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General Anesthetics Sensitize the Capsaicin Receptor TRPV1

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Running title: **TRPV1 and general anesthetics**

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Abbreviations: AMG9810, [(E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide], capsaicin, [*N*-(4-hydroxy-3-methoxybenzyl)-8-methylnon-6-eneamide], capsazepine, *N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2-H-2-benzazepine-2-carbothioamide; GAs, general anesthetics; TRP, Transient Receptor Potential; VGAs, volatile general anesthetics

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Abstract

General anesthetics (GAs) are central nervous system depressants that render patients unresponsive to external stimuli. In contrast, many of these agents are also known to stimulate peripheral sensory nerves raising the possibility that they may exacerbate tissue inflammation. Recently, we found that pungent GAs excite sensory neurons by directly activating the TRPA1 ion channel. Here, we show that GAs also sensitize the capsaicin receptor TRPV1, a key ion channel expressed in nociceptive neurons. Clinically-relevant concentrations of isoflurane, sevoflurane, enflurane and desflurane sensitize TRPV1 to capsaicin and protons and reduce the threshold for heat activation. Further, isoflurane directly activates TRPV1 following stimulation of protein kinase C. Similarly, isoflurane excites TRPV1 and sensory neurons during concomitant application of bradykinin, a key inflammatory mediator formed during tissue injury. Thus, GAs can enhance the activation of TRPV1 that occurs during surgically-induced tissue damage. These results support the hypothesis that some GAs, through direct actions at TRP channels, increase post-surgical pain and inflammation.

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Introduction

Volatile general anesthetics (VGAs) are a diverse group of volatile and gaseous substances with the shared ability to suppress CNS activity (Franks, 2008). In addition to hypnosis or unconsciousness, most of these drugs also cause a varying degree of amnesia, muscle relaxation and blunting of sympathetic responses (Miller et al., 2002). Although the precise molecular mechanisms are unresolved several studies suggest that GAs can inhibit CNS activity by sensitizing or activating γ-aminobutyric acid ($GABA_A$) receptors (Jones et al., 1992; Nakahiro et al., 1991; Wakamori et al., 1991) and background K^+ channels (Franks and Lieb, 1988; Gray et al., 1998; Patel et al., 1999) or by inhibiting glutamate receptors (Hollmann et al., 2001; Yamakura and Harris, 2000) and presynaptic transmitter release (van Swinderen et al., 1999).

In contrast to their effects in the CNS, several VGAs are known to excite peripheral nociceptive ("pain-sensing") neurons (Mutoh and Tsubone, 2003; Mutoh et al., 1998) and are perceived as pungent. Indeed, inhalation of desflurane (greater than 1 MAC) and isoflurane (greater than 1.5 MAC) produces airway irritation in humans and consequently these GAs are avoided as induction agents (Eger, 1995; Kong et al., 2000). We have recently shown that these pungent GAs excite sensory neurons by directly activating the Transient Receptor Potential (TRP) channel, TRPA1 (mustard-oil receptor) (Matta et al., 2008). Further, we found that TRPA1-dependent neurogenic inflammation is greater during anesthesia with pungent, compared with non-pungent, anesthetics. Thus, we have proposed that GAs, by modulating TRPA1, could exacerbate surgical pain and inflammation.

TRPA1 is expressed in 25% of sensory neurons and notably is almost always found co-expressed with another "pain"-sensing ion channel, capsaicin receptor TRPV1 (Bautista et al., 2006). TRPV1 imbues sensory neurons with sensitivity to the plant irritant, capsaicin [*N*-(4-hydroxy-3-methoxybenzyl)-8-methylnon-6-eneamide], and is more widely distributed than TRPA1; approximately 50 % of dorsal root, nodose and trigeminal ganglia express TRPV1 based on mRNA or capsaicin-sensitivity (Bautista et al., 2006; Kobayashi et al., 2005; Nagy et al., 2004). Targeted disruption of the TRPV1 gene in mice profoundly attenuates thermal hyperalgesia (Caterina et al., 2000; Davis et al., 2000) supporting a central role for TRPV1 in inflammatory

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pain. A number of diverse chemical and physical stimuli activate TRPV1, including heat, protons, cations and inflammatory fatty acids (Pingle et al., 2007). Characteristically, these noxious stimuli act in a co-operative or synergistic manner to enhance TRPV1 channel activity. Indeed, protons and ethanol, act primarily by sensitizing the channel to other stimuli, as opposed to being direct agonists (Tominaga et al., 1998; Trevisani et al., 2002). We therefore considered the possibility that GAs, while not directly activating TRPV1, could nonetheless sensitize TRPV1. Our results show that clinically relevant concentrations of VGAs sensitize TRPV1 to heat, protons and capsaicin. Furthermore, inflammatory stimuli, including protein kinase C and bradykinin, enhance the stimulatory effects of VGAs. These results suggest that VGAs, by their collective actions at TRPA1 and TRPV1 ion channels, may exacerbate peripheral nociceptive signaling in the context of surgery.

Materials and Methods

HEK cell and sensory neuron electrophysiology. HEK 293F cells (Invitrogen) were cultured in DMEM supplemented with 1% non-essential amino acids and 10% fetal calf serum. Cell cultures were maintained at 37ºC with 5% CO_2. Cells were transfected with rat TRPV1 (gift of David Julius), and GFP cDNA using Lipofectamine™ Transfection Reagent (Invitrogen) and used 24-48 h after transfection. Nodose ganglia were obtained from adult mice (C57Bl6/J and TRPV1-null), cut, digested with collagenase, and cultured in Neurobasal + 2% B-27 medium (Invitrogen), 0.1% L-glutamine and 1% penicillin/streptomycin on poly-D-lysine-coated glass coverslips at 37°C in 5% CO_2. Neurons were used within 24-36 hr of culture. Whole-cell and single-channel patch clamp recordings were performed using an EPC8 amplifier (HEKA). The current signal was low-pass filtered at 1-3 kHz and sampled at 4 kHz. Currents were further filtered for display purposes. For whole-cell and excised patch recordings the bath solution contained (in mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 1 EGTA, 10 HEPES, 10 glucose pH 7.3 (290 mOsm). The pipette solution contained (in mM): 140 CsCl, 10 NaCl, 10 HEPES, 5 EGTA, 2 MgATP and 0.03 GTP, pH 7.3. The peak amplitudes measured either

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during the prepulse or the tail current (within 1 ms) were plotted as a function of the test potential and normalized to the maximal current obtained from the following Boltzmann function:

$$I_{Tail} = \frac{I_{\max} - I_{\min}}{1 + \exp((V - V_{1/2})/s)} + I_{\min}$$

Where $V_{½}$ is the potential that elicits half maximal activation, s is the slope factor, and I_{min} is the minimum current observed.

Oocyte electrophysiology. Defolliculated *Xenopus laevis* oocytes (harvested from adult females anesthetized with 0.5 g/l tricaine methanesulfonate) were injected with ~10 ng of wild-type rat TRPV1 cRNA or mutant S502A/S800A TRPV1 cRNA (gift of Makoto Tominaga). Oocytes were placed in a Perspex chamber and continuously superfused (5 ml min^{-1}) with Ca^{2+}-free solution containing (in mM): 100 NaCl, 2.5 KCl, 5 HEPES, 1 $MgCl_2$ and titrated to pH 7.3 with ~5 mM NaOH. For solutions < pH 6.0, HEPES was replaced with either 5 mM MES or 5mM sodium citrate. Oocytes were routinely voltage-clamped at –60 mV at 22-23°C. For heat activation, bath temperature was raised from ~22-50ºC over ~100 s using an in-line solution heater (Warner Instruments). The temperature was continuously monitored with a probe placed within 2 mm of the oocyte. The temperature-activation threshold was defined as a 20% increase in current above baseline.

Volatile general anesthetics. Saturated stock solutions of volatile general anesthetics (VGAs) were prepared in gas-tight bottles by dissolving excess anesthetic agents in bath solutions and stirring vigorously overnight. From these stock solutions fresh dilutions were made up every 40-60 minutes. Concentrations of volatile anesthetics in the bath solutions were verified using a modified head-space gas chromatography method. The gas chromatograph (Carlo Erba, Milan, Italy) was equipped with a flame ionization detector (FID) and mass spectrometer. Standards were prepared from a mixture of halothane, isoflurane, and sevoflurane dissolved in acetonitrile with enflurane as an internal standard. The equivalent minimum alveolar concentration (MAC) were calculated using published conversion factors reported for halothane (1 MAC, 0.27 mM), isoflurane (1 MAC, 0.31 mM) and sevoflurane (1 MAC, 0.35 mM) in rat at 37°C (Franks and Lieb, 1996).

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Chemicals. Capsazepine, PDBu, bradykinin and staurosporine were obtained from Sigma. Capsaicin and AMG9810 were purchased from Tocris Cookson (Ellisville, MO). Drugs were prepared as stock solutions in DMSO or ethanol and diluted into physiological solution prior to experiments.

Statistical analysis. Data are given as mean ± S.E.M. and statistical significance was evaluated using ANOVA or Student's t-test.

Results

Volatile anesthetics sensitize TRPV1 to capsaicin and protons

Although our previous data showed that VGAs do not directly activate TRPV1 we considered the possibility that they could nonetheless sensitize TRPV1 to other modes of activation. Indeed, a diverse array of physical and chemical stimuli activate TRPV1 (Pingle et al., 2007) and these stimuli produce synergistic effects when applied together. In sensory neurons, isoflurane (0.9mM or ~2.9 minimum alveolar concentration, MAC) enhanced by approximately 3-fold whole-cell currents evoked by capsaicin (30 nM, Fig. 1A, n = 5). Further, isoflurane increased capsaicin-evoked single channel activity in cell-free, outside out patches (Fig. 1B). We found that VGAs produced a similar sensitization of TRPV1 to protons. In TRPV1-expressing oocytes, isoflurane (0.9 mM) significantly enhanced by approximately 10-fold the currents evoked by a pH 5.5 solution (Fig 2A, n = 4). Dose-response analyses show that isoflurane reduced the half-maximal concentration required for activation by capsaicin and protons (Figs. 1C&2B); the capsaicin EC_{50} was reduced from ~1.6 to 0.8 μM ($P<0.01$) and the proton pEC_{50} was increased from 4.95 to 5.23 ($P<0.01$). In addition, isoflurane enhanced the maximal proton-evoked current by~3 fold.

Anesthetics enhance voltage and thermal sensitivity of TRPV1

TRPV1 is a voltage sensitive channel; membrane depolarization gates TRPV1 and half-maximal activation ($V_{1/2}$) is seen at ~120 mV (at 25°C) (Voets et al., 2004). Although, these membrane potentials are supraphysiologic, agonists enhance the sensitivity of TRPV1 to voltage such that the channel responds to

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voltage in the physiologic range. In addition, agonists increase the maximal voltage–evoked current (Matta and Ahern, 2007). Similarly, we found that application of isoflurane (0.9mM) enhanced the currents evoked by depolarization in HEK293 cells expressing TRPV1 (Fig. 3A). Figure 3B shows the Boltzmann fits to these data. Isoflurane reduced the $V_{1/2}$ by 23.0± 6.2 mV and enhanced the maximal current by 15±6% (n=5).

TRPV1 is characteristically gated by heat with an activation threshold of ~42-43°C in mammalian cells and ~46°C in oocytes (Caterina et al., 1997). We asked whether isoflurane could alter this temperature sensitivity. In TRPV1-expressing oocytes isoflurane significantly reduced the temperature threshold in a dose-dependent manner (Figs. 3C&D); the thresholds for control, 0.5 mM and 0.9 mM isoflurane respectively were ~46°C, 43°C and 40°C.

Clinical concentrations of Diverse VGAs regulate TRPV1

Next, we explored whether VGAs could effectively modulate TRPV1 at clinically-relevant concentrations. Figure 4 shows that isoflurane (0.1 to 2 mM) enhanced proton-evoked responses in a dose-dependent manner and a significant potentiation occurred between 0.1 to 0.9 mM (corresponding to ~0.3 to 3 MAC). Thus isoflurane, at concentrations achieved during maintenance anesthesia, is capable of enhancing TRPV1 activity. Next, we explored the effects of different VGAs. Figure 5 show that VGAs (0.6mM) with the most pungency, desflurane and enflurane, enhanced proton-evoked currents significantly more than the less pungent agents isoflurane and sevoflurane. Therefore, similar to our earlier data with TRPA1 (Matta et al., 2008), there is a correlation, albeit less pronounced, between VGA pungency and TRPV1 sensitization.

PKC and bradykinin enhance VGA activation of TRPV1

Many inflammatory mediators engage G-protein coupled receptors expressed on sensory neurons, leading to the activation of protein kinase C (PKC). In turn, PKC produces a marked sensitization of TRPV1 (Numazaki et al., 2002; Premkumar and Ahern, 2000; Vellani et al., 2001). We found that PKC significantly

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enhanced heat activation of TRPV1 by VGAs. After application of phorbol 12, 13 dibutyrate (PDBu), isoflurane (0.9 mM) reduced the temperature threshold further from 45.9±0.2 to 32.8 ± 0.8 ºC, whereas PDBu alone reduced it to 39.3 ± 2.3 ºC (Fig. 3D). In contrast, PDBu did not produce a significant effect in oocytes expressing mutant TRPV1 receptors that lack essential PKC-phosphorylation sites (S502A/S800A, 39.4±1.9°C and 37.3±0.7°C for isoflurane (n=5) and PDBu + isoflurane (n=3) respectively), indicating that PDBu produces its effects through direct phosphorylation of TRPV1. This effect of PKC was more dramatic in mammalian cells. After PDBu treatment, VGAs (0.9 mM) evoked inward currents at room temperature (25°C) in both TRPV1-expressing HEK293 cells (Fig. 6A&C, n = 7) and in capsaicin-sensitive sensory neurons (Fig. 6B-D, n=6). Further, isoflurane activated single TRPV1 channel activity in neurons after PDBu treatment. These responses were completely inhibited by the TRPV1-specific antagonist, AMG9810 ([(E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide]; see Fig. 6B&D, n=3), indicating the selective activation of TRPV1.

Surgery is associated with tissue injury and the release of numerous inflammatory mediators that can activate/sensitize sensory neurons. One key "pain" signaling molecule is bradykinin (BK). BK acting through its type two receptor can activate/sensitize TRPV1 (Cesare and McNaughton, 1996; Chuang et al., 2001; Premkumar and Ahern, 2000; Shin et al., 2002) and TRPA1 (Bandell et al., 2004; Bautista et al., 2006) via multiple signaling pathways. We found that BK enhanced the responses of sensory neurons to isoflurane (Fig. 7C&D). Under control conditions isoflurane produced negligible responses, but after BK treatment, there was a marked increase in current (n=7, P<0.01). These neurons were all insensitive to AITC, excluding a contribution of TRPA1, and the TRPV1 blocker, capsazepine (*N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2-H-2-benzazepine-2-carbothioamide; 1μM), completely inhibited responses to isoflurane (in 3 of 3 cells), indicating that the major effect of BK was to recruit previously quiescent TRPV1 channels. These responses are sufficient to drive membrane excitability; under current-clamp, co-application of isoflurane and BK depolarized neurons and initiated sustained spike discharge (Fig. 7D, n=3). Taken together, these data

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provide strong support for the hypothesis that tissue injury can amplify the excitatory actions of VGAs on sensory neurons.

Volatile anesthetics interact directly with TRPV1 channels

VGAS could potentially alter TRPV1 activity by altering cellular signaling cascades. However, our data showed that VGAs retained their effect on TRPV1 in cell-free patches indicating a membrane-delimited effect. To examine whether VGAs regulate TRPV1 by directly interacting with the TRPV1 protein, as opposed to effects on membrane fluidity, we investigated the action of long chain alcohols. The results of several studies indicate that VGAs and alcohols bind directly to $GABA_A$ and glycine receptors, at a common binding site located between transmembrane domains 2 & 3 (Mascia et al., 2000; Mihic et al., 1997). Further, alcohols exhibit a carbon chain-length "cutoff"; the potency of alcohols increase with carbon chain length up until this cutoff, after which further increases in molecular size no longer increase alcohol potency (Mascia et al., 2000; Mihic et al., 1997). These data are consistent with alcohols binding to a "pocket" on these channels of finite molecular volume. Figure 8A-D shows that n-alcohols (2-12 carbons) enhanced voltage-dependent activation of TRPV1 in proportion to carbon chain length. Shifts in the $V_{1/2}$ and increases in maximal conductance reached a maximum with octanol, thereafter, decanol produced a smaller response and dodecanol was without effect. Next, we examined whether alcohols and VGAs act at similar binding site(s) on TRPV1. We found that isoflurane (0.9 mM) produced negligible effects on TRPV1 when applied together with an apparent saturating dose of octanol (1.8 mM) (Fig. 8E). This result was not due to a "ceiling effect" because the responses to octanol were submaximal (~40% of that produced by 10 μM capsaicin at 200 mV, data not shown). Therefore, these non-additive effects are consistent with VGAs and alcohols acting at the same site(s).

Discussion

GAs generally suppress cell excitability in the CNS and this may occur through a concerted action on many targets including sensitization/activation of K^+ channels (Franks and Lieb, 1988); (Gray et al., 1998; Patel et al., 1999) and GABA receptors (Jones et al., 1992; Nakahiro et al., 1991; Wakamori et al., 1991) and

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inhibition of NMDA receptors (Hollmann et al., 2001; Yamakura and Harris, 2000). Therefore, it may appear counter-intuitive that GAs can excite peripheral sensory neurons. However, there is abundant evidence that GAs can activate C-fibers innervating the skin (Campbell et al., 1984), cornea (MacIver and Tanelian, 1990), airways (Mutoh and Tsubone, 2003; Mutoh et al., 1998) and vasculature (Doenicke et al., 1996; Picard and Tramer, 2000; Tan and Onsiong, 1998). Our recent data show that the TRPA1 ion channel mediates these acute noxious effects of GAs (Matta et al., 2008). Our results here show that GAs also sensitize TRPV1, a principal nociceptive ion channel highly expressed in sensory nerves. VGAs at pharmacological concentrations sensitize TRPV1 to activation by various stimuli including capsaicin, protons, heat and voltage. Significantly, this effect is greater with pungent compared with non-pungent VGAs. Furthermore, VGAs have a more marked effect under inflammatory signaling conditions; following stimulation of PKC, isoflurane alone activates TRPV1 at room temperature. Similarly, isoflurane directly activates TRPV1 and excites sensory neurons after treatment with the inflammatory mediator, bradykinin.

VGAs appear to regulate TRPV1 directly and several lines of evidence support this idea. First, VGAs were effective in cell-free patches indicating a membrane-delimited action. Second, although VGAs can alter membrane fluidity this effect is unlikely to explain their modulating TRPV1; we have observed that treatment with an agent that increases membrane fluidity, Triton-X, produces the opposite effect to isoflurane on TRPV1 function (Matta et al., 2007). In addition, the differential modulation of TRPV1 by smooth versus pungent VGAs does not correlate with their lipid solubility, arguing against non-specific effects on the membrane. Third, long chain alcohols replicated the effects of VGAs and exhibited a carbon-chain length cut-off, consistent with these agents binding directly to a protein site. Alcohols and VGAs similarly modulate the TRPA1 channel (Matta et al., 2008). Taken together, these results suggest that VGAs interact directly with the TRPV1 protein. The precise molecular sites(s) are unclear. In addition to VGAs, several other ligands (for example, polyunsaturated fatty acids and 2-Aminoethoxydiphenyl borate) are capable of activating both TRPV1 and TRPA1. Thus, it is possible that VGAs interact with common binding sites(s) on these channels. Interestingly, local anesthetics have recently been shown to activate and sensitize TRPV1 (Leffler et al., 2008).

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This effect is attenuated in TRPV1 mutants lacking capsaicin sensitivity, suggesting that local anesthetics interact, in part, at capsaicin binding sites. In the future, studies with mutagenesis and TRP chimeric proteins may be prove useful in identifying potential anesthetics binding sites(s). This approach was used in a recent study of the two-pore K^+ channel, TASK3, to reveal an essential role for a single amino acid (M159) in the third transmembrane domain; mutation of this residue abolished anesthetic activation (Andres-Enguix et al., 2007).

Our finding that VGAs can activate TRPV1 during inflammatory signaling has significant implications. First, in the laboratory setting, studies of central sensitization in the spinal cord are routinely performed on anesthetized animals. The use of VGAs in these experiments and subsequent facilitation of TRPV1 (and TRPA1) signaling may influence these measurements. Second, in the clinical setting our data support the hypothesis that VGAs may augment nociceptive signaling arising from surgical insults (see Model, Figure 9). Tissue injury leads to the release of an array of inflammatory mediators, including ATP, protons, serotonin, bradykinin and prostaglandins. Protons directly regulate TRPV1. The other mediators act at G-protein coupled receptors leading to the formation of arachidonic acid metabolites that can gate TRPV1 (Hwang et al., 2000), and/or the stimulation of protein kinases. PKC-mediated phosphorylation, in particular, profoundly sensitizes TRPV1 to chemical and thermal activation (Premkumar and Ahern, 2000; Sugiura et al., 2002; Vellani et al., 2001). Our data show that PKC stimulation enhances the effects of isoflurane leading to the activation of TRPV1 at room temperature. Moreover, we show that isoflurane acts in a synergistic fashion with the inflammatory mediator, bradykinin, to activate TRPV1 currents. Bradykinin-signaling also activates TRPA1, and this may occur downstream of TRPV1 activation (Bautista et al., 2006). Importantly these results suggest that VGAs could enhance the activation of TRPV1 and TRPA1 in the context of tissue injury.

The activation/sensitization of TRPV1 by VGAs has two broad consequences. First, it may lead to increased release of neuropeptides from peripheral terminals, culminating in greater neurogenic inflammation (see Figure 9). Indeed, we have shown that mustard oil (TRPA1-dependent)-evoked inflammation is greater in animals anesthetized with pungent compared with non-pungent VGAs (Matta et al., 2008). The higher expression of TRPV1 in sensory nerves suggests that VGAs acting at TRPV1 would produce even more marked

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effects. Second, it should lead to enhanced C-fiber discharge. Since central sensitization is dependent on C-fiber spike frequency (Ji et al., 2003) then stimulation of TRPV1 by VGAs could lead to a medium to long-lasting facilitation of nociceptive processing in the spinal cord. Thus, through these TRP-dependent mechanisms, VGAs could augment post-surgical pain and inflammation.

How could one avoid the sensitization of TRPs by VGAs? One strategy is through the use of non-pungent agents such as sevoflurane; these non pungent VGAs have no activity at TRPA1 (Matta et al., 2008) and significantly less activity at TRPV1 compared with pungent VGAs. Another strategy is the use of pre-emptive analgesia with local anesthetics and opioids (Gottschalk et al., 2002; Wilder-Smith, 2000). However, while these drugs would inhibit C-fiber discharge and transmission in the spinal cord they would not prevent direct activation of TRPV1 and TRPA1 and the resultant neuropeptide secretion at nerve terminals. Finally, the use of selective TRP antagonists may have utility by inhibiting the sensitizing effects of GAs as well as the generalized excitation of nociceptors by inflammatory mediators.

In summary, our results show that clinically-relevant concentrations of volatile anesthetics activate and sensitize the TRPV1 channel. These results suggest that these VGAs may enhance peripheral nociceptive signaling in the context of surgery.

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Figure legends

Figure 1. Isoflurane enhances capsaicin-evoked TRPV1 currents. A, Representative current trace from a voltage-clamped neuron treated sequentially with isoflurane (0.9mM), capsaicin (30 nM) and isoflurane plus capsaicin. B, Upper trace, Continuous recording of capsaicin sensitive channels in an outside/out patch (holding potential of +50mV) in the presence of capsaicin (30nM) or capsaicin plus isoflurane (0.9 mM). Lower traces (i-iii), Expanded sections of recording from indicated timepoints. C, Dose-response curves in oocytes for activation of TRPV1 by capsaicin with or without isoflurane (0.9mM, n = 3-7 for each data point). The smooth curves are fits to a Hill function yielding EC_{50} values of 1.64±0.12 μM and 0.81±0.04 μM for control and isoflurane respectively.

Figure 2. Isoflurane enhances the sensitivity of TRPV1 to protons. A, Current trace from a TRPV1-expressing oocyte treated with pH 5.5 and pH 5.5 plus isoflurane (0.9mM) solutions. B, Dose-response curves in oocytes for activation of TRPV1 by protons in the absence or presence of isoflurane (0.9mM, n = 3-7 for each data point). The smooth curves are fits to a Hill function yielding pEC_{50} of 4.95±0.15 and 5.23±0.10 for control and isoflurane respectively. Isoflurane also increased the maximal response from 14.3% to 32.2% of 10μM capsaicin ($P<0.01$)

Figure 3. Volatile GAs increase the sensitivity of TRPV1 to voltage and heat. A, TRPV1 currents activated by a family of voltage steps (-90 to 210 mV) under control conditions and with isoflurane (0.9 mM). B, Plots of tail current versus voltage-prepulse for control and isoflurane. Smooth lines are best fits to a Boltzmann function yielding $V_{1/2}$ values of 161.5±2.7 mV and 129.0±3.6 mV. C, Current versus temperature plots in TRPV1-expressing oocytes for control (black), 0.5mM (blue), or 0.9mM (red) isoflurane. Currents are normalized to the maximum current evoked at 47°C. D, Mean thresholds of heat activation for control, 0.5 mM

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isoflurane, 0.9 mM isoflurane, PDBu (200 nM, 3 minutes) and PDBu + isoflurane (0.9 mm), *P<0.01 compared with control, or versus PDBu alone. Data are mean of 4-5 oocytes.

Figure 4. Isoflurane modulates TRPV1 at clinically-relevant concentrations. The mean fold increase in proton-evoked currents in oocytes produced by co-application with varying concentrations of isoflurane (0.1-2 mM, 0.3-3 MAC) or a second application of pH 5.5 alone. Data are the mean of 3-4 oocytes, * P<0.01 compared with pH 5.5 alone.

Figure 5. Modulation of TRPV1 by diverse volatile anesthetics. The relative potentiation of proton (pH5.5)-evoked currents by 0.6 mM concentrations of sevoflurane, isoflurane, enflurane and desflurane (n=3-4 for each data point). * P<0.01 compared with control. ** P<0.01 between designated groups of VGAs (one-way ANOVA)

Figure 6. Isoflurane activates TRPV1 in a PKC-dependent manner. A, After pretreatment with PDBu (500 nM) halothane (0.9mM) and isoflurane (0.9mM) activate currents in TRPV1-expressing HEK293 cells. B, AMG9810 (1 μM) inhibits the current evoked by isoflurane (0.9mM) in a sensory neuron (pretreated with PDBu). C, Mean current evoked by isoflurane in TRPV1-expressing HEK293 cells and capsaicin-sensitive sensory neurons, with or without PDBu treatment. Data are normalized to responses evoked by a saturating capsaicin concentration (5 μM), and the number of cells are given in parentheses. D, Single TRPV1 channel activity in an outside-out patch from a sensory neuron in response to isoflurane (0.9mM) and AMG9810 (1μM). The holding potential was +60 mV.

Figure 7. Isoflurane and bradykinin synergistically excite TRPV1 and sensory neurons. A, Bradykinin (BK, 10 μM) enhances capsaicin (30 nM)-evoked currents in sensory neurons. B&C, Co-application of BK and isoflurane (0.9mM) induces inward currents in sensory neurons and these currents are inhibited by capsazepine

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(1 μM). D, Co-application of BK and isoflurane depolarizes a capsaicin-sensitive sensory neuron under current clamp. The arrow indicates -60 mV.

Figure 8. Volatile anesthetics interact directly with TRP channels. **A,** Representative TRPV1 current traces in response to voltage steps in the presence of various-alcohols. B**,** Boltzmann fits to the conductance measured at the end of test potential. C&D**,** Summary of changes in TRPV1 $V_{1/2}$ and maximal conductance induced by alcohols (n=4-7 cells). Concentrations were: ethanol (508 mM), hexanol (3mM), octanol (1mM), decanol (0.6mM) and dodecanol (0.1 mM) and were chosen according to the solubility limitations of these alcohols as described previously (Peoples and Weight, 1995) E**,** Octanol (1.8 mM) and isoflurane (0.9 mM) modulate TRPV1 in a non-additive fashion.

Figure 9. Model of synergistic activation of TRPs by anesthetics and inflammatory mediators in sensory nerves. Tissue injury leads to accumulation of inflammatory mediators such as proteases and bradykinin which engage their respective G-protein coupled receptors (protease receptor, PAR; bradykinin receptor, BKR) expressed on sensory nerves. In turn, this leads to sensitization of TRPV1 and TRPA1 via phospholipase C-dependent pathways. VGAs act directly on TRPs to further enhance their activity. Finally, depolarization and Ca^{2+} entry via TRPs evokes release of inflammatory peptides including substance P (SP) and calcitonin gene-related peptide (CGRP).

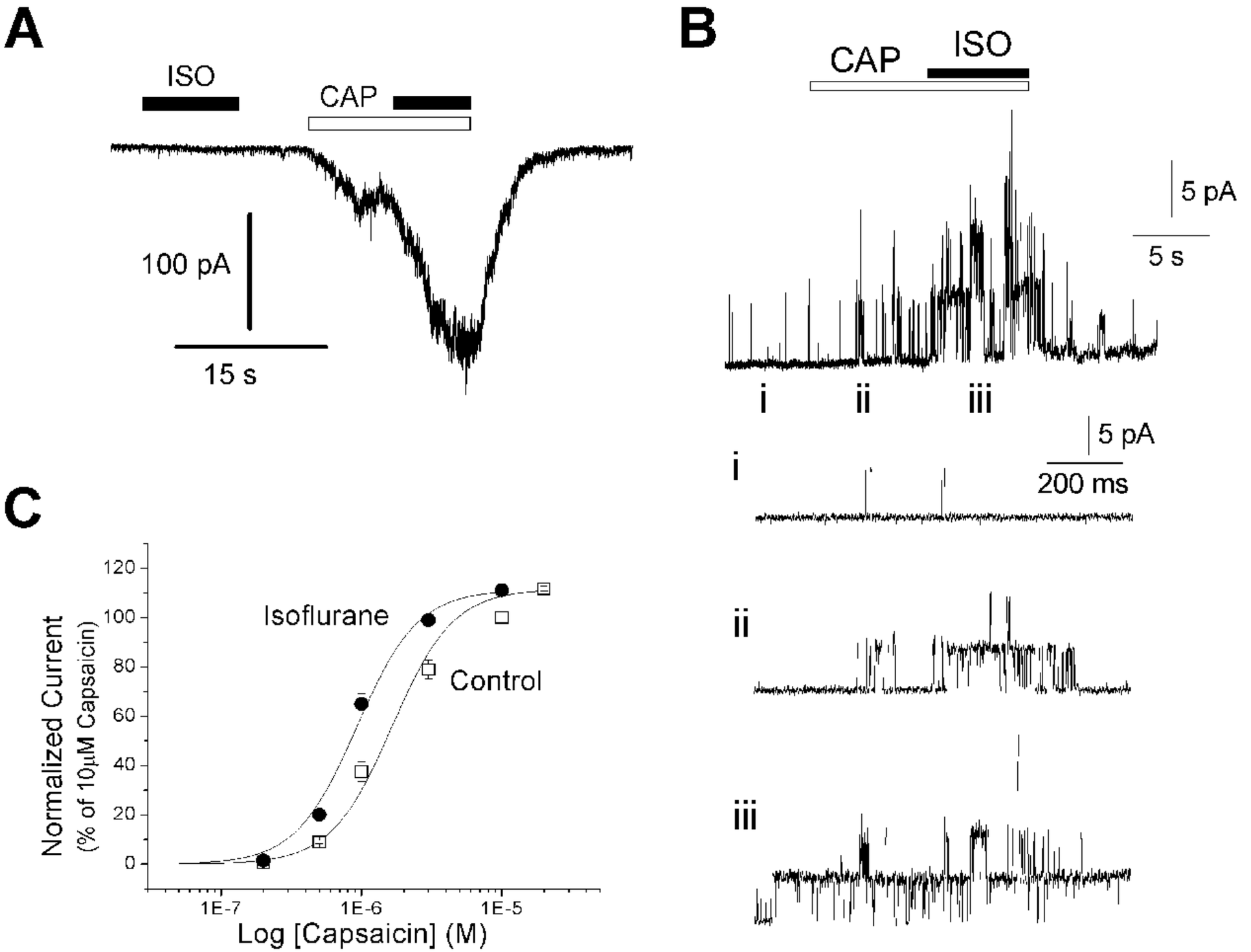
A
ISO
CAP
100 pA
15 s
B
CAP
ISO
5 pA
5 s
i
ii
iii
5 pA
200 ms
i
ii
iii
C
Isoflurane
Control
Normalized Current
(% of 10μM Capsaicin)
120
100
80
60
40
20
0
1E-7
1E-6
1E-5
Log [Capsaicin] (M)

Figure 1

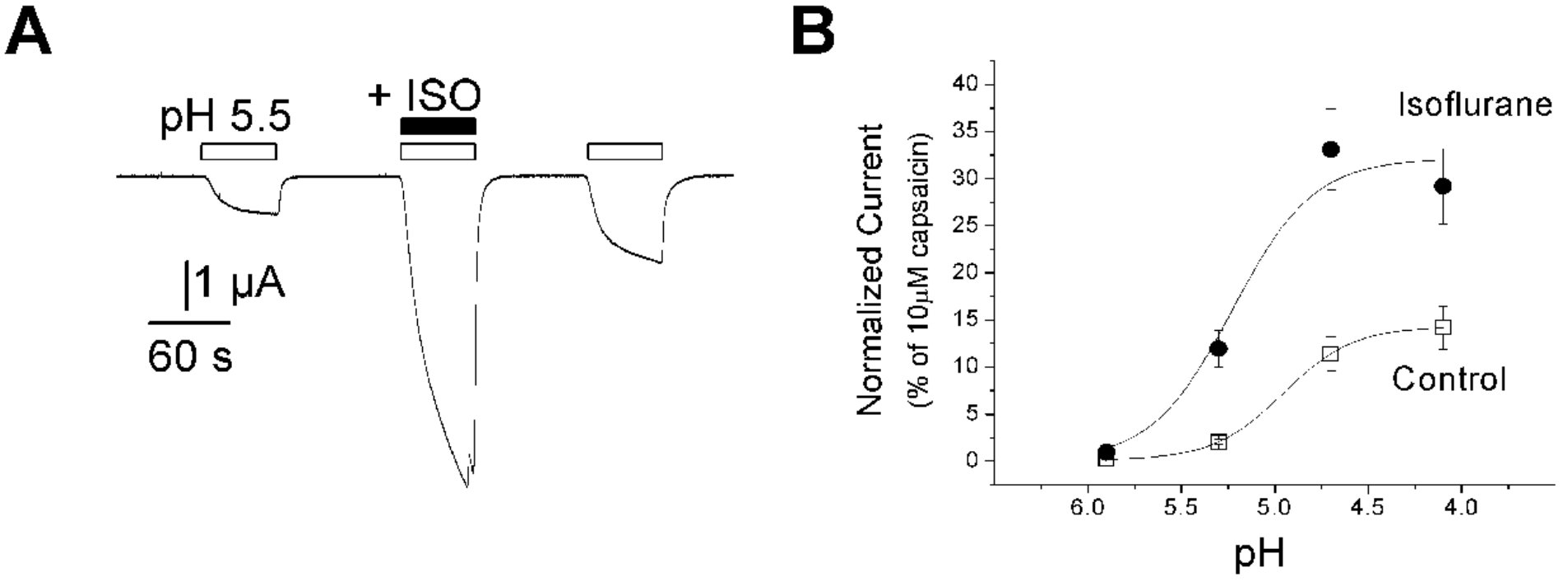
A
pH 5.5
+ ISO
1 µA
60 s
B
Normalized Current
(% of 10µM capsaicin)
Isoflurane
Control
pH
6.0
5.5
5.0
4.5
4.0
0
5
10
15
20
25
30
35
40

Figure 2

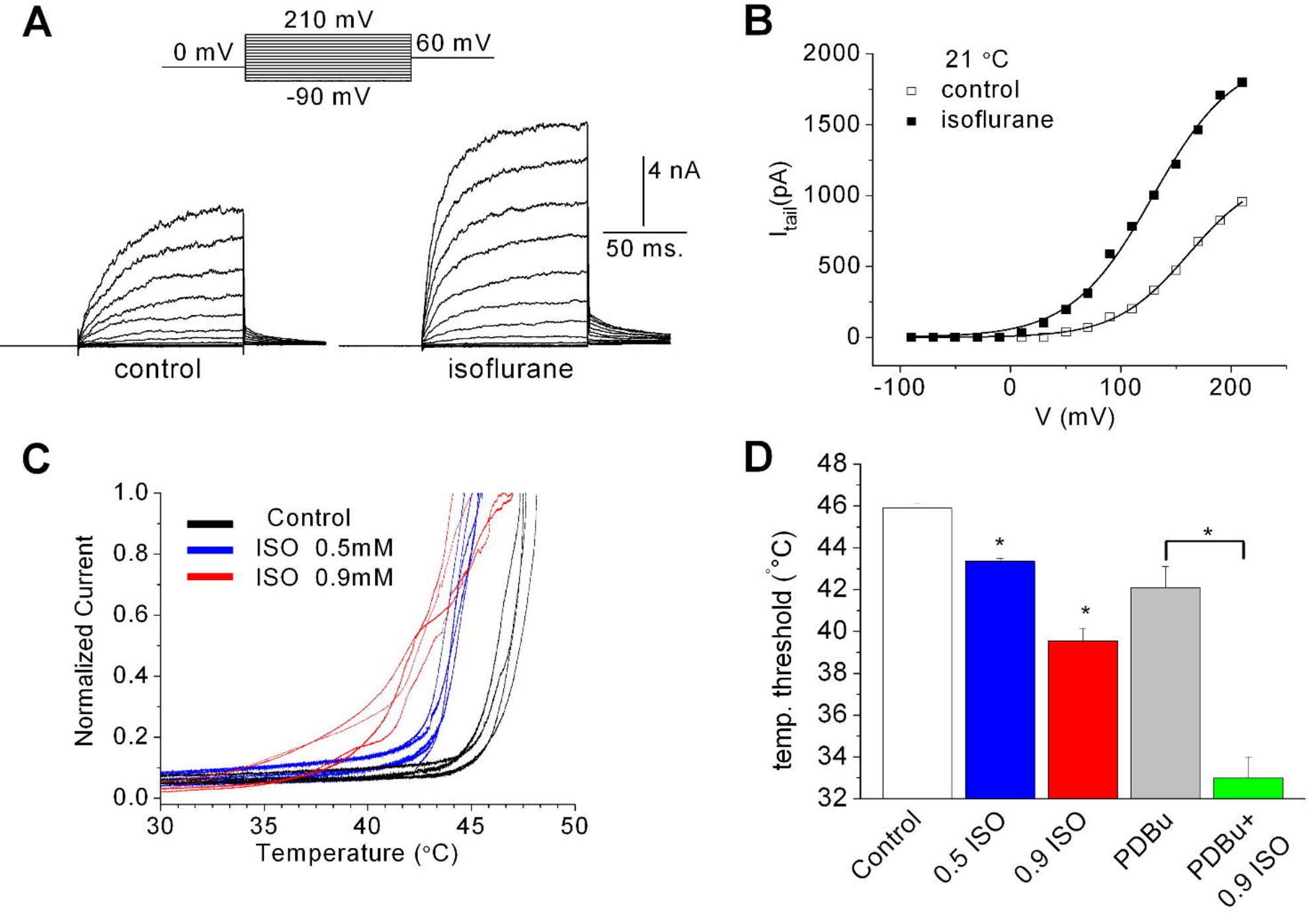
A
210 mV
0 mV
60 mV
-90 mV
4 nA
50 ms.
control
isoflurane
B
21 °C
control
isoflurane
I_{tail}(pA)
2000
1500
1000
500
0
-100
0
100
200
V (mV)
C
Control
ISO 0.5mM
ISO 0.9mM
Normalized Current
1.0
0.8
0.6
0.4
0.2
0.0
30
35
40
45
50
Temperature (°C)
D
temp. threshold (°C)
48
46
44
42
40
38
36
34
32
*
*
*
Control
0.5 ISO
0.9 ISO
PDBu
PDBu+
0.9 ISO

Figure 3

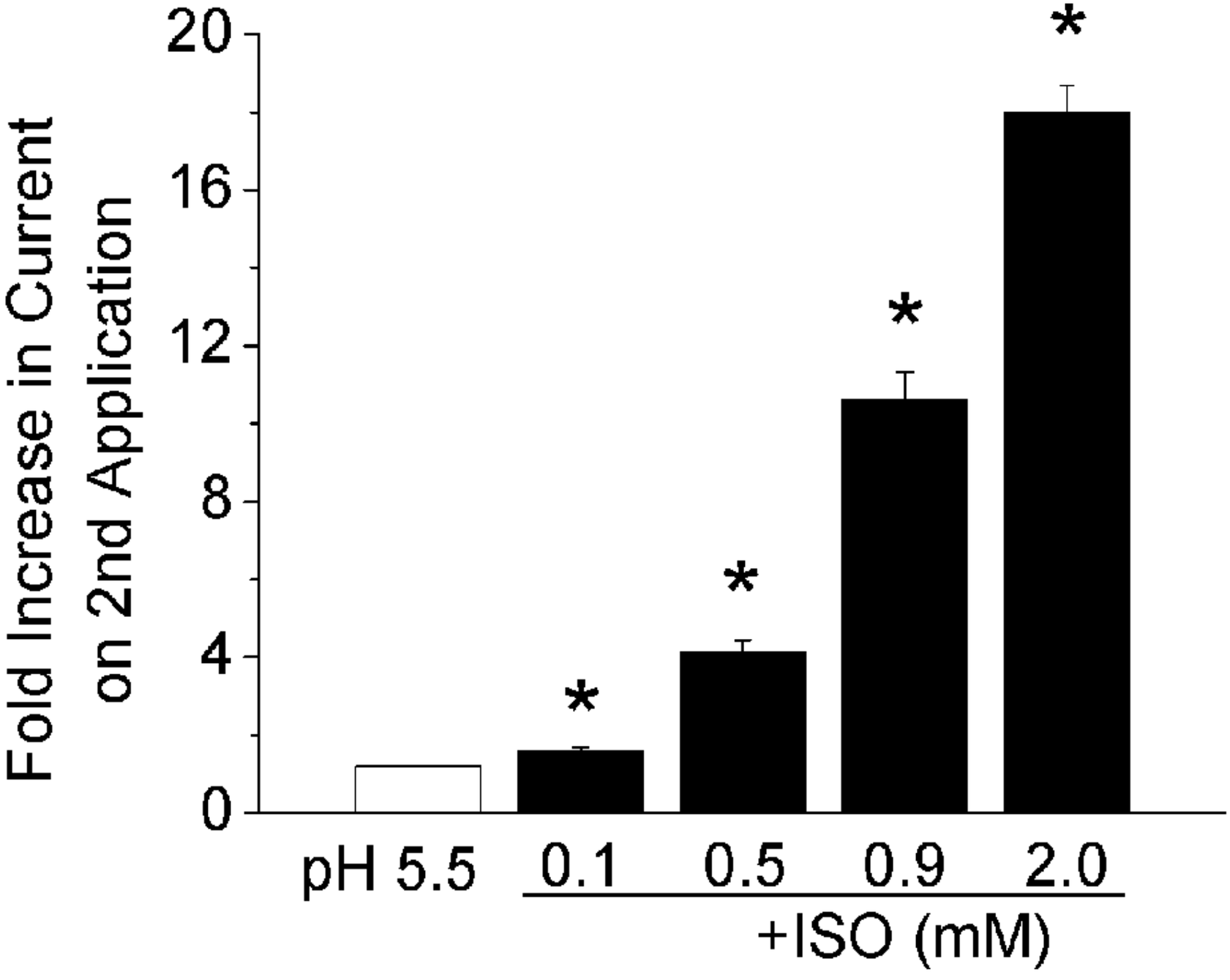
Fold Increase in Current on 2nd Application
20
16
12
8
4
0
*
*
*
*
pH 5.5
0.1
0.5
0.9
2.0
+ISO (mM)

Figure 4

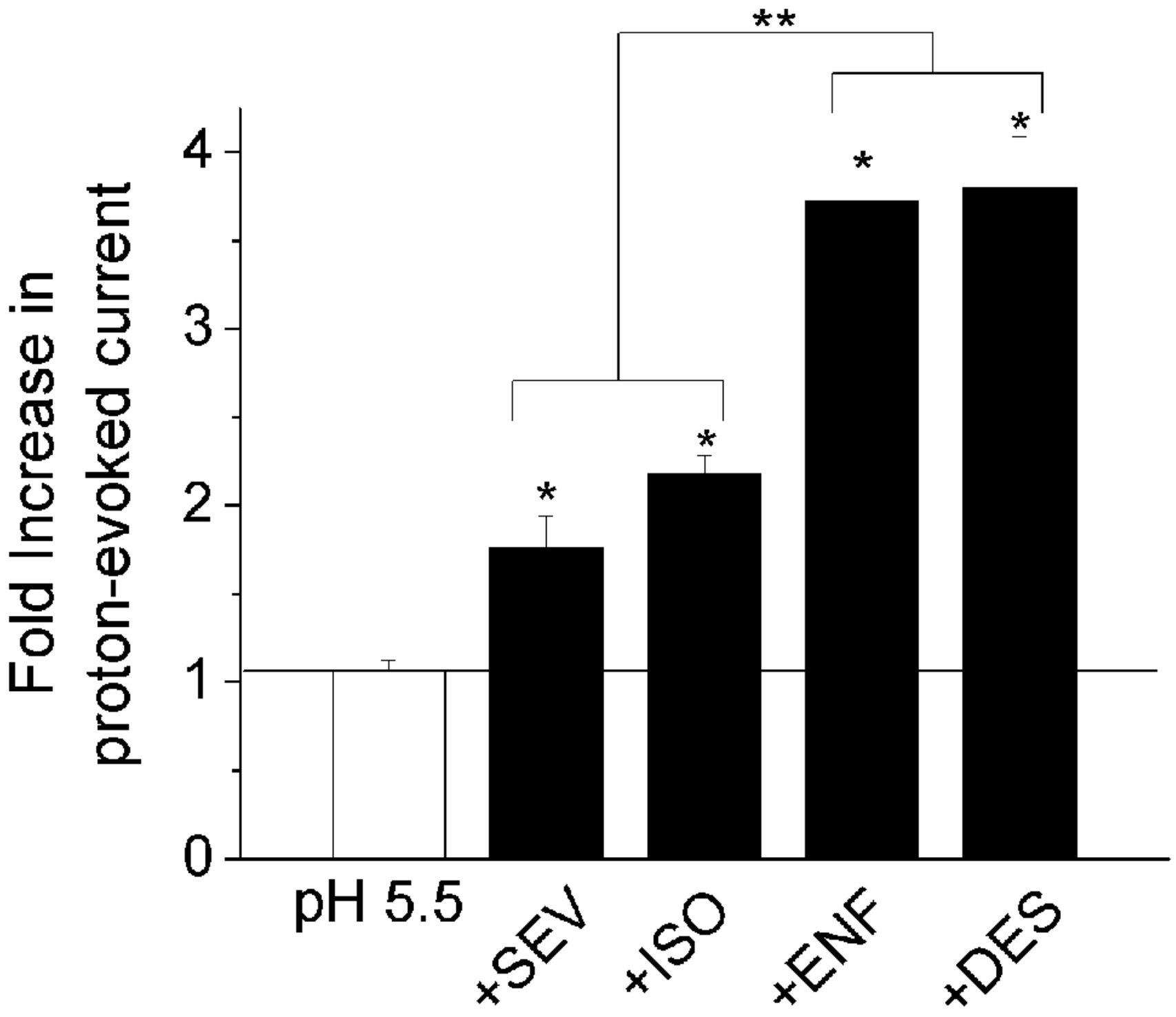
Fold Increase in proton-evoked current
0
1
2
3
4
**
*
*
*
*
pH 5.5
+SEV
+ISO
+ENF
+DES

Figure 5

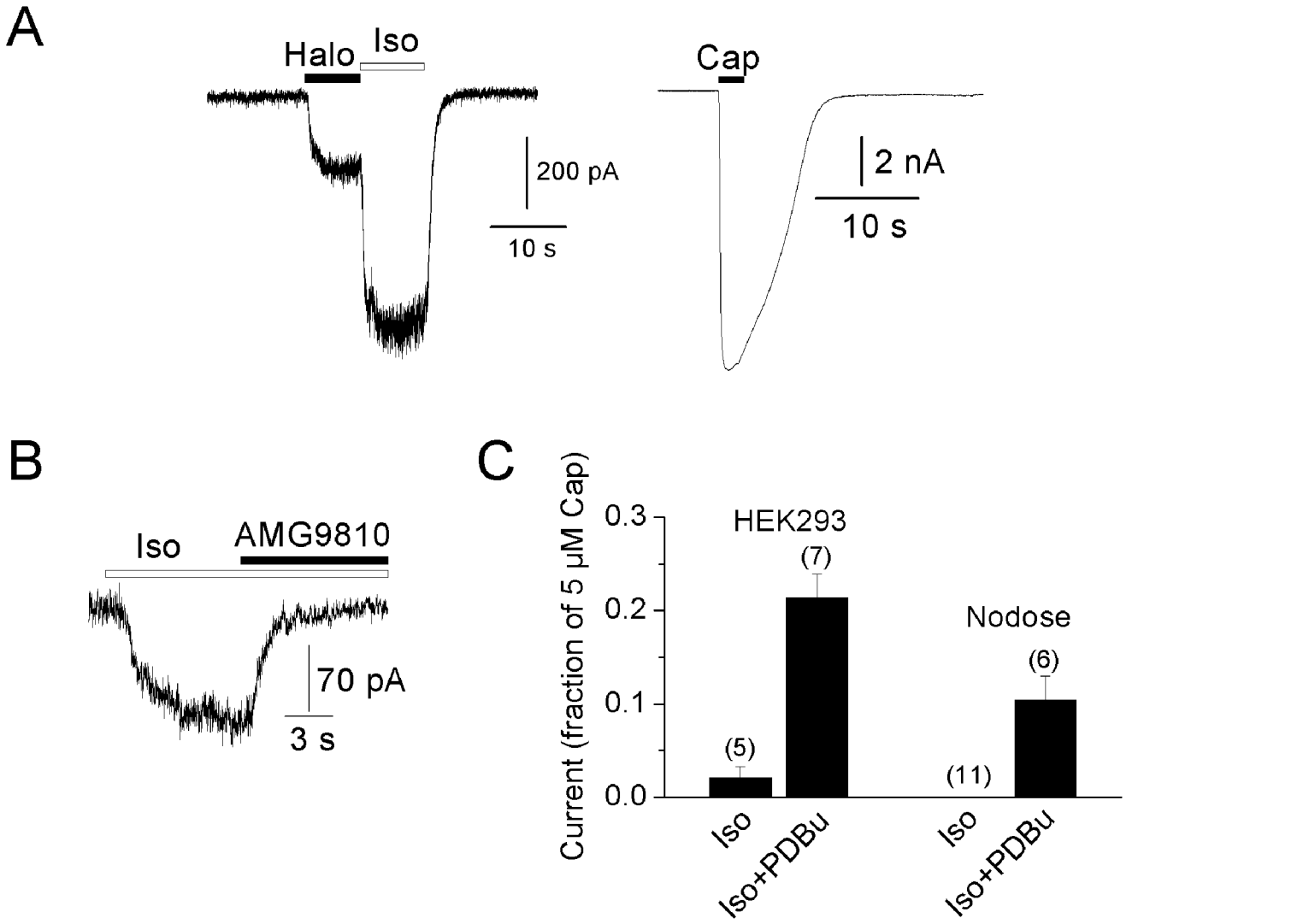

Figure 6

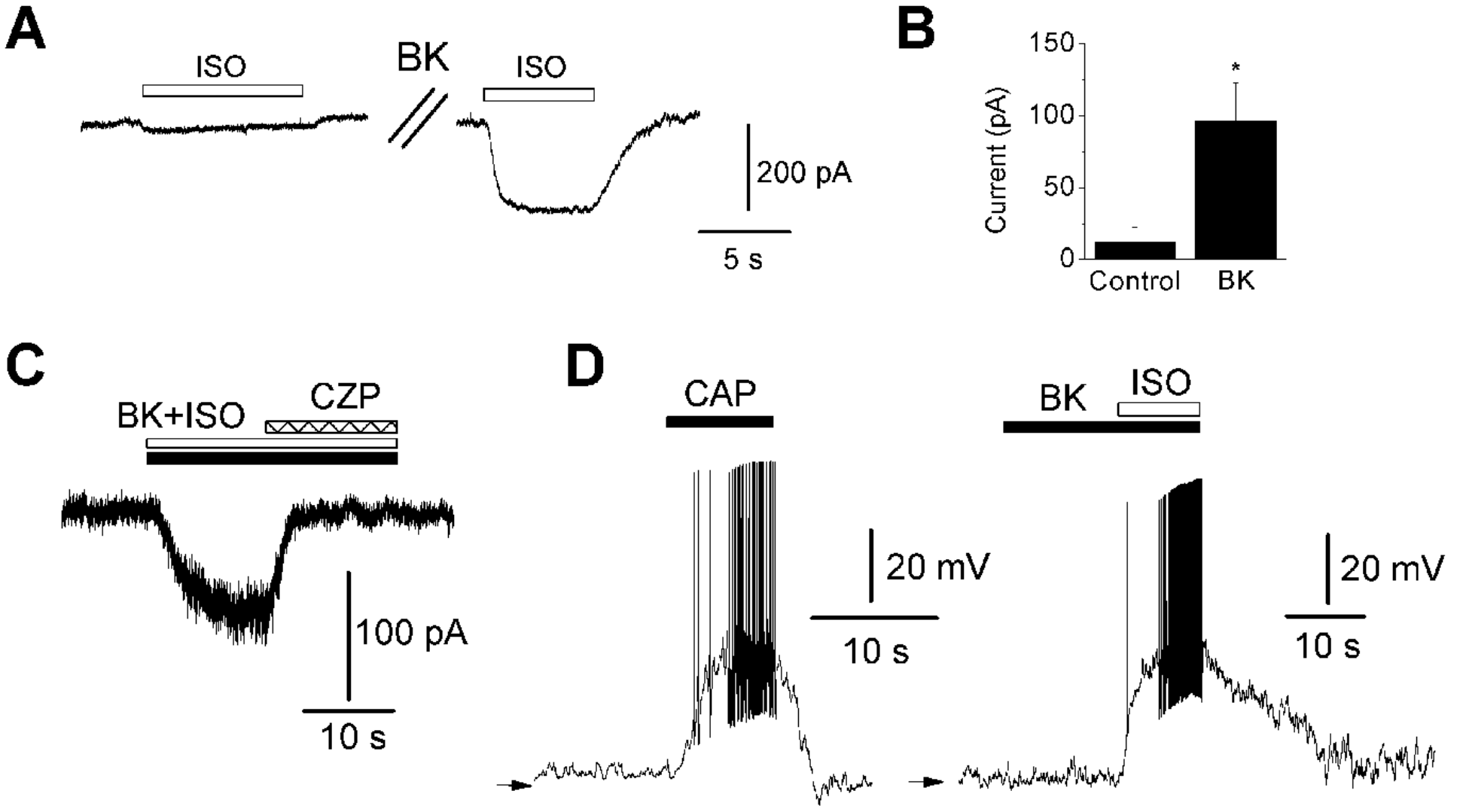
A
ISO
BK
ISO
200 pA
5 s
B
150
100
50
0
Current (pA)
*
Control
BK
C
BK+ISO
CZP
100 pA
10 s
D
CAP
20 mV
10 s
BK
ISO
20 mV
10 s

Figure 7

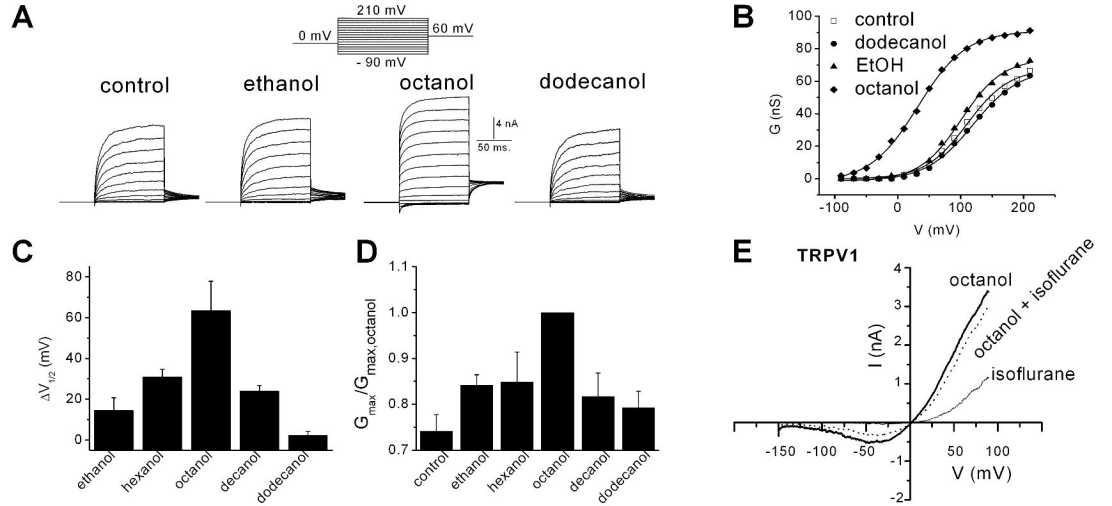
A
210 mV
0 mV
60 mV
- 90 mV
control
ethanol
octanol
dodecanol
4 nA
50 ms.
B
control
dodecanol
EtOH
octanol
G (nS)
V (mV)
C
ΔV1/2 (mV)
ethanol
hexanol
octanol
decanol
dodecanol
D
Gmax/Gmax,octanol
control
ethanol
hexanol
octanol
decanol
dodecanol
E
TRPV1
octanol
octanol + isoflurane
isoflurane
I (nA)
V (mV)

Figure 8

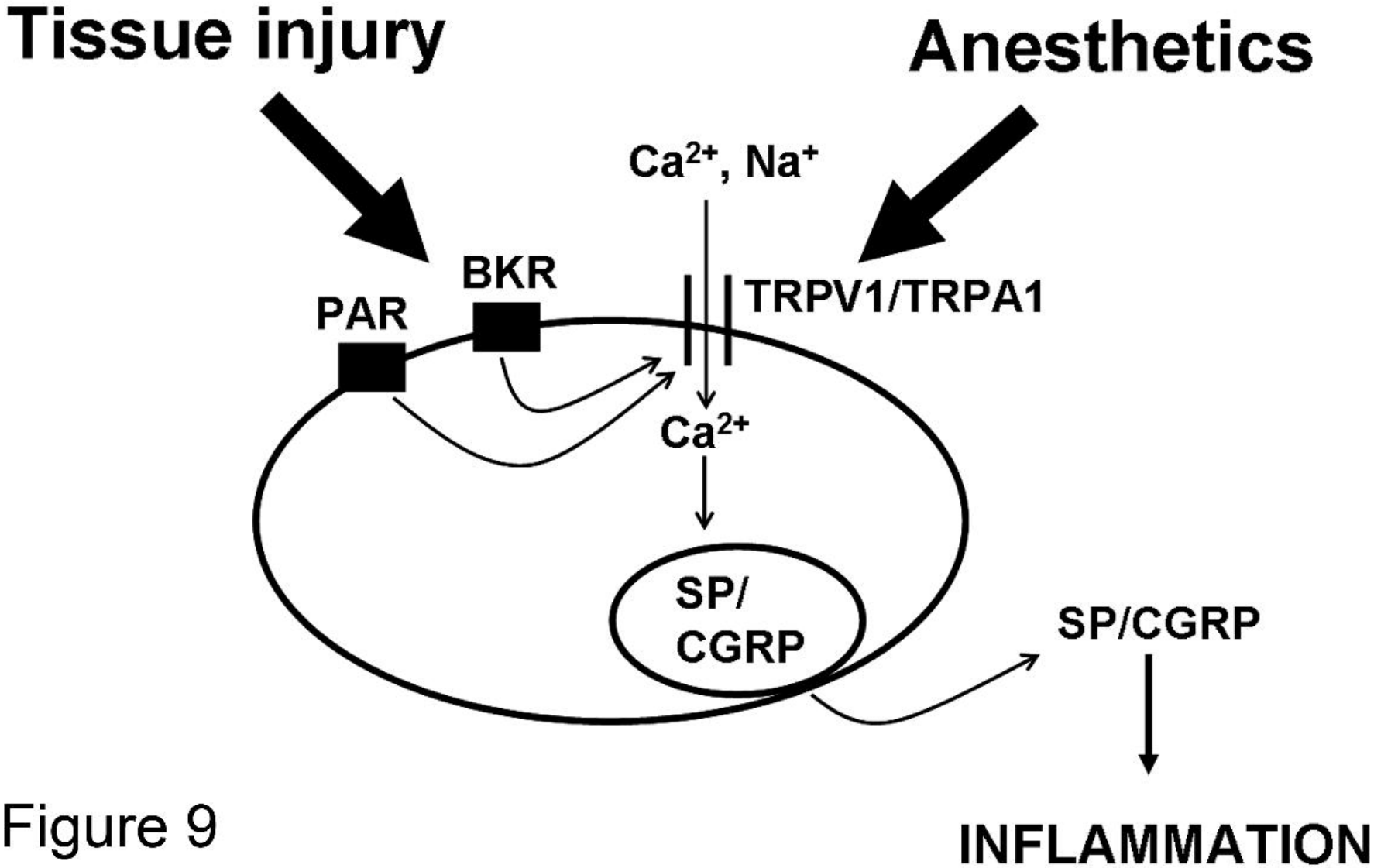
Tissue injury
Anesthetics
Ca2+, Na+
BKR
PAR
TRPV1/TRPA1
Ca2+
SP/
CGRP
SP/CGRP
INFLAMMATION

Figure 9