1521-0111/12/8105-689–700$25.00
MOLECULAR PHARMACOLOGY
Copyright © 2012 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 81:689–700, 2012

Vol. 81, No. 5
76000/3764223

Agonist-Dependent Potentiation of Vanilloid Receptor Transient Receptor Potential Vanilloid Type 1 Function by Stilbene Derivatives[S]

Xuan Zhang, Xiao-Na Du, Guo-Hong Zhang, Zhan-Feng Jia, Xing-Juan Chen, Dong-Yang Huang, Bo-Yi Liu, and Hai-Lin Zhang

Department of Pharmacology, Hebei Medical University, Shijiazhuang, China

Received October 12, 2011; accepted February 7, 2012

ABSTRACT

Transient receptor potential vanilloid type 1 (TRPV1) is a nonselective cation channel activated by capsaicin, low pH, and noxious heat and plays a key role in nociception. Understanding mechanisms for functional modulation of TRPV1 has important implications. One characteristic of TRPV1 is that channel activity induced by either capsaicin or other activators can be sensitized or modulated by factors involving different cell signaling mechanisms. In this study, we describe a novel mechanism for the modulation of TRPV1 function: TRPV1 function is modulated by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and its analogs. We found that, in rat dorsal root ganglion neurons, although DIDS did not induce the activation of TRPV1 per se but drastically increased the TRPV1 currents induced by either capsaicin or low pH. DIDS also blocked the tachyphylaxis of the low pH-induced TRPV1 currents. 4-Acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), a DIDS analog, failed to enhance the capsaicin-evoked TRPV1 current but increased the low pH-evoked TRPV1 currents, with an effect comparable with that of DIDS. SITS also blocked the low pH-induced tachyphylaxis. DIDS also potentiated the currents of TRPV1 channels expressed in human embryonic kidney 293 cells, with an effect of left-shifting the concentration-response curve of the capsaicin-induced TRPV1 currents. This study demonstrates that DIDS and SITS, traditionally used chloride channel blockers, can modify TRPV1 channel function in an agonist-dependent manner. The results provide new input for understanding TRPV1 modulation and developing new modulators of TRPV1 function.

Introduction

Since the first vanilloid (capsaicin) receptor, transient receptor potential vanilloid 1 (TRPV1), was cloned (Caterina et al., 1997), transient receptor potential channels, especially TRPV1, have been a focus of intensive studies (Jung et al., 1999; Jordt et al., 2000;Bhave et al., 2003; Karai et al., 2004; Patwardhan et al., 2010). TRPV1 is a nonselective cation channel and is expressed predominantly in nociceptive sensory neurons. As a polymodal receptor, TRPV1 is activated by vanilloids (such as capsaicin), low pH (pH <5.8), and noxious heat (>43°C) (Caterina et al., 1997) and a number of endogenous ligands, such as certain inflammatory lipoxygenase products and the endocannabinoid anandamide (Zygmunt et al., 1999; Hwang et al., 2000; Jordt et al., 2000; Ahern, 2003). TRPV1 is considered to be one of the major molecular mechanisms for nociception (Szolcsányi, 1996; Davis et al., 2000; Bölcskei et al., 2005).

The general topology of the vanilloid channel, structurally similar to the Shaker-related voltage-gated potassium channels, includes an intracellular N- and C-terminal region and six transmembrane (TM) domains with a short pore-forming stretch between TM5 and TM6 (Caterina et al., 1997). The structure of TRPV1 has been studied by using electron cryomicroscopy. The channel protein appears to exhibit 4-fold symmetry and comprises two distinct regions: a large open basket-like domain, likely corresponding to the cytoplasmic N- and C-terminal portions,

This work was supported by the National Natural Science Foundation of China [Grant 30730031] (to H.-L.Z.); and the National Basic Research Program [Grant 2007CB512100].

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

http://dx.doi.org/10.1124/mol.111.076000.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: TRPV1, transient receptor potential vanilloid type 1; AEA, anandamide/arachidonylethanolamide; ASIC, acid-sensing ion channel; CAP, capsaicin; DADS, 4,4′-diaminostilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DMEM, Dulbecco's modified Eagle's medium; DNDS, 4,4-dinitro-2,2-stilbenedisulfonic acid; DRG, dorsal root ganglion; HEK, human embryonic kidney; PIP_2, phosphatidylinositol bisphosphate; PKC, protein kinase C; RR, ruthenium red; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; TM, transmembrane.

and a more compact domain, corresponding to the TM portion (Moiseenkova-Bell et al., 2008). Important regions or amino acids in TRPV1 possibly involved in capsaicin binding, proton and noxious heat activation, sensitization/desensitization, and phosphorylation of the channel have been described previously (Koplas et al., 1997; Jordt et al., 2000; Sutton et al., 2005; Lishko et al., 2007). TRPV1 is believed to be activated by stimuli with different mechanisms. For example, capsaicin was found to bind to the intracellular site, whereas protons were suggested to bind to the extracellular site of the channel (Jung et al., 1999; Jordt et al., 2000).

One striking feature of TRPV1 is that the activated currents can be sensitized and desensitized. This feature implies that TRPV1 function is under great modulation, which has significant implications for the involvement of TRPV1 in physiological and pathophysiological conditions. Some inflammatory mediators in damaged tissues sensitize TRPV1 to its agonists. Whereas protons sensitize TRPV1 protein directly, most of the mediators work through receptor pathways that include G protein-coupled receptors and receptors with intrinsic tyrosine kinase activity. Protein kinase C (PKC)-dependent phosphorylation can sensitize TRPV1 to capsaicin, protons, or heat (Vellani et al., 2001; Bhave et al., 2003).

The activated TRPV1 currents exhibit two types of desensitization: acute desensitization and tachyphylaxis. In acute desensitization, the current amplitude diminished during prolonged agonist application, whereas in tachyphylaxis, current amplitude diminished in response to repeated agonist applications. It is still an issue of debate whether these two processes are related or independent. Some studies suggest that these two desensitization processes operate independently to modulate TRPV1 functions (Koplas et al., 1997). Others suggest that tachyphylaxis can be explained as a failure of recovery from acute desensitization without invoking an additional, slowly desensitizing state (Liu et al., 2005). In either case, both types of desensitization seem to be sensitive to the concentration of free internal Ca^{2+} (Koplas et al., 1997). Further studies suggest that phosphatidylinositol bisphosphate (PIP_2) depletion, Ca^{2+}/calmodulin binding, and dephosphorylation may contribute to tachyphylaxis (Numazaki et al., 2002; Liu et al., 2005; Lishko et al., 2007; Vyklický et al., 2008).

Stilbene derivatives, such as 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), are known to inhibit Cl^- channels and transporters (Jessen et al., 1986; Dietrich and Lindau, 1994; Lai et al., 1996; Lane et al., 1999; Matulef and Maduke, 2005). In addition, DIDS also was reported to be able to inhibit TRP canonical 4 (Walker et al., 2002) and TRP melastatin 4 (Morita et al., 2007) currents. In this study, we describe a novel function for these stilbene derivatives in the modulation of TRPV1 function. DIDS and its analog SITS enhance the agonist-induced TRPV1 current amplitude and reduced the tachyphylaxis of the activated currents in adult rat dorsal root ganglion (DRG) neurons. DIDS also potentiated the currents of TRPV1 channels expressed in HEK293 cells, with an effect of left-shifting the concentration-response curve of capsaicin-induced TRPV1 currents.

Materials and Methods

Rat DRG Cell Culture. The use of animals in this study was approved by the Animal Care and Ethical Committee of Hebei Medical University (Shijiazhuang, China). DRGs were extracted from all of the spinal levels of 6- to 8-week-old Sprague-Dawley rats. Ganglia were placed in modified D-Hanks' solution and digested at 37°C with collagenase (2 mg/ml; Worthington Biochemicals, Freehold, NJ) for 50 min, followed by another 20 min of digestion with trypsin (2.5 mg/ml; Sigma-Aldrich, St. Louis, MO). They were suspended subsequently at least twice in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (PAA Laboratories GmbH, Linz, Austria) to stop digestion. Ganglia then were dissociated into a suspension of individual cells and plated on poly-D-lysine-coated glass coverslips in 24-well tissue culture plates. Cells were incubated at 37°C with a 5% CO_2 and 95% air atmosphere. Neurons were used for recording within 72 h.

cDNAs. Plasmids encoding rat TRPV1 (Gene ID: 83810) were subcloned in pcDNA3. TRPV1(E600Q), TRPV1(E648A), and TRPV1(S502A) mutants were produced by *Pyrococcus furiosus* DNA polymerase with a QuikChange kit (Stratagene, La Jolla, CA). The structures of the mutants were confirmed by DNA sequencing.

HEK293 Cell Culture and Transfection. HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics in a humidified incubator at 37°C (5% CO_2). Cells were seeded on glass coverslips in a 24-well plate and transfected when 60 to 70% confluence was reached. For the transfection of six wells of cells, a mixture of 3 μg of TRPV1 pERFP cDNAs and 3 μl of Lipofectamine 2000 reagent (Invitrogen) was prepared in 1.2 ml of DMEM and incubated for 20 min. The mixture then was applied to the cell culture wells and incubated for 4 h. Recordings were made 24 h after cell transfection, and cells were used within 72 h.

Electrophysiology. Perforated whole-cell patch recordings were performed on DRG neurons and HEK293 cells expressing TRPV1 channels. Recordings were made at room temperature (23–25°C). Pipettes were pulled from borosilicate glass capillaries and had resistances of 1.5 to 2.5 MΩ when filled with internal solution. Currents were recorded using a MultiClamp 700B amplifier and pCLAMP 10.0 software (Molecular Devices, Sunnyvale, CA) and were filtered at 2 kHz. For perforated patch recording, a pipette first was front-filled with the standard internal solution, then backfilled with the same internal solution containing amphotericin B (250 μg/ml). The external solution used to record TRPV1 currents contained: 160 mM NaCl, 2.5 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 8 mM glucose (pH 7.4). The internal solution for perforated patch recording contained: 150 mM KCl, 5 mM $MgCl_2$, and 10 mM HEPES (pH 7.4).

Chemicals. Capsaicin, DIDS, SITS, 4,4′-diaminostilbene-2,2′-disulfonic acid (DADS), 4,4-dinitro-2,2-stilbenedisulfonic acid (DNDS), anandamide, and ruthenium red were purchased from Sigma-Aldrich. A stock solution for capsaicin was made in ethanol, and all of the other drugs were made in dimethyl sulfoxide. The stock solutions were stored at −20°C and diluted in the appropriate solution immediately before use. The final concentration of dimethyl sulfoxide or ethanol was <0.1%.

Data Analysis and Statistics. Currents were analyzed and fitted using Clampfit 10.2 (Molecular Devices) and Origin 7.5 (OriginLab Corp., Northampton, MA) software. Results are expressed as mean ± S.E.M. Differences were analyzed with Student's paired or unpaired t tests when appropriate and were considered significant at $p < 0.05$.

Results

DIDS Enhances Capsaicin-Evoked TRPV1 Current in Adult Rat DRG Neurons. DIDS (Supplemental Fig. 1A) long has been used as a chloride channel and anion transporter blocker. In preliminary experiments, we found that

DIDS was able to potentiate capsaicin-induced currents in rat DRG neurons. Thus, we decided to conduct a detailed investigation into this unexpected effect of DIDS. For this, adult rat DRG neurons and perforated patch clamp techniques were used.

It has been shown that a long interval (1 h) between two sequential capsaicin applications is necessary to avoid capsaicin-induced desensitization (Schnizler et al., 2008). Obviously, such a long time frame is not practical in most of the patch clamp experiments. To reliably assess the effects of DIDS on activated TRPV1 currents, we first quantified the desensitization of capsaicin-induced currents. In the presence of 5 mM extracellular Ca^{2+}, prominent reductions in the amplitudes of capsaicin-activated currents (tachyphylaxis) were observed after the first application of 1 μM capsaicin when administrated with short durations (20 s) and multiple times (2-min intervals) in DRG neurons (Fig. 1A). The average capsaicin-induced tachyphylaxis from multiple experiments is shown in Fig. 1B; in this case, the current amplitudes at each of the repetitive applications of capsaicin were normalized to the current amplitude obtained with the first capsaicin application (Fig. 1B).

When DIDS was coapplied with capsaicin after the capsaicin-induced currents had gone through substantial tachyphylaxis, the capsaicin-induced currents were greatly increased (Fig. 1, C and D); this increase resulted from the potentiation of the capsaicin-induced currents, because DIDS did not induce any currents when applied alone (100 μM; Fig. 1C). This also was true for DIDS at a higher concentration of 1 mM (n = 6; data not shown). In some cases, the desensitized capsaicin-induced currents were potentiated by DIDS to levels that were greater than the current amplitudes before the tachyphylaxis (Fig. 1, C and D). The effects of DIDS on the potentiation of the capsaicin-induced current were concentration dependent, with an EC_{50} of 4.66 $\pm$ 0.77 μM (Fig. 1, D and E). On average, at saturating concentrations,

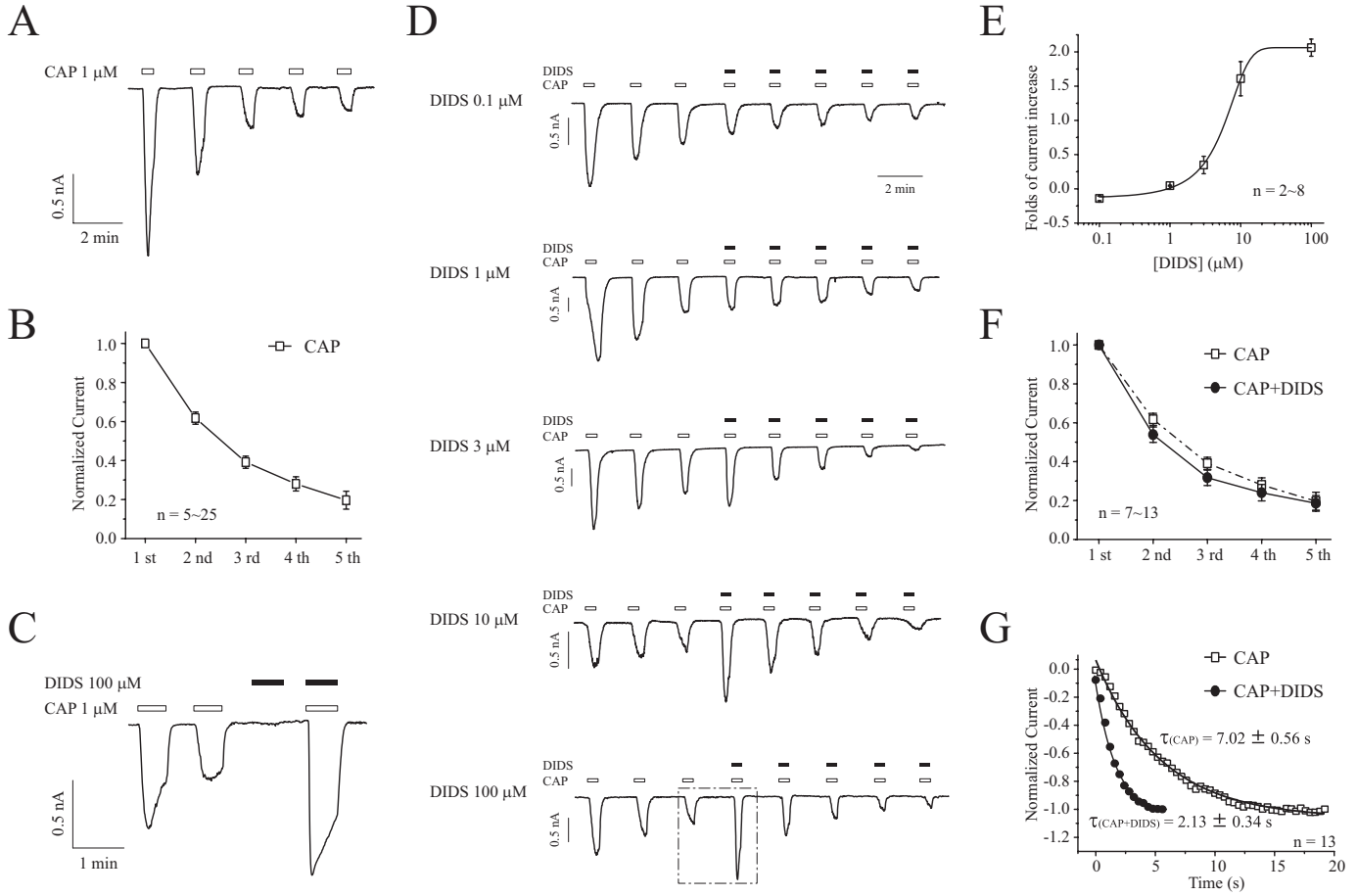

Fig. 1. DIDS enhances the capsaicin-evoked TRPV1 current in adult rat DRG neurons. Whole-cell currents from rat adult DRG neurons were recorded at a holding potential of −60 mV, using a perforated patch clamp technique, in the presence of 5 mM extracellular Ca^{2+}. Timing of the applications of the drugs is indicated by bars. Intervals between the drug applications were 2 min. A, representative trace of the TRPV1 currents induced by short (20 s), repetitive application of 1 μM capsaicin. B, normalized currents induced by capsaicin (1 μM) versus the number of capsaicin applications. The amplitudes of the activated currents decreased with repetitive applications of capsaicin (tachyphylaxis). The current amplitudes were normalized to the current amplitude obtained with the first capsaicin application. C, DIDS (100 μM), on its own, did not activate TRPV1 currents in capsaicin-sensitive neurons. D, concentration dependent potentiation of TRPV1 currents by DIDS. The dotted square box outlines the two current amplitudes used for the quantitative analysis shown in E. E, concentration-response curve for DIDS-induced potentiation of TRPV1 currents. The EC_{50} is 4.66 $\pm$ 0.77 μM. DIDS always was coapplied with capsaicin at the fourth application of capsaicin. At this point, the capsaicin-induced current amplitude without DIDS (CAP) was calculated based on the current amplitude with the third application of capsaicin and the percentage of tachyphylaxis shown in B. Then the current amplitude induced by the coapplication of capsaicin and DIDS (CAP + DIDS) was normalized to this calculated current amplitude. F, effects of DIDS on the tachyphylaxis of the capsaicin-induced TRPV1 currents. The current amplitudes were normalized to the current amplitudes obtained with the first coapplication of capsaicin and DIDS. G, effects of DIDS on activation kinetics of capsaicin-induced currents. The rising phases of the capsaicin-activated currents in the absence and presence of DIDS were fitted with a single exponential function.

DIDS increased the capsaicin-induced currents by >2-fold in comparison with the capsaicin-induced current (Fig. 1E) after taking into account of the current tachyphylaxis (Fig. 1B).

The tachyphylaxis of the capsaicin-induced currents in the presence of DIDS is shown in Fig. 1F and is compared with that in the absence of DIDS (dotted line). The percentage of current reduction evoked by repetitive application of capsaicin without or with DIDS was not significantly different, indicating that DIDS did not affect the capsaicin-induced tachyphylaxis (Fig. 1F), which does not depend on the concentrations of capsaicin used (Supplemental Fig. 2). DIDS did not affect the capsaicin-induced TRPV1 current recovery from tachyphylaxis (Supplemental Fig. 3).

DIDS accelerated the activation of the capsaicin-induced currents (Fig. 1D). The activation process of the capsaicin-induced currents can be fitted by a single exponential function with a time constant of 7.02 ± 0.56 s, which was shortened significantly by the application of DIDS (2.13 ± 0.34 s, $n = 13$, $p < 0.01$) (Fig. 1G).

Effects of DIDS on Capsaicin-Induced TRPV1 Currents in the Absence of Extracellular Ca^{2+}. Ca^{2+} influx through the TRPV1 channel plays an important role in the acute desensitization and tachyphylaxis of TRPV1 currents (Koplas et al., 1997). Thus, in the absence of extracellular Ca^{2+}, TRPV1 currents are virtually void of desensitization (Koplas et al., 1997; Mohapatra and Nau, 2005). To study whether DIDS could also activate the TRPV1 currents that were not being desensitized, we studied the effects of DIDS on capsaicin-induced TRPV1 currents in the absence of extracellular Ca^{2+}.

In Ca^{2+}-free extracellular solution, capsaicin-activated TRPV1 currents did not desensitize and did not present tachyphylaxis upon repetitive application of capsaicin (Fig. 2A, top). Under this circumstance, DIDS still potenti-

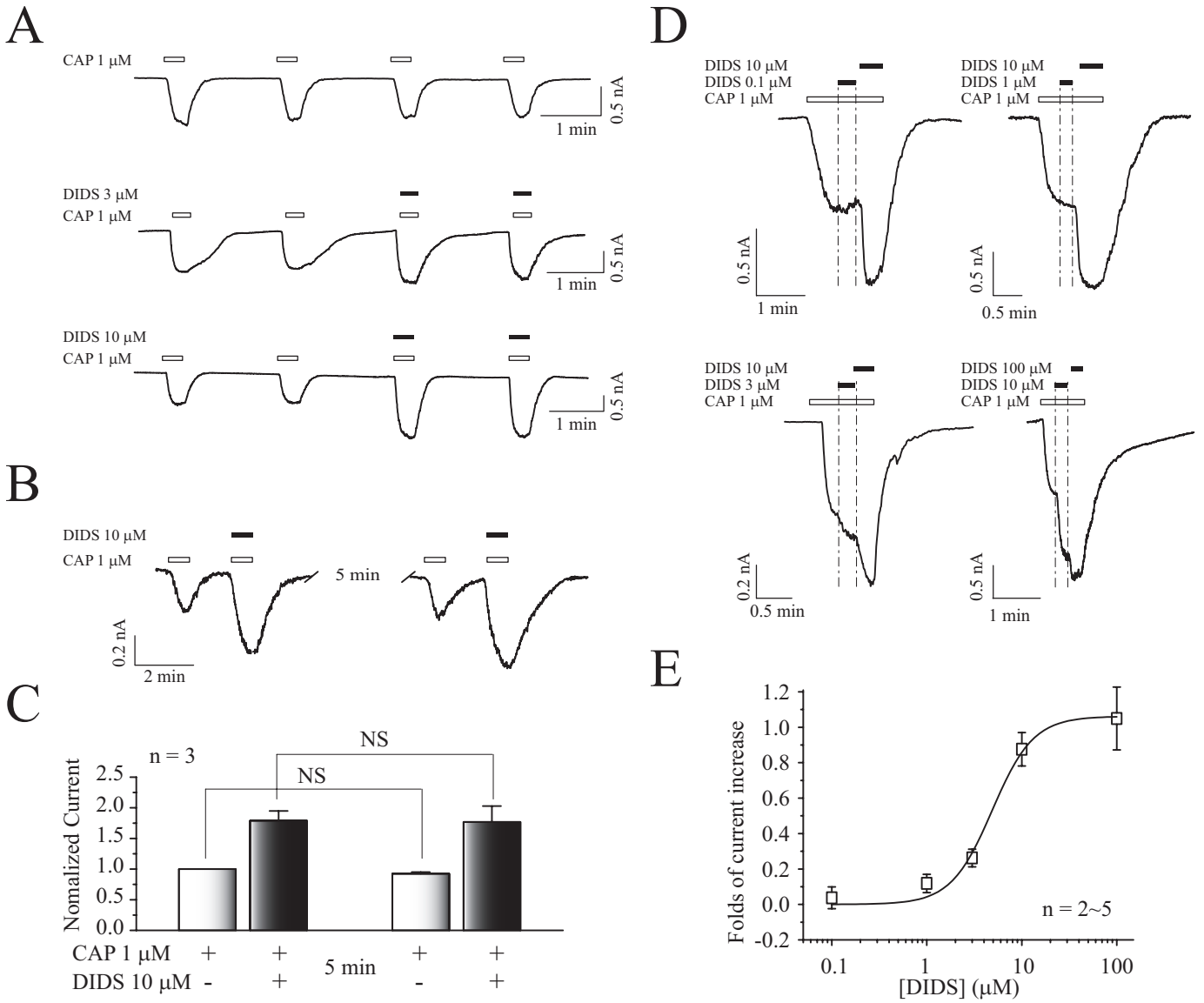

Fig. 2. DIDS enhances the capsaicin-induced TRPV1 currents in the Ca^{2+}-free extracellular solution. Whole-cell currents were recorded at a holding potential of −60 mV in the Ca^{2+}-free extracellular solution. A, representative trace of the TRPV1 current by short (20 s), repetitive application of 1 μM capsaicin or with coapplication of 3 or 10 μM DIDS. B, effects of DIDS were totally reversible. Five minutes indicates the time for washout of drugs. C, a summary of the data in B. D, concentration-dependent effects of DIDS on the capsaicin-induced currents in the Ca^{2+}-free extracellular solution. E, concentration-response curve for DIDS-induced potentiation of TRPV1 currents in the Ca^{2+}-free extracellular solution. The EC_{50} is 4.88 ± 0.74 μM.

ated the capsaicin-induced currents (Fig. 2A, middle and bottom). The nondesensitizing characteristic of the TRPV1 current evoked by capsaicin in Ca^{2+}-free extracellular solution provided a reliable way to test whether the effect of DIDS was reversible. As shown in Fig. 2B, the effects of DIDS indeed were reversible. Thus, capsaicin evoked similar amplitudes of TRPV1 currents before their potentiation by DIDS and after washout of DIDS (Fig. 2C).

The concentration-response relationship was established for DIDS in the absence of extracellular Ca^{2+} (Fig. 2, D and E); DIDS potentiated the capsaicin-induced currents with an EC_{50} of 4.88 ± 0.74 μM (Fig. 2E), which is similar to the EC_{50} in the presence of extracellular Ca^{2+} (Fig. 1E).

DIDS Enhances the TRPV1 Current Induced by Endovanilloid Anandamide. Anandamide (arachidonylethanolamide) is a powerful vasodilator and was isolated originally from brain as an endogenous cannabinoid receptor ligand (Zygmunt et al., 1999). In electrophysiology experiments, anandamide induces a capsazepine-sensitive inward current in TRPV1-expressing HEK cells and primary sensory neurons, which indicates that the vanilloid receptor thus may be another molecular target for endogenous anandamide (Zygmunt et al., 1999). This provides a likely molecular mechanism for anandamide and/or structurally related lipids to participate in the regulation of nociception and vasodilation as vanilloid receptor ligands.

We tested whether DIDS also could enhance the anandamide-induced TRPV1 currents in DRG neurons. Anandamide evoked a much smaller inward TRPV1 current at a concentration of 10 μM compared with that evoked by capsaicin, and the current amplitude varied between cells (Fig. 3, A–C). DIDS (10 μM; Fig. 3, A–C) strongly enhanced the anandamide-induced TRPV1 currents and did not affect desensitization (Fig. 3, C and D).

DIDS Enhances Low pH-Evoked TRPV1 Currents and Blocks the Tachyphylaxis of Low pH-Induced Currents. Low pH of the extracellular milieu regulates TRPV1 function in two primary ways. First, low pH increases the potency of heat or capsaicin as TRPV1 agonists and lowers the channel activation threshold. Second, lower pH (pH <5.8) activates the channel directly as an agonist of TRPV1 at room temperature (Caterina et al., 1997; Jordt et al., 2000). We tested whether DIDS could affect low pH-evoked TRPV1 currents in DRG neurons.

Low pH-induced currents also showed similar tachyphylaxis on repetitive application of low pH (Fig. 4, A and B) and also were sensitive to DIDS (Fig. 4A). DIDS concentration-dependently increased the low pH-induced current with an EC_{50} of 1.83 ± 0.29 μM (Fig. 4D). The low pH-induced currents were more sensitive to DIDS modulation than the capsaicin-induced currents; thus, the low pH-induced currents were increased by 448 ± 69% of the control (the control current amplitude was calculated based on the percentage desensitization shown in Fig. 4B) with coapplication of 100 μM DIDS (Fig. 4D), whereas the capsaicin-evoked currents were increased by 216 ± 13% of the control with coapplication of 100 μM DIDS (Fig. 1E). Furthermore, DIDS almost completely abolished the tachyphylaxis of currents evoked by low pH (Fig. 4, A and B), in sharp contrast with the lack of effects of DIDS on the tachyphylaxis of the capsaicin-induced currents (Fig. 1, D and F). This effect of DIDS was maintained even in a long recording with more repetitive application of pH + DIDS (Supplemental Fig. 4).

Similar to the activation process of the capsaicin-induced currents, the activation process of low pH-induced currents also was accelerated by DIDS (Fig. 4E). The time constant for the activation process of the low-pH-induced currents was reduced from 2.93 ± 0.32 to 1.26 ± 0.11 s ($n = 5, p < 0.01$) by coapplication of DIDS (Fig. 4E).

Acid-Sensitive Currents Are Not Involved in the DIDS-Potentiated Currents Induced by Low pH. There are acid-sensitive currents in DRG neurons, including the currents through acid-sensing ion channels (ASICs). ASICs are ligand-gated ion channels activated by extracellular pro-

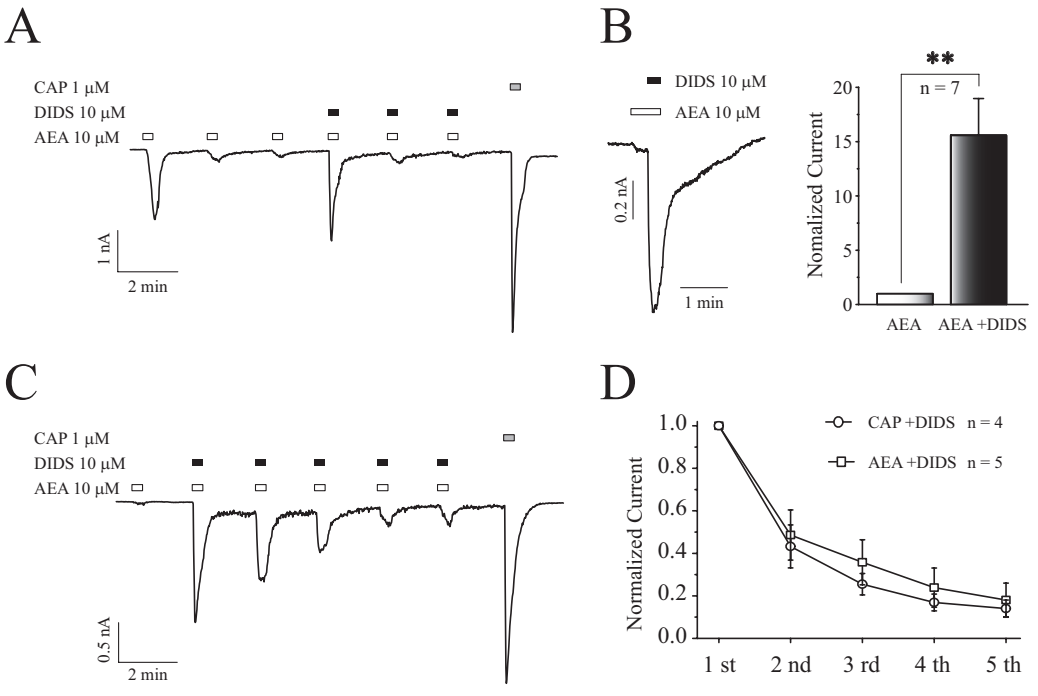

Fig. 3. DIDS enhances the TRPV1 currents evoked by endovanilloid anandamide. Whole-cell currents were recorded at a holding potential of −60 mV in the presence of 5 mM extracellular Ca^{2+}. A, representative trace of the TRPV1 current induced by 10 μM anandamide (AEA) or by coapplication of 10 μM DIDS and 10 μM anandamide. The TRPV1 current evoked by anandamide could be enhanced by DIDS in the capsaicin-sensitive DRG neurons. B, folds of the current increase by 10 μM DIDS. C, coapplication of DIDS with anandamide increased the currents but did not prevent the desensitization of the currents. D, summarized effects of DIDS (10 μM) on the desensitization of the anandamide-induced TRPV1 currents. The current amplitudes were normalized to the current amplitudes obtained with the first coapplication of DIDS with capsaicin or anandamide.

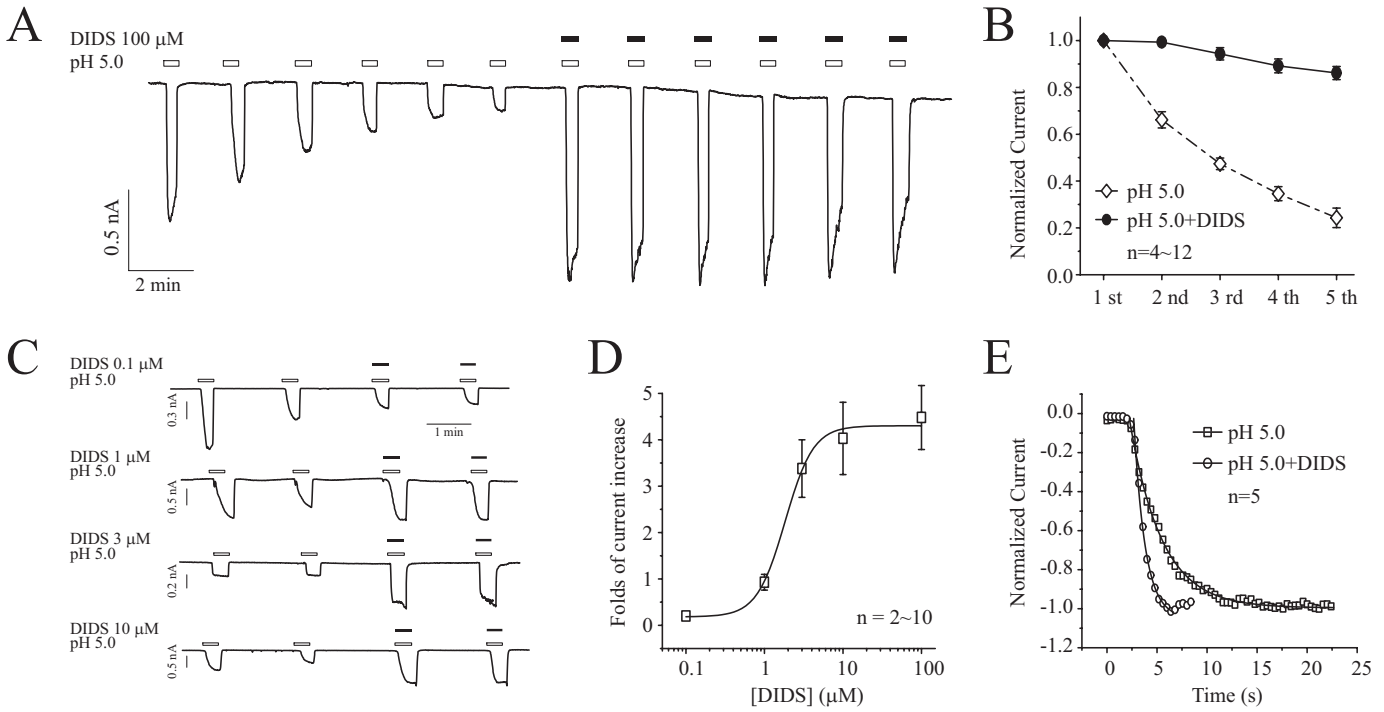

Fig. 4. DIDS enhances the current amplitudes and abolishes the tachyphylaxis of the low pH-evoked TRPV1 currents. Whole-cell currents were recorded at a holding potential of −60 mV in the presence of 5 mM extracellular Ca^{2+}. A, representative trace of the TRPV1 currents induced by short (20 s), repetitive applications of low pH solution (pH 5.0) and the effects of DIDS (100 μM). Coapplication of DIDS with capsaicin increased the currents evoked by low pH and abolished the desensitization of the currents with repetitive application of low pH (tachyphylaxis). B, effects of DIDS on the desensitization of the low pH-induced TRPV1 currents. The current amplitudes were normalized to the current amplitude obtained with the first coapplication of low pH and DIDS. C, concentration-dependent potentiation of the low pH-induced TRPV1 currents by DIDS. D, concentration-response curve for the DIDS-induced potentiation of TRPV1 currents. The EC_{50} is 1.83 ± 0.29 μM. E, effects of DIDS on the activation kinetics of the low pH-induced currents. The rising phases of the low pH-activated currents in the absence and presence of DIDS were fitted with a single exponential function.

tons. To date, six members of the ASIC family have been identified. They are expressed widely in the peripheral and central nervous system, and all of the ASIC subunits are present in DRG neurons (Alvarez de la Rosa et al., 2002; Poirot et al., 2006). Because acid-sensitive currents can be activated by low pH at the range that we used in the above experiments, we proceeded to test whether the currents through ASICs and other acid-sensitive components could contribute to the effects of DIDS. For this, ruthenium red (RR), a broad-spectrum Ca^{2+} channel blocker, was used to block the TRPV1 currents to isolate the acid-sensitive currents. As shown in Fig. 5, A and B, RR almost abolished the capsaicin-induced currents (97.3 ± 0.01%, n = 5) (Fig. 5B). We found that, as we reported earlier, only a subpopulation of DRG neurons responded to capsaicin stimulation (Liu et al., 2010). Among the cells that we studied, 51.7% of the cells were capsaicin sensitive, and another 48.3% of neurons did not respond to capsaicin with the development of measurable currents (total number of cells was 118). However, low pH (pH 5.0) always induced inward currents regardless whether the neurons were capsaicin sensitive or insensitive (Fig. 5, C and D). However, in the capsaicin-sensitive cells, low pH induced large currents, which were mostly inhibited by RR (Fig. 5C), whereas in the capsaicin-insensitive neurons, low pH induced small currents that were not inhibited by RR (Fig. 5D). These results suggest that the residual currents left in the capsaicin-sensitive neurons after RR inhibition and the currents in the capsaicin-insensitive cells are the acid-sensitive currents. The average amplitudes of these two currents were not greatly different (146.7 ± 13.2 versus 222 ± 16 pA; Fig. 5, C and D). Thus, in the capsaicin-sensitive neurons, low pH activated the acid-sensitive currents in addition to the TRPV1 currents, which were absent in the capsaicin-insensitive neurons. The acid-sensitive currents in the capsaicin-insensitive neurons were inhibited by DIDS (39.2 ± 0.18% of the level before DIDS administration, n = 4; Fig. 5, E and F), and the TRPV1 currents in the capsaicin-sensitive neurons were potentiated (566 ± 88% of the level before DIDS administration; Fig. 5, G and H). These data clearly demonstrate that DIDS selectively sensitizes TRPV1 currents but not the acid-sensitive currents induced by low pH.

SITS Potentiates Low pH-Evoked But Not Capsaicin-Evoked Currents. We next investigated whether SITS, an analog of DIDS, also could act similarly to DIDS. There is only a minor structural difference between these two compounds (Supplemental Fig. 1, A and C). One of the isothiocyano groups in DIDS is replaced with an acetamido group in SITS. However, SITS (100 μM) did not potentiate the capsaicin-evoked TRPV1 currents (Fig. 6, A and B), and it did not alter the tachyphylaxis of the capsaicin-induced currents (Fig. 6, C and D). However, SITS strongly and concentration-dependently potentiated the low pH-induced currents (Fig. 6, E and F). Similar to DIDS, SITS alone did not induce any currents, even at a high concentration of 1 mM (n = 6; data not shown). The efficacy of SITS on the low pH-induced currents is comparable with that of DIDS (421 ± 68 versus 448 ± 69% for low pH + SITS and low pH + DIDS over the

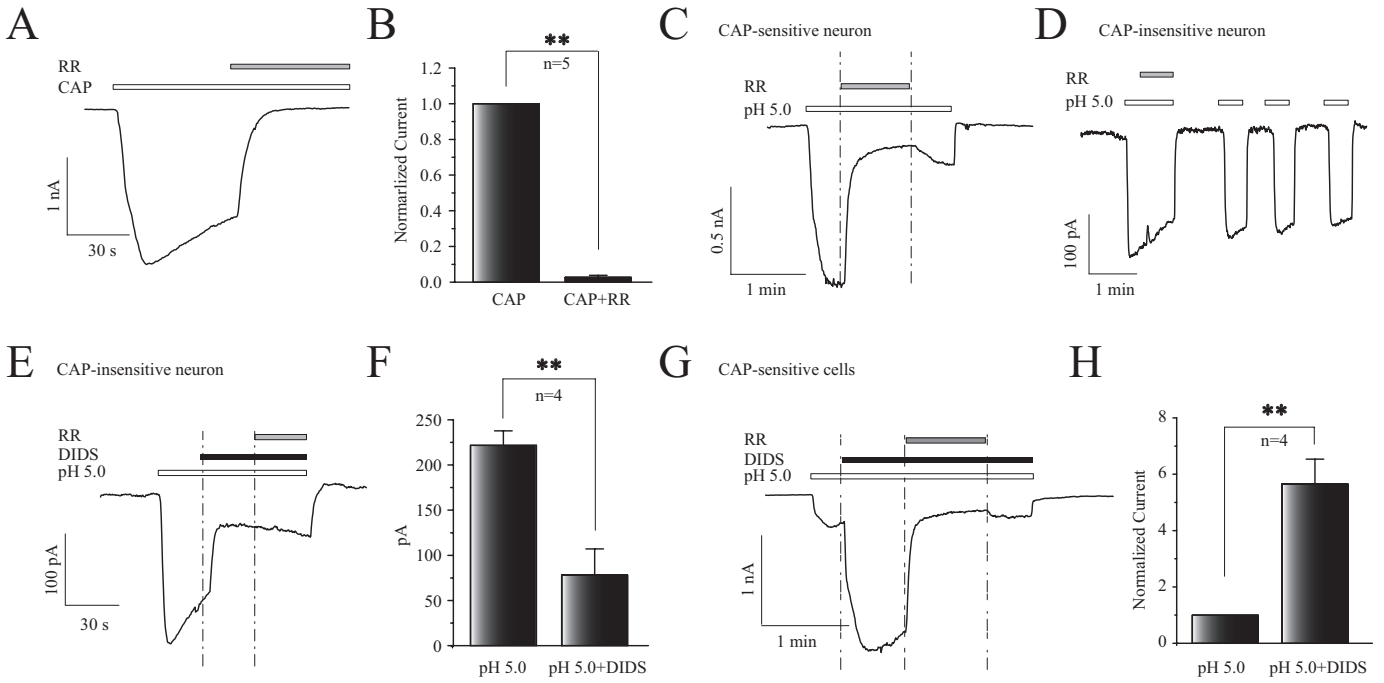

Fig. 5. The acid-sensitive currents are not involved in the effects of DIDS on the low pH-induced currents. Whole-cell currents were recorded at a holding potential of −60 mV in the presence of 5 mM extracellular Ca^{2+}. A, capsaicin-induced currents were inhibited greatly by ruthenium red (RR) (10 μM). B, a summary of the data in A. The currents were normalized to the capsaicin-induced currents. C, effects of RR on the low pH (pH 5.0)-induced currents from the capsaicin-sensitive neurons. D, effects of RR on the low pH-induced currents from the capsaicin-insensitive neurons. RR did not inhibit the currents. E, DIDS (100 μM) markedly inhibited the low pH-induced currents from the capsaicin-insensitive neurons. F, summary of DIDS effects as shown in E. G, DIDS (100 μM) markedly enhanced the low pH-induced currents from the capsaicin-sensitive neurons, which were inhibited by RR. H, summary of DIDS (100 μM) effects as shown in G. The currents were normalized to the currents evoked by low pH solution. In all panels, **, $P < 0.01$.

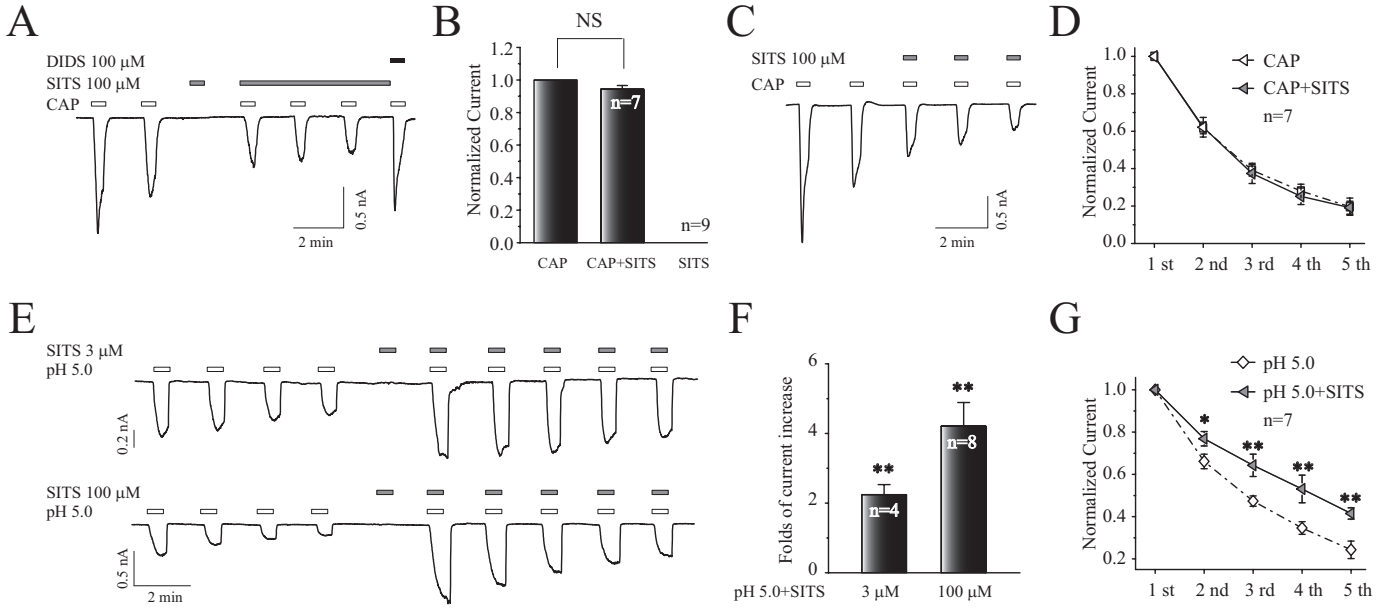

Fig. 6. SITS potentiates the low pH- but not the capsaicin-induced currents. Whole-cell currents were recorded at a holding potential of −60 mV in the prescence of 5 mM extracellular Ca^{2+}. A, SITS did not enhance the capsaicin-induced currents. B, a summary for the effects of SITS as shown in A. C and D, SITS did not affect the tachyphylaxis of the capsaicin-induced currents. E, SITS concentration-dependently potentiated the TRPV1 currents induced by the low pH. F, summary data for the effects of SITS shown in E. **, $P < 0.01$. G, SITS partially blocked the tachyphylaxis of the low pH-induced currents. *, $P < 0.01$.

controls, respectively). SITS also partially blocked the tachyphylaxis of the low pH-evoked currents (Fig. 6G).

DIDS Enhances Currents of TRPV1 Expressed in HEK293 Cells. To confirm that TRPV1 is the direct target of modulation by DIDS, we studied the effects of DIDS on the currents of TRPV1 expressed in HEK293 cells. As in rat DRG neurons, the TRPV1 currents induced by capsaicin also underwent substantial tachyphylaxis on repetitive applications of capsaicin. DIDS, as it did in DRG neurons, potentiated the capsaicin-evoked TRPV1 current but did not affect the tachy-

phylaxis of the capsaicin-induced currents ($n = 4$; Fig. 7A). SITS did not affect the capsaicin-induced currents (8 ± 6%, $n = 4$). As in DRG neurons, DIDS also potentiated the TRPV1 currents in the absence of extracellular Ca^{2+} ($n = 4$; Fig. 7B). The effects of DIDS were concentration dependent (Fig. 7, C and D); DIDS potentiated the capsaicin-induced currents in the absence of Ca^{2+} with an EC_{50} of 3.21 ± 0.10 μM (Fig. 7D).

A lack of tachyphylaxis of the capsaicin-induced TRPV1 currents in the absence of extracellular Ca^{2+} enables us to study the effects of DIDS under repetitive applications of capsaicin. Thus, the effects of DIDS on the concentration-dependent activation of TRPV1 by capsaicin were tested. Coapplication of DIDS with capsaicin led to a leftward shift of the concentration-response curve for capsaicin activation of TRPV1 expressed in HEK293 cells ($n = 6$–8; Fig. 8, A and B).

To help to understand the possible mechanism of the DIDS-mediated potential of TRPV1, we tested the effect of DIDS on voltage-dependent properties of TRPV1 currents. TRPV1 was reported to be partially voltage dependent, and agonists of TRPV1 (protons and capsaicin) could increase the voltage sensitivity of TRPV (Voets et al., 2004; Matta and Ahern, 2007). Figure 8C shows representative current traces of TRPV1 expressed in HEK293 cells that resulted from a series of voltage steps either under control conditions or in the presence of capsaicin (100 nM) or with coapplication of 100 μM DIDS. The conductance and voltage (*G-V*) curves were fitted with a Boltzmann function. DIDS (100 μM) shifted $V_{1/2}$ from 40 ± 5 mV and −42 ± 7 mV (Fig. 8D). However, the slope factors for these two fittings were not different (63 mV for capsaicin and 69 mV for capsaicin plus DIDS).

DIDS Does Not Potentiate TRPV1 Currents through Known Modulation Sites. Both low pH and activation of PKC were reported to be able to potentiate the TRPV1 currents activated by agonists such as capsaicin (Jordt et al., 2000; Numazaki et al., 2002; Bhave et al., 2003). The effects of low pH and PKC are mediated through specific sites in TRPV1. We decided to investigate whether the effects of DIDS also were mediated through these sites. A Glu residue (Glu600) in TRPV1 has been suggested to be the key regulatory site for low pH-mediated modulation of TRPV1 function, whereas the residue Glu648 may serve as the key site for low pH activation of TRPV1 (Jordt et al., 2000), and two Ser residues, Ser502 and Ser800, have been suggested to be involved in the potentiation of the TRPV1 current via PKC-mediated phosphorylation (Numazaki et al., 2002; Bhave et al., 2003). We tested the effects of DIDS on TRPV1 mutants involving the above-mentioned sites expressed in HEK293 cells. The data shown in Fig. 8 suggested that, for the two low pH-related mutants, TRPV1(E600Q) and TRPV1(E648A), the capsaicin-induced currents were potentiated by DIDS to same level as the wild-type TRPV1 current (Fig. 9, A and C), although their responses to low pH were reduced greatly (Fig. 9, A and

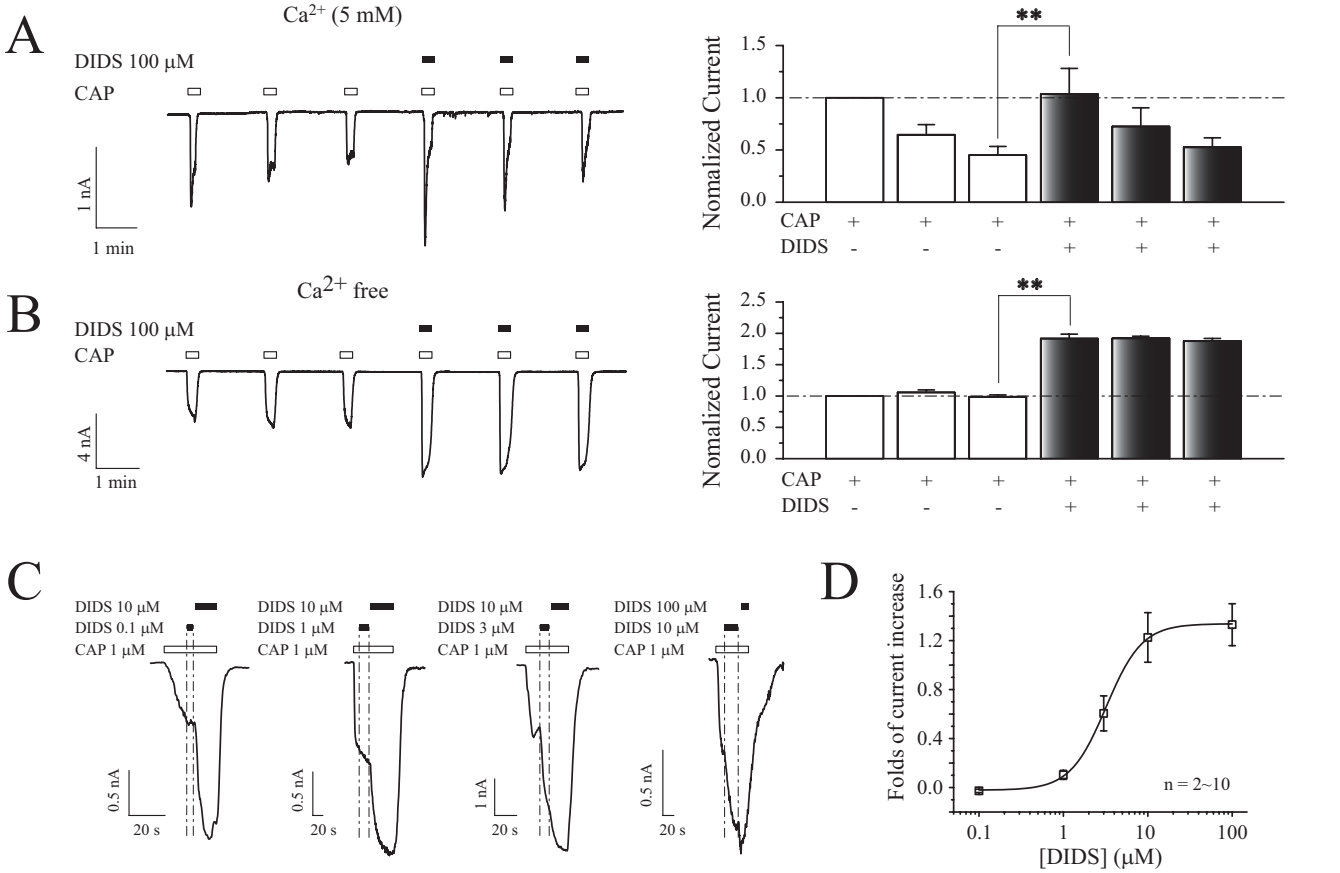

Fig. 7. The effects of DIDS on the currents of TRPV1 expressed in HEK293 cells. Whole-cell currents were recorded at a holding potential of −60 mV. A and B, representative traces of the TRPV1 current induced by short (10 s), repetitive applications of 1 μM capsaicin or coapplication of 1 μM capsaicin and 100 μM DIDS in the presence of 5 mM Ca^{2+} (A) or in the absence of Ca^{2+} (B) extracellular solution (left). Normalized summary data are shown on the right. C, concentration-dependent potentiation of the capsaicin-induced currents by DIDS in the Ca^{2+}-free extracellular solution. D, concentration-response curve for the effects of DIDS. The EC_{50} is 3.21 ± 0.10 μM.

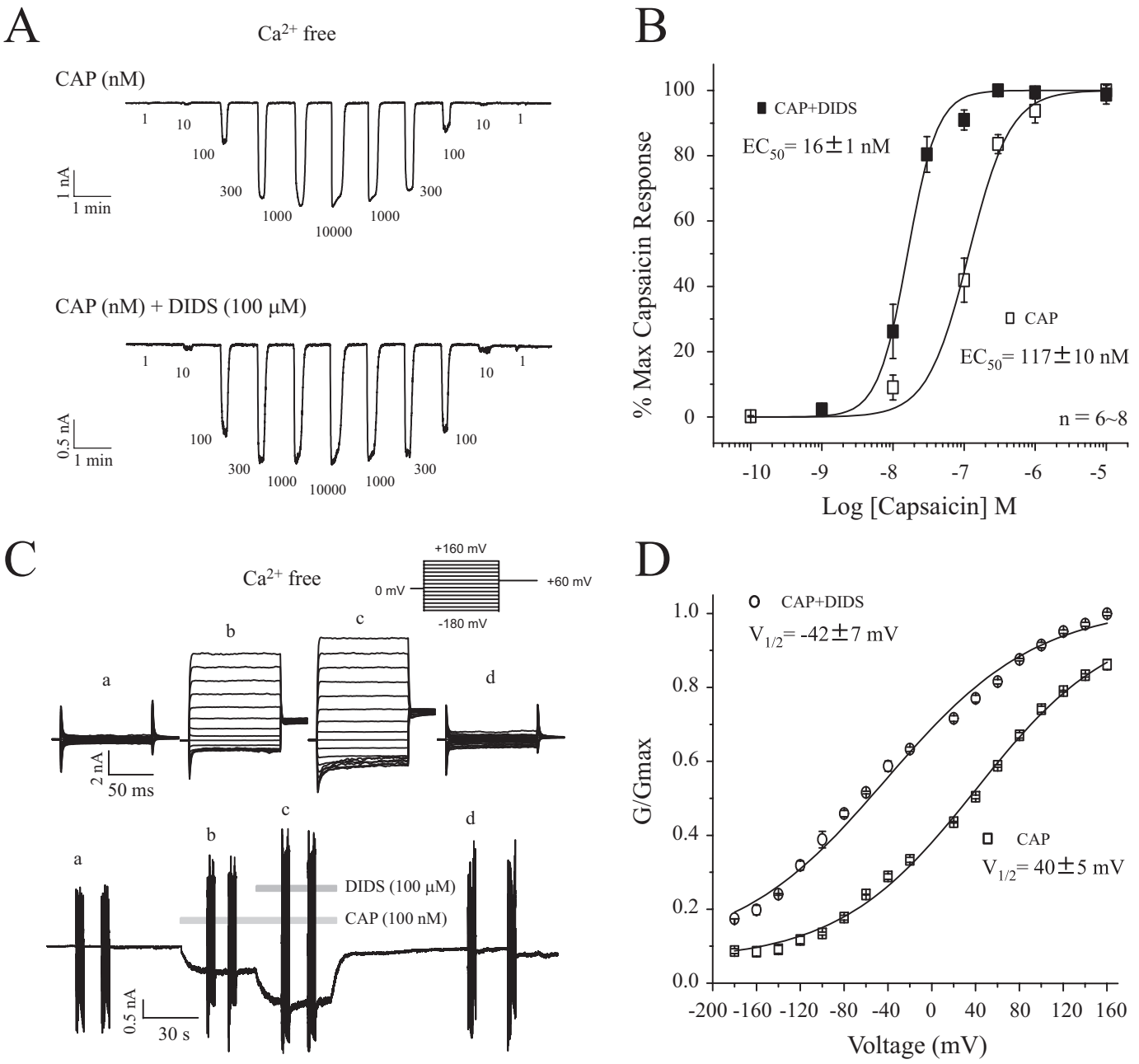

Fig. 8. DIDS sensitizes the effects of capsaicin on the activation of TRPV1 and potentiates the TRPV1 currents in a voltage-dependent manner. The capsaicin-induced TRPV1 currents were recorded in HEK293 cells. Whole-cell currents were recorded at a holding potential of −60 mV in the Ca^{2+}-free extracellular solution. A, representative traces of the currents induced by different concentrations of capsaicin in the absence (top) or presence (bottom) of 100 μM DIDS in the Ca^{2+}-free extracellular solution. B, concentration-response relationships for capsaicin with or without coapplication of 100 μM DIDS. Data are fitted with the Hill equation (n = 6–8). The EC_{50} values are shown. The Hill coefficient is 1.48 for capsaicin alone and 2.08 for capsaicin + DIDS. C, cells were held at −60 mV, and the holding currents at −60 mV were recorded continuously. A family of voltage steps (using the voltage protocol shown above the current traces) were applied at a to d, and the representative traces of the control, in the presence of 100 nM capsaicin or with coapplication of 100 μM DIDS, induced by the protocol are shown in the upper panel. D, Boltzmann fits to the conductances obtained from steady-state currents at the end of the pulse. $V_{1/2}$ values for 100 nM capsaicin or with coapplication of 100 μM DIDS are shown. The slope factor is 63 mV for capsaicin alone and 69 mV for capsaicin + DIDS.

B). DIDS also potentiated the low pH-induced currents of the E648A mutant to a similar extent as it did for the currents of wild-type TRPV1 (Fig. 9, D and E). We also tested the effect of DIDS on TRPV1(S502A), which should abolish the PKC-mediated TRPV1 potentiation. As shown in Fig. 9, F and G, the capsaicin-induced TRPV1(S502A) currents were potentiated by DIDS similarly to the wild-type TRPV1 currents. Thus, DIDS potentiates the TRPV1 currents through a mechanism different from the known mechanism.

Discussion

TRPV1, as a polymodal integrator, is activated by vanilloids (capsaicin) and low pH (pH <5.8). We studied here the effects of stilbene derivatives on TRPV1 currents activated by these two prototypical stimuli in DRG neurons as well as the capsaicin-activated currents of TRPV1 expressed in HEK293 cells. The results demonstrate that DIDS potentiated the TRPV1 currents evoked by capsaicin or low pH,

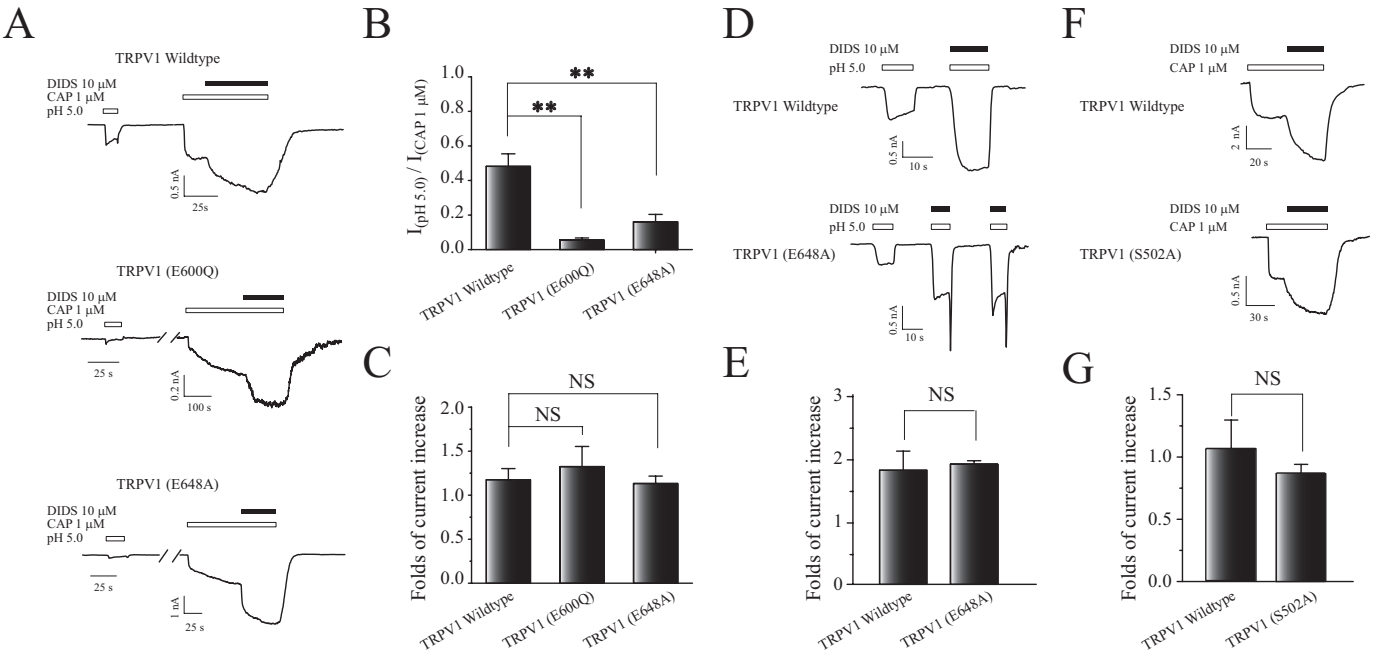

Fig. 9. Mutations that affect the pH- and PKC-mediated modulation of TRPV1 currents do not affect the DIDS-mediated potentiation of the TRPV1 currents. Whole-cell currents were recorded at a holding potential of −60 mV in the Ca^{2+}-free extracellular solution. A, residues of TRPV1 (Glu600, Glu648) related to pH modulation were not involved in DIDS potentiation of capsaicin-induced TRPV1 currents. B, ratio of low pH (pH 5.0)- versus capsaicin (1 μM)-activated currents. **, $P < 0.01$. C, a summary of the data for the effects of DIDS shown in A. D and E, the residue (Glu648) of TRPV1 related to pH modulation was not involved in DIDS potentiation of pH-induced TRPV1 currents. F and G, the residue (Ser502) of TRPV1 related to PKC modulation was not involved in DIDS potentiation of capsaicin-induced TRPV1 currents.

whereas SITS selectively enhanced the TRPV1 currents induced by low pH. Furthermore, DIDS greatly reduced the tachyphylaxis of the TRPV1 currents induced by low pH but not by capsaicin. To our knowledge, this is the first report that stilbene derivatives are direct modulators of activated TRPV1 function besides their well known blocking effects on chloride channels. The characterization of the effects of DIDS sheds some new insight into the modulation of TRPV1 function.

It is striking that the agonist (capsaicin or low pH)-induced TRPV1 currents are potentiated greatly by the stilbene derivatives even when substantial tachyphylaxis of the currents has developed. In fact, all of the effects of DIDS and SITS were observed when the TRPV1 currents have been desensitized to <30% of their initial values (Fig. 1B). Under this condition, the desensitized currents were not only increased but in some cases increased to a higher level than the currents before desensitization. This is truly remarkable considering that the desensitized channels rarely respond to stimuli and they need long time to recover from desensitization. Clearly, DIDS does not merely recover the desensitized TRPV1 channels, because the nondesensitizing TRPV1 currents in the absence of extracellular Ca^{2+} also are potentiated by DIDS (Figs. 2A and 7B). Actually, as shown in Fig. 1F, DIDS does not alter the tachyphylaxis of capsaicin-induced TRPV1 currents. It has been reported that Ca^{2+}/calmodulin binding, membrane PIP_2 hydrolysis, and channel dephosphorylation may contribute to the repetitive stimuli-induced TRPV1 tachyphylaxis (Liu et al., 2005; Lishko et al., 2007). DIDS is not likely to affect cellular Ca^{2+}/calmodulin, membrane PIP_2 hydrolysis, or channel phosphorylation. If any of these mechanisms indeed are involved in the tachyphylaxis of TRPV1 seen in this study, DIDS should potentiate the agonist-induced TRPV1 currents independent of these mechanisms. Indeed, as shown in Fig. 7, when the capsaicin-activated TRPV1 currents are devoid of tachyphylaxis due to the absence of extracellular Ca^{2+}, DIDS still greatly potentiates the TRPV1 currents. Activation of PKC can sensitize the TRPV1 currents activated by capsaicin, low pH, or heat in DRG neurons and in HEK293 cells (Vellani et al., 2001). However, the TRPV1 mutant that lacks the suggested phosphorylation site still responds to DIDS similarly to the wild-type TRPV1 (Fig. 9, F and G). Thus, it is likely that DIDS uses a novel mechanism to potentiate the TRPV1 currents. It is possible that binding of DIDS to the TRPV1 channel alters the conformation of TRPV1 so that the channel now is not only more sensitive to the agonists but also can be activated to a greater extent.

Although it has been reported that TRPV1 could be activated by strong membrane depolarization in the absence of agonists (Voets et al., 2004; Matta and Ahern, 2007), we could not record appreciably sized TRPV1 currents in HEK293 cells when the membranes were depolarized to up to 160 mV (Fig. 8C). Furthermore, this property of TRPV1 was not changed by the application of DIDS alone. Nonetheless, as it was reported previously (Matta and Ahern, 2007), there was a voltage-dependent component of the TRPV1 currents activated by capsaicin (Fig. 8D). DIDS shifted the voltage-dependent activation of the capsaicin-induced currents to a more negative potential without affecting the slope factor of the *G-V* curve. Capsaicin also could concentration-dependently shift the voltage-dependent TRPV1 currents to more negative potentials (Matta and Ahern, 2007). We interpreted the effect of DIDS as increasing the sensitivity of TRPV1 to capsaicin, which is consistent with the above discussion.

DIDS potentiated the desensitized capsaicin-activated TRPV1 currents but did not affect the process of current tachyphylaxis (Fig. 1F). However, DIDS potentiated the low pH-activated TRPV1 currents and abolished the current tachyphylaxis (Fig. 4, A and B). These selective effects of DIDS again support our above argument that DIDS modulates TRPV1 function with novel mechanisms that are distinct from the current known mechanisms. For example, membrane PIP_2 is believed to be important for both low pH- and capsaicin-induced TRPV1 tachyphylaxis (Liu et al., 2005). Furthermore, although SITS potentiated the low pH-induced but not the capsaicin-induced TRPV1 currents in this study, PKC-mediated sensitization of the TRPV1 channel is nonselective for these two stimuli (Vellani et al., 2001). Thus, overall, we have the following hypothesis for the mechanisms involved in DIDS and SITS modulation of TRPV1 function. Capsaicin and low pH should use different mechanisms to activate TRPV1, thus creating different conformation states of activated channels, and the difference is small enough to accommodate the binding and action of DIDS on both conformation states but large enough to discriminate for the selective binding and action of SITS on the pH-induced activated state of TRPV1. Likewise, the mechanisms of tachyphylaxis for capsaicin- and low pH-activated TRPV1 currents also could be different. Thus, it is not too difficult to understand why DIDS only abolished the tachyphylaxis of pH-activated but not capsaicin-activated TRPV1 currents. The above hypothesis is supported by evidence from electrophysiological and mutagenesis studies, which demonstrate that capsaicin binds to the intracellular domain of TRPV1 (Tyr511 and Ser512, located at an intracellular loop and TM3), whereas protons bind to extracellular sites (Glu648 in the pore-forming stretch between TM5 and TM6) (Jordt et al., 2000).

Two lines of evidence suggest that the isothiocyanate groups in stilbene derivatives are important for the modulation of TRPV1 functions. First, when both of the isothiocyanate groups in DIDS are replaced by primary amines, as for DADS (Supplemental Fig. 1B), or replaced by nitro groups, as for DNDS (Supplemental Fig. 1D), the capability to modulate TRPV1 function is lost (Supplemental Fig. 5). Second, when one of the two isothiocyanate groups in DIDS is replaced by an acetamido group, as for SITS (Supplemental Fig. 1C), the capability to potentiate capsaicin-activated TRPV1 currents is lost, leaving only the ability to potentiate the pH-activated currents. These features of the structural specificity of stilbene derivatives further support the notion that specific structure-related interactions between the stilbene derivatives and the TRPV1 channels are necessary for the modulation of TRPV1. It has been reported that the multimers formed from DADS have greater potency in inhibiting Cl^- channel function than DIDS (Matulef et al., 2008; Wulff, 2008), which suggests that the isothiocyanate groups are not needed for the function of stilbene derivatives as Cl^- channel blockers. It is thus possible to develop new compounds with TRPV1 potentiation actions but void of Cl^- channel-blocking actions.

It has been reported recently that a variety of 1,4-dihydropyridine derivatives can enhance capsaicin-induced TRPV1 activity but exhibit minimal or no intrinsic agonist activity of their own (Roh et al., 2008). Thus, as we show here for stilbene derivatives, functions of the TRPV1 channel can be modulated by small molecular compounds with different structures. This will have at least two important implications. First, it will help to understand the mechanisms underlying TRPV1 activation, desensitization, and other properties of channel activities. Because the structure of TRPV1 is not available, these functional tools will be very valuable. Second, it will help to develop new modulators of TRPV1 function, which have great clinical therapeutic potential. In fact, agonists of TRPV1 have been used and are being evaluated to be used as analgesic agents (Mason et al., 2004). The logic behind this seemly paradoxical scenario is that the sustained opening of the TRPV1 channel would allow a large influx of Ca^{2+}, which results in Ca^{2+} excitotoxicity that occurs selectively in TRPV1-expressing, primary afferent nociceptive neurons (Karai et al., 2004). In this regard, compounds such as DIDS could be very valuable. First, it greatly enhances the influx of Ca^{2+} through the activated TRPV1 channel and in some cases may abolish the desensitization of the activated TRPV1 currents. Thus, overall Ca^{2+} excitotoxicity to nociceptive neurons will be increased. Second, because DIDS and SITS do not activate TRPV1 on their own and only potentiate the agonist-activated TRPV1 currents, they would be efficacious only at nerve endings where TRPV1 is activated by pain-inducing substances rather than at all of the accessible nociceptors. Thus, selective, localized actions may be achieved but without the need for local administration and without blockade or inhibition of the important protective aspects of pain elsewhere in the body, as might be encountered with an antagonist of TRPV1.

Acknowledgments

We greatly appreciate the gift of rat TRPV1 cDNA from Dr. Sven-Eric Jordt (Yale University, New Haven, Connecticut). We thank Bing-cai Guan and Hong-chao Men for expert technical assistance and helpful discussions.

Authorship Contributions

Participated in research design: X. Zhang, Liu, and H.-L. Zhang.
Conducted experiments: X. Zhang, Du, G.-H. Zhang, Jia, Chen, and Huang.
Performed data analysis: X. Zhang and H.-L. Zhang.
Wrote or contributed to the writing of the manuscript: X. Zhang and H.-L. Zhang.

References

Ahern GP (2003) Activation of TRPV1 by the satiety factor oleoylethanolamide. *J Biol Chem* **278:**30429–30434.
Alvarez de la Rosa D, Zhang P, Shao D, White F, and Canessa CM (2002) Functional implications of the localization and activity of acid-sensitive channels in rat peripheral nervous system. *Proc Natl Acad Sci USA* **99:**2326–2331.
Bhave G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS, and Gereau RW 4th (2003) Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc Natl Acad Sci USA* **100:**12480–12485.
Bölcskei K, Helyes Z, Szabó A, Sándor K, Elekes K, Németh J, Almási R, Pintér E, Petho G, and Szolcsányi J (2005) Investigation of the role of TRPV1 receptors in acute and chronic nociceptive processes using gene-deficient mice. *Pain* **117:**368–376.
Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, and Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389:**816–824.
Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, et al. (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* **405:**183–187.
Dietrich J and Lindau M (1994) Chloride channels in mast cells: block by DIDS and role in exocytosis. *J Gen Physiol* **104:**1099–1111.
Hwang SW, Cho H, Kwak J, Lee SY, Kang CJ, Jung J, Cho S, Min KH, Suh YG, Kim D, et al. (2000) Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. *Proc Natl Acad Sci USA* **97:**6155–6160.

Jessen F, Sjøholm C, and Hoffmann EK (1986) Identification of the anion exchange protein of Ehrlich cells: a kinetic analysis of the inhibitory effects of 4,4′-diisothiocyano-2,2′-stilbene-disulfonic acid (DIDS) and labeling of membrane proteins with 3H-DIDS. *J Membr Biol* **92:**195–205.
Jordt SE, Tominaga M, and Julius D (2000) Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc Natl Acad Sci USA* **97:**8134–8139.
Jung J, Hwang SW, Kwak J, Lee SY, Kang CJ, Kim WB, Kim D, and Oh U (1999) Capsaicin binds to the intracellular domain of the capsaicin-activated ion channel. *J Neurosci* **19:**529–538.
Karai L, Brown DC, Mannes AJ, Connelly ST, Brown J, Gandal M, Wellisch OM, Neubert JK, Olah Z, and Iadarola MJ (2004) Deletion of vanilloid receptor 1-expressing primary afferent neurons for pain control. *J Clin Invest* **113:**1344–1352.
Koplas PA, Rosenberg RL, and Oxford GS (1997) The role of calcium in the desensitization of capsaicin responses in rat dorsal root ganglion neurons. *J Neurosci* **17:**3525–3537.
Lai ZF, Liu J, and Nishi K (1996) Effects of stilbene derivatives SITS and DIDS on development of intracellular acidosis during ischemia in isolated guinea pig ventricular papillary muscle in vitro. *Jpn J Pharmacol* **72:**161–174.
Lane M, Baltz JM, and Bavister BD (1999) Bicarbonate/chloride exchange regulates intracellular pH of embryos but not oocytes of the hamster. *Biol Reprod* **61:**452–457.
Lishko PV, Procko E, Jin X, Phelps CB, and Gaudet R (2007) The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. *Neuron* **54:**905–918.
Liu B, Linley JE, Du X, Zhang X, Ooi L, Zhang H, and Gamper N (2010) The acute nociceptive signals induced by bradykinin in rat sensory neurons are mediated by inhibition of M-type K+ channels and activation of Ca2+-activated Cl- channels. *J Clin Invest* **120:**1240–1252.
Liu B, Zhang C, and Qin F (2005) Functional recovery from desensitization of vanilloid receptor TRPV1 requires resynthesis of phosphatidylinositol 4,5-bisphosphate. *J Neurosci* **25:**4835–4843.
Mason L, Moore RA, Derry S, Edwards JE, and McQuay HJ (2004) Systematic review of topical capsaicin for the treatment of chronic pain. *BMJ* **328:**991.
Matta JA and Ahern GP (2007) Voltage is a partial activator of rat thermosensitive TRP channels. *J Physiol* **585(Pt 2):**469–482.
Matulef K, Howery AE, Tan L, Kobertz WR, Du Bois J, and Maduke M (2008) Discovery of potent CLC chloride channel inhibitors. *ACS Chem Biol* **3:**419–428.
Matulef K and Maduke M (2005) Side-dependent inhibition of a prokaryotic ClC by DIDS. *Biophys J* **89:**1721–1730.
Mohapatra DP and Nau C (2005) Regulation of Ca2+-dependent desensitization in the vanilloid receptor TRPV1 by calcineurin and cAMP-dependent protein kinase. *J Biol Chem* **280:**13424–13432.
Moiseenkova-Bell VY, Stanciu LA, Serysheva II, Tobe BJ, and Wensel TG (2008) Structure of TRPV1 channel revealed by electron cryomicroscopy. *Proc Natl Acad Sci USA* **105:**7451–7455.
Morita H, Honda A, Inoue R, Ito Y, Abe K, Nelson MT, and Brayden JE (2007) Membrane stretch-induced activation of a TRPM4-like nonselective cation channel in cerebral artery myocytes. *J Pharmacol Sci* **103:**417–426.
Numazaki M, Tominaga T, Toyooka H, and Tominaga M (2002) Direct phosphorylation of capsaicin receptor VR1 by protein kinase Cepsilon and identification of two target serine residues. *J Biol Chem* **277:**13375–13378.
Patwardhan AM, Akopian AN, Ruparel NB, Diogenes A, Weintraub ST, Uhlson C, Murphy RC, and Hargreaves KM. (2010) Heat generates oxidized linoleic acid metabolites that activate TRPV1 and produce pain in rodents. *J Clin Invest* **120:**1617–1626.
Poirot O, Berta T, Decosterd I, and Kellenberger S (2006) Distinct ASIC currents are expressed in rat putative nociceptors and are modulated by nerve injury. *J Physiol* **576(Pt 1):**215–234.
Roh EJ, Keller JM, Olah Z, Iadarola MJ, and Jacobson KA (2008) Structure-activity relationships of 1,4-dihydropyridines that act as enhancers of the vanilloid receptor 1 (TRPV1). *Bioorg Med Chem* **16:**9349–9358.
Schnizler K, Shutov LP, Van Kanegan MJ, Merrill MA, Nichols B, McKnight GS, Strack S, Hell JW, and Usachev YM (2008) Protein kinase A anchoring via AKAP150 is essential for TRPV1 modulation by forskolin and prostaglandin E2 in mouse sensory neurons. *J Neurosci* **28:**4904–4917.
Sutton KG, Garrett EM, Rutter AR, Bonnert TP, Jarolimek W, and Seabrook GR (2005) Functional characterisation of the S512Y mutant vanilloid human TRPV1 receptor. *Br J Pharmacol* **146:**702–711.
Szolcsányi J (1996) Capsaicin-sensitive sensory nerve terminals with local and systemic efferent functions: facts and scopes of an unorthodox neuroregulatory mechanism. *Prog Brain Res* **113:**343–359.
Vellani V, Mapplebeck S, Moriondo A, Davis JB, and McNaughton PA (2001) Protein kinase C activation potentiates gating of the vanilloid receptor VR1 by capsaicin, protons, heat and anandamide. *J Physiol* **534(Pt 3):**813–825.
Voets T, Droogmans G, Wissenbach U, Janssens A, Flockerzi V, and Nilius B (2004) The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature* **430:**748–754.
Vyklický L, Nováková-Tousová K, Benedikt J, Samad A, Touska F, and Vlachová V (2008) Calcium-dependent desensitization of vanilloid receptor TRPV1: a mechanism possibly involved in analgesia induced by topical application of capsaicin. *Physiol Res* **57(Suppl 3):**S59–S68.
Walker RL, Koh SD, Sergeant GP, Sanders KM, and Horowitz B (2002) TRPC4 currents have properties similar to the pacemaker current in interstitial cells of Cajal. *Am J Physiol Cell Physiol* **283:**C1637–C1645.
Wulff H (2008) New light on the "old" chloride channel blocker DIDS. *ACS Chem Biol* **3:**399–401.
Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sørgård M, Di Marzo V, Julius D, and Högestätt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400:**452–457.

Address correspondence to: Prof. Hai-Lin Zhang, Department of Pharmacology, Hebei Medical University, No. 361 East Zhongshan Rd., Shijiazhuang, Hebei 050017, China. E-mail: zhanghl@hebmu.edu.cn