicating a nonselective current. The properties of this current described here are highly consistent with those of TRPA1 observed by other researchers, supporting that NPPB activates TRPA1 channels. The effect of NPPB was quickly and completely reversible upon removal of the compound (Fig. 2C). The repetitive application of NPPB resulted in nearly identical responses ($n = 3$), indicating that NPPB activated TRPA1 likely through a non-cysteine modification mechanism since the reactive chemicals such as AITC modified cysteine by covalently chemical reaction generally in slower on-and-off rates [8,24]. This result is also consistent with the nonreactive chemical property of NPPB.

How does NPPB as a nonreactive compound activate TRPA1 channel? To acquire further details for the activation mechanism of the compound, we performed single channel recording using patch clamp technique. In cell-attached configuration, we were able to record a single channel current in most of the cells tested (6 out of 8). This current opened occasionally with a very short opening duration that could be only captured from a long period of recording time, suggesting that the channel remained largely closed at resting state. As shown in Fig. 3A, the single channel current amplitudes measured at certain membrane voltages possessed a linear relation with membrane voltage. The single channel conductance calculated from linear fitting of the current amplitude resulted in 84 ± 6.8 pS ($n = 4$), close to the value published for TRPA1 previously [14]. Fig. 3B shows the effect of NPPB on this single channel current. The representative cell-attached membrane patch contained a very few channel openings under the control condition. NPPB at 10 μM significantly increased the open frequency of the channels on the patch. Application of Ca^{2+} drastically potentiated the opening and then completely inactivated the channels, consistent with the Ca^{2+} potentiation and inactivation as described for the whole cell current. The NPPB elicited single channel current was observed in every patch we studied ($n = 6$). The current traces shown in the stretched time panel in Fig. 3B clearly demonstrated that NPPB caused more frequent openings and longer stays at open state. Ca^{2+} induced more frequent open-and-close transitions during inactivation process. No significant change in single channel conductance was observed after application of either NPPB (86.3 ± 4.6, $n = 3$) or Ca^{2+} (81.4 ± 5.3, $n = 3$). The NPPB effect at single channel level was also readily reversible. As shown in Fig. 3C, consecutively applied NPPB induced nearly identical responses in this representative patch containing a large number of channel proteins. The fact that NPPB quickly activated the channel and the effect was completely reversible highly suggested that NPPB was able to enter into membrane and access the binding site close to the surface. To test if NPPB activates the channel in a membrane-delimited manner, we performed single channel recording in excised inside-out configuration. As shown in Fig. 3D, NPPB induced a significant increase in single channel activity, highly suggesting that NPPB directly acts on the TRPA1 channel protein to evoke the channel activation.

3.3. Mutations affecting TRPA1 channel activation modified NPPB action

We next performed mutagenesis studies to compare NPPB with other known TRPA1 agonists and tried to identify the molecular basis for NPPB action. Several studies have demonstrated the critical amino acid residues for channel activation by different agonists. We decided to make these mutations and to examine the

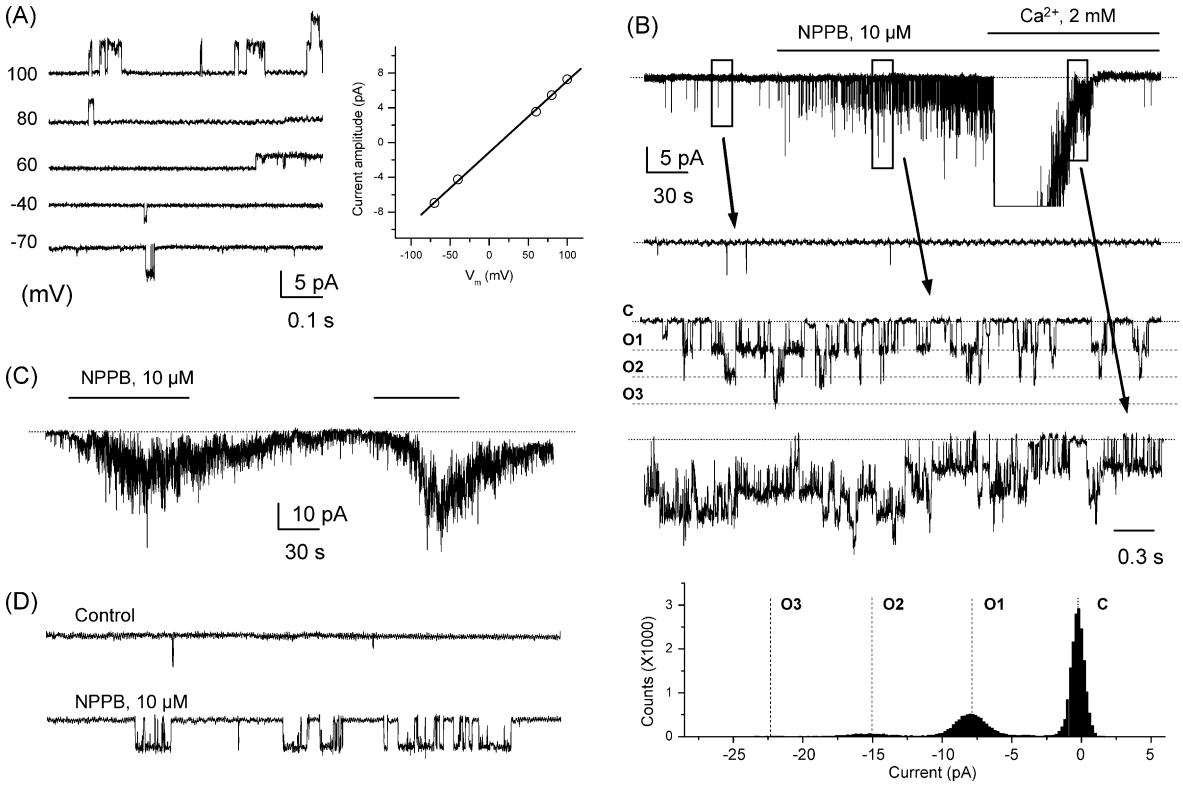

Fig. 3. Activation of TRPA1 channel by NPPB in single channel recording. (A) Cell-attached single channel recording detected a very few channel openings in the absence of NPPB. The amplitudes of the single channel currents captured in this patch at several voltage levels were plotted on the right and fitted with linear equation, $y = a + bx$, where b is the single channel conductance. (B) Cell-attached single channel recording shows the effect of NPPB and Ca^{2+} on TRPA1 channel activity. The time scale in the boxed regions is stretched and shown in the individual windows below the time-compressed trace. The dotted lines represent the closed state and the dashed lines represent the various open states. The current from the second boxed area was used for the construction of distribution graph on the bottom. (C) Single channel current from another cell-attached patch shows the reversibility of the NPPB effect. The repetitive application of NPPB induced nearly identical responses. Note that this patch contained a larger number of the active channels. (D) The current was recorded from an excised inside-out patch in the absence (Control) and the presence of NPPB. The scale bars are the same in the stretched window in (B).

effect of NPPB on the mutant channels. As illustrated in Fig. 4A, we chose the mutations from three separate studies: C621A, C641A and C665A located on the N-terminus of the channel between the ankyrin repeat domain and the first transmembrane domain (S1) reportedly responsible for the action of AITC [13]; double mutant S873V/T874L located on the inner segment of the fifth transmembrane domain (S5) reportedly responsible for menthol action [17]; double mutant S943A/I946M located on the outer segment of the sixth transmembrane domain (S6) responsible for the species-specific blocking action of CMP1 [28]. The mutant channels as well as the wild-type channel were transiently expressed in HEK-293 cells and assayed on FLIPR platform. We compared NPPB with three known agonists: AITC, menthol and FTS. The results are presented in Fig. 4B and the potencies derived from four independent experiments are summarized into Table 1. The individual mutant of three cysteines (C621, C641, C665), which are reportedly potential binding (modifying) sites for AITC, reduced potency of AITC activation. S5 mutations (S873V/T874L) significantly increased EC_{50} of menthol activation. These data were consistent with the previous reports [13,17]. The S6 mutations (S943A/I946M) reduced the potencies of all four agonists, suggesting that these residues are in general critical for channel activation. The S5 mutations were more specific for menthol activation, although they slightly increased the potencies of the other three agonists. More strikingly, one of the cysteine mutations, C665A, significantly increased menthol potency and changed the menthol action into a bell shaped response, an action pattern of menthol on rodent TRPA1 channels [17]. Like S6 mutations, C641A caused a decrease in potencies of all agonists, suggesting that it is another key residue involved in general activation. Only C621A mutation was specific to AITC without significant modification in potencies of the other agonists. All mutations tested here caused no or moderate change in FTS potency, suggesting that they are not key residues for FTS action. Although the mutations induced slightly greater change in NPPB potency, the pattern of the mutant response to NPPB was very similar to FTS, suggesting that these two compounds may have a similar molecular basis of the action.

3.4. NPPB effect was associated with its chemical structure

NPPB is a nonreactive compound that is hypothesized not to form covalent bond with the target residue. The activation through covalent modification has been shown to be structure-unspecific. We tested if NPPB activation was consistent with this hypothesis. A group of NPPB analogs was used to explore the structure–activity relation of this compound scaffold. As shown in Fig. 5, NPPB could be viewed as a benzoic acid with a phenylalkanamine group at position 2 and a nitro group at position 5. The phenylalkane group was essential since the removal (ANB) or substituent propionamide (NPAB) abolished the activity. The alkane chain linking phenyl and amine groups was not essential but important for compound potency. A positive correlation between the length of the alkane chain and the activity was observed. The benzoic acid was another critical group since the benzamide (NPBA) or benzonitrile (NPBN) could not recover the activity. The nitro group seemed important as well since the omission (PPB) lost the activity. The location of this group was tolerable, as the shift in

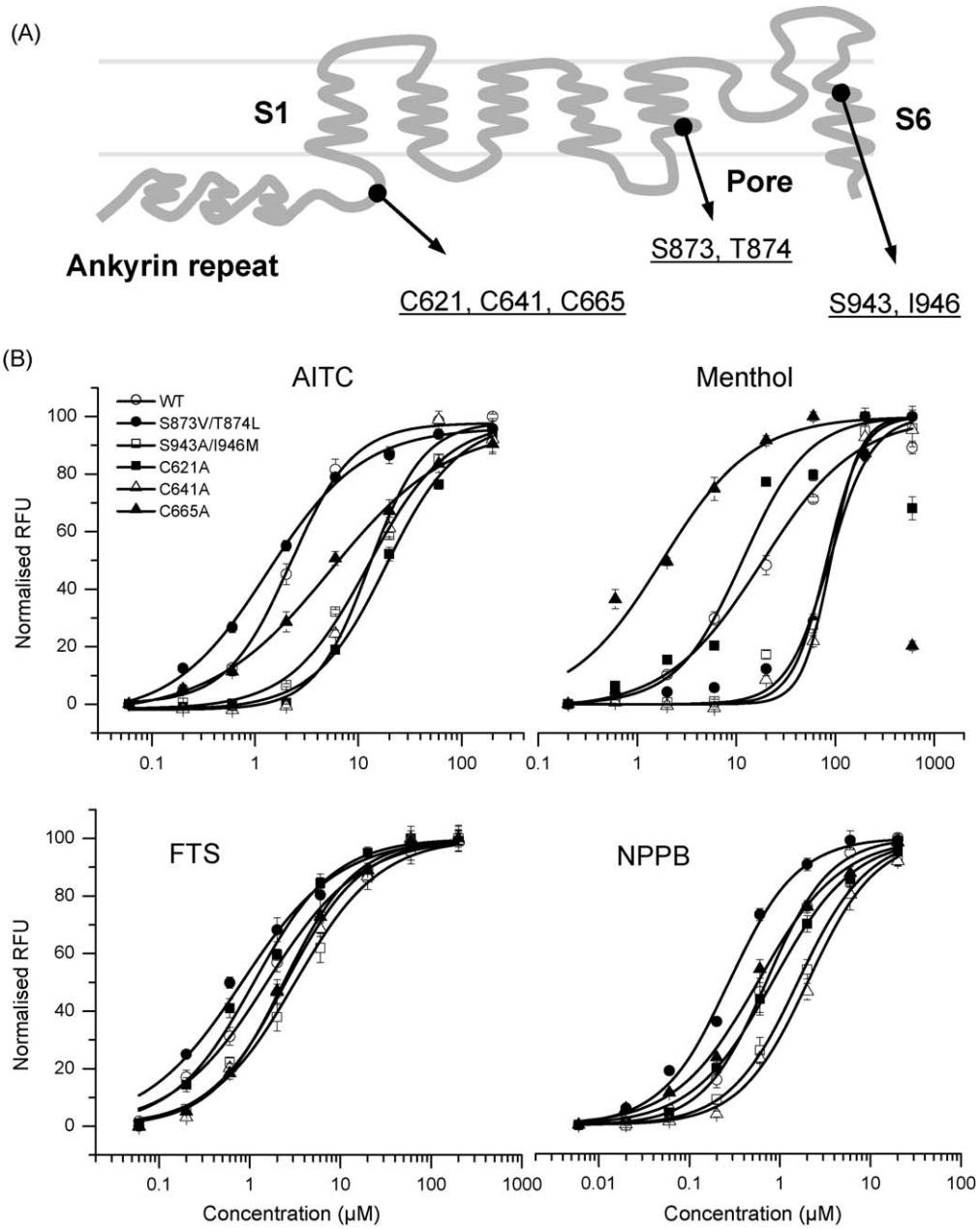

Fig. 4. Mutagenesis studies of molecular mechanisms for the activation of different TRPA1 agonists. (A) Predicted membrane topology of TRPA1 channel is illustrated with six-transmembrane domains (S1–S6) spanning across membrane. The amino acid residues mutated in the study are labeled within three separate regions. (B) The potencies of the structurally different agonists were assayed in FLIPR on HEK-293 cells transiently expressing TRPA1 wild-type and the mutants. Data were normalized to the maximal response for each compound and presented in concentration–response relation. The smooth lines represent fitting curves using Hill equation. Note that for the bell shaped response in menthol panel, the fitting was performed using the data acquired at the concentrations lower than and equal to that eliciting the maximal response. For each data point, $n = 12$ from 4 determinations in triplicate.

Table 1
Activation potencies (EC_{50}, μM) of the agonists on hTRPA1 and the mutants.

	AITC		Menthol		FTS		NPPB	
WT	2.2	0.27 (1)	18	6.2 (1)	1.54	0.1 (1)	0.71	0.03 (1)
S873V/T874L	1.3	0.25 (0.6)	**87.1	7.9 (4.8)	**0.82	0.14 (0.5)	**0.28	0.02 (0.4)
S943A/I946M	**12.5	1.6 (5.7)	**82.2	15.2 (4.6)	**3.16	0.25 (2.1)	**1.59	0.07 (2.2)
C621A	**18.6	2.2 (8.5)	11.6	2.3 (0.6)	1.11	0.12 (0.7)	0.84	0.04 (1.2)
C641A	**12.8	2.2 (5.8)	**90.2	6.3 (5.0)	**2.5	0.2 (1.6)	**2	0.13 (2.8)
C665A	*5.4	0.65 (2.5)	**1.8	0.43 (0.1)	*2.37	0.1 (1.5)	0.57	0.04 (0.8)

EC_{50} was determined using FLIPR Ca^{2+} assay. Data are expressed as means SEM for $n = 4$ determinations in triplicate. The number in parentheses represents the ratio of EC_{50} compared with the wild-type (WT). The significant difference in EC_{50} values from WT was determined using Student's *t*-test.

* $p < 0.05$.

** $p < 0.01$.

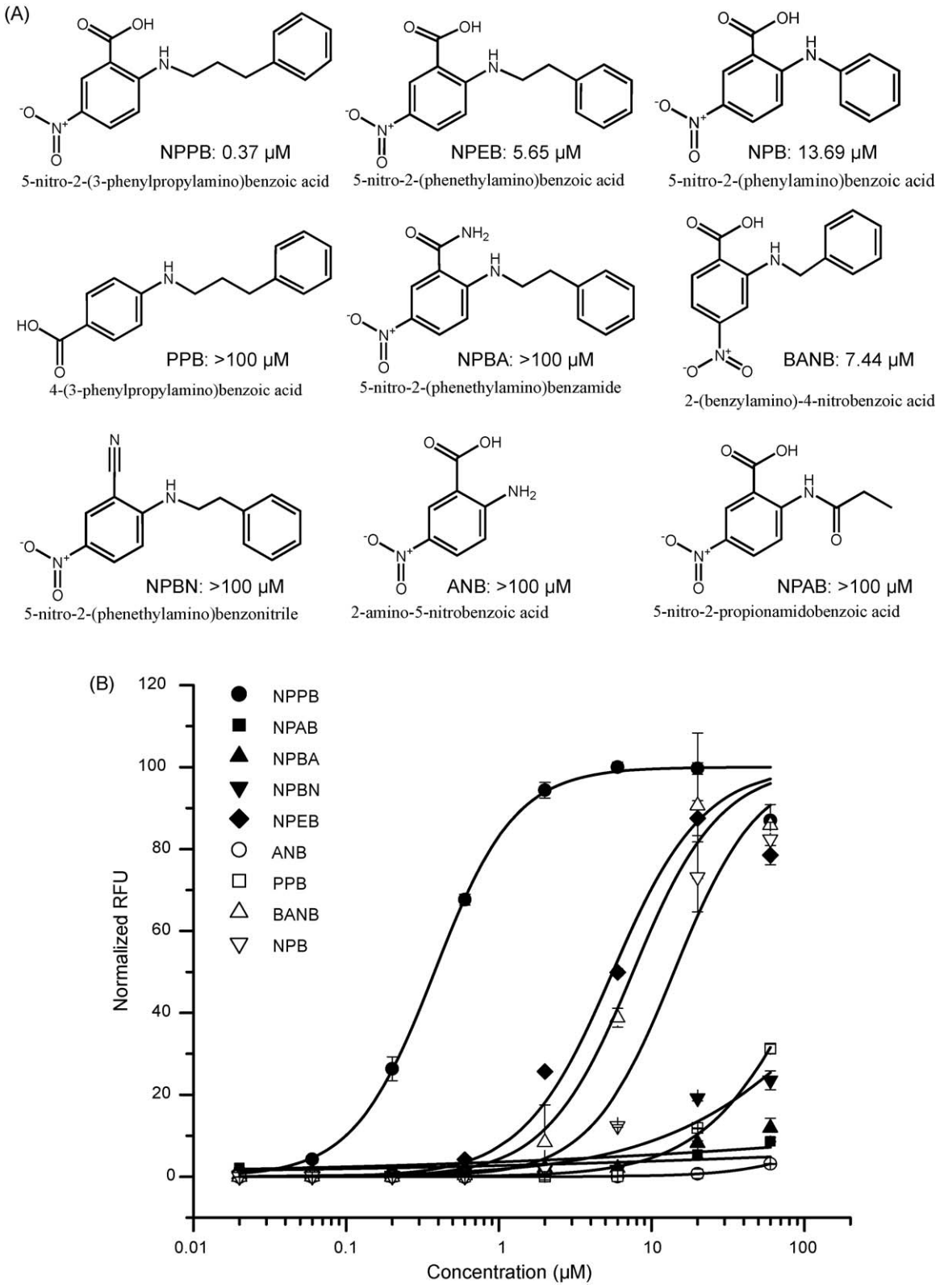

Fig. 5. Structure–activity relationship of NPPB analogs. (A) The chemical structures of NPPB analogs used in this study are presented with the EC_{50}s derived from FLIPR Ca^{2+} assay. (B) Concentration–response relation for NPPB analogs were fitted with Hill equation. The data were normalized to the maximal response of NPPB. For each data point, $n = 12$.

BANB retained the activity. We also tested if any analog possessed antagonizing effect since the modification in some compound series did switch the activity on TRPA1 [29]. However, we did not observe any blocking effect using AITC (20 μM) as an agonist (data not shown). The results shown here demonstrate a clear structure and activity relationship of NPPB analogs, indicating that NPPB activates TRPA1 through a classic structure-specific interaction with the channel protein.

4. Discussion

In the present study, we have demonstrated that NPPB is a potent and specific TRPA1 channel agonist. In FLIPR assay, NPPB possesses sub-micromolar activity, which is currently one of the most potent known TRPA1 agonists. The activation effect has been confirmed using patch clamp recording at both whole cell and single channel levels. Although we cannot fully exclude the possibility that NPPB affects TRPA1 channel through an intermediate component such as a regulatory protein, the effect retained in excised inside-out patch highly suggests a direct modulation of the channel by NPPB. Several lines of evidence consistently support that NPPB activates the channel through a different mechanism from AITC. First, NPPB is a nonreactive compound that is not expected to react with the target residues. Second, NPPB effect is fast, fully reversible and replicable, contrary to the nature of covalent reaction. Third, the mutations affecting AITC effect are not critical for NPPB. Only C641A nonspecifically reduces the potency of NPPB and other agonists as well. Lastly, NPPB activation is clearly structure-specific, providing strong evidence that NPPB acts as a classic compound with "key and lock" mechanism other than the covalent modification by AITC.

NPPB has long been used as a Cl^- channel blocker for identification and characterization of Cl^- channels in cells, tissues, and animals. A large body of literature is in place using NPPB as a studying agent. It has been shown that NPPB blocks various Cl^- channels including CFTR, ClC, CaCC and volume regulated Cl^- channels [22]. In general, NPPB effect on Cl^- channels is of low potency. Hundreds of micromolar sometimes millimolar concentration is needed in order to consistently block Cl^- channel activities. However, due to the suitable pharmacokinetic profile of the compound, it has been used by some researchers in in vivo studies to examine Cl^- channel functions in certain disease models. One interesting finding is the hyperalgesic effect of NPPB in a rodent neuropathic pain model [30]. NPPB administered via i.p. significantly reduced the threshold or latency of paw lifting response to mechanical, radiant heat and cold allodynia stimulation. The Cl^- channel agonist had no effect on these parameters, suggesting that the hyperalgesic effect is irrelevant to Cl^- channel functions. Our data in the present study provide a plausible explanation that the activation of TRPA1 channels by NPPB may cause the hyperalgesia. These results support the conclusion that TRPA1 is involved in pain sensation. On the other hand, NPPB effect observed in in vivo studies can be completely irrelevant to Cl^- channel activities. Recently, Cl^- channel has been paid significant attention for its important roles identified in many physiological processes. Drug discovery on Cl^- channel targets has been initiated. As a classic Cl^- channel blocker, NPPB will continue to be used as a tool compound for such studies. However, based on our findings in this study, cautions should be taken when this compound is used in cells or tissues where TRPA1 channel might be also sufficiently expressed. Conversely, future studies employing NPPB as a TRPA1 agonist should be interpreted in light of its broad actions on Cl^- channel functions.

Up to now, a number of structurally diverse compounds have been discovered as TRPA1 channel agonists. The existence of highly diverse agonist structures can be a good argument that TRPA1 is a chemical sensor for sensory neurons. However, it raises the question how the channel proteins accommodate such a diversity to conform in accordance. The finding that TRPA1 can be activated through covalently chemical reaction at certain cysteine residues provides the answer to some extent, since such compounds require no structure similarity except a reactive group to be functional. However, there are still an increasing number of nonreactive compounds found to activate TRPA1. Molecular elucidation of the activation mechanism for nonreactive compounds can be potentially useful for designing pharmaceutical chemicals that activate or block the channels in a more specific manner. Our results from mutagenesis study provide a refreshed view on the residues reportedly responsible for agonist action. Of three cysteine mutations, C621 is specific for AITC action, supporting that it may be an AITC modifying residue. Mutation at C641 reduces the potencies of all four agonists in a similar manner, suggesting that this residue is involved in a common mechanism for channel activation. The most intriguing residue is C665. Mutation to alanine at this site slightly reduces the potency of AITC but significantly increases the potency of menthol. Menthol activation has been reported to be determined by the residues located within S5 (S873, T874) [17]. Our results indeed confirm that the mutations of these two residues significantly reduce the potency of menthol activation. However, C665 is located on a separate domain segment from S5. To explain the impact of such mutations on menthol activation, C665 must closely interact with S5 domain in order to be involved in menthol action. Together with the important role of C641 in channel activation, we speculate that the sequence domain between ankyrin repeat and S1 lies right underneath S5 or S4–S5 linker. The interaction between the residues in these two separate domains is critical for activation of the channel by AITC and menthol.

Herein, we have shown a novel activation effect of NPPB on TRPA1 channels. We demonstrate that this effect is nonreactive and structure-specific, in contrast to the structure-unspecific cysteine modifying action of AITC on this channel. The results of mutagenesis studies reveal the possible interaction between S5 and N-terminal domain, shedding light on elucidation of molecular basis of TRPA1 channel activation.

Conflict of interest

None.

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References

[1] Clapham DE, Runnels LW, Strubing C. The TRP ion channel family. Nat Rev Neurosci 2001;2:387–96.
[2] Flockerzi V. An introduction on TRP channels. Handb Exp Pharmacol 2007;1–19.
[3] Venkatachalam K, Montell C. TRP channels. Annu Rev Biochem 2007;76:387–417.
[4] Minke B, Cook B. TRP channel proteins and signal transduction. Physiol Rev 2002;82:429–72.
[5] Nilius B, Voets T. Diversity of TRP channel activation. Novartis Found Symp 2004;258:140–9.
[6] Broad LM, Mogg AJ, Beattie RE, Ogden AM, Blanco MJ, Bleakman D. TRP channels as emerging targets for pain therapeutics. Expert Opin Ther Targets 2009;13:69–81.
[7] Bandell M, Story GM, Hwang SW, Viswanath V, Eid SR, Petrus MJ, et al. Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron 2004;41:849–57.
[8] Nagata K, Duggan A, Kumar G, Garcia-Anoveros J. Nociceptor and hair cell transducer properties of TRPA1, a channel for pain and hearing. J Neurosci 2005;25:4052–61.
[9] Bautista DM, Movahed P, Hinman A, Axelsson HE, Sterner O, Hogestatt ED, et al. Pungent products from garlic activate the sensory ion channel TRPA1. Proc Natl Acad Sci USA 2005;102:12248–52.
[10] Macpherson LJ, Geierstanger BH, Viswanath V, Bandell M, Eid SR, Hwang S, et al. The pungency of garlic: activation of TRPA1 and TRPV1 in response to allicin. Curr Biol 2005;15:929–34.
[11] Trevisani M, Siemens J, Materazzi S, Bautista DM, Nassini R, Campi B, et al. 4-Hydroxynonenal, an endogenous aldehyde, causes pain and neurogenic inflammation through activation of the irritant receptor TRPA1. Proc Natl Acad Sci USA 2007;104:13519–24.

[12] McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, et al. TRPA1 mediates formalin-induced pain. Proc Natl Acad Sci USA 2007;104:13525–30.
[13] Hinman A, Chuang HH, Bautista DM, Julius D. TRP channel activation by reversible covalent modification. Proc Natl Acad Sci USA 2006;103:19564–8.
[14] Macpherson LJ, Dubin AE, Evans MJ, Marr F, Schultz PG, Cravatt BF, et al. Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. Nature 2007;445:541–5.
[15] Namer B, Seifert F, Handwerker HO, Maihofner C. TRPA1 and TRPM8 activation in humans: effects of cinnamaldehyde and menthol. Neuroreport 2005;16:955–9.
[16] Karashima Y, Damann N, Prenen J, Talavera K, Segal A, Voets T, et al. Bimodal action of menthol on the transient receptor potential channel TRPA1. J Neurosci 2007;27:9874–84.
[17] Xiao B, Dubin AE, Bursulaya B, Viswanath V, Jegla TJ, Patapoutian A. Identification of transmembrane domain 5 as a critical molecular determinant of menthol sensitivity in mammalian TRPA1 channels. J Neurosci 2008;28:9640–51.
[18] Maher M, Ao H, Banke T, Nasser N, Wu NT, Breitenbucher JG, et al. Activation of TRPA1 by farnesyl thiosalicylic acid. Mol Pharmacol 2008;73:1225–34.
[19] Meseguer V, Karashima Y, Talavera K, D'Hoedt D, Donovan-Rodriguez T, Viana F, et al. Transient receptor potential channels in sensory neurons are targets of the antimycotic agent clotrimazole. J Neurosci 2008;28:576–86.
[20] Fajardo O, Meseguer V, Belmonte C, Viana F. TRPA1 channels: novel targets of 1,4-dihydropyridines. Channels (Austin) 2008;2:429–38.
[21] Hu H, Tian J, Zhu Y, Wang C, Xiao R, Herz JM, et al. Activation of TRPA1 channels by fenamate nonsteroidal anti-inflammatory drugs. Pflugers Arch 2009. doi: 10.1007/s00424-009-0749-9.
[22] Verkman AS, Galietta LJ. Chloride channels as drug targets. Nat Rev Drug Discov 2009;8:153–71.
[23] Zhang ZR, Zeltwanger S, McCarty NA. Direct comparison of NPPB and DPC as probes of CFTR expressed in xenopus oocytes. J Membr Biol 2000;175: 35–52.
[24] Liu K, Samuel M, Harrison KR, Paslay JW. Rb^+ efflux assay for assessment of non-selective cation channel activities. Assay Drug Dev Technol 2010. doi: 10.1089/adt.2009.0243.
[25] Witte DG, Cassar SC, Masters JN, Esbenshade T, Hancock AA. Use of a fluorescent imaging plate reader-based calcium assay to assess pharmacological differences between the human and rat vanilloid receptor. J Biomol Screen 2002;7:466–75.
[26] Zurborg S, Yurgionas B, Jira JA, Caspani O, Heppenstall PA. Direct activation of the ion channel TRPA1 by Ca^{2+}. Nat Neurosci 2007;10:277–9.
[27] Wang YY, Chang RB, Waters HN, McKemy DD, Liman ER. The nociceptor ion channel TRPA1 is potentiated and inactivated by permeating calcium ions. J Biol Chem 2008;283:32691–703.
[28] Chen J, Zhang XF, Kort ME, Huth JR, Sun C, Miesbauer LJ, et al. Molecular determinants of species-specific activation or blockade of TRPA1 channels. J Neurosci 2008;28:5063–71.
[29] Defalco J, Steiger D, Gustafson A, Emerling DE, Kelly MG, Duncton MA. Oxime derivatives related to AP18: agonists and antagonists of the TRPA1 receptor. Bioorg Med Chem Lett 2009.
[30] Ramteke VD, Tandan SK, Kumar D, Aruna Devi R, Shukla MK, Ravi Prakash V. Increased hyperalgesia by 5-nitro-2,3-(phenylpropylamino)-benzoic acid (NPPB), a chloride channel blocker in crush injury-induced neuropathic pain in rats. Pharmacol Biochem Behav 2009;91:417–22.