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The effect of the electrophilic fatty acid nitro-oleic acid on TRP channel function in sensory neurons

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Abstract

Nitro-oleic acid (NO_2-OA) and related nitroalkenes are electrophilic fatty acid derivatives that are present in normal tissues at nanomolar concentrations and can increase significantly during inflammation. These substances can suppress multiple intracellular signaling pathways contributing to inflammation by reversible Michael addition reactions with nucleophilic residues such as cysteine and histidine leading to post-translational modification of proteins. NO_2-OA also can influence inflammation and pain by acting on transient receptor potential (TRP) channels in primary sensory neurons. TRPV1, TRPA1 and TRPC can respond to electrophilic fatty acids because they have ankyrin-like repeats in their N terminus that are rich in cysteine residues that react with electrophiles and other thiol modifying species. NO_2-OA acts on TRP channels to initially depolarize and induce firing in sensory neurons followed by desensitization and suppression of firing. *In vivo* experiments revealed that pretreatment with NO_2-OA reduces nociceptive behavior evoked by local administration of a TRPA1 agonist (AITC) to the rat hind paw. These results raise the possibility that NO_2-OA might be useful clinically to reduce neurogenic inflammation and certain types of painful sensations by desensitizing TRPA1 expressing nociceptive afferents.

Keywords

Transient Receptor Potential Vanilloid 1 (TRPV1); Transient Receptor Potential Ankyrin 1 (TRPA1); Transient Receptor Potential Canonical (TRPC); Nitro-oleic acid; afferent nerves; Electrophilic fatty acids; Dorsal root ganglia (DRG); Capsaicin; Allyl isothiocyanate (AITC); Urinary bladder

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Conflict of Interest

Authors have no conflict of interest to disclose.

1. Introduction

Nitro-oleic acid (NO_2-OA) and related nitroalkenes are electrophilic fatty acid derivatives formed by nitric oxide- or nitrite-mediated redox reactions. These species are present in normal tissues at nanomolar concentrations and can increase significantly during injury or inflammation [1–3]. Fatty acid nitroalkenes induce a variety of pharmacological effects including: (1) activation of peroxisome proliferator-activated receptor γ (PPARγ) [2], (2) activation of the Keap1-Nrf2 pathway [4,5], (3) upregulation of heme oxygenase 1 (HO-1) expression [6], (4) inhibition of NF-κB-dependent gene expression [7,8], (5) inhibition of platelet or neutrophil function [9,10] and (6) inhibition of proinflammatory cytokine secretion by macrophages [7]. These actions can all be ascribed to the post-translational modification of functionally significant proteins by the reversible Michael addition reactions that target nucleophilic residues such as cysteine and histidine [1,7,11–15].

In addition to actions on intracellular signaling pathways, NO_2-OA also activates transient receptor potential (TRP) channels in primary sensory neurons [16–18]. These channels are members of a large superfamily of six transmembrane domain cation channels which produce membrane depolarization and firing of sensory neurons when activated. In mammals, this superfamily is divided into 6 subfamilies (TRPA, TRPC, TRPM, TRPML, TRPP, TRPV), with each sub-family consisting of a varying number of members (see partial list in Table 1) [19–21]. TRP channels respond to chemical and mechanical stimuli and detect changes in temperature, pH and osmotic pressure [20,22].

The sensitivity of TRP channels to natural products facilitated the isolation and chemical characterization of these channels. For example, TRPV1 is activated by vanilloids (Table 1) such as capsaicin, the pungent ingredient in hot pepper, which induces a burning sensation when applied to the skin or ingested with food. It was later discovered that TRPV1 expressed in nociceptive sensory neurons is responsible the burning sensation induced by high temperatures [20]. TRPM8, which is activated by the cooling substance menthol, responds to moderate cold temperatures [23], while TRPA1 responds to extreme cold temperatures which induce pain [24]. The role of various TRP channels in sensory mechanisms including nociception has raised the possibility that drugs might be developed to selectively target these channels to treat pain and other pathological conditions.

Several members of the TRP family, including TRPV1, TRPA1 and TRPC, have ankyrin-like repeats in the N terminus that are rich in cysteine residues (Fig. 1A) [23,25] that react with electrophiles and other thiol modifying species (Fig. 1B) [23–29] and therefore respond to electrophilic fatty acids. This paper will review the effects of NO_2-OA on TRP channels in sensory neurons and the possible role of electrophilic fatty acids in the modulation of nociceptive afferent pathways.

2. Effect of 9-NO_2-OA on human TRPA1 channels and TRPA1 channels in mouse vagal and trigeminal sensory neurons

HEK293 cells stably transfected with human TRPA1 respond to low concentrations (EC_{50}, 1 uM) of 9-NO_2-OA in calcium imaging assays, while cells transfected with human TRPV1

do not respond [16]. The potency of 9-NO_2-OA is 10-fold greater than that of a TRPA1 agonist (allyl isothiocyanate, AITC). Human TRPA1 in which three cysteines ($Cys_{619,639,663}$) are mutated to serine and Lys_{708} is mutated to glutamine do not respond to 9-NO_2-OA [17], indicating that interaction with these nucleophilic residues is necessary for activation of the channel. The effects of 9-NO_2-OA are not mimicked by oleic acid in high concentrations (5 mM) and are not blocked by the nitric oxide (NO) scavenger cPTIO, indicating that the parent compound is the active agent [17].

Calcium imaging and patch clamp recording in dissociated sensory neurons revealed that 9-NO_2-OA (1–10 μM) activates a subset of mouse vagal and trigeminal nociceptive neurons that respond to agonists for TRPA1 (AITC) and TRPV1 (capsaicin, CAPS) [17]. The responses are eliminated in neurons obtained from TRPA1 (−/− mice) and are suppressed by a selective TRPA1 antagonist (HC-030031), indicating that the effect of 9-NO_2-OA on these neurons is mediated primarily by activation of TRPA1 channels. Extracellular recordings from mouse capsaicin-sensitive bronchopulmonary afferents revealed that 9-NO_2-OA evokes a robust discharge of action potentials in these afferents, a response inhibited by a TRPA1 antagonist. It was concluded that 9-NO_2-OA induces firing in visceral nociceptive afferent nerves in the mouse by directly activating TRPA1 but not TRPV1 channels.

3. Effect of NO_2-OA on TRPA1 and TRPV1 channels in rat lumbosacral dorsal root ganglion neurons

NO_2-OA also activates TRP channels in sensory neurons dissociated from dorsal root ganglia (DRG) of rats [16]. NO_2-OA (3.5–35 μM) elicits Ca^{2+} transients in 20–40% of DRG neurons (Fig. 2A), the majority of which (60–80%) are likely to be nociceptive because they also respond to TRPA1 (AITC, 1–50 μM) and TRPV1 (CAPS, 0.5 μM) agonists (Fig. 2A, B). The NO_2-OA–evoked Ca^{2+} transients are reduced by a TRPA1 antagonist HC-030031 (5–50 μM) (Fig. 2B) or a TRPV1 antagonist (capsazepine, 10 μM) (Fig. 2C).

NO_2-OA also depolarizes and induces inward currents in 62% of DRG neurons. The NO_2-OA currents are: (1) elicited by concentrations ≥5 nM, (2) blocked by dithiothreitol (DTT, 10 mM) a nucleophile that quenches free NO_2-OA and removes any nitroalkene adducts already present through β-elimination, (3) not mimicked by oleic acid in high concentrations (5 mM), (4) not blocked by the nitric oxide (NO) scavenger cPTIO and (5) reduced by TRPV1 (diarylpiperazine, DPA, 5 μM) or TRPA1 (HC-030031, 5 μM) antagonists. These observations suggest that the effects of NO_2-OA, like those of various pungent compounds and AITC [24,25,27,28], are mediated by an electrophilic interaction with cysteine residues of TRP channel proteins. However, concentrations of NO_2-OA ≥5 nM, also reduce action potential (AP) overshoot, increase AP duration, inhibit firing induced by depolarizing current pulses and suppress Na^+ currents [16]. Thus, endogenous NO_2-OA generated at sites of inflammation may initially activate multiple TRP channels on nociceptive afferent nerves, evoke afferent nerve activity and release of afferent neurotransmitters but later suppress afferent firing.

4. Effect of NO_2-OA on TRPC channels in guinea pig lumbosacral dorsal root ganglion neurons

Calcium imaging and patch clamp recording revealed that another TRP channel mediates the effects of NO_2-OA on sensory neurons in the guinea pig [30]. NO_2-OA increases intracellular Ca^{2+} (EC_{50}, 8.8 μM, Fig. 3A,B) in 60–80% of cultured neurons from lumbosacral DRGs, a considerably higher percentage than the percentage of neurons in rat or mouse responding to NO_2-OA (Fig. 3C,D). 1-oleoyl-2acetyl-sn-glycerol (OAG), a TRPC 3/6/7 agonist, elicits responses in 36% of NO_2-OA sensitive neurons while capsaicin or AITC elicit responses in only 16% and 10%, respectively, of these neurons [30]. A TRPV1 antagonist (DPA, 5 μM,) in combination with a TRPA1 antagonist (HC-030031, 30 μM) does not change the amplitude of the Ca^{2+} transients or the percentage of neurons responding to NO_2-OA; however, DTT (50 mM) or La^{3+} (50 μM), which is known to block TRPC channels, completely abolishes the NO_2-OA responses (Fig. 3E,F). Because nitrated fatty acids may induce some of their biological effects by the release of NO [31], the potential role of NO_2-OA as an NO donor was also examined. The NO donor SNAP does not elicit detectable increases in intracellular calcium and pretreatment with the NO scavenger, cPTIO, does not prevent the NO_2-OA induced calcium transients [30], suggesting that the effects of NO_2-OA are not due to NO mediated nitrosylation of TRP channels, which has also been shown to occur when stimulated directly with NO donors [32].

Patch clamp recording revealed that NO_2-OA also induces a transient inward current associated with a membrane depolarization followed by a prolonged outward current and hyperpolarization in 80% of neurons [30]. The reversal potentials of inward and outward currents are approximately −20 mV and −60 mV, respectively. Inward current is reduced in zero extracellular Na^+, but is unchanged by niflumic acid (100 μM), a Cl^- channel blocker. Outward current is abolished by either zero extracellular Ca^{2+} or a combination of two Ca^{2+} activated K^+ channel blockers (iberiotoxin, 100 nM and apamin, 1 μM). BTP2 (1 or 10 μM), a broad spectrum TRPC antagonist, or La^{3+} (50 μM) completely abolish NO_2-OA currents. Thus, outward currents are likely induced indirectly by influx of calcium through TRPC channels. RT-PCR revealed the expression of mRNA for the seven subtypes of TRPCs in guinea pig DRGs [30]. These results suggest that NO_2-OA activates TRPC channels in addition to TRPV1 and TRPA1 channels already known to be targets in rat and mouse sensory neurons.

5. Effects of NO_2-OA on visceral afferent nerves in the urinary bladder of rats and guinea pigs

Nociceptive afferent nerves expressing TRP channels and neuropeptides are widely distributed in all organs and have a role in various pathological conditions [33]. Stimulation of TRP channels in afferent nerves releases neuropeptides which can induce inflammation and pain as well as modulate organ motility, blood flow, capillary permeability and glandular secretion by acting on nearby smooth muscle, nerves, epithelial cells or mast cells [18,33–36]. The effects of NO_2-OA on visceral afferent nerves were examined indirectly by measuring the spontaneous contractile activity of bladder muscle strips *in vitro* (Fig. 4A)

[18,37]. Application of capsaicin or AITC to rat bladder strips elicits increases in basal tone and contractile activity (Fig. 4 B & C) which are blocked, respectively, by TRPV1 (DPA) and TRPA1 (HC-3) receptor antagonists (Fig. 4E) or by a combination of antagonists for neurokinin receptor subtypes 1, 2 and 3, indicating that the agonists act on afferent nerves to release substance P (SP) or neurokinin A which then stimulate receptors on smooth muscle (Fig. 4E) [18]. NO_2-OA (1–33 μM) mimics the effect of TRP agonists producing a concentration-dependent increase in the amplitude of phasic bladder contractions and baseline muscle tone (Fig. 4A and D) which are suppressed by a combination of the TRPV1 and TRPA1 antagonists or by the combination of the three neurokinin receptor antagonists [18]. The amplitude of phasic contractions prior to administration of NO_2-OA is reduced 12–25% by the antagonists indicating that tonic activation of TRP channels and neuropeptide release occurs under basal conditions. It is not known if this activation of TRP channels is mediated by endogenous electrophilic nitro-fatty acids.

Given that NO_2-OA can activate TRPC channels in guinea pig DRGs, experiments were conducted to determine if activation of TRPC channels also modulates the activity in the guinea pig bladder [37]. Unexpectedly, NO_2-OA decreased the amplitude of phasic bladder contractions, the opposite effect of what occurred in rat bladder. This effect was reversed in the presence of La^{3+} but not by specific TRPV1/TRPA1 antagonists, suggesting that NO_2-OA acts primarily on TRPC channels and not TRPV1 or TRPA1 channels in the guinea pig bladder. NO_2-OA-mediated inhibition is dependent on the release of calcitonin gene-related peptide (CGRP) from bladder afferent nerve terminals, as it is prevented following application of olcegepant, a selective CGRP antagonist [37].

The difference in the effect of NO_2-OA in rat and guinea pig bladders might be due to multiple factors. Studies on dissociated DRG cells suggest that a lower percentage of guinea pig neurons respond to capsaicin or AITC than rat neurons (TRPA1: 5% vs 41%, TRPV1: 21% vs 70%) [30], suggesting that any excitatory effects of NO_2-OA on TRPV1 or TRPA1 channels in guinea pig bladder may be masked by a dominant CGRP signal mediated by TRPC channel activation. It is also possible that differences in the affinity/efficacy of NO_2-OA at each TRP channel varies between species due to differences in amino acid sequences of the receptors. Regardless, it appears that electrophilic fatty acids may play an important role in bladder function and hence may be an attractive target for treatment of bladder pathology.

6. NO_2-OA desensitization of TRPA1 and TRPV1 channels in rat sensory neurons

Sensitization of TRPV1 and TRPA1 channels in sensory neurons is involved in the development of hyperalgesia (hypersensitivity to noxious stimuli) in inflammatory pain models [38,39]; while desensitization is an important mechanism for down-regulation of channel activity and reducing nociceptor function. Capsaicin activates and subsequently desensitizes TRPV1 channels (homologous desensitization) and also reduces the effect of AITC on TRPA1 channels (heterologous desensitization) [40,41]. AITC also elicits homologous and heterologous (TRPV1) desensitization [40,41]. Experiments on rat

dissociated dorsal root ganglion cells using Ca^{2+} imaging and patch clamp techniques [42] and on sensory nerves in the rat hindpaw using pain behavioral testing *in vivo* [42] revealed that NO_2-OA produces heterologous desensitization of responses to TRP agonists. A 5–10 min exposure to NO_2-OA reduces by 40–60% the magnitude of the calcium signals and currents as well as the percentage of cells responding to AITC or capsaicin. However, deltamethrin, a phosphatase inhibitor, which reduces the AITC induced heterologous desensitization of capsaicin or NO_2-OA does not suppress the NO_2-OA induced desensitization of AITC or capsaicin, indicating that heterologous desensitization induced by NO_2-OA and AITC occur by different mechanisms.

Subcutaneous injection of a small volume (35 μL) of AITC (10 mM) or NO_2-OA (2.5 mM) into a rat hind paw induces nociceptive behaviors (licking or repeated withdrawals of the injected paw) [42]. Homologous desensitization occurs when AITC is applied at 10 minute intervals, but does not occur when NO_2-OA is applied at 30 minute intervals. Pretreatment with NO_2-OA 30 minutes before AITC reduces by 50% the AITC-evoked nociceptive behaviors but does not alter capsaicin induced nociceptive responses [42]. These results raise the possibility that NO_2-OA might be useful clinically to reduce neurogenic inflammation and certain types of painful sensations by desensitizing TRPA1 expressing nociceptive afferents.

7. Conclusions

Nitrated fatty acids, which are generated at sites of inflammation, may target multiple TRP channels in nociceptive sensory neurons producing an initial excitation followed by desensitization of these channels and a reduction in afferent activity. Thus endogenous nitrated fatty acids may have a role in modulating the activity of nociceptive afferents and, in turn, influence the development and/or resolution of inflammatory responses and pain. The demonstration that oral administration of nitrated fatty acids or their precursors can produce significant blood, tissue and urine levels of these substances [3,43–45] raises the possibility that nitro-lipid therapies may be developed to target pathophysiological mechanisms and disorders which involve TRP channels [46] and that passage of these substances from the urine into the bladder wall might be effective in treating certain types lower urinary tract dysfunctions.

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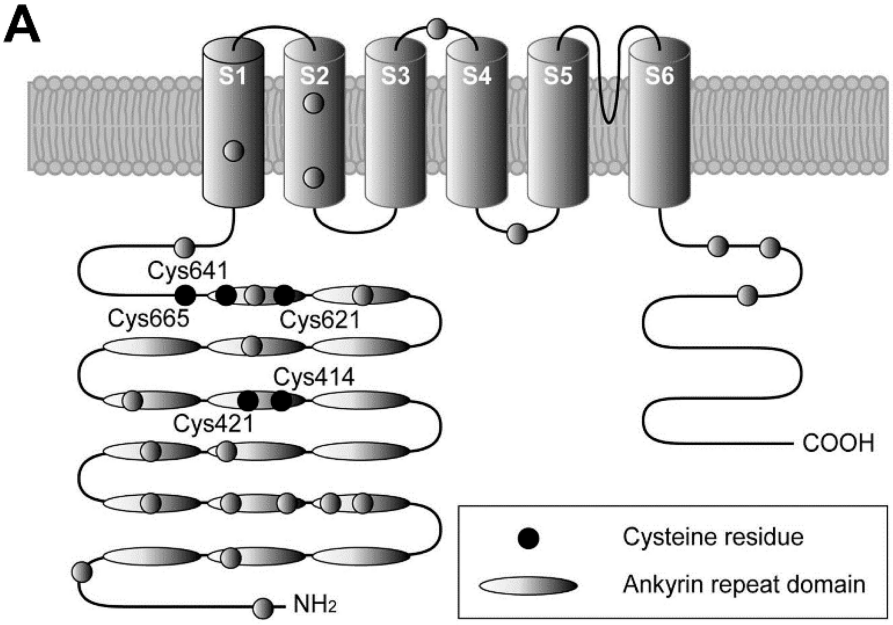
Highlights

- Nociceptive sensory neurons express multiple TRP channels (TRPV1, TRPA1, TRPC)
- TRP channels have N terminals rich in cysteine residues that react with electrophiles
- NO_2-OA, an electrophilic fatty acid, activates TRP channels in sensory neurons
- NO_2-OA also elicits a secondary desensitization of TRP channels in sensory neurons
- TRP channel desensitization by NO_2-OA may contribute to suppression of inflammation

A



B

Agonist	Targeted cysteine(s)
H_2O_2	Cys621, Cys641, Cys665
	Cys421, Cys641, Cys665
Hypochlorite	Cys621, Cys641, Cys665
Nitric Oxide	Cys421, Cys641, Cys665
NO_2-OA	Cys621, Cys641, Cys665
15d-PGJ_2	Cys621, Cys641, Cys665
	Cys421, Cys621
4-HNE	Cys621, Cys641, Cys665
AITC	Cys621, Cys641, Cys665
	*Cys415, *Cys422, *Cys622
Cinnamaldehyde	*Cys415, *Cys422, *Cys622

**Cys415, *Cys422, and *Cys622 in mouse TRPA1.*

Figure 1.

(A) Structure of the TRPA1 channel highlighting the N-terminus enriched with ankyrin repeats (indicated by ovals) and nucleophilic cysteine residues (filled circles) identified as crucial sites for covalent modification of TRPA1. (B) Table of cysteine residues on TRPA1 channels that have been shown to be covalently modified through redox chemistry with the listed agonists. This figure and table are modified from Takahashi and Mori [47].

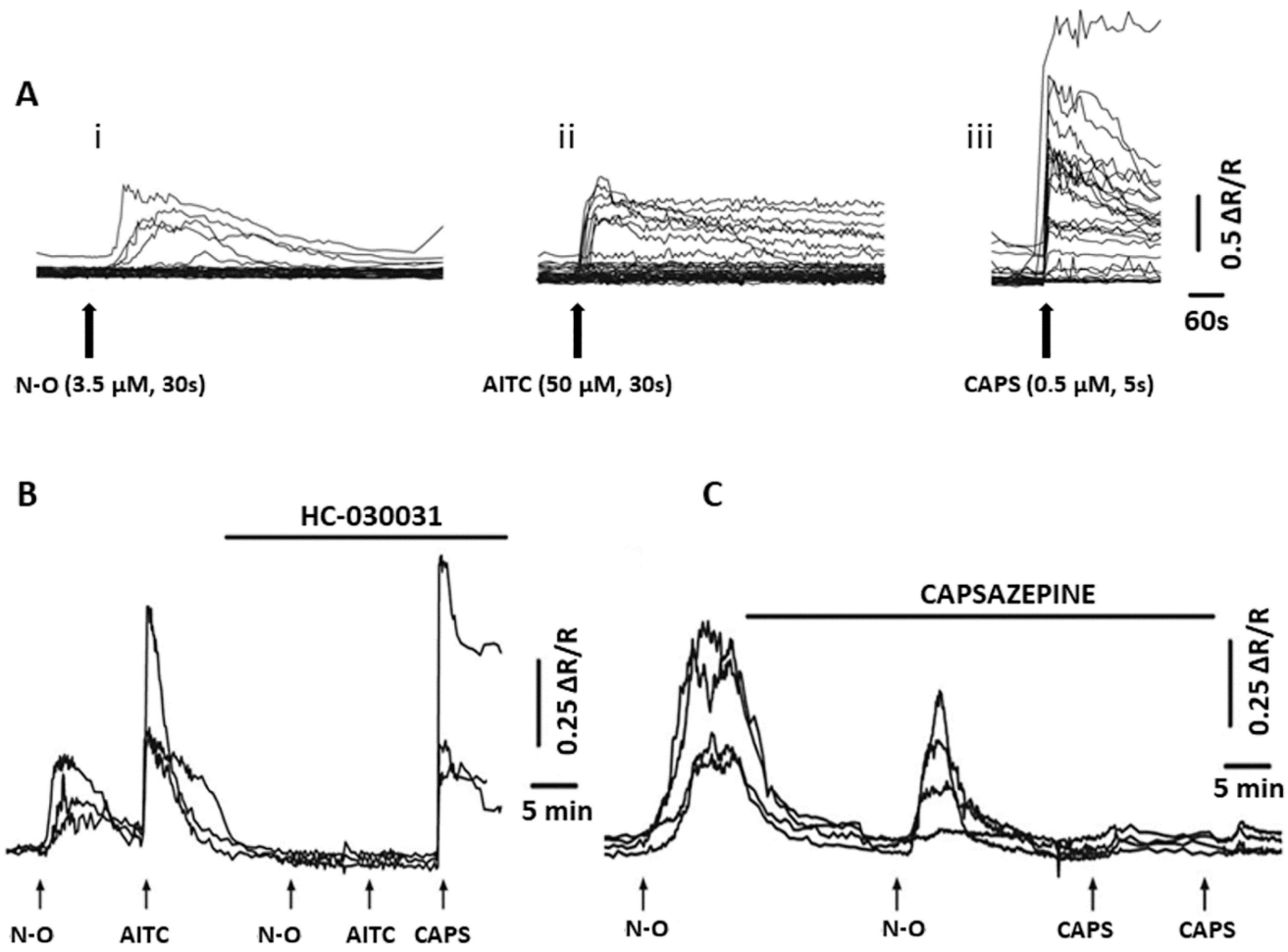


Figure 2.

(A) Intracellular Ca^{2+} transients (Ca^{2+}_i) induced by NO_2-OA (N-O, 3.5 μM, 30-s duration) (i), allyl isothiocyanate (AITC, 50 μM, 30-s duration) (ii), and capsaicin (CAPS, 0.5 μM, 5-s duration) (iii) in rat DRG neurons. Each line represents the response from one DRG neuron. The fluorescence signal measured at 340 nm, divided by the fluorescence signal measured at 380 nm is proportional to $[Ca^{2+}_i]$ and is represented by the ratio (R). The records are shown as percentage increase of R (ΔR/R) above resting levels of $[Ca^{2+}_i]$. (B and C) inhibition of agonist induced Ca^{2+}_i transients in DRG neurons by a TRPA1 (HC-030031) or a TRPV1 (capsazepine) antagonist. B, examples from several cells showing responses to NO_2-OA (N-O, 3.5 μM; 30-s duration) and AITC (10 μM; 30-s duration) in the absence and in the presence of HC-030031 (50 μM). HC-030031 completely blocked NO_2-OA and AITC responses but did not affect the CAPS response. C, examples from several cells showing responses to NO_2-OA (3.5 μM; 30-s duration) in the absence and in the presence of capsazepine (10 μM). Capsazepine reduced NO_2-OA responses and completely blocked CAPS responses (0.5 μM; 5-s duration). Reproduced with permission from Sculptoreanu et al., 2010 [16].

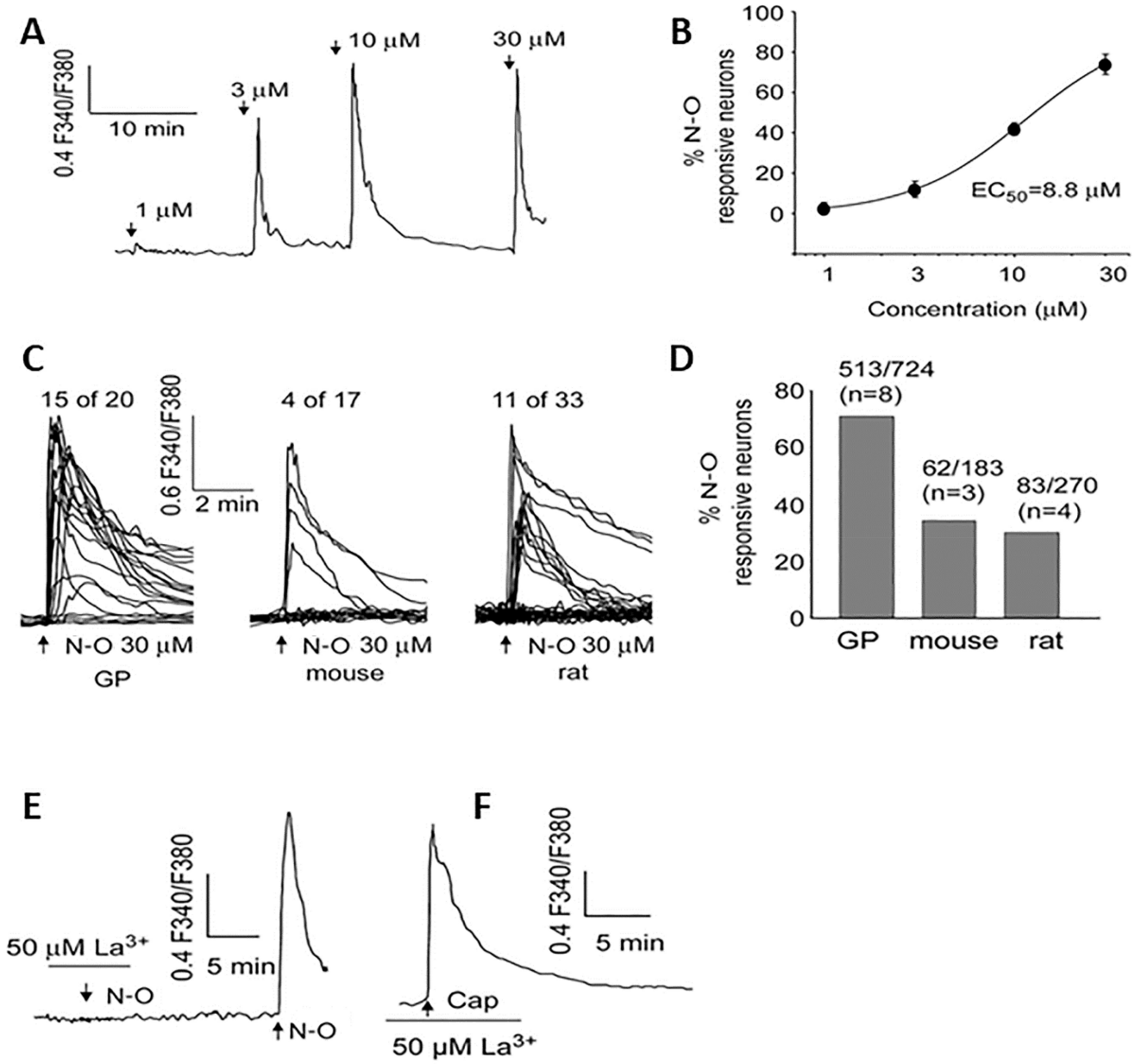


Figure 3.

Comparison of NO_2-OA (N-O) evoked Ca_i^{2+} transients in guinea-pig (GP), mouse and rat DRG neurons. Ca_i^{2+} increase is expressed as the ratio of fluorescence at 340 and 380 nm (F_{340}/F_{380}). NO_2-OA at concentrations of 1, 3, 10 and 30 μM applied for 30 s evoked a concentration-dependent increase in both the amplitude (recording from one neuron) (A) and percentage of responsive neurons (summary data from 85 neurons from 6 coverslips) (B). The curve in (B) was fitted with a Hill equation with EC_{50} = 8.8 μM and Hill coefficient = 1.2. (C and D) NO_2-OA (30 μM) applied for (30 s) evoked an increase in a higher percentage of guinea-pig neurons than in rat and mouse neurons. Scales are the same for all the traces in (C); the number of cells and animals (in parentheses) are indicated above each bar in (D). (E and F) analysis of the role of TRPA1 and TRPV1 channels in the NO_2-OA

evoked Ca_i^{2+} increase in guinea-pig DRG neurons. (E) NO_2-OA (30 μM) did not evoke an increase in Ca_i^{2+} in the presence of 50 μM La^{3+} but did evoke a normal response after washout of La^{3+}. (F) La^{3+} did not block the capsaicin response. Reproduced with permission from Zhang et al., 2014 [30].

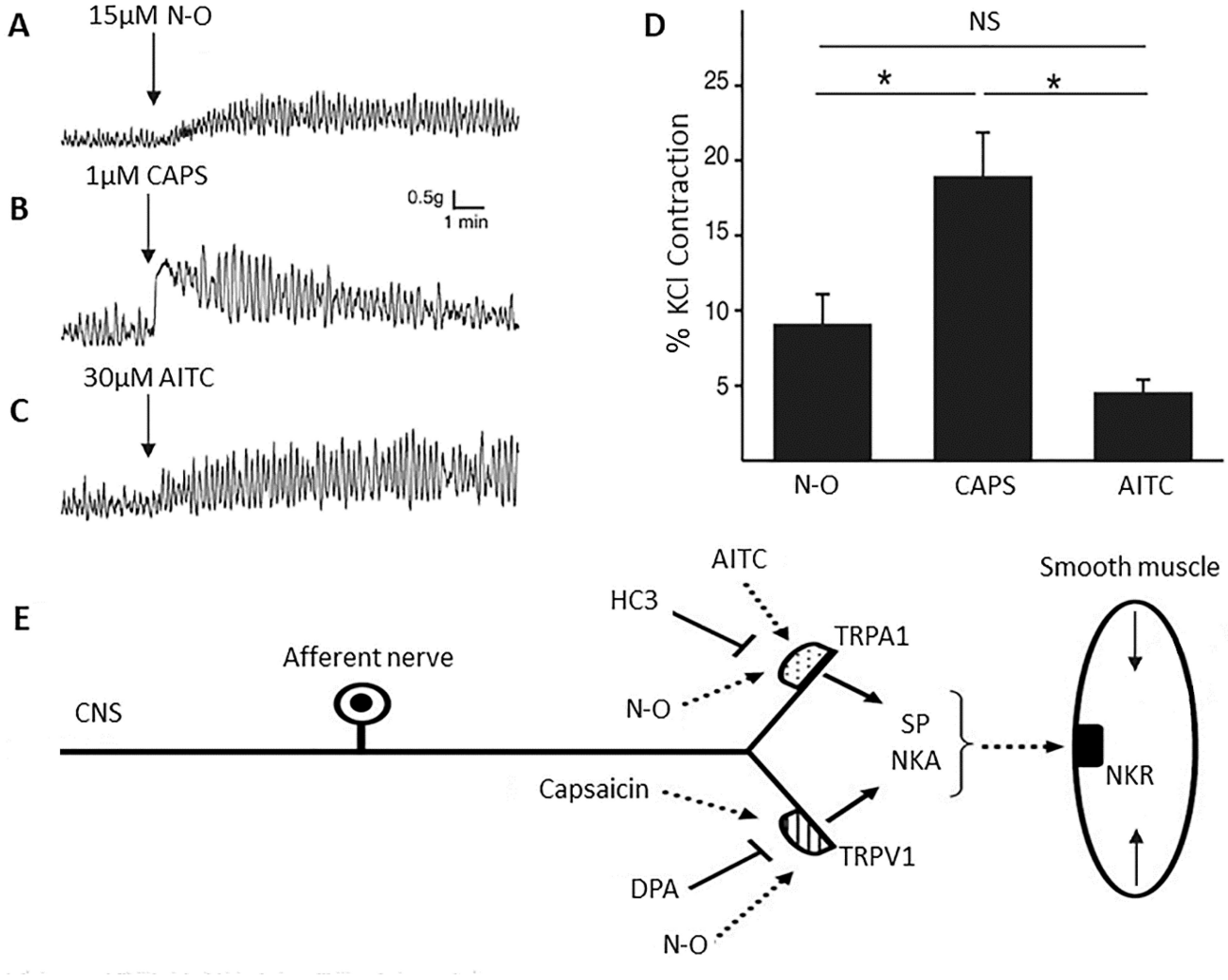


Figure 4.

Examples of agonist-induced phasic contractions in rat bladder strips showing that NO_2-OA (N-O) mimics the effects induced by activation of TRPV1 or TRPA1 receptors. D. Arrows indicate time of application of NO_2-OA (15μM, A), CAPS (1μM, B) and AITC (30μM, C). (D) Summary data showing the amplitude of agonist-induced increases in phasic contraction amplitude normalized to KCl-evoked contraction). * $p < 0.05$, NS $p > 0.05$ by one-way ANOVA, n = 9 to 13. E, Schematic of NO_2-OA mechanism of action. NO_2-OA activates TRPV1 and TRPA1 channels on capsaicin-sensitive afferent nerve terminals to trigger the release of neurokinins that act on postjunctional receptors to induce smooth muscle contractions. SP – substance P, NKA – neurokinin A. NKR – neurokinin receptor. Reproduced with permission from Artim et al., 2011 [18].

Table 1

Partial list of known TRP channels in vertebrates, their activators and known distribution. This list is updated from tables previously published by Venkatachalam and Montell (2011) [21] and is not comprehensive due to space constraints.

Subfamily	Members	Possible Activators	Tissue Expression
TRPA	TRPA1	Cold temperature, icilin, isothiocyanates, allicin, DAG, PUFAs, bradykinin, cinnamaldehyde, acrolein, cannabinoids	DRG, hair cells, ovary, spleen, testis
TRPC	TRPC1	Ca^{2+} store depletion, conformational coupling, mechanical stretch	Heart, brain, testis, ovary, liver, spleen
	TRPC2	DAG	Vomeronasal organ, testis
	TRPC3	Ca^{2+} store depletion, conformational coupling, DAG	Brain
	TRPC4	Ca^{2+} store depletion	Brain, endothelia, testis, adrenal grand, retina
	TRPC5	Ca^{2+} store depletion, sphingosine-1-phosphate	Brain
	TRPC6	Conformational coupling, DAG, PIP_3	Lung, brain, placenta, ovary
	TRPC7	Ca^{2+} store depletion, DAG	Eye, heart, lung
TRPV	TRPV1	Heat, vanilloids, anandamide, camphor, piperine, allicin, ethanol, pH, PIP_2, phosphorylation, nitrosylation, resiniferanoids	TG, DRG, neurons, urinary bladder, testis
	TRPV2	Heat, hypoosmotic solution	DRG, spinal cord, brain, spleen, intestine
	TRPV3	Warm temperature, PUFAs, menthol, monoterpenes from oregano, cloves, thyme	DRG, spinal cord, brain, keratinocytes, TG
	TRPV4	Warm temperature, hypoosmotic solution, Epoxyeicosatrienoic acid	DRG, kidney, lung, spleen, testis, heart, liver
	TRPV5	Low extracellular Ca^{2+}	Kidney, intestine, pancreas, placenta
	TRPV6	Ca^{2+} store depletion	Small intestine, pancreas, placenta

Abbreviations: DRG: dorsal root ganglion, TG, Trigeminal ganglion, DAG: diacylglycerol, PUFAs: poly unsaturated fatty acids, PIP_2: Phosphatidylinositol 4,5-bisphosphate, PIP_3: Phosphatidylinositol (3,4,5)-trisphosphate, NAD: Nicotinamide adenine dinucleotide, EGF: Epidermal growth factor.