Etodolac Activates and Desensitizes Transient Receptor Potential Ankyrin 1

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The transient receptor potential ankyrin 1 (TRPA1) channel is well known as a sensor to environmental irritant compounds, cold, and endogenous proalgesic agents. TRPA1 is expressed on sensory neurons and is involved in pain modulation. Etodolac is a cyclooxygenase (COX)-2 inhibitor that belongs to the class of nonsteroidal anti-inflammatory drugs (NSAIDs). A recent study indicates that etodolac inhibits allyl isothiocyanate (AITC)-induced calcium influx in heterologous HEK293 cells and sensory neurons. To examine whether and how etodolac modulates the TRPA1 channels, we applied etodolac to TRPA1-transfected HEK293 cells or rat dorsal root ganglion (DRG) neurons and recorded the currents using the whole-cell patch clamp technique. We found that etodolac at higher doses could activate and then desensitize TRPA1 channels in heterologous expressing HEK293 cells as well as in DRG neurons. The etodolac-induced currents were significantly attenuated in cysteine residues mutated human TRPA1-transfected HEK293 cells. Interestingly, application of etodolac at drug plasma levels in clinical usage did not induce significant TRPA1 currents but reduced the subsequent AITC-induced currents to 25% in HEK293 cells expressing TRPA1. Moreover, no modulatory effect of etodolac on TRPA1 was detected in the cysteine mutant cells. These data indicate a novel mechanism of the anti-inflammatory and analgesic clinical effects of etodolac, which may be involved with its direct activation and the subsequent desensitization of TRPA1 through the covalent modification of cysteine residues.

Key words: pain; NSAIDs; desensitization

The transient receptor potential ankyrin 1 (TRPA1) channel is expressed by a subset of small sensory neurons (Kobayashi et al., 2005; Nagata et al., 2005; Anand et al., 2008). TRPA1 has been reported to be activated by many irritants, such as some noxious, pungent chemicals; cold stimuli; and inflammatory factors (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004; Macpherson et al., 2005; Fujita et al., 2007), as well as by several endogenous components (Trevisani et al., 2007; Cruz-Orengo et al., 2008; Fujita et al., 2008; Materazzi et al., 2008; Ohkawara et al., 2012). Most of these compounds activated TRPA1 through covalent modification of cysteine residues (Hinman et al., 2006; Macpherson et al., 2007). TRPA1 has been reported to be involved in the mediation of formalin-induced pain (McNamara et al., 2007) and shown to be an important component of the transduction machinery through which environmental irritants and endogenous proalgesic agents activate nociceptors to elicit inflammatory pain (Bautista et al., 2006; Kwan et al., 2006; André et al., 2008; Lapointe and Altier, 2011; Lennertz et al., 2012). Inhibition of TRPA1 significantly attenuated mechanical hypersensitivity in inflammation and neuropathy (Eid et al., 2008). Overall, it is clear that this channel is one of the important transducers of noxious stimuli in primary afferents.

Prostaglandins (PGs) are produced from oxidation of arachidonic acid (AA) by COX-1 or COX-2. COX-1 is responsible for the baseline levels of PGs, whereas COX-2 produces PGs after stimulation. Selective inhibition of COX-2 has been considered to reduce the levels of inducible PGs in inflammation but to have no effect on constitutive PGs. Most nonsteroidal anti-inflammatory drugs (NSAIDs) act as nonselective inhibitors of COX. Selective COX-2 inhibitors have been developed as alternatives to COX-2 inhibitors, which may be involved with its direct activation and the subsequent desensitization of TRPA1 through the covalent modification of cysteine residues.

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Etodolac, as a selective COX-2 inhibitor, is widely used for the treatment of inflammatory disorders and painful conditions such as rheumatoid arthritis (Tirunagari et al., 2009; Sancheti et al., 2010; Colebatch et al., 2011).

A recent study shows that etodolac induced Ca\(^{2+}\) influx in TRPA1-expressing cells and sensory neurons and that this increase in intracellular calcium was blocked by a TRPA1-selective antagonist. In addition, etodolac reduced allyl isothiocyanate (AITC)-induced nociceptive behavior (Inoue et al., 2012). To investigate further whether and how etodolac activates TRPA1, we tested the effects of etodolac on AITC-induced currents and found that etodolac not only selectively activated TRPA1 but also desensitized it. Moreover, covalent modification of cysteine residues was involved in the modulation of TRPA1 by etodolac.

**MATERIALS AND METHODS**

**Mammalian Cell Culture**

Human embryonic kidney-derived (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamax, penicillin, and streptomycin. HEK293 cells were transfected with a wild-type (WT) human TRPA1 (WT hTRPA1) or a triple cysteine mutant of hTRPA1 (cysteine-serine substitution at C621/C641/C665; 3C hTRPA1; Komatsu et al., 2012) cDNA or rat TRPV1 (rTRPV1) cDNA by using Lipofectamine LTX and Plus Reagent (Invitrogen, Carlsbad, CA). An enhanced green fluorescence protein reporter plasmid (BD Bioscience, San Jose, CA) was cotransfected with TRP channels. The WT hTRPA1, 3C hTRPA1, and rTRPV1 cDNAs were generous gifts from Prof. Makoto Tominaga. For primary culture of dorsal root ganglion (DRG) neurons, DRGs were collected from adult Sprague-Dawley rats (100–150 g) using sterile techniques and placed in ice-cold Earle’s balanced salt solution (EBSS; Sigma, St. Louis, MO). Adhering fat and connective tissue were removed, and each DRG was placed immediately in a medium consisting of 2 ml EBSS and 1.25 mg/ml collagenase P (Sigma) and kept at 37°C for 60 min, with occasional agitation. After dissociation of the DRG cells, this cell suspension was centrifuged for 5 min at 1,000 rpm, and the cell pellet was resuspended in EBSS supplemented with 10% FBS, 2 mM glutammax, MEM vitamin, penicillin, and streptomycin. Recombinant rat nerve growth factor (NGF; 100 ng/ml; Sigma) was added to the medium.

**Electrophysiology**

Whole-cell patch-clamp recordings were performed 2 days after transfection of hTRPA1 (WT or 3C) cDNA or 1 day after transfection of rTRPV1 cDNA to HEK293 cells or at 1 day after dissociation of the DRG neurons. Voltage-clamp experiments were performed at a −60 mV holding potential, and recordings were sampled at 5 kHz and filtered at 2 kHz. The current–voltage relationships were determined by using a 200-msec voltage step from −100 mV to +100 mV. Drugs were applied until the evoked currents underwent a desensitization. The current magnitude was quantified by peak current amplitude in all experiments. In experiments with DRG neurons, after AITC application, capsaicin (1 μM) was applied at the end of recording to identify whether the AITC-induced current was mediated by TRPA1 channels (Kwan et al., 2006). Data were obtained in the DRG neuron was sensitive to both AITC and capsaicin application. Standard bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, and 10 mM glucose, pH 7.4 (adjusted with NaOH). The pipette solution contained 140 mM KCl, 5 mM MgATP, 5 mM EGTA, and 10 mM HEPES, pH 7.2 (adjusted with KOH). All patch clamp experiments were performed at room temperature (RT; ~25°C). The solutions containing drugs were applied to the chamber (1 ml) by a gravity system at a flow rate of 3–4 ml/min.

**Compounds**

Etodolac, capsaicin, capsazeine, and 2-aminoethoxy diphenyl borate (2APB) were purchased from Sigma-Aldrich; in some experiments, etodolac was provided by Nippon Shinyaku Co.; AITC was purchased from Nacalai Tesque (Kyoto, Japan); HC 030031 was from Tocris Bioscience (Ellisville, MO). Glutamax, FBS, penicillin-streptomycin, MEM vitamin solution, and Opti-MEM were from Invitrogen (Carlsbad, CA).

**Statistical Analysis**

All results are expressed as mean ± SEM. An unpaired t-test was used to compare the electrophysiological data between the two groups. A difference was accepted as significant if the probability was less than 5% (P < 0.05).

**RESULTS**

**Etodolac Activated TRPA1 but Not TRPV1 in Heterologous HEK293 Cells**

We used whole-cell patch clamp to investigate the etodolac-induced currents (I\(_{ETO}\)) in hTRPA1-transfected HEK293 cells. We found that etodolac at concentrations from 0.5 to 200 μM did not induce obvious inward currents in hTRPA1-transfected HEK293 cells (Fig. 1A,C). However, etodolac at 300 μM induced a significant inward current that underwent desensitization (Fig. 1A). I\(_{ETO}\) were saturated at a concentration of 500 μM. The EC50 value for I\(_{ETO}\) was 296.5 μM (Fig. 1C). In contrast, etodolac at 500 μM did not induce any currents either in nontransfected (data not show) or in rTRPV1–transfected HEK293 cells (Fig. 1B). The I–V relationships showed that I\(_{ETO}\) and a TRPA1 potent agonist, AITC–induced currents (I\(_{AITC}\)), had identical reversal potentials and rectification properties (Fig. 1D). Together, the results suggest that etodolac, like other TRPA1 agonists, activates TRPA1 with nonselective cation permeability and undergoes desensitization.

**Etodolac Activated TRPA1 Through Covalent Modification of Cysteine Residues in the N-Terminal**

Many electrophilic agonists activate TRPA1 through covalent modification of cysteine residues in the N-terminal.

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Nonelectrophilic compounds, such as 2APB or icilin, might activate TRPA1 via a conventional ligand–receptor interaction. We tested whether etodolac activates TRPA1 through covalent modification of cysteine residues. The cysteine mutant of hTRPA1, 3C hTRPA1, was transfected to HEK293 cells, and then the agonists-induced currents were compared with those in WT hTRPA1-transfected HEK293 cells. Consistent with a previous study, 2APB (400 μM) induced significant currents (\(I_{2APB}\)) in the 3C hTRPA1-transfected HEK293 cells as well as in the WT-transfected cells (Fig. 2A). Application of etodolac at 500 μM induced significant \(I_{ETO}\) in the WT-transfected cells but did not cause obvious currents in the 3C hTRPA1-transfected HEK293 cells. Consistent with previous studies, 2APB (400 μM) induced significant currents (\(I_{2APB}\)) in the 3C hTRPA1-transfected HEK293 cells as well as in the WT-transfected cells (Fig. 2A). Application of etodolac at 500 μM induced significant \(I_{ETO}\) in the WT-transfected cells (Fig. 2B,C; −70.1 ± 20.3 pA/pF, n = 11 for WT hTRPA1-transfected HEK293 cells; −0.7 ± 0.2 pA/pF, n = 5 for 3C hTRPA1-transfected HEK293 cells). These results suggest that etodolac activates TRPA1 through covalent modification of cysteine residues.

**Long-Term, Constant Pretreatment With Etodolac Suppressed \(I_{AITC}\) in HEK293 Cells**

Etodolac is a well-known drug used for anti-inflammation and management of pain. We investigated whether etodolac suppresses \(I_{AITC}\) in vitro. When etodolac was used as an NSAID, following oral administration of 200 mg, the plasma concentration reached a peak (~77 μM) within 1–2 hr and was eliminated with a half-life between 6 and 8 hr (Brocks and Jamali, 1994). We confirmed that etodolac at 50 μM (the drug plasma level) did not induce obvious currents in hTRPA1-transfected HEK293 cells (Fig. 1A,C). Then, etodolac at 50 μM was added to the culture medium for 3–6 hr to carry out the next experiments. We found that \(I_{AITC}\) was significantly suppressed after this etodolac pretreatment (Fig. 3A,B; −134.7 ± 43.2 pA/pF, n = 11 for TRPA1 currents in the absence of etodolac; −69.9 ± 14.6 pA/pF, n = 11 for TRPA1 currents in the presence of etodolac; \(t\)-test, \(P < 0.05\)). These results suggest that etodolac at a lower concentration, near the plasma level following oral administration in common clinical treatment, might not fully activate TRPA1 but might significantly suppress its activity.

**Short Preapplication of Etodolac Desensitized \(I_{AITC}\) Through TRPA1 Cysteine Residues in HEK293 Cells**

Sensitization or desensitization is a vital mechanism for modulation of channel activity. We thus investigated whether...
etodolac desensitizes TRPA1. A short preapplication with either AITC 100 µM or etodolac 50 µM attenuated subsequent $I_{\text{AITC}}$ in hTRPA1-transfected HEK293 cells by 95% or 75%, respectively ($-7.7 \pm 0.7$ pA/pF, $n = 6$ for $I_{\text{AITC}}$ following AITC application; $-41.0 \pm 2.9$ pA/pF, $n = 5$ for $I_{\text{AITC}}$ following etodolac application, vs. $-164.1 \pm 38.7$ pA/pF, $n = 6$ for $I_{\text{AITC}}$ without any preapplication; $t$-test, $P < 0.01$ or $P < 0.05$; Fig. 4A,B). $I_{2\text{APB}}$ was also suppressed by a short preapplication of etodolac in hTRPA1-transfected HEK293 cells ($-7.6 \pm 2.7$ pA/pF, $n = 8$ for $I_{2\text{APB}}$ following 2APB application; $-5.4 \pm 1.3$ pA/pF, $n = 5$ for $I_{2\text{APB}}$ following etodolac application, vs. $-55.3 \pm 15.0$ pA/pF, $n = 8$ for $I_{2\text{APB}}$ without any preapplication; $t$-test, $P < 0.05$; Fig. 5A,B). These data revealed that etodolac suppression of TRPA1 was not ligand specific. Moreover, this suppression was not observed in HEK293 cells expressing the 3C hTRPA1, a cysteine residue mutated hTRPA1 (Fig. 5C), suggesting that the etodolac-induced inhibition of TRPA1 is a sequent phenomenon of the channel activation, which can be called desensitization.

**Etodolac Activated and Desensitized TRPA1 Channels in DRG Neurons**

Because cell types differ in their membrane composition, we next tested whether etodolac could activate TRPA1 channels in sensory neurons. We performed voltage-clamp experiments in cultured rat DRG neurons and found that etodolac induced significant currents in some small neurons, which were also sensitive to AITC and capsaicin. HC030031, a specific antagonist for TRPA1 (Andrè et al., 2008; Eid et al., 2008), completely and reversibly inhibited $I_{\text{ETO}}$ in DRG neurons (Fig. 6A). By contrast, capsazepine, a specific antagonist of TRPV1,
blocked the capsaicin-induced currents but had no effect on $I_{ETO}$ (Fig. 6B). Therefore, etodolac could activate TRPA1 but not TRPV1 both in heterologous transfected cells and in sensory neurons.

We also tested whether etodolac desensitizes TRPA1 in DRG neurons. Short preapplication with etodolac 50 µM suppressed subsequent $I_{AITC}$ ($-100.9 \pm 15.6$ pA/pF, $n = 6$ for $I_{AITC}$ without any preapplication; $-49.2 \pm 13.1$ pA/pF, $n = 6$ for $I_{AITC}$ following etodolac application; $t$-test, $P < 0.05$; Fig. 6C). These data suggest that this suppression is observed not only in heterologous transfected cells but also in sensory neurons.
DISCUSSION

Multibiochemical pathways can contribute to TRPA1 activation by environmental or endogenous stimuli. In addition to a conventional ligand–receptor interaction mechanism (2APB and icilin that nonelectrophilicity activate TRPA1 via this pathway; Hinman et al., 2006; Macpherson et al., 2007), many pungent chemicals and environmental irritants activate TRPA1 through covalent modification of the cysteine residues and/or lysine residue at the N-terminal of the TRPA1. These agonists, such as cinnamaldehyde, AITC, acrolein, and iodoacetamide, despite their structural heterogeneity, share a similar feature showing electrophilicity (Hinman et al., 2006; Macpherson et al., 2007). In the present study, we found that etodolac, at higher concentrations, could activate TRPA1 (but not TRPV1) channels in heterologous expressing HEK293 cells as well as in DRG neurons. The etodolac-induced activation of TRPA1 was almost completely eliminated by mutation of the cysteine residues (C621, C641, and C665), which could be covalently modified by several electrophilic TRPA1 agonists. These findings indicate that etodolac, like other electrophilic agonists, could activate TRPA1 through the covalent modification of cysteine residues. The concentration–response curve (Fig. 1C) showed that the EC50 of etodolac for TRPA1 activation was 296.5 μM, and application of etodolac at concentrations from 0.5 to 200 μM did not produce obvious TRPA1 currents but reduced the subsequent I_{AITC} to 25% in HEK293 cells expressing hTRPA1 (Fig. 4). These doses include those that produce the plasma concentration of etodolac following oral administration of a common oral dose in clinical usage (Brocks and Jamali, 1994). These findings reveal that etodolac at the oral dose may suppress TRPA1 activity without causing significant TRPA1 activation.

Etodolac, because of its highly selective inhibition of COX-2, which is involved in PG synthesis and metabolism in inflammation (Dvornik, 1997; Bennett and Tavares, 2001). AA and a number of AA derivatives, including several PGs, have also been shown to active TRPA1 (Bandell et al., 2004; Cruz-Orengo et al., 2008; Maher et al., 2008; Materazzi et al., 2008; Takahashi et al., 2008; Taylor-Clark et al., 2008). Although the effect of etodolac on TRPA1 receptors may be a result of its activity on COX2, we do not consider that this mechanism is working on the present study. In our experiment, the onset of suppression of I_{AITC} was recorded within 3 min after etodolac application (Fig. 3), which is too short for COX-2-mediated PG synthesis and metabolism, indicating that a direct regulation mechanism may be involved in the inhibition of TRPA1. Moreover, we did not detect any regulatory effect of etodolac on TRPA1 in the cysteine mutant-transfected HEK293 cells (Fig. 4C), revealing that activation of TRPA1 is an essential requirement for the channel suppression, which means desensitization.

The mechanism of desensitization of TRPA1 by etodolac is not clear. Hargreaves’s group has reported that TRPA1 was desensitized by homologous (AITC) and heterologous (capsaicin) agonists via calcium-independent and calcium-dependent pathways, respectively (Akopian et al., 2007). The present study demonstrates that etodolac shares the same mechanism of AITC on TRPA1 activation, through covalent modification of cysteine residues. Etodolac at lower concentrations could desensitize nerve ligation model in rats, which were not alleviated by other COX inhibitors (Suyama et al., 2004; Inoue et al., 2009). On the other hand, TRPA1 is upregulated by inflammation, as well as neuropathy. Inhibition of TRPA1 attenuated both inflammatory and neuropathic mechanical hypersensitivity (Eid et al., 2008). Given that COX inhibition is generally considered to have no effect on neuropathic pain (Baron and Binder, 2004), our findings support idea that the reported alleviation of neuropathic pain by etodolac may be a result of its ability to suppress TRPA1.

Etodolac selectively inhibits COX-2, which is involved in PG synthesis and metabolism in inflammation (Dvornik, 1997; Bennett and Tavares, 2001). AA and a number of AA derivatives, including several PGs, have also been shown to active TRPA1 (Bandell et al., 2004; Cruz-Orengo et al., 2008; Maher et al., 2008; Materazzi et al., 2008; Takahashi et al., 2008; Taylor-Clark et al., 2008). Although the effect of etodolac on TRPA1 receptors may be a result of its activity on COX2, we do not consider that this mechanism is working on the present study. In our experiment, the onset of suppression of I_{AITC} was recorded within 3 min after etodolac application (Fig. 3), which is too short for COX-2-mediated PG synthesis and metabolism, indicating that a direct regulation mechanism may be involved in the inhibition of TRPA1. Moreover, we did not detect any regulatory effect of etodolac on TRPA1 in the cysteine mutant-transfected HEK293 cells (Fig. 4C), revealing that activation of TRPA1 is an essential requirement for the channel suppression, which means desensitization.

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TRPA1 without causing obvious currents. The intracellular solution used in the present study was tightly buffered by 5 mM EGTA, a calcium chelator. All this evidence supports the case that the etodolac-induced TRPA1 desensitization is unlikely to be calcium dependent. Etodolac may influence the covalent modification of cysteine residues because of a lack of response in the 3C hTRPA1-transfected HEK293 cells. However, etodolac also reduced the 2APB-induced TRPA1 response, suggesting that the etodolac-induced TRPA1 inhibition is due to receptor desensitization (activation-induced desensitization), and cysteine modification is not a possible explanation for this desensitization. One possible mechanism of the desensitization may be internalization of the TRPA1 channels. TRPA1 may cycle between the plasma membrane and intracellular compartments, and the balance between membrane insertion and retrieval determines its surface abundance and activity. However, determining the detailed mechanism of etodolac-mediated desensitization of TRPA1 requires further investigation.

The pharmacological desensitization of receptors is a fundamental mechanism for regulating the activity of neuronal systems. Desensitization of TRP channels shows therapeutic value on pain relief (Brederson et al., 2013). Several TRPV1 agonists have been tried for pain therapy in clinic practice (Knotkova et al., 2008; Moran et al., 2011). This study shows that etodolac at plasma concentrations poorly activated TRPA1 but strongly desensitized the channel with a consequent suppression of the agonist-induced response either during constant pretreatment or after a tonic preappplication with etodolac. Overall, our study indicates that etodolac might possess a characteristic, acting as a selective activator and blocker of TRPA1, in addition to its putative inhibition of COX-2.

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The pharmacological desensitization of receptors is a fundamental mechanism for regulating the activity of neuronal systems. Desensitization of TRP channels shows therapeutic value on pain relief (Brederson et al., 2013). Several TRPV1 agonists have been tried for pain therapy in clinic practice (Knotkova et al., 2008; Moran et al., 2011). This study shows that etodolac at plasma concentrations poorly activated TRPA1 but strongly desensitized the channel with a consequent suppression of the agonist-induced response either during constant pretreatment or after a tonic preappplication with etodolac. Overall, our study indicates that etodolac might possess a characteristic, acting as a selective activator and blocker of TRPA1, in addition to its putative inhibition of COX-2.

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