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Title page

Agonist-dependent Potentiation of Vanilloid Receptor TRPV1 Function by Stilbene Derivatives

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.

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Running title page

Potentiation of TRPV1 by DIDS

Corresponding author: Prof. Hailin Zhang. Department of Pharmacology, Hebei Medical University, No.361 East Zhongshan Road, Shijiazhuang, Hebei, 050017, China. Tel.: 86 0311 86265562; Fax: 86 0311 86057291; E-mail: zhanghl@hebmu.edu.cn, z.hailin@yahoo.com

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tables----------0

figures----------9

references----------38

words in the *Abstract*----------224

words in the *Introduction*----------683

words in the *Discussion*----------1402

The abbreviations used are: CAP, capsaicin; DRG, dorsal root ganglia; TRPV1, transient receptor potential vanilloid type 1; AEA, anandamide/arachidonylethanolamide; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; SITS, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid; DADS, 4,4'-diaminostilbene-2,2'-disulfonic acid; DNDS, 4,4-dinitro-2,2-stilbenedisulfonic acid; HEK, human embryonic kidney; RR, Ruthenium Red;

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Abstract

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 modulation of TRPV1 function: TRPV1 function is modulated by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and analogs. We found that, in rat dorsal root ganglion (DRG) neurons, although DIDS did not induce activation of TRPV1 *per se* but increased drastically 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 to the effect of DIDS. SITS also blocked the low pH-induced tachyphylaxis. DIDS also potentiated the currents of TRPV1 channels expressed in HEK293 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 development of new modulator of TRPV1 function.

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Introduction

Since the first vanilloid (capsaicin) receptor, transient receptor potential vanilloid 1, (TRPV1) was cloned (Caterina et al., 1997), TRP channels, especially TRPV1, have been a focus of intensive studies (Bhave et al., 2003; Jordt et al., 2000; Jung et al., 1999; Karai et al., 2004; Patwardhan et al.). TRPV1 is a nonselective cation channel and is predominantly expressed 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 endocannibinoid anandamide (Ahern, 2003; Hwang et al., 2000; Jordt et al., 2000; Zygmunt et al., 1999). TRPV1 is considered to be one of the major molecular mechanisms for nociception (Bolcskei et al., 2005; Davis et al., 2000; Szolcsanyi, 1996).

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 exhibits fourfold symmetry and comprises two distinct regions: a large open basket-like domain, likely corresponding to the cytoplasmic N- and C-terminal portions, and a more compact domain, corresponding to the transmembrane 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 (Jordt et

al., 2000; Koplas et al., 1997; Lishko et al., 2007; Sutton et al., 2005). TRPV1 is believed to be activated by stimuli with different mechanisms. For example, capsaicin was found to bind to the intracellular site whereas proton was suggested to bind to the extracellular site of the channel (Jordt et al., 2000; Jung et al., 1999).

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 which include G protein-coupled receptors and receptors with intrinsic Tyr kinase activity. PKC-dependent phosphorylation can sensitize TRPV1 to capsaicin, protons or heat (Bhave et al., 2003; Vellani et al., 2001).

The activated TRPV1 currents exhibit two types of desensitization: acute desensitization and tachyphylaxis. In acute desensitization, the current amplitude diminished during a prolonged agonist application, whereas in tachyphylaxis, current amplitude diminished in the response to repeated agonist applications. It is still a debating issue in regard 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). Anyway, both types of desensitization seem to be sensitive to the concentration of free internal calcium (Koplas et al., 1997). Further studies suggest PIP_2 depletion, Ca^{2+}/calmodulin binding and

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dephosphorylation may contribute to tachyphylaxis (Lishko et al., 2007; Liu et al., 2005; Numazaki et al., 2002; Vyklicky et al., 2008).

The stilbene derivatives such as DIDS and SITS are known to inhibit Cl^- channels and transporters (Dietrich and Lindau, 1994; Jessen et al., 1986; Lai et al., 1996; Lane et al., 1999; Matulef and Maduke, 2005). Besides, DIDS were also reported to be able to inhibit TRPC4 (Walker et al., 2002) and TRPM4 (Morita et al., 2007) currents. In this study, we describe a novel function for these stilbene derivatives in 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 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**.**

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Materials and Methods

Rat DRG cell culture. Use of animal in this study was approved by Animal Care and Ethical Committee of Hebei Medical University (Shijiazhuang, China). DRGs were extracted from all 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) for 50 min, followed by another 20 min digestion with trypsin (2.5 mg/ml, Sigma). They were subsequently suspended at least twice in DMEM medium plus 10% fetal bovine serum (PAA, Austria) to stop digestion. Ganglia were then 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 hr.

cDNAs. Plasmids encoding rat TRPV1 (Gene ID: 83810) is subcloned in pcDNA3. TRPV1(E600Q), TRPV1(E648A) and TRPV1(S502A) mutants were produced by *Pfu* DNA polymerase with a QuickChange kit (Stratagene, La Jolla, CA, USA). The structure of the mutants was confirmed with DNA SEQUENCING.

HEK293 cell culture and transfection. HEK293 cells were cultured in Dulbecco's modified Eagle's mediums (DMEM) supplemented with 10% fetal bovine serum and antibiotics in a humidified incubator at 37^{o}C (5% CO_2). Cells were seeded on glass coverslips in a 24-multiwell plate and transfected when 60-70% confluence was reached. For transfection of 6 wells of cells, a mixture of 3 μg TRPV1, pERFP cDNAs and 3 μl Lipofectamine 2000 reagent (Invitrogen) were prepared in 1.2 ml of DMEM and incubated

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for 20 min. The mixture was then applied to the cell culture wells and incubated for 4 hr. Recordings were made 24 hr after cell transfection and cells were used within 72 hr.

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 1.5~2.5 MΩ when filled with internal solution. Currents were recorded using an Axon patch 700B amplifier and pClamp 10.0 software (Axon Instruments, CA), and were filtered at 2 KHz. For perforated patch recording, a pipette was first 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 (in mM): 160 NaCl, 2.5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 8 glucose, pH 7.4. The internal solution for perforated patch recording consisted of (in mM): 150 KCl, 5 $MgCl_2$, 10 HEPES, pH 7.4.

Chemicals. Capsaicin (CAP), DIDS, SITS, DADS, DNDS, anandamide (AEA), ruthenium red (RR) were all purchased from Sigma (St. Louis, MO, USA). Stock solution for capsaicin was made in ethanol, and all other drugs were made in DMSO. The stock solutions were stored at -20°C and diluted in the appropriate solution immediately before use. The final concentration of DMSO or ethanol was less than 0.1%.

Data analysis and statistics. Currents were analyzed and fitted using Clampfit 10.2 (Axon Instrument) and Origin 7.5 (Originlab Corporation) software. Results were expressed as mean ± S.E.M. Differences were analyzed with Student's paired/unpaired t test when appropriate, and were considered significant at $p < 0.05$.

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Results

DIDS enhances capsaicin-evoked TRPV1 current in adult rat DRG neurons. DIDS (Fig. S1A) has long been used as the chloride channel and anion transporter blocker. In preliminary experiments, we found DIDS to be able to potentiate capsaicin-induced currents in rat DRG neurons. Thus we decided to have a detailed investigation into this unexpected effect of DIDS. For this, adult rat DRG neurons and perforated patch clamp technique were used.

It has been shown that a long (1 hr) interval 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. In order to have a reliable assessment of DIDS effects 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 amplitude of capsaicin-activated currents (tachyphylaxis) was observed following the first application of 1 μM capsaicin when administrated with short duration (20 s) and multiple times (2 min interval) in DRG neurons (Fig. 1A). The average capsaicin-induced tachyphylaxis from multiple experiments was 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 co-applied with capsaicin after the capsaicin-induced currents had gone through substantial tachyphylaxis, the capsaicin-induced currents were greatly increased (Fig. 1C, 1D); this increase resulted from the potentiation of the capsaicin-induced currents because

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DIDS did not induce any currents when applied alone (100 μM, Fig. 1C). This was also 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 which were greater than the current amplitudes before the tachyphylaxis (Fig. 1C, 1D). The effects of DIDS on potentiation of capsaicin-induced current were concentration dependent, with an EC_{50} of 4.66 ± 0.77 μM (Fig. 1D, 1E). On average, at saturating concentration DIDS increased capsaicin-induced currents by more than two folds 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 was shown in Fig. 1F, and was compared with that in the absence of DIDS (dotted line). The percentage current reduction evoked by repetitive application of capsaicin without or with DIDS was not significantly different, indicating that DIDS do not affect the capsaicin-induced tachyphylaxis (Fig. 1F), which does not depend on the concentrations of capsaicin used (Fig. S2). DIDS did not affect the capsaicin-induced TRPV1 current recovery from tachyphylaxia (Fig. S3).

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 significantly shorten by the application of DIDS (2.13 ± 0.34 s, n=13, $p < 0.01$) (Fig. 1G).

The effects of DIDS on capsaicin-induced TRPV1 currents in the absence of extracellular Ca^{2+}. Ca^{2+} influx through the TRPV1 channel play important role in the acute

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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 TRPV1 currents not being desensitized, we study 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, the top panel). Under this circumstance, DIDS still potentiated the capsaicin-induced currents (Fig. 2A, the middle and the bottom panels). The non-desensitizing characteristic of 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, indeed the effects of DIDS were reversible. Thus capsaicin evoked similar amplitude 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. 2D, 2E); DIDS potentiated the capsaicin-induced currents with an EC_{50} of 4.88 ± 0.74 μM, Fig. 2E), similar to the EC_{50} in the presence of extracellular Ca^{2+} (Fig. 1E).

DIDS enhances TRPV1 current induced by endovanilloid anandamide. Anandamide (arachidonylethanolamide) is a powerful vasodilator and was originally isolated from brain as an endogenous cannabinoid receptor ligand (Zygmunt et al., 1999). In electrophysiology experiments, anandamide induces a capsazepine-sensitive inward current in

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TRPV1-expressing HEK cells and primary sensory neurons, which indicates that the vanilloid receptor may thus be another molecular target for endogenous anandamide (Zygmunt et al., 1999). This provides a likely molecular mechanism of anandamide and/or structurally-related lipids participating in the regulation of the nociception and vasodilation as vanilloid receptor ligands.

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

DIDS enhances low pH-evoked TRPV1 current and blocks the tachyphylaxis of the low pH-induced currents. Low pH 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. Secondly, lower pH (pH < 5.8) activates channel directly as agonist of TRPV1 at room temperature (Caterina et al., 1997; Jordt et al., 2000).

In this part, we tested whether DIDS could affect the low pH-evoked TRPV1 currents in DRG neurons.

Low pH-induced currents also showed similar tachyphylaxis on repetitive application of low pH (Fig. 4A, 4B) and were also 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). Actually, the low pH-induced currents were more sensitive to DIDS

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modulation than the capsaicin-induced currents were; 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 co-application of 100 μM DIDS (Fig. 4D) whereas the capsaicin-evoked currents were increased by 216 ± 13% of the control with co-application of 100 μM DIDS (Fig. 1E). Furthermore, DIDS almost completely abolished the tachyphylaxis of currents evoked by low pH (Fig. 4A, 4B), in sharp contrast with the no-effects of DIDS on the tachyphylaxis of the capsaicin-induced currents (Fig 1D, 1F). This effect of DIDS was maintained even in a long recording with more repetitive application of pH+DIDS (Fig. S4).

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

The acid-sensitive currents are not involved in the DIDS-potentiated currents induced by low pH. There exist acid-sensitive currents in DRG neurons, among them including the currents through acid-sensing ion channels (ASICs). ASICs are ligand-gated ion channels activated by extracellular proton. To date, six members of ASICs family have been identified. They are widely expressed in the peripheral and central nervous system and all ASIC subunits are present in DRG neurons (Alvarez de la Rosa et al., 2002; Poirot et al., 2006). Since acid-sensitive currents can be activated by low pH at the range as we used in above experiments, we proceeded to test whether currents through ASICs and other acid-sensitive

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components could contribute to the effects of DIDS. For this, broad-spectrum Ca^{2+} channel blocker, ruthenium red (RR) was used to block TRPV1 currents to isolate the acid-sensitive currents. As shown in Fig. 5A and 5B, RR almost abolished capsaicin-induced currents (97.3 ± 0.01%, n = 5) (Fig. 5B). We found, as we reported earlier, only subpopulation of DRG neurons responded to capsaicin stimulation (Liu et al., 2010). Among the cells we studied, 51.7% cells were the capsaicin-sensitive cell and other 48.3% neurons did not respond to capsaicin with the development of measurable currents (total numbers of cells were 118). On the other hand, low pH (pH 5.0) always induced inward currents regardless whether the neurons were capsaicin sensitive or insensitive (Fig. 5C, 5D). 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 which 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 from the acid-sensitive currents. The average amplitudes of these two currents were not greatly different (Fig. 5C and Fig. 5D, 146.7 ± 13.2 pA *vs* 222 ± 16 pA). Thus in the capsaicin sensitive neurons, low pH activated the acid-sensitive currents on top of the TRPV1 currents which were absent in the capsaicin insensitive currents. The acid-sensitive currents in the capsaicin insensitive neurons were inhibited by DIDS (Fig. 5E, 5F; 39.2 ± 0.18% of pre-DIDS level, n = 4), and the TRPV1 currents in the capsaicin sensitive neurons were potentiated (Fig. 5G, 5H, 566 ± 88 % pre-DIDS level). These data clearly demonstrates DIDS selectively sensitizes TRPV1 currents but not the acid-sensitive currents induced by low pH.

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SITS potentiates the low pH-evoked but not the capsaicin-evoked currents. We next investigated whether SITS, an analog of DIDS, could also act similarly as DIDS does. There is only minor structure difference between these two compounds (Fig. S1A, S1C). One of isothiocyano in DIDS is replaced by acetamido in SITS. However, SITS (100 μM) did not potentiate the capsaicin-evoked TRPV1 currents (Fig. 6A, 6B), neither did it alter the tachyphylaxis of the capsaicin-induced currents (Fig. 6C, 6D). On the other hand, SITS strongly and concentration dependently potentiated the low pH-induced currents (Fig. 6E, 6F). Similar to DIDS, SITS alone did not induce any currents, even at high concentration of 1 mM (n = 6, data not shown). Efficacy of SITS on the low pH-induced currents is comparable with that of DIDS (421 ± 68% *vs*. 448 ± 69%, for low pH + SITS and low pH + DIDS over the 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. In order to confirm 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 went substantial tachyphylaxis on repetitive application of capsaicin. DIDS, as it did in DRG neurons, potentiated the capsaicin-evoked TRPV1 current, but did not affect the tachyphylaxis of the capsaicin-induced currents (Fig. 7A, n=4). 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+} (Fig. 7B, n=4). The effects of DIDS were concentration dependent (Fig. 7C, 7D); DIDS potentiated the

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capsaicin-induced currents in the absence of Ca^{2+} with an EC_{50} of 3.21 ± 0.10 μM (Fig. 7D).

Lack of the tachyphylaxis of the capsaicin-induced TRPV1 currents in the absence of extracellular calcium enables us to study the effects of DIDS under repetitive application of capsaicin. Thus the effects of DIDS on the concentration-dependent activation of TRPV1 by capsaicin were tested. Co-application DIDS with capsaicin led to a leftward shift of the concentration-response curve for capsaicin activation of TRPV1 expressed in HEK293 cells (Fig. 8A, 8B, n=6~8).

To help understand the possibly mechanism for 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 (proton and capsaicin) could increase the voltage sensitivity of TRPV(Matta and Ahern, 2007; Voets et al., 2004). Fig. 8C shows representative current traces of TRPV1 expressed in HEK 293 cells, resulting from a family of voltage steps either under control conditions or in the presence of capsaicin (100 nM) or with co-application of 100 μM DIDS. The conductance and voltage (G-V) curves were fitted by Boltzmann function. 100 μM DIDS shifted the $V_{1/2}$ from 40±5 mV and -42±7 mV (Fig. 8D). However, the slope factors from 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 the agonists such as capsaicin (Bhave et al., 2003; Jordt et al., 2000; Numazaki et al., 2002). The effects of low pH and PKC are mediated through specific sites in TRPV1. We

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decided to investigate whether the effects of DIDS were also mediated through these sites. A Glu residue (E600) in TRPV1 was suggested to be the key regulatory site for low pH mediated modulation of TRPV1 function whereas the residue E648 may serve as the key site for low pH activation of TRPV1 (Jordt et al., 2000). And two Ser residues, S502 and S800 were suggested be involved in the potentiation of the TRPV1 current via PKC-mediated phosphorylation (Bhave et al., 2003; Numazaki et al., 2002). We tested the effects of DIDS on TRPV1 mutants involving above mentioned sites expressed in HEK293 cells. The data shown in Figure 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 compared with the wild type TRPV1 current (Fig. 9A, 9C), although their responses to low pH were greatly reduced (Fig. 9A, 9B). DIDS also potentiated the low pH-induced currents of E648A mutant to the similar extent as it did for the currents of wild type TRPV1 (Fig. 9D, 9E). We also tested the effect of DIDS on TRPV1(S502A), which should abolish the PKC-mediated TRPV1 potentiation. As shown in Fig 9F and 9G, the capsaicin-induced TRPV1(S502A) currents were potentiated by DIDS similarly as the wild type TRPV1 currents. Thus, DIDS potentiates the TRPV1 currents through a mechanism different from the known mechanism.

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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 on 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, whereas SITS selectively enhanced the TRPV1 currents induced by low pH. Furthermore, DIDS greatly reduced the tachyphylaxis of TRPV1 current 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 DIDS effects sheds some new insights into the modulation of TRPV1 function.

It is striking that the agonists (capsaicin or low pH)-induced TRPV1 currents are greatly potentiated by the stilbene derivatives even substantial tachyphylaxis of the currents has developed. In fact, all of the effects of DIDS and SITS were observed when the TRPV1 currents have desensitized to less than 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 the desensitization. This is truly remarkable in considering that the desensitized channels rarely respond to stimuli and they need long time to recover from the desensitization. Clearly, DIDS does not merely to recover the desensitized TRPV1 channels, since the non-desensitizing TRPV1 currents in the absence of extracellular Ca^{2+} are also potentiated by DIDS (Fig. 2A, 7B). Actually, as shown in Fig 1F, DIDS does not

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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 (Lishko et al., 2007; Liu et al., 2005). DIDS is not likely to affect cellular Ca^{2+}/calmodulin, membrane PIP_2 hydrolysis or channel phosphorylation. If any of these mechanisms are indeed 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 the 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, TRPV1 mutant that lacks the suggested phosphorylation site still responds to DIDS similarly as the wild type TRPV1 did (Fig. 9F, 9G). Thus it is likely that DIDS employs a novel mechanism in potentiating the TRPV1 currents. It is possibly that binding of DIDS to 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 (Matta and Ahern, 2007; Voets et al., 2004) we could not record appreciable size of TRPV1 currents expressed in HEK 293 cells when membrane was depolarized to up to 160 mV (Fig 8C). Furthermore, this property of TRPV1 was not changed by application of DIDS alone. Nonetheless, as it was reported before(Matta and Ahern, 2007) there was a voltage-dependent component of TRPV1 currents activated by capsaicin (Figure 8D). DIDS shifted the voltage dependent activation of capsaicin-induced

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currents to more negative potential without affect the slope factor of the G-V curve. In consideration that capsaicin could also concentration-dependently shifted the voltage-dependent TRPV1 currents to more negative potential(Matta and Ahern, 2007). we interpreted the effect of DIDS as increasing the sensitivity of TRPV1 to capsaicin, consistent with above discussion.

DIDS potentiated the desensitized capsaicin-activated TRPV1 currents but did not affect the process of current tachyphylaxis (Fig. 1F). On the other hand, DIDS potentiated the low pH-activated TRPV1 currents and abolished the current tachyphylaxis (Fig. 4A, 4B). 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 the low pH and the capsaicin-induced TRPV1 tachyphylaxis (Liu et al., 2005). Furthermore, while SITS potentiated the low pH-induced but not the capsaicin-induced TRPV1 currents in this study, the PKC-mediated sensitization of TRPV1 channel are non-selective for these two stimuli (Vellani et al., 2001). Thus, overall, we have following hypothesis for 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 big enough to discriminate for the selective binding and action of SITS on pH-induced activated state of TRPV1. Similarly, the mechanisms of tachyphylaxis for capsaicin- and low pH-activated TRPV1 currents could also be different. Thus it is not too difficult to understand why DIDS only abolished the tachyphylaxis of

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pH-activated but not capsaicin-activated TRPV1 currents. The above hypothesis are supported by the evidence from electrophysiological and mutagenesis studies, which demonstrate capsaicin binds to the intracellular domain of TRPV1 (Y511 and S512, located at an intracellular loop and TM3), whereas protons bind to the sites localized extracellularly (E648 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 their actions on modulation of TRPV1 functions. First, when both of two isothiocyanate groups in DIDS are replaced by primary amines, as for DADS (Fig. S1B), or replaced by nitro, as for DNDS (Fig. S1D), the capability to modulate TRPV1 function is lost (Fig. S5). Second, when one of the two isothiocyanate groups in DIDS is replaced by acetamido, as for SITS (Fig. S1C), the capability to potentiate capsaicin-activated TRPV1 currents, leaving only the ability to potentiate the pH-activated currents. These features of structural specificity for stilbene derivatives further support the notion that specific structure-related interaction between the stilbene derivatives and TRPV1 channels are necessary for 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 suggest that the isothiocyanate groups are not needed for 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 (DHP) 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,

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functions of 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 mechanisms underlying TRPV1 activation, desensitization and other properties of channel activities. Since 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 potentials. In fact, agonists of TRPV1 have been used and are evaluated to be used as analgesic agents (Mason et al., 2004). The logic behind this seemly paradoxical scenario is that the sustained opening of 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 like DIDS could be very valuable. First, it enhances greatly 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 noceceptive neurons will be increased. Secondly, since DIDS and SITS do not activate TRPV1 on their own and only potentiate the agonists-activated TRPV1 currents, they would be efficacious only at nerve endings where TRPV1 is activated by pain-inducing substances, rather than at all 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.

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Authorship Contributions

Participated in research design: X. Zhang, Liu, H. Zhang

Conducted experiments: X. Zhang, Du, G. Zhang, Jia, Chen, Huang

Performed data analysis: X. Zhang, H. Zhang

Wrote or contributed to the writing of the manuscript: X. Zhang, H. Zhang

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References

Ahern GP (2003) Activation of TRPV1 by the satiety factor oleoylethanolamide. *J Biol Chem* **278**(33):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 U S A* **99**(4):2326-2331.

Bhave G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS and Gereau RWt (2003) Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc Natl Acad Sci U S A* **100**(21):12480-12485.

Bolcskei K, Helyes Z, Szabo A, Sandor K, Elekes K, Nemeth J, Almasi R, Pinter E, Petho G and Szolcsanyi J (2005) Investigation of the role of TRPV1 receptors in acute and chronic nociceptive processes using gene-deficient mice. *Pain* **117**(3):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**(6653):816-824.

Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A and Sheardown SA (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* **405**(6783):183-187.

Dietrich J and Lindau M (1994) Chloride channels in mast cells: block by DIDS and role in

exocytosis. *J Gen Physiol* **104**(6):1099-1111.

Hwang SW, Cho H, Kwak J, Lee SY, Kang CJ, Jung J, Cho S, Min KH, Suh YG, Kim D and Oh U (2000) Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. *Proc Natl Acad Sci U S A* **97**(11):6155-6160.

Jessen F, Sjoholm 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**(3):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 U S A* **97**(14):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**(2):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**(9):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**(10):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**(2):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**(2):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**(6):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**(4):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**(19):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**(7446):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**(7):419-428.

Matulef K and Maduke M (2005) Side-dependent inhibition of a prokaryotic ClC by DIDS. *Biophys J* **89**(3):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**(14):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 U S A* **105**(21):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**(4):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**(16):13375-13378.

Patwardhan AM, Akopian AN, Ruparel NB, Diogenes A, Weintraub ST, Uhlson C, Murphy RC and Hargreaves KM Heat generates oxidized linoleic acid metabolites that activate TRPV1 and produce pain in rodents. *J Clin Invest* **120**(5):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**(20):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**(19):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**(5):702-711.

Szolcsanyi 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**(7001):748-754.

Vyklicky L, Novakova-Tousova K, Benedikt J, Samad A, Touska F and Vlachova 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**:S59-68.

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**(6):C1637-1645.

Wulff H (2008) New light on the "old" chloride channel blocker DIDS. *ACS Chem Biol* **3**(7):399-401.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di Marzo V, Julius D and

Hogestatt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400**(6743):452-457.

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Footnote

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Legends for figure

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 perforated patch clamp technique in the presence of 5 mM extracellular Ca^{2+}. Timing of the drugs application was 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) *vs* the numbers of capsaicin applications. The amplitudes of the activated currents decreased with the repetitive application 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 the capsaicin-sensitive neuron. D, The 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, The concentration-response curve for the DIDS-induced potentiation of TRPV1 currents. The EC_{50} is 4.66 ± 0.77 μM. DIDS was always co-applied 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 tachyphylaxis shown in B. Then the current amplitude induced by co-application of capsaicin and DIDS (CAP+DIDS) was normalized to this calculated current amplitude. F, The effects of DIDS on the tachyphylaxis of the capsaicin-induced TRPV1 currents. The current amplitudes were normalized to the current amplitudes obtained with the

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first co-application of capsaicin and DIDS. G, The effects of DIDS on the activation kinetics of the 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.

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, The representative trace of the TRPV1 current by short (20 s), repetitive application of 1 μM capsaicin or with co-application of 3 or 10 μM DIDS. B, The effects of DIDS were totally reversible. 5 min indicates the time for washout of drugs. C, The summary data of B. D, The concentration dependent effects of DIDS on the capsaicin-induced currents in the Ca^{2+}-free extracellular solution. E, The 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.

Fig. 3. DIDS enhances the TRPV1 currents evoked by endovanillold anandamide. Whole-cell currents were recorded at a holding potential of -60 mV in the presence of 5 mM extracellular Ca^{2+}. A, The representative trace of the TRPV1 current induced by 10 μM anandamine (AEA) or by co-application of 10 μM DIDS and 10 μM anandamine. 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, Co-application of DIDS with anandamide increased the currents, but did not prevent the desensitization of the currents. D, The summarized effects of DIDS (10 μM) on the desensitization of the

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anandamide-induced TRPV1 currents. The current amplitudes were normalized to the current amplitudes obtained with the first co-application of DIDS with capsaicin or anandamine.

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 application of low pH solution (pH 5.0) and the effects of DIDS (100 μM) were shown. Co-application of DIDS with capsaicin increased the currents evoked by low pH, and abolished the desensitization of the currents with repetitive application of low pH (tachyphylaxia). B, The 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 co-application of the low pH and DIDS. C, The concentration dependent potentiation of the low pH-induced TRPV1 currents by DIDS. D, The concentration-response curve for the DIDS-induced potentiation of TRPV1 currents. The EC_{50} is 1.83 ± 0.29 μM. E, The effects of DIDS on the activation kinetics of the low pH-induced currents. The rising phases of low pH-activated currents in the absence and presence of DIDS were fitted with a single exponential function.

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, The capsaicin-induced currents were greatly inhibited by ruthenium red (RR, 10 μM). B, Summary data for A. The currents were

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normalized to the capsaicin-induced currents. C, The effects of RR on the low pH (pH 5.0)-induced currents from the capsaicin-sensitive neuron. D, The effects of RR on the low pH-induced currents from the capsaicin-insensitive neuron. RR did not inhibit the currents E, DIDS (100 μM) markedly inhibited the low pH-induced currents from the capsaicin-insensitive neuron. F, Summary of DIDS effects as shown in E. G, DIDS (100 μM) markedly enhanced the low pH-induced currents from the capsaicin-sensitive neuron, 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.

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 Ca^{2+}-free extracellular solution. A, SITS did not enhance the capsaicin-induced currents. B, 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 show in E. $^{**}P < 0.01$. G: SITS partially blocked the tachyphylaxis of the low pH-induced currents.

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, The representative trace of the TRPV1 current induced by short (10 s), repetitive application of 1 μM capsaicin or co-application 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 panel). Normalized summary

data were shown in the right panel. C, The concentration dependent potentiation of the capsaicin-induced currents by DIDS in the Ca^{2+}-free extracellular solution. D, The 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 activation of TRPV1 and potentiates the TRPV1 currents in a voltage dependent manner. The capsaicin-induced TRPV1 currents were recorded in HEK 293 cells. Whole-cell currents were recorded at a holding potential of -60 mV in the Ca^{2+}-free extracellular solution. A, The representative traces of the currents induced by different concentration of capsaicin in the absence (upper panel) or presence (lower panel) of 100 μM DIDS in the Ca^{2+}-free extracellular solution. B, The concentration–response relationships for capsaicin with or without co-application of 100 μM DIDS. Data are fitted with Hill equation (n = 6~8). EC_{50} are shown. The Hill coefficient is 1.48 for capsaicin alone and 2.08 for capsaicin+DIDS. C, The 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-d, and the representative traces of control, in the presence of 100 nM capsaicin or with co-application of 100 μM DIDS induced by the protocol were shown in the upper panel. D, Boltzmann fits to the conductances obtained from steady state currents at the end of the pulse. The $V_{1/2}$ values for 100 nM capsaicin or with co-application of 100 μM DIDS were shown. The slope factor is 63 mV for capsaicin alone and 69 mV for capsaicin+DIDS

Fig. 9. Mutations that affect the pH and PKC mediated modulation of TRPV1 currents

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do not affect the DIDS mediated potentiation of TRPV1 currents. Whole-cell currents were recorded at a holding potential of -60 mV in the Ca^{2+}-free extracellular solution. A, The residues of TRPV1 (E600, E648) related to pH modulation were not involved in DIDS potentiation of capsaicin-induced TRPV1 currents. B, The ratio of low pH (pH 5.0)- vs capsaicin (1 μM)-activated currents. C, The summary data for the effects of DIDS shown in A. D and E, The residue (E648) of TRPV1 related to pH modulation were not involved in DIDS potentiation of pH-induced TRPV1 currents. F and G, The residue (S502) of TRPV1 related to PKC modulation were not involved in DIDS potentiation of capsaicin-induced TRPV1 currents.

Figure 1

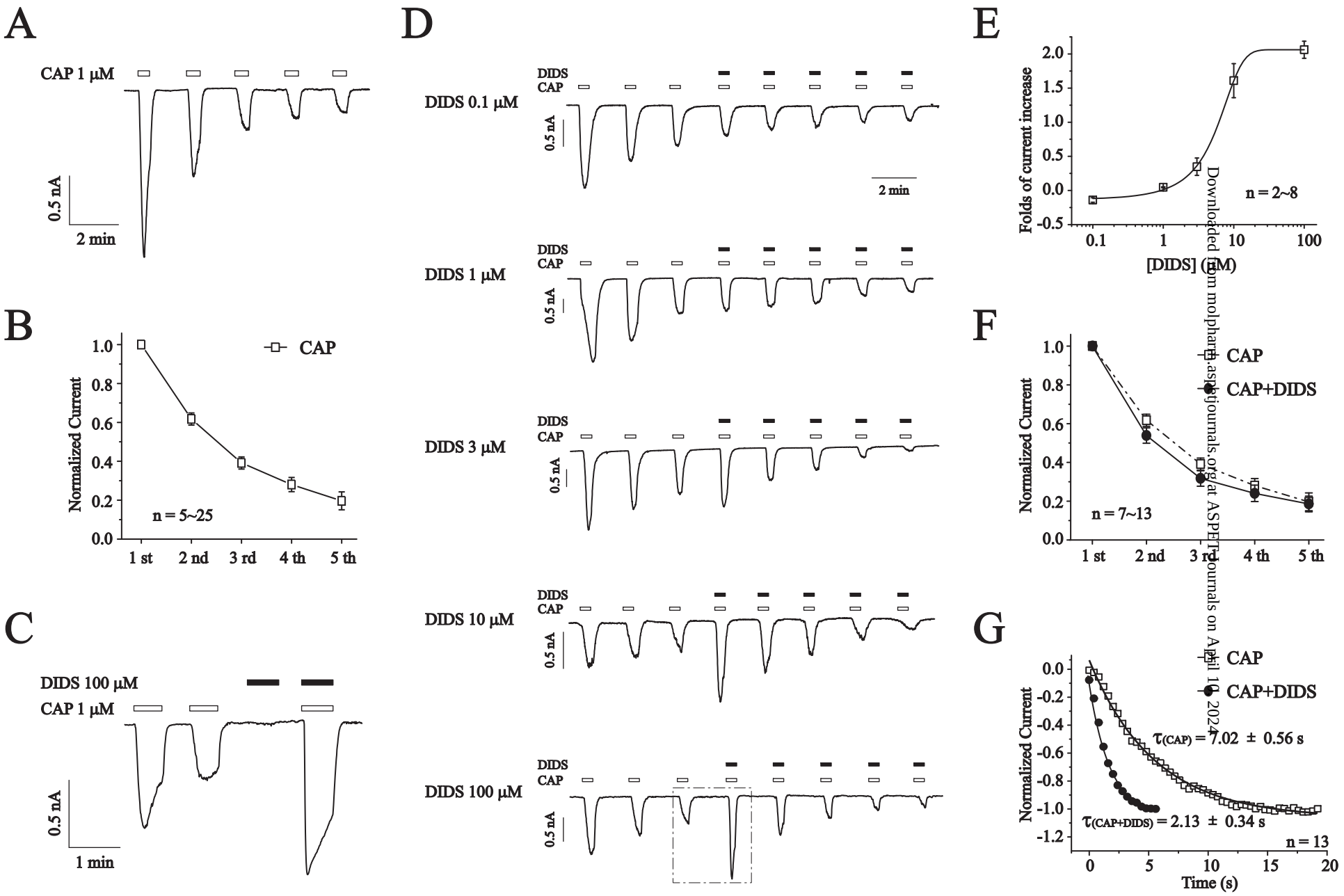
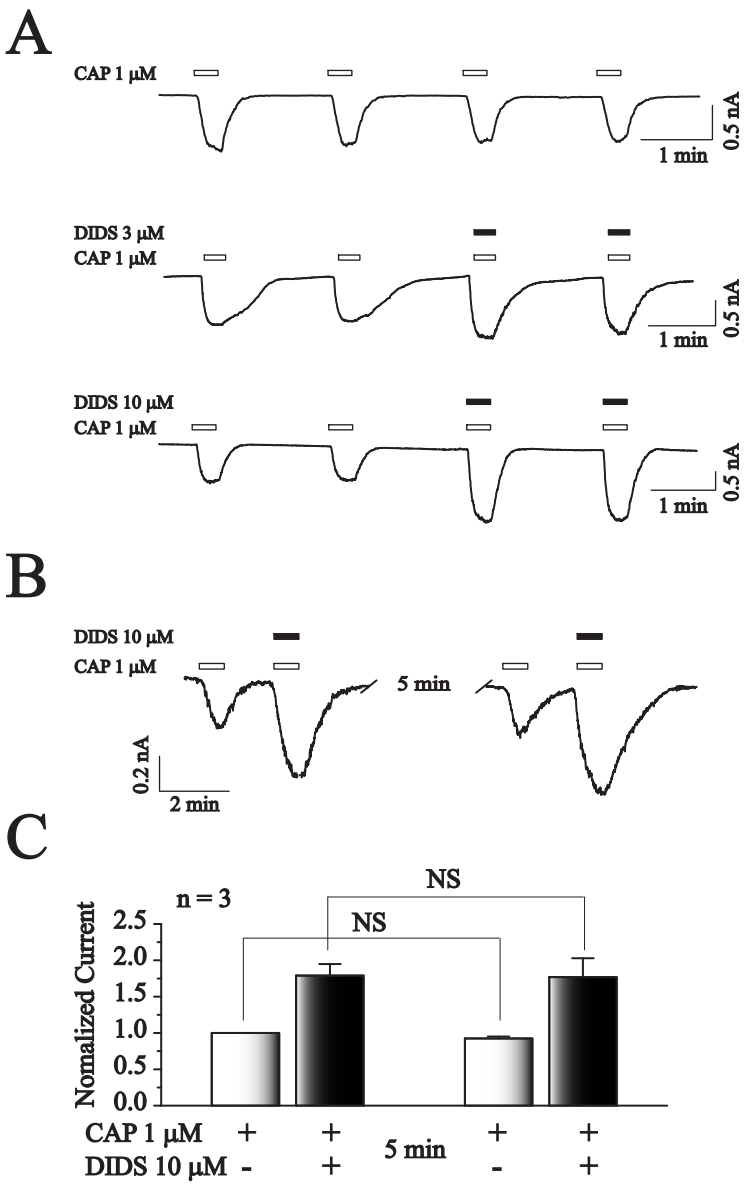


Figure 2



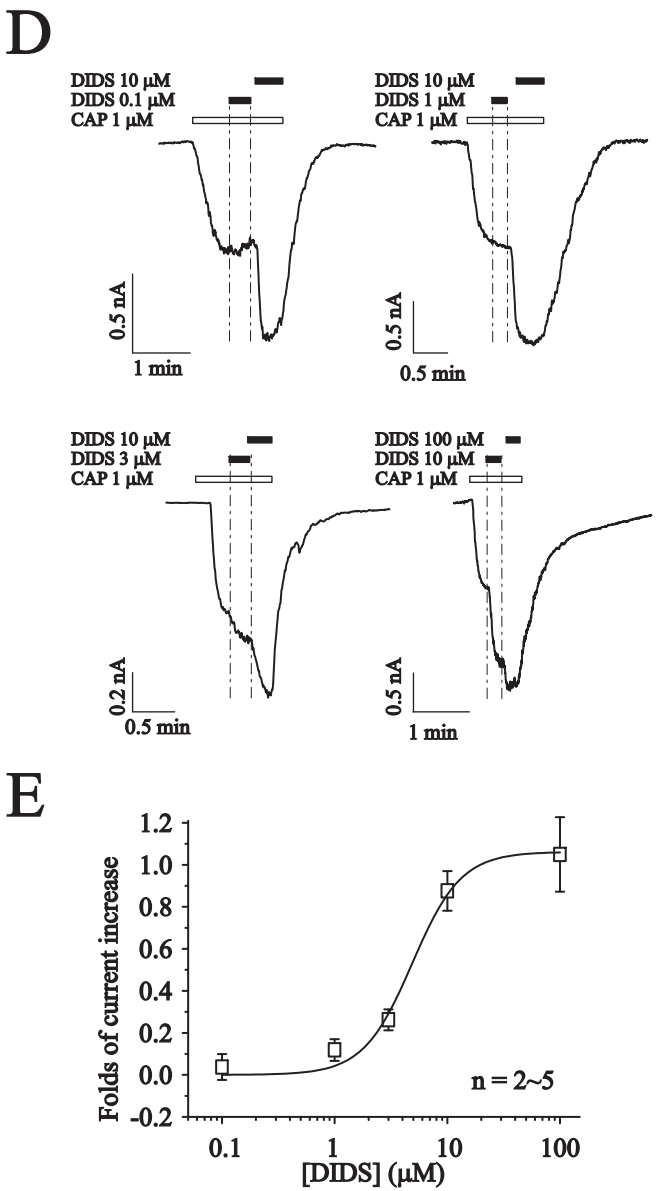


Figure 3

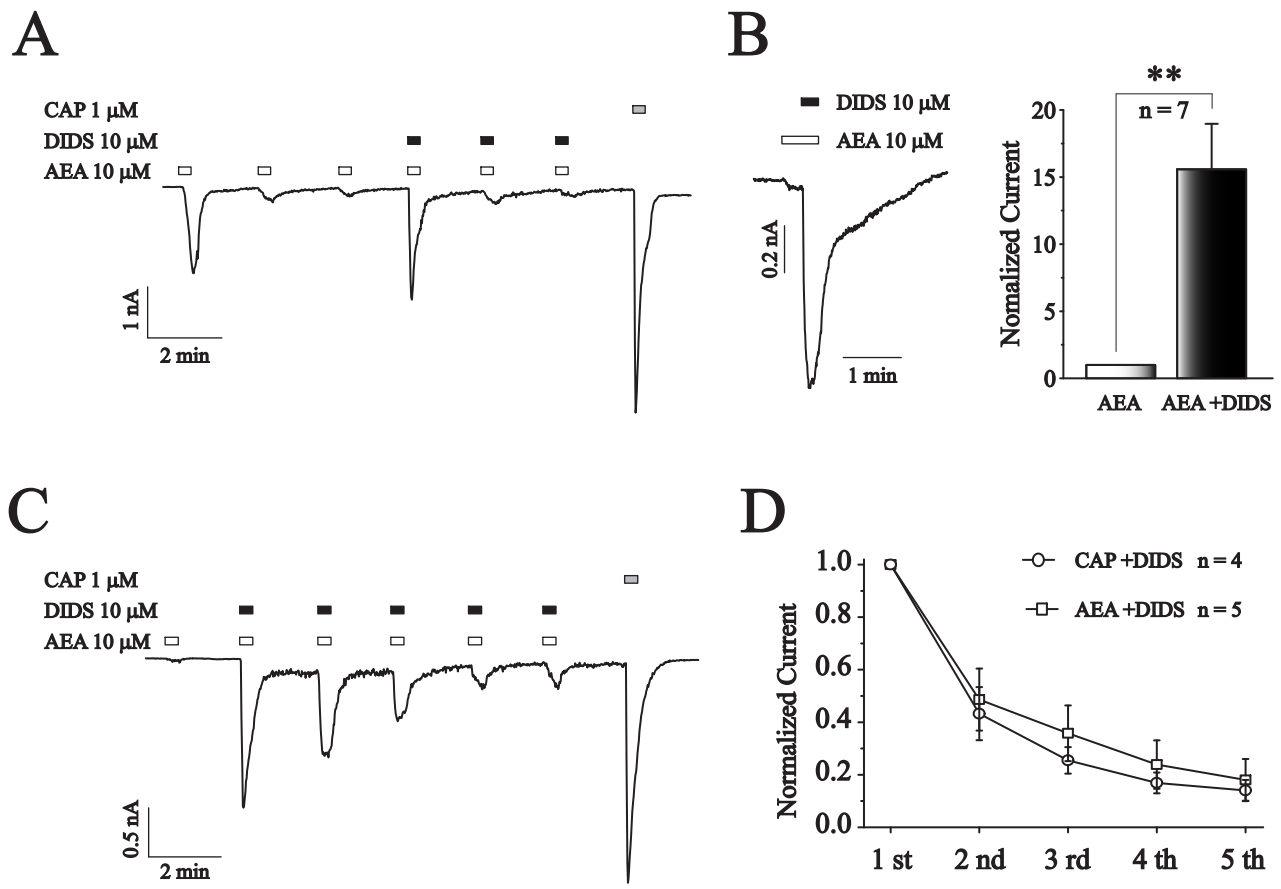


Figure 4

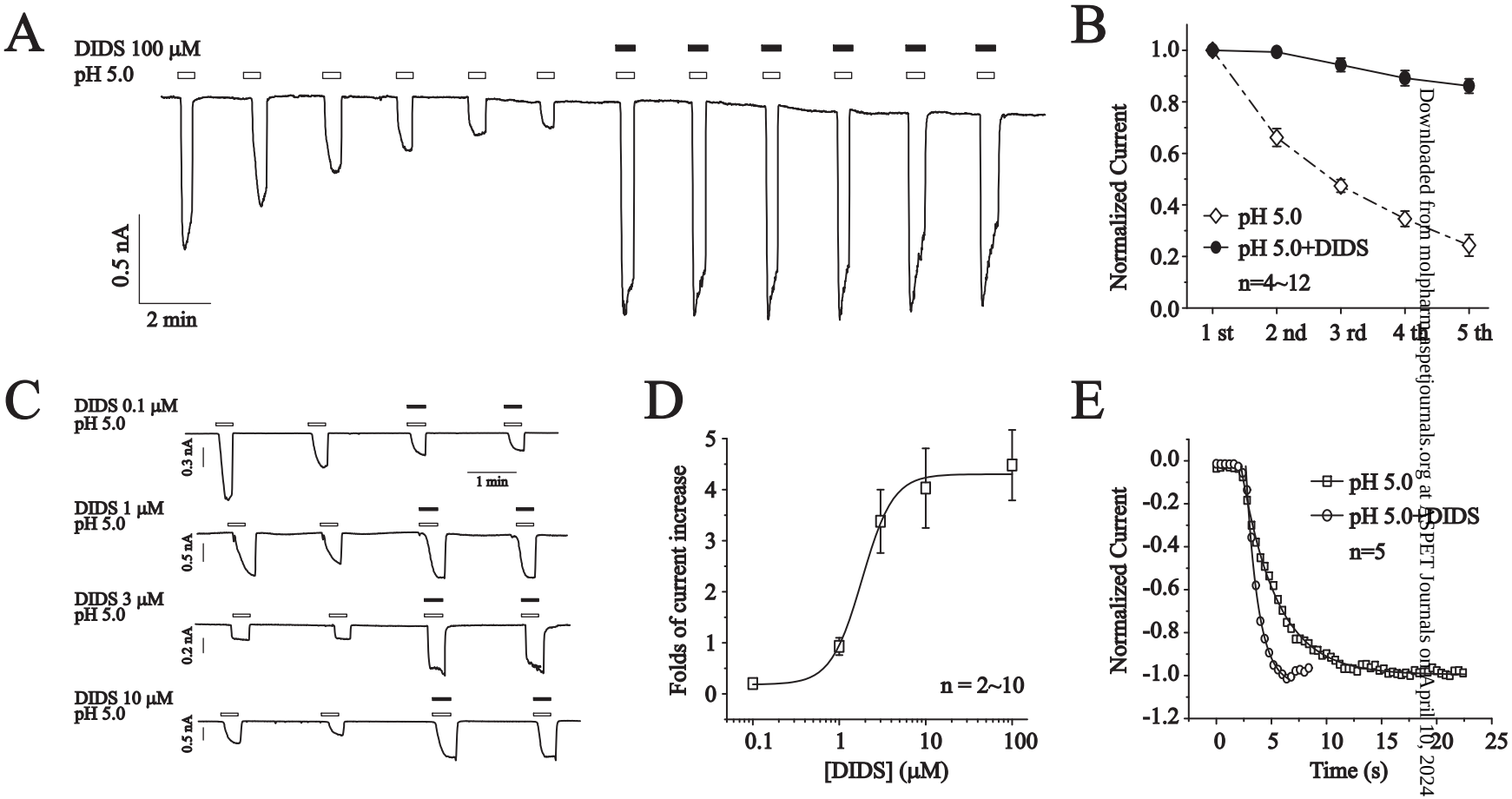


Figure 5

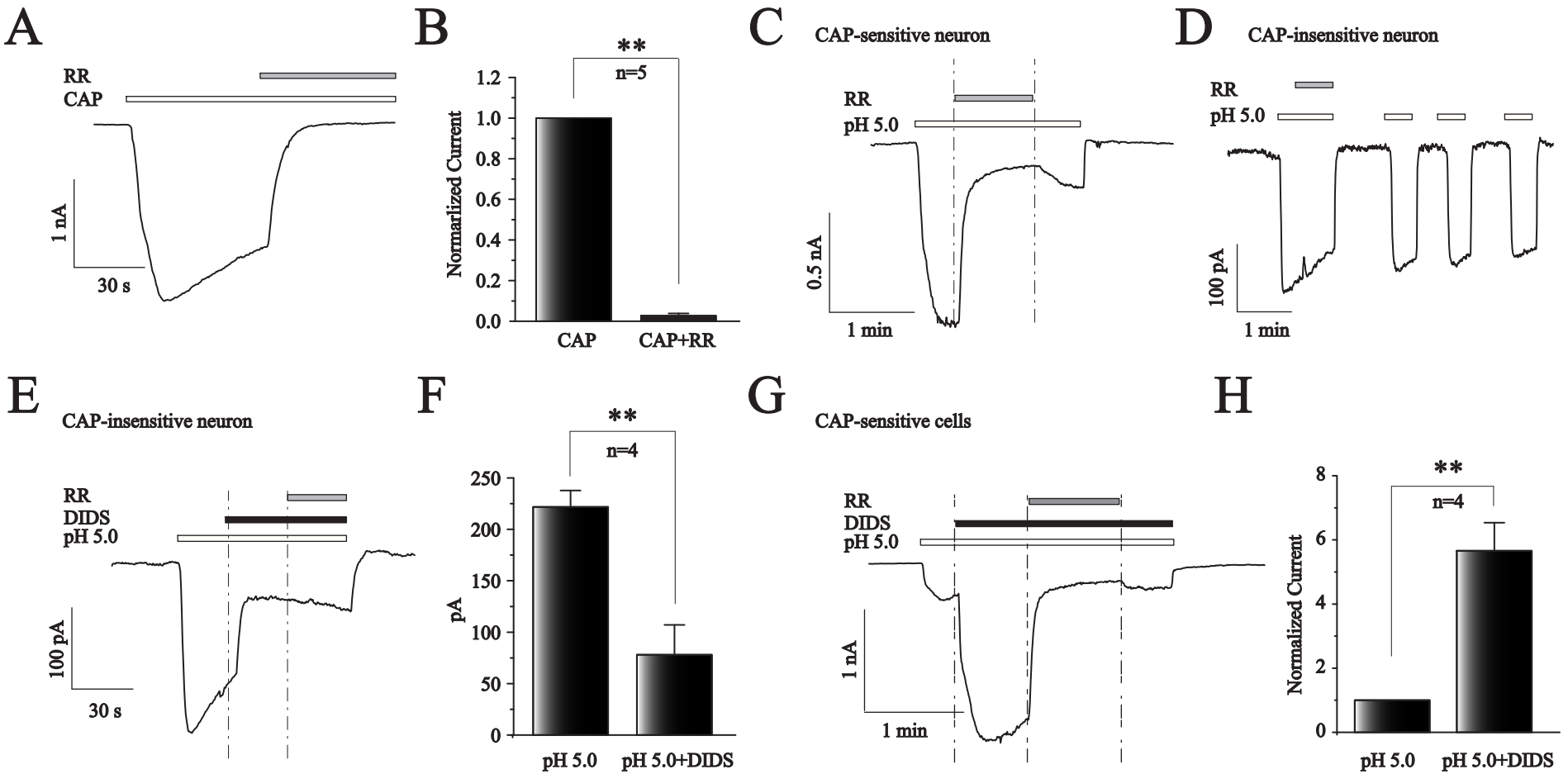

A
RR
CAP
1 nA
30 s
B
**
n=5
Normarlized Current
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CAP
CAP+RR
C
CAP-sensitive neuron
RR
pH 5.0
0.5 nA
1 min
D
CAP-insensitive neuron
RR
pH 5.0
100 pA
1 min
E
CAP-insensitive neuron
RR
DIDS
pH 5.0
100 pA
30 s
F
**
n=4
pA
250
200
150
100
50
0
pH 5.0
pH 5.0+DIDS
G
CAP-sensitive cells
RR
DIDS
pH 5.0
1 nA
1 min
H
**
n=4
Normalized Current
8
6
4
2
0
pH 5.0
pH 5.0+DIDS

Figure 6

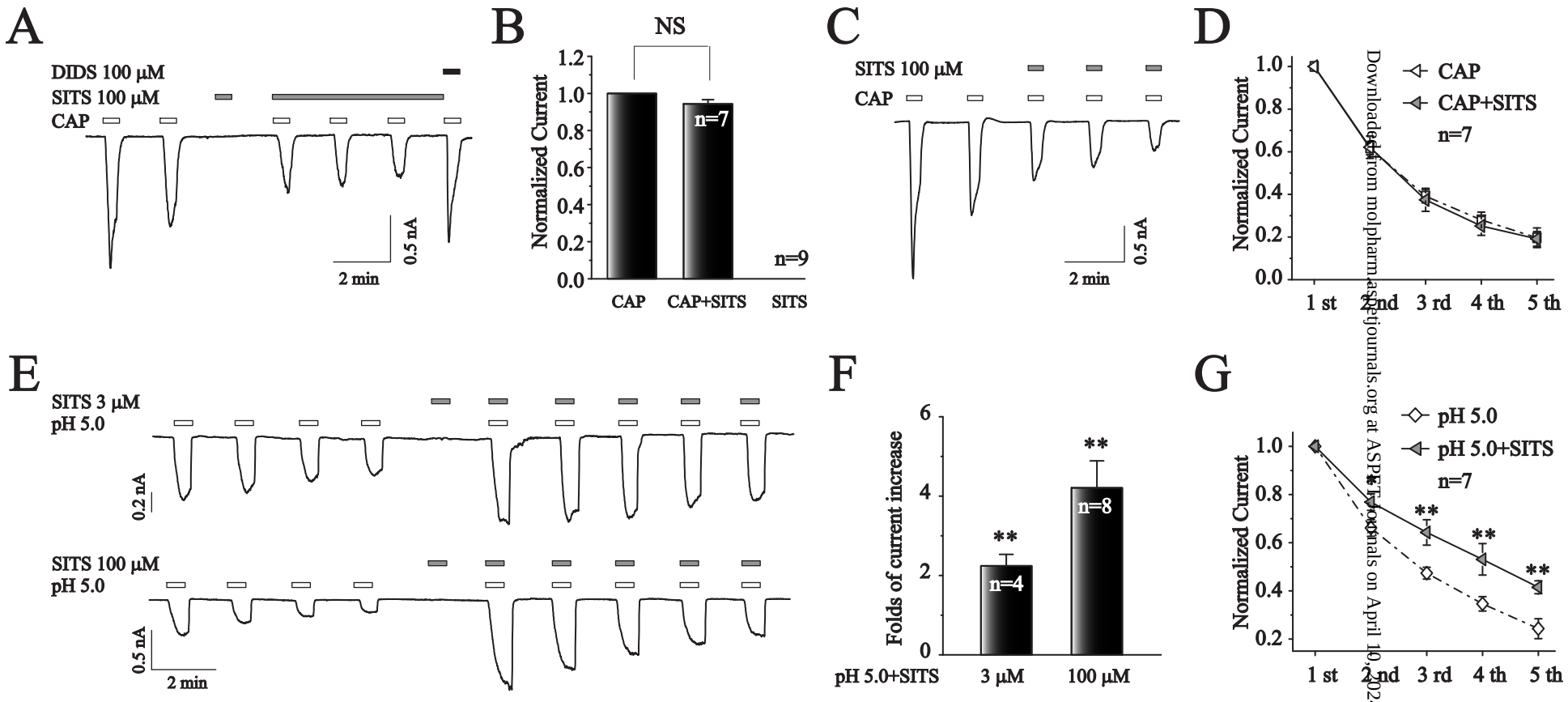


Figure 7

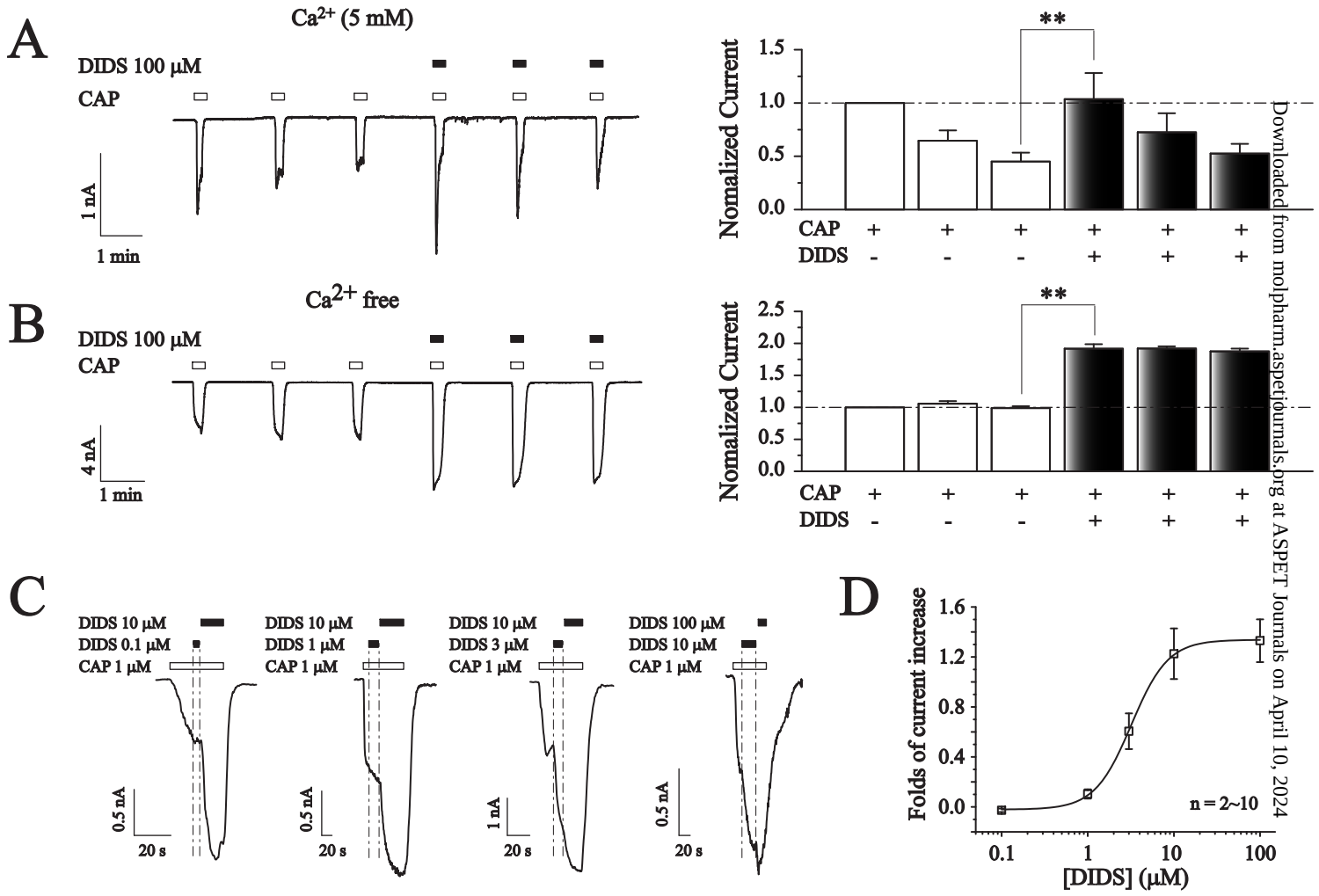


Figure 8

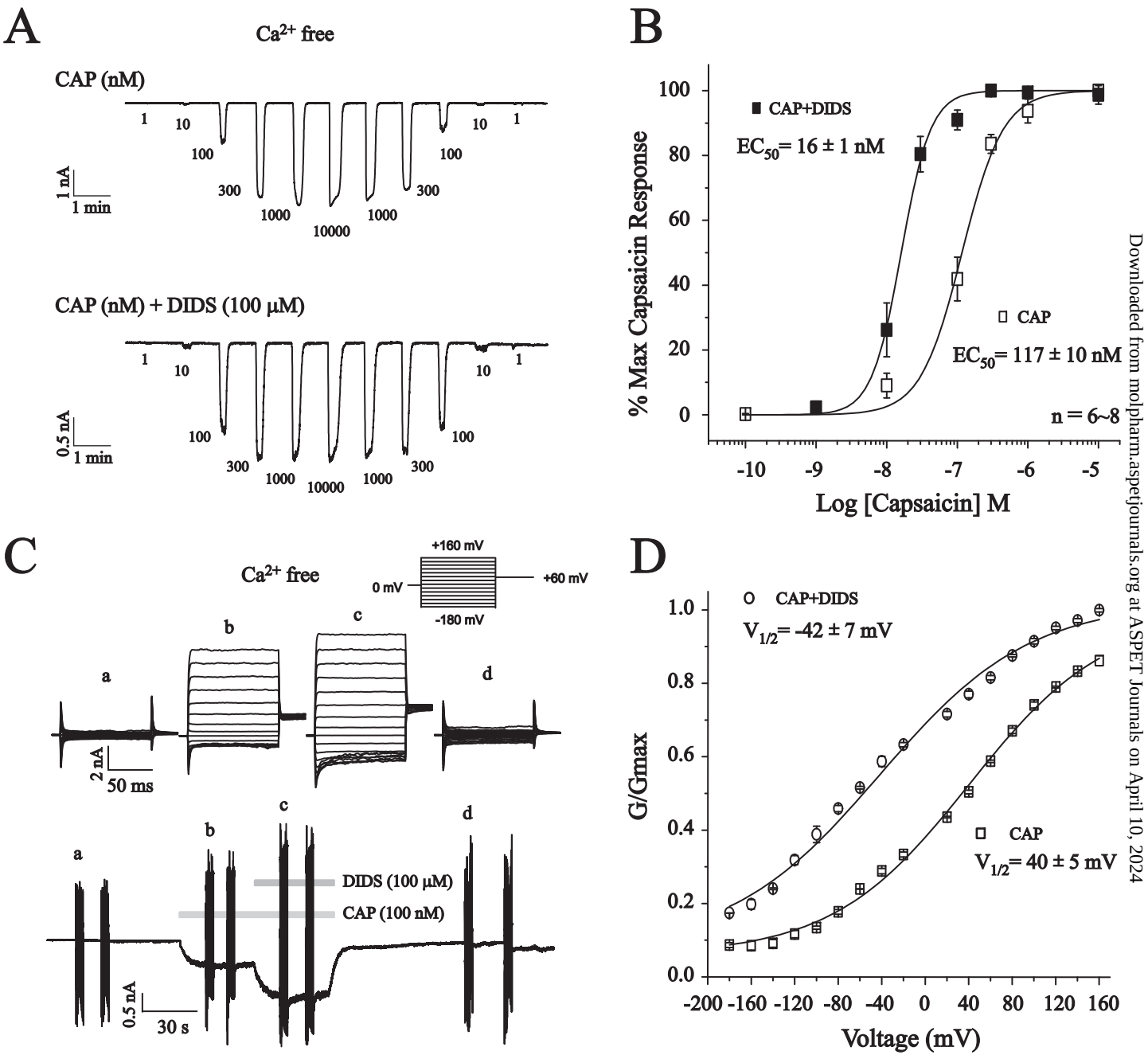


Figure 9