TRPV1 is a novel target for omega-3 polyunsaturated fatty acids

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Omega-3 (*n*-3) fatty acids are essential for proper neuronal function, and they possess prominent analgesic properties, yet their underlying signalling mechanisms are unclear. Here we show that *n*-3 fatty acids interact directly with TRPV1, an ion channel expressed in nociceptive neurones and brain. These fatty acids activate TRPV1 in a phosphorylation-dependent manner, enhance responses to extracellular protons, and displace binding of the ultrapotent TRPV1 ligand [^{3}H]resiniferatoxin. In contrast to their agonistic properties, *n*-3 fatty acids competitively inhibit the responses of vanilloid agonists. These actions occur in mammalian cells in the physiological concentration range of 1–10 μM. Significantly, docosahexaenoic acid exhibits the greatest efficacy as an agonist, whereas eicosapentaenoic acid and linolenic acid are markedly more effective inhibitors. Similarly, eicosapentaenoic acid but not docosahexaenoic acid profoundly reduces capsaicin-evoked pain-related behaviour in mice. These effects are independent of alterations in membrane elasticity because the micelle-forming detergent Triton X-100 only minimally affects TRPV1 properties. Thus, *n*-3 fatty acids differentially regulate TRPV1 and this form of signalling may contribute to their biological effects. Further, these results suggest that dietary supplementation with selective *n*-3 fatty acids would be most beneficial for the treatment of pain.

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Omega-3 polyunsaturated fatty acids (*n*-3 PUFAs) are essential dietary fatty acids containing a double bond on the third carbon from the methyl end, and consist of docosahexaenoic acid (DHA; 22:6 *n*-3), eicosapentaenoic acid (EPA; 20:5 *n*-3), and linolenic acid (LNA; 18:3 *n*-3). The *n*-3 PUFAs are obtained primarily from fish oils, vegetables and breast milk. DHA and EPA are also obtained through bioconversion of the shorter chain precursor LNA. DHA is highly concentrated in the brain and constitutes ∼50% of membrane in the retina (Fliesler & Anderson, 1983). The abundance of DHA in the brain suggests essential roles for this fatty acid in neuronal function (for review see Marszalek & Lodish, 2005). DHA deprivation results in neurological defects, primarily in the memory, visual and sensory systems (Salem *et al.* 2001). Furthermore, dietary supplementation with DHA is beneficial in various psychiatric disorders, including Alzheimer's disease (Barberger-Gateau *et al.* 2002), attention deficit hyperactivity disorder (Richardso & Puri, 2002), autism (Vancassel *et al.* 2001), schizophrenia (Assies *et al.* 2001), anxiety (Mamalakis *et al.* 1998), bipolar disorder (Stoll *et al.* 1999) and depression (Logan, 2003). Interestingly, *n*-3 PUFAs also appear to have pronounced analgesic properties. Several studies have shown that an increased intake of *n*-3 PUFAs is associated with reduced pain in rheumatoid arthritis (Calder, 2001), inflammatory bowel disease (Belluzzi *et al.* 2000) and dysmenorrhoea (Harel *et al.* 1996). In addition, rats treated with a high *n*-3/*n*-6 PUFA ratio exhibit a markedly increased threshold for thermal pain (Yehuda & Carasso, 1993).

Although the underlying mechanisms are unclear, many of these effects have been attributed to the ability of *n*-3 PUFAs to compete with arachidonic acid as substrates for cyclo- and lipoxygenase (Lee *et al.* 1984; von Schacky *et al.* 1993), thus reducing the production of inflammatory eicosanoids. However, recently Shapiro (2003) suggested *n*-3 PUFAs might relieve pain by directly modulating the properties of neurones, because it is known that *n*-3 PUFAs can reduce cell excitability in a number of systems. For example, *n*-3 PUFAs can inhibit cardiac and hippocampal voltage-gated Na^+ channels and L-type Ca^{2+} channels (Xiao *et al.* 1995; Vreugdenhil *et al.* 1996) and activate background K^+ channels in the brain (Lauritzen *et al.* 2000). These effects lead to a reduction in cell excitability and may have antiarrhythmic and neuroprotective actions, respectively. Currently, there is no evidence for a direct action of *n*-3 PUFAs at sensory neurones for alleviating pain.

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One potential neuronal target for *n*-3 PUFAs is the capsaicin receptor or transient receptor potential vanilloid subtype 1 (TRPV1). TRPV1 is a non-selective cation channel that plays a critical role in inflammatory pain signalling (Szallasi & Blumberg, 1999; Caterina *et al.* 2000; Davis *et al.* 2000). In addition, TRPV1 is also expressed in the brain (Mezey *et al.* 2000), suggesting a broader role for this receptor in modulating behavioural states. In peripheral nociceptive neurones, TRPV1 is gated by various noxious stimuli including heat (>43°C) and capsaicin (Caterina *et al.* 1997), protons (<pH 6) (Tominaga *et al.* 1998) and cations (Ahern *et al.* 2005). Significantly, lipids and their derivatives also activate TRPV1, including *n*-arachidonoyldopamine (NADA) (Huang *et al.* 2002), *n*-oleoyldopamine (Chu *et al.* 2003), anandamide (Zygmunt *et al.* 1999; Smart *et al.* 2000), oleoylethanolamide and other *N*-acylethanolamines (Ahern, 2003; Movahed *et al.* 2005; Wang *et al.* 2005) and lipoxygenase derivatives (Hwang *et al.* 2000). Interestingly, PUFAs have been shown to activate TRP homologues of *Caenorhabditis elegans* and *Drosophila* (Chyb *et al.* 1999; Kahn-Kirby *et al.* 2004). Although these studies indicate a direct effect of PUFAs at homologues of TRPV channels, it is not known if *n*-3 PUFAs interact with the mammalian TRPV1. TRPV1 plays a critical role in pain/inflammation and is expressed in nerve terminals in the brain that are highly enriched with DHA. Thus, determining the interaction of PUFAs with TRPV1 has potentially important biological implications.

Here we demonstrate differential modulation of TRPV1 by specific *n*-3 PUFAs. These fatty acids directly activate TRPV1 in a protein kinase C (PKC)-dependent manner, with DHA exhibiting significantly greater efficacy compared with EPA and LNA. In contrast, *n*-3 PUFAs also inhibit TRPV1 currents evoked by vanilloid agonists; surprisingly EPA and LNA are significantly more potent and effective inhibitors than DHA. Similarly, EPA attenuates capsaicin-induced pain-related behaviour in mice more potently than DHA. These distinct actions of *n*-3 PUFAs at TRPV1 may contribute to the biological regulation of the channel in discrete tissue environments.

Methods

All experimental procedures involving animals were approved by the Georgetown University Animal Care and Use Committee and conform to NIH guidelines.

Oocyte electrophysiology

Xenopus laevis oocytes were harvested from adult females anaesthetized with 0.5 g l^{-1} tricaine methanesulphonate. Animals were allowed to recover for 2 months between surgeries and were killed by an overdose of anaesthetic after the sixth procedure. Defolliculated ooctyes were injected with ∼10 ng of rat TRPV1 cRNA (gift from David Julius, University of California, SF, USA). Double electrode voltage clamp was performed using a Warner amplifier (Warner Instruments; OC725C). Oocytes were superfused (5 ml min^{-1}) with Ca^{2+}-free solution containing (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 5 mM Mes.

Sensory neurone and HEK 293F cell culture and electrophysiology

Adult mice (C57Bl6/J) were killed by CO_2/decapitation. Nodose ganglia were 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. Neurones were used within 24–36 h of culture. HEK 293F cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% non-essential amino acids and 10% fetal calf serum, and were cultured on poly-D-lysine-coated glass coverslips. Cells were transfected with rat TRPV1 (gift of David Julius) and GFP cDNA using Lipofectamine Transfection Reagent (Invitrogen) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Whole-cell patch-clamp and excised patch recordings were performed using an EPC8 amplifier (HEKA). Pipette resistances were in the range 3–4 MΩ and series resistance was routinely compensated by ∼30–50%. The current signal was low-pass filtered at 1–3 kHz and sampled at 4 kHz. The bath solution contained (mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 1 EGTA, 10 Hepes, 10 glucose pH 7.3 (290 mosmol l^{-1}). The pipette solution contained (mM): 140 CsCl, 10 NaCl, 10 Hepes, 5 EGTA, 2 MgATP and 0.3 GTP, pH 7.3.

[^{3}H]Resiniferatoxin binding

Displacement of tritiated resiniferatoxin by fatty acids was performed in HEK 293F cell membrane fraction transfected with rat TRPV1 cDNA (gift of David Julius). Cell lysate was centrifuged at 20 817 *g* for 15 min at 4°C to obtain membrane fraction. Various concentrations of EPA, DHA, or TX-100 were incubated for 1 h at 37°C with 1 nM [^{3}H]resiniferatoxin ([^{3}H]-RTX) and ∼50 μg of membrane fraction. Non-specific binding tubes contained 250 nM unlabelled resiniferatoxin. The reaction was stopped on ice and after 10 min, α1-acid glycoprotein (0.2 mg ml^{-1}) was added to decrease non-specific binding of RTX. Samples were centrifuged at 20 817 *g* for 15 min and the radioactive content of the pellet was determined using a Beckman Instruments (Fullerton, CA, USA) liquid scintillation counter (LS5801).

Calcium imaging

HEK 293 cells transfected with TRPV1 were loaded with 1 μM acetoxymethyl ester (AM) form of Fluo4 (Molecular Probes, Eugene, OR, USA) for 20 min and washed for a further 10–20 min prior to recording. The dye was excited at 488 ± 15 nm. Emitted fluorescence was filtered with a 535 ± 25 nm bandpass filter, captured by a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) and read into a computer. Analysis was performed off-line using Simple PCI software (Compix Inc., Cranberry Township, PA, USA). Drugs were applied via a micropipette (100 μm diameter) positioned at a distance of 0.5 mm from the cells of interest via a pressure-ejection system. Data are expressed as fluorescence (F) or change in fluorescence (ΔF), divided by basal fluorescence (F_0). Data were normalized to responses evoked by a saturating dose of capsaicin (10 μM).

Acute capsaicin test

Mice were purchased from The Jackson Laboratory (Bar Harbour, ME, USA) and were either bred in house or allowed to acclimate at least 10 days prior to experimentation. C57Bl6 male mice (5–8 weeks old) received an intraplantar injection (20 μl, using 30 gauge needle) of capsaicin (50 ng), capsaicin plus EPA (20 or 40 μg) or capsaicin plus DHA (21.7 or 43.4 μg) into the left hindpaw. Drugs were dissolved in Hank's buffer pH 8.1 and the final solution contained <0.01% ethanol. Mice were immediately placed in observation chamber and behaviour was recorded with a digital camera. The total time mice spent shaking, licking or biting the injected paw over a period of 3 min was measured by a blind observer.

Chemicals

Phorbol 12,13-dibutyrate (PDBu), and bovine α1-acid glycoprotein were obtained from Sigma. Anandamide (AEA), capsazepine (CZP) and capsaicin (CAP) were obtained from Tocris Cookson (Ellisville, MO, USA). NADA, EPA, DHA, stearic acid, oleic acid, linoleic acid, arachidonic acid and linolenic acid (LNA) were obtained from Cayman Laboratories (Ann Arbor, MI, USA). [^{3}H]-RTX was obtained from Perkin Elmer (Boston, MA, USA). Drugs were prepared as stock solutions in ethanol and diluted into physiological solution prior to experiments; final ethanol was always <0.03%, which was found to have no effect on TRPV1 activity. In some experiments BSA (0.01%) was included to improve fatty acid solubility. At higher concentrations (greater than 0.1%) BSA inhibited fatty-acid-evoked TRPV1 currents, presumably by binding free fatty acids as previously described (Itoh *et al.* 2003).

Voltage-dependence analysis

A family of test potentials ranging from −90 to 210 mV for 100 ms duration was used to study the voltage-dependent activation property of TRPV1. After the test potentials, the voltage was stepped to 60 mV for a duration of 50 ms to measure the tail currents. The peak amplitudes within 1 ms of the 60 mV step were plotted as a function of the test potential and normalized to the maximal current obtained from the following Boltzmann function:

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

where $V_{1/2}$ is the potential that elicits half-maximal activation, and s is the slope factor.

Statistical analysis

Data are given as means ± S.E.M. Student's t test was used to determine significant differences between two groups and one-way analysis of variance (ANOVA) for larger numbers of groups, ANOVAs were followed with Tukey's HSD to confirm significance. Statistical comparisons of non-linear regression fit parameters were performed using GraphPadSoftware Inc. In all cases, significance was evaluated at $P < 0.05$.

Results

Direct activation of TRPV1 by fatty acids

We tested the ability of *n*-3 PUFAs to activate TRPV1 expressed in *Xenopus* oocytes. Under control conditions, EPA and DHA (50 μM) failed to evoke any membrane current. However, after stimulation of PKC, which is known to sensitize TRPV1 to agonists (Premkumar & Ahern, 2000; Vellani *et al.* 2001), we found that fatty acids evoked currents in all oocytes tested ($n > 50$, Fig. 1*A*). These currents exhibited pronounced outward rectification, a reversal potential of about −10 mV (Fig. 1*B*) and were blocked by the TRPV1 inhibitor, capsazepine (5 μM), indicating selective activation of TRPV1 channels. To characterize further the requirement of TRPV1 phosphorylation for activation by *n*-3 PUFAs, we studied a mutant TRPV1 lacking key PKC phosphorylation sites (S502A/S800A) (Numazaki *et al.* 2002). In oocytes expressing this mutant, *n*-3 PUFAs evoked little to no current after activation of PKC with PDBu (Fig. 1*D*). These results demonstrate a requirement of TRPV1 phosphorylation for activation by *n*-3 PUFAs. The efficacies of anandamide (Premkumar & Ahern, 2000), NADA (Premkumar *et al.* 2004) and oleoylethanolamide (Ahern, 2003) are also known to be very low in the absence of PKC signalling.

Significantly we found that much lower concentrations of *n*-3 PUFAs were capable of activating TRPV1 in mammalian cells. In TRPV1-expressing HEK 293 cells, 10 μM DHA, LNA and EPA evoked large capsazepine-sensitive inward currents after PDBu treatment (Fig. 1*C* and *D*). However, the responses were much smaller and less frequent responses when cells were not treated with PDBu. Plasma concentrations of free fatty acids are in the range 0.01–10 μM (Spector & Hoak, 1975). Thus, these data indicate that physiological concentrations of *n*-3 PUFAs are capable of activating TRPV1.

Several studies indicate TRPV1 is sensitive to transmembrane voltage (Piper *et al.* 1999; Gunthorpe *et al.* 2000; Ahern & Premkumar, 2002; Vlachova *et al.* 2003; Voets *et al.* 2004) and depolarization alone can activate the channel (Voets *et al.* 2004). At room temperature (25°C), the voltage necessary to elicit half the maximal response ($V_{1/2}$) is ~100 mV, but it shifts to more physiologically relevant potentials as temperature increases or in the presence of capsaicin (Voets *et al.* 2004). We found that DHA (10 μM) markedly enhanced the voltage-dependent activation of TRPV1 in whole-cell recordings (25°C, Fig. 2*A*). DHA reduced the $V_{1/2}$ by ~50 mV (control, 111.9 ± 8.9 mV; DHA, 62.9 ± 2.3 mV; $n = 7$). Furthermore, DHA (10 μM) elicited TRPV1 currents at positive potentials ($V_h = +50$ mV) in outside-out excised patches in the absence of PKC stimulation (Fig. 2*C*). Thus, DHA can activate TRPV1 in mammalian cells in a voltage- and membrane-delimited manner.

Next, we tested the ability of a range of saturated and unsaturated fatty acids to activate TRPV1 expressed in oocytes. Following PKC stimulation, 50 μM concentrations of these fatty acids activated TRPV1 in a structure-specific manner (Fig. 3*A* and *B*). Saturated

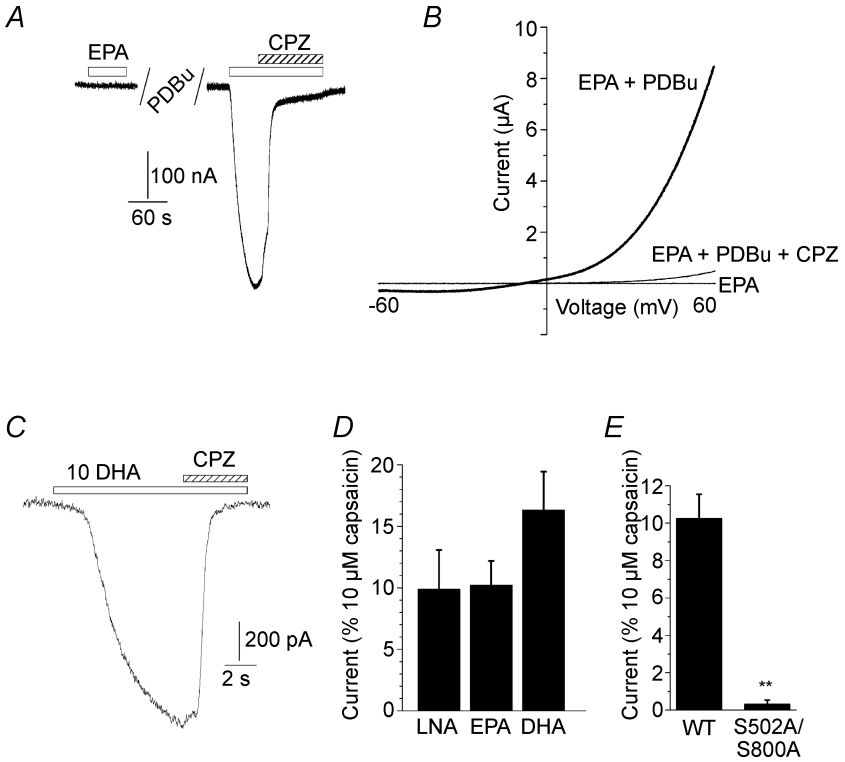

Figure 1. *n*-3 PUFAs activate TRPV1 expressed in heterologous systems after stimulation of protein kinase C

A, eicosapentaenoic acid (EPA; 50 μM) evoked inward currents in TRPV1-expressing oocytes after treatment with the protein kinase C (PKC) activator phorbol 12,13-dibutyrate (PDBu) (200 nM, 3 min, $n > 50$). Currents were blocked by the selective TRPV1 inhibitor capsazepine (CZP; 5 μM; $n = 4$). *B*, current–voltage relationship for the response evoked by EPA (50 μM) yielded reversal potentials of ~−5 to −10 mV ($n = 38$) and subsequent block by CZP (5 μM). No response was elicited before PDBu treatment. *C*, docosahexaenoic acid (DHA; 10 μM) evoked inward currents in voltage-clamped TRPV1-expressing HEK 293F cells (pretreated with PDBu, 200 nM, 3 min; $n = 5$). The response to DHA was completely blocked by CZP (5 μM). *D*, mean currents activated by 10 μM linolenic acid (LNA), EPA and DHA (after PDBu pretreatment) normalized to 10 μM capsaicin ($n = 4$–7). *E*, currents evoked by 50 μM DHA (after PDBu treatment) were absent in oocytes expressing TRPV1 receptors lacking PKC phosphorylation sites (S502A/S800A). Data were measured at +60 mV and normalized to maximal capsaicin (10 μM) responses ($n = 5$, $^{**}P < 5 \times 10^{-5}$) in voltage ramps as in Fig. 1*B*.

stearic fatty acid and mono-unsaturated oleic fatty acid minimally activated TRPV1 as compared with the *n*-3 PUFA, EPA, whereas activation was generally enhanced with increasing unsaturation (stearic acid 18:0; oleic acid 18:1; linoleic acid 18:2 *n*-6; arachidonic acid 20:4 *n*-6; LNA 18:3 *n*-3; EPA 20:5 *n*-3; DHA 22:6 *n*-3; Fig. 3*A*). To investigate further the structural features of PUFAs for TRPV1 activation, we tested a range of PUFAs with differences in the degree of unsaturation and location of double bonds (*n*-3 *versus* *n*-6). Figure 3*B* shows that both α-linolenic acid (18:3 *n*-3) and omega-3 arachidonic acid (20:4 *n*-3) elicited larger TRPV1 currents than the corresponding omega-6 PUFAs, γ-linolenic acid (18:3 *n*-6) and arachidonic acid (20:4 *n*-6), respectively. We also tested whether the degree of unsaturation had an effect on TRPV1 activation. Figure 3*B* shows that activation of TRPV1 by PUFAs containing 20 carbons (eicosatrienoic acid 20:3 *n*-3; *n*-3 arachidonic acid 20:4 *n*-3; and EPA 20:5 *n*-3) tended to increase with the number of double bonds. In contrast, no such correlation was observed with PUFAs containing 18 carbons (α-linolenic acid 18:3 *n*-3; and stearidonic acid 18:4 *n*-3). Thus, the position of double bonds and the degree of unsaturation – at least for the 20-carbon PUFAs – appear to be important structural features of fatty acids relevant to TRPV1 activation. Figure 3*C* shows that this structural requirement is not a consequence of differences in fatty acid solubility, since similar results were observed when DHA and stearic acid were dissolved in a buffer solution containing BSA (0.01%, see Methods). Figure 3*D* shows that the *n*-3 PUFAs, DHA and EPA, activated TRPV1 in a dose-dependent manner. DHA evoked a considerably greater maximal response compared with EPA (~12% *versus* 4% of 10 μM capsaicin responses, +60 mV) whereas both fatty acids exhibited a similar potency (DHA $EC_{50} = 36.0 \pm 3.3\ \mu$M, EPA $EC_{50} = 29.7 \pm 9.6\ \mu$M). Thus, these data show that at 22°C, *n*-3 PUFAs act as partial TRPV1 agonists and that DHA has significantly greater efficacy compared with EPA and LNA (Fig. 2*B* and *D*). It is important to note that the efficacy and potency of *n*-3 PUFAs are significantly greater in mammalian cells (see Fig. 1) and would be enhanced at physiological temperature (37°C).

To confirm a direct interaction of *n*-3 PUFAs with the TRPV1 protein, we performed competition binding assays using the radiolabelled form of the ultra-potent TRPV1 agonist resiniferatoxin ([^{3}H]-RTX) (Szallasi & Blumberg, 1999). DHA and EPA displaced binding of [^{3}H]-RTX to TRPV1 expressed in HEK 293 cells in a dose-dependent manner with a K_i values of 3.1 ± 0.2 and $10.2 \pm 1.2\ \mu$M, respectively (Fig. 3*E*). The micelle-forming detergent, Triton X-100 is known to increase membrane fluidity, which can alter the properties of ion channels. However, Triton X-100 (100 μM) only minimally displaced [^{3}H]-RTX (93.2 ± 18.0% of maximal binding, Fig. 3*E*). Thus, these data are consistent with *n*-3 PUFAs directly binding to TRPV1 as opposed to non-specific effects on the membrane bilayer (see Fig. 6).

Omega-3 PUFAs enhance TRPV1 responses to protons but inhibit responses to vanilloids

TRPV1 is a polymodal receptor capable of integrating a variety of physical and chemical stimuli. The vanilloids

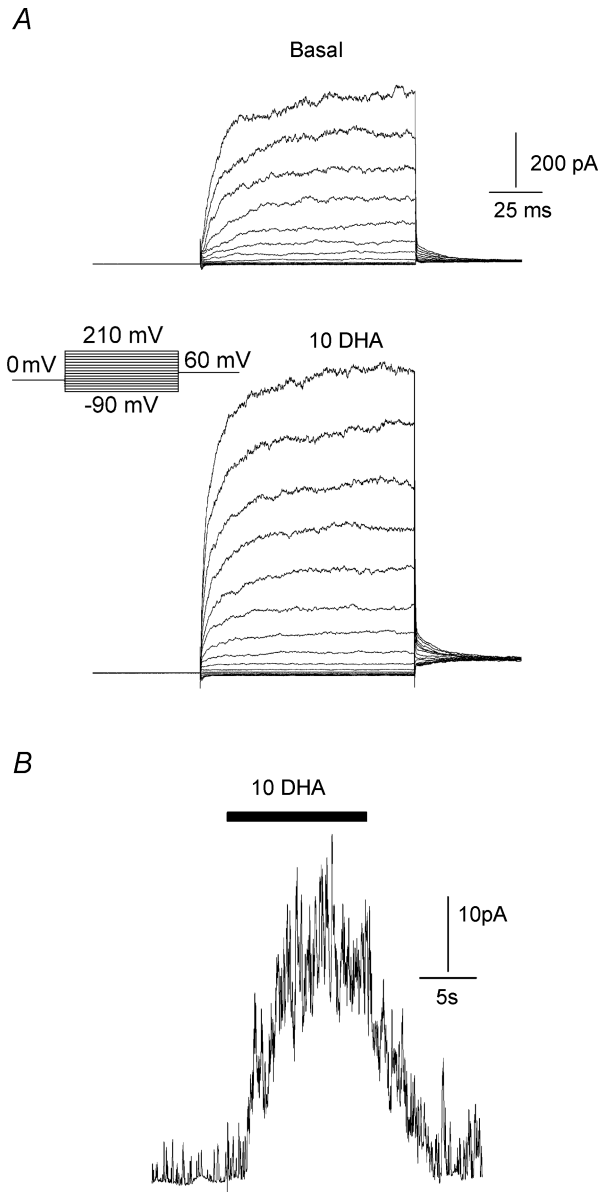

Figure 2. DHA enhances the voltage-dependent activation of TRPV1
A, voltage-dependent activation of TRPV1 in HEK 293F cells in response to a family of voltage steps from −90 to 210 mV in 20 mV increments ($n = 5$) in the presence and absence of DHA (10 μM). Voltages for half-maximal activation derived from a Boltzmann function are given in the text. *B*, DHA (10 μM) activated TRPV1 channel activity in an excised outside-out patch from a TRPV1-expressing HEK 293F cell (holding potential, +50 mV).

(capsaicin, anandamide and NADA) are thought to act at intracellular site(s) (Jung *et al.* 1999; Hwang *et al.* 2000; Chou *et al.* 2004), while protons and cations act extracellularly (Jordt *et al.* 2000; Ahern *et al.* 2005). To explore further the action of *n*-3 PUFAs at TRPV1, we tested their effects on agonist-evoked responses. Figure 4*A* and *C* shows that EPA enhanced the current evoked by protons (pH 5.5) in a dose-dependent manner and this is consistent with activation via an intracellular 'vanilloid' site. In contrast, the mono-unsaturated oleic acid (50 μM) failed to potentiate proton-evoked responses, reflecting the poor agonistic properties of this lipid at TRPV1 (Fig. 4*B* and *C*). Similarly, stearic acid, dissolved in a solution containing 0.01% BSA, failed to enhance the proton-evoked responses (Fig. 4*C*). Thus, the poor agonistic effects of the more saturated fatty acids are not due to limited solubility. The other *n*-3 PUFAs, LNA and DHA, also potentiated the proton-evoked currents (Fig. 4*C*). To determine a physiological relevance of synergism between *n*-3 PUFAs and protons, we tested the effect of lower concentrations of PUFAs in HEK 293 cells. Figure 3*D* shows that 5 μM DHA significantly increased proton-evoked responses by nearly twofold ($n = 5$). This potentiation did not depend on PKC-mediated phosphorylation because DHA produced a similar effect in cells pretreated with the specific PKC

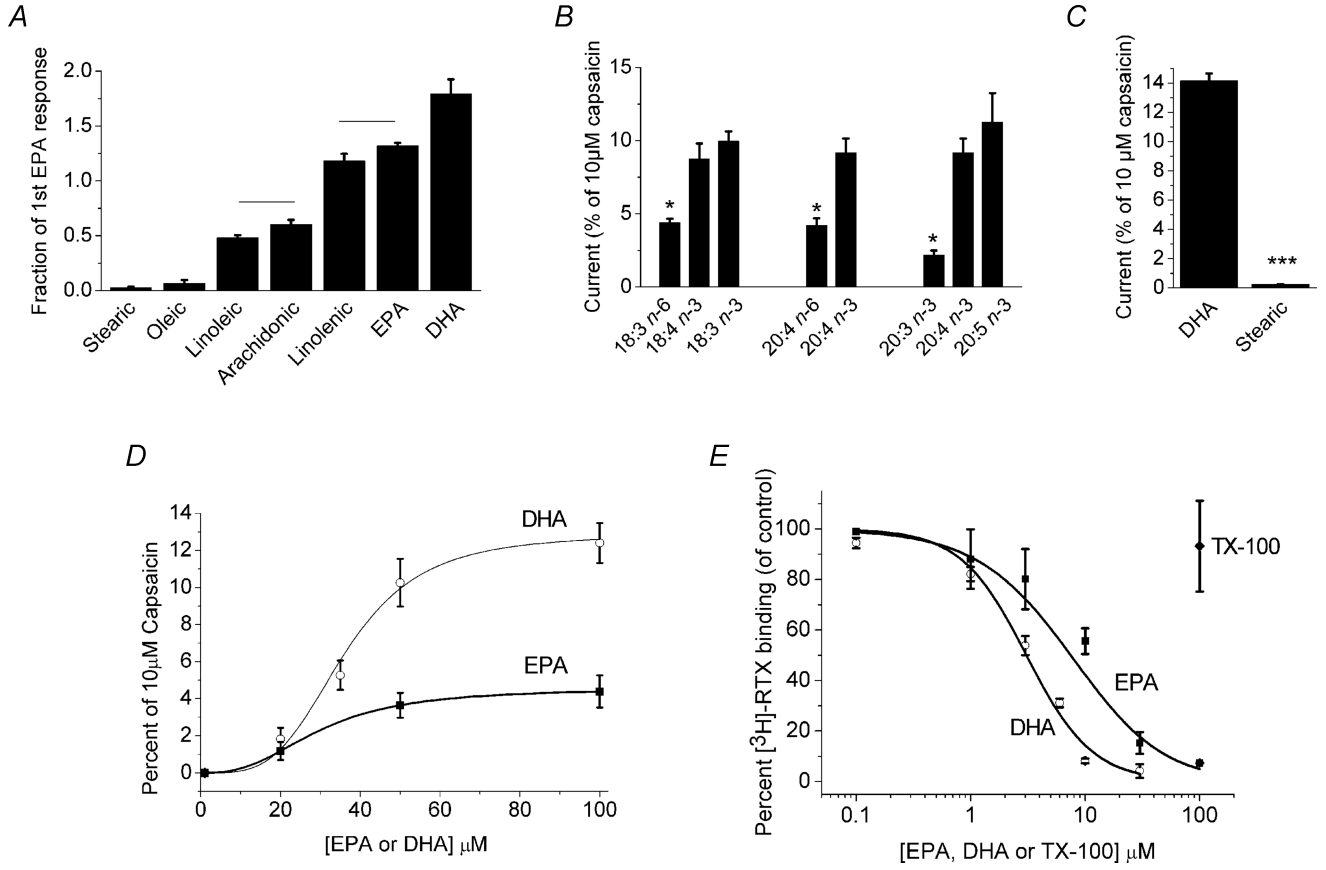

Figure 3. Unsaturated fatty acids activate TRPV1 and displace [^{3}H]resiniferatoxin binding
A, a variety of fatty acids (50 μM) activated TRPV1 after PKC stimulation with PDBu (200 nM, 3 min) in TRPV1-expressing oocytes. Data are shown as the relative response of fatty acids compared with an initial EPA (50 μM) application ($n = 3$–6, horizontal lines over bars indicate no significant difference between groups, otherwise, $P < 0.05$ by one-way ANOVA, Tukey's HSD). *B*, structure correlation for TRPV1 activation by varying unsaturation and location of double bonds. Responses were measured at +60 mV during a voltage ramp (see Fig. 1*B*) and normalized to maximal capsaicin (10 μM) response. Data are the means of 4–6 eggs. *C*, summary of fatty acid (50 μM)-evoked currents when dissolved in 0.01% BSA buffer solution in oocytes ($n = 3$; $P = 4.14 \times 10^{-6}$). Responses were measured and normalized as in Fig. 3B. *D*, dose–response curve for activation of TRPV1 by EPA and DHA in oocytes. Responses were measured and normalized as in Fig. 3B; data are the means of 3–5 eggs (DHA $E_{max} = 12.4 \pm 1.1\%$, EPA $E_{max} = 4.4 \pm 0.9\%$; $P = 0.0006$). Both of the *n*-3 PUFAs showed similar potencies (DHA $EC_{50} = 36.0 \pm 3.3$ μM, EPA $EC_{50} = 29.7 \pm 9.6$ μM). *E*, DHA and EPA inhibited binding of [^{3}H]resiniferatoxin ([^{3}H]-RTX) to TRPV1 receptors expressed in HEK 293 cells in a dose-dependent manner (K_i 10.23 ± 1.15 and 3.07 ± 0.20 μM for EPA and DHA, respectively; $P < 0.001$). TX-100 (100 μM) minimally displaced [^{3}H]-RTX. Data are means ± S.E.M. of 3–5 separate experiments.

inhibitor bisindolylmaleimide I (GF-109203X, 500 nM; control, 1.95 ± 0.11; PKC inhibitor, 1.94 ± 0.10; $n = 4$ for both and 1.2 mM extracellular Ca^{2+}).

Next, we explored the effect of *n*-3 PUFAs on TRPV1 responses to vanilloid/endovanilloids in HEK 293 cells. In contrast to the effect observed with protons, low concentrations of LNA blocked the currents evoked by capsaicin and NADA (Fig. 5*A*, *B* and *G*). LNA (1 and 10 μM) inhibited capsaicin-evoked currents (CAP, 30 nM) by ∼22 and 82%, respectively, and NADA-evoked currents (0.5 μM) by ∼46 and 84%, respectively. EPA (10 μM) also blocked capsaicin (30 nM)-evoked responses by ∼48% (Fig. 5*C* and *G*). In oocytes expressing TRPV1, EPA (30 μM) was also an effective blocker of anandamide (15 μM)-evoked responses, reducing the currents by 70 ± 4% at −60 mV ($n = 8$, $P < 0.001$, paired *t* test, data not shown). In these experiments, we were able to distinguish this blockade from TRPV1 desensitization because capsaicin applications failed to rapidly desensitize the channel in Ca^{2+}-free extracellular solution. We observed a similar blockade in sensory neurones expressing native TRPV1 channels: LNA (10 μM) blocked capsaicin (30 nM)-evoked currents by ∼73% (Fig. 5*E*). In contrast to the antagonistic effects seen with LNA and EPA, DHA (10 μM) either increased the capsaicin-evoked responses or had little effect depending on the extracellular Ca^{2+} concentration. In Ca^{2+}-free conditions DHA increased the currents evoked by capsaicin (30 nM) by ∼49% (Fig. 5*D*), whereas DHA was without effect when tested in a medium containing Ca^{2+} (1.2 mM, Fig. 5*G*). LNA blocked capsaicin-evoked currents under both Ca^{2+}-free and Ca^{2+}-rich conditions (Fig. 5*E* and *G*). We also tested whether LNA could inhibit the current activated by DHA (10 μM, after PDBu treatment). However, we found that LNA (10 μM) did not inhibit but instead enhanced these currents (1.43 ± 0.11-fold, $n = 4$). This effect is consistent with LNA being an agonist under these conditions (see Fig. 1*D*).

We next explored the ability of other fatty acids to antagonize capsaicin-evoked currents (Fig. 5*G*). The *n*-6 PUFAs γ-linolenic and arachidonic acid inhibited capsaicin-evoked currents to a similar extent as the *n*-3 PUFAs α-linolenic and EPA, respectively. Oleic acid and stearic acid produced considerably less block. Finally, to explore further the underlying mechanism for inhibition by fatty acids, we studied the effects of linolenic acid on the maximal capsaicin response. In HEK 293 cells, LNA failed to inhibit currents activated by a saturating concentration of capsaicin (10 μM) (Fig. 5*F*). Thus, inhibition by fatty acids is likely to occur in a competitive manner and this is consistent with *n*-3 PUFAs binding to a vanilloid site. This inhibition of TRPV1 constitutes a novel mechanism by which certain *n*-3 PUFAs may reduce sensory C-fibre excitability and produce analgesia. Furthermore, these data reveal clear differences in the inhibitory properties of these *n*-3 PUFAs.

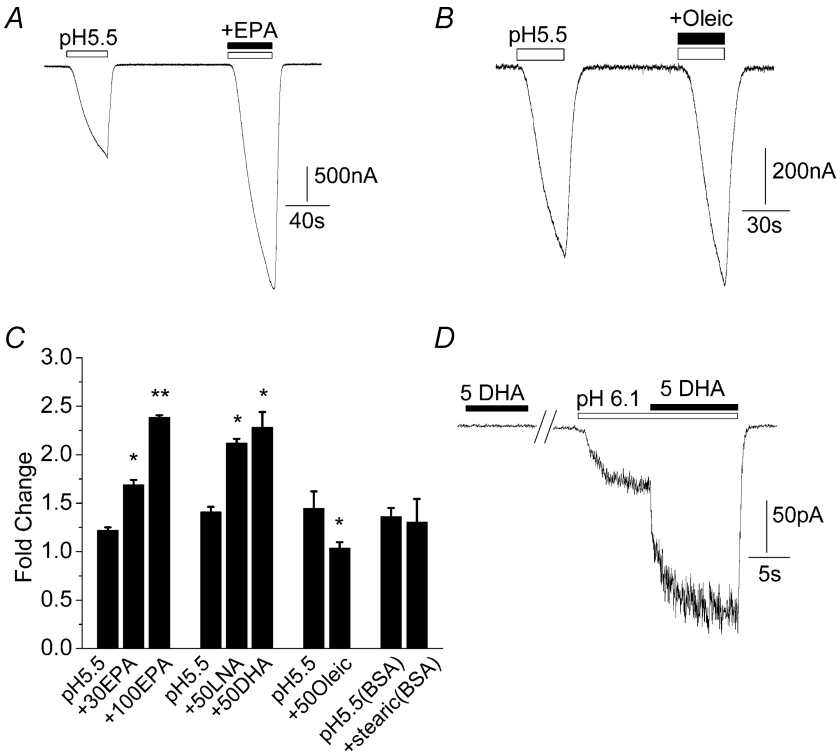

Figure 4. *n*-3 PUFAs enhance proton-evoked TRPV1 currents
A, proton (pH 5.5)-evoked currents in the presence or absence of EPA (30 and 100 μM) in oocytes. *B*, proton (pH 5.5)-evoked currents in the presence or absence of oleic acid (50 μM) in oocytes. *C*, summary of effects of EPA (30 and 100 μM, $n = 3$–4), LNA (50 μM, $n = 4$), DHA (50 μM, $n = 5$), oleic acid (50 μM, $n = 6$) and stearic acid (50 μM, $n = 4$) on proton (pH 5.5)-evoked currents in oocytes. Note that stearic acid was dissolved in BSA (0.01%) to improve solubility (*$P = 4 \times 10^{-4}$, **$P = 4.5 \times 10^{-5}$ compared with pH 5.5 alone). *D*, proton (pH 6.1)-evoked responses before and after coapplication with DHA (5 μM) in HEK 293F cells expressing TRPV1 (1.81 ± 0.21-fold of original response, $n = 5$, $P = 0.005$, paired *t* test).

Omega-3 PUFAs modulate TRPV1 independently of membrane fluidity

Fatty acids may modulate ion channels in a non-specific manner through changes in membrane elasticity and fluidity (Bolotina *et al.* 1989; Chang *et al.* 1995; Lundbaek & Andersen, 1999; Levitan *et al.* 2000; Santiago *et al.* 2001; Liu *et al.* 2003; Lundbaek *et al.* 2004). Indeed, *n*-3 PUFAs may inhibit voltage-gated Na^+ and Ca^{2+} channels by altering membrane elasticity and this effect is mimicked by the micelle-forming detergent Triton X-100 (Leaf *et al.* 2003;

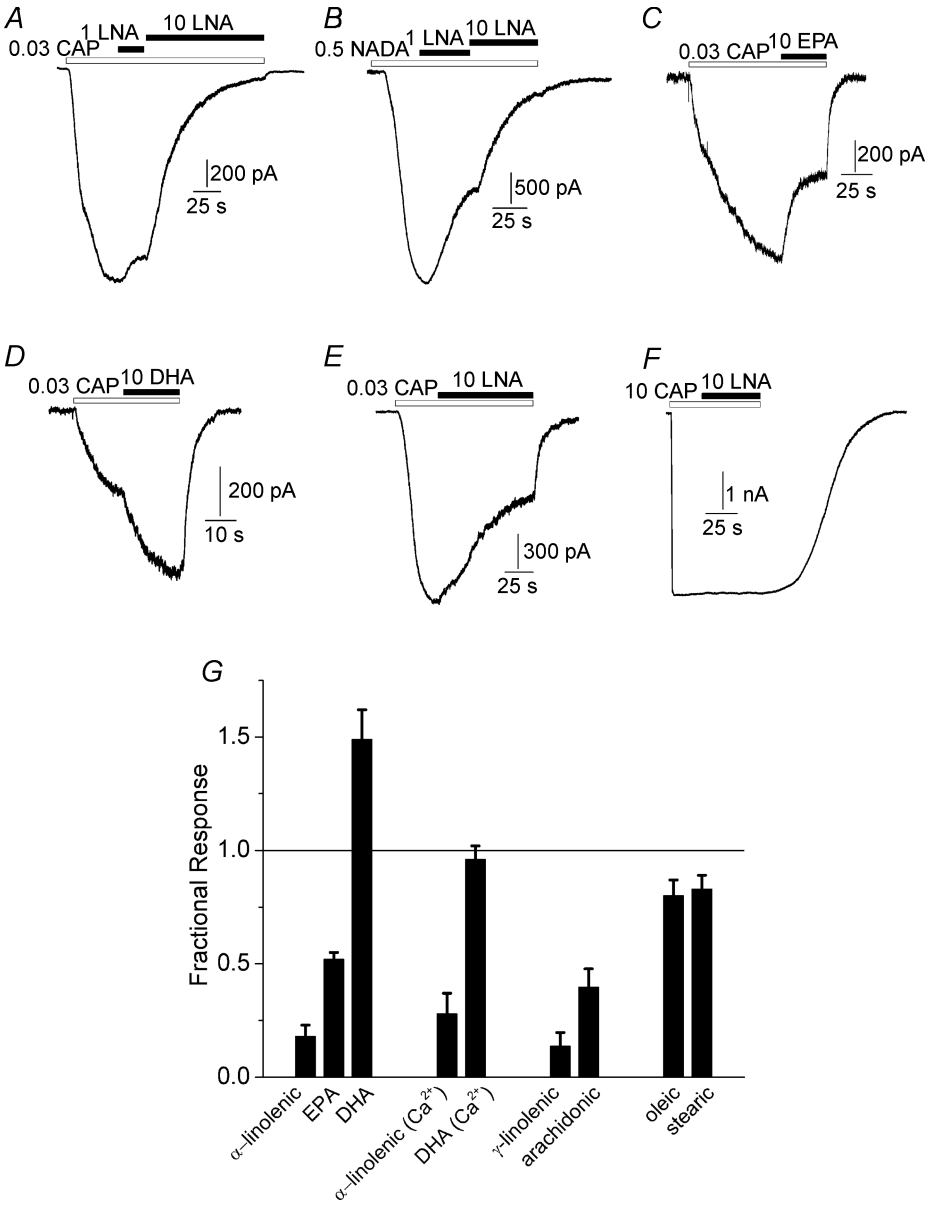

Figure 5. LNA and EPA inhibit vanilloid-evoked TRPV1 currents in HEK 293 cells and sensory neurones
A and *B*, LNA (1 μM) blocked responses to capsaicin (CAP, 30 nM; 0.78 ± 0.02-fold of the original response; $n = 10$, $P = 0.01$) and *N*-arachidonoyldopamine (NADA, 0.5 μM; 0.54 ± 0.04-fold of the original response, $n = 10$, $P = 0.001$). To a greater extent, LNA (10 μM) blocked capsaicin-evoked responses (0.18 ± 0.05-fold of the original response; $n = 10$, $P = 0.007$) and NADA-evoked responses (0.16 ± 0.02-fold of the original response; $n = 3$, $P = 0.04$) *C*, similarly, EPA (10 μM) blocked capsaicin (30 nM)-evoked responses (0.52 ± 0.03-fold of the original response; $n = 7$, $P = 0.016$). *D*, in contrast, DHA (10 μM) potentiated the capsaicin (30 nM) response by 1.49 ± 0.13-fold of the original response ($n = 5$, $P = 0.032$). *E*, LNA (10 μM) blocked capsaicin-evoked (30 nM) responses in sensory neurones (0.27 ± 0.05-fold of the original response; $n = 5$, $P < 0.05$). *F*, LNA (10 μM) did not block the maximal capsaicin (10 μM) response in HEK 293 cells expressing TRPV1. *G*, summary of the block by various fatty acids (10 μM) for capsaicin (30 nM)-evoked responses ($n = 4$–5). All *P* values were obtained using a paired *t* test.

Lundbaek *et al.* 2004). Therefore to explore this possibility with TRPV1, we tested whether Triton X-100 (TX-100) could reproduce the actions of *n*-3 PUFAs. We found that TX-100 at 30 μM (a concentration that produces approximately equivalent changes in membrane fluidity as 10 μM DHA; Leaf *et al.* 2003) partially blocked the current evoked by 30 nM and 10 μM capsaicin by ∼34 and ∼15%, respectively (Fig. 6*A* and *B*). However, this inhibition was not strongly competitive and thus differed from the block produced by *n*-3 PUFAs. Further, TX-100 produced only minimal activation of TRPV1 before or after PDBu treatment in both oocytes (Fig. 6*C*) and HEK 293 cells (0.19 ± 0.01 fractional response of 10 μM DHA; $n = 5$; data not shown). Finally, in contrast to *n*-3 PUFAs, TX-100 (30 μM) blocked TRPV1 currents activated by protons (0.64 ± 0.11 of original response, Fig. 6*D* and *E*). Taken together, these data demonstrate that non-specific changes in membrane fluidity play only a small part in the regulation of TRPV1 by *n*-3 PUFAs.

LNA inhibits TRPV1-mediated Ca^{2+} transients at 37°C

The *n*-3 PUFAs directly activate TRPV1 after PKC activation, and potentiate proton-evoked responses; thus, these fatty acids may activate TRPV1 at the physiological temperature of 37°C, which would limit their antagonistic properties. To determine the predominant effect of LNA at 37°C, we examined Ca^{2+} transients in TRPV1-expressing HEK 293 cells evoked by NADA (500 nM) and capsaicin (10 μM) in either the absence or presence of LNA (10 μM). Figure 7 shows that LNA alone evoked only a small increase in $[Ca^{2+}]$, ∼6% of the maximal capsaicin response. In contrast, NADA evoked a much larger response ∼58% of that produced by capsaicin. Significantly, coapplication of LNA reduced this NADA-evoked response to ∼15% of that produced by capsaicin. These results suggest that at 37°C, the predominant effect of LNA is to block activation by endovanilloids.

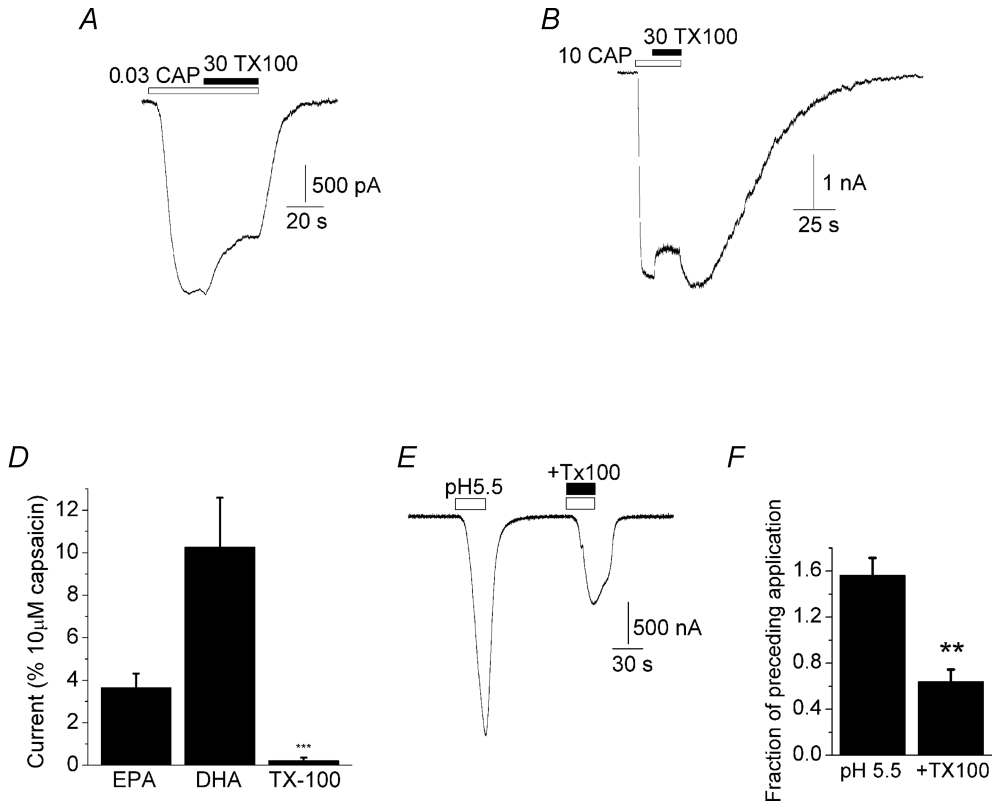

Figure 6. *n*-3 PUFAs modulate TRPV1 channel activity independently of changes in membrane fluidity
A and *B*, capsaicin (30 nM and 10 μM, *A* and *B*, respectively)-evoked currents before and after TX-100 (30 μM; fractional response after TX-100 application in *A* and *B*: 0.67 ± 0.03 and 0.86 ± 0.02, respectively) in HEK 293 cells expressing TRPV1. *C*, summary of 50 μM EPA- and DHA-evoked currents, and 100 μM TX-100-evoked currents, in oocytes expressing TRPV1 after PDBu treatment (TX-100-evoked currents recorded at +60 mV in voltage ramp as in Fig 1*B*, $n = 4$, 0.2 ± 0.1% of maximum capsaicin current, $^{***}P = 1.7 \times 10^{-4}$ compared with DHA). *D* and *E*, proton (pH 5.5)-evoked currents in the presence and absence of TX-100 (100 μM, $^{**}P = 0.01$ paired *t* test).

EPA inhibits capsaicin-induced pain-related behaviour in mice

In order to confirm the physiological relevance of *n*-3 PUFAs at TRPV1, we examined their effects in a nociceptive assay. Intraplantar injection of DHA (41.60–86 μg, $n = 6$) did not evoke any detectable pain-related behaviour (licking, biting and shaking of the injected paw). This is consistent with DHA being a weak or 'non-pungent' TRPV1 agonist under non-inflammatory conditions. We next tested whether *n*-3 PUFAs could modulate capsaicin-induced pain (Sakurada *et al.* 2003) (Fig. 8). Intraplantar injection of capsaicin produced characteristic pain-related behaviour that persisted for ~55 s. Coinjection of EPA (20 and 40 μg) markedly reduced this time by $55 \pm 7.2\%$ and $90.4 \pm 5.6\%$, respectively, whereas coinjection of equimolar concentrations of DHA (21.7 and 43.4 μg) was much less effective, reducing the time by only $12.4 \pm 9.7\%$ (not significant) and $47 \pm 10.5\%$, respectively. Thus, this ability of EPA and DHA to inhibit capsaicin-evoked pain in mice corresponds with their potency as TRPV1 antagonists.

Discussion

Activation of TRPV1 by PUFAs

In this study we identify the TRPV1 ion channel as a novel target for PUFAs. Significantly, we show that PUFAs can directly activate TRPV1 and that this occurs in a structure-specific manner. Activation of TRPV1 by PUFAs was greater with *n*-3 than with *n*-6 PUFAs. The degree of unsaturation also influenced activation: among 20-carbon fatty acids, the rank order of activation tended to correlate with the degree of unsaturation

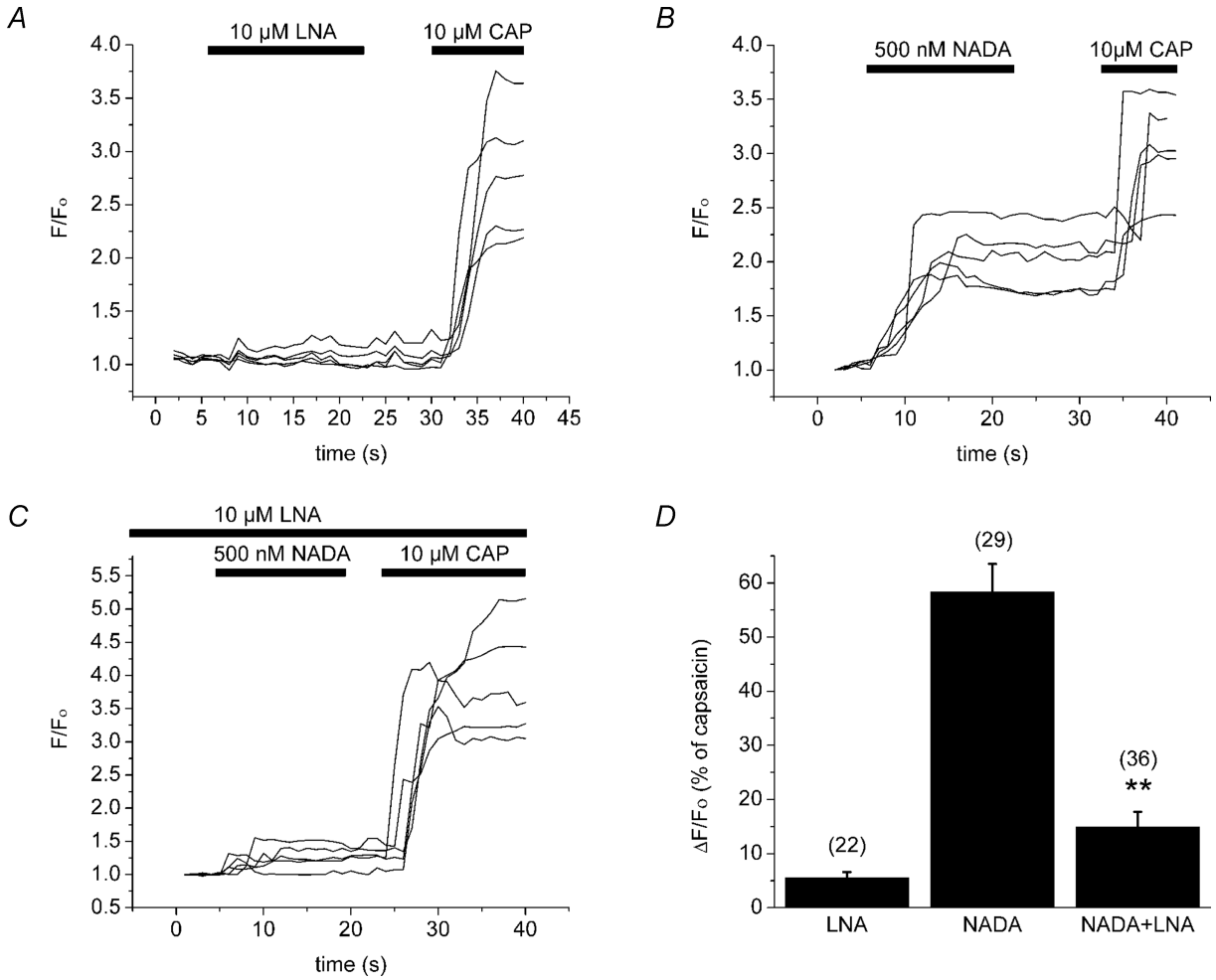

Figure 7. LNA inhibits NADA-evoked Ca^{2+} transients at 37°C in HEK 293 cells
A–C, increases in intracellular [Ca^{2+}] evoked by LNA (10 μM, $n = 22$), NADA (500 nM, $n = 29$) and NADA in the presence of LNA ($n = 36$). At the end each experiment all cells were challenged with 10 μM capsaicin. *D*, summary of normalized responses (percentage of 10 μM capsaicin, $^{**}P < 0.001$).

(20:5*n*-3 ≥ 20:4*n*-3 ≫ 20:3*n*-3). Furthermore, the monounsaturated fatty acid oleic acid, and the saturated fatty acid stearic acid, were comparatively weak agonists. Other structural features of PUFAs may also contribute to TRPV1 activation, including the position of the first double bond from the carboxyl terminus.

Activation of TRPV1 by PUFAs requires stimulation of PKC and phosphorylation of TRPV1. This was confirmed using mutant TRPV1 channels lacking critical PKC phosphorylation sites (S502A, S800A). These receptors were essentially unresponsive to *n*-3 PUFAs following PKC stimulation with phorbol ester. PKC-dependent phosphorylation is known to increase the sensitivity of TRPV1 to various ligands (Premkumar & Ahern, 2000; Vellani *et al.* 2001). In addition, PKC stimulation is essential for activation of TRPV1 by the lipid oleoylethanolamide (Ahern, 2003) and this also appears to be the case for activation by *n*-3 PUFAs. In addition to direct TRPV1 activation, we show that DHA and EPA are able to displace binding of the ultrapotent TRPV1 ligand RTX. Furthermore, EPA, DHA and LNA potentiate the proton-evoked responses as expected for vanilloid agonists.

Importantly, we show that activation occurs with physiological concentrations of fatty acids. In mammalian cells 10 μM DHA activated TRPV1 currents and 5 μM DHA potentiated proton-evoked responses. Furthermore, these experiments were performed at 22°C, and TRPV1 responses would be greatly enhanced at physiological temperature, 37°C. Thus, DHA may represent a novel endogenous TRPV1 ligand. Indeed, a biological role for PUFAs in TRPV activation is suggested by a recent study in *C. elegans* in which animals with naturally occurring mutations in PUFA biosynthesis exhibited deficits in TRPV-mediated signalling and nocifensive behaviour (Kahn-Kirby *et al.* 2004).

Antagonistic properties of PUFAs

Interestingly, in contrast to direct activation of TRPV1, we show that PUFAs can inhibit currents evoked by the vanilloids, capsaicin, NADA and anandamide. Among *n*-3 PUFAs, EPA and LNA are the most effective antagonists of capsaicin-evoked currents, whereas DHA does not possess antagonistic properties. Low micromolar concentrations of LNA and EPA inhibited TRPV1 responses in HEK 293 cells (at room temperature and 37°C) and in sensory neurones. Furthermore, in animals, EPA markedly reduced capsaicin-evoked pain-related behaviour, whereas DHA was considerably less effective. Similarly, *n*-6 PUFAs were as effective as EPA and LNA in blocking capsaicin-evoked currents, but the monounsaturated and saturated fatty acids oleic acid and stearic acid were considerably less effective antagonists.

The mechanism underlying inhibition of TRPV1 by PUFAs seems competitive. These fatty acids are partial TRPV1 agonists and therefore could potentially inhibit TRPV1 by competitively displacing vanilloids. In support of this hypothesis, *n*-3 PUFAs displaced RTX binding to TRPV1, and inhibition of capsaicin-evoked currents by LNA was abolished with saturating capsaicin concentrations. Interestingly, DHA, in contrast to other *n*-3 PUFAs, did not inhibit capsaicin-activated currents, indicating a degree of structural specificity underlying inhibition. Under Ca^{2+}-free conditions, DHA enhanced capsaicin-evoked currents. In contrast, in the presence of Ca^{2+}, DHA did not alter capsaicin-evoked currents, whereas LNA still displayed antagonistic properties. These different effects of DHA observed in the absence or presence of Ca^{2+} may be due to differences in the phosphorylation state of the channel. As previously observed (Vellani *et al.* 2001), TRPV1 is sensitized under Ca^{2+}-free conditions and it is proposed that this may be due to removal of Ca^{2+}-dependent dephosphorylation mediated by calcineurin (Mohapatra & Nau, 2005).

Alternatively, inhibition may arise via alterations in the fluidity or elasticity of the lipid bilayer. Indeed, Andersen and colleagues have proposed that changes in the composition and elasticity of the bilayer membrane may represent a generalized mechanism for ion channel modulation (Lundbaek & Andersen, 1999; Lundbaek *et al.* 2004). We believe that this is not the case with TRPV1: 30 μM Triton X-100 (a concentration shown to form micelles and increase membrane elasticity; Leaf *et al.* 2003) could only partly inhibit capsaicin-evoked currents in a non-competitive fashion. Thus, these results suggest that

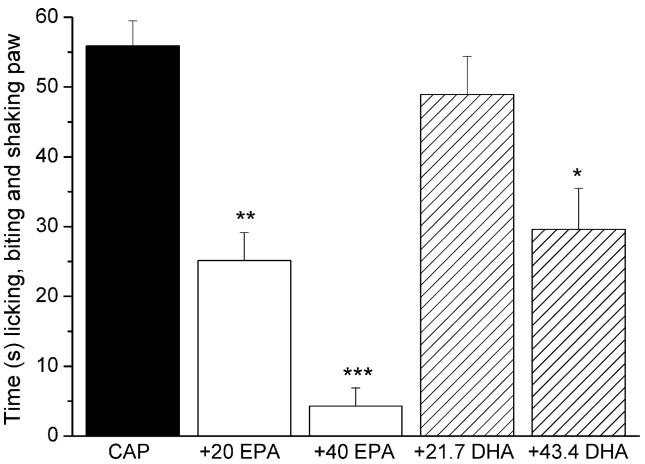

Figure 8. EPA inhibits capsaicin-evoked pain-related behaviour more effectively than DHA
Mean time spent licking, biting and shaking paw after injection of capsaicin (50 ng) or capsaicin in combination with EPA (20 and 40 μg) or DHA (equivalent molar concentration of 21.7 and 43.4 μg). Data are means ± S.E.M. of at least nine experiments, $^{*}P = 5 \times 10^{-4}$, $^{**}P = 9 \times 10^{-5}$, $^{***}P = 4 \times 10^{-7}$.

EPA and LNA block TRPV1 predominantly through direct interactions with TRPV1.

Evidence for a direct interaction between *n*-3 PUFAs and TRPV1

Although PUFAs are known to influence membrane protein function by altering the lipid bilayer properties, several lines of evidence support direct interaction of *n*-3 PUFAs with TRPV1. First, EPA and DHA displace binding of the ultrapotent TRPV1 ligand RTX. Second, EPA, LNA and DHA enhance proton-gated currents in a synergistic manner. This mimics the synergy observed between protons and vanilloid/endovanilloid compounds (Tominaga *et al.* 1998; Ahern, 2003) and is consistent with EPA, LNA and DHA interacting at vanilloid-binding site(s). Third, LNA blocks capsaicin-evoked currents in a competitive manner. Fourth, DHA is a poor antagonist of capsaicin-evoked currents, thus changes in membrane fluidity cannot account for the antagonistic effects of EPA and LNA. Fifth, TX-100 (a detergent known to increase membrane fluidity) (Klausner *et al.* 1980) minimally activated TRPV1, blocked capsaicin-evoked currents in a non-competitive manner, failed to synergize with protons, and did not displace RTX binding.

Potential physiological relevance

Our results suggest that TRPV1 may be a novel biological target for *n*-3 PUFAs. Physiological concentrations of fatty acids in plasma are 0.2–1.7 mM, but most are bound to serum albumin so that the free fatty concentration is in the range 0.01–10 μM (Spector & Hoak, 1975). We show that PUFAs can regulate TRPV1 in mammalian cells within this range (1–10 μM). First, we show that *n*-3 PUFAs (in particular DHA) can directly activate TRPV1. This effect may be important in the brain, where DHA is especially abundant (Marszalek & Lodish, 2005). Numerous brain regions also express TRPV1, including the hypothalamus, locus ceruleus, cerebellum, hippocampus and substantia nigra (Mezey *et al.* 2000). Like their counterparts in peripheral neurones, these central TRPV1 receptors may be localized in presynaptic terminals. This is supported by observations that TRPV1 ligands, such as capsaicin, can increase the frequency of spontaneous miniature EPSCs as well as the firing rate of dopaminergic neurones of the substantia nigra and ventral tegmental area, respectively (Marinelli *et al.* 2003, 2005). In addition, administration of capsaicin directly into the hypothalamus and substantia nigra produces hypothermia (Jancso-Gabor *et al.* 1970) and hyperlocomotor behaviours (Dawbarn *et al.* 1981), respectively. Despite strong evidence for TRPV1 function in the brain, it is unclear how these receptors may be activated since they are unlikely to directly encounter noxious stimuli. The endocannabinoid anandamide represents one potential endogenous ligand (Al-Hayani *et al.* 2001) because it is synthesized extensively at central synapses. In this regard, it is notable that DHA is also highly enriched at synapses (Breckenridge *et al.* 1972), localizing this fatty acid in close proximity to TRPV1. DHA is incorporated into the membrane phospholipid and is liberated by phospholipase A2 (Piomelli & Greengard, 1990). Free DHA in concentrations sufficient to activate TRPV1 may therefore be released in the near membrane space. The potential for DHA signalling via central TRPV1 channels provides an exciting new approach for exploring the physiological role of TRPV1 in the brain.

In addition to actions in the CNS, DHA may also regulate TRPV1 in peripheral sensory nerves. However, we found that despite being a TRPV1 agonist, DHA did not induce pain-related behaviours when injected at high concentrations into the mouse footpad. It is possible that the efficacy of DHA is too low, or alternatively that the kinetics of activation by DHA are too slow to effectively excite C-fibres. Indeed, high lipophilicity and slow membrane transport may explain the 'non-pungent' qualities of several vanilloid analogues (Dickenson *et al.* 1990; Liu *et al.* 1997; Lazar *et al.* 2006). Nonetheless, even in the absence of impulse activity, the local activation of TRPV1 in nerve terminals by DHA may modulate secretion of the vasodilatory neuropeptide calcitonin-gene-related peptide (CGRP), and thereby regulate vascular resistance. Interestingly, anandamide is proposed to play an important role in the regulation of vascular tone through the activation of TRPV1 and subsequent release of neuropeptides (Zygmunt *et al.* 1999). The abundance of DHA in neuronal membranes suggests that this fatty acid could also participate in this process, and such a mechanism could contribute to the beneficial circulatory effects of *n*-3 fatty acids.

Second, our data indicate that EPA and LNA may directly suppress TRPV1 activity and resultant C-fibre excitability. We demonstrate that EPA and LNA, but not DHA, markedly reduce vanilloid-evoked TRPV1 currents both at room temperature and 37°C. Further, EPA profoundly inhibits capsaicin-evoked pain-related behaviour in mice, whereas DHA is much less effective. It should be pointed out that *n*-6 PUFAs also suppress capsaicin-evoked currents. However, the effectiveness of *n*-6 PUFAs *in vivo* would be limited since, unlike *n*-3 PUFAs, they are converted into pro-inflammatory metabolites. TRPV1 plays a critical role in inflammatory pain signalling (Szallasi & Blumberg, 1999; Caterina *et al.* 2000; Davis *et al.* 2000), and a variety of lipid metabolites and eicosanoids produced during tissue injury are capable of activating TRPV1 including 12-(*S*)-hydroperoxyeicosatetraenoic acid (HPETE), -15-(*S*) HPETE, 5-(*S*)-hydroxyeicosatetraenoic acid (HETE) and leukotriene B4 (Hwang *et al.* 2000). Thus, EPA and LNA could effectively block TRPV1 currents

gated by these 'endovanilloid' compounds. This represents a novel mechanism by which *n*-3 PUFAs could mediate analgesia. Interestingly, our data suggest that selective dietary supplementation with LNA (found in flaxseed oils) would be most beneficial for pain treatment.

In summary, we show that structurally distinct *n*-3 PUFAs can differentially activate TRPV1 and inhibit agonist-evoked TRPV1 currents. The *n*-3 PUFAs may therefore be important biological regulators of TRPV1 channels, and this form of regulation may contribute to the pharmacological actions of these fatty acids.

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