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Mechanisms of TRPV1 activation and sensitization by allyl isothiocyanate

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List of non-standard abbreviations:

$[Ca^{2+}]_i$: Intracellular Ca^{2+} concentration

AITC: Allyl isothiocyanate

Caps: Capsaicin

CCP: Complementary cumulative probability

CGRP: Calcitonin gene-related peptide

CPZ: Capsazepine

DRG: Dorsal root ganglion

GDNF: Glial cell line-derived neurotrophic factor

GFP: Green fluorescent protein

HEK: Human embryonic kidney

hTRPV1: Human transient receptor potential vanilloid 1

PCR: Polymerase chain reaction

mTRPV1: Mouse transient receptor potential vanilloid 1

MTSEA: 2-aminoethyl methanethiosulfonate hydrobromide

NT4: Neurotrophic factor 4

rTRPV1: Rat transient receptor potential vanilloid 1

TRP: Transient receptor potential

TRPA1: Transient receptor potential ankyrin 1

TRPV1: Transient receptor potential vanilloid 1

WT: Wild type

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Abstract

Allyl isothiocyanate (AITC, aka mustard oil) is a powerful irritant produced by *Brassica* plants as a defensive trait against herbivores and confers pungency to mustard and wasabi. AITC is widely used experimentally as inducer of acute pain and neurogenic inflammation, which are largely mediated by the activation of nociceptive cation channels TRPA1 and TRPV1. Although it is generally accepted that electrophilic agents activate these channels through covalent modification of cytosolic cysteine residues, the mechanism underlying TRPV1 activation by AITC remains unknown. Here we show that, surprisingly, AITC-induced activation of TRPV1 does not require interaction with cysteine residues, but is largely dependent on S513, a residue that is involved in capsaicin binding. Furthermore, AITC acts in a membrane-delimited manner and induces a shift of the voltage dependence of activation towards negative voltages, which is reminiscent of capsaicin effects. These data indicate that AITC acts through reversible interactions with the capsaicin binding site. Additionally, we show that TRPV1 is a locus for cross sensitization between AITC and acidosis in nociceptive neurons. Furthermore, we show that residue F660, which is known to determine the stimulation by low pH in human TRPV1, is also essential for the cross sensitization of the effects of AITC and low pH. Taken together, these findings demonstrate that not all reactive electrophiles stimulate TRPV1 via cysteine modification and help understanding the molecular bases underlying the surprisingly large role of this channel as mediator of the algesic properties of AITC.

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Introduction

TRPV1 and TRPA1, two members of the Transient Receptor Potential (TRP) superfamily of cation channels, are expressed in sensory nerve endings, where they act as molecular sensors of noxious thermal and chemical stimuli (Bautista et al., 2006; Caterina et al., 2000; Caterina et al., 1997; Jordt et al., 2004; Kwan et al., 2006). TRPV1 is activated by heat, low pH and by a variety of endogenous and exogenous compounds, including vanilloids such as the hot pepper compound capsaicin (Caterina et al., 1997; Tominaga et al., 1998). TRPA1 is directly activated by cold (Karashima et al., 2009; Sawada et al., 2007; Story et al., 2003) and by a wide variety of pungent chemicals, including menthol (Karashima et al., 2007), nicotine (Talavera et al., 2009), isoflurane (Matta et al., 2008), among many others (Nilius et al., 2012). TRPA1 is also activated by highly reactive electrophilic compounds such as cinnamaldehyde (Bandell et al., 2004), allyl isothiocyanate (AITC; aka mustard oil) (Jordt et al., 2004), acrolein (Bautista et al., 2006), endogenous aldehydes (Andersson et al., 2008; Macpherson et al., 2007; Trevisani et al., 2007) and aldehydes contained in cigarette smoke (Andre et al., 2008).

Capsaicin and AITC have been extensively used to probe the *in vivo* function of TRPV1 and TRPA1, respectively (see for example: Bautista et al., 2006; Caterina et al., 2000; Kwan et al., 2006). However, while the specificity of capsaicin for TRPV1 remains unchallenged, recent studies demonstrated that TRPA1 is not the sole molecular target of AITC. For instance, Kwan *et al.* (2006) showed that *Trpa1* KO mice have significant aversion towards AITC and residual nociceptive response to intraplantar injection of this compound. Furthermore, Ohta *et al.* (2007) reported that AITC activates porcine TRPV1 and suggested that inflammatory and nociceptive responses to this compound *in vivo*

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could be partly mediated by TRPV1 activation. Finally, it was recently showed that AITC activates mouse and human TRPV1, especially in recording conditions that better resembled experimental settings *in vivo*, such as prolonged application of pharmacologically relevant concentrations at physiological temperatures (Everaerts et al., 2011). Consistently with these data, it was also found that TRPV1 has a large relative role in several effects of AITC *in vivo* and *ex vivo*, including bladder irritation, CGRP release and acute pain. In contrast, TRPA1 was shown to be more important for aversion responses in a drinking test and for AITC-induced acute inflammation (Everaerts et al., 2011).

Although these data strongly suggest that both TRPV1 and TRPA1 are required for normal nociceptive responses to AITC in mice, they also indicate that more studies are needed to clarify the precise role of these channels in different experimental paradigms over a wide range of pharmacologically relevant concentrations. In addition, two other questions remain open, the first regarding the mechanisms underlying AITC-induced activation of TRPV1 and the second concerning the possibility that AITC sensitizes TRPV1 to low extracellular pH. The latter question is particularly important, as it relates to the mechanisms by which AITC induces neurogenic inflammation (Grant et al., 2005; Inoue et al., 1997; Jancso et al., 1968), where extracellular acidosis is a key feature (Holzer, 2009; Julius and Basbaum, 2001). We therefore set out to investigate the mechanism by which AITC activates TRPV1, and whether AITC interacts with low pH. We provide compelling evidence that AITC activates TRPV1 in a membrane-delimited manner by interacting with the binding site for capsaicin and demonstrate that TRPV1 is a locus for cross sensitization between AITC and acidosis in sensory neurons.

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Materials and Methods

Culture and transfection of HEK-293 cells

Human embryonic kidney cells, HEK-293, were seeded on 18 mm glass coverslips coated with poly-L-lysine (0.1 mg/ml) and grown in Dulbecco's modified Eagles medium containing 10% (v/v) fetal calf serum, 2 mM L-glutamine, 2 U/ml penicillin and 2 mg/ml streptomycin at 37 °C in a humidity controlled incubator with 10% CO_2. HEK-293 cells were transiently transfected with either human (ENSG00000196689) or mouse (ENSMUSG00000005952) TRPV1 cDNAs cloned into the pCAGGSM2-IRES-GFP vector (Meseguer et al., 2008), using Trans-IT-293 reagents (Mirus, Madison, MI, USA). All TRPV1 mutants were obtained using the standard PCR overlap extension technique (Ho et al., 1989) and verified by sequencing. For rat TRPV1 experiments, HEK293 cells were co-transfected with pcDNA3.1 vector expressing corresponding rat TRPV1 cDNA (ENSRNOG00000019486) (Caterina et al., 1997) or the $C18^-$ rat TRPV1 mutant (Salazar et al., 2008) and the pEGFP-C1 vector. For the human TRPV1 and the F660A and F660S mutants we used the vectors constructed by Aneiros *et al.* (2011). In all experiments transfected cells were identified by GFP expression and sensitivity to the TRPV1 agonist capsaicin or low extracellular pH.

Patch-clamp experiments

For current recordings, coverslips with cells were placed in the stage of an inverted microscope (Olympus IX70) and rinsed for a few minutes with Krebs (stabilization) solution, containing (in mM): 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, 10 HEPES and titrated to pH 7.4 with 1N NaOH. TRPV1 currents were recorded in the whole-cell and inside-out configurations, using an extracellular solution containing (in

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mM): 150 NaCl, 5 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES and was titrated to 7.4 with NaOH. The intracellular solution contained (in mM): 150 NaCl, 10 HEPES, 5 EGTA, 1 $MgCl_2$ and was titrated to pH 7.2 with NaOH.

Currents were recorded using the patch-clamp technique using an EPC-7 (LIST Electronics, Darmstadt, Germany) amplifier and filtered with an eight-pole Bessel-filter (Kemo, Bekenham, UK). For control of voltage-clamp protocols and data acquisition, we used an IBM-compatible PC with a TL-1 DMA interface (Axon Instruments) and the software pCLAMP (Version 9.0, Axon Instruments, Foster City, CA, USA). Bath solutions were perfused by gravity via a multi-barreled pipette. Patch pipettes were pulled from Vitrex capillary tubes (Modulohm, Herlev, Denmark) using a DMZ-Universal puller (Zeitz-instruments, Augsburg, Germany). An Ag-AgCl wire was used as reference electrode. Adequate voltage control was achieved by using low pipette resistances (1-2.5 MΩ) and series resistance compensation to the maximum extent possible (40-50%). Membrane capacitive transients were electronically compensated. Current traces were filtered at 2.5-5 kHz and digitized at 5-10 kHz. The temperature of the bath solution was controlled as previously described (Voets et al., 2004).

Currents were routinely recorded every 2 s during the application of 500 ms-lasting voltage ramps from to -100 mV to +160 mV with a holding potential of 0 mV. The voltage dependence of channel activation was studied using a voltage-step protocol consisting of increasing depolarizations in the range from -100 to +200 mV from a holding potential of 0 mV followed by an invariant step to +60 mV (or -60 mV for experiments with capsaicin). Activation curves were obtained from instantaneous currents at the beginning of the pulse to +60 mV (-60 mV) determined using mono-

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exponential fits (Talavera and Nilius, 2011). These curves were fitted with Boltzmann function of the form: $I_{inst}(+60) = \frac{I_{Max}}{1+\exp\left(-\left(V-V_{act}\right)/s_{act}\right)}$, where I_{Max} is the current at very positive potentials, V_{act} is the voltage for half-maximal activation and s_{act} is the slope factor. The lack of saturation of the open probability at positive potentials in control conditions made it impossible to estimate the fitting parameters independently. To overcome this limitation, the value of I_{Max}, which is proportional to the maximal whole-cell conductance for TRPV1, and s_{act}, were assumed not to be affected by AITC. In practice, data in control and AITC were simultaneously fit with values for I_{Max} and s_{act} being shared in both conditions. Data were analyzed using WinASCD (G. Droogmans; KU Leuven, Belgium).

Animals

All animal experiments were carried out in accordance to the European Community Council guidelines and were approved by the local ethics committee of the Katholic University Leuven. WT 8-14 weeks old C57Bl/6 mice were obtained from Janvier (France). TRPA1 (Karashima et al., 2009) and TRPV1 mice were backcrossed at least 10 generations in C57Bl6/J background. Double *Trpa1/Trpv1* KO mice were generated by crossing these single *Trpa1* and *Trpv1* KO mice. The mice were maintained under standard conditions at a 12-hour light/12-hour dark cycle with free access to food pellets and tap water.

Isolation and culture of dorsal root ganglion neurons

Dorsal root ganglion (DRG) neurons from adult (postnatal weeks 8–12) from wild type (WT) C57Bl/6J and *Trpa1* KO mice were cultured using a variant of a method previously described (Descoeur et al., 2011). In brief, for each series of experiments 3 to 4 mice

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were killed by cervical dislocation and DRGs were bilaterally excised under a dissection microscope. The ganglia were washed in 10% fetal calf serum Neurobasal A medium (basal medium) and then incubated at 37 °C in a mix of collagenase 1 mg/ml (Gibco) and dispase 2.5 mg/ml (Gibco) for 45 min. Digested ganglia were gently washed twice with basal medium and mechanically dissociated by mixing with syringes fitted with increasing needle gauges. Neurons were seeded on poly-L-ornithine/laminin-coated glass bottom chambers (Fluorodish WPI) and cultured for 12-18 hours at 37 °C in B27 supplemented Neurobasal A medium (Invitrogen) containing GDNF 2 ng/ml (Invitrogen) and NT4 10 ng/ml (Peprotech).

Microfluorimetric intracellular Ca^{2+} imaging experiments

Before the measurements cells were incubated with 2 μM Fura-2AM ester for 30 min at 37 °C. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured on an Olympus (Tokyo, Japan) Cell Mˆ system. Fluorescence was measured during excitation at 340 and 380 nm, and after correction for the individual background fluorescence signals, the ratio of the fluorescence at both excitation wavelengths (*F*340/*F*380) was monitored. Experiments were performed using the standard Krebs solution (see above). The data were classified semi-automatically using a function programmed in MATLAB (MathWorks, MA) and analyzed with Origin 7.0 (OriginLab Corporation).

Drugs

AITC was kept as a 10 M stock solution (4 °C) and fresh dilutions were prepared daily. Solutions containing capsaicin were prepared from an ethanol-based 1 mM stock solution that was kept for not longer than 1 month at -20 °C. Both AITC and capsaicin were purchased from Sigma-Aldrich (Bornem, Belgium). Allicin was purchased from LKT

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Laboratories (St. Paul, USA) and kept as a 60 mM stock solution at -80 °C and fresh dilutions were prepared every 4 hours.

Statistical analysis

Statistical analyses were performed with Origin 7.0. Data presented as mean ± S.E.M. Paired and unpaired t-tests were performed whenever appropriate and $P < 0.05$ was considered for statistical significance.

Results

We first investigated whether activation of TRPV1 by AITC requires an intracellular signal transduction pathway, by testing if this effect is preserved in cell-free inside-out patches. Application of 3 mM AITC to the cytosolic side of membrane patches of TRPV1-expressing HEK-293 cells resulted in robust and reversible stimulation of outwardly rectifying currents (Figure 1A,B). As previously observed for whole-cell currents (Everaerts et al., 2011), the amplitudes of AITC-activated inward and outward currents were several-fold smaller than those upon application of 1 μM capsaicin (Figure 1A-C). These data indicate that AITC activates TRPV1 in a membrane-delimited manner. It has been reported previously that the activation of TRPA1 by AITC and various other electrophiles occurs via covalent binding to cysteine residues (Hinman et al., 2006; Macpherson et al., 2007). Similarly, the activation of rat TRPV1 by allicin, a pungent electrophilic component of raw garlic, or by MTSEA, a cysteine-reactive reagent, requires a single N-terminal cysteine residue (C157) (Salazar et al., 2008). Thus, we tested whether AITC activates TRPV1 via covalent modification of cysteine residues on the channel.

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We found that, similar to the rat orthologue, wild type mouse TRPV1 was activated by allicin (6.5 ± 1.0 fold current increase at -75 mV; n = 3; P = 0.017; Figure 2A,C), whereas the corresponding C158A mutant was not (n = 4; Figure 2B,C). However, the C158A mutation did not affect AITC-induced stimulation (5.4 ± 0.6 fold increase at -75 mV compared to 5.8 ± 1.2 for WT mTRPV1; n = 5-6; P = 0.74; Figure 2B-D). Since TRPV1 contains multiple cysteine residues, these data do not exclude that AITC activation of TRPV1 is mediated via covalent modification of other cysteine residues on the channel. We therefore analyzed the effect of AITC on $C18^-$ rTRPV1, a cysteine-less rat TRPV1 mutant that was previously shown to be insensitive to allicin and MTSEA (Salazar et al., 2008). We found that the $C18^-$ rTRPV1 channel was activated by AITC (3.6 ± 0.4 fold current increase at -75 mV; n = 8; P = 0.0025; Figure 3A,B), similar to WT rTRPV1 (4.3 ± 0.6 fold current increase at -75 mV; n = 7; Figure 3B). We therefore conclude that, in contrast to allicin and MTSEA, AITC activates TRPV1 via a mechanism independent of covalent linkage of AITC to cysteine residues on the channel.

Next, we explored the possibility that residues important for the stimulatory effects of capsaicin are also involved in the response to AITC. Mutation S512Y in rat TRPV1 results in a selective ablation of capsaicin-induced activation, whereas activation by low pH or heat was largely preserved (Jordt and Julius, 2002). We studied the corresponding mutant in mouse TRPV1 (S513Y) using Ca^{2+}-imaging and whole-cell patch-clamp recordings and confirmed that it had a strongly reduced sensitivity to capsaicin, whereas responses to low pH were conserved (Figure 4A,B). Interestingly, mutations in other positions, Y512A and T551I, which correspond to mutants previously reported to significantly impair the response to capsaicin in rat (Gavva et al., 2004; Jordt and Julius,

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2002) and human TRPV1 (Gavva et al., 2004), did not have significant effect on the responses to AITC or capsaicin in the mouse isoform (Figure 4A). Surprisingly, cells expressing the S513Y mutant mTRPV1 also showed strongly reduced responses to 3 mM AITC (Figure 4A,B).

Activation of TRPV1 by capsaicin, heat or low pH involves a shift of the voltage dependence of activation towards more negative potentials (Aneiros et al., 2011; Voets et al., 2004). Thus, we tested whether the mutation S513Y interferes with this mechanism by recording mTRPV1 currents during the application of a voltage step protocol that allows estimating the steady-state TRPV1 open probability at different membrane potentials (see Materials and methods). Capsaicin induced a leftward shift of the voltage dependence of activation in the S513Y mutant (55 ± 10 mV; Figure 4C,D), but in a much smaller extent that in mTRPV1 WT (225 ± 18 mV).

Application of 3 mM AITC caused a substantial leftward shift of the activation curve by an estimated 210 ± 13 mV (n = 5; Figure 5A,C), resulting in significant inward currents in the physiological voltage range (Figure 5D). These data demonstrate that AITC acts on TRPV1 as in a similar way to capsaicin (Voets et al., 2004) and raised the possibility that both compounds have common interaction sites on the channel. Consistently with this contention, AITC caused a much smaller leftward shift of the voltage-dependent activation curve in the S513Y mutant than in the WT (Figure 5E,F). This explains the limited effects of AITC and capsaicin on calcium influx and inward currents at physiological potentials (Figures 4A and 5D).

Considering that local extracellular acidosis is a hall mark of neurogenic inflammation (Holzer, 2009; Julius and Basbaum, 2001) and a well-known TRPV1 sensitizer (Aneiros

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et al., 2011; Tominaga et al., 1998), we determined whether there is cross sensitization between AITC and low pH. We found that mild acidification to pH 6.8 caused a modest but significant stimulation of hTRPV1 (1.7 ± 0.2 fold current increase at -75 mV; n = 6; P = 0.038, Figure 6) and that this response was significantly larger in the presence of 100 µM AITC (3.3 ± 0.4 fold current increase at -75 mV; n = 8; P = 0.002; Figure 6C). Conversely, Ca^{2+}-imaging experiments revealed that application of 100 µM AITC evoked responses in TRPV1-expressing HEK-293 cells at extracellular pH 6.8 but not at pH 7.4 (data not shown).

To investigate the molecular basis for the cross sensitization of the effects of AITC and low pH on TRPV1, we tested the effects of mutations at position F660, which are known to ablate stimulation by low pH in human TRPV1 (Aneiros et al., 2011). We found that mutants F660A and F660S responded to high concentrations of AITC in a similar way to WT hTRPV1, but fully lacked the cross sensitization between AITC and pH 6.8 (Figure 6C).

Finally, we tested whether the TRPV1-mediated cross sensitization between AITC and low extracellular pH is also present in sensory neurons. For this we performed microfluorimetric intracellular Ca^{2+} imaging in primary cultures of dorsal root ganglion (DRG) neurons isolated from *Trpa1* KO mice, to avoid interference with the AITC responses mediated by this channel. In line with previous results (Everaerts et al., 2011), we found that extracellular application of 300 µM AITC at pH 7.4 induced responses in 3.9% (21 out of 537) of DRG neurons with a mean amplitude of 330 ± 50 nM (Figure 7A,C). These responses were strongly potentiated when AITC was applied at

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extracellular pH 6.8 (16.2%, 117 out of 721; mean amplitude 490 ± 30 nM; Figure 7B-C), indicating that acidosis sensitizes TRPV1 for the AITC effect.

Conversely, pre-application of AITC strongly enhanced the responses of *Trpa1* KO mouse neurons to acidification (Figure 7D-F). Indeed, lowering the extracellular pH from 7.4 to 6.8 induced responses in 3.9% (17 out of 432) with a mean amplitude of 270 ± 40 nM, whereas when acidosis was applied in the presence of 100 μM AITC the responses were observed in 24.4% of the cells (144 out of 589), with a mean amplitude of 350 ± 20 nM. Notably, the amplitude of the responses to acidification in the presence of AITC largely correlated with the amplitudes of the responses to capsaicin ($R = 0.72$, $P < 0.0001$). The responses were strongly reduced by application of 20 μM capsazepine, a TRPV1 inhibitor (10.5%, 38 out of 361; mean amplitude 280 ± 20 nM; Figure 7F), and were totally absent in neurons isolated from double *Trpa1/Trpv1* KO mice (0 out of 240).

Discussion

AITC is a natural compound largely responsible for the pungent effect of mustard and wasabi and has been widely used for research purposes to induce experimental irritation and local inflammation (see for example Albin et al., 2008; Bautista et al., 2006; Carstens and Mitsuyo, 2005; Caterina et al., 2000; Dunham et al., 2010; Garcia-Martinez et al., 2002; Kwan et al., 2006; McMahon and Abel, 1987; Merrill et al., 2008; Reeh et al., 1986; Sawyer et al., 2009; Simons et al., 2004). Until recently, these responses were attributed to the nociceptor channel TRPA1 (Bautista et al., 2006). However, it has been recently shown that AITC activates another key nociceptor cation channel, TRPV1 (Everaerts et al., 2011; Ohta et al., 2007). Moreover, it was shown that the genetic

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ablation of TRPV1 reduced some of the responses to AITC *in vivo* (Everaerts et al., 2011). This surprising result was partly explained in terms of the differences between the effects of AITC on these channels. While AITC interacts covalently with cysteine residues of TRPA1 (Hinman et al., 2006; Macpherson et al., 2007), leading to a strong and long-lasting desensitization of this channel (Macpherson et al., 2007), this compound induces a sustained but quickly reversible activation of TRPV1. These fundamental differences prompted us to investigate the mechanism whereby AITC activates TRPV1 in more detail.

We found that activation of TRPV1 by AITC is conserved in cell-free inside-out patches, which implies that the underlying mechanism does not involve soluble cytosolic component and suggests that AITC acts directly on the channel. The installation of effects of AITC in this recording configuration was faster (~30 s) than previously reported for the extracellular application in the whole-cell configuration (> 1 min, Everaerts et al., 2011), which suggests for an intracellular access to the active site(s). Yet, AITC effects in the inside-out patches were rather slow, which in turn indicates a restricted access to the site(s) and fits with the relatively low apparent affinity of AITC for the channel (300 µM at 35 °C and , Everaerts et al., 2011).

Next, we found that the sensitivity to AITC is not altered by mutation of C158, a residue that is necessary and sufficient to confer sensitivity to cysteine-modifying agents such as MTSEA and allicin (Salazar et al., 2008). Even more strikingly, sensitivity to AITC was conserved in the completely cysteine-less $C18^-$ rTRPV1 mutant. These data demonstrate that AITC-induced activation of TRPV1 does not critically depend on covalent cysteine modification.

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On the other hand, we found that, surprisingly, AITC-induced activation of TRPV1 is in many aspects similar to activation by capsaicin. First, AITC induces a shift of the voltage dependence of channel activation towards negative voltages, therefore acting as some other TRP channel agonists (Nilius et al., 2005; Talavera et al., 2008; Voets et al., 2005). Second, TRPV1 sensitivity to both AITC and capsaicin critically depends on the residue S513, which has been suggested to form part of the capsaicin binding site (Jordt and Julius, 2002). It should be noted, however, that the apparent affinity of TRPV1 for AITC is several orders of magnitude lower than for capsaicin (Everaerts et al., 2011), and that maximal AITC response amplitudes are significantly lower. Finally, as for capsaicin, the effect of AITC cross-sensitizes with that of low extracellular pH.

The latter aspect is of particular importance, as it helps explaining the surprisingly large relevance of TRPV1 (relative to that of TRPA1) as a AITC receptor *in vivo* (Everaerts et al., 2011). Indeed, this potentiating interaction renders TRPV1 even more sensitive to AITC in real life and experimental scenarios, since this compound is typically present in acid food (mustard, vinaigrettes) and acidosis is a typical feature of local inflammation (Holzer, 2009; Julius and Basbaum, 2001).

Our analysis of the effects of AITC on the gating properties of TRPV1 indicates that the mechanism of cross sensitization between acidosis and AITC is the same as the one between heat and capsaicin, i.e. the induction of additive shifts of the voltage dependence of channel activation (Voets et al., 2004). Indeed, the sensitizing effect of extracellular acidosis on the response to AITC was fully ablated by the same mutations that determine the shift of the activation curve of TRPV1 by low pH (Aneiros et al., 2011).

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In conclusion, we have provided evidence that AITC activates TRPV1 in a membrane-delimited manner via an interaction with the capsaicin-binding site. Hence, TRPV1 can be activated by highly reactive electrophilic compounds either through cysteine-dependent (allicin and MTSEA) and -independent (AITC) mechanisms. In addition, our results unveil molecular mechanisms that help to understand the complex effects of AITC as an inflammatory and sensitizing agent.

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Authorship Contributions

Participated in research design: Gees, Alpizar, Boonen, Everaerts, Owsianik, Nilius, Voets, and Talavera.

Conducted experiments: Gees, Alpizar, Boonen, Sanchez, Xue, Janssens, Owsianik and Talavera.

Contributed new reagents or analytic tools: Segal.

Performed data analysis: Gees, Alpizar, Boonen, Sanchez, and Talavera.

Wrote or contributed to the writing of the manuscript: Gees, Alpizar, Everaerts, Owsianik, Nilius, Voets, and Talavera.

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Footnotes

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Figure Legends

Figure 1. AITC activates TRPV1 in cell-free inside-out patches

A) Current traces of mTRPV1 in inside-out patches. Currents were evoked by a voltage ramp from -100 to +150 mV (holding potential of 0 mV) in control condition and in the presence of 3 mM AITC or 1 μM capsaicin in the bath solution. Traces are labelled to indicate the correspondence with the filled data points shown in panel B. Inset: magnification of the currents at negative potentials.

B) Example of the time course of the effects of application of 3 mM AITC or 1 μM capsaicin on the amplitude of mTRPV1 currents at +75 mV and -75 mV.

C) Average increase of mTRPV1 inside-out currents during AITC (3 mM) and capsaicin (1 μM) application at -75 mV and +75 mV (n = 5).

Figure 2. The mTRPV1 C158A mutant is activated by AITC

A and B) Examples of the time course of the amplitude of wild type mTRPV1 and mTRPV1 C158A mutant currents at +75 mV and -75 mV in control or in the presence of extracellular allicin (200 μM) or AITC (3 mM). The letters next to the filled symbols indicate the time points when the traces shown on the right were recorded.

C) Increase of amplitude for wild type mTRPV1 and C158A mutant mTRPV1 currents during application of AITC (3 mM) and allicin (200 μM) (n = 3-6). The asterisks (*) denote $P < 0.05$ for the comparison of current amplitudes measured before and during stimulation.

D) Effect of extracellular application of 3 mM AITC on $[Ca^{2+}]_i$ in HEK-293 cells expressing the C158A mutant mTRPV1. Transfected cells were identified by the

expression of GFP and their responsiveness to 1 μM capsaicin (upper traces, n = 68). Non-transfected cells did not respond to either AITC or capsaicin (lower traces, n = 26). Thick traces represent the means and dashed traces represent the means ± the corresponding s.e.m.

Figure 3. The cysteine-less rTRPV1 mutant $C18^-$ is activated by AITC

A) Example of the effects of extracellular application of 3 mM AITC and 1 μM capsaicin on the amplitude of currents through the $C18^-$ rTRPV1 mutant expressed in HEK-293 cells. The letters next to the filled symbols indicate the time points when the traces shown on the right were recorded.

B) Average current increase for rTRPV1 and $C18^-$ rTRPV1 induced by application of 3 mM AITC and 1 μM capsaicin (n = 7).

Figure 4. Stimulation of TRPV1 by AITC is reduced in the S513Y mutant

A) Intracellular Ca^{2+} imaging experiments showing the average effects of AITC, capsaicin and low extracellular pH on HEK-293 cells over-expressing WT mTRPV1 or the mutants S513Y, Y512A or T551I. Transfected cells were detected by the expression of GFP and their responsiveness to pH 5.4.

B) Example of the effects of extracellular application of 3 mM AITC, 1 μM capsaicin and pH 5.4 of the time course of the amplitude of mTRPV1 S513Y mutant currents measured at +75 mV and -75 mV. The letters next to the filled symbols indicate the time points when the traces shown on the right were recorded.

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C) Example of S513Y mutant current traces recorded during the application of a voltage-step protocol in control and in the presence of 1 μM capsaicin.

D) Average voltage dependence of the normalized whole-cell conductance of the S513Y mutant in control and in the presence of 1 μM capsaicin (n = 4).

Figure 5. Effects of AITC on the voltage dependence of mTRPV1 and the mutant S513Y

A,B) Examples of current traces recorded during the application of a voltage-step protocol in HEK-293 cells transfected with mTRPV1 (A) or the S153Y mutant (B) in control and in the presence of 3 mM extracellular AITC.

C) Average voltage dependence of amplitude of steady-state currents recorded for mTRPV1 (n = 5) and the S153Y mutant (n = 4) in control conditions and in the presence of 3 mM extracellular AITC. For each cell data were normalized to the value obtained in control at +200 mV. Note the lack of difference between the data sets recorded in control.

D) Zoom of the data shown in panel C in the range of negative voltages. Substantial inward currents were only observed for WT mTRPV1 in the presence of AITC.

E) Average voltage dependence of the amplitude of tail currents measured in control and in the presence of 3 mM AITC for wild type TRPV1 (n = 5) and the S513Y mutant (n = 4). For each cell data were normalized to the value obtained in control at +200 mV. Note the lack of difference between the data sets recorded in control.

F) Comparison of the shift of the voltage for half-maximal activation induced by application of 3 mM AITC or 1 μM capsaicin in WT mTRPV1 (n = 5) and the S513Y mutant (n = 4) in HEK-293 cells. The asterisks (*) denote $P < 0.05$ for the comparison of values for wild type mTRPV1 and the S513Y mutant.

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Figure 6. Stimulation of hTRPV1 by extracellular acidosis is potentiated by AITC

A) Whole-cell hTRPV1 currents were evoked by a voltage ramp from -100 to +120 mV (holding potential of 0 mV) in control condition and during extracellular acidification to pH 6.8 in the absence and in the presence of 100 µM AITC. Traces are labelled to indicate the correspondence with the filled data points shown in panel B.

B) Time course of the effects of extracellular acidification to pH 6.8 in the absence and in the presence of 100 µM AITC on the amplitude of hTRPV1 currents at +75 mV and -75 mV.

C) Increase of amplitude of currents obtained for hTRPV1 and the F660A and F660S mutants induced by acidosis (pH 6.8), by AITC 100 µM application (at pH 7.4 and 6.8) or by AITC 3 mM application (n = 6-8). The asterisks (*) denote $P < 0.05$ for the comparison of data obtained in control during stimulation. The # symbol denotes $P < 0.05$ for the comparison of the amplitude of currents recorded at pH 6.8 in the absence or in the presence of AITC.

Figure 7. Cross sensitization of the effects of AITC with and acidosis in mouse sensory neurons

A, B) Examples of the responses in intracellular Ca^{2+} concentration elicited in *Trpa1* KO DRG neurons by extracellular application of 300 µM AITC at pH 7.4 (panel A) or at pH 6.8 (panel B).

C) Comparison of the complementary cumulative probability (CCP) distributions of the amplitude of the responses induced by 300 µM AITC at pH 7.4 (n = 528) and 6.8 (n = 690). The data in the ordinates correspond to the percentage of neurons responding with

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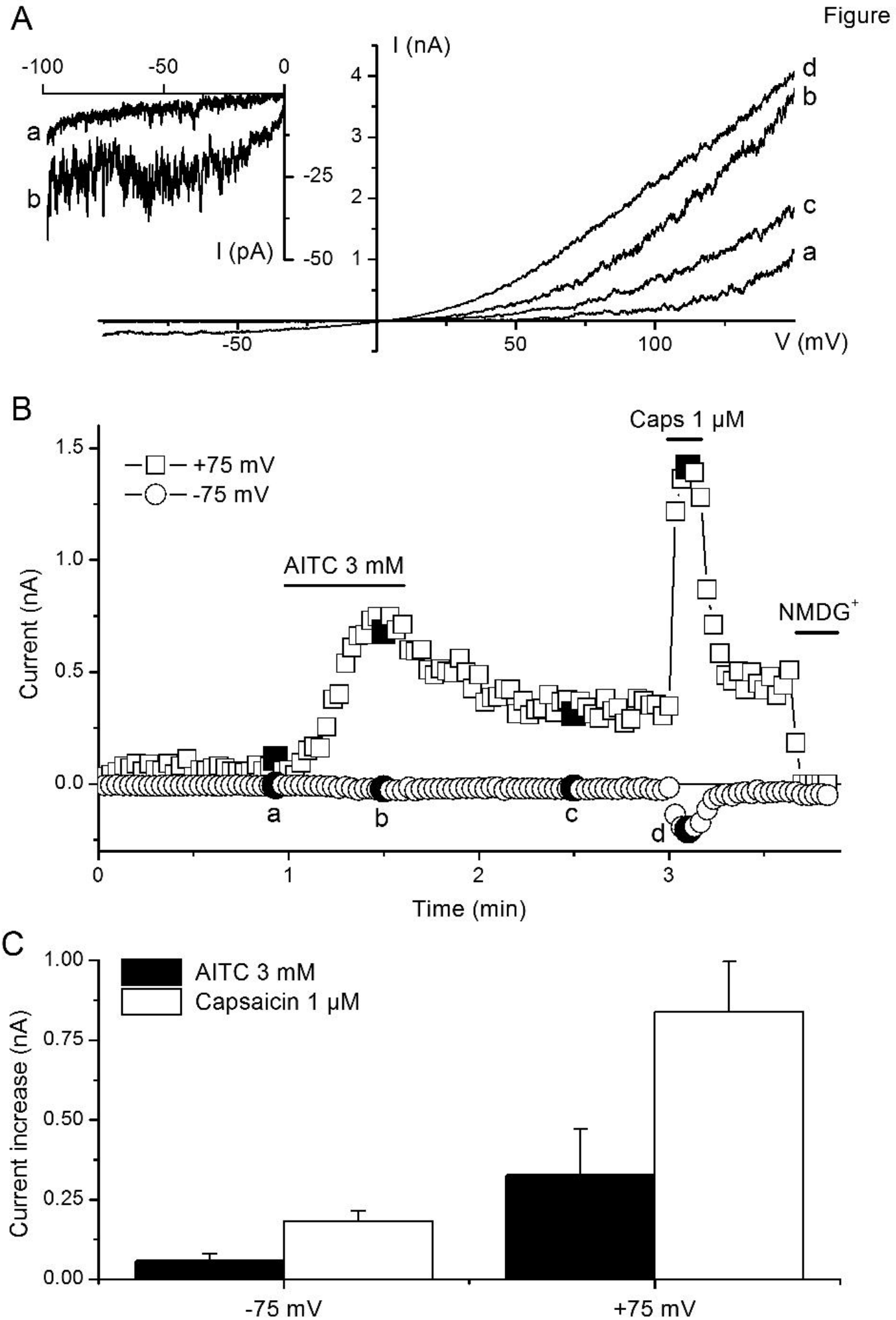
amplitude larger than the change in intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]$) of the corresponding abscissa. The values of CCP at $\Delta[Ca^{2+}]$ = 0.05 µM correspond to the percentage of neurons responsive to AITC in each condition. The mean amplitudes of the responses correspond to the abscissa of the filled circles.

D, E) Examples of the responses in intracellular Ca^{2+} concentration elicited in *Trpa1* KO DRG neurons by extracellular acidification from pH 7.4 to 6.8 in control (panel D) or during extracellular application of 300 µM AITC (panel E).

F) Comparison of the complementary cumulative probability distributions of the amplitude of the responses of *Trpa1* KO DRG neurons induced by acidification from pH 7.4 to 6.8 in control (n = 431), in the presence of 300 µM AITC (n = 585) and in the presence of 300 µM AITC and capsazepine (CPZ, 20 µM, applied from 1 min till 8 min, n = 361). The mean amplitudes of the responses correspond to the abscissa of the filled circles.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I (pA)
-50
4
3
2
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d
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-50
50
100
V (mV)
B
Caps 1 μM
+75 mV
-75 mV
AITC 3 mM
NMDG+
Current (nA)
1.5
1.0
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a
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Time (min)
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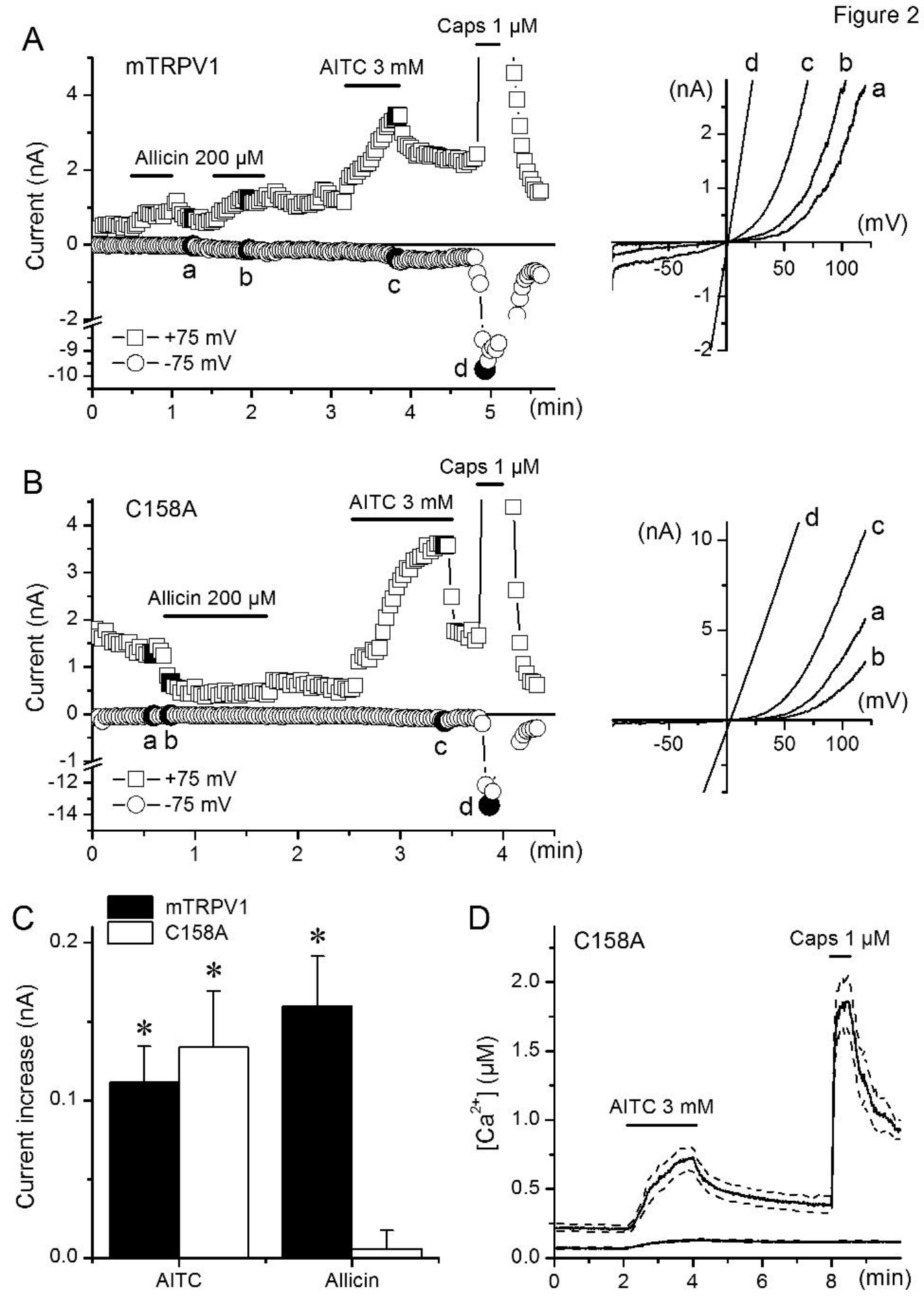
+75 mV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Allicin 200 μM
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c
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-75 mV
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(nA)
(mV)
B
C158A
C
mTRPV1
C158A
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Allicin
D
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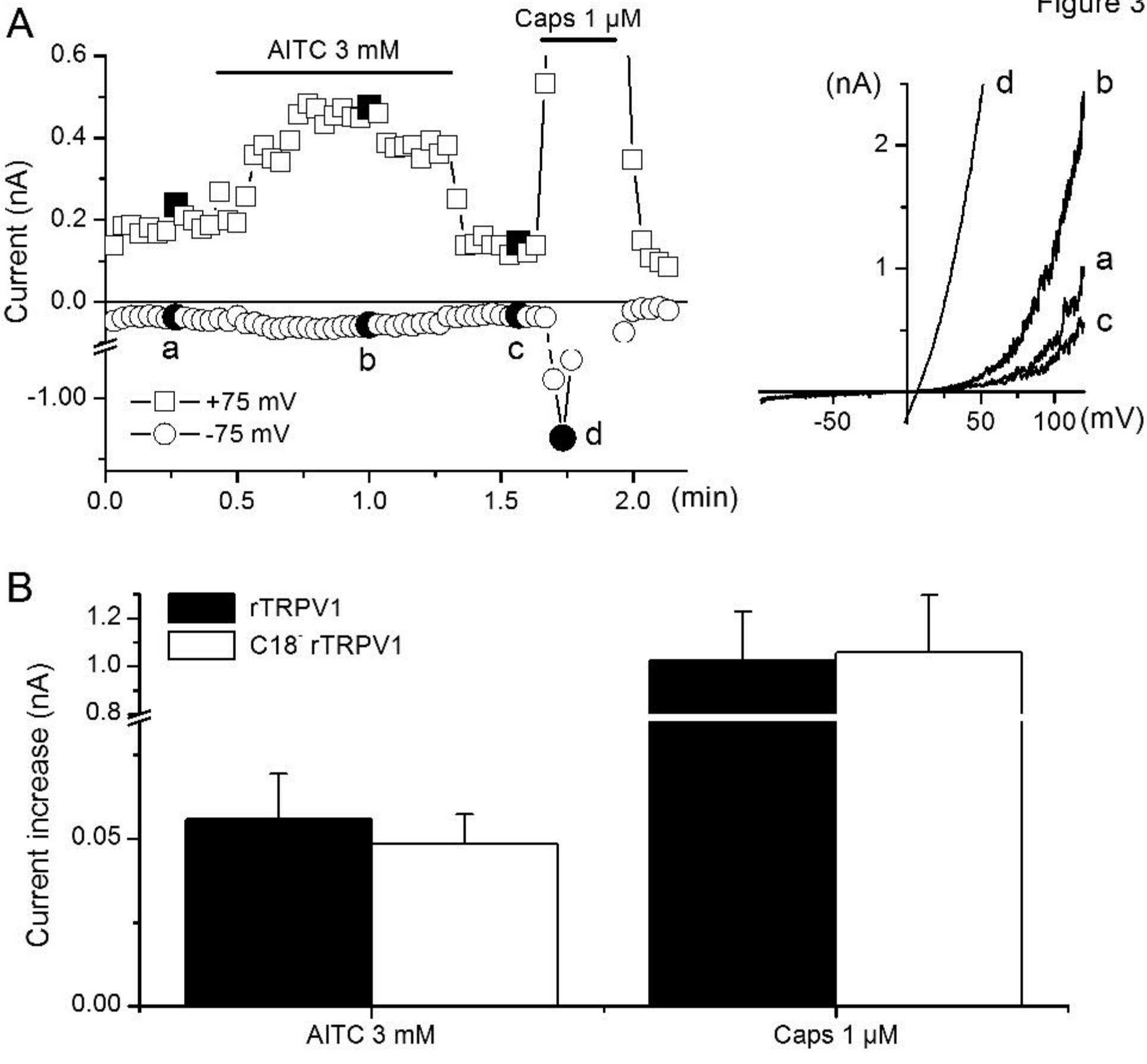
[Ca2+] (μM)
(min)

Figure 3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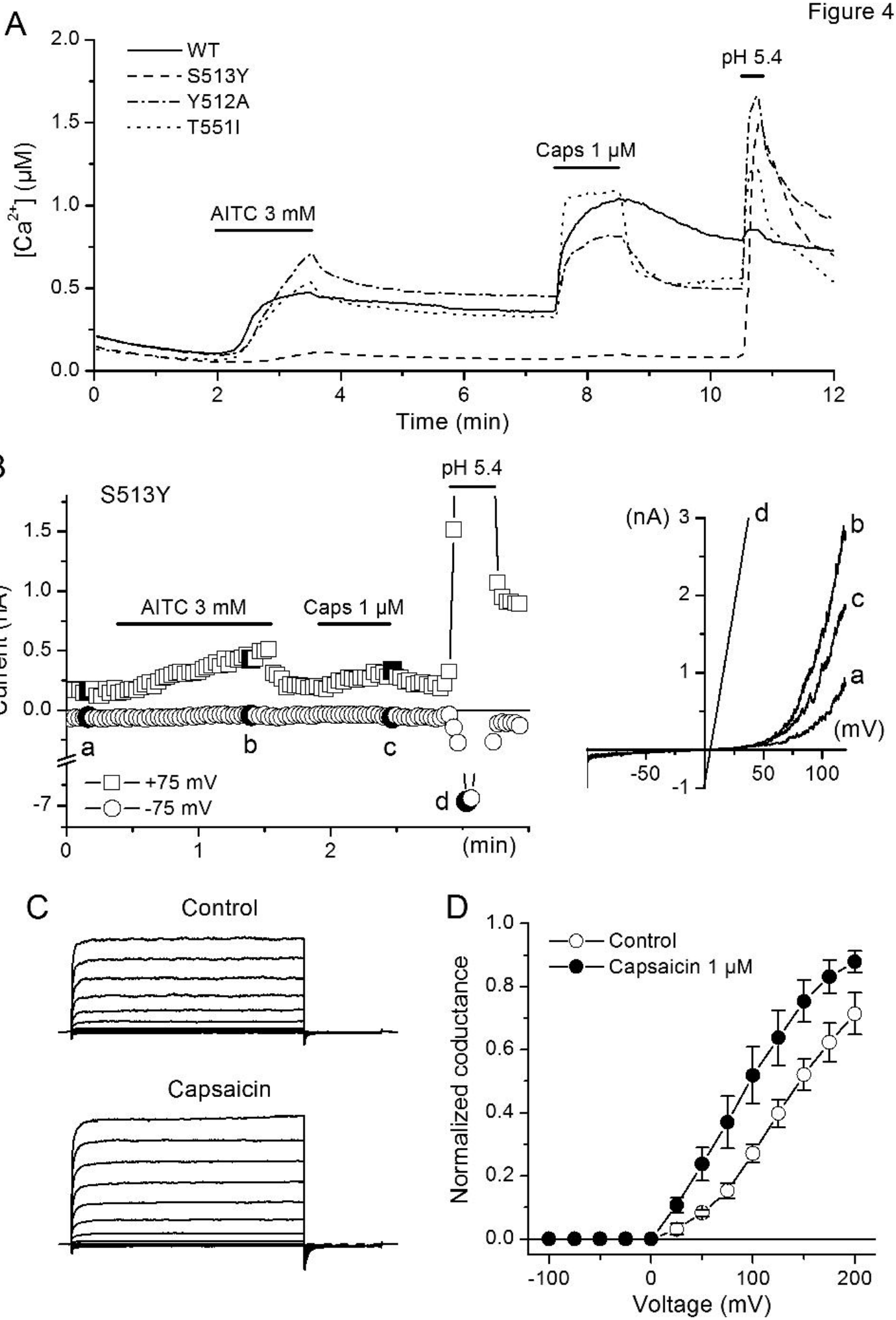
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pH 5.4
AITC 3 mM
Caps 1 μM
Current (nA)
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-75 mV
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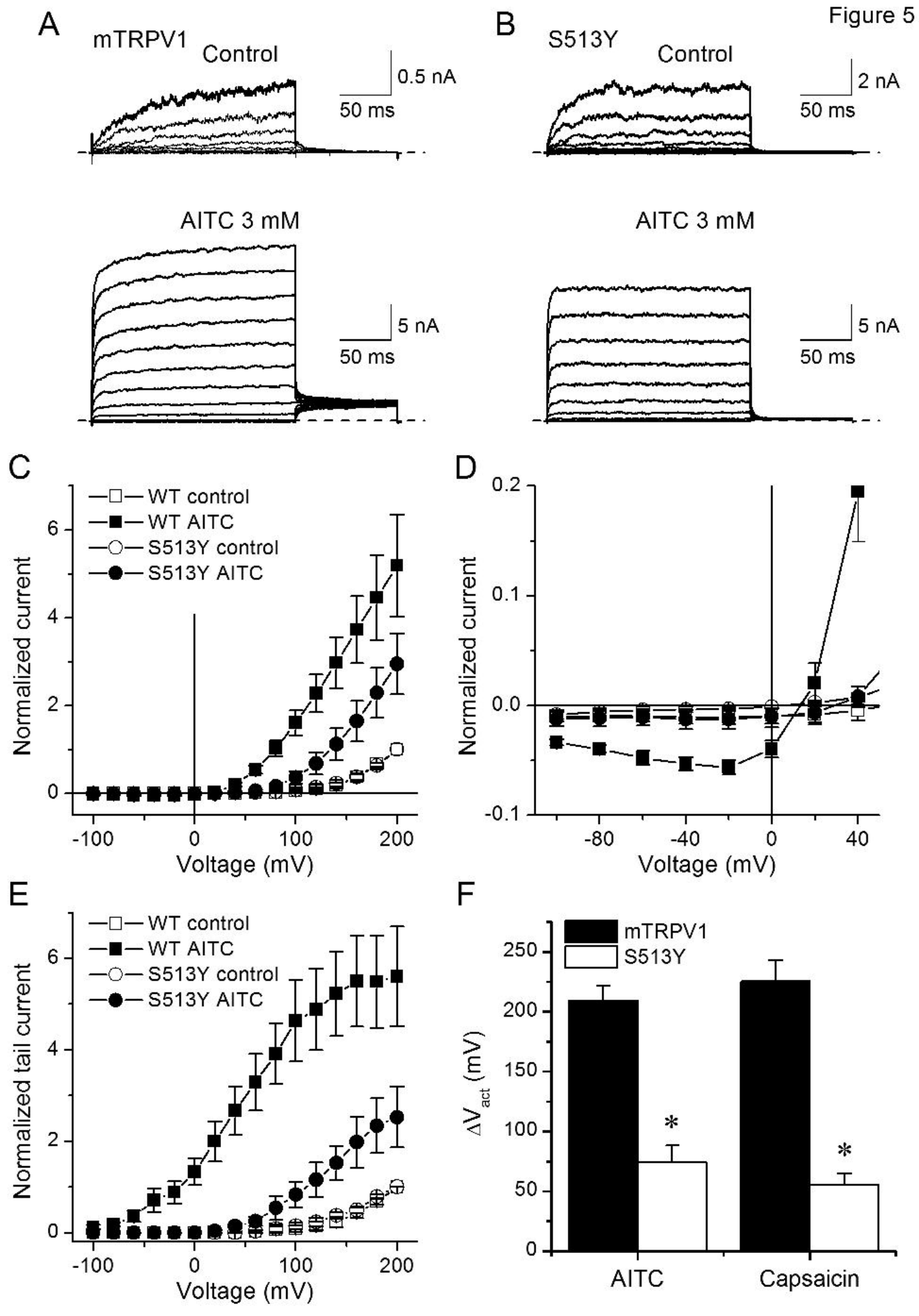
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5 nA
50 ms
C
WT control
WT AITC
S513Y control
S513Y AITC
Normalized current
Voltage (mV)
D
Normalized current
Voltage (mV)
E
WT control
WT AITC
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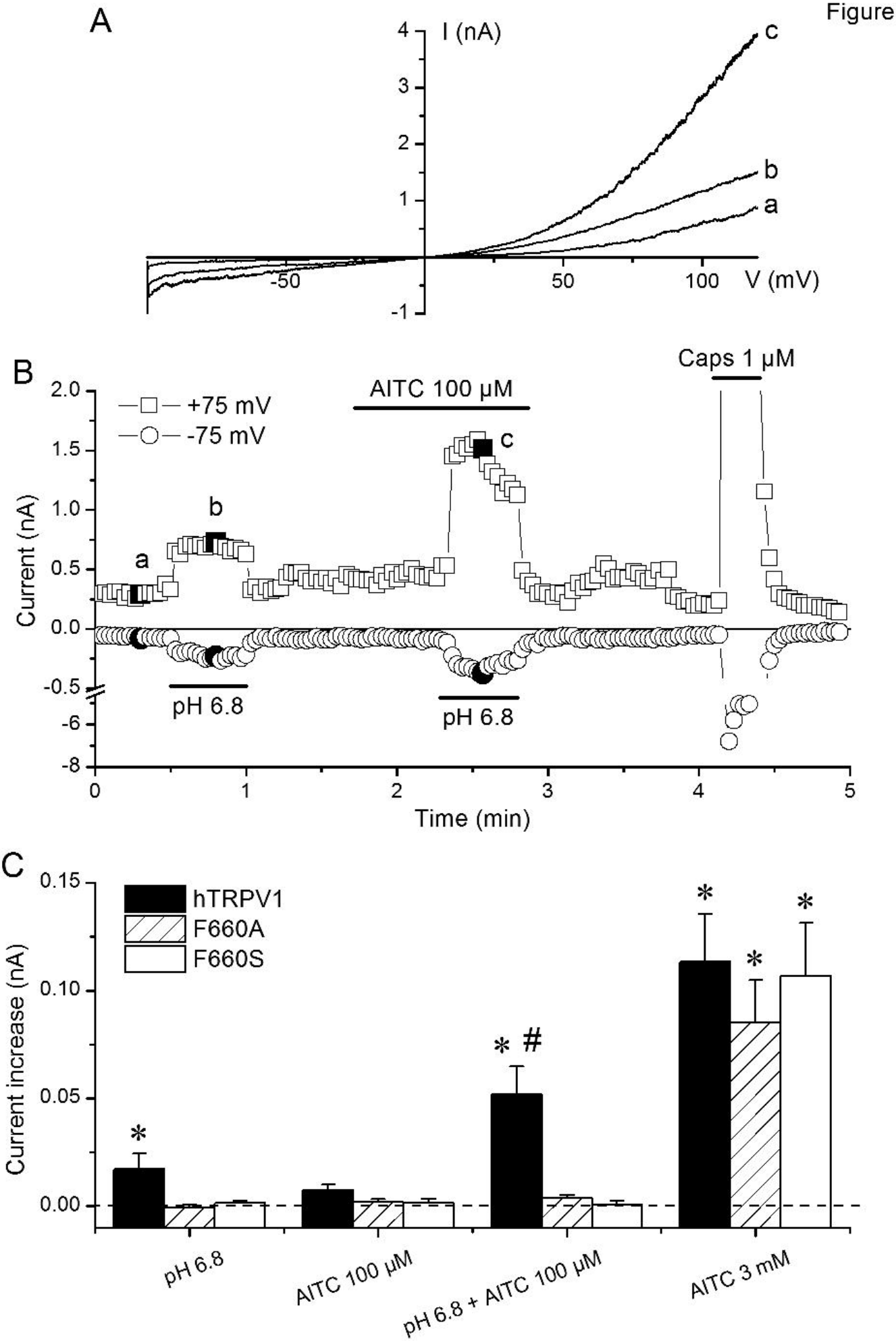
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Caps 1 μM
AITC 100 μM
+75 mV
-75 mV
Current (nA)
2.0
1.5
1.0
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0.0
-0.5
-6
-8
pH 6.8
pH 6.8
0
1
2
3
4
5
Time (min)
C
hTRPV1
F660A
F660S
Current increase (nA)
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pH 6.8 + AITC 100 μM
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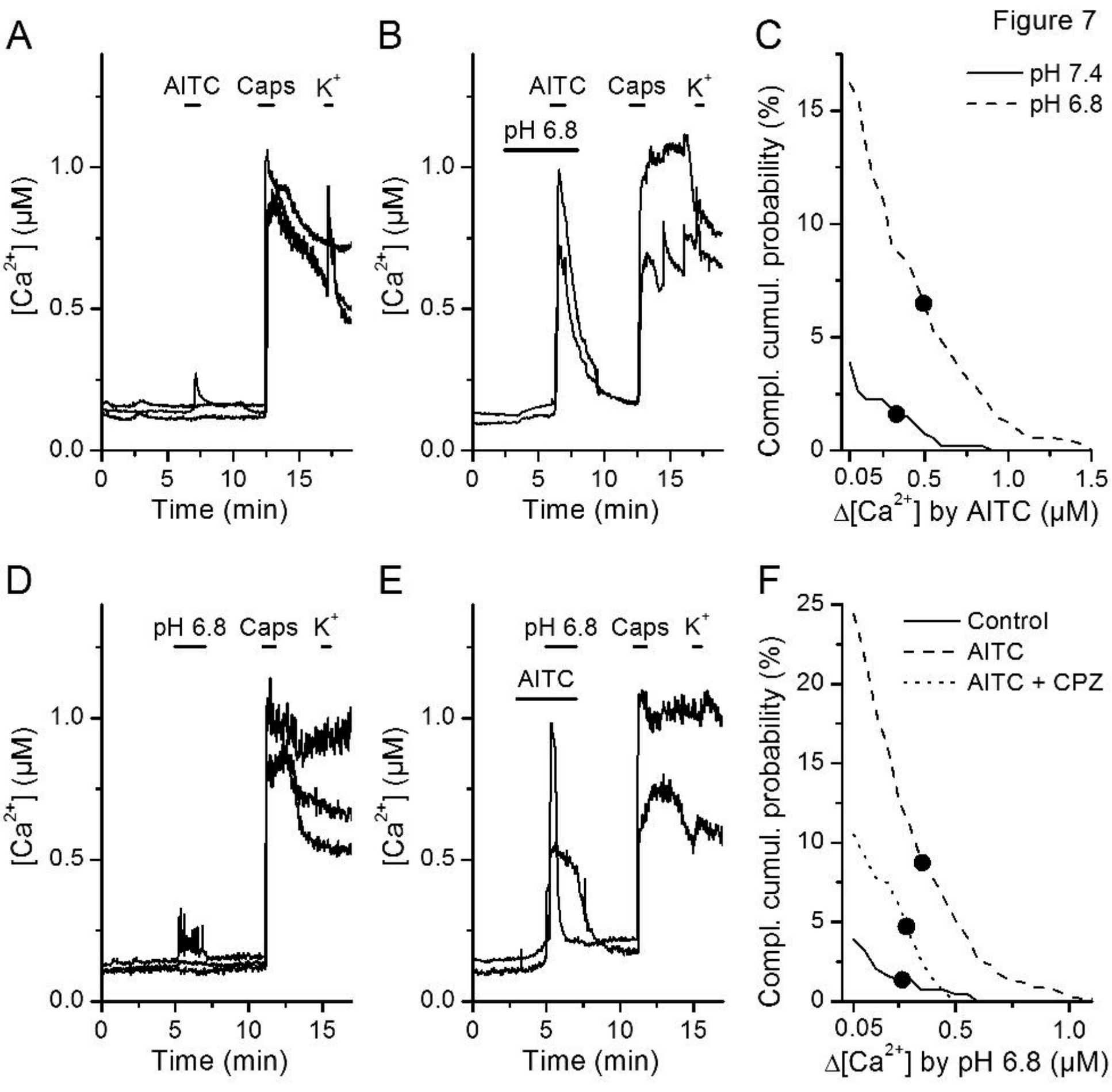
Figure 7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Time (min)
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C
pH 7.4
pH 6.8
Compl. cumul. probability (%)
Δ[Ca2+] by AITC (μM)
D
pH 6.8
E
F
Control
AITC
AITC + CPZ
Δ[Ca2+] by pH 6.8 (μM)